Chapter 2

PURE Technology

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Abstract

The *Escherichia coli*-based reconstituted cell-free protein synthesis system, which we named the PURE (Protein synthesis Using Recombinant Elements) system, provides several advantages compared with the conventional cell-extract-based system. Stability of RNA or protein is highly improved because of the lack of harmful degradation enzymes. The system can be easily engineered according to purposes or the proteins to be synthesized, by manipulating the components in the system. In this chapter, we describe the construction and exploitation of the PURE system. Methods for preparing and assembling the components composing the PURE system for the protein synthesis reaction are shown.

**Key words:** Cell-free protein synthesis system, In vitro translation system, PURE system, Ribosome, tRNA, Translation factor

1. Introduction

A cell-free protein synthesis system is a useful tool for the protein production or protein engineering (1). Since proteins can be synthesized only by the addition of the template DNA to the reaction mixtures, it provides an easy way for the rapid expression of various proteins. Furthermore, several applied technologies utilizing this system enable the synthesis of active membrane proteins (2, 3), incorporation of unnatural amino acids into the proteins (4), or screening of proteins with desired function from the large library pool (5). These features ensure its availability in the field of protein sciences or biotechnology.

Generally, the system is constructed with cell extract, so-called S30 fraction, which contains essential components for the protein synthesis. Through the recent development of the S30-directed cell-free protein synthesis system, milligram quantity of the protein production has been achieved (6, 7). However, the use of such crude extract for the basis of the system encounters several problems.
Rapid shortage of energy sources or degradation of nucleic acids and proteins results in poor reproducibility of the system. Lack of information on the ingredients of the system causes low reliability upon the extension to the applied technologies.

To develop reliable system, we developed the cell-free protein synthesis system reconstituted with purified factors and enzymes required for the translation (8). This system, designated as the PURE (Protein synthesis Using Recombinant Elements) system, is based on the *E. coli* translation apparatus. Purified ribosomes, tRNAs, translation factors, aminoacyl-tRNA synthetases, and several other enzymes for the accomplishment of the protein synthesis composes this system. In contrast to the S30 system, all of the components of the system are identified and, therefore, it is more easily controlled. Reduced nucleases are advantageous for the screening system utilizing the cell-free protein synthesis system (9). Expanded or reconfigured genetic code can be achieved by regulating the codon of mRNA and tRNA or a specific release factor (4, 8, 10). In this report, we describe how to construct the PURE system. Methods for preparation of PURE system components and protein synthesis using this system are described.

### 2. Materials

2.1. Preparation of His-Tagged PURE System Components

1. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Wako). Dissolve at 100 mM and store at −20°C (see Note 1).
2. Buffer A: 50 mM Hepes-KOH, pH 7.6, 1 M ammonium chloride, 10 mM magnesium chloride, 7 mM 2-mercaptoethanol. Store at 4°C (see Note 2).
3. Buffer B: 50 mM Hepes-KOH, pH 7.6, 100 mM potassium chloride, 10 mM magnesium chloride, 500 mM imidazole, pH 7.6, 7 mM 2-mercaptoethanol. Store at 4°C.
4. HT buffer: 50 mM Hepes-KOH, pH 7.6, 100 mM potassium chloride, 10 mM magnesium chloride, 7 mM 2-mercaptoethanol. Store at 4°C.
5. Stock buffer: 50 mM Hepes-KOH, pH 7.6, 100 mM potassium chloride, 10 mM magnesium chloride, 30% glycerol, 7 mM 2-mercaptoethanol. Store at 4°C.
6. 100 mM nickel sulfate.
7. HiTrap chelating column (2 × 5 mL, GE Healthcare) (see Note 3).
8. Protein assay kit based on Bradford method (Bio-Rad).
9. 2 mg/ml bovine serum albumin (BSA) standard for the protein assay (Pierce).
10. Digital Sonifier (Branson).
2.2. Preparation of Ribosome

1. Suspension buffer: 10 mM Hepes-KOH, pH 7.6, 50 mM potassium chloride, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol. Store at 4°C.

2. Buffer C: 20 mM Hepes-KOH, pH 7.6, 1.5 M ammonium sulfate, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol. Store at 4°C.

3. Buffer D: 20 mM Hepes-KOH, pH 7.6, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol. Store at 4°C.

4. Cushion buffer: 20 mM Hepes-KOH, pH 7.6, 30 mM ammonium chloride, 10 mM magnesium acetate, 30% sucrose, 7 mM 2-mercaptoethanol. Store at 4°C.

