Cell-Free Protein Synthesis Kit Technical Manual

202605 V1.0

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Contents

1 Product Information

1.1 Kit Specifications

Cat. No.	Product Name	Size	Application
EG24301S	Cell-Free Protein Synthesis Kit	20 reaction	Standard expression system
EG24301M		100 reaction	
EG25301S	Cell-Free Protein Synthesis Kit Pro	20 reaction	Enhances protein folding & solubility
EG25301M		100 reaction	
EG25302S	Cell-Free Protein Synthesis Kit Max	20 reaction	For disulfide bond-rich proteins
EG25302M		100 reaction	

1.2 Kit Components

Reagent	Size (20 reaction)	Size (100 reaction)	Description	Storage
Cell-free System Solution A	300 µl	1500 µl	Cell lysate	≤-80℃
Cell-free System Solution B	600 µl	3000 µl	Contains amino acids, nucleotides, energy substrates, salts, etc.	≤-80℃
Control Plasmid	2 µg	2 µg	pJL1-sfGFP (positive control, ~100 ng/µl)	≤-20℃

Storage Note: Solution A and B must be stored at -80℃ after use; aliquot to avoid repeated freeze-thaw cycles.

1.3 Aliquoting Method

The kit is stable at -80℃, while repeated freeze-thaw cycles reduce activity. For small-volume use, reagent aliquoting is recommended.

Thaw Solution A/B on ice or at 4℃ (avoid prolonged thawing) and mix thoroughly. Flash-freeze aliquots in liquid nitrogen, then store at -80℃.

1.4 Precautions

For scientific research only. Not for clinical diagnosis, therapy, food or drug use.

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2 Cell-Free Protein Synthesis

2.1 Overview

This kit uses E. coli cell lysate for in vitro protein synthesis. It reconstitutes a transcription-translation system with ribosomes, translation factors, enzymes, energy sources, nucleotides, amino acids and salts, expressing proteins from DNA/RNA templates without living cells.

Key advantages:

a. Rapid expression: Target protein detected in 1~2 h; maximal yield in 8~24 h

b. High yield: Up to 3 mg/ml.

c. Simple operation: Add template DNA/RNA and incubate in tubes or 96-well plates.

2.2 Experimental Workflow

The procedure for protein synthesis using a cell-free protein synthesis kit is shown in Figure 2-1. Please select an appropriate cell-free expression kit based on the properties of the target protein.

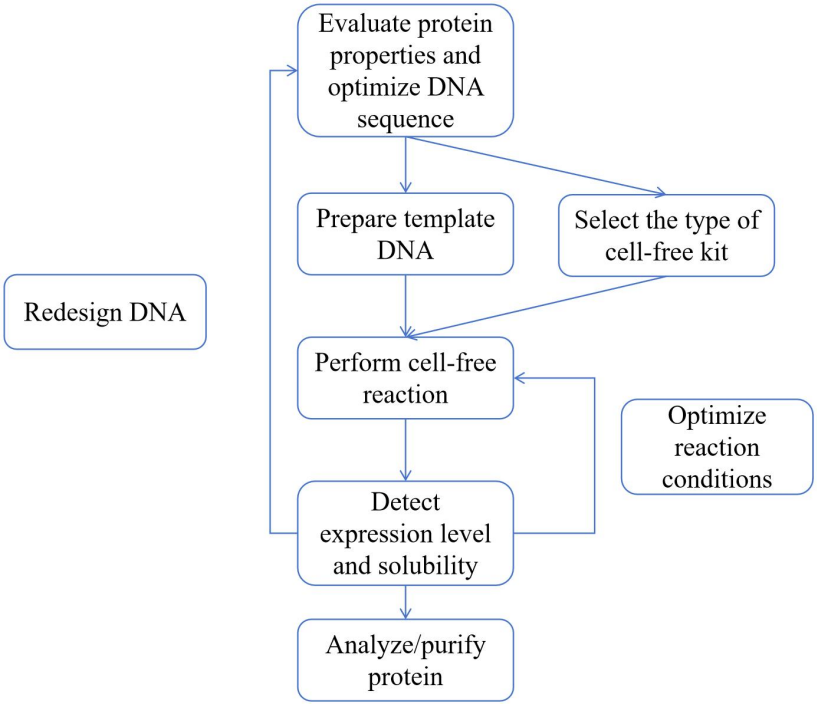

Figure 2-1 Conventional workflow of cell-free protein

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3 Template DNA Preparation

3.1 Design of the Target Gene

3.1.1 Codon Optimization

In addition to protein properties, the DNA sequence greatly affects protein yield and solubility. This kit is based on E. coli—ribosomes, tRNAs, and translation factors are all from E. coli. Therefore, the gene sequence should ideally be codon-optimized for E. coli. For optimal expression, it is recommended to design multiple DNA sequences.

3.1.2 Tags

The N-terminal sequence initiates translation and significantly affects protein yield. Adding commonly used tags such as MBP, Ub, or SUMO to the N-terminus may enhance expression. Therefore, when protein expression is poor, consider adding an N-terminal tag.

3.2 Flanking Sequence Requirements

The upstream region of the target gene must contain the T7 promoter, ribosome binding site (RBS), and other 5' UTR elements, while the downstream region must contain the T7 terminator.

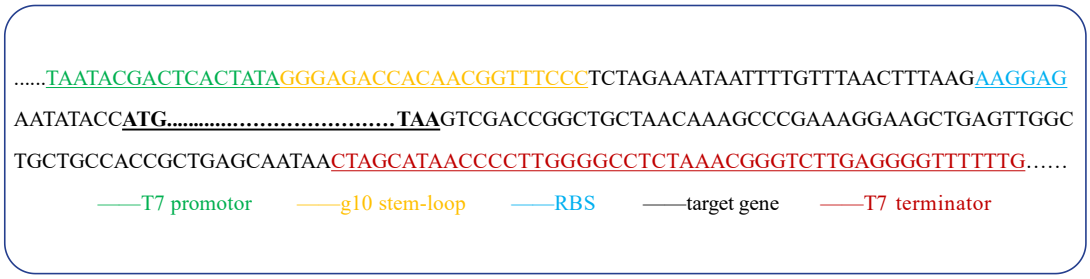

Figure 3-1 Schematic diagram of the upstream and downstream sequences of the target

3.3 DNA Template Types

Cell-free reaction templates can be plasmids, linear DNA, or RCA products.

Plasmid: Highest and most stable expression yield; requires time-consuming bacterial culture and plasmid extraction.

