Characterizing and Improving Reaction Times for *E. coli*-based Cell-Free Protein Synthesis

Journal:	*ACS Synthetic Biology*
Manuscript ID	sb-2021-00195u.R2
Manuscript Type:	Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	Burrington, Logan; California Polytechnic State University College of Science and Mathematics, Biochemistry Watts, Katharine; California Polytechnic State University College of Science and Mathematics, Chemistry and Biochemistry Oza, Javin; California Polytechnic State University, Chemistry & Biochemistry

Characterizing and Improving Reaction Times for *E. coli*-based Cell-Free Protein Synthesis

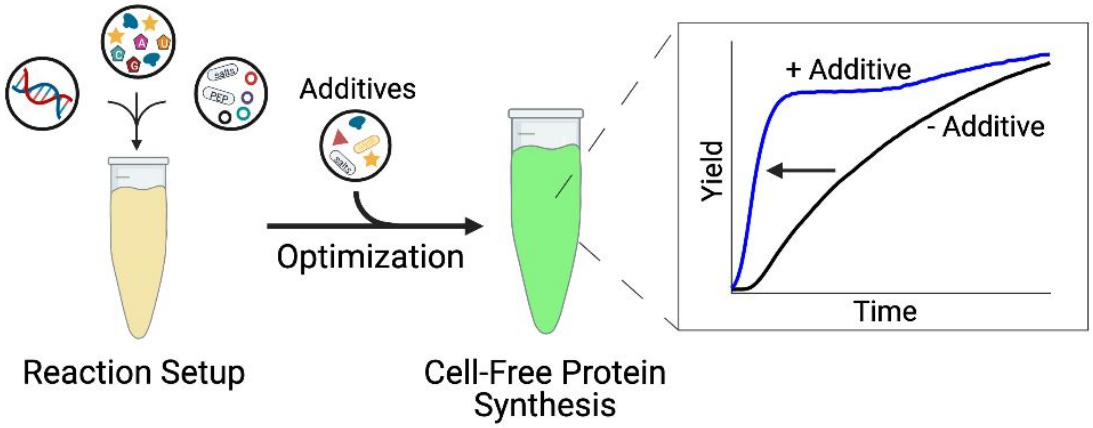
Logan R. Burrington, Katharine R. Watts, and Javin P. Oza*

Chemistry and Biochemistry Department, California Polytechnic State University, 1 Grand Avenue, San Luis Obispo, California 93407, USA
Center for Applications in Biotechnology, California Polytechnic State University, 1 Grand Avenue, San Luis Obispo, California 93407, USA

*Corresponding author: Javin P. Oza; joza@calpoly.edu; (805) 756-2265.

Abstract

Cell-free protein synthesis (CFPS) is a platform biotechnology that has enabled the on-demand synthesis of proteins for a variety of applications. Numerous advances have improved the productivity of the CFPS platform to result in high-yielding reactions; however, many applications remain limited due to long reaction times. To overcome this limitation, we first established the benchmarks for reaction times for CFPS across in-house *E. coli* extracts and commercial kits. We then set out to fine-tune our in-house extract systems to improve reaction times. Through the optimization of reaction composition and titration of low-cost additives, we have identified formulations that reduce reaction times by 30-50% to obtain high titers for biomanufacturing applications, and reduce times by >50% to reach the sfGFP detection limit for applications in education and diagnostics. Under optimum conditions, we report the visible observation of sfGFP signal in less than 10 minutes. Altogether, these advances enhance the utility of CFPS as a rapid, user-defined platform.



Keywords: Cell-free protein synthesis, *in vitro* transcription/translation, synthetic biology, rapid, rate, CFAI

Cell-free protein synthesis (CFPS) is a biotechnology platform that provides a robust user-defined strategy for the on-demand production of proteins to support a variety of applications.[1–13] Innovations of the CFPS platform have led to a large array of compatible extract types, energy systems, and DNA templates capable of producing high protein titers.[14–21] Despite these advancements, slow reaction rates remain a bottleneck for design-build-test-learn cycles, and also limit the effectiveness of CFPS in applications such as diagnostics and education in which rapid readouts are desired.

As we sought to implement CFPS reactions with the quickest readouts, we observed that while most publications report end-point measurements for CFPS reactions, time-course data is seldom collected, reported, or optimized. The dearth of time course data in literature poses a challenge in selecting the optimal extract types or reaction conditions for reaction time optimization. In this work we first sought to establish time-course data for a variety of *E. coli*-based CFPS systems, including both in-house reagents and commercial kits. We then sought to identify additives that speed up protein production for our CFPS systems.

Towards our first goal we chose to evaluate *E. coli*-based cell extracts due to their low-cost, reproducibility, and general prevalence as a broadly utilized extract type.[1,22,23] However, even among *E. coli* extracts, the preparation method and energy system can impact transcriptional, translational, protein expression, and folding rates, as well as overall protein yield.[15] Therefore, we quantified CFPS reaction times using three different in-house *E. coli* extracts prepared from cells grown in 2xYTPG media to OD_{600} 2.5 (2xYTPG), CFAI (cell-free autoinduction) media grown to OD_{600} 10 (CFAI 10), and CFAI media grown to OD_{600} 2.5 (CFAI 2.5) with CFPS reactions run using the PANOx-SP energy system. We also selected five commercially available *E. coli* extract-based kits that are capable of supporting T7-RNAP based expression.

We then focused our efforts on improving protein synthesis rates in 2xYTPG- and CFAI-based CFPS reactions by titrating 12 additives that have been previously reported to support transcription, translation, energy metabolism, or CFPS in general. As a result, we have identified formulations that notably improve the rate of protein synthesis in *E. coli*-based CFPS reactions (Figure 1). Our optimized reaction condition has a limit of detection by eye of 9.9 ± 0.6 min, offering a >80% time improvement over our traditional reaction setup. We believe these findings will be transformative for field applications such as diagnostics and education, in which CFPS is

used under time constraints. We also reduce the time required to produce high titers of protein product by >30%, supporting faster design-build-test-learn cycles and biomanufacturing applications. Together, the work reported herein provides useful benchmarks for the CFPS community as well as actionable information for improving reaction times.

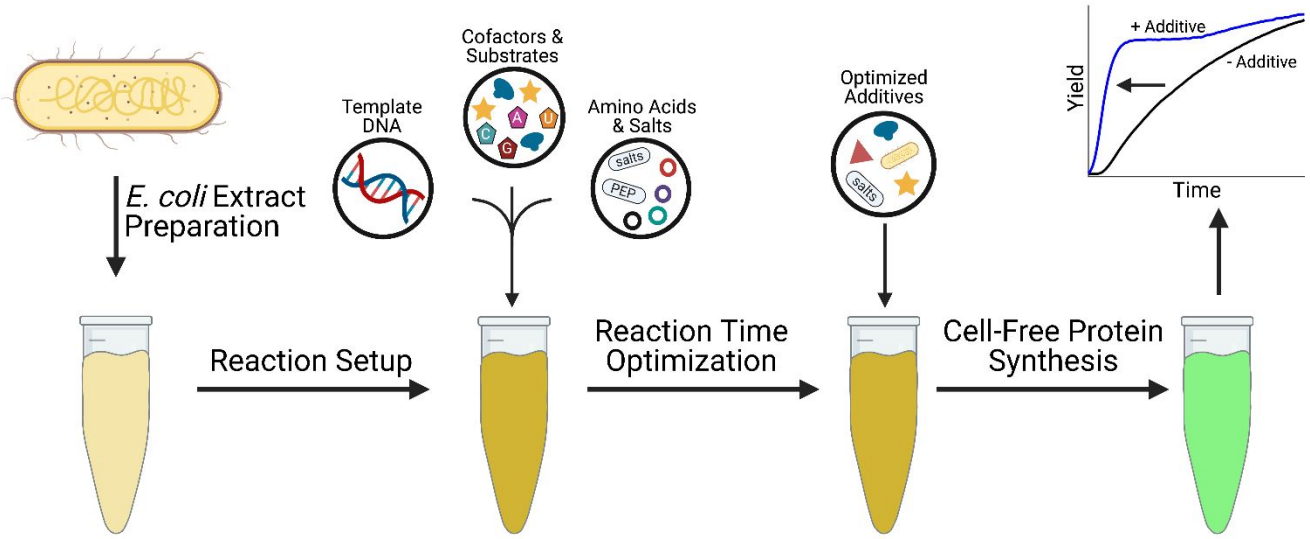


Figure 1. Generalized workflow for Cell-Free Protein Synthesis (CFPS) limit of detection optimization. *E. coli* extract is prepared and combined with the appropriate cofactors, substrates, amino acids, salts, and template DNA to initiate *in vitro* transcription and translation of sfGFP. The reaction mixture is then supplemented with additive(s) capable of decreasing the time to sfGFP detection by eye under ambient light (200µg/mL sfGFP) and blue light (100 µg/mL sfGFP) conditions.

