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From Cells to Cell-Free Protein Synthesis Within 24 Hours using Cell-Free Autoinduction Workflow

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TITLE

From Cells to Cell-Free Protein Synthesis Within 24 Hours using Cell-Free Autoinduction Workflow

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SUMMARY:

This work describes the preparation of cell extract from *Escherichia coli (E. coli)* followed by cell-free protein synthesis (CFPS) reactions in under 24 hours. Explanation of the cell-free autoinduction (CFAI) protocol details improvements made to reduce researcher oversight and increase quantities of cell extract obtained.

ABSTRACT:

Cell-free protein synthesis (CFPS) has grown as a biotechnology platform that captures transcription and translation machinery *in vitro*. Numerous developments have made the CFPS platform more accessible to new users and have expanded the range of applications. For lysate based CFPS systems, cell extracts can be generated from a variety of organisms, harnessing the unique biochemistry of that host to augment protein synthesis. Within the last 20 years, *Escherichia coli* (*E. coli)* has become one of the most widely used organisms for supporting CFPS due to its affordability and versatility. Despite numerous key advances, the workflow for *E. coli* cell extract preparation has remained a key bottleneck for new users to implement CFPS for their applications. The extract preparation workflow is time-intensive and requires technical expertise to achieve reproducible results. To overcome these barriers, we previously reported the development of a 24 h cell-free autoinduction (CFAI) workflow that reduces user input and technical expertise required. The CFAI workflow minimizes the labor and technical skill required to generate cell extracts while also increasing the total quantities of cell extracts obtained. Here we describe that workflow in a step-by-step manner to improve access and support the broad implementation of *E. coli* based CFPS.

INTRODUCTION:

The use of cell-free protein synthesis (CFPS) for biotechnology applications has grown substantially over the past few years[1–3]. This development can be attributed in part to increased efforts in understanding the processes that occur in CFPS and the role of each component[4,5]. Additionally, reduced costs attributed to optimized set-ups and alternative energy sources have made cell-free technology easier to implement for new users[6–9]. In order to implement the necessary transcription and translation factors for protein synthesis, a cell extract is often used to drive cell-free reactions[10]. Recently published user guides have provided simple protocols for producing functional extract, making it easier to implement for new and experienced users alike[1,11–14]. Cell extract is usually obtained through the lysis of a cell culture, which can be grown using different organisms depending on the specific use desired[1,15,16].

Escherichia coli (*E. coli*) has rapidly become one of the most commonly used host organisms for producing functional extracts[17]. The BL21 Star (DE3) strain is preferred because it removes the proteases from the outer membrane (OmpT protease) and the cytoplasm (Lon protease), providing an optimal environment for the recombinant protein expression. Additionally, the DE3 contains the λDE3 that carries the gene for T7 RNA polymerase (T7 RNAP) under the control of the lacUV5 promoter; the star component contains a mutated RNaseE gene which prevents cleavage of mRNA[4,14,18,19]. Under the lacUV5 promoter, isopropyl-thiogalactopyranoside (IPTG) induction allows the expression of T7 RNAP[20,21]. These strains are used to grow and harvest cells, which give raw material for extract preparation. Cell lysis can be performed using a variety of methods, including bead beating, French press, homogenization, sonication, and nitrogen cavitation[1,11,12,22].

The process of bacterial culture and harvesting is consistent across most platforms when using *E. coli,* but requires multiple days and intense researcher oversight[1,11,13]. This process generally starts with an overnight seed culture in LB broth, which upon overnight growth is then inoculated into a larger culture of 2xYTPG (yeast, tryptone, phosphate buffer, glucose) the next day. The growth of this larger culture is monitored until it reaches the early-to-mid log phase, at an optical density (OD) of 2.5[14,20]. Constant measurement is required as the components of transcription and translation have been previously demonstrated to be highly active in the early-to-mid log phase[23,24]. While this process can create reproducible extract, our lab has recently developed a new method using Cell-Free Autoinduction (CFAI) Media, which reduces researcher oversight, increases the overall yield of extract for a given liter of cell culture, and improves access to *E. coli*-based extract preparation for both experienced and new users (**Figure 1**). Here we provide the step-by-step guide for implementing the CFAI workflow, to go from a streaked plate of cells to a completed CFPS reaction within 24 hours.

