ProteoLiposome PLUS Expression Kit

For small-scale expression of membrane proteins as proteoliposomes

(8 reactions on 240 µl bilayer reaction scale)

Instruction manual for proteoliposome preparation using wheat germ cell-free protein expression system and liposomes from asolectin

(Catalog No. CFS-EDX-PLUS-PLE)

CellFree Sciences Co., Ltd.

Store kit at -80°C

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.

For the ProteoLiposome PLUS Expression Kit, CellFree Sciences developed a reaction format based on WEPRO®9240 with added liposomes from asolectin. Asolectin from soybeans is a mixture of polyunsaturated phospholipids that is often used for the preparation of proteoliposomes and functional analysis of membrane proteins. In combination with the ready-to-use WEPRO®9240, protein expression reactions can easily be set up with few pipetting steps, and no fresh liposomes have to be prepared for each experiment. The ProteoLiposome PLUS Expression Kit uses a small-scale bilayer reaction format for testing the formation of proteoliposomes for a membrane protein of interest, and to possibly doing some functional testing on the protein obtained from an expression reaction. The reaction conditions used in this kit have been tested for the expression of various membrane proteins, where a 240 µl bilayer expression reaction yields for example for the G Protein-Coupled Taste Receptor T1R1 about 10 µg of protein in the purified proteoliposome fraction.

The ProteoLiposome PLUS Expression Kit provides all necessary reagents to perform eight protein expression reactions. In addition, the kit provides the pEU-E01-MCS expression vector for wheat germ cell-free protein expression system, and an expression vector for T1R1 as positive control.

For more information on the use of our wheat germ cell-free protein expression system 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 (included in this kit) 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 or contact us directly for more information on all our expression vectors, which can be obtained from CellFree Sciences (contact information provided at the end of the manual).

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 so-called "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. When using PCR products in the transcription reaction, make sure that the PCR yields the necessary DNA concentration of 1.0 µg/µl.

We do not recommend the use of PCR templates for preparative protein expression; do use cloned expression templates that have been properly characterized.

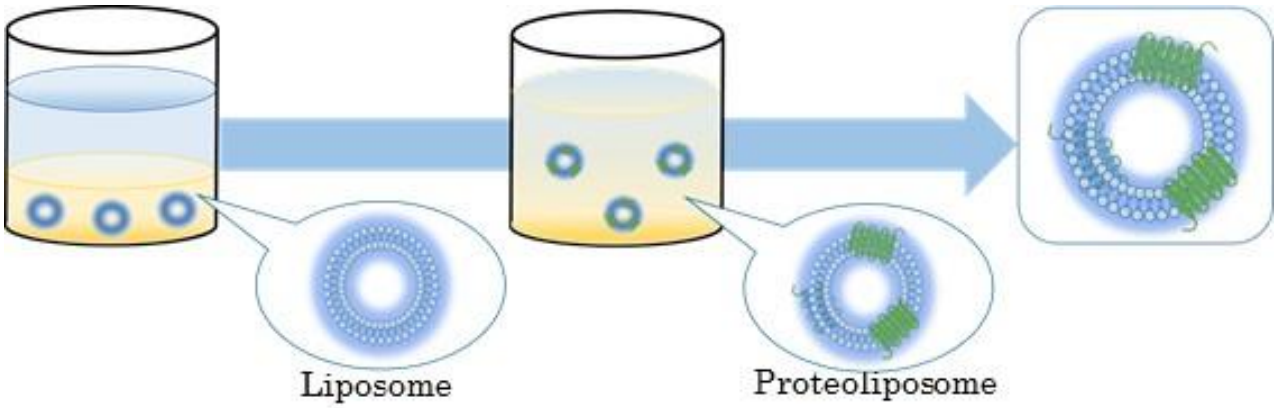
We strongly advise to test expression templates in small-scale expression reactions before doing any large-scale expression experiments. For such an initial vector testing, we recommend to perform for example some 240 µl bilayer reactions as included into this kit. Alternatively, expression vectors can also be tested in the absence of liposomes to just see whether the protein can be made. 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.

