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TITLE:

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

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SUMMARY:

This protocol details the steps, costs, and equipment necessary to generate highly-productive *E. coli*-based cell extract and implement high-throughput *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 in 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 the user to access these applications. Here we describe methods to prepare highly productive 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 high-throughput protein expression capable of producing 900 µg/mL or more of superfolder green fluorescent protein (sfGFP) in just 5 hours 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 $0.02 per µg of protein produced. Additionally, our protein expression methods mirror the ease of reaction setup seen in commercially available systems such as PureExpress due to optimization of reagent pre-mixes, while maintaining low reaction costs. 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 cost-effective technology that has opened a number of new opportunities for protein production, functional genomics, and metabolic engineering 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) removal of genomic DNA and the introduction of a template DNA encoding our gene(s) of interest channels all of the systemic energy within the reaction to the production of the protein(s) of interest; 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. 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.[11–15] As a result of these advantages, CFPS has uniquely enabled a variety of biotechnology applications such as the production of proteins that are difficult to solubly

express *in vivo*,[16–19] detection of disease,[20–22] on demand biomanufacturing,[17, 23–26] and education,[27, 28] all of which show the power and flexibility 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.[29] 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.[30–34]

The methods presented herein enable non-experts with basic laboratory skills to implement cell growth, extract preparation, and reaction setup for an *E. coli*-based CFPS system. This approach is time and cost-effective for establishing a highly productive CFPS platform, with applications both in the laboratory and in the field. Our methods combine growth in enriched media and baffled flasks, with relatively rapid and reproducible methods of cell lysis through sonication, and fast CFPS reaction setup through the utilization of optimized premixes.[35] 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, homogenizer, bead beaters, lysozyme and other biochemical or physical disruption methods.[35–38] Using this approach, we are able to produce, on average, 900 µg/mL of the reporter superfolder green fluorescent protein (sfGFP) at a cost of $0.02/µg of protein produced, excluding the cost of labor and equipment (Figure 1). Starting from scratch, this method can be implemented in 4-days, and repeat CFPS experiments can be completed within hours (Figure 2). Importantly, this approach can be implemented by non-experts, and only requires basic laboratory skills. For these reasons, *E. coli* CFPS is primed for broad usage.

PROTOCOL:

The following protocol will outline a three-part procedure capable of being completed by one person that includes: 1) media preparation and cell growth (steps 1.1-1.26), 2) cell extract preparation (steps 2.1-2.10), and 3) cell-free protein synthesis (steps 3.1-4.4). The first part of this procedure is divided into 3 days for convenience and is capable of producing ~2 mL of crude cell extract per liter of cells, which can support four hundred 15 µL CFPS reactions, each producing ~900 µg/mL of reporter sfGFP protein from plasmid pJL1-sfGFP, with slight variation seen from batch to batch of extract. The protocol can be scaled in volume to suit the user's needs. All reagent recipes and storage conditions can be found in the Supplemental Information.

Day 1

1. Media Preparation and Cell Growth

1.1. Streak a BL21*DE3 strain from -80°C stock onto an LB agar plate and incubate for at least 18 hr at 37°C or until colonies are readily visible.

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

Day 2

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

1.4. 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 minutes at 121°C.

1.5. Both sterile solutions should be stored at 37°C if cell growth is being performed next day to maximize growth rates upon inoculation. Solutions can be stored at 4°C for 1-2 days if needed, though the 2x YTP enriched media is highly prone to contamination.

1.6. Start an overnight culture of BL21*DE3 by inoculating 50 mL LB media with a single colony of BL21*DE3 using a sterilized loop in a laminar flow hood to avoid contamination.

1.7. Place the 50 mL BL21*DE3 LB culture from step 1.6 into a 37°C 250 rpm shaking incubator and grow overnight for 15-18 hours.

1.8. Prepare and sterilize all materials required for day 3, including: 2 1L centrifuge bottles, 4 cold 50mL conical tubes (weigh and record masses of three), and 1.5 mL microfuge tubes.

Day 3

1.9. Remove the 50 mL overnight culture of BL21*DE3 LB ~~culture~~ from the shaking incubator and measure the OD_{600} on the OceanOptics spectrophotometer using a 1:10 dilution with LB media. Calculate the volume necessary to add to 1L 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 1L of 2x YTPG; $C_1V_1=C_2V_2$)

1.10. Remove the warmed 2x YTP media and D-glucose solutions from the 37°C incubator along with the 50 mL LB culture, a serological pipet and a sterile tip, and bring all items to a sterile laminar flow hood.

1.11. Carefully pour the 37°C D-glucose solution into the 2x YTP media (avoiding the sides of the baffled flask) and then inoculate the 1L 2x YTPG solution with the appropriate amount of the 50 mL culture to begin the 1L culture at a 0.1 OD_{600} using the serological pipet and sterile tip. Immediately place the inoculated 1L culture into the 37°C shaking incubator at 200 rpm.

1.12. Take the first OD_{600} reading after the first hour of growth (lag phase typical takes 1 hour). No dilution is needed. Continue taking OD_{600} measurements until OD_{600} reaches 0.6.

1.13. Upon reaching OD_{600} = 0.6, inoculate the 2x YTPG culture with 1 mL of 1M IPTG (final concentration of solution = 1 mM). Ideal induction OD_{600} is 0.6; however, anywhere from 0.6-0.8 is acceptable.

1.14. After induction, measure the OD_{600} until it reaches 3.0 (usually requires a measurement every 20 minutes).

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

1.15. Once the OD_{600} reaches 3.0, pour the culture into a cold 1L centrifuge bottle that is placed into an ice-water bath. During this time, weigh a water-filled 1L centrifuge bottle to be used 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, we recommend the user to evaluate and optimize this variable as a troubleshooting measure. In our observations, larger spectrophotometers may result in relatively lower OD_{600} readings compared to smaller cuvette-based spectrophotometers such as the OceanOptics and NanoDrop instruments.

