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A minimal-component E. coli cell-free transcription–translation system: implications for democratization

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Abstract

In vitro transcription-translation (TX-TL) systems are central to synthetic biology as platforms for protein production outside living cells; however, most current protocols rely on multicomponent formulations that increase costs and limit accessibility. Our objective was not to develop a high-yield TX-TL cell-free platform based on a complex energy buffer formulation, but rather to establish a simple system with a minimal set of reaction components capable of synthesizing three monomeric proteins: dihydrofolate reductase (DHFR, ~ 20 kDa), green fluorescent protein (GFP, ~ 27 kDa), and the bacterial chitinase ChiA74 (~ 70 kDa). Using a crude extract obtained by glass mini-beadbeater lysis and a fixed DNA template concentration (250 ng/µL), we evaluated a reduced TX-TL buffer formulation, including a minimal buffer and a minimal energy buffer supplemented with phosphoenolpyruvate. The optimized formulation reduced the buffer composition by 46% relative to a low-cost reference protocol. DHFR was synthesized as a protein of ca. 18 kDa, achieving 50% the reference system´s yield. DHFR production was sensitive to nucleotide concentration and the DNA purification method. While DHFR synthesis was performed without an energy regeneration compound, the synthesis of GFP and ChiA74 required the addition of phosphoenolpyruvate. GFP accumulated to 0.021 mg/mL, and the chitinase exhibited enzymatic activity up to 35,540 relative fluorescent units. These results demonstrate that functional protein synthesis, including large enzymes with disulfide bonds, can be achieved using a simplified TX-TL platform, providing a valuable entry point for training and educating researchers in cell-free systems, promoting the democratization of these technologies in academic and research environments with limited resources.

Introduction

Many proteins are produced in microbial cell factories for human use. However, *in vivo* expression is limited by culture requirements, growth times, inclusion-body formation, heterologous-protein toxicity, and protein-purification costs [1]. Fortunately, with the development of new technologies, those disadvantages can be avoided by synthesizing proteins outside cells using cell-free protein synthesis (CFPS) platforms [2–7]. Generally, two types of CFPS have been developed: the Protein Synthesis Using Recombinant Elements (PURE) system and the *in vitro* transcription-translation (TX-TL, IVTT) system [8, 9].

The PURE systems are constructed using a bottom-up approach with purified recombinant components that support transcription and translation, along with an energy regeneration solution [9–11]. Currently, the PURE systems are marketed under various trademarks. Alternatively, the TX-TL system consists of a biological crude extract containing the transcription and translation machinery, supplemented with an energy buffer containing chemical compounds to generate energy (e.g., phosphoenolpyruvate PEP, 3-phosphoglycerate 3-PGA, acetyl phosphate, glucose, maltodextrin), ions to maintain ionic strength for enzyme activity (e.g., magnesium acetate, oxalic acid), buffers to stabilize pH (HEPES, Bis-Tris), and cofactors to enhance enzyme function (NAD, CoA). Additionally, components necessary for transcription include a DNA template encoding the protein of interest, RNA polymerases (native or T7 RNAP), nucleotides (NTPs, NMPs), crowding agents to improve molecular interactions (polyethylene glycol PEG), RNA-stabilizing components, and, if needed, regulatory elements (IPTG, cAMP). For translation, essential supplements include tRNA to link amino acids, polyamines (spermidine, putrescine) to stabilize RNA and regulate gene expression, amino acids, folinic acid (fMet precursor), reducing agents (DTT) to stabilize proteins with free sulfhydryl groups, or reduced glutathione to promote disulfide bonds [2, 12–14] (Fig. 1).

Currently, multiple cell-free protein synthesis (CFPS) platforms have been developed that produce high yields and proteins with multiple applications [2, 8–12, 15]; however, they often rely on complex formulations that increase costs and can be inaccessible to low-income laboratories that would like to learn how to perform cell-free systems. In addition, their implementation in academia, especially in resource-limited and industrial settings, remains challenging. To make CFPS an inclusive and democratized technology, it is important to develop robust, affordable in-house systems for cell-free synthesis [16, 17] that lower per-reaction costs and improve accessibility. In countries like Mexico, commercial

ready-to-use cell-free kits are more expensive because chemicals are mainly produced in the United States and arrive at Mexican labs through intermediaries, which increases the product cost and limits use in labs with limited funding. Additionally, if kits containing biological compounds are not transported in accordance with proper cold-chain protocols, they may arrive inactive. Furthermore, we face additional challenges; most chemicals used in TX-TL are imported, costs are rising, and it can take several months to receive the compounds [1, 17].

In contrast, proteins found in nature exhibit extensive structural and functional diversity, which can differentially challenge the applicability of cell-free platforms. Consequently, the requirements and performance of cell-free reactions are highly dependent on the target proteins. In this regard, distinct considerations arise from small proteins that require post-translational maturation and for large proteins containing disulfide bonds. Illustrative examples include *E. coli* dihydrofolate reductase (DHFR) [18], the green fluorescent protein (GFP) [19], and the bacterial chitinase ChiA74 [20, 21]. DHFR and GFP are monomeric proteins that lack disulfide bonds, with molecular masses of about 18 kDa (159 amino acids) and 27 kDa (238 aa), respectively. DHFR converts dihydrofolate (DHF) into tetrahydrofolate (THF) [18], and GFP is a fluorescent protein naturally produced by the jellyfish *Aequorea victoria*, with various applications, including translational and transcriptional fusions. The GFP chromophore is formed by Ser65, Tyr66, and Gly67, whose spontaneous oxidation and cyclization form the fluorescent center [19]. ChiA74 is a chitinolytic enzyme produced by *Bacillus thuringiensis*, one of the most important microbial insecticides worldwide. The enzyme is a monomeric protein with four disulfide bonds, with a molecular mass of 74 kDa (676 aa) in its unprocessed form and 70 kDa (642 aa) after processing and loss of the signal peptide [20, 21].

Several *E. coli* TX-TL protocols have shown that protein synthesis can be achieved with fewer reaction components, promoting more cost-effective cell-free systems [12, 14, 22, 23]. Inspired by this, we aimed to determine whether it is possible to reduce the components in a TX-TL cell-free reaction while still effectively producing proteins, with the goal of enhancing accessibility and reproducibility. Here, we developed a cell-free transcription-translation system with minimal components that supports the synthesis of proteins such as DHFR, GFP, and the chitinase ChiA74, aiming to promote broader adoption and democratization of synthetic biology tools, especially in labs with limited resources.

Methods

Protein genes and molecular protocols

The chitinase *chiA74* gene (GenBank accession number AF424979) from *B. thuringiensis* was previously cloned into pColdI (GenBank accession number AB186388) (Takara Bio Inc, Otsu, Shiga, Japan, Cat. 3361) by our group [20, 21]. The green fluorescent protein (GFP) gene was amplified from the pGLO plasmid (BioRad, 1660405EDU) as previously described [24], cloned into pColdI (Takara Bio Inc., Otsu, Shiga, Japan), and transformed into *E. coli* Top10 (Invitrogen, C4040-03) by electroporation at 2.5 kV using an E. coli pulser (BioRad, Hercules, CA, USA). The dihydrofolate reductase (DHFR), controlled by the T7 promoter, was contained in a plasmid obtained from the PURExpress *in vitro* protein synthesis kit (New England Biolabs, NEB, E6800S), and was used as a positive control in the assays. The DHFR sequence was amplified by PCR using primers designed to introduce 5' end restriction sites. The forward primer (gcgc**aagctt**atgatcagtctgattgcggcg) included an additional sequence containing the HindIII recognition site (AAGCTT), followed by the gene's ATG start codon. The reverse primer (gcgc**ctgcag**ttacgcgtcgatcgt) incorporated the PstI restriction site (CTGCAG). Extra nucleotides were added to the 5' ends of both primers to improve enzyme digestion efficiency. The amplified product was then digested with both enzymes and cloned into the previously linearized pColdI vector. This recombinant plasmid was introduced into *E. coli* Top10 as previously described. In all cases, recombinant *E. coli* Top10 strains were cultured in LB medium supplemented with ampicillin (100 µg/mL) at 37°C overnight with shaking at 200 rpm. Five milliliters of the bacterial culture were used for minipreps (QIAGEN, 27104; Omega, D6942-02), and 100 milliliters for midipreps (Omega, D6904-03), following the manufacturers' protocols.

