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# Escherichia coli-based cell-free protein synthesis: Protocols for a robust, flexible, and accessible platform technology

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September 6, 2018

Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Phillip Steindel,

Re: JoVE58882 "Escherichia coli-based cell-free protein synthesis: protocols for a robust, flexible, and accessible platform technology".

We appreciate the thorough review of our manuscript and we thank you and the reviewers for providing insightful and constructive comments. Based on the reviewer comments, we have made several changes to the text and figures in the manuscript and conducted the additional experiments that the reviewers requested. We believe that the reviewer's concerns are now directly addressed and that their suggestions have greatly improved the clarity and quality of the manuscript. We feel that our strengthened manuscript will make an essential and unique contribution *JoVE's* broad readership. Specifically, as reviewer #1 has pointed out in their comments, cell-free protein synthesis has not been a traditionally accessible biotechnology for non-experts. Our improved manuscript combined with the visualization of the experimentation through *JoVE* will enable us to breakdown this barrier. Therefore, we believe that *JoVE* is the best outlet for maximizing the impact of this work as we aim to democratize the cell-free protein synthesis platform.

Below, we provide detailed responses and revisions to the manuscript on a point-by-point basis. We thank both you and the reviewers for your time and effort. We look forward to hearing from you in due course.

Sincerely,

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**TITLE:**

*Escherichia coli*-Based Cell-Free Protein Synthesis

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**KEYWORDS:**

Cell-free protein synthesis, CFPS, *in vitro* protein expression, High-throughput protein synthesis, TX-TL, Synthetic biology, Cell-free metabolic engineering, CFME

**SUMMARY:**

This protocol details the steps, costs, and equipment necessary to generate *E. coli*-based cell extracts and implement *in vitro* protein synthesis reactions within 4 days or less. To leverage the flexible nature of this platform for broad applications, we discuss reaction conditions that can be adapted and optimized.

**ABSTRACT:**

Over the last 50 years, Cell-Free Protein Synthesis (CFPS) has emerged as a powerful technology to harness the transcriptional and translational capacity of cells within a test tube. By obviating the need to maintain the viability of the cell, and by eliminating the cellular barrier, CFPS has been foundational to emerging applications in biomanufacturing of traditionally challenging proteins, as well as applications in rapid prototyping for metabolic engineering, and functional

genomics. Our methods for implementing an *E. coli*-based CFPS platform allow new users to access many of these applications. Here, we describe methods to prepare extract through the use of enriched media, baffled flasks, and a reproducible method of tunable sonication-based cell lysis. This extract can then be used for protein expression capable of producing 900 µg/mL or more of super folder green fluorescent protein (sfGFP) in just 5 h from experimental setup to data analysis, given that appropriate reagent stocks have been prepared beforehand. The estimated startup cost of obtaining reagents is $4,500 which will sustain thousands of reactions at an estimated cost of $0.021 per µg of protein produced or $0.019 per µL of reaction. Additionally, the protein expression methods mirror the ease of the reaction setup seen in commercially available systems due to optimization of reagent pre-mixes, at a fraction of the cost. In order to enable the user to leverage the flexible nature of the CFPS platform for broad applications, we have identified a variety of aspects of the platform that can be tuned and optimized depending on the resources available and the protein expression outcomes desired.

**INTRODUCTION:**

Cell-free Protein Synthesis (CFPS) has emerged as a technology that has unlocked a number of new opportunities for protein production, functional genomics, metabolic engineering, and more within the last 50 years[1,2]. Compared to standard *in vivo* protein expression platforms, CFPS provides three key advantages: 1) the cell-free nature of the platform enables the production of proteins that would be potentially toxic or foreign to the cell[3–6]; 2) inactivation of genomic DNA and the introduction of a template DNA encoding the gene(s) of interest channel all of the systemic energy within the reaction to the production of the protein(s) of interest; and 3) the open nature of the platform enables the user to modify and monitor the reaction conditions and composition in real time[7,8]. This direct access to the reaction supports the augmentation of biological systems with expanded chemistries and redox conditions for the production of novel proteins and the tuning of metabolic processes[2,9,10]. Direct access also allows the user to combine the CFPS reaction with activity assays in a single-pot system for more rapid design-build-test cycles[11]. The capacity to perform the CFPS reaction in small volume droplets or on paper-based devices further supports high-throughput discovery efforts and rapid prototyping[12–16]. As a result of these advantages and the plug and play nature of the system, CFPS has uniquely enabled a variety of biotechnology applications such as the production of proteins that are difficult to solubly express *in vivo*[17–20], detection of disease[21–23], on demand biomanufacturing[18,24–27], and education[28,29], all of which show the flexibility and utility of the cell-free platform.

CFPS systems can be generated from a variety of crude lysates from both prokaryotic and eukaryotic cell lines. This allows for diverse options in the system of choice, each of which have advantages and disadvantages depending on the application of interest. CFPS systems also vary greatly in preparation time, cost, and productivity. The most commonly utilized cell extracts are produced from wheat germ, rabbit reticulocyte, insect cells, and *Escherichia coli* cells, with the latter being the most cost-effective to date while producing the highest volumetric yields of protein[30]. While other CFPS systems can be advantageous for their innate post-translational modification machinery, emerging applications using the *E. coli*-based machinery are able to bridge the gap by generating site-specifically phosphorylated and glycosylated proteins on demand[31–35].

CFPS reactions can be run in either batch, continuous-exchange cell-free (CECF) or continuous-flow cell-free (CFCF) formats. The batch format is a closed system whose reaction lifetime is limited due to diminishing quantities of reactants and the accumulation of inhibitory byproducts of the reaction. CECF and CFCF methods increase the lifetime of the reaction, and thereby result in increased volumetric protein yields compared to the batch reaction. This is accomplished by allowing the byproducts of protein synthesis to be removed from the reaction vessel while new reactants are supplied throughout the course of the reaction[2]. In the case of CFCF, the protein of interest can also be removed from the reaction chamber, while in CECF, the protein of interest remains in the reaction chamber comprised of a semi-permeable membrane[36,37]. These methods are especially valuable in overcoming poor volumetric yields of difficult-to-express proteins of interest[38–43]. The challenges in implementing the CECF and CFCF approaches are that 1) while they result in more efficient use of the bio machinery responsible for transcription and translation, they require notably larger quantities of reagents that increases overall cost and 2) they require more complex reaction setups and specialized equipment compared to the batch format[44]. In order to ensure accessibility for new users, the protocols described herein focus on the batch format at reaction volumes of 15 µL with specific recommendations for increasing the reaction volume to the milliliter scale.

The methods presented herein enable non-experts with basic laboratory skills (such as undergraduate students) to implement cell growth, extract preparation, and batch format reaction setup for an *E. coli*-based CFPS system. This approach is cost-effective compared to commercially available kits without sacrificing the ease of kit-based reaction setup. Furthermore, this approach enables applications in the laboratory and in the field. When deciding to implement CFPS, new users should thoroughly evaluate the efficacy of conventional protein expression systems for startup investment, as CFPS may not be superior in every case. The CFPS methods described here enable the user to directly implement a variety of applications, including functional genomics, high-throughput testing, the production of proteins that are intractable for *in vivo* expression, as well as field applications including biosensors and educational kits for synthetic biology. Additional applications such as metabolic engineering, tuning of protein expression conditions, disease detection, and scale-up using CECF or CFCF methods are still possible but may require experience with the CFPS platform for further modification of reaction conditions. Our methods combine growth in enriched media and baffled flasks, with relatively rapid and reproducible methods of cell lysis through sonication, followed by a simplified CFPS reaction setup that utilizes optimized premixes[45]. While the cellular growth methods have become somewhat standardized within this field, methods for cell lysis vary widely. In addition to sonication, common lysis methods include utilization of a French press, a homogenizer, bead beaters, or lysozyme and other biochemical and physical disruption methods[46–49]. Using our methods, approximately 2 mL of crude cell extract are obtained per 1 L of cells. This quantity of cell extract can support four hundred 15 µL CFPS reactions, each producing ~900 µg/mL of reporter sfGFP protein from the template plasmid pJL1-sfGFP. This method costs $0.021/µg of sfGFP produced ($.019/µL of reaction), excluding the cost of labor and equipment (**Supplemental Figure 1**). Starting from the scratch, this method can be implemented in 4 days by a single person and repeat CFPS reactions can be completed within hours (**Figure 1**). Additionally, the protocol

can be scaled up in volume for larger batches of reagent preparation to suit the user's needs. Importantly, the protocol presented here can be implemented by laboratory trained non-experts such as undergraduate students, as it only requires basic laboratory skills. The procedures described below, and the accompanying video have been specifically developed to improve accessibility of the *E. coli* CFPS platform for broad usage.

**PROTOCOL:**

## 1. Media Preparation and Cell Growth

### 1.1. Day 1

1.1.1. Streak *E. coli* BL21*(DE3) cells from a glycerol stock onto an LB agar plate and incubate for at least 18 h at 37 °C.

1.1.2. Prepare 50 mL of LB media and autoclave the solution on a liquid cycle for 30 min at 121 °C. Store at room temperature.

### 1.2. Day 2

1.2.1. Prepare 750 mL of 2x YTP media and 250 mL of 0.4 M D-Glucose solution as described in the supplemental information.

1.2.2. Pour the 2x YTP media into an autoclaved 2.5 L baffled flask and the D-Glucose solution into an autoclaved 500 mL glass bottle. Autoclave both solutions on a liquid cycle for 30 min at 121 °C.

1.2.3. Ensure that both sterile solutions are stored at 37 °C if cell growth is being performed on the next day, to maximize growth rates upon inoculation. Do not combine solutions until inoculation.

Note: Solutions can be stored at 4 °C for 1-2 d if needed, though the 2x YTP media is highly prone to contamination.

1.2.4. Start an overnight culture of BL21(DE3) by inoculating 50 mL of LB media with a single colony of BL21(DE3) using a sterilized loop and sterile technique to avoid contamination.

1.2.5. Place the 50 mL of BL21*(DE3) LB culture into a 37 °C 250 rpm shaking incubator and grow overnight for 15-18 h.

1.2.6. Prepare and sterilize all materials required for days 3 and 4, including: two 1 L centrifuge bottles, 4x cold 50 mL conical tubes (weigh and record masses of three), and many 1.5 mL microfuge tubes.

### 1.3. Day 3

1.3.1. Remove the 50 mL overnight culture of BL21*(DE3) in LB from the shaking incubator and measure the $OD_{600}$ on a spectrophotometer using a 1:10 dilution with LB media. Calculate the volume of overnight culture necessary to add to 1 L of media for a starting $OD_{600}$ of 0.1 (For example, if an $OD_{600}$ of a 1:10 dilution is read as 0.4, inoculate 25 mL of the undiluted $OD_{600}$ = 4.0 overnight culture into 1 L of 2x YTPG).

1.3.2. Remove the warmed 2x YTP media and D-Glucose solutions from the 37 °C incubator along with the 50 mL of LB culture. Using sterile technique, carefully pour the D-Glucose solution into the 2x YTP media (avoiding the sides of the baffled flask).

Note: Addition of D-Glucose completes the recipe for 1 L of 2x YTPG.

1.3.3. Maintaining sterile technique, inoculate the 1 L of 2x YTPG solution with the appropriate amount of the 50 mL culture to begin the 1 L culture at a 0.1 $OD_{600}$. Immediately place the inoculated 1 L culture into a 37 °C shaking incubator at 200 rpm.

1.3.4. Take the first $OD_{600}$ reading after the first hour of growth (lag phase typical takes 1 h). Do not dilute the culture. Continue taking $OD_{600}$ measurements approximately every 20-30 min until $OD_{600}$ reaches 0.6.

1.3.5. Upon reaching $OD_{600}$ = 0.6, add 1 mL of 1M IPTG (final concentration in 1 L culture = 1 mM) to the 2x YTPG culture with.

Note: Ideal induction $OD_{600}$ is 0.6; however, a range of 0.6-0.8 is acceptable. Induction by IPTG is for endogenous production of T7 RNA Polymerase (T7RNAP).

1.3.6. After induction, measure the $OD_{600}$ approximately every 20-30 min until it reaches 3.0.

Note: Cool down the centrifuge to 4 °C during this time. Prepare cold S30 buffer as detailed in the Supplementary Information. If the S30 buffer is prepared in advance, ensure that DTT is not added until the day of use.

1.3.7. Once the $OD_{600}$ reaches 3.0 (**Figure 2A**), pour the culture into a cold 1 L centrifuge bottle in an ice-water bath. Prepare a water-filled 1 L centrifuge bottle of equal weight to be used as a balance in the centrifuge.

Note: Absorbance values vary from instrument-to-instrument. While the $OD_{600}$ of harvest of BL21(DE3) is not a sensitive variable, it is recommended that the user evaluate and optimize this variable as a troubleshooting measure. Larger spectrophotometers may result in relatively lower $OD_{600}$ readings compared to smaller cuvette-based spectrophotometers.

1.3.8. Centrifuge the 1 L bottles for 10 min at 5000 x g and 10 °C to pellet cells.

1.3.9. Slowly pour off the supernatant and dispose of it according to the institution's biological waste procedures. Place the pellet on ice.

1.3.10. Using a sterile spatula, scrape the cell pellet from the centrifuge bottle and transfer it to a cold 50 mL conical tube.

1.3.11. Add 30 mL of cold S30 buffer to the conical tube and resuspend the cell pellet by vortexing with short bursts (20 - 30 s) and rest periods (1 min) on ice until fully resuspended with no chunks.

1.3.12. Once the pellet is fully resuspended, use another 50 mL conical tube with water as a balance and centrifuge for 10 min at 5000 x g and 10 °C (pre-cooled to 4 °C).

Note: This completes the 1st of 3 washes required when harvesting the cells.

1.3.13. Pour out the supernatant and dispose of it according to the institution's biological waste procedures. Resuspend the pellet with 20-25 mL of cold S30 buffer and centrifuge for 10 min at 5000 x g and 10 °C (pre-cooled to 4 °C).

Note: This completes the 2nd of 3 washes.

1.3.14. Again, pour out the supernatant and dispose of it according to the institution's biological waste procedures. Add exactly 30 mL of S30 buffer and vortex again to resuspend the pellet.

1.3.15. Using the 3 pre-weighed, cold 50 mL conical tubes and a serological pipette filler with a sterile pipette, aliquot 10 mL of resuspended pellet/S30 buffer mixture into each of the 3 conical tubes.

Note: Splitting the cells into 3 tubes is not required, but this step results in smaller cell pellets (~ 1 g) for increased convenience at later steps.

1.3.16. Centrifuge all tubes, using appropriate balances as needed, for 10 min at 5000 x g and 10 °C (pre-cooled to 4 °C).

Note: This completes the final wash step.

1.3.17. Pour out the supernatant and dispose of it according to the institution's biological waste procedures. Remove the excess S30 buffer by carefully wiping the inside of the conical tube and cap with a clean tissue; avoid touching the pellet.

