Remove the kit from the dry ice box promptly after its delivery.
Do not store it in a freezer where dry ice is also kept.
Excessively long exposure to dry ice (carbon dioxide) may damage the kit.

ENDEXT® Technology

Premium ONE Expression Kit

Wheat germ cell-free protein synthesis manual

(Catalog No. CFS-EDX-ONE)

CellFree Sciences Co., Ltd.

Table of contents

1. About this kit

This kit is for convenient confirmation of whether the target protein can be synthesized by the wheat germ cell-free protein synthesis system.

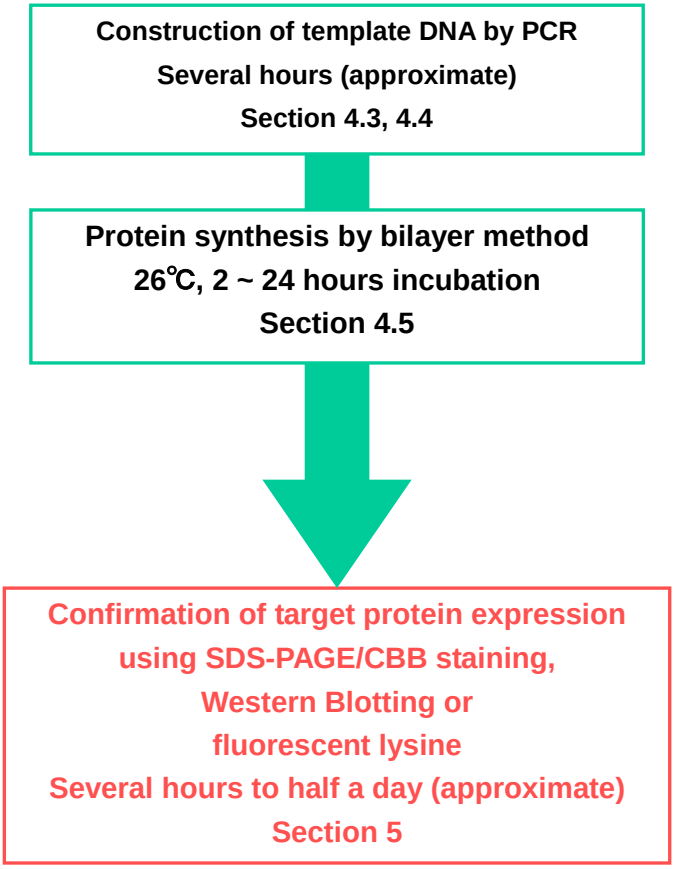
You can choose synthesis confirmation method from the following.

- Confirmation of protein synthesis by Western Blotting (protein synthesis reaction time of minimum 2 hours)
- Confirmation of protein synthesis using FluoroTect™ $Green_{Lys}$ tRNA (tRNA with fluorescently labeled lysine base added (fluorescent lysine)) (protein synthesis reaction time of the shortest 2 hours)
- Confirmation of protein synthesis by SDS-PAGE/CBB staining (protein synthesis reaction time of 12 to 24 hours)

In this kit, protein synthesis is performed using the PCR product prepared by the split primer PCR method (*) as the template DNA.
If you need a lot of protein, please use our other kit ("Premium PLUS Expression Kit", "WEPRO 7240 Expression Kit" etc.). Please see our website for details.
URL: http://www.cfsciences.com

(*) In addition to the primer attached to the kit, you need to have two kinds of custom primers. See section 4.3 for details.

2. Protocol overview



3. Kit and required reagents

3.1. Storage conditions of the kit

All reagents should be stored at – 80°C.

3.2. Kit contents

This kit contains reagents for 24 protein synthesis reactions.

Reagent name	Quantity	Volume	Concentration	Container color	Container type
WEPRO TTmix	1	70 µl	—	Transparent	0.5 ml Tube
SUB-AMIX TT	1	1.3 ml	—	Transparent	2 ml Tube
SPU Primer	1	100 µl	1 µM	Orange	0.2 ml PCR Tube
deSP6E01 Primer	1	100 µl	10 nM	Purple	0.2 ml PCR Tube
pEU-E01-DYKDDDDK-DHFR	1	10 µl	15 ng/µl	Transparent	0.2 ml PCR Tube
FluoroTect™ $Green_{Lys}$ tRNA (*)	1	4 µl	—	Black	0.5 ml Tube

(*) Sourced and manufactured by Promega Corp.

