ENDEXT® Technology

ProteoLiposome BD Expression Kit

For preparative expression of membrane proteins
New high-yield reaction format for preparation of proteoliposomes

(6 reactions on 2.5 ml BD reaction scale)

Instruction manual for protein synthesis using wheat germ cell-free expression system

(Catalog No. CFS- TRI-PLE-BD)

CellFree Sciences Co., Ltd.

Contents

1. Introduction

Although nearly a third of all eukaryotic genes encode membrane proteins, their expression and functional analysis still offers many challenges. This is mostly caused by complications to express those proteins in standard protein expression systems, where membrane proteins can be toxic to the cell system or form insoluble aggregates. These problems can be addressed by using the wheat germ cell-free protein expression system offered by CellFree Sciences. By adding liposomes, artificially-prepared spherical lipid vesicles, to the translation reaction, membrane proteins are directly inserted into the lipid bilayer of the liposome to form proteoliposome complexes. Proteoliposomes can be easily isolated by centrifugation and offer convenient tools to study protein functions. Moreover, proteoliposomes can be directly used in immunization experiments to prepare antibodies directed against membrane proteins.

CellFree Sciences developed a new reaction BD ("bilayer and dialysis") reaction format for high-yield preparation of proteoliposomes, which combines our bilayer protein expression method with a dialysis reaction. During our tests on the expression of a number of membranes proteins, we found that the new BD reaction format yielded about up to four times the protein amounts of a standard bilayer reaction. This new method is suitable to obtain for many membrane proteins the necessary protein yields for antigen preparation. The reaction conditions used in this kit have been tested for the expression of various membrane proteins, where a 2.5 ml BD expression reaction yields for example for the G Protein-Coupled Taste Receptor T1R1 about 600 µg of protein in the purified proteoliposome fraction.

The ProteoLiposome BD Expression Kit provides all necessary reagents to perform six preparative 2.5 ml BD reactions. For easy use, the kit contains lyophilized liposomes prepared from asolectin from soybeans. After rehydration those liposomes can be directly used in the translation reaction, thus avoiding organic solvents involved in classical liposome preparation steps.

For more information on the use of our wheat germ expression system in combination with liposomes for the preparation of membrane proteins, refer to the references at the end of the manual.

2. General Information on Working with Wheat Germ System

To perform the protein expression experiment, a template DNA is required that has a SP6 RNA polymerase promoter and a suitable enhancer. We advise to clone your cDNA into expression vector pEU-E01-MCS to express the native protein. CellFree Sciences can provide other expression vectors for working with tagged proteins using the His- or GST-tag. Refer to our homepage for more information on all our expression vectors, which can be obtained from CellFree Sciences. We provide below further directions on how to prepare DNA templates for use in our expression system.

Optionally, you can also prepare a DNA template by PCR. Please contact CellFree Sciences for more information on how to prepare protein expression templates by the "Split-PCR" method. However, we only recommend the use of PCR templates in small-scale reactions for example for screening expression vectors, or when working with a large number of DNA templates. We do not recommend the use of PCR templates for preparative protein expression; do use cloned expression templates that have been properly characterized.

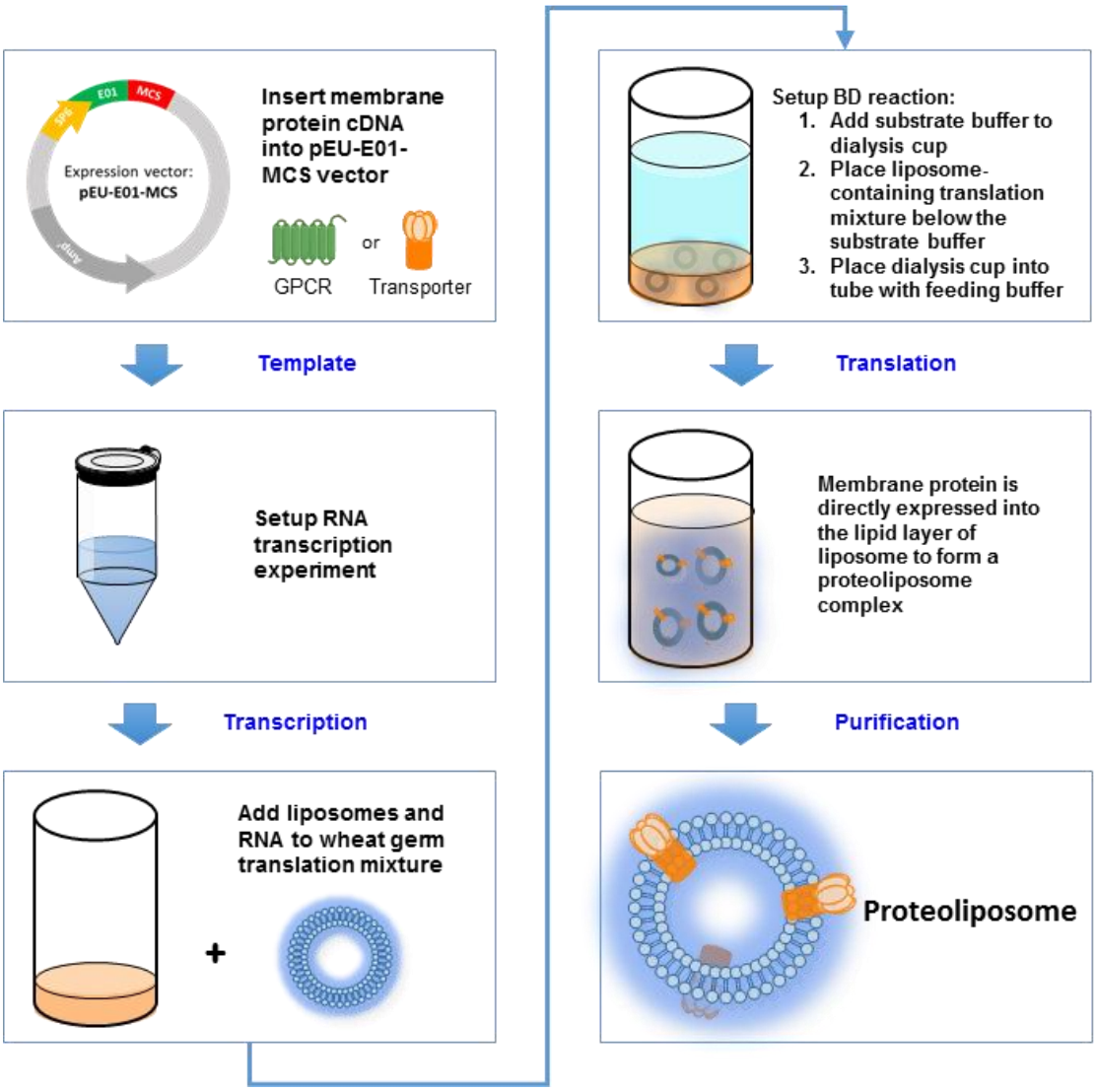
We strongly advise to test expression templates in small-scale expression reactions before doing any large-scale expression experiments. For such an initial vector testing, we recommend to perform a standard 227 µl bilayer reaction without the addition of liposomes. Even though membrane proteins are most likely insoluble under these expression conditions, the small-scale expression reaction can still be used to confirm the functionality of an expression vector and to get a first impression on the expected protein yields. In the following protocol, we provide also directions on how to set up a small-scale 227 µl bilayer reaction. The ProteoLiposome BD Expression Kit provides sufficient reagents to perform up to 20 small-scale 227 µl bilayer reactions besides the six 2.5 ml BD reactions.

