# CellFree Sciences

The natural power of wheat driving science

*High Performance Cell-Free Wheat Germ Protein Expression System*

# INSTRUCTION MANUAL

## ProteoLiposome BD Kit

This kit provides reagents to prepare proteoliposomes in cell-free protein expression experiments

Product Number(s): CFS-CPLE-BD

Version/date: Version 2.3_eng/May 2021

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

CFS products are for research use only.

*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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# 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. Prepare aliquots of the wheat germ extract on first use if you want to keep making more expression experiments later; refer to the protocol below on how much extract is needed per reaction. Do not freeze/thaw the wheat germ extract more than three times.

## 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/

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 transcription and translation 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 ProteoLiposome BD Kit 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 during the translation reaction to form proteoliposomes. Proteoliposomes can be easily isolated by centrifugation and offer convenient tools to study protein functions (in case highly purified proteoliposomes are required, refer to the literature on how to purify them by gradient centrifugation). Moreover, proteoliposomes can be directly used in immunization experiments to prepare antibodies directed against membrane proteins. More details on the use of the wheat germ cell-free system for making membrane proteins can be found in the literature. Refer to reference list on our homepage.

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

CellFree Sciences provides dedicated expression vectors optimized for use with our wheat germ cell-free protein expression system. The preparation of proteoliposomes requires no affinity tag. However, the His-tag can be used while making proteoliposomes whereas we would advise against using the GST-tag. We recommend using our expression vectors for template preparation. It is also possible to prepare expression templates by PCR methods for rapid expression testing and high-throughput studies. Note that using linear DNA templates from PCR reactions will commonly lead to lower protein yields as linear DNA is less stable than circular plasmid DNA.

Refer to our homepage or contact our support team for more information on how to use our cell-free protein expression system. The contact information is given at the end of the manual.

# Kit Contents

The following tables summarize the reagents provided with this product: The reagents are shipped in three boxes.

Expression Reagent Kit (white box):

| Item | Quantity | Volume | Vial | Vial Color |
|---|---|---|---|---|
| **WEPRO®7240** | 1 | 1000 µl | 2.0 ml tube | Clear |
| **5xTranscription Buffer LM** | 1 | 240 µl | 1.5 ml tube | Yellow |
| **NTP Mix (25 mM)** | 1 | 120 µl | 1.5 ml tube | Blue |
| **RNase Inhibitor (80 U/µl)** | 1 | 15 µl | 1.5 ml tube | Orange |
| **SP6 RNA Polymerase (80 U/µl)** | 1 | 15 µl | 1.5 ml tube | Green |
| **Creatine Kinase (20 mg/ml)** | 1 | 20 µl | 1.5 ml tube | Red |
| **40xSUB-AMIX® SGC S-1** | 1 | 600 µl | 1.5 ml tube | Purple |
| **40xSUB-AMIX® SGC S-2** | 1 | 600 µl | 1.5 ml tube | White |
| **40xSUB-AMIX® SGC S-3** | 1 | 600 µl | 1.5 ml tube | Brown |
| **40xSUB-AMIX® SGC S-4** | 1 | 600 µl | 1.5 ml tube | Gray |

40xSUB-AMIX® SGC Translation Buffer (white box):

| Item | Quantity | Volume | Vial | Vial Color |
|---|---|---|---|---|
| **40xSUB-AMIX® SGC S-1** | 1 | 7 ml | 15 ml tube | Purple |
| **40xSUB-AMIX® SGC S-2** | 1 | 7 ml | 15 ml tube | White |
| **40xSUB-AMIX® SGC S-3** | 1 | 7 ml | 15 ml tube | Brown |
| **40xSUB-AMIX® SGC S-4** | 1 | 7 ml | 15 ml tube | Gray |

Lyophilized liposomes (green box):

| Item | Quantity | Amount | Vial | Vial Color |
|---|---|---|---|---|
| **Asolectin Liposome, lyophilized** | 6 | 10 mg | Brown glass vials | Purple |