1.3.18. Reweigh the tubes on an analytical balance and record the final pellet weight on each tube.

Note: The protocol can be paused at this point. The pellets can be flash frozen in liquid nitrogen and stored at –80 °C for up to a year until needed for extract preparation.

## 2. Crude Cell Extract Preparation - Day 4

2.1. For extract preparation, keep cells cold on ice during each step. Add 1 mL of cold S30 buffer per 1 g of cell mass of the pellet. Ensure that dithiothreitol (DTT) has been supplemented to the S30 buffer to a final concentration of 2 mM.

Note: Cool down the microcentrifuge to 4 °C during this time.

2.2. Resuspend the cell pellet by vortexing with short bursts (20 - 30 s) and rest periods (1 min) on ice until fully resuspended. If resuspension is difficult, leave the pellets on ice for 30 min to defrost.

2.3. Transfer 1.4 mL of resuspended cells into a 1.5 mL microfuge tube.

2.4. Place one 1.5 mL tube containing 1.4 mL of resuspended cells into an ice water bath in a beaker. Sonicate for 45 s on followed by 59 s off for 3 total cycles, with amplitude set at 50%. Close and invert the tubes to gently mix during the off periods. In total, deliver 800-900 J of energy to each 1.5 mL microfuge tube containing 1.4 mL of resuspended cells (**Figure 3A & 3B**).

Note: This step is sensitive to the sonicator type and model used and should be optimized if equipment is different than listed for this procedure. Two complementary approaches can be used to scale-up the amount of extract prepared during this step: 1) multiple 1.5 mL microfuge tubes can be sonicated in parallel, and/or 2) larger volumes can be sonicated in conical tubes (up to 15 mL of cell resuspension per tube), scaling the amount of energy delivered as previously described[29,45].

2.5. Immediately after sonication is complete, add 2.8 µL of 1 M DTT (supplementing an additional 2 mM DTT) into the 1.4 mL of lysate and invert several times to mix. Place the tube on ice. Repeat steps 2.4 and 2.5 for any additional tubes of resuspended cells before proceeding to centrifugation.

2.6. Microcentrifuge samples at 18,000 x g and 4 °C for 10 min (**Figure 3C**).

2.7. Pipette the supernatant into a new 1.5 mL microfuge tube. Do not disturb the pellet; it is preferable to leave some supernatant behind to maintain purity than to disrupt the pellet in efforts to maximize yield.

2.8. Incubate the supernatant from the previous step at 250 rpm and 37 °C for 60 min by taping the tubes to the shaking platform of the incubator (this is the runoff reaction).

2.9. Microcentrifuge samples at 10,000 x g and 4 °C for 10 min.

2.10. Remove the supernatant without disturbing the pellet and transfer it to a new tube. Create many 100 µL aliquots of extract for storage.

Note: The protocol can be paused here, and the extract can be flash frozen in liquid nitrogen and stored at –80 °C for up to a year until needed for CFPS reactions. At least 5 freeze-thaw cycles can be undergone without detriment to extract productivity (**Figure 4**).

## 3. Cell-Free Protein Synthesis Batch Format Reactions

3.1. Thaw Solutions A and B, DNA template, BL21(DE3) extract (if frozen), T7RNAP, and an aliquot of molecular grade water.

Note: CFPS reaction template can be found in the **Supplementary Information**. Solutions A and B recipes are provided in the **Supplementary Information** and correspond to specific concentrations for numerous reagents to support the PANOx-SP based energy system for CFPS. The role of each reagent and acceptable variation in these reagent concentrations that can support CFPS have been determined[50]. A T7RNAP purification protocol can be found in the Supplementary Information[51]. Supplemental T7RNAP can increase volumetric yields but is not necessary if T7RNAP is induced during cell growth. Plasmid DNA template (pJL1-sfGFP) can be prepared using a maxiprep kit with two washes using the wash buffer in the kit, followed by a post-processing DNA-cleanup using a PCR purification kit (**Figure 2B**). Linear DNA templates can also be used in CFPS reactions.

3.2. Label the necessary amount of microfuge tubes needed for CFPS reactions.

Note: Reactions can be performed in various vessel sizes, but a smaller vessel can decrease volumetric protein yields (**Figure 2C**). Scaling up a reaction in the same size vessel may also reduce volumetric yields, as a function of decreasing the oxygen exchange, due to a decrease in the surface area to volume ratio. When increasing reaction volume above 100 µL, it is recommended to use flat bottom well plates[31,37,52].

3.3. Add 2.2 µL of Solution A, 2.1 µL of Solution B, 5 µL of BL21*(DE3) extract, 0.24 µg of T7RNAP (16 µg/mL final concentration), 0.24 ng of DNA template (16 ng/mL final concentration), and water to bring the final volume to 15 µL.

Note: Vortex Solutions A and B frequently during reaction setup to avoid sedimentation of components and ensure that each reaction receives a homogenous aliquot of each solution. Avoid vortexing the extract, instead invert the tube to mix.

3.4. After all reagents have been added to the reaction, mix each tube by pipetting up and down or gently vortexing while ensuring that the final reaction mixture is combined into a single 15 µL bead at the bottom of the 1.5 mL microfuge tube.

3.5. Place each reaction into the 37 °C incubator without shaking for 4 h, or 30 °C overnight.

Note: Successful reactions can be qualitatively assessed visually based on the green color of the sfGFP product within the CFPS reaction mixture (**Figure 3D**). Expression of the protein of interest can also be confirmed by SDS-PAGE (**Supplemental Figure 2**).

4. **Quantification of the Reporter Protein, [sfGFP]**

4.1. Load 48 µL of 0.05 M HEPES, pH 8, into each well needed for quantification (usually performed in triplicate per reaction tube).

4.2. Remove reactions from incubator. Pipette up and down to mix each reaction, then transfer 2 µL of reaction into the 48 µL of 0.05 M HEPES, pH 8. Pipet up and down again in the well to mix.

4.3. Once all reactions are loaded and mixed, place the 96 well plate into the fluorometer and measure the sfGFP endpoint fluorescence.

Note: Excitation and emission wavelengths for sfGFP fluorescence quantification are 485 and 510, respectively.

4.4. Using a previously generated standard curve, determine the [sfGFP] from the obtained fluorescence readings.

Note: Instructions for generating a standard curve of sfGFP concentration versus fluorescence intensity are provided in Supplementary Information (**Supplementary Figure 3**). Users will need to establish a standard curve for their instrument since instrument sensitivity may vary.

**REPRESENTATIVE RESULTS:**

We have presented a sonication-based *E. coli* extract preparation protocol that can be completed over a four-day span, with **Figure 1** demonstrating the procedural breakdown over each day. There is malleability to the steps that can be completed in each day with various pausing points, but we have found this workflow to be the most effective to execute. Additionally, both the cell pellets (step 1.3.18) and fully prepared extract (step 2.10) are stable at -80 °C for at least a year, allowing the user to create larger stocks of each to save for use at a later time[17]. Not only is the extract stable over long time periods, but the extract can also undergo at least five freeze thaw cycles without a significant loss of productivity (**Figure 4**). This allows for larger aliquots of extract to be stored for multiple uses if freezer storage space is limited. However, we recommend multiple smaller aliquots (~100 µL) of extract whenever possible.

With every new extract preparation, we recommend that the user performs a magnesium titration in order to determine the optimal amount of magnesium for that batch of extract. Users can quantify batch-to-batch variability in total protein concentration of the cell extract by Bradford assay. For higher performing extracts, we typically see total protein concentrations of

30-50 mg/mL, and within this range there is no obvious correlation between total protein concentrations and cell extract performance. Therefore, we recommend that users tune magnesium concentrations accordingly to ensure that protein and nucleic acid functionality are maximized for each extract batch. Magnesium levels are important for proper DNA replication, transcription and translation, but excessive levels can be detrimental to these processes[53]. In order to demonstrate this dependency, we have performed a co-titration of magnesium and extract volume to determine the optimal combination that minimizes the amount of extract necessary, while maintaining a productive reaction (**Figure 5**). From this experiment, we recommend using 5 µL of extract and 10 mM $Mg^{2+}$ for extract with a total protein content of 30 mg/mL, in order to obtain over 1000 µg/mL of sfGFP.

Our experience with CFPS has also allowed us to determine steps within the protocol that can be varied without detriment to the overall productivity of the system, and others that are integral for a high performing CFPS system. Most notably, the final $OD_{600}$ of cell harvest does not significantly affect the final output of the CFPS reaction, and cells can feasibly be harvested anywhere from 2.7 - 4.0 $OD_{600}$. This represents the early exponential phase of growth where ribosome concentration per cell is the highest and the translational machinery is the most active to support rapid growth. This observation allows users flexibility to optimize their own procedures. We recommend harvesting at approximately 3.0 $OD_{600}$ in order to capture the cells at an $OD_{600}$ closer to 3.3 by the time harvesting is complete (**Figure 2A**). Variables that do impact CFPS yields include template DNA quality, reaction vessel size, and the relative quantities of cell extract and magnesium ion present in the reaction. We have found DNA quality to have notable batch-to-batch variation. In order to resolve this, we recommend that users purify DNA via a midi or maxi prep, followed by an additional DNA cleanup step either on the DNA purification column used in the maxiprep, or post-purification using an additional DNA cleanup kit. This improves the reproducibility in DNA quality for CFPS reactions and results in more robust protein production (**Figure 2B**). The reaction vessel also impacts volumetric yields, such that the protein production of identical reaction setups in varying vessel volumes can differ up to 40%. It has been theorized that the boost in in volumetric yield observed in larger vessels is due to an increased surface area of the reaction mixture, allowing for better oxygen exchange (**Figure 2C**), and others have further boosted volumetric yields by running CFPS reactions in large flat-bottom plates, which we recommend for reactions over 100 µL[17,31,37,52].

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Timeline for culture growth, production of cell extract, setup and quantification of CFPS reactions.** The user can implement the CFPS platform for their research applications through this four-day workflow. Reagent preparation represents the primary time and cost investment for the first round of this experiment and diminishes substantially after reagents stocks are established. Additionally, cell pellets and prepared cell extract can be stored for over a year at -80 °C, allowing the user to begin the timeline at various steps for faster results. The user can also pause at various steps to modify the timeline of this workflow.

**Figure 2: Modifiable conditions for CFPS and the effects on volumetric reaction yields. A.** Extract productivity comparison based upon harvesting BL21(DE3) cells at various $OD_{600}$ readings. Based on this plot, we recommend harvesting at an $OD_{600}$ of 3.3 to produce at least 1000 µg/mL of target protein. Reactions were performed at a 15 µL scale in 1.5 mL microfuge tubes. **B.** Comparison of two DNA maxiprep wash protocols with and without post-purification DNA-cleanups. pJL1-sfGFP plasmids underwent a maxiprep with one or two washes followed by a post-purification cleanup by PCR purification kit. To achieve ~900 µg/mL of protein expression, we suggest performing a post-purification DNA cleanup regardless of the number of maxiprep washes. Reactions were performed at a 15 µL scale in 1.5 mL microfuge tubes. **C.** 15 µL CFPS reactions performed in various vessels ranging from 2 mL to 0.6 mL microfuge tubes. "Neg" represents a negative control where no DNA template was added to the reaction. All error bars represent 1 standard deviation of three independent reactions for each condition, each of which was quantified in triplicate.

**Figure 3: Key procedural setups and outcomes for creating productive extract. A.** Proper setup of sonication ice water bath to ensure cooling of sample while heat is generated during sonication. **B.** 1.5 mL microfuge tube containing resuspended cell pellet pre (left) and post (right) sonication. The resulting lysate should display a darker hue compared to resuspended cell pellet. **C.** Proper separation of the supernatant and pellet of cell lysate after 18,000 x g centrifugation. **D.** CFPS reactions after 4 h of incubation at 37 °C. 1.5 mL microfuge tube on the right (successful reaction) shows visible fluorescence of the sfGFP reporter protein at ~900 µg/mL. The negative control tube on the left, lacking template DNA and simulating an unsuccessful reaction, displays a clear solution with no fluorescence.

**Figure 4: Change in protein expression over 5 freeze-thaw cycles for CFPS extract**. Extract prepared from the same growth underwent five freeze thaw cycles via liquid nitrogen flash freezing followed by thawing on ice. No significant changes in extract productivity for expressing sfGFP were seen over the five freeze-thaw cycles. Reactions were performed at a 15 µL scale in 1.5 mL microfuge tubes. "Neg" represents a negative control where no DNA template was added to the reaction. All error bars represent 1 standard deviation of three independent reactions for each condition, each of which was quantified in triplicate.

**Figure 5: CFPS for reactions with varying [$Mg^{2+}$] and extract volumes versus [sfGFP]**. [$Mg^{2+}$] ranged from 8 mM to 14 mM with 2 mM increments and extract volumes ranged from 3 µL to 7 µL with 1 µL increments. The color code represents [sfGFP] produced from high (red) to low (purple). To maximize reagent efficiency while maintaining high protein production, we recommend using 5 µL of extract and 10 mM $Mg^{2+}$ for extracts that have a total protein content of ~30 mg/mL, as determined by Bradford assay. Original points to generate the contour plot were based off endpoint fluorescence of three independent reactions for each condition, each of which was measured in triplicate. Reactions were performed at a 15 µL scale in 1.5 mL microfuge tubes.

**Supplementary Figure 1: Cost per microgram of protein produced and per microliter of reaction across six cell-free protein synthesis platforms.** Our platform is compared to five different cell

free protein synthesis kits/platforms with varying productivity and pricing. Our sonication based CFPS platform is more cost-effective in both $/μg of protein and $/μL of reaction than most commercial kits and provides the ease of a kit for reaction setup, while remaining cost comparable to other academic CFPS platforms.

**Supplementary Figure 2: SDS-PAGE of sfGFP expression in CFPS.** Cell-free protein synthesis reactions with (+ DNA) and without (- DNA) DNA template for sfGFP were run on a 12% SDSPAGE gel to demonstrate the expression of sfGFP observed at 27 kDa (black arrow). Traditional SDS-PAGE techniques were used. Each sample loaded onto the gel included 18 μg of total protein based on Bradford assay quantification of total protein in the cell extract. Based on fluorescence intensity measurements and our standard curve, we estimate that the "+ DNA" lane contains 0.42 μg of sfGFP. In order to obtain these samples, CFPS reactions were run at a 15 μL scale in 1.5 mL microfuge tubes producing volumetric yields consistent with **Figure 3C**.

**Supplementary Figure 3: Standard curve for sfGFP on Cytation 5.** This curve was determined using the methods outlined above. All data collected for this manuscript was converted from endpoint fluorescence readings to [sfGFP] in μg/mL using this standard curve.