In the following protocol, we provide directions on how to set up a small-scale 240 µl bilayer reaction using the premixed reagents provided with the ProteoLiposome PLUS Expression Kit. For conducting more 227 µl bilayer reactions without added liposomes, you can purchase a Protein Research Kit (S), Product Number: CFS-PRK-24, from CellFree Sciences. This kit provides the WEPRO®9240 premixed reagents to perform 24 small-scale 227 µl bilayer expression reactions. For large-scale preparation of proteoliposomes, CFS further offers a ProteoLiposomes BD Expression Kit, Product Number: CFS-TRI-PLE-BD, for preparation of proteoliposomes on a 2.5 ml bilayer-dialysis format yielding a few hundred microgram of membrane protein per reaction. For more information regarding these and other 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 an 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 in the wheat germ cell-free protein expression system



4. Use of ProteoLiposome PLUS Expression Kit

The ProteoLiposome PLUS Expression Kit provides sufficient reagents to do all steps required to test the expression of your membrane protein of interest on a small-scale prior to preparing proteoliposomes on a preparative scale. In addition, the kit provides expression vector pEU-E01-MCS to prepare an expression vector for the protein of interest. In the following protocol, we are providing some advice on the preparation of an expression vector, template preparation, and give reaction conditions to perform the protein expression reactions. Proteins can be analyzed on a SDS-PAGE to see whether a protein of the correct size has been made.

5. Materials Provided by the Kit

The ProteoLiposome PLUS 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.

5.1. Kit Content

Item	Quantity	Concentration	Volume	Vial	Vial Color
pEU-E01-T1R1	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Green
pEU-E01-MCS	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Red
Transcription Premix LM*	8	1 x	18 µl	Strip of eight 0.2 ml PCR tubes	Blue
WEPRO®9L*	8		21 µl	Strip of eight 0.2 ml PCR tubes	Violet
SUB-AMIX® SGC*	8	1 x	210 µl	Strip of eight with wells	Clear
Aluminum seals	2	-	-	-	-

*Use total volume within each vial per one reaction. Cut off individual vials from the strips of eight wells, when doing less reactions. Do not thaw vials that are not used.

5.2. Instructions on Use of Reagents

Item	Description
pEU-E01-T1R1	Expression vector for human T1R1 This is a positive control for protein expression testing
pEU-E01-MCS	Expression vector for cloning your gene of interest Refer to Appendix A for more details on the vector
Transcription Premix LM	Premixed SP6 RNA polymerase transcription reagent
WEPRO®9L	WEPRO®9240 wheat germ extract with liposomes from asolectin
SUB-AMIX® SGC	High performance translation buffer
Aluminum seal	Seals to cover the wells during translation reaction Cut into appropriate size to cover well

6. Materials to Be Prepared by User

6.1. Reagents for optional Plasmid DNA Purification

Purify plasmid DNA by a commercial DNA purification kit. The following reagents are needed for further phenol extraction of the plasmid DNA (see Section 7.3).

Reagents	Description
Phenol/Chloroform	phenol:chloroform:isoamyl alcohol (25:24:1 v/v), 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 use DEPC treated water!

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

Consumable	Description
Nuclease-free water	DNase, RNase free. We DO NOT use DEPC treated water!
PBS	Phosphate buffered saline, pH7.5
Incubator	Temperature range 15 to 37°C
Centrifuge	For 1.5 ml tubes
SDS-PAGE	SDS-PAGE*, gel electrophoresis apparatus and power supply

*Use of commercially available SDS-PAGE can give better results and avoids the risk of working with toxic chemicals.

7. Protocols

For your safety:

Read this protocol carefully before starting the experiment. Do not drink or eat in the laboratory. Do take precautions to work under RNase free conditions following standard laboratory 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 got 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.

Inform yourself about necessary precautions for performing SDS-PAGE experiments using high voltage, and toxic chemicals in case you wish to prepare your own gels.

Material Safety Data Sheets (MSDS) for our products can be downloaded at:

http://www.cfsciences.com/eg/msds.html

Contact CellFree Sciences for further support and advice if you have any questions on the experiments described here and materials provided by CellFree Sciences.

