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Article

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Title: BioBits™ Health: Classroom activities exploring engineering, biology, and human health with fluorescent readouts

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Abstract:

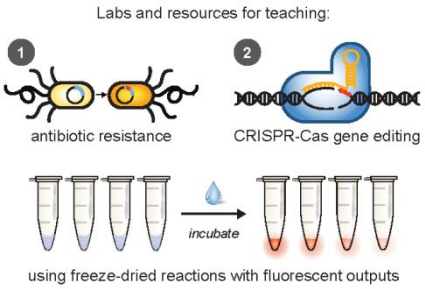
Recent advances in synthetic biology have resulted in biological technologies with the potential to reshape the way we understand and treat human disease. Educating students about the biology and ethics underpinning these technologies is critical to empower them to make informed future policy decisions regarding their use and to inspire the next generation of synthetic biologists. However, hands-on, educational activities that convey emerging synthetic biology topics can be difficult to implement due to the expensive equipment and expertise required to grow living cells. We present BioBits™ Health, an educational kit containing lab activities and supporting curricula for teaching antibiotic resistance mechanisms and CRISPR-Cas9 gene editing in high school classrooms. This kit links complex biological concepts to visual, fluorescent readouts in user-friendly freeze-dried cell-free reactions. BioBits™ Health represents a set of educational resources that promises to encourage teaching of cutting-edge, health-related synthetic biology topics in classrooms and other non-laboratory settings.

Keywords: cell-free protein synthesis, fluorescence, antibiotic resistance, CRISPR-Cas9, STEM curriculum, biological engineering education, synthetic biology

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Introduction:

Synthetic biological technologies promise to enable paradigm-shifting advances in human health and disease. To date, the field of synthetic biology has made meaningful progress toward biomanufacturing of antimicrobials and other medicines [1-4], cellular diagnostics and therapies [5-8], and human gene editing technologies [9-12]. These potentially transformative technologies offer rich opportunities for hands-on biology education, as they require students to confront real-world, interdisciplinary problems at the intersection of diverse disciplines including biology, chemistry, engineering, math, design, policy, and ethics. Such cross-cutting educational activities align closely with the objectives of K-12 STEM (Science, Technology, Engineering, and Mathematics) education and priorities identified by the National Academy of Engineering to enable students to apply, adapt, and connect fundamental principles across multiple disciplines [13]. Moreover, educating students about the science and ethics associated with biological technologies will empower them to make informed policy decisions in the future about how such technologies should be used and regulated.

However, due to the cutting-edge nature of these technologies, their translation into educational activities has been limited. In fact, hands-on resources for teaching molecular and synthetic biology have been limited in general, despite evidence that hands-on science activities have been shown to improve student understanding and academic performance [14]. Synthetic biology-based educational efforts such as BioBuilder Educational Foundation, the International Genetically Engineered Machines competition, Amino Labs, and The ODIN have made great strides toward integration of cutting-edge, hands-on biology research into classrooms. However, these resources rely on cell-based experimentation, which requires (i) expensive equipment and specialized expertise to grow and engineer cells [15, 16], (ii) extended instructor prep time and in-class time due to the time scales associated with cell growth [17], and (iii) compliance with biosafety regulations that can limit the ability to work with cells outside of a laboratory setting [18, 19].

To address these issues, we previously reported adaptation of synthetic biology lab activities into two portable, low-cost, and user-friendly educational kits: BioBits™ Bright [20] and BioBits™ Explorer [17]. These kits are made possible through the use of freeze-dried cell-free (FD-CF) technology, which harnesses an ensemble of catalytic components (*e.g.*, RNA polymerases, ribosomes, aminoacyl-tRNA synthetases, translation initiation and elongation factors, etc.) from cell lysates to synthesize proteins *in vitro* [21]. FD-CF reactions circumvent many of the biosafety and biocontainment regulations that exist for living cells because they use cell lysates, rather than intact cells, to synthesize proteins. Further, FD-CF reactions eliminate the need for specialized equipment or experimental expertise as they are shelf-stable [22] and can be activated simply by adding water and other desired inputs (*e.g.*, DNA, small molecules, enzymes) to a freeze-dried pellet of reagents. In addition, we showed that FD-CF reactions are robust teaching tools, evidenced by the fact that K-12 students and teachers running FD-CF reactions for the first time were able to obtain the intended experimental results [20]. Together, these features make BioBits™ kits a welcome complement to existing educational kits for classrooms or other non-laboratory settings. Despite these developments, activities that capture the recent advances and impacts of biological technologies on human health are still limited. If FD-CF technology could be used to develop educational modules about these technologies, it could significantly lower the barrier to entry for teaching emerging health-related topics.

Here, we describe BioBits™ Health, an educational kit that links complex biological experiments to fluorescent readouts in easy-to-use FD-CF reactions (**Figure 1**). We and others have demonstrated that fluorescent or chromoproteins are ideal instructional tools because a wide variety of these proteins have been developed which produce colors and/or fluorescence visible to the naked eye, enabling easy qualitative or semi-quantitative data collection without the need for expensive analytical equipment [20, 23]. To enable BioBits™ Health, we developed two educational modules designed for high school classrooms with wet lab activities that investigate antibiotic resistance and CRISPR-Cas9 gene editing technology. These labs use FD-CF reactions

with just a few simple inputs (water, DNA, small molecules, and enzymes). For both modules, we show that the fluorescent results from the various lab activities can be analyzed qualitatively by eye using a low-cost blue light imager [20], demonstrating the ability to run and assay reactions without sophisticated laboratory equipment. Each lab activity can be set up in a single 1-hour class period and produces results that can be analyzed as soon as the following day. The simplified nature of reaction setup and analysis minimizes both the amount of in-class time and out-of-class instructor preparation time required to incorporate hands-on lab activities, which have been cited as limiting factors for high school biology teachers [24, 25]. In addition, we show that the labs can be run successfully by Chicago high school students and teachers, representing our target audience. Though the primary goal of this work was to develop and validate the lab activities, we also worked with Chicago high school teachers to develop a set of curriculum and pre-lab lecture slides (**Curriculum S1-S8**; **File S1-S3**) to provide an example framework for teaching using the BioBits™ Health kit. Overall, the BioBits™ Health kit uniquely offers hands-on, cross-cutting educational activities with example supporting curricula that convey some of the most recent health-related advancements in synthetic biology in an interactive way. These resources promise to encourage hands-on education at the high school level about biological technologies for treating and understanding human health and disease.

