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TITLE:

Cell-Free Production of Proteoliposomes for Functional Analysis and Antibody Development Targeting Membrane Proteins

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KEYWORDS:

membrane protein, proteoliposome, cell-free protein synthesis, wheat germ extract, antibody development, GPCR, ion channel, transporter

SUMMARY:

This protocol describes an efficient cell-free method for production of high-quality proteoliposome by bilayer-dialysis method using wheat cell-free system and liposomes. This method provides suitable means for functional analysis of membrane proteins, drug targets screening, and antibody development.

ABSTRACT:

Membrane proteins play essential roles in a variety of cellular processes and perform vital functions. Membrane proteins are medically important in drug discovery because they are the targets of more than half of all drugs. An obstacle to conducting biochemical, biophysical, and structural studies of membrane proteins as well as antibody development has been the difficulty in producing large amounts of high-quality membrane protein with correct conformation and activity. Here we describe a “bilayer-dialysis method” using a wheat germ cell-free system, liposomes, and dialysis cups to efficiently synthesize membrane proteins and prepare purified proteoliposomes in a short time with a high success rate. Membrane proteins can be produced as much as in several milligrams, such as GPCRs, ion channels, transporters, and tetraspanins. This cell-free method contributes to reducing the time, cost and effort for preparing high-quality proteoliposomes, and provides suitable means for functional analysis of membrane proteins, drug targets screening, and antibody development.

INTRODUCTION:

Membrane proteins are one of the most important drug targets in diagnosis and therapeutics. Indeed, half of small compound drugs target are membrane proteins, such as G-protein-coupled receptors (GPCRs) and ion channels[1]. Over the years, researchers have been working on

biochemical, biophysical, and structural studies of membrane proteins to elucidate their structure and function[2,3]. Development of monoclonal antibodies against membrane proteins is also performed actively in order to accelerate functional and structural studies and to develop therapeutic and diagnostic applications[4–9]. All these studies require a large amount of high quality membrane proteins[10]. For example, several milligrams of purified membrane proteins with natural conformation are needed for antibody development. A much larger quantity of highly purified membrane proteins are required for X-ray crystallography. However, mass production of membrane proteins remains a bottleneck in membrane protein research[11]. Membrane proteins have complicated structures with one or more transmembrane helices and play important roles in cell homeostasis. Heterologous overexpression of membrane proteins leads to multiple obstacles such as aggregation of membrane proteins that accumulate at high local concentrations or disturbance of cellular signal pathways. Even if the expression is successful, subsequent steps of sample preparation also face difficulty. For instance, preparation of proteoliposome, requires high-level skills and professional experiences in solubilization, purification, and stabilization of membrane proteins, and costs much effort and time as well[12,13].

On the other hand, some advanced technologies have emerged in recent decades to produce proteins without the use of living cells[14–18]. Cell-free protein synthesis technology reconstitutes translation reaction in a test tube. Since there are no limitations that the cellular expression system has, cell-free systems have potential to synthesize a variety of proteins that are difficult to express or show toxicity in cells. Purified cell extract or reconstituted translational machinery is mixed with template mRNAs, amino acids, and energy sources, and recombinant proteins are synthesized in a short time. Regarding the membrane protein synthesis, some kinds of scaffolds composed of lipids or amphiphiles, such as liposomes, bicelles, nanodiscs, or copolymers are added to the cell-free reaction[19–24]. Synthesized membrane proteins interact with the scaffolds and can be stabilized in water. Cell-free synthesized membrane proteins are used widely in functional studies and antibody production[25–31].

In this protocol, we describe an efficient cell-free method of proteoliposome production using wheat cell-free system and liposomes. Wheat cell-free protein synthesis system is a powerful in vitro translation system using extract from wheat germ[15,32,33]. Wheat germ contains a large amount of translational machinery and few translation inhibitors. The translational machinery in wheat, a member of eukaryotes, is suitable for translating eukaryotic proteins, and its translation efficiency is hardly affected by codon usage of the template mRNA. Using wheat cell-free system, we have synthesized a variety of proteins including protein kinases[34,35], ubiquitin ligases[36], transcription factors[37], and membrane proteins with high success rates. For membrane protein production, we add lipid vesicle liposome into the translation mixture as scaffold[19,38]. Hydrophobic domains of membrane protein interact with lipid bilayer and are spontaneously integrated with liposome. Density gradient centrifugation is used to strictly separate proteoliposome from endogenous wheat proteins, even though a common centrifugation of the translation reaction mixture is sufficient for a simple purification of proteoliposome[20]. Many kinds of integral membrane proteins have been synthesized using wheat cell-free system and applied for various researches and developments[25,28,38–44]. Moreover, we developed the “bilayer-dialysis method” for large scale production[45,46]. In this method, cup-type dialysis device is

immersed in the substrate feeding buffer, and two layers of translation reaction mixture and substrate feeding buffer are formed in the cup as shown in **Figure 1**. Continuous supply of substrates and removal of the byproduct can be efficiently conducted at both the top and the bottom of the reaction mixture for a long time, which leads to excellent translation efficacy (**Figure 2A** and **Figure 2B**)[45].

PROTOCOL:

1. Preparation of pEU expression plasmid

NOTE: pEU expression plasmid should include start codon, open reading frame of target membrane protein, and stop codon in the fragment (see **Figure 1**). Add detection/purification tag sequence(s) at the appropriate position when required. Either restriction enzyme digestion or seamless cloning is applicable for subcloning. Here we describe a protocol using a seamless cloning method.

1.1. Prepare insert DNA fragment.

1.1.1. Amplify the gene of interest by PCR using the cDNA template, primer 1 and primer 2. Primer 1 and primer 2 contain 15 bp overlaps for seamless cloning, respectively (see the **Table of Materials**).

