Reports

Streamlined extract preparation for *Escherichia coli*-based cell-free protein synthesis by sonication or bead vortex mixing

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Escherichia coli-based cell extract is a vital component of inexpensive and high-yielding cell-free protein synthesis reactions. However, effective preparation of *E. coli* cell extract is limited to high-pressure (French press-style or impinge-style) or bead mill homogenizers, which all require a significant capital investment. Here we report the viability of *E. coli* cell extract prepared using equipment that is both common to biotechnology laboratories and able to process small volume samples. Specifically, we assessed the low-capital-cost lysis techniques of: (i) sonication, (ii) bead vortex mixing, (iii) freeze-thaw cycling, and (iv) lysozyme incubation to prepare *E. coli* cell extract for cell-free protein synthesis (CFPS). We also used simple shake flask fermentations with a commercially available *E. coli* strain. In addition, RNA polymerase was overexpressed in the *E. coli* cells prior to lysis, thus eliminating the need to add independently purified RNA polymerase to the CFPS reaction. As a result, high-yielding *E. coli*-based extract was prepared using equipment requiring a reduced capital investment and common to biotechnology laboratories. To our knowledge, this is the first successful prokaryote-based CFPS reaction to be carried out with extract prepared by sonication or bead vortex mixing.

Cell-free protein synthesis (CFPS) is an in vitro protein transcription/translation system in which cell maintenance and growth is detached from protein synthesis. CFPS enables direct control and optimization of protein synthesis by performing the reaction in a test tube wherein the transcription, translation, and protein folding machinery provided by cell extract are combined with energy sources to catalyze the synthesis of only the target protein. Hence, viable cell extract is a vital constituent of effective cell-free reactions, and cell lysis is a key unit operation in cell extract preparation. Due to the superior control and direct engineering that CFPS affords over protein synthesis, many independent researchers have developed, simplified and optimized CFPS reactions and cell extract preparation procedures (1–10). However, methods for inexpensive and high-yielding *Escherichia coli*-based CFPS still require specialized cell lysis

equipment, resulting in a significant capital investment. In this work, we assess the use of cell lysis techniques with common biotechnology equipment requiring a smaller capital investment to prepare viable *E. coli*-based CFPS extract.

CFPS is an open system devoid of a membrane barrier and is thus amenable to direct engineering. CFPS has been of great use when toxic proteins (11,12), virus-like particles (12–14), membrane proteins (15–17), and proteins containing unnatural amino acids (18–21) are to be produced. Additionally, CFPS complements other applications including high-throughput functional genomics (22), protein evolution (23–25), and structural proteomics and genomics (17,26). The open nature of the CFPS allows for manifold manipulations of the system, including adjustment of energy, cofactor, and genetic template concentrations, as well as the cell extract itself. For example,

many different energy sources such as phosphoenolpyruvate (27), phosphocreatine (28,29), glucose (30,31), and fructose-1,6 bisphosphate (32) have been successfully incorporated into CFPS, and *E. coli* central metabolism and oxidative phosphorylation have been activated (33,34). Additionally, PCR-generated linear DNA templates have been incorporated in CFPS, mitigating the labor- and time-intensive processes of gene cloning (22,35,36). To enable more scientist and engineers to reap the benefits that CFPS has to offer, a simple, robust, convenient, and high-yielding cell extract preparation method is needed.

The *E. coli*-based system is the least expensive, the highest yielding, and the most time efficient CFPS system (37). The *E. coli* extract preparation protocol for CFPS dates back to an article from Nirenberg in 1963 (10), which was further modified by Zubay (2) and Pratt (3). More recently, Kigawa et al.

Figure 1. Schematic comparison of different extract preparation methods developed over the last 50 years. Methods with a star by the cell strain require the addition of independently purified RNA polymerase to the final CFPS reaction. The bead vortex mixing and sonication methods are reported in this paper.

