ENDEXT® Technology

Premium PLUS Expression Kit

Instruction manual for protein synthesis
with wheat germ cell-free system

(Catalog No. CFS-EDX-PLUS)

CellFree Sciences Co., Ltd.

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1. Introduction

Premium PLUS Expression Kit is a starter kit to ascertain the feasibility of producing your target proteins in the Wheat Germ Cell-Free System. The kit can be used either with plasmid DNA or with PCR DNA (*1) constructed by the split-primer PCR method (*2).

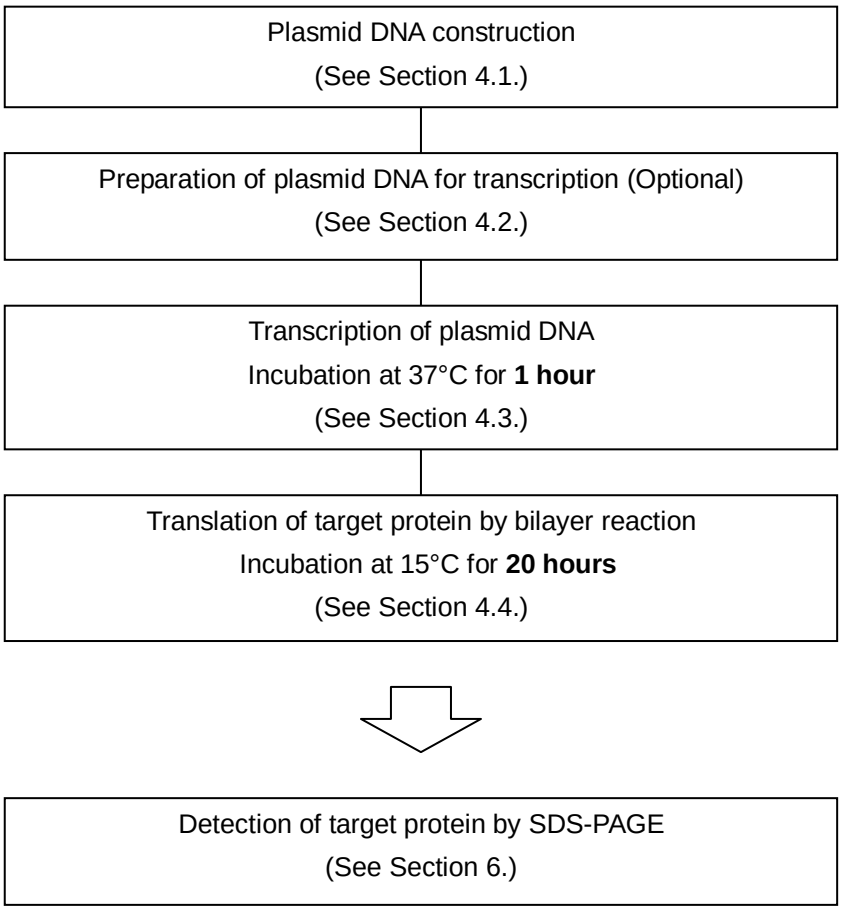
(Note)

*1 With PCR DNA, the protein yield is reduced to about 70% of that with plasmid DNA.

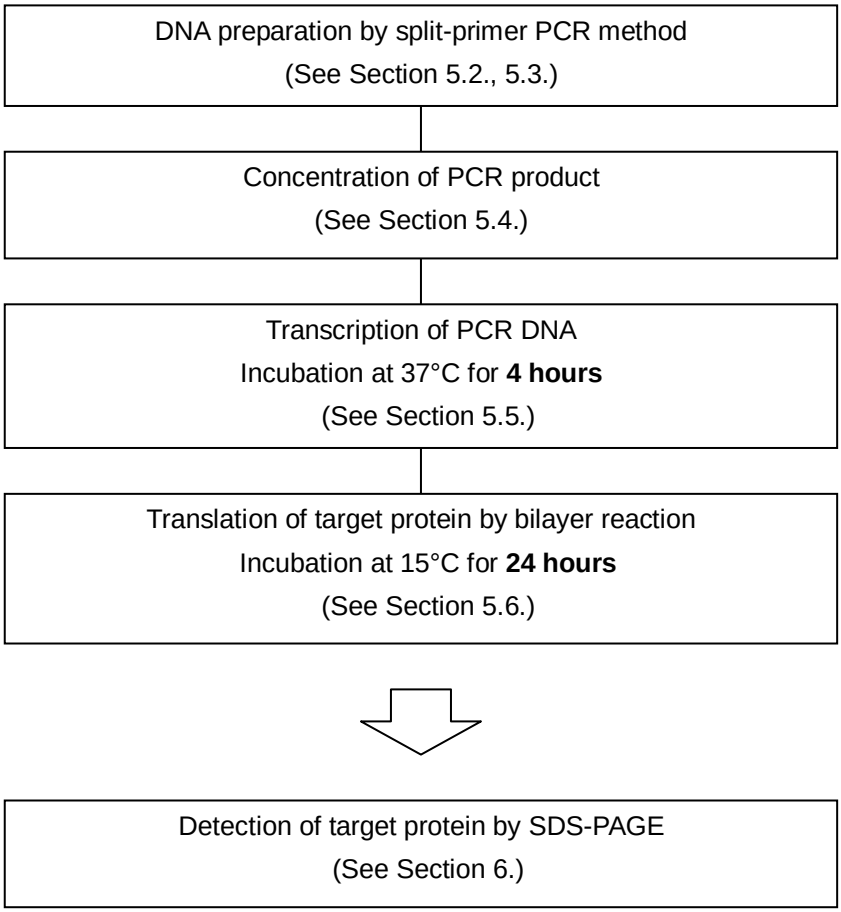
*2 Two kinds of custom primers are needed except for the two primers included in the kit.

