Accessible and easy-to-use educational tools to teach molecular and synthetic biology using freeze-dried, cell-free technology

By

Ally Huang

B.S. Biomedical Engineering
Johns Hopkins University, 2012

Submitted to the Department of Biological Engineering in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Biological Engineering
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2019

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ABSTRACT

Hands-on demonstrations greatly enhance the teaching of STEM concepts and foster engagement and exploration in the sciences. While numerous chemistry and physics classroom demonstrations exist, few biology demonstrations are practical and accessible due to the challenges and concerns of growing living cells in classrooms. Here I introduce a platform to develop hands-on molecular and synthetic biology educational activities based on easy-to-use, shelf-stable, freeze-dried, cell-free (FD-CF) reactions, which are simply activated by water. By using fluorescent proteins as a visual output, I created a variety of engaging modules using this platform that can teach the central dogma of biology, how certain cellular functions work, and other basic molecular biology topics that are otherwise difficult to easily teach in a hands-on manner. By expanding the platform to other non-visual outputs (such as smell or touch), as well as further incorporating components, such as RNA switches, I also developed modules that can teach more advanced biology topics, such as biochemistry, biomaterials, and synthetic biology, as well as basic laboratory skills such as pipetting, experimental design, and the scientific method. Pilot testing of a prototype kit based on these elements were tested in classrooms across the country and initial results suggest that the activities are accessible, easy to use, educational, and engaging for high school students. Overall, the platform introduces low-cost, user-friendly, and hands-on activities that can be used in classrooms to improve the quality of biology education and open the door for student-driven, independent explorations in the life sciences.

Thesis Supervisor: James. J. Collins

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Acknowledgements

I started my Ph.D. the fall of 2014 as a bright-eyed, optimistic young scientist who thought she wanted to do antibody engineering for cancer therapeutics and now am (hopefully) ending it as a more realistic (although still optimistic) experienced scientist creating low-cost education kits to teach molecular and synthetic biology. This journey has been filled with unexpected twists and turns but I would not change anything – every challenge and obstacle has been a valuable learning experience. And I certainly would not be here without the support of everyone in my life, both in lab and out.

My interest in biology first stated back in tenth grade, where my high school biology teacher, Kevin Engstrom opened my eyes to the wonderful and intricate world of molecular biology. My speech and debate coach, Todd Hering, taught me how to stand up and talk about anything without tripping over my words. When I got to college, my undergraduate lab advisor, Bill Matsui, took the time to mentor me about how to properly design experiments and interpret my results. Zeshaan Rasheed, the post-doc I worked under in the lab, (who somehow kept me on after I broke a serological pipette on my very first day in lab) taught me the basics of molecular biology and cell culture. At my post-graduate job at Genentech, all my supervisors – Aaron Miller, Kara Calhoun, Ambrose Williams, and Bob Kelley – convinced me it would be worth giving up my job to go back to graduate school (and they were right!).

Here at MIT, I was overwhelmed with the amount of support available. To Jim Collins – thank you for taking me on as a lost third-year graduate student and giving me the opportunity to carry out this unconventional thesis project. Thank you for pulling me back from the minute details of the work and helping me see the big picture, as well as providing invaluable advice and mentorship about the business and career side of things. To Doug Lauffenburger, Forest White, and Mark Bathe, for guiding me through the toughest point of my Ph.D. and helping me navigate the thorny subject of switching labs halfway through my graduate career. To the entire Collins lab for being a friendly and welcoming environment to work in. Peter Nguyen – your dedication and work ethic on your projects both inspire and terrify me and working on the education project with you has been such a rewarding experience. Also, I can only wish to match your Illustrator skills one day. Melissa Takahashi – thank you for teaching me everything you know about toeholds and cell-free, helping me grind out figures for education papers, and serving as a role model of how one should precisely organize experiments in lab. Aaron Dy – for being a friend, classmate, and labmate and willing to take on the more weird side of my project, where you sequenced strawberries and squished up bananas and kiwis in the name of science education. Nina Donghia – for teaching me everything about how to run freeze-dried reactions and proving that scientists can be rocker cool as well. Tom Ferrante – for being the amazing engineering wizard you are, designing all the equipment for the project, and teaching me how to solder, saw, and drill without losing any of my fingers. To Keith Pardee – even though we never met in person, your advice over email and the phone – not to mention starting this whole freeze-dried, cell-free branch in the lab and all the

extremely helpful papers that I constantly cite in this work – has been the reason that this project can even exist. To Chad Johnston, Nichole Daringer, Atti English, and Raph Gayet for all the great and amusing conversation we've had in the office. To my fellow Collins lab grad students, past and present – Dana Braff, Ning Mao, Sarah Bening, Bernie Cervantes, Ian Andrews, Nico Angenent-Mari, Luis Soenksen, Erika Zheng – for always having great advice in subgroup meetings and fun conversations at lunch. To my awesome team of undergraduate and high school research assistants, Sara Corchado, Audrey Ory, and Abby Mauermann, for quickly learning and carrying out experiments for me. To Michelle Morrison and Kostis Psimopoulos for being 100% responsible for keeping the lab at all functional and for keeping us all well-fed and happy. And to everyone else in the lab – at IMES, at the Broad, and at the Wyss – being always being friendly, helpful, and supportive and making the Collins lab an amazing place to work at.

To our collaborators at the Jewett lab at Northwestern – Jess Stark, it is a testament to this project that when we found out we were working on the same project, we decided to work together rather than race to scoop each other. Thank you for teaching me how to (properly) make cell-free extracts and for being such a dedicated and passionate member of this project (and for introducing me to the Bachelor, which may have been a mistake…). To Mike Jewett – for being so enthusiastic about science education and supportive of the project's efforts and for hosting me for a week at Northwestern to learn all your research secrets.

To my thesis committee, for not only agreeing to be on a committee with an unconventional project, but also being fully supportive of it and guiding me on the right path. Angela Koehler, you are my female role model for all things STEM - thanks for being a great thesis committee chair, as well as mentoring me both on things in and out of lab. Pete Dedon – fellow Minnesotan, thank you for sticking with me when I transitioned labs and remaining on my thesis committee, as well as understanding the importance of an education-applied project.

To all the resources that MIT provides – to Dalia Fares, Sue Jaskela, Aran Parillo, and Dan Darling for being the backbone of the Biological Engineering department. You all made sure that we all got paid, our posters got printed, we took the right classes, and could find jobs after graduation. To my funding sources – thanks to Claudia Urrea, Eric Klopfer, and Angela Belcher as part of MIT's Abdul Latif Jameel World Education Lab, Alison Hynd, Ariana Ricarte and the rest of the Priscilla King Gray Public Service Center at MIT, Ashley Richard and the Community Service Fund, Babi Mitra and Will Dickson and the Sandbox Innovation Fund Program, Penny Devoe at New England Biolabs, Beth Knudson at Eppendorf, and Lorna Bosworth at Beckman Coulter for making it even financially and logistically possible that I could develop curriculum and a prototype kit to send out to schools.

To each and every teacher and student that I worked with to develop the curriculum and test the prototype kit in their schools. Specifically, thanks to Helen Shao, Elizabeth Genovese, Crystal Shah, Jacqueline Theoharidis, and Faith Blake for taking time out of their busy lives teaching our

future bright minds and helping me design worksheets and instructional manuals for the prototype kits.

To Sebastian Kraves and Zeke Alvarez-Saavedra at miniPCR for having the same philosophy about accessible education as me, taking a chance on my ideas, and offering me my dream job – I can't wait to start developing novel educational kits with you both and the rest of the miniPCR team to teach molecular and synthetic biology to students

To the BE class of 2014 – from studying in the dungeon during first year to giant group dinners at Dumpling House to lounging on the beach in Puerto Rico to celebrate post-quals, every one of you have made grad school fun as we struggled and celebrated through it together. To Erika Handly for being a stellar roommate for our first three years, to Ross Jones and Brett Geiger for all those Catan games, to Alex Brown for binging TV and playing Minecraft with me, to Manu Kumar for our cooking/Game of Thrones/Westworld get-togethers. To all the other grad students in the department for making this truly the best bioengineering graduate program in the country.

To my roommates, George Sun and Jared Kehe, for all our late-night discussions at home, planning the interior decorating of our house, and of course, discovering the wonderful world of RuPaul's Drag Race together. If you can't love yourself, how the hell you gonna love somebody else?

To all the amazing providers at MIT Medical for helping me keep my anxiety in check so I could survive grad school. To the MIT Club Golf team (especially Kim Dinh and Alex Triassi) for giving me an outlet when lab got tough. To the External Affairs Board at MIT and the MIT Museum, for giving me more opportunities to give back to the community. To my board gaming group for teaching me so many new board games – sorry I haven't been around lately, but will be backing to gaming once I finish this thesis!

And lastly, but certainly not least – to my amazing, wonderful family for all their unconditional love and support. For my parents, Frank and Alisa, for always encouraging me educationally, sacrificing and working so hard to provide the best opportunities for me, paying for college so I could be debt-free, and just being there for me, no matter what. I don't know if I've ever been have – or be able to - properly express my appreciation and gratitude for all that you have done for me, so I'll just keep it short and sweet: 謝謝你為我做的一切. 你是世界上最好的父母. 我愛你們.

To Katy – my baby sister, who has always been there for me, who understands me better than anyone else in this world, and proving that we're sisters by chance but friends my choice. And to our late beloved family Pomeranian, Doodle – sadly, you passed away three months shy of my Ph.D. defense, but you certainly did brighten our lives with your sassy personality while you were still with us.

In short, to everyone in my life – thank you for everything.

Table of Contents

CHAPTER ONE: BACKGROUND AND INTRODUCTION

This chapter is in part adapted from:

Huang, A., Nguyen, P.Q., Stark, J.C., Takahashi, M.K., Donghia, N., Ferrante, T., Dy, A.J., Hsu, K.J., Dubner, R.S., Pardee, K., et al. (2018). BioBits™ Explorer: A modular synthetic biology education kit. Sci. Adv. *4*, eaat5105.

Stark, J.C., Huang, A., Nguyen, P.Q., Dubner, R.S., Hsu, K.J., Ferrante, T.C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J.S., et al. (2018). BioBits™ Bright: A fluorescent synthetic biology education kit. Sci. Adv. *4*, eaat5107.

1.1 Introduction to synthetic biology

Synthetic biology is a rapidly advancing field that utilizes engineering concepts to harness the power and diversity of biology. As a highly interdisciplinary field, synthetic biology combines a number of different disciplines beyond just bioengineering, including biophysics, chemical engineering, electrical engineering and computer science/engineering, for a variety of different applications. Through understanding and re-purposing naturally occurring biological processes, synthetic biologists are able to create artificial biological systems, either through 1) engineering living cells to take on new functions or 2) building novel *in vitro* biological systems comprised of abiotic biological parts for a specific application/function. At the base of these applications is the ability to control gene expression in a predictable manner, which is often accomplished through synthetic gene networks. To build these networks, synthetic biologists have developed modular parts to control and tune the processes of transcription and translation (Medema et al. 2010; Purnick and Weiss 2009). Using these processes, the field has been able to provide new methods

for chemical and drug manufacturing (Smanski et al. 2016; Eisenstein 2016; Medema et al. 2010), clinical diagnostics (Gootenberg et al. 2017; Pardee et al. 2017), cell therapies (Fesnak et al. 2016; Roybal et al. 2017), as well as advanced fuels (Liu et al. 2016). In short, the field of synthetic biology has so far yielded many cutting-edge innovations, but has to potential to deliver even more.

1.2 Freeze-dried, cell-free (FD-CF) protein synthesis

1.2.1 Overview of FD-CF systems

For the synthetic biology advancements made in novel *in vitro* systems, cell-free protein synthesis systems are often utilized. Cell-free systems use essential cellular machinery, including polymerases, ribosomes, and transcription factors, in an *in vitro* setting to carry out the processes of transcription and translation, circumventing the need for specialized, sterile equipment and media to culture living cells. Moreover, the lack of living cells eliminates concerns of biocontainment. There are two general types of cell-free systems: crude extracts, where the required cellular components are harvested from whole-cell bacterial lysis (Kwon and Jewett 2015), and commercial purified recombinant systems, such as PURExpress®, (Shimizu et al. 2001), where each individual component is produced recombinantly, purified, and mixed together (**Figure 1**). In both system types, a separate buffer is included that contains essential components such as nucleotides and amino acids. Cell-free systems can be used to produce proteins and other biomolecules, as well as build and execute synthetic biology circuits (Gootenberg et al. 2017; Pardee et al. 2014; Pardee, Green, et al. 2016, Pardee et al. 2017; Carlson et al. 2012).

Due to the simplicity of the whole-cell lysate process, crude extracts are inexpensive to produce – as low 1¢/μL (see Chapter Seven: Materials and Methods, **Table 11**). Crude extracts can be derived from *E. Coli*, wheat germ, or mammalian cells, but the focus of this work here will be with *E. Coli*

derived crude extracts. Crude extracts derived from *E. Coli* also have the highest protein yield of all the cell-free systems (Villarreal and Tan 2017). The drawback to the crude system is that despite the high yield, the expressed proteins tend to be lower quality due to issues like aggregation (Gagoski et al. 2016). Furthermore, since the *E. Coli* crude extract is a prokaryotic system, expression of eukaryotic proteins can be challenging. For example, the prokaryotic crude extracts lack certain post-translation modifications that can cause improper protein folding. Finally, as the name implies, crude extracts are not purified or processed post-lysis, resulting in many cellular components, like nucleases and proteases, that will negatively interfere with protein expression processes (Villarreal and Tan 2017). Some innovations have been made to address these challenges, such as incorporating glycoprotein synthesis into crude cell-free systems to allow for expressed eukaryotic proteins to be glycosylated (Jaroentomeechai, Stark, et al 2018).

Commercial systems addresses the other drawbacks of the crude system. In this work, the focus will be on the PURExpress® system (noted from this point as "PURE"). Since each component of the PURE system is individually expressed, purified and combined, it only contains the cellular machinery that is needed for transcription and translation, so even though the protein yield is not as high as in the crude extracts, the protein quality is higher (Hillebrecht and Chong 2008). The major limitation of the PURE system is the cost – the commercial cost of PURE is nearly $1/μL, which is 100 times more costly than the crude system. Recent improvements to the efficiency of the PURE production system though suggest that its cost could be reduced to that of the crude system (Villarreal et al. 2017; Shepherd et al. 2017).

Importantly, cell-free systems can be freeze-dried along with genetic elements to form pellets that are stable at room temperature and can be easily transported (Pardee et al. 2014). The shelf-stable nature of the freeze-dried, cell-free (FD-CF) pellets eliminates the need for cold-chain transport or

dedicated freezers. Additionally, FD-CF reactions do not require specialized equipment, making them especially ideal for low-resource environments. Reactivation of the FD-CF components just requires the end user to rehydrate the reaction – or in even simpler terms, to "just add water."

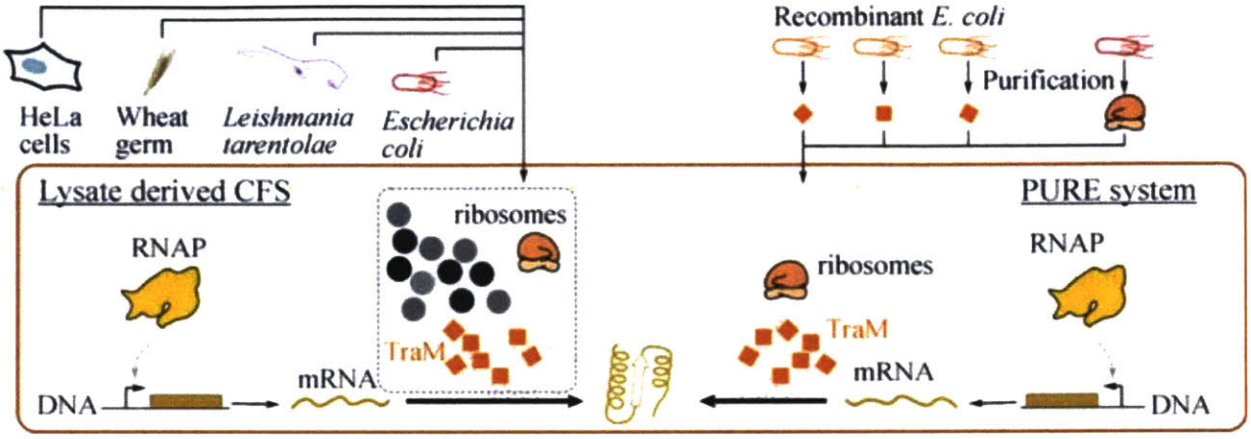


Figure 1: Schematic of cell-free systems.

Schematic depicting the two main types of cell-free systems, lysates derived from whole cells (left) and individually purified and combined proteins (right). Figure taken from Villarreal and Tan 2017.

1.2.2 FD-CF for diagnostic applications

The standard for accurately detecting diseases, such as in the recent case of the Zika virus, is often nucleic acid-based methods of amplifying DNA, such as PCR (de M Campos et al., 2016), but these techniques require specialized equipment and expertise that many low-resource regions cannot afford. Thus, the ease of use and low cost of FD-CF reactions make them an attractive system for these kinds of diagnostic applications. Previous work in our lab group focused on applying FD-CF to diagnostics by 1) developing programmable RNA sensors (toehold switches) to allow detection and reporting of specific nucleic acid sequences (Green et al, 2014), and 2) incorporating these sensors into the FD-CF platform in a way that they could be deployed and used outside of a laboratory setting (Pardee et al., 2014; Pardee, Green, et al., 2016).

Toehold switch sensors are programmable synthetic riboregulators that allow protein expression only when a specific trigger RNA is present. A toehold switch sensor is an mRNA designed to include a hairpin structure that blocks gene translation in cis by sequestration of the ribosome binding site (RBS) and start codon. Upon hybridization to a complementary trigger RNA, sequestration of the RBS and start codon is relieved, allowing for ribosomal translation of an output gene (**Figure 2**). Toehold switch sensors provide a means for controlling the translation of a protein, usually a reporter protein (such as green fluorescent protein or a colorimetric b-galactosidase enzyme-mediated color change) that can be visualized as a diagnostic output. Unlike previous similar methods, toehold switches do not have any sequence constraints for the trigger, allowing a wide variety of applications (Green et al, 2014).

These toehold switches can be used in FD-CF systems, where the plasmid constructs encoding for the toehold switch sensors are freeze-dried along with the cell-free components and rehydrated with a patient sample containing the target virus or bacteria. The specific trigger sequence would then be a specific sequence from the virus or bacteria, so if the reporter protein is observed, then the patient sample contains the target pathogen. For further ease of use, the cell-free reactions were embedded on paper discs prior to freeze-drying to enable distribution and make handling these reactions much simpler (**Figure 3**; Pardee et al, 2014).

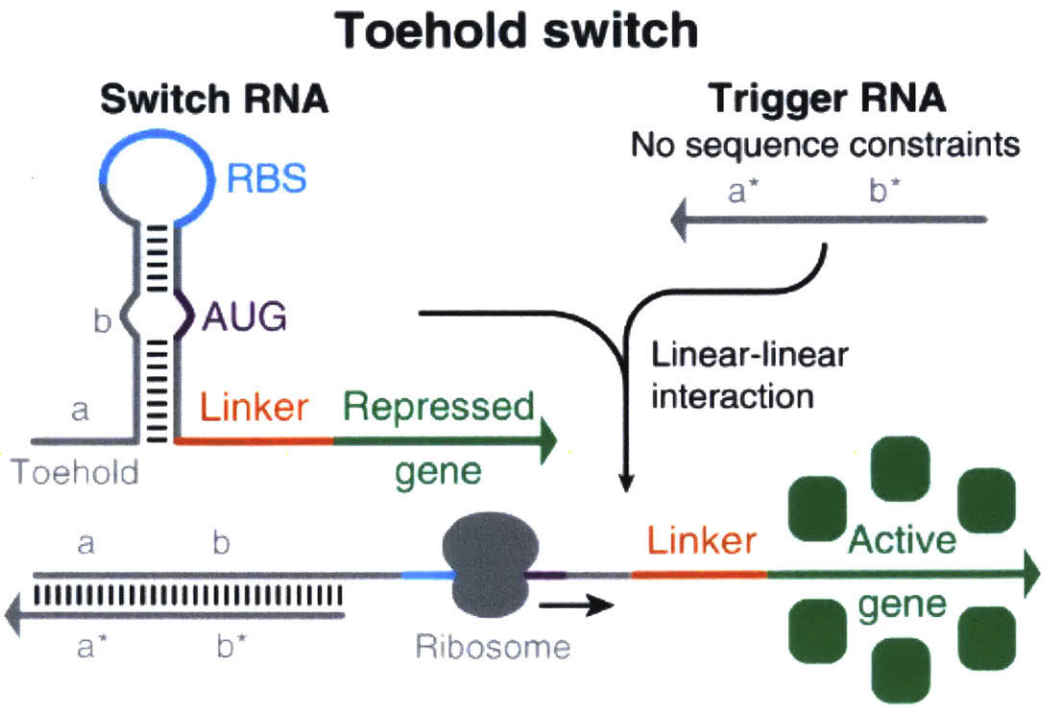


Figure 2: Schematic of the toehold switch sensor.

Schematic depicting the toehold switch sensor. Hairpin structure in the switch RNA blocks gene translation by sequestration of the ribosome binding site (RBS) and start codon. Hybridization to a complementary trigger RNA relieves the s RBS and start codon, allowing for ribosomal translation of an output gene. Figure taken from Green et al 2014.

Combing the toehold switches and the paper-based FD-CF platform, the lab group was able to show a rapidly prototyped proof of concept by developing sensors specific to the Ebola virus (**Figure 4**; Pardee et al. 2014). The group then further developed this proof of concept platform to incorporate sample handling to extract and amplify the specific RNA sequences needed to improve detection, this time using the Zika virus as the model disease. At less than $1/test, the "just add water/sample" protocol, and a result turnaround time of three hours, this platform is well-suited for the limited medical infrastructure and/or expertise in developing areas (Pardee, Green, et al. 2016).

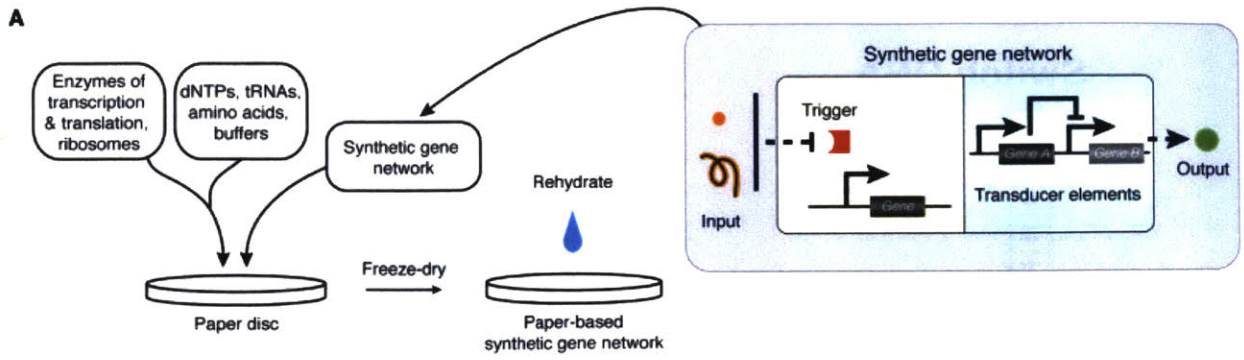


Figure 3: Schematic of the paper-based FD-CF system

Schematic depicting the paper-based FD-CF system. The cell-free system of choice (containing all necessary materials for transcription and translation) is freeze-dried on a paper disc with a synthetic gene network. Upon rehydration with water, the synthetic gene network will be activated, producing an output based on a given input. Figure taken from Pardee et al 2014.

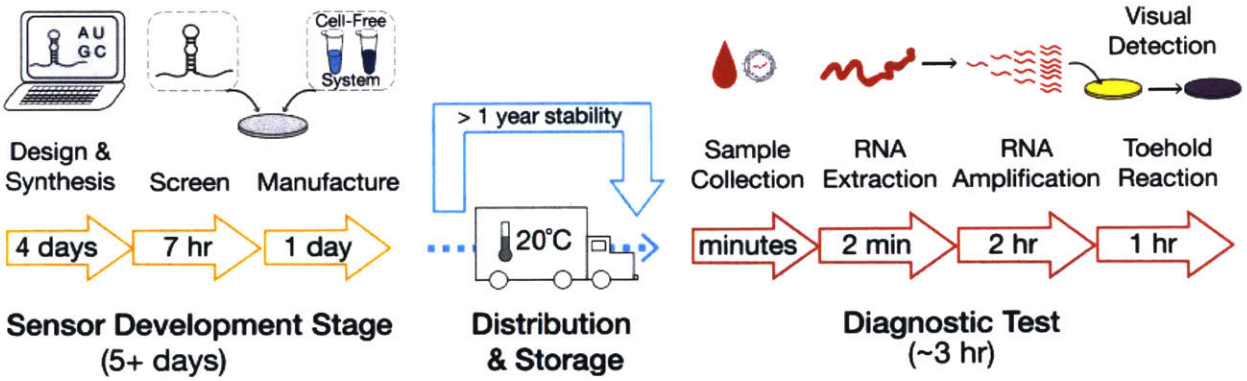


Figure 4: Workflow to develop a novel diagnostic system

Workflow depicting how this system can be used to develop a novel diagnostic. The design and synthesis of the toehold sensors can be completed in less than a week. Once completed and applied to the paper-based FD-CF system, the new diagnostic test would only take about 3 hours to run and includes steps to process and amplify the RNA for toehold detection. Figure taken from Pardee, Green, et al 2016.

The paper-based FD-CF platform was also applied to detecting certain species, host biomarkers, and toxins within the gut microbiome as a more inexpensive and rapid technique to analyze patient microbiome samples (**Figure 5;** Takahashi et al 2018). This proof of concept further demonstrates the wide applicability in diagnostics of the FD-CF platform, as well as its potential as an easy-to-use research tool.

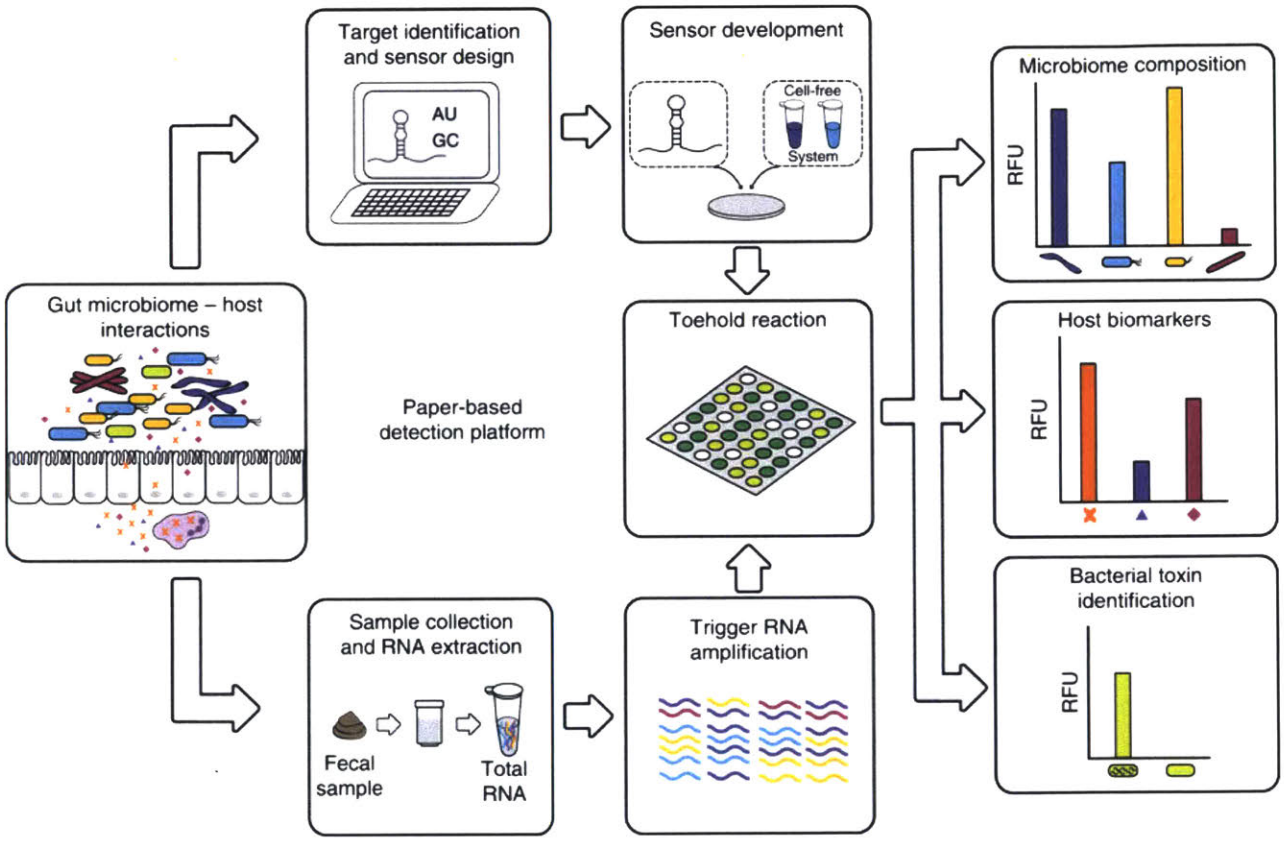


Figure 5: Application of paper-based FD-CF to the gut microbiome.

The paper-based FD-CF diagnostic workflow (Figure 4) can be applied to create a diagnostic and research tool to identify microbiome composition, host biomarkers, and bacterial toxins in the gut microbiome. Figure taken from Takahashi et al 2018.

The FD-CF platform can also be embedded on other materials besides paper as well. Current ongoing work by the lab group includes running FD-CF reactions on fabrics and other materials

to create wearable diagnostics that could be deployed in the battlefield on solider uniforms to detect nerve agents and other biochemical threats or in hospitals on hospital gowns/bracelets to detect spread of specific diseases (**Figure 6**). The same fluorescent or colorimetric diagnostics outputs can be used here, but development of an electronic based sensor (also embedded in the clothing) to sense and display results is also ongoing.

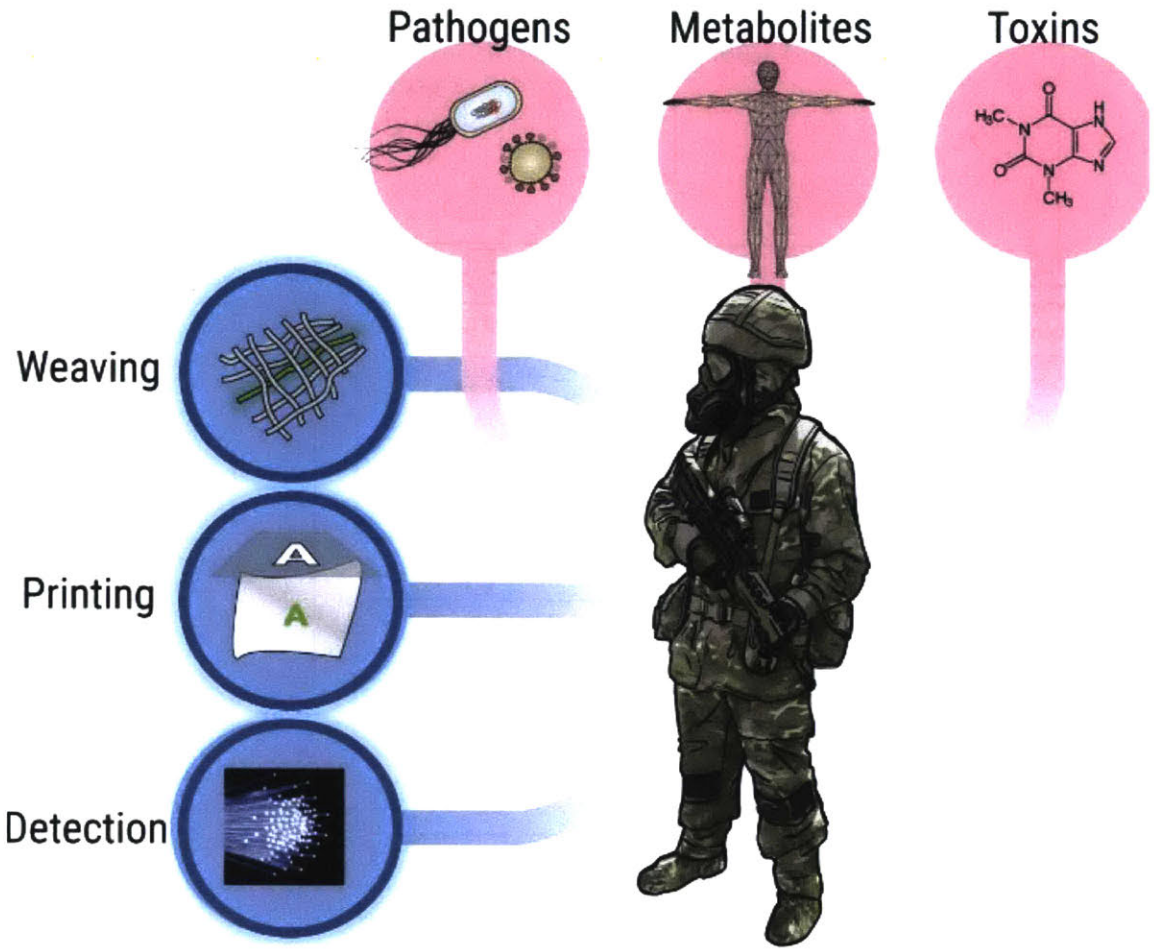


Figure 6: Application of FD-CF to the wearable applications.

The FD-CF diagnostic workflow (Figure 4) can be applied to create a diagnostic tool embedded on fabric to detect various pathogens, metabolites, and toxins (i.e., on a soldier's uniform in the battlefield). Figure taken from Nguyen et al. 2019 (unpublished work).

1.2.3 FD-CF for portable bio-manufacturing

Besides diagnostics, FD-CF systems also have potential to be used in expressing proteins for therapeutic and medical applications (**Figure 7**). Besides cost and ease-of-use, FD-CF systems also have the advantage that they will only express a specified protein, unlike living cultures that will express other proteins besides the one of interest. Since FD-CF systems will only express the proteins encoded by the added DNA constructs, this makes purifying and isolating the protein of interest easier. The lab group demonstrated the use of FD-CF to produce on-demand, small quantities of antimicrobial peptides to be used as defense molecules against infection and as an alternative to antibiotics, vaccines antigens to eliminate cold-chain vaccine distribution challenges to the developing world, antibody analogs for their research and therapeutic benefits, and biosynthesis of small molecules for therapeutic applications (Pardee, Slomovic, et al. 2016). The advantages of this FD-CF system allows expression of needed therapeutic proteins without need for a centralized lab with specialized equipment or expertise, meaning that proteins can be expressed on-demand in developing areas without having to worry about distribution, storage, or biosafety concerns.

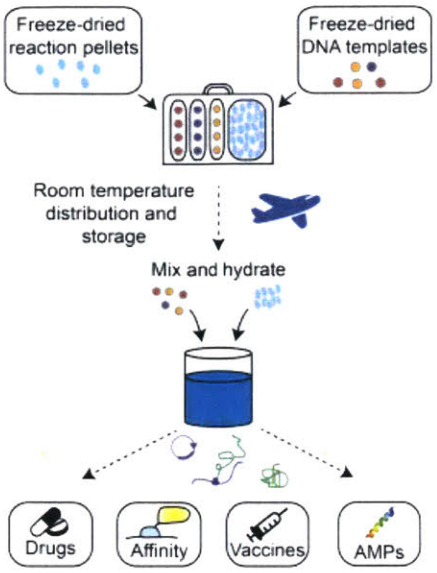


Figure 7: Application of FD-CF to the wearable applications.

The FD-CF diagnostic workflow (Figure 4) can be applied to create a diagnostic tool embedded on fabric to detect various pathogens, metabolites, and toxins (i.e., on a soldier's uniform in the battlefield). Figure taken from Pardee, Slomovic, et al. 2016.

1.3 Status quo of molecular biology education in secondary schools

Although these technologies were designed with clinical and medical applications in mind, they also have great potential as educational tools to teach molecular and synthetic biology concepts. The current status quo of STEM (Science, Technology, Engineering, and Math) education in the United States seems to be less than ideal. An in-depth study conducted by the American Association for the Advancement of Science (AAAS) in 2015 reveal that while both the general public and scientists highly regard the country's scientific achievements, most consider the country's K-12 STEM education as only average or below average **(Figure 8**; Funk et al. 2015).

Indeed, U.S. students' test scores in science rank quite low against those of other countries, with many countries surpassing the U.S. average (**Figure 9**; National Science Board 2012), which indicates that improvements could be made to our science education system.

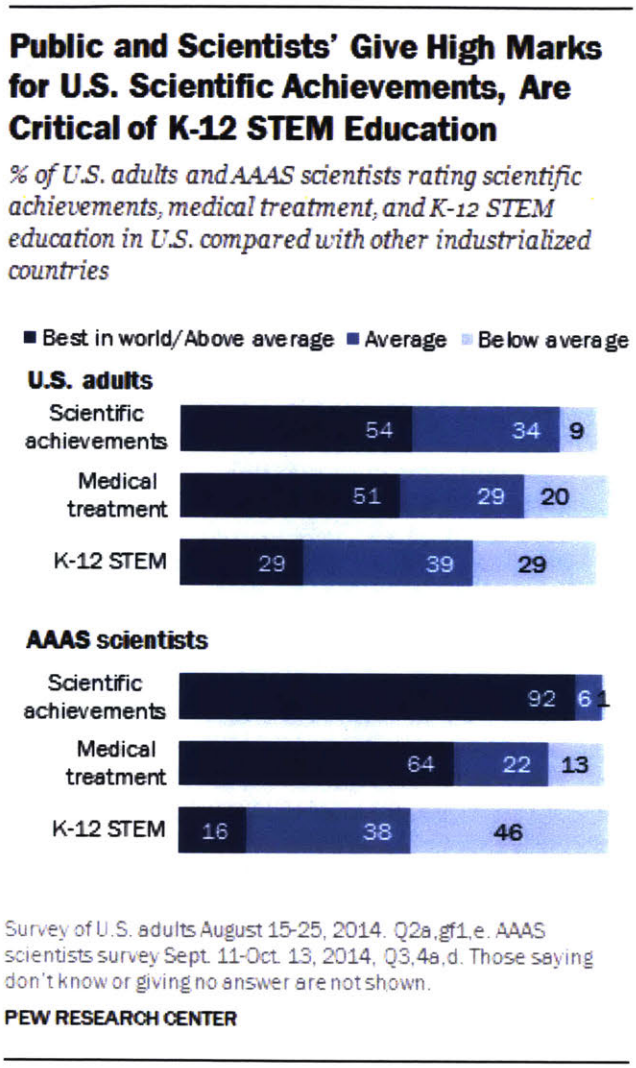


Figure 8: Country's scientific achievements highly viewed, but K-12 STEM education are criticized. A survey of both US scientists and non-scientists adults to rate the scientific achievements and K-12 STEM education on a scale of below average, average, or above average. Figure taken from Funk et al. 2015.

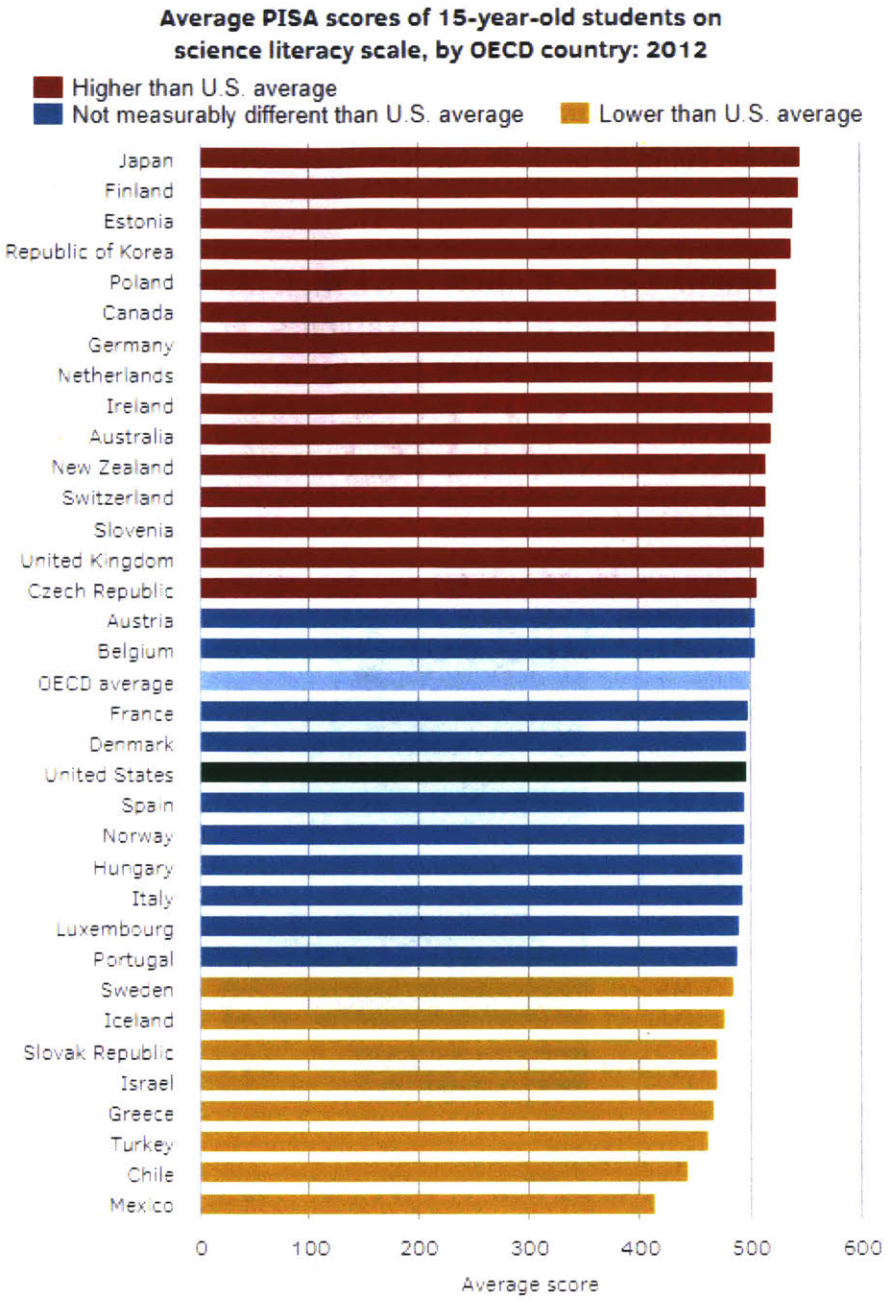


Figure 9: Average PISA (Program for International Student Assessment) scores by country

Average Program for International Student Assessment (PISA) scores of 15-year old students from Organisation for Economic Co-operation and Development (OECD) countries. Graph color-coded to indicate which countries scored above, the same, or below the U.S. Figure taken from National Science Board 2012.

A lack of proficiency in basic science knowledge appears to trend after high school as well, with large gaps between scientists and non-scientists about their views on science-related issues. For example, 88% of the scientists surveyed for the study believe the genetically modified organisms (GMOs) are safe to eat (as they understand the basic science behind genetic modifications), while only 37% of non-scientist adults agree. Similar trends are found with other science issues, such as animal research, human evolution, and vaccines (**Figure 10**).

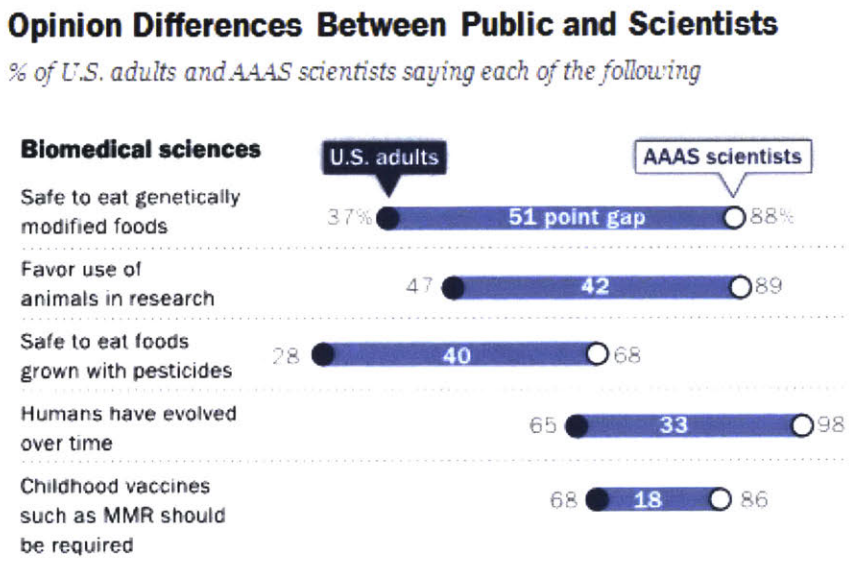


Figure 10: Differences between the public and scientists concerning biomedical issues.

A survey of both US scientists and non-scientists adults on if they agree with the list of biomedical science issues. Graph color-coded to highlight the % point difference between U.S. scientists and the general public. Figure taken from Funk et al. 2015.

Furthermore, the majority of scientists view the fact that the public has limited knowledge about science as a major problem and cite "not enough K-12 STEM" as a major reason why there is limited public science knowledge, with lack of interest in the public and media as a secondary reason (**Figure 11**; Funk et al. 2015). Since some of these issues, like GMO's and vaccines, have

real-world implications and impacts, it is critical that our public understands the fundamental science behind these topics, so they can make informed personal and policy decisions.

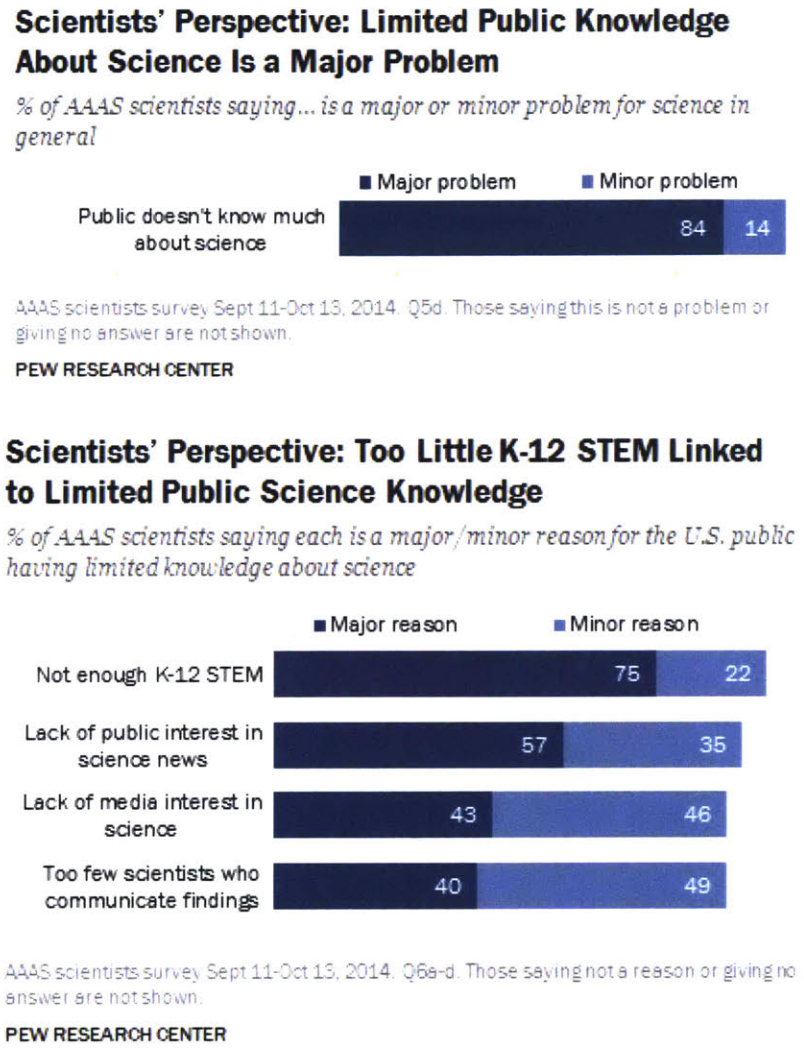


Figure 11: Scientists' perspective on the impact of K-12 STEM on public science knowledge.

A survey of US scientists on if they view limited public science knowledge as a minor or major problem, and what the major or minor reason for that limited knowledge is. Figure taken from Funk et al. 2015.

Many scientists can trace their initial fondness for the sciences to formative experiences with hands-on exploratory kits, such as chemistry sets, and research has supported that hands-on experiences enhances learning, especially in the science fields (Riskowski et al. 2009; Dhanapal and Shan 2014; Kontra et al 2015). In fact, it is estimated that classrooms that utilize frequent hands-on learning activities will outperform classrooms who do not by more than 40% of a grade level in science (National Center for Education Statistics 2011). This trend has expanded today to include a spectrum of educational kits that teach subjects such as physics, electronics, programming, or robotics (Benitti 2012; Resnick et al. 2009; Clark et al. 2016). However, there are few successful and engaging systems for teaching molecular or synthetic biology concepts in a hands-on manner (National Research Council 2013, Freeman et al. 2014). The efforts that do exist, such as the BioBuilder Education Foundation (Dixon and Kuldell 2011; Dixon and Kuldell 2012) and the International Genetically Engineered Machines (iGEM) competition (iGEM Team EPFL 2017; Kelwick et al. 2015), have had positive impact on its participants. These include self-reported academic gains, high level of student engagement, and increased self-identification as scientists or engineers (Campbell 2005; Mitchell and Kuldell 2015; Mitchell, Dori, and Kuldell 2011).

Unfortunately, these efforts involve traditional biology experimentation, which requires a cold chain to prevent the biological components from spoiling, sterile equipment and media to prevent contamination, specialized instruments such as shaking incubators, and concerns with the biocontainment of recombinant microorganisms. This limits these opportunities to schools who have the funding, time, and expertise to be able to implement these programs for their students, or the students who are fortunate enough to be near biotechnology-heavy cities (i.e. Boston, San Francisco). While there are some states who heavily invest in their students' education, other states

do not **(Figure 12;** National Science Board 2018). Students and schools in these more rural or low-resource areas will not be able to experience these hands-on molecular/synthetic biology activities as often, and thus, not gain the benefits associated with them.

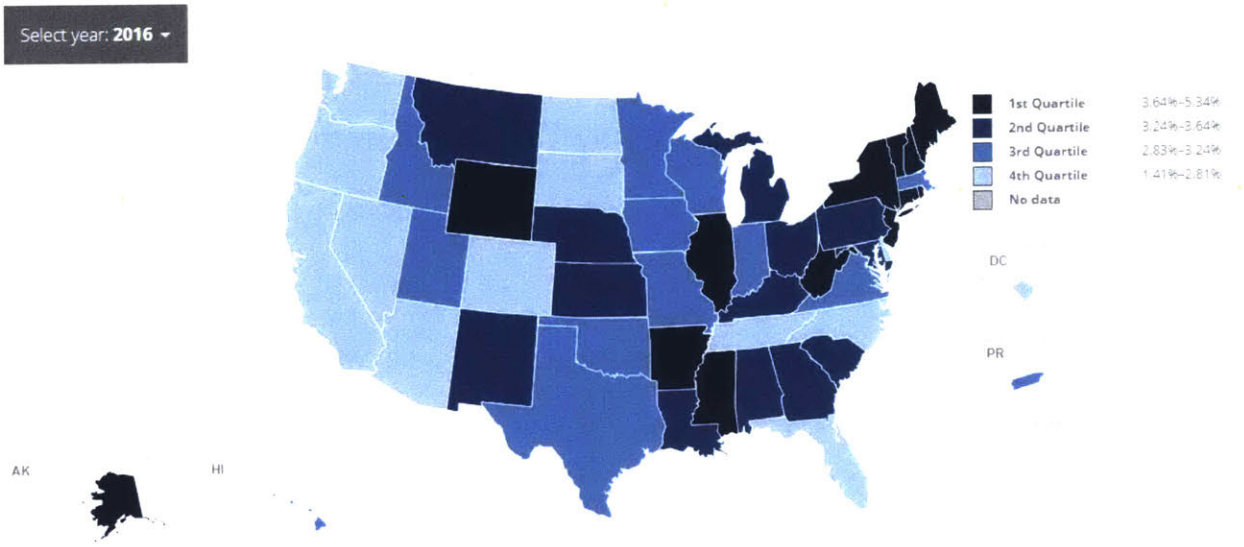


Figure 12: Public School Expenditures as a Percentage of Gross Domestic Product (GDP)

Representation by state on what percentage of each state's GDP is spent on elementary and secondary public schools. Figure taken from National Science Board 2018

1.4 Dissertation overview

Here, I present the use of the FD-CF synthetic biology platform to circumvent these educational challenges, resulting in a shelf-stable and affordable educational kit for demonstrating advanced biological concepts with inquiry-based learning (**Figure 13**). The same advantages that make FD-CF a powerful tool for diagnostics and bio-manufacturing in low resource areas (i.e. low cost, ease of use) also make it a potentially beneficial addition into high school biology curriculum. I will cover the fundamental development using the FD-CF platform for educational applications, including utilizing fluorescent protein as engaging outputs for students, considering storage

logistics in the classroom, and providing some ideas of hands-on activities to teach molecular biology concepts (Chapter 2). Then, I will delve deeper into one of these biological concepts, the central dogma biology, present a specialized genetic circuit I developed to visually illustrate each step of the central dogma, and suggest accompanying inquiry-based learning activities that can be run with this genetic circuit (Chapter 3). I then describe a series of other modules that teach a variety of other concepts, including some that utilize other sensory outputs (non-fluorescent), such as smell and touch (Chapter 4). Moving beyond basic molecular biology, I then develop a series of modules to create a "biological breadboard" to introduce students to basic synthetic biology concepts and techniques and their real-world applications (Chapter 5). Finally, I conclude by translating this work into actual classroom: first by developing a prototype kit with the help of high school biology teachers, testing them with high school students in their classroom, and gathering feedback from both the teachers and students to determine how effective the kits were in teaching biological concepts, inspiring interest in biology/engineering, and addressing common classroom challenges (Chapter 6).

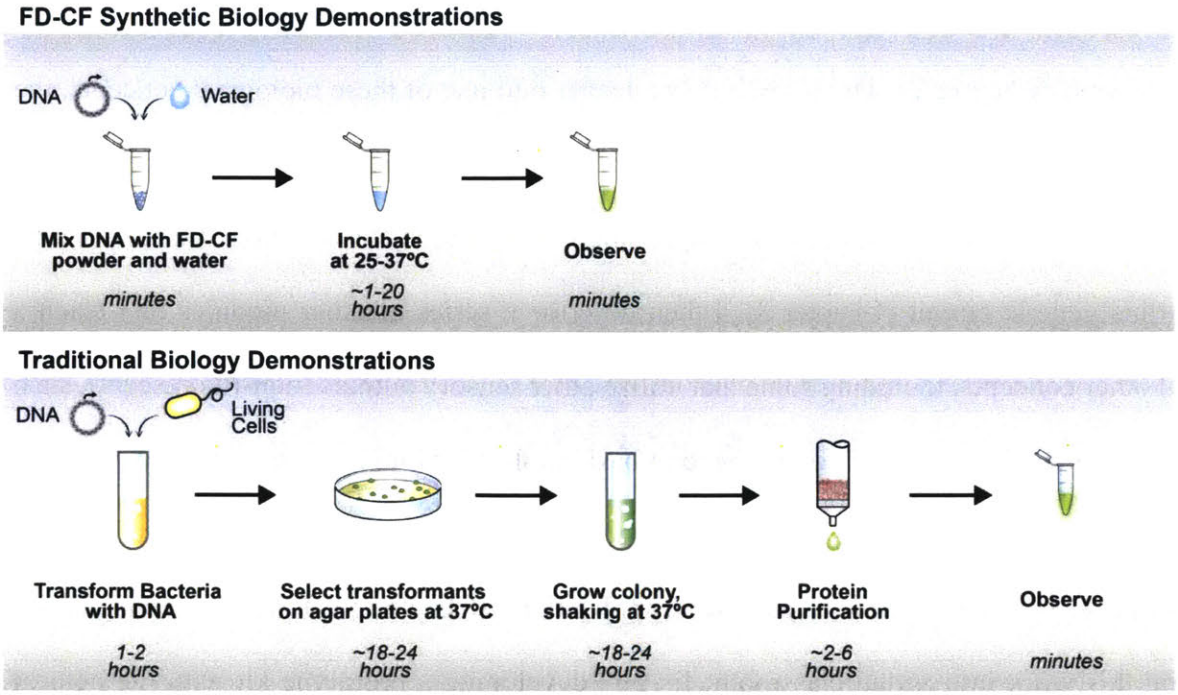


Figure 13: Traditional vs. FD-CF Biology Demonstrations

FD-CF demonstrations require only the addition of water to the supplied reactions and incubation for 2-20 hours at 25- 37°C for observation and analysis by students. In contrast, traditional biology experiments require substantial time, resources, and specialized equipment. Figure taken from Huang, Stark, Nguyen et al. 2018.

CHAPTER TWO: FUNDAMENTAL DEVELOPMENT OF THE EDUCATION KIT

This chapter is in part adapted from:

Huang, A., Nguyen, P.Q., Stark, J.C., Takahashi, M.K., Donghia, N., Ferrante, T., Dy, A.J., Hsu, K.J., Dubner, R.S., Pardee, K., et al. (2018). BioBits™ Explorer: A modular synthetic biology education kit. Sci. Adv. *4*, eaat5105.

Stark, J.C., Huang, A., Nguyen, P.Q., Dubner, R.S., Hsu, K.J., Ferrante, T.C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J.S., et al. (2018). BioBits™ Bright: A fluorescent synthetic biology education kit. Sci. Adv. *4*, eaat5107.

2.1 Introduction

The unique characteristics of FD-CF reactions that allow for efficient storage and usage also make them well suited for creating hands-on, educational activities for molecular biology concepts. The combination of FD-CF technology and a toolbox of synthetic biology parts provides the foundation for the creation of a hands-on biology educational kit that demonstrate a variety of outputs (**Figure 14**). Such a kit could be used to demonstrate both the breadth of synthetic biology activities that can be developed with FD-CF technology and how these platforms can increase student involvement, illustrate core concepts in molecular and synthetic biology, and provide opportunities for independent, student-directed research in the life sciences.

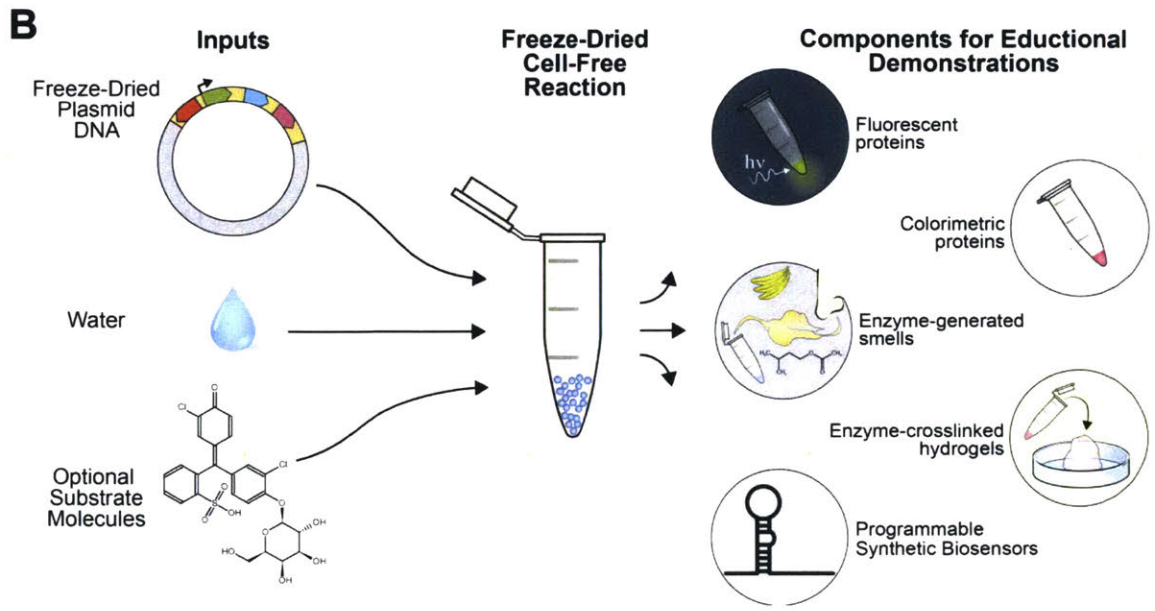


Figure 14: Schematic for a freeze-dried, cell-free educational kit.

With the DNA template and any substrate molecules provided with the FD-CF reaction, the students just have to add water to run a number of bioscience activities and demonstrations. Figure taken from Huang, Stark, Nguyen et al. 2018.