(4), Liu et al. (5), Kim et al. (6), and Yang et al. (7) have sought to streamline the extract preparation protocol. Kim et al. (6) eliminated unnecessary steps and reduced the reagent cost and processing time for extract preparation by up to 80% when compared with the protocol established by Pratt (3). In addition, Kigawa et al. (4) and Yang et al. (7) have reported the use of shake flask fermentation to simplify the cell growth. Kim et al. (6) also reported the use of the commercial BL21 (DE3) strain (Invitrogen, Carlsbad, CA, USA) to overexpress the T7 RNA polymerase during cell extract preparation and eliminate the need to add independently purified T7 RNA polymerase to the CFPS reaction as required by other protocols (4,5,7). More recently, the same research laboratory reported the use of a BL21 Star (DE3; Invitrogen) strain that contains an RNA stabilizing mutation and will be used in this work (38). Figure 1 provides an overview of these developments. As shown in Figure 1, all of the aforementioned protocols use a specialized bead mill or high-pressure homogenizer (French press-style or impinge-style) for cell disruption, requiring a significant capital investment before research laboratories can assess the efficacy of *E. coli*-based CFPS for their protein of interest or application. Inspired by the

successful use of freeze-thaw cycling for insect-based CFPS (8) and an unsuccessful attempt at using sonication for *E. coli*-based CFPS (4), here we explore more capital-cost-effective cell lysis techniques for *E. coli*-based CFPS. We also will explore streamlining the extract preparation method by combining the simple shake flask fermentation with the use of the commercial BL21 Star (DE3) *E. coli* strain and the highly simplified lysate processing protocol reported by Kim et al. (6).

Materials and methods

Shake flasks cell culture

Cell growth for extract preparation was performed using BL21 Star (DE3) cells harboring the pEVOL-*pPrF* plasmid (39) in 2.5-L baffled tunair flasks (IBI Scientific, Peosta, IA, USA). Cells were cultured at 37°C with 280 rpm agitation in a rotary incubator shaker (value in *g* not specified for all incidences of rpm). The fermentation was performed with and without the presence of 100 mM 3-morpholinopropanesulfonic acid (MOPS) in 2× yeast extract and tryptone (YT) growth media. The fermentations were inoculated with 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.02% (w/v) L-arabinose at an optical density (OD₆₀₀) of 0.6. Cells were harvested at

mid to late logarithmic growth phase at an OD₆₀₀ of 2.7 to 3.8 4 h after induction by centrifugation at 8000 rpm at 4°C for 30 min. Cells were then washed by suspending in 10 mL ice-cold buffer A (10 mM Tris base, 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol) per gram of cell and centrifuged at 6000 rpm at 4°C for 30 min and subsequently resuspended in 1 mL ice-cold buffer A per gram of cell in preparation for cell lysis. Finally, the cell suspension was flash-frozen in liquid nitrogen and stored at -80°C prior to lysis. Note that the pEVOL-*pPrF* plasmid and its induction with arabinose are not necessary for extract preparation. However, its inclusion in the *E. coli* cells used for extract preparation enables the option of cell-free production of proteins containing unnatural amino acids.

Cell lysis and extract preparation

High-pressure homogenization.

Thawed cell suspensions were lysed with three passes through an Emulsiflex-B15 French press-style high-pressure homogenizer (Avestin, Ottawa, ON, Canada) at 24,000 psi with sample cooling for 1 min in an ice-water bath after the second pass. The lysate was centrifuged at 12,000× *g* for 10 min at 4°C, and the pellet was discarded. The supernatant was carried forward for a run-off

Figure 2. Protein production with extracts prepared from cells lysed with sonication, bead vortex mixing, and high-pressure homogenization. Protein production yields from extracts prepared by lysing cells with 10-min sonication burst with 2-min 4°C cooling cycles (A), 20-min sonication burst with 2-min 4°C cooling cycles (B), 10-min bead vortex mixing with 1-min 4°C cooling cycles (C), and French press-style high-pressure homogenization (D), represented as HP in the figure. The dark and light bars represent the protein yields from CFPS reactions using extracts prepared from cells grown in fermentations with and without MOPS, respectively. Error bars represent the standard deviation from triplicate CFPS reactions. The x-axis represents the total amount of sonication time or bead vortex mixing time applied to the cells for lysis and excludes the time spent cooling. Each time point effectively represents an additional lysis and cooling cycle.

reaction by incubating at 37°C with 280 rpm agitation for 30 min. The extract was flash-frozen in liquid nitrogen and stored at -80°C until use.