1.16. Centrifuge the 1L bottles for 10 minutes at 5000 x g and 10°C.

1.17. Gradually pour off the supernatant and dispose of it with 10% bleach while keeping the pellet on ice.

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

1.19. Add 30 mL of cold S30 buffer to the conical tube and resuspend the cell pellet by vortexing with short bursts (20- 30 seconds) and rest periods (1 minute) on ice until fully resuspended.

Note: S30 buffer is made up of the following in a 100 mL volume: 14 mM $Mg(OAC_2)$, 10 mM Tris(OAc) pH 8.2, 60 mM KOAc, and 2mM DTT (added during the day of cell harvest).

1.20. Once fully resuspended, use another conical tube with water as a balance and centrifuge for 10 minutes at 5000 x g at 10°C (pre-cooled to 4°C).

1.21. Pour out the supernatant and dispose of with 10% bleach and resuspended the pellet with 20-25 mL cold S30 buffer and centrifuge again for 10 minutes at 5000 x g at 10°C (pre-cooled to 4°C).

1.22. Again, pour out the supernatant and dispose with 10% bleach. Add exactly 30 mL S30 buffer and vortex again to resuspend the pellet.

1.23. Using the 3 pre-weighed cold 50 mL conical tubes and a serological pipet with a sterile tip, transfer 10 mL of resuspended cell/S30 buffer mixture into each of the 3 conical tubes.

1.24. Centrifuge all tubes (may need a 4th tube for a balance) for 10 minutes at 5000 x g at 10°C (pre-cooled to 4°C).

1.25. Pour out supernatant and dispose according to your institution's biological waste procedures. Remove excess S30 buffer by wiping the inside of the tube and cap with a KimWipe tissue, without touching the pellet.

Note: While sterile technique is not necessary, ensure cleanliness during this step by using clean tissues and gloves.

1.26. Reweigh the tubes on an analytical balance to get final pellet weight and place the conical tubes in liquid nitrogen to flash freeze the cells.

Note: Protocol can be paused at this point. The pellets can be stored at –80°C until needed for extract preparation.

Day 4

2. Crude Cell Extract Preparation

2.1. For extract preparation, cells should be kept cold on ice for each step. For each pellet to be prepared, add 0.8 mL of cold S30 buffer per 1g of cell mass. Ensure that DTT has been supplemented to the S30 buffer.

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

2.2. Resuspend cell pellet by vortexing with short bursts (20- 30 seconds) and rest periods (1 minute) on ice until fully resuspended.

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

2.4. Place tubes in an ice water bath in a beaker and sonicate for 45 seconds followed by 59 seconds OFF for 3 total bursts with amplitude set at 50%. Invert the tubes to gently mix during the off cycles. In total, 800-900 J of energy should be delivered to each 1.5 mL microfuge tube containing 1.4 mL of resuspended cells (Figures 3A & 3B).

Note that this step is sensitive to the sonicator type and model used and should be optimized if equipment is different than listed for this procedure.

2.5. Immediately after sonication is complete, add 4.5 µL 1M dithiothreitol (DTT) into the lysate and invert several times to mix.

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

2.7. Pipette supernatant into a new 1.5 mL microfuge tube. Do not disturb pellet; it is okay to leave some supernatant behind.

2.8. Incubate the supernatant from the previous step at 250 rpm, 37°C for 60 minutes (this is the runoff reaction).

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

2.10. Transfer supernatant to a new tube and flash freeze by liquid nitrogen.

Note: The protocol can be paused here and the extract can be frozen and stored at –80°C.

3. Cell-Free Protein Synthesis

The reaction mixture for CFPS contains the following reagents calculated to a total volume of 15 µL: 2.21 µL 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 20 amino acids, and 0.03 M Phosphoenolpyruvate (PEP)). Plasmid or linear template (pJL1-sfGFP) that will be expressed should be prepared for this step using the Invitrogen High Pure Plasmid Maxiprep Kit with two washes using the wash buffer in the kit followed by a post-processing DNA-cleanup using the ThermoScientific GeneJET PCR Purification Kit, along with an aliquot of purified T7 RNA polymerase (T7 RNAP), and nanopure/molecular grade water.[39, 40] 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. Yields will decrease wen using smaller vessels as seen in Figure 4C. CFPS reactions can also be scaled up in volume in order to increase the total protein yield for a single condition.

Note: CFPS reaction template and Solution A and B recipes can be found in Supplementary Information.

3.1. Thaw Solutions A and B, plasmid or linear DNA template, BL21*DE3 extract (if frozen), and an aliquot of nanopure water/molecular grade water.

3.2. Label the necessary amount of microfuge tubes (tubes of greater volume result in higher protein yields) needed for CFPS and add 2.2 µL Solution A, 2.1 µL Solution B, the appropriate amount of T7 RNAP to yield 16 µg/mL in a 15 µL volume (0.24 µg), the appropriate volume of DNA template to yield 16 ng/mL in a 15 µL volume (0.24 ng), and enough 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. After all reagents have been added to the reaction, ensure that it is well mixed and combined into a single 15 µL solution at the bottom of the 1.5 mL microfuge tube.

3.3. Place each reaction into the 37°C incubator for 4 hours, or 30°C overnight (Figure 3D).

4. Quantification of reporter protein, [sfGFP]

Instructions for creation of [sfGFP] standard curve are present in Supplementary Information. Reagents/materials needed for quantification are 0.05M HEPES buffer, pH 8, a 96-well half area black plate, and a fluorometer.

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

4.2. Carefully remove reactions from incubator and pipet 2 µL of reaction into the 48 µL of 0.05 HEPES pH 8 with gentle mixing via pipetting up and down.