Reagent name	Description
WEPRO TTmix	Pre-mixed wheat germ extract for protein synthesis reaction. Avoid unnecessary freeze-thawing of the wheat germ extract! When melting the first time, prepare aliquots depending on your needs, e.g. 10 µl per aliquot. Avoid freeze-thawing the wheat germ extract more than 3 times.
SUB-AMIX TT	Translation reaction buffer
SPU	2nd PCR sense primer
deSP6E01	2nd PCR sense primer
pEU-E01-DYKDDDDK-DHFR	Expression vector for DYKDDDDK-tagged DHFR protein (Positive control)
FluoroTect™ $Green_{Lys}$ tRNA	Fluorescent tRNA that can be used to confirm protein expression (light shielding required)

3.3. Required reagents, consumables, laboratory instruments not included in kit

Reagent name	Description
Gene specific primer (1st PCR sense primer)	Please refer to section 4 for details
Antisense primer (used in 1st PCR and 2nd PCR)	Please refer to section 4 for details
PCR reagents	DNA polymerase, reaction buffer, dNTP required for PCR are not provided. Here, we give examples using PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc. or Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher to conduct PCR experiments.
Ultra-pure water	DNase, RNase free DEPC treated water is not recommended
RNase A (10 μg/μl) stock solution	RNase A is needed when confirming protein synthesis using fluorescently labeled tRNA to reduce background on SDS-PAGE.

Consumables · Laboratory instrument names	Description
0.2ml PCR tube	DNase, RNase free
Thermocycler	Required for PCR reactions
Incubator	For protein expression at 26°C. * Can also be done in a thermocycler for PCR reactions
SDS-PAGE related equipment	Used for confirming the synthesis of the target protein
Devises to conduct Western Blotting experiment	Used for confirming the synthesis of the target protein
Fluorescent gel scanner	Used for detection of target protein after fluorescent lysine incorporation. Please use equipment which can measure at excitation wavelength 502 nm and detection wavelength 510 nm

4. Protein synthesis protocol

4.1. PCR Overview

This PCR protocol adds the SP6 promoter and E01 translation enhancer sequence at the 5' end of the open reading frame. The open reading frame is extended at the 3' end to stabilize the expression template. The split primer PCR method requires 2 steps for preparing the final expression template. Only in the second PCR, the SP6 promoter and E01 translation enhancer sequences are added to the template.