2.2 Selection of basic set of fluorescent proteins plasmids

My first goal was to select a set of protein outputs that would be easily detected without any specialized equipment and decided on visual outputs that could be easily seen by eye or with the help of inexpensive equipment. Fluorescent proteins was selected as the visual output due to their vibrant colors and efficient expression in FD-CF systems (determined from prior uses of FD-CF fluorescent proteins).

First, a panel of 12 different fluorescent proteins (readily available from previous projects) was screened in fresh PURE reactions (**Figure 15, top**). The resulting protein outputs were visually analyzed for their brightness/intensity and reaction kinetics.

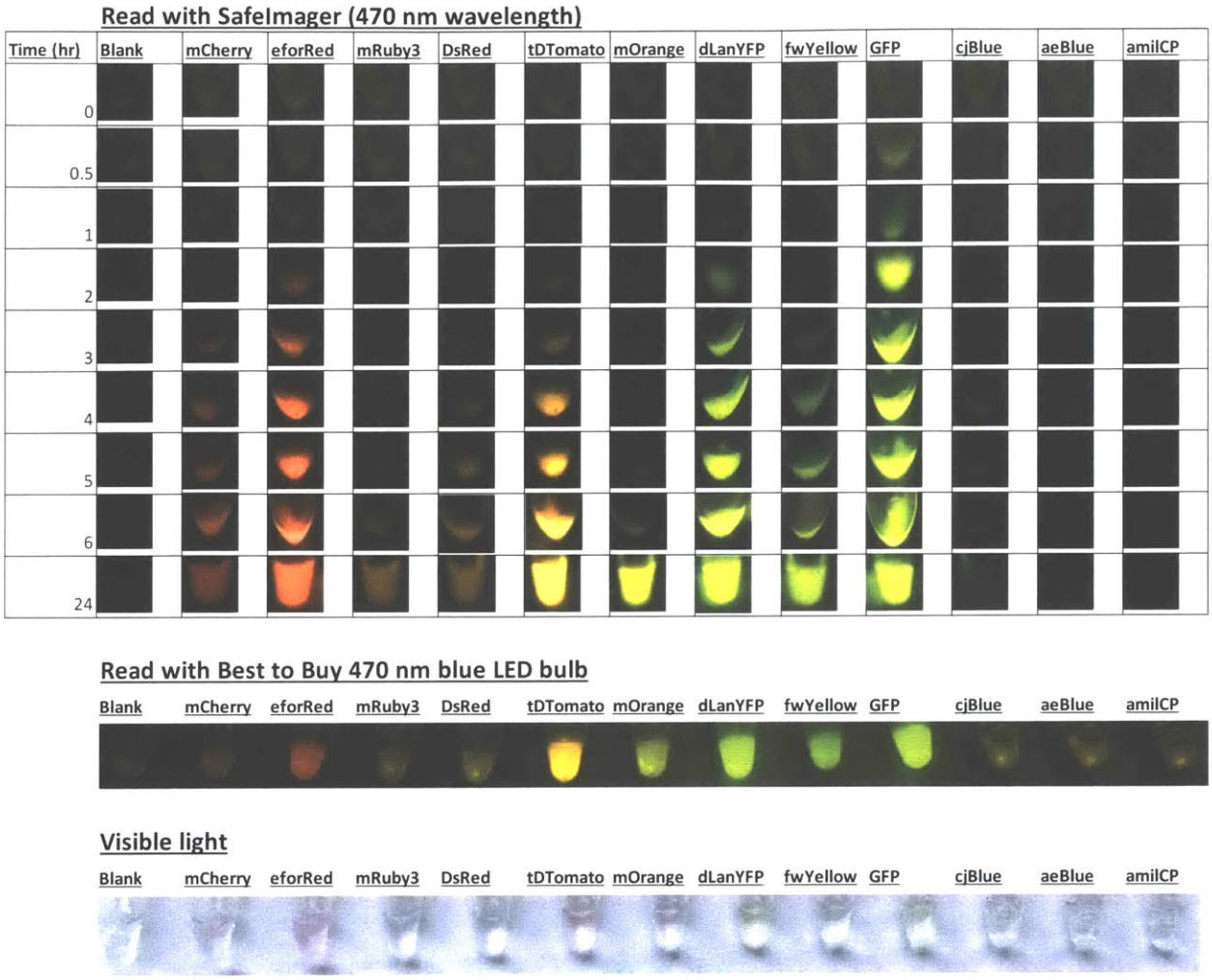


Figure 15: Screen of a fluorescent protein panel to determine set for kit

Plasmids containing fluorescent proteins were linearized, added to PURExpress reactions at 20 uL volume at saturating DNA conditions, and incubated at 30°C for 24 hours. Timecourse images were taken on a 470 nm SafeImager transilluminator. At 24 hours, an endpoint image was taken with the SafeImager (top), an inexpensive blue LED bulb (middle), and by eye on white paper under ambient light (bottom).

From this screen, I chose the brightest/fastest fluorescent protein from each color family to cover a spectrum of colors comprised of a red (eforRed; Alieva et al. 2008; ten Buren et al. 2014), an orange (tdTomato; Bianchi et al. 2015; Campbell et al. 2002), a yellow (mOrange; Bayle et al. 2008), and a green (GFP). Although the dLanYFP was much brighter and faster than mOrange,

dLanYFP appeared green and was too similar to GFP. mOrange, while slower, still is bright after 24 hours and appears more yellow.

I also tested if the fluorescent proteins could be visualized with an inexpensive blue LED bulb, instead of the more expensive laboratory transilluminator (**Figure 15, middle**) and saw that the 24 hour endpoint image was visually comparable. Finally, the fluorescent proteins can also be visualized by eye under ambient light (**Figure 15, bottom**), although in the PURExpress system, the colors were very faint.

Notably, none of the blue fluorescent proteins could be visualized, as blue fluorescent proteins are difficult to visualize due to their shorter wavelength. After some experimentation with these and alternate blue fluorescent proteins, I selected Aqua (Aquamarine; Erard et al. 2013) (**Figure 16**). Although not as brightly expressed as the other proteins in the set, it is different enough from the GFP to be considered a blue-green, but still can be visualized under the same excitation wavelengths as the other proteins in the set. I also replaced the regular GFP with a superfolder GFP (sfGFP; Heim and Tsien 1996) for a brighter and faster green output (**Figure 16**).

To validate that the fluorescent proteins could be expressed after the cell-free reaction was freeze-dried, FD-CF pellets including DNA encoding the five proteins were rehydrated and incubated overnight (~20 hours) at 30°C. The fluorescent proteins expressed robustly (**Figure 16, top**), and because this particular expression was done in crude extract, they were also easily visible by eye even without fluorescent excitation (**Figure 16, bottom**) (Huang, Stark, Nguyen et al. 2018).

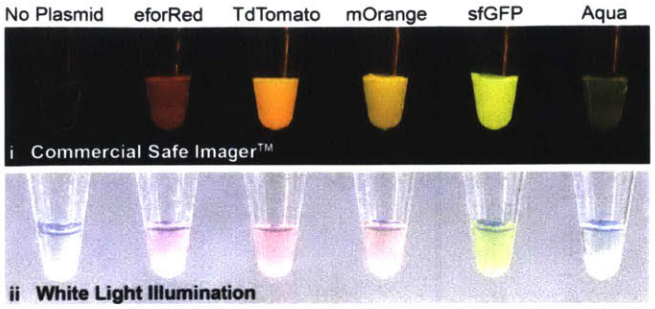


Figure 16: Fluorescent proteins as visual outputs.

A set of fluorescent proteins were expressed by FD-CF expression in crude extract and visualized with (i) a laboratory transilluminator (Safe Imager at 470 nm excitation) and a (ii) white light epi-illumination. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

The resulting fluorescent proteins were also quantified by both protein yield (measured by 14C-Leucine incorporation; **Figure 17**) and by relative fluorescent units (RFU) (at optimal excitation and emission spectra for each fluorescent protein; **Figure 18**). Both quantification methods showed that the fluorescent proteins were highly expressed in the FD-CF system and confirms the visual data (Huang, Stark, Nguyen et al. 2018).

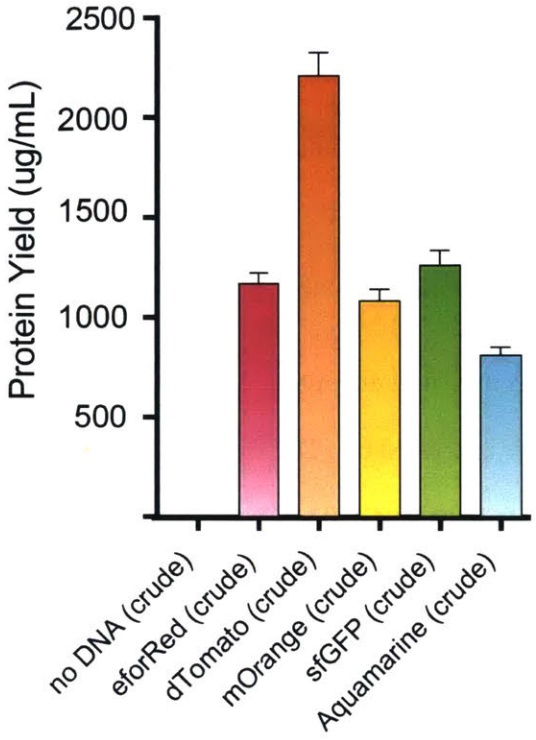


Figure 17: Quantification of fluorescent proteins expressed in crude FD-CF.

All of the FD-CF expressed fluorescent proteins used in the demonstration experiments had high soluble yields in crude extract (between 500 and >1000 μg/mL), as measured by 14C-Leucine incorporation (done by Jessica Stark). Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

2.3 Selection of cell-free system

As discussed in Chapter One, there are two general types of cell-free systems: crude extracts, where the required cellular components are harvested from bacterial lysis (Kwon and Jewett 2015), and the commercial PURE system (Shimizu et al. 2001), where each individual component is produced recombinantly and mixed together. In both system types, a separate buffer is included that contains essential components such as nucleotides and amino acids.

While screening and testing the fluorescent proteins, the PURE system was initially used because it had been validated in prior projects and was readily available for use. However, since the crude extracts are up to 100 times more inexpensive than PURE, economically, it would be the better system to use to develop a low-cost molecular biology kit. Furthermore, as noted above, the fluorescent proteins were also easily visible by eye even without fluorescent excitation in the crude system (**Figure 16, bottom**), but very faintly visible by eye in the PURE system (**Figure 15, bottom**). This was further quantifiably verified by measuring the relative fluorescent units (RFUs) of the fluorescent proteins in the PURE vs. crude system (**Figure 18**), where there was up to a 10-fold difference between the two systems (Huang, Stark, Nguyen et al. 2018).

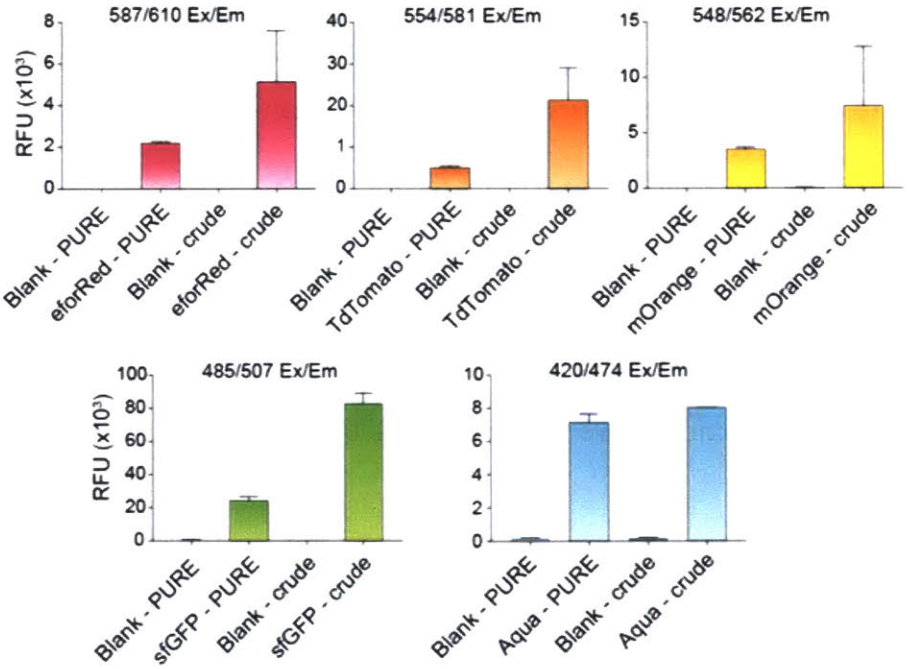


Figure 18: Fluorescent proteins expressed in the PURE and crude extract systems.

Endpoint fluorescent readouts of all expressed proteins in the commercial PURE or in-house crude system. Values represent averages and error bars represent average errors of n≥2 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

Since the crude system is more inexpensive and has higher expression of the fluorescent proteins, I selected it as the primary cell-free system for developing my educational kits. Unless specifically noted, all experiments from this point on were done in the crude extract system. (For development of some of the other outputs besides constitutively expressed fluorescent proteins, PURE was used if the protein expression was low in the crude extracts).

2.4 Storage and stability of FD-CF

One of the advantages of FD-CF is that due to the freeze-dried conditions, the cell-free reactions do not need to be shipped or stored at -80°C, which makes it optimal for a classroom setting. Previous data show that PURE can be stored in a vacuum-sealed Mylar bag under nitrogen for up to a year with minimal loss of activity (Pardee et al. 2014). To help reduce kit production costs, I tested the stability of FD-CF reactions made with crude extract under conditions that were more inexpensive and realistic for a classroom setting.

For these stability studies, 5 uL FD-CF reaction pellets in PCR tubes were stored in vacuum-sealed bags and tested at each timepoint in triplicate with 1 ng/uL of sfGFP. After a 24 hour incubation at room temperature, the reactions were visualized (at 470 nm) and quantified on a plate reader for RFU (ex/em 485/520).

Any oxygen that remains in the vacuum bag and any water that remains bound in the reaction after freeze-drying can negatively affect the stability of the FD-CF pellets by degrading the reactions. To help mitigate these elements, I first tested the stability of the pellets at room temperature that had a cardboard desiccant card (Dri-Card, ~0.08g of Dri-Card per 5 uL pellet) and/or an oxygen scrubber packet (one packet per 3 x 5 uL pellets) sealed with the pellets in the vacuum-sealed bag.

Without any storage components, the pellets at room temperature last for less than a week (the one week timepoint shows a ~85% loss in activity and a significant drop in visual signal). With the addition of Dri-Cards, the pellets at room temperature last up to three weeks before losing ~95% activity by week 4. Although there was a ~50% loss of activity during weeks 1-3, this loss of activity does not correspond to a visual loss of signal. With the addition of both Dri-Cards and oxygen scrubber packets however, the pellets at room temperature show the same degradation rate as the pellets without any addition of storage components. This means that either the oxygen scrubber packet negatively affects the Dri-Card and prevents it from functioning properly or that the oxygen scrubber packet negatively affects the pellets themselves (**Figure 19**). From this study, I decided to include Dri-Cards, but not oxygen scrubber packets, with the pellets to extend their shelf life to three weeks at room temperature. As shipping typically will take less than three weeks, this will allow me to ship the kit at room temperature without any ice packs or dry ice, which will further reduce kit costs.

While a three-week shelf life at room temperature alleviates shipping concerns, many teachers would have to buy kits ahead of time and may not necessary use it within three weeks. To help extend the shelf life of the kit even further, I tested the stability of the pellets (with Dri-Cards added) at 4°C. At 4°C, the shelf life of the pellets is extended to 6 months. At 6 months, there is a ~50% loss of activity, but this loss of activity does not correspond to a visual loss of signal (**Figure 20**). (Note: this stability study is actually still ongoing, so the shelf life of the pellets at 4°C is potentially even longer than 6 months).

Since any water that remains bound in the reaction after freeze-drying can negatively affect the stability of the FD-CF pellets, I tested the stability of FD-CF pellets that had been freeze-drying for one week (instead of the overnight freeze-dry typically done in all other experiments) and

stored at room temperature with Dri-Cards. Although it does appear that a longer freeze-dry delays the loss in activity of the pellets (ex: 0% loss of activity vs. 50 % loss of activity at 2 weeks), it does not visually seem to make a significant visual difference (**Figure 21**). Combined with the fact that the pellets can be stored at 4°C to significantly extend shelf life, I decided that freeze-drying for a week was an unnecessary and time-consuming step.

With all of these stability studies, the sfGFP DNA plasmid used to test the pellets were stored at the same temperature that its corresponding pellets was stored at, in order to test overall kit stability (as the teachers will likely store the pellets and DNA together). However, I did test to see how long the DNA plasmid could be stored at room temperature (independent of the pellets) by testing the stored plasmid on freshly made FD-CF pellets. I found that the DNA plasmid has a similar shelf life to the FD-CF pellets, where at one month, there is a ~25% loss of activity and a significant visual drop in signal **(Figure 22)**. However, as demonstrated by the other stability studies, this loss of activity can be mitigated by also storing the DNA plasmid at 4°C with the pellets.

From all these stability studies, I conclude that the FD-CF pellets (vacuum-sealed with Dri-Cards) and DNA plasmids that make up the education kit can be shipped at room temperature (with stability up to 3 weeks) and stored at 4°C (with stability up to 6 months). This should be a feasible storage condition as regular fridges have an equivalent temperature and are common in schools.

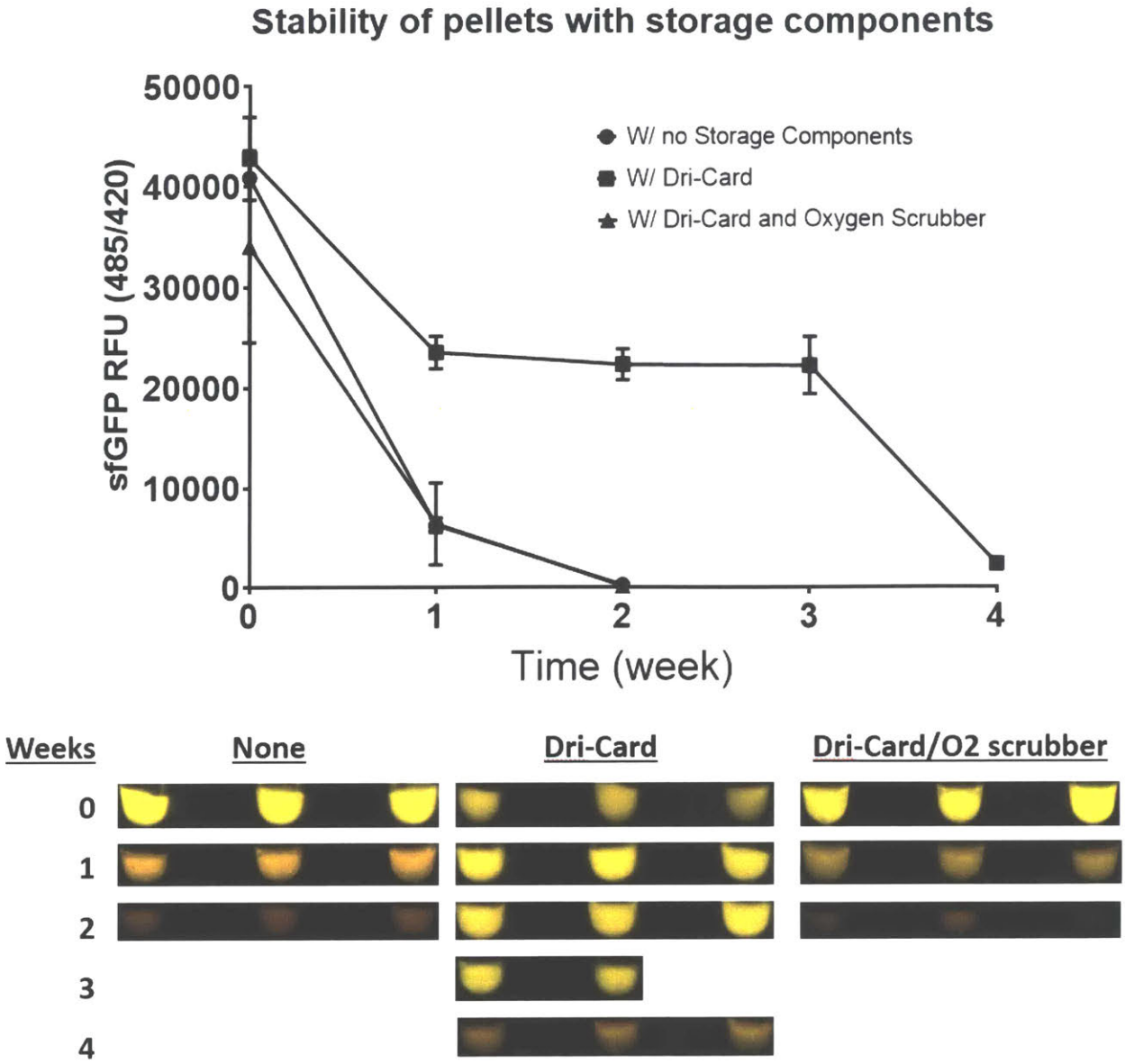


Figure 19: Stability of FD-CF reaction pellets stored at room temperature with additional storage components.

5 uL FD-CF reaction pellets in PCR tubes were stored in vacuum-sealed bags with Dri-Cards, oxygen scrubbers, or no additional components at room temperature. The pellets were tested at each timepoint in triplicate with 1 ng/uL of sfGFP (also stored at room temperature). After a 24 hour incubation at room temperature, the reactions were visualized (470 nm) and quantified on a plate reader for RFU (ex/em 485/520). Note that the 3 week timepoint was done in duplicate (third pellet was lost).

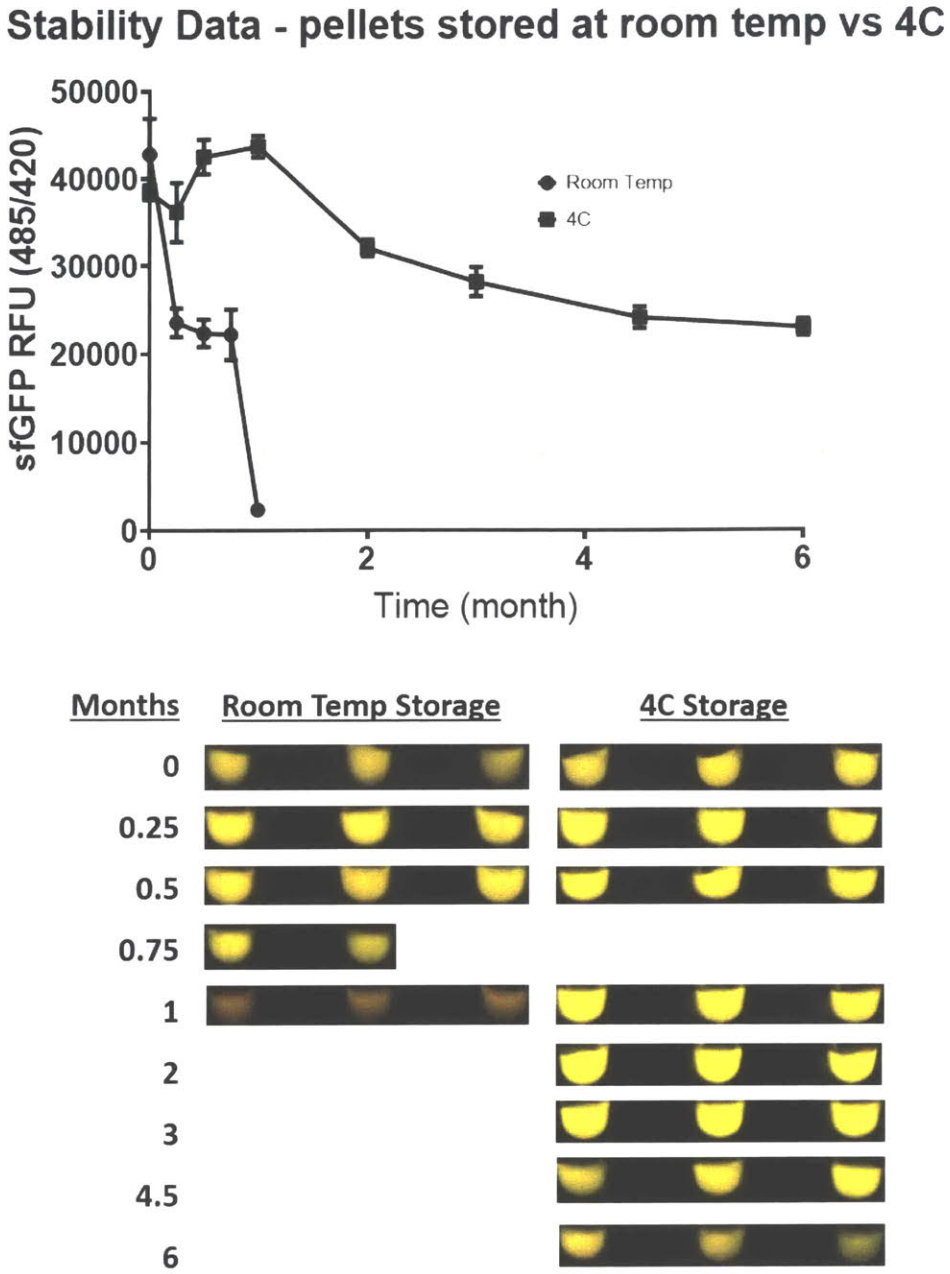


Figure 20: Stability of FD-CF reaction pellets stored at 4°C.

5 uL FD-CF reaction pellets in PCR tubes were stored in vacuum-sealed bags with Dri-Cards at either room temperature or 4°C. The pellets were tested at each timepoint in triplicate with 1 ng/uL of sfGFP (also stored at room temperature or 4°C). After a 24 hour incubation at room temperature, the reactions were visualized (470 nm) and quantified on a plate reader for RFU (ex/em 485/520). Note that the 0.75 month timepoint was done in duplicate (third pellet was lost).

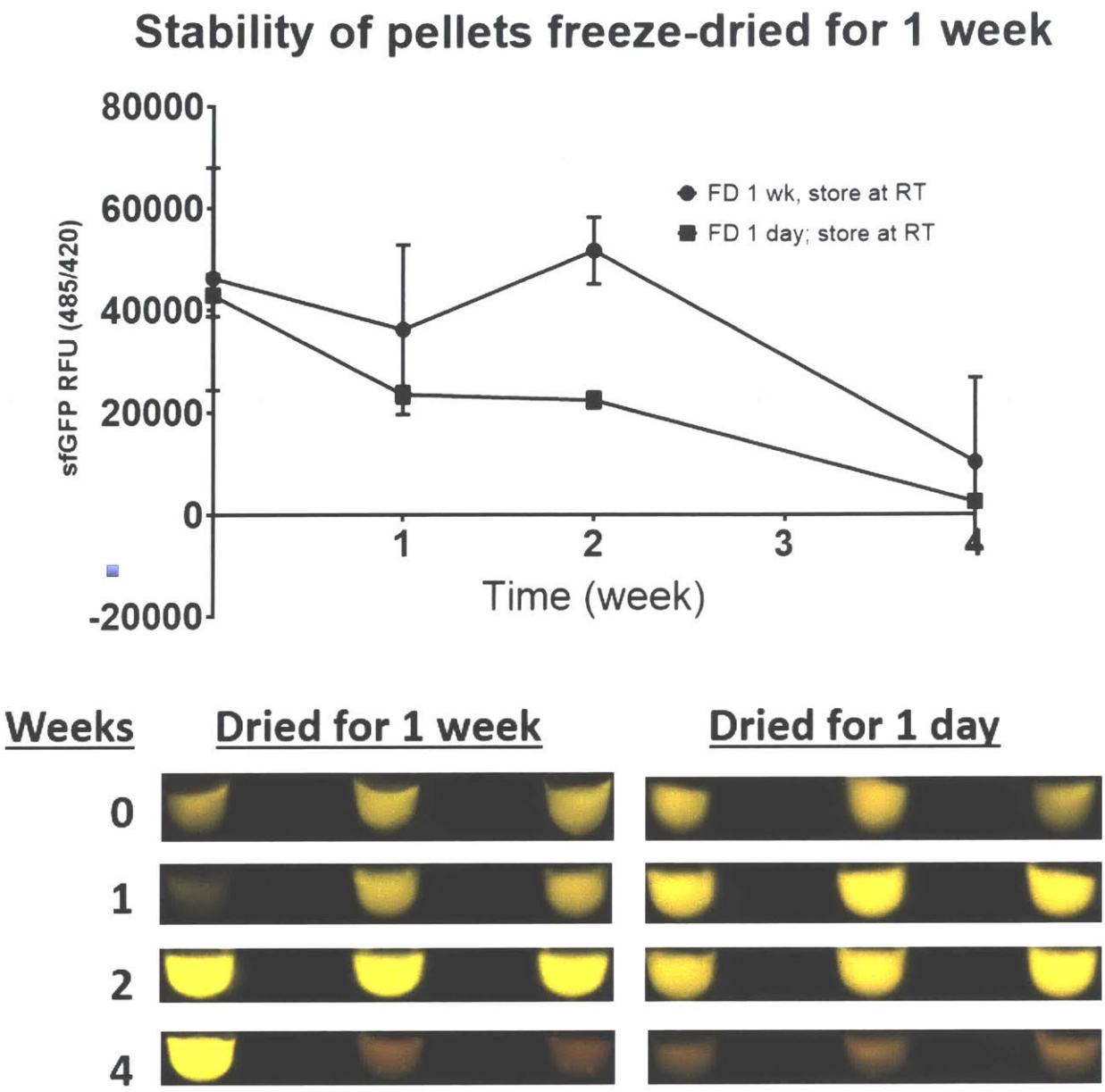
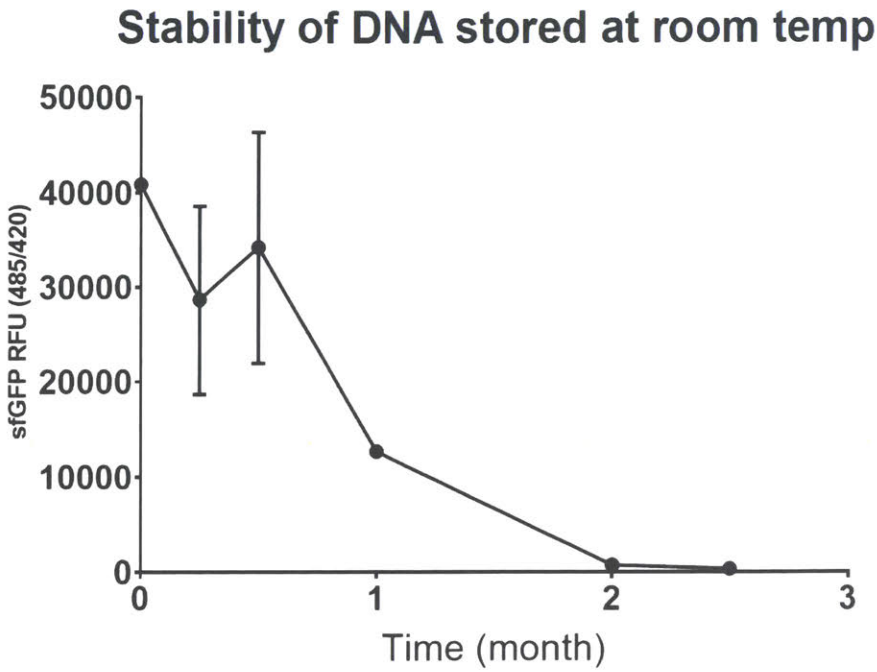


Figure 21: Stability of FD-CF reaction pellets freeze-dried for one week.

5 uL FD-CF reaction pellets in PCR tubes were freeze-dried for either one week or one day and stored in vacuum-sealed bags with Dri-Cards at room temperature. The pellets were tested at each timepoint in triplicate with 1 ng/uL of sfGFP (also stored at room temperature). After a 24 hour incubation at room temperature, the reactions were visualized (470 nm) and quantified on a plate reader for RFU (ex/em 485/520).



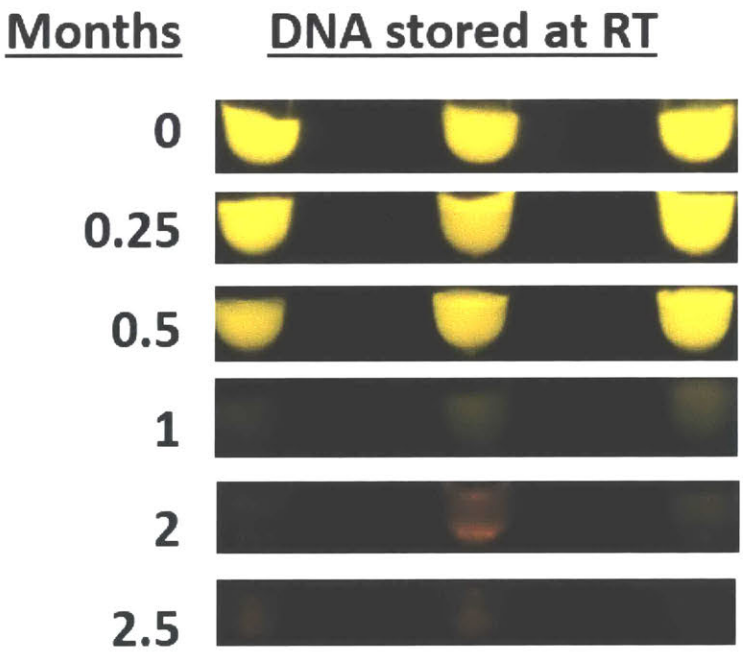


Figure 22: Stability of DNA plasmid stored at room temperature.

sfGFP plasmid was stored at room temperature. Freshly made FD-CF pellets were tested at each timepoint in triplicate with 1 ng/uL of stored sfGFP. After a 24 hour incubation at room temperature, the reactions were visualized (470 nm) and quantified on a plate reader for RFU (ex/em 485/520).

2.5 Development of accompanying hardware

While the FD-CF system circumvents the need for most expensive and specialized equipment, there are still some equipment that are needed to properly carry out and visualize fluorescent protein expression in a classroom setting. Even though the fluorescent proteins are dimly visible by eye without any fluorescent excitation, the colors under fluorescent excitation are much more vibrant and engaging for the students to observe.

Since most schools do not have laboratory transilluminiators, I worked with collaborators at the Wyss Institute and Northwestern University to develop a portable version that accessible for all classrooms (Stark, Huang and Nguyen 2018). We built a handheld, battery-powered imager capable of visualizing the full set of fluorescent proteins. A single 450 nm LED light excites the fluorescent proteins, while transparent orange acrylic is used to filter out the blue light of the LED. The 8-well version of the imager (**Figure 23a**) cost about $15 to build, while the 96-well version (**Figure 23b**) cost about $32 to build.

While the FD-CF reactions can be run overnight at room temperature, some classrooms may want a faster reaction due to time constraints. Heating the reaction to 30°C or 37°C will decrease the time needed before fluorescent proteins are visible. Since most schools do not have laboratory incubators, we also built a portable, USB-powered incubator that can heat to either 30°C or 37°C (**Figure 23c**). Using this portable incubator (costing less than $20 to build), our collaborators at Northwestern found at least 50% of the proteins yields of their fluorescent protein set (which is slightly different from my set but contains the same color families), compared to a traditional laboratory incubator (Stark, Huang and Nguyen 2018).

The CAD files involved in building these portable pieces of equipment are available open-access online (Stark, Huang, Nguyen et al. 2018), allowing teachers and students to build their own imager or incubator to create an interdisciplinary curriculum that ties engineering, biology, fabrication, and electronics together.

2.6 Running FD-CF reactions in a "lab-free" environment

All of these elements (the set of DNA plasmids encoding fluorescent proteins, FD-CF pellets, and the portable imager and incubator) form the basis of the educational kit to teach molecular biology. Paired with either micropipettes or disposable transfer pipettes (**Figure 24a**), this kit is self-contained and classrooms do not need any other reagents or materials to successfully run their own FD-CF reactions. Furthermore, the kit components are easy to use, even for first-time users. Samples run by a PhD student (with extensive laboratory experience), a high school student (with little laboratory experience), and a professor/teacher (with little laboratory experience) using all of these lab-free elements are comparable to samples run with normal laboratory equipment and conditions (**Figure 24b**). Even if there are variations in the reactions due to individual operator (i.e., the professor sample), the sample still results in visible fluorescent proteins, meaning that the kit has a reasonable margin of error for operator error (Stark, Huang, Nguyen et al. 2018).

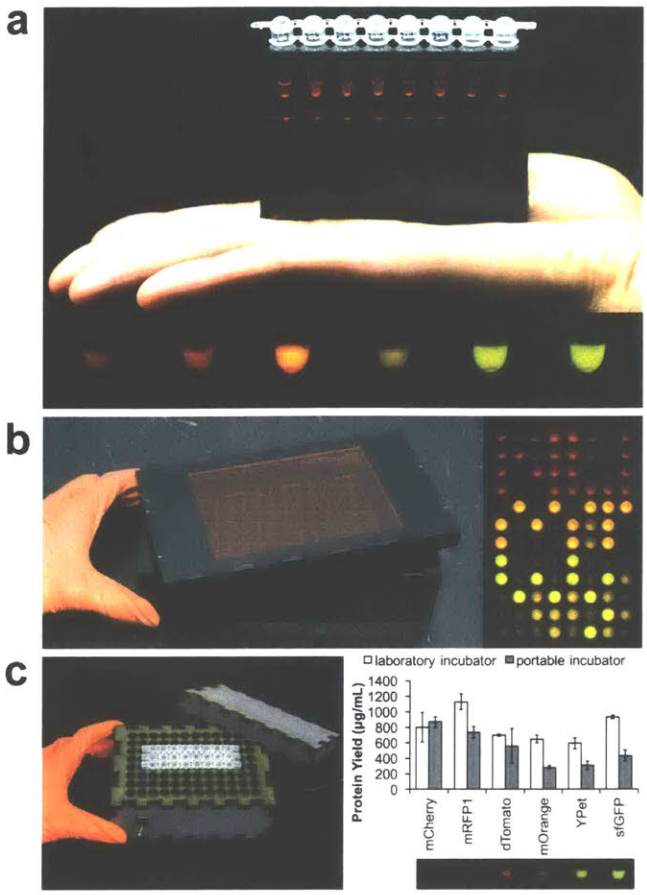


Figure 23: Portable, low-cost lab equipment for teaching outside of the laboratory.

(a) The 8-well imager is handheld and battery operated for easy use (top), and can be used to image the 6-member fluorescent library (bottom). We show FD-CF reactions expressing, from left to right, mCherry, mRFP1, dTomato, mOrange, YPet, and sfGFP. (b) The 96-well imager is also battery-powered and has a removable lid for easy use (left). In vitro biological programs can be imaged using our custom 96-well imager with similar performance as a laboratory imager (right). (c) The portable incubator accommodates up to 96 standard PCR tubes and has a removable, insulating lid for maintaining reaction temperature at its two set points, 30°C and 37°C (left). Fluorescent protein yields using our incubator set at 30°C are at least 50% of those achieved using a laboratory incubator (top right) and produce fluorescence that is clearly visible in our handheld 8-well imager (bottom right). Figure and caption taken from Stark, Huang, Nguyen et al. 2018.

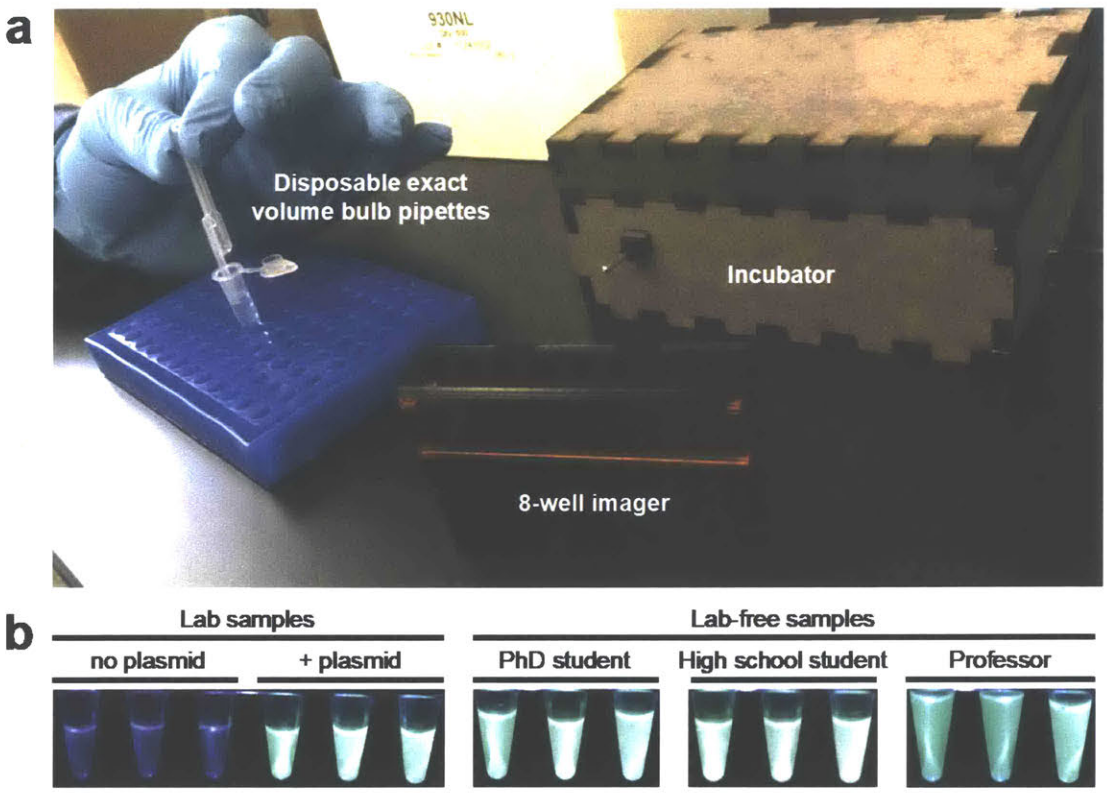


Figure 24: FD-CF reactions can be run in a "lab-free" environment using low-cost, portable imagers and incubator.

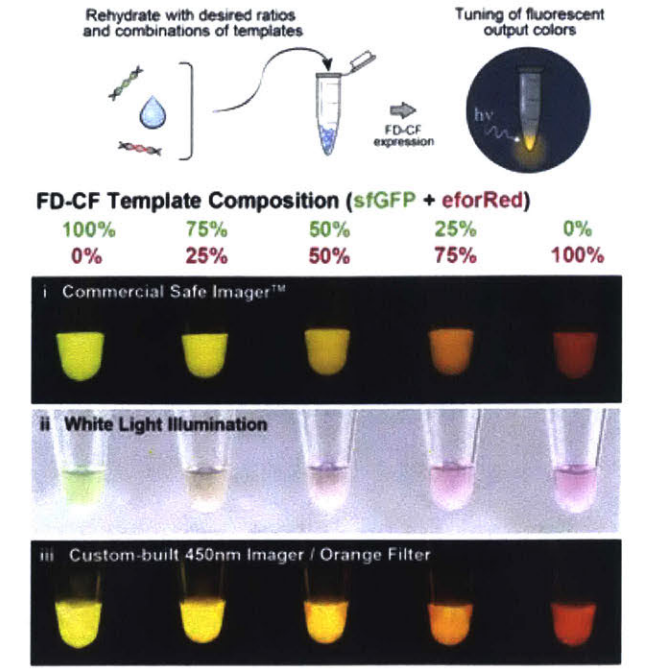
(a) Equipment used in "lab-free" experiments, including disposable 50 μL transfer pipettes, a portable imager, and a portable incubator. (b) sfGFP expression is visually consistent across different experiments and different operators. All tubes images are scaled identically; the volume variations of the reactions are due to individual operator pipetting differences. Figure and caption taken from Stark, Huang, Nguyen et al. 2018.

2.7 Examples of activities using the fluorescent proteins

As an example of how these fluorescent protein outputs can be used to teach molecular biology concepts, we created a demonstration designed to convey the concept of tuning gene expression, a key aspect of synthetic biology. Here we used FD-CF crude extract reactions to co-express two different fluorescent proteins, sfGFP and eforRed, simultaneously in a single reaction. FD-CF

pellets, containing different ratios of each DNA template, were rehydrated with water to achieve a range of intermediate colors from green to red visible both by eye under white light and fluorescently (**Figure 25;** Huang, Stark, Nguyen et al. 2018). This activity can be replicated with DNA templates for any other fluorescent protein pairs in the kit, providing students the freedom to choose the combination of visual outputs they would like to engineer. This activity also provides the opportunity to teach students the concept of the design-build-test cycle, a common paradigm used by synthetic biologists when developing new circuits (Pham et al. 2017). Once students choose the visual output they would like to engineer, they can design an experiment to mix fluorescent proteins in different ratios to achieve their goal. In this example, the build step would involve obtaining FD-CF pellets with the appropriate DNA concentrations. Students would then test their experimental design, evaluate the results, and iterate the process as desired.

Another example of an activity that can be run with this education kit setup to teach a design-build-test cycle is to give students a 96-well plate with FD-CF reactions and the set of DNA plasmids encoding different fluorescent proteins. Students would then draw upon what they have learned from prior activties/curriculum to design a picture using the different fluorescent encoding DNA to obtain different colors and different concentrations of DNA to obtain different color intensities. After designing their pictures on paper, the students then will build their designs by carrying out a series of FD-CF reactions and then test their designs by visualizing their plates using the portable 96-well imager. **Figure 26** shows a few examples of successful designs done by actual students at Northwestern and represents an activity that ties both science and art together, incorporating interdisciplinary STEAM ideas (Stark, Huang, Nguyen et al. 2018).



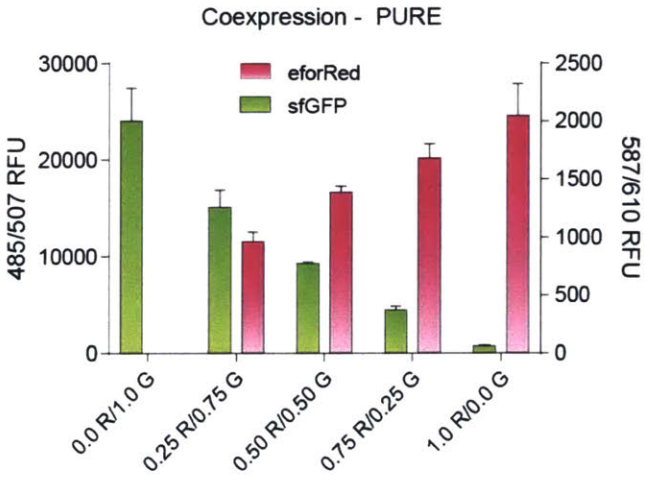


Figure 25: Co-expression of fluorescent proteins.

sfGFP and eforRed fluorescent proteins were expressed at a range of different combinations (by ratio of template DNA added) in FD-CF crude extract and (top) visualized with (i) the SafeImager, (ii) white light, and (iii) the portable handheld imager and (bottom) quantified with endpoint fluorescent readouts. Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

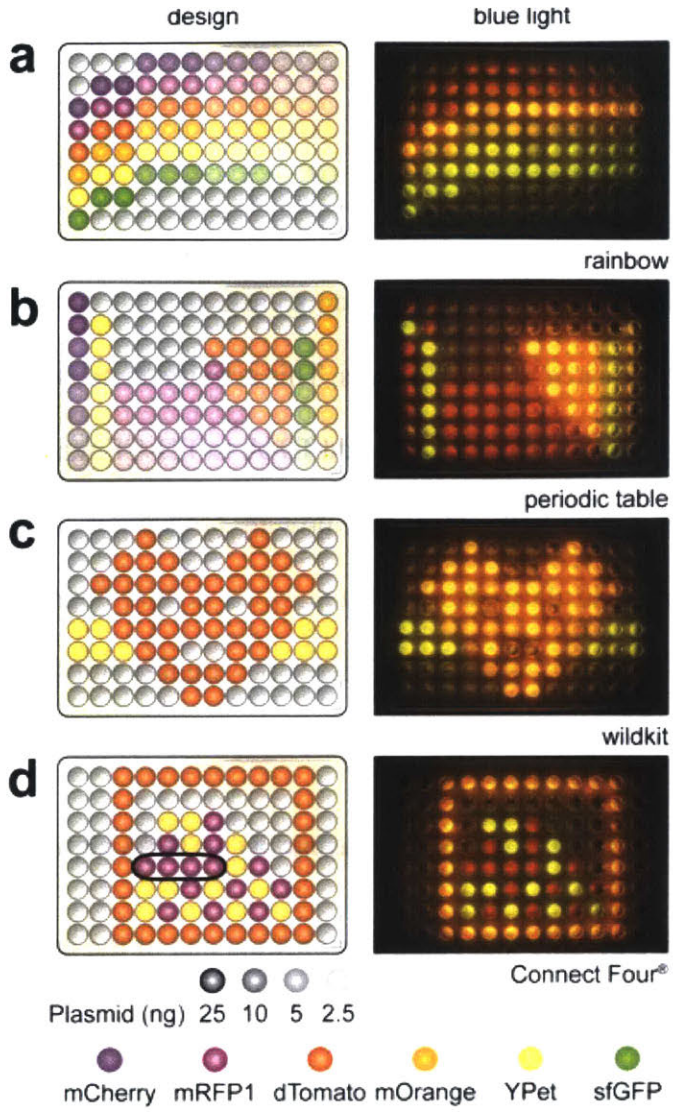


Figure 26: Design and execution of in vitro programs.

Participants were asked to design, build, and test their own in vitro program with DNA in a 96-well PCR plate. Designs could include the mCherry, mRFP1, dTomato, mOrange, YPet, or sfGFP plasmids at concentrations between 0 and 25 ng, denoted with corresponding colors and opacity in the pictured designs (legend, bottom left). Successful designs included (a) a rainbow, (b) a periodic table, (c) a wildkit (the Evanston Township High School mascot), and (d) a game of Connect Four®. These biological programs were designed, built, and tested by untrained operators, demonstrating the potential of this lab for use in a classroom setting. Figure and caption taken from Stark, Huang, Nguyen et al. 2018.

CHAPTER THREE: MODULE TO ILLUSTRATE THE CENTRAL DOGMA OF BIOLOGY

3.1 Introduction

Here, I expand on the FD-CF platform to develop an activity that delve deeper into commonly taught biological concepts related to what DNA is. This activity demonstrates the basic concept of central dogma of biology, where students can add DNA to FD-CF reactions and observe visual fluorescent outputs that signal both RNA and protein production. It is directed towards an introductory biology class and illustrates each step of the central dogma of biology to help the students understand DNA, RNA, and proteins. These user-friendly activities allow demonstrations of specific DNA concepts to help facilitate the understanding of these ideas.

3.2 Development of an aptamer-containing plasmid as an RNA signal

My goal was to develop a module that could demonstrate one of the most fundamental concepts of molecular biology: the central dogma, where DNA transcribes into RNA (transcription) and RNA translates into protein (translation). While there are a few classroom activities available that allow students to transform DNA into cells to express proteins (i.e., a visible green fluorescent protein) (BIO-RAD Laboratories), none of these activities emphasize an RNA-related step. I wanted to develop a module that visually highlighted all the steps of the central dogma, and not just the beginning and end.

3.2.1 Screening aptamer-fluorescent protein pairs

In order to develop a visual signal that indicate transcription, I cloned and screened a few candidate aptamer sequences into my existing T7-driven pJL1-eforRed or-sfGFP plasmids (Stark, Huang,

and Nguyen 2018; Huang, Stark, and Nguyen 2018), between the T7 promoter and the fluorescent protein ribosome binding site. When these plasmid constructs are added to FD-CF reactions, the DNA is transcribed into RNA. The RNA sequence corresponding to the aptamer folds into a specific secondary structure that allows it to bind to conformation-sensitive fluorophore. Once bound by the aptamer, the fluorophore will produce fluorescence. The RNA sequence corresponding to the fluorescent protein gene will then be translated into the fluorescent protein, which fluoresces another color. Ideally, the aptamer and fluorescent protein paired together in the plasmid construct will have 1) distinct output colors so students can easily differentiate the two signals, and 2) have distinct kinetics between the two signals that are visually separated, so students can easily identify the transcription step and the translation step.

Four different aptamers were screened in fresh cell-free PURE reactions: Orange Broccoli, which binds DFHO to produce an orange signal (Song et al. 2017), Mango, which binds T01 to produce a yellow/orange signal (Autour et al. 2018), Red Broccoli, which binds to DFHO to produce a red signal (Song et al. 2017), and Broccoli, which binds to DFHBI-1T to produce a green signal (Filonov et al 2014). These four aptamers were paired and screened with two fluorescent proteins: eforRed (red fluorescence) and sfGFP (green fluorescence). All possible combinations of aptamer and fluorescent proteins were screened, with the exception of Broccoli + sfGFP and Red Broccoli + eforRed because the two signals would be from the same color family.

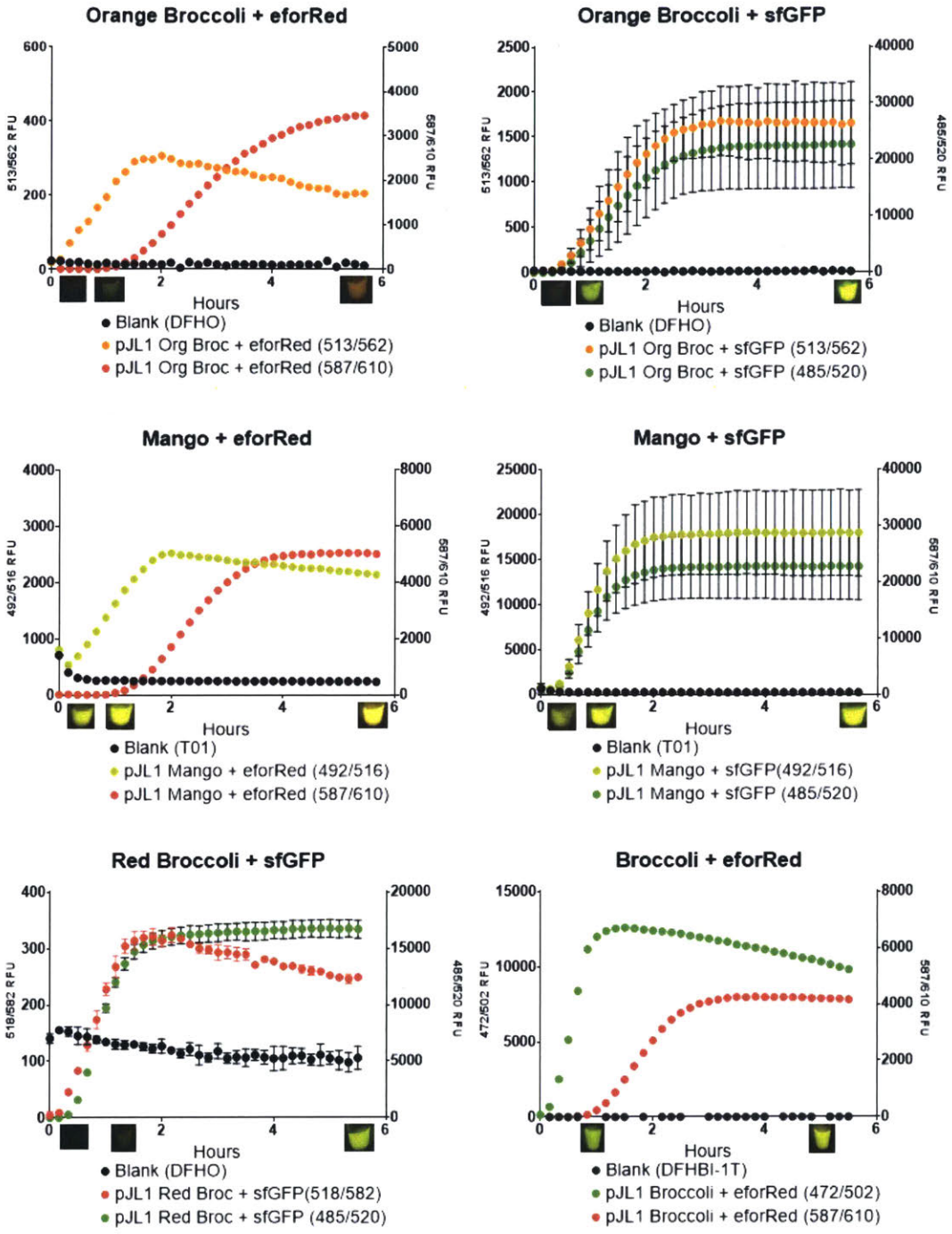


Figure 27: Screening aptamer + fluorescence pairs.

Four aptamers (Broccoli, Orange Broccoli, Red Broccoli, and Mango) were paired with two fluorescent proteins (eforRed and sfGFP) and tested for both their visual and kinetic separation in output signals.

The initial screen shows that sfGFP is not an ideal fluorescent partner – because it expresses so quickly, there is little to no kinetic separation between the green sfGFP signal and the aptamer signal it is paired with. On the other hand, eforRed expresses more slowly, allowing kinetic separation between the aptamer signal and the fluorescent signal (**Figure 27**). Of the three aptamers paired with eforRed, the Mango and the Broccoli were selected for further testing. Orange Broccoli had a very weak signal that was barely visible, while both Mango and Broccoli were bright enough to be visibly detected after only 15-30 minutes.

As this initial screen was completed with fresh cell-free reactions, I tested the two remaining aptamers in an FD-CF system with PURE. While the Broccoli aptamer showed activity in the freeze-dried settings, the Mango aptamer lost all activity once freeze-dried (**Figure 28**). Based on these results, and the fact that Broccoli + eforRed has a greater visual separation than Mango + eforRed, I selected Broccoli + eforRed as the final construct to proceed developing the central dogma activity.

3.2.2 Optimization of the central dogma activity

The schematic of the central dogma activity is shown in **Figure 29a**: the RNA sequence corresponding to the Broccoli aptamer folds into a specific secondary structure that allows it to bind to conformation-sensitive fluorophore called DFHBI. Once bound by the Broccoli aptamer, DFHBI will produce green fluorescence. The RNA sequence corresponding to the eforRed gene will then be translated into the eforRed protein, which fluoresces red.

As the initial tests of the Broccoli + eforRed construct were performed in PURE, I re-optimized the concentration of the construct and DFHBI in the FD-CF crude extracts and determined that 25

ng/uL of Broccoli + eforRed construct and 50 uM of DFHBI provided an optimal visual signal of both the green transcription and the red translation signals.

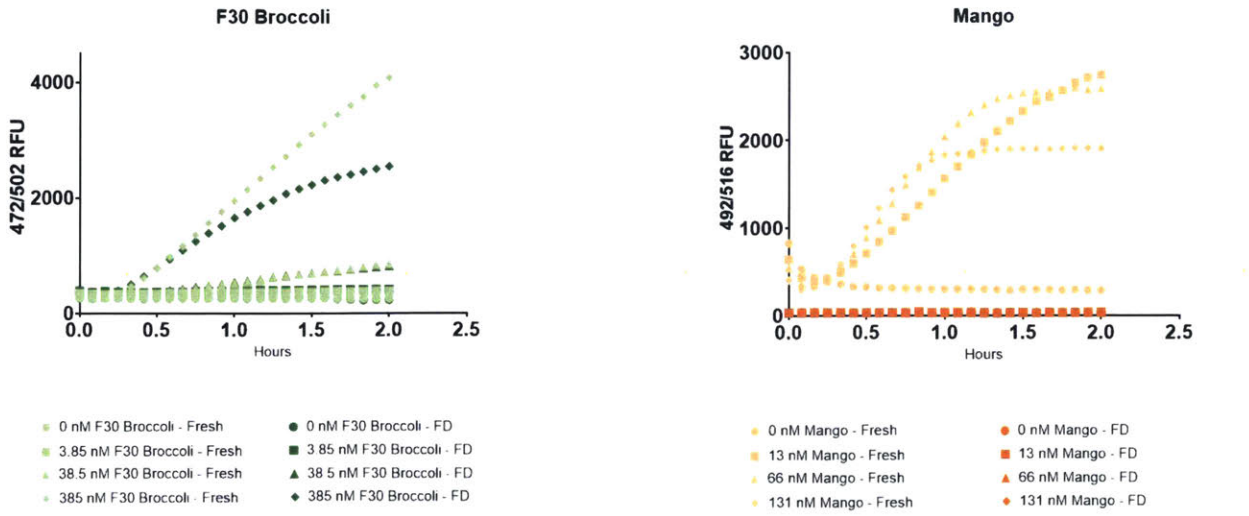


Figure 28: Impact of freeze-drying on aptamer reactions.

FD-CF PURE reactions containing the fluorophores for Broccoli or Mango were made, rehydrated with constructs containing various concentrations of Broccoli or Mango constructs, and compared to fresh CF PURE reactions at the same concentrations.