PROTOCOL:

1. Media growth

1.1. Prepare 960 mL of CFAI media as described in **Table 1** and adjust the pH to 7.2 using KOH.

1.2. Transfer culture media to a 2.5 L baffled flask and autoclave for 30 min at 121 °C.

1.3. Prepare a 40 mL sugar solution as described in **Table 1**. Filter-sterilize the solution into a separate autoclaved glass container.

NOTE: The sugar solution can be stored in a 30 °C incubator until further use.

1.4. Allow the media to completely cool to below 40 °C after autoclaving.

1.5. Prior to inoculation of the CFAI media, add the sugar solution directly to the CFAI media.

1.6. To inoculate the media, swipe a loopful of colonies from a previously streaked *E. coli* BL21 Star (DE3) plate and insert directly into the media. Swirl the loop into the media but avoid touching the sides of the container. Ensure that the streak plate is fresh, with viable cells.

1.7. Place the flask with the inoculated media in a 30 °C incubator while shaking at 200 rpm. Allow the culture to grow overnight. If cells are inoculated in the evening, the culture will reach an approximate optical density, measured at 600 nm (OD_{600}), of 10 the subsequent morning. Inoculation and harvest times can be adjusted as needed.

2. Cell harvest

2.1. Prepare S30 Buffer in advance and keep it cold. Prepare the S30 buffer according to **Table 2**, to a final pH of 8.2.

NOTE: The S30 buffer can be prepared days prior to the cell harvest. If this is done, prepare without dithiothreitol and store at 4 °C. Add dithiothreitol just before use.

2.2. From this point forward, keep all solutions and materials on ice. Transfer 1 L of media into a 1 L centrifuge bottle and centrifuge at 5,000 *x g* between 4-10 °C for 10 min. Decant and dispose of the supernatant. Using a sterile spatula, transfer the pellet to a pre-chilled and previously weighed 50 mL conical tube.

2.3. Wash once with 30-40 mL of cold S30 buffer by resuspending the pellet *via* vortexing in 30 s bursts with rest periods on ice.

NOTE: Due to a higher volume of pellet, splitting the cell pellet into two 50 mL conical tubes can be helpful for the wash step. Storing cells in smaller aliquots also provides flexibility for the downstream processing.

2.4. Centrifuge the cell resuspension at 5000 *x g* between 4-10 °C for 10 min.

2.5. Dispose of the supernatant and wipe any excess from the inside walls of the 50 mL conical tube using a clean tissue, avoid touching the pellet itself. Weigh and flash freeze the pellet in liquid nitrogen. Store at –80 °C until further use.

NOTE: Pellets may not require flash-freezing if the user plans to continue with the extract preparation protocol.

3. Extract preparation

3.1. Combine the frozen pellet with 1 mL of S30 buffer for every 1 g of the cell pellet and allow to thaw on ice for approximately 30-60 min. Resuspend thawed pellet via vortexing in bursts of 30 s with rest periods on ice. Vortex until there are no visible clumps of cells remaining.

NOTE: Smaller clumps can be resuspended by mixing using a pipette.

3.2. Transfer aliquots of 1.4 mL of cell resuspension into 1.5 mL microfuge tubes for cell lysis. Sonicate each tube with a frequency of 20 kHz and 50% amplitude for three bursts of 45 s with 59 s of rest per cycle surrounded by an ice bath. Invert the tube between cycles and immediately add 4.5 µL of 1 M dithiothreitol after the last sonication cycle.

NOTE: Due to the heat released from sonication, it is extremely important to make sure all aliquots are kept on ice when not being sonicated. The ice bath should be constantly replenished or large enough to stay cool throughout the entire sonication process.

3.3. Centrifuge each tube at 18,000 *x g* and 4 °C for 10 min. Retrieve the supernatant and aliquot into 1.5 mL microfuge tubes in 600 µL aliquots. Flash freeze aliquots and store at –80 °C until further use. Care should be taken to pipette only the supernatant.

4. Cell-free protein synthesis

4.1. Thaw one aliquot of extract from the previous step to perform 15 µL of cell-free protein synthesis reactions in 1.5 mL microfuge tubes in quadruplicate.

4.2. Prepare each reaction by combining 240 ng of DNA (16 µg/mL final concentration), 2.20 µL of Solution A, 2.10 µL of Solution B, 5.0 µL of extract, and a varying volume of molecular-grade water to fill the reaction to 15 µL. This reaction can be scaled to higher volumes. See **Table 3** for ratios.

