A simplified and highly efficient cell-free protein synthesis system for prokaryotes

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Abstract

Cell-free protein synthesis (CFPS) systems are a powerful platform with immense potential in fundamental research, biotechnology, and synthetic biology. Conventional prokaryotic CFPS systems, particularly those derived from *Escherichia coli* (*E. coli*), often rely on complex reaction buffers containing up to thirty-five components, limiting their widespread adoption and systematic optimization. Here, we present an optimized *E. coli* cell-free protein synthesis (*e*CFPS) system, significantly streamlined for high efficiency. Through systematic screening, we successfully reduced the essential core reaction components from 35 to 7. The thorough optimization of these seven key components ensured that protein expression levels were not only maintained but even improved. Furthermore, we developed a much simpler procedure for preparing the bacterial cytosolic extracts, enhancing the overall accessibility and robustness of *e*CFPS. This optimized and user-friendly *e*CFPS is poised to become a powerful tool, facilitating diverse applications in basic life science research and beyond.

26 **Introduction**

27 Cell-free protein synthesis (CFPS) offers a powerful and flexible platform for biological
28 research and biotechnological applications by reproducing the cellular protein synthesis
29 process in an *in vitro* environment[1,2]. Compared to traditional cell-based expression
30 systems, CFPS possesses several notable advantages, including rapid reaction kinetics,
31 ease of operation, independence from cell culture, high tolerance to toxic proteins, and
32 facile incorporation of non-canonical amino acids[3–5]. These characteristics position CFPS
33 for broad applications in protein engineering, high-throughput screening, synthetic biology,
34 and diagnostic reagent development[6,7].

35 Prokaryotic CFPS systems, especially those based on *E. coli* lysates, have garnered
36 significant attention for their high protein synthesis yields and compatibility with genetic
37 manipulations[8–12]. They have played a pivotal role in early molecular biology research
38 and continue to be relevant in industrial protein production and biosensor
39 development[1,13,14]. However, for a long time, most prokaryotic CFPS protocols have relied
40 on complex buffer systems containing a large number of auxiliary components[15,16]. A
41 comprehensive analysis of existing literature reveals that the composition of reaction
42 buffers differs widely between protocols, both in the number of optional components and
43 in the concentrations of individual components (**Table S1**). These complex systems, while
44 effective, often lead to high costs, laborious preparation procedures, and potential
45 interactions among components, posing significant challenges for further optimization
46 and standardization, thereby limiting their widespread adoption in resource-constrained
47 laboratories.

48 In recent years, the field of eukaryotic CFPS has seen remarkable progress in system
49 optimization and simplification[17,18]. Highly optimized human *in vitro* translation systems
50 have demonstrated that high-efficiency protein synthesis can be achieved with a minimal
51 number of core components[17]. Recognizing the potential of such systematic optimization
52 approaches, we hypothesized that similar strategies could be equally applicable and
53 beneficial for *E. coli* CFPS systems by thoroughly analyzing the functions of existing
54 components and integrating insights from previous reports on prokaryotic protein
55 synthesis mechanisms. This study aimed to develop a highly simplified yet efficient *E. coli*

56 cell-free protein synthesis system (*e*CFPS). We conducted a systematic component
57 reduction screening of traditional *e*CFPS systems, successfully streamlining the core
58 components from thirty-five to just seven. The subsequent meticulous optimization of
59 these seven key components not only maintained but even improved protein expression
60 levels. Furthermore, we developed a much simpler procedure for preparing the bacterial
61 cytosolic extracts, enhancing the overall accessibility and robustness of *e*CFPS. This
62 optimized *e*CFPS is poised to become a powerful tool, facilitating diverse applications in
63 basic life science research and beyond.

64

65 **Results**

66 **Streamlining *e*CFPS: removal of dispensable components**

67 To develop a more streamlined and efficient *e*CFPS system, we performed a systematic
68 screening of auxiliary components commonly found in traditional prokaryotic CFPS
69 protocols[15,19]. Our objective was to identify dispensable components while maintaining or
70 enhancing protein synthesis efficiency, thereby simplifying system preparation and
71 reducing costs. Starting with a comprehensive reaction mixture containing up to thirty-five
72 components[15], we iteratively evaluated the contribution of individual constituents through
73 luciferase reporter assays. Throughout this optimization, essential core components such
74 as creatine phosphate (CrP), creatine kinase (CrK), ATP, GTP, magnesium, and
75 potassium were maintained in the base reaction mixture[3,8,17]. Our systematic approach
76 allowed us to precisely determine the impact of each component on overall protein
77 synthesis yield, leading to the identification of both dispensable and critical factors.