NOTE: Do not include sequences to be processed and removed from mature protein in the cells (e.g., signal sequence). Processing of synthesized protein is not performed in wheat cell-free system. Do not add Kozak sequence. pEU-E01-MCS vector has E01 translation enhancer.

1.1.2. Add 1/25 volume of *Dpn*I restriction enzyme to the PCR product to remove template plasmid DNA. Incubate for 30 min at 37 °C.

1.1.3. Use a PCR purification kit to purify the PCR product and adjust concentration at 20–50 ng/µL.

1.2. Linearize pEU-E01-MCS vector.

1.2.1. Conduct inverse PCR using pEU-E01-MCS, primer 3, and primer 4.

1.2.2. Add 1/25 volume of *Dpn*I restriction enzyme to the inverse PCR product. Incubate for 30 min at 37 °C.

1.2.3. Use a PCR purification kit to purify the PCR product as per the manufacturer's recommendation. Adjust the concentration at 20–50 ng/µL.

1.3. Mix 2 µL of insert DNA fragment, 2 µL of linearized vector, and 4 µL of 2x seamless cloning enzyme mixture.

1.4. Transform *Escherichia coli* strain JM109 with the seamless cloning product. Using a spreader, spread the bacterial suspension on a LB-ampicillin agar plate.

NOTE: pEU vector has an ampicillin resistance marker.

1.5. Confirm the sequence of the expression plasmid constructed using primer 5 and primer 6 from 5' and 3' side of MCS in pEU plasmid, respectively.

1.6. Amplify and purify the expression plasmids.

1.6.1. Culture the plasmid-transformed *E. coli* strain JM109 in 150 mL of LB-ampicillin medium at 37 ˚C and 125 strokes per minute shaking overnight.

1.6.2. Extract and purify the plasmids using commercially available plasmid prep midi kit. Dissolve plasmids in 500 µL of TE buffer.

CAUTION: Do not use a mini prep kit for plasmid extraction. It does not provide sufficient quality and quantity of plasmid.

1.6.3. Add 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1). Mix vigorously for 5 min, and centrifuge for 5 min at 21,600 *x g* and room temperature. Transfer the upper plasmid solution to a new tube.

CAUTION: Wear disposable gloves to protect the skin from phenol and chloroform.

NOTE: In order to remove contamination of RNase from plasmid extraction kit, purify the plasmids using phenol-chloroform purification.

1.6.4. Add 500 µL of chloroform to remove phenol completely. Mix vigorously for 5 min, and centrifuge for 5 min at 21,600 *x g* and room temperature. Transfer the upper plasmid solution to a new tube.

1.6.5. Add 2.5 volume of ethanol and 1/8 volume of 7.5 M ammonium acetate, and store at -30 °C for 1 h.

1.6.6. Centrifuge at 21,600 *x g* at 4 °C for 10 min. Wash the pellet with 500 µL of 70% ethanol. Remove the supernatant carefully and leave the pellet for 5 min to dry up.

1.6.7. Dissolve the expression plasmids in 100 µL of ultrapure water completely. Measure the concentration of plasmids with absorbance at 260 nm. Adjust the concentration to 1 mg/mL.

2. In vitro transcription

CAUTION: Use DNase and nuclease-free plastic tubes and tips in steps of transcription and translation. Avoid autoclaving plastic wares to prevent contamination.

2.1. Harvest ultrapure water in a new plastic tube.

CAUTION: Do not use DEPC-treated water because residual DEPC strongly inhibits the reaction. Use freshly purified ultrapure water for transcription and translation.

2.2. Prepare transcription reaction mix by mixing 115.2 µL of ultrapure water, 40 µL of Transcription Buffer LM, 20 µL of NTP mix, 2.4 µL of 80 U/µL RNase inhibitor, 2.4 µL of 80 U/µL SP6 polymerase, and 20 µL of 1 mg/mL pEU expression plasmids. Mix the reagents gently by inverting. Perform a quick spin.

2.3. Incubate the transcription reaction at 37 °C for 6 h.

2.4. Mix the reaction gently by inverting and quickly spin down. Use it immediately for translation, otherwise freeze and store at -80 °C.

2.5. Confirm the transcription product by electrophoresis.

2.5.1. Mix 100 mL of 1x TAE buffer and 1 g of agarose. Heat the suspension in a microwave oven to prepare 1% agarose TAE gel.

2.5.2. Take 1 µL of transcription reaction and mix with 3 µL of water and 4 µL of 2x loading dye.

NOTE: Denaturing of RNA is not required.

2.5.3. Load 4 µL of the mixture and 2 µL of DNA ladder marker to the agarose TAE gel.

2.5.4. Electrophorese at 100 V for 20 min.

2.5.5. Stain the gel in ethidium bromide for 30 min. Check the ladder band pattern of mRNA using UV transilluminator and gel imager.

NOTE: When a smeared band of less than 500 bp is observed, mRNA degradation is suspected.

3. Preparation of materials for translation

3.1. Prepare the translation buffer.

3.1.1. Mix 27 mL of freshly prepared ultrapure water and 0.75 mL of each 40x stock solution for S1, S2, S3, and S4, respectively in a 50 mL tube.

NOTE: Modulate the amounts of materials according to the final required amount of 1x

translation buffer.

CAUTION: Do not store or refreeze the excess 1x translation buffer after use.

3.2. Prepare creatine kinase stock solution. Dissolve lyophilized creatine kinase in ultrapure water to a final concentration of 20 mg/mL. Dispense the solution in small amounts (10 to 50 µL each) in 0.2 mL 8-strip PCR tubes. Freeze the tubes in liquid nitrogen, and store at -80 °C.