The distinct kinetics of the two fluorescent signals allows them to be visually separated, so students can easily identify the transcription step and the translation step. The green fluorescent signal activated by bound DFHBI is quickly visible after only ~5-10 minutes of incubation at 37°C. The red fluorescent signal activated by synthesis of the eforRed protein is visible after 2 hours of incubation at 37°C, due to the time required for protein folding and fluorescence maturation (**Figure 29b,c**). The Broccoli aptamer and the eforRed protein do not impact each other's function because their sequences are distinct from each other on the hybrid plasmid construct, they. Notably, there is no significant difference in the green fluorescent signal output between the FD-CF reactions with a plasmid that only contains the Broccoli aptamer and the FD-CF reactions with the

hybrid plasmid (**Figure 29b**). Similarly, there is no significant difference in the red fluorescent signal output between the FD-CF reactions with the hybrid plasmid with or without the DFHBI fluorophore. Visually, the green and red fluorescent signals, when combined in a single reaction, appear orange, but the difference in the kinetics of transcription and translation allows for one to monitor the progression of the reaction by tracking the change in the color gradient from green to red (**Figure 29c**).

3.3 Testing the central dogma activity in actual classrooms

Teachers and educators can use this easy-to-use activity module to illustrate the central dogma of molecular biology. To test how simple this activity is to perform, we had groups of actual high school students test it in a classroom setting. After a simple addition of the DNA to the FD-CF reaction, the students incubated their reactions at 37°C using the previously developed USB-powered portable incubator (Stark, Huang, and Nguyen 2018). After just 15 minutes of incubation, students used the previously developed portable LED-based illuminator (Stark, Huang, and Nguyen 2018) to detect the green fluorescent signal. Seeing the green signal indicated to the students that the DNA they added was transcribed into RNA. The reactions then was incubated overnight in the 37°C incubator, and when the students returned to class the following day, they used the illuminator to detect the red fluorescent signal (which appeared slightly orange due to the green fluorescent signal also in the reaction). Seeing the change in color towards red indicated to the student that the RNA was translated into the red fluorescent protein. To help the students gauge baselines for these reactions, they also ran the Broccoli aptamer + eforRed reaction alongside a Broccoli aptamer only condition (to visualize what green looks like by itself) and a DFHBI-free condition (to visualize what red looks like by itself). These students were able to perform the activity successfully (**Figure 30**) with only minor differences in the results due to human error.

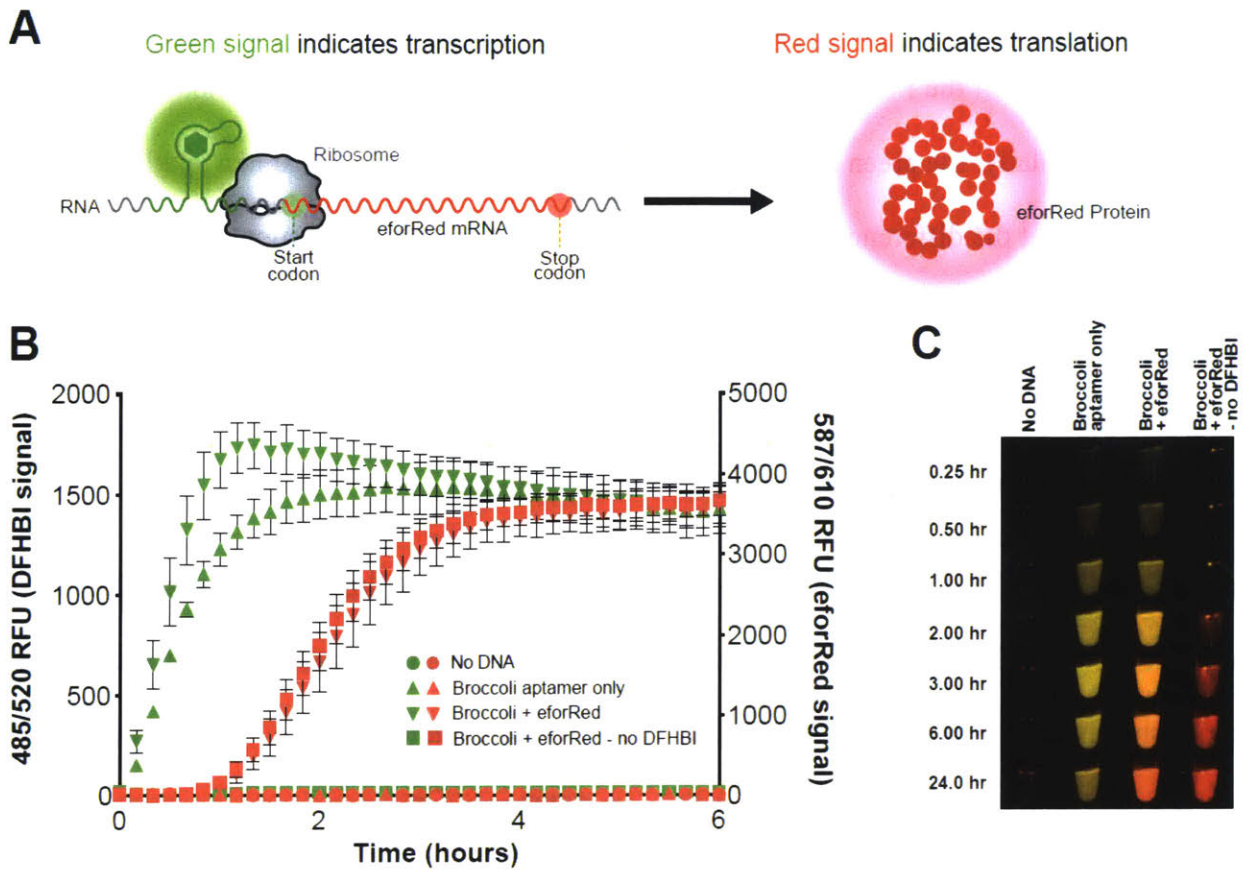


Figure 29: Activity to demonstrate the central dogma.

(A) The Broccoli aptamer RNA sequence folds so it can bind to DFHBI and activate green fluorescence to indicate transcription. The eforRed RNA sequence translates into eforRed protein, which appears as a red fluorescence to indicate translation. (B) Relative fluorescent readouts (n=3) showing the kinetics of all expressed aptamers and/or proteins quantified by plate reader. Values represent averages and error bars represent standard deviations of n=3 biological replicates. (C) Visual timecourse of all the expressed aptamers and/or proteins. Images shown are from a custom-built 450nm handheld imager with an orange acrylic filter.

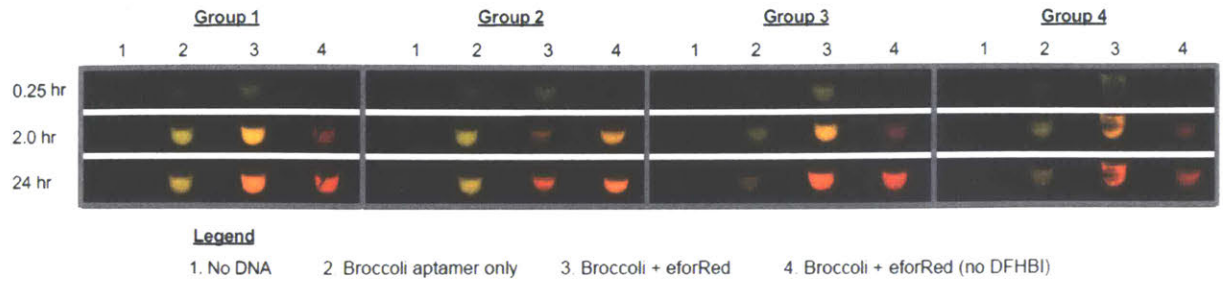


Figure 30: Field testing of central dogma activity.

Four groups of high school students were given the freeze-dried reactions the corresponding DNA and basic instructions of this activity. Shown are the images taken of all the expressed aptamers and/or proteins of the student groups' actual results. Images shown are from a custom-built 450nm handheld imager with an orange acrylic filter.

Feedback for this activity from the test group of students was very positive, with the majority of them commenting that they specifically gained a better understanding of RNA's role in the central dogma that they previously did not have before. The teachers and educators may also want to point out that the kinetics of the central dogma have been engineered to be slower in this particular experiment for demonstration purposes, and that in reality, transcription and translation happen at a much faster timescale.

By creating a construct that contains distinct Broccoli aptamer and eforRed sequences, we were able to develop a module that illustrated each main step of the central dogma of molecular biology. This module will help students break down and conceptualize each step of the process to facilitate in their understanding of this fundamental biology concept.

3.4 Addition of inquiry-based learning elements

Although feedback to this central dogma activity was very positive, there were some concerns that activity was too "black box" – i.e., that the process of transcription and translation is overly simplified in the FD-CF setup, making it difficult for students to conceptually grasp what is happening in their reactions to result in the red and green signals. To alleviate these concerns, I developed a few extensions to the central dogma activity that allow students to manipulate and target specific processes in the reactions. This potentially can provide more context to what is happening in their FD-CF reactions at a molecular/cellular level in an inquiry-based learning context.

3.4.1 Inhibition of transcription by targeting promoter sequences

The main three methods that transcription can be specifically inhibited in the FD-CF reactions is by either 1) targeting the RNA polymerase, 2) targeting the promoter sequence in the plasmids, or 3) targeting the DNA. Of these methods, targeting the RNA polymerase would be logistically difficult from a production perspective because the crude extracts are produced with overexpressed T7 RNA polymerase (Kwon and Jewett 2015). Omitting or altering the T7 RNA polymerase expression in the crude extracts would require a separate batch of extracts that need to be produced and labeled in the kit. While DNA could be easily targeted with the addition of an enzyme, such as DNAse or a specific restriction enzyme, those enzymes would not be stable to ship at room temperature and would add an additional cost to the kit.

On the other hand, targeting the promoter sequence would only require cloning new DNA constructs, which can be easily and inexpensively produced. All of the plasmids used in our crude FD-CF platform contain a T7 promoter, corresponding to the T7 RNA polymerase in the FD-CF

reactions. To demonstrate that a specific pairing of the promoter and RNA polymerase is needed, I cloned versions of the sfGFP construct that contained different promoter sequences, a T3 and an SP6 sequence (Jorgensen et al. 1991). I then tested these new constructs against the original T7-sfGFP construct in crude FD-CF reactions.

After 24 hours of incubating at room temperature, the reaction containing the T7 construct show a strong sfGFP signal, but the reactions containing the T3 or SP6 constructs do not show any sfGFP signal at all (**Figure 31**). As an extension of the central dogma activity, students could be given the sequences of these three constructs (unlabeled) and tasked with researching the promoter sequences of T7, T3, and SP6 to determine which construct to use in their FD-CF reaction to obtain a sfGFP signal (**Figure 32**).

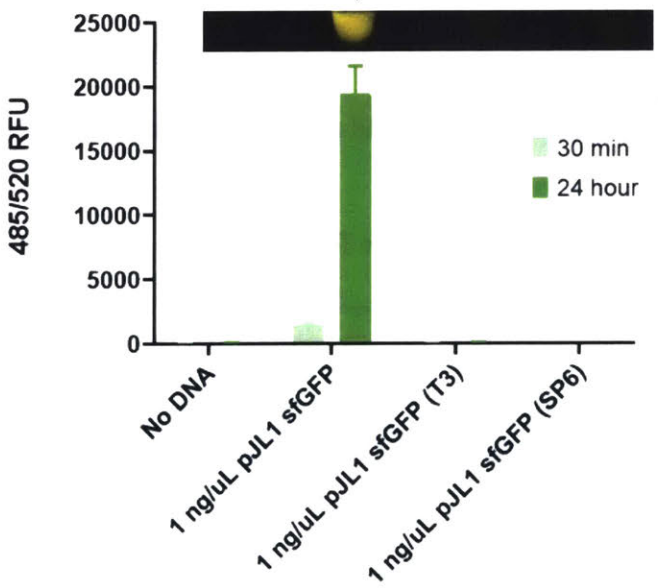


Figure 31: Testing non-T7 promoter sequences in the T7 FD-CF crude system.

sfGFP constructs containing either T7, T3, or SP6 promoter sequences upstream of the sfGFP sequence were added to 5 uL FD-CF reactions at 1 ng/uL, incubated at room temperature overnight, quantified at 30 minutes, and visualized/quantified the next day.

T3	AATTAACCCTCACTAAAGGGAGA
T7	TAATACGACTCACTATAGGGAGA
SP6	ATTTAGGTGACACTATAGAAGNG

Figure 32: Table of different bacteria promoter sequences.

Table containing the different promoter sequences for T7, T3, and SP6. Students can be given this information and tasked with determining which one will work in the FD-CF system. Sequences taken from Jorgensen et al. 1991.

A follow-up activity could also have the students add T3 or SP6 RNA polymerases to the FD-CF reactions to obtain a sfGFP signal with the T3 or SP6 constructs. The addition of the appropriate RNAP does help "rescue" the reaction and allow for sfGFP expression (**Figure 33**), although further optimization will be needed to determine reaction conditions that will bring the sfGFP expression up to similar levels of the T7 construct. I also note that those polymerases would not be stable to ship at room temperature and would add an additional cost to the kit.

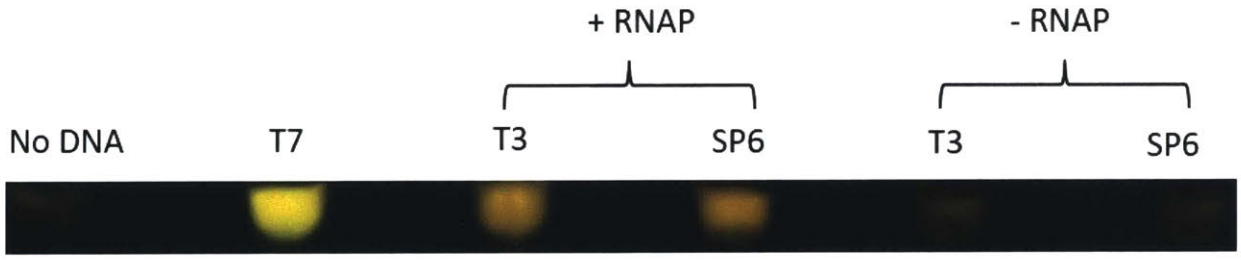


Figure 33: "Rescuing" non-T7 promoter sequences in the T7 FD-CF crude system by adding RNAP

sfGFP constructs containing either T7, T3, or SP6 promoter sequences upstream of the sfGFP sequence were added to 5 uL FD-CF reactions at 1 ng/uL with or without the appropriate RNAP at 2.5 u/uL (T3 RNAP for T3 and SP6 RNAP for SP6), incubated at room temperature overnight and visualized the next day.

To demonstrate that this concept could also be done in the central dogma activity, I cloned versions of the broccoli-eforRed construct that contained different promoter sequences, a T3 and an SP6 sequence. I then tested these new constructs against the original T7-broccoli-eforRed construct in crude FD-CF reactions.

After 30 minutes of incubating at 37C (typical classroom length of time), the reaction containing the T7 construct show a strong DFHBI (green) signal, but the reactions containing the T3 or SP6 constructs do not show any green signal at all. After 24 hours of incubating at room temperature, the reaction containing the T7 construct show a strong eforRed signal, but the reactions containing the T3 or SP6 constructs do not show any eforRed signal at all (**Figure 34**). This can be used to demonstrate how the promoter sequence impacts transcription and how that also impacts translation, as translation is sequential after transcription.

Another method to target the RNA polymerase – promoter sequence interaction is to introduce a competitive element into the FD-CF reactions that would sequester the RNA polymerase away from the construct of interest. To test this, I ran FD-CF reactions containing both the sfGFP construct as well as another construct that contained the T7 promoter sequence upstream of a non-fluorescent protein (in this case, a sortase enzyme) or a construct that contained the T7 promoter sequence with no protein coding. An increase in the amount of competitive T7 promoter-sortase and T7-promoter only construct decreases the sfGFP expression, both within 30 minutes of the reaction and overnight (**Figure 35**). The T7-sortase construct decreases the sfGFP expression more than the T7-only construct at the same DNA concentrations. This is likely due to the fact that the T7-sortase construct competes with both transcription resources (i.e., the RNA polymerase) as well as translation resources (i.e., ribosomes), while the T7-only construct solely competes with the transcription resources and thus will have less of a negative impact on sfGFP expression. For the

purpose of this educational activity however, I would choose to include the T7-only construct over the T7-sortase construct because it is a better representation of transcription repression.

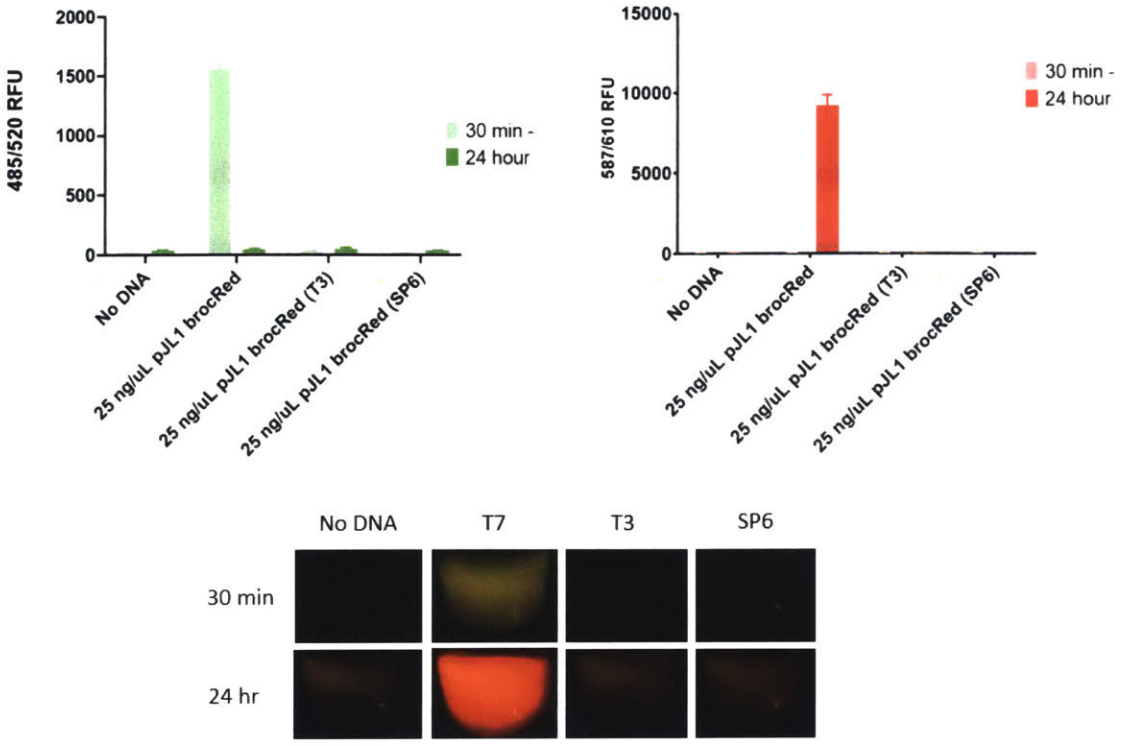


Figure 34: Testing non-T7 promoter sequences in the T7 FD-CF crude system in the central dogma activity.

broccoli-eforRed (brocRed) constructs containing either T7, T3, or SP6 promoter sequences upstream of the broccoli-eforRed sequence were added to 5 uL FD-CF reactions at 25 ng/uL, quantified/visualized at 30 minutes (following incubation at 37C) and at 24 hours (following incubation at 37C).

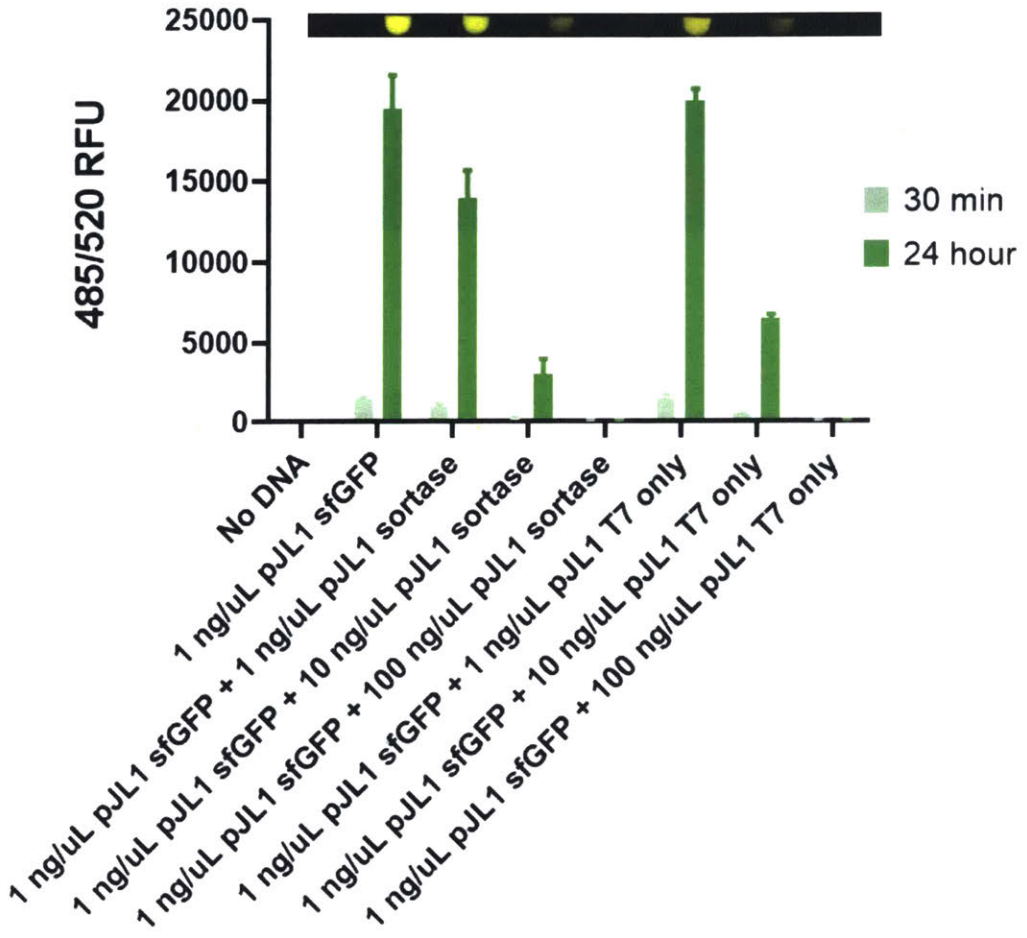


Figure 35: Inhibition of sfGFP expression with competitive constructs.

The sfGFP construct was added to FD-CF reactions with an increasing concentration of a competitive construct that also contained a T7 promoter sequence and coded for a non-fluorescent protein (sortase) or a construct that contained T7 promoter sequence with no downstream coded protein. The reactions were incubated at room temperature and (top) quantified at 485/520 ex/em at 30 minutes and (bottom) quantified at 485/520 ex/em and visualized after 24 hours.

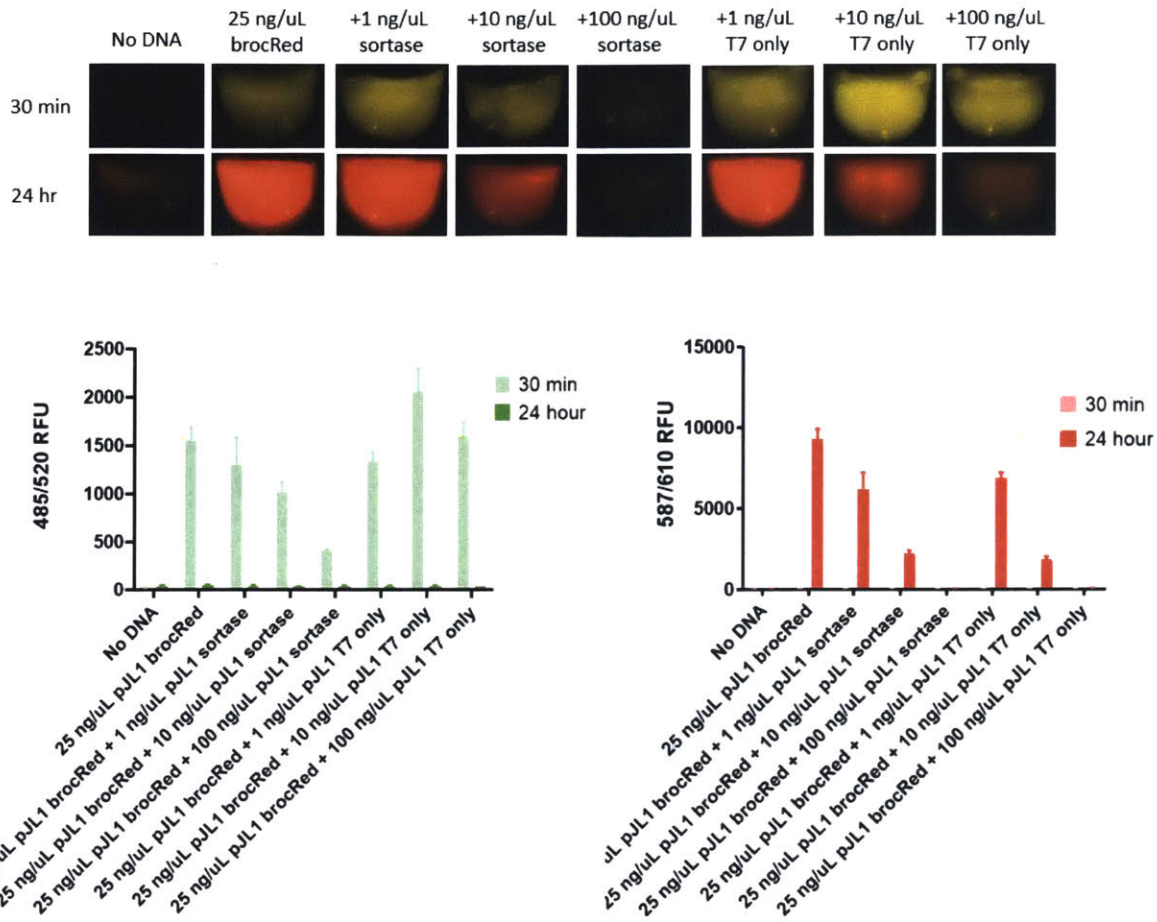


Figure 36: Inhibition of brocRed expression with competitive constructs.

The brocRed construct was added to FD-CF reactions with an increasing concentration of a competitive construct that also contained a T7 promoter sequence and coded for a non-fluorescent protein (sortase) or a construct that contained T7 promoter sequence with no downstream coded protein. The reactions were incubated at 37C for 30 minutes and visualized/quantified at 485/520 and 587/610 ex/em and then incubated overnight at room temperature and visualized/quantified at 485/520 and 587/610 ex/em.

To demonstrate that this concept could also be done in the central dogma activity, I ran FD-CF reactions with the same competition constructs, except with the brocRed construct. After 30 minutes of incubating at 37C (typical classroom length of time), all of the reaction containing the brocRed construct show a strong DFHBI (green) signal, regardless of how much competition

construct was added, except for the condition where 100 ng/uL of sortase construct is added. After 24 hours of incubating at room temperature, an increase in the amount of competitive T7 promoter-sortase and T7-promoter only construct decreases the eforRed expression, with no significant difference between the rate of decreased expression between the two competitive constructs (**Figure 36**). Additional exploration and optimization will have to be made with this experiment to determine what is happening from a transcription and translation standpoint (i.e., why the T7 competition constructs seem to only be affecting translation, not transcription), so that can be incorporated into the central dogma activity.

3.4.2 Inhibition of translation with antibiotics

The main three methods that translation can be specifically inhibited in the FD-CF reactions are by either 1) targeting the ribosomes, 2) targeting the ribosome binding sequence in the plasmids, or 3) targeting the RNA. Of these methods, while RNA could be easily targeted with the addition of an enzyme, such as RNAse, those enzymes would not be stable to ship at room temperature and would add an additional cost to the kit.

Ribosomes can be targeted with the addition of specific ribosome-targeting antibiotics and the ribosome binding sequence can be targeted by cloning new DNA constructs. Here, I chose to target the ribosomes with antibiotics because of the potential to tie real-world issues, like antibiotic resistance, into the extension activity.

Kanamycin and carbenicillin were selected as the two antibiotics to test because kanamycin specifically targets ribosomes, and thus should disrupt translation in FD-CF, while carbenicillin targets cell wall synthesis, and thus should have no effect because FD-CF reactions do not have cell walls. FD-CF reactions with the Broccoli + eforRed construct were incubated at room

temperature with various concentrations of kanamycin and carbenicillin. I expected to not see any impact on the transcription (i.e., the green aptamer signal) with the addition of the antibiotics because neither of them target the transcription process. However, increased kanamycin in the FD-CF reactions did seem to have a negative impact on the aptamer signal **(Figure 37)**. This could be due to off-target effects of kanamycin at higher concentrations. The lower concentrations (0.1 uM – 1 uM) do not have a significant impact on the aptamer signal and can be used to prevent off-target targeting of the aptamer or transcription. Carbenicillin, at any of the concentrations tested, did not have a significant impact on the aptamer signal (**Figure 37**).

As expected, the addition of kanamycin to the FD-CF reactions inhibited the expression of eforRed expression (**Figure 37**). 0.1 uM of kanamycin was enough to completely inhibit any visible eforRed fluorescent signal. Also as expected, the addition of carbenicillin (at 0.1-1 uM) to the FD-CF reactions did not inhibit the expression of eforRed expression. There appeared to be some inhibition at 10 uM carbenicillin from the quantification, but visually, there was no significant difference (**Figure 37**).

This extension activity can be used to 1) highlight the translation process in the FD-CF reactions and how it can be inhibited and 2) introduce antibiotics, their mechanisms of action, and their real-world applications and issues.

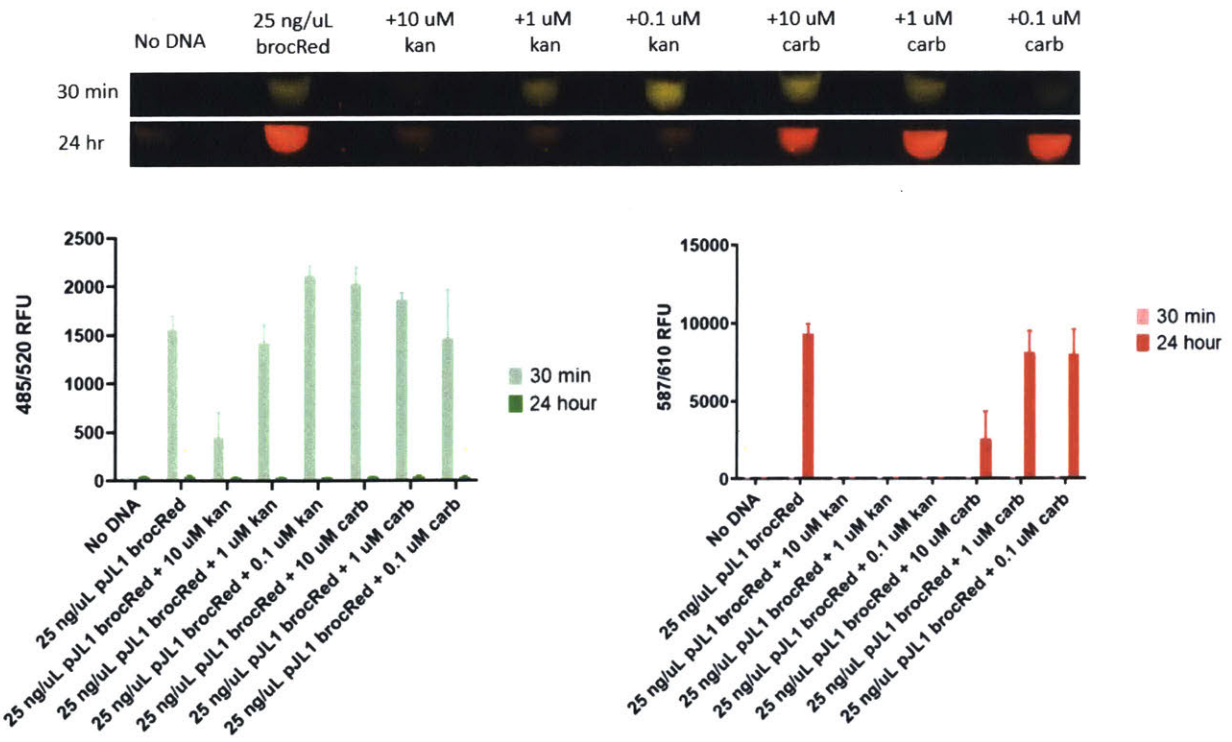


Figure 37: Impact of antibiotics on FD-CF broccoli aptamer eforRed reactions. FD-CF reactions were rehydrated in triplicate with 25 ng/uL of pJL1 broccoli+eforRed plasmid and varying concentrations of kanamycin or carbenicillin. During the 30 minutes, the reactions were incubated at 37°C and quantifiied/visualized on a plate reader at 485/520 ex/em. Afterwards, the reactions were incubated at room temperature and quantified/visualized the next day at the 24 hours timepoint.

CHAPTER FOUR: MODULES TO ILLUSTRATE OTHER BASIC CONCEPTS

This chapter is in part adapted from:

Huang, A., Nguyen, P.Q., Stark, J.C., Takahashi, M.K., Donghia, N., Ferrante, T., Dy, A.J., Hsu, K.J., Dubner, R.S., Pardee, K., et al. (2018). BioBits™ Explorer: A modular synthetic biology education kit. Sci. Adv. *4*, eaat5105.

Stark, J.C., Huang, A., Nguyen, P.Q., Dubner, R.S., Hsu, K.J., Ferrante, T.C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J.S., et al. (2018). BioBits™ Bright: A fluorescent synthetic biology education kit. Sci. Adv. *4*, eaat5107.

4.1 Introduction

Besides teaching the central dogma, the FD-CF fluorescent outputs can also be used to teach other basic molecular biology concepts, as well as tie into real-world issues. Here, I developed an activity that explores protein structure/function and an activity that demonstrates antibiotic resistance. While the fluorescent protein outputs rely on sight, I also wanted to engage as many of the five senses as possible to pique students' interest in the activities. I developed a set of demonstrations designed to engage smell, and touch, through the expression of proteins in FD-CF reactions that produce enzyme-generated fragrances and large-scale hydrogels, respectively. These outputs can be used to create activities to teach fundamentals of protein expression, enzyme catalysis, and properties of biomaterials. These activities can be run on their own or in sequence with the other developed FD-CF activities, which are paired with low-cost, portable lab equipment and supporting curriculum in a kit.

4.2 Protein structure/function relationship

One of the concepts included in the Next Generation Science Standards (NGSS) (National Research Council 2012) and the Advanced Placement (AP) Biology requirements (College Board 2015) is the idea of the protein structure/function relationship, i.e., that the structure of the protein is essential to its function. The initial fluorescent protein activities already touch upon this concept – students add different DNA plasmids to the FD-CF reactions and end up with different fluorescent proteins. Teachers can help explain that the different colors they see is due to the different structures of each of the fluorescent proteins.

Here, I developed a potential module that explores this idea more closely. The set of fluorescent proteins that I used before included sfGFP (super folder green fluorescent protein), which has been specifically engineered prevent protein misfolding, and thus, expresses faster and brighter than regular GFP (Heim and Tsien 1996). I cloned a regular GFP sequence into the plasmid backbone and compared the expression of GFP vs sfGFP in FD-CF. After less than one hour of incubation at room temperature, sfGFP has a much higher fluorescent signal than GFP. After 24 hours of incubation at room temperature, this trend still holds, with sfGFP visually and quantitatively more fluorescent than GFP (**Figure 38**).

A suggested activity designed around this experiment would be to give the students the DNA and/or protein sequences to both GFP and sfGFP and have them align the sequences in an open-source gene editor or protein database. This will allow the students to see all the different changes made in the GFP sequence in order to create sfGFP. After seeing the sequence and the protein scaffolding model (**Figure 39**) of GFP (Pédelacq et al. 2016), students then can carry out their own sfGFP vs. GFP FD-CF experiments to see the difference in protein function (in this case,

fluorescence) first hand. Discussions can also be included regarding how protein structure-function relationships can play a role in disease, therapeutic development, and other relevant topics.

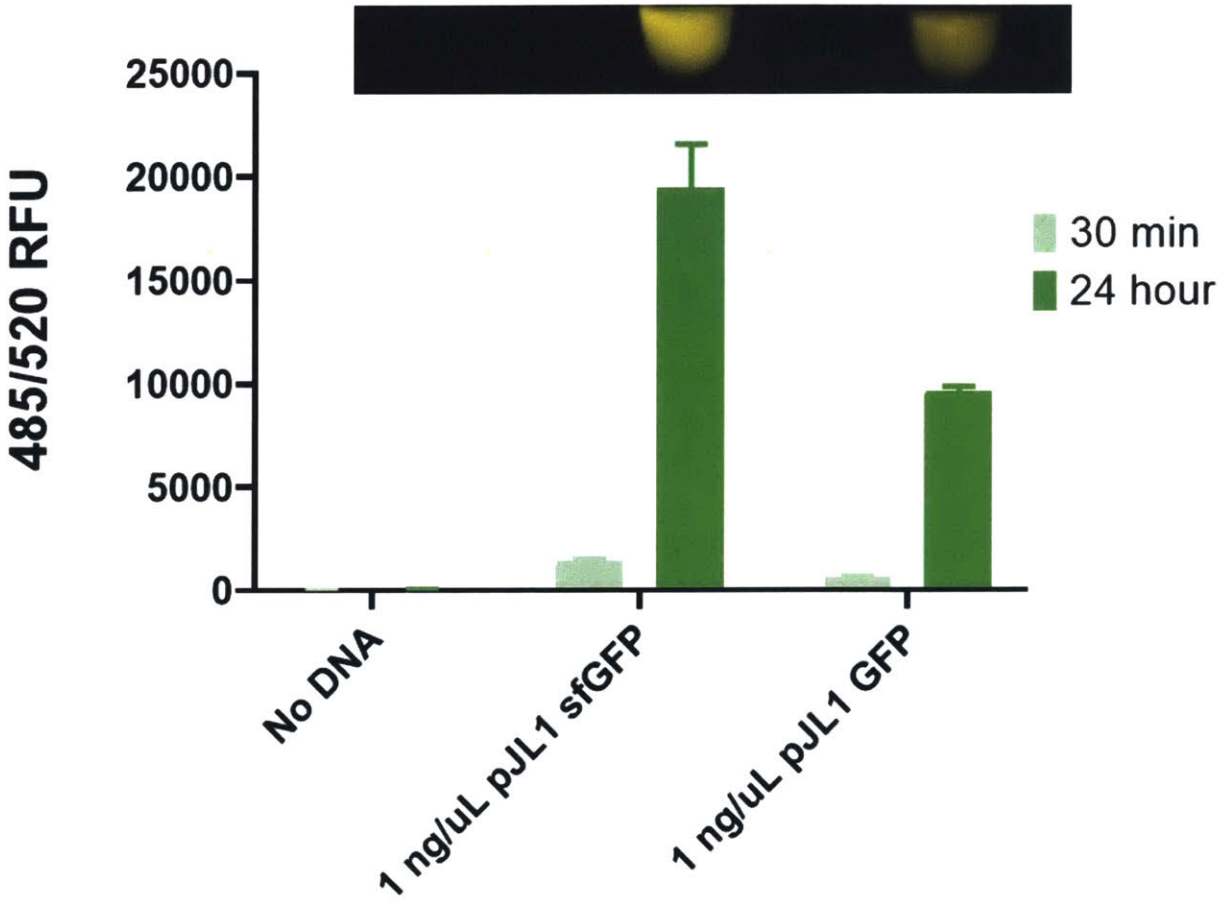
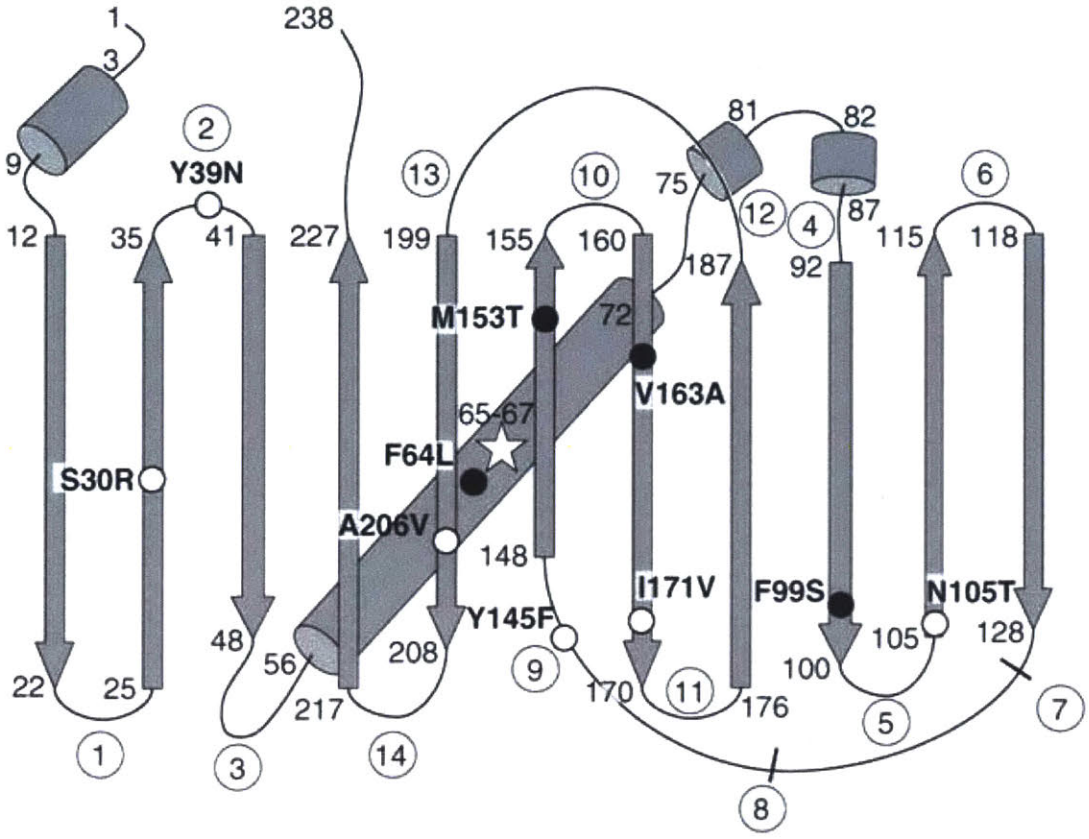


Figure 38: FD-CF reactions of sfGFP vs. GFP to demonstrate protein structure-function relationships.

Varying concentrations of sfGFP or GFP encoding DNA plasmids were added to 5 uL FD-CF reactions in triplicate. Reactions were incubated at room temperature and quantified on a plate reader (485/520 ex/em) at 30 minutes. Following an overnight incubation at room temperature, reactions were quantified and visualized.

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GFP       MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTL 60
sfGFP     MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATIGKLTLKFICTTGKLPVPWPTL 60
          ***************************** ******** ********************

GFP       VTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLV 120
sfGFP     VTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGKYKTRAVVKFEGDTLV 120
          ************************************ *****:***** *********

GFP       NRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLAD 180
sfGFP     NRIELKGTDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFTVRHNVEDGSVQLAD 180
          ******* ***************:******* ********.**.:***:*********

GFP       HYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEK--RDHMVLLEFVTAAGITHGMDELYK 238
sfGFP     HYQQNTPIGDGPVLLPDNHYLSTQTVLSKDPNEKGTRDHMVLHEYVNAAGITWSHPQFEK 240
          ************************:.******** ****** *:*.***** .  :: *
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Figure 39: Schematic and sequences of a folded sfGFP protein.

(Top) A schematic such as this one can be used to show students what the protein sequence will look like and identify the specific spots where a mutation was engineered in the original GFP to create sfGFP. Figure taken from Pédelacq et al. 2016. (Bottom) Students can align the given protein sequences to identify where all the mutations were made in the sequence.

4.2 Demonstrating antibiotic resistance

In Chapter Three, I demonstrated the impact of adding antibiotics to the FD-CF reactions, allowing students to understand translation more in-depth by providing an inquiry-based learning context. Here, my collaborators from Northwestern and I build on this module by adding additional hands-on experiments that introduce the concept of antibiotic resistance. Antibiotic resistance is a current relevant health issue, as it is projected to result in about 10 million deaths by 2050 (O'Neill 2014), therefore it is important for students to understand what antibiotic resistance is and the potential mechanisms of action. Since antibiotic resistance can occur by two different methods (horizontal gene transfer and chromosomal DNA mutations), we developed two different FD-CF experiments that demonstrate each of these two methods (Stark et al. 2019).

To demonstrate horizontal gene transfer, we used FD-CF to express two enzymes that confer antibiotic resistance: aminoglycoside O-phosphotransferase (NeoR), which confers kanamycin resistance, and streptomycin 3'-adenylytransferase (AadA), which confers streptomycin resistance. We then used another FD-CF reaction to express a fluorescent protein (dTomato) with various concentrations of kanamycin or streptomycin, as well as the pre-expressed NeoR or AadA enzymes. The reactions without the antibiotic resistance enzymes show decrease in dTomato expression at higher antibiotic concentrations, but the addition of the enzymes can "rescue" the FD-CF reaction from the effects of the antibiotic and allow for dTomato expression (**Figure 40**; Stark et al 2019).

Figure 40: Demonstrating horizontal gene transfer of antibiotic resistance in FD-CF reactions. Kanamycin (neoR) and streptomycin (aadA) resistance genes were pre-expressed in FD-CF reactions for 20 hours at 30°C 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 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 (done by Jessica Stark). When representative FD-CF reactions from are imaged using a low-cost blue-light imager, differences in results using wild-type and resistant reactions can be distinguished by eye. Figure and caption from Stark et al. 2019.

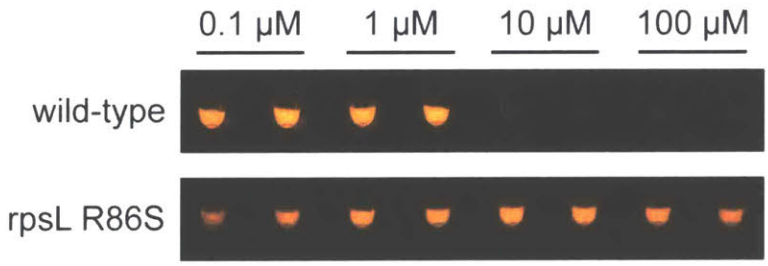


Figure 41: Demonstrating genetic mutation of antibiotic resistance in FD-CF reactions. We generated lysate from cells with an R86S mutation in the rpsL gene, which are resistant to streptomycin. FD-CF reactions with the resistant lysates also retain the ability to synthesize dTomato in the presence of up to 100 μM streptomycin (done by Jessica Stark). 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 and caption from Stark et al. 2019.

To demonstrate genetic mutation, we engineered a R86S mutation into the *rpsL* gene (codes for ribosomal protein S12) in the E.Coli cells that we typically use to make crude extract. This mutation prevents streptomycin from binding to the ribosome and inhibiting translation (Edgar et al. 2012). Using these mutant cells, we generated crude extract to make FD-CF reactions that were streptomycin resistant. When streptomycin is added to these FD-CF reactions to express dTomato, the mutation appears to block the effects of the antibiotic and allow for dTomato expression (**Figure 41**; Stark et al 2019).

These extension modules on the antibiotic activity not only demonstrates the mechanisms of action of antibiotic resistance, it also allows for classroom discussion about the broader issues surrounding antibiotic use and misuse.

4.3 Fragrance-generating enzymes as olfactory outputs

Next, I developed an output that would engage students' sense of smell. To do so, I expressed the alcohol acetyltransferase (ATF1) enzyme in FD-CF PURE and crude reactions overnight (20 hours) at 37°C (**Figure 43**). ATF1 is a key enzyme in aroma biochemistry (Dixon and Kuldell 2011, Jordán et al. 2001) that converts isoamyl alcohol to isoamyl acetate, which imparts a strong banana fragrance (**Figure 42a**). FD-CF reactions expressing ATF1 were then mixed at a 1:10 dilution into a buffered reaction containing 25mM isoamyl alcohol and 5mM acetyl-CoA. The enzymatic reactions were allowed to proceed 20 hours at room temperature, after which we were able to detect strong banana scents by smell.

To verify this objectively, I set up identical enzymatic reactions in FD-CF PURE during which the vapor phase of the reaction was collected and analyzed by GC-MS. GC-MS analysis confirmed the presence of isoamyl acetate at ~5.3 ppm (volatile phase), which is well within the odor

detection threshold for this compound (0.00075 – 366 ppm), but below the permissible exposure limit of 100 ppm (**Figure 42b**) (Murnane, Lehocky, and Owens 2013). Thus, this FD-CF generated aromatic can be readily detected by nose in a classroom by the average student. ATF1 can convert various long-chain alcohol substrates to the corresponding acetylated esters, which have different fragrances (Yoshioka and Hashimoto 1981, Malcorps and Dufour 1992). Incubation of ATF1-expressing FD-CF reactions with the substrates hexanol and octanol, for example, could generate volatile products that encompass pear and citrus smells, respectively (**Figure 42a**).

Production of these olfactory outputs can be used to teach students about basic enzymatic reactions, and provides a great connection to lessons learned in their chemistry classes. For example, here, we set up the enzymatic reactions containing isoamyl alcohol with and without FD-CF-produced ATF1 to show that the enzyme must be present to generate a smell. ATF1 could also be mixed with non-reactive substrates with different chemical functional groups to demonstrate that the enzyme only catalyzes a specific reaction. Moreover, these experiments can be put into a real-world context by noting that there are synthetic biology companies that work with enzymes in engineered microbes to produce fragrances and other commodity chemicals (Mandal, Chakraboty, and Dev 2010, Wackett 2013).

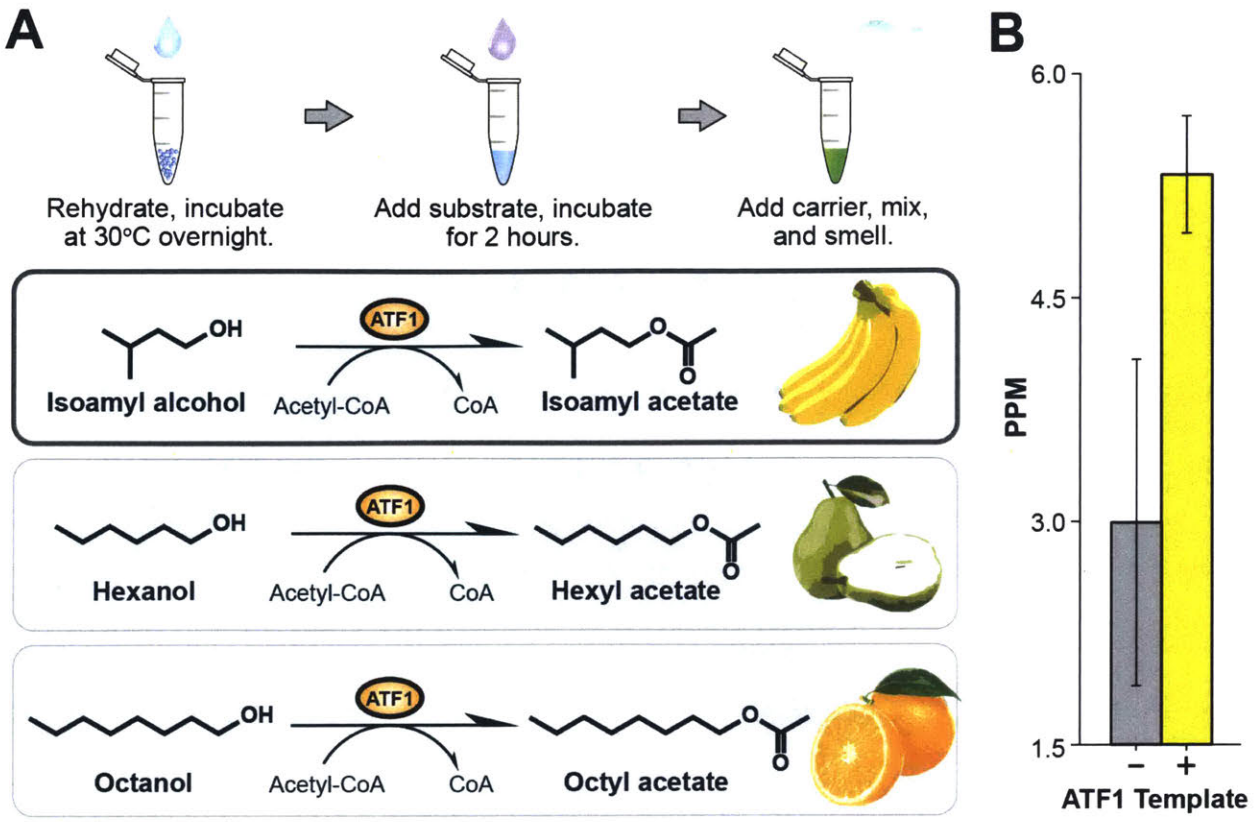


Figure 42: Fragrance-generating enzymes as olfactory outputs.

(A) Using FD-CF reactions, enzymes are manufactured that can generate various smells from the S. cerevisiae acetyltransferase ATF1. (B) Production of fragrance molecules after substrate addition to overnight FD-CF reactions of ATF1, as detected by headspace GC-MS (done by Peter Nguyen). Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

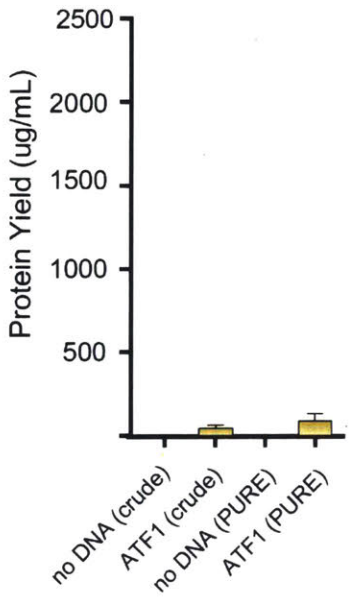


Figure 43: Quantification of ATF1 expressed in FD-CF.

The FD-CF expressed ATF1 used in the demonstration experiments had soluble yields (<100 μg/mL) in crude and PURE, as measured by 14C-Leucine incorporation (done by Jessica Stark). Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

4.4 Hydrogel-generating enzymes as tactile outputs

Next, I created a product using FD-CF reactions that students could feel. To do so, I drew inspiration from engineered hydrogel materials that have been developed for biomedical and biotechnological applications (34–36). Like the olfactory outputs, hydrogels can be produced by an enzymatic reaction (**Figure 44a**). Sortase is an enzyme that recognizes and covalently links specific peptide sequences (GGG and LPRT) through a transpeptidation reaction (Valdez et al 2017). We expressed sortase in FD-CF crude and PURE reactions overnight at 37°C (**Figure 45**) and used it to crosslink a solution of 8-arm polyethylene glycol (PEG) molecules modified with GGG or LPRT peptides (8% w/v). We observed hydrogel formation within 30 minutes of

incubation at 37°C (**Figure 44b**, **44c**, **46**). In contrast, PEG solutions incubated with FD-CF reactions that contained no template DNA did not exhibit a phase change and remained in liquid form.

In addition to the sortase-catalyzed hydrogel, we developed a method to use FD-CF PURE reactions to produce fibrin-based hydrogels. Fibrinogen is a glycoprotein found in blood, that when enzymatically converted to fibrin, leads to the formation of a blood clot (Davie and Ratnoff 1964). Outside the context of blood, the proteases ecarin and batroxobin (Trx-Bx) have been shown to cleave fibrinogen, which leads to the self-assembly of fibrin molecules into a hydrogel (Dyr, Blombäck, Kornalik 1983, Wedgwood, Freemont, Tirelli 2013). FD-CF reactions were used to produce ecarin and batroxobin (Trx-Bx) (**Figure 45**), which were added to resolubilized fibrinogen. Fibrin-based hydrogels were observed after overnight incubation at room temperature, while the enzyme-free reaction remained unpolymerized (**Figure 44b, 44c, 46**).

Once formed, the hydrogels can be manipulated by hand, allowing students to experience another enzyme-catalyzed biochemical reaction, this time resulting in an output that they can feel. These basic demonstrations can be used to introduce advanced biological concepts such as blood clotting or how cells use similar processes to form human tissue. As a follow-up activity, students can be introduced to the notion of engineering gene expression to create new biomaterials with tunable properties, a current aim of synthetic biology research (Jeon et al. 2009, Partlow et al. 2014). Specifically, the mechanical properties of the hydrogel can be tuned by varying the concentration the substrates to create a range of materials from a viscous slime to a stiff hydrogel (**Figure 44d**, **44e**). The hydrogels can also be cast into shapes using molds and/or combined with the fluorescent protein outputs to create fluorescent hydrogels (**Figure 44f**).

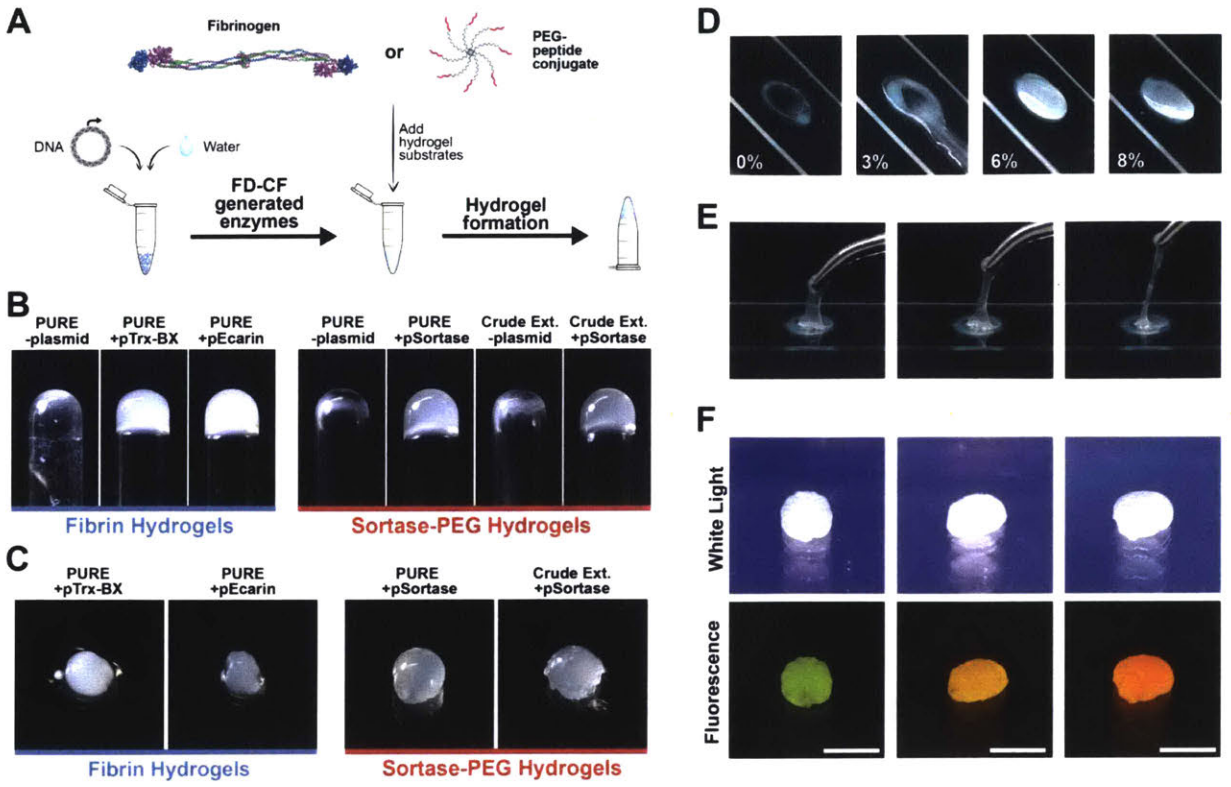


Figure 44: Hydrogel-generating enzymes as tactile outputs.