78 Through this systematic screening, we first identified several components that could be
79 entirely removed from the reaction mixture without compromising protein synthesis
80 efficiency (**Figure 1**). Dithiothreitol (DTT), a reducing agent commonly included in both
81 transcription and translation systems to maintain protein sulfhydryl groups in a reduced
82 state and prevent aggregation, was found to be unnecessary within our specific system,
83 as its removal did not affect the final protein expression levels (**Figure 1A**). Similarly,
84 cyclic adenosine 3′,5′-monophosphate (cAMP), a regulator implicated in transcriptional
85 regulation[20–22], was also found to be dispensable for efficient protein synthesis (**Figure**

86 **1B**). Furthermore, we systematically evaluated other auxiliary components, including the
87 molecular crowder polyethylene glycol 8000 (PEG8000), used to enhance
88 macromolecular crowding[24–28]; ammonium ions (NH_4^+), typically included as an osmolyte
89 and for its role in maintaining protein stability and solubility in *e*CFPS reactions[28–32]; and
90 folinic acid, which serves as a crucial cofactor for nucleotide synthesis[8,32]. Our results
91 consistently showed that removing these components had a negligible impact on overall
92 protein synthesis performance (**Figure 1C-E**). These findings collectively demonstrate
93 that a substantial portion of the auxiliary components in traditional *e*CFPS protocols can
94 be eliminated, paving the way for a more streamlined and cost-effective system.

95 An evaluation of various concentrations of amino acids and tRNA was conducted, as
96 these components are fundamental building blocks for protein synthesis[33–35]. Previous
97 studies on *e*CFPS have highlighted the rapid degradation of certain amino acids, such as
98 arginine, cysteine, and tryptophan, necessitating their replenishment for prolonged
99 synthesis[36,37]. Our results showed that while these components are critical for achieving
100 high yields, protein synthesis still occurs in their absence (**Figure 1F-G**). This suggests
101 that while they are optimizable, tRNA and amino acids are not strictly essential for the
102 reaction to proceed, likely due to residual amounts within the cell lysate (**Figure 1F-G**).
103 Furthermore, we evaluated the role of nucleoside triphosphates (NTPs) in our system,
104 which are essential for coupled transcription-translation. Interestingly, we found that
105 protein synthesis could proceed without the addition of CTP or UTP (**Figure 1H-I and**
106 **Figure S1**). While adding either ATP or GTP alone resulted in a very weak reaction, the
107 presence of both ATP and GTP together recovered the reaction to approximately 40% of
108 the complete NTPs mix (**Figure S1B-C**). This highlights the critical role for ATPase-
109 dependent chaperones (e.g., DnaK) and GTPase-dependent elongation factors (e.g., EF-
110 Tu and EF-G)[38], which are crucial for proper protein folding during synthesis[39].

111 Ultimately, this comprehensive screening allowed us to successfully reduce the core
112 reaction components from thirty-five to just seven (**Figure 1J**).
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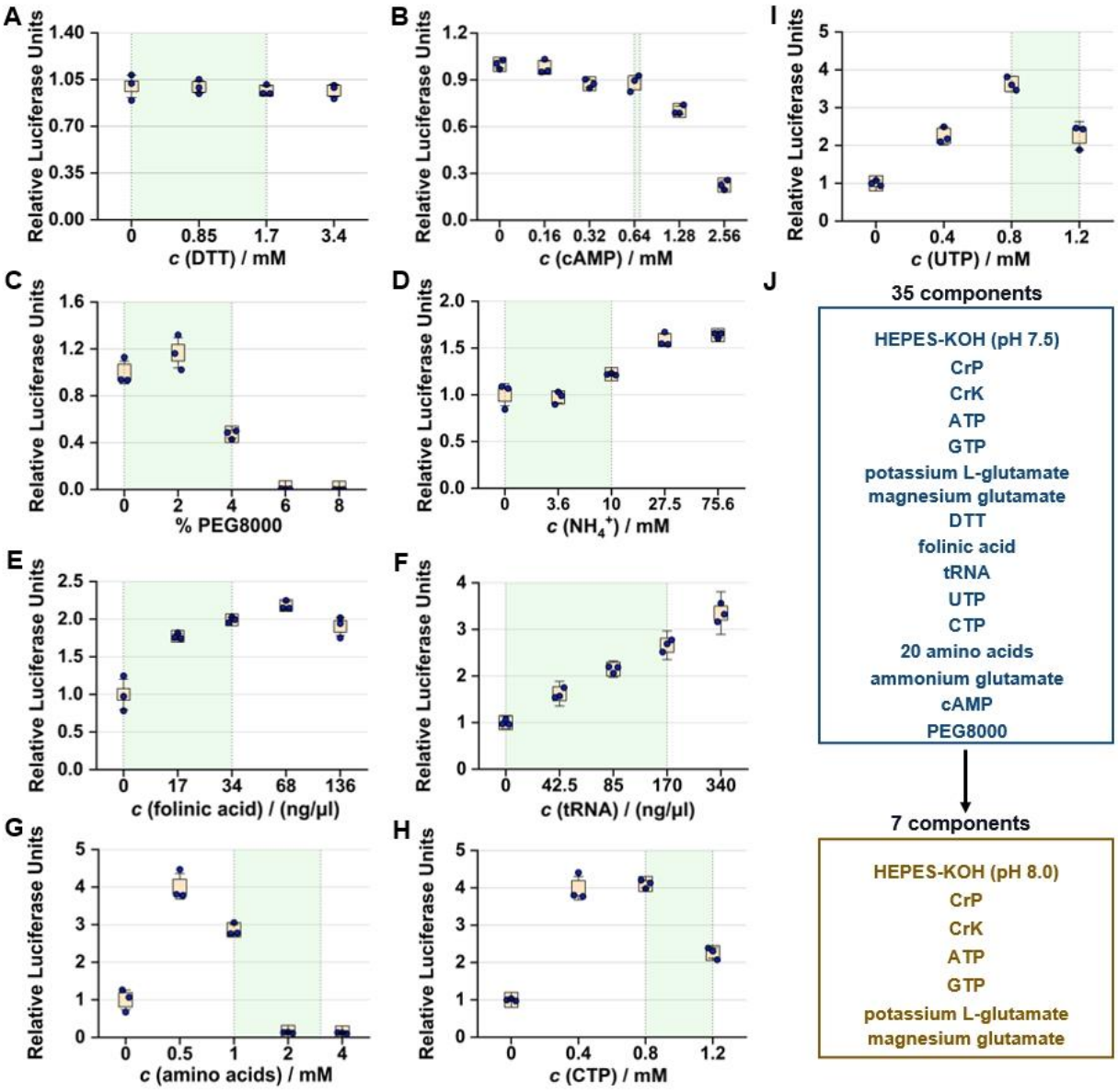