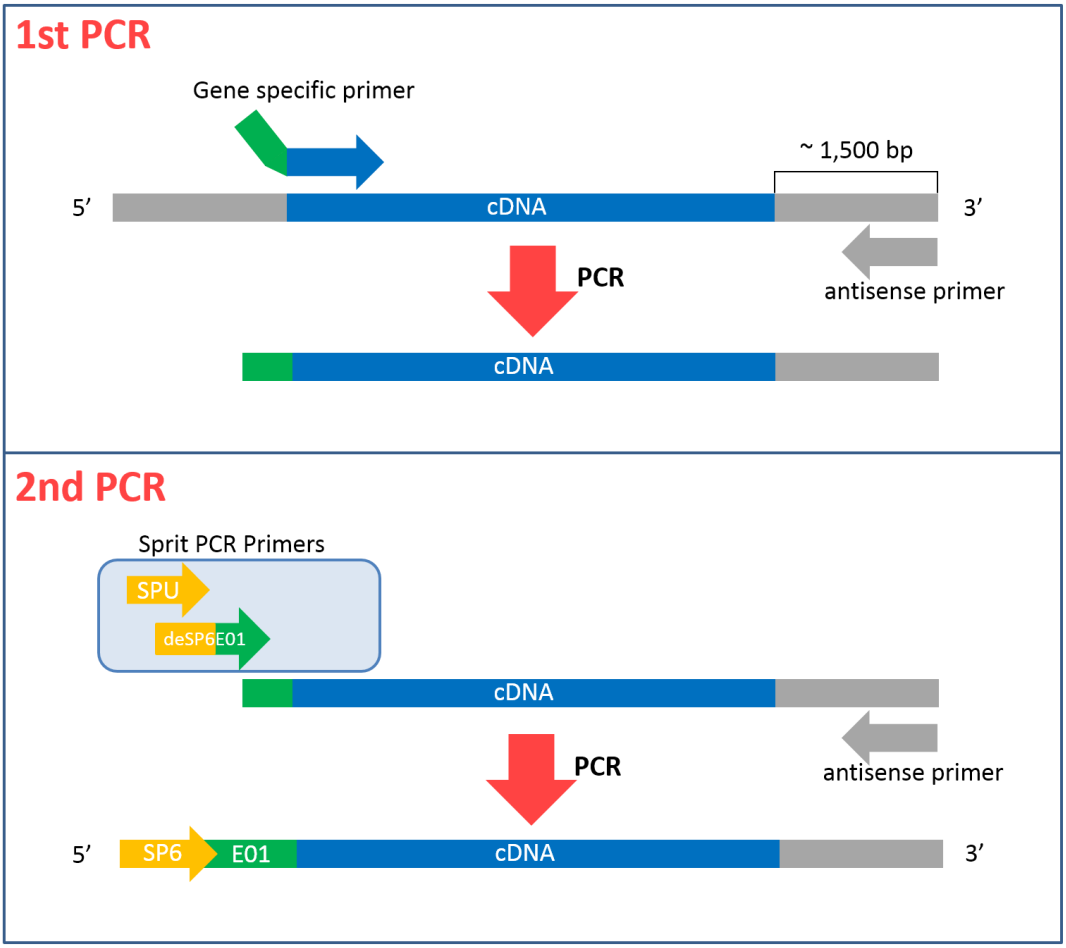


Fig.1. Schematic drawing of 2-step PCR method

(*) If the insertion direction of the gene (cDNA) is opposite, the orientation of each primer is reversed.

4.2. Notes

I. We noticed that target genes inserted into vectors of the pET-24 series or pET-28 series may not yield any PCR product. We further advise to make sure that your vector does not have any SP6 promoter sequence; such sequences

may cause mispriming. In both cases, we recommend subcloning of the inserts into one of the pEU-E01 vectors from CellFree Sciences. Expression vectors based on the pEU-E01 vectors can be directly used in our expression system and no further PCR step is required. Find out more information on our vectors at:

http://www.cfsciences.com/products/vector

II. In this protocol, we use PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc. or Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher as examples for high-fidelity PCR DNA polymerases that have a low error rate while replicating DNA. Even if you wish to use another DNA Polymerase, we strongly recommend the use of a high-fidelity PCR DNA polymerase.

4.3. 1st PCR

4.3.1. Primers design for 1st PCR

Primer	gene specific primer
Length (bp)	About 35 bases
Array	5'- CCACCCACCACCACCAATGNNNNNNNNNNNNNNNN -3'
Description	Green: Part of E01 translation enhancer sequence (Overlapping sequence with primer "deSP6E01" used for 2nd PCR) Red: Start codon Blue: Gene specific sequence of interest (use about 20 bases)

Primer	antisense primer (1st PCR and 2nd PCR)
Length (bp)	About 20 bases
Array	Based on vector sequence into which the target gene was inserted
Description	Please design with a length of about 20 bases in the downstream region about 1.5 kb or more away from the 3' end of the target gene. **This primer is also used for 2nd PCR step.**

4.3.2. 1st PCR protocol

1) Prepare the following reaction mixture using 0.2 ml PCR tube.

≪In the case of PrimeSTAR® GXL DNA Polymerase≫

Reagent name	Volume	Concentration
Ultra-pure water	16 µl	-
Template plasmid DNA (250 pg/µl)	4 µl	25 pg/µl
5x PrimeSTAR GXL Buffer	8 µl	1x
2.5 mM dNTP	3.2 µl	0.2 mM
100 nM gene specific primer	4 µl	10 nM
100 nM antisense primer	4 µl	10 nM
PrimeSTAR GXL DNA Polymerase	0.8 µl	0.025 U/µl
Total	40 µl	

