Development of a Freeze-Dried CRISPR-Cas12 Sensor for Detecting Wolbachia in the Secondary Science Classroom

Grant A. Rybnicky, Radeen A. Dixon, Robert M. Kuhn, Ashty S. Karim, and Michael C. Jewett*

ABSTRACT: Training the future synthetic biology workforce requires the opportunity for students to be exposed to biotechnology concepts and activities in secondary education. Detecting Wolbachia bacteria in arthropods using polymerase chain reaction (PCR) has become a common way for secondary students to investigate and apply recombinant DNA technology in the science classroom. Despite this important activity, cutting-edge biotechnologies such as clustered regularly interspaced short palindromic repeat (CRISPR)-based diagnostics have yet to be widely implemented in the classroom. To address this gap, we present a freeze-dried CRISPR-Cas12 sensing reaction to complement traditional recombinant DNA technology education and teach synthetic biology concepts. The reactions accurately detect Wolbachia from arthropod-derived PCR samples in under 2 h and can be stored at room temperature for over a month without appreciable degradation. The reactions are easy-to-use and cost less than $40 to implement for a classroom of 22 students including the cost of reusable equipment. We see these freeze-dried CRISPR-Cas12 reactions as an accessible way to incorporate synthetic biology education into the existing biology curriculum, which will expand biology educational opportunities in science, technology, engineering, and mathematics.

KEYWORDS: CRISPR, Cas12, nucleic acid detection, secondary science education, Wolbachia

INTRODUCTION

The global market for synthetic biology products is estimated to grow to over $30 billion USD by 2026 from a current $9.5 billion USD in 2021. To support this growth, a workforce of graduating students must be inspired and trained to work in synthetic biology and its associated science, technology, engineering, and mathematics (STEM) disciplines. This requires student exposure to, and formative hands-on experiences with, STEM educational activities.

Many secondary schools in the United States have adopted STEM-related frameworks to teach science and engineering practices. For example, thousands of secondary science classrooms integrate recombinant DNA technology into curriculum through activities such as the Wolbachia Project. The Wolbachia Project teaches ecology, biotechnology, and bioinformatics concepts through surveying native arthropods for Wolbachia infection. Given the widespread range of these bacteria globally, the detection of Wolbachia in the secondary education classroom has become an accessible model for learning about recombinant DNA biotechnologies. The project includes collection and identification of arthropods from the students' local environment, bulk DNA extraction from the specimen, polymerase chain reaction (PCR) amplification of a Wolbachia specific amplicon, and gel electrophoresis-based detection of the PCR amplicon (Figure 1A). By carrying out these activities, students learn about the existence of standard molecular biotechnology techniques as well as practice them in an engaging inquiry-based way. While curriculum such as this teaches foundational techniques important to synthetic biology, translation of the most cutting-edge synthetic biology concepts has been limited.

A major barrier to teaching synthetic biology and other cutting edge STEM concepts is that education standards can make it difficult to interject new topics not explicit in the standard curriculum. Despite this barrier, recent synthetic biology-based educational efforts such as BioBits, the BioBuilder Educational Foundation, the international Genetically Engineered Machines (iGEM) competition, Amino Labs, and The ODIN are making progress. However, there are opportunities to do more. A particularly exciting area for expansion is in portable cell-free diagnostics. In recent years, point-of-use, cell-free diagnostics based on clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) technologies have emerged to detect everything from human parasites to SARS-CoV-2. A key feature of CRISPR-based diagnostics is the ability to use Cas12 or Cas13 to convert DNA or RNA binding into an
observable signal, respectively (Figure 1B). Incorporating synthetic biology innovations such as this into existing biology lab activities could lead to improved classroom activities and the integration of synthetic biology education into the educational infrastructure that already exists.

