ENDEXT® Technology

ProteoLiposome Expression Kit

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

(Catalog No. CFS-TRI-PLE)

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 cell system used 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.

The ProteoLiposome Expression Kit from CellFree Sciences provides researchers with all necessary reagents to prepare proteoliposome complexes for their proteins of interest. The kit contains lyophilized liposomes prepare from soybean asolectin for easy reaction setup.

This kit had been designed to perform protein expression reactions on a 4 ml reaction scale using 6-well plates.

The reaction conditions used in this kit have been tested for the expression of various membrane proteins. A 4 ml reaction yielded for example for the G Protein-Coupled Taste Receptor T1R1 about 120 µg of protein in the purified proteoliposome fraction. For additional examples on membrane proteins that were prepared by using our wheat germ expression system in combination with liposomes, refer to the references at the end of the manual.

For initial testing of your expression vectors, we recommend to use our Protein Research Kit (S), which contains ready to use reagents to perform 227µl bilayer reactions. Even though membrane proteins may be insoluble under standard expression conditions used in the Protein Research Kit (S), the 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.

This kit does not contain any expression vector for the wheat germ cell-free protein expression system. If you do not have a suitable expression vector, contact us for more information. CellFree Sciences will provide expression vectors for our expression system free of charge.

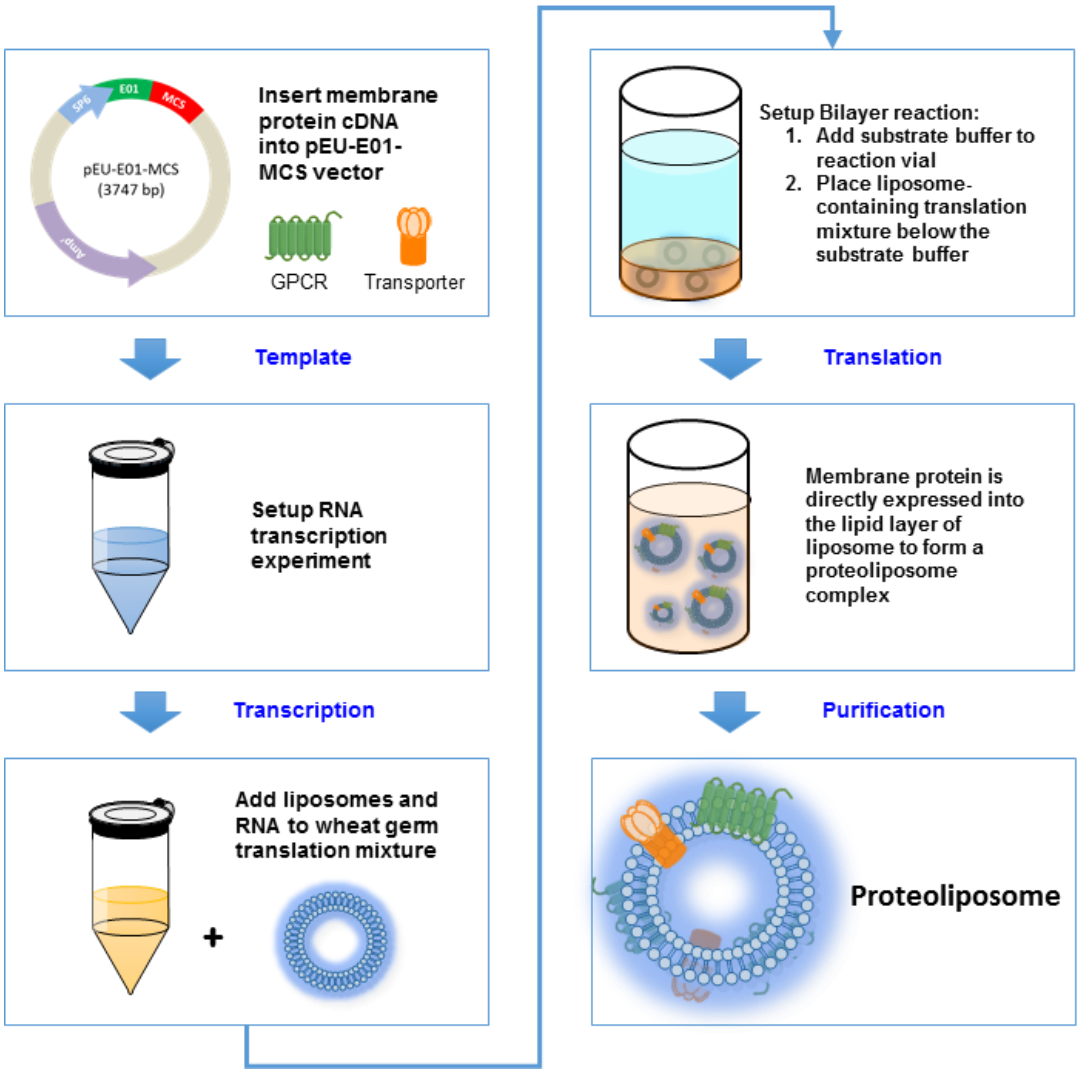
2. Protocol Overview

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 (separately available from CellFree Sciences). 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”.

Protein synthesis is carried out by preparing first the RNA template in a transcription reaction followed by protein synthesis in a translation reaction step.

Figure 1: Illustration of proteoliposome preparation



3. Materials Provided by Kit

The ProteoLiposome Expression Kit is shipped on dry ice. The reagents are provided in two boxes marked by different colors. Upon arrival, store both boxes immediately at -80°C.