Figure 1 Optimization of *e*CFPS components. Protein expression levels from the *e*CFPS system were measured using an Nanoluciferase (NLuc) reporter DNA. Green area in the graphs indicate the common concentration range used in published protocols for *e*CFPS. Error bars represent the standard error (SE) of at least three independent reactions. (**A-E**) Protein expression levels of the *e*CFPS system supplemented with different concentration of DTT (**A**), cAMP (**B**), PEG8000 (**C**), NH_4^+ (**D**), and folinic acid (**E**). (**F-I**) Protein expression levels of *e*CFPS with various concentrations of tRNA (**F**), amino acids (**G**), CTP (H) and UTP (**I**). (**J**) A summary of the supplement components before and after optimization.

Optimization of essential *e*CFPS components

125 While a core set of seven components was found to be sufficient for protein synthesis,
126 we conducted further fine-tuning to maximize the system's performance. First, we
127 optimized the concentrations of key salts and energy components (**Figure 2**). Our findings
128 reveal that the final protein expression level is highly dependent on the concentrations of
129 both magnesium (Mg^{2+}) and potassium ions (K^{+}), which are fundamental for the structural
130 integrity and catalytic activity of ribosomes, and various enzymatic reactions critical to
131 *e*CFPS[40,41]. Through a detailed matrix-based optimization, we first identified the optimal
132 concentrations of Mg^{2+} and K^{+} to achieve maximum protein expression (**Figure 2A**).
133 Similarly, we conducted a separate screen to optimize the concentrations of Mg^{2+} and
134 PEG8000 as previous reports have suggested a cooperative relationship between them[15]
135 (**Figure 2B**).

136 A comprehensive evaluation of the core energy components was also performed. We
137 specifically focused on co-optimizing the concentrations of ATP and GTP, which serve as
138 both energy sources and building blocks[42–44], to maximize the yield of protein synthesis
139 (**Figure 2C**). Additionally, the energy regeneration system, primarily composed of CrP
140 and CrK, is vital for maintaining sustained ATP levels[45,46]. We found that CrP is crucial,
141 with no reaction occurring in its absence (**Figure S2A**). Initial experiments showed while
142 CrK addition significantly boosted translation efficiency early on, reactions without
143 exogenous CrK could achieve a protein expression level approximately four times higher
144 at later time points (2 hours or more), suggesting the presence of an endogenous CrK-
145 like enzyme in the lysate (**Figure S2B**). A co-optimization screen for CrP and CrK
146 concentrations was also performed, and our results showed that optimizing the
147 concentrations of these components is critical for achieving and sustaining high protein
148 expression (**Figure 2D**). Furthermore, we optimized the pH and concentration of the
149 HEPES buffer, finding that a specific range was critical for maintaining stable and high
150 protein expression (**Figure 2E**). These multi-faceted optimizations ensured that each
151 component was present at its ideal concentration, leading to a synergistic effect that
152 significantly boosted the system's overall performance.