CAUTION: Do not re-freeze creatine kinase solution after thawing.

3.3. Wash dialysis cups (0.1 mL size) to remove glycerol from the dialysis membrane.

NOTE: There are several different-sized dialysis cups. Small-sized cups (0.1 mL) are used for small-scale test (section 5.4), and large-sized cups (2 mL) for large-scale production (section 5.5), respectively. The washing step of dialysis membrane of large-size cups is avoidable.

3.3.1. Put 1 mL of ultrapure water into a new 1.5 mL tube. Insert small-sized dialysis cup (0.1 mL) to the tube. Add 0.5 mL of ultrapure water into the cup.

3.3.2. Incubate for more than 30 min at room temperature.

4. Preparation of liposomes

NOTE: Here we describe two protocols for preparation of liposomes. One uses ready-to-use lyophilized liposomes (section 4.1), while the other produces liposomes by hydrating a thin lipid film (section 4.2).

4.1. Prepare liposomes using lyophilized liposomes.

NOTE: An easier way to produce proteoliposome is to use commercially available Asolectin liposome. Asolectin is a kind of natural lipid extracted from soybeans.

4.1.1. Open the vial containing 10 mg of lyophilized Asolectin liposomes (see the **Table of Materials**) and add 200 µL of translation buffer (section 3.1) to the bottom of the vial. Seal the vial and incubate for 10 min.

4.1.2. Mix vigorously by putting the vial on the vortex-mixer for 1 min.

4.1.3. Insert the vial into a 50 mL tube. Spin down the tube by centrifuging at 500 *x g* for 1 min.

4.1.4. Using a pipette, transfer the Asolectin liposome suspension (50 mg lipid/mL) to a new 1.5 mL tube. Use liposome immediately for translation, otherwise freeze in liquid nitrogen and store at -80 °C.

4.2. Prepare liposomes by hydrating a thin lipid film.

4.2.1. If a lipid is sold in powder form, dissolve in chloroform or appropriate organic solvent to 10-100 mg/mL concentration.

NOTE: A thin lipid film can be prepared using purified and/or synthesized amphiphilic lipids. The purification method of asolectin is previously described[38]. Functionally modified lipids, such as biotinylated lipids, fluorescent lipids, and adjuvant lipids, can be added to the basal lipids to produce functional liposomes.

4.2.2. Transfer the lipid solution containing 50 mg of lipid(s) to an evaporation flask.

4.2.3. Using a rotary evaporator, evaporate solvent and evenly spread the lipid on the wall of the flask bottom to form a thin film of lipid.

4.2.4. Put the flask in a vacuum desiccator and leave under negative pressure overnight to remove the solvent completely.

4.2.5. Add 1 mL of translation buffer to the evaporation flask. Rotate the flask to spread the buffer over the thin lipid film. Incubate for 5 min to hydrate the film.

4.2.6. Sonicate the flask with an ultrasonic homogenizer or ultrasonic cleaner. Change the angle of the flask occasionally to allow the solution to touch the film thoroughly. Ensure that the thin lipid film is peeled from the bottom and emulsified completely and homogenously.

NOTE: Electron micrograph of biotinylated lipids containing liposomes is shown in **Figure 1**.

4.2.7. Transfer the liposome suspension (50 mg lipids/mL) to a new 1.5 mL tube. If it is not to be used immediately, freeze the liposomes in liquid nitrogen, and store at -80 °C.

5. In vitro translation

5.1. Thaw the wheat germ extract quickly by floating the tubes on water at room temperature for a few minutes. After thawing, immediately mix gently by inverting the tubes, spin down, and chill on ice until use.

NOTE: Freeze wheat germ extract in liquid nitrogen after use, and store at -80 °C. It withstands several freeze/thaw cycles.

5.2. Thaw 20 mg/mL creatine kinase stock solution. Mix 5 µL of stock solution and 45 µL of translation buffer to prepare 2 mg/mL creatine kinase working solution.

CAUTION: Refreezing creatine kinase is not recommended.

5.3. Thaw liposomes or mRNAs when required.

5.4. Conduct a small-scale protein translation.

5.4.1. Remove water from both the tube and the dialysis cup (0.1 mL), as prepared in step 3.3.2.

5.4.2. Inject 1 mL and 300 µL of translation buffer in the tube and the dialysis cup, respectively.

CAUTION: In case the bottom of the dialysis cup does not reach the surface of translation buffer in the 1.5 mL tube, inject an additional 50–100 µL of buffer to the tube.

5.4.3. Prepare translation reaction mixture by mixing 15.6 µL of translation buffer, 2.4 µL of 2 mg/mL creatine kinase, 12 µL of 50 mg/mL liposomes, 15 µL of wheat germ extract, and 15 µL of mRNA. Mix gently by inverting the tubes, and spin down.

5.4.4. Aspirate 60 µL of the translation reaction mixture using a 200 µL pipette.

5.4.5. Insert the pipette tip into the undersurface of translation buffer in the dialysis cup. Pipette out the reaction mixture slowly and gently. Cover the dialysis cup with a lid to prevent evaporation.

NOTE: The reaction mixture sinks naturally to the bottom of the cup and forms a bilayer. Do not disturb the bilayer by mixing or shaking the cup.

5.5. Conduct a large-scale translation (**Figure 1**).

5.5.1. Pour 22 mL of translation buffer into a 25 mL tube. Insert a large-sized dialysis cup (2 mL) into the tube and add 2 mL of translation buffer in the cup.