Linear DNA: Yield reaches 60%–70% of plasmid; easily prepared by PCR.

RCA product: Yield similar to linear DNA; prepared by isothermal amplification, ideal for large-volume reactions.

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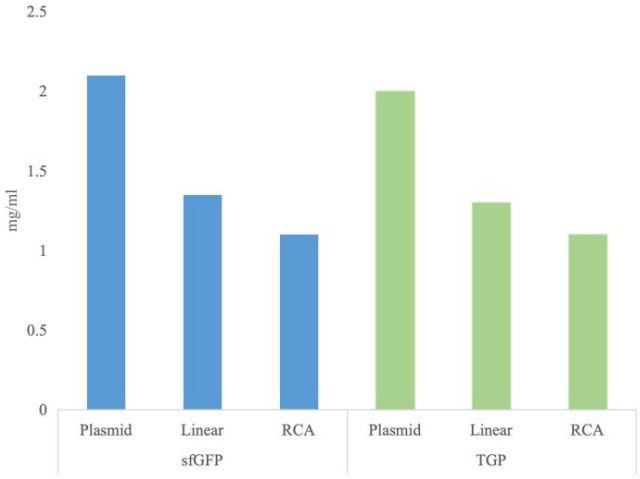

Figure 3-2 Expression results of different DNA templates

Select the appropriate template based on your experimental requirements.

3.4 Template DNA Solution Requirements

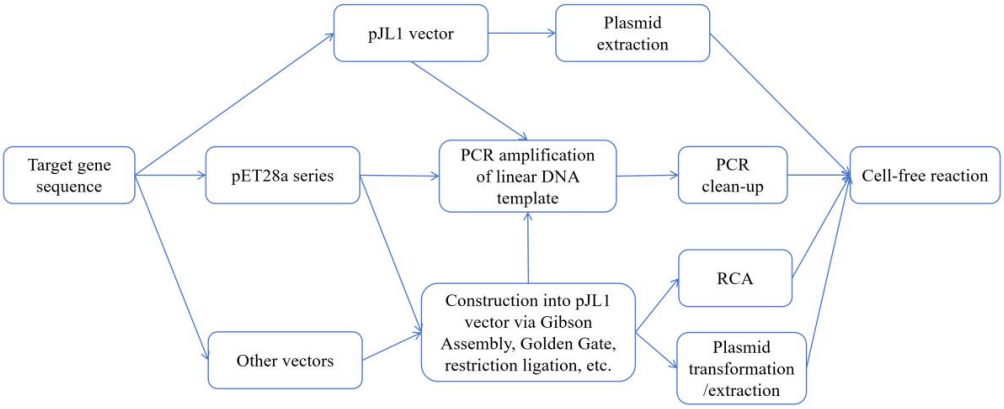

Figure 3-3 Strategies for construction and preparation of cell- free DNA templates.

EDTA in TE buffer chelates Mg^{2+}, potentially inhibiting Mg^{2+}-dependent enzymes and affecting downstream applications. Therefore, it is recommended to dissolve the template DNA in ddH_2O. In addition, RNase degrades RNA (including mRNA and tRNA) in the reaction system. Thus, use RNase-free water and reagents, clean labware, and wear gloves and a mask during the experiment.

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3.5 Circular DNA Template – Plasmid

3.5.1 Plasmid Vector Requirements

Vectors such as pET28a carry lac operator sequences that may limit cell-free protein yield. It is recommended to use vectors lacking the lac operon, such as pJL1, pET9a, or pET23a. The positive control plasmid pJL1-sfGFP (Figure 3-4) provided with this kit can be used as a cloning vector for the target gene. Direct synthesis of the target gene into the pJL1 vector is recommended for cell-free reactions.

If the target gene is already in another vector, subclone it into pJL1 using restriction ligation, seamless cloning, or similar methods.

Figure 3-4 Schematic diagram of the positive control plasmid pJL1-sfGFP structure and selected restriction sites

3.5.2 Example: Plasmid Construction by Restriction Enzyme Digestion and Ligation

3.5.2.1 Enzyme Site Selection

The positive control plasmid pJL1-sfGFP contains a multiple cloning site (MCS) for restriction ligation of the target gene into the expression vector pJL1. The restriction sites within the MCS are shown in Figure 3-5. When selecting restriction enzymes, ensure they have no recognition sites outside the open reading frame of pJL1.

For example, NcoI and BsrGI can be used to replace the existing gene in positive control with the target gene. When using restriction ligation, verify that the target gene lacks internal NcoI and BsrGI sites. If present, eliminate them via synonymous codon substitution, or use alternative methods such as seamless cloning.

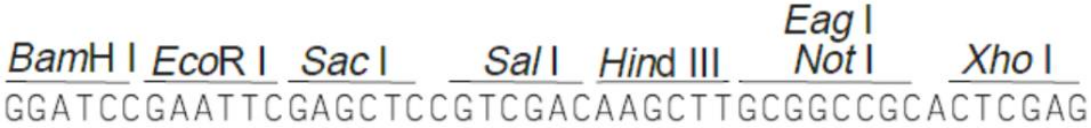

Figure 3-5 MCS sequence

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3.5.2.2 Digestion and Ligation

Follow standard protocols for digestion/ligation.

3.5.2.3 Transformation, Sequencing and Extraction

Perform transformation of the ligation product following standard E. coli transformation procedures. pJL1 is a high-copy vector lacking the lac operon. Avoid DE3 lysogenic strains (e.g., BL21(DE3)); leaky expression may affect plasmid stability. Cloning strains such as DH5α, T1, or TOP10 are recommended.

After transformation, submit colonies for sequencing of the region flanking the target gene using the universal primer pair T7/T7_ter (T7: 5′-TAATACGACTCACTATAGG-3′; T7_ter: 5′-GCTAGTTATTGCTCAGCGG-3′) to confirm the correct sequence.

Residual nucleases from plasmid DNA extraction can inhibit protein synthesis. Ensure the final purified product is nuclease-free. It is recommended to use a high-purity plasmid kit and elute with ddH_2O. If the plasmid is obtained from a gene synthesis company, request column-purified material; otherwise, re-extract the plasmid before use in cell-free reactions.