RESULTS AND DISCUSSION

In developing a CFPS system that produces reporter sfGFP as rapidly as possible, we first evaluated a titration range of superfolder green fluorescent protein (sfGFP) to assess the minimum concentration that appears visually distinct to the eye compared to the negative control in ambient light conditions (Figure S1). We chose this condition to establish the limit of detection by eye since obviating the need for technical instrumentation makes this work most broadly applicable. While the limit of detection by eye can vary based on numerous factors, we observed 200 µg/mL (~2x10^6 RFU) of sfGFP to be the lower limit of detection for an average user observing sfGFP by eye under ambient indoor lighting. Given that portable, field-deployable fluorescence illuminators have been recently developed, we also establish a second benchmark concentration using the low-cost Fold-Illuminator device.[10,24–26] Illuminated using a blue light paired with a yellow acrylic filter, we subjectively established 100 µg/mL (~1x10^6 RFU) sfGFP as the lower limit of detection by eye when using the Fold-Illuminator. This lower limit of

detection is likely also relevant for users utilizing a hand-held blue light combined with a hand-held acrylic filter.[24] For these reasons, we use the time required to produce 200 μg/mL and 100 μg/mL of sfGFP as our two benchmarks for evaluating CFPS reaction rates. Since laboratory equipment can be significantly more sensitive for detecting sfGFP fluorescence, relative comparisons reported amongst extract types and the optimization advancements presented here will translate to lower limits of detection when instrumentation is available.

E. coli-based cell extracts are a well-established, low-cost, high yielding, and broadly adopted extract type that supports numerous CFPS applications.[22,23] For this reason, we sought to examine CFPS reaction time courses using three separate in-house *E. coli*-based extracts. Among these are extracts from cells grown in 2xYTPG (yeast, tryptone, PEP, glycerol) media to OD_{600} 2.5 (referred to as "2xYTPG"), CFAI (cell-free autoinduction) media grown to OD_{600} 10 (referred to as "CFAI 10"), and CFAI media grown to OD_{600} 2.5 (referred to as "CFAI 2.5"), each of which have been previously used to achieve high protein titers >1000 μg/mL sfGFP.[15] We had previously demonstrated that each cell-growth method results in unique metabolism within the cell extracts, here we evaluated whether these differences affect CFPS reaction rates (Figure 2A-G). For consistency, all experiments were performed with supplementation of the PANOx-SP energy system.[21] We observed that 2xYTPG extract produced sfGFP the fastest with 44.7 ± 2.7 min to 200 μg/mL and 29.4 ± 2.0 min to 100 μg/mL, followed by CFAI 2.5 extract reaching the benchmarks at 60.4 ± 2.4 and 39.4 ± 1.8 mins respectively, with the CFAI 10 extract being the slowest to reach our benchmarks at 84.2 ± 3.9 and 52.3 ± 0.7 mins (Figure 2A, 2B, Table S1-5). For broader utility to the cell-free community, we also purchased and tested five commercially available kits to establish the reaction rates among different *E. coli*-based extract systems, and their respective energy systems (Table 1). For 2xYTPG, CFAI 10, and CFAI 2.5, we titrated 12 components including: extract, circular DNA template, T7 RNA Polymerase, magnesium, polyethylene glycol 8000 (PEG8K), ATP, GTP, PEP, maltose, Triton X-100, trehalose, and betaine.

We provide our rationale for selecting specific additives based on prior works, however, the mechanisms by which they function are likely to be far more complex. To evaluate whether our reaction composition was limiting transcription we titrated the circular DNA template pJL1-sfGFP, T7 RNA Polymerase (T7-RNAP), and magnesium. Additionally, it has been shown that polyethylene glycol 8000 (PEG8k), a large organic molecule commonly used as a macromolecular crowding agent in CFPS reactions, both increases transcription rates and decreases translation rates in wheat germ CFPS systems.[20,25,27] If the CFPS reaction time is transcription-limited due to a specific reaction component, then increasing the concentrations of

that component should lead to faster mRNA output, and in turn more protein production per time. However, our data are not able to directly evaluate this hypothesis since we are only quantifying reporter protein production in this work. To evaluate whether our reaction composition was limiting translation, we directed our efforts to the PANOx-SP energy system components used in our reactions. The PANOx-SP (*P*EP, *A*mino acids, *N*AD, *Ox*alic acid, *S*permidine, *P*utrescine) system was developed in 2003 to produce high sfGFP titers through an ATP-regenerating metabolic cycle.[21] This system uses phosphoenolpyruvate (PEP) to actively regenerate adenosine triphosphate (ATP), the main energy source in protein synthesis alongside guanosine triphosphate (GTP).[28] Additionally, maltose is a sugar that has been shown to participate in an ATP-regenerating metabolic cycle, which also actively regenerates inorganic phosphate.[29] We hypothesized that if translation is energy-limited, the incorporation of higher ATP, GTP, PEP, and/or maltose concentrations would allow for faster protein synthesis reaction times. Lastly, we considered that there may be a rate-limiting process beyond transcription and translation. To address this, we screened additives that have demonstrated positive influences on CFPS yields and/or rate.[14,25,30,31] For example, Triton X-100 is a non-ionic detergent that has been found to have a stabilizing effect on protein.[32] Previous work has found that using Triton X-100 in an *E. coli*-based cell-free system is able to improve the production rate of Cecropin P1 over a two hour reaction time, and results in a 1.8x yield increase.[33] This same work also demonstrated that translation, rather than transcription, led to these marginal benefits. We also examined trehalose and betaine, two additives that have been previously shown to benefit cell extracts.[25]

2xYTPG Results. Here we report our optimization results using extract from cells grown in 2xYTPG (*Y*east, *T*ryptone, *P*hosphate, *G*lucose) to OD_{600} 2.5. 2xYTPG media is a traditional *E. coli* extract preparation method capable of supporting high protein titers in CFPS and has become the standard growth medium for many applications of *E. coli*-based CFPS.[15] For this reason, we wanted to establish a benchmark reaction time course for CFPS with 2xYTPG extract using the PANOx-SP energy system. CFPS reactions commonly consist of 33% v/v cell extract; under these standard conditions, our positive control reactions using 2xYTPG extract reach our 200 μg/mL sfGFP limit of detection in 45.1 ± 2.1 min. Increasing extract % within the CFPS reactions resulted in a notable improvement in the rate of protein production, reducing the time to 200 μg/mL sfGFP to 36.0 ± 1.0 min. Reaction rates did not improve when extract % was increased beyond 40% (Figure 2A, Table S1). We also observed increased overall yield (end-point measurement after 18 hours) of sfGFP as extract % increased, with 40% v/v yielding 112% as compared to the positive control and 53% v/v attaining 119%. With our positive

controls comprising the standard 33% extract, all subsequent additives optimizations were done with 40% extract. Notably, our extracts contain T7-RNAP which is expressed during cell growth; therefore, increasing extract concentration within a CFPS reaction increases both transcription and translation machinery.

We observed that supplementing circular DNA template pJL1-sfGFP or T7-RNAP did not notably improve the rate of protein production or the overall yield of sfGFP (Table S1). These results suggest that the reaction components necessary for transcription are not limiting. We then titrated various components of the PANOx-SP energy system. Here, we did not observe notable improvements in the rate of protein production upon titrating GTP or PEP, leading to the conclusion that GTP and PEP are all already rate-optimized in our reaction conditions (Table S1). PEP concentrations higher than 41.25 mM had a detrimental effect on reaction rate, but these slower reactions resulted in an increase in overall yield at the endpoint, producing 110% of the control with 57.75 mM PEP. We did observe notable improvements in protein production upon supplementation of ATP. We found a final reaction concentration of 2.4 mM ATP to be optimal, reducing the time to 200 μg/mL sfGFP to 27.1 ± 2.4 mins along with an increase in overall yield of 110% as compared to the 1.2 mM ATP control (Figure 2B). Maltose also did not improve the rate of protein production. We conclude that the system is energy-limited in the short-term; addressing this limitation allows for a more rapid reaction without negatively affecting the characteristic long-lived reactions supported by the PANOx-SP system while still maintaining the production of high protein titers.

Lastly, we observed that supplementing PEG8K to a final concentration of 0.5% w/v reduced the time to 33.2 ± 1.5 mins for producing 200 μg/mL sfGFP (Figure 2C). Triton X-100 also improved the time required to reach 200 μg/mL sfGFP, reaching the limit of detection in 26.9 ± 2.2 mins with 50 CMC (11.50 mM) (Figure 2D). However, the faster reaction upon Triton X-100 supplementation comes at a trade-off with total yield, resulting in a ~60% drop in overall sfGFP observed at the reaction end-point. In terms of utility, Triton X-100 ultimately forces the user to consider whether the improved reaction speed obtained with Triton X-100 is worth the decrease in total yield for their specific application.

