



ENDEXT[®] Technology

Wheat Germ Expression Premium Kit

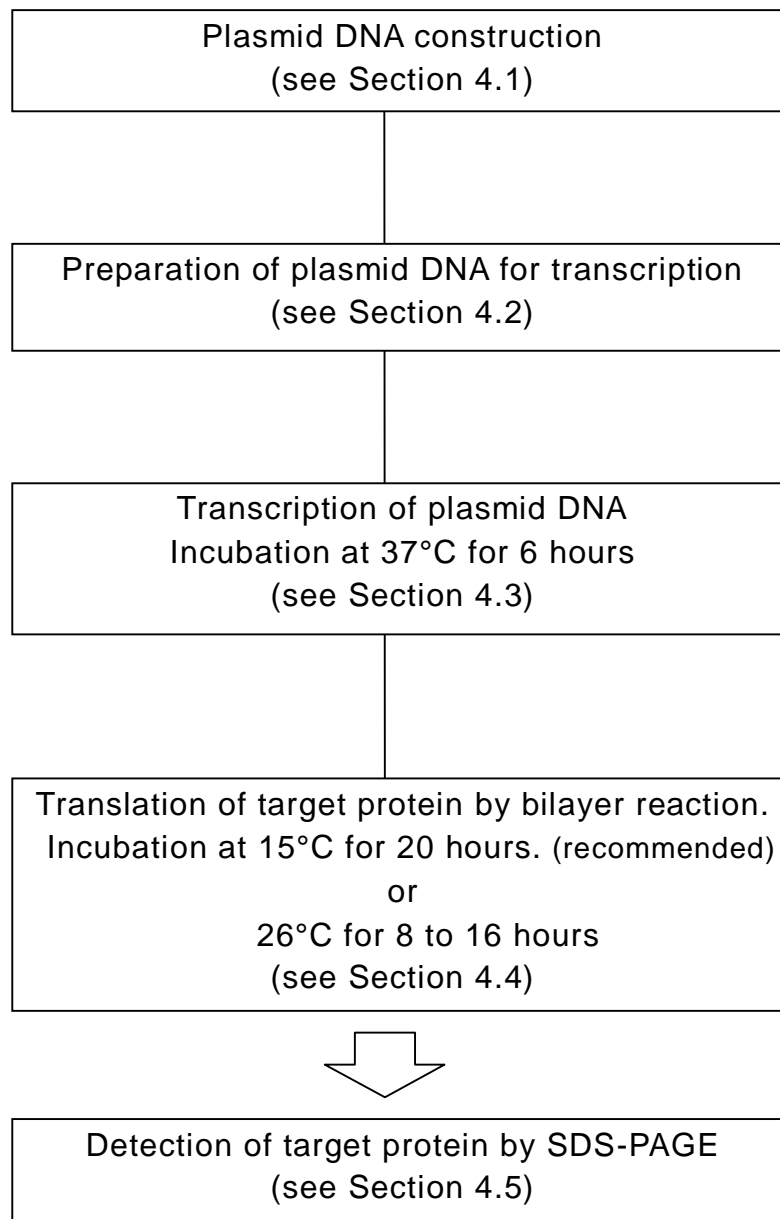
Ver 1.7

CellFree Sciences Co., Ltd.

1. Purpose

“Wheat Germ Expression Premium Kit” is a starter kit to ascertain if the Wheat Germ Cell-Free System is a candidate expression system for producing of your targeted proteins.

2. Protocol Overview



3. Materials

3.1 Storage of Wheat Germ Expression Premium Kit

Store all reagents at -80°C .