Refer to the table below for more information on how to handle and store the reagents:

| Item | Description | Storage |
|---|---|---|
| **WEPRO®7240** | WEPRO®7240 wheat germ extract is temperature sensitive! Immediately after thawing the extracts place them on ice. Upon thawing for the first time, prepare aliquots for later use and store them at -80°C. Do not freeze/thaw the wheat germ extract more | -80°C |

| | | |
|---|---|---|
| | than three times. We recommend using liquid nitrogen to freeze the extracts. | |
| **40xSUB-AMIX® SGC (S-1, S-2, S-3, S-4)** | The 40xSUB-AMIX® SGC translation buffer is provided in four separate master mixes (S-1, S-2, S-3, S-4) to avoid the precipitation of some amino acids. It is possible to prepare onetime a large volume of the 1xSUB-AMIX® SGC translation buffer, and to store aliquots at -80°C for later use. Do not mix the 40xSUB-AMIX® SGC translation buffers directly as some amino acids will precipitate. Avoid unnecessary freeze/thawing of the 1xSUB-AMIX® SGC translation buffer.<br>You will need the 40xSUB-AMIX® SGC translation buffers provided in the Expression Reagent Kit and the separate buffers to perform 6 BD reactions on a 2.5 ml scale. | -80°C |
| **5xTranscription Buffer LM** | After thawing for the first time, divide 5xTranscription Buffer LM into appropriate aliquots convenient for later use. The buffer can be stored at -20°C. | -20°C |
| **NTP Mix** | The NTP Mix contains ATP, GTP, CTP, and UTP at a concentration of 25 mM. The NTP Mix can be stored at -20°C. | -20°C |
| **SP6 RNA Polymerase** | Provided in 50% glycerol, and can be stored at -20°C. | -20°C |
| **RNase Inhibitor** | Provided in 50% glycerol, and can be stored at -20°C. | -20°C |
| **Creatine Kinase (*1)** | Creatine Kinase is temperature sensitive, and we recommend to always use fresh enzyme preparations. The Creatine Kinase in the kit is provided at a concentration of 20 mg/ml. This concentration can be directly used to set up translation reactions on a BD reaction scale. The enzyme must be diluted with nuclease-free water to 1 mg/ml for setting up SMALL scale translation reactions for testing protein expression. | -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 outer and inner cover of the vial containing the asolectin liposomes. Slowly add 200 µl of 1x SUB-AMIX® SGC at the center of bottom of the vial. Close the vial with inner cover and let it stay at room temperature for 10 min. Mix the liposomes by vortexing (30 seconds to 1 minute). Transfer from the vials to a 50 ml tube and centrifuge them at 500x g for 1 min. Take out the vials from the 50 ml tube and transfer the rehydrated liposomes to a 1.5 ml tube. We advise to sonicate the liposome solution before use in a water bath until the solution becomes clear and the liposomes form uniform single lamella. The clear solution will have a yellowish color. Rehydrated liposomes are for single use, and we recommend not to freeze/thaw the liposomes after rehydration. | -80°C |

*1: Creatine Kinase can be purchased from Sigma-Aldrich, Catalog No. 10127566001.

# Required Reagents, Consumables, and Instruments

| Consumable | Description |
|---|---|
| **Nuclease-free water** | DNase, RNase free. We DO NOT recommend DEPC treated water |

| | |
|---|---|
| **PBS** | Phosphate buffered saline |
| **96-well plate (only for testing expression on 227 µl scale)** | Cell culture plate, flat bottom, non-treated. |
| **Cover tape** | To seal well of 96-well plate. |
| **Slide-A-Lyzer™ MINI Dialysis Devices, 10K MWCO** | Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Devices, 10K MWCO from Thermo SCIENTIFIC, Product No. 88404. |
| **Incubator** | Temperature range 15 to 37°C. |
| **Centrifuge** | Suitable for 1.5 ml tube and 50 ml tube |
| **Sonicator bath (optional)** | Needed to prepare liposome solution |
| **SDS-PAGE** | Gel electrophoresis apparatus and power supply |