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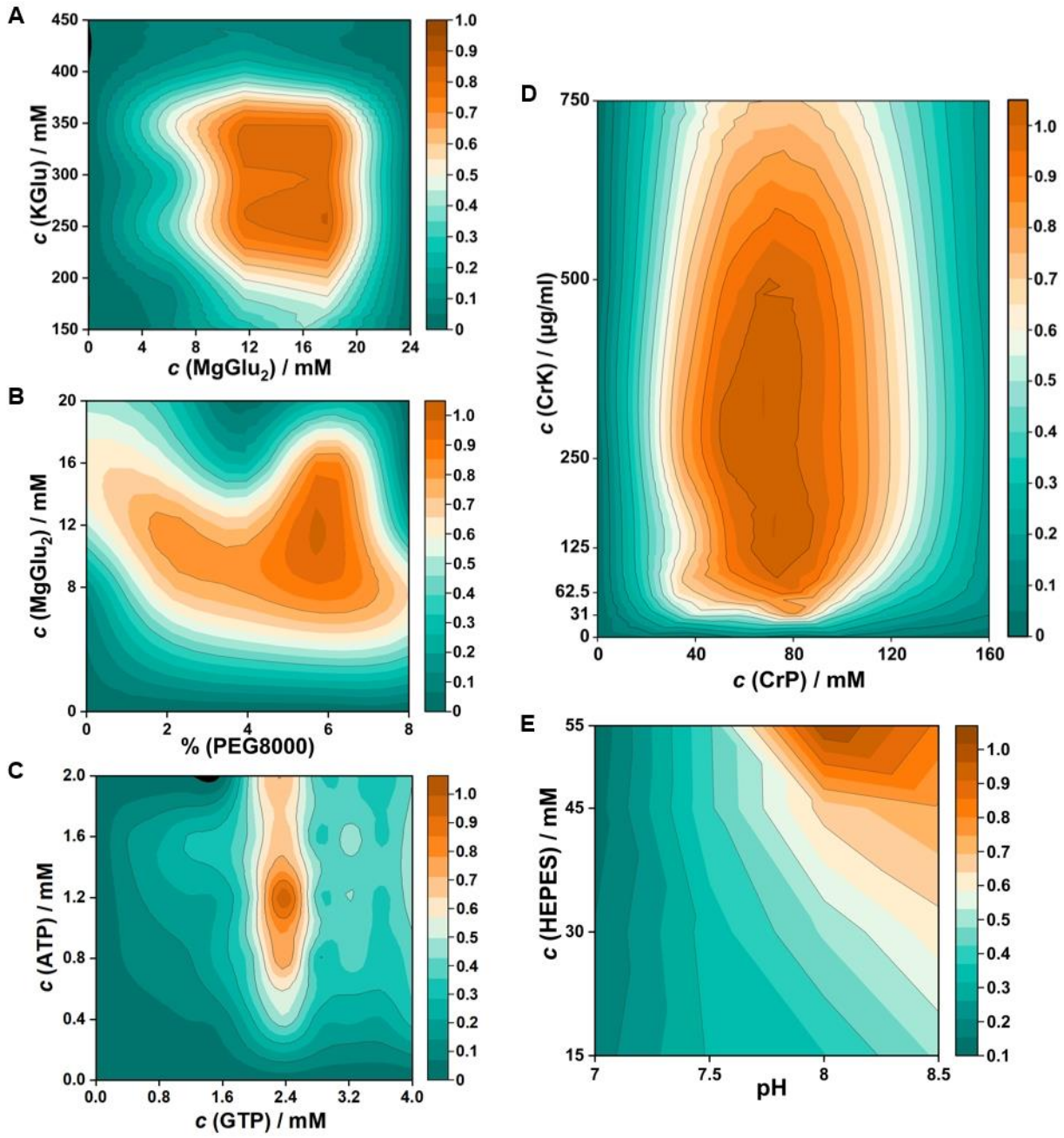
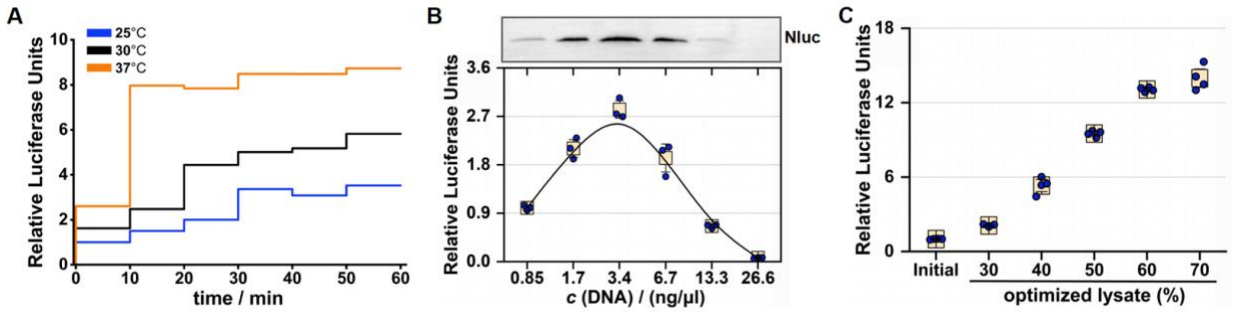


Figure 2 Optimization of essential components for *e*CFPS system. (**A**) Protein expression levels of the *e*CFPS system measured at varying concentrations of KGlu and $MgGlu_2$. (**B**) Protein expression levels of the *e*CFPS system measured at varying concentrations of $MgGlu_2$ and PEG8000. (**C**) Protein expression levels of the *e*CFPS system measured at varying concentrations of ATP and GTP. (**D**) Protein expression levels of the *e*CFPS system measured at varying concentrations of CrK and CrP. (**E**)

161 Protein expression levels of the *e*CFPS system measured at varying pH and buffer
162 concentrations. Data from all panels present mean ± SE, n = 3.

