CellFree Sciences

The natural power of wheat driving science

High Performance Cell-Free Wheat Germ Protein Expression System

INSTRUCTION MANUAL

ProteoLiposome PLUS Expression Kit

This kit provides premixed reagents for 8 linked transcription-translation reactions to express membrane proteins as proteoliposomes

Product Number(s): CFS-EDX-PLUS-PLE

Version/date: Version 2.0_eng/April 2022

This Product has a shelf life of 1 year being safely stored at -80°C.

CFS products are for research use only.

All our manuals are available for download in PDF versions on our homepage at: https://www.cfsciences.com/eg/. Work with electronic versions where possible to reduce paper waste.

Our products are produced under a strict quality management system offering high-quality reagents including wheat germ extracts from wheat obtained by natural farming in Japan.

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Web: https://www.cfsciences.com/eg/ Follow us on Twitter: @CFSciences

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Important Information

Shipment and Storage

Our products are shipped on dry ice. Wheat germ extracts are temperature sensitive and must always be kept frozen. Store kit at -80°C right upon arrival and only thaw reagents when needed. Avoid repeated freeze/thawing cycles.

Safety

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.

Read the protocol carefully before starting the experiment.

Do not drink or eat in the laboratory, and always wear gloves and a lab coat while working in the lab.

Wash hands before and after doing an experiment. If you get any reagent(s) 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.

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

Safety Data Sheets (SDS) for our products can be downloaded from our homepage at:

https://www.cfsciences.com/eg/

Look up your product to find the matching SDS.

Contact CellFree Sciences for further support and advice if you have any questions on the experiments described herein and materials provided with this product. Contact information is given at the end of this manual.

For your convenience

CellFree Sciences is providing short versions of our protocols (“Bench Notes”). Use these Bench Notes to setup your experiments at your workplace. 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 correctly.

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 new WEPRO®9L, protein expression reactions can easily be set up with few pipetting steps, and no fresh liposomes must 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.

To prepare proteoliposomes on a larger scale purchase our ProteoLiposome BD Kit (CFS-CPLE-BD). This kit uses the new BD (Bilayer-Dialysis) reaction format to achieve higher protein yields. The reagents provided with the kit allow to perform 6 bilayer-dialysis reactions on a 2.5 ml reaction scale.

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.

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 many 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.

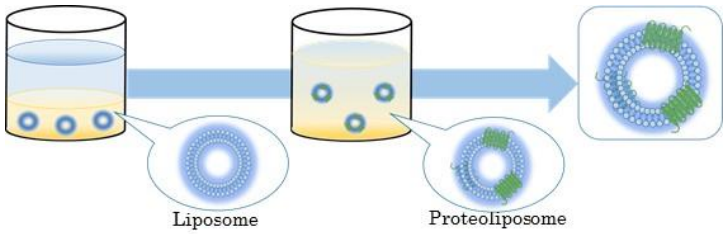
We strongly advise to test expression templates in small-scale expression reactions before doing any large-scale expression experiments. For such an initial vector testing, we recommend performing for example some 240 µl bilayer reactions as included into this kit. Alternatively, expression vectors can 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 Premium PLUS Expression Kit, Product Number: CFS-EDX-PLUS, or the larger Protein Research Kit S16, Product Number: CFS-PRK-S16, from CellFree Sciences. These kits provide the same WEPRO®9240 premixed reagents to perform 8 or 16 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 micrograms 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.

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



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 embedded into proteoliposomes can be analyzed on an SDS-PAGE to see whether a protein of the correct size has been made.

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-thaw cycles. The wheat germ extract will rapidly lose activity when kept above -80°C!

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.

Instructions on Use of Reagents

Item	Description
Transcription Premix LM	Pre-mixed transcription reaction mixture including SP6 RNA polymerase. Avoid unnecessary freeze-thawing of the Transcription Premix LM!
WEPRO®9L	Pre-mixed wheat germ extract with added creatine kinase and liposomes from asolectin for linked protein expression system. The liposomes added to the wheat germ extract have a particle size with a peak distribution at about 150 nm based on scattered light intensity measurement. These liposomes should be close to a monodispersed distribution. Avoid freeze-thawing of the wheat germ extract; just remove the tubes needed for each experiment. Store buffer at -80 °C.
SUB-AMIX® SGC	Translation reaction buffer for protein expression reactions. Store buffer at -80 °C.
pEU-E01-MCS	Standard expression vector for use in wheat germ cell-free protein expression system, refer to Appendix A for more details on the vector.
pEU-E01-T1R1	Expression vector for human T1R1: This is a positive control for protein expression testing.
Aluminum seals	Seals to cover the wells during translation reaction. Cut into appropriate size to cover well.