≪*in the case of* ***Platinum™ SuperFi™ DNA Polymerase***≫

Reagent name	Volume	Concentration
Ultra-pure water	10.8 µl	-
Template plasmid DNA (250 pg/µl)	4 µl	25 pg/µl
5x SuperFi™ Buffer	8 µl	1x
5x SuperFi™ GC Enhancer	8 µl	1x
10 mM dNTP mix	0.8 µl	0.2 mM
100 nM gene specific primer	4 µl	10 nM
100 nM antisense primer	4 µl	10 nM
Platinum™ SuperFi™ DNA Polymerase	0.4 µl (*1)	0.02 U/µl
Total	40 µl	

2) Place the tube into the thermocycler and run reaction under the following conditions.

≪*In case of* ***PrimeSTAR® GXL DNA Polymerase***≫

Temp	Time	
98°C	30 sec	
98°C	10 sec	30~35 cycles
55°C (*2)	15 sec	
68°C	1 min/kb (*3)	
68°C	1 min/kb (*3)	
20°C	∞	

≪*In case of* ***Platinum™ SuperFi™ DNA Polymerase***≫

Temp	Time	
98°C	30 sec	
98°C	10 sec	
55°C (*2)	10 sec	30~35 cycles
72°C	15–30 sec/kb (*3)	
72°C	15–30 sec/kb (*3)	
20°C	∞	

3) 2 µl of the PCR product can be loaded onto an agarose gel to confirm that a single band of the desired product size can be detected (*4).

（Remarks)

(*1) The required amount of the DNA polymerase is small. Therefore, it can be helpful to work with a master mix in case you run multiple PCR reactions at the same time.

(*2) Please set the optimal annealing temperature based on the Tm value of the primers.

(*3) Please set an extension time suitable for the expected size of the PCR product.

(*4) Even if amplification of the PCR product cannot be confirmed, proceed to the next step. The target sequence may be amplified during 2nd PCR step.

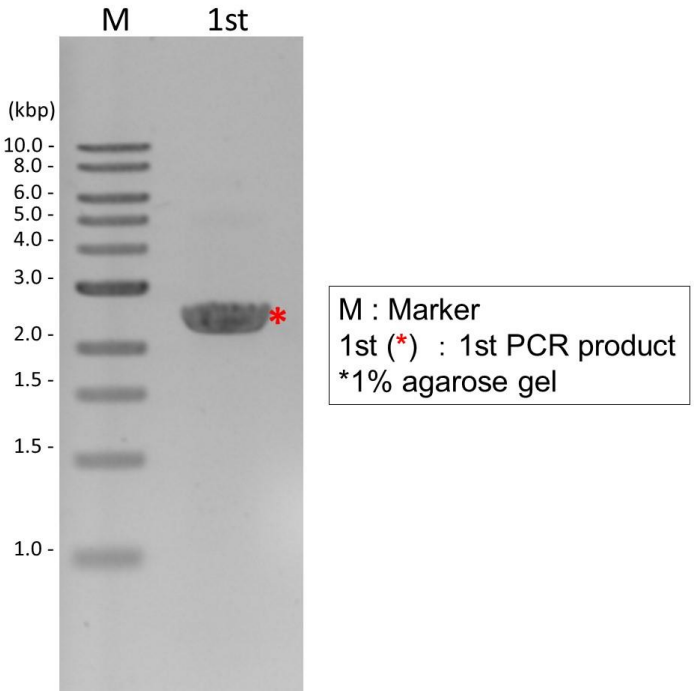


Fig. 2 Agarose gel electrophoresis detecting 1st PCR product

4.4. 2nd PCR

4.4.1. Primers for 2nd PCR

Use the same antisense primer as used already during the 1st PCR step. Refer to Section 4.3.1 for more details.
The sense primers used in the 2nd PCR are included in the kit: SPU and deSP6E01.

Primer	SPU
Length (bp)	21 bases
Array	5'- GCGTAGCATTTAGGTGACACT -3'
Description	Yellow: 5' end of SP6 promoter sequence

Primer	deSP6E01
Length (bp)	100 bases
Array	5'-GGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCACTAACCACCTATCTACATCACCACCCACCACCACCAATG-3'
Description	Yellow: 3' end of SP6 promoter sequence Green: E01 translation promoting sequence (Underline: Overlapping sequence with the 1st PCR product) Red: Start codon

4.4.2. 2nd PCR protocol

1) Prepare the following reaction mixture using 0.2 ml PCR tube.