For conducting more 227 µl bilayer reactions, you can purchase a Protein Research Kit (S), Product Number: CFS-PRK-24, from CellFree Sciences, which provides premixed reagents to perform 24 small-scale expression reactions. We also offer a ProteoLiposomes Expression Kit (Product Number: CFS-TRI-PLE) for preparation of proteoliposomes on a bilayer format. For further information regarding our products, refer to our homepage or contact us directly using the contact information at the end of the manual.

3. Protocol Overview

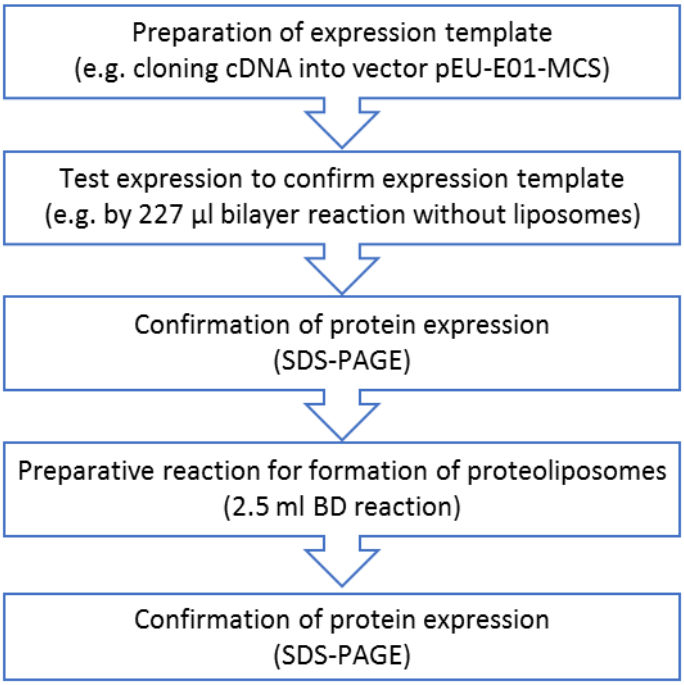
Protein synthesis is carried out by preparing first a RNA from the DNA template in a transcription reaction. The RNA is then used in the following translation reaction for protein synthesis. In the presence of liposomes, membrane proteins are incorporated into the lipid bilayer to form proteoliposomes. Thereafter, the proteoliposomes can be isolated from the reaction mixture in a simple centrifugation step.

Figure 1: Illustration of proteoliposome preparation



4. Use of ProteoLiposome BD Expression Kit

The Proteoliposome BD Expression Kit provides sufficient reagents to do all steps required to test the expression of your protein of interest on a small-scale prior to preparing proteoliposomes on a preparative scale. If one 2.5 ml BD reaction does not provide the needed protein yields, conduct multiple expression reactions and pool the protein from multiple reactions to obtain a conform protein mixture.



The expression template should first be tested in a small-scale 227 µl reaction in the absence of added liposomes to see on a SDS PAGE whether a protein of the correct size can be made. We advise again to always first test each expression template before spending time and reagents on a large-scale expression experiments in the presence of liposomes.

Once the expression of the protein has been confirmed in the initial test expression, the reaction scale can be increased and the formation of a proteoliposome complex can be performed using preparative BD reactions with the liposomes provided with the kit. Performing an SDS-PAGE on the proteins from the isolated proteoliposomes should confirm the presence of a protein of the correct molecular weight.

In the following protocol we are providing some advice on the preparation of an expression template using expression vector pEU-E01-MCS and later template preparation, and give reaction conditions to perform protein expression reactions on the two different reaction formats.

5. Materials Provided by the Kit

The ProteoLiposome BD Expression Kit is shipped on dry ice. Upon arrival, store the box immediately at -80°C. Do not thaw reagents at any time until starting the actual experiment. Avoid unnecessary freeze/thawing cycles and prepare aliquots of the reagents as indicated below.

5.1. Contents of the Kit

Item	Quantity	Concentration	Volume	Box Color
WEPRO®7240	1	240 OD	1 ml	White
5x Transcription Buffer LM	1	5x	0.4 ml	
NTP 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	
pEU-E01-T1R1 plasmid	1	1 mg/ml	30 µl	
SUB-AMIX® SGC S1	1	40x	12.5 ml	White
SUB-AMIX® SGC S2	1	40x	12.5 ml	
SUB-AMIX® SGC S3	1	40x	12.5 ml	
SUB-AMIX® SGC S4	1	40x	12.5 ml	
Asolectin Liposome, lyophilized	6	(10 mg)	Lyophilized	Green

5.2. Instructions for the Use of Reagents

Item	Description	Storage
WEPRO®7240	WEPRO®7240 (wheat germ extract) is sensitive to warm temperatures 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. To avoid multiple freeze-thawing cycles, subdivide it into appropriate aliquots in separate containers. Store them at -80°C for later use. Do not subject wheat germ extracts to three 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 wheat germ extracts. When using the reagent, mix	-80°C

	it gently by pipetting several times. Avoid bubbling. We recommend to prepare the following aliquots upon first time use of the wheat germ extract: 20 times 10 µl (227 µl test reactions) 6 times 130 µl (2.5 ml proteoliposome expression reactions)	
SUB-AMIX® SGC (S1, S2, S3, S4)	This product consists of a set of four buffer components (S1, S2, S3, S4) at 40x concentration. Store all four reagents at -20°C or below, e.g. at -80°C, along with the wheat germ extract. No change in their reaction efficiency has been observed after 10 freeze-thawing cycles. To prepare 1x SUB-AMIX® SGC buffer use nuclease-free water, and add the high concentration buffers to the water. If the four high concentration reagents are mixed first, precipitation may occur. Once this happens, it takes time to dissolve the precipitates. To avoid multiple freeze-thawing cycles, subdivide 1x SUB-AMIX® SGC mixture into appropriate aliquots in separate containers and store them at -80°C. Do not unnecessarily subject 1x SUB-AMIX® SGC mixture to multiple freeze-thawing cycles. Decrease in the reaction efficiency may occur, the degree of which depends on the way of handling.	-80°C
5x Transcription Buffer LM	After thawing, subdivide 5x Transcription Buffer LM into appropriate aliquots 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
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 aliquots 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	Note storage buffer contains 50% glycerol. Keep on ice.	-20°C
RNase Inhibitor	Note storage buffer contains 50% glycerol. Keep on ice.	-20°C
Creatine Kinase (*1)	Avoid multiple freeze-thawing cycles; otherwise the activity of Creatine Kinase will decrease. For small-scale reaction format on a 227 µl scale, dilute Creatin Kinase stock solution by 20	-80°C

	times using nuclease-free water. The final concentration should be 1 mg/ml. Keep on ice.	
Asolectin Liposome, lyophilized	Lyophilized asolectin liposomes are provided in a specially sealed vial to avoid any air contact, and are stable at room temperature or below. Open outer and inner cover of the vial containing the asolectin liposomes. Slowly add 200 µl of 1x SUB-AMIX® SGC at the center of bottom of the vial. Close the vial with inner cover and let it stay at room temperature for 10 min. Mix the liposomes by vortexing (30 seconds to 1 minute). Transfer the vials to a 50 ml tube and centrifuge them at 500x g for 1 min. Take out the vials from 50 ml tube and transfer the rehydrated liposomes to a 1.5 ml tube. Rehydrated liposomes are for single use, and we recommend not to freeze/thaw the liposomes after rehydration.	room temperature or below

(Notes)

(*1) Creatine Kinase can be purchased from Roche Applied Science, Catalog No. 10127566001. Dissolve it with nuclease-free water to make a 20 mg/ml stock solution. For convenience, subdivide the solution into smaller aliquots and store them at -80°C. Avoid multiple freeze-thawing cycles; otherwise the activity of Creatine Kinase will decrease.