Sonication. Thawed cell suspensions were lysed using a Vibra-cell VCX 400 probe sonicator with a CV 26 probe (tip diameter of 3 mm; Sonics & Materials, Newtown, CT, USA) at a frequency of 20 kHz. The sample vial was kept in an ice-water bath to prevent significant heating in the sample during sonication. The lysate was centrifuged for 30 min at 12,000× *g* at 4°C, and the run-off reaction was performed by incubating the supernatant at 37°C with 280 rpm agitation for 30 min. The extract was flash-frozen in liquid nitrogen and stored at -80°C until use. Sonication was performed by cycling at the sonication and cooling intervals as shown in Table 1.

Bead vortex mixing. Glass beads of 0.1 mm diameter (Scientific Industries, Bohemia, NY, USA) at 10%, 20%, 50% or 80% (w/v) beads-to-cell suspension ratio were used for cell lysis in 1.5- or 2-mL microcentrifuge tubes. The cell and bead suspension was vortex mixed on the table top vortex mixer, Fisher Vortex Genie 2 (Scientific Industries), at 3200 rpm for different time intervals with

a 1-min cooling period between each vortex mixing. The lysate was centrifuged twice at 12,000× *g* at 4°C for 30 min with the supernatant retained each time. The run-off reaction was performed at 37°C and 280 rpm shaking for 30 min. The extract was flash-frozen in liquid nitrogen and stored at -80°C until use.

Lysozyme incubation. Hen egg white lysozyme (Sigma-Aldrich, St. Louis, MO, USA) was added to the thawed cell suspension at a concentration of 0.1 mg/mL and incubated at 37°C with gentle shaking (80 rpm) for 3 h, similar to as reported previously (40). The lysate was centrifuged at 12,000× *g* at 4°C for 10 min. The run-off reaction was performed by incubating the supernatant at 37°C and 280 rpm shaking for 30 min. The extract was flash-frozen in liquid nitrogen and stored at -80°C until use.

Freeze-thaw cycling. Thawed cell suspensions of 500 μL in 1.5-mL microcentrifuge tubes were subjected to three freeze-thaw cycles by freezing the cell suspensions on liquid nitrogen or dry ice for 15 min and thawing for 15 min in a water bath at 25°C, similar to previously reported methods (41,42). The

lysate was centrifuged at 12,000× *g* at 4°C for 10 min. The run-off reaction was performed by incubating the supernatant at 37°C and 280 rpm shaking for 30 min. The extract was flash-frozen in liquid nitrogen and stored at -80°C until use.

Cell-lysis efficiency determination

The concentration of the *E. coli* cells used for extract preparation was determined using the wet-weight as measured following cell harvest (1 trillion *E. coli* cells per gram wet weight). Following lysis and before clarification by centrifugation, the lysate was diluted 50, 2500, 120,000, and 24,000,000 fold. Twenty microliters from the dilutions were plated on sterile Luria-Bertani agar Petri dishes (without antibiotics) and incubated overnight at 37°C. The lysis efficiency was determined by comparing the numbers of colonies on each plate to the total number of cells that would be present in the corresponding same volume and dilution if the cells were not lysed.

Cell-free protein synthesis reaction

PANOx-SP energized cell-free reactions were performed on a flat bottomed 96-well black microtiter plate (Fisher

Figure 3. Temperature of sample during sonication and bead vortex mixing. Blue diamonds represent 10 min continuous bead vortex mixing with 1 min cooling in an ice-water bath. Green triangles and red squares represent continuous sonication for 10 min and 20 min with 2 min cooling time in between each burst. Sonication and cooling were performed in an ice-water bath. Red dots represent the temperature of the sample at different stages during French press-style high-pressure homogenization with the order of pass above or beside the dot. Legend for high-pressure homogenization: 1, temperature of the sample at the beginning; 2, temperature of the lysate after the first pass; 3, temperature of the sample after the second pass; 4, temperature of the sample after cooling for a 1-min ice-water bath in a steel beaker; and 5, temperature of the lysate after the third pass. The error bar with the corresponding colors represents the standard deviation of triplicate temperature measurements.