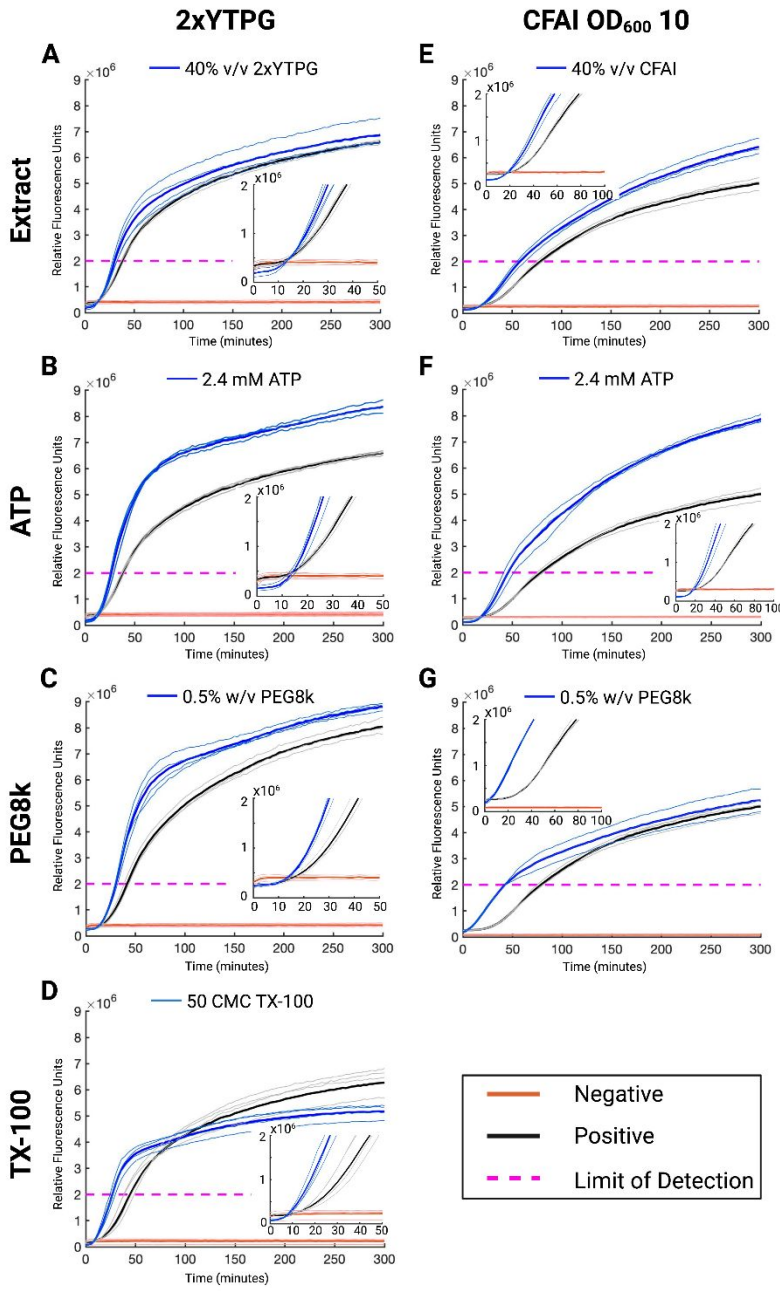


Figure 2. Time-course fluorescence readings for 2xYTPG (left) and CFAI OD_{600} 10.0 (right). Traditional CFPS (**A, B**), and 40% v/v extract with the indicated additive (**C-G**) (blue), with a negative control (orange). The 200 µg/mL ($2x10^6$ RFU) limit of detection is depicted (dash). Insets provide a zoom-in view of the control and treatment conditions until the limit of detection is reached. Each panel lists the corresponding additive and its final concentration in the reaction. Data is plotted as an average of a minimum of N=3, maximum N=4 experimental replicates. The average of each data set is indicated by a darker color. Outliers were identified by a Grubbs' Test on time to $2x10^6$ RFU at a 90% confidence interval. Negative conditions have no pJL1-sfGFP DNA template or additive present. CFAI 2.5 is not included due to no observed rate benefits from any additive (other than higher % v/v extract).

Following single-additive tests, we combined the best single-additive conditions to determine whether CFPS rates would improve in a combinatorial manner. For this analysis, we selected 2.4 mM ATP, 0.5% w/v PEG8k, and 50 CMC Triton X-100 since they individually resulted in the most notable improvements to CFPS rates. Combining 2.4 mM ATP and 0.5% w/v PEG8k reduced the time to the 200 μg/mL sfGFP limit of detection to 23.6 ± 2.5 mins, or 15.7 ± 1.9 mins to 100 μg/mL sfGFP (Figure 3A, 3B, Table S2). Combining 2.4 mM ATP and 0.5% w/v PEG8k with 50 CMC Triton X-100 further reduced the time to 200 μg/mL sfGFP to 19.8 ± 1.4 mins, and 13.3 ± 0.8 mins to 100 μg/mL sfGFP. We also observed that shaking the reaction helps improve CFPS rates. With plate shaking at 567 cpm, we are able to achieve the 100 μg/mL sfGFP limit of detection within 12.2 ± 0.4 mins upon ATP and PEG8k supplementation, and within 9.9 ± 0.6 mins upon ATP, PEG8k, and Triton X-100 supplementation. 2xYTPG extracts supplemented with ATP and PEG8k also resulted in 114% of the positive control when quantified at the reaction end-point. When 2xYTPG is supplemented with the combination of ATP, PEG8k, and Triton X-100, improved reaction rates come at the cost of reduced protein yield, producing only 47% sfGFP compared to the positive control at the end-point.

The optimization of 2xYTPG extract %, ATP and PEG8k concentrations reduced the time to reach the limits of detection of sfGFP from 45.1 ± 2.1 to under 20 mins. We considered that CFPS is a multifaceted process with covariates affecting the observed rate of sfGFP fluorescence. As we modified the reaction composition, we sought to evaluate whether other reaction conditions had shifted from their respective optima to becoming rate limiting, restricting further optimization. Starting with 40%v/v 2xYTPG extract containing 2.4 mM ATP and 0.5% w/v PEG8k, we re-optimized magnesium concentrations and T7-RNAP. Here, we did not observe additional improvements to CFPS rates (Table S2).