4.2.1. Prepare Solution A and B according to **Table 4**. Each solution can be prepared in batches of 100 µL to 1 mL, aliquoted, and stored at –80 °C until further use.

NOTE: The DNA amount can vary depending on the protein of interest. In this case, the plasmid used, pJL1-sfGFP, has been optimized to perform at 16 µg/mL, or 597 µM.

4.3. Let the reactions run for at least 4 h at 37 °C.

5. Quantification of reporter protein, super folder green fluorescent protein (sfGFP)

5.1. Using a half area 96-well black polystyrene plate, combine 2 µL of each cell-free protein synthesis reaction product with 48 µL of 0.05 M HEPES buffer at pH 7.2. Three to four replicates of each reaction tube are recommended.

5.2. Quantify the fluorescence intensity of the sfGFP with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

5.3. For conversion of relative fluorescence units to volumetric yield (µg/mL) of sfGFP, establish a standard curve using purified pJL1-sfGFP.

REPRESENTATIVE RESULTS:

When preparing CFAI media, glucose was exchanged for an increase in lactose and glycerol as the main energy substrate in the media. Additionally, the buffering capacity of the CFAI media was increased as well. These specific components are given in **Table 1**.

The cells were then grown to both an OD_{600} of 10 and the standard 2.5 in CFAI media to show consistency with extract quality despite varying extract quantities. The 2.5 OD_{600} CFAI media was grown after inoculating from a seed culture in LB broth at 37 °C, 200 rpm, while the OD_{600} 10 culture was inoculated directly from a plate**.** Each batch of CFAI media was then monitored and harvested at their respective OD_{600}. The growth to an OD_{600} of 10 led to an increase in higher amount of cell pellet and overall extract obtained, as it produced 9.60 mL of extract versus the 2.10 mL of extract obtained from the growth to 2.5 OD_{600} (**Figure 2**). Further analysis of total protein concentration demonstrated no significant difference in overall protein in each extract (**Table 5**). Even though they were grown to different levels of optical density, both batches of extract demonstrated similar results in cell-free reactions using sfGFP (**Figure 3**). This suggests that the combination of the increased buffering capacity, the use of lactose and glycerol as the main carbon source and implementing lactose instead of IPTG for T7RNAP induction help stabilize extract growths to any OD_{600} below 10.

FIGURE AND TABLE LEGENDS:

Table 1: CFAI components. Components for CFAI media and sugar solutions with their respective amounts. The media should be stirred throughout the addition of each component and the sugar solution filter sterilized. Each solution should be added to a separate sterile container prior to inoculation.

Table 2: S30 buffer components: Components for S30 Buffer were added with their respective amounts into a sterile 50 mL conical tube.

Table 3: CFPS reaction ratios: Relative volume percentages for Solution A, Solution B, and extract. The DNA volume can vary depending on the specific plasmid's concentration and may need to be optimized for the user's specific plasmid being used.

Table 4: Solution A and B components. Stock concentrations for the components for Solution A and B were added with their respective amounts, each in a 1.5 mL microfuge tube.

Table 5: Total extract protein yields. Analysis of the total protein of different cell extract growths. Total protein concentration was determined using a Bradford Assay. Each concentration was determined from triplicates using a 1:40 dilution.

Figure 1: Comparison of CFAI and typical workflow from cells to CFPS: Comparison of the overall timeline from cells to CFPS using the (**A**) CFAI workflow (left, red) versus the (**B**) previously established method (green, right). The comparison demonstrates the reduced researcher oversight and timeline when performing CFPS using the CFAI workflow.

Figure 2: CFAI pellet size comparison. Comparison of CFAI media pellets after cell harvest at different OD_{600}. The media grown to an OD_{600} of 2.5 produced a 2.23 g cell pellet (left) and the media grown to an OD600 of 10 produced a 9.49 g cell pellet (right).

Figure 3: Effects of growth on CFPS reaction yields. (**A**) Comparison of CFPS reaction yields between growths to 2.5 OD_{600} and 10 OD_{600}, with (**B**) images of each CFPS reaction above their respective yield. Cell-free reactions were performed in a 1.5 mL microfuge tube and quantified after 24 h of incubation at 37 °C using a standard curve to correlate fluorescence to sfGFP concentration. The "Negative" corresponds to the set of negative control reactions in which no template DNA was added. The traditional 2xYTPG media (the positive control) and the CFAI extracts are of similar quality as demonstrated through their high CFPS yields.

