CHAPTER TWO

Protocol for preparation of cell–free system

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Abstract

Cell-free protein synthesis (CFPS) has emerged as a revolutionary process for producing proteins quickly and in a modular way, with the optionality of not having all of the restraints of living cells. As described in this chapter, we provide full protocols for preparing cell extracts with high activity, optimizing the reaction systems for protein expression, and establishing assays to characterize and evaluate protein expression and function. We included a detailed stepwise protocol for host strain selection, mechanical lysis, extract clarification, and optimization of energy and ionic balance. We evaluated several analytical techniques including SDS-PAGE and Western blot analysis and fluorescence assays as protocols for evaluating yield and functionality of the protein. For troubleshooting yield losses, we discussed some of the more common bottlenecks such as degradation of mRNA, protein misfolding, and contamination. CFPS offers the opportunity to expand applications for novel protein production and applications in synthetic biology, diagnostic fields, and on-demand biomanufacturing processes. CFPS can potentially produce proteins that are toxic, unstable, or non-natural. The future integrated process with combinations of machine learning and CFPS in freeze-dried formats are promising in that they will create scalable, mobile, and potentially deployable CFPS systems that can be utilized for food and healthcare applications as well as potential uses in industry. This work appears to be a suitable balancing act between serving as a protocol for synthesis of proteins, while also serving as a model and foresight for potential growth of a platform by researchers.

1. Introduction

Cell-free protein synthesis (CFPS) is a very strong in vitro platform for synthesizing proteins in the absence of any living cells. CFPS is transcribing and translating in a controlled environment with cellular machineries derived from lysed organisms, usually mammalian cells, wheat germ or insect cells, or *Escherichia coli*.[1] The key components of a CFPS system are cofactors,

ribosomes, tRNA, energy sources, amino acids, and enzymes necessary for gene expression. CFPS systems are important tools for molecular biology, synthetic biology, and biotechnology since CFPS provides fast and flexible protein synthesis using linear or plasmid DNA templates.[2]

Then CFPS began in the 1960's, and Nirenberg and Matthaei made breakthroughs experiments showing in vitro protein synthesis that ultimately led to the deciphering of the genetic code[3] (Table 1). A lot has changed since

Table 1 Cell-free systems: development and significant events.

Year	Significant event	Development	References
1961	In vitro protein synthesis was discovered using *E. coli* extracts	Nirenberg and Matthaei demonstrated that polypeptides could be synthesized in cell-free extracts and also deciphered the first codon of the genetic code.	3
1973	S30 extract was developed from *E. coli*	Zubay simplified the extraction of *E. coli* cells in order to produce cell extracts, and was able to conduct reproducible experiments using the extracts to produce proteins.	4
1999	The commercialization of the wheat germ cell-free system	Scalable wheat germ-based CFPS systems were developed that have high translational efficiency.	5
2001	Coupled transcription–translation systems were developed	The inclusion of the T7 RNA polymerase also allowed for continuous coupled transcription–translation reactions to occur in CFPS.	6
2004	High-throughput CFPS platforms emerged	Automated and miniaturized CFPS also enabled parallel expression and screening of expressed proteins.	7
2012	Synthetic circuits were integrated into CFPS	Cell-free systems were subsequently developed in synthetic biology to engineer and characterize gene circuits in vitro that otherwise are incorporated in vivo.	8

(continued)

Table 1 Cell-free systems: development and significant events. (*cont'd*)

Year	Significant event	Development	References
2020	CFPS was utilized for rapid diagnostics (e.g. SARS-CoV-2 detection)	Rapid, on-demand diagnostics produced utilizing paper-based CFPS techniques, predominantly during the COVID−19 pandemic.	Pardee et al.
2021	Cell-free glycoprotein synthesis	Developers also produced cell-free systems with intrinsic post-translational modifications for the in vitro synthesis of glycoproteins.	9
2022	Expansion into freeze-dried CFPS technologies	CFPS kits could be freeze-dried, producing relatively decent stability, deployability in the field, and long-term shelf-life prospects for biotechnology and healthcare-based applications.	10
2023	Hybrid systems that combined prokaryotic and eukaryotic components	Hybrid systems produced higher yields with improved protein folding and post-translational modification for complex protein production.	11
2024	AI-based optimization done for CFPS protocol.	Machine learning techniques have begun to optimize reaction parameters, and to optimize component compositions in cell-free systems.	12

then in terms of the development of CFPS from a basic science and academic tool, to a scalable, commercially applicable technology. Key developments include the betterment of extract prepration protocols, better energy regeneration systems, and the use of molecular biology methodologies to optimize yield and stability in different conditions.[13,14] Comparison with in-vivo expression systems, CFPS gives several distinct benefits. First, it eliminates the need for maintaining cell viability, allowing toxic or unstable proteins to be synthesized with ease.[15] Second, CFPS is an open reaction environment and does not involve any limitations in reaction conditions, concentrations of components, unnatural amino acids and cofactors.[13] One

Table 2 Comparative features between in-vivo and cell-free system.[2,13]

Feature	In vivo system	Cell-free system
Reaction Environment	Compartmental and sealed	Open and manipulated
Time spent Turnaround	A few days	Within hours
Toxic Protein Expression	Limited	Readily possible
Non-canonical AA	Difficult	Enabled
Scale	Large, scalable	Miniaturize scalable
Downstream Processing	Complicated	Streamlined

of the major strengths of CFPS lies in its modularity. Researchers can tailor the reaction environment by adjusting magnesium concentration, nucleotides, cofactors, and energy substrates without concern for cell viability. Additionally, CFPS facilitates the synthesis of toxic, unstable, or non-natural proteins with challenging in vivo expression, thereby expanding the protein expression toolbox[16] (Table 2).

In addition, CFPS allows for fast protein generation, often within hours, thereby considerably decreasing the design-build-test cycle in biotechnology. Moreover, unlike cell-based systems, CFPS is a lyophilized system that can be rehydrated and used on demand or taken into the field for uses such as development of synthetic biology circuits, vaccine candidates, biosensors, and personalized medicine.[17] The viability of lyophilized CFPS kits has made protein synthesis technology portable away from the laboratory setting including point-of-care applications in low-resource places.

With the rate at which the demand is increasing for rapid, reproducible, and scalable protein production platforms, it is important for researchers to develop and establish efficient protocols for preparing CFPS in a standardized manner. This review outlines the range of classification for CFPS systems, protocols for preparation of cell extracts from a variety of organisms, optimization strategies, and approaches with respect to emerging applications.