Materials to Be Prepared by User

Reagents for optional Plasmid DNA Purification

Plasmid DNA should be prepared by a commercial DNA purification kit. The following reagents are only needed for an optional phenol extraction of the plasmid DNA. A phenol extraction is not mandatory but can help to remove impurities from standard plasmid DNA preparations when a vector gives low protein yields.

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. - DO NOT use DEPC treated water!

Other Consumables and Instruments Required for Membrane Protein Expression

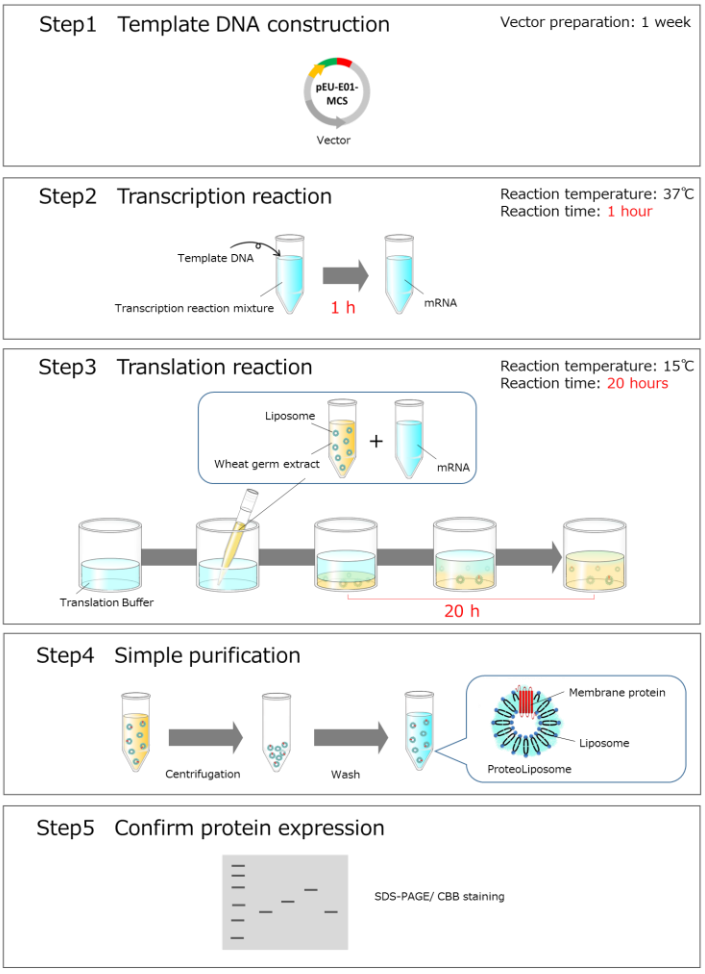
Consumable	Description
Incubator	Temperature set to 15 to 37°C, the transcription reaction can be performed in a thermocycler
1.5 ml Tubes	Required to purify proteoliposomes
Centrifuge	For 1.5 ml tubes
Nuclease-free water	DNase, RNase free. We DO NOT use DEPC treated water!
PBS	Phosphate buffered saline, pH7.5
SDS-PAGE	SDS-PAGE*, gel electrophoresis apparatus and power supply

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

Protocols

Time Requirements

Refer to the chart below on the estimated time per protein expression and purification steps. Preparing a new expression vector may take up to one week.