Results and Discussion:

Module I: Investigating mechanisms of antibiotic resistance

Antibiotic resistance is a pressing global issue, projected to threaten up to 10 million lives per year by 2050 [26]. To help address this growing worldwide problem, it is important to educate students about how resistance occurs. To meet this educational need, we developed a classroom module that aims to teach (i) antibiotic mechanisms of action, (ii) mechanisms by which pathogenic bacteria can develop resistance, and (iii) how human behaviors can accelerate the development of resistance. As early as the 1960s, cell-free systems have been used to interrogate

the mechanisms of action of antibiotics that inhibit the ribosome [27, 28]. In this module, we show that it is possible to use synthesis of the orange fluorescent protein dTomato in FD-CF reactions as a reporter of antibiotic efficacy (**Figure 2a**). In the first activity, students use a panel of six antibiotic ribosome inhibitors, including large (50S) and small (30S) subunit inhibitors, to inhibit protein expression in FD-CF reactions (**Figure 2b**). Antibiotic-mediated inhibition of protein synthesis is visible by eye when reactions are viewed using the BioBits™ portable blue light imager [20] (**Figure 2c**). This activity is analogous to running a minimum inhibitory concentration assay, a common technique used to determine the potency of antibiotics against pathogenic bacteria [29], but without the complications and biosafety concerns of using live pathogenic cells. This first lab can be used as part of an inquiry-based classroom activity in which students are tasked with identifying the mechanisms of action for various classes of antibiotics (*e.g.*, **Curriculum S1**; **File S1**). Students can investigate ribosome inhibitors, which will inhibit protein synthesis, as well as other antibiotic classes, such as cell wall biosynthesis inhibitors, which have no effect on cell-free protein synthesis (**Figure 2b**). In order to successfully identify the mechanism of action of the latter class, students will have to demonstrate understanding of cellular architecture and which cellular components are present in the cell-free system (**Curriculum S1**). This particular curriculum example further offers the opportunity to introduce students to image analysis and biological statistics using ImageJ, facilitated by our step-by-step ImageJ tutorial for quantifying fluorescence in cell-free reactions (**File S2**). These represent important analytical skills for students interested in pursuing careers in biology-related fields.

If class time allows, this activity can be extended with an additional lab that guides students through an investigation of two potential mechanisms of antibiotic resistance: horizontal gene transfer and genetic mutation. To mimic horizontal gene transfer (**Figure 3a**), we show that FD-CF reactions can be used to express the aminoglycoside O-phosphotransferase (NeoR) and streptomycin 3'-adenylytransferase (AadA) enzymes that confer kanamycin and streptomycin resistance, respectively (**Figure S1**). When added to fresh FD-CF reactions expressing dTomato,

we observe that these pre-expressed enzymes can "rescue" the FD-CF reactions from the effects of their target antibiotic (**Figure 3b**), and that these effects are visible by eye compared to reactions lacking the enzymes using our portable blue light imager (**Figure 3c**). Similarly, to explore the idea of genetic mutation (**Figure 3d**), we made lysate from BL21 Star™ (DE3) cells in which we introduced an R86S mutation in the *rpsL* gene, which codes for ribosomal protein S12. This mutation has been previously reported to result in resistance to streptomycin, likely by preventing the antibiotic from binding to the ribosome [30]. When the mutant cell extract is used in FD-CF reactions, dTomato expression is observed even in the highest concentrations of streptomycin tested (**Figure 3e**), and the level of expression can again be easily distinguished from reactions with wild-type lysate using the BioBits™ imager (**Figure 3f**). We also show that similar levels of protein expression are achieved in both wild-type and resistant reactions assembled by high school students and teachers (**Figure S2a**). We developed a protocol with pre- and post-lab questions for implementing this lab in high school classrooms (**Curriculum S2**; **File S1**), with the option for students to quantify their results with ImageJ (**File S2**) and carry out a statistical analysis of their results. In sum, this module offers rich, inquiry-guided educational experiences that can be used to meet educational standards for high school biology (**Table S1**) by exploring antibiotic mechanisms of action, demonstrating multiple ways in which antibiotic resistance can be acquired, and offering opportunities to expose students to biological statistics and real-world data analysis methods. Further, understanding mechanisms of antibiotic resistance opens 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, as well as broader policy decisions about antibiotic use and misuse (**Curriculum S2**; **File S1**).

Module II: Fundamentals of CRISPR-Cas9 gene editing

CRISPR-Cas systems for editing DNA promise to reshape the way we understand and treat human diseases and genetic disorders [31, 32]. For example, researchers recently showed that a CRISPR-based therapy could restore function of the protein dystrophin in mouse [33] and canine [34] models of Duchenne muscular dystrophy, and this therapy is now advancing toward human clinical trials. With gene editing therapies starting to make their way into the clinic and garnering broad public interest [35, 36], especially in light of the recent controversial report of CRISPR editing of humans in China [4], it is important to educate students about the fundamentals of gene editing in order to help them make informed decisions about the politics and ethics surrounding CRISPR and other gene editing technologies. To support this goal, we developed a classroom module that requires students to (i) demonstrate understanding of the biological mechanism of CRISPR-Cas9 gene editing and (ii) consider the ethical implications of using CRISPR or other gene editing technologies to address societal problems.