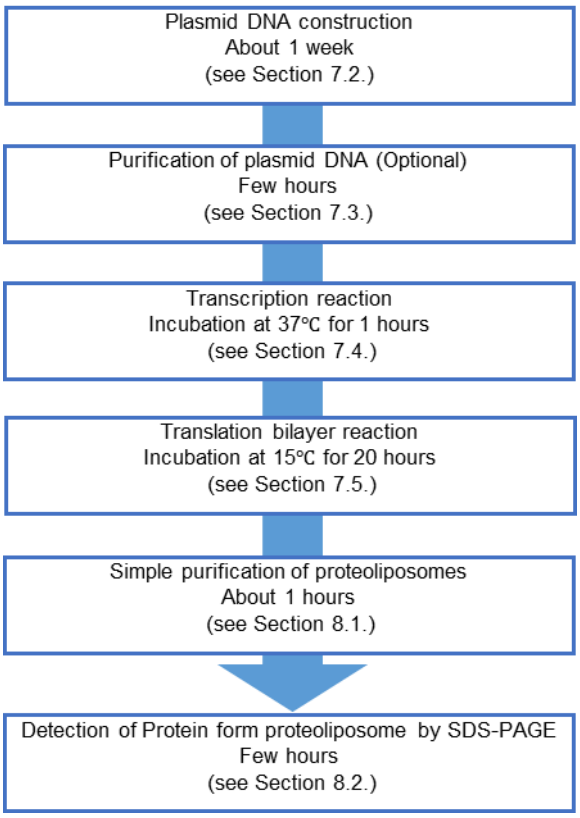
Laboratory standards:

This kit can be used in a regular molecular biology laboratory. We strongly 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 a short version of the protocol ("Bench Note"). Use the Bench Note to setup your transcription and translation experiments at the bench. They only contain the basic information needed for setting up the experiments. Use the checkmarks in the Bench Note to assure that all pipetting steps have been completed.

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 on a preparative scale.

In the following we give some brief advice on the use of our expression vector. Those vectors, including pEU-E01-MCS included in this kit, contain a SP6 promoter, an E01 translational enhancer, and an ampicillin resistance gene. 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. Noncoding regions flacking the ORF have to be removed when preparing an expression vector. Refer to a handbook for more information on how to conduct DNA cloning experiments.

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 information on the multi cloning site (MCS) 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 an ATG start at the 5' end and a stop codon at the 3' end. Vector pEU-E01-MCS does not provide any stop codons to terminate protein synthesis.**

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. Grow transformed bacteria on Lysogeny Broth (LB) medium plus ampicillin at a concentration of 100 µg/mL.
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.

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 at http://www.cfsciences.com/eg/vector.html.

(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.

7.3. Preparation of Plasmid DNA Templates

We DO NOT recommend the use of DNA mini-preparation methods by the alkaline elution procedure without any further purification for direct use in our expression system. Instead prepare plasmid DNA from *E. coli* cultures using a commercially available DNA purification kit for use in the protein expression experiment. 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.

A highly purified plasmid DNA is essential for successful transcription and subsequent translation reactions. The protein synthesis may not proceed properly, if the plasmid DNA does not have a proper A260/A280 ratio (proteins remained in the DNA preparation). Remaining proteins in the DNA

preparation may lead to a low quality of RNA transcripts, or poor RNA yields. 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:

1. Add an equal volume of phenol/chloroform (see section 6.1) to the purified plasmid DNA solution and mix well (*1).
2. Centrifuge the mixture at 15,000 rpm for 5 min at room temperature.
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 at room temperature.
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. Mix solutions and store at -20°C for 10 min.
9. Centrifuge at 15,000 rpm for 20 min at 4°C.
10. Remove the supernatant. Do not disturb the DNA pellet.
11. Add 800 µl of 70% ethanol to wash the remaining DNA pellet in the tube.
12. Centrifuge the tube at 15,000 rpm for 10 min at 4°C.
13. Remove the supernatant. Do not disturb the DNA pellet.
14. Dry the DNA pellet for 10 to 20 min (do not dry pellet for extended period of time).
15. Add an appropriate volume of TE buffer to resuspend the DNA pellet.
16. 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).
17. Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer (*3).

You need 2 µg purified plasmid DNA per 240 µl translation reaction. In case you later want to upscale protein expression, 13 µg purified plasmid DNA are needed per 2.5 ml BD translation reaction provided with the ProteoLiposome BD Expression Kit.

Plasmid DNA can be stored for a long time at -20°C. We advise to keep aliquots of the vector DNA for later use.