Considerations on Obtaining cDNA Templates for Proteins of Interest

Gather information on your protein of interest before preparing your expression template. Expression templates can be easily made by standard cloning methods or gene synthesis, which may be further used to do some codon optimization for expression in a wheat system. However, codon optimization is not required for using our expression system. Otherwise, for many protein coding genes cDNA clones are available in the public domain. There are large cDNA collections from which cDNA clones encoding for proteins of interest may be available. These clones are commonly distributed through clone distributors or public depositories. Searching such cDNA collections can be an easy and convenient way to find cDNAs clones other than requesting published materials from other researchers. In general, for inquires on certain genes you can make a search at "Gene" on NCBI homepage at:

https://www.ncbi.nlm.nih.gov/gene/

Gene holds information on reference sequences from RefSeq, maps, pathways, variations, phenotypes, and links to genome-, phenotype-, and locus-specific resources worldwide. While the sequence information may be useful for using gene synthesis services, the links to worldwide resources are most useful to look for matching cDNA clones.

At the very end of the page, you will find "Additional links", where you must get the list under "Gene LinkOut" (you may have to click on the + sign to see the complete list). NCBI allows suppliers to put there their links to products and services on the specified gene shown in the output page.

Click on the links for getting more information on each product to see whether this is what you want to obtain. For making a protein, you require information on available cDNAs for cloning into an expression vector. There had been several large cDNA cloning projects to provide ORF clones for most human protein coding genes. Look under "Research Materials" to see those resources, where you commonly will find various providers distributing academic clone collections like the ones offered by the "NITE Biological Resource Center" (a Japanese National Project), or the international ORFeome Collaboration clones.

As an alternative to searching Gene at NCBI, you may consider to directly searching the databases of different clone providers and public depositories. They commonly provide comprehensive information on their clones and the resources they are offering.

Note, that a cDNA clone just represents one possible isoform. However, for most transcripts from higher organisms there are multiple splice variants that commonly encode for different proteins. As an alternative to searching matching cDNA clones, gene synthesis services offer a convenient way to get access to ready to use expression vectors. Working with a gene synthesis provider allows you to fully avoid any cloning experiments. However, you should be careful about selecting the correct sequence information and correct insertion into the expression vector (see below). Gene synthesis may also be used to design templates for fusion proteins.

For more information on your protein of interest, refer to the UniProtKB database (https://www.uniprot.org/). Basic physical and chemical parameters for a protein can be calculated by the ProtParam tool (https://web.expasy.org/protparam/).

Remarks on Expression Vector Construction

We recommend preparing an expression vector 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 for continuous protein production and to up-scale protein production to a preparative scale.

In the following we give some brief advice on the use of our expression vectors. This kit contains expression vector pEU-E01-MCS (this vector does not encode any tag; refer to Appendix A for more details; red vial). The pEU-E01-MCS vector, and the positive control vector included in the kit, contain a SP6 promoter, an E01 translational enhancer,

and an ampicillin resistance gene. If you obtained a standard cDNA clone, it may be necessary to isolate the coding region (Open Reading Frame or "ORF") for later cloning into any of our expression vectors. Noncoding regions flacking the ORF must be removed when preparing an expression vector. Additional considerations apply when expressing protein fragments to assure proper use of the starting methionine and stop codon. Refer to a cloning handbook for more information on how to conduct vector design and DNA cloning experiments.

1. Insert the coding region for your protein of interest into the multiple cloning site (MCS) of the vector using one or two restriction enzyme sites properly selected according to the information on the MCS in the vector map for vector pEU-E01-MCS (Appendix A) (*1). The protein will be translated from the first start codon, an ATG, up to the first in frame stop codon in your cDNA.
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 added ampicillin at a concentration of 100 µg/mL; the same ampicillin concentration can be used to growing bacteria transformed with the positive control vector included into the kit. Our vectors are high-copy vectors and should commonly give good yields for DNA preparations in line with the yield ranges expected for a plasmid DNA purification kit.
3. Once you have selected an expression vector having the correct insert with the correct orientation and reading frame, we advise to prepare glycerol stocks from the transformed bacteria, and to store bacteria for future use. It is also possible to store DNA aliquots of the expression vector.

We recommend confirming correct insertion of the cDNA into the expression vector by at least end-sequencing of the insert and both cloning sites. In case the cDNA insert was prepared by PCR, sequencing of the entire insert is recommended to exclude inserts with PCR errors. Refer to Appendix B for more information on sequencing primers for our vectors. The vector sequence information for vector pEU-E01-MCS and all our other vectors can be downloaded from our homepage at http://www.cfsciences.com/eg/vector.html.