2. Cell-free system's historical context

(Table 3)

Table 3 Comparative features between sources.[18,19,43]

Source for CFS	Cell degradation techniques	Metabolic systems	Pros	Cons	Implements	References
PROKARYOTIC SYSTEM						
E. coli extract	1. High-pressure homogenization 2. sonication 3. bead-beating	1. Phosphoenolpyruvate (PEP) 2. creatine phosphate or maltodextrin	1. A high yield of protein 2. Easy cultivation, quick cell division and lysate production 3. Economical 4. Simple genetic engineering 5. Reputable	1. Minimal post-translational changes 2. The generation of integral membrane proteins is not supported by endogenous membrane structures. 3. Eukaryotic proteins may not fold correctly since there are only native prokaryotic chaperones accessible.	1. Non-canonical amino acid incorporation which leads to protein engineering for the creation of ribosome display techniques, customized enzymes, and human medicines. 2. Maxium volume production up to 100 L. 3. Manufacturing antibody-drug conjugates on an industrial scale.	1,2,13, 20–22,51
Archaeal extract	1. Sonication, 2. French press	1. ATP-regenerating systems similar to bacterial extracts	1. Extreme synthesis circumstances, such as high temperatures, which result in less secondary structures in mRNA inhibiting translation 2. Thermostable proteins that are appropriately folded	1. Low yield of protein	1. Production of proteins that are thermostable	2,22,23

EUKARYOTIC SYSTEM

Yeast extract	1. Bead-beating, 2. high-pressure homogenization	1. Creatine phosphate, 2. PEP	1. Capacity to carry out post-translational modifications such as glycosylation 2. Easy and quick cell culture for preparation of lysate 3. A well-known in-vivo system, which makes cell engineering techniques well-established	1. Low yield of protein 2. No post-translational changes resembling those in mammals	1. Making virus-like particles for research on antiviral drugs 2. Making bioreactors with bioethanol and (S)-l-acetoxyalkan-2-ol without cells	2,22,24
Wheat germ extract	1. Grinding embryo-rich wheat grains, 2. filtration	1. Creatine phosphate, 2. phosphocreatine	1. A cell-free system that works very well, so it makes a lot of complex proteins. 2. Systems that can make proteins with disulphide bridges 3. The right way to fold a variety of proteins, which makes them very soluble 4. A popular system	1. costly and time-consuming lysate preparation 2. There is no space for post-translational changes 3. Absence of endogenous membranes 4. Poor production of protein in comparison to wheat germ and prokaryotic systems	1. Malaria protein synthesis to evaluate new vaccine candidates 2. Protein synthesis on-chip 3. High-throughput uses 4. Manufacturing monoclonal antibodies directed against GPCRs 5. Examining translational processes (ribosome conformation analysis)	2,22,25,26

(continued)

Table 3 Comparative features between sources.[18,19,43] (*cont'd*)

Source for CFS	Cell degradation techniques	Metabolic systems	Pros	Cons	Implements	References
Tobacco BY-2 extract	1. Protoplast preparation 2. sonication or nitrogen 3. decompression	1. Creatine phosphate	1. A quick and simple method for preparing lysate 2. It is possible for glycosylation and disulfide-bridge formation to occur. 3. Yield on par with extracts from wheat germ	1. There aren't many system assessments available. 2. Contains endogenous amino acids, making it challenging to achieve a high protein yield.	1. A new system without cells 2. High possibility for use in the future	2,22,27
Insect cell extract	1. Dounce homogenization, 2. nitrogen cavitation	1. Creatine phosphate, 2. PEP	1. Simple and quick lysate preparation 2. Glycosylation, disulphide bridge formation, lipidation, signal peptide cleavage, and phosphorylation are examples of post-translational modifications that may occur. 3. The availability of endogenous microsomes 4. Direct membrane protein synthesis and integration	1. Expensive cultivation	1. GUV formation for studying biological processes using a membrane protein model 2. Protein engineering incorporating non-canonical AA. 3. Automated membrane protein synthesis	2,28

Rabbit reticulocyte extract	1. Hypotonic lysis of reticulocytes, 2. freeze-thaw	1. Creatine phosphate	1. A proven system 2. System of mammals 3. Protein synthesis is feasible when supplemented, heterogeneous microsomes (such as canine pancreatic microsomes) are present.	1. Low yield of proteins 2. Supplementing with exogenous microsomes is the only way to achieve post-translational modifications 3. The process of treating live animals needed to prepare lysate	1. Microarray technologies for proteins 2. Research on the interactions between proteins and molecules 3. Technology for displays Technologies used for screening	2,18,26,29,30
CHO cell extract	1. Nitrogen 2. cavitation 3. sonication	1. Creatine phosphate	1. A well-recognized and characterized cell line (usually used for validation in pharmaceutical in-vivo protein production base) 2. Contain endogenous microsomal-like structures 3. Mammalian post-translational modifications 4. Direct membrane proteins production 5. Increase in protein yield by IRES-mediated translational initiation	1. Low protein yield compared to prokaryotic CFS 2. Cost is high for cultivation 3. Strong and healthy cell line	1. A new process without cells 2. Excellent prospects for applications in the future	2,19

(continued)

Table 3 Comparative features between sources.[18,19,43] (*cont'd*)

Source for CFS	Cell degradation techniques	Metabolic systems	Pros	Cons	Implements	References
human cell lines culture extract	1. Nitrogen 2. cavitation 3. sonication	1. Creatine phosphate	1. The ideal setting for human protein folding and assembly 2. Include natural microsomal structures 3. The possibility of human-like post-translational changes 4. Modified codon usage to make high-molecular-weight protein synthesis easier	1. minimal protein yield in contrast to Cell-free prokaryotic systems 2. Cultivation is expensive	1. Examining the mechanisms of viral replication 2. creation of antiviral medications	2,31,32
PURE (Protein synthesis Using Recombinant Elements) SYSTEM						
Pure system	Instead of using lysates from damaged cells, PURE uses purified translation components.	Needs an outside source of energy-regenerating systems (like creatine phosphate) as well as nucleotides, amino acids, tRNAs, etc.	1. A well-defined system devoid of background RNAs, proteases, and nucleases 2. Diminished expression of the background 3. Perfect for synthetic biology, such as genetic circuits 4. Non-canonical amino acids can be easily incorporated 5. Beneficial for aggregating or toxic proteins 6. Facilitates in-depth mechanistic research	1. Pricey reagents (ribosomes, purified proteins) 2. Yields are lower than with cell-extract systems 3. Intricacy of system setup 4. Limited capacity to scalable	1. Prototyping genetic circuits 2. Including non-canonical amino acids 3. Display of ribosomes 4. The uses for diagnosis (e.g. biosensors) 5. Growth of synthetic minimal cells	20,43

3. Classification of cell-free systems

System Type	Scalability	Adaptability	Yield of protein	Price
E. coli Extracts	High (up to liters)	Moderate (bacterial proteins)	High (mg/mL range)	Low
Wheat Germ Extract	Moderate	High (eukaryotic proteins, PTMs)	Moderate (μg–mg/mL)	Moderate
Rabbit Reticulocyte extract	Low	Moderate (mammalian proteins)	Low	Moderate to High
CHO Extracts	Moderate	High (mammalian proteins with PTMs)	Moderate	High
PURE System	Low to Moderate	vast	Moderate	High

4. CELL-free system design considerations

4.1 Selection criteria for host strains

To optimize Cell-free protein synthesis (CFPS), the hosting strain is critical.