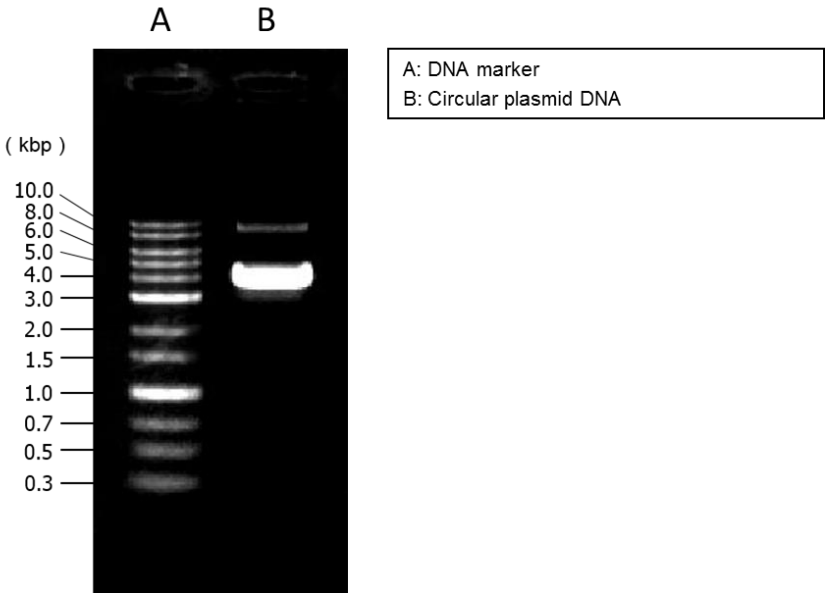
(Notes)

*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 have an A260/A280 ratio 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.

*3: Plasmid DNA quality can be further confirmed by agarose gel electrophoresis using some 0.1 to 0.2 µg of DNA.

Figure 2: Example for a 1 % agarose gel showing circular plasmid DNA for vector pEU-E01-T1R1



7.4. Transcription from Plasmid DNA Template

We recommend to use positive control plasmid, pEU-E01-T1R1 (green vial), in a separate expression experiment to make sure that the experiment has been setup correctly. Per reaction perform the following steps:

1. Thaw your template DNA before the experiment. You need 2 µg of plasmid DNA per reaction.
2. Take one vial with Transcription Premix LM (blue vial) per reaction from storage at -80 ˚C (*1).
3. Thaw required number of Transcription Premix LM vials on ice. Do not thaw unneeded vials and wells. Put the remaining vials and wells back into the freezer and store them at -80°C immediately. After thawing, spin the vials briefly to collect the entire volume at the bottom of the vial. Mix the reagent gently before use. Place reagents on ice and keep it cold at all times.

4. Add 2 µl of highly purified plasmid DNA (1.0 µg/µl) to each vial with the Transcription Premix LM shown in the table below. Then mix gently by pipetting up and down.

Table 1: Preparation of transcription mixture

Reagents	Working Volume	Final Concentration
Transcription Premix LM	18 µl	1x
Plasmid (circular DNA, 1.0 µg/µl)	2 µl	100 ng/µl
Total	20 µl	

5. Incubate at 37°C for 1 hours in incubator (*2).
6. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis loading 0.5 µl of the reaction mixture (*4). Refer to a cloning handbook for more information on how to perform RNA gel electrophoresis.

(Notes)

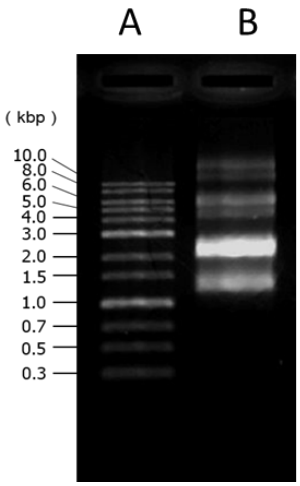
*1: The strip holding the Transcription Premix LM vials can be cut into individual tubes by bending or cutting. Hold the vials firmly so that they do not pop open while separating them.

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

*3: A smear or RNAs smaller than 500 bases indicate possible degradation of the RNA probably caused by RNases. 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.

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

Figure 3: Example for RNA transcripts analyzed on a 1% agarose gel



A: RNA marker
B: Transcription reaction mixture

7.5. Translation Reaction Using Bilayer Method

1. Take a single vial with WEPRO®9L (violet vial) and a single well (clear well) containing SUB-AMIX® SGC from storage at -80°C per reaction (*1). Do not thaw unneeded vials and wells. Put the remaining vials and wells back into the freezer and store them at -80°C immediately. WEPRO®9L losses its activity if not kept at -80°C.
2. Thaw reagents on ice. After thawing, spin down each vial with WEPRO®9L briefly to collect the reagent at the bottom of the vial. Avoid excessive centrifugation of WEPRO®9L!
3. Resuspend SUB-AMIX® SGC by pipetting gently up and down in the well (*2).
4. Let the transcription mixture from the transcription reaction(s) cool down to room temperature. DO NOT forcibly cool it down on ice or in a refrigerator. Resuspend the transcription mixture by pipetting gently up and down (*3).
5. Prepare "translation mixture" by adding 9 µl of "transcription mixture" containing the RNA template to the vial containing the WEPRO®9L. Mix gently by pipetting up and down, avoid bubbles.