6. Materials to Be Prepared by User

6.1. Reagents for optional Plasmid DNA Purification

The following reagents are needed if a further purification step for the plasmid DNA becomes necessary (see Section 7.2 and 7.3).

Reagents	Description
Phenol/Chloroform	phenol:chloroform:isoamyl alcohol = 25:24:1 in volume, pH 7.9
Chloroform	> 99%
Ethanol	100%
Ethanol	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 nuclease-free water when preparing TE buffer. - We DO NOT recommend DEPC treated water.

6.2. Reagents, Consumables, and Instruments Required for Membrane Protein Expression

Consumable	Description
Nuclease-free water	DNAase, RNase free. We DO NOT recommend DEPC treated water
PBS	Phosphate buffered saline
96-well plate	Cell culture plate, flat bottom, non-treated.
Cover tape	To seal well of 96-well plate.
Slide-A-Lyzer MINI Dialysis Devices, 10K MWCO	Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Devices, 10K MWCO from Thermo SCIENTIFIC, Product No. 88404.
Incubator	Temperature range 15 to 37°C.
Centrifuge	1.5 ml tube and 50 ml tube
SDS-PAGE	Gel electrophoresis apparatus and power supply

7. Protocols

For your safety:

Do not drink or eat in the laboratory. Do take precautions to work under RNase free conditions following standard lab procedures. Ware gloves and a lab coat at all times, and keep reagents on ice while setting up the reactions.

Wash hands before and after doing an experiment. If you have any reagent in your eyes or on your skin, wash eyes or skin immediately with water. Although this kit does not contain any hazardous reagents, do not take any risk. Note, that Phenol and Chloroform are toxic chemicals that may be used during the optional plasmid DNA purification step.

Read this protocol carefully before starting the experiment. Contact CellFree Sciences for further support and advice if you have any questions on the experiments described here and materials provided from CellFree Sciences.

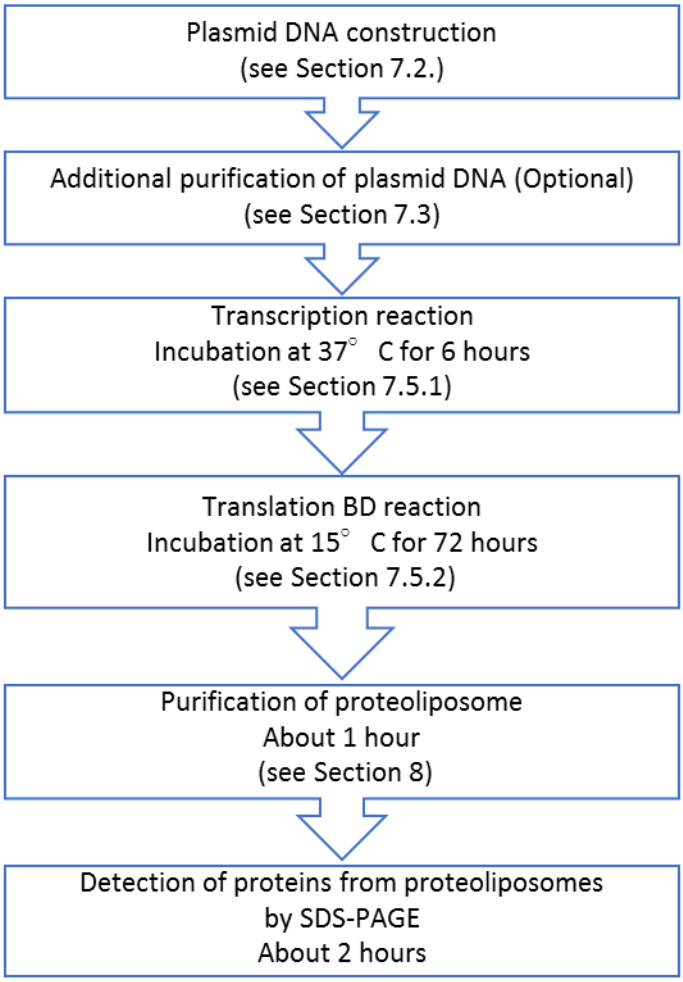
Laboratory standards:

This kit can be used in a regular molecular biology laboratory. We advise to work under RNase free conditions. Refer to a laboratory handbook for more information on how to work under RNase free conditions.

For your convenience:

With this manual we are providing short versions of the protocols ("Bench Notes"). Use the Bench Notes to setup your transcription and translation experiments in the lab. They only contain the basic information needed for setting up the experiments. Use the checkmarks in the Bench Notes to assure that all pipetting steps have been completed. Separate Bench Notes are provided for the 227 µl test reaction, as well as the 2.5 ml BD reaction scale for the formation of proteoliposomes.

7.1. Time Requirements



7.2. Remarks on Expression Vector Construction

We recommend to prepare an expression template for your protein(s) of interest prior to doing the protein expression experiments. While PCR products can be used in a cell-free protein expression system, plasmid DNA templates commonly provide better yields and give more reproducible results. Plasmid DNA templates are essential to up-scale protein production to a preparative scale. In the following we give some brief advice on the use of our expression vector. Refer to a handbook for more information on how to conduct cDNA cloning experiments.

Note, for many protein coding genes cDNA clones are available in the public domain. However, it may be necessary isolate the coding region (Open Reading Frame or "ORF") for later cloning into an expression vector.

1. Insert the coding region for your protein of interest into the multiple cloning site (MCS) of vector pEU-E01-MCS (or another expression vector for our expression system) using one or two restriction enzyme sites properly selected according to the MCS information in the vector map for pEU-E01-MCS (Appendix A) (*1). The protein is translated from the first start codon, an ATG, to a stop codon in your cDNA inserted in the MCS. Note that your cDNA must have a stop codon at the 3' end. Vector pEU-E01-MCS does not provide any stop codons to terminate protein synthesis. pEU-E01-MCS contains a SP6 promoter, an E01 translational enhancer, and an ampicillin resistance gene as illustrated in the vector map.
2. After the ligation step transform a suitable *E. coli* strain (e.g. JM109) with the vector DNA containing the cDNA-inserted into the expression vector.
3. Once you have selected an expression vector having the correct insert with the correct orientation, we advise to prepare glycerol stocks of the transformed bacteria and to store bacteria for future use. It is also possible to store DNA aliquots of the expression vector.
4. Extract the plasmid DNA from *E. coli* and purify it using a commercially available DNA purification kit. We recommend a QIAGEN Plasmid Midi Kit (catalog No. 12143) or QIAGEN Plasmid Maxi Kit (catalog No. 12163), which has commonly worked well in combination with our expression system. We DO NOT recommend the use of DNA mini-prep methods by the *alkaline elution* procedure without any further purification for direct use in our expression system.
5. After plasmid DNA 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 (*2).
6. Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer.
7. Plasmid DNA can be stored for a long time at -20°C. We advise to keep aliquots of the vector for later use.
8. You need 2 µg purified plasmid DNA per 227 µl translation reaction, 13 µg purified plasmid DNA per 2.5 ml BD translation reaction.