Protein Type:

- Type of Protein: Bacterial proteins are generally best expressed with prokaryotic CFPS systems derived from *Escherichia coli*. For complex eukaryotic proteins, especially those involving Post Translational Modifications (PTMs) such as formation of disulphide bonds and glycosylation, use eukaryotic cell free systems, such as yeast (*Saccharomyces cerevisiae*), insect cells (*Spodoptera frugiperda*, e.g., Sf9 cell extract), and wheat germ (*Triticum aestivum*).[13]
- Bacterial proteins: Use *E. coli* lysates that are optimized for low-protease activity and high ribosome density.
- Examples: BL21 (DE3) a strain that carries the T7 RNA polymerase (with IPTG-inducible control), allowing genes that are cloned under a T7 promoter to be expressed at high levels. Provides low protease background and BL21 Star has a mutation in the gene for RNaseE (rne131), providing a decrease in the degradation of mRNA, enhancing stability of mRNA, which provides for potentially higher protein expression in CFPS.[34]

- Glycosylated/disulfide-rich eukaryotic proteins: Use insect, mammalian or wheat-germ lysates, or modified strains of *Escherichia coli* that express chaperones or disulfide isomerases (e.g., DsbC) to promote correct folding.[35]

Genetic modifications:

- Knockout strains that lack nucleases and/or proteases (rne–, Δlon, ΔompT) or release factors (RF1), among others, can facilitate higher yield and improved overall stability of the template.
- Overexpress tRNAs (for example, Rosetta strains provide rare tRNAs to enhance translation of heterologous genes that contain rare codons, which is useful for E. coli to express eukaryotic proteins. A19 is a ribosome-rich strain which has low RNase and protease activity resulting in enhanced translation capability) or proteins such as DsbC, FkpA, peptidyl prolyl isomerases can also help facilitate proper folding.[33]

Alternative chassis:

- *Vibrio natriegens*: an extremely fast-growing bacterium, provides rapid extract preparation and likely higher yield in CFPS.
- *Bacillus subtilis*: A GRAS organism, useful for certain industrial enzymes and for CFPS applications due to low endotoxin.
- *Corynebacterium glutamicum*: a non-pathogenic organism, increasingly examined as a chassis for CFPS in biomanufacturing, known to give high yields

Present possibilities as CFPS sources for new classes of proteins or large-scale systems.[13,34,35]

Post-Translational Modifications (PTMs):

- Eukaryotic systems will be better to produce proteins that rely on glycosylation or disulfide bonds[13]

Yield and Cost:

- Engineered strains that over-express that have content with higher ribosomes and lower nucleases, proteases (for example, *E. coli* strain A19, Rosetta-gami) can improve productive yield

Special Features:

- If strains demonstrated mutations (higher nucleases/proteases vs lower) or were knockouts designed to express other molecular chaperones (DnaK, GroEL/ES) as a way to promote folding and an apparent improvement in functional folding of the recombinant protein.[16]

4.2 Selection of energy regeneration routes

ATP regeneration should be efficient and sustainable.

- **Traditional high-energy donors:** PEP, creatine phosphate, 3-phosphoglycerate are traditional donors that provide quick ATP through substrate level phosphorylation, but produce inhibitory inorganic phosphate as by-products.
- **Phosphoenolpyruvate (PEP)**: Commonly used but will produce inorganic phosphate waste that binds to divalent (Mg^{2+}), thus inhibiting translation.[36]
- **Creatine Phosphate**: Used mainly in eukaryotic CFPS systems; supports action for longer lengths of time.[37]

Glycolytic intermediates & PANOxSP:

- Inexpensive, and applicable to continuous systems; Extended activity shown by intermediates like fructose-1,6-bisphosphate and glucose-6-phosphate in CECF formats.[38]
- Utilizing G6P or 3-PG with enzymes hexokinase, enolase, pyruvate kinase can sustain glucose regenerative pathway based ATP, as these also recycle phosphate.

Cytomim system:

- A novel *E. coli*-based CFPS format that utilizes native metabolic pathways (e.g., oxidative phosphorylation) that utilizes substrates like glutamate or glucose to continually regenerate ATP to extend reactions beyond 6 h.
- Preparation of *E. coli* S30 extract using no PEG (no added PEG is preservation of cytoplasmic enzymes and inverted membrane vesicles).
- Provide a mix of pyruvate or glutamate substrates, Mg^{2+}/K^{+} salts, NTPs, tRNAs, amino acids, DTT, and cofactors (NAD^{+}, CoA, oxalate, etc.).
- The oxidative phosphorylation is derived through vesicle-embedded ETC that has been confirmed with sensitivity to certain inhibitors and an oxygen requirement.
- Yields ~700 μg/mL CAT in 6 h; long-form goes up to ~1200 mg/L in 2 h.[39]

Substrate energy lifetimes in CFPS:

- PEP: The reaction typically produces translation for one to two hours before reactions slow by inhibition from phosphate accumulation due to Mg2 + chelation.
- Creatine phosphate: Unlike PEP, which produces less inhibitory byproducts, the reaction generally produces translation and lasts about three to four hours.

- Glycolytic Intermediates (e.g. glucose-6-phosphate and maltodextrin) have substrates that can extend the duration of CFPS activity to 6–10 h via an exchange-based system with recycling of intermediates and reduced phosphates in their cycle.
- Cytomim (glutamate, pyruvate or alternatively forms of metabolism) can provide increased translation rates for at least 6 h, with some reports of outputs at 8–10 h in optimised systems.[38,39]

4.3 Types of DNA templates and preparation

The format of the DNA template will determine the ease of reaction, stability, and yield.

- **Plasmid DNA:** These DNA forms being circular DNA are very stable, offer high yield capacity but is a cloning and purification step and express highly resistant to exonuclease digestion.[40]
- **Linear DNA:** Offers speed of preparation potential through PCR is useful to avoid cloning but degrades via the exonuclease digestion phase. Protective methods exist such as chi-site sequences, terminal modification or use of GamS protein inhibitors.[41]
- **mRNA Templates:** Allows for direct translation in PURE systems and skips transcription, less stable, more expensive to produce.[42]

4.4 Reaction formats

The selection of the reaction format impacts scalability, productivity, and use of resources.

- **Batch Systems:** Simple one-pot reactions that are limited on time due to depletion of substrates and accumulation of byproducts. Mix extract, energy substrate, and templates in one pot; incubate 1–4 h (easy, but can't change substrates once depleted).[13]
- **Continuous Exchange Cell-Free (CECF):** Assemble reaction in dialysis bag/membrane chamber, with a separate reservoir; substrates diffuse into the mixture, waste products diffuse out. We can extend the reaction time to 6−10 h with yield rates up to ~10 mg/mL, making for a longer reaction and greater yield of protein.[38]
- **Microfluidic Platforms:** Using miniature chambers or droplets for parallelized, controlled CFPS, ideal for screening, low-volume prototyping, or single molecules. Can be integrated with automated pumps located outside of the microliter scale, and sensors can allow for greater control of reagents.[17]

5. Materials, reagents, and tools

5.1 Essential reagents

Buffers: Tris-HCl or HEPES at pH 7.5–8.0.

Buffers such as Tris-HCl and HEPES play an important role in maintaining pH during biochemical reactions. Tris-HCl is used heavily because it effectively buffers the pH range of 7–9 which is necessary for the activity of enzymes and to maintain the structural stability of many biomolecules. HEPES is useful in some cell-free systems because it has a lower dependence of pKa on temperature and does not interfere with biological reactions.

Ions: Magnesium (10–15 mM) and Potassium Salts (100–150 mM).

In many biological reactions, magnesium ions are cofactors required for transcriptional and translational mechanism, and serves to stabilise ribosomal structure. potassium ions are also important for ribosome activity and to maintain osmotic balance.

Nucleotides: ATP, GTP, CTP, UTP.

They provide energy carriers during translation and provide the building blocks for RNA synthesis during transcription. ATP and GTP are especially important when driving ribosomal translocation and enzymatic reactions (Spirin, 1988).