5.5.2. Prepare a translation reaction mixture by mixing 130 µL of translation buffer, 20 µL of 2 mg/mL creatine kinase, 100 µL of 50 mg/mL liposome, 125 µL of wheat germ extract, and 125 µL of mRNA. Mix gently by pipetting.

5.5.3. Aspirate all the translation reaction mixture (500 µL) using a 1,000 µL pipette. Inject translation reaction mixture into the dialysis cup in the same way as described in step 5.4.5. Cover dialysis cup with a lid to prevent evaporation.

5.6. Incubate the reactions at 15 °C for 24 h.

5.7. Mix the reaction well in the dialysis cup by pipetting. Transfer the crude proteoliposome suspension to a new tube.

NOTE: A flat-bottomed 1.5 mL tube is recommended to collect the proteoliposome from the small-scale translation. After centrifugation, liposomes form compact and easily visible pellet on

the bottom corner of the tube.

6. Purification of proteoliposomes

6.1. Centrifuge the tube containing crude proteoliposome suspension at 21,600 *x g* at 4 °C for 10 min.

6.2. Remove the supernatant. Suspend the proteoliposome pellet in PBS (small scale: 1 mL, large scale: 10 mL) by pipetting.

6.3. Repeat the centrifugation and washing of proteoliposomes for another two circles.

6.4. After washing, add a small amount of PBS and re-suspend proteoliposome pellet well by pipetting. Measure the volume of suspension using a micro pipette. Add PBS to adjust the volume to 60 µL (small scale) or 500 µL (large scale). Transfer the suspension to a new 1.5 mL tube.

6.5. Transfer 10 µL of proteoliposome suspension to a new PCR tube for SDS-PAGE. Divide the rest of the samples into smaller portions for use when necessary. Freeze in liquid nitrogen and store at -80 °C.

7. SDS-PAGE and CBB staining

7.1. Add 70 µL of water and 40 µL of 3x SDS-PAGE sample buffer to 10 µL of proteoliposome suspension.

CAUTION: Do not boil the SDS-PAGE sample, as membrane proteins aggregate, and hardly penetrate into acrylamide gel in electrophoresis. Also, add enough reducing agent to SDS-PAGE sample buffer (e.g., 2-mercaptoethanol at 3% final concentration) to prevent oxidation.

7.2. Set a 5%–20% gradient SDS-PAGE gel in an electrophoresis chamber. Load 3 µL, 6 µL, 12 µL of proteoliposome samples, 2 µL of protein size marker, and BSA standard series as well.

7.3. Electrophorese at 52 mA, 400 V for 30 min.

7.4. Stain the gel with CBB dye for 1 h. Decolorize in hot water and scan the gel image.

7.5. Using NIH Image J software (https://imagej.nih.gov/ij/), quantify the band intensity of membrane protein in each lane. Estimate the amount of synthesized membrane proteins with BSA standard series.

REPRESENTATIVE RESULTS:

Using this protocol, partially purified proteoliposomes can be obtained in a short time. Representative results are shown in **Figure 2A**. Twenty five GPCRs of Class A, B, and C were successfully synthesized using the bilayer-dialysis method (small scale) and partially purified by

centrifugation and buffer wash. Although the amount of synthesized proteins varies according to the type of protein, 50 to 400 µg of membrane proteins usually can be synthesized per reaction when large dialysis cups are used. Several milligrams of membrane proteins can be easily produced by increasing the number of reactions, due to the high scalability of wheat cell-free system. A pre-test using a small dialysis cup is sufficient to determine the production efficacy of the target protein in bilayer-dialysis method. According to the obtained productivity, the amount of the target protein to be produced using large dialysis cups can be estimated.

This protocol is suitable for expression of membrane proteins, particularly for those with multiple transmembrane helices. In most cases, membrane proteins with three or more transmembrane helices are easily incorporated into proteoliposomes after synthesis (**Figure 2B**), which makes a good productivity of proteoliposomes. Single-transmembrane-helix proteins are usually synthesized smoothly; however, they hardly integrate into liposomes due to the small hydrophobic region. Regarding proteins with two transmembrane helices, whether or not they are anchored to liposomes is dependent on the way their transmembrane helices are exposed.

Synthesized proteoliposomes are collected by simple centrifugation, and partially purified with a washing buffer, which greatly shortens the purification process of membrane proteins. Although both biological membranes and membrane proteins have been removed from wheat germ extracts beforehand, small amounts of wheat proteins are sometimes co-precipitated by binding to liposomes or membrane proteins synthesized (**Figure 2A**). Such protein contaminants are difficult to remove by simple centrifugation and buffer wash. When a highly purified membrane protein is required, it is necessary to solubilize the partially purified proteoliposomes with a surfactant and purify them by column chromatography.

FIGURE LEGENDS:

Figure 1: Scheme of cell-free proteoliposome production. SP6, SP6 promoter sequence; E01, E01 translation enhancer sequence; Amp^r, ampicillin resistance gene; DTT, dithiothreitol. Electron micrograph shows immunogold labeling of biotinylated lipid containing liposome. Bar, 0.2 µm. This electron micrograph image was from **Figure 1D** in Takeda et al., 2015[45].

Figure 2: Representative results of proteoliposome production by bilayer-dialysis method. (**A**) SDS-PAGE image of cell-free synthesized GPCRs. Twenty-five selected GPCRs were synthesized by the bilayer-dialysis method. Proteoliposomes were partially purified and applied to SDS-PAGE and CBB staining. Arrowheads indicate target GPCRs. (**B**) Comparison of membrane protein productions between different translation methods. Dopamine receptor D1 (DRD1) protein was synthesized by each method in the same ratio of wheat germ extract, liposomes, and mRNA, respectively. DRD1 proteoliposome was partially purified by centrifugation, and subjected to SDS-PAGE and CBB staining. (**C**) Immunogold labeling of DRD1-biotin/liposome complex. DRD1 was enzymatically biotinylated by BirA biotin ligase. Bar, 0.2 µm. Blank arrowheads indicate DRD1-biotin on liposomes. This figure was modified from **Figure 1** in Takeda et al., 2015[45].