Here, we present a cell-free, tube-based, and freeze-dried CRISPR-Cas12 (FD-CC12) DNA sensing reaction that can be directly integrated into a popular recombinant DNA technology educational activity developed by the Wolbachia Project to visualize the presence of Wolbachia DNA in arthropod-derived samples (Figure 1A). In contrast to gel electrophoresis-based visualization, FD-CC12 reactions do not require specialized electrophoresis equipment, pipetting into small wells, DNA staining or band interpretation, and are ready to interpret in two or fewer hours. FD-CC12 reactions are less technically challenging to perform than gel electrophoresis, and a positive result is easily interpreted as fluorescence visible in the tube after incubation at 37 °C for 2 h is reported. Error bars represent standard deviation of four replicates. Purified synthetic DNA with the same sequence as the consensus Wolbachia amplicon was used as an activator.

### RESULTS

**Development of a Cas12 Diagnostic to Detect Wolbachia.** We set out to develop a CRISPR-Cas12 diagnostic for Wolbachia detection to implement in secondary classrooms. First, we developed a functional CRISPR-Cas12 diagnostic. Cas12, similar to other CRISPR nucleases, binds and cleaves DNA at a specific sequence encoded by a CRISPR RNA (crRNA). Once activated, Cas12 indiscriminately cleaves single-stranded DNA (ssDNA) in solution (Figure 1B). This indiscriminate cleavage activity can be visualized within a diagnostic in a variety of ways, including the use of a ssDNA chemically modified with a fluorophore on one end and a fluorescence quencher on the other. In the uncleaved state, the ssDNA probe emits no fluorescence upon excitation as the quencher molecule is near the fluorophore and absorbs the fluorescent emission from the fluorophore. Once cleaved, the ssDNA probe is no longer intact, and the fluorescence quencher is not close enough to the fluorophore to absorb the fluorescent emission. To test for CRISPR-Cas12 activity, we screened all possible crRNAs that can target the consensus 483 bp Wolbachia amplicon previously developed for the Wolbachia Project. Of the five locations with the appropriate TTTV protospacer adjacent motif (PAM) (supplementary Figure S1), all possible crRNAs mediated detection of the Wolbachia amplicon (Figure 1C). Although all were functional, reaction endpoint fluorescence varied with different crRNA constructs. crRNA 286R yielded the highest endpoint fluorescence as well as fold activation. Thus, we selected crRNA 286R for further development.

**Characterization of Freeze-Dried Cas12 Diagnostics.** With a functional CRISPR-Cas12 diagnostic for Wolbachia detection in hand, we next wanted to assess the possibility of
freeze-drying the system. Previous works have shown that cell-free systems, including CRISPR-based diagnostics, can be freeze-dried for increasing stability and portability.\textsuperscript{27,28} Such features would be advantageous for preparation and delivery in the classroom setting. We freeze-dried CRISPR-Cas12 cell-free reactions in a VirTis BenchTop Pro lyophilizer at $\leq 100 \text{ mtorr}$ and $-80^\circ\text{C}$ overnight or until fully freeze-dried. We then compared CRISPR-Cas12 reactions prepared from fresh reagents and these pre-assembled, lyophilized reactions to determine whether the CRISPR-Cas12 formulation is stable through the lyophilization process. Unfortunately, when lyophilized, the CRISPR-Cas12 formulation demonstrated no activity in the presence of activating DNA (Figure 2A). We hypothesized that the lack of activity was due to degradation of the Cas12 protein or the crRNA.

To address this bottleneck and improve the stability of the reactions, we evaluated the diagnostic activity when reactions were lyophilized in the presence of different lyoprotectant formulations (Figure 2B). Specifically, we tested sucrose, trehalose, and dextran (70 kDa average weight) individually and in combination. We found that the activity of reactions supplemented with sucrose and trehalose individually increased with concentration, but dextran exhibited a concentration optimum of 25 mg/mL in the final reaction. Although combining 25 mg/mL each of sucrose and trehalose achieved similar endpoint fluorescence as 50 mg/mL of each respective lyoprotectant, combining 25 mg/mL of either sugar with 25 mg/mL dextran increased the retained activity. Combining 25 mg/mL each of sucrose and dextran exhibited approximately 75% recovery of the prelyophilization signal.