Scientific, Waltham, MA, USA) with reaction volume of 15 μ L at 37°C for 3 h with the following modifications: (i) radiolabeled leucine was not added to the reaction mixture, (ii) cell extract was used at 24%–25% (w/v) concentration, and (iii) BL21 Star DE3 *E. coli* cell strain was used for extract preparation, eliminating the need for addition of purified T7 RNA polymerase (13). Phosphoenolpyruvate and *E. coli* tRNA mixture were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). All other reagents were obtained from Sigma-Aldrich. The super-folder green fluorescent protein (sfGFP) expression plasmid used in this work has been reported previously (18). The protein yield was determined by diluting the reaction volume to 60 μ L with MilliQ water (Millipore, Billerica, MA, USA) in flat-bottomed 96-well black microtiter plates and measuring the fluorescence with a SYNERGY MX microplate reader (BioTek Instruments, Winooski, VT, USA) at a sensitivity setting of 50 and excitation/emission wavelengths of 485 and 510 nm, respectively. A calibration curve was used to determine

sfGFP concentration (see Supplementary Figure S2).

Results and discussion

Shake flask cell culture

The use of shake flask fermentation and a common commercial *E. coli* strain, BL21 Star (DE3) for extract preparation simplifies the fermentation and eliminates the need to add independently purified RNA polymerase to the reaction or the acquisition of a specialized cell strain. While effective at producing *E. coli* cells viable for producing CFPS extract (7), the nutrient concentrations and pH are not directly controlled during shake flask fermentation. To assess the effect of the pH change during fermentation on extract viability, *E. coli* cells were fermented with and without the presence of 100 mM MOPS. Although, over the course of the fermentation, the pH exhibited a smaller change with MOPS (Supplementary Figure S1), the average sfGFP production yields from the extract prepared from cells grown in MOPS was within a standard deviation of the extract prepared from cells grown

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Table 1. Sonication time and cooling time intervals in between for extract preparation using sonication.

Sonication Burst Time	Cooling Time	sfGFP Yield ($\mu\text{g/mL}$)	Variability
10 s	30 s	587 ± 178	High
20 s	60 s	588 ± 66	High
60 s	90 s	731 ± 66	High
3 min	2 min	761 ± 208	High
10 min	2 min	1004 ± 82^a	Low ^a
20 min	2 min	1010 ± 194^a	Low ^a

^aData obtained beyond 100 min of total sonication time was not included in the analysis. sfGFP yield results shown in Figure 3 for 10 min and 20 min burst times, with all other burst time data shown in Supplementary Figure S3.

Yield represents the highest obtained active sfGFP yield with corresponding standard deviation ($n = 3$). High variability is defined as variation of greater than 25% in protein production yields when identical conditions for preparing the CFPS extract were repeated. Low variability is defined as variation less than 25% in protein production yields when identical conditions for preparing the CFPS extract were repeated.

without MOPS (see Figure 2D). Thus the inclusion of MOPS in the fermentation did not significantly affect extract performance.

Performance of extract prepared with sonication

The purpose of using sonication cell lysis as opposed to the previously reported lysis by bead mill and high-pressure homogenizers is to reduce the capital cost and enable researchers without access to these specialized equipment the opportunity to assess and use CFPS for their desired application. The capital cost of the bead mill or high-pressure homogenizers used in this work and reported by others to produce CPFS extract is approximately \$10,000 to \$40,000 (Supplementary Table S1) compared with the commonly available sonicator, which costs about \$4000 (Supplementary Table S1).

For cell lysis, the sonication burst periods and 4°C cooling periods were initially selected based upon commonly reported protocols for cell lysis, which use 10- to 60-s sonication burst periods and 3–10 cycles as shown in Table 1 (43–46). However, following these sonication protocols for cell lysis, the resulting CFPS extract produced protein at yields <25% of that achieved using a high-pressure homogenizer (Supplementary Figure S3). Also, different from the simplified Kim et al. (6) protocol using a French press-style high-pressure homogenizer (Figure 1), centrifugation for 30 min was needed to clarify the extract to appear similar to that obtained by high-pressure homogenization and 10 min of centrifugation.