4.3. Once all reactions are loaded and mixed, place the 96 well plate into the fluorometer and measure the [sfGFP] endpoint fluorescence. Excitation and emission wavelengths for sfGFP fluorescence quantification are 485 and 510, respectively.

4.4. Using the equation on the standard curve (Supplementary Figure 1), solve for the slope value by using the fluorescence excitation value generated by the fluorometer as the y value.

Note: Users will need to establish a standard curve for their instrument since instrument sensitivity may vary.

REPRESENTATIVE RESULTS:

We have presented a sonication-based extract preparation protocol that can be completed over a four-day span, with Figure 2 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 1.26) and fully prepared extract (step 2.10) are stable at -80°C for at least a year,

allowing the user to create larger stocks at each to save for use at a later time.[17] In addition to extract stability over long time periods, 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 freezer storage space is limited. However, we recommend multiple smaller aliquots (~100 µL) of extract whenever possible.

When performing this protocol, there are a few key considerations that impact reaction yields as well as indicators associated with poorly performing extract. In order to ensure proper lysis and to obtain 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 3B (Qsonica Q125 sonicator, 3.175 mm diameter probe, frequency 20kHz, 50% amplitude) is adaptable to a range of volumes from 100 µL to 1400 µL of resuspended cells. To accomplish this, the user can adjust the amount of Joules delivered for lysis of smaller volume of cells.[35] 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) and the pellet (undesired cellular components such as the cell membrane, genomic DNA, precipitated proteins) (Figure 3C). While this centrifugation step is often performed at lower relative centrifugal forces (RCF) by other labs, we have found that increasing to 18,000 x g improves the separation for improved reproducibility without compromising extract performance. For convenience we recommend using a table-top refrigerated centrifuge, capable of achieving a minimum of 12,000 x g. When removing the 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 versus quantity of extract results in more productive extracts. When the procedure is executed successfully, CFPS reaction tubes should turn visibly green upon sfGFP expression following a 4-hour CFPS reaction at 37°C (Figure 3D).

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, and 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.[41] 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 highly productive reaction (Figure 5). From this experiment, we recommend using 5 µL of extract and 10 mM Mg^{2+}, in order to obtain over 1 mg/mL of protein, while minimizing reagent consumption as much as possible.

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 (Figures 5 & 6). 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} representing 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 6A). Variables that impact CFPS yields include template DNA quality, reaction vessel size, as well as the relative quantities of cell extract and magnesium ion present in the reaction. We have found the 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 ensures more reproducibility in DNA quality for CFPS reactions and results in more robust protein production (Figure 6B). The reaction vessel also impacts yields, such that the protein production of identical reaction setups in varying vessel volumes can differ up to 25%. It has been theorized that this boost is attributed to an increased surface area of the reaction mixture, allowing for better oxygen exchange,[42] and others have further boosted yields by running CFPS reactions in large flat-bottom plates (Figure 6C).[9, 30]

FIGURE AND TABLE LEGENDS:

Figure 1: **Cost per microgram of protein produced across six cell-free protein synthesis platforms.** Our platform is compared among five different cell-free protein synthesis kits/platforms with varying productivity and pricing. Our sonication-based CFPS platform is much more cost-effective than most commercial kits and provides the ease of a kit while remaining cost-comparable to with other platforms like that of TX-TL (Z. Z. Sun).[43]

Figure 2: **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 for each following round. 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 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 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 four hours of incubation at 37°C. 1.5 mL microfuge tube on the right (successful reaction) shows visible fluorescence of

the sfGFP reporter protein at ~1 mg/mL. The negative control tube on the left lacking template DNA (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. 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 8mM to 14mM with 2mM deviations and extract volumes ranged from 3 µL to 7 µL with 1 µL deviations. The color code represents amount of protein 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+}. 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.

Figure 6: **Modifiable conditions for CFPS and the effects on reaction yields. A.** Extract productivity comparison based upon harvesting BL21*DE3 cells at various OD_{600} readings. Based on this plot, we recommend a harvest at an OD_{600} of 3.3 to produce at least 1 mg/mL of target protein. **B.** Comparison of two DNA maxiprep wash protocols with and without post-purification DNA-cleanups. pJL1-sfGFP plasmids underwent DNA maxiprep using the Invitrogen High Pure Plasmid Maxiprep Kit with one or two washes followed by a post-purification DNA-cleanup using the ThermoScientific GeneJET 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. **C.** 15 µL CFPS reactions performed in various vessels ranging from 2 mL to 0.6 mL microfuge tubes. Error bars for all data represent endpoint fluorescence quantified after 4 hours for three independent reactions, each measured in triplicate. All error bars represent 1 standard deviation of three independent reactions for each condition, each of which was quantified in triplicate.

DISCUSSION:

Cell-free protein synthesis has emerged as a powerful 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 (Figure 2). Additionally, once a stock of reagents and extract are produced, subsequent CFPS batch reactions can be set up, incubated, and quantified in just 5-6 hours. A single, 1 L cell growth can result in enough extract for at least 360 15 µL CFPS reactions, while preparations of the other cell-free reagents

can provide for hundreds to thousands of reactions. Our CFPS platform costs $0.021/µg protein or less (excluding the cost of labor and equipment), compared to $4.95/µg of the PURExpress® In Vitro Protein Synthesis Kit (NEB), and $0.014/µg for the TX-TL system presented in Sun, Z.Z. et al. (2013),[43] making our system competitively priced, highly productive, and accessible for new users (Figure 1).

We estimate startup costs to be ~$4500 for all reagents, not including specialized equipment. Person hours to complete this procedure is estimated to be ~26 hours for all reagent prep from the ground up. However, after large stocks of reagents have been prepared, demands on labor diminish substantially and costs per reaction are low. Additionally, as experience with the platform is gained, we recommend scaling up the size of the growth, extract preparation, and reagent preparation to maximize time efficiency. Given the large initial buy in cost compared to the low cost per reaction, we recommend the CFPS platform for applications in synthetic biology, high-throughput efforts, and reaction conditions that would conflict with the cell's biochemistry and viability.