2. Protocol Overview

2.1. Plasmid DNA-Based Protein Synthesis



2.2. PCR DNA-Based Protein Synthesis



3. Materials

3.1. Storage of Premium PLUS Expression Kit

Store all reagents at -80 °C or below.

3.2. Contents of Premium PLUS Expression Kit

The kit contains 8 reactions for protein expression:

Item	Tube type & color	Quantity	Volume	Description
pEU-E01-MCS	0.2 ml tube, Red	1	5.0 µl (1.0 µg/µl)	Expression vector for subcloning your gene of interest. See Section 4.1. in detail.
pEU-E01-DHFR	0.2 ml tube, Green	1	5.0 µl (1.0 µg/µl)	Expression vector encoding dihydrofolate reductase (DHFR) gene derived from *E. coli*. It works as positive control for protein expression.
SPU	0.2 ml PCR Tube, Orange	1	100 µl (1µ M)	Sense primer for 2nd PCR. See Section 5.3.2.
deSP6E01	0.2 ml PCR Tube, Violet	1	100 µl (10 nM)	Sense primer for 2nd PCR. See Section 5.3.2.
Transcription Premix LM*	0.2 ml tube, Blue	8	18 µl	Premixed transcription reagent
WEPRO®9240*	0.2 ml tube, Yellow	8	10 µl	WEPRO7240 wheat germ extract mixed with creatine kinase
SUB-AMIX® SGC*	Single-break strip well, Clear	8	206 µl	Translation buffer
Aluminum seal		2		Seal to cover the well during translation. Cut it in an appropriate size to cover the well. See Section 4.4. or 5.6.

* Use the entire content of each tube for 1 reaction.

3.3. Materials to be Prepared by Users

3.3.1. Materials for plasmid DNA-based protein synthesis

(See Section 4.2. for the details.)

Reagents	Description
Phenol/Chloroform	phenol:chloroform:isoamyl alcohol = 25:24:1 in volume, pH 7.9
Chloroform	> 99%
Ethanol	2 grades: > 99% and 70 %
Sodium acetate	3 M, pH 5.2
TE buffer	10 mM Tris, 1 mM EDTA, pH 8.0. Sterilized. It is highly recommended to use DNase-RNase free water when you prepare TE buffer. We DO NOT recommend homemade DEPC treated water.

3.3.2. Materials for PCR DNA-based protein synthesis

(See Section 5.2. for the details.)

Reagents	Description
Gene specific primer (Sense primer for 1st PCR)	Please design an appropriate primer for target gene. See Section 5.2.2.
Antisense primer (Antisense primer for 1st PCR in common with 2nd PCR)	Please design an appropriate primer for vector containing target gene. See Section 5.2.2.
PCR reagents	Taq DNA Polymerase, buffer and dNTP are necessary for PCR. The protocol shown below uses TaKaRa *ExTaq*® (*) and a related buffer from TaKaRa Bio Incorporation.
Ethanol	2 grades : > 99% and 70 %
Sodium acetate	3 M, pH 5.2
Nuclease-free water	DNase, RNase free. We DO NOT recommend homemade DEPC water.

* TaKaRa *ExTaq*® is a trademark of TaKaRa Bio Incorporated.

4. Protocol for Plasmid DNA-Based Protein Synthesis

4.1. Plasmid DNA Construction

1) Insert your cDNA into the multiple cloning site (MCS) of the pEU-E01-MCS vector with restriction enzymes properly selected according to the MCS information given on next page (*1, *2). Protein is translated from the first start codon ATG to stop codon in your cDNA inserted in the MCS. Please note that pEU-E01-MCS contains SP6 promoter, E01 translational enhancer, and ampicillin resistance gene as illustrated on next page.
2) Cultivate *E. coli* containing the cDNA-inserted pEU-E01-MCS.
3) Extract the plasmid DNA from *E. coli* and purify it with a commercially available kit, for example, one from QIAGEN. We recommend QIAGEN Plasmid Midi Kit (catalog No. 12143) or QIAGEN Plasmid Maxi Kit (catalog No. 12163). We DO NOT recommend mini-prep method for the present purpose.
4) After the purification, determine the concentration of the DNA with a spectrophotometer at the wavelengths of 260 nm and 280 nm. Absorbance at 260 nm represents the concentration of DNA. The ratio of absorbance at 260 nm to that at 280 nm represents the purity of the DNA (*3).
5) Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer.