Amino Acids: All 20 Canonical Amino Acids (~1 mM each).

In order to complete protein synthesis, a full set of canonical amino acids will be necessary. Balanced concentrations are important for the prevention of depletion in long in vitro reactions.[43]

Cofactors: tRNAs, Aminoacyl-tRNA Synthetases, Chaperones, Folding Catalysts.

- tRNAs bring amino acids to the ribosome.
- Aminoacyl-tRNA synthetases charge tRNAs with their amino acid substrate.
- Chaperones, and folding catalysts such as GroEL-GroES, assist in protein folding, which is particularly important in cell-free systems to obtain functional proteins.

Energy Substrates: PEP, Creatine Phosphate, Glycolytic Intermediates.

Energy recovery systems are important for maintaining ATP levels during cell-free protein synthesis. Either phosphoenolpyruvate (PEP) or creatine phosphate can serve as phosphate donors for ATP recovery.[44]

Reducing Agents: DTT.

Dithiothreitol (DTT) creates a reducing environment by preventing disulfide bonds from forming in proteins that need to stay reduced, maintaining activity and folding of the enzyme.

5.2 Important tools

Centrifuge.

Used to remove debris from extracts following lysis and for cell harvesting, which involves pelleting cells from cell culture media.[45]

Sonicator or French Press.

- Mechanical methods for cell wall and membrane rupturing causes releasing the internal contents of cells.
- Sonication breaks cells using ultrasonic waves.
- The French press ruptures cells by forcing them through a tiny valve with high pressure.

Incubator/Shaker.

Provides cell culture at controlled temperature and agitation to facilitate the best growth conditions and for uniform mixing during reactions.

Spectrophotometer/Fluorometer.

Measuring the concentration of proteins or nucleic acids (A260/A280 ratios), monitoring fluorescent reporter signals (reaction kinetics) in cell-free reactions.[46]

SDS-PAGE and Western Blot.

- Western blots allow for the detection of specific proteins based on antibody binding to confirm successful synthesis in cell-free systems.
- SDS-PAGE can be used to separate proteins by molecular weight making it possible to analyze expression and purity.[47]

5.3 Safety, sterility, and quality control

- Nuclease-free reagents inhibit degradation of nucleic acids during reactions.
- Regularly assay extract activity (e.g., efficiency of coupled transcription-translation) to make sure it performs consistently.[43]
- Aliquoting extracts and storing at −80 °C suppresses degradation related to freeze-thaw cycles.[36]
- Use biosafety procedures for handling genetically modified materials or recombinant DNA to follow regulatory compliance by NIH Guidelines.

6. Overview for preparation of cell-free system

6.1 Host culture and growth

Choosing optimized strains: *E. Coli* BL21(DE3), BL21 are engineered to have a larger ribosome volume and lower levels of protease and nuclease[42] to maximize translational machinery, culture in rich media (e.g., 2 × YT or TB) at 34–37 °C with shaking (~250 rpm) to mid-log (OD_{600} 2–3) grow[42] (Fig. 1).

6.2 Cell culture harvesting, washing, and buffer exchange

- To prevent enzyme inactivation, harvest cells using centrifugation at 5000 × g for 10–15 min at 4 °C.[42]
- To remove any remaining media, wash cell pellets thrice with ice-cold S30 (or Buffer A in addition to 10 mM Tris-acetate pH 8.2, 14 mM Mg-acetate, 60 mM K-glutamate, and 2 mM DTT). Using a buffer with

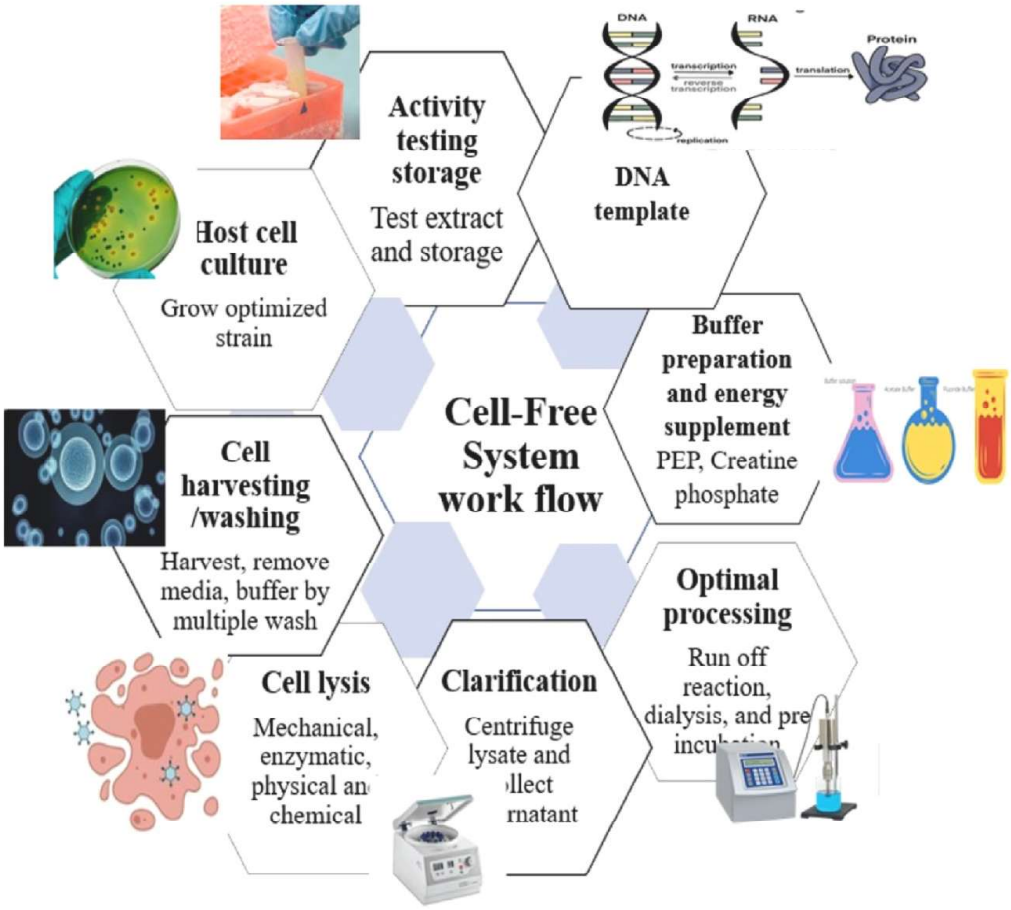

Fig. 1 Overview for preparation of cell-free system.

ionic conditions similar ~14 mM Mg^{2+}, 60-130 mM K^{+}, pH 8.0, resuspend cells in lysis buffer at a volume of ~1 mL buffer per g wet mass.[42]

6.3 Cell lysis methods

Uses Mechanical lysis for successful lysis try using a French press (~20–25 kpsi), or sonication (20 kHz, ~50 % amplitude, on ice; approx. ~537 J/mL).[42] Chemical lysis should be avoided, since chemical lysis will likely produce a negative effect on any translation assays. Note as well that there could be nuclease contamination associated with lytic enzymes (e.g., lysozyme). The suggestion would be to use mechanical means of lysis.