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.[44] The differences that we have observed in extract productivity are generally diminished upon supplementation with T7 RNA Polymerase (RNAP) to a final concentration of approximately 16 µg/mL to each reaction. While BL21*DE3 is capable of expressing T7 RNAP during growth to augment the transcriptional machinery of the extract, exogenous addition of T7 RNAP is common among CFPS reactions to support optimal protein expression.[35, 36] Other variations to protein expression can be due to differences in the size, structure, and codon usage of the protein of interest, ribosome binding site, and the type of expression vector.[45, 46]

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.[47–49] 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.[44, 50, 51]

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Figure1

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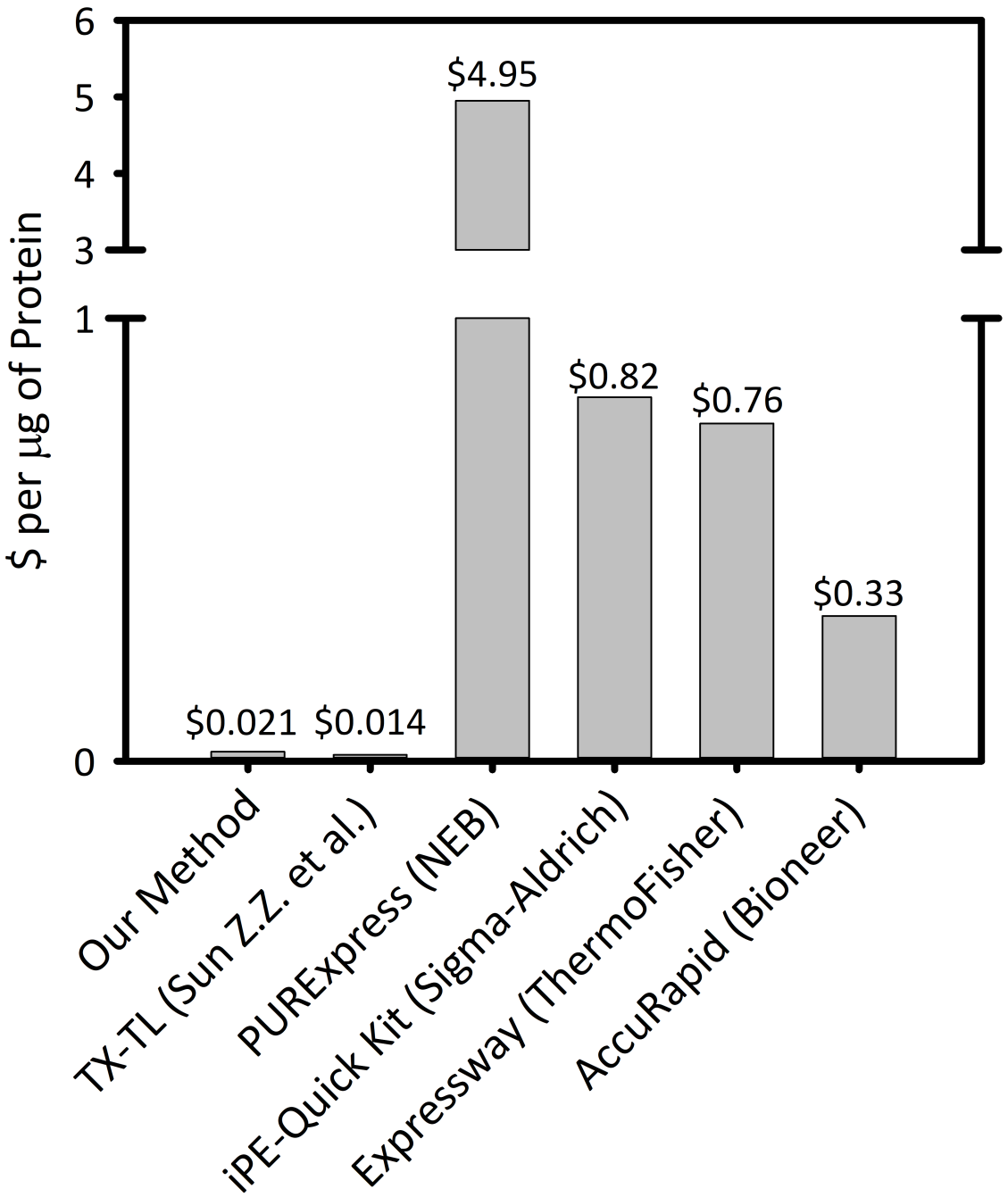

Figure2

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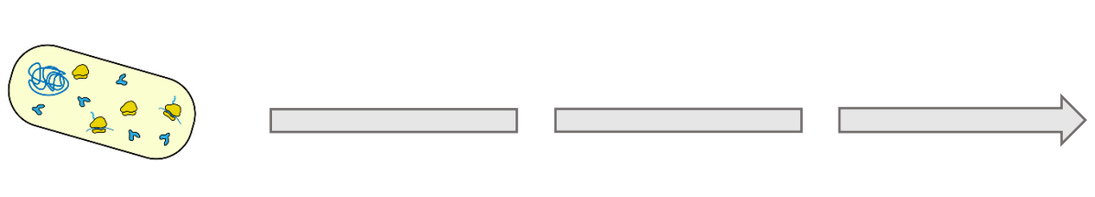