Plasmid DNA purification using different commercial kits and the CTAB method

Recombinant *E. coli* Top10-pColdI-DHFR, *E. coli* Top10-pColdI-GFP, *E. coli* Top10-pColdI-ChiA74 were cultured in LB medium with ampicillin (100 µg/mL) at 37°C with shaking (200 rpm) for 16 hours. Subsequently, the cultures were centrifuged at 4,000 rpm for 15 minutes, and the cell pellet was used to purify plasmids with the QIAgen Spin Miniprep Kit (Qiagen, 27104), E.Z.N.A Plasmid DNA Midi Kit (Omega Bio-Tek, D6904-03), E.Z.N.A Plasmid DNA Mini Kit (Omega Bio-Tek, D6942-02), and the CTAB (cetyltrimethylammonium bromide) protocol [25]. The concentration and purity of the DNA obtained by each method were determined by spectrophotometry (A260/A280 and A260/A230). The integrity of the plasmid DNA was assessed by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. Plasmids were used as templates (250 ng/µL) in cell-free reactions to synthesize the corresponding proteins.

Strain and extract preparation

A sample of *E. coli* GoldDLacZ (DE3) lacking Lon and Ompt proteases and deficient in LacZ and nuclease [22, 26] was taken from a glycerol stock and grown on LB agar overnight at 37°C. A bacterial colony was isolated and inoculated into 5 mL of 2xYTP (5 g sodium chloride, 16 g tryptone, 10 g yeast extract, 7 g monobasic potassium phosphate, and 3 g dibasic potassium phosphate per liter) and incubated at 37°C with shaking at 200 rpm for about 16 hours. The next day, the 5 mL culture was transferred into 50 mL of fresh 2xYTP and grown overnight at 37°C with shaking at 200 rpm. On the third day, the optical density of the 50 mL culture was measured. A portion of this preculture was transferred to fresh 2xYTP medium for a final volume of 500 mL and an optical density of 0.1 at 600 nm. The culture was then grown at 37°C with shaking at 200 rpm until the absorbance reached 0.6 at 600 nm. At this point, 500 µL of 1 M IPTG was added, and the culture was incubated at 37°C with shaking at 200 rpm for approximately 3.5 hours to reach an optical density of 1.5 to 2.0.

Cellular lysis

Bacterial cell lysis was carried out following previously reported protocols with some modifications [12, 22]. The cultures were transferred to 250 mL centrifuge bottles and centrifuged at 5,000 rpm for 15 minutes at 4°C to pellet the bacterial cells. Following centrifugation, the supernatant was discarded. The bottles containing the cell pellet were kept on ice and washed three times. For the first wash, 150 mL of S30A buffer (14 mM Mg-glutamate, 60 mM K-glutamate, 100 mM Tris-base, 0.1 mM DTT, pH 7.7 adjusted with acetic acid) was added to each cell pellet, mixed thoroughly, and centrifuged at 5,000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed again as described previously. For the third wash, 130 mL of S30A buffer containing 1 mM DTT was added to the pellet; the suspension was centrifuged at 2,000 rpm for 8 minutes at 4°C, then the supernatant was discarded. The pellet was centrifuged once more at 2,000 rpm for 15 minutes to remove any remaining S30A buffer. The mass of the pellet was determined, and a suspension was prepared in the ratio of 1 g pellet to 5 g mini-beadbeaters glass (0.1–0.15 mm) (Biospec Products, 11079101) and 0.9 mL of S30A buffer with 1 mM DTT. To ensure a homogeneous suspension, the mixture was thoroughly vortexed to produce a uniform pellet, resulting in a thick mass. This homogeneous mass was transferred to 2 mL microtubes with caps, and cell lysis was performed for 30 seconds at 46 rpm by bead beating (MP Biomedicals, Santa Ana, CA, USA). Immediately afterward, the tubes were placed on ice. Once lysed, the microtubes were connected to a Micro Bio-Spin chromatography column (Biorad, Hercules, CA, USA, 7326204) and placed inside a 15 mL Falcon tube. The mixture was centrifuged at 10,000 *g* for 5 minutes at 4°C to separate the extract from the microspheres. The supernatants were then collected to ensure clarity, and the extracts were incubated for 1 hour at 37°C with shaking at 200 rpm. Afterward, the samples were centrifuged at 10,000 *g* for 10 minutes at 4°C, and the supernatants were collected. These supernatants were dialyzed in S30B buffer (14 mM Mg-glutamate, 60 mM K-glutamate, 1 mM DTT, pH

8.2 adjusted with 2 M Tris-base) for one hour using a 3 kDa dialysis membrane. The extracts were then collected, aliquoted into 0.5 mL tubes, and stored at -80°C for future use (Fig. 2).

Cell-free transcription-translation reactions

The TX-TL reaction consists of 5 µL of cellular extract (obtained as previously described), 4 µL of minimal energy buffer (or minimal buffer) (Tables 1, 2, 3), 250 ng of template closed DNA plasmid (pColdI-DHFR, pColdI-GFP, or pColdI-ChiA74) per reaction, and sterile deionized water to bring the total volume to 15 µL. If the buffer used in the reaction did not contain PEP, it was called the minimal buffer; if it did, it was named the minimal energy buffer. During the synthesis reactions, the template and crude extract levels were kept constant, while the minimal buffer or minimal energy buffer was varied to optimize conditions for synthesizing DHFR, GFP, and ChiA74.

Table 1
Minimal buffer formulations lacking PEP, compared with minimal energy buffers.

REAGENT	**FORMULATION**													
mM	**GCh***	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Mg glutamate	20	-	4	4	-	4	-	4	4	4	4	4	4	4
K glutamate	175	-	-	-	-	-	-	150	150	150	150	150	150	150
ATP	1.5	-	1.2	2.4	-	1.2	2.4	-	1.2	2.4	1.2	2.4	1.2	2.4
GTP	0.86	-	0.9	1.8	-	0.9	1.8	-	0.9	1.8	0.9	1.8	0.9	1.8
CTP	0.86	-	0.9	1.8	-	0.9	1.8	-	0.9	1.8	0.9	1.8	0.9	1.8
UTP	0.86	-	0.9	1.8	-	0.9	1.8	-	0.9	1.8	0.9	1.8	0.9	1.8
Amino acids	1.5	-	-	-	-	-	-	1.5	1.5	1.5	1.5	1.5	1.5	1.5
3-PGA	-	-	2	2	-	-	-	-	-	-	3	3	-	-
PEG-8000	-	-	-	-	-	-	-	4	4	4	4	4	4	4
PEP	33	-	-	-	-	-	-	-	-	-	-	-	33	33
Acetil-CoA	0.27	0.263	-	0.263	-	0.263	-	-	-	-	-	-	-	-
Putrescine	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Spermidine	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-
tRNA(µg/mL)	172	-	-	-	-	-	-	-	-	-	-	-	-	-
Folinic acid (µg/mL)	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-
NH_4^+ glutamate	100	-	-	-	-	-	-	-	-	-	-	-	-	-
K oxalate	27	-	-	-	-	-	-	-	-	-	-	-	-	-

*Energy buffer reported in Guzman-Chavez et al., 2022 [12], with slight modifications.

