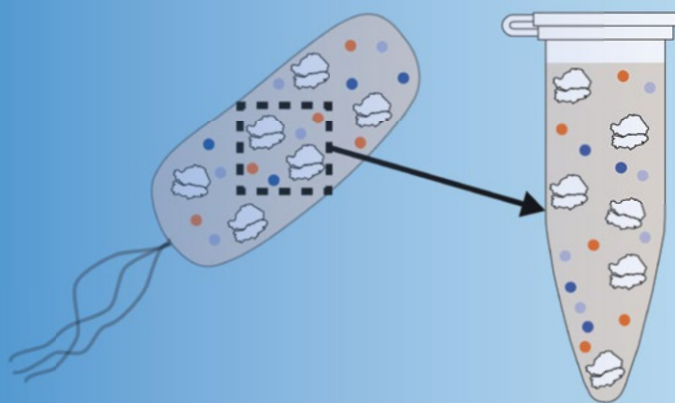


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Ashty S. Karim
Michael C. Jewett
Editors



Cell-Free Gene Expression

Methods and Protocols

 Humana Press

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Cell-Free Gene Expression

Methods and Protocols

Edited by

Ashty S. Karim

Department of Chemical and Biological Engineering, Chemistry of Life Processes Institute, and Center for Synthetic Biology, Northwestern University, Evanston, IL, USA

Michael C. Jewett

Department of Chemical and Biological Engineering, Chemistry of Life Processes Institute, Center for Synthetic Biology, Robert H. Lurie Comprehensive Cancer Center, and Simpson Querrey Institute, Northwestern University, Evanston, IL, USA

Editors

Ashty S. Karim
Department of Chemical and Biological
Engineering, Chemistry of Life
Processes Institute, and Center for
Synthetic Biology
Northwestern University
Evanston, IL, USA

Michael C. Jewett
Department of Chemical and Biological Engineering, Chemistry
of Life Processes Institute, Center for Synthetic Biology, Robert
H. Lurie Comprehensive Cancer Center, and Simpson Querrey
Institute
Northwestern University
Evanston, IL, USA

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Preface

A recent technical renaissance in cell-free expression (CFE) systems, built on over five decades of fundamental research, has resulted in highly productive CFE systems and multiplexed strategies for rapidly assessing biological design. This volume of *Methods in Molecular Biology* explores perspectives and methods using CFE to enable next-generation synthetic biology applications. The volume is organized into two major parts. The first part focuses on tools for CFE systems. These include a primer on DNA handling and reproducibility (Chapter 1), methods for cell extract preparation from diverse organisms (Chapters 2–6), and enabling high-throughput cell-free experimentation (Chapters 7–10). The second part provides an array of applications for CFE systems. These chapters highlight some of the most-promising applications including metabolic manipulation (Chapters 11–13), membrane-based and encapsulated CFE (Chapters 14–18), cell-free sensing and detection (Chapters 19–23), and a research education and instructional tool (Chapters 24–25). Collectively, this volume provides technical methods to current aspects of CFE and related applications, serving as a versatile cell-free synthetic biology experimental handbook.

Part I: Tools for Cell-Free Expression Systems

CFE systems offer barrier-free control over biological processes to directly probe function, making it an attractive approach for prototyping genetic parts and designs as well as a suite of point-of-use applications. Direct access to the reaction environment, however, comes with a degree of variability with and sensitivity to added components. Thus, having quality control standards for reagents is essential. With this in mind, Chapter 1 offers a suite of tactics for DNA handling and reproducibility in CFE systems. This serves as the start to this section of chapters focused on tools for the wide variety of CFE applications.

Variability in extract quality also has an impact on CFE system performance. Tried and true methods of extract preparation ensure reliability in CFE experimentation. Chapter 2 shares a simple extract preparation procedure for *Escherichia coli*, taking notes from the past 20 years of method development. The time at which cells are harvested prior to extract preparation has a lasting effect on extract quality, with typical extracts being made from early- to mid-exponential growth. Chapter 3 provides a method for extract preparation from non-growing *Escherichia coli* A19 cells, demonstrating how to maintain quality lysate from cells harvested later in growth. With many applications exploring the prototyping of genetic parts and designs for cellular systems, mimicking the reaction environment of cellular systems could improve the predictive power of CFE prototypes. CFE preparation protocols have been developed for non-model genera like *Pichia*, *Streptomyces*, and a few mammalian systems, among others. By having a diversity of available extracts, researchers can match the CFE system with cellular species of interest for potentially enhanced predictive power. Chapter 4 shares a procedure for making *Pichia pastoris* extracts; Chapter 5 provides an approach to making extracts from *Streptomyces* cells; and Chapter 6 offers a method for HeLa cell-based extracts and their use for membrane-bound protein production. Whatever the application these methods provide a framework for reliable extract preparation and highlight key parameters for extending extract preparation to new species.

A key advantage of CFE is the ability to prepare reactions rapidly and in high throughput, making CFE ideal for use of liquid-handling systems. The non-extract reagents can be quite challenging, though, as they come in many varieties and the way in which these reagents are assembled into a CFE reaction can dramatically affect the application of interest. The energy source, cofactors, DNA, nucleotides, and tRNAs can all play an important role in cell-free activity. The next set of chapters provide tools for high-throughput CFE. Chapter 7 demonstrates CFE systems' amenability to automation, providing tips for automating cell-free experimentation. Toward high-throughput CFE and screening, Chapter 8 shows how DNA brushes can be used as the DNA templates for CFE. Chapter 9 describes the use of *in vitro* transcribed, modified tRNAs for enhanced CFE. Rounding out this section, Chapter 10 delivers an approach to measure transcription, translation, and enzymatic processes using PERSIA, an RNA-based method for quantification.

Part II: Applications of Cell-Free Expression Systems

Beyond prototyping genetic parts and designs, CFE can enable applications in biomanufacturing, artificial cells, sensing, and education. Part II of this volume catalogs an array of these applications and their methods. Fueling transcription, translation, and other biological functions is metabolism. Cell-free systems necessitate the appropriate biochemical environment to meet ATP and redox demand. CFE systems support highly integrated, multistep metabolic networks that can be understood, modified, and controlled. Controlling native metabolic pathways and implementing new ones enables a new application space for CFE. Toward powering biochemical transformations, Chapter 11 introduces a method for operating a noncanonical redox cofactor system; Chapter 12 outlines an approach for using CFE to express enzymes and assemble complete biosynthetic pathways for testing; and Chapter 13 demonstrates the use of gas chromatography for the measurement and analysis of cell-free biochemical processes. Together these chapters provide the important methods and concepts for cell-free biomanufacturing applications.

CFE systems are ideally suited for the building of artificial cells integrating multiple genetic and metabolic designs. Many laboratories have recapitulated cellular functions by encapsulating CFE systems and synthetic gene circuits inside liposomes. One challenging aspect of this work is developing the right formulation and process to make these compartments. Thus, Chapter 14 offers a strategy using a 3D-printed microcapillary-based apparatus for robust and tunable liposome preparation. A complementary approach to producing cell-mimics using a microfluidic apparatus is provided in Chapter 15. Hybrid lipid and polymer formulations are used to enable high-yielding protein synthesis in vesicles in Chapter 16. Monitoring CFE activity in vesicles can be challenging. To address this, Chapter 17 allows for assessment of co-translational folding of proteins within lipid membranes. Beyond creating and assessing these encapsulated systems, controlling synthetic cells through self-assembling structures and information is important. Chapter 18 creates RNA nanostructures from double crossover tiles for use with CFE systems. These methods have and will allow for compartmentalization of CFE with applications in energy production, synthetic "cell-cell" communication, reproduction, and uncovering the rules of life.

CFE has had its point-of-use-application success in cell-free biosensing. Cell-free reactions can sense an analyte of interest by programmed expression of a reporter output (usually a protein) only when the tested sample contains the target analyte. The advantages of cell-free sensing are that they can detect cytotoxic analytes, the sensors are not subject

to evolutionary changes, and they are not attendant to constraints on genetically modified organisms (GMOs). With these technological advances, key progress in cell-free biosensing thus far has been made for the detection of chemical contaminants, disease-causing viruses and bacteria, and biological functions, in strategies that are both rapid and inexpensive. Chapter 19 applies artificial intelligence to cell-free biosensor design allowing for improved sensing functions. Chapter 20 provides a method for using purified allosteric transcription factors that bind chemical contaminants to regulate expression of a fluorescent RNA aptamer in a freeze-dried cell-free transcription-only reaction (ROSALIND). Chapter 21 switches the input of the sensing function to changes in light for optical sensing applications. Chapter 22 looks at a strategy for nucleic acid detection in the gut microbiota using toehold switches. In a complementary approach for nucleic acid detection, Chapter 23 uses isothermal nucleic acid amplification for the detection of Norovirus using paper-based cell-free sensors.

Beyond sensing, CFE is a powerful tool to understand biology and features of biological processes for both research and instructional purposes. On the research side, Chapter 24 explores the detection and identification of PAM sequences for CRISPR-Cas systems. Discovering new PAM sequences can expand the scope of CRISPR applications. On the instructional side, the ease-of-use, inexpensive assembly, and open reaction environment of CFE systems make them prime for molecular and synthetic biology education. Chapter 25 provides a manual of sorts for hands-on BioBits cell-free educational kits to train the next generation of scientists and engineers.

The cell-free synthetic biology community is growing, and with each new technological advance comes a swath of new and improved cell-free applications. This volume, which we dedicate to James R. Swartz from Stanford University who is a leading pioneer and founder of the field, catalogs some of the most compelling methods for cell-free expression systems from some of the top experts in the field. We gratefully thank each of our contributing authors for their work in providing cell-free tips and tricks from their laboratories. We hope these methods provide you with clarity and inspiration for your endeavors in the cell-free synthetic biology community.

Evanston, IL, USA

*Ashty S. Karim
Michael C. Jewett*

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Contributors

- KATARZYNA P. ADAMALA • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*
- KHALID K. ALAM • *Stemloop, Inc., Evanston, IL, USA*
- NINA ALPEROVICH • *National Institute of Standards and Technology, Gaithersburg, MD, USA*
- EZEQUIEL ALVAREZ-SAAVEDRA • *miniPCR bio™, Cambridge, MA, USA*
- KAZUAKI AMIKURA • *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, USA*
- NOA AVIDAN • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- ROCHELLE AW • *Department of Chemical Engineering, Imperial College London, London, UK; Imperial College Centre for Synthetic Biology, Imperial College London, London, UK*
- IMRE BANLAKI • *Max Planck Institute for Terrestrial Microbiology, Marburg, Germany*
- ROY H. BAR-ZIV • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- CHASE L. BEISEL • *Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz-Centre for Infection Research (HZI), Würzburg, Germany; Medical Faculty, University of Würzburg, Würzburg, Germany*
- WILLIAM B. BLACK • *Department of Chemical and Biomolecular Engineering, University of California Irvine, Irvine, CA, USA*
- PAULA J. BOOTH • *Department of Chemistry, King's College London, London, UK*
- BRUCE BRYAN • *miniPCR bio™, Cambridge, MA, USA*
- PETER A. CARR • *MIT Lincoln Laboratory, Lexington, MA, USA; Synthetic Biology Center at MIT, Cambridge, MA, USA*
- SHIRLEY S. DAUBE • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- CHRISTOPHER DEICH • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*
- YIFTACH DIVON • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- AARON J. DY • *Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA; Broad Institute of MIT and Harvard, Cambridge, MA, USA; Health Advances LLC, Newton, MA, USA*
- FRANK ENGLERT • *Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz-Centre for Infection Research (HZI), Würzburg, Germany*
- JUREK FAILMEZGER • *Institute of Biochemical Engineering, Universität Stuttgart, Stuttgart, Germany*
- ZECONG FANG • *Department of Biomedical Engineering, University of California, Davis, CA, USA; Institute of Biomedical and Health Engineering, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China; Shenzhen Engineering Laboratory of Single-Molecule Detection and Instrument Development, Shenzhen, China*

- JEAN-LOUP FAULON • *Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France; Université Paris-Saclay, Systems & Synthetic Biology Lab (iSSB), UMR, Evry, France; Manchester Institute of Biotechnology, SYNBIOCHEM Center, School of Chemistry, The University of Manchester, Manchester, UK*
- LÉON FAURE • *Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France*
- ELISA FRANCO • *Mechanical and Aerospace Engineering, Bioengineering, and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA, USA*
- ALEXANDER A. GREEN • *Department of Biomedical Engineering, Boston University, Boston, MA, USA*
- FERDINAND GREISS • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- ALESSANDRO GROAZ • *Baylor College of Medicine, Houston, TX, USA*
- NICOLA J. HARRIS • *Department of Chemistry, King's College London, London, UK*
- JOSEPH M. HEILI • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*
- KEITA HIBI • *Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan*
- FLORIAN HIERING • *Institute of Biochemical Engineering, Universität Stuttgart, Stuttgart, Germany*
- YEN-YU HSU • *University of Michigan, Ann Arbor, MI, USA*
- ALLY HUANG • *miniPCR bio™, Cambridge, MA, USA*
- MIRANDA L. JACOBS • *Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA; Interdisciplinary Biological Sciences Program, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA*
- MICHAEL C. JEWETT • *Department of Chemical and Biological Engineering, Chemistry of Life Processes Institute, Center for Synthetic Biology, Robert H. Lurie Comprehensive Cancer Center, and Simpson Querrey Institute, Northwestern University, Evanston, IL, USA*
- JAERYOUNG K. JUNG • *Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA; Center for Water Research, Northwestern University, Evanston, IL, USA*
- NEHA P. KAMAT • *Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA*
- ASHTY S. KARIM • *Department of Chemical and Biological Engineering, Chemistry of Life Processes Institute, and Center for Synthetic Biology, Northwestern University, Evanston, IL, USA*
- SEBASTIAN KRAVES • *miniPCR bio™, Cambridge, MA, USA*
- MAISH KUSHWAHA • *Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France*
- FRANÇOIS-XAVIER LEHR • *Max Planck Institute for Terrestrial Microbiology, Marburg, Germany*
- MICHAEL LEVY • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*

- HAN LI • *Department of Chemical and Biomolecular Engineering, University of California Irvine, Irvine, CA, USA*
- JIAN LI • *School of Physical Science and Technology, ShanghaiTech University, Shanghai, China*
- ALLEN P. LIU • *University of Michigan, Ann Arbor, MI, USA*
- WAN-QIU LIU • *School of Physical Science and Technology, ShanghaiTech University, Shanghai, China*
- MARJORIE L. LONGO • *Department of Chemical Engineering, University of California, Davis, CA, USA*
- YUAN LU • *Key Laboratory of Industrial Biocatalysis, Ministry of Education, Department of Chemical Engineering, Tsinghua University, Beijing, China*
- JULIUS B. LUCKS • *Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA; Center for Water Research, Northwestern University, Evanston, IL, USA; Stemloop, Inc., Evanston, IL, USA; Interdisciplinary Biological Sciences Graduate Program, Northwestern University, Evanston, IL, USA*
- CONARY MEYER • *Department of Biomedical Engineering, University of California, Davis, CA, USA*
- APRIL M. MIGUEZ • *School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA*
- HOSSEIN MOGHIMIANAVVAL • *University of Michigan, Ann Arbor, MI, USA*
- ALISSA C. MULLIN • *Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, CA, USA*
- HENRIKE NIEDERHOLTMEYER • *Max Planck Institute for Terrestrial Microbiology, Marburg, Germany*
- JAVIN P. OZA • *Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, CA, USA*
- TINGRUI PAN • *Department of Biomedical Engineering, University of California, Davis, CA, USA; Institute of Biomedical and Health Engineering, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China; Shenzhen Engineering Laboratory of Single-Molecule Detection and Instrument Development, Shenzhen, China; Suzhou Institute for Advanced Research, University of Science and Technology of China, Suzhou, China; Department of Precision Machinery and Precision Instrumentation, University of Science and Technology of China, Hefei, China*
- KAREN M. POLIZZI • *Department of Chemical Engineering, Imperial College London, London, UK; Imperial College Centre for Synthetic Biology, Imperial College London, London, UK*
- BLAKE J. RASOR • *Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA; Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA*
- EAMONN READING • *Department of Chemistry, King's College London, London, UK*
- EUGENIA F. ROMANTSEVA • *National Institute of Standards and Technology, Gaithersburg, MD, USA*
- DAVID ROSS • *National Institute of Standards and Technology, Gaithersburg, MD, USA*
- WAKANA SATO • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*

- YOSHIHIRO SHIMIZU • *Laboratory for Cell-Free Protein Synthesis, RIKEN Center for Biosystems Dynamics research (BDR), Osaka, Japan*
- MARTIN SIEMANN-HERZBERG • *Institute of Biochemical Engineering, Universität Stuttgart, Stuttgart, Germany*
- TAYLOR SLOUKA • *Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, CA, USA*
- PAUL SOUDIER • *Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France*
- ALEX J. SPICE • *Department of Chemical Engineering, Imperial College London, London, UK; Imperial College Centre for Synthetic Biology, Imperial College London, London, UK*
- JESSICA C. STARK • *Department of Chemistry and Stanford ChEM-H, Stanford University, Stanford, CA, USA*
- JAIME MARIE STEWART • *Division of Engineering and Applied Science, California Institute of Technology, Pasadena, CA, USA*
- ELIZABETH A. STRYCHALSKI • *National Institute of Standards and Technology, Gaithersburg, MD, USA*
- MARK P. STYCZYNSKI • *School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA*
- HARI K. K. SUBRAMANIAN • *ChromaCode, Inc., Carlsbad, CA, USA*
- DREW S. TACK • *National Institute of Standards and Technology, Gaithersburg, MD, USA*
- MELISSA K. TAKAHASHI • *Department of Biology, California State University Northridge, Northridge, CA, USA*
- CHEEMENG TAN • *Department of Biomedical Engineering, University of California, Davis, CA, USA*
- XIAO TAN • *Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA; Division of Gastroenterology, Massachusetts General Hospital, Boston, MA, USA; Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA, USA; Broad Institute of MIT and Harvard, Cambridge, MA, USA*
- ORION M. VENERO • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*
- BASTIAN VÖGELI • *Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA; Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA; Center for Synthetic Biology, Northwestern University, Evanston, IL, USA*
- OHAD VONSHAK • *Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel*
- SCOTT WICK • *MIT Lincoln Laboratory, Lexington, MA, USA; Synthetic Biology Center at MIT, Cambridge, MA, USA*
- FRANZISKA WIMMER • *Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz-Centre for Infection Research (HZI), Würzburg, Germany*
- KAIYUE WU • *Department of Biomedical Engineering, Boston University, Boston, MA, USA; Molecular Biology, Cell Biology and Biochemistry Program, Graduate School of Arts and Sciences, Boston University, Boston, MA, USA*
- HUILING XU • *School of Physical Science and Technology, ShanghaiTech University, Shanghai, China*
- JUNZHU YANG • *Key Laboratory of Industrial Biocatalysis, Ministry of Education, Department of Chemical Engineering, Tsinghua University, Beijing, China*

YAN ZHANG • *School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA*

CHUQING ZHOU • *Department of Biomedical Engineering, University of California, Davis, CA, USA*

Part I

Tools for Cell-Free Expression Systems



Chapter 1

Best Practices for DNA Template Preparation Toward Improved Reproducibility in Cell-Free Protein Production

Eugenia F. Romantseva, Drew S. Tack, Nina Alperovich, David Ross, and Elizabeth A. Strychalski

Abstract

Performance variability is a common challenge in cell-free protein production and hinders a wider adoption of these systems for both research and biomanufacturing. While the inherent stochasticity and complexity of biology likely contributes to variability, other systematic factors may also play a role, including the source and preparation of the cell extract, the composition of the supplemental reaction buffer, the facility at which experiments are conducted, and the human operator (Cole et al. *ACS Synth Biol* 8:2080–2091, 2019). Variability in protein production could also arise from differences in the DNA template—specifically the amount of functional DNA added to a cell-free reaction and the quality of the DNA preparation in terms of contaminants and strand breakage. Here, we present protocols and suggest best practices optimized for DNA template preparation and quantitation for cell-free systems toward reducing variability in cell-free protein production.

Key words Cell-free systems, Cell-free protein production, TX-TL, DNA template preparation, Automated DNA extraction, Automated DNA purification

1 Introduction

Cell-free systems are increasingly used for in vitro protein production and offer a promising alternative to traditional, cell-based biomanufacturing [1–6]. At the state-of-the-art, cell-free systems can provide a high-throughput platform for on-demand biomanufacturing of therapeutics [3, 7–9], antibodies [10–13], antimicrobials [1, 14], and vaccines [15–18]. Additionally, cell-free systems can accelerate the design-build-test-learn cycle in engineering biology workflows through rapid prototyping of genetic circuits [19–26], expansion of the genetic toolbox [27–34], and development

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and optimization of biosensors [35–38]. All of these applications rely on high-quality DNA templates for robust cell-free protein production. However, common DNA preparation techniques may yield damaged or contaminated DNA. Here, we present detailed protocols for extraction, purification, and quantitation of circular DNA templates intended specifically for cell-free protein production. While linear templates are becoming more popular, especially for prototyping genetic elements in cell-free systems [40, 41], DNA is typically circularized prior to insertion *in vivo*, and the methods here may still apply to the preparation and quantitation of those circularized templates. Although our protocols are optimized for use with *E. coli*-derived transcription-translation cell-free systems—myTXTL, or its equivalent—the methods described herein are applicable for cell-free systems prepared from nonmodel organisms [10, 15, 42] and reconstituted cell-free systems, such as PURExpress [43, 44].

Starting with two common commercial kits for DNA extraction for cell-based systems, we optimized and modified the protocols to yield high concentration, intact DNA templates for use with cell-free systems. We selected a rapid and simple method that uses filter columns in a bind-wash-elute procedure to prepare high-grade plasmid DNA for routine applications, including PCR, such as the QIAprep Spin Miniprep kit, or its equivalent. Optimization of this approach was of particular interest to us, as inconsistent results in the quality and quantity of the DNA prepared with mini prep kits, as opposed to midi and maxi prep kits, have been reported anecdotally by the cell-free community [45]. We selected a facile magnetic bead-based approach to DNA extraction for high-quality endotoxin-free plasmid DNA, such as the Mag-Bind Ultra Pure Plasmid DNA 96 kit, or its equivalent. Optimization of this approach was of particular interest to us, as a methodology that lacks centrifugation-dependent steps, which can produce high shear forces and lead to breakage especially of larger plasmids and genomic DNA [46].

Beyond these two kits, we added a downstream cleanup step for the extracted DNA templates in the form of a DNA purification kit. We selected a rapid and high-yield method that uses filter columns to remove contaminants, including primers, dNTPs, enzymes, and salts, from PCR amplicons, such as the PureLink PCR Purification kit, or its equivalent. Optimization of this approach was of particular interest to us, as this kit has been reported previously to improve the quality of DNA templates for cell-free applications [47].

All prepared DNA templates were quantitated using a fluorometric assay, such as the Quant-iT Broad-Range dsDNA kit, or its equivalent. While more labor intensive than ultraviolet spectrophotometry, fluorometric quantitation yields more accurate results for DNA concentration, especially when a calibration curve is constructed using freshly prepared samples. Physical damage to the

DNA template was assessed using gel electrophoresis, with specific attention to breakage and shearing resulting from, for example, centrifugation, pipetting, vortexing, and passage through a separation column.

Because DNA template preparation can be time-consuming and labor-intensive, we include protocols for automation (Subheadings 3.13, 3.15, 3.17, 3.19) to increase throughput. We adapted manual DNA extraction and quantitation methods (Subheadings 3.12, 3.14, 3.16, 3.18, 3.20) to automation protocols for use with common biofoundry equipment, such as automated liquid handlers, positive pressure filter press modules, heater-shaker modules, multimode plate readers, and robotic centrifuges. Specifically, we used the matching counterpart to the manual DNA template preparation and quantitation described above, for example the QIAprep 96 Turbo Miniprep kit, or its equivalent, the Mag-Bind Ultra Pure Plasmid DNA 96 kit, or its equivalent, the PureLink Pro 96 PCR Purification kit, or its equivalent, and the Quant-iT Broad-Range dsDNA assay kit, or its equivalent. We caution that automating DNA preparation, while higher throughput, may yield damaged or less concentrated templates compared to manual approaches. We offer the protocols for automation presented here as starting points for additional optimization.

The DNA template accounts for nearly 10% of the total reaction volume in a typical cell-free reaction [45]. Producing high concentration, intact, purified DNA templates can reduce or remove contaminants that may accumulate throughout the cell-free workflow and contribute to variability in cell-free protein production [39]. Removing DNA preparation as a source of variability in protein expression can free resources to further optimize other processes and measurements typical to cell-free workflows.

2 Materials

Certain commercial entities, equipment, or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the entities, materials, or equipment are necessarily the best available for the purpose.

Should the recommended materials become unavailable, we offer additional guidance to aid in the selection of alternate materials. Where possible, NIST traceable calibrants should be chosen, to inform measurement assurance and troubleshooting. Plate seals should meet the needs of the reagents and measurements. For example, aluminum seals protect light-sensitive samples, while seals with low autofluorescence mitigate background signal during

fluorescence measurements. 96- and 384-well plates, for example for DNA quantitation and cell-free protein production, should be sterile and nuclease-free to avoid contamination or degradation of the DNA. Additionally, use black plates for fluorescence measurements to reduce photodegradation of samples during storage and measurements of adjacent wells. For optical measurements from the bottom, rather than the top of the plate, use plates with flat, clear bottoms.

The selection of labware for automation must meet further criteria. Limited space on the deck of a liquid handler may require reservoirs that minimize the use of deck spaces. When selecting reservoirs or multiwell plates, consider the volume of reagent or sample needed for each step of the automation protocol as the volume will differ, for example between single, two-, and six-column reservoirs, and 96-well plates and 96-well deep well plates. Consider centrifugation stability of any plates that will be used, for example to pellet cell culture. Consider the shapes of the opening and the bottom of the wells when selecting labware to accommodate the types of pipette tips and liquid handling parameters used in the automated method. For example, use plates with square well openings with wide-bore tips to avoid collisions between the tips and the labware. Use plates with V-bottom wells where sample recovery is critical, for example for complete and efficient resuspension of the cell pellet. Be aware that labware selection may ultimately depend on the size, function, and location of the different modules on the deck of a liquid handler. For example, the location and distribution of the magnets in the magnetic plate will determine the size and shape of the wells in the multiwell plate best suited for DNA extraction with magnetic beads.

Although specific numbers of technical and biological repeats are stated here, an appropriate number of technical and biological replicate measurements should be used to address the needs of the user and the specific goals of the cell-free experiment.

2.1 Calibration

1. Digital-bottle traceable thermometer.
2. NIST human DNA quantitation standard.
3. λ DNA standards (0 ng/ μ L, 5 ng/ μ L, 10 ng/ μ L, 20 ng/ μ L, 40 ng/ μ L, 60 ng/ μ L, 80 ng/ μ L, and 100 ng/ μ L).
4. Fluorescence calibration plate for plate reader.
5. Fluorescein, NIST traceable.
6. Aluminum foil seals for multiwell plates.
7. Low autofluorescence seals for multiwell plates.
8. 96-well calibration plates: blank, nuclease-free water, and 10 nmol/L fluorescein. Prepare the blank plate by sealing an empty 96-well, black, flat bottom, optically clear bottom plate

with an aluminum foil seal to prevent dust and particulates from depositing into the wells. Prepare the nuclease-free water plate by adding 200 μ L of nuclease-free water to each well of a 96-well, black, flat bottom, optically clear bottom plate. Seal the water plate with an aluminum foil seal. Prepare the 10 nmol/L fluorescein plate by adding 200 μ L of 10 nmol/L fluorescein to each well of a 96-well, black, flat bottom, optically clear bottom plate. Seal the fluorescein plate with an aluminum foil seal. Spin down the water and fluorescein plates at $2500 \times g$ for 15 s to ensure all the liquid is at the bottom of the wells. Keep the fluorescein plate protected from light at all times. Store the calibration plates wrapped in foil at 4 °C for up to 1 week or at -20 °C for up to 1 month.

9. 384-well calibration plates: blank, nuclease-free water, and 1 μ mol/L fluorescein. Prepare the blank plate by sealing an empty 384-well, black, sterile, flat-bottom, glass-bottom plate with an aluminum foil seal to prevent dust and particulates from depositing into the wells. Prepare the nuclease-free water plate by adding 15 μ L of nuclease-free water to each well of a 384-well, black, sterile, flat-bottom, glass-bottom plate. Seal the water plate with a low autofluorescence seal. Prepare the 1 μ mol/L fluorescein plate by adding 15 μ L of 1 μ mol/L fluorescein to each well of a 384-well, black, sterile, flat-bottom, glass-bottom plate. Seal the fluorescein plate with a low autofluorescence seal. Spin down the water and fluorescein plates at $2500 \times g$ for 15 s to ensure all the liquid is at the bottom of the wells. Keep the fluorescein plate protected from light at all times. Store the calibration plates wrapped in foil at 4 °C for up to 1 week or at -20 °C for up to 1 month.
10. Recombinant eGFP standard.
11. Qubit assay tubes, or any 500 μ L thin-walled PCR tubes.
12. Hamilton Liquid Verification Kit, or its equivalent.

2.2 Cell Growth

1. pEFR40019 plasmid (Addgene, 40019 [48]) glycerol stock or bacterial stab (*see* **Note 1**).
2. 2.0 mL per well, sterile, square well, deep well, 96-well plates.
3. Sterile, clear polystyrene lids for multiwell plates.
4. Ampicillin stock solution, 100 mg/mL: Dissolve 1 g of ampicillin salt in ultrapure water to a final volume of 10 mL. Using a sterile, disposable 5 mL Luer-Lok syringe with a 0.2 μ m polyether sulfone syringe filter, filter the ampicillin solution into a 15 mL sterile, polypropylene centrifuge tube. You can further aliquot the ampicillin stock solution in 1 mL aliquots into 1.5 mL sterile, polypropylene microcentrifuge tubes for ease of use. Store the ampicillin stock solutions at -20 °C for up to 6 months.

5. LB-ampicillin agar culture plates: Dissolve 12.5 g LB powder in ultrapure water to a final volume of 450 mL. Add 7.5 g agar powder and ultrapure water to a final volume of 500 mL. Autoclave the LB agar in a 1 L glass bottle. Once the autoclave cycle is complete, transfer the bottle of LB agar to a 55 °C water bath and allow to equilibrate for at least 30 min (*see Note 2*). Add 100 mg/mL ampicillin to the LB agar for a final concentration of 100 µg/mL. Prepare culture plates by pouring approximately 25 mL of LB-ampicillin agar into each 100 mm diameter plate (*see Note 3*). Allow the covered plates to cool at room temperature overnight before storing at 4 °C, wrapped in foil and bagged, for up to 6 months.
6. Enriched M9 media with 4% glycerol and 2% casamino acids: Prepare stock solution of 5× M9 minimal media by dissolving 56.4 g of 5× M9 minimal salts in ultrapure water to a final volume of 1 L; autoclave to sterilize. Prepare stock solution of 40% glycerol by adding 400 mL ultrapure glycerol to ultrapure water for a final volume of 1 L; autoclave to sterilize. Prepare stock solution of 4% casamino acid by dissolving 40 g of Omni-Pur casamino acids in ultrapure water to a final volume of 1 L (*see Note 4*); filter-sterilize using a sterile bottle-top vacuum filter system with cellulose acetate membrane. Prepare stock solution of 100 mmol/L CaCl₂ by dissolving 2.7745 g of CaCl₂ in ultrapure water to a final volume of 250 mL; filter-sterilize by using a sterile bottle-top vacuum filter system with cellulose acetate membrane. Prepare stock solution of 1 mol/L MgSO₄ by dissolving 61.6175 g of MgSO₄ 7 H₂O in ultrapure water to a final volume 250 mL; filter-sterilize by using a sterile bottle-top vacuum filter system with cellulose acetate membrane. Assemble the enriched M9 media aseptically by adding 200 mL of 5× M9 minimal media, 100 mL of 40% glycerol, 500 mL of 4% casamino acids, 1 mL of 100 mmol/L CaCl₂, and 2 mL of 1 mol/L MgSO₄ to ultrapure water for a final volume of 1 L. Store at room temperature for up to 1 year.

2.3 DNA Extraction

1. 2.0 mL, sterile, polypropylene microcentrifuge tubes.
2. 25 mL, disposable, sterile, nonpyrogenic, RNase-free reagent reservoirs.
3. 1.5 mL, PCR clean, DNA LoBind tubes.
4. Aluminum foil seals for multiwell plates.
5. QIAprep Spin Miniprep kit with complete set of buffers, reagents, and consumables, or its equivalent.
6. QIAprep 96 Turbo Miniprep kit with complete set of buffers, reagents, and consumables, or its equivalent.
7. Mag-Bind Ultra Pure Plasmid DNA 96 kit with complete set of buffers, reagents, and consumables, or its equivalent.

8. Magnetic separation rack.
9. Universal 96-well magnet plate.
10. 2.0 mL per well, sterile, deep well, 96-well plates.
11. 300 mL, sterile, single cavity, 12-column reservoirs.
12. 47 mL per column, sterile, 6-column reservoirs.
13. 146 mL per column, sterile, 2-column reservoirs.
14. 1.2 mL per well, sterile, 96-well storage plates.
15. 500 μ L per well, PCR clean, 96-well DNA LoBind plates.
16. Sterile, clear polystyrene lids for multiwell plates.

2.4 DNA Purification

1. 1.5 mL, PCR clean, DNA LoBind tubes.
2. PureLink PCR Purification kit with complete set of buffers, reagents, and consumables, or its equivalent.
3. PureLink Pro 96 PCR Purification kit with complete set of buffers, reagents, and consumables, or its equivalent.
4. 500 μ L per well, PCR clean, 96-well DNA LoBind plates.
5. Sterile, clear polystyrene lids for multiwell plates.
6. 47 mL per column, sterile, six-column reservoirs.
7. 146 mL per column, sterile, two-column reservoirs.

2.5 DNA Quantitation

1. Quant-iT Broad-Range dsDNA assay kit with complete set of buffers, reagents, and consumables, or its equivalent.
2. Qubit assay tubes, or any 500 μ L thin-walled PCR tubes.
3. Black, nuclease-free, flat bottom, optically clear bottom, 96-well plates.
4. 300 mL, sterile, black, single cavity, 12-column reservoirs.
5. Sterile, black polystyrene lids for multiwell plates.

2.6 Cell-Free Protein Production

1. myTXTL Sigma 70 Master Mix, 5 mL single lot, or its equivalent.
2. 1 \times phosphate buffered saline (PBS), RNase-free.
3. Recombinant eGFP.
4. 200 μ L, nuclease-free, PCR inhibitor-free, PCR 8-tube strip with caps.
5. Black, sterile, flat-bottom, glass-bottom, 384-well plates.
6. Low autofluorescence seals for multiwell plates.

2.7 Gel Electrophoresis

1. 200 μ L, nuclease-free, PCR inhibitor-free, PCR 8-tube strip with caps.
2. 1.2% agarose, precast gels for electrophoresis.
3. 1 kb ready-to-use DNA ladder.

3 Methods

Carry out all procedures at room temperature, unless otherwise specified. Diligently follow proper labeling procedures, biosafety precautions, and all waste disposal regulations. Always use filter pipette tips for manual and automated liquid handling to minimize contamination risk.

3.1 Instrument Calibration for Benchtop Incubators for Plate and Liquid Culture Growth

Perform this calibration at least quarterly.

1. Set the incubator to the desired temperature and shaking speed.
2. Place the digital-bottle traceable thermometer into the incubator and allow to equilibrate for at least 1 h. If planning on using the shaking function, for example for liquid culture growth, ensure shaking is enabled while the calibrant equilibrates.
3. Record the temperature of the calibrant, and adjust temperature setting on incubator as necessary until the desired temperature is reached by the calibrant.

3.2 Manufacturer's Calibration for Fluorescence Plate Reader

Perform this calibration at least monthly and the day of any plate reader measurements. Avoid using plate readers that fail the manufacturer's calibration.

1. Set the temperature of the plate reader to 23 °C.
2. Using 70% isopropanol and a lint free wipe, wipe the carrier of the plate reader on all sides.
3. Remove fluorescence calibration plate from its storage case (*see Note 5*) and examine for any dust particles or debris.
4. Remove any dust or debris by using the dust blower bulb included in the storage case. Holding the bulb at an angle, squeeze and release the bulb several times to blow any dust or debris from the plate wells and filters. If particulates remain, use the cleanroom swabs included in the storage case, and gently press and release the affected area.
5. Using the software that came with the plate reader and the protocols supplied with the fluorescence calibration plate, build the desired imaging experiments. We recommend at least verifying fluorescence intensity with the top and bottom monochromators, and fluorescence polarization.
6. Run each imaging experiment, rotating the fluorescence calibration plate when instructed. Save each experiment and export the collected data in .xls format.
7. Return the fluorescence calibration plate to its storage case immediately after all experiments are finished. Avoid touching any plate wells and filters (*see Note 6*).

8. To determine if the plate reader passed the calibration, open the exported data .xls file and the Calibration_Certificate_Template.xls included with the fluorescence calibration plate. Copy the contents in the dashed box of the Calibration_Certificate_Template.xls and paste into the “FTLP_Calibration_Cert” tab of the exported data .xls file. This will generate pass or fail messages in the other tab(s) of the exported data .xls file.

3.3 Calibration for Fluorescence Plate Reader for Automated DNA Quantitation

Perform this calibration the day of any automated DNA quantitation with fluorometry. The resulting data can be used to identify any systematic bias in the measurement across the plate, for example, due to carrier misalignment.

1. Remove 96-well calibration plates (blank, nuclease-free water, and 10 nmol/L fluorescein) from storage and allow to come to room temperature for at least 1 h, wrapped in aluminum foil to protect from light. Spin down the water and fluorescein plates at $2500 \times g$ for 15 s to ensure all liquid is at the bottom of the wells. Keep plates wrapped in foil to protect from light.
2. Using the software that came with the plate reader, build the imaging protocol and associated experiments for fluorometric quantitation of DNA with the Quant-iT Broad-Range dsDNA assay (*see* **Note 7** and **Table 1**).
3. Run each calibration plate, making sure to remove the seal from the plate just prior to inserting the plate into the plate reader. Reseal each plate when it comes out of the reader with a fresh aluminum foil seal. Store the calibration plates wrapped in foil at 4 °C for up to 1 week or at –20 °C for up to 1 month.

Table 1

Gen5 protocol settings for DNA quantitation using fluorometry and the Quant-iT Broad-Range dsDNA assay in a BioTek Neo2 plate reader

Setting name	Setting value
Temperature	23 °C
Plate	Greiner 96 flat bottom; no seal
Excitation wavelength	485/20 nm
Emission wavelength	528/20 nm
Optics	Bottom
Gain	75; no autogain adjustment
Read speed	Normal
Delay after plate movement	0 ms
Measurement per data point	10
Lamp energy	Low (fast)
Read height	4.50 mm

3.4 Calibration for Fluorometer for Manual DNA Quantitation

Perform this calibration the day of any manual DNA quantitation with fluorometry. The resulting data can be used to verify the performance of the fluorometer.

1. Remove the NIST human DNA quantitation standard from storage at 4 °C and allow to come to room temperature for at least 30 min, protected from light. Vortex each tube for 5 s, and spin down for 3 s. Protect tubes from light by keeping them in their storage container when not in use.
2. Remove the Quant-iT Broad-Range dsDNA assay kit from storage at 4 °C and allow to come to room temperature for at least 30 min, protected from light.
3. Vortex Component A, Component C 0 ng/μL λ DNA, and Component C 100 ng/μL λ DNA for 5 s, and then spin down for 3 s. Keep tubes wrapped in aluminum foil to protect from light.
4. Prepare the Quant-iT Broad-Range dsDNA working solution in a 15 mL centrifuge tube or other disposable plastic container by combining 16 μL of Component A with Component B to a final volume of 3.2 mL (*see Notes 8 and 9*). Vortex the working solution for 10 s and wrap in aluminum foil to protect from light. The working solution is stable for up to 3 h. Return Component A and Component B to storage at 4 °C.
5. Prepare DNA standards in Qubit assay tubes. To prepare Standard #1, add 10 μL of Component C 0 ng/μL λ DNA to 190 μL of Quant-iT Broad-Range dsDNA working solution, vortex for 5 s, and then spin down for 3 s (*see Note 9*). To prepare Standard #2, add 10 μL of Component C 100 ng/μL λ DNA to 190 μL of Quant-iT Broad-Range dsDNA working solution, vortex for 5 s, and then spin down for 3 s. Incubate tubes for 2 min at room temperature, protected from light. Return Component C 0 ng/μL λ DNA and Component C 100 ng/μL λ DNA to storage at 4 °C.
6. Prepare DNA calibration samples in Qubit assay tubes, 5 technical repeats of each component in the NIST human DNA quantitation standard. Add 5 μL of the NIST human DNA quantitation standard Component A, B, or C to 195 μL of Quant-iT Broad-Range dsDNA working solution, vortex for 5 s, and then spin down for 3 s (*see Note 9*). Repeat this until you have five DNA replicates for each component in the NIST human DNA quantitation standard. Incubate tubes for 2 min at room temperature, protected from light. Return all components of the NIST human DNA quantitation standard to storage at 4 °C.

7. Read the DNA standards on the fluorometer. For example, if using the Qubit Fluorometer 3.0, from the Home screen, select “DNA,” then “dsDNA Broad Range,” then “Read Standards.” Lift the chamber lid, insert the Qubit assay tube containing Standard #1, close the chamber lid, and press “Read.” When sample reading is complete, lift the chamber lid, discard the tube containing Standard #1, insert the Qubit assay tube containing Standard #2, close the chamber lid, and press “Read.” When sample reading is complete, discard tube with Standard #2, and close the chamber lid.
8. Read the DNA calibration samples on the fluorometer. For example, if using the Qubit Fluorometer 3.0, from the Home screen, select “DNA,” then “dsDNA Broad Range,” then “Read Samples.” Set the sample volume to 5 μL and select the desired units for output sample concentration. Lift the chamber lid, insert the Qubit assay tube containing NIST human DNA quantitation standard Component A technical replicate 1, close the chamber lid, and press “Read.” Record the measured DNA concentration. When sample reading is complete, lift the chamber lid, discard the tube containing NIST human DNA quantitation standard Component A technical replicate 1, insert the Qubit assay tube containing NIST human DNA quantitation standard Component A technical replicate 2, close the chamber lid, and press “Read.” Record the measured DNA concentration. Repeat these steps until all five technical replicates for each component in the NIST human DNA quantitation standard have been measured.

**3.5 Calibration
for Spectrophotometer
for Manual
Absorbance
Measurements of DNA
Using
Spectrophotometry**

Perform this calibration the day of any manual absorbance measurements of DNA quantity and quality with spectrophotometry. The resulting data can be used to verify the performance of the spectrophotometer.

1. Remove the NIST human DNA quantitation standard from storage at 4 $^{\circ}\text{C}$, and allow to come to room temperature for at least 30 min, protected from light. Once at room temperature, vortex each tube for 5 s, and then spin down for 3 s. Protect tubes from light by keeping them in their storage container when not in use.
2. Select the appropriate assay on the spectrophotometer. For example, if using the DeNovix, from the home screen select the “dsDNA” application.
3. Wash the sample stage and lid of the spectrophotometer with DI water and wipe dry using Kimwipes.
4. Run a blank by adding 1 μL buffer consisting of 10 mmol/L Tris chloride 0.1 mmol/L EDTA (*see Note 10*) to the sample stage, closing the lid, and pressing “Run.” Open the lid, and use Kimwipes to clean the sample stage and lid.

5. Add 1 μL of the NIST human DNA quantitation standard Component A to the sample stage, close the lid, and press “Run.” Record the measured DNA concentration, measured absorbance at 260 nm, ratio of absorbance measured at 260 nm to 230 nm, and ratio of absorbance measured at 260 nm to 280 nm. Open the lid, and use Kimwipes to clean the sample stage and lid.
6. Repeat **step 5** for a total of five technical repeats for each component in the NIST human DNA quantitation standard.
7. Return the NIST human DNA quantitation standard to storage at 4 °C.

3.6 Calibration for Fluorescence Plate Reader for Cell-Free Protein Production

Perform this calibration the day of any fluorescence measurements of cell-free protein production. The resulting data can be used to identify any systematic bias in the measurement across the plate, for example, due to carrier misalignment.

1. Remove 384-well calibration plates (blank, nuclease-free water, and 1 $\mu\text{mol/L}$ fluorescein) from storage and allow to come to room temperature for at least 1 h, wrapped in aluminum foil to protect from light. Spin down the water and fluorescein plates at $2500 \times g$ for 15 s to ensure all liquid is at the bottom of the wells. Keep plates wrapped in foil to protect from light.
2. Using the software that came with the plate reader, build the imaging protocol and associated experiments for fluorescence measurements of cell-free protein production (*see* **Note 7** and **Table 2**).
3. Run each sealed calibration plate and immediately wrap the plate in foil to protect it from light when it comes out of the reader. Save each experiment and export the data in the desired format. Store the calibration plates wrapped in foil at 4 °C for up to 1 week or at -20 °C for up to 1 month.

3.7 Daily Maintenance of Automated Liquid Handler with Positive Pressure Module

Perform this maintenance the day of any automated liquid handling (*see* **Note 37**).

1. If using the Hamilton Microlab STAR: From the Desktop of the computer controlling the STAR, select the “Microlab STAR Maintenance & Verification” application. Check the “Execute” box for “Daily maintenance” and press the green triangle to execute the “Run Process” command. Follow the user prompts to empty the waste container(s) and perform tightness checks on the channel pipettors. Follow the user prompts to load the Maintenance Tool 96 TADM onto the deck with the “Maintenance” side facing up. Follow the user prompts to complete daily maintenance of the STAR.

Table 2

Gen5 protocol settings for fluorescence measurement of eGFP produced with the myTXTL Sigma 70 Master Mix in a BioTek Neo2 plate reader

Setting name	Setting value
Temperature	30 °C
Plate	Nunc 384 flat bottom with seal
Excitation wavelength	485/20 nm
Emission wavelength	516/20 nm
Optics	Bottom
Gain	55; no autogain adjustment
Read speed	Normal
Delay after plate movement	0 ms
Measurement per data point	3
Lamp energy	Low (fast)
Read height	8.00 mm

2. If using the Hamilton Microlab STAR with [MPE]² module: Manually add 1 mL of water to at least half the wells of a 2 mL per well, sterile, 96-well filter plate. Run the “MPE test.Agilent FP” method and follow the method prompt to manually load the filter plate onto the positive pressure module. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to push the water through the filter plate into waste. Run the positive pressure module at 448,159 Pa (65 psi) for 600 s to dry the columns in the filter plate. Follow the method prompt to manually unload the filter plate from the positive pressure module. Using Kimwipes, dry any water remaining on the bottom stage of the positive pressure module. Follow the method prompt to complete maintenance of the positive pressure module.

3.8 Liquid Class Development for Automated Methods Using a Gravimetric Approach

Perform liquid class development for any relevant buffers and reagents, if using an automated liquid handler for DNA extraction, purification, and quantitation.

1. Install the Liquid Verification Kit (LVK) on the deck of the automated liquid handler (*see Note 11*). If using the Hamilton Microlab STAR: Load the balance carrier onto the deck as indicated on the layout in the “LiquidClassVerificationKit_STAR” method. Connect the data cable to the control box and the balance. Connect the RS232-to-USB cable to the control box and the computer controlling the Hamilton STAR. Connect the power cable to the control box and a

power outlet. Place the balance on the STAR deck so it sits on the 4 pins on the carrier. Place the air-shield on top of the balance.

2. Set up a new experiment in the LVK application for a new liquid class: Open the LVK application on the Desktop and select “Create New Test.” Specify the run mode, instrument, and pipetting channels. Specify number of pipetting channels to test, tip size, tip type, liquid type, vial type, liquid class, density of the buffer or reagent, ambient temperature, and ambient humidity. Specify the number of pipetting loops, the dispense volume, tip handling after each pipetting cycle, pipetting settings for aspiration and dispensation, and acceptance criteria for precision and accuracy (*see Note 12*).
3. Run the experiment in the LVK application: With only the air-shield on the balance, press the “Start” button in the LVK application. Follow the prompts to load the holder and vessel containing the buffer or reagent, then press “Okay” to begin testing. Adjust the parameters of the designated custom liquid pipetting class until all pipetting criteria have been met (*see Note 13*).

3.9 Plate Culture

1. Remove LB-ampicillin agar culture plates from storage at 4 °C and equilibrate for at least 30 min in an incubator set to 30 °C (*see Note 14*).
2. Using a sterile loop(s) and working in a biosafety cabinet, touch the bacteria containing plasmid pEFR40019 in the bacterial stab or glycerol stock and streak it across the plate (*see Note 15*).
3. Incubate the culture plate at 30 °C for at least 16 h (*see Note 16*).
4. Store culture plates at 4 °C, wrapped in plastic wrap, for up to 1 month (*see Note 17*).

3.10 Liquid Culture

1. Remove plate culture from storage at 4 °C and equilibrate for at least 30 min in an incubator set to 30 °C.
2. Remove 100 mg/mL ampicillin stock from storage at –20 °C and allow to come to room temperature for at least 15 min.
3. Prepare media for the starter liquid culture: Working in a biosafety cabinet, pipet 12 mL of enriched M9 media with 4% glycerol and 2% casamino acids into a 15 mL centrifuge tube. Add 100 mg/mL ampicillin stock for a final concentration of 100 µg/mL. Mix the contents of the tube by vortexing for 5 s. Aliquot 5 mL of enriched M9 media with 4% glycerol, 2% casamino acids, and 100 µg/mL ampicillin into two 14 mL culture tubes (*see Note 18*).

4. Inoculate the starter liquid culture with cells containing pEFR40019 plasmid: Using a 10 μ L sterile filter pipette tip, select a single colony from the culture plate (*see Note 19*). Drop the tip with the colony into the culture tube, place the cap onto the tube, and swirl the tube to mix for 5 s. Repeat for all culture tubes.
5. Incubate the starter culture at 30 °C for at least 16 h with 300 RPM shaking (*see Note 20*).
6. Prepare large liquid culture: Working in a biosafety cabinet, pipet 60 mL of enriched M9 media with 4% glycerol and 2% casamino acids into a 250 mL Erlenmeyer culture flask (*see Note 21*). Add 100 mg/mL ampicillin stock for a final concentration of 100 μ g/mL. Mix the contents of the flask by swirling it for 5 s. Add 60 μ L of starter liquid culture, place the cap on the flask, and swirl the flask for 5 s to mix the contents.
7. Incubate the large liquid culture at 30 °C with 300 RPM shaking for at least 16 h (*see Note 22*).

3.11 Pellet Cell Culture

1. Briefly cold shock the cells to stop growth by placing the culture flask into 4 °C for 15 min.
2. Aliquot the cell culture in 2.0 mL microcentrifuge tubes or 96-well deep well plate(s) by adding 1.8 mL of culture per tube or well (*see Note 23*).
3. Pellet the cells in tubes or plates by centrifugation at $4500 \times g$ for 15 min (*see Note 24*).
4. Discard the supernatant from the tubes by inverting the tubes over a waste container. Alternatively, aspirate the supernatant from each well in the 96-well plate(s) using an automated liquid handler (*see Note 25*).
5. Store the pelleted cells for at least 16 h and up to 1 week at -20 °C (*see Note 26*).

3.12 Manual DNA Extraction with Filter Columns: QIAprep Spin Miniprep Kit, or its Equivalent

1. Prepare your work area (*see Note 27*): In the biosafety cabinet, prepare a storage rack with labeled QIAprep 2.0 spin columns, a storage rack with labeled 1.5 mL DNA LoBind tubes for DNA elution, an ice bath, a mini vortex mixer, a dry bath at 65 °C for warming the water, and a waste container. Use a benchtop centrifuge for all centrifugation steps.
2. Remove the pelleted cells in tubes from storage at -20 °C and allow to come to room temperature for at least 30 min.
3. Prepare buffers (*see Note 28*): Vortex the vial of RNase A for 5 s, and add the entire contents of the vial to Buffer P1 for a final concentration of 100 μ g/mL. Mix Buffer P1 by swirling the bottle, and store at 4 °C if not using immediately (*see Note 29*). Add the appropriate volume of (96–100) % ethanol

to Buffer PE as indicated on the bottle label. Check Buffer P2 and Buffer N3 for salt precipitation. Redissolve any precipitate by warming the buffers to 37 °C in a dry bath. Thoroughly chill Buffer N3 at 4 °C for at least 30 min (*see* **Note 30**). Keep N3 on ice during the extraction process. Aliquot nuclease-free water in 2.0 mL microcentrifuge tubes, and place into a 65 °C dry bath for the duration of the extraction.

4. Resuspend the pelleted cells by adding 250 µL of Buffer P1 to each tube (*see* **Note 31**), and vortexing each tube for at least 30 s (**Note 32**).
5. Lyse the cells by adding 250 µL of Buffer P2 to each tube, and mix the contents of each tube thoroughly by inverting the tubes. Do not allow the lysis to proceed for more than 4 min (*see* **Note 33**).
6. Neutralize the cells by adding 350 µL of cold Buffer N3 to each tube, and mix the contents of each tube thoroughly by inverting the tubes. Place tubes on ice for 5 min (*see* **Note 34**).
7. Pellet the cell debris by centrifuging the tubes at 18,000 × *g* for 30 min.
8. Transfer 800 µL of supernatant from each tube to the center of a QIAprep 2.0 spin column. Discard the culture tubes (*see* **Note 35**).
9. Centrifuge each spin column at 18,000 × *g* for 60 s and discard the flow through into a waste container.
10. Add 500 µL of Buffer PB to each spin column.
11. Centrifuge each spin column at 18,000 × *g* for 60 s and discard the flow through into a waste container.
12. Wash each spin column by adding 750 µL of Buffer PE to each spin column.
13. Centrifuge each spin column at 18,000 × *g* for 60 s and discard the flow through into a waste container.
14. Dry each spin column by centrifugation at maximum speed for 60 s.
15. Elute the DNA: Place each spin column into a 1.5 mL DNA LoBind tube. Add 55 µL of warm nuclease-free water to the center of each spin column. Incubate the spin columns at room temperature for 5 min. Centrifuge each spin column at 18,000 × *g* for 60 s. Discard the spin columns. Store the eluted DNA at 4 °C for up to 1 week (*see* **Note 36**).

3.13 Automated DNA Extraction with Filter Plates: QIAprep 96 Turbo Miniprep Kit, or its Equivalent

Use this method to extract DNA using filter plates with an automated liquid handler as an alternative to manual DNA extraction using filter columns (Subheading 3.12). All liquid handling, plate movement, and lid movement steps are automated unless otherwise specified (*see* **Note 37**). Always perform the daily maintenance of

the automated liquid handler and positive pressure module (Subheading 3.7) ahead of performing this method. Use custom liquid classes developed for handling the buffers and reagents specific to this method (Subheading 3.8).

1. Remove a 96-well plate with pelleted cells from storage at -20°C , and allow to come to room temperature for at least 30 min. Remove the foil seal and discard, but keep the plate covered with the lid. Load the covered 96-well plate with pelleted cells onto the deck of the liquid handler.
2. Prepare buffers (*see Note 28*): Vortex the vial of RNase A for 5 s, and add the entire contents of the vial to Buffer P1 for a final concentration of $100\text{ }\mu\text{g/mL}$. Mix Buffer P1 by swirling the bottle, and store at 4°C if not using immediately (*see Note 29*). Add the appropriate volume of (96–100) % ethanol to Buffer PE as indicated on the bottle label. Check Buffer P2 and Buffer N3 for salt precipitation. Redissolve any precipitate by warming the buffers to 37°C in a dry bath. Thoroughly chill Buffer N3 at 4°C for at least 30 min (*see Note 30*). Aliquot nuclease-free water into a 15 mL centrifuge tube, and place into a 65°C dry bath for at least 30 min.
3. Prepare deck of automated liquid handler (*see Note 38*): Add 15 mL of Buffer P1 with RNase A to column 1 of a 6-column reservoir (BufferRes 1), cover the reservoir with a sterile lid, and return Buffer P1 with RNase A to storage at 4°C . Add 20 mL of Buffer P2 to each column of a 2-column reservoir (BufferRes 2), and cover the reservoir with a sterile lid. Add 25 mL of Buffer N3 to each column of a 2-column reservoir (BufferRes 3), and cover the reservoir with a sterile lid. Store BufferRes 3 at 4°C until prompted by the automation method to add it to the deck of the liquid handler (**step 5** below). Add 100 mL of Buffer PB to column 1 and 100 mL of Buffer PE to column 2 of a 2-column reservoir (BufferRes 4). Cover BufferRes 4 with a sterile lid. Load BufferRes 1, BufferRes 2, BufferRes 4, an empty 6-column reservoir with lid (BufferRes 9), an empty storage plate with lid, and an empty DNA LoBind plate with lid onto the deck of the liquid handler. Place the filter plate on top of the binding plate onto the deck of the liquid handler. Load the pipette tips for the automated method (*see Note 39*).
4. Resuspend the pelleted cells: Add $200\text{ }\mu\text{L}$ of Buffer P1 to each well of the 96-well plate containing pelleted cells, and resuspend the cells by pipetting up and down (*see Note 40*). Transfer resuspended cells to the storage plate. Transfer the storage plate to the heater-shaker module.
5. Lyse the cells by adding $250\text{ }\mu\text{L}$ of Buffer P2 to each well of the storage plate, and mix the contents of the wells by shaking the

plate at 90 RPM for 120 s (*see Note 41*). Follow the method prompt to manually load BufferRes3 with cold Buffer N3 onto the deck. Do not allow the lysis to proceed for more than 4 min.

6. Neutralize the cells by adding 350 μ L of cold Buffer N3 to each well of the storage plate, and mix the contents of the wells by shaking the plate at 90 RPM for 120 s (*see Note 41*). Follow the method prompt to manually load the filter and binding plates onto the positive pressure module. Transfer the contents of the storage plate into the filter plate (*see Note 42*), and discard the storage plate.
7. Clear the lysate: Incubate the filter plate for 5 min to allow the cell debris to rise to the top of the wells. Run the positive pressure module at 34,473.8 Pa (5 psi) for 420 s, followed by 448,159 Pa (65 psi) for 60 s to pass the lysate through the filter plate into the binding plate (*see Note 43*). Follow the method prompt to manually discard the filter plate, leaving just the binding plate on the positive pressure module. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to pass the cleared lysate through the binding plate to waste.
8. Add 900 μ L of Buffer PB to each well of the binding plate. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to pass the buffer through the binding plate to waste.
9. Wash the DNA with Buffer PE: Add 900 μ L of Buffer PE to each well of the binding plate. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to pass the buffer through the binding plate to waste.
10. Run the positive pressure module at 448,159 Pa (65 psi) for 600 s to dry the columns of the binding plate (*see Note 43*).
11. Elute the DNA: Follow the method prompt to manually unload the binding plate from the positive pressure module, and place the binding plate on top of the DNA LoBind plate (*see Note 44*). Follow the method prompt to manually pour 15 mL of warm nuclease-free water into column 1 of BufferRes 9. Add 110 μ L of warm nuclease-free water to each well of the binding plate. Follow the method prompt to manually transfer the binding plate on top of the DNA LoBind plate to the positive pressure module. Incubate the plates for 5 min. Run the positive pressure module at 448,159 Pa (65 psi) for 600 s to elute the DNA from the binding plate into the DNA LoBind plate. Follow the method prompt to manually discard the binding plate and transfer the DNA LoBind plate to its storage location on the deck of the liquid handler. Cover the DNA LoBind plate, and store the eluted DNA at 4 °C for up to 1 week (*see Note 36*).

**3.14 Manual DNA
Extraction
with Magnetic Beads:
Mag-Bind Ultra Pure
Plasmid DNA 96 Kit,
or its Equivalent**

1. Prepare your work area (*see Note 27*): In the biosafety cabinet, prepare a storage rack with labeled 2.0 mL microcentrifuge tubes, a storage rack with labeled 1.5 mL DNA LoBind tubes for DNA elution, an ice bath, a mini vortex mixer, magnetic separation rack(s), a dry bath at 65 °C for warming the water, a dry bath at 75 °C for drying the magnetic particles, and a waste container.
2. Remove the pelleted cells in tubes from storage at –20 °C, and allow to come to room temperature for at least 30 min.
3. Prepare buffers: Vortex the vial of RNase A for 5 s, and add the entire contents of the vial to Solution I. Mix Solution I by swirling the bottle, and store at 4 °C if not using immediately (*see Note 45*). Add the appropriate volume of 100% ethanol to VHB Buffer as indicated on the bottle label. Add the appropriate volume of 100% ethanol to SPM Wash Buffer as indicated on the bottle label. Check Solution II and N3 Buffer for salt precipitation. Redissolve any precipitate by warming the buffers to 37 °C in a dry bath. Thoroughly chill N3 at 4 °C for at least 30 min. Keep N3 on ice during the extraction process (*see Note 30*). Remove Mag-Bind Particles RQ from storage at 4 °C, and allow to come to room temperature for at least 30 min. Vortex the particles for 60 s to ensure contents are thoroughly resuspended. Aliquot 1 mL of well-mixed particles into a 2.0 mL microcentrifuge tube, and return the stock bottle of particles to storage at 4 °C. Aliquot nuclease-free water in 2.0 mL microcentrifuge tubes and place into a 65 °C dry bath for the duration of the extraction.
4. Resuspend the pelleted cells by adding 250 µL of Solution I to each tube, and vortexing the tubes for at least 30 s (*see Notes 31 and 32*).
5. Lyse the cells by adding 250 µL of Solution II to each tube, and mix the contents of each tube thoroughly by inverting the tubes. Do not allow the lysis to proceed for more than 4 min (*see Note 33*).
6. Neutralize the cells by adding 125 µL of cold N3 Buffer to each tube, and mix the contents of each tube thoroughly by inverting the tubes. Place tubes on ice for 5 min (*see Note 34*).
7. Pellet the cell debris by centrifuging the tubes at $18,000 \times g$ for 30 min in a benchtop centrifuge.
8. Transfer 500 µL of supernatant from each tube containing cell debris into a 2.0 mL microcentrifuge tube (*see Note 46*).
9. Add ETR Binding buffer and Mag-Bind Particles RQ: Add 500 µL of ETR Binding Buffer to each tube. Vortex the aliquot of Mag-Bind particles for 10 s to ensure particles are

resuspended, and add 30 μL of particles to each tube (*see Note 47*). Vortex each tube for 10 s to ensure lysate, binding buffer, and particles are fully mixed. Incubate the tubes at room temperature for 5 min. Place the tubes in the magnetic separation rack, and incubate the tubes for 5 min to clear the particles. Aspirate the clear supernatant without disturbing the particles and discard into a waste container. Remove the tubes from the magnetic separation rack.

10. Wash DNA with ETR Wash Buffer: Add 500 μL ETR Wash Buffer to each tube. Vortex each tube for 6 s to completely resuspend the magnetic particles in solution. Place the tubes in the magnetic separation rack, and incubate for 90 s to clear the particles. Aspirate the clear supernatant without disturbing the particles and discard into a waste container. Remove the tubes from the magnetic separation rack.
11. Wash DNA with VHB Buffer: Add 700 μL VHB Buffer to each tube. Vortex each tube for 3 s to completely resuspend the magnetic particles in solution. Place the tubes in the magnetic separation rack and incubate for 90 s to clear the particles. Aspirate the clear supernatant without disturbing the particles and discard into a waste container. Remove the tubes from the magnetic separation rack.
12. Repeat **step 11** for a total of two washes with the VHB Buffer (*see Note 48*).
13. Wash DNA with SPM Wash Buffer: Add 700 μL SPM Wash Buffer to each tube. Vortex each tube for 3 s to completely resuspend the magnetic particles in solution. Place the tubes in the magnetic separation rack, and incubate for 90 s to clear the particles. Aspirate the clear supernatant without disturbing the particles and discard into a waste container (*see Note 49*). Remove the tubes from the magnetic separation rack.
14. Dry the magnetic particles: Place each tube into the dry bath at 75 °C for 7 min. Do not over dry the particles (*see Note 50*). Immediately remove the tubes from the dry bath and proceed to the next step.
15. Elute the DNA: Add 60 μL of warm nuclease-free water to each tube (*see Note 51*). Vortex each tube for 6 s to completely resuspend the magnetic particles in solution. Incubate the tubes at room temperature for 5 min. Place the tubes in the magnetic separation rack, and incubate for 90 s to clear the particles. Using wide-bore filter pipette tips and pipetting slowly (*see Note 52*), aspirate the cleared supernatant containing DNA from each tube into a 1.5 mL DNA LoBind tube. Store the eluted DNA at 4 °C for up to 1 week (*see Note 36*).

**3.15 Automated DNA
Extraction
with Magnetic Beads:
Mag-Bind Ultra Pure
Plasmid DNA 96 Kit,
or its Equivalent**

Use this method to extract DNA using magnetic particles with an automated liquid handler as an alternative to manual DNA extraction using magnetic particles (Subheading 3.14). All liquid handling, plate movement, and lid movement steps are automated unless otherwise specified (*see* **Note 37**). Always perform the daily maintenance of the automated liquid handler and positive pressure module (Subheading 3.7) ahead of performing this method. Use custom liquid classes developed for handling the buffers and reagents specific to this method (Subheading 3.8).

1. Remove a 96-well plate with pelleted cells from storage at -20°C , and allow to come to room temperature for at least 30 min. Remove the foil seal and discard, but keep the plate covered with the lid. Load the covered 96-well plate with pelleted cells onto the deck of the liquid handler.
2. Prepare buffers and reagents: Vortex the vial of RNase A for 5 s, and add the entire contents of the vial to Solution I. Mix Solution I by swirling the bottle, and store at 4°C if not using immediately (*see* **Note 45**). Add the appropriate volume of 100% ethanol to VHB Buffer as indicated on the bottle label. Add the appropriate volume of 100% ethanol to SPM Wash Buffer as indicated on the bottle label. Check Solution II and N3 Buffer for salt precipitation. Redissolve any precipitate by warming the buffers to 37°C in a dry bath. Thoroughly chill N3 at 4°C for at least 30 min (*see* **Note 30**). Remove Mag-Bind Particles RQ from storage at 4°C , and allow to come to room temperature for at least 30 min. Vortex the particles for 60 s to ensure contents are thoroughly resuspended. Aliquot 500 μL of well-mixed particles into five 1.5 mL microcentrifuge tubes. Return stock bottle of particles to storage at 4°C . Aliquot nuclease-free water into a 15 mL centrifuge tube, and place into a 65°C dry bath for at least 30 min.
3. Prepare deck of automated liquid handler (*see* **Note 38**): Add 15 mL of Solution I with RNase A to column 2, 30 mL of ETR Binding Buffer to column 5, and 30 mL of ETR Wash Buffer to column 6 of a 6-column reservoir (BufferRes 1). Cover BufferRes1 with a sterile lid, and return Solution I with RNase A to storage at 4°C . Add 20 mL of Solution II to each column of a two-column reservoir (BufferRes 5), and cover the reservoir with a sterile lid. Add 15 mL of N3 Buffer to each column of a two-column reservoir (BufferRes 6), and cover the reservoir with a sterile lid. Store BufferRes 6 at 4°C until prompted by the automation method to add it to the deck of the liquid handler (**step 5** below). Add 40 mL of VHB Buffer to columns 1 and 2, and 40 mL SPM Wash Buffer to column 6 of a 6-column reservoir (BufferRes 7). Cover BufferRes 7 with a

sterile lid. Load BufferRes 1, BufferRes 5, BufferRes 6, BufferRes7, an empty 6-column reservoir with lid (BufferRes 9), two empty storage plates with lids (preparation plate and lysate plate), an empty single cavity, 12-column reservoir with lid (waste plate), an empty DNA LoBind plate with lid, the universal magnetic plate, and the 32-tube carrier with 1.5 mL tube inserts onto the deck of the liquid handler. Place the filter plate on top of a deep well plate onto the deck of the liquid handler. Load the pipette tips for the automated method (*see Note 39*).

4. Resuspend the pelleted cells with the automated liquid handler: Add 200 μ L of Solution I to each well of the 96-well plate containing pelleted cells, and resuspend the cells by pipetting up and down (*see Note 40*). Transfer resuspended cells to the preparation plate. Transfer the preparation plate to the heater-shaker module.
5. Lyse the cells by adding 250 μ L of Solution II to each well of the preparation plate, and mix the contents of the wells by shaking the plate at 90 RPM for 120 s (*see Note 41*). Follow the method prompt to manually load BufferRes 6 with cold N3 Buffer onto the deck. Do not allow the lysis to proceed for more than 4 min.
6. Neutralize the cells by adding 125 μ L of cold N3 to each well the preparation plate, and mix the contents of the wells by shaking the plate at 90 RPM for 120 s (*see Note 41*). Follow the method prompt to manually load the filter plate on top of the deep well plate onto the positive pressure module. Transfer the contents of the preparation plate into the filter plate, and discard the preparation plate (*see Notes 42 and 53*).
7. Clear the lysate: Incubate the filter plate for 5 min to allow the cell debris to rise to the top of the wells. Run the positive pressure module at 34,473.8 Pa (5 psi) for 420 s, followed by 448,159 Pa (65 psi) for 60 s to pass the lysate through the filter plate into the deep well plate (*see Note 43*). Follow the method prompt to manually discard the filter plate, and move the deep well plate to its storage location on the deck of the liquid handler.
8. Transfer 500 μ L of cleared lysate from each well of the deep well plate into the corresponding wells of the lysate plate. Discard the deep well plate.
9. Add ETR Binding buffer and Mag-Bind Particles RQ: Add 500 μ L of ETR Binding Buffer to each well of the lysate plate. Follow the method prompt to manually vortex the tubes containing the magnetic particles for 10 s ensuring particles are well mixed. Load the tubes into the 32-tube carrier, and open the tube lids. Add 30 μ L of particles to each well of the lysate plate, and resuspend the contents of the lysate plate

by pipetting (*see* **Note 54**). Transfer the lysate plate to the magnetic plate, and incubate for 10 min to clear the particles. Aspirate the cleared supernatant without disturbing the particles and discard into the waste plate (*see* **Note 55**). Transfer the lysate plate from the magnetic plate to the heater-shaker module.

10. Wash DNA with ETR Wash Buffer: Add 500 μ L of ETR Wash buffer to each well of the lysate plate, and resuspend the contents of the lysate plate by pipetting and shaking (*see* **Note 56**). Transfer the lysate plate to the magnetic plate, and incubate for 120 s to clear the particles. Aspirate the cleared supernatant without disturbing the particles, and discard into the waste plate. Transfer the lysate plate from the magnetic plate to the heater-shaker module.
11. Wash DNA with VHB Buffer: Add 700 μ L of VHB buffer to each well of the lysate plate, and resuspend the contents of the lysate plate by pipetting and shaking (*see* **Note 56**). Transfer the lysate plate to the magnetic plate, and incubate for 120 s to clear the particles. Aspirate the cleared supernatant without disturbing the particles, and discard into the waste plate. Transfer the lysate plate from the magnetic plate to the heater-shaker module.
12. Repeat **step 11** for a total of 2 washes with the VHB Buffer (*see* **Note 48**).
13. Wash DNA with SPM Wash Buffer: Add 700 μ L of SPM Wash Buffer to each well of the lysate plate, and resuspend the contents of the lysate plate by pipetting and shaking (*see* **Note 56**). Transfer the lysate plate to the magnetic plate, and incubate for 120 s to clear the particles. Aspirate the cleared supernatant without disturbing the particles, and discard into the waste plate. Transfer the lysate plate from the magnetic plate to the heater-shaker module.
14. Dry the magnetic particles (*see* **Note 57**): Dry the lysate plate on the heater-shaker module at 75 °C for 7 min. Do not over dry the particles (*see* **Note 50**). Follow the method prompt to manually pour 15 mL of warm nuclease-free water into column 3 of BufferRes 9.
15. Elute the DNA: Add 70 μ L of warm nuclease-free water to each well of the lysate plate, and incubate the plate for 5 min (*see* **Note 58**). Resuspend the contents of the lysate plate by shaking at 900 RPM for 60 s. Transfer the lysate plate to the magnetic plate, and incubate for 120 s to clear the particles. Aspirate the cleared supernatant without disturbing the particles and transfer to the DNA LoBind plate. Store the eluted DNA at 4 °C for up to 1 week (*see* **Note 36**).

3.16 Manual DNA Purification with Filter Columns: PureLink PCR Purification Kit, or its Equivalent

1. Prepare your work area (*see Note 27*): In the biosafety cabinet, prepare a storage rack with labeled PureLink PCR spin columns with collection tubes, a storage rack with labeled 1.5 mL DNA LoBind tubes for mixing extracted DNA with Binding Buffer B2, a storage rack with labeled 1.5 mL DNA LoBind tubes for elution, and a waste container (*see Note 24*). Use a benchtop centrifuge for all centrifugation steps.
2. Remove extracted DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min.
3. Prepare buffers: Add the appropriate volume of 100% isopropanol to B2 Binding Buffer as indicated on the bottle label. Add the appropriate volume of (96–100) % ethanol to W1 Wash Buffer as indicated on the bottle label. Aliquot nuclease-free water in 2.0 mL microcentrifuge tubes and place into 65 °C dry bath for the duration of the purification.
4. Combine extracted DNA with Binding Buffer B2: Using wide-bore filter pipette tips and pipetting slowly, transfer 50 µL of extracted DNA from each tube of extracted DNA into a 1.5 mL DNA LoBind tube (*see Note 41*). Return remaining extracted DNA to storage at 4 °C. Add 200 µL of Binding Buffer B2 to each tube, and mix thoroughly by inverting the tubes 10× (*see Note 28*). Using wide-bore filter pipette tips and pipetting slowly, add the contents of each tube to the center of a PureLink PCR spin column with collection tube.
5. Centrifuge each spin column with collection tube at $10000 \times g$ for 60 s and discard the flow through into a waste container.
6. Add 650 µL of Wash Buffer W1 to each spin column with collection tube.
7. Centrifuge each spin column with collection tube at $10,000 \times g$ for 60 s and discard the flow through into a waste container.
8. Dry each spin column with collection tube by centrifugation at maximum speed for 3 min. Discard the collection tube attached to each spin column.
9. Elute the DNA: Place each spin column into a clean 1.5 mL DNA LoBind tube. Add 50 µL of warm nuclease-free water to the center of each spin column. Incubate the spin columns at room temperature for 5 min. Centrifuge each spin column at maximum speed for 2 min. Discard the spin column. Store the eluted DNA at 4 °C for up to 1 week (*see Note 33*).

3.17 Automated DNA Purification with Filter Plates: PureLink Pro 96 PCR Purification Kit, or its Equivalent

Use this method to purify extracted DNA using filter plates with an automated liquid handler as an alternative to manual DNA purification using filter columns (Subheading 3.16). All liquid handling, plate movement, and lid movement steps are automated unless otherwise specified (*see Note 37*). Always perform the daily

maintenance of the automated liquid handler and positive pressure module (Subheading 3.7) ahead of performing this method. Use custom liquid classes developed for handling the buffers and reagents specific to this method (Subheading 3.8).

1. Remove 96-well DNA LoBind plate(s) with extracted DNA (prepared in Subheadings 3.13 or 3.15) from storage at 4 °C, and allow to come to room temperature for at least 15 min. Load the DNA LoBind plate onto the deck of the liquid handler.
2. Prepare buffers: Add the appropriate volume of 100% isopropanol to B2 Binding Buffer as indicated on the bottle label. Add the appropriate volume of (96–100) % ethanol to W1 Wash Buffer as indicated on the bottle label. Aliquot nuclease-free water into a 15 mL centrifuge tube, and place into a 65 °C dry bath for at least 30 min.
3. Prepare deck of automated liquid handler: Add 25 mL of B2 Binding Buffer to column 1, and 75 mL of W1 Wash Buffer to column 2 of a 2-column reservoir (BufferRes 8). Cover BufferRes 8 with a sterile lid. Load BufferRes 8, an empty 6-column reservoir with lid (BufferRes 9), two empty DNA LoBind plates with lids (preparation plate and elution plate), and the filter plate onto the deck of the liquid handler. Load the pipette tips for the automated method (*see* **Note 39**).
4. Combine extracted DNA with Binding Buffer B2: Transfer 50 µL of extracted DNA from the DNA LoBind plate(s) to each well of the preparation plate (*see* **Note 59**). Add 200 µL of Binding Buffer B2 to each well of the preparation plate. Mix the contents of preparation plate by shaking at 300 RPM for 120 s. Follow the method prompt to manually load the filter plate onto the positive pressure module. Transfer the contents of the preparation plate into the filter plate, and discard the preparation plate.
5. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to bind the DNA to the columns in the filter plate and pass the buffer to waste.
6. Wash the DNA with Wash Buffer W1: Add 600 µL of Wash Buffer W1 to each well of the filter plate. Run the positive pressure module at 275,790 Pa (40 psi) for 60 s to pass the buffer through the filter plate to waste.
7. Run the positive pressure module at 448,159 Pa (65 psi) for 600 s to dry the columns of the filter plate (*see* **Note 43**).
8. Elute the DNA: Follow the method prompt to manually unload the filter plate from the positive pressure module, and place the filter plate on top of the elution plate (*see* **Note 60**). Follow the method prompt to manually pour 15 mL of warm

nuclease-free water into column 5 of BufferRes 9. Add 60 μL of warm nuclease-free water to each well of the filter plate. Follow the method prompt to manually transfer the filter plate on top of the elution plate to the positive pressure module. Incubate the plates in the positive pressure module for 5 min. Run the positive pressure module at 448,159 Pa (65 psi) for 600 s to elute the DNA from the filter plate into the elution plate. Follow the method prompt to manually discard the filter plate and transfer the elution plate to its storage location on the deck of the liquid handler. Store the eluted DNA at 4 °C for up to 1 week (*see* **Note 36**).

3.18 Manual DNA Quantitation with Fluorometry: Quant-iT Broad-Range dsDNA Assay Kit, or its Equivalent

Always perform calibration for fluorometer for manual DNA quantitation (Subheading 3.4) ahead of taking any measurements.

1. Remove prepared DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min.
2. Remove Component A and Component B of the Quant-iT Broad-Range dsDNA assay kit from storage at 4 °C, and allow to come to room temperature for at least 30 min, protected from light.
3. Prepare the Quant-iT Broad-Range dsDNA working solution: Vortex Component A for 5 s, and spin down for 3 s. Keep Component A protect from light at all times.
4. In a 15 mL centrifuge tube, combine 33 μL of Component A with Component B to a final volume of 6.6 mL (*see* **Notes 8 and 61**). Vortex the tube for 10 s, and wrap in foil to protect from light. The working solution is stable for up to 3 h. Return Component A and Component B to storage at 4 °C.
5. Prepare DNA samples for quantitation: In Qubit assay tubes, add 5 μL of DNA to 195 μL of Quant-iT Broad-Range dsDNA working solution. Vortex the tubes for 5 s, and spin down for 3 s (*see* **Note 9**). Incubate the tubes for 2 min, protected from light. Return all stock prepared DNA to storage at 4 °C.
6. Measure DNA concentration in fluorometer (*see* **Notes 62 and 63**). For example, if using the Qubit Fluorometer 3.0, from the Home screen, select “DNA,” then “dsDNA Broad Range,” then “Read Samples.” Set the sample volume to 5 μL , and select the desired units for output sample concentration. Lift the chamber lid, insert the tube containing the DNA sample, close the chamber lid, and press “Read.” Record the measured DNA concentration. When sample reading is complete, lift the chamber lid, discard the tube that was just measured, insert the next DNA sample tube, close the chamber lid, and press “Read.” Record the measured DNA concentration. Repeat these steps until all DNA samples have been measured.

3.19 Automated DNA Quantitation with Fluorometry: Quant-iT Broad-Range dsDNA Assay Kit, or its Equivalent

Use this method to quantify the prepared DNA with an automated liquid handler and fluorescence plate reader as an alternative to manual DNA quantitation with fluorometry (Subheading 3.18). All liquid handling, plate movement, and lid movement steps are automated unless otherwise specified (*see Note 37*). Always perform the daily maintenance of the automated liquid handler and positive pressure module (Subheading 3.7) ahead of performing this method. Use custom liquid classes developed for handling the buffers and reagents specific to this method (Subheading 3.8). Always perform the manufacturer's calibration for fluorescence plate reader (Subheading 3.2) and the calibration for fluorescence plate reader for automated DNA quantitation (Subheading 3.3) ahead of taking any measurements.

1. Remove DNA LoBind plates with prepared DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min (*see Note 64*).
2. Remove Component A, Component B, and Component C λ DNA standards (0 ng/ μ L, 5 ng/ μ L, 10 ng/ μ L, 20 ng/ μ L, 40 ng/ μ L, 60 ng/ μ L, 80 ng/ μ L, and 100 ng/ μ L) of the Quant-iT Broad-Range dsDNA assay kit from storage at 4 °C, and allow to come to room temperature for at least 30 min, protected from light.
3. Using the software that came with the plate reader, build the imaging protocol and associated experiments for fluorometric quantitation of DNA with the Quant-iT Broad-Range dsDNA assay (*see Note 7* and Table 1).
4. Prepare buffers and reagents: Vortex Component A and all Component C λ DNA standards for 5 s, and spin down for 3 s. Keep Component A protect from light at all times. In a 250 mL Nalgene polyethylene terephthalate glycol (PETG) bottle or other disposable plastic container, combine 600 μ L of Component A with Component B to a final volume of 200 mL (*see Notes 8* and *65*). Mix the Quant-iT Broad-Range dsDNA working solution by swirling the bottle for 10 s, and wrap the bottle in foil to protect it from light. The working solution is stable for up to 3 h. Return Component A and Component B to storage at 4 °C.
5. Prepare deck of automated liquid handler: Transfer the Quant-iT Broad-Range dsDNA working solution to a black, single cavity, 12-column reservoir, and cover with a black sterile lid (BufferRes 10). Load all Component C λ DNA standards into a 32-tube carrier with 2.0 mL tube inserts. Load BufferRes 10, DNA LoBind plates containing prepared DNA, tube carrier with λ DNA standards, and up to four empty, black, 96-well plates with black lids (quantitation plates), onto the

deck of the liquid handler. Load the pipette tips for the automated method (*see* **Note 39**). Turn off any lights above the automated liquid handler.

6. Aliquot Quant-iT Broad-Range dsDNA working solution into the quantitation plate(s): Add 190 μL of working solution to each well in columns 1 and 12 of the quantitation plate(s), and 195 μL of working solution to each well in columns 2–11 of the quantitation plate(s) (*see* **Note 66**).
7. Add 10 μL of Component C λ DNA standards to each well in columns 1 and 12 of the quantitation plate(s) (*see* **Note 67**).
8. Add 5 μL of DNA from DNA LoBind plates to each well in columns 2–11 of the quantitation plate(s) (*see* **Note 67**).
9. Mix the contents of the quantitation plate(s) by shaking at 200 RPM for 120 s. Return all prepared DNA to storage at 4 °C.
10. Measure DNA concentration in the fluorescence plate reader: Remove the lid from quantitation plate, and load the plate into the fluorescence plate reader. Run the experiment on the plate reader, and when completed discard the measured quantitation plate. Repeat these steps until all quantitation plates have been measured.

3.20 Manual DNA Qualification with Spectrophotometry

Always perform calibration for spectrophotometer for manual absorbance measurements of DNA using spectrophotometry (Subheading 3.5) ahead of taking any measurements.

1. Remove prepared DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min.
2. Select the appropriate assay on the spectrophotometer. For example, if using the DeNovix, from the home screen select the “dsDNA” application.
3. Wash the sample stage and lid of the spectrophotometer with DI water, and wipe dry using Kimwipes.
4. Run a blank by adding 1 μL nuclease-free water to the sample stage, closing the lid, and pressing run. Open the lid, and use Kimwipes to clean the sample stage and lid.
5. Add 1 μL of DNA to the sample stage, close the lid, and press run. Record the measured DNA concentration, measured absorbance at 260 nm, ratio of absorbance measured at 260 nm to 230 nm, and ratio of absorbance measured at 260 nm to 280 nm. Open the lid, and use Kimwipes to clean the sample stage and lid.
6. Repeat **step 5** until all DNA samples have been measured.
7. Return all prepared DNA to storage at 4 °C.

3.21 Gel Electrophoresis of DNA Templates

1. Prepare your work area (*see Note 27*): In the biosafety cabinet, prepare a storage rack with labeled 200 μ L PCR tubes, 1.2% agarose, precast gels for electrophoresis, a mini vortex mixer, and a mini centrifuge.
2. Remove prepared DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min.
3. Prepare DNA samples for gel electrophoresis: Aliquot 15 μ L of nuclease-free water into 200 μ L PCR tubes; prepare one tube for each individual prepared DNA template. Add 5 μ L of DNA to each PCR tube. Mix the contents of the PCR tubes by gently tapping the bottom of each tube with your finger. Spin down each tube for 3 s.
4. Load the precast gel: Add 20 μ L of DNA ladder to lanes 1 and 12 of the gel. Add 20 μ L of each DNA sample from **step 2** above into all the other lanes. Any empty lanes can be filled with 20 μ L of nuclease-free water.
5. Run the precast gel for 45 min to ensure the lanes with DNA are well resolved, then image. Discard the gel or store at 4 °C for future imaging (*see Note 68*).

3.22 Cell-Free Protein Production: myTXTL Sigma 70 Master Mix, or its Equivalent

Always perform the manufacturer's calibration for fluorescence plate reader (Subheading 3.2) and the calibration for fluorescence plate reader for cell-free protein production (Subheading 3.6) ahead of taking any measurements.

1. Using the software that came with the plate reader, build the imaging protocol and associated experiments for fluorescence measurements of cell-free protein production (*see Note 7* and Table 2). Preheat the plate reader to 30 °C.
2. Prepare your work area (*see Note 27*): In the biosafety cabinet, prepare a storage rack with labeled 200 μ L PCR tubes, an ice bath, a mini vortex mixer, and a mini centrifuge.
3. Remove prepared DNA from storage at 4 °C, and allow to come to room temperature for at least 15 min.
4. Prepare reagents (*see Note 27*): Remove myTXTL Sigma 70 Master Mix and recombinant eGFP standard from storage in -80 °C, and defrost on ice, protected from light. Aliquot 1 mL of nuclease-free water into a 2.0 mL microcentrifuge tube, and store on ice. Aliquot 1 mL of 1 \times PBS into a 2.0 mL microcentrifuge tube, and store on ice.
5. Measure the concentration of recombinant eGFP using spectrophotometry: Select appropriate assay on the spectrophotometer. For example, if using the DeNovix, from the home screen select the "Fluoro Protein" application. In the "Sample Type List," select the "+" to bring up the "Add New Sample Type" menu. Fill in the sample name, the molecular mass (Da),

and the extinction coefficient ($M^{-1} \text{ cm}^{-1}$) (*see* **Note 69**). Select “OK” to save. Wash the sample stage and lid of the spectrophotometer with DI water, and wipe dry using Kimwipes. Run a blank by adding 1 μL $1\times$ PBS to the sample stage, closing the lid, and pressing run. Open the lid, and use Kimwipes to clean the sample stage and lid. Vortex the tube of recombinant eGFP for 5 s, and spin down for 3 s; keep the eGFP protected from light at all times. Add 1 μL of recombinant eGFP standard to the sample stage, close the lid, and press run. Record the measured eGFP concentration. Measure the concentration of recombinant eGFP four more times, for a total of five technical repeats. Return the recombinant eGFP to storage on ice, protected from light.

6. Calculate the mean concentration of recombinant eGFP standard using measurements from **step 5**. Calculate the volumes recombinant eGFP standard and $1\times$ PBS for each concentration of eGFP in the calibration curve: 0 $\mu\text{mol/L}$, 1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$, 20 $\mu\text{mol/L}$, and 30 $\mu\text{mol/L}$ (*see* **Note 70**).
7. Calculate volume of DNA and nuclease-free water to add to each cell-free sample for a total concentration of 5 nmol/L DNA per sample using the measured concentration of DNA from Subheading 3.18 (manual) or Subheading 3.19 (automated) (*see* **Note 71**).
8. Prepare blank samples in tubes (*see* **Note 72**): Aliquot 36 μL of nuclease-free water into three 200 μL PCR tubes. Aliquot 36 μL of $1\times$ PBS into three 200 μL PCR tubes. Store the PCR tubes on ice.
9. Prepare recombinant eGFP calibration curve in tubes: Vortex the stock recombinant eGFP for 5 s, and spin down for 3 s. Using volumes calculated in **step 6** above, prepare each concentration of recombinant eGFP in triplicate in 200 μL PCR tubes. For example, Table 3 details the volumes of the stock recombinant eGFP and $1\times$ PBS for each point on the calibration curve if the concentration of the stock recombinant eGFP is 30 $\mu\text{mol/L}$. Vortex the PCR tubes for 5 s, and spin down for 3 s. Keep the PCR tubes on ice and protected from light. Return any unused of recombinant eGFP standard to storage at -80°C .
10. Prepare the negative control in tubes (*see* **Note 73**): Vortex the tube(s) of myTXTL Sigma 70 Master Mix for 5 s, and spin down for 3 s. Aliquot 27 μL of myTXTL Master Mix into three 200 μL PCR tubes. Add 9 μL of nuclease-free water to each PCR tube. Vortex the PCR tubes for 5 s, and spin down for 3 s. Keep the myTXTL Master Mix and PCR tubes on ice and protected from light.

Table 3
Volumes for stock recombinant eGFP (reGFP) and 1× PBS for each point on a 6-point calibration curve, if the stock reGFP concentration is 30 μM. The precision of volumes in columns 2 through 4 is limited by the pipettor

reGFP calibration curve concentration (μmol/L)	Volume reGFP Stock (μL)	Volume 1× PBS (μL)	Total volume (μL)
0	0.0	36.0	36.0
1	1.2	34.8	36.0
5	6.0	30.0	36.0
10	12.0	24.0	36.0
20	24.0	12.0	36.0
30	36.0	0.0	36.0

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0 μM eGFP	0 μM eGFP	0 μM eGFP																					
B	1 μM eGFP	1 μM eGFP	1 μM eGFP																					
C	5 μM eGFP	5 μM eGFP	5 μM eGFP																					
D	10 μM eGFP	10 μM eGFP	10 μM eGFP																					
E	20 μM eGFP	20 μM eGFP	20 μM eGFP																					
F	30 μM eGFP	30 μM eGFP	30 μM eGFP																					
G	H2O Blank	H2O Blank	H2O Blank								0 μM eGFP	0 μM eGFP	0 μM eGFP											
H	1X PBS Blank	1X PBS Blank	1X PBS Blank								1 μM eGFP	1 μM eGFP	1 μM eGFP											
I	- Control	- Control	- Control								5 μM eGFP	5 μM eGFP	5 μM eGFP											
J											10 μM eGFP	10 μM eGFP	10 μM eGFP											
K											20 μM eGFP	20 μM eGFP	20 μM eGFP											
L											30 μM eGFP	30 μM eGFP	30 μM eGFP											
M											H2O Blank	H2O Blank	H2O Blank											
N											1X PBS Blank	1X PBS Blank	1X PBS Blank											
O											- Control	- Control	- Control											
P																								

Fig. 1 Example of the plate layout and locations of calibration wells, blank wells, and negative control wells for measurements of cell-free protein production in a 384-well plate. The cell-free samples can be added to any unused well of the plate

11. Prepare cell-free sample(s) in tubes: Vortex the tube(s) of myTXTL Sigma 70 Master Mix for 5 s and spin down for 3 s. Aliquot 27 μL of myTXTL Master Mix into 200 μL PCR tubes; prepare three tubes for each prepared DNA sample for triplicate replicates of each. Using the volumes of DNA and nuclease-free water calculated in **step 7** above, add the appropriate volume of DNA to each tube, and then the appropriate volume of nuclease-free water to each tube (*see Note 74*). Vortex the PCR tubes for 5 s, and spin down for 3 s. Keep the PCR tubes on ice and protected from light. Return any unused myTXTL Master Mix to storage at −80 °C. Return prepared DNA to storage at 4 °C.
12. Load cell-free samples into a 384-well plate: Pipet 10 μL into each well of a 384-well plate. For example, add the samples to the plate as shown in Fig. 1 with triplicates of the calibration curve, blanks, and negative control in 3 locations of the plate, and cell-free samples distributed across the rest of the plate. Seal the plate with a low autofluorescence seal, and spin down

the plate at $2500 \times g$ for 15 s to ensure all liquid is at the bottom of the wells. Immediately transfer the plate to a pre-heated plate reader and initiate the experiment (*see* **Note 75**).

4 Notes

1. We recommend using the pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 plasmid, referred to as pEFR40019 in this protocol, as a positive control for the myTXTL Sigma 70 Master Mix cell-free system. pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 was a gift from Vincent Noireaux (Addgene plasmid # 40019; RRID:Addgene 40,019 <http://n2t.net/addgene:40019>). We have found that the manual and automated protocols in this chapter yield up to 100 nmol/L and 70 nmol/L of DNA, respectively.
2. The LB agar needs to cool for at least 30 min at 55 °C to prevent the antibiotic from degrading upon addition.
3. Pour the LB-ampicillin agar until it just coats the bottom of the culture plate, then move to the next plate. Any remaining agar can be spread among all the plates evenly. Use a hand torch to remove bubbles by moving the flame across the surface of the liquid in each plate. If necessary, plates can be cooled quickly in the biosafety cabinet for 20 min with the lid off.
4. We have found some lots of casamino acid are more soluble than others. However, all lots should be at least soluble enough to prepare a 4% stock solution.
5. We used the Fluorescence Test Plate (BioTek Instruments Inc.) as the fluorescence calibration plate. Always wear clean, powder-free gloves when handling the fluorescence calibration plate to avoid leaving residue and particulates on the plate.
6. The fluorescence calibration plate may require regular recalibration by the manufacturer to ensure that the performance of the instrument is evaluated accurately. We recommend this recalibration is done annually.
7. Set the plate reader to the recommended temperature for the kit or reagent. Excitation and emission wavelengths are specific to the fluorescent reporter being assayed; this information is available from the product manual or manufacturer's website. Gain may need to be optimized after additional testing and should be identical for all subsequent experiments to ensure comparability. Autogain should always be disabled. Read speed can impact the precision of the measurement and may need to be optimized after additional testing. Delay in the plate movement corresponds to the time between the end of the plate carrier movement (plate is in the read position) and the

beginning of data acquisition. We typically set this to the shortest recommended time. Measurement per data point corresponds to the number of measurements the plate reader makes for each data point. The time to perform each measurement is on the order of milliseconds, and the value reported by the plate reader at each data point is the mean of all the measurements taken at that data point. Lamp energy affects processing time and measurement sensitivity and may need to be optimized after additional testing. Read height corresponds to the height of the probe based on the detection method and plate dimensions. Often this setting cannot be adjusted for applications involving reads made from the bottom of the plate.

8. The Quant-iT Broad-Range dsDNA assay kit manual recommends adding 100 μL of Component A to 20 mL of Component B when preparing the working solution for 100 reactions. However, we recommend being precise with the volumes of Component A and B to combine, in this case adding 100 μL of Component A to 19.9 mL of Component B, to maintain the 1:200 ratio of Component A to B as this leads to a more accurate measurement of DNA concentration and may affect reproducibility.
9. Component B of the Quant-iT Broad-Range dsDNA assay kit contains surfactants. Vigorous shaking or aggressive pipetting should be avoided when preparing the working solution, the DNA standards, and DNA samples as this will generate bubbles. Any bubbles in Qubit assay tubes will interfere with the fluorometric reading and may produce inaccurate results. If needed, spin down the tubes for a longer time to get rid of any bubbles, or remake the standard or sample if the bubbles persist.
10. The DNA in the NIST human DNA quantitation standard is prepared in 10 mmol/L Tris chloride and 0.1 mmol/L EDTA buffer. If working with a different DNA standard, run a blank sample in the spectrophotometer with the buffer or reagent in which the DNA standard is prepared.
11. There are several different methods for validating liquid transfers and verifying pipetting performance, accuracy, and repeatability, including gravimetry, fluorescence measurements, and absorbance measurements. The Liquid Verification Kit (Hamilton Robotics) uses the gravimetric approach to tune the pipetting of liquids of known density. If the density of a buffer or reagent is not listed in the Safety Data Sheet, it can be measured in the lab. To do this, we recommend working with larger volumes of (200–1000) μL to reduce user error, and measuring at least five different volumes, with three technical replicates per volume. Temperature of the reagent or buffer will affect its density.

12. We recommend testing all the pipetting channels that will be used for liquid handling in the automated method, with at least three technical replicates per channel, to fully verify and tune pipetting performance. We recommend an acceptance criterion for CV and R of (1–3) % for larger volumes of (100–1000) μL . We do not recommend using positive pressure displacement liquid handlers for pipetting volumes less than 5 μL , as we have found that pipetting precision and accuracy at these volumes can vary by as much as (50–150) %, even after optimization.
13. Liquid classes for Hamilton liquid handlers are created and modified using the Hamilton CO-RE Liquid Editor application. Specific parameters that can be optimized include flow rate ($\mu\text{L}/\text{s}$), mix rate ($\mu\text{L}/\text{s}$), air transport volume (μL), blow out volume (μL), swap speed (mm/s), settling time (s), over-aspirate volume (μL), clot retract height (mm), stop flow rate ($\mu\text{L}/\text{s}$), stop back volume (μL), pressure liquid level detection sensitivity, and max height different (mm) for both aspirate and dispense steps. For the methods described in this chapter, we recommend developing custom liquid classes for QIAprep buffers P1, P2, cold N3, PB, and PE, Mag-Bind buffers Solution I, Solution II, cold N3, ETR Binding Buffer, Mag-Bind Particles RQ, ETR Wash Buffer, VHB Buffer, and SPM Wash Buffer, PureLink buffers B2 and W1, warm nuclease-free water, and Quant-IT Broad-Range working solution and Component C λ DNA standards.
14. Equilibrating the culture plates for at least 30 min at 30 °C avoids temperature shocking the cells when they are streaked and may lead to faster growth.
15. Dip the loop into the bacterial stab or glycerol stock and streak the plate in a zig-zag pattern starting from one end of the plate and ending at the other; avoid puncturing the surface of the agar. If using glycerol stock, work quickly and avoid letting it defrost, as multiple freeze–thaw cycles will damage the cells and may affect growth. If streaking more than five plates, consider using several different aliquots of glycerol stock and working sequentially.
16. The pEFR40019 plasmid is optimized for growth at 30 °C. Growing culture containing this plasmid at 37 °C leads to very slow or poor growth (data not shown). If working with a different plasmid, check the manufacturer’s specification for growth conditions, including temperature, shaking, media, and antibiotic. We grow the pEFR40019 plasmid for 24 h to ensure the colonies are sufficiently large for inoculation with a 10 μL filter pipette tip. Always incubate the culture plates upside-down to prevent condensation from dripping onto the agar.

17. Limit storage of plate cell culture at 4 °C to 1 month, as using old colonies to prepare liquid culture may lead to poor growth.
18. We recommend preparing at least two starter liquid culture tubes for each large liquid culture to ensure working with the starter culture with the highest OD₆₀₀.
19. We use a 10 µL filter pipette tip to pick the colonies because the colony is clearly visible when it enters the tip. Use a fresh tip for each colony and work carefully to avoid puncturing the agar in the culture plate(s).
20. We typically grow starter liquid culture for 20 h to OD₆₀₀ (4.0 to 4.5) or cell densities of roughly (3.2×10^9 to 3.6×10^9) cells/mL. Check the OD₆₀₀ of the cells after overnight growth and extend growth time as necessary. Ensure the cap on the culture tube is not too tight to permit proper aeration. Experiment with shaking speed, and consider growing the cultures at an angle to increase growth rate and reduce growth time.
21. The ratio of the culture volume to flask volume should be at least (1 to 4) to permit proper aeration. For example, 60 mL of liquid culture should be grown in a 250 mL culture flask. 60 mL of liquid culture is enough to prepare 33 DNA samples, including five technical replicates for each manual extraction and purification methodology described in this protocol.
22. We typically grow large liquid culture for 24 h to OD₆₀₀ (9.0–10.0) or cell densities of roughly (7.2×10^9 to 8.0×10^9) cells/mL. Check the OD₆₀₀ of the cells after overnight growth and extend growth time as necessary. Experiment with shaking speed to increase growth rate and reduce growth time.
23. The liquid culture should be well-mixed prior to aliquoting to ensure all aliquots contain a comparable cell density. If working by hand, swirl the flask for 10 s to mix the cells on every fifth aspiration. If using an automated liquid handler, include a mixing step on aspiration; we aliquot 1.8 mL in two steps, and include five cycles of mixing at 800 µL.
24. If preparing a single 96-well plate of cells, use a counterbalance plate filled with water to balance the centrifuge. Prepare the counterbalance plate in advance by filling the wells with 1.8 mL of water. Store the counterbalance plate sealed at room temperature to prevent evaporation. In our case, we made sure the two plates were within 2 g to ensure proper centrifugation.
25. We found the best way to remove the supernatant without disturbing the cell pellet was to position the pipette tip at a fixed height just above the pellet and aspirate the total volume of supernatant in two separate steps of 1000 µL and 800 µL, respectively.

26. We found freezing the cell pellet overnight damages the cell membrane, improves lysis, and leads to higher yields of extracted DNA (data not shown). If working with 96-well plates, seal each plate containing pelleted cells with an aluminum foil seal and cover with a sterile polystyrene lid to prevent evaporation and dust or particulates from entering the wells.
27. We recommend all pipetting steps for DNA extraction, DNA purification, gel electrophoresis, assembly of cell-free samples, and loading of cell-free samples into 384-well plate(s) be performed in a biosafety cabinet to minimize the risk of contamination that may affect reproducibility.
28. The QIAprep Buffer P1, Buffer P2, and Buffer PE can be made in-house to reduce reagent cost. Prepare Buffer P1 (50 mmol/L Tris-HCl, 10 mmol/L EDTA, 100 µg/mL RNase A): Combine 50 mL of ultrapure 1 mol/L Tris-HCl, pH 8.0 (Thermo Fisher Scientific), 20 mL of 0.5 mol/L RNase-free EDTA, pH 8.0 (Thermo Fisher Scientific), and ultrapure water to a final volume of 1 L. Filter-sterilize using a bottle-top vacuum filter system. Add 100 mg/mL RNase A (QIAGEN) for a final concentration of 100 µg/mL, mix well, and store at 4 °C for up to 6 months. Prepare Buffer P2 (200 mmol/L NaOH, 1% SDS): Combine 40 mL of 5 mol/L NaOH (Millipore Sigma), 100 mL of 10% RNase-free SDS solution (Thermo Fisher Scientific), and ultrapure water to a final volume of 1 L. Filter-sterilize using a bottle-top vacuum filter system and store at room temperature for up to 6 months. Prepare Buffer PE (8 mmol/L Tris-HCl, pH 7.5, 98.4% ethanol): Combine 8 mL of ultrapure 1 mol/L Tris-HCl, pH 7.5 (Thermo Fisher Scientific) with 176 mL ultrapure water. Filter-sterilize using a bottle-top vacuum filter system. Add 200 proof ethanol (Fisher Scientific, Inc.) to a final volume of 1 L and store at room temperature for up to 6 months.
29. Store QIAprep Buffer P1 at 4 °C once RNase A has been added. If preparing this buffer in advance, allow the buffer to come to room temperature for at least 30 min before using.
30. We recommend neutralizing lysed cells with cold N3, because this helps the cell debris clump together (data not shown). Aliquot N3 in a 50 mL centrifuge tube in advance, and store at 4 °C for up to 12 h. Prolonged storage of N3 at 4 °C will cause the buffer to precipitate, and the N3 will need to be warmed to 37 °C for at least 30 min to redissolve the precipitate.
31. When working with more than five samples at a time, consider using a multichannel pipettor and pouring the buffers into sterile disposable reagent reservoirs (Vista Lab Technologies, Inc.) for ease of pipetting. This will decrease the time to complete each pipetting step and may reduce user error.

32. Pelleted cells should be fully resuspended prior to lysis. We recommend vortexing each tube for 30 s. If any cell clumps remain, pipet the contents of the tube up and down 10× with a 1000 µL pipettor set to 400 µL. Always process all samples in the same way to ensure comparability.
33. Limit the lysis step to 4 min to avoid the risk of degrading the DNA. Once the lysis buffer has been added, close the tube and invert it ten times to gently mix the contents of the tube. Avoid aggressive pipetting or vortexing as this can lead to degraded DNA. If working with many samples at once, lyse no more than ten tubes of cells at a time, then immediately neutralize the cells in those tubes before moving on to lysing and neutralizing the cells in the next ten tubes. We found the easiest way to invert multiple tubes simultaneously is to place the tubes into a storage rack, cover the tubes with another storage rack, and invert the rack–tube–rack stack ten times.
34. Once the neutralization buffer has been added, close the tube and invert it 20 times to gently mix the contents of the tube. Avoid aggressive pipetting or vortexing, as this can lead to degraded DNA. We found the easiest way to invert multiple tubes simultaneously is to place the tubes into a storage rack, cover the tubes with another storage rack, and invert the rack–tube–rack stack 20 times. Incubating the neutralized cells on ice for 5 min helps the cell debris clump together and rise to the top of the tube (data not shown).
35. Consider increasing centrifugation time by 10 min if the amount of supernatant is less than 800 µL. Alternatively, use a smaller volume of supernatant for subsequent steps, though this will reduce the concentration of eluted DNA. Always use the same volume of supernatant for all samples to ensure comparability.
36. We recommend eluting DNA into DNA LoBind tubes or plates, which are designed for long-term storage of nucleic acids and limit adhesion of DNA to the container walls. Teflon tubes or plates can also be used, but they are generally more expensive and available in larger volumes than practical for storing DNA solutions. We recommend eluting DNA in nuclease-free water instead of elution buffer. The constituents of elution buffers, such as EDTA and high concentrations of salt, may affect the buffering capacity of the cell-free system, whether purchased commercially or prepared in-house. Warm water rehydrates the filter column better than room temperature water and can lead to higher concentrations of eluted DNA (data not shown). Eluted DNA can be stored for up to 5 days at 4 °C without significant change in concentration (Fig. 2). However, after 1 month, DNA concentration can

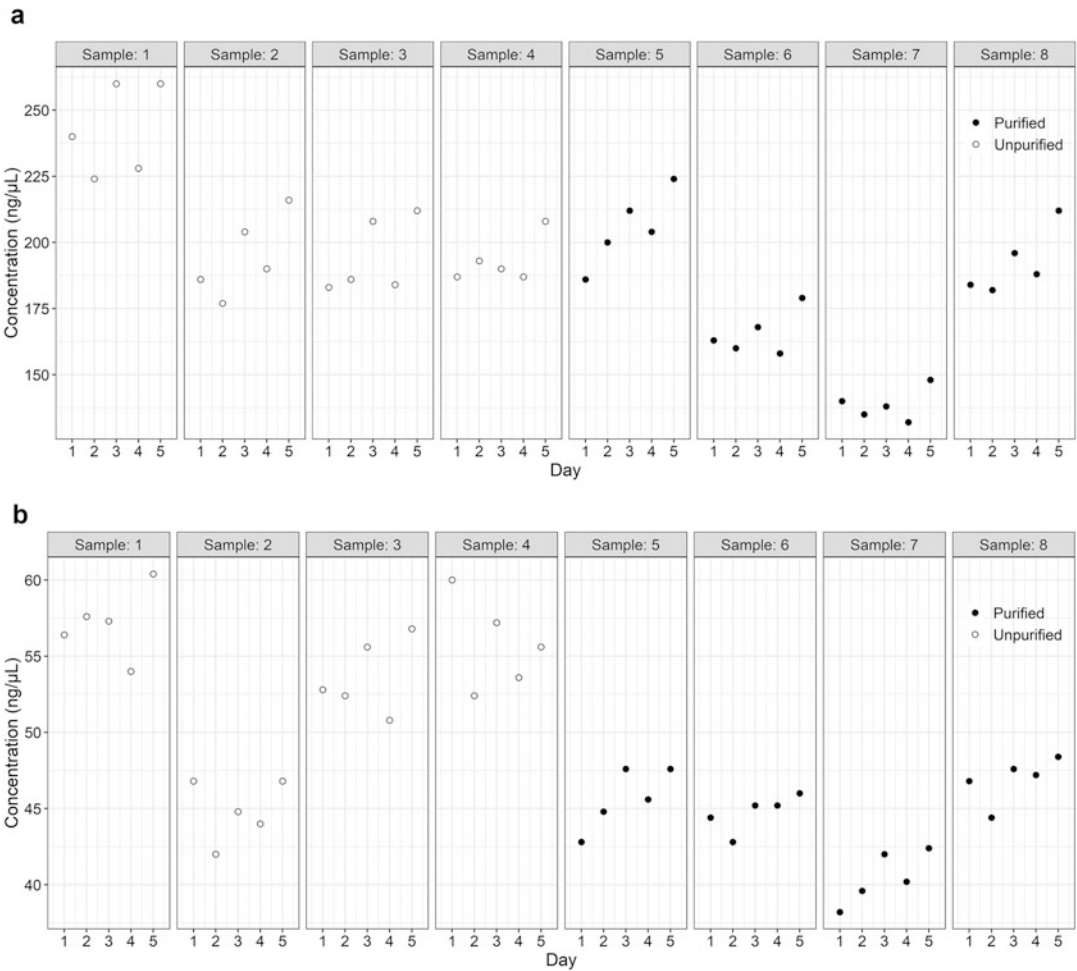


Fig. 2 DNA concentration measured daily over 5 days for eight DNA samples stored at 4 °C, where (a) is DNA extracted manually with filter columns and (b) is DNA extracted manually with magnetic particles. Samples 5 through 8 in both (a) and (b) were also purified with a filter column PCR purification kit. The data indicates that eluted DNA can be stored for up to 5 days at 4 °C without significant change in concentration. Each data point represents a single measurement. Note the difference in scale of the y axis in (a) and (b)

decrease by up to 30% (data not shown). For short-term storage of DNA in LoBind plates up to 4 h at room temperature, use sterile environmental lids, such as MicroClime lids (Labcyte), to prevent evaporation from the wells.

37. This method was developed for the Hamilton Microlab STAR with onboard [MPE]² (positive pressure) module and Thermoshake (heater-shaker) module and is available upon request from the authors. The STAR is housed in a BSL-1 enclosure comparable to a biosafety cabinet. Movement of plates to and from the positive pressure module are performed manually, due to the limitations of the hardware and the positioning of the modules on the STAR deck.

38. Work in a biosafety cabinet to add buffers and reagents to reservoirs to minimize the risk of contamination. Keep all plates and reservoirs covered with sterile lids when not in use, on the benchtop, in the biosafety cabinet, and on the deck of the automated liquid handler. Use the deck layout and user dialogs at the start of the method to verify the location of any pipette tips, plates, and reservoirs.
39. We recommend writing a separate method to automate pipette tip loading onto the deck of the liquid handler. In general, we use conductive tips that enable the use of the liquid level detection and liquid level following features of the liquid handler.
40. We add less resuspension buffer than recommended in the kit manual, because the automated removal of supernatant from deep well plates containing cell pellets leaves approximately 50 μL of media in each well. This ensures that the final volume of the resuspended cells is 250 μL per well. We resuspend cells using 300 μL tips, which have a smaller orifice than 1000 μL tips, and include a mixing step of 25 cycles to ensure the cells are fully resuspended. We use two aspirate-dispense steps to ensure the full volume of resuspended cells is transferred to the prep plate.
41. Lysis and neutralization buffers are added sequentially using the 96-channel pipetting head, to ensure that cells are lysed or neutralized in all wells at the same time, respectively. After the addition of lysis and neutralization buffers, respectively, cells are mixed by shaking instead of pipetting to minimize shearing the DNA. Shaking parameters, such as duration and RPM, can be optimized using a color indicator, such as LyseBlue reagent (QIAGEN), to help visualize sample mixing. In general, we recommend limiting the lysis step to 4 min to avoid the risk of degrading the DNA.
42. We recommend using wide-bore filter pipette tips and low flow rates for aspiration and dispensation, when transferring the lysate from preparation plate to filter plate to minimize shearing the DNA.
43. Pressure and duration parameters for pushing the buffers or reagents through the filter or binding plate using the positive pressure module will depend on the pore size of the columns in each plate and may require further optimization. When passing lysed cells through the filter plate, aim for clear lysate in the binding plate or deep well plate, cell debris in the filter plate, and avoid pressures that clog the columns in the filter plate. When drying a plate, avoid pressures and duration that over dry the filter column, as this may reduce the amount of extracted

DNA in the elution step. Any remaining drops at the end of the columns in either filter or binding plate can be dislodged by a short high pressure burst, such as 448,159 Pa (65 psi) for 60 s.