(Notes)

*1: To efficiently express the target protein, it is recommended to select a restriction enzyme site as close as possible to the E01 translational enhancer when cloning into vector pEU-E01-MCS. For cloning cDNAs into this vector, do not add a Kozak consensus sequence. The E01 translational enhancer is sufficient to induce translation.

Preparation of Plasmid DNA Template

We do not recommend the use of DNA mini-preparation methods based on alkaline elution procedures lacking any further purification step. Those may not work when directly using the vector in our expression system. Instead prepare plasmid DNA from *E. coli* cultures 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 have commonly worked well in combination with our expression system. Comparable products from another provider may as well provide suitable results.

A highly purified plasmid DNA is essential for successful transcription and subsequent translation reactions. The protein synthesis may not proceed well, 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 that can remove proteins and some other contaminations:

1. Add an equal volume of phenol/chloroform to the 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 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 longer period).
15. Add an appropriate volume of TE buffer to resuspend the DNA pellet.
16. Determine the concentration of the DNA with a spectrophotometer at wavelengths of 260 nm and 280 nm. Absorbance at 260 nm represents the concentration of DNA. The ratio of absorbance at 260 nm and at 280 nm indicates the purity of the DNA (*2).
17. Adjust the DNA concentration to 1 µg/µl by adding an appropriate volume of TE buffer (*3).

You need 2 µl purified plasmid DNA per 240 µl expression reaction according to this kit manual. In principle, plasmid DNA can be stored for a long time at -20°C. We advise to keep aliquots of the vector DNA for later use.

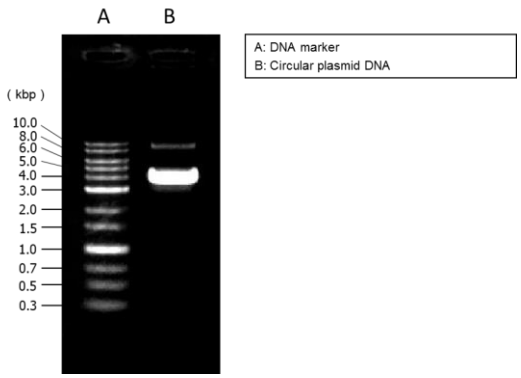
(Notes)

*1: Phenol and chloroform are hazardous chemicals and should only be handled with appropriate care and precautions. Note that phenol and chloroform must 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 this range indicate that the plasmid DNA is still not suitable for conduction the expression experiment. In that case, repeat the phenol extraction from the beginning.

*3: Plasmid DNA quality can be further confirmed by agarose gel electrophoresis loading some 0.1 to 0.2 µg of DNA on a standard or small agarose gel.

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



Protein Expression from DNA Template

The linked cell-free protein expression is performed by two separate reactions, where first an RNA transcript is prepared from the DNA template encoding for the protein of interest. The RNA transcripts are then used in the following translation reaction.

Transcription Reaction Using DNA Template

We recommend using 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 (*1).
2. Take one vial with Transcription Premix LM (blue vial) per reaction from storage at -80 ˚C (*2). Put the remaining vials and wells back into the freezer and store them at -80°C immediately.
3. Thaw required number of Transcription Premix LM vials on ice. Do not thaw unneeded vials. 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 always keep it cold.
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.

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

5. Incubate at 37°C for 1 hour in an incubator (*3).
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)

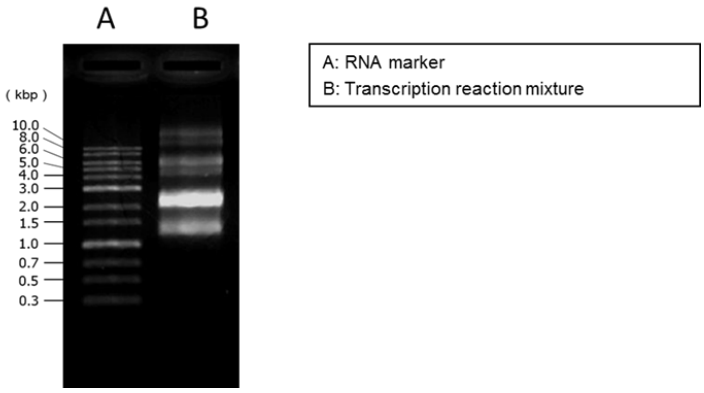
*1: Commonly plasmid DNA prepared by a commercial DNA purification kit is suitable for use in protein expression experiments. Do not use plasmid DNA from alkaline lysis without further purification.

