September 6, 2018

Journal of Visualized Experiments  
1 Alewife Center, Suite 200

Cambridge, MA 02140

Dear Dr. Phillip Steindel,

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

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

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

Sincerely,

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

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

**Summary**

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

**Reviewers' comments:**

***Reviewer #1:*** *Major Concerns:*  
*In general, I have problems with two particular issues. First, the system is propagated as being highly productive, giving yields of 900 µg/mL. However, the protocol is described for 15 µl total volume and it is shown that larger reaction vessels have a strong negative effect on expression efficiency.*

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

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

**Response:**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**Response:** We agree with the reviewer that CFPS is a niche system that can be useful when *E. coli* expression is not sufficient for desired applications. To clarify this for users, we have added the following text:  
 “Given the startup costs, we recommend the CFPS platform for applications in synthetic biology, high-throughput efforts, and protein expression conditions that are incompatible with traditional protein expression platforms due to conflict with the cell’s biochemistry and viability constraints. In these specialized cases where the desired technique is enabled by the CFPS platform, the greater cost of CFPS over *in vivo* expression is justified.”

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

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

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

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

***Reviewer #1:*** *Specific comments:*  
*44, 56: highly productive, relative to what?*

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

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

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

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

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

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

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

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

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

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

***Reviewer #1:*** *114: non experts? Perhaps the authors mean to use an established protocol for GFP expression and finally pipet anything together can be done by non-experts. Potentially true for any technique, but the strength of CFPS is rather to modify it for new targets. I would say to produce a new protein in acceptable quality and stability by tuning the system accordingly clearly requires some deeper expert know how. Best to delete this sentence.*  
**Response:** We agree that the capacity to modify the platform is one of the advantages of CFPS, and modifying the platform for specific applications requires experience and expertise. We have clarified this in the main text as follows:

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

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

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

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

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

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

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

“Note: Induction by IPTG is for endogenous production of T7 RNA Polymerase (T7RNAP).”

and

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

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

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

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

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

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

***Reviewer #1:*** *416-419: Oxygen access is mentioned as being important  
I would expect that this is either confusing a reader or it is overseen. The protocol should be very clear on that and should make very clear which production efficiency can be expected in which volume and vessel. Please also indicate in which reaction vessels the demonstrated expressions have been performed.***Response:** We appreciate this feedback, we have clarified the text and figures at numerous locations to indicate the reaction size and vessel that have been used, along with the vessels we recommend for specific volume ranges to maintain consistent volumetric yields of the reaction. .

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

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

***Reviewer #1:*** *375: genomic DNA is not completely removed by this procedure as it is disintegrated into smaller pieces by the sonication process, which then stay in the supernatant. Please make the reader aware of this potential problem.***Response:** We agree that this is important to clarify for the reader.We have removed all language that suggested that genomic DNA is removed, and we have indicated that fragmented genomic DNA remains.

***Reviewer #1:*** *Fig. 1: Please make a cost per µl/mL reaction calculation. Comparison with commercial kits is not very meaningful anyhow, as no labor costs, tax, company costs etc. are included. So better present comparisons with other published calculations of academic systems.***Response:** We have added the cost per reaction volume calculation. We also include cost comparison with an extract preparation method that is the most different from the one we present. We respectfully disagree about the meaningfulness of the comparison with commercial kits. We posit that cost comparison to commercial kits may be useful for many users who are deciding the most accessible path toward utilizing CFPS in their labs based on their funding situation, which is generally independent of a company’s costs and profit margins. We agree that our calculations do not include labor since the actual cost of labor varies by position and salary standards, which differ greatly. In lieu of including cost of labor, we have identified that the initial startup of the platform presented in this manuscript requires ~26 hours of time. Our timeline (Figure 1) gives more details on the specific time required for any particular task in the methods. Users can calculate their daily wage rates and can add this to the costs we present in order to evaluate which CFPS procedure or commercial kit is most effective for their time/cost constraints.

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

**Response:**We have included a Coomasie stained SDS-PAGE of GFP production in CFPS in the supplemental information (Supplemental Figure 2) along with details on the quantities loaded.

We apologize for the quality of the photograph demonstrating GFP expression, we agree that the green color was not captured effectively. We have retaken the photograph for this figure (see Figure 3D).