≪*In case of PrimeSTAR® GXL DNA Polymerase*≫

Reagent name	Volume	Concentration
Ultra-pure water	12 µl	-
1st PCR product	4 µl	1/10 vol.
5x PrimeSTAR GXL Buffer	8 µl	1x
2.5 mM dNTP	3.2 µl	0.2 mM
1 µM SPU	4 µl	100 nM
10 nM deSP6E01	4 µl	1 nM
1 µM antisense primer	4 µl	100 nM
PrimeSTAR GXL DNA Polymerase	0.8 µl	0.025 U/µl
Total	40 µl	

≪*In case of Platinum™ SuperFi™ DNA Polymerase*≫

Reagent name	Volume	Concentration
Ultra-pure water	6.8 µl	-
1st PCR product	4 µl	1/10 vol.
5x SuperFi™ Buffer	8 µl	1x
5x SuperFi™ GC Enhancer	8 µl	1x
10 mM dNTP mix	0.8 µl	0.2 mM
1 µM SPU	4 µl	100 nM
10 nM deSP6E01	4 µl	1 nM
1 µM antisense primer	4 µl	100 nM
Platinum™ SuperFi™ DNA Polymerase	0.4 µl (*1)	0.02 U/µl
Total	40 µl	

2) Place the tube into the thermocycler and run reaction under the following conditions.

≪*In case of PrimeSTAR® GXL DNA Polymerase*≫

Temp	Time	
98°C	30 sec	
98°C	10 sec	5 cycles
55°C	30 sec	
68°C	1 min/kb (*2)	
98°C	10 sec	35 cycles
60°C	40 sec	
68°C	1 min/kb (*2)	
68°C	1 min/kb (*2)	
20°C	∞	

≪*In case of Platinum™ SuperFi™ DNA Polymerase*≫

Temp	Time	
98°C	30 sec	
98°C	10 sec	5 cycles
55°C	10 sec	
72°C	15–30 sec/kb (*2)	
98°C	10 sec	35 cycles
60°C	40 sec	
72°C	15–30 sec/kb (*2)	
72°C	15–30 sec/kb (*2)	
20°C	∞	

3) 2 µl of the PCR product can be loaded onto an agarose gel to confirm that a single band of the desired product size is detected.

(Remarks)

(*1) The required amount of the DNA polymerase is small. Therefore, it can be helpful to work with a master mix in case you run multiple PCR reactions at the same time.

(*2) Please set an extension time suitable for the expected size of the PCR product.

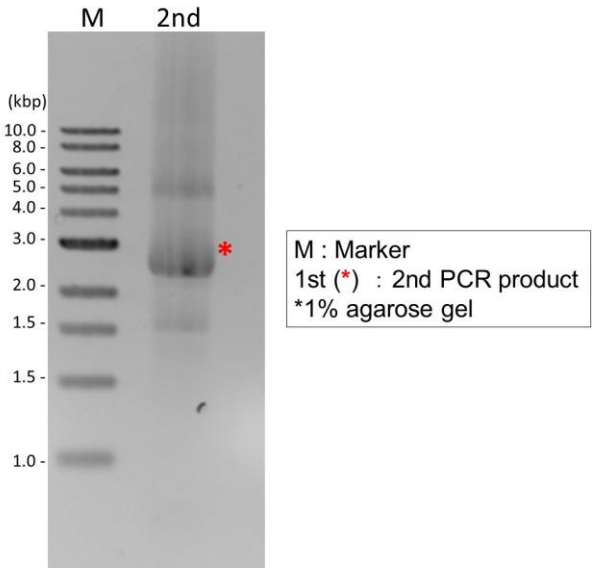


Fig. 3 Agarose gel electrophoresis detecting 2nd PCR product

4.5. Protein expression by bilayer method

" Important point "
*** Keep reagents on ice at all times.**
*** Please use 0.2 ml PCR tube for each reaction.**
*** Mix reagents with care. Do not vortex reagents.**
*** Do not mix the two layers when setting up bilayer reactions! Handle with care after setting up the reaction to avoid any accidental mixing of the two layers.**