Figure 3: Application of functional proteoliposomes. (**A**) Immunization of adjuvant lipid-containing proteoliposome. **(B)** Biotinylated liposome-based interaction assay (BiLIA). Interaction

between membrane protein and anti-membrane protein antibody was detected by AlphaScreen.

DISCUSSION:

The presented protocol provides a method of producing membrane proteins at a high success rate. This protocol is simple, highly reproducible, and easy to scale up. It also has the potential to reduce the time and cost of experiments that consume a large amount of membrane proteins. The bilayer-dialysis method improves the productivity by 4–10 times compared with bilayer method or dialysis method (**Figure 2B)**[45]. In an extreme case, the yield of an ion channel and a transporter increased 30 and 20 times, respectively, with bilayer-dialysis method than that with bilayer method (data not shown). High productivity of this protocol is an advantage in antigen production for immunization. Proteoliposomes are often used as immunizing antigens for development of anti-membrane protein antibodies. Highly concentrated and purified membrane proteins imbedded in proteoliposome effectively stimulate immune response and induce antibodies[41,47]. Using this bilayer-dialysis method, proteoliposomes carrying several milligrams of membrane proteins for immunization purpose can be easily prepared in a few days. Indeed, our group has synthesized GPCRs, ion channels, and claudins using this protocol and immunized mice with the products to obtain monoclonal antibodies against them[31,41,45]. Some of the monoclonal antibodies obtained were verified as functional antibodies, such as high affinity antibodies, conformation-sensitive antibodies, flow cytometry applicable antibodies, and inhibitory antibodies, which indicates that this protocol is capable of producing membrane proteins with functionally correct conformations.

Another attractive benefit of this protocol is to allow the production of proteoliposomes that are assigned specific functions using modified lipids, such as biotinylated lipids, fluorescent lipids, or adjuvant lipids. Prepared proteoliposomes with specific functions are useful and applicable to a wide range of experiments. For example, adjuvant lipid-containing proteoliposomes, such as lipid A[48] or monophosphoryl lipid A (MPLA)[49], make convenient immunizing antigens, because they can be administered directly to immunize mice without emulsion. Adjuvant lipids effectively stimulate immune response in host animals, inducing antibodies against target membrane proteins (**Figure 3A**). Indeed, we have successfully induced flow cytometry applicable antibodies by immunizing mice with MPLA-containing proteoliposome[31]. Also, proteoliposomes prepared from biotinylated lipids are ideal probes for screening assays. We developed a high-throughput screening method to select anti-membrane protein antibodies using biotinylated proteoliposomes and AlphaScreen (BiLIA method) (**Figure 3B**)[45]. Sandwich ELISA is also able to be easily constructed using biotinylated proteoliposomes and streptavidin-coated plates.

Finally, there are two important caveats that should be addressed when using this method. First, formation of disulfide bonds may be insufficient due to the high concentrations of DTT in the translation buffer, which possibly affect the structure of some kinds of membrane proteins[15]. Although disulfide bonds are able to form after the reductant is removed during the purification process, they possibly form into different type rather than the natural ones. The other one is that membrane proteins are not glycosylated. The enzymes required for glycosylation are theoretically absent in the cell-free system because during the process when wheat germ extract is produced, biomembranes, including Golgi and ER, have been removed. Since the lack of

disulfide bonds and glycosylation may cause different conformations, careful consideration and evaluation should be given to the experimental design, particularly when post-translational modifications are critical for the functional expression of proteins according to the experimental purposes.

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DISCLOSURES:

The authors have nothing to disclose.