Additionally, all formulations containing dextran exhibited beneficial changes in freeze-dried pellet consistency. Rather than a powdery pellet subject to movement by static electricity, all lyophilized dextran formulations exhibited a bulky, solid
pellet unaffected by static charges. This made manipulation of pellets much easier and decreased the risk of static electricity ejecting portions of the pellet from the tube. In making the pellet easier to work with, the inclusion of dextran in the lyoprotectant formulation made the FD-CC12 reactions more amenable to use in secondary science classrooms. Moving forward, we chose to use 25 mg/mL each of sucrose and dextran to formulate our freeze-dried reactions.

We next characterized the final formulation on freeze-dried CRISPR-Cas12 (FD-CC12) reaction stability over time. After lyophilization, we stored reactions at either 20 or 37 °C in vacuum sealed bags with a desiccant card for 2 months, monitoring activity over time. When stored at room temperature, the lyophilized reactions retained approximately 75% activity in the on state after 2 months of storage without an appreciable leak in the off state (Figure 2C). When stored at 37 °C for the same amount of time, the reactions retained over 40% activity in the on state without leak.

Assessment of Freeze-Dried Cas12 Diagnostics in the Classroom. With stable and robust FD-CC12 reactions established, we next wanted to test the compatibility with the Wolbachia Project educational framework. We first wanted to make sure that Wolbachia DNA from arthropod-derived samples could be detected. When rehydrated with a PCR reaction that used bulk DNA from a Wolbachia-infected Nasokia vitripennis wasp as template, the FD-CC12 reaction achieved endpoint yields comparable to those observed in the PCR kit screen (Figure 3A). Using a Wolbachia-free N. vitripennis wasp-derived PCR, the FD-CC12 reaction showed signal above background observable by fluorescent plate reader but not by eye.

Once validated with control insects, we next assessed the ability of the workflow to be completed in a high school classroom setting by a high school student. FD-CC12 reactions were prepared at Northwestern University and shipped to Centennial High School (CHS) in Roswell, Georgia. At CHS, a student collected and identified 22 arthropods across six orders (supplementary Table S2), extracted bulk DNA from each specimen, and performed PCR to amplify the Wolbachia amplicon as is standard practice in the Wolbachia Project workflow. Those PCR products were then used to rehydrate FD-CC12 reactions and were also visualized using gel electrophoresis. Out of the 22 samples tested, 21 agreed between FD-CC12 reaction and gel electrophoresis (Figure 3B). For sample ID# 11, the gel indicated Wolbachia infection, while the FD-CC12 reaction indicated no infection. Upon sequencing the Wolbachia amplicon for sample ID# 11, it was revealed that there were five mutations within the protospacer region the crRNA 286R targeted (supplementary Figure S2). As Wolbachia are a diverse genus of bacteria, it is not unreasonable to expect that some isolates would evade detection by any single crRNA. Using multiple crRNAs in the same reaction to create an OR gate logic, it may be possible to detect a wider diversity of isolates. Likewise, FD-CC12 reactions that generate false negatives may be useful in teaching the fundamental differences in mechanism between analysis by gel electrophoresis and CRISPR-based diagnostics.
1A). We identified Wolbachia infection in arthropod-derived samples, successful completion by a high school student, and high accuracy of sensing reactions, this demonstrates that FD-CC12 reactions can be integrated into school student, and high accuracy of sensing reactions, this demonstrates that FD-CC12 reactions can be integrated into the Wolbachia Project laboratory activities.