**DISCUSSION:**

Cell-free protein synthesis has emerged as a powerful and enabling technology for a variety of applications ranging from biomanufacturing to rapid prototyping of biochemical systems. The breadth of applications is supported by the capacity to monitor, manipulate, and augment cellular machinery in real-time. In spite of the expanding impact of this platform technology, broad adaptation has remained slow due to technical nuances in the implementation of the methods. Through this effort, we aim to provide simplicity and clarity for establishing this technology in new labs. Toward this end, our protocol for an *E. coli*-based cell-free protein synthesis platform can be achieved within a startup time of four-days by laboratory trained non-experts, such as undergraduate students (**Figure 1**). Additionally, once a stock of reagents and extract are produced, subsequent CFPS batch reactions can be set up, incubated, and quantified in just 5 h. A single, 1 L cell growth can result in enough extract for four hundred 15 μL CFPS reactions, while single batch preparations of the other cell-free reagents can support thousands of reactions. Reagent preparations can also be scaled up if an even larger stock is needed. The CFPS reactions can be setup in a high-throughput manner, by using a 96-well plate or PCR tubes for testing of a variety of conditions in parallel. Volumetric yields will decrease when using smaller vessels as seen in **Figure 2C**. CFPS reactions can also be scaled up from microliters to tens of milliliters of total reaction volume in order to increase the total protein yield for a single condition. When scaling up volume, the primary consideration is that volumetric reaction yields decrease as the surface area-to-volume ratio of the reaction decreases[37,52]. In order to scale-up while maintaining similar volumetric yields of protein expression, users should split the reaction volume into numerous reaction vessels and/or increase vessel size. For reaction scales ranging from 15 μL – 100 μL in volume, numerous 15 μL reactions in parallel are recommended. For reactions exceeding 100 μL in volume, flat-bottom 24-well plates are recommended, and 12-well plates are recommended for reaction volumes exceeding 600 μL. Such pairings of reaction volumes and vessels provide consistency in volumetric reaction yields upon scale-up[17,31,37,52].

Scaling up beyond these volumes can be accomplished by utilizing multiple wells of the plate in parallel. Using this format, the reaction can be scaled to over 10 mL total volume. Optimizing the reaction volume-reaction vessel combination can support applications of biomanufacturing without sacrificing the productivity of the reaction.

When performing this protocol, there are a few key considerations that impact volumetric reaction yields as well as indicators associated with poorly performing extract. In order to ensure proper lysis and to prevent denaturation of functional transcription/translation machinery, it is important to mitigate the heat produced during lysis. Immerse the cell resuspension in an ice water bath during sonication to rapidly dissipate heat during sonication (**Figure 3A**). An indicator of effective cell lysis is the emergence of a darker appearance of the cell lysate compared to pre-sonicated samples (**Figure 3B**). For user flexibility, the sonicator and probe shown in **Figure 3A** is adaptable to a range of volumes from 100 µL to 15 mL of resuspended cells. To accomplish this, the user can adjust the number of joules delivered for lysis of the desired volume of cells. Additionally, larger volumes of extract can be prepared through two complementary approaches. Users can sonicate multiple tubes in parallel, and/or sonicate larger volumes of cell resuspension, scaling the amount of energy proportionally with the volume as previously described[29,45]. Another step that indicates extract quality is the centrifugation step following cell lysis. Post cell lysis, we recommend centrifugation at 18,000 x g to provide a clear division between the supernatant (transcription/translation machinery, fragmented genomic DNA which no longer functions to template transcription/translation) and the pellet (undesired cellular components such as the cell membrane and precipitated proteins) (**Figure 3C**). We have found that centrifugation at 18,000 x g improves separation, resulting in improved reproducibility compared to spins at lower speeds such as 12,000 x g. For convenience, we recommend using a table-top refrigerated centrifuge, capable of achieving a minimum of 12,000 x g. This step is also commonly performed at 30,000 x g, which should be considered if the appropriate equipment is available[54–60]. Extract performance is not affected by centrifugation speeds at this step given that proper separation is achieved. When removing the desired supernatant, it is best to avoid any cloudy materials that exist at the boundary between the supernatant and pellet since this contamination will reduce the productivity of the extract. Aiming for purity of the supernatant results in more productive extracts and is worth the reduced quantity of extract obtained for new users.

It is important to note that while the methods we have presented are reproducible and can be executed by scientists with minimal expertise, there can be batch-to-batch and reaction-to-reaction variation. This may be attributed to variation in the proteomic composition of the lysate post-sonication[61]. The batch-to-batch variability that we have observed is generally diminished upon supplementation with T7RNAP and optimization of magnesium concentrations. Exogenous addition of T7RNAP is common among CFPS reactions to support optimal protein expression, and we find that having two sources of T7RNAP – endogenous expression in BL21*(DE3) and the supplemental T7RNAP to a final concentration of 16 µg/mL – improves batch-to-batch reproducibility for new users[45,46]. With experience, users can modify their experiments to utilize only a single source of T7RNAP if desired. Quantification of total protein content of a new batch of extract and appropriate adjustment of $Mg^{2+}$ concentration may also help to diminish batch-to-batch variation in volumetric protein expression yields. Variations in protein expression can also

be due to differences in the size and structure of the protein of interest, the codon usage of the gene and its corresponding ribosome binding site of the gene of interest, as well as the type of expression vector used[62,63]. For these reasons, some proteins may not express as well as the model protein sfGFP, resulting in reduced volumetric yields from CFPS reactions.

Limitations of the presented CFPS technique include that it may not be directly suitable to all applications of cell-free, such as metabolic engineering and tuning of expression conditions, without additional modifications to the protocols. However, we believe that this protocol will provide a basis for establishing the CFPS platform in new laboratories and provide non-experts with the ability to implement introductory cell-free reactions in their labs. After initial implementation, researchers can experiment with the platform to make their own modifications for more specific applications based on other literature in the field.

The CFPS platform costs $0.021/µg protein (excluding the cost of labor and equipment), making our system competitively priced with commercial kits without compromising ease of reaction setup. Assessments of comparative costs per µL of reaction show similar trends (**Supplemental Figure 1**). We estimate startup costs to be ~$4,500 for all reagents, and an additional $3,200 for specialized equipment, such as a sonicator. Person hours to complete this procedure are estimated to be ~26 h for all reagent prep from the ground up. However, once large stocks of reagents have been prepared, demands on labor diminish substantially. Additionally, as experience with the platform is gained, we recommend scaling up the size of the cell growth, extract preparation, and reagent preparation to maximize time efficiency. Given the startup costs, we recommend the CFPS platform for applications in synthetic biology, high-throughput efforts, and protein expression conditions that are incompatible with traditional protein expression platforms due to conflict with the cell's biochemistry and viability constraints. In these specialized cases where the desired technique is enabled by the CFPS platform, the greater cost of CFPS over *in vivo* expression is justified.

Continued development of the CFPS platform is likely to provide broader utility to biotechnology efforts such as the metabolic engineering of enzymatic pathways, production and characterization of traditionally intractable proteins, nonstandard amino acid incorporation and unnatural protein expression, stratified medicine manufacturing, and expanding beyond the laboratory into the classroom for STEM education[64–66]. These efforts will be further supported by the ongoing efforts for detailed characterization of the CFPS platform. A better understanding of the composition of the cell extract will lead to continued refinement toward improved reaction yields and flexibility in reaction conditions[61,67,68].

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**DISCLOSURES:**

The authors declare that they have no competing financial interests or other conflicts of interest.

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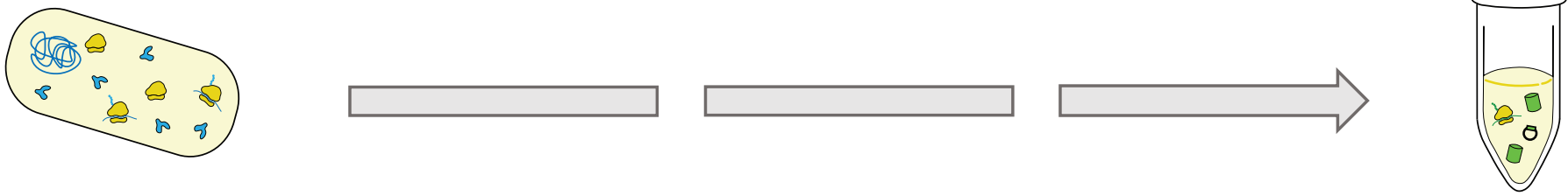


## Day One

Streak a plate with desired strain
**10 min, wait 18 h**
*Step 1.1*

Prepare LB for overnight culture (autoclave)
**2 h**
*Step 1.2*

## Day Two

Prepare 2x YPT and Glucose for 1 L growth (autoclave)
**2.5 h**
*Step 1.3 – 1.5*

Inoculate 50 mL LB overnight culture from plate
**10 min, wait 20 h**
*Steps 1.6 – 1.8*

## Day Three

Inoculate and grow 1 L to final OD of approximately 3.0
**3 h**
*Steps 1.9 – 1.15*

Pellet, wash, and freeze cells
**1.5 h**
*Steps 1.16 – 1.26*

## Day Four

Prepare extract
**2.5 h**
*Steps 2.1 – 2.10*

Set up CFPS batch reactions
**10 min per 4 rxns, wait 4 h**
*Steps 3.1 – 3.5*

Quantification of protein production
**5 min per 4 rxns**
*Steps 4.1 – 4.4*

These reagents must be prepped intermittently *(See Supplemental Info)*:

- Solutions A and B and their components
- DNA by maxi prep
- S30 buffer components