3.2 Contents of Wheat Germ Expression Premium Kit

The kit contains 8 reactions for protein expression:

Item	Tube type & color	Quantity	Volume	Description
pEU-E01-MCS	0.2 ml tube, <u>Red</u>	1	5.0 μl (1.0 $\mu\text{g}/\mu\text{l}$)	Expression vector for subcloning your gene of interest. See section 4.1 in detail.
pEU-E01-DHFR	0.2 ml tube, <u>Green</u>	1	5.0 μl (1.0 $\mu\text{g}/\mu\text{l}$)	Expression vector encoding dihydrofolate reductase (DHFR) gene derived from E. coli. It works as positive control for protein expression.
Transcription buffer*	0.2 ml tube, <u>Blue</u>	8	18 μl	Premixed transcription buffer
WEPRO [®] 3240*	0.2 ml tube, <u>Yellow</u>	8	10 μl	Premixed wheat germ extract
SUB-AMIX ^{®*}	Single-break strip well, <u>Clear</u>	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.

* Use the entire content of the tube in 1 reaction.

3.3 Materials to Be Prepared by the User

The following reagents are necessary for preparing plasmid DNA for transcription (see section 4.2):

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.

4. Protocols

4.1 Plasmid DNA Construction

- 1) Insert your cDNA into the multiple cloning site (MCS) of the vector "pEU-E01-MCS" with restriction enzymes properly selected according to the MCS information shown below (*1, *2). Protein is translated from the first start codon "ATG" to stop codon in your cDNA inserted in the MCS. Please note that the pEU-E01-MCS contains SP6 promoter, E01 translational enhancer, and ampicillin resistance gene as illustrated below.
- 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, the one from Qiagen.

(Multiple cloning site information)

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      EcoRV   SpeI   XhoI   SacI   KpnI                               NotI
74-GATATCACTAGTTCTCGAGCTCGGTACCTGTCCGGTCCGCGACGTACGCGGGCGGCCGCC
      CTATAGTGATCAAGAGCTCGAGCCATGGACAGGCCAGCGCTGCATGCGCCCGCCGGCGGG
      BamHI   SmaI   SalI   NcoI
ATAAATTGGATCCATATATAGGGCCCGGGTTATAATTACCTCAGGTCGACGTCCCATGG-193
TATTTAACCTAGGTATATATCCCGGGCCCAATATTAATGGAGTCCAGCTGCAGGGTACC
  
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pEU-E01-MCS sequence

SP6 promoter:-17~ 1

Translational enhancer(E01): 16~ 72

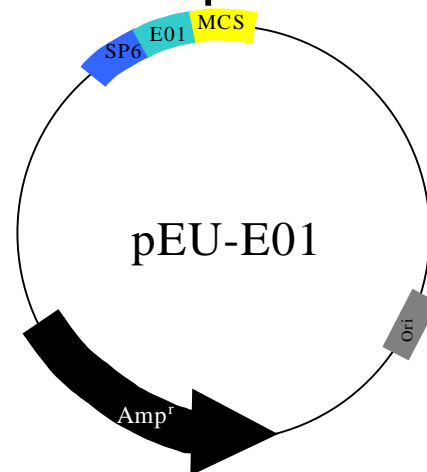
Multiple Cloning Site:74~193

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



(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 restriction enzyme site, use *Sal* I as well in combination with *Xho* I site.

4.2 Preparation of Plasmid DNA for Transcription

A highly purified plasmid DNA is required for the transcription and subsequent translation. It is therefore mandatory to further purify the plasmid DNA that has been extracted from *E. coli* and purified with a commercially available kit. This additional purification is accomplished by extraction first with phenol/chloroform and then with chloroform, and by ethanol precipitation 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 (*2).

(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.
- *2 Concentration of plasmid DNA should be within:
1.0 µg/µl +/- 0.05 µg/µl.