In this module, we link activity of the *Streptococcus pyogenes* Cas9 nuclease to a fluorescent readout through the design of a synthetic guide RNA (gRNA) targeting the red fluorescent protein mRFP1, enabling straightforward investigation of the mechanism of action of this CRISPR-Cas9 system. First, we showed that full-length *Streptococcus pyogenes* Cas9 nuclease could be expressed in FD-CF reactions (**Figure S3**). Next, we designed a gRNA construct to target a 20 base pair sequence in the first 50 nucleotides of the *mRFP1* gene (**Figure S4a**) and adjacent to an NGG protospacer adjacent motif (PAM) site that is required for *S. pyogenes* Cas9 activity (**Figure 4a**; **Figure S4b**). To test whether our anti-mRFP1 gRNA could effectively repress mRFP1 expression and fluorescence, we rehydrated fresh FD-CF reactions encoding mRFP1 with gRNA plasmid, pre-expressed Cas9 nuclease, or both gRNA and pre-expressed Cas9. When both gRNA and Cas9 are present, we observe an approximately 4-fold reduction in fluorescent signal, indicative of Cas9 cleavage of the mRFP1 DNA template and silencing of fluorescent protein synthesis (**Figure 4b**). Similar levels of repression are observed in reactions assembled by high school students and teachers (**Figure S2b**). This experiment

represents a simple lab activity that could be paired with a sorting and classification exercise like the one we developed (**Curriculum S3**) to help students understand the functions of the necessary components for Cas9 activity (*i.e.*, nuclease, gRNA, target, PAM site).

Having demonstrated the activity of our gRNA construct against mRFP1, we designed a second lab activity that challenges students to discover which sequence of DNA this gRNA targets by screening its activity against a set of six fluorescent proteins. We measured production of each of the six fluorescent proteins in reactions containing the target DNA, pre-expressed Cas9, and with or without gRNA plasmid. With the exception of its target, mRFP1, our gRNA construct did not greatly impact expression of the other fluorescent proteins, although our results suggest that there may be a low level of off-target activity against mOrange and YPet (**Figure 4c**). This is not surprising given that the *morange* and *ypet* gene sequences have homologies of ~79% and ~44% to *mrfp1*, respectively. This off-target activity offers the opportunity to discuss how off-target effects are possible and need to be thoroughly characterized to ensure the safety of using CRISPR technologies for clinical applications. Despite this low amount of off-target activity, repression of mRFP1 expression could be observed by eye when the reactions were imaged with our portable blue light imager, while fluorescence of the other target proteins was not visibly affected (**Figure 4d**). We developed pre-lab slides as well as a protocol with pre- and post-lab questions for running this lab in a high school classroom (**File S3**; **Curriculum S4**). Like the labs in Module I, this lab activity also offers the option for students to quantify their results with ImageJ (**File S2**) and carry out a statistical analysis of their results. This activity could open the door to additional exercises that investigate how gene editing technologies can be used to treat human diseases and how they compare to existing treatments. For example, we developed an assignment to help students investigate a variety of gene editing technologies (*i.e.*, CRISPR, zinc finger nucleases, etc.) for treating Huntington's disease (**Curriculum S5**), as well as a process oriented guided inquiry learning (POGIL) activity that explores a variety of possible solutions to treat a patient with sickle cell anemia (**Curriculum S6**). Finally, all of the activities described will

prepare students for independent research projects in which they can investigate a problem of their choice that could be solved with CRISPR technology (**Curriculum S7**), and consider the ethics involved in such interventions (**Curriculum S8**). Overall, this module offers diverse, student-guided educational activities that can be used to meet educational standards for high school biology (**Table S1**) and that are centered on an important, cutting-edge topic in synthetic biology. These activities have the potential to empower students with the knowledge to make informed ethical and policy decisions about how CRISPR-Cas gene editing technology could be used and regulated to yield the most benefit for society.

Conclusion:

We present here the BioBits™ Health educational kit and an accompanying collection of curriculum and data for teaching health-related synthetic biology topics in high school classrooms. To enable BioBits™ Health, we designed wet lab activities with fluorescent readouts that can be run in FD-CF reactions with just a few simple inputs. These activities are organized into two lab modules that investigate antibiotic resistance and CRISPR-Cas9 gene editing technology. For both modules, we show that experimental results can be analyzed qualitatively by eye and successfully reproduced by high school students and teachers using the kit for the first time (**Figure S2**), demonstrating the utility of these resources for use by untrained operators without sophisticated laboratory equipment. Each lab activity can be set up in a single 1-hour class period and results can be analyzed as soon as the following day, limiting the class time required to run hands-on activities. Finally, we developed a set of example supporting curriculum for facile integration of these activities into high school STEM classrooms. In pre- and post-assessments collected while beta-testing the BioBits™ Health labs with high school students, we found that students reported statistically significant increased confidence in their understanding of the mechanisms of antibiotic resistance and CRISPR-Cas genome editing, as well as increases in their self-identification as engineers after running BioBits™ Health labs (**Figure S5**). While these

data suggest that positive educational outcomes are possible using the BioBits™ Health resources, longitudinal studies will be needed to more fully assess educational benefits. It should also be noted that recent work has described educational activities for teaching CRISPR genome editing using cell-free systems [37], highlighting broad interest from educators in integrating this topic into educational curriculum. However, these resources are designed for undergraduate students, rely on expensive commercial cell-free kits, and require access to expensive laboratory equipment such as temperature-controlled spectrophotometers and -80°C freezers. Thus, our work meets a need for economical and accessible hands-on biology activities designed to teach cutting-edge health-related topics in high school classrooms.