DISCUSSION:

Researcher oversight is traditionally needed for two key actions during cell growth: the induction of T7 RNAP and harvesting cells at a specific OD_{600}. CFAI obviates both of those requirements to decrease the researcher's time and technical training required in order to prepare high quality cell extracts. Auto-induction of T7 RNAP is achieved by replacing glucose with lactose as the primary sugar in the media, obviating the previous need to actively monitor the growth and then induce with isopropyl-thiogalactopyranoside (IPTG) at a precise point during cell growth. The need to actively monitor cell cultures to harvest at a specific OD_{600} is also obviated, untethering the researcher from the cell culture. This adds to the recent work which have also demonstrated production of quality extracts harvested at non-traditional times[13,25,26]. The new media formulation improves buffering capacity and carbon sources to support active energy metabolism even as the cell culture approaches stationary phase. The capacity to obtain robust cell extracts from high OD_{600} cultures allows the researcher to harvest the cultures at their convenience[27]. The workflow we prefer and recommend is to inoculate the culture in the evening and returning to harvest the next morning.

Harvesting cells at a higher OD_{600} also results in a significantly larger quantity of cells obtained for extract preparation. For experienced researchers, it is worth noting that the cell pellet is much darker in color compared to cells grown in 2xYTPG media, even when harvested at an OD_{600} of 2.5. It is also important to note that if the entire cell pellet is being processed at once, the large

amount of resuspension obtained per cell pellet when performing lysis via sonication will take some time. Hence, it is important to keep all aliquots cold during this process[11,13,14]. The increase in extract volume per growth decreases cost proportionally and supports biomanufacturing applications. With the improvements demonstrated, the CFAI workflow provides an easier protocol for new and experienced users of cell-free technology to produce reproducible, functional *E. coli* extract.

Despite the advantages of the provided CFAI media, there are limitations to this method. The primary challenge is the nascent nature of the workflow. While metabolomics analysis has shed light on the differences in CFAI OD_{600} 10 extracts as well as reaction products compared to 2xYTPG, the implications of these differences on specific applications remain uncharacterized[27]. Additionally, this workflow has been developed for BL21 *E. coli*-based lysate. It is unclear whether the media reformulation would support robust extract preparation from other *E. coli* strains, such as the genomically recoded strains of *E. coli*[28,29]. It is possible that the CFAI approach could be utilized for generating extracts from other bacterial organisms, but it is unlikely to support extract preparation for eukaryotic organisms such as Chinese Hamster Ovary or rabbit reticulocyte; however, these have their own established methods[30,31]. We anticipate that the simplicity of the CFAI workflow will reduce the barriers and incentivize the cell-free community to characterize and evaluate its utility for the broad range of applications that CFPS supports.

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DISCLOSURES:

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

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Figure 1

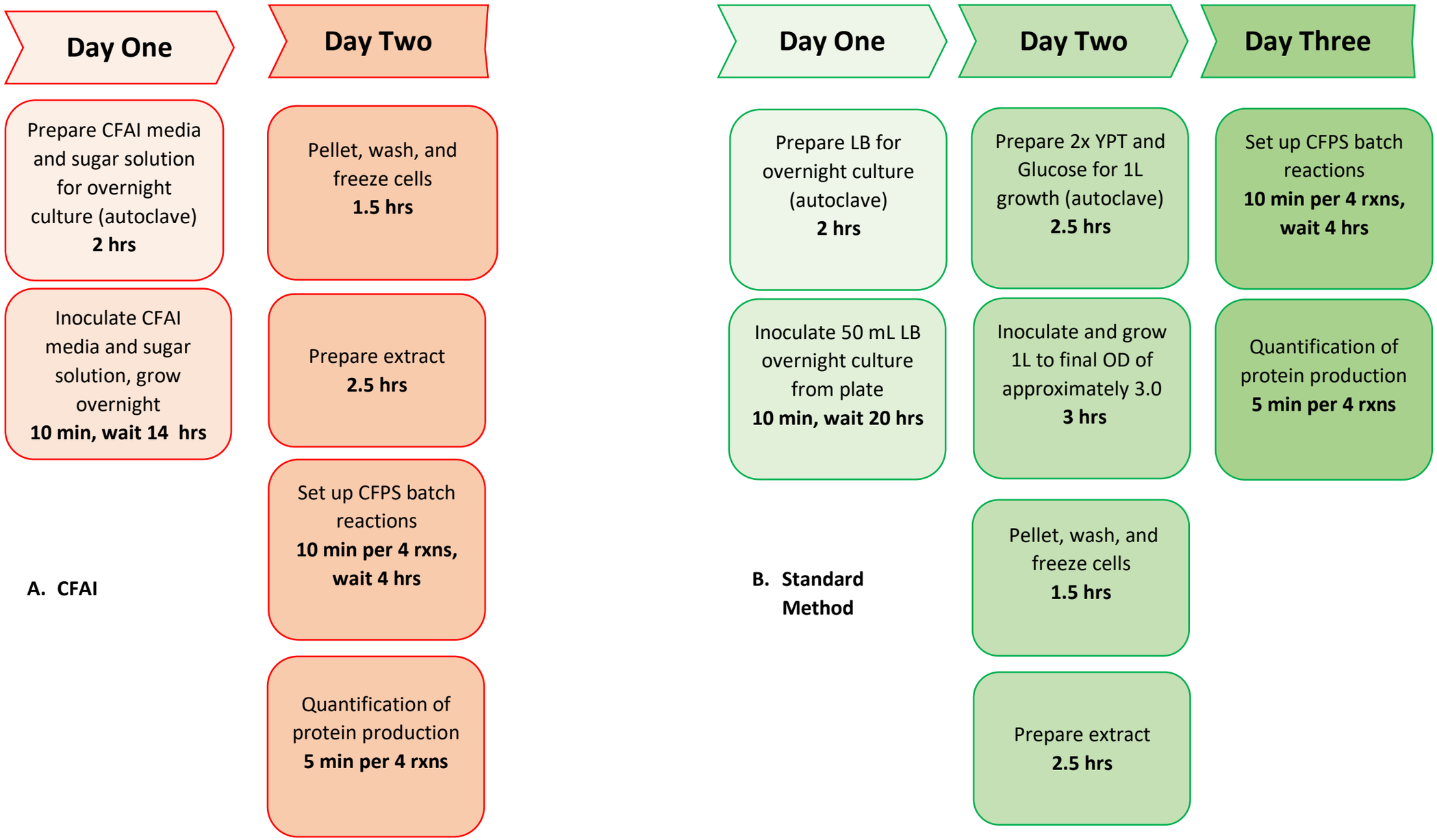

Figure 2

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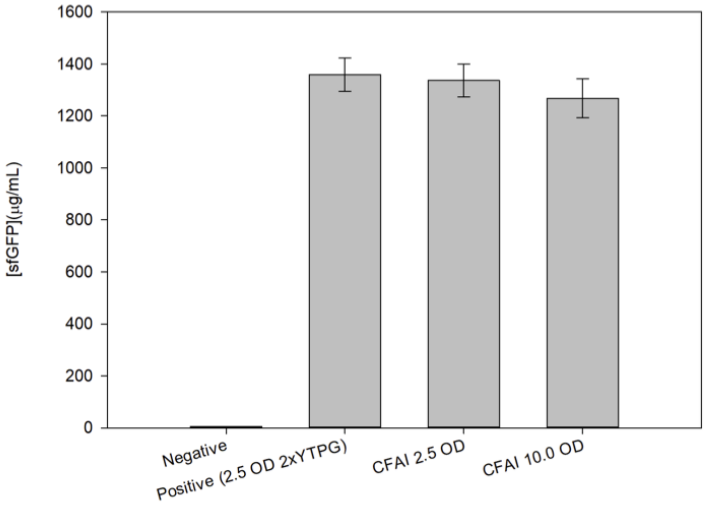
Figure 3



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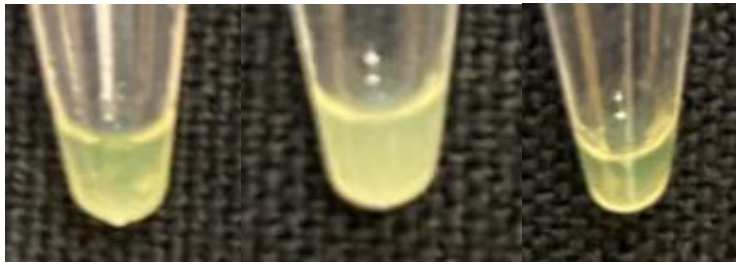