44. The fit between the binding plate and DNA LoBind plate may be tight. Ensure that the binding plate is inserted snugly into the DNA LoBind plate on all sides to avoid punching through the filter columns in the binding plate with pipette tips during dispensation of the water. We have found that rehydrating the columns in the QIAprep binding plate with 110 μ L per well yields approximately 60 μ L of eluted DNA solution from each well.
45. Store Solution I at 4 °C once RNase has been added. If preparing this buffer in advance, allow the buffer to come to room temperature for at least 30 min before using.
46. We recommend using 2.0 mL microcentrifuge tubes for DNA extraction with magnetic particles. The bottom of 2.0 mL tubes is less tapered as compared to 1.5 mL or 1.7 mL tubes. This leads to a tighter particle pellet when placed on the magnetic separation base, decreases the time to fully resuspend the particles during washing steps, and leaves more space to aspirate the supernatant without disturbing the particles during washing and elution steps.
47. Fully resuspend the magnetic particles prior to aliquoting into smaller volumes or adding to the DNA sample. This will ensure a comparable number of particles is added to each DNA sample and may improve reproducibility. When pipetting manually, resuspend the particles by pipetting up and down ten times on every fifth aspiration. Alternatively, include a mixing step on aspiration if using an automated liquid handler (*see Note 54*). We recommend adding 30 μ L of particles to each DNA sample, instead of 20 μ L as recommended in the kit manual, as this yields higher concentrations of eluted DNA (data not shown).
48. We recommend performing two consecutive washes with VHB Buffer, as this leads to higher concentrations of eluted DNA (Fig. 3).
49. Remove all the supernatant from the tube prior to drying, as this may contribute to how evenly the tubes dry and can affect reproducibility. First aspirate the supernatant using a 1000 μ L pipettor set to 1000 μ L, and then aspirate any remaining droplets at the bottom of the tube with a 200 μ L pipettor set to 200 μ L. The 200 μ L tips have a smaller orifice than the 1000 μ L tips and can better collect any leftover droplets at the bottom of the tube.
50. Limit the dry time to 7 min, as longer times lead to overdried magnetic particles that are difficult to resuspend and may yield reduced concentrations of eluted DNA (Fig. 3). The particles

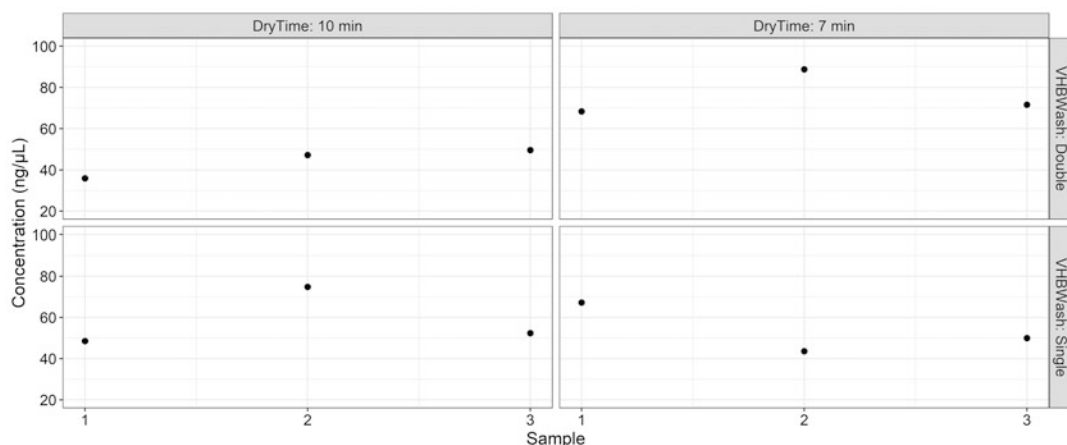


Fig. 3 Optimizing manual DNA extraction using magnetic particles, including the number of washes with VHB Wash Buffer and the time to dry the particle pellet. Each data point represents a single measurement

will change color as they dry from dark brown when wet, to light brown as they dry, to tan and crumbly when overdried.

51. Dispense the nuclease-free water directly over the particles during the elution step. This helps rehydrate the particles and leads to faster resuspension times. We found that rehydrating the particles with 60 μL of nuclease-free water yields 55 μL of eluted DNA solution.
52. We recommend using wide-bore filter pipette tips and pipetting slowly, whenever aspirating or dispensing DNA, to avoid shearing the plasmid. For automated liquid handling of DNA, the wide-bore filter tips were only available for large volumes (1000 μL), had poor pipetting precision and accuracy at small volume (5–10 μL), and were too wide for magnetic particle extraction methods without disturbing the particles during DNA elution.
53. Filter plates recommended by the kit manufacturer had very narrow round wells with loose tolerance in the spacing of the wells from plate to plate. This caused collisions between the wide-bore filter tips and the filter plate during the transfer of lysate from preparation plate to filter plate. Instead, we optimized the method for use with QIAprep filter plates for clearing the lysate of cell debris. In general, filter plates with square rather than round wells reduce the chances of collision between plate and wide-bore filter tips. Additional optimization of the positive pressure module parameters may be required based on the pore size of the columns in the filter plates.
54. We included ten cycles of mixing at 150 μL on every aspirate step from the tubes containing magnetic particles, to ensure that the particles were fully resuspended and that an equal

volume of particles was added to each well in the lysate plate. After the addition of ETR Binding buffer and magnetic particles, wide-bore filter tips were used to mix the contents of the lysate plate to minimize shearing the DNA.

55. We had the most success with aspirating supernatant without disturbing the magnetic particles from plates with wells that had square openings and round bottoms. When placed on the universal magnetic plate, the magnetic particles form a wide ring around the bottom of the well, leaving enough space to aspirate the supernatant from the bottom of each well.
56. We used a combination of mixing by pipetting and shaking to fully resuspend the particles. After the addition of ETR Wash Buffer, particles were resuspended by ten cycles of mixing at 400 μL per well, followed by shaking at 800 RPM for 60 s. After the addition of VHB Buffer, particles were resuspended by ten cycles of mixing at 600 μL per well, followed by shaking at 700 RPM for 60 s. After the addition of SPM Wash Buffer, particles were resuspended by ten cycles of mixing at 500 μL , followed by shaking at 700 RPM for 60 s. We suspect that the DNA on the particles was partially sheared by the pipetting steps and recommend further optimization of automated DNA extraction using magnetic particles to avoid this problem.
57. Preheat the heater-shaker module for 10 min at 75 $^{\circ}\text{C}$ prior to moving the lysate plate to the heater-shaker module for drying. This can be initiated during the previous step, in which the SPM supernatant is removed from the lysate plate.
58. Ideally, nuclease-free water should be dispensed directly over the dried particles to help rehydrate and resuspend them faster. If using a different magnetic plate that causes the magnetic particles to clump at the side of the wells, adjust the position of the tips during dispensation for side touch-off pipetting. We found that rehydrating the particles with 70 μL of warm nuclease-free water yields approximately 55 μL of eluted DNA solution. We used 1000 μL filter tips (*see Note 52*) and a custom liquid class that reduced the flow rate of aspiration and dispensation to 2 $\mu\text{L}/\text{s}$ to aspirate the DNA from the lysate plate and dispense into the DNA LoBind plate. This likely leads to some shearing of the DNA and requires further optimization.
59. We used 1000 μL filter tips (*see Note 52*) and a custom liquid class that reduced the flow rate of aspiration and dispensation to 2 $\mu\text{L}/\text{s}$ to aspirate the extracted DNA from the DNA LoBind plate and dispense into the preparation plate. This likely leads to some shearing of the DNA and requires further optimization. We used wide-bore filter tips to transfer the contents of the preparation plate into the filter plate.

60. Ensure that the binding plate is inserted snugly into the DNA LoBind plate on all sides to avoid punching through the columns in the filter plate with pipette tips during the dispensation of the water. We have found that rehydrating the columns in the filter plate with 60 μL of warm nuclease-free water per well yields approximately 35 μL of eluted DNA solution from each well.
61. The indicated volumes of Component A and B are for preparing enough working solution to measure 33 DNA samples.
62. Always leave the lid of the fluorometer closed when not in use, as this will prevent dust and particulates from entering the chamber and affecting the measurement. If necessary, use compressed air to gently blow any particulates from the chamber and a Kimwipes wetted with 70% ethanol to remove any stubborn particulates that may have accumulated inside.
63. Measure the concentration of prepared DNA no more than 1 day prior to using that DNA for cell-free protein production. This will help to ensure an accurate measurement of DNA concentration and volume to add to each cell-free sample.
64. This automated method can be used to quantify DNA samples prepared in tubes. Using wide-bore filter pipette tips and pipetting slowly, transfer the DNA from each tube into individual wells in a DNA LoBind plate and cover the plate with a sterile lid.
65. The indicated volumes of Component A and B are for preparing enough working solution to measure 200 DNA samples. There needs to be a minimum volume of working solution in BufferRes 10 to avoid triggering a low-volume error when aspirating from BufferRes 10 with the automated liquid handler.
66. Columns 1 and 12 are used to generate the calibration curve for DNA concentration. They are prepared by combining 190 μL of Quant-iT Broad-Range dsDNA working solution and 10 μL of Component C λ DNA standard in each well. Columns 2 through 11 are used to quantify the concentration of the prepared DNA samples. These columns are prepared by combining 195 μL of Quant-iT Broad-Range dsDNA working solution and 5 μL of the DNA sample in each well.
67. Adding mixing on the aspiration step when pipetting Component C λ DNA standards and DNA samples ensures the same amount of DNA is added to each well of the quantitation plates and helps reduce measurement variability (data not shown). We included 3 cycles of mixing at 45 μL for Component C λ DNA standards and 3 cycles of mixing at 30 μL for the DNA samples. We used 50 μL filter pipette tips (*see* **Note 52**) and a

custom liquid class that reduced the flow rate of aspiration, dispensation, and mixing to 2 $\mu\text{L/s}$. This likely leads to some shearing of the DNA and requires further optimization.

68. Precast gels can be stored at 4 °C for up to 1 week for further analysis, by first wrapping the lanes with Parafilm to prevent evaporation and then wrapping the whole gel in aluminum foil to protect it from light. Plasmids will often yield several bands, because they can exist in multiple conformations, such as uncoiled and supercoiled. Smears indicate broken, damaged, or contaminated DNA. Using broken, damaged, or contaminated DNA in cell-free reactions may lead to poor protein yields (data not shown).
69. Information on the molecular mass, extinction coefficient, and excitation and emission wavelengths is generally provided by the manufacturer in the calibration certificate. We recommend always checking the concentration of the recombinant fluorescent reporter in-house, as we have found some discrepancy in the concentration from lot to lot.
70. A template for calculating the molar concentration of recombinant eGFP, and volumes of eGFP and 1 \times PBS (Supplementary File 1) for making the calibration curve is available in the Electronic Supplementary Materials. We recommend at least a 5-point calibration curve that spans at least one order of magnitude in concentration above and below what the cell-free sample will produce. This may be limited by the stock concentration of the recombinant fluorescent reporter. We recommend making triplicate measurements of each point in the calibration curve in three separate locations on the cell-free plate, especially if significant variation across the length or width of the plate is possible.
71. A template for calculating the volume of DNA and water to add to each cell-free sample (Supplementary File 2), based on the measured concentration of the prepared solution of DNA and the minimal recommended DNA concentration of 5 nmol/L per sample, is available in the Electronic Supplementary Materials. We recommend making triplicate measurements of each cell-free sample to validate the performance of the cell-free reactions and calculate relevant statistical metrics such as mean, standard deviation, and uncertainty.
72. We recommend making triplicate measurements of each blank sample in three separate locations on the plate, especially if significant variation across the length or width of the plate is possible.
73. The negative control samples contain water in place of the DNA template. We recommend making triplicate measurements of any negative control sample in three separate

locations on the plate to validate the performance of the cell-free reactions and calculate relevant statistical metrics such as mean, standard deviation, and uncertainty. Keep all samples on ice to prevent the reactions from activating prematurely.

74. We recommend using an electronic pipettor with adjustable aspiration and dispensation flow rates for adding DNA solution to the cell-free sample(s). This minimizes the risk of shearing the DNA due to aggressive pipetting. Pay close attention to the order in which the cell-free samples are assembled, as this can affect performance. For the myTXTL Sigma 70 Master Mix, the Master Mix is added first, then the DNA, then the water. Other commercially available cell-free kits or in-house lysates may require the order to be reversed. Contact the manufacturer or whomever prepared the lysate for more information on the recommended assembly order for the reactions. Keep all samples on ice to prevent the reactions from activating prematurely.
75. We recommend using an adjustable well plate stand (Thomas Scientific) and an electronic multichannel pipettor with adjustable aspiration and dispensation flow rates for loading the 384-well plate(s) to minimize pipetting error. This ensures high-throughput loading of the plate, while minimizing aggressive pipetting of the cell-free samples. If measurements are delayed, the sealed plate can be stored on ice and wrapped in foil for up to 1 h. Spin down the plate at $2500 \times g$ for 15 s prior to loading it into the plate reader to ensure all liquid is at the bottom of the wells. If preparing a single 384-well plate of cell-free reactions, use a counterbalance plate filled with water to balance the centrifuge. This counterbalance plate can be prepared in advance by filling the wells with DI water. Store the counterbalance plate sealed at room temperature to prevent evaporation. In our case, we made sure the two plates were within 2 g to ensure proper centrifugation.

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Simple Extract Preparation Methods for *E. coli*-Based Cell-Free Expression

Alissa C. Mullin, Taylor Slouka, and Javin P. Oza

Abstract

Cell-free protein synthesis (CFPS) is a powerful platform for synthetic biology, allowing for the controlled expression of proteins without reliance on living cells. However, the process of producing the cell extract, a key component of cell-free reactions, can be a bottleneck for new users to adopt CFPS as it requires technical knowledge and significant researcher oversight. Here, we provide a detailed method for implementing a simplified cell extract preparation workflow using CFAI media. We also provide a detailed protocol for the alternative, 2x YPTG media-based preparation process, as it represents a useful benchmark within the cell-free community.

Key words Cell-free protein synthesis (CFPS), Cell-free expression (CFE), Synthetic biology, In vitro transcription/translation (TX-TL), Cell-free autoinduction (CFAI), 2xYTPG

1 Introduction

The cell-free protein synthesis (CFPS) platform has undergone significant development over the last 60 years and has become an essential component of the biotechnology portfolio, supporting a broad range of applications [1, 2]. CFPS, also referred to as in vitro transcription/translation (TX-TL), allows for the expression of a protein of interest without a reliance on living cells. Instead, CFPS utilizes cell extract which harnesses cells' transcription and translation machinery and their metabolic processes that support protein synthesis for use in vitro. The cell extract is stable at -80°C for extended periods of time, and when combined with a DNA or mRNA template encoding the gene of interest, the necessary cofactors, and energy reagents, protein synthesis can be initiated on-demand. This approach has numerous documented advantages, namely the production of toxic, complex, or otherwise burdensome proteins which would be detrimental to living cells [1]. The open nature of the platform has made it suitable for a vast array of

applications in the medicinal and biological fields, as improvements in the technology have led to advances in biomaterials synthesis [3, 4], point-of-care diagnostics [5], an expansion in the genetic code [3, 4, 6, 7], and better understanding of viruses and virus-like particles [8, 9]. Further innovations have enabled the manipulation of the cell-free redox environment, expanding the utility of CFPS to in vitro synthesis of proteins containing disulfide bonds [10–12]. CFPS has been utilized for the large scale and rapid synthesis of functional antibodies [12–14] as well as antibody–drug conjugates [15]. CFPS is also useful as an educational platform as it enables students to directly engage with the transcription and translation processes [16, 17]. The advancements that have led to these applications show the versatility of CFPS and its potential for further capabilities.

Given the broad utility of cell-free protein synthesis, there is growing interest among new researchers to learn how to effectively employ CFPS for their work. A common barrier for new CFPS users is the reproducible production of cell extracts. Since CFPS can be implemented using lysates from a variety of cell types, the resulting cell extract contains that cell type’s unique biochemical milieu [1]. Matching the cell extract with the desired application is important, however, unless the user requires specific metabolism or biochemical processes, we posit that *E. coli*-based extracts are a great starting point for most users interested in on-demand gene expression.

In recent years, significant efforts have been made in the scientific community to improve the process of producing cell extracts. The introduction of technologies such as sonication [18, 19] and bead beating for lysis [19] as well as baffled flasks for cell culturing [18, 20] have significantly improved the ease of producing cell extracts as well as the quality of extract. Most recently, we reported the development of an autoinduction media specifically optimized for cell-free protein synthesis, cell-free autoinduction (CFAI) media, which significantly reduces the amount of researcher experience and effort necessary for successful extract preparation [21]. The CFAI media alleviates the need for a researcher to monitor the culture for induction of T7 RNAP, as the ratio of sugars in the media causes the induction of the lac operon by the lactose to only occur once the log phase of cell growth has been reached [22]. Importantly, the CFAI media formulation also supports robust cellular metabolism into the stationary phase of cell growth, thus enabling a researcher to leave the culture to grow overnight, decoupling the quality of the resulting extract from the harvest OD₆₀₀. As an additional benefit, higher OD₆₀₀ harvests result in a much larger mass of cells, and therefore a larger volume of extract for the same effort [21]. For further convenience, the media is robust enough to support inoculation directly from cell colonies from a fresh streak plate, obviating the need for a seed culture. The

elimination of these steps also increases the reproducibility of generating highly productive extract as there are fewer opportunities for user error.

Here we provide detailed instructions for generating robust *E. coli* cell extracts with a focus on the CFAI workflow. The CFAI workflow is simple and ideal for new users as well as experienced researchers looking to streamline their extract preparation. Since new users may also be interested in establishing the 2x YTPG media-based method as a benchmark, we also include details for this more widely used approach. A reader may choose to follow either the CFAI or 2xYTPG procedures below. Be advised that since we are providing two alternative methodologies, the reader should pay close attention to the section headings and will not need to complete every listed step. If the reader intends to follow the CFAI media method, materials and methods labelled with “CFAI,” Subheadings 2.2 and 3.2, should be completed and sections listed “2x YTPG,” should be excluded, and vice versa for those looking to implement 2x YTPG. Both methods share the following steps in common: materials pertaining to agar plates, cell harvest, extract preparation, and CFPS, and should be completed irrespective of the media type.

2 Materials

Deionized water with a resistivity of 18 M Ω -cm should be used for all solutions unless otherwise noted. Molecular grade free of DNase and RNase will also be required. Materials may be stored at room temperature, unless otherwise stated.

2.1 Agar Plate

1. LB Plate: Suspend 40 g of LB Agar powder in deionized water with a final volume of 1 L in a 2 L Erlenmeyer flask (*see Note 1*). Cover top of flask with aluminum foil. Autoclave for 30 min at 121 °C. Move the liquid agar to a bead or water bath set to 50 °C and allow temperature to acclimate. In a laminar flow hood, pour the agar into sterile polystyrene petri dishes, till the bottom of the dish is covered [23] (*see Note 2*). Stack the plates in the laminar flow hood and allow to cool until the agar has solidified. Store at 4 °C.
2. BL21*(DE3) *E. coli*: Glycerol stock should be stored at –80 °C.

2.2 CFAI Workflow— Media Preparation

1. CFAI Media: Combine 5.0 g of NaCl, 20.0 g of tryptone, 5.0 g of yeast extract, 14.0 g of potassium phosphate dibasic, and 6.0 g of potassium phosphate monobasic with ~500 mL of deionized water to dissolve completely. Adjust pH to 7.2

using 5.0 M KOH. Fill with additional deionized water to reach a final volume of 960 mL. Autoclave for 30 min at 121 °C (*see Note 3*).

2. CFAI Sugar Solution: Combine 6.0 mL of 100% glycerol, 4.0 g of D-lactose, 0.5 g of D-glucose, and deionized water a final volume of 40 mL. Mix until homogeneous. Sterilize the sugar solution using a 0.22 µm syringe filter into a sterile container (*see Note 4*).

2.3 2x YTPG

Workflow—Media Preparation

1. LB Broth: Suspend 25 g of LB Broth powder in deionized water to a final volume of 1 L. Autoclave for 30 min at 121 °C.
2. 2x YTP media: Combine 5.0 g NaCl, 16.0 g tryptone, 10.0 g yeast extract, 7.0 g of potassium phosphate dibasic, and 3.0 g of potassium phosphate monobasic in ~500 mL deionized water and mix until dissolved. Adjust pH to 7.2 using 5 M KOH. Fill with additional deionized water to reach a final volume of 750 mL. Autoclave for 30 min at 121 °C.
3. 2× “G” Glucose Solution: Dissolve 18.0 g of D-glucose in deionized water to a final volume of 250 mL. Autoclave for 30 min at 121 °C.
4. 1 M IPTG Stock Solution: Dissolve 2.38 g of isopropyl β-D-1-thiogalactopyranoside (IPTG) powder in deionized water to reach final volume of 10 mL. Use a micropipette to separate the solution into ten 1 mL aliquots in 1.5 mL microfuge tubes. Store at −20 °C.

2.4 Cell Harvest

1. 1 M DTT Stock Solution: Dissolve 1.54 g of dithiothreitol (DTT) powder in deionized water to a final volume of 10 mL. Use a micropipette to separate solution into ten 1 mL aliquots in 1.5 mL Eppendorf tubes. Store at −20 °C.
2. S30 buffer: A solution of 14 mM Mg(OAc)₂, 10 mM Tris (OAc), 60 mM KOAc can be prepared in advanced, and supplemented with 2 mM DTT when the buffer is ready for use. Combine 0.50 g magnesium acetate, 0.45 g tris-acetate, and 1.47 g potassium acetate with ~250 mL deionized water to dissolve. Adjust pH to 8.2 using 5 M KOH. Fill with more deionized water to reach a final volume of 500 mL. Filter sterilization is optional. Store at 4 °C.

2.5 CFPS Reaction

1. Stock Solution A: Dissolve 6.09 mg ATP, 4.45 mg GTP, 4.12 mg UTP, 4.11 mg CTP, 0.315 mg folinic acid, 1.71 mg tRNA, 2.65 mg nicotinamide adenine dinucleotide (NAD), 2.07 mg coenzyme A (CoA), 3.60 mg oxalic acid, 0.88 mg putrescine, 2.18 mg spermidine, and 136.6 mg HEPES in enough molecular grade water to reach a final volume of 10 mL and mix till homogeneous. Pipet out as 1 mL aliquots and store at −80 °C.

2. Stock Solution B: Dissolve 38.9 mg $\text{Mg}(\text{Glu})_2$, 18.1 mg $\text{NH}_4(\text{Glu})$, 240.8 mg $\text{K}(\text{Glu})$, 1.78 mg alanine, 3.48 mg arginine, 2.64 mg asparagine, 2.66 mg aspartate, 2.42 mg cysteine, 2.94 mg glutamate, 2.92 mg glutamine, 1.50 mg glycine, 3.10 mg histidine, 2.62 mg isoleucine, 2.62 mg leucine, 2.92 mg lysine, 2.98 mg methionine, 3.30 mg phenylalanine, 2.30 mg proline, 2.10 mg serine, 2.38 mg threonine, 4.08 mg tryptophan, 3.62 mg tyrosine, 2.34 mg valine, and 0.05 mg phosphoenolpyruvate (PEP) in molecular grade water to reach a final volume of 10 mL. Given the solubility limits of some amino acids, this solution will not become homogeneous, and it is advised to add the hydrophilic components first till dissolved, and add the hydrophobic amino acids last. Mix till a uniform heterogeneous solution is obtained, and pipet out 1 mL aliquots, with intermittent mixing to ensure the heterogeneous mixture remains uniform. Store aliquots at -80°C .
3. Cell Extract: Protocol outlined below. Store at -80°C .
4. DNA or mRNA template of choice. For DNA templates, pJLI-sfGFP (Addgene plasmid # 102634) purified using a midi or maxi-scale prep is recommended as a positive control.

3 Methods

All procedures can be performed at room temperature while keeping specified materials on ice.

3.1 Agar Plate Inoculation

1. Streak an LB agar plate with *E. coli* BL21*(DE3) that has been stored as a glycerol stock at -80°C . Gently scrape the top of the glycerol stock with an inoculating loop. Streak the cells onto the plate by moving the inoculating loop in a zig-zag motion over one quadrant of the plate. Pull the inoculating loop through the streaked quadrant once and repeat the zig-zag motion over a new, second quadrant. Repeat until all four quadrants of the plate are streaked (Illustrated in Fig. 1).
2. Place the plate upside down (lid down, agar up) in an incubator set to 37°C for at least 18 h or until colonies are visible on the plate.
3. Plate can be wrapped with Parafilm and stored at 4°C for later use.
4. Continue to either Subheadings 3.2 or 3.3 depending on which method is preferred.

3.2 CFAI Workflow—Inoculation and Cell Growth

1. Combine the 960 mL of CFAI broth and 40 mL of CFAI sugar solution in a sterile 2.5 L baffled flask (see Note 5).
2. Inoculate a loopful of colonies (see Note 6) directly into media (Fig. 1).

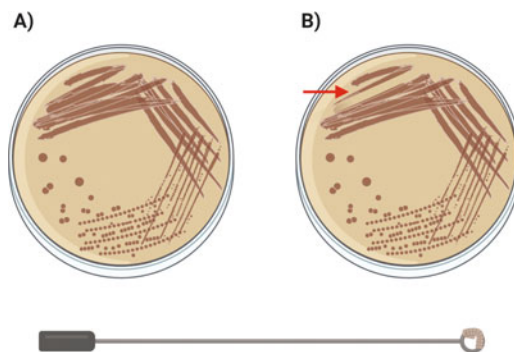


Fig. 1 Schematic representation of what a “loopful” of colonies for inoculation may look like when taken from a BL21*(DE3) streak plate. **(a)** A streak plate before colonies are harvested for inoculation. **(b)** Red arrow indicates where colonies have been taken from plate so that they are now on the inoculating loop

3. Place culture in an incubator set to 30 °C and shaking at 200 rpm overnight (approximately 15 h).
4. In the morning, record the final OD₆₀₀ (*see Note 7*).
5. Continue to Subheading 3.4.

3.3 2x YTPG

Workflow—

Inoculation and Cell Growth

1. Select a single colony from the streak plate using an inoculating loop and inoculate 50 mL of LB broth. Incubate overnight at 37 °C while shaking at 250 rpm.
2. Prewarm the 2x YTPG broth by incubating at 37 °C overnight, without shaking.
3. The next morning, in approximately 15 h, combine the 750 mL of 2x YTPG broth and 250 mL of 2× G solution in a sterile 2.5 L baffled flask. This mixture will result in 1 L of 2x YTPG.
4. Measure the OD₆₀₀ of the overnight culture: Blank your spectrophotometer with LB broth. Pipet 900 µL LB broth and 100 µL of the overnight seed culture into a cuvette (*see Note 8*). Ensure your cuvette is well mixed by pipetting up and down thoroughly prior to reading. Place cuvette in the spectrophotometer and read absorbance at 600 nm (*see Note 9*). Once the OD₆₀₀ of the dilution is measured, multiply by 10 to determine the OD₆₀₀ of your overnight culture.
5. Determine the appropriate volume of the overnight seed culture that should be added to the 1 L of 2x YTPG media so that the growth has a starting OD of 0.1 (*see Note 10*).
6. Inoculate the growth media by adding the amount of overnight you determined in **step 5** into the baffled flask containing the 2x YTPG media (*see Note 11*).
7. Incubate the culture at 37 °C and shaking at 250 rpm. You will be monitoring the OD₆₀₀ of this culture as it grows.

8. First OD₆₀₀ measurement should be collected at 1 h after inoculation. These measurements should not require dilutions, and 1 mL aliquot of the culture can be measured directly in the cuvette. Subsequent measurements should be taken approximately every 20–30 min, in preparation of inducing T7 RNAP expression at OD₆₀₀ of 0.6–0.8 (*see Note 12*). As the culture nears this range, measurements should be taken more frequently to ensure induction window is not missed.
9. Once OD₆₀₀ 0.6–0.8 has been reached, induce expression of T7 RNA polymerase with the addition of 1 mL of 1 M of IPTG stock to the 1 L growth culture.
10. After induction, continue to incubate the culture while shaking, and measure OD₆₀₀ every 20–30 min until an OD₆₀₀ of 2.5 is reached. After induction, cell culture aliquots will require dilutions for OD₆₀₀ measurements to ensure that the values are within the linear range of detection, generally between OD₆₀₀ = 0.1 to 0.8.
11. Once OD₆₀₀ of 2.5 is reached, continue to Subheading 3.4.

3.4 Cell Harvest

1. Cool down the standing centrifuge: attach the appropriate rotor and set the centrifuge to 4 °C. Spin at lowest allowed rpm for 10 min to expedite the cooling process (*see Note 13*).
2. Obtain an ice bucket or tray large enough to accommodate the baffled flask, and prepare an ice–water bath.
3. Prepare the S30 buffer by adding DTT and keep on ice. Prepare by pipetting 1 mL of 1 M DTT stock solution for every 500 mL of buffer you are preparing (*see Note 14*). You will generally use 50–150 mL per 1 L of cell culture, see wash steps below.
4. Once the cell culture has reached the OD₆₀₀ = 2.5, immediately transfer the flask to the ice–water bath to halt further cell growth. Transfer the 1 L cell growth to a cold, sterile 1 L centrifuge bottle, keeping the culture on ice (*see Note 15*).
5. Centrifuge at 5000 × *g* and 10 °C for 10 min.
6. Decant and collect the supernatant containing growth media (*see Note 16*).
7. Record weight of an empty, cold 50 mL conical tube (*see Note 17*). Transfer the cell pellet into the cold, 50 mL Falcon tube using a sterile laboratory spatula (*see Note 18*).
8. Add 40–50 mL of cold S30 buffer to the Falcon tube.
9. Resuspend the cell pellet by vortexing in short intervals, allowing for rest periods on ice.
10. Centrifuge resuspension at 5000 × *g* and 10 °C for 10 min.
11. Decant supernatant (*see Note 19*). **Steps 8–11** represent one wash, which is sufficient for the CFAI workflow; for the 2x YTPG workflow, two additional washes are advised.

12. Weigh the conical tube with the washed cell pellet. Determine the weight of pellet by calculating the difference between the current weight and the weight of the empty Falcon tube.
13. Flash freeze the pellet by submerging pellet in liquid nitrogen.
14. Pellet may be stored at -80°C for a year.
15. All liquid and solid waste that came into contact with cells should be sterilized and disposed of properly, accordingly to institutional guidelines.

3.5 Extract Preparation

1. Prepare S30 buffer by adding DTT. Pipet in 1 mL of 1 M DTT stock solution for every 500 mL of buffer you are preparing (*see Note 15*).
2. For every 1 g of pellet, add 1 mL of cold S30 buffer to the Falcon tube and allow to thaw on ice (*see Note 20*).
3. Resuspend pellet by vortexing in intervals, allowing for rest periods on ice.
4. Pipet 1.4 mL aliquots of the resuspension into 1.5 mL microfuge tubes for sonication (*see Note 21*).
5. Prepare an ice–water bath in a 500 mL glass beaker, densely packed with ice. Flatten the top and push the microfuge tube containing cell resuspension into the ice so that it is being held straight up and down. If the tube is floating up in the ice–water bath, decant excess water.
6. Clean the sonicator probe with 70% ethanol solution and allow to dry. Lower the probe so it is suspended approximated half-way down into the microfuge tube of cell resuspension. Ensure the probe is not hitting the walls of the tube (Fig. 2).
7. Set the sonicator to 20 kHz and 50% amplitude. Adjust the sonicator settings to bursts of 45 s on, and 59 s off.
8. Lyse cells with three rounds sonication, each comprising 45 s on and 59 s off (*see Note 22*). Optional: During the “off” periods, quickly adjust the height of the sonicator to remove the probe from the tube. Cap the tube, invert a few times, and return the tube to its position in the beaker before the next “on” period begins, this helps ensure the extract is well mixed and the lysis is uniform (*see Note 23*).
9. After sonication, add 4.5 μL of 1 M DTT to each tube. Invert gently to mix.
10. Centrifuge cell lysate in tabletop centrifuge at $18,000 \times g$ at 4°C for 10 min.
11. The supernatant represents the desired cell extract. Collect it through careful pipetting and transfer into new 1.5 mL microfuge tubes (*see Note 24*).

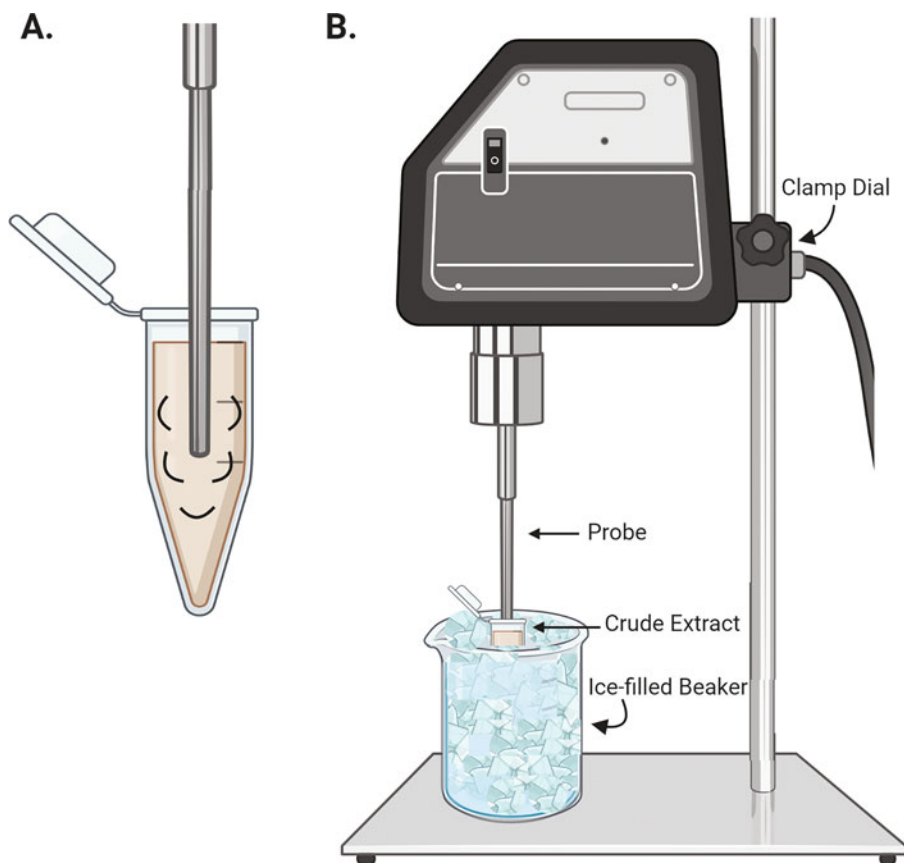


Fig. 2 Setup of sonicator and tube of crude cell extract. (a) Illustration of how sonicator probe should be positioned within the 1.5 mL tube. Ensure the probe is fully submerged and suspended in the center of the tube so it does not touch the sides. (b) Illustration of sonicator setup. Utilize the clamp dial to adjust the sonicator's height

12. The cell extract can now be aliquoted in the desired volumes, anticipating that fewer freeze-thaw cycles are preferred for extract stability. Flash freeze the cell extract by submerging the tubes in liquid nitrogen and store at -80°C .

3.6 CFPS Reaction

1. The reaction described here will be 15 μL total performed in a 1.5 mL microfuge tube (*see Note 25*). The reaction volume can be scaled linearly by adjusting all components accordingly. The volume of water and template DNA or mRNA will vary depending on the stock concentration of the template DNA or mRNA. The reaction will be composed of 2.20 μL of Solution A, 2.10 μL of Solution B (*see Note 26*), 5.0 μL of cell extract, 16 ng/ μL (*see Note 27*) of template DNA or mRNA, and molecular grade water to reach a final reaction volume of 15 μL .

2. Allow Solutions A and B, template DNA, and the cell extract to thaw on ice. Solution B will remain heterogeneous, be sure to vortex regularly to maintain a uniform mixture for reproducible cell-free reactions.
3. Prepare the CFPS reaction by pipetting the reagents in the order of the largest volume first and smallest volume last. Mix by pipetting up and down (*see* **Note 28**).
4. Place reactions in incubator at 37 °C for 4 h, or 30 °C for 20 h.

4 Notes

1. Whenever you autoclave, use a large enough container that your solution constitutes a maximum of 75% of the total volume (based on manufacturer guidelines). Otherwise, the heat and pressure of the autoclave could cause your solution to boil over.
2. This may be accomplished by pouring the agar directly into the dish and estimating the volume or by using a serological pipette to dispense at least 8 mL of agar into each dish [23]. Be aware that this step must be done quickly as the agar will begin to solidify.
3. When autoclaving containers with lids, ensure they are only loosely capped, so the pressure generated from autoclaving has a means by which to escape. Once the containers are removed from the autoclave, caps can be tightened to preserve sterility.
4. Due to the viscosity of the sugar solution, there will be a fair amount of pressure and resistance when pressing down on plunger to syringe filter. Do not attempt to rush the sterilization by forcing the plunger down quickly as this may result in the rupturing of the seal between the syringe and the filter. If possible, use smaller volume syringes to make the filtration process easier, and repeat the filtration process in aliquots until all 40 mL have been sterilized.
5. For best results warm the media in an incubator and allow to acclimate to 30 °C in advance.
6. It is conventional to select a single colony from a plate for inoculation. But since the plate has been created from an isogenic glycerol stock, concern of genetic variation by selecting multiple colonies is minimized. Robustness of this methodology has been demonstrated [21].
7. Anticipate the OD₆₀₀ to reach approximately 10.
8. Standard spectrophotometers only operate accurately between absorbances of 0.1–1. Thus, a dilution of the overnight culture must be performed to accurately read the OD₆₀₀. Typically,

this can be achieved with a 1:10 dilution into the cuvette (100 μ L overnight, 900 μ L LB broth). An OD₆₀₀ reading of 3–5 is typical of an overnight seed culture. If the absorbance reading is out of range, further dilutions may be necessary.

9. Since the overnight was diluted 1:10 in the cuvette, the absorbance reading must be multiplied by 10 to determine the true OD₆₀₀ of the overnight culture. Any dilution performed to obtain OD₆₀₀ readings must be accounted for in this way.
10. Utilize the dilution formula, $C_1 V_1 = C_2 V_2$, to evaluate how much of the overnight should be added. C_2 is 0.1, the desired starting absorbance of the growth culture. V_2 is 1000 mL, the total volume of your growth culture. C_1 is the concentration of your overnight, and V_1 is what you will solve for, the volume of overnight that should be combined with 2x YTPG to reach a starting volume of 1 L.
11. Remaining overnight culture may be transferred to a secondary “biological waste” container and treated with 10% bleach solution before disposal.
12. Ideally, these intervals will constitute a doubling of the culture’s OD₆₀₀, but if the culture is growing a bit slower than this, that should not be a reason for alarm.
13. For “Cell Harvest” and “Extract Preparation,” it is important to be diligent about keeping all materials cold for best results. Ensure all materials are kept on ice throughout the processes.
14. Only add DTT to as much S30 buffer as will be used in the protocol, as the buffer should not be stored once DTT is added. Keep the buffer cold.
15. It is helpful to place the sterile 1 L centrifuge tube in the fridge the night before.
16. Supernatant may be poured into a secondary “biological waste” container and treated with 10% bleach before disposal.
17. It would be advantageous to weigh the tube and place it in the freezer the night before.
18. It may be easiest to use two sterile laboratory spatulas so that you may use them to scrap cell pellet off one another. Some users have observed that the cell pellets from CFAI growths appear “goopy” or “slimy” compared to those from 2x YTPG growths. If you notice this, it should not be a point of concern as this appears to be an inconsistent observation and does not appear to be correlated with the productivity of the resulting extract.
19. Supernatant may be poured into a secondary “biological waste” container and treated with 10% bleach before disposal.
20. Now is a good time to cool down the tabletop centrifuge. Set centrifuge to 4 °C. Spin at lowest allowed rpm for 10 min.

21. If there is remaining resuspension that amounts to less than 1.4 mL, pipet that into an Eppendorf and fill with S30 buffer so the final volume amounts to 1.4 mL. Sonicate this tube for less time, proportionate to the fraction of the tube's volume which is made up of resuspension.
22. The most common issue that may arise when sonicating is the mispositioning of the probe, which may cause the cell extract to foam. If you start to see bubbles forming, stop your sonicator and assess the state of your setup. Foaming is likely caused by the probe hitting the walls of the tube or not being fully submerged. Reposition if necessary and try again.
23. When done properly, expect to notice a slight shift in shade of the extract after sonicating. It should become slightly darker, more of a brown tone rather than the tan tone of the un-lysed suspension. Ensure tubes are kept cold during sonication; it may be necessary to replace the ice in the beaker between tubes of extract. Continue to discard excess water as needed.
24. Quality should be valued over quantity. Be careful to only pipet out pure supernatant. If pellet appears to be shifting and starting to draw into pipette, stop—even at the cost of some supernatant. Once supernatant is removed, remaining pellets can be disposed of as biological waste.
25. Using large tubes relative to the volume of the reaction is important, as it has been demonstrated that increasing the surface area available for oxygen exchange significantly improves CFPS reaction yields [1, 18].
26. Be sure to vortex solution A and B often through setup process to ensure solution is homogeneous. Components, particularly the hydrophobic amino acids, tend to settle at the bottom. Thus, it is essential to vortex solution A and B directly before they are added to the reaction to ensure every reaction setup contains the necessary amino acids for protein synthesis.
27. Utilize the $C_1 V_1 = C_2 V_2$ dilution formula to determine the volume of stock template DNA should be added to the reaction. This is similar to the calculation described in **Note 10**. C_1 = concentration of stock of DNA in ng/ μ L, C_2 = 16 ng/ μ L, and V_2 = 15 μ L.
28. Again, vortex Solution A and B during setup. Be sure to pipet the template DNA or mRNA and the cell extract up and down before adding them to the reaction to ensure those solutions are homogeneous. After everything has been added, if there are any droplets on the sides of the tube, centrifuge briefly to ensure all components are gathered at the bottom as a 15 μ L reaction.

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Preparation and Screening of Cell-Free Extract from Nongrowing *Escherichia coli* A19 Cells

Florian Hiering, Jurek Failmezger, and Martin Siemann-Herzberg

Abstract

Cell-free extracts have been researched and continuously streamlined for around 50 years. It is believed that these extracts work best when routinely obtained from exponentially growing cells to capture the most active translation system. Here we report on an active cell-free extract derived from *E. coli* A19 that was harvested under nongrowing, stressed conditions. Although this process is based on the conventional routine process for the production of S30-extracts, our process is less labor intensive and reduces variability between extracts.

Key words Stationary phase, Growing conditions, Cell-free protein synthesis, Ribosome, S30 lysate, Cell extract, In vitro transcription and translation, TX-TL, Stress, Synthetic biology

1 Introduction

Cell-free extracts have proven to be a powerful and versatile tool for synthetic biology and producing complex proteins. Based upon the pioneering work of Nirenberg and Matthai [1], the first protocols for extract preparation were established by Zubay [2], and since then, they were constantly streamlined by various groups (for most recent reviews *see* [3, 4]). However, one dogma remained—well performing cell-free extracts can only be obtained from highly active (i.e., rapidly growing) cells. The reason for this dogma is that ribosome content is correlated with growth rates [5]. Since ribosomes are the heart of protein synthesis, this couples high protein synthesis to high growth rates. For this reason, all current protocols harvest at early- or mid- exponential growth phase (Fig. 1a). This coupling also applies to extremely fast growing organisms like *Vibrio natriegens*, which also have a very high ribosome content [6, 7]. Nevertheless, the rate of synthesis and the amount of synthesized protein from *V. natriegens* extracts hardly

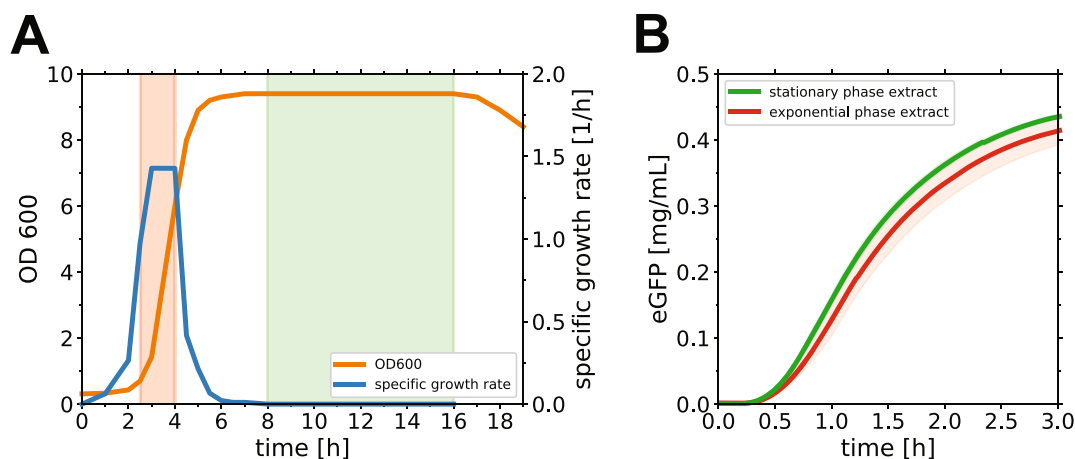


Fig. 1 Possible harvest points for highly active cell-free extract and resulting kinetic curve. **(a)** The orange line resembles a typical *E. coli* growth curve. The blue line shows the corresponding specific growth rate, negative growth rates have been neglected. The red area depicts the ideal point of harvest in current protocols while the green area depicts the possible harvest time according to our protocol. **(b)** Green and red line resemble the kinetics of extracts from bacteria that were harvested in the stationary and exponential phases, respectively. The shaded areas represent one standard deviation

differ from well-performing *E. coli* extracts [8]. Thus, the ribosome content alone cannot be the determinant for extract performance. This finding opens up the search for alternative sources of cell-free extracts. Our work shows that a well performing extract can be obtained from cells that have entered the stationary phase [9]. This approach makes the extract process less labor-intensive, increases yield, and reduces variability between extracts (Fig. 1b). In this chapter we describe how to prepare a stationary phase-related extract and screen it for further experiments.

2 Materials

The amounts used in this protocol are based on 1.6 L of culture divided into five 2-L Erlenmeyer-flasks (400 mL of media per flask). In total, this should add up to about 20 g of wet cell mass, making about 5 mL of extract. Unless otherwise stated, deionized water (dH₂O) is used for dissolving reagents.

2.1 Growth and Harvest of *E. coli*

1. *E. coli* A19 glycerol stock (*see* **Note 1**).
2. 2x YT agar plate: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 20 g/L agar.
3. 2x YT medium for preculture, 150 mL: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl.

4. 2x YTPG medium, 1000 mL: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 22 mM KH_2PO_4 , 40 mM K_2HPO_4 , 100 mM glucose (*see Note 2*).
5. Five 3-L Erlenmeyer-flasks with four baffles and appropriate filter plugs.
6. Centrifuge and flasks with appropriate volume for harvesting.

2.2 S30 Extract Preparation

1. Cooled Avestin Emulsiflex homogenizer (4 °C).
2. Cold S30 buffer, 2 L for dialysis and additional 200 mL for resuspension and homogenizer priming: 14 mM magnesium acetate, 60 mM potassium acetate, 10 mM Tris(HCl) pH 8.0, 2 mM DTT (*see Note 3*).
3. Presoaked, sterile, and cooled dialysis membrane with 6–8 kDa molecular weight cutoff.
4. 20-mL Luer-lock syringe.
5. Prewighted falcon tubes.

2.3 Cell-Free Protein Synthesis

All compounds in this section are dissolved in autoclaved double-distilled water or HPLC water (*see Notes 4 and 5*). The following compounds refer to a standard reaction using the creatine phosphotransferase system for energy regeneration. However, there are several other regeneration systems, a good overview can be found in [10]. Store the compounds at –20 °C and the ion solutions at 4 °C.

1. Amino acid-mix with 25 mM of each amino acid (*see Note 6*).
2. 250 mM magnesium glutamate.
3. 4 M potassium glutamate.
4. 200 mM ammonium glutamate.
5. Tenfold PEG-Mix: with 20% PEG8000 (w/v), 800 mM HEPES-KOH.
6. 20 mM dithiothreitol (DTT).
7. 25-fold dNTP-Mix (with 40 mM ATP, 30 mM GTP, UTP, CTP each, 850 µg/mL folinic acid) (*see Note 7*).
8. 1.5 M creatine phosphate.
9. 10 mg/mL creatine kinase.
10. 200 U/µL T7-RNA polymerase (T7-RNAP).
11. Plasmid.
12. S30-extract.

3 Methods

3.1 Growth and Harvest of *E. coli*

1. Streak *E. coli* on 2x YT agar plate from a glycerol stock. Cultivate overnight at 37 °C.
2. Preculture: Inoculate 50 mL of 2x YT medium into a 500 mL baffled flask with a single colony picked from the agar plate. Cultivate overnight at 37 °C with vigorous shaking (120 rpm).
3. Main culture: Fill 400 mL of 2x YTPG medium in each of the five 2 L baffled flasks and inoculate each of them with 100 µL of the preculture. Cultivate for 8–16 h at 37 °C with medium shaking (120 rpm). The OD600 should be between 8 and 10 (*see* **Notes 8** and **9**).
4. Harvest the culture by transferring it into prechilled centrifugation flasks. Centrifuge at $4750 \times g$ for 15 min at 4 °C.
5. Discard the supernatant and transfer the biomass to pre-weighted falcon tubes. Then weigh the tubes and immerse them in liquid nitrogen. Store at –80 °C (*see* **Note 10**).

3.2 S30 Extract Preparation

1. Calculate the amount of wet cells in each tube by subtracting the preweight from the weight with biomass. Add 1.2 mL of S30 buffer per gram of wet cells. If possible, keep the biomass on ice until the run-off step.
2. Resuspend cells by vortexing (*see* **Notes 11** and **12**).
3. Attach the 20-mL Luer-lock syringe to the output of the homogenizer.
4. Prime the homogenizer by passing a few milliliters of S30 buffer through it.
5. Empty the syringe.
6. Pass the resuspended cell mass with 15–20k psi through the homogenizer. Discard the first 6 mL (*see* **Notes 13** and **14**).
7. Pass the resuspended cell mass through the homogeniser a second time at 15–20k psi.
8. Fill the lysed cells in centrifugation tubes and centrifuge them at $30,000 \times g$ for 30 min at 4 °C.
9. Transfer the upper two-thirds of the supernatant to a new tube and repeat the centrifugation process (*see* **Note 15**).
10. Run-off step. Transfer the upper four-fifths of the crude extract to a falcon tube and incubate for 80 min at 37 °C.
11. Transfer the crude extract to a dialysis membrane and dialyze for 3 h at 4 °C against 2 L of S30 buffer (*see* **Notes 16** and **17**).
12. Centrifuge the extract at $4000 \times g$ for 20 min at 4 °C.
13. Aliquot the supernatant as needed and flash-freeze in liquid nitrogen before storing at –80 °C (*see* **Note 18**).

3.3 Cell-Free Protein Synthesis

Both Mg^{2+} and K^{+} concentrations have a high influence on extract performance, with Mg^{2+} being the most important. The optimal concentrations for these two ions can vary from extract to extract and must therefore be optimized for each new batch. In this section we will briefly show how to perform a quick “ Mg^{2+} screen” on the extract. We use GFP fluorescence as a detection method to track the kinetics during the reaction. The plasmid and T7-RNAP concentrations are optimized on our in-house standard plasmid pJOE4056.2 [11]. These concentrations must also be optimized for each new type of plasmid used.

1. Thaw all the necessary reagents. Keep them on ice while pipetting.
2. Mix the reagents in a 1.5-mL reaction tube in the same order and volumes as shown in Table 1, with the exception of the S30 extract and water.
3. To setup the screening, pipet everything into the wells of a standard 96-well plate as shown in Table 2, with the exception of the master-mix.
4. Pipet the S30 extract into the 1.5-mL reaction tube to complete the master-mix. Mix thoroughly by vortexing.
5. Immediately add the appropriate volume of the master-Mix to the wells according to Table 2.
6. Incubate for 3–4 h at 37 °C.

Table 1

Recipe for an 860 μL master mix. If screening is not desired, add 86 μL of dH_2O to get a full mix

Reagent name	Stock conc.	Final conc.	Unit	Volume (μL)
Amino acid mix	25	1	mM	34
Mg^{2+} glutamate	250	10	mM	34
K^{+} glutamate	4000	90	mM	19
NH_4^{+} glutamate	200	20	mM	86
PEG mix	10	1	Fold	86
DTT	20	2	mM	86
dNTP mix	25	1	Fold	34
Creatine phosphate	1500	120	mM	69
Creatine kinase	10	0.24	mg/mL	21
T7-RNAP	200	3.9	$\text{U}/\mu\text{L}$	17
Plasmid	500	37	$\mu\text{g}/\text{mL}$	64
S30 extract ^a		26	%	224

^aAdd the S30-extract at **step 3**

Table 2
Mg²⁺-Screening setup for ten individual 80 μ L batch reactions

Well No.	1	2	3	4	5	6	7	8	9	10
Final Mg ²⁺ concentration (mM)	10	12	14	16	18	20	22	24	26	28
ddH ₂ O (μ L)	8.0	7.4	6.7	6.1	5.4	4.8	4.2	3.5	2.9	2.2
Mg ²⁺ glutamate (μ L)	0	0.64	128	1.92	2.56	3.2	3.84	4.48	5.12	5.76
Master mix (μ L)	72	72	72	72	72	72	72	72	72	72

7. In order to analyze at which Mg²⁺ concentration the extract performs best, the kinetics can be tracked or a simple endpoint measurement can be performed.

4 Notes

1. Growing *E. coli* to high ODs to obtain a highly active extract works for the A19 strain and standard 2x YTPG media. There have been different reports for other *E. coli* strains. Unsuccessful attempts have been made by Kim et al. [12] for a K12 strain. Successful growth to high ODs has been demonstrated by Levine et al. [13] for the BL21Star-(DE3) strain, although they used a self-developed media for cultivation.
2. Do not autoclave glucose to avoid caramelization. It is advisable to dissolve the other components in 950 mL of distilled H₂O and autoclave, while glucose is dissolved in 50 mL of dH₂O and sterile filtered. Add the glucose solution after autoclaving.
3. The standard procedure for preparing the S30 buffer uses three separate concentrates and ddH₂O. The three solutions are 2 M Tris-HCl pH 8.0, 1.4 M magnesium-acetate and 6 M potassium-acetate. They are usually sterile filtrated for stability, but autoclaving should work too. The pH of the final S30 buffer is usually not checked.
4. It is recommended to use HPLC-water, but ddH₂O should work too. The reason is that HPLC-water is RNase-free. If too much RNase gets into the cell-free reaction, it will result in bad extract performance.
5. It is also recommended to divide the compounds into disposable (single-use) aliquots (excluding plasmid). This ensures that all reagents are in an optimal state when they are used.
6. Tyrosine is not soluble in this mixture. This will not affect performance as long as the mixture is mixed thoroughly just before use. It is advisable to prepare at least a 50 mL of stock

solution, as the amino acids are difficult to weigh with smaller stock volumes.

7. Each of the reactants should be made up as a single stock solution and then mixed together.
8. It is very important to ensure that the filter-plug does not get wet during cultivation, otherwise gas exchange will be restricted, resulting in poor extract performance. In our laboratory we use a standard benchtop shaker with a shaking radius of 5 cm and glass flasks with four small baffles. Baffled Tunair flasks (2.5-L) will work as well.
9. It is easiest to cultivate overnight, thus inoculating the main culture in the evening. However, if the culture is to be grown during the day, the main culture can be inoculated with about 5 mL of preculture and cultivated for 8 h.
10. Use a large and a small spoon/spatula to transfer the biomass. Also use centrifugation tubes with a large opening, as this makes it easier to reach the cell pellet. In addition, it is recommended to not put more than 10 g of cell mass in each falcon tube as this would unnecessarily delay the resuspension process. It is best to aim for 5–7 g of cell mass per tube.
11. After adding the S30 buffer, the tubes can be held in hand for a few minutes. The heat helps peeling off the biomass from the tube, making the resuspension process easier and faster. Alternatively, the tubes can be placed in a warm water bath for a few minutes.
12. For the homogenization process it is vital that the cells are very well resuspended. If in doubt, pipet the resuspended biomass up and down a few times with a 1-mL pipette. If the pipette is not clogged, the process can be started.
13. This volume is discarded to prevent the extract from being diluted by the buffer that is still in the machine. The amount of volume that needs to be discarded depends on the specific machine used and may need to be adjusted.
14. It is very important to avoid bubbles or foam getting into the homogenizer as this can affect homogenization and thus the performance of the extract. Most likely, some foam has formed during the resuspension process. Most of it can be removed with centrifugation at $1000 \times g$ for 30 s at 4 °C. Other cell-lysis methods have also been used to disrupt the cells, mainly French press and bead milling.
15. The cell debris affects the performance of the extract. Therefore, only the top two-thirds of the centrifuged crude extract are used for the next step. Also, when inserting and removing the centrifugation tubes, be careful not to disturb the cell pellet.

16. Make sure that no air remains in the dialysis membrane, as this would dilute the extract.
17. Most extract preparation protocols include a dialysis step, the details of which can vary widely. Liu et al. [14] showed that this step can be omitted entirely without affecting the performance of the extract. (However, in our lab this step is still carried out for sake of comparability to previous work.)
18. When stored at -80°C , the extracts are stable for at least a year and can be thawed and refrozen for at least two cycles without affecting the performance of the extract. Nevertheless, the preparation of single-use aliquots is strongly recommended.

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Cell-Free Protein Synthesis Using *Pichia pastoris*

Alex J. Spice, Rochelle Aw, and Karen M. Polizzi

Abstract

Pichia pastoris (syn. *Komagataella phaffii*) is an industrially relevant recombinant protein platform that has been used to produce over 5000 proteins to date. Cell-free protein synthesis can be used as a screening tool before strain development or for the production of proteins that are difficult or toxic to make in vivo. Here we describe the methods for generating an active cell lysate from *P. pastoris* using high pressure homogenization and an improved reaction mix which results in high yields of reporter proteins such as luciferase, and complex proteins such as human serum albumin and virus-like particles.

Key words Cell-free protein synthesis, In vitro transcription translation, *Pichia pastoris*, *Komagataella phaffii*, Rapid prototyping, Synthetic biology, Eukaryotic cell-free system

1 Introduction

Pichia pastoris (syn. *Komagataella phaffii*) is the second most popular recombinant protein expression platform after *Escherichia coli* [1]. It has many advantages including the ability to grow to high cell densities, which results in high volumetric productivity, the capability to perform post-translational modifications such as glycosylation, and stable genomic integration of recombinant DNA via homologous recombination [2]. The ability to generate a cell-free protein synthesis (CFPS) workflow for such a pivotal industrial protein expression platform, enables the production of toxic products, the development of stratified medicines, and screening vaccine targets [3–5]. CFPS allows the decoupling of cell growth from protein production and can overcome stress responses such as the unfolded protein response that often limit the yield of secreted recombinant proteins [6]. To date, cell extracts from four different strains of *P. pastoris* have been used to produce CFPS extracts: a wild-type strain (X33), a protease-deficient strain (SMD1163), and

Alex J. Spice and Rochelle Aw contributed equally to this work.

two strains that were engineered for the higher ribosome content through overexpression of transcription regulators, *FHL1* or *HMO1* [7, 8]. Overexpression of *FHL1* led to a three-fold increase in protein production compared to X33 and optimization of the reaction mix using Design of Experiments (DoE) led to a further increase of approximately 3.5-fold [9]. The production of complex proteins such as human serum albumin, with yields up to 116 µg/mL, and ~6.4 µg/mL of fully assembled Hepatitis B core antigen virus-like particle (VLP) have also been demonstrated [9, 10]. Herein we provide details of our optimized workflow using high pressure homogenization to generate an active cell free extract and an improved reaction mix that enhances the production of the model protein luciferase.

2 Materials

2.1 Preparation of *Pichia pastoris* Lysate

1. Stock of desired *Pichia pastoris* strain.
2. YPD liquid broth: 1% yeast extract, 2% peptone, 2% dextrose.
3. YPD agar: 1% yeast extract, 2% peptone, 2% dextrose, 2% agar, add antibiotic as appropriate for strain selection.
4. Buffer A: 20 mM HEPES KOH (pH 7.4), 100 mM potassium acetate, 2 mM magnesium acetate. Prepare fresh and keep ice cold.
5. Dry ice (optional).
6. Lysis buffer A: 20 mM HEPES KOH, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.5 mM PMSF. Prepare fresh and keep ice cold.
7. Bradford assay kit (or another suitable protein quantification kit).
8. 1-L baffled glass flask
9. Orbital shaking incubator, 30 °C.
10. UV/visible spectrophotometer.
11. 1.5 mL cuvettes
12. 50-mL centrifuge tubes
13. High-speed refrigerated centrifuge.
14. High-pressure cell disruptor (e.g., CF1 model, Constant Systems Ltd., Daventry, England) precooled in a 4 °C refrigerator and One-Shot Head Adapter (for processing volumes up to 10 mL) (Constant Systems Ltd.)
15. 10 mL serological pipettes
16. Syringe with 18-gauge needle.
17. 3.5 K MWCO Slide-A-Lyzer™ G2 dialysis cassette, or equivalent.

2.2 Coupled In Vitro Transcription and Translation

Unless otherwise noted, all components should be stored at -20°C .

1. 40 nM DNA template (plasmid) prepared by extraction with Qiagen Plasmid Maxi Kit. DNA template resuspended in TE buffer: 100 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).
2. 1 M HEPES-KOH (pH 7.4), filter-sterilized and stored at 4°C
3. 1 M magnesium glutamate, filter-sterilized and stored at 4°C
4. 0.1 M dithiothreitol (DTT), filter-sterilized
5. Amino Acid Mix: 0.75 mM leucine, 0.9 mM each of the other 19 standard amino acids, stored at -80°C .
6. NTP solution set, 100 mM each.
7. 500 mM creatine phosphate, filter-sterilized
8. 4 mg/mL creatine phosphokinase in 25 mM glycylglycine (pH 7.5), filter-sterilized
9. 40 U/ μL murine RNase inhibitor
10. T7 polymerase, HC, 200 U/ μL .
11. Cell lysate, stored at -80°C (from Subheading 3.1).
12. Water bath or heat block, 60°C .

2.3 Determining Luciferase Production from Cell-Free Reactions

1. CFPS reaction product (from Subheading 3.2).
2. 200 mM tricine, pH 7.6
3. 25 mM ATP
4. 50 mM MgSO_4
5. 10 mM MgCO_3
6. 10 mM EDTA, pH 7.6
7. 500 mM DTT
8. 25 mM D-luciferin or luciferin salt
9. 1.5 mL microcentrifuge tubes
10. 96-well, half-area, flat-bottom white microtiter plates
11. Plate reader for luminescence.

3 Methods

3.1 Preparation of *Pichia pastoris* Lysate

A graphical overview of the entire process is illustrated in Fig. 1.

1. Prior to preparation of each cell-free lysate, streak a fresh YPD agar plate with a stock of the desired strain and incubate at 30°C for 3–5 days (*see* **Note 1**).
2. Pick a single colony from a freshly prepared YPD agar plate and inoculate 5 mL YPD medium in a 50 mL centrifuge tube.

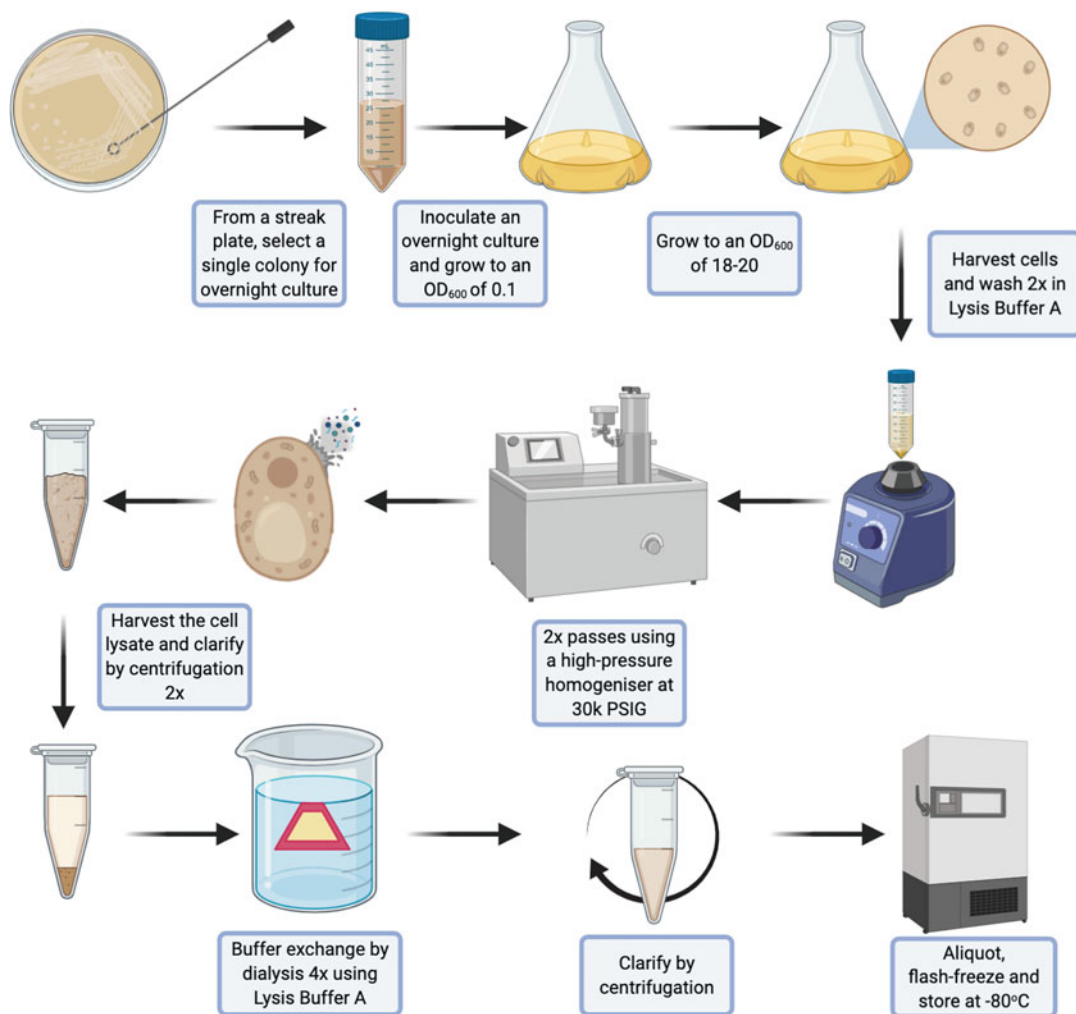


Fig. 1 Schematic representation of the cell-free extract preparation process. Made using biorender.com

Incubate overnight at 30 °C with shaking at 250 rpm. Antibiotic is encouraged to be used in both the agar and liquid culture should selection of a specific strain be required.

3. Use the overnight culture to inoculate 200 mL YPD medium in a 1-L baffled glass flask to an OD₆₀₀ of 0.1 (*see Note 2*).
4. Regularly measure the OD₆₀₀ of the culture, and harvest cells once an OD₆₀₀ of 18.0–22.0 has been achieved.
5. Weigh an empty 50 mL centrifuge tube and record the weight of the tube. Store this tube and three additional 50 mL centrifuge tubes on ice until use.
6. Once the desired OD₆₀₀ has been achieved, decant the cultures into four 50 mL centrifuge tubes, ensuring cells and reagents are kept on ice as much as possible (*see Note 3*).

7. Centrifuge the tubes for 10 min at $3000 \times g$, 4°C .
8. Remove the supernatant and resuspend the cells in a total of 50 mL ice-cold buffer A. Split equally among the four 50-mL centrifuge tubes. Combine the resuspended cultures in the 50-mL centrifuge tube that was previously weighed.
9. Centrifuge for 10 min at $3000 \times g$, 4°C .
10. Wash the cells by resuspending in 20 mL ice-cold buffer A.
11. Centrifuge for 10 min at $3000 \times g$, 4°C and pour off the supernatant.
12. Repeat **steps 10** and **11**.
13. Blot the tubes on a towel in order to remove excess buffer.
14. Weigh the 50 mL centrifuge tube containing the cell pellet. Wet cell weight can be determined by subtracting the original weight of the centrifuge tube (*see Note 4*).
15. At this point in the protocol, there is a natural break point where it is possible to flash-freeze the pellet to continue the process later (*see Note 5*), or alternatively, continue with lysis.
16. To continue with lysis, resuspend the pellet in 1 mL ice-cold lysis buffer A per 1 g wet cell weight.
17. Perform two passes using a high-pressure homogenizer at 30,000 psi (or according to the manufacturer's instructions). Ideally, the homogenizer should be precooled in advance by refrigeration (*see Note 6*). All samples should be kept as cold possible throughout and collected on ice. If available, a one-shot head adapter can be employed to process smaller volumes (≤ 10 mL). Use a 10 mL serological pipette to transfer the lysate to the high-pressure homogenizer, and to remove the lysate before re-adding it for the second pass.
18. Lysis efficiency can be determined by the total protein concentration of the resultant extract post-lysis (*see Note 7*).
19. After lysis, collect the sample in a 50-mL centrifuge tube and centrifuge for 30 min at $18,000 \times g$, 4°C .
20. After the first centrifugation, transfer the supernatant to a fresh 50 mL centrifuge tube and repeat centrifugation for 30 min at $18,000 \times g$, 4°C .
21. Pipet the supernatant from the lysis into a 3.5 K MWCO Slide-A-Lyzer™ G2 dialysis cassette. The cassette must be properly rehydrated prior to use, according to the manufacturer's guidelines (*see Note 8*).
22. Buffer exchange the sample four times, 30 min each, with a minimum of 50 volumes of lysis buffer A at 4°C .
23. Remove the sample from the dialysis cassette using a serological pipette, micropipette, or needle and syringe, and transfer to a 50-mL centrifuge tube.

24. Further clarify the lysate by centrifugation for 1 h at $18,000 \times g$, 4 °C.
25. We advise the 1.5-mL microcentrifuge tubes for aliquots be prechilled for a minimum of 30 min in a –80 °C freezer.
26. Immediately divide the supernatant into aliquots in the prechilled 1.5-mL microcentrifuge tubes. Aliquots are frozen for single-use and the volumes chosen should be determined accordingly for individual in the context of the number of replicates desired per single-use aliquot (*see Note 9*).
27. Once aliquoted, immediately flash-freeze the samples using a dry ice and methanol bath before transfer to a –80 °C freezer as soon as possible.
28. If desired, retain a single aliquot to determine total protein concentration of the lysate using a Bradford assay (or other protein determination assay of choice) according to the manufacturer's instructions. Using this lysis method, we would expect lysis to yield a total protein concentration of 15–25 mg/mL.

3.2 Coupled In Vitro Transcription and Translation

The protocol described here is uses an improved reaction mix that was developed using a Design of Experiments approach [9] (*see Note 10*). All components should be defrosted on ice. Ensure they are fully thawed prior to use. It is essential that the amino acid mix is fully dissolved before use (*see Note 11*). This can be achieved by heating at 60 °C for 5 min once fully thawed. Allow the solution to cool to room temperature before use. Note that the solution should not be placed on ice to avoid precipitation.

1. Extract DNA plasmid template using Qiagen Plasmid Maxi Kit (*see Note 12*).
2. The following components (*see Note 13*) should be combined in the exact order detailed below (*see Note 14*), on ice, in a 1.5-mL centrifuge tube, to a total volume of 25 µL for a single reaction (*see Note 15*):
 - 3 µL 1 M HEPES-KOH, pH 7.4
 - 0.3 µL 1 M magnesium glutamate
 - 1 µL 100 mM DTT
 - 7.5 µL amino acid mix
 - 0.5 µL 100 mM ATP
 - 0.5 µL 100 mM GTP
 - 0.5 µL 100 mM UTP
 - 0.5 µL 100 mM CTP
 - 4.5 µL 500 mM creatine phosphate
 - 3.38 µL 4 mg/mL creatine phosphokinase (*see Note 16*)

- 0.25 μL 40 U/ μL RNase inhibitor
 - 0.5 μL 200 U/ μL T7 polymerase
 - 1 μL Plasmid DNA (40 nM) (*see* **Note 17**).
3. Flick the tube to ensure complete mixing and centrifuge briefly; vortexing is not recommended.
 4. Add 25 μL *Pichia pastoris* cell lysate to the mix.
 5. Flick the tube and centrifuge again briefly to ensure all the components are combined at the bottom of the tube.
 6. The reaction tubes can be placed in either a water bath or a static incubator at 21 °C (*see* **Note 18**). Vast differences in ambient/tube temperature may result in evaporation within the reaction tubes and may negatively impact the reproducibility of the reactions.
 7. In general, the reaction will take 2–8 h. This is dependent on the protein of interest as folding of more complex proteins (e.g., greater in size or those containing post-translational modifications) may take longer. Depending on the protein of interest, a period of troubleshooting and optimisation to determine the optimal reaction time may be required.
 8. Once protein synthesis has ceased, the reaction products can be analysed immediately using the relevant analytical methodology (*see* **Note 19**), or alternatively, certain proteins may be amenable to freezing for later analysis.

3.3 Determining Luciferase Production from Cell-Free Reactions

The protocol here describes use of a luminescence assay to determine luciferase production (*see* **Note 20** for amino acid sequence), which can be employed for establishing and troubleshooting the platform (*see* **Note 21**). The luciferase assay is conducted in 96-well, half-well white microtiter plates to allow for smaller sample sizes.

1. Combine the following components of the luciferase assay buffer on ice for total volume of 600 μL (*see* **Note 22**):
 - 50 μL 200 mM tricine
 - 5 μL 25 mM ATP
 - 26.7 μL 50 mM MgSO_4
 - 53.4 μL 10 mM MgCO_3
 - 5 μL 10 mM EDTA
 - 17 μL 500 mM DTT
 - 5 μL 25 mM D-luciferin
 - 337.9 μL water.
2. Pipet 30 μL luciferase assay buffer into a clean 96-well half-area flat-bottom white microtiter plate and add 5 μL of CFPS reaction.

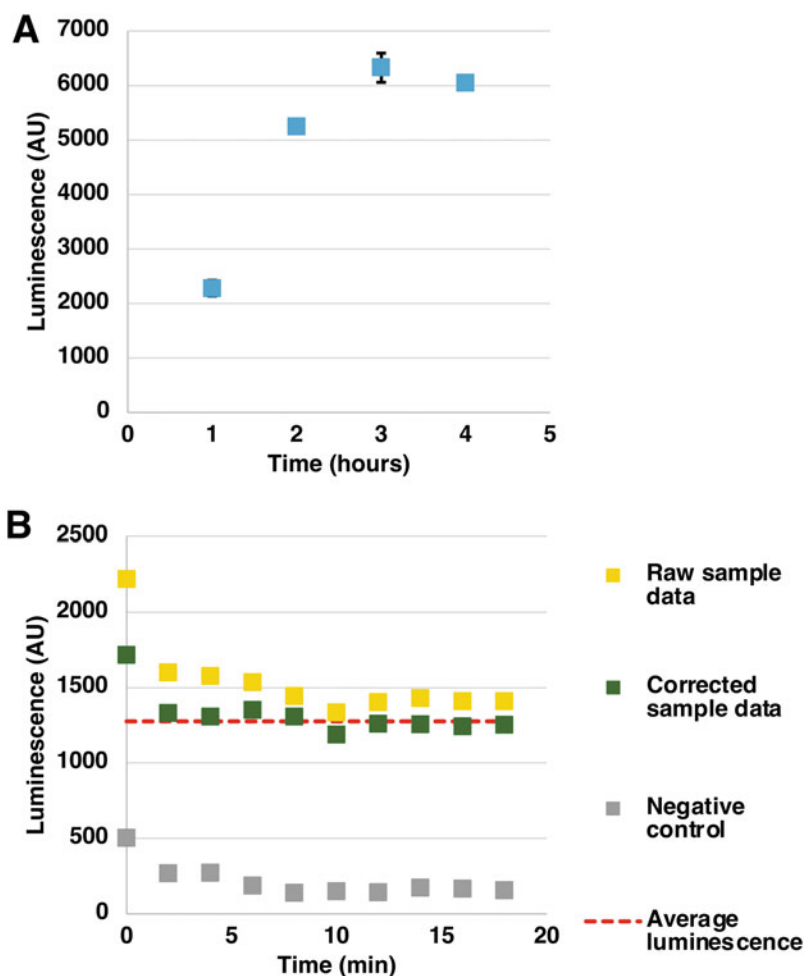


Fig. 2 (a) An example time course of luciferase production over time using the coupled in vitro transcription/translation protocol and the improved reaction mix composition, extract derived from the FHL1 strain, and a DNA template containing the cricket paralysis virus (CrPV) IRES. Luciferase production was measured over a period of 5 h. Error bars represent the standard deviation of the mean of three biological repeats (corrected for the negative control reactions) and are calculated using error propagation. **(b)** Example data from a luciferase assay from a single CFPS time point. A negative control reaction was also set up containing all components of the reaction mix aside from the DNA template, where the volume was substituted with deionised water. Ten readings were taken over a period of 20 min. The luminescence signal from the negative control reaction (grey) was used to subtract background luminescence signal from the raw sample data (yellow) yielding the corrected sample data (green). Readings 2–10 of the corrected sample data were then averaged to determine the average luminescence (red dashed line)

- Using a plate reader able to detect luminescence, read immediately after combining the luciferase assay buffer and CFPS reaction sample (*see Note 23*). Ten readings should be taken over a period of 20 min. For an example of a typical luciferase expression time-course and an example of data taken during a single timepoint, *see Fig. 2*.

4 Notes

1. It is recommended to use a fresh YPD agar plate and that growth is static for 3–5 days. A plate prepared in this way may be reused for a period of up to 2 weeks, if stored at 4 °C for that period of time.
2. It is possible to scale the culture volume; however, the experimentalist must be mindful that subsequent protocol steps must be scaled accordingly. It is also possible to divide larger cultures into smaller 200 mL batches in order to overcome scaling latter parts of this protocol. All further information pertaining to the protocol are in reference to the handling of a single 200 mL batch.
3. During the entire extract preparation process, experimentalists should aim to maintain the temperature of the extract consistently cold throughout if possible to limit potential damage to the cellular machinery.
4. It is critical to accurately record the empty weight of the 50 mL tube and the wet cell weight (WCW) to calculate the optimum volume of lysis buffer in which to resuspend the harvested cells. This ensures the highest possible total protein concentration of the extract post-lysis.
5. Given that the 200 mL cultures are grown overnight, and cell lysate processing takes a full working day, there is a break point after washing the cells that enables the procedure to be split into 2 days, if necessary. We would recommend taking this approach when preparing the lysate for the first time. To flash freeze the pellet, use a dry ice and methanol bath. Hold the tube in the dry ice and methanol bath until the colour has visibly changed throughout the whole pellet. It may also be possible to observe a raised point in the centre of the pellet surface indicating complete freezing throughout. The pellet should be stored at –80 °C. It is worth noting that if using a flash-frozen pellet, the pellet must be fully defrosted on ice prior to continuing with lysis; this can take at least 30 min.
6. Prior to conducting lysis, it is strongly recommended to pre-cool the sample cooling jacket of the cell disruptor, if possible, to help mitigate increases in the sample temperature when processing as this may have a negative impact on the viability of the extract.
7. If low total protein yield is observed after lysate preparation, the most likely cause is insufficient or incomplete lysis. Lysis conditions may need further optimization depending on the chosen method and make and type of cell disruptor. It may be

possible to include a higher number of passes through the disruptor when processing the extract or use of higher pressures. However, please note this may result in an increase in the sample temperature that could negatively impact lysate activity.

8. An 18-gauge needle will be required to remove excess air from the Slide-A-Lyzer™ G2 dialysis cassette.
9. Storing the extract in small-volume aliquots is recommended in order to minimize freeze-thaw cycles, which could negatively impact the viability of the extract over multiple uses. In our workflows, based on six standard runs, aliquots are generally made in 175 μL volume. Stability of the extract for up to 1 year at $-80\text{ }^{\circ}\text{C}$ has been observed.
10. An alternate or “standard” reaction mix is also viable for linked or coupled transcription/translation using the *P. pastoris* cell free system, and was used for its initial development [7]. The alternate reaction mix should be set up in the same manner as outlined in the protocol described here, using the components and concentrations detailed below:
 - 1.25 μL 1 M HEPES-KOH, pH 7.4
 - 2 μL 2 M potassium glutamate
 - 1 μL 1 M magnesium glutamate
 - 1 μL 100 mM DTT
 - 5 μL amino acid mix (0.9 mM each of 19 amino acids, 0.75 mM leucine)
 - 0.75 μL 100 mM ATP
 - 1 μL 100 mM GTP
 - 1 μL 100 mM UTP
 - 1 μL 100 mM CTP
 - 2.5 μL 500 mM creatine phosphate
 - 6.75 μL 4 mg/mL creatine phosphokinase
 - 0.25 μL 40 U/ μL RNase inhibitor
 - 0.5 μL 200 U/ μL T7 polymerase
 - 1 μL Plasmid DNA (40 nM).
11. We obtain higher expression yields when the amino acid mixture is obtained from a commercial source (e.g., RTS Amino Acid Sampler from biotechrabbit, GmbH), although it is possible to make the mixture from individual amino acids in house.
12. In vitro transcription/translation using the protocols described here requires preparation of template DNA containing a T7 promoter and terminator, an internal ribosome entry site (IRES) upstream of the gene of interest to facilitate

cap-independent translation (in addition to a Kozak sequence), and poly(A) tail to promote mRNA stability. Typically, we use a Cricket Paralysis virus (CrPV) IRES, however this can be replaced with another IRES, if desired. All template DNA should be extracted using the Qiagen Plasmid Maxi Kit and resuspended using TE buffer to a concentration of 40 nM. While it may be possible to modify the concentration of DNA template used, the reaction mix component concentrations outlined within this protocol have been optimized for use with 40 nM template. Be mindful that the properties of the DNA template in the cell-free reaction can have a dramatic impact on yield of protein synthesis. This includes the quality of the DNA template, its design, and the quantity added to the reaction.

13. Most of the reaction mix components are water soluble. However, the amino acid mixture is not and will require further care during preparation.
14. The reaction mix is a critical component of the cell-free reactions and so particular attention and care must be taken when formulating it. In our experience, the order of addition of reaction mix components described in the reaction mix protocol must be followed exactly as described. Failure to do so may result in failed or significantly decreased protein synthesis.
15. Our well-established method for preparing the reaction mix involves creating a larger volume master mix, which is then used to create multiple reactions. All components except for the lysate are combined into a single tube, which is mixed by flicking the tube multiple times as described in the main protocol. Individual replicates are then prepared from this mixture by aliquoting into separate tubes. Preparing a master mix helps to alleviate introduction of error as a result of pipetting small volumes. While the downside of this method is the use of more reagent than is strictly necessary for any given number of replicates (for example, for four replicates, we would prepare a master mix containing the volume sufficient for five replicates), we would strongly recommend adopting it to improve reproducibility and decrease error.
16. In our experience, creatine phosphokinase is particularly sensitive to multiple freeze/thaw cycles which can result in decreased enzyme activity and lower cell-free reaction yields. To overcome this problem, we recommend aliquoting the creatine phosphokinase reagent stock into single-use tubes.
17. A negative control reaction should be run simultaneously for each condition tested, consisting of the full reaction mix described above, but with the volume of the template DNA replaced with nuclease-free water.

18. It was shown in a recent paper, that increasing the incubation temperature of *P. pastoris* cell-free protein synthesis reactions could lead to higher yields [8]. We have found that incubating at higher temperatures has a negligible impact on protein synthesis and can be deleterious at temperatures as high as 30 °C. The difference may be due to the use of a protease-deficient strain in Zhang et al.
19. To characterise and troubleshoot the cell-free system, we recommend using luciferase as a reporter protein in the first instance. The system is also capable of producing other proteins of interest, the yield of which can be determined by fluorescence, luminescence, or a protein-specific assay. In general, it will not be possible to visualize proteins synthesised from the cell-free reaction via an SDS-PAGE gel due to the high concentration of contaminating host proteins present in the lysate. This may be overcome by purifying the protein of interest or using an alternate method of visualization such as Western blotting or an assay for protein activity.
20. Amino acid sequence for firefly luciferase used as a reporter enzyme in this protocol.
 >tr|Q27758|Q27758_PHOPY Firefly luciferase OS=Photinus pyralis OX=7054 GN=luc PE=4 SV=1
 MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVP
 GTIAFTDAHIEVNITYAEYFEMSVRLAEAMKRYGLN
 TNHRIVVCSNSLQFFMPVLGALFIGVAVAPANDIY
 NERELLNSMNISQPTVVVFSKKGLQKILNVQKKLPII
 QKIIIMDSKTDYQGFQSMYTFVTSHLPPGFNEYDFV
 PESFDRDKTIALIMNSSGSTGSPKGVALPHRTACVRF
 SHARDPIFGNQIIPDTAILSVPFHHGFGMFTTLGY
 LICGFRVVLMYRFEEELFLRSLQDYKIQSALLVPTLF
 SFFAKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVAK
 RFHLPGIRQGYGLTETTSAILITPEGDDKPGAVGK
 VVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMS
 GYVNDPEATNALIDKDGWLHSGDIAYWDEDEHFFI
 VDRLKSLIKYKGCQVAPAELESILLQHPNIFDAGVAG
 LPGDDAGELPAAVVVLEHGKTMTEKEIVDYVASQV
 TTAKKLRGGVVFVDEVPKGLTGKLDARKIREILIKA
 KKGKSKL.
21. Inconsistent yields across replicates of the luciferase assay are a likely indicator of incomplete reaction mix homogenization or evaporation occurring within the cell-free reaction tube. Ensure that the master mixes are prepared in large enough volumes to minimise pipetting errors, mix the components fully by flicking the tube and spinning down the reaction components before aliquoting, and try to ensure minimal

temperature differences between the incubation temperature within the tubes and the ambient environmental temperature. If reactions lose productivity over time or a noticeable drop in yield occurs, fresh reagent stocks should be prepared. Certain reagents, for example, creatine phosphate and creatine phosphokinase can deteriorate over time even when stored at -20°C .

22. Batches of the luciferase assay buffer can be prepared in advance and stored on ice. However, it is important to protect the buffer from light sources. This can easily be achieved using foil. For each experiment, fresh DTT and ATP solutions should be prepared and kept on ice. All other components of the luciferase assay buffer can be prepared as stock solutions beforehand and stored at room temperature, apart from D-luciferin, which should be stored at -20°C and protected from light.
23. The initial reading within a luciferase assay can show variability due to the instantaneous photon generation and time of exponential decay once the cell-free reaction sample and luciferase assay buffer are combined. The initial values may vary based on the time taken to transfer the plate from bench to plate reader and commence the luminescence reading. To overcome this, we recommend taking an average of the luminescence values beginning from the second timepoint, after which the exponential decay of the luminescent signal has plateaued [11] (Fig. 2). Each hourly timepoint therefore consists of an average of 9 readings taken over a period of 20 min, for three biological repeats, minus the average of the negative control reactions for that particular condition. The standard deviation for each hourly timepoint is therefore the deviation of the 9 readings taken over a period of 20 min for 3 biological replicates taking into account the standard deviation of the negative control reactions using error propagation.

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A *Streptomyces*-Based Cell-Free Protein Synthesis System for High-Level Protein Expression

Huiling Xu, Wan-Qiu Liu, and Jian Li

Abstract

With the rapid development of cell-free biotechnology, more and more cell-free protein synthesis (CFPS) systems have been established and optimized for protein expression in vitro. Here, we aim to improve the productivity of a newly developed *Streptomyces*-based CFPS system. Protein translation in CFPS systems depends on the entire endogenous translation system from cell lysates. However, lysates might lack such translation-related elements, limiting the efficiency of protein translation and therefore the productivity of CFPS systems. To address this limitation, we sought to add protein translation related factors to CFPS reactions. By doing this, the protein yield of EGFP was significantly improved up to approximately 400 µg/mL. In this chapter, we mainly describe the preparation of *Streptomyces* cell extracts, expression and purification of nine translation related factors, and optimization of the *Streptomyces*-based CFPS system for enhanced protein expression.

Key words Cell-free protein synthesis, *Streptomyces*, Translation related factors, Protein expression, Synthetic biology

1 Introduction

Cell-free protein synthesis (CFPS) systems are emerging as effective and powerful platforms for in vitro protein expression. Such systems are widely used because they can separate cell growth from protein synthesis, allow direct control and manipulation, and enable high-yielding production. So far, a broad range of proteins have been expressed by using CFPS systems, including, but not limited to, therapeutic proteins, membrane proteins, metalloproteins, unnatural amino acid modified proteins, and large nonribosomal peptide synthetases [1–5]. While many CFPS systems have been developed on the basis of prokaryotic and eukaryotic organisms, the *Escherichia coli*-based CFPS system is the most robust platform for many applications [6–8]. However, the *E. coli* CFPS system might not be always suitable to express all kinds of proteins, for example, those from *Streptomyces* microorganisms coded by

high GC-content genes. In this context, new CFPS systems derived from several *Streptomyces* strains have recently been developed, albeit the productivity of these platforms is still lower than that of the well-developed *E. coli* CFPS system (often ~1000 µg/mL) [9–11].

In CFPS reaction systems, cell extracts (or lysates) contain all of the necessary components (e.g., ribosomes, translation initiation and elongation factors, release factors, and ribosome recycling factors) for the *in vitro* translation. However, the amounts of these translation related elements might not be enough to support a highly efficient protein translation, leading to a low productivity. Therefore, if this is true, supplementation of such translation related factors to CFPS systems could increase the level of protein expression. In this chapter, we aim to improve the productivity of a crude extract based *Streptomyces* CFPS system. To this end, we describe our standard protocols in details for the preparation of *Streptomyces* cell extracts, expression and purification of translation related factors (i.e., initial factors (IF1, IF2, and IF3), elongation factors (EF-Tu, EF-Ts, and EF-G), release factors (RF1 and RF2), and the ribosome recycle factor (RRF)), and optimization of the *Streptomyces*-based CFPS system for enhanced protein expression.

2 Materials

2.1 Preparation of *Streptomyces* Cell Extracts

1. The *Streptomyces lividans* TK24 strain (*see Note 1*).
2. Sterilized yeast extract–malt extract (YEME) medium: yeast extract 3 g/L, peptone 5 g/L, malt extract 3 g/L, glucose 10 g/L, sucrose 340 g /L, MgCl₂ 5 mM (*see Note 2*).
3. S30 washing buffer: 10 mM HEPES–KOH pH 7.5, 10 mM MgCl₂, 1 M NH₄Cl, 5 mM β-mercaptoethanol. S30 buffer: 50 mM HEPES–KOH pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, 5 mM β-mercaptoethanol. S30 buffer plus 10% (v/v) glycerol: 50 mM HEPES–KOH pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, 5 mM β-mercaptoethanol, and 10% (v/v) glycerol.
4. Baffled flasks: 100 mL, 500 mL, and 2.5 L.
5. Orbital shaker.
6. Centrifuge.
7. Vortex mixer.
8. pH meter.
9. Q125 Sonicator (Qsonica, Newtown, CT).
10. Liquid nitrogen.
11. –80 °C refrigerator.

Table 1
The primer sequences of nine translation related proteins^a

Name	Primer sequence (5'→3')
IF-1_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GCCAAGAAGCAAGGTGCCATCGAGATCGAA</i>
IF-1_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACTTGTACCGGTAGACGATCCGGCCACG</i>
IF-2_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GCTAAGGTCCGGGTCTACGAACTCGCCAAG</i>
IF-2_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACACCCGCGGCTTCTCGCGCATC</i>
IF-3_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>AGCGCCGAGCCCCGCATCAACGACC</i>
IF-3_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACGCTTCGGCAGGTGCCTCGGCCGG</i>
EF-Tu_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GCGAAGGCGAAGTTCGAGCGGACTAAG</i>
EF-Tu_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACTTGTGTGATCTTGTTGACCTGGCCGGC</i>
EF-Ts_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GCGAACTACACCGCCGCGGACGTCA</i>
EF-Ts_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTAGATGCCGACCTTGATGCGCGAGAAGCG</i>
EF-G_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GCTACCACCTTCACTTGACCTGGCCAAGGTC</i>
EF-G_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACTCGCCCTTGGCCTTCGCGATGATC</i>
RF-1_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>TTCGAGGCCGTCGAGGAACTCGTCGCCGAG</i>
RF-1_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACGCGGCGGCGAGCTTGGCCGCC</i>
RF-2_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>GAGTCGATCGAGGCCGTCCTGGACCTCGAC</i>
RF-2_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTACTTCTCCCCTGCTTGCGCCAGCGAAT</i>
RRF_F	TGCCGCGCGGCAGCC <u>CATATG</u> <i>ATCGAAGAGACCCTCCTCGAGGCCGAG</i>
RRF_R	GCGGCCGCAAGCTT <u>GTCGAC</u> <i>TTAGACCTCGAGCAGCTCCGCTTCCTTGTG</i>

^aHomologous arms are bolded. Restriction enzyme sites are underlined. Sequences flanking each gene are italic

2.2 Expression and Purification of Protein Translation
Related Factors: IF1, IF2, IF3, EF-Tu, EF-Ts, EF-G, RF1, RF2, and RRF

1. Genome Extraction SET buffer: 75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5.
2. Lysozyme (50 mg/mL in water).
3. The primers used for gene amplification are listed in Table 1. All gene sequences are available from NCBI (GenBank: CP009124).
4. SDS-PAGE gel electrophoresis systems.
5. GenGreen.
6. *E. coli* DH5α and *E. coli* BL21 Star(DE3).
7. Plasmid of pET-28a.
8. Water bath.
9. Restriction enzymes (NdeI and SalI).
10. ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China).

11. Luria–Bertani (LB) medium and Terrific Broth (TB) medium (*see* **Note 3**).
12. Isopropyl- β -D-thiogalactopyranoside (IPTG) and antibiotics.
13. Ni-NTA column.
14. Lysis buffer: 40 mM Tris, 50 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8.0.
15. Washing buffer: 40 mM Tris, 50 mM NaCl, 10 mM/30 mM/50 mM imidazole, 10% glycerol, pH 8.0 (*see* **Note 4**).
16. Elution buffer: 40 mM Tris, 200 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0.
17. Desalting buffer: 40 mM Tris–HCl, 25 mM NaCl, pH 8.0 (*see* **Note 4**).
18. Ultrafiltration columns (3, 10, 30, and 50 K cutoff).

2.3 Detection of Protein Yields

1. The reporter protein enhanced green fluorescent protein (EGFP).
2. Bovine serum albumin (BSA).
3. Quick Start™ Bradford reagent (Bio-Rad).
4. qPCR instrument.
5. Microplate reader, capable of fluorescence measurements.
6. 96-well plates with flat bottom.

3 Methods

3.1 Cultivation of *S. lividans*

1. Inoculate 600 μ L of the glycerol stock of *S. lividans* into 6 mL of YEME medium in a test tube and incubate at 30 °C in an orbital shaker at 220 rpm for 24 h.
2. Inoculate 1 mL of the preculture to 25 mL of fresh YEME medium in a 100 mL flask, followed by 24 h cultivation at 30 °C and 220 rpm.
3. Inoculate 2 mL of the culture to 50 mL of fresh YEME medium in a 500 mL baffled flask for 24 h cultivation at 30 °C and 220 rpm.
4. Then, the *Streptomyces* cells are grown in 1 L of YEME medium in a 2.5-L baffled flask by inoculation of 20 mL of culture from the last step cultivation for 16 h.

3.2 Collection and Washing of Cells

1. After 16 h cultivation (mid-exponential growth phase), the cells are harvested by centrifugation in 4 \times 250 mL centrifuge tubes at 7000 $\times g$ and 4 °C for 20 min (*see* **Note 5**).
2. Cell pellets in each 250 mL tube are resuspended with 200 mL of cold S30 washing buffer. Centrifuge at 7000 $\times g$ and 4 °C for 15 min. Repeat this step for another wash (*see* **Note 6**).

3. Transfer the whole pellet to a 50 mL centrifuge tube. Resuspend cell pellets with 40 mL of cold S30 buffer and then centrifuge at $7000 \times g$ for 10 min at 4 °C. Repeat this step (*see Note 7*).
4. Discard supernatant and measure the wet weight of cell pellets (*see Note 8*).
5. Add S30 buffer plus 10% (v/v) glycerol to the tube and resuspend the pellets with vortex. At this step, 1 g of wet cells are resuspended with 1 mL of S30 buffer plus 10% glycerol.

3.3 Disruption of Cells

1. Place the 50 mL centrifuge tube with suspended cells in an ice-water bath.
2. Use Q125 Sonicator to disrupt cells with a probe (3 mm diameter) (*see Note 9*).
3. Disrupt the cells with the program: 45 s on, 59 s off, and 50% amplitude (*see Note 10*). The total time of disruption depends on the input energy (Joules) and usually around 3.5 g of wet cells are disrupted with an input energy of ~1000 J.
4. Transfer 1 mL of lysed cells to new 1.5 mL centrifuge tubes. Then immediately centrifuge the lysates at $16,000 \times g$ for 30 min at 4 °C. During the centrifugation, a 15 mL centrifuge tube and several new 1.5 mL centrifuge tubes are prepared and placed on ice for precooling.
5. After centrifugation, transfer all supernatant to a chilled 15 mL centrifuge tube and gently mixed (*see Note 11*).
6. Aliquot 500 μ L of crude cell extracts from the last step into each chilled 1.5 mL centrifuge tube.
7. Flash-freeze them in liquid nitrogen for at least 2 min. Then store them at -80 °C until use.

3.4 Construction of Plasmids for the Expression of Nine Translation Related Factors

The translation-related genes are inserted into the pET-28a backbone between the restriction sites of NdeI and SalI. Enzyme digestion and homologous recombination are used for the cloning. The steps for generating nine expression vectors are summarized in Fig. 1 and performed as follows:



Fig. 1 The process for constructing expression vectors of nine translation related proteins

1. Extract the *Streptomyces* genome as reported previously [12] with slight modifications. Briefly, grow cells in 30 mL of YEME medium at 30 °C overnight and harvest cells by centrifugation (7000 × *g*, 15 min, 4 °C). Wash the pellet with 15 mL of SET buffer and then centrifuge (7000 × *g*, 10 min, 4 °C). Resuspend the pellet with 5 mL of SET buffer. Add 100 µL of lysozyme (a final concentration of 1 mg/mL) and incubate at 37 °C for 0.5–1 h. Then add 300 µL of 20% SDS and incubate at 55 °C with occasional inversion for 2 h. Add 2 mL of 5 M NaCl, mix thoroughly by inversion and cool to 37 °C. Add equal volume (here is 7.4 mL) of phenol-chloroform-isoamyl alcohol mixture (25:24:1) and incubate at room temperature for 0.5 h with frequent inversion. Centrifuge at 12,000 × *g* for 0.5 h. Transfer the aqueous phase to a new tube and add equal volume of chloroform. Mix by inversion at room temperature for 20 min and then centrifuge (12,000 × *g*, 0.5 h). Transfer the aqueous phase to a fresh tube, add 0.6 volume of isopropanol (*see* **Note 12**) and gently invert the tube to precipitate DNA. Transfer the DNA to a new tube and rinse with 70% ethanol, dry with air and dissolve in nuclease-free water at 55 °C. The extracted genome is stored at –20 °C.
2. Design forward and reverse primers of nine target genes with homologous arms and restriction enzyme sites. The framework of primer design is shown in Fig. 2.
3. Nine target genes are PCR amplified from genomic DNA of the *S. lividans* strain with forward and reverse primers (Table 1). PCR reactions are performed using Q5[®] High-Fidelity DNA Polymerase. The plasmid pET-28a is digested by NdeI and SalI to generate a linear backbone. The PCR reaction mixture and program are listed in Table 2. The digestion reaction is performed as shown in Table 3.
4. Use agarose gel electrophoresis to determine whether the linear target gene sequences and the digested plasmid have correct sizes (*see* **Note 13**).
5. PCR amplified nine gene fragments are directly purified with a PCR purification kit. The plasmid backbone is purified by a PCR gel purification kit. Purified DNA products are stored at –20 °C.

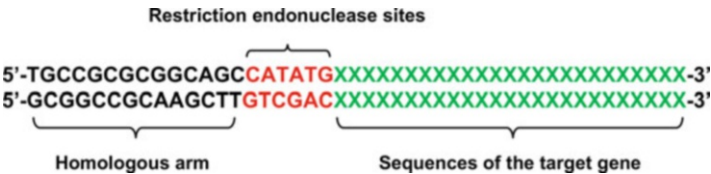


Fig. 2 The framework of primer design. The first and second row of sequence represent a forward and a reverse primer, respectively

Table 2
PCR reaction mixture and protocol

PCR reaction mixture		
Component	25 μ L reaction	Final concentration
5 \times Q5 reaction buffer	5 μ L	1 \times
2.5 mM dNTPs	2 μ L	200 μ M
10 μ M forward primer	1.25 μ L	0.5 μ M
10 μ M reverse primer	1.25 μ L	0.5 μ M
Template DNA	2.5 ng (plasmid) 500 ng (genomic DNA)	0.1 ng/ μ L (plasmid), 20 ng/ μ L (genomic DNA)
Q5 high-fidelity DNA polymerase	0.25 μ L	0.02 U/ μ L
5 \times Q5 high GC enhancer (option)	(5 μ L)	(1 \times)
Nuclease-free water	To 25 μ L	/

PCR reaction program		
Step	Temperature	Time
Initial denaturation	98 $^{\circ}$ C	30 s
35 cycles	98 $^{\circ}$ C	5–10 s
	72 $^{\circ}$ C	10–30 s
	72 $^{\circ}$ C	20–30 s/kb
Final extension	72 $^{\circ}$ C	10 min
Hold	4 $^{\circ}$ C	/

6. Use ClonExpress II One Step Cloning Kit to ligate the plasmid backbone and each fragment of nine target genes.
7. Transform ligated products into competent cells of *E. coli* DH5 α and plate the cells on LB agar plates with kanamycin. Incubate the plates at 37 $^{\circ}$ C for 16 h.
8. Pick single colonies to make index plates and incubate at 37 $^{\circ}$ C for 12 h.
9. Verify the correct size of each gene by colony PCR. The PCR reaction mixture and program are listed in Table 4.
10. Prepare plasmids for DNA sequencing to confirm the correctness of each plasmid.
11. Make glycerol stocks of each *E. coli* DH5 α strain with corresponding plasmid. All stocks are stored at -80° C.

Table 3
Fast digestion reaction mixture and protocol

Fast digestion (NdeI and SalI) mixture		
Component	Volume	
MilliQ water	To 20 µL	
10 × FastDigest green buffer	2 µL	
DNA	1 µg	
FastDigest enzyme (NdeI and SalI)	1 µL	

Fast digestion conditions		
Step	Temperature	Time
1	37 °C	1 h
2	65 °C	20 min

Table 4
Colony PCR reaction mixture and protocol

Colony PCR reaction mixture		
Component	50 µL reaction	Final concentration
DreamTaq green PCR master mix (2×)	25 µL	1×
10 µM forward primer	2.5 µL	0.5 µM
10 µM reverse primer	2.5 µL	0.5 µM
Template DNA	10 pg ~ 1 µg	/
Nuclease-free water	To 50 µL	/

PCR reaction program		
Step	Temperature	Time
Initial denaturation	95 °C	1–3 min
25 cycles	95 °C	30 s
	72 °C	30 s
	72 °C	1 min/kb
Final extension	72 °C	10 min
Hold	4 °C	/

3.5 Expression and Purification of Nine Translation Related Factors

1. Transform the constructed expression vectors into competent cells of *E. coli* BL21 Star(DE3), respectively, and plate them on LB agar with kanamycin at 37 °C for 16 h.
2. Select single colony from each plate and cultivate them in 25 mL of LB overnight at 37 °C and 250 rpm.
3. Transfer 10 mL of overnight culture into 500 mL of fresh LB/TB medium and incubate at 37 °C and 250 rpm for about 2.5–3 h when the OD₆₀₀ reaches 0.6–0.8.
4. Add IPTG with a final concentration of 0.5 mM to induce protein expression and reduce the cultivation temperature to 30 °C for additional incubation at 250 rpm for 8 h (*see Note 14*).
5. Cells are collected by centrifugation at $5000 \times g$ and 4 °C for 10 min and discard supernatant. Then transfer the pellets (around 3 g) to 50 mL centrifuge tubes.
6. The pellets are washed twice with lysis buffer. Then add about 30 mL of lysis buffer to the tubes and mix by vortex.
7. Disrupt the cells by sonication with the program: 10 s on, 10 s off at 50% amplitude for 15 min.
8. Centrifuge the disrupted cells at $12,000 \times g$ and 4 °C for 30 min. Collect the supernatant in a new 50 mL centrifuge tube and then centrifuge again for 20 min.
9. Collect the supernatant in new centrifuge tubes and place the tubes on ice.
10. Filter the crude protein solution through 0.22 µm filter membrane by syringe.
11. Purify proteins by Ni-NTA columns. Elute the column using buffers with 10, 30, and 50 mM imidazole, respectively. Finally, target proteins are eluted with elution buffer containing 500 mM imidazole.
12. Mix the purified proteins and desalting buffer in a volume ratio of 2:3. Desalt and concentrate the proteins by ultrafiltration tubes with different molecular weight cutoffs.
13. Run SDS-PAGE gels to verify each protein's purity (Fig. 3).

3.6 Make Protein Standard Curve and Detect the Concentration of Nine Proteins

1. Dilute the BSA protein (1 mg/mL) to 0.2, 0.4, 0.6, and 0.8 mg/mL with desalting buffer. Mix 20 µL of diluted protein solution and 1 mL of Bradford reagent and detect the absorption (A) at 595 nm by spectrophotometer. The standard curve is shown in Fig. 4.
2. Measure the concentration of each purified protein.
3. Dilute nine proteins each with concentrations at 1, 2, and 4 µM.

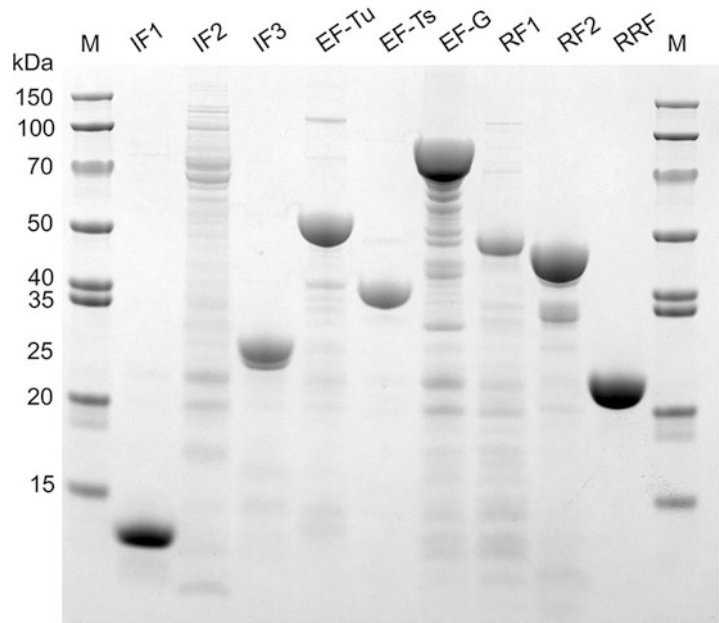


Fig. 3 SDS-PAGE analysis of nine purified proteins. IF1 (8.4 kDa), IF2 (105.7 kDa), IF3 (24.1 kDa), EF-Tu (43.7 kDa), EF-Ts (29.8 kDa), EF-G (77.7 kDa), RF1 (39.5 kDa), RF2 (39.4 kDa), and RRF (20.8 kDa). Note that IF2 was not successfully purified. (Reprinted (adapted) with permission from ref. [13]. Copyright © 2020 American Chemical Society)

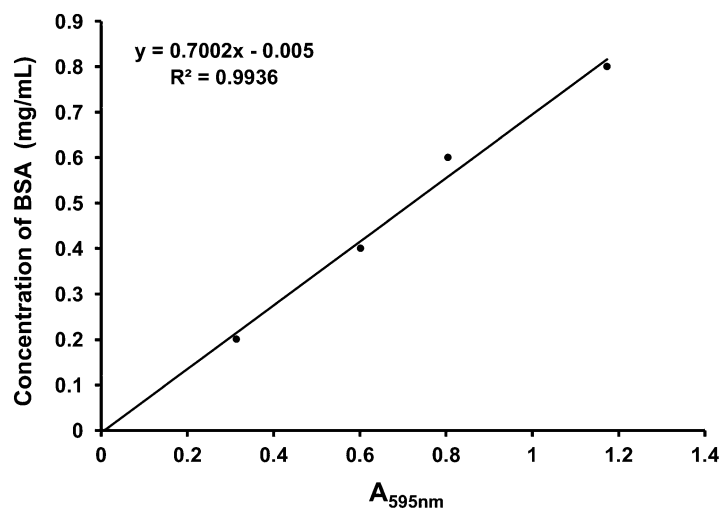


Fig. 4 The stand curve of BSA protein

Table 5
***Streptomyces*-based CFPS reaction mixture and components**

Composition of the <i>Streptomyces</i> -based CFPS reaction mixture	
Component	Volume
S30 buffer ^a	3 μ L
Mg(OAc) ₂	0.7 μ L
Synthesis mix ^a	4 μ L
Template	200 ng
T7 RNA polymerase ^a	0.3 μ L
Cell extract	5 μ L
Nuclease-free water	To 15 μ L

^aThe components of S30 buffer and synthesis mix are shown below. T7 RNA polymerase is a commercial product (Thermo Scientific™, EP0113)

3.7 Translation
Related Factors
Enhance EGFP
Expression In Vitro

The reaction components and their final concentrations of the *Streptomyces*-based CFPS system are listed in Table 5 (*see Note 15*). The reporter protein is EGFP. The plasmid used in CFPS reactions is pJL1-EGFP [9].