5. Ribosome buffer: 20 mM Hepes-KOH, pH 7.6, 30 mM potassium chloride, 6 mM magnesium acetate, 7 mM 2-mercaptoethanol. Store at 4°C.

6. GD/X syringe filter (0.45 mm, PVDF, Whatman).

7. HiTrap Butyl FF column (2 × 5 mL, GE Healthcare).

8. High-pressure homogenizer (EmulsiFlex-C5, Avestin).

2.3. Preparation of Template DNA for the Protein Synthesis

1. Template-specific forward primer: 5’-AAGGAGATATACCA ATGNNNNNNNNNNNNNNNNNNNNNNNNNNN-3’. The underlined is a nucleotide sequence identical to a 5’-terminus of the target gene. The Italicized represents an initiation codon.

2. Template-specific reverse primer: 5’-TATTCATTA NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3’. The underlined is a nucleotide sequence complementary to a 3’-terminus of the target gene. The Italicized represents a termination codon.


4. TaKaRa Ex Taq (Takara-Bio).

5. Wizard SV Gel and PCR Clean-Up System (Promega).

2.4. Translation Reaction

1. 1 mM each 20 amino acids. Dissolve 20 amino acids in water and store at −20°C.

2. 700 OD\textsubscript{260}/ml \textit{E. coli} tRNA mixtures. Dissolve \textit{E. coli} tRNA mixtures (Roche) in water and store at −20°C (see Note 4).

3. 100 mM ATP, GTP, UTP, CTP. NTPs are dissolved in water and the pH is adjusted to 7.0 by the addition of sodium hydroxide. Store at −20°C.

4. 500 mM creatine phosphate. Creatine phosphate (sodium salt) is dissolved in water and stored at −20°C.
5. 1 mg/ml formyl donor. 25 mg Folinic acid (calcium salt) is dissolved in 2 mL of 50 mM 2-mercaptoethanol. After the addition of 220 μL hydrochloric acid, the solution is incubated for 3 h at room temperature. The solution is diluted to 1 mg/ml by water and store at −20°C.

6. 1 M Hepes-KOH, pH 7.6. Store at 4°C.

7. 1 M magnesium acetate. Store at room temperature.

8. 2 M potassium glutamate. Store at −20°C in aliquots.

9. 500 mM spermidine. Store at −20°C in aliquots.

10. 500 mM DTT. Store at −20°C in aliquots.


13. SYPRO Orange protein gel stain (Bio-Rad).

14. 7.5% acetic acid.

15. Typhoon 9410 (GE Healthcare).

3. Methods

3.1. Preparation of His-Tagged PURE System Components

1. Construction of overexpression plasmids of the His-tagged PURE system components is shown in Table 2.1. Amplify each gene by PCR from corresponding genome and clone into an appropriate vector described in Table 2.1. Transform the plasmids into an *Escherichia coli* strain BL21/DE3 (pET vector) or BL21/pREP4 (pQE vector).

2. Grow cells to an $A_{600nm} = 0.7$ in 2 L of LB broth at 37°C. Add IPTG to a final concentration of 0.1 mM and additionally grow for 3 h. Harvest cells by centrifugation (see Note 5).

3. Resuspend the cells in buffer A and disrupt them by sonication. Remove cell debris by centrifugation at 20,000 × g.

4. Apply the supernatant to a 10 mL Ni²⁺ column (see Note 3). Wash the column with 100 mL of buffer A–buffer B (95:5 (vol/vol); 25 mM imidazole) with a 2 mL/min flow rate. Elute His-tagged protein with a linear gradient from buffer A–buffer B (95:5 (vol/vol); 25 mM imidazole) to buffer A–buffer B (20:80 (vol/vol); 400 mM imidazole) with a 1 mL/min flow rate for 80 min.