3.1. Contents of the Kit

Item	Quantity	Concentration	Volume	Box Color
WEPRO®7240	1	240 OD	1 ml	White
SUB-AMIX® SGC S1	1	40x	1.1 ml	
SUB-AMIX® SGC S2	1	40x	1.1 ml	
SUB-AMIX® SGC S3	1	40x	1.1 ml	
SUB-AMIX® SGC S4	1	40x	1.1 ml	
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	
Asolectin Liposome, lyophilized	6	(10 mg)	Lyophilized	Green

3.2. 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 3 or more freeze-thawing cycles. After the third freeze-thawing	-80°C

	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 it gently by pipetting several times. Avoid bubbling.	
SUB-AMIX® SGC (S1, S2, S3, S4)	This product consists of a set of 4 reagents (S1, S2, S3, S4) at 40x concentration. Store all 4 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 4 ml of 1x SUB-AMIX® SGC mixture that is used for 1 reaction of 4 ml scale translation, add 0.1 ml each of S1 through S4 to 3.6 ml of nuclease-free water while agitating the latter. If the 4 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 -20°C or -80°C. Do not 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.	-20°C or -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	Buffer contains 50% glycerol.	-20°C

RNase Inhibitor	Buffer contains 50% glycerol.	-20°C
Creatine Kinase (*1)	Avoid multiple freeze-thawing cycles; otherwise the activity of Creatine Kinase will decrease.	-80°C
Asolectin Liposome, lyophilized	Lyophilized Asolectin liposomes are provided in a specially sealed vial to avoid any air contact, and are stable at -80°C. Open inner and outer cover of the vial containing the Asolectin liposomes. Slowly add 200 µl of 1x SUB-AMIX® SGC to 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 vial to a 50 ml tube and centrifuge them at 500x g for 1 min. Take out the vial from 50 ml tube and transfer the rehydrated liposomes to a 1.5 ml tube. Rehydrated liposome is for single use only.	-80°C

(Notes)

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.

4. Materials to Be Prepared by User

4.1. Reagents for Plasmid DNA Preparation

The following reagents are necessary to prepare plasmid DNA for the transcription reaction (see Section 5.2 and 5.3).

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.