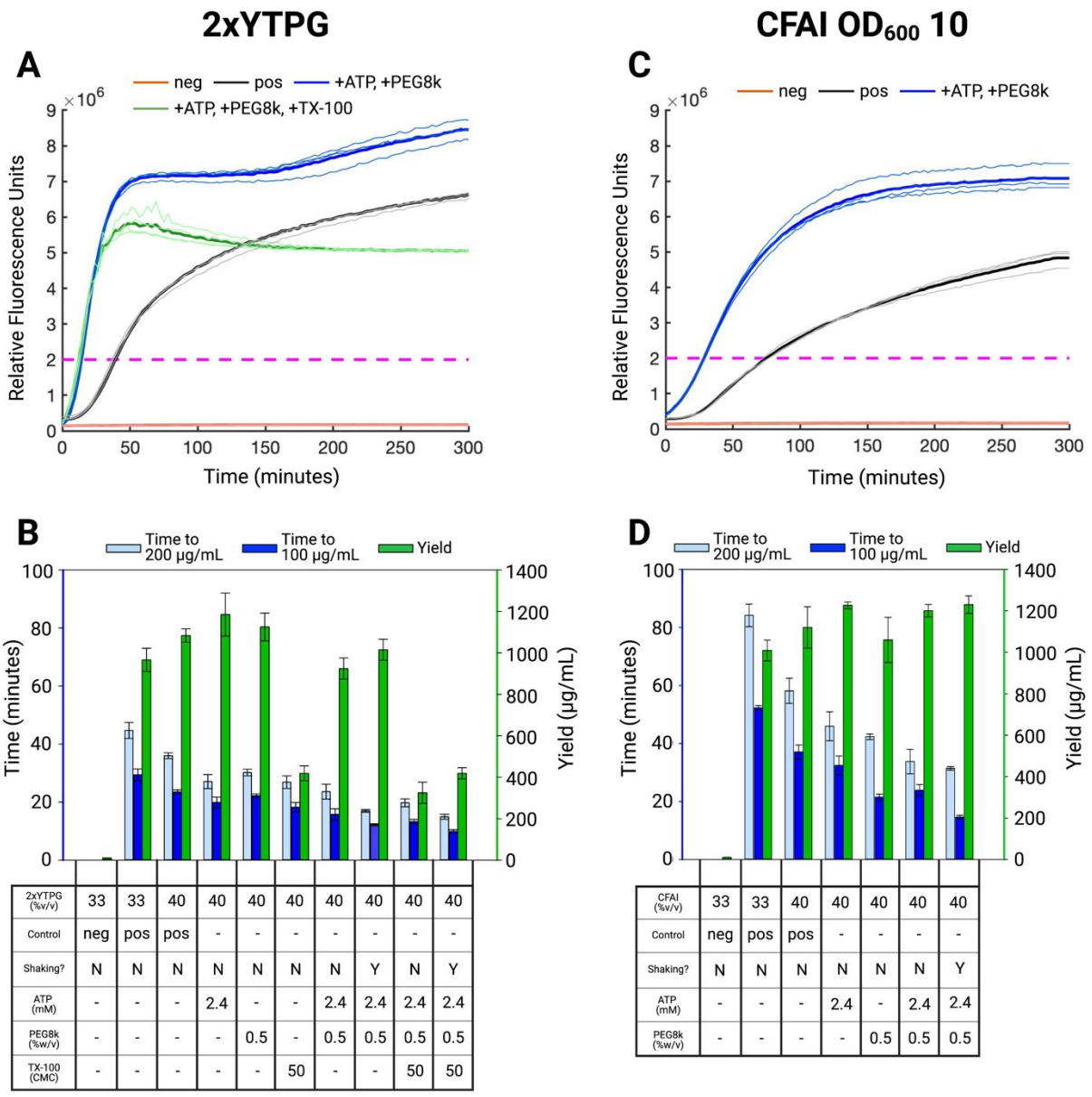


Figure 3. Single and multi-additive titrations that resulted in faster times to the limit of detection for **A/B)** 2xYTPG extract and **C/D)** CFAI OD_{600} 10 extract. **A/C)** 300 minute time-course fluorescence data from our traditional 33% v/v extract setups (black) as compared to our most optimal rate-boosting setups (blue, green). The 200 µg/mL limit of detection standard is depicted (dash). The average of each data set is indicated by a darker color. **B/D)** Left Y-axis represents time to 100µg/mL and 200 µg/mL sfGFP from reaction start; right Y-axis represents total reaction yield 18 hours after reaction start. Listed concentrations are representative of the final CFPS reaction. Negative conditions have no pJL1-sfGFP DNA template or additive present. All data is presented with a minimum of N=3, maximum N=4 experimental replicates.

CFAI Results. Here we report our titration results using extracts from cells grown in cell-free autoinduction (CFAI) media. Cells grown in CFAI media to OD_{600} 10 (CFAI 10) is a recently developed *E. coli*-based extract preparation method. CFAI provides an 'Instant Pot' type utility for the growth of cells suitable for extract preparation, where the higher OD_{600} harvest results in

a >400% increase in extract production without sacrificing CFPS productivity.[15] The reduced production costs and improved reproducibility that CFAI provides makes the method well suited for supporting education and diagnostics applications, especially in resource-limited environments, while also being compatible with biomanufacturing applications. In our optimization efforts, we first optimized extract concentration in a titration range of 33-53% v/v CFAI 10 in CFPS reactions. Interestingly, while we observed end-point yields to be similar between 2xYTPG and CFAI 10 reactions, CFAI 10 required notably more time to reach our limits of detection (Figure 2E, 3C, 3D, Table S1, S3). CFAI 10 extract at 33% v/v reached the 200 μg/mL sfGFP limit of detection in 84.2 ± 3.9 mins, or 52.3 ± 0.7 mins to 100 μg/mL. As with 2xYTPG, reaction rate increased with increasing extract % to an optimum of 40% v/v extract, reaching 200 μg/mL sfGFP in 58.2 ± 4.3 mins and 100 μg/mL in 37.1 ± 2.4 mins. All subsequent optimizations were performed using 40% v/v CFAI 10 extract.

As with 2xYTPG, CFAI 10 reaction rates did not improve upon the titration of circular pJL1-sfGFP DNA template, T7-RNAP, GTP, PEP, maltose, trehalose, or betaine (Table S3). However, we did observe an increase in overall yield of 137% compared to the positive control with 20 mM trehalose and 330 mM betaine in combination. Unlike 2xYTPG, CFAI 10 did not benefit from Triton X-100 supplementation. Consistent with 2xYTPG, reaction rates of CFAI 10 improved upon optimization of ATP and PEG8k (Figure 2F, 2G, 3D). The optimal final reaction concentration of ATP was 2.4 mM, which improved the time to reach 200 μg/mL sfGFP to 45.97 ± 5.08 mins; 100 μg/mL was reached in 32.4 ± 3.3 mins. The optimal concentration of PEG8k was 0.5% w/v, improving the time to 42.3 ± 1.0 mins to reach the 200 μg/mL sfGFP limit of detection, or 21.5 ± 1.0 mins to 100 μg/mL.

We then combined the best single-additive conditions to determine whether CFPS rates would improve in a combinatorial manner with CFAI 10 as we observed with 2xYTPG. For this analysis, we selected 40% v/v CFAI 10 extract, 2.4 mM ATP, and 0.5% w/v PEG8k, which in combination reduced the time to reach 200 μg/mL sfGFP to 33.8 ± 4.2 mins, and 23.9 ± 1.9 mins to reach 100 μg/mL sfGFP (Figure 3C, 3D, Table S4). Reaction shaking also improved reaction rates for CFAI 10 extracts, reducing the time to reach 200 μg/mL sfGFP to 31.4 ± 0.6 mins, and 14.67 ± 0.5 mins to reach 100 μg/mL sfGFP. For total sfGFP yield quantified 18 hours after reaction start, the optimized reaction conditions produced 113% sfGFP compared to the CFAI 10 positive control. As with 2xYTPG, we re-optimized magnesium concentrations and T7-RNAP. Again, titrations of each did not lead to any additional improvements to the CFPS reactions.

While the utility of CFAI is maximized for high OD harvests, it can also support workflows developed for 2xYTPG by harvesting cells at OD_{600} 2.5. Since some may find utility in CFAI 2.5, we sought to establish its reaction time course and optimize its reaction rate. CFAI 2.5 at 33% v/v reached the 200 μg/mL sfGFP limit of detection in 60.4 ± 2.4 mins, or 39.4 ± 1.8 mins for 100 μg/mL. CFAI 2.5 also benefited from increased extract concentration, with an optimum at 40% v/v that reduced the times to 50.7 ± 3.3 and 32.2 ± 2.3 mins, respectively. (Table S5). Following the same optimization process as with 2xYTPG and CFAI 10, we were surprised to observe that none of our additives had any notable effect on the reaction time of CFAI 2.5, including ATP and PEG8k. We had previously performed metabolomics analysis to characterize the metabolism of CFAI extract in CFPS.[15] While we identified unique differences amongst 2xYTPG, CFAI 2.5, and CFAI 10, our current understanding is not sufficient to propose why the rate limiting conditions would be distinct for CFAI 2.5 compared to CFAI 10 or 2xYTPG.

Commercial Kit Results. Since many researchers may opt to purchase their *E. coli*-based CFPS reagents, we evaluated five commercially available extract kits from Arbor Biosciences, Bioneer, Invitrogen, NEB, and Promega in an attempt to establish their reaction rates. The specific kits were selected because they utilize *E. coli*-based extracts, and support T7-RNAP driven transcription for compatibility with our circular DNA template pJL1-sfGFP. These kits are likely to vary in their energy systems, which are proprietary. Therefore, our goal here was simply to establish benchmarks for the broader community and not to improve kit performance. We implemented each kit based on their specifications; when methodological details were absent, we selected parameters to match those of our 2xYTPG reactions (Table S6). As expected, there is a large variation in reaction rates, ranging in time to 200 μg/mL sfGFP from 24.7 ± 0.5 to 106.8 ± 6.8 mins, and time to 100 μg/mL ranging from 12.1 ± 0.6 to 51.3 ± 0.5 mins (Figure S2, Table 1). Total sfGFP yield also ranged from 276 ± 16 to 964 ± 69 μg/mL. We anticipate that establishing these benchmarks will allow researchers to select a kit that is suitable for their applications when producing 2xYTPG or CFAI extracts in-house is not an option.

Table 1. Evaluation of Commercial *E. coli*-based Extract Kits*

Company	Arbor	Bioneer	Invitrogen	NEB	Promega
Kit	MyTxTl	AccuRapid Midi Protein Expression	Expressway Mini Expression	Cell-free *E. coli* Protein Synthesis	*E. coli* S30 Extract System
Time to 200 µg/mL (min)	113.3 ± 14.2	42.8 ± 4.9	29.7 ± 2.1	24.7 ± 0.5	23.3 ± 0.6
Time to 100 µg/mL (min)	51.3 ± 0.5	27.3 ± 3.0	18.0 ± 1.3	12.1 ± 0.6	16.0 ± 0.2
Yield (µg/mL)	964 ± 69	611 ± 25	276 ± 16	833 ± 58	661 ± 43
Shaking**	No	No	Yes	Yes	Yes

* All reactions were run with circular pJL1-sfGFP plasmid as the DNA template for consistency. This may have deviated from specific manufacturer recommendations for template choice, which are highly variable, or non-existent.
** Shaking was dictated by manufacturer's instructions.

