Video Article

Escherichia coli-Based Cell-Free Protein Synthesis: Protocols for a robust, flexible, and accessible platform technology

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/58882/

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[3,4,5,6]; 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[12,13,14,15,16]. 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*[17,18,19,20], detection of disease[21,22,23], on demand biomanufacturing[18,24,25,26,27], 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[31,32,33,34,35].

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[38,39,40,41,42,43]. 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[46,47,48,49]. 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. **Day 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.
    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.

2. **Day 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.
    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.
    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.
    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.
    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.
    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.

3. **Day 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).
    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.

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.
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.
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.
   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).
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.
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.
8. Centrifuge the 1 L bottles for 10 min at 5,000 x g and 10 °C to pellet cells.
9. Slowly pour off the supernatant and dispose of it according to the institution's biological waste procedures. Place the pellet on ice.
10. Using a sterile spatula, scrape the cell pellet from the centrifuge bottle and transfer it to a cold 50 mL conical tube.
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.
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.
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.
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.
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.
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.
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.
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

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. 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.
3. Transfer 1.4 mL of resuspended cells into a 1.5 mL microfuge tube.
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].
5. Immediately after sonication is complete, add 4.5 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.
6. Microcentrifuge samples at 18,000 x g and 4 °C for 10 min (**Figure 3C**).
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.
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).
9. Microcentrifuge samples at 10,000 x g and 4 °C for 10 min.
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

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.
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. 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.
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.
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]

1. Load 48 µL of 0.05 M HEPES, pH 8, into each well needed for quantification (usually performed in triplicate per reaction tube).
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.
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 nm and 510 nm, respectively.
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 1,000 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].

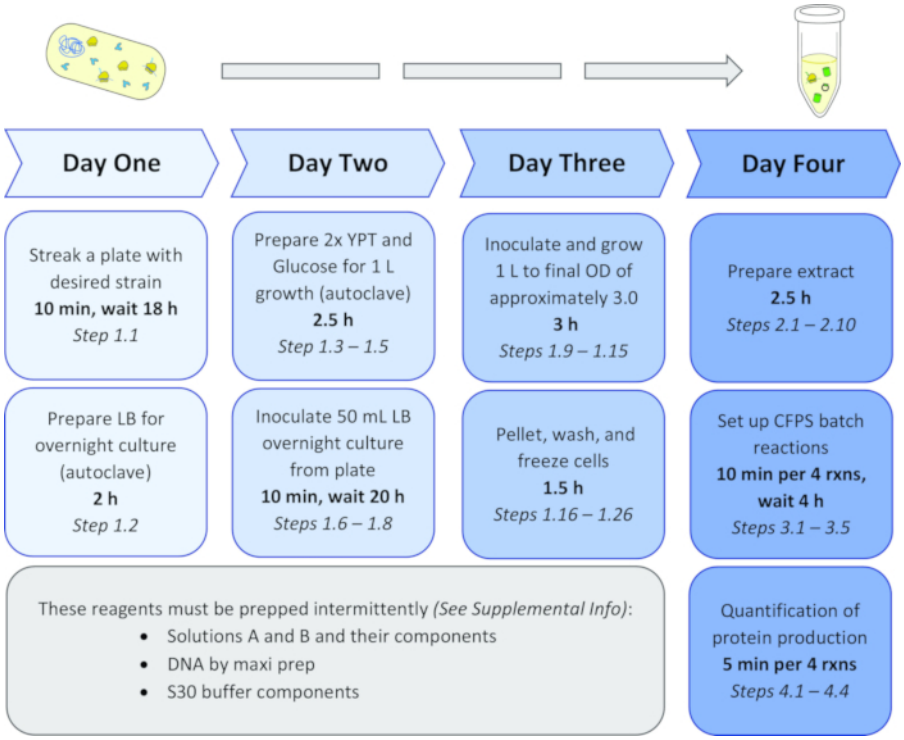

Figure 1: Timeline for culture growth, production of cell extract, setup and quantification of CFPS reactions. The user can implement the CFPS platform for their research applications through this four-day workflow. Reagent preparation represents the primary time and cost investment for the first round 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. Please click here to view a larger version of this figure.