*2: 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.

*3: White precipitate may occur during incubation. This is magnesium pyrophosphate, which will not interfere with the following translation experiment. Use the whole reaction mixture including the precipitate in the next step.

*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



Translation Reaction Using RNA Prepared from DNA Template

After completion of the transcription reaction, let the reaction mixture cool down to room temperature. Do not forcibly cool it down on ice or in a refrigerator. Per reaction perform the following steps to set up translation reaction:

1. Per reaction take a single vial with WEPRO®9L (violet vial) and a single well (clear well) containing SUB-AMIX® SGC (*1) from storage. 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 loses its activity if not kept at -80°C!
2. Thaw reagents on ice. After thawing, briefly spin down each vial with WEPRO®9L 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. Resuspend the "transcription mixture" from previous step by pipetting gently up and down (*3).
5. Prepare "translation mixture" by adding 9 µl of the "transcription mixture" containing the RNA template to the vial containing the WEPRO®9L as indicated in the table below. Mix gently by pipetting up and down, avoid bubbles.

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 a form bilayer with the translation mixture in the lower layer and SUB-AMIX® SGC in the upper layer. Refer to the figure at the end of this section on how to setup a bilayer reaction: Go with the pipette tip to the bottom of the well, and slowly release the translation mixture below the reaction buffer. Because of the yellowish color of the wheat germ extract, you can distinguish the translation mixture from the translation buffer in the well.

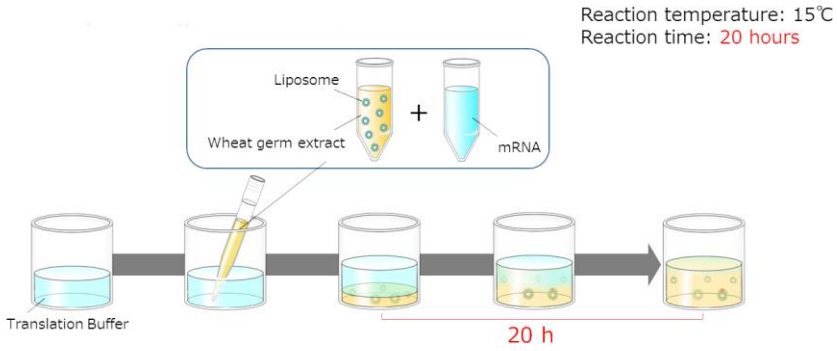
Reagents	Working Volume	Final Concentration
SUB-AMIX® SGC	210 µl	1 x
Translation mixture	30 µl	-
Total	240 µl	-

7. Seal the well with an aluminum seal included in the kit to avoid evaporation (*4).
8. Incubate at 15˚C for 20 hours in an incubator (*5). Be careful that the well stably stands on a flat surface.
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 particular care to keep the wells with SUB-AMIX® SGC upright at all times. They easily flip 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 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 the seal onto the well. Press down the seal onto the well to make sure it covers the entire surface of the well. The seal can easily be removed after the completion of the reaction by simply pulling it up. Save the remaining seals for later use. Be careful not to disturb the bilayer while placing the seal onto the well.
*5: Extend incubation time to 24 hours when using PCR products as expression templates to achieve higher yields.

Figure 4: Illustration on how to setup bilayer reaction



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 to transfer the remaining reaction mixture.
3. Centrifuge reaction mixture in 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 reaction well with the remaining reaction mixture (Step 2) 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 the pellet by pipetting up and down.
9. Repeat washing steps 6 to 8 two more times (in total washing the pellet 3 times).