4.5.1. Standard expression reaction without added label (Detecting protein by SDS-PAGE/CBB staining or Western Blotting)

1) Thaw the WEPRO TTmix and SUB-AMIX TT on ice. After thawing, lightly spin the tubes collect all reagents at the bottom of the tube (avoid excessive centrifugation).
2) Add 50 µl of SUB-AMIX TT to a 0.2 ml PCR tube.
3) In in a separate tube, mix 2.5 µl of WEPRO TTmix and 2.5 µl of PCR product (This reaction mixture is used to set up translation reaction). (*1)
   (*) Use 2.5 µl of pEU-E01-DYKDDDDK-DHFR instead of PCR product for positive control.
4) Setup bilayer:
   Carefully place the entire reaction mixture at the bottom of PCR tube containing SUB-AMIX TT (50 µl) to form a bilayer. As shown in Fig. 4, the reaction mixture forms the lower layer while the SUB-AMIX TT forms the upper layer. (*2)
   Do not mix these reagents in the tube by pipetting or any other means (**Important!!!**).
5) Incubate at 26°C for **2 to 24 hours.**
   (*) It is preferable to maintain the temperature more than 12 hours and 2 to 4 hours for SDS-PAGE/CBB staining and Western Blotting, respectively.
6) After completion of the protein expression reaction, the reaction mixture should be gently mixed by pipetted up and down before use in subsequent experiments.

(Remarks)
(*1) Please suspend gently to avoid any air bubbles.
(*2) Please be note that bubbles can reduce protein yields.

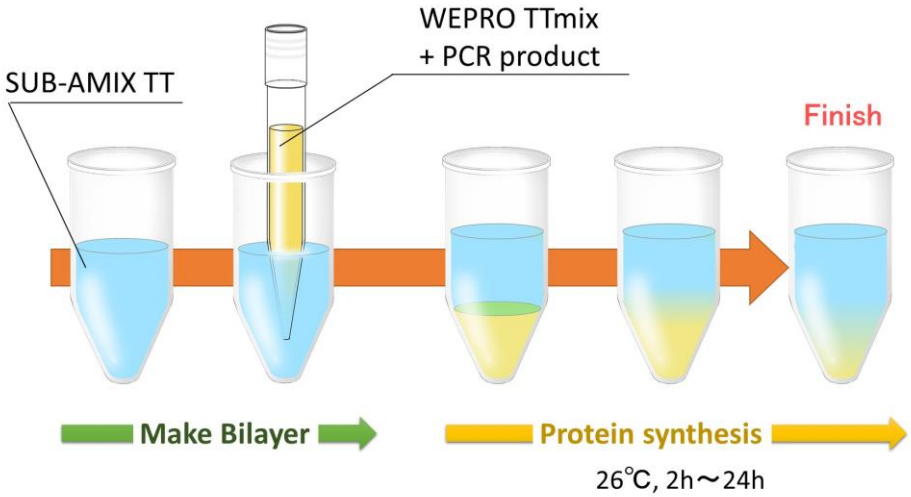


Fig. 4 Bilayer formation and protein synthesis reaction

4.5.2. When detecting with fluorescent lysine

By adding fluorescent lysine (FluoroTect™ $Green_{Lys}$ tRNA) to the synthesis reaction, the target protein can be detected already after a short synthesis time (fluorescent gel scanner is necessary for detection).
Please confirm that your protein contains some lysine amino acids to achieve incorporation into the target protein.
If there is no lysine in the target protein, this method cannot be applied.
Also, there is no activity on proteins with fluorescent lysine added.