3.5.3 Example: Plasmid Construction by Seamless Cloning

3.5.3.1 Primer Design

Figure 3-6 Schematic diagram of subcloning the target gene into pJL1 using seamless cloning

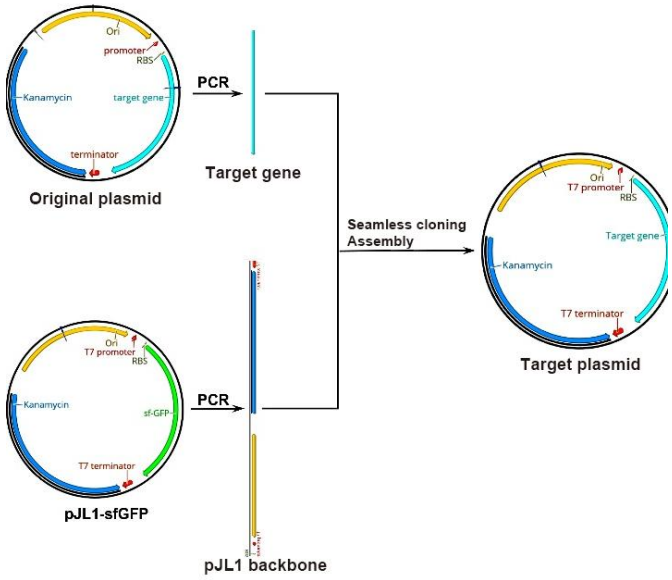

Figure 3-6 Schematic diagram of subcloning of the target gene using seamless cloning.

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Based on the target gene and pJL1-sfGFP sequence, design primers containing homology arms. Use a high-fidelity polymerase to amplify the target gene and the pJL1 vector separately, obtaining the corresponding PCR fragments. Then assemble the two fragments into the desired plasmid via seamless cloning. Software is recommended for primer design; for manual design, refer to the two schemes in Figure 3-7. Customers may adjust the primers according to the specific sequence of the target gene to achieve optimal amplification and assembly.

Using Scheme 1, the vector-backbone primers remain unchanged for different gene

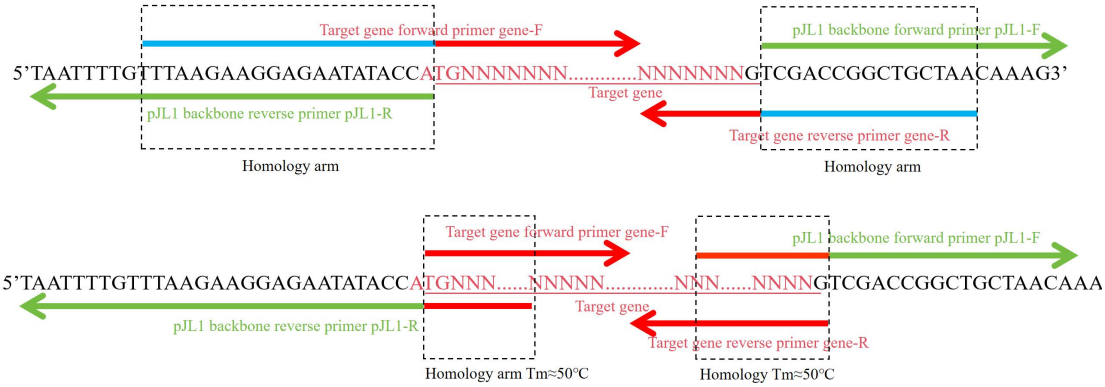

Figure 3-7 Reference for primer design schemes for seamless cloning

constructs—only the target gene primers need to be designed. If customers already have primers for amplifying the target gene, Scheme 2 can be adopted. Primer sequences are listed in Table 3-1.

Table 3-1 Primer sequences

Scheme	Purpose	Primer name	Sequence
1	Amplify target gene	gene-F1	5'-TTTAAGAAGGAGAATATACCATGNNNNNNNNNNNNNNN-3'
		gene-R1	5'-TAGCAGCCGGTCGACNNNNNNNNNNNNNNNNN-3'
	Amplify pJL1 backbone	pJL1-F1	5'-GTCGACCGGCTGCTAACAAAG-3'
		pJL1-R1	5'-GGTATATTCTCCTTCTTAAAGTTAAACAAAAT-3'
2	Amplify target gene	gene-F2	5'-ATGNNNNNNNNNNNNNNN-3'
		gene-R2	5'-NNNNNNNNNNNNNNNNN-3'
	Amplify pJL1 backbone	pJL1-F2	5'-NNNNNNNNNNNNNNNGTCGACCGGCTGCTAACAAAG-3'
		pJL1-R2	5'-NNNNNNNNNNNNCATGGTATATTCTCCTTCTTAAAGTTAAACAAAAT-3'

Note: Green indicates sequences complementary to the pJL1 plasmid backbone; red indicates sequences complementary to the target gene.

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3.5.3.2 Seamless Cloning

We recommend using DNA Assembly Mix Ultra seamless cloning kit. Prepare the reaction system as listed in Table 3-2 according to the instruction manual. Add all required reagents for seamless cloning into a PCR tube, mix well, and incubate at 50 °C for 15 min. Briefly centrifuge, then either keep the product at 4 °C or proceed directly to the transformation step.

Table 3-2 Gibson Assembly reaction system

Reagent	Amount
DNA Assembly Mix Ultra	5 μl
Gene fragment	10~200 ng
Vector (pJL1) fragment	40 ng
ddH_2O	To 10 μl

Note: For the specific amount of gene fragment to add, please refer to the instruction manual of our company's DNA Assembly Mix Ultra product.

3.5.3.3 Transformation, sequencing, and plasmid extraction

Section 3.5.2.3 for plasmid transformation and extraction

3.6 Linear DNA Template – PCR Product

3.6.1 PCR Preparation

Linear DNA templates can be amplified from plasmids or seamless cloning products by PCR. It is recommended to add 200 bp of protective bases upstream of the T7 promoter and downstream of the T7 terminator. For the pJL1 vector, the primers shown in Table 3-3 can be used.

Table 3- 3 Primer sequences

Primer name	Sequence
pJL1-CFE forward	5'-GGCTACCACCTGCCAGGCTATAATACGACTCACTATAGGGAGAC-3'
pJL1-CFE reverse	5'-TGGCAGGTGGTAGCCGGCTACAAAAAACCCCTCAAGACCCG-3'

High-fidelity polymerase is required for PCR amplification of linear DNA templates. We recommend using our company's 2× S705 HiFi Master Mix, which contains S705 High-Fidelity DNA Polymerase, dNTPs, and an optimized reaction buffer. Simply add template, primers, and water to perform high-fidelity PCR.