Schematic of (A) fibrin hydrogels created from FD-CF-generated batroxobin / ecarin proteases that activate fibrinogen by cleavage or PEG-peptide hydrogels crosslinked by FD-CF-generated sortase enzymes that induce crosslinking by transpeptidase activity. (B) Inverted glass tubes to demonstrate formation of hydrogels. (C) Close-up images of the formed hydrogels that can be manipulated by hand. (D) Tuning the mechanical properties of the hydrogel by varying the % PEG to create a range of materials with varying viscosities. (E) An 8% crude FD-CF PEG hydrogel is highly elastic. (F) Casting the hydrogels into shapes using molds and mixing with crude FD-CF fluorescent protein reactions to obtain shaped fluorescent hydrogels. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

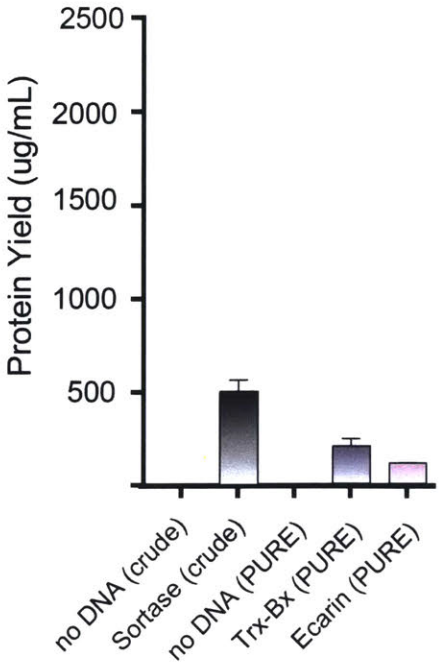


Figure 45: Quantification of hydrogel enzymes expressed in FD-CF.

The FD-CF expressed hydrogel enzymes used in the demonstration experiments had soluble yields (<100 μg/mL) in crude and PURE, as measured by 14C-Leucine incorporation (done by Jessica Stark). Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

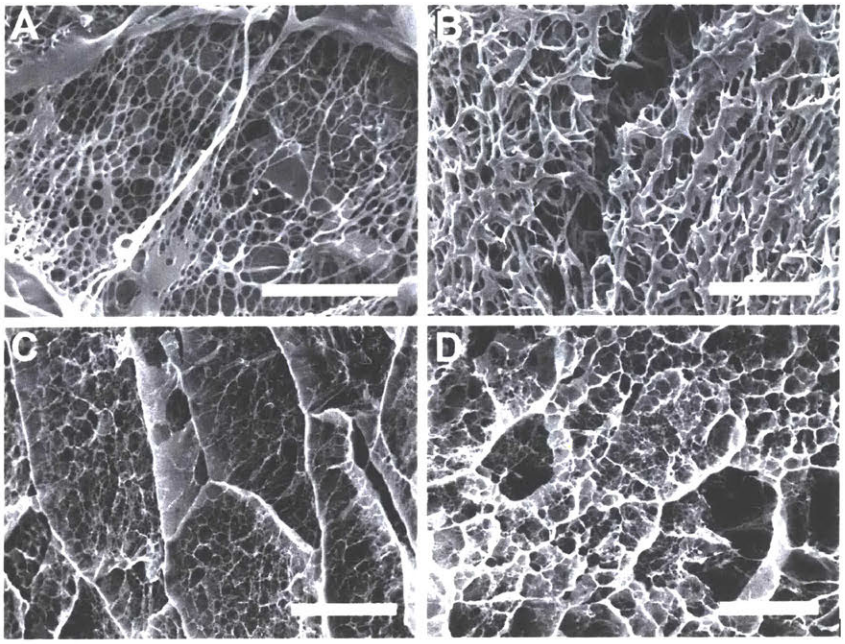


Figure 46: Representative SEM images of hydrogel ultrastructures generated with FD-CF enzymes. PEG hydrogels crosslinked by FD-CF expressed sortase in (A) PURE and (B) crude extract. Fibrin hydrogels crosslinked by FD-CF expressed (C) ecarin in PURE and (D) batroxobin in PURE. All scale bars are 10 microns. Imaged by Peter Nguyen. Figure and caption taken from Huang, Stark, Nguyen et al. 2018.

CHAPTER FIVE: MODULES TO ILLUSTRATE ADVANCES IN SYNTHETIC BIOLOGY

This chapter is in part adapted from:

Huang, A., Nguyen, P.Q., Stark, J.C., Takahashi, M.K., Donghia, N., Ferrante, T., Dy, A.J., Hsu, K.J., Dubner, R.S., Pardee, K., et al. (2018). BioBits™ Explorer: A modular synthetic biology education kit. Sci. Adv. *4*, eaat5105.

5.1 Introduction

While the previous modules aim to teach concepts within molecular biology, the FD-CF reactions also have the potential to be used to introduce synthetic biology concepts to students. With more advancements being made in the synthetic biology field and ideas like GMOs and CRISPR being discussed within the general public, it is more important than ever for our students to have a basic understanding of these concepts. Here, I describe several modules that specifically focus on teaching concepts from synthetic biology.

5.2 Performing ligation reactions in the FD-CF system

5.2.1 Development of a ligation activity in FD-CF

I sought to develop a more advanced module that could be used in an AP Biology classroom or an advanced biotechnology class to introduce the concept of cloning and how it is applied to biotechnology. One of the required topics in AP Biology is how genetic engineering techniques can be used to manipulate DNA for biotechnological applications (The College Board 2015). Typically, instructors have students do hands-on activities such as plasmid transformation or

restriction enzyme digest analysis of DNA through electrophoresis, but all of these activities require specialized equipment and reagents, as well as extensive preparation time for the instructor(s) (BIO-RAD; The College Board 2012). To create a module using our FD-CF platform that could be an easier alternative to these activities, I focused on the concept of ligations to illustrate one of the techniques commonly used in genetic engineering and cloning (Ford 2016). The main component used in this module is our previously reported pJL1-sfGFP plasmid (Huang, Stark, and Nguyen 2018; Stark, Huang, Nguyen 2018), which has an XbaI restriction site between the T7 promoter and the sfGFP gene and a StyI restriction site within the T7 terminator sequence. Using PCR and restriction enzyme digest techniques, I created two plasmid "parts" – one that contained the pJL1 backbone (including the T7 promoter) and one that contained the sfGFP sequence (without the T7 promoter). Due to the restriction enzyme digestion, both parts contain unpaired nucleotides on either ends of the segments, which are the homologous "sticky ends" that correspond to the XbaI and StyI sequences (**Figure 47a**). The two parts were combined with a T4 ligase enzyme and freeze-dried. Once rehydrated, the ligation reaction was incubated at room temperature overnight before being added to a FD-CF reaction for synthesis of the sfGFP protein.

When combined, freeze-dried, and subsequently rehydrated, the sticky ends of the pJL1 and sfGFP parts are ligated by the ligase to form a complete pJL1 sfGFP plasmid that is capable of being transcribed and translated into sfGFP (**Figure 47a**). The resulting green fluorescent signal is nearly as strong as an undigested pJL1 sfGFP plasmid at the same DNA molar concentration (**Figure 47b**). Importantly, both parts are required in order for the ligation to occur. Both the pJL1 part and sfGFP part alone do not result in any signal, which shows that the gene requires a promoter in order to be transcribed and provides an educational opportunity to discuss both natural and synthetic genetic parts. Notably, a low level of sfGFP signal when pJL1 and sfGFP are combined

even without the addition of ligase, which could be due to transient interactions arising from the XbaI and StyI homologous sticky ends bonding to each other (**Figure 47b-c**).

5.2.2 Testing ligation activity in classroom settings

Similar to the central dogma activity, we had the same groups of students test this activity in a classroom setting. After just adding water to rehydrate the ligation reaction, the students incubated their reactions at room temperature overnight. The following day, they used their ligation reactions to rehydrate FD-CF reactions and incubated their reactions at 37°C using our previously developed USB-powered portable incubator (Stark, Huang, Nguyen et al 2018). After 3 hours of incubation, students used our previously developed portable LED-based illuminator to detect the green fluorescent signal 9. Seeing the green signal indicated to the students that they successfully ligated DNA parts together into a functional plasmid that could be used in protein expression. To help the students gauge baselines for these reactions, they also ran the DNA parts separately (as a visual negative control) and a condition with undigested pJL1-sfGFP (as a visual positive control).

The students were able to replicate the activity (**Figure 48**) with only minor differences in the results due to human error Feedback for this activity was positive and it particularly inspired experimental curiosity in the students. Many of them had ideas to test out additional variables in future experiments, such as varying incubation temperature or time, and asked many follow-up questions to the activity that indicated good understanding of the biology concepts.

This activity was specifically designed to be used as an introduction to cloning. Students are not only able to combine two distinct parts of a plasmid and use ligase to form a complete plasmid, but then they can test their plasmid in the FD-CF system to see how effective their ligation was. This gives students a glimpse into how DNA is similarly manipulated and tested in the

biotechnology industry for a variety of different applications, such as creating novel proteins – like antibodies used for cancer therapeutics. Since this topic, demonstrated in a hands-on way, is required in the AP Biology curriculum (The College Board 2015), this module can a simpler alternative to the traditional restriction enzyme based cloning activities. Beyond its potential use in advanced biology courses, this module could even be adapted for more introductory biology courses by focusing on fundamental concepts involved, such as the fact that the sfGFP gene requires a promoter upstream of its sequence in order to be transcribed (another detail important to the central dogma). Observing the difference between the conditions with and without ligase could also be used to demonstrate the function of enzymes and how they catalyze reactions to make them more effective. This particular activity is modular and flexible, allowing instructors to integrate the activity into their classroom as needed.

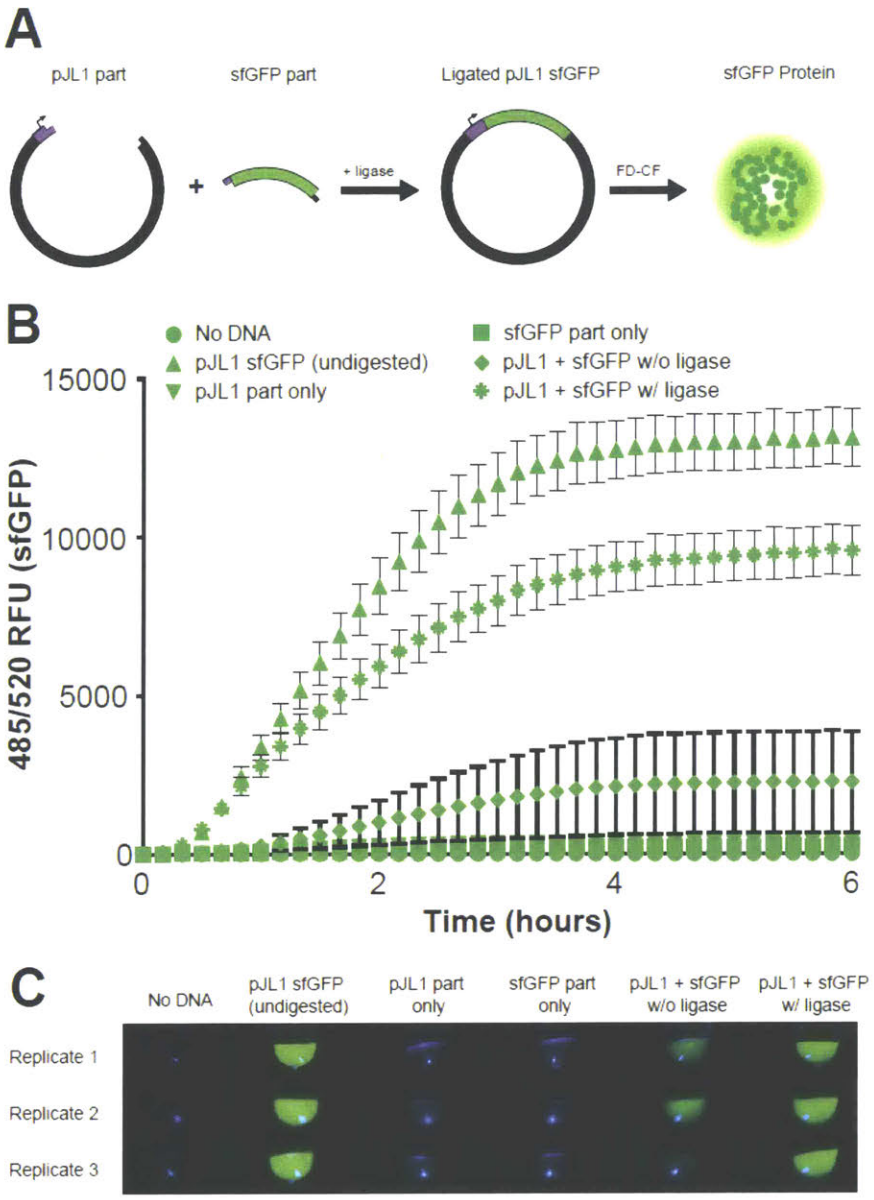


Figure 47: Activity to demonstrate ligations.

(A) The pJL1 part and sfGFP part have corresponding sticky restriction sites and can be ligated in a freeze-dried reaction. The ligated products then can be used in a FD-CF expression to express sfGFP. (B) Relative fluorescent readouts (n=3) showing the kinetics of the sfGFP expressed from the ligated products quantified by plate reader. Values represent averages and error bars represent standard deviations of n=3 biological replicates. (C) Visual endpoints of the sfGFP expressed from the ligated products. Images shown are from a custom-built 450nm handheld imager with a yellow acrylic filter.

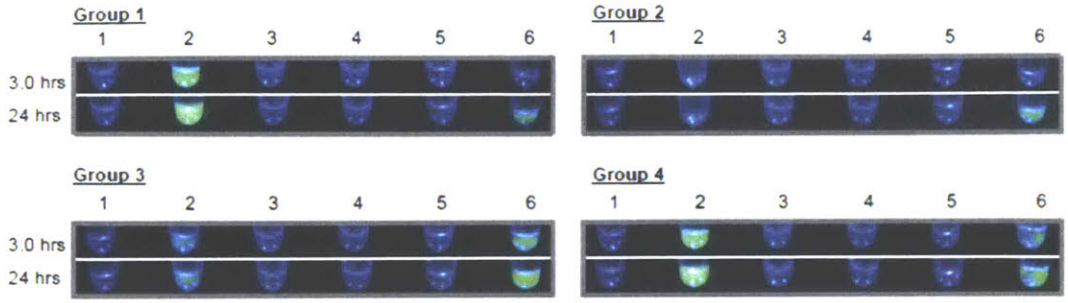


Figure 48: Field testing of ligation activity.

Four groups of high school students were given the freeze-dried reactions the corresponding DNA and basic instructions of this activity. Shown are the images taken of all the expressed proteins of the student groups' actual results. Images shown are from a custom-built 450nm handheld imager with a yellow acrylic filter.

5.2.3 Further optimization and improvement of ligation activity

One potential drawback to this activity is the inclusion of a commercial T4 ligase, as recombinant enzymes tend to be expensive. To help keep the costs of the activity low, I cloned a pJL1 T4 ligase sequence, so that the T4 ligase could be expressed in the FD-CF reactions and subsequently used in the ligation activity. FD-CF reactions with PURE were incubated with the T4 ligase plasmid overnight before being added to the FD-CF ligation reaction the next day.

Although the sfGFP signal that resulted was a bit lower than the commercial T4 ligase (New England Biolabs M0202S), it is still a robust signal that can be visualized with the portable imager (**Figure 49**). Further optimization will be done so that the ligase expression can be done in crude FD-CF (rather than PURE) to lower the cost of this activity even more.

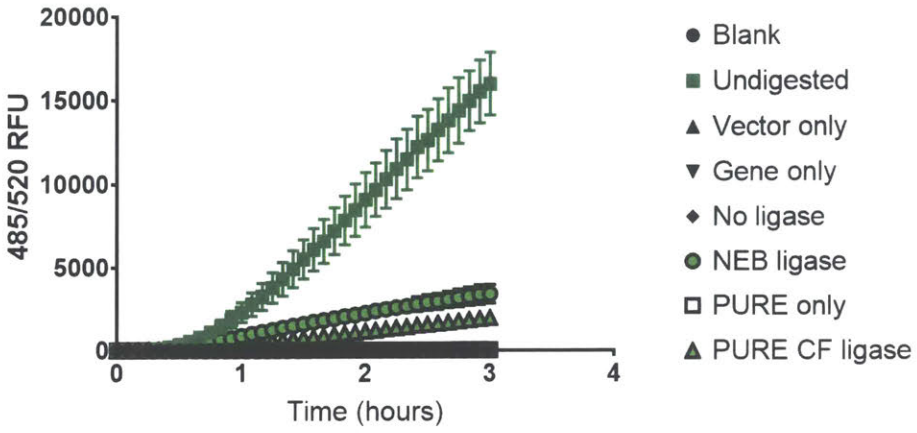


Figure 49: Using FD-CF to express ligase to be used in the ligation activity

Ligase was expressed in a PURE FD-CF reaction before used to ligate the sfGFP plasmid. Relative fluorescent readouts (n=3) showing the kinetics of the sfGFP expressed from the ligated sfGFP quantified by plate reader. Values represent averages and error bars represent standard deviations of n=3 biological replicates.

I anticipate that this activity will provide a solid foundation for students in their core biology classes to teach advanced scientific exploration of the synthetic biology.

5.3 Development of synthetic biology breadboard

While the previous modules provide students with an FD-CF toolkit of sensory outputs from simple DNA inputs, I wanted to expand the kit to include the ability to control these outputs and build genetic circuits in a user-defined fashion. To create an FD-CF "biological breadboard," I, along with other collaborators in the lab, turned to modular components previously developed in our lab, toehold switch sensors, that allow for the creation of programmable logic gates based on the principles of nucleic acid hybridization.

5.3.1 Toehold switches in FD-CF

Toehold switch sensors are programmable synthetic riboregulators that allow protein expression only when a specific trigger RNA is present (Pardee et al 2017, Green et al 2014, Green et al. 2017). A toehold switch sensor is an mRNA designed to include a hairpin structure that blocks gene translation in cis by sequestration of the ribosome binding site (RBS) and start codon. Upon hybridization to a complementary trigger RNA, sequestration of the RBS and start codon is relieved, allowing for ribosomal translation of an output gene (**Figure 2, 50**). Toehold switch sensors provide a means for controlling the translation of any of the sensory outputs described previously (fluorescence, enzymes for fragrance, and enzymes for hydrogels).

To demonstrate programmable gene expression, we used a set of four toehold switches designed in previous work (Green et al. 2014), that each have a unique trigger RNA to control the expression of four different fluorescent outputs. DNA encoding the sensor-fluorescent proteins and trigger RNAs were expressed in FD-CF PURE reactions to determine both the ability of the trigger RNAs to activate gene expression and the specificity for their respective targets. We tested all 16 sensor and trigger combinations. After overnight incubation (20 hours) at 37°C, we observed that all four triggers demonstrated specificity for their sensors, such that expression of the respective fluorescent proteins only occurred in the presence of their designed triggers (**Figure 50, 51**).

An important property of toehold switch sensors is that multiple switches can be composed together to create genetic logic gates, which are a popular tool used by synthetic biologists to engineer and control cellular behavior. Genetic logic gates provide the ability to process multiple input signals from the environment when deciding which proteins to express, and are analogous to logic gates found in personal electronic devices, such as laptop computers and smart phones. To

illustrate these concepts, we utilized previously developed in vivo toehold switch logic gates (Green et al. 2017) a two-input AND gate and a two-input OR gate to control the expression of sfGFP in FD-CF PURE reactions, demonstrating that these logic gates could function in a cell-free context as well (**Figure 50c-d**). For the AND gate, sfGFP expression was only visible when the DNA for both the A and B trigger RNAs were present in the FD-CF reaction. In contrast, for the OR gate, sfGFP was expressed when either or both triggers were present.

The toehold switch reactions here were done in the PURE system because there is some inherent auto-fluorescence from the crude extract that made visualization of the fluorescent outputs more difficult (**Figure 52**), especially since the fluorescent signal from the toehold switch is usually lower than a constitutively expressed fluorescent protein. In the future, the toehold switches could be optimized to increase their output expression, which would allow use of the inexpensive crude extract. Additionally, crude extracts could be optimized to improve expression of specific enzymes by using different bacterial strains. Recent improvements to the efficiency of the PURE production system also suggest that its cost could be reduced to that of the crude system (Villarreal et al. 2017, Shepherd et al. 2017). These optimizations would reduce the cost of the kits even further.

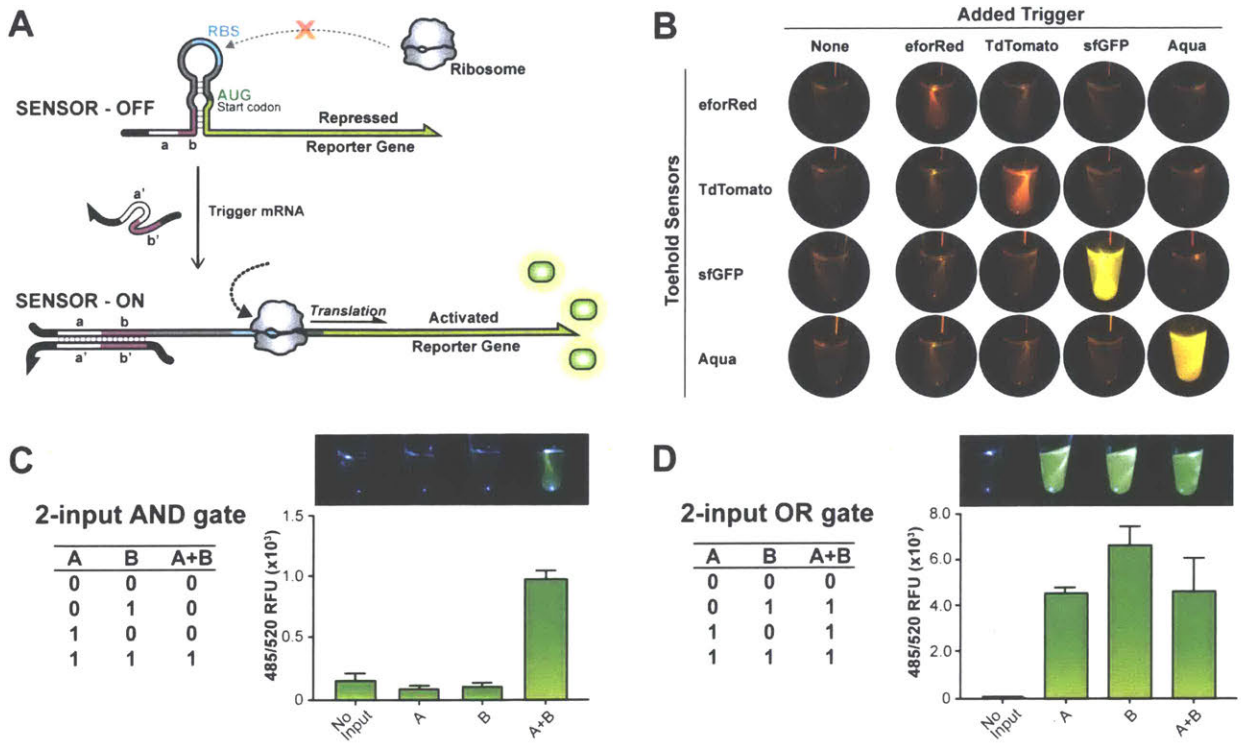


Figure 50: Synthetic biology breadboard for prototyping genetic logic.

(A) Schematic of a toehold switch sensor. Upon the presence of a trigger RNA, strand invasion melts the secondary structure, allowing ribosomal translation to occur. (B) A set of four unique toehold switch sensors with their corresponding RNA triggers were expressed in FD-CF to demonstrate four well-designed toeholds that are orthogonal to each other, imaged in the custom-built 450 nm handheld imager with an orange acrylic filter. A set of toehold switch sensors that are designed to be either (C) an AND gate or (D) an OR gate were expressed in FD-CF reactions with the corresponding inputs to demonstrate the concept of logic gates, imaged in the custom-built 450 nm handheld imager with an yellow acrylic filter and quantified by plate reader at 485 excitation and 520 emission (done in part by Melissa Takahashi). Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure taken from Huang, Stark, Nguyen et al. 2018.

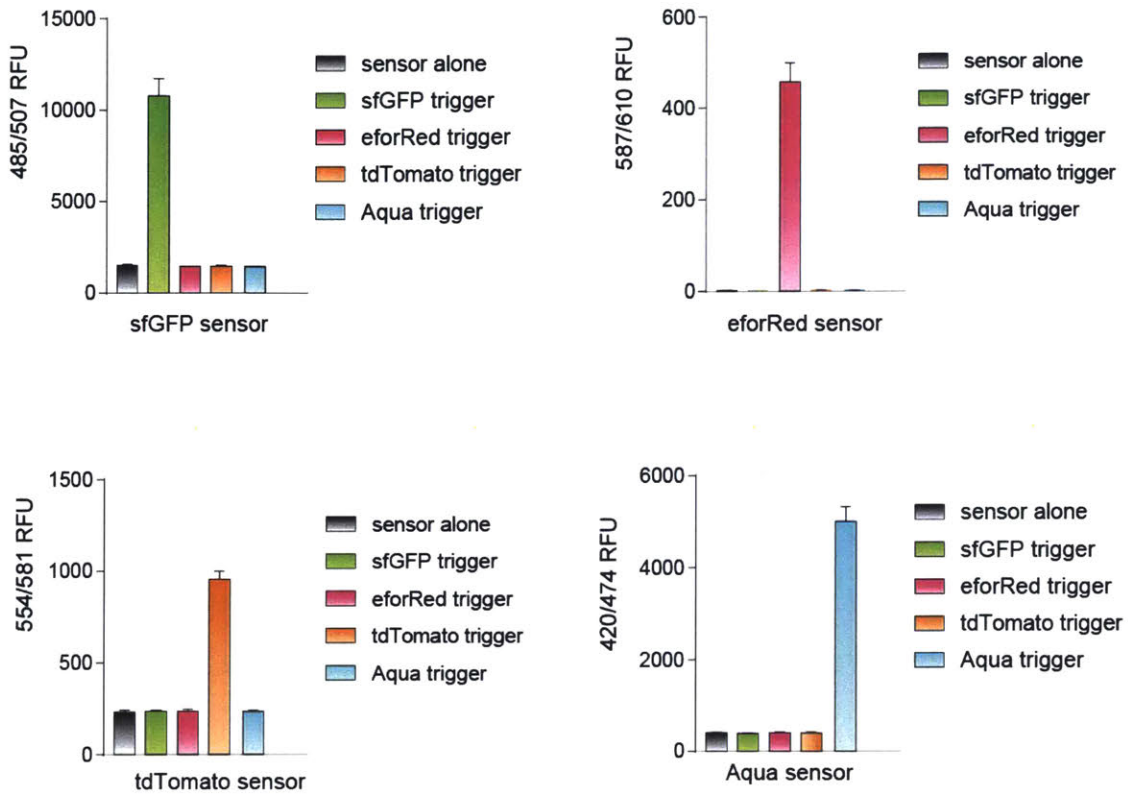


Figure 51: Quantitative analysis of toehold outputs.

Endpoint fluorescent readouts of all combinations of toehold sensor and triggers, demonstrating orthogonality within the toehold set. All FD-CF reactions were performed in NEB PURExpress. Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure taken from Huang, Stark, Nguyen et al. 2018.

This module provides an interactive means for students to learn and understand core programming and circuit concepts in synthetic biology, and could be used to engage students in the design-build-test cycle by enabling them to connect different toehold switch elements to different outputs (such as fragrance or hydrogel generating enzymes) and predict the outcomes of the reactions.

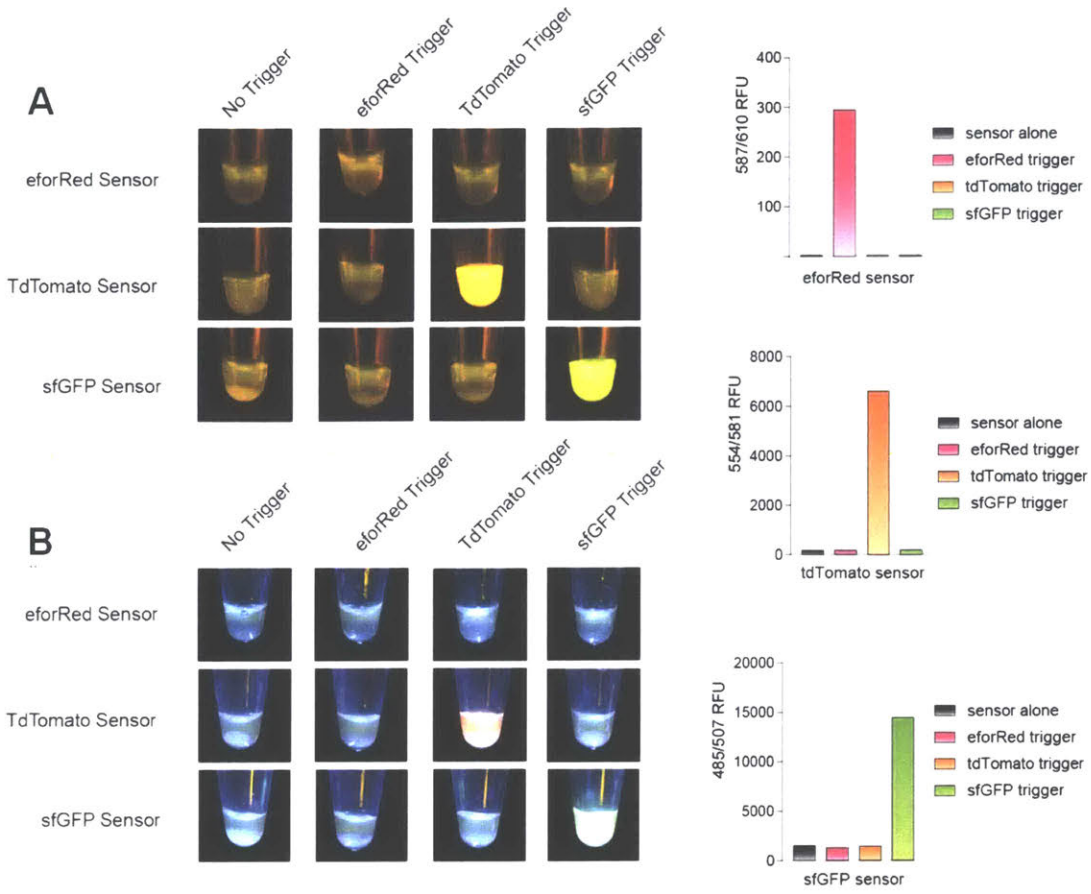


Figure 52: Demonstration of toeholds in FD-CF crude extracts.

Subset of the orthogonal toehold set expressed in FD-CF crude extracts to demonstrate portability over to the crude system in (A) a laboratory transilluminator (SafeImager at 470 nm excitation) and (B) a portable and inexpensive (< $15 USD) 450 nm classroom illuminator with a yellow acrylic filter. (C) Endpoint fluorescent readouts (n=1) of all expressed proteins. Done in part by Melissa Takahashi. Figure taken from Huang, Stark, Nguyen et al. 2018.

5.3.2 Developing modularity for the toehold system in FD-CF

In order to create a more modular "biological breadboard" that allows students to mix and match genetic pieces and give them more control over their designs, I combined the work from the previous toehold module with the work from the ligation activity (**Chapter 5.2**).

Similar to the ligation activity, I used a specialized toehold-gated sfGFP plasmid with an EcoRI restriction site between the T7 promoter/toehold sequence and the sfGFP gene and a BamHI restriction site after the T7 terminator sequence. Using PCR and restriction enzyme digest techniques, I created two plasmid "parts" – one that contained the backbone (including the T7 promoter and the toehold sequence) and one that contained the sfGFP sequence (without the T7 promoter or toehold sequence). Due to the restriction enzyme digestion, both parts contain unpaired nucleotides on either ends of the segments, which are the homologous "sticky ends" that correspond to the EcoRI and BamHI sequences. The two parts were combined with a T4 ligase enzyme and freeze-dried. Once rehydrated, the ligation reaction was incubated at room temperature overnight before being added to a FD-CF reaction with the proper toehold trigger for synthesis of the sfGFP protein.

When combined, freeze-dried, and subsequently rehydrated, the sticky ends of the vector and sfGFP insert parts are ligated by the ligase to form a complete toehold-gated sfGFP plasmid that is capable of being transcribed and translated into sfGFP with the addition of the appropriate trigger plasmid. The preliminary results show that the resulting green fluorescent signal is as strong as an undigested toehold-gated sfGFP plasmid at the same DNA molar concentration (**Figure 53**). Importantly, both parts are required in order for the ligation to occur. Both the vector part and sfGFP insert part alone do not result in any signal, which shows that the gene requires a promoter in order to be transcribed and provides an educational opportunity to discuss both natural and synthetic genetic parts.

The signal is lower than a regular sfGFP reaction and can be difficult to visualize, so additional optimization will be needed to adapt this reaction to a classroom-friendly setup. Once optimized,

this design can be expanded to the full set of toehold plasmids and additional toehold sequences that can detect a specific sequence (i.e., a disease or specimen).

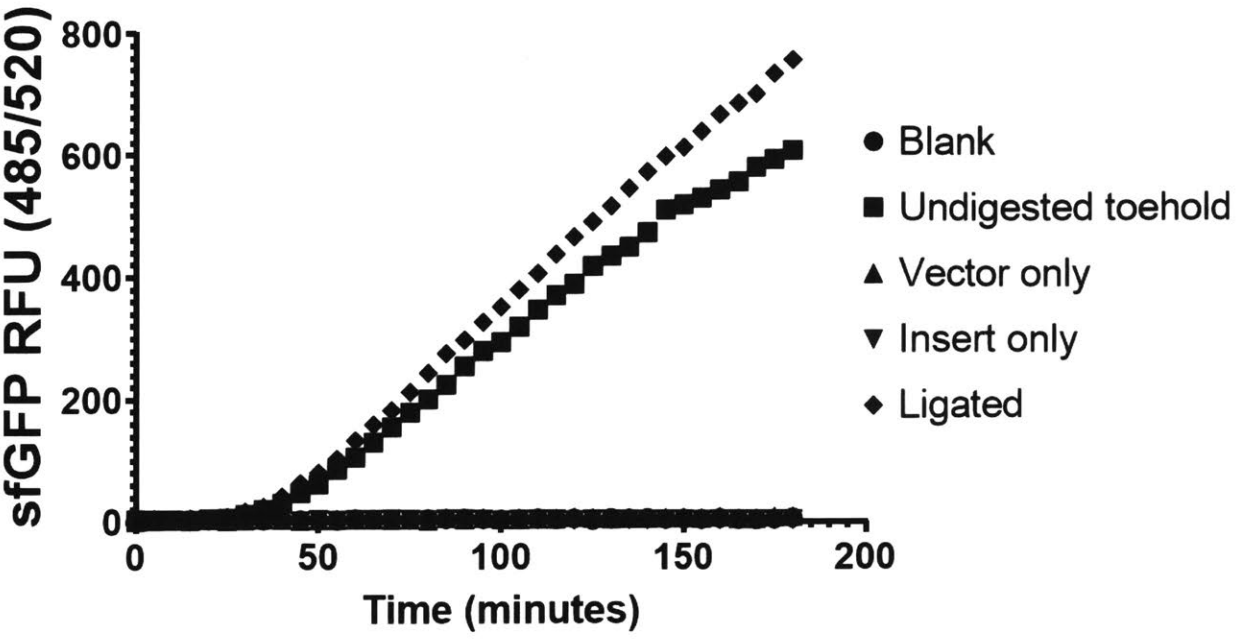


Figure 53: Preliminary experiments to use FD-CF to ligate toehold components

A freeze-dried reaction was used to ligate the toehold-gated sfGFP plasmid before being added to a FD-CF reaction for a toehold sfGFP readout. Relative fluorescent readouts (n=1) showing the kinetics of the sfGFP expressed from the ligated sfGFP quantified by plate reader. Done in part by Sara Corchado.

5.4 Developing diagnostics for educational use in FD-CF

One approach to inspire a proactive, inquisitive mindset in students is to provide them with the means to interrogate the world around them. In this demonstration, I developed FD-CF-based tools that allow students to probe real-world biological samples using the toehold switch sensor technology. Here, we expand on the common classroom activity of isolating DNA from fruits (Sweeney 2003) to allow students to detect DNA signatures of a specific fruit and couple it to a fluorescent output (**Figure 54a**).

I designed toehold switch sensors to recognize specific sequences from either the banana genome (*rbcL* gene from *Musa acuminata* or *Musa balbisiana*) or the kiwi genome (5.8S ribosomal RNA gene from *Actinidia deliciosa*) and produce sfGFP as an output (Christelová et al. 2011, Taguchi et al. 2007). Since the toehold switch sensors recognize RNA and not DNA, I included a recombinase polymerase amplification (RPA) (Piepenburg 2006) step to generate short DNA amplicons from fruit genomic DNA that incorporate a T7 promoter for transcription of RNA triggers in FD-CF reactions (**Figure 55**). The RPA reaction components can also be freeze-dried, conforming to the shelf-stable aspect of the kits. To demonstrate the specificity of these toehold switch sensors, I isolated DNA from banana, kiwi, or strawberry (**Figure 56**), using a simple classroom procedure that utilizes common inexpensive supplies such as dish soap, table salt, rubbing alcohol, and coffee filters. Diluted DNA from each of the fruits was used to rehydrate RPA reactions and incubated overnight at 37°C. Completed RPA reactions were diluted 1:4 in water and used to rehydrate FD-CF PURE reaction pellets containing the banana or kiwi toehold switch sensor. Upon overnight incubation at 37°C, activation of the toehold switch sensors was only observed from the reaction containing the specific fruit DNA (banana DNA for the banana sensor and kiwi DNA for the kiwi sensor) and not from the reactions containing other fruit DNA (**Figure 54b-c**).

The biosensor module described here utilizes the detection capability of the toehold switches to provide a hands-on introduction to the concept of nucleic acid-based diagnostics (Gootenberg et al. 2017, Pardee et al. 2017). This module could be readily expanded to include additional toehold switch sensors designed to detect other fruits and vegetables. Sensors could also be designed to identify specific animal species (e.g., cats, dogs), enabling students to test DNA from their pets. Additonally, the initial experiments showing the modularity of the toehold reactions would allow

students to further utilize the existing components to create synthetic biology circuits. For example, they could combine multiple outputs (e.g., a fluorescent protein and a fragrance-generating enzyme for an output that is colorful and fragrant) or pair any input with any output (e.g., detection of banana DNA to a yellow fluorescent protein or banana-smell generating output).

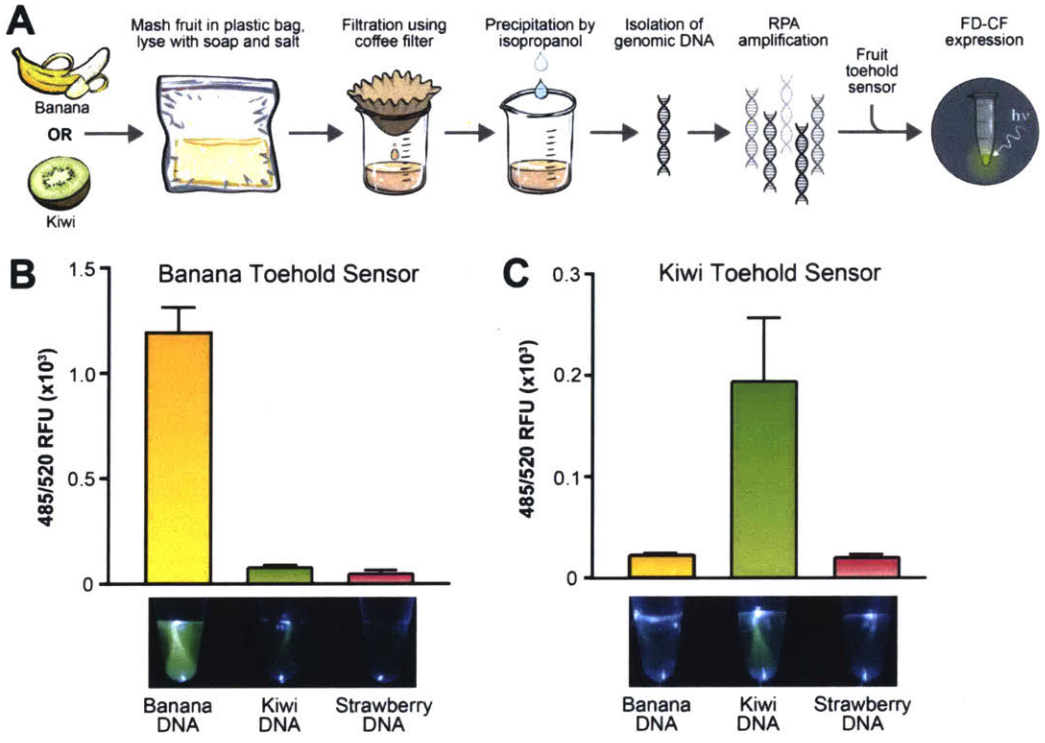


Figure 54: Toehold-based environmental sensing demonstrations.

(A) Schematic of activity that allows extracted DNA from banana or kiwi fruit to be processed and detected by a toehold switch sensor in FD-CF. (B) The banana or (C) kiwi toehold switch sensor produces a clear fluorescence output (sfGFP) when exposed to extracted and amplified DNA of the relevant fruit, but not when exposed DNA sequences from other fruits. Images shown are from a custom-built 450nm handheld imager with a yellow acrylic filter and quantified by plate reader at 485 excitation and 520 emission. Done in part by Aaron Dy. Values represent averages and error bars represent standard deviations of n=3 biological replicates. Figure taken from Huang, Stark, Nguyen et al. 2018.

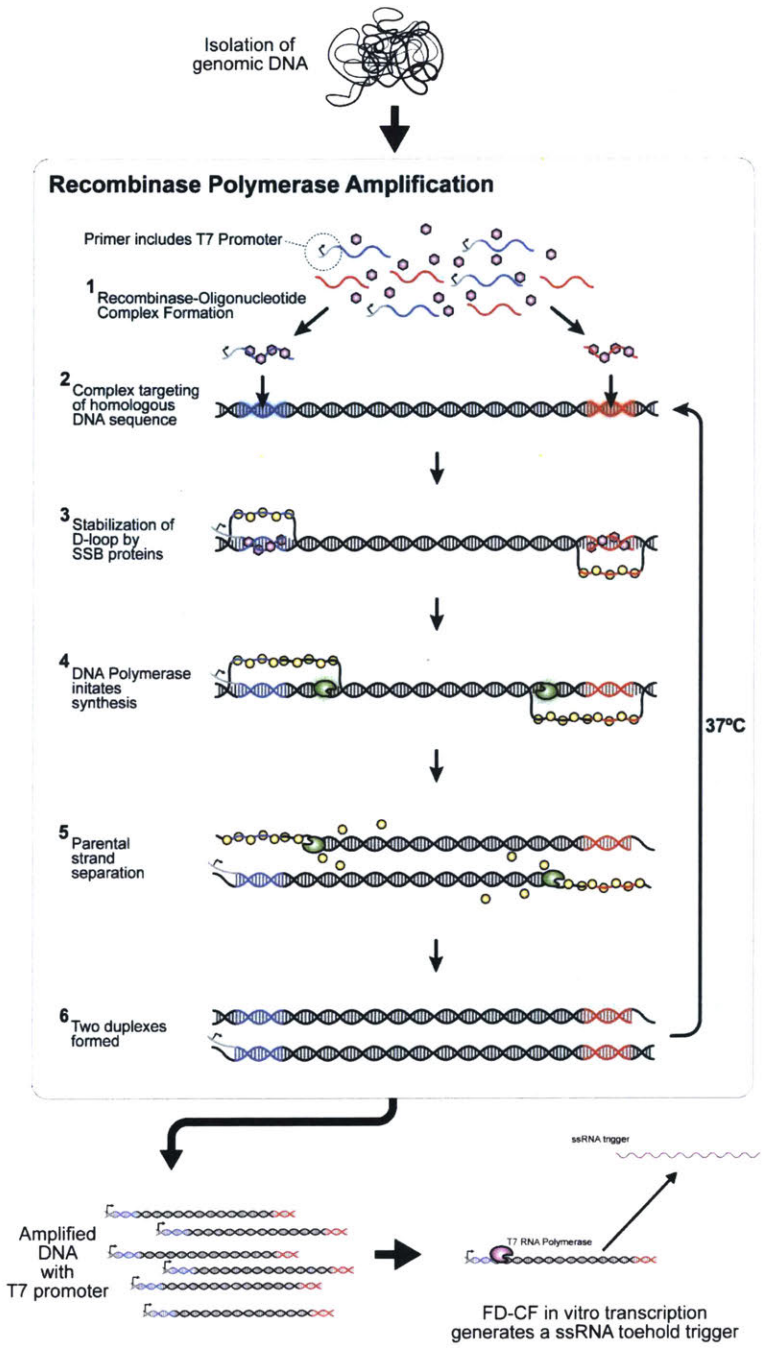


Figure 55: Schematic of RPA reaction.

From a genomic DNA sample, a specific region is isothermally amplified using Recombinase Polymerase Amplification. The primer includes a T7 promoter, such that the amplicons act as a template to generate a large amount of RNA trigger molecules when added to a FD-CF reaction. This results in signal amplification for toehold sensor activation. Figure taken from Huang, Stark, Nguyen et al. 2018.

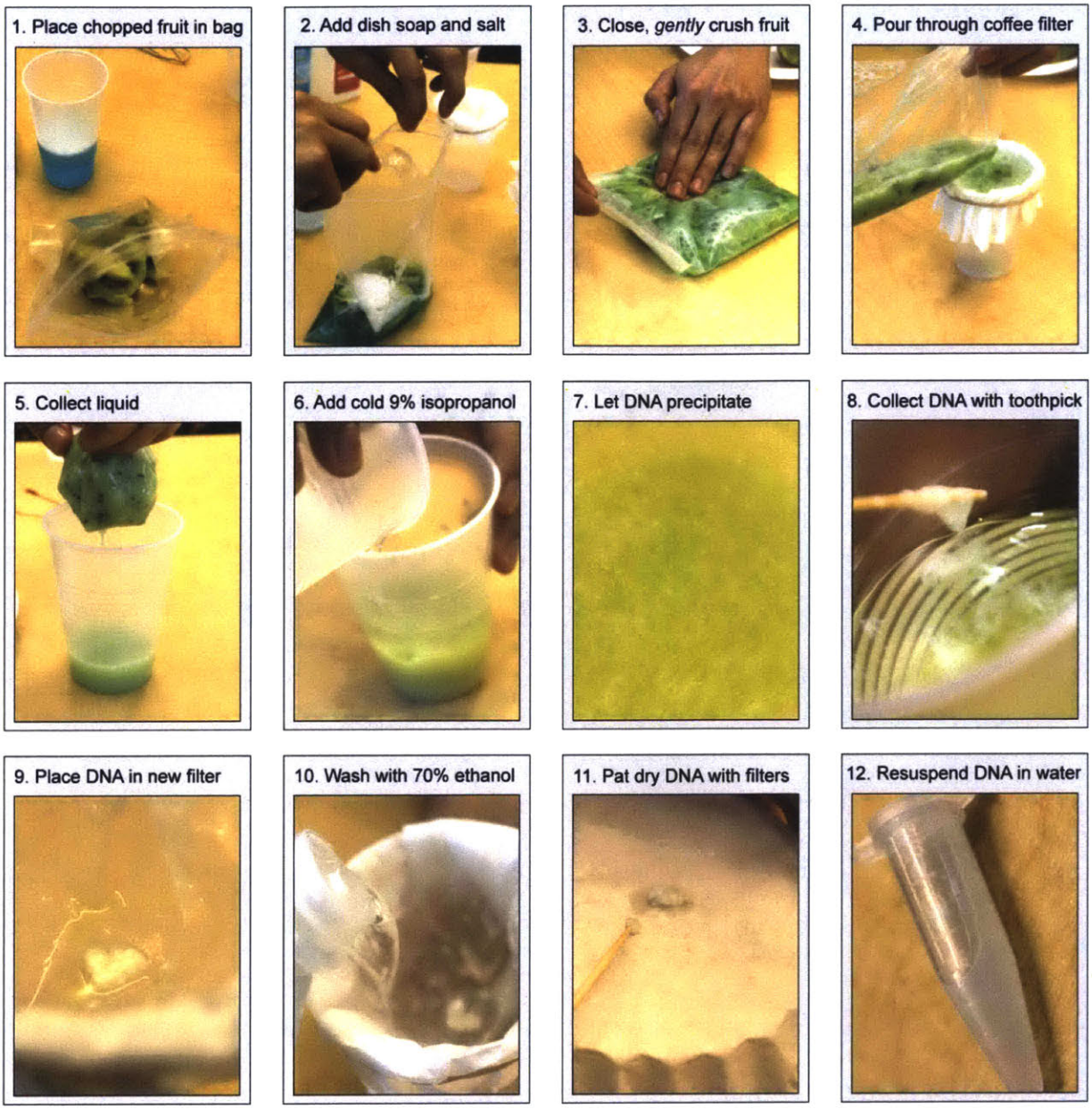


Figure 56: Detailed steps for isolating genomic DNA from fruits for environmental sensing activity.

Photographs showing the DNA extraction process from fruit. Done in part by Aaron Dy. Figure taken from Huang, Stark, Nguyen et al. 2018.

5.5 Developing modules to teach CRISPR in FD-CF

With all the recent and rapid advancements in CRISPR and its potential role in researching and treating diseases (Huang, Lee, and Doudna 2018; Xiong et al. 2016), it will be important to provide students with a basic understanding of gene editing, so they are able to make informed decisions concerning the ethics surrounding this topic. Here, alongside my collaborator, Jessica Stark, at Northwestern, a pair of modules are currently in development to demonstrate 1) the mechanism of action of CRISPR, and 2) a potential application of CRISPR.

5.5.1 Development of a module to introduce how CRISPR works

In this first module, we demonstrate how CRISPR can be used to cut DNA at a specific site on the target DNA. In our case, the target DNA is an mRFP1 plasmid and the target site is a 20 base pair sequence with the appropriate protospacer adjacent motif (PAM) site towards the start of the *mRFP1* gene. A CRISPR-Cas9 system was developed by first designing a gRNA construct that targets the target cut site and pairing the gRNA construct with a *Streptococcus pyogenes* Cas9 nuclease.

As the inclusion of a recombinant Cas9 would increase the cost of the kit, we first showed that a functional Cas9 nuclease could be expressed using the FD-CF system. When this pre-expressed Cas9 is mixed with the mRFP1-targeting gRNA in subsequent FD-CF reactions to express fluorescent proteins, we see that the mRFP1 reactions are repressed by the CRISPR-Cas9 system, while the other fluorescent proteins (which were not targets of the gRNA) show no significant repression of the fluorescent signal (**Figure 57**).

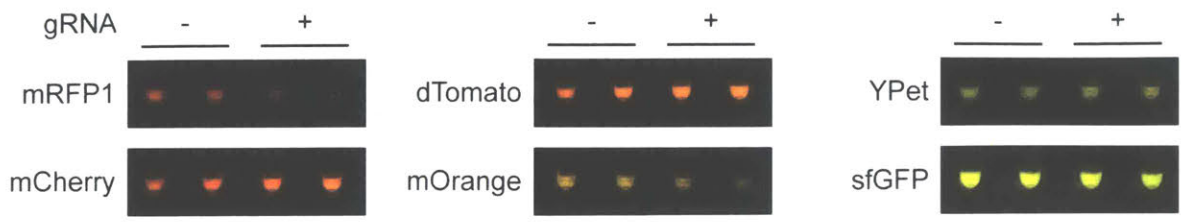


Figure 57: Interrogating the mechanism of a CRIPSR-Cas9 nuclease system using fluorescence. We designed a synthetic guide RNA construct that targets the gene for the red fluorescent protein. This makes it possible to use repression of mRFP1 fluorescence as a reporter of Cas9 activity in FD-CF reactions. We 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. Blue light images of representative FD-CF reactions 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. Done by Jessica Stark. Figure and caption from Stark, Huang, et al. 2019.

This experimental setup can be used to develop an introductory CRISPR activity, where students are given the sequence of the gRNA construct, as well as the sequences of a set of fluorescent protein, and tasked with using gene editor software to identify which fluorescent protein sequence they think the gRNA is targeting. Once they have formed their hypothesis, they can carry out the experimental procedure described above to test their hypothesis.

5.5.2 Development of a module to demonstrate an application of CRISPR

After the students understand how CRISPR actually works, they can explore different applications of CRISPR. Here, I show preliminary development data of a potential activity, showing how CRISPR can be used as a diagnostic. While much of the discussion of CRISPR in the news has

been around its therapeutic potential in treating human diseases, CRISPR also has much potential in improving research techniques and/or in diagnostics. Thus, I wanted to create an activity that highlights this application of CRISPR.

The basis of this activity is the SHERLOCK platform that was developed previously in the lab. In this platform, a characteristic of the Cas13 enzyme (which targets RNA, not DNA) is exploited, where once the Cas13 enzyme locates and cuts its specific RNA target, it begins nonspecifically cutting all the other RNA nearby. In the SHERLOCK system, a target sequence/site, corresponding to a known marker of a disease, is amplified from the sample (i.e., a patient sample containing a virus or other pathogen). Once sufficiently amplified, the CRISPR-Cas13 system is added, causing a specific cut at the target site. Once this targeted cut happens, the Cas13 will then start nonspecifically cutting all other nearby RNA. In this case, the nonspecific RNA will all be conjugated to a signaling molecule, which is released once the RNA is cleaved by the Cas13. Detection of the signaling molecule (i.e. fluorescence) will then indicate if the target sequence was present in the initial sample (**Figure 58**; Gootenberg et al. 2017).

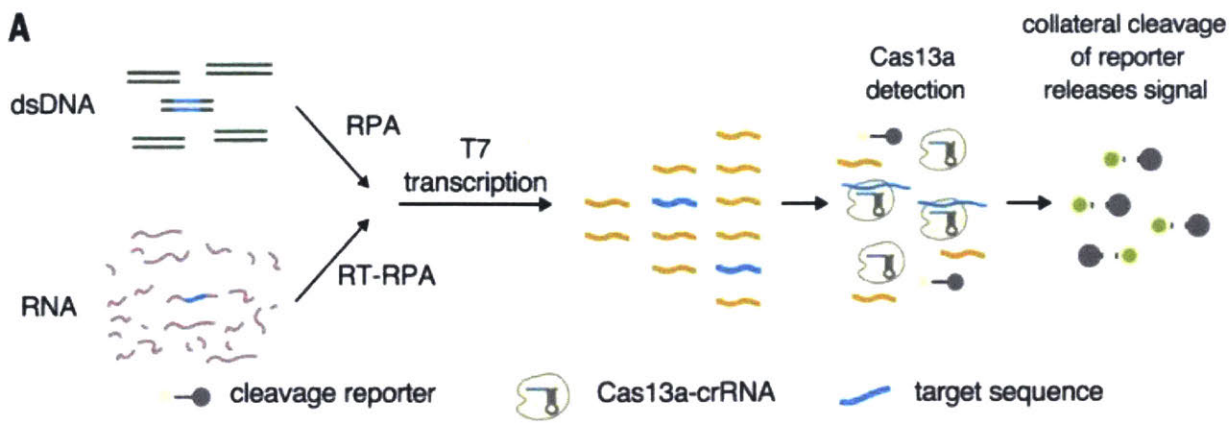


Figure 58: Schematic of the SHERLOCK diagnostics platform.

DNA or RNA samples are amplified with RPA and used to induce Cas13a nonspecific cutting activity to release signal molecules. Figure taken from Gootenberg et al. 2017.

Since typical healthcare privacy restrictions would prevent diagnostic screening of relevant health conditions in students, I decided to develop a classroom diagnostic, in collaboration with members in the lab, based on the SHERLOCK platform to screen student samples for a more innocuous genetic marker, the rs4481887 marker. This genetic marker determines if an individual is able to detect an unfamiliar odor in their urine after eating asparagus. Those with a GG genetic variant are less likely to detect the asparagus urine odor, while those with the AG or AA genetic variant are more likely to detect the odor (Eriksson et al. 2010).

We first designed four potential gRNAs that would target the AA genetic variant on the gene and cause Cas13 to nonspecifically cut and release the signaling molecules (**Figure 59**). We then ran each of the potential gRNAs in the SHERLOCK platform against synthetic target RNA with either AA or the GG variant or a target with a nonrelevant sequence. For screening purposes, we started with RNA samples that already had been pre-amplified (see Materials and Methods for more experimental detail). The ideal gRNA would result in high fluorescent output against the AA target

RNA and little to no fluorescent output against the nonspecific target. Ideally, it would also have low fluorescent output against the GG target RNA, but for this first pass of gRNA design, having a high positive signal is prioritized over a low negative signal (as we can engineer additional mutations to lower the negative fluorescent signal).

Specific gRNA sequences against rs4481887	
Asparagus_1	ATGTAGCCCATTCCTTTATTATA
Asparagus_2	TGTAGCCCATTCCTTTATTATAC
Asparagus_3	GTAGCCCATTCCTTTATTATACT
Asparagus_4	AGCCCATTCCTTTATTATACTTG

Figure 59: Table of potential gRNA's screened against rs4481887.

List of 4 sequences screened against rs4481887 in the SHERLOCK platform. Done in part by Aaron Dy, Audrey Ory, and Abby Mauermann.

Based on this criteria, we selected gRNA 3. gRNA 3 had a high signal for the AA variant and a low signal for the nonspecific target (**Figure 60**). The other potential gRNA had either high signals for the GG variant (higher than the AA variant), a higher nonspecific signal, or a low AA variant signal. We then engineered a secondary mutation into the existing gRNA construct – introducing a secondary mutation to the gRNA sequence will somewhat lower the signal output against the target AA variant RNA, but should drastically lower the signal output against the GG variant target RNA. This allows the signal to noise ratio to increase between the positive and negative conditions, as we want a visually detectable difference between the AA and GG variants.

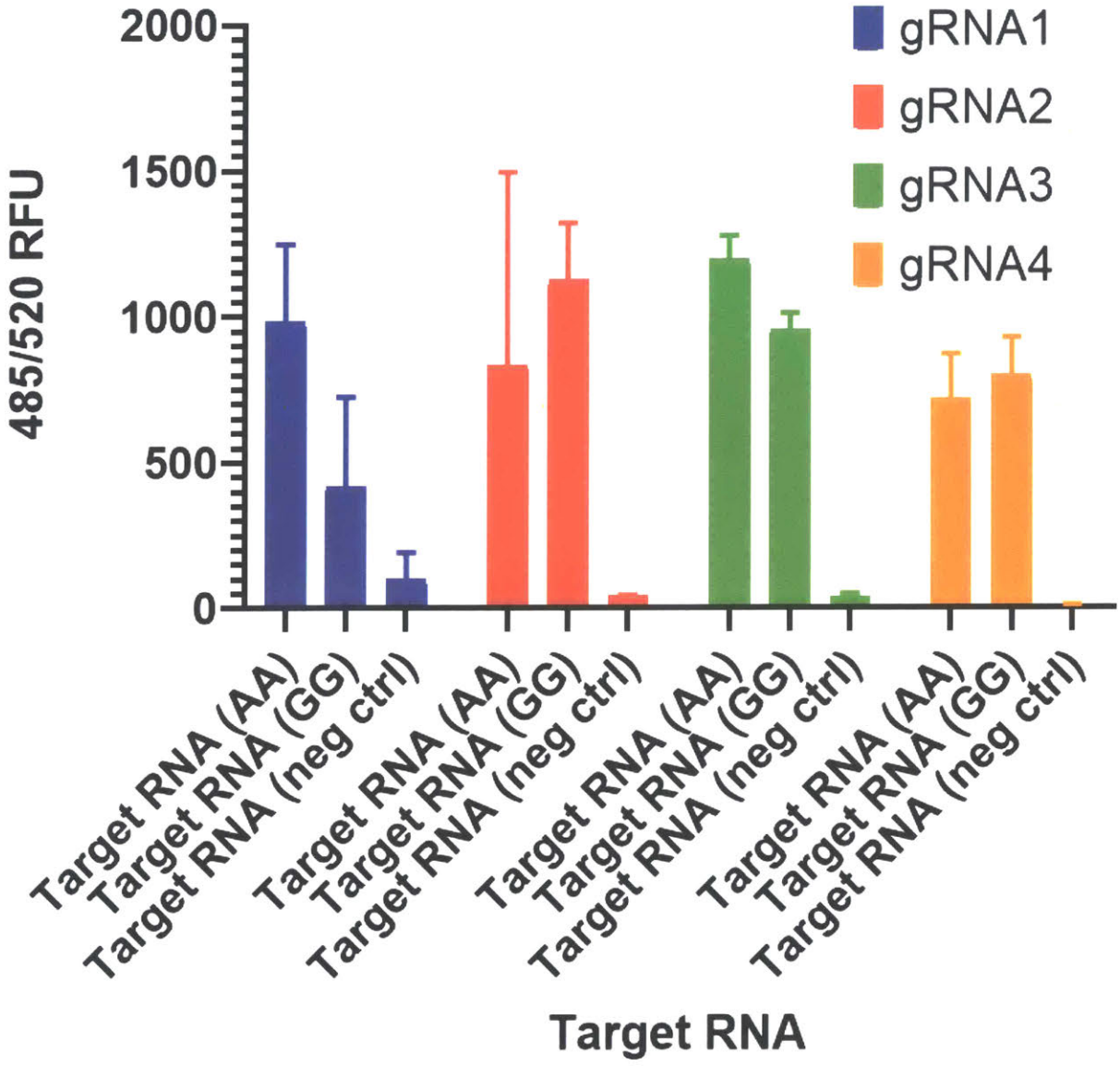


Figure 60: Preliminary screen of potential gRNAs for rs4481887

Results of 4 potential gRNA's screened against rs4481887 in the SHERLOCK platform (see Materials and Methods). Samples were run at n=2 and quantified at 485/520 ex/em. Done in part by Aaron Dy, Audrey Ory, and Abby Mauermann.