We recommend to confirm correct incorporation of the cDNA into the expression vector by end-sequencing of the insert and cloning sites. Refer to Appendix B for more information on sequencing primers for vector pEU-E01-MCS. The entire vector sequence for pEU-E01-MCS is given in Appendix C or can be downloaded from our homepage.

Refer to any cloning handbook or manual for more information on how to clone a cDNA into a vector, and how to propagate plasmid DNA.

(Notes)

*1: In order to efficiently express the target protein, it is recommended to select a restriction enzyme site as close as possible to the E01 translational enhancer. For cloning into vector pEU-E01-MCS you do not have to add a Kozak consensus sequence.

*2: Purity of plasmid DNA should be such that the A260/A280 ratio ranges between 1.70 and 1.85. Ratios outside of this range indicate that the plasmid DNA may contain remaining proteins or other contaminations. In that case, further purify the plasmid DNA as described in Section 7.3.

7.3. Optional Extra Purification Step for Plasmid DNA Templates

A highly purified plasmid DNA is essential for successful transcription and subsequent translation. If the plasmid DNA purified with a commercially available kit did not have a proper A260/A280 ratio, or the quality of RNA transcripts made with the plasmid DNA is low, the protein synthesis may not be succeed properly. Therefore we recommend in such cases a further purification of the plasmid DNA by phenol/chloroform extraction, which can remove proteins and some other contaminations.

This additional purification, which is optional if plasmid DNA has been prepared properly, can be accomplished by extracting the DNA first with phenol/chloroform and then with chloroform to remove remaining phenol. Afterwards the DNA is precipitated with ethanol (*1). Perform the following steps using your plasmid DNA:

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 7.2.) 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. Do not take the intersection.
4. Add an equal volume of chloroform into the tube with the aqueous phase 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. Do not take the intersection.
7. To this aqueous solution, add 2.5 times the volume 100% ethanol, and 3M sodium acetate (pH 5.2) at a 1/10 of the volume of the aqueous phase to precipitate the DNA.
8. Store 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 (do not dry pellet for extended period of time).
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 (*2).
16. Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer.

(Notes)

*1: Phenol and chloroform are hazardous chemicals and should only be handled with appropriate care and precautions. Note that phenol and chloroform have to be discarded as special chemical waste.

*2: 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 is still not suitable for conduction the expression experiment. In that case, repeat Section 7.3 from the beginning.

7.4. Small-Scale Test Expression Experiment

We advise to test each expression vector prior to working on a preparative scale. The ProteoLiposome BD Expression Kits contains sufficient reagents to perform up to 20 times a 227 µl bilayer reaction in addition to the preparative BD reactions. Do not add liposomes to the test reactions since they are not needed to confirm protein expression.

7.4.1. Transcription from Plasmid DNA Template

1. Thaw 5x Transcription Buffer LM and NTP Mix on ice. After thawing, spin the tubes for a short time to drop down reagent staying on the tube wall or on the cap. To avoid uneven concentrations, mix the reagent gently before using it. Place and keep all reagents on ice during handling.
2. Prepare transcription mixture on ice according to the table shown below using a 1.5 ml tube, and mix gently by pipetting up and down.

Reagents	Volume	Final concentration
Nuclease-free water	11.5 µl	-
5x Transcription Buffer LM	4 µl	1x
NTP Mix (25 mM)	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 µg/µl)	2 µl	100 ng/µl
Total	20 µl	

3. Incubate at 37°C for 6 hours in incubator (*1).
4. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis using 1 µl of the reaction mixture (*2). Refer to a cloning handbook for more information on RNA gel electrophoresis.

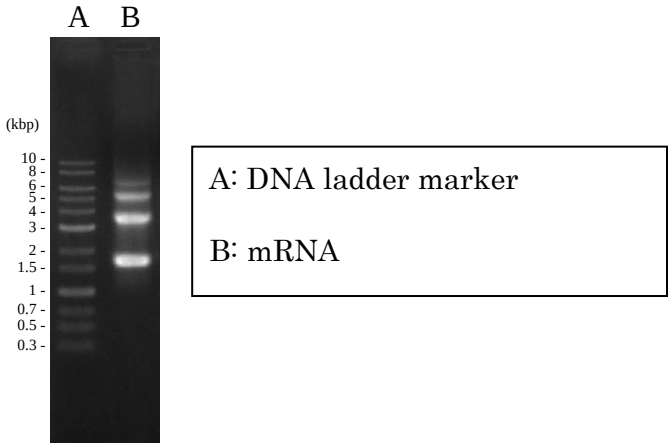
(Notes)

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

*2: A smear or pattern of less than 500 bases indicates possible degradation of mRNA probably caused by an RNase. In that case, further purification of the plasmid DNA as described in Section 7.3 is required. Confirm further that you are working under RNase free conditions.

*3: An example for high quality mRNA expression products is shown below. Note that the size difference between the RNA bands should be in the range of the plasmid sequence length.

Figure 2: Example for RNA agarose gel



7.4.2. Preparation of Translation Buffer

Prepare 4 ml of the translation buffer (1x SUB-AMIX® SGC) for doing the translation reaction. Mix buffer components of SUB-AMIX® SGC according to the table below and mix gently. Add the water first before adding the buffer components to avoid precipitations. See Section 5.2 for more information.

Reagent	Volume	Final Concentration
Nuclease-free water	3.6 ml	
40x SUB-AMIX® SGC (S1)	100 µl	1x
40x SUB-AMIX® SGC (S2)	100 µl	1x
40x SUB-AMIX® SGC (S3)	100 µl	1x
40x SUB-AMIX® SGC (S4)	100 µl	1x
Total	4 ml	

Keep buffer on ice until use. The buffer can be frozen for reuse.

7.4.3. Translation Reaction Using Bilayer Method

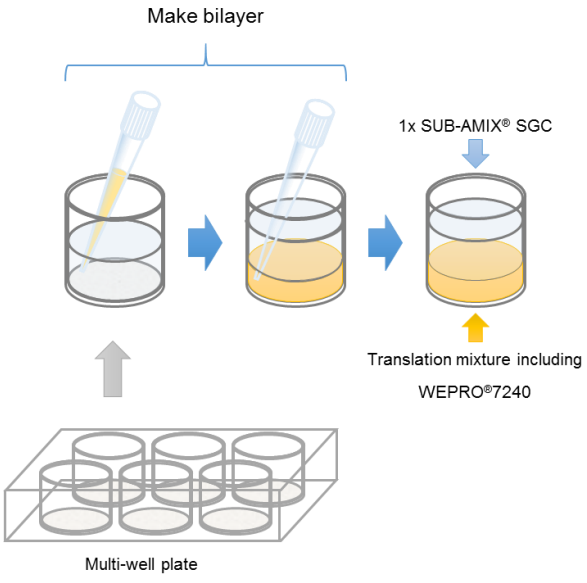
1. Let the mRNA reaction cool down to the room temperature. DO NOT forcibly cool it on ice or in the refrigerator. Before adding mRNA into the translation mixture, resuspend the white pellet (magnesium pyrophosphate) in the tube by pipetting gently up and down.
2. Thaw WEPRO®7240 under running water while thawing Creatine Kinase on ice, and immediately after thawing, place them on ice. After thawing the reagents, spin the tubes for a short time to drop down any reagent staying on the tube wall or on the cap. Avoid excessive centrifugation. To avoid uneven concentrations, mix the reagent gently before using it. Avoid bubbling.
3. The bilayer reaction is best setup in a 96-well plate for 227 µl reactions, where we recommend to use uncoated cell-culture plates.
4. Add 206 µl of 1x SUB-AMIX® SGC (see Section 7.4.2) to one well of a 96-well plate to perform 227 µl test reaction without liposomes.
5. Prepare translation mixture in 1.5 ml tube on ice according to the table shown below and mix gently by pipetting up and down. Avoid bubbling.