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PCR system and procedure are as follows：

Table 3- 4 PCR reaction system

Reagent	Amount
2× S705 HiFi Master Mix	25 μl
pJL1-CFE forward(10 μM)	2 μl
pJL1-CFE reverse(10 μM)	2 μl
Template (plasmid/seamless cloning product)	2~20 ng/2 μl
ddH_2O	To 50 μl

Add each reagent to a PCR tube according to the table above, mix well, and run following the program in the table below

Table 3- 5 PCR amplification program

Temperature	Time	Cycles
95℃	3 min	
95℃	10 s	35 cycles
60℃	15 s	
72℃	30 s/kb	
72℃	5 min	

After PCR amplification, if non-target bands are detected by electrophoresis, optimize the PCR conditions. Non-specific products can also serve as transcription templates, thereby affecting the synthesis efficiency of the target protein. If non-specific products remain after optimization, purify the target band with a gel extraction kit. Avoid UV illumination during gel excision, as it may damage DNA and affect transcription efficiency. It is recommended to use a blue light transilluminator and minimize the exposure time.

3.6.2 Example: Procedure for obtaining a linear DNA template from pET series vectors via one‑ step PCR

3.6.2.1 Overview

For recombinant expression in E. coli, vectors containing the lactose operon (e.g., pET28a,

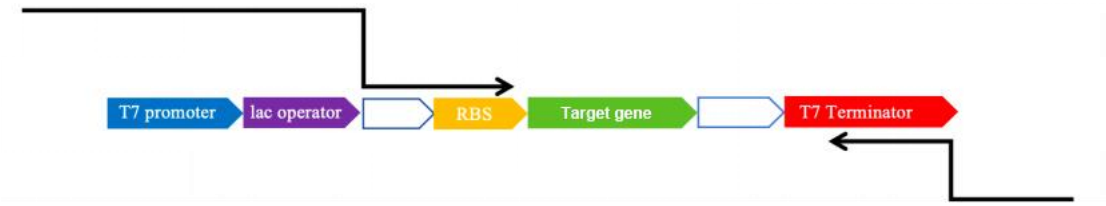

Figure 3-8 One-step PCR amplification of a cell-free linear DNA template from a pET vector

pET32a) are commonly used. However, the operon in such vectors greatly reduces the efficiency

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of cell-free expression. To address this, we designed forward and reverse primers to amplify the target gene, generating templates directly suitable for cell-free reactions (see Figure 3-8 for the schematic). The forward primer includes the T7 promoter and RBS, while the reverse primer includes the T7 terminator. The linear DNA template obtained by one-step PCR with these primers is compatible with cell-free expression kits.

The primer sequences are shown in Table 3-6.

Table 3- 6 Primer sequences for obtaining linear DNA template from pET vector.

Primer name	Sequence
T7Pro-SD primer forward	5'-GGCTACCACCTGCCAGGCTATAATACGACTCACTATAGGGAGAC CACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAG-3'
T7Ter primer reverse	5'-TGGCAGGTGGTAGCCGGCTACAAAAAACCCCTCAAGACCCG-3'

3.6.2.2 Procedure

The PCR system and program are as follows:

Table 3- 7 PCR reaction system

Reagent	Amount
2× S705 HiFi Master Mix	25 μl
T7Pro-SD primer forward(10μM)	2 μl
T7Ter primer reverse(10μM)	2 μl
Template (pET series plasmids)	2~20 ng
ddH_2O	To 50 μl

Table 3- 8 PCR amplification program

Temperature	Time	Cycles
95℃	3 min	
95℃	10 s	35 cycles
55℃	15 s	
72℃	30 s/kb	
72℃	5 min	

After PCR, take 2 μl sample for agarose gel electrophoresis to check the amplification of the target band. If satisfactory, purify the product using a PCR purification kit, store at 4°C for short-term or at -20°C for long-term.

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3.7 RCA Product – phi29 Amplification Product

3.7.1 Overview

Rolling Circle Amplification (RCA) is an isothermal nucleic acid amplification technique that uses a circular DNA template. It works by annealing a short DNA primer to the circular template, and under the catalysis of phi29 DNA polymerase, robust and efficient amplification of the circular DNA is achieved. phi29 DNA polymerase possesses exceptional strand displacement and processive synthesis capabilities, allowing it to generate DNA fragments up to 70 kb in length, with a fidelity higher than that of most currently available DNA polymerases. RCA products have been applied in doggybone DNA technology, completely bypassing fermentation steps and enabling the rapid production of GMP-grade DNA.

Seamless cloning techniques such as Gibson Assembly and Golden Gate assembly allow modular DNA assembly, handling from two to over ten fragments without leaving residual scars. By assembling DNA into circular forms using seamless cloning and then amplifying the circles via RCA, large quantities of cell-free compatible DNA templates can be obtained. This approach eliminates time-consuming and labor-intensive steps such as bacterial culture and plasmid extraction, enabling both DNA template construction and protein expression to be carried out entirely in vitro.

For example, the gene of interest (one or multiple fragments) and the pJL1 plasmid backbone are first assembled into a circle using Gibson Assembly, followed by RCA amplification with phi29 DNA polymerase. The amplified product is then examined and used in a cell-free reaction system. A schematic workflow is shown in Figure 3-9.

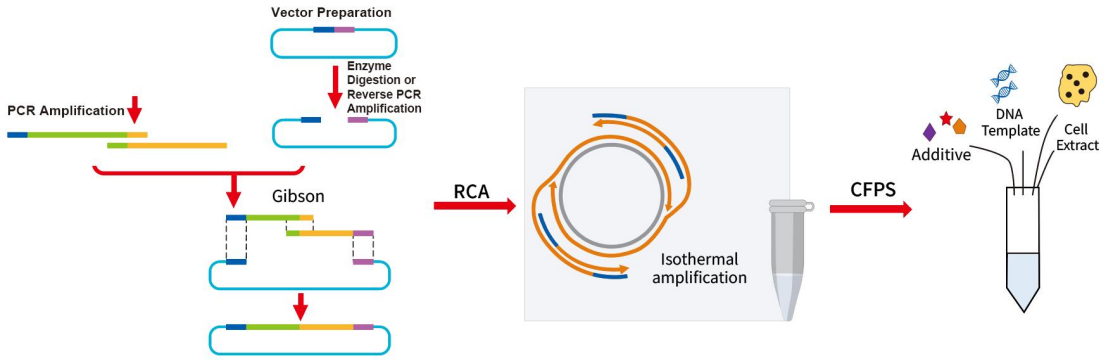

Figure 3-9 Schematic diagram of the workflow for preparing template DNA by rolling circle amplification.