4.3 Transcription of Plasmid DNA into mRNA

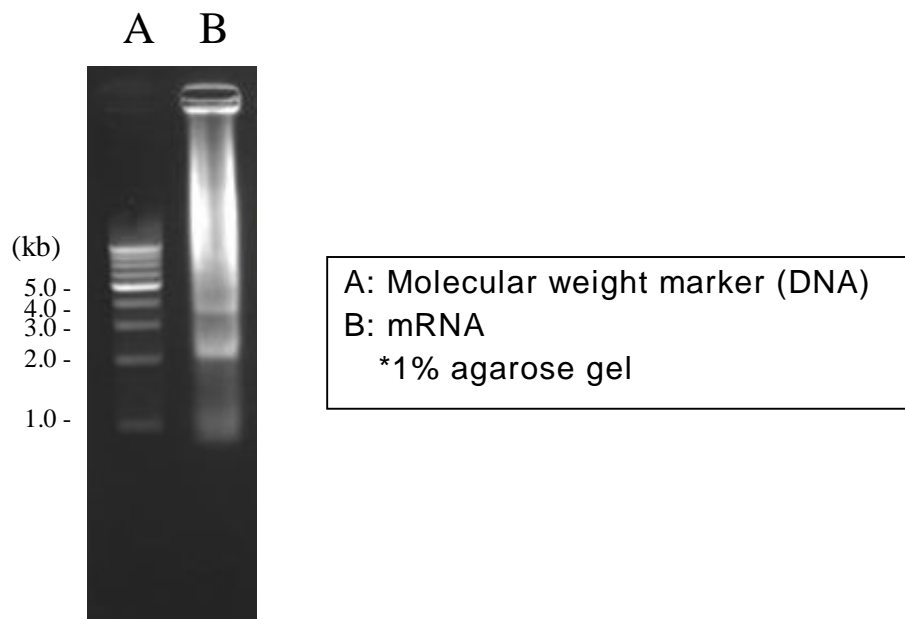
It is recommended to use pEU-E01-DHFR as positive control and express DHFR protein.

- 1) Remove from storage (-80°C) a necessary number of transcription buffer tubes(*1). Keep the remaining tubes in storage at -80°C.
- 2) Thaw the transcription buffer on ice.
- 3) Add 2 µl of the high purity plasmid DNA (1.0 µg/µl) to each tube of the transcription buffer and then mix gently by pipetting.
- 4) Incubate at 37°C for 6 hours in a thermal cycler or an incubator (*2).
- 5) After the incubation, inspect the mRNA quality by the ordinary method of agarose gel electrophoresis (*3).

(Notes)

- *1 The set of transcription buffer tubes can be separated into individual tubes by bending or cutting.
- *2 White pellet that appears during the incubation is magnesium pyrophosphate.
- *3 A smear or ladder pattern, especially that of mRNA of a small molecular weight (50-500 bases), 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:



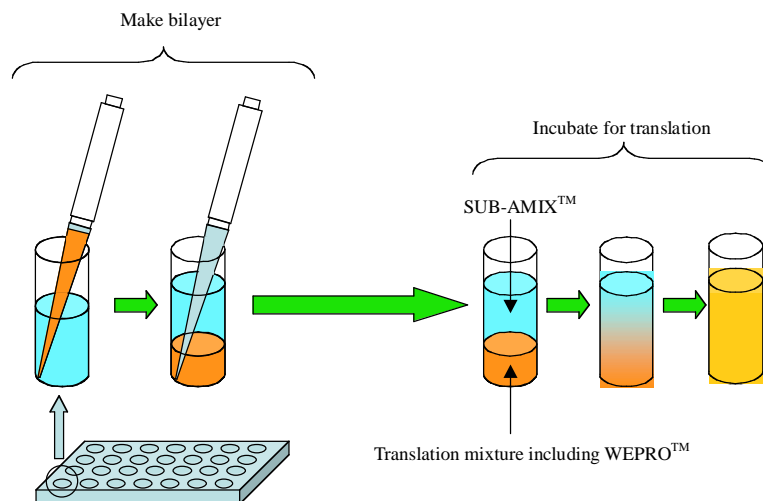
4.4 Translation of Target Protein

- 1) Remove from storage (-80°C) a necessary number of WEPRO[®] 3240 tubes and single-break strip wells containing SUB-AMIX[®] (*1).
Keep the remaining tubes and wells in storage at -80°C
- 2) Thaw the two reagents on ice.
- 3) Cool the mRNA tube(s) down to the room temperature and resuspend white pellet(s) by pipetting gently.
- 4) Resuspend SUB-AMIX[®] by pipetting gently in the well after thawing.
- 5) Add 10 µl of resuspended mRNA into WEPRO[®] 3240 and then mix gently by pipetting. Avoid bubble formation.
- 6) **Carry out bi-layer reaction.**
Carefully transfer the whole mixture (20µl) of WEPRO[®] 3240 and mRNA (206µl) to form bi-layer with WEPRO[®] mixture in the lower layer and SUB-AMIX[®] in the upper layer as illustrated below. (Important !!)
- 7) Seal the well with aluminum seal attached on this kit to avoid evaporation (*2).
- 8) Incubate the mixture at 15°C for 20 hours (recommended) or 26°C for 8 to 16 hours. Higher activity with proper folding would be expected for lower temperature.

(Notes)

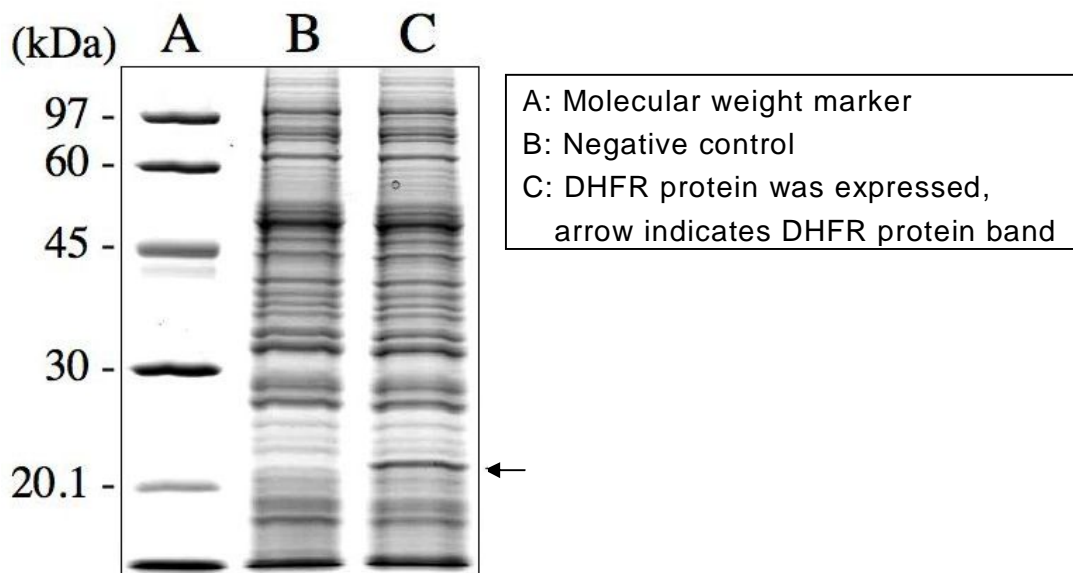
- *1 The sets of WEPRO[®] 3240 tubes and SUB-AMIX[®] wells can be separated into individual tubes and wells by bending or cutting.
- *2 Cut aluminum seal in an appropriate size to cover the wells. Save the remaining seal.

Bi-layer reaction system



4.5 Detection of the Expressed Protein

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 μ l 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 protein used as positive control is expressed as approx. 20 kDa protein. Typical CBB-stained gel data is shown below. The yield of DHFR protein is expected to be approx. 15 μ g/well.





5. Label License Policy

By opening the cap of any of the reagents listed in the above Section 3.2, the buyer of the Wheat Germ Expression Premium 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 6869774, US Patent 6905843 and other pending patents or equivalent patents in the US and other foreign countries regarding bi-layer reaction system (see section 3.2), WEPRO[®], and vectors.

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.

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7. Others

All specifications are subject to change without prior notice.



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