1. Thaw all of the reagents required for the CFPS on ice (*see Note 16*).
2. Set up CFPS reactions by adding nuclease-free water first, followed by addition of other reagents in the order shown in Table 5.
3. Transfer 13 μ L of reaction mixture to qPCR tubes (*see Note 17*). All reactions are incubated in a qPCR instrument for 8 h at 23 °C. The fluorescence is recorded every 5 min.
4. After the reaction, EGFP yields are detected by a microplate reader. Two microliters of the CFPS sample are mixed with 48 μ L of nuclease-free water and placed in a 96-well plate with flat bottom. Then, measurements of the EGFP fluorescence are carried out with excitation and emission wavelength at 485 and 528 nm, respectively. The fluorescence of EGFP is converted to concentration (μ g/mL) according to a linear standard curve made in house. All measurements are performed at least in triplicate.
5. Optimize tRNA concentration per CFPS reaction. Add tRNA at 0, 2, 3, 4, or 5 μ g to each 15 μ L of reaction. The result shows that the yield of EGFP reaches the highest when adding 3 μ g of tRNA to 15 μ L of CFPS reaction (Fig. 5a).
6. Investigate the effects of nine translation related factors on EGFP expression. Each purified factor is added to the reaction with concentrations from 1 to 4 μ M. The data indicate that five

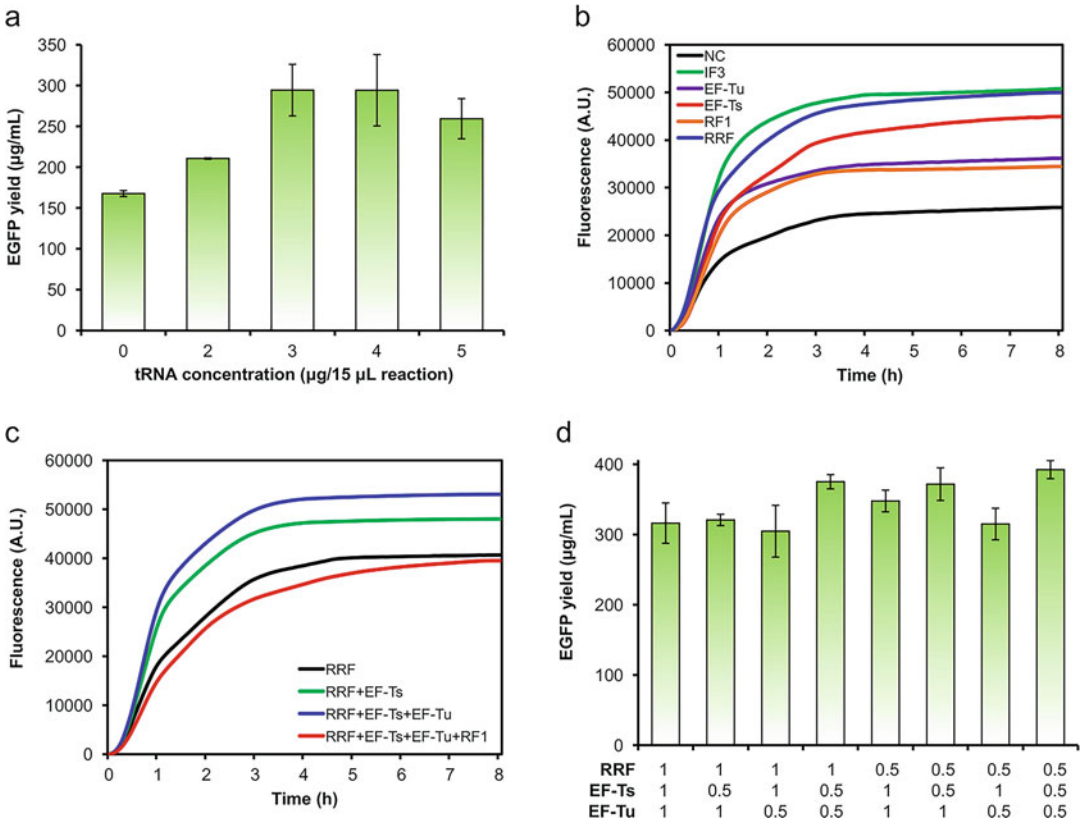


Fig. 5 (a) Effect of tRNA concentration on cell-free synthesis of EGFP. Representative time courses of EGFP synthesis with (b) five positive single factors and (c) combined factors. NC, negative control without addition of any single factor. (d) Optimization of the concentration (μM) of RRF, EF-Ts, and EF-Tu on EGFP synthesis. Values show means with error bars representing standard deviations (S.D.) of at least 3 independent experiments. (Reprinted (adapted) with permission from ref. [13]. Copyright © 2020 American Chemical Society)

- out of the nine translation factors (IF3, EF-Tu, EF-Ts, RF1, and RRF) have obviously positive effects on EGFP synthesis when supplemented with 1 μM of each factor (Fig. 5b). Of note, IF3 and RRF are the most effective factors to enhance EGFP expression.
- Investigate the synergistic effect of the five positive factors. The result indicates that the best combination is RRF, EF-Ts, and EF-Tu each with 1 μM per reaction, giving rise to the highest EGFP expression (Fig. 5c).
 - Optimize the concentrations of RRF, EF-Ts, and EF-Tu (0.5 and 1 μM) per CFPS reaction. The optimum combination of each factor is found to be 0.5 μM, generating the highest EGFP yield of 392.4 ± 12.9 μg/mL (Fig. 5d).

Components of S30 buffer and Synthesis Mix		
Component	Stock concentration	Final concentration
<i>S30 buffer</i>		
HEPES–KOH, pH 7.5	1 M	50 mM
MgCl ₂	1 M	10 mM
NH ₄ Cl	4 M	500 mM
β-Mercaptoethanol	/	0.035% (v/v)
Nuclease-free water	/	To the total volume
<i>Synthesis mix</i>		
HEPES–KOH, pH 8.2	2 M	195 mM
DTT	0.55 M	6.7 mM
ATP	38 mM	4.6 mM
CTP/UTP/GTP	88 mM	3.2 mM
Phosphoenolpyruvate (PEP)	0.42 M	102.5 mM
Amino acids	50 mM	1.2 mM
PEG8000	40%	7.30%
Folinic acid	2.7 mg/mL	0.13 mg/mL
Pyruvate kinase	1408 U/mL	234.3 U/mL
NH ₄ OAc	4.2 M	136.3 mM
KOAc	8.4 M	272.6 mM
tRNA (option)	50 mg/mL	0, 0.5, 0.75, 1, 1.25 mg/mL
Nuclease-free water	/	To the total volume

4 Notes

1. The glycerol stock of *Streptomyces lividans* TK24 is stored at -80°C .
2. To prepare the liquid YEME medium, sucrose is gradually added to water and continuously stirred until completely dissolved. MgCl₂ is separately prepared and sterilized by filtration through a 0.22 μm membrane. Before inoculation of *S. lividans* to the medium, MgCl₂ is added to YEME at a final concentration of 5 mM.
3. TB is used to cultivate *E. coli* for recombinant protein expression if the protein cannot be well expressed using the LB medium.

4. For a better purification of each protein, the concentration gradient of imidazole in the washing buffer as well as the concentration of NaCl in the desalting buffer can be adjusted according to different proteins.
5. Carefully discard the supernatant and avoid the loss of cell pellets.
6. To resuspend cell pellets, use hands to shake the centrifuge tube without using a vortex mixer because the *Streptomyces* pellet is loose.
7. Perform this step the same as in **Note 6**.
8. The empty centrifuge tube is weighed in advance. The centrifuge tube with cell pellets is placed upside down on a piece of clean paper to completely remove supernatant. We often obtain around 5 g cell pellets from 1 L of culture.
9. Before disruption, the probe needs to be cleaned in the order of MilliQ water, 75% ethanol, and MilliQ water. The sonication is performed directly in the 50 mL tube, because resuspended cells are very sticky and hard to be distributed to small tubes (e.g., 1.5 mL tubes).
10. During the sonication process, keep the 50 mL tube always in an ice–water bath.
11. Carefully transfer the supernatant and avoid pipetting out the bottom precipitate.
12. When isopropanol is added, using a pipette tip to stir and collect precipitated DNA.
13. Usually, 1% agarose gels are used for the analysis of DNA fragment size.
14. The cultivation conditions for protein expression are optimized with a final IPTG concentration of 0.5 mM and a cultivation temperature of 30 °C. The cultivation time is usually 8 h.
15. As reported previously, the optimum plasmid template concentration is 300 ng and the cell extract volume is 6 μ L per 15 μ L of CFPS reaction [9, 10]. However, in order to provide enough space for adding protein translation related factors, we use 200 ng plasmid and 5 μ L of cell extract to run each CFPS reaction in this work.
16. It often takes 30–60 min to thaw all frozen reagents. After thawing, vortex each reagent before adding to the final reaction mixture. Do not vortex cell extract, T7 RNA polymerase, and each purified translation related proteins.
17. Carefully transfer each 13 μ L of mixture and avoid introducing air bubbles to qPCR tubes.

Acknowledgments

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Chapter 6

In Vitro Reconstitution Platforms of Mammalian Cell-Free Expressed Membrane Proteins

Hossein Moghimianavval, Yen-Yu Hsu, Alessandro Groaz, and Allen P. Liu 

Abstract

Membrane proteins are essential components in cell membranes and enable cells to communicate with their outside environment and to carry out intracellular signaling. Functional reconstitution of complex membrane proteins using cell-free expression (CFE) systems has been proved to be challenging mainly due to the lack of necessary machinery for proper folding and translocation of nascent membrane proteins and their delivery to the supplied synthetic bilayers. Here, we provide protocols for detergent-free, cell-free reconstitution of functional membrane proteins using HeLa-based CFE system and outline assays for studying their membrane insertion, topology, and their orientation upon incorporation into the supported lipid bilayers or bilayers of giant unilamellar vesicles as well as methods to isolate functional translocated cell-free produced membrane proteins.

Key words Cell-free expression, Membrane proteins, In vitro reconstitution, HeLa-based cell-free expression, SUN, Linker of nucleoskeleton and cytoskeleton complex, Encapsulation, Giant unilamellar vesicle

1 Introduction

Bottom-up in vitro reconstitution into biological membranes is a promising approach to demystify complex interactions between different proteins in isolated cellular functions [1]. Despite recent advances, using purified membrane proteins or detergent-mediated reconstitution approaches still have certain drawbacks [2–5]. For instance, the expression of recombinant proteins in conventional cell culture systems might cause growth retardation or lysis of the host cells. Also, toxic effects might be attributed to the overproduction of heterologous proteins. Additionally, residual detergents in membranes can affect protein function and membrane integrity of liposomes. Moreover, arbitrary protein insertion into the lipid

Hossein Moghimianavval and Yen-Yu Hsu contributed equally to this work.

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membrane hinders the probing of intracellular and extracellular structure and functions of membrane proteins. To overcome these challenges, cell-free methods that focus on production of proteins outside the cellular environment offer promising solutions. Cell-free expression (CFE) systems that rely on de novo synthesis of proteins using the translation–transcription (TXTL) machinery of cell lysates [6] are becoming popular. Using such platforms, TXTL machineries are fully devoted to expressing the protein(s) of interest. This yields an efficient expression system and as an in vitro system, there is a high degree of control over its components. Therefore, CFE systems have significant advantages over detergent-free reconstitution and can circumvent the limitations mentioned above [7–10].

Membrane proteins play essential roles in different cellular contexts by providing communication pathways between the intra- and extra-cellular environments through receptors, transporters, and channels. Despite their importance, CFE platforms for studying membrane proteins have not been straightforward. The main barrier is the inability for common CFE systems, including *E. coli*, to produce functional eukaryotic membrane proteins due to the lack of necessary components such as molecular chaperons and post-translational modifications. Even though prokaryotic CFE systems have gained significant interest and are well established in literature, there is increasing attention to eukaryotic CFE systems [11, 12]. A few eukaryotic CFE systems have been utilized for detergent-free production and incorporation of membrane proteins. For instance, microsomal bodies in the cell-free lysates of Sf-21 insect cells have been isolated for the incorporation of pH-sensitive bacterial membrane protein KcsA by Dondopati et al. [13]. Among mammalian CFE systems, HeLa-based cell-free protein synthesis system (available commercially) is a great candidate for de novo production of complex proteins since it contains endogenous microsomal structures that mediate the direct translocation and post-translational modifications of nascent proteins.

While the cell-free production of membrane proteins is a milestone toward a robust platform for in vitro reconstitution of functional membrane proteins, establishing a proper environment in which the cell-free synthesized membrane proteins reside remains challenging. Depending on the application, several platforms have been developed to study membrane proteins topology, function, and biophysical properties [14]. Supported lipid bilayers, for instance, are popular platforms for analyzing the functionality of reconstituted membrane proteins, specifically ion channels since they feature lipid tunability and easy channel recording process [15]. Also, recently, giant unilamellar vesicles (GUVs) have gained attention as they can confine purified components or CFE reactions in cell-sized compartments that allows reconstitution of cellular

mechanisms in a micron-sized space [16–18] as well as creation of synthetic cells that can sense and respond to external stimuli through their membrane proteins [19, 20].

Here, we outline several platforms for reconstitution of the linker of nucleoskeleton and cytoskeleton complex (LINC) assembly membrane proteins SUN1 and SUN2 as our model membrane proteins [21]. The approaches are translatable to other membrane proteins. We describe a HeLa-based CFE system and illustrate that endogenous microsomes that contain translocated membrane protein can be isolated. Additionally, we offer a strategy to investigate the localization of membrane proteins on the supplied lipid membranes by supplementing the CFE reaction with supported lipid bilayers with excess reservoirs (SUPER templates) [22]. The orientation of membrane proteins inserted into the lipid bilayer is further analyzed using protease digestion. Moreover, we introduce a method to encapsulate the CFE reactions in GUVs to visualize the localization of membrane proteins in the membrane of GUVs (Fig. 1).

The protocols described here are not unique to the HeLa-based CFE system and can provide rapid assays to investigate the ability of other mammalian CFE systems in the reconstitution of

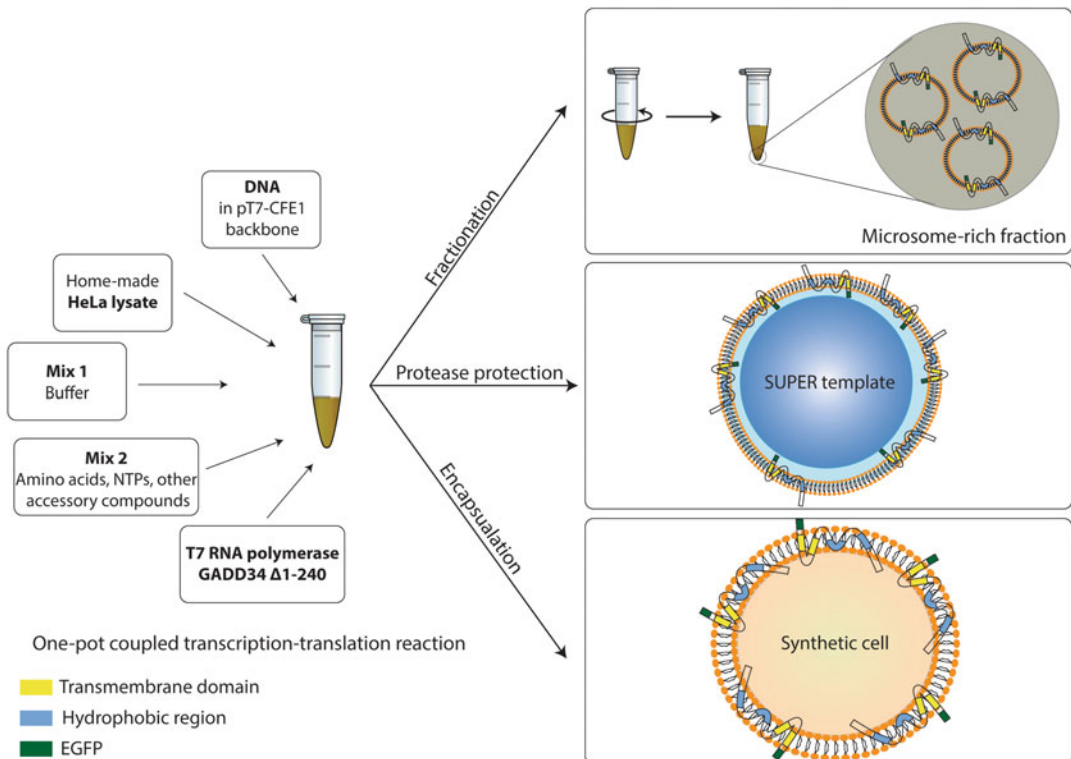


Fig. 1 Schematics of HeLa CFE system depicting in vitro platforms for isolating translocated membrane proteins and reconstituting the cell-free synthesized membrane proteins into membranes

membrane proteins. Given the rising attention toward utilizing CFE technologies to recreate certain cellular processes in cell-sized compartments, the need for functional reconstitution of membrane proteins is inevitable. The assays we present here provide a powerful toolbox for characterizing cell-free membrane protein synthesis and their functional reconstitution.

2 Materials

2.1 Cell-Free Expression

2.1.1 HeLa Cell Culture

1. Cell culture medium: DMEM, 10% fetal bovine serum, $1 \times$ GlutaMAX, 100 IU/mL penicillin, 100 μ g/mL streptomycin. Store at 4 °C.
2. D-PBS.
3. 0.05% trypsin–EDTA. Store at 4 °C.
4. Tissue culture petri dishes (150 mm diameter).

2.1.2 HeLa Lysate Preparation

1. Washing buffer: 35 mM HEPES–KOH, pH 7.5, 140 mM NaCl, and 11 mM glucose. Store at 4 °C.
2. Extraction buffer: 20 mM HEPES–KOH, pH 7.5, 45 mM potassium acetate, 45 mM KCl, 1.8 mM magnesium acetate, 1 mM dithiothreitol (DTT). Store at 4 °C without DTT. Add DTT just before use.
3. High K buffer: 20 mM HEPES–KOH, pH 7.5, 945 mM potassium acetate, 945 mM KCl, 1.8 mM magnesium acetate, 1 mM DTT. Store at 4 °C without DTT. Add DTT just before use.
4. BeadBug bead-beater (Benchmark Scientific) or equivalent.
5. Bead-beating tubes containing 0.1 mm zirconium beads (Benchmark Scientific, item number: D1032-01, conical bottom if using BeadBug).

2.1.3 Assembling TX-TL Coupled CFE Reaction

1. HeLa lysate.
2. Plasmid DNA containing the gene of interest after an internal ribosome entry site (IRES) sequence. The recommended commercial product is the expression vector pT7-CFE1 from Thermo Fisher Scientific (*see Note 1*).
3. Amino acid mixture: 5–100 mM amino acid mixture (Sigma Aldrich). Store in aliquots at –80 °C.
4. Mixture 1: 50 mM magnesium acetate ($\text{Mg}(\text{OAc})_2$), 170 mM K-HEPES (pH 7.5). Store in aliquots at –80 °C.
5. Mixture 2: 7.8 mM K-HEPES (pH 7.5), 12.5 mM ATP, 8.36 mM GTP, 8.36 mM CTP, 8.36 mM UTP, 200 mM creatine phosphate, 0.6 mg/mL creatine kinase, $1 \times$ amino acid mixture, 5 mM spermidine. Store in aliquots at –80 °C.

6. T7 RNA polymerase and GADD34 are expressed in *E. coli* and purified as previously reported [23]. Store in aliquots at -80°C .

2.2 Airfuge Fractionation Assay

1. Plasmid DNA containing the gene of interest under the T7 promoter after an IRES sequence.
2. Extraction buffer: 20 mM HEPES-KOH, pH 7.5, 45 mM potassium acetate, 45 mM KCl, 1.8 mM magnesium acetate. Store at 4°C .
3. Low volume clear flat bottom 384-well plate.
4. Polyacrylamide gels, use precast polyacrylamide gels with a gradient concentration of 4–20% if desired.
5. Ultracentrifuge.

2.3 Membrane Protein Incorporation Assay

2.3.1 SUPER Template Preparation

1. Lipids: 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS), L- α -phosphatidic acid (egg, chicken) (sodium salt) (Egg-PA), and cholesterol.
2. 100 nm liposome extruder.
3. 5 M NaCl solution, sterile-filtered.
4. Silica beads (preferably $5\ \mu\text{m}$).

2.3.2 Incorporation and Protease Protection Assay

1. DNA plasmids (stock concentration of 500 ng/mL): pT7-CFE1-EGFP-SUN1 and pT7-CFE1-EGFP-SUN2.
2. $1\times$ phosphate buffer saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 .
3. Glass slides and coverslips.
4. Double-sided tape.
5. Lyophilized *S. griseus* Pronase.

2.4 Vesicle Encapsulation System

1. DNA plasmids (stock concentration of 500 ng/mL): pT7-CFE1-EGFP-SUN2 and pT7-CFE1-EGFP-YAP.
2. OptiPrep.
3. Lipids: stock concentration of 0.4 mM with 69.9% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 30% cholesterol, and 0.1% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE) in a 4:1 mixture of silicone oil and mineral oil.
4. Outer solution stock solution: 115 mM HEPES, 23 mM MgCl_2 , 1.15 M KCl, 770 mM glucose.

3 Methods

3.1 Preparation of HeLa-Based CFE System

We describe how to produce and utilize a HeLa-derived cell-free protein synthesis system. The procedure described herein is a modification of the protocol described by Mikami et al. [23] The method we present does not require specialized equipment (e.g., spinner flasks) for cell culture, and can be carried out in conventional culture dishes. The use of a bead beater during the cell lysis step can be substituted by any other nonchemical lysis procedure (e.g., French press, nitrogen cavitation).

3.1.1 HeLa Cell Culture

1. Prewarm all media and solutions to 37 °C.
2. If cells are frozen in a liquid nitrogen stock, thaw the frozen cells in a 37 °C water bath by swirling the tube until thawed (*see Note 2*).
3. Transfer the content of the cryotube into a 15 mL-tube and add 1 mL of warm cell culture medium drop by drop to the tube.
4. Continue to add warm medium to 10 mL.
5. Centrifuge at $250 \times g$ for 5 min and discard supernatant.
6. Resuspend the cell pellet into 20 mL of warm cell medium.
7. Transfer the cell suspension to a 150-mm petri dish (*see Note 3*).
8. Cross-shake the dish and place in the incubator at 37 °C with 5% CO₂.
9. When the cells reach ~80% confluency, aspirate the medium and wash with DPBS once. Aspirate the washing solution.
10. Add 2 mL 0.05% trypsin–EDTA and incubate at 37 °C for 10 min.
11. Add 10 mL of warm cell culture medium and pipet up and down to break down clumps of cells. Gently swirl the dish to detach all the cells.
12. Add 0.5 mL of the cell suspension in each of 4 × 150 mm petri dishes.
13. Add 5 mL of culture medium, cross-shake and incubate at 37 °C with 5% CO₂.
14. After cells reach ~80% confluency, repeat the steps 3–6 and split cells in a total of 8 × 150-mm dishes. Grow cells at 37 °C with 5% CO₂ until each dish reaches ~90% confluency (*see Note 4*).

3.1.2 HeLa Lysate Preparation

1. Prechill washing buffer, extraction buffer and high K buffer on ice for 20 min.
2. Aspirate the medium from 150-mm dishes and wash the cells with DPBS once.

3. Add 2 mL of 0.05% trypsin–EDTA to each dish and incubate at 37 °C for 10 min.
4. Add 10 mL per dish of warm culture medium and break cell clumps by pipetting up and down several times.
5. Pool the content of the 8 petri dishes into two 50-mL conical tubes. Centrifuge tubes at $250 \times g$ for 5 min and use 20 mL of cold washing buffer to resuspend and pool cells into a single tube.
6. Centrifuge at $1200 \times g$ for 5 min and discard the supernatant. Wash for a total of three times using the cold washing buffer (*see Note 5*).
7. Resuspend the cell pellet in 10 mL of cold extraction buffer, and check cell viability with trypan blue (expect ~95% viability). Centrifuge at $1200 \times g$ for 5 min and discard the supernatant. Weigh the cell pellet.
8. Add extraction buffer to the pellet in a 1:1 ratio of buffer (in mL):pellet weight (measured in grams).
9. Fill a bead-beating tube with the cell suspension. Place some suspension in the cap of the bead-beating tube and tightly screw the cap, make sure that no bubbles are present (*see Note 6*).
10. Place the bead-beating tubes in the bead-beater and start the apparatus at a speed of 3500 rpm for 20 s, then keep the tubes on ice for 2 min.
11. Repeat **step 10** for 7 times.
12. Add high-K buffer of a volume of 1/29 of the homogenate volume and carefully mix by pipetting.
13. Transfer the homogenate to 1.5 mL microcentrifuge tubes and centrifuge at $1200 \times g$ for 10 min at 4 °C. Recover the supernatant and repeat the centrifugation once more.
14. Divide the supernatant (cell lysate) into aliquots and snap-freeze them in liquid nitrogen. Store at –80 °C (*see Note 7*).

3.1.3 Assembling Coupled Cell-Free Expression Reaction

1. Prechill tubes on ice. Thaw aliquots of HeLa lysate, mixture 1 (Mix 1), mixture 2 (Mix 2), T7 RNA polymerase and GADD34 and keep them on ice.
2. Assemble a reaction by mixing 5 μ L of HeLa lysate, 1 μ L of Mix 1, 1 μ L GADD34 (final concentration of 310 nM), 1 μ L Mix 2, 0.5 μ L T7 RNA polymerase (final concentration of 450 nM), 5 nM plasmid DNA, and RNase-free water to a total volume of 10 μ L in a 1.5-mL microcentrifuge tube. Keep the tubes on ice while assembling the reaction, and carefully mix by pipetting after each addition (*see Note 8*).

3. Incubate the reaction at 30 °C for 4–5 h. Synthesized protein can be detected either in a plate reader in real time if using a fluorescence protein or using Western blot analysis (*see Note 9*).

3.2 Airfuge Fractionation Assay

1. Assemble a coupled CFE reaction as described in Subheading 3.1.3 (*see Note 10*). Run two control reactions one with EGFP and one without DNA. Transfer the reactions to the low-volume 384-well plate and use a fluorescence plate reader to monitor protein synthesis.
2. Once the CFE reaction is complete (as assessed by a plateau in fluorescence reading), remove the plate and add 30 μ L of extraction buffer to the CFE reaction. This ensures that the concentrations of soluble proteins in both pellet and supernatant fractions subsequently are the same.
3. Use the Airfuge to centrifuge the mixture at $\sim 100,000 \times g$ for 15 min at room temperature (refer to the manual for the exact pressure settings). Recover 20 μ L of the supernatant and transfer to a 1.5-mL microcentrifuge tube. Pipet up and down the remaining 20 μ L in the CFE reaction tube to make sure the pellet (*see Note 11*) is resuspended in the pellet fraction. Transfer the pellet fraction to another 1.5-mL microcentrifuge tube (*see Note 12*).
4. Following the recovery of fractions, cell-free expressed proteins in each fraction can be visualized using Western blot using appropriate antibodies after SDS-PAGE or directly on an SDS-PAGE gel for a fluorescent protein. The difference in the intensity of lanes containing supernatant and pellet indicates the amount of membrane proteins that are translocated into the membrane of microsomes (*see Note 13*). Figure 2 shows the presence of microsomes in the pellet fraction by comparing the localization of ER-tracker stain in the puncta in pellet fraction and no localization of the stain in the supernatant fraction.

3.3 Membrane Protein Incorporation Assay

3.3.1 SUPER Template Preparation

1. Mix appropriate aliquots of lipids with the mole percentage of 45% DOPC, 27% DOPE, 9% DOPS, 2.2% Egg-PA, and 16.8% cholesterol for a final concentration of 1 mM in a glass test tube (*see Note 14*).
2. Dry the lipid film under a gentle stream of nitrogen gas.
3. To make sure that any residuals of organic solvent are removed from the mixture, desiccate the dried lipid film for at least 1 h under vacuum.
4. Add Milli-Q water to a final lipid concentration of 1 mM and mix thoroughly by vortexing.

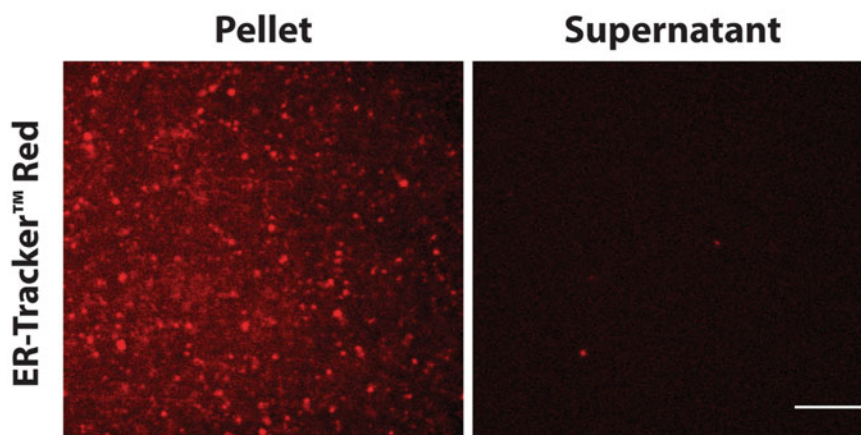


Fig. 2 The enrichment of microsomes in the pellet fraction after ultracentrifugation can be detected by staining with a fluorescent ER-tracker. The localization of ER-tracker signals shows the presence of microsomes in the pellet fraction in HeLa lysate. Scale bar: 10 μm

5. Pass the mixture through the liposome-extruder (*see Note 15*) 11 times to generate SUV solution. Store the 1 mM SUV stock solution at 4 °C. The stock solution is stable at 4 °C for about 2 weeks.
6. While the CFE reaction is being incubated, calculate the concentration of bead stock solution using a Neubauer counting chamber.
7. In a 1.5-mL microcentrifuge tube, mix 20 μL of SUV stock solution, 20 μL of 5 M NaCl solution, and 5×10^6 beads. Bring the mixture to a final volume of 100 μL with Milli-Q water.
8. Incubate the mixture at room temperature for 30 min. Flick the tube gently and occasionally to make sure that the beads are suspended and mixed.
9. Add 900 μL of Milli-Q water to the mixture, and centrifuge at $100 \times g$ for 2 min. Remove 970 μL of supernatant carefully (*see Note 16*) and mix by gently flicking until the pellet is completely resuspended.
10. Repeat **step 4** three times. The final mixture is the stock solution of SUPER templates (30 μL) that is stable at room temperature for 2–3 h (*see Note 17*).

3.3.2 Membrane Protein Incorporation

1. Assemble a coupled CFE reaction as described in Subheading 3.1.3. Run two control reactions one with EGFP and one without DNA. Use a fluorescence plate reader to monitor protein synthesis.
2. Once the CFE reaction has reached completion (typically in about 4 h), add 1 μL of the SUPER template stock solution to

the reaction in the well-plate (if assembling a CFE reaction with higher volumes, the SUPER template to the reaction volume ratio is kept at 1:10). Alternatively, the solution can be transferred to a 1.5-mL microcentrifuge tube and the beads are added.

3. Incubate the plate at 30 °C for 30 min or on a heat block if using microcentrifuge tubes.
4. Transfer the CFE reaction to a 1.5-mL microcentrifuge tube and centrifuge at $200 \times g$ for 5 min. Carefully remove the supernatant (the pellet is hardly visible, and care should be taken when recovering the supernatant, as it can be used for other purposes such as Airfuge fractionation assay or an SDS-PAGE).
5. Resuspend the pellet in 1 mL of $1 \times$ PBS and wash the beads by centrifuging at $200 \times g$ for 5 min. Repeat once.
6. Resuspend the pellet in 40 μ L of $1 \times$ PBS.
7. Prepare an imaging flow chamber by adhering a glass coverslip and a glass slide using double-sided tape and transfer the mixture to the chamber for imaging. The solution will creep into the chamber by capillary action. Fluorescence imaging can be carried out by confocal or epifluorescence imaging. Figure 3a shows membrane incorporation of membrane proteins EGFP-SUN1 and EGFP-SUN2.

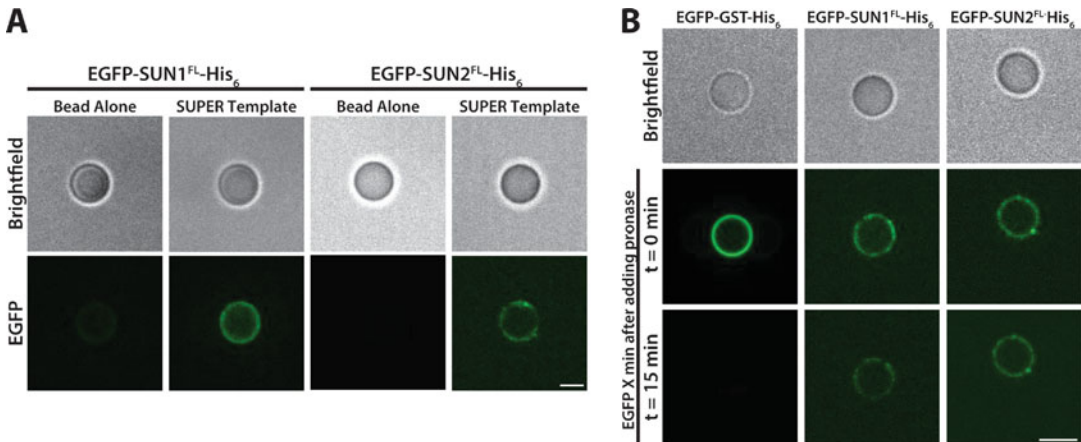


Fig. 3 (a) Localization of cell-free expressed EGFP-SUN1 and EGFP-SUN2 on the SUPER templates provides visual evidence of membrane protein incorporation into the supported lipid bilayer **(b)** The protease protection assay can help reveal membrane protein topology. The EGFP signal for SUN1 and SUN2 after addition of protease remains on SUPER templates, indicating that the N-terminal EGFP is in the space between the bead and the lipid bilayer where the protease cannot reach. (Adapted with permission from Majumder et al. [21] from *Journal of Cell Science*. Scale bar: 5 μ m)

3.3.3 Protease Protection Assay

Protease protection assay can be utilized to study the topology of membrane protein insertion into the lipid bilayer. Additionally, protease protection assay can confirm the incorporation of the integral membrane protein into the bilayer and determine if the protein is peripherally associated with the membrane. To investigate the topology of membrane protein insertion, the protein of interest is labeled fluorescently. In the case of fluorescent protein fusion, the reporter (e.g., EGFP) can be fused to either terminus of the protein to investigate the topology of insertion. Using this strategy, we have previously identified an additional transmembrane domain in SUN1 and a membrane-associated hydrophobic region in both SUN1 and SUN2 [21].

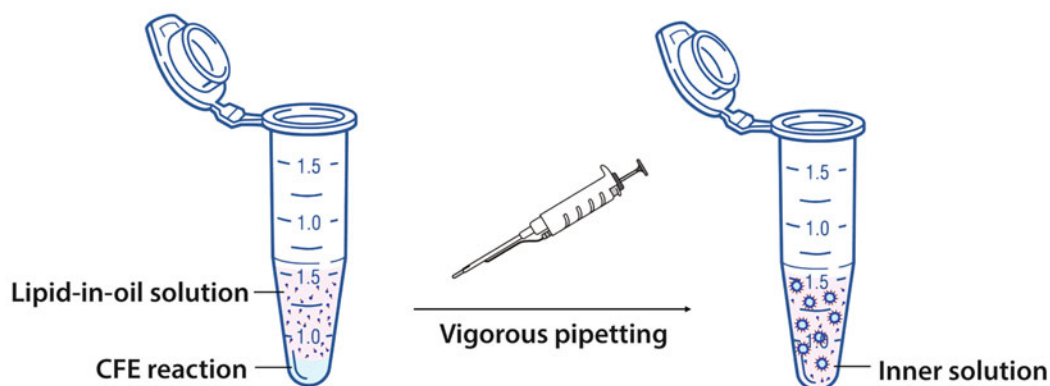
1. Prepare a 6 mg/mL stock solution of lyophilized *S. griseus* Pronase in Milli-Q water. Store at 4 °C for a maximum of 3 days.
2. After resuspending beads in 20 μ L of 1 \times PBS and loading the sample into an imaging flow chamber as described in the previous section, add the appropriate amount of Pronase to the final concentration of 2 mg/mL.
3. Incubate for at least 15 min at room temperature before imaging. Figure 3b shows strong association of EGFP-SUN1 and EGFP-SUN2 with SUPER template which implies that N-terminal EGFP is located between the bead and the lipid membrane and thus protected from protease digestion. However, the loss of fluorescence after addition of protease to the His₆-GST-EGFP (bound to Ni-NTA containing SUPER template) shows its peripheral association with the membrane of SUPER templates.

3.4 Vesicle Encapsulation System

Vesicles were generated by a modification of the continuous droplet interface crossing encapsulation (cDICE) method [18, 24] (Fig. 4). A rotor chamber was 3D-printed with clear resin by using a 3D printer (Formlabs) and mounted on the servo motor of a benchtop stir plate and rotated at 700 rpm. The custom chamber has a capped hole near the outer edge where samples can be retrieved.

1. Assemble a coupled CFE reaction as described in Subheading 3.1.3. Run two control reactions one with EGFP and one without DNA.
2. Dilute 1 μ L of CFE reaction with 9 μ L Milli-Q water and measure the osmolarity. Use triplicates to determine the final osmolarity of the saturated CFE reaction before encapsulation. Add 5% OptiPrep to the CFE reaction and keep in ice as the inner solution (IS).
3. Make 1 mL of aqueous outer solution (OS) by diluting OS stock with DI water until the osmolarity matches that of the IS (see Note 18).

Single emulsion generation



cDICE

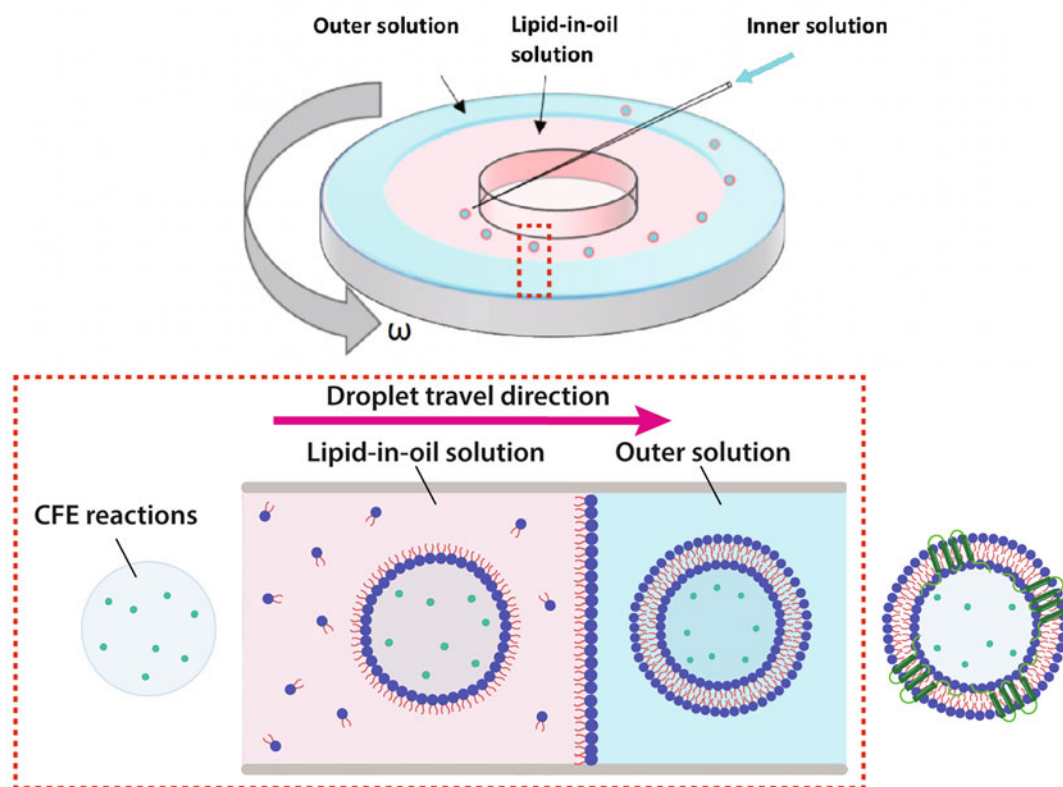


Fig. 4 The schematic for generating vesicles encapsulating CFE reactions by cDICE method. **(Top)** The inner solution is made by vigorous pipetting of the CFE reaction with lipid-in-oil solution (LOS) solution. **(Bottom)** The cDICE contains a cylindrical chamber with a lid. Through the hole in the lid, the outer solution (OS) and LOS are sequentially introduced. The IS droplets are ejected from the pipette tip into the chamber and are then centrifuged radially toward the LOS–OS interface to form the final lipid bilayer vesicles containing CFE reactions. (The top schematics were sourced from <https://scidraw.io/>, and the cDICE device schematic is adapted with permission from Bashirzadeh et al. [18])

4. Make lipid-in-oil solution (LOS) by mixing lipids together in a glass test tube with the desired mole percentage and chloroform (e.g., DOPC:DOPE:cholesterol = 40:30:30) and a total of 0.4 mM lipids (*see Note 19*). Mix the lipids with the desired volume of 1:4 silicon oil and mineral oil by vortexing for at least 10 s (*see Note 20*).
5. Gently add 500 μL LOS on top of encapsulated CFE reaction in the 1.5-mL microcentrifuge tube. The interface between the LOS and the encapsulated CFE reaction should be clearly visible because the difference in density between the two solutions results in the formation of two distinct layers with a vertical water–oil interface at their boundary.
6. Make water-in-oil emulsion by vigorously pipetting $\sim 8 \mu\text{L}$ of CFE reaction in 500 μL of LOS (*see Note 21*).
7. Sequentially add 700 μL of aqueous OS, 5 mL of LOS and the water-in-oil emulsion generated in step 6 into the cDICE chamber rotating at 700 rpm (Fig. 4) (*see Note 22*).
8. After 3 min of rotation, there are two layers of solutions inside the chamber. Vesicles will accumulate in the OS near the chamber wall. Carefully collect the vesicles by gently retrieving the OS (*see Note 23*). Figure 5 shows the localization of membrane proteins EGFP-SUN1 in the lipid bilayer membrane of vesicles generated by cDICE method. EGFP-YAP (Yes-Associated Protein), a transcription factor involved in mechanotransduction, is shown as a negative control as it does not localize to the membrane.

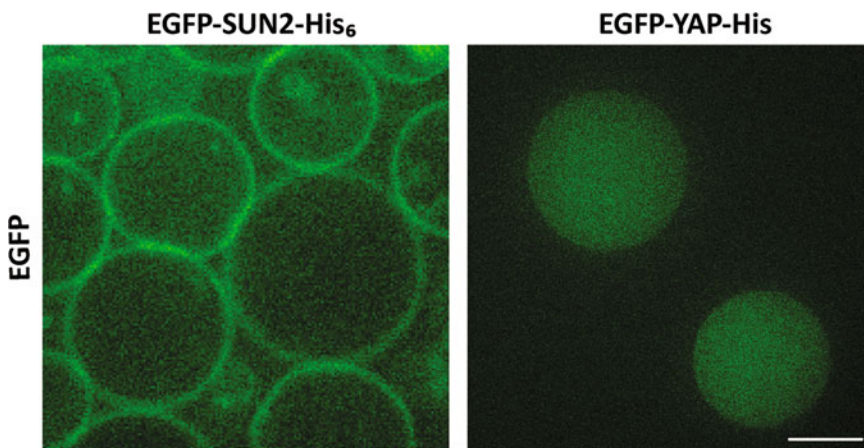


Fig. 5 Localization of cell-free expressed EGFP-SUN2 and EGFP-YAP in vesicles created with cDICE. Membrane protein SUN2 localizes to the lipid bilayer vesicle membrane whereas YAP is uniformly distributed inside vesicles. Scale bar: 10 μm

4 Notes

1. The use of high-quality fresh plasmid preparations is strongly encouraged.
2. Transfer the cryotube to the tissue culture hood when a small piece of ice is still present in the tube. It will thaw before starting the procedure.
3. If the initial stock concentration of cells is low, cells can be grown in a 100-mm petri dish and split when they reach ~100% confluency to two 150-mm petri dishes.
4. Do not let the cells overgrow, as their metabolic state may change and lower the transcription/translation efficiency of the lysate.
5. It is important to keep the “cold chain” in this and the following steps. Centrifuge at 4 °C if possible, keep the solutions and the tubes in ice.
6. It is crucial that no air bubbles are present during the bead-beating step. When the bead-beating tube is full, tap the tube on a hard surface to make the bubbles emerge to the surface, and pop the bubbles with a needle if needed. Then place a small amount of solution in the cap of the tube, tightly close the assembly, and tap on a hard surface. If bubbles appear, unscrew the cap and repeat the process.
7. Repeated freeze-thaw cycles severely impair the transcription/translation efficiency of the lysate. Ideally, a single aliquot should be used for no more than 2–3 experiments. Plan the aliquot volume accordingly.
8. To ensure consistency in the results in the case of multiple reactions, consider preparing a master mix. Be aware that the lysate efficiency varies from batch to batch and may be dependent on other factors such as the quality of the plasmid, the purity of T7 and GADD34, and the absence of RNase in the solutions.
9. Depending on the fluorescence reporter, the incubation time can vary. For instance, due to the slower folding of mCherry, CFE reactions synthesizing the protein of interest fused with mCherry take a longer time to reach completion.
10. We used a final DNA concentration of 5 nM in the CFE reactions. Performing a DNA titration assay to obtain the optimal concentration of the DNA to have the highest reaction efficiency is recommended.
11. After fractionation, the pellet fraction is not visible. Therefore, it is crucial to carefully recover the supernatant without disturbing the pellet.

12. Even though we have recommended the addition of the beads directly to the CFE reaction in this protocol, one can add beads to each fraction to assess the membrane incorporation efficiency of the soluble fraction of cell-free synthesized proteins.
13. For quantification purposes, one can use a fluorescent reporter such as EGFP with a certain concentration in the SDS-PAGE to quantify the amount of translocated cell-free produced membrane proteins.
14. This lipid composition mimics the composition of nuclear membrane that has been used for SUN protein reconstitution. One can use other lipid compositions as desired.
15. Alternatively, one can use an Avanti mini-extruder with a 100 nm filter.
16. The pellet is visible and if it is hard to see, make sure to flick the tube enough to resuspend the beads homogeneously, and repeat the centrifugation.
17. SUPER templates should be made fresh right before adding to the CFE reaction.
18. The difference of the osmolarity between the OS and encapsulated CFE reaction should be less than 50 mOsm. If the osmolarity of the OS is larger than the encapsulated CFE reaction, the vesicles shrink. On the other hand, when the osmolarity of the OS is smaller than the encapsulated CFE reaction, the vesicles might burst. As compared to encapsulated CFE systems, a bit larger osmolarity of the OS is better than less osmolarity.
19. The LOS can be used immediately or stored at 4 °C for a maximum of 2 days.
20. The composition and concentration of lipids are critical for the reconstitution of membrane proteins. The insertion (solubilization) efficiency and quality of membrane protein can depend on the lipid composition of the provided membrane with a preference for negatively charged lipids.
21. The density of the encapsulated solution inside vesicles should be a bit larger than the OS so that the vesicles can settle down to facilitate imaging. The addition of 5% OptiPrep, a density gradient medium, is helpful.
22. For the use of cDICE, there are some crucial factors that could determine the success of the formation of lipid bilayer vesicles. First, the thickness of the LOS layer and its corresponding flight time should be large enough so that the lipids from LOS have enough time to saturate the surface of droplets. Second, the inertia of the droplets should not significantly deform the interface and the slight density difference between the encapsulated solution and the outer fluid is needed to help the vesicles to detach from the lipid monolayer.

23. To collect vesicles, tilt the 3D-printed chamber at 45° and the interface between LOS and OS with vesicles should be clear. When collecting vesicles, try to avoid taking the oil layer on top of the OS; otherwise, oil droplets will obstruct imaging.

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High-Throughput Experimentation Using Cell-Free Protein Synthesis Systems

Conary Meyer , Chuqing Zhou , Zecong Fang , Marjorie L. Longo , Tingrui Pan , and Cheemeng Tan

Abstract

Cell-free protein synthesis can enable the combinatorial screening of many different components and concentrations. However, manual pipetting methods are unfit to handle many cell-free reactions. Here, we describe a microfluidic method that can generate hundreds of unique submicroliter scale reactions. The method is coupled with a high yield cell-free system that can be applied for broad protein screening assays.

Key words High throughput, Cell-free protein synthesis, Droplet printing, Automation, Protein screening, Microfluidic adaptive printing, Low-volume liquid handling

1 Introduction

Cell-free protein synthesis (CFPS) systems have gained tremendous interest from the development of applications including the synthesis of toxic and challenging proteins [1, 2], high-throughput screening [3], a priori prediction of genetic circuits [4, 5], in vitro metabolic pathways construction for biomanufacturing [6], and noncanonical amino acid incorporation [7]. While substantial progress has been made in this field, the application of these systems often requires high-throughput exploration of numerous parameters. These parameters include a wide range of different salt, reactant, and protein concentrations, leading to millions of possible combinations [8, 9]. Extensive exploration of this parameter space is expensive, error-prone, and prohibitively laborious using conventional pipetting methods [10].

To address the shortcomings of traditional liquid handling, numerous automated liquid handling robots have come to market (e.g., Fluent™ from Tecan and the OT-2™ from Opentrons). While these systems can minimize the involvement of researchers,

they still incur the issue of inaccuracy at submicroliter volumes. This issue requires precious and expensive biological reagents to be used at a scale far greater than is necessary. Microfluidic adaptive printing technologies have been developed to allow for ultralow-volume liquid handling, effectively overcoming this critical problem [11–14]. These systems have been utilized in several examples of combinatorial screens since their inception [15–17]. Recently, a new pipette-free robotic-dispensing interface utilizing a simple microfluidics-enabled container has been developed [18]. The microfluidic cap-to-dispense (μ CD) system integrates high accuracy robotic motion and droplet dispensing with nanoliter resolution to test hundreds to thousands of unique reactions rapidly and accurately.

The core of the μ CD system is a disposable multilayer microfluidic container composed of a 3D printed reservoir linked to a microfluidic printing nozzle with two pneumatic control channels that can be reversibly connected to a robotic arm. This robotic arm is customized to enable reliable linking between the pneumatic controllers and the microfluidic device. The modular and disposable design of the μ CD system allows for the inclusion of limitless reagents in a multicomponent experiment as the microfluidic devices can be swapped in and out without the risk of cross-contamination.

We will detail the setup and use of the μ CD system, along with the production of the recently developed highly productive CFPS system described in [19]. This novel CFPS system utilizes a coculture of seven different strains, each overexpressing a subset of the eleven translation factors [20]. This overexpression both increases the concentration of the components needed for efficient translation and triggers a global proteome shift, which adapts the CFPS system to be more amenable to protein synthesis. The construction of the required strains and the method of whole-cell extract preparation are described below. However, this system can be utilized with any form of a cell-free system or in other applications requiring the manipulation of submicroliter volumes.

2 Materials

2.1 Strain and Plasmid Construction for Whole-Cell Extract

1. BL-7S plasmids (Addgene https://www.addgene.org/Cheemeng_Tan/).
2. pLysS plasmid (Novagen, cat# 69659).
3. BL21(DE3) chemically competent cells.

2.2 Whole-Cell Extract Preparation

1. 2 \times YTP: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, 7 g/L Sodium phosphate dibasic, 3 g/L disodium phosphate monobasic.

2. 2×YTP agar plates: 2×YTP supplemented with 15 g/L agar.
3. Washing buffer: 10 mM Tris acetate, 14 mM Magnesium acetate, 60 mM Potassium Gluconate, pH to 7.6 using Sodium hydroxide.
4. BL21(DE3) pLysS Chemically Competent.
5. Sonication buffer: Washing buffer supplemented with 2 mM Dithiothreitol.
6. Carbenicillin (Carb).
7. Chloramphenicol (Cam).
8. Kanamycin (Kan).
9. Isopropyl β -D-1-thiogalactopyranoside (IPTG).
10. Tabletop centrifuge.
11. 250-mL flat bottom centrifuge bottles.
12. Plate reader.
13. Q125 Sonicator with a 2-mm diameter probe (Qsonica model CL-18).

2.3 Preparation of Cell-Free Reaction Supplement

1. Amino acid solutions as indicated in Table 1, mixed together (*see* Notes 1 and 2).
2. Stock solutions specified in Table 2, mixed together to make the cell-free reaction supplement.

2.4 Cell-Free Reaction Supplement Preparation

1. Ribonucleotide triphosphates (ATP, GTP, UTP, and CTP).
2. Folinic acid.
3. *E. coli* tRNA mixture from *E. coli* MRE600.
4. 20 standard amino acids.
5. Nicotinamide adenine dinucleotide (NAD).
6. Coenzyme-A.
7. Spermidine.
8. Potassium glutamate.
9. Magnesium glutamate.
10. HEPES.
11. Creatine phosphate.
12. Creatine kinase.
13. Cyclic adenosine monophosphate (cAMP).
14. Polyethylene glycol 8000 (PEG8000).
15. Dithiothreitol.

Table 1
Composition of Amino Acid mixture

Amino acid	Molecular weight	Stock conc. (M)	Solution	Volume (mL)	Mass (mg)
Alanine	89.09	0.01	Water	0.02	17.8
Arginine	210.66	0.01	Water	0.02	42.1
Asparagine	132.12	0.01	0.5 M HCl	0.02	26.4
Aspartic acid	133.1	0.01	0.5 M HCl	0.02	26.6
Cysteine	157.62	0.01	Water	0.02	31.5
Glutamic acid	147.13	0.01	1 M HCl	0.02	29.4
Glutamine	146.14	0.01	0.5 M HCl	0.02	29.2
Glycine	75.07	0.01	Water	0.02	15.0
Histidine	209.63	0.01	Water	0.02	41.9
Isoleucine	131.17	0.01	Water	0.02	26.2
Leucine	137.17	0.01	Water	0.02	27.4
Lysine	182.65	0.01	Water	0.02	36.5
Methionine	149.21	0.01	0.5 M HCl	0.02	29.8
Phenylalanine	165.19	0.01	5 M KOH	0.02	33.0
Proline	115.13	0.01	Water	0.02	23.0
Serine	105.09	0.01	Water	0.02	21.0
Threonine	119.12	0.01	Water	0.02	23.8
Tryptophan	204.23	0.01	0.5 M HCl	0.02	40.8
Tyrosine	181.19	0.01	1 M HCl	0.02	36.2
Valine	117.15	0.01	Water	0.02	23.4

2.5 Printing Platform Equipment and Consumables

1. PMMA sheets (75 μ m thick).
2. Double-sided adhesive membrane (80 μ m thick).
3. CO2 laser cutter.
4. 85 μ m nozzle in 75 μ m thick PMMA.
5. SLA 3D printer (Form 3 Formlabs).
6. Clear Resin (Formlabs).
7. Analytical Balance (U.S Solid 1 mg or ideally 0.1 mg precision).
8. Robotic arm (Dobot Magician).
9. Two mini solenoid valves (LHDA122111H, Lee Co.)
10. Manifold (Lee Co.)
11. Automated pressure controller (PeciGenome Pressure/Flow controller light version, PG-MFC-LT2CH-X).

Table 2
Composition of cell-free reaction supplement

Component	Molecular weight (g/mol)	Working concentration	Stock concentration
HEPES-KOH pH 7.6	238.3	0.05 M	2 M
tRNA	N/A	0.17 mg/mL	17
Folinic acid	N/A	0.034 mg/mL	6.8 mg/mL
Magnesium glutamate	388.61	0.012 M	1.5 M
Potassium glutamate	203.23	0.18 M	5 M
Polyethelene Glycol 8000	N/A	2%	40%
Dithiothreitol	154.25	0.002 M	1 M
Spermidine	145.25	0.004 M	0.5 M
Creatine phosphate	327.2	0.067 M	2 M
Creatine kinase	N/A	0.08 mg/mL	34.4 mg/mL
Nicotinamide adenine dinucleotide	663.4	0.33 mM	175 mM
Cyclic adenosine monophosphate	329.21	0.64 mM	640 mM
Coenzyme-A	767.53	0.26 mM	65 mM
20 amino acid mixture	N/A	2 mM	10 mM
ATP	507.18	1.2 mM	100 mM
GTP	523.18	1.2 mM	100 mM
CTP	483.16	0.8 mM	100 mM
UTP	484.14	0.8 mM	100 mM

12. Microcontroller (Arduino Uno).
13. 12.5 V Power supply (BK precision programmable DC power supply)
14. Thin tubing (1/16" and 1/8" Tygon[®] S3[™] E-3603 Non--DEHP Laboratory Tubing).
15. Circuit components.
 - (a) Diode 1 N4005.
 - (b) Zener Diode 1 N4757 51 V.
 - (c) Transistor MJH11022G.
 - (d) Resistor 330 Ω .
 - (e) Connector wires.
 - (f) Breadboard.

3 Methods

3.1 Construction of BL-7S Strains

1. Order the plasmids listed in Table 3 from Addgene.
2. Purify the plasmids listed using a standard miniprep plasmid purification kit.
3. Transform the plasmids along with pLysS into BL21(DE3) chemically competent cells. Thaw 50 μ L of chemically competent cells on ice for 10 min. Add 1 ng of each plasmid to the cells and incubate on ice for 30 min. Heat shock at 42 $^{\circ}$ C for exactly 10 s and return to ice for 5 min. Recover the cells by adding 950 μ L of room temperature SOC to the solution. Incubate for 60 min at 37 $^{\circ}$ C with shaking. Mix the tubes and streak onto 2 \times YTP agar plates containing 100 μ g/mL Carb, 34 μ g/mL Cam, and 30 μ g/mL Kan. Incubate the plates overnight at 37 $^{\circ}$ C.

3.2 Preparation of BL-7S Whole-Cell Extract

1. Inoculate 3 mL of 2 \times YTP supplemented with 100 μ g/mL Carb, 34 μ g/mL Cam, and 30 μ g/mL Kan in a 15-mL culture tube with a single colony from each fresh plate.
2. Incubate the cultures overnight at 37 $^{\circ}$ C with shaking at 250 rpm.
3. Combine the cultures using the percentages specified in Table 3 and maintain a final OD600 of 1.0 and transfer 1.8 mL of the mixture to 2 L Erlenmeyer flask with 300 mL of 2 \times YTP supplemented with 100 μ g/mL Carb and 30 μ g/mL Kan (*see Note 3*).
4. Incubate the flask at 30 $^{\circ}$ C with 250 rpm shaking until the absorbance at 600 nm (OD600) of the culture reaches 0.15 (*see Note 4*). Once the desired concentration is reached, induce protein expression using a final concentration of 0.5 mM IPTG.

Table 3
Strain construction and inoculation proportion

Strain number	pET15b backbone	pSC101 backbone	Relative density (%)
1	B1-4	B1-5	57.8
2	B7-5	B3-1	16.6
3	B7-1	B5-5	3.8
4	B4-1	B3-2	8.3
5	B4-4	B3-4	0.5
6	B1-3	B3-4	5.1
7	B7-1	Empty	7.9

5. Continue to incubate the flask until the OD600 reaches 1.0 (*see Note 4*).
6. Transfer the cultures to 300 mL to flat bottom centrifugation bottles.
7. Centrifuge at $4000 \times g$ for 20 min at 4 °C.
8. Decant the supernatant of each tube and resuspend with 20 mL of Wash Buffer (*see Note 5*).
9. Repeat the wash step.
10. Weigh a 50-mL conical tube for each 300 mL of culture and transfer the resuspended culture to the tubes.
11. Centrifuge at $4000 \times g$ for 10 min at 4 °C.
12. Decant the supernatant (*see Note 6*).
13. Weigh the pellet and subtract the original mass of the tube to find the pellet mass.
14. Resuspend the pellet with 1 mL of Sonication Buffer per 1 g of wet cell mass (*see Note 7*).
15. Aliquot 500 μ L of the resuspended pellet to 1.5-mL microfuge tubes.
16. Sonicate the cells at a frequency of 20 kHz and 50% amplitude while the tube is inside an ice-water slurry. Sonication is carried out with the Q125 Sonicator in 10 s ON/10 s Off intervals until the input energy is about 1000 J. This generally takes 27 cycles (*see Note 8*).
17. Centrifuge the cell lysate at $12,000 \times g$ for 20 min at 4 °C.
18. Collect the supernatant and aliquot into individual tubes.
19. Incubate the tubes at 30 °C for 30 min (*see Note 9*).
20. Store the aliquots at -80 °C.

3.3 Printing Platform Assembly

The printing platform is comprised of several different components. The three-dimensional positioning and loading of printer heads are achieved using the Dobot Magician robotic arm with the gripper attachments. The printing and refuel pressures are regulated by the PreciGenome pressure controller. The Arduino microcontroller unit (MCU) and valves are used to apply the pressures in bursts for printing and refueling. The assembled system is shown in Fig. 1. The code, which is freely available at [<https://github.com/ccmeyer/printing-platform>], integrates and controls all these components for simple and robust use.

1. The required files for 3D printing and laser cutting the necessary components to construct the system are also available at [<https://github.com/ccmeyer/printing-platform>] (*see Note 10*).

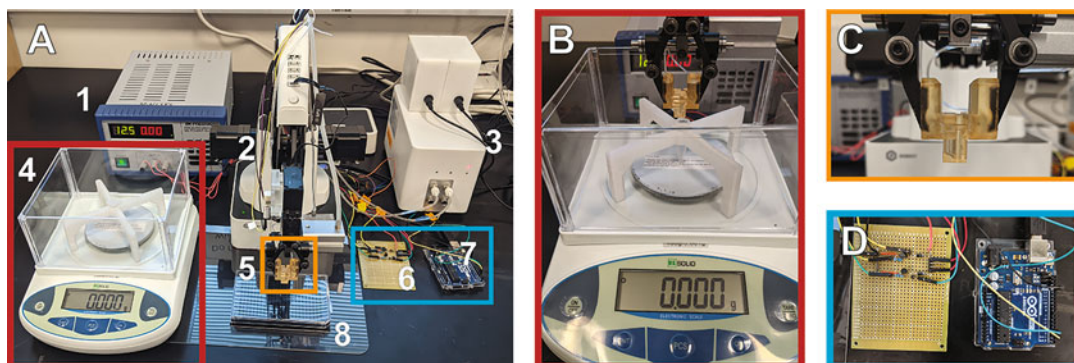


Fig. 1 Diagram of the complete printing platform. (a) The image of the full platform. (1) 12.5 V DC power supply, (2) Dobot robotic arm, (3) Precigenome pressure regulator, (4) Analytical balance, (5) Gripper holding a printer head, (6) Valve control circuit, (7) Arduino Uno microcontroller, (8) Well plate. (b) Close-up of the printer head positioned over the reagent tube during calibration. (c) Dobot gripper with the 3D printed gripper adapters holding a printer head. (d) Valve control circuit connected to an Arduino

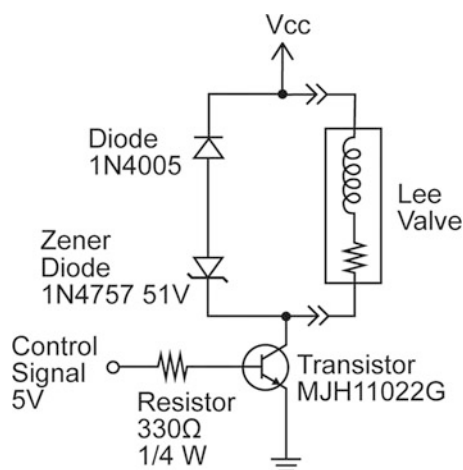


Fig. 2 The Arduino circuit design. The circuit is used to regulate the valves that control the pressure flow

2. Connect the gripper attachment to the Dobot and attach the 3D printed gripper adapters to the end effector.
3. Screw each valve into the manifold and attach it to the Dobot near the gripper (*see Note 11*).
4. Connect the gripper adapters to the manifolds using thin tubing. Then connect the manifold inlets to the corresponding pressure outlets on the PreciGenome pressure controller.
5. Construct the circuit shown in Fig. 2 and connect the Arduino, the two valves, and the power source to it (*see Note 12*).
6. Set the voltage of the power source to 12.0 V or the required voltage for the valves.

7. Laser-cut the provided template and place it around the Dobot to provide consistent placement of the well plate and balance in the Dobot's Cartesian coordinate system.
8. Connect the robotic arm, the pressure controller, and the Arduino to your computer.
9. Upload the provided Arduino program to the Arduino.
10. 3D-print the tube stand and place it on top of the scale.

3.4 Printer Head Fabrication

1. To fabricate the layer with the small nozzle, a UV-laser is used to ablate a small hole (65–85 μm diameter) into a PMMA sheet (*see* **Notes 13** and **14**).
2. The channel layer can be fabricated using a CO₂ laser to ablate microchannels in double-sided membrane adhesive. The chosen channel width was 200 μm (*see* **Note 15**).
3. The bulk component of the printer head, which contains the connections to the pressure inlets and the reagent reservoir, is fabricated by 3D printing (*see* **Note 16**). The Solidworks models are available on GitHub (*see* **Note 17**).
4. The full printer head is assembled stepwise. Peel off one side from the adhesive layer containing the channel and adhere it to the bottom of the 3D printed component. Line up the entry and exit holes of the channel with the corresponding openings on the 3D printed part.
5. Peel off the other side of the adhesive layer and place the nozzle layer on top. Ensure that the small nozzle is lined up with the exit hole of the channel. (*see* **Note 18**).

3.5 Printing Calibration

Due to the inherent variability between the physical properties of different reagents and imperfections in the fabrication process, the flow resistance through the channel and the nozzle will vary. If not accounted for, the droplet volume will differ from the intended volume. This section details how to rapidly calibrate the printer head with the desired reagent to achieve printing with nanoliter precision.

3.5.1 Calibration Theory

- (a) The fundamental idea of this calibration process is to leverage the fixed and tunable parameters to both determine and then account for the unknown parameters. The parameters of the system are Unknown—Channel and nozzle resistances; Fixed—Overflow chamber volume; Tunable—Refuel and printing pressures.
- (b) The unknown channel and nozzle resistances can be calculated using a simple procedure. Using the known dimensions of the overflow channel shown in Fig. 3, the volume displaced during the refuel and printing steps can be measured by

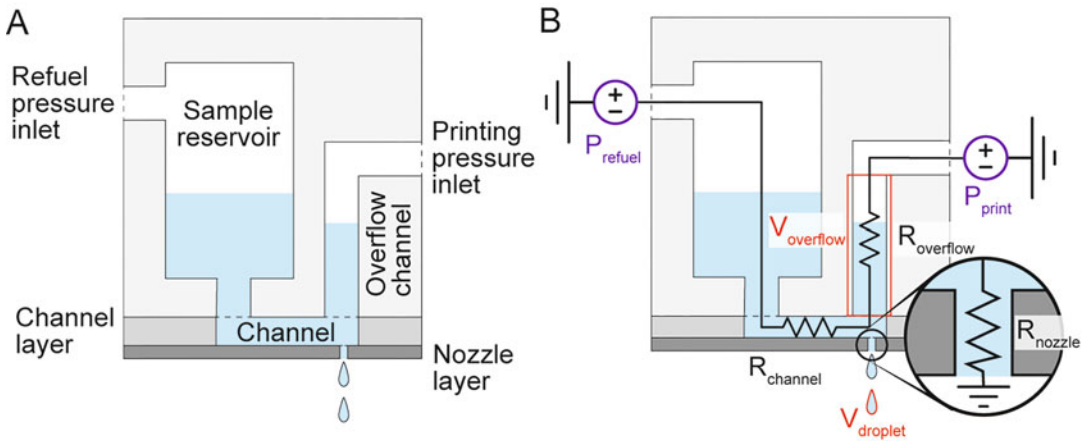


Fig. 3 Schematic depicting the printer head design. (a) The components of the printer head. (b) Key control parameters of the system. Tunable parameters: purple. Fixed parameters: red. Unknown parameters: black

monitoring the fluid level in the channel. The displaced volume and the known magnitude and duration of the applied pressure can be used to calculate the total flow of the system. The droplets that are ejected during the printing step are collected such that the volume dispensed can be calculated from the measured mass. This volume can be used to calculate the partial flow out of the nozzle. Using Eqs. 1–3 the individual resistances can be calculated using the magnitude of the pressure and the time the pressure was applied. In the equations below R represents resistance, P is pressure, t is time the pressure is applied, V is volume, ρ is density, and m is mass.

$$R_{\text{channel}} = \frac{P_{\text{refuel}} t_{\text{refuel}}}{V_{\text{chamber}}} \quad (1)$$

$$R_{\text{total}} = \frac{P_{\text{print}} t_{\text{pulse}}}{V_{\text{chamber}}} \quad (2)$$

$$R_{\text{nozzle}} = \frac{P_{\text{print}} t_{\text{pulse}} \rho_{\text{reagent}}}{m_{\text{droplet}}} \quad (3)$$

- (c) Once the resistances are known, the refuel and printing pressures can be adjusted to both allow for the generation of the desired droplet volume and ensure that the flow out of the overflow channel matches the flow in for continuous printing. The desired pressures can be calculated using Eqs. 4 and 5 where f_{print} represents the frequency of printing and t_{width} represents the duration of each printing pulse.

$$P_{\text{print}} = \frac{R_{\text{nozzle}} V_{\text{ejected}}}{f_{\text{print}} t_{\text{width}}} \quad (4)$$

$$P_{\text{refuel}} = \frac{P_{\text{print}} t_{\text{print}} R_{\text{channel}}}{R_{\text{total}} (1 - t_{\text{print}})} \quad (5)$$

3.5.2 Method for Individual Printer Head Calibration

1. Measure the density of the reagent by finding the mass of 100 μL of sample. Divide the number of milligrams by the volume dispensed to find the density in $\text{mg}/\mu\text{L}$.
2. Pipet the desired amount of reagent into an assembled printer head and load the printer head into the printing assembly (*see Note 19*).
3. Place the reagent tube into the tube holder on top of the scale and tare the scale. Position the Dobot holding the printer head directly above the tube and initiate the calibration protocol in the provided API.
4. Start by filling the printer head overflow chamber by applying refuel pressure until the liquid level reaches the first notch in the printer head. This is where the calibration starts.
5. Continue to apply refuel pressure until the overflow chamber fills to the second notch and hit continue.
6. Apply printing pulses until the liquid level in the overflow chamber reaches the first notch.
7. Repeat this process two more times to get replicates of the measurements.
8. Enter the new mass into the program, and it will automatically calculate and display the resultant resistances for the flow into and out of the overflow chamber as well as the nozzle resistance.
9. The program will then determine the needed refuel and printing pressures needed to continuously print the desired droplet volume with that printer head and reagent (*see Note 20*).

4 Notes

1. The different solutions used in the resuspension help to dissolve the amino acids.
2. It is recommended that a static gun, such as the Zerostat 3 from Sigma-Aldrich, be used to remove excess static from the tubes and scale while weighing the amino acids.
3. We exclude Cam as it is responsible for the maintenance of the pLysS plasmid, which is not needed at this step.
4. The 600 nm absorbance measurement was taken in a plate reader with a clear bottom 96-well plate with 200 μL of culture. The path length is roughly 0.5 cm compared to the

standard 1cm pathlength used in OD600 measurements. Samples were diluted such that the absorbance reading was under 0.25 to maintain linearity.

5. The wash step is carried out on ice by swirling the Wash Buffer around to resuspend the pellet.
6. After decanting the supernatant, the centrifugation step can be repeated to remove excess liquid.
7. Use a laboratory spatula with a rounded end to mechanically resuspend the pellet.
8. The cell paste will turn more transparent and slightly pink if using wildtype BL21(DE3). However, if using the BL-7S strains, it will not significantly change color or turbidity.
9. This is a run-off reaction to allow time for the ribosomes to finish reading through the RNA that they are bound to. It is not necessarily required.
10. To download all the files and scripts required to build and operate the system, it is recommended to clone the Git repository.
11. It is critical to have a tight seal between the manifold and the valve to ensure no loss of pressure.
12. Provide enough extra tubing and wires to run them along the arm to not restrict its motion while in use.
13. A range of nozzle diameters can be used to give different sized droplets.
14. The nozzles can be fabricated by the following companies: Jestar Mold Tech Co., Ltd. (China) and Micron Laser Technology Inc. (USA).
15. A wide range of channel widths can be used. Choose the width based on the desired channel resistance.
16. We used stereolithography (SLA) printing with surgical guide photosensitive resin to generate the printer heads and the gripper adapters.
17. It is critical that these printer heads are watertight, transparent, and have smooth channels for proper use. These qualities are very difficult to achieve with standard fused deposition modeling (FDM) printers as there are many imperfections at the junctions between layers.
18. Firmly press the layers down to make sure that there are no air pockets.
19. The standard printer head design holds 200 μ L of liquid.

20. Depending on the available lasers, the nozzle size might differ from the ones used in this instantiation of the system. The difference in nozzle size will impact the available range of droplet volumes that are possible. The pressure will correlate linearly with the droplet volume. However, the pressure must stay high enough to pinch off the droplet, which sets the floor for the possible droplet size.

Acknowledgments

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Cell-Free Gene Expression from DNA Brushes

Michael Levy, Ohad Vonshak, Yiftach Divon, Ferdinand Greiss, Noa Avidan, Shirley S. Daube, and Roy H. Bar-Ziv

Abstract

Linear double-stranded DNA polymers coding for synthetic genes immobilized on a surface form a brush as a center for cell-free gene expression, with DNA density 10^2 – 10^3 fold higher than in bulk solution reactions. A brush localizes the transcription-translation machinery in cell extracts or in cell-free reconstituted reactions from purified components, creating a concentrated source of RNA and proteins. Newly synthesized molecules can form circuits regulating gene expression in the same brush or adjacent ones. They can also assemble into functional complexes and machines such as ribosomal units, then analyzed by capture on prepatterned antibodies or by cascaded reactions. DNA brushes are arranged as a single center or multiple ones on a glass coverslip, in miniaturized compartments carved in silicon wafers, or in elastomeric microfluidic devices. Brushes create genetically programmable artificial cells with steady-state dynamics of protein synthesis. Here, we provide the basic procedure for surface patterning, DNA immobilization, capture of protein products on antibody traps and fluorescent imaging. The method of DNA brush surface patterning enables simple parallelization of cell-free gene expression reactions for high throughput studies with increased imaging sensitivity.

Key words Cell-free protein expression, DNA brush, DNA chip, Fluorescent microscopy, Surface confinement, Macromolecular machine assembly

1 Introduction

Over the past several years, cell-free gene expression from dense DNA brushes immobilized on surfaces has become an enabling methodology for spatial–temporal control of RNA and protein synthesis [1, 2]. Gene immobilization leads to dynamic localization of the gene expression machinery, namely RNA polymerases (RNAPs) and ribosomes, to the DNA brush [3]. Driven by energy consumption, DNA brushes become sources of high local concentration of RNA and protein products, which can be selectively captured on the surface surrounding the DNA brushes [4] (Fig. 1).

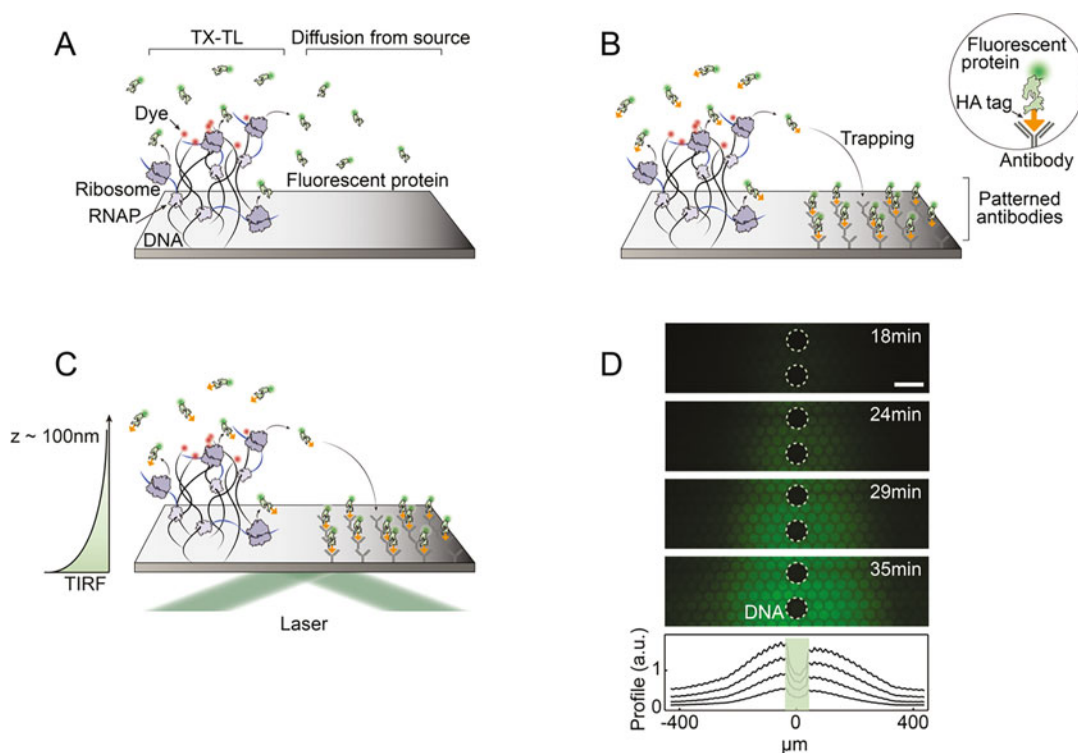


Fig. 1 Protein expression and trapping on the chip. (a–c) Schemes of the three options A, B and C. (a) Option A: Transcription and translation colocalize on the DNA brush, forming a local source of gene expression products. Nascent proteins diffuse from the source. DNA is labeled in red and proteins in green. The setup is compatible with epifluorescence microscopy. (b) Option B: Nascent proteins fused to an HA peptide tag (orange) are trapped on patterned antibodies. The setup is compatible with epifluorescence microscopy. (c) Option C: Similar to option B but on a setup compatible with TIRF microscopy. (d) Top: Images of the TIRF signal that builds up in time symmetrically on two sides of a line of brushes coding for the ribosomal protein S15 fused to GFP and an HA tag: S15-GFP-HA. Time $t = 0$ corresponds to the instance when the temperature crossed 30°C in its rise from 17°C to 37°C . Bottom: profiles of the signal. (Reproduced from ref [5] with permission from ACS. Further permissions related to the material excerpted should be directed to the ACS.)

High local concentration of newly synthesized RNA and proteins promote association interactions that could not be realized in a dilute regime of bulk reactions. This key feature allowed us recently to demonstrate autonomous synthesis and assembly of the *E. coli* small ribosomal subunit (SSU) [6]. The entire assembly line of this complex macromolecular machine, composed of twenty ribosomal proteins (r-proteins) and one ribosomal RNA (r-RNA), was reconstructed using patterned DNA brushes on boundary-free surfaces in contact with a large reservoir of bulk solution. DNA brushes could also be enclosed in micron-scale silicon compartments with characteristic dimensions of live cells for further confinement and increased local concentrations, allowing for spatial resolution of assembly intermediates along quasi-one-dimensional diffusion profiles [4]. Microfluidic silicon compartments patterned

with DNA brushes achieve steady-state protein synthesis and allow for the realization of complex dynamics such as coupled genetic oscillators with cell-cell communication [7–9]. Finally, microfluidic polydimethylsiloxane (PDMS) compartments have been used to realize biochemical decision-making using a synthetic genetic switch [10].

In addition to the high throughput and reproducibility of this surface approach, DNA brushes reduce the overall quantity of DNA required for the cell-free reaction from typically nanomolar to picomolar concentrations. The resulting DNA brush, with a typical surface density of ~ 1000 molecules/ μm^2 [11], has an effective micromolar local DNA concentration, creating a boundary-free subcompartment with favorable conditions for biomolecular interactions.

Here, we present the basic procedure to realize cell-free gene expression from immobilized DNA brushes using either cell-free *E. coli* extracts [12] or minimal transcription-translation reactions from purified components [13]. We describe the steps required to assemble DNA brushes, starting with coating of a silicon dioxide (SiO_2) surface with a biocompatible monolayer, followed by patterning it using UV lithography, and finally DNA brush buildup and on-surface gene expression. We discuss three experimental options compatible with the platform (Fig. 1). Gene products synthesized from DNA brushes can either be free to diffuse and interact with each other in the reaction volume (option A), or captured on antibodies patterned on the surface (option B). Gene expression can be monitored using regular epifluorescence microscopy (options A and B) or total internal reflection fluorescence (TIRF) microscopy (option C) that images fluorescent objects close to the surface with high signal-to-noise values. This basic protocol can be applied to any SiO_2 surface, whether boundary-free, enclosed in silicon or in PDMS compartments.

2 Materials

Use deionized water in all steps of chip fabrication, and nuclease-free water in all steps involving DNA and cell-free reactions.

2.1 DNA Solutions

1. Plasmids used as templates for generating linear PCR fragments are at a stock concentration of $10 \text{ ng}/\mu\text{L}$. The gene of interest should be cloned under control of a promoter (T7 or *E. coli* according to the RNA polymerase involved in the cell-free reaction) and an *E. coli* ribosome binding site. Coding sequences for an N- or C-terminal high-affinity peptide tag (e.g., HA peptide YPYDVPDYA) can be inserted in the gene of interest for trapping the protein on high affinity surface

bound biotinylated antibodies. Plasmids should be stored at -20°C .

2. High-fidelity polymerase chain reaction (PCR) amplification kit.
3. Forward primer and 5'-biotin reverse primer at a concentration of $10\text{ }\mu\text{M}$. Primers should be stored at -20°C . The forward primer may be fluorescently labeled at the 5' end for imaging the DNA brushes.
4. A PCR cleanup kit.
5. Aliquots of $20\text{--}30\text{ }\mu\text{L}$ of streptavidin at a typical concentration of $5\text{ }\mu\text{M}$. The aliquots should be stored at -20°C .
6. Dulbecco's magnesium- and calcium-free phosphate-buffered saline (PBS) $10\times$.
7. Glycerol.

2.2 Cleaning Slides with Base Piranha

1. Slides with an SiO_2 surface layer: glass slides, fused silica slides (best for option C), coverslips or silicon wafers coated with 50 nm of SiO_2 [4, 7] (options A and B).
2. Teflon slide holder.
3. Two beakers.
4. 25% Ammonia solution.
5. 30% Hydrogen peroxide.
6. Ultrasonic cleaner.
7. Aluminum foil.

2.3 Coating Slides with a Photosensitive and Biocompatible Polymer Monolayer

1. The polymer that is used to coat SiO_2 surfaces is composed of a polyethylene glycol (PEG) backbone with a protected amine at one end and a triethoxysilyl group at the other end [14] (*see Note 1*), which forms a self-assembled monolayer covalently bound to the surface. The protected amine group can be activated by UV light (365 nm). Prepare aliquots of $\sim 0.3\text{ mg}$ in a toluene-resistant tube (e.g., polypropylene). Avoid any glassware as the polymer would coat the glass vessel, reducing its solution concentration. Aliquots can be stored at 4°C in a desiccator for a few months. For long term storage (more than a year), keep in a freezer inside a glovebox to avoid contact with humidity.
2. Toluene or acetonitrile (HPLC grade, preferably anhydrous).
3. 5- and 10-mL syringe with Luer Lock tip, needle and filter ($0.20\text{ }\mu\text{m}$, hydrophobic PTFE).
4. Glass petri dish.
5. Plastic petri dish.

6. Aluminum foil.
7. Nitrogen storage box.

2.4 Biotin Surface-Patterns

1. 50 μL aliquots of Biotin N-hydroxysuccinimidyl ester in DMSO at a concentration of 5 mg/mL. The aliquots should be stored at -20°C .
2. Optional for surface blocking: 50 μL Aliquots of methyl-PEG4-NHS in DMSO at a concentration of 5 mg/mL. The aliquots should be stored at -20°C .
3. 250 mM Borate buffer pH 8.6.
4. Equipment for UV lithography at 365 nm with intensity $2.5\text{ J}/\text{cm}^2$: UV laser writer, mask aligner, or microscope equipped with UV filter. Photomasks can be ordered from specialized companies.

2.5 Preparation of the Reaction Chamber

1. Thin PDMS film, UV curable adhesive, Scotch tape (*see Note 2*).
2. Option C: Prism for prism-based TIRF microscopy.
3. Option C: Double sided adhesive spacer to fix the chip on the prism and index-matching liquid.

2.6 Assembly of DNA Brushes and Deposition of Antibodies

1. $1\times\text{PBS}$.
2. 50 mM potassium-Hepes buffer (pH 7).
3. Chamber with humidity control.
4. Options B and C: biotinylated Anti-HA-biotin antibodies stored at -20°C in 20–50 μL aliquots at a typical concentration of 500 nM.
5. Optional for precise DNA deposition: droplet dispensing system.

2.7 Gene Expression Reaction

1. Cell-free protein expression reaction: PURE System or *E. coli* cell extract (commercial or homemade). The latter must be supplemented with the protein Gam from λ bacteriophage or with short double-stranded oligonucleotides containing χ sequences in order to inhibit the recBCD nuclease that degrades linear DNA fragments [15, 16].
2. Optional: Complementary molecules for labeling may be required depending on the fluorescent marker used in the experiment. For example, the gene expression reaction may be supplemented by unnatural fluorescent amino acids in order to in situ label proteins carrying an engineered codon [17]. RNA products may be visualized by inserting RNA aptamers, such as RNA Broccoli [18], into their coding sequences, necessitating supplementation of the gene expression reaction with the appropriate aptamer-binding dye.

3. Optional for investigating the gene expression machinery on DNA brushes: Fluorescently labeled RNAPs or ribosomes [3].
4. Optional for macromolecular assembly reactions: PEG 8000 to supplement the cell-free reaction at a final concentration of 4% (*see Note 3*).
5. Slide holder with temperature control.
6. Dehumidifier.
7. Low-retention pipette tips.

2.8 Imaging

1. Options A and B: Fluorescence microscope.
2. Option C: prism-based TIRF microscope.

3 Methods

3.1 DNA Solutions

1. PCR amplify linear double stranded DNA fragments from the corresponding template plasmid. Use a forward primer positioned ~200 bp upstream to the promoter (*see Note 4*) and 5'-biotin reverse primer positioned downstream to the transcription terminator (or to the translation stop codon as in run-off transcription) (*see Note 5*, Fig. 2a). The forward primer may be fluorescently labeled (*see Note 6*).
2. Purify the PCR reaction mix using a commercial kit to ensure that almost no biotinylated primer remains in the solution (*see Note 7*). Two consecutive purifications may be required. The final DNA concentration after purification should be ~400 nM in order to reach the desired concentration required for the following steps: **step 3** and Subheading 3.6, **step 1**.
3. Conjugate the biotinylated linear DNA to streptavidin at a 1:1.4 ratio [19]: Mix the DNA solution, 10× PBS, glycerol, and water, then add streptavidin to reach final concentrations of ~300 nM DNA, 1× PBS, and 5–7% glycerol (the latter is added to reduce evaporation during DNA surface deposition). Pipette thoroughly. The resulting solution of streptavidin-conjugated DNA (SA-DNA) can be further diluted with 1× PBS, 5–7% glycerol depending on downstream application and stored at 4 °C for about 1 year (*see Note 8*).
4. For preparation of DNA brushes containing several different types of DNA molecules, conjugate each type of DNA separately and then mix. The molar ratios between DNA types in the mixtures will reflect the ratios in brushes (*see Note 9*).

3.2 Cleaning Slides with Base Piranha

Perform the cleaning procedure in a fume hood.

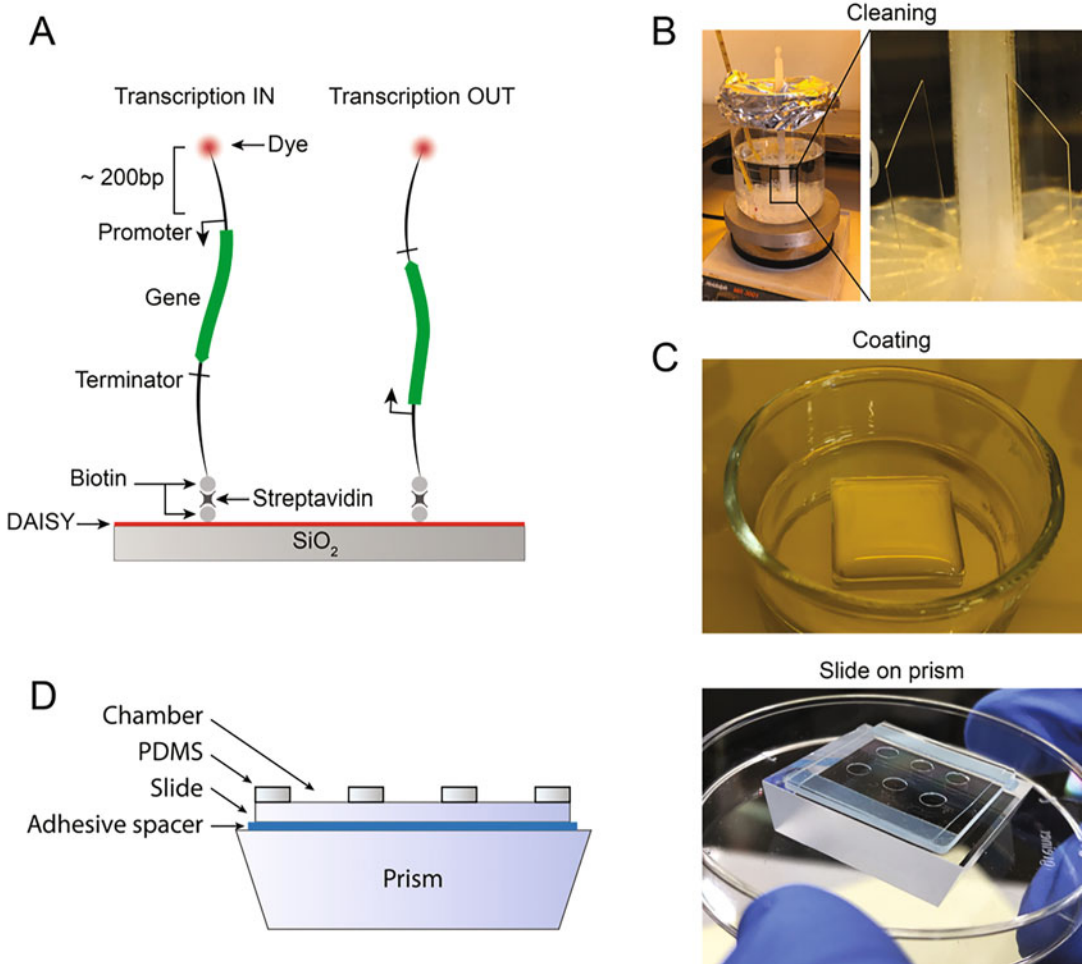


Fig. 2 Illustration of the protocol. **(a)** Scheme of two DNA constructs immobilized on the surface. The position of the promoter, determined by the biotinylated primer (reverse or forward), dictates the direction of transcription (IN: toward the surface or OUT: away from the surface). **(b)** Left: Slides positioned in a holder inside a beaker on a hot plate during the cleaning procedure. Temperature is monitored with a regular mercury thermometer immersed next to the slides. The cleaning solution should cover the slides. Right: Zoom on coverslips inserted in the holder. **(c)** A slide positioned inside a glass petri dish is covered with a solution of the biocompatible polymer dissolved in toluene to assemble a coating monolayer on the SiO₂ surface. During incubation, the glass petri dish should be closed to avoid evaporation of toluene. The step is performed under yellow light. **(d)** Scheme and picture of a slide fixed on a prism with double sided adhesive spacers (in blue) for TIRF microscopy (option C). Six circular chambers are cut into a thin PDMS film (~150 μm height) on the slide

1. Place the slides (glass slides, fused silica slides, coverslips, or silicon wafers coated with SiO₂) in the slide holder, inside a beaker.
2. Add >95% ethanol to fully immerse the slides and cover the beaker with aluminum foil. Boil for 10 min on a hot plate (Fig. 2b).

3. Sonicate in an ultrasonic cleaner for 10 min (*see Note 10*).
4. In a new beaker mix 3 volumes of double-distilled water and 1 volume of a 25% ammonia solution. The final volume should cover the slides. Heat to 70 °C on a hot plate.
5. Transfer the slide holder with the slides from the beaker with ethanol to the second beaker with the hot ammonia solution (from **step 4**).
6. Add 1 volume of hydrogen peroxide. Warning: the reaction is highly exothermic. Personal protective equipment including gloves, goggles and lab coat is required. Let boil for 10 min, keeping the temperature around 70 °C. The beaker may need to be removed from the hot plate if the temperature rises too much.
7. Rinse the slides by dipping the slide holder in two consecutive beakers full of double-distilled water. Keep the slides in water and cover the beaker to avoid dust contamination.

3.3 Coating Slides with a Photosensitive and Biocompatible Polymer Monolayer

Perform this step under yellow light and if possible, in a clean room.

1. Dry the slides and place them inside individual glass petri dishes.
2. Add 1.5 mL of anhydrous toluene per coating polymer aliquot (final concentration 0.2 mg/mL). Anticipate using about 250 µL of this solution per slide of 24 mm × 24 mm. Vortex the tube until the powder is dissolved (*see Note 11*).
3. Filter the solution, pushing it through a filter resistant to toluene (0.20 µm, hydrophobic PTFE). Collect the filtered solution in a toluene-resistant tube. Alternatively, acetonitrile (HPLC grade) may replace toluene to dissolve the coating polymer for applications that should avoid toluene, such as coating PDMS compartments [10].
4. Use a 200-µL pipette to cover each slide with the coating solution. Close the glass petri dish as soon as the slide is covered to avoid toluene evaporation (Fig. 2c).
5. Incubate for 20 min. Regularly check that the toluene solution still covers the full surface of the slides. If not, add more solution or toluene.
6. Wash the slides by dipping them in two consecutive beakers full of AR grade toluene. Remove the slides from the toluene and allow them to dry by leaning them against a clean glass vessel.
7. Place each slide in an individual plastic petri dish with the coated side up. Wrap the petri dish in aluminum foil to protect it from light and store in a nitrogen box (*see Note 12*). Slides can be stored for more than a year.

3.4 Biotin Surface-Patterns

Perform this section under yellow light.

1. Optional: The polymer might have a residual fraction of unprotected amines after synthesis (no more than a few % as estimated by NMR). These unprotected amines can be blocked by conjugation to NHS-PEG in order to reduce conjugation of NHS-biotin to undesignated areas.

Dilute the Methyl-PEG4-NHS to 0.5 mg/mL with 250 mM borate buffer, pH 8.6 and apply on the coated surface in order to block any residual unprotected amines present within the monolayer prior to UV illumination [10]. Incubate for 5 min. Rinse with water.

2. Expose the slide to UV with a device for UV lithography such as a laser writer or a mask aligner, according to the pattern of choice. Saturating UV dose (365 nm) to fully deprotect the surface amines is 2.5 J/cm². Exposure with a lower dose leads to partial deprotection, generating patterns of variable density of deprotected amine groups over a full grayscale [14].
3. Immediately prior to surface deposition, dilute an aliquot of NHS-Biotin in borate buffer to a final concentration of 0.5 mg/mL.
4. Quickly cover the exposed slide with the solution of NHS-Biotin. The NHS-Biotin covalently binds to the deprotected amine groups, forming a biotin pattern on the surface.
5. Incubate from 20 min to a few hours in an environment protected from ambient light, although the NHS is no longer reactive after the initial 20 min.
6. Rinse the slides by dipping them in two consecutive beakers full of double-distilled water (*see Note 13*).
7. Dry the slides with a flow of N₂ gas and store them in a nitrogen box (*see Note 14*). They can be stored for about a week.

3.5 Preparation of the Reaction Chamber

1. Option C: Fix the slide on a prism with double sided adhesive spacers. Keep openings to add index-matching liquid between the slide and prism (Fig. 2d).
2. Cut chambers of desired dimensions in PDMS film (*see Note 15*) or scotch tape.
3. Place the PDMS film on top of the slide with patterned biotins and gently press. Alternatively, use UV curable adhesive with PDMS molds to reach more precise and reproducible chamber designs (*see Note 16*). Chambers can be etched on a silicon wafer as well, using standard nanofabrication techniques (options A and B). In that case, the wafer should be coated

after etching with 50 nm of SiO₂ for the biocompatible coating polymer to form a monolayer covalently bound to the surface.

3.6 Assembly of DNA Brushes and Deposition of Antibodies

1. Deposit the solutions of SA-DNA on the slide. For high density DNA brushes use solutions of 150–300 nM SA-DNA. If brushes of different compositions are required on a single slide, deposit small droplets of the different DNA solutions. This can be achieved with a simple pipette (*see Note 17*) or with a droplet dispensing system (*see Note 18*).
2. Incubate from 1 h to overnight in a chamber with around 60% controlled humidity. During incubation, the SA-DNA binds to the biotinylated surface and forms a brush of density about 10³ DNA/μm².
3. Wash the droplets by applying a solution of 1×PBS to the chamber and pipetting up and down vigorously (*see Note 19*). Repeat 3–4 times. The slide can be stored at 4 °C for a few days in a chamber filled with PBS.
4. Options B and C: Conjugate anti-HA-biotin antibodies to streptavidin at a 1:1.4 ratio in 1×PBS. Incubate 30 min on ice. Dilute to a final concentration of ~50 nM in 1×PBS. The solution can be stored at 4 °C for a few days.
5. Perform the DNA droplet washing step (**step 3**) with the solution of SA-conjugated anti-HA-biotin antibodies in 1×PBS (*see Note 20*), then incubate for an hour at 4 °C. Wash the excess antibodies by exchanging with 1xPBS in the chamber. Repeat 3–4 times, never drying the chamber. Once antibodies are bound to the surface, the slide cannot be stored more than about a day at 4 °C.
6. Replace 1×PBS with potassium-Hepes buffer inside the chamber (*see Note 21*).

3.7 Gene Expression Reaction

1. Option C: Add index-matching liquid in the slit between prism and slide.
2. To measure dynamics of gene expression: Position the functionalized slide under the fluorescent microscope on a temperature-controlled holder. Set the temperature to 15–17 °C to hinder protein expression when setting up the experiment (*see Notes 22 and 23*).

To measure the cell-free reaction at a single timepoint: the gene expression reaction does not have to take place under the microscope. Incubate the slide in an enclosed chamber with humidity at 15–17 °C to hinder protein expression when setting up the experiment (*see Notes 22 and 23*).

3. Fix a temperature probe close to the slide and track its temperature during the experiment.

4. Prepare the cell-free protein expression reaction by assembling all the components of either the PURE System or the *E. coli* cell extract. If required, supplement the reaction with the required molecules for crowding or fluorescence-labeling. Supplement the reaction with labeled machinery (RNA polymerase or ribosome) if needed. Keep on ice.
5. Remove as much potassium-Hepes buffer from the chamber as possible without drying any region of the chamber.
6. Add the protein expression reaction solution by exchanging four times with fresh reaction. Each time, pipette carefully to mix the chamber (*see* **Note 20**).
7. Seal the chamber with a glass coverslip.
8. To measure dynamics of gene expression: *see* Subheading 3.8.

To measure the cell-free reaction at a single timepoint: Increase the temperature of the holder to 30 °C or 37 °C to start the *E. coli* extract or PURE reaction, respectively. Stop the reaction after reaching the timepoint of interest by lowering the temperature to 15–17 °C. For options B and C, when the product of interest is immobilized on antibodies, it is possible to open the chamber and wash it with 1× PBS to remove the fluorescent background caused by unbound molecules (*see* **Notes 24** and **25**).

3.8 Imaging

1. To image the cell-free reaction at a single timepoint: place the slide under the fluorescent microscope, find the regions of interest, and image.
2. To measure dynamics of gene expression: Increase the temperature of the holder to 30 °C or 37 °C to start the *E. coli* extract or PURE reaction, respectively. A typical experiment lasts between 1 and 4 h.

4 Notes

1. The photosensitive and biocompatible polymer is not commercially available. It can be synthesized according to the protocol published in [14].
2. Thin chambers enable confinement of the reaction in the axis perpendicular to the surface, which can be critical when studying interactions between gene products. A simple way to make a thin PDMS film (of thickness about 100–200 μm) is to cure PDMS between two plastic slides separated by thin spacers such as coverslips. Another possibility is to cut a chamber inside regular Scotch tape (thickness ~50 μm).
3. Addition of PEG to the protein expression reaction increases crowding and may facilitate machine assembly [20].

4. The forward primer should be positioned ~200 bp upstream to the promoter to protect the promoter from DNA degradation that can occur, especially in cell extract [15, 20]. The reverse primer may be positioned either downstream to the terminator or downstream to the translation stop codon (upstream to the transcription terminator).
5. The biotinylated primer controls the direction of transcription relative to the surface (Fig. 2a). A biotinylated forward primer directs transcription outward in the DNA brush and a biotinylated reverse primer directs transcription inward. Inward transcription was shown to be more efficient [21].
6. Avoid a 5' guanosine nucleotide in a 5'-fluorescently labeled primer. The guanine base promotes quenching of the fluorophore.
7. Excess of biotinylated primer might undesirably bind streptavidin and thus lower the efficiency of the subsequent surface binding of SA-DNA.
8. Resolve a sample of the SA-DNA by agarose gel electrophoresis and compare to non-SA-DNA in an adjacent lane. The migration of the SA-DNA should be slightly retarded by the conjugation to streptavidin. A second band corresponding to DNA doublets (two DNA molecules bound to the same streptavidin) is frequently seen. If a third band representing DNA triplets is visible, repeat the conjugation step with a higher concentration of streptavidin until only singlets and doublets are present.
9. The composition of a DNA mixture reflects the brush composition when DNA molecules of the same size are mixed. This is not true for DNA molecules of different sizes. Short DNA molecules bind the surface at a higher density than long ones [11].
10. When working with thin coverslips, skip the sonication step to avoid breaking the coverslips.
11. Using a vortex machine to dissolve the powder in toluene may take a few minutes. Ideally, no aggregate visible by eye should remain. However, if the powder was in contact with humidity, irreversible polymerization might occur, resulting in persistent aggregates. These aggregates will be filtered in a following step. In general, the dissolved polymers are at sufficiently high concentration to build a monolayer and cover the surface of the slide despite these aggregates.
12. Branched structures, visible under the microscope, appear on the coated slide when stored outside of a nitrogen box for a few months, most likely due to humidity.

13. The surface with the biotin pattern should be very hydrophobic as seen by the contact angle between slide surface and the droplet.
14. From the moment excess biotin is rinsed off the surface, there is no need to protect it from ambient light.
15. Thin PDMS films of thickness 100–200 μm have the tendency to fold on themselves and are therefore hard to manipulate. One solution is to apply the PDMS film on a clean metallic cylinder, and then apply the film on the slide by gently rolling the cylinder over the slide.
16. It is useful to prepare a small chamber for optical calibration. This chamber could be filled with fluorescently labeled streptavidin and used to find the right focus and calibrate the TIRF microscope if needed.
17. Circular brushes of diameter smaller than a millimeter can be achieved with a regular pipette.
18. Circular brushes of diameter between 50 and 100 μm can be achieved with picoliter droplet dispensing techniques. Smaller brushes can be achieved through lithography, by activating only a small region of the photoactivable monolayer [10]. The brush dimensions that can be generated with this photolithography approach are limited by the diffraction of UV light. Electron-beam lithography was also shown to activate the monolayer and facilitated brush dimensions smaller than optical resolution [22]. Using this technique, DNA molecules were immobilized on lines of width ~ 100 nm. When the dimension of the brush is defined by lithography techniques instead of droplet deposition, it becomes essential to reduce nonspecific adsorption with surface-blocking treatments (*see* Subheading 3.4, step 1).
19. Use a large amount of $1\times\text{PBS}$ when washing the droplets of DNA solutions and pipette vigorously to prevent excess unbound DNA molecules from binding nonspecifically on the slide. If antibodies are added to the washing solution, their binding to surface biotins removes nonspecifically bound DNA by competition.
20. To limit the formation of bubbles during the washing procedure with antibodies or when exchanging the protein expression reaction, use low retention pipette tips.
21. Residual $1\times\text{PBS}$ might inhibit gene expression.
22. In the case of a humid climate, condensation may form on the slide and on the prism when the temperature of the slide holder is set to 15–17 $^{\circ}\text{C}$. If so, use a dehumidifier in the room.
23. Transcription still occurs at 15–17 $^{\circ}\text{C}$. Translation does not.

24. The chamber should not dry out during the washing process. One way to ensure that is to open the chamber only when immersed in PBS.
25. The washing process may be aggressive and should be used only when studying stable complexes on antibodies.

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Efficient and Precise Protein Synthesis in a Cell-Free System Using a Set of In Vitro Transcribed tRNAs with Nucleotide Modifications

Kazuaki Amikura, Keita Hibi, and Yoshihiro Shimizu

Abstract

Reconstitution of a complicated system with a minimal set of components is essential for understanding the mechanisms of how the input is reflected in the output, which is fundamental for further engineering of the corresponding system. We have recently developed a reconstituted cell-free protein synthesis system equipped only with 21 in vitro transcribed tRNAs, one of the minimal systems for understanding the genetic code decoding mechanisms. Introduction of several nucleotide modifications to the transcribed tRNAs showed improvement of both protein synthesis efficiency and its fidelity, suggesting various combinations of tRNAs and their modifications can be evaluated in the developed system. In this chapter, we describe how to prepare this minimal system. Methods for preparing the transcribed tRNAs, their modifications, and the protein production using the set of prepared tRNAs are shown.

Key words tRNA, Cell-free protein synthesis system, PURE system, Genetic code, In vitro transcription, Nucleotide modification, Synthetic biology

1 Introduction

In principle, codons in mRNA are tightly linked to 20 canonical amino acids, which is well illustrated in a universal genetic code table. The decoding, that is, translation of codons to a sequence of synthesized proteins, is achieved by a specific interaction of aminoacyl-tRNA with each codon on a ribosome. In a cellular translation machinery, aminoacyl-tRNA synthetases (ARSs) corresponding to each amino acid also function for precise decoding processes, where they specifically recognize and aminoacylate each target tRNA. Thus, tRNAs, ARSs, and the ribosome are key elements for precise translation of genes encoded in genome of each organism.

Changing this coordination can artificially redesign or expand the genetic code table [1]. For example, transplanting an identity element for alanyl-tRNA synthetase into other tRNA species can alter the association between the codon and amino acid, which can rewrite the genetic code [2, 3]. Utilization of a chemical aminoacylation method [4], ARSs mutants [5], and ribozymes known as flexizyme [6] can further expand the variety of amino acids apart from 20 canonical amino acids, which provides proteins with physically, chemically, or biologically novel properties [7, 8].

Although the genetic code expansion is currently available in living cells, a cell-free protein synthesis system is fundamental to this field [9], in a sense that complicated relationships between codons, tRNAs, and ARSs can be evaluated in a test tube. More comprehensive understanding of such relationships, including nucleotide modifications introduced to the tRNAs [10, 11] enables the development of more sophisticated controlling of the decoding processes in terms of efficiency and fidelity. Such information is also essential to identify minimal requirements for constructing a minimal cell [12], one of the major goals in the field of synthetic biology [13].

To provide a fundamental platform system for studying the genetic code decoding, we recently developed a reconstituted cell-free protein synthesis system [14] equipped only with 21 in vitro transcribed tRNAs (iVT tRNAs) [15]. Introduction of several nucleotide modifications have shown to improve translation efficiency and fidelity, which was comparable to the system using native tRNA mixtures. Thus, the system is one of the minimal systems with sufficient efficiency and fidelity, which can be utilized for a comprehensive study of decoding processes as described. In this chapter, we provide detailed descriptions including preparation of iVT tRNAs and tRNA modification enzymes, and the protein synthesis experiment using prepared iVT tRNAs.

2 Materials

2.1 Affinity Purification of Proteins

1. Plasmids encoding TsaB, TsaC, TsaD, TsaE, TrmD, GlyA, MnmC, GidA, and C5 protein cloned from *E. coli* genome [15, 16].
2. BL21 (DE3) competent cells.
3. 100 mg/mL ampicillin.
4. 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG).
5. Lysis buffer: 50 mM HEPES-KOH, pH 7.6, 1 M NH_4Cl , 10 mM MgCl_2 , and 7 mM 2-mercaptoethanol.
6. cOMplete His-tag Purification Resin.
7. Econo-Pac[®] Chromatography Columns.

8. Elution Buffer: 50 mM HEPES–KOH, pH 7.6, 400 mM KCl, 10 mM MgCl₂, 400 mM imidazole, and 7 mM 2-mercaptoethanol.
9. Amicon Ultra-15 Centrifugal Filter Unit, 3 kDa, 10 kDa, and 30 kDa.
10. Stock Buffer: 50 mM HEPES–KOH, pH 7.6, 500 mM KCl, 10 mM MgCl₂, 7 mM 2-mercaptoethanol, 30% (v/v) glycerol.
11. Ulp1p active domain (403-621) (*see Note 1*).
12. Cleavage Buffer 1: 50 mM HEPES–KOH, pH 7.6, 100 mM KCl, 10 mM MgCl₂, and 7 mM 2-mercaptoethanol.
13. Cleavage Buffer 2: 50 mM HEPES–KOH, pH 7.6, 500 mM KCl, 10 mM MgCl₂, and 7 mM 2-mercaptoethanol.
14. Cleavage Buffer 3: 50 mM HEPES–KOH, pH 7.6, 500 mM KCl, 10 mM MgCl₂, and 7 mM 2-mercaptoethanol, 30% (v/v) Glycerol.
15. HiTrap Q HP anion exchange chromatography column, 5 mL.
16. IEX Buffer: 50 mM HEPES–KOH, pH 7.6, 100 mM KCl, 10 mM MgCl₂, 7 mM 2-mercaptoethanol.
17. C5 suspension buffer 1: 50 mM Tris–HCl, pH 7.6, 1 M NaCl, 5 mM EDTA, 10% (v/v) glycerol.
18. Ammonium sulfate, powder.
19. C5 suspension buffer 2: 50 mM Tris–HCl, pH 7.6, 250 mM NaCl, 5 mM EDTA, 7 mM 2-mercaptoethanol.
20. C5 purification buffer 1: 50 mM HEPES–KOH, pH 7.6, 100 mM NH₄Cl, 6 M urea, 7 mM 2-mercaptoethanol.
21. 0.45 µm Millex-HV filter.
22. HiTrap SP HP cation exchange chromatography column, 5 mL.
23. C5 purification buffer 2: 50 mM HEPES–KOH, pH 7.6, 2 M NH₄Cl, 6 M urea, 7 mM 2-mercaptoethanol.
24. C5 storage buffer: 50 mM HEPES–KOH, pH 7.6, 800 mM NH₄Cl, 10 mM MgCl₂, 7 mM 2-mercaptoethanol.
25. Compact Lab Homogenizer UP50H or equivalent.
26. Rotator RT-50 or equivalent.
27. Chromatography system AKTA pure 25 or equivalent.

2.2 In Vitro Transcription of iVT tRNA

1. Plasmids encoding a set of 21 tRNAs (Table 1) and M1 RNA (*see Note 2*).
2. Oligo DNA primers (Table 2).
3. C5 protein purified by the method described in Subheading 3.2.6.