163 **Performance characterization of the optimized *e*CFPS**

164 To thoroughly evaluate the performance of our optimized *e*CFPS, we conducted a series
165 of experiments. We first investigated the kinetics of protein synthesis measuring the
166 expression over time at different temperatures (25°C, 30°C, and 37°C). Our results
167 demonstrate that the system exhibits robust protein synthesis across this range, with the
168 highest expression rates observed at the physiological temperature of 37°C (**Figure 3A**).
169 Next, we assessed the system's sensitivity to DNA concentration, finding that it could
170 efficiently utilize a wide range of reporter DNA templates to produce quantifiable protein
171 products, which were validated by both luminescence assays and western blot analysis
172 (**Figure 3B**). Furthermore, we investigated the impact of varying cell lysate volume ratios,
173 comparing our initial system with the newly optimized *e*CFPS. Our findings indicate that
174 the optimized system maintains superior protein expression even at different lysate-to-
175 buffer ratios (**Figure 3C**).



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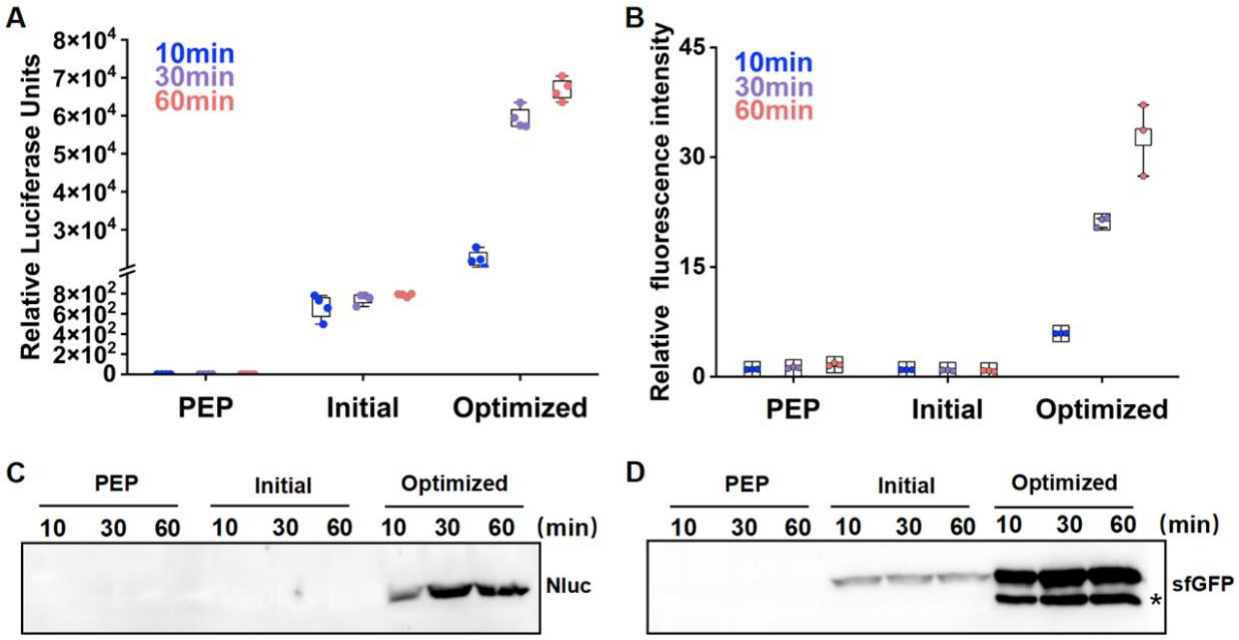
177 **Figure 3 Characterization of the optimized *e*CFPS system.** (**A**) Kinetics of protein
178 synthesis at 25℃, 30℃ and 37℃ over a 60-minute period. Data present mean ± SE, n =
179 3. (**B**) Protein expression levels of the *e*CFPS system measured at varying DNA
180 concentrations for a reporter encoding a FLAG-tagged NLuc. The protein product was
181 quantified via a luminescence assay and confirmed by western blotting. Data present
182 mean ± SE, n = 3. (**C**) Comparison of protein expression levels from the initial and
183 optimized *e*CFPS systems at various cell extract volume ratios. Data present mean ± SE,
184 n = 4.