5. Subject the eluted fractions to SDS–PAGE and recover the fractions containing His-tagged protein (Fig. 2.1).

6. Dialyze the recovered fractions against HT buffer (2 × 3 h) and stock buffer (1 × 3 h). Determine the concentration of purified His-tagged protein by Bradford method using BSA as a standard.
### Table 2.1
**Construction of overexpression plasmids of the His-tagged PURE system components**

<table>
<thead>
<tr>
<th>Components</th>
<th>Source</th>
<th>Expression vector</th>
<th>Terminus for His-tag attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanyl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pQE30 (SphI/HindIII)</td>
<td>N</td>
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<td>Arginyl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET16b (NdeI/BamHI)</td>
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<td><em>E. coli</em></td>
<td>pET16b (NdeI/BamHI)</td>
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<td>Aspartate-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
</tr>
<tr>
<td>Cysteiny1-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
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<tr>
<td>Glutaminyl-tRNA synthetase</td>
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<tr>
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<td>pET21a (NdeI/XhoI)</td>
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<tr>
<td>Glycyl-tRNA synthetase</td>
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<td>C</td>
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<td>Histidyl-tRNA synthetase</td>
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<td><em>E. coli</em></td>
<td>pET21a (NdeI/HindIII)</td>
<td>N</td>
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<tr>
<td>Leucyl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (XbaI/XhoI)</td>
<td>C</td>
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<tr>
<td>Lysyl-tRNA synthetase</td>
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<td>pET21a (NdeI/XhoI)</td>
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<td>C</td>
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<tr>
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<td>pQE30 (SphI/HindIII)</td>
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<tr>
<td>Prolyl-tRNA synthetase</td>
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<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
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<tr>
<td>Seryl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (XbaI/XhoI)</td>
<td>C</td>
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<tr>
<td>Threonyl-tRNA synthetase</td>
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<td>Tryptophanyl-tRNA synthetase</td>
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<tr>
<td>Tyrosyl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
</tr>
<tr>
<td>Valyl-tRNA synthetase</td>
<td><em>E. coli</em></td>
<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
</tr>
<tr>
<td>Methionyl-tRNA formyltransferase</td>
<td><em>E. coli</em></td>
<td>pET21a (NdeI/XhoI)</td>
<td>C</td>
</tr>
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<td>Initiation factor 1</td>
<td><em>E. coli</em></td>
<td>pQE30 (BamHI/HindIII)</td>
<td>N</td>
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<tr>
<td>Initiation factor 2</td>
<td><em>E. coli</em></td>
<td>pQE30 (BamHI/HindIII)</td>
<td>N</td>
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<tr>
<td>Initiation factor 3</td>
<td><em>E. coli</em></td>
<td>pQE30 (BamHI/HindIII)</td>
<td>N</td>
</tr>
<tr>
<td>Elongation factor G</td>
<td><em>E. coli</em></td>
<td>pQE60 (MunI/BglII)</td>
<td>C</td>
</tr>
<tr>
<td>Elongation factor Tu</td>
<td><em>E. coli</em></td>
<td>pQE60 (EcoRI/BglII)</td>
<td>C</td>
</tr>
<tr>
<td>Elongation factor Ts</td>
<td><em>E. coli</em></td>
<td>pQE60 (NcoI/BglII)</td>
<td>C</td>
</tr>
<tr>
<td>Release factor 1</td>
<td><em>E. coli</em></td>
<td>pQE60 (BamHI/HindIII)</td>
<td></td>
</tr>
<tr>
<td>Release factor 3</td>
<td><em>E. coli</em></td>
<td>pQE30 (EcoRI/BamHI)</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
1. Grow *Escherichia coli* A19 strain to mid-log phase in LB broth at 37°C. Harvest the cells by centrifugation (see Note 5).

2. Suspend the cells in an equal volume of suspension buffer and disrupt them by high-pressure homogenizer at 7,000–10,000 psi. Remove the cell debris by centrifugation at 20,000 \( \times g \) for 30 min.

3. Add ammonium sulfate to the supernatant to a final concentration of 1.5 M (see Note 6) and dissolve it with stirring at 4°C for 30 min. Remove the precipitated fraction by centrifugation at 20,000 \( \times g \). Filter the supernatant through a 0.45 \( \mu \)m membrane to completely eliminate the precipitated fraction.