Future development of the BioBits™ Health kit will involve scaling its production, further developing and beta-testing supporting curriculum, and implementation in high school classrooms where both short- and long-term educational impact can be quantitatively measured. We recently launched a website (www.mybiobits.org) that we plan to use as the online home for an open-source community centered around the BioBits™ kits. The website currently houses a curriculum database, where the BioBits™ curricula can be freely accessed. In the future, we plan to provide information on the website about how (i) educators can order kits or participate in pilot programs as these become available, (ii) kit users can share data, and (iii) researchers and educators can upload new lab activities or curriculum pieces that use FD-CF reactions as the community of BioBits™ users and developers grows. We also think there are many exciting future directions for health-related educational modules using FD-CF technology. In particular, RNA toehold switches [17, 38] or CRISPR-based RNA [39] or DNA [40] sensing technologies could be adapted to detect infectious agents (viruses, pathogenic bacteria) in environmental samples, or cancer-causing mutations in mock patient samples. Implementing such tools in FD-CF reactions could support classroom activities on infectious disease and cancer, respectively.

We have purposely designed the BioBits™ Health kits to be economically accessible, priced at less than $150 per 30-person classroom (**Table S2**) and within the range of prices

teachers reported being willing to pay for such a kit (**Figure S6**). This includes a complete set of reagents to run all of the labs described here, as well as the custom, low-cost blue light imager and incubator that we developed as part of the BioBits™ Bright kit [20]. We also show that FD-CF reactions are stable for 2 weeks at room temperature and at least 6 months in a commercial refrigerator using packaging that could easily and economically be replicated for shipping to classrooms (**Figure S7**). This allows for ambient temperature shipping and short-term storage of kits, as well as longer term refrigerated storage if necessary. Due to the highly portable, cost-effective, and user-friendly nature of the reagents and lab activities, the BioBits™ Health kit has utility both inside and outside of a formal classroom or laboratory setting. We anticipate that these resources will increase access to cell-free technologies, enhance basic biology education through integration of cutting-edge health topics, and help the next generation of students make informed decisions about new and transformative biological technologies.

Materials and Methods:

Bacterial strains and plasmids

E. coli NEB® 5-alpha (NEB) was used in plasmid cloning transformations and for plasmid preparation. Wild-type or *rpsL* R86S *E. coli* BL21 Star™ (DE3) cells (Thermo Fisher Scientific) were used for preparation of cell-free extracts. Gibson assembly was used for seamless construction of plasmids used in this study (**Table S3**). For cloning, the pJL1 vector (Addgene 69496) was digested using restriction enzymes NdeI and SalI-HF® (NEB). Each gene was amplified *via* PCR using Phusion® High-Fidelity DNA polymerase (NEB) with forward and reverse primers designed with the NEBuilder® Assembly Tool (nebuilder.neb.com) and purchased from IDT. The DNA construct encoding the anti-mRFP1 gRNA was also purchased from IDT. PCR products were gel extracted using an EZNA Gel Extraction Kit (Omega Bio-Tek), mixed with Gibson assembly reagents and incubated at 50°C for 1 hour. Plasmid DNA from the Gibson assembly reactions were transformed into *E. coli* NEB 5-alpha cells and circularized constructs

were selected on LB-agar supplemented with kanamycin at 50 μg mL^{-1} (Sigma). Sequence-verified clones were purified using an EZNA Plasmid Midi Kit (Omega Bio-Tek) for use in FD-CF reactions.

Construction of rpsL R86S mutant strain

The *rpsL* R86S strain of BL21 Star™ (DE3) was generated using a mutagenic oligonucleotide that was designed to introduce the C256A mutation into the *rpsL* gene via a single-cycle of MAGE [41]. Briefly, BL21 Star™ (DE3) cells were transformed with the pKD46 plasmid encoding the lambda Red recombinase system [42]. Transformants were grown to an OD_{600} of 0.5-0.7 in 200 mL LB-Lennox media (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} NaCl) with 50 μg mL^{-1} carbenicillin at 30°C, harvested and washed three times with 40 mL ice-cold 10% glycerol to make them electrocompetent, and resuspended in a final volume of 200 μL 10% glycerol. Electrocompetent cells were combined with the *rpsL* MAGE oligonucleotide for a final concentration of 37.5 mM DNA, transformed via electroporation, and plated on LB-agar with 50 μg mL^{-1} streptomycin for selection of resistant colonies. Plates were grown at 37°C to cure cells of the pKD46 plasmid. Colonies that grew on streptomycin were confirmed to have the desired mutation via colony PCR and DNA sequencing and to have lost the pKD46 plasmid via replica plating on LB agar plates with carbenicillin and streptomycin. All primers used for construction and validation of this strain are listed in **Table S4**.

Cell-free extract preparation

Cell-free extract was prepared by sonication as previously reported [43]. Briefly, *E. coli* BL21 Star™ (DE3) (Thermo Fisher Scientific) cells were grown in 2xYTPG media (5 g L^{-1} NaCl, 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 7 g L^{-1} potassium phosphate dibasic, 3 g L^{-1} potassium phosphate monobasic, 18 g L^{-1} glucose) pH 7.2 at 37°C. BL21 Star™ (DE3) *rpsL* R86S cells were grown in 2xYTPG media supplemented with 50 μg mL^{-1} streptomycin. T7 polymerase expression was

induced at an OD_{600} of 0.6-0.8 with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cells were grown at 37°C to a final OD_{600} of 3.0, at which point cells were pelleted by centrifugation at 5,000xg for 15 min at 4°C. Cell pellets were then washed three times with cold S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, and 60 mM potassium acetate) and pelleted at 5,000xg for 10 min at 4°C. After the final wash, cells were pelleted at 7,000xg for 10 min at 4°C, weighed, flash frozen in liquid nitrogen, and stored at -80°C. For lysis, cell pellets were suspended in 1 mL of S30 buffer per 1 g of wet cell mass. The cell-buffer suspension was transferred into 1.5 mL microcentrifuge tube and placed in an ice-water bath to minimize heat damage during sonication. The cells were lysed using a Q125 Sonicator (Qsonica) with 3.175 mm diameter probe at 20 kHz and 50% amplitude. The input energy was monitored and 640 J was used to lyse 1 mL of suspended cells. The lysate was then centrifuged once at 12,000xg at 4°C for 10 min. Cell extract was aliquoted, flash-frozen on liquid nitrogen, and stored at -80°C. Alternatively, for classroom settings where it is not practical to generate or obtain FD-CF reactions, similar cell-free systems are available commercially from companies such as Promega® (L1130).