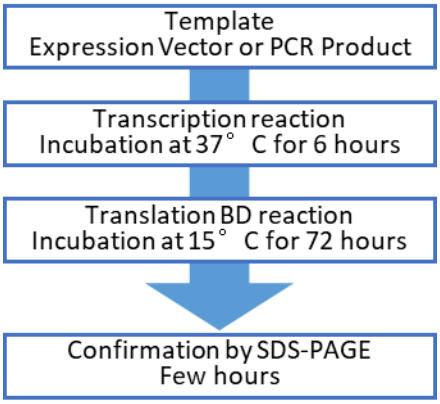
# Protocol

The ProteoLiposome BD Kit provides enough reagents to do all steps required to test the expression of your protein of interest on a small-scale (up to 24 reactions) prior to preparing proteoliposomes on a preparative scale (6 reactions). Initial expression tests should be made on a 227 µl bilayer translation reaction scale in the absence of added liposomes. These reactions must be set up in flat-bottom vials! While individual flat-bottom vials are available, multiple reactions can be set up in multi-well plates. Multi-well plates are available with different dimensions to match the translation reaction size, where 96-well plates can be used for setting up 227 µl bilayer translation reactions. For first time use, we recommend including a positive control to make sure that the experiment has been set up correctly. Contact CFS on available control vectors that can be used in a separate expression experiment. Preparative expression for the preparation of proteoliposomes is done on a 2.5 ml BD reaction scale. If one 2.5 ml BD reaction does not provide the needed protein yields, conduct multiple expression reactions and pool the protein from multiple reactions to work with a homogenous protein mixture.

Successful protein expression should be confirmed before using any protein in your later experiments. Protein expression can be analyzed by SDS-PAGE to see whether a protein of the correct size has been made. It can be helpful to compare your protein in a crude expression reaction mixture to a negative control reaction prepared without added expression vector. The negative control reaction will only show the background proteins in the wheat germ extract. We advise to use known amounts of a BSA standard in the SDS-PAGE experiment to estimate protein yields. As an alternative, protein expression can also be confirmed by Western blotting using an antibody against the target protein or an affinity tag. When working with small protein amounts, Western blotting and labeling methods offer more sensitive protein detection than protein staining in SDS-PAGE gels. Those methods further provide background free detection as commonly proteins in the wheat germ extract should not be recognized by a specific antibody, nor is there any background when labeling proteins during expression experiments.

## Time Requirements

Refer to the flowchart below on the estimated time per reaction step.




# Small-Scale Test Expression Experiment

The following protocol uses a small-scale 227 µl bilayer reaction without added liposomes to test the expression of your target protein(s) in the wheat germ system. Do this test before scaling up to a 2.5 ml BD reaction with added liposomes for preparation of proteoliposomes.

## Transcription Reaction Using DNA Template

1. Thaw your template DNA before the experiment. You need 1 µg of purified plasmid DNA (*1).
2. Take vials with 5xTranscription Buffer LM (yellow vial), NTP Mix (blue vial), RNase Inhibitor (orange vial) and SP6 RNA polymerase (green vial) out from storage at -80°C.
3. Thaw the reagents in a water bath at ~25°C. After thawing, spin the vials briefly to collect the entire volume at the bottom of the vial. Mix the reagents gently before use. Always keep reagents on ice.
4. Set up transcription experiment as shown in the table below for three different reaction sizes. Then mix gently by pipetting up and down.

| Reagents | Volume | Final Concentration |
|---|---|---|
| **Nuclease free water*** | 5.75 µl | - |
| **5xTranscription Buffer LM** | 2 µl | 1x |
| **NTP Mix (25 mM)** | 1 µl | 2.5 mM |
| **RNase Inhibitor (80 U/µl)** | 0.125 µl | 1 U/µl |
| **SP6 RNA Polymerase (80 U/µl)** | 0.125 µl | 1 U/µl |
| **Plasmid (circular DNA, 1.0 µg/µl)** | 1 µl | 100 ng/µl |
| **Total** | 10 µl | |

***Do not use DEPC treated water; we recommend using commercially available pure water.**

5. Incubate at 37°C for 1 hour in an incubator (*2).
6. After completion of the transcription reaction, leave reaction mixture at room temperature until later use in the translation reaction. Do not cool the reaction mixture, nor store it on ice.
7. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis taking out 0.5 µl from the reaction mixture for loading onto the gel. Refer to a cloning handbook for more information on how to perform RNA gel electrophoresis. Successful

RNA expression reactions yield in multiple transcripts that can be seen on the gel. Do not use an RNA preparation if you see an RNA smear because it indicates that your RNA had been degraded.