Reagents	Volume	Final concentration
Comment:	No Liposomes	
1x SUB-AMIX® SGC	-	
mRNA	10 µl	1/2 vol.
WEPRO®7240 (240 OD)	10 µl	60 OD
Creatine Kinase (1 mg/ml)	0.8 µl (*)	40 ng/µl
Asolectin Liposomes (50 mg/ml)	-	10 mg/ml
Total	20.8µl	

(*) use Creatine Kinase diluted to 1 mg/ml; the original stock in the kit is 20 mg/ml!

6. Carry out bilayer reaction: Carefully transfer the translation mixture (20.8 µl) to the bottom of the well containing 1x SUB-AMIX® SGC (206 µl) to form bilayer with the translation mixture in the lower layer and 1x SUB-AMIX® SGC in the upper layer. Bring pipette tip close to the bottom of the well, and slowly add the reaction mixture. Because of the higher density of the wheat germ extract, the reaction mixture will form a second layer below the reaction buffer. Do not disturb the layers when removing the pipette tip. **DO NOT mix the reagents in the well by pipetting or any other means. (Important!!).** See Figure 3 for more information.
7. Seal the well with cover tape to avoid evaporation.
8. Incubate at 15˚C for 20 hours.

Figure 3: Illustration on how to setup bilayer reaction



7.4.4. Detection of Target Protein by SDS-PAGE

Confirm the expression of your target protein using SDS-PAGE gel electrophoresis. Since membrane proteins can be insoluble when expressed without liposomes, make sure that you mix the reaction mixture well prior to taking the aliquot for the analysis. Resuspend any precipitate.

Run a SDS-PAGE followed by coomassie blue staining to identify the expressed protein following a standard protocol. SDS-PAGE requires high resolution and an appropriate gel concentration to distinguish the expressed protein from background proteins originated from the wheat germ extract. We advise to use commercially available gradient gels of the appropriate concentration range suitable for the size of your target protein. Load 3 µl of reaction mixture when using a standard SDS-PAGE mini gel. If the volume is too high or too low to identify the protein, change the volume to obtain a clear result. **Take care not to boil the SDS–PAGE sample before loading.** We have observed that proteins may not enter the gel after they had been boiled in the SDS sample buffer (for example for a 2x SDS sample buffer containing 150 mM Tris-HCl (pH6.8), 1.2% SDS, 24% glycerol, 0.1% bromophenol blue, 4% 2-mercaptoethanol).

7.5. Preparation of ProteoLiposomes using BD Reaction Format

7.5.1. Transcription from Plasmid DNA Template

1. Thaw 5x Transcription Buffer LM and NTP Mix on ice. After thawing, spin the tubes for a short time to drop down reagent staying on the tube wall or on the cap. To avoid uneven concentrations, mix the reagent gently before using it. Place and keep all reagents on ice during handling.
2. Prepare transcription mixture on ice according to the table shown below using a 1.5 ml tube, and mix gently by pipetting up and down.

Reagent	Volume	Final Concentration
Nuclease-free water	74.8 µl	-
5x Transcription Buffer LM	26 µl	1x
NTP Mix (25 mM)	13 µl	2.5 mM
RNase Inhibitor (80 U/µl)	1.62 µl	1 U/µl
SP6 RNA Polymerase (80 U/µl)	1.62 µl	1 U/µl
Plasmid (circular DNA, 1 µg/µl)	13 µl	100 ng/µl
Total	130 µl	

3. Incubate at 37°C for 6 hours in incubator (*1).

4. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis using 1 µl of the reaction mixture (*2). Refer to a cloning handbook for more information on RNA gel electrophoresis.

(Notes)

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

*2: A smear or pattern of less than 500 bases indicates possible degradation of mRNA probably caused by an RNase. For more information refer to section 7.4.1, and Figure 2 in there.

7.5.2. Preparation of Feeding Buffer for BD Reaction

Prepare 48 ml of feeding buffer (1x SUB-AMIX® SGC) for the dialysis reaction and the translation reaction. Mix buffer components of SUB-AMIX® SGC according to the table below and mix gently. Add the water first before adding the buffer components to avoid precipitations. See Section 5.2 for more information.

Reagent	Volume	Final Concentration
Nuclease-free water	43.2 ml	
40x SUB-AMIX® SGC (S1)	1.2 ml	1x
40x SUB-AMIX® SGC (S2)	1.2 ml	1x
40x SUB-AMIX® SGC (S3)	1.2 ml	1x
40x SUB-AMIX® SGC (S4)	1.2 ml	1x
Total	48 ml	

Keep buffer on ice until use.

7.5.3. Rehydration of lyophilized Asolectin Liposomes

1. To setup a 2.5 ml BD reaction, one vial with lyophilized asolectin liposomes is needed (Section 5.2).
2. Open outer and inner cover of the vial containing the asolectin liposomes.
3. Slowly add 200 µl of 1x SUB-AMIX® SGC to the center of bottom of the vial.
4. Close the vial with inner cover and let it stay at room temperature for 10 min.
5. Mix the liposomes by vortexing (30 seconds to 1 minute).
6. Transfer the vial to a 50 ml tube and centrifuge them at 500x g for 1 min.
7. Take out the vial from 50 ml tube and transfer the rehydrated liposomes to a 1.5 ml tube.

7.5.4. Preparation of Translation Reaction Mixture

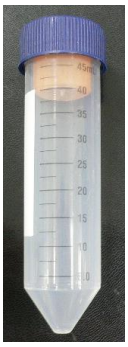
1. Let the mRNA reaction cool down to the room temperature. DO NOT forcibly cool it on ice or in the refrigerator. Before adding mRNA into the translation mixture, resuspend the white pellet (magnesium pyrophosphate) in the tube by pipetting gently up and down.
2. Thaw WEPRO®7240 under running water while thawing Creatine Kinase on ice, and immediately after thawing, place them on ice. After thawing the reagents, spin the tubes for a short time to drop down any reagent staying on the tube wall or on the cap. Avoid excessive centrifugation. To avoid uneven concentrations, mix the reagent gently before using it. Avoid bubbling.
3. Set up a 500 µl translation reaction following the directions of the table below. Keep reagents on ice at all times. Mix reaction mixture gently by pipetting up and down. Avoid air bubbles.