Table 2: Preparation of translation mixture

Reagents	Working Volume	Final Concentration
Transcription mixture (mRNA)	9 µl	0.3 vol.
WEPRO®9L	21 µl	60 OD
Total	30 µl	-

6. Carefully transfer the translation mixture (30 µl) to the bottom of a single well containing SUB-AMIX® SGC (210 µl) to form bilayer with the translation mixture in the lower layer and SUB-AMIX® SGC in the upper layer. Refer to Figure 4 on how to setup bilayer reaction: Go with the pipette tip the bottom of the well, and slowly release the translation mixture below the reaction buffer. You can distinguish the translation mixture from the translation buffer in the well, because of its yellowish color.

   DO NOT mix the reagents in the well by pipetting or any other means! It will reduce the yield of the reaction.

Table3. Bilayer reaction

Reagents	Working Volume	Final Concentration
SUB-AMIX® SGC	210 µl	1 x
Translation mixture	30 µl	
Total	240 µl	-

7. Seal the well with the aluminum seal included in the kit to avoid evaporation (*4).
8. Incubate at 15˚C for 20 hours.
9. After completion of the translation reaction, mix the bilayer reaction gently by pipetting up and down.

(Notes)

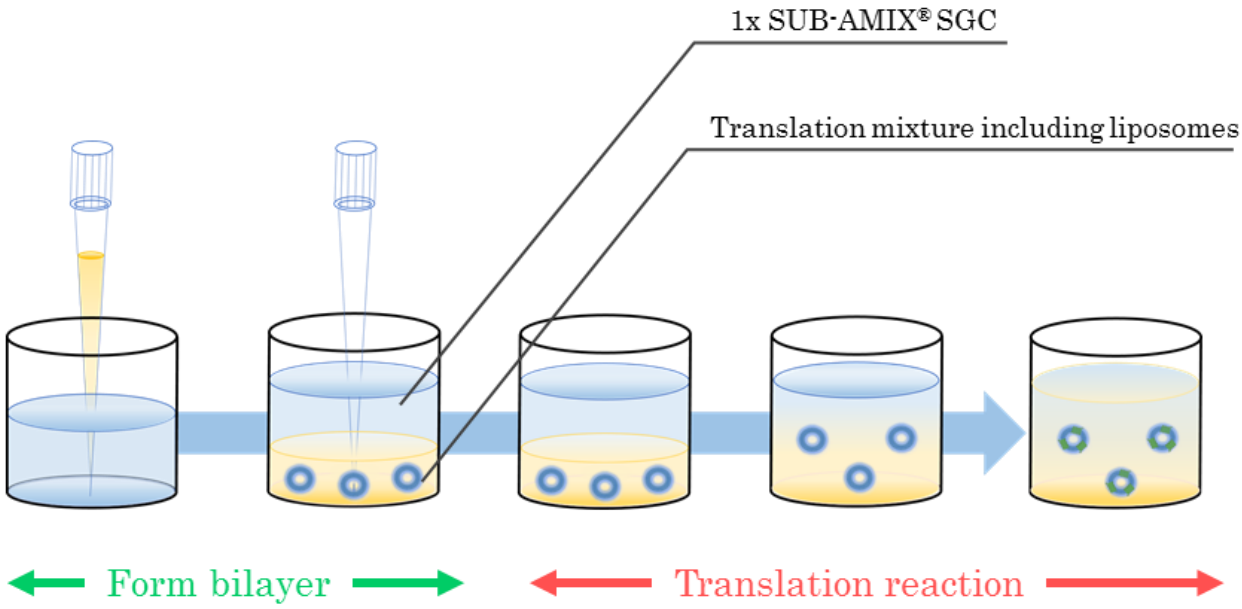
*1: The strips with the WEPRO®9L vials and SUB-AMIX® SGC wells can be split into individual vials or wells by bending or cutting. Hold the vials with the WEPRO®9L firmly so that they do not pop open while separating them.