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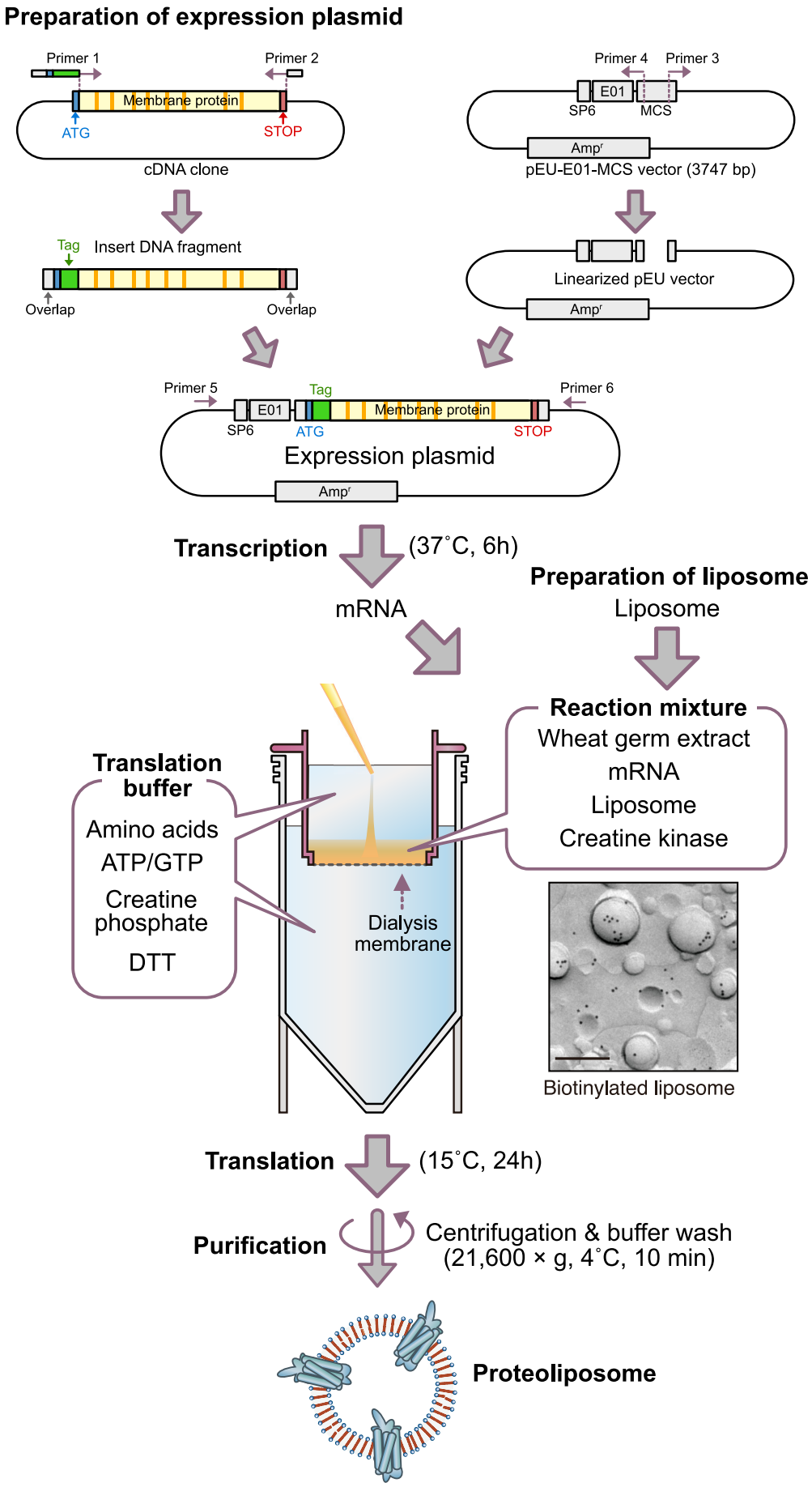


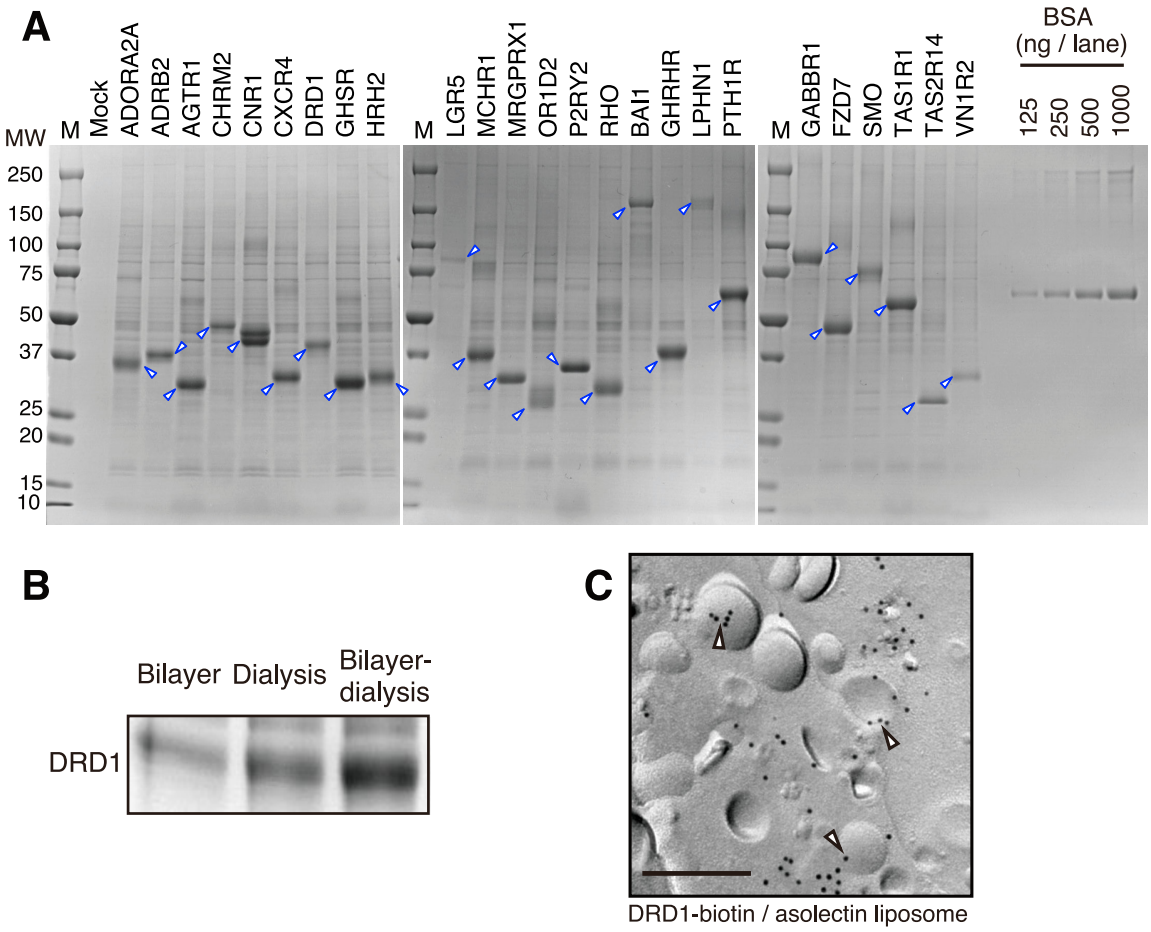
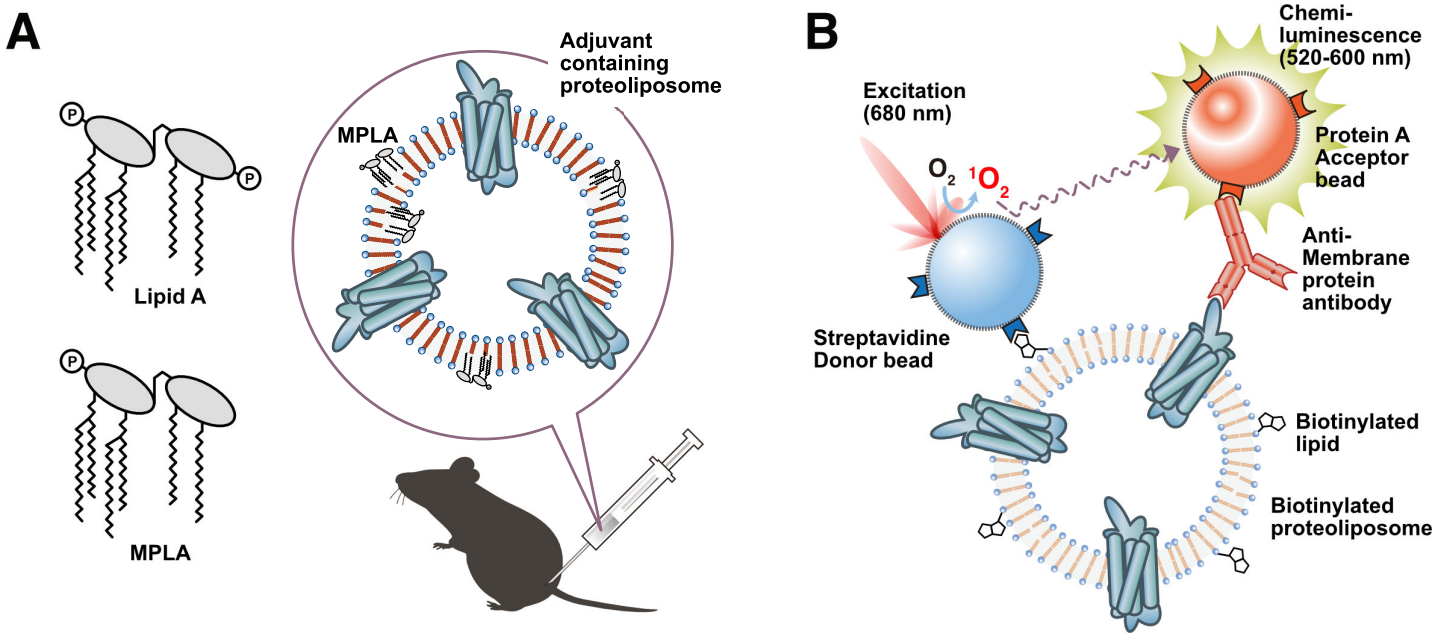