**DISCUSSION**

We developed a FD-CC12 Wolbachia DNA sensing reaction that can be used to teach synthetic biology concepts in the secondary science classroom. As designed, the sensing reactions detect a PCR amplicon commonly used to diagnose Wolbachia infection in field-collected arthropods as a demonstration of recombinant DNA technologies (Figure 1A). We identified and evaluated five functional crRNAs that can be used to detect this amplicon with Cas12 (Figure 1C). Additionally, we identified a lyoprotectant additive formulation that both recovers about 75% of reaction endpoint fluorescence after lyophilization (Figure 2A,B) and is stable at room temperature for over a month without appreciable degradation (Figure 2C). By stabilizing reactions with lyophilization for storage at room temperature prior to use in the classroom, FD-CC12 reactions are poised for centralized manufacturing and distribution without cold chain shipping. Using this formulation, we evaluated the reaction’s compatibility with commonly used PCR kits, finding that the freeze-dried reactions could be rehydrated with virtually any unpurified PCR product and still distinguish the presence of the Wolbachia amplicon in the sample (Figure 2D). To validate that the reaction can be applied to the established classroom activity, we rehydrated freeze-dried reactions with arthropod-derived samples. Nasonia wasps with known Wolbachia infection states yielded results as expected (Figure 3A). Screening of 22 field-collected arthropods across six orders for Wolbachia infection by a high school student using gel electrophoresis and FD-CC12 reactions yielded a 95% accuracy rate (Figure 3B).

Table 1. Comparison of DNA Visualization Method Equipment and Consumable Costs

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*abolished equipment to cast gels, generate current, house the gel in buffer, and visualize the gel as well as initial reagents cost ~$1000 or ~$300 for the same-sized class for traditional or alternative the molecular biology research lab, gel electrophoresis poses a few technical hurdles to implementation that science educators may face. Foremost, gel electrophoresis is intimidating to educators who have not had hands-on training in a laboratory or elsewhere. The ability to properly prepare and cast an agarose gel, pipette samples into the well of a gel, and consistently stain and visualize gels takes hands-on practice before it becomes routine. Collectively, these technical hurdles may cause teachers to seek more expensive alternatives (e.g., precast gels) or to avoid implementation of gel electrophoresis altogether. Alternatively, the FD-CC12 reactions contain all necessary components within a single tube, are easier to pipette analyte into, and are visualized by handheld blue light imager without the addition of stains. The use of freeze-dried cell-free reactions has the potential to increase teacher confidence in their laboratory skills and encourage them to attempt laboratory activities they may have been hesitant to try before such as gel electrophoresis. Similarly, the initial monetary investment required to implement FD-CC12 sensing in the classroom is lower than implementing gel electrophoresis. Requiring only incubation at 37 °C and a blue light imager such as the miniPCR bio P51 Molecular Fluorescence Viewer, the FD-CC12 reactions have a startup cost of ~$38 for a 22-student class. Alternatively, the equipment to cast gels, generate current, house the gel in buffer, and visualize the gel as well as initial reagents cost ~$1000 or ~$300 for the same-sized class for traditional or

While we recommend that FD-CC12 sensing reactions be used to complement gel electrophoresis in recombinant DNA technology education, we do recognize that specific features of our sensing reactions may make them a more attractive readout than gel electrophoresis to secondary science educators. The FD-CC12 reactions primarily lower two barriers to DNA visualization in the classroom: (1) FD-CC12 reactions are less technically difficult to set up and run than gel electrophoresis and (2) FD-CC12 reactions are less expensive. While commonplace in the molecular biology research lab, gel electrophoresis poses a few technical hurdles to implementation that science educators may face. Foremost, gel electrophoresis is intimidating to educators who have not had hands-on training in a laboratory or elsewhere. The ability to properly prepare and cast an agarose gel, pipette samples into the well of a gel, and consistently stain and visualize gels takes hands-on practice before it becomes routine. Collectively, these technical hurdles may cause teachers to seek more expensive alternatives (e.g., precast gels) or to avoid implementation of gel electrophoresis altogether. Alternatively, the FD-CC12 reactions contain all necessary components within a single tube, are easier to pipette analyte into, and are visualized by handheld blue light imager without the addition of stains. The use of freeze-dried cell-free reactions has the potential to increase teacher confidence in their laboratory skills and encourage them to attempt laboratory activities they may have been hesitant to try before such as gel electrophoresis. Similarly, the initial monetary investment required to implement FD-CC12 sensing in the classroom is lower than implementing gel electrophoresis. Requiring only incubation at 37 °C and a blue light imager such as the miniPCR bio P51 Molecular Fluorescence Viewer, the FD-CC12 reactions have a startup cost of ~$38 for a 22-student class. Alternatively, the equipment to cast gels, generate current, house the gel in buffer, and visualize the gel as well as initial reagents cost ~$1000 or ~$300 for the same-sized class for traditional or
education optimized gel electrophoresis equipment, respectively (Table 1). Assuming one sample per student and a class size of 22 students, the CRISPR-Cas12-based activity is cheaper to run for 848 students or fewer than the education optimized gel electrophoresis setup. The price of FD-CC12 reactions can be further reduced about 10x by applying state-of-the-art cell-free technology such as the use of paper instead of tubes. That being said, gel electrophoresis systems are generalizable for visualizing almost any DNA sample, while the FD-CC12 sensing reaction presented is specific to Wolbachia amplicon detection given the crRNA used.

