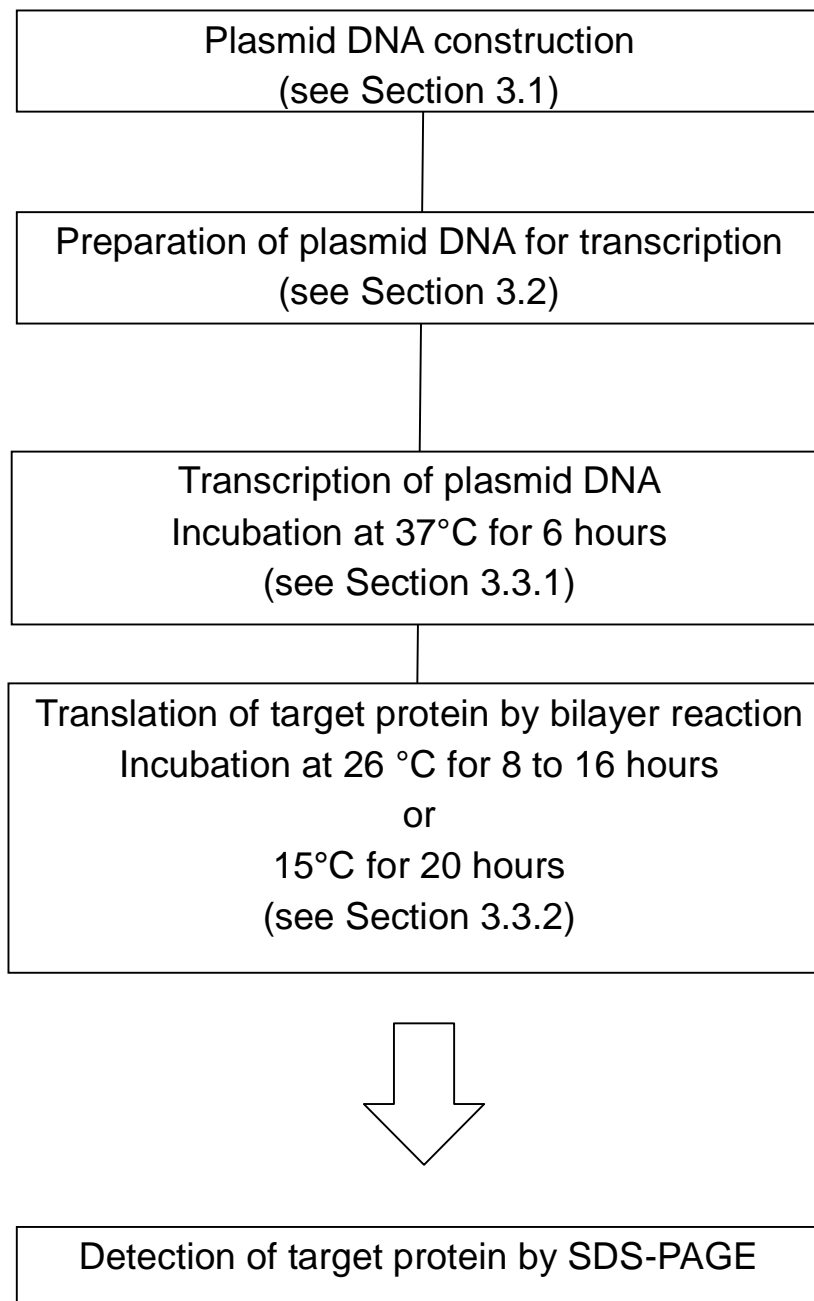




ENDEXT[®] Technology

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

1 Protocol Overview



2 Materials

2.1 Contents of the Kit

| Item | Quantity | Concentration | Volume |
|---------------------------|----------|---------------|--------|
| WEPRO [®] 1240G | 2 | 240 OD/ml | 1 ml |
| SUB-AMIX [®] S-1 | 1 | 40x | 1.1 ml |
| SUB-AMIX [®] S-2 | 1 | 40x | 1.1 ml |
| SUB-AMIX [®] S-3 | 1 | 40x | 1.1 ml |
| SUB-AMIX [®] S-4 | 1 | 40x | 1.1 ml |
| 5x Transcription buffer | 1 | 5x | 0.4 ml |
| NTPs mix | 1 | 25 mM | 0.2 ml |
| SP6 RNA polymerase | 1 | 80 U/μl | 30 μl |
| RNase inhibitor | 1 | 80 U/μl | 30 μl |
| Creatine kinase | 1 | 20 mg/ml | 20 μl |