We designed three potential gRNAs based on the gRNA 3 sequence with an additional secondary mutation (**Figure 61**). The ideal gRNA would result in high fluorescent output against the AA target RNA, a low signal output against the GG target RNA, and little to no fluorescent output against the nonspecific target. Based on these criteria, gRNA 3C (**Figure 62**) appears the most promising because there is a 2:1 ratio between the positive AA signal vs the negative GG signal while the other potential secondary mutations have a much lower signal to noise ratio.

Specific gRNA sequences with secondary mutations (highlighted in red)	
Asparagus_3	GTAGCCCATTCCTTTATTATACT
Asparagus_3A	GTAGCCCATTCCTTTATTAAACT
Asparagus_3B	GTAGCCCATTCCTTTATAATACT
Asparagus_3C	GTAGCCCATTCCTTTAATATACT

Figure 61: Table of potential secondary mutations in gRNA3 screened against rs4481887.

List of 3 potential sequences (compared to the original gRNA3 sequence) containing secondary mutations in the gRNA3 sequeence screened against rs4481887 in the SHERLOCK platform. The secondary mutation is highlighted in red. Done in part by Aaron Dy and Abby Mauermann.

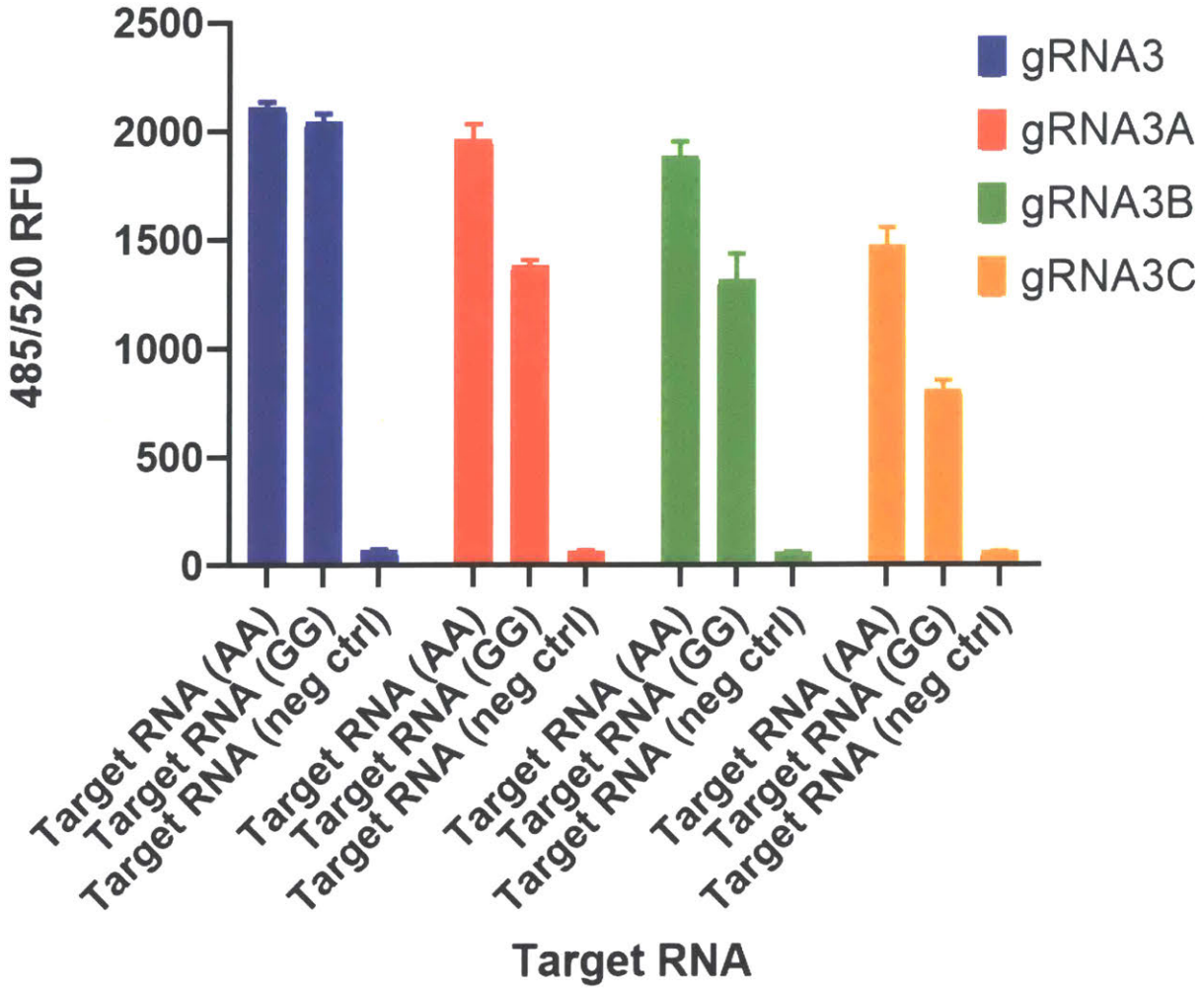


Figure 62: Preliminary screen of potential gRNA3 with secondary mutations for rs4481887

Results of 4 potential gRNA3 with secondary mutations screened against rs4481887 in the SHERLOCK platform (see Materials and Methods). Samples were run at n=2 and quantified at 485/520 ex/em. Done in part by Aaron Dy and Abby Mauermann.

After identifying the gRNA sequence to target the rs4481887 marker with the AA variant (over the GG variant), additional development and optimization will still be needed to complete the development of this CRISPR activity. Specifically, we still need to 1) further increase the signal to noise ratio (to the point that we can visually detect the difference between a positive and negative signal) by varying the concentration of the target RNA and/or the gRNA, 2) develop and optimize

the amplification (PCR)/transcription (*in vitro* transcription) reaction of DNA to RNA, and 3) develop and optimize the sample prep step of the process so that actual DNA extracted from human saliva could be used instead of synthetic target DNA/RNA. Once fully developed, this module could also be expanded to other genetic markers, such as rs72921001 (Eriksson et al. 2012; responsible for cilantro taste) or the TAS2R38 gene (Calancie et al. 2002; responsible for detecting a bitter chemical called PTC).

CHAPTER SIX: TESTING OF EDUCATION KIT MODULES IN CLASSROOMS

6.1 Introduction

Since I have designed all of these modules and activities to be used in high school classrooms, the next step was to test whether or not this FD-CF setup could be packaged and deployed in schools. All of the previous student data was done with students who were visiting our lab and were guided by myself or other lab scientists. For this to be useful as a classroom kit however, it needs to be something that a teacher (who may not have a background in lab research or in-depth knowledge of molecular biology) could use and teach their students with in their regular classrooms. To learn how these activities would fit into a typical classroom, and to also identify any areas of improvement in the technical aspects of a FD-CF kit, I developed a prototype kit (complete with curriculum) and sent them to schools across the country for beta-testing.

6.2 Prototype kit development

6.2.1 Designing set of classroom activities

The primary goal of this pilot beta-test was to identify any technical areas of improvement that needed to be made to the FD-CF kit platform (ex any portion of the protocol that might not be feasible for a classroom that I did not realize). The secondary goal of this pilot beta-test was to determine if the FD-CF kit had any value in 1) teaching students molecular biology concepts in a visual manner, 2) teaching students about the scientific method and how to conduct experiments, and 3) inspiring student interest in the field of molecular biology. While I had designed a variety of different modules and activities for the kit, I decided to focus on just the basic concepts for this prototype kit. The prototype kit contained four distinct activities, each building on the lessons learned from the previous activity. Each activity was accompanied with a student worksheet that

provided background information relevant to the lab, the protocol, and ended with lab analysis questions to be answered by the students (see **Appendix** for copies of each worksheet). The whole prototype kit was also accompanied with a Teacher Instructional Manual to provide more in-depth details about the activities and protocol, an answer key to the worksheet questions, a guide for the teacher in terms of classroom setup, and a troubleshooting manual (see **Appendix** for a copy of the teacher manual). These documents were developed in conjunction with a team of local high school biology teachers, who provided their expertise as educators to help build a protocol that made sense to a non-scientist and structured lab questions that aligned with typical high school biology classroom standards. The teachers also assisted me in developing feedback forms to gather data and feedback from both the teachers and the students who test the kits (see **Appendix** for the feedback forms).

The first activity is entitled "Observing Fluorescent Proteins" and its purpose is for students to produce fluorescent proteins that they can visualize, as well as familiarize the teachers and the students with how to handle the components in the kit and the techniques (i.e. pipetting) they will need. In this activity, students add different plasmids (encoding different fluorescent proteins) to FD-CF pellets, wait overnight, and then observe their results the next day in the illuminator. Teachers can use these results to explain what proteins are, how they have unique structures, and how that structure determines its unique function (in this case, fluorescence). This activity also helps acclimate the students and teachers to working with smaller volumes (~5 uL), micropipettes, and PCR tubes.

The second activity is entitled "From DNA to Proteins" and its purpose is to illustrate the central dogma of biology to the students (DNA to RNA to proteins). The process is broken down by step, allowing students to visualize each aspect of the central dogma process using the RNA-aptamer

based module developed in Chapter Three. Students add different plasmids (one encoding just red fluorescent protein, one encoding just the RNA aptamer, and one encoding both) to the FD-CF pellets and then warm the reactions either in an incubator (if available in the classroom) or in their hands for 15-20 minutes. At this time, the RNA aptamer sequence should have been transcribed to the point that the DFHBI green signal is visible in the illuminator – indicating transcription. Students then wait overnight and then observe their results to visualize the red fluorescent signal to indicate translation. The advantage of this activity is the fact that it highlights the transcription step and the synthesis of RNA, which is often glossed over in other hands-on activities.

The first two activities were designed as an introductory to basic molecular biology, so the third and fourth activity were designed for students to apply what they learned to and design experiments according to the central dogma. The third activity is entitled "How to Control Protein Expression – Part 1" and the purpose of this activity is to guide students to experiment with a single variable that impacts their protein expression. This will teach them about tuning protein expression as well as basics of experimental design. Students add varying amounts of DNA (by serially diluting the stock given to them) to the pellets and observe the effect of DNA amount on the brightness of their protein expression the next day. Even though DNA is something that can be reused, the higher amount of DNA the students add to this reaction, the more protein will be expressed in a given amount of time because there is more starting material for the reaction to work with before its energy source runs out. This activity also introduces experimental concepts, like positive and negative controls, and takes students through the scientific method.

The fourth activity is entitled "How to Control Protein Expression – Part 2" and the purpose of this activity is to allow students to freely experiment with a variable of their choice that might impact protein expression. This will allow students to apply everything they learned (both

biological concepts and basic experimental skills) from Activities 1-3 and design their own experiments. Students brainstorm a list of possible variables they would like to test and select one for their experiments. They will design an experiment to test their hypothesis with one negative control, at least one positive control, and three test conditions. This allows the students to think critically about the past activities, what they learned, and formulate ideas of other variables that might impact protein expression. They then get to apply inquiry-based learning strategies to design their own experiments and carry them out.

6.2.2 Optimization of reaction conditions

After selecting a set of fluorescent proteins to be used in the activities (eforRed, sfGFP, broccoli-eforRed), I next optimized the reaction conditions (DNA concentration and temperature) for classroom demonstrations. The prior experiments used a saturating concentration of DNA, but I wanted to determine the minimum amount of DNA plasmid that to add to a FD-CF experiment and still get robust expression. **Figure 63** shows that for sfGFP, only 5 ng of encoding plasmid is needed to generate a yield comparable to adding 50 ng of plasmid in a 5 uL reaction. Similar experiments were done for the other plasmids included in the prototype kit to determine the minimum concentration and the resulting concentrations are shown in **Table 10** in the Chapter Seven: Materials and Methods.

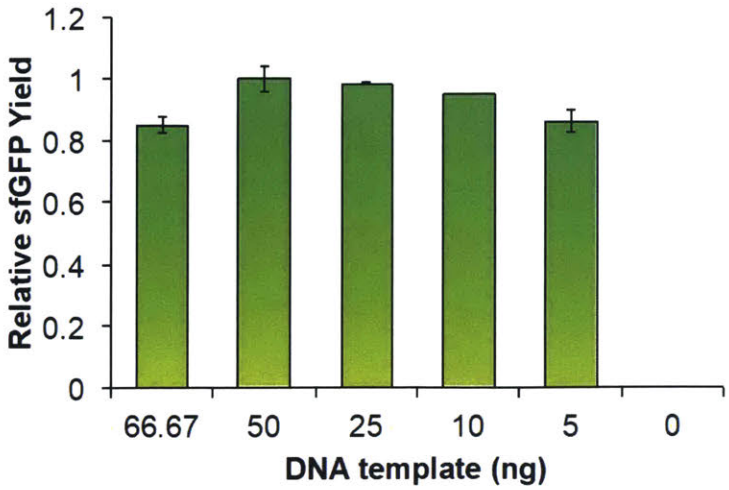


Figure 63: Optimal DNA plasmid concentrations for fluorescent proteins.

FD-CF reactions containing DNA template encoding sfGFP were incubated at 30°C for 20 hours. Endpoint yields for sfGFP synthesis measured via fluorescence at 20 hours show that protein synthesis is not limited by DNA template concentration. Done by Jessica Stark. Values represent averages and error bars represent average errors of N≥2 biological replicates. Figure taken from Stark, Huang, Nguyen et al. 2018.

For Activity 3, where the DNA is serially diluted before using, I needed to determine concentrations at which a visual difference could seen between the different concentrations. Using a similar experimental setup as above, I diluted both sfGFP and eforRed plasmids down below the minimum concentration that was previously determined and from a visual test, concluded that diluting both plasmids down from the minimum concentrations 5-fold twice (to obtain a three-point serial dilution) was sufficient to result in different visual results (**Figure 64**).

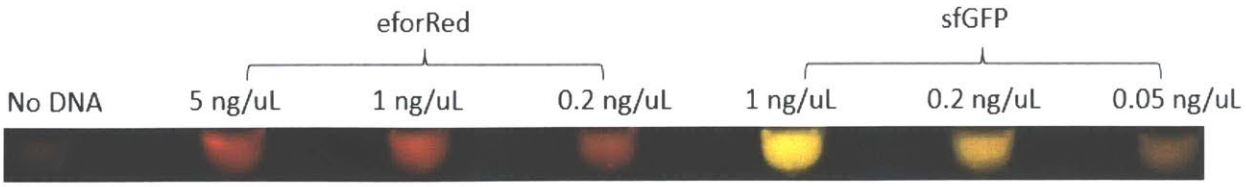


Figure 64: Diluted DNA plasmid concentrations for differences in fluorescent protein visualization.

FD-CF reactions containing DNA template encoding sfGFP and eforRed at serially diluted concentrations (1:5) were incubated at ambient temperature for 24 hours before visualized.

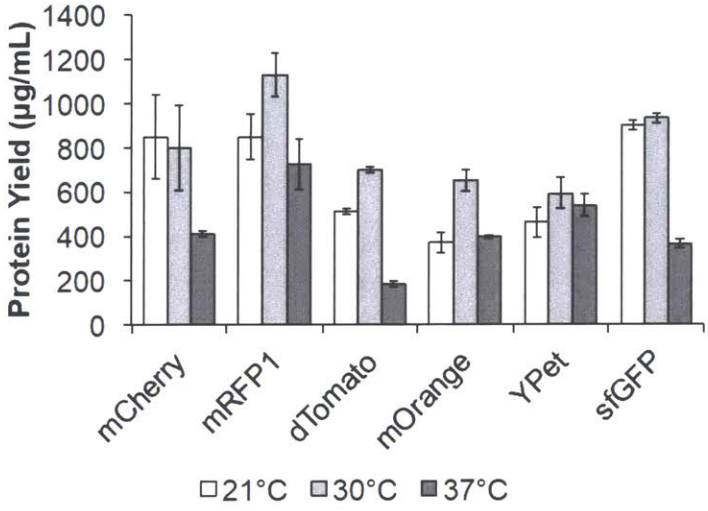


Figure 65: FD-CF reactions tolerate a range of incubation temperatures.

FD-CF reactions containing DNA template encoding mCherry, mRFP1, dTomato, mOrange, YPet, sfGFP were incubated at 37°C, 30°C, or 21°C. Reactions incubated at 37°C and 30°C were run for 20 hours, while reactions incubated at 21°C were run for 40 hours. Done by Jessica Stark. Values represent averages and error bars represent standard deviations of N=3 biological replicates. Figure taken from Stark, Huang, Nguyen et al. 2018.

Optimization was also done to determine what temperatures the FD-CF reactions could be done at, since many classrooms may not have access to a 37°C incubator. As seen in **Figure 65**, the FD-CF reactions appear to have the most protein expression at 30°C, although the protein expression

at room temperature is also comparable. From this, I decided to set the incubation temperature at room temperature to make it easier for the classroom and cut the kit costs even further by not having to include the portable incubator that we built.

Finally, I determined the maximum volume of DNA plasmid that could be added to the 5 uL FD-CF reaction to still obtain a visible signal. As many high school students would not have much experience pipetting, I anticipate many mistakes in pipetting that would result in the wrong volume being added – especially adding too much volume, since students might have trouble differentiating between the first and second stop on the pipette. To test the margin of error on these FD-CF experiments, I added increasing amounts of pJL1 eforRed to 5 uL FD-CF pellets. I used eforRed over sfGFP, as eforRed overall is a less efficient fluorescent protein.

As expected, the red fluorescent signal drops off sharply as the volume of plasmid added increases (diluting out the reaction). The signal is barely visible at 15 uL and completely gone by 20 uL. From this, it appears that the margin of acceptable error for these experiments is 10 uL (twice the target volume of 5 uL), as that still results in an output that can be readily seen.

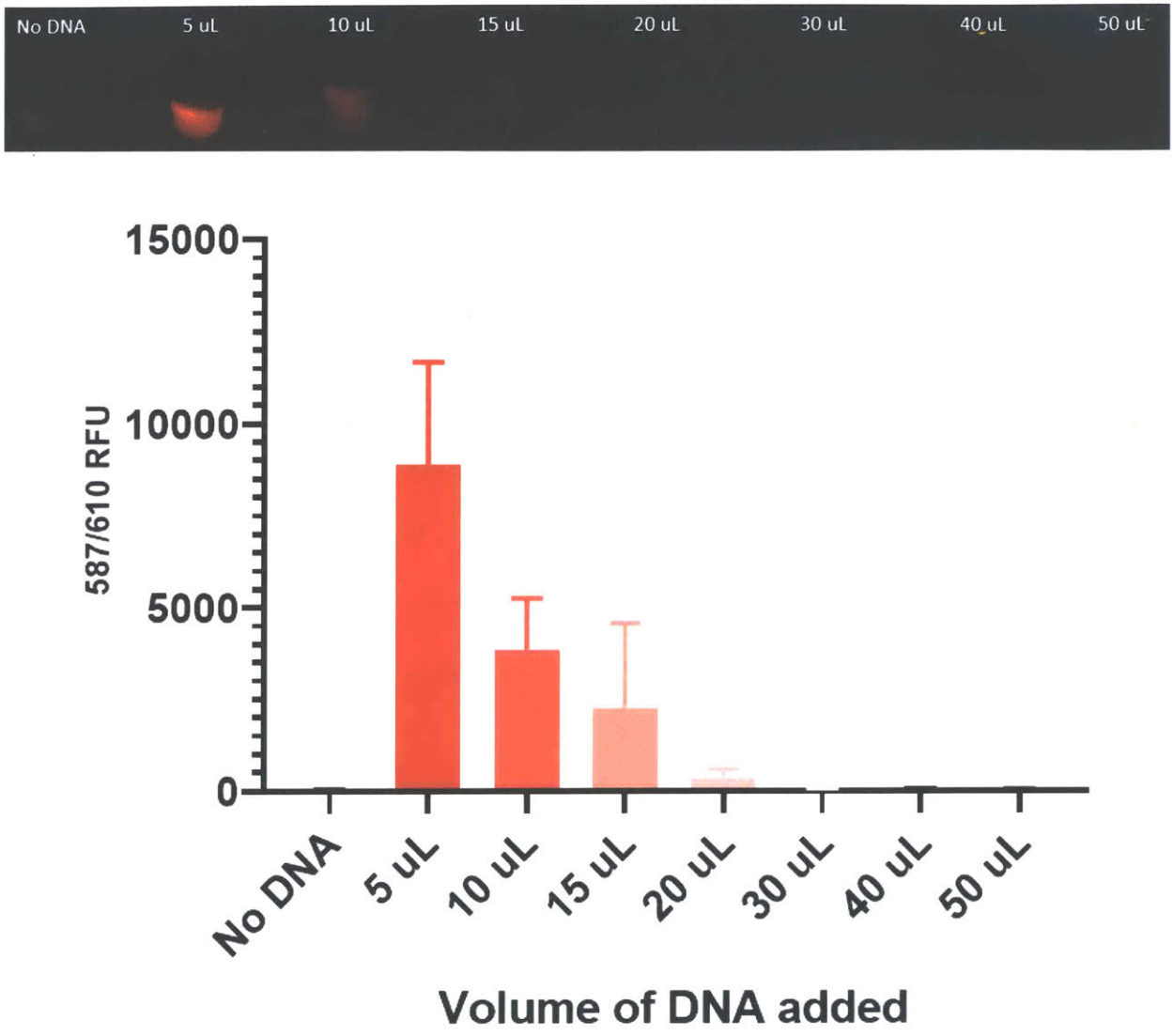


Figure 66: Determining the margin of error of volume added to FD-CF reactions.

5 uL FD-CF pellets were rehydrated with pJL1 eforRed DNA template at various volumes ranging from 5-50 uL and were incubated at room temperature overnight before visualized (top) and quantified (bottom) at 587/610 ex/em on a plate reader. Values represent averages and error bars represent standard deviations of N=3 biological replicates.

6.3 Setup of pilot study

After the initial publications outlining our FD-CF based kits for molecular biology education (Huang, Stark, Nguyen, et al. 2018; Stark, Huang, Nguyen, et al. 2018), many teachers and educators from around the country and world contacted my team and I to request kits. As these kits are not yet in commercial production, I was unable to fulfill these requests, but I did offer these teachers the opportunity to participate in my pilot beta-testing program. To recruit for this pilot study, I contacted all of the teachers who had reached out and were in the United States (to avoid international shipping logistics) and screened them to specifically identify the high school teachers, as the curriculum had been designed for that knowledge level. Ideally, I would have selected schools that fit the project's target user of low-resource high schools, but as the primary goal of the pilot study was to identify any technical areas of improvement, I recruited all types of high schools. From there, I had 51 high school teachers from 46 different schools. From December 2018 to April 2019, I sent each of these teachers a kit that contained all the materials they needed for their classroom, as well as the relevant documentation. Ideally, teachers would review the material, have the students fill out the pre-activity feedback form, have the students do all four activities in sequence, and then have them and their students fill out the post-activity feedback form.

As of writing, I have feedback from 26 of the teachers from 23 of the high schools. There are still 20 teachers who are still currently beta-testing and their feedback will be collected later. 2 of 26 the teachers completed the pilot study and provided their feedback failed to collect data from their students, so their classrooms were excluded from analysis (though their teacher feedback was still included). The remaining 5 teachers either dropped out of the pilot study partway through due to scheduling issues or stopped responding to requests for feedback. The teachers sorted based on 1)

their perceived resource level of their school (how much STEM funding they had, how many hands-on opportunities and equipment their students had access to, etc.) compared to other schools, and 2) what knowledge level of students they tested their kids on (either intro/basic biology or advanced/AP/honors biology). The breakdown is shown in the **Table 1**. (Note: unfortunately, I was not able to recruit an even distribution across all the categories).

Resource level of school/knowledge level tested on	Number of schools/classrooms
Low resource/intro or basic biology	5
Average resource/intro or basic biology	11
High resource/intro or basic biology	0
Low resource/advanced biology	2
Average resource/advanced biology	4
High resource/advanced biology	5

Table 1: Breakdown of the teachers/schools/classrooms that participated in the pilot study. Of the 26 teachers who completed the feedback at the time of writing, they were sorted to the categories displayed above. The total number adds up to 27 because one teacher tested the kits in both their basic and their advanced biology classrooms.

Of 26 teachers who provided feedback, not all of them were able to complete all four activities. **Table 2** displays the number of students who completed each activity and provided feedback (note: some classrooms did not complete the pre-activity feedback forms, but did provide post-activity feedback. In these cases, these classrooms were excluded from the quantitative analysis, but their qualitative responses were still analyzed). Time permitting, the teachers were also interviewed over the phone about their pilot study experience to probe further into their feedback and gain insights into what worked and what did not work for them.

Resource level of school/knowledge level tested on	Total number of students who participated	Number of students who completed Activity 1	Number of students who completed Activity 2	Number of students who completed Activity 3	Number of students who completed Activity 4
Low resource/intro or basic biology	47	45	42	26	23
Average resource/intro or basic biology	136	103	92	61	50
High resource/intro or basic biology	0	0	0	0	0
Low resource/advanced biology	24	22	24	13	14
Average resource/advanced biology	68	67	66	67	68
High resource/advanced biology	116	109	102	96	97

Table 2: Breakdown of the students that participated in the pilot study. The number of students who provided feedback who completed each of the different activities.

6.4 Results of pilot study

From the feedback forms collected, the responses were either a numerical score (i.e., a score from 1-5, a price, or a time in minutes) or written response to an open response question. For the written responses, I categorized each response into a larger category bin and counted the number of responses that fell in each category (per question) to convert these qualitative responses to a semi-

quantitative ones. When analyzing the data, I compiled all of the responses broken down by either school resource level or classroom knowledge level when relevant.

The first portion of the teacher feedback form asked them to indicate if they agree or disagree with these statements in **Table 3** regarding the prototype kit overall.

Question Code	Question (1-5 scale; 1=Strongly Disagree/3=Neutral/5=Strongly Agree)
TQ1	All of the kit components were easy to find and identify.
TQ2	The instructional manual as a whole was easy to follow.
TQ3	The kit provided educational value to my students.
TQ4	The kit inspired interest in biology in my students.
TQ5	I would be interested in using the kit again in my class.
TQ6	I would be interested in future new activities.

Table 3: Question codes for the agree/disagree statements for the kit overall. Each teacher was asked to indicate if they agree or disagree with the statements (on a scale of 1-5, with 1 being strongly disagree, 3 neutral, and 5 strongly agree). Each statement was assigned a question code for analysis and graphing purposes.

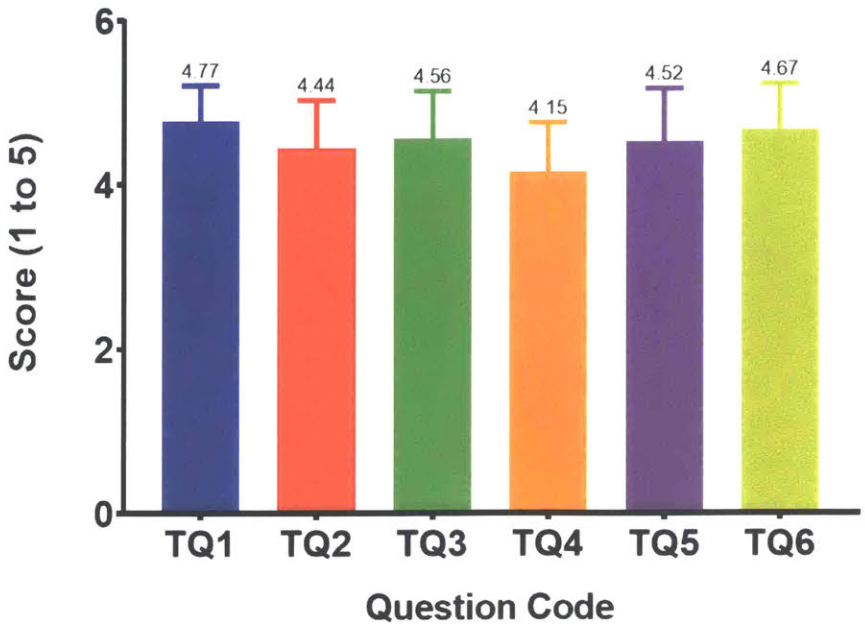


Figure 67: Results of teacher feedback – TQ1-TQ6

Results of the teacher feedback for questions TQ1-TQ6 (from Table 3). Values (displayed above each bar) represent averages and error bars represent standard deviations of N=26 teacher participants.

Nearly all teacher participants either agreed or strongly agreed on all of the statements listed in **Table 3** – that the kit was easy to use (TQ1 and TQ2), the kit provided educational value (TQ3), the kit inspired interest in biology (TQ4), and that they would be interested in doing these or similar new activities again (TQ5 and TQ6). The statement that had the lowest agreement score was "The kit inspired interest in biology in my students" (TQ4) with 4.15. While that is still a relatively high score (as 4 = Agree), it indicates that there may be room to improve the kit to make it more engaging for the students.

The teachers were then asked a series of open-response questions about the kit overall. For these responses, I sorted them into categorical bins (as many of the responses fell into a few overarching categories) and counted the number responses per bin per question. Some open responses listed several items that fell into different bins/categories (i.e., a teacher listing three distinct elements

that they liked about the kits), so a single response may result adding counts to multiple bins/categories. To gain more insight about how well the kit worked across different knowledge levels and school resource levels, I also analyzed the number of responses by those breakdowns. On the graphs below, percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level. The percentage breakdown by bin/category then can be compared to the percentage breakdown of all total respondents to identify any trends that are found more in specific classroom knowledge levels or school resource levels. Note that due to the small and uneven sample size of the teacher feedback, statistically significant conclusions are not possible; however, I can still use the data to make informal conclusions to help guide designs/improvements to future kits.

When asked "What was your favorite part of the kits?," the top response was that the labs in the kit were easy to use, regardless of classroom knowledge level or school resource level (**Figure 68**). When speaking to teachers prior to designing the prototype kit, many of them brought up that their biggest challenge was the complexity of existing molecular biology classroom kits. For example, the pGLO kit (BIO-RAD) that is commonly done requires quite a bit of teacher preparation – they have to make cell media, grow cells, and obtain equipment (water bath, incubator, etc.) not included in the kit beforehand. This can take a teacher up to a week in prep time to accomplish, and the teachers I spoke to said that due to limited class and prep time (even at high resource schools), this causes them to cut this lab from their schedule, even if they are able to afford it. As one teacher put it:

> *"I appreciated how easy it was to set up the activities and how very little equipment was needed to complete the lab."*

Besides ease of use, the other responses was that the labs were engaging for their students and helped teach biological concepts and basic lab skills (**Figure 68**). Specifically, one phrase – that the students felt like scientists doing the labs – kept repeating so often that it was placed in its own bin/category. As one teacher said:

> *"The students felt like scientists and doctors by touching and using the laboratory materials provided during the [lab] demo. Even the students that didn't seem engaged were secretly paying attention and wanted to take their turn pipetting. They knew they were part of something really cool and so we were able to connect with them in a way that was new to them."*

There did not seem to be a huge difference in these responses between intro vs. advanced biology classes, although when broken down by school resource level, it appears that low resource schools more appreciated using the labs as a way to teach basic biology concepts (67% of those who reported this were low resource, compared to 59% of total low resource respondents), while average or high resource schools more appreciated using the labs as a way to teach basic lab skills and show students what being a research scientist is like (83-85% of those who reported these were average resource, compared to 41% of total average resource respondents).

One final response that a few teachers brought up was the reliability of the lab/kit:

> *"It worked! That doesn't always happen with biology labs."*

Which helped support that this prototype overall worked from a technical perspective and could be used by teachers and students who may not have a background in molecular biology labs.

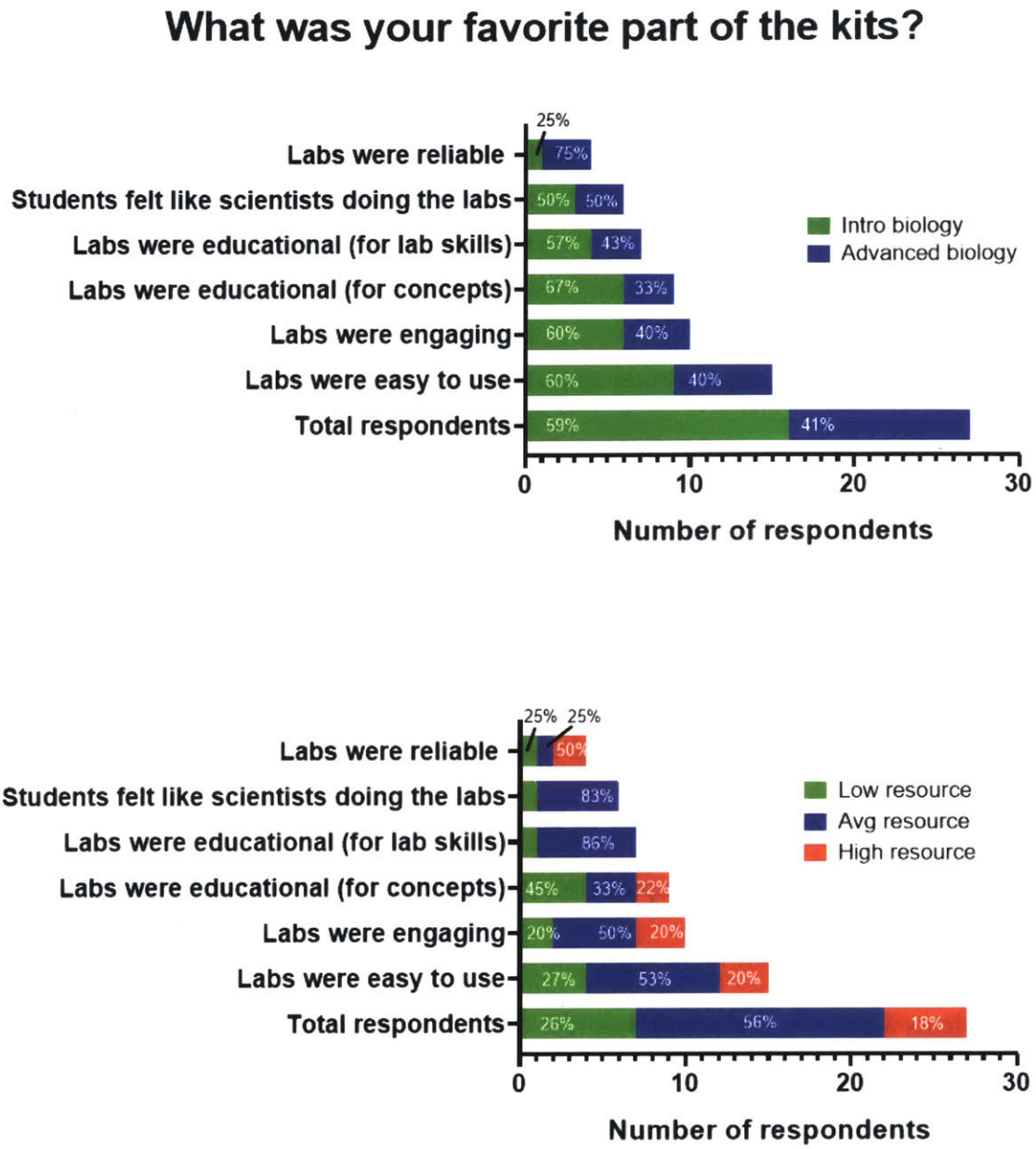


Figure 68: Results of teacher feedback – open response to "What was your favorite part of the kits?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was your least favorite part of the kits?," the top response was regarding the curriculum – either that some steps of the protocol were not entirely clear (which I will describe in more detail below for each individual activity) or that they wanted more background information about the labs they were doing with the students, regardless of classroom knowledge level or school resource level (**Figure 69**).

> *"My students come from many different schools and different levels of knowledge, perhaps a bit of background information [is needed]. Since this was my first time using the kit it might have been nice to know how much the students are expected to know before we started the activity."*

The advanced biology classrooms and the high resource classrooms also reported the labs being overall too easy for their students (**Figure 69**). To help improve the kits on these points, one major change would be to design curriculum to fit each type of biology class. In this pilot test, I only had one set of curriculum for all participants (regardless of if there were an introductory or an advanced class), which may have resulted in the curriculum being too advanced for the introductory classes and too basic for the advanced classes. I will continue to work closely with educators to develop more curriculum to fit the needs of individual classrooms further.

Another challenge was getting the students to pipette precisely (**Figure 69**). To save overall kit costs, each FD-CF pellet included was only 5 uL, and for an inexperienced user, this was a difficult amount to grasp. When I followed up and interviewed the students, I did find out that those teachers, who spent an entire class period or more having the students practice pipetting with food coloring and seeing how large the volumes would be, reported less issues of precise pipetting than those teachers who started their students straightaway with the labs. It also appears that most of the

classrooms that struggled with pipetting were either intro level classrooms or low/average resource classrooms, which was expected, as advanced or high resource classrooms may have more opportunities to practice pipetting in other labs. To help with this, I plan on making it more clear in the teacher manual that some level of pipetting practice is highly recommended, and not just suggested, in order for more successful results for their students.

One final major drawback to the kit was that, due to the simplicity of the experiment (just adding water to the pellets), it was difficult for the students to tell what was happening at the molecular level. As one teacher put it:

> *"For some the students, the project seemed more like magic than science. Put liquid into pellet and colors appear. I think having everything all ready packaged together, they had a difficult time parsing what was happening."*

This was a response mentioned regardless of knowledge or resource level, and will be one of the main areas of improvement in the future (**Figure 69**). One strategy to help overcome this issue would be to incorporate more inquiry-based learning explorations into the lab activities, so instead of passively adding water and observing the result, the students can actively manipulate specific variables to obtain results that will help inform them of what is happening at the molecular level. For example, incorporating some of the work shown in Chapter 3 to add specific elements to inhibit transcription or translation and observing the results can help better illustrate the central dogma. Another strategy would be linking these activities more to real-world examples. During pilot testing, I had the opportunity to visit some of the classrooms and talk about my work in the lab. In doing so, I realized that the students were interested in how FD-CF was being applied to other fields, such as diagnostics or protein production for medicine. Including these kind of examples in

the curriculum will help provide more context for what is happening, and hopefully, engage more of the students and help them learn.

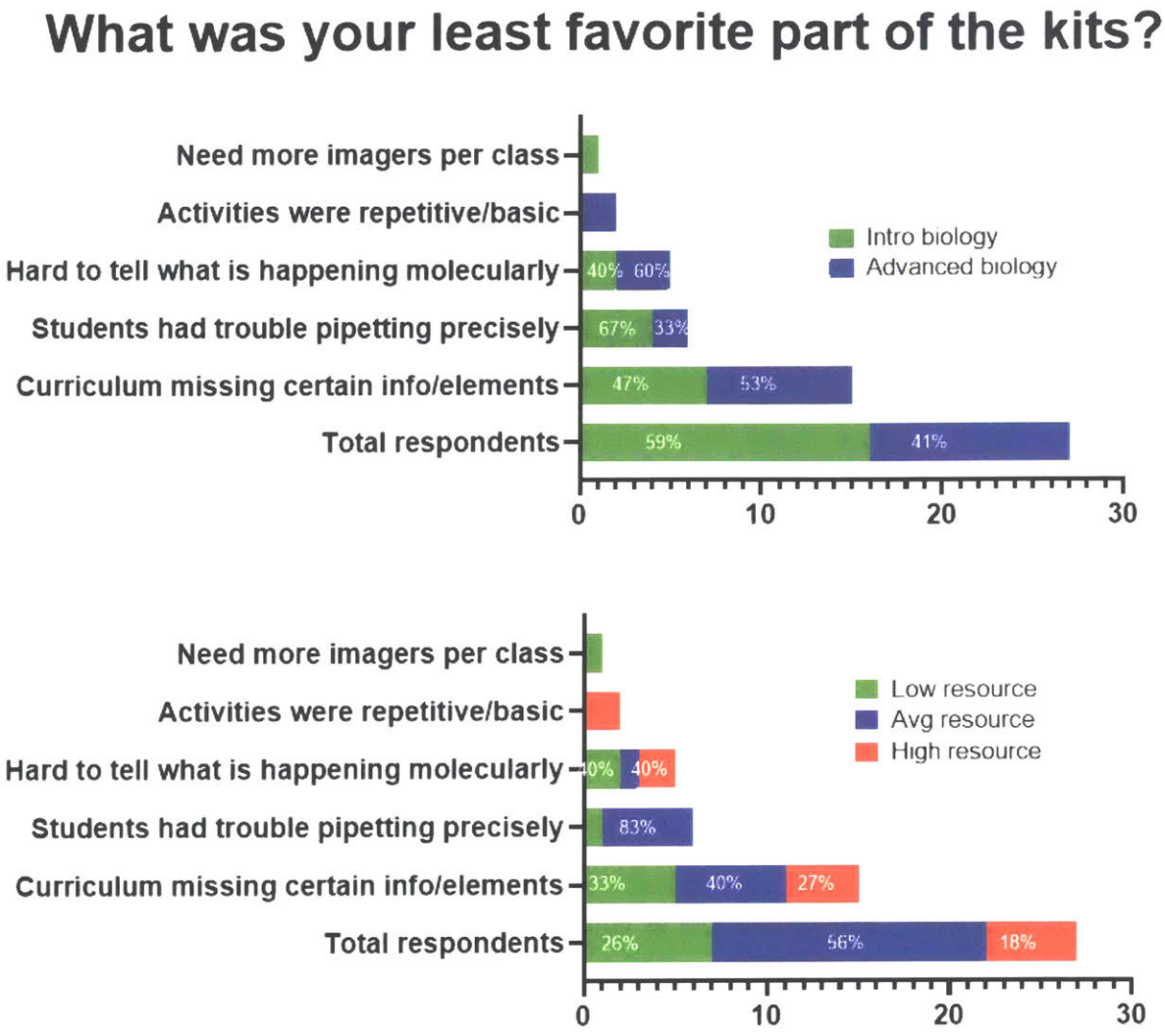


Figure 69: Results of teacher feedback – open response to "What was your least favorite part of the kits?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "How would you explain these kits to a fellow teacher?," the top responses were that they were educational and/or easy to use, regardless of classroom knowledge level or school resource level (**Figure 70**). This helps support my primary goal of having these kits easily functional in a classroom to help teach molecular biology concepts. Some examples of how teachers would explain the kits are:

> *"I told my biology colleagues that it was a very easy to use lab that produced great results."*
>
> *"It's a kit that allows you to bring molecular biology into a classroom in a safe and surprisingly fun way!"*
>
> *"[The kit] offers a fun and quick way to allow students to visualize protein synthesis. It's hands on, simple, quick and requires no lab equipment and yet, students can make modifications to increase or decrease protein expression."*
>
> *"A great way to get in another transcription/translation lab with little prep and consistent results for students to see."*

Many teachers also would explain the kits as enjoyable/engaging. Most of these teachers were either teaching introductory level biology classes (70% of respondents who said enjoyable were from intro level classes, versus 59% of total intro level respondents) or from low resource schools ((50% of respondents who said enjoyable were from low resource schools, versus 26% of total low resource respondents; **Figure 70**). This could be due to that in more advanced classes or schools with more resources, the teachers and students have more opportunities to do hands-on molecular biology experiments and that these activities were not as novel to them.

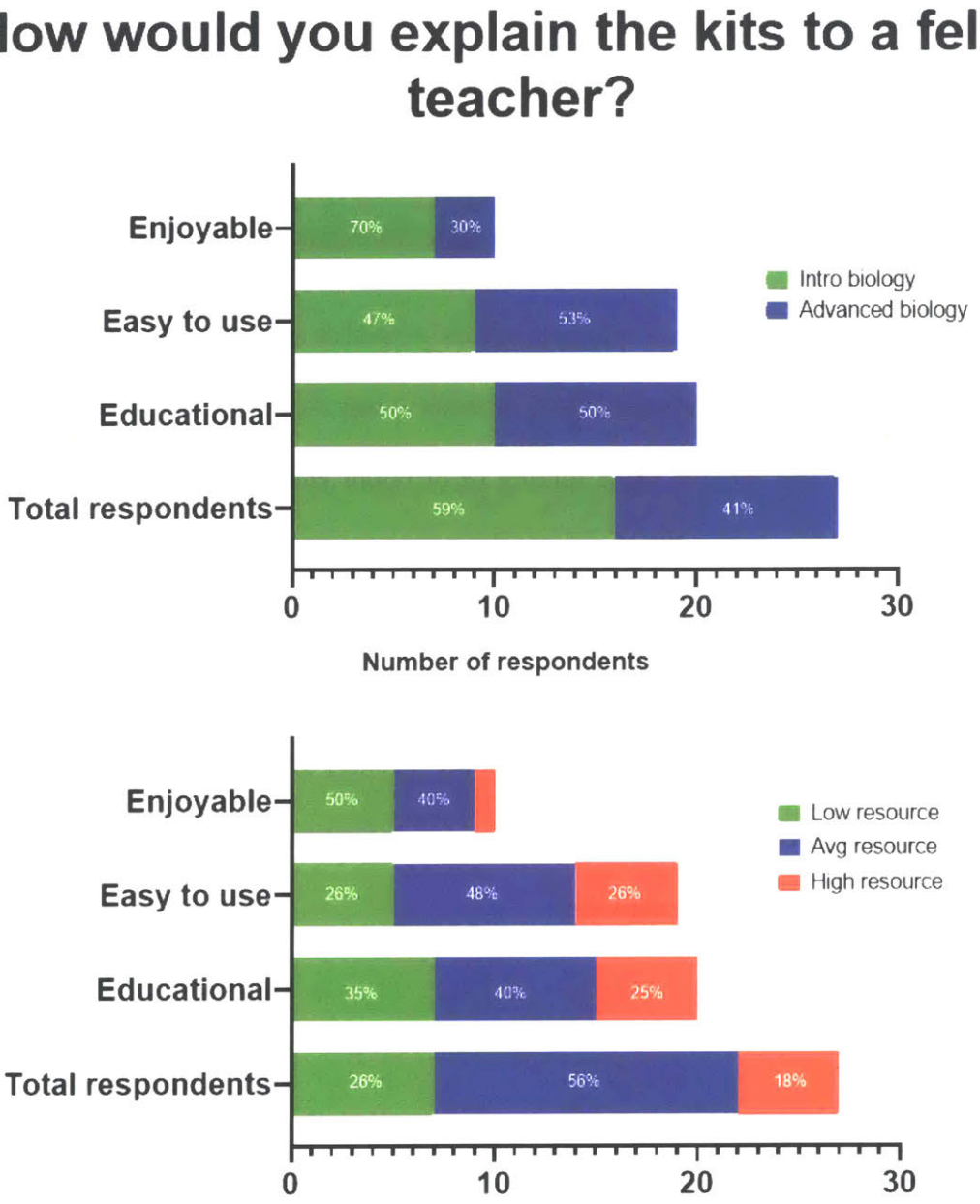


Figure 70: Results of teacher feedback – open response to "How would you explain the kits to fellow teacher?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

Teachers were also asked how long each of the activities took to run in their classrooms. Because classes often run on strict timetables, I wanted to make sure the activities could be completed in a given class time. From my interviews with teachers prior to the study, I found that biology classes are at least 40 minutes long (with some classes as long as 90 minutes, depending on the school).

Activity 1 and 2 (which illustrate basic concepts and only involve adding the DNA plasmid to the pellets) took most of the teachers between 20-60 minutes to run. Activity 3 and 4 (which illustrate basic scientific method and involve more mixing of different elements before adding it to the pellets) took most of the teachers between 40-60 minutes to run (**Figure 71**). This timeframe includes both the time it takes on the first day to setup, as well as some time the next day for observation and discussion. Based on this feedback, it seems that the activities can fit within a typical class timeframe. When I spoke with some of the teachers afterwards, they also mentioned that the minimal prep involved also saves them quite a bit of time to prepare and allows them even more time in class to allocate for the activities.

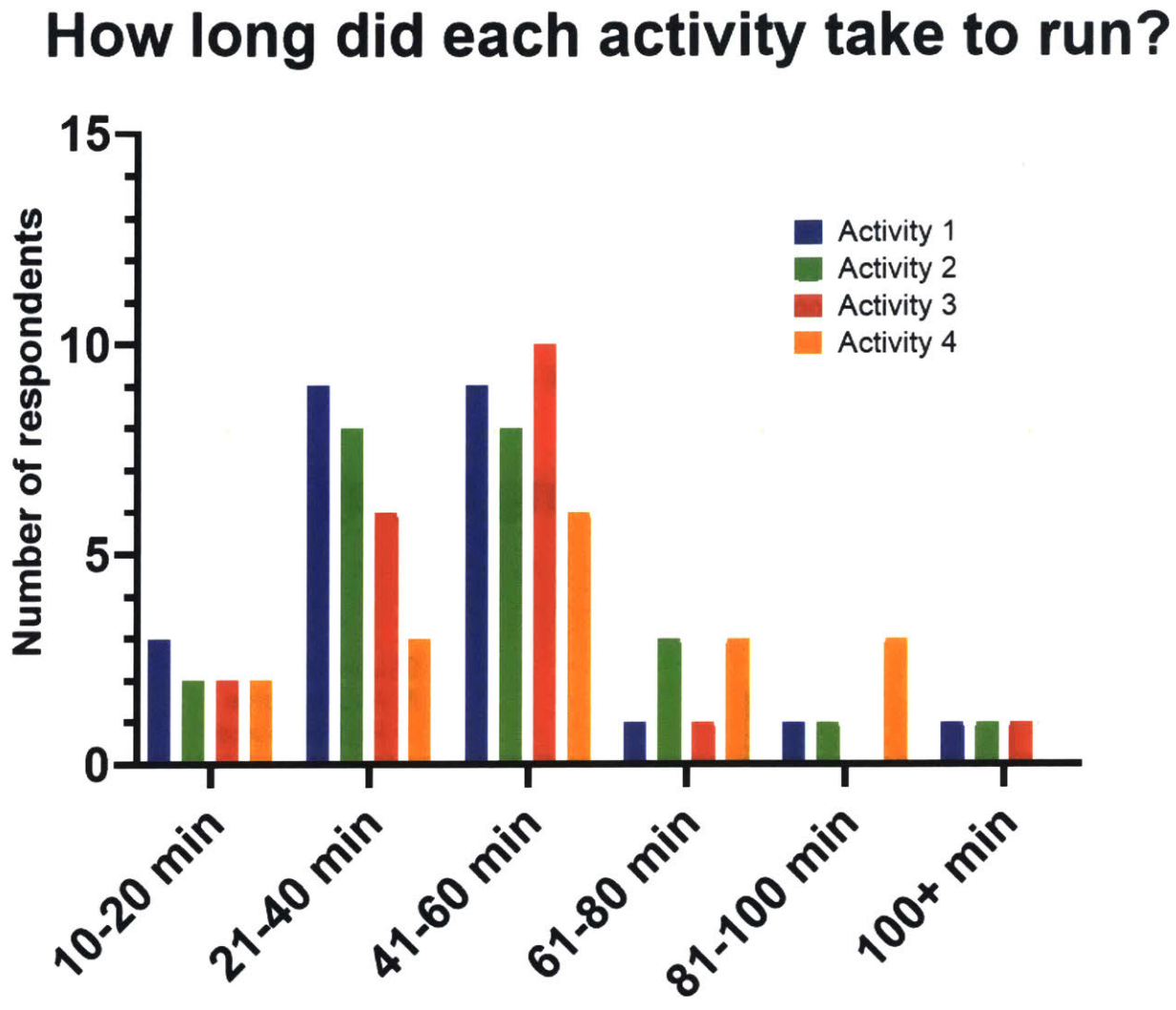


Figure 71: Results of teacher feedback – numerical response to "How long did each activity take to run"

Teachers were asked this question for each activity. The responses to each activity were binned into the ranges shown above. N=26 teacher participants.

Teachers were also asked what they would be willing to pay or expect the kit to cost. My target kit cost is between $75-100 (based on how much it costs to manufacture the kit). There appears to be a bimodal distribution (again, not significant due the number of responses), with a cluster of teachers who would pay $50-75 per kit and a cluster of teachers who would pay $100+ for the kit (**Figure 72**). When the kit is commercialized, I will attempt to keep the costs within these ranges, as my primary goal with this project is to make the kit as accessible to as many schools as possible.

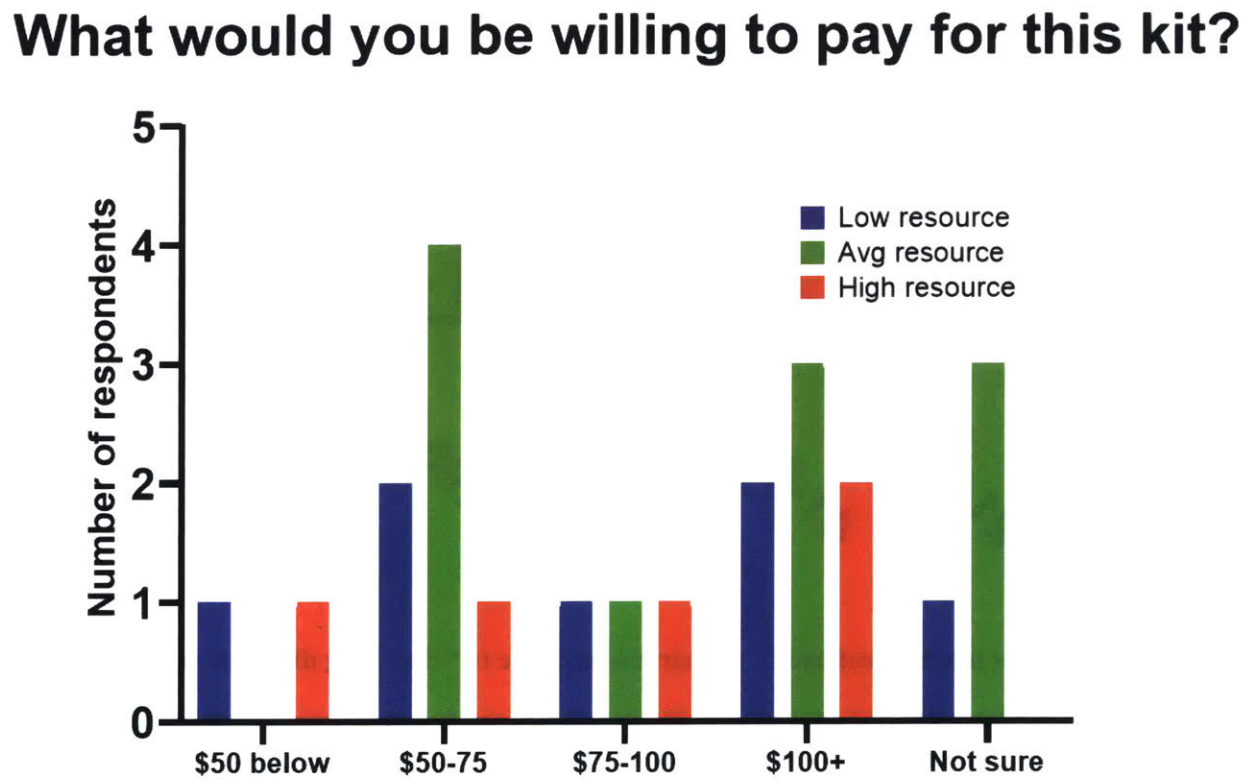


Figure 72: Results of teacher feedback – numerical response to "What would you be willing to pay for this kit?"

Teachers were asked this question for each activity. The responses to each activity were binned into the ranges shown above. N=23 teacher participants (some teachers chose not to answer this question).

Question Code	Question (1-5 scale; 1=Strongly Disagree/3=Neutral/5=Strongly Agree)
TQ1.X	Activity X was easy to setup for my classroom.
TQ2.X	Activity 1 was easy to teach and run in a classroom
TQ3.X	The teacher manual for Activity X was easy to follow.
TQ4.X	The worksheet for Activity X was easy to follow.
TQ5.X	I, as a teacher, learned new things from Activity X
TQ6.X	Activity X was fun and engaging for the students
TQ7.X	Activity X was useful to teach biological concepts
TQ8.X	Activity X was useful to teach scientific inquiry and skills
TQ9.X	I would be interested in using Activity X again in my class

Table 4: Question codes for the agree/disagree statements for the specific activities. Each teacher was asked to indicate if they agree or disagree with the statements (on a scale of 1-5, with 1 being strongly disagree, 3 neutral, and 5 strongly agree). Each statement was assigned a question code for analysis and graphing purposes. The X in the code refers to the activity number (1, 2, 3, or 4), as the teachers were asked the same set of statements for each specific activity.

The first portion of the teacher feedback form asked them to indicate if they agree or disagree with these statements in **Table 4** regarding the specific individual activities in the kit. The teacher participants either agreed or strongly agreed on nearly all of the statements listed in **Table 4** –the activity provided educational value (TQ5.X, TQ7.X, Q8.X), and that they would be interested in doing this activity again (TQ9.X). The results are displayed in **Figure 73**.

All 4 of the activities had an agreement score of at least 4.25 for the questions pertaining to how easy the activity was to use (TQ1.X, TQ2.X, TQ3.X, TQ4.X), although Activity 3 and 4 did have slightly lower scores than 1 and 2. This is expected as Activity 3 and 4 have a slightly more

complicated protocol, but the open-response feedback discussed below identified potential areas to help improve this.

All 4 activities had lower agreement scores for the question pertaining to if the teachers themselves learned anything new (TQ5.X). For Activity 1, 3, and 4, the scores were less than 4.0, meaning the teachers were more neutral on this statement. This is expected, as these activities cover topics that teachers typically already teach, so while the technology might be novel to them, the actual concepts taught are not. Activity 2 did have a slightly higher agreement score of 4.15, which is likely due to the inclusion of the RNA aptamer in the experiment, which teachers may not have heard of prior to this activity. All 4 activities had an agreement score of at least 4.40 for the question pertaining to how fun and engaging the activities were (TQ6.X).

Activity 2, 3, and 4 had an agreement score of at least 4.40 for being useful to teach biological concepts (TQ7.X) and at least 4.41 for being useful to teach scientific inquiry and skills (TQ8.X). Activity 1 had lower agreement scores for both of these statements (4.26 and 3.96, respectively), which is likely due to that activity being an introductory activity to the kit and focuses more on acclimating students to the kit than teaching much concrete concepts.

Despite this, Activity 1 still had a relatively high agreement score (4.41) for if the teachers would like to repeat this activity in the future (TQ9.X). Even though it was simplistic, teachers commented that it was very helpful as a foundational activity for the students to build upon when they did the other activities. Activity 2 and 4 also had high agreement scores (4.44 and 4.47, respectively) for this statement. Activity 3 had a slightly lower agreement score (4.28) for this statement. Teachers commented while they enjoyed it, they felt that Activity 3 could be included in Activity 4 (with DNA dilution from Activity 3 as one of the potential variables tested in Activity

4 rather than its own standalone activity), especially if the teacher was short on time and did not room for all 4 of these activities. Specific areas of improvement for each activity are discussed further below with the results from the teachers' open responses about each activity.

As the plan is to commercialize this kit in the future to make it more accessible, I also utilized a commonly used metric in business and management, the Net Promoter Score (NPS), a metric that measures a customer's "loyalty" or satisfaction with a product. Customers (or in this case, the teacher participants), are asked how likely (on a scale of 0-10) that they would recommend this product (the prototype kit) to a friend or colleague. Responses of 9 or 10 are labeled as Promoters, 7 or 8 as Passives, and 6 or below are Detractors. The Net Promoter Score is calculated by subtracting the percent of Detractors from the percent of Promoters, resulting in a score that ranges from -100 to 100 (**Figure 74, top**). Scores from 0-50 are considered good, 50-70 are considered excellent, and 70+ are considered exceptional. This score is often used in the business and marketing world to measure customer satisfaction of a product (Kaplan 2016).

As seen in **Figure 74,** bottom, many teachers would recommend this kit to a fellow teacher. In fact, the lowest score given in the feedback was a 7, meaning that there were no Detractors. In fact, the majority of the teachers answered 9 or 10, making them Promoters. When I break down the NPS by resource level, it reveals that low and average resource schools resulted in an NPS rating in the "exceptional" zone, while ratings by the high resource schools were only in the "good" zone. This is expected because as mentioned above, high resource schools likely have more opportunities for their students, so a kit like this one is likely not novel or needed for their schools. When breaking down the NPS ratings within each resource level by knowledge level, there is no difference between intro or advanced groups – both still fall in the "exceptional" zone.