Although we focused on the detection of Wolbachia in the secondary science classroom, we note that the technology described has potential applications beyond the scope presented. Within science education, the DNA that is detected can easily be changed by altering the crRNA in the reaction. There are numerous well established laboratory activities that can be implemented in the classroom. This can lead to applications such as tracking of specific organisms in the environment by DNA detection, identification of visibly indistinguishable species, or monitoring the spread of mobile genetic elements in the environment.

Taken together, FD-CC12 reactions have the potential to be a simple, cost-effective companion to gel electrophoresis that provide a hands-on way to teach CRISPR-based nucleic acid detection and concepts of synthetic biology. In integrating this activity with the established recombinant DNA technology workflow designed by the Wolbachia Project, we anticipate the widespread adoption of CRISPR-Cas12-based detection into the classroom, further strengthening the effort to expose school children to synthetic biology concepts and priming the future STEM workforce.

## METHODS

### Cas12 Sensing Reactions

Fresh Cas12 reactions were assembled by mixing reaction buffer (40 mM Tris-HCl, 60 mM NaCl, 6 mM MgCl2, pH 7.3), 45 nM crCas12a V3 (IDT), 45 nM crRNA (IDT), 1 μM ssDNA Fluorophore—Quencher (FQ) Probe (IDT), and lyprotecan. Prior to the addition of FQ Probe and lyprotecan, the mixture was equilibrated at 37 °C for 15 min. Upon addition of the analyte, the reaction was incubated at 37 °C and fluorescence was measured (490 nm excitation, 525 nm emission) using a Synergy H1 microplate reader (BioTek, USA) and Gen5 v. 2.09 (BioTek) software. Step-by-step instructions for assembling and running fresh Cas12 reactions are provided in Supplementary File 1.

Lyhophilized Cas12 reactions were assembled as described above and then flash frozen with liquid nitrogen in PCR strip tubes (Thermo Scientific, AB2000) with a small hole melted in the cap of each tube. The reactions were lyophilized using a VirTis BenchTop Pro lyophilizer (SP Scientific) at ≤100 mtorr and −80 °C overnight or until fully freeze-dried. Following lyophilization, tubes were packaged in Food Saver bags with a desiccant card, vacuum sealed, and stored at the indicated temperature. When ready for use, tubes were removed from the Food Saver bags and rehydrated with the same volume of analyte as the mixture prior to lyophilization. Reactions were incubated at 37 °C and fluorescence was measured as described above.

### Arthropod Collection and Wolbachia DNA Amplification

Arthropods belonging to six different orders (supplemental Table S2) were collected from Roswell, GA and Brevard, NC. Each specimen was preserved in 75% EtOH in a 1.5 mL collection tube and frozen at −20 °C. Samples were visually identified to taxonomic order and later identified further by COI barcoding. PCR using the primers described by Folmer was performed and the product was Sanger sequenced (GeneWiz). Consensus sequences for each specimen were obtained using DNA Subway (CyVerse/DNA learning Center), and NIH nucleotide BLAST was used to taxonomically identify the arthropod. Wolbachia-infected and Wolbachia-free N. vitripennis wasps were obtained from the Wolbachia Project through the Bordenstein Lab at Vanderbilt University. Bulk DNA was extracted from each specimen by manual grinding using sterile, plastic minipestles for 2 min in a 1.5 mL microcentrifuge tube containing 200 μL of lysis buffer/EDTA mix and subsequent purification with the Monarch Genomic DNA Purification Kit (catalog #T3010) as directed. A 100 μL DNA elution was collected and stored at −20 °C until used for PCR.