Although the sonication-produced extract was less productive than that produced by high-pressure homogenization, continued sonication burst-cooling cycles, and thus longer total sonication times, did in most cases result in a more productive CFPS extract (Supplementary Figure S3). However, a significant limitation was the variation in extract productivity observed among replicates (Table 1 and Supplementary Figure S3). This result was not surprising given that Kigawa et al. (4) claimed from their tests that sonication was not suitable for *E. coli*-based CFPS extract preparation. Although Kigawa et al. did not report the method used or the actual data from testing sonication; it is likely they also used common sonication protocols for *E. coli* cell lysis.

Based upon the trend of higher yields from both longer sonication burst periods and longer total sonication time (Table 1 and Supplementary Figure S3), we increased the sonication burst period to 10 or 20 min with a 2-min 4°C cooling period. For a direct comparison to the traditional extract preparation protocol with a high-pressure homogenizer, cell aliquots from the same shake flask fermentations (with and without MOPS) reported in Supplementary Figure S1 were used with sonication. The protein production yields obtained with CFPS extracts prepared using the 10- or 20-min sonication burst and a 2-min cooling period are shown in Figure 2, A and B. Similar results were obtained from cells harvested from either fermentation (with or without MOPS). The extracts prepared by sonication were also suitable for CFPS

using linear DNA templates (see Supplementary Figure S5). Also, continuing the sonication burst-cooling cycle beyond the first sonication burst-cooling cycle was not necessary to obtain a CFPS extract as productive as that obtained from high-pressure homogenization (Figure 2, A, B, and D). In addition, the CFPS extract productivity remained fairly constant over 100 min total sonication time (10 cycles for the 10-min sonication burst, and 5 cycles for the 20-min sonication burst) (Figure 2, A and B).

One concern of lysis by sonication is sample heating, and Kigawa et al. (4) postulated that sample heating is a possible reason why in their tests sonication was not suitable for preparing CFPS extract. The temperature of cell samples was measured over 120 min with continuous cycling between the 10- or 20-min sonication burst and 2 min cooling. The temperature of three independent experiments was measured by a K-type mini thermocouple, and the average remained below 15°C throughout the 120-min run (Figure 3, triangle and square data). In comparison, the average temperature of the cell samples processed by high-pressure homogenization was much higher with the maximum approaching 34°C. Also, sonicated CFPS extract productivity did not significantly decrease over 100 min of sonication, suggesting the extract was not damaged by heat.

The efficiency of lysis by sonication was also assessed relative to a French press-style high-pressure homogenizer by plating dilutions of lysate on LB broth agar Petri dishes. Lysis efficiency

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increased with total sonication time, with 10 cycles of 10 s sonication resulting in 98.898% lysis, 40 cycles of 10 s sonication resulting in 99.789% lysis, and 10 min of continuous sonication resulting in 99.988% lysis. The high-pressure homogenizer had the highest efficiency at 99.9996%. By comparing lysis efficiency alone, it appears that very high lysis efficiency (>99.98%) is needed for the consistent preparation of productive *E. coli* extract for CFPS. Although, other factors also play a role, such as the formation of soluble inverted membrane vesicles to facilitate oxidative phosphorylation CFPS, as reported with extracts prepared using a high-pressure homogenizer (34,47).

Performance of cell extract prepared using bead vortex mixing

With simplifying the extract preparation as our ultimate goal, we also sought to simplify the bead milling process for extract preparation by using a table top vortex mixer with 0.1-mm diameter glass beads. This method is significantly more economical than the commercial bead mill method (approximately \$10,000–\$40,000 for a commercial bead mill; Supplementary Table S1) compared with about \$350 for the commonly available table top vortex mixer (Supplementary Table S1). Commercial bead milling equipment has been regularly used for protein purification (48,49), DNA extraction (50), cell-free extract preparation (4,51), and lipid extraction (52).