(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: 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.

## 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. Resuspend the transcription mixture by pipetting gently up and down before use in translation reaction (*1).

Per reaction perform the following steps to set up translation reaction:

1. Take the vial with WEPRO®7240 wheat germ extract (clear vial). Thaw the extract in a water bath at ~25°C. After thawing, briefly spin the vial with wheat germ extract to collect it at the bottom of the vial. Avoid excessive centrifugation of wheat germ extracts. Mix the extract gently before use. Keep wheat germ extract on ice at all time. After use, immediately freeze the wheat germ extract in liquid nitrogen and put it back into the freezer for storage at -80°C. The wheat germ extract losses its activity if not kept at -80°C!
2. Thaw Creatine Kinase (red vial) in a water bath at ~25°C. After thawing, briefly spin the vial with Creatine Kinase to collect it at the bottom of the vial. Mix the enzyme gently before use. Dilute Creatine Kinase stock solution to 1 mg/ml with RNase free water before used for SMALL scale reaction setup! We strongly recommend not to freeze/thaw Creatine Kinase as it will rapidly lose activity (*2).
3. Prepare “translation mixture” as indicated in the table below. Mix gently by pipetting up and down and avoid any bubbles.

| Reagents | Volume | Final Concentration |
|---|---|---|
| **Transcription Reaction** | 10 µl | 1/2 volume |
| **Diluted Creatine Kinase (1mg/ml)** | **0.8 µl** | 40 ng/µl |
| **WEPRO®7240 Extract** | 10 µl | 120 OD/ml |
| **Total** | 20.8 µl | |

4. Prepare 1xSUB-AMIX® SGC translation buffer and add the required amount of buffer to a flat-bottom vial or well. Refer to the table below on the required volumes for the different reaction sizes (*3).

| Reagents | Volume | Final Concentration |
|---|---|---|
| **Translation Mixture** | 20.8 µl | - |
| **1xSUB-AMIX® SGC** | 206 µl | 1x |
| **Total** | 227 µl | |

5. Carefully transfer the translation mixture from step 3 to the bottom of a single vial or well containing 1xSUB-AMIX® SGC translation buffer to form the bilayer reaction with the translation mixture in the lower layer and the 1xSUB-AMIX® SGC translation buffer in the upper layer. Refer to figure at the end of this section on how to setup a bilayer reaction: Go with the pipette tip to the bottom of the vial or well, and slowly release the translation mixture below the 1xSUB-AMIX® SGC translation buffer. Because of the yellowish color of the wheat germ extract, you can distinguish the translation mixture from the translation buffer. The wheat germ extract has a higher density than the buffer and therefore will form easily the lower layer.
   **Do not mix the reagents in the vial or well by pipetting or any other means! It will reduce the yield of the reaction.**
6. Close the vial or seal the well to avoid evaporation.
7. Incubate at 15˚C for 20 hours in an incubator. Be careful that the vial stably stands on a flat surface.
8. After completion of the translation reaction, mix the bilayer reaction gently by pipetting up and down.
9. Place reaction mixture on ice to protect your protein before later use.
10. Commonly some 2 to 3 µl of the crude translation reaction mixture are enough to detect the expressed protein on SDS-PAGE. It can be helpful to load different amounts of the crude reaction mixture to get a better estimation of the protein yields.

(Notes)

*1: If you notice a white precipitate after the transcription reaction, resuspend the precipitate by pipetting gently up and down before mixing with the wheat germ extract. There is no need to remove the precipitate.

*2: We recommend to always use fresh Creatine Kinase. Creatine Kinase is required for the energy supply of the translation reaction, and a loss of Creatine Kinase activity will reduce protein yields.

*3: Do not mix directly the 40xSUB-AMIX® SGC translation buffers; always add them nuclease-free water for dilution. Do not use the 1xSUB-AMIX® SGC translation buffer if you observe any precipitation.