B. 2.5 OD 2xYTPG CFAI 2.5 OD CFAI 10.0 OD

Table 1

Autoclaved CFAI Media:

Components	Amount
Sodium Chloride	5.0 g
Tryptone	20.0 g
Yeast Extract	5.0 g
Potassium Phosphate, monobasic	6.0 g
Potassium Phosphate, dibasic	14.0 g
Nanopure™ Water	Fill to a total of 960 mL

Filter Sterilized Sugar Solution:

Components	Amount
D-Glucose	0.50 g
D-Lactose	4.0 g
80% v/v Glycerol	7.5 mL
Nanopure™ Water	28.0 mL

Table 2

S30 Buffer	
Components	Concentration
Tris Acetate pH 8.2 at room temperature	10 mM
Magnesium Acetate	14 mM
Potassium Acetate	60 mM
Dithiothreitol	2 mM

Table 3

Component	Amount
Solution A	2.20 µL
Solution B	2.1 µL
Extract	5 µL
DNA Template	Volume for 16 µg/mL final
Water	Fill to a total of 15 µL

Table 4

Solution A		Solution B	
Components	Concentration	Components	Concentration
ATP	1.2 mM	Magnesium Glutamate	10 mM
GTP	0.850 mM	Ammonium Glutamate	10 mM
UTP	0.850 mM	Potassium Glutamate	130 mM
CTP	0.850 mM	Phosphoenolpyruvate (PEP)	30 mM
Folinic Acid	31.50 µg/mL	L-Valine	2 mM
tRNA	170.60 µg/mL	L-Tryptophan	2 mM
Nicotinamide Adenine Dinucleotide (NAD)	0.40 mM	L-Isoleucine	2 mM
Coenzyme A	0.27 mM	L-Leucine	2 mM
Oxalic Acid	4.00 mM	L-Cysteine	2 mM
Putrescine	1.00 mM	L-Methionine	2 mM
Spermidine	1.50 mM	L-Alanine	2 mM
HEPES Buffer pH 7.5	57.33 mM	L-Arginine	2 mM
		L-Asparagine	2 mM
		L-Aspartic Acid	2 mM
		L-Glutamic acid	2 mM
		L-Glycine	2 mM
		L-Glutamine	2 mM
		L-Histidine	2 mM
		L-Lysine	2 mM
		L-Proline	2 mM
		L-Serine	2 mM
		L-Threonine	2 mM
		L-Phenylalanine	2 mM
		L-Tyrosine	2 mM

Table 5

Extract	Total Protein Concentration (µg/mL)	Standard Deviation
2xYTPG 2.5 OD	30617	3745
CFAI 2.5 OD	30895	2254
CFAI 10.0 OD	27905	3582

Response to Editor

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response to Comment1: The paper has been proofread and all abbreviations are defined at first use. Thank you.

Comment 2: Please reduce the word count of the summary to be 10-50 words.

Response to Comment 2: The summary has been reduced to below 50 words. Thank you.

Comment 3: For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Response to Comment 3: This change has been made. Thank you.

Comment 4: Please consider providing reaction set-ups and solution composition as Tables (like your Table 1) in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text. Remove these solution recipes from the text.

Response to Comment 4: We have taken the editor's advice and added tables for the compositions of Solution A, Solution B, S30 Buffer, and the CFPS reaction set up.

Comment 5: Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response to Comment 5: These changes have been made. Thank you.

Comment 6: Consider replacing or reducing the figures in your paper.
i) The figure legend for Figure 1 would be more interesting to see in the video. Consider removing figure 1 from the paper.
ii) Figure 3 also is a bit redundant and uninformative. Three sets of tubes with identical-looking reactions does not make much of an impact. If the reaction does have some significance, consider including just one tube of each reaction type pasted above the respective data bars in Figure 4 and delete Figure 3. But the legend for figure 4 (which becomes figure 2) will include all these details (replicates, reaction details etc).

Response to Comment 6: We have taken the editor's advice and removed figure one and combined figures 3 and 4. We also added an additional figure comparing the workflow of the

method described in this manuscript compared to the standard method as recommended by reviewer 1 to help aid new CF users.

Comment 7: Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Ref 3 does not have any page numbers.

Comment 8: Please sort the Materials Table alphabetically by the name of the material.

Response to Comment 7 and 8: We have made these changes to the submission. Thank you.