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

Reagents	Description
Nuclease-free water	DNase, RNase free. We DO NOT recommend DEPC treated water
PBS	Phosphate buffered saline, pH 7.5
6-well plate	Cell culture plate, flat bottom, non-treated
Cover tape	To seal well of 6-well or 24-well plate
Incubator	37˚C, 15˚C.
Centrifuge	1.5 ml tube, 50 ml tube
SDS PAGE	Gel electrophoresis apparatus and power supply

5. 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.
>
> 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 available from CellFree Sciences.

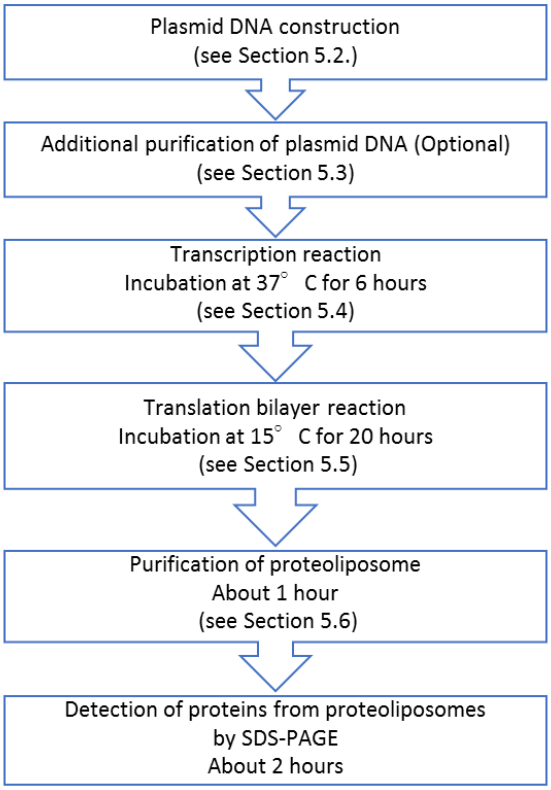
Laboratory standards:

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:

Use the Bench Notes provided with this manual to setup your transcription and translation experiments. They only contain the information needed for setting up the experiments.

5.1. Time Requirements



5.2. Plasmid DNA Construction

1. Insert your cDNA into the multiple cloning site (MCS) of vector pEU-E01-MCS using restriction enzymes properly selected according to the MCS information vector map (Appendix A) (*1). Protein is translated from the first start codon ATG to stop codon in your cDNA inserted in the MCS. Please note that pEU-E01-MCS contains SP6 promoter, E01 translational enhancer, and ampicillin resistance gene as illustrated in the vector map. Note further 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.
2. Transform a suitable *E. coli* strain (e.g. JM109) with the vector containing the cDNA-inserted pEU-E01-MCS.
3. Prepare glycerol stocks of the transformed bacteria to store your vector for future use.
4. Extract the plasmid DNA from *E. coli* and purify it using a commercially available kit, for example, one from QIAGEN. We recommend QIAGEN Plasmid Midi Kit (catalog No. 12143) or QIAGEN Plasmid Maxi Kit (catalog No. 12163). We DO NOT recommend DNA mini-prep methods by the *alkaline elution* procedures for the present purpose.

5. After the plasmid 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 20 μg purified plasmid DNA per 4 ml translation reaction.

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 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 the range indicate that the plasmid DNA has been contaminated. In that case, further purify the plasmid DNA as described in Section 5.3.

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 is given in Appendix C.

5.3. Preparation of Plasmid DNA for Transcription

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

This additional purification, which is optional if plasmid DNA has been prepared properly, can be accomplished by extraction first with phenol/chloroform and then with chloroform, followed by precipitation with ethanol (*1). Perform the following steps using your plasmid DNA 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 5.2.) 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 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 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 (*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.

*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 has been contaminated. In that case, repeat Section 5.3 from the beginning.

5.4. Transcription with Plasmid DNA as Template

1. Thaw 5x Transcription Buffer LM and NTP Mix on ice. After thawing, spin down the tubes for a short time to drop down the reagent staying on the tube wall or on the cap. To avoid localized concentration, mix the reagent gently before using it. Place and keep all reagents on ice during handling.
2. Prepare 200 µl of transcription mixture using 1.5 ml tube on ice according to the mixing formula shown below and mix gently by pipetting.