*2: Take special care to keep SUB-AMIX® SGC wells upright at all times. They easily tip over, which would disturb the bilayer.

*3: If you notice a white precipitate after the transcription reaction, resuspend the precipitate by pipetting gently up and down before mixing with the . WEPRO®9L. There is no need to remove the precipitate.

*4: Cut off aluminum seals of an appropriate size to cover the wells. Peel off the brown cover on the back of the seals and glue them on the well. Press down the seal onto the well to make sure it covers the entire surface of the well. The seal can be easily removed after the completion of the reaction by simply pulling it up. Save the remaining seal.

Figure 4: Illustration on how to setup bilayer reaction



8. Simple Purification of Proteoliposomes

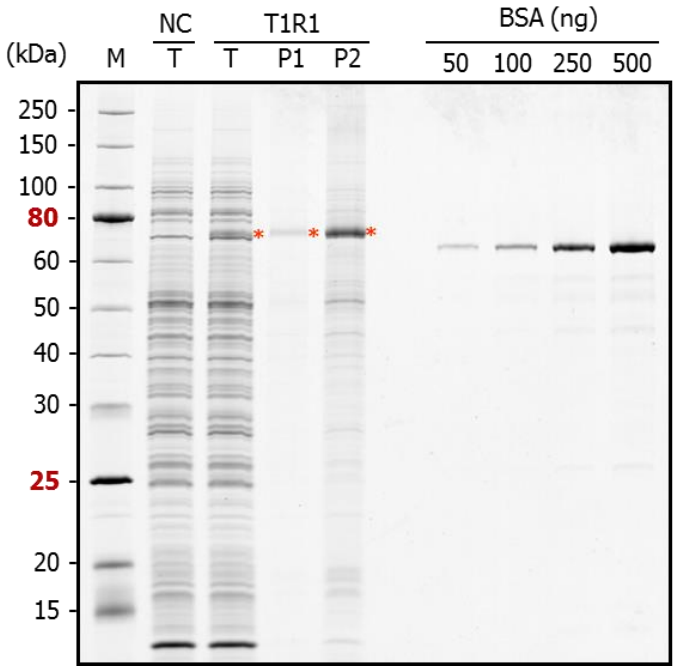
8.1. Purification of Proteoliposomes

1. Mix the reaction mixture in the well by pipetting up and down. Then transfer 200 µl of the reaction mixture to a 1.5-ml tube. Avoid U-bottom shaped tubes at this step because the proteoliposome pellet is easily detaching from bottom of the tube.
2. Add 170 µl of PBS to the well from the translation mixture. This PBS will be used later.
3. Centrifuge the 1.5 ml tube at 15,000 rpm, 4˚C, for 10 min.
4. Remove supernatant. Do not disturb the pellet (it should be visible as a small white pellet). Take care not to remove whole supernatant, leave a small volume of supernatant in the tube to protect the pellet at the bottom of the tube.
5. Mix the PBS in the well by pipetting up and down. Use the PBS to wash the surface of the well. Transfer the PBS to the 1.5 ml tube a containing proteoliposome pellet and suspend the pellet well.
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 170 µl of PBS to the 1.5 ml tube and resuspend it by pipetting.
9. Repeat washing steps 6 to 8 two more times (in total washing the pellet 3 times).
10. After the last centrifugation, remove supernatant completely, do not disturb the pellet. Resuspend proteoliposome pellet in a total of 200 µl PBS. Resuspend pellet completely by pipetting up and down.
11. For later use, you can freeze proteoliposomes in liquid nitrogen and store them in PBS at -80 ˚C. Avoid repeated freeze/thawing of the proteoliposomes.

8.2. Confirmation of Protein Expression and Proteoliposome Formation

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 samples before loading.** We have observed that the protein may not enter the gel after the sample 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 6: SDS-PAGE showing example data for the G Protein-Coupled Taste Receptor T1R1



M:	Molecular weight marker
NC:	Negative control
T1R1:	G protein-coupled taste receptor
T:	Total fraction (crude reaction mix)
P1:	Proteoliposome (1 µl)
P2:	Proteoliposome (5 µl)
*	: Detected protein

9. Troubleshooting

The experiments require correct and accurate pipetting during reaction setup. Any mistakes in the volumes added to the reactions, mixing the reagents, or forgetting any of the reagents will lead to wrong results. Therefore carefully check the number on each reagent tube prior to starting the pipetting step.

Mark in your protocol each pipetting step you have completed, or use the Bench Notes for your records.