Response to Reviewers

Reviewer #1:

Comment 1: Line 71-73: "transcription and translation are thought to be most active due to the highest rate of growth of the culture in the early-to-mid log phase". Though this does seem to be mostly the case (and may always be the case for BL21*), there is literature which shows different results, e.g.:
Kim, J., et al., A Crude Extract Preparation and Optimization from a Genomically Engineered Escherichia coli for the Cell-Free Protein Synthesis System: Practical Laboratory Guideline. Methods Protoc, 2019. 2(3).
Failmezger, J., et al., Cell-free protein synthesis from non-growing, stressed Escherichia coli. Sci Rep, 2017. 7(1): p. 16524.
Whilst others sometimes show less significance for the IPTG exposure time, e.g.:
Dopp, J.L. and N.F. Reuel, Process optimization for scalable E. coli extract preparation for cell-free protein synthesis. Biochemical Engineering Journal, 2018. 138: p. 21-28.

Response to Comment 1: We thank the reviewer for pointing out the unclarity. We have re-worded this statement to note that harvesting cultures at an OD of 2.5 yield have been shown to yield highly productive extract. The publications you noted have indeed shown that it is possible that alternative harvesting times can also be sufficient.

Comment 2: Line 158: DNA template amount might be better given in final molar concentration or moles.

Response to Comment 2: We thank the reviewer for pointing this out. We have added a note, giving the molar concentration of our DNA template, pJL1-sfGFP and have noted that each cell-free system may need to be optimized for the specific plasmid being implemented into the reaction.

Comment 3: For new CF users (a major target audience) there may be value in including a simple diagram comparing this method to the standard.

Response to Comment 3: We thank the reviewer for pointing this out. We have added a figure comparing the workflow using CFAI to the standard method as Figure 1.

Comment 4: Could the authors indicate the final protein concentration of cell extracts prepared from CFAI 2.5 and 10 OD600 and 2xYTPG 2.5 OD600 (around line 199)

Response to Comment 4: Yes, we were able to determine the protein concentration using a Bradford Assay and have included that information in the Results section, as well as Table 5. Thank you for this suggestion.

Comment 5: In the legend of Fig 3 it is mentioned: '..CFAI (2.5 OD600), and 10.0 CFAI (2.5 OD600)', I am a bit confused … wouldn't this be CFAI (10 OD600)

Response to Comment 5: Yes, this was a mistake in labeling on our end. We ended up removing this figure and combined it with the bar graph demonstrating the sfGFP yields of each extract. We have also fixed the annotations throughout the manuscript.

Comment 6: Fig 3 needs annotations or maybe the authors could find a way to pull together figs 3 and 4? These figs are showing pretty much the same thing (one being qualitative and the ~~other~~ the measurements of GFP concentration in CFPS).

Response to Comment 6: We thank the reviewer for pointing this out. This is a helpful suggestion and we have combined figures 3 and 4 into a two-panel figure. We agree that this is better for the reader.

Comments 7- 13, 17, 20:

BL21*(DE3) BL21 Star (DE3)
Line 62-63: "These strains are then used to grow and harvest cells for extract." to "These strains are grown and cells harvested to give raw material for extract."
Line 64: "Extract preparation" to "Cell lysis"
Line 66: "harvesting" to "culture and harvesting"
Line 68: "overnight seed culture," to "overnight seed culture in LB broth," ?
line 71. 2.5.. 2.5.

Line 175: "carry out" - "run"
Line 214: "at" - ""

Response to Comments 7-13, 17, 20: These changes have been made. Thank you.

Comment 14: Line 130-131: Flash freezing the pellet may not be needed. This is not part of our protocol.

Response to Comment 14: We thank the reviewer for pointing this out. We generally flash freeze all our pellets since we generate more cells than we use for one extract prep, but we agree it is not required if cells are being lysed immediately for extract preparations. Therefore, we have added a note to the procedure to inform the reader.

Comment 15: Line 148-149: You don't flash freeze your extracts? Flash freezing the aliquots of extract (on first freezing) is part of our protocol.

Response to Comment 15: We do indeed flash freeze our extracts. We thank the reviewer for pointing out this mistake. This step has been added into our protocol.

Comment 16: Line 148-149: It might be useful to state here what volume is anticipated

Response to Comment 16: We thank the reviewer for pointing this out. We thought that it would be best to point out the expected volume in our representative results section rather than in the methods section for consistency with other expected yields, such as sfGFP yield and pellet size.