Table 1
List of 21 in vitro transcribed tRNA

tRNA	Gene sequence (5' - 3'; underlined characters correspond to the anticodon)
tRNA ^{Phe} _{GAA}	GCCCCGATAGCTCAGTCGGTAGAGCAGGGGATT <u>GAA</u> AATCCCCGTGTCCT TGGTTCGATTCCGAGTCCGGGCACCA
tRNA ^{Leu} _{CAG}	GCGAAGGTGGCGGAATTGGTAGACGCGCTAGCTT <u>CAG</u> GTGTTAGTGTCTCT TACGGACGTGGGGGTTCAAGTCCCCCCCCCTCGCACCA
tRNA ^{Ile} _{GAU}	AGGCTTGTAAGCTCAGGTGGTTAGAGCGCACCCCT <u>GAT</u> AAGGGT GAGGTCGGTGGTTCAAGTCCACTCAGGCCTACCA
tRNA ^{fMet} _{CAU}	CGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCT <u>CATA</u> ACCCGAA GATCGTCGGTTCAAATCCGGCCCCCGCAACCA
tRNA ^{mMet} _{CAU}	GGCTACGTAGCTCAGTTGGTTAGAGCACATCACT <u>CATA</u> ATGATGGGGTCA CAGGTTCTGAATCCCGTCGTAGCCACCA
tRNA ^{Val} _{GAC}	GCGTCCGTAGCTCAGTTGGTTAGAGCACACCTT <u>GAC</u> ATGGTGGGGGTCTG GTGGTTCGAGTCCACTCGGACGCACCA
tRNA ^{Ser} _{GGA}	GGTGAGGTGTCCGAGTGGCTGAAGGAGCACGCCT <u>GGA</u> AAGTGTGTA TACGGCAACGTATCGGGGGTTCGAATCCCCCCCCCTCACC GCCA
tRNA ^{Pro} _{GGG}	CGGCACGTAGCGCAGCCTGGTAGCGCACCGTCAT <u>GGG</u> GTGTCTGGGGGTCTC GGAGGTTCAAATCCTCTCGTGCCGACCA
tRNA ^{Thr} _{GGU}	GCTGATATGGCTCAGTTGGTAGAGCGCACCCCTT <u>GGT</u> AAGGGTGAGGTCCC CAGTTCGACTCTGGGTATCAGCACCA
tRNA ^{Ala} _{GGC}	GGGGCTATAGCTCAGCTGGGAGAGCGCTTGCAT <u>GGC</u> ATGCAAGAGGT CAGCGGTTTCGATCCCGCTTAGCTCCACCA
tRNA ^{Tyr} _{GUA}	GGTGGGGTTCCTCGAGCGGCCAAAGGGAGCAGACT <u>GTA</u> AATCTGCCGTCA CAGACTTCGAAGGTTTCGAATCCTTCCCCCACCACCA
tRNA ^{His} _{GUG}	GGTGGCTATAGCTCAGTTGGTAGAGCCCTGGATT <u>GTG</u> ATTCCAGTTGtCGT GGGTTCTGAATCCCATAGCCACCCCA
tRNA ^{Gln} _{CUG}	TGGGGTATCGCCAAGCGGTAAGGCACCGGATT <u>CTG</u> ATTCCGGCATTCC GAGGTTCTGAATCCTCGTACCCCAGCCA
tRNA ^{Asn} _{GUU}	TCCTCTGTAGTTCAGTCGGTAGAACGGCGGACT <u>GTT</u> AATCCGTATGT CACTGGTTCGAGTCCAGTCAGAGGAGCCA
tRNA ^{Lys} _{CUU}	GGGTCGTTAGCTCAGTTGGTAGAGCAGTTGACT <u>CTT</u> AATCAATTGGTCTG CAGGTTCTGAATCCTGCACGACCCACCA
tRNA ^{Asp} _{GUC}	GGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCT <u>GTC</u> ACG CAGGGGGTCTCGGGGTTTCGAGTCCCGTCCGTTCCGCCA
tRNA ^{Glu} _{CUC}	GTCCCCCTTCGTCTAGAGGCCACAGGACACCGCCCT <u>CTC</u> ACGGCGGTAA CAGGGGTTCTGAATCCCCTAGGGGACGCCA
tRNA ^{Cys} _{GCA}	GGCGCGTTAAACAAAGCGGTTATGTAGCGGATT <u>GCA</u> AATCCGTC TAGTCCGGTTCGACTCCGGAACGCGCCTCCA
tRNA ^{Trp} _{CCA}	AGGGGCGTAGTTCAATTGGTAGAGCACCGGTCT <u>CCA</u> AAACCGGGTGtTGGG AGTTCGAGTCTCTCCGCCCTGCCA

(continued)

Table 1
(continued)

tRNA	Gene sequence (5' - > 3'; underlined characters correspond to the anticodon)
tRNA ^{Arg} _{CCG}	GCGCCCGTAGCTCAGCTGGATAGAGCGCTGCCCT <u>CCG</u> GAGGCGAGAGGTCT CAGGTTCTGAATCCTGTCTGGGCGCGCCA
tRNA ^{Gly} _{CCC}	GCGGGAATAGCTCAGTTGGTAGAGCACGACCTT <u>GCCA</u> AGGTCGGGGTCTCGC GAGTTCGAGTCTCGTTTCCCGCTCCA
tRNA ^{Glu} _{UUC}	GTCCCTTCGTCTAGAGGCCAGGACACCGCCCTT <u>TTC</u> ACGGCGGTAA CAGGGGTTCTGAATCCCCTAGGGGACGCCA

Table 2
List of oligo DNA primers used for the preparation of DNA templates for iVT tRNA and M1 RNA transcription

Gene name	Reverse primer (5' - > 3')	Forward primer (5' - > 3')
tRNA ^{Phe} _{GAA}	TGGTGCCCGGACTCGGAA	CCGCGTAATACGACTCACTATAG
tRNA ^{Leu} _{CAG}	TGGTGCGAGGGGGGGGA	CCGCGTAATACGACTCACTATAG
tRNA ^{Ile} _{GAU}	TGGTAGGCCTGAGTGGACTTG	CCGCGTAATACGACTCACTATAG
tRNA ^{fMet} _{CAU}	TGGTTGCGGGGGCCGGA	CCGCGTAATACGACTCACTATAG
tRNA ^{mMet} _{CAU}	TGGTGGCTACGACGGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Val} _{GAC}	TGGTGCGTCCGAGTGGACTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Ser} _{GGA}	TGGCGGTGAGGGGGGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Pro} _{GGG}	TGGTCGGCACGAGAGGATTT	CCGCGTAATACGACTCACTATAG
tRNA ^{Thr} _{GGU}	TGGTGCTGATACCCAGAGTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Ala} _{GGC}	TGGTGGAGCTAAGCGGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Tyr} _{GUA}	TGGTGGTGGGGGAAGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{His} _{GUG}	TGGGGTGGCTAATGGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Gln} _{CUG}	TGGCTGGGGTACGAGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Asn} _{GUU}	TGGCTCCTCTGACTGGACTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Lys} _{CUU}	TGGTGGGTCTGTCAGGATT	CCGCGTAATACGACTCACTATAG
tRNA ^{Asp} _{GUC}	TGGCGGAACGGACGGGACTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Glu} _{CUC}	TGGCGTCCCCTAGGGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Cys} _{GCA}	TGGAGGCGCGTTCCGGAGTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Trp} _{CCA}	TGGCAGGGGCGGAGAGACTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Arg} _{CCG}	TGGCGCGCCCCGACAGGATTCG	CCGCGTAATACGACTCACTATAG
tRNA ^{Gly} _{CCC}	TGGAGCGGGAAACGAGAC	CCGCGTAATACGACTCACTATAG
tRNA ^{Glu} _{UUC}	TGGCGTCCCCTAGGGGATTCG	CCGCGTAATACGACTCACTATAG
M1 RNA	AGGTGAAACTGACCGATA	CCGCGTAATACGACTCACTATAG

4. *Taq* DNA Polymerase with ThermoPol Buffer.
5. Phenol–chloroform–isoamyl alcohol (25: 24: 1, v/v/v).
6. Chloroform–isoamyl alcohol (10:1, v/v).
7. 3 M KOAc, pH 5.2.
8. Reagents for a reaction mixture: 1 M HEPES–KOH, pH 7.6, 500 mM MgCl₂, 500 mM spermidine, 1 M DTT, 100 mM ATP, UTP, CTP, and GTP, T7 RNA polymerase (*see Note 3*), and Inorganic pyrophosphatase (Roche).
9. Water-saturated phenol.
10. HiTrap Q HP anion exchange chromatography column, 5 mL.
11. tRNA purification buffer 1: 20 mM HEPES–KOH, pH 7.6, 200 mM KCl.
12. tRNA purification buffer 2: 20 mM HEPES–KOH, pH 7.6, 1000 mM KCl.

2.3 Nucleotide Modification of iVT tRNA

1. Reagents for a reaction mixture: 1 M HEPES–KOH, pH 7.6, 2 M KCl, 500 mM MgCl₂, 1 M DTT, 500 mM NaHCO₃, 0.1 mM CaCl₂, 100 mM ATP, 100 mM Thr, 1 mM S-adenosyl methionine (SAM), 10 mM Flavin adenine dinucleotide (FAD), 10 mM tetrahydrofolate, 100 mM GTP, 100 mM NADH, 10 mM pyridocal-5'-phosphate, 100 mM Ser, 100 mM Gly, recombinant RNase inhibitor (TAKARA Bio Inc.).
2. 3 M KOAc, pH 5.2.
3. Water-saturated phenol.
4. Chloroform–isoamyl alcohol (10:1, v/v).

2.4 Protein Synthesis and Measurement of the Activity of Synthesized Product

1. Plasmids encoding dihydrofolate reductase (DHFR) [15].
2. T7 promoter primer (5'-GAAATTAATACGACTCACTATAG-3') and terminator primer (5'-CCCGTTTAGAGGCCCAAG-3') for PCR amplification.
3. QIAquick PCR purification kit or equivalent.
4. PURE frex[®] 2.0.
5. PURE frex[®] 2.0, Solution I without tRNA mixture (*see Note 4*).
6. Native tRNA mixtures from *E. coli* MRE 600 dissolved in Milli-Q water.
7. Reagents for a reaction mixture used in DHFR activity measurement: 1 M MES–NaOH, pH 7.0, 1 M Tris–HCl, pH 7.0, 3 M NaCl, 1 M ethanolamine, 2-mercaptoethanol, and 100 mM EDTA.
8. Dihydrofolic acid.
9. Dihyronicotinamide-adenine dinucleotide phosphate (NADPH).

3 Methods

3.1 Plasmid Designs

The design of plasmids encoding tRNAs for in vitro transcription, M1 RNA and C5 protein for 5'-end processing of iVT tRNAs, and tRNA modification enzymes (TsaB, TsaC, TsaD, TsaE, TrmD, GlyA, MnmC, MnmE, and GidA) were described previously [15, 16]. Key elements and sequences are illustrated in Fig. 1 and Table 1.

3.2 Affinity

Purification of Proteins

The procedure depends on the property of each protein, as summarized in Fig. 2 and Table 3. Most proteins are expressed in a His-tagged small ubiquitin-like modifier (SUMO) protein-fused form, in a similar way as a previous study [18]. The SUMO protein is designed to be removed with a SUMO-specific protease (Ulp1p) [19] (Table 4).

3.2.1 Purification of TsaB and TrmD

1. Transform *E. coli* BL21(DE3) cells with a plasmid encoding a protein to be purified.
2. Cultivate the transformed cells in >10 mL of LB medium containing 100 µg/mL ampicillin at 37 °C overnight.
3. Transfer 10 mL of the overnight sub-culture into 1 L of LB medium containing the same antibiotics. Grow the culture at 37 °C to an optimal density at OD₆₀₀ = 0.6–1.0. Add 0.1–1.0 mM IPTG (Table 3) to induce the protein expression. Additionally, grow the cells for 3 h and then, harvest cells by centrifugation at 5000 × *g* for 10 min at 4 °C (*see Note 5*).
4. Resuspend cell pellets in 40 mL of Lysis buffer and disrupt them by sonication on ice.
5. Centrifuge the cell lysate at 12,000 × *g* for 30 min at 4 °C and recover the supernatant. Add 5 mL of cComplete His-tag Purification Resin, which is equilibrated with Lysis buffer, into the supernatant and gently mix with a rotator for 1 h.
6. Transfer the mixture to Econo-Pac Chromatography column for gravity-flow chromatography.
7. Wash the column with 20 bed volumes of Lysis buffer.

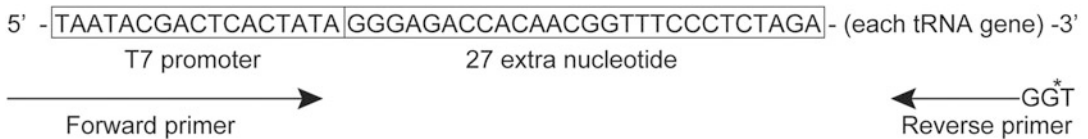


Fig. 1 Design of DNA templates of iVT tRNAs and oligo DNA primers. T7 RNA polymerase transcribes tRNA fused with extra 27 nucleotides at its 5'-terminus, which is processed with RNase P to form mature tRNA. *All reverse primers contained a 2'-methoxy modification at the second nucleotide from the 5'-terminus to prevent template-independent addition of an extra nucleotides [17]

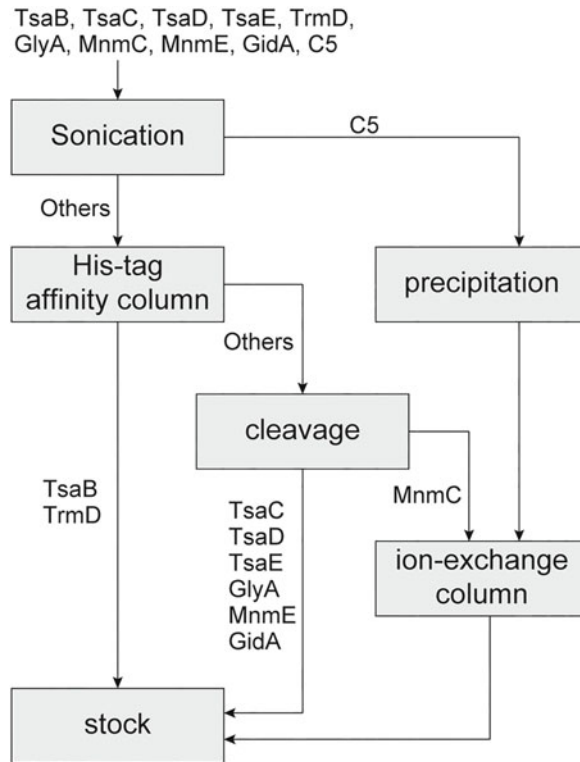


Fig. 2 Schematic flow chart of protein purification

8. Elute the target proteins with 5 bed volumes of Elution buffer. Collect fractions for SDS-PAGE analysis.
9. Analyze all collected fractions by SDS-PAGE and combine the fractions containing target proteins.
10. Concentrate the sample by Amicon Ultra-15 Centrifugal Unit with a cutoff of appropriate molecular weight (Table 3).
11. Dialyze the solution against Stock buffer at 4 °C.
12. Recover the dialyzed sample and flash freeze with liquid nitrogen. Store the protein at −80 °C until use.

3.2.2 Purification of *TsaC* and *TsaE*

1. Follow 1–9 steps in Subheading 3.2.1.
2. Combine the fractions containing target proteins and add Ulp1p to a final concentration of 23 µg/mL to remove the SUMO moiety. Dialyze the mixture against Cleavage buffer 1 at 4 °C.
3. Add 5 mL of cComplete His-tag Purification Resin, which is equilibrated with Cleavage buffer 1, into the dialyzed sample and gently mix with a rotator for 1 h.

Table 3
Conditions of each protein purification procedure

Protein	Affinity tag	IPTG ^a (mM)	MWCO ^b (kDa)	Notes
TsaB	His	1	3	His-tagged SUMO fused TsaB was not cleaved by Ulp1p. The tertiary structure of TsaB might inhibit the cleavage
TsaC	His-SUMO	1	10	
TsaD	His-SUMO	1	10	Low protein solubility; buffer containing urea is used in all procedures
TsaE	His-SUMO	1	3	
TrmD	His	1	3	
GlyA	His-SUMO	0.1	10	
MnmC	His-SUMO	0.1	30	Nonspecifically bind to His-tag purification resin
MnmE	His-SUMO	0.1	10	
GidA	His-SUMO	0.1	10	
C5		0.1	3	

^aFinal concentration of IPTG to induce protein expression

^bAppropriate molecular weight cutoff (MWCO) of Amicon filter and dialysis membrane for concentration and dialysis

- Transfer the mixture to Econo-Pac Chromatography column for gravity-flow chromatography and recover the flow-through fraction.
- Follow 10–12 steps in Subheading 3.2.1.

3.2.3 Purification of MnmE and GlyA

MnmE and GlyA purification method is the same as those described in Subheading 3.2.2 except that Cleavage buffer 2 is used for MnmE and Cleavage buffer 3 is used for GlyA, respectively, instead of Cleavage buffer 1.

3.2.4 Purification of TsaD

TsaD (YgjD) has been reported to be insoluble after sonication [20]. Therefore, the purification is performed under the condition containing urea.

- Follow 1–4 steps in Subheading 3.2.1.
- Centrifuge the cell lysate at $20,400 \times g$ for 45 min at 4 °C and remove the supernatant.
- Resuspend insoluble pellets in Lysis buffer containing 4% Triton X-100. Repeat the previous step.

Table 4

DNA sequence of Ulp1p active domain (403-621), His-SUMO protein, M1 RNA, and DHFR. His-tag and spacer sequence are underlined. SUMO sequence is described as lowercase

Protein to be synthesized	Gene sequence (5' - > 3')
Ulp1p	ATGCTTGTTTCCTGAATTAAATGAAAAAGACGATGACCAAGTACAAAAAGCTT TGGCATCTAGAGAAAATACTCAGTTAATGAATAGAGATAATATAGAGATAA CAGTACGTGATTTTAAAGACCTTGGCACCACGAAGATGGCTAAATGACACT ATCATTGAGTTTTTTATGAAATACATTGAAAAATCTACCCCTAATACAGTG GCGTTTAATTCATTTTTCTATACCAATTTATCAGAAAGGGGTATCAAGGC GTCCGGAGGTGGATGAAGAGAAAAGAAGACACAAATTGATAAACCTTGATAA AATCTTTACACCAATAAATTTGAACCAATCCCACTGGGCGTTGGGCATAA TTGATTTAAAAAAGAAAACCTATAGGTTACGTAGATTCAATTATCGAATGGTC CAAATGCTATGAGTTTCGCTATACTGACTGACTTGCAAAAATATGTTATGG AGGAAAGTAAGCATACAATAGGAGAAGACTTTGATTTGATTCATTTAGATT GTCCGCAGCAACCAAATGGCTACGACTGTGGAATATATGTTTGTATGAAT ACTCTCTATGGAAGTGCAGATGCGCCATTGGATTTTGATTATAAAGATGCG ATTAGGATGAGAAGATTTATTGCCCATTTGATTTTAAACCGACGCTTTAAAA <u>CTCGAGCACCACCACCACCACCTGA</u>
His-SUMO fused modification enzymes	ATGGGCGGCAGGC <u>CATCATCATCATCATCACAGCAGCGGC</u> Catgtcggactcagaag tcaatcaagaagctaagccagaggtcaagccagaagtcaagcctgagactcacatcaattaaaggtgtccgatgcatctt cagagatcttcttcaagatcaaaaagaccactccttaagaaggctgatggaagcgttcgctaaaagacagggttaaggaa atggactccttaagattctgtacgacgggtattagaattcaagctgatcagaccctgaagatttgacatggaggataac gatattattgaggctcacagagaacagattgggtgt-(modification enzyme sequence)
M1 RNA	GAAGCTGACCAGACAGTCGCCGCTTCGTCGTCGTCCTC TTCGGGGGAGACGGGCGGAGGGGAGGAAAGTCCGGGGCTCCA TAGGGCAGGGTGCCAGGTAACGCCTGGGGGGGAAACCCACGACCAG TGCAACAGAGAGCAAACCGCCGATGGCCCGCGCAAGCGGGATCAGG TAAGGGTGAAAGGGTGCGGTAAGAGCGCACCGCGCGGCTGGTAACAG TCCGTGGCACGGTAAACTCCACCCGGAGCAAGGCCAAATAGGGGTTC TAAGGTACGGCCCGTACTGAACCCGGGTAGGCTGCTTGAGCCAG TGAGCGATTGCTGGCCTAGATGAATGACTGTCCACGACAGAACCCGGC TTATCGGTTCAGTTTCACCT
DHFR	ATGATTTCTCTGATTGCTGCTCTGGCTGTTGACCGGGTTATTGGTA TGGAGAACGCTATGCCTTGGAACCTGCCTGCTGACCTGGCTTGG TTTAAGCGGAACACTCTGAACAAGCCTGTTATTATGGGTGCGCACAC TTGGGAGTCTATTGGTCGGCCTCTGCCTGGTCGGAAGAACATTATTCTG TCTTCTCAGCCTGGTACTGACGACCGGGTACTTGGGTAAAGTCTG TTGACGAGGCTATTGCTGCTTGTGGTGACGTTCTGAGATTATGGTTA TTGGTGTTGGTCGGGTTTACGAGCAGTTTCTGCCTAAGGCTCAGAAGC TGTACCTGACTCACATTGACGCTGAGGTTGAGGGTGACACTCACTTTCC TGACTACGAGCCTGACGACTGGGAGTCTGTTTTTTCTGAG TTTCACGACGCTGACGCTCAGAACTCTCACTCTTACTGTTTTGAGATTC TGGAGCGGCGGTAA

4. Resuspend pellets in Lysis buffer containing 6 M Urea.
5. Centrifuge the cell lysate at $20,400 \times g$ for 45 min at 4 °C and recover the supernatant.
6. Subsequent procedures are same as the MnmE purification except that all buffers contain 2 M urea.

3.2.5 Purification of MnmC

For MnmC preparation, all steps until removal of His-tagged SUMO protein are same as GlyA preparation. Because MnmC nonspecifically binds to the His-tag purification resin, the purification with anion-exchange chromatography is selected to purify MnmC without the SUMO moiety.

1. Follow 1–7 steps in Subheading 3.2.1. using Cleavage buffer 3 instead of Lysis buffer.
2. Add Ulp1p to a final concentration of 23 $\mu\text{g}/\text{mL}$ into the resin-containing mixture for cleavage between the SUMO moiety and MnmC. Gently rotate with a rotator during the cleavage.
3. Elute the target proteins with 2 column volumes (CVs) of Lysis buffer containing 50 mM imidazole, 1 CV of Lysis buffer containing 100 mM imidazole, 1 CV of Lysis buffer containing 200 mM imidazole, and 3 CVs of Lysis buffer containing 400 mM imidazole. Analyze all fractions by SDS-PAGE and recover those containing MnmC.
4. Equilibrate 5 mL HiTrap Q HP anion exchange chromatography column with IEX buffer and then, inject the sample to the column.
5. Wash the column with 5 bed volumes of IEX buffer.
6. Elute MnmC using two mobile phases A (IEX buffer) and B (IEX buffer with 1 M KCl) with a sequential gradient (0–11% B for 3 CV, 11–22% B for 5 CV, and 22–24% B for 3CV).
7. Follow 9–12 steps in Subheading 3.4.1.

3.2.6 Purification of C5 Protein

The procedure is a modified protocol described in the previous study [16].

1. Follow 1–3 steps in Subheading 3.2.1.
2. Resuspend harvested cells in 40 mL of C5 suspension buffer 1 and disrupt them by sonication on ice.
3. Centrifuge the cell lysate at $14,400 \times g$ for 45 min at 4 °C and recover the supernatant.
4. Add ammonium sulfate to reach 50% saturation and stir at 4 °C until dissolved.
5. Centrifuge the solution at $12,000 \times g$ for 30 min at 4 °C and recover the supernatant.
6. Add ammonium sulfate to reach 80% saturation and stir at 4 °C until dissolved.
7. Centrifuge the solution at $12,000 \times g$ for 30 min at 4 °C and remove the supernatant.
8. Dissolve the pellet in C5 suspension buffer 2.
9. Dialyze the solution against C5 suspension buffer 2 at 4 °C (*see Note 6*).

10. Centrifuge the dialyzed solution at $10,000 \times g$ for 10 min at 4 °C and remove the supernatant. Add 500 μ L of C5 suspension buffer 2 to wash the pellet. Repeat this procedure twice.
11. Centrifuge the solution at $10,000 \times g$ for 10 min at 4 °C and remove the supernatant. Add 20 mL of C5 purification buffer 1 containing 10 mM DTT instead of 2-mercaptoethanol to dissolve the pellet.
12. Dialyze the solution against C5 purification buffer 1 containing 10 mM DTT instead of 2-mercaptoethanol at 4 °C. Filter the dialyzed solution through 0.45 μ m Millex-HV filter.
13. Equilibrate 10 mL HiTrap SP HP cation exchange chromatography column with C5 purification buffer 1 and then, inject the sample to the column.
14. Wash the column with 8 CV with C5 purification buffer 1-2 (90:10 [vol/vol]).
15. Elute C5 protein using two mobile phases A (C5 purification buffer 1) and B (C5 purification buffer 2) with a sequential gradient (10–14.5% B for 2 CV, 14.5% B for 2 CV, and 14.5–100%B for 2 CV).
16. Analyze all collected fractions by SDS-PAGE.
17. Recover the fractions containing C5 protein. Dialyze against C5 storage buffer containing 2 M urea at 4 °C.
18. Transfer the dialysis tube to C5 storage buffer and further dialyze against C5 storage buffer at 4 °C.
19. Recover the dialyzed sample and concentrate by Amicon Ultra-15 Centrifugal Unit with a cutoff of 3 kDa. Dialyze the sample against C5 storage buffer containing 50%(v/v) glycerol at 4 °C.
20. Recover the sample and store at –30 °C.

3.3 In Vitro Transcription of iVT tRNA

3.3.1 Preparation of DNA Template

1. Amplify DNA templates for in vitro run-off transcription by PCR using appropriate primer sets (Fig. 1 and Table 2) (*see Note 7*).
2. Add equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and vortex. Centrifuge the mixture at $13,000 \times g$ for 10 min and recover the upper water layer. Repeat this procedure twice.
3. Add 0.1 volume of 3 M KOAc, pH 5.2 and 2.5 volume of ethanol.
4. Centrifuge the mixture at $13,000 \times g$ at 4 °C for 15 min.
5. Remove the supernatant and add 70% ethanol.
6. Centrifuge the mixture at $13,000 \times g$ at 4 °C for 15 min.
7. Remove the supernatant.

8. Dissolve the pellet in Milli-Q water.
9. Measure the absorbance at 260 nm to determine the concentration of DNA. Store the purified DNA templates at -20°C .

3.3.2 In Vitro

Transcription of iVT tRNA

This procedure is also applicable for the preparation of M1 RNA. Skip the tRNA processing step and change the elution step in the column chromatography to a linear gradient from 200 mM to 1 M KCl for 2 CV using tRNA purification buffer 1 and 2.

1. Prepare reaction mixtures ($2\text{ mL} \times 10$) containing 40 mM HEPES-KOH, pH 7.6, 20 mM MgCl_2 , 1.5 mM Spermidine, 5 mM DTT, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 30 nM T7 RNA polymerase, 0.2 U/mL inorganic pyrophosphatase, and 20 μg DNA template (*see Note 8*).
2. Incubate at 37°C for 3 h.
3. Add 150 nM RNase P components composed of C5 protein and M1 RNA to the reaction mixtures and further incubate them at 37°C for 1 h.
4. Combine the reaction mixtures in one tube.
5. Add 1/10 volume of 3 M KOAc, pH 5.2 and equal volume of water-saturated phenol, and then vortex. Centrifuge the mixture at $14,200 \times g$ at 4°C for 15 min.
6. Recover the upper water layer and add equal volume of chloroform-isoamyl alcohol (10:1, v/v) and then vortex. Centrifuge the mixture at $14,200 \times g$ at 4°C for 10 min.
7. Recover the upper water layer.
8. Equilibrate 10 mL HiTrap Q HP anion exchange chromatography column with tRNA purification buffer 1 and inject the sample to the column.
9. Wash the column with 3 CV of tRNA purification buffer 1.
10. Elute transcribed and RNase P-processed iVT tRNA using two mobile phases A (tRNA purification buffer 1) and B (tRNA purification buffer 2) with a sequential gradient (0–55% B for 3 CV, 55–70% B for 6 CV, and 100%B for 2 CV).
11. Analyze all collected fractions by Urea PAGE. Combine fractions including target iVT tRNA.
12. Add 1/10 volume of 3 M KOAc, pH 5.2 and equal volume of 2-propanol, and then vortex.
13. Centrifuge the mixture at $14,200 \times g$ at 4°C for 20 min.
14. Remove the supernatant and dissolve the pellet with 500 μL of Milli-Q water.
15. Repeat **steps 12 and 13**.
16. Remove the supernatant. Add 500 μL of 70% ethanol.
17. Centrifuge the mixture at $20,400 \times g$ at 4°C for 10 min.

18. Remove the supernatant.
19. Dissolve the pellet in Milli-Q water.
20. Measure the absorbance at 260 nm to determine the concentration of DNA. Store the purified iVTtRNA at -20°C .

3.4 Nucleotide Modification of iVT tRNA

1. Prepare reaction mixtures. A mixture for $t^6\text{A}37$ modification contains 50 mM HEPES-KOH (pH 7.6), 300 mM KCl, 20 mM MgCl_2 , 5 mM DTT, 50 mM NaHCO_3 , 1 μM CaCl_2 , 1 mM ATP, 1 mM Thr, 5 μM TsaC, 5 μM TsaB, 5 μM TsaD, 5 μM TsaE, and 4 A_{260} unit/mL $\text{tRNA}^{\text{Ile}}_{\text{GAU}}$ or $\text{tRNA}^{\text{Asn}}_{\text{GUU}}$ or $\text{tRNA}^{\text{Phe}}_{\text{GAA}}$. A mixture for $m^1\text{G}37$ modification contains 50 mM HEPES-KOH (pH 7.6), 200 mM KCl, 10 mM MgCl_2 , 36.4 μM S-adenosyl methionine (SAM), 1.5 μM TrmD, and 10 A_{260} unit/mL $\text{tRNA}^{\text{Pro}}_{\text{GGG}}$ or $\text{tRNA}^{\text{Phe}}_{\text{GAA}}$. A mixture for $\text{mnm}^5\text{U}34$ modification contains 50 mM HEPES-KOH (pH 7.6), 150 mM KCl, 12.5 mM MgCl_2 , 5 mM DTT, 500 μM FAD, 1 mM tetrahydrofolate, 4 mM GTP, 2.5 mM NADH, 0.2 mM pyridocal-5'-phosphate, 1 mM Ser, 1 mM Gly, 36.4 μM SAM, 0.1 unit/ μL recombinant RNase inhibitor, 10 μM GlyA, 3 μM MnmE, 2.5 μM MnmC, and 6 A_{260} unit/mL $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$ (*see* **Notes 9** and **10**).
2. Incubate the reaction mixtures at 37°C for 2 h for $t^6\text{A}37$ and $m^1\text{G}37$ modification. Incubate the reaction mixtures at 37°C for 4 h for $\text{mnm}^5\text{U}34$ modification.
3. Follow 5–7 steps in Subheading 3.3.2.
4. Follow 12–19 steps in Subheading 3.3.2.
5. Store the modified iVT tRNA at -80°C .

3.5 Protein Synthesis with the PURE System

1. Prepare DNA templates for cell-free expression of DHFR in the PURE system (Table 4) (*see* **Note 11**). Amplify by PCR using T7 promoter and terminator primers. Purify the product using QIAquick PCR purification kit according to the manufacturer's instructions.
2. Prepare reaction mixtures. Mix PURE frex[®] 2.0 Solution I (Buffer mix without tRNAs*), Solution II (Enzymes), Solution III (Ribosomes), 4 nM PCR-amplified DNA template for DHFR expression, and 40 A_{260} unit/mL of native tRNA mixtures or 60 A_{260} unit/mL iVT tRNA mixtures with or without the nucleotide modifications (*see* **Notes 12** and **13**).
3. Incubate the reaction mixtures at 30°C for 12 h.

3.6 Measurement of Synthesized DHFR Activity

1. Prepare 1 mL of the mixture containing 100 mM MES-NaOH, pH 7.0, 50 mM Tris-HCl, pH 7.0, 200 mM NaCl, 50 mM ethanolamine, 20 mM 2-mercaptoethanol, and 0.2 mM EDTA. Add 0.96 mL of MilliQ water. Incubate at 37°C for 15 min.

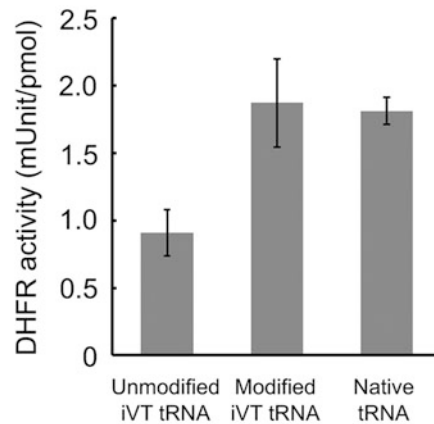


Fig. 3 Specific activity of synthesized DHFR. (The figure is reproduced from a previous manuscript [15] under a Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>))

2. Transfer the mixture to a cuvette for spectrophotometer.
3. Add 20 μL of 10 mM dihydrofolic acid dissolved in 100 mM NaOH.
4. Add 10 μL of reaction mixture of PURE system that synthesized DHFR.
5. Mix with a stir bar at 37 °C for 10 min.
6. Add 20 μL of 20 mM NADPH.
7. Measure the absorbance at 340 nm with a spectrophotometer every 5 seconds for 10 minutes. Calculate the activity based on the rate of absorbance decrease under different concentrations of NADPH (Fig. 3). One unit of DHFR is defined as the amount of enzyme required to process 1 μmol of dihydrofolic acid in 1 min at 37 °C.

4 Notes

1. The active domain (403-621) of Ulp1p (SUMO protease 1) was cloned into pET29a as a C-terminal His-tagged form (Table 4). Ulp1p was purified according to the previous study [19].
2. M1 RNA was cloned in a similar way as a set of tRNAs but directly downstream of the T7 promoter without any extra nucleotides (Fig. 1, Table 4).
3. T7 RNA polymerase is purified as described previously [21].

4. GeneFrontier Corporation, a distributor of this kit in Japan, accepts the order of the Solution I without tRNA mixture. Also you can prepare the system by yourself according to the previously described methods [21].
5. Unless proteins are immediately purified, the cells can be frozen with liquid nitrogen and stored at -80°C .
6. C5 protein is gradually precipitated in the membrane during this process, which is recovered by the centrifugation in the next step.
7. DNA purification steps using phenol and chloroform as described from the next step is for large-scale preparation of the DNA templates. The use of commercially available PCR purification kits such as QIAquick PCR Purification Kit (QIAGEN) can also be selected as a substitution.
8. Large-scale transcription reaction is necessary for preparing sufficient amount of iVT tRNA. If the small-scale reaction is enough for the purpose, purification with column chromatography can be replaced by the extraction from the PAGE gels [22].
9. We notice that only $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$ can be modified. Also, In the case of $\text{mnm}^5\text{U34}$ modification of $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$, the reaction is better to be performed again using recovered tRNA in order to maximize the modification level. After the phenol and chloroform extraction steps (**step 3** in Subheading 3.4.2), the sample is desalted by MicroSpin G-25 Columns (GE Healthcare) and recovered by 2-propanol precipitation (**step 4** and **5** in Subheading 3.4.2).
10. Modification levels in each iVT tRNA can be measured with radioisotope labeled substrates according to the procedures described previously [15].
11. The gene for DHFR expression comprises of restricted codons because limited species of iVT tRNA are used [15]. Only TTT (Phe), CTG (Leu), ATT (Ile), ATG (Met), GTT (Val), TCT (Ser), CCT (Pro), ACT (Thr), GCT (Ala), TAC (Tyr), CAC (His), CAG (Gln), AAC (Asn), AAG (Lys), GAC (Asp), GAG (Glu), TGT (Cys), TGG (Trp), CGG (Arg), and GGT (Gly) are used. They are arranged to code DHFR and synthesized with commercially available gene synthesis technology [23].
12. Equally abundant iVT tRNA mixtures are used. Various compositions of iVT tRNAs can be evaluated including artificial tRNAs for genetic code redesigning studies [15].
13. The anticodon of tRNA^{Glu} is UUC for mixtures with nucleotide modifications. However, CUC is used for mixtures without nucleotide modifications, because the Glu codon GAG is not supposed to be decoded by unmodified $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$.

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Measurement of Transcription, Translation, and Other Enzymatic Processes During Cell-Free Expression Using PERSIA

Scott Wick and Peter A. Carr

Abstract

We developed the PERSIA technique with an interest in quantifying proteins as they are being produced during a cell-free synthesis reaction. A short 6-amino acid sequence added to a protein of interest reacts with a fluorogenic reagent (ReAsH), yielding a measure of protein concentration in close to real time. We combine this measurement with simultaneous fluorescent detection of mRNA production, quantifying both transcription and translation. Alternatively, we combine simultaneous measurement of protein synthesis and that protein's enzymatic activity. We have found these simple capabilities enabling for multiple applications, including sequence–structure–function studies and target-specific assessment of drug candidate compounds.

Key words Cell-free synthesis, Protein quantitation, PERSIA, Fluorescent label, Transcription, Translation

1 Introduction

The PERSIA method was developed to provide real-time quantitative analysis of cell-free transcription and translation processes, as well as to characterize enzymatic processes associated with a protein target of interest [1]. The acronym is derived from the original components of the system (PUREExpress—ReAsH—Spinach—In vitro Assay). Here we detail the protocol for a PERSIA reaction quantitating cell-free transcription and translation outputs. Additionally, we provide the protocol for an alternate application where the enzymatic output of a protein of interest (a protease) is characterized concurrently with the translation of that protein.

Cell-free synthesis (CFS) reactions are often used to produce data that support later in vivo experiments. They are also used in assays to detect the presence of chemicals or biological agents, and

are increasingly being employed for biomanufacturing. Applications of *in vitro* transcription/translation reactions include, but are not limited to evaluating fluctuations arising from natural or designed changes of the genetic code, probing sequence and function relationships associated with changes in amino acid composition of a gene product, prototyping genetic circuits and nucleic acid-based biosensors, and synthesis of proteins that are difficult to produce in an intact whole-cell production scheme.

There are many advantages of using cell-free extracts, or synthetic cell-free systems (such as the PURE system [2, 3]), over intact whole cell systems for the examples given above—expediency in design, building, and testing thousands of prototype genetic or protein variants to a particular application; the ability to supplement or deplete CFS components to maximize the robustness of the desired experimental processes; reduced constraints of nucleic acid and gene product toxicity; directing consumption of basic biological building blocks (NTPs, amino acids, energy) to building and analyzing the prototype end-products of interest (nucleic acids, protein) instead of other biological processes associated with growth and replication.

Analysis of these experimental methods often includes expression of fluorescent proteins, or enzymes capable of a reporting activity (e.g., luciferases for a luminescent output, or β -lactamase for colorimetric conversion output). This process can vary in efficiency and can take several minutes to report [4, 5]. Short peptide tags, such as the 6-amino acid tetracysteine (TC) tag [6], are capable of binding small molecules (e.g., FAsH, ReAsH) to yield a fluorescent output immediately after translation. This fluorescent output provides a way to quantify the target of interest with minimal use of reaction resources (e.g., amino acids).

Characterization of transcriptional outputs can also be linked to the translation of a nucleic acid encoding a protein of interest. In this process, monitoring transcription is a secondary process which can also inform interpretation of translation and protein folding. Problems in the formation of reporting polypeptides can obviate the actual data of the transcriptional process of interest. The development of aptamer nucleic acid sequences like Spinach [7], Broccoli [8], Mango [9], and the malachite green aptamer [10] provide a tool for directly measuring the transcribed RNA of interest. These sequences are designed to fold around specific small molecule chemicals (like DFHBI, DFHBI-1T, TO1/TO3, and malachite green) that alone are minimally fluorescent—but convert to an excitable fluorescent state when bound to the designed aptamer sequence. The aptamer sequences are often attached as noncoding sequences at the 3' end of an RNA transcript.

The nucleic acid encoding your gene of interest can be in plasmid form, a synthetic linear DNA (services available from many commercial vendors such as IDT, GenScript, and Twist

Biosciences), PCR-amplified DNA, or ssRNA. It has been reported that the use of commercial plasmid and PCR purification kits do not remove all nucleases during their procedures, reducing the efficiency of the PURExpress reaction, although we have not noticed substantial differences in using DNA from commercial kits versus DNA prepared through phenol–chloroform extraction and alcohol precipitation. If you are using ssRNA as a starting material synthesized from a commercial vendor or prepared via in vitro transcription kits, great caution must be taken to use RNase-free solutions in preparation of your template, an RNase-free working area on your benchtop, and RNase-free water in preparing the PURExpress mixture.

The PURExpress system requires a T7 promoter sequence (P_{T7}) and ribosome binding site (RBS) upstream of the initiating methionine for your gene of interest. The common T7 promoter and RBS sequence for most of our work with the PERSIA platform (Fig. 1) exists in many popular plasmids such as pUC57, the pET series, pT7, pRSET, and others. The specific coding sequence of your gene of interest may cause wide variations in transcription and translation output during your in vitro reaction. The RNA secondary structure of your design (within the coding sequence, or between the coding sequence and the upstream RBS sequence) can strongly affect the polymerase and ribosome complexes. Codon optimization algorithms are readily available from commercial vendors and open-source offerings. In addition, we have successfully used the RBS Calculator [11, 12] on a number of occasions to design and examine the effects of varying RBS sequences on transcription and translation products.

Other sequences included in the design of transcripts include:

1. A DNA sequence encoding the tetracycline (TC) tag placed at the 3' end of the gene of interest, in-frame with the coding region so the 6-amino acid tag (CCPGCC) is incorporated at the C-terminus of the translated protein product. We include a 2 amino acid spacer element (Glycine-Serine) between the gene coding region and the TC tag sequence.
2. One or more stop codons (TAA, TAG, TGA) immediately following the last cysteine codon of the TC tag.
3. A Spinach aptamer tRNA sequence immediately after the stop codon that will be included in the mRNA transcription product made during the PERSIA/PURExpress reaction.
4. A standard T7 terminator sequence immediately following the Spinach aptamer 3' untranslated region.

For all of our work, we have also included two additional nucleic acid sequences to our templates: a 38-base sequence upstream of the T7 promoter that includes the universal M13 forward primer, and a 57-base sequence downstream of the T7

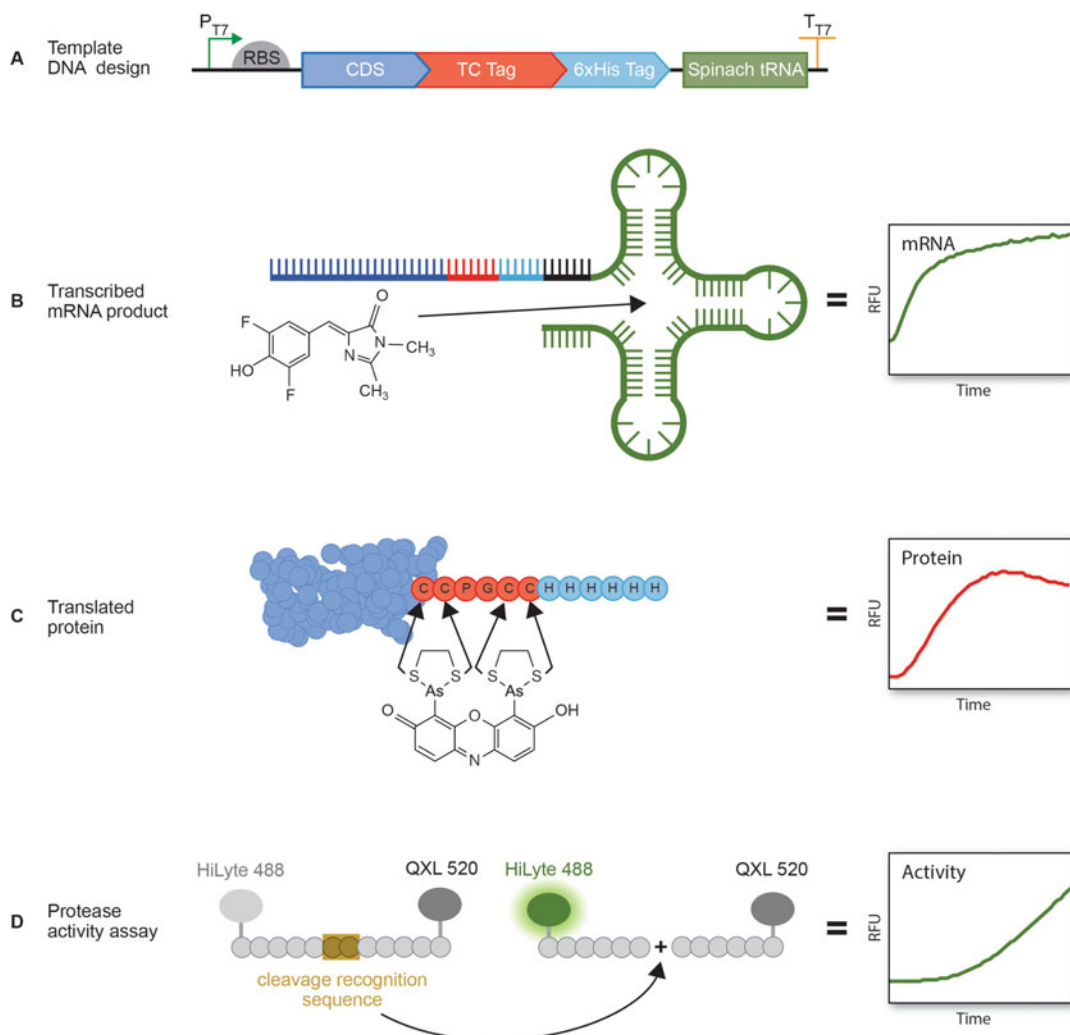


Fig. 1 Overview of PERSIA and its key components. (a) DNA templates used in PERSIA reactions include a T7 promoter (P_{T7}), ribosome binding site (RBS), gene of interest coding sequence (CDS), tetracysteine coding region (TC-tag), Spinach RNA aptamer, and T7 terminator (T_{T7}). Purification tags (i.e., 6 × His, Strep-II, etc.) can be incorporated at the C-terminal end of the coding sequence, after the TC-tag if purification of the protein product is desired after the reaction is completed. (b) Template DNA results in transcribed mRNA as shown, with the Spinach aptamer folding to bind DFHBI permitting fluorescent excitation and emission for quantitating mRNA production. (c) Transcribed mRNA is translated into protein which harbors the TC-tag that binds ReAsH-EDT₂, allowing fluorescent excitation and emission for quantitating protein output. (d) If desired, measurement of a protein's enzymatic activity can be determined by addition of fluorogenic assay substrates. In this example, HIV protease activity is observed using a quenched labeled peptide (Sensolyte 520 HIV protease substrate, AnaSpec). All graphs show actual data, reflecting the delays from assay start to appearance of mRNA, protein, and proteolytic cleavage product respectively, over a 3-h time course

terminator that includes the universal M13 reverse primer. These two sequences are used for PCR amplification of our transcripts to produce the required amounts of template DNA, and to provide stability by serving as a potential buffer against accidental

exonuclease activity. Table 1 provides sequence details for the composition of a typical template DNA, including an example sequence we have used for PERSIA with HIV protease.

Table 1
Sequence components for a PERSIA DNA template

Segment name	Sequence	Comments
M13 forward PCR primer	GTAAAACGACGGCCAGTGA	Priming site to amplify template DNA by PCR
Spacer	GAGGTAGCACATCTCGA TGCCGCGAAA	
T7 promoter	TTAATACGACTCACTATAGGGAGA	Recruits T7 RNA polymerase transcriptional to template DNA in PURExpress reaction
Spacer sequence	CCACAACGGTTTCCCTCTAGAAATAA TTTTGTTTAACTTT	
Ribosome binding site (RBS)	AAGAAGGAGATATACC	Recruits translational machinery to mRNA transcript in PURExpress reaction
Coding sequence (CDS)	ATGTCCTGGTCCTCAGGTCACTCTTT GGCAACGACCCCTCGTCACAATAA AGATAGGGGGGCAACTAAAGGAA GCTCTATTAGATACAGGAGCAGAT GATACAGTATTAGAAGAAATGAGT TTGCCAGGAAGATGGAAACCAAAA ATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAG ATACTCATAGAAATCTGTGGACAT AAAGCTATAGGTACAGTATTAGTA GGACCTACACCTGTCAACATAATT GGAAGAAATCTGTTGACTCAGATT GGTTGCACTTTAAATTTT	Gene of interest (HIV protease in this example)
Protein spacer	GGATCC	Glycine-serine spacer sequence between protein of interest and tags; intended to provide greater flexibility and accessibility of fluorophores labels
Tetracysteine (TC) tag	TGTTGCCCGGGTTGCTGT	Cysteine side chains react with ReAsH-EDT2 (or FAsH-EDT2) yielding red (or green) fluorescent signal
Purification tag	CATCACCATCACCATCAC	Optional 6× histidine tag shown (we have also used a strep II tag); allows postreaction purification of protein
Stop codons	TAATAA	Signals end of translation
Spacer	TCTAG	

(continued)

Table 1
(continued)

Segment name	Sequence	Comments
Spinach aptamer	ACGCGACCGAAATGGTGAAGGACG GGTCCAGTGCTTCGGCACTGTTGA GTAGAGTGTGAGCTCCGTAAGTGG TCGCGTC	RNA element includes and tRNA-like folding; provides binding site for DFHBI, yielding green fluorescent signal
Spacer	AGCTTAT	
T7 terminator	AACCCCTTGGGGCCTCTAAACGGG TCTTGAGGGGTTTTTGTCTGAAAGG AGGAACTATATCCGC TCAGCAGTCAGT	Signals termination of transcription
M13 reverse PCR primer	CCATGGTCATAGCTGTTTCC	Priming site to amplify template DNA by PCR
Full combined sequence	GTAAAACGACGGCCAGTGAAGAGGT AGCACATCTCGATGCCGCGAAATT AATACGACTCACTATAGGGAGACC ACAACGGTTTTCCCTCTAGAAATAA TTTTGTTTAACTTTAAGAAGGAGAT ATACCATGTCTGGTCCTCAGGTCAC TCTTTGGCAACGACCCCTCGTCACA ATAAAGATAGGGGGGCAACTAAA GGAAGCTCTATTAGATACAGGAGC AGATGATACAGTATTAGAAGAAAT GAGTTTGCCAGGAAGATGGAAACC AAAAATGATAGGGGGAATTGGAG GTTTTATCAAAGTAAGACAGTATG ATCAGATACTCATAGAAATCTGTG GACATAAAGCTATAGGTACAGTAT TAGTAGGACCTACACCTGTCAACA TAATTGGAAGAAATCTGTTGACTC AGATTGGTTGCACTTTAAATTTTGG ATCCTGTTGCCCCGGTTGCTGTGGA TCCCATCACCATCACCATCACTAAT AATCTAGACGCGACCGAAATGGTG AAGGACGGGTCCAGTGCTTCGGCA CTGTTGAGTAGAGTGTGAGCTCCG TAACTGGTCGCGTCAGCTTATAAC CCCTTGGGGCCTCTAAACGGGTCTT GAGGGGTTTTTGTCTGAAAGGAGG AACTATATCCGCTCAGCAGTCAGT CCATGGTCATAGCTGTTTCC	

Sequences are listed in contiguous order, with no other bases or spacers. In designing a new template for PERSIA, one can replace the coding sequence below, ensuring that it is in frame with the tetracysteine tag to allow for incorporation during translation. The ribosome binding site sequence can be customized for higher or lower expression rates using the Ribosome Binding Site calculator [11, 12]. Sequences have been colored corresponding to Fig. 1a

2 Materials

1. Template nucleic acid in the form of plasmid DNA, synthetic linear DNA, PCR amplified DNA, or ssRNA purified using a commercial purification kit of the scientists' preference. Sequence elements required for PERSIA are described above, in Fig. 1 and in Table 1 (*see Note 1*). The work documented here routinely used purification products from Qiagen. The protocols below employ a DNA stock at 10× the final concentration used in the PERSIA reaction. For the majority of our work, 12.5 nM is typically the final DNA concentration (*see Note 2*).
2. PURExpress In Vitro Protein Synthesis kit (*see Note 3*). This reagent is stored at −80 °C.
3. Autoclaved deionized water.
4. TC-ReAsH II In-cell tetracysteine tag Detection Kit reaction substrate (*see Note 4*): this reagent is a 2 mM stock made in DMSO and is stored at −20 °C. For PERSIA, this is a 400× stock solution.
5. DFHBI substrate ((5Z)-5-[(3,5-difluoro-4-hydroxyphenyl)methylene]-3,5-dihydro-2,3-dimethyl-4H-imidazol-4-one, (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one) available from several commercial vendors (*see Notes 5 and 6*). Reagent should be dissolved in DMSO to a final concentration of 10 mM and stored at −20 °C. For PERSIA, this is a 200× stock.
6. For measurement of HIV protease function within a PERSIA assay, enzymatic activity is monitored through fluorescence measurement of the substrate Component A found in the SensoLyte 520 HIV protease Assay Kit available from AnaSpec. This reagent is a 100× stock made in DMSO and is stored at −20 °C.
7. Black-wall, clear-bottom assay plates, available from multiple vendors (*see Note 7*).
8. Optical adhesive plate covers.
9. Fluorescence plate reader (*see Note 8*). We have employed a Spectramax M5 plate reader for our PERSIA assay development. The plate reader is also used to mix nucleic acid template with PURExpress reagents at the beginning of a PERSIA experiment (*see Note 9*).

3 Methods

3.1 Basic PERSIA
Method to Measure
Real-Time
Transcription/
Translation from a
Target Nucleic Acid

1. Template nucleic acid dilutions should be prepared in autoclaved deionized water at a 10× final concentration. Nucleic acid solutions should be made and stored on ice.
2. Thaw tubes of PURExpress Component A and Component B on ice. A pulse spin of 2500 × *g* for 5 s is acceptable to get all liquid to the bottom of the tubes.
3. Thaw stocks of ReAsH and DFHBI at room temperature.
4. If multiple tubes of PURExpress Components A and B are needed, each component should be mixed separately by micropipette and combined into separate component tubes prior to making the master mix (e.g., 5 tubes of Component A mixed into one tube, 5 tubes of Component B mixed into a separate tube), at the manufacturer’s recommendation. Refer to their manual regarding how to mix Components A and B. Store both on ice.
5. Prepare PERSIA Master mix (if any additives are to be included, reduce the volume of water accordingly; *see Note 10*) by combining PERSIA reagents into a single master mix tube on ice in the order of addition in Table 2 (the components are shown both for a single reaction solution, and for preparation of an entire single tube of PURExpress components).
6. Place 384-well/96-well plate on ice prior to addition of reagents.

Table 2
PERSIA master mix for Subheading 3.1

Component	Volume (μL) per 15 μL rxn	Volume (μL) per 100 μL tube of component A
PURExpress component A	6	100
PURExpress component B	4.5	75
Sterile deionized water	2.9	45
TC ReAsH (400× stock) ^a	0.036	0.6
DFHBI (200× stock) ^a	0.075	1.25
DNA sample (1/10th reaction volume of a 10× stock)	–	–
Total	13.5	221.9 (>16 aliquots of 13.5 μL each)

^aVolumes given for ReAsH and DFHBI in a single reaction are too small to measure by standard pipettor, but reflect the proportions we typically use per 100 μL tube of Component A. A user performing a small number of these reactions can adjust for larger volumes of more dilute stocks, reducing the amount of water to keep total reaction volume the same

7. Add 1/10th volume of template nucleic acid to the bottom of each assay well. For example, add 1.5 μL of template to the bottom of each well for a 15 μL reaction in a 384-well plate. For in-plate calibration purposes, DNA template can be substituted with known concentrations of a purified corresponding RNA or protein sample (*see* **Note 11**).
8. Add PERSIA master mix into each assay well while plate is still on ice. For example, add 13.5 μL master mix to 1.5 μL DNA in a 384-well plate. Do not mix template and master mix at this stage. They will be mixed by the plate reader to begin the reaction.
9. Adhere optical adhesive film and ensure proper seal, wipe plate bottom to remove ice/water.
10. Reading assay plate.
 - (a) Preheat plate reader to desired reaction temperature (*see* **Note 12**).
 - (b) Position plate in plate reader as recommended by manufacturer.
 - (c) Program excitation at 470 nm and emission read at 500 nm for analysis of mRNA production via DFHBI/Spinach aptamer interaction.
 - (d) Program excitation at 590 nm and emission read at 615 nm for analysis of target protein production via ReAsH/TC-tag interaction.
 - (e) Program a mixing step between interval reads, both in order to thoroughly combine the DNA template with the PERSIA master mix, and to minimize settling of components (*see* **Note 13**).
 - (f) Each read should be performed with a kinetic read program, taking measurements of both fluorophores at a designated interval in time. We routinely take readings every 3–8 min. The time period was dependent upon the plate reader processing time to excite and read the designated number of samples on the plate. Reactions were typically monitored for 3–5 h (using between 1 and 25 nM of nucleic acid).

3.2 PERSIA Method Measuring Real-Time Transcription/ Translation and HIV Protease Enzymatic Activity in Parallel Reactions

1. Follow Subheading **3.1 steps 1–3** regarding dilution and thawing of reagents. SensoLyte Component A peptide substrate should be thawed at room temperature.
2. If multiple tubes of PURExpress Components A and B are needed, each component should be mixed separately by micropipette and combined into separate component tubes prior to making the master mix (e.g., five tubes of Component A mixed into one tube, five tubes of Component B mixed into a separate tube). Store both on ice.

Table 3
PERSIA master mix for Subheading 3.2

Component	Per 100 μL tube of component A
PURExpress component A	100 μL
PURExpress component B	75 μL
Sterile deionized water	45 μL
TC ReAsH (400 \times stock)	0.6 μL
Total	220.6 μL

Table 4
Separated PERSIA Master Mixes for real-time transcription/translation and HIV protease enzymatic activity

Component	Enzymatic assay—Translation reaction	Transcription—Translation reaction
PERSIA—ReAsH only master mix	110 μL	110 μL
SensoLyte component A substrate	1.25 μL	–
DFHBI (200 \times stock)	–	0.625 μL
DMSO	–	0.625 μL
Total	111.25 μL (> 8 aliquots of 13.5 μL each)	111.25 μL (> 8 aliquots of 13.5 μL each)

- Combine PERSIA reagents into a Master mix tube on ice in the order of addition in Table 3 (the components are shown for a single tube each of PURExpress components). Divide this mixture in half into separate Eppendorf tubes.
- In one tube add 0.625 μL of DFHBI (200 \times stock) and 0.625 μL DMSO, for a standard PERSIA reaction measuring RNA transcription and protein translation (Table 4).
- In the other tube add 1.25 μL of SensoLyte Component A substrate, providing a reaction measuring protein translation and HIV protease enzyme activity (Table 4).
- Complete steps 6 through 10 of Subheading 3.1. Each unique DNA sample should be added to 2 wells for parallel analysis for each sample. One well should have the enzymatic PERSIA master mix added (second column Table 4); to the other well add the standard PERSIA master mix (third column Table 4).

7. Plate reads need to be performed as follows:

(a) Wells containing Enzyme Assay/Translation reaction.

- ReAsH—590 nm excitation, 615 nm emission, 610 nm cutoff.
- SensoLyte—488 nm excitation, 525 nm emission, 515 nm cutoff.

(b) Wells containing Transcription/Translation reaction.

- ReAsH—590 nm excitation, 615 nm emission, 610 nm cutoff.
- DFHBI—470 nm excitation, 500 nm emission, 495 nm cutoff.

4 Notes

1. We have performed PERSIA with 15 different proteins and over 100 variant sequences of those proteins, ranging in size from 7 to 37 kDa. Most of these are monomeric proteins, though HIV protease forms a homodimer.
2. Increasing amounts of template nucleic acid will increase the rate of mRNA and protein production, as more polymerase molecules and ribosome complexes are used to produce the end products of cell-free expression. However, one expects a threshold at which the addition of more nucleic acid saturates the transcription/translation capacity of the system, and may result in more early termination events due to depletion of the nucleotides and amino acids in the PURExpress reaction [13]. We advise performing multiple reactions PERSIA with different amounts of template nucleic acid to determine what is optimal for your specific scientific application. We have used between 1 and 25 nM final concentration of template nucleic acid in our PERSIA applications, but a majority of our work uses 12.5 nM DNA in the final PERSIA reaction.
3. While we have not tested other sources of the PURE transcription/translation system (e.g., PUREfrex 2.0 from GeneFrontier and Magic PURE system from Creative Biolabs) we expect these to also be compatible with the PERSIA method.
4. The TC-tag labeled with either FAsH or ReAsH survives standard protein gel electrophoresis conditions (sodium dodecyl sulfate, denaturation, β -mercaptoethanol) allowing specific visualization of the protein product via SDS-PAGE.
5. Unpublished data from our development of PERSIA demonstrated that the alternative substrate DFHBI-1T ((Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-

trifluoroethyl)-1H-imidazol-5(4 H)-one), also available from Lucerna (410-10MG) can be used as a substitute for DFHBI for the detection of mRNA transcripts.

6. Although we have not examined the use of other RNA aptamer sequences, we expect that the incorporation of other RNA aptamers and their corresponding small molecule ligands could replace the Spinach-DFHBI combination documented in the original PERSIA publication and described in this protocol.
7. PERSIA was developed with the goal of being scalable to high-throughput approaches. Use in 96-well and 384-well plates indicates general compatibility with standard liquid-handling robotics. PERSIA is also compatible with microfluidic devices [1], with parallel reactions performed at the nanoliter scale.
8. Plate reader settings: ReAsH measurements were obtained using 590 nm excitation and 615 nm emission using a 610 nm cutoff filter. DFHBI measurements were obtained using 470 nm excitation and 500 nm emission using a 495 nm cutoff filter. SensoLyte measurements were obtained using 488 nm excitation and 528 nm emission using a 515 nm cutoff filter. Fluorescence measurements were made by excitation through the optical film cover on top of the plate, reading fluorescence output through the bottom of the clear plate wells.
9. Mixing: we have performed mixing of DNA template with PERSIA master mix within the plate reader, in order to synchronize initiation of the cell free reactions. For a plate reader without a mixing function, it should also be feasible to mix the reagents by pipettor (preferably a multichannel pipettor) before inserting the plate into the plate reader.
10. Additives: The product of the reaction between the TC-tag and ReAsH is very stable in salt concentrations up to 1 M, and DTT concentrations 1 mM or less [14]. However, in our PERSIA reactions, we have observed the addition of certain chloride-based salts causes diminished TC-ReAsH fluorescence [1]. It is our interpretation that the PURExpress transcription/translation reactions are being adversely affected, and the diminished fluorescence is not a result of TC-ReAsH interference. Alternatively, stabilizing or solubility additives such as albumin and detergents increase background fluorescence, interpreted as nonspecific binding of the fluorophores to the additives. Fluorescence background reactions without template DNA should be included in any analysis to ensure the observed fluorescence is specific to transcription and translation.
11. Calibration: Serial dilutions of purified, quantitated ssRNA have been spiked into template-free PERSIA reactions to

serve as quantitative standards for RNA production in the same multiwell plate. Similarly, recombinant TC-tagged protein purified from expression in *E. coli* can be spiked into template-free PERSIA reactions to serve as quantitative protein standards.

12. Temperature: we have successfully performed PERSIA at temperatures of 30 and 37 °C. NEB PURExpress instructions recommend 37 °C for optimal protein yields.
13. We routinely mix the assay plate between interval reads, as ribosomes can otherwise settle in the reaction over the course of the experiment. Not all plate readers have this capability, and we have not explored the behavior of this assay on stationary samples over time.

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Part II

Applications of Cell-Free Expression Systems



Cell-Free Noncanonical Redox Cofactor Systems

William B. Black and Han Li

Abstract

Noncanonical redox cofactor systems utilize nicotinamide adenine dinucleotide (phosphate), NAD(P)H, mimics to perform biotransformation reactions. Compared to systems utilizing native NAD(P)H, these noncanonical redox cofactors can offer decreased cost of cofactor supply, improved system activities, and can even supply reducing power directly to targeted reactions in complex biological environments. When these systems are operated in cell-free settings, the high level of user control afforded by direct access to the reaction system enables specific tuning of cofactor parameters, enzyme activity, and reaction progression to maximize system productivity. In this chapter, we will describe methods for constructing these cell-free noncanonical redox cofactor systems. Specifically, methods, design concepts, and system adaptation will be discussed for applying noncanonical redox cofactors to both purified protein-based and crude lysate-based biotransformation systems.

Key words Noncanonical redox cofactor, Cell-free biotransformation, Biomimetic cofactor, Crude lysate

1 Introduction

Recently, noncanonical redox cofactor-based biotransformation has emerged as a promising alternative to traditional cell-free reductive biotransformation [1–4]. Noncanonical redox cofactors are mimics of the native nicotinamide adenine dinucleotide (phosphate), NAD(P)(H), redox cofactors found in biology. These noncanonical cofactors typically retain the pyridine ring of NAD(P)(H), retaining their catalytic capabilities. However, they deviate structurally from native redox cofactors in the lower moiety “handle” used to bind the cofactor, and in some cases, the electron-withdrawing group extending off the pyridine ring is also modified [1–3, 5, 6]. The structural deviations in these noncanonical cofactors have offered advantages in cell-free biotransformation including decreased cost of cofactor supply [7, 8], improved cofactor mass transfer rates [9], and even improved reaction rates compared to their native counterparts [5, 9, 10]. Furthermore, when used in systems with high

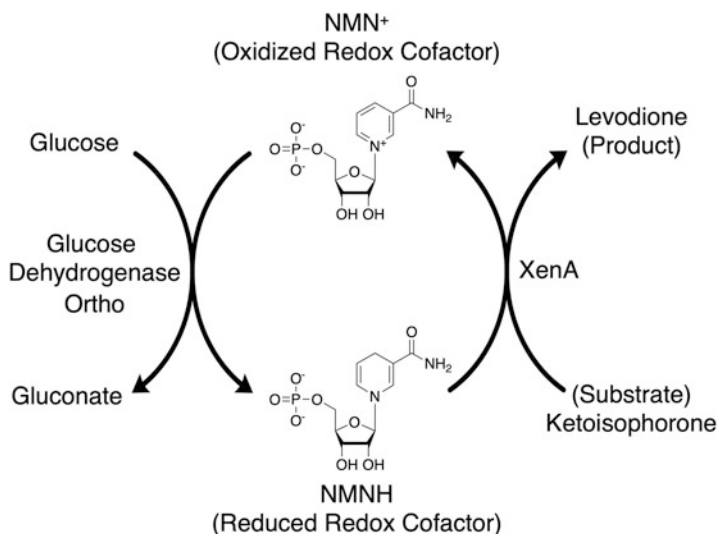


Fig. 1 Enzymatic noncanonical redox cofactor cycling system schematic. The reduced noncanonical redox cofactor nicotinamide mononucleotide, NMNH, is used by XenA to reduce ketoisophorone to levodione. Oxidized nicotinamide mononucleotide, NMN⁺, is recycled back to NMNH by an engineered glucose dehydrogenase, forming a redox cofactor cycle

cellular background, like crude lysate-based biotransformation systems, noncanonical redox cofactors have been demonstrated to function orthogonally to the native redox systems, allowing the specific control of reducing power to targeted reactions [1, 3, 4, 6, 11]. Cell-free noncanonical redox systems have been used as valuable tools to rapidly prototype and develop noncanonical redox cofactors systems for in vivo applications [4].

These systems are regularly comprised of two modules, a cofactor reduction module and a cofactor oxidation module. The two modules are used to form a cofactor recycling system, where one module makes the desired product of interest and consumes the cofactor, while the other module recycles the cofactor back to its original redox state, Fig. 1 [2, 3, 11–13]. Similar to natural redox systems, these cycling systems can theoretically be expanded to have multiple components oxidizing and reducing the noncanonical cofactor pool, forming an increasingly complex noncanonical redox network.

In these methods, a model system is built around the noncanonical redox cofactor nicotinamide mononucleotide, NMN⁺, Fig. 1. In this system, the xenobiotic reductase, XenA, from *Pseudomonas putida* is used to produce levodione, through the reduction the C=C double bond of ketoisophorone, using reduced nicotinamide mononucleotide (NMNH) as a reducing source. NMNH is regenerated using an engineered glucose dehydrogenase from *Bacillus subtilis*, GDH Ortho [3], forming the redox cofactor cycle.

This chapter will focus on the application and methods for developing cell-free noncanonical redox cofactor-based biotransformation systems. Specific methods will be discussed for applications in purified protein-based systems and crude lysate-based systems. Finally, this chapter will discuss system adaptation and design considerations when applying noncanonical redox cofactor systems.

2 Materials

2.1 Purified Protein-Based Systems

2.1.1 Purified Protein Preparation

1. Sterile 2× YT culture medium containing 100 mg/L of ampicillin: 16 g/L tryptone, 10 g/L yeast extract, 100 mg/L ampicillin.
2. *Escherichia coli* strain BL21 (*see Note 1*) harboring plasmid pEK102, an expression plasmid for the *Pseudomonas putida* xenobiotic reductase, XenA [3].
3. *E. coli* strain BL21 (*see Note 1*) harboring plasmid pLZ216, an expression plasmid for the engineered *Bacillus subtilis* glucose dehydrogenase, GDH Ortho [3].
4. 0.5 M Isopropyl β-D-1-thiogalactopyranoside, IPTG.
5. 50% volume/volume glycerol in dH₂O.
6. His-tagged protein purification kit.
7. Bradford assay kit.

2.1.2 Purified Protein Reaction System

1. 1 M potassium phosphate at pH 7.5.
2. 120 mM oxidized β-nicotinamide mononucleotide, NMN⁺.
3. Ketoisophorone.
4. 1 M D-glucose.
5. 2 mL screw-cap glass vials with PTFE-lined caps.
6. Gas chromatography-grade chloroform containing 200 mg/L octanol (*see Note 2*).
7. Gas chromatograph equipped with a flame-ionized detector, GC-FID (*see Note 3*).

2.2 Crude-Lysate Based Systems

2.2.1 Crude-Lysate Preparation

1. Crude lysate cell culture medium: Dissolve 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 10 g of D-mannitol (*see Note 4*) in 800 mL of water in a 1 L volumetric flask. Once fully dissolved, add dH₂O up to the 1 L mark. Mix well. Filter-sterilize through a 0.2 μm filter. Add sterile ampicillin to a final concentration of 100 mg/L.
2. *E. coli* strain MX102 (BW25113 Δ*pncC* Δ*pgi* Δ*zwf* Δ*gntK::kan*) [3] (*see Note 5*) harboring the *P. putida* XenA expression plasmid, pEK102 [3].

3. *E. coli* strain MX102 (*see* **Note 5**) [3] harboring the engineered *B. subtilis* GDH Ortho expression plasmid, pLZ216 [3].
4. 0.5 M IPTG.
5. Cell wash buffer: 120 mM potassium acetate, 28 mM magnesium acetate, and 20 mM tris-base at pH 8.2.
6. French Press (*see* **Note 6**).
7. Bradford assay kit.

2.2.2 Determining the Molar Concentration of the Enriching Protein in the Crude Lysate

1. 1 M potassium phosphate at pH 7.5.
2. 40 mM reduced β -nicotinamide adenine dinucleotide phosphate (NADPH) (*see* **Note 7**).
3. Ketoisophorone.
4. 1 M Tris-Cl at pH 8.0.
5. 120 mM NMN⁺.
6. 1 M D-glucose.
7. Purified *P. putida* XenA, prepared as described in Subheading 3.1.1.
8. Purified *B. subtilis* GDH Ortho, prepared as described in Subheading 3.1.1.
9. XenA-enriched crude-lysate, prepared as described in Subheading 3.2.1.
10. GDH Ortho-enriched crude lysate, prepared as described in Subheading 3.2.1.

2.2.3 Crude-Lysate Reaction System

1. 1 M potassium phosphate at pH 7.5.
2. 1 M D-glucose.
3. 120 mM NMN⁺.
4. Ketoisophorone.
5. XenA-enriched crude-lysate, prepared as described in Subheading 3.2.1.
6. GDH Ortho-enriched crude lysate, prepared as described in Subheading 3.2.1.
7. 2 mL screw-cap glass vial with PTFE-lined cap.
8. Gas chromatography-grade chloroform containing 200 mg/L octanol (*see* **Note 2**).
9. Gas chromatograph equipped with a flame-ionized detector (GC-FID) (*see* **Note 3**).

3 Methods

3.1 Purified Protein-Based Systems

Purified protein-based systems are one of the simplest forms of a noncanonical redox cofactor cycling system. In this system, purified proteins are supplied for the cofactor reducing and oxidizing modules. Since only reaction-essential components are supplied to the system, this system is capable of fine tuneability and optimization [2, 14].

The following subsections will discuss the preparation of the purified proteins and a method for performing noncanonical redox cofactor biotransformation with the purified proteins.

3.1.1 Purified Protein Preparation

1. Culture *E. coli* BL21 (*see Note 1*) cells expressing XenA or GDH Ortho in 250 mL of 2× YT medium in a 500-mL baffled shake flask at 37 °C while shaking at 250 rpm until early log-phase growth is reached.
2. Reduce the incubator temperature to 30 °C.
3. Induce protein expression by adding IPTG to a final concentration of 0.5 mM in the media. Continue shaking at 30 °C for an additional 24 h.
4. Process cells according to the His-tagged protein purification kit to yield purified XenA and purified GDH Ortho.
5. Measure the protein concentration using the Bradford assay kit.
6. Mix the purified proteins with ice-cold 50% glycerol to a final concentration of 20% glycerol.
7. Distribute 100 µL aliquots of the protein–glycerol mixture into prechilled microcentrifuge tubes.
8. Store the samples at –80 °C until use.

3.1.2 Purified Protein-Based Biotransformation

1. Generate the reaction buffer master mix by mixing 5 mL of 1 M potassium phosphate at pH 7.5, 10 mL of 1 M D-glucose, 2.5 mL of 120 mM NMN⁺, 10 mL of 5 M NaCl, 250 mg of ketoisophorone, and dH₂O to a final volume of 40 mL. Mix well. Prewarm the reaction buffer at 30 °C (*see Notes 8 and 9*).
2. Thaw the purified proteins on ice.
3. Prepare the protein mixture at a fivefold concentration on ice, 58.5 µM of GDH Ortho, and of 94.2 µM XenA (*see Notes 10 and 11*). Dilute the thawed proteins with the elution buffer used with the His-tagged protein purification kit.
4. Distribute 800 µL of prewarmed assay buffer into each 2-mL glass screw-cap vial.
5. Spike 200 µL of protein mixture into the vial to initiate the reaction. Mix well.

6. Seal the vial with a PTFE-lined screw-cap (*see* **Note 12**).
7. Incubate at 30 °C (*see* **Note 13**).
8. Sample intermittently over the course of the reaction to determine conversion (*see* **Note 14**). In a fume hood, remove 100 µL of sample and mix well with an equivalent volume of chloroform containing 200 mg/L octanol in a microcentrifuge tube (*see* **Notes 15** and **16**). Centrifuge to separate the aqueous and organic fractions. Transfer the organic fraction to a gas chromatography vial for GC-FID analysis.
9. Compare the GC response of each sample to a standard curve of known substrate and product concentrations to determine conversion.
10. Repeat the reaction process, varying the protein supply and master mix conditions, until desired production characteristics are achieved (*see* **Note 17**).

3.2 Crude Lysate-Based Systems

Crude lysate-based biotransformation is performed similarly to the purified protein-based system described above. However, instead of using purified proteins, crude cell lysates are used to perform the enzymatic conversion. While using crude lysates reduces the total processing requirements when compared to purified protein-based systems, the presence of the endogenous enzymes in the crude lysates introduces increased system complexity.

The following subsections will discuss the preparation of crude cell lysates enriched with XenA or GDH Ortho, a method for determining the molar concentration of the enriching protein in each lysate, and a method for performing noncanonical redox cofactor biotransformation with the crude cell lysates.

3.2.1 Crude Lysate Generation

1. Prechill the French Press chamber at 4 °C.
2. Culture *Escherichia coli* strain MX102 [3] (*see* **Notes 4** and **18**) harboring a plasmid expressing XenA or GDH Ortho at 30 °C in 250 mL of crude lysate cell culture medium (*see* **Note 19**) in a 500-mL baffled shake flask while shaking at 250 rpm for 4 h.
3. Induce protein expression by adding 0.5 M IPTG to a final concentration of 0.5 mM in the media.
4. Shake the cultures for an additional 10 h at 30 °C.
5. Chill the cell culture in an ice water bath for 15 min.
6. Pellet the cells by centrifugation for 15 min at 4 °C at 4000 RCF.
7. Resuspend the pellet in 250 mL of ice-cold cell wash buffer. Repellet the cells.
8. Repeat the wash step two additional times.

9. Resuspend the washed cells in 0.7 mL of ice-cold cell wash buffer per 1 g of wet cell weight of cell pellet.
10. Lyse the cells in the prechilled French press (*see Note 6*).
11. Clarify the lysate by centrifugation at 20,000 RCF at 4 °C for 30 min (*see Note 20*). The supernatants in this step yield ready-to-run crude lysates enriched with their respective recombinant protein.
12. Measure the total protein concentration in the lysate by Bradford assay (*see Note 21*).
13. Aliquot the lysate into 1.5-mL microcentrifuge tubes and store at –80 °C until use.

3.2.2 Determining the Molar Concentration of the Enriching Protein in the Crude Lysate

The molar concentration of XenA or GDH Ortho in the crude lysates can be determined by comparing the specific activity of a standard curve of known XenA or GDH Ortho purified proteins to the specific activity of their respective crude lysates. It is important to include no-substrate controls of these reactions to determine the nonspecific activity rate under each condition [4].

1. Generate purified protein standards of XenA and GDH Ortho by purifying the proteins as described in Subheading 3.1.1. Serially dilute the purified proteins with the elution buffer used in the His-tagged protein purification kit to create a series of purified protein standards.
2. Generate the XenA and GDH Ortho-specific activity master mixes. For the XenA activity, mix 10 mL of 1 M potassium phosphate at pH 7.5, 250 μ L of 40 mM NADPH, and 37 μ L of ketoisophorone in a 50 mL volumetric flask. Add dH₂O up to the 50 mL mark. Mix well. Prewarm the master mix at 37 °C. For the GDH Ortho master mix, mix 350 μ L of 1 M Tris-Cl at pH 8.0, 250 μ L of 120 mM NMN⁺, and 1.4 mL of 1 M D-glucose in a 10 mL volumetric flask. Mix well. Add dH₂O up to the 10 mL mark. Mix well.
3. To generate the XenA and GDH Ortho activity standard curve, spike 20 μ L of the purified protein dilution series into 180 μ L of their respective master mixes. Measure the rate of change in light absorption at 340 nm. For XenA, the initial linear rate in which light absorption decreases at 340 nm correlates to the rate of enzyme activity via the consumption of NADPH by XenA. For GDH Ortho, the initial linear rate in which light absorption increases at 340 nm correlates to the rate of enzyme activity via the production of NMNH by GDH Ortho.
4. To determine the molar concentration of XenA or GDH Ortho in the crude lysates, spike 20 μ L of lysate into 180 μ L of the respective master mix, as described in the previous step (*see Note 22*). Measure the rate of light absorption at 340 nm.

5. To determine the nonspecific background activity of the lysates, spike the lysates into master mix devoid of substrate as a no-substrate control. Subtract this initial rate from the rate observed in the previous step to determine the protein activity-specific rate of cofactor consumption or production.
6. Compare the initial linear rate of light absorption at 340 nm of the crude lysate samples, adjusted with the no substrate control, to the purified protein standard curve to determine the molar concentration of the enriching protein in the lysate.

3.2.3 Crude Lysate Biotransformation

1. Remove the lysates from the -80°C freezer, and thaw them on ice.
2. Generate the reaction master mix by mixing 10 mL of 1 M potassium phosphate at pH 7.5, 10 mL of 1 M D-glucose, 2 mL of 5 M NaCl, 417 μL of 120 mM NMN⁺ (*see* **Notes 23** and **24**), 243 μL of ketoisophorone, and 22.34 mL of dH_2O . Mix well. Prewarm by incubating at 30°C .
3. Generate a tenfold concentrated lysate mixture (151 μM XenA and 58 μM GDH Ortho) by mixing the concentrated lysates on ice, based on the molar concentrations determined in Subheading 3.2.2. (*see* **Note 25**).
4. Distribute 800 μL of prewarmed reaction master mix into each 2 mL glass vial.
5. Spike 200 μL of crude lysate mixture into each vial to initiate the reaction. Mix well.
6. Seal each tube with a PTFE-lined screw-cap (*see* **Note 12**).
7. Incubate samples at 30°C .
8. Sample the vials intermittently to determine conversion, as described in Subheading 3.1.2.
9. Analyze samples with GC-FID, as described in Subheading 3.1.2.
10. Repeat the reaction process, varying the lysate supply and master mix conditions, until desired production characteristics are achieved (*see* **Note 26**).

3.3 System Adaptation and Design Considerations

The model system presented in this chapter, Fig. 1, represents just one concept of how a noncanonical redox cofactor system can be constructed. The modular nature and high level of user control of cell-free systems make noncanonical redox cofactor systems highly user adaptable. Therefore, the cofactor, product-forming enzyme, and recycling component can be exchanged to fit many different system conformations. However, when designing these systems, it is important to consider how each component works together, since the function of each component is largely dependent on the others.

When selecting the noncanonical redox cofactor, one should consider not just the cofactor properties, like solubility, cost, and stability, but also which enzymes have been demonstrated to have activity with the cofactor, and which substrate classes are accessible when using the cofactor. To date, a large number of noncanonical redox cofactors have been derived through chemical synthesis or from natural NAD(P)(H) intermediates [1, 2, 6, 10, 12, 13]. However, no single cofactor has presented itself as superior. In addition to NMNH, 1-benzylnicotinamide, BNAH [1, 2, 5, 13, 15], nicotinamide cytosine dinucleotide, NCDH [6, 11, 16], and 3-carbamoyl-1-phenethylpyridin-1-ium, P2NAH [17, 18] have all demonstrated promising activity and compatibility with a variety of enzymes. However, many other candidate cofactors have shown encouraging activity, but still require more extensive investigation with additional enzymes [5, 10, 19]. Nitroreductases [3, 10], alcohol dehydrogenases [13, 20], monooxygenases [3, 15, 19, 21, 22], azoreductases [23], and enoate reductases [1–5, 24, 25] have all demonstrated appreciable activity with these cofactors. Encouragingly, protein engineering efforts have begun to further broaden the substrate scope accessible to these systems [13].

Additionally, the compatibility between the cofactor and the recycling component is also important to consider. The model system described in this chapter, Fig. 1, utilizes an enzymatic recycling component. However, the reduction of noncanonical redox cofactors has proven to be difficult, with extensive protein engineering being required to produce enzyme candidates with appreciable activity to match the rate of cofactor consumption by the other side of the system [3, 9, 11, 14, 17, 26–28]. Alternatively, metal catalysts have been used as an effective means for cofactor recycling [5, 13, 15, 29]. For example, Okamoto and coworkers developed a noncanonical redox cofactor system using a biotinylated iridium-pianostool cofactor docked into a streptavidin enzyme scaffold to successfully reduce a variety of cofactors, including 1-benzyl-3-acetylpyridium and BNA⁺, forming a redox cofactor cycle [2].

When designing crude lysate-based systems, additional factors to take into account are how the cofactor recycling component interacts with the native cofactors in the lysate and how the noncanonical redox cofactor interacts with the lysate background. Ideally, the noncanonical redox cofactor recycling component is specific to the noncanonical cofactor, with minimal activity towards the native cofactors, NAD⁺, and NADP⁺ [3, 4, 16, 27]. This ensures that the resources allocated to noncanonical cofactor regeneration are only used by the desired system. In the model system, *B. subtilis* GDH Ortho is an engineered enzyme which was designed to yield NMN⁺ activity and to effectively eliminate NAD⁺ and NADP⁺ activity [3]. Similarly, the noncanonical redox cofactor supplied to the system should have minimal activity with the background of the

lysate. Cofactors which exhibit minimal activity with the lysate background but high activity towards the target reactions will enable simpler system control, optimization, and specific reducing power delivery [3, 4, 6, 11].

4 Notes

1. Any *E. coli* strain typically used for protein expression should be sufficient for this step. Ensure compatibility with the plasmid's selection marker and protein expression induction system.
2. Octanol is used as an injection internal standard to enable GC response standardization across all samples.
3. Choose an analytical device which is appropriate for the system being designed. Ensure the device is sensitive in the range of substrate and product concentrations which are being investigated.
4. Mannitol is used to improve the growth of the MX102 strain [4]. Mannitol addition is not essential for the function of the noncanonical redox cofactor system.
5. *E. coli* strain MX102 contains gene knockouts to prevent the consumption of glucose and NMN⁺ by the endogenous enzymes in the lysate background [3].
6. Any lysis technique or instrumentation can be used, as long as the resulting lysate is active and compatible with the biotransformation system.
7. NADPH is prone to degradation. Make fresh if possible. If storing, aliquot into small volumes and store at -80°C .
8. Prewarming the reaction buffer may aid in solubilizing the substrate if concentrations are near the solubility limit.
9. Use cofactor concentrations which best match your system's kinetics. When working in cell-free biotransformation systems, the high level of user control enables fine tunability of parameters, like cofactor concentration, to best support efficient biocatalysis [3, 14]. In the model system discussed, Fig. 1, GDH Ortho has a K_M of 5.9 mM [3]. Therefore, NMN⁺ was supplied at 6 mM to increase the rate of reduced cofactor regeneration.
10. We typically start varying the protein concentration ratio on either side of an approximate 1:1 specific activity ratio of each protein with the noncanonical redox cofactor. The total amount of protein added to the system highly depends on the activity of the system with the noncanonical redox cofactor and the desired kinetics. If the protein activity is low with the noncanonical cofactor, protein concentration can be increased to compensate.

11. If the purified protein concentrations are too low to reach a fivefold concentrated mixture, a larger volume of protein can be supplied. Ensure that a proportional amount of water is removed from the reaction master mix to compensate for the increase in protein volume.
12. Performing the reactions in a well-sealed container will aid in reducing loss of volatile compounds. If the compounds in the system are not volatile, other containers and closures are sufficient.
13. Vary the reaction temperature to best suit the components supplied to the system. For example, if using thermostable enzymes, take advantage of their potentially improved kinetics and stability at high temperatures [14].
14. The sampling volume and chloroform volume can be adjusted to meet experimental needs. Sampling smaller volumes of liquid can greatly increase the number of time points which can be taken for each vial. Extracting those smaller volumes with larger volumes of chloroform will aid in consistent and clean transfer of the organic fraction to the GC vial.
15. Test the extraction of the substrate and product prior to beginning the assay. Change the solvent used in the liquid–liquid extractions if your analytes do not move well across the phases.
16. Ensure samples are well mixed during liquid–liquid extraction. Vortexing samples individually can be time consuming when processing large sample sets. We have found that taping the microcentrifuge tubes horizontally to a plate shaker platform and shaking for 2 min at a high speed enables high sample throughput and consistent extractions at a lab scale.
17. Conditions to consider varying include, but are not limited to, buffer type, pH, cofactor concentration, enzyme loading, enzyme ratio, enzyme mutants, and substrate.
18. The cell strain selected for this step will comprise the background of the crude cell lysate. Ensure the appropriate cell strain is used to support proper and efficient biotransformation.
19. Scale cell cultivation as necessary to produce more lysate per round of cell growth.
20. Additional lysate clarification by ultracentrifugation may aid in overall system performance [4]. Apply as necessary. Centrifuge the supernatant at 30,000 RCF at 4 °C for an additional 30 min.
21. This measurement will indicate the total amount of protein in the lysate. This gives a rough estimate to the relative amount of enriching protein present on top of the background proteins in the lysate [4]. See Subheading 3.2.2. for determination of the molar concentration of the enriching protein in the lysate.

22. Test a dilution series of each lysate to ensure the initial rate of the reaction is comparable to the standard curve. Dilute the crude lysates with the elution buffer supplied with the His-tagged protein purification kit as necessary.
23. The amount of noncanonical redox cofactor added to crude lysate-based systems highly varies on the goals of your system. For example, crude lysate-based biotransformation systems have been shown as a powerful tool to rapidly prototype *in vivo* systems. Maintaining cofactor concentrations similar to achievable intracellular noncanonical redox cofactor levels is informative to the expected *in vivo* performance of the system [4].
24. It has been previously demonstrated that increasing the concentration of noncanonical redox cofactor in a crude lysate-based system can increase nonspecific background activity of the lysate with the cofactor [4]. Test different cofactor concentrations to determine the optimum cofactor supplementation in the system being examined.
25. The total amount of lysate to add to the system varies on the recombinant protein concentration in the lysate and the overall activity of the system. Adjust the concentration and ratio as necessary to achieve the desired system performance.
26. Conditions to consider varying include, but are not limited to: buffer type, pH, cofactor concentration, enzyme loading, enzyme ratio, and the stain background in the lysates.

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Cell-Free Protein Synthesis for High-Throughput Biosynthetic Pathway Prototyping

Blake J. Rasor, Bastian Vögeli, Michael C. Jewett, and Ashty S. Karim

Abstract

Biological systems provide a sustainable and complimentary approach to synthesizing useful chemical products. Metabolic engineers seeking to establish economically viable biosynthesis platforms strive to increase product titers, rates, and yields. Despite continued advances in genetic tools and metabolic engineering techniques, cellular workflows remain limited in throughput. It may take months to test dozens of unique pathway designs even in a robust model organism, such as *Escherichia coli*. In contrast, cell-free protein synthesis enables the rapid generation of enzyme libraries that can be combined to reconstitute metabolic pathways in vitro for biochemical synthesis in days rather than weeks. Cell-free reactions thereby enable comparison of hundreds to thousands of unique combinations of enzyme homologs and concentrations, which can quickly identify the most productive pathway variants to test in vivo or further characterize in vitro. This cell-free pathway prototyping strategy provides a complementary approach to accelerate cellular metabolic engineering efforts toward highly productive strains for metabolite production.

Key words Cell-free, Metabolic engineering, TX-TL, High-throughput screening, In vitro, Enzyme assay, Biosynthetic pathways

1 Introduction

Metabolic engineering seeks to establish efficient biological platforms for chemical production by increasing flux through desired pathways through optimizing expression of heterologous enzyme variants and combinations while downregulating competing native pathways [1]. This process is slowed by the constant need to balance product formation with cell growth and viability, as well as the large time and labor investment required for genetic manipulations [2]. Engineering cellular metabolism with these constraints limits the ability to thoroughly screen pathway variants comprising different enzyme homologs, combinations, and expression levels. Cell-free systems, on the other hand, enable biosynthesis in the absence of genetic regulation or biomass generation,

providing the opportunity for direct modulation of the reaction environment outside normal physiological conditions or viability constraints [3–6]. These cell-free systems are also amenable to immobilization, compartmentalization, and high-throughput assembly through automated liquid handling [7, 8]. Purified enzymatic reactions are the most common example of cell-free biochemical synthesis (Fig. 1a). This approach enables pathway design from the ground up with precise control of enzyme concentrations and has led to a wide range of products, from simple acids [9, 10] and alcohols [11, 12] to more complex terpenes [13], bioplastics [14], and cannabinoids [15]. However, the time and cost associated with purifying enzymes often limit the number of homologs tested at each step in the pathway, and the fully synthetic environments of purified reactions lack native metabolism, including cofactor regeneration. These factors limit the ability to effectively screen purified enzyme libraries and reduce applicability to *in vivo* systems that contain other pathways competing for substrate and cofactor pools [3–5].

Extract-based cell-free metabolic engineering (CFME) provides a cheaper and faster route to test heterologous enzymes in the context of native metabolism, although these platforms do sacrifice some of the extensive control afforded by purified systems [3, 16]. Crude cell extracts have been generated from a diversity of organisms by growing cells to a desired density, lysing them through physical or chemical disruption, and removing insoluble components by centrifugation [17]. Among these extract-based cell-free platforms, *Escherichia coli* extracts have been extensively optimized and modified for an array of applications, including metabolite synthesis [18]. One key benefit of these cell-free extracts is that native *E. coli* glycolysis can convert the carbon substrate (most commonly glucose) to the appropriate central carbon metabolite for the investigated biosynthetic pathway while recycling the cofactors ATP and NADH [3]. Studies have demonstrated that *E. coli* extract generated from cells expressing the pathway for 2,3-butanediol biosynthesis results in high-yielding CFME reactions that are more tolerant to toxic substrates than cells [19, 20]. While this approach may be useful for biomanufacturing, it does not increase the ability to screen enzymes for cellular biosynthesis. Greater control over CFME reactions can be attained by generating extracts from several strains that each overexpress one enzyme homolog from the desired pathway, providing the ability to mix-and-match enzymes at different relative concentrations (Fig. 1a). The extract mixing approach has successfully produced mevalonate [21, 22], terpenes [23, 24], and butanol [25, 26]. However, the scope of this technique is limited by the need to grow separate strains for expression of each target enzyme, and the concentration of heterologous enzymes must be estimated relative to native enzymes. A recent advance combined cell-free protein

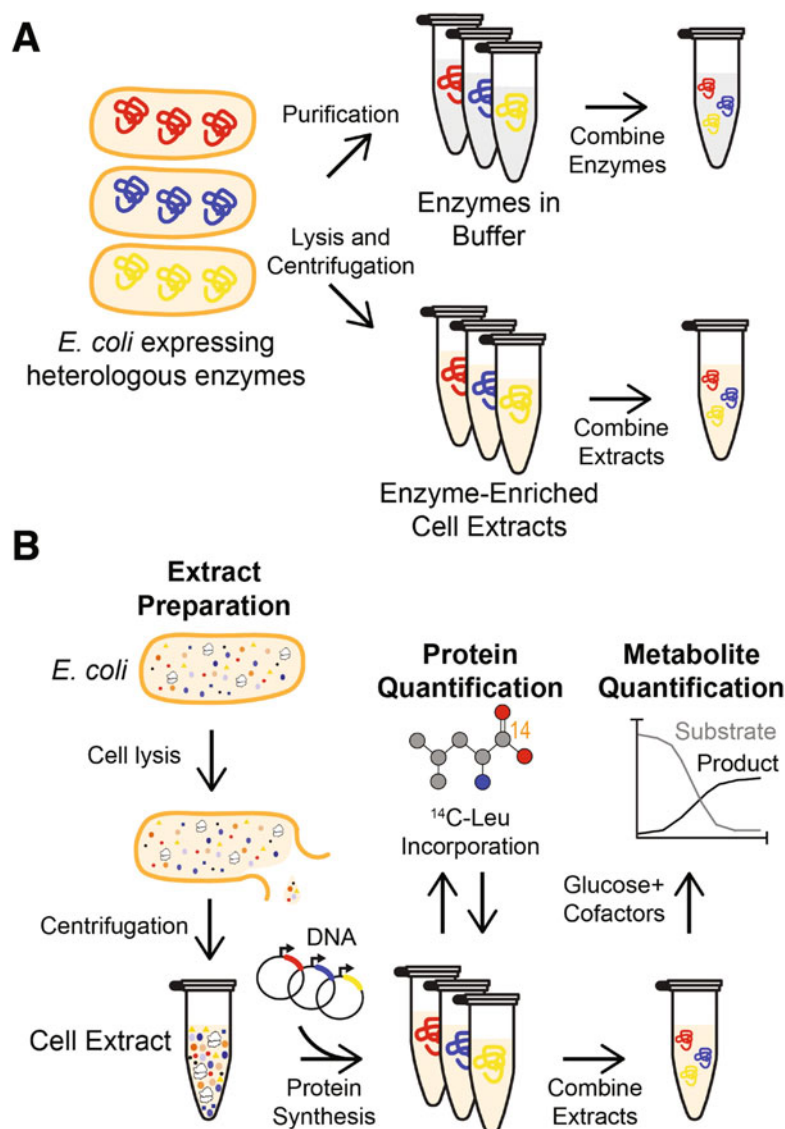


Fig. 1 Methods for cell-free metabolite synthesis. (a) Enzymes expressed in vivo can be purified for precise control of concentration or obtained in a crude cell extract to retain native metabolism prior to mixing in vitro. (b) Combining cell-free protein synthesis with in vitro chemical synthesis results in a high-throughput method that includes quantitative determination of protein expression and native *E. coli* catabolism and cofactor regeneration. Cell extract is generated from *E. coli* through physical lysis and centrifugation. Enzymes are expressed individually using plasmid DNA of linear expression templates, and expression is quantified by radioactive leucine incorporation. Enzymes are then combined at defined concentrations with substrate and cofactors to recapitulate metabolic pathways in vitro, followed by biochemical analysis

synthesis (CFPS) and CFME for pathway prototyping using extract from a wildtype strain of *E. coli* to generate enzymes for butanol biosynthesis in a single reaction, to which glucose and cofactors were added to activate the pathway [27]. This provides greater flexibility for enzyme expression by simply exchanging plasmids, without any additional growth steps. Although relative enzyme levels can be modulated by adjusting plasmid DNA concentrations, inevitable resource competition reduces the ability to precisely tune the pathway composition [28].

Here, we describe a two-pot approach to cell-free protein synthesis-driven metabolic engineering (CFPS-ME) that enables high-throughput expression and testing of enzyme variants, combining the benefits of endogenous metabolism in cell extract for cofactor regeneration with the quantitative control over enzyme concentrations normally afforded by purified systems (Fig. 1b). First, cell extract is generated from a highly productive *E. coli* strain for CFPS [29]. Second, proteins are expressed *in vitro* from plasmid DNA or linear expression templates [30]. ^{14}C -leucine incorporation enables quantification of only the heterologous protein expressed during CFPS, which increases the precision of this CFME technique compared to other extract-based approaches. Third, enzyme-enriched extracts are combined with the desired substrate and catalytic concentrations of cofactors to activate native *E. coli* catabolism and the heterologous anabolic pathway. Biosynthesis can then be quantified via chromatography, chemical assays, or biosensors to indicate the most active enzyme combinations and reaction conditions. This method provides a high-throughput platform for individual and combinatorial enzyme screening with unique flexibility, as illustrated by the rapidly produced acetone biosynthesis dataset below (Fig. 2). While enzyme libraries can be generated through CFPS to screen pathways of interest, other cell-free approaches may be used in tandem to add well-characterized accessory enzymes (e.g., purified enzymes [31] and overexpression extracts [23]). Applications of CFPS-ME to date include the biosynthesis of 3-hydroxybutyrate [32], butanol [33], styrene [34], valinomycin [35], and indole alkaloids [36] as well as the combinatorial assessment of glycosylation pathways [31]. Coupling the high-throughput workflows of two-pot CFPS-ME with machine learning algorithms can reduce the experimental test space of combinatorial pathway prototyping to quickly identify effective enzyme combinations and ratios [33, 37]. The ability to use linear expression templates additionally carries the potential for direct screening of enzyme libraries without the need for cellular transformations [38] and production of enzyme homologs from inexpensive gene blocks within 24 h [39].

Pairing cell-free protein synthesis with the combinatorial assembly of metabolic pathways *in vitro* compounds recent developments in cell-free synthetic biology to provide a powerful tool for

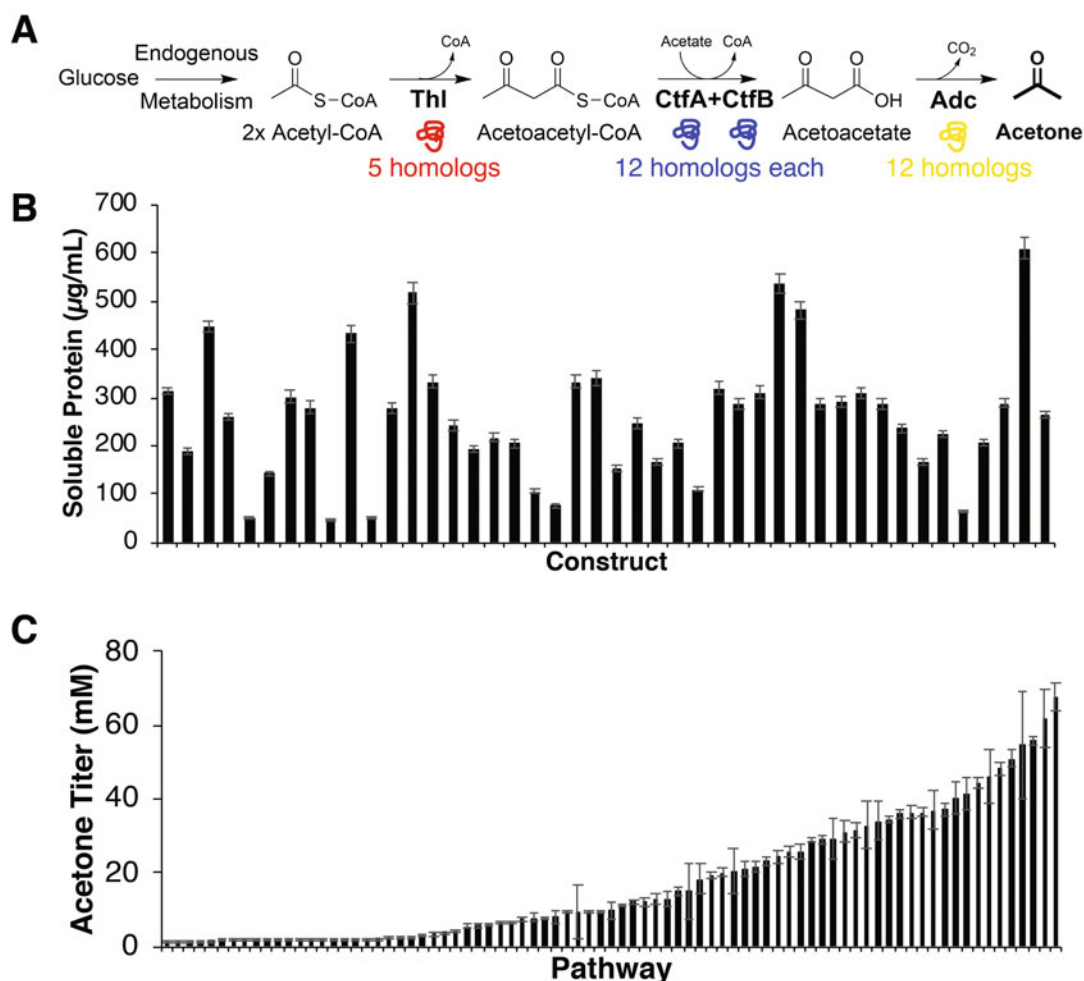


Fig. 2 Sample data from CFPS-ME workflow. (a) Pathway diagram for conversion of glucose to acetone, utilizing native enzymes from *E. coli* cell extract and homologs of 4 heterologous enzymes. (b) CFPS with radioactive incorporation from 44 linear expression templates provides accurate protein yields in 2 days. Oxalic acid was omitted to enhance acetone titers in downstream reactions (see **Note 10**), resulting in ~60% of the sfGFP reporter yields (construct 43) seen in previous CFPS optimizations. (c) Combinatorial assembly of 81 pathway combinations (varying homologs and enzyme concentrations of 0.05, 0.1, and 0.5 µM) produces in vitro acetone titers ranging from the limit of detection to 67 mM in a matter of days, with HPLC analysis as the limiting step

high-throughput pathway prototyping [28]. The modular nature of this approach allows for the design of tailored strategies for each optimization problem. For example, extract preparation methods may be altered based on available equipment for lysis, and utilizing knockout strains can prime cell-free metabolism for different pathways through rerouted carbon flux. Additionally, linear expression templates may be used for CFPS to avoid cellular transformation bottlenecks, and reaction composition can be altered to improve

protein and/or metabolite yields. Overall, the CFPS-ME framework is poised to facilitate multiplexed study of biosynthetic pathways at an unprecedented speed and throughput in order to inform cellular engineering efforts [33] and eventually large-scale cell-free biosynthesis platforms [40] with implications for producing commodity chemicals [3], biomaterials [41], and natural products [42].

2 Materials

2.1 Cell Extract Preparation

1. LB broth and LB agar plates: Combine 10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl with deionized water to 1 L and dissolve completely. If making agar plates, add 15 g of agar. Autoclave or filter to sterilize.
2. 2xYTPG media: Combine 16.0 g of tryptone, 10.0 g of yeast extract, 5.0 g of NaCl, 7.0 g of K_2HPO_4 , and 3.0 g of KH_2PO_4 with deionized water to 750 mL. Dissolve completely and adjust pH to 7.2 using 5 N NaOH or KOH. Dissolve 18.0 g of glucose in 250 mL of deionized water. Autoclave separately to avoid Maillard reaction and combine prior to growth. Alternatively, combine all reagents in water to 1 L and sterile filter after adjusting pH.
3. 1.0 M Isopropyl β -D-1-thiogalactopyranoside (IPTG).
4. S30 buffer: Prepare 1.0 M Tris-base (adjusted to pH 8.2 with glacial acetic acid), 1.4 M magnesium acetate, and 6.0 M potassium acetate. Sterile-filter stock solutions and combine 10 mL of each solution with 970 mL of water for final concentrations of 10 mM Tris base, 14 mM magnesium acetate, and 60 mM potassium acetate. Add 2 mL of 1.0 M dithiothreitol (DTT) per L of buffer immediately prior to use.
5. BL21 StarTM (DE3) or desired knockout strain.
6. pJL1-derived plasmids or linear templates (Addgene plasmid # 102634) containing genes of interest for biosynthetic pathways.
7. 250-mL baffled flask.
8. 2.5-L Tunair flask.
9. High-speed centrifuge with 1-L centrifuge bottles.
10. Tabletop centrifuge with 50-mL conical tubes.
11. Lysing instrument: Sonicator or homogenizer.
12. 10–30 mL Luer lock syringes.
13. Liquid nitrogen.
14. Vortex.

2.2 Cell-Free Reactions

1. 15× salt solution: 120 mM magnesium glutamate, 150 mM ammonium glutamate, and 2.01 M potassium glutamate.
2. 15× nucleotide master mix: 18 mM adenosine triphosphate (ATP), 12.75 mM guanosine triphosphate (GTP), 12.75 mM cytidine triphosphate (CTP), 12.75 mM uridine triphosphate (UTP), 0.51 mg/mL folinic acid, and 2.559 mg/mL *E. coli* tRNAs.
3. 1 M phosphoenolpyruvate (PEP).
4. 50 mM amino acid solution (all 20 canonical amino acids at 50 mM).
5. 100 mM nicotinamide adenine dinucleotide (NAD).
6. 50 mM coenzyme A (CoA).
7. 250 mM putrescine.
8. 250 mM spermidine.
9. 1.0 M oxalic acid.
10. 1.0 M HEPES buffer (pH 7.2).
11. 2.2 M glucose.
12. 1.0 M Bis-Tris buffer.
13. 100 mM ATP.
14. 50 mg/mL kanamycin.
15. 10% (w/v) trichloroacetic acid (TCA) or another quenching reagent.
16. Echo 550 liquid handling robot (Labcyte Inc., CA, USA).

2.3 Protein Quantification via Radioactivity

1. 0.32 mM ^{14}C -Leucine.
2. 0.5 N KOH.
3. 5% (w/v) TCA.
4. Styrofoam blocks with the center cut out wrapped in aluminum foil.
5. 0.5 mm diameter metal pins with glass heads.
6. Micro Beta scintillation counter or other means of protein quantification.
7. Filtermat A (Perkin Elmer).
8. Meltilex A scintillation wax sheets (Perkin Elmer).
9. Adjustable heating block.
10. Geiger counter.

3 Methods

3.1 Cell Extract Preparation

3.1.1 Cell Growth and Harvest

1. Streak glycerol stock of BL21 Star™ (DE3) or desired strain (*see* **Notes 1** and **2**) on LB-agar plate, including antibiotic if necessary. Incubate plate overnight at 37 °C.
2. Inoculate 30 mL of LB broth with a single colony from **step 1**. Incubate at 37 °C with shaking at 220 rpm for 16–18 h.
3. Ensure that centrifuge rotors, S30 buffer, and centrifuge bottles are cooling to 4 °C (*see* **Note 3**).
4. Use the overnight culture to inoculate 1 L of 2xYTPG (*see* **Note 4**) with an initial OD₆₀₀ of 0.05–0.1 in a 2.5-L Tunair flask. Incubate at 37 °C with shaking at 220 rpm.
5. For BL21 Star™ (DE3), induce T7 expression with 0.5 mL of 1 M IPTG at OD₆₀₀ of 0.5–0.6.
6. Continue monitoring cell density, diluting culture as necessary to be within the linear range of your spectrophotometer.
7. Harvest the cells in mid-exponential phase (OD₆₀₀ 3–3.5) by transferring the culture to a 1-L centrifuge bottle or dividing the culture into two bottles. Centrifuge for 5 min at 8000 × *g* and 4 °C.
8. Carefully discard the supernatant and immediately place the centrifuge bottle(s) on ice.
9. Transfer the pellet to two 50-mL conical tubes per liter of cell culture using a spatula. Keep the tubes on ice.
10. Wash the cell pellets with 25 mL of S30 buffer (with DTT added to a final concentration of 2 mM) using a vortex to resuspend the cells. Ensure that the slurry remains cold by alternating with 15 s on the vortex and 15 s on ice.
11. When the pellets are completely resuspended and no cell clumps are visible, centrifuge at 4 °C for 2 min at 10,000 × *g*.
12. Carefully discard the supernatant.
13. Repeat **steps 10–12** twice more for a total of three washes.
14. Dry the interior and exterior of the conical tubes with Kim-wipes, taking care not to disturb the pellet.
15. Measure and record the cell mass in each tube.
16. Flash-freeze the cell pellets in liquid nitrogen and store at –80 °C until lysis. Alternatively, omit flash-freezing and immediately begin lysis and clarification steps.

3.1.2 Lysis and Clarification

1. If cell pellets were frozen, thaw for 1 h on ice. Otherwise, proceed directly to **step 2**.
2. Add 1 mL of S30 buffer per gram of cell pellet.

3. Resuspend the cell pellet using a vortex at max speed, alternating with 15 s intervals on the vortex and on ice to keep cells cold and metabolism slow. Continue until the cell pellet is fully resuspended and no clumps remain.
4. A layer of foam may form atop the cell suspension, particularly if the cells were frozen. Allow the cells to rest on ice for 15 min so that foam can dissipate.
5. Lyse the cells via sonication or high-pressure homogenization (*see Note 5*).
 - (a) Sonication:
 - Transfer 1 mL aliquots of cell suspension to 1.5-mL microcentrifuge tubes.
 - Lyse each aliquot using a QSonica Q125 Sonicator with 3.175 mm diameter probe at 20 kHz and 50% amplitude, keeping the tube in an ice water bath. Input 680 J for 1 mL aliquots, alternating 15 s on and 15 s off to reduce overheating.
 - Immediately place tubes of cell lysate on ice.
 - (b) Homogenization:
 - Transfer resuspended cells without foam to a syringe using an 18-gauge needle to break up any remaining cell clumps.
 - Lyse the cells using an Avestin EmulsiFlex-B15 homogenizer with a single pass at a pressure of 20,000–25,000 psi. Maintain a controlled flow rate for consistent pressure to maximize the percentage of cells lysed.
 - Immediately transfer cell lysate to a clean conical tube on ice.
 - Aliquot lysate into 1.5-mL microcentrifuge tubes on ice.
6. Optional: Add 3 mM DTT (3 μ L of 1 M DTT per 1 mL lysate) to the lysate and mix by inverting the tube (*see Note 6*).
7. Centrifuge tubes of lysate for 10 min at $12,000 \times g$ and 4°C (*See Notes 7 and 8*).
8. Carefully remove the clarified supernatant (now referred to as cell extract) without disturbing the pellet of insoluble cell debris and transfer to fresh 1.5-mL microcentrifuge tubes on ice.
9. Centrifuge tubes of extract again for 10 min at $12,000 \times g$ and 4°C to remove any residual insoluble debris. This will produce a small pellet, if any.

10. Transfer final extract to a conical tube and mix to ensure homogeneity in the batch.
11. Aliquot cell extract in desired volume in either 200- μ L PCR tubes or 1.5-mL microcentrifuge tubes on ice.
12. Flash-freeze aliquots in liquid nitrogen and store at -80°C until use.
13. Determine the bulk protein content of your cell extract using a Bradford assay or similar protein quantification method, expecting $\sim 50\text{--}60$ mg/mL.

3.2 Cell-Free Protein Synthesis (CFPS)

1. Prepare pJL1 expression templates for proteins of interest using either plasmids or linear templates.
 - (a) Purify plasmids using high-quality midi- or maxi-prep kits. Ethanol precipitation may increase CFPS yields by removing residual salts.
 - (b) Amplify linear expression templates using a high-fidelity polymerase, such as Q5.
 - Forward primer: 5'-ctgagatactacagcgtgagc-3'.
 - Reverse primer: 5'-cgctcactcatggtgatttctcacttg-3'.
2. If using extract from a strain without chromosomally induced T7 polymerase, add purified T7 polymerase to a final concentration of 0.1 mg/mL (*see Note 9*).
3. Add 1 μ L of purified expression template to 2-mL tubes to reach final concentrations of 5–10 nM. Use 2 μ L of linear expression template when using unpurified PCR products to avoid transcriptional limitation. Ensure that the droplet containing expression template is at the bottom of the tube (*see Note 10*).
4. Aliquot 14 μ L of CFPS master mix (Table 1) to each tube with expression template and gently mix at the bottom of the tube.
5. Incubate CFPS reactions at 30°C for 6–20 h.

3.3 Protein Quantification

1. Set up CFPS reactions in triplicate as described above with the addition of 10 μM ^{14}C -leucine and incubate for desired time. Include a blank reaction (using water instead of expression template) to determine background radioactivity, and express green fluorescent protein as a positive control. For alternatives to radiolabeling, *see Note 12*.
2. Prepare PCR strips with 5 μ L of 0.5 N KOH to dissolve total and soluble fractions of each CFPS reaction.
3. Add 5 μ L from your reaction to a PCR tube and pipette to mix with base. This is the total fraction. Incubate samples at 37°C for 20 min.