185 **Comparative analysis of energy regeneration systems**

186 To validate the efficacy of our optimized *e*CFPS, we benchmarked its performance
187 against two widely used systems: the classical Phosphoenolpyruvate (PEP)-based
188 energy system, a common alternative for ATP regeneration [6,7], and an initial CrP/CrK-
189 based system (**Figure 4**). We utilized NLuc, representing a broadly applicable protein,
190 and super-folder green fluorescent protein (sfGFP), with codons optimized for *E. coli* as
191 reporter proteins and monitored their expression over time (**Figure 4A-B, Figure S3A**).
192 Our results show that the optimized *e*CFPS consistently outperforms both the classical
193 and initial systems, achieving significantly higher protein expression levels in a shorter
194 period (**Figure 4A-B**). The robust expression was further confirmed by western blot
195 analysis, which provided clear visual evidence of the superior protein yield from our
196 optimized system (**Figure 4C-D, Figure S3B-C**). Finally, we validated the function of our
197 system through antibiotic-mediated inhibition assays. Our results demonstrate that
198 protein expression in the optimized *e*CFPS can be effectively inhibited by standard
199 antibiotics, confirming its robust and native-like translation machinery (**Figure S3D**).



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201 **Figure 4 Benchmarking the optimized *e*CFPS system with different DNA templates.**
202 (**A**) Protein expression over time using NLuc as DNA templates for different energy
203 regeneration systems. Data present mean ± SE, n = 4. (**B**) Protein expression over time

204 using sfGFP as DNA templates for different energy regeneration systems. Data present
205 mean ± SE, n = 3. (**C-D**) Western blot validation of protein expression for NLuc (**C**) and
206 sfGFP (**D**) from the different *e*CFPS system shown in (**A-B**). The asterisk (*) indicates a
207 non-specific band.

208 **A simplified method for bacterial lysate preparation**

209 In addition to optimizing the reaction buffer, we sought to simplify the entire *e*CFPS
210 procedure by developing an easy-to-use method for preparing the bacterial cell lysate.
211 During lysate making, traditional methods, which often rely on time consuming runoff and
212 dialysis procedures[19,8], were replaced with a high-pressure homogenizer for efficient cell
213 disruption, eliminating the need for additional dialysis. Compared to traditional
214 biochemical methods such as ultrasonication, lysozyme treatment, or freeze-thaw
215 cycles[47], this approach is faster and more convenient (**Figure 5A**). Additionally,
216 endogenous T7 RNA polymerase in the optimized *e*CFPS lysate obviates exogenous
217 addition, simplifying preparation and reducing costs while maintaining high translation
218 efficiency (**Figure 5A**).

219 We validated this new method by testing lysates prepared from cells harvested at various
220 optical densities at 600 nm (OD_{600}). While the density did not significantly impact protein
221 expression levels, a harvest optical density of OD_{600}=2 yielded the best performance
222 (**Figure 5B**). The quality of the ribosomes was further confirmed by sucrose gradient
223 centrifugation (**Figure S4A**) and negative-staining transmission electron microscopy
224 (TEM) images (**Figure S4B**).

225 To further simplify the protocol, we evaluated the necessity of the traditional runoff and
226 dialysis steps[8] (**Figure 5A**). Our results from sucrose gradient analysis and TEM imaging
227 confirmed that ribosomes remained intact, and the *e*CFPS expression signal was
228 significantly higher without them (**Figure 5C-D, Figure S4B**). These findings confirm that
229 both the runoff and dialysis steps are dispensable for our system, allowing for a
230 significantly simplified and faster preparation. Additionally, our reliance on endogenously
231 expressed T7 RNA polymerase yields a significantly higher expression signal,
232 outperforming previously reported eCFPS systems by up to four-fold (**Figure 5D**).

Our optimized lysate preparation method yielded highly active and stable extracts. It is capable of sustaining protein synthesis for up to 120 minutes (**Figure S4C**) and maintains the protein synthesis efficiency even after multiple freeze-thaw cycles, making it highly suitable for routine laboratory use (**Figure 5E**).