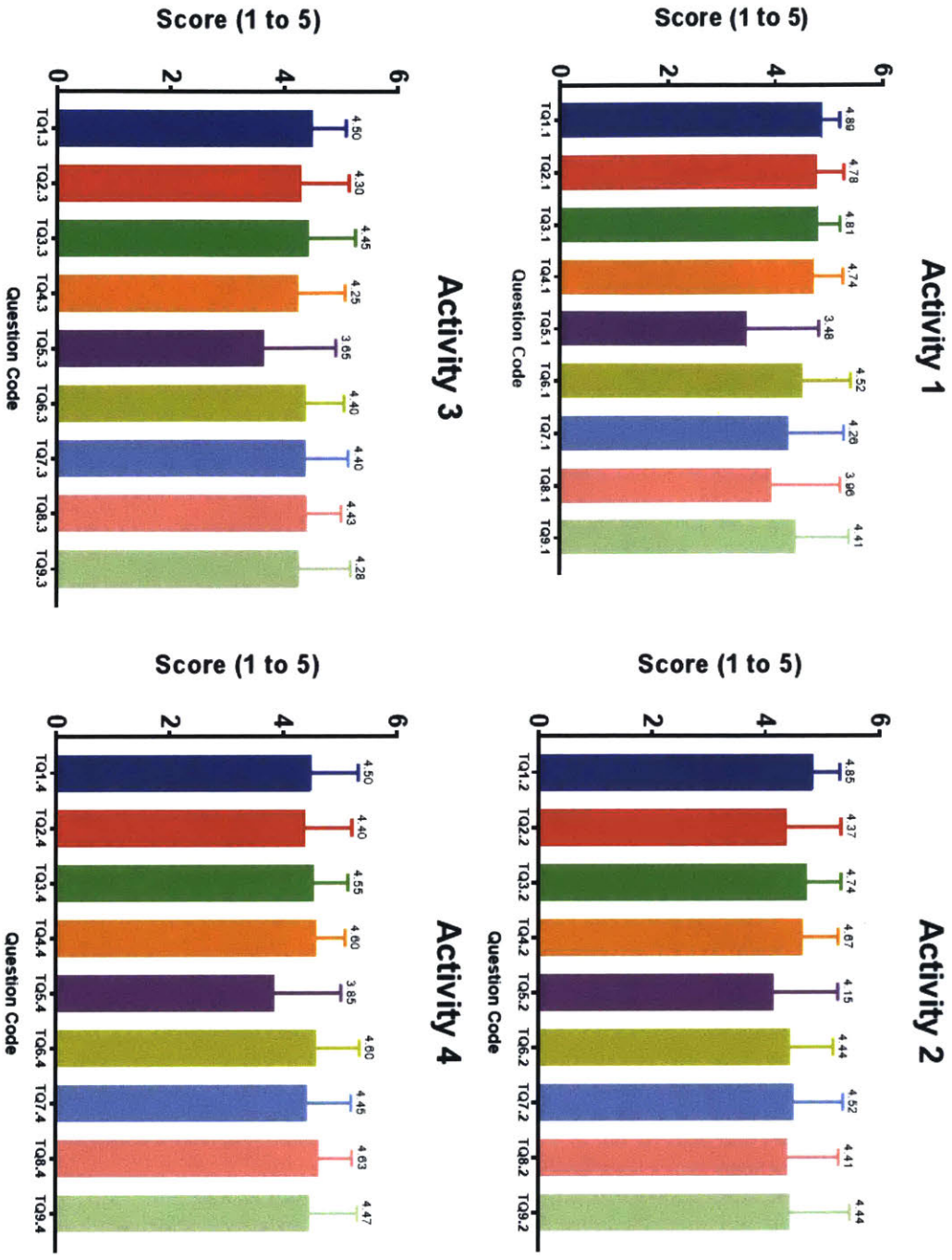
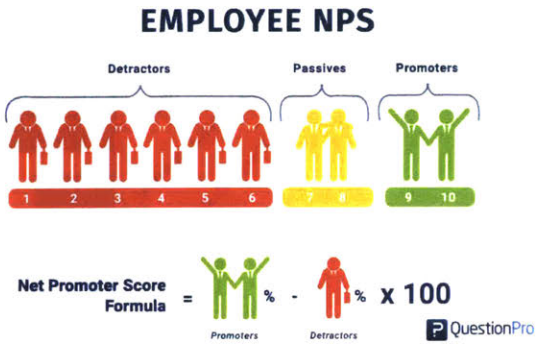


Figure 73: Results of teacher feedback – specific for each activities

Results of the teacher feedback for questions (from Table 4) for each individual activity. Values (displayed above each bar) represent averages and error bars represent standard deviations of N=26 teacher participants.



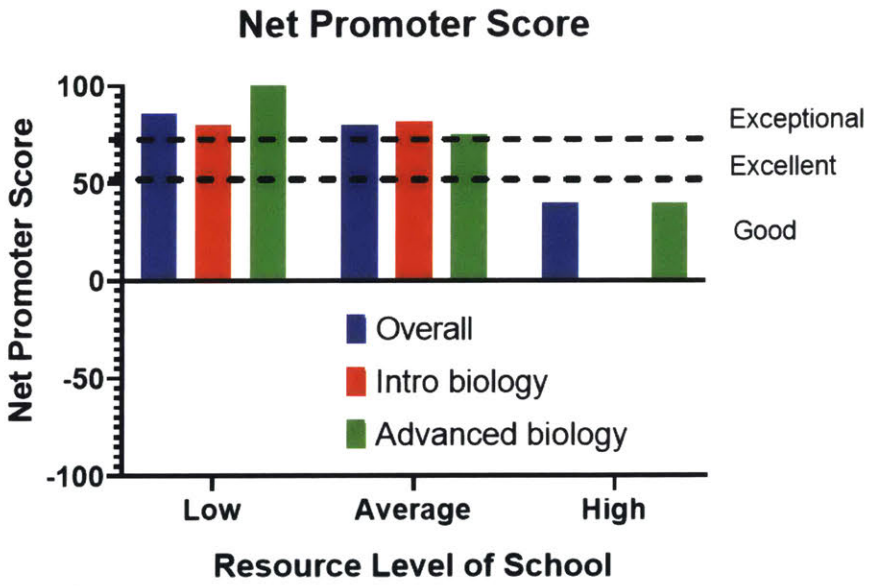


Figure 74: Net promoter score for the overall prototype kit.

(Top) Graphical definition of the net promoter score (NPS) taken from QuestionPro.com. (Bottom) NPS calculated from teacher feedback from N=26 teacher participants. NPS scores were further broken down by classroom knowledge level (intro or advanced) and resource level of school (low, average, or high).

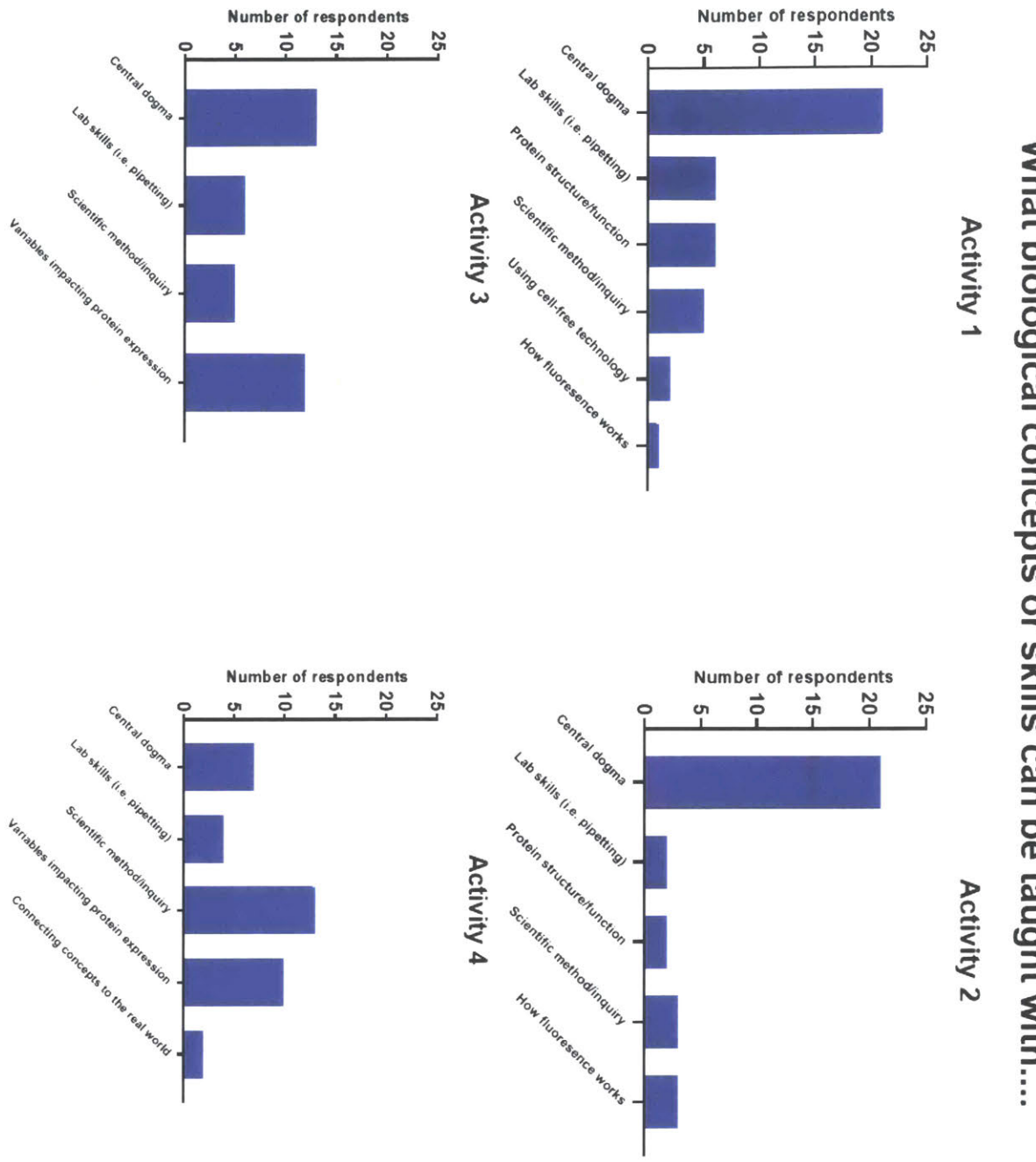


Figure 75: Results of teacher feedback – open response to "What biological concepts or skills can be taught with Activity X?"

Responses from teachers were binned into larger categories and quantified. N=26 teacher participants.

Teachers were then asked open-response questions for each specific activity. **Figure 75** shows their responses to "What biological concepts or skills can be taught with Activity X?" The majority of respondents said "central dogma of biology" for Activity 1 and 2, while also adding "variables impacting protein expression" and "scientific method/inquiry" for Activity 3 and 4. Since Activities 1 and 2 were developed to teach the central dogma and 3 and 4 to teach manipulating protein expression through the scientific method, the prototype kit was successful in getting these teaching goals across to the teachers through the curriculum.

When asked "What was you or your students' favorite part of Activity 1?," the top response was viewing the fluorescent protein results at the end of the lab, regardless of classroom knowledge level or school resource level (**Figure 76**). In fact, 23 out of the 26 respondents listed this as their response. Since I chose fluorescent proteins as an output for this system to be engaging and eye-catching, these results seem to support that the fluorescent proteins were successful in this regard.

"They loved how vibrant the colors were."

"Seeing the results, definitely. The oohs and ahhs were awesome."

Another common response to the question was being able to use the micropipettes (for the first time for some of the students) and the process of carrying out the lab, though this response was more popular with introductory biology classrooms or low/average resource classrooms. This is likely because these classrooms have not gotten many opportunities to do hands-on molecular biology labs prior to this. Again, in the responses, the phrase "made students feel like real scientists" was commonly used.

"Using the micropipettes seemed to be everyone's favorite activity. That made the students feel like "real" scientists."

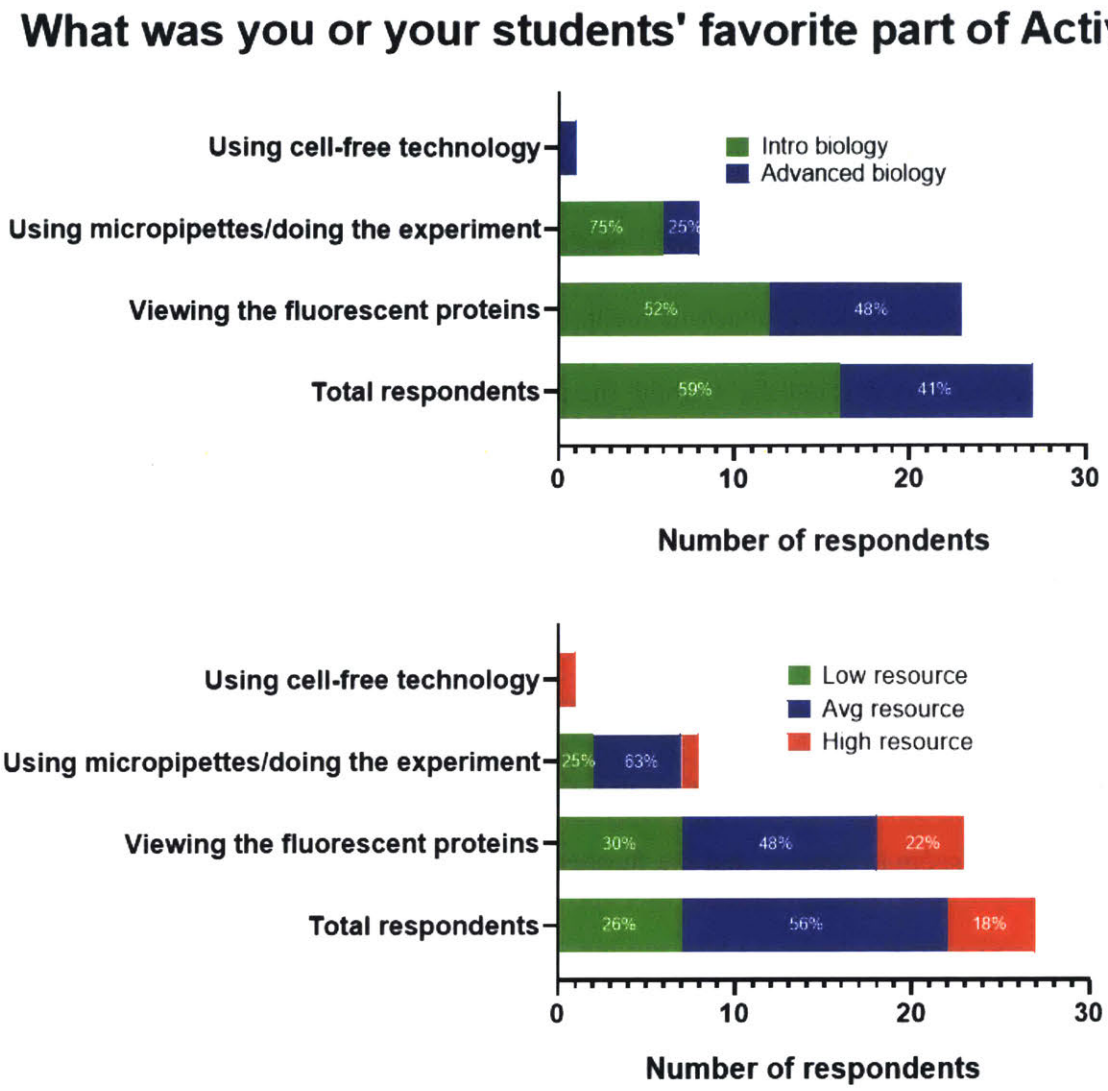


Figure 76: Results of teacher feedback – open response to "What was you or your students' favorite part of Activity 1?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was the most challenging part of Activity 1?," the responses were similar to the responses from overall "What was your least favorite part of the kit?" question, where students had trouble pipetting precisely and the difficulty in determining what is happening at the molecular level in such a simple system (**Figure 77**). Again, the pipetting issue can be addressed by emphasizing the importance of pipette practice prior to doing the activities, and the experiments can be explain more at the molecular level by incorporating more inquiry-based learning explorations and concrete real world-applications for the students.

One technical issue that was brought up was that some teachers wanted more accessories in the kit to help with carrying out the experiment. For example, only one imager per kit was included, but teachers wanted more per classroom so the entire class did not have to share. This can easily be remedied by offering more imagers for the teachers to buy as a kit add-on, as well as encouraging them to explore making their own with the open-source CAD files that were published on this work (Stark, Huang, Nguyen et al. 2018). Another item that teachers wanted were tube racks to hold both the Eppendorf tubes (containing the DNA plasmids) and the PCR tubes (containing the FD-CF pellets). I had assumed that these were not necessary because the small volumes contained in the tubes will not pour out even when lying on the side due to surface tension. However, when following up with teachers afterwards, I realized that students, especially those who have not handled these items before, needed racks to hold the tubes while they pipetted to help stabilize. When commercializing the kits, I will be sure to take this into consideration.

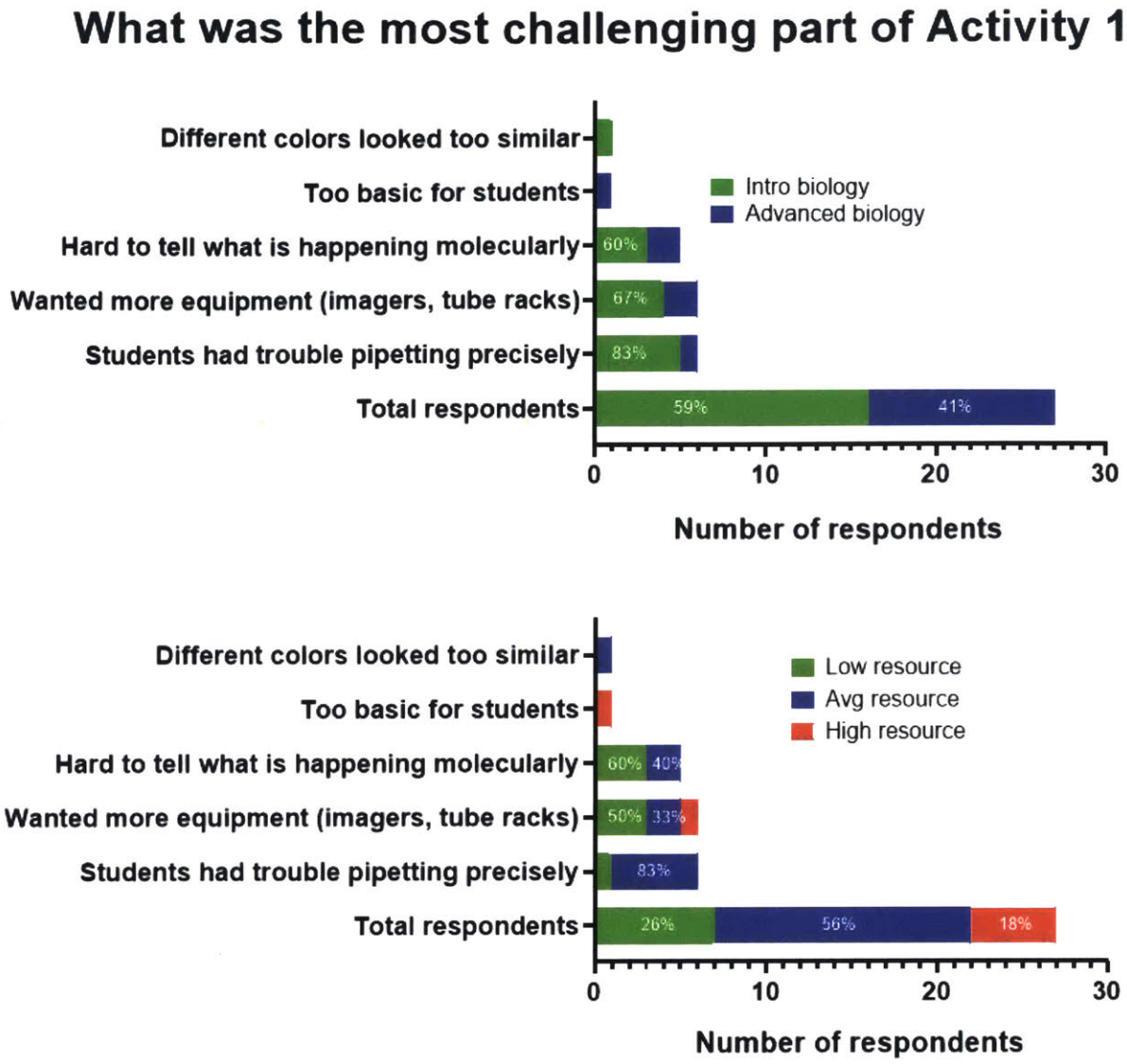


Figure 77: Results of teacher feedback – open response to "What was the most challenging part of Activity 1?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was you or your students' favorite part of Activity 2?," the top response was again viewing the fluorescent protein results at the end of the lab, regardless of classroom knowledge level or school resource level (**Figure 78**). Furthermore, the majority of teachers specified that it was viewing the central dogma stepwise – being able to see the green signal that corresponded to transcription and the red signal that corresponded to translation. When interviewing the teachers afterwards, some of them emphasized that no other molecular biology labs that they have used before actually highlight RNA or the transcription process, and they really appreciated that this activity did.

> *"I like how it visually separates the transcription and translation process - students don't often see them as two distinct processes taking some time."*

> *"Students actually liked that is was a two step process and they could see results on the first day and still observe something different on the next day."*

Again, using micropipettes to actually carry out the experiment was another common response, especially for introductory level or low/average resource classrooms.

> *"Using the pipettes. I also think they liked seeing the results. I heard a lot of "that's cool"."*

Some teachers also liked how the experiment inspired class discussions about what was actually happening because discussions can further enhance students' learning.

> *"They had the hardest time with this section, [but] they eventually figured it out and the ones who made reasonable guesses were very satisfied with the results."*

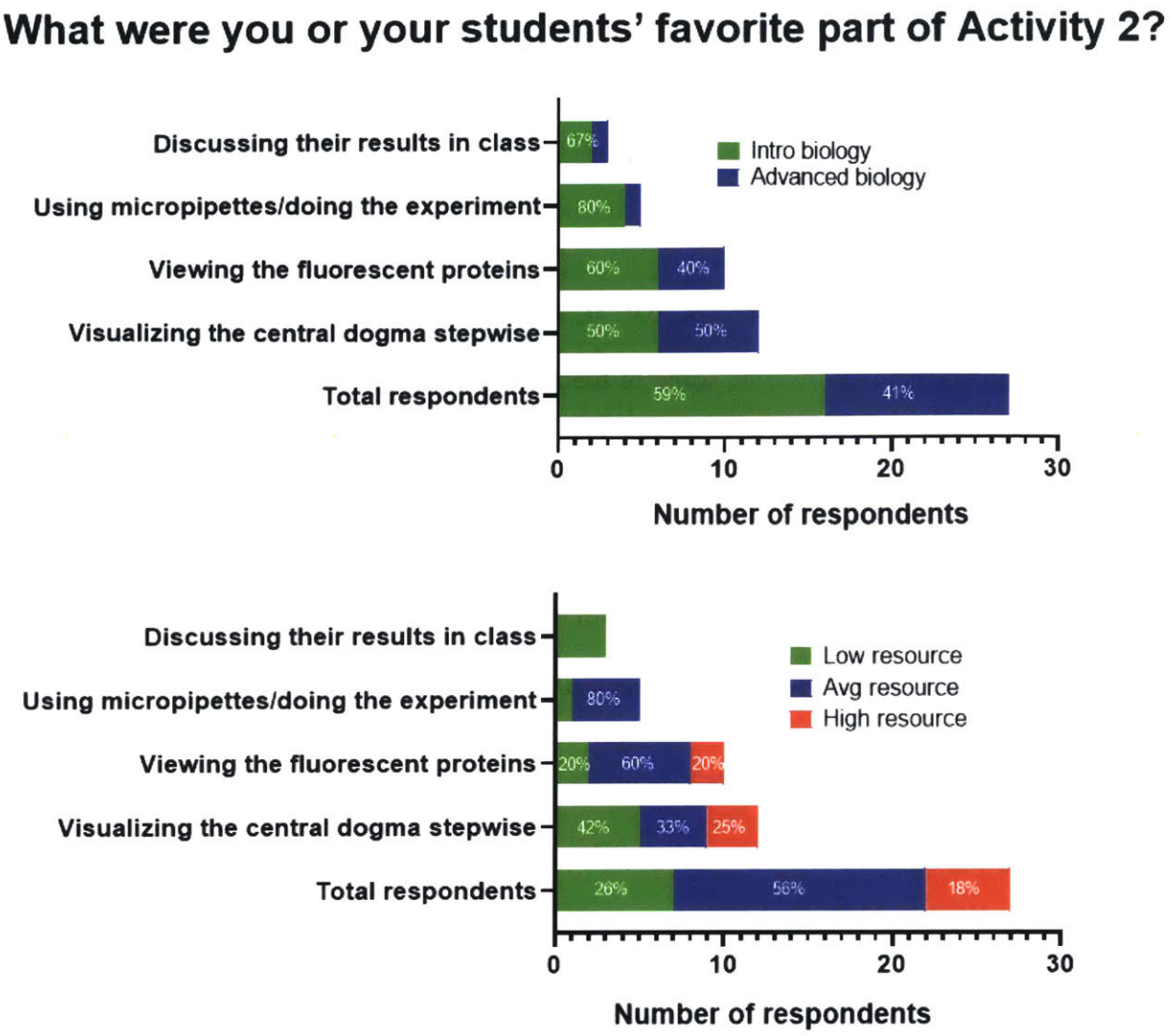


Figure 78: Results of teacher feedback – open response to "What was you or your students' favorite part of Activity 2?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

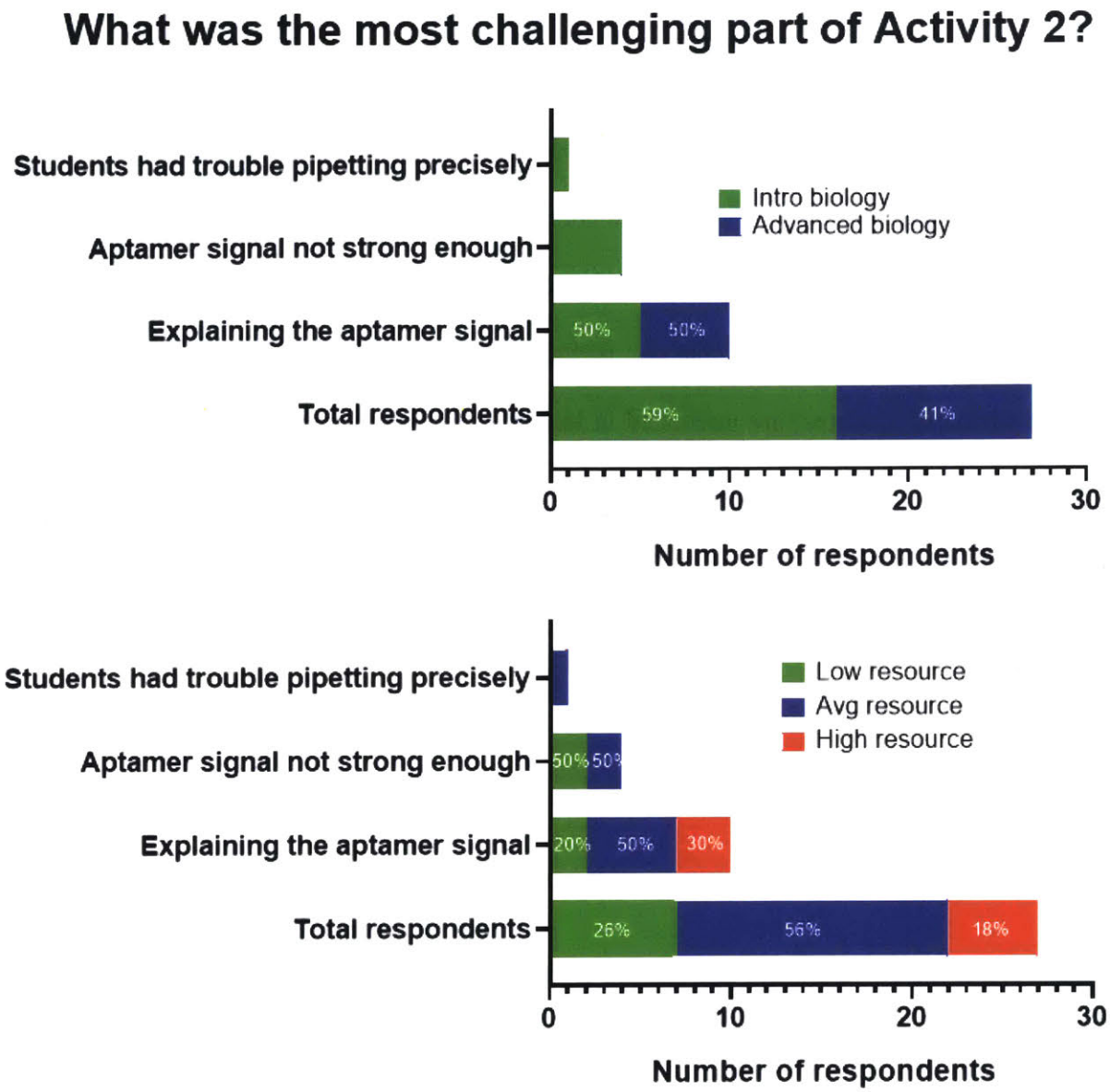


Figure 79: Results of teacher feedback – open response to "What was the most challenging part of Activity 2?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was the most challenging part of Activity 2?," the top response was that teachers had difficulty explaining to the students the aptamer-based transcription signal and why there was even a green signal to indicate transcription. Many teachers that I talked to afterwards expressed that because this was an unfamiliar concept to them, it made it difficult to try and explain it to their students. There are two ways to help address this issue. First, I plan on including more scientific background on the RNA aptamer, including links to scientific papers if the teachers (and the more advanced students) are interested in learning more about how it works. Second, for the more introductory level biology classes, I plan on simplifying this aspect of the student worksheet – perhaps simply informing them that the RNA in this reaction has been specially engineered to output a green signal to indicate transcription, without getting too deep into the scientific detail.

> *"My lack of understanding of the mechanisms behind visualizing transcription – students had questions I couldn't answer well."*
>
> *"This concept was not as easy to explain to students with very little science background."*
>
> *"The aptamer with a fluorophore was confusing for students. It was difficult to teach. I just reduced it to green means transcription and red means translation but the students did not understand why. Next time I would teach more about RNA signaling and how fluorophores work."*

Another response was that the RNA aptamer signal itself was actually not strong enough – this was a response I saw in the first wave of pilot participants, and from this feedback, I was actually able to determine that my DFHBI stock that I had been using had gone bad. After replacing the stock and checking for quality control, the later waves of pilot participants reported no issue with being able to detect the green signal.

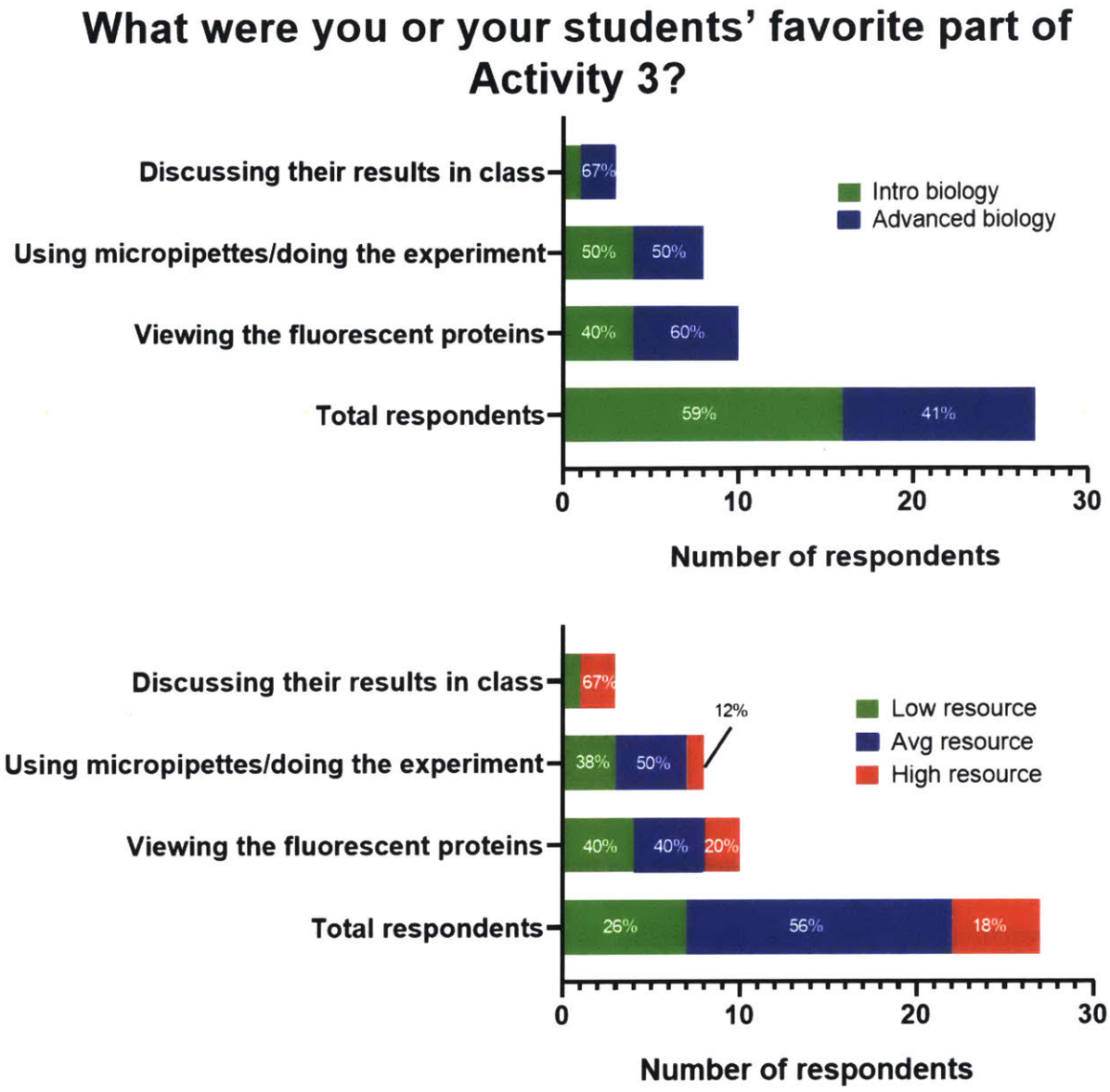


Figure 80: Results of teacher feedback – open response to "What was you or your students' favorite part of Activity 3?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was you or your students' favorite part of Activity 3?," the top responses were again viewing their fluorescent protein results and the process of carrying out the experiments.

> *"It was cool to see the direct correlation of concentration corresponding with decreasing intensity of signal."*

> *"They really enjoyed seeing the gradient of colors. Each group was satisfied in their confirmed hypothesis."*

> *"They loved seeing if they had been accurate in their pipetting. This concept was easily understood - the results were obvious."*

Several teachers also highlighted the fact that this activity led to class discussions about what happened – while DNA is "re-used" in our bodies as a template, this activity seemed to indicate otherwise (as less DNA resulted in less fluorescent protein signal). This allowed teachers to discuss concepts, like the role of energy in carrying out these processes, and unlike in live cells, how these cell-free reactions do not have the ability to regenerate energy to continue carrying out transcription and translation.

> *"Great discussion about whether DNA could be reused and if doubling it actually mattered."*

> *"They were totally surprised by the results. This led into our discussion on gene expression and regulation."*

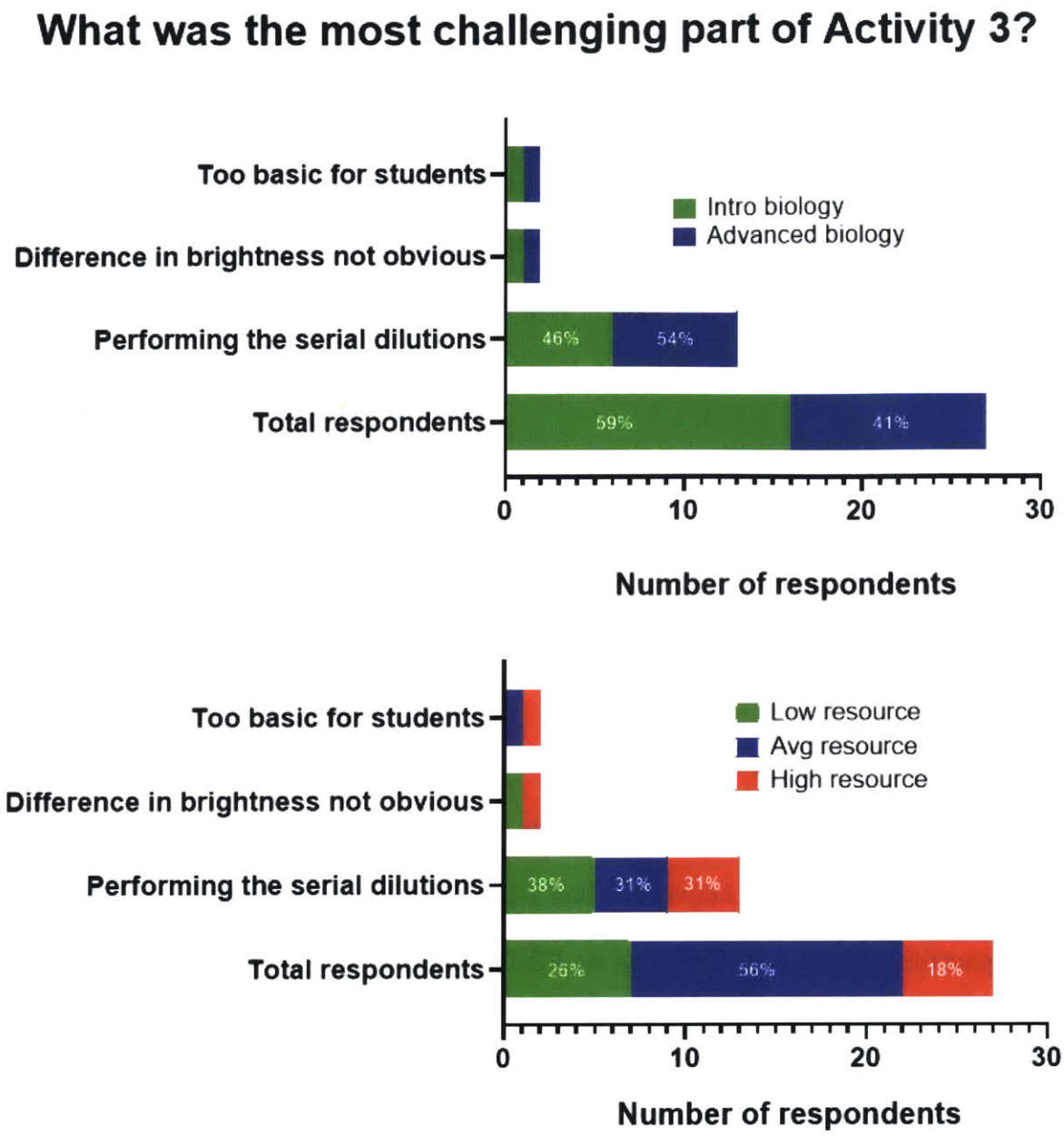


Figure 81: Results of teacher feedback – open response to "What was the most challenging part of Activity 3?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was the most challenging part of Activity 3?," the top response was that students had difficulty understanding and performing the serial dilutions necessary for the activity (and this was the case regardless of classroom type) (**Figure 81**).

> *"The directions for the dilution process could be a bit more clear. The students have not done many dilutions like this before, especially since we haven't done many labs with biotech."*
>
> *"This was a challenging lab for us because our micropipettes only went to 4 uL when the lab called for 2 uL. We had to improvise. I think my students were really proud of themselves for adjusting the lab protocol to meet our equipment restrictions. Again, this made them feel like "real" scientists."*

This issue can be addressed by including diagrams in the protocol for the serial dilution steps (as the current curriculum does not include them), as well as bold or highlight the steps that differ from the previous protocols of Activities 1 and 2 (i.e., that you have to create the dilutions in a separate spare tubes before adding them to the FD-CF pellets).

Some teachers also indicated that the difference in brightness between the tubes was not obvious This can be addressed by increasing the dilution from a 1:5 to a 1:10 dilution to increase the difference in brightness. This will be something that I will have to further optimize to determine the best dilution series. Finally, some teachers indicated that this activity was too basic for their students. As mentioned above, curriculum will need to be tailored for an introductory versus an advanced biology class, which should help create activities that are more suited for the knowledge level of each class.

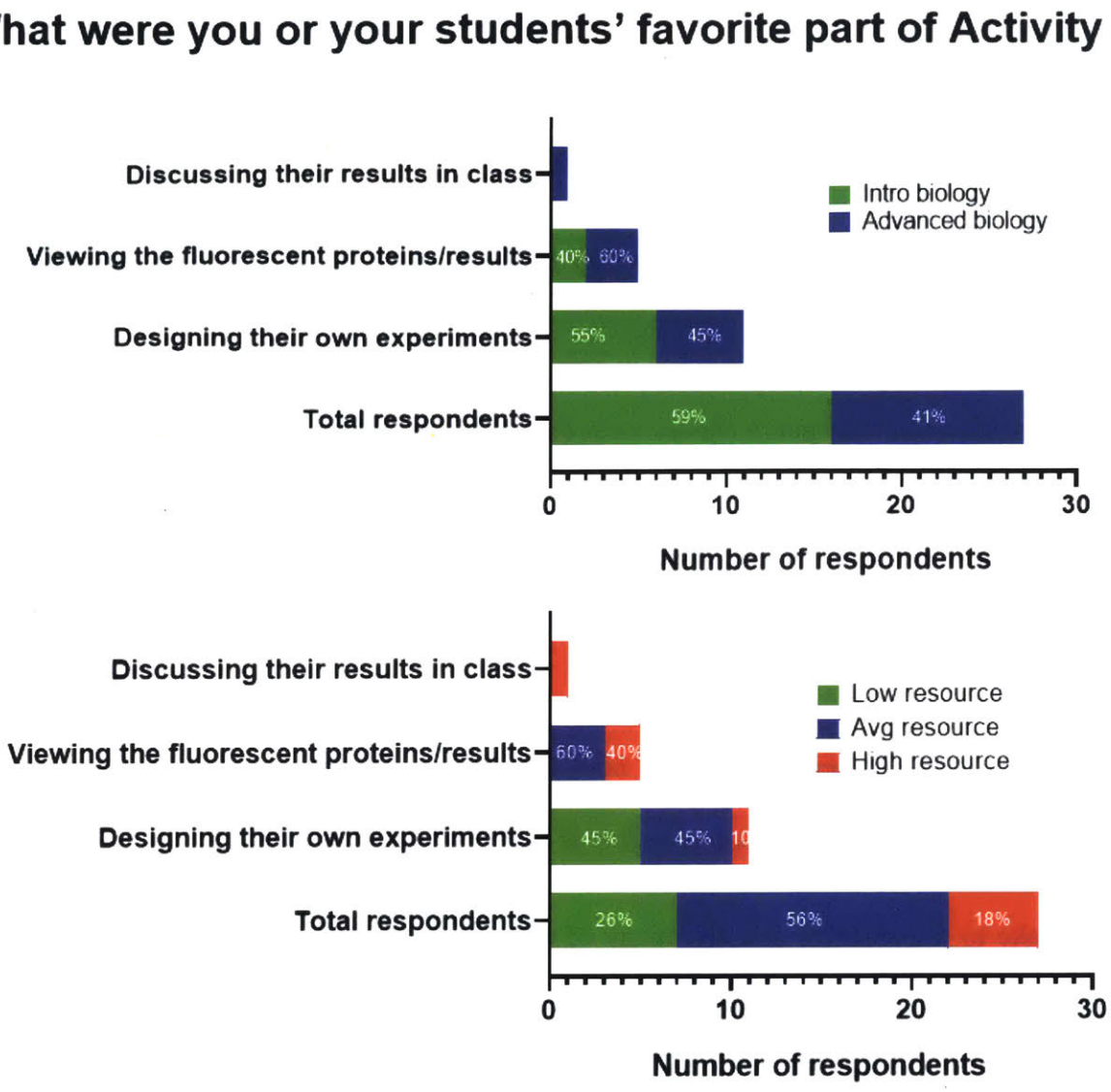


Figure 82: Results of teacher feedback – open response to "What was you or your students' favorite part of Activity 4?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was you or your students' favorite part of Activity 4?," the top response was that the students were given the freedom to design their own experiments, and again, this was across all different types of classrooms (**Figure 83**).

> *"They got to be really creative and try different reagents. They had control over the experiment."*
>
> *"They liked that they got to choose what they changed with this one!"*

Viewing their results, especially from an experiment the students got to create, was also a common response. Again, being able to discuss this results to figure out what and why happened was also mentioned.

> *"[The students] made connections back to factors that affect enzyme function - great collaboration among lab partners - lots of good question[s] generated."*

When I spoke to the teachers directly, many of them brought up the fact that other molecular biology labs do not have much inquiry-based elements built into them – students simply have to follow the given protocol. They liked that this particular activity allowed the students to think about what they have learned before, identify some variables that they think may impact these reactions, and design an experiment to test their hypotheses. Many teachers told me that they think inquiry-based labs, such as this one, are one of the better ways for students to truly understand the concepts learned.

When asked "What was the most challenging part of Activity 4?," the top response was that some students felt they had too much freedom in designing their experiments and wanted some guidance on what to do (even at the more advanced level classes).

> *"I think it would be OK to provide more guidance as to factors to test without compromising inquiry"*

> *"Students had a difficult time selecting only one variable to test and including their negative and positive control. In the future I would do a mini-lesson on the scientific method."*

Part of the difficulty in designing their own experiments was the fact that scientific method is not a commonly taught or emphasized concept in classrooms. Teachers did not realize the importance of preparing the students about concepts like positive or negative controls.

> *"The lab identified a "hole" in our knowledge. If I had reacted better to their questioning I could have used this opportunity to make sure they understood what the roles of the two controls were."*

One way to address this issue is emphasize in the teacher manual that the students should be briefed on the scientific method prior to performing this activity. Another strategy – that also addresses another response about not having enough real-world connections – is to provide students guidance on specific processes/cellular elements to target in the FD-CF reactions. For example, the activity can ask the students to brainstorm variables that might specifically affect the ribosomes in the reaction (rather than just brainstorm random variables). This will force the students to come up

with a biologically relevant experiment and further enforce their understanding of processes like transcription and translation.

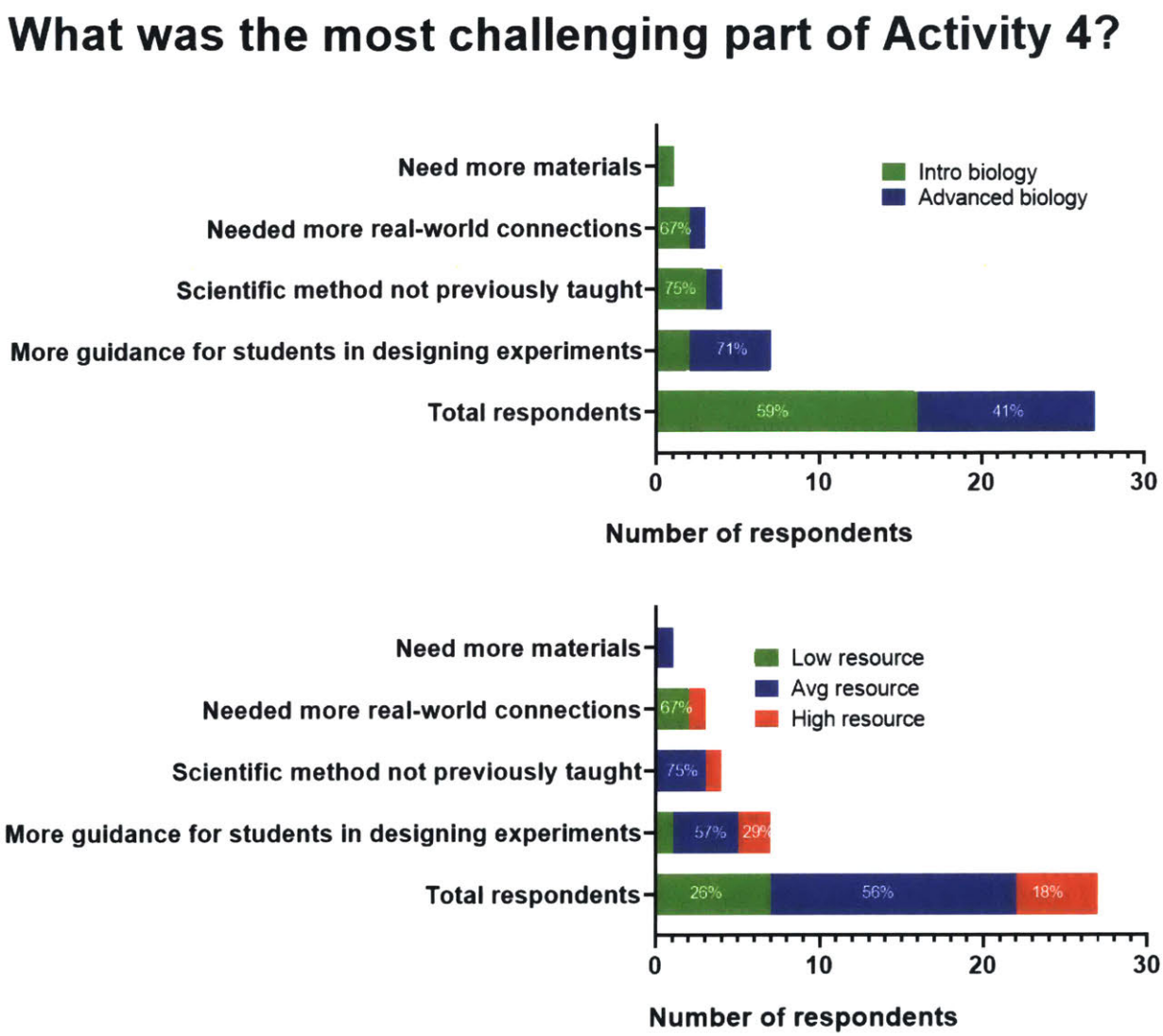


Figure 83: Results of teacher feedback – open response to "What was the most challenging part of Activity 4?"

Responses from teachers were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=26 teacher participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

Feedback was also collected from the students, both before and after they completed the activities (**Appendix**). The first portion of the student feedback form (both before and after) asked them to indicate if they agree or disagree with these statements in **Table 5** regarding their knowledge about molecular biology. They were also asked to answer two free-response questions about these biological concepts (**Table 5**). The free response questions were graded based on a rubric (**Table 6**). All the scores were compared before and after with a two-tailed t-test (parametric, unpaired, assuming both populations have the same standard deviations, p-value set to 0.05), performed in GraphPad Prism 8.0.1. The results of the responses were graphed and the significance level indicated by the symbols (ns = not significant, * = <0.05, ** = <0.005, *** = <0.0005, and **** = <0.0001).

Question Code	**Question (1-5 scale; 1=Strongly Disagree/3=Neutral/5=Strongly Agree)**
Q1	I understand how proteins are made from DNA
Q2	I can explain to others how to make proteins from DNA
Q3	I understand how to control or engineer DNA systems
Question Code	**Free Response Question (graded on a 1-5 scale)**
FR1	Please describe how cells use DNA to synthesize (make) proteins.
FR2	Please describe how you can control protein expression (i.e., what variables can you change).

Table 5: Question codes for the feedback questions for the students (pre and post activity). Each student was asked to indicate if they agree or disagree with the statements (on a scale of 1-5, with 1 being strongly disagree, 3 neutral, and 5 strongly agree). They were also asked two free-response questions. Each statement was assigned a question code for analysis and graphing purposes.

Score	FR1 - Please describe how cells use DNA to synthesize (make) proteins.
1	Students say they don't know and do not offer any attempt at explanation.
2	Students offer an explanation, but it's not accurate (missing DNA, RNA, or protein)
3	Students can describe DNA to RNA to proteins but little to no additional details (no mention of transcription and translation)
4	Students can describe DNA to RNA to proteins with some details (mention of transcription and translation and mention of 1 or 2 of nucleotides/amino acids/nucleus/ribosomes/polymerases/etc.)
5	Students can describe DNA to RNA to proteins with advanced details (mention 3+ of nucleotides, amino acids, nucleus, ribosomes)
Score	**FR2 - Please describe how you can control protein expression (i.e., what variables can you change).**
1	Students say they don't know and do not offer any attempt at explanation.
2	Students offer an explanation, but it's not accurate
3	Students can list at least one or two variables that impacts protein expression but no additional details
4	Students can list at least one or two variables that impacts protein expression with some explanation of how and/or why
5	Students can identify 3+ variables that impacts protein expression with some explanation of how and/or why

Table 6: Rubric to grade student free-responses on biological concepts. For each free response from the student, it was graded according to the rubric and assigned a score from 1-5.

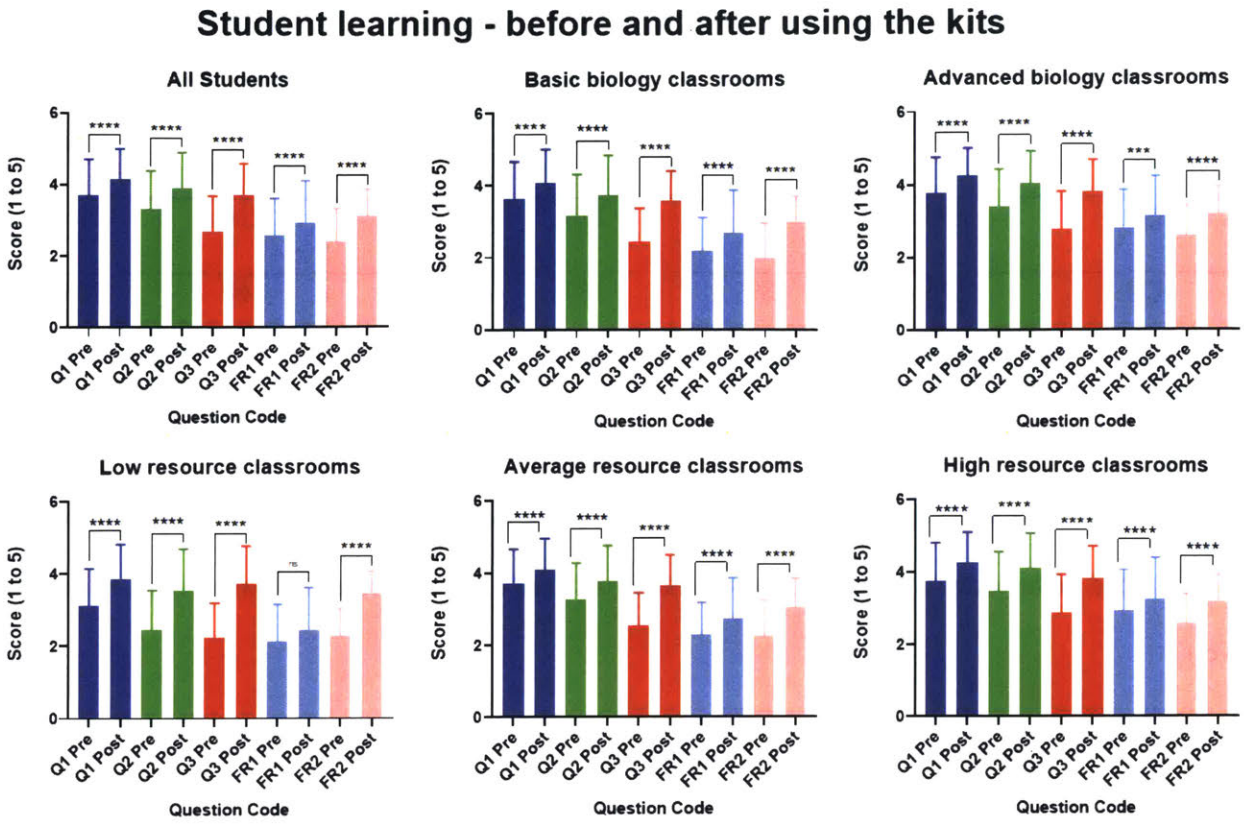


Figure 84: Analysis of student responses – before and after completing prototype activities. Student responses were quantified and a two-tailed t-test (parametric, unpaired, assuming both populations have the same standard deviations, p-value set to 0.05) was performed in GraphPad Prism 8.0.1 between the responses pre and post activities within each question. The results of the responses were graphed and the significance level indicated by the symbols (ns = not significant, * = <0.05, ** = <0.005, *** = <0.0005, and **** = <0.0001).

Question Code	All students	Basic biology	Advanced biology	Low Resource	Average Resource	High Resource
Q1	364	140	224	57	216	216
Q2	364	140	224	57	216	216
Q3	276	89	187	22	160	160
FR1	359	138	221	57	214	214
FR2	272	87	185	23	158	158

Table 7: Number of students per category/responses. Table indicating the number of students who were included in the analysis for each question and across each classroom category (knowledge, resource level)

The number of students analyzed per response is displayed in **Table 7** – some responses were omitted either due to the fact that some students did not complete all 4 activities (and wouldn't be able to properly answer Q3 and FR2) or that their response was completely irrelevant or unrelated to the question asked. Overall, while there was a large range in the responses, there was still a significant increase between what the students reported that they knew and what they demonstrated what they knew before and after completing the activities (**Figure 84**). This trend was true across all classrooms, regardless of knowledge level or resource level. The only question that did not have a significant increase was the first free-response question *(Please describe how cells use DNA to synthesize (make) proteins.)* for the low resource classrooms, although this is likely due the t-test not having enough power to determine significance from the small sample size.

These results suggest that there is educational value to the students completing the activities, especially in their understanding of the central dogma and protein expression. I note that the students' assessment of their own understanding of the concepts (Q1-3) is overall higher than my

assessment of their understanding (FR1 and FR2) but within each question, there is still a significant increase.

I will also note that some assumption were made in the statistical analysis (i.e., assuming each standard deviation across all data sets) and that a larger-scale longitudinal study will likely be needed to properly measure the educational value of the activities. There are also some variables that I could not control for (for example, some classrooms might have already learned about these topics prior to doing the activities). For an initial prototype test however (where the primary goal was to assess the functionality of the kit, and the education value second), these results are promising. Paired with the positive feedback from the teachers that this kit is educationally useful for them, these preliminary results suggest that there is some educational value to these kits, especially once improvements are made to the worksheets and other curriculum.

The next portion of the student feedback form (both before and after) asked them to indicate if they agree or disagree with these statements in **Table 8** regarding their opinions and interest in biology and engineering. The number of students who responded to each of these questions are the same as the numbers for Q1 in **Table 7**.

Question Code	Question (1-5 scale; 1=Strongly Disagree/3=Neutral/5=Strongly Agree)
Q4	I find biology interesting
Q5	I find engineering (testing and manipulating different variables) interesting
Q6	I could imagine becoming a scientist one day
Q7	I could imagine becoming an engineer one day

Table 8: Question codes for the feedback questions for the students (pre and post activity). Each student was asked to indicate if they agree or disagree with the statements (on a scale of 1-5, with 1 being

strongly disagree, 3 neutral, and 5 strongly agree). Each statement was assigned a question code for analysis and graphing purposes.

All the scores were compared before and after with a two-tailed t-test (parametric, unpaired, assuming both populations have the same standard deviations, p-value set to 0.05), performed in GraphPad Prism 8.0.1. The results of the responses were graphed and the significance level indicated by the symbols (ns = not significant, * = <0.05, ** = <0.005, *** = <0.0005, and **** = <0.0001).

Overall, there were not any significant increases between the interest that the students reported before and after completing the activities (**Figure 85**). This is not surprising, as I did not expect just a few activities to drastically change a student's opinion on biology and engineering. Again, I will also note that some assumption were made in the statistical analysis (i.e., assuming each standard deviation across all data sets) and that a larger-scale longitudinal study will likely be needed to properly measure the value of the activities to inspire interest in students. There are also some variables that I could not control for (for example, the teacher in the classroom and how they presented these activities to the students).