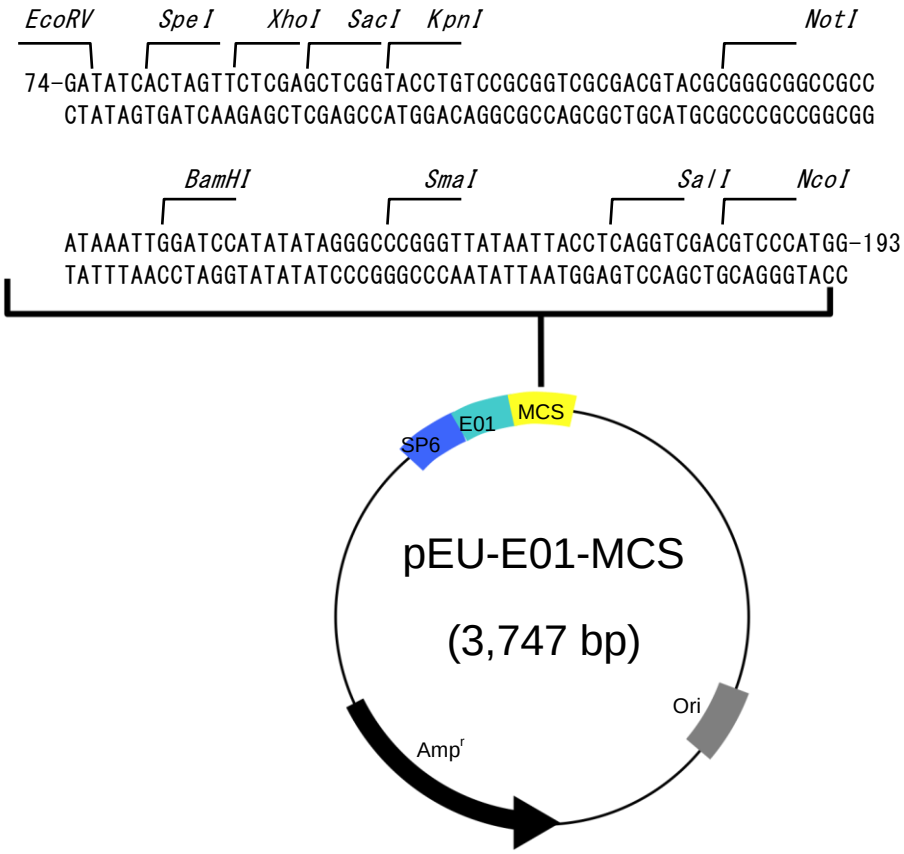
(Notes)

*1 In order to efficiently express the target protein, it is recommended to select a restriction enzyme site as close as possible to E01 translational enhancer.

*2 It is NOT recommended to select *Xho* I restriction enzyme site alone, because self-ligation may occur. Should you use *Xho* I site, use *Sal* I site in combination with *Xho* I site.

*3 Purity of plasmid DNA should be such that the A260/A280 ratio ranges between 1.70 and 1.85. Ratios outside the range indicate that the plasmid DNA has been contaminated. In that case, further purify the plasmid DNA as described in Section 4.2.

(Multiple cloning site information)



pEU-E01-MCS sequence

SP6 promoter: -17~1

Translational enhancer (E01): 16~72

Multiple Cloning Site: 74~193

Origin: 1190~1830

Ampicillin resistance gene: 1974~2838

Position 1 is located at the final G
(underlined in the following sequence)
of SP6 promoter: ATTTAGGTGACACTATAG

4.2. Preparation of Plasmid DNA for Transcription

A high purity plasmid DNA is essential for successful transcription and subsequent translation. If the plasmid DNA purified with a commercially available kit is contaminated or the quality of transcripts made with the plasmid DNA is low, protein synthesis may not be successful. In that case, further purification of the plasmid DNA may be necessary.

This additional purification, which is optional if plasmid DNA has been prepared properly, is accomplished by extraction first with phenol/chloroform and then with chloroform, and by precipitation with ethanol as described below:

1) Add an equal volume of phenol/chloroform (phenol:chloroform:isoamyl alcohol = 25:24:1, pH 7.9) to the purified plasmid DNA solution (see Section 4.1) and mix well.
2) Centrifuge the mixture at 15,000 rpm for 5 min.
3) Carefully transfer the upper aqueous phase to a new tube.
4) Add an equal volume of chloroform into the tube and mix well.
5) Centrifuge this mixture at 15,000 rpm for 5 min.
6) Carefully transfer the upper aqueous phase to another new tube.
7) To this upper aqueous solution, add 100% ethanol, 2.5 times the volume, and 3M sodium acetate (pH 5.2), 1/10 of the volume, to precipitate the DNA.
8) Hold at -20°C for 10 min.
9) Centrifuge at 15,000 rpm for 20 min at 4°C.
10) Remove the supernatant. Add 800 µl of 70% ethanol to wash the remaining DNA pellet in the tube.
11) Centrifuge the tube at 15,000 rpm for 10 min at 4°C.
12) Remove the supernatant.
13) Dry the DNA pellet for 10 to 20 min.
14) Add an appropriate volume of TE buffer to resuspend the DNA pellet.
15) Determine the concentration of the DNA with a spectrophotometer at the wavelengths of 260 nm and 280 nm. Absorbance at 260 nm represents the concentration of DNA. The ratio of absorbance at 260 nm to that at 280 nm represents the purity of the DNA (*1).
16) Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer.

(Notes)

*1 Purity of plasmid DNA should be such that the A260/A280 ratio ranges between 1.70 and 1.85. Ratios outside the range indicate that the plasmid DNA has been contaminated. In that case, repeat Section 4.2 from the beginning.

4.3. Transcription of Plasmid DNA into mRNA

It is recommended to use pEU-E01-DHFR to express DHFR protein as positive control.

1) Remove from storage (-80 °C) required number of Transcription Premix LM tubes (*1). Keep the remaining tubes in storage at -80 °C.
2) Thaw the Transcription Premix LM on ice. After thawing, spin down each tube for a short time to drop down the reagent staying on the tube wall or on the cap.
3) Add 2 µl of high purity plasmid DNA (1.0 µg/µl) to each tube of Transcription Premix LM and then mix gently by pipetting.
4) Incubate at 37 °C for **1 hour** in a thermal cycler or an incubator (*2).
5) After the incubation, inspect mRNA quality by the ordinary method of agarose gel electrophoresis (*3).

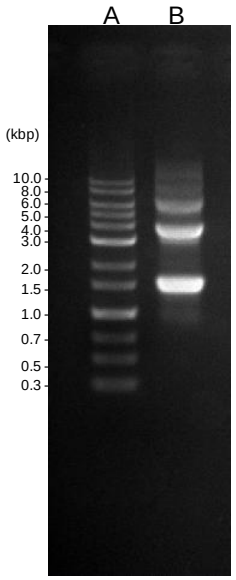
(Notes)

*1 The set of Transcription Premix LM tubes can be split into individual tubes by bending or cutting. Hold the tubes firmly so that they may not pop when they are separated.