Table 2
Evaluation of different NTP concentrations in the energy buffer for the optimization of a cell-free system.

REAGENT	**FORMULATION**							
mM	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Magnesium glutamate	4	4	4	4	4	4	4	4
Potassium glutamate	150	150	150	150	150	150	150	150
ATP	1.8	1.5	1.32	1.2	0.96	0.72	0.6	0.36
GTP	1.2	1	0.88	0.8	0.64	0.48	0.4	0.24
CTP	1.2	1	0.88	0.8	0.64	0.48	0.4	0.24
UTP	1.2	1	0.88	0.8	0.64	0.48	0.4	0.24
Amino acids	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.24
3-PGA	-	-	-	-	-	-	-	-
PEG-8000	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
PEP		-	-	-	-	-	-	-
Acetil-CoA	-	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-	-
Spermidine	-	-	-	-	-	-	-	-
tRNA(µg/mL)	-	-	-	-	-	-	-	-
Folinic acid (µg/mL)	-	-	-	-	-	-	-	-
NH_4^+ glutamate	-	-	-	-	-	-	-	-
Potassium oxalate	-	-	-	-	-	-	-	-

Table 3
Minimal energy buffer formulations for use in protein synthesis in a cell-free system

REAGENT	FORMULATION												
mM	GCh*	1	2	3	4	5	6	7	8	9	10	11	12
Mg glutamate	20	5	10	15	5	10	15	5	10	15	5	10	15
K glutamate	175	100	100	100	100	100	100	100	100	100	100	100	100
Tris-HCl pH8	-	150	150	150	150	150	150	150	150	150	150	150	150
ATP	1.5	2	2	2	2	2	2	2	2	2	2	2	2
GTP	0.86	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CTP	0.86	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
UTP	0.86	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Amino acids	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
3-PGA	-	-	-	-	-	-	-	-	-	-	-	-	-
PEG-8000	-	2	2	2	4	4	4	2	2	2	4	4	4
PEP	33	10	10	10	15	15	15	10	10	10	15	15	15
Acetil-CoA	0.27	-	-	-	-	-	-	-	-	-	-	-	-
Putrescine	1	-	-	-	-	-	-	-	-	-	-	-	-
Spermidine	1.5	-	-	-	-	-	-	-	-	-	-	-	-
tRNA(µg/mL)	172	-	-	-	-	-	-	-	-	-	-	-	-
Folinic acid (µg/mL)	0.07	-	-	-	-	-	-	-	-	-	-	-	-
NH_4^+ glutamate	100	-	-	-	-	-	-	-	-	-	-	-	-
K oxalate	27	-	-	-	-	-	-	-	-	-	-	-	-

* Energy buffer reported in Guzman-Chavez et al., [12], with slight modifications

To synthesize DHFR, the pColdI-DHFR was used as a template, and thirteen minimal buffer formulations were tested (Table 1) and compared with an energy buffer based on the components reported by Guzman-Chavez et al. (2022) [12], excluding NAD and PEG-8000 (see Table 1, formulation GCh). In the negative control reaction, pColdI was used as a template, while the positive control contained pColdI-DHFR. To synthesize fluorescent proteins GFP and the chitinase ChiA74, several minimal energy buffers containing phosphoenolpyruvate (PEP) were added (Table 3). Mix reactions were prepared at 4°C (on ice) in a final volume of 15 µL, then loaded into microtubes or individual wells of 384-well plates (Greiner Bio-One, NC, USA). Plates were sealed and incubated at 37°C in a Synergy HTX microplate reader (Winooski, VT) to monitor protein synthesis over time.

Assessing various total NTP concentrations in the energy buffer formulation

To determine the minimum nucleotide concentration required for effective protein synthesis in the TX-TL system, different amounts of ATP, GTP, CTP, and UTP were used to prepare the minimal buffer formulation while keeping all other

components constant (Table 2). Each formulation contained 5 µL of cellular extract, 4 µL of minimal buffer (Table 2), 250 ng of pColdI-DHFR as template, and sterile deionized water to a total volume of 15 µL. The DHFR synthesis was then analyzed by SDS-PAGE. Expression levels were measured across various NTP concentrations to identify the lowest concentration at which detectable and reproducible protein synthesis occurred.

Protein expression analysis by SDS-PAGE

The dihydrofolate reductase (DHFR) synthesis was visualized on polyacrylamide gels under denaturing conditions (SDS-PAGE) (Tris-Tricine SDS-PAGE) [27]. Fifteen microliters of the cell-free reaction mixture were combined with 15 µL of Laemmli loading buffer (2X) [28] and vortexed. The samples were boiled for 5 minutes and then centrifuged at 13,000 rpm for 10 minutes. Next, 15 µL of supernatant was loaded onto two 15% continuous SDS-PAGE gels and run at 120 V for about 2 hours. The gels were stained with a Coomassie Blue solution (0.25% Coomassie Blue, 45% methanol, 45% acetic acid diluted with distilled water) for approximately 1 hour, then destained (10% acetic acid diluted with water) for 3 hours, after which they were analyzed. Scanning of protein bands was carried out with the Image Lab Software version 6.1 (BioRad, Hercules, CA, USA).

GFP expression assessed by fluorescence Assay

The synthesis of green fluorescent protein (GFP) in a cell-free reaction was determined by measuring the relative fluorescence (excitation at 485 nm, emission at 520 nm) in a microplate reader (Synergy™ HTX, Biotek™). Twelve energy buffer formulations were prepared with varying concentrations of phosphoenolpyruvate (PEP) and polyethylene glycol 8000 (PEG8000) (Table 3). Reactions containing 5 µL of cellular extract, 4 µL of minimal buffer (Table 3), 250 ng of pColdI-GFP as template, and sterile deionized water to a total volume of 15 µL were placed in 384-well microplates and incubated for 6 h at 37°C. Fluorescence was recorded every 30 minutes for 8 hours in kinetic assays in a Synergy HTX microplate reader (Winooski, VT). GFP concentration was determined using a standard curve constructed with purified GFP at known concentrations, which showed a linear relationship evaluated by the coefficient of determination (R^2). Fluorescence values, previously corrected for the blank, were interpolated into the standard curve equation to estimate the protein concentration. This approach is based on established principles of fluorescence spectroscopy [29]. At least duplicate experiments were conducted, and results were reported as the mean ± standard deviation. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

Chitinase activity assay

Mixtures containing 5 µL of cellular extract, 4 µL of minimal buffer (Table 3), 250 ng of pColdI-ChiA74 as template, and sterile deionized water to a total volume of 15 µL were prepared, and then placed in 384-well microplates, and incubated for 6 h at 37°C. Chitinase enzymatic activity was measured using 4-methylumbelliferyl-β-D-N,N',N"-triacetylchitotrioside [(4MU-$(GlcNAc)_3$] as a fluorogenic substrate, which, when hydrolyzed, releases the fluorescent compound 4-methylumbelliferone (4-MU). Reactions were carried out in 384-well microplates to a final volume of 20 µL. Each reaction contained 16.9 µL of 20 mM phosphate buffer (pH 7.0); 2.5 µL of [(4-$(MU)_3$] substrate at a final concentration of 10 µM; and 3 µL of ChiA74 generated in a cell-free reaction. Reactions were incubated for 1 hour at 37°C. The concentrations of the methylumbelliferone (MU) released from the substrate were measured spectrofluorometrically (excitation at 360 nm and emission at 455 nm) with a Synergy HTX microplate reader (Winooski, VT, USA) [20, 30]. At least duplicate experiments were conducted, and results were reported as the mean ± standard deviation. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

Results

Testing a minimal buffer for protein synthesis

To synthesize the dihydrofolate reductase (DHFR) protein, we prepared an energy buffer based on the components reported by [12]. This energy buffer contains potassium, ammonium, and magnesium glutamate, putrescine, spermidine, the four nucleotides, CoA, *E. coli* tRNA, folinic acid, PEP, and amino acids (Table 1, column GCh), which were used to compare with our in-house cell-free reactions. To explore whether DHFR could be produced with fewer components, we prepared 13 different buffer combinations by varying the concentration of each component (Table 1). The minimal buffer with the best results contained potassium glutamate, magnesium glutamate, nucleotides, amino acids, and PEG-8000. This approach reduced the chemicals content of the buffer composition in [12] by 46%. After incubating the in-house cell-free reactions, samples from each reaction were separated by SDS-PAGE. We observed that DHFR was not synthesized in the negative control, but the positive control produced a protein of approximately 18 kDa corresponding to dihydrofolate reductase. Interestingly, in the thirteenth reaction, the DHFR was synthesized; however, the strongest signal was observed using the minimal buffer formulation 8 (Table 1), corresponding to about 50% of the intensity relative to the positive control (Fig. 3).