Reagent	Volume	Final Concentration
mRNA	125 µl	1/4 vol.
1x SUB-AMIX® SGC	149 µl	
WEPRO®7240 (240 OD)	125 µl	60 OD
Creatine Kinase (20 mg/ml)	1 µl	40 µg/ml
Asolectin liposome (50 mg/ml)	100 µl	10 mg/ml
Total	500 µl	

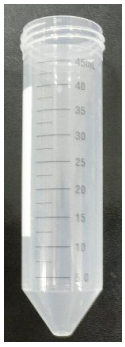
Keep translation reaction mixture on ice until use.

7.5.5. Preparation of BD Reaction

The BD reaction format combines the dialysis method with a bilayer reaction setup in the dialysis cup. For setting up a 2.5 ml BD reaction, we recommend using a Slide-A-Lyzer MINI Dialysis Devices, 10K MWCO from Thermo SCIENTIFIC, Product No. 88404. We have tested the reaction format using these dialysis cups, but similar products from other providers may work as well. Slide-A-Lyzer MINI Dialysis Devices, 10K MWCO comes with a 50 ml tube and a dialysis cup having a membrane at the bottom:



Assembled device



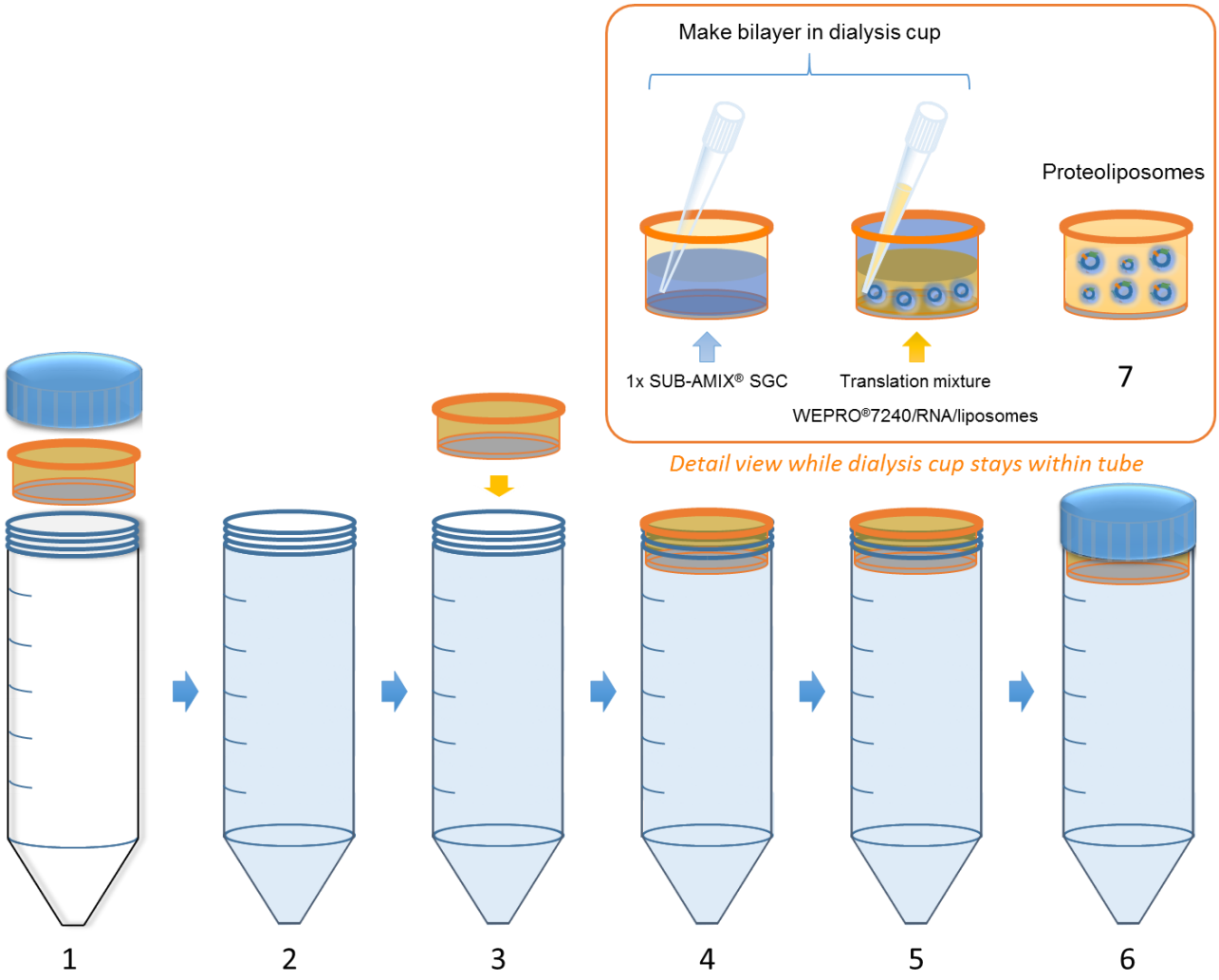
50 ml tube

Dialysis cup

1. Remove dialysis cup from Slide-A-Lyzer MINI Dialysis Devices, 10K MWCO and add 43 ml of 1x SUB-AMIX® SGC feeding buffer to the 50 ml tube.
2. Add 4 ml of nuclease-free water to the dialysis cup, and rinse the dialysis membrane. Conform there is no leakage from membrane. Decant the water and shake the cup to remove remaining water. **Do not dry the membrane.**
3. Place the dialysis cup back to the 50 ml tube. Make sure the membrane in the dialysis cup is within the feeding buffer.
4. Add 2 ml of 1x SUB-AMIX® SGC to the dialysis cup. **Take care not to damage dialysis membrane.**
5. Set up a bilayer in the dialysis cup placed in the 50 ml tube with the feeding buffer.
6. Carefully transfer the translation mixture (500 µl) to the bottom of the dialysis cup containing 1x SUB-AMIX® SGC (2 ml) to form bilayer with the translation mixture in the lower layer and 1x SUB-AMIX® SGC in the upper layer. Bring pipette tip close to the bottom of the dialysis cup, and slowly add the reaction mixture. Because of the higher density of the wheat germ extract, the reaction mixture will form a second layer below the reaction buffer. Do not disturb the layers when removing the pipette tip. **Take care not to damage dialysis membrane. DO NOT mix the reagents in the well by pipetting or any other means. (Important!!) See Figure 4 above for more information.**
7. Close the 50 ml tube and tighten the cap on the tube. Be careful not to shake the tube with the bilayer reaction in the dialysis cup.
8. Place the tube in an incubator and incubate the translation reaction for up to 72 hours at 15°C. It is not necessary to exchange the feeding buffer during the incubation time.

9. We do not recommend to change the feeding buffer during the incubation to avoid disturbing the bilayer reaction. Note, the kit contains enough feeding buffer to do 10 times a 2.5 ml BD reaction. You can purchase additional SUB-AMIX® SGC (Product Number: CFS-SUB-AMX) from CellFree Sciences.