2.2 Instruction for the Use of Reagents

| Item | Description | Storage |
|---|---|----------------------|
| WEPRO [®] 1240G | WEPRO [®] 1240G (wheat germ extract) is sensitive to temperature and vibration. Immediately after thawing under running water, place the reagent on ice. Upon thawing for the first time, separate the portion that is not used immediately, and to avoid multiple freeze-thawing exposures, subdivide it into appropriate volumes in separate containers. Store them at -80°C for later use. Do not subject it to 3 or more freeze-thawing cycles. After the third freeze-thawing cycle, it is possible that protein synthesis activity decreases, the degree of which depends on the way of handling. Use of liquid nitrogen is recommended for re-freezing. When using the reagent, mix it gently by pipetting several times. Avoid bubbling. | -80°C |
| SUB-AMIX [®] (S-1, S-2, S-3, S-4) | This product consists of a set of 4 reagents (S-1, S-2, S-3, S-4) at 40x concentration. Store all 4 reagents at -20°C or below. No change in their reaction efficiency has been observed after 10 freeze-thawing cycles. To prepare 40 ml of 1x SUB-AMIX [®] , add 1 ml each of S-1 through S-4 to 36 ml of nuclease-free water while agitating the latter. If 4 reagents are mixed first, precipitation may occur. Once it happens, it takes time to dissolve the precipitates. To avoid multiple freeze-thawing exposures, subdivide 1x SUB-AMIX [®] into appropriate volumes in separate containers and store them at -80°C. Do not subject 1x SUB-AMIX [®] to multiple freeze-thawing cycles. Decrease in the reaction efficiency may occur, the degree of which depends on the way of handling. | -20°C or -80°C |

(Continued)

(continued from Section 2.2)

| Item | Description | Storage |
|-------------------------|---|---------|
| 5x Transcription buffer | After thawing, subdivide 5x Transcription buffer into appropriate volumes convenient for your use. It has been confirmed that the freeze-thawing cycle for this product can be repeated up to 10 times. | -20°C |
| 25mM NTP mix | ATP, GTP, CTP, and UTP in this NTP mix have all been prepared at a concentration of 25 mM. After thawing, subdivide the NTP mix into appropriate volumes convenient for your use. It has been confirmed that the freeze-thawing cycle for this product can be repeated up to 10 times. | -20°C |
| SP6 RNA polymerase | 50% glycerol is included. | -20°C |
| RNase inhibitor | 50% glycerol is included. | -20°C |
| Creatine kinase (*1) | Creatine kinase is required for protein expression. Avoid multiple freeze-thawing exposures; otherwise, the activity of creatine kinase may decrease. The concentration of the starting creatine kinase solution is 20 mg/ml. Use it as it is for preparing the translation mixture for large scale translation. For small scale translation (see Section 3.3.2), dilute it with nuclease-free water to 1 mg/ml. In both cases, the final concentration of creatine kinase in translation mixture should be 40 ng/μl. | -80°C |

Notes *1:

Creatine kinase can be purchased from Roche, Catalog No. 127566. Dissolve it with nuclease-free water to make a 20 mg/ml solution. For convenience, subdivide the solution into smaller volumes and store them at -80°C. Avoid multiple freeze-thawing cycles; otherwise, the activity of creatine kinase may decrease.

2.3 Materials to Be Prepared by the User

These reagents are necessary to prepare plasmid DNA for transcription (see Section 3.2).