Reagents	Working vol.	Final conc.
Nuclease-free water	115 µl	-
5x Transcription Buffer LM	40 µl	1x
NTP Mix (25 mM)	20 µl	2.5 mM
RNase Inhibitor (80 U/µl)	2.5 µl	1 U/µl
SP6 RNA Polymerase (80 U/µl)	2.5 µl	1 U/µl
Plasmid (circular DNA, 1 µg/µl)	20 µl	100 ng/µl
Total	200 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 loading 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 RNase. In that case, further purification of the plasmid DNA as described in Section 5.3 is required. Confirm further that you are working under RNase free conditions.

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

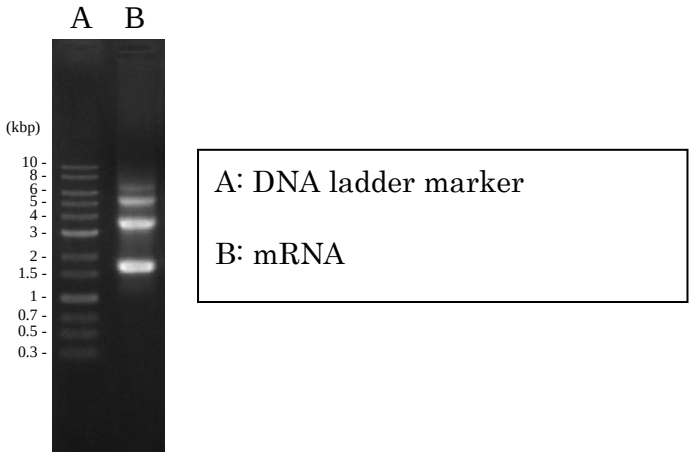


Figure 2: Example for RNA agarose gel

5.5. Translation by 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.
2. Thaw WEPRO®7240 and Creatine Kinase under running water, and immediately after thawing, place it on ice. After thawing the reagents, spin down the tubes for a short time to drop down the reagent staying on the tube wall or on the cap. Avoid excessive centrifugation. To avoid localized concentration, mix the reagent gently before using it. Avoid bubbling.
3. Add 3.5 ml of 1x SUB-AMIX® SGC (see Section 3.2) to a well of a 6-well plate to perform standard reaction.
4. Prepare translation mixture in 1.5 ml tube on ice according to the mixing formula shown below and mix gently by pipetting. Avoid bubbling. For rehydration of Asolectin liposomes refer to Section 3.2.

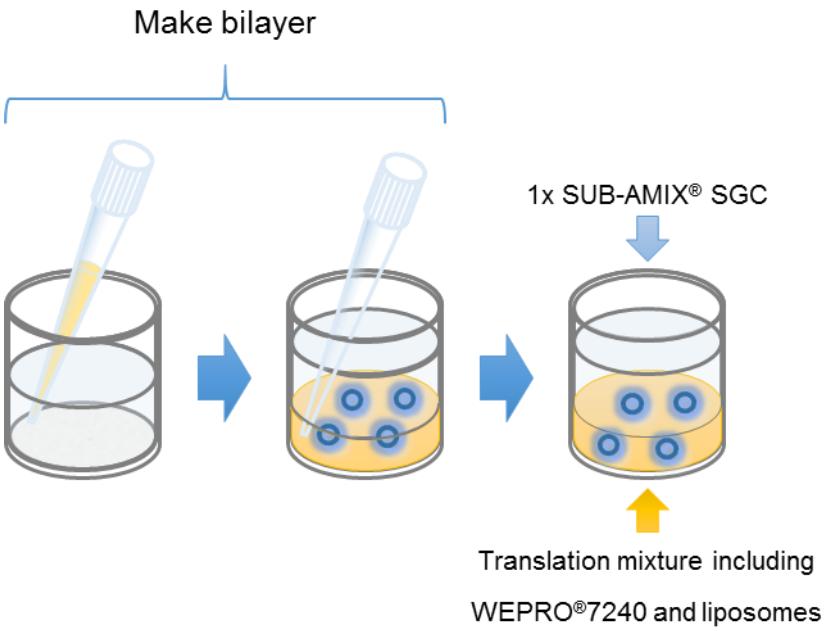
Reagents	Working vol.	Final conc.
1x SUB-AMIX® SGC	123 µl	
mRNA	150 µl	1/2 vol.
WEPRO®7240(240 OD)	125 µl	60 OD
Creatine Kinase (20 mg/ml)	2 µl	40 ng/µl
Asolectin Liposomes (50 mg/ml)	100 µl	10 mg/ml
Total	500 µl	