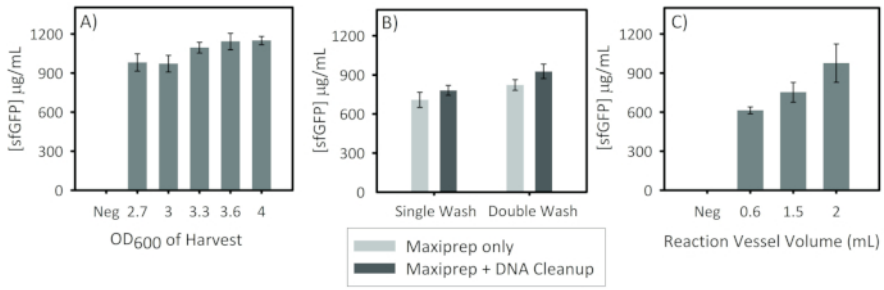

Figure 2: Modifiable conditions for CFPS and the effects on volumetric reaction yields.A. Extract productivity comparison based upon harvesting BL21(DE3) cells at various OD_{600} readings. Based on this plot, we recommend harvesting at an OD_{600} of 3.3 to produce at least 1000 µg/mL of target protein. 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. Please click here to view a larger version of this figure.

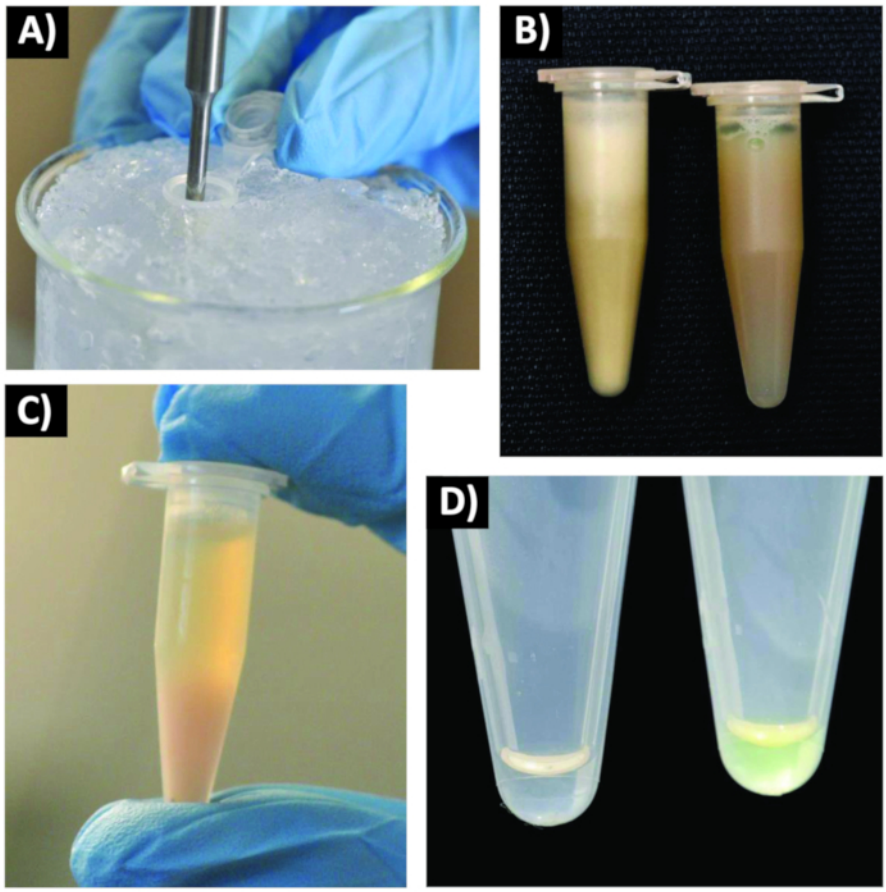

Figure 3: Key procedural setups and outcomes for creating productive extract. A. Proper setup of sonication ice water bath to ensure cooling of sample while heat is generated during sonication. **B.** 1.5 mL microfuge tube containing resuspended cell pellet pre (left) and post (right) sonication. The 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. Please click here to view a larger version of this figure.