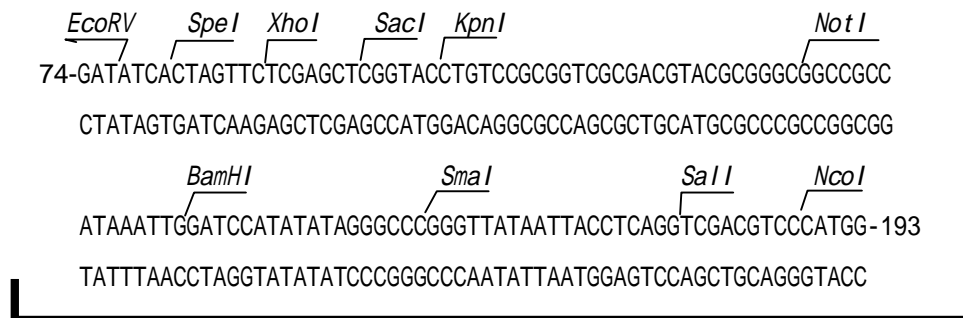
| Reagents | Description |
|---------------------|---|
| Nuclease free water | DNase, RNase free |
| Phenol/Chloroform | phenol:chloroform:isoamyl alcohol = 25:24:1 in volume, pH 7.9 |
| Chloroform | > 99% |
| Ethanol | 2 grades: > 99% and 70% |
| Sodium acetate | 3 M, pH 5.2 |
| TE buffer | 10 mM Tris, 1 mM EDTA, pH 8.0. Sterilized. It is highly recommended to use DNase-RNase free water when you prepare TE buffer. |

3 Protocols

3.1 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 given 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)



pEU-E01-MCS sequence

SP6 promoter:-17~ 1

Translational enhancer(E01): 16~ 72

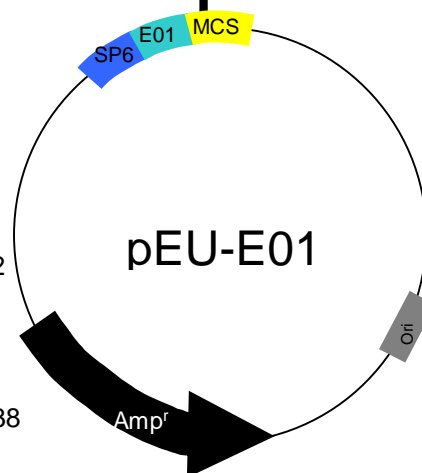
Multiple Cloning Site:74~193

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



Note*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.

Note*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.

3.2 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. As described below, this additional purification is accomplished by extraction first with phenol/chloroform and then with chloroform, and by ethanol precipitation:

- 1) To the purified plasmid DNA solution (see Section 3.1), add an equal volume of phenol/chloroform (phenol:chloroform:isoamyl alcohol = 25:24:1, pH 7.9) 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 of the solution, and 3 M 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 $\mu\text{g}/\mu\text{l}$ by adding an appropriate volume of TE buffer (*2).

Notes*1: Purity of plasmid DNA should be such that the A_{260}/A_{280} ratio ranges between 1.70 and 0.85. Ratios outside the range indicate that the plasmid DNA has been contaminated. In that case, repeat Section 3.2 from the beginning.

Notes*2: Concentration of plasmid DNA should be within 1.0 $\mu\text{g}/\mu\text{l}$ \pm 0.05 $\mu\text{g}/\mu\text{l}$.

3.3 Small Scale Protein Expression

The following description is for small scale protein expression using a standard 96 multi-well plate. It is for a translation reaction volume of 226.8 μl per well.

3.3.1 Transcription

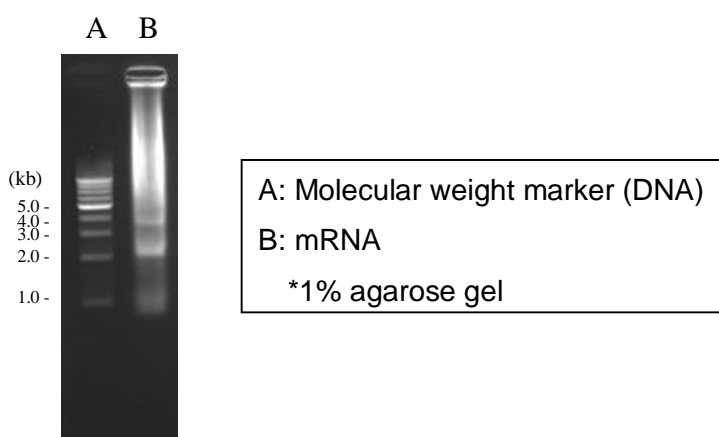
- 1) Thaw 5x transcription buffer and 25 mM NTP mix on ice. Place and keep all reagents on ice during handling. Prepare 20 μl of transcription mixture on ice according to the mixing formula shown below and mix gently by pipetting.