Cell-free protein synthesis (CFPS)

FD-CF reactions were carried out in PCR tubes (5 µL reactions) or 1.5 mL microcentrifuge tubes (15 µL reactions). The CFPS reaction mixture consists of the following components: 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 34.0 µg mL^{-1} L-5-formyl-5, 6, 7, 8-tetrahydrofolic acid (folinic acid); 170.0 µg mL^{-1} of *E. coli* tRNA mixture; 130 mM potassium glutamate; 10 mM ammonium glutamate; 8 mM magnesium glutamate; 2 mM each of the 20 amino acids; 0.4 mM nicotinamide adenine dinucleotide (NAD); 0.27 mM coenzyme-A (CoA); 1.5 mM spermidine; 1 mM putrescine; 4 mM sodium oxalate; 33 mM phosphoenolpyruvate (PEP); 57 mM HEPES; 13.3 µg mL^{-1} plasmid; and 27% v/v of cell extract [44]. If pre-expressed antibiotic resistance enzymes or Cas9 nuclease were also added, 1 µL of the soluble fraction

of CFPS reactions encoding these enzymes was used along with water and plasmid to rehydrate fresh FD-CF reactions. For CRISPR reactions, reactions contained all of the ingredients above, except with 2 μg mL^{-1} target plasmid (mRFP1 or other fluorescent protein constructs). If anti-mRFP1 gRNA plasmid was also added, it was supplied at 6.66 μg mL^{-1}. For quantification of fluorescent protein yields *via* radioactive leucine incorporation, 10 μM of L-^{14}C-leucine (11.1 GBq $mmol^{-1}$, PerkinElmer) was added to the CFPS mixture. All reagents required to make cell-free extracts and assemble FD-CF reactions are listed in **Table S5**.

Lyophilization of cell-free reactions

FD-CF reactions were prepared according to the recipe above, but without plasmid added. CFPS reactions and plasmids were separately lyophilized using a VirTis BenchTop Pro lyophilizer (SP Scientific) at 100 mTorr and -80°C overnight or until fully freeze-dried. Following lyophilization, plasmids were rehydrated with nuclease-free water (Ambion) and added to FD-CF reaction pellets at a final concentration of 13.3 μg mL^{-1} unless otherwise noted. FD-CF reactions were carried out at 30°C for 20 hours after rehydration unless otherwise noted. In a classroom setting, reactions can be incubated in the BioBits™ portable incubator [20] at 30°C or in a 30°C water bath in an insulated container (Styrofoam, plastic cooler, etc.) for 20 hours or at room temperature for 24-48 hours.

Quantification of in vitro synthesized protein

Active-full length protein synthesis was measured continuously via fluorescence using a CFX96 Touch Real-Time PCR Detection System (BioRad). If fluorescence saturated the RT-PCR detector, endpoint fluorescence was measured in 96 well half-area black plates (CoStar 3694; Corning Incorporated) using a Synergy2 plate reader (BioTek). Excitation and emission wavelengths used to measure fluorescence of each protein construct were as follows: mCherry, eforRed, mRFP1, dTomato ex 560-590 nm, em 610-650 nm; mOrange ex 515-535 nm, em 560-

580 nm; YPet, sfGFP, mTFP1, CyPet, Aquamarine, mTagBFP2, mKalama1, eBFP2 ex 450-490, em 510-530 nm. Fluorescence units were converted to concentrations using a standard curve as previously described [20, 45]. For assessing yields of the antibiotic resistance enzymes and the Cas9 nuclease, reaction soluble fractions were analyzed directly by incorporation of ^{14}C-leucine into trichloroacetic acid (TCA)-precipitable radioactivity using a liquid scintillation counter as described previously [46]. The soluble fractions were also run on a Coomassie-stained SDS-PAGE gel and exposed by autoradiography. Autoradiographs were imaged with a Typhoon 7000 (GE Healthcare Life Sciences).

For quantification without a spectrophotometer, reactions can be semi-quantitatively analyzed *via* imaging using the BioBits™ 8-well blue light imager [20] and subsequent fluorescence analysis in ImageJ, a free image analysis program, using our step-by-step ImageJ tutorial (**File S2**). Images of FD-CF reactions were taken with a DSLR and arranged in Adobe Illustrator. Protein production can also be qualitatively assessed with the naked eye under white light or blue or black light using our portable blue light imager or others (*e.g.,* Bio-Rad® UV pen lights #1660530EDU, Walmart® black light bulb with fixture #552707607, Home Science Tools® portable UV black light #OP-BLKLITE, miniPCR blueBox™ transilluminator #QP-1700-01).

Statistical analysis

Statistical parameters and analytical techniques including the definitions and values of N, standard deviations, and/or average errors are reported in the Figures and corresponding Figure Legends.

Author Contributions:

J.C.S. designed research, performed research, analyzed data, and wrote the paper. A.H. designed research, designed curriculum, and edited the paper. J.F., T.M., S.M., F.R., and M.W.,

performed research and designed curriculum. R.S.D., K.J.H., B.H., R.J., R.K., V.K., and W.W. performed research. G.A.R. and P.Q.N. aided in research design, designed curriculum, and edited the paper. T.M. aided in curriculum design. M.C.J. directed research, analyzed data, and wrote the paper.

Conflict of Interest:

The authors declare no competing interests.

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Research, Defense Threat Reduction Agency, or the U.S. Government. All plasmid constructs developed in this study are deposited on Addgene (constructs 117048, 117050, 117051, 117052). Reagents available by request from M.C.J.