Figure 1

Click here to access/download;Figure;(1) timeline peer review.eps

Figure 2

Click here to access/download;Figure;(2) comp fig.eps

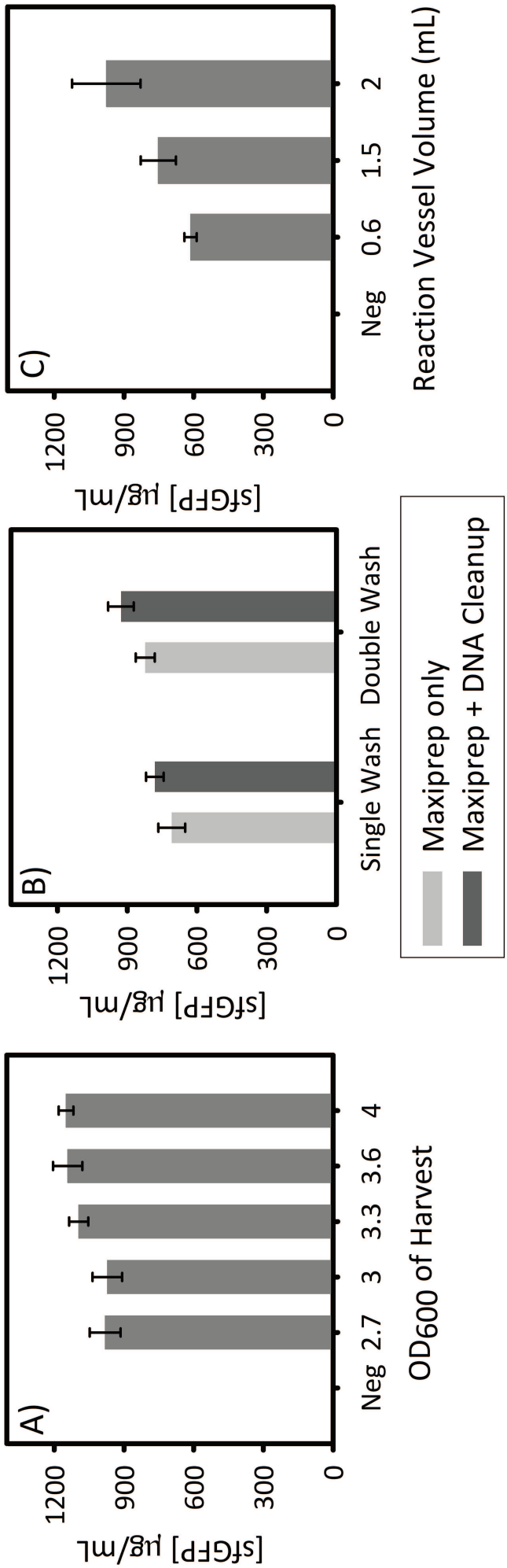


Figure 3



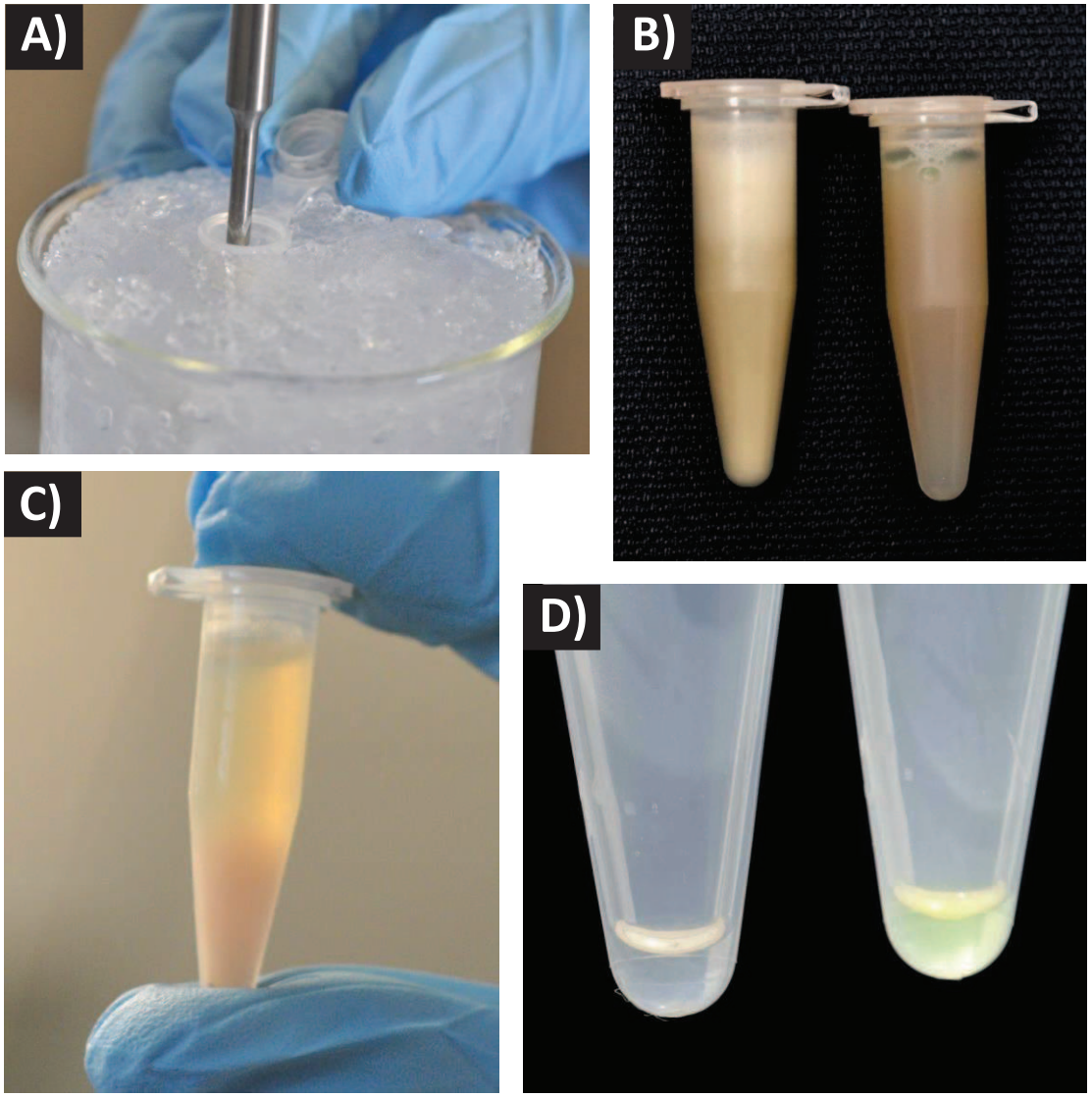


Figure 4



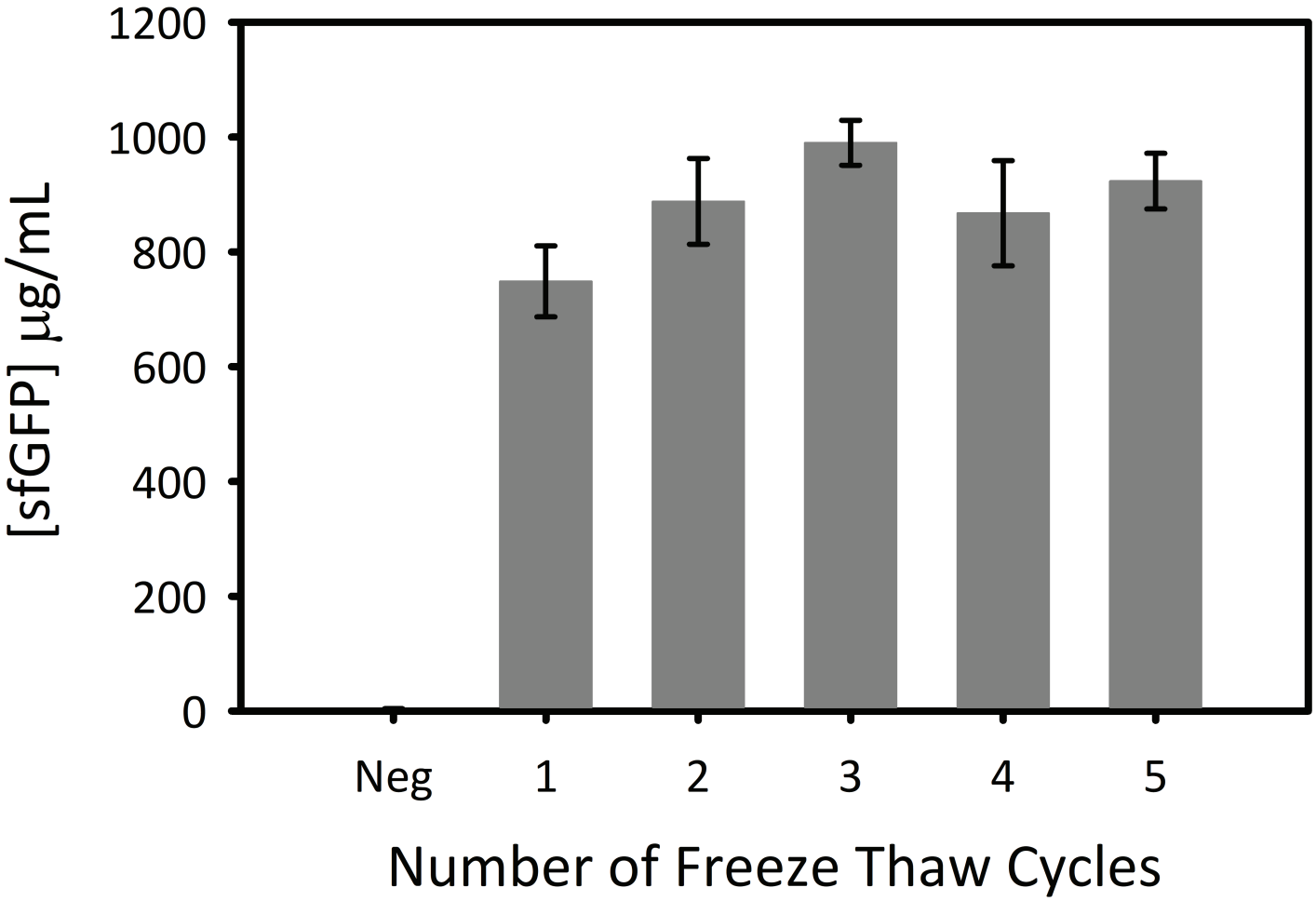
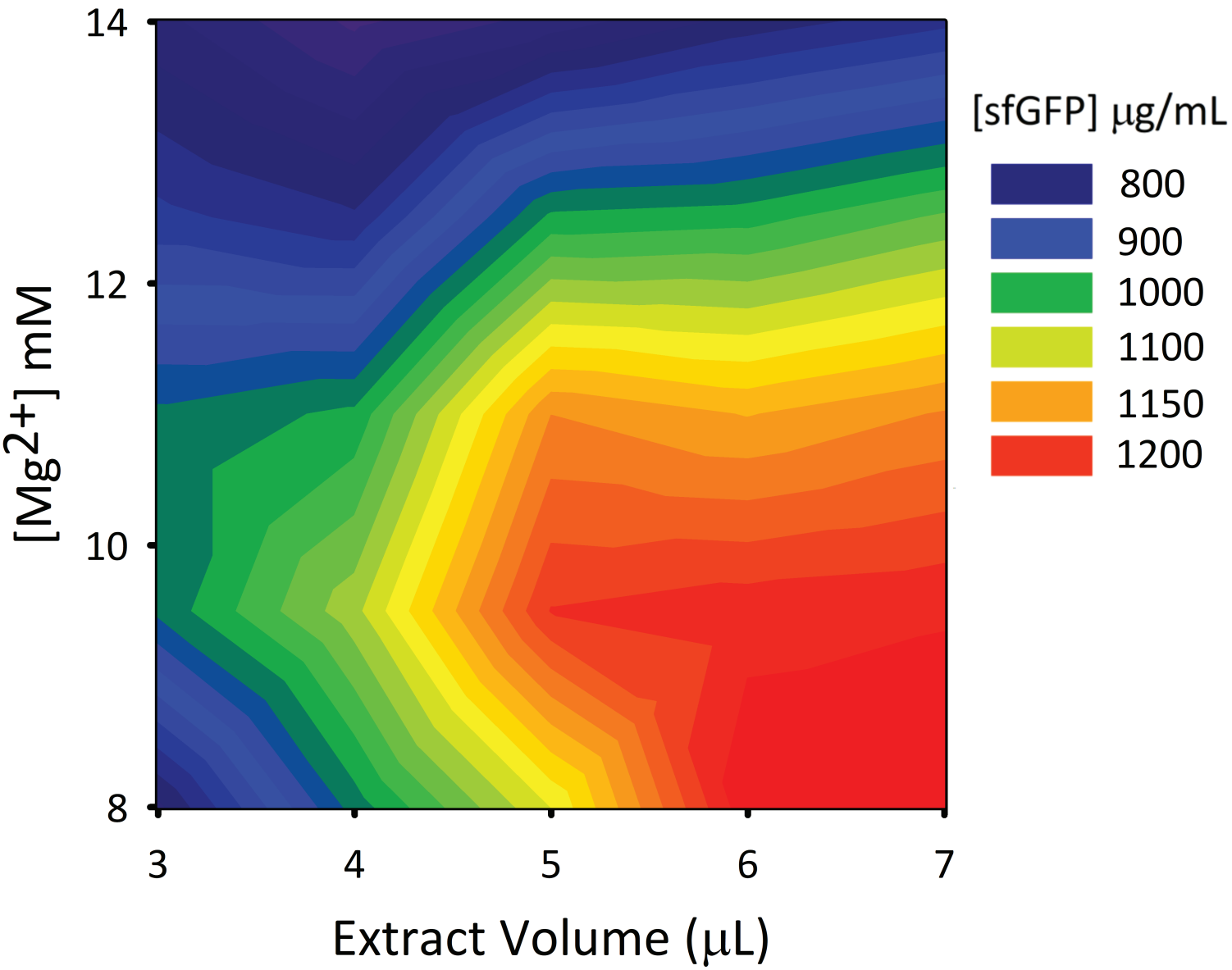


Figure 5

Click here to access/download;Figure;(5) contour mg extract.eps



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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Luria Broth | ThermoFisher | 12795027 | |
| Tryptone | Fisher Bioreagents | 73049-73-7 | |
| Yeast Extract | Fisher Bioreagents | 1/2/8013 | |
| NaCl | Sigma-Aldrich | S3014-1KG | |
| Potassium Phosphate Dibasic | Sigma-Aldrich | 60353-250G | |
| Potassium Phosphate Monobasic | Sigma-Aldrich | P9791-500G | |
| D-Glucose | Sigma-Aldrich | G8270-1KG | |
| KOH | Sigma-Aldrich | P5958-500G | |
| IPTG | Sigma-Aldrich | I6758-1G | |
| Mg(OAc)2 | Sigma-Aldrich | M5661-250G | |
| K(OAc) | Sigma-Aldrich | P1190-1KG | |
| Tris(OAc) | Sigma-Aldrich | T6066-500G | |
| DTT | ThermoFisher | 15508013 | |
| tRNA | Sigma-Aldrich | 10109541001 | |
| Folinic Acid | Sigma-Aldrich | F7878-100MG | |
| NTPs | ThermoFisher | R0481 | |
| Oxalic Acid | Sigma-Aldrich | P0963-100G | |
| NAD | Sigma-Aldrich | N8535-15VL | |
| CoA | Sigma-Aldrich | C3144-25MG | |
| PEP | Sigma-Aldrich | 860077-250MG | |
| K(Glu) | Sigma-Aldrich | G1501-500G | |
| NH4(Glu) | MP Biomedicals | 02180595.1 | |
| Mg(Glu)2 | Sigma-Aldrich | 49605-250G | |
| Spermidine | Sigma-Aldrich | S0266-5G | |
| Putrescine | Sigma-Aldrich | D13208-25G | |
| HEPES | ThermoFisher | 11344041 | |
| Molecular Grade Water | Sigma-Aldrich | 7732-18-5 | |
| L-Aspartic Acid | Sigma-Aldrich | A7219-100G | |
| L-Valine | Sigma-Aldrich | V0500-25G | |
| L-Tryptophan | Sigma-Aldrich | T0254-25G | |
| L-Phenylalanine | Sigma-Aldrich | P2126-100G | |
| L-Isoleucine | Sigma-Aldrich | I2752-25G | |

| | | | |
|---|---|---|---|
| L-Leucine | Sigma-Aldrich | L8000-25G | |
| L-Cysteine | Sigma-Aldrich | C7352-25G | |
| L-Methionine | Sigma-Aldrich | M9625-25G | |
| L-Alanine | Sigma-Aldrich | A7627-100G | |
| L-Arginine | Sigma-Aldrich | A8094-25G | |
| L-Asparagine | Sigma-Aldrich | A0884-25G | |
| Glycine | Sigma-Aldrich | G7126-100G | |
| L-Glutamine | Sigma-Aldrich | G3126-250G | |
| L-Histadine | Sigma-Aldrich | H8000-25G | |
| L-Lysine | Sigma-Aldrich | L5501-25G | |
| L-Proline | Sigma-Aldrich | P0380-100G | |
| L-Serine | Sigma-Aldrich | S4500-100G | |
| L-Threonine | Sigma-Aldrich | T8625-25G | |
| L-Tyrosine | Sigma-Aldrich | T3754-100G | |
| Fisherbrand Premium Microcentrifuge Tubes: 2.0 mL | Fisher Scientific | 05-408-138 | |
| Fisherbrand Premium Microcentrifuge Tubes: 1.5 mL | Fisher Scientific | 05-408-129 | |
| Fisherbrand Premium Microcentrifuge Tubes: 0.6 mL | Fisher Scientific | 05-408-120 | |
| PureLink HiPure Plasmid Prep Kit | ThermoFisher | K210007 | |
| Ultrasonic Processor | QSonica | Q125-230V/50Hz | 3.175 mm diameter probe |
| Avanti J-E Centrifuge | Beckman Coulter | 369001 | |
| JLA-8.1000 Rotor | Beckman Coulter | 366754 | |
| 1L Centrifuge Tube | Beckman Coulter | A99028 | |
| Tunair 2.5L Baffeled Shake Flask | Sigma-Aldrich | Z710822 | |
| Microfuge 20 | Beckman Coulter | B30134 | |
| New Brunswick Innova 42/42R Incubator | Eppendorf | M1335-0000 | |
| Cytation 5 | BioTek | | |
| Strep-Tactin XT Starter Kit | IBA | 2-4998-000 | |
| pJL1-sfGFP | Addgene | 69496 | |
| | | | |
| BL21(DE3) | New England BioLabs | | |

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Title of Article: Escherichia Coli-based Cell-free Protein Synthesis: Protocols for a robust, flexible, and accessible platform technology.

Author(s): Max Z. Levine, Nicole E. Gregorio, Michael C. Jewett, Katharine R. Watts, Javin P. Oza.

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**CORRESPONDING AUTHOR**

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| Institution: | California Polytechnic State University, San Luis Obispo |
| Title: | Assistant Professor |
| Signature: |  |
| Date: | 7/31/18 |

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College of Science and Mathematics
*Chemistry & Biochemistry Department*

September 6, 2018

Journal of Visualized Experiments
1 Alewife Center, Suite 200
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Dear Dr. Phillip Steindel,

Re: JoVE58882 "Escherichia coli-based cell-free protein synthesis: protocols for a robust, flexible, and accessible platform technology".

We appreciate the thorough review of our manuscript and we thank you and the reviewers for providing insightful and constructive comments. Based on the reviewer comments, we have made several changes to the text and figures in the manuscript and conducted the additional experiments that the reviewers requested. We believe that the reviewer's concerns are now directly addressed and that their suggestions have greatly improved the clarity and quality of the manuscript. We feel that our strengthened manuscript will make an essential and unique contribution *JoVE's* broad readership. Specifically, as reviewer #1 has pointed out in their comments, cell-free protein synthesis has not been a traditionally accessible biotechnology for non-experts. Our improved manuscript combined with the visualization of the experimentation through *JoVE* will enable us to breakdown this barrier. Therefore, we believe that *JoVE* is the best outlet for maximizing the impact of this work as we aim to democratize the cell-free protein synthesis platform.

Below, we provide detailed responses and revisions to the manuscript on a point-by-point basis. We thank both you and the reviewers for your time and effort. We look forward to hearing from you in due course.

Sincerely,

Javin P. Oza, Ph.D.
joza@calpoly.edu
Assistant Professor
Chemistry & Biochemistry Department
California Polytechnic State University, SLO

**Response to reviewer's comments for JoVE58882 "Escherichia coli-based cell-free protein synthesis: protocols for a robust, flexible, and accessible platform technology"**

The reviewers' comments are *italicized* and our responses follow. New or modified text in the manuscript is also provided here in green to ensure that the reviewers' comments are addressed.