*2 White precipitate may appear during the incubation. This is magnesium pyrophosphate. Use the whole mixture including precipitate in the next step.

*3 A smear or ladder pattern of less than 500 bases of mRNA indicates possible degradation of mRNA probably caused by RNase. In that case, repeat the preparation of plasmid DNA as described in Section 4.2.

Example of mRNA obtained in high quality:



A: Molecular weight marker (DNA)
B: Transcript
*1% agarose gel

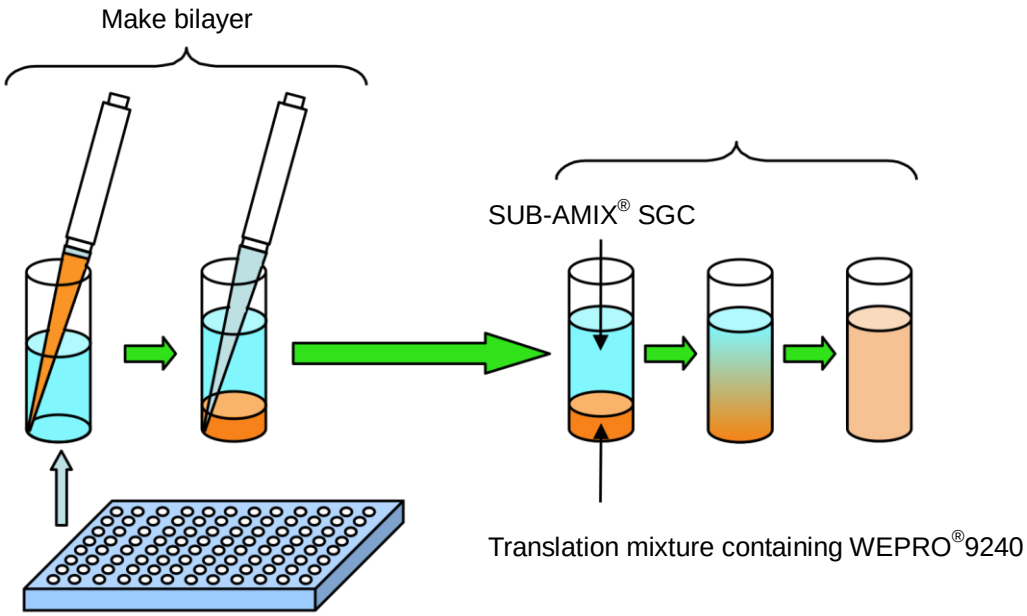
4.4. Translation of Target Protein

1) Remove from storage (-80 °C) required number of WEPRO®9240 tubes and single-break strip wells containing SUB-AMIX® SGC (*1). Keep the remaining tubes and wells in storage at -80 °C
2) Thaw the two reagents on ice. After thawing, spin down each tube containing WEPRO®9240 for a short time to drop down the reagent staying on the tube wall or on the cap. Avoid excessive centrifugation. Resuspend SUB-AMIX® SGC by pipetting gently in the well (*2).
3) Let the mRNA tube(s) cool down to the room temperature. DO NOT forcibly cool it on ice or in the refrigerator. Resuspend the mRNA by pipetting gently (*3).
4) Add 10 µl of resuspended mRNA into WEPRO®9240 and then mix gently by pipetting. Avoid bubble formation.
5) **Carry out bilayer reaction.**
   Carefully transfer the whole mixture (20 µl) of WEPRO®9240 and mRNA to the bottom of the single-break strip well containing SUB-AMIX® SGC (206 µl) to form bi-layer with WEPRO® mixture in the lower layer and SUB-AMIX® SGC in the upper layer as illustrated on next page. DO NOT mix the reagents in the well by pipetting or any other means. (Important !!)
6) Seal the well with aluminum seal included in the kit to avoid evaporation (*4).
7) Incubate at 15 °C for **20 hours**.
8) After translation, mix the bilayer reaction gently by pipetting.

(Notes)

*1 The sets of WEPRO®9240 tubes and SUB-AMIX® SGC wells can be split into individual tubes and wells by bending or cutting. Hold the tubes or wells firmly so that they may not pop when they are separated.
*2 Take special care to keep SUB-AMIX® SGC wells upright, since they easily tip over.
*3 If there is white precipitate in mRNA tube, resuspend the mRNA together with the precipitate by pipetting gently.
*4 Cut aluminum seal in an appropriate size to cover the wells. Save the remaining seal.

Bilayer reaction system



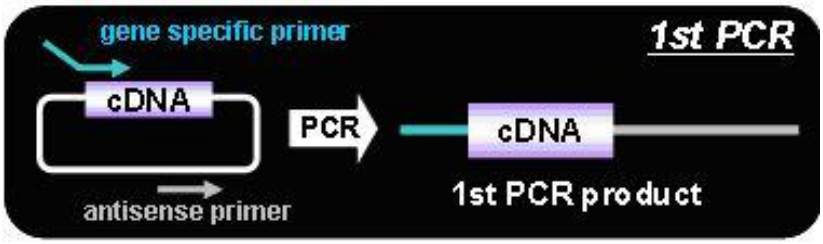
5. Protocol for PCR DNA-Based Protein Synthesis

5.1. Precaution

1) If cDNA is inserted in a vector of pET-24 series or pET-28 series, PCR products may not be obtained. The vector containing the cDNA of target protein should not contain SP6 promoter sequence. If it does, it is recommended to change the vector.
2) The pEU-E01-vector is not suitable to be used with the split-primer PCR method to add an affinity tag to PCR products, since the resultant products would be a mixture of some with the tag and others without.
3) With PCR DNA, the protein yield is reduced to about 70% of that with plasmid DNA.