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

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

***Reviewer #1:*** *At the beginning, 96 well plates have been propagated for the described 15 µl volume reactions. As these hold only 300 µl, the maximum yield of GFP is then less than 500 mg/mL and not 900 µg(mL as always mentioned? Please show in addition the expression efficiency in reactions in same vessel but with increasing volumes up to 1 mL.***Response:** Yes, we discuss the utility of the 96-well plates for higher-throughput applications. To clarify, we do not recommend using 96-well plates for scaling up volume for biomanufacturing; the main text specifies that if the user intends to scale up the volume of the reaction, larger vessels should be utilized. Scaling of CFPS reactions to 1+ mL has been shown in previous publications which we reference. To assist the readers, we have also added the following text to provide guidance in executing larger volume reactions:   
“When scaling up volume, the primary consideration is that volumetric reaction yields decrease as the surface area-to-volume ratio of the reaction decreases.37, 52 In order to scale-up while maintaining similar volumetric yields of protein expression, users should 1) split the reaction volume into numerous reaction vessels and/or 2) increase vessel size. For reaction scales ranging from 15 µL – 100 µL in volume, numerous 15 µL reactions in parallel are recommended. For reactions exceeding 100 µL in volume, flat-bottom 24-well plates are recommended, and 12-well plates are recommended for reaction volumes exceeding 600 µL. Such pairings of reaction volumes and vessels provide consistency in volumetric reaction yields upon scale-up.17, 31, 37, 52 Scaling up beyond these volumes can be accomplished by utilizing multiple wells of the plate in parallel. Using this format, the reaction can be scaled to over 10 mL total volume.”

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

***Reviewer #1:*** *Protocol/materials: I would find it helpful, to add comments on the action and tolerance range of the individual compounds as well. If even non-expert readers are addressed, this would be essential to give them more confidence for setting up such a system*.  
**Response:** We agree that this information will provide new users with more confidence in setting up CFPS in their labs. We have added the following text within the protocol section:  
“Note 3.1.B: Solutions A and B recipes are provided in the Supplementary Information and correspond to specific concentrations for numerous reagents to support the PANOx-SP based energy system for CFPS. The role of each reagent and acceptable variation in these reagent concentrations that can support CFPS have been determined.49”

***Reviewer #2:*** *Major Concerns:*  
*The manuscript lacks the view of the quality of the prepared cell extract. Although they describes "Users can quantify batch-to-batch variability in total protein concentration of the cell extract by Bradford assay" (in lines 388-389), there is no description of how to use quantified values for evaluating the variability. For example, they only describes the volume of the extract in experiments in Figure 5. I think they should at least describe the concentration of the extract used in Figure 5 experiments as an indicator for the reader to evaluate the quality of extract.*

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

“Users can quantify batch-to-batch variability in total protein concentration of the cell extract by Bradford assay. For higher performing extracts we typically see total protein concentrations of 30-50 mg/mL, and within this range there is no obvious correlation between total protein concentrations cell extract performance. Therefore, we recommend that users tune magnesium concentrations accordingly to ensure that protein and nucleic acid functionality are maximized for each extract batch.”  
  
***Reviewer #2:*** *Minor Concerns:*  
*-- Some references are in incomplete format (e.g., 15, 35, 36). Please check the correctness of all the references.*

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

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

**Response:** We have updated our table of materials. We agree that details of the DNA template are crucial to successful CFPS reactions. Given that the pJL1-sfGFP vector and its plasmid map are available on Addgene.com, we have simply listed the catalog number in the table of materials instead of providing the genetic structure of the plasmid in supplemental information. We hope this is satisfactory to the reviewer.

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

“(For example, if an OD600 of a 1:10 dilution is read as 0.4, inoculate 25 mL of the undiluted OD600 = 4.0 overnight culture into 1 L of 2x YTPG).”

***Reviewer #2:*** In line 304, Figure 4C does not exist. "wen using smaller"?   
**Response:** We apologize for these errors, we have updated the figure numbers and resolved the typos in the revised manuscript. The new figure number is 2C.

***Reviewer #2:*** In lines 314-318, it lacks the most essential ingredients, cell extract. It would be helpful if they provide here the information of the cell extract concentration.  
**Response:** We thank the reviewer for identifying this missing detail. The updated text reads as follows:

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

***Reviewer #2:*** In lines 446-451 (the legend for Figure 4), There is no explanation for "Neg".  
**Response:** Figure captions have been modified to improve clarity of the labels used. Text for one such example appears as follows:

“’Neg’ represents a negative control where no DNA template was added to the reaction.”

***Reviewer #2:*** In Table of Materials  
"Glucose" is described as "D-glucose" in the text. I think it's better to unify the notation to avoid any confusion.  
**Response:** All instances of glucose have been changed to D-glucose.

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

**Response:** We have clarified this mismatch to indicate that only NH4(Glu) is used.

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

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

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

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