**Summary**

Below is our point-by-point response to the reviewers' comments. Overall, the reviewers' comments were helpful and implementing the recommended changes has made our manuscript stronger. Specifically, we have re-written or added text as to address the reviewer's comments and to clarify their concerns. We also carried out the requested follow-up experiments, and have updated figures accordingly.

**Reviewers' comments:**

***Reviewer #1:*** *Major Concerns:*

*In general, I have problems with two particular issues. First, the system is propagated as being highly productive, giving yields of 900 µg/mL. However, the protocol is described for 15 µl total volume and it is shown that larger reaction vessels have a strong negative effect on expression efficiency.*

**Response:** This appears to be a misinterpretation of our data. We have improved the layout of Figure 2C to resolve this issue. Consistent with literature, our data show that expression efficiency increases with larger reaction vessels at a constant reaction volume.

***Reviewer #1:*** *Applications such as the mentioned biomanufacturing or protein characterization are to my opinion out of scope of such systems. 96well microplates and similar are for screening purpose and the batch system may be excellent for that application. However, if one wants to isolate larger amounts of purified proteins, e.g. for structural or biochemical characterization, reaction volumes of 1 mL or higher are usually desired. The introduction is therefore misleading and the real applications, but also limitations, of the propagated system should be better emphasized.*

**Response:**

In order to ensure the reader is not misled about the applications and limitations of our methods, we have modified the language within the introduction to state the following:

"The CFPS methods described here enable the user to directly implement a variety of applications, including functional genomics, high-throughput testing, the production of proteins that are intractable for *in vivo* expression, as well as field applications including biosensors and educational kits for synthetic biology. Additional applications such as metabolic engineering, tuning of protein expression conditions, disease detection, and scale-up using CECF or CFCF methods are still possible but may require experience with the CFPS platform for further modification of reaction conditions."

Regarding the capacity to biomanufacturer larger quantities of protein, our experience, which is consistent with the literature, does not match the reviewer's opinion that such applications are 'out of scope of such systems'. These authors have successfully scaled the CFPS reaction to a mL scale, and have used this platform for the characterization of protein function. This can be accomplished without modification to the extract preparation procedure or CFPS reaction setup as described in this manuscript. Primary modifications for scaling up involve the volume of the reaction and selection of the reaction vessels. In order to reduce the threshold for new users to adopt the CFPS platform in their labs and classrooms, this manuscript is focused on the details of cell extract preparation and reaction set-up at a 15 uL scale. Details for scaling up the reaction are well characterized in literature, and we have provided the relevant references for users interested in such applications. To ensure clarity of the manuscript and utility for the reader, we also have added additional text regarding vessel selection for reaction volume scale up. If the reviewer prefers, demonstration of a scaled-up reaction setup can be included in the video production accompanying this manuscript.

"CFPS reactions can also be scaled up from microliters to tens of milliliters of total reaction volume in order to increase the total protein yield for a single condition. When scaling up volume, the primary consideration is that volumetric reaction yields decrease as the surface area-to-volume ratio of the reaction decreases.[37, 52] In order to scale-up while maintaining similar volumetric yields of protein expression, users should 1) split the reaction volume into numerous reaction vessels and/or 2) increase vessel size. For reaction scales ranging from 15 µL – 100 µL in volume, numerous 15 µL reactions in parallel are recommended. For reactions exceeding 100 µL in volume, flat-bottom 24-well plates are recommended, and 12-well plates are recommended for reaction volumes exceeding 600 µL. Such pairings of reaction volumes and vessels provide consistency in volumetric reaction yields upon scale-up.[17, 31, 37, 52] Scaling up beyond these volumes can be accomplished by utilizing multiple wells of the plate in parallel. Using this format, the reaction can be scaled to over 10 mL total volume. Optimizing the reaction volume-reaction vessel combination can support applications of biomanufacturing without sacrificing the productivity of the reaction."

***Reviewer #1:*** *Furthermore, do not mix up concentrations and yields.*

**Response:** We agree with the reviewer that *µg/ml* is a unit of concentration, however we had listed this as 'yield' to remain consistent with the academic literature and commercial products for the benefit for the reader. In order to improve clarity while remaining consistent, we have replaced all occurrences of "yield" with "volumetric yield" within the text.

***Reviewer #1:*** *The given 900 µg/mL is only a concentration obtained in 15 µl, but it is questionable whether it can be obtained really as yield in higher volumes.*

***Reviewer #1:*** *125: The protocol can be scaled up. Please give examples or a range of reaction volume scales and make a clear statement which GFP concentration are routinely obtained in higher reaction volumes.*

***Reviewer #1:*** *304: Yields decrease in smaller vessels, comment on increase in larger volumes.*

***Reviewer #1:*** *408: reaction vessel size is given as major impact on production efficiency.*

**Response:** Our experience along with that of others reported in literature has demonstrated that the volumetric yield of the CFPS reaction obtained in 15 µl can in fact be obtained in higher volumes. These include references #9, 30, 36, 51. While the reviewer's experience may vary from this, we would like to point out that the scope of this manuscript is to reduce the barrier for new users to establishing CFPS in their laboratories; once they are able to implement the reaction at the 15 µl, they will have the foundation to experiment with reaction scale-ups as characterized in literature. We agree that this is important to clarify for the reader since increasing the reaction volume in a fixed vessel size will result in diminishing volumetric yields. To further reduce the barrier for new users to scale-up the reaction, we have added the following text:

"CFPS reactions can also be scaled up from microliters to tens of milliliters of total reaction volume in order to increase the total protein yield for a single condition. When scaling up volume, the primary consideration is that volumetric reaction yields decrease as the surface area-to-volume ratio of the reaction decreases. In order to scale up while maintaining similar volumetric yields of protein expression, users should 1) split the reaction volume into numerous reaction vessels or 2) increase vessel size. For reaction scales ranging from 15 µL – 100 µL in volume, numerous 15 µL reactions in parallel are recommended. For reactions exceeding 100 µL in volume flat-bottom 24-well plates and are recommended, and 12-well plates for reaction volumes exceeding 600 µL in order to maintain robust volumetric reaction yields. Scaling up beyond these volumes can be accomplished by utilizing multiple wells of the plate in parallel. Using this format, the reaction can be scaled to over 10 mL total volume. Optimizing the reaction volume-reaction vessel combination can support applications of biomanufacturing without sacrificing the productivity of the reaction."

***Reviewer #1:*** *Second, the system is propagated even for non-experts and being cost-effective. Working now quite some time in that field, the most complains I heard at conferences was that CFPS is quite tricky and very expensive. Regardless which system was used. So I would refrain from selling it as cheap and easy, as this is simply not the reality.*

**Response:** The reviewer has identified a significant challenge in the broad dissemination of the CFPS platform in spite of broad interest within the field. In fact, this is the challenge we have sought to overcome with this manuscript, and we are confident that visualizing the procedures through *JoVE* will help address many of the complaints the reviewer has heard at conferences.

We stand by our claim that the approach presented herein is accessible to non-expert users (given basic laboratory techniques). We have established this confidence over two years of optimizing and implementing these procedures at a primarily undergraduate institution. Over 6 novice students who had less than 3 months of laboratory experience have successfully generated *E. coli* based cell extracts for CFPS in this time. In fact, the lead authors of this manuscript include a master's student and an undergraduate student who started as a Freshman, both of whom successfully generated cell extracts within weeks-to-months of joining in the lab and have collected all data shown within the manuscript. They were able to accomplish this with little more than our lab's written procedures and demonstrations of basic methods required for the implementation of these procedures. Making CFPS accessible through broad dissemination of our process is exactly what this manuscript and its accompanying video through *JoVE* will accomplish.

***Reviewer #1:*** *For standard proteins, CFPS is clearly not competitive to conventional E. coli expression. It is a niche system with benefits for difficult proteins, labelling or many new special applications.*

**Response:** We agree with the reviewer that CFPS is a niche system that can be useful when *E. coli* expression is not sufficient for desired applications. To clarify this for users, we have added the

following text:

"Given the startup costs, we recommend the CFPS platform for applications in synthetic biology, high-throughput efforts, and protein expression conditions that are incompatible with traditional protein expression platforms due to conflict with the cell's biochemistry and viability constraints. In these specialized cases where the desired technique is enabled by the CFPS platform, the greater cost of CFPS over *in vivo* expression is justified."

***Reviewer #1:*** *For the cost calculations, the price is given based on µg synthesized GFP, probably synthesized in 15 ml volumes in optimized vessels. This makes no sense. GFP is super-expressed and most other targets from users will express at significant lower levels. The calculation is therefore misleading and should be rather given for one µl or mL of reaction in order to become independent of expression efficiency and reaction volume. 489: Please compare price per reaction volume.*

**Response:** We apologize that the context for our cost calculation was not clear enough to make sense to the reviewer. We have calculated the cost of the reaction per *µl* as requested, and we agree that this may have utility to many users in deciding to pursue the CFPS platform. Supplemental Figure 1 now includes the updated values. We have concurrently retained the $/*µg* along with the *$/µL* calculation as both metrics match the cost analysis in prior literature, and evaluation of cost using both metrics will be useful for the readers to compare academic platforms and commercial products as they decide to implement the CFPS platform.

Furthermore, the reviewer's comment regarding variation in protein expression levels is an important and unresolved issue in every expression platform. In order to provide clarity on this issue, to adjust user expectation, and importantly – to not mislead the users, we have added additional language within the main text:

"Variations in protein expression can also be due to differences in the size and structure of the protein of interest, the codon usage of the gene and its corresponding ribosome binding site of the gene of interest, as well as the type of expression vector used.[62, 63] For these reasons, some proteins may not express as well as the model protein sfGFP, resulting in reduced volumetric yields from CFPS reactions."

***Reviewer #1:*** *Specific comments:*

*44, 56: highly productive, relative to what?*

**Response**: We agree that this is ambiguous. We have removed the use of the phrase "highly productive" from the text.

***Reviewer #1:*** *59: 900 µg/mL or more, concentration, but not amount*

**Response**: We agree. We have modified the language to reflect that this is a "volumetric yield".

***Reviewer #1:*** *cost-effective? Compared to what?*

**Response**: Relative to commercially available kits that many readers may be considering or using. We have clarified this within the main text:

"This approach is cost-effective compared to commercially available kits without sacrificing the ease of kit-based reaction setup"

***Reviewer #1:*** *110: 900µg yield in which volume?*

**Response:** in 15 µl. We have clarified this throughout the manuscript also.

***Reviewer #1:*** *111: cost should be better given for 1 µl or mL of reaction!*

**Response:** We now provide cost per volume in Supplemental Figure 1 and in the main text when we discuss costs.

***Reviewer #1:*** *114: non experts? Perhaps the authors mean to use an established protocol for GFP expression and finally pipet anything together can be done by non-experts. Potentially true for any technique, but the strength of CFPS is rather to modify it for new targets. I would say to produce a new protein in acceptable quality and stability by tuning the system accordingly clearly requires some deeper expert know how. Best to delete this sentence.*

**Response:** We agree that the capacity to modify the platform is one of the advantages of CFPS, and modifying the platform for specific applications requires experience and expertise. We have clarified this in the main text as follows:

"Additional applications such as metabolic engineering, tuning of protein expression conditions, disease detection, and scale-up using CECF or CFCF methods are still possible but may require experience with the CFPS platform for further modification of reaction conditions."

Regarding the experience-level required to implement CFPS, the authors' intent is as stated – to enable non-expert users to adpt the entirety of the protocol described in this manuscript, not simply the final pipetting for GFP expression. We have validated this to be true with Freshmen and Sophomore level undergraduate students who started with little-to-no laboratory experience. We are confident that this manuscript and its accompanying video will enable users at a college level and up to establish the CFPS platform in their labs and classrooms. After initial implementation, these users will have some experience with the platform and can refer to other literature for guidance and begin experimenting with advanced methods, reaction setup and conditions.

***Reviewer #1:*** *141: Indicate concentration of D-glucose.*

**Response:** The text has been clarified to include this detail:

"Prepare 750 mL of 2x YTP media and 250 mL of 0.4 M D-Glucose solution as described in Supplemental Information."

***Reviewer #1:*** *185: Please state what you induce with IPTG. If it is the T7RNAP, please make a comment why you still have to add additional polymerase at line 303. Is induction or using a DE3 strain necessary at all if purified T7RNAP has to be added later anyhow?*

**Response:** The reviewer is correct in identifying that the IPTG induction is for T7RNAP, we observe that supplementing T7RNAP at the recommended concentration helps ensure consistency for *in vitro* transcription and subsequent translation at the concentration of DNA template we recommend. The user can modify their system to utilize only a single source of T7RNAP if they choose. We have added the following clarifying text:

"Note: Induction by IPTG is for endogenous production of T7 RNA Polymerase (T7RNAP)."
and
"Exogenous addition of T7RNAP is common among CFPS reactions to support optimal protein expression, and we find that having two sources of T7RNAP – endogenous expression in BL21*(DE3) and the supplemental T7RNAP to a final concentration of 16 μg/mL – improves batch-to-batch reproducibility for new users.[45, 46] With experience, users can modify their experiments to utilize only a single source of T7RNAP if desired."

***Reviewer #1:*** *263: 1.4 mL tubes, small scale extract preparation is given only. How can this be scaled up?*

**Response:** We have added the following clarifying text:

"Two complementary approaches can be used to scale-up the amount of extract prepared during this step: 1) multiple 1.5 mL microfuge tubes can be sonicated in parallel, and/or 2) larger volumes can be sonicated in conical tubes (up to 15 mL of cell resuspension per tube), scaling the amount of energy delivered as previously described.[29, 45]"

***Reviewer #1:*** *301: specify "aliquot" of T7RNAP*

**Response:** Language has been modified to reduce ambiguity.

***Reviewer #1:*** *416-419: Oxygen access is mentioned as being important*

*I would expect that this is either confusing a reader or it is overseen. The protocol should be very clear on that and should make very clear which production efficiency can be expected in which volume and vessel. Please also indicate in which reaction vessels the demonstrated expressions have been performed.*

**Response:** We appreciate this feedback, we have clarified the text and figures at numerous locations to indicate the reaction size and vessel that have been used, along with the vessels we recommend for specific volume ranges to maintain consistent volumetric yields of the reaction. .

***Reviewer #1:*** *325: Incubation with or without shaking?*

**Response:** Without shaking has been clarified within the protocol.

***Reviewer #1:*** *375: genomic DNA is not completely removed by this procedure as it is disintegrated into smaller pieces by the sonication process, which then stay in the supernatant. Please make the reader aware of this potential problem.*

**Response:** We agree that this is important to clarify for the reader. We have removed all language

that suggested that genomic DNA is removed, and we have indicated that fragmented genomic DNA remains.