1) Thaw the WEPRO TTmix and SUB-AMIX TT on ice. After thawing, lightly spin the tubes collect all reagents at the bottom of the tube (avoid excessive centrifugation).
2) Add 50 µl of SUB-AMIX TT to a 0.2 ml PCR tube.
3) In in a separate tube, mix 2.5 µl of WEPRO TTmix, 2.5 µl of PCR product and **0.5 µl of FluoroTect™ $Green_{Lys}$ tRNA** (This reaction mixture is used to set up translation reaction). (*1)
   (*) Use 2.5 µl of pEU-E01-DYKDDDDK-DHFR instead of PCR product for positive control.
4) Setup bilayer:
   Carefully place the entire reaction mixture at the bottom of PCR tube containing SUB-AMIX TT (50 µl) to form a bilayer. As shown in Fig. 5, the reaction mixture forms the lower layer while the SUB-AMIX TT forms the upper layer. (*2)
   Do not mix these reagents in the tube by pipetting or any other means (**Important!!!**).
5) Incubate at 26°C for **2 to 24 hours.**
6) After completion of the protein expression reaction, the reaction mixture should be gently mixed by pipetted up and down before use in subsequent experiments.
7) Transfer 5 µl of the reaction mixture to another tube, add 1 µl of RNase A (10 µg/µl) and incubate at 26° C for 30 minutes. Do not expose reaction mixture to light. (*3)
8) Add 6 µl of 2x SDS sample buffer and heat at 70° C for 3 min.
9) Perform SDS-PAGE analysis in the dark. Apply 10 µl of sample application amount.
10) Analyze with fluorescent gel scanner.

(Remarks)
(*1) Please suspend gently to avoid any air bubbles.
(*2) Please be note that bubbles can reduce protein yields.

(*3) Unless RNase A treatment is carried out, multiple bands other than the protein band are emitted in a molecular weight of 30,000 or less. If the target sample has a molecular weight of 30,000 or less, be sure to perform RNase A treatment. RNase A should destroy remaining labeled tRNA and thus reduces these background signals.

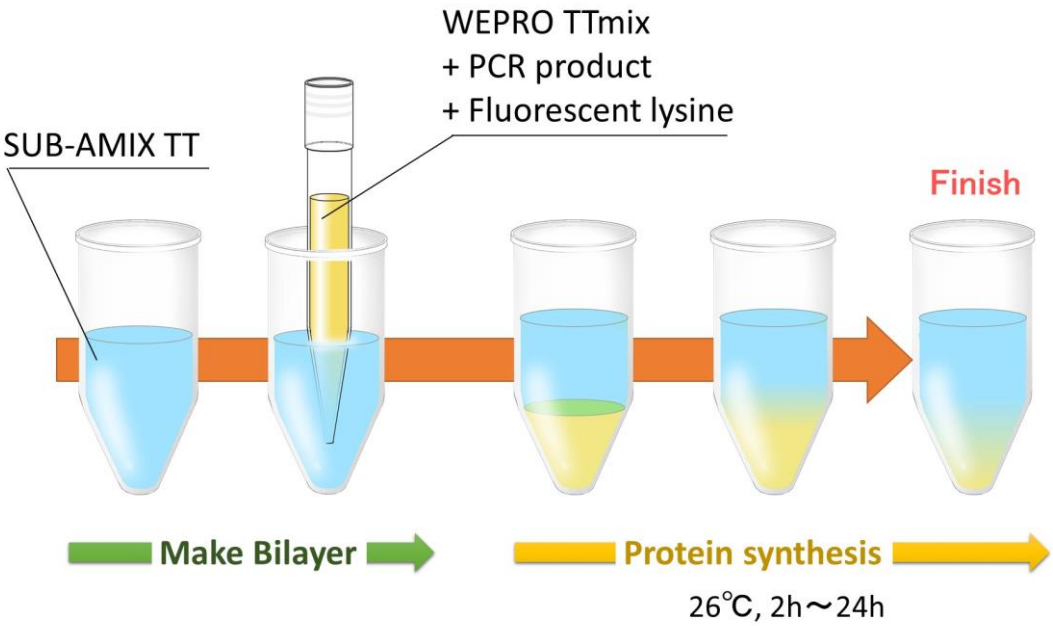


Fig. 5 Bilayer formation and protein synthesis reaction using fluorescent label

5. Confirmation of protein synthesis by SDS-PAGE

The synthesized target protein can be confirmed by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining, Western Blotting, or fluorescent lysine detection.