Figure 4: Setup of BD reaction



1: Dialysis device comprising 50 ml tube, dialysis cup, and lid

2: Prepare tube and add feeding buffer

3: Insert dialysis cup into tube with feeding buffer

4: Add feeding buffer into dialysis cup within the tube

5: Setup bilayer reaction in the dialysis cup within the tube. Refer to drawings within the orange box for more details on how to place the translation mixture below the feeding buffer

6: Put lid onto tube with dialysis cup and conduct translation reaction

7: Proteoliposomes will be formed within the dialysis cup

8. Purification of Proteoliposomes

8.1. Purification of Proteoliposomes

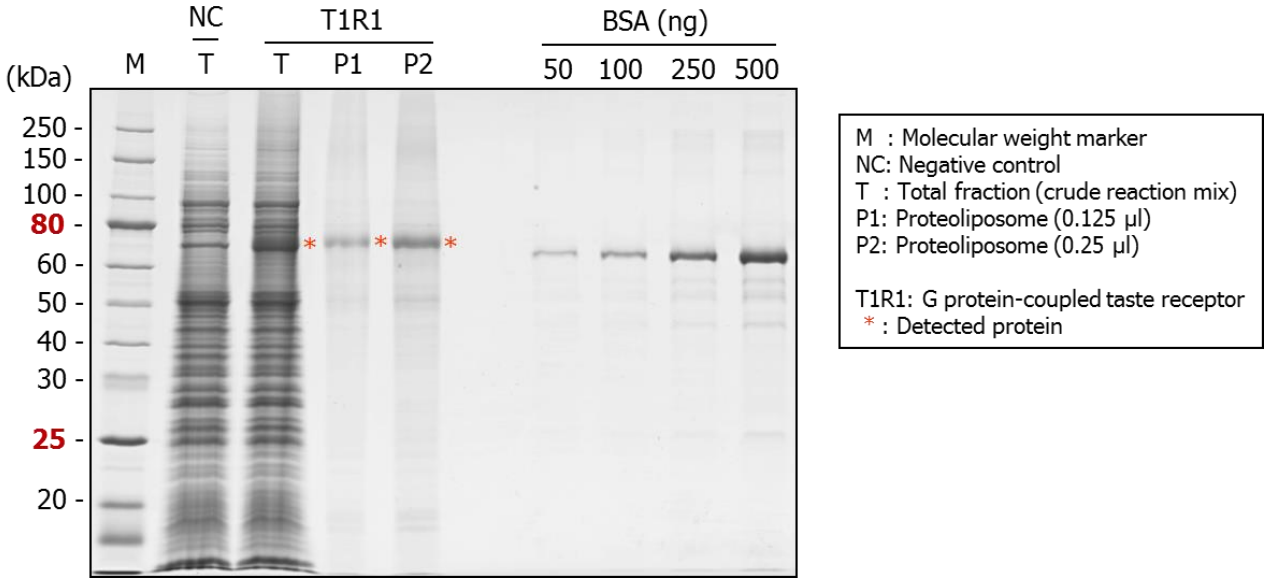
1. Mix the reaction mixture in the dialysis cup by pipetting up and down. Then transfer 0.83 ml of the reaction mixture to a 1.5-ml tube; repeat this step three-times to transfer the entire reaction mixture into three 1.5-ml tubes. Avoid U-bottom shaped tubes at this step because the proteoliposome pellet is easily detaching from bottom of the tube. **Take care not to damage dialysis membrane.**
2. Add 1 ml of PBS to the dialysis cup that contained protein product. This PBS will be used later.
3. Centrifuge the 1.5 ml tubes at 15,000 rpm, 4˚C, for 10 min.
4. Remove supernatant. Take care not to remove whole supernatant, leave a small volume of supernatant in the tube because the pellet is attached loosely at the bottom of the tube.
5. Mix the PBS in the dialysis cup by pipetting up and down. Use the PBS to wash the surface of the cup. Transfer the PBS to the first 1.5 ml tubes a containing proteoliposome pellet and suspend it well. Continue to gather the pellets from the remaining 3 tubes and collect the entire PBS in one tube.
6. Centrifuge the 1.5 ml tube at 15,000 rpm, 4˚C, for 10 min.
7. Remove supernatant. Take care not to remove whole supernatant.
8. Add 1 ml of PBS to the tube and resuspend it by pipetting.
9. Repeat washing steps 6 to 8 two more times (in total washing pellet 3 times).
10. After the last centrifugation, remove supernatant and add appropriate volume of PBS to the pellet. Resuspend proteoliposomes in a total of 500 µl PBS. Resuspend pellet completely by pipetting up and down.

Store proteoliposmes in PBS at -80 ˚C.

8.2. Confirmation of Protein Expression into Proteoliposomes

Proteins contained in the proteoliposomes can be analyzed by SDS-PAGE gel electrophoresis. Load some 0.2 to 4 µl of the forgoing proteoliposome preparation per well for protein detection. **Take care not to boil the SDS–PAGE sample before loading.** We have observed that protein may not enter the gel after they had been boiled in the SDS sample buffer (for example for a 2x SDS sample buffer containing 150 mM Tris-HCl (pH6.8), 1.2% SDS, 24% glycerol, 0.1% bromophenol blue, 4% 2-mercaptoethanol).

Figure 5: SDS PAGE showing example data for the G Protein-Coupled Taste Receptor T1R1



9. References

The following studies have used our wheat germ expression system in combination with liposomes for the preparation of different membrane proteins:

Production of monoclonal antibodies against GPCR using cell-free synthesized GPCR antigen and biotinylated liposome-based interaction assay.

Takeda H, Ogasawara T, Ozawa T, Muraguchi A, Jih PJ, Morishita R, Uchigashima M, Watanabe M, Fujimoto T, Iwasaki T, Endo Y, Sawasaki T. (2015) *Sci Rep.* 5, 11333.

High-throughput synthesis of stable isotope-labeled transmembrane proteins for targeted transmembrane proteomics using a wheat germ cell-free protein synthesis system.

Takemori N, Takemori A, Matsuoka K, Morishita R, Matsushita N, Aoshima M, Takeda H, Sawasaki T, Endo Y, Higashiyama S. (2015) *Mol Biosyst.* 11(2), 361-5.

The ligand binding ability of dopamine D1 receptors synthesized using a wheat germ cell-free protein synthesis system with liposomes.

Arimitsu E, Ogasawara T, Takeda H, Sawasaki T, Ikeda Y, Hiasa Y, Maeyama K. (2014) *Eur J Pharmacol.* 745C, 117-122.

Modifications of wheat germ cell-free system for functional proteomics of plant membrane proteins.

Nozawa A, and Tozawa Y. (2014) *Methods Mol Biol.* 1072, 259-72.

Cell-free protein synthesis of membrane (1,3)-ß-d-glucan (curdlan) synthase: co-translational insertion in liposomes and reconstitution in nanodiscs.

Periasamy A, Shadiac N, Amalraj A, Garajová S, Nagarajan Y, Waters S, Mertens HD, and Hrmova M. (2013) *Biochim Biophys Acta.* 1828(2), 743-57.

Function of Shaker potassium channels produced by cell-free translation upon injection into Xenopus oocytes.

Jarecki BW, Makino S, Beebe ET, Fox BG, and Chanda B. (2013) *Sci Rep.* 3, 1040.

A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters.

Norawa A, Nanamlya H, Mlyata T, Linka N, Endo Y,Weber AP, and Tozawa Y. (2007) *Plant Cell Physiol*, 48, 1815-1820.

Bench Notes for 227 µl Test Expression Reaction Scale

Print this Bench Note for setting up your experiments. Mark each step in the protocol after completion.