6.4 Clarification

Used to clean up your lysate, Centrifuge the cell lysate at 30,000–40,000 x g in 4 °C for 30–60 min to generate a clarified lysate. Collect the supernatant to made a crude extract.[42]

6.5 Extract processing

- **Run-off reactions:** incubate the crude extract at 37 °C shaking at 250 rpm for 30-60 min to degrade endogenous mRNAs and free ribosomes.[42]
- **Dialysis:** dialyse the post-lysis extract against buffer (10 mM Tris, 14 mg 60 K and 1 mM DTT) using a 10 kDa dialysis cassette to eliminate minor inhibitors and resolve salts that want to exchange the buffer three times for at least 45 min each time at 4 °C before storing at −80 °C.
- **Pre-incubation:** Translation capacity can be enhanced by driving metabolic and enzymatic activities with heat and agitation.

6.6 Activity testing, storage and aliquoting

- **Test activity of extract:** include a reporter (i.e., sfGFP or CAT) in a mini scale CFPS test in a small volume reaction (~15 μL) and assess presence of expressed protein using a reporter enzyme assay to confirm expression using radiolabel assay or fluorescence.[42]
- **Aliquot and flash-freeze:** freeze the aliquots on liquid nitrogen to limit ice crystal formation, and then store aliquots at −80 °C to help preserve extract activity.[42]
- **For extended storage:** This will preserve extract activity; lyophilization is another route at room temperature or for point-of-care function.

7. Cell-free reaction assembly

7.1 Reaction buffer preparation

A common cell-free system should have a buffer developed in order to closely mimic a cellular cytoplasmic environment.

7.1.1 pH-7.5

pH is a key factor in ensuring the activity of enzymes during cell-free transcription-translation (TX-TL) states pH ranges of 7.4–7.6 provide for optimal ribosome function in a supernatant extract from *E. coli*.[48]

7.1.2 Magnesium ions (~10–15 mM)

To help stabilize ribosomes, tRNAs, and the RNA-ribosome complex. Too much Mg^{2+} could cause nucleotide precipitation which could inhibit translation. Too little Mg^{2+} may lead to dissociation of ribosomes.[16]

7.1.3 Potassium ions (~100–150 mM)

Potassium glutamate or potassium acetate would give the cytosol's ionic strength., which is essential for proper ribosomal function and for the folding faster.[14]

7.1.4 Reducing agents (e.g. DTT ~1–2 mM)

DTT is helpful in keeping cysteine residues in proteins from oxidizing so that the enzymes remain active.[4]

7.1.5 Crowding agents (e.g. PEG 3350 at 2–5 % w/v)

PEG adds macromolecular crowding, which can increase the efficiency of folding rates, and also creates the viscosity observed in cells.[49]

Translation speed is enhanced by macromolecular crowding, as the crowded environment high macromolecule concentration is a good mimic of the cytoplasm environment of a cell because it provides sufficiently high effective macromolecule concentrations. Macromolecular crowding increases the rate of biochemical reactions by increasing effective molecular concentrations, as well as promoting correct macromolecule folding such that function is not disturbed in an otherwise crowded system. Crowding agents such as PEG have been shown in CFPS systems to help stabilize ribosome-mRNA interactions, as well as provide a higher overall translational efficiency.[50]

7.2 Energy systems and supplementation

Continuous CFS protein synthesis may require significant turnover of ATP. There are a number of different ways to do this are,

Energy Substrates.

- **Phosphoenolpyruvate (PEP):** It is the most common choice to use as an ATP regeneration system using pyruvate kinase. This method works well, but it generates inorganic phosphate which, can chelate the Mg^{2+} needed in TX-TL systems added activity over time.[51]
- **Creatine phosphate:** It is a differential energy source used with creatine kinase and less inorganic phosphate is generated, while still maintaining more reaction time.[52]
- **Glycolytic intermediates:** Using glucose-6-phosphate or maltodextrin allows for a respectful reaction lifetime because phosphate is added more gradually.[53]

All 20 amino acids (~1 mM each). All 20 amino acids (at about 1 mM each), don't want rapid depletion of low abundance amino acids to halt translation by achieving an appropriate balance of amino acids.[44]

NTPs (ATP, GTP, CTP, UTP). ATP and GTP are needed for translation (for peptide elongation and tRNA charging) and transcription; CTP and UTP are needed for RNA production. Typical NTP concentrations are the following ATPs and GTPs at ~1–2 mM; CTPs and UTPs at ~0.5–1 mM.[16]

Cofactors.

- **tRNAs:** Required to have a good chance of doing translation well; either endogenous tRNA populations or supplemented purified tRNA populations.[51]
- **Chaperones (e.g. GroEL/GroES):** enhancement of proper folding, especially for complicated proteins.[54]

7.3 Addition of DNA templates and optimal regulatory elements

- **Plasmid or Linear DNA (10–50 ng/μL).** Plasmid DNA offers greater stability, while linear templates offer a unique option for rapid prototyping. Linear DNA methods are also prone to degradation by nucleases, unless protected in some way.[55]
- **T7 RNA Polymerase.** T7 RNA Polymerase. T7 polymerase's greater efficiency leads to rapid transcription of target genes. T7 is also probably the most commonly used polymerase for coupled transcription-translation reactions.[16]
- **Ribosome Binding Site Enhancers.** Optimizing your RBS (like strong Shine-Dalgarno sequences) is one way to enhance translation.[56]
- **Regulatory Proteins.** Also include regulatory factors (e.g. LacI, AraC) to allow us to control logic in our synthetic circuits.[57]

- **Purification of products:**

  These are the techniques used for purification:
- Affinity purification: His-tag/Ni-NTA chromatography and Strep-tag/Strep-Tactin resin
- Ion-exchange chromatography separation based on charge
- Size-exclusion chromatography separation based on size (Swartz et al., 2001).

7.4 Incubation conditions

- **Temperature (30–37 °C).** *E. coli* system have an operational temperature of 30 −37 °C. We may improve the folding of some proteins by using lower temperatures, but at the expense of limiting the rate of translation.[45]
- **Incubation Time (1–4 h).** Standard reactions will typically have measurable protein at 1–2 h post-transcription: high yield systems could run up to 4-8 h and could be fuelled with energy regeneration systems.[20]
- **Reaction Volume.** Reactions are scalable from (~10–50 μL) microliter to (1–10 mL) milliliter scale, with microfluidics enabling high-throughput screens.[58]
- **Gentle Shaking or Mixing.** Mixing is beneficial for oxygen transfer which is critical for oxidative phosphorylation in some energy pathways and to provide a homogenous distribution of components.[51]

8. Analytical methods for product assessment

8.1 Protein expression analysis

SDS-PAGE:

- Purpose: Separates proteins according to their molecular weight under denaturing conditions. Proteins unfold upon binding to SDS and have a uniform charge-to-mass ratio.[45]
- Resolution is from ~5 kDa to ~250 kDa.[59]

Western Blot:

- Purpose: Verify the identity of the protein using specific antibodies.
- Process: Transfer proteins from SDS-PAGE to PVDF or nitrocellulose membranes. Block non-specific binding using either milk or BSA. Probe with primary antibody specific for target protein. Detect with labeled secondary antibody.[47]
- Sensitive detection of protein amounts in nanograms.[60]

Coomassie Staining.