5.2. 1st PCR

5.2.1. Outline of 1st PCR



※ When the direction of insertion of a cDNA is reversed,
the direction of each primer is also reversed.

5.2.2. Preparation of primers for 1st PCR

Primer	Gene specific primer
Length (bp)	About 35
Sequence	5'- ccacccaccaccaccaATGNNNNNNNNNNNNNNN -3'
Description	Small letter: It is to bind with deSP6E01 sequence. ATG: Start codon NNN: cDNA specific sequence consisting of about 20 bases

Primer	Antisense primer (common to 2nd PCR)
Length (bp)	About 20
Sequence	The sequence is derived from the plasmid.
Description	Design about 20 bases primer which binds with a site about 1.6 kbp away from the 3' end of the ORF.

5.2.3. Method of 1st PCR

1) Using a 0.2-ml PCR tube, prepare a reaction solution of the following composition.

	Volume per sample (µl)	Final conc.
Template plasmid DNA (250 pg/µl)	2	25 pg/µl
10x *ExTaq*® Buffer	2	1x
2.5 mM dNTP	1.6	0.2 mM
100 nM gene specific primer	2	10 nM
100 nM antisense primer	2	10 nM
Nuclease-free water	10.35	-
TaKaRa *ExTaq*® (5 U/µl)	0.05	0.0125 U/µl
Total	20 (*1)	

2) Place the reaction tube in the PCR thermal cycler, program the cycler for the following condition, and run the first PCR campaign.

°C	Time	
98	1 min	
98	10 sec	
55	1 min	35 cycles
72 (*2)	*x* min (*3)	
72 (*2)	*x* min (*3)	
20	∞	

3) Run agarose gel electrophoresis with 2 µl of the PCR product solution to see if the target product is detected as a single band (*4).

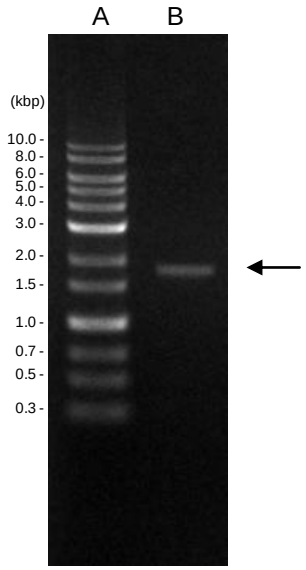
(Notes)

*1 Since the volume of required Taq polymerase is small, mix enough amounts of the reagents for at least 10 samples to measure out the correct volume of the polymerase.

*2, 3 To adjust the elongation time and temperature according to the DNA polymerase and the length of the gene, follow the supplier's instruction for the DNA polymerase.

*4 Proceed with 2nd PCR even if 1st PCR product is not detected, because 2nd PCR may still yield the product.

Example of 1st PCR Product



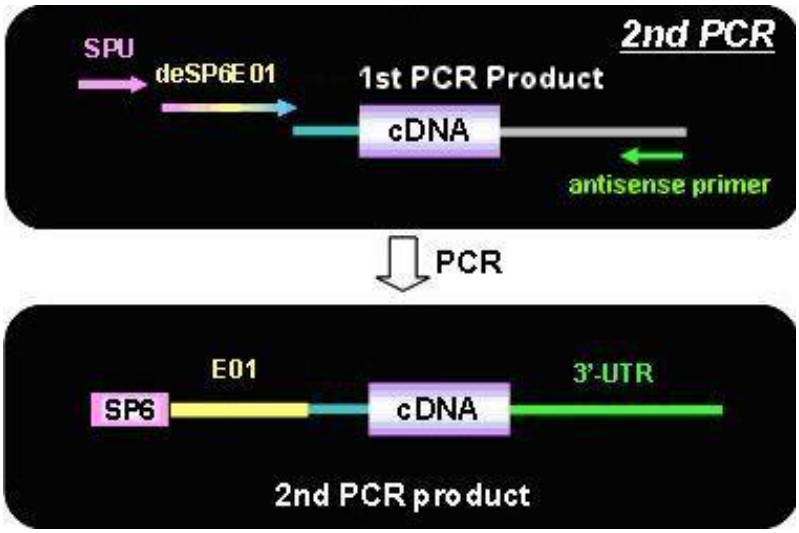
A: Molecular weight marker (DNA)

B: 1st PCR product

*1% agarose gel

5.3. 2nd PCR

5.3.1. Outline of 2nd PCR



5.3.2. Primers for 2nd PCR

Antisense primer for the 2nd PCR is the same as the one used in 1st PCR (See Section 5.2.2).
2nd PCR uses 2 sense primers, SPU and deSP6E01. They are included in the kit.

Primer	SPU
Length(bp)	21
Sequence	5'- GCGTAGCatttaggtgacact -3'
Description	Small letter: 5' part of the SP6 promoter

Primer	deSP6E01
Length(bp)	100
Sequence	5'-ggtgacactatag*AACTCACCTATCTCCCCAACACCTAATAACATTCAAT CACTCTTTCCACTAACCACCTATCTACATCA***CCACCCACCACCACCA** ATG-3'
Description	Small letter: 3' part of the SP6 promoter Italic capital letter: It contains the E01 enhancer sequence. Bold capital letter: It is the part that overlaps with the tip of the 1st PCR product ATG: Start codon

5.3.3. Method of 2nd PCR

1) Prepare a reaction solution of the following composition.