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3.7.2 Example of RCA Template DNA Preparation

3.7.2.1 DNA Fragment Preparation and Gibson Assembly

Design primers with homology arms using software (SnapGene, etc.). Amplify the gene of interest and the pJL1 backbone by PCR. Use the DNA Assembly Mix Ultra kit to assemble the PCR fragments into a circular product as described in section 3.5.3.2.

3.7.2.2 RCA

Use the phi29 polymerase. Set up the reaction as shown in Table 3-9. Add all components except phi29 to a PCR tube, mix, and incubate at 95 °C for 3 min (with 105 °C heated lid). Cool completely on ice, then add 1 µl phi29 (10 U/µl), mix, incubate at 37 °C for 2 h, then 65 °C for 10 min. The RCA product can be stored at 4 °C (short-term) or -20 °C (long-term).

Table 3-9 RCA reaction system

Reagent	Amount
10× phi29 buffer	2 µl
Random hexamer primer (100 µM, 3' thio-modified)	10 µl
dNTPs (10 mM)	2 µl
phi29 II DNA polymerase (10 U/µl)	1 µl
Template (seamless cloning product)	2 µl
ddH_2O	To 20 µl

3.7.2.3 Detection and Verification of RCA Product

RCA products have high molecular weight and are difficult to resolve by direct electrophoresis. Digest an aliquot with a restriction enzyme (e.g., BsaI) before electrophoresis. Set up the digestion as shown in Table 3-10, incubate at 37 °C for 1 h, then 80 °C for 20 min. Run 10 µl of the digest on an agarose gel to estimate concentration using a DNA marker.

Table 3-10 Restriction digestion of RCA product

Reagent	Amount
10× CutOne® Buffer	2 µl
LightNing® BsaI	1 µl
phi29 product	1 µl
ddH_2O	16 µl

If sequencing of the RCA product is required, consult the sequencing provider about direct sequencing. If direct sequencing is not possible, digest the RCA product as above and submit the digested product for sequencing.

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4 Cell-Free Reaction

4.1 Preparation

Thaw Solution A and Solution B from the kit on ice. After thawing, vortex to mix and briefly centrifuge to collect liquid at the bottom of the tube. The table below shows an example of a 50 µl cell-free expression reaction. The reaction volume can be adjusted flexibly by scaling each component proportionally. The amount of template should be optimized. For a negative control, replace the template with ddH_2O.

Table 4- 1 Cell-free reaction system

Reagent	Amount
Solution A	15 µl
Solution B	30 µl
Template	200~500 ng
ddH_2O	To 50 µl

RNase contamination degrades transcripts, tRNA, and other RNAs, inhibiting protein synthesis and greatly reducing yield. Use RNase-free tubes and tips and wear gloves during experiments. For first-time cell-free protein synthesis, include the pJL1-sfGFP positive control provided with the kit.

4.2 DNA Template Amount Requirements

4.2.1 Plasmid DNA

The final concentration of plasmid DNA in the reaction should be around 2 nM, equivalent to 1~3 ng/µl per 1 kb of plasmid length. For example, for a 3 kb plasmid (with a 1 kb gene of interest), the final template concentration should be (1~3)×3 = 3~9 ng/µl. Use the full plasmid length, not just the gene length.

4.2.2 Linear DNA

The final concentration of linear DNA template should be around 4 nM, 2–6 ng/µl per 1 kb. Unpurified PCR reaction mixture can be used directly, but purification improves yield. When adding unpurified product, limit the volume to ⩽10% of the total reaction volume; excess salts may disrupt ionic strength and reduce transcription/translation efficiency. Purify the PCR product with a DNA purification kit. If the PCR product concentration is low, concentrate it using a vacuum concentrator.

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4.2.3 RCA Product

RCA product can be used directly without purification. Estimate its concentration (e.g., by restriction digest) and add to a final concentration of 6~10 ng/μl in the cell-free reaction.

4.3 Reaction Vessel and Equipment

The cell-free system requires oxygen for energy metabolism. For a 50 μl reaction, a round-bottom 2 ml EP tube is recommended; for 5 ml, use a 50 ml centrifuge tube with a breathable cap; for 50 ml, use a 500 ml Erlenmeyer flask. For other volumes, adjust the container size – the reaction volume should be ≤10% of the vessel capacity.

To ensure oxygen supply, place the reaction in a temperature-controlled shaker or oscillator at 200~250 rpm.

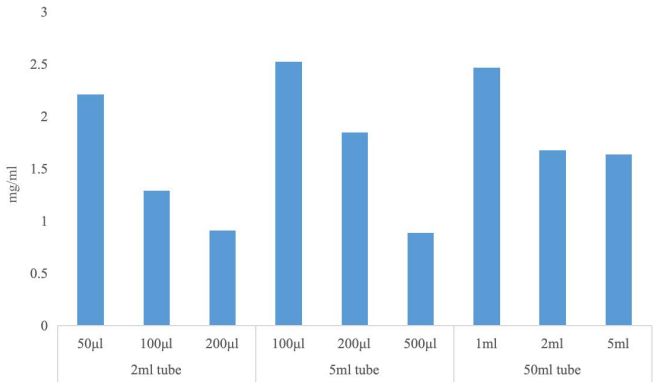

Figure 4- 1 sfGFP expression results with different reaction volumes and vessels.

4.4 Reaction Temperature and Time

Besides volume and oxygen, temperature and time are important factors. Optimize these for each protein. For first-time experiments, use 26 °C for 16 h. If expression is high but solubility is poor, try 18 °C for 24 h. Lower temperature improves solubility but reduces yield, so extend the reaction time.

5 Detection of Cell-Free Expression Products

5.1 SDS-PAGE

SDS-PAGE is generally used to check whether the target protein is successfully expressed. The total reaction mixture after cell-free expression is used to assess overall protein expression, while the supernatant after centrifugation is used to evaluate the soluble fraction. Approximately 1 μl of the reaction mixture and 1 μl of the post-centrifugation supernatant are subjected to SDS-PAGE. By comparing with a reaction lacking the DNA template, observe whether a specific band appears near the molecular weight of the target protein. The SDS-PAGE procedure can be performed as follows:

a. Mix 1 μl of the reaction sample, 2.5 μl of 4× protein loading buffer, and 6.5 μl of ddH_2O in a PCR tube.

b. Heat denature at 95 °C for 10 minutes.

c. Place the gel in an electrophoresis tank, add running buffer, and load the entire 10 μl of each mixed sample into the wells. Run at an appropriate voltage, adjusting the duration according to the protein molecular weight.

d. After electrophoresis, stain the gel with Coomassie Brilliant Blue to visualize the target protein band (the positive control sfGFP appears at approximately 33 kDa).