Day One

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

Prepare LB for overnight culture (autoclave)
2 hrs
Step 1.2

Day Two

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

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

Day Three

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

Pellet, wash, and freeze cells
1.5 hrs
Steps 1.16 – 1.26

Day Four

Prepare extract
2.5 hrs
Steps 2.1 – 2.10

Set up CFPS batch reactions
10 min per 4 rxns, wait 4 hrs
Steps 3.1 – 3.3

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

Figure3

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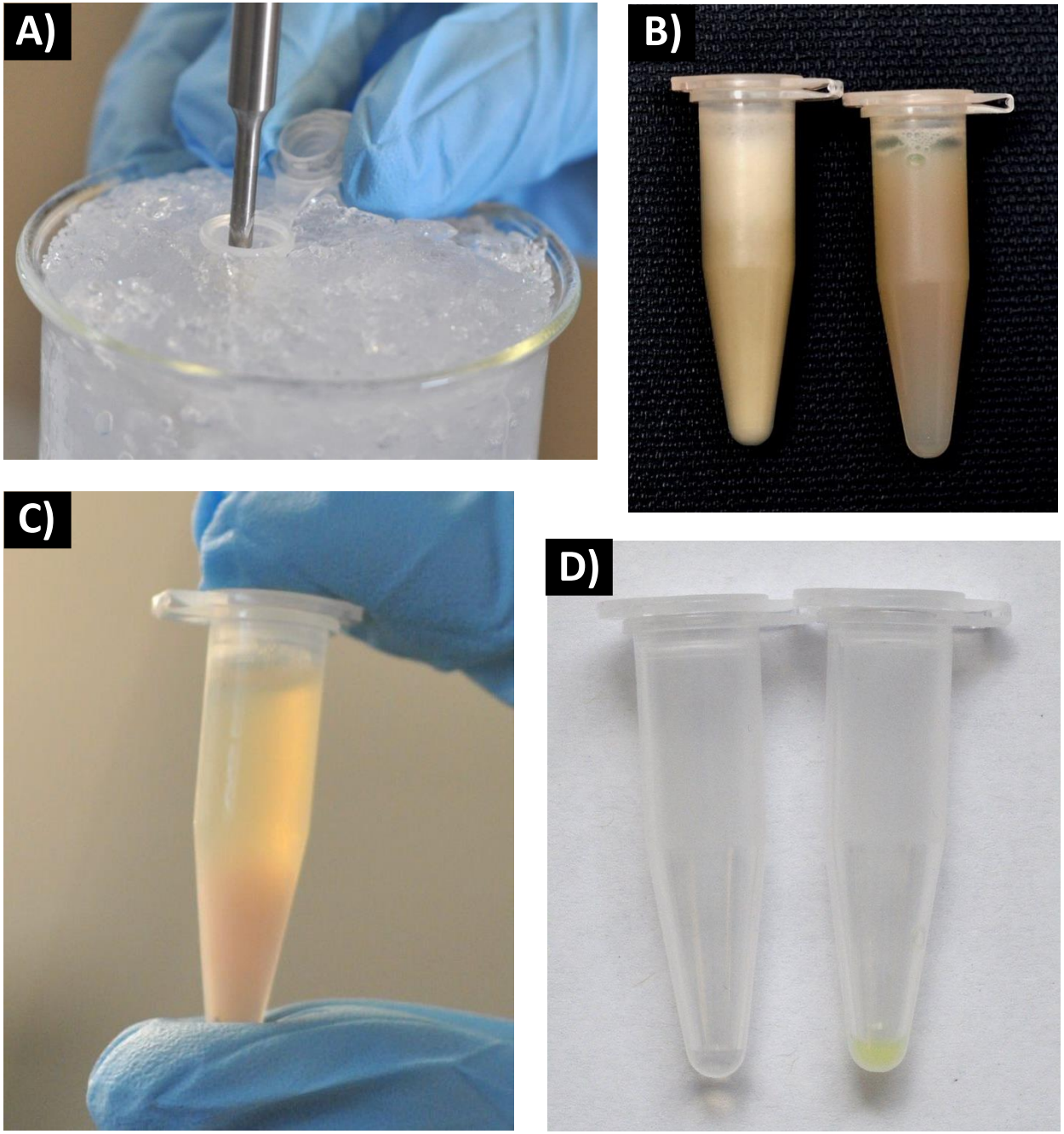