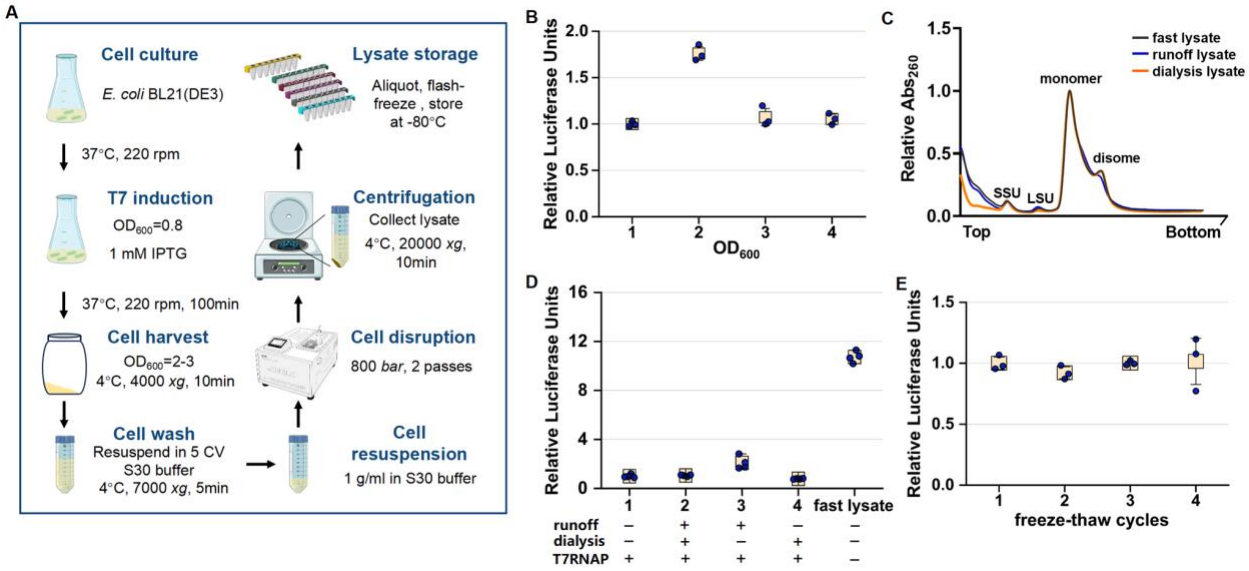

Figure 5 Preparation of *e*CFPS from cultured *E. coli* Cells. (**A**) Flowchart of the *e*CFPS preparation procedures. (**B**) Comparison of reaction efficiency in *e*CFPS using lysate with bacteria cells harvested at different optical density. Data present mean ± SE, n = 3. (**C**) Sucrose gradient sedimentation analysis of different lysates used for *e*CFPS, revealing the presence of ribosome monomers. (**D**) Comparison of protein expression levels in *e*CFPS system using lysates prepared by runoff, dialysis, and rapid endogenous T7 RNA polymerase induction. Data present mean ± SE, n = 4. (**E**) Comparison of reaction efficiency in *e*CFPS using lysates after different numbers of freeze-thaw cycles. Data present mean ± SE, n = 3.

Discussion

In this study, we successfully developed a highly streamlined and efficient *e*CFPS system. By systematically eliminating dispensable components from traditional protocols, we reduced the complexity of the reaction mixture from thirty-five to a core set of just seven components. This simplification represents a significant advancement over conventional systems by not only drastically lowering the cost and effort of preparing the reaction buffer,

but also by minimizing potential inhibitory interactions between numerous auxiliary components, thereby making the system more accessible for a wider range of researchers and laboratories.

The thorough optimization of the seven core components was a critical step in achieving high protein expression levels. Our detailed screening of key factors like salts, energy components, and buffer conditions revealed that precise concentrations are essential for optimal performance. The synergistic effect of these optimized components, particularly the energy source and regeneration system, is based on CrK coupled with CrP[36]. A notable finding from our optimization was the ability of our system to function effectively without certain components traditionally considered essential. For example, the removal of dithiothreitol (DTT) did not negatively impact protein expression, suggesting that our lysate preparation procedure may maintain sufficient reducing conditions or that the high expression levels are transient enough that protein aggregation is not a major issue. Similarly, the ability to achieve high protein yields with minimal or even no added tRNA or CTP/UTP highlights the remarkable robustness and inherent capacity of our lysate, likely due to residual components retained during our simplified preparation method. We also observed that the protein synthesis signal was significantly higher without traditional runoff and dialysis steps, suggesting that these steps may remove essential components that are beneficial for efficient protein synthesis. This led to a significant improvement in protein synthesis yield compared to both classical and initial systems. The benchmarking experiments clearly demonstrated that our optimized eCFPS system provides higher protein expression levels compared to these traditional systems while offering faster kinetics, which is a key advantage for high-throughput applications.

Our simplified lysate preparation method, which eliminates the time-consuming runoff and dialysis steps, further distinguishes this work. We demonstrate that a highly active lysate can be prepared rapidly using a high-pressure homogenizer, which enhances the overall accessibility and robustness of our *e*CFPS system. The ability to use the lysate directly after a single centrifugation step, coupled with the reliance on endogenous T7 RNA polymerase, makes our protocol one of the most straightforward and rapid for producing high-quality *e*CFPS lysates. This streamlined preparation procedure is a significant step

toward making CFPS a more routine and scalable tool for a variety of applications, from educational settings to industrial-scale bioproduction.