CONCLUSION

In this work, we sought to map the reaction time courses of *E. coli* extract-based CFPS reactions. CFPS has proven capable of producing high protein titers *in vitro*; however, real-world applications of this biotechnology including biosensing and education remain limited due to the slow reaction times of the biochemical processes involving transcription, translation, and energy metabolism. In order to achieve a time-optimized CFPS setup, we established the reaction times of 2xYTPG, CFAI 10, and CFAI 2.5 extracts driven by the PANOx-SP energy system, and screened several additives for improving reaction rates while also evaluating their impacts on overall yield. Our initial efforts involved mapping the reaction rates of the 2xYTPG, CFAI 10, and CFAI 2.5 extracts, where we discovered that 40% v/v extract generated the most optimal rates for each case. Through metabolomics analysis, we had previously characterized the metabolic differences between 2xYTPG and CFAI extracts, as well as their resulting CFPS reactions.[15] Based on that work, we were not surprised that CFAI 10 at 33% v/v was notably slower than 2xYTPG. We do find it interesting that their respective optima were achieved using similar additives formulations, possibly due to the overlap in the PANOx-SP system. While studying the impact of various additives on CFPS reaction times, we observed that increased ATP

concentrations of up to 2.4 mM and a PEG8k concentration of 0.5% w/v notably improved the reaction times individually and in combination for both 2xYTPG and CFAI 10. We also report a reaction time improvement when supplementing 50 CMC Triton X-100 into 2xYTPG extract, attaining our fastest time to the sfGFP limit of detection at 9.9 ± 0.6 mins when using 40% v/v 2xYTPG extract, 2.4 mM ATP, 0.5% w/v PEG8k, and 50 CMC Triton X-100. The benefits of Triton X-100 on reaction times correspond to notable decreases in overall yield of the CFPS reactions. In terms of overall cost, these additives are highly cost-efficient, costing less than a penny/30 µL reaction for 0.5% w/v PEG8k and 50 CMC Triton X-100, and under $0.05/30 µL reaction for 2.4 mM ATP. (Table S7).

The discoveries and advancements presented here improve the utility of CFPS as a rapid, low-cost, and accessible platform capable of producing high protein titers on-demand. Our screening of 12 additives provides some insights into rate limiting reagents for the PANOx-SP energy system and rate limiting steps for CFPS. Based on the observations that ATP, PEG8k and Triton X-100 were the most impactful additives, we posit that energy and molecular crowding were the primary limitations in the systems we tested. It is possible that translation rates of the ribosome may have also increased, but our data are not sufficient to validate or nullify this hypothesis. To the extent that translation rates are affected, it is important to note that faster protein synthesis rates could affect protein folding, having undesired effects on the protein of interest which may in turn limit some applications.[34–37] As such, future work will benefit the platform by taking a more in-depth look at a variety of translation-regulating additives, including protein-based translation factors, to further improve the reaction rates of cell-free protein synthesis.

Methods

Extract Growth

For CFAI extract OD_{600} 10 preparation, an overnight culture of BL21* (DE3), started from a loopful of colonies on an LB agar plate, was inoculated into a 2 L baffled flask containing 1 L CFAI Media (14 g K2HPO4, 6 g KH2PO4, 20 g Tryptone, 5 g Yeast extract, 5 g NaCl, pH 7.2/960 mL, 6 mL 100% Glycerol, 0.5 g Glucose, 4 g Lactose/40 mL). Growth was then incubated at 30°C, 250 rpm overnight for ~14 hours to OD_{600} 10. CFAI OD_{600} 2.5 extract was prepared by inoculating a single BL21* (DE3) colony into a 125 mL Erlenmeyer flask containing 50 mL LB broth, followed by incubation at 37 °C, 200 rpm for ~12 hours. This seed culture was

then supplemented into 1 L CFAI Media to OD_{600} 0.1, and incubated at 30 °C, 250 rpm to OD_{600} 2.5. Cells were then harvested.

For 2xYTPG extract preparation, an overnight culture of BL21* (DE3), started from a single colony on an LB agar plate, was inoculated into a 2 L baffled flask containing 1 L 2xYPTG media (5 g NaCl, 16 g Tryptone, 10 g Yeast extract, 7 g KH_2PO_4, 3 g $KHPO_4$, pH 7.2/750 mL, 18 g Glucose/250 mL) to OD_{600} 0.1. Growth at 37 °C and 200 rpm was monitored until OD_{600} 0.6, whereupon T7-RNAP expression was induced by addition of IPTG to a final concentration of 1 mM. Once OD_{600} 2.5 was reached, cells were harvested.

Extract Harvest

1 L cell cultures were transferred into cold 1 L centrifuge bottles, and centrifuged at 4°C, 5,000 xg, for 10 mins. Pellets were transferred into cold, tared, 50 mL falcon tubes, and resuspended in 45 mL S30 buffer (10mM Tris OAc, pH 8.2, 14mM $Mg(OAc)_2$, 60 mM KOAc, 2 mM dithiothreitol) by intermittent vortexing while on ice. The resuspension was then centrifuged at 4 °C, 5,000 xg, for 10 minutes, followed by supernatant decanting. The remaining pellet was flash frozen with liquid nitrogen and stored at –80 °C until extract preparation.

Extract Preparation

Frozen cell pellets were resuspended in 1 mL S30 buffer per 1 g of cell mass. Resuspended cells were moved into 1.5 mL centrifuge tubes in 1.4 mL aliquots. The resuspension was sonicated using a Qsonica Q125 Sonicator with a 3.175 mm probe, with the cell resuspension resting in an ice water bath. Three pulses of 45s on, 59s off at 50% amplitude were performed, with inversion during the off cycles. Immediately after sonication, 4.5 µL dithiothreitol was spiked into the extract and the tube was inverted several times to mix. 2xYTPG extract was centrifuged at 4 °C and 12,000 xg for 10 min, and CFAI extract was centrifuged at 4 °C and 18,000 xg for 10 min. The supernatant from each 1.5 mL tube was then collected, flash frozen using liquid nitrogen, and stored at –80 °C until ready for use in CFPS reactions.

DNA Purification

pJL1-sfGFP was purified from DH5α cells using an Invitrogen PureLink™ HiPure Plasmid Maxiprep Kit. The DNA was eluted with ultra-pure water rather than the provided TE buffer to increase compatibility with CFPS reactions.

CFPS Reactions

Each condition was performed in experimental triplicate or quadruplicate. In-house pre-mixes containing the necessary energy system and cofactors were used. Unless noted otherwise, the final CFPS reaction contained the following concentrations of each reagent: 33.33% v/v cell extract, 16 ng/μL pJL1-sfGFP plasmid, 1.20 mM ATP, 0.850 mM GTP, 0.850 mM UTP, 0.850 mM CTP, 22.67 mg/mL Folinic Acid, 113.73 mg/mL tRNA, 0.33 mM Nicotinamide Adenine Dinucleotide (NAD), 0.27 mM Coenzyme A (CoA), 4.00 mM Oxalic Acid, 1 mM Putrescine, 1.50 mM Spermidine, 57 mM HEPES buffer, 12 mM $Mg(Glu)_2$, 10 mM $NH_4(Glu)$, 130 mM K(Glu), 2 mM of each canonical amino acid, 33 mM Phosphoenolpyruvate, and ultra-pure water to a final volume of 30 μL. Water was replaced by additives for all additive testing. Commercial extract kit testing was performed with the pJL1-sfGFP plasmid according to the manufacturers instructions. Reagents were pipetted directly into a cold, half-volume black, opaque bottomed 96-well plate. pJL1-sfGFP template was added last, to minimize the time between reaction start and real-time tracking. To minimize evaporation, ~200 μL distilled water was added into each open spacing between plate wells, a lid was placed on the 96-well plate, and the wells lining the edges of the plate were not used. Reactions were then quantified at 37°C in real-time for five hours, removed, left at room temperature for 13 hours, and end-point quantified.

Quantification and Data Analysis

Reactions were quantified every three mins for five hours in a Cytation^{TM} 5. Fluorescence intensity was read at an absorption wavelength of 485 nm and an emission wavelength of 510 nm. For reactions that required shaking, linear plate shaking was performed at a frequency of 567 cpm. The resulting absorbance values were graphed as a function of time, where the four replicates per condition were averaged to generate one absorbance versus time trendline per condition. Times to $2x10^6$ relative fluorescence units (RFU) were then determined based on these curves and averaged for each condition replicate; outliers were identified and removed using a Grubbs Test for both minimum and maximum values at a 90% confidence interval. Each condition sustained a minimum of N=3 and a maximum of N=4 samples. The ensuing times and standard deviations were noted. This procedure was repeated for determining the time to $1x10^6$ RFU.

End-point quantification of sfGFP took place in half-volume black, opaque bottomed 96-well plates. In each well, 2 µL of reaction solution was added to 48 µL of 50 mM HEPES buffer, pH 7.2. Each individual reaction was quantified in triplicate, giving 12 readings per condition. Fluorescence intensity was read with an excitation wavelength of 485 nm and emission wavelength of 510 nm on a Cytation™ 5. A previously established standard curve was used to determine sfGFP concentration from the fluorescence measurements (Figure S3). sfGFP concentration values were then averaged for each reaction, resulting in four values for each tested condition. Outliers were identified and removed using a Grubbs Test for both minimum and maximum values at a 90% confidence interval. Each condition sustained a minimum of N=3 samples and a maximum of N=4 samples. The remaining values were averaged and the standard deviation was determined.