Effects of nucleotide concentration on protein synthesis

To determine whether changing the nucleotide concentration affects DHFR synthesis while keeping all other components of the cell-free system constant, we adjusted nucleotide levels and tested eight different NTPs concentrations as shown in Table 2. The cell-free reactions were performed, and the protein synthesis was analyzed by SDS-PAGE (Fig. 4). Among the eight different minimal buffer combinations, formulation 2 (4 mM magnesium glutamate, 150 mM potassium glutamate, 1.5 mM ATP, 1 mM GTP, CTP, UTP, 1.5 mM amino acids, and 0.1 mM PEG-8000) (Table 2) produced the highest DHFR levels, which are observed as a protein of ~18 kDa. No DHFR was detected in the negative control using the pColdI template.

Effect of DNA protocol on cell-free protein synthesis

Three protocols were employed to extract plasmid DNA from the bacterial chassis: a Qiagen commercial kit, an Omega Bio-Tek kit, and an in-house CTAB-based protocol. After extraction, the plasmid DNA was quantified and normalized to the same concentration. The closed plasmids (pCold-DHFR) served as templates and were mixed with the minimal buffer of formulation 8 (Table 1) (i.e., 4 mM Mg glutamate, 150 mM K glutamate, 1.2 mM ATP, 0.9 mM GTP, CTO, UTP, 1.5 mM amino acids, 4 mM PEG-8000) and the crude extract, as described in the materials and methods. Since the aim was to determine whether the DNA templates extracted using different protocols affect DHFR synthesis, we used a DHFR plasmid obtained directly from the PURExpress *in vitro* protein synthesis kit as a positive control, and a no-template plasmid as a negative control. The highest DHFR synthesis (~ 18 kDa) was observed with the positive control, followed by the cell-free reaction using the pCold-DHFR plasmid obtained with the Omega kit as a template, which produced approximately 40% of the DHFR produced in the positive control. We did not observe DHFR synthesis using DNA templates obtained by the Qiagen or CTAB protocols (Fig. 5). These data indicate that the method of template DNA preparation may influence protein synthesis in a cell-free system.

Synthesis of green fluorescent protein (GFP) in a cell-free system

When we tested formulation 8 from Table 1 as a minimal buffer in the cell-free reaction to synthesize GFP, we could not amplify this protein. To synthesize the GFP, we included PEP, and also a buffer (Tris-HCl) to stabilize the pH of the cell-free reaction and adjusted the nucleotide concentration (formulations 1 to 12, Table 3). With this modification, we

prepared 12 distinct minimal-energy buffer formulations. We observed variations in GFP expression levels, indicating that GFP synthesis was enhanced by the addition of PEP and the buffer (Fig. 6). Although in all twelve cell-free reactions GFP was produced, the highest yield was obtained with formulation 3 (0.021 mg/mL), which was considered to provide more favorable energy conditions. This mix reaction contains the cell extract, the template pColdI-GFP, and the buffer energy containing 15 mM Mg glutamate, 100 mM potassium glutamate, 150 mM Tris-HCl pH 8.0, 2 mM ATP, 0.5 mM GTP, CTO, UTP, 1.5 mM amino acids, 2 mM PEG-8000, and 10 mM PEP (Table 3, formulation 3).

Synthesis of chitinase ChiA74 by a cell-free system

The synthesis of ChiA74 in a cell-free reaction was achieved using the same energy buffer formulation employed for GFP synthesis (Table 3). The enzymatic activity of the chitinase ChiA74 produced in the cell-free system was evaluated by measuring the fluorescence of the MU released from the (4MU-$(GlcNAc)_3$ after the hydrolytic activity of the chitinase. Although reactions six and ten showed the highest hydrolytic activity (~ 36450 relative fluorescence units), no statistical difference was observed in chitinase activity across the twelve reactions (Fig. 6). The energy buffers number 6 and 10 have the same components of the optimal energy buffer used to amplify the GFP (formulation 3), only varied in the magnesium glutamate and PEP concentrations (Table 3).

Discussion

Our objective was not to develop a high-yield TX-TL cell-free platform based on a complex energy buffer formulation, but rather to establish a simple system with minimal components, suitable for implementation in labs with limited resources and in academic laboratories, thereby contributing to the democratization of cell-free platforms and providing a foundation for entering this field. In this regard, we developed a simple, TX-TL cell-free system with a few components, compared to other protocols [12, 22, 31], sufficient to synthesize the DHFR, GFP, and ChiA74 proteins [18–21].

The quality of the crude extract plays an important role in the performance the TX-TL system, and it depends on several factors, including the bacterial strain, the lysis method, and the time at which cells are harvested [2, 32]. Taking this into account, we tested various protocols to prepare it, including sonication under different conditions and combinations with lysozyme [33–35]; however, the most reproducible protocol in this work was using mini-bead beaters with glass in a FastPrep-24. It is likely that, using this equipment and the manufacturer's recommendations, most components in the crude extract were functional. We used an *E. coli* strain derived from BL21(DE3) to prepare the bacterial extract. This strain is deficient in proteases, nucleases, and LacZ, but can synthesize T7 RNAP and the endogenous RNAP-σ^{70} [22, 26]. In BL21(DE3), induction with IPTG allows the synthesis of T7RNAP, which is dependent on the LacI repressor. In contrast, the cold-shock promoter (cspA) present in pColdI is induced by low temperatures, but the presence of the lac operator provides additional regulation dependent on LacI, which is inactivated by IPTG. In this regard, effective induction of the *cspA* promoter requires a decrease in temperature and the release of the LacI repressor; accordingly, the extract should contain T7RNAP and the endogenous RNAP, two different transcription systems. As DHFR, GFP, and ChiA74 were under the control of a cold-shock promoter (*cspA*) (Takara Bio Inc, Otsu, Shiga, Japan, 3361), the transcription of the three genes depended on the endogenous RNAP and not on T7RNAP [31]; however, the extract was suitable for transcribing genes under the control of T7RNAP.