Figure 5-1 shows the SDS-PAGE gel results of three target proteins, with boxes indicating the positions of the target protein bands. "Total" refers to the total cell-free reaction mixture, and "Supernatant" refers to the supernatant after centrifugation of the total reaction mixture.

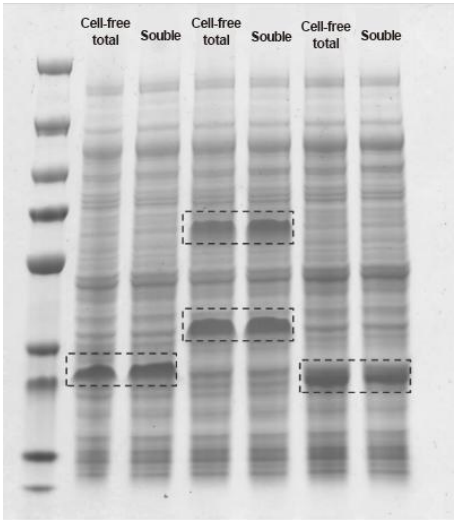

Figure 5- 1 Example of SDS-PAGE result.

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5.2 Other Detection Methods

The sfGFP positive control yields 2~3 mg/ml, clearly visible by SDS-PAGE. However, expression varies greatly among proteins. When the yield is below 0.1 mg/ml, SDS-PAGE may not resolve the band. Use alternative methods based on protein properties: fluorescence (for GFP or fusion proteins), Western blot, activity assay, or ELISA.

6 Purification of Cell-Free Expression Products

The cell-free system contains numerous proteins involved in transcription and translation. For functional assays, it is advisable to purify the target protein. Choose a purification strategy based on protein tags or properties: Ni affinity for polyhistidine tags, streptavidin for Strep-tag II, Protein A for antibodies.

For small volumes (≤1 ml), magnetic beads are convenient and effective. For larger volumes (≥5 ml), use column purification with appropriate resins.

Example using Ni magnetic beads:

a. Centrifugation: Centrifuge the cell-free reaction at 15,000×g, 4 °C for 10–20 min. Keep the supernatant.
b. Bead preparation: Wash the required volume of Ni beads 3× with 5 volumes of ddH_2O using a magnetic stand.
c. Equilibration: Add wash buffer containing ~200 mM salt to the beads, mix, place on magnet, discard supernatant.
d. Binding: Mix the supernatant from step 1 with the beads. For large volumes, rotate at 4 °C for ~1 h; for small volumes, incubate on ice for ~1 h, mixing every 5~10 min.
e. Collect flow-through: Place on magnet, collect supernatant (Flow) and keep a sample.
f. Wash: Add low-imidazole buffer (e.g., 20 mM imidazole), mix on magnet, collect supernatant (Wash) and keep a sample.
g. Elution: Add high-imidazole buffer (100~400 mM), mix, place on magnet, collect eluate (Elution) and keep a sample.
h. SDS-PAGE: Analyze samples from supernatant after centrifugation, Flow, Wash, and Elution.

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i. If the eluted protein is sufficiently pure, dialyze against the appropriate storage buffer to remove imidazole.

7 Cell-Free Expression Examples

7.1 Conventional Proteins

7.1.1 mCherry Green Fluorescent Protein sfGFP and Red Fluorescent Protein mCherry

Expression of sfGFP and mCherry using the Cell-free Protein Synthesis Kit. Reactions were performed in 2 ml EP tubes with a 50 µl reaction volume at 26°C for 16 hours. The resulting appearance is shown in Figure 7-1 (left to right: negative control, sfGFP, mCherry).

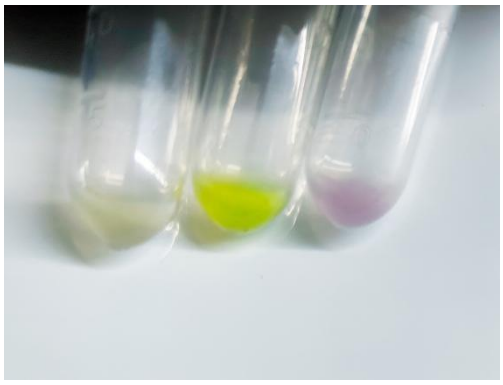

Figure 7- 1 Cell-free expression of sfGFP and mCherry

7.1.2 Other Conventional Proteins

Expression of five proteins of different molecular weights using the Cell-free Protein Synthesis Kit. Reaction volume: 50 µl, 26°C for 16 hours. 1 µl of each reaction was analyzed by SDS-PAGE (Figure 7-2). Red arrows indicate the target protein bands.

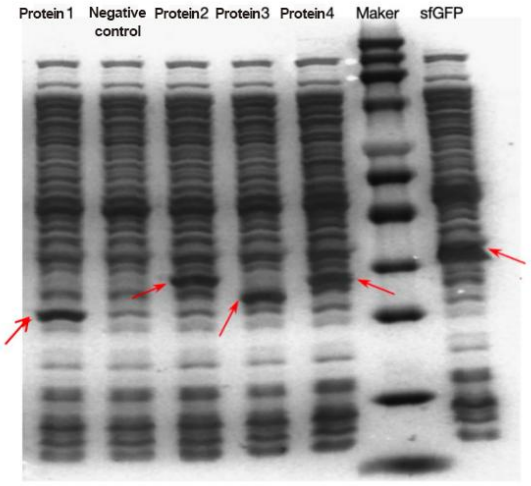

Figure 7- 2 Cell-free expression of multiple proteins

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7.2 Proteins Containing Disulfide Bonds

7.2.1 Gaussia Luciferase

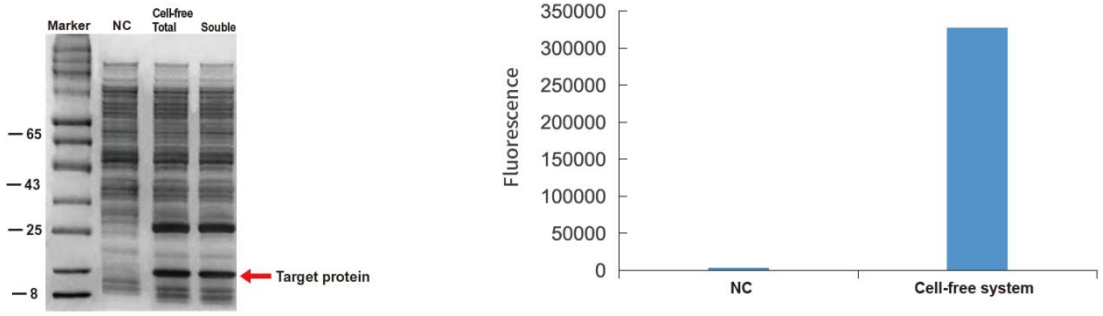

Figure 7- 3 Cell-free expression of Gaussia luciferase and activity assay results

Expression of Gaussia luciferase (containing multiple disulfide bonds) using the Cell-free Protein Synthesis Kit Max. Reaction volume: 50 µl, 26°C for 16 hours. SDS-PAGE and activity assay results are shown in Figure 7-3.