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Figures and Figure Legends:

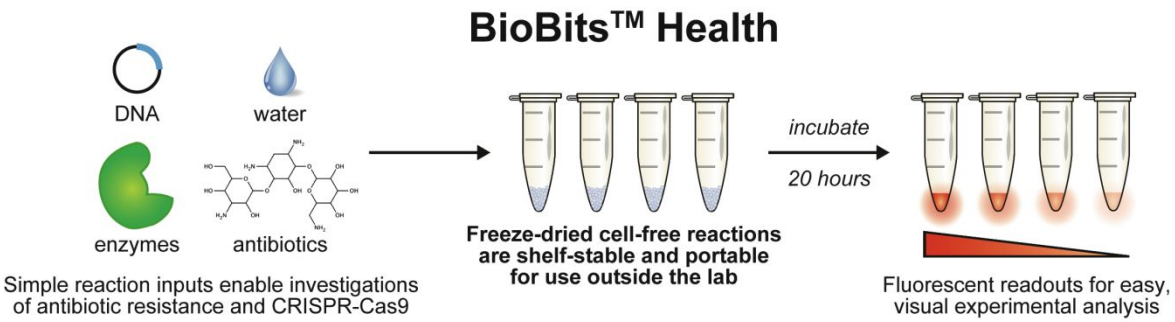


Figure 1. BioBits™ Heath is a set of classroom activities and curriculum that links cutting-edge, health-related biology experiments to visual, fluorescent readouts. Using freeze-dried cell-free (FD-CF) reactions with simple inputs (DNA, antibiotics, enzymes, and water), BioBits™ Health labs enable hands-on, inquiry-guided educational activities focused on antibiotic resistance and CRISPR-Cas9 genome engineering. FD-CF reactions are shelf-stable and can be run and analyzed without expensive equipment, making them well suited for use in classrooms or other non-laboratory settings.

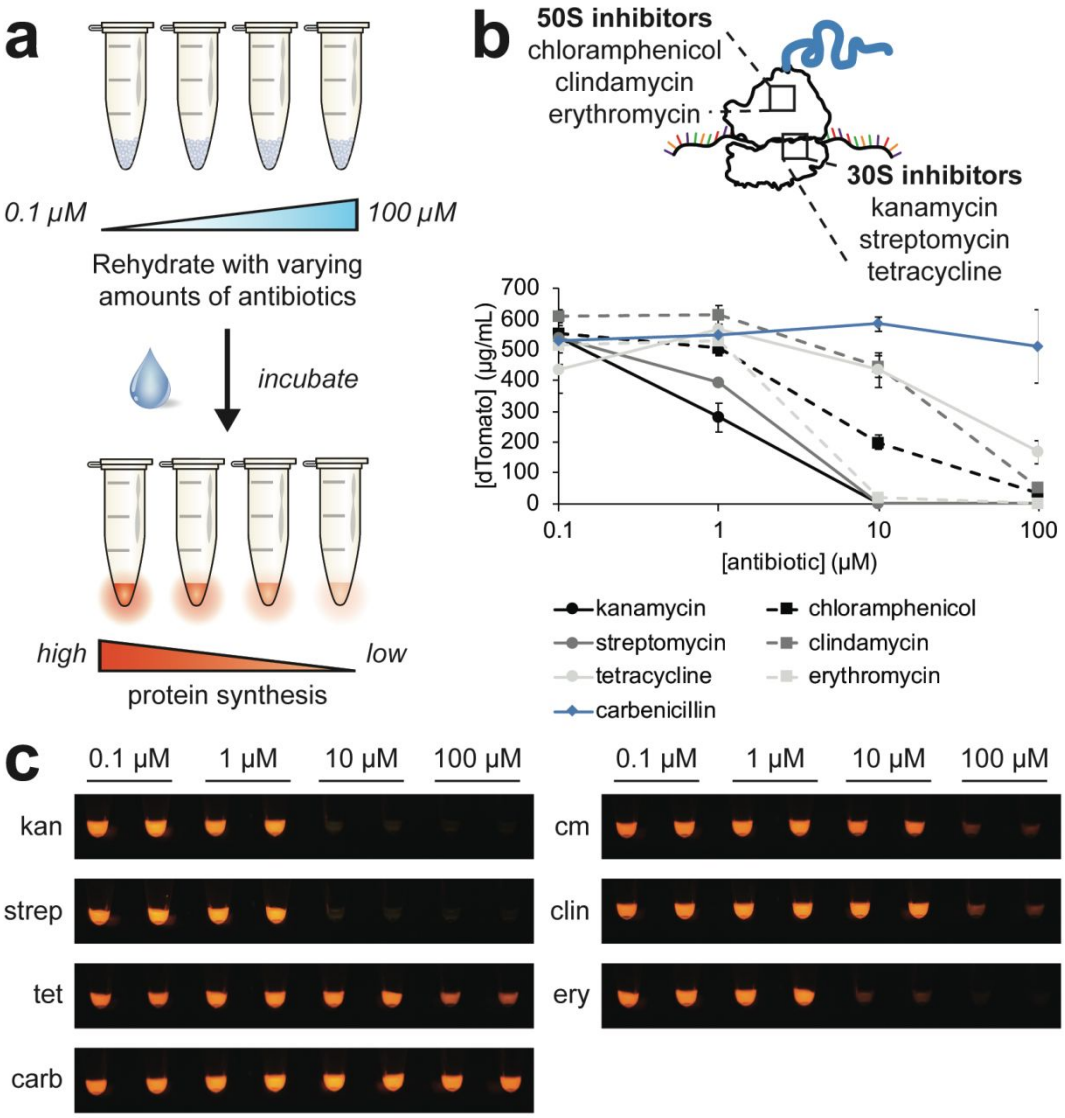


Figure 2. Fluorescent protein expression in FD-CF reactions can be used to assay antibiotic potency for a variety of antibiotic ribosome inhibitors. (**a**) By adding varying amounts of antibiotic ribosome inhibitors to FD-CF reactions expressing a fluorescent protein, fluorescence can be used as a reporter of antibiotic efficacy. (**b**) Cell-free protein synthesis of dTomato was carried out in FD-CF reactions containing 0.1-100 μM antibiotic. Antibiotics tested included the 50S inhibitors chloramphenicol (cm), clindamycin (clin), and erythromycin (ery) and the 30S inhibitors kanamycin (kan), streptomycin (strep), and tetracycline (tet) (**top**). For all antibiotics tested, except for the cell wall biosynthesis inhibitor carbenicillin (carb), protein synthesis was suppressed with increasing levels of antibiotics (**bottom**). Values represent averages and error bars represent standard deviations of $n \geq 3$ biological replicates. (**c**) When representative FD-CF reactions from (**b**) are imaged using a low-cost blue-light imager, inhibition of protein synthesis can be observed by eye.