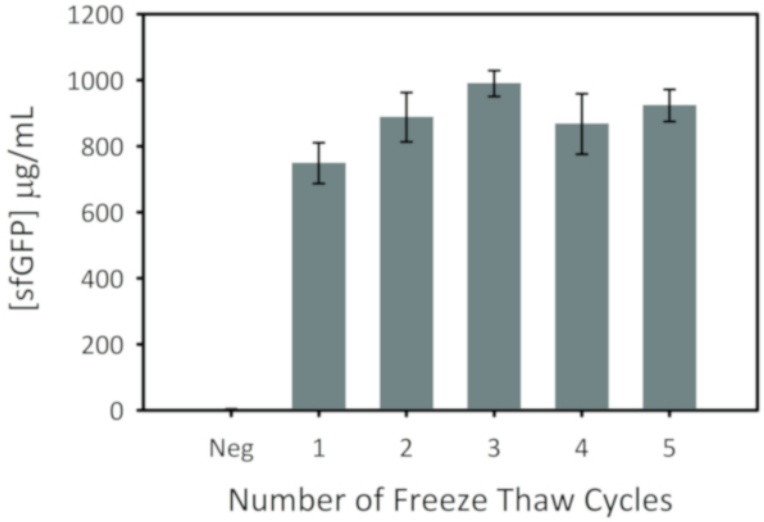

Figure 4: Change in protein expression over 5 freeze-thaw cycles for CFPS extract. Extract prepared from the same growth underwent five freeze thaw cycles via liquid nitrogen flash freezing followed by thawing on ice. No significant changes in extract productivity for expressing sfGFP were seen over 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. Please click here to view a larger version of this figure.

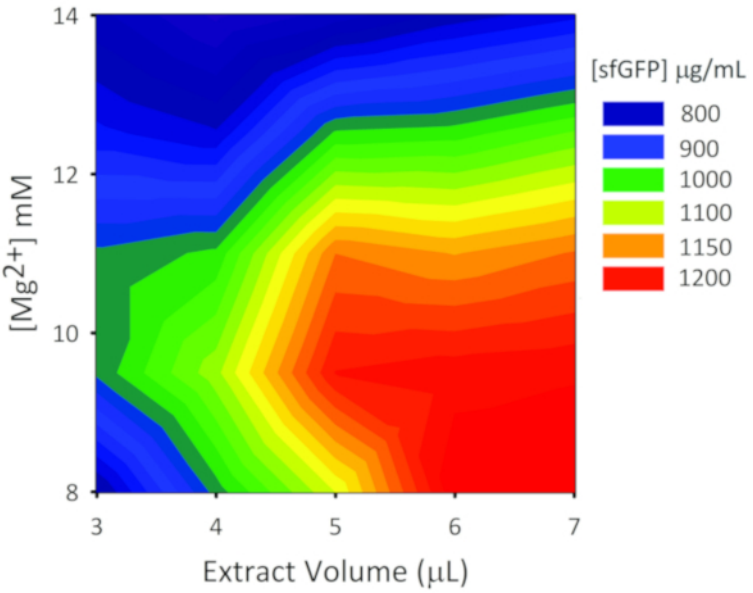

Figure 5: CFPS for reactions with varying [Mg^{2+}] and extract volumes versus [sfGFP]. [Mg^{2+}] ranged from 8 mM to 14 mM with 2 mM increments and extract volumes ranged from 3 µL to 7 µL with 1 µL increments. The color code represents [sfGFP] produced from high (red) to low (purple). To maximize reagent 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. Please click here to view a larger version of this figure.

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. Please click here to view a larger version of this figure.

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**. Please click here to view a larger version of this figure.

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. Please click here to view a larger version of this figure.

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[54,55,56,57,58,59,60]. 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[64,65,66]. 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].

Disclosures

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

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