7.2.2 Nanobodies

Expression of two nanobodies using the Cell-free Protein Synthesis Kit Max. Reaction volume: 50 µl, 26°C for 16 hours. 1 µl was analyzed by SDS-PAGE (Figure 7-4). After magnetic bead purification, the yield was approximately 0.2 mg/ml. Functional assays confirmed the activity of these GFP nanobodies. (Reference: Kirchhofer A, Helma J, Schmidthals K, et al. Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol*. 2010;17(1):133-138.)

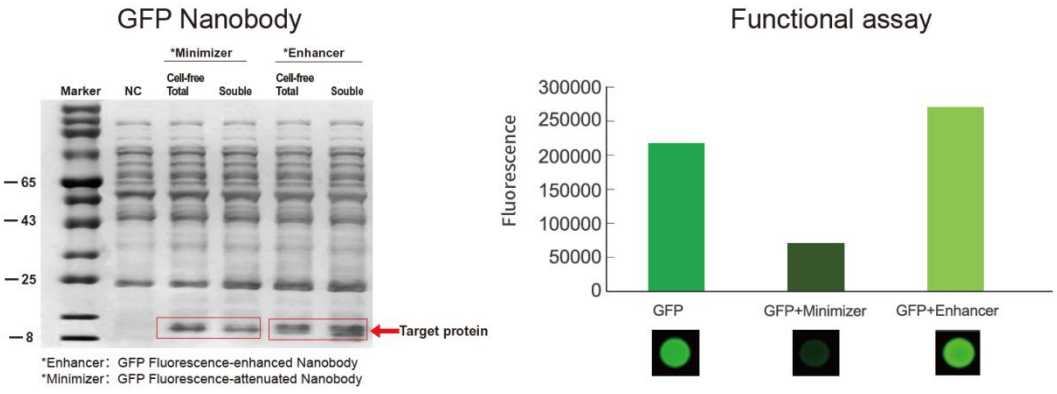

Figure 7- 4 Cell-free expression of nanobodies and activity assay results

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7.2.3 vtPA

Expression of vtPA (containing 9 disulfide bonds) using the Cell-free Protein Synthesis Kit Max. Reaction volume: 50 µl, 26°C for 16 hours. Enzyme activity was measured by adding 10 µl supernatant, 70 µl buffer, and 20 µl 5 mM S-2288 substrate, incubating at 37°C for 1 hour, and measuring absorbance at 405 nm. The activity assay (Figure 7-5) shows that the Cell-free Protein Synthesis Kit Max produces correctly folded and functional vtPA.

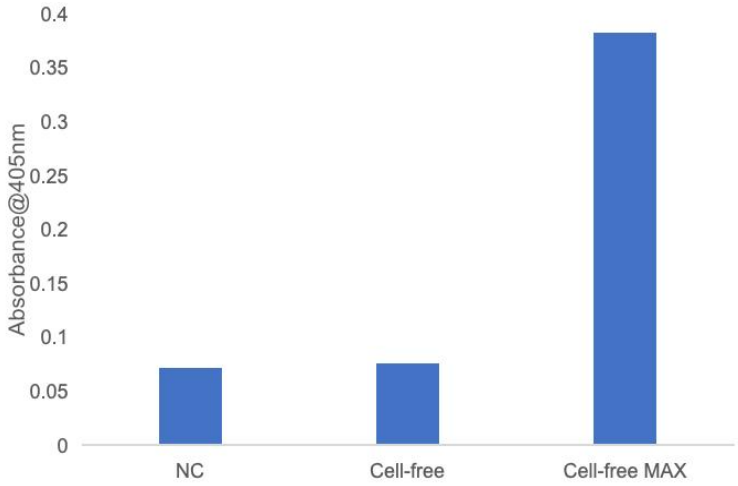
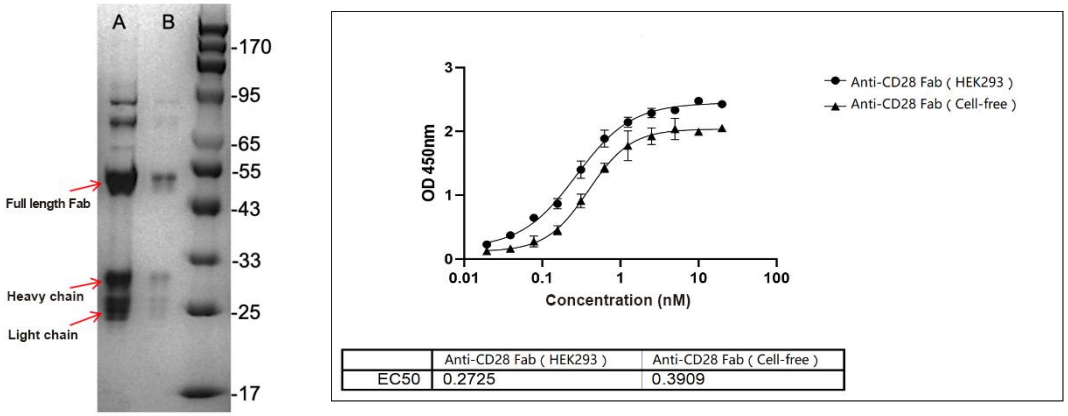


Figure 7- 5 Activity assay of cell-free expressed vtPA

7.2.4 Fab

Expression of Anti-CD28 Fab using the Cell-free Protein Synthesis Kit Max. Reaction volume: 50 µl, 26°C for 16 hours. Heavy and light chain DNA inputs were 8 ng/µl each.

The supernatant was purified in one step using Ni-IDA magnetic beads. ELISA was used to compare the affinity of cell-free expressed Anti-CD28 Fab with that expressed in eukaryotic cells (Figure 7-6). The results show comparable activity.