Our first challenge was to synthesize DHFR, a small protein of ca. 159 amino acids [18]. The best result was achieved with a minimal buffer that lacked an energy-regeneration compound and contained only potassium and magnesium glutamates, nucleotides, amino acids, and PEG-8000 (Table 1, formulation 8). This minimal buffer contains fewer components than previously reported reduced-cell-free formulations [12, 22, 31] and yields approximately 50% of the DHFR produced by the protocol of Guzman-Chavez et al., 2022 [12]. The minimal buffer of this work contains only salts to maintain ionic strength, nucleotides for transcription, and a crowding agent to stabilize RNA and increase

macromolecular interactions [2]. On the other hand, the absence of a precursor of ATP, such as PEP or 3-PEG, suggests that protein synthesis is performed in the absence of ATP regeneration, and although the residual ATP in the extract could have been consumed rapidly, it was enough for a visible DHFR synthesis [36, 37]. It is important to note that the robustness of the TX-TL system generated here may be low because it lacks an ATP regeneration pathway and energy donors. Besides, the system may also be highly sensitive to small changes in magnesium or potassium concentrations, nucleotide quality, and DNA template concentration, among other factors, as previously reported [31]. In this regard, we observed that a small variation in nucleotide concentration can affect DHFR synthesis (Table 2, Fig. 4). Higher or lower NTP concentrations may affect transcription efficiency, and this effect can vary depending on the protein being synthesized. The high availability of NTPs can lead to a lack of Mg^{2+} or to rapid accumulation of inhibitory products, including inorganic phosphate [31, 38], whereas low NTPs availability can lead to competition between transcription and translation for metabolic resources [39]. In addition, the quality and concentration of the DNA template were observed to influence the efficiency of *in vitro* transcription (Fig. 5). Previous studies have shown that contaminants co-purified with DNA, such as proteins, salts, or phenolic compounds, can inhibit downstream processes, including amplification, transcription, and cell-free protein synthesis [40, 41]. Although commercial purification kits generally yield DNA of high integrity and purity, alternative extraction methods, such as CTAB-based protocols [25], may leave residual contaminants that negatively affect protein synthesis. In the present study, we observed that high concentrations of template DNA (i.e., 250 ng) used in the assay, purified with the Omega kit, supported DHFR production in the cell-free system, whereas template DNA purified with a Qiagen kit did not. At present, we lack experimental evidence to explain this result. One possible explanation is the presence of residual inhibitory compounds associated with the Qiagen-purified DNA under the tested conditions, which may have impaired transcription or translation efficiency.

When we tried to synthesize GFP using the minimal buffer, we obtained negative results, so we considered it was important to add an energy precursor and a buffer. Although several glycolytic intermediates, including glucose-6-phosphate, fructose-1,6-biphosphate, 3-phosphoglycerate, and phosphoenolpyruvate, as well as compounds such as glucose, maltose, maltodextrin, and starch, are used as energy sources [2, 22], we added PEP and a buffer. PEP serves as a direct phosphate donor to ADP via pyruvate kinase, rapidly generating ATP and pyruvate and thereby supporting a continuous energy supply without reliance on complex or multi-step metabolic pathways [38], whereas other lower-cost energy sources, such as maltodextrin, require multiple enzymes to be converted into high-energy intermediates [2, 22, 42]. PEP-based energy regeneration maximizes performance and robustness but has a higher cost; however, although maltodextrin is low-cost, with the few components used in this work, the system efficiency might be compromised. For the purposes of this work, we prioritized developing a method with few components; however, balancing cost–performance considerations remains a central challenge in developing scalable and economically viable cell-free biomanufacturing platforms. With the addition of PEP and the buffer, we synthesized GFP (Fig. 6, Table 3). This suggests that, unlike DHFR, whose synthesis probably requires less total energy, GFP production requires ATP regeneration [36, 39] and tighter pH control to extend the functional lifetime of the reaction to complete transcription, translation, folding, and chromophore maturation, enabling GFP accumulation to detectable levels. Although the yield of GFP production (0.021 mg/mL) is lower than that of eGFP synthesized by a TX-TL cell-free system using the endogenous RNAP of *E. coli*, with promoters dependent on σ^{70} (0.65 mg/mL) [31] or by a new-generation TXTL 3.0 whose transcription depends on the 7TNAP (4 mg/mL) [43], this work demonstrates that functional protein synthesis can be achieved with a substantially reduced set of components, reinforcing the potential of this system for educational purposes and implementation in laboratories with limited infrastructure funding.

Alternatively, we examined whether this simple TX-TL cell-free system, which produces proteins of around 20 kDa (i.e., DHFR, GFP), could support the synthesis of more complex proteins, such as the chitinase ChiA74 (ca. 70 kDa, 642 aa) containing four disulfide bonds [20, 30]. This chitinase, composed of four domains, shows synergistic activity with the insecticidal Cry proteins and activity against phytopathogenic fungi [20, 24, 30]. This is the first time a chitinase from the microbial insecticide *B. thuringiensis* has been produced by a cell-free platform. Using the same formulations that

support GFP synthesis, supplemented with PEP and Tris, we obtained biologically active ChiA74 capable of hydrolyzing the fluorogenic substrate 4MU-$(GlcNAc)_3$, thereby producing fluorescence upon methylumbelliferone release (Fig. 7, Table 3). This result suggests that, like GFP, the synthesis of the enzyme ChiA74 requires ATP regeneration, likely due to its larger size, extended translation time, and increased folding. Importantly, the detection of chitinase activity suggests that disulfide bond formation can occur even in the minimal system, probably supported by residual redox-active components retained in the crude *E. coli* extract [14, 36, 44].

Conclusions

We present a simple TX-TL platform featuring a minimal-energy buffer supplemented with an energy precursor. Despite its reduced biochemical complexity, the system supports the synthesis of DHFR, GFP, and the chitinase ChiA74. Although the platform performed well with the three proteins tested, its broader applicability remains to be demonstrated. Future work should assess performance with larger, multimeric, and membrane-associated proteins. In addition, reliance on a single energy precursor (PEP) may limit scalability and increase costs relative to alternative energy regeneration systems. It will also be necessary to benchmark yield and reproducibility against established TX-TL platforms. Reliance on a crude extract prepared with specific bead-beating equipment may affect reproducibility across laboratories. However, by minimizing the number of components, this approach provides a valuable entry point for training and educating researchers in cell-free systems, thereby promoting the democratization and broader adoption of TX-TL technologies in academic and research environments with limited resources.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

M.F. M-A. conceptualization, conducted the experiments, analyzed the data, drafted the manuscript, and revised the final manuscript. L.E. C-V. contributed to data analysis, interpretation, and manuscript revision. F. F. trained M.F., analyzed the data, and revised the final manuscript. S. C. trained M.F and revised the final manuscript. M.L. P-Z. discussed democratization aspects and contributed to drafting and revising the final manuscript. J.E. B-C. conceived the study, secured funding, supervised the research, interpreted the data, and contributed to drafting and revising the final manuscript.

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Data Availability

All data supporting the findings of this study are included within the manuscript.

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Figures

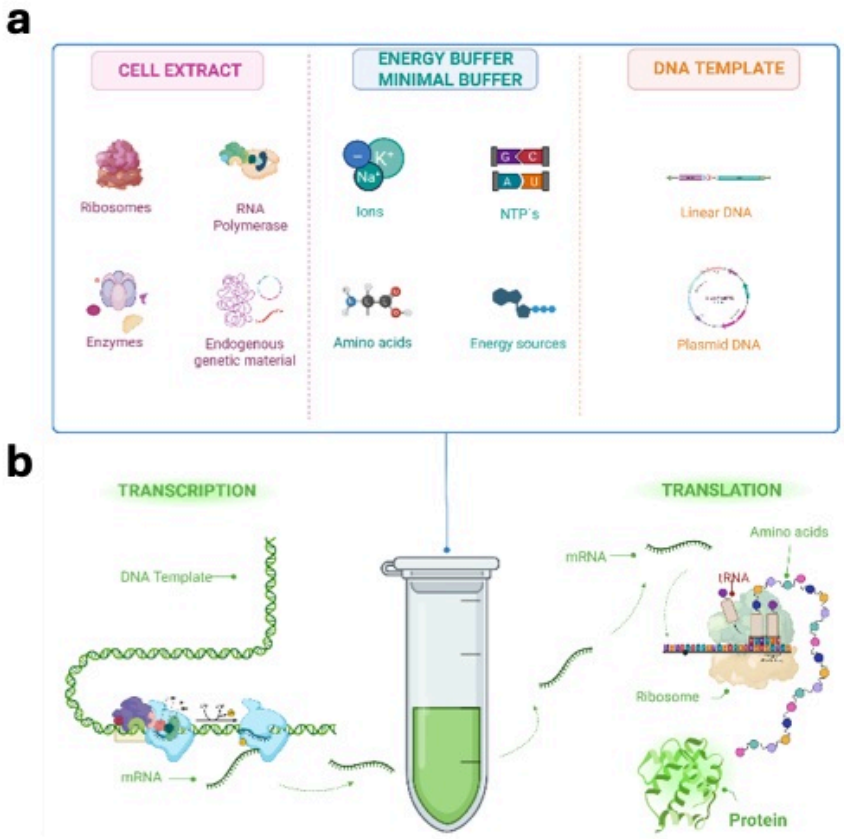


Figure 1

Schematic overview of a cell-free system that allows *in vitro* protein synthesis from extracts and DNA templates. (a) Core components required for cell-free reactions, including the cell extract, an energy buffer supplying energy sources, and a

DNA template. (b) Representation of the transcription and translation processes leading to protein synthesis, eliminating the need for living cells.