4. Use butyl column for the subsequent hydrophobic chromatography. Equilibrate 10 mL butyl column with buffer C. Load 1,000 U at OD\(_{260}\) of the recovered supernatant onto the column and wash with 100 mL of buffer C–buffer D (80:20

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### 3.2. Preparation of Ribosome Components

<table>
<thead>
<tr>
<th>Components</th>
<th>Source</th>
<th>Expression vector</th>
<th>Terminus for His-tag attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosome recycling factor</td>
<td><em>E. coli</em></td>
<td>pQE60 (EcoRI/BamHI)</td>
<td>C</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>Rabbit</td>
<td>pET15b (NdeI/BamHI)</td>
<td>N</td>
</tr>
<tr>
<td>Myokinase</td>
<td>Yeast</td>
<td>pET15b (NdeI/BamHI)</td>
<td>N</td>
</tr>
<tr>
<td>Nucleotide diphosphate kinase</td>
<td><em>E. coli</em></td>
<td>pQE30 (BamHI/HindIII)</td>
<td>N</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td><em>E. coli</em></td>
<td>pET15b (NdeI/BamHI)</td>
<td>N</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>T7 phage</td>
<td>pQE30 (BamHI/HindIII)</td>
<td>N</td>
</tr>
</tbody>
</table>

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**Notes**

- Note 5: Harvest the cells by centrifugation at 20,000 \( \times g \) for 30 min.
- Note 6: Add ammonium sulfate to the supernatant to a final concentration of 1.5 M and dissolve it with stirring at 4°C for 30 min. Remove the precipitated fraction by centrifugation at 20,000 \( \times g \). Filter the supernatant through a 0.45 \( \mu \)m membrane to completely eliminate the precipitated fraction.

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**Fig. 2.1.** SDS–PAGE analysis of the eluted His-tagged protein. Eluted fractions containing His-tagged protein by the Ni\(^{2+}\) column chromatography is analyzed by SDS–PAGE. Samples with high homogeneity and high concentration are recovered as a purified PURE system component. The figure shows the result of His-tagged EF-Tu purification.
(vol/vol); 1.2 M ammonium sulfate) with a 2 mL/min flow rate. Elute the fraction containing ribosome with 35 mL of buffer C–buffer D (50:50 (vol/vol); 0.75 M ammonium sulfate) with a 2 mL/min flow rate and recover the eluate (see Note 7).

5. Overlay the recovered fraction onto 35 mL of cushion buffer in a polycarbonate tube (void volume: 70 mL) for the Beckman type 45 Ti rotor. Pellet down the ribosome by the ultracentrifugation at 36,000 rpm (100,000 × g) for 16 h.

6. After ultracentrifugation, dissolve the pelleted ribosome in 200–300 mL of ribosome buffer. Store the recovered ribosome at −80°C in aliquots until use (see Note 8).

3.3. Preparation of Template DNA for the Protein Synthesis

1. The outline of the method to prepare template DNA for the protein synthesis is illustrated in Fig. 2.2 (see Note 9). Using template-specific forward primer and reverse primer, amplify the gene encoding target protein by PCR.

2. Further amplify the gene by PCR using universal primer and template-specific reverse primer.

3. Purify the amplified gene and dilute it to 0.2 pmol/μL.

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**1st step PCR**

[Diagram of 1st step PCR]

**2nd step PCR**

[Diagram of 2nd step PCR]

Fig. 2.2. Preparation of template DNA for the protein synthesis. A DNA template is prepared by two-step PCR. An ORF of the target protein is amplified by first step PCR and T7 promoter, epsilon sequence originated from bacteriophage T7 (12), and SD (Shine–Dalgarno) sequence are attached by second step PCR.
1. Prepare solution A: Solution A contains 0.2 mM each 20 amino acids, 108 OD$_{260}$/ml E. coli tRNA mixtures, 4 mM ATP, 4 mM GTP, 2 mM CTP, 2 mM UTP, 40 mM creatine phosphate, 20 µg/ml formyl donor, 100 mM Hepes-KOH, pH 7.6, 200 mM potassium glutamate, 26 mM magnesium acetate, 4 mM spermidine, 2 mM DTT.

2. Prepare solution B: Solution B contains 690 µg/mL alanyl-tRNA synthetase, 20 µg/mL arginyl-tRNA synthetase, 220 µg/mL asparaginyl-tRNA synthetase, 80 µg/mL aspartate-tRNA synthetase, 12 µg/mL cysteinyl-tRNA synthetase, 38 µg/mL glutaminyl-tRNA synthetase, 126 µg/mL glutamyl-tRNA synthetase, 96 µg/mL glycyl-tRNA synthetase, 8 µg/mL histidyl-tRNA synthetase, 400 µg/mL isoleucyl-tRNA synthetase, 40 µg/mL leucyl-tRNA synthetase, 64 lysyl-tRNA synthetase, 21 µg/mL methionyl-tRNA synthetase, 170 µg/mL phenylalanyl-tRNA synthetase, 100 µg/mL prolyl-tRNA synthetase, 19 µg/mL seryl-tRNA synthetase, 63 µg/mL threonyl-tRNA synthetase, 11 µg/mL tryptophanyl-tRNA synthetase, 6 µg/mL tyrosyl-tRNA synthetase, 18 µg/mL valyl-tRNA synthetase, 200 µg/mL methionyl-tRNA formyltransferase, 100 µg/ml initiation factor 1 (IF1), 400 µg/ml initiation factor 2 (IF2), 100 µg/ml initiation factor 3 (IF3), 500 µg/ml elongation factor G (EF-G), 1,000 µg/ml elongation factor Tu (EF-Tu), 500 µg/ml elongation factor Ts (EF-Ts), 100 µg/ml release factor 1 (RF1), 100 µg/ml release factor 3 (RF3), 100 µg/ml ribosome recycling factor (RRF),