5. Carry out bilayer reaction: Carefully transfer the translation mixture (500 µl) to the bottom of the well containing 1x SUB-AMIX® SGC (3.5 ml) to form bilayer with the translation mixture in

the lower layer and 1x SUB-AMIX® SGC in the upper layer as illustrated in the figure below. It becomes easy by tilting a pipetting tip to add translation mixture. **DO NOT mix the reagents in the well by pipetting or any other means. (Important!!)**

6. Seal the well with cover tape to avoid evaporation.
7. Incubate at 15˚C for 20 hours.

Figure 3: Illustration on how to setup bilayer reaction



6. Purification of Proteoliposomes

6.1. Purification of Proteoliposomes

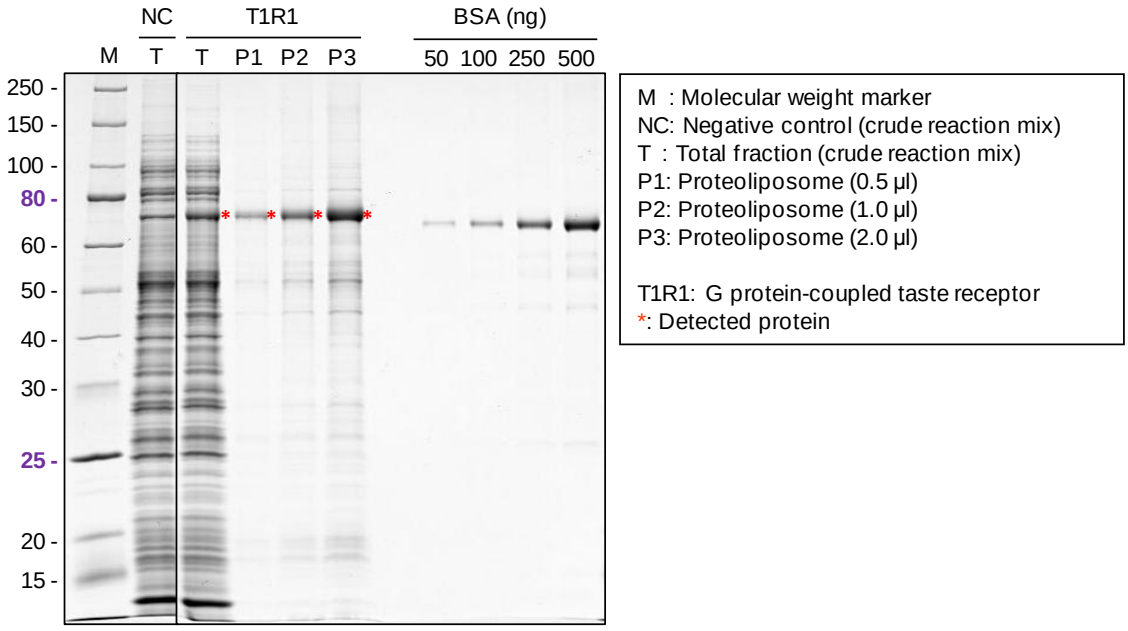
1. Mix the reaction mixture in the well of the 6-well plate by pipetting up and down, then transfer 1 ml each of the reaction mixture to a 1.5-ml tube. You will need 4 1.5-ml tubes for an entire 4 ml translation reaction. Avoid U-bottom tubes at this step because the proteoliposome pellet is easily detaching from bottom of the tube.
2. Add 1 ml of PBS to the well of 6-well plate 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 well of 6-well plate by pipetting up and down. Use the PBS to wash the surface of the well. 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 step 6 to 8 two times (in total washing pellet 3 times).
10. After the last centrifugation, remove supernatant and add appropriate volume of PBS to the pellet. Resuspend proteoliposome from 4 ml reaction in a total of 500 µl PBS. Resuspend pellet completely by pipetting up and down.