Real-Time Data Plotting

All real-time raw data was collected using a Cytation™ 5 plate reader, and plotted using MATLAB ver. R2020b. All plots were then transferred to Biorender.com for figure creation.

Supporting Information

- Titration range of sfGFP; commercial extract kits time-course data; standard curve for sfGFP quantification; 2xYTPG single-additive results; 2xYTPG multi-additive results; CFAI 10 single-additive results; CFAI 10 multi-additive results; CFAI 2.5 single-additive results; commercial extract kit information
- Pre-Grubbs' Raw Data

Author Information

Corresponding Author

Javin P. Oza − Chemistry and Biochemistry Department and Center for Applications in Biotechnology, California Polytechnic State University, San Luis Obispo, California 93407, United States; orcid.org/0000-0002-2902-6939; Phone: 805-765-2265; Email: joza@calpoly.edu

Authors

Logan R. Burrington − Chemistry and Biochemistry Department and Center for Applications in Biotechnology, California Polytechnic State University, San Luis Obispo, California 93407, United States; orcid.org/0000-0001-8335-6800

Katharine R. Watts − Chemistry and Biochemistry Department and Center for Applications in Biotechnology, California Polytechnic State University, San Luis Obispo, California 93407, United States; orcid.org/0000-0002-6421-8370

Author Contributions

J.P.O. and L.R.B. conceived the project. L.R.B. designed and performed the experiments, collected and interpreted the data, and generated all figures. L.R.B and J.P.O wrote the paper. K.R.W. edited the manuscript.

Competing Interests

The authors declare no competing financial interests.

Acknowledgments

The authors would like to acknowledge Andrea Laubscher, Dr. Jennifer VanderKelen, Celine diBernardo, and Kevin Dunham for technical support; as well as Max Z. Levine, Nicole E. Gregorio, Wesley Y. Kao, Layne C. Williams, Philip E. J. Smith, Byungcheol So, Elizabeth J. Vojvoda, and Aiden Greengard for helpful feedback. All figures were created with Biorender.com. Research was supported by the William and Linda Frost Fund in the Cal Poly College of Science and Mathematics, and the National Science Foundation (NSF-1708919) grant awarded to K.R.W. and J.P.O.

References

(1) Gregorio, N. E.; Levine, M. Z.; Oza, J. P. A User's Guide to Cell-Free Protein Synthesis. *Methods Protoc.* **2019**, *2* (1), 24. https://doi.org/10.3390/mps2010024.

(2) Khambhati, K.; Bhattacharjee, G.; Gohil, N.; Braddick, D.; Kulkarni, V.; Singh, V. Exploring the Potential of Cell-Free Protein Synthesis for Extending the Abilities of Biological Systems. *Frontiers in Bioengineering and Biotechnology*. Frontiers Media S.A. October 11, 2019, p 248. https://doi.org/10.3389/fbioe.2019.00248.

(3) Dondapati, S. K.; Stech, M.; Zemella, A.; Kubick, S. Cell-Free Protein Synthesis: A Promising Option for Future Drug Development. *BioDrugs*. Adis June 1, 2020, pp 327–348. https://doi.org/10.1007/s40259-020-00417-y.

(4) Collias, D.; Marshall, R.; Collins, S. P.; Beisel, C. L.; Noireaux, V. An Educational Module to Explore CRISPR Technologies with a Cell-Free Transcription-Translation System. *Synth. Biol.* **2019**, *4* (1). https://doi.org/10.1093/synbio/ysz005.

(5) DeVries, J. K.; Zubay, G. DNA-Directed Peptide Synthesis. II. The Synthesis of the Alpha-Fragment of the Enzyme Beta-Galactosidase. *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *57* (4), 1010–1012. https://doi.org/10.1073/pnas.57.4.1010.

(6) Williams, L. C.; Gregorio, N. E.; So, B.; Kao, W. Y.; Kiste, A. L.; Patel, P. A.; Watts, K. R.; Oza, J. P. The Genetic Code Kit: An Open-Source Cell-Free Platform for Biochemical and Biotechnology Education. *Front. Bioeng. Biotechnol.* **2020**, *8*, 941. https://doi.org/10.3389/fbioe.2020.00941.

(7) NIRENBERG, M. W.; MATTHAEI, J. H. The Dependence of Cell-Free Protein Synthesis in E. Coli upon Naturally Occurring or Synthetic Polyribonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **1961**, *47* (2), 1588–1602. https://doi.org/10.1073/pnas.47.10.1588.

(8) Silverman, A. D.; Karim, A. S.; Jewett, M. C. Cell-Free Gene Expression: An Expanded Repertoire of Applications. *Nature Reviews Genetics*. Nature Research March 1, 2020, pp 151–170. https://doi.org/10.1038/s41576-019-0186-3.

(9) Huang, A.; Nguyen, P. Q.; Stark, J. C.; Takahashi, M. K.; Donghia, N.; Ferrante, T.; Dy, A. J.; Hsu, K. J.; Dubner, R. S.; Pardee, K.; Jewett,

M. C.; Collins, J. J. Biobits™ Explorer: A Modular Synthetic Biology Education Kit. *Sci. Adv.* **2018**, *4* (8), 5105–5106. https://doi.org/10.1126/sciadv.aat5105.

(10) Stark, J. C.; Huang, A.; Nguyen, P. Q.; Dubner, R. S.; Hsu, K. J.; Ferrante, T. C.; Anderson, M.; Kanapskyte, A.; Mucha, Q.; Packett, J. S.; Patel, P.; Patel, R.; Qaq, D.; Zondor, T.; Burke, J.; Martinez, T.; Miller-Berry, A.; Puppala, A.; Reichert, K.; Schmid, M.; Brand, L.; Hill, L. R.; Chellaswamy, J. F.; Faheem, N.; Fetherling, S.; Gong, E.; Gonzalzles, E. M.; Granito, T.; Koritsaris, J.; Nguyen, B.; Ottman, S.; Palffy, C.; Patel, A.; Skweres, S.; Slaton, A.; Woods, T. R.; Donghia, N.; Pardee, K.; Collins, J. J.; Jewett, M. C. BioBits™ Bright: A Fluorescent Synthetic Biology Education Kit. *Sci. Adv.* **2018**, *4* (8), 33. https://doi.org/10.1126/sciadv.aat5107.

(11) Stark, J. C.; Huang, A.; Hsu, K. J.; Dubner, R. S.; Forbrook, J.; Marshalla, S.; Rodriguez, F.; Washington, M.; Rybnicky, G. A.; Nguyen, P. Q.; Hasselbacher, B.; Jabri, R.; Kamran, R.; Koralewski, V.; Wightkin, W.; Martinez, T.; Jewett, M. C. BioBits Health: Classroom Activities Exploring Engineering, Biology, and Human Health with Fluorescent Readouts. *ACS Synth. Biol.* **2019**, *8* (5), 1001–1009. https://doi.org/10.1021/acssynbio.8b00381.

(12) Pardee, K.; Green, A. A.; Takahashi, M. K.; Braff, D.; Lambert, G.; Lee, J. W.; Ferrante, T.; Ma, D.; Donghia, N.; Fan, M.; Daringer, N. M.; Bosch, I.; Dudley, D. M.; O'Connor, D. H.; Gehrke, L.; Collins, J. J. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **2016**, *165* (5), 1255–1266. https://doi.org/10.1016/j.cell.2016.04.059.

(13) Soltani, M.; Davis, B. R.; Ford, H.; Nelson, J. A. D.; Bundy, B. C.

Reengineering Cell-Free Protein Synthesis as a Biosensor: Biosensing with Transcription, Translation, and Protein-Folding. *Biochem. Eng. J.* **2018**, *138*, 165–171. https://doi.org/10.1016/j.bej.2018.06.014.

(14) Des Soye, B. J.; Davidson, S. R.; Weinstock, M. T.; Gibson, D. G.; Jewett, M. C. Establishing a High-Yielding Cell-Free Protein Synthesis Platform Derived from Vibrio Natriegens. *ACS Synth. Biol.* **2018**, *7* (9), 2245–2255. https://doi.org/10.1021/acssynbio.8b00252.

(15) Levine, M. Z.; So, B.; Mullin, A. C.; Fanter, R.; Dillard, K.; Watts, K. R.; La Frano, M. R.; Oza, J. P. Activation of Energy Metabolism through Growth Media Reformulation Enables a 24-Hour Workflow for Cell-Free Expression. *ACS Synth. Biol.* **2020**, *9* (10), 2765–2774. https://doi.org/10.1021/acssynbio.0c00283.

(16) Chiao, A. C.; Murray, R. M.; Sun, Z. Z. Development of Prokaryotic Cell-Free Systems for Synthetic Biology. https://doi.org/10.1101/048710.