***Reviewer #1:*** *Fig. 1: Please make a cost per µl/mL reaction calculation. Comparison with commercial kits is not very meaningful anyhow, as no labor costs, tax, company costs etc. are included. So better present comparisons with other published calculations of academic systems.*

**Response:** We have added the cost per reaction volume calculation. We also include cost comparison with an extract preparation method that is the most different from the one we present. We respectfully disagree about the meaningfulness of the comparison with commercial kits. We posit that cost comparison to commercial kits may be useful for many users who are deciding the most accessible path toward utilizing CFPS in their labs based on their funding situation, which is generally independent of a company's costs and profit margins. We agree that our calculations do not include labor since the actual cost of labor varies by position and salary standards, which differ greatly. In lieu of including cost of labor, we have identified that the initial startup of the platform presented in this manuscript requires ~26 hours of time. Our timeline (Figure 1) gives more details on the specific time required for any particular task in the methods. Users can calculate their daily wage rates and can add this to the costs we present in order to evaluate which CFPS procedure or commercial kit is most effective for their time/cost constraints.

***Reviewer #1:*** *Fig. 3D: Please show a Coomassie stained SDS-PAGE with indicated volumes of applied reaction for better illustration of GFP production. This is more reliable and a widely accepted standard for demonstrating expression yields and the reader can better draw his/her own conclusion. To my opinion, the shown faint green color does hardly represents 1mg/mL GFP concentration. As mentioned in the text, calibration curves and spectrometer parameters may largely influence the concentration determination.*

**Response:** We have included a Coomasie stained SDS-PAGE of GFP production in CFPS in the supplemental information (Supplemental Figure 2) along with details on the quantities loaded. We apologize for the quality of the photograph demonstrating GFP expression, we agree that the green color was not captured effectively. We have retaken the photograph for this figure (see Figure 3D).

***Reviewer #1:*** *Fig. 6C: What is the geometry of the indicated reaction vessels? A significant decrease in yield depending on vessel volume is indicated and oxygen access is mention as important parameter.*

**Response:** We agree that this is an important consideration. We use standardized microfuge tubes from Fisher Scientific. We have provided catalog numbers to reduce ambiguity for the user in selecting the vessels. Users are able to find the dimensions of these standardized vessels within the manufacturer's documentation.

***Reviewer #1:*** *At the beginning, 96 well plates have been propagated for the described 15 µl volume reactions. As these hold only 300 µl, the maximum yield of GFP is then less than 500 mg/mL and not 900 µg(mL as always mentioned? Please show in addition the expression efficiency in reactions in same vessel but with increasing volumes up to 1 mL.*

**Response:** Yes, we discuss the utility of the 96-well plates for higher-throughput applications. To clarify, we do not recommend using 96-well plates for scaling up volume for biomanufacturing; the main text specifies that if the user intends to scale up the volume of the reaction, larger vessels should be utilized. Scaling of CFPS reactions to 1+ mL has been shown in previous publications which we reference. To assist the readers, we have also added the following text to provide guidance in executing larger volume reactions:

"When scaling up volume, the primary consideration is that volumetric reaction yields

decrease as the surface area-to-volume ratio of the reaction decreases.[37, 52] In order to scale-up while maintaining similar volumetric yields of protein expression, users should 1) split the reaction volume into numerous reaction vessels and/or 2) increase vessel size. For reaction scales ranging from 15 µL – 100 µL in volume, numerous 15 µL reactions in parallel are recommended. For reactions exceeding 100 µL in volume, flat-bottom 24-well plates are recommended, and 12-well plates are recommended for reaction volumes exceeding 600 µL. Such pairings of reaction volumes and vessels provide consistency in volumetric reaction yields upon scale-up.[17, 31, 37, 52] Scaling up beyond these volumes can be accomplished by utilizing multiple wells of the plate in parallel. Using this format, the reaction can be scaled to over 10 mL total volume."

***Reviewer #1:*** *376: More common are even higher g-forces of 30,000 x g. Please check the literature and comment on tha*

**Response:** We have modified the main text as follows:

"For convenience we recommend using a table-top refrigerated centrifuge, capable of achieving a minimum of 12,000 x g. This step is also commonly performed at 30,000 x g, which should be considered if the appropriate equipment is available.[54–60] "

***Reviewer #1:*** *Introduction/Discussion: A general introduction in the major variations of E. coli based CFPS is missing, while this would be very helpful for the reader to better evaluate the presented system. So please explain the batch versus two-compartment exchange configuration. What are the differences in applications?*

**Response:** We had not initially included commentary semicontinuous and continuous reaction configurations since we are presenting a methods article targeted toward new CFPS users and the two-compartment exchange configurations require more expertise and specialized equipment. However, we agree that readers unfamiliar with the CFPS literature may benefit from this scope. We have added the following text to the introduction:

"CFPS reactions can be run in either batch, continuous-exchange cell-free (CECF) or continuous-flow cell-free (CFCF) formats. The batch format is a closed system whose reaction lifetime is limited due to diminishing quantities of reactants and the accumulation of inhibitory byproducts of the reaction. CECF and CFCF methods increase the lifetime of the reaction, and thereby result in increased volumetric protein yields compared to the batch reaction. This is accomplished by allowing the byproducts of protein synthesis to be removed from the reaction vessel while new reactants are supplied throughout the course of the reaction.[2] In the case of CFCF, the protein of interest can also be removed from the reaction chamber, while in CECF, the protein of interest remains in the reaction chamber comprised of a semi-permeable membrane.[36, 37] These methods are especially valuable in overcoming poor volumetric yields of difficult-to-express proteins of interest.[38–43] The challenges in implementing the CECF and CFCF approaches are that 1) while they result in more efficient use of the biomachinery responsible for transcription and translation, they require notably larger quantities of reagents which increases overall cost and 2) they require more complex reaction setups and specialized equipment compared to the batch format.[44] In order to ensure accessibility for new users, the protocols described herein focus on the batch format at reaction volumes of 15 μL with specific recommendations for increasing the reaction volume to the milliliter scale."

***Reviewer #1:*** *Furthermore, numerous protocols on how to set-up E. coli based CFPS have been published. It would therefore be valuable for the paper to refer also to other protocols for preparation of similar E. coli extracts.*

**Response:** We have added numerous additional references. We are happy to add more if the reviewer feels that a specific paper is missing. Here is a list of references that have been added to the revised version of the manuscript:

Krinsky, N. *et al. A Simple and Rapid Method for Preparing a Cell-Free Bacterial Lysate for Protein Synthesis*. doi: 10.1371/journal.pone.0165137. (2016).

Pratt, J.M. *Transcription and Translation: A Practical Approach*. IRL Press. New York. (1984).

Kim, D.-M., Kigawa, T., Choi, C.-Y., Yokoyama, S. A Highly Efficient Cell-Free Protein Synthesis System from Escherichia coli. *European Journal of Biochemistry*. **239** (3), 881–886, doi: 10.1111/j.1432-1033.1996.0881u.x (1996).

Shin, J., Noireaux, V. Efficient cell-free expression with the endogenous E. Coli RNA polymerase and sigma factor 70. *Journal of Biological Engineering*. **4** (1), 8, doi: 10.1186/1754-1611-4-8 (2010).

Zubay, G. In Vitro Synthesis of Protein in Microbial Systems. *Annual Review of Genetics*. **7** (1), 267–287, doi: 10.1146/annurev.ge.07.120173.001411 (1973).

Kigawa, T. *et al.* Preparation of Escherichia coli cell extract for highly productive cell-free protein expression. *Journal of Structural and Functional Genomics*. **5** (1/2), 63–68, doi: 10.1023/B:JSFG.0000029204.57846.7d (2004).

Liu, D. V., Zawada, J.F., Swartz, J.R. Streamlining Escherichia Coli S30 Extract Preparation for Economical Cell-Free Protein Synthesis. *Biotechnology Progress*. **21** (2), 460–465, doi: 10.1021/bp049789y (2008).

Yang, W.C., Patel, K.G., Wong, H.E., Swartz, J.R. Simplifying and streamlining Escherichia coli-based cell-free protein synthesis. *Biotechnology Progress*. **28** (2), 413–420, doi: 10.1002/btpr.1509 (2012).

Chekulayeva, M.N., Kurnasov, O. V., Shirokov, V.A., Spirin, A.S. Continuous-Exchange Cell-Free Protein-Synthesizing System: Synthesis of HIV-1 Antigen Nef. *Biochemical and Biophysical Research Communications*. **280** (3), 914–917, doi: 10.1006/BBRC.2000.4188 (2001).

Hong, S.H. *et al.* Improving Cell-Free Protein Synthesis through Genome Engineering of *Escherichia coli* Lacking Release Factor 1. *ChemBioChem*. **16** (5), 844–853, doi: 10.1002/cbic.201402708 (2015).

Endo, Y., Otsuzuki, S., Ito, K., Miura, K. Production of an enzymatic active protein using a continuous flow cell-free translation system. *Journal of Biotechnology*. **25** (3), 221–230, doi: 10.1016/0168-1656(92)90157-5 (1992).

Volyanik, E.V., Dalley, A., Mckay, I.A., Leigh, I., Williams, N.S., Bustin, S.A. Synthesis of Preparative Amounts of Biologically Active Interleukin-6 Using a Continuous-Flow Cell-Free Translation System. *Analytical Biochemistry*. **214** (1), 289–294, doi: 10.1006/ABIO.1993.1490 (1993).

Martin, G.A., Kawaguchi, R., Lam, Y., DeGiovanni, A., Fukushima, M., Mutter, W. High-yield, in vitro protein expression using a continuous-exchange, coupled transcription/ translation system. *BioTechniques*. **31** (4), 948–50, 952–3, at <http://www.ncbi.nlm.nih.gov/pubmed/11680726> (2001).

Stech, M., Quast, R.B., Sachse, R., Schulze, C., Wüstenhagen, D.A., Kubick, S. A Continuous-Exchange Cell-Free Protein Synthesis System Based on Extracts from Cultured Insect Cells. *PLoS ONE*. **9** (5), e96635, doi: 10.1371/journal.pone.0096635 (2014).

Quast, R.B., Sonnabend, A., Stech, M., Wüstenhagen, D.A., Kubick, S. High-yield cell-free synthesis of human EGFR by IRES-mediated protein translation in a continuous exchange cell-free reaction format. *Scientific Reports*. **6** (1), 30399, doi: 10.1038/srep30399 (2016).

Thoring, L., Dondapati, S.K., Stech, M., Wüstenhagen, D.A., Kubick, S. High-yield production of "difficult-to-express" proteins in a continuous exchange cell-free system based on CHO cell lysates. *Scientific Reports*. **7** (1), 11710, doi: 10.1038/s41598-017-12188-8 (2017).

Hoffmann, M., Nemetz, C., Madin, K., Buchberger, B. Rapid translation system: A novel cell-free way from gene to protein. *Biotechnology annual review*. **10**, 1–30, doi: 10.1016/S1387-2656(04)10001-X (2004).

***Reviewer #1:*** *Protocol/materials: I would find it helpful, to add comments on the action and tolerance range of the individual compounds as well. If even non-expert readers are addressed, this would be essential to give them more confidence for setting up such a system*.

**Response:** We agree that this information will provide new users with more confidence in setting up CFPS in their labs. We have added the following text within the protocol section:

"Note 3.1.B: Solutions A and B recipes are provided in the Supplementary Information and correspond to specific concentrations for numerous reagents to support the PANOx-SP based energy system for CFPS. The role of each reagent and acceptable variation in these reagent concentrations that can support CFPS have been determined.[49]"

***Reviewer #2:*** *Major Concerns:*

*The manuscript lacks the view of the quality of the prepared cell extract. Although they describes "Users can quantify batch-to-batch variability in total protein concentration of the cell extract by*

*Bradford assay" (in lines 388-389), there is no description of how to use quantified values for evaluating the variability. For example, they only describes the volume of the extract in experiments in Figure 5. I think they should at least describe the concentration of the extract used in Figure 5 experiments as an indicator for the reader to evaluate the quality of extract.*

**Response:** We appreciate the reviewer's feedback and we agree with their assessment. We have provided more complete details including the total protein concentration of the extract used in Figure 5. We have also modified the main text for more clarity:

"Users can quantify batch-to-batch variability in total protein concentration of the cell extract by Bradford assay. For higher performing extracts we typically see total protein concentrations of 30-50 mg/mL, and within this range there is no obvious correlation between total protein concentrations cell extract performance. Therefore, we recommend that users tune magnesium concentrations accordingly to ensure that protein and nucleic acid functionality are maximized for each extract batch."

***Reviewer #2:*** *Minor Concerns:*

*-- Some references are in incomplete format (e.g., 15, 35, 36). Please check the correctness of all the references.*

**Response:** We apologize for this oversight, we have resolved these issues.

***Reviewer #2:*** *BL21*DE3 and pJL1-sfGFP are not supplied in the table of materials. Especially, the genetic structure of the template DNA for CFPS is very important for the synthesis efficiency and I think that they should provide information of the 5'- and 3'- UTR of the pJL1-sfGFP.*

**Response:** We have updated our table of materials. We agree that details of the DNA template are crucial to successful CFPS reactions. Given that the pJL1-sfGFP vector and its plasmid map are

available on Addgene.com, we have simply listed the catalog number in the table of materials instead of providing the genetic structure of the plasmid in supplemental information. We hope this is satisfactory to the reviewer.

***Reviewer #2:*** In line 169, C1, V1, C2 and V2 are not defined. I think this formula is not necessary because the previous explanation is sufficient.

**Response:** We have removed the formula and kept the following text:

"(For example, if an $OD_{600}$ of a 1:10 dilution is read as 0.4, inoculate 25 mL of the undiluted $OD_{600}$ = 4.0 overnight culture into 1 L of 2x YTPG)."

***Reviewer #2:*** In line 304, Figure 4C does not exist. "wen using smaller"?

**Response:** We apologize for these errors, we have updated the figure numbers and resolved the typos in the revised manuscript. The new figure number is 2C.

***Reviewer #2:*** In lines 314-318, it lacks the most essential ingredients, cell extract. It would be helpful if they provide here the information of the cell extract concentration.

**Response:** We thank the reviewer for identifying this missing detail. The updated text reads as follows:

"Add 2.2 µL of Solution A, 2.1 µL of Solution B, 5 µL of BL21(DE3) extract, 0.24 µg of T7RNAP (16 µg/mL final concentration), 0.24 ng of DNA template (16 ng/mL final concentration), and water to bring the final volume to 15 µL."

***Reviewer #2:*** In lines 446-451 (the legend for Figure 4), There is no explanation for "Neg".

**Response:** Figure captions have been modified to improve clarity of the labels used. Text for one such example appears as follows:

"'Neg' represents a negative control where no DNA template was added to the reaction."

***Reviewer #2:*** In Table of Materials

"Glucose" is described as "D-glucose" in the text. I think it's better to unify the notation to avoid any confusion.