Change the pipetting tip after each pipetting step. Do not use the same pipetting tip to pipette different reagents or reaction mixtures.

Leaving out the plasmid template will always yield negative results.

Do not mix the two layers during setup of the bilayer translation reaction. Mixing both layers will sharply reduce the protein yields as the reaction will run dead within few hours.

If protein is not expressed, check reaction conditions, reagents and DNA template in a small-scale expression reaction to confirm their integrity. If the results are unclear, you can check the performance of the transcription and translation reactions separately to narrow down the problem. Use the positive control vector provided with the kit to make sure that all reagents work, and the experiment is done correctly. The kit provides enough vector DNA to conduct two control experiments.

Make sure you are working under RNase free conditions.

Make sure that the wheat germ extract had been keep frozen at all time before. Avoid repeated freeze/thawing; it will inactivate the extract.

Store wheat germ extract at -80°C; storage at higher temperature will lead to low activity or even total loss of activity.

Proteoliposomes can be frozen and stored at -80°C. However, for functional analysis of membrane proteins, you should first test the proteoliposomes directly without freezing before working with proteoliposomes that had been frozen down.

Contact the technical support of CellFree Sciences for further help.

10. References

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

A Barley Efflux Transporter Operates in a Na+-Dependent Manner, as Revealed by a Multidisciplinary Platform. Nagarajan Y, Rongala J, Luang S, Singh A, Shadiac N, Hayes J, Sutton T, Gilliham M, Tyerman SD, McPhee G, Voelcker NH, Mertens HD, Kirby NM, Lee JG, Yingling YG, Hrmova M. Plant Cell. 2016 Jan;28(1):202-18.

Cell-Free Synthesis of a Functional Membrane Transporter into a Tethered Bilayer Lipid Membrane. Zieleniecki JL, Nagarajan Y, Waters S, Rongala J, Thompson V, Hrmova M, Köper I. Langmuir. 2016 Mar 15;32(10):2445-9.

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.

11. Others

Label License Policy

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

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Others

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All specifications are subject to change without prior notice.

Bench Note

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

Setup transcription reaction:

Reagent	Volume	Final Concentration	Checkmark
Transcription Premix LM	18 µl	1x	☐
Plasmid (circular DNA, 1.0 µg/µl)	2 µl	100 ng/µl	☐
Total	20 µl	INCUBATE 1h at 37°C	☐

Setup translation reaction:

Add transcription mixture to wheat germ extract and mix gently by pipetting up and down.

Reagent	Volume	Final Concentration	Checkmark
Transcription mixture (mRNA)	9 µl		☐
WEPRO®9L	21 µl	60 OD	☐
Total	30 µl		☐

Place translation reaction mixture below the reaction buffer to form bilayer. DO NOT MIX LAYERS!

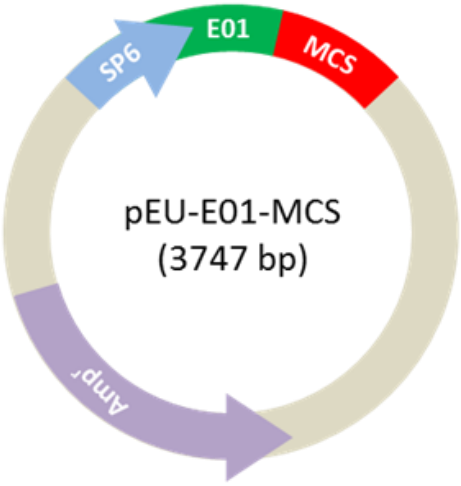
Reagent	Volume	Final Concentration	Checkmark
SUB-AMIX® SGC	210 µl	1x	☐
WEPRO®9L plus Transcription mixture (mRNA)	30 µl	-	☐
Total	240 µl	INCUBATE 20 h at 15°C	☐

Purification of proteoliposomes:

1. Transfer 200 µl of the reaction mixture into four 1.5 ml tube
2. Spin down proteoliposomes in the 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 pellet
4. Add 170 µl PBS to the reaction well used for the translation reaction; wash well with the PBS buffer
5. Remove PBS buffer from well and resuspend pellet in the first 1.5 ml tube
6. Spin down proteoliposomes in the PBS buffer by centrifuge at 15,000 rpm, 4˚C, for 10 min
7. Carefully remove supernatant; do not remove all liquid, do not disturb the pellet
8. Wash the pellet two more times with 170 µl PBS buffer per washing step (in total 3 washing steps)
9. Resuspend the pellet after the final showing step in 200 µl PBS buffer
10. Optimally, freeze proteoliposomes in liquid nitrogen and store them in PBS at -80 ˚C. Avoid repeated freeze/thawing of the proteoliposomes