(17) Cole, S. D.; Miklos, A. E.; Chiao, A. C.; Sun, Z. Z.; Lux, M. W. Methodologies for Preparation of Prokaryotic Extracts for Cell-Free Expression Systems. *Synthetic and Systems Biotechnology*. KeAi Communications Co. December 1, 2020, pp 252–267. https://doi.org/10.1016/j.synbio.2020.07.006.

(18) Kim, D.-M.; Swartz, J. R. Regeneration of Adenosine Triphosphate from Glycolytic Intermediates for Cell-Free Protein Synthesis. *Biotechnol. Bioeng.* **2001**, *74* (4), 309–316. https://doi.org/10.1002/bit.1121.

(19) Kim, H. C.; Kim, D. M. Methods for Energizing Cell-Free Protein Synthesis. *Journal of Bioscience and Bioengineering*. J Biosci Bioeng

July 2009, pp 1–4. https://doi.org/10.1016/j.jbiosc.2009.02.007.

(20) Dopp, B. J. L.; Tamiev, D. D.; Reuel, N. F. Cell-Free Supplement Mixtures: Elucidating the History and Biochemical Utility of Additives Used to Support in Vitro Protein Synthesis in E. Coli Extract. *Biotechnology Advances*. Elsevier Inc. January 1, 2019, pp 246–258. https://doi.org/10.1016/j.biotechadv.2018.12.006.

(21) Jewett, M. C.; Swartz, J. R. Mimicking the Escherichia Coli Cytoplasmic Environment Activates Long-Lived and Efficient Cell-Free Protein Synthesis. *Biotechnol. Bioeng.* **2004**, *86* (1), 19–26. https://doi.org/10.1002/bit.20026.

(22) Zemella, A.; Thoring, L.; Hoffmeister, C.; Kubick, S. Cell-Free Protein Synthesis: Pros and Cons of Prokaryotic and Eukaryotic Systems. *ChemBioChem* **2015**, *16* (17), 2420–2431. https://doi.org/10.1002/cbic.201500340.

(23) Batista, A. C.; Soudier, P.; Kushwaha, M.; Faulon, J. Optimising Protein Synthesis in Cell-free Systems, a Review. *Eng. Biol.* **2021**, *5* (1), 10–19. https://doi.org/10.1049/enb2.12004.

(24) Heili, J. M.; Gomez-Garcia, J.; Gaut, N. J.; Cash, B. W.; Aufdembrink, L. M.; Heffron, B. A.; Shirley, J. D.; Carlson, E. E.; Adamala, K. P.; Engelhart, A. E. Real-Time Visualization of in Vitro Transcription of a Fluorescent RNA Aptamer: An Experiment for the Upper-Division Undergraduate or First-Year Graduate Laboratory. *J. Chem. Educ.* **2018**, *95* (10), 1867–1871. https://doi.org/10.1021/acs.jchemed.7b00735.

(25) Gregorio, N. E.; Kao, W. Y.; Williams, L. C.; Hight, C. M.; Patel, P.; Watts, K. R.; Oza, J. P. Unlocking Applications of Cell-Free Biotechnology through Enhanced Shelf Life and Productivity of E. Coli

Extracts. *ACS Synth. Biol.* **2020**, *9* (4), 766–778. https://doi.org/10.1021/acssynbio.9b00433.

(26) Burrington, L. R.; Baryal, E.; Hui, K.; Lambert, E.; Harding, S. T.; Oza, J. P. The Fold-Illuminator: A Low-Cost, Portable, and Disposable Incubator-Illuminator Device. *Synth. Syst. Biotechnol.* **2021**, *6* (2), 95–101. https://doi.org/10.1016/j.synbio.2021.04.003.

(27) Kai, L.; Dötsch, V.; Kaldenhoff, R.; Bernhard, F. Artificial Environments for the Co-Translational Stabilization of Cell-Free Expressed Proteins. *PLoS One* **2013**, *8* (2), e56637. https://doi.org/10.1371/journal.pone.0056637.

(28) Jewett, M. C.; Miller, M. L.; Chen, Y.; Swartz, J. R. Continued Protein Synthesis at Low [ATP] and [GTP] Enables Cell Adaptation during Energy Limitation. *J. Bacteriol.* **2009**, *191* (3), 1083–1091. https://doi.org/10.1128/JB.00852-08.

(29) Caschera, F.; Noireaux, V. Synthesis of 2.3 Mg/Ml of Protein with an All Escherichia Coli Cell-Free Transcription-Translation System. *Biochimie* **2014**, *99* (1), 162–168. https://doi.org/10.1016/j.biochi.2013.11.025.

(30) Simperler, A.; Kornherr, A.; Chopra, R.; Bonnet, P. A.; Jones, W.; Motherwell, W. D. S.; Zifferer, G. Glass Transition Temperature of Glucose, Sucrose, and Trehalose: An Experimental and in Silico Study. *J. Phys. Chem. B* **2006**, *110* (39), 19678–19684. https://doi.org/10.1021/jp063134t.

(31) Moriizumi, Y.; Tabata, K. V.; Miyoshi, D.; Noji, H. Osmolyte-Enhanced Protein Synthesis Activity of a Reconstituted Translation System. *ACS Synth. Biol.* **2019**, *8* (3), 557–567. https://doi.org/10.1021/acssynbio.8b00513.

(32) Koley, D.; Bard, A. J. Triton X-100 Concentration Effects on Membrane Permeability of a Single HeLa Cell by Scanning Electrochemical Microscopy (SECM). *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (39), 16783–16787. https://doi.org/10.1073/pnas.1011614107.

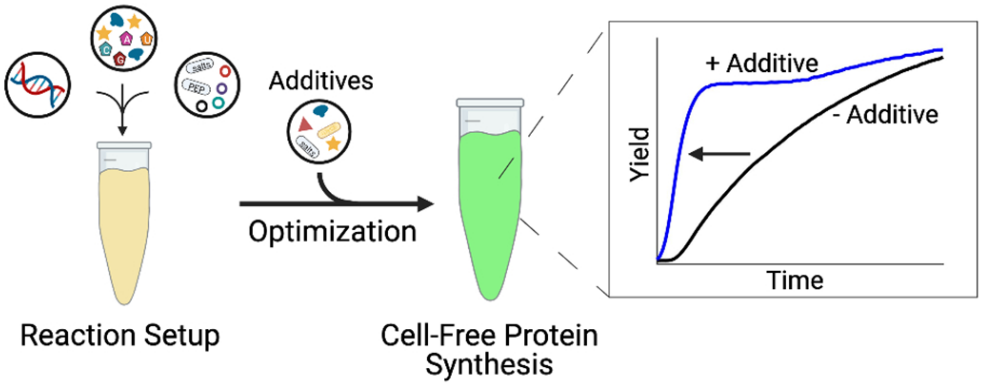
(33) Go, S. Y.; Lee, K. H.; Kim, D. M. Detergent-Assisted Enhancement of the Translation Rate during Cell-Free Synthesis of Peptides in an Escherichia Coli Extract. *Biotechnol. Bioprocess Eng.* **2018**, *23* (6), 679–685. https://doi.org/10.1007/s12257-018-0418-y.

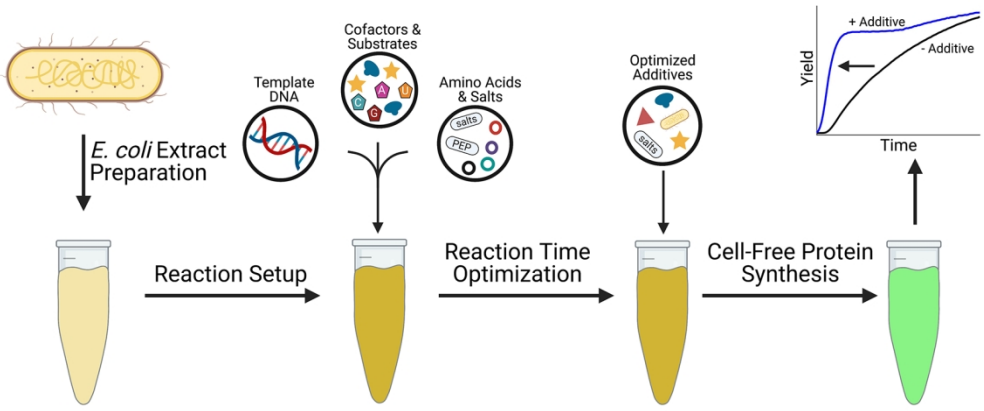
(34) Pechmann, S.; Willmund, F.; Frydman, J. The Ribosome as a Hub for Protein Quality Control. *Molecular Cell*. NIH Public Access February 7, 2013, pp 411–421. https://doi.org/10.1016/j.molcel.2013.01.020.

(35) O'Brien, E. P.; Vendruscolo, M.; Dobson, C. M. Prediction of Variable Translation Rate Effects on Cotranslational Protein Folding. *Nat. Commun.* **2012**, *3* (1), 1–9. https://doi.org/10.1038/ncomms1850.