- Purpose: Visualizes total protein bands after SDS-PAGE.
- Process: This binds to aromatic and basic amino acids. Sensitivity is approximately 100 ng per band.[61]
- More straightforward but less sensitive than silver staining.
- The Coomassie Brilliant Blue staining detection limit is generally 50–100 ng protein/band on SDS-PAGE, but varies slightly with staining methods. Coomassie dye is much less sensitive than silver staining (detection limits of ~0.1−1 ng/band).[47,62]

8.2 Quantitative protein yield estimation

Bradford Assay (Dye-binding method):

Coomassie Brilliant Blue absorbs at 465 nm (red) and changes to 595 nm (blue) upon binding proteins.[46] Fast and easy but sensitive to detergents and components in buffer.

BCA Assay (Bicinchoninic acid) Assay:

Spectrophotometric Techniques based on the reduction of Cu^{2+} to Cu^{+} by proteins, this forms a purple complex with BCA at 562 nm. Less affected by detergents than the Bradford assay.[63]

Fluorescence Detection.

This technique is used for proteins that have fluorescence properties, i.e. GFP or RFP. Fluorescence detection allows for measurement of the fluorscent emission intensity when exposed to exciting light under a set of specific wavelengths (for ex: GFP: Ex: ~488 nm, Em: ~510 nm). High sensitivity, good for unlabelled, kinetic studies.[64]

Radiolabeling.

Radioactive Amino Acids can include radioactive amino acids (e.g. [^{35}S]-methionine) incorporated into newly synthesized proteins and later detect them with autoradiography, or liquid scintillation counting.[71] Measuring radioactively labeled proteins with these techniques can allow for very small protein yields to be accurately measured.[51]

8.3 Activity and functional assays

8.3.1 Enzymatic assays

Use to measure specific catalytic activity e.g. β-galactosidase and hydrolyzes ONPG to yield a yellow product and measured at 420 nm.[65] ATPases are detected via phosphate release assay. It is valuable to confirm the expressed protein are properly folded and functional.[66]

8.3.2 Binding assays

Used To measure protein, ligand or protein and protein interactions. ELISA to quantitatively analyzing the binding.[67] Surface Plasmon Resonance (SPR) measures the kinetic rates of binding in real time.[68]

8.3.3 Structural characterization

Circular dichroism (CD) spectroscopy provides a basic estimate of secondary structure.[69] NMR or x-ray crystallography original structures or high-resolution structures.[70]

8.4 Advanced detection methods

8.4.1 Radiolabeling for kinetic studies

Use radiolabeled amino acids to measure translation rates, assess degradation kinetics, enabled very sensitive time-course experiments.[51,71]

8.4.2 Luminescent reporters

Uses Luciferase assay for Good for rapid, high-throughput quantification. An ATP-dependent reaction generates light that is detectable using luminometers. De Wet.[72]

8.4.3 Fluorescent tags

Employs chemically based fluorophores or genetically encoded fluorescent proteins (such as GFP and mCherry) which enables Facilitates high-throughput screening, localisation studies, and real-time protein expression monitoring.[64]

9. Troubleshooting and optimization

9.1 Ionic strength modifications

9.1.1 Magnesium

- Magnesium ions (Mg^{2+}) are essential for Ribosome subunit association. tRNA charging. Stabilizing mRNA-ribosome complexes. Low Mg^{2+} results in ribosomal disassociation; high Mg^{2+} results in non-specific RNA folding and inhibition of enzyme activity.[16] Generally, optimal Mg^{2+} concentrations are between 8-15 mM depending on the system, and the DNA template concentration.[39]

9.1.2 Potassium

- Buffers of potassium glutamate or potassium acetate: Approximate the ionic strength of the cytoplasm. Stabilize functional ribosome structures.

Standardize the activities of enzymes, such as RNA polymerase and aminoacyl-tRNA synthetases.[39,51] Typical K^+ concentrations for cell-free reactions are ~60−130 mM.[16]

9.1.3 DTT (Dithiothreitol)

- Maintains a reducing environment which Avoids non-specific disulfide bonds of proteins. Protects cysteines in enzymes from oxidation. High DTT concentrations may cause inhibit enzyme activity by destabilizing the metals contained within cofactors required for proper enzymatic activity. Produce poorly or incompletely folded proteins requiring disulfide bonds.[54]

9.2 Protein solubility and folding

9.2.1 Molecular chaperones

Molecular chaperones, including GroEL/GroES and DnaK/DnaJ/GrpE which prevent aggregates of newly synthesized polypeptides. Refold wrongly folded proteins. Help enhance solubility of poorly soluble proteins. GroEL/GroES are especially useful for folding larger or complex protein structures.[73]

9.2.2 Folding catalysts by PDI (protein disulfide isomerase)

- Promotes the creation and isomerisation of disulphide bonds in proteins. Imperative for correct folding of proteins that have more than one disulfide bond.[54]
- Including PDI has been shown to greatly improve yields of bioactive therapeutic proteins constructed in cell-free systems.[74]

9.3 Temperature and incubation time

- Lower temperatures (20–30 °C) slow down translation: Give proteins time to properly fold. Less aggregation for challenging targets.[48]
- Optimizing reaction time prevents: Over degradation of the template and build-up of challenging by-products.[39]

9.4 Template stability and yield protecting

9.4.1 Protecting DNA modifications

- Linear DNA is susceptible to the nucleases (RecBCD in *E. coli* extracts). Protective actions Phosphorothioate linkages at the ends of the DNAs limit exonuclease degradation.
- 5′ or 3′ protective sequences can also be used to increase stability.[75]

9.4.2 Nuclease inhibition+

- Nuclease inhibitors such as GamS protein: Bind RecBCD complex. Protect linear DNA from degradation.[75]
- Alternatively, engineer E. coli strains that lack nucleases: BL21 Star (DE3) strain has lower RNase activity, leads to better mRNA stability.[42]

9.4.3 Purity of the DNA template

Impurities (salts, residual solvent) decrease transcription/translation. Recommended to use the best plasmid purification kits and avoid residual phenol or ethanol from DNA extraction.

9.5 Reducing inhibitory contaminants

9.5.1 Protease and nuclease elimination

- Proteases degrade proteins during and after synthesis. Nucleases breakdown mRNA and DNA templates.
- Solutions: Careful extract preparation, Cold handling, Fast clarification, Protease inhibitors during extract preparation.[42]

9.5.2 Dialysis and further purification

- Dialysis removes: Low-molecular-weight inhibitors, Excess salts, Residual small molecules that are toxic to enzymes.[39]
- Typical dialysis involves Typical dialysis: Buffer with 10 mM Tris, 14 mM Mg^{2+}, 60 mM K^{+}, 1 mM DTT. At 4 °C, use 3 exchanges of 45 min.

9.5.3 Endotoxin and contaminant testing

- Endotoxins (lipopolysaccharides) from E. coli extracts may inhibit the proteins synthesis enzymes will affect downstream applications in particular, therapeutics.[48]
- Endotoxin removal: Endotoxin-free reagents purify the extracts using affinity or anion-exchange resins.[76]

10. Future directions

CFPS is looking to expand in multiple interdisciplinary ways into the future:

AI-Optimized: Machine learning and artificial intelligence can provide a model to automate reaction condition tuning, and identify optimal extract compositions for maximum yield and fidelity.

Synthetic Minimal Cells: CFPS combined with synthetic compartments and chassis can allow bottom-up assembly of minimal cells, facilitating an avenue of artificial cell technologies.

On-Demand Platforms and Deployed Production: Freeze-dried CFPS kits could broaden access to protein synthesis in medicine, particularly in low-resource locations, point-of-care diagnostics, and space biotechnology.