Table 1
CFPS master mix composition

Reagent	Stock concentration	Final concentration
Salt solution (<i>see Note 3</i>)	15×	1×
Nucleotide master mix	15×	1×
PEP	1 M	33.33 mM
Canonical amino acid mix	50 mM	2 mM
NAD	100 mM	0.4 mM
CoA	50 mM	0.27 mM
Putrescine	250 mM	1 mM
Spermidine	250 mM	1.5 mM
Oxalic acid (<i>see Note 11</i>)	1 M	4 mM
HEPES (pH 7.2)	1 M	57 mM

- During the incubation, centrifuge remaining CFPS volume for 10 min at $12,000 \times g$ and 4 °C to isolate the soluble fraction.
- Add 5 μ L of supernatant to PCR tubes and pipette to mix with base. Take care not to disturb the pellet, which will be wide in a 2-mL tube. Incubate samples from soluble fraction at 37 °C for 20 min.
- Prepare 2 Filtermats. One will be washed, and the other will not. Pin them to the foil-wrapped Styrofoam blocks with the center cut out to ensure your samples remain elevated and do not touch the surface. This reduces error substantially.
- After incubation, place 4 μ L from each sample onto corresponding wells of the washed and unwashed Filtermats. Avoid air bubbles to ensure the same volume of each sample is placed on each Filtermat.
- Allow Filtermats to dry under the heat lamp for 20 min.
- Take the Filtermat designated for washing and place in a plastic tray. Cover with cold 5% TCA, shake gently, and place at 4 °C for 15 min. Carefully pour liquid into the appropriate radioactive waste container, and repeat this step for a total of 3 washes.
- Cover the washed Filtermat with 100% ethanol, shake gently, and incubate at room temperature for 15 min. Carefully pour liquid into the appropriate radioactive waste container.
- Place the Filtermat back on the Styrofoam block and allow to dry under the heat lamp for 20 min.
- Turn on the heating block to 80–90 °C and apply a clean transparency sheet on top to contain melted wax.

13. Carefully place dry Filtermat on the transparency and melt scintillation wax to align with the edges of the sample wells. Use metal forceps in both hands to keep the mat flat for even melting. Once the wax has soaked into the Filtermat, lift it up with forceps on both sides and gently blow on it until the wax becomes opaque again.
14. Place the waxed Filtermat in a plastic plate holder, taking care to align the holes with wells on the Filtermat.
15. Load your washed and unwashed mats into the MicroBeta and run desired program for scintillation counting.
16. Determine the amount of protein produced in each reaction:
 - (a) Calculate the percent of leucine incorporated into proteins by dividing counts from the washed sample by counts from the corresponding unwashed sample.
 - (b) Subtract the background incorporation percent (quantified in the blank reaction) from all samples.
 - (c) Divide the corrected incorporation percent by the number of leucine residues in the expressed protein and multiply by the total concentration of leucine in the reaction.
 - (d) Divide this value by the molecular weight for each protein expressed to obtain micromolar concentrations.

$$\begin{aligned} & [((\text{Washed count} - \text{Background count}) / \text{Unwashed count}) \\ & \quad * [\text{Leu}] \mu\text{M} * (\text{MW Protein } \mu\text{g}/\mu\text{mol})] \\ & \quad / [(\text{Leu residues in protein}) * (1000 \text{ mL/L})] = \mu\text{g/mL}. \end{aligned}$$

3.4 Cell-Free Metabolic Engineering (CFME)

3.4.1 CFME Assembly by Hand

1. Reconstitute biosynthetic pathways in vitro by combining desired μM concentrations of enzymes produced by CFPS. Correct for different amounts of CFPS added by adding spent CFPS reaction without plasmid template or with sfGFP template to normalize the concentrations of reagents and native enzymes between samples.
2. Combine enzyme-enriched CFPS reactions with reagents listed in Table 2 in nuclease-free water to 20 μL in 1.5-mL microcentrifuge tubes (*see* **Notes 13** and **14**).
3. Incubate CFME reactions at 30 °C for 6–24 h and quench prior to analysis (*see* **Note 15**).

3.4.2 Assembly Using an Echo 550 Automated Liquid-Handling Robot

1. Set up CFPS as described previously, calculating in the dead volume needed for the Echo source plate (20 μL for PP⁺ 384 plates and 2 μL for LDV plates).
2. Combine CFPS reactions in 96- or 384-well plate using 384PP/LDV_AQ_CP as a fluid type on an Echo 550 liquid handling robot (Labcyte Inc., CA, USA). Normalize CFPS

Table 2
CFME reaction mix composition

Reagent	Stock concentration	Final concentration
Salt solution (<i>see Note 3</i>)	15×	1×
Bis-Tris buffer	1 M	100 mM
Glucose	2.2 M	50–200 mM
ATP	100 mM	0–10 mM
NAD	100 mM	0–10 mM
CoA	50 mM	0–10 mM
Kanamycin	50 mg/mL	1.25 mg/mL
Fresh <i>E. coli</i> extract	~50 mg/mL	8 mg/mL

volume added to each combination using spent CFPS reaction without plasmid template or with sfGFP template.

3. Start reactions by adding the same amount of a master mix containing the above listed components dissolved in nuclease-free water to each well using 384PP_AQ_BP as a fluid type on the Echo or an Integra VIAFLO 96/384 multichannel pipette.
4. Seal plate, incubate at 30 °C, and optimize concentrations as stated in Subheading 3.4.1.

4 Notes

The modularity and adaptability of CFPS-ME to fit the specific needs of the pathways under investigation are major advantages of the system. This does, however, make it difficult to write a generalizable protocol that can be used for any case. Here, we offer some of the adaptations to the above protocol that might benefit or enable certain experiments, and we include comments about common pitfalls and sensitive steps of the outlined protocol above.

1. Endogenous enzymes in the BL21 Star™ (DE3) strain can affect product yields by diverting the starting substrate or interacting with other metabolic pathways. Extracts from strains containing knockouts of these enzymes can be made for CFPS-ME experiments, and this workflow can even be used to accelerate the characterization of a gene knockout's impact on a metabolic pathway. However, these knockouts may impact the growth of cells for high-yielding CFPS extract preparation. For this reason, we suggest optimization of the harvest OD₆₀₀ (Subheading 3.1.1, step 7) by comparing timepoints in early, mid, and late exponential growth.

2. As an alternative to knockout strains, recent studies have shown that undesired enzymes can be removed or reduced post-lysis by adding protease sites [43] or affinity tags [44] (adding an additional processing step). This reduces the detrimental effect on cell growth caused by some gene knockouts and could enable highly productive CFPS-ME extracts even when essential genes are targeted for removal.
3. Organic salts used in S30 lysate buffer (Subheading 3.1.1, step 3), in the CFPS reaction mix (Subheading 3.2, step 2), and during CFME reactions (Subheading 3.4.1, step 2) can directly influence yields of the CFME reaction depending on the pathway under investigation. For example, acetate has been shown to enter cell-free metabolism during synthesis of hydroxymethylglutaryl-CoA and mevalonate [21]. Glutamate undergoes deamination and enters the TCA cycle at α -ketoglutarate, which eventually leads to a build-up of succinate. Acetate or glutamate can be used interchangeably in all three steps depending on pathway demands.
4. The growth medium (and especially the carbon source) can directly influence metabolism of the extract. The addition of glucose to 2xYTP does not influence CFPS yields in our experience, but it increases glycolytic activity in cell extracts. Alternative carbon sources for growth and/or cell-free biosynthesis might benefit CFPS-ME for pathways relying on different parts of central carbon metabolism.
5. Lysis through sonication or homogenization requires specialized equipment, such as the QSonica Q125 Sonicator with 3.75 mm tip or Avestin EmulsiFlex-B15 Homogenizer. Less-expensive methods (including bead-beating, French press, and chemical lysis) have successfully produced functional cell extracts as well [17, 46].
6. Addition of 3 mM DTT to lysed cells prior to centrifugation is included in some published protocols, but empirically this has shown little difference in CFPS yields. DTT added to S30 buffer should provide a sufficiently reducing environment.
7. A run-off reaction after lysis and initial centrifugation (after Subheading 3.1.2, step 7), which consists of a 60–80 min incubation at 37 °C, can improve CFPS yields for some strains, including K strains of *E. coli* [29].
8. Centrifugation speed during extract preparation (Subheading 3.1.2, step 7) influences diameter, size distribution, and concentration of inverted membrane vesicles in the extract. This may influence oxidative phosphorylation (and therefore ATP regeneration) in both CFPS and CFME reactions [47, 48]. This dependence on respiration makes the reactions sensitive to oxygen availability, so surface area to volume ratios can impact efficiency.

9. Induction of T7 polymerase is not possible in all strains, and purified T7 polymerase can be added to those extracts during CFPS (Subheading 3.2, **step 2**). Alterations to this method are required for robust expression from native *E. coli* promoters [45].
10. Linear expression templates are stable in extracts derived from BL21 Star™ (DE3), but stability varies between extracts of different strains based on exonuclease activity. GamS nuclease inhibitor can stabilize linear templates when added in the CFPS mix at 30 µg/mL (Subheading 3.2, **step 3**) [30].
11. Addition of oxalic acid to CFPS master mix increases protein production, but it can reduce titers of some target metabolites [33].
12. Alternative methods for quantifying expression levels from CFPS include reporter protein domains (e.g., split-GFP [49], cleavable N-terminal GFP fusion [50], or NanoLuc [51]) and chemical labeling kits (e.g., FluoroTect™ or Transcend™ from Promega [52]).
13. Reaction pH and buffer choice may be optimized to balance pH optima of desired catabolic and anabolic enzymes [26, 34].
14. CFME reactions require optimization of substrate, cofactor, and enzyme concentrations to maximize product titers and/or reaction rates. Additional cofactors, such as NADP(H), may be beneficial for some pathways. Optimize incubation time and temperature for each investigated pathway.
15. Use an appropriate quenching method for the downstream analytic method to precipitate proteins in the CFME reaction or otherwise stop metabolic activity. For liquid chromatography, add a common quenching reagent such as 5–10% acid (trifluoroacetic, trichloroacetic, or formic), 50% acetonitrile, or 50% methanol. Centrifuge samples at $10,000 \times g$ for 10 min and transfer supernatants to appropriate vials or plates for your instrument. For gas chromatography, extract samples with an appropriate solvent (e.g., ethyl acetate, hexane, dodecane), adding 6.25% H₂SO₄ and 7.5% NaCl to precipitate proteins if desired. After extraction, further derivatization may be appropriate for target molecule detection.

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Metabolomics Analysis of Cell-Free Expression Systems Using Gas Chromatography-Mass Spectrometry

April M. Miguez, Yan Zhang, and Mark P. Styczynski

Abstract

Metabolomics is the systems-scale study of the biochemical intermediates of metabolism. This approach has great potential to uncover how metabolic intermediates are used and generated in cell-free expression systems, something that is to date not well understood. Here, we present a detailed metabolomics protocol for characterization of the small molecules in cell-free systems. We specifically focus on the analysis of *Escherichia coli* lysate-based cell-free systems using gas chromatography coupled to mass spectrometry. Measuring and monitoring the metabolic changes in cell-free systems can provide insight into the ways that metabolites affect the productivity of cell-free reactions, ultimately allowing for more informed engineering and optimization efforts for cell-free systems.

Key words Metabolomics, Gas chromatography-mass spectrometry, Cell-free expression systems, Metabolites, *Escherichia coli*

1 Introduction

Metabolomics is a systems-scale approach to measuring metabolites, which are the small molecule intermediates of the biochemical reactions by which cells take in nutrients and turn them into energy and the building blocks for more cells (metabolism). It is a well-established technique to study the metabolism of living systems ranging from unicellular prokaryotes to multicellular eukaryotes [1–5]. It typically entails the use of sophisticated detection techniques like nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry to identify and quantify the amounts of different small molecules in a sample. Often, an upstream separation step is used to allow resolution of more molecules than would otherwise be possible to distinguish strictly on the basis of the detection instrument alone.

However, the use of metabolomics is a relatively new approach to study cell-free expression (CFE) systems [6–8]. The application of metabolomics has great potential in this field because, like whole cells, CFE systems contain small molecules that are consumed and created over time, which can be measured and monitored. Characterization of the metabolic changes in these systems may help reveal the roles and impacts of small molecules in CFE reactions, thus providing insights for optimization efforts. Here, we present a workflow for assembly and metabolomics analysis of an *E. coli*-based CFE system using gas chromatography coupled to mass spectrometry (GC-MS).

An example of an *E. coli*-specific CFE reaction assembly protocol is used here, as has been previously described [9]. However, the details of reaction assembly for metabolomics analysis of CFE systems will largely depend on the chassis organism of the lysate and specific experimental needs. Regardless of the assembly method used, metabolomics analyses can be applied to all CFE reactions and extracts. The general metabolomics pipeline for CFE sample analysis includes sample acquisition, sample preparation, data acquisition, and data processing. Sample acquisition and preparation steps will depend on the analytical instrument used, but in general, all metabolic activity needs to be rapidly stopped at the time of collection with minimal damage to metabolites. This is followed by removal of the proteins and large nucleic acid molecules to leave behind the small molecules from the CFE mixture. To do this, we present a simple yet effective precipitation method that is modified from a methanol-based serum protein precipitation protocol [10].

Although the protocol described in this chapter uses a GC-MS for data acquisition, other instruments such as liquid chromatography-mass spectrometry or NMR spectroscopy can be used in place of a GC-MS. Selection of the instrument often depends heavily on availability or logistical convenience but should also account for the types of molecules expected to be of interest, since different instruments are better at measuring different classes of molecules. Each platform has its own set of advantages and limitations that should all be considered when designing an experiment if multiple options for instruments are available [11]. Due to the complexity of metabolomics data, multiple data processing steps and potentially even multiple software platforms may be required. We present here an example of steps for processing GC-MS data, but the details of the data processing procedure will depend on the instrument and software available. Ultimately, the results obtained from metabolomics analyses will enable identification of changes of key metabolites involved in primary pathways and aid optimization efforts of CFE systems. Although the results will be specific to the system and experimental conditions studied, the metabolomics procedure presented here is highly generalizable and can be applied to all CFE systems analyzed via GC-MS.

2 Materials

This protocol produces biohazardous and hazardous chemical waste; follow all waste disposal regulations carefully.

2.1 Cell-Free Reaction Materials

1. *E. coli* cell-free extract and reagents prepared as described by Kwon and Jewett [9].
2. Midi-prepped plasmid coding for superfolder GFP expression (*see* **Note 1**).
3. Black-bottomed 384-well plate.
4. Microplate reader.

2.2 Protein Precipitation Materials

1. 100% methanol.
2. Centrifugal concentrator.

2.3 GC-MS Data Acquisition

1. GC-MS instrument.
2. Autosampler vials with caps and low-volume inserts.
3. MeOx solution: 40 mg/mL methylhydroxyamine hydrochloride (MeOx), pyridine (*see* **Notes 2** and **3**). Store at 4 °C in flammable-safe storage.
4. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) plus 1% 2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl)-acetamide, chlorotrimethylsilane (TMCS) silylation reagent. Store at 4 °C in flammable-safe storage (*see* **Note 2**).

2.4 Data Processing Software

1. Spectral deconvolution and identification software, such as Automated Mass Spectral Deconvolution and Identification System (AMDIS [12], freely available at <http://chemdata.nist.gov/mass-spc/amdis/downloads/>) or instrument manufacturer's peak deconvolution software.
2. Peak Alignment Software such as SpectConnect [13] (freely available at <http://spectconnect.mit.edu/>) or MetPP [14] (designed for GCxGC-TOF-MS data, freely available at <http://metaopen.sourceforge.net>).
3. Other data processing software as desired such as Metaboanalyst [15] (freely available at <https://www.metaboanalyst.ca/>).

3 Methods

3.1 CFE Reaction Assembly

1. Thaw cell-free reagents described in Table 1 on ice for approximately 15 min. Set vortex mixer to medium-high speed for mixing.

Table 1
Composition of a cell-free reaction for metabolomics analysis

Reagents	Stock solution		Volume (μL): 1 ×	Final concentration	
15 × SS	15	×	14.00	1.00	×
<i>Mg</i> (<i>GLU</i>) ₂	180	mM		12	mM
<i>NH</i> ₄ (<i>GLU</i>)	150	mM		10	mM
<i>K</i> (<i>GLU</i>)	1950	mM		130	mM
15 × master mix	15	×	14.00	1.00	×
<i>ATP</i>	18	mM		1.2	mM
<i>GTP</i>	12.8	mM		0.85	mM
<i>UTP</i>	12.8	mM		0.85	mM
<i>CTP</i>	12.8	mM		0.85	mM
<i>Folinic acid</i>	0.51	mg/mL		0.034	mg/mL
<i>tRNA</i>	2.56	mg/mL		0.171	mg/mL
12 × reagent mix	12	×	17.50	1.00	×
<i>NAD</i>	100	mM		0.333	mM
<i>CoA</i>	33	mM		0.267	mM
<i>Oxalic acid</i>	1000	mM		4	mM
<i>Putrescine</i>	250	mM		1	mM
<i>Spermidine</i>	250	mM		1.5	mM
<i>HEPES</i>	1000	mM		57	mM
20 amino acids	50	mM	8.4	2.00	mM
PEP	1	M	6.3	0.03	M
<i>E. coli</i> BL21 extract	1	×	56.07	0.27	×
P _{J23100} -sfGFP	200	nM	12.60	12	nM
Nuclease-free water	–		81.13	–	
<i>V</i> _{Total} =			210.00		

2. Assemble the cell-free reaction. Always add water first, then small molecule mixes, plasmid DNA, and lysate last. Make sure to vortex the mixture after addition of each reagent for homogeneous mixing. If assembling multiple cell-free reactions at once, it is best to prepare a master mix of reagents and aliquot into smaller reactions to reduce pipetting errors among reactions (*see* **Note 4**).
3. Incubate assembled reactions in a 37 °C incubator for the desired amount of time (typically ranging from 30 min to 6 h).

4. After incubation has completed, immediately place the reactions on ice. Aliquot 10 μL of the reaction into a 384-well plate for fluorescence analysis (*see Note 5*). Then place the rest of the reaction in the -80°C freezer to stop metabolic reactions and save the sample for later metabolomics analysis.
5. For fluorescence analysis, program the plate reader to detect GFP fluorescence at 485 nm excitation, 510 nm emission (*see Note 6*), and a gain setting of 70.

3.2 Protein Precipitation for Metabolomics Analysis

1. Fill an ice bucket with ice.
2. Thaw all samples on ice. (This should take approximately 20–30 min) (*see Note 7*).
3. If pooled quality control (QC) samples are desired, vortex thawed samples, remove a set volume from each tube, and pool together (*see Notes 8 and 9*).
4. Add methanol to each sample and to the pooled QC at a 1:2 ratio of sample to methanol.
5. Vortex each tube for approximately 5 s.
6. Incubate tubes at -20°C for at least 20 min.
7. Centrifuge tubes at 11,600 rcf for 30 min at room temperature (*see Note 10*).
8. Transfer the supernatant from each sample and from the pooled QC into clean microcentrifuge tubes.
9. If a pooled quality control was collected, aliquot a set volume of the pooled QC supernatant into an appropriate number of clean microcentrifuge tubes (*see Note 11*).
10. Dry the collected supernatants completely in a centrifugal concentrator under vacuum at 40°C .
11. Dried samples and split QCs can then be derivatized for subsequent GC-MS analysis or stored at -80°C for later use. If stored, place the samples back into the centrifugal concentrator under vacuum at 40°C for 10–15 min when ready for GC-MS analysis to ensure there is no water in the sample.

3.3 Derivatization and Data Acquisition

1. Ensure the samples and QCs are completely dry before derivatization (*see Subheading 3.2, step 11*). In a chemical fume hood, add 10 μL MeOx solution to each sample and scrape the sides of the tube with the pipette tip to submerge the entire pellet (*see Note 12*).
2. Incubate the samples at 30°C for 90 min (*see Note 13*).
3. While the samples are incubating, label autosampler vials and add low-volume inserts into each vial.

4. In a chemical fume hood, add 90 μL of MSTFA plus 1% TMCS reagent to each tube and incubate the samples at 37 °C for 30 min.
5. Centrifuge the samples at 10,000 rcf or more at room temperature for 3 min.
6. In a chemical fume hood, transfer 50 μL of each sample into the low-volume insert in the appropriate autosampler vial and cap each vial tightly. Be sure not to collect any solid matter from the bottom of the tube, as this will clog the instrument's syringe.
7. Spike a retention index standard into samples, if desired (*see Note 14*).
8. Place the vials into the autosampler of the GC-MS and begin instrument run (*see Note 15*).

3.4 Data Processing

1. Perform peak deconvolution on all data to compute spectra for each peak. This is typically done using the instrument manufacturer's software but can be done using free software such as AMDIS. The specific steps and parameter values used for this process will vary based on the software package, but key considerations typically include expected peak width, minimum signal to noise ratio, and baseline offset.
2. Perform peak alignment on all data to adjust for drifts in retention time across samples. This can be done using software such as SpectConnect or MetPP. Again, note that the details in executing this step will vary based on the software used, but key considerations typically include expected retention time deviation and selection of alignment standards. Peak filtering can also be done after the alignment to ensure reproducibility, if desired.
3. Use a mass spectral compound library to help identify analytes and allow for biological data interpretation [16–18].
4. Conduct statistical analyses as desired. Tools such as principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) can be used to reduce the dimensionality of the data set into a more manageable set of variables that allows for easier data visualization and overall interpretation. *T*-tests and analysis of variance (ANOVA) can be used to determine metabolites with statistically significant differences between two groups of samples and between three or more groups of samples, respectively. To determine the specific portions of metabolism that are most significantly varying between groups or across samples, metabolite pathway enrichment analysis (MPEA) can be used. Software such as the freely available Metaboanalyst can be used to conduct all of the aforementioned analyses.

4 Notes

1. For successful cell-free protein expression, we recommend using midi-prepped (rather than mini-prepped) plasmids followed by DNA precipitation. To ensure plasmid purity and reduce batch-to-batch differences in quality, always digest the plasmid and analyze it on a gel after each preparation to check for genomic DNA contamination.
2. Pyridine and MSTFA are highly hazardous, flammable chemicals that should only be handled in a chemical fume hood with appropriate gloves.
3. If the MeOx does not easily dissolve in pyridine, mild heating at 37 °C using an incubator or water bath can resolve this issue.
4. We have found that 200 μ L of a reaction is the optimal volume of sample to run on our instrument that maximizes the number of analytes detected without detector saturation. This volume should be optimized based on the analytical instrument selected as well as the practical experimental needs. If measuring fluorescence, we recommend preparing a total reaction volume that is 10 μ L more than required for metabolomics analysis, so 10 μ L reaction aliquots can be used for fluorescence measurements.
5. When running experiments with multiple time points, it is best to save the 10 μ L reaction aliquot at each time point in a 0.5 mL microcentrifuge tube at -80°C until all the time points have been collected, so the fluorescence at all time points can be analyzed at once.
6. We have found a gain setting of 70 for GFP measurement avoids signal overflow but allows sufficient sensitivity. This gain setting may need to be optimized on different models of plate readers.
7. Thawing on ice is essential to minimize any residual metabolic activity in the samples that would change the metabolic profile from what it was when the sample was acquired.
8. If derivatizing samples, note that the derivatization reaction will proceed while samples are queued and running. This will intensify the “instrument drift” in the data set, which is a common challenge in GC-MS data analysis resulting from changes in metabolite detection over the duration of sample analysis. Due to this, samples should ideally be derivatized and run in batches (run time per batch should be no more than 12 h). If multiple batches of samples are run, pooled quality control (QC) samples are critical to adjust for this variability and should consist of a small volume of every sample to be run on the instrument. The QC sample measurements can then be

used for data adjustment and alignment to account for the drift. Each batch should have pooled QC samples that are run every 4–8 injections, with at least one at the beginning and at least one at the end of the batch. After precipitation, the pooled QC sample will be equally split into aliquots so that there is one aliquoted QC sample for each batch, which will be derivatized and analyzed with that batch.

9. The collection for the pooled QC sample can be done after the protein precipitation of each sample and supernatant transfer, but we have found that collecting at the beginning of the protein precipitation protocol helps prevent additional experimental error such as sample loss during pipetting.
10. Once the proteins have been precipitated out, metabolic activity should cease and thus samples can be worked with at room temperature.
11. The number of aliquoted split QC samples should be equal to the number of batches required. Ideally, each split QC sample volume is equal to the volume of each regular sample. For example, if there are 18 samples each with a volume of 200 μL in each sample and two batches are required (to keep the run time per batch under 12 h), then 20 μL would need to be removed from each sample, pooled together, and then split between two tubes. This would result in a volume of 180 μL for all samples and each of the split QCs. However, due to sample loss during pipetting, it is best to collect a slightly larger volume than calculated from each sample. For this example, instead of collecting 20 μL , it would be best to collect 30 μL and transfer 170 μL of the pooled QC into each of the split QC sample tubes. This will ensure that there is enough pooled QC sample to be equally split while maintaining equal volumes among samples.
12. Derivatization is a GC-MS sample preparation step used to volatilize otherwise nonvolatile compounds, as well as alter analyte properties to improve compound separation and detector responsivity. MeOx and MSTFA plus 1% TMCS are common derivatization reagents that react with the analytes in the sample to enhance their volatility and thermal stability.
13. A heating block with agitation is preferable for derivatization, but a heating block without agitation or a water bath may also be used.
14. Retention index standards are reference chemicals with defined chromatographic retention times that can be used during data processing to align samples and ultimately enhance the identification of analytes. They are ideally not part of the metabolome of interest, they do not coelute with the metabolome of

interest, and they span the entirety of the sample run time. We typically add 0.25 μL of a previously described mixture of fatty acid methyl esters (FAME markers) to our samples [16].

15. Operation and method will vary based on the instrument and metabolites of interest. In general, the main priority is high-quality separation of compounds before they reach the mass spectrometer. This can obviously be enabled by selecting a suitable GC column that is amenable to separating the molecules of interest, but optimal separation also typically requires a suitable oven temperature and ramp speed. These parameters will need to be adjusted for the instrument used and the types of molecules of interest. We typically use a starting oven temperature of 70 °C with a ramp rate of 10 °C per minute until the oven reaches 315 °C; however, varying ramp rates throughout the analysis may be desirable if there is a mix of regions that are densely populated and sparsely populated with analytes in the resulting chromatogram, indicating poor separation using a single ramp rate. An additional parameter that can affect the quality of peak separation is the sample concentration. Analysis of samples that are too concentrated may result in detector saturation or peak broadening. This can easily be resolved by adjusting the front inlet mode from splitless to split. Splitless mode allows for all the injected sample to enter the column, whereas split mode only allows a defined fraction of the injected sample to enter the column, reducing the number of molecules reaching the column and detector. We typically use splitless mode and adjust the amount of sample collected for analysis, but if split mode is preferred, the split ratio will need to be adjusted for optimal compound measurement.

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Chapter 14

Liposome Preparation by 3D-Printed Microcapillary-Based Apparatus

Orion M. Venero, Wakana Sato, Joseph M. Heili, Christopher Deich, and Katarzyna P. Adamala

Abstract

Liposomal encapsulation serves as the basis for the engineering of biomimetic and novel synthetic cells. Liposomes are normally formed using such methods as thin film rehydration (TFH), density-mediated reverse emulsion encapsulation (REE), or one of many microfluidics-based approaches—with the latter of these two methods being used mainly for the encapsulation of various lumen constituents such as cell-free protein expression reactions. Here, we describe the simultaneous formation and encapsulation of liposomes and various cell-mimetic lumen chemistries, respectively, using a 3D-printable microcapillary-based microfluidics device based off of the droplet-shooting and size-filtration (DSSF) liposome preparation method.

Key words Liposomal encapsulation, Synthetic cells, 3D-printable, Microfluidics, Giant unilamellar vesicles

1 Introduction

Synthetic cells composed of bilayer membranes act as liposomal microreactors, which enable the observation of diverse lumen chemistries and components in a biomimetic system with less background noise as compared to living cells [1, 2]. The lumen of synthetic minimal cells contains cell-free protein expression system TxTl and serves as the synthetic cytoplasm of the minimal cell [3]. In order to emulate natural cell membranes, the membrane encapsulating synthetic minimal cells is, most commonly, a unilamellar phospholipid bilayer membrane comprised of lipids and cholesterol as commonly found in natural cell membranes [4].

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The encapsulation of functional biochemical processes inside phospholipid membranes provides numerous challenges [5]. While several microfluidics-based methods exist, capable of efficiently creating, with the proper equipment and settings, unilamellar liposomes, of uniform size with good encapsulation rates, the main associated limitation is that trace amounts of carrier organic solvent remain present in the membrane [6, 7]. A popular method bypasses the problem of the presence of residual solvent by first, evaporating all solvent which creates a thin film of lipids used to prepare liposomes through subsequent methods [8]. However, most protocols which utilize this methodology of thin film preparation do not result in unilamellar, uniform liposome formulations [9, 10]. Here we present a simplified, highly efficient, method of liposome formation based off of thin film and microfluidics-based logics, which results in transcriptionally and translationally active synthetic cells.

This method can be used to encapsulate transcription and translation machinery, as it does not require high-temperature incubation for lipid formation. Similar in logic to the droplet-shooting and size-filtration (DSSF) method of liposome formation [11], this preparation described herein avoids the tier alignment and complex manufacturing issues associated with DSSF by making use of a 3D-printed chassis which greatly streamlines the liposome preparation process and, in turn, increases experiment repeatability. Additionally, this method can also be used to create liposomes encapsulating other enzymes and reaction circuits in phospholipid bilayer membranes.

2 Materials

Prepare all aqueous solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 °C). For nonpolar preparations, use glass pipettes, adhere to proper safety precautions regarding volatile agents, and follow all waste disposal regulations when disposing of waste materials.

2.1 3D-Printed Microfluidics Device

1. 3D-printed microcapillary holder (Fig. 1): SLA-printed microcapillary holder, printed in Accura 60 material. Printed in high-resolution (XY plane: $\pm 0.005''$ for the first inch, plus $\pm 0.002''$ for every inch thereafter. Z plane: $\pm 0.010''$ for the first inch, plus $\pm 0.002''$ for every inch thereafter) (*see Note 1*).
2. Glass microcapillary: 1 mm OD microcapillary with 10 μ m pulled tip.
3. Glass microcapillary scoring file: Blade-shaped medium grit ruby degussit abrasive file.
4. Conical centrifuge tube: 1.7 mL conical microcentrifuge tube with 2 cm throat length and 9 mm ID.

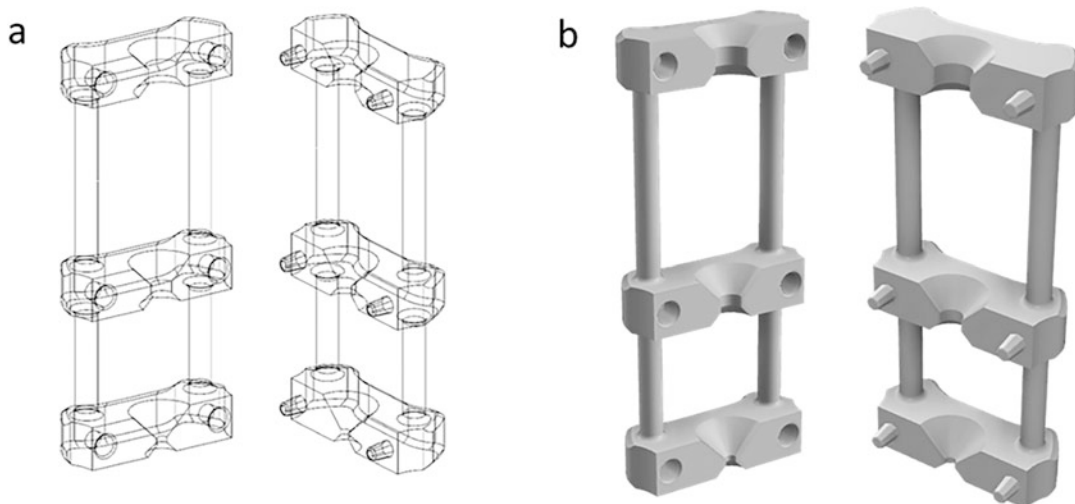


Fig. 1 The 3D-printed microcapillary holder. Microcapillary is seated within the central channel and is held in place by channel geometry. (a) Wire frame model, (b) plastic model

2.2 Lipid–Oil Solution

Lipid–oil solution used in this demonstration was: 4.925 mM POPC, 4.925 mM DOPC, and 0.15 mM fluorescently labeled PE (NBD-PE, *N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine).

Prepare a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) stock solutions by adding a nonspecific amount of each powdered lipid species to separate massed glass vials. Mass the vials to determine the amount of each lipid present within their respective vials. Dissolve the powdered lipids within the vial in 5 mL of CHCl_3 and record the measured concentrations of each solution using the observed mass of each lipid. Use these solutions of known concentrations to prepare a singular 1:1 9.85 mM DOPC/POPC solution; however, do not adjust this solution to its final volume yet.

2.3 Biomimetic Lumen Chemistries

The chemistry of lumen reactions should not include large amounts of hydrophobic molecules that could destabilize the membrane [12]. Here we present examples of two different lumen reactions: transcription as well as transcription-translation (TxTl). The Transcription reaction was visualized using Broccoli, a fluorescent RNA aptamer [13].

1. The Transcription-Translation reaction was visualized using green fluorescent protein (GFP).

The reaction was prepared as described before, using TxTl lysate of *E. coli* Rosetta DE3 strain and sonication protocol [14].

2. The reaction was prepared as previously described [15], with the final concentration of all reagents: 4 mM each NTP, 40 mM tris pH 7.9, 42 mM MgCl₂, 100 mM KCl, 2 mM spermidine, 1 mM DTT, 2 μ L 1 mM DFHBI-1T Broccoli aptamer ligand, 2 μ L 5 μ M Oligonucleotide Broccoli DNA template duplex, 2 μ L 10 \times T7 RNA polymerase, and 2 μ L 10 \times inorganic pyrophosphatase.

The Broccoli template used in this work was the sense strand: d(TAA TAC GAC TCA CTA TAG GAG ACG GTC GGG TCC AGA TAT TCG TAT CTG TCG AGT AGA GTG TGG GCT C).

3. The final concentration in the reaction mixture was 500 mM HEPES pH 8, 15 mM ATP and GTP, 9 mM CTP and UTP, 2 mg/mL of *E. coli* tRNA mixture, 0.68 mM folinic acid, 3.3 mM nicotinamide adenine dinucleotide (NAD), 2.6 mM coenzyme-A (CoA), 15 mM spermidine, 40 mM sodium oxalate, 7.5 mM cAMP, 300 mM 3-PGA, 12 mM Mg-glutamate, 140 mM K-glutamate, 1 mM DTT, 2 mM each of 20 amino acids, 10 nM GFP plasmid, RNase inhibitor Murine 40 U/ μ L 1 \times , 1 μ M T7 RNA polymerase, cell-free lysate 0.33 \times total reaction volume [16].

2.4 Liposome Preparation Buffer

1. To make liposome preparation buffer: Mix 100 mM HEPES and 900 mM Glucose, pH 7.5.
2. Add about 100 mL water to a 1-L graduated cylinder or glass beaker.
3. Weigh 23.83 g HEPES and transfer to cylinder. Weigh 162.14 g glucose and transfer to cylinder.
4. Add water to a volume of 900 mL.
5. Mix and adjust pH with HCl and NaOH, respectively. Make up to 1 L with water.
6. Filter-sterilize the buffer. Store at 4 °C.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of Lipid–Oil Solution

1. Add 0.15 mM of the desired fluorescently labeled lipid to the DOPC/POPC lipid solution (prepared in Subheading 2.2) and subsequently bring the solution up to its final volume. At this point, one should be left with a 10 mM 1:1 DOPC/POPC solution doped to 1.5 mol% with a fluorescently labeled lipid.
2. Aliquot 20 μ L (for a 1 mM lipid–oil solution) or 10 μ L (for a 0.5 mM lipid–oil solution) of this solution into amber HPLC glass vials.

3. Leave to desiccate inside a fume hood for minimum 24 h, or desiccate under gas flow (*see* **Notes 2** and **3**).
4. Add 200 μL of mineral oil to desiccated lipid films (*see* **Note 5**).
5. Sonicate the mineral the lipid–oil solution to expedite solvation. Sonicate for two periods of 30 min with intermediate high-intensity vortexing before and after each period of sonication.

3.2 Assembly of 3D-Printable Microfluidics Device

1. Test fit male and female subunits of 3D-printed microcapillary collet chassis and ensure proper subunit fit-up (*see* **Note 5**).
2. When using 1.7-mL microcentrifuge tubes with a 2-cm throat length, score microcapillaries and break to a length of approximately 24 mm using a ruby degussit abrasive file (*see* **Note 6**).
3. Carefully, as to not damage the delicate pulled tip of the microcapillary, seat the glass capillary in the central channel of the male subunit using a forceps to manipulate the microcapillary with the subunit resting on an elevated flat surface. Once the capillary is in place within the central channel, assemble the two subunits together by registering the pins of the male subunits to the ports of the female subunit (*see* **Note 7**).
4. Aliquot 30 μL of liposome preparation buffer into the bottom of a 1.7-mL microcentrifuge tube making sure that the volume ends up in the posterior terminus of the conic (*see* **Note 8**).
5. Layer 100 μL of the lipid–oil solution on top of the 30 μL of liposome preparation buffer by aliquoting the 100 μL while carefully swirling the tip of the pipette circularly around the internal circumference of the microcentrifuge tube (*see* **Note 9**).
6. Cap and let equilibrate for 30 min at STP (*see* **Note 10**).
7. Carefully insert the 3D-printed microcapillary collet into the microcentrifuge tube (Fig. 2).

3.3 Liposome Preparation

1. Taking advantage of capillary action, use a pipette to carefully fill the seated microcapillary, within the 3D-printed microfluidics device, with the desired lumen chemistry (*see* Subheading 2.3).
2. Cap assembly and subsequently centrifuge at 16,000 RCF for 3 min.
3. After centrifugation, remove the 3D-printed collet and microcapillary from the microcentrifuge tube using serrated forceps.
4. Aspirate off 29 μL of the aqueous bottom layer.
5. Carry out data collection, using method specific to the lumen chemistry.

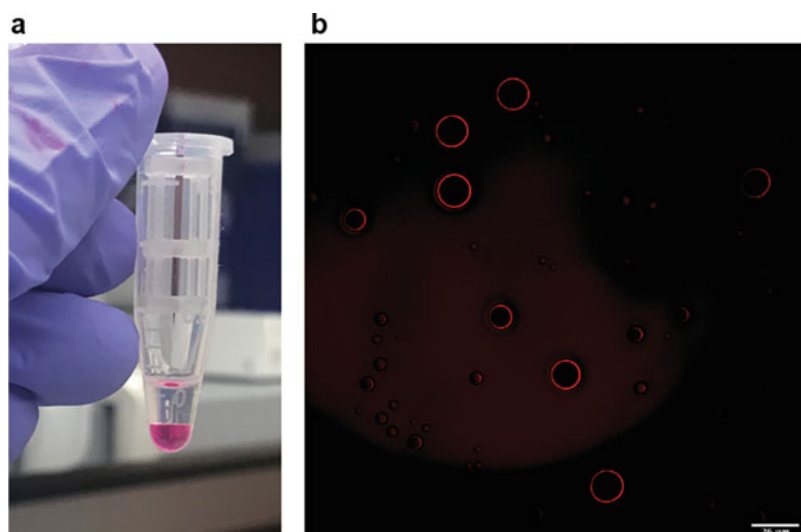


Fig. 2 (a) Assembled microfluidics device loaded with solutions for liposome based TxTL composed of 3D-printed microcapillary collet, pulled microcapillary, and 1.7 mL Eppendorf tube. (b) The liposomes with NBD-PE-labeled membrane. Scale bar is 26 μm

3.4 Example Data

1. Transcription reactions were incubated at STP (approx. 20 °C). Time-lapse video in the FITC channel was captured on a Nikon Ti-E using a 20 \times objective; video was analyzed using Nikon Elements software to automatically record the following data within a stationary FOV over the course of 100 min: mean fluorescent intensity, fluorescent intensity standard deviation, and object count. See Fig. 3 for fluorescence data and see Electronic Supplementary Video for transcription time-lapse.
2. The translation was performed at 30 °C [17]. The data was collected using a fluorescent plate reader, with an excitation of 488 nm and emission 509 nm (Fig. 4).
3. Each fluorescent measurement performed on plate reader should have internal calibration. For each set of experiments and controls, one well was filled with 1 μM fluorescein solution in water, to provide steady fluorescence calibration marker (*see Note 12*).

4 Notes

1. If you wish to use the device, we can send you an injection molded microcapillary holder for free. To receive the device, please email the corresponding author of this chapter. Please attach to the email a printable shipping label for FedEx Envelope or UPS Express Envelope. Shipping should be paid by recipient, and the address listed for the institutional affiliation

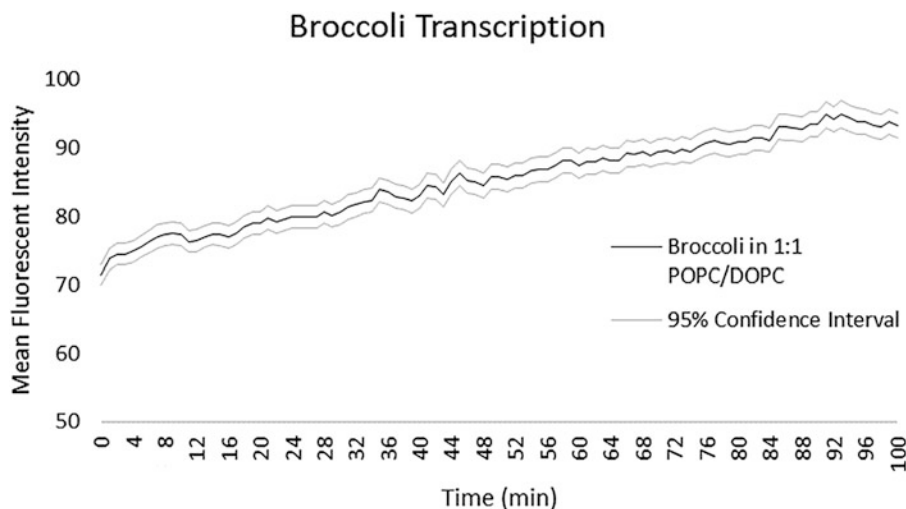


Fig. 3 Broccoli aptamer transcription inside phospholipid liposomes. The collected data was used to calculate a 95% confidence interval for Broccoli transcription via quantifying fluorescence from the transcription video. See Electronic Supplementary Video for transcription time-lapse

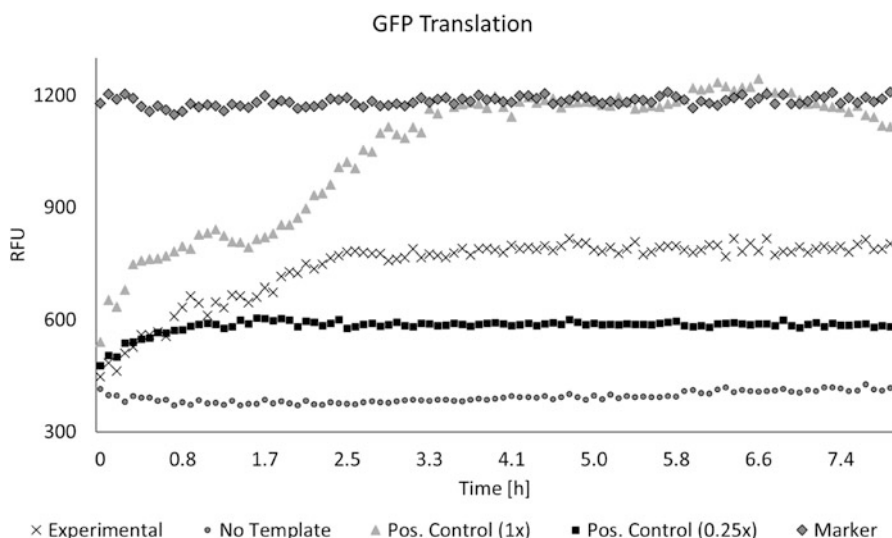


Fig. 4 The time course of TxTI GFP expression. Experimental: inside liposomes. No template: negative control, without GFP DNA plasmid [17]. Pos. Control (1 \times): the same GFP reaction in solution, without encapsulation. Pos. Control (0.25 \times): the same GFP reaction in solution, without encapsulation, at quarter volume (see Note 11). Marker: 1 μ M fluorescein solution in water (see Note 12)

in this article should be used for the sender information. Alternatively, 3D-printed parts can be ordered from Xometry (<https://www.xometry.com>) or any other 3D-printing service. STEP file is attached as supplementary information with this article.

2. Preparing lipid stock solutions can aid in the preparation of lipid solutions as it can be difficult to accurately measure and handle lipid powders due to their unguent physical properties. Additionally, working with volumes of CHCl_3 in volumes of 5 mL or more can decrease the concentration skew associated with evaporation. Moreover, a (2:1) mixture of CHCl_3 and CH_3OH may be used to prepare nonaqueous lipid solutions.
3. Glass syringes (e.g., Hamilton 1700 Series Syringes) should be used for the handling of lipid solutions dissolved in nonpolar solvents. Syringes should be appropriately cleaned and sterilized in order to prepare accurate solutions.
4. Our experimentation has shown that mixed weight mineral oil seems to be ideal for liposome preparation using this method (i.e., not light or heavy mineral oil).
5. Seating and unseating the male and female subunits can help mold the pin geometry and facilitate a proper fit-up.
6. Using your finger as a stop to gently glide the file around the circumference of the microcapillary can help to create a less jagged edge; doing this not only creates a safer edge but is itself also a safer process as it results in less microcapillary fragmentation.
7. The seating of a glass microcapillary can somewhat retract in the superior direction prior to the mating of the two subunits as once the subunits are combined the capillary can be fully seated by applying light axial pressure downwards from the superior terminus of the microcapillary.
8. A benchtop centrifuge may be used to ensure that the droplet of buffer is fully in the bottom of the microcentrifuge tube.
9. Gel loading tips can greatly aid in this step as their flexibility makes the swirling motion easier.
10. Equilibration is necessary for the oil solution to fully settle down the side of the microcentrifuge tube.
11. During encapsulation, the TxTl solution is diluted (accounting for losses from incomplete encapsulation). Also, the lumen of liposomes only comprises of fraction of the volume of the whole sample. Therefore, equal volume comparison for encapsulated and nonencapsulated TxTl solutions will not provide reliable indication of performance of the encapsulated solution. We provide $1\times$ (=liposome sample volume) and $0.25\times$ (=quarter of liposome sample volume) unencapsulated controls.
12. The fluorescein solution should remain stable over the time of the experiment. Any change in fluorescence from the marker solution indicates evaporation from incompletely sealed plate, or some other technical problem.

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Chapter 15

Microfluidic Production of Porous Polymer Cell-Mimics Capable of Gene Expression

Imre Banlaki, François-Xavier Lehr, and Henrike Niederholtmeyer

Abstract

Engineering simple, artificial models of living cells allows synthetic biologists to study cellular functions under well-controlled conditions. Reconstituting multicellular behaviors with synthetic cell-mimics is still a challenge because it requires efficient communication between individual compartments in large populations. This chapter presents a microfluidic method to produce large quantities of cell-mimics with highly porous, stable, and chemically modifiable polymer membranes that can be programmed on demand with nucleus-like DNA-hydrogel compartments for gene expression. We describe expression of genes encoded in the hydrogel compartment and communication between neighboring cell-mimics through diffusive protein signals.

Key words Artificial cells, Synthetic multicellular systems, Communication, DNA hydrogel, Cell-free transcription and translation, Microfluidics, Double emulsions

1 Introduction

Multicellular tissues and biofilms coordinate collective behaviors through chemical signals. This, in turn, enables a variety of functions that go beyond the capabilities of individual cells such as spatial organization, distribution of tasks, or synchronization. Synthetic compartments programmed with regulatory networks provide an opportunity to study these emergent properties of multicellular systems under simplified and controlled conditions [1–3]. For the assembly of synthetic multicellular systems, it is still a challenge to generate large quantities of molecularly programmed compartments and to engineer communication between individual compartments. Microfluidic methods have emerged as useful tools to generate large quantities of uniform, cell-like

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Ashty S. Karim and Michael C. Jewett (eds.), *Cell-Free Gene Expression: Methods and Protocols*, Methods in Molecular Biology, vol. 2433, https://doi.org/10.1007/978-1-0716-1998-8_15,
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compartments [4–6]. However, communication between compartments with phospholipid membranes has generally been limited to small molecule signals [1, 7, 8]. Additionally, the encapsulation of transcription and translation (TXTL) reagents in microfluidically generated compartments is still a challenge because synthesis activities degrade rapidly. Here, we present a microfluidic method to produce porous polymer cell-mimics that can be stored and filled with DNA templates for cell-free expression as needed. Cell-mimics' polymer membranes are highly permeable, stable, and can be chemically modified. Within cell-mimics, DNA templates are immobilized in a clay–DNA hydrogel. Upon adding TXTL reagents externally, cell-mimics have the ability to produce, release, and sequester protein signals [9], which we observe by fluorescence microscopy. The uniform size, density, high contrast, and circularity of cell-mimics facilitate the implementation of automatic image analysis tools. For laboratories without experience in microfluidics, it is often a challenge to implement microfluidic methods. We therefore present a detailed protocol of all the necessary steps, from fabricating microfluidic chips to demonstrating gene expression and communication in cell-mimics.

2 Materials

2.1 *Fabrication of SU-8 Patterned Silicon Wafers*

1. Silicon wafers (4" diameter, thickness: 525 ± 25 μm , Type/Dopant: P/B).
2. High-resolution film photomask.
3. 4" recycled soda lime glass plate.
4. SU-8 2015 photoresist.
5. SU-8 developer.
6. Mask aligner.
7. Spin coater.
8. Precision hotplates (2 \times).
9. Wafer handling tweezers.
10. 120-mm glass petri dish.
11. 115-mm crystallization dishes (3 \times).
12. Nitrogen gas gun.

2.2 *Production of Microfluidic PDMS Chips*

1. Polydimethylsiloxane (PDMS) (184 Silicone Elastomer Kit).
2. Patterned silicon wafer with 43 μm high SU-8 features (Fig. 1).
3. 120-mm glass petri dishes.
4. Aluminum foil.
5. Chamber oven.

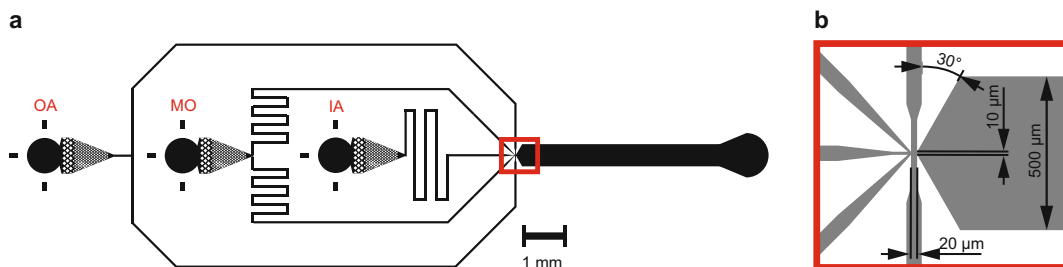


Fig. 1 Microfluidic chip design. **(a)** Outline of the microfluidic device. OA, MO, and IA indicate inlets for the outer aqueous, middle organic, and inner aqueous phases, respectively. **(b)** Magnified view of the flow focusing junction indicating important dimensions

6. Trimethylchlorosilane (TMCS).
7. Vacuum desiccator.
8. Vacuum pump.
9. Spin coater.
10. Dust removal tape.
11. Plasma system with 40 kHz generator, connected to an oxygen bottle and vacuum pump.
12. Glass slides.
13. Biopsy hole puncher, 0.5 mm (World Precision Instruments).

2.3 Polyvinyl Alcohol (PVA) Treatment

1. Inverted microscope with brightfield illumination and camera, connected to a computer.
2. Polyvinyl alcohol (PVA) (MW ~25,000, 88 mol% hydrolyzed).
3. Tygon microbore tubing, 0.51 mm ID × 1.52 mm OD.
4. 1-mL disposable plastic syringes.
5. Blunt stainless steel dispensing needles, gauge 23.
6. Chamber oven.

2.4 Generation of Double Emulsion Droplets and Polymerization

1. Glycerol.
2. Kolliphor P188.
3. Ethoxylated trimethylol-propane triacrylate, $M_n \sim 428$ (ETPTA).
4. Glycidyl methacrylate (GMA).
5. 2,2-dimethoxy-1,2-diphenylethanone (photoinitiator).
6. 1-decanol.
7. Sorbitan Monooleate (Span 80).
8. Syringe pumps (×3).
9. Light mineral oil.

10. Inverted microscope with brightfield illumination and camera connected to a computer.
11. Tygon microbore tubing, 0.51 mm ID \times 1.52 mm OD.
12. 1-mL disposable plastic syringes.
13. 100- μ L glass syringe.
14. Blunt stainless steel dispensing needles, gauge 23.
15. Weigh boat.
16. UV lamp with 365 nm LEDs (200 mW/cm²).

2.5 PEG Treatment

1. Potassium carbonate.
2. Amino-PEG12-alcohol.

2.6 Membrane Staining (Optional)

1. CF 633 amine.

2.7 DNA Loading

2. Midiprep kit.
3. Laponite[®] XLG (BYK Additives).
4. Plasmid PRS316-240xtetO (Addgene plasmid #44755) [10].
5. Plasmid pTNT-pT7-TetR-sfGFP (Addgene plasmid #140868) [9].
6. HEPES, free-acid.

2.8 Cell-Free Expression (TXTL)

1. S30 T7 High-yield protein expression system (Promega).
2. Inverted microscope with brightfield illumination, fluorescence filters according to experiment, and camera connected to a computer.
3. Hydrophobic, gas permeable membrane dish (Lumox[®] dish 35 for suspension cells, Sarstedt).
4. 18 \times 18 mm cover glass.
5. High-vacuum silicone grease.

2.9 Stock Solutions

1. PVA 50 mg/mL in water, filtered.
2. Kolliphor 188, 200 mg/mL in water.
3. 50% (v/v) glycerol in water.
4. 70% (v/v) glycerol in water.
5. 70% (v/v) ethanol in water.
6. 1 M KOH.
7. 2 M HEPES pH 8 in water, use KOH to adjust pH.
8. 200 mM K₂CO₃ pH 10 in water.
9. 500 mM amino-PEG12-alcohol in water, pH 10.
10. 5 mM CF 633 amine in water (optional).

3 Methods

3.1 Fabrication of SU-8 Patterned Silicon Wafers

1. Order a custom-printed film photomask with the chip design (Fig. 1), with a resolution to resolve a minimum feature size of 10 μm . A CAD design file of the device is available at: <https://metafluidics.org/devices/double-emulsion-chip/>. Specify polarity as darkfield (negative), emulsion down.
2. The following steps should be carried out in a clean room (*see Note 1*).
3. Preheat two hot plates to 65 and 95 $^{\circ}\text{C}$.
4. Program the spin coater to achieve a photoresist thickness of approximately 40 μm height according to the data sheet of the photoresist SU-8 2015. Set a 10 s ramp up to 1000 rpm (acceleration of 100 rpm/s), followed by 30 s at 1000 rpm.
5. Center the silicon wafer on the chuck of the spin coater.
6. Pour photoresist SU-8 2015 on the center of the wafer to cover an area of about 4 cm in diameter and avoid making air bubbles. Start the spin coater.
7. When the program is finished, place the wafer on the hot plate set to 65 $^{\circ}\text{C}$ and soft bake it for 20 min. While the wafer is baking, heat up a glass petri dish to 65 $^{\circ}\text{C}$.
8. When the soft bake time is up, remove the glass dish from the hot plate and place the wafer into the glass dish so that it can slowly cool down to room temperature.
9. While the wafer is cooling down, prepare the mask assembly. Cut the film photomask to a slightly smaller size than the glass plate. Tape the film photomask to the glass plate with the ink side facing up.
10. When the wafer has cooled down, center it on the mask assembly with the photoresist facing the film photomask. Avoid sliding it around to avoid damaging the surface. Tightly tape it in place at the corners of the glass plate (*see Note 2*).
11. Slide the mask-wafer assembly into the mask aligner and expose it with an exposure energy of 160 mJ/cm^2 according to the SU-8 2015 data sheet.
12. Place the wafer on the 95 $^{\circ}\text{C}$ hot plate for a 6 min post-exposure bake. While the wafer is baking, heat up a glass petri dish to 95 $^{\circ}\text{C}$.
13. When the post exposure bake time is up, remove the glass dish from the hot plate and place the wafer into the glass dish so that it can slowly cool down to room temperature.
14. While the wafer is cooling down, prepare three glass dishes in the solvent wet bench or fume hood. Fill two of them about 1 cm deep with SU-8 developer.

15. Place the wafer in the first developer bath for approximately 5 min. Swirl the dish gently and watch the development. When the patterns appear clearly and the unexposed photoresist has been dissolved, move the wafer to the second developer bath to wash away all residues of dissolved photoresist.
16. On top of the third empty dish, use a spray bottle filled with isopropanol to thoroughly rinse the wafer from both sides.
17. Use a nitrogen gun to dry the wafer (*see Note 3*).
18. Place the wafer in a glass dish and hard bake it for 30 min at 120 °C in an oven. Let it cool down slowly.

3.2 Production of Microfluidic PDMS Chips

1. Place the patterned silicon wafer into a box inside a fume hood.
2. Add a small cap inside the box and add a drop of TMCS into the cap. Close the box and expose the wafer to the TMCS vapor for approximately 20 min (*see Note 4*).
3. Preheat the oven to 80 °C.
4. For one wafer and 4–6 slides, mix 50 g PDMS resin with 5 g curing agent (10:1). Mix well, before placing it inside a desiccator to remove all bubbles from the resin.
5. Cut three round pieces of aluminum foil to fit inside glass petri dishes.
6. In the fume hood, take out the wafer and pack it in aluminum foil, so that the bottom is covered and a 1–2 cm ridge around the wafer is formed. Gently press down the foil on the wafer edges to form a mold that can hold the liquid PDMS.
7. Pour the PDMS resin on the wafer, until it is covered with a 0.5 cm deep layer. Avoid generating air bubbles (*see Note 5*).
8. Place the wafer in a petri dish and bake it at 80 °C for 1 h to harden the PDMS.
9. With the leftover resin, spin coat four to six clean, dust-free glass slides. Place each slide, one after the other, in the spin coater and cover it with a line of PDMS resin. Spin each slide at 1000 rpm for approximately 30 s until fully covered (*see Note 6*).
10. Place the slides on the aluminum foil in the prepared petri dishes and bake for 1 h at 80 °C.
11. When the PDMS is fully cured on the wafer, gently separate it from the master.
12. Use a scalpel to cut out the individual chips.
13. With the biopsy needle, punch out the access holes for the three inlets and one outlet.
14. Clean the surface with the features three times using tape. Cover the features with tape to protect them from dust (*see Note 7*).

15. Clean the spin-coated glass slides with tape as well. Protect the cleaned surfaces with tape until further use.
16. For plasma bonding, place the chips and a coated glass slide for each chip in the chamber of the plasma machine. The surfaces to be bonded (feature-side and PDMS coating) should face upwards. Remove the tape and start the plasma machine.
17. Adjust the oxygen pressure to 0.5 mbar and set the power to 75 W. Plasma treat the chips for 5 s.
18. Immediately after plasma treatment, carefully place chips on the PDMS of the glass slides by flipping them so that the features face down. Gently apply some pressure and remove any trapped air bubbles. Avoid too much pressure, which might collapse the channels.
19. Bake the chips in the oven at 120 °C for 1 h to bond (*see Note 8*).

3.3 PVA Treatment

The PVA treatment is a critical step in the fabrication of functional chips (*see Note 9* and Electronic Supplementary Video) [4, 11].

1. Prepare your microscope station to have a vacuum pump and a source of compressed air with a pressure regulator.
2. Make three 90° angled metal pins, by bending blunt needle inserts with two pliers.
3. Connect a line of Tygon tubing to the vacuum pump so that it can reach the microscope station. Insert an angled metal pin to later connect to the chip.
4. Connect two lines of Tygon tubing to the pressure regulator, for example, through a manifold or a Y-fitting. Both lines should be long enough to reach the microscope station. Insert 90° angled metal pins for connection to the chip.
5. Following the post-plasma bake, check chips under the microscope. Discard chips with dust particles trapped in channels or if channels are not fully bonded.
6. Set the air pressure to 3 psi (0.2 bar) and check that compressed air is flowing from the two connected Tygon tubing lines.
7. Connect the pressurized air lines to the inner (IA) and middle (MO) inlet (Fig. 1). Also connect a piece of Tygon tubing to the outlet via a metal pin.
8. Place the end of the outlet tube in a glass flask to collect waste. Make sure that the flask is below the microscope, so flow is not hindered by gravity.
9. Center the microscope on the junction.
10. Connect a 15–20 cm Tygon tube to a 1-mL syringe and insert an angled metal pin at the end.

11. Use the syringe to fill the first 7–10 cm of Tygon tubing with PVA solution (*see Note 10*).
12. Connect the PVA-filled tubing to the outer inlet (OA) of the chip (*see Note 11*).
13. Disconnect the syringe from the tubing. Quickly pull back the plunger halfway. Reconnect the syringe to the tubing.
14. Hold the syringe in a way that you can comfortably move the plunger back and forth. See Electronic Supplementary Video.
15. Start increasing the pressure by gently pushing the plunger until you see the PVA solution appearing at the junction and flowing towards the outlet (*see Note 12*).
16. Further increase the pressure to bring the two fluid streams closer to each other (Fig. 2a, **step 2**).
17. Give the liquid some time to close the gap in between and use short pumps of pressure to connect the two streams in the center (*see Note 13*).
18. After the streams have joined and bubbles start forming (Fig. 2a, **step 3**), decrease the pressure (*see Note 14*).
19. Check the tubing for the flow rate and PVA solution left. Check in the microscope for the bubble patterns forming four or three parallel streams. Check whether the whole channel is being coated (*see Note 15*).
20. After ~30 s, or before the PVA solution runs out, start to decrease pressure by moving the plunger back in small steps.

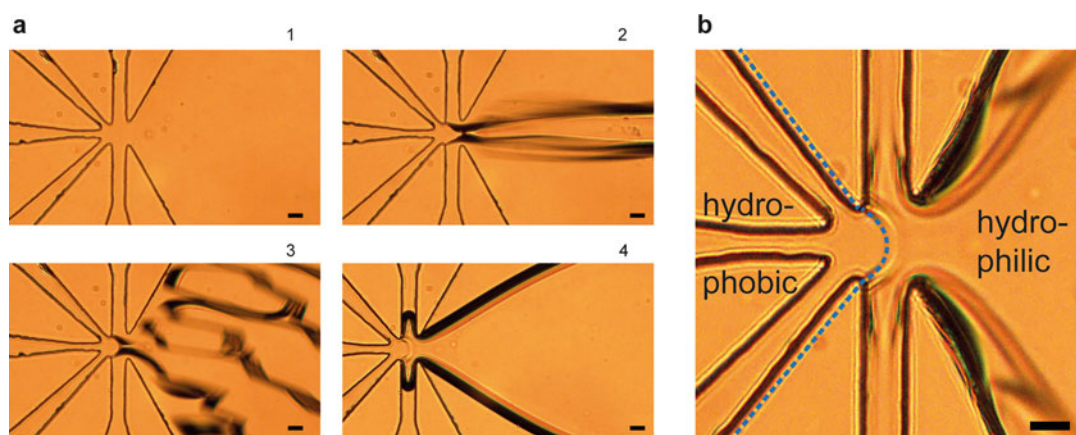


Fig. 2 PVA treatment of microfluidic chips. **(a) Step 1:** Junction of chip before PVA treatment. **Step 2:** Two streams of PVA solution flow from the OA inlet towards the outlet, barely touching. **Step 3:** The merged PVA streams form bubbles of pressurized air flowing in through the MO and IA channels. At the junction, an air pocket forms, creating a crescent PVA wetting. **Step 4:** The PVA solution is aspirated back, clearing the narrow tapering of the OA channel. **(b)** PVA-treated junction with crescent coating of PVA. The part of the junction to the left of the dotted line remains hydrophobic, while the right side is hydrophilic. Scale bars: 20 μm

21. Observe the change in bubble formation. Once single-lane, large, oscillating bubbles are formed, give it a few seconds to run-off excess PVA.
22. Now start to pull back the plunger in slightly larger steps, until the two streams oscillate between touching and disconnecting (*see Note 16*).
23. If you observe this oscillation, move the plunger back in even larger steps to clear the tapering at the junction (Fig. 2a, **step 4**; *see Note 17*). Keep moving the plunger back all the way in constant steps until all the PVA solution has disappeared from the junction (Fig. 2b).
24. Quickly disconnect the PVA tubing from the OA inlet and the outlet. Leave the compressed air lines connected. Attach vacuum to the OA inlet to remove any residual liquid.
25. After 10–15 s, change the vacuum to the outlet and wait another 10–15 s. Some bubbles will remain at the inlet and outlet.
26. Mark the chip as PVA coated and bake it overnight at 120 °C (*see Note 18*).

3.4 Generation of Double Emulsion Droplets and Polymerization

1. Prepare the outer aqueous phase: 600 μL 50% (v/v) glycerol, 250 μL Kolliphor P188 solution, and 1150 μL of water (*see Note 19*).
2. Prepare the middle organic phase: 480 μL of GMA, 320 μL of ETPTA, 200 μL of 1-decanol, 2.5 μL of Span 80, and 20 mg of 2,2-dimethoxy-1,2-diphenylethanone (for 2% w/v) (*see Note 19*).
3. Prepare the inner aqueous phase: 95 μL of 70% (v/v) glycerol and 5 μL of Kolliphor P 188 (*see Note 19*).
4. Set up the microscope with a PVA-coated chip. Connect the outlet to the waste. Prepare a second tube to swap with the outlet and collect droplets.
5. Set the syringe pumps next to the microscope and mark them “IA,” “MO,” and “OA,” for the inner aqueous, middle organic, and outer aqueous phases.
6. Set the flow rate adjustment range to correspond to the expected flow rates. Between 4 and 11 $\mu\text{L}/\text{h}$ for the inner, 20 and 60 $\mu\text{L}/\text{h}$ for the middle, and 300 and 370 $\mu\text{L}/\text{h}$ for the outer phase (*see Note 20*).
7. Fill the 100- μL glass syringe with mineral oil. Prepare a piece of Tygon tubing with a straight metal pin at one end and a blunt dispensing needle at the other end. Fill the tubing and the connected needle with mineral oil. Connect the syringe with the oil-filled tubing. Avoid trapping air bubbles in the syringe and the line of tubing (*see Note 21*).

8. Place the oil-filled 100- μ L syringe in the syringe pump for the inner phase.
9. Turn on the pump and let it run until some mineral oil is pushed out of the straight metal pin.
10. Prepare three-angled metal pins and connect them to two approximately 15 cm long and to one 5–10 cm long pieces of Tygon tubing.
11. Take two 1-mL syringes connected to blunt dispensing needles and fill one with the outer and one with the middle phase.
12. Turn them upside, and flick all the bubbles into the tip with your fingers. Use a tissue and push out the bubbles collected in the tip.
13. Connect the two syringes to the longer tubing. Push out the air in the tubing and place them in their respective syringe pumps. Let the pumps run until fluid is pushed out of the metal pins (*see Note 22*).
14. Pipet between 50 and 100 μ L of inner phase into the cap of a microcentrifuge tube. Using a syringe, fill the front section of the shorter piece of Tygon tubing with the inner phase and avoid bubbles (*see Note 23*).
15. Quickly insert the pin of the Tygon tubing into the inner phase inlet of the chip (*see Note 24*).
16. Run the inner phase syringe pump again, to make sure the mineral oil did not recede. Turn the pump off again.
17. Make out the end of the inner phase in the tube connected to the chip. Cut the tube 1–2 mm above, so no air is left in the tube. Connect the straight pin from the inner phase syringe pump to the inner phase tube already connected to the chip.
18. Now run the middle and outer phase pumps again, to make sure the fluids are at the front of the tubes. Turn the pumps off again.
19. Connect the tubes, via pin, to the chip inlets for the middle and outer phase, respectively.
20. Set initial flow rates for inner phase to 30–50 μ L/h, middle phase to 100–200 μ L/h, and outer phase to 300–600 μ L/h to purge the air in the chip efficiently. Start the pumps simultaneously.
21. Observe in the microscope. Once a phase has flushed the chip, reduce its flow rate (*see Note 25*).
22. Adjust the flow rate to the expected range for double emulsification: 4–11 μ L/h for the inner, 20–60 μ L/h for the middle, and 300–370 μ L/h for the outer phase.
23. Wait for the system to equilibrate and observe the changes (*see Note 26*).

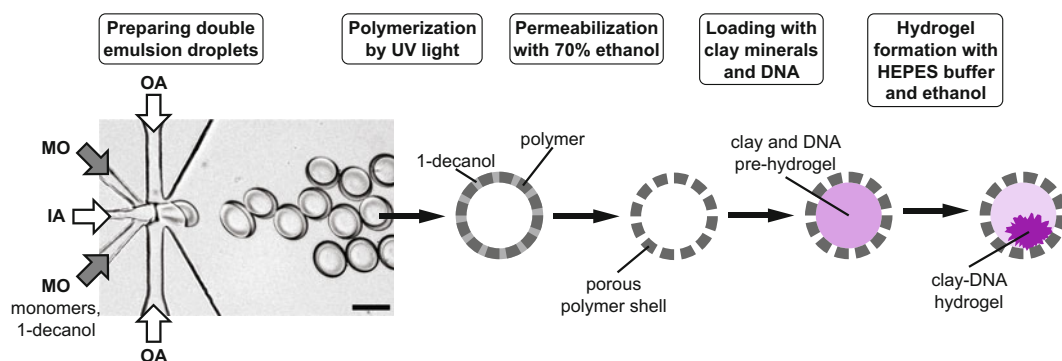


Fig. 3 Overview of the production of cell-mimics. Water-in-oil-in-water double emulsion droplets are produced microfluidically and have an organic shell containing the acrylate monomers that will form the cell-mimics' polymer shells. Double emulsion droplets are collected and treated with UV-light to form porous polymer membrane shells by phase separation of 1-decanol [9, 13]. Polymer membrane shells are permeabilized with ethanol that removes 1-decanol from the pores of the membrane. Microcapsule shells are then sequentially loaded with clay minerals and with DNA templates. Formation of the clay–DNA hydrogel nucleus is induced by the sequential addition of HEPES buffer and ethanol. Scale bar: 50 μm

24. Droplet production should transform from a jetting regime to a dripping regime for the middle phase (*see Note 27*).
25. Adjusting the outer phase will change overall flow velocity and size of the middle phase droplets (*see Note 28*).
26. Adjusting the middle phase will change the size of middle phase droplets and shell thickness (*see Note 29*).
27. Adjusting the inner phase will change shell thickness and filling of the middle phase droplets (*see Note 30*).
28. Find a stable double emulsification regime, with the desired size and shell thickness of the droplets (Fig. 3).
29. When a setting is found, exchange the outlet tube with the prepared collection tube and start collecting droplets into a microcentrifuge tube.
30. When enough droplets have been collected, use the leftover outer phase or water to dilute the collection ($\sim 1:1$). Carefully resuspend the double emulsion droplets that have collected on the top and pipet the emulsion on a weigh boat so that a thin liquid layer is formed.
31. Directly illuminate the droplets from the top for 30 s with the UV lamp at an exposure energy of $200 \text{ mW}/\text{cm}^2$.
32. Add pure ethanol to create a solution of $\sim 70\%$ (v/v) ethanol (*see Note 31*).
33. Recover the suspension of polymerized microcapsule shells into a microcentrifuge tube.
34. Place at -20°C for further use.

3.5 PEG Treatment

1. Take the desired amount of empty shells and wash them with 200 mM K_2CO_3 at pH 10 by centrifugation at $1500 \times g$. Remove the entire supernatant to leave a dense pellet of microcapsules on the bottom of the tube.
2. Resuspend them in 1:1 ethanol and 500 mM PEG in water. A small amount of treatment solution is sufficient (e.g., 5 μ L of ethanol +5 μ L of PEG for a small pellet).
3. Carefully flick the tube, to bring all shells in contact with the PEG solution but avoid spreading them on the walls of the tube where they are not in contact with the solution (*see Note 32*).
4. Place at 37 °C overnight.
5. Wash the pellet with water for immediate use or store in 70% (v/v) ethanol at –20 °C.

3.6 Membrane Staining (Optional)

1. During PEG treatment, add 1 μ L of 5 mM CF 633 to 10 μ L of PEG treatment solution.
2. Incubate at 37 °C overnight.
3. Wash 5–10 times with 30 μ L water to remove unbound dye (*see Note 33*). Use immediately or store in 70% (v/v) ethanol at –20 °C.

3.7 DNA Loading

1. Prepare plasmid DNA templates for cell-free gene expression within cell-mimics. Plasmid DNA needs to be at a high purity, high concentration, and dissolved in water.
2. To prepare 10 mL 2% (w/v) clay mineral dispersion, pipet 10 mL of water in a glass vial and place it on a magnet stirrer at high rpm, so it makes a vortex.
3. Weigh 200 mg of Laponite[®] XLG clay powder.
4. Slowly add the clay powder into the water vortex. If the solution turns turbid, stop and wait for it to clear up before adding more (*see Note 34*).
5. Leave the liquid to stir until the solution turns completely clear again, approximately for 20 min. The clay dispersion can be used for up to 2 days and should be discarded when it starts gelling or after 48 h.
6. Take some empty microcapsule shells and wash them three times with 30 μ L of water by centrifugation at $1500 \times g$ (*see Note 35*).
7. Resuspend the pellet in a solution of 4 μ L of 2% (w/v) clay mineral dispersion and 10 μ L of water.
8. Incubate overnight at room temperature.
9. Remove the supernatant and wash once with water (*see Note 36*).

10. Resuspend the shells in 14 μL of aqueous solution containing plasmid DNA templates for cell-free expression (e.g., 57 nM PRS316-240xtetO and 140 nM pTNT-pT7-TetR-sfGFP). Incubate for 5 min (*see Note 37*).
11. Remove the supernatant and resuspend in 30 μL of 100 mM HEPES buffer. Incubate for 10 min (*see Note 38*).
12. Remove the supernatant and resuspend in 30 μL of 70% (v/v) ethanol containing 100 mM HEPES buffer. Incubate for 10 min (*see Note 39*).
13. Wash the cell-mimics three times with 30 μL of 100 mM HEPES buffer.

3.8 Cell-Free Expression (TXTL)

1. Preheat the microscope to 37 °C.
2. Prepare a Lumox[®] dish (*see Note 40*). Place a vacuum grease border, a bit smaller than the size of a cover glass, in the middle of the membrane.
3. Work on ice. Prepare tubes for each reaction and for the TXTL master mix.
4. Calculate a master mix for $(n + 1)$ 5- μL reactions consisting of T7 S30 extract, premix, water, and 1 μL of cell-mimics per reaction (not added in the master mix).
5. Spin down the S30 T7 Extract for 1 min at $17,000 \times g$ (*see Note 41*).
6. Mix the TXTL master mix.
7. Add 1 μL of cell-mimics in each reaction tube.
8. Top the cell-mimics with 4 μL of TXTL master mix. Mix gently.
9. Spot 4 μL of each reaction (up to five reactions per dish) inside the grease border on the Lumox dish.
10. Protect the reactions from evaporation by covering them with a cover glass on top of the grease border. Gently press down the cover glass until sealed and all droplets make contact with the glass.
11. Place in the microscope and image the samples at 37 °C for 3 h using the appropriate fluorescence channels according to the experiment (Fig. 4).

3.9 Automatic Image Analysis

1. Open the script (*see Note 42*) in MATLAB[®] (2018b or newer versions). The script is available at <https://github.com/HN-lab/cell-mimics->.
2. Replace the following variables: (1) “Path” with the address location of the image stack file to analyze. (2) “Frame_to_analyze” with the most suitable frame for segmentation (the brighter the better). You may use the brightfield or

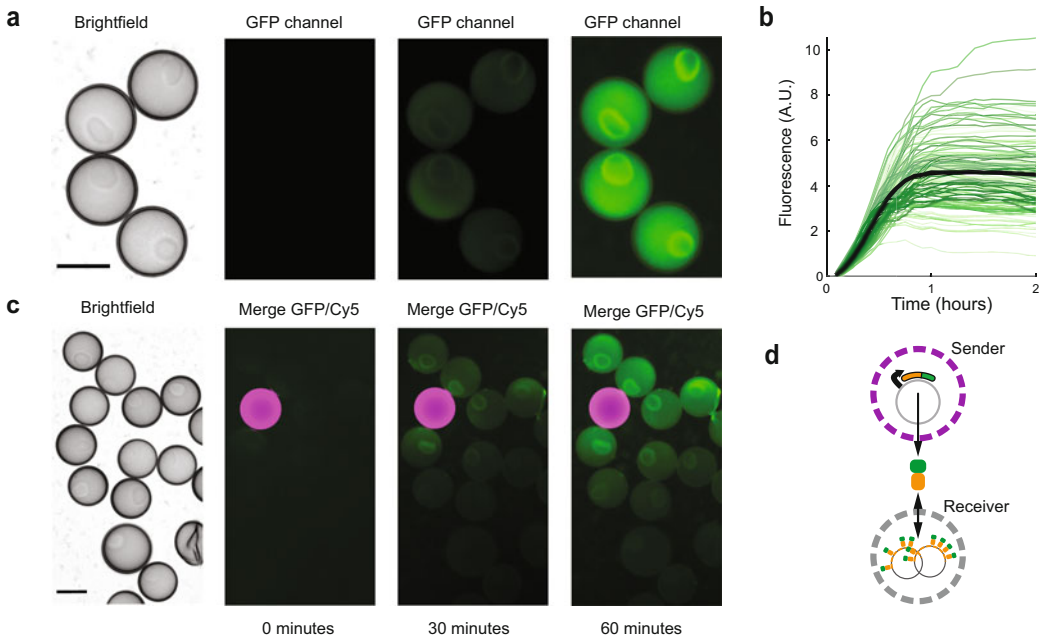


Fig. 4 Gene expression in cell-mimics in time-lapse microscopy experiments. **(a)** Images of brightfield and GFP channels over time with cell-mimics expressing TetR::sfGFP in presence of tetO array. **(b)** Fluorescence time traces of cell-mimics co-expressing TetR::sfGFP in the presence of the tetO array ($n = 131$ segmented cell-mimics, average shown in bold). **(c)** Cropped images of brightfield and merged GFP and Cy5 channels at different time points with two genetically distinct populations of cell-mimics. The cell-mimics stained with CF 633 (magenta) express TetR::sfGFP while the nonstained population contains the tetO array plasmid. Scale bars: 40 μM

fluorescence channels according to your microscope setup or image quality (*see Note 43* for details about the implementation methods).

3. Run the script. A panel of processed images will appear and show a succession of morphological functions applied to the raw image (*see Note 44*). You may need to tune the morphological structural elements (“se” and “se2”) according to your image size and quality.
4. You can now write the title of the processed image you want to use in the displayed dialog box. (e.g., “contrast-1” for the first enhancement) (*see Note 44*).
5. The script will ask you to draw a circle on the last displayed image. Select a region corresponding to the background. This will automatically subtract the background fluorescence from the cell-mimics fluorescence for every frame.
6. You can now observe the resulting segmented cell-mimics which will be analyzed in the next step (*see Note 45*).

7. You should now be able to observe the displayed fluorescence traces (Fig. 4b). The resulting time-course fluorescence data is stored in the variable “Full_data” and can be saved or exported for further analysis.

4 Notes

1. If no clean room facility is available, custom-made SU-8 patterned silicon wafers can be obtained commercially from different companies.
2. The mask aligner we use can only hold 3" wafers on the wafer chuck. We prefer to fabricate silicon wafers with a 4" diameter to be able to produce more chips at the same time. To do so, we have to tape the wafer onto the mask in order to place it in the mask aligner. We found that the quality of the molds we generate this way is sufficient. If a different mask aligner is used that can process larger substrates, the wafer can be placed on the wafer chuck for alignment and making contact with the mask.
3. If white residue remains on the wafer after drying it, briefly place it back into the second developer bath and repeat the isopropanol rinse and drying.
4. TMCS treatment makes the wafer surface hydrophobic to facilitate peeling off the cured PDMS. The treatment should be repeated each time before preparing PDMS chips. TMCS is toxic. TMCS treatment should be carried out in a fume hood.
5. If some bubbles form, place the mold in the desiccator to remove them.
6. To fully coat the slides, pour the resin along an imagined middle line so half of the slide area is covered with PDMS.
7. Use some pressure with your fingers, to clean the inside of the channels as well.
8. The baking of the chips covalently bonds the PDMS chip to the PDMS coated slide. Baking also partially removes the hydrophilic silanol surface created by the plasma. This process is time and temperature dependent as it is caused by the diffusion of polymer chains. Different baking times will change the wettability of PVA solution or other fluids. Equilibrium is generally reached after 1 day at room temperature.
9. PVA treatment generates a hydrophilic surface in a defined area of the chip, downstream of the flow focusing junction. PVA treatment is a critical step and requires some practice. If there are problems with double emulsion generation, it is likely that the PVA treatment was not done correctly. See Electronic Supplementary Video for a video of a successful PVA treatment.

10. Pay attention not to create any bubbles. Also, do not move the PVA solution back and forth in the tube as it changes the flow behavior. Use a new, dry piece of Tygon tubing for each attempt. You can reuse the pin and syringe after removing the residual solution.
11. By holding the syringe above the pin, you prevent the PVA solution to creep back in the tube, creating bubbles.
12. During PVA treatment, always watch the fluidic junction and the flow of the PVA solution with the microscope. Be careful when increasing the pressure to join the streams of PVA solution and avoid using too much pressure. If at any point in the PVA treatment the PVA solution comes in contact with the upstream side of the flow focusing junction, discard the chip. The upstream side of the junction has to stay hydrophobic or the production of double emulsions will be impossible.
13. During this phase, it may appear like some PVA is building up between the two streams. Gently apply short pumps of pressure to dissolve this build up and close the gap. Ideally, there is a wide crescent of PVA formed within the junction after the lanes merge (Fig. 2a, **step 3**; Fig. 2b). Increasing the pressure will shrink this crescent.
14. To reduce pressure, you may even reverse the plunger a few millimeters. The bubbles form lanes depending on the flow and pressure. The higher the pressure, the more lanes with smaller bubbles are formed. Ideally there are three or four lanes of bubbles at this step.
15. You should have enough PVA solution left in the tube. To prevent running out, you may reduce pressure and flow, giving you more time.
16. Keep this step brief as it can clog up the channels with PVA.
17. At this step, you have to be decisive. See the Electronic Supplementary Video. If not all PVA is sucked out of the tight section, it will clog up. If that happens, a careful retreatment of the chip may save it. Be sure to dissolve the clogs patiently, as too much pressure will splash the whole junction with PVA. After the lanes are cleared, you can reduce suction to keep up a steady backflow. If you arrive at the end of the syringe and some PVA is still left in the large channel, quickly disconnect the inlet and outlet and move to the next step.
18. Baking it at 120 °C assures efficient bonding of PVA to silanol groups. We found that baking at 80 °C is not sufficient to create a functional chip.
19. The outer, middle, and inner phase can be stored in the fridge and used for up to a month. We store the monomer mix without photoinitiator. When photoinitiator is added to the monomer mix, protect it from light.

20. Make sure to adjust the pumps to the correct syringe diameter.
21. After the first time, refilling can be done by slowly aspirating oil from a microcentrifuge tube through the connector pin. Make sure to prevent bubbles because air pockets function as pressure dampeners, preventing exact flow control.
22. To make this process quick, you may run the pumps on the highest setting, but make sure to reverse them to slower speed before connecting them to the chip.
23. Having bubbles in any syringe or tubing will not only prevent accurate control of flows but air in the system will disrupt droplet production. This is unavoidable while starting the experiment and filling the chip initially but should not happen during droplet production.
24. Hold the syringe above the pin, while you connect it, to prevent the liquid creeping back towards the syringe in the tube. Once it is connected, you can place the syringe next to the chip while setting up the connection to the pump.
25. At the start, there is still air in the chip, which will create a bit of a mess. This is not a problem. However, make sure the flow rates are such that no phase is pushed back in a channel supposed to run another phase. This primarily happens with the MO being pushed into the IA channel since that is the weakest flow. This can be caused by either a large flow disparity between the two or by a too strong flow of the OA phase.
26. At high flow rates, all phases are jetting. That means, the OA will form two outer lanes along the channel, encompassing a single lane of MO phase. Depending on flow, the MO may break up closer or further from the junction. Similarly, the IA phase streams inside the MO phase, breaking up into droplets. Reducing the flow rates should destabilize the stream of MO phase, if the PVA treatment was successful. If for some reason the IA phase curves and connects to the OA phase within the junction, try increasing the IA flow to straighten it out, before slowly decreasing it again.
27. Further flow reduction of MO should create large MO droplets encapsulating one or several IA droplets. IA flow influences size and frequency of IA droplets.
28. A high OA flow decreases the MO droplet size and increases overall flow velocity. It may be used to decrease shell thickness.
29. Increasing the MO flow rate increases the size of the droplets. If the IA flow remains constant, it will thicken the organic shell of the double emulsion. While thin shells are preferred, a too thin shell will destabilize encapsulation.
30. The flow rate of the IA phase can be used to increase overall size and shell thickness of the droplets.

31. The ethanol will dissolve the 1-decanol and open up the pores of the now polymerized shells (Fig. 3).
32. Depending on the liquid the shells are in, they become more or less sticky. Mix them gently.
33. The dye also stains the clay hydrogel so thorough washing is essential before further use.
34. Sometimes some aggregates are formed when adding the last bit of clay powder. These should dissolve without issues. Use a dry glass to prepare the solution. Isolated water drops may accumulate clay particles while the powder is being added and create clumps.
35. In water, the shells are particularly sticky. Spin them very briefly multiple times, rotating the tube between spins, to collect a dense pellet at the tube bottom.
36. Without washing, there will be many clay fragments left outside the shells after gelling. However, keep the washing step brief to avoid losing clay minerals from inside the cell-mimics.
37. To create genetically distinct sender and receiver cell-mimics, fill one batch with PRS316-240xtetO and a second batch with pTNT-pT7-TetR-sfGFP plasmid. Optionally, stained shells may be used for the senders (Fig. 4c).
38. Electrolytes gel the clay minerals within the shells, immobilizing the added plasmids inside cell-mimics (Fig. 3) [9, 12].
39. The addition of ethanol collapses the loose hydrogel into a nucleus-like structure (Fig. 3).
40. Performing cell-free expression and imaging on gas permeable membranes such as Lumox[®] dishes ensures that fluorescence will be evenly distributed in the sample.
41. Spinning the S30 T7 extract removes particles but does not decrease protein production.
42. The script only performs segmentation and does not include a dynamic tracking procedure since we did not observe significant movement of the cell-mimics during the time-course experiments (likely due to the high density of the cell-mimics). However, image registration is necessary in case of sample drifting.
43. The segmentation method is based on the circle Hough transform since the high level of circularity and contrast of the cell-mimics make them perfect candidates for this simple detection method. Brightfield images with contrast enhancement can often be good enough to obtain a high percentage of correctly segmented cell-mimics.
44. If samples are extremely dense or a highly contrasted bright-field image is not available, the fluorescence channel can be

used. In this case, using one of the processed images treated with the morphological functions is often necessary to improve segmentation accuracy.

45. If you need to remove some of the segmented cell-mimics, add the corresponding label numbers in the variable “mimics_not_segmented” (list) and rerun the script.

Acknowledgments

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Cell-Free Membrane Protein Expression into Hybrid Lipid/Polymer Vesicles

Miranda L. Jacobs and Neha P. Kamat

Abstract

Hybrid membranes comprised of diblock copolymers, and phospholipids have gained interest due to their unique properties that result from blending natural and synthetic components. The integration of membrane proteins into these synthetic membranes is an important step towards creating biomembrane systems for uses such as artificial cellular systems, biosensors, and drug delivery vehicles. Here, we outline a technique to create hybrid membranes composed of phospholipids and diblock copolymers. Next, we describe how membrane proteins can be co-translationally integrated into hybrid lipid/polymer membranes using a cell-free reaction. We then outline a method to monitor insertion and folding of a membrane-embedded channel protein into the hybrid membrane using a fluorescent-protein reporter and dye release assay, respectively. This method is expected to be applicable for a wide range of membrane proteins that do not require chaperones for co-translational integration into vesicles and provides a generalized protocol for expressing a membrane protein into a membrane mimetic.

Key words Membrane protein, Vesicles, Cell-free protein synthesis, Diblock copolymer, Hybrid membranes

1 Introduction

Hybrid-membrane vesicles, self-assembled from phospholipids and diblock copolymers, display an array of unique properties. These properties can include increased stability of the vesicle and enhanced and directed integration of cell-free-expressed membrane proteins [1–3]. Together, these properties should enhance the performance of biosensors, drug delivery vehicles, as well as artificial cellular systems [4–10]. While membranes serve as a critical scaffold to incorporate a variety of amphiphiles and proteins, the membrane proteins themselves allow a range of functionalities to be introduced through behaviors such as the transport of specific molecules, inducible permeability, and enzymatic activity [11].

To add to the functionalities imparted by proteins to membrane systems, nonnatural molecules such as diblock copolymers have recently gained interest as a membrane mimetic. These synthetic molecules offer increased stability and chemical flexibility compared to natural lipids [3, 12–15]. Diblock copolymers can be blended with phospholipids to form hybrid membranes that display both the biocompatibility of a lipid membrane and the increased stability of a polymer membrane [16, 17]. Recently, diblock copolymers have also been shown to impart unique physical properties to phospholipid membranes that, in turn, enhance the integration of certain channel proteins [1].

Cell-free protein expression enables the co-translational integration of membrane proteins into synthetic membrane mimetics such as synthetic vesicles. Unlike traditional protein purification and reconstitution techniques required to integrate membrane proteins into vesicles, this system requires limited equipment and is faster and less labor-intensive than traditional expression and purification techniques. The cell-free expression of membrane proteins has the additional benefits of reduced aggregation of proteins and decreased cytotoxicity that results when the desired protein is made within live cells prior to purification [18]. The PURExpress system for *in vitro* protein expression is particularly useful as it is commercially available and is extremely user-friendly [19]. In addition, the PURExpress system lacks proteases, which allows for the production of proteins that would typically be signaled for degradation in a cellular context [20]. Cell-free expression systems offer ease-of-use and have the ability to overcome many challenges in membrane protein production.

Traditionally, reconstitution-based techniques to integrate membrane proteins into synthetic vesicles require detergent solubilization of both proteins and lipids followed by reconstitution and removal of excess detergent. While methods using detergent-based reconstitution are more commonly used to integrate membrane proteins, complete detergent removal after protein insertion into vesicles is not possible and residual detergent in the membrane would affect membrane physical properties and subsequently may interfere with protein activity and membrane stability [21–23]. Similarly, the cell-free expression of membrane proteins in cell-free systems made from crude cellular extract is subject to lipid contamination from residual cellular lipids. However, the PURExpress system lacks significant lipid contamination. This purity allows the user to better control the composition of membrane mimetics to which they will co-translationally integrate membrane proteins, ultimately providing more control over experimental studies of protein functionality assays.

An added benefit to using cell-free protein expression techniques to integrate membrane proteins into synthetic vesicles is the detection of proteins as they are being produced. Green fluorescent

protein (GFP) can be used as a protein tag for the observation of protein folding in a cell-free reaction [24, 25]. In this method, we outline the use of green fluorescent protein as a C-terminal tag on a membrane protein of interest, the mechanosensitive channel of large conductance (MscL). This tag enables the detection of protein production and folding through a fluorescent readout [1, 24] and could be included as a chimera on a wide variety of proteins to detect protein expression and folding during the reaction.

Here, we outline a protocol for the formation of these hybrid membranes that can be used for any application. Cell-free membrane protein expression into polymer or lipid vesicles has been used to reconstitute a variety of membrane proteins. However, many of the applications of such systems have yet to be realized. This protocol outlines a streamlined method to insert membrane proteins into hybrid lipid/polymer membranes and observe protein production, while the reaction is taking place. In addition, we outline a method to demonstrate the functional folding of a channel protein by observation of dye transport through an open channel. This method can be applied to the formation of hybrid vesicles for any application and can be used to assess the expression of membrane proteins into any membrane mimetic. Together, these methods should help scientists rapidly assess the assembly of hybrid vesicle membranes containing integral membrane proteins for a variety of subsequent applications.

2 Materials

All organic solvents should be handled in a fume hood using glass syringes and containers.

2.1 Hybrid Vesicle Preparation

1. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (786.1 g/mol): 50 mg/mL (63.6 mM) in HPLC-grade chloroform (*see Note 1*).
2. Poly(1,2-butadiene)-b-poly(ethyleneoxide) (PEO₁₄-b-PBD₂₂) (1800 g/mol): 40 mM in HPLC-grade methylene chloride (*see Note 2*).
3. 20-mL glass vial.
4. Nitrogen gas.
5. Vacuum oven (*see Note 3*).
6. Calcein: 100 mM Calcein in ultrapure water (*see Note 4*).
7. 60 °C oven.
8. Vortex.
9. Miniextruder set: 2 O-rings, 2 gastight syringes, heat block, and filter supports.
10. Polycarbonate membrane filters: 100 nm pore size.

2.2 Vesicle Purification

1. Gravity flow purification column: Chromatography column packed with 4 mL Sepharose 4B resin, hydrated in water (*see Note 5*).
2. 96-well plates.
3. Fraction collector, compatible with 96-well plates (*see Note 6*).

2.3 Cell-Free Expression of a GFP-Membrane Protein into Hybrid Vesicles

1. Membrane protein expression plasmid: Any bacterial expression plasmid containing a T7 promoter and membrane protein of interest, 200 ng/ μ L in ultrapure water (*see Note 7*). A C-terminal GFP fusion may be included for the observation of protein expression (*see Note 8*).
2. PURExpress kit (*see Note 9*).
3. Microplate reader with fluorescence and kinetic scan capabilities.
4. Glass-bottom 384-well plate.
5. Plate sealers.

2.4 Assessment of Membrane Protein Activity

1. Osmometer.
2. 800 mOsm PBS: Phosphate-buffered saline (PBS tablets) dissolved to 800 mOsm (*see Note 10*).
3. MTSET solution: (2-(Trimethylammonium)ethyl methanethiosulfonate bromide) dissolved in ultrapure water to 21 mM.
4. Glass-bottom 96-well plate.
5. Triton-X 100: 10% by volume in ultrapure water.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Hybrid Vesicle Preparation

1. Using a glass syringe (*see Note 11*), mix 369.2 μ L of DOPC stock (63.6 mM) and 65 μ L of PEO-b-PBD stock (40 mM) (*see Note 12*) into a large (~20 mL) glass vial. Gently swirl the vial to mix. This will later equate to vesicles with 26.09 mM total amphiphile concentration in 1 mL (90 mol% DOPC, 10 mol% PEO-b-PBD) (*see Note 13*).
2. Dry chloroform from the sample by tilting at a 45° angle and slowly rotating the vial under a nitrogen stream until no chloroform is visible. An even, thin film should be deposited on the wall of the vial (*see Note 14*).
3. Remove residual chloroform by incubating the sample in a vacuum oven at -0.8 Pa for >4 h at room temperature.

4. Add 1 mL of ultrapure water to the dried film, being careful not to disturb the film. Cap tightly to limit evaporation of water and incubate at 60 °C overnight (*see* **Note 15**).
5. Vortex hydrated films until homogenous (5–10 s).
6. Assemble the minixtruder [26]. Hydrate filter supports in water and place on either side of the open mini extruder inside the O-ring. Place a 100 nm polycarbonate filter over one side of the extruder O-ring and filter support. Close the extruder with the O-rings facing each other, separated by the filter. Hydrate the extruder cavity by filling one of the extruder syringes with water and inserting on the right side of the extruder. Use a kimwipe to catch the water on the left port and push the water through the extruder.
7. Extrude vesicles. Fill the syringe with your sample and place the syringe in the right extruder port. Place an empty syringe in the left extruder port. Slowly push the sample through the extruder into the other syringe, this equals one pass. Repeat for seven passes so the sample is removed on the opposite side of the extruder from where it began, remove the left syringe from the extruder, and collect extruded vesicles in a 1.5-mL Eppendorf tube (*see* **Note 16**).

3.2 Vesicle Purification

3.2.1 Purification with an Automated Fraction Collector

1. Rinse fraction collector column connectors and tubing with 3 mL of water then reattach to the bottom of the columns.
2. Use a drop-to-drop connection and connect a gravity size-exclusion column to the line. Raise the column above the collector to initiate gravity flow (Fig. 1a), and rinse the column with one column volume of purification solution (*see* **Note 17**).
3. Lower the column height below the fraction collector and add 100 μ L of calcein-containing vesicles carefully to the resin (Fig. 1b).
4. Move the column height above the fraction collector and start collecting fractions. Collect drops at 0.4 min/well into one row of two 96-well plates.
5. Add buffer dropwise to the resin until the calcein has completely entered the column and a clear layer of resin is visible above the calcein.
6. Gently fill the column with one column volume of water and collect 24 fractions.
7. When all fractions are collected, rinse with one column volume of buffer. Lower the columns below the fraction collector height to stop gravity flow and cap both ends of each column to store.

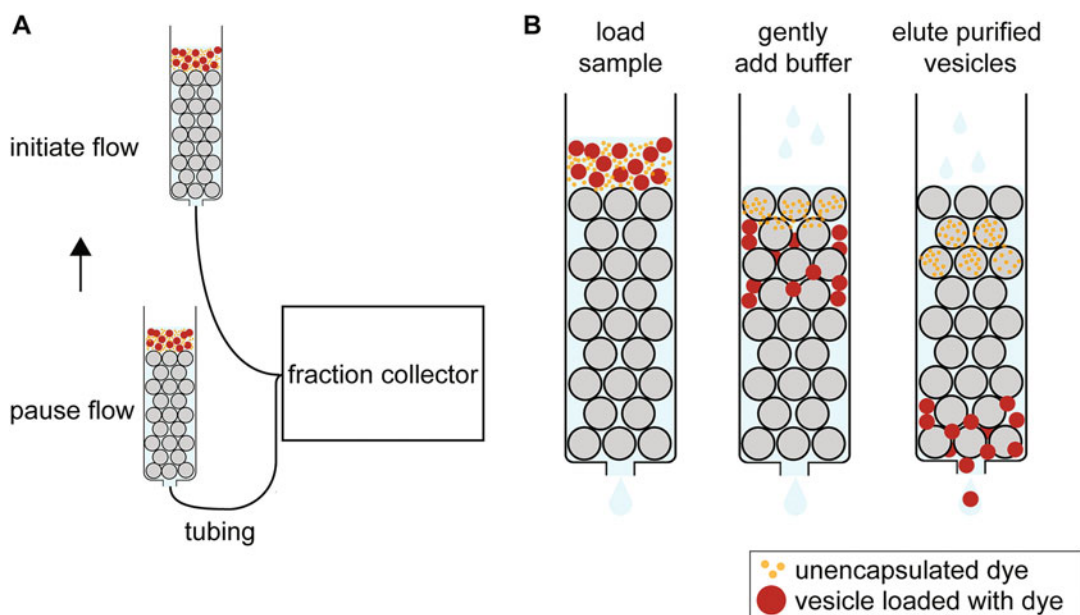


Fig. 1 Vesicle purification schematic. **(a)** Gravity size-exclusion chromatography using a fraction collector. Altering the height of the column relative to the fraction collector controls flow within the column. Fraction collector use is optional, and samples can be collected manually by placing a tube under a dripping column as described in Subheading 3.2.2. **(b)** Vesicles are isolated from unencapsulated dye using gravity size-exclusion chromatography as described in Subheading 3.2

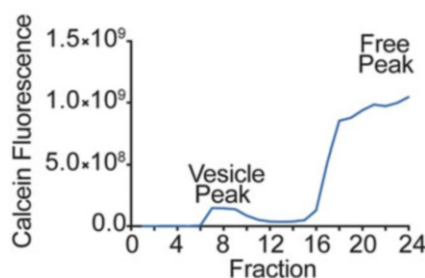


Fig. 2 Sample vesicle purification elution curve. After calcein encapsulation, vesicles are purified to remove free calcein. The vesicle fraction is detected first, in the column void fraction, followed by the free calcein peak. Calcein fluorescence is plotted against the collected fraction number

- To determine which fractions contain vesicles, measure the fluorescence of each well using a plate reader (Fig. 2) with excitation 480 nm and emission 520 nm (see **Note 18**).

3.2.2 Purification Without the Assistance of a Fraction Collector

- Secure the column by clamping onto a stand or placing in holder such that a 96-well plate can be moved easily underneath the column. Add vesicle samples as described above and collect an equivalent number of drops (3–4 drops) per well (see **Note 19**).

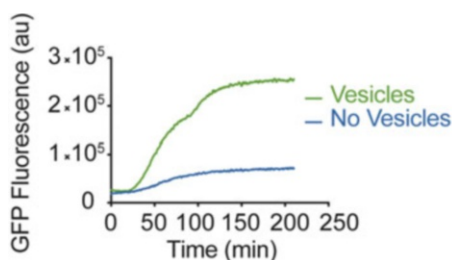


Fig. 3 GFP fluorescence allows the observation of protein production and folding. MscL-GFP expression is monitored during a cell-free reaction by observation of GFP fluorescence. A comparison of MscL-GFP expression in the presence of hybrid lipid/polymer (10 mol% PEO-b-PBD, 90 mol% DOPC) vesicles compared to no vesicles

3.3 Cell-Free Expression of a GFP-Membrane Protein into Hybrid Vesicles

1. Heat a plate reader to 37 °C.
2. Thaw all PURExpress kit components on ice for 30 min. Mix tube A by vortexing for several seconds, if precipitation does not dissolve, mix by pipetting. Do not vortex tube B. Briefly centrifuge tubes A and B.
3. Add 10 μ L of tube A, 7.5 μ L of tube B, 1 μ L (200 ng/ μ L) of DNA (*see Note 20*), and 11.5 μ L of extruded vesicles to a 0.5 mL tube. Mix by pipetting 15 times (*see Note 21*).
4. Aliquot each sample into a glass-bottom 384-well plate and seal with a plate sealer. Insert the plate into the plate reader and begin reading GFP fluorescence.
5. Allow the reaction to incubate for 3.5 h at 37 °C while reading GFP fluorescence every 5 min (*see Note 22*). GFP fluorescence should increase over time as protein is produced in the presence of vesicles (Fig. 3). If vesicles contain calcein for nonselective channel activity, proceed to Subheading 3.4, and GFP fluorescence will not be able to be detected in the presence of calcein.

3.4 Assessment of Membrane Protein Activity

1. Repurify vesicles as described in Subheading 3.2, using 800 mOsm PBS as a purification buffer instead of water (*see Note 23*). Equilibrate the prepared column that was used for the initial vesicle purification in 800 mOsm PBS by washing with two column volumes (*see Note 24*).
2. Using the vesicle fraction from **step 1** with the highest amount of calcein (Fig. 2), transfer 75 μ L of each sample in duplicate into a glass-bottom 96-well plate (*see Note 25*).
3. Read the background calcein fluorescence of the aliquoted samples using a plate reader with excitation 480 nm and emission 520 nm.
4. Add 3.57 μ L of ultrapure water or 3.57 μ L of 21 mM MTSET to each sample for a final concentration of 1 mM MTSET (*see Note 26*).

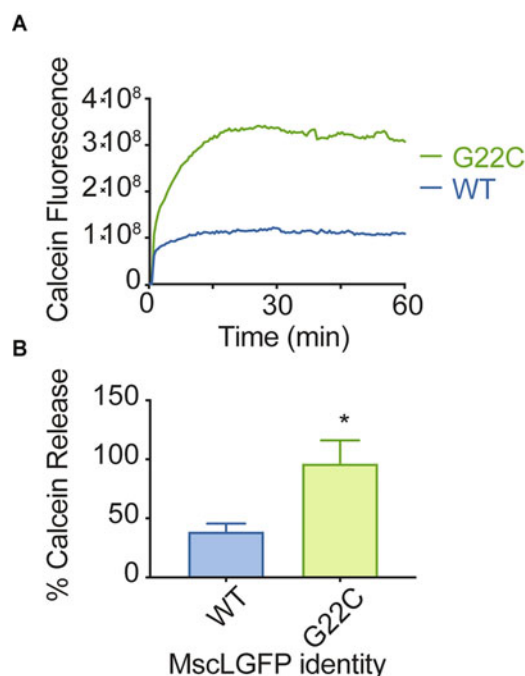


Fig. 4 MTSET calcein release assay. **(a)** MscLGFP is activated by the addition of MTSET to initiate channel opening, and activity is observed using calcein fluorescence activation upon release from vesicles. Increased calcein fluorescence demonstrates release from vesicles in the presence of an activatable mutant (G22C) and a wildtype negative control. **(b)** Calcein release is greater in the presence of chemically activatable MscL compared to wildtype. Final % calcein release is compared to the wildtype negative control, * $p < 0.5$, significance values are determined by the student's unpaired t -test. $n = 3$, error bars represent standard error of the mean

5. Observe calcein release immediately by measuring fluorescence on a plate reader with excitation 480 nm and emission 520 nm, taking measurements every 30 s for a total release time of 30 min (Fig. 4a) (see Note 27).
6. To determine calcein fluorescence upon complete release from vesicles, add 1 μ L of 10% triton-X 100 solution to each well and mix using a shaker for 5 min to ensure total destruction of vesicles.
7. Measure total calcein release on a plate reader with excitation 480 nm and emission 520 nm.
8. Calculate channel activity through percent calcein release using the following equation, $(F_{\text{MTSET}} - F_{\text{initial}})/(F_{\text{triton}} - F_{\text{initial}})$ (Fig. 4b).
9. Compare % release between MTSET and control samples, and perform an unpaired t -test to determine if the channel is active.

4 Notes

1. Using a metal spatula, weigh 100 mg DOPC powder in a glass vial (*see Note 11*). Add 2 mL HPLC-grade chloroform with a glass syringe and vortex to dissolve. If the lipids are predissolved in chloroform from the manufacturer, the solution may be dried under a stream of N₂ and rehydrated to the proper concentration. It is helpful to use small volumes of chloroform stock solution when drying mixtures of lipids and polymers into a thin film.
2. Weigh 144 mg PEO-b-PBD on a balance. Using a metal spatula, transfer polymer to a glass vial by scraping small amounts of polymer from a pure stock of polymer and depositing the polymer on the inside of the glass vial near the bottom. The polymer should be viscous and sticky. Add 2 mL HPLC-grade methylene chloride with a glass syringe, cap, and vortex for 3–5 s to dissolve.
3. A vacuum that can hold up to -0.8 mPa is preferred, use at room temperature.
4. Add 1245 mg calcein powder to ~ 18 mL of ultrapure water, Add NaOH or KOH pellets, one at a time, until calcein is dissolved. This step may take longer than expected and should be performed in advance to calcein usage. Adjust pH to 8.0 using HCl. Adjust final volume to 20 mL with ultrapure water. The pH measurements will likely dye your pH probe. If possible, it is nice to designate a pH probe in the lab for measurements in fluorescent solutions such as this. Prepared calcein may be stored at 4 °C for 6 months and should be discarded if aggregates are observed.
5. Snap the stopper off the end of a poly-prep chromatography column and wash with one column volume of ultrapure water. Add 7 mL of Sepharose 4B resin slurry and let the ethanol flow out by gravity as the resin settles. The resin rapidly settles—be sure to mix the Sepharose 4B slurry in its original container well before addition to the column to ensure you are extracting approximately the same amount each time you prepare a column. The final column volume will settle to ~ 4 mL. Wash with three column volumes of ultrapure water to remove residual ethanol by gravity flow. To wash a column, it is best to add a small layer of liquid above the column first, let that liquid fully enter the column, then add a larger volume of water or buffer. To add one column volume of water (4 mL in this case), you would add ~ 200 μ L of water to the column, let that water enter the column completely but not allow the column to remain dry for more than 5 s, and then add the remaining 3.8 mL of water. Repeat 2 \times . Allowing the resin to remain dry for more than

5–10 s will cause cracking of the column, however, not allowing column solutions to completely enter the column prior to the addition of more water/buffer results in continual dilutions of that solution opposed to its complete removal.

6. We use a Gilson FC204 fraction collector; however, fractions may be collected manually as well.
7. Any plasmid containing a T7 promoter is suitable for protein expression in the PURExpress system (e.g., Addgene #165097).
8. A membrane protein fused with GFP at the C-terminus may be used to observe expression and folding of most proteins [24]. This tag must be fused C-terminally to ensure the full length of the desired membrane protein is translated when GFP is detected. However, this tag is a large soluble protein that is expected to impact protein orientation. If a protein with a different desired orientation is required, alternate methods may be used to assess expression and folding (e.g., Western blotting, CD spectra, NMR, EPR).
9. Any cell-free protein synthesis system can be used; however, PURExpress contains purified transcription and translation machinery and does not contain lipid contamination, which is preferred for downstream applications including assaying the effect of membrane composition on membrane protein behavior.
10. If an osmometer is not available, $3\times$ PBS is acceptable to use as a purification buffer to prevent vesicle bursting after exposing the vesicles to the cell-free reaction.
11. Glass syringes are necessary when handling organic solvents since plastic is soluble in chloroform. Wash glass syringes at least three times in clean chloroform prior to handling samples and between each sample.
12. To determine an optimal molar ratio of lipids and polymers, a range of ratios may be tested. We have used 10, 15, 25, 50, 75, and 100% PEO-b-PBD and formed bilayer membranes. Some diblock copolymers may not form bilayers depending on the size of their hydrophobic and hydrophilic blocks, and the polymer we use was purchased from Polymer Source and contains ~14 PEO groups linked to 22 PBD groups. PEO-b-PBD polymers with a similar ratio of hydrophilic to hydrophobic regions should form bilayers. The successful formation of bilayers can be determined by observing giant vesicles using microscopy techniques, more coarsely by performing dynamic light scattering (DLS) techniques to measure particle size, and most accurately by performing cryoTEM analysis. DLS should never be used as the sole method to assess vesicle formation as aggregates also form particles in the size range of vesicles.

Unextruded vesicles should be visible using phase-contrast microscopy with a 20 \times objective or higher and can range from <1 to 80 μ m. If bilayers do not form, the amphiphiles may form micelles, which are too small to visualize using microscopy (*see* **Note 13**) but can result in a DLS peak around 10 nm.

13. When using microscopy for the determination of bilayer formation, a larger vial and a lower concentration of lipids should be used during the thin film hydration procedure. For example, we find that 20 mL glass vials containing 1 mL of a 150 μ M solution of amphiphiles is best. It is also important to dilute vesicles in an osmolarity matched, but less-dense solution to aid imaging after vesicle formation. For example, we hydrate our vesicles in ~300 mOsm sucrose and dilute these vesicles in PBS. Pipette a small amount of formed vesicles into a larger microscope chamber containing equiosmolar PBS. The vesicles should sink to the bottom of the chamber and be more readily visible.
14. It is important to be careful while drying mixtures of lipid and polymer films. If a uniform film is not distributed with maximum surface area within the vial, we have observed visible aggregation of the amphiphiles after hydration. Aggregation is also an indication that the amphiphile concentration needs to be reduced. Thin film formation is critical for hybrid lipid/polymer vesicle formation as some methods may cause independent lipid and polymer vesicles or domain formation [17, 27]. By slowly spinning the vial as the solvent is removed, a uniform and visible thin film will coat the glass surface of the vial and the lipids and polymers should remain mostly mixed upon rehydration. Other inert gasses such as argon are also acceptable for drying lipids and polymers.
15. For fluorescent protein detection, hydrate vesicles in ultrapure water. For channel activity assays, hydrate vesicles in 100 mM calcein. Vesicle addition to the cell-free reaction takes the place of water, it is important to avoid the introduction of excess salt to the reaction during the addition of vesicles as salt changes affect PURExpress reaction efficiency. Accordingly, the vesicles must be hydrated in water, or hydrated in calcein, then purified in water. Calcein release from vesicles is a straightforward method to measure channel activity. This method is applicable to any nonselective membrane channel protein that can be activated using a chemical additive. We used a chemically activatable mutant of the mechanosensitive channel of large conductance, MscLGFP (G22C), which opens with the addition of MTSET [28]. Upon channel opening, calcein is released from the vesicles to the external solution, which increases calcein fluorescence and provides a fluorescent readout for channel activity.