Figure4

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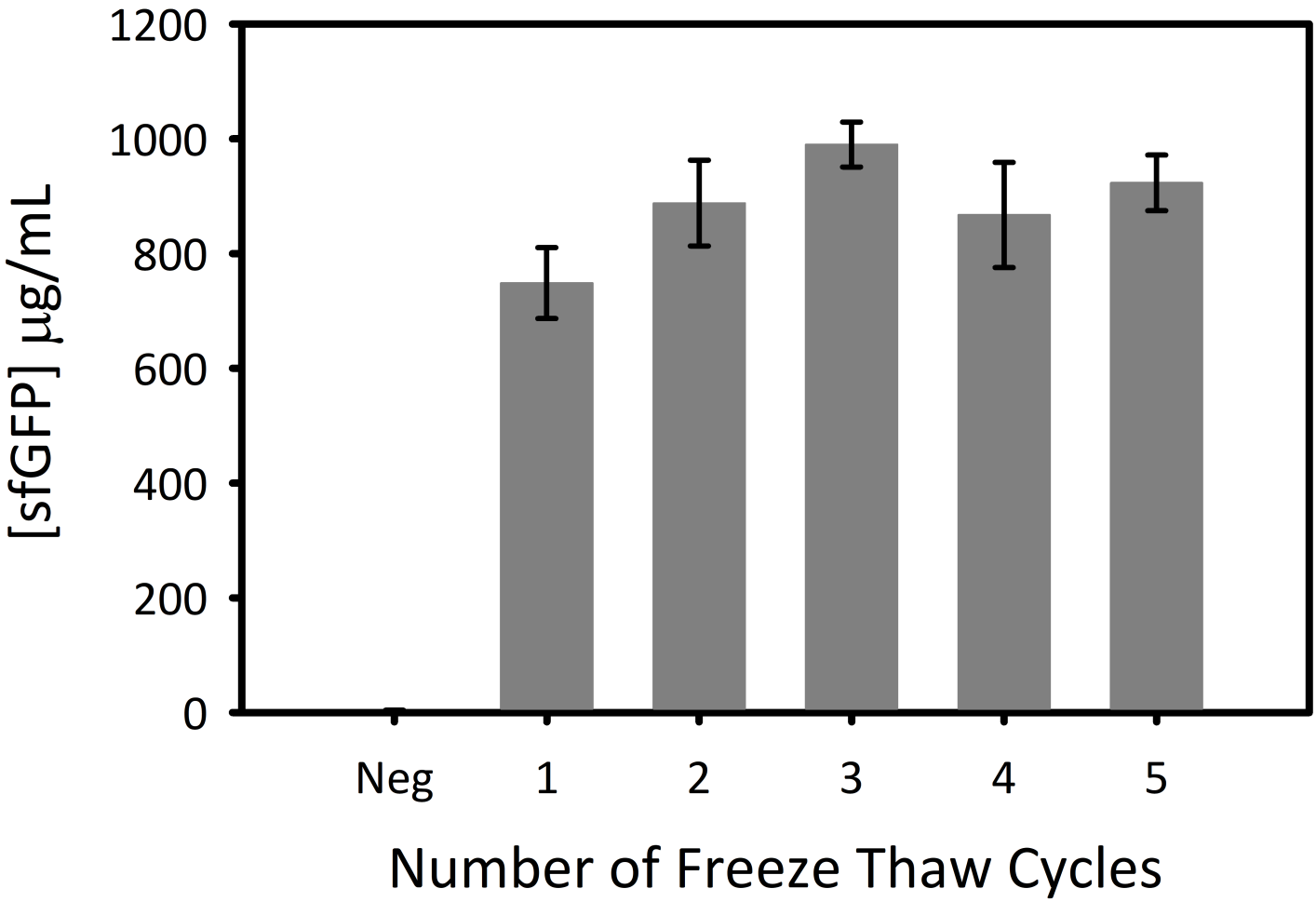

Figure5

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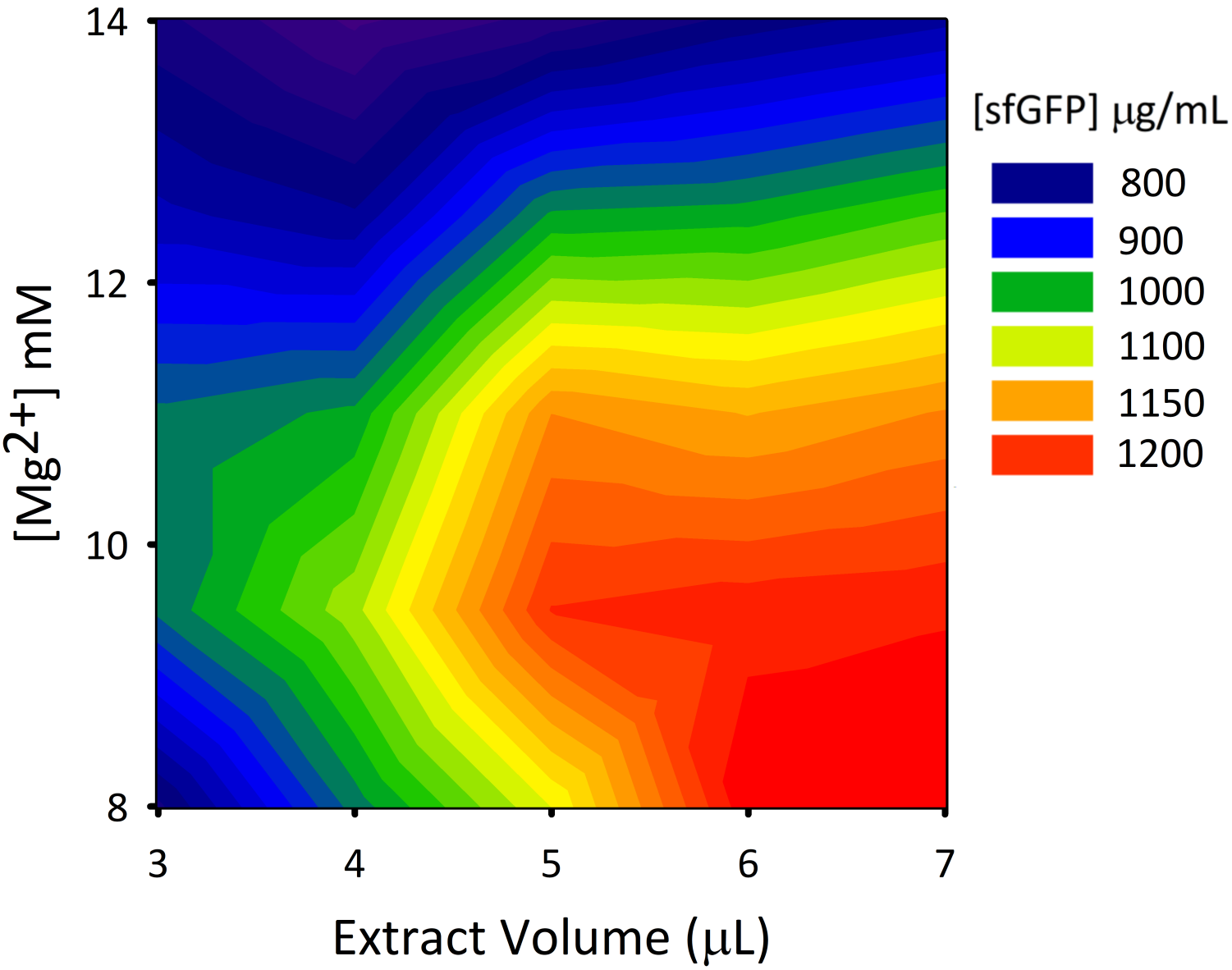