| Reagents | Working vol. | Final conc. |
|--|--------------------|-----------------------|
| Nuclease free water | 11.5 μl | - |
| 5x transcription buffer | 4 μl | 1x |
| 25 mM NTP mix | 2 μl | 2.5 mM |
| RNase inhibitor (80 U/ μl) | 0.25 μl | 1 U/ μl |
| SP6 RNA Polymerase (80 U/ μl) | 0.25 μl | 1 U/ μl |
| Plasmid (circular DNA, 1 $\mu\text{g}/\mu\text{l}$) | 2 μl | 100 ng/ μl |
| Total | 20 μl | |

- 2) Incubate at 37°C for 6 hours in a thermal cycler or incubator (*1).
- 3) After incubation, confirm the mRNA quality by the ordinary method of agarose gel electrophoresis (*2).

Note*1: White pellet that appears during incubation is magnesium pyrophosphate.

Note*2: 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 3.2. An example of mRNA produced in high quality is shown below:



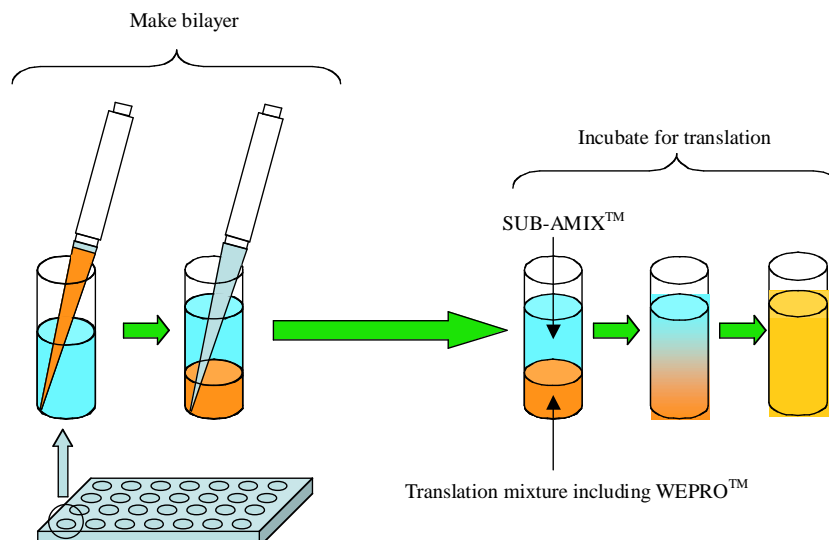
3.3.2 Translation

- 1) Cool the mRNA tube down to the room temperature. Thaw WEPRO[®]1240G under running water, and immediately after thawing, place it on ice. Thaw creatine kinase on ice. Before adding mRNA into the translation mixture, resuspend the white pellet (magnesium pyrophosphate) in the tube by pipetting gently. Prepare 20.8 μ l of translation mixture on ice according to the mixing formula shown below and mix gently by pipetting. Avoid bubbling.

| Reagents | Working vol. | Final conc. |
|--------------------------------------|--------------|----------------|
| mRNA | 10 μ l | 1/2 vol. |
| Creatine kinase (1 mg/ml) | 0.8 μ l | 40 ng/ μ l |
| WEPRO [®] 1240G (240 OD/ml) | 10 μ l | 120 OD/ml |
| Total | 20.8 μ l | |

- 2) Thaw 1x SUB-AMIX[®] on ice and mix gently by pipetting. Pipet out 206 μ l of 1x SUB-AMIX[®] and add it into a well of a 96 multi-well, flat bottom plate.
- 3) **Carry out bilayer reaction:** Carefully transfer the translation mixture into the bottom of the well containing SUB-AMIX[®] to form bilayer with the translation mixture in the lower layer and SUB-AMIX[®] in the upper layer as illustrated below. (Important !!)
- 4) Seal the well with Parafilm to avoid evaporation.
- 5) Incubate at 26°C for 8 to 16 hours. Higher activity, solubility, and productivity are expected at lower temperatures (ex. 15°C for 20 hours).

Bilayer reaction system



3.4 Large Scale Protein Expression

The following description is for large scale protein expression using a standard 6 multi-well plate. It is for a translation reaction volume of 6ml per well.