PCR was performed using 2 μL of eluted DNA, Wspec-F (5'-CAT ACC TAT TCG AAG GGA TAG-3') and Wspec-R (5'-AGC TTC GAG TGA AAC CAA TTC-3') primers, and miniPCR bio EZ PCR Master Mix (catalog RG-1000-01). miniPCR bio Mini8 thermocyclers were used for PCR with the following protocol: initial denaturation at 94 °C for 120 s and 34 cycles of denaturation 95 °C for 30 s, annealing 55 °C for 45 s, extension 72 °C for 60 s, and final extension 72 °C for 600 s. PCR product was frozen at −20 °C for further use.

### Gel Electrophoresis and Cas12 Detection of Wolbachia DNA in the Classroom

Gel electrophoresis assays were completed in a secondary science education classroom at CHS in Roswell, GA using the miniPCR bio blueGel electrophoresis system with a built-in transilluminator. Gels were poured using GelGreen Agarose Tabs to a concentration of 2% agarose. 5 bp amplicon using the built-in blue light transilluminator. Freeze-dried Cas12 reactions were assembled and packaged at Northwestern University in Evanston, IL. The reactions were then shipped at room temperature to CHS in Roswell, GA. Reactions were stored at room temperature for 9 days prior to rehydration. PCR strip tubes containing a freeze-dried pellet of the CRISPR-Cas12 system were rehydrated using 10 μL of PCR product. Tubes were spun briefly at 10,000 rpm and moved to a heat block to incubate at 37 °C for 2 h. Fluorescence was detected using a miniPCR bio p51 fluorescence viewer and photographs were taken using a blue light transilluminator.
**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00503.

Instructions for setting up and running fresh CRISPR-Cas12 reactions (PDF)

List of nucleic acids used in the study, summary field-collected arthropods, cost analysis of CRISPR-Cas12 reactions, and expanded detail of crRNAs used in the study (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Michael C. Jewett — Chemistry of Life Processes Institute, Center for Synthetic Biology, and Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Robert H. Lurie Comprehensive Cancer Center and Simpson Querrey Institute, Northwestern University, Chicago, Illinois 60611, United States; orcid.org/0000-0003-2948-6211; Phone: 847-467-5007; Email: m-jewett@northwestern.edu

**Authors**

Grant A. Rybnicky — Chemistry of Life Processes Institute, Center for Synthetic Biology, and Interdisciplinary Biological Sciences Graduate Program, Northwestern University, Evanston, Illinois 60208, United States; orcid.org/0000-0002-0198-4596

Radeen A. Dixon — Centennial High School, Roswell, Georgia 30076, United States

Robert M. Kuhn — Centennial High School, Roswell, Georgia 30076, United States; Innovation Academy Fulton County Schools STEM Magnet High School, Alpharetta, Georgia 30009, United States

Ashly S. Karim — Chemistry of Life Processes Institute, Center for Synthetic Biology, and Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00503

**Author Contributions**


**Notes**

The authors declare the following competing financial interest(s): M.C.J. is a cofounder of SwiftScale Biologics, Stemloop, Inc., Design Pharmaceuticals, and Pearl Bio. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies.

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**ABBREVIATIONS**

PCR, polymerase chain reaction; CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR associated protein; FD-CC12, freeze-dried CRISPR-Cas12; STEM, science, technology, engineering, and mathematics; K-12, Kindergarten through Twelfth Grade; ssDNA, single-stranded DNA; FQ, fluorophore quencher; PAM, protospacer adjacent motif; crRNA, CRISPR RNA

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