![Fig. 2.3. Detection of the synthesized protein. Synthesized protein is analyzed by SDS-PAGE. The figure shows the result of dihydrofolate reductase (DHFR) synthesis.](image-url)
40 μg/ml creatine kinase, 30 μg/ml myokinase, 11 μg/ml nucleotide diphosphate kinase, 10 μg/ml pyrophosphatase, T7 RNA polymerase, and 12 μM ribosome.

3. Synthesize the protein. Mix 25 μL solution A, 19 μL water, 5 μL solution B, and 1 μL 0.2 pmol/μL in a reaction tube and incubate it at 37°C for 1–2 h (see Note 10).

4. Apply all the reaction mixtures to Microcon YM-100 (see Note 11).

5. Centrifuge at 1,500 × g at 4°C for 30 min (see Note 12).

6. Subject the permeate to the SDS–PAGE (see Note 13). Stain the gel with SYPRO Orange protein gel stain, wash with 7.5% acetic acid, and visualize with Typhoon 9410 (Fig. 2.3) (see Note 14).

4. Notes

1. Unless otherwise specified, all solutions should be prepared in water that has a resistivity of 18.2 MΩ·cm.

2. 2-Mercaptoethanol should be added to the prechilled buffer before use.

3. Before column chromatography, apply 15 mL of 100 mM nickel sulfate solution to the column with a syringe and wash away the unbound solution with water. After the column chromatography, the column can be restored by washing with the buffer containing 20 mM sodium phosphate, pH 7.2, 500 mM sodium chloride, and 50 mM ethylenediamine-N,N′,N″-tetraacetic acid (EDTA). When you reuse the column, it is important to wash away EDTA with water before immobilization of the nickel ion.

4. Put in the water directly into the vial and dissolve RNA in it. Do not use spatula since the RNA is sensitive to the nucleases.

5. Unless harvested cells are immediately processed for purification, freeze the cells with liquid nitrogen and store at −80°C.

6. Total amount of the ammonium sulfate (g) is the value that the volume of supernatant (mL) is multiplied by 0.222. If the volume of supernatant is 100 mL, add 22.2 g ammonium sulfate into the supernatant.

7. Although the total volume of the eluate is limited by the void volume of a polycarbonate tube used in the following ultracentrifugation, all the ribosome adsorbed on the column can be eluted with this volume.
8. Recovery rate is about 20%, which means 200 U at OD$_{260}$ of the ribosome (24 nmol) is recovered from 1,000 U at OD$_{260}$ of the supernatant after the ammonium sulfate precipitation.

9. A plasmid DNA can also be used for the template DNA. The DNA should encode T7 promoter, SD sequence, the ORF of the protein, and T7 terminator. If the plasmid does not encode T7 terminator, a digested plasmid by specific restriction enzyme at a position downstream of the ORF of the protein can be used.

10. If the detection of the synthesized protein by SDS–PAGE is not necessary for your purpose, following protocol can be abbreviated.

11. This protocol is to remove the ribosome proteins from the reaction mixtures that may hinder the detection of the synthesized protein on the SDS–PAGE. Other methods such as the use of radioisotope-labeled amino acids and BODIPY-Lys-tRNA$_{Lys}$ (Promega) are also available for the detection of the synthesized protein (8, 11).

12. Insoluble or extremely large proteins cannot pass through the membrane. If the synthesized proteins possess such characteristics, other methods described in Note 11 are recommended to try.

13. It is recommended to prepare a negative control sample that does not contain the template DNA or mRNA.

14. Any other equipment that can detect the fluorescence can be used alternatively.

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Cell-Free Protein Production
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