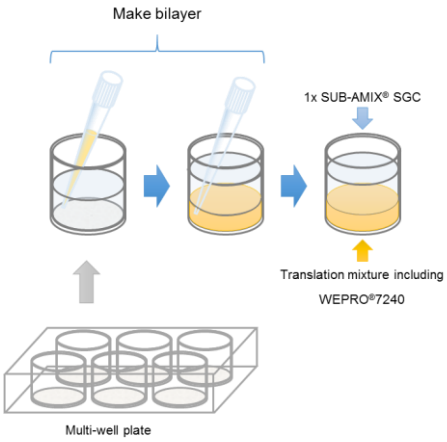



*Illustration on how to setup bilayer reaction in multi-well plate*

# Preparation of Proteoliposomes using BD Reaction Format

## Transcription Reaction Using DNA Template

1. Thaw your template DNA before the experiment. You need 13 µg of purified plasmid DNA for a 2.5 ml BD reaction scale (*1).
2. Take vials with 5xTranscription Buffer LM (yellow vial), NTP Mix (blue vial), RNase Inhibitor (orange vial) and SP6 RNA polymerase (green vial) out from storage at -80°C.
3. Thaw the reagents in a water bath at ~25°C. After thawing, spin the vials briefly to collect the entire volume at the bottom of the vial. Mix the reagents gently before use. Always keep reagents on ice.
4. Set up transcription experiment as shown in the table below for three different reaction sizes. Then mix gently by pipetting up and down.

| Reagents | Volume | Final Concentration |
|---|---|---|
| **Nuclease free water*** | 74.8 µl | - |
| **5xTranscription Buffer LM** | 26 µl | 1x |
| **NTP Mix (25 mM)** | 13 µl | 2.5 mM |
| **RNase Inhibitor (80 U/µl)** | 1.62 µl | 1 U/µl |
| **SP6 RNA Polymerase (80 U/µl)** | 1.62 µl | 1 U/µl |
| **Plasmid (circular DNA, 1.0 µg/µl)** | 13 µl | 100 ng/µl |
| **Total** | 130 µl | |

***Do not use DEPC treated water; we recommend using commercially available pure water.**

5. Incubate at 37°C for 6 hours in an incubator (*2).
6. After completion of the transcription reaction, leave reaction mixture at room temperature until later use in the translation reaction. Do not cool the reaction mixture, nor store it on ice.
7. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis taking out 0.5 µl from the reaction mixture for loading onto the gel. Refer to a cloning handbook for more information on how to perform RNA gel electrophoresis. Successful RNA expression reactions yield in multiple transcripts that can be seen on the gel. Do not use an RNA preparation if you see an RNA smear because it indicates that your RNA had been degraded.

### (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: 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.

## Preparation of Translation Buffer for BD Reaction

Prepare 48 ml of feeding buffer (1x SUB-AMIX® SGC) for the dialysis reaction and the translation reaction. This buffer is also needed to rehydrate the lyophilized liposomes (*1). Mix buffer components of SUB-

AMIX® SGC according to the table below. Add the water first before adding the buffer components to avoid precipitations. Mix the final buffer after all reagents have been added.

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

Keep buffer on ice until use.

(Notes)

*1: Do not mix directly the 40xSUB-AMIX® SGC translation buffers; always add them nuclease-free water for dilution. Do not use the 1xSUB-AMIX® SGC translation buffer if you observe any precipitation.

## Rehydration of lyophilized Liposomes

To setup a 2.5 ml BD reaction, one vial with lyophilized asolectin liposomes (*1) is needed. We advise to sonicate the liposome solution when using the proteoliposomes for functional analysis. After sonification most liposomes will form a uniform single lamella. If you do not have a sonication apparatus, rehydrated liposomes can be used directly in the subsequent protein synthesis reaction. However, they are non-uniform in size and form multi-lamella. Sonication is also not needed if the proteoliposomes are later used for example as antigens in immunization experiments.