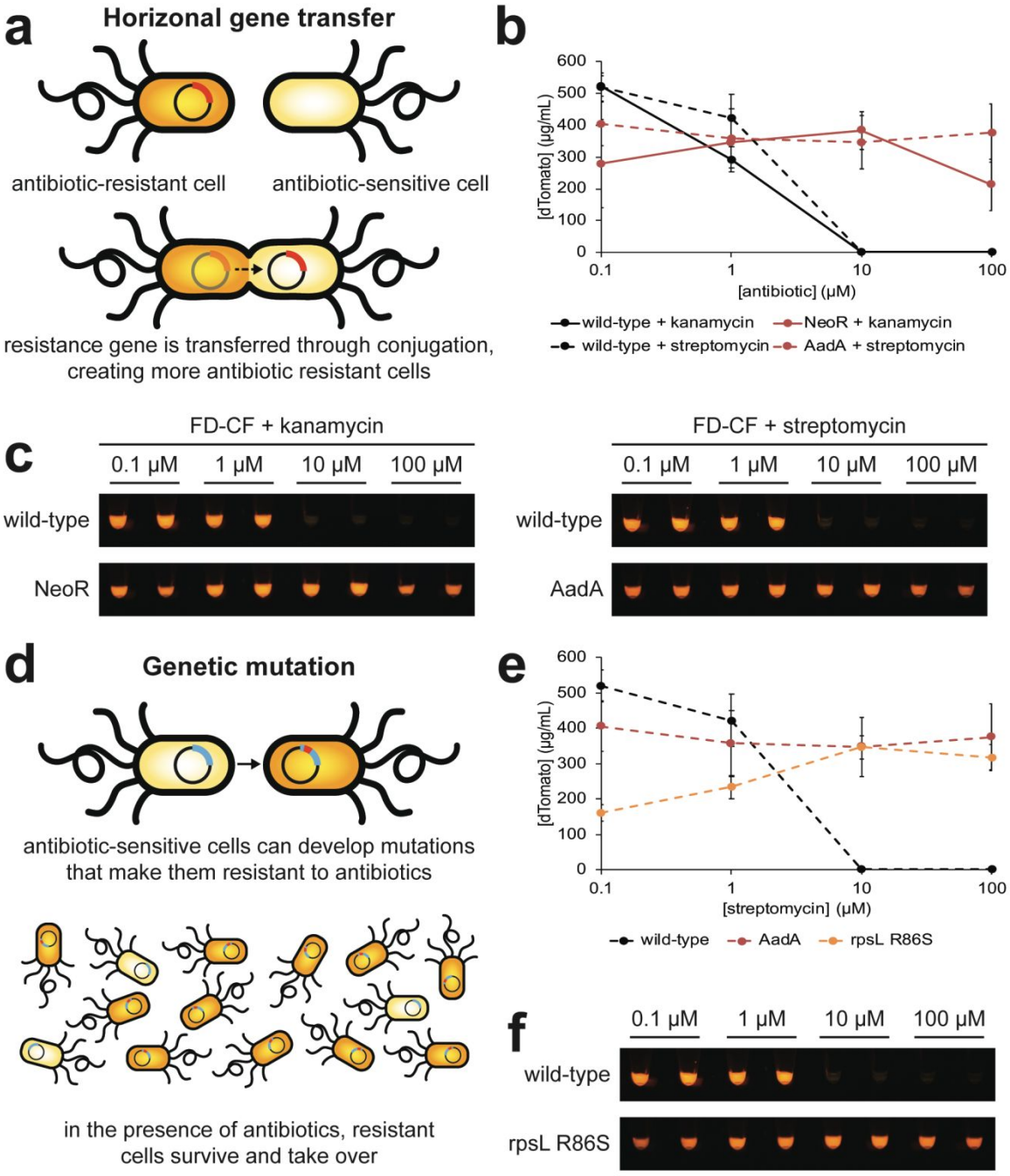


Figure 3. Demonstrating mechanisms of antibiotic resistance in FD-CF reactions. (**a**) To demonstrate the concept of horizontal gene transfer, kanamycin (*neoR*) and streptomycin (*aadA*) resistance genes were pre-expressed in FD-CF reactions for 20 hours at 30°C (**Figure S1**) and 1 μL of the soluble fraction was added to fresh FD-CF reactions encoding dTomato and containing 0.1-100 μM of either kanamycin or streptomycin. (**b**) Following cell-free protein synthesis for 20 hours at 30°C, reactions containing the resistance enzymes retain the ability to synthesize protein, even in the highest concentrations of antibiotics tested. Values represent averages and error bars represent standard deviations of $n \geq 3$ biological replicates. (**c**) When representative FD-CF reactions from (**b**) are imaged using a low-cost blue-light imager, differences in results using wild-type and resistant reactions can be distinguished by eye. (**d**) To illustrate genetic mutation and

selection, we generated lysate from cells with an R86S mutation in the *rpsL* gene, which are resistant to streptomycin. (**e**) FD-CF reactions with the resistant lysates also retain the ability to synthesize dTomato in the presence of up to 100 μM streptomycin. Values represent averages and error bars represent standard deviations of $n \geq 3$ biological replicates. (**f**) Images of representative FD-CF reactions from (**e**) imaged using a low-cost blue-light imager show that the difference between resistant and wild-type reactions can be observed qualitatively with the naked eye.