16. Vesicles may be stored at 4 °C for up to 2 weeks. If vesicles were hydrated in calcein, purification is required immediately prior to protein expression, proceed to Subheading 3.2 for vesicle purification. If vesicles were hydrated in water, proceed to Subheading 3.3.
17. Purification solution for calcein vesicles prior to expression is ultrapure water. After protein expression, the purification solution may be any buffer near 800 mOsm, such as 3× PBS. Once the buffer is level with the top of the resin, being careful not to allow the resin to dry and crack, lower the column height to below the fraction collector to pause the column flow. It is easiest to add samples to multiple columns while gravity flow is paused.
18. The vesicle peak should be within the first 10 fractions, and the free peak should be present after fraction 12. The vesicle peak should exhibit less fluorescence than the free peak since calcein is present at a self-quenching concentration within the vesicles. If high concentrations of vesicles are used, calcein in the vesicle and free fractions will be visible by eye as well.
19. Purification without a fraction collector is useful if a fraction collector is not available [29]. As this method requires the user to manually collect samples, it is more prone to user error, and it may be helpful to tune the number of drops per well to the desired elution volume. We find that 200–300 µL elution volume per well is optimal for vesicle purification.
20. The optimal plasmid concentration varies and may be optimized. Any plasmid containing a T7 promoter is suitable for expression using the PURExpress system. Many membrane proteins display the ability to fold into their native conformation without the need for chaperones [30–32]. This phenomenon may allow for the production of difficult-to-express membrane proteins [33]. Therefore, the present method is likely applicable to a wide range of membrane proteins for the functionalization of model membranes. A method to determine the optimal phospholipid for a given protein is to form pure lipid vesicles at equal concentrations and observe fluorescence during expression. The composition which exhibits the highest fluorescence should be used as the phospholipid in a lipid/polymer blended vesicle.
21. Vesicle addition to the PURExpress reaction takes the place of water, which differs from the manufacturer's protocol. The volume of vesicles is limited to 11.5 µL per reaction in order to maintain the proper concentration of reaction components. If more vesicles per reaction are desired, increasing the lipid concentration during vesicle preparation is preferred over

adding more vesicle volume to the cell-free reaction. Vesicles composed of DOPC and diblock copolymer will form up to 60 mM, or higher if polymer is present at a lower mol%. If aggregation is observed after vesicle hydration, the concentration of amphiphile must be reduced. If a no-vesicle control is desired, the vesicle volume in the cell-free reaction should be replaced with ultrapure water.

22. A membrane protein expressed as a fusion protein with a C-terminal GFP tag may be used for the observation of protein expression and folding during the cell-free reaction [24, 34]. While this protocol is applicable to the expression of any membrane protein, tagged or untagged; using a GFP folding reporter allows the observation of protein production and allows the quantification of protein folding. If calcein is encapsulated in vesicles, GFP fluorescence will not be detected as calcein fluorescence occurs at a similar wavelength as GFP.
23. After protein expression, vesicle purification is necessary to remove any calcein that may have diffused out of the vesicles during protein insertion. In addition to removal of calcein, vesicle purification can also be used to purify the expressed membrane protein away from the PURExpress components that may disrupt downstream applications of the functional vesicles.
24. The osmolarity of the vesicle internal and external solutions must match to prevent vesicle bursting and to retain the remaining encapsulated components. In this step, in order to retain calcein for the activity assay, the osmolarity of the purification buffer solution after cell-free expression must be near 800 mOsm.
25. Free calcein will have higher fluorescence than encapsulated calcein. It is important to be careful to use the vesicle fraction which is the first small peak instead of the large peak of unencapsulated dye (Fig. 2).
26. Ultrapure water is used as a control for the osmotic disruption of vesicles when MTSET is added, and some calcein leakage is expected with water addition. An additional important control for studies like this is to include vesicles that do not have any membrane protein to evaluate the extent to which water or MTSET nonspecifically induces calcein release.
27. Calcein is a self-quenching dye. Fluorescence becomes increasingly quenched past a certain concentration of calcein, which we encapsulated in vesicles. Upon release from vesicles, the local concentration of calcein is reduced and an increase in fluorescence is observed. Depending on the ratio of the concentration of vesicles used compared to the volume of the

extravesicular solution, the encapsulated concentration of calcein may be optimized to maximize the increased signal in calcein fluorescence upon vesicle disruption or permeabilization. The optimal range of calcein concentration for vesicle formation may range from 10 to 200 mM.

Acknowledgments

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Cell-Free Synthesis Strategies to Probe Co-translational Folding of Proteins Within Lipid Membranes

Nicola J. Harris, Eamonn Reading, and Paula J. Booth

Abstract

In order to comprehend the molecular basis of transmembrane protein biogenesis, methods are required that are capable of investigating the co-translational folding of these hydrophobic proteins. Equally, in artificial cell studies, controllable methods are desirable for in situ synthesis of membrane proteins that then direct reactions in the synthetic cell membrane. Here we describe a method that exploits cell-free expression systems and tunable membrane mimetics to facilitate co-translational studies. Alteration of the lipid bilayer composition improves the efficiency of the folding system. The approach also enables membrane transport proteins to be made and inserted into artificial cell platforms such as droplet interface bilayers. Importantly, this gives a new facet to the droplet networks by enabling specific transport of molecules across the synthetic bilayer against a concentration gradient. This method also includes a protocol to pause and restart translation of membrane proteins at specified positions during their co-translational folding. This stop–start strategy provides an avenue to investigate whether the proteins fold in sequence order, or if the correct fold of N-terminal regions is reliant on the synthesis of downstream residues.

Key words Cell-free transcription/translation, In vitro co-translational folding, Membrane proteins, Lipid bilayers, Translation pausing, Artificial cells, Active transport

1 Introduction

Folding is a fundamental process during the biogenesis of all proteins, with correct folding underlying function and misfolding decreasing cell viability and frequently triggering disease [1]. In an effort to uncover molecular, mechanistic detail on membrane protein folding, many studies have focused on denaturation in detergent micelle systems of over-expressed and purified full-length protein chains [2–6]. Although obviously far from the natural situation where most proteins fold as they are synthesized and not as full-length chains, these artificial denaturation studies have enabled significant insight into the kinetics, thermodynamics, and mechanisms of folding to be attained. Recently, work has pro-

gressed to co-translational folding, but the majority of detailed folding work in this area to date has addressed water-soluble proteins [7–11] with far fewer studies on membrane proteins [12–15]. This shortfall in co-translational folding investigations constitutes a significant issue for the dominant and ubiquitous class of α -helical integral membrane proteins, as the vast majority of these proteins insert and fold during translation and thus as their polypeptide chain elongates from the N- to C-terminus.

Fundamental questions relating to membrane protein folding remain unresolved; how does structure evolve as the polypeptide chain elongates? Do transmembrane (TM) helices form and pack in sequence order? Can the N-terminal helices fold correctly by themselves, or do they have to wait for later sections of the protein to be synthesized? How does the lipid bilayer influence co-translational TM folding? In order to answer these questions, it is important to work in lipid environments as membrane proteins are highly dependent on their surrounding lipids [5, 16–22]. The convenience of detergents mean they are frequently used as a method of choice, but they do not reproduce many aspects of lipid membranes. We have developed a series of methods to probe co-translational folding in lipids and provide means to address the above prevailing questions. We have exploited the fact that α -helical integral membrane proteins have been shown to insert and fold into lipid mixtures co-translationally in vitro without the Signal Recognition Particle or translocon [23–26]. A synthetic bilayer can be used to optimize the yield of folded functional protein [12, 13, 27–33], although our protocols are also amenable to incorporation of the translocon and other cellular factors.

Cell-free expression has emerged as a useful method to synthesize proteins for a variety of studies. Current methods for successful cell-free expression of membrane proteins are tailored towards the production of protein for structural and functional analysis of the final folded protein [28, 30, 34], or investigating the forces governing TM insertion into a bilayer [24, 35, 36], rather than for study of co-translational folding per se. Generally, cytosolic cellular extracts such as rabbit reticulocyte, insect cell, wheat germ, or *Escherichia coli* (*E. coli*) cell extracts are used to produce protein, and these are mixed with membrane extracts such as *E. coli* inner membrane vesicles or ER microsomes [28, 32, 34–45]. Membrane proteins have also been successfully expressed using the PURE system [46–53], which contains purified components for protein synthesis. We have used these fruitful cell-free approaches as a basis to develop a complementary, controllable cell-free expression system for investigating co-translational folding. Our idea is to use a minimal synthetic system to increase molecular mechanistic detail and time resolution beyond that currently achievable with biochemical methods and more intricate cellular samples. We will describe a

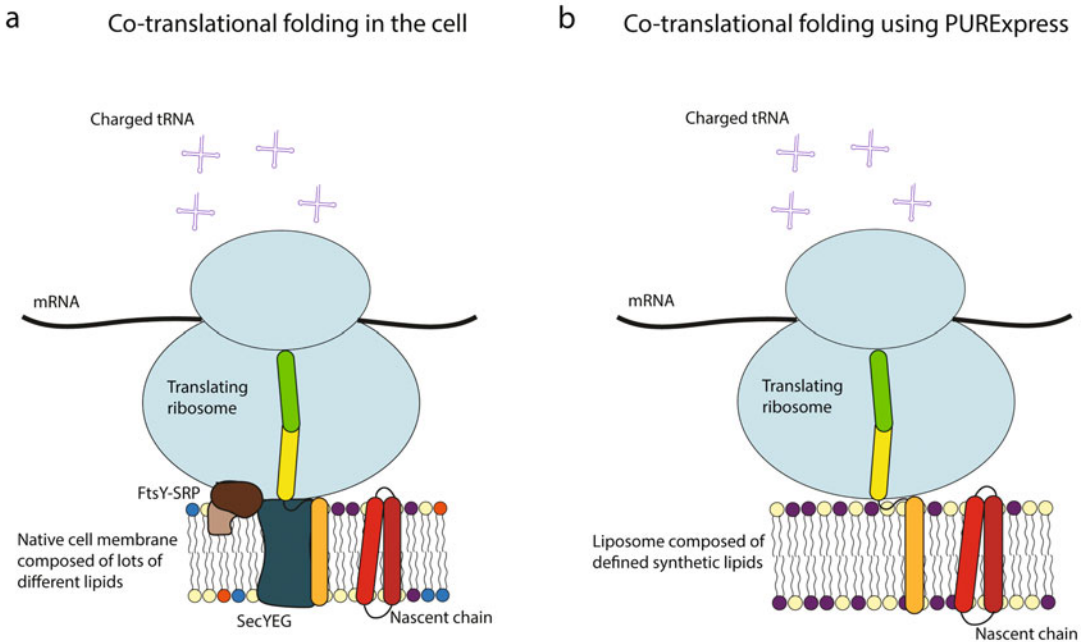


Fig. 1 Schematic of TM insertion in *E. coli* in vivo (a) vs. using our protocol with PURExpress (b). In the cell, TM helices are inserted into the membrane with the aid of the translocon SecYEG, with the Signal Recognition Particle and FtsY. The cell membrane is composed of many different types of lipids. When using PURExpress and our protocol (b), the ribosome translates the nascent chain which inserts spontaneously into a supplied lipid bilayer. Only purified translation components are present, in contrast to the more complex cytosol in vivo

method using PURExpress and lipid mixtures, which creates a “cleaner” system than cell-free systems based on cell extracts (Fig. 1).

We will also describe a method we have introduced to pause and restart translation at defined points during protein synthesis. Our ability to restart synthesis significantly increases the scope for more detailed folding investigations. Earlier stalling approaches have employed stop or arrest sequences, such as SecM [54–56] or TnaC [57, 58], to give truncated polypeptide chains on the ribosome. These nascent chains may not represent an actual folding intermediate, as significant time elapses between translation and the analysis of the stalled nascent chain, during which the nascent chain structure may rearrange. Equally it is not possible to continue translation to the full-length protein following stalling to see if overall folding is successful. Our stop–start method exploits the opportunity afforded by PURExpress to omit particular amino acids from the cell-free reaction to pause or stop translation [13, 59]. The excluded amino acid can thus be added to restart translation after a specified time period. The approach is further aided by the fact that PURExpress contains solely proteins for transcription/translation and does not contain any of the cellular mechanisms which rescue stalled ribosomes [60].

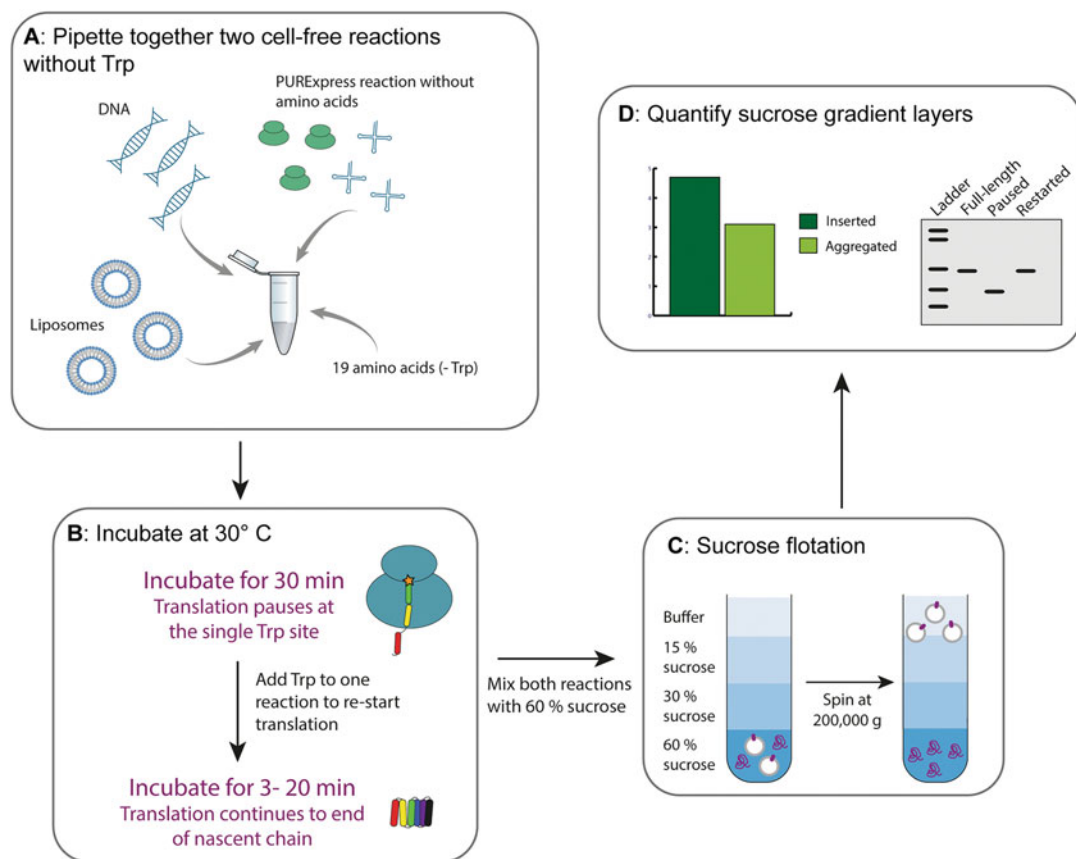


Fig. 2 The pausing and restarting translation experiment (Subheading 3.7). This schematic uses Trp as the omitted amino acid, but an alternative amino acid could be used instead. First (a), two PURExpress cell-free reactions are pipetted together but with 19 amino acids instead of the usual 20. Then (b), these reactions are incubated for 30 min. Trp is added to one of the reactions and incubated further to finish translation. Both reactions are mixed with 60% sucrose for the sucrose flotation (c). The layers are separated and quantified, or analyzed by SDS-PAGE (d)

This stop–start method is outlined in Fig. 2. A single amino acid mutant of the target protein is made, and the location chosen for the single site depends on the structural region of interest. When the ribosome reaches the codon for the amino acid, translation pauses. Translation can then be restarted by adding the amino acid into the cell-free reaction, allowing elongation to continue. As approximately 60 residues fit into the ribosome exit tunnel if they are α -helical (approximately 2 TM helices [61]), the site chosen to pause translation should be around 60 residues after the structural element of interest (*see* Fig. 3 for a schematic and example). A crystal structure aids in the design of constructs, although topology analysis can also be used using tools such as MPEx [62]. As an example, we have been able to create a Trp-free construct of LacY and create single-Trp mutants at specified sites to omit Trp from the cell-free reaction [13].

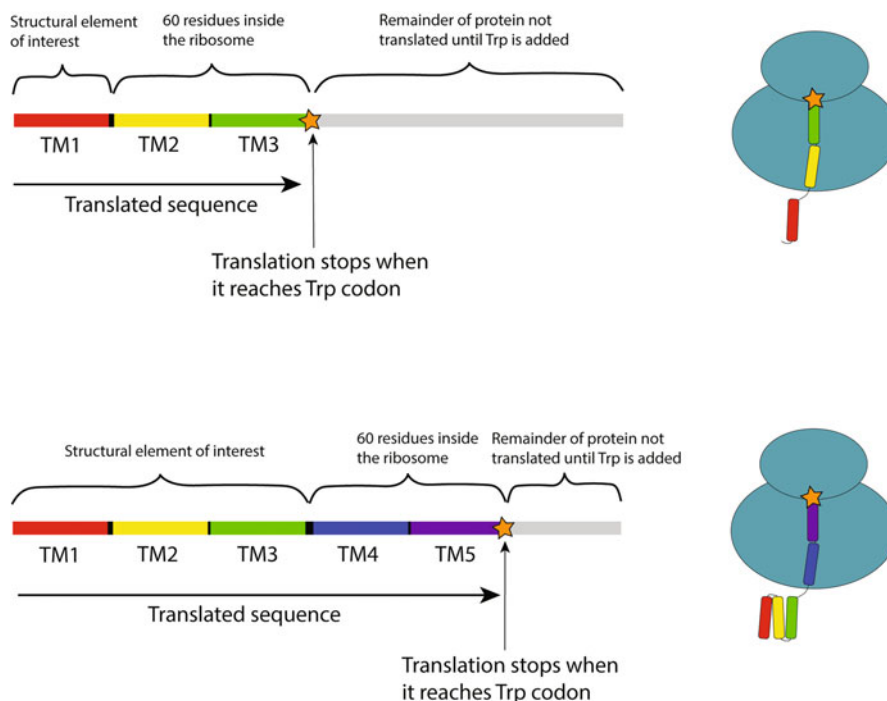


Fig. 3 Examples of constructs used to pause translation are shown. Each construct has a Trp codon at a different position. Trp is omitted from the cell-free reaction so translation stops when the Trp codon is reached. The examples show two different constructs: one stops after 1 TM helix and the other after 3 TM helices have exited the ribosome. In this schematic, the location of the translation pause is shown as an orange star

Another advantage of these tunable cell-free methods is the potential to exploit them in artificial cells. Nature encapsulates reactions within membrane-bound compartments, and droplet interface bilayers are proving a valuable platform to mimic this key biological feature in artificial systems. A major issue, however, is manipulating flow across the synthetic bilayers. The cell-free expression approach outlined here enables membrane transporters to be synthesized in a droplet and inserted into the bilayer to enable specific transport of biomolecules, and notably transport against a concentration gradient, across the droplet interface bilayer. We have demonstrated the approach using an archetypal member of the ubiquitous major facilitator superfamily [63], thus paving the way for controllable transport of sugars, metabolites, and other essential biomolecules.

There are many practical issues involved in studying membrane protein folding and manipulating membrane protein synthesis in artificial systems. We have aimed for reliable, generally applicable and adaptable approaches. The purpose of these procedures is to establish working experimental systems to probe co-translational

folding at greater structural and temporal resolution than has been possible. Our procedures can be combined with techniques such as surface enhanced infrared spectroscopy [12] to attain mechanistic detail. We have used our protocol on 12 TM major facilitator superfamily transporters [13], as well as a 6 TM membrane protease and a 4 TM disulfide bond formation protein [12]. These targets show how our methods work well for *E. coli* proteins ranging from 4 to 12 transmembrane helices and from small, stable membrane proteins to those that are dynamic and comprise more than one domain.

Our methods enable a user to: (1) create a robust method for studying co-translational folding of membrane proteins, (2) investigate the effect of the lipid bilayer on co-translational folding, (3) stop and start translation to ascertain whether folding of the initially synthesized N-terminal regions requires translation of later sections of the protein, and (4) perform on-board synthesis of membrane proteins in artificial cells for specific, active transport of molecules into or out of the cell.

2 Materials

Reagents should be of the highest grade possible and only used within their expiry date (where provided). Specific suppliers are in brackets. Ultrapure water (for example, Milli-Q or another alternative) with a resistivity of 18.2 M Ω -cm should be used for all buffers to minimize RNase contamination.

2.1 Preparation of Liposomes

1. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), as powder from Avanti Polar lipids (*see* **Note 1**).
2. Cyclohexane.
3. Liquid N₂.
4. Freeze drier.
5. Glass vials.
6. Mini-extruder kit from Avanti Polar Lipids with 100 nm polycarbonate filters, *see* **Note 2**.
7. Buffer for liposomes: 40 mM HEPES-KOH pH 7.6.

2.2 Cell-Free Expression

1. The gene of interest in a suitable vector: The vector should have a T7 promoter 20–100 nucleotides upstream of the start codon, with a T7 terminator downstream of the gene. There should also be a suitable detection tag on the N- or C-terminus (*see* **Note 3**). The plasmid DNA should be used at a concentration of 1 mg/mL (*see* **Note 4**).

2. The PURE system: PURExpress In Vitro Protein Synthesis Kit and Δ aa, tRNA Kit (New England Biolabs).
3. L-Amino acids: Using nuclease-free H_2O throughout, make a 1 M stock of KOH and a 1 M stock of HCl. Make 100 mM stock solutions of each amino acid by dissolving Ala, Arg, Gly, His, Lys, Met, Val, Thr, Ser, and Pro in dH_2O . Ile and Phe should be dissolved in 1 M KOH. Gln, Cys, Asp, Asn, Glu, Trp, Tyr, and Leu should be dissolved in 1 M HCl. Mix all amino acids but Trp (i.e., 19 amino acids, called 19AA, *see Note 5*) together with dH_2O to make a final concentration of 3 mM each. This 19AA amino acid mix will be diluted $10\times$ in the cell-free reaction, giving the same final concentration as those supplied in PURExpress (0.3 mM). A separate 7.5 mM Trp stock in dH_2O should also be made to be added separately as appropriate. Amino acids should be stored at -70°C for up to 1 month.
4. Heat block.
5. RNase-free pipette tips and microcentrifuge tubes.

2.3 Protein Analysis

1. Sucrose buffers: Prepare 60% and 30% (w/v) sucrose solutions with 4 M urea in 40 mM HEPES-KOH (pH 7.6). Also prepare a 4 M solution of urea in 40 mM HEPES-KOH (pH 7.6) without sucrose. The buffers may take time to dissolve in solution, particularly the 60% sucrose. Brief heating (not higher than 37°C for a few mins) may be required. The solutions are stable at room temperature but replace them if the sucrose gradients are not working as expected (*see Note 6*).
2. Sucrose buffers for pausing experiments: Prepare a second set of sucrose buffers for translation pausing experiments. In this case, prepare 60%, 30%, and 15% (w/v) sucrose solutions and sucrose buffers with 4 M urea, 10 mM $\text{Mg}(\text{OAc})_2$, and 40 mM HEPES-KOH (pH 7.6). Also prepare a 4 M solution of urea in 10 mM $\text{Mg}(\text{OAc})_2$ and 40 mM HEPES-KOH (pH 7.6).
3. 10% (w/v) *N*-Dodecyl-b-D-Maltopyranoside (DDM). This can be stored at -20°C for months.
4. Electrophoresis system and 12% acrylamide gels for SDS-PAGE, including running buffers and SDS loading buffer.
5. Semidry transfer unit (for example, from GE Healthcare, cat. no. TE 77 PWR).
6. PVDF or nitrocellulose membrane (*see Note 7*).
7. Benchtop ultracentrifuge capable of spinning up to $400,000 \times g$.
8. Thermolysin: Dissolved in PBS at 1 mg/mL and used to make a serial dilution to a final concentration of 10 $\mu\text{g}/\text{mL}$. Stocks can be frozen and stored at -20°C for months.

9. Transfer buffer: 25 mM Tris, 192 mM glycine, 1.5 mM SDS, and 10% (v/v) methanol. Can be stored at room temperature.
10. Reagents for Western blots: Make 1 L of 1× PBS following standard procedures. Add 500 µL of tween-20 and mix well to make 0.05% of PBS-T. Use this PBS-T to make a 5% (w/v) milk solution (approx. 20 mL per membrane is needed). The chosen antibody should be prepared according to the manufacturer's instructions; the amount of antibody used and the incubation time may need to be tested empirically.
11. Gel imager for visualizing Western blots (*see Note 8*).

3 Methods

The methods will describe how to perform a cell-free reaction using PURExpress, followed by a procedure for visualizing synthesized protein by Western blot. A method for protease digestion will then be described, to help verify that cell-free expressed protein is inserted across the bilayer. Subheadings 3.6 and 3.7 (pausing and restarting translation) will describe the method to pause and restart translation by omitting an amino acid. It is recommended that all experiments are repeated at least three times to verify reproducibility (Fig. 4).

3.1 Liposome Preparation

1. Dissolve DOPC, DOPE, and DOPG in cyclohexane in glass vials at 50 mg/mL at 45 °C (*see Note 9*).
2. Once dissolved, allow the lipid solutions to cool to room temperature before mixing them together at the desired ratios in glass vials.
3. Freeze the lipids in liquid N₂, loosen the lids on the glass vials, and place in a freeze drier. Apply the vacuum overnight to remove the solvent and produce a lipid film. The lipid film should appear white and powdery (*see Note 10*).
4. After drying overnight, tighten up the lids on the vials and wrap parafilm around the lids and store at −20 °C. Lipids can be stored for up to 2 weeks.
5. To make liposomes, dissolve the lipid film in 40 mM HEPES-KOH (pH 7.6) to a lipid concentration of 10 mg/mL by vortexing and stirring for at least 20 min at room temperature. The lipid suspension should be cloudy.
6. The lipids are then extruded using the mini-extruder by passing through the 100 nm filter 31 times. The lipids should become visibly less opaque after extruding (*see Note 11*).

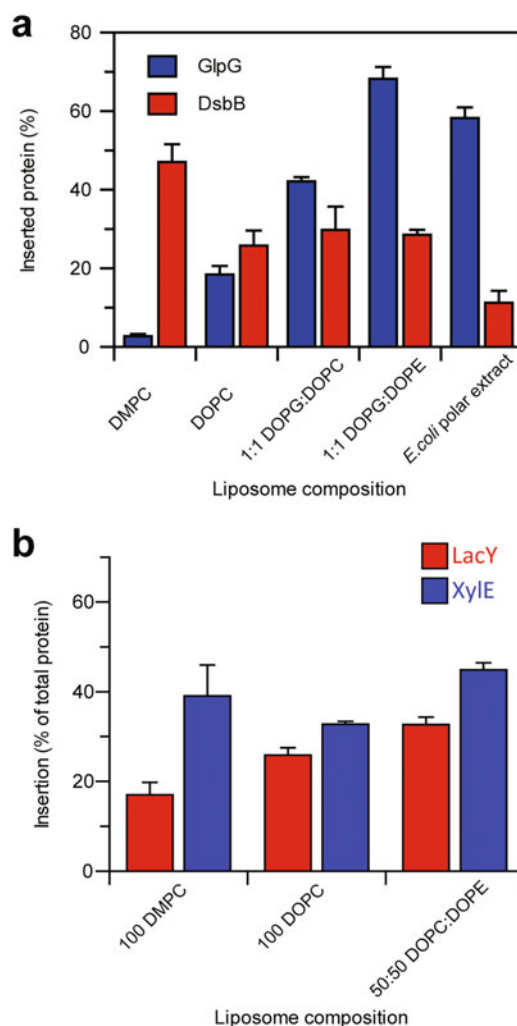


Fig. 4 Lipids affect the insertion yield. The membrane proteins GlpG and DsbB (a), and the transporters LacY and XylE (b) were expressed following the protocol in Subheadings 3.1-3.3 with liposomes of different lipid compositions. The amount of protein inserted into the liposomes varied depending on the lipids used and was different for each protein, indicating the importance of the liposome composition selected for cell-free expression. The insertion yield was quantified via LSC of incorporated [^{35}S] Met (GlpG, LacY, XylE) or [^{14}C] Leu (DsbB). (Part (a) adapted from [12] with permission under CC BY 4.0 <http://creativecommons.org/licenses/by/4.0/>. Part (b) adapted from [13] with permission under CC BY 4.0 <http://creativecommons.org/licenses/by/4.0/>)

3.2 Cell-Free Expression

1. Follow the PURExpress instructions: Pipette together 10 μL of solution A with 7.5 μL of solution B (**Note 12**), with 6.5 μL of the prepared liposomes (*see Notes 13 and 14*).

2. Start the reaction by addition of 1 μL of DNA template (at 1 mg/mL) to produce a final volume of 25 μL . Incubate the reaction at 30 °C for 1–4 h (*see* **Notes 15** and **16**).

3.3 Sucrose Flotation

1. Mix the 25 μL cell-free reaction with 80 μL of the 60% sucrose/4 M urea buffer in an ultracentrifuge tube.
2. Carefully layer on top 100 μL of the 30% sucrose/4 M urea buffer, followed by 50 μL of the 4 M urea buffer.
3. Balance the tubes carefully on a fine balance.
4. Spin the sucrose gradients at room temperature for 30 min at $200,000 \times g$ (*see* **Note 17**).
5. Carefully remove the top (125 μL) and bottom (130 μL) layers of the sucrose gradient by pipetting into separate tubes. The top layer of the sucrose gradient can be used in a functional assay to determine correct protein folding (*see* **Notes 18** and **19**).

3.4 SDS-PAGE Analysis

1. Mix 20 μL of each sucrose gradient layer with 20 μL of SDS loading buffer. Do not boil the samples.
2. Load the samples onto a 12% SDS-PAGE gel and run according to the manufacturer's instructions.
3. Proceed with a Western blot against the chosen tag to detect protein. Transfer the gel onto either PVDF or nitrocellulose membrane. Follow the manufacturer's instructions for the chosen transfer system to transfer the SDS-PAGE onto the membrane.
4. Follow standard procedure for Western blot with antibody against the chosen affinity tag (*see* **Note 3**). For example, block the membrane on a rocker from 1 h (at room temperature) to overnight (at 4 °C) in 5% milk powder in PBS-T. Incubate in the chosen antibody, in 5% milk powder and PBS-T, for 1 h at RT. Wash the membrane in PBS-T with at least six changes of PBS-T, for 2–15 min each. Incubate the membrane in a Western blotting detection reagent according to the manufacturer's instructions and develop using a gel imager. *See* Fig. 5 for example results and **Notes 20** and **21**.

3.5 Protease Digestion of Inserted Protein

1. Pipette 20 μL of sample from the top fraction of the sucrose gradient into separate tubes for different timepoints, with 5 μL of buffer.
2. Pipette an identical sample together with 5 μL 10% DDM, to solubilize the liposomes prior to incubation with thermolysin (*see* **Note 22**).
3. Add 20 ng thermolysin and incubate at room temperature.

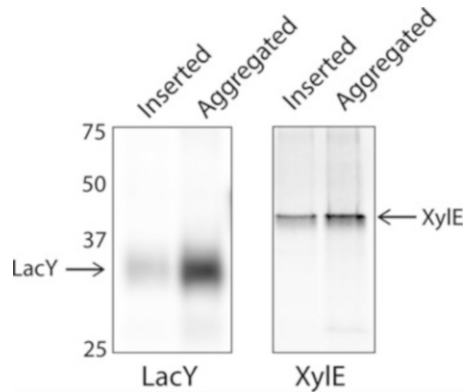


Fig. 5 Example data of cell-free synthesis. The *E. coli* MFS sugar transporters LacY and Xyle were synthesized using this protocol (following the protocol in Subheadings 3.1-3.3) in the presence of 25:50:25 DOPC:DOPE:DOPG (mol ratio) 100 nm liposomes. The top (inserted) and bottom (aggregated) fractions of the sucrose gradient flotation were analyzed by SDS-PAGE (Subheading 3.5) and detected by Western blot via an anti-HA tag (LacY) or phosphorimaging of incorporated [^{35}S]Methionine (Xyle). (Adapted from [13] with permission under CC BY 4.0 <http://creativecommons.org/licenses/by/4.0/>)

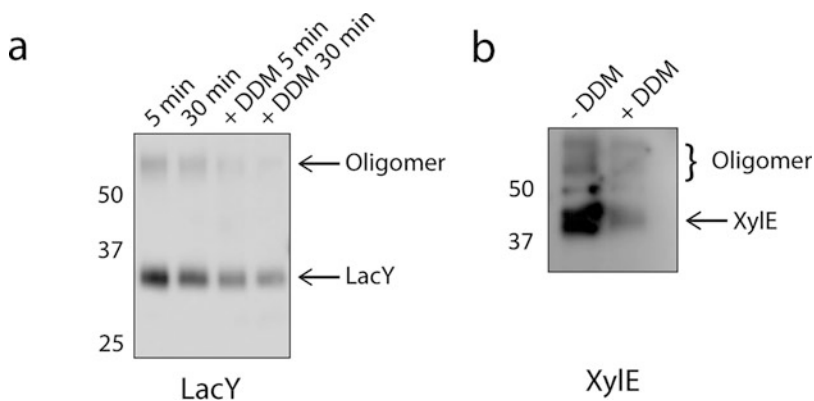


Fig. 6 Example data for protease digestion of cell-free expressed protein. LacY (a) and Xyle (b) digested by thermolysin following cell-free expression (Subheading 3.5). Each protein was incubated with thermolysin with or without 1% DDM. More digestion occurred when solubilized in DDM than when in liposomes. Protection from proteolysis when in liposomes indicates integration of the protein across the bilayer. Protein was detected by Western blot using an anti-His or anti-HA tag antibodies. LacY was incubated with 20 ng thermolysin for 5 min or 30 min, and Xyle required an incubation at 4 °C overnight with 10 ng thermolysin for digestion of the DDM-solubilized protein to occur. (This figure is adapted from [13] with permission under CC BY 4.0 <http://creativecommons.org/licenses/by/4.0/>)

4. Stop digestion by flash-freezing in liquid N_2 at 1 min, 5 min, 10 min, 20 min, and 30 min.
5. Analyze samples by SDS-PAGE (using the same procedure as that described above). See Fig. 6 for example results.

3.6 Pausing and Restarting Translation: Determining How Long It Takes to Make Full-Length Protein

1. For the remaining steps, the protein construct with a single-site amino acid at a chosen site (*see* **Note 23**) should be used.
2. Pipette together a PURExpress reaction, but double the volumes to create a reaction volume of 50 μ L (20 μ L of solution A, 15 μ L of solution B, 13 μ L of liposomes, and 2 μ L of DNA).
3. Incubate at 30 °C (*see* **Note 24**).
4. During incubation, remove 5 μ L of samples at 5 min intervals using a sterile RNase-free pipette tip, mix with 5 μ L of SDS loading buffer, and place on ice.
5. Follow the SDS-PAGE and Western blot protocol described in Subheading 3.4 to determine when full-length protein appears. *See* Fig. 7 for example results.

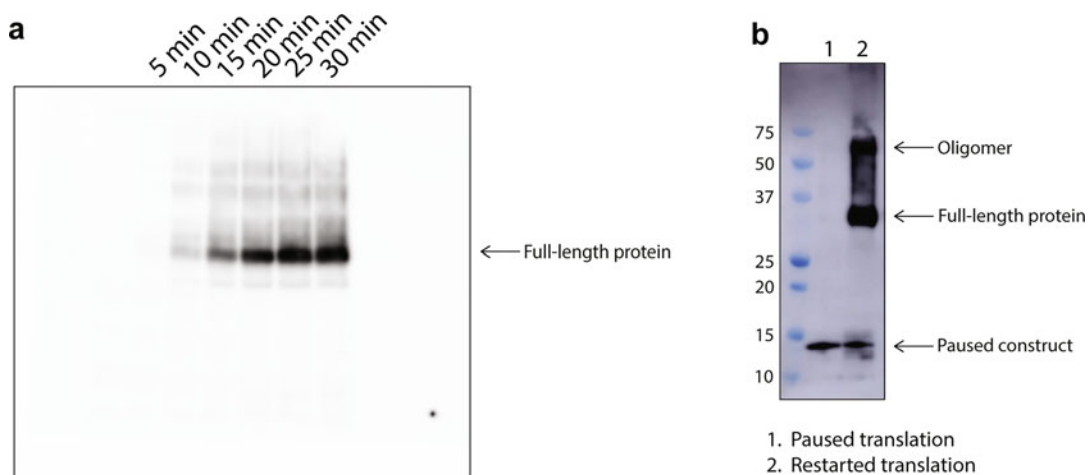


Fig. 7 Example data of translation pausing. **(a)** Example data for determining when a protein band corresponding to full-length protein appears on SDS-PAGE, following the protocol in Subheading 3.6. Protein was detected by Western blot using an anti-HA tag antibody to an N-terminal HA tag. The example in this figure shows the single Trp LacY construct NC(TM1); a band corresponding to full-length protein first appears in 5–10 min. An increase in the band intensity over time occurs as more total protein is produced. Example data for pausing and restarting translation is shown in **(b)**. A Western blot of translation of the LacY construct NC(TM1) is shown, where translation was stopped by omitting Trp from the cell-free expression reaction (lane 1, paused translation), or paused for 30 min before Trp was added back in and incubated for a further 8 min (lane 2, restarted translation) according to the protocol in Subheading 3.7. Each cell-free reaction was floated on a sucrose gradient containing urea to establish whether the protein made was liposome associated. The floated, i.e., liposome associated, fraction was analyzed by SDS-PAGE. Whether or not the truncated, ribosome-attached construct floats with the liposome (lane 1) helps to ascertain the co-translational folding pathway. (Figure adapted from [13] with permission under CC BY 4.0 <http://creativecommons.org/licenses/by/4.0/>)

Table 1
PURExpress reaction mixture for pausing and restarting translation

	Paused translation	Restarted translation
A	5	5
B	7.5	7.5
19AA	2.5	2.5
tRNA	2.5	2.5
Lipids	6.0	6.0
H ₂ O	1	–
DNA	1	1

3.7 Pausing and Restarting Translation

1. Pipette together the PURExpress Δaa, tRNA Kit according to the manufacturer's instructions (*see* Table 1 for volumes). Replace the provided amino acid mix with the preprepared 19 amino acid (19AA) mix. Pipette together two reactions per construct—one for paused translation and one for restarted translation.
2. Start the cell-free reaction by addition of 1 μL of plasmid DNA, and incubate at 30 °C for 30 min.
3. Add 1 μL of Trp (0.3 mM) to one of the reactions per construct—this is the restarted translation sample (*see* Note 25).
4. Incubate for a further 3–20 min (as determined in Subheading 3.6, Note 24), and then place on ice to stop translation.
5. Mix the liposomes with 80 μL of 60% sucrose, and layer 50 μL of 30% sucrose on top, followed by 50 μL of 15% sucrose and 50 μL of buffer (4 M urea, 40 mM HEPES-KOH pH 7.6, and 10 mM Mg(OAc)₂ in all sucrose layers).
6. Spin the sucrose gradients at $200,000 \times g$ for 30 min at room temperature to float the liposomes to the 15% sucrose: buffer interface.
7. Pipette off the sucrose layers carefully in 50 μL steps into separate tubes, either to be loaded directly onto an SDS-PAGE gel (following the same procedure as described above) or to be used for further analysis (*see* Note 26).

4 Notes

1. The lipids listed here may not be those most suitable for the chosen protein. Multiple considerations must be taken into account when selecting the lipids for insertion or folding of the target protein. PURExpress is suitable for liposomes

composed of a wide variety of different lipids such as those with charged headgroups and nonbilayer forming lipids. There may be lipids which are required for correct protein function [18–21, 64, 65], and these are not necessarily the lipids which aid insertion and folding. Liposomes can be either empty, therefore the proteins will insert into the bilayer spontaneously, or can be preconstituted with an insertase such as SecYEG (for which cardiolipin is necessary [64, 65]). *See Fig. 4* for example data showing the effect that different lipids can have on the insertion yield.

2. Different sized liposomes can be made by changing the filter pore size; 50 nm up to 400 nm liposomes are recommended.
3. PURExpress contains His-tagged proteins, so it may be necessary to clone in an alternative detection tag either at the N- or C-terminus of the target protein in order to detect protein by Western blot. Truncated constructs will require the detection tag to be at the N-terminus if a specific protein antibody is not available.
4. Linear and circular DNA as well as RNA can all be used with the PURExpress kit; however, more reproducible and consistent data is produced from high-quality circular plasmid DNA.
5. Trp is used in this protocol as an example, but any other amino acid (apart from Met) can be used instead.
6. Go straight from solid powder to make the desired concentrations of sucrose and urea for each buffer, and use 1 M Mg (OAc)₂ and 1 M HEPES-KOH stocks. This ensures the correct concentrations of each buffer component.
7. Different proteins prefer different membranes; this may need to be tested empirically.
8. The gel imager used depends on the preferred detection method: HRP-conjugated antibodies are detected by chemiluminescence, and fluorescent antibodies will need an imager capable of detecting fluorescence. A phosphorimager is required for detection of radioactive amino acids, or a scintillation counter if liquid scintillation counting will be used.
9. Cyclohexane is the preferred choice for making lipid films as it is removed more efficiently than chloroform during lyophilization. However, some lipids are not soluble in cyclohexane. In this case, dissolve them in chloroform and then prior to placing them in the freeze drier, remove the chloroform from the dissolved lipid by drying under a stream of nitrogen gas before placing the lipids in the freeze drier to remove the last of the solvent.

10. Times less than overnight may result in incomplete removal of the solvent. Lipid films made using chloroform may appear glassy rather than powdery.
11. Do not reuse lipids on subsequent days, always prepare fresh liposomes from lipid films. The liposome size distribution can be confirmed by dynamic light scattering, if available. Poor quality liposomes can affect downstream steps as the lipid bilayer may be leaky.
12. PURExpress comes as two tubes which need to be mixed together—solution A and B. Full instructions for this kit are on the New England Biolabs website.
13. It is essential to use RNase-free tips and wear gloves during pipetting to avoid contaminating the cell-free reaction with RNases.
14. A radioactive amino acid (such as L-[³⁵S] Methionine or L-[¹⁴C] Leucine) can be added at this step to the cell-free reaction for quantification of the protein produced. While not essential for these experiments, addition of radioactive amino acids can greatly aid in quantifying the amount of protein made and aids troubleshooting. Quantification of incorporated labeled amino acids via liquid scintillation counting (LSC) is a very accurate method for determining protein yields within cell-free systems.
15. A 2-h incubation produces a sufficient yield for most proteins, and a 4-h incubation will produce more total protein but potentially more aggregation of the target protein.
16. The cell-free reaction can be adapted to be performed within a synthetic cell (for example, a droplet interface bilayer [63] or a giant unilamellar vesicle [52, 66]), in a cuvette or on a planar surface (for example, for measurement by surface-enhanced infrared spectroscopy). If there will be no temperature control during cell-free expression when adapting the reaction, then a 5 min preincubation at 37 °C must be done to initiate transcription–translation prior to incubation at room temperature [63], otherwise PURExpress will not produce protein [63, 67].
17. A thick-walled ultracentrifuge tube is recommended and should be a suitable size for the sample volume. Be very careful to avoid mixing the layers when pipetting. Inclusion of 4 M urea in the sucrose gradient is necessary to ensure that floated protein is fully integrated across the bilayer and not merely associated with the outside of the liposome. When separating the layers, the top fraction is referred to as inserted protein, and the bottom fraction as aggregated protein. The bottom fraction also contains the PURExpress system proteins.

18. It is important to measure the function of the protein in the top (inserted) fraction of the sucrose gradient to ensure the synthesized protein is folded correctly. Alternatively, the protease assay described in Subheading 3.5 (protease digestion of inserted protein) can be used to verify the protein is inserted across the bilayer.
19. Freezing the sucrose gradient fractions at -70°C overnight is acceptable if SDS-PAGE will be done as the next step; however, it is highly recommended that any activity, topology, or protease assays are done immediately without a freeze-thaw cycle to ensure the liposomes have not been disrupted.
20. The Western blot can be done with multiple pause points as long as the membrane is kept at 4°C .
21. A lack of SDS-PAGE bands can be a result of many problems. It can be due to poor DNA quality, the wrong lipid composition causing aggregation/misfolding, poor-quality liposomes, or RNase contamination. Systematic optimization and troubleshooting will be required to eliminate these possibilities.
22. Protease digestion of cell-free expressed protein is recommended to verify that synthesized protein is inserted across the lipid bilayer. A control sample is also prepared, with DDM to solubilize the liposomes. Protease digestion of the protein in this DDM control should be observed and reproducible, if the protein target is not digested in DDM then the incubation time and temperature can be altered. Alternatively, a different protease can be chosen (for example, proteinase K [33] or chymotrypsin [13]).
23. Pausing and restarting translation is recommended for proteins which have been characterized fully by the previous steps in the method. It is important to ascertain whether any mutations made in the target protein adversely affect folding and function. This could be done by producing the construct cell-free and comparing it with wildtype protein. Alternatively, the construct could be overexpressed and purified from cells and an appropriate functional assay performed. Measurements of the thermal or chemical stability of the construct could also be performed using techniques such as circular dichroism. An alternative amino acid can be chosen if necessary, but mutagenesis should be kept to a minimum to avoid impact upon folding and function.
24. The purpose of this step is to determine how long it takes for full-length protein to be translated. The length of time it takes to produce full-length protein will be used to determine the incubation time in step 3.7.4. For example, if it takes 10 min to produce full-length protein, it will take <10 min to finish translation once it has resumed.

25. Note that the final volume is over 25 μ L; PURExpress does not decrease in activity when up to 20% over volume.
26. A corresponding construct with a stop codon at the pause/stop site should also be made, as a control to compare to the paused version and to aid data interpretation. A lack of SDS-PAGE bands corresponding to paused protein may be a sign of protein aggregation or of construct instability.

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Assembly of RNA Nanostructures from Double-Crossover Tiles

Jaimie Marie Stewart, Hari K. K. Subramanian, and Elisa Franco 

Abstract

Artificial self-assembling RNA scaffolds can be produced from many types of RNA motifs that are rationally designed. These scaffolds are of interest as nanoscale organizers, with applications in drug delivery and synthetic cells. Here we describe design strategies, production methods, and imaging of micrometer-sized RNA nanotubes and lattices that assemble from RNA tiles comprising multiple distinct strands.

Key words RNA, RNA assembly, RNA nanotubes, RNA lattices

1 Introduction

RNA molecules spontaneously fold into a variety of structural motifs due to their base-pairing interactions. In living cells, natural motifs include aptamers, siRNA, miRNA, and ribozymes that can have functional properties such as catalysis and gene regulation. In nanotechnology, folding of artificial RNA molecules has been exploited to design a multitude of artificial scaffolds with demonstrated functionality in biomedical applications such as gene silencing and targeted therapeutic delivery [1, 2].

The synthesis of increasingly complex and large artificial RNA scaffolds has been sought after with the goal of building programmable molecular machines and organelles. While the first efforts in this direction take advantage of conserved tertiary motifs [3], recent work has taken inspiration from the success of design methods developed in DNA nanotechnology [4–6]. The tiling approach originally developed for DNA components can be adapted to RNA yielding structures that reach micrometers in size like their DNA counterparts [7–9]. RNA structures demonstrated with this approach include hollow nanotubes, filaments, and lattices, which can be obtained by specifying design parameters of double-crossover (DX) tiles (Fig. 1) [8, 9]. These tiles include three to

five short, distinct RNA strands that can be assembled either by gel-purification of individual strands followed by annealing or transcription of all strands in one-pot and annealing [8]. While RNA tile components can be expressed and interact inside cells, the formation of lattices and nanotubes in vivo remains challenging to demonstrate [10]. These RNA assemblies show promise as a vehicle for therapeutic delivery [7]. Understanding RNA design principles to build large assemblies will make it possible to build customizable scaffolds for drug delivery and for the spatial arrangement of components in synthetic cells.

Here we provide protocols for the design, production, and imaging of self-assembled RNA structures from RNA DX tile motifs.

2 Materials

Nuclease-free water and RNase removal agent (e.g., RNase Zap) are used throughout the Methods.

2.1 RNA Transcription

1. Synthetic PAGE purified template DNA strands from Integrated DNA Technologies (Coralville, IA, USA).
2. 10× TE buffer: 100 mM, 10 mM EDTA, pH 8.0 at 25 °C.
3. AmpliScribe™ T7-Flash™ Transcription Kit (Lucigen): T7 RNA Polymerase, 10× Transcription Reaction Buffer, ATP (100 mM), GTP (100 mM), CTP (100 mM), UTP (100 mM), DTT (100 mM), RiboGuard RNase Inhibitor (40 U/μL), nuclease-free water, control template DNA (0.5 μg/μL), RNase-free DNase I (1 U/μL), store at −20 °C.

2.2 Polyacrylamide Gel Components

1. 10× TBE buffer: 890 mM Tris–borate, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0–8.5 at 25 °C.
2. Acrylamide/Bis 19:1, 40% (w/v) solution, store at 5 °C.
3. Disposable gel cassettes.
4. Urea.
5. Ammonium persulfate (APS): 10% (w/v) solution in water.
6. *N,N,N',N'*-Tetramethylethylenediamine (TEMED), store at 5 °C.

2.3 RNA Extraction, Elution, and Precipitation

1. Razor blades.
2. Plastic wrap.
3. Aluminum TLC plate, silica gel coated with fluorescent indicator F₂₅₄.
4. Sodium acetate.
5. Glycogen, store at −20 °C.

2.4 Assembly Buffer Components

1. Magnesium chloride.
2. 10× TAE: 400 mM Tris–acetate, and 10 mM EDTA, pH 8.5, store at 25 °C.

3 Methods

3.1 RNA Tile Design

1. Choose one of the tile designs in Fig. 1 (*see Note 1*), and choose a set of parameters from Table 1 (*see Notes 2 and 3*). Using the parameters of choice, the sequence of the RNA strands required for assembly can be designed using the DNA Design Toolbox [11]. While this toolbox is primarily for DNA nanostructure design, it can be used as a first-pass filter to design sequences that do not have bad secondary structures (both intra and inter-strand) at a specified temperature.
2. Confirm the absence of unwanted secondary structures in the designed RNA strands using NUPACK [12].
3. The number of sticky ends can be changed if one needs to tune the melting temperature of the nanotube assembly. Changing

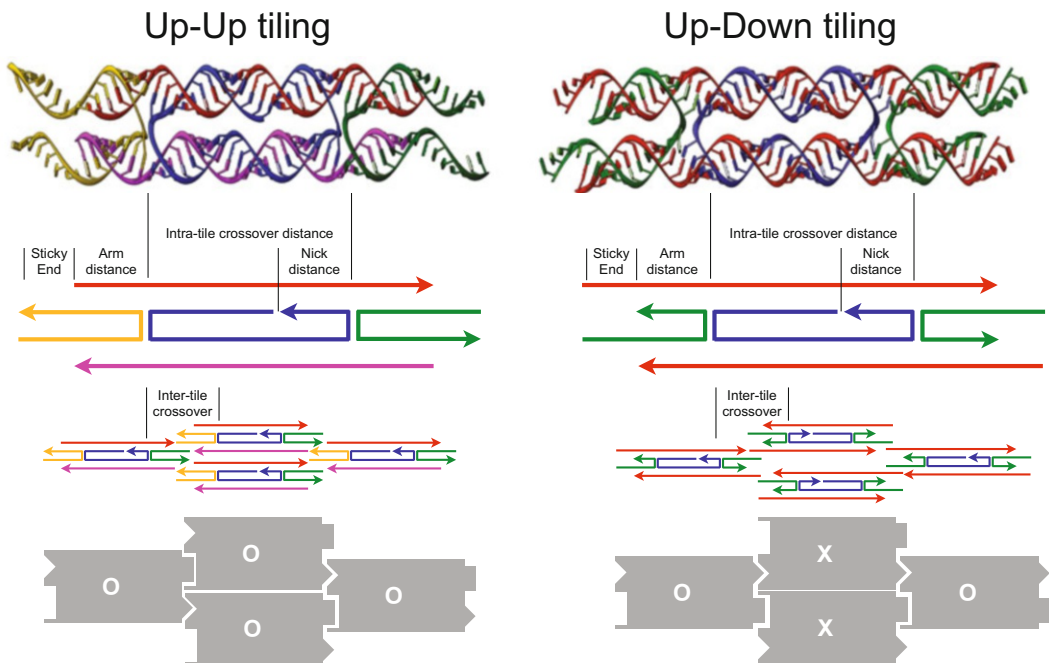


Fig. 1 RNA-based tilings considered in this work. The “up-up” tiling has all the tiles showing the same side up and the “up-down” tiling has every other column of tiles in the assembly showing the same side up. The top row of the figure shows the 3D model of monomer tiles for each tiling. The second row shows an abstraction of the monomer tile that identifies tile domains that influence the assembly outcome. The third row illustrates how the monomers assemble to form an array and the bottom row shows the arrangement of faces of the monomers in the array in each tile type

Table 1
Parameters for RNA tile designs

Tiling type	Inter-tile crossover distance	Sticky end	Nick distance	Arm distance	Predominant structure formed	Avg. nanotube length
Up-up	22	6	8	8	2D arrays	NA
Up-up	23	7	8	8	Nanotubes	1.5 μm
Up-up	24	8	8	8	Nanotubes	1.5 μm
Up-up	25	9	8	8	2D filaments	NA
Up-up	26	10	8	8	2D filaments	NA
Up-down	28	5	9	11	Nanotubes	0.5 μm
Up-down	28	5	13	11	Nanotubes	0.5 μm
Up-down	28	5	11	11	No structures	NA

sticky-end length without adjusting any other design parameter will change the inter-tile crossover distance and result in different types of structures formed as shown in Table 1.

4. If parameters not listed in Table 1 are going to be tried, it is important to build 3D computer model of the design to ensure that the crossovers positions provided work with the A-form geometry of the RNA-RNA duplex (*see* **Note 4**). The angle with which the bases tilt with respect to the central helical axis can make crossovers at some pairs of positions impossible [4]. A 2D line drawing as shown in Fig. 1 does not capture this detail so a 3D computer model is needed.
5. RNA needed for the assembly can either be purchased directly from a vendor or instead the DNA needed for in vitro transcription of RNA strands can be purchased from a vendor. RNA synthesis by in vitro transcription can be done as described in Subheadings 3.5 and 3.6. The DNA templates will be ordered with a T7 RNA polymerase promoter [7].

3.2 DNA and RNA Quantitation

UV absorbance quantifies nucleic acids by measuring the absorbance of light through a DNA or RNA liquid solution to determine the concentration of molecules.

1. DNA or RNA purchased from the vendor is resuspended in nuclease-free water and vortexed until completely dissolved (*see* **Note 5**).
2. Determine the concentration of DNA and RNA by measuring the absorption at 260 nm according to Beer Lambert’s law using sequence-specific extinction coefficients.

3.3 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) provides useful information by separating DNA and RNA molecules by their electrophoretic mobility.

3.3.1 Denaturing

Denaturing PAGE separates DNA and RNA molecules based on size and is used to determine sequence length of strands.

1. Prepare a 100 mL 8% (w/v) 19:1 acrylamide:bis and 7 M urea premix solution. Add 42 g of urea to 25 mL of nanopure water. Heat the solution until the urea is completely dissolved (*see Note 6*). Then add 10 mL of 10× TBE, and the appropriate volume for the desired polyacrylamide percentage. Typically, we prepare 10% polyacrylamide gel by adding 25 mL of the 40% acrylamide/bis 19:1, and then fill the remaining volume to 100 mL.
2. Cast the gel by adding in a 50-mL falcon tube 8 mL of the premix solution, 48 μ L 10% APS, and 3.2 μ L TEMED. Gently rock the tube avoiding the creation of bubbles.
3. Slowly pour the solution in an upright empty 8 cm \times 8 cm with 1 mm width cassette.
4. Carefully add the gel comb, avoiding the creation of bubbles.
5. Allow the gel to polymerize for 2 h (*see Note 8*).
6. After the gel has polymerized, carefully remove the comb as not to disturb the wells.
7. Rinse the wells with running buffer, 1× TBE, to remove any residual unpolymerized remains.
8. Place the gel in the electrophoresis chamber and add 1× TBE buffer.
9. Prerun the gel at 10 V/cm at room temperature or 45 °C in 1× TBE for 10 min.
10. Carefully pipette the sample and loading buffer into the wells.
11. Run the gel for 50–60 min at room temperature or 45 °C.

3.4 Annealing dsDNA Templates

1. Dilute PAGE-purified DNA strands in nuclease-free water.
2. Mix the template (t) and non-template (nt) strands at a target concentration of 20 μ M in a buffer of 10 mM Tris, 20 mM NaCl, and 1 mM EDTA.
3. Apply a heat ramp on a thermocycler or PCR machine. Heat the sample up to 95 °C, holding the maximum temperature for 5 min, and cooling to 20 °C at a rate of -1 °C/min.

3.5 RNA Transcription

1. In a 0.5-mL reaction tube, mix:
 - (a) 1× T7-Flash reaction buffer.
 - (b) 9 mM each NTPs.
 - (c) 10 mM DTT.

- (d) 1 μ g DNA template.
 - (e) 20 units of RiboGuard RNase Inhibitor.
 - (f) 1 unit of AmpliScribe T7-Flash enzyme solution.
2. Incubate the transcription mixture at 37 °C for 4 h (*see Note 10*).
 3. After the transcription reaction, to remove the DNA template, add 1 unit of RNase-free DNase I and incubate at 37 °C for 10 min.

3.6 RNA Purification

Purified RNA can be chemically synthesized and ordered directly from a vendor. Alternatively, one can produce RNA via an enzymatic reaction and purify the RNA.

1. Assemble a transcription mix (typically 20 μ L) as described in AmpliScribe™ T7-Flash™ Transcription Kit instructions.
2. Incubate the reaction mix at 37 °C for 4 h.
3. Add 1 μ L DNase I and incubate at 37 °C for 10 min to remove DNA templates.
4. Add equal volume of 2 \times denaturing loading buffer, then load it on a 10–15% denaturing gel.
5. After the run, place an aluminum TLC plate, and silica gel coated with fluorescent indicator F254 and cover with plastic wrap.
6. Crack open the gel cassette and place the gel on the plastic wrap on the aluminum TLC plate.
7. Use a short-wave UV (~254 nm) light source to observe RNA transcripts in the gel.
8. Excise the RNA bands of interest, and chop them into small gel pieces. Collect the pieces in a 0.5-mL tube, filling about half the tube.
9. Add 350 μ L of 0.3 M sodium acetate (pH: 5.2–5.3) to completely submerge the pieces and incubate at 42 °C overnight.
10. Transfer the supernatant to a fresh tube and add 100 μ L of the 0.3 M sodium acetate (pH: 5.2–5.3) to the gel pieces. Vortex the content and transfer the 100 μ L of buffer to the other 350 μ L in the fresh 1.5-mL tube.
11. Add 1 mL of freezer cold ethanol (–20 °C) to the supernatant and 1 μ L of glycogen (20 μ g/ μ L) and vortex.
12. Incubate the sample at –20 °C overnight.
13. Spin the tube at >13,500 rpm at 4 °C for 15 min in a centrifuge.

14. Decant the supernatant with a pipette, then add 500 μL of 70% freezer cold ethanol, and spin (19,400 rcf) at 4 °C for 5 min.
15. Repeat **step 14**.
16. Remove as much supernatant as you can without disturbing the pellet.
17. Open tubes and place in vacufuge and allow to spin at room temperature for 10 min.
18. Resuspend the pellet in nuclease-free water and determine the concentration.
19. Store extracted and purified RNA at -20 °C.

3.7 Assembling RNA Nanotubes by Standard Anneal

1. In a 0.5-mL reaction tube, mix:
 - (a) $1\times$ TAE.
 - (b) 12.5 mM MgCl_2 .
 - (c) 1 μM of each strand.
2. Apply a heat ramp on a thermocycler or PCR machine. Heating the sample up to 70 °C, holding the maximum temperature for 5 min, and cooling to room temperature at a constant rate over 24 h.

3.8 Assembling RNA Nanotubes by One-Pot Transcription and Anneal

1. In a 0.5-mL reaction tube, mix:
 - (a) $1\times$ TAE.
 - (b) 22.5 mM MgCl_2 .
 - (c) 2.25 mM of each NTP.
 - (d) 10 mM DTT.
 - (e) 1/6 volume dilution of AmpliScribe T7-Flash Enzyme Solution.
 - (f) 0.5–1 μg of each DNA template.
2. Apply a heat ramp on a thermocycler or PCR machine. Incubate the sample at 37 °C for 15 min, then heat the sample up to 70 °C, holding the maximum temperature for 5 min, and cooling to room temperature at a constant rate over 24 h.

3.9 Atomic Force Microscopy

RNA nanotubes can be imaged in buffer with nanometer resolution using atomic force microscopy.

1. Place metal specimen disc on a hot plate and melt a small amount of a hot glue stick.
2. Place a mica disc that has been cut into a square and firmly secure it on the metal specimen disc.
3. Cleave the mica with a piece of tape.
4. Place 5 μL of sample on the freshly cleaved mica and allow it to incubate on the surface for 30 s.

5. Add 25 μL of the sample buffer on top of the sample on the mica.
6. Add 25 μL of the sample buffer to the AFM tip.
7. Sharp Nitride Lever (SNL) tips from Bruker with a nominal spring constant of 0.24 N/m are used for imaging, with a drive frequency of 9–10 kHz (*see* **Note 12**). The AFM data are collected with a Digital Instruments Multimode AFM, equipped with a Nanoscope III controller and quantitated using ImageJ.

3.10 Fluorescence Microscopy

1. Add 1–2 μL of a fluorescent labeled sample (*see* **Note 13**) on a precleaned coverslip.
2. Carefully place a clean glass slide on the sample on the coverslip.
3. Place one drop of immersion oil on a 60 \times oil immersion objective and position the assembled slide on the stage.
4. Select the correct filter and focus on the sample.
5. Images are obtained with a NIKON TI-E inverted fluorescence microscope. Cy3 filter was used to image all tiles with Cy3 labeled S3 strand. A 60 \times oil immersion objective is used to collect all images, with a standard exposure time of 600 ms.

4 Notes

1. There are two DX tile designs that have been shown to produce nanostructures. Figure 1 shows their 3D structure, strand composition, and tiling pattern. The “up-up” tiling system self-assembles with all the monomers having the same side up. The “up-down” tiling system self-assembles with tiles alternatingly facing up and down as shown in Fig. 1. For “up-up” tiling systems, monomer tiles with five unique strands have been shown to work [9]. For “up-down” tiling systems, monomer tiles with as low as three unique strands have been shown to work [8]. While the “up-down” design is more parsimonious with only three distinct strands, the “up-down” tiling designs are more versatile both in terms of the number of uniquely modifiable strands and resultant morphologies.
2. The important parameters to determine before the start of the design are: (a) inter-tile crossover distance, (b) sticky-end length, (c) nick distance, and (d) arm distance. These parameters are shown in Fig. 2.
3. Table 1 shows the resulting nanostructures and their characteristics based on the choice of the parameters listed above. Choose the tiling type and parameter values depending on the type and characteristics of nanostructures desired. The parameter combinations not listed in this table have not yet been tested.

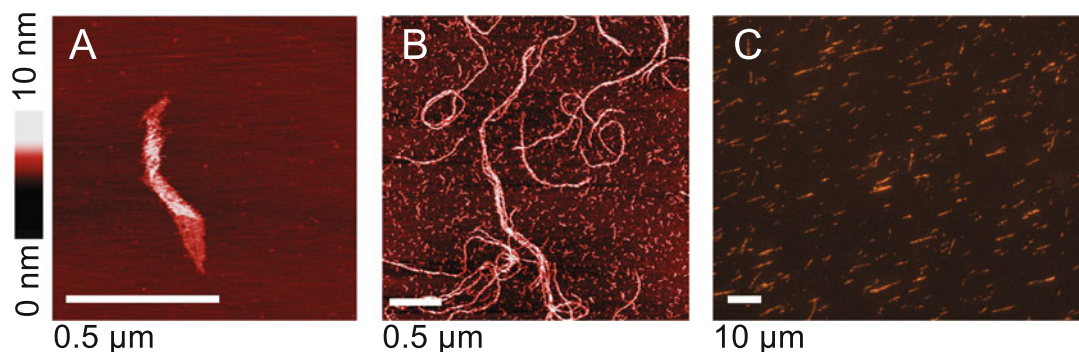


Fig. 2 Example AFM and fluorescence microscopy images of RNA tile-based assemblies. (a) AFM image of up-down tile assembly. (b) AFM image of up-up tile assembly. (c) Representative fluorescence microscopy image of up-up tile assembly

4. Multiple tools are available to build 3D models: (a) PDB models can be built using NAMOT software [13] and edited and rendered using Chimera software [14]. (b) You can also build PDB models using simRNA [15] and export the PDB file to oxDNA [16, 17] if you want to run simulations with the model. PDB files can be converted to oxDNA using the TacoxDNA server [17]. Additionally, caution should be taken when using the TacoxDNA server for manipulating RNA as it currently does not support the import of uracil bases
5. Lyophilized DNA or RNA can also be resuspended in tris-buffer for longer storage time; however, the downstream application should be taken into account when selecting the storage buffer.
6. To quickly dissolve urea in water, microwave the solution in 10 s increments, careful not to let the solution boil.
7. Denaturing polyacrylamide premix can be stored at 4 °C for up to 4 weeks [18].
8. Gels can be cast and stored for later use in buffer wrapped with plastic wrap 4 °C for up to a week.
9. We recommend using a 10-basepair dsDNA ladder (Invitrogen) as a size marker and to stain gels with staining solution, SYBR gold (Molecular Probes) for quantitation of DNA and RNA molecules [19].
10. To increase the amount of transcripts produced, the incubation temperature can be changed from 37 °C to 42 °C.
11. According to the manufacturer, transcription reactions can be scaled linearly to increase yield of RNA products.
12. When imaging via AFM, sometimes samples will aggregate on the mica surface, so you may have to search the surface for the sample.

13. When preparing fluorescently labeled RNA strands, adding more than 25% will result in a lack of assemblies or in malformed assemblies. Adding 10% of fluorescently labeled strands is optimal.

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Cell-Free Biosensors and AI Integration

Paul Soudier, Léon Faure, Maish Kushwaha, and Jean-Loup Faulon

Abstract

Cell-free biosensors hold a great potential as alternatives for traditional analytical chemistry methods providing low-cost low-resource measurement of specific chemicals. However, their large-scale use is limited by the complexity of their development.

In this chapter, we present a standard methodology based on computer-aided design (CAD) tools that enables fast development of new cell-free biosensors based on target molecule information transduction and reporting through metabolic and genetic layers, respectively. Such systems can then be repurposed to represent complex computational problems, allowing defined multiplex sensing of various inputs and integration of artificial intelligence in synthetic biological systems.

Key words Metabolite biosensors, Transcription factors, Machine learning, CAD, Artificial neural networks, Perceptron

1 Introduction

Metabolite biosensors emerged as a major application of synthetic biology. Repurposing biological systems naturally present in various organisms enabled the development of synthetic sensing devices [1] giving rapid and inexpensive point of care measurement of various molecules of interest levels in a broad range of samples with applications in health [2], environment [3], industries, and fundamental research [4]. The potential of these types of devices lies in the fact that they are able to deliver information on the presence and levels of certain chemicals without the need for expensive reagents, trained operators, and large-size facilities. Transcription factor-based biosensors are a subset of those sensing devices repurposing transcription factor and inducible promoter for the quick detection of molecules of interest.

Cell-free biosensors emerged as an alternative for traditional whole-cell biosensors, solving several issues carried by these systems [5]. Cell-free biosensors are efficient, generating big signal over noise ratios [6]. They are suitable for the detection of molecules

toxic for bacterial growth or molecules that are not able to cross the cell membrane. Their ability to be freeze dried on paper enables long-term storage at room temperature without loss of activity pushing their use as point-of-care devices, especially in low resource communities [7]. The absence of living organisms in it is also facilitating their industrial developments with reduced regulatory issues and low biosafety concerns. Finally, the ability to express each gene from distinct DNA fragments (plasmids or linear fragments) [8] and to fine-tune their expression levels by varying their respective concentrations enable a fast development and optimization of new biosensor candidates or more complex devices relying on them. This central property of straightforward tuning of gene expression is necessary for the development of the complex information processing systems that will be described in this chapter.

The main issue encountered in the development of transcription factor (TF)-based biosensors is the limited number of molecules for which an interacting transcription factor has been described. The list of these metabolites known to trigger transcriptional response, either in natural systems or in the context of synthetic biosensors, has been described in various databases [9, 10] and compiled in a dataset of small molecules triggering transcriptional and translational cellular responses [11].

In order to detect molecules for which associated transcription factors are not available, new methodologies have been developed relying on the use of metabolic pathways. It has indeed been shown that enzymes could be used to convert nondetectable molecules into molecules known to regulate a characterized transcription factor [12]. This framework has been formalized and showcased as the sensing-enabling metabolic pathway (SEMP) concept [13]. This shifted the problem from finding a TF binding the molecule of interest to finding any potential enzymatic route between the molecule of interest and the known set of detectable molecules. This was addressed by repurposing bioinformatic tools traditionally used for retrosynthesis [14] into a platform able to find potential SEMP for any molecule of interest. This web-service called SENSIPATH is a CAD program predicting pathways of up to two steps allowing detection for any query compounds [15]. This approach was then used for the development of a plug and play cell-free workflow where enzymes formalized as metabolic transducers were used as interchangeable modules converting various chemicals into detectable molecules called effectors [6]. Such biosensors were found to be highly efficient, the cell-free system potentialities allowing to reach optimal response for each biosensor by a systematic tuning of each component involved (reporter, transcription factor, and enzyme encoding DNA). They were also applied for the commercially relevant sensing of molecules of interest in complex real-world samples (preservative in beverage, disease biomarker and drug in clinical samples).

In addition to its biosensing potential, this framework has also then been applied to the field of information processing. Indeed, the constructed devices sensing various molecules can also be conceptualized as information processing systems, converting an input signal (the concentration of detected molecules) into an output one (the level of protein synthesized by the reporter plasmid). This processing device uses the continuous concentration of input metabolites as analog signals able to be processed by the metabolic or the genetic layer featured in the system. Multiple molecules can be continuously converted into the same effector at the same time allowing the construction of multi-input devices derived from analog adders.

Starting from this basis, we have implemented a machine learning architecture that allows integration of AI computations in cell-free systems [16]. One of the simplest architectures for signal processing is the perceptron [17]. A single-layer neural network transforms an input vector into an output value through the application of a weighted sum of the input followed by a thresholding function on the obtained result. Input data can be integrated as categorical or numerical (discrete or continuous). This signal-processing unit has been implemented in a cell-free system to enhance its multiplex sensing abilities [16]. This system is indeed capable of classifying complex samples in two categories (above or below a threshold of the reporter gene expression) based on the recognition of certain patterns of molecules present in those samples. A theoretical perceptron was first constructed and trained “*in silico*” to compute the optimal set of weights required to solve a defined problem of classification. The perceptron was then implemented in cell-free using a molecular model, giving concentrations of enzymes coding DNA that correspond to the weight of each input (*see* Fig. 1). The thresholding applied through the effector biosensor dose–response curve.

Different elements of the computing unit have a defined terminology: e.g., transducer, adder, actuator. A transducer, in cell-free terms, means one or more enzymes transforming one molecule into another. An adder consists of several reactions having different substrates but yielding the same compound. An actuator is simply a way for the cell-free system to report its activity, through the expression of a reporter gene, for example. Theoretically, many more parameters of a cell-free reaction could be implemented in such signal-processing manner. The diversity of elements can be increased, as well as the number of layers in the perceptron, and the number of detectable molecules.

Here we describe a straightforward methodology that details how to use CAD tools to identify potential new biosensors for a chosen molecule, how to build and test these biosensor candidates, and finally how to repurpose these cell-free biosensors into signal processing devices to implement neural computing in biological systems.

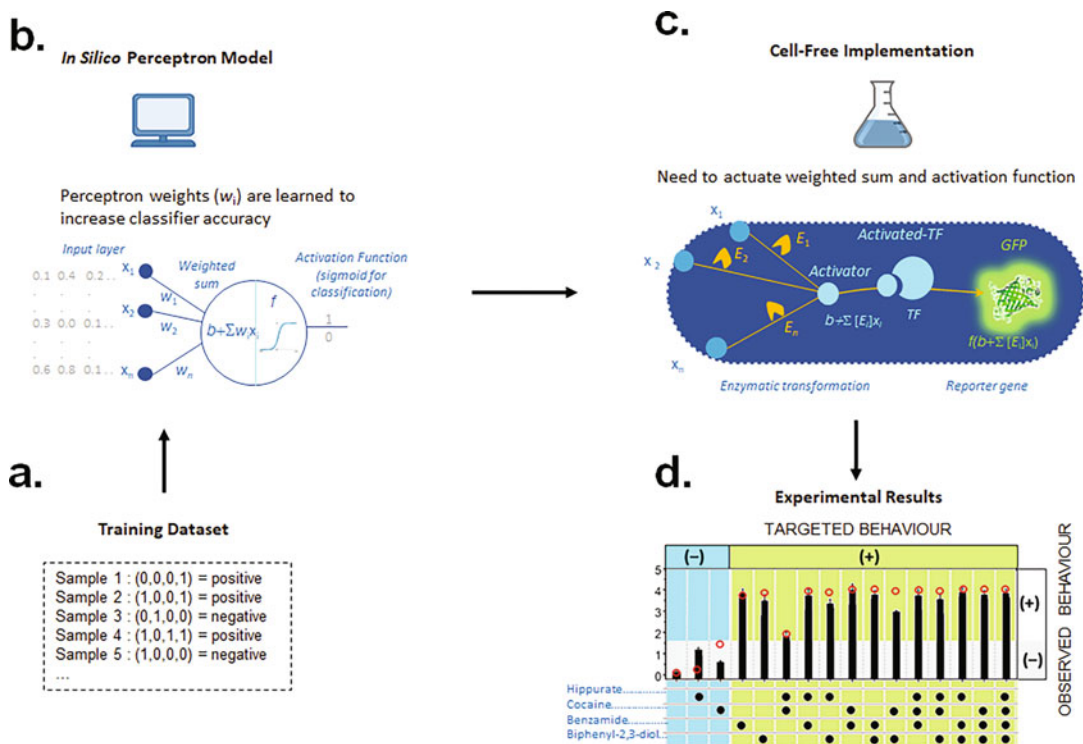


Fig. 1 Implementation of a clustering problem in a biological system through the metabolic perceptron: to obtain a genetic device operating multiplex sensing on a set of defined clustered samples an in silico model (**b**) is first trained with the input dataset (**a**). The results give information on the best set of DNA concentration to implement the problem in a cell free environment (**c**). The device is evaluated for response to a set of samples with various compositions (**d**)

This method to this date has been applied for the construction of a single layer 4-input perceptron but is easily scalable for more inputs and adaptable for more complex designs including multi-layer perceptron and other types of computational architectures. An example of potential implementation of a multilayer perceptron using this system can be found in the supplementary figure 14 of the original metabolic perceptron paper [16].

2 Materials

2.1 Preparation of Cell-Free Extract and Buffer

1. Extract preparation equipment.

In addition to common lab equipment, materials for the preparation of cell extract include large-volume centrifugation equipment (for 1 L bottles) and a French press.

2. Extract preparation media and buffers.

Cells are grown in 2YTP media (31 g/L 2xYT, 40 mM potassium phosphate dibasic, 22 mM potassium phosphate monobasic).

S30A and S30B buffers are also required for the preparation of the cell extract.

They are prepared with the following components:

- (a) S30A = (14 mM Mg glutamate, 60 mM K-glutamate, 50 mM Tris, pH 7.7).

S30A is titrated using acetic acid and 2 mM DTT is added just before use.

- (b) S30B = (14 mM Mg glutamate, 60 mM K-glutamate, ~5 mM Tris, pH 8.2).

S30B is titrated using 2 M Tris and 1 mM DTT is added just before use.

3. Chemicals for the reaction buffer.

- (a) Solutions of the 20 canonical amino acids, 19 of them concentrated at 168 mM, except leucine that is concentrated at 140 mM.
- (b) Chemicals for the energy solution, including individual solutions of: HEPES pH 8, ATP, GTP CTP, UTP, tRNA, CoA, NAD, cAMP, folinic acid, Spermidine, and 3-PGA, all of them concentrated close to their limit of solubility.
- (c) Additional components for the reaction buffer including K-glutamate solution at 3000 mM, Mg-glutamate solution at 100 mM, PEG8000 solution at 40% w/w, and DTT solution at 1 M.
- (d) Reference plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 (Addgene #40019) for reaction buffer calibration experiments

2.2 Cloning of DNA Parts and Production of Plasmids

Additional materials for the construction and production of plasmid encoding the elements for biosensors include:

1. High-fidelity PCR material (Q5 High-Fidelity 2× Master Mix or equivalent).
2. Template plasmid for backbone amplifications (plasmids #114597 and #114598 from Addgene) and respective primers:

FWD primer Backbone for CDS:

FBC: cccGGTCTCtGCTTactttatctgagaatagtc.

REV primer Backbone for CDS:

RBC: cccGGTCTCtCATCcatatctcttcttaaagttaaac.

FWD primer Backbone for Promoter:

FBP:cccGGTCTCtATGCgtaaaggcgaagagctgttc.

REV primer Backbone for Promoter: RBP:
cccGGTCTCtTAAGaatagtaatacaggatccgaatcgtttcag.

3. Thermocycler.
4. TAE buffer, 50×.
5. 1% agarose gel with SYBR™ Safe DNA Gel Stain or equivalent.
6. Monarch® DNA Gel Extraction Kit or equivalent.
7. Thermo Scientific NanoDrop 1000 for DNA concentration determination or equivalent.
8. Thermo Scientific™ Savant™ DNA SpeedVac™ Concentrator Kits to concentrate DNA if required.
9. BsaI enzyme.
10. T4 DNA ligase.
11. T4 DNA ligase buffer.
12. DH5α competent cells.
13. Lysogenic broth liquid medium for cell culture.
14. Lysogenic broth + agar solid medium and ampicillin for cell plating.
15. Monarch® Plasmid Miniprep Kit or equivalent.
16. Macherey-Nagel™ Kits NucleoBond™ Xtra Maxi for plasmid purification.
17. Sequencing plasmids seqF: gataggttaaggaacgg and seqR: ttgatgcctggcaccaac for sequencing verifications of the inserts.

2.3 Running Cell-Free Reactions

1. Plate reader equipment: Typically, Synergy HTX Multi-Mode Reader from Biotek®.
2. 384-well black plate, optically clear polymer bottom.
3. Adhesive PCR plate seals.
4. Analytical-grade chemical powder or solution for any sensed effector and target molecule.

2.4 In Silico Materials

The R programming language (version 3.2.3) was used for fitting experimental data from actuator and transducers, as well as for defining and solving classification tasks. One can perform calculations described in Subheading 3.6. with regular computational power, and computational clusters are not required. Other programming languages can be used, but we recommend to use R in order to take advantage of our git repository containing all files from Pandi et al. work [16]. The repository is freely available here: https://github.com/brsynth/metabolic_perceptrons/tree/master/cell_free.

3 Methods

3.1 Identifying Sensing Routes for a Target Molecule (Fig. 2)

1. Choose a target molecule to sense. Check that the molecule you want to sense is not one of the components of the cell-free buffer you are using (*see Note 1*).
2. Identify the InChi identifier or your target. To do so, you can either search it by name on the database Pubchem (<https://pubchem.ncbi.nlm.nih.gov/>) or search for the InChi in the section Identifier. The other possibility is to go on Pubchem sketcher: (<https://pubchem.ncbi.nlm.nih.gov/edit3/index.html>) and to draw the molecule to retrieve its InChI by replacing SMILES by StdInChI.
3. Go to <http://sensipath.micalis.fr/> to access the online platform. Use the section “Query with a Standard InChI” and paste the InChI of your molecule.
4. Run the tool first for one step pathways. If the results are not satisfying, you can run a second attempt for two step pathways.

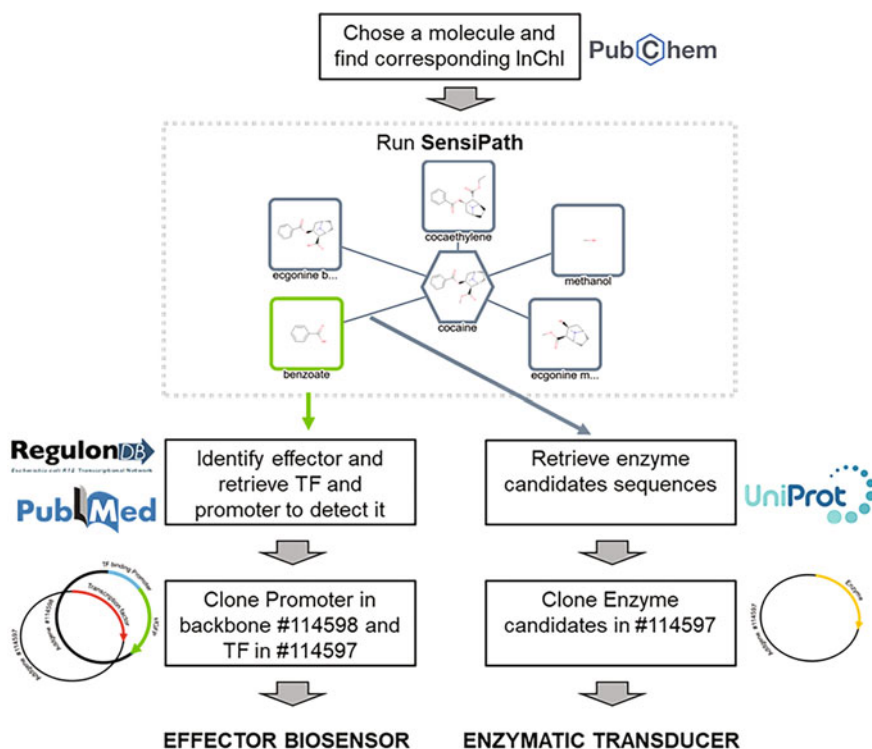


Fig. 2 CAD pipeline for Cell-Free biosensor Design. Main steps of the biosensor design and construction process are presented, from the identification of the chemical identifiers for the target molecule, to the cloning of the DNA parts in plasmids. The sensipath results showed here are the output given for a query of 1 step detection potentialities for cocaine

This can be the case if no pathway to a detectable molecule (in green on the graph view) is identified or if the identified detectable molecule is unsuitable for cell-free biosensors (components of the cell-free buffer) (*see Note 2*).

5. Identify the promising effector molecules from the “Pathways view” section.

If the molecule you want to sense appears in green, it means it is directly detectable without need for enzymatic conversion. For developing a biosensor for it you can skip the enzyme-related steps.

6. Download the database of detectable molecules from: https://github.com/brsynth/detectable_metabolites and isolate the lines corresponding to the identified effector. First identify transcription factor that interacts with your component. You can use column E to retrieve the names of it or, if this information is not available, use the column B that contains literature reference of papers describing potential sensing mechanisms for the molecule.
7. Once you find a TF of interest, you have to find the promoters that are potentially regulated by it. To do so, you can search for it in databases such as RegulonDB (<http://regulondb.ccg.unam.mx/>) for *E. coli* and Subtiwiki (<http://subtiwiki.uni-goettingen.de/>) for *B. subtilis*. You can also try a naive bibliographic search for this regulator to identify features linked to it like regulated promoters but also mechanism of action and possible existing design of biosensors using it.
8. Identify the enzymes converting the molecule you want to sense into the effector for which you found a TF. By clicking on the edges from the SENSIPATH graph view, you can retrieve the references associated with the enzymatic reaction of interest. From this point using cross-references between databases or identifiers such as EC number, you can find enzyme candidates in UNIPROT with described catalyzed reactions matching your expectations. You can also use the computational tool Selenzyme (<http://selenzyme.synbiochem.co.uk/>) with SENSIPATH provided references to find potential hits for other enzymes candidates (*see Note 3*).
9. Retrieve DNA sequences for the identified parts. TF and enzymes sequences can be codon optimized using any of the available tools. Promoter sequence is often defined as the 200 nucleotides before the start codon of a regulated gene. For troubleshooting purposes, various size promoters can be synthesized to test for their response to the TF and their transcription initiation ability (*see Note 4*).

3.2 Constructing Candidate Biosensors Plasmids

1. Synthesize the previously isolated sequence of your transcription factor, its regulated promoter, and any enzyme required to convert the molecule you want to detect into the TF binding effector.
2. Design and orders primers for golden gate cloning of the synthesized parts. Genes (TF and enzymes) require overhangs in the format:

FWD: ccGGTCTCtGATG.... REV: ccGGTCTCtAAGC....

Promoters require overhangs in the format:

FWD: ccGGTCTCtCTTA.... REV: ccGGTCTCtGCAT....

3. Run high fidelity PCRs to amplify with the correct golden gate overhangs the vectors and the synthesized inserts. The reaction typically consists of pipetting 25 μ L of 2 \times Q5[®] polymerase master mix, 2.5 μ L of FW and RV primer at 10 μ M, 1 μ L of template DNA (at concentration around 100 ng/ μ L), and 19 μ L of water.

This mix is then typically incubated in a thermocycler applying the following program: 30 s at 98 °C then 35 cycles with (10 s at 98 °C, 30 s at $T_m + 3$ °C, 30 s/kb at 72 °C) then 2 min at 72 °C. T_m being the lowest melting temperatures of the two primers and kb being the size of the amplicon in kilo bases. Use primer pairs FBP/RBP on the template plasmid 114598 to reamplify the linearized reporter backbone and the primers FBC/RBC on the template plasmid 114597 to reamplify the linearized backbone for enzyme or TF. Use the newly designed primers from **step 2** on the synthesized parts from **step 1** to reamplify the inserts.

4. Run an electrophoresis on the PCR product on a 1% agarose gel stained with SYBR safe using an appropriate DNA ladder to be able to discriminate your amplicon by its size. After an approximate time of 30 min at 100 V, identify and cut the band of the expected size by imaging on a blue-light transilluminator.
5. Recover the DNA from the Gel using a DNA Gel extraction kit, following the kit's instructions to purify your DNA fragment. Determine the titer of purified DNA using a NanoDrop spectrophotometer.
6. Prepare a golden gate reaction to insert each fragment in its respective backbone (*see Note 5*). To do so, you need to calculate the molarity of your DNA. You can use the tool available at <https://nebiocalculator.neb.com/#!/dsdnaamt> using the NanoDrop determined concentrations of each purified DNA fragment. For the golden gate reaction, incubate 100 fmol of insert with 50 fmol of linearized backbone in a tube with 1 μ L T4 DNA ligase, 1 μ L BsaI enzyme, 2 μ L T4

DNA ligase buffer, and water to adjust the volume to 20 μL . Incubate the mix at the 37 °C for 1 h and then 16 °C for 5 min (*see Note 6*).

7. Transform DH5 α competent cells with the golden gate reaction product. Incubate 5 μL of golden gate product with 50 μL chemically competent cells at 4 °C for 30 min.
8. Heat-shock at 42 °C for 45 s.
9. Incubate at 4 °C for 3 min.
10. Add 300 μL LB media and incubate at 37 °C for 1 h.
11. Finally spread 100 μL of the final mix on LB agar + ampicillin (100 $\mu\text{L}/\text{mL}$) plates and incubate overnight at 37 °C.
12. Small-scale culture for screening:
 - (a) Select 4 colonies per assembly and inoculate them in 3 mL LB medium + ampicillin (100 $\mu\text{L}/\text{mL}$) overnight.
 - (b) Purify the plasmids from 2 mL the bacterial cultures using a plasmid miniprep kit and use the primers seqF and seqR to check the integrity of each cassette by Sanger sequencing.
 - (c) Save one correct clone per construction by freezing the rest of the liquid culture at -80 °C after addition of glycerol to final concentration of 25%.
13. Large-scale culture for plasmid production. You will require big quantities (>100 μg) of each plasmid to run the cell-free reactions for the characterization and optimization of each biosensor candidate (*see Note 7*). To do so, you have to realize large-scale culture and plasmid extraction for each construction.
 - (a) Inoculate 300 mL of LB + ampicillin (100 $\mu\text{L}/\text{mL}$) from the -80 °C glycerol stock and grow the cells overnight.
 - (b) Pellet the cells by spinning them at $6000 \times g$ for 15 min at 4 °C.
 - (c) Use the Maxiprep kit to recover plasmid DNA from the pellet.
 - (d) After the last step of your purification, resuspend the precipitated DNA in 200 μL pure water in order to have a final solution at high concentration.
 - (e) Measure the final concentration using a nanodrop and adjust it at 1 μM by either diluting it with water or concentrating it using a SpeedVac machine.

3.3 Preparing in House Cell-Free Extract and Buffer

The method briefly described here is adapted from a widely used protocol [18] of 3-PGA powered cell-free mix with minor modifications mostly concerning the lysis method and the starting strain.

3.3.1 Extract Preparation

1. Inoculate BL21* cells from an overnight culture in 4 L of 2YTP medium.
2. Stop the culture at OD 2 and pellet the cells by centrifugation for 12 min at $5000 \times g$ at 4 °C in 4-L bottles.
3. Rinse the cells twice by successive resuspension/centrifugation steps with 250 mL of S30A buffer.
4. Resuspend the pellets in 40 mL of S30A buffer and transfer the suspension to preweighed 50-mL Falcon tubes.
5. Centrifuge the tubes at $2000 \times g$ at 4 °C during 8 min, discard the supernatant, and after weighing the pellets freeze them overnight at -80 °C.
6. Thaw the cell-pellet on ice, weigh the pellet, and resuspend them in 1 mL of S30A buffer per gram of cell pellet.
7. Lyse the cells by passing the whole flow once through a French press at 15,000 psi (*see Note 8*).
8. Centrifuge the lysate at $12,000 \times g$ at 4 °C during 30 min.
9. Incubate the supernatant for 1 h at 37 °C with a 220 rpm shaking before a second centrifugation at $12,000 \times g$ at 4 °C during 30 min.
10. Transfer the supernatant to a 12–14 kDa MWCO dialysis cassette and incubate the cassette overnight in 2 L S30B buffer at 4 °C (*see Note 9*).
11. After a last centrifugation at $12,000 \times g$ 30 min 4 °C, aliquot the supernatant (500 μ L in 1.5-mL tubes) and flash-freeze them in liquid nitrogen before storing at -80 °C.

3.3.2 Buffer Preparation

1. Starting from individual solutions of chemicals dissolved in pure water (described in detail in the original paper [18]), prepare an amino acid (at 4 \times concentration) and an energy solution mix (at 14 \times concentration) that will be used for buffer preparation. The amino acid mix has to be prepared by mixing all the 20 canonical amino acids at a final concentration of 6 mM except for leucine at 5 mM. Prepare energy solution with HEPES pH 8 700 mM, ATP 21 mM, GTP 21 mM, CTP 12.6 mM, UTP 12.6 mM, tRNA 2.8 mg/mL, CoA 3.64 mM, NAD 4.62 mM, cAMP 10.5 mM, folinic Acid 0.95 mM, Spermidine 14 mM, and 3-PGA 420 mM. Store each mix at -80 °C.
2. Using previously prepared extract, amino acid mix, and the energy solution, you will have to evaluate in this order the best concentration of: (a) Mg-glutamate, (b) K-glutamate, (c) DTT, and (d) PEG8000 to add to your buffer to optimize the protein production of your extract. You will run four consecutive calibration experiments of 8 h cell-free reactions. For each calibration, prepare a master mix for 12 reactions by

mixing 88 μL extract, 66 μL amino acid mix, 18.86 μL energy solution, and 13.2 μL of the Addgene plasmid #40019 concentrated at 200 nM. Add the remaining three components that you are not calibrating for from the following four (Mg-glutamate, K-glutamate, PEG8000, and DTT) at either a starting concentration (if the optimum has not been determined yet) or the optimal concentration determined in a previous step. Finally, add water to this mix to reach a final volume of 237.6 μL . Prepare each calibration reaction by mixing in PCR tubes 19.8 μL of the master mix with 2.2 μL of the tested component concentrated at 20 \times . Then, take 20 μL from each PCR tube and pipette them inside individual wells of a 384-well microplate before incubating the plate for 8 h while measuring GFP signal produced (ex: 458 nm, em: 528 nm).

The concentration that leads to the highest GFP signal at 8 h is identified as the optimal one to be used for future calibrations and run.

The starting concentrations for each component are the following ones: (6 mM for Mg-glu, 80 mM for K-glu, 0 for DTT, and 2% for PEG8000).

The tested concentrations for each component are the following:

Mg-glu: {0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mM}.

K-glu: {0, 20, 40, 60, 80, 100, 120, 140, 160, 180 mM}.

DTT: {0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mM}.

PEG8000 {0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4%}.

3. Prepare the final buffer mix that will be used for running the cell-free reactions. The following quantities are for 1 tube of 650 μL (you need to scale up to have two tubes of buffer for 1 mL of final extract produced): per tube of buffer, add 128.97 μL of amino acid mix, 110.54 μL of energy solution, PEG8000, K-glu, Mg-glu, and DTT according to the best determined concentration and pure water to adjust the volume to 650 μL .

3.4 Running Cell-Free Reactions

All the cell-free experiments in the following parts should be run according to the same methodology. This method is for the preparation of N number of 20- μL cell-free reactions. All the following reactions have to be prepared on ice and have to be run in technical triplicate.

1. Thaw your cell-free reagents (extract and buffer) on ice.
2. Predilute your DNA and inducer stock: You will prepare 22- μL reactions each with 7.33 μL of extract, 9.17 μL of buffer, and 5.5 μL of other components (DNA plasmids, inducer(s) and water to adjust). The easiest way to proceed if you have less

than five different components to add per reaction is to make 20 \times solutions of the plasmids and inducers you want to use to add 1.1 μ L of those to the reaction.

3. Prepare your cell-free master mix for $(N + 15\%)$ reactions to compensate for the pipetting loss. Add $(7.33 * (N + 15\%))$ μ L of cell extract and $(9.17 * (N + 15\%))$ μ L of buffer to a single 1.5-mL tube.
4. Mix by briefly vortexing.
5. Pipette 16.5 μ L of that mix in N PCR tubes (in strips).
6. Add the respective other inputs (plasmids, inducers, water...) from the 20 \times stocks, to each PCR tube up to 22 μ L.
7. Close these tubes and mix them by briefly vortexing and bench centrifugation.
8. Pipette 20 μ L from each tube to a well of a black 384-well plate prechilled.
9. Then cover the plate with a transparent sealing film.
10. Using a plate reader, monitor the green fluorescence (ex: 458 nm, em: 528 nm) at various gains during a 12-h kinetic run.

3.5 Cell-Free Biosensors Characterizations and Optimizations

In order to obtain the best possible biosensor response for our target, we need to first develop an efficient biosensor for the TF binding effector, before optimizing the enzymatic conversion of our target molecule into our effector.

The first step is aimed to screen for any potential response of designed candidate biosensors and to answer two questions: first, is there any interaction between the TF expression and the level of expression of the reporter, and then is this interaction modulated in any way by the effector that we are trying to detect. To answer those, you need to characterize the behavior of the reporter plasmid in a cell-free reaction in presence or absence of transcription factor and in presence or absence of effector. If you have identified multiple candidates for the sensing of the same molecule, this first experiment can also be used to decide what are the TFs and the promoters most promising for the next steps.

1. Run cell-free reactions varying the quantity of added plasmids and chemicals in the following possibilities: Reporter DNA concentration at 0 or 30 nM, TF DNA concentration at 0 or 30 nM, and inducer concentration at 0, 100 μ M, or 1 mM.
2. Use end point kinetics results at 8 h to evaluate the potential mechanism of your TF (activation or repression) and a possible response of your system to the inducer.
3. Find the optimal plasmid concentration for the reporter and TF that results in a maximal response for your biosensor. With

three concentrations of inducer (0 μM , 100 μM , and 1 mM), test a combination of concentrations gradients for the added plasmid DNA varying reporter and TF DNA concentration on a logarithmic scale (0, 0.1, 0.3, 1, 3, 10, 30, and 100 nM).

4. Using the previously determined best pair of plasmid concentration, evaluate the inducer dose–response of the constructed sensor. Run a cell-free experiment with inducers concentration at: 0 nM, 0.5 nM, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, and 1000 nM. Dose–response curve is obtained by plotting (fluorescence at concentration \times / fluorescence at concentration 0) for each datapoint. Use the final results obtained to assess the performance of the developed sensor and its conformity with your objective of sensitivity (*see* **Note 10**).
5. Once you have a satisfying sensor for your effector, you can start screening for enzymes candidates to convert your target into this effector. The first experiment to do consists of screening for any potential activity on each of the selected enzymes candidates. Prepare a cell-free experiment with the previously identified best concentration for reporter and TF DNA, varying enzyme DNA concentration at 0 nM or 10 nM, and the molecule of interest at 0 mM or 1 mM.
6. Select the best enzyme candidate and optimize the expression of the enzyme on a dose–response curve (same concentrations as in **step 3**) by varying the DNA concentration coding for the enzyme on the scale of 0, 0.1, 0.3, 1, 3, 10, 30, and 100 nM.

3.6 Design, Build, and Test a Perceptron

1. Define the architecture of the perceptron based on input molecules. There are two possibilities depending on the level of constraints you have with the input molecules. If you want to implement a computing device with a defined behavior but without any constraint on the nature of the inputs, you can reprogram the already developed benzoate-based perceptron [16] to implement your desired computational function.
2. If you are interested in multiplexed sensing for defined molecules, you need to identify a common effector they can be converted into. You can use SENSIPATH with a reaction length of 2 to increase the chance to find a product accessible from the two or more substrates. Some central molecules (lactate, acetate, hydrogen peroxide, ammonium...) may be useful for that purpose but a specific attention has to be put on the development of biosensors with high dynamic range for these molecules with potential high-noise issues related to their central positions on the metabolic networks.
3. Create and fit the cell-free model. It can be decomposed in two parts: actuators and transducers.

4. An actuator is modeled with a modified Hill function; commonly used in biochemistry for simulating the binding of ligands to proteins according to the ligand concentration.

$$\text{Actuator}(\text{total}) = \left(\frac{(\text{total})^{\text{hill}_a}}{(K_M)^{\text{hill}_a} + (\text{total})^{\text{hill}_a}} \times \text{fc} + 1 \right) \times \text{basal} + \text{lin} \\ \times 0.0001 \times \text{total}$$

Function description:

- total: Concentration of input metabolite in μM .
 - K_M : Concentration of input metabolite yielding half of the maximum induction of the system (also called IC50).
 - hill_a : Hill coefficient characterizing the cooperativity of the induction system.
 - fc: Stands for fold-change, corresponds to the dynamic range (in Arbitrary Units) of the system.
 - basal: Basal GFP fluorescence signal without input, i.e., the background noise of the system.
 - lin: Accounts for the linearity of the system when dealing with concentrations saturating the Hill transfer function.
5. A transducer is also modeled with a particular Hill function:

$$\text{Transducer}(\text{input}) = \text{range}_{\text{enzyme}} \times \left(\frac{(E)^{n_E}}{(K_E)^{n_E} + (E)^{n_E}} \right) \\ \times \left(\frac{(\text{input})^{n_{\text{input}}}}{(K_I)^{n_{\text{input}}} + (\text{input})^{n_{\text{input}}}} \right)$$

Function description:

- input: Input metabolite concentration in μM .
- range_enzyme: Coefficient characterizing the capacity of the enzyme to transduce the signal (dimensionless).
- E : enzyme concentration in nM .
- K_E : Hill constant for enzyme concentration E .
- n_E : Hill constant for enzyme concentration E .
- K_I : Hill constant for input metabolite input.
- n_{input} : Hill constant for input metabolite input.

We recommend at least some inspiration from our fitting process described in the notes section (*see* **Note 11**) as it puts an emphasis on tackling the loss of signal of the actuator when the whole system is modeled then implemented. Otherwise, one can adapt another fitting process for a particular project's needs.

6. Measure the model's performance with different metrics: root mean square deviation (RMSD); R^2 ; weighted R^2 ; error percentage.

Ensuring high metrics (e.g., above 0.9 for scores between 0 and 1) on many experimental data points guarantee a robust model for predicting the weights schemes for each classification task.

7. Define your classification problem. Start by defining a set of tasks (here, classifications), which outputs either 0 (OFF) or 1 (ON); as well as a set of weights to be tested (equivalent to a range of possible enzyme concentrations in the cell-free experiment, for our work it was between 0.1 and 10 nM).
8. Continue by sampling uniformly input values for your problem, that is to be resolved by the perceptron. For example, in the case of a binary classification of solutions composed of hippurate and cocaine, sample points in a given range, either for a "low" concentration or a "high" concentration for each of the compounds. Here, let us assume we can sample between 0 and 2 μM for low concentrations and between 80 and 100 μM for high concentrations.
9. For each of the two clusters, choose to sample either in the low or high range for each compound.
10. Then, two clusters have been produced and a binary classifier can be easily defined on these points: the perceptron set of weights and its corresponding fluorescence threshold. Please find a visual example of the definition of a classification task on Fig. 3. Further is detailed how to find the set of weights and the fluorescence "decision threshold."
11. Predict the best set of weights for solving this problem (aka "train" the perceptron on a classification task). Using the previously fitted (part 2) and benchmarked (part 3) model, simulate all possible input combinations with all possible sets of weights.
12. Screen for performant set of weights, i.e., those enabling a sharp threshold between the output states of the system ("ON" or "OFF"). Several thresholds can be tested for considering an output value as ON or OFF, for all possible simulations.
13. Manually select the best set of weights and corresponding threshold, i.e., those that show the highest and clearest difference between ON and OFF behaviors, and in most scenarios. Also, prefer those showing low enzyme concentrations (to avoid resource competition). One can also test several possibilities (several sets of weights and/or thresholds) in the following cell-free experiments.

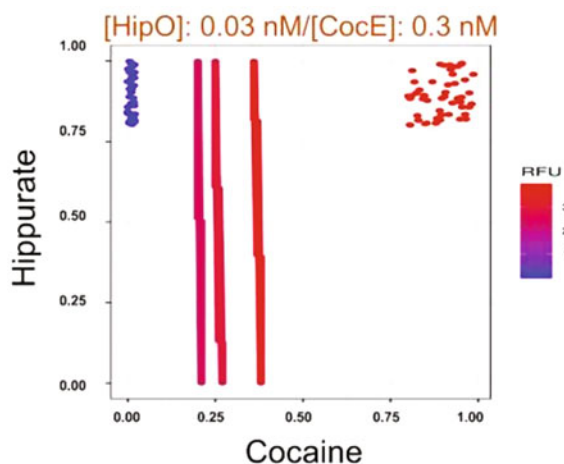


Fig. 3 Visual example of a binary clustering with a metabolic perceptron. This figure has been copied from supplementary material of Pandi et al. [16] with authorization from the authors. The X-axis shows the normalized concentration of cocaine in a sample, and the Y-axis shows the normalized concentration of Hippurate in a sample. Here, the blue points drop into the first cluster (“high Hippurate, low cocaine”) and the red ones drop into the second cluster (“high cocaine, high hippurate”). Colors of the points correspond to the predicted RFU values by the fitted perceptron (after Subheading 3.6, part 3 is done). The three vertical lines correspond to three isofluorescence lines; equivalent to three fluorescence thresholds, each of them being associated with one set of weights of the perceptron (i.e., [HipO]: 0.03 nM and [CocE]: 0.3 nM)

14. Implement the designs predicted as best ones, to test it in a cell-free reaction. Start by drawing a test set of chemical (input) combinations from the compositions used for the training of the in silico perceptron. Be cautious to evaluate enough points from the space of possibilities to capture the behavior of your system. If your perceptron is designed to solve the particular problem of binary inputs sample classifications, you can eventually build a complete test set with all the possible combinations of inputs (*see* Fig. 1d).
15. The designed test set can be prepared at $20\times$ concentration in PCR tube strips to be evaluated on various perceptron implementations. We advise you to prepare a single master mix with the cell-extract, the buffer, and the DNA coding for the reporter system and the various transducers at the desired concentration for each implementation of the perceptron that you designed.
16. Use this master mix to evaluate the response of the system on the chemical test set previously designed. If the perceptron does not have the expected behavior from in silico analysis, try another set of weights.

4 Notes

1. Most of the molecules present in the cell-free buffer are in high concentration masking any potential response for a biosensor designed to detect them. This list includes all the 20 amino acids, HEPES, ATP, GTP, UTP, CTP, tRNA, CoA, NAD, cAMP, folinate, spermidine, 3-PGA, magnesium, potassium, and DTT.
2. If you cannot find satisfying results with the software SENSIPATH, you can try to find other SEMP using the retrosynthesis workflow Retropath [14]. It has the advantage to allow prediction for pathways with more than two steps or pathways using promiscuous activity of enzymes to find potential new reactions. To run Retropath for biosensor design, use the list of detectable molecules [11] as Sink, the molecule you want to detect as source and the reaction rules in the forward direction. Results coming from this workflow have to be taken with more care as it relies on less-reliable predictions.
3. The Selenzyme tool is predicting potential enzymes catalyzing a defined reaction based on similarity of sequences, reactions or other features existing between enzymes from a well-annotated database and enzymes potentially catalyzing the query reaction. The predictions should be manually checked in published literature/datasets to evaluate if the identified enzyme can likely catalyze the given reaction.
4. The methodology described here is a standard to be used in the case where the identified promoter has limited annotation or features described. You are strongly encouraged to search for existing biosensing projects described in the literature that uses the same transcription factor with defined size promoters or synthetic ones (built by inserting TF-binding sequences in another promoter) as these promoters may show a better response than the natural ones.
5. Golden gate assembly was chosen over other methods like Gibson assembly as it allows reusing the same primers to reamplify backbone for every new insert cloned.
6. This thermocycler protocol for golden gate is a variation of the fast golden gate assembly protocol (1 h 37 °C, 10 min 55 °C) that is adapted to backbone plasmid containing internal *BsaI* cut sites. Removing the last 55 °C step and adding one at 16 °C for 5 min avoid cutting the final assembly containing the *BsaI* site.
7. DNA batch can have an influence on its expression level in cell-free limiting reproducibility of results from one maxiprep to another. You are advised to purify each plasmid in a big enough

quantity for running all your experiment with a single batch. If necessary, run multiple maxiprep in parallel and mix the resulting DNA to have a sufficiently large quantity of plasmid.

8. Sonication and autolysis have also been successfully tested as lysis methods on this protocol for the development of cell-free biosensors with good results. The French press method has the advantage of being easily scalable for the production of large quantity of extract but if you lack the equipment for it you can adapt the protocol to any other lysis method.
9. In our experience, the dialysis does not show a major impact on behavior of the final extract. For optimization purposes, dialyzed and nondialyzed extract can be screened to find the condition giving the best response for specific biosensors.
10. If you plan to use this effector biosensor for indirect detection (using metabolic transducers) or for multiplex sensing (through a perceptron like architecture), you may want to have it optimized for the detection of inducers present at a lower concentration than what you need for your target molecule as the transduction of the signal through the enzymatic layer often goes with a decrease of sensitivity and an increase of potential noise.
11. Once these functions are encoded in R, the actual fitting can happen.

First, fit the actuator experimental data (effector dose-response curve obtained in part 3.5 Subheading 3) to the Hill function model.

To do so, fit 100 times the actuator experimental values to the actuator model. Let all parameters be able to vary. Use ordinary least-square error or the R “optim” function (that uses the Limited-memory Broyden Fletcher Goldfarb Shanno algorithm) as the objective function for the data fitting. Keep the seed of the fitting process in memory so it ensures reproducibility. Retrieve fitted parameters for each fitting (a population of 100 sets of fitted parameters will be produced). From this population, save the mean, standard deviation, standard error, and confidence interval; for each parameter.

To account for the decrease of signal in experimental actuator data when the whole system is implemented in cell-free, one needs to fit the transducer model in a specific way.

To fit the transducers models, we need to actually fit the whole model to the whole system’s cell-free experimental data.

In order to have a coherent perceptron, we will actually constrain the actuator’s parameters, then fit all transducers *and* the actuator together.

To do so, start by constraining each of the actuator's previously fitted parameters with bounds corresponding to the 95% confidence interval; or \pm one standard deviation from the mean, in the case of following parameters: the fold-change, termed "fc," and the baseline, termed "basal."

We call "pseudo-experimental" the data used to fit the whole system, but that originates from specific components' experimental data. These individual data were aggregated as described in the whole-system model: combine transducers data by simply adding their output and feeding the sum to the actuator. Note that in the cell-free experimental system, the same process happens, e.g., benzoate concentrations are added as a result of all transducers yielding benzoate, and the actuator takes this aggregated benzoate concentration as an input. As a result, we obtain "pseudo-experimental" data for the whole cell-free system, with experimental data only available for each component separately.

Once your whole system is modeled and you have constrained your actuator's parameters, you can fit your transducer parameters. Initialize the whole model by drawing actuator parameters values according to a Gaussian distribution centered on the mean of the parameter estimations, with a standard deviation equal to the standard error of this parameter estimation. Let all transducer parameters vary to fit the whole system to the previously described "pseudo-experimental" data.

Acknowledgments

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ROSALIND: Rapid Detection of Chemical Contaminants with In Vitro Transcription Factor-Based Biosensors

Jaeyoung K. Jung, Khalid K. Alam, and Julius B. Lucks

Abstract

ROSALIND (RNA Output Sensors Activated by Ligand Induction) is an in vitro biosensing system that detects small molecules using regulated transcription reactions. It consists of three key components: (1) RNA polymerases, (2) allosteric protein transcription factors, and (3) synthetic DNA transcription templates that together regulate the synthesis of a fluorescence-activating RNA aptamer. The system can detect a wide range of chemicals including antibiotics, small molecules, and metal ions. We have demonstrated that ROSALIND can be lyophilized and transported at ambient conditions for water testing on-site. Here, we describe how to set up a ROSALIND reaction for detecting various chemical contaminants in water using a model transcription factor as well as how to build a new ROSALIND sensor.

Key words Cell-free synthetic biology, Allosteric transcription factors, Biosensors, In vitro transcription, RNA aptamer, Water quality monitoring

1 Introduction

For decades, synthetic biologists have been harnessing the diversity of allosteric transcription factors (aTFs) to engineer microorganisms to serve as whole-cell biosensors [1]. In a genetic circuit, these aTFs trigger the production of a detectable reporter molecule in response to binding of their cognate ligand(s). Changing the aTF allows whole-cell biosensors to detect a range of contaminants including metals, aromatic compounds, and antibiotics [1]. However, whole-cell biosensors must maintain cell viability, have difficulties with sensing toxic contaminants at high concentrations, suffer from membrane transport limitations, and present biocontainment and regulatory concerns for deploying these bioengineered organisms in the field [2].

Recently, there has been a growing effort to overcome these challenges with cell-free gene-expression technologies. Cell-free platforms decouple gene expression from cell viability, enable faster

design-build-test cycles, provide greater tunability of individual components, and greatly reduce the biocontainment and regulatory concerns [3]. In addition, the ability to lyophilize cell-free reaction components for easy storage and distribution at ambient temperatures has enabled deploying these technologies at the point-of-need [4].

There are two main approaches for using cell-free reaction systems in sensing applications. One approach involves the use of a cellular extract where aTFs are expressed *in vivo* and enriched in the extract [5] or expressed *in situ* in the extract through the introduction of the aTF-encoding DNA [6]. In either case, a plasmid encoding a reporter protein that is regulated by the enriched aTF can be added to the extract to create a biosensor. The second approach is a reconstituted *in vitro* system where individual components necessary for gene expression are purified and assembled into the reaction [7, 8]. The defined nature of the reconstituted *in vitro* system reduces batch-to-batch variability and provides a greater control over reaction mixtures.