	Volume per sample (µl)	Final conc.
1st PCR product	10	1/10 vol.
10x *ExTaq*® Buffer	10	1x
2.5 mM dNTP	8	0.2 mM
1 µM SPU	10	100 nM
10 nM deSP6E01	10	1 nM
1 µM antisense primer	10	100 nM
Nuclease-free water	41.75	-
TaKaRa *ExTaq*® (5 U/µl)	0.25	0.0125 U/µl
Total	100 (*1)	

2) Divide the solution into two 50-µl aliquots, each in a 0.2 ml PCR tube.

3) Place the two reaction tubes in the PCR thermal cycler, program the cycler for the following condition, and run the 2nd PCR campaign.

°C	Time	
98	1 min	
98	10 sec	
55	1 min	5 cycles
72 (*2)	*x* min (*3)	
98	10sec	
60	40sec	35 cycles
72 (*2)	*x* min (*3)	
72 (*2)	*x* min (*3)	
20	∞	

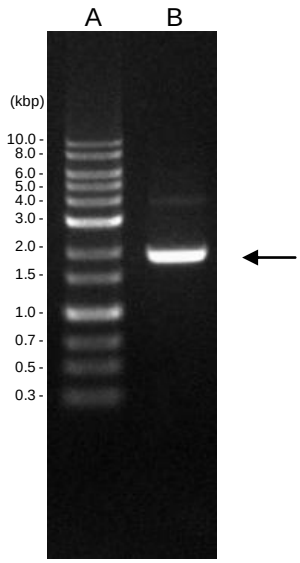
4) Run agarose gel electrophoresis with 2 µl of the PCR product solution to see if the target product is detected as a single band.

(Notes)

*1 Since the volume of required Taq polymerase is small, mix enough amounts of the reagents for at least 2 samples to measure out the correct volume of the polymerase.

*2, 3 To adjust the elongation time and temperature according to the DNA polymerase and the length of the gene, follow the supplier's instruction for the DNA polymerase.

Example of 2nd PCR product



A: Molecular weight marker (DNA)
B: 2nd PCR product
*1% agarose gel

5.4. Concentration of PCR Product

1) Transfer the whole content of 2nd PCR product solution into a 1.5-ml tube.
2) To this 2nd PCR product solution, add 100% ethanol, 2.5 times the volume of the solution, and 3 M Sodium acetate, one tenth of the volume (*1).
3) Mix the solution well and incubate for 10 minutes at -20 °C.
4) Centrifuge the mixture at 15,000 rpm for 15 minutes at 4 ˚C.
5) Remove the supernatant and add 300 µl of 70% ethanol to it.
6) Centrifuge the mixture at 15,000 rpm for 5 minutes at 4 ˚C.
7) Remove the supernatant as much as possible and dry it for 15 minutes (*2).
8) Add 1/10 volume (of the 2nd PCR product solution when transferred into a 1.5-ml tube in the first step) of nuclease-free water. Let the tube stand for 10 minutes to loosen the DNA pellet, and then resuspend the pellet gently by pipetting (*3).
9) Run agarose gel electrophoresis with 0.1 µl of the solution to confirm the presence of the concentrated PCR product. Two microliter of the concentrated PCR product is required for the transcription reaction.

(Notes)

*1 Do not dilute the 2nd PCR product solution before adding ethanol and sodium acetate.
*2 The DNA pellet should not be too dry to resuspend.
*3 It may be possible to increase the protein synthesis yield by increasing the concentration of PCR product.

5.5. Transcription of PCR DNA into mRNA

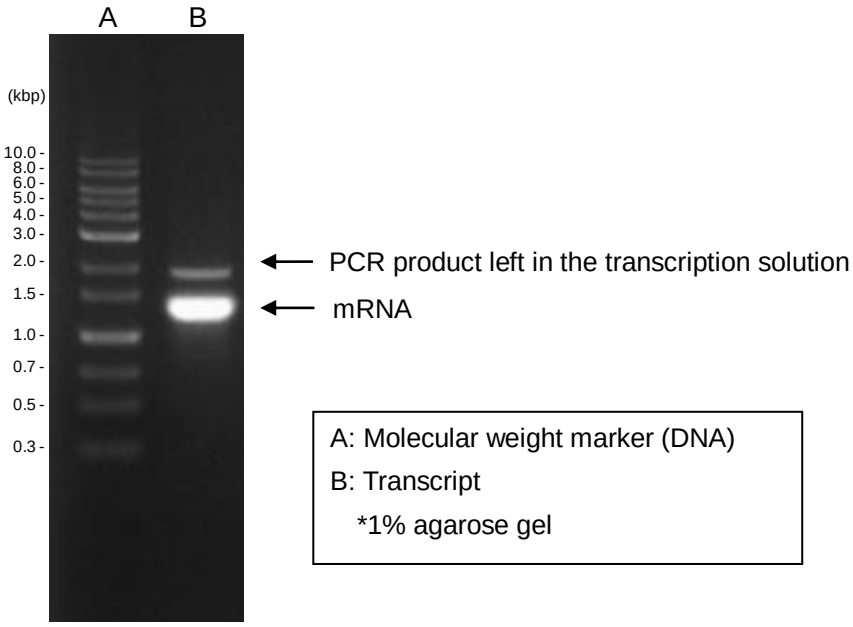
It is recommended to use pEU-E01-DHFR to express DHFR protein as positive control.