Broader Post-Translational Possibilities: Engineering CFPS systems capable of high order post-translational modification could lead to in vitro syntheses of even fully functional glycosylated or membrane-bound eukaryotic proteins.

Sustainable Biomanufacturing: CFPS has created a path towards sustainable biomanufacturing. Bio-circuit prototyping could have recycling capabilities and CFPS could be incorporated into cell-free enzymatic cascade production systems that eliminates waste during production.

11. Conclusion

Cell-free protein synthesis (CFPS) has grown from a tool for basic research to a valuable technology in various areas including biotechnology, diagnostics and synthetic biology. Since the 1960s – when the genetic code was first deciphered – CFPS has offered rapid, flexible and scalable protein production capability for diverse applications including personalized medicine, rapid diagnostics and point-of-care therapeutics. This document provides guidance and standard operating procedures (SOPs) to optimize CFPS including strain selection, lysis, extract clarification, energy systems, and reaction conditions. Many advantages – such as access to open environments, incorporation of non-canonical amino acids, and the ability to produce toxic and complex proteins – make CFPS extremely advantageous for rapid prototyping and high-throughput screening. Much progress has been made since the 1960s, but hurdles to reproducibility and yield, template integrity, and proper folding remain. Emerging innovations such as hybrid systems, lyophilized kits and artificial intelligence (AI) optimized platforms are focused on overcoming these issues to make CFPS robust, automated, and portable. In conclusion, CFPS is foundational to 21st century biomanufacturing and synthetic biology. Ongoing advances in extract engineering, platform systems, and computing will enable full realization of CFPS applications extending medicine, research, and sustainable manufacturing well into the future.

References

1. Silverman AD, Karim AS, Jewett MC. Cell-free gene expression: an expanded repertoire of applications. *Nat Rev Genet*. 2020;21(3):151–170. https://doi.org/10.1038/s41576-019-0186-3
2. Zemella A, Thoring L, Hoffmeister C, Kubick S. Cell-free protein synthesis: pros and cons of prokaryotic and eukaryotic systems. *Chembiochem*. 2015;16(17):2420–2431. https://doi.org/10.1002/cbic.201500340
3. Nirenberg M, Matthaei JH. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc Natl Acad Sci*. 1961;47(10):1588–1602.
4. Zubay G. In vitro synthesis of protein in microbial systems. *Annu Rev Genet*. 1973;7(1):267–287. https://doi.org/10.1146/annurev.ge.07.120173.001411 PMID: 4593305.
5. Endo Y, Sawasaki T. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnol Adv*. 2003;21(8):695–713. https://doi.org/10.1016/s0734-9750(03)00105-8 PMID: 14563476.
6. Kim DM, Choi CY. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnol Prog*. 2000;16(3):385–390. https://doi.org/10.1021/bp0000261
7. Kigawa T, Yabuki T, Yoshida Y, et al. Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett*. 1999;442(1):15–19. https://doi.org/10.1016/s0014-5793(98)01620-2
8. Niederholtmeyer H, Sun ZZ, Hori Y, et al. Rapid cell-free forward engineering of novel genetic ring oscillators. *eLife*. 2015;4:e09771. https://doi.org/10.7554/eLife.09771
9. Jaroentomeechai T, Stark JC, Natarajan A, et al. Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery. *Nat Commun*. 2018;9(1):2686. https://doi.org/10.1038/s41467-018-05110-x
10. Nguyen PQ, Soenksen LR, Donghia NM, et al. Wearable materials with embedded synthetic biology sensors for biomolecule detection. *Nat Biotechnol*. 2021;39(11):1366–1374. https://doi.org/10.1038/s41587-021-00950-3
11. Xu H, et al. Hybrid prokaryotic–eukaryotic CFPS systems for post-translational modifications. *Front Bioeng Biotechnol*. 2023;11:10842.
12. Li M, et al. Machine learning-guided optimization of cell-free protein synthesis. *Biotechnol Adv*. 2024;64:108112.
13. Carlson ED, Gan R, Hodgman CE, Jewett MC. Cell-free protein synthesis: applications come of age. *Biotechnol Adv*. 2012;30(5):1185–1194. https://doi.org/10.1016/j.biotechadv.2011.09.016
14. Jewett MC, Swartz JR. Mimicking the E. Coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnol Bioeng*. 2004;86(1):19–26. https://doi.org/10.1002/bit.20026
15. Hodgman CE, Jewett MC. Cell-free synthetic biology: thinking outside the cell. *Metab Eng*. 2012;14(3):261–269. https://doi.org/10.1016/j.ymben.2011.09.002
16. Shin J, Noireaux V. Efficient cell-free expression with the endogenous E. Coli RNA polymerase and sigma factor 70. *J Biol Eng*. 2010;4:8.
17. Pardee K, et al. Rapid, low-cost detection of zika virus using programmable biomolecular components. *Cell*. 2016;165(5):1255–1266. https://doi.org/10.1016/j.cell.2016.04.059
18. Hino M, Kataoka M, Kajimoto K, et al. Efficiency of cell-free protein synthesis based on a crude cell extract from escherichia coli, wheat germ, and rabbit reticulocytes. *J Biotechnol*. 2008;133(2):183–189.
19. Schloßhauer JL, Cavak N, Zemella A, Thoring L, Kubick S. Cell engineering and cultivation of Chinese hamster ovary cells for the development of orthogonal eukaryotic cell-free translation systems. *Front Mol Biosci*. 2022;9:832379.