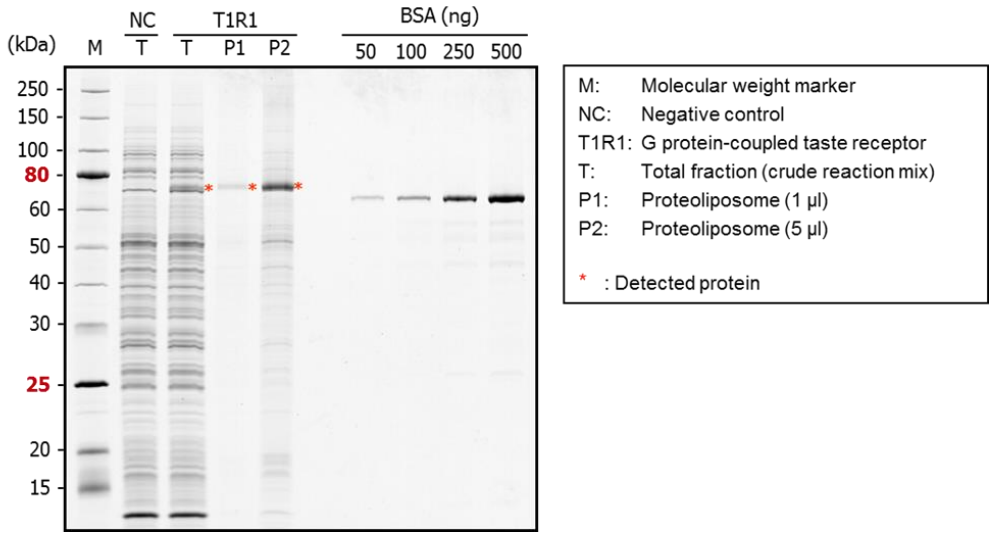
10. After the last centrifugation, remove supernatant completely, do not disturb the pellet. Resuspend proteoliposome pellet in a total of 200 µl PBS. Resuspend pellet completely by pipetting up and down.
11. For later use, you can freeze proteoliposomes in liquid nitrogen and store them in PBS at -80 ˚C. Avoid repeated freeze/thawing of the proteoliposomes. 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.

Confirmation of Protein Expression

We recommend confirming protein expression before use in any other experiments. Below we show example data for demonstrating the expression of the positive control included in the Proteoliposome PLUS Expression Kit. The synthesized T1R1 protein is a 93 kDa protein that may show a different molecular weight around 70 kDa under standard conditions using SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining.

Proteins contained in the proteoliposomes can be analyzed by SDS-PAGE gel electrophoresis. Load some 1 to 5 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 5: SDS-PAGE showing example data for the G Protein-Coupled Taste Receptor T1R1.



Troubleshooting

Sometimes the amounts of the expressed proteins are difficult to detect especially when just loading the crude reaction mixtures and using stained SDS-PAGE. Therefore, this kit includes an expression vector for a T1R1 protein that can be used as a control on how the system works. Refer to the Figure 5 above for reference on the expected signal after a 20 h reaction time; you should be able to see the T1R1 protein on SDS-PAGE after an overnight reaction. The positive control can be used to confirm that the expression reactions are handled correctly and that all reagents work properly. When loading crude reaction mixtures on SDS-PAGE, the wheat germ proteins in the extract may make it difficult to assign the right band for the overexpressed protein. After purifying the proteoliposomes, however,

the protein band should become clearer as the background proteins from the wheat germ extract should have been removed. As an alternative, also Western Blotting can be used to better detect the expressed protein as the wheat germ proteins in the extract should not be detected by the antibody. Follow the advice given below if you have problems to see expression of the control or your proteins of interest.

The experiments require correct and accurate pipetting during reaction setup. Avoid air bubbles as they will reduce protein yields. Any mistake 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 label for each reagent prior to starting the pipetting step. The good protein yields of the expression reactions are dependent on accurate use of the DNA template and setting the reaction temperature preferably to 15°C. However, the wheat germ extract can be used at temperatures of up to 25°C to test other expression conditions. At even higher temperatures, the wheat germ extract will lose its activity leading to low protein yields.

Working with an expression vector commonly gives better protein yields and more reproducible results. For routinely making the same protein, we advise to prepare an expression vector using one of the vectors provided by CellFree Sciences for use in our expression system. There are other expression vectors on the market and in public depositories for use in a wheat germ cell-free protein expression system. We have not confirmed how expression vectors from other providers work in combination with our expression system. When using expression vectors from other sources, always make sure that the vector has an SP6 RNA Polymerase promoter. The reagents for the Proteoliposome PLUS Expression Kit contain the SP6 RNA Polymerase and other RNA polymerase promoters are not supported.