1) Remove from storage (-80 °C) required number of Transcription Premix LM tubes (*1). Keep the remaining tubes in storage at -80 °C.
2) Thaw the Transcription Premix LM on ice. After thawing, spin down the tube for a short time to drop down the reagent staying on the tube wall or on the cap.
3) Add 2 µl of the concentrated PCR product to each tube of the Transcription Premix LM and then mix gently by pipetting (*2).
4) Incubate at 37 °C for **4 hours** in a thermal cycler or an incubator (*3).
5) After the incubation, inspect mRNA quality by the ordinary method of agarose gel electrophoresis (*4).

(Notes)

*1 The set of Transcription Premix LM tubes can be split into individual tubes by bending or cutting. Hold the tubes firmly so that they may not pop when they are separated.
*2 Use 2 µl of pEU-E01-DHFR (1.0 µg/µl) when it is used as positive control.
*3 White precipitate may appear during the incubation. This is magnesium pyrophosphate. Use the whole mixture including precipitate in the next step.
*4 A smear or ladder pattern of less than 500 bases of mRNA indicates unsuccessful PCR reaction or the possible degradation of mRNA probably caused by RNase.

Example of mRNA obtained in high quality:



A: Molecular weight marker (DNA)
B: Transcript
*1% agarose gel

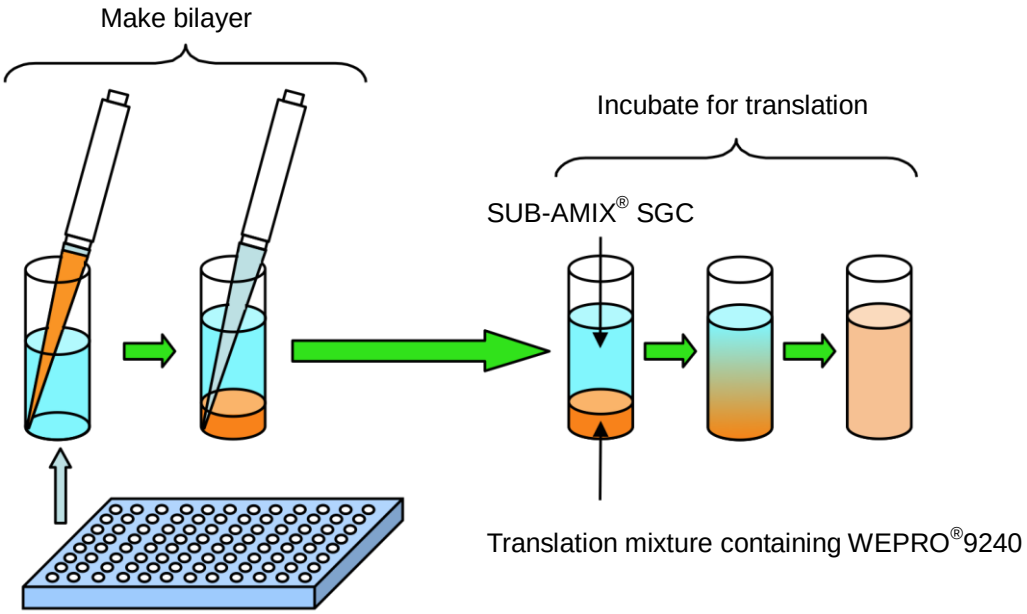
5.6. Translation of Target Protein

1) Remove from storage (-80 °C) required number of WEPRO®9240 tubes and single-break strip wells containing SUB-AMIX® SGC (*1). Keep the remaining tubes and wells in storage at -80 °C
2) Thaw the two reagents on ice. After thawing, spin down the tube containing WEPRO®9240 for a short time to drop down the reagent staying on the tube wall or on the cap. Avoid excessive centrifugation. Resuspend SUB-AMIX® SGC by pipetting gently in the well (*2).
3) Let the mRNA tube(s) cool down to the room temperature. DO NOT forcibly cool it on ice or in the refrigerator. Resuspend the mRNA by pipetting gently (*3).
4) Add 10 µl of resuspended mRNA into WEPRO®9240 and then mix gently by pipetting. Avoid bubble formation.
5) **Carry out bilayer reaction.**
   Carefully transfer the whole mixture (20 µl) of WEPRO®9240 and mRNA to the bottom of the single-break strip well containing SUB-AMIX® SGC (206 µl) to form bilayer with WEPRO® mixture in the lower layer and SUB-AMIX® SGC in the upper layer as illustrated on next page. DO NOT mix the reagents in the well by pipetting or any other means. (Important !!)
6) Seal the well with aluminum seal included in the kit to avoid evaporation (*4).
7) Incubate at 15 °C for **24 hours**.
8) After translation, mix the bilayer reaction gently by pipetting.

(Notes)