20. Jewett MC, Swartz JR. Substrate replenishment extends protein synthesis with an in vitro translation system. *Biotechnol Bioeng.* 2004;87(4):465–472.
21. Albayrak C, Swartz JR. Cell-free co-production of an orthogonal transfer RNA activates efficient site-specific non-natural amino acid incorporation. *Nucleic Acids Res.* 2013;41(11):5949–5963.
22. Yue K, Chen J, Li Y, Kai L. Advancing synthetic biology through cell-free protein synthesis. *Comput Struct Biotechnol J.* 2023;21:2899–2908.
23. Hethke C, Bergerat A, Hausner W, Forterre P, Thomm M. Cell-free transcription at 95: thermostability of transcriptional components and DNA topology requirements of pyrococcus transcription. *Genetics.* 1999;152(4):1325–1333.
24. Hodgman CE, Jewett MC. Optimized extract preparation methods and reaction conditions for improved yeast cell-free protein synthesis. *Biotechnol Bioeng.* 2013;110(10):2643–2654.
25. Harbers M. Imaging techniques: SDS-PAGE and coomassie staining. *Protein Electrophoresis*. Springer; 2014:29–44. https://doi.org/10.1007/978-1-62703-706-8_3
26. Jérôme V, Thoring L, Salzig D, Kubick S, Freitag R. Comparison of cell-based versus cell-free mammalian systems for the production of a recombinant human bone morphogenic growth factor. *Eng Life Sci.* 2017;17(10):1097–1107.
27. Buntru M, Vogel S, Stoff K, Spiegel H, Schillberg S. A versatile coupled cell-free transcription–translation system based on tobacco BY-2 cell lysates. *Biotechnol Bioeng.* 2015;112(5):867–878.
28. Stech M, Brödel AK, Quast RB, Sachse R, Kubick S. Cell-free systems: functional modules for synthetic and chemical biology. *Fundamentals Appl. N Bioproduction Syst.* 2013:67–102.
29. Taylor JM, Schimke RT. Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. *J Biol Chem.* 1973;248(22):7661–7668.
30. Anastasina M, Terenin I, Butcher SJ, Kainov DE. A technique to increase protein yield in a rabbit reticulocyte lysate translation system. *Biotechniques.* 2014;56(1):36–39.
31. Brödel AK, Sonnabend A, Roberts LO, Stech M, Wüstenhagen DA, Kubick S. IRES-mediated translation of membrane proteins and glycoproteins in eukaryotic cell-free systems. *PLoS ONE.* 2013;8(12):e82234.
32. International Stem Cell Initiative Consortium, Akopian V, Andrews PW, et al. Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *Vitro Cell Dev. Biol.-Animal.* 2010;46:247–258.
33. Yam A, Groff D, Rivers P, Thanos CD. U.S. Patent No. 10,190,145. Washington, DC: U.S; 2019.
34. Hong SH, Kwon YC, Martin RW, et al. Improving cell-free protein synthesis through genome engineering of escherichia coli lacking release factor 1. *ChemBioChem.* 2015;16(5):844–853.
35. Groff D, Armstrong S, Rivers PJ, et al. Engineering toward a bacterial "endoplasmic reticulum" for the rapid expression of disulfide-bonded proteins in a cytoplasmic E. Coli extract. *ACS Synth Biol.* 2019;8(6):1239–1250. https://doi.org/10.1021/acssynbio.8b00510
36. Kim DM, Swartz JR. Prolonging cell-free protein synthesis by selective reagent additions. *Biotechnol Bioeng.* 1999;66(3):180–188. https://doi.org/10.1002/(SICI)1097-0290(1999)66:33.0.CO;2-N
37. Madzak C, et al. Eukaryotic cell-free systems: recent advancements and expanding potential for industrial protein production. *Front Bioeng Biotechnol.* 2019;7:248. https://doi.org/10.3389/fbioe.2019.00248
38. Zawada JF, et al. Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines. *Biotechnol Bioeng.* 2011;108(7):1570–1578. https://doi.org/10.1002/bit.23103

39. Jewett MC, Swartz JR. Substrate replenishment extends protein synthesis with an in vitro translation system designed to mimic the cytoplasm. *Biotechnol Bioeng.* 2004;87(4):465–472. https://doi.org/10.1002/bit.20139
40. Spirin AS, Swartz JR. *Cell-Free Protein Synthesis: Methods and Protocols.* Wiley-VCH; 2008.
41. Sun ZZ, Hayes CA, Shin J, Caschera F, Murray RM, Noireaux V. Protocols for implementing an escherichia coli based TX-TL cell-free expression system for synthetic biology. *J Visualized Exp.* 2013(79):e50762.
42. Kwon YC, Jewett MC. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci Rep.* 2015;5:8663. https://doi.org/10.1038/srep08663
43. Shimizu Y, Inoue A, Tomari Y, et al. Cell-free translation reconstituted with purified components. *Nat Biotechnol.* 2001;19(8):751–755.
44. Kim DM, Swartz JR. Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis. *Biotechnol Bioeng.* 2001;74(4):309–316.
45. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680–685.
46. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–254.
47. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci.* 1979;76(9):4350–4354. https://doi.org/10.1073/pnas.76.9.4350
48. Swartz JR. Developing cell-free biology for industrial applications. *J Ind Microbiol Biotechnol.* 2001;28(3):133–138.
49. Matsuura T, Yamaguchi A, Shimizu Y, Ueda T. *Synthesis of functional proteins by using the PURE system. Cell-Free Protein Production.* Springer; 2009:167–182. https://doi.org/10.1007/978-1-59745-525-1_12
50. Matsuura T, Kazuta Y, Aita T, Adachi J, Yomo T. Quantifying epistatic interactions among the components constituting the protein translation system. *Mol Syst Biol.* 2009;5(1):297.
51. Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science.* 1988;242(4882):1162–1164.
52. Kim DM, Kigawa T, Yokoyama S. *Methods for high-level synthesis of proteins in cell-free systems. Cell-Free Protein Expression.* Springer; 2007:1–16.
53. Calhoun KA, Swartz JR. An economical method for cell-free protein synthesis using glucose and nucleoside monophosphates. *Biotechnol Prog.* 2005;21(4):1146–1153.
54. Yin G, Swartz JR. Enhancing multiple disulfide bonded protein folding in a cell-free system. *Biotechnol Bioeng.* 2004;86(2):188–195.
55. Sun ZZ, Yeung E, Hayes CA, Noireaux V, Murray RM. Linear DNA for rapid prototyping of synthetic biological circuits in an *Escherichia coli* based TX-TL cell-free system. *ACS Synth Biol.* 2013;3(6):387–397. https://doi.org/10.1021/sb400131a
56. Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. *Nat Biotechnol.* 2009;27(10):946–950.
57. Siegal-Gaskins D, Tuza ZA, Kim J, Noireaux V, Murray RM. Gene circuit performance characterization and resource usage in a cell-free 'breadboard'. *ACS Synth Biol.* 2014;3(6):416–425.
58. Caschera F, Noireaux V. A cost-effective polyphosphate-based metabolism fuels an all E. Coli cell-free expression system. *Metab Eng.* 2015;27:29–37.
59. Schägger H, von Jagow G. Tricine-SDS-PAGE for the separation of proteins in the range from 1 to 100 kda. *Anal Biochem.* 1987;166(2):368–379.

60. Burnette WN. "Western blotting": electrophoretic transfer of proteins from SDS-PAGE gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem.* 1981;112:195–203.
61. Neuhoff V, et al. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity. *Electrophoresis.* 1988;9(6):255–262.
62. Harbers M. Wheat germ systems for cell-free protein expression. *FEBS Lett.* 2014;588(17):2762–2773.
63. Smith PK, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985;150(1):76–85.
64. Chalfie M, et al. Green fluorescent protein as a marker for gene expression. *Science.* 1994;263(5148):802–805.
65. Miller JH. Experiments in molecular genetics. *Cold Spring Harb Laboratory.* 1972.
66. Lanzetta PA, et al. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem.* 1979;100(1):95–97.
67. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). *Immunochemistry.* 1971;8(9):871–874.
68. Rich RL, Myszka DG. Survey of the year 2000 commercial optical biosensor literature. *J Mol Recognit.* 2000;13(6):388–407.
69. Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc.* 2006;1(6):2876–2890.
70. Wüthrich K. *NMR of Proteins and Nucleic Acids.* Wiley; 1986.
71. Pelham HRB, Jackson RJ. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem.* 1976;67(1):247–256.
72. De Wet JR, et al. Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol.* 1985;5(11):2934–2944.
73. Hofmann H, et al. The protein folding machinery in E. Coli. *Microbiol Mol Biol Rev.* 2012;76(2):252–288.
74. Goerke AR, Swartz JR. Development of cell-free protein synthesis platforms for disulfide bonded proteins. *Biotechnol Bioeng.* 2009;103(4):718–729.
75. Marshall R, Noireaux V. Synthetic biology with an all E. Coli TXTL system: methods and applications. *ACS Synth Biol.* 2018;7(12):2414–2426.
76. Liu S, et al. Endotoxin removal techniques in biopharmaceutical production. *Biotechnol J.* 2010;5(1):38–44.