Appendix A: Vector Map for pEU-E01-MCS

Map:



Multi cloning site (MCS):

Vector elements:

SP6 Promoter (SP6): -17-1

Translation Enhancer E01 (E01): 1-73

Multi cloning site (MCS): 74-193

Origin: 1190-1830

Ampicillin resistance gene (AMP^r): 1974-2838

Position 1 is located at the final G of the SP6 Promoter: ATTTAGGTGACACTATA**G**

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

Standard M13 sequencing primers are available from different providers. Alternatively, customized sequencing primers can be prepared by DNA synthesis. All sequencing primers should be purified by gel electrophoresis or HPLC.

pUC/M13 Sequencing Primers

The pUC/M13 Primers are designed for sequencing inserts cloned into the M13 vectors and pUC plasmids. These primers can also be used for sequencing other *lacZ*-containing plasmids such as the pGEM®-Z and pGEM®-Zf Vectors.

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

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

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

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

For 5' end sequence: SP6 Primer

5'-ATTTAGGTGACACTATAGAA-3'

For 3' end sequence

5'-CCTGCGCTGGGAAGATAAAC-3'

Appendix C: Vector Sequence of pEU-E01-MCS

ATTTAGGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCA
CTAACCACCTATCTACATCACCAAGATATCACTAGTTCTCGAGCTCGGTACCTGTCCGCGGTCGCG
ACGTACGCGGGCGGCCGCCATAAATTGGATCCATATATAGGGCCCGGGTTATAATTACCTCAGGTC
GACGTCCCATGGTTTTGTATAGAATTTACGGCTAGCGCCGGATGCGACGCCGGTCGCGTCTTATCC
GGCCTTCCTATATCAGGCGGTGTTTAAGACGCCGCCGCTTCGCCCAAATCCTTATGCCGGTTCGAC
GACTGGACAAAATACTGTTTATCTTCCCAGCGCAGGCAGGTTAATGTACCACCCCAGCAGCAGCCG
GTATCCAGCGCGTATATACCTTCCGGCGTACCTTTGCCCTCCAGCGATGCCCAGTGACCAAAGGCG
ATGCTGTATTCTTCAGCGACAGGGCCAGGAATCGCAAACCACGGTTTCAGTGGGGCAGGGGCCTCT
TCCGGCGATTCTTACTAGCTAGTATGCATAGGTGCTGAAATATAAAGTTTGTGTTTCTAAAACACA
CGTGGTACGTACGATAACGTACAGTGTTTTTCCCTCCACTTAAATCGAAGGGTAGTGTCTTGGAGC
GCGCGGAGTAAACATATATGGTTCATATATGTCCGTAGGCACGTAAAAAAAGCGAGGGATTCGAAT
TCCCCCGGAACCCCCGGTTGGGGCCCACGCCTCGATCGAGCAAAAAAAAAAAAAAAGAAAAAAAAA
AAAAAAAAAAGCTTTCCCGCGGCCAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAAT
TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCC
TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTG
TCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT
TCCGCTTCCTCACTCACTGACTCGCTGCGCTCGGTCGCTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAA
GGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCC
CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGA
TACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTC
AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC
TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCA
GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGG
TGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC
TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTT
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GGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGG
ATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAA
ACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGT

TCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGC
CCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAG
CCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAAT
TGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCT
ACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCA
AGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTT
GTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACT
GTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAG
CGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGA
ACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTG
TTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACC
AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG
AAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTC
ATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
CGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT
ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTC
CCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCA
GCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTG
CACCATTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCC
GTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCAC
GGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTC
CCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCAC
GATGCGTCCGGCGTAGAGGATCTGGCTAGCGATGACCCTGCTGATTGGTTCGCTGACCATTTCCGG
GTGCGGGACGGCGTTACCAGAAACTCAGAAGGTTCGTCCAACCAAACCGACTCTGGCGGCAGTTTA
CGAGAGAGATGATAGGGTCTGCTTCAGTAAGCCAGATGCTACACAATTAGGCTTGTACATACTGTC
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.

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ProteoLiposome PLUS Expression Kit,_E_ver. 1.1, Jul. 25, 2018.