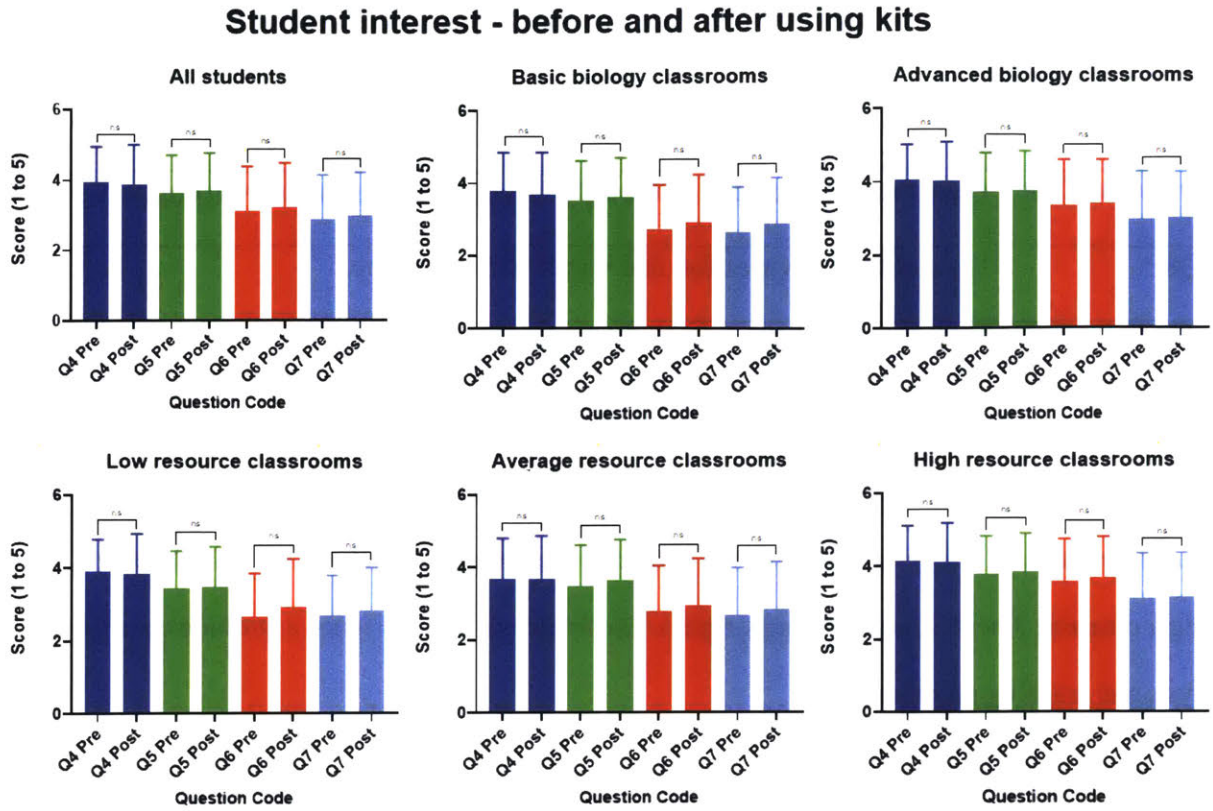


Figure 85: Analysis of student responses – before and after completing prototype activities. Student responses were quantified and a two-tailed t-test (parametric, unpaired, assuming both populations have the same standard deviations, p-value set to 0.05) was performed in GraphPad Prism 8.0.1 between the responses pre and post activities within each question. The results of the responses were graphed and the significance level indicated by the symbols (ns = not significant, * = <0.05, ** = <0.005, *** = <0.0005, and **** = <0.0001).

In the post-activity feedback, the students were also asked some questions about the overall prototype kit (**Table 9**).

Question Code	Question (1-5 scale; 1=Strongly Disagree/3=Neutral/5=Strongly Agree)
Q8	I found the activities fun and worth our class time.
Q9	After the activities, I think differently about biology
Q10	After the activities, I think differently about engineering

Table 9: Question codes for the feedback questions for the students (post activity only). Each student was asked to indicate if they agree or disagree with the statements (on a scale of 1-5, with 1 being strongly disagree, 3 neutral, and 5 strongly agree). Each statement was assigned a question code for analysis and graphing purposes.

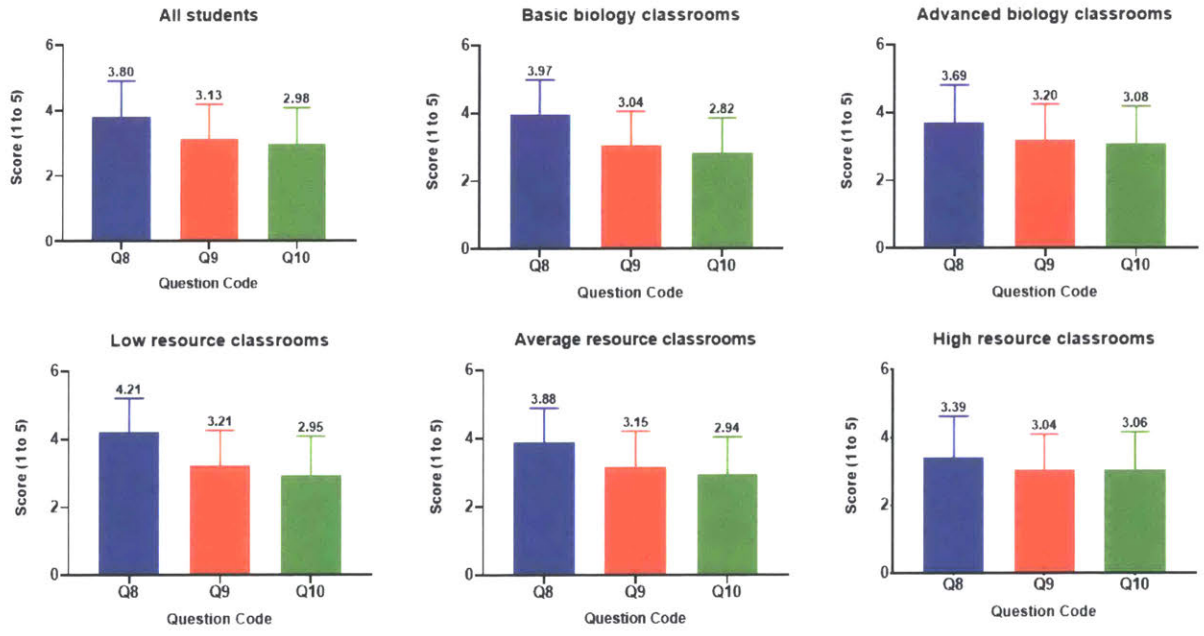


Figure 86: Results of student feedback – overall for entire prototype kits. Results of the student feedback for questions (from **Table 9**). Values (displayed above each bar) represent averages and error bars represent standard deviations of N=X participants, where the number for each category is listed in **Table 7.**

Overall, students were neutral about if the activities were fun and worth the class time and if they think about biology or engineering afterwards (**Figure 86**). When broken down by classroom category however, it seems that the basic biology classroom and the low resource classrooms had a higher agreement score (3.97 and 4.21, respectively) than the advanced or average/high resource classrooms (3.69, 3.88, and 3.39, respectively) about if the activities were fun and worth class time. Again, this trend aligns with the fact that those classrooms likely have less opportunities to conduct hands-on activities, while on the other hand, these activities may be too basic for more advanced or resourced classrooms. For Q9 and Q10 (thinking differently about biology or engineering), there was no major difference between the classroom types. This again is expected, as I did not expect these activities to drastically change the students' opinions about biology or engineering.

The students were then asked a series of open-response questions about the kit overall. For these responses, I sorted them into categorical bins (as many of the responses fell into a few overarching categories) and counted the number responses per bin per question. Some open responses listed several items that fell into different bins/categories (i.e., a student listing three distinct elements that they liked about the kits), so a single response may result adding counts to multiple bins/categories. To gain more insight about how well the kit worked across different knowledge levels and school resource levels, I also analyzed the number of responses by those breakdowns. On the graphs below, percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level. The percentage breakdown by bin/category then can be compared to the percentage breakdown of all total respondents to identify any trends that are found more in specific classroom knowledge levels or school resource levels. Note that due to the uneven sample size of the student feedback,

statistically significant conclusions are not possible; however, I can still use the data to make informal conclusions to help guide designs/improvements to future kits.

When asked "What was the most interesting thing you learned from all the activities?," the top responses depended on the classroom type (**Figure 87**). For basic biology or low resource classrooms, the students tended to respond learning about the central dogma of biology and basic lab skills. For more advanced and high resource classrooms, students tended to respond learning about variables that impact protein expression and the cell-free technology. This is useful feedback for me to design more tailored activities for different types of the classrooms. For example, for younger students (introductory biology), the activities can focus on more basic biological concepts and getting experience with doing hands-on experiments.

"I enjoyed using the micro [pipettes] and viewing the proteins under a black light."

"The most interesting thing I learned from this experiment is the procedure for typical experiments"

"It was most interesting to see tangible results from the DNA processes."

"I had never heard of the central dogma before, it was an interesting thing to learn about."

On the other hand, for the older students (advanced/honors/AP biology), the activities can focus more on manipulating the cell-free system and learning about how the cell-free reaction works and is applied in the real-world (rather than just use it as a substitute for cells).

"That science has advanced this far to harness the machinery of a cell and improve lives through a method that initially seems to be impossible to do."

"I found the applications of [cell-free] very interesting, like testing for Ebola or Zika, or using the technology in military uniforms to detect toxins."

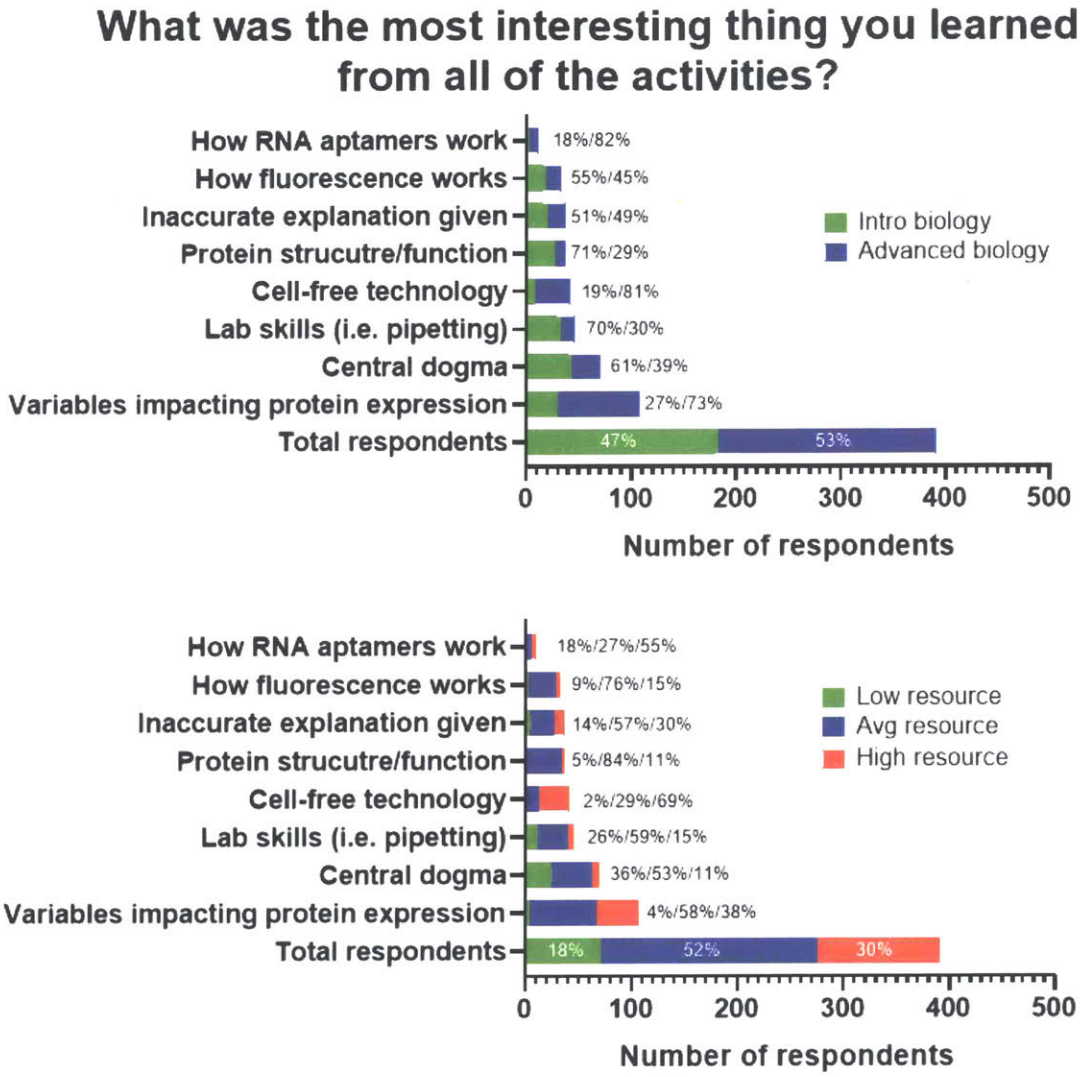


Figure 87: Results of student feedback – open response to "What was the most interesting thing you learned from all the activities?"

Responses from students were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=364 student participants.

Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was your favorite part of all the activities?" the top responses was carrying out the experiment and then seeing the fluorescent protein results (**Figure 88**). These responses were across all classroom types. Since one of my initial goals in designing these activities was to use an output that would excite and engage the students, this feedback seems to indicate that the fluorescent proteins (and the process for the students of making them) were successful in achieving that goal.

> *"My favorite part was looking at the florescence in the black light – it was really interesting and cool looking."*

> *"My favorite part was using the equipment. I would describe as a fun activity that immerses you into what top scientists are currently doing."*

The more advanced or resourced classrooms also reported they enjoyed using the scientific method to create their own experiments and using the experiments to learn biological concepts. This supports the conclusion made in the earlier feedback question about tailoring the activities to the classrooms, and having more advanced classrooms focus on manipulation of the variables.

> *"I enjoyed making my own experiment because it was fun to think of things to change to see what would happen."*

> *"Picking a variable to change was the most fun. I would describe it as becoming a scientist and researcher for a couple of days."*

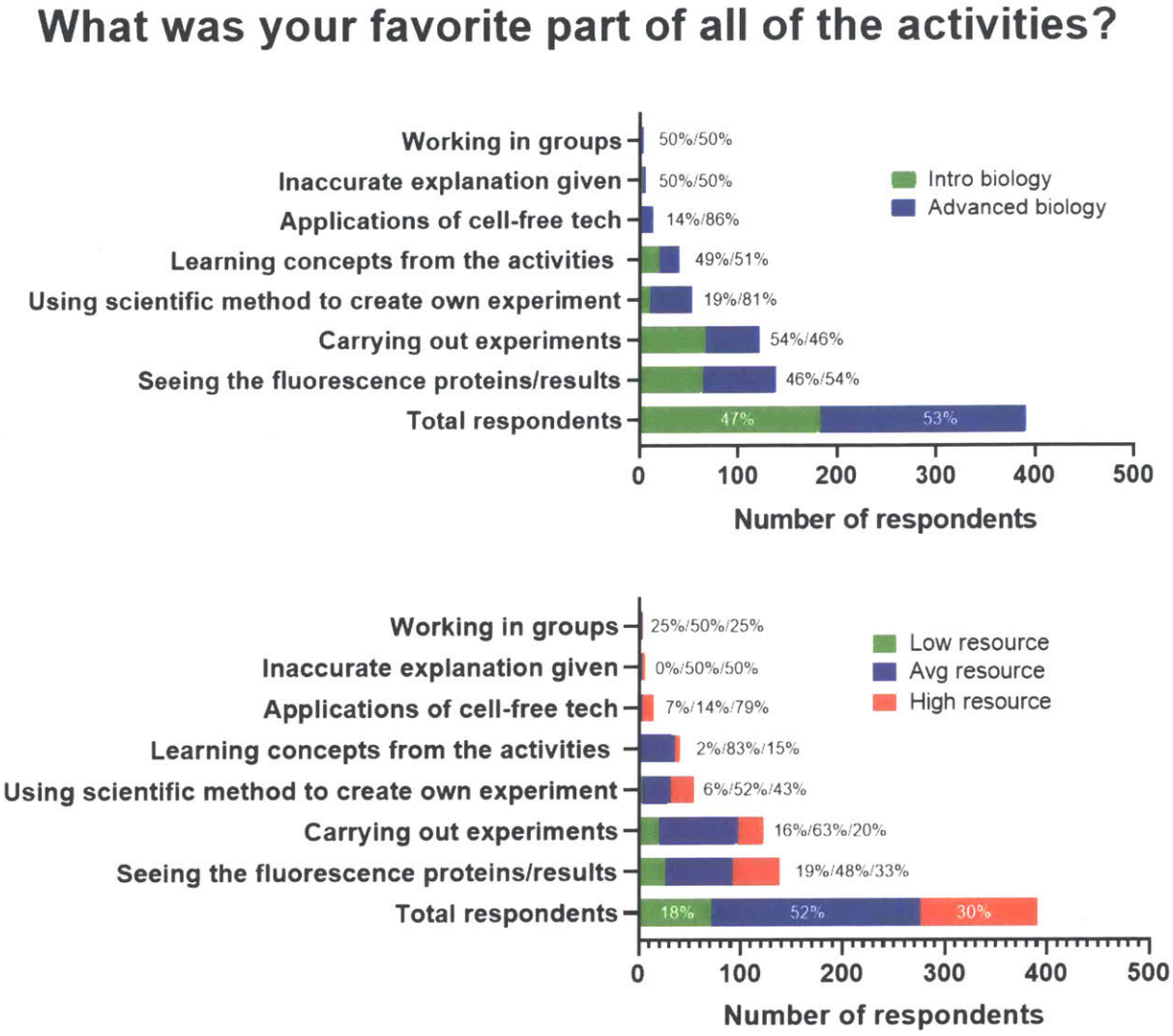


Figure 88: Results of student feedback – open response to "What was your favorite part of all the activities?"

Responses from students were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=364 student participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

When asked "What was your least favorite part of all the activities?" the top responses was that the worksheets were too basic or repetitive for the students (**Figure 89**). While the students as a

whole enjoyed the hands-on learning aspect of the kit, many of them disliked having to fill out pre and post-lab analysis questions and felt like the worksheets were just "busy work."

> *"While I didn't necessarily enjoy answering the questions at the end of each activity, I understood their purpose but would suggest leaving them out."*
>
> *"The writing was my least favorite part. Mostly because I am a hands on learner."*
>
> *"Just answering the questions, which is to be expected for any science experiment and I realize that."*

I realize that while some students may dislike worksheets, I will still need to include them in some form to give context and framework for the activities, as well as provide a way to measure learning for the teachers. For future curriculum however, I will continue working closely with teachers to develop worksheets that are not only informative, but also more engaging for the students. For example, we can develop curriculum that provides more real-world context to the questions to make them more interesting/relevant to the students.

Specifically for the older/more advanced students, they felt like the experiments were too simple for their knowledge level.

> *"The activities were clearly designed for students earlier along in their studies of biotech, and a more advanced option for later education is needed."*
>
> *"Possibly making more intricate and engaging kits for older students, I think that these kits would absolutely blow the minds of elementary and middle schoolers, but I found them a bit dull."*

Again, this can be addressed by creating activities that are more advanced for more advanced students, such as some of the other modules that I have developed in previous chapters (i.e., the enzyme-based labs to create hydrogels, the modules to teach synthetic biology, the antibiotic labs, etc.).

Another response was that there was not enough background on what was actually happening in the tubes. This aligns with one of the teachers' main concerns – that the experiments were so simple that it is hard to tell what is happening at the molecular level. As mentioned above, this can be addressed by incorporating more inquiry-based learning explorations into the lab activities and linking these activities more to real-world examples to help provide context for what is happening at a biological level.

> *"Not being able to see the process of it transforming, but just seeing the results and not being able to see what it looks like up close."*

> *"What is the DNA from? What is the [pellet] made of and what does it do? What does it look like at a microscopic level?"*

> *"There should be an explanation as to why a process that usually takes place within a cell is able to occur outside of it."*

Other responses that was commonly brought up was that the students disliked how small the tubes/volumes/pellets were, as well as having to wait overnight to see their results.

> *"Working with such tiny concentrations of DNA and enzymes made it hard to keep track of everything."*

"The [pipette] provided and its tips were difficult to use to measure out the very small amounts of DNA and RNA."

"I didn't like the waiting but you can't really improve that."

When I inquired to some of the teachers about these issues in our follow-up interviews, the teachers actually said they liked how the results had to develop overnight, as that allowed the activities to fit in better to their classroom schedules. Due to this, I will defer to the teachers' preferences (as they are the eventual customer) and maintain the overnight development of the activities, although I will suggest that if they have an incubator, warming up the reactions may allow them to see results by the end of class time. I also asked teachers to comment on if they thought the volumes/tubes were too small for the students, and the majority of the teachers said that while there was a learning curve at the beginning for the students to acclimate to the materials, most students were able to handle them by the end of the activities. They also commented that this was a realistic look at the kinds of experiments scientists will have to do in the lab and that we should not change the activities to make it further "easier" for the students, as the cell-free aspect of the reaction has already simplified the labs quite a bit. Again, I will defer to the teachers' preferences, but look into providing supplemental supplies that might help the students with performing the experiments, such as tube racks.

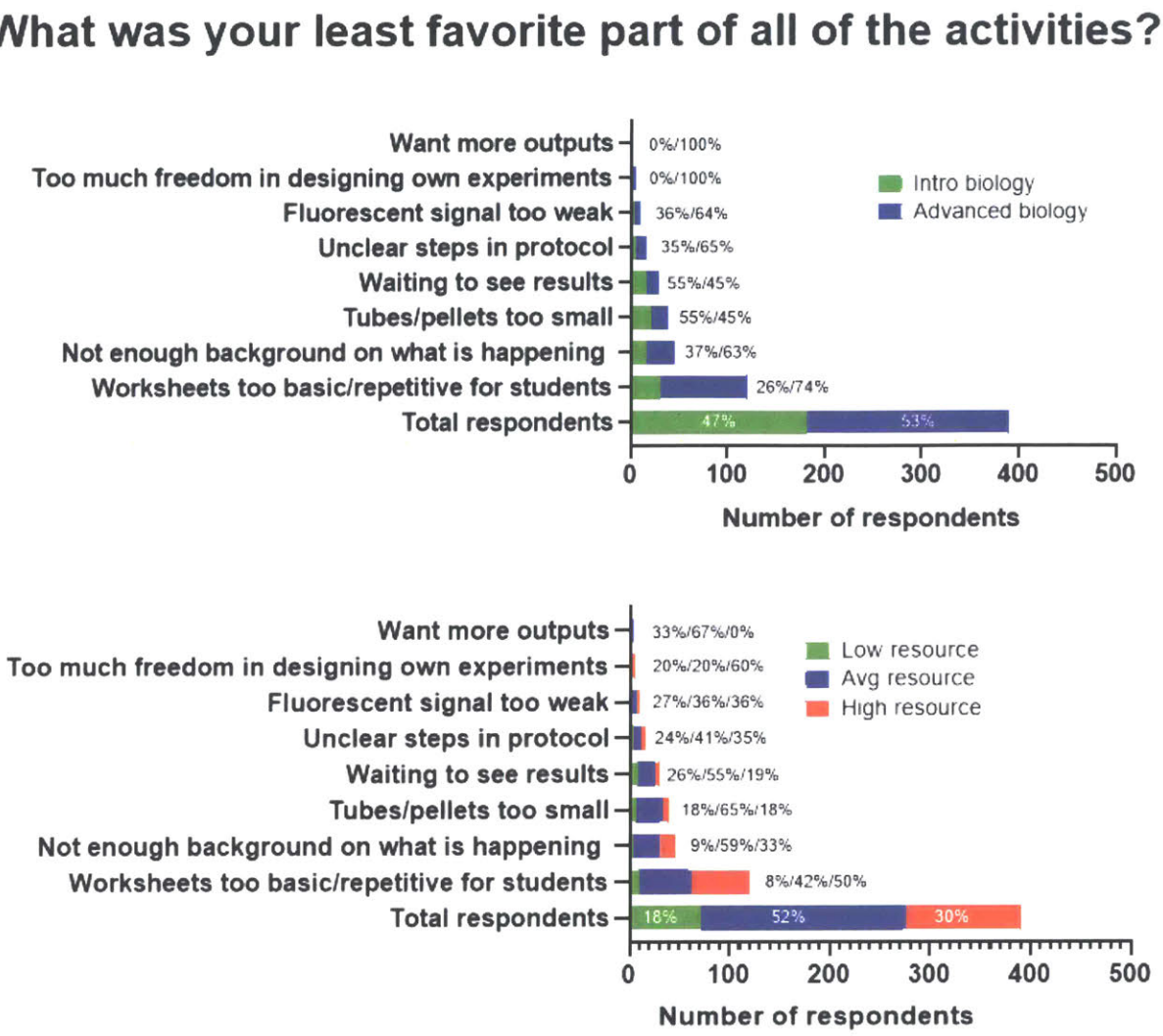


Figure 89: Results of student feedback – open response to "What was your least favorite part of all the activities?"

Responses from students were binned into larger categories and quantified by either knowledge level (intro vs. advanced biology) or school resource level (low, average, or high). N=364 student participants. Percentages are displayed that indicate the percent of respondents within that bin/category (not total respondents) that are either a certain knowledge or resource level.

\\

6.5 Future Directions

In this work, I have presented a platform using FD-CF that can be applied to education to make hands-on activities more accessible to schools. I will continue to analyze data from the pilot study, as half of the teachers are still currently testing the prototype kit, and use the results from the study to help inform improvements or additions to make to future versions of the kit. I will also continue to develop the modules for classroom use to expand my current set of classroom-ready activities that can be used by incorporating the non-visual output activities and the synthetic biology focused activities. While those activities currently work in a research lab setting, optimization will need to be done to determine the best conditions and workflow for the activities to function in a classroom setting.

As of writing, I am currently exploring commercialization options for this educational platform to bring these kits to market in order to make them more accessible for schools across the country and across the world.

6.6 Conclusions

The platform demonstrated here engage students by using illustrative demonstrations to introduce and cover a wide range of molecular and synthetic biology concepts in classrooms. Both proof of concept data and pilot study data support the platform as an easy-to-use and accessible resource to bring molecular and synthetic biology into classrooms. This work contains just a few examples of the potential activities that can be developed using FD-CF reactions, and as noted above, new modules could be created to teach additional biological concepts. I hope that other scientists, upon reading this work, will be inspired to view their own research from an educational perspective and

think of ways to apply their work to education as well. My hands-on biology educational kits promise to engage more students in the life sciences and biotechnology.

CHAPTER SEVEN: MATERIALS AND METHODS

This chapter is in part adapted from:

Huang, A., Nguyen, P.Q., Stark, J.C., Takahashi, M.K., Donghia, N., Ferrante, T., Dy, A.J., Hsu, K.J., Dubner, R.S., Pardee, K., et al. (2018). BioBits^{TM} Explorer: A modular synthetic biology education kit. Sci. Adv. *4*, eaat5105.

Stark, J.C., Huang, A., Nguyen, P.Q., Dubner, R.S., Hsu, K.J., Ferrante, T.C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J.S., et al. (2018). BioBits^{TM} Bright: A fluorescent synthetic biology education kit. Sci. Adv. *4*, eaat5107.

General template design and preparation

DNA sequences encoding for all genes sequences were derived from the literature, codon optimized for E. coli and synthesized as gBlocks or oligos by Integrated DNA Technologies (IDT). All toehold sequences were obtained from previous publications (10, 43, 44). Cloning and plasmid propagation were performed using either Mach1 (ThermoFisher; C862003) or NEB Turbo (New England Biolabs; C2984H) competent E. coli cells. All sequences were cloned into a T7-expression plasmid system called pJL1, which was gifted from the Michael Jewett lab at Northwestern (Addgene plasmid #69496) Gibson assembly (51) or blunt-end ligation. All template DNA preps of the plasmids were done with the E.Z.N.A. Plasmid Midi Kit (Omega Bio-Tek; # D6904) or QIAprep HiSpeed Plasmid Maxi Kit (Qiagen; #12662) for crude extract reactions or the QIAprep Spin Miniprep Kit (Qiagen; #27106) for PURE reactions.

PURE cell-free reaction preparation and lyophilization protocol

For cell-free reactions performed in the PURExpress In Vitro Protein Synthesis Kit (New England Biolabs; E6800S), the reactions consisted of: NEB Solution A (40%) and B (30%), RNase inhibitor (Roche, 03335402001; 0.5%), and the template DNA (10-50 nM). For the FD-CF expression of ATF1, the Disulfide Bond Enhancer (New England Biolabs; E6820S) was added into the reactions as per the manufacturer's instructions, before lyophilization. The reactions were then flash-frozen in liquid nitrogen, lyophilized overnight at < 0.01 mBar (FreeZone® 2.5 L Benchtop Freeze Dryers, Labconco # 700201000) to obtain the freeze-dried reaction, and stored at room temperature. The reactions were reconstituted with nuclease-free water to the original reaction volume and incubated at 30° or 37°C.

In-house crude cell-free extract preparation and lyophilization protocol

Cell extract was prepared as described previously (14). Briefly, E. coli BL21 Star (DE3) cells (ThermoFisher) were grown in 150 ml of LB at 37°C at 250 rpm. T7 RNAP expression was induced with 1 mM IPTG at OD600 ~1.5 and cells were harvested in mid-exponential growth phase (OD600 ~2-3), and cell pellets were washed three times with ice-cold Buffer A containing 10 mM Tris-Acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium glutamate, and 2 mM DTT, flash-frozen and stored at -80°C. Briefly, cell pellets were thawed and resuspended in 1 mL of Buffer A per 1g of wet cells and sonicated in an ice-water bath. Total sonication energy to lyse cells was determined by using the sonication energy equation for BL21 Star (DE3), [Energy] = [[Volume (uL)] – 33.6] * 1.8-1. A Q125 Sonicator (Qsonica) with 3.174 mm diameter probe at a frequency of 20 kHz was used for sonication. An amplitude of 50% in 10 s on/off intervals was applied until the required input energy was met. Lysate was then centrifuged at 12,000 rcf for 10

min at 4°C, and the supernatant was incubated at 37°C at 300 rpm for 1 hr. The supernatant was centrifuged again at 12,000 rcf for 10 min at 4°C, flash-frozen and stored at -80°C until use. The 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; 12 mM magnesium glutamate; 2 mM each of 20 amino acids; 0.33 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); 1-100 μg mL−1 plasmid (optional; can also be added post-freeze-dry); and 27% v/v of cell extract. The reactions were then flash-frozen in liquid nitrogen, lyophilized overnight at < 0.01 mBar (FreeZone® 2.5 L Benchtop Freeze Dryers, Labconco # 700201000) to obtain the freeze-dried reaction, and stored at room temperature. The reactions were reconstituted with nuclease-free water to the original reaction volume and incubated at 30° or 37°C.

Fluorescent protein production and characterization

After incubation at the appropriate temperature/time/DNA concentration (experiment dependent, see **Table 10**), the cell-free, freeze-dried synthesized fluorescent proteins visualized using either a Safe Imager 2.0 Blue-Light Transilluminator (Thermo Fisher), white light, or the inexpensive imager developed as part of the educational kits. Images were taken with a DSLR, adjusted and cropped in Adobe Photoshop. For quantitative analysis, cell-free reactions were transferred to a 384-well clear bottom, black-walled plate and relative fluorescent units were read on a SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices). Excitation and emission wavelengths used to measure fluorescence of each protein construct were as follows: eforRed 587/610 ex/em; tdTomato 554/581 ex/em; mOrange 548/562 ex/em; sfGFP or F30xd Broccoli

485/507-520 ex/em; and Aquamarine 420/474 ex/em. For assessing protein yields, reaction samples were analyzed directly by incorporation of 14C-leucine into trichloroacetic acid (TCA)-precipitable radioactivity using a liquid scintillation counter.

Stability studies of FD-CF reactions – final storage conditions

5 uL FD-CF reaction pellets in PCR tubes were stored in vacuum-sealed bags (NutriChef 8"X10' 4 mil Commercial Grade Vacuum Sealer Food Storage Rolls using a NutriChef Vacuum Sealer PKVS20STS) with Dri-Cards at ambient temperature or 4°C. The pellets were tested at each timepoint in triplicate with 1 ng/uL of sfGFP (stored at same temperature as the pellets). After a 24 hour incubation at room temperature, the reactions were visualized (450-470 nm) and quantified on a plate reader for RFU (ex/em 485/520).

Construction of portable imagers and incubators

To design our portable lab equipment, we used the open source 3D CAD modeling software FreeCAD. Open-source tutorials for FreeCAD are also available on their website (freecadweb.org). Designed acrylic or wood components were laser cut to desired specifications (Bright paper citation) and assembled using adhesive (SCIGRIP Weld-On 16 for acrylics or Gorilla Wood Glue for wood components). Individual acrylic or wood parts were gently pressed together by hand for about a minute and left to cure overnight. Electronic components were soldered and heat shrink was applied as necessary. Once the incubator circuit was assembled (Bright paper citation), it was mounted on to incubator with 0.25" screws through laser cut and/or pre-drilled pilot holes.

After the incubator was assembled, the set temperature was calibrated. For the switch version of the incubator, various resistors or resistor combinations were tested to achieve the two desired temperature set points (30 and 37°C). For the dial version of the incubator, the potentiometer

position was adjusted to reach the desired set points. In both cases, the temperature was monitored using an Arduino and, once determined, the set positions were labeled and temperatures were verified through additional temperature monitoring.

Smell production and characterization

Freeze-dried, cell-free reactions for the expression of ATF1 enzyme were incubated at 37°C for 20 hours in the cell-free reaction. The completed FD-CF reaction containing the enzymes was then added into a separate freshly prepared catalysis reaction. The total catalysis reaction volume was 300 µL and included 50 mM HEPES, pH 7.5, 100 mM KCl, 5 mM EDTA, the relevant substrates (25 mM isoamyl alcohol for ATF1), freshly prepared cofactor (5 mM Acetyl-CoA for ATF1), and 10% of the volume was the FD-CF reaction containing the enzyme. These reactions were allowed to proceed 20 hours in capped vials at room temperature. For GC-MS analysis, the Stirbar Sorptive Extraction method (53, 54) was used. Polydimethylsiloxane stirbars (GERSTEL 011222-001-00) were held in the headspace of the reaction vial by a magnet during the catalysis reaction to absorb volatile components. After the completion of the reaction, the stirbar was added to a headspace vial containing 100 µL of dodecane:ethanol (10:1) and analyzed on a GC-MS headspace sampler (Agilent 7697A) to confirm the identity of the converted product. The GC-MS total ion count signal was converted to PPM by generating a standard curve using the same process described above, but the FD-CF reactions did not contain DNA template or substrates but were spiked instead with known PPM concentrations of the product, isoamyl acetate.

Hydrogel production and characterization

For the sortase hydrogel peptides, 8-arm PEG vinyl sulfone MW 20,000 Da (PEG-VS) was purchased from JenKem Technology (Beijing). The cross-linking peptides GCRELPRTGG and

GGGSGRC were custom-synthesized by CPC Scientific (Sunnyvale, CA). 8 mM of each peptide was separately conjugated to 1 wt% PEG, dialyzed, lyophilized, and then reconstituted to 30 wt%. Freeze-dried, cell-free reactions were used to generate enzymatically cross-linked hydrogels using a two-step process. First, FD-CF reactions containing a sortase, ecarin, or Trx-Bx-encoding template were reconstituted with nuclease-free water and incubated at 37°C. Following incubation, the hydrated sortase reaction was added to a solution of 0-8% PEG-GCRELPRTGG, 0-8% PEG-GGGSGRC in reaction buffer (50 mM HEPES, 150 mM NaCl, 10 mM CaCl2, pH 7.9) and incubated at 37°C for 30 minutes. The hydrated ecarin or Trx-Bx reactions were added separately to a solution of 17.5 mg/mL bovine fibrinogen, 2.3 TIU/mL aprotinin, and 20 mM CaCl2, and incubated overnight at room temperature. The hydrogels were transferred to glass vials and inverted to demonstrate the hydrogel properties. Images were taken with a DSLR camera and adjusted for size and contrast in Adobe Photoshop. For ultrastructural analysis of the resulting hydrogels, the samples were extensively washed with nuclease-free water, snap-frozen in liquid nitrogen, and lyophilized to remove all water. The hydrogel samples were then sputter-coated with 5nm of Pt/Pd before imaging using a Zeiss Supra55VP FE-SEM.

Freeze-dried ligations

To prepare the DNA segments used in the ligation activity, primers were designed (Integrated DNA Technologies) to amplify the segments from the pJL1-sfGFP or toehold-GFP plasmid. Restriction sites with homologous sticky ends were created by double digesting the segments with XbaI and StyI (for pJL1-sfGFP; New England Biolabs; R0145S and R0500S, respectively) or with BamHI and EcoRI (for toehold-sfGFP; New England Biolabs; R0136S and R0101S, respectively) and purified with gel extraction. The DNA segments (100-300 nM) were added in a 1:3 pJL1 vector:insert molar ratio and combined with 200 units of T4 DNA Ligase and 1x T4 DNA Ligase

Buffer (New England Biolabs; M0202S). The ligations were then flash-frozen in liquid nitrogen, lyophilized overnight to obtain the freeze-dried reaction, and stored at room temperature. The ligations were reconstituted with nuclease-free water to the original reaction volume and incubated at room temperature overnight. The ligations were then used to rehydrate the PURE FD-CF reactions, as described above.

DNA extraction and processing from fruit

Household dish soap was diluted 1:10 in water along with 1 gram of table salt and then added to a plastic bag containing chopped fruit (banana, kiwi, or strawberry). The fruit was then gently crushed in the soap and salt mixture by hand until homogenous. The resulting mixture was strained through a household coffee filter into a cup. A pre-chilled 25mL volume of 91% isopropyl alcohol (rubbing alcohol) was added to the strained liquid. The mixture was left undisturbed for 5 minutes to allow phase separation to occur. The upper white layer containing extracted DNA was removed, placed on a clean coffee filter, and washed with 70% ethanol (ethyl rubbing alcohol). The resulting extracted DNA was then patted with paper towels to remove any excess extraction liquid. The DNA was then diluted in water until it dissolved (at around 100 ng/uL) and added to an isothermal recombinase polymerase amplification (RPA) at 20 ng/uL, according to the manufacturer's protocol (TwistAmp® Basic RT; Twist Dx; Figure S8), with primers that were complementary to one section of the banana or kiwi genome (Table S1). The primers also incorporated a T7 promoter for transcription in FD-CF. The resulting RPA product was then added 1:3.75 to a rehydrated FD-CF reaction containing a linearized toehold complementary to the amplified RPA product and run according to the FD-CF methods described above.

Reaction	**DNA plasmid(s)**	**ng/uL**	**Temp**	**Time**

eforRed	pJL1 eforRed	5	RT	Overnight
tdTomato	pJL1 tdTomato	50	RT	Overnight
mOrange	pJL1 mOrange	50	RT	Overnight
GFP	pJL1 GFP	1	RT	Overnight
sfGFP	pJL1 sfGFP (and all variants)	1	RT	Overnight (Visible at 30 minutes)
"Orange"	pJL1 eforRed pJL1 sfGFP	3.75 0.25	RT	Overnight
"Yellow"	pJL1 eforRed pJL1 sfGFP	1.25 0.75	RT	Overnight
Aquamarine*	pJL1 Aquamarine	50	RT	Overnight
RNA aptamer + red fluorescence	pJL1 broccoli eforRed	25	37C RT	15-30 min Overnight
RNA aptamer	pJL1 F30-2xdBroccoli	25	37C	15-30 min
Fluorophore for RNA aptamer	DFHBI-1T	50 uM	37C	15-30 min
ATF1 (PURE only)*	pJL1 ATF1	50	37C	Overnight
Sortase*	pJL1 sortase	25	37C	Overnight
Trx-Bx (PURE only)*	pJL1 Trx-Bx	50	37C	Overnight
Ecarin (PURE only)*	pJL1 ecarin	50	37C	Overnight
Ligation – vector	pJL1	12.5	RT	Overnight
Ligation – insert	sfGFP	15	RT	Overnight

Toehold –switch (PURE)	(various in pJL1)	5 nM	37C	Overnight
Toehold – switch (PURE for fruit)	(various in pJL1)	10 nM	37C	Overnight
Toehold – triggers (PURE)	(various in pJL1)	12 nM	37C	Overnight
Toehold – logic gate triggers (PURE)	(various in pJL1)	10 nM	37C	Overnight

Table 10: DNA concentration and incubation times for FD-CF reactions.

Reactions with a (*) indicate an un-optimized reaction.

SHERLOCK detection assays

Detection assays were performed with 45 nM purified LwCas13a, 22.5 nM crRNA, 125 nM quenched fluorescent RNA reporter (RNAse Alert v2, Thermo Scientific), 2 μL murine RNase inhibitor (New England Biolabs), 100 ng of background total human RNA (purified from HEK293FT culture), and 1 uM input nucleic acid target, unless otherwise indicated, in nuclease assay buffer (40 mM Tris-HCl, 60 mM NaCl, 6 mM MgCl2, pH 7.3). Reactions were incubated overnight at 37°C and then quantified at 485/520 ex/em on a plate reader (Gootenburg et al. 2017).

Statistical analysis

Statistical parameters including the definitions and values of N, standard deviations, and/or standard errors are reported in the figures and corresponding Figure legends. T-tests completed in Chapter Six (parametric, unpaired, assuming both populations have the same standard deviations, p-value set to 0.05) were performed in GraphPad Prism 8.0.1. The results of the responses were graphed and the significance level indicated by the symbols (ns = not significant, * = <0.05, ** = <0.005, *** = <0.0005, and **** = <0.0001).

Cost analysis of kit components

From the supplementary materials of:

Stark, J.C., Huang, A., Nguyen, P.Q., Dubner, R.S., Hsu, K.J., Ferrante, T.C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J.S., et al. (2018). BioBits™ Bright: A fluorescent synthetic biology education kit. Sci. Adv. *4*, eaat5107.

Cost analysis of portable imagers and incubators. The total cost to build working prototypes of our portable 8-well imager, 96-well imager, and incubator (switch and dial versions) are calculated below. Purchasing information for materials used to construct the prototypes are also included. Compiled by Jessica Stark and Tom Ferrante.

Component	Cost ($/unit)	Manufacturer	Product No
8-well Imager Total	**14.13**		
Black acrylic	0.95	Inventables	24112-03
Transparent yellow acrylic	0.12	Inventables	24165-03
Transparent orange acrylic	0.12	Inventables	24164-03
SCIGRIP Weld-On 16 Cement	0.12	TAP Plastics	10886
448 nm blue LED	6.97	LuxeonStarLEDs	SP-05-V4
Thermal adhesive tape for LEDs	0.94	LuxeonStarLEDs	LXT-R-10
Aluminum	0.35	McMaster-Carr	9146T38
Battery pack (3 AAA) with switch	1.56	Digi-Key	SBH431-1AS-ND
AAA rechargeable batteries	3.00	Amazon	RFQ420
96-well Imager Total	**32.01**		
Black acrylic	7.05	Inventables	24112-01
Transparent yellow acrylic	0.85	Inventables	24165-03
Transparent orange acrylic	0.85	Inventables	24164-03
SCIGRIP Weld-On 16 Cement	0.29	TAP Plastics	10886
448 nm blue LEDs (2)	13.94	LuxeonStarLEDs	SP-05-V4
Thermal adhesive tape for LEDs	3.77	LuxeonStarLEDs	LXT-R-10
Aluminum	0.70	McMaster-Carr	9146T38
Battery pack (3 AAA) with switch	1.56	Digi-Key	SBH431-1AS-ND
AAA rechargeable batteries	3.00	Amazon	RFQ420
Switch Incubator Total	**19.79**		
Black medium-density fiberboard	5.67	Inventables	30463-04
Screw, 0.25 in	1.20	Inventables	25297-01
Gorilla® wood glue	0.20	Inventables	26032-01
Mini solder-able breadboard	2.95	Sparkfun	PRT-12702
N-channel MOSFET	0.95	Sparkfun	COM-10213
Heating pad	3.95	Sparkfun	COM-11288
microB USB breakout	1.95	Sparkfun	BOB-12035
10K resistor	0.10	Digi-Key	10.0KXBK-ND
1-20K resistor (2x)	0.20	Digi-Key	variable

110K resistor (2x)	0.20	Digi-Key	110KXBK-ND
Temperature sensor	1.84	Digi-Key	TC622VAT-ND
Switch	0.58	Digi-Key	EG1903-ND
Dial Incubator Total	**19.67**		
Black medium-density fiberboard	5.67	Inventables	30463-04
Screw, 0.25 in	1.20	Inventables	25297-01
Gorilla® wood glue	0.20	Inventables	26032-01
Mini solder-able breadboard	2.95	Sparkfun	PRT-12702
N-channel MOSFET	0.95	Sparkfun	COM-10213
Heating pad	3.95	Sparkfun	COM-11288
microB USB breakout	1.95	Sparkfun	BOB-12035
10K resistor	0.10	Digi-Key	10.0KXBK-ND
110K resistor	0.10	Digi-Key	110KXBK-ND
Temperature sensor	1.84	Digi-Key	TC622VAT-ND
Potentiometer	0.76	Digi-Key	987-1726-ND

Cost analysis of FD-CF reactions. The total cost to assemble FD-CF reactions is ~$0.01 per μL. This comes out to ~$0.05 per 5 μL reaction used in the DNA titration module, or ~$1.00 per 96 well plate used in the design-build-test module. In the table, amino acid cost accounts for 2 mM each of the 20 canonical amino acids purchased individually from Sigma. Extract cost is based on a single employee making 50 mL lysate from a 10 L fermentation, assuming 30 extract batches per year and a 5-year equipment lifetime. Component source is also included in the table if it is available to purchase directly from a supplier. Compiled in by Jessica Stark.

Component	Cost ($/μL rxn)	Supplier	Product No
$Mg(Glu)_2$	negligible	Sigma	49605
NH_4Glu	negligible	MP	02180595
KGlu	negligible	Sigma	G1501
ATP	negligible	Sigma	A2383
GTP	0.00026	Sigma	G8877

UTP	0.00022	Sigma	U6625
CTP	0.00019	Sigma	C1506
Folinic acid	0.00002	Sigma	47612
tRNA	0.00021	Roche	10109541001
Amino acids	negligible	Sigma	
PEP	0.00178	Roche	10108294001
NAD	negligible	Sigma	N8535-15VL
CoA	0.00033	Sigma	C3144
Oxalic acid	negligible	Sigma	P0963
Putrescine	negligible	Sigma	P5780
Spermidine	negligible	Sigma	S2626
HEPES	negligible	Sigma	H3375
Extract	0.00737	homemade	
Total	**0.01**	**$/µL rxn**	
	0.05	**$/5 µL rxn**	
	1.00	**$/96 well plate**	

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APPENDIX

Welcome!

We can't wait to get your students started with exploring the world of molecular biology! This kit was developed as a cost-effective and easier alternative to the existing molecular biology classroom activities. Our cutting-edge cell-free, freeze-dried technology allows for this kit to be cheap and easy - and best of all, no prep work for the instructor. Everything comes ready to use and pre-packaged, straight out of the box!

This kit comes with four different activities for you and your students - one to introduce proteins, one to illustrate the central dogma of biology, and two to allow students to freely experiment with the variables involved with tuning protein expression. Our goal is to not only teach some basic concepts in molecular biology, but also introduce students to the idea of scientific experimentation and how to properly set up and carry out scientific protocols.

We hope you all have fun learning!

Table of Contents

Quick Start Guide

We ***strongly*** suggest that you read through this entire manual carefully, but if you are in a rush or just need a refresher, here's the quick-start version!

1. **Put your kit in the fridge**

   The fluorescent imager can stay out at room temperature.

2. **Print the worksheets for the activities you want to do**

3. **Read through the activity protocols and identify the kit components you will need.**

4. **Put the students in groups of 2-4 and hand each group a set of materials.**

5. **Remove the cell-free pellets from their packaging and tap the tubes on the table to make the pellets are all at the bottom.**

6. **Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.**

7. **Transfer 5 uL of each DNA sample to corresponding tubes of cell-free pellets**
   Be careful not to introduce air or bubbles to the reaction.

8. **Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom of the tubes.**

9. **The reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!**

10. **The next day (or next time class meets), students can take turns placing their tubes in the fluorescent imager and observe the resulting colors!**
    Observation can take place anywhere from 1-10 days after re-hydrating the pellets, as the fluorescent proteins are stable for a while after expression!

OVERVIEW OF KIT

Kit Contents *(enough for 10 repeats of each activity)*

- **180** cell-free pellets (already divided up by each activity in individual vacuum sealed pouches)
- **DNA plasmids** (**10** of each of Red, Orange, Yellow, Green and RNA Green DNA) – *there is enough for all activities, even though it may not look like it!*
- **10** tubes of **Nuclease-free water**
- **1** bag of spare strips of extra empty tubes
- **1** Fluorescent imager with orange and yellow filters and batteries (to be shared by whole class)
- (If requested), **200** disposable plastic transfer pipettes

Recommended Additional Supplies

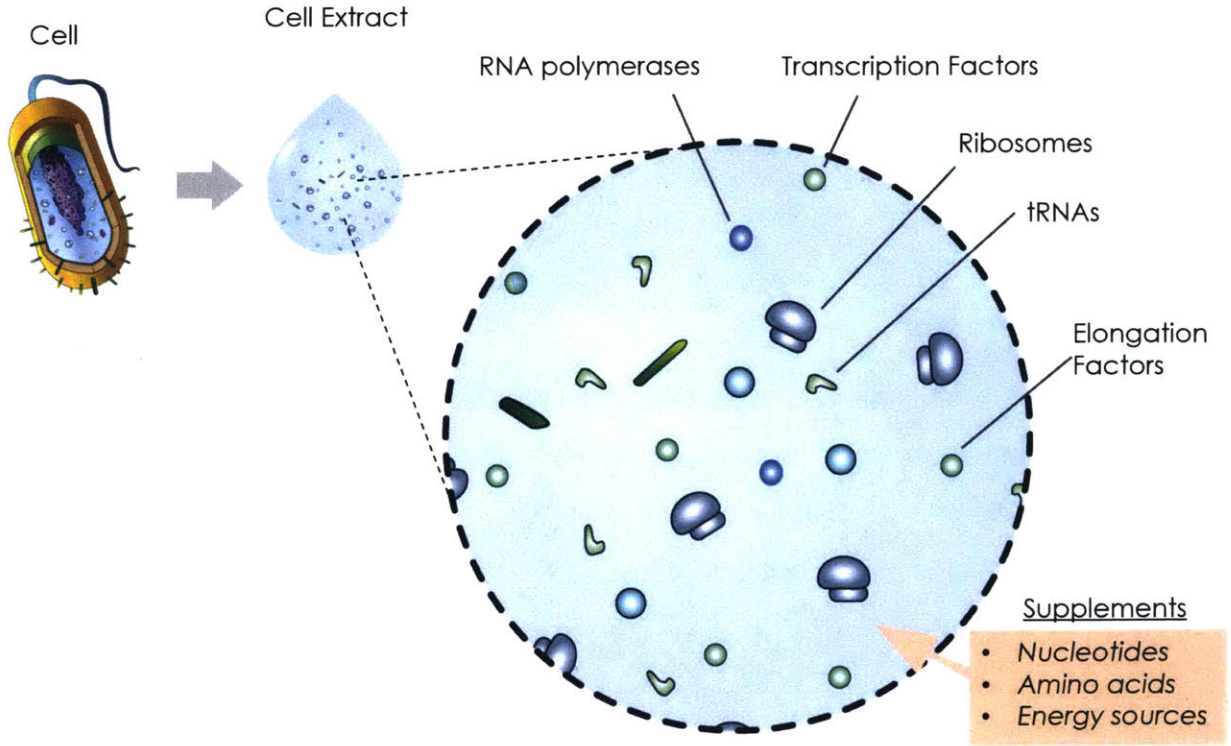
- P5, P10 or P20 pipettes (any with 5 uL capability) with tips – ***highly recommended!***
- Lab gloves (to keep hands clean)
- Permanent markers
- Food coloring in water (for pipetting practice, if needed)
- Incubator (for advanced extension activities)

Storage

- Store your kit (apart from the imager) in the fridge (not freezer).
- For best results, we suggest using the kit within 3 months of receipt.
- Try to keep the pellets in the sealed vacuum bags right up until needed for the activities. If you do need to remove them, be sure to store them in an airtight container with the provided Dri-Card desiccant card.
- If you need to split a strip of the tubes to get the right number per group, use a pair of scissors to cut the tubes apart with the caps on.

How Does It Work?

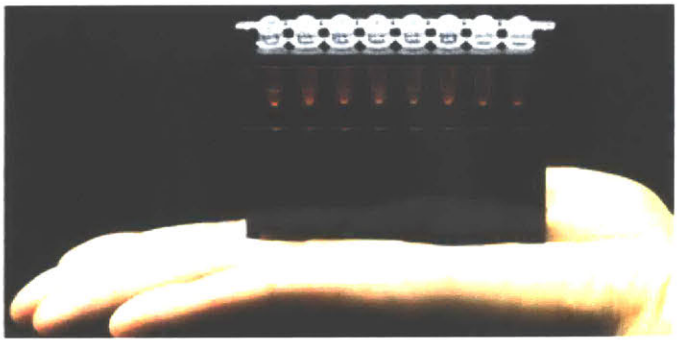
Most other hands-on molecular biology labs involve live cell cultures, which requires extensive prep work for the teachers, expensive reagents and equipment, and complicated protocols. With our kit, **we have extracted all the cellular machinery needed for transcription and translation** (such as the ribosomes, transcription factors, tRNAs, etc.) from the cells into an cell-free extract. The extract is then supplemented with a buffer that contains nucleotides, amino acids, an energy source, and other chemical components needed for protein expression. This reaction is freeze-dried into a pellet so that it's stable at either room temperature (for two weeks) or in a normal fridge (for up to 3 months) until you need it! All the students need to do is add the DNA (provided in solution) to rehydrate the cell-free pellets, and then process of transcription and translation will take place to express fluorescent proteins.



Using Your Kit

- We recommend a group size of 2-4 students.
- Pellets can be dislodged from the bottom of the tube during shipping and handling. Tap the tubes gently on the table before opening to ensure pellets are at the bottom to prevent pellet loss.
- We strongly recommend that the kit be used with pipettes, like P5, P10, or P20, for ease of use. If your classroom doesn't have any pipettes, don't worry – you can still use the kits. We've included disposable plastic transfer pipettes that can take the place of traditional pipettes (although less accurate)
- Read the **Troubleshooting Guide** and **FAQ** at the end to learn about the common mistakes and how to avoid them.

Fluorescent Imager



- Your kit comes with a fluorescent imager (batteries included)
- To use, place the tubes in the holes on top, slot the orange or yellow filter in the front, and turn on the imager with the switch.
- For the majority of activities, use the orange filter unless indicated otherwise. The red, orange, and yellow colors appear best under the orange filter. The green color appears best under the yellow filter.
- Turn off imager when not in use to preserve battery life and prevent overheating of the imager, which can burn out the LEDs inside.

- To change batteries, slide the compartment in the back open.

Safety and Waste Disposal

- We highly recommend the students wear gloves while doing the experiments to keep the samples from being contaminated.
- Please follow all general lab safety rules, such as no eating near the experiments and keep all reagents away from the eyes, nose, and mouth.
- To dispose of the used reagents, please follow your school's or building's biological waste disposal guidelines.
- If you do not have these guidelines in place, please just disinfect all the used reagents and plastics with 10% bleach and dispose into the regular trash.
- For the imager, do not point the holes on the top towards your or anyone else's eyes when you are turning it on or using it. The LED is bright and should not be shown directly into eyes.
- Turn off imager when not in use to preserve battery life and prevent overheating of the imager, which can burn out the LEDs inside. (*Note: if you have a push-button version of the imager, it automatically turns off when the button is not being pushed).*

ACTIVITIES

Pre-Activity: Pipetting Practice

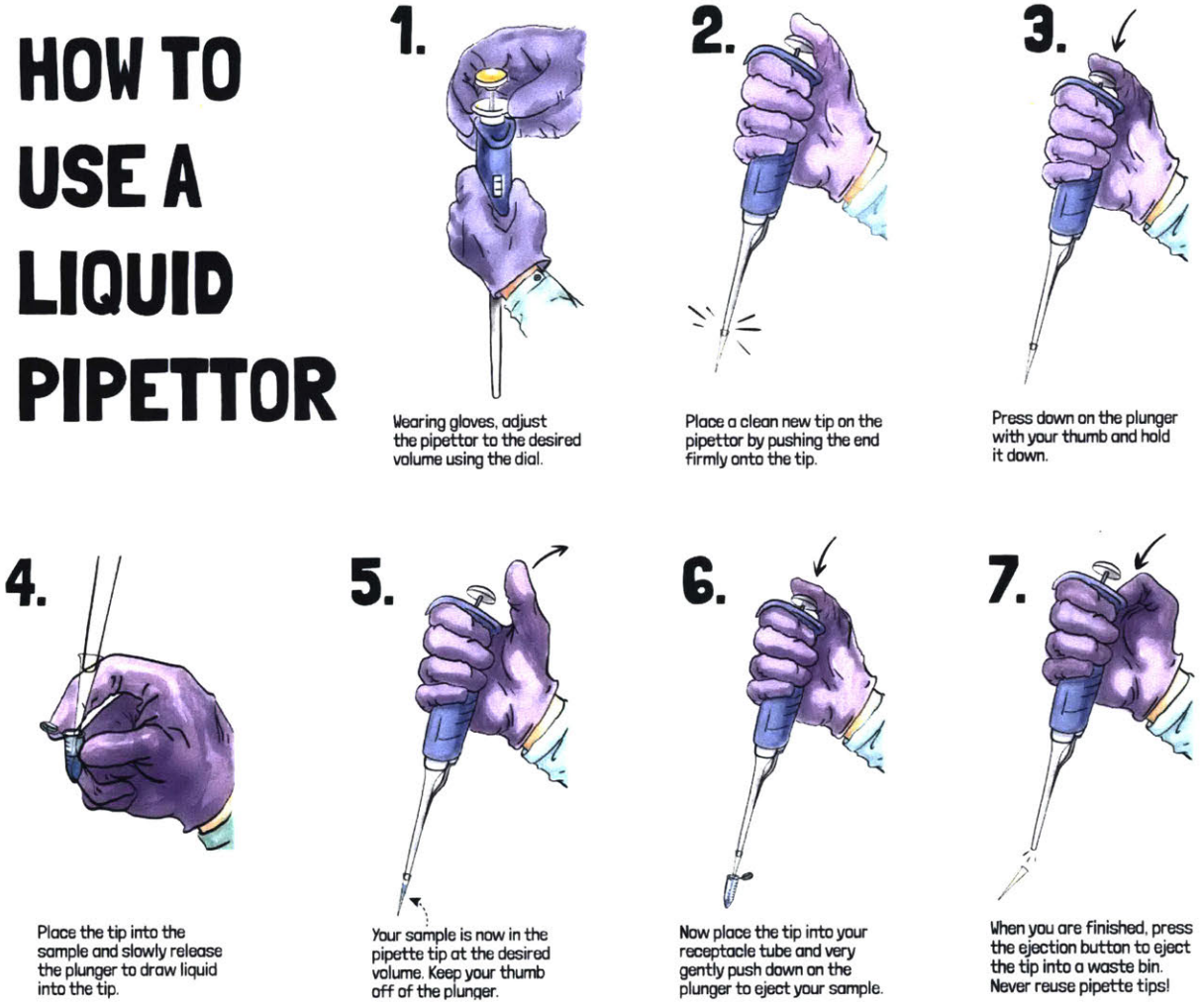
If students are unfamiliar or uncomfortable with pipetting, the teacher may want to have the students do the quick pipetting practice activity below. If students are comfortable with pipetting, skip to **Activity 1** below. If your students need written directions for how to pipette, print the **Pipetting Protocol** for them to reference.

While we highly recommend the use of regular micro-pipettes, as they are much easier and more accurate to use, we recognize that not every classroom will have them. As an alternative, we have provided plastic transfer pipettes (upon request) in your kits. If you are using the transfer pipettes, please be sure to provide your students with the correct **Pipetting Protocol** as well as the transfer pipette version of all the activity worksheets. Be sure to practice with food coloring first to get the feel for how to use the transfer pipettes properly.

Pipetting Protocol - Regular Pipettes

Each group will get:

- A pair of gloves per student
- A P5, P10 or P20 pipette (any with 5 uL capability) with tips
- A tube with food coloring in water
- Empty tubes



If you have regular pipettes (recommended):

1. Hold the pipette so that the curved handle is resting comfortably on your index finger and your thumb is on the plunger.

    a. When you press down on the plunger, you will feel two different "stopping" points. The first stop sets the amount of liquid the pipette will pick up. You can push down harder on the plunger to reach the second stop. This helps push all the liquid out of the tip.
2. To use the pipette, place the end of the pipette into one tip and gently press down to attach the tip to the pipette.

3. To pick up liquid, press the plunger down to the first stop and hold it there. Place the tip of your pipette into the liquid. Slowly release the plunger and you should see the liquid get pulled up into the tip. It's important to do it slowly to prevent air bubbles. Once you have completely released the plunger, remove the tip from the liquid.

4. To release the liquid into a new tube, place the tip of your pipette into the tube. Slowly push the plunger down all the way to the first stop, then the second stop to make sure all solution has been pushed out of the tip. While keeping your thumb pressed all the way down on the plunger, pull the pipette out of the tube

    a. If you let go too soon, all the liquid you just released will just be pulled up again.

5. Discard the tip by hitting the ejector button. It is always a good idea to change tips every time to prevent the different liquids from accidentally contaminating each other.

6. Have the students practice pipetting the food coloring (5-10 uL volumes) into the empty tubes until they are comfortable with transferring those small volumes.