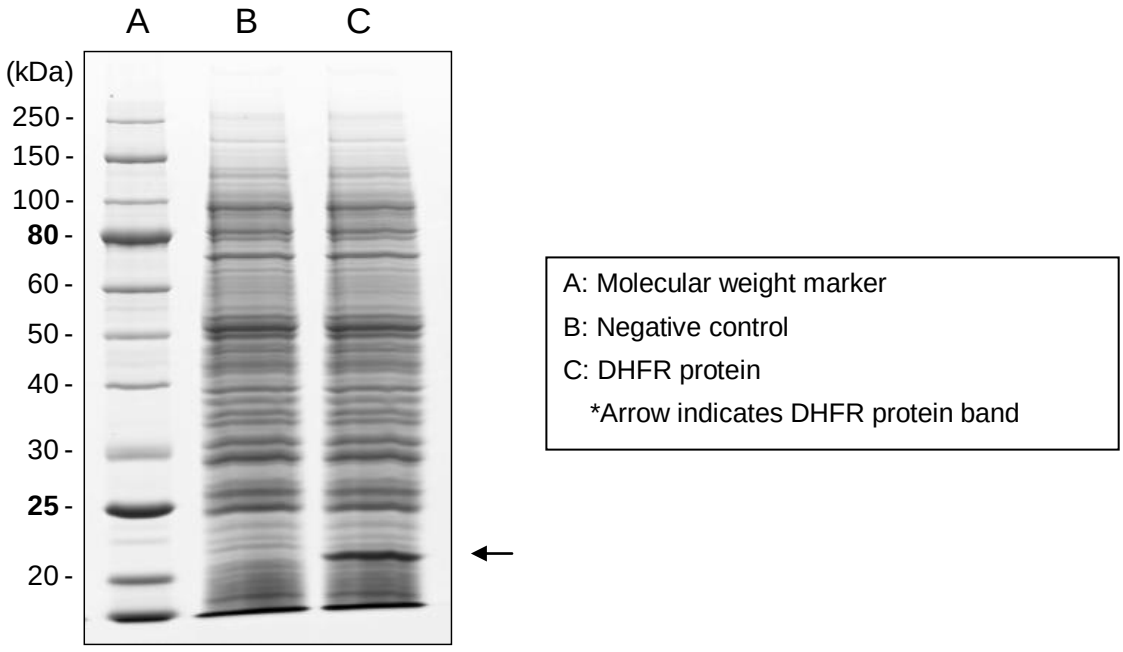
*1 The sets of WEPRO®9240 tubes and SUB-AMIX® SGC wells can be split into individual tubes and wells by bending or cutting. Hold the tubes or wells firmly so that they may not pop when they are separated.
*2 Take special care to keep SUB-AMIX® SGC wells upright, since they easily tip over.
*3 If there is white precipitate in mRNA tube, resuspend the mRNA together with the precipitate by pipetting gently.
*4 Cut aluminum seal in an appropriate size to cover the wells. Save the remaining seal.

Bilayer reaction system



6. Detection of Protein Expression

Run SDS-PAGE followed by CBB staining to identify the expressed protein. SDS-PAGE requires high resolution and an appropriate gel concentration to distinguish the expressed protein from background proteins originating from wheat germ. Load 3 µl of sample for SDS-PAGE. If the volume is too high or too low to identify the protein, change the volume to obtain a clear result. DHFR inserted in pEU-E01 vector, which is used as positive control, is expressed as approx. 20 kDa protein. Typical CBB-stained gel data is shown below.



7. Appendix

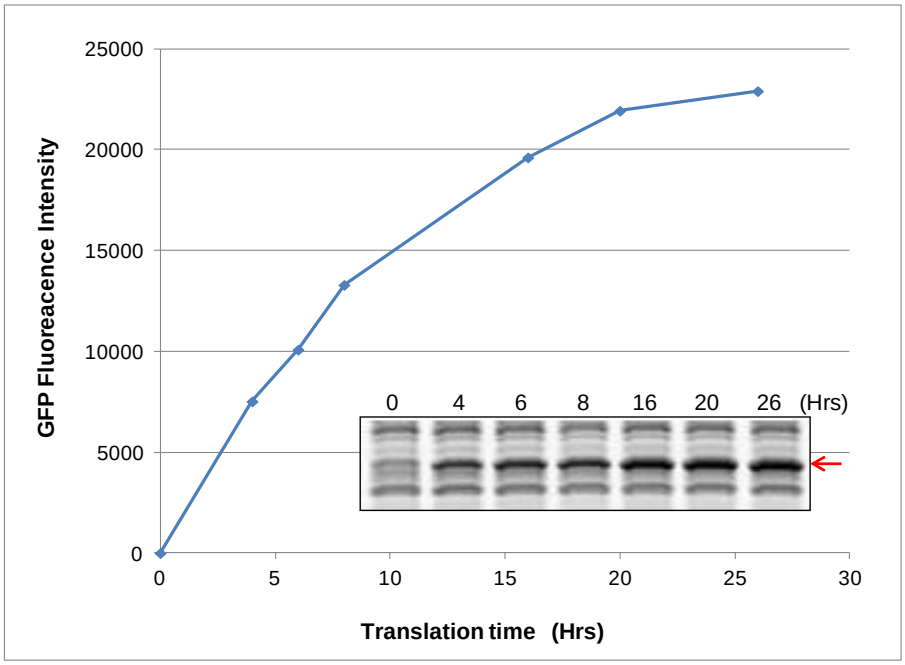
7.1. Example of Plasmid DNA-Based Protein Synthesis

Green fluorescent protein (GFP) was synthesized using plasmid DNA, and the synthesis was confirmed and quantified by fluorescence intensity and SDS-PAGE.

Experimental Condition

Transcription: 1 hour at 37℃

Translation: 15℃



8. Others

8.1. Label License Policy

By opening the cap of any of the reagents listed in the above Section 3.2, the buyer of the Premium PLUS Expression Kit is agreeing to be bound by the terms of the following Label License Policy.

<< Label License Policy>>
ENDEXT® technology and products are covered by US Patent Nos. 6905843, 6869774 and 7919597, and other pending or equivalent patents

The purchase of the products conveys to the buyer the non-transferable right to use the purchased products and components of the products in research conducted by the buyer. The buyer cannot sell or otherwise transfer (a) the products (b) their components (c) materials made using the products or their components to a third party or otherwise use the products or their components or materials made using the products or their components for commercial purposes. The buyer may transfer information or materials made through the use of the products to a scientific collaborator, provided that such transfer is not for any commercial purposes, and that such collaborator agrees (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for commercial purposes.

For information on purchasing a license to products for purposes other than research, contact Intellectual Property Department of CellFree Sciences Co., Ltd. located at the address shown on page 28.

8.2. Trademarks

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8.3. Others

All specifications are subject to change without prior notice.

9. Contact Us

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