1. By CBB staining:

In order to clearly distinguish the background proteins derived from the wheat embryo extract and the protein of interest, please use a polyacrylamide gel with a high resolution and appropriate concentration (see the figure on next page). Normally, if you load 5 µl per sample, you can check the band of the protein of interest. However, please increase or decrease the amount of sample according to the result and repeat the SDS-PAGE analysis. As shown in example (Fig.5), the DHFR protein used as a positive control is synthesized as a protein of about 25 kDa.

2. By Western Blotting:

Please perform Western Blotting with suitable antibody against the target protein. Since the DYKDDDDK tag sequence is added to the positive control DHFR protein included in this kit, an anti-DYKDDDDK tag antibody can be used for detection.

3. In the case of fluorescent lysine labeling:

Run SDS-PAGE in the dark. Then detect fluorescent signal in the gel with a fluorescent gel scanner.

See next page for each result.

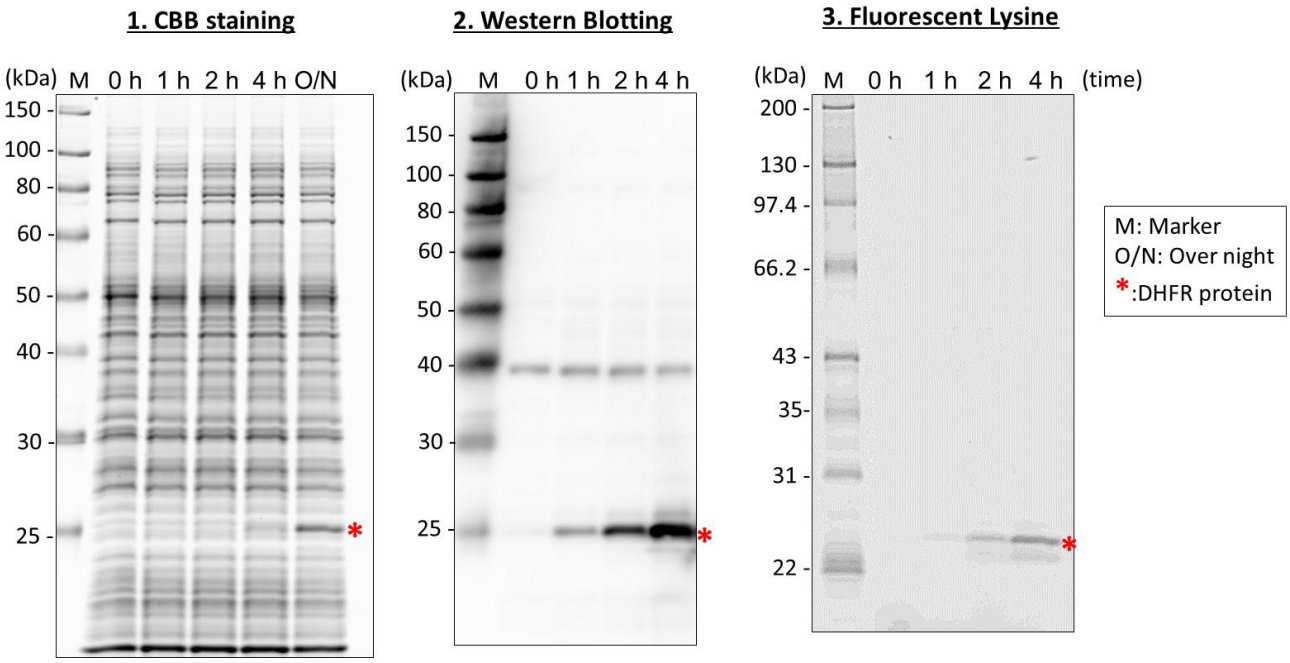


Fig. 6 Synthesis result of positive control over time. Protein synthesis was confirmed by CBB staining (1), Western Blotting using anti-DYKDDDDK antibody (2), fluorescent lysine (3)

6. Others

6.1. Label license policy

By opening the cap of any of the reagents listed in the above Section 3.2, the buyer of the Premium ONE 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.

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Platinum™, SuperFi™ are trademarks of Thermo Fisher Scientific Corporation.
FluoroTect™ is a trademark of Promega Corporation.
Other company names and product names mentioned herein are the trademarks of each company as stated above.

6.3. Others

Product specifications are subject to change without prior notice.

7. Contact information

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