We recently developed an *in vitro* transcriptional sensing platform called ROSALIND that uses aTFs to detect chemical contaminants for various applications [9]. This technology was enabled by the development of RNA reporters—such as the fluorescence-activating RNA aptamer Broccoli—that can bind to a specific chemical dye and generate a fluorescent signal [10]. ROSALIND consists of three components: (1) T7 RNA polymerase (RNAP), (2) aTFs, and (3) synthetic DNA templates that together regulate the transcription of a fluorescence-activating RNA aptamer (Fig. 1). In the absence of a chemical contaminant, an aTF binds to the transcription template and blocks RNAP from transcribing the RNA aptamer. In its presence, however, the contaminant binds to its cognate aTF, releasing the aTF from the DNA template and enabling RNAP to produce the RNA aptamer. The fluorescence-activating RNA aptamer used in this platform is an engineered variant of a broccoli aptamer called three-way junction dimeric broccoli (3WJdB), which is brighter and structurally more stable [11].

Here we describe in detail how to prepare individual components in ROSALIND and to assemble them into sensing reactions. We also describe a method to lyophilize and package ROSALIND reactions for long-term storage and field-deployment. Although we focus this protocol on the construction of a tetracycline sensor using the model aTF TetR, we briefly discuss how to extend this platform to new aTFs of interest for the creation of novel ROSALIND reactions.

While not described in this protocol, ROSALIND is extensible to several different formulations. For example, a different phage RNAP such as T3 and SP6 can be used in place of T7 RNAP with corresponding changes to *in vitro* transcription (IVT) buffer

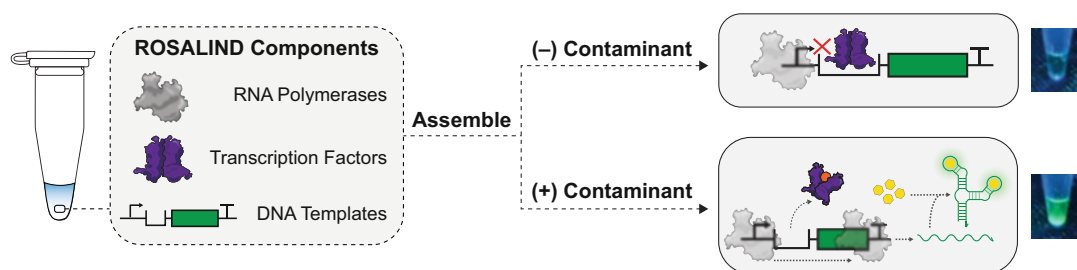


Fig. 1 The ROSALIND platform. ROSALIND consists of three programmable components: RNA polymerases, transcription factors, and DNA templates. The DNA templates are designed to encode a promoter sequence, followed by an operator sequence for transcription factor binding and a downstream sequence encoding a fluorescence-activating RNA aptamer. In the absence of a target contaminant, a transcription factor binds to the operator, blocking RNA polymerase and preventing transcription. In the presence of a target contaminant, the contaminant-transcription factor complex releases from the DNA template, allowing RNA polymerase to transcribe the aptamer that then binds to the dye DFHBI-1T to generate a signal. The fluorescent signal then can be visualized using blue light. Elements of this figure were adapted from the original manuscript [9]

compositions and synthetic transcription templates. Various types of downstream RNA circuitry such as logic gates and feedback loops can also be built into ROSALIND to improve sensor specificity and sensitivity [9]. Finally, ROSALIND can be used for screening of poorly characterized aTFs to determine their cognate ligands and putative operator sequences with straightforward adaptations of this protocol.

2 Materials

Prepare all solutions using ultrapure water (18 MΩ-cm at room temperature) and analytical-grade reagents.

2.1 Preparing a Linear Transcription Template

A ROSALIND transcription template typically encodes in the 5' to 3' direction, T7 promoter—aTF operator—3WJdB—T7 terminator (Fig. 2). It is prepared by PCR-amplifying the segment including the aforementioned sequences from a ROSALIND plasmid.

1. A ROSALIND plasmid: For testing an existing sensor, obtain a plasmid with the appropriate operator sequence from Addgene (see Table 1). For constructing a plasmid for a new sensor, perform inverse PCR off of the unregulated plasmid (Addgene # 87308) to insert the operator sequence in between the promoter and 3WJdB sequences (see Notes 1 and 2).
2. Forward and reverse primers (Fig. 2c) (see Note 3).
3. Phusion DNA polymerase, Phusion HF buffer, and 10 mM of dNTPs, or equivalent.
4. A thermocycler.

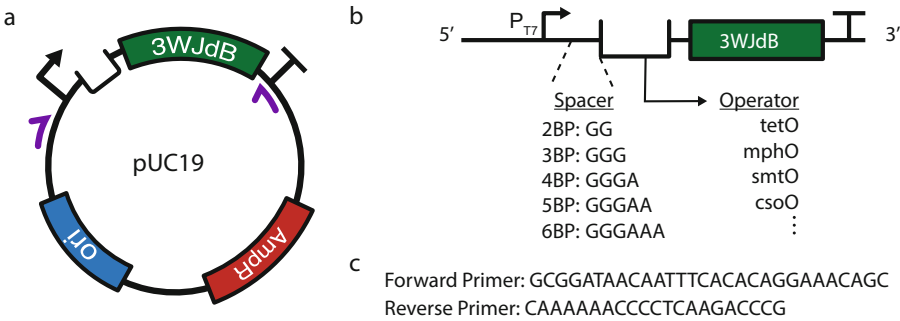


Fig. 2 Construction and Design of a ROSALIND transcription template. **(a)** A plasmid map containing a ROSALIND transcription template. The purple arrows indicate the forward and reverse primers that can be used to generate a linear transcription template through PCR amplification. **(b)** A linear transcription template consists of T7 promoter, a 2–6BP spacer, an operator sequence for aTF binding, the 3WJdB aptamer, and a T7 terminator. **(c)** Sequences of the forward and reverse primers that can be used to generate the linear DNA template shown in **(b)**

Table 1
ROSALIND plasmids

Addgene #	Description	Regulated by	Sensor for
140374	T7 promoter— <i>tetO</i> —3WJdB—T7 terminator	TetR	Tetracyclines
140382	T7 promoter— <i>otrO</i> —3WJdB—T7 terminator	OtrR	Oxytetracycline
140384	T7 promoter— <i>ctcO</i> —3WJdB—T7 terminator	CtcS	Chlortetracycline
140386	T7 promoter— <i>mphO</i> —3WJdB—T7 terminator	MphR	Macrolides
140388	T7 promoter— <i>mobO</i> —3WJdB—T7 terminator	MobR	3-hydroxy benzoic acid
140390	T7 promoter— <i>qacA</i> —3WJdB—T7 terminator	QacR	Benzalkonium chloride
140392	T7 promoter— <i>ttgO</i> —3WJdB—T7 terminator	TtgR	Naringenin
140394	T7 promoter— <i>hucO</i> —3WJdB—T7 terminator	HucR	Uric acid
140396	T7 promoter— <i>smtO</i> —3WJdB—T7 terminator	SmtB	Zinc
140398	T7 promoter— <i>csoO</i> —3WJdB—T7 terminator	CsoR	Copper
140399	T7 promoter— <i>cadO</i> —3WJdB—T7 terminator	CadC	Lead, cadmium

5. A NanoDrop or Qubit for measuring DNA concentrations.
6. 2% Tris-acetate-EDTA (TAE) agarose gel: Dissolve 1 g of agarose in 50 mL of TAE buffer using a microwave, quickly add 1–5 μ L of a dye such as GelRed for visualization (see supplier instructions), and solidify the solution in a gel-casting stand at room temperature.

**2.2 Assembling
Fresh ROSALIND
Reactions**

1. A microplate reader capable of green fluorescence measurements or a blue-light illuminator (see **Note 4**).
2. IVT buffer (10 \times): 400 mM Tris-HCl pH 8, 80 mM MgCl₂, 100 mM DTT, 200 mM NaCl, and 20 mM Spermidine.

Prepare and filter-sterilize each solution separately at the following concentrations: 1 M Tris-HCl pH 8, 1 M MgCl₂, 1 M DTT, 4 M NaCl, and 1 M spermidine. Make DTT fresh whenever you make new buffer aliquots. Typically, 10 mL of the 10× buffer lasts for a few months. For 10 mL of the 10× IVT buffer, mix 4 mL of 1 M Tris-HCl pH 8, 0.8 mL 1 M MgCl₂, 1 mL of 1 M DTT, 5 mL of 4 M NaCl, 0.2 mL of 1 M spermidine, and 3.5 mL of H₂O. Store at −20 °C.

3. Linear transcription template. For preparing this reagent, *see* Subheading 3.1. Store at 4 °C for immediate use or at −20 °C for long-term storage.
4. T7 RNA Polymerase: This can be commercially obtained or purified in-house. Store at −20 °C.
5. 100 mM Tris-buffered NTPs (25 mM each NTP), pH 7.5 (*see Note 5*): This can be commercially obtained or prepared in-house. For preparing them in-house, make 100 mM stock of each NTP (ATP, GTP, CTP, UTP) by titrating to pH 7.5 using Tris-base. Mix equal volume of each NTP to make 100 mM Tris-buffered total NTPs stocks. Store at −20 °C.
6. DFHBI-1T: Prepare the DFHBI-1T solution in 100% DMSO to make a 40 mM or 20 mM stock. Protect from light when not in use. Store at −20 °C.
7. Inorganic pyrophosphatase: This can be commercially obtained and is optional but recommended for efficient transcription. Store at −20 °C.
8. Purified transcription factor. A protocol to purify a model aTF, TetR, is provided in Subheading 3.6. Store at −20 °C.
9. Ligand of interest in solution (*see Note 6*). Some ligands are more stable than the others, but we recommend making a ligand stock fresh for every experiment. Here, we include a method to prepare anhydrotetracycline (aTc) solution as an example. Measure out 1–2 mg of aTc in a 1.7 mL Eppendorf tube and add up to 1 mL of 100% ethanol to fully dissolve (aTc has a solubility of 2 mg/mL in ethanol). Dilute it in ultrapure water to make 10–200 μM aTc solution.
10. A black, clear-bottom 384-well plate.
11. Clear plate sealing tape.
12. A multichannel pipette: This is useful for setting up hundreds of reactions simultaneously but is not needed.

2.3 Freeze-Drying ROSALIND Reactions

1. Lyophilizer (2.5-L, or similar, capable of ultra-low temperature condenser temperature and 0.04 mbar of pressure).
2. Lyophilizer flasks: They are not necessary if the lyophilizer has a shelf instead of a manifold.

3. 8-tube flat-cap PCR strips.
4. A metal block that holds PCR tubes.
5. A pin or thumbtack.
6. Liquid nitrogen and dewar. Keep it sealed and cold until usage.
7. Aluminum foil.
8. 1 M sucrose or trehalose (*see Note 7*): Prepare the solution by dissolving the appropriate amount of sucrose or trehalose in ultrapure water and filter-sterilize. Store at room temperature.
9. 780 mM D-mannitol: Prepare the solution by dissolving the appropriate amount of D-mannitol in ultrapure water and filter-sterilize. Store at room temperature.

2.4 Packaging the Freeze-Dried ROSALIND Reactions

1. Light-protected Mylar bags.
2. DriCard desiccants.
3. Vacuum sealer: Any vacuum sealer that is used to package food can be used for this application.
4. Argon canister. This does not need to be a laboratory-grade canister. We have found that canisters intended for the preservation of wine work well.

2.5 Rehydrating the Freeze-Dried ROSALIND Reactions

1. Exact volume (20- μ L) transfer pipettes (*see Note 8*).
2. 37 °C portable incubator (optional) (*see Note 9*).

2.6 Purifying an Allosteric Transcription Factor, TetR (See Note 10)

1. An aTF expression plasmid (*see Note 11*). For expressing and purifying TetR, obtain the plasmid # 140371 from Addgene.
2. Rosetta 2 (DE3) pLysS *E. coli* or equivalent strain for protein overexpression.
3. 37 °C shaking incubator for bacterial transformation and expression cultures.
4. A centrifuge and rotor capable of high-speed centrifugation (e.g., Thermo Scientific Sorvall Lynx 4000 with Fiberlite™ F10-4 \times 1000 LEX Rotor).
5. 1 L of sterile Luria Broth. If the protein of interest does not express well, prepare a larger volume of cell culture.
6. Inducer for induction of protein overexpression (e.g., 1 M IPTG for the pET-28-c plasmid system). Filter-sterilize and store at -20 °C.
7. Ni-NTA resin for affinity purification of 6X-His-tagged protein.
8. An empty gravity flow column.
9. Lysis buffer: 10 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM TCEP, and protease inhibitor (cOmplete EDTA-free Protease

Inhibitor Cocktail or equivalent). Depending on the size of the cell pellet, prepare 25–100 mL of the lysis buffer (*see* **Note 12**) for immediate use. Store at 4 °C.

10. Equilibration buffer: 10 mM Tris–HCl pH 8, 500 mM NaCl, 1 mM TCEP, and 10 mM Imidazole. Make 1 M of imidazole stock solution in a dark bottle, filter-sterilize, and use it for making the buffers with imidazole (*see* **Note 13**). Prepare 10 mL of the equilibrating buffer solution. Store at 4 °C.
11. Wash buffers: 10 mM Tris–HCl pH 8, 500 mM NaCl, 1 mM TCEP, and 20–50 mM Imidazole (*see* **Note 14**). Prepare 10 mL of each washing buffer solution. Store at 4 °C.
12. Elution buffer: 10 mM Tris–HCl pH 8, 500 mM NaCl, 1 mM TCEP, and 250 mM Imidazole. Make 20 mL of the elution buffer. Store at 4 °C.
13. Storage buffer without glycerol (2×): 50 mM Tris–HCl pH 8, 200 mM NaCl, and 2 mM TCEP. Store at 4 °C.
14. Centrifugal concentration columns (10 kDa MWCO for TetR or larger depending on the size of the aTF of interest) to concentrate protein.
15. 100% glycerol to store proteins in a final concentration of 50% glycerol (v/v).
16. Tube rotator/nutator to gently mix glycerol into protein stock solution.
17. SDS-polyacrylamide gel electrophoresis (PAGE) gel: A pre-made gel can be commercially obtained.

3 Methods

3.1 Preparing Linear Transcription Template

1. Assemble a ~1 mL of PCR with the ROSALIND plasmid as a starting DNA material. We recommend using less than 200 ng total of the starting plasmid in a total volume of a 1 mL of PCR.
2. Run the PCR using a thermocycler with the appropriate primer annealing temperature and extension time depending on the polymerase of choice. The amplified DNA segment is around 300 base pairs long. If using Phusion polymerase and the primers listed in Fig. 2c, run the following program: 98 °C for 3 min—(98 °C for 30 s, 71 °C for 30 s, 72 °C for 15 s) × 25 cycles—72 °C for 10 min—hold at 12 °C.
3. Make a 2% TAE agarose gel during the PCR run.
4. Perform PCR purification and elute it in 50–100 µL of ultra-pure water.
5. Measure the concentration of DNA either on NanoDrop or on Qubit.

6. Run ~50–100 ng of the purified DNA template on a 2% TAE agarose gel to confirm the size and purity of the product.
7. Store at 4 °C for immediate use or at –20 °C for long-term storage.

3.2 Assembling a 20 μ L ROSALIND Reaction for a Microplate Reader Assay

This section is written specifically for setting up a TetR-regulated ROSALIND reaction. However, virtually any ROSALIND sensors can be tested using the same overall workflow described below by swapping the transcription template, aTF, and the ligand of interest.

1. Set the microplate reader temperature to 37 °C. For ROSALIND reactions generating the Broccoli aptamer, excitation and emission wavelengths should be set to 472 and 507 nm, respectively. For kinetic measurement, we recommend taking reads every 3 min over the course of a 4-h reaction.
2. Mix the components shown in Table 2 at room temperature to make a 15- μ L reaction master mix, to which 5 μ L of activator mix containing T7 RNAP +/– aTc will be added later (*see Note 15*). The final concentrations of the following components can be adjusted from the values listed in Table 2: tris-buffered NTPs, DFHBI-1T, DNA template (*see Note 16*), and TetR (*see Note 17*). However, for the maximum transcription efficiency, we recommend following the NTPs and DFHBI-1T concentrations listed in Table 2 (*see Note 18*). In particular, the concentration of DFHBI-1T greatly impacts the transcription efficiency (*see Note 19*; Fig. 3).
3. Incubate the master mix at 37 °C for 10–15 min to allow TetR to equilibrate with the DNA template.
4. While the reaction master mix is incubating, mix the components listed in Table 3 in a separate tube to make an activator mix (*see Notes 20 and 21*).

Table 2
Components and concentration of a ROSALIND reaction master mix

Reaction master mix component	Final concentration	Volume per reaction (μ L)
10 \times IVT buffer	1 \times	2
100 mM Tris-buffered NTPs	11.4 mM	2.28
2 U/ μ L inorganic pyrophosphatase	0.015 U/ μ L	0.15
1 μ M IVT linear DNA template	5–50 nM	Vary
40 mM DFHBI-1T	0.2 mM	0.1
210 μ M TetR dimer	0.25–2.5 μ M dimer	Vary
H ₂ O	–	Up to 15

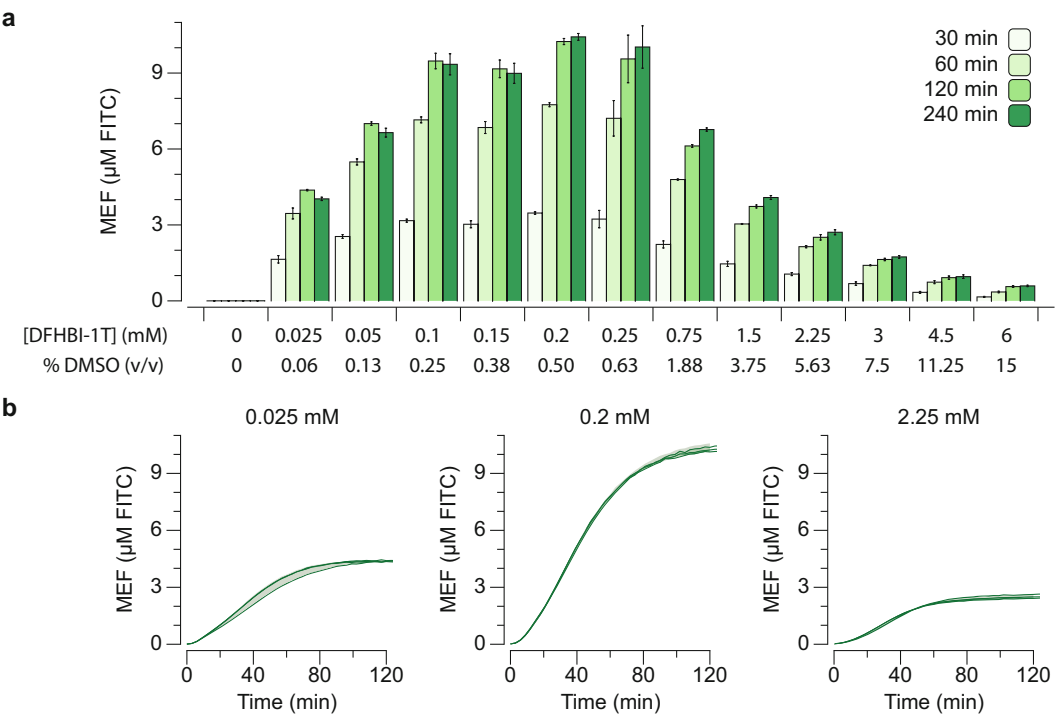


Fig. 3 Optimizing ROSALIND for DFHBI-1T concentrations. Unregulated ROSALIND reactions lacking a transcription factor were setup and run to study the effects of dye concentration on the output signal. **(a)** Titration of DFHBI-1T at a fixed DNA concentration measured at 30, 60, 120, and 240 min. The DMSO concentration (% v/v) in the final reaction, due to carryover from the dissolved dye, is indicated. **(b)** Kinetic traces of the reactions shown in **(a)** for three different dye concentrations (0.025 mM, 0.2 mM, and 2.25 mM)

Table 3
Components and concentration of a ROSALIND activator mix

Activator mix component	Final concentration	Amount per reaction (μL)
1 mg/mL T7 RNAP	10 ng/ μL	0.2 μL
100 μM aTc	10 μM	2 μL
H ₂ O	–	2.8 μL (up to 5 μL)

5. Ensure the microplate reader is ready to read, then add 5 μL of the activator mix to 15 μL of the master mix and gently pipette up and down several times to mix, being careful to avoid the introduction of air bubbles. If setting up multiple reactions simultaneously, use a multichannel pipette to add the activator mix to the master mix to ensure each reaction activates at the same time.
6. Immediately transfer the reaction onto a 384-well plate, seal the plate to prevent evaporation, and start the microplate reader run.

3.3 Freeze-Drying ROSALIND Reaction

1. Turn on the lyophilizer and set condenser temperature to the lowest setting (-85°C) and vacuum pressure to 0.04 mbar.
2. Before assembling reaction components, chill the metal block at -80°C . A cold block ensures that the reactions remain frozen during transfer into and within the lyophilizer.
3. Using a thumbtack or a small pin, poke three holes in each cap and set them aside (Fig. 4). These perforations allow for lyophilization.
4. Assemble the master mix components as described in Table 2 in a strip of PCR tubes but add H_2O up to $10\ \mu\text{L}$ instead of $15\ \mu\text{L}$. The extra volume will be used to add lyoprotectants.
5. Incubate the master mix at 37°C for 10–15 min to equilibrate binding of aTFs and DNA templates.
6. While the master mix is incubating, mix the components listed in Table 4 to make the activator mix with the lyoprotectants.
7. Add the activator mix to the master mix on ice and gently mix by pipetting up and down several times.
8. Remove the chilled metal block from the -80°C freezer. Immediately transfer the PCR tubes from ice onto the chilled metal block (see Note 22).

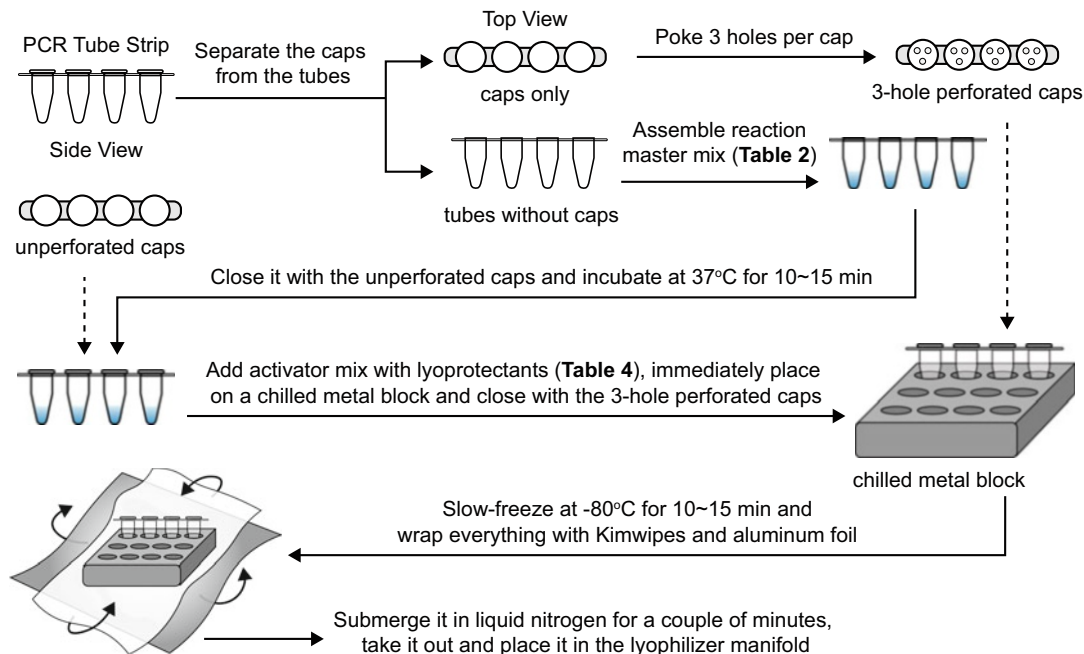


Fig. 4 Lyophilization of ROSALIND. The diagram demonstrates the process of preparing a ROSALIND reaction for overnight lyophilization. For a more detailed instruction, please see Supp. Video 2 from the original manuscript [9]

Table 4
Components and concentration of a ROSALIND activator mix for freeze-drying

Activator mix component	Final concentration	Amount per reaction (μL)
1 mg/mL T7 RNAP	10 ng/μL	0.2 μL
1 M sucrose or trehalose	50 mM	1 μL
780 mM D-mannitol	250 mM	6.4 μL
H ₂ O	–	2.4 μL (up to 10 μL)

9. Close the tubes with the three-hole perforated caps and transfer the block of reactions into the -80°C freezer to slow-freeze (Fig. 4).
10. While the reactions are slow-freezing, prepare liquid nitrogen. Liquid nitrogen can cause frostbite or cryogenic burns if not handled properly. Make sure to wear proper PPE and cryogloves when handling it.
11. Once reactions are frozen, loosely wrap the metal block holding the tubes with Kimwipe and aluminum foil (Fig. 4).
12. Submerge the aluminum foil/Kimwipe-wrapped metal block in liquid nitrogen for a couple of minutes.
13. Place the aluminum foil/Kimwipe-wrapped metal block in an amber lyophilizer flask and attach the flask to the lyophilizer manifold or transfer to a lyophilizer shelf depending on the instrument type.
14. Turn on the vacuum pump.
15. Let the reactions freeze-dry overnight.

3.4 Packaging Freeze-Dried ROSALIND Reactions

1. Turn off the vacuum and condenser.
2. Remove the lyophilizer flask/shelf from the manifold.
3. Take the metal block out of the lyophilizer flask/shelf.
4. Place a DriCard desiccant inside of a Mylar bag (Fig. 5a).
5. Take the strips of tubes out of the metal block and place them inside of the Mylar bag.
6. Purge the bag with Argon.
7. Immediately impulse heat-seal the bag.
8. Store them in a cool, shaded area until usage.

3.5 Rehydrating a Freeze-Dried ROSALIND Reaction

1. Set the microplate reader temperature, or an optional portable incubator if on-site, to 37°C .
2. Using either a micropipette or an exact volume transfer pipette if on-site, add 20 μL of the sample of interest to the freeze-dried reaction (Fig. 5b).

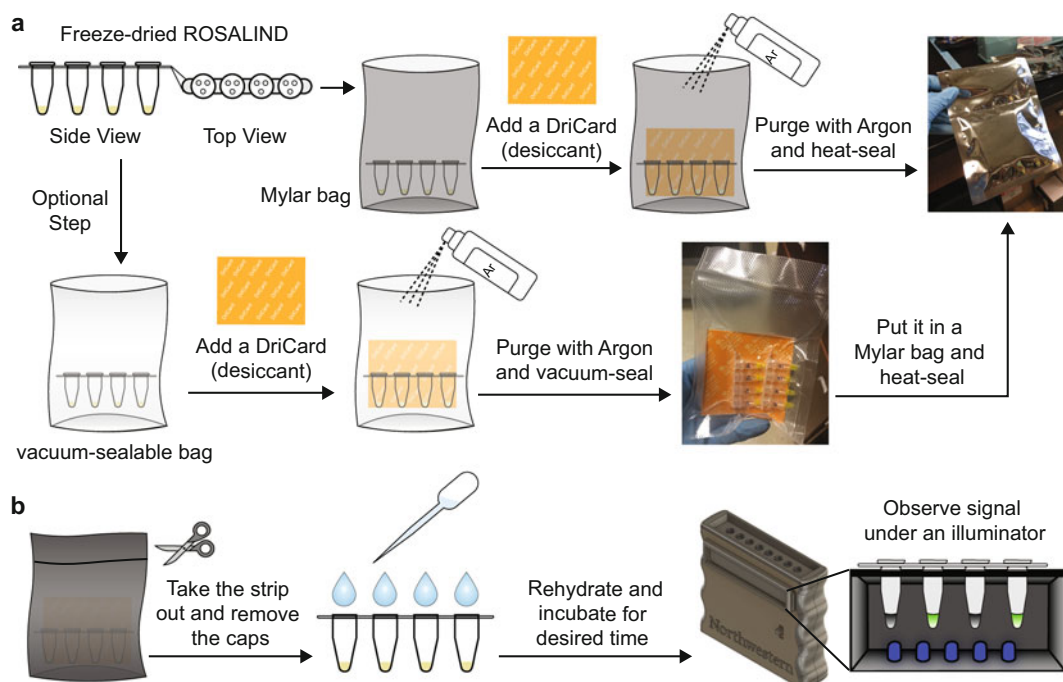


Fig. 5 Packaging and rehydration of lyophilized ROSALIND reactions. **(a)** Packaging of freeze-dried ROSALIND reactions. There is an optional step of vacuum-sealing the tubes with a desiccant before placing it inside of a Mylar bag. Alternatively, the tubes can be placed with a desiccant directly in a Mylar bag. The photograph image in the upper right corner was adapted from the original manuscript [9]. For a more detailed instruction, please see Supp. Video 2 from the original manuscript [9]. **(b)** Rehydrating of freeze-dried ROSALIND reactions

3. If you are using a micropipette, gently mix by pipetting up and down a couple of times while avoiding any bubble formation. If using a disposable transfer pipette, close the tube and flick gently to mix.
4. If you are using a microplate reader for signal observation, carefully load each reaction onto a 384-well plate using a multichannel pipette, seal the plate, and immediately start the microplate reader using the same setting used in Subheading 3.2.
5. If you are using a handheld illuminator for signal observation, place the tubes in the illuminator and check for fluorescent signal every 5 min. If possible, incubate the reactions at 37 °C using a portable incubator as it will speed up the reactions.

3.6 Purifying an Allosteric Transcription Factor, TetR

1. Transform the expression plasmid into the Rosetta 2 (DE3) pLysS strain.
2. Pick a colony from the plate and start a 10 mL overnight seed culture with the appropriate antibiotics.

3. Add 10 mL of the overnight culture into 1 L of Luria Broth with the appropriate antibiotics (*see* **Note 23**).
4. Grow the culture in a shaking incubator (~220–250 rpm) at 37 °C.
5. Induce the culture with 0.5 mM IPTG (or appropriate inducer) once the cultures reach OD₆₀₀ of 0.5 (*see* **Note 24**).
6. Let the culture grow for additional 4–5 h at 37 °C (*see* **Note 25**).
7. Spin down the cell culture at $5000 \times g$ for 20 min, discard the supernatant, and store the cell pellet at –80 °C until purification.
8. Carry out all the subsequent steps at 4 °C. Resuspend the cell pellet with the lysis buffer.
9. Lyse the cells through six rounds of ultrasonication on ice at 50% duty cycle for 20 s with 40 s rest in between rounds. Additional rounds of ultrasonication can be done if needed.
10. Spin down the sonicated cell pellet at $13,000 \times g$ for 30 min to remove any cellular debris and collect the supernatant lysate.
11. During the centrifugation, prepare a nickel affinity column by loading 2–4 mL of Ni-NTA resin to an empty gravity flow column (*see* **Note 26**).
12. Equilibrate the nickel column with the equilibrating buffer and allow the column to empty by gravity flow.
13. Add the lysate to the column and allow the protein of interest to bind to the column by gravity flow. Collect the flowthrough for the SDS-PAGE analysis.
14. Wash the column with the washing buffer, starting with the lowest imidazole buffer, by gravity flow. Collect the flowthroughs for the SDS-PAGE analysis.
15. Elute the protein of interest with the elution buffer by gravity.
16. Concentrate the eluted protein using the centrifugal concentration tube (*see* **Note 27**). Collect the flowthrough for the SDS-PAGE analysis.
17. Perform buffer exchange in the centrifugal concentration tube by adding 10 mL of the $2\times$ storage buffer without glycerol and letting it concentrate in the storage buffer (*see* **Note 28**). Repeat 3–4 times. Collect the flowthroughs for the SDS-PAGE analysis.
18. Collect the protein of interest.
19. Run the protein of interest and the flowthroughs that were collected in **steps 13, 14, 16, and 17** on an SDS-PAGE gel to confirm the size and purity of the purified protein.

20. Measure the concentration of the protein either on NanoDrop or on Qubit.
21. Add an equal volume of 100% glycerol to the protein of interest to make the final concentration of the storage buffer 1× and 50% glycerol (*see* Table 2 for a target final concentration).
22. Tumble the protein at 4 °C on a tube rotator/shaker to gently mix the glycerol and the protein solution overnight.
23. Store the protein at −20 °C.

4 Notes

1. The nucleotide spacing between the minimal 17BP T7 promoter sequence (TAATACGACTCACTATA) and the operator sequence must balance the need for efficient transcription initiation to generate strong fluorescence with the need for robust repression by the aTF. We have found that 2–6 BP spacing enables strong repression in the absence of a ligand and efficient transcription in the presence of a ligand (Fig. 2b). For efficient transcription, we recommend using guanine-rich sequence (GGG/A) for this space [12].
2. A different plasmid backbone can be used to construct a ROSALIND plasmid as long as it includes T7 promoter— aTF operator—3WJdB—T7 terminator sequences. If a different backbone is used, design new PCR primers to generate a linear transcription template.
3. Our primer sets are designed so that the forward primer binds to a sequence 91 BP upstream of the T7 promoter start site, in the pUC19 backbone, and the reverse primer binds to the T7 terminator sequence, resulting in a 5′ DNA leader sequence and no 3′ DNA trailer sequence after the terminator. While not needed, including the T7 terminator sequence improves the speed as well as the magnitude of fluorescence signal [9].
4. If you intend to use the reactions for field-testing, you can observe fluorescence activation using a 3D-printed handheld illuminator (Fig. 5b). The CAD files needed for 3D-printing and the instruction to assemble the 3D-printed parts can be found in the original manuscript [9].
5. While unbuffered NTPs can be used, we have found that the transcription efficiency is improved by using Tris-buffered NTPs.
6. Each ligand has different solubility limits in various solvents. When making the ligand solutions, keep in mind that some organic solvents are toxic to IVT above a certain threshold value (please refer to Supp. Fig. 4 of the original manuscript [9] for IVT toxicity limits of various ligands and solvents).

7. Trehalose is believed to offer better long-term stability, but sucrose is more cost-effective and provides comparable lyoprotection.
8. These pipettes (Thomas Scientific, Cat. No. 1207F80) are useful when rehydrating ROSALIND reactions on-site with minimal equipment. However, they are not as accurate as micropipettes.
9. ROSALIND reactions work well at room temperature. However, incubating them at 37 °C will speed up the response.
10. Here, we are describing a simple gravity flow column method for purifying TetR. However, there are different variations in this method that can be used for different aTFs. Some proteins such as CadC and AdcR that are sensitive to mis-metalation might require different and/or additional purification steps and a different storage buffer composition [13]. FPLC can be used for any aTFs to automate this process and to increase purity of the protein sample as well.
11. Many of the aTFs used in ROSALIND can be purified using affinity chromatography by fusing the protein with 6XHis sequence and a TEV cleavage sequence in between using a vector such as pET-28-c. We recommend checking the crystal structure of the aTF of interest, if available, to determine the location of these tags (N-terminus vs. C-terminus) that would interfere the least with its function (i.e., ligand and/or DNA operator site binding).
12. Most aTFs used in ROSALIND express well. For 1-L cell culture, expect ~5 g of the cell pellet. For every gram of the cell pellet, prepare 10 mL of the lysis buffer.
13. Imidazole can absorb light and turn yellow, thereby interfering with the absorbance spectra on FPLC. To prevent this, keep the imidazole stock solution in a dark bottle and protect from light.
14. Prepare four different washing buffers with the following imidazole concentrations: 20 mM, 30 mM, 40 mM, and 50 mM. The purpose of these washing buffers is to remove any impurities that are bound to the nickel column with low imidazole washes of increasing stringency. Thus, these imidazole concentrations can be adjusted accordingly depending on the number of impurities and desired stringency.
15. The final volume of a master mix and an activator mix can be adjusted as needed as long as the two mixes sum to the final volume of 20 µL.
16. This can be adjusted depending on the sensor performance. Increasing the concentration of the DNA template will improve the overall maximum ON signal as well as the speed

at which we see fluorescence activation. However, it will require more aTF to efficiently regulate IVT, which in turn could result in poorer sensitivity for the intended ligand.

17. If you are building a new sensor, and the purified aTF has not been previously tested in ROSALIND, perform a titration of aTF at a fixed DNA template concentration to determine the minimum aTF concentration to prevent transcription.
18. The system is designed so that the amount of MgCl_2 (8 mM final concentration) in the reaction is 70% of the amount of Tris-buffered NTPs (11.4 mM final concentration) for maximum transcription efficiency [14].
19. The concentration of the chemical dye, DFHBI-1T, can be adjusted as needed. However, at a high DFHBI-1T concentration, the reactions are impacted as the solvent used to dissolve it, DMSO, can inhibit IVT (Fig. 3a).
20. If the stock concentration of T7 RNAP is different, adjust the volume to add 0.2 μg total of T7 RNAP.
21. Here, we included a concentration of aTc that should fully induce TetR and generate fast and robust signal. However, this value can be adjusted to test for the sensor's limit of detection. We also recommend running a negative control where no ligand is added to the activator master mix every time an induced reaction is run.
22. To prevent reactions from starting to activate, add the activator mix one strip at a time and place the strip onto the chilled metal block before proceeding to the next strip of tubes.
23. If growing a larger volume of cell culture, add the overnight culture into fresh Luria Broth in 1:100 ratio.
24. If the protein is toxic to the cell culture, induce with a lower IPTG concentration (0.1–0.5 mM).
25. Similar to using a lower IPTG concentration, if the protein is toxic to the cell culture, the cell culture can be grown at 30 °C or at a lower temperature before harvesting.
26. Ni-NTA resin is typically provided in ethanol in 1:1 ratio. Loading 2 mL of Ni-NTA resin will result in 1 mL total of Ni-NTA after the ethanol flows through. Depending on how concentrated your lysate is with your protein of interest, more Ni-NTA resin can be added. Thus, check for the resin capacity to prevent oversaturating the column and losing your protein of interest in the flowthrough.
27. A higher protein concentration is desirable as it minimizes the amount of glycerol, which can inhibit transcription, in a ROSALIND reaction. We recommend concentrating it down to a volume less than 1 mL.

28. Some aTFs might stick to the membrane of the concentrator. To prevent this, carefully wash the membrane with 200–500 μ L of the 2 \times storage buffer before adding the rest in each round.

Acknowledgments

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Competing Interests *J.K.J., K.K.A. and J.B.L. have submitted a US provisional patent application (no. 62/758,242) relating to regulated IVT reactions and a US provisional patent application (no. 62/838,852) relating to the preservation and stabilization of IVT reactions. K.K.A. and J.B.L. are founders and have a financial interest in Stemloop, Inc. The latter interests are reviewed and managed by Northwestern University in accordance with their conflict-of-interest policies.*

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Optical Sensing in Cell-Free Expression

Junzhu Yang and Yuan Lu

Abstract

Light can be used as a control switch for gene expression with potential advantages, avoiding the defects induced by chemical substances. By transplanting components capable of emitting light at a specific wavelength from cells into a cell-free synthesis system, controlled gene expression can be achieved in vitro. Here, we describe an effective method to achieve optical sensing in cell-free protein synthesis (CFPS) based on *Escherichia coli* crude extract containing the two-component system (TCSs) YF1/FixJ, which was able to respond to blue light. Plasmids capable of interacting with the photosensitive components were constructed, and the fluorescent protein mCherry was used as a reporter. This protocol provides a detailed procedure guiding how to construct the blue-light sensing system in CFPS.

Key words Cell-free protein synthesis, Optical sensing, Two-component system

1 Introduction

Cell-free protein synthesis (CFPS) has developed rapidly in recent years [1], which activates biological machinery without living cells and allows direct control of transcription, translation, and metabolism in vitro [2]. For CFPS, the synthesis of different proteins has specific requirements in time and space. Therefore, one of the major challenges for CFPS is appropriate spatial and temporal control of the transcription or translation.

Traditional methods generally use chemical inducers to regulate gene-expression reaction, but the toxicity and instability of the inducers cannot be neglected [3, 4]. In addition, chemical induction cannot achieve “on/off” control without adding inhibitors. Light possesses many advantages as an ideal genetic control switch, including fast input speed, good spatiotemporal conversion, low toxicity, and low cost of light source device [5–8]. Photosensitive components that respond to specific wavelengths of light have also been developed to regulate the transcription and translation of genes. Here, we demonstrate that the two-component system (TCSs) YF1/FixJ is effective in CFPS [9, 10]. Two plasmids

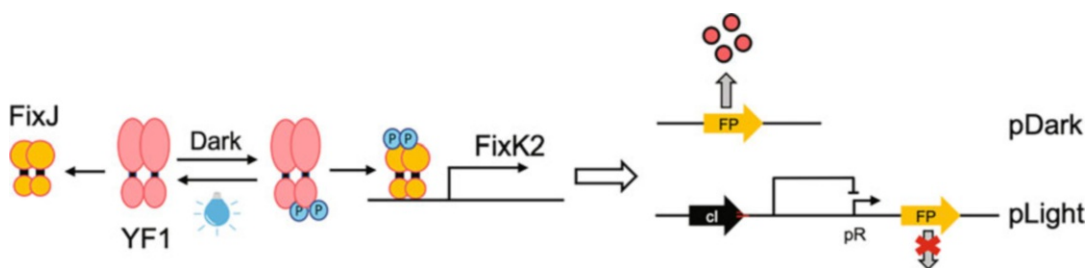


Fig. 1 The mechanism of blue-light sensing. Blue light ($\lambda = 470$ nm) was used to create input signals in the cell-free system. YF1 autophosphorylates in the dark and transfers phosphate to the cognate response regulator FixJ. Under blue light, YF1 dimer is dephosphorylated and converted to phosphatase for FixJ \sim P. pDark is capable of gene expression in the dark. pLight was obtained by inserting a reversal polarity circuit on the basis of pDark. In pLight, the YF1/FixJ system drives the expression of the λ phage repressor *cl* from the FixK2 promoter, which, in turn, represses the expression from the strong λ promoter pR, so pLight enables gene expression under blue light

named pDark and pLight that respond to blue light were constructed to achieve cell-free light-controlling gene expression with *Escherichia coli* extract under darkness and blue light in the batch, respectively (see Fig. 1) [11].

In this study, the red fluorescent protein mCherry was used as the reporter protein, and the effect of controlling gene expression was determined by the difference in fluorescence values between blue-light exposure and dark conditions. This experiment was carried out in three steps. First, plasmids expressing the proteins of YF1/FixJ were constructed and transferred into *E. coli* to prepare the crude extract. Second, the *E. coli* cell extract containing YF1/FixJ was prepared, and pDark and pLight used for cell-free reaction were extracted. Third, the cell-free reaction was started under different conditions, including under blue light and darkness.

2 Materials

2.1 Preparation of *E. coli* Strains Containing YF1/FixJ

1. Plasmid pET23a-YF1 and pET23a-FixJ (see Note 1).
2. *E. coli* B21 (DE3) strain (see Note 2).

2.2 Preparation of Expression Template

1. Plasmid DNA Maxi Kit.
2. The *E. coli* strain containing pDark-mCherry and pLight-mCherry gene (see Note 3).
3. Plasmid Mini kit.
4. Thermocycler.

2.3 Preparation of *E. coli* Extract Containing YF1/FixJ

1. *E. coli* B21 (DE3) strain containing pET23a-YF1 and pET23a-FixJ plasmids.
2. 2× Yeast extract-Tryptone (YT)-Phosphate (P)-Ampicillin (Am) medium: 10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl, 40 mM K₂HPO₄, 22 mM KH₂PO₄, and 1.5% agar for plate use if needed. Autoclaved.
3. S30 buffer A: 14 mM L-Glutamic acid hemimagnesium salt tetrahydrate, 60 mM potassium L-glutamate, 50 mM Tris-HCl, pH 7.7, titrated with acetic acid. Add 1,4-Dithio-DL-threitol (DTT) to 2 mM just before use. Sterile-filter and store at 4 °C.
4. S30 buffer B: 14 mM L-Glutamic acid hemimagnesium salt tetrahydrate, 60 mM potassium L-glutamate, pH 8.2, titrated with Tris. Add DTT to 1 mM just before use. Sterile-filter and store at 4 °C.

2.4 Preparation of Reaction Mixture

1. Reaction mixture: 175 mM potassium glutamate, 10 mM ammonium glutamate, 2.7 mM potassium oxalate, 33 mM phosphoenolpyruvate (PEP), 50 mM HEPES (pH 8), 1.5 mM ATP and GTP, 0.9 mM UTP and CTP, 0.26 mM Coenzyme A (CoA), 0.2 mg/mL tRNA, 0.75 mM cAMP, 1 mM spermidine, 0.06 mM folinic acid, 2 mM of each of the 19 amino acids, 0.33 mM NAD, 1 mM GSH, 2.5% PEG 8000, and 30% (volume) of *E. coli* extract.
2. Prepared expression template in the cell-free system, 3 nM.

3 Methods

3.1 Preparation of *E. coli* Strains Containing YF1/FixJ

1. Add 1–2 µL pET23a-YF1/pET23a-FixJ [9] plasmid solution into 100 µL BL21(DE3) competent cells (*see* **Note 4**) and put them on ice for 30 min (*see* **Note 5**).
2. Add LB medium about 400 µL and culture them at 37 °C and 220 rpm about 30 min (*see* **Note 6**).
3. Coat ~100 µL suspension on the LB plate (*see* **Note 7**).
4. Pick up two single colonies to sequence.
5. Select the correct strain and use the strain to prepare cell extracts in Subheading 3.3.

3.2 Preparation of Expression Template

1. Select the correct strain and extract the plasmid with Plasmid Maxi Kit (*see* **Note 8**).
2. Purify pDark and pLight [9] gene by ethanol precipitation.
3. Dilute the pDark and pLight to 2 mg/mL and store at –20 °C.

3.3 Preparation of *E. coli* Extract Containing YF1/FixJ

1. Cultivate *E. coli* BL21 (DE3) strain containing genes-expressing YF1/FixJ protein (*see Note 9*) on 2× YT-P-Am solid medium with 100 mg/L ampicillin (Am), and then incubate overnight at 37 °C.
2. Select a single colony and transfer it to 10 mL liquid 2× YT-P medium in 50 mL flask with Am (*see Note 10*). Incubate overnight at 37 °C with 220 rpm.
3. Transfer 10 mL overnight culture into 200 mL fresh 2× YT-P medium in 1 L flask at 37 °C with 220 rpm for 5 h.
4. Transfer the cultured bacterial solution to 800 mL 2× YT + P medium at a dilution of 1:20 with Am (*see Note 11*). Control the fermentation conditions at 37 °C and 220 rpm stirring.
5. Add 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the OD600 reaches 0.3–0.4 (*see Note 12*) to induce.
6. After induction, shake cells at 16 °C, 16 h, and then harvest the cells quickly at 4 °C.
7. Wash the cell pellets with 100 mL S30 buffer A at least twice (*see Note 13*).
8. Resuspend the pellets in 1 mL of S30 buffer A per gram of biomass on the ice.
9. Subject the suspension to a high-press crusher twice at 15,000–20,000 psi (*see Note 14*).
10. Centrifuge the lysed cells at 4 °C and 13,000 × *g* for at least 30 min.
11. Incubate the supernatant at 37 °C with 120 rpm for 80 min.
12. Centrifuge the extract at 4 °C and 13,000 × *g* for at least 30 min.
13. Transfer the supernatant to 6–8 kDa MWCO dialysis tubing and dialyze in 100 times volume of supernatant S30 buffer B overnight at 4 °C (or 4 h twice).
14. Centrifuge the extract at 4 °C with 13,000 × *g* for 30 min.
15. Collect and transfer the supernatant to 1.5-mL Eppendorf tubes on ice.
16. Determine the concentrations of two photoresponsive proteins in cell extracts: Perform SDS-PAGE on the prepared two cell extracts and BSA with known concentration for comparison. Then, subject the target band from SDS-PAGE gel to gray analysis. The standard curve was determined by gray analysis of BSA, and the concentration of the two proteins YF1/FixJ in the cell extract can be determined.
17. Flash-freeze the extracts in liquid nitrogen and store them at –80 °C (*see Note 15*).

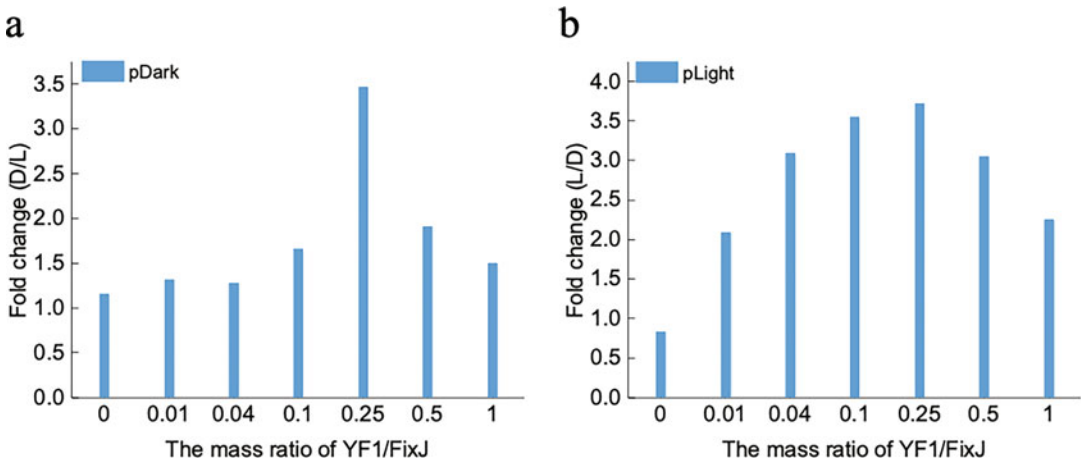


Fig. 2 Expression ratio curve under different conditions (L: blue-light exposure condition, D: dark condition). (a) pDark expression under blue light and darkness. The highest fold change (D/L) of fluorescent is under the mass ratio of YF1/FixJ = 0.25. (b) pLight expression under blue light and darkness. The highest fold change (L/D) of fluorescent is under the mass ratio of YF1/FixJ = 0.25

3.4 Cell-Free Reaction

1. Thaw the CFPS and expression templates on the ice.
2. Incubate 20- μ L cell-free reaction mixture at 30 °C (*see Note 16*) for 16 h under blue light (*see Note 17*) or darkness.
3. Dilute 5-microliter samples from each reaction mixture with 195 μ L ddH₂O, and measure the fluorescence intensity of these diluted samples with F587 excitation and F615 emission filters using Microplate reader. The obtained expression differences under blue-light irradiation and dark conditions are shown in Fig. 2.

4 Notes

1. More detailed information about the two plasmids can be referred to [9]. The vector to express protein is plasmid pET23a.
2. The plasmid used to induce protein expression is transferred to *E. coli* BL21 (DE3).
3. The DNA sequence of plasmid pDark-mCherry and pLight-mCherry can be referred to [9]. When building the blue light-sensing system, the sequence was validated by knocking out the sequence that expressed the YF1/FixJ protein.
4. Competent cells BL21 (DE3) need to be kept strictly at -80 °C, which need to be taken out and thawed on the ice before mixing with plasmid. Due to ultra-low temperature, the competent cells need to take anti-frostbite measures, such as wearing low-temperature resistant gloves.

5. It is important to be sure to leave the mixture on ice for 30 min.
6. This step is the resuscitating process. For plasmids resistant to ampicillin, since only the plasmids are transferred to the competent cells BL21 (DE3), this step can be omitted, and the mixture is directly coated on the plate.
7. Solid LB medium containing ampicillin (100 mg/L) was used here, and the plate was uniformly coated.
8. First, reaction plasmids pDark and pLight were transferred to receptive cell *E. coli* DH5 α , and then plasmids extraction was performed after extensive culture.
9. The plasmid used for expressing YF1/FixJ, pET23a-YF1, and pET23a-FixJ, which are transferred in BL21 (DE3), is stated in the previous step.
10. The concentration of ampicillin is 100 mg/mL, and the volume of ampicillin is generally one thousandth of the total volume of the medium. In the following steps, ampicillin is added to the medium in this way.
11. In this step, 800 mL medium is evenly distributed into 4 L flasks. The cultured bacterial solution in last step is transferred to each flask at a dilution of 1:20. To ensure that the bacterial growth is approximately the same in each flask, the cultured bacterial solution should be fully blown by a pipette before being transferred.
12. In this step, the volume of IPTG added to the medium is one thousandth of the total volume of the medium.
13. The bacteria were washed twice with S30A to remove the residual culture medium. If the cleaning effect was not good, washed the cell pellet more than twice. After being washed, the pellet could be stored at 4 °C overnight or at -80 °C for a long time.
14. The high-pressure crusher produces a lot of heat, which needs to be removed in time to ensure the biological activity of the cell extract. And large quantities of ice water mixture and ice packs need to be added to the tank of the crusher.
15. It is necessary to wear antifreeze gloves to prevent frostbite during flash-freezing.
16. It is necessary to control the ratio of cell extracts containing two kinds of proteins YF1/FixJ during the preparation, and the ratios will play a key role in controlling the expression effect.
17. Blue light is provided by an LED light source capable of providing a wavelength of 470 nm.

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Cell-Free Paper-Based Analysis of Gut Microbiota and Host Biomarkers

Melissa K. Takahashi, Xiao Tan, and Aaron J. Dy

Abstract

The gut microbiome and its interactions with the host have been shown to affect several aspects of human health and disease. Investigations to elucidate these mechanisms typically involve sequence analysis of fecal samples. To support these studies, we present methods to design RNA toehold switch sensors to detect microbial and host transcripts. The sensors are embedded in paper-based, cell-free reactions that enable affordable and rapid analysis of microbiome samples.

Key words Gut microbiota, Host biomarkers, RNA toehold switch, Biosensors, Paper-based diagnostics, NASBA

1 Introduction

The gut microbiome has been shown to contribute to several aspects of human health ranging from gastrointestinal function [1, 2] to cardiovascular disease [3], to cancer immunotherapy [4, 5]. In addition to microbiome composition, host-microbiome interactions play a key role in these health conditions [6, 7]. Typical methods for analyzing the gut microbiome involve deep sequencing coupled with high-throughput bioinformatics. However, the cost, time, and expertise required to execute these methods can limit the ability to perform the large-scale studies necessary to determine the role of microbial changes in these conditions. Furthermore, on-demand analysis of select microbial and host transcripts could be useful for analysis of patient symptoms, disease stratification, or treatment selection [8, 9].

Recently, a cell-free, paper-based platform was developed for detection of biologically relevant RNAs [10–13] that could address the need for affordable, on-demand analysis of microbiome

Melissa K. Takahashi, Xiao Tan and Aaron J. Dy contributed equally with all other contributors.

samples. The platform combines two synthetic biology technologies: RNA toehold switches and a cell-free transcription-translation system. RNA toehold switches are programmable riboregulators that can be designed to bind and detect nearly any RNA sequence [14]. Cell-free systems allow for rapid execution of genetic circuits, including RNA toehold switches. In addition, cell-free systems can be freeze-dried, along with a genetic circuit, onto paper discs, and stored at room temperature. The combination of these two technologies enables rapid development and deployment of biological sensors. By adding an isothermal amplification step such as NASBA (nucleic acid sequence based amplification) [15], low femtomolar concentrations of RNA can be detected [11–13]. Furthermore, by creating a calibration curve, amplification by NASBA can be quantified using RNA toehold switches that approximate the performance of RT-qPCR [12].

In this chapter, detailed methods are presented for analysis of gut microbiota and host biomarkers utilizing RNA toehold switches embedded in cell-free paper-based reactions based on work originally presented by Takahashi et al. [12]. Included are guidelines for identifying target RNAs, designing RNA toehold switches, designing NASBA primers, and developing a calibration curve to quantify NASBA amplification. The methods described here refer to running fresh cell-free reactions without lyophilization. For information on freeze-dried reactions, refer to Pardee et al. [10].

2 **Materials**

2.1 **Toehold Switch
Sensor Assembly
and Purification**

- 1. DNA containing the RNA toehold switch sensor (or DNA to assemble the RNA toehold switch sensor) designed in Sub-heading 3.3. The construct should have the following format: T7 promoter—toehold switch sensor—GFPmut3b—T7 terminator (*see* **Note 1**).

T7 promoter sequence	5'-TAATACGACTCACTATAGG-3'
GFPmut3b sequence	5'- ATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTG AAGGTGATGCAACATACGGAAAACCTACCCTTAAATTTATTTGCACTACTGGAAAACCTGTTCCGTGGCCAACACTTGTCACTACTTTCGGTTATGGT GTTCAATGCTTTGCGAGATACCCAGATCACATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTACGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACACAAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAG

(continued)

	ATGGAAACATTCTTGGACACAAATTGGAATACAA CTATAACTCACACAATGTATACATCATGGCAGAC AAACAAAAGAATGGAATCAAAGTTAACTTCAAAA TTAGACACAACATTGAAGATGGAAGCGTTCAACT AGCAGACCATTATCAACAAAATACTCCGATTGG CGATGGCCCTGTCTTTTACCAGACAACCATTA CCTGTCCACACAATCTGCCCTTTCGAAAGATCC CAACGAAAAGAGAGACCACATGGTCCTTCTTGA GTTTGTAACCGCTGCTGGGATTACACATGGCAT GGATGAACTATACAAA
T7 terminator sequence	5'- CTAGCATAACCCCTTGGGGCCTCTAAACGGGT CTTGAGGGGTTTTTTG-3'

2. Oligonucleotides to PCR amplify the RNA toehold switch sensor.
3. PCR kit (polymerase, dNTPs, buffer).
4. MinElute PCR Purification kit or equivalent.
5. Qubit dsDNA BR assay kit or equivalent.

2.2 RNA Synthesis and Purification

1. DNA containing the T7 promoter upstream of the 36-nt trigger RNA (Subheading 3.3).
2. DNA containing the T7 promoter upstream of the mRNA sequence (Subheading 3.1).
3. Oligonucleotides to PCR amplify T7-RNA constructs.
4. PCR kit (polymerase, dNTPs, buffer).
5. HiScribe T7 High-Yield RNA Synthesis kit or equivalent
6. RNA Clean and Concentrator kit or equivalent
7. TURBO DNA-free DNase kit or equivalent
8. Qubit RNA BR Assay kit or equivalent

2.3 RNA Toehold Switch Cell-Free Assay

1. 42.5-mm Whatman Filter paper discs (*see* Note 2).
2. Bovine serum albumin.
3. 2-mm biopsy punch.
4. RNase Zap.
5. PURExpress In Vitro Protein Synthesis Kit.
6. Protector RNase Inhibitor.
7. 384-well microtiter plate (Corning 3544).
8. Plate sealing film.
9. Plate reader capable of temperature control.

2.4 NASBA (*See* Note 3)

1. NASBA primers (*see* Note 4).
2. 3× NASBA reaction buffer.

3. 6× nucleotide mix.
4. NASBA enzyme cocktail wet mix.
5. Protector RNase Inhibitor.
6. Yeast tRNA.

2.5 Stool Sample Processing

1. Stainless steel mortar.
2. Ceramic pestle.
3. RNeasy PowerMicrobiome kit.
4. Vortex Genie 2 with microtube adapter.
5. TURBO DNA-free DNase kit or equivalent.
6. Qubit RNA BR Assay kit or equivalent.

3 Methods

3.1 Identification of Unique Bacterial mRNA Sequences

The following steps describe the workflow for identifying unique bacterial mRNA sequences from species known to exist in the human gut that can be used to develop RNA toehold switches and NASBA primers. For a general description of the considerations taken in this approach, please refer to **Note 5**. All commands provided below are for a Linux environment (Ubuntu 20.04 LTS) as single line commands. The BLAST-related commands are identical across platforms. For small numbers of markers, using the NCBI BLAST website at <https://blast.ncbi.nlm.nih.gov/> is often easier and faster. However, for querying larger numbers of markers or when using custom databases, running locally installed command-line BLAST is necessary.

3.1.1 Metaphlan In-Group Marker Extraction

1. Access the Metaphlan 3.0 page on Github (*see Note 6*): <https://github.com/biobakery/MetaPhlAn/wiki/MetaPhlAn-3.0>.

Select and download the latest version of the Metaphlan marker database file using the direct links to managed shared folders containing the marker files on Dropbox or Google Drive (*see Note 7*). <https://www.dropbox.com/sh/7qze7m7g9fe2xjg/AADHWzATSQcI0CNFD0sk7MAga> or https://drive.google.com/drive/folders/1_HaYl6mT7mZ_Z8JtesH8zCfG9ikWcLXG?usp=sharing.

2. Extract Metaphlan markers for desired target species. To provide an example, the remaining steps will include commands to find markers for *Faecalibacterium prausnitzii*. A text search of the marker file for s__*Faecalibacterium prausnitzii* using a tool such as Awk will find all markers for this species based on the labeled headers (*see Note 8*):

```
awk 'BEGIN {RS=">"} /s__Faecalibacterium_prausnitzii/{print ">" $0}' mpa_v30_CHOCOPhlAn_201901.fna > Faecalibacterium_prausnitzii_metaphlan_markers.fasta
```

3.1.2 Confirm the Presence of Preliminary Markers in the Target Species (See **Note 9**)

1. Refer to the NCBI instructions for installing BLAST locally at <https://www.ncbi.nlm.nih.gov/books/NBK279671/>.
2. Obtain sequences of the desired in-group, for example, a target species. Potential sources include the NCBI microbial genomes website: <https://www.ncbi.nlm.nih.gov/genome/microbes/>.
3. Download the appropriate in-group genome FASTA files (see **Note 10**).
4. Combine these separate genome files into a single FASTA file:

```
cat *.fna > combined_f_prausnitzii_genomes.fasta
```

5. To confirm that the previously extracted Metaphlan markers for *F. prausnitzii* are actually found in the eight genomes we downloaded, we will run a BLAST search between the Metaphlan markers and the combined genomes (see **Note 11**):

```
blastn -task dc-megablast -query Faecalibacterium_prausnitzii_metaphlan_markers.fasta -subject combined_f_prausnitzii_genomes.fasta -dust no -evaluate 0.1 -max_target_seqs 5000 -outfmt 5 -out Faecalibacterium_prausnitzii_metaphlan_markers_genomes_blast.xml
```

6. Analyze the BLAST output and select preliminary markers based on the desired marker size and degree of conservation (see **Note 12**).

3.1.3 Check Marker Expression Levels in Human Stool (See **Note 13**)

1. Access the NCBI SRA at <https://www.ncbi.nlm.nih.gov/sra> and search for the appropriate transcriptomic data (see **Note 14**).
2. Select the desired datasets and download the list of accession numbers.
3. If not already installed, install NCBI SRA tools from <https://github.com/ncbi/sra-tools>. The location for downloaded files may be selected by running the virtual database configuration command.

```
./vdb-config -i
```

4. Download the desired datasets using the prefetch tool included with SRA tools and the accession numbers separated as below (*see Note 15*). We will continue the example of assessing potential markers for *F. prausnitzii* by using a few whole-stool metatranscriptomic datasets.

```
./prefetch SRR3313094 SRR7464977 SRR7464979 SRR9118110
SRR9118120 SRR9118121
```

This will download six SRA archive files into the SRA subdirectory of the folder selected during the configuration step above.

5. Perform BLAST directly using the blastn_vdb tool included with SRA Tools and the SRR reads as databases to search for hits to potential markers (*see Note 16*).

```
./blastn_vdb -task dc-megablast -query Faecalibacterium_prausnitzii_metaphlan_markers.fasta -db "SRR3313094 SRR7464977 SRR7464979 SRR9118110 SRR9118120 SRR9118121" -dust no -evaluate 0.1 -max_target_seqs 1000000 -outfmt 5 -out Faecalibacterium_prausnitzii_metaphlan_markers_SRA_blast.xml
```

6. Quantify the BLAST hits for each marker against the SRR datasets in order to get an approximate estimate of relative expression levels of each marker, and select markers that have the highest overall expression levels (*see Note 17*).

3.1.4 Assess Marker Specificity (*See Note 18*)

1. Download the latest NCBI nucleotide database by changing to the directory where the BLAST databases will be stored and running update_blastdb.pl (*see Note 19*).

```
update_blastdb.pl nt --decompress
```

2. Obtain the NCBI taxonomy ID (taxid) for target species to use in BLAST search filtering (*see Note 20*). This may be done by visiting the NCBI Taxonomy database at <https://www.ncbi.nlm.nih.gov/taxonomy>, searching for a species, and obtaining the top level taxid. For example, searching for *F. prausnitzii* leads to a result page and taxid 853. Specific strains will also have their own taxids. Because BLAST sometimes does not propagate sublevel taxids, it is necessary to use the get_species_taxids.sh script from <https://www.ncbi.nlm.nih.gov/books/NBK546209/> using the top species-level taxid, which yields a plain text file that contains all taxids for all strains of *F. prausnitzii*.

```
get_species_taxids.sh -t 853 > F_prausnitzii_full_taxid_list.txt
```

3. The taxid file is then used with the `-negative_taxidlist` flag as a filter when BLAST is used with the nt database (*see* **Note 21**).

```
blastn -task blastn -query Faecalibacterium_prausnitzii_
metaphlan_markers.fasta -db nt -outfmt 5 -out F_prausnitzii_
metaphlan_markers_vs_nt.xml -dust no -evalue 0.1 -num_threads
30
```

4. Jointly analyze the results and select markers with the desired combination of inclusivity for the target in-group, relatively high-level expression in human stool, and specificity for the target species (*see* **Note 22**).

3.2 Selection of Host Transcripts

For identification of eukaryotic host transcripts, refer to procedures used to select target regions for RT-qPCR assays reviewed in Bustin and Huggett [16] (*see* **Note 23**). Manually selected markers may also be assessed for specificity using the same workflow described above in Subheading 3.1.4 using taxid 9606 for *Homo sapiens* for BLAST filtering.

3.3 RNA Toehold Switch Design

1. The following steps utilize the series B toehold switch design from Pardee et al. [11] (Fig. 1).
2. Replace the “source mRNA” sequence in the NUPACK script (*see* **Note 24**) with the sequence determined in Subheadings 3.1 or 3.2 and run the script using the NUPACK website design interface [17]. <http://nupack.org/design/new> (*see* **Note 25**).
3. Check for and eliminate toehold switch sequences with stop codons in frame with the downstream start codon.
4. Use the analysis feature in NUPACK with the maximum complex size set to one to view the predicted secondary structure of toehold switch designs. Select designs with the least amount of secondary structure in the toehold region of the switch to screen.

3.4 Toehold Switch Sensor Assembly and Purification

1. Assemble double-stranded DNA containing the RNA toehold switch sensor using standard techniques.
2. PCR amplify the toehold switch sensor to create a linear DNA template.
3. Purify the PCR using the MinElute PCR Purification kit according to manufacturer’s protocol.
4. Measure DNA concentration using the Qubit DNA BR Assay kit.

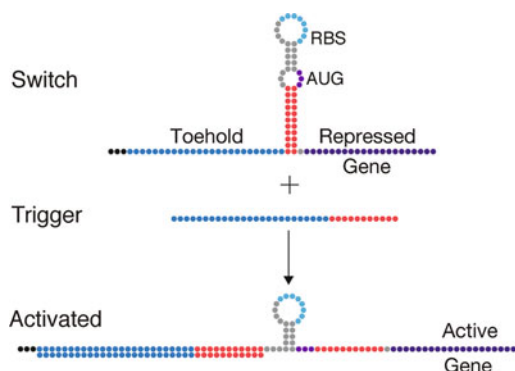


Fig. 1 RNA toehold switch structure. In the initial RNA toehold switch structure, the ribosome is unable to access the ribosome-binding site (RBS) and start codon (AUG). The trigger RNA binds to the toehold region of the switch and activates translation of the downstream gene. The switch, trigger, and activated structures correspond to the structures in the NUPACK design code

3.5 Trigger RNA and mRNA Standard Synthesis

1. Assemble double-stranded DNA containing the T7 promoter upstream of the trigger or mRNA sequence using standard techniques.
2. PCR amplify to create a linear DNA template.
3. In vitro transcribe RNA using the HiScribe T7 High Yield RNA Synthesis kit or equivalent following the manufacturer's protocol.
4. Purify using the Zymo RNA Clean and Concentrator kit following the manufacturer's protocol.
5. Perform DNase digestion using the TURBO DNA-free DNase kit for 1 h following the manufacturer's protocol.
6. Measure RNA concentration using the Qubit RNA BR Assay kit.

3.6 Preparation of Paper Substrate

1. Block paper disc substrate overnight in 5% bovine serum albumin (BSA) at 4 °C.
2. Rinse the blocked paper with nuclease-free water three times.
3. Prepare a clean, RNase-free surface on a hot plate by covering the hot plate with aluminum foil and wiping down with RNase Zap.
4. Place the rinsed paper on the hot plate and heat at 65 °C for approximately 15 min to dry, flipping the paper halfway to ensure even drying.
5. Store paper in a dry, sterile container such as a 50-mL conical tube.

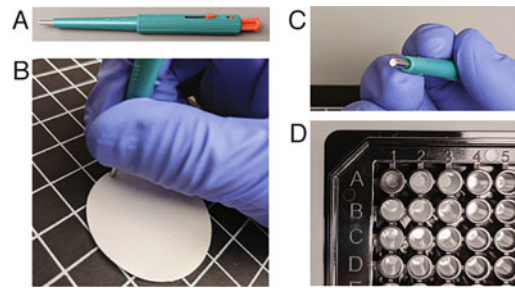


Fig. 2 Cutting paper discs. A 2-mm biopsy punch (a) is used to cut discs out of the BSA-blocked paper (b). Cut discs (c) are placed directly into the wells of a 384-well microtiter plate (d)

3.7 Microtiter Plate Setup for RNA Toehold Switch Cell-Free Assay

1. Use a black 384-well microtiter plate with round wells and clear bottom to perform the paper-based reactions.
2. To ensure best results in a non-humidified environment, add 20 μL of water to all perimeter wells of the plate (76 wells for a 384-well plate).
3. Punch 2-mm discs from the blocked paper (Subheading 3.6) using a biopsy punch and place the punched discs into the wells of the 384-well microtiter plate (Fig. 2) (*see Note 26*).

3.8 RNA Toehold Switch Cell-Free Assay

1. In general, the cell-free reaction contains the following components from the PURExpress Kit: NEB A (40%), NEB B (30%), linear toehold switch sensor DNA (1.875 nM), RNase Inhibitor (0.5%, Protector RNase), nuclease-free water, and RNA sample (*see Note 27*).
2. Keep all components on ice throughout reaction setup. This is critical to prevent differences in output caused by pipetting order or evaporation.
3. Thaw cell-free components NEB A and NEB B on ice.
4. Prepare RNA sample such that 1 μL will be used in the reaction.
5. The following describes a 5.5 μL of cell-free reaction, which is sufficient volume for triplicate paper-based reactions. Reactions can also be run in a liquid-only format without a paper matrix but requires more volume (*see Note 28*).

NEB A	2.20 μL
NEB B	1.65 μL
RNase inhibitor	0.0275 μL
Sensor DNA	1.875 nM
Sample	1.0 μL
Nuclease-free water	Volume to 5.5 μL

6. Two controls should be run with each experiment: a blank and a sensor-only control. The blank contains the components described in **step 5** replacing both the sensor DNA and sample with nuclease-free water. The sensor-only control utilizes nuclease-free water as the sample.
7. It is recommended to prepare a master mix of all components excluding the sample. Include 10% extra volume of each component to account for volume loss during pipetting. For example, for a set of eight reactions, the master mix would be a total of 39.6 μL with 19.35 μL of NEB A, 14.52 μL of NEB B, 0.24 μL of RNase inhibitor, and the remaining 5.48 μL is made up of nuclease-free water and linear toehold switch sensor DNA.
8. Gently vortex the master mix and briefly spin down.
9. Pipette 4.5 μL of master mix into PCR tubes (on ice).
10. Add 1.0 μL of sample or NASBA reaction product to each PCR tube to create a final volume of 5.5 μL .
11. Gently vortex the reaction tubes and briefly spin down.
12. Prepare a separate 5.5- μL reaction for the blank according to **step 6**.
13. From each 5.5- μL reaction mix, apply 1.4 μL directly to three separate paper discs in the 384-well microtiter plate to create technical triplicates for the reaction.
14. Be sure that the paper disc is firmly pressed to the bottom of the well to ensure even imaging. Use the pipette tip to press down the disc.
15. Check for and remove any bubbles in the wells by pressing down on the discs using a pipette tip.
16. Seal the microtiter plate with a clear sealing film, making sure that it is firmly attached to prevent evaporation.
17. Place the microtiter plate in a plate reader preheated to 37 °C for time-course measurements.
18. Measure fluorescence from the bottom of the plate using appropriate wavelengths for the fluorescent reporter of choice (e.g., 485/520 nm for GFP) with 5-min intervals over a 2-h period.

3.9 Analysis of Kinetic Fluorescence Data

1. Compute the average of the blank condition at each time point.
2. Subtract off the blank average from each reaction condition.
3. Adjust each fluorescence trace to start at 0 RFU at time zero. For an individual reaction, calculate the average of the first three time points (0, 5, 10 min). Subtract the average value from all time points for that individual reaction.

4. Fit the zero-adjusted data to the following equation:

$$\text{RFU}(\text{zero adjusted}) = \frac{a}{e^{-bt} + c}.$$
5. Calculate the slope of the fitted curve at $t = 50$ min (*see Note 29*).

3.10 RNA Toehold Switch Screening

1. Screen RNA toehold switches designed in Subheading 3.3 using 2 μM trigger in vitro transcribed in Subheading 3.5.
2. Upon completion of data analysis described in Subheading 3.9, compare the ON (trigger added) and OFF (sensor only) values for each candidate sensor.
3. Select the design with the lowest OFF and highest ON/OFF ratio.
4. Additional testing to determine and compare sensitivity of RNA toehold switch designs can be done (*see Note 30*).

3.11 NASBA Reaction

1. Create a primer mix containing 6.25 μM of both the forward and reverse primers diluted in nuclease-free water.
2. RNA sample: For in vitro transcribed RNA standards, perform a serial dilution of the RNA in a 50 ng/ μL yeast tRNA solution to match the total RNA complexity of samples. For total RNA samples, dilute the sample to 50 ng/ μL using nuclease-free water.
3. The following volumes describe an individual 5- μL NASBA reaction.

Reaction buffer	1.675 μL
Nucleotide mix	0.825 μL
Primer mix	0.2 μL
RNase inhibitor	0.025 μL
Nuclease-free water	0.03 μL
Enzyme cocktail	1.25 μL
Sample	1.0 μL

4. To avoid pipetting very small volumes, it is recommended to create a master mix for the total number of NASBA reactions being run that includes the reaction buffer, nucleotide mix, RNase inhibitor, nuclease-free water, and primer mix. Include 10% extra volume of each component to account for volume loss during pipetting.
5. Distribute 2.75 μL of the master mix from **step 4** into PCR tubes.
6. Add 1 μL of your sample to the PCR tube and mix.
7. Heat denature secondary structure and dimers at 95 °C for 2 min and immediately follow with a 10-min incubation at 41 °C.

8. Remove from heat. Add 1.25 μL of NASBA enzyme cocktail to each tube and mix.
9. Incubate at 41 $^{\circ}\text{C}$ for 30–180 min (*see* **Note 31**).
10. After incubation, transfer 1.0 μL of completed NASBA reaction to the toehold switch reaction.

3.12 NASBA Primer Evaluation

1. Use an in vitro transcribed RNA as the sample. The sample RNA should be the unique RNA determined in Subheadings 3.1 or 3.2. Prepare a 30 pM sample diluted in 50 ng/ μL yeast tRNA.
2. Set up NASBA reactions according to Subheading 3.11, except in **steps 3** and **5**, do not include the primer mix in the master mix and distribute 2.55 μL into PCR tubes. Add 0.2 μL of each primer pair to separate PCR tubes.
3. Test the NASBA products in toehold switch reactions.
4. Select the primer set that provides the largest activation of the toehold switch.

3.13 Development of NASBA/Toehold Switch Calibration Curve

1. Prepare a serial dilution of standards diluted in 50 ng/ μL yeast tRNA (30 pM, 3 pM, 300 fM, 30 fM, 3 fM).
2. Set up NASBA reactions according to Subheading 3.11.
3. Test a range of incubation times (30–180 min), and determine the NASBA incubation time that results in a linear response when plotting toehold switch output vs NASBA RNA standard concentration (*see* **Note 32**).
4. Once the proper incubation time has been determined, perform calibration runs. Each run should consist of three independent NASBA reactions per standard concentration. Perform a total of three calibration runs.
5. Analyze toehold switch data according to Subheading 3.9.
6. For each calibration run, take the average GFP production (RFU/min) for the middle concentration RNA standard. Normalize all other data points to this average value. Repeat this for each calibration run and average the results for each RNA standard across all three runs.
7. Fit the normalized data to the equation: $\text{GFP production} = A * \ln(\text{RNA concentration}) + B$.
8. To estimate the concentration of an RNA in an unknown sample, concurrently perform NASBA and toehold switch reactions for the RNA standard used to normalize the calibration curve. Normalize data as in **step 6** and use the equation in **step 7** to determine the concentration of the RNA in the unknown sample.

3.14 Stool Sample Homogenization and RNA Extraction

1. Store stool samples at -80°C without any stabilization reagents.
2. Within a biosafety cabinet, place individual stool samples into a stainless-steel mortar along with liquid nitrogen to maintain a completely frozen sample.
3. With liquid nitrogen keeping the stool frozen, grind each stool sample into a fine powder using a mortar and pestle [18].
4. Transfer pulverized stool to a sterile container such as 50-mL conical tube and place on dry ice to keep cold. Be sure to minimize time that sample is not on dry ice (*see Note 33*).
5. Using a sterile spatula, weigh out between 0.15 and 0.20 g of homogenized stool sample directly into a glass bead tube provided in the RNeasy PowerMicrobiome kit (*see Note 33*). Weighing into a secondary container such as weigh boat can cause thawing and sample loss during transfer to the glass bead tube. Be sure to tare the scale with the specific tube into which the stool will be added. Weighed samples can be stored at -80°C prior to RNA extraction.
6. Repeat homogenization and weighing process for any additional stool samples being sure to thoroughly clean and sterilize the mortar and pestle to avoid cross-contamination.
7. Thaw homogenized and weighed stool sample prior to starting RNA extraction.
8. For RNA extraction, use the RNeasy PowerMicrobiome kit following the manufacturer's instructions.
9. Include the extended on-column DNase step of 15 min.
10. Elute with 100 μL of nuclease-free water.
11. Measure total RNA concentration.
12. Dilute the RNA solution so that it is no more than 200 ng of nucleic acid per μL .
13. Perform a secondary DNase digestion using TURBO DNA-free DNase kit following manufacturer's instructions. Utilize the method of adding half of the TURBO DNase for an initial 30-min incubation at room temperature then adding the other half of the DNase for another 30-min incubation (*see Note 34*).
14. Measure the concentration of the RNA sample using the Qubit RNA BR Assay kit.
15. Dilute the RNA solution to 50 ng per μL with nuclease-free water.
16. Store the purified RNA solution at -80°C if not being used immediately as an input to the NASBA reaction.

4 Notes

1. The GFPmut3b sequence is provided here, but other fluorescent or enzymatic reporters can be utilized as well.
2. In this application, Whatman 1442-042 paper discs were used. It is possible to utilize other paper substrates that are first tested for compatibility with the cell-free system and reporter protein.
3. NASBA reagents were from Life Sciences Advanced Technologies. The volumes listed in Subheading 3.11 are specific to these reagents and should be modified if alternate reagents are used. The NASBA enzyme cocktail is sensitive to freeze–thaw cycles. It is recommended to aliquot the enzyme cocktail upon first thaw.
4. The NASBA primer design rules from Deiman et al. recommend an amplified RNA length between 120 and 250 nt [19]. In Takahashi et al., it was found that amplified RNAs as small as 72 nt function well [12]. In general, shorter NASBA amplicons with minimal secondary structure are desired to activate the RNA toehold switch sensor. Design NASBA primers according to these modified rules. Use a primer design tool such as Primer-BLAST: (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Enter the sequence found in Subheading 3.1 or 3.2 along with the primer design parameters. Screen at least three primer pairs to start.
 - (a) Hybridizing regions of both primers should be 20–30 nt.
 - (b) The GC content of the hybridizing regions should be 40–60%.
 - (c) Amplified RNA should be between 70 and 180 nt.
 - (d) Avoid identical runs of four or more nucleotides (AAAA, CCCC, GGGG, TTTT).
 - (e) Avoid stretches of pyrimidines in the first 10 nt downstream of the T7 promoter sequence.
 - (f) If possible, the 5' end of the hybridizing region should be GC rich, while the 3' end of the hybridizing region should be AT rich.
 - (g) The T7 promoter sequence should be added to the 5' end of the forward primer:
 5'-AATTCTAATACGACTCACTATAGGG-3'
5. The performance of any diagnostic is critically dependent on the selection of nucleic acids to use as targets for detection. Sensitivity, which describes how inclusive a sequence is for a given target, and specificity, which describes how well a given sequence can differentiate between target and nontarget nucleic acids, are two critical parameters that will determine

the expected best-case performance for any selected nucleic acid sequence. Real-world performance will additionally depend on a number of experimental factors as detailed below. In general, the goal is to choose a sequence that is only found in a target in-group and not in any other organism (the out-group). The in-group may consist of specific strains, species, or other taxonomic levels, but all members must contain common sequence(s) that differentiate them from the out-group.

The complexity of out-group analysis depends on the stringency required and the expected complexity of the sample matrix. For example, in biomedical testing, human blood is a sterile space, and only a few organisms are known to cause human disease [16, 17]. An important task is to eliminate sequences with unintentional identity to human nucleic acids as well as organisms that may be human-associated. Stool contains much greater microbial diversity, and there may be additional organisms encountered in such samples through dietary intake or environmental exposure during collection. Because there is not a well-established curated list of all known organisms that are associated with the human gut environment, the current practice is to design assays that are not triggered by all nontarget sequences using comprehensive nucleotide databases.

The general steps to nucleic acid selection are applicable to the design of any nucleic acid-based test. There are some additional considerations for this particular application given that its amplification method, NASBA, performs best with RNA input. The overall workflow consists of collecting the genomes to be used as the in-group, performing the search for specific markers in comparison to the out-group while filtering for sequences that are highly expressed in human stool samples and confirming the sensitivity and specificity of the markers. These sequences are then used for downstream RNA toehold switch and NASBA primer design. The tools for this workflow were selected to minimize the number of packages and steps needed, although numerous alternatives exist.

The workflow starts with in-group specific sequences extracted from databases generated for use with Metaphlan [18, 19], a computational package designed for metagenomic profiling. Metaphlan and its related packages attempt to identify and catalog clade-specific marker genes at the strain, species, genus, and additional phylogenetic levels. There are currently 13.5 k species represented and 1.1 M markers. Its focus on identifying specific portions of coding regions and its frequently updated database [20] make it an ideal starting point for the extraction of potential species-specific in-group

markers. There may be some species that are not represented in the Metaphlan databases, for which alternative approaches are needed such as k-mer-based differential genomic comparisons [21]. Metaphlan is actively developed and maintained by the Segata Lab [<http://segatalab.cibio.unitn.it/tools/metaphlan/index.html>]. Only the marker database is needed, which is made separately available as a compressed FASTA file with full phylogenetic species labels. If a specific bacterial transcript such as a toxin is the target, then the Metaphlan marker step is not needed and the transcript sequence should be used directly for downstream steps (*see* Subheadings 3.1.2–3.1.4).

6. Although it is possible to install the Metaphlan 3 package and have it automatically download the database, this is not necessary and sometimes fails. It is easier to download the database directly from the cloud sources listed as the last installation step on the Metaphlan 3 Github page.
7. The file mpa_latest is a plain text file that contains the name of the latest version of the marker database. At the time of writing, the latest version is mpa_v30_CHOCOPhlan_201901. Therefore, the correct marker file is: mpa_v30_CHOCOPhlan_201901.tar located in the same folder. Downloading and decompressing this tar and bzip2 compressed file yields a 1.2 GB FASTA file called mpa_v30_CHOCOPhlan_201901.fna with labeled markers. The taxonomic labels are of the format: k__Kingdom|p__Phylum|c__Class|o__Order|f__Family|g__Genus|s__Species.
8. This command uses the Linux tool Awk to parse the Metaphlan marker database FASTA file using > as the record separator, selects only the records that contain the string s__Faecalibacterium_prausnitzii, and then prints these records to the specified filename. An additional > is inserted in the print command because Awk removes the field separators by default and > is needed to maintain the standard FASTA heading. One can search for other species by replacing Faecalibacterium_prausnitzii with another species name being sure to use an underscore between the genus and species. In this example, the output from these operations is a FASTA nucleic acid file with genomic markers from the Metaphlan database that are supposed to be specific for *F. prausnitzii*, and we have 41 sequences with a median length of 708 bp. However, new genomes are incorporated into comprehensive databases frequently, and the inclusivity and specificity of the Metaphlan markers for any given species may change over time. We therefore must perform some confirmatory tests (*see* Subheadings 3.1.2–3.1.4).
9. A common goal is to create species-specific markers, although the in-group can also be defined as a collection of strains or other taxonomic clades. The steps involve obtaining genomes

of the target in-group and running homology searches to assess the presence of the potential markers in these genomes. We use BLAST to find partial homology so that sections of potential markers may still be used.

10. Search for any target species, and click on the Genome Assembly and Annotation report link. The genomes selected for analysis depend on the goal of the user. We will review the process for *F. prausnitzii* as an example. At the time of writing, there are 136 separate sequencing assemblies for various strains of *F. prausnitzii*. Sorting by scaffold in ascending order shows that only 8 of these are considered completely or mostly assembled. The rest have between 4 and 1135 scaffolds and have many breaks and overlaps. For most tasks, using only the completed assemblies is sufficient. Since most markers are typically tested on pure cultures of the target species, we recommend including the genome of a commercially available strain. Here, *F. prausnitzii* A2-165/JCM 31915/DSM 17677 is obtainable from both the Japan Microbe Collection and the DSMZ-German Collection of Microorganisms and Cell Cultures. The FTP column of the Genome Assembly and Annotation report contains links to RefSeq (R) or Genbank (G) FTP directories for the genome assemblies. We recommend using the RefSeq sources. Download the _genomic.fna.gz files and decompress to obtain FASTA nucleic acid files for each genome assembly. These steps may also be automated with scripts such as Batch Entrez, which allows easy web-based batch downloading at <https://www.ncbi.nlm.nih.gov/sites/batchentrez>.
11. Here, we are using the option -task dc-megablast because we are not interested in small bits of homology. The dc-megablast task tolerates some breaks but searches for larger contiguous chunks instead of focusing on every small island of identity. The mpa_v30_CHOCOPhAn_201901 version of the database contains 1.1 million markers with median size of 852 bp and an interquartile range of 606–1260 bp.
12. The BLAST output can be of any standard format. Please refer to the NCBI BLAST e-book for additional information https://www.ncbi.nlm.nih.gov/books/NBK279684/#appendices.Options_for_the_commandline_a. XML or CSV is more easily used with software such as BLASTGrabber [20], a Java-based platform-independent BLAST output analysis package, or Biopython [21]. Plain text alignments are helpful for human readability. Numerous limiting filters are also available. The limits for inclusivity are dependent on the specific goals of the user. For example, the *F. prausnitzii* marker 853__A0A3E2U7E6 of length 2157 bp is 96–98% conserved for 5 of 8 *F. prausnitzii* genomes and 92% conserved for the other 3 genomes, whereas marker 853__A0A173XRA8 of

length 2355 bp has high-percentage identity to only 2 of 8 *F. prausnitzii* genomes. This may or may not be acceptable depending on the usage scenario. In general, the goal is to find regions that are at least 150–200 bp long so that there is sufficient genomic space to design different potential amplification primers and RNA toehold switches. Given the size of most Metaphlan markers, a handful of markers is sufficient to test, depending on additional filtering such as expression levels in stool (see below). One can alter the BLAST output to include data in tabular format (-outfmt 6) with customizable data such as the query length, alignment length, hit score, and other values that are sortable. Please refer to the NCBI Blast e-book listed above. An alternative approach is to limit the BLAST output using flags such as percent identity (-perc_identity 98) and percent query coverage per high-scoring segment pair (-qcov_hsp_perc 98). This significantly simplifies subsequent alignment analysis but also greatly reduces the number of reported alignments. This also needs to be considered concurrently with expression levels in stool (see below). For example, using the 98% filters for percent identity and query coverage, the only marker with significant hits to 6 of 8 *F. prausnitzii* genomes is marker 853__E2ZJT1 of length 588 bp, which has 100% identity in 4 genomes and 99% in 2 genomes for the entire length of the marker. There are no reported matches with the marker 853__A0A2A7B1Y7 of length 3195 bp. When no percent identity filter is used, the same marker 853__A0A2A7B1Y7 yields hits across its entire length with >92% identity for 5 of 8 genomes and two hits per genome indicating that there are two copies of this marker in these strains.

13. Since NASBA targets RNA, it is helpful to determine whether there is high-level expression of the potential target marker in microbes in human stool in order to create the most sensitive diagnostic. Several transcriptomic studies have been performed on human stool, and the raw sequencing data is often available from publicly accessible databases such as the NCBI Sequencing Read Archive (SRA). One can tally the reads as a rough approximation of expression levels with caveats described below. The steps involve finding and downloading transcriptomic data from the archives, performing BLAST homology search using these datasets versus potential markers, and counting and comparing the resulting hits.
14. Using the search term “human adult gut metagenome” is a helpful limit. Many experiments are targeted to mucosa or biopsy-associated sequencing, which may be excluded by using the search term “NOT mucosa.” The results may be sent to the RunSelector, which provides an interactive interface

that allows filtering by experimental type, such as Library-Source (metatranscriptomic), and sorting by dataset size. The datasets also have associated metadata, which may be used to further guide data selection. If the target is a specific bacterial transcript such as a toxin, then a search may be performed for metatranscriptomic data from cultures of the specific bacterium or disease condition, which is typically much more useful for these targets than nontargeted whole-stool metatranscriptomic data given the typical paucity of toxin-specific reads in these studies.

15. The data may be downloaded directly without charge by installing NCBI SRA tools locally. The sequencing reads may also be delivered directly to cloud-based computing instances on Amazon Web Services or Google Cloud, which require active accounts on these platforms and associated payment plans.
16. Here, we have significantly increased the `max_target_seqs` because each sequencing read is a potential hit. The default limit is 500 hits, and BLAST does not record additional hits per query beyond this limit unless directed otherwise.
17. Quantitation of the hits provides an estimate of relative expression levels between the different markers within the experimental conditions of the pooled stool metatranscriptomes. This may help selection of markers that balance broad coverage of the in-group (see above) and high expression levels in stool. Convenient interactive visualization is available through BlastGrabber by importing the XML file, selecting sequence viewer under sequence selection, and indicating the entire dataset. This will display all markers and all hits in a sortable interactive table. Clicking any marker will also load a map of the marker and all matching sequencing reads. For example, using the above search parameters, marker `853__A0A2A7B1Y7` (3195 bp) has the highest number of hits at 27,481 roughly evenly distributed throughout the marker, whereas marker `853__A0A173T8T3` (531 bp) has only 16 hits. Normalization methods such as reads/fragments per kilobase per million or transcripts per million are often used as methods to quantitate RNAseq data and enable cross-experiment comparisons [22]. However, the goal here is to obtain a rough approximation through the combination of all reads of multiple similar experiments into one pool and then compare the relative abundance within this single pool. This can be simplified by only normalizing the number of hits relative to marker length, say 1000 nt (i.e., per kb). In this example, marker `853__A0A2A7B1Y7` has 8601 reads per kb whereas `853__A0A173T8T3` has 30 reads per kb. Since the same SRA databases are used for all potential markers, the relative expression comparisons should remain valid.

18. The selection of potential markers is based on both inclusivity for the in-group of genomes and the expression level in stool transcriptomic studies. To obtain the best performance, it may be necessary to look at the alignments and only use subsections of the potential markers. Once promising marker regions have been identified, an additional check is needed in order to ensure specificity. This is accomplished by performing a BLAST search using potential markers against relevant BLAST databases. It is possible to generate a complete microbial database by downloading all microbial RefSeq sequences as described at <https://www.ncbi.nlm.nih.gov/genome/doc/ftpfaq/#allcomplete> and creating a local BLAST database with the `makeblastdb` command <https://www.ncbi.nlm.nih.gov/books/NBK279688/>. However, this is generally not necessary and lacks any eukaryotic genomes. The nonredundant nt database from NCBI is typically sufficient and may be conveniently downloaded using the `update_blastdb.pl` script included with BLAST.
19. The `update_blastdb.pl` Perl script will download the nt database archive files, decompress them, and delete the archive files to save space. Running the same command in the same directory will only download additional files if the specified BLAST database has been updated on the NCBI servers and will automatically overwrite outdated files. Please refer to NCBI instructions at <https://www.ncbi.nlm.nih.gov/books/NBK52640/> for setting up BLAST including the BLASTDB environmental variable to indicate the directory of BLAST database files.
20. In order to identify regions of the preliminary markers that may be present in organisms other than *F. prausnitzii*, it is useful to perform a BLAST search against the nt database but exclude sequences from *F. prausnitzii*. This is achieved through taxonomy ID (taxid) filtering against the numbers indicating *F. prausnitzii* strains.
21. Here, the `blastn` task is used since smaller sections of identity are still useful to find. Using `dc-megablast` may not be sensitive enough to discover subsections of preliminary markers with identity to non-*F. prausnitzii* sequences. Contrarily, the nt database is comprehensive, and setting a high e-value (default value 10) would yield huge numbers of small hits that make further selection highly challenging. In general, using the `blastn` task and an e-value of 0.1 maintains a good balance between sensitivity and avoiding excessive numbers of small hits for query input sizes typical of Metaphlan markers. This step is also significantly accelerated by using the `-num_threads` flag for multithreaded operation and selects the number of processor cores to dedicate to the program.

22. For example, analyzing the Metaphlan markers for *F. prausnitzii*, marker 853__A0A2A7B1Y7 has high-expression levels in stool and is found in triple copies in all 8 *F. prausnitzii* strains, but there are 229 hits that span the entirety of the marker sequence to non-*F. prausnitzii* species including *Enterococcus faecium* isolates. This marker would be a poor choice based on lack of specificity. Marker 853__D4KAK8__C4N21_13380 is 540 bp is expressed at 8387 reads per kb and is >93% identical for the length of the marker in 5 of 8 *F. prausnitzii* strains. It has no significant hits to other species and would be a highly specific marker for *F. prausnitzii*. One may also use subsections of the markers to avoid limited areas of identity with other species. For example, marker 853__C7H1R8__DW905_06675 is 1401 bp and has 85% identity in nt 1–353 to an uncultured organism clone. There were no other non-*F. prausnitzii* hits, and it is unclear whether this uncultured organism would be found in human stool. One could ignore this or one could delete the first 353 nt of marker 853__C7H1R8__DW905_06675 and use the rest of the marker for subsequent design steps.
23. Eukaryotic genes are generally much larger, typically contain introns, and may have many varied isoforms. Similar to the in-group selection process, it will be important to select a target region that is present in isoforms expressed in the tissues and conditions desired. Additionally, a commonly used strategy to avoid the impact of DNA contamination is to select regions on mRNA transcripts that bridge exon-exon junctions and would be too large to amplify from immature unspliced transcripts or DNA genomic source. The availability of preexisting expression data generally motivates the selection and design of assays for specific host transcripts, but large databases such as the European Bioinformatics Institute's Gene Expression Atlas at <https://www.ebi.ac.uk/gxa> may also be used. There are several automated web-based tools for marker design and quality checking for specific hosts such as Primer-BLAST at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>.
24. Copy the following text into the NUPACK design interface and replace source mRNA sequence.

```
# Template for toehold design for mRNA trigger
material = rna
temperature[C] = 37 # optional units: C (default) or K
trials = 10
sodium[M] = 1.0 # optional units: M (default), mM, uM, nM, pM
dangles = some
allowmismatch = true
#target structure
```

```

structure switch = U28 D11 (U3 D5 U12 U3) U22
structure trigger = U36
structure activated = D36 (+ U3) U3 D5 (U12) U36

#sequence domains
#series B conserved sequence GGACUUUAGAACAGAGGAGAUAAAGAUG
#Green et al linker AACCUGGCGGCAGCGCAAAAGAUGCGUAAA
domain a = N11
domain b = N25
domain g = GGG
domain s = GGACUUUAGAACAGAGGAGAUAAAGAUG
domain l = N1 AACCUGGCGGCAGCGCAAAAG

#source sequence
source MRNA = TATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAA
TACCTAGAGATAGTGGACGTTACTCGC

#windows from sources
window rrna_window = a b
rrna_window.source = MRNA
switch.seq = g b* a* s a l
trigger.seq = a b
activated.seq = a b g b* a* s a l
switch.stop = 10.0
prevent = AAAA, CCCC, GGGG, UUUU

```

25. The NUPACK design script provided here is written for the website interface. Modifications can be made to run the command-line version of NUPACK. The maximum number of designs that the website interface provides is ten. The script can be run multiple times to obtain more potential solutions. The “prevent” line in the script eliminates toehold switch designs with a run of four identical nucleotides in a row. If the sequence determined in Subheadings 3.1 or 3.2 includes runs of individual nucleotides, the corresponding nucleotide can be removed from the “prevent” line. It is recommended to run the script once with the complete “prevent” line and once with the specific nucleotide removed and to test toehold switch designs made with and without this 4 N restriction.
26. To punch paper discs, it is recommended to use a cutting mat cleaned first with 10% bleach, followed by a wipe down with 70% ethanol and RNase Zap.
27. Use of the NEB PURExpress system, which consists of reconstituted individually purified transcription and translation components, is described here. Other cell-free systems including whole-cell lysate/extract-based systems can be used. If using an extract-based system with a GFP reporter, an alternate paper

substrate should be used. The Whatman 1442-042 paper exhibits high-autofluorescence background when used with extract-based cell-free systems. Additionally, if using a whole-cell lysate/extract-based system, intact circular plasmid DNA constructs should be used instead of the PCR amplified, linear products described in Subheading 3.5 due to linear DNA degradation in extract-based systems.

28. Reactions can also be run in a well without any paper matrix, but these liquid-only reactions require larger volumes to cover the well adequately and avoid significant evaporation. For a 384-well plate, it is recommended that each well contains at least 10 μ L of cell-free reaction volume, rather than 1.4 μ L, when run without paper or another porous matrix.
29. The time at which fluorescence data is analyzed can be optimized. Identify the time at which the rate of fluorescence production is at a maximum or constant.
30. Limit of detection for RNA toehold switch sensors varies by design. High-performing sensors can detect \sim 100 pM–1 nM trigger RNA without amplification.
31. The NASBA reaction time will depend on the intent of the experiment. For initial primer screening, an incubation of 120 min is recommended. For quantification, a range of incubation times should be tested (30–180 min). For a presence or absence test where assay speed is not critical, an incubation of 120–180 min is recommended to maximize sensitivity.
32. The NASBA RNA standard concentrations can be adjusted and do not have to include all five concentrations. The concentrations recommended here serve as a starting point and should be adjusted for each individual application.
33. Weighing and distribution of stool sample should be done quickly to avoid thawing of the sample. Once the stool thaws, it will no longer be in powder form, which will make it difficult to weigh or manipulate.
34. The TURBO DNA-free DNase digestion was performed to ensure a DNA-free sample for RT-qPCR. **Steps 11–13** in Subheading 3.14 can be skipped if not performing RT-qPCR since NASBA has much higher preference for amplifying RNA than DNA.

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Detection of Norovirus Using Paper-Based Cell-Free Systems

Kaiyue Wu and Alexander A. Green

Abstract

Norovirus infections are the leading cause of foodborne illness and human gastroenteritis, afflicting hundreds of millions of people each year. Molecular assays with the capacity to detect norovirus without expensive equipment and with high sensitivity and specificity represent useful tools to track and contain future outbreaks. Here we describe how norovirus can be detected in low-cost paper-based cell-free reactions. These assays combine freeze-dried, thermostable cell-free transcription-translation reactions with toehold switch riboregulators designed to target the norovirus genome, enabling convenient colorimetric assay readouts. Coupling cell-free reactions with synbody-based viral enrichment and isothermal amplification enables detection of norovirus from clinical samples down to concentrations as low as 270 zM. These diagnostic tests are promising assays for confronting norovirus outbreaks and can be adapted to a variety of other human pathogens.

Key words Paper-based diagnostics, Isothermal amplification, Cell-free systems, In vitro protein synthesis, Norovirus detection, Toehold switches, Riboregulators, NASBA

1 Introduction

Norovirus is the most common cause of gastroenteritis worldwide and is estimated to infect nearly 700 million individuals each year, leading to 219,000 deaths [1]. The virus is extremely contagious and can be transmitted from person to person or by contact with contaminated surfaces. Although norovirus infections are usually self-limiting, they can lead to severe symptoms in children and the elderly and each year cause billions of dollars of costs to healthcare systems and economies worldwide [1, 2]. While diagnostics such as real-time quantitative reverse transcriptase PCR (qRT-PCR) and enzyme-linked immunosorbent assays (ELISAs) have been developed for norovirus detection, they either require high-cost instruments and trained technicians or lack specificity and sensitivity [3–5]. Tests that are fast, cost-effective, and can detect noroviruses with high specificity and sensitivity are still needed. In this chapter,

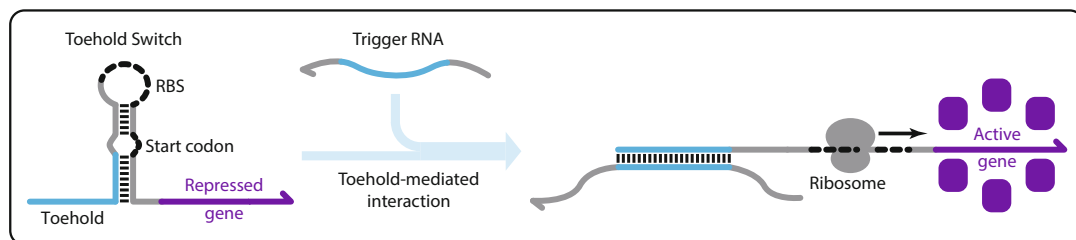


Fig. 1 Toehold switch RNA detection mechanism. The toehold switch riboregulator contains a 5' single-stranded toehold region, a hairpin that conceals the ribosome binding site (RBS) and the start codon, and a downstream reporter gene. In the presence of the trigger RNA, the toehold switch hybridizes with the trigger through a toehold-mediated strand-displacement reaction to unwind the hairpin, thereby exposing the RBS and the start codon and activating downstream gene expression. This figure is adapted from [7]

we demonstrate how cell-free systems coupled with toehold switch riboregulators can be applied for detection of norovirus. This method provides a rapid, low-cost, and sensitive norovirus diagnostic by examining viral nucleic acids.

Toehold switches are a class of de novo-designed riboregulators that control translation through defined RNA secondary structures and RNA–RNA interactions [6]. A typical toehold switch contains a single-stranded region known as a toehold at its 5' end (Fig. 1). The hairpin positioned after the toehold region conceals both the ribosome binding site (RBS) and the start codon in a strong secondary structure. As a result, the downstream reporter gene is strongly repressed. In the presence of a triggering RNA molecule, the toehold switch can hybridize with the trigger through a toehold-mediated strand-displacement reaction to unwind the hairpin, thereby exposing the RBS and the start codon and activating downstream gene expression. Toehold switches can be used for mRNA and endogenous RNA detection in *Escherichia coli* and can also be used to regulate endogenous gene expression through chromosomal integration [6]. More complex cellular logic circuits have also been developed using toehold switches to enable sophisticated biocomputing [8].

Use of toehold switches for in vitro applications has been made possible with cell-free transcription-translation systems. In these applications, toehold switches are designed to activate downstream reporter expression only when a pathogen RNA or DNA of interest is present. Sequence-specific toehold switches have been demonstrated for a variety of pathogens including the Ebola virus [9], Zika virus [10], norovirus [7], SARS-CoV-2 [11], and *Salmonella typhi* [11]. Other types of riboregulators such as translational repressors [12], which turn off translation in response to a trigger RNA, and ultraspecific riboregulators termed SNIPRs [13], which detect single-base changes, have been developed and expand the potential in vitro applications of riboregulators.

To enable easier test deployment, diagnostic assays using toehold switches and cell-free reactions can be hosted on paper substrates. Paper provides a low-cost, field-deployable, sensitive, and specific platform for diagnostic reactions. Importantly, cell-free reactions on paper substrates can be freeze-dried for long-term storage while maintaining their activity [9]. Following an initial demonstration detecting high concentrations of Ebola virus RNA [9], a more systematic workflow integrating isothermal amplification was developed for detection of the Zika virus in a rapid, low-cost, and sensitive format amid the Zika outbreak of 2016 [10]. More recently, paper-based toehold switches have been used for gut microbiome host biomarker detection and monitoring though detection of bacterial 16S rRNA or mRNA [14]. SNIPRs have also been applied in paper-based reactions to achieve single-nucleotide specificity for identification of drug resistance and cancer-causing mutations [13].

In this chapter, we detail the application of paper-based cell-free systems for norovirus detection using toehold switches (Fig. 2). The overall assay employs norovirus-binding synthetic antibodies (synbodies) to capture and enrich norovirus particles from a sample, followed by heat-driven lysis and isothermal amplification to generate large amounts of norovirus RNA without thermal cycling. The amplified RNA is then applied to paper-based cell-free reactions where norovirus-specific toehold switches detect the viral RNA and generate the reporter enzyme β -galactosidase, which generates a yellow-to-purple color change

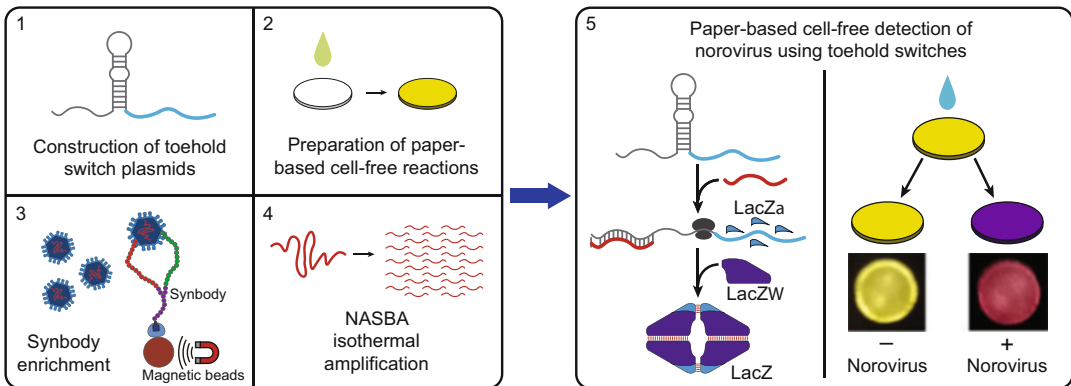


Fig. 2 Workflow for detection of norovirus using cell-free systems and toehold switches. Prior to beginning the assay, plasmids for expressing norovirus-specific toehold switches are constructed (1) and embedded in freeze-dried paper-based cell-free reactions (2). Norovirus particles in a sample are enriched using synbodies (3) and subsequently lysed through a brief heating step. The released norovirus RNA is then amplified using NASBA (4) and applied to paper-based cell-free reactions (5). Norovirus-specific toehold switches produce a LacZ α reporter in response to norovirus RNA amplicons to generate a visible purple color change after complementation with LacZ Ω and cleavage of the substrate CPRG. This figure contains elements adapted from [7]

by cleaving a chromogenic substrate. Herein, we describe the construction of plasmids encoding the toehold switches and the preparation of freeze-dried paper-based cell-free reactions for expressing the riboregulators. We further detail how norovirus RNA can be purified, enriched, and amplified using synbody-based magnetic bead concentration in combination with isothermal nucleic acid sequence-based amplification (NASBA) reactions. Lastly, we describe the procedure for detection of amplified norovirus RNA using the paper-based toehold switch cell-free reactions. By combining all the above elements, this method enables detection of norovirus with visible signals and a limit of detection down to 270 zM from clinical samples [7]. We expect that the procedures described in this chapter can be applied to a variety of other viral pathogens by using amplification primers and toehold switches targeted to the pathogen of interest, with improved sensitivity obtained using pathogen-specific antibodies or synbodies as necessary.

2 Materials

2.1 Preparation of Toehold Switch Plasmids

DNase-free, RNase-free, Ultrapure 18.2 M Ω -cm water is used for all PCR and cell-free reactions.

1. High-fidelity DNA polymerase master mix (e.g., Phusion, Q5) or equivalent.
2. PCR primers and toehold switch insert templates are purchased as synthetic DNA oligonucleotides. Strands are initially dissolved in 10 mM Tris, 0.1 mM EDTA, pH 8.0 at concentrations of 2–100 μ M. The primer and the toehold switch sequences used in Subheading 3 are listed in Table 1.
3. The vector backbone used for toehold switch expression is pCOLADuet-1, which is commercially available from EMD Millipore. The reporter for the toehold switches, the β -galactosidase α peptide (LacZ α), is preinserted into pCOLADuet-1 to generate the template plasmid for vector backbone PCR. A plasmid containing LacZ α that provides the necessary backbone template is available on Addgene as pDM_noro_A2_lacZA (Addgene ID 118819).
4. 2 \times Gibson assembly enzyme mix: 8 units/ μ L Taq DNA ligase, 0.05 units/ μ L Phusion DNA polymerase, 0.008 units/ μ L T5 exonuclease, 10% PEG-8000, 200 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 20 mM DTT, 0.4 mM each of the four dNTPs, and 2 mM NAD⁺ in water. Gibson assembly enzyme mix (2 \times) can also be purchased from commercial vendors. The concentration of T5 exonuclease in the reaction mix specified above is recommended for overlap domains shorter than 150 bp [15].

Table 1
List of DNA sequences used for construction of plasmids for toehold switch expression

Name	Sequence
Insert_fwd	CTAGTAAATTCGCGTTTCTACGGTAGCCGGGCGCTAATACGACTCACTA TAGGG
Insert_rev	GACGGCCAGTGAATCCGTAATCATGGTCATCTTCTTGCGCTGCCGCCAGG TT
Backbone_fwd	ATGACCATGATTACGGATTCACTGGCCGTC
Backbone_rev	CCGGCTACCGTAGAAACGCGAATTTACTAGCATAAGGGAGAGCGTCGAGA TC
Toehold switch S2	GCGCTAATACGACTCACTATAGGGATCGCCCTCCCACGTGCTCAGATC TGAGAATCTCATGGACTTTAGAACAGAGGAGATAAAGATGATGAGATTC TCAACCTGGCGGCAGCGCAAGAAGATG

5. DpnI enzyme: 20,000 units/mL.
6. Plasmid DNA miniprep kits.
7. 0.5× TBE buffer.
8. Sterile glass beads for spreading bacteria onto agar plates. Glass beads are 4 mm in diameter and are made of soda-lime glass. Beads are autoclaved at 121 °C for 30 min and stored at room temperature in a clean and sealed plastic container.
9. 14-mL cell culture tubes with round bottoms and snap-on caps are used for bacterial cell culture.
10. Antibiotics. Plasmids based on pCOLADuet-1 vectors confer kanamycin resistance. The working concentration for kanamycin is 50 µg/mL for *E. coli*.
11. DH5α competent *E. coli* cells.
12. LB media used to inoculate bacteria are prepared with LB broth and ultrapure H₂O and are sterilized by autoclaving at 121 °C for 30 min.
13. Sterile toothpicks.
14. Agar plates.

2.2 Preparation of Paper-Based Cell-Free Reactions

1. Cell-free transcription-translation kit (e.g., PURExpress).
2. Bovine serum albumin (BSA) is used to block the filter papers.
3. RNase inhibitor is added to the cell-free reactions to inhibit RNase activity.
4. Chromatography filter papers (e.g., Whatman Grade 42 filter papers).
5. Disposable biopsy punches. The biopsy punches are used to create 2-mm-diameter paper disks.