**Response:** All instances of glucose have been changed to D-glucose.

***Reviewer #2:*** NH4(Glu) is used in the text whereas NH4(OAc) is supplied in the table. Which is true?

**Response:** We have clarified this mismatch to indicate that only $NH_4$(Glu) is used.

***Reviewer #2:*** Mg(Glu)2 appears twice**.**

**Response:** The duplicate has been removed.

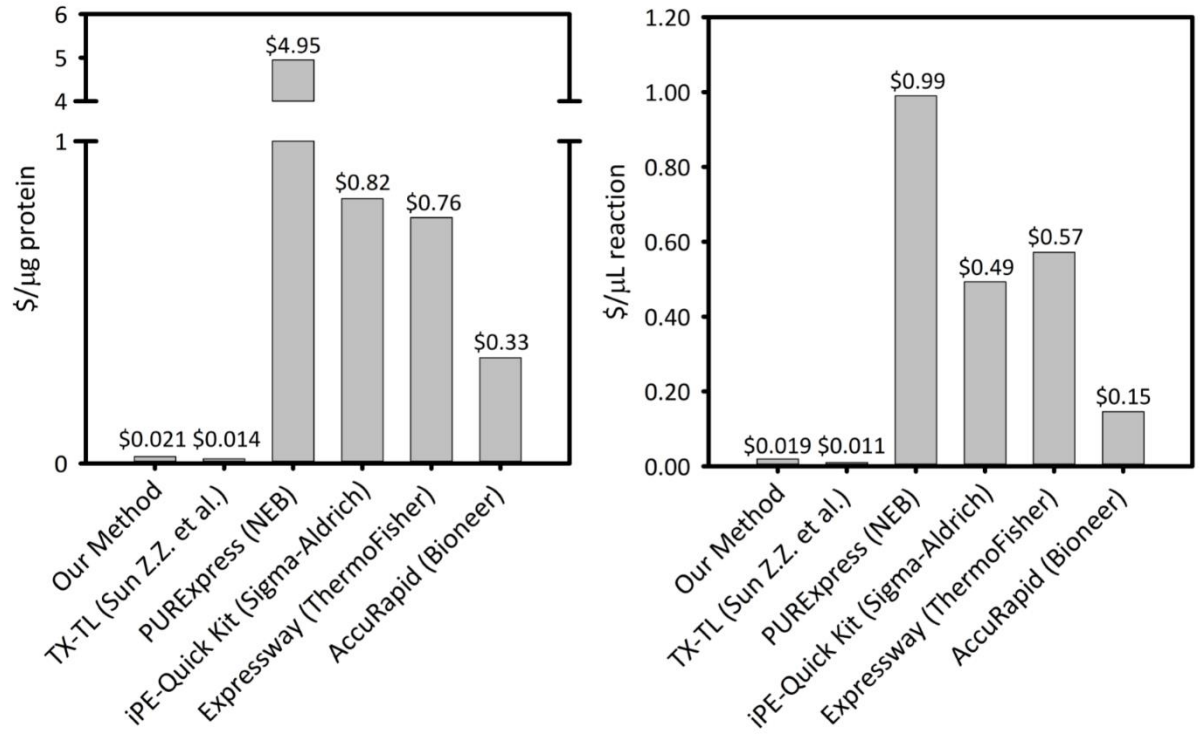
***Reviewer #2:*** In Materials Preparation, I found that pH of PEP solution is adjusted to 7.0. How about Putrescine, Spermidine, HEPES, and NTPs?

**Response:** We have clarified that pH of HEPES is adjusted and the pH value is specified. Putrescine, spermidine, and the NTPS are not adjusted for pH in our methods.

# Supplemental Information

**Table of Contents:**

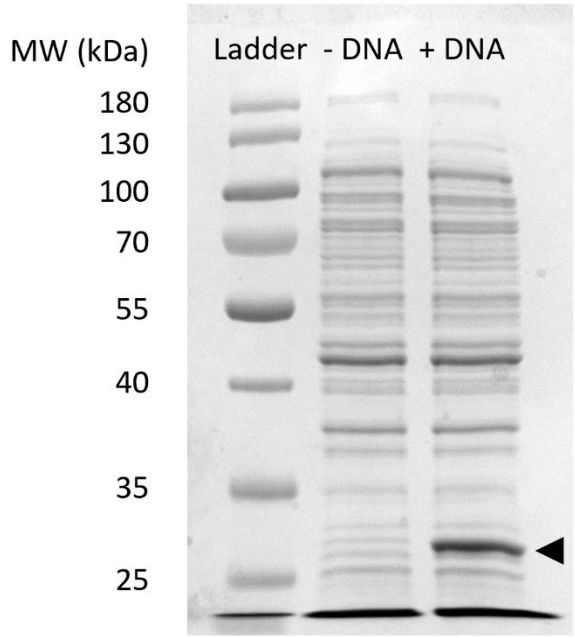
**Cost Comparison of Our Method with Commercial and Academic Options**




Supplemental Figure 1: **Cost per microgram of protein produced and per microliter of reaction across six cell-free protein synthesis platforms.** Our platform is compared to five different cell-free protein synthesis kits/platforms with varying productivity and pricing. Our sonication-based CFPS platform is more cost-effective in both $/µg of protein and $/µL of reaction than most commercial kits and provides the ease of a kit for reaction setup, while remaining cost-comparable to other academic CFPS platforms.

*An excel sheet detailing how the cost calculation for our platform was made is provided.

**SDS-PAGE of sfGFP Expression in CFPS**

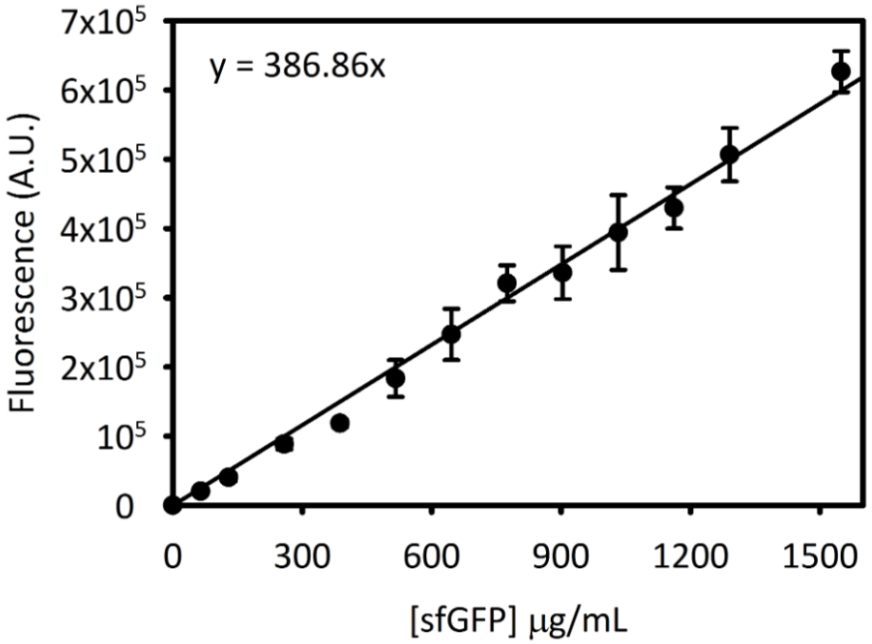



Supplemental Figure 2. **SDS-PAGE of sfGFP expression in CFPS.** Cell-free protein synthesis reactions with (+ DNA) and without (- DNA) DNA template for sfGFP were run on a 12% SDS-PAGE gel to demonstrate the expression of sfGFP observed at 27 kDa (black arrow). Traditional SDS-PAGE techniques were used. Each sample loaded onto the gel included 18 µg of total protein based on Bradford assay quantification of total protein in the cell extract. Based on fluorescence intensity measurements and our standard curve, we estimate that the "+ DNA" lane contains 0.42 µg of sfGFP. In order to obtain these samples, CFPS reactions were run at a 15 µL scale in 1.5 mL microfuge tubes producing volumetric yields consistent with Figure 3C. Ladder represents the PageRuler Prestained Protein Ladder (Thermo Scientific).

**Methods for Preparation of an sfGFP Standard Curve**

To prepare a standard curve for quantification of sfGFP, a single colony of BL21* pJL1-sfGFP was inoculated into 5 mL of LB with kanamycin in a round bottom test tube and grown overnight at 37 °C and 250 rpm. After 15-18 h, this culture was centrifuged at 10,000 x g for 5 min in a 15 mL falcon tube, with the appropriate balance. The supernatant was discarded, and the pellet was flash frozen in liquid nitrogen and stored at –80 °C. Strep-tag purification was then carried out as follows using a strep-tag affinity purification column. The pellet was retrieved from the –80 °C and 500 µL of wash buffer was added to the falcon tube. The mixture was placed on ice for 20-30 minutes to thaw. The pellet was completely resuspended through gentle vortexing, with resting periods on ice, to minimize bubble formation. The resuspended mixture was transferred to a 1.5 mL microfuge tube and sonicated for 10 s on, 10 s off, until the total J delivered was ~200 J. The sample was centrifuged at 10,000 x g for 5 min, and the supernatant was collected and saved in a separate 1.5 mL microfuge tube. Next, the column was prepared by allowing the storage buffer to drip through and equilibrating the column with 2 column volumes of wash buffer (400 µL). 500 µL of supernatant was then applied to the column, and the flow through was collected. The column was washed with 5 column volumes of wash buffer (1000 µL). Elution was performed by adding 7 separate 0.5 column volumes of elution buffer (100 µL each), and collecting the elution in 7 separate 1.5 mL microfuge tubes. Lastly, the column was washed with 3 column volumes of 10 mM NaOH (600 µL) followed by 10 column volumes of wash buffer (2000 µL). The column was then capped and wash buffer was added for storage at 4 °C. SDS-PAGE was performed on all collected samples to determine purity, and those samples with high yields of pure sfGFP were combined. The combined stock was then used to create dilutions in HEPES buffer (0.05 M, pH 7.0), and the absorbance at 280 nm was obtained to determine the concentration of the stock sfGFP solution, given that the extinction coefficient for strep tagged sfGFP is 24410 $M^{-1}$ $cm^{-1}$. Serial dilutions of the stock sfGFP were prepared, ranging from 0 to 1500 µg/mL of protein. Each dilution was then quantified via a multi well plate fluorometer as follows: 48 µl 0.05 M HEPES, pH 7.0 buffer and 2 µL of the respective sfGFP dilution were added to each well of a flat bottom 96-well half area black plate and each dilution was quantified in triplicate. Excitation and emission wavelengths for sfGFP fluorescence quantification were 485 nm and 510 nm, respectively. A standard curve was created from the collected data in order to convert from fluorescence readings to concentration of sfGFP in µg/mL.

**Standard curve for sfGFP on Cytation 5**




Supplemental Figure 2. **Standard curve for sfGFP on Cytation 5.** This curve was determined using the methods outlined above. All data collected for this manuscript was converted from endpoint fluorescence readings to [sfGFP] in µg/mL using this standard curve.

**Methods for T7 RNAP Preparation**

T7 RNA Polymerase was purified by affinity tag chromatography as previously described.[51]

**Reagent Preparation Instructions**

- Tris(OAc): Prepare by weighing out 6.057 g Tris Base and bringing volume up to 50 mL. pH with Glacial Acetic Acid to pH 8.2. Store at room temperature.
- $Mg(OAc)_2$: Prepare by weighing out 15.01 g $Mg(OAc)_2$ and bringing final volume to 50 mL using nanopure water. Store at room temperature.
- K(OAc): Prepare by weighing out 29.442 g K(OAc) and bringing final volume to 50 mL using nanopure water. Store at room temperature.
- DTT: Prepare by weighing out 1.54 g of DTT and bringing final volume to 10 mL using nanopure water. Aliquot 1 mL of solution into each 1.5 mL microfuge tube and store at –80 °C.
- S30 components: Prepare by adding 1 mL Tris(OAc), 1 mL $Mg(OAc)_2$, 1 mL K(OAc), and 0.200 mL DTT and bringing volume to 100 mL using nanopure water. Do not add DTT until day of use and store at 4 °C.
- 2x YTP Media: Prepare by weighing out 5 g NaCl, 16 g Tryptone, 10 g Yeast Extract, 7 g Potassium Phosphate Dibasic, and 3 g Potassium Phosphate Monobasic and bringing volume to 375 mL using nanopure water. Adjust pH to 7.20 using 5 M KOH. Dilute solution to a final volume of 750 mL. Autoclave in 2.5 L baffled flask at liquid 30 setting. Store at 37 °C until use.
- D-Glucose Solution: Prepare by weighing out 18 g D-Glucose and bringing volume to 250 mL using nanopure water. Autoclave solution in a glass bottle at liquid 30 setting. Store at 37 °C until use. Combine with 2x YTP media prior to inoculation of 2x YTPG media with overnight BL21*DE3 culture.
- IPTG: Prepare by weighing out 2.38 g IPTG and bringing final volume to 10 mL using nanopure water. Aliquot 1 mL of solution into each 1.5 mL microfuge tube and store at –80 °C.

For the following stocks, we recommend keeping log sheets for each batch. Over time, this will help identify batch-to-batch variation in reaction performance.

- NAD: Prepare by weighing out 0.050 g NAD and bringing final volume to 0.750 mL using molecular grade water. Store at –80 °C
- PEP: Prepare by weighing out 0.206 g PEP and bringing volume to 0.500 mL using molecular grade water. pH solution to 7.0 by adding 10 M KOH. Bring final volume to 1 mL using molecular grade water. Store at –80 °C.
- CoA: Prepare by weighing out 0.010 g CoA and bringing final volume to 0.260 mL using molecular grade water. Store at –80 °C.

- Putrescine: Prepare by weighing out 0.011 g Putrescine and bringing final volume to 0.500 mL using molecular grade water. Store at –80 °C.
- Spermidine: Prepare by weighing out 0.018 g Spermidine and bringing final volume to 0.500 mL molecular grade water. Store at –80 °C.
- HEPES: Prepare by weighing out 2.38 g HEPES and bringing volume to 5 mL using molecular grade water. pH solution to 7.5 by adding 10 M KOH. Bring final volume to 10 mL using molecular grade water. Store at –80 °C.
- Folinic Acid: Prepare by weighing out 0.015g folinic acid and bringing final volume to 1.5 mL using molecular grade water. Store at –80 °C.
- tRNA: Prepare by weighing out 0.050g tRNA and bringing final volume to 1 mL using molecular grade water. Store at –80 °C.
- 15X MasterMix: Prepare by combining 180 µL ATP, 127.5 µL GTP, 127.5 µL CTP, 127.5 µL UTP (NTPs were purchased at a stock concentration of 100 mM), 47.22 µL folinic acid, and 51.18 µL tRNA. Store at –80 °C.
- 15X Salt Solution: Prepare by weighing out 0.290 g of Magnesium Glutamate, 0.120 g of Ammonium Glutamate, and 1.98 g of Potassium Glutamate and bringing final volume to 5 mL using molecular grade water. Store at –80 °C.
- Oxalic Acid: Prepare by weighing out 0.92 g and bringing final volume to 5 mL using molecular grade water. Store at –80 °C.
- 20 Amino Acids: Prepare by weighing out 0.234 g L-Valine, 0.408 g L-Tryptophan, 0.330 g L-Phenylalanine, 0.262 g L-Isoleucine, 0.262 g L-Leucine, 0.242 g L-Cysteine, 0.298 g L-Methionine, 0.178 g L-Alanine, 0.348 g L-Arginine, 0.264 g L-Asparagine, 0.266 g L-Aspartic Acid, 0.406 g L-Glutamic Acid Potassium Salt Monohydrate, 0.150 g Glycine, 0.292 g L-Glutamine, 0.308 g L-Histidine, 0.365 g L-Lysine, 0.230 g L-Proline, 0.210 g L-Serine, 0.238 g L-Threonine, 0.362 g L-Tyrosine and adding molecular grade water to a final volume to 40 mL. Shake for 15 min in 37 °C incubator. pH of solution should be ~6.7. Store at –80 °C.