Pipetting Protocol - Transfer Pipettes

Each group will get:

- A pair of gloves per student
- Plastic transfer pipettes
- A tube with food coloring in water
- Empty tubes

If you are using the plastic transfer pipettes provided:

1. Gently squeeze the bulb on the top and place the tip into the liquid. As you let go of the bulb, the liquid will be pulled up.
2. To release the liquid, just gently squeeze the bulb again until the liquid is all pushed out. While keeping the bulb squeezed, pull the pipette out of the tube
    a. If you let go too soon, all the liquid you just released will just be pulled up again).
3. For the kit, you only have to pull liquid up to right below the first shelf (see diagram) to get the right amount of about 5 uL (referred to as the "**first shelf volume**"). You **barely** need to squeeze the bulb to do this, so be gentle!
4. It is always a good idea to change pipettes every time to prevent the different liquids from accidentally contaminating each other.
5. Have the students practice pipetting the food coloring into the empty tubes until they are comfortable with transferring the small volume needed.

Activity 1: Observing Fluorescent Proteins

The purpose of this activity is for students to produce fluorescent proteins that they can visualize, as well as familiarize the teachers and the students with how to handle the components in the kit and the techniques (i.e. pipetting) they will need.

Activity 1 Setup and Protocol:

Each group will get:

- One worksheet per student
- A pair of gloves per student
- A P5, P10 or P20 pipette (any with 5 uL capability) with tips or plastic transfer pipettes
- A permanent marker for labeling tubes
- **5** cell-freepellets (***1*** *strip of 5 tubes from the Activity 1 bag*)
- **1** tubes **DNA plasmids** each of
  - Red
  - Orange
  - Yellow
  - Green
- **1** tube of **Nuclease-free water**
- Fluorescent imager (to be shared by whole class) - with the orange filter

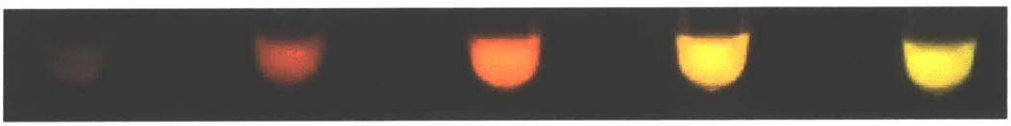
1. Remove the cell-free pellets from their packaging and tap the tubes on the table to make the pellets are all at the bottom. Label each tube (near the top) 1 through 5 and the group name with the permanent marker.
2. Transfer 5 uL of each **DNA plasmid** to corresponding tubes #1-4 of cell-free pellets.
   a. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.

b. Be careful not to introduce air or bubbles to the reaction.
c. If using plastic transfer pipettes, add 1 of the "first shelf volume"

3. To the control condition (tube #5), add 5 uL of nuclease-free water.
   a. This is the negative control
4. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.
5. The reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!
6. The next day (or next time class meets), students can take turns placing their tubes in the fluorescent imager with the orange filter and observe the resulting colors!

What You Should See

The students should see colors similar to the image below:



The color comes from the fluorescent proteins that were produced in the reaction. Each protein has a unique structure, which allows the protein to perform its unique function. These fluorescent proteins here all have different structures, which is why they are all different colors. We humans have a huge variety of different proteins in us - sadly, no fluorescent ones! These proteins are found in creatures like jellyfish or coral that need fluorescence to help with their survival. In the next activity, students will learn how proteins are made from the central dogma of biology (DNA to RNA to proteins).

Having trouble? See **Troubleshooting Guide**.

Answer Guide for Activity 1 Worksheet *(exact answers may depend on your class curriculum and student level)*

1. Briefly summarize the structure of DNA and its function in living things.

   Structure = double strand/helix made up of 4 bases, bases follow the base pair rules (A with T; C with G) and form the "ladder rungs" in the middle of the double helix

   Function = genetic instructions for making all the proteins in your body

2. Why is DNA plasmid not added to tube 5? Why is this critical for a successful scientific experiment?

   It is the negative control – it provides the baseline for what the reaction looks like when it doesn't work. This helps provide a helpful frame of reference when students are trying to determine if their reactions did work or not.

3. **How** did the appearance of each tube vary?

   Each tube should be a different color fluorescent protein.

4. **Why** did the color expression vary the way it did in the 5 tubes? Use the terms DNA, protein, fluorescent, and water.

The structure of the protein will determine its fluorescent color (extra credit: based on light wavelengths). The structure of the protein is based on the DNA sequence that is added as the instructions for building the protein. We added a different DNA plasmid/sequence to each tube, so each sequence codes for a different protein, resulting in different colors. The negative control has no color because only water (no DNA) was added, so no fluorescent proteins were made.

5. Find another group in the class to compare results with. What were their results? Do they confirm your results or contradict your results?

   If everyone did everything correctly, all the results should be the same. This question helps students determine if a technical error was made during the lab (i.e., the caps were not on tight enough and the reaction evaporated).

6. ***Extend your thinking***:
   a. Predict what would happen if you added two kinds of DNA plasmid to one tube.

      Students should get credit for attempting to answer this question, regardless of accuracy. Ideally, they would predict that both fluorescent proteins would be made and the resulting color would be a combination of the two colors (extra credit for using the light color wheel properties rather than the regular color wheel).

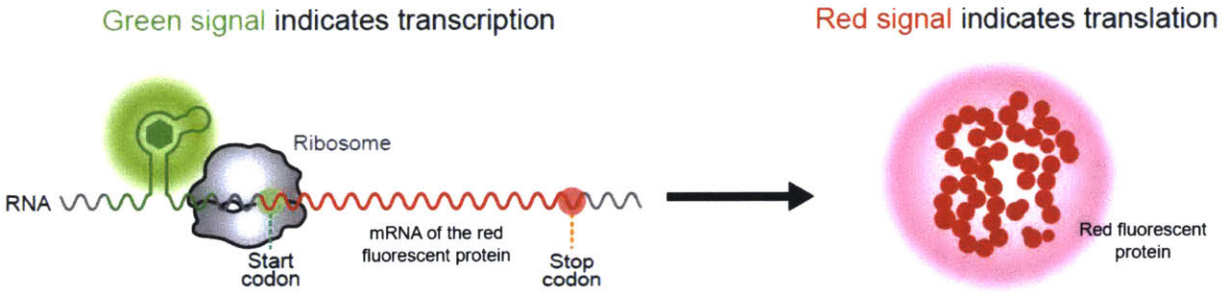
b. Predict what would happen if you added twice as much DNA plasmid to each tube.

Students should get credit for attempting to answer this question, regardless of accuracy. Ideally, they would predict that by doubling DNA plasmid ***concentration****, the brightness of the fluorescent protein will also be doubled or increased. (extra credit for distinguishing between adding twice as much DNA in terms of* ***concentration vs. volume*** *– If twice as much* ***volume*** *is added, the brightness of the fluorescent protein will be halved or decreased.)*

Activity 2: From DNA to Proteins

The purpose of this activity is to illustrate the central dogma of biology to the students (DNA to RNA to proteins). The process is broken down by step, allowing students to visualize each aspect of the central dogma process.

Students will be adding a specialized DNA sequence to the cell-free pellets. The DNA sequence contains two parts 1) an RNA aptamer and 2) a red fluorescent gene. When the sequence is in the RNA format, the RNA aptamer will form a structure that will pocket a signaling molecule called a fluorophore, allowing green fluorescence to be visible – this will indicate transcription. When the red fluorescent gene is translated, red fluorescent protein will be visible – this will indicate translation. See the FAQ for more detail about the science behind this.



Activity 2 Setup and Protocol:

Each group will get:

- One worksheet per student
- A pair of gloves per student
- A P5, P10 or P20 pipette (any with 5 uL capability) with tips or plastic transfer pipettes
- A permanent marker for labeling tubes
- **4** cell-free pellets (***1*** *strip of 4 tubes from the Activity 2 bag*)
- **1** tubes **DNA plasmids** each of:

- ➢ Red
- ➢ Green
- ➢ RNA Green

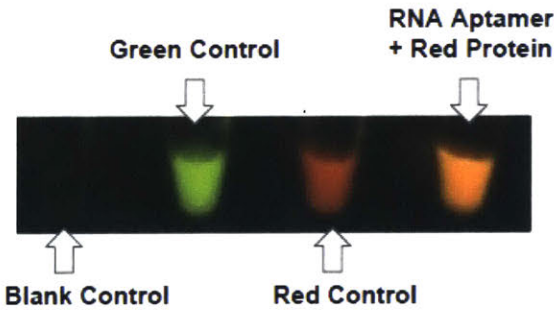
- ❖ **1** tube of **Nuclease-free water**
- ❖ **1** fluorescent imager (to be shared by whole class) - with the orange filter
- ❖ Incubator set at 37C (optional)

1. Remove the cell-free pellets from their packaging and tap the tubes on the table to make the pellets are all at the bottom. Label each tube (near the top) 1 through 4 and the group name with the permanent marker.
2. Transfer 5 uL of each **DNA plasmid** to corresponding tube of cell-free pellets.
    a. If using plastic transfer pipettes, add 1 of the "first shelf volume"
    b. Careful not to introduce air or bubbles to the reaction.
    c. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    d. To the control condition, add 5 uL of nuclease-free water
    e. The DNA for Green represents a green-only condition and the DNA for Red represents a red-only condition - these tubes will be used as the positive controls for the green and red colors.
3. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.
4. Place the tubes in the incubator set at 37C.
    a. If you don't have an incubator, you can use body heat (i.e., your hands) to warm the tubes.

5. After 15 minutes, students can take turns placing their tubes in the fluorescent imager with the orange filter and observing what they see so far.
    a. The RNA aptamer reacts very quickly, so the green should be visible after just a few minutes. If it isn't, keep warming it!
6. The rest of the reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!
7. The next day (or next time class meets), students can take turns placing their tubes in the imager with the orange filter and observe the resulting colors!
    a. The red fluorescent protein needs the overnight incubation time to mature.

What You Should See

The students should see colors similar to the image below:



The Green only tube should just have green signal in it because it just contains green fluorescent protein (as a positive control for the green signal). The Red tube should just have red signal (as a positive control for the red signal) in it because it does not contain the RNA aptamer for the green signal (both transcription and translation happened, but the transcription was not labeled with a signal). The tube on the far right has the RNA Green + Red plasmid added to it. It should be a red-orange color because there is both green signal from the RNA aptamer to

indicate transcription and there is red signal from the red fluorescent protein to indicate translation.

Having trouble? See **Troubleshooting Guide**.

Answer Guide for Activity 2 Worksheet *(exact answers may depend on your class curriculum and student level)*

PRE-LAB:

1. In your own words, describe the processes of transcription and translation. What are the products of these processes?

   Transcription = when DNA is used as a template to transcribe and create mRNA within the nucleus of the cell

   Translation = when mRNA moves from the nucleus to the cytoplasm and gets translated by a ribosome into different codons that correspond to different amino acids (using tRNAs) to create a protein chain of amino acids.

2. In this lab, you are not using real cells. However, our cell-free pellets contain everything we need for transcription and translation! What do you think these pellets contain?

   Ribosomes, RNA polymerases, nucleotides/bases, and amino acids (extra credit: transcription factors, tRNAs, elongation factors).

HYPOTHESIS: Based on the information given in the Background Information, what do you think your set up will look like before transcription and translation occur? After? Between? Write your hypothesis below.

Students should get credit for attempting to answer this question, regardless of accuracy. Ideally, they would predict that the set up will have no fluorescence before transcription and translation occur, the RNA Green tube will have green fluorescence after transcription happens, and after transcription/translation happens, the Red tube will be red, Green will be green, and the RNA green will be a combination of red and green (yellow).

ANALYSIS:

1. After the first 15 minutes of having the DNA plasmids in the pellets, what colors were visible and in which tubes? Why do you think you saw this color?

   Green should be visible in the RNA Green tube. You see this color because transcription has happened and there is RNA present in the tube. The specialized RNA aptamer has formed, bound to the fluorophore, and is giving off a green signal. The students may also see green in the regular Green tube – the green fluorescent protein matures very quickly and can be seen after about 15-20 minutes, depending on the incubation temperature. You may want to specify the difference between the two green signals – one is a signal for transcription occurring, and one is a signal for both transcription and translation occurring (as well as a positive control for the green signal).

2. What process of the central dogma occurred after these 15 minutes? How do you know?

   Transcription has happened because we see the green signal in the RNA Green tube. This means that the RNA must be present, because you need RNA to create that green signal from the special aptamer/fluorophore binding. For RNA to be made, transcription had to happen.

3. The next day, you should have seen a new color. Which tube did you see this in? What process has occurred at this point? How do you know?

   Translation has happened because we see the corresponding fluorescent protein in all tubes (red in Red and RNA green; green in Green). This means that the protein must be present, because you need fluorescent protein to see that fluorescent signal. For protein to be made, translation had to happen.

4. Why do you think you did not see this color after 15 minutes?

   While transcription and translation happens on the order of minutes in our bodies, the actual protein has some post-translational modifications that have to happen first, such as folding. In our artificial system, this takes a few hours. (The green fluorescent protein matures quickly, which is why you can see it after just 20 minutes).

5. The three tubes with DNA plasmids all had different color expression. Can you explain why the tubes were all different?

   The Red tube only had the DNA sequence for a red fluorescent protein, so

you see red. The Green tube only had the DNA sequence for a green fluorescent protein, so you see green Those tubes are used as positive controls so we know what a red only and a green only signal looks like. The RNA Green tube had both the DNA sequence for the special RNA aptamer and the red fluorescent protein, so you see green at first, and then the tube turns more yellow/orange as more red fluorescent protein is made.

6. Why was DNA not added to the control tube? Why is this important?

It is the negative control – it provides the baseline for what the reaction looks like when it doesn't work. This helps provide a helpful frame of reference when students are trying to determine if their reactions did work or not.

Activity 3: How to Control Protein Expression – Part I

The purpose of this activity is to guide students to experiment with a single variable that impacts their protein expression. This will teach them about tuning protein expression as well as basics of experimental design.

Students will be adding varying amounts of DNA to the pellets and observe the effect of DNA amount on the brightness of their protein expression.

Activity 3 Setup and Protocol:

Each group will get:

- One worksheet per student
- A pair of gloves per student
- A P5, P10 or P20 pipette (any with 5 uL capability) with tips or plastic transfer pipettes
- A permanent marker for labeling tubes
- **4** cell-free pellets (***1*** *strip of 4 tubes from the Activity 3 bag*)
- **1** tube of **DNA plasmid** of your choice (Red, Orange, Yellow, or Green)
- **1** tube of **Nuclease-free water**
- **1** 3-tube strip of spare empty tubes
- Fluorescent imager (to be shared by whole class) - with the orange or yellow filter

1. Remove the cell-free pellets from their packaging and tap the tubes on the table to make the pellets are all at the bottom. Label each tube (near the top) 1 through 4, the intended color, and the group name with the permanent marker.
2. For the **DNA plasmid** used, dilutions will be created to vary the amount of DNA. (*This can be done ahead of time by the teacher or part of the activity done by the students.)*

a. Label 3 tubes in the strip of spare empty tubes A through C.
b. Add 8 uL of **Nuclease-free water** to empty tubes B and C.
    i. If using plastic transfer pipettes, add 4 of the "first shelf volume"
c. Add 12 uL of **DNA plasmid** to empty tube A.
    i. If using plastic transfer pipettes, add 4 of the "first shelf volume"
d. Add 2 uL of the contents of tube A to tube B. Pipette up and down gently to mix (try to avoid air bubbles).
    i. If using plastic transfer pipettes, add 1 of the "first shelf volume"
e. Add 2 uL of the contents of tube B to tube C. Pipette up and down gently to mix (try to avoid air bubbles).
    i. If using plastic transfer pipettes, add 1 of the "first shelf volume"

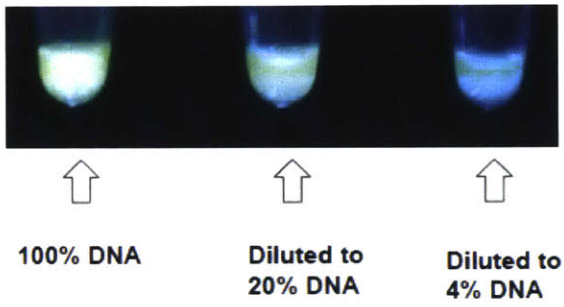
3. Transfer 5 uL of each diluted **DNA plasmid** (from the spare tubes labeled A through C) to corresponding 3 tubes of cell-free pellets (the tubes labeled 1-4)
    a. If using plastic transfer pipettes, add 1 of the "first shelf volume"
    b. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    c. Careful not to introduce air or bubbles to the reaction.
    d. To the control condition, add 5 uL of nuclease-free water to the remaining cell-free pellet.
4. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.
5. The reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!

6. The next day (or next time class meets), students can take turns placing their tubes in the imager and observe the resulting colors!

    a. If using Green, we suggest using the yellow filter. For all other colors, we suggest using the orange filter.

What You Should See

The students should see a gradient in colors similar to the image below:



Even though DNA is something that can be reused (as it is in our bodies), the higher amount of DNA you add to this reaction, the more protein you will get in a given amount of time because there is more starting material for the reaction to work with before its energy source runs out. Sometimes, our bodies need an exact amount of a protein, so being able to control protein expression like this is very important. Having trouble? See **Troubleshooting Guide**.

Answer Guide for Activity 3 Worksheet *(exact answers may depend on your class curriculum and student level)*

1. Why was no DNA plasmid added to tube 4? Why is this critical for a successful scientific experiment?

    It is the negative control – it provides the baseline for what the reaction looks like when it doesn't work. This helps provide a helpful frame of

reference when students are trying to determine if their reactions did work or not.

2. **How** did the color expression change with the varying amount of each DNA Plasmid?

   The lower the concentration of DNA plasmid that was added, the less bright the fluorescent protein was.

3. **Why** did the color expression change for the different amount of each DNA Plasmid? Use the terms DNA, mRNA, transcription, and protein.

   Even though DNA is something that can be reused (as it is in our bodies), the higher amount of DNA you add to this reaction, the more mRNA will be made during transcription and the more protein will be made during translation because there is more starting material for the reaction to work with before its energy source runs out.

4. Was your hypothesis supported or rejected? Why or why not?

   Students will answer this according to what their original hypothesis was. Ideally, students will use the results of this experiment to provide evidence to back up their answer, such as the lower the concentration of DNA plasmid that was added, the less bright the fluorescent protein was.

5. Find at least one group that tested a different DNA plasmid than your group. What were their results? Do they confirm your results or contradict your results?

If everyone did everything correctly, all the trends should be the same (just in different colors, depending on the plasmids chosen). This question helps students determine if a technical error was made during the lab (i.e., the caps were not on tight enough and the reaction evaporated).

6. Write a conclusion paragraph to answer the essential question (*How does the amount of DNA affect protein expression?*)

   Look for a combination of the answers to question #2, #3, and #4.

7. How does your conclusion relate to the central dogma?

   The central dogma of biology is DNA → RNA → protein, and this experiment provides evidence for that, since by changing the DNA concentration, the resulting protein brightness was directly changed as well.

8. ***Extend your thinking:*** Haplosufficiency is when an individual has only one functional copy of the gene because of a mutation on one copy of the gene. For example, Classical Ehlers–Danlos syndrome is caused by a mutation in a gene that codes for the structure of the protein collagen. Collagen provides structure and strength to tissues like skin. People with this syndrome have overly stretchy skin. Based on your results, how could this mutation lead to a disease?

   If an individual only has functional copy of a gene, that means they have less functional DNA template to begin with. Just like how in this activity, if we add less DNA in the reaction, there are less fluorescent proteins (which

we know because they are less bright), we would expect that a person with only one functional collagen gene will produce less collagen. If they have less collagen (which provides structure/strength to skin), then their skin will be less structured/strong and therefore overly stretchy.

Activity 4: How to Control Protein Expression – Part II

The purpose of this activity is to allow students to freely experiment with a variable of their choice that might impact protein expression. This will allow students to apply everything they learned (both biological concepts and basic experimental skills) from Activities 1-3 and design their own experiments.

Students will brainstorm a list of possible variables they would like to test and select one for their experiments. They will design an experiment to test their hypothesis with one negative control (i.e., to compare the test conditions to see if they did not work; usually just a water-only condition), at least one positive control (i.e., to compare the test conditions to see if they did work, usually just adding the DNA plasmid at a normal concentration under normal incubation conditions), and three test conditions.

Have your students complete the **Pre-Activity Experimental Design** portion of the worksheet before starting the actual lab portion of the activity (ex: could be a homework assignment the day before).

While we encourage the students to come up with their own variable that they would like to test, here are some suggestions:

- Effects of different incubation temperatures
- Effects of light exposures
- Effects of adding salt solutions, ethanol, or any other chemicals available in your classroom
- Effects of adding antibiotics
- Effects of adding your own saliva/tears (please be careful and avoid contact of the reagents with your nose/eyes/mouth)
- Effects of mixing two different **DNA plasmids** together
- Effects of different incubation times

Activity 4 Setup and Protocol *(note: this will vary depending on what variable the students decide to test)*

Each group will get:

- One worksheet per student
- A pair of gloves per student
- A P5, P10 or P20 pipette (any with 5 uL capability) with tips or plastic transfer pipettes
- A permanent marker for labeling tubes
- **5** cell-free pellets (***1*** *strip of 5 tubes from the Activity 4 bag*)
- **1** tube of **DNA plasmid(s)** of your choice (Red, Orange, Yellow, or Green)
- **1** tube of **Nuclease-free water**
- **1** 5-tube strip of spare empty tubes
- Fluorescent imager (to be shared by whole class) - with the orange filter

1. Remove the cell-free pellets from their packaging and tap the tubes on the table to make the pellets are all at the bottom. Label each tube (near the top) 1 through 5 and the group name with the permanent marker.
2. Label the tubes in the strips of spare empty tubes A through E. The students can use these tubes to prepare their reactions, whether that's mixing two **DNA plasmids** together, diluting the **DNA plasmids,** or adding another chemical (not included) into the mix.
    a. Having students create a table (see Activity 4 worksheet) will be helpful in tracking how much of each reagent they need to add.
    b. Be sure to remind the students that if they add an external chemical to test, this will dilute out their **DNA plasmids** and they should adjust their reactions accordingly to make sure the same amount of **DNA plasmids** get added to each reaction.

3. Transfer 5 uL of each reaction mixture (from tubes A through E) to corresponding 5 tubes of cell-free pellets.
    a. If using plastic transfer pipettes, add 1 of the "first shelf volume"
    b. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    c. Careful not to introduce air or bubbles to the reaction.
    d. The negative control condition will almost always be 5 uL nuclease-free water.
4. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.
5. The reactions will occur overnight at room temperature (unless you are testing temperature as a variable) - you can leave them in a tube rack or just laying flat on the table!
6. The next day (or next time class meets), students can take turns placing their tubes in the imager and observe the resulting colors!
    a. If using Green, we suggest using the yellow filter. For all other colors, we suggest using the orange filter.

What You Should See

This will completely depend on what the students have decided to test! Here are some general guidelines to help you figure out what your students may see.

- The optimal temperature range for these reactions is between 25-30C. Higher temperatures cause degradation of the materials in the pellets and lower temperatures lower the kinetic energy to let these reactions happen.
- Exposure to light may cause degradation of the materials in the pellets.

- Certain antibiotics (i.e., those that impact ribosome function) will disrupt the reaction. Other antibiotics (i.e., those that impact cell wall synthesis) will not, since this cell-free reaction does not contain cells.
- Addition of salt will throw off the optimal salt concentration in the reaction. Addition of other chemicals may or may not impact the reaction, depending on the chemical.
- Adding saliva or tears is likely to disrupt the reaction, due to a combination of the salt in these fluids throwing off the salt balance and the enzymes contained in these fluids (i.e. RNAses, DNAses) that will break down the DNA and RNA within the reaction and prevent protein expression.

If you like, you may have the students complete a lab report (according to your usual specifications) instead of completing the rest of the Activity 4 worksheet. If you also have leftover pellets at the end of all the activities, they could be used for the students to explore more variables or to investigate their variable even further.

Having trouble? See **Troubleshooting Guide**.

Answer Guide for Activity 4 Worksheet *(exact answers may depend on your class curriculum and student level)*

PRE-LAB:

1. Brainstorm a list of at least 3 variables that you think may impact protein expression in the cell-free system. For each variable, write down how you think it might impact protein expression (i.e. increase or decrease protein expression?) and why you think that will happen.

   Look for students using prior knowledge to predict what will happen with each of these variables.

2. From your list, circle the variable in the table that your group would like to test. Why do you think you are only testing one variable at a time?

   We are only testing one variable at a time, so that if we see a change in protein expression, we know it was that variable that caused the change. If we tested more than one variable at the same time, then we would not know which variable was responsible for changes in protein expression that we might see.

3. Write down your hypothesis, using the "**If……, then……**" format.

   Look for students using the IF…THEN…format (i.e., if we increase the volume of the reaction, then the brightness of the proteins will be more dim).

4. What is a negative control? In your experiment, what would your negative control be?

   The negative control is the condition where we know the reaction will not work. It provides the baseline for what the reaction looks like when it doesn't work. This helps provide a helpful frame of reference when students are trying to determine if their reactions did work or not. Usually, the negative control will be a water-only condition (with no DNA plasmid added)

5. What is a positive control? In your experiment, what would your positive control be?

   The positive control is the condition where we know the reaction will work. It provides a helpful frame of reference when students are trying to determine if their reactions did work or not. Usually, the positive control will be a reaction run under "normal" conditions – in this case, volume at 5 uL, temperature at room temperature, overnight incubation, etc. Note: if the students add less DNA to the other test reactions because they are diluted with other compounds they are testing, the positive control should also be diluted accordingly with water to maintain consistency across samples.

6. You will get 5 pellets to test. One would be your negative control and one would be your positive control. The remaining three will be your different test conditions. Please fill out the table below with your experimental details.

   Look for students only testing a single variable – all other variables (volume, temperature, etc.) should be constant across all samples.

ANALYSIS

1. Did you prove or disprove your hypothesis? Back up your answer with evidence from your results table.

   Students will answer this according to what their original hypothesis was. Ideally, students will use the results of this experiment to provide evidence to back up their answer.

2. Scientifically, please offer a possible explanation for the results that you saw.

   Students should get credit for attempting to answer this question, regardless of accuracy. Ideally, they would use their prior knowledge to offer a possible explanation.

3. Why was including the negative control important for your experiment?

   The negative control is the condition where we know the reaction will not work. It provides the baseline for what the reaction looks like when it doesn't work. This helps provide a helpful frame of reference when students are trying to determine if their reactions did work or not.

4. Why was including the positive control important for your experiment?

   The positive control is the condition where we know the reaction will work. It provides a helpful frame of reference when students are trying to

determine if their reactions did work or not.

5. What would you do differently about this experiment, if you were to run it again?

   Answers may range from improving on technical mistakes that were made or reaction setups that were not scientifically sound (i.e. contained more than one variable changing at a time). Can also go into what they want to test next, which is the next question.

6. Design and describe an experiment where you investigate your variable further. (For example, you could design an experiment where you test your possible explanation from question #2).

   Look for students drawing on the results from this experiment and using that was evidence to why they want to investigate further. It may be trying out a different range of conditions or testing a whole new concept altogether, depending on what they have discovered.

Troubleshooting Guide

We see mistakes and errors as a learning opportunity for the students! A good debriefing activity would be for the whole class to come together and share their data (i.e., images of their proteins) so everyone can see everyone else's results and come to the conclusion all together about what they were supposed to see. Anything unexpected or out of the ordinary can be an extension question for the students to think about why that happened and how they can prevent it in the future.

Here is a list of common issues and how to avoid or fix them.

Issue	**Solution**
We accidentally added way too much DNA/liquid to the reactions!	This is the most common issue that students have when doing the activities. **We strongly recommend that students spent time practicing pipetting (either with the regular pipettes or the transfer pipettes) with food coloring until they are comfortable with the techniques.** Once the liquid is mixed with the pellets, it can't be undone. Try looking at the reactions the next day anyways - you might be able to see some color, depending on how much you added.

There doesn't seem to be much DNA in the tubes you provided.	We provide 100 uL in each tube of DNA. This should be more than enough for the activities – each activity only requires 5 uL of each DNA. Note that 100 uL is a very small amount and may look like a single drop to the untrained eye. If the liquid has shifted in shipping, tap the tube to collect it all at the bottom.
The reaction liquid has disappeared from the tube the next day.	The caps likely weren't capped tightly enough and the reactions evaporated. Make sure the caps are completely capped on the tubes.
The reaction is still there, but there is no visible fluorescent proteins	Possibility 1 - if too much DNA was added (too much liquid), the reaction will get diluted out and the protein will be more weakly expressed and may not be visible at all. Be sure you add 5 uL only to each reaction. Possibility 2 - the DNA and the regular water look the same (clear liquid). Make sure you are adding the DNA (check the labels on the tubes) and not the water, which will result in no proteins.

	Possibility 3 - if the pellets were previously removed from the sealed bag and/or not properly stored in the fridge, moisture from the air may have gotten into the pellets and spoiled them. Possibility 4 - if the pellets were stored at room temperature for more than two weeks or in the fridge for more than 3 months, they may have degraded. Possibility 5 - If the students are not careful when opening the tubes, the pellets can fly out when the tube is opened. Before opening any pellet tube, students should tap the tubes on the table so the pellet falls to the bottom, and slowly open the tubes.
The fluorescent proteins are very dim.	Possibility 1 – If too much DNA was added (too much liquid), the reaction will get diluted out and the protein will be more weakly expressed and may not be visible at all. Be sure you add 5 uL only to each reaction. Possibility 2 - if the pellets were stored

	at room temperature for more than two weeks or in the fridge for more than 3 months, they may have degraded.
The reaction has way too many bubbles in it.	Bubbles arise when too much air is forced into the tube during pipetting. To avoid this, you can try having students avoid pressing the pipette down to the second stop. To get rid of bubbles, students can try to tap the tubes on the table. Incubating the reactions at a higher temperature (i.e. 37C) can also help pop some bubbles overnight. Bubbly reactions will still work and produce the fluorescent proteins - they just might be slightly harder to see the next day due to all the bubbles in the way.
I don't see my pellet in the tube!	Sometimes the pellet will dislodge from the bottom and be hidden from view in the cap. Before opening any pellet tube, students should tap the tubes on the table so the pellet falls to the bottom. This will prevent the pellet from flying out when the tube is

	opened.
The pellet is stuck on the side of the tubes and tapping does not work.	If the pellet will not budge from the side of the tube, simply use your 5 uL of DNA or water to "wash" it off the sides.
All liquid is stuck on the sides of the tube.	Tap the tube gently on the table and the liquid will collect down into the bottom. If this does not work, you can pipette up the liquid from the side and re-dispense it at the bottom of the tube.
The tubes are too small to handle/label/cut!	To split the tubes easier, you can try using a razer blade to cut them apart. To make labeling easier, you can mark the left-most tube with a simple mark (like a dot) to designate it as Tube #1. All the subsequent tubes will then be in numerical order. Make sure to have the students note this notation in their worksheets if they do this.

Frequently Asked Questions (FAQ)

1. There doesn't seem to be much DNA in the tubes you provided.

We provide 100 uL in each tube of DNA. This should be more than enough for the activities – each activity only requires 5 uL of each DNA. Note that 100 uL is a very small amount and may look like a single drop to the untrained eye. If the liquid has shifted in shipping, tap the tube to collect it all at the bottom.

To help your students, you may want to pipette 5 uL of just water into a spare tube (provided) to give them a frame of reference of what 5 uL looks like.

2. What is nuclease-free water?

Nucleases are enzymes that can break down DNA and RNA and will interfere with your reactions because of that. We provide nuclease-free water to prevent nucleases from getting into your reactions (and suggest students wear gloves), as nucleases are everywhere in our environment!

3. I (or my students) want to know more about the freeze-dried, cell-free pellets! What is in them and how does it work?

Please refer to the description and diagram on page 5 to see what is in the pellets.

You can also explain to your students that this technology is being used to develop affordable and easy diagnostics in developing countries (to detect disease like Zika or Ebola without needing fancy equipment).

4. Can you explain more about what is going in Activity 2 about the aptamer and fluorophore?

An **RNA aptamer** is a special sequence of RNA that can bind to a target molecule. In this case, the RNA aptamer in Activity 2 targets a chemical called DFHBI, which gives off a green fluorescent light when it is bound. Because of this fluorescence,

it is known as a **fluorophore**. In Activity 2, the "RNA Green" DNA that is used has this particular RNA aptamer cloned right between the promoter and the red fluorescent protein ribosome binding site. When this DNA is added to a pellet, the DNA is transcribed into RNA. The RNA sequence corresponding to the RNA aptamer folds into a specific secondary structure that allows it to bind to the fluorophore, DFHBI. Once bound by the aptamer, DFHBI will produce green fluorescence.

For more information, you can read about this aptamer in this published paper.

5. In Activity 2, why does transcription and translation take so long? I thought it happened faster!

While transcription and translation happens on the order of minutes in our bodies, the actual fluorescent protein has some post-translational modifications that have to happen first, such as folding, before being visible. The reaction also has to make enough fluorescent protein that can be detected with a naked eye, which takes some time. That and because we are using a cell-free artificial system, this takes up to a few hours. (The green fluorescent protein matures more quickly than the red, which is why you can see it after just 20 minutes).

6. In Activity 3, why does adding less DNA result in less protein expression? I thought DNA was a template that could be re-used!

Even though DNA is something that can be reused (as it is in our bodies), the higher amount of DNA you add to this reaction, the more protein you will get in a given amount of time because there is more starting material for the reaction to work with before its energy source runs out. Because we are working with a cell-free reaction, there is limited resources in the tube (ATP for energy, nucleotides, amino acids, etc.) In fact, after about 4-6 hours, your reaction will run out of energy and stop making proteins.

Activity 1 Worksheet – Observing Fluorescent Protein:

Observing Fluorescent Proteins

Essential Question:

How can we use lab techniques to make and observe fluorescent proteins?

Objective:

Students will make fluorescent proteins by following a lab protocol in order to familiarize themselves with lab techniques.

Materials: One worksheet per student in group

- One pair of gloves per student
- P5, P10 or P20 pipette (any with 5 µL capability) with tips
- Fluorescent imager with orange filter
- Permanent Marker for labeling tubes
- 5 **cell-free pellets**
- 1 tube **DNA plasmids** each of
    - Red
    - Orange
    - Yellow
    - Green
- 1 tube **Nuclease-free water**

Background Information:

Deoxyribo**n**ucleic **A**cid, or DNA, is a molecule found in living things that acts as a code. This code consists of four different building blocks, known as nucleotides, arranged in a specific order and connected into a very long chain. This code provides instructions for cells to build proteins that perform different functions in living things. Some functions, or jobs, of proteins include making up the structure of your hair, muscles, and enzymes that help chemical reactions occur. Each unique protein in your body comes from its own unique DNA code, similar to how each item at the grocery store is marked with a unique barcode.

Some proteins are shaped in a particular way that makes them appear brightly colored to the human eye. Proteins that produce these bright colors are called "fluorescent." Although our DNA does not code for fluorescent proteins, some animals such as jellyfish can produce fluorescent proteins to help with their survival. In this lab, you will be observing several different proteins that each produce a unique fluorescent color. Their unique, bright colors will allow you to easily see them, as well as tell them apart from each other. You will practice your pipetting techniques to use 4 different DNA plasmids, each of which has a unique code that makes a different fluorescent color protein. You will also use a "control" tube to combine the cell-free pellet with just water to see what happens if no DNA is added.

Activity Protocol:

1. Use the permanent marker to label each tube (near the top) 1 through 5 and the group name.
   Tube #1 contains **DNA plasmid** Red

   Tube #2 contains **DNA plasmid** Orange

Tube #3 contains **DNA plasmid** Yellow

Tube #4 contains **DNA plasmid** Green

Tube #5 contains **Nuclease-free water** (control condition - **NO** DNA plasmid)

2. Transfer 5 µL of each **DNA plasmid** to the corresponding tube of **cell-free pellets**.
    a. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    b. Use the pipetting protocol provided by your teacher or the ell-free Instructional Video.
    c. Be careful not to introduce air or bubbles to the reaction.

3. To the control condition (**NO** DNA plasmid), add 5 µL of **nuclease-free water** to the **cell-free pellet**.

4. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.

5. The reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!The next day, place your tubes in the fluorescent imager with the orange filter and observe the resulting colors!

Results:

After 24 hours of incubation use the fluorescent imager to record observations of the colors you see in each tube in Table 1 below.

Table 1. Observations after 24 Hours

Tube #	Describe Your Observations
1	
2	
3	
4	
5	

Analysis Questions:

7. Briefly summarize the structure of DNA and its function in living things.

8. Why is DNA plasmid not added to tube 5? Why is this critical for a successful scientific experiment?

9. **How** did the appearance of each tube vary?

10. **Why** did the color expression vary the way it did in the 5 tubes? Use the terms DNA, protein, fluorescent, and water.

11. Find another group in the class to compare results with. What were their results? Do they confirm your results or contradict your results?

12. ***Extend your thinking***:

    a. Predict what would happen if you added two kinds of DNA plasmid to one tube.

    b. Predict what would happen if you added twice as much DNA plasmid to each tube.

From DNA to Proteins

Essential Question:

How can we understand and visualize the processes that lead to the formation of proteins?

Objective:

Students will understand the central dogma by conducting an experiment that will allow them to visualize the processes of transcription and translation in order to describe how proteins are formed.

Materials:

- One worksheet per student in group
- One pair of gloves per student
- P5, P10 or P20 pipette (any with 5 µL capability) with tips
- Fluorescent imager with orange filter
- Permanent marker for labeling tubes
- 4 **cell-free pellets**
- 3 tube **DNA plasmids** each of:
    - Red
    - Green
    - RNA Green

- 1 tube **Nuclease-free water**
- Incubator set at 37°C (optional)

Background Information:

Deoxyribo**n**ucleic **A**cid, or DNA, are instructions to build proteins. You might not know how to do this, but the cells of living things certainly do! Cells contain everything they need, including DNA, ribosomes, and amino acids, to build protein using the DNA as instructions. In this lab, we have the goal of building a specific protein: red fluorescent protein! As the name implies, if our experiment is successful, we will see the red protein under the fluorescent imager. Instead of using cells, we will have our cell-free pellets, which contain all of the cell parts needed for the protein-making process to occur.

But what is the protein-making process? In Activity 1, "Observing Fluorescent Proteins," you were able to see a few proteins the fluoresced, or glowed, a different color based on what type of DNA plasmid we used. But how did this happen? Take a look at Figure 1 below. It illustrates what is called the central dogma, which states that proteins are made through a two-step process. The first step is called transcription, which is when a cell part called RNA Polymerase reads the DNA code and builds a material called **R**ibo**n**ucleic **A**cid, or RNA. The second step is called translation, which is when a cell part called a ribosome reads the RNA copy and builds the correct protein out of building blocks called amino acids.

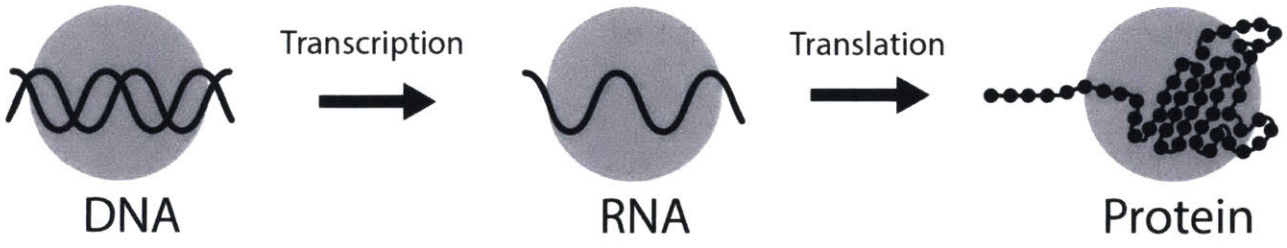


Figure 1. The central dogma states that through a two-step process, we can use DNA to make proteins.

Can we use our fluorescent proteins to see what this looks like? In this activity, we will explore and look at transcription and translation up close! You will be using the Red and Green DNA plasmids from the previous activity, which make red and green fluorescent proteins, respectively. You will also have a special DNA plasmid called RNA Green – this plasmid actually contains two DNA sequences in one! One is a special sequence called an **aptamer,** which means that when it is in its RNA format, it folds up into a specific shape, binds to a certain chemical called a **fluorophore**, causing green fluorescence to be released. The other DNA sequence is the regular sequence for the red fluorescent protein.

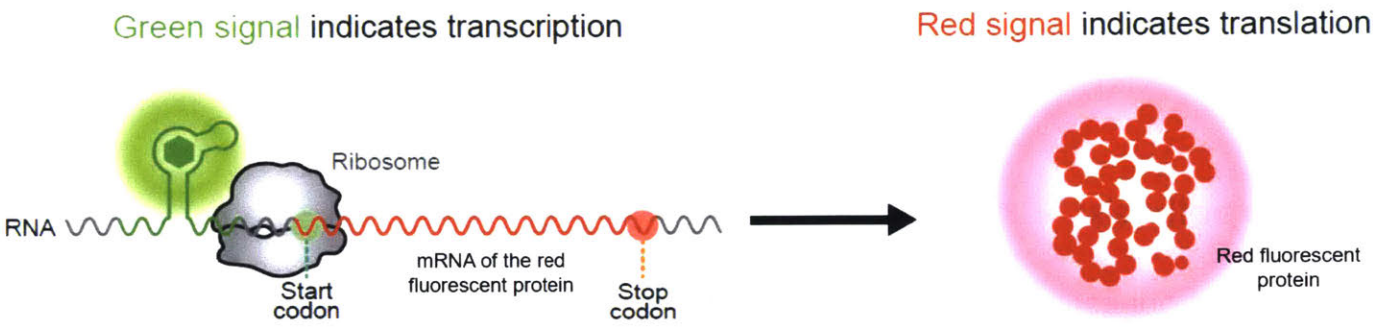


Figure 2. We will be using a special DNA plasmid that is capable of creating two different colors in a single reaction. Each color represents a different step of the central dogma.

Pre-Lab Questions:

3. In your own words, describe the processes of transcription and translation. What are the products of these processes?

4. In this lab, you are not using real cells. However, our cell-free pellets contain everything we need for transcription and translation! What do you think these pellets contain?

Hypothesis:

Recall our essential question: ***How can we understand and visualize the processes that lead to the formation of proteins?*** Based on the information given in the Background Information, what do you think your set up will look like before transcription and translation occur? After? Between? Write your hypothesis below.

Lab Protocol:

1. Remove the **cell-free pellets** from their packaging. Label each tube (near the top) 1 through 4 and the group name with the permanent marker.
   Tube #1 contains **DNA plasmid** RED
   Tube #2 contains **DNA plasmid** Green

Tube #3 contains **DNA plasmid** RNA Green
Tube #4 contains **Nuclease-free water** (control condition - **NO** DNA plasmid)

2. Using your micropipette, set to 5 uL, with the appropriate tip, transfer 5 µL of each **DNA plasmid** to corresponding tube of **cell-free pellets** according to the list above.
    a. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    b. Careful not to introduce air or bubbles to the reaction.

3. To Tube #4, add 5 µL of **nuclease-free water** (*Remember: This tube is your control. Why do we add water?*)

4. Cap the tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.

5. Place the tubes in the incubator set at 37°C.
    a. If you don't have an incubator, you can use body heat (i.e., your hands) to warm the tubes.

6. After 15 minutes, place your tubes in the fluorescent imager with the orange filter and observe what you see so far in Table 1 below. If you don't see anything – keep warming it!

Table 1. Initial Observations

Tube	Initial Observations
1	
2	
3	
4	

7. The rest of the reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!

8. The next day, place your tubes in the imager with the orange filter and observe the resulting colors!

Results:

Below, record the final color expression in each tube.

Table 2. Final Observations

Tube #	Color Expressions
1	
2	
3	
4	

Analysis:

7. After the first 15 minutes of having the DNA plasmids in the cell-free pellets, what colors were visible and in which tubes? Why do you think you saw this color?

8. What process of the central dogma occurred after these 15 minutes? How do you know?

9. The next day, you should have seen a new color. Which tube did you see this in? What process has occurred at this point? How do you know?

10. Why do you think you did not see this color after 15 minutes?

11. The three tubes with DNA plasmids all had different color expression. Can you explain why the tubes were all different?

12. Why was DNA not added to the control tube? Why is this important?

How to Control Protein Expression – Part I

Essential Question:

How does the amount of DNA affect protein expression?

Objective:

Students will understand the central dogma by investigating how the amount of DNA impacts protein expression in order to write a conclusion.

Materials:

- One worksheet per student in group
- One pair of gloves per student
- P5, P10 or P20 pipette (any with 5 µL capability) with tips
- Fluorescent imager with orange or yellow filter
- Permanent marker for labeling tubes
- One 3-tube strip of empty tubes
- 4 **cell-free pellets**
- 1 tube **DNA plasmid**, your choice:
    - Red
    - Orange
    - Yellow
    - Green
- 1 tube **Nuclease-free water**

Background Information:

Our bodies need specific amounts of certain proteins. Additionally, the amount of protein synthesized depends on the amount of the starting materials in the reaction. Our bodies are synthesizing proteins all the time!

In this experiment, you will be adding varying amounts of DNA to the cell-free pellets and observe the effect of DNA amount on the brightness of their protein expression. Remember the central dogma of life.

Write a summary of the central dogma below (refer to Activity #2 "From DNA to Proteins" if you need help):

Hypothesis:

Choose one DNA plasmid to vary in amount (see materials list):

The DNA plasmid that my group will test is _____.

Testable Question:

How does the amount of the _____ DNA plasmid affect protein expression?

Hypothesis:

Activity Protocol:

1. Remove the **cell-free pellets** from their packaging. Label each tube (near the top) 1 through 4, the intended color, and the group name with the permanent marker.

2. Obtain the DNA plasmid from your teacher in three different concentrations: 100%, 20%, and 4% -- **OR** --

   Create dilutions to vary the amount of DNA:

   a. Label 3 tubes in the strip of the *empty* tubes A, B, and C.
   b. Add 8 µL of **Nuclease-free water** to empty tubes B and C.
   c. Add 12 µL of **DNA plasmid** to empty tube A.
   d. Add 2 µL of the contents of tube A to tube B. Pipette up and down gently to mix (try to avoid air bubbles).
   e. Add 2 µL of the contents of tube B to tube C. Pipette up and down gently to mix (try to avoid air bubbles).

3. Transfer 5 µL of each diluted **DNA plasmid** (from spare tubes A through C) to corresponding 3 tubes of **cell-free pellets** (#1-3). (There will be extra dilution mixes leftover)

   a. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
   b. Careful not to introduce air or bubbles to the reaction.

4. To the control condition (#4), add 5 µL of **nuclease-free water** to the remaining **cell-free pellet**.

5. Cap the cell-free pellets tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.

6. The reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!

7. The next day, place your cell-free pellets tubes in the imager and observe the resulting colors! If using Green, we suggest using the yellow filter. For all other colors, we suggest using the orange filter.

Results:

Table 1. Final Observations for _____ *DNA Plasmid* *(Write name of plasmid)*

Tube #	Amount of Plasmid Added	Color Expressions
1		
2		
3		
4	Control	

Analysis Questions:

9. Why was no DNA plasmid added to tube 4? Why is this critical for a successful scientific experiment?

10. **How** did the color expression change with the varying amount of each DNA Plasmid?

11. **Why** did the color expression change for the different amount of each DNA Plasmid? Use the terms DNA, mRNA, transcription, and protein.

12. Was your hypothesis supported or rejected? Why or why not?

13. Find at least one group that tested a different DNA plasmid than your group. What were their results? Do they confirm your results or contradict your results?

14. Write a conclusion paragraph to answer the essential question.

15. How does your conclusion relate to the central dogma?

16. ***Extend your thinking:*** Haplosufficiency is when an individual has only one functional copy of the gene because of a mutation on one copy of the gene. For example, Classical Ehlers–Danlos syndrome is caused by a mutation in a gene that codes for the structure of the protein collagen. Collagen provides structure and strength to tissues like skin. People with this syndrome have overly stretchy skin. Based on your results, how could this mutation lead to a disease?

How to Control Protein Expression – Part II

Essential Question:

How do I set up an experiment to test a variable that might affect protein expression?

Objective:

Students will be able to freely experiment with a variable of their choice that may affect protein expression. This will allow students to apply everything they learned (both biological concepts and basic experimental skills) from Activities 1-3 and design their own experiments.

Materials:

- One worksheet per student in group
- One pair of gloves per student
- P5, P10 or P20 pipette (any with 5 µL capability) with tips
- Fluorescent imager
- Permanent marker for labeling tubes
- **1** 5-tube strips of empty tubes
- **5 cell-free pellets** tubes
- **1** tube of each **DNA plasmid** of your choice
- **1** tube of **Nuclease-free water**

Background Information:

From the previous cell-free activities, you have learned what proteins are, how to make them using DNA, and one way of controlling protein expression. Now, you get a chance to put your new knowledge to the test and become a scientist/engineer!

When scientists or engineers want to see what happens when they change something about their system that they are studying, they form a hypothesis about that variable and design an experiment to test it. Since experiments go wrong all the time in science, either due to human error or just bad luck, it's important for scientists or engineers to have the proper benchmarks, or controls, in place, so they know if a result is actually because of the variable they were testing or if it was a technical error.

In the last activity, you saw what happened when you changed the amount of DNA that was added to the protein expression reaction. There are many more variables that can impact protein expression, and we'll be exploring a variable of your choice in this activity!

Pre-Activity Experimental Design

Please complete this section with your group prior to doing the activity in class.

7. Brainstorm a list of at least 3 variables that you think may impact protein expression in the cell-free system. For each variable, write down how you think it might impact protein expression (i.e. increase or decrease protein expression?) and why you think that will happen.

	Variable	Impact on Proteins	Why?
1			
2			
3			

8. From your list, circle the variable in the table that your group would like to test. Why do you think you are only testing one variable at a time?

9. Write down your hypothesis, using the "**If....., then.....**" format.

10. What is a negative control? In your experiment, what would your negative control be?

11. What is a positive control? In your experiment, what would your positive control be?

12. You will get 5 cell-free pellets to test. One would be your negative control and one would be your positive control. The remaining three will be your different test conditions. Please fill out the table below with your experimental details.

    Be sure to make sure that your test variable is the only thing that is changing between all the test conditions. That means you should make sure that volume and amount of DNA per reaction are the same across all reactions.

Tube #	Test Condition	Amount (in uL) and type of DNA to add	Amount (in uL) and type of other components to add	Predict what you will see
1/A	Negative control			

2/B	Positive control			
3/C				
4/D				
5/E				

Activity Protocol:

1. Remove the **cell-free pellets** tubes from their packaging. Label each tube (near the top) 1 through 5 and the group name with the permanent marker.

1. Label the strip of empty tubes A through E and your group name with the permanent marker.

2. Use the table you created to transfer and mix the appropriate amount of **DNA plasmid** and/or other components to the empty tubes labeled A-E.

3. Transfer 5 µL of your completed reaction mixture from the spare tubes (labeled A-E) to the corresponding tube of the **cell-free pellet** (labeled 1-5)
    a. Remove the caps of the cell-free pellets tubes carefully so the pellets inside don't fall out.
    b. Be careful not to introduce air or bubbles to the reaction.

4. Cap the cell-free pellet tubes tightly (should feel it "click" into place). To mix, gently tap the tubes against the table or flick the tubes with your finger until the pellets are all dissolved and all the liquid has settled to the bottom.

5. If you are not testing temperature or time, then reactions will occur overnight at room temperature - you can leave them in a tube rack or just laying flat on the table!

6. The next day, place your cell-free pellet tubes in the fluorescent imager with the appropriate filter. If using Green, we suggest using the yellow filter. For all other colors, we suggest using the orange filter. Observe and record the results in the table (next page).

Results:

Tube #	Test Condition	Record your observations – what do you see?
1	Negative control	
2	Positive control	
3		
4		
5		

Analysis Questions:

7. Did you prove or disprove your hypothesis? Back up your answer with evidence from your results table.

8. Scientifically, please offer a possible explanation for the results that you saw.

9. Why was including the negative control important for your experiment?

10. Why was including the positive control important for your experiment?

11. What would you do differently about this experiment, if you were to run it again?

12. Design and describe an experiment where you investigate your variable further. (For example, you could design an experiment where you test your possible explanation from question #2).

Teacher Feedback Form

Kits User Feedback – Teachers and Educators

After you have used our kits, we could greatly appreciate it if you could fill out this feedback form – it'll help us improve our kits!

Name: _____
Email: _____
School/Program: _____
Location of school (state): _____
Size of school (# of students): _____
Size of classroom (# of students): _____
Type of class: _____
Activities completed: 1 ___ 2___ 3___ 4___

School demographics *(as our kit is targeted at low resource schools, we would appreciate if you provide any relevant demographics for the school as a whole, such as % minorities, % students on reduced lunch, details on STEM funding, etc.)*

Please complete the feedback sections for the corresponding activities that you did in your class, as well as the overall feedback section!

Overall (The kit as a whole)	Strongly Disagree		Neutral		Strongly Agree
All of the kit components were easy to find and identify.	1	2	3	4	5
The instructional manual as a whole was easy to follow.	1	2	3	4	5
The kit provided educational value to my students.	1	2	3	4	5

The kit inspired interest in biology in my students.	1	2	3	4	5
I would be interested in using the kit again in my class.	1	2	3	4	5
I would be interested in future new activities.	1	2	3	4	5

What was your favorite part of the kits?

What was your least favorite part of the kits (or what could be improved?)

Compared to other biology kits you have purchased before, what would you be willing/able to pay for this kit?

How would you explain this kit to a fellow teacher?

On a scale of 0 to 10, how likely are you to recommend this kit to a friend or a colleague?

(Not at all likely) 0 1 2 3 4 5 6 7 8 9 10 (Extremely likely)

Any anecdotes or observations you would like to share regarding what your students learned from this experience?

Any other suggestions?

For Activity 1 (Observing Fluorescent Proteins)	Strongly Disagree		Neutral		Strongly Agree
Activity 1 was easy to setup for my classroom.	1	2	3	4	5
Activity 1 was easy to teach and run in a classroom	1	2	3	4	5
The teacher manual for Activity 1 was easy to follow.	1	2	3	4	5
The worksheet for Activity 1 was easy to follow.	1	2	3	4	5
I, as a teacher, learned new things from Activity 1	1	2	3	4	5
Activity 1 was fun and engaging for the students	1	2	3	4	5
Activity 1 was useful to teach biological concepts	1	2	3	4	5
Activity 1 was useful to teach scientific inquiry and skills	1	2	3	4	5
I would be interested in using Activity 1 again in my class	1	2	3	4	5

How long did it take your class to run Activity 1? (i.e., how many minutes over how many days)

Which biological concepts or skills would you teach using Activity 1? Where would this fit well in the curriculum?

What were you or your students' favorite part(s) of the Activity 1?

What were the most challenging parts of Activity 1 (or what could be improved, how would you teach this the next time, etc.)

For Activity 2 (From DNA to Proteins)	Strongly Disagree		Neutral		Strongly Agree
Activity 2 was easy to setup for my classroom.	1	2	3	4	5
Activity 2 was easy to teach and run in a classroom	1	2	3	4	5
The teacher manual for Activity 2 was easy to follow.	1	2	3	4	5
The worksheet for Activity 2 was easy to follow.	1	2	3	4	5
I, as a teacher, learned new things from Activity 2	1	2	3	4	5
Activity 2 was fun and engaging for the students	1	2	3	4	5
Activity 2 was useful to teach biological concepts	1	2	3	4	5
Activity 2 was useful to teach scientific inquiry and skills	1	2	3	4	5
I would be interested in using Activity 2 again in my class	1	2	3	4	5

How long did it take your class to run Activity 2? (i.e., how many minutes over how many days)

Which biological concepts or skills would you teach using Activity 2? Where would this fit well in the curriculum?

What were you or your students' favorite part(s) of the Activity 2?

What were the most challenging parts of Activity 2 (or what could be improved, how would you teach this the next time, etc.)

For Activity 3 (Protein Expression – Part I)	Strongly Disagree		Neutral		Strongly Agree
Activity 3 was easy to setup for my classroom.	1	2	3	4	5
Activity 3 was easy to teach and run in a classroom	1	2	3	4	5
The teacher manual for Activity 3 was easy to follow.	1	2	3	4	5
The worksheet for Activity 3 was easy to follow.	1	2	3	4	5
I, as a teacher, learned new things from Activity 3	1	2	3	4	5
Activity 3 was fun and engaging for the students	1	2	3	4	5
Activity 3 was useful to teach biological concepts	1	2	3	4	5
Activity 3 was useful to teach scientific inquiry and skills	1	2	3	4	5
I would be interested in using Activity 3 again in my class	1	2	3	4	5

How long did it take your class to run Activity 3? (i.e., how many minutes over how many days)

Which biological concepts or skills would you teach using Activity 3? Where would this fit well in the curriculum?

What were you or your students' favorite part(s) of the Activity 3?

What were the most challenging parts of Activity 3 (or what could be improved, how would you teach this the next time, etc.)

For Activity 4 (Protein Expression – Part II)	Strongly Disagree		Neutral		Strongly Agree
Activity 4 was easy to setup for my classroom.	1	2	3	4	5
Activity 4 was easy to teach and run in a classroom	1	2	3	4	5
The teacher manual for Activity 4 was easy to follow.	1	2	3	4	5
The worksheet for Activity 4 was easy to follow.	1	2	3	4	5
I, as a teacher, learned new things from Activity 4	1	2	3	4	5
Activity 4 was fun and engaging for the students	1	2	3	4	5
Activity 4 was useful to teach biological concepts	1	2	3	4	5
Activity 4 was useful to teach scientific inquiry and skills	1	2	3	4	5
I would be interested in using Activity 4 again in my class	1	2	3	4	5

How long did it take your class to run Activity 4? (i.e., how many minutes over how many days)

Which biological concepts or skills would you teach using Activity 4? Where would this fit well in the curriculum?

What were you or your students' favorite part(s) of the Activity 4?

What were the most challenging parts of Activity 4 (or what could be improved, how would you teach this the next time, etc.)

Student Feedback Form (Pre-Activity)

Kit User Feedback - Student Pre-Activities

We could greatly appreciate it if you could fill out this feedback form right before (i.e., the day before) doing the activities – it'll help us improve our kits!

Unique Identifier: _____

(each individual student will choose a random word and put it on both the pre and post feedback forms. This lets us pair up each individual student's pre and post forms without using your name. Remember your word!)

Teacher Name: _____

Location of school (state): _____

Grade: _____

Type of class: _____

	Strongly Disagree		Neutral		Strongly Agree
I understand how proteins are made from DNA	1	2	3	4	5
I can explain to others how to make proteins from DNA	1	2	3	4	5
I understand how to control or engineer DNA systems	1	2	3	4	5
I find biology interesting	1	2	3	4	5
I find engineering (testing and manipulating different variables) interesting	1	2	3	4	5
I could imagine becoming a scientist one day	1	2	3	4	5
I could imagine becoming an engineer one day	1	2	3	4	5

Please describe how cells use DNA to synthesize (make) proteins.

Please describe how you can control protein expression (i.e., what variables can you change).

Student Feedback Form (Post-Activity)

Kit User Feedback - Student Post-Activities

After you have used our kits, we could greatly appreciate it if you could fill out this feedback form – it'll help us improve our kits!

Unique Identifier: _____

(use the same word/identifier you used in the pre-activity feedback form!)

Teacher Name: _____

Location of school (state): _____

Grade: _____

Type of class: _____

Please complete the feedback sections for the corresponding activities that you did in your class, as well as the overall feedback section!

Overall (the kit as a whole)	Strongly Disagree		Neutral		Strongly Agree
I understand how proteins are made from DNA	1	2	3	4	5
I can explain to others how to make proteins from DNA	1	2	3	4	5
I understand how to control or engineer DNA systems	1	2	3	4	5
I find biology interesting	1	2	3	4	5
I find engineering (testing and manipulating different variables) interesting	1	2	3	4	5
I could imagine becoming a scientist one day	1	2	3	4	5
I could imagine becoming an engineer one day	1	2	3	4	5
I found the activities fun and worth our class time.	1	2	3	4	5
After the activities, I think differently about biology	1	2	3	4	5
After the activities, I think differently about engineering	1	2	3	4	5

Please describe how cells use DNA to synthesize (make) proteins. Use the results of your experiments to provide evidence of your description.

Please describe how you can control protein expression (i.e., what variables can you change). Use the results of your experiments to provide evidence.

What was the most interesting thing you learned from all of the activities?

What was your favorite part of all of the activities? How would you describe the kit to your friends?

On a scale of 0 to 10, how likely are you to recommend the kit to a friend?

(Not at all likely) 0 1 2 3 4 5 6 7 8 9 10 (Extremely likely)

What was your least favorite part of all of the activities (or what could be improved?)

Any other comments/suggestions?