Preparation of liposomes without sonication step:

1. Open outer and inner cover of the vial containing the asolectin liposomes.
2. Slowly add 200 µl of 1x SUB-AMIX® SGC to the center of bottom of the vial.
3. Close the vial with inner cover and let it stay at room temperature for 10 min.
4. Mix the liposomes by vortexing (30 seconds to 1 minute). After vortexing, the solution will appear cloudy, and liposomes will have a multi-dispersed having a non-uniform particle size.
5. Transfer the vial to a 50 ml tube and centrifuge them at 500x g for 1 min.
6. Take out the vial from 50 ml tube and transfer the rehydrated liposomes to a 1.5 ml tube for later use.

Preparation of liposomes with sonication step:

1. Open outer and inner cover of the vial containing the asolectin liposomes.
2. Slowly add 200 µl of 1x SUB-AMIX® SGC to the center of bottom of the vial.
3. Close the vial with inner cover and let it stay at room temperature for 10 min.
4. Sonicate the liposome solution using a sonication apparatus until the solution becomes clear. The clear solution will have a yellowish color. For example, when using a Digital Sonifier equipped with a horn cup (BRANSON) the liposome solution becomes clear within 2 to 5 minutes at 20-30% output power. After sonification, 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.

5. Centrifuge the clear liposome solution at 20,000g for 5 min to remove debris.
6. Use the supernatant for the subsequent protein synthesis.

(Notes)

*1: The asolectin liposomes provided with the kit can be replaced by biotinylated asolectin liposomes sold separately (Product number CFS-ASL-BT).

## 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. Resuspend the transcription mixture by pipetting gently up and down before use in translation reaction (*1).

Per reaction perform the following steps to set up translation reaction:

1. Take the vial with WEPRO®7240 wheat germ extract (clear vial). Thaw the extract in a water bath at ~25°C. After thawing, briefly spin the vial with wheat germ extract to collect it at the bottom of the vial. Avoid excessive centrifugation of wheat germ extracts. Mix the extract gently before use. Keep wheat germ extract on ice at all time. After use, immediately freeze the wheat germ extract in liquid nitrogen and put it back into the freezer for storage at -80°C. The wheat germ extract losses its activity if not kept at -80°C!
2. Thaw Creatine Kinase (red vial) in a water bath at ~25°C. After thawing, briefly spin the vial with Creatine Kinase to collect it at the bottom of the vial. Mix the enzyme gently before use. We strongly recommend not to freeze/thaw Creatine Kinase as it will rapidly lose activity (*2).
3. Prepare “translation mixture” as indicated in the table below. Mix gently by pipetting up and down and avoid any bubbles.

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

Keep reaction mixture on ice until use.

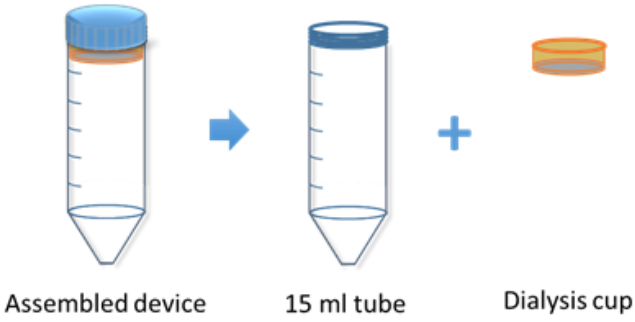
(Notes)

*1: If you notice a white precipitate after the transcription reaction, resuspend the precipitate by pipetting gently up and down before mixing with the wheat germ extract. There is no need to remove the precipitate.

*2: We recommend to always use fresh Creatine Kinase. Creatine Kinase is required for the energy supply of the translation reaction, and a loss of Creatine Kinase activity will reduce protein yields.