3.4.1 Transcription

- 1) Thaw 5x transcription buffer and 25 mM NTP mix on ice. Place and keep all reagents on ice during handling. Prepare 250 μ l of transcription mixture on ice according to the mixing formula shown below and mix gently by pipetting.

| Reagents | Working vol. | Final conc. |
|--|----------------|-----------------|
| Nuclease free water | 143.75 μ l | - |
| 5x transcription buffer | 50 μ l | 1x |
| 25 mM NTP mix | 25 μ l | 2.5 mM |
| RNase inhibitor (80 U/ μ l) | 3.125 μ l | 1 U/ μ l |
| SP6 RNA Polymerase (80 U/ μ l) | 3.125 μ l | 1 U/ μ l |
| Plasmid (circular DNA, 1 μ g/ μ l) | 25 μ l | 100 ng/ μ l |
| Total | 250 μ l | |

- 2) Incubate at 37°C for 6 hours in a thermal cycler or incubator (*1).
- 3) After incubation, confirm the mRNA quality by the ordinary method of agarose gel electrophoresis (*2).

Note*1: White pellet that appears during incubation is magnesium pyrophosphate.

Note*2: 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 3.2.

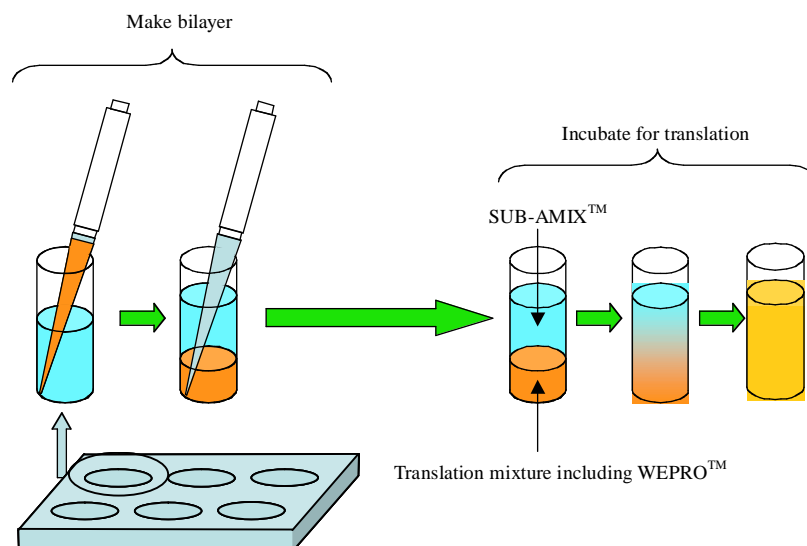
3.4.2 Translation

- 1) Cool the mRNA tube down to the room temperature. Thaw WEPRO[®]1240G under running water, and immediately after thawing, place it on ice. Thaw creatine kinase on ice. Before adding mRNA into the translation mixture, resuspend the white pellet (magnesium pyrophosphate) in the tube by pipetting gently. Prepare 501 μ l of translation mixture on ice according to the mixing formula shown below and mix gently by pipetting. Avoid bubbling.

| Reagents | Working vol. | Final conc. |
|--------------------------------------|--------------|----------------|
| mRNA | 250 μ l | 1/2 vol. |
| Creatine kinase (20 mg/ml) | 1 μ l | 40 ng/ μ l |
| WEPRO [®] 1240G (240 OD/ml) | 250 μ l | 120 OD/ml |
| Total | 501 μ l | |

- 2) Thaw 1x SUB-AMIX[®] on ice and mix gently by pipetting. Pipet out 5.5 ml of 1x SUB-AMIX[®] and add it into a well of a 6 multi-well, flat bottom plate.
- 3) **Carry out bilayer reaction:** Carefully transfer the translation mixture into the bottom of the well containing SUB-AMIX[®] to form bilayer with the translation mixture in the lower layer and SUB-AMIX[®] in the upper layer as illustrated below. (Important !!)
- 4) Seal the well with Parafilm to avoid evaporation.
- 5) Incubate at 26°C for 8 to 16 hours. Higher activity, solubility, and productivity are expected at lower temperatures (ex. 15°C for 20 hours).

Bilayer reaction system



4 Label License Policy

By opening the cap of any of the reagents listed in the above Section 3, the buyer of the Protein 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 6869774, US Patent 6905843 and other pending patents or equivalent patents in US and other foreign countries regarding bilayer 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, and (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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6 Others

All specifications are subject to change without prior notice.

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