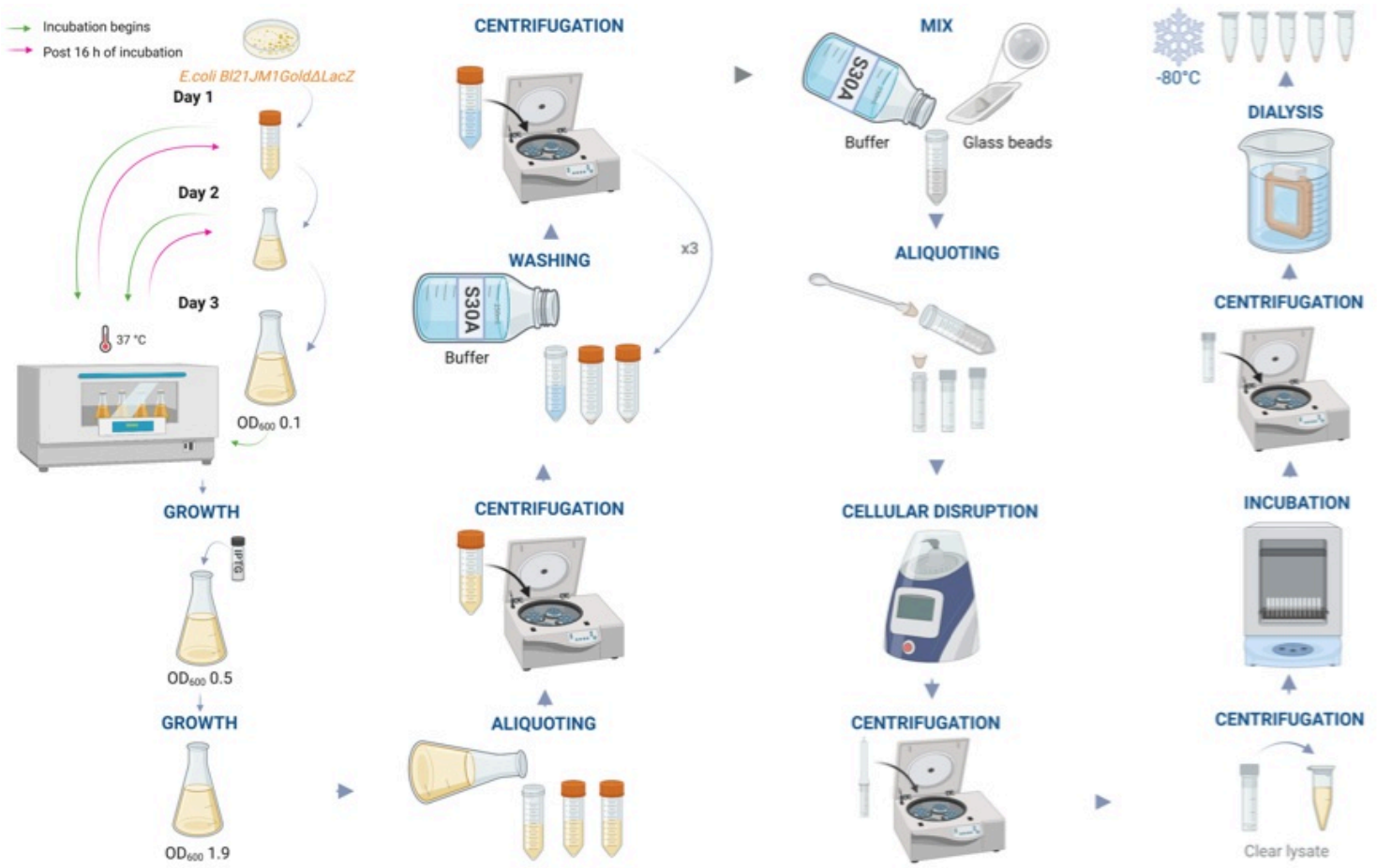


Figure 2

Schematic overview of the cell extract preparation process, including the cell growth, lysis, and lysate clarification, resulting in a functional system for *in vitro* protein synthesis.