## Preparation of BD Reaction

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




1. Remove dialysis cup from Slide-A-Lyzer™ MINI Dialysis Devices, 10K MWCO and add 43 ml of 1x SUB-AMIX® SGC feeding buffer to the 50 ml tube.
2. Add 4 ml of nuclease-free water to the dialysis cup and rinse the dialysis membrane. Confirm there is no leakage from membrane. Decant the water and shake the cup to remove remaining water. **Do not dry the membrane.**
3. Place the dialysis cup back to the 50 ml tube. Make sure the membrane in the dialysis cup is within the feeding buffer.
4. Add 2 ml of 1x SUB-AMIX® SGC to the dialysis cup. **Take care not to damage dialysis membrane.**
5. Set up a bilayer in the dialysis cup placed in the 50 ml tube with the feeding buffer.
6. Carefully transfer the translation mixture (500 µl) to the bottom of the dialysis cup containing 1x SUB-AMIX® SGC (2 ml) to form bilayer with the translation mixture in the lower layer and 1x SUB-AMIX® SGC in the upper layer. Bring pipette tip close to the bottom of the dialysis cup, and slowly add the reaction mixture. Because of the higher density of the wheat germ extract, the reaction mixture will form a second layer below the reaction buffer. Do not disturb the layers when removing the pipette tip. **Take care not to damage dialysis membrane. Do not mix the reagents in the well by pipetting or any other means. Refer to the figure below for more information on how to set up the reaction.**
7. Close the 50 ml tube and tighten the cap on the tube. Be careful not to shake the tube with the bilayer reaction in the dialysis cup.
8. Place the tube in an incubator and incubate the translation reaction for up to 72 hours at 15°C. It is not necessary to exchange the feeding buffer during the incubation time.
9. We do not recommend changing the feeding buffer during the incubation to avoid disturbing the bilayer reaction. Note, the kit contains enough feeding buffer to do 6 times a 2.5 ml BD reaction, and no translation buffer is provided to change the buffer during the translation reaction. You can purchase additional SUB-AMIX® SGC (Product Number: CFS-SUB-AMX) from CellFree Sciences.