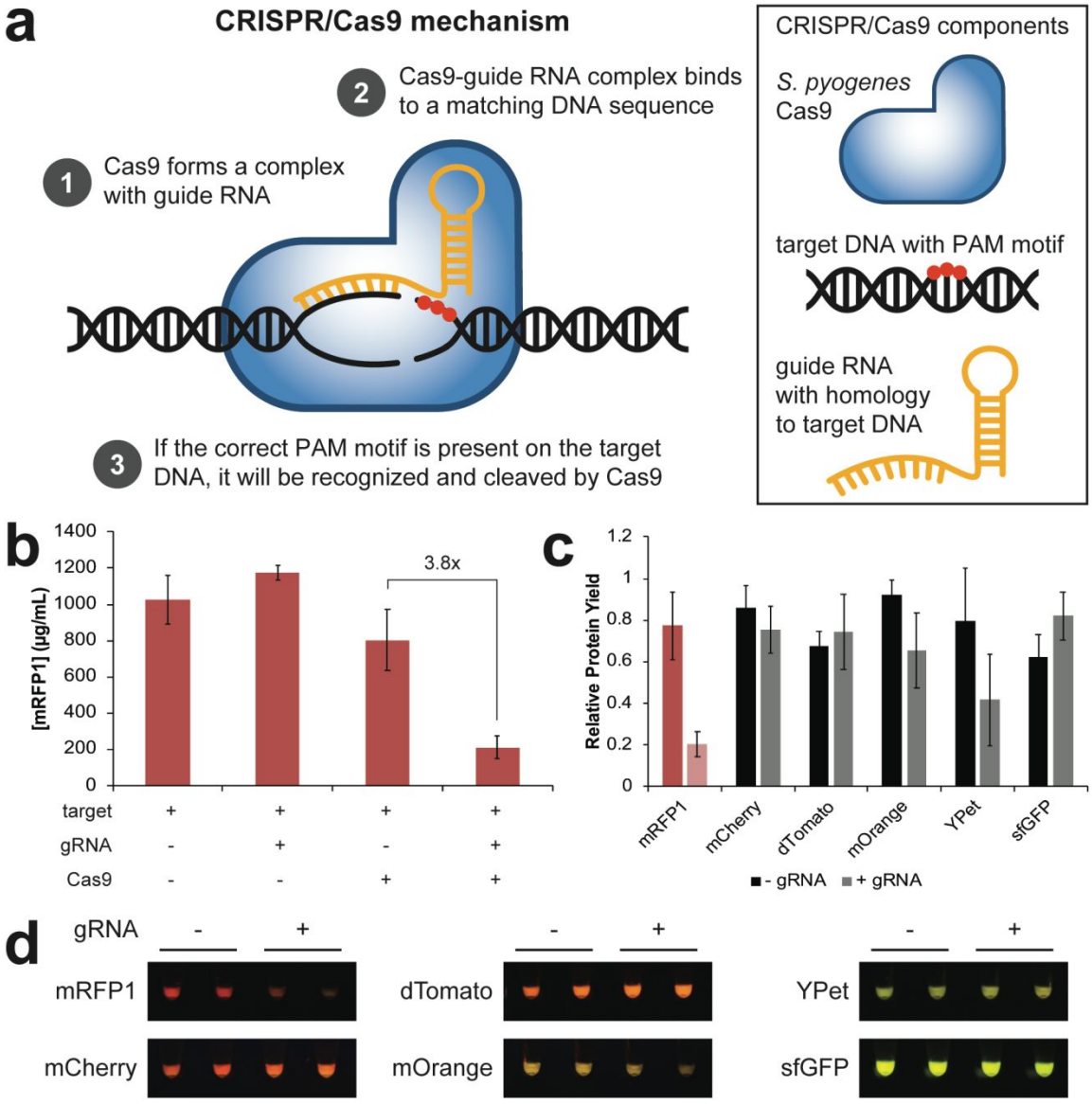


Figure 4. Interrogating the mechanism of a CRIPSR-Cas9 nuclease system using fluorescence. (**a**) To illustrate the mechanism of action of a CRISPR-Cas9 system (**left**) and outline the components required for Cas9 nuclease activity (**right**), we designed a synthetic guide RNA construct that targets the gene for the red fluorescent protein. This makes it possible to use repression of mRFP1 fluorescence as a reporter of Cas9 activity in FD-CF reactions. (**b**) To test whether our anti-mRFP1 gRNA construct could effectively repress mRFP1 expression, we added gRNA plasmid, Cas9, or both gRNA plasmid and Cas9 to FD-CF reactions expressing mRFP1.

The Cas9 nuclease from *S. pyogenes* was pre-expressed in FD-CF reactions for 20 hours at 30°C (**Figure S3**) and 1 µL of the soluble fraction was added to the mRFP1 reactions. When both gRNA plasmid and Cas9 are added to the reaction, ~4 fold repression of mRFP1 fluorescence was observed after incubation for 20 hours at 30°C. Values represent averages and error bars represent standard deviations of $n \geq 3$ biological replicates. (**c**) We next tested the orthogonality of our anti-mRFP1 gRNA construct by screening for Cas9 activity against a set of five other fluorescent proteins. Reactions contained template for the fluorescent protein of interest and pre-expressed Cas9, with or without anti-mRFP1 gRNA plasmid. Expression of the other fluorescent proteins tested is not significantly repressed by the anti-mRFP1 gRNA. Values represent averages and error bars represent standard deviations of $n \geq 3$ biological replicates. (**d**) Blue light images of representative FD-CF reactions from (**c**) show that repression of mRFP1 can be observed with the naked eye, while fluorescence of the other protein targets is retained in the presence of gRNA.