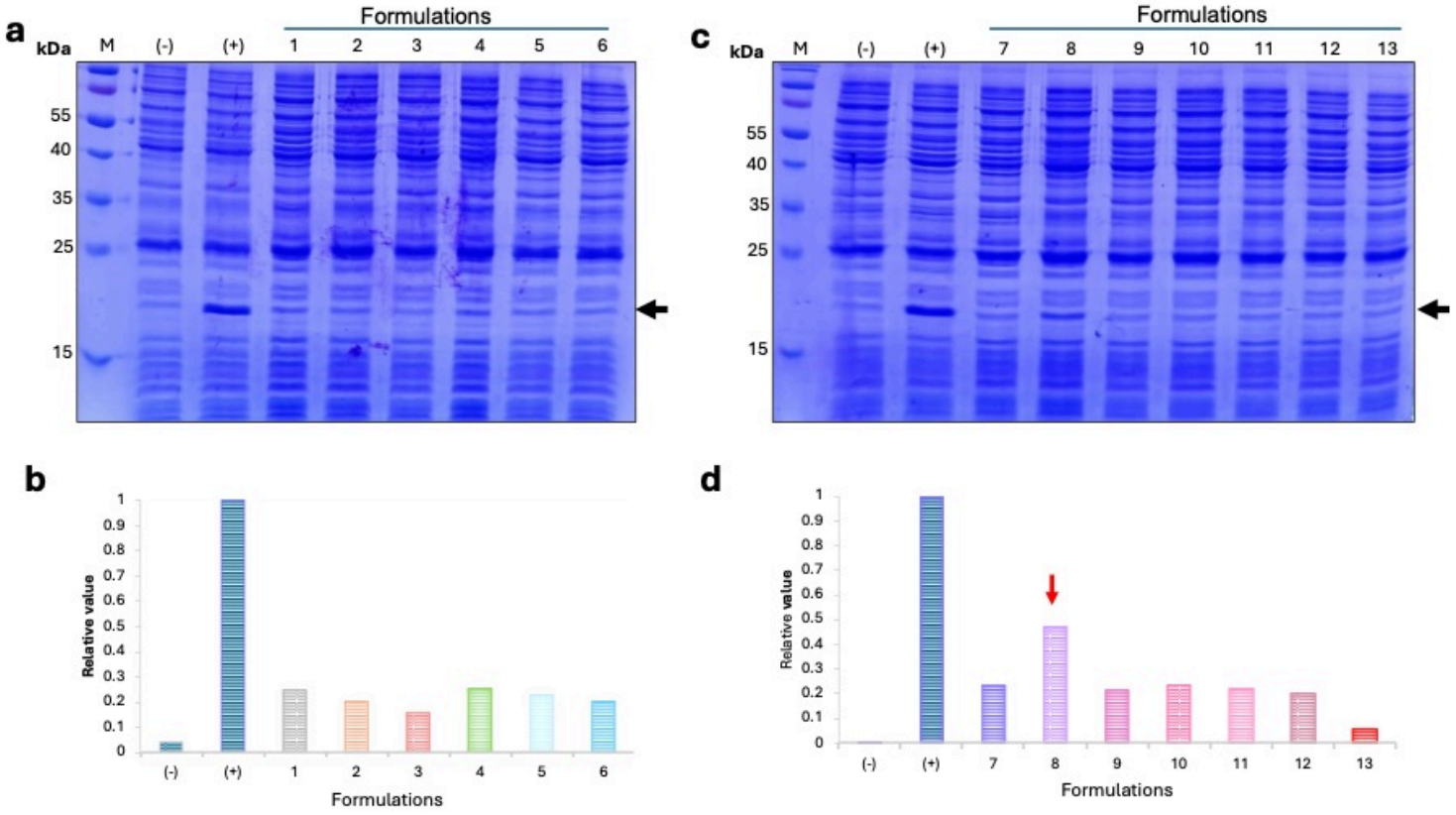


Figure 3

Synthesis of dihydrofolate reductase in a cell-free reaction with minimal components. (a), (c) Cell-free reaction products were analyzed by SDS-PAGE to detect the presence of the DHFR. (b), (d) DHFR was scanned with the Image Lab Software 6.1, and the relative values compared to the positive control were analyzed. The (-) control contains a cell-free product using the pColdI vector as a template. The (+) control well is a positive control used with the pColdI-DHFR as the template. Wells 1 to 13 represent the cell-free reactions, in which the energy buffer is formulated as listed in Table 1. The black arrow indicates the position of DHFR, a protein of approximately 18 kDa. The red arrow indicated that formulation 8 showed the highest DHFR production. The positive (+) and negative (-) controls use the energy buffer reported in [12] with slight modifications (formulation GCh, Table 1). Each well number in (a) and (c) corresponds to formulations in (b) and (d), which are shown in Table 1.

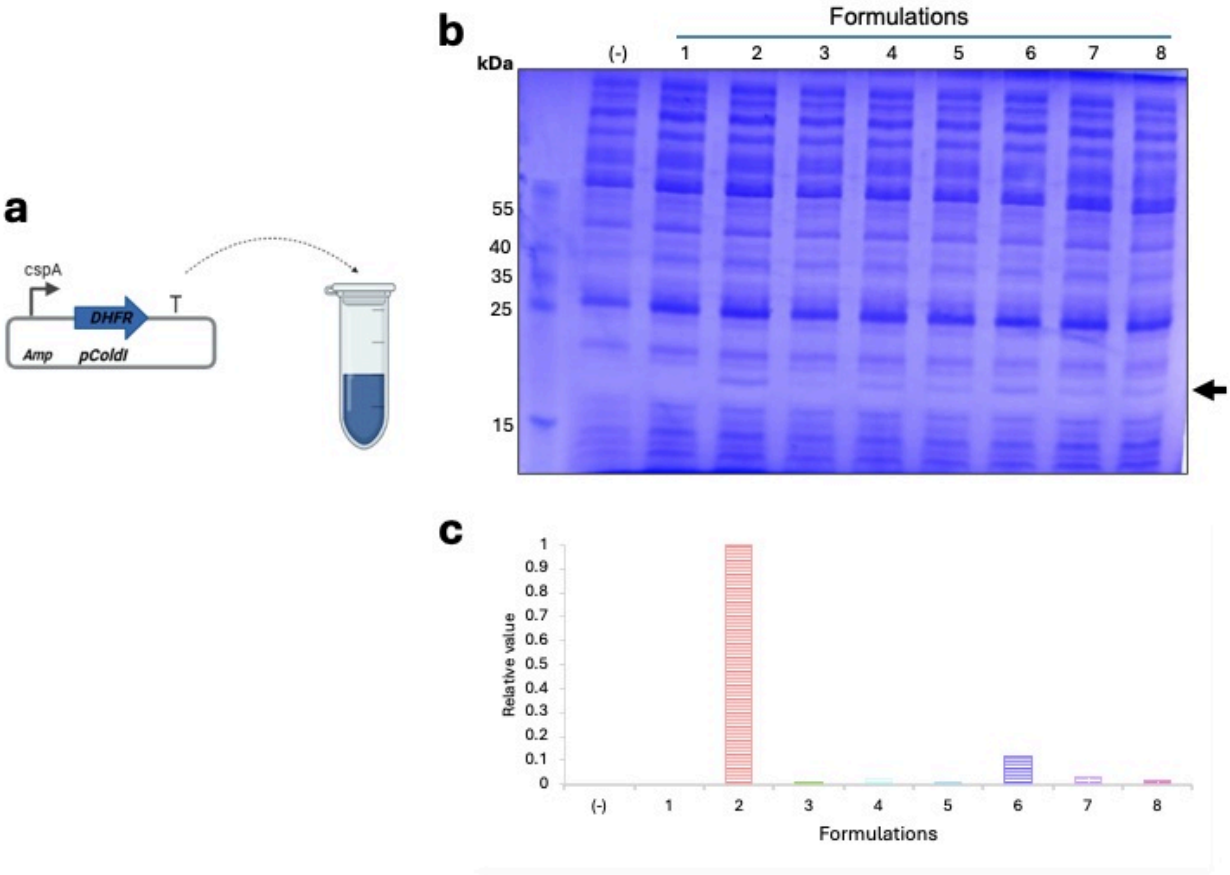


Figure 4

Synthesis of dihydrofolate reductase in a cell-free reaction, modifying the NTP concentration. (a) The DHFR gene present in the pColdI was used as a template in the cell-free reaction. (b) SDS-PAGE. The black arrow indicates the position of DHFR, a protein of approximately 20 kDa. (c) Scanning of the DHFR protein using the Image Lab Software 6.1. Wells 1 to 8 represent the cell-free reactions with the energy buffer shown in Table 2. For the negative control, pCold was used as the template, using the energy buffer from formulation 8 shown inTable 1.