If you cannot obtain the desired protein, please confirm the following as general advice for using the wheat germ expression system:

- Leaving out the DNA template will always yield negative results. The same applies if there is a mistake in the expression vector e.g., leaving out the starting ATG, forgetting a stop codon, or having a frame shift error.
- Confirm that your expression vector is correct and has a start and stop codon in line with the reading frame for the protein. Refer to our vector map primer information in Appendix A and B on more information on suitable sequencing primers to confirm the sequence of your expression vector.
- Confirm the DNA quality and concentration if the protein yields are low. Low RNA yields during the transcription reaction will also reduce the protein yields. Perform a phenol/chloroform extraction on the template DNA if RNA yields are low and ensure working under RNase-free conditions. An OD260/280 ratio of ~1.8 for your plasmid DNA preparations is commonly considered as pure enough for use in protein expression experiments. Lower ratios may indicate remaining proteins and/or other contaminations absorbing near 280 nm. Note that the actual values for the OD260/280 ratio can vary from vector to vector as the actual OD values depend also on the nucleotide composition of your DNA vector. It is important to confirm the OD260/280 ratio for your vector DNA before use in protein expression experiments because low DNA purity prevents RNA and protein expression. We advise in the manual on doing an additional phenol/chloroform extraction if the OD260/280 ratio is not correct or poor protein yields had been obtained.
- Confirm the concentration of your DNA template especially when working with linear DNA prepared by PCR. Expression vectors do not have to be linearized for use in the protein expression reactions. Make sure that the vector was used at a concentration of 1 µg/µl. Lower DNA concentrations can reduce protein yields.
- Make sure to run the transcription and translation reactions at the indicated reaction temperatures. The wheat germ extract will lose activity above 25°C. If possible, we recommend using a thermocycler as they are more accurate on keeping the set temperature, but the cups provided with this kit can be incubated in an incubator as well.
- Make sure not to miss any reagents: Mark in your protocol each pipetting step you have completed.
- The experiment must be done under RNase-free conditions as any loss of the RNA template will prevent protein expression.

- Change the pipetting tip after each pipetting step. Do not use the same pipetting tip to pipette different reagents or reaction mixtures.
- For the protein expression reactions, do not mix the two layers during setup of the bilayer translation reaction. Mixing both layers will sharply reduce the protein yields. Let both layers slowly mix by diffusion without any further manipulation. The translation reaction can be maintained for up to 24 hours.
- It is possible to work with linear templates in cell-free protein expression experiments, which can be easily prepared by PCR methods. Working with PCR products, however, can reduce protein yields. PCR products are used to quickly find the best expression construct or to test expressing different protein fragments. If the yields obtained with PCR products are too low, it may be worthwhile to prepare an expression vector already for doing the test expression experiments. Always use expression vectors for large-scale protein production to have stable and reproducible conditions.
- If the protein is not expressed, check reaction conditions, reagents, and DNA template in an expression reaction with the positive control vector provided with the kit to confirm their integrity. Reconsider the design of your expression template to improve protein yields if all the forgoing steps do not explain low yields for your target protein while the positive control works.
- Make sure that the wheat germ extract was always keep frozen before use. 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. Clearly follow the recommendations on reagent storage and handling given in this manual.

Working with membrane proteins and proteoliposomes:

- Membrane proteins are commonly insoluble when expressed in the wheat germ system and therefore special reaction conditions are commonly applied for their expression. In the case of the ProteoLiposome PLUS Expression Kit liposomes are added to the translation reactions for integrating the membrane proteins into a lipid bilayer. Proteoliposomes are insoluble and can therefore be enriched after completion of the expression reaction in a simple centrifugation step. Afterwards, washing steps with PBS allow for further purification of the proteoliposomes. However, highly purified proteoliposomes can only be obtained by gradient centrifugation. Protocol for gradient purification steps can be found in the literature.
- Keep all fractions obtained during the purification process. The pellet should be visible, but the pellet size may vary between different experiments. In case you lost the pellet by mistake, the proteoliposomes may still be in your washing buffer. Test the different fractions, if the protein could be seen in the crude reaction mixture bit no longer in the enriched proteoliposomes fraction.
- Protein yields per reaction can vary and will be dependent on the protein; it is also possible that the lipid composition may affect protein yields. Asolectin has given good results for many proteins but testing more lipids may give benefits for some proteins or applications. However, for some assays the protein may be removed from the proteoliposomes for further testing.
- While we assume that most proteins in the proteoliposome may have a defined orientation, proteins incorporated into proteoliposomes can have both orientations.
- While the purification steps will separate soluble proteins from insoluble ones, insoluble proteins may copurify with the proteoliposomes. To separate insoluble proteins from the proteoliposomes, a gradient centrifugation is required. Commonly, the proteins in the wheat germ extract are soluble and only few background proteins should be seen in the purified proteoliposome fraction.
- The number of proteins per proteoliposome may vary. We cannot give a general answer on how many copies of a protein are found per proteoliposome.
- Proteoliposome size will most likely vary at the end of the expression and purification experiment. During the production process of the kit, the liposomes are added to the pre-mixed wheat germ extract. The particle size of the liposomes in the solution commonly has a peak distribution at about 150 nm based on scattered light intensity measurement, and the liposomes should be close to a monodispersed distribution.

- Refer to the literature for more advice on the use of proteoliposomes in different experimental settings.
- We commonly freeze and later store proteoliposomes in PBS buffer. However, for functional analysis of your protein of interest, we would advise to analyze the protein directly before freezing down samples. Test for each protein what are the best storage conditions if PBS only is not suitable for storing your protein.
- Some membrane proteins may have special requirements and do not express well under standard conditions. Gather information on your target protein before the expression experiments to see whether additional considerations are needed. Contact us for more information on how to modify cell-free protein expression experiments. CellFree Sciences is offer additives for preparing proteins having disulfide bonds.

Contact the technical support of CellFree Sciences for further help using the contact information on the last page of the manual.

References

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

1. 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.
2. 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.
3. 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.
4. 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.
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.
6. 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.
7. Cell-free protein synthesis of membrane (1,3)-β-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.
8. 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.
9. 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.

A general introduction for using the wheat germ cell-free protein expression system can be found in the following review article, which is freely available on the internet:

1. Easy Synthesis of Complex Biomolecular Assemblies: Wheat Germ Cell-Free Protein Expression in Structural Biology. Fogeron ML, Lecoq L, Cole L, Harbers M, and Böckmann A. (2021) Front Mol Biosci Mar 25;8:639587. doi: 10.3389/fmolb.2021.639587.

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!

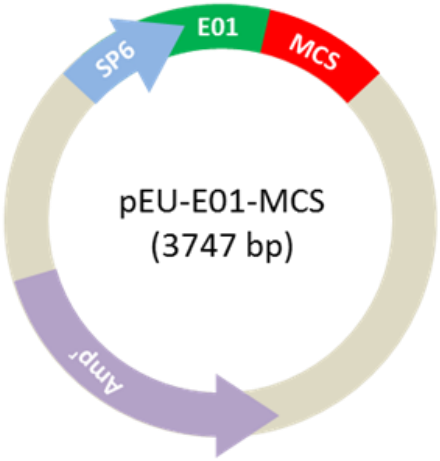
Reagent	Volume	Final Concentration	Checkmark
SUB-AMIX® SGC	210 µl	1x	☐
WEPRO®9L plus Transcription mixture (mRNA)	30 µl	-	☐
Total	240 µl	INCUBATE 20 h at 15°C	☐

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 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:

Vector elements:

SP6 Promoter:	-17-1
Translation Enhancer E01:	1-73
Multi cloning site:	74-193
Origin:	1190-1830
Ampicillin resistance gene:	1974-2838

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

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.

Appendix B: Sequencing Primers for Vectors of pEU-E01-MCS Family

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 *lac*Z-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'

Customer Information

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Our products are covered by the US patents Nos. 7838640, 7981617, 8734856, and equivalent pending or granted patents in other countries. Please contact us at tech-sales@cfsciences.com if you indent to make commercial use of our expression system.

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- Using the product for offering paid services.
- Reselling the product or any of its components.

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Others

All product specifications and information in the manual may be changed without prior notice.

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