Store proteoliposmes in PBS at -80 ˚C.

6.2. Confirmation of Protein Expression into Proteoliposomes

Proteins contained in the proteoliposomes can be analyzed by SDS-PAGE gel electrophoresis. Load some 0.5 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 (Example of 2x SDS sample buffer: 150 mM Tris-HCl (pH6.8), 1.2% SDS, 24% glycerol, 0.1% bromophenol blue, 4% 2-mercaptoethanol).

Figure 4: SDS PAGE showing example data for the G Protein-Coupled Taste Receptor T1R1



7. References

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

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.

Eur J Pharmacol. 2014 Oct 16;745C:117-122.

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.; Mol Biosyst. 2014 Nov 28. [Epub ahead of print]

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 Note

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	115 µl	-	☐
5x Transcription Buffer LM	40 µl	1x	☐
NTP Mix (25 mM)	20 µl	2.5 mM	☐
RNase Inhibitor (80 U/µl)	2.5 µl	1 U/µl	☐
SP6 RNA Polymerase (80 U/µl)	2.5 µl	1 U/µl	☐
Plasmid (circular DNA, 1 µg/µl)	20 µl	100 ng/µl	☐
Total	200 µl	INCUBATE 6 h at 37°C	☐

Setup translation buffer SUB-AMIX SGC®:

Add 3.5 ml 1x SUB-AMIX SGC® to a well on a 6-well plate for setting bilayer reaction. Place translation reaction mix below the reaction buffer to form bilayer. DO NOT MIX THE TWO LAYERS.

Setup translation reaction mix:

Reagent	Volume	Final Concentration	Checkmark
mRNA	150 µl	1	☐
1x SUB-AMIX SGC®	123 µl		☐
WEPRO®7240 (240 OD)	125 µl	60 OD	☐
Creatine Kinase (20 mg/ml)	2 µl	80 µg/ml	☐
Asolectin Liposomes (50 mg/ml)	100 µl	10 mg/ml	☐
Total	500 µl	INCUBATE 20 h at 15°C	☐

Purification of proteoliposomes:

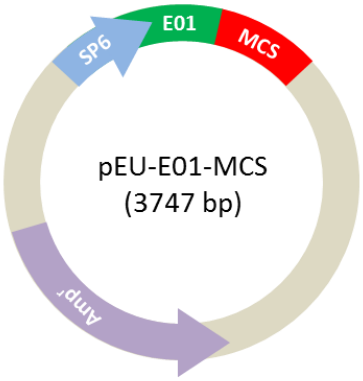
1. Transfer 1 ml each of the reaction mixture into four 1.5 ml tubes
2. Spin down proteoliposomes in each tube by centrifuge at 15,000 rpm, 4˚C, for 10 min
3. Carefully remove supernatant; do not remove all liquid, do not disturb the pellets

4. Add 1 ml PBS to the reaction well on the 6-well plate; wash well with the PBS buffer
5. Remove PBS buffer from well 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 *lacZ*-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
CTAACCACCTATCTACATCACCAAGATATCACTAGTTCTCGAGCTCGGTACCTGTCCGCGGTCGCG
ACGTACGCGGGCGGCCGCCATAAATTGGATCCATATATAGGGCCCGGGTTATAATTACCTCAGGTC
GACGTCCCATGGTTTTGTATAGAATTTACGGCTAGCGCCGGATGCGACGCCGGTCGCGTCTTATCC
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CGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT
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CellFree Sciences can provide the vector sequence as a MS Word file on request.

8. Others

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