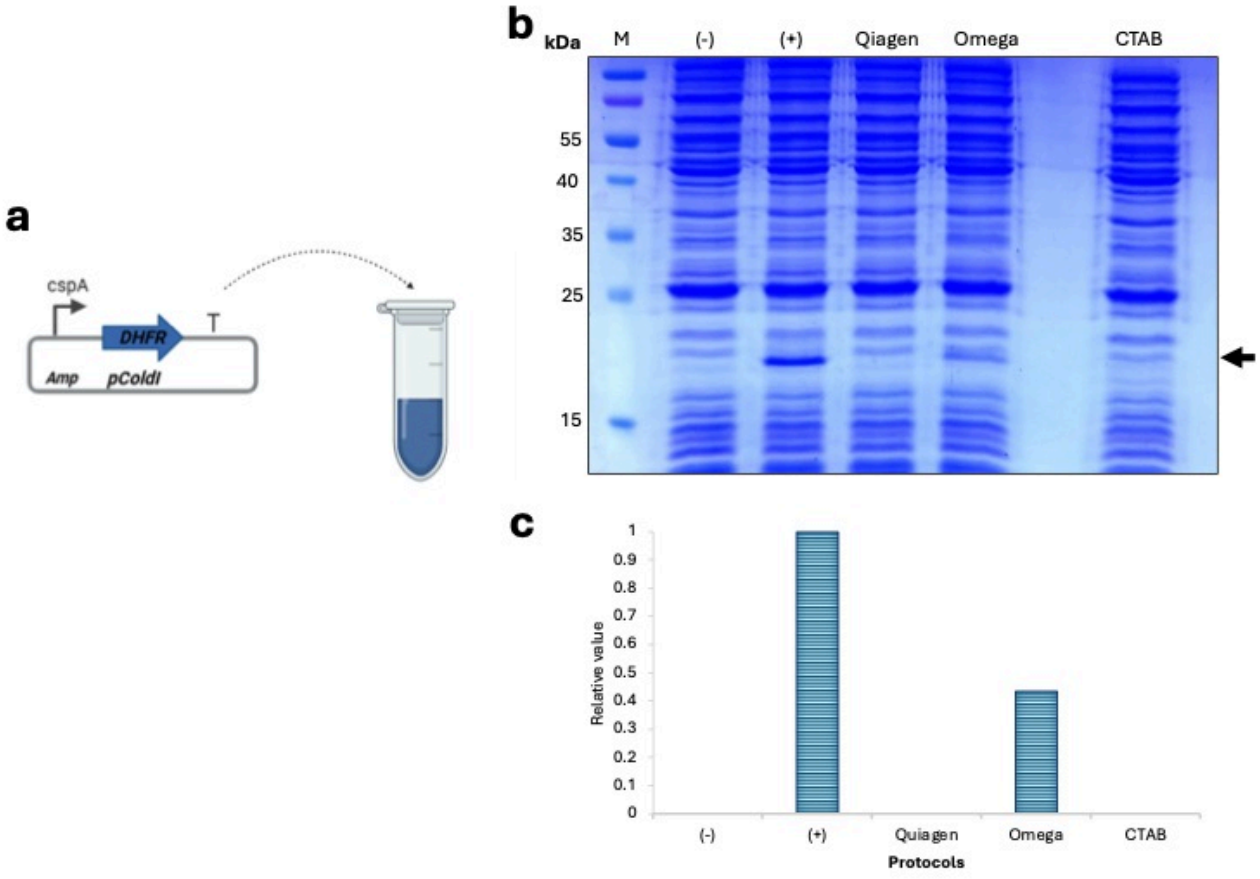


Figure 5

Synthesis of dihydrofolate reductase in a cell-free reaction using a closed plasmid obtained through different protocols as a template. (a) The DHFR gene present in the pColdI was used as a template in the cell-free reaction. (b) Polyacrylamide gel electrophoresis. The black arrow indicates the position of DHFR, a protein of approximately 18 kDa. (c) Scanning of the DHFR protein. All reactions were conducted using energy buffer formulation 8, as shown in Table 1. The three protocols extracted DNA from *E. coli*-pColdDHFR, and the plasmid was used as the DNA template for the cell-free reaction. In the positive reaction, DHFR plasmid was obtained directly from the PURExpress *in vitro* protein synthesis kit (NEB, E6800S) and used at 250 ng/mL. In the negative control, the cell-free reaction contained no plasmid as a template.

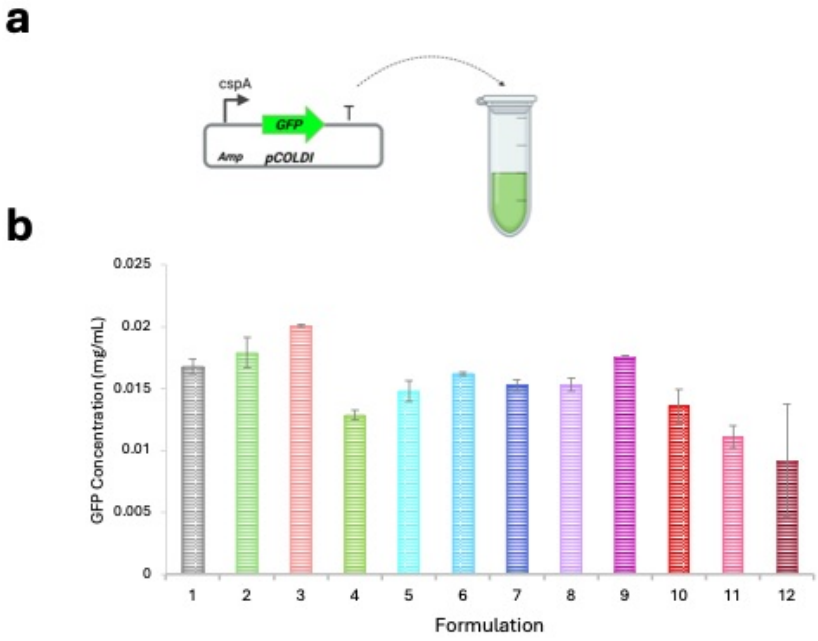


Figure 6

Synthesis of green fluorescent protein (GFP) in a cell-free system using different energy buffer formulations. (a) The pColdI-GFP was used as a template in the cell-free reaction. (b) The number in each column corresponds to the formulations presented in Table 3. The negative (-) controls use the energy buffer reported in Guzman-Chavez et al., 2022 [12], with slight modifications (formulation GCh, Table 3). In the negative control, the cell-free reaction contained no plasmid as a template. Fluorescence obtained in each sample was subtracted from that of the negative control. Data represent mean ± standard deviation from three independent biological replicates (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

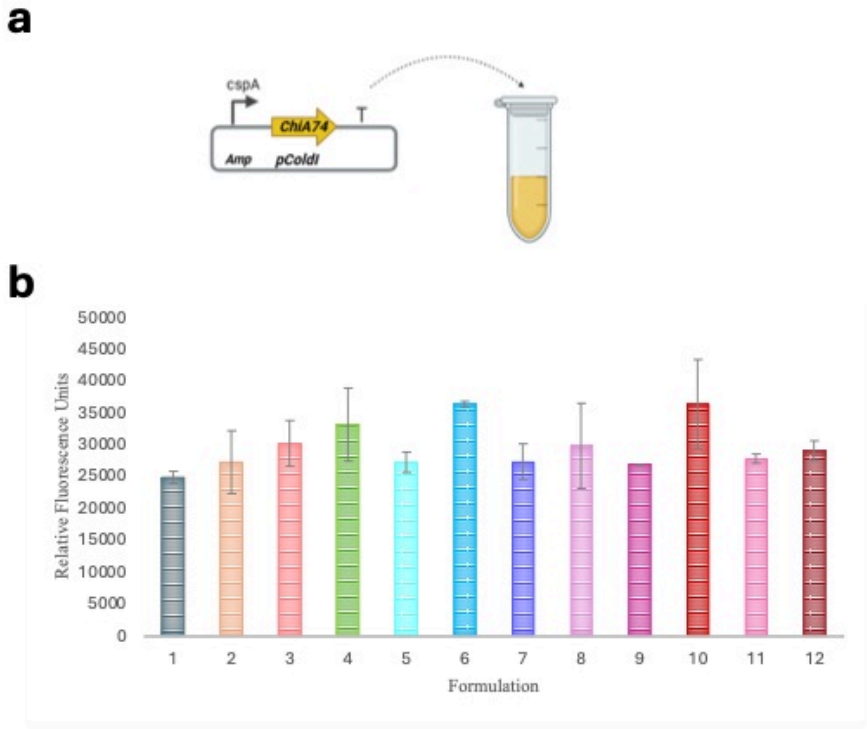


Figure 7

Synthesis of the chitinase ChiA74 in a cell-free system using different energy buffer formulations. a) The *chiA74* gene present in the pColdI was used as a template in the cell-free reaction. (b) The number in each column corresponds to the formulations presented in Table 3. The relative fluorescence units represent the value of the fluorescence reactions minus the fluorescence of the negative control. The negative (-) controls use the energy buffer reported in Guzman-Chavez et al., 2022 [12], with slight modifications (formulation GCh, Table 3). In the negative control, the cell-free reaction contained no plasmid as a template. Fluorescence obtained in each sample was subtracted from that of the negative control. Data represent mean ± standard deviation from three independent biological replicates (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$).