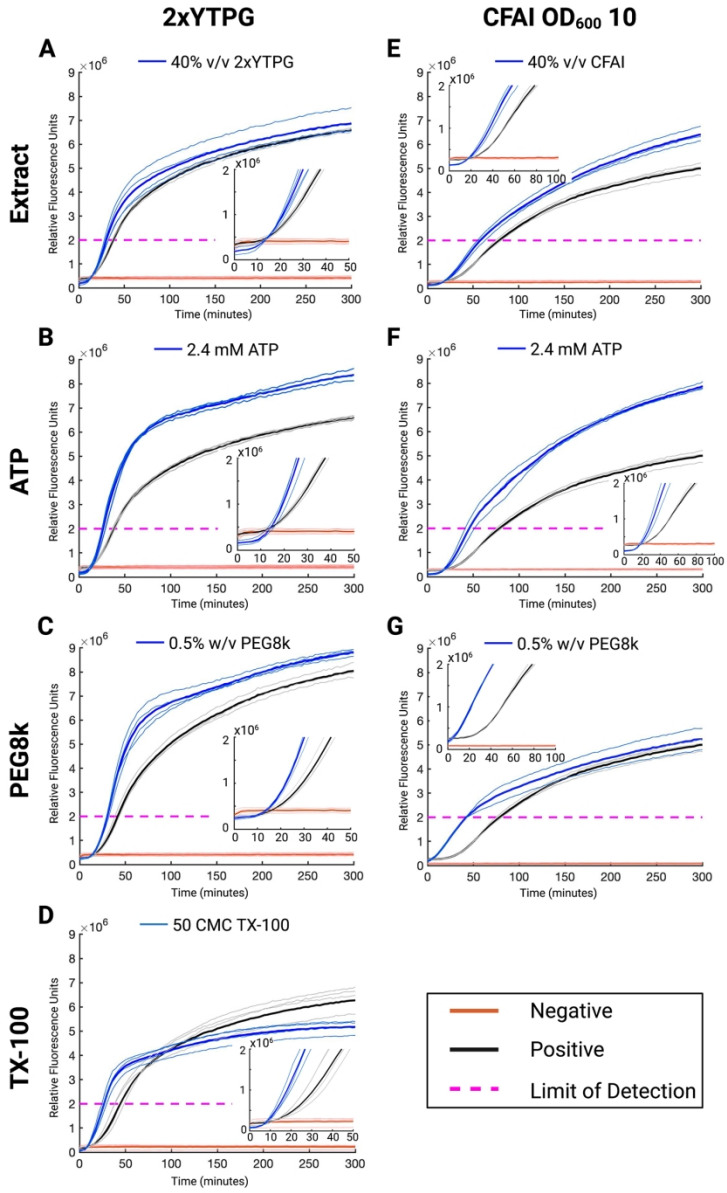
(36) Spencer, P. S.; Siller, E.; Anderson, J. F.; Barral, J. M. Silent Substitutions Predictably Alter Translation Elongation Rates and Protein Folding Efficiencies. *J. Mol. Biol.* **2012**, *422* (3), 328–335. https://doi.org/10.1016/j.jmb.2012.06.010.

(37) Kaiser, C. M.; Liu, K. Folding up and Moving on—Nascent Protein Folding on the Ribosome. *Journal of Molecular Biology*. Academic Press October 26, 2018, pp 4580–4591. https://doi.org/10.1016/j.jmb.2018.06.050.


Additives
Optimization
+ Additive
- Additive
Yield
Time
Reaction Setup
Cell-Free Protein Synthesis

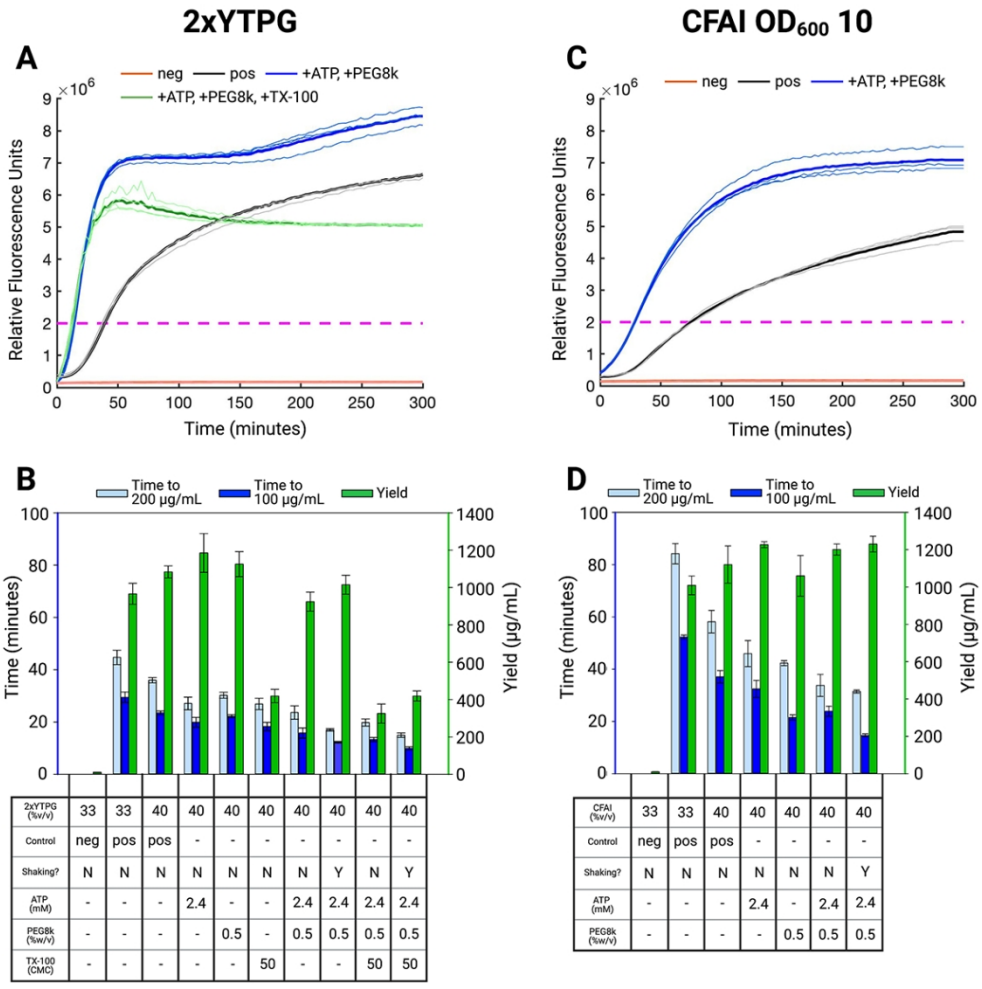


Generalized workflow for Cell-Free Protein Synthesis (CFPS) limit of detection optimization. *E. coli* extract is prepared and combined with the appropriate cofactors, substrates, amino acids, salts, and template DNA to initiate in vitro transcription and translation of sfGFP. The reaction mixture is then supplemented with additive(s) capable of decreasing the time to sfGFP detection by eye under ambient light (200μg/mL sfGFP) and blue light (100 μg/mL sfGFP) conditions.



Time-course fluorescence readings for 2xYTPG (left) and CFAI OD600 10.0 (right). Traditional CFPS (**A, B**), and 40% v/v extract with the indicated additive (**C-G**) (blue), with a negative control (orange). The 200 µg/mL (2x106 RFU) limit of detection is depicted (dash). Insets provide a zoom-in view of the control and treatment conditions until the limit of detection is reached. Each panel lists the corresponding additive and its final concentration in the reaction. Data is plotted as an average of a minimum of N=3, maximum N=4 experimental replicates. The average of each data set is indicated by a darker color. Outliers were identified by a Grubbs' Test on time to 2x106 RFU at a 90% confidence interval. Negative conditions have no pJL1-sfGFP DNA template or additive present. CFAI 2.5 is not included due to no observed rate benefits from any additive (other than higher % v/v extract).

125x203mm (300 x 300 DPI)



Single and multi-additive titrations that resulted in faster times to the limit of detection for A/B) 2xYTPG extract and C/D) CFAI OD600 10 extract. A/C) 300 minute time-course fluorescence data from our traditional 33% v/v extract setups (black) as compared to our most optimal rate-boosting setups (blue, green). The 200 µg/mL limit of detection standard is depicted (dash). The average of each data set is indicated by a darker color. B/D) Left Y-axis represents time to 100µg/mL and 200 µg/mL sfGFP from reaction start; right Y-axis represents total reaction yield 18 hours after reaction start. Listed concentrations are representative of the final CFPS reaction. Negative conditions have no pJL1-sfGFP DNA template or additive present. All data is presented with a minimum of N=3, maximum N=4 experimental replicates.

101x100mm (300 x 300 DPI)