In conclusion, our optimized and streamlined *e*CFPS system represents a significant advancement in the field of prokaryotic cell-free protein synthesis. By drastically reducing the number of reaction components and simplifying the lysate preparation procedure, we have created a highly efficient, cost-effective, and user-friendly platform. This system is poised to accelerate fundamental research and facilitate high-throughput protein engineering, compound screening, and diagnostic development. However, we acknowledge that our system, like all CFPS platforms, has limitations, such as the synthesis of complex membrane proteins and glycosylation, which would require the addition of specific accessory components. Future work will focus on further characterizing the endogenous factors in the lysate that enable the high efficiency of our system and exploring its use in novel synthetic biology applications.

298 **Methods**

299 **Plasmid construction**

300 All plasmids and primers used in this study are detailed in **Table S2**. The NLuc and sfGFP
301 reporter genes, each fused with a FLAG-tag, were cloned into a T7-driven expression
302 vector. The sfGFP gene was optimized for *E. coli* codon usage to ensure efficient
303 translation. The vector backbone was constructed using standard molecular biology
304 techniques.

305 ***E. coli* cell culture and lysate preparation**

306 *E. coli* S30 cell lysate for CFPS was prepared using a simplified protocol adapted from a
307 previously reported method[8]. Briefly, *E. coli* BL21(DE3) was cultured in LB medium at
308 37°C with shaking. Once the OD_{600} reached 0.8, endogenous T7 RNA polymerase
309 expression was induced with 1mM IPTG. The culture was then grown for an additional 2
310 hours before being harvested (when the OD_{600} reached 2-3). The cells were collected by
311 centrifugation at 3500 *g* for 15 minutes at 4°C. The cell pellet was washed with 5 volumes
312 of cold S30 buffer (10 mM HEPES-KOH pH7.5, 14 mM $Mg(OAC)_2$, 60 mM KOAc) and
313 then resuspended in S30 buffer at a ratio of 1 mL per gram of wet cell paste. Cell
314 disruption was performed using a high-pressure homogenizer at 800 *bar* for two passes.
315 The lysate was then clarified by centrifugation at 20,000 *g* for 10 minutes at 4°C, and the
316 resulting supernatant, designated as the fast lysate, was collected. Finally, the fast lysate
317 was aliquoted and flash-frozen in liquid nitrogen before being stored at -80°C until use.

318 ***e*CFPS reactions**

319 The optimized *e*CFPS reaction was performed in a 10 μL total volume containing the
320 components listed in **Table S3** and **Table S4**. Plasmid of NLuc reporter gene was used
321 as the template.

322 **Protein quantification and analysis**

323 Protein expression levels were quantified using NLuc and sfGFP reporter systems. NLuc
324 activity was measured with the Nano-Glo luciferase assay system (Promega). Reaction
325 mixtures were diluted as specified in the source data to prevent signal saturation and then
326 analyzed on a microplate luminometer (BERTHOLD, Centro XS3 LB 960) to obtain the

327 luminescence units as described priously[48,49]. SfGFP fluorescence was used to quantify
328 protein synthesis following the methods reported[50]. Briefly, 5 μL of the reaction mixture
329 was added to 195 μL of fluorescence assay buffer (20mM HEPES-KOH pH7.5, 100mM
330 NaCl, 5mM magnesium glutamate). Fluorescence was measured on a Cell Imaging
331 Multimode Reader (BioTek, Cytation 5) with an excitation wavelength of 485 nm and an
332 emission wavelength of 528 nm.

333 Western blotting analysis was performed to confirm protein synthesis of the *e*CFPS
334 reactions. Synthesized proteins were detected using a primary anti-FLAG (MBL Life
335 Sciences, Cat#: M185-3L). Chemiluminescence was detected using a Gel Imaging
336 System (Tanon).

337 **Sucrose density gradient (SDG) analysis**

338 A volume of 170–180 μL of lysate was loaded onto a 10%–40% (w/w) SDG. The gradient
339 buffer contained 10 mM HEPES-KOH (pH 7.5), 14 mM $Mg(OAc)_2$, 60 mM KOAc. The
340 gradient was prepared using a simple diffusion method (Biocomp) and centrifugation at
341 38,000 rpm for 2.5 hours at 4°C in a Beckman SW41Ti rotor. Following centrifugation, the
342 gradient was fractionated using a density gradient fractionation system (Biocomp), with
343 continuous monitoring of the absorbance at 260 nm.

344 **Negative-staining transmission electron microscopy (TEM)**

345 Samples from the SDG were prepared for TEM. Fresh, glow-discharged carbon-coated
346 grids were immersed in 3 μL of the corresponding sample for 2 minutes. Excess solvent
347 was removed, and the grids were stained with uranyl acetate for 1 minute and air dried.
348 Samples were observed using a Talos F200C transmission electron microscope at a
349 nominal magnification of 92,000.

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Contributions

X.L.,C.Z. and J.L. were responsible for obtaining experimental data and performing data processing. X.L. and C.Z. conducted biochemical experiments. X.L., C.Z., Z.Z. and W.L. analyzed the data and created charts. W.L. wrote the initial draft of the paper. All authors edited the paper.

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