Initial attempts with bead vortex mixing were performed with 10%, 20%, 50%, and 80% (w/v) bead. Of the different combinations, only the bead concentration recommended by the manufacturer [80% (w/v) bead-to-cell buffer ratio] produced a significant amount of protein (results not shown) and was therefore chosen for further experiments. Also, cell lysates obtained by bead vortex mixing were observed to have a higher viscosity, and two centrifugation steps of 30 min each were required to adequately clarify the lysate. The protein yield obtained with extract prepared by cycling between bead vortex mixing and cooling in ice water resulted in lower protein production yields and higher extract-to-extract variability (Figure 2C and Supplementary Figure S4) as compared with protein production yields from extracts prepared by high-pressure homogenization and 10+ min of sonication. Lysis efficiency following two and five cycles of 10-min bead vortex mixing was 99.378% and 99.479%, respec-

tively, which is lower than that observed with the high-pressure homogenization and 10 min of sonication. The bead vortex mixing lysis efficiency observed is similar to that observed at lower sonication times, which also resulted in CFPS extract with lower protein yields and higher variability between replicate extract preparations. Increasing the vortex mixing time beyond 80 min seemed unreasonable for a streamlined extract preparation method. Also of note is the large temperature swings observed by cycling between bead vortex mixing and cooling, although temperatures higher than that obtained with the high-pressure homogenizer were not observed (Figure 3). Although there are challenges associated with this method, yields up to 600 µg/mL sfGFP production were obtained from CFPS extract prepared with bead vortex mixing.

Performance of cell extract prepared by freeze-thaw and lysozyme

As part of this work, the simple cell lysis methods of freeze-thaw and enzymatic lysis by lysozyme were also assessed for preparing CFPS extract. However, the extracts prepared using these techniques did not produce measurable amounts of protein in CFPS reactions (results not shown). Lysis efficiencies of 99.609% to 99.976% for freeze-thaw and 99.991% for lysozyme were observed. These efficiencies are comparable to the lysis efficiency obtained after 10 min of sonication (99.988%), suggesting insufficient lysis was not the reason for the unviable extract. In contrast to lysis by a high-pressure homogenization or sonication, the freeze-thaw and lysozyme lysis methods do not involve mechanical shearing, which may be important for viable *E. coli* cell extract (40,53). However, freeze-thaw lysis has been reported as a viable method to produce insect cell extract for CFPS (8), which suggests that with further engineering such a method could be developed for *E. coli* cell extract preparation.

In this work, we have sought to simplify the cell extract preparation method using equipment common to biotechnology laboratories, thus eliminating the need for specialized growth and lysis equipment. This was accomplished by performing cell growth in shake flasks and assessing the alternative *E. coli* disruption techniques of: (i) sonication, (ii) bead vortex mixing, (iii) enzymatic lysis, and (iv) freeze-thaw cycling. Using sonication, we were able

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to reproducibly produce high-yielding CFPS extract and, in terms of capital equipment cost, sonication is approximately 60% to 90% less expensive than the developed techniques shown in Figure 1. Also, sonication is well suited for laboratories with access to a sonicator but not a bead mill or a high-pressure homogenizer. Another benefit of sonication is that sample volumes

as low as 150 μ L can be processed, and 96-well plate sonicators are available for high-throughput applications (Supplementary Table S1). Extracts produced by sonication could also be engineered for other cell-free configurations, such as continuous exchange CFPS, which enables extended reaction durations and higher productivities. For researchers without access to a sonicator, bead

vortex mixing can also be used for extract preparation, although the CFPS yields obtained through this lysis method are lower than those obtained using sonication or high-pressure homogenization with higher extract-to-extract variability. In conclusion, the combination of shake flask cell culture, sonication or bead vortex mixing cell lysis technique, and streamlined extract preparation protocol described in this work can significantly reduce the time, effort, and capital cost expended in initial proof-of-concept experiments with CFPS. We believe this simple extract preparation technique could become an economically sound milestone for extract preparation and will enable more scientists and engineers to test CFPS for their desired application.

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Competing interests

The authors declare no competing interests.

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