Figure6

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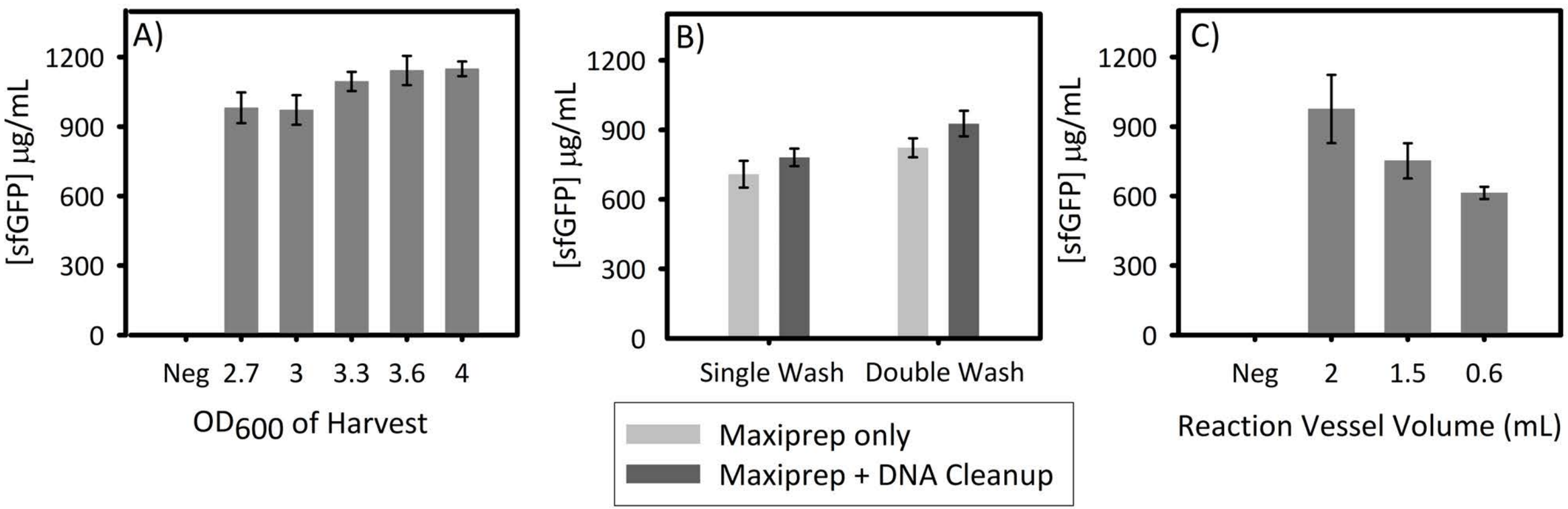

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Luria Broth	ThermoFisher	12795027	
Tryptone	Fisher	73049-73-7	
Yeast Extract	Fisher	1/2/8013	
NaCl	Sigma-Aldrich	S3014-1KG	
Potassium Phosphate Dibasic	Sigma-Aldrich	60353-250G	
Potassium Phosphate Monobasic	Sigma-Aldrich	P9791-500G	
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(OAc)	Sigma-Aldrich	09689-250G	
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
Mg(Glu)2	Sigma-Aldrich	49605-250G
PureLink HiPure Plasmid Prep Kit	ThermoFisher	K210007

Ultrasonic Processor	QSonica	Q125-230V/50HZ
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

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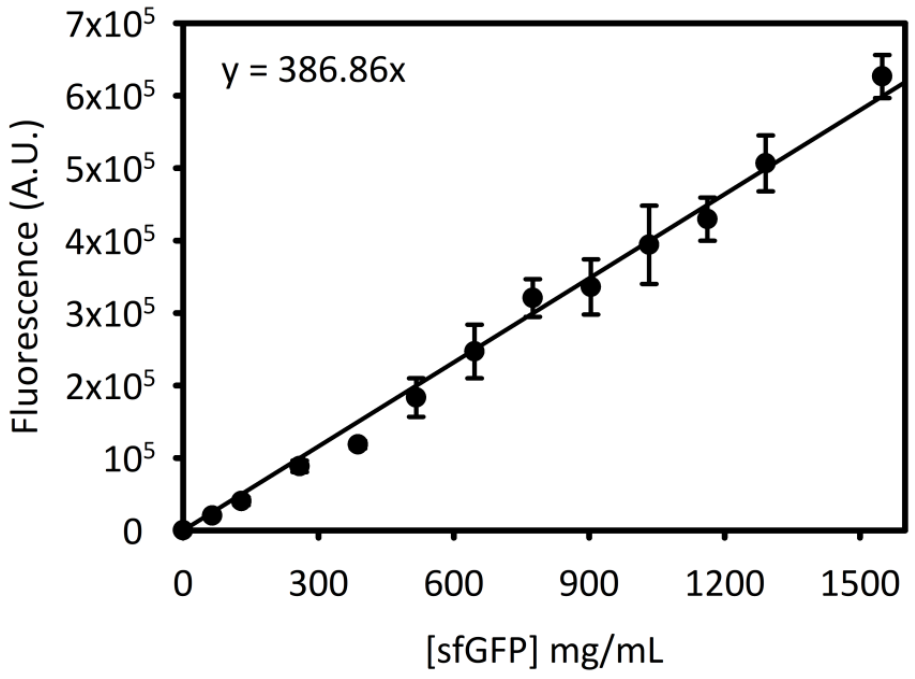
Preparation of a sfGFP Standard Curve