Figure 3 combined

Click here to access/download;Figure;Fig3_combined.eps



Name of Material/ Equipment	Company	Catalog Number
×3 SDS-PAGE sample buffer		
5-20% gradient SDS-PAGE gel	ATTO	E-D520L
70% ethanol		
Agarose	Takara Bio	
Ammonium acetate	Nakalai tesque	02406-95
Ampicillin Sodium	Nakalai tesque	02739-74
Asolectin Liposome, lyophilized	CellFree Sciences	CFS-PLE-ASL
BSA standard		
CBB gel stain		
cDNA clone of interest		
Chloroform	Nakalai tesque	08402-84
Cooled incubator		
Creatine kinase	Roche Diagnostics	04524977190
Dialysis cup (0.1 mL)	Thermo Fisher Scientific	69570
Dialysis cup (2 mL)	Thermo Fisher Scientific	88404
DNA ladder marker	Thermo Fisher Scientific	SM0311
Dpn I	Thermo Fisher Scientific	FD1703
E. coli strain JM109		
Electrophoresis chamber	ATTO	
Ethanol (99.5%)	Nakalai tesque	14713-95

Ethidium bromide		
Evaporation flask, 100 mL		
Gel imager		
Gel scanner		
LB broth		
Lipids of interest	Avanti Polar Lipids	
Micro centrifuge	TOMY	MX-307
NTP mix	CellFree Sciences	CFS-TSC-NTP
Nuclease-free 25 mL tube	IWAKI	362-025-MYP
Nucrease-free plastic tubes	Watson bio labs	
Nucrease-free tips	Watson bio labs	
PBS buffer		
PCR purification kit	MACHEREY-NAGEL	740609
pEU-E01-MCS vector	CellFree Sciences	CFS-11
Phenol/chloroform/isoamyl alcohol (25:24:1)	Nippon Gene	311-90151
Plasmid prep Midi kit	MACHEREY-NAGEL	740410
Primer 1	Thermo Fisher Scientific	Custom oligo synthesis
Primer 2	Thermo Fisher Scientific	Custom oligo synthesis

Primer 3	Thermo Fisher Scientific	Custom oligo synthesis
Primer 4	Thermo Fisher Scientific	Custom oligo synthesis
Primer 5	Thermo Fisher Scientific	Custom oligo synthesis
Primer 6	Thermo Fisher Scientific	Custom oligo synthesis
Protein size marker	Bio-Rad	1610394
Rotary evaporator		
seamless cloning enzyme mixture	New England BioLabs	E2611L
SP6 RNA Polymerase & RNase Inhibitor	CellFree Sciences	CFS-TSC-ENZ
Submarine Electrophoresis system		
TAE buffer		
Transcription Buffer LM	CellFree Sciences	CFS-TSC-5TB-LM
Translation buffer	CellFree Sciences	CFS-SUB-SGC
Ultrapure water		