Setup transcription reaction:

Reagent	Volume	Final Concentration	Checkmark
Nuclease-free water	11.5 µl	-	□
5x Transcription Buffer LM	4 µl	1x	□
NTP Mix (25 mM)	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 µg/µl)	2 µl	100 ng/µl	□
Total	20 µl	INCUBATE 6 h at 37°C	□

Setup translation buffer SUB-AMIX® SGC:

Reagent	Volume	Checkmark
Nuclease-free water	3.6 ml	□
40x SUB-AMIX® SGC (S1)	100 µl	□
40x SUB-AMIX® SGC (S2)	100 µl	□
40x SUB-AMIX® SGC (S3)	100 µl	□
40x SUB-AMIX® SGC (S4)	100 µl	□
Total	4 ml	

Add 206 µl 1x SUB-AMIX® SGC to a well on a 96-well plate for setting bilayer reaction.

Setup translation reaction mix:

Reagent	Volume	Final Concentration	Checkmark
mRNA	10 µl	1	□
WEPRO®7240 (240 OD)	10 µl	60 OD	□
Creatine Kinase (1 mg/ml)	0.8 µl	40 µg/ml	□
Total	20.8 µl	INCUBATE 20 h at 15°C	□

Place translation reaction mix below the reaction buffer to form bilayer. DO NOT MIX THE TWO LAYERS.

SDS-PAGE:

1. Mix reaction mixture well before taking aliquot for analysis on SDS-PAGE
2. Use 3 µl of the crude reaction mixture to perform SDS-PAGE gel electrophoresis

Bench Notes for 2.5 ml BD Reaction Scale

Print this Bench Note for setting up your experiments. Mark each step in the protocol after completion.

Setup transcription reaction:

Reagent	Volume	Final Concentration	Checkmark
Nuclease-free water	74.8 µl	-	☐
5x Transcription Buffer LM	26 µl	1x	☐
NTP Mix (25 mM)	13 µl	2.5 mM	☐
RNase Inhibitor (80 U/µl)	1.62 µl	1 U/µl	☐
SP6 RNA Polymerase (80 U/µl)	1.62 µl	1 U/µl	☐
Plasmid (circular DNA, 1 µg/µl)	13 µl	100 ng/µl	☐
Total	130 µl	INCUBATE 6 h at 37°C	☐

Rehydrate liposomes:

1. Slowly add 200 µl of 1x SUB-AMIX® SGC lyophilized liposomes.
2. Incubate at room temperature for 10 min and then mix by vortexing up to 1 min.
3. Spin at 500 g for 1 min before use.

Setup translation buffer SUB-AMIX® SGC:

Reagent	Volume	Final Concentration	Checkmark
Nuclease-free water	43.2 ml		☐
40x SUB-AMIX® SGC (S1)	1.2 ml	1x	☐
40x SUB-AMIX® SGC (S2)	1.2 ml	1x	☐
40x SUB-AMIX® SGC (S3)	1.2 ml	1x	☐
40x SUB-AMIX® SGC (S4)	1.2 ml	1x	☐
Total	48 ml		

Setup translation reaction:

1. Rinse dialysis membrane with 4 ml of nuclease-free water.
2. Add 43 ml of 1x SUB-AMIX® SGC to tube of the dialysis device.
3. Prepare translation reaction mixture:

Reagent	Volume	Final Concentration	Checkmark
mRNA	125 µl	1/4 vol.	☐
1x SUB-AMIX® SGC	149 µl		☐
WEPRO®7240 (240 OD)	125 µl	60 OD	☐
Creatine Kinase (20 mg/ml)	1 µl	40 µg/ml	☐
Asolectin liposome (50 mg/ml)	100 µl	10 mg/ml	☐
Total	500 µl		

3. Add 2 ml of 1x SUB-AMIX® SGC to a dialysis cup.
4. Setup bilayer with translation reaction in the dialysis cup by adding 500 µl translation reaction mixture.
5. Incubate for 72 hours at 15°C.

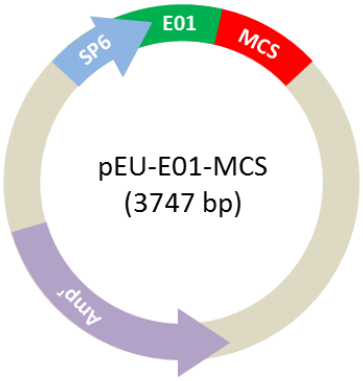
Purification of proteoliposomes:

1. Transfer 0.83 ml each of the reaction mixture into three 1.5 ml tubes
2. Spin down proteoliposomes in each tube by centrifuge at 15,000 rpm, 4˚C, for 10 min
3. Carefully remove supernatant; do not remove all liquid, do not disturb the pellets
4. Add 1 ml PBS to the reaction dialysis cup; wash well with the PBS buffer
5. Remove PBS buffer from dialysis cup and resuspend pellet in the first 1.5 ml tube
6. Use the same PBS buffer to resuspend all the pellets to unite the reaction products in one tube
7. Spin down proteoliposomes in the PBS buffer by centrifuge at 15,000 rpm, 4˚C, for 10 min
8. Carefully remove supernatant; do not remove all liquid, do not disturb the pellet
9. Wash the pellet two more times with 1 ml PBS buffer per washing step (in total 3 washing steps)
10. Resuspend the pellet after the final showing step in 500 µl PBS buffer
11. Store proteoliposomes at -80°C

Appendix A: Vector Map for pEU-E01-MCS

pEU-E01-MCS vector

Multiple cloning site information



EcoRV *SpeI* *XhoI* *SacI* *KpnI* *NotI*

74- GATATCACTAGTTCTCGAGCTCGGTACCTGTCCGCGGTCGCGACGTACGCGGGCGGCCG

BamHI *SmaI* *SalI* *NcoI*

CCATAAATTGGATCCATATATAGGGCCCGGGTTATAATTACCTCAGGTCGACGTCCCATGG -193

SP6 Promoter: -17~1
Translational Enhancer (E01):1~73
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

Appendix B: Sequencing Primers for Vector pEU-E01-MCS

For 5' end sequence: SP6 Primer

5'-ATTTAGGTGACACTATAGAA-3'

For 3' end sequence

5'-CCTGCGCTGGGAAGATAAAC-3'

pUC/M13 Sequencing Primers

The pUC/M13 Primers are designed for sequencing inserts cloned into the M13 vectors and pUC plasmids developed by Messing. These primers also can be used for sequencing other *lac*Z-containing plasmids such as the pGEM®-Z and pGEM®-Zf Vectors. The primers are purified by gel electrophoresis or HPLC.

Primer Sequences

Forward (17mer): 5´-d(GTTTTCCCAGTCACGAC)-3´

Reverse (17mer): 5´-d(CAGGAAACAGCTATGAC)-3´

Reverse (22mer): 5´-d(TCACACAGGAAACAGCTATGAC)-3´

Forward (24mer): 5´-d(CGCCAGGGTTTTCCCAGTCACGAC)-3´

Appendix C: Vector Sequence of pEU-E01-MCS

ATTTAGGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCA
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GTTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACG

CellFree Sciences can provide the vector sequence as a text file. For downloading vector maps and sequences visit our homepage at: http://www.cfsciences.com/eg/vector.html.

10. Others

Label License Policy

By opening the cap of any of the reagents listed in the above Section 5.1, the buyer of the ProteoLipoome Expression Kit is agreeing to be bound by the terms of the following Label License Policy.

<< Label License Policy>>

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ProteoLiposome BD Expression Kit_E_ver. 1.2, Jul. 12, 2018.