To prepare a standard curve for quantification of sfGFP, a single colony of BL21* PY71 sfGFP was inoculated into 5 mL of LB with Kanamycin in a round bottom test tube and grown overnight. The next day, this culture was centrifuged at 10,000 x g for 5 minutes 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 Purification column (Strep-TactinXT, IBA, Göttingen, Germany). The pellet was retrieved from the -80°C and 500uL of diluted Buffer W (provided with column) was added to the falcon tube, and the mixture was placed on ice for 20-30 minutes to thaw. The pellet was then completely resuspended through gentle vortexing, with resting periods, to minimize bubble formation. The resuspended mixture was transferred to an Eppendorf tube and sonicated for 10 seconds on then 10 seconds off, until the total Joules delivered was about 200J. The sample then centrifuged at 10,000 xg for 5 minutes, and the supernatant was collected and saved in a separate Eppendorf tube. Next, the column was prepared by allowing the storage buffer to drip through and equilibrating the column with 2 column volumes of Buffer W (400 uL). 500 uL of the supernatant was then applied to the column, and the flowthrough was collected. The column was washed with 5 column volumes of Buffer W (1000 uL). Elution was performed by adding 7 separate 0.5 column volumes of Buffer BXT (100 uL each), and collecting the elution in 7 separate Eppendorf tubes. Lastly, the column was washed with 3 column volumes of 10 mM NaOH (600uL) followed by 10 column volumes of buffer W (2000 uL). The column was then capped and buffer W was added for storage at 4°C. SDS-PAGE was performed on all collected samples to determine purity, and those sample with 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 His tagged sfGFP is 18910 1/M*cm. Serial dilutions of the stock sfGFP were then prepared, ranging from 0 to 800 ug/mL of protein. Each dilution was then quantified via a multi well plate fluorometer (Cytation5, BioTek, Winwooski, VT) as follows: 48ul 0.05 M HEPES pH 7.0 buffer and 2 uL of the respective sfGFP dilution were added to each well of a flat bottom 96-well half area black plate (Corning Incorporated, Kennebunk, ME), and each dilution was quantified in triplicate. Excitation and emission wavelengths for sfGFP fluorescence quantification were 485 and 510, respectively. A standard curve was created from the data of known concentrations in order to convert from fluorescence readings to concentration of sfGFP in ug/mL.



Supplemental Figure 1. **Standard curve for sfGFP on Cytation 5.** This curve was determined using the methods outlined above.

T7 RNAP Preparation

T7 RNA Polymerase was purified by affinity tag chromatography as previously described.[40]

Materials Preparation

- Tris(OAc): Prepare 6.057 g Tris Base and bring volume up to 50 mL and pH with Glacial Acetic Acid to pH 8.2.
- $Mg(OAc)_2$: Prepare 15.01 g $Mg(OAc)_2$ and bring final volume to 50 mL using nanopure water.
- K(OAc): Prepare 29.442 g K(OAc) and bring final volume to 50 mL using nanopure water.
- DTT: Prepare 1.54 g of DTT and bring final volume to 10 mL. Aliquot 1 mL of solution per tube and store at -80°C.
- S30 components: Prepare 1 mL Tris(OAc), 1 mL $Mg(OAc)_2$, 1 mL K(OAc), and 0.200 mL DTT and bring volume to 100 mL using nanopure water. Do not add DTT until day of use and store at 4°C.
- 2x YTP Media: Prepare 5 g NaCl, 16 g Tryptone, 10 g Yeast Extract, 7 g Potassium Phosphate Dibasic, and 3 g Potassium Phosphate Monobasic and bring volume to 375 mL using nanopure water. Adjust pH to 7.200 using 5 M KOH. Dilute solution to 750 mL. Autoclave in 2.5 L Tunair Baffled Flask at liquid 30 setting. Store at 37°C until use.

- D-Glucose Solution: Prepare 18 g D-Glucose and bring volume to 250 mL using nanopure water. Autoclave solution in 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 2.38 g IPTG and bring final volume to 10 mL using nanopure water. Store 1 mL aliquots 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 0.050 g and bring volume to 0.750 mL molecular grade water. Store at -80°C
- PEP: Prepare 0.206 g and bring 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 0.010 g and bring volume to 0.260 mL using molecular grade water. Store at -80°C.
- Putrescine: Prepare 0.011 g and bring final volume to 0.500 mL using molecular grade water. Store at -80°C.
- Spermidine: Prepare 0.018 g and bring final volume to 0.500 mL molecular grade water. Store at -80°C.
- HEPES: Prepare 2.38 g HEPES and bring volume to 10 mL using molecular grade water. Store at -80°C.
- Folinic Acid: Prepare 0.015g folinic acid and bring volume to 1.5 mL using molecular grade water. Store at -80°C.
- tRNA: Prepare 0.050g tRNA and bring volume to 1 mL using molecular grade water. Store at -80°C.
- 15X MasterMix: 180 uL ATP, 127.5 uL GTP, 127.5 uL CTP, 127.5 uL UTP (NTPs were purchased at a stock concentration of 100 mM), 47.22 uL folinic acid, and 51.18 uL tRNA. Store at -80°C.
- 15X Salt Solution: Prepare 0.290 g of Magnesium Glutamate, 0.120 g of Ammonium Glutamate, and 1.98 g of Potassium Glutamate and bring volume to 5 mL using molecular grade water. Store at -80°C.
- Oxalic Acid: Prepare 0.92 g and bring volume to 5 mL using molecular grade water. Store at -80°C.
- 20 Amino Acids: Prepare 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 add molecular grade water to a

final volume to 40 mL. Shake 15 min in 37°C incubator. pH of solution should be ~6.7. Store at -80°C.

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 * 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 * Add 2.1 µL to each 15 µL reaction	

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)

	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	4.70	2.20	2.10	5.00	1.00
Experimental	4.70	2.20	2.10	5.00	1.00