Comment 18: Line 177: Perhaps best to state that this is given as the "control reaction". It would also be useful to state the specific plasmid used (as performance can vary).

Response to Comment 18: We were not entirely sure what the reviewer meant by “control reaction” but we specified the reporter protein, and noted the plasmid used as a note in step 4.2 when discussing the cell-free reaction set up. We hope the reviewer is OK with this.

Comment 19: Line 190-192: This information is useful in understanding what has been done, and should be shared earlier in the document.

Response to Comment 19: We agree about the importance of this information. We debated its placement and landed on the results section as the appropriate place to maintain the focus of

this manuscript as a methods guide. While important, this information is not necessary for the technical implementation of the CFAI workflow. We hope the reviewer is OK with keeping it as is.

Reviewer #2:

Comment 1: Line 64-65 - I think that the transition from paragraph 2 to paragraph 3 is not logical enough. I would move the sentence about the general methods of cell lysis to the previous paragraph. I would then start the next paragraph by putting forward the reported drawbacks of the current protocol for E. coli cell extract preparation, then move to describing this 24-hr CFAI workflow and its advantages.

Response to Comment 1: We thank the reviewer for pointing this out. We re-arranged this structure per the suggestion. It does indeed provide a more logical transition.

Comment 2: Please provide the reference here to the published manuscript that developed and applied the CFAI formulation.

Response to Comment 2: We have updated the reference, thank you.

Comments 3-8:

Line 86 - Change "the solution" to "culture media"
Line 87 - "the media" repetitive - remove.
Line 89 - Please specify that it is a v/v ratio.
Line 96 - Sentence structure, "Prior to inoculation of the CFAI media, add the sugar solution directly to it."
Line 111-112 - Please provide the non-abbreviated molecular names of the salts since they are mentioned here for the first time.
Line 112 - Please specify the required pH for this buffer.

Response to Comments 3-8: These changes have been made. Thank you.

Comment 9: Line 117 and 127 - Conventionally, cells are harvested at 4°C. Is 10°C selected for practicality or for a specific reason?

Response to Comment 4: We thank the reviewer for pointing this out. We have updated the manuscript to reflect a temperature range of 4-10°C. We strive to maintain 4°C, however, at colder temperatures, the humidity can sometimes affect our centrifuge electronics. Therefore, we set 10°C as the upper limit on refrigeration as a practical solution.

Comments 10-12:

Line 118 - Change "cold" to "pre-chilled" to emphasise the need for sufficiently cooled vessels prior to transferring the cells.
Lines 143-144 - Due to sensitivity of cell extract to over-heating, I would add a comment on keeping the tubes in ice-water during sonication process and when changing tubes to make sure the lysates do not warm up (even though this is mentioned in the Discussion section).
Line 169 - Please provide the full molecular name of the glutamate salts: "magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate…"

Response to Comments 10-12: These changes have been made. Thank you.

Comments 14-21:

Line 196-197 - "different from the OD600 10 culture, which was inoculated directly from a plate… This CFAI media was then monitored and harvested at 2.5 OD600". This is confusing, please rephrase this part.
Line 203 - It might be useful to remind that D-lactose is used to induce T7 RNAP directly, instead of using the analog, IPTG.
Line 216 - "cell harvest at different OD600"
Lines 216 and 217 - Please approximate to two decimal places.
Line 228 - "similar quality as demonstrated by their high CFPS yields."
Line 248 - Sentence structure not easily readable. "…some time. Hence, it is important to keep…"In Table 1, I assume that it is a 100% (v/v) glycerol, but please specify for completeness.
In the table of material/equipment, details for glutamic acid are missing.

Response to Comments 14-21: These changes have been made. Thank you.

Typos:
Line 50: a cell extract (word missing)
Line 63: extract preparation (word missing)
Line 71: additional punctuation mark (remove)
Line 113: in (remove)
Line 131: "…flash-freeze the pellet in liquid nitrogen" (wording)
Line 214: at (remove)
Line 231: use ":" instead of "," (wrong punctuation used)
Line 234: "is achieved by replacing" (change word)
Line 236: "at a precise point during cell growth." (Change word)

Line 238: "The new media formulation improves" (change word to avoid repetition)
Last page (material/equipment list): baffled (typo)

Response to Typos: These errors have been corrected. Thank you.