Table 2
Sequences of primers used for isothermal amplification of norovirus genomic RNA

Name	Sequence
NASBA_fwd	AATTCTAATACGACTCACTATAGGGAGAAGGCAGGCAAG AGCCAATGTTTCAGA
NASBA_rev	CTCATTGTTGACCTCTGGGA

- 6. Chlorophenol red- β -D-galactopyranoside (CPRG): The CPRG is dissolved in ultrapure water to obtain a stock solution of 24 mg/mL.
- 7. A LacZ Ω expression plasmid is used to produce LacZ Ω protein under the T7 promoter. This plasmid is available on Addgene as pDM_T7_HisLacZomega (Addgene ID 118815).
- 8. Liquid nitrogen.
- 9. A float rack is used to hold the tubes when freezing with liquid nitrogen.

**2.3 Processing
Norovirus Samples,
Synbody Enrichment,
and Isothermal
Amplification**

- 1. Isothermal amplification kit (e.g., recombinase polymerase amplification (RPA) kit, nucleic acid sequence-based amplification (NASBA) kit).
- 2. Primers required for isothermal amplification of norovirus are purchased as DNA oligonucleotides. The sequences of the primers are included in Table 2.
- 3. Streptavidin-coated magnetic beads.
- 4. 1 \times phosphate-buffered saline (PBS).
- 5. Magnetic bead washing solution (PBST): 1 \times PBS with 0.05% Tween 20.
- 6. Blocking solution: 3% BSA in PBST (*see Note 1*).
- 7. Stool samples with or without norovirus.
- 8. Biotin-labeled anti-norovirus synthetic antibody [16].
- 9. Silica gel desiccation packages.

2.4 Equipment

- 1. Thermocycler: For performing PCR and isothermal amplification. A heat block can also be used for isothermal amplification.
- 2. Microplate reader: Enables measurements of optical density or fluorescence resulting from the cell-free reaction.
- 3. Gel electrophoresis system: For analysis of DNA products.
- 4. Hot plate: For quick drying of blocked filter papers.
- 5. Lyophilizer: For freeze-drying the cell-free components on the paper disks.
- 6. Shaker: For incubation and shaking of bacterial cell cultures.

3 Methods

3.1 Preparation of Toehold Switch Plasmids

To prepare toehold switch sensors for norovirus detection, we first constructed plasmids for expression of toehold switches in cell-free reactions. The T7 promoter was used to initiate transcription by T7 RNA polymerase, and a T7 terminator was used as the termination signal, both of which can be found on the source vector pDM_nor-o_A2_lacZA. We followed routine molecular cloning procedures and used Gibson assembly to ligate the designated switch to the vector backbone. The detailed steps are described as follows:

3.1.1 Preparing Toehold Switch Insert Sequence

1. Order the toehold switch sequence as a synthetic DNA oligonucleotide and the necessary PCR primers (*see* Table 1 for primer and toehold switch sequence information). Although toehold switch S2 is used in this protocol, the amplification method can be applied to all toehold switch sequences containing the same primer-binding sites.
2. Dilute the toehold switch S2 template with ultrapure H₂O to a final concentration of 20 nM. Dilute the primers to 10 μM for use (*see* Note 2).
3. Prepare a 20-μL PCR master mix in a PCR tube with the components and volumes indicated in Table 3.
4. Pipette the PCR mixture up and down to mix the solution.
5. Place the tube into a thermal cycler and start the PCR with the program indicated below.
 - (a) **Step 1:** 98 °C, 1 min.
 - (b) **Step 2:** 98 °C, 10 s; 57 °C, 10 s; 72 °C, 15 s. Repeat this step 35 times (*see* Note 3).
 - (c) **Step 3:** 72 °C, 3 min.
 - (d) **Step 4:** 4 °C, forever.

Table 3
Reaction components and volumes used for toehold switch insert PCR

	Volume (μL)
Insert_fwd (10 μM)	2
Insert_rev (10 μM)	2
Toehold switch S2 template (20 nM)	0.2
Ultrapure H ₂ O	9.4
2× PCR master mix	6.4
<i>Total reaction volume</i>	20

Table 4
Reaction components and volumes used for vector backbone PCR

	Volume (μL)
Backbone_fwd (10 μM)	10
Backbone_rev (10 μM)	10
pDM_noro_A2_lacZA (1–5 ng μL^{-1})	1
Ultrapure H_2O	29
2 \times PCR master mix	50
<i>Total reaction volume</i>	100

- Analyze the toehold switch insert PCR product by gel electrophoresis using a 2–2.5% agarose gel. To identify successful amplification, expect a band between 100 and 200 nt in length depending on the toehold switch design.

3.1.2 Preparing Vector Backbones

- Order the primer sequences used to amplify the backbone vector: Backbone_fwd and Backbone_rev (*see* Table 1 for sequence information). pDM_noro_A2_lacZA, which contains LacZ α , can be used as the template for vector backbone PCR.
- Dilute the backbone vector with ultrapure H_2O to a final concentration of 1–5 ng/ μL . Dilute the primers to 10 μM for PCR use (*see* Note 2).
- Prepare a 100- μL PCR master mix in a PCR tube with the components and volumes indicated in Table 4.
- Pipette the PCR mixture up and down to mix the solution.
- Place the tube into a thermocycler and start the PCR with the program indicated below.
 - Step 1:** 98 $^{\circ}\text{C}$, 1 min.
 - Step 2:** 98 $^{\circ}\text{C}$, 10 s; 57 $^{\circ}\text{C}$, 10 s; 72 $^{\circ}\text{C}$, 120 s. Repeat this step 35 times (*see* Note 3).
 - Step 3:** 72 $^{\circ}\text{C}$, 3 min.
 - Step 4:** 4 $^{\circ}\text{C}$, forever.
- To eliminate residual template plasmid DNA, add 1 μL of DpnI enzyme (20 units) to the completed PCR.
- Pipette up and down to mix the solution.
- Incubate at 37 $^{\circ}\text{C}$ for at least 30 min (*see* Note 4).
- Heat-inactivate the DpnI enzyme by incubating at 80 $^{\circ}\text{C}$ for 5 min.
- Analyze vector backbone PCR product by gel electrophoresis using a 0.8–1% agarose gel. To identify successful backbone amplification, expect a band at around 3.5 kb.

3.1.3 Gibson Assembly

1. After generating the toehold switch insert sequence and the vector backbone sequence, combine 2 μL of the backbone PCR product, 2 μL of the insert PCR product, and 4 μL 2 \times Gibson assembly mix in a PCR tube (*see Note 5*).
2. Gently pipette the solution to mix.
3. Incubate the reaction at 50 °C for 30 min.
4. Remove the Gibson assembly reaction from the thermal cycler and place on ice.
5. The resulting Gibson assembly reaction products are now ready for transformation.

3.1.4 Transformation of Chemically Competent *E. coli* Cells

1. Take chemically competent *E. coli* cells from the –80 °C freezer and leave them to thaw on ice for 10 min. A cloning strain such as DH5 α is recommended for plasmid construction.
2. Add a 50- μL aliquot of competent cells into a prechilled PCR strip tube.
3. Pipette 2 μL of the Gibson reaction into the aliquot of chemically competent cells (*see Note 6*).
4. Mix by pipetting up and down.
5. Incubate the cells on ice for 10–15 min.
6. Quickly transfer the tube with competent cells to a 42 °C water bath and incubate for 36 s. Make sure the cells are completely submerged in the water.
7. Replace the tube on ice and chill for 5 min.
8. Prepare a 1.7-mL centrifuge tube with 0.5 mL to 1 mL of antibiotic-free LB.
9. Transfer all transformed competent cells to the 1.7-mL centrifuge tube.
10. Place the tube in a shaker and incubate at 250 rpm, 37 °C for at least 60 min.
11. After shaking, harvest the cells by centrifuging at 3000 $\times g$ for 5 min. Discard the supernatant by gently pouring the solution into a biohazardous waste container. Use care to ensure the cell pellet is not discarded.
12. Resuspend the cell pellet in 100 μL of antibiotic-free LB by pipetting it up and down carefully. Otherwise, the remaining LB from the supernatant can be used for resuspension.
13. Fetch a clean and freshly made agar plate containing the appropriate antibiotics.
14. Add sterile glass beads to the plate.
15. Transfer all the resuspended cells to the designated agar plate (*see Note 7*).

16. Evenly spread cells onto the plate with glass beads. Let the plates stand with lid side up at room temperature for 10–15 min.
17. Discard glass beads and leave plates with lid side down at 37 °C overnight. Colonies should be visible after 12–16 h of incubation.
18. Identify successful toehold switch constructs using Sanger sequencing.

3.1.5 Production of Toehold Switch Plasmid DNA

1. Prepare a 14-mL culture tube with 6 mL of LB media and kanamycin at 50 µg/mL.
2. Select the colony with a correct toehold switch sequencing result by using a sterile toothpick and transfer the bacteria colony into the LB-containing culture tube by dipping in the toothpick.
3. Place the tube in a shaker and incubate overnight at 250 rpm and 37 °C.
4. The next day, centrifuge the tube at $3000 \times g$ for 10 min. Discard the supernatant into a biohazardous waste container.
5. Miniprep the plasmids.
6. Use a UV/Vis spectrophotometer to determine the concentration (ng/µL) of the plasmids. The concentration will be needed for preparation of cell-free reaction mix.

3.2 Preparation of Freeze-Dried Cell-Free Reactions on Paper

3.2.1 Cell-Free Production of LacZΩ Protein

LacZΩ is the complementary fragment to the toehold switch reporter LacZα, which collectively constitute the full-active β-galactosidase (LacZ) that catalyzes colorimetric reactions with substrate CPRG. We recommend producing LacZΩ protein with the cell-free system in advance and adding the protein as a supplement in the preparation of paper-based toehold switch cell-free reactions.

1. Remove the cell-free solutions from the −80 °C freezer and the RNase inhibitor and LacZΩ plasmids from the −20 °C freezer. Thaw on ice.
2. Prepare a 20-µL cell-free reaction mix for lacZΩ expression in a PCR tube with the following components: PURExpress cell-free solution A, 40%; PURExpress cell-free solution B, 30%; and RNase Inhibitor, 2%. The expression template LacZΩ plasmids are added at a final concentration of 20 ng/µL. The remaining volume is filled with ultrapure H₂O to a total volume of 20 µL.
3. Place the assembled cell-free reaction in a thermocycler or an incubator for incubation at 37 °C for 4 h.

4. Store the cell-free reaction products in a -20°C freezer for subsequent use. The cell-free expressed LacZ Ω protein can be directly added to the cell-free reactions without purification.

3.2.2 Preparation of Paper Substrates

1. Block filter papers overnight at 4°C using 5% BSA. Make sure all filter papers are fully submerged in the BSA solution.
2. After overnight blocking, rinse the papers with ultrapure H_2O for 5 min, three times in total. Discard water waste into a hazardous waste container.
3. Place the rinsed filter papers into suitable container to place on the hot plate. Dry the filter papers at 50°C for 5 h or until fully dried.
4. Prepare paper disks to host the cell-free reactions by using a biopsy punch to cut the filter papers into 2-mm-diameter disks. Use tweezers to collect the cut paper disks and place them into PCR strips. The paper disks should be placed at the bottom of the PCR tubes (*see Note 8*).

3.2.3 Preparation of Freeze-Dried Cell-Free Reactions

1. Remove the cell-free reaction components from the -80°C freezer. Remove the RNase inhibitor, stock CPRG solution (24 mg mL^{-1}), and lacZ Ω protein produced from cell-free reactions from the -20°C freezer.
2. Thaw all the solutions on ice.
3. After thawing, pipette up and down to gently and fully mix the solutions (*see Note 9*).
4. Pulse-spin the solutions and return them to the ice.
5. Prepare a cell-free reaction mix for toehold switch norovirus detection. Here, we use PURExpress in vitro protein synthesis system as a demonstration (*see Note 10*). Assemble the following cell-free reaction mix on ice: PURExpress cell-free solution A, 40%; PURExpress cell-free solution B, 30%; RNase Inhibitor, 2%; CPRG (24 mg mL^{-1}), 2.5%; and LacZ Ω protein produced from cell-free reaction, 12%. The remaining volume is reserved for toehold switch sensor DNA and H_2O . Toehold switch DNA from plasmids is added to the cell-free reaction mix at a final concentration of $30\text{ ng}/\mu\text{L}$. Toehold switch DNA directly from a PCR can also be used with a final concentration of 33 nM (*see Note 11*).
6. Pipette up and down to fully mix the solution. Use care to avoid formation of bubbles (*see Note 12*).
7. Transfer $1.8\text{ }\mu\text{L}$ of cell-free reaction mix onto each paper disk.
8. Pulse-spin the strip containing the paper disks. This step encourages all the cell-free reaction solution to absorb into the paper.

9. Flash-freeze the PCR strips containing the paper disks using liquid nitrogen by placing the strips onto a float rack and placing the rack onto liquid nitrogen. Make sure the paper disk and the cell-free solution are fully submerged. Leave for 30 s.
10. Promptly transfer the strips to a lyophilizer for freeze-drying overnight (*see* **Note 13**). Freeze-dried paper-based cell-free reactions are now prepared.
11. Store the freeze-dried paper-based cell-free reactions sealed under nitrogen, shielded from light, and in the presence of silica gel desiccation packages.

3.3 Norovirus Sample Processing and Amplification

3.3.1 Concentration and Extraction of Norovirus RNA

To achieve the highest sensitivity, we employed magnetic beads coated with norovirus-binding synthetic antibodies known as synbodies to capture and concentrate norovirus particles from stool samples. This same procedure can be applied to other pathogens using antibodies or synbodies with affinity for these pathogens. In addition, RNA can be released from the norovirus particles using a 2-min 95 °C heating step without use of magnetic beads prior to isothermal amplification, albeit with a higher limit of detection.

1. Prepare streptavidin-coated magnetic beads equal to 2.1×10^8 to 3.6×10^8 beads in a low protein binding centrifuge tube.
2. Discard bead storage solution and wash the beads three times with 1 mL of 1× phosphate-buffered saline with 0.05% Tween 20 (PBST). Discard the PBST after each wash.
3. Block the washed magnetic beads with 3% BSA in PBST at 4 °C overnight.
4. On the following day, discard the blocking buffer and resuspend the beads with fresh 3% BSA in PBST and block at 4 °C for an additional 2 h.
5. Discard the blocking buffer.
6. Wash the beads with PBST for three times. Discard the PBST each time.
7. Resuspend the beads with 1× PBS. The blocked magnetic beads are now prepared.
8. Dilute the stool samples with or without norovirus by adding 1 µL of stool sample to 49 µL PBS in PCR strips, respectively. Pipette to mix.
9. Add the biotin-labeled anti-norovirus synthetic antibody to the diluted samples at a final concentration of 1 µM.
10. Incubate the resulting solution with shaking at 15–20 rpm at room temperature for 1 h.
11. Add the solution to the prepared streptavidin-coated magnetic beads and shake for an additional 15 min at room temperature.

12. Wash the beads three times with PBST and one time with PBS, 5 min for each wash.
13. Heat the beads at 95 °C for 2 min to lyse the norovirus particles.
14. The norovirus RNA samples are now prepared. Place the samples on ice for subsequent use in NASBA amplification or at −80 °C freezer for long-term storage.

3.3.2 NASBA Isothermal Amplification Reactions

In our experiments, we found that both NASBA and RT-RPA can work to amplify norovirus RNA [7]. Here, we detail the procedure for NASBA to demonstrate the concept of isothermal amplification since NASBA provided significantly better sensitivity than RT-RPA for detection.

1. Remove the NASBA kits from the −80 °C freezer and thaw the components on ice, including reaction buffer, 6× nucleotide mix, and enzyme mix. Place the samples acquired from Sub-heading 3.3.1 on ice (*see Note 14*).
2. After thawing, assemble the NASBA reaction on ice in PCR tubes with the components and volumes indicated in Table 5.
3. Pipette up and down to mix.
4. Place the tubes onto a thermal cycler and incubate at 65 °C for 2 min, followed by incubation at 41 °C for 10 min.
5. Promptly add 1.25 µL enzyme mix to each reaction, which brings the total reaction volume to 5 µL. Briefly mix the solution by pipetting up and down.
6. Replace the strips back to the thermal cycler or hot plate and incubate at 41 °C for 2 h. The amplification products can then be used for downstream detection (*see Note 15*). For immediate detection, place the amplified products on ice. Otherwise, store the amplification products in a −80 °C freezer.

Table 5
Reaction components and volumes used for NASBA reactions (excluding the enzyme mix)

	Volume (µL)
Reaction buffer	1.675
6× nucleotide mix	0.825
RNase inhibitor	0.025
NASBA_fwd (12.5 µM)	0.1
NASBA_rev (12.5 µM)	0.1
RNA sample (e.g., stool samples)	1
Ultrapure water	0.025
<i>Total volume</i>	3.75

3.4 Paper-Based Cell-Free Detection of Norovirus

1. Carefully transfer the paper disks containing freeze-dried cell-free components from the original PCR strips to a low-volume black 384-well assay microplate with round wells and clear bottoms using tweezers. Make sure the paper disks are fully attached to the bottom of the wells (*see Note 16*).
2. Dilute the amplified products from isothermal amplification at a ratio of 1:5 in ultrapure H₂O. For example, add 5 μ L ultrapure H₂O to 1 μ L of amplified products.
3. Pipette up and down to mix the diluted products thoroughly.
4. Use a pipette to apply 1.8 μ L of each diluted amplified product onto the paper disks. Carefully apply the solution to avoid formation of air bubbles.
5. After the addition of diluted solution, fill the surrounding empty wells with ultrapure H₂O, using approximately 40 μ L per well. This step is essential to prevent excessive evaporation of the cell-free reactions.
6. Cover the low-volume black 384-well assay plate with a clear plastic lid.
7. Promptly transfer to a microplate reader with the lid on.
8. Set up a kinetic measurement program for the microplate reader with the following steps and parameters:
 - (a) Preheat the plate reader to 37 °C.
 - (b) Continuously shake at 200–300 rpm and measure optical density (OD) at 575 nm. Record OD at 575 nm every 1 min for 6 h.
 - (c) Turn off the heating module after completion of the program.
9. Alternatively, place the 384-well plate in a 37 °C incubator and monitor the color by eye or with a smartphone.

4 Notes

1. We recommend making fresh PBST and 3% BSA in PBST each time before use.
2. The toehold switch sequence is usually synthesized as a single ultramer oligonucleotide and is provided by the manufacturer at a 2–4 μ M concentration. Primers are usually provided at a 100 μ M concentration.
3. The 57 °C annealing temperature is determined based on the primers used in this experiment and can be adjusted based on the T_m of the actual primers used. The 72 °C polymerization

extension time can be adjusted depending on the length of the final DNA product and the polymerization rate specified by the manufacturer.

4. Sufficient incubation time is critical to ensure complete removal of residual template DNA plasmids as the antibiotic resistance they confer can lead to production of unwanted colonies without the desired insert. DpnI treatment digests methylated DNA present in the template DNA plasmids originating from bacteria but not the desired DNA products from PCR.
5. It is not necessary to clean up the PCR products prior to use in the Gibson assembly reaction. More than two DNA fragments can be ligated during one Gibson reaction, provided they have suitable homologous domains.
6. Gibson products do not need to be purified prior to addition to the competent cells.
7. Add the resuspended cells directly onto the glass beads to avoid accidental spillage of the cells.
8. Proceed with caution to ensure the paper disks are placed at the bottom of the tubes. In this way, the cell-free solution added can be fully absorbed by the paper disks.
9. PURExpress cell-free solution B contains a very high concentration of enzymes. Gentle pipetting avoids the formation of bubbles.
10. We have tested several different recombinant-enzyme-based cell-free systems and found PURExpress to work best with toehold switches.
11. A cell-free reaction master mix excluding the toehold switch DNA can be assembled first if different switches are used in the cell-free reactions.
12. The use of wide-orifice pipette tips is helpful for preventing formation of bubbles.
13. After processing with liquid nitrogen, the paper disks containing cell-free reactions must be promptly transferred to the lyophilizer to prevent liquefaction of the cell-free reactions, which could compromise the performance of the paper-based cell-free reactions.
14. NASBA enzymes are also available in a lyophilized thermostable format.
15. For isothermal amplification, it is highly recommended to perform the preamplification steps and postamplification steps on separate laboratory benches to avoid cross-contamination.
16. When placing the paper disks in the assay plate, remember to leave empty wells that will be filled with water to provide moisture for the paper-based cell-free reactions.

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A TXTL-Based Assay to Rapidly Identify PAMs for CRISPR-Cas Systems with Multi-Protein Effector Complexes

Franziska Wimmer, Frank Englert, and Chase L. Beisel

Abstract

Type I CRISPR-Cas systems represent the most common and diverse type of these prokaryotic defense systems and are being harnessed for a growing set of applications. As these systems rely on multi-protein effector complexes, their characterization remains challenging. Here, we report a rapid and straightforward method to characterize these systems in a cell-free transcription-translation (TXTL) system. A ribonucleo-protein complex is produced and binds to its target next to a recognized PAM, thereby preventing the targeted sequence from being cleaved by a restriction enzyme. Selection for uncleaved targeted plasmids leads to an enrichment of recognized sequences within a PAM library. This assay will aid the exploration of CRISPR-Cas diversity and evolution and help contribute new systems for CRISPR technologies and applications.

Key words CRISPR-Cas systems, PAM, TXTL, Positive screen, Multi-protein complex, Binding assay

1 Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas (CRISPR-associated) systems are adaptive immune systems present in many bacteria and most archaea [1, 2]. Adaptive immunity is conducted in three general steps: acquisition, expression, and immunity. In the first step (acquisition), foreign DNA or RNA is recognized, and short fragments, called protospacers, are integrated into the CRISPR array as spacers separated by conserved repeats [3]. The selected protospacers are normally flanked by a protospacer adjacent motif (PAM) unique to each system and used to differentiate the invader-associated sequence from the spacer integrated into the CRISPR array [4, 5]. In the second step (expression), CRISPR arrays are transcribed and processed into mature CRISPR RNAs (crRNAs) [6]. These crRNAs then form a complex with the Cas effector nuclease. In the third step

(immunity), the resulting ribonucleoprotein complex screens DNA or RNA present in the cell for sequences complementary to the spacer and flanked by the PAM [7]. Upon recognition, the complex cleaves the recognized target through different mechanisms, completing the CRISPR-Cas system's task as an adaptive immune system [8].

Despite the three general steps of adaptive immunity, CRISPR-Cas systems are highly diverse. To date, two classes, six types, and more than 30 subtypes have been classified. Class I and II systems are divided based on the presence of a multi-protein effector complex or a single-effector protein, respectively [9]. Type I systems are part of Class I and represent the majority of all CRISPR-Cas systems, yet remain understudied compared to Class II systems. This is in part due to Type I systems involving four to eight proteins in the effector complex at different stoichiometries (*see* Fig. 1a). Almost all of these proteins form a complex with the crRNA called Cascade (CRISPR-associated complex for antiviral defense) and screen invading DNA for its target. Upon target recognition, Cas3 is recruited to nick the nontarget strand and processively degrade this strand in the 3'-to-5' direction [10, 11]. While harnessing this complex as a technology is far more challenging than harnessing a Class II single-effector protein (e.g., Cas9 from Type II systems), applications with Type I CRISPR-Cas systems are now emerging. In particular, Type I systems have been employed for genome editing and gene regulation in bacteria, archaea, and eukaryotes or as tailored-spectrum antimicrobials against bacterial pathogens [12–28]. To further explore the extensive diversity of CRISPR biology and advance the existing suite of CRISPR technologies, there is an opportunity to accelerate the characterization of Type I systems.

Cell-free transcription-translation (TXTL) systems offer a convenient way to rapidly characterize CRISPR-Cas systems. TXTL systems are typically based on an *E. coli* cell lysate with the transcriptional and translational machinery being retained. With TXTL, DNA can be added to produce RNAs and proteins in minutes to hours, allowing for subsequent biochemical assays without protein purification or cell culturing [29]. So far, TXTL-based methods have been established to characterize the expression and immunity steps of CRISPR [30–33]. CRISPR arrays are efficiently transcribed and processed in TXTL, and processing can be visualized via northern blotting or RNA-Seq analyses [30, 31]. The targeting activity of Cas nucleases and crRNAs can also be assessed with TXTL by directing the CRISPR effector complex to target a fluorescence reporter plasmid, inhibiting fluorescence production [32, 33]. Targeting activity can also be used to screen for anti-CRISPR proteins (Acrs) that inhibit different steps of CRISPR immunity [33–37]. Within these various characterization approaches, arguably the most important is determining the PAM. A number of *in vivo*

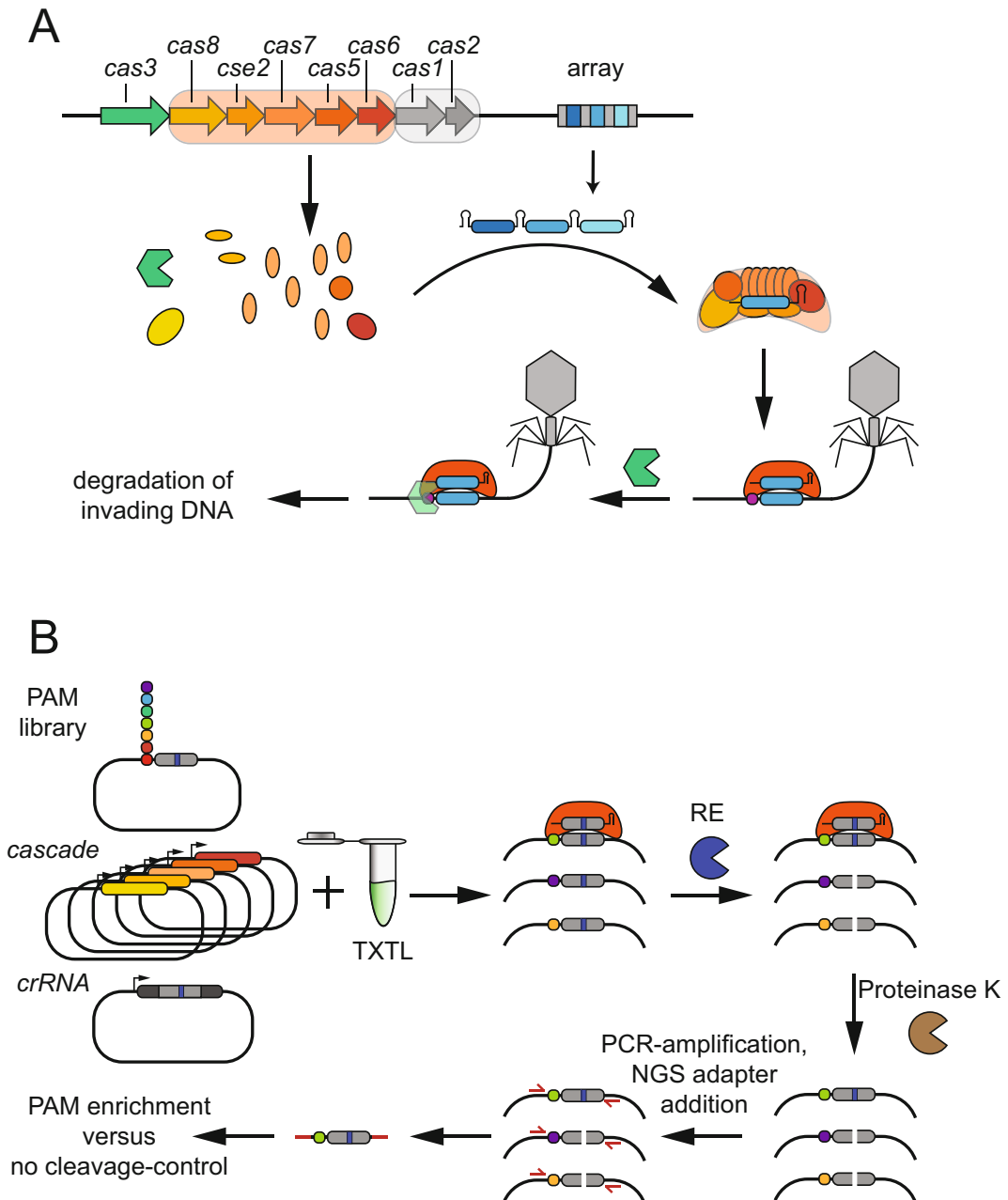


Fig. 1 (a) Overview of DNA targeting with Type I CRISPR-Cas systems. The Type I-E CRISPR-Cas system from *E. coli* is used as a representative example. The *cas* genes encoding the proteins that form the Cascade complex are encircled in orange. The genes responsible for spacer acquisition are encircled in gray. As part of adaptive immunity, the system expresses the Cas proteins and transcribes the CRISPR array. The transcribed array is then processed into individual crRNAs that form a multi-protein ribonucleoprotein complex called Cascade. This complex screens DNA for protospacers comprising complementary sequences to the spacer (blue) and flanked by a PAM (purple). After Cascade binds a protospacer, it recruits Cas3 (green) to degrade the DNA. When the target is located in an invader such as a phage, recognition leads to DNA degradation and clearance of the invader. (b) Overview of the TXTL-based PAM determination assay. As part of the assay, three

and in vitro PAM determination methods have been reported [38], although they have typically been limited to single-effector nucleases. Here, we report a binding-based PAM assay in TXTL that is well suited for multi-subunit CRISPR effectors 46. This assay differs from our previously performed TXTL-PAM assay [33, 39], as it relies on target binding rather than cleavage. Furthermore, the assay enriches rather than depletes recognized PAM sequences, reducing the required sequencing depth to identify even weakly recognized PAMs. Overall, the reported PAM assay enables a fast and easy method to rapidly determine PAM requirements of CRISPR-Cas systems with multi-protein effector complexes.

2 Materials

2.1 Reagents and Kits

1. 3 M sodium acetate, pH 5.2: 3 M sodium acetate, adjust pH with CH₃COOH.
2. 70% ethanol (EtOH): prepare EtOH solution in dH₂O by measuring EtOH and dH₂O separately before combining.
3. Arbor Biosciences myTXTL Sigma 70 Master Mix kit.
4. LB-medium: 1% tryptone, 0.5% yeast extract, 86.6 mM NaCl, autoclave solution.
5. NGS library purification kit (e.g., AMPure beads).
6. Nuclease appropriate for NGS library preparation.
7. PCR purification kit.
8. Plasmid Midiprep kit.
9. Proteinase K (20 mg/μL).
10. Restriction Enzyme (here PacI).
11. SOC medium: SOB medium, 10 mM MgCl₂, 20 mM glucose, sterilize by passing solution through a 0.2 μm filter.
12. SOB medium: 2% tryptone, 0.5% yeast extract, 8.6 mM NaCl, 2.5 mM KCl, adjust pH to 7.0 with 5 N NaOH, autoclave solution.

Fig. 1 (continued) sets of plasmids are added to the TXTL mix: a plasmid encoding a PAM library (pGFP-PacI-5N) next to a targeted sequence (gray) that contains a restriction enzyme (RE) recognition site (blue), plasmids encoding for Cascade (pEcCas8, pEcCse2, pEcCas7, pEcCas5, pEcCas6) and a plasmid encoding a crRNA (pEc-crRNA1). The ribonucleoprotein complex (orange) is produced and binds to targets flanked by a recognized PAM. A subsequent digestion step with the RE results in cleavage of target sequences not bound by Cascade. Proteinase K (brown) is then used to remove all proteins. Adapters for NGS are added to the undigested PAM-containing plasmids by PCR. NGS sequencing results in the enrichment of recognized PAMs in the digested samples in comparison to samples without RE digestion

2.2 Equipment

1. 96-well V bottom plate.
2. Cover mat for 96-well plate.
3. Plate Reader.
4. Real-Time PCR thermocycler.
5. Thermocycler.

3 Methods

This binding-based PAM assay in TXTL results in an enrichment of positive PAMs (*see* Fig. 1b). The CRISPR-Cas multi-protein complex is expressed, and the crRNA is transcribed. An effector complex is formed and binds at its target region flanked by recognized PAMs within a PAM library. Thus, the targeted plasmid is protected from digestion of a restriction enzyme (RE) (here PacI) that has its recognition site within the crRNA complementary region. The TXTL reaction is then digested with the RE, and a proteinase K digestion is performed. The remaining DNA is extracted by ethanol precipitation and sent for next-generation sequencing (NGS). We also provide a protocol for a quality check with Sanger sequencing or quantitative PCR (qPCR) before sending the samples for NGS. Finally, we provide an example by characterizing the PAM requirements for the well-known *E. coli* Type I-E CRISPR-Cas system. All plasmids and primers that are used are stated in Tables 1 and 2, respectively.

3.1 PAM Library Construction

The PAM library should be at least one nucleotide longer than the expected PAM. We use five nucleotides (1024 combinations) in the example here, as the demonstration system from *E. coli* traditionally has a 3-nt PAM. For the PAM library, choose a plasmid with a unique RE recognition site within an untranscribed region. Our construct utilizes a unique restriction site recognized by PacI and additionally encodes for deGFP (pGFP-PacI). Make sure the RE can be heat inactivated. If introduction of a unique RE recognition site is necessary, this can be done by PCR mutagenesis of the chosen plasmid with primers including the recognition site at the 5' end of either one or both primers followed by circularization and template removal such as with a Kinase-Ligase-DpnI (KLD) enzyme mix (*see* Notes 1 and 2).

The region targeted by the CRISPR-Cas system of interest should be chosen to span the RE recognition site. The PAM library (pGFP-PacI-5N) is then constructed adjacent to the targeted region (*see* Fig. 2a). For introduction of the PAM library, mutagenic primers are used (pr-01, pr-02) that amplify the whole plasmid at the desired site and include randomized nucleotides at their 5' end. The resulting PCR product is then circularized, and the original DNA template is removed, such as with a Kinase-Ligase-DpnI (KLD) enzyme mix (*see* Note 1).

Table 1
List of plasmids used

Name	Internal number	How to obtain	Benchling link
pEc-crRNA1	CBS-1272	Addgene # 170088	https://benchling.com/s/seq-TnglgDNlecSHLmvamOql
pEc-crRNA2	CBS-2206	Addgene # 170089	https://benchling.com/s/seq-zsn7pzxiagaIAfG4kjO6
pEc-crRNAnt	CBS-212	[33]	https://benchling.com/s/seq-DFDGGZdbiIESw3ElXz2iy
pEcCas5	CBS-189	Addgene # 170090	https://benchling.com/s/seq-RVJdQ9UfNPxyvcInvD6l
pEcCas6	CBS-186	Addgene # 170091	https://benchling.com/s/seq-jbB5Es4jNHNTyKvR9Ctj
pEcCas7	CBS-194	Addgene # 170092	https://benchling.com/s/seq-YC8qRHGsEstzLtMu5liU
pEcCas8	CBS-196	Addgene # 170093	https://benchling.com/s/seq-FCnUAatortgKbxfUOAtK9
pEcCse2	CBS-184	Addgene # 170094	https://benchling.com/s/seq-eEYwschE6o4eaH5yKHw5
pGFP-ATAAC	CBS-2816	Addgene # 170095	https://benchling.com/s/seq-KiDRmcAwnw7WybwoZuK5
pGFP-CAAAG	CBS-2188	Addgene # 170096	https://benchling.com/s/seq-2s53R7nPYcmkAgsq2EQg
pGFP-CAATG	CBS-2190	Addgene # 170097	https://benchling.com/s/seq-rnN1p3zVbrooInMHWVoV
pGFP-GTAAT	CBS-2762	Addgene # 170098	https://benchling.com/s/seq-f63QZs2FKTHAxS2EonHz
pGFP-GTATT	CBS-2754	Addgene # 170099	https://benchling.com/s/seq-VpH65rIVdKZQ8hkPj6f8
pGFP-PacI	CBS-332	Addgene # 170100	https://benchling.com/s/seq-TWxPKgiuHeuxelvn0WzG
pGFP-PacI-5N	CBS-1851	Constructed based on pGFP-PacI	https://benchling.com/s/seq-6r4nLvHPddju0vBhANN5
pT7RNAP	CBS-344	Addgene # 170101	https://benchling.com/s/seq-PDEMzFwKICs9iRDTFTJ

1. Transform library construct (pGFP-PacI-5N) in competent *E. coli*.
2. Recover transformed cells in 1 mL SOC medium without antibiotics for 1 h.
3. Add the recovered cell suspension in 50 mL LB medium with appropriate antibiotics and incubate overnight at an appropriate temperature.

Table 2
List of primers used

Name	Sequence	Purpose	Source
pr-01	5'-AATTCTGGCGAATCCTTTAATTAACTGAC-3'	PAM library introduction	This study
pr-02	5'-NNNNNAGACGAAAGGGCCTCGTGATAC-3'	PAM library introduction	This study
pr-03	5'-GGCGACACGGGAAATGTTGAAT-3'	Sanger sequencing primer	This study
pr-04	5'-GCTGCAACCATTATCACCGC-3'	Sanger sequencing primer	This study
pr-05	5'-TATCACGAGGCCCTTTTCGTC-3'	qPCR primer PAM library	This study
pr-06	5'-TCTGAATTGCAGCATCCGGT-3'	qPCR primer PAM library	This study
pr-07	5'-AACGTGGCGAGAAAGGAAGG-3'	qPCR primer pT7RNAP	This study
pr-08	5'-CGCTCGCGTATCGGTGATTC-3'	qPCR primer pT7RNAP	This study
pr-09	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATCACGAGGCCCTTTTCGT*C-3'	NGS library generation	This study
pr-10	5'-GTGACTGGAGTTTCAGACGCTGTGCTCTTCCGATCtCGTTTTCTGGCTGGTCAGTT*A-3'	NGS library generation	This study
pr-11	5'-AATGATACGGCGACACCCGAGATCTACACTTGGACTTACACTCTTTCCCTACACGAC*G-3'	NGS library generation	This study
pr-12	5'-CAAGCAGAAAGACGGGCATACGAGATGCGTTGGAGTGACTGGAGTTCAGACGTG*T-3'	NGS library generation	This study

*Indicates phosphorothioate bond at the 3' end of the primer to prevent degradation by polymerases with a strong proofreading activity

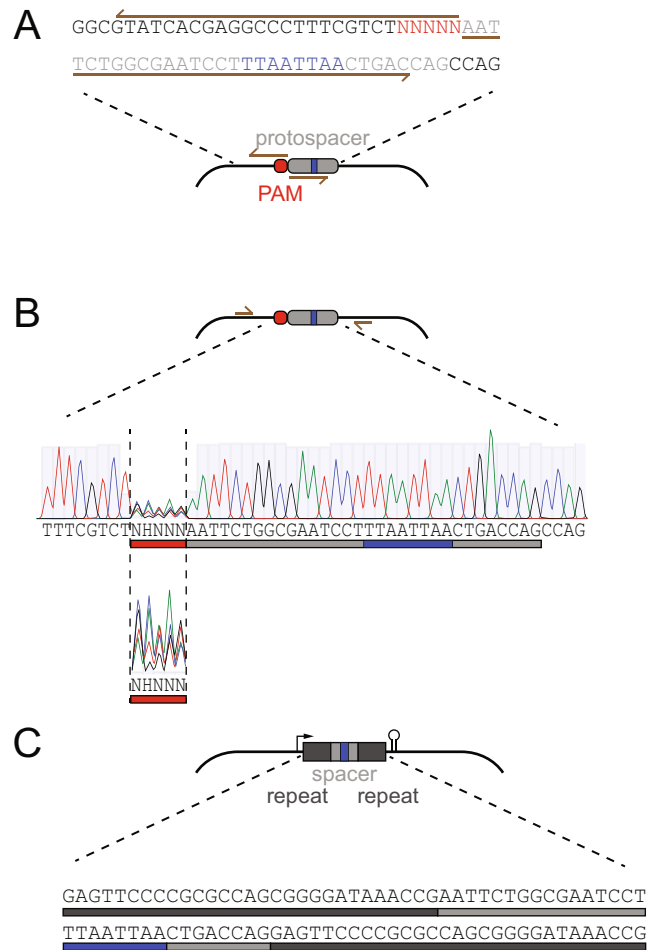


Fig. 2 Construction of the PAM library and crRNA expression construct. **(a)** Overview of the PAM library containing plasmid (pGFP-Pacl-5N). The PAM (red) consists of five random nucleotides and is located upstream of the protospacer (gray) that includes the RE recognition site (blue). Primers for library construction (pr-01, pr-02) are shown in brown. **(b)** Preliminary check of PAM library cloning by Sanger sequencing. To verify the cloned PAM library, a region containing the PAM library is amplified (primers are shown in brown) (pr-03, pr-04) and sent for Sanger sequencing. A representative sequencing trace is shown with nucleotide peaks. Below: a zoomed-in view of the library region. The nucleotide distribution does not need to be entirely equal, as enriched sequences are normalized to sequences in undigested samples as part of the NGS analysis. **(c)** The designed array sequence (pEc-crRNA1) from the I-E system in *E. coli* that is designed to target the PAM library-flanked target. The array is flanked by an upstream promoter and a downstream terminator. Repeats are shown in dark gray and the spacer is shown in light gray. The crRNA spacer spans the RE recognition site (blue)

4. Use a plasmid Midiprep kit to isolate the library plasmid from the 50 mL culture.
5. Re-clean and concentrate the plasmid DNA, if necessary, by a PCR purification kit.
6. Amplify the library plasmid (pGFP-PacI-5N) with primers spanning the PAM library (pr-03, pr-04) and send for Sanger sequencing to check for library quality (*see* Fig. 2b).

3.2 CRISPR-Cas Plasmid Design and Preparation

Cas proteins that form the Cascade can either be cloned on separate plasmids each (pEcCas8, pEcCse2, pEcCas7, pEcCas5, pEcCas6) or as an operon on one plasmid (*see* **Notes 3** and **4**). Use a protein expression vector with a strong promoter, an appropriate ribosomal binding site, and a terminator as the backbone (*see* **Note 5**). CRISPR arrays are cloned as repeat-spacer-repeat (pEc-crRNA1) with a strong promoter and flanked by a terminator (*see* Fig. 2c). The spacer sequence herein is identical to the sequence downstream of the PAM library, covering the RE-recognition site. Transform all plasmids in competent *E. coli* and isolate the plasmids with a plasmid Midiprep kit followed by a PCR purification step.

3.3 PAM Assay

1. Prepare TXTL reaction on ice (*see* Table 3) (*see* **Note 8**).
2. Carefully vortex the reactions and spin them down shortly.
3. Incubate at 29 °C for 16 h (*see* **Notes 8** and **9**).
4. Dilute 3 µL of the TXTL reaction in 1,197 µL of nuclease-free water (*see* **Note 10**).
5. Prepare RE digestion reaction (*see* Table 4), always prepare a control reaction with nuclease-free water instead of the RE.
6. Digest for 1 h at appropriate temperature (37 °C for PacI).
7. Heat inactivate RE (20 min at 65 °C for PacI).
8. Prepare Proteinase K digestion (*see* Table 5) and incubate for 1 h at 45 °C.
9. Inactivate Proteinase K for 5 min at 95 °C.

3.4 DNA Extraction

DNA is extracted by EtOH precipitation (*see* **Note 11**). All centrifugation steps are done at maximum speed and at 4 °C.

1. Divide each sample in two equal parts of 280 µL so the following EtOH precipitation can be carried out in 1.5-mL tubes.
2. Add 3 M sodium acetate, pH 5.2 and ice-cold 100% EtOH to your samples (*see* Table 6).
3. Mix by vortexing and spin down quickly.
4. Immediately store at −80 °C in a pre-cooled rack for at least 20 min (*see* **Note 12**).
5. Spin your samples for 15 min (*see* **Note 13**).

Table 3
Components for the TXTL reaction of the PAM determination assay

Component	Volume (μL)	Initial concentration (nM)	Final concentration (nM)
TXTL	4.5	–	–
<i>E. coli</i> Cascade plasmids (pEcCas8, pEcCse2, pEcCas7, pEcCas5, pEcCas6) (<i>see</i> Note 7)	0.4	45	3
crRNA plasmid (pEc-crRNA1)	0.5	12	1
PAM library plasmid (pGFP-PacI-5N)	0.4	15	1
IPTG	0.06	50,000	500
T7RNAP (pT7RNAP)	0.1	12	0.2
Water	0.04	–	–

T7 RNA polymerase plasmid can be added if a T7 promoter is used, and an inducer can be added if necessary (*see* **Note 6**). Prepare at least two replicates for each reaction

Table 4
Components for the RE digestion reaction

Component	Volume (μL)	Initial concentration	Final concentration
TXTL dilution	500	–	–
RE Buffer	56.1	10×	1×
RE (PacI)/water	5	10 units/μL	0.09 units/μL

Prepare one reaction with RE and one reaction with water instead of RE

Table 5
Components for the Proteinase K digestion reaction

Component	Volume (μL)	Initial concentration (mg/mL)	Final concentration (mg/mL)
RE digestion reaction	561.1	–	–
Proteinase K	1.4	20	0.05

Table 6
Components for ethanol precipitation reaction

Component	Volume (μL)
Proteinase K digestion reaction	280
100% EtOH, ice-cold	700
3 M sodium acetate, pH 5.2	28

Split Proteinase K digestion reaction into two equal volumes of 280 μL so the reaction can be performed in 1.5-mL tubes

6. Carefully remove liquid by decanting.
7. Add 200 μ L of ice-cold 70% EtOH to your samples.
8. Spin for 10 min (*see* **Note 13**).
9. Repeat **steps 6–8**.
10. Remove liquid completely with a pipette, being careful to not touch the side of your tube with the DNA pellet.
11. Evaporate the remaining liquid by placing the tube at 50 °C with an open lid.
12. Add 10 μ L of nuclease-free water to the tube, be careful not to touch the pellet.
13. Incubate at 65 °C for 10 min.
14. Vortex vigorously.
15. Combine your divided samples.

3.5 Quality Check

The final output of the PAM assay requires NGS. However, there are some methods to estimate if the assay was successful and that provide some indication of the expected PAMs. Specifically, Sanger sequencing of the PAM library containing region gives indication of the recognized PAMs if compared to sequencing results of samples lacking RE-digestion. Analysis of protection of RE-digestion by qPCR can provide an idea if the Cascade-crRNA complex was able to bind to its target.

3.5.1 Sanger Sequencing

1. PCR-amplify a region containing the PAM library from the targeted plasmid in the digested and the undigested (control) sample (pr-03, pr-04).
2. Use a PCR purification kit to purify the amplicons.
3. Send the amplicons for Sanger sequencing using one or both primers used for amplification.
4. A noticeable difference in the sequencing results within the PAM library region between the digested and undigested samples indicates recognition of specific PAMs and thus enrichment of the recognized PAMs in the Sanger sequencing file (*see* Fig. 3a).

3.5.2 Quantitative PCR (qPCR)

1. Design two primer pairs. One amplifies a 100–250 bp long region containing the PAM library (pr-05, pr-06). The other amplifies a plasmid that is added to every single reaction but is not digested by your RE (pT7RNAP, pr-07, pr-08) and generates a similar amplicon size.
2. Use a qPCR kit without a reverse-transcription step to amplify the DNA obtained with the PAM assay with the primer pairs from **step 1**.

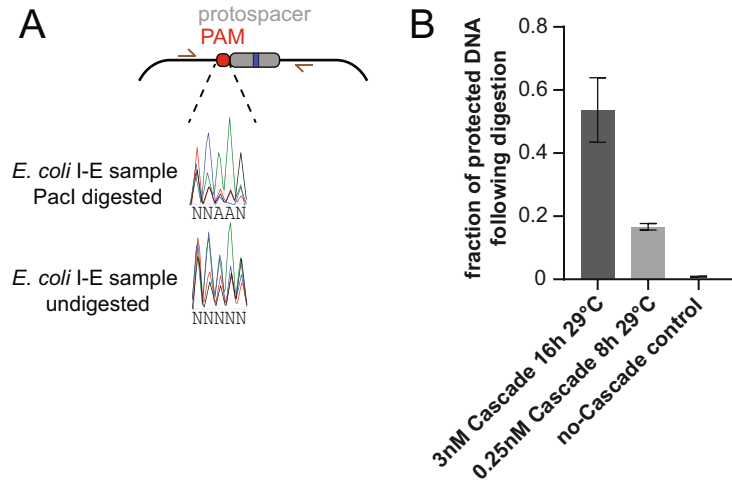


Fig. 3 Preliminary assessment of a PAM assay readout. **(a)** Preliminary assessment by Sanger sequencing. An amplicon is generated from the targeted plasmid (pGFP-Pacli-5N) that includes the PAM library region (red) and the targeted region (gray) with the RE recognition site (blue). Primers (pr-03, pr-04) are shown in brown. The nucleotide peaks from Sanger sequencing are shown for a sample that was digested with Pacl and a sample that was not digested with Pacl. The digested sample shows some increased peaks compared to the undigested sample, suggesting successful PAM recognition during the PAM assay. **(b)** Preliminary assessment with qPCR. The relative amount of uncleaved DNA in a digested sample is compared to the undigested control. The fraction of protected DNA is then determined based on $2^{-\Delta\Delta C_t}$ values from qPCR. An amplicon from the plasmid without the RE recognition site (pT7RNAP) was used as the reference sequence. Results are shown after applying the assay to the *E. coli* I-E Cascade following a TXTL reaction with 3 nM Cascade plasmid for 16 h or 0.25 nM Cascade plasmid for 8 h. Less protection, which can produce a stricter PAM profile, is achieved when using a lower Cascade plasmid concentration or a shorter incubation time (see **Note 8**). A control reaction without the Cascade or crRNA plasmids in the TXTL reaction shows negligible protection. Bars reflect the mean and error bars reflect the standard deviation from the duplicate reactions

3. Determine the fold change between digested and undigested samples. Calculate $2^{-\Delta\Delta C_t}$ using the undigested sample as the control sample and the amplicon from the plasmid without RE recognition site (pT7RNAP) as the reference sequence. The fold change shows how much of your targeted plasmid is protected by the CRISPR-Cas systems and not prone to RE digestion. A fold change of 0.01 or lower indicates no binding of the CRISPR-Cas system (see Fig. 3b).

3.6 NGS Library Preparation

1. Amplify the EtOH precipitated DNA from Subheading 3.4 with a nuclease appropriate for NGS library preparation adding Illumina sequencing primer binding sites on both ends of the

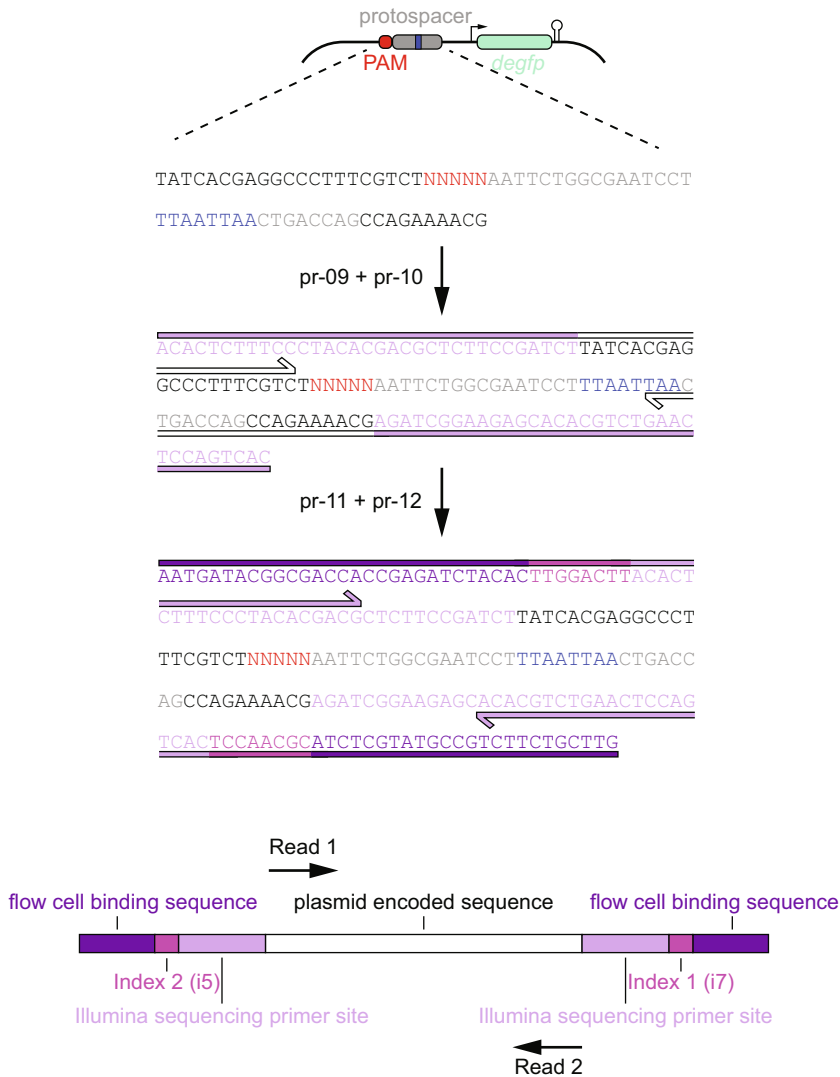


Fig. 4 NGS library preparation. A region of the targeted plasmid is amplified that spans the PAM region (red) and the protospacer region (gray) including the RE recognition site (blue). Primers (pr-09, pr-10) are used that add the Illumina sequencing primer sites (light purple). Flow cell binding sites (dark purple) and the i7 and i5 indices are added in a second PCR (pr-11, pr-12). Directions of Read1 and Read2 generated by NGS are shown

amplicons containing the PAM library region (pr-09, pr-10) (see Fig. 4).

2. Check the amplicons on an agarose gel.
3. Purify the amplicon with an appropriate method, e.g., use AMPure beads.
4. Add the flow cell binding sequences and unique dual indices to both sides of the amplicon by amplifying the amplicon at the Illumina sequencing primer binding sites with a nuclease appropriate for NGS library preparation (pr-11, pr-12) (see Fig. 4).

5. Check the amplicons on an agarose gel.
6. Purify the amplicon with an appropriate method, e.g., use AMPure beads.
7. Sequence the amplicon with a NovaSeq 6000 Sequencing System with 50 bp paired-end reads.

3.7 NGS Data Analysis

Data analysis is done according to Leenay et al. [40]. A detailed protocol can be found there. The analysis starts with raw .fastq files. The following protocol is based on the sequence of Fig. 4 and can be adjusted according to the plasmid encoding the PAM library.

1. List the nucleotides from the randomized region with the following code:
 Read 1:

```
grep '[TCAG][TCAG][TCAG][TCAG][TCAG]AATTC
TGGCGAATCCTTTAATTAA' Sample1.fastq | cut -c 22-26 |
sort | uniq -c | sort -nr | less > Sample1List.txt
```

 Read 2:

```
grep 'TTAATTAAAGGATTCGCCAGAATT[TCAG]
[TCAG][TCAG][TCAG][TCAG]' Sample1.fastq | cut -c
43-47 | sort | uniq -c | sort -nr | less > Sample1List.txt
```
2. Import the .txt list into Microsoft Excel and sort it with the Sort and Filter tool.
3. Calculate PAM enrichment with the following formula:

$$\text{Enrichment} = \frac{\text{total reads non-digested sample}}{\text{total reads digested sample}} \cdot \frac{\text{reads digested sample}}{\text{reads non-digested sample}}$$

4. Use the calculated PAM enrichments and the PAM sequences to generate Krona Plots by adding them to the KronaExcel-Template (available at <https://github.com/marbl/Krona/wiki>) and using Category 1 for the nucleotide adjacent to the protospacer (*see Note 14*) (*see Fig. 5*).
5. The generated Krona Plot can be viewed as a .html file with any web browser. The file can be downloaded and modified (*see Fig. 6*) (*see Note 15*).

3.8 Data Validation

TXTL can be also used to validate the PAMs that were found in the Krona Plot. A convenient method for this is to target the promoter region of a deGFP encoding plasmid (pGFP-PacI) (*see Fig. 7a*). Clone each PAM-of-interest upstream of the –35 region of the promoter with the targeted sequence covering parts of the promoter (*see Fig. 7b*). Recognition of the PAM and thus binding of the Cascade-crRNA complex results in repression of deGFP expression, halting the build-up of fluorescence. Fold changes between a

Enrichment	Category 1	Category 2	Category 3	Other categories	5' to 3'
1.28074176	A	A	A	A	AAAAA
2.29633284	C	A	A	A	AAAAC
2.66738671	G	A	A	A	AAAAG
1.19200502	T	A	A	A	AAAAT
0.25554045	A	C	A	A	AAACA
0.04757172	C	C	A	A	AAACC
0.30977032	G	C	A	A	AAACG
0.05010758	T	C	A	A	AAACT
2.05049297	A	G	A	A	AAAGA
0.85070642	C	G	A	A	AAAGC
2.72242137	G	G	A	A	AAAGG
0.67600828	T	G	A	A	AAAGT
0.66324258	A	T	A	A	AAATA
1.02993144	C	T	A	A	AAATC
2.55832849	G	T	A	A	AAATG
0.14667648	T	T	A	A	AAATT
0.05604735	A	A	C	A	AACAA
0.14719521	C	A	C	A	AACAC
1.48221946	G	A	C	A	AACAG

Fig. 5 Populating the Krona Excel template to generate the PAM wheel. Positioning of the calculated PAM enrichments and the sorted PAMs in the Excel template file are shown. The PAM sequences are depicted in their 5' to 3' direction. To achieve a 5' to 3' order from the outer circle to the inner circle in the PAM wheel, the nt in the -1 position is placed in Category 1, the nt in the -2 position in Category 2, and so on

reaction containing a CRISPR array with a nontargeting spacer and a reaction containing a CRISPR array targeting the *degfp*-promoter region indicate the functionality of the chosen PAM.

1. Prepare TXTL reactions on ice (*see* Table 7) (*see* **Note 16**). Always include a background control consisting of TXTL and water only.
2. Carefully vortex the reactions and spin them down shortly.
3. Incubate reactions at 29 °C for 4 h (*see* **Note 9**).
4. Add reporter plasmid (here pGFP-CAAAG, pGFP-CAATG, pGFP-ATAAC, pGFP-GTAAT or pGFP-CTATT) to TXTL reaction (*see* Table 7), add water to your background control instead.
5. Carefully vortex and briefly spin down your reactions.
6. Load at least technical duplicates of 5 µL each in a 96-well plate with V-shaped bottom.
7. Seal the loaded plate with a cover mat to prevent evaporation over time.
8. Measure deGFP fluorescence (Ex 485 nm, Em 528 nm) (*see* **Note 17**) in a plate reader pre-warmed to 29 °C (*see* **Note 9**). Measure fluorescence every 3 min for up to 16 h.

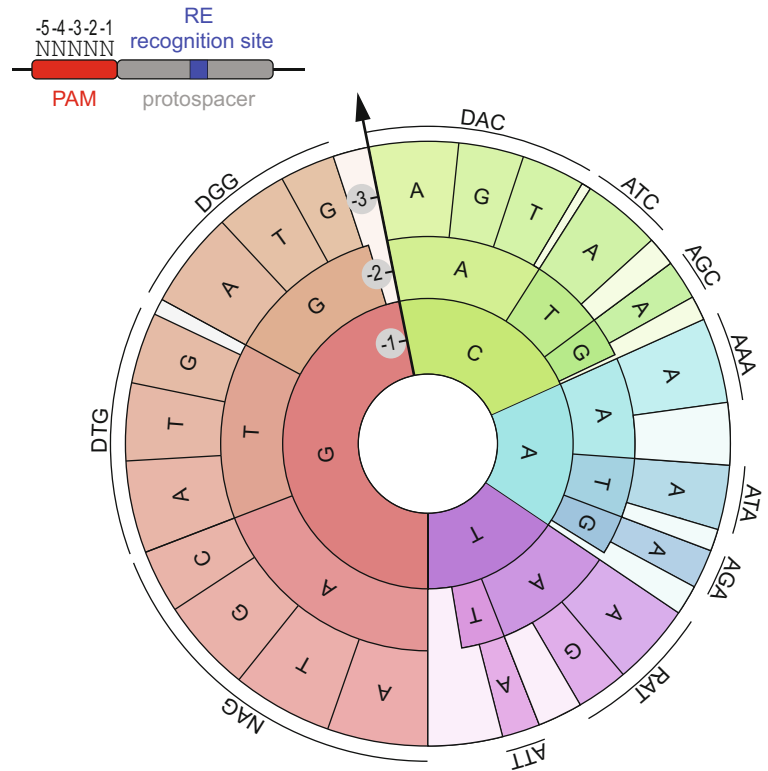


Fig. 6 PAM wheel for the PAM determination assay conducted with Cascade from *E.coli*'s Type I-E system. The Krona Plot representing recognized PAMs generated from the example data with 3 nM Cascade and an incubation time of 16 h is shown. Thereby, only three nts are depicted as positions –4 and –5 did not show any nucleotide specificity. The section size of a PAM sequence is proportional to its enrichment during the PAM assay

9. Subtract the values of the background control from your samples for every measured timepoint.
10. Calculate fold-changes by dividing the fluorescence of the nontargeting control by the targeting control. High fold-changes represent highly recognized PAMs, low/no fold changes represent low/no recognition (see Fig. 7c).

4 Notes

1. Online tools can be used to design the mutagenic primers, e.g., NEBase Changer.
2. A deGFP-expressing plasmid can be used for introduction of the unique RE recognition site for an easy visual control of the functionality of the TXTL reaction and for use in later validation (here pGFP-PacI). A slightly green reaction mix after the

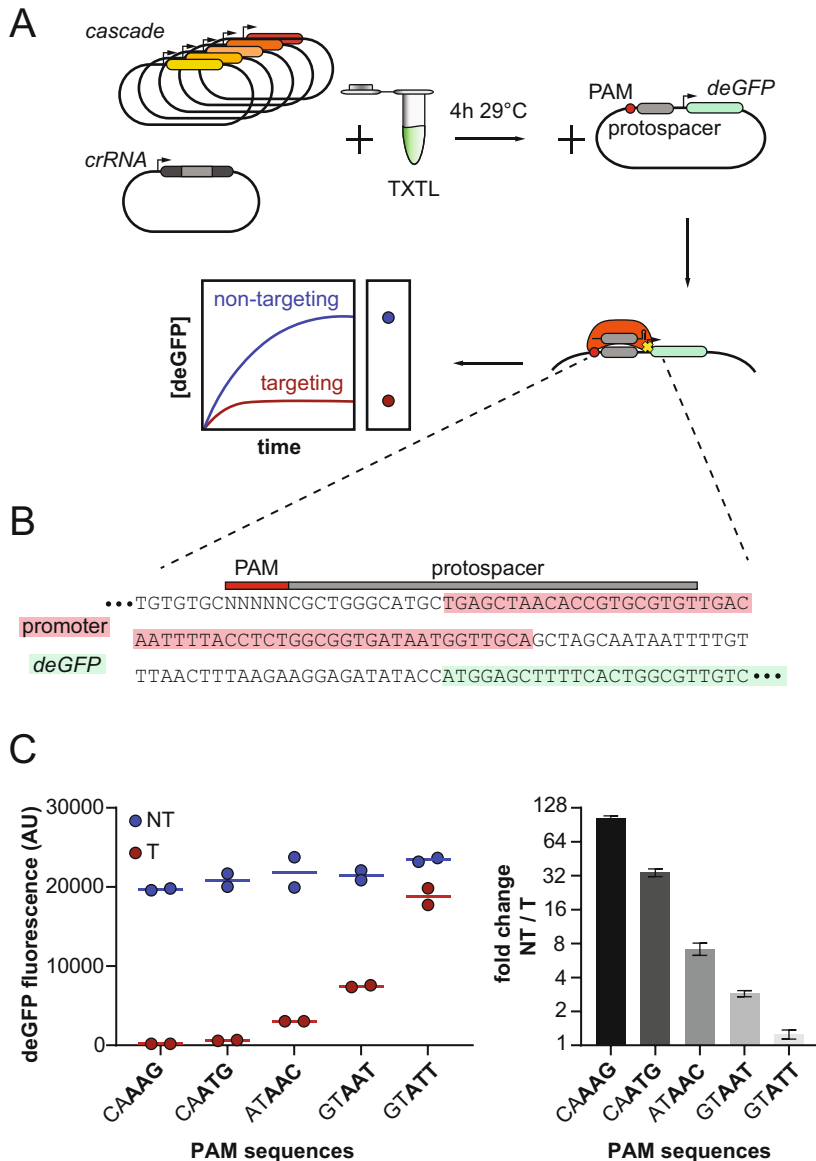


Fig. 7 Validation of enriched PAM sequences using a TXTL-based reporter assay. **(a)** Overview of the reporter assay. Plasmids encode the Cascade proteins (pEcCas8, pEcCse2, pEcCas7, pEcCas5, pEcCas6) as well as a crRNA targeting the *deGFP* promoter (pEc-crRNA2) or a nontargeting crRNA (pEc-crRNAnt). These components are pre-expressed in TXTL at 29 °C for 4 h to allow for ribonucleoprotein complex formation prior to expressing the reporter. Addition of a targeted deGFP reporter plasmid (pGFP-CAAAG, pGFP-CAATG, pGFP-ATAAC, pGFP-GTAAT or pGFP-CTATT) leads to binding of the protospacer by the ribonucleoprotein complex with the targeting crRNA, blocking transcription of the reporter. The rate of binding and the efficiency of transcriptional blocking impacts the accumulation of deGFP and the resulting fluorescence of the TXTL reaction. Under this setup, better-recognized PAMs result in less deGFP accumulation and fluorescence. **(b)** Sequence of the targeted plasmid. A region within the targeted plasmids containing the PAM (red), the protospacer (gray), the promoter (light red), and beginning of the *deGFP* coding region (light green) is shown. The PAM sequence is located upstream of the promoter, limiting interference with *deGFP* expression on its own and can be replaced with any sequence-of-interest. **(c)** deGFP expression levels and fold changes based on endpoint fluorescence. deGFP fluorescence levels of reactions with a targeting crRNA (T) or a nontargeting crRNA (NT) are shown on

incubation time indicates active protein expression (*see* **Note 17**).

3. Cloning all Cas proteins on separate plasmids allows for the addition of Cas protein encoding plasmids in a stoichiometric manner, while cloning Cascade proteins as an operon on one plasmid facilitates handling.
4. Plasmids can be exchanged with linear DNA if GamS is added to the TXTL reaction (final concentration 2 μ M) or if χ sites are included in the linear construct to prevent RecBCD-induced DNA degradation [41, 42].
5. If a protein-of-interest is toxic, inducible promoters such as T7 promoter or IPTG-inducible promoters can be used.
6. We recommend preparing a MasterMix with TXTL, water and inducer and/or T7 RNA polymerase plasmid if necessary. We do not recommend including any other component (e.g., PAM library plasmid) into the MasterMix to ensure highest independence between replicates.
7. If Cas proteins are encoded on separate plasmids, prepare a MasterMix combining all Cas-encoding plasmids in one sample. Add every plasmid according to the stoichiometry of the encoded Cas protein (For *E. coli* type I-E: Cas8₁-Cse2₂-Cas7₆-Cas5₁-Cas6₁). Concentration of all Cas-encoding plasmids should result in an initial concentration of 45 or 12 nM. If Cas proteins are encoded on one plasmid, prepare a stock concentration of 45 or 12 nM.
8. If less Cascade protein production is required, e.g., to only select for strong PAMs, add less plasmids encoding for Cas proteins and/or shorten the incubation time.
9. 29 °C is commonly used for TXTL reactions and optimal for deGFP production, although the temperature can be varied between 25 and 42 °C [43] and can impact the expression and activity of some Cas nucleases [33].
10. A 1:400 dilution of the TXTL reaction is optimized for the RE PacI. If other enzymes are used, different dilutions of TXTL can be tested for optimal results.
11. We recommend using EtOH precipitation over column-based purification due to the small amount of plasmid DNA remaining in the reaction and the lower DNA recovery of column-based purification compared to EtOH precipitation.

Fig. 7 (continued) the left. The bigger the difference in fluorescence between NT and T, the better the PAM is recognized. Fold changes represent the ratio of deGFP fluorescence for the nontargeting reaction over the targeting reaction. Bars reflect the mean and error bars reflect the standard deviation from the duplicate reactions

Table 7
Components for the TXTL reporter assay to validate identified PAMs

Component	Volume (μL)	Initial concentration (nM)	Final concentration (nM)
TXTL	9	–	–
<i>E. coli</i> Cascade plasmids (pEcCas8, pEcCse2, pEcCas7, pEcCas5, pEcCas6) (<i>see Note 7</i>)	0.5	12	0.5
crRNA plasmid (pEc-crRNA2 or pEc-crRNAnt)	1	12	1
Reporter plasmid (pGFP-CAAAG, pGFP-CAATG, pGFP-ATAAC, pGFP-GTAAT or pGFP-CTATT) (added later)	0.5	24	1
IPTG	0.12	50,000	500
T7RNAP (pT7RNAP)	0.2	12	0.2
Water	0.68	–	–

The T7 RNA polymerase plasmid can be added if a T7 promoter is used. An inducer can also be added if necessary. Prepare one reaction with a targeting crRNA (pEc-crRNA2) and one reaction with a nontargeting crRNA (pEc-crRNAnt) (*see Note 18*). The reporter plasmid is added after 4 h incubation at 29 °C (*see Note 9*)

12. This step can proceed overnight.
13. Always place the tube in the centrifuge in the same orientation (e.g., lid pointing toward the center of the rotor) so you know where your DNA pellet is located.
14. People with programming experience can also use the source code and run it on a local machine (*see* <https://github.com/marbl/Krona/wiki> for more information).
15. Other visualization methods besides Krona Plots can be used. Examples are sequence logos or motif plots [40, 44, 45].
16. Determination of the optimal Cascade plasmid concentration may be required if overall fluorescence is low.
17. deGFP can be exchanged with other fluorescence reporters.
18. We recommend preparing a MasterMix with TXTL, water, Cascade plasmids, crRNA plasmid and inducer and/or T7 RNA polymerase plasmid if necessary.

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Implementing Hands-On Molecular and Synthetic Biology Education Using Cell-Free Technology

Ally Huang, Bruce Bryan, Sebastian Kraves, Ezequiel Alvarez-Saavedra, and Jessica C. Stark

Abstract

Active, hands-on learning has been shown to improve educational outcomes in STEM subjects. However, implementation of hands-on activities for teaching biology has lagged behind other science disciplines due to challenges associated with the use of living cells. To address this limitation, we developed BioBits[®]: biology education activities enabled by freeze-dried cell-free reactions that can be activated by just adding water. Here, we describe detailed protocols for labs designed to teach the central dogma, biomaterial formation, an important mechanism of antibiotic resistance, and CRISPR-Cas9 gene editing via cell-free synthesis of proteins with visual outputs. The activities described are designed for a range of educational levels and time/resource requirements, so that educators can select the demonstrations that best fit their needs. We anticipate that the availability of BioBits[®] activities will enhance biology instruction by enabling hands-on learning in a variety of educational settings.

Key words Synthetic biology, Cell-free biology, STEM education, Active learning, Biology demonstrations, Central dogma, Biomaterials, Antibiotic resistance, CRISPR-Cas9

1 Introduction

Hands-on science activities are known to improve student performance and engagement, reduce achievement gaps, and spark interest in STEM careers [1–4]. However, implementation of hands-on biology activities in classrooms has traditionally been challenging due to the expensive equipment and expertise required to grow cells. To address this limitation, we developed BioBits[®]: a suite of activities that facilitate hands-on molecular and synthetic biology activities in classrooms and other nonlaboratory settings [5–7]. These kits are powered by freeze-dried, cell-free (FD-CF) reactions that can be 1) activated by just adding water and desired

reagents and 2) run without expensive laboratory equipment. By coupling biological processes with cell-free synthesized visual or tactile outputs (e.g., fluorescent proteins, hydrogels), BioBits[®] activities allow students to engage and interact with otherwise abstract concepts. We have developed a number of classroom resources designed to teach students about the cell-free technology that makes BioBits[®] activities possible as well as its real-world applications (*see Note 1*).

Here, we provide detailed protocols for classroom implementation of four hands-on lab activities enabled by FD-CF reactions. These activities are designed to teach the central dogma of biology, hydrogel formation, a primary mechanism of antibiotic resistance, and CRISPR-Cas9 gene editing. These activities showcase the diversity of topics that can be taught using BioBits[®] resources, spanning a range of different educational levels, time requirements, and resource requirements (Table 1). The central dogma activity is available as a commercial kit and represents an optimized, off-the-shelf option for hands-on experimentation. The other activities described are prototypes; however, they can be run using FD-CF reactions from the commercial kit by adding the appropriate reagents, as described in detail below (*see Note 2*). To help educators integrate these activities into their classrooms, we developed freely available supporting curriculum, which can be found in the notes for each methods section. We further provide ideas and resources for curriculum scaffolding that can be used to tailor activities to various educational levels by conveying a variety of additional molecular and synthetic biology concepts and cutting-edge applications. Finally, solutions to common challenges faced during implementation of activities in classrooms are discussed.

Because they contain all of the necessary reagents for running labs with easy-to-use reactions, we anticipate that the BioBits[®] activities will significantly lower the barrier to entry to educational hands-on biology activities. In fact, we have already leveraged the portability of the BioBits[®] platform to help educators run hands-on experiments from home to support remote learning during the COVID-19 pandemic [8, 9]. Overall, BioBits[®] resources have the potential to enhance biology education by offering a user-friendly alternative to cell-based biology experimentation. We expect that BioBits[®] activities will help inspire the next generation of biomedical scientists and engineers from diverse backgrounds by increasing access to high-quality, hands-on biology education.

Table 1
BioBits® activities at a glance

Activity	Output	Stage of development	Topics	Level	Time required	Instructor prep time	Approx. reagent cost ^a
Central dogma	Fluorescence (visual)	Commercial kit	Central dogma of molecular biology, transcription, translation, gene expression	General biology, AP biology, introductory college	One 40-min class period, 5–10 min to observe results the next day	10–20 min to aliquot, distribute reagents	\$100
Hydrogel	Hydrogel (tactile)	Prototype	Biomaterials, hydrogels, enzymatic reactions	AP biology, introductory college	One 90-min class period or two 40 min class periods	3 days (3–4 h hands-on time)	\$50
Antibiotic resistance	Fluorescence (visual)	Prototype	Mechanisms of antibiotics, antibiotic resistance	AP biology, AP environmental science, introductory college	One 60-min class period, 5–10 min to observe results the next day	10–20 min to start resistance enzyme reactions	\$100
CRISPR	Fluorescence (visual)	Prototype	Gene editing, CRISPR-Cas systems	AP biology, introductory college	One 60-min class period, 5–10 min to observe results the next day	10–20 min to start Cas9 reaction	\$100

^aEstimated cost of reagents for a 32 student classroom. Cost of reagents for prototype activities may vary. Estimates do not include costs of equipment for reagent preparation (e.g., hydrogel activity) and experiment implementation (e.g., pipettes, blue light illuminator)

2 Materials

2.1 Central Dogma Activity

For this activity, educators should purchase the commercially available BioBits[®] Central Dogma kit from miniPCR bioTM; see **Notes 2 and 3**. Kits contain the materials listed below.

Per experiment/group:

1. 4× FD-CF reactions.
2. DNA A in nuclease-free water (at least 15 µL).
3. DNA B in nuclease-free water (at least 10 µL).
4. Kanamycin solution in nuclease-free water (at least 10 µL).

2.2 Hydrogel Activity

Per experiment/group:

1. 8× FD-CF reactions (miniPCR bioTM; see **Notes 2 and 3**).
2. 25 ng/µL pJL1 sortase plasmid DNA in nuclease-free water (at least 50 µL; Addgene #106288).
3. 2× freeze-dried hydrogel monomers prepared as described in Subheading 3.2.

For the preparation of freeze-dried hydrogel monomers:

- (a) 1 g eight-arm PEG vinyl sulfone 20,000 Da MW (PEG-VS) (JenKem Technology).
- (b) 250 mg of each cross-linking peptides GCRELPRTGG and GGGSGRC (CPC Scientific or similar peptide synthesis provider).
- (c) 100 mM HEPES, pH 7.9 (at least 100 mL).
- (d) Dialysis cassettes and relevant accessories (i.e., syringes, 4 L beakers, stir bars).
- (e) Reaction buffer: 50 mM HEPES, 150 mM NaCl, and 10 mM CaCl₂, pH 7.9.
- (f) Lyophilizer.

2.3 Antibiotic Resistance Activity

Per experiment/group:

1. 10× FD-CF reactions (miniPCR bioTM; see **Notes 2 and 3**).
2. 250 µM streptomycin in nuclease-free water (at least 10 µL).
3. 250 µM kanamycin in nuclease-free water (at least 10 µL).
4. 13.33 ng/µL AadA plasmid DNA in nuclease-free water (at least 10 µL; Addgene #117050).
5. 13.33 ng/µL NeoR plasmid DNA in nuclease-free water (at least 10 µL; Addgene #117048).
6. 33.33 ng/µL dTomato plasmid DNA in nuclease-free water (at least 30 µL; Addgene #102631).

2.4 CRISPR Activity

Per experiment/group:

1. 10× FD-CF reactions (miniPCR bio™; *see* **Notes 2 and 3**).
2. 13.33 ng/μL Cas9 plasmid DNA in nuclease-free water (at least 20 μL; Addgene #117051).
3. 5 ng/μL mRFP1 plasmid DNA in nuclease-free water (at least 20 μL; Addgene #102630).
4. 33.33 ng/μL gRNA plasmid DNA in nuclease-free water (at least 10 μL; Addgene #117052).

3 Methods

3.1 Observing Transcription and Translation in Real Time

The central dogma of molecular biology—the flow of genetic information from DNA to RNA to protein—is the foundation for understanding molecular biology as a whole. However, the central dogma of biology is difficult to demonstrate using cells, as the molecular machinery that carries out transcription and translation is obscured behind cell membranes. To help convey this foundational concept, we developed and commercialized an educational activity that uses FD-CF reactions to enable visualization of RNA and protein synthesis in real-time using fluorescence (Fig. 1a). This is possible via cell-free transcription and translation of a synthetic construct that encodes two functional motifs. The first is a gene encoding an RNA aptamer, a specific RNA sequence that binds a small molecule present in the FD-CF reactions and emits green fluorescence upon binding. The second is a gene encoding a red fluorescent protein (Fig. 1b). Hence, green fluorescence of the RNA aptamer is used to report transcription of mRNA from DNA and red fluorescence reports subsequent translation of mRNA to protein. To our knowledge, this represents the first educational activity that allows students to visualize the central dogma in real time and with authentic DNA, RNA, and proteins, enabling enhanced understanding of an otherwise abstract concept.

This lab has students carry out four reactions as part of a guided, hands-on exploration of the central dogma (Fig. 1c). The first reaction serves as a negative control, to which no DNA is added. The second reaction is primed with the complete aptamer-fluorescent protein fusion. The third reaction is identical to the second, but students also add kanamycin, an antibiotic that inhibits protein translation. Finally, the fourth reaction contains a mystery DNA construct. Students are then challenged to predict what they will observe in the first three reactions and deduce what occurred in the fourth reaction based on their observations and understanding of the central dogma (for freely available supporting curriculum, *see* **Note 4**). A recorded experimental demonstration as well as optional extension activities focused on RNA aptamers, mRNA vaccines, and quantification of mRNA/protein production are also available (*see* **Note 5**). Overall, this activity serves as an

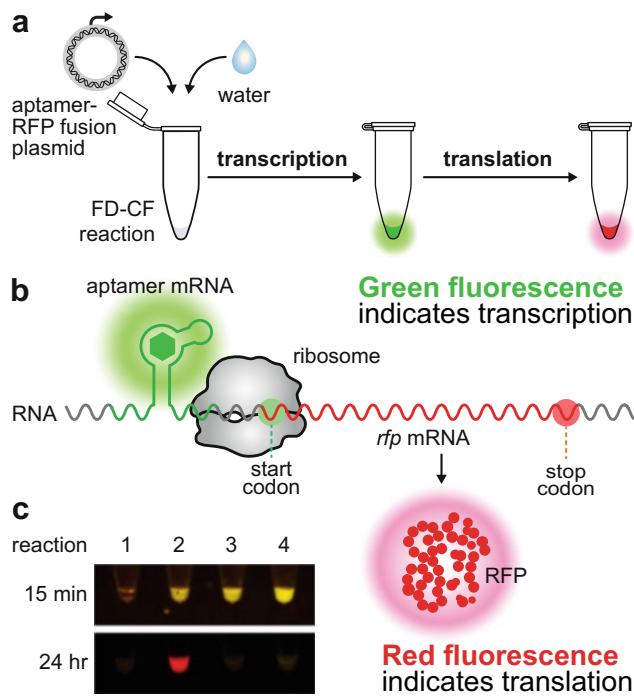


Fig. 1 Using FD-CF reactions to visualize the central dogma in real time. (a) Rehydration of FD-CF reactions with water and a plasmid encoding an RNA aptamer-fluorescent protein fusion construct allows students to observe cell-free transcription and translation in real time via fluorescence. (b) Structure of the RNA aptamer-fluorescent protein fusion construct synthesized in the BioBits® central dogma lab. (c) Green fluorescence is observed as an indicator of mRNA transcription at early reaction timepoints and red fluorescence is observed as an indicator of protein synthesis after 24 h of incubation

interactive tool for teaching the central dogma of molecular biology and exposes students to cutting-edge cell-free technology.

Classroom Protocol for Central Dogma Activity

Day 1

1. Add DNA, antibiotic, and/or nuclease-free water to the each of the FD-CF reactions as shown in Table 1 (*see* Subheading 3.5 for guidance for successful FD-CF reaction assembly).

Tube	Reaction	Water (μL)	DNA A (μL)	DNA B (μL)	Kanamycin (μL)
1	Negative control	7	0	0	0
2	DNA A	2	5	0	0
3	DNA A + kanamycin	0	5	0	2

(continued)

Tube	Reaction	Water (μ L)	DNA A (μ L)	DNA B (μ L)	Kanamycin (μ L)
4	DNA B	2	0	5	0

2. Incubate the reactions at 37 °C for 15–20 min. Body heat (e.g., placing reactions in pocket or hand) can be used to incubate reactions if an incubator is not available.
3. Use a blue light illuminator to visualize reaction fluorescence. Take a photo or otherwise record observations.
 - (a) At this point, green fluorescence, indicating successful transcription, should be visible in tubes 2, 3, and 4. Tube 1 serves as a negative control (Fig. 1c).
4. Incubate reactions overnight at room temperature.

Day 2

5. Use a blue light illuminator to visualize reaction fluorescence. Take a photo or otherwise record observations.
 - (a) After overnight incubation, red fluorescence, indicating successful translation, should be visible in tube 2. No fluorescence will be visible in tubes 1, 3, and 4 due to the absence of protein translation (Fig. 1c). Further explanation of results can be found in the teacher guide (*see Note 4*).

3.2 Making Biomaterials with Hydrogel-Generating Enzymes

Hydrogels are programmable, biocompatible materials with exciting applications as scaffolds for drug delivery, cell-based therapies, and tissue regeneration and replacement [10–12]. To introduce students to hydrogel technology, we developed a prototype activity that uses FD-CF reactions to enzymatically synthesize hydrogels. Sortase A is an enzyme that recognizes and covalently links specific peptide sequences (G_{1-5} and LPXTGX, where X is any amino acid) via a transpeptidation reaction [13]. In this activity, students use FD-CF reactions to express sortase and use it to crosslink a solution of eight-arm polyethylene glycol (PEG) molecules modified with GGG or LPRTGG-containing peptides (Fig. 2a). Hydrogel formation can be observed within 30 min of incubation at 37 °C (Fig. 2b). Optional extension activities investigating how one might tune hydrogel stiffness or use hydrogels as delivery vehicles can also be implemented (*see Note 6*). This activity can further be paired with discussion of fundamental biological concepts such as the properties of extracellular matrix, as well as the translational applications of hydrogels in drug delivery, cell and tissue engineering, and regenerative medicine [10–12].

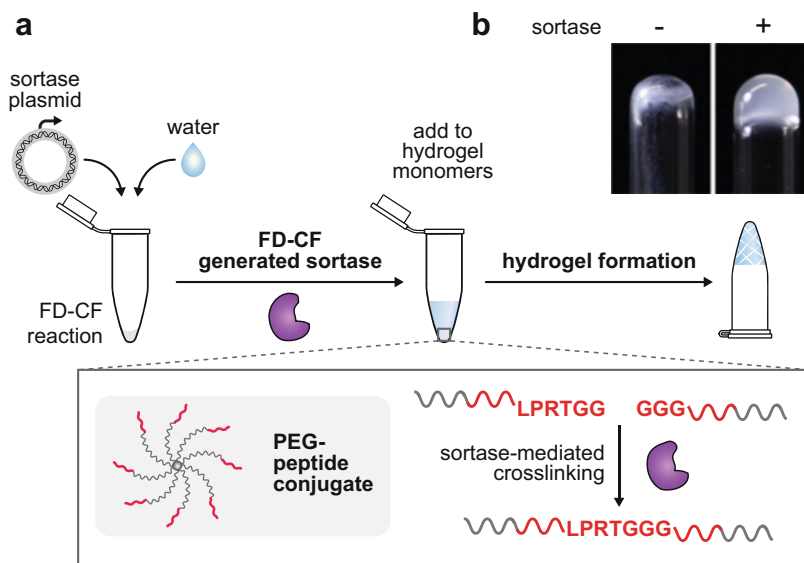


Fig. 2 Making biomaterials in FD-CF reactions. **(a)** Sortase A synthesized in FD-CF reactions crosslinks PEG molecules modified with LPRTGG- and GGG-containing peptides by catalyzing a transpeptidation reaction. **(b)** Following the addition of sortase, crosslinked PEG-peptide conjugates form a hydrogel.

3.2.1 Hydrogel Monomer Preparation

1. Make a 1 weight% (wt%) solution of the PEG-VS in 100 mM HEPES buffer pH 7.9 (i.e., dissolving 1 g of the PEG-VS in 100 mL of buffer).
2. Divide the dissolved PEG-VS in half (i.e., into two solutions of 50 mL each) in 50-mL Falcon tubes.
3. Calculate the amount of GCRELPRTGG peptide needed for a final concentration of 8 mM in a 50 mL solution, using the exact molecular weight reported by the manufacturer. Weigh out this amount and dissolve directly into one of the PEG-VS solutions.
4. Repeat **step 3** with the GGGSGRC peptide and dissolve in the second PEG-VS solution.
5. Incubate the solutions at room temperature for 3 h.
6. Transfer the solutions to separate dialysis cassettes and dialyze into 4 L MilliQ water with gentle stirring. Change the MilliQ water once an hour for 3 h. After the third exchange, allow the solutions to dialyze overnight.
7. The next day, change the MilliQ water once an hour for 2 h.
8. Measure and record the weights of two empty 50-mL Falcon tubes.
9. Transfer each of the dialyzed solutions from the cassettes into a separate pre-weighed 50-mL Falcon tube.

10. Freeze-dry until all water has been removed (approximately 48 h). Weigh the tubes and subtract the empty tube weight to calculate the weight of freeze-dried PEG-peptide conjugate.
11. Calculate and add MilliQ water to make a 20 wt% solution.
12. Make a 20 μ L solution containing 8% PEG-GCRELPRTGG and 8% PEG-GGGSGRC in reaction buffer (50 mM HEPES, 150 mM NaCl, and 10 mM CaCl_2 , pH 7.9) by further diluting the 20 wt% stock. Lyophilize overnight to obtain the freeze-dried hydrogel monomer reagent for use in the below lab activity.

3.2.2 Classroom Protocol for Hydrogel Activity

1. Reconstitute four FD-CF reactions with 5 μ L nuclease-free water (*see* Subheading 3.5 for guidance for successful FD-CF reaction assembly).
2. Reconstitute the other four FD-CF reactions with 5 μ L sortase plasmid.
3. Incubate the reactions at 37 °C for 30 min–24 h or at room temperature for 24–48 h (*see* Note 7).
4. Transfer all four of the nuclease-free water FD-CF reactions into one of the freeze-dried hydrogel monomer tubes, for a total volume of 20 μ L.
5. Transfer all four of the sortase FD-CF reactions into the other freeze-dried hydrogel monomer tube, for a total volume of 20 μ L.
6. Incubate the reactions at 37 °C for 30–60 min.
7. Periodically remove the tubes from the incubator and check gel formation by touching the reaction with a pipette tip or toothpick. A hydrogel has formed if the solution can no longer be pipetted as a liquid.
 - (a) Hydrogel formation should be observed in the tube containing sortase, but not in the tube with negative control FD-CF reactions (Fig. 2b).

3.3 Investigating a Mechanism of Antibiotic Resistance

Antibiotic resistance is a pressing global issue, projected to threaten up to ten million lives per year by 2050 [14]. As a result, it is important to educate students about how resistance occurs. To address this educational need, we developed a hands-on activity that demonstrates horizontal gene transfer as a mechanism of antibiotic resistance. This activity uses synthesis of the orange fluorescent protein dTomato in FD-CF reactions as a reporter of antibiotic efficacy (Fig. 3a, b). Following addition of antibiotic ribosome inhibitors, such as streptomycin and kanamycin, dTomato translation was inhibited and no fluorescence was observed (Fig. 3c, d). To mimic horizontal gene transfer, we showed that FD-CF reactions can be used to express the aminoglycoside O-

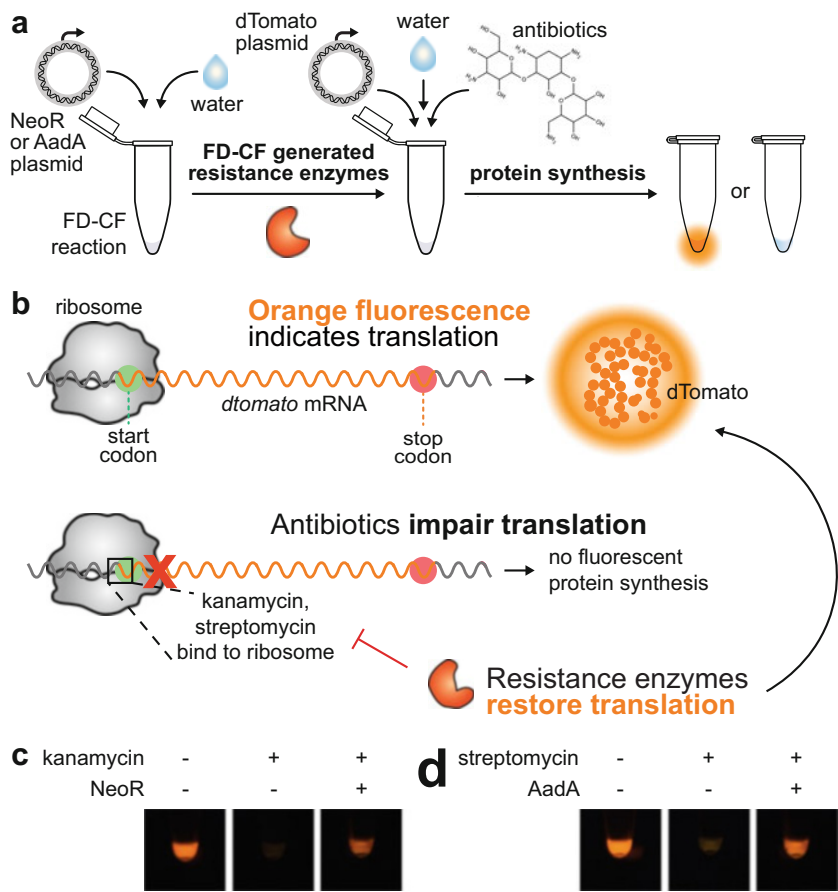


Fig. 3 Observing antibiotic resistance using FD-CF reactions. **(a)** Horizontal gene transfer can be illustrated in vitro by adding pre-synthesized antibiotic resistance enzymes to FD-CF reactions synthesizing the fluorescent protein dTomato. **(b)** In the presence of the antibiotic ribosome inhibitors kanamycin or streptomycin, translation is impaired, resulting in loss of fluorescent protein synthesis. Addition of the NeoR **(c)** or AadA **(d)** resistance enzymes “rescue” dTomato synthesis from the effects of their cognate antibiotics, and fluorescent protein synthesis is observed

phosphotransferase (NeoR) and streptomycin 3'-adenylyltransferase (AadA) enzymes that confer kanamycin and streptomycin resistance, respectively, by chemically modifying their target antibiotics [15, 16]. When added to FD-CF reactions expressing dTomato, we observed that these pre-expressed enzymes “rescued” the FD-CF reactions from the effects of their target antibiotic (Fig. 3c, d). This experiment is designed to illustrate how bacteria that acquire resistance enzymes can continue to synthesize proteins, and thus grow and survive, even in the presence of antibiotics.

In the activity described below, students are challenged to discover which antibiotic resistance enzyme confers resistance to each of two antibiotic ribosome inhibitors. This lab demonstrates the development of antibiotic resistance through the acquisition of resistance genes (for open-access supporting curriculum, see Note 8). Optional extension activities that ask students to investigate the mechanisms of action for various classes of antibiotics and/or

quantify dTomato synthesis in each of their reactions can also be implemented (*see* **Note 9**). These activities open the door to classroom discussions or independent research projects that could help students make informed choices about the use of antimicrobial consumer products in their own lives.

Classroom Protocol for Antibiotic Resistance Activity

Day 1

1. Add 5 μL of AadA (enzyme A) plasmid DNA to an FD-CF reaction (*see* Subheading 3.5 for guidance for successful FD-CF reaction assembly).
2. Add 5 μL of NeoR (enzyme B) plasmid DNA to a second FD-CF reaction.
3. Incubate reactions for 24 h at 30 °C or at room temperature for 24–48 h. These reactions will pre-express the streptomycin resistance protein AadA or the kanamycin resistance enzyme NeoR that will be used in the experiment.
4. Prepare 250 μM stock solutions of streptomycin and kanamycin in nuclease-free water (*see* **Note 10**). Vortex or pipet the solution up and down to mix.

Day 2

5. Obtain two sets of four FD-CF reactions. Label one set of four reactions as “strep” for streptomycin and the other set “kan” for kanamycin.
6. Table 2 shows the volumes of antibiotic, resistance enzyme reaction mixture, dTomato plasmid, and nuclease-free water you will add to each FD-CF reaction. Begin with the first set of four tubes and add the below ingredients, using streptomycin as the antibiotic.
7. Repeat **step 6** using your second set of four FD-CF reactions and kanamycin as the antibiotic.

Table 2
Reaction volumes for antibiotic resistance activity

Tube	Reaction	Antibiotic (μL)	Enzyme		dTomato plasmid (μL)	Water (μL)
			Enzyme A reaction (μL)	Enzyme B reaction (μL)		
1	No antibiotic control	0	0	0	2	3
2	Antibiotic	2	0	0	2	1
3	Resistance enzyme A	2	1	0	2	0
4	Resistance enzyme B	2	0	1	2	0

8. Incubate reactions for 24 h at 30 °C or at room temperature for 24–48 h.

Day 3

9. Use a blue light illuminator to visualize reaction fluorescence. Take a photo or otherwise record observations.
 - (a) dTomato fluorescence should be visible in the no antibiotic controls and the tubes containing streptomycin + enzyme A and kanamycin + enzyme B, but not in tubes containing antibiotic and the noncognate resistance enzyme (Fig. 3c, d).

3.4 Exploring CRISPR-Cas9 Gene Editing

CRISPR-Cas systems for editing DNA promise to reshape the way we understand and treat human diseases and genetic disorders [17]. Recent advances include the first report of efficacy of CRISPR-engineered cells for treatment of sickle cell disease and transfusion-dependent β -thalassemia, [18] as well as the launch of a clinical trial testing CRISPR therapy delivered directly into patients for the first time [19]. In recognition of the transformative effects that CRISPR has had on biological science and human health, Professors Jennifer Doudna and Emmanuelle Charpentier were awarded the 2020 Nobel Prize in chemistry for its discovery and application as a gene editing technology. With CRISPR therapies starting to make their way into the clinic and garnering broad public interest [20, 21], it is important to educate students about the fundamentals of CRISPR technology.

To support this goal, we developed a classroom module that guides students through an exploration of the biological mechanism of CRISPR-Cas9 gene editing. To do this, we linked activity of the *Streptococcus pyogenes* Cas9 nuclease to a fluorescent readout through the design of a synthetic guide RNA (gRNA) targeting the gene encoding red fluorescent protein mRFP1 (Fig. 4a, b). When both gRNA and Cas9 are added to FD-CF reactions synthesizing mRFP1, we observed an approximately four-fold reduction in fluorescent signal, indicative of Cas9 cleavage of the *mrfp1* DNA template and silencing of fluorescent protein synthesis (Fig. 4c). This fluorescent reporter enables straightforward investigation of the mechanism of action of CRISPR-Cas9.

The below activity is designed to help students understand the roles of the biological components necessary for CRISPR-Cas9 gene editing (i.e., the Cas9 nuclease, target DNA, and guide RNA). This hands-on experiment could be paired with a variety of supporting and extension activities to help students understand the mechanism of CRISPR gene editing, how CRISPR compares to existing therapeutic modalities, and the ethics associated with CRISPR technology (see **Note 11**). Students can also quantify the production of mRFP1 in each of their reactions via fluorescence (see

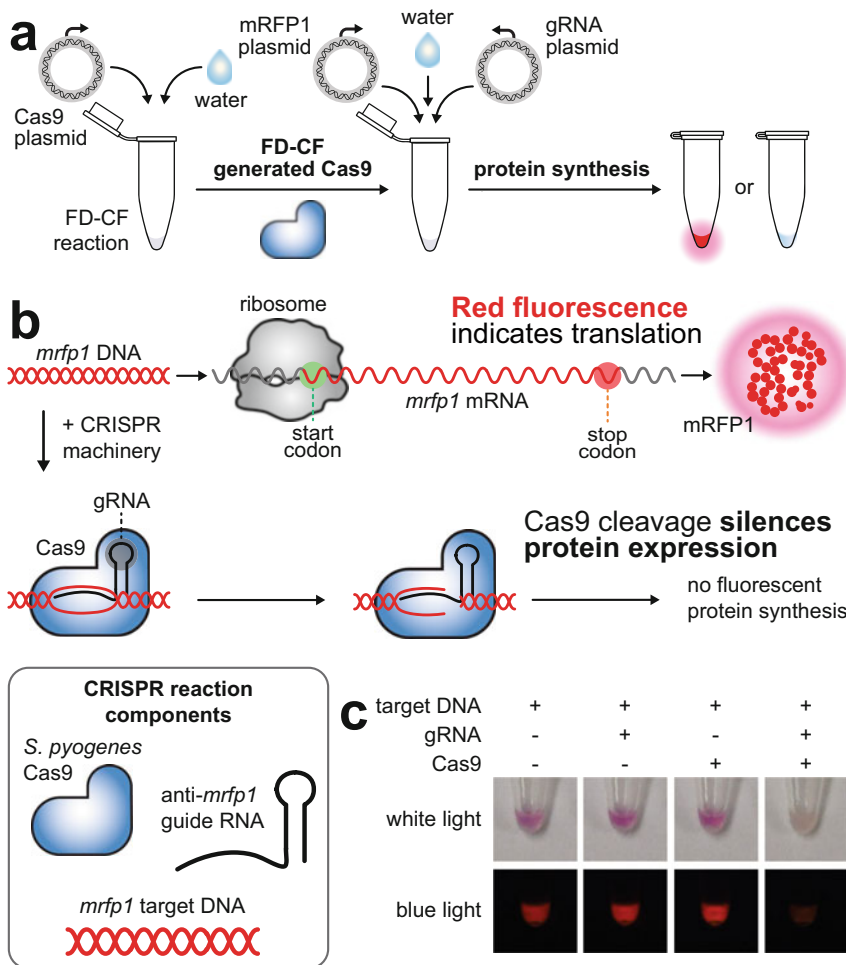


Fig. 4 Teaching the mechanism of CRISPR-Cas9 gene editing with FD-CF reactions. **(a)** This activity links CRISPR-Cas9 activity to a fluorescent readout through the use of a gRNA that targets the *mrfp1* gene encoding a red fluorescent protein. **(b)** When CRISPR machinery is added to the reaction, synthesis of the red fluorescent protein mRFP1 is silenced due to cleavage of the *mrfp1* DNA template by Cas9. **(c)** Silencing of protein synthesis is observed as the loss of red color and fluorescence in reactions containing Cas9, gRNA, and target DNA

Note 11). Overall, these activities and resources represent diverse, student-guided learning modules centered on a cutting-edge biological technology with broad applications for human health.

Classroom Protocol for CRISPR Activity

Day 1

1. Rehydrate two FD-CF reactions by adding 5 μ L of Cas9 plasmid DNA (see Subheading 3.5 for guidance for successful FD-CF reaction assembly). Incubate for 24 h at 30 $^{\circ}$ C or on

Table 3
Reaction volumes for CRISPR activity

Tube	Reaction	Water (μL)	Cas9 reaction (μL)	gRNA plasmid (μL)	mRFP1 plasmid (μL)
1	Target DNA	3	0	0	2
2	Target + gRNA	1	0	2	2
3	Target + Cas9	2	1	0	2
4	Target + gRNA + Cas9	0	1	2	2

the benchtop at room temperature for 24–48 h. These reactions will pre-express the Cas9 protein that will be used in the experiment.

Day 2

2. Add the appropriate amounts of Cas9 reaction mix, gRNA plasmid, mRFP1 plasmid, and nuclease-free water as shown in Table 3 below to each of 4 FD-CF reactions.
3. Seal the FD-CF reactions and incubate for 24 h at 30 °C or on the benchtop at room temperature for 24–48 h.

Day 3

4. Place reactions in front of a piece of white paper. Take a photo or otherwise record observations.
5. Use a blue light illuminator to visualize reaction fluorescence. Take a photo or otherwise record observations.
 - (a) mRFP1 fluorescence will be present in all tubes, but reduced color/fluorescence intensity should be observed in the tube containing all components for the CRISPR-Cas9 reaction (Cas9 nuclease + gRNA plasmid + target DNA) (Fig. 4c).

**3.5 Addressing
Common Challenges**

The following are tips to prevent common technical challenges observed during classroom implementation of BioBits® activities:

1. Micropipette technique: Students performing BioBits® labs should be familiar with proper micropipette technique and be able accurately to pipette volumes in the 2–5 μL range. This activity will introduce students to proper pipetting technique and have them practice pipetting a variety of volumes: <http://www.minipcr.com/micropipetting/>. Alternatively, have students practice pipetting by transferring small volumes of food coloring.

2. **RNAses:** Contaminating RNAses can be introduced into FD-CF reactions from skin or water that has not been screened for nuclease activity. These RNAses will digest mRNA transcripts in FD-CF reactions, inhibiting cell-free protein synthesis. For this reason, we strongly encourage the use of nuclease-free water for rehydration of FD-CF reactions, as noted in each experimental protocol. Wearing gloves while handling FD-CF reactions will further prevent the introduction of contaminating RNAses from skin. Finally, RNase Zap can be used to clean work surfaces or pipettes that are suspected to be contaminated with RNAses.
3. **Plasmid DNA purity:** Plasmid DNA impurities can reduce cell-free protein synthesis yields. We strongly encourage the use of midi- or maxi-prep kits to purify plasmid DNA of sufficient quality for use in FD-CF reactions.
4. **Handling FD-CF reactions:** When uncapping FD-CF reactions, freeze-dried pellets can be dislodged or even ejected from the tubes if they are not handled carefully. To avoid this, gently tap tubes on the work surface to collect pellets at the bottom before opening. Then, carefully remove each cap in the strip one at a time, taking care not to dislodge the pellets to the greatest extent possible.
5. **Reagent addition:** When adding reagents to FD-CF reactions, students should not touch their pipette tip to the pellet or the pellet may get stuck inside the tip. Instead, touch the pipette tip to the side of the tube while transferring liquid to avoid disrupting the pellet at the bottom. Then, tap the tube so the pipetted liquid collects at the bottom of the tube and dissolves the pellet. Further, because the reaction volumes are small, formation of bubbles in the reaction tubes should be avoided. We advise against using the second stop on your micropipette and also against pipetting up and down to mix, as these actions tend to introduce bubbles.
6. **Avoiding reagent/reaction contamination:** Students should be sure to use a new pipet tip for addition of each reagent to FD-CF reactions.
7. **Pipetting FD-CF reaction mixtures:** The rehydrated FD-CF reaction mixtures are viscous and can be hard to pipet. When pipetting reactions synthesizing sortase, antibiotic resistance enzymes, or Cas9, students should do their best to transfer all of the volume indicated, and try not to leave any of the pipetted reaction in the pipet tip. Touching the pipette tip to the side of the tube while ejecting liquid can help facilitate complete transfer.
8. **Reaction incubation:** After adding reagents, students should cap their reaction tubes tightly to avoid sample evaporation.

They should feel the caps “click” into place if they are closed tightly. Before incubating reactions, students should be sure the FD-CF pellet is fully dissolved and the reaction volume is collected at the bottom of the tube. If necessary, students can gently flick the reaction tube to mix and dissolve the pellet after all reagents are added, and the tube is firmly capped. Use a flick of the wrist or briefly centrifuge to ensure all reagents collect at the bottom of the tube before incubating. Reaction tubes can be incubated in a PCR thermocycler, in a tube rack or laid flat in the incubator (for heated incubations), or on a lab bench or table (for room temperature incubations).

9. Observing reaction fluorescence: When observing fluorescence resulting from FD-CF reactions, make sure that the blue light from the illuminator is filtered out with an orange filter. Dimming ambient lights can also help students better visualize and/or document reaction fluorescence.

4 Notes

1. Educational resources and activities related to cell-free protein synthesis technology:
 - (a) Cell-free protein synthesis: BioBits® activities uses cell-free protein synthesis technology to enable low-cost and easy-to-use hands-on laboratory experiments. Read more about how cell-free reactions work and their real-world applications: <https://dnadots.minipcr.com/dnadots/cell-free-technology>.
 - (b) Introduction to cell-free technology: This webinar discusses cell-free technology and how it is applied to the field of synthetic biology: (<https://www.minipcr.com/news/cell-free-technology-webinar-biobits/>).
 - (c) Genes in Space: Genes in Space™ is a free national science competition in which school and high school students propose pioneering DNA experiments that address a space exploration challenge. Students only need to submit a proposed idea—they do not need to do any experiments to enter the contest. Winners have their experiments launched to the International Space Station where they are carried out by astronauts using miniPCR™ biotechnology tools. BioBits® FD-CF reactions are part of the toolkit that students may use to propose experiments. More information at: <https://www.genesinspace.org/>.
2. We strongly recommend purchasing FD-CF reactions through BioBits® at miniPCR bio™ (included in miniPCR bio™ KT-1910-01 or KT-11102-01). These commercially available reactions have been optimized for classroom use, reduce

instructor prep time and effort, and may be more cost-effective for instructors due to the equipment, reagents, and facilities required to prepare FD-CF reactions. At the time of this publication, there is no other commercial source for FD-CF reactions, to our knowledge. For the central dogma activity, purchase of the commercial kit is required. For the prototype activities, instructors may choose to prepare their own FD-CF reactions, using previously described methods [5–7]. A low-cost blue light illuminator can also be purchased from miniPCR bio™ for visualization of fluorescent reaction outputs (miniPCR bio™ QP-1900-01).

3. FD-CF reactions are stable for up to 3 months at 4 °C (fridge) and up to 6 months at –20 °C (freezer) from date of receipt. Once opened, store any unused pellets in the resealable bag including the supplied desiccant card. Store under these conditions until immediately before use.
4. This protocol is adapted from the existing accompanying curriculum in the commercially available BioBits® Central Dogma Kit. The full teacher guide for this activity, along with a student guide, study questions, figures, and teacher notes is freely available for download: <https://www.minipcr.com/product/biobits-central-dogma/>.
5. Optional extension activities related to the central dogma activity are intended to provide curriculum scaffolding for students at a variety of educational levels:
 - (a) Recorded demo: A demonstration of the full Central Dogma lab may be used to guide instructors in implementing labs or shown to students if hands-on classroom labs are not feasible: <https://www.youtube.com/watch?v=FCwJyRvpDOU>.
 - (b) RNA aptamers: The green signal associated with RNA and transcription in this lab is made possible with a structure called an RNA aptamer. This supporting activity asks students to read and answer questions about RNA aptamers (page 32 of the Central Dogma Teacher Lab Guide: <https://www.minipcr.com/product/biobits-central-dogma/>).
 - (c) mRNA vaccines: This extension activity helps students understand how mRNA vaccines work, using their understanding of the central dogma as a scaffold: <https://www.minipcr.com/wp-content/uploads/BioBits-Central-Dogma-mRNA-add-on-student-guide.pdf>. Students can also learn more about mRNA vaccines through a supporting written assignment: <https://dnadots.minipcr.com/dnadots/mrna-vaccines> and/or webinar: <https://www.youtube.com/watch?v=SMODg6ZwKDK>.

- (d) Students can quantify mRNA and protein produced in each reaction by taking photos and carrying out green and red fluorescence analysis with ImageJ, using the BioBits[®] ImageJ tutorial (File S2 in the supplementary material of our previous, open-access publication [7]). They can plot their and/or their classmates' results and carry out statistical analysis using Excel or a similar data analysis program.
6. Optional extension activities related to the hydrogel activity are intended to provide curriculum scaffolding for advanced students:
 - (a) The idea of creating biomaterials with tunable properties can be explored. Specifically, the mechanical properties of the hydrogel can be tuned by varying the concentration of the PEG-peptide monomers to create a range of materials from a viscous slime to a stiff hydrogel [22]. Example concentrations are given in our previous, open-access publication.
 - (b) Sortase can be coexpressed with fluorescent proteins and added to freeze-dried hydrogel monomers to create fluorescent hydrogels. To do this, add a plasmid encoding a fluorescent protein of your choice (e.g., the dTomato or mRFP1 plasmids used in other BioBits[®] activities) with the sortase plasmid to the initial FD-CF reaction (see our previous, open-access publication for examples) [22]. This experiment could be used to introduce the concept of hydrogels as delivery devices for controlled release of small molecule or protein cargo.
7. This incubation represents an optional pause point to allow educators to spread the lab over two class periods, if desired. Sortase can also be pre-expressed the day before the lab to accelerate the experimental protocol.
8. Supporting curriculum pieces for the antibiotic resistance activity including pre- and post-lab questions and slides introducing antibiotic resistance mechanisms can be found in Curriculum S2 and File S1 of our previous, open-access publication [7].
9. Optional extension activities related to the antibiotic resistance activity are intended to provide curriculum scaffolding for students at a variety of educational levels:
 - (a) An optional extension activity guides students through an investigation of various antibiotic mechanisms of action via a research project and hands-on lab (Curriculum S1 and File S1 of our previous, open-access publication [7]).
 - (b) Students can quantify the amount of dTomato produced in each reaction by taking photos and carrying out

fluorescence analysis with ImageJ, using the BioBits[®] ImageJ tutorial (File S2 in the supplementary material of our previous, open-access publication [7]). They can plot their and/or their classmates' results and carry out statistical analysis using Excel or a similar data analysis program.

10. Antibiotic stocks can be prepared ahead of time and refrigerated for short-term storage (days) or frozen at -20°C for long-term storage.
11. Optional extension activities related to the CRISPR activity are intended to provide curriculum scaffolding for students at a variety of educational levels:
 - (a) This lab activity is designed to be paired with a sorting and classification exercise to help students understand the functions of the molecular components necessary for Cas9 activity (nuclease, guide RNA, target DNA, proto-spacer adjacent motif) and slides introducing CRISPR technology (Curriculum S4 and File S3 in our previous, open-access publication [7]).
 - (b) We developed an assignment to help students investigate a variety of gene editing technologies (i.e., CRISPR, zinc finger nucleases) for treating Huntington's disease (Curriculum S5 in our previous, open-access publication [7]).
 - (c) A process-oriented guided inquiry learning (POGIL) activity allows students to explore a variety of possible solutions to treat a patient with sickle cell anemia (Curriculum S6 in our previous, open-access publication [7]).
 - (d) Students can carry out independent research projects in which they can investigate a problem of their choice that could be solved with CRISPR gene editing approaches and consider the ethics associated with the use of this technology (Curriculum S7 and S8 in our previous, open-access publication [7]).
 - (e) Students can quantify the amount of mRFP1 produced in each reaction by taking photos and carrying out fluorescence analysis with ImageJ, using the BioBits[®] ImageJ tutorial (File S2 in the supplementary material of our previous, open-access publication [7]). They can plot their and/or their classmates' results and carry out statistical analysis using Excel or a similar data analysis program.
 - (f) Webinars, readings, paper models, and more resources for teaching CRISPR in the classroom are available at <https://www.minipcr.com/products/minipcr-learning-labs/crispr/#library>.

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