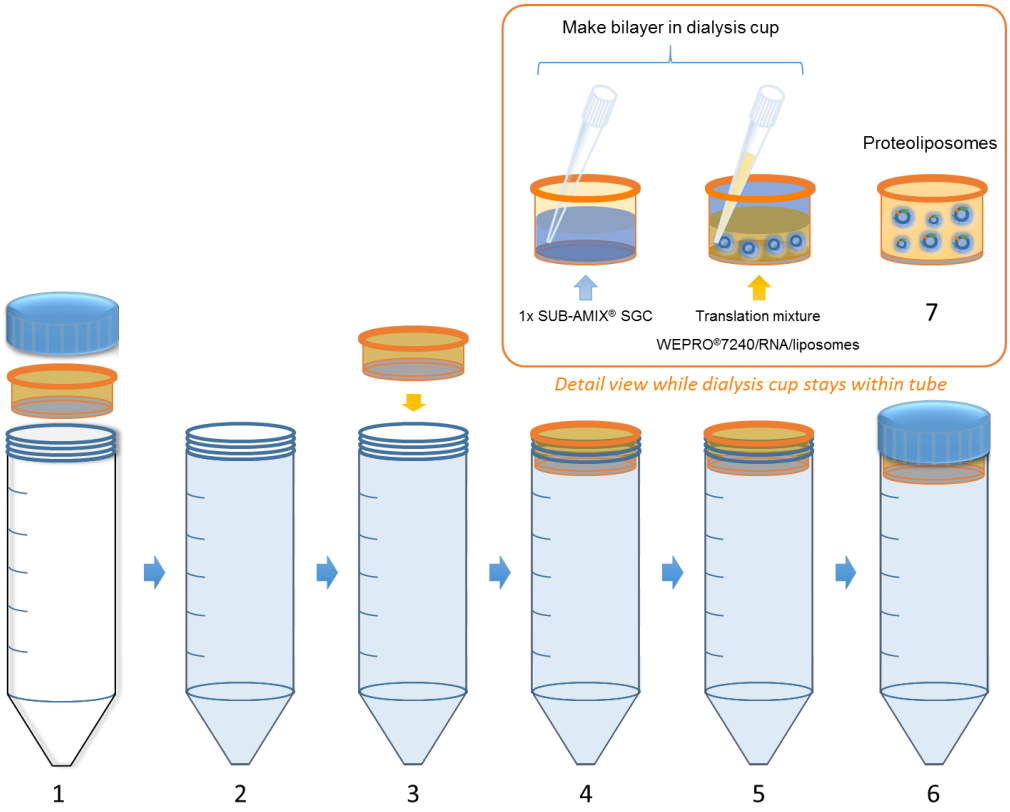



*Illustration on how to setup of BD reaction: 1: Dialysis device comprising 50 ml tube, dialysis cup, and lid; 2: Prepare tube and add feeding buffer; 3: Insert dialysis cup into tube with feeding buffer; 4: Add feeding buffer into dialysis cup within the tube; 5: Setup bilayer reaction in the dialysis cup within the tube. Refer to drawings within the orange box for more details on how to place the translation mixture below the feeding buffer; 6: Put lid onto tube with dialysis cup and conduct translation reaction; 7: Proteoliposomes will be formed within the dialysis cup*

## Purification of Proteoliposomes

Proteoliposomes can be purified by an easy centrifugation and washing step:

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

7. Remove supernatant. Take care not to remove whole supernatant.
8. Add 1 ml of PBS to the tube and resuspend it by pipetting.
9. Repeat washing steps 6 to 8 two more times (in total washing pellet 3 times).
10. After the last centrifugation, remove supernatant and add appropriate volume of PBS to the pellet. Resuspend proteoliposomes in a total of 500 µl PBS. Resuspend pellet completely by pipetting up and down.

Store proteoliposomes in PBS at -80 ˚C.

### Confirmation of Protein Expression into Proteoliposomes

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

## Troubleshooting

The protein expression experiments require correct and accurate pipetting during reaction setup. 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.

- Note, proteoliposomes can only be prepared for proteins having a trans membrane domain. Some other proteins with an affinity to lipid layers may also associate with liposomes.
- The experiment must be done under RNase-free conditions as any loss of the RNA template will prevent protein expression.
- Mark in your protocol each pipetting step you have completed.
- Change the pipetting tip after each pipetting step. Do not use the same pipetting tip to pipette different reagents or reaction mixtures. Always change the pipetting tip after use.
- Leaving out the plasmid 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 maps on more information on suitable sequencing primers to confirm the sequence of your expression vector.
- Confirm the DNA and RNA quality if the protein yields are low. Low RNA yields during the transcription reactions 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 recommend to always using fresh Creatine Kinase. Do not freeze/thaw Creatine Kinase as it will rapidly lose activity. Creatine Kinase is required for the energy supply of the translation reactions, and a loss of Creatine Kinase activity will reduce protein yields.
- For the translation reaction, do not mix the two layers during setup of the bilayer or BD translation reaction. Mixing both layers will sharply reduce the protein yields of the translation reaction as the reaction will run dead within few hours. A slow mixing of both layers is required to maintain the translation reaction.
- 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 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 a positive control vector to make sure that all reagents work, and the experiment is done correctly. Reconsider the design of your expression template to improve protein yields if all the forgoing steps do not explain low protein yields.
- 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. Follow clearly the recommendations on reagent storage and handling.
- Keep all fractions during protein purification until you have confirmed the recovery of the purified proteins. If you are not able to recover the protein from the resin during the purification experiment, check whether the protein can be found in the flow through or the washing fractions.
- Do not boil samples before loading onto SDS-PAGE when analyzing proteoliposomes.
- Some 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.

# Additional Information

Certain proteins may require changes to the expression reactions, where we have dedicated expression kits to prepare isotope-labeled proteins for use in MS or NMR studies. We can provide more information on the use of other additives such as detergents or ions in our cell-free protein expression system. Visit the homepage of CellFree Sciences for more information on other products and how to use our protein expression system.

Contact the technical support of CellFree Sciences for more information and further help. The contact information is given on the last page of the manual.

# Patent

CellFree Sciences' ENDEXT® technology and products are covered by US Patent Nos. 6869774, 7981617, 8734856 and other pending or equivalent patents.

# Customer Information

## Product-related Information

All our products are for research purpose only; not for use in diagnostic testing and use in human. Contact us for more information and help on the use of our products.

## Trademarks

ENDEXT®, WEPRO® and SUB-AMIX® are registered trademarks of CellFree Sciences Co., Ltd.

## Others

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

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