	Anti-CD28 Fab (HEK293)	Anti-CD28 Fab (Cell-free)
EC50	0.2725	0.3909

Figure 7- 6 Cell-free expression of Fab and activity assay results

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7.3 Non-Canonical Amino Acids

7.3.1 Overview

Non-canonical amino acid (ncAA) is synthetic amino acid derivatives that offer unique chemical properties and biological activities, opening up many possibilities for protein engineering. By using termination codons to insert ncAA into proteins (Figure 7-7), the structure and function of proteins can be greatly expanded.

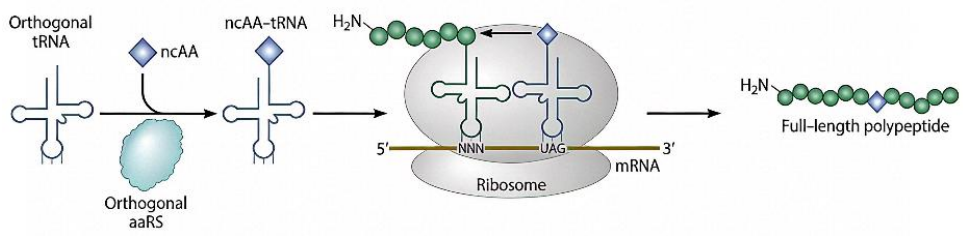

Figure 7- 7 Insertion of ncAA into a protein using a termination codon

Incorporating ncAA requires overcoming challenges such as geneic code expansion and tRNA/aminoacyI-tRNA synthetase (aaRs) pair optimization. Traditional cell-based expression faces problems including cytotoxicity and inefficient transport. Cell-free systems offer greater flexibility and customizability, allowing precise control and efficient incorporation of ncAA. Moreover, using cell-free systems for ncAA incorporation significantly reduces costs.

BestEnzymes Biotech has developed unique ncAA cell-free kits. Advantages: the kit includes separate aaRS, tRNA, and ncAA components, allowing optimization of amounts for different proteins; simple operation (users only need to provide DNA containing a TAG codon); high incorporation efficiency (single-site incorporation efficiency for pAcF and pAzF exceeds 60%). BestEnzymes Biotech offers three ncAA cell-free kits for site-specific incorporation of 4-acetyl-L-phenylalanine (pAcF), 4-azido-L-phenylalanine (pAzF), or Nε-crotonyl-L-lysine (Kcr) via the amber stop codon TAG.

7.3.2 Example: Incorporation of pAzF and pAcF into sfGFP

Four sfGFP sites (Y74, Y106, Y143, Y182) were individually mutated to TAG codons. DNA templates were expressed using the cell-free kit (pAzF or pAcF). The full-length sfGFP containing ncAA is shown in Figure 7-8.

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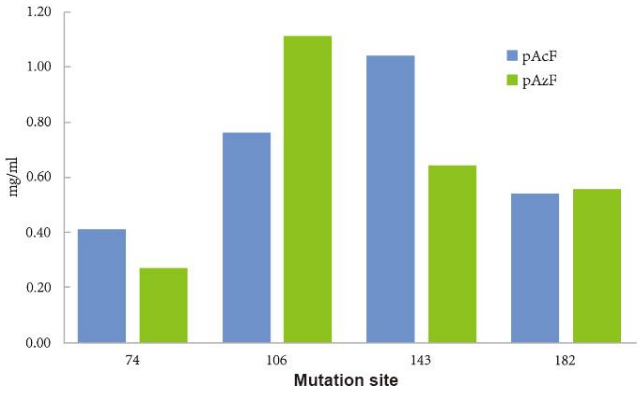

Figure 7- 8 Incorporation of ncAA pAzF or pAcF at different sites in sfGFP

8 Troubleshooting

Problem	Cause	Solution
No expression of positive control	Kit inactive	Store kit at -80°C (lyophilized product at -20°C); minimize freeze-thaw cycles.
	Nuclease contamination	Wear gloves; use nuclease-free water, tips, and tubes.
Low or no target protein yield	Gene structure	Ensure correct sequence design upstream/downstream of target gene
	DNA sequence	Perform codon optimization for *E. coli*; Add or change fusion tag at N-terminus
	DNA template preparation	Remove ethanol, RNase; Use RNase-free ddH_2O for final elution; Avoid nonspecific amplicons when using PCR-prepared templates.
	DNA template amount	Quantify accurately and optimize the amount added.
Insoluble target protein	Disulfide bonds	Use Max version (basic kit leads to inclusion body formation).
	Too fast expression	Use lower temperature (e.g., 16°C) and longer reaction time (e.g., 24 h); Choose Pro version with chaperones.

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9 Related Products for Cell-Free Kits

Product Name	Cat. No.	Function
2× S705 HiFi Master Mix	EG24110S	PCR
	EG24110M	
	EG24110L	
LightNing® DpnI	EG15585S	Template plasmid digestion
DNA Assembly Mix Ultra	EG24204S	Gibson assembly
Golden Gate Assembly Kit (BpiI)	EG25209V	Golden gate
	EG25206S	
phi29 II DNA Polymerase	EG25102S	RCA
	RG25102M	
LightNing® BsaI	EG15518S	RCA product digestion
LightNing® NcoI	EG15550S	Plasmid digestion
LightNing® BsrGI	EG23505S	Plasmid digestion
T4 DNA Ligase (Fast)	EG15205S	DNA fragment ligation
1kb DNA Ladder	EG21909M	DNA marker
Color Prestained Protein Marker (8~250 kDa)	EG23302S	Protein marker
	EG23302M	
Cell-Free Protein Synthesis Kit (Lyophilized)	EG24302S	Basic cell-free kit, lyophilized
	EG24302M	
Cell-Free Protein Synthesis Kit Pro (Lyophilized)	EG25303S	Cell-free kit Pro (solubility-enhanced), lyophilized
	EG25303M	
Cell-Free Protein Synthesis Kit Max (Lyophilized)	EG25304S	Cell-free kit Max (disulfide bond-enhanced), lyophilized
	EG25304M	
Cell-Free Protein Synthesis Kit (pAcF)	EG25330S	ncAA pAcF incorporation kit
Cell-Free Protein Synthesis Kit (pAzF)	EG25331S	ncAA pAzF incorporation kit
Cell-Free Protein Synthesis Kit (Kcr)	EG25332S	ncAA Kcr incorporation kit

Note: Click on the product name to go to the product details page.

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