**Solution A and B Recipes**

Solutions A and B are generated upon mixing the aforementioned stock solutions as described below:

| Solution A | |
|---|---|
| **Reagent** | **Amount** |
| Master mix | 1000 µL |
| NAD | 60 µL |
| CoA | 80 µL |
| Oxalic Acid | 60 µL |
| Putrescine | 60 µL |
| Spermidine | 90 µL |
| HEPES | 855 µL |
| Total Volume | 2205 µL |
| *Makes enough for one thousand 15 µL reactions<br>* Add 2.2 µL to each 15 µL reaction | |

| Solution B | |
|---|---|
| **Reagent** | **Amount** |
| 15x SS | 1000 µL |
| 20 Amino Acids | 600 µL |
| PEP | 495 µL |
| Total Volume | 2095 µL |
| *Makes enough for one thousand 15 µL reactions<br>* Add 2.1 µL to each 15 µL reaction | |

The final CFPS reaction will contain the following concentrations of each reagent: Solution A (1.2 mM ATP, 0.850 mM GTP, 0.850 mM UTP, 0.850 mM CTP, 31.50 µg/mL Folinic Acid, 170.60 µg/mL tRNA, 0.40 mM Nicotinamide Adenine Dinucleotide (NAD), 0.27 mM Coenzyme A (CoA), 4.00 mM Oxalic Acid, 1.00mM Putrescine, 1.50 mM Spermidine, and 57.33 mM HEPES buffer), 2.20 µL Solution B (10 mM $Mg(Glu)_2$, 10 mM $NH_4(Glu)$, 130 mM K(Glu), 2 mM each of the 20 amino acids, and 0.03 M Phosphoenolpyruvate (PEP)).

**CFPS Reaction Setup Guide** (excel sheet provided)

**Name:**
**Date:**
**Purpose:**
**Reaction Size (uL):** 15

**Reagent Information**

| Cell Extract | | | | |
|---|---|---|---|---|
| Cell Type | Growth Date | Extract Preparation Date | Volume per Reaction (µL) | Note |
| BL21* DE3 | 7/26/2018 | 7/27/2018 | 5.00 | |

| Solution A | | Solution B | | |
|---|---|---|---|---|
| Lot # | Volume per Reaction (µL) | Lot # | Volume per Reaction (µL) | Note |
| 1 | 2.20 | 1 | 2.10 | |

| DNA Template | | | | |
|---|---|---|---|---|
| Template Name | Stock concentration (ng/µL) | Final concentration in reaction (ng/µL) | Volume per Reaction (µL) | Note |
| pJL1-sfGFP | 240 | 16 | 1.00 | Must be less than volume of water in negative control |

**Reaction Set Up**
(perform each in triplicate)

| | | | | | |
|---|---|---|---|---|---|
| **Negative** | Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
| | 5.70 | 2.20 | 2.10 | 5.00 | 0.00 |

| | | | | | |
|---|---|---|---|---|---|
| **Positive** | Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
| | 4.70 | 2.20 | 2.10 | 5.00 | 1.00 |

| | | | | | |
|---|---|---|---|---|---|
| **Experimental** | Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
| | 4.70 | 2.20 | 2.10 | 5.00 | 1.00 |

**Name:**
**Date:**
**Purpose:**
**Reaction Size (uL):** 15

## Reagent Information

| Cell Extract | | | | |
|---|---|---|---|---|
| Cell Type | Growth Date | Extract Preparation Date | Volume per Reaction (µL) | Note |
| BL21* DE3 | 7/26/2018 | 7/27/2018 | 5.00 | |

| Solution A | | Solution B | | |
|---|---|---|---|---|
| Lot # | Volume per Reaction (µL) | Lot # | Volume per Reaction (µL) | Note |
| 1 | 2.20 | 1 | 2.10 | |

| DNA Template | | | | |
|---|---|---|---|---|
| Template Name | Stock concentration (ng/µL) | Final concentration in reaction (ng/µL) | Volume per Reaction (µL) | Note |
| pJL1-sfGFP | 240 | 16 | 1.00 | Must be less than volume of water in negative control |

## Reaction Set Up

(perform each in triplicate)

| | Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
|---|---|---|---|---|---|
| **Negative** | | | | | |

| | | | | |
|---|---|---|---|---|
| 5.70 | 2.20 | 2.10 | 5.00 | 0.00 |

**Positive**

| Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
|---|---|---|---|---|
| 4.70 | 2.20 | 2.10 | 5.00 | 1.00 |

**Experimental**

| Molecular Grade Water (µL) | Solution A (µL) | Solution B (µL) | Cell Extract (µL) | DNA Template (µL) |
|---|---|---|---|---|
| 4.70 | 2.20 | 2.10 | 5.00 | 1.00 |

Cost Analysis

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Cost analysis Calculation.xlsx

| Reagent | Company | Purchase quantity (g) | Purchase Price ($) |
|---|---|---|---|
| L-ASPARTIC ACID | Sigma Aldrich | 100 | 50.50 |
| L-VALINE | Sigma Aldrich | 1 | 14.20 |
| L-TRYPTOPHAN | Sigma Aldrich | 1 | 14.50 |
| L-PHENYLALANINE | Sigma Aldrich | 100 | 79.50 |
| L-ISOLEUCINE | Sigma Aldrich | 1 | 16.20 |
| L-LEUCINE | Sigma Aldrich | 25 | 23.75 |
| L-CYSTEINE | Sigma Aldrich | 25 | 50.50 |
| L-METHIONINE | Sigma Aldrich | 5 | 11.30 |
| L-ALANINE | Sigma Aldrich | 1 | 13.70 |
| L-ARGININE | Sigma Aldrich | 25 | 24.75 |
| L-ASPARAGINE | Sigma Aldrich | 25 | 28.00 |
| GLYCINE | Sigma Aldrich | 100 | 25.75 |
| L-GLUTAMINE | Sigma Aldrich | 100 | 64.50 |
| L-HISTIDINE | Sigma Aldrich | 5 | 13.50 |
| L-LYSINE | Sigma Aldrich | 1 | 91.30 |
| L-PROLINE | Sigma Aldrich | 100 | 76.00 |
| L-SERINE | Sigma Aldrich | 1 | 11.20 |
| L-THREONINE | Sigma Aldrich | 1 | 15.10 |
| L-GLUTAMIC ACID MONOPOTASSIUM SALT | Sigma Aldrich | 100 | 53.50 |
| L-TYROSINE | Sigma Aldrich | 50 | 73.50 |
| 1,4-DIAMINOBUTANE (PUTRESCINE) | Sigma Aldrich | 25 | 28.70 |
| TRNA, FROM E.COLI MRE | Sigma Aldrich | 0.1 | 180.00 |
| FOLINIC ACID CALCIUM SALT HYDRATE | Sigma Aldrich | 0.1 | 76.50 |
| AMMONIUM GLUTAMATE | MP Biomedicals | 100 | 83.25 |
| POTASSIUM OXALATE MONOHYDRATE | Sigma Aldrich | 100 | 43.50 |
| L-GLUTAMIC ACID HEMIMAGNESIUM SALT | Sigma Aldrich | 250 | 55.50 |
| POTASSIUM HYDROXIDE | Sigma Aldrich | 500 | 69.50 |
| SODIUM CHLORIDE | Sigma Aldrich | 1000 | 62.50 |
| POTASSIUM PHOSPHATE DIBASIC | Sigma Aldrich | 250 | 86.50 |
| POTASSIUM PHOSPHATE MONOBASIC | Sigma Aldrich | 500 | 85.00 |
| D-(+)-GLUCOSE | Sigma Aldrich | 1000 | 43.50 |
| BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE | Sigma Aldrich | 0.05 | 257.00 |
| COENZYME A SODIUM SALT HYDRATE | Sigma Aldrich | 0.025 | 87.00 |
| TRIZMA(R) BASE | Sigma Aldrich | 500 | 90.50 |
| PHOSPHO(ENOL)PYRUVIC ACID MONOPOTASSIUM | Sigma Aldrich | 0.25 | 103.00 |
| ISOPROPYL B-D-THIOGALACTOPYRANOSIDE | Sigma Aldrich | 5 | 287.00 |
| SPERMIDINE | Sigma Aldrich | 1 | 44.25 |
| TRYPTONE | Fisher Bioreagents | 1000 | 214.36 |
| YEAST EXTRACT | Fisher Bioreagents | 1000 | 272.82 |
| LB BROTH | ThermoFisher | 500 | 82.00 |
| MAGNESIUM ACETATE | Sigma Aldrich | 500 | 40.00 |
| POTASSIUM ACETATE | Sigma Aldrich | 100 | 28.75 |

| | | | |
|---|---|---|---|
| DITHIOTHREITOL | ThermoFisher | 5 | 129.00 |
| NTPs | ThermoFisher | 250 uL each | 124.00 |
| HEPES | ThermoFisher | 1000 | 542.00 |
| MOLECULAR GRADE WATER | Sigma Aldrich | 1 L | 57.56 |
| MAXI PREP KIT | ThermoFisher | 10 preps | 235.00 |

| Quantity required per batch of stock prepared (g) | Number of batches of stock solution possible per total quantity purchased | Volume of stock solution prepared (uL) | Volume of stock solution used per rxn (uL) per 15 uL reaction |
|---|---|---|---|
| 0.266 | 375.94 | 40000 | 0.600 |
| 0.234 | 4.27 | 40000 | 0.600 |
| 0.408 | 2.45 | 40000 | 0.600 |
| 0.33 | 303.03 | 40000 | 0.600 |
| 0.262 | 3.82 | 40000 | 0.600 |
| 0.262 | 95.42 | 40000 | 0.600 |
| 0.242 | 103.31 | 40000 | 0.600 |
| 0.298 | 16.78 | 40000 | 0.600 |
| 0.178 | 5.62 | 40000 | 0.600 |
| 0.348 | 71.84 | 40000 | 0.600 |
| 0.264 | 94.70 | 40000 | 0.600 |
| 0.15 | 666.67 | 40000 | 0.600 |
| 0.292 | 342.47 | 40000 | 0.600 |
| 0.308 | 16.23 | 40000 | 0.600 |
| 0.365 | 2.74 | 40000 | 0.600 |
| 0.23 | 434.78 | 40000 | 0.600 |
| 0.21 | 4.76 | 40000 | 0.600 |
| 0.238 | 4.20 | 40000 | 0.600 |
| 2.386 | 41.91 | 40000 | 1.467 |
| 0.362 | 138.12 | 40000 | 0.600 |
| 0.011 | 2272.73 | 500 | 0.060 |
| 0.025 | 4.00 | 500 | 0.051 |
| 0.015 | 6.67 | 1500 | 0.047 |
| 0.12 | 833.33 | 5000 | 0.067 |
| 0.92 | 108.70 | 5000 | 0.060 |
| 0.36 | 694.44 | 5000 | 0.067 |
| 280.52 | 1.78 | 1000000 | 8.330 |
| 5 | 200.00 | 1800 | 5.000 |
| 7 | 35.71 | 1800 | 5.000 |
| 3 | 166.67 | 1800 | 5.000 |
| 18 | 55.56 | 1800 | 5.000 |
| 0.05 | 1.00 | 500 | 0.060 |
| 0.01 | 2.50 | 260 | 0.080 |
| 121.14 | 4.13 | 1000000 | 0.001 |
| 0.206 | 1.21 | 1000 | 0.490 |
| 2.383 | 2.10 | 10000 | 0.556 |
| 0.018 | 55.56 | 500 | 0.090 |
| 16 | 62.50 | 1800 | 5.000 |
| 10 | 100.00 | 1800 | 5.000 |
| 1.25 | 400.00 | 50000 | 27.770 |
| 300.23 | 1.67 | 1000000 | 0.001 |
| 588.9 | 0.17 | 1000000 | 0.001 |

| | | | |
|---|---|---|---|
| 154.254 | 0.03 | 1000000 | 0.149 |
| 180 | 1.39 | 1000 | 1.000 |
| 2.38 | 420.17 | 10000 | 0.855 |
| - | - | - | 5.000 |
| - | 10.00 | 400 | 1.000 |

To

| Number of 15 uL reactions possible per total quantity purchased | Cost per 15 uL reaction ($) |
|---|---|
| 25062657 | 0.00000201 |
| 284900 | 0.00004984 |
| 163399 | 0.00008874 |
| 20202020 | 0.00000394 |
| 254453 | 0.00006367 |
| 6361323 | 0.00000373 |
| 6887052 | 0.00000733 |
| 1118568 | 0.00001010 |
| 374532 | 0.00003658 |
| 4789272 | 0.00000517 |
| 6313131 | 0.00000444 |
| 44444444 | 0.00000058 |
| 22831050 | 0.00000283 |
| 1082251 | 0.00001247 |
| 182648 | 0.00049987 |
| 28985507 | 0.00000262 |
| 317460 | 0.00003528 |
| 280112 | 0.00005391 |
| 1142772 | 0.00004682 |
| 9208103 | 0.00000798 |
| 18939394 | 0.00000152 |
| 39078 | 0.00460620 |
| 211775 | 0.00036123 |
| 62468766 | 0.00000133 |
| 9057971 | 0.00000480 |
| 52057305 | 0.00000107 |
| 213974 | 0.00032481 |
| 72000 | 0.00086806 |
| 12857 | 0.00672778 |
| 60000 | 0.00141667 |
| 20000 | 0.00217500 |
| 8333 | 0.03084000 |
| 8125 | 0.01070769 |
| 5981820052 | 0.00000002 |
| 2477 | 0.04158728 |
| 37737 | 0.00760520 |
| 308642 | 0.00014337 |
| 22500 | 0.00952711 |
| 36000 | 0.00757833 |
| 720202 | 0.00011386 |
| 2413608504 | 0.00000002 |
| 246098720 | 0.00000012 |

| | |
|---|---|
| 217544 | 0.00059298 |
| 1389 | 0.08928000 |
| 4914246 | 0.00011029 |
| 200000 | 0.00028780 |
| 4000 | 0.05875000 |

| | |
|---|---|
| Total cost per 15 uL reaction ($) | 0.275 |
| tal start-up cost for reagents ($) | 4159.94 |