Ultrasonic homogenizer	Branson	SONIFIER model 450D-Advanced
UV transilluminator		
Vacuum desiccator		
Wheat germ extract	CellFree Sciences	CFS-WGE-7240

Comments/Description
Containing 10% 2-mercaptoethanol
Diluted ethanol by ultrapure water.
As this reagent is deliquescent, dissolve all of it in water once opened and store it at -30˚C.
A vial contains 10 mg of lyophilized liposomes.
1000 ng, 500 ng, 250 ng, 125 ng BSA / 10 µL ×1 SDS-PAGE sample buffer
Plasmid harboring cDNA clone or synthetic DNA fragment
Temperature ranging from 0 to 40˚C or wider.
Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.1 mL
Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 2 mL
GeneRuler 1 kb DNA Ladder
FastDigest *Dpn* I

We use document scanner and LED immuninator as a substitute.

Mixture of ATP, GTP, CTP, UTP, at 25 mM each

Do not autoclave. Use them separately from other experiments.
Do not autoclave. Use them separately from other experiments.

NucleoSpin Gel and PCR Clean-up

NucleoBond Xtra Midi

5’-CCAAGATATCACTAGnnnnnnnnnnnnnnnnnnnnnnn-3’
Gene specific primer, forward. Upper case shows overlap sequence to be added for seamless cloning. Lower case nnnn…. (20-30 bp) shows gene specific sequence.

5'-CCATGGGACGTCGACnnnnnnnnnnnnnnnnnnnnnnn-3’
Gene specific primer, reverse. Upper case shows overlap sequence to be added for seamless cloning. Lower case nnnn…. (20-30 bp) shows gene specific sequence.

5'-GTCGACGTCCCATGGTTTTGTATAGAAT-3'
Forward primer for vector linearization. Underline works as overlap in seamless cloning.
5'-CTAGTGATATCTTGGTGATGTAGATAGGTG-3'
Reverse primer for vector linearization. Underline works as overlap in seamless cloning.
5’-CAGTAAGCCAGATGCTACAC-3’
Sequencing primer, forward
5’- CCTGCGCTGGGAAGATAAAC-3’
Sequencing primer, reverse
Precision Plus Protein Standard

Gibson Assembly Master Mix
Other seamless cloning reagents are also avairable.

SUB-AMIX SGC (×40) stock solution (S1, S2, S3, S4).
Prepare ×1 translation buffer before use by mixing stock S1, S2, S3, S4 stock and ultrapure water.

We recommend to prepare ultrapure water by using ultrapure water production system every time you do experiment. Do not autoclave.
We preparaed ultrapure water by using Milli-Q Reference and Elix10 system.
Commercially available nuclease-free water (not DEPC-treated water) can be used as a substitute. Take care of contamination after open the bottle.

Ultrasonic cleaner can be used as a substitute.

WEPRO7240

Dear Dr. Bajaj,

Thank you very much for reviewing our manuscript.
The pertinent comments and constructive suggestions from you and the other 2 reviewers have significantly helped us to improve the paper. We revised our manuscript following your comments as below.

We hope that our revised manuscript is now suitable for publication in *JoVE*. We are looking forward to hearing from you soon.

Sincerely,

Hiroyuki Takeda
Bunkyocho 3, Matsuyama, Ehime 791-8577, Japan.
Tel: +81-89-927-8285
e-mail: Takeda.hiroyuki.mk@ehime-u.ac.jp

Response to the editorial comments.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript and made corrections where necessary. Due to the wide range of revisions, we provide you with another manuscript showing the revision history (please find JoVE_manuscript_ZhouTakeda_revision history.docx).

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We checked the manuscript and made sure that the manuscript is in correct format.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “This protocol describes…”

We rephrased the Summary as suggested. The length of the Summary is 38 words.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

We checked the Abstract and added some more words. The length of the abstract is 152 words.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Gibson Assembly, NucleoBond Xtra Midi kit, Slide-A-Lyzer MINI Dialysis Device, CellFree Science, WEPRO7240, etc.

We removed all the commercial language from the former manuscript, and replaced them with generic terms. Instead, we put the commercial language and specific product names in the Table of Material and Reagents (please find JoVE_Materials_ZhouTakeda.xls).

The commercial languages were revised as below.
Gibson Assembly >> seamless cloning or seamless cloning enzyme mixture
NucleoBond Xtra Midi kit >> plasmid prep Midi kit
Slide-A-Lyzer MINI Dialysis Device >> dialysis cup
CellFree Science >> removed from the manuscript
WEPRO7240 >> wheat germ extract
SUB-AMIX SGC >> translation buffer

6. Please reword lines 186-188, 243-244, 283-284, 330-333 as it matches with previously published literature.
We rephrased the sentences you kindly pointed out as below.

(Previous, L186-188)
Dissolve lyophilized creatine kinase in freshly prepared ultrapure water to a final

concentration of 20 mg/mL. Dispense the solution into small portions in PCR tubes (10 to 50 µL each). Freeze the tubes using liquid nitrogen, and store at -80˚C. Avoid re-freezing after thawing.

(Revised, L230-232)

Dissolve lyophilized creatine kinase in ultrapure water to a final concentration of 20 mg/mL. Dispense the solution in small amounts (10 to 50 µL each) in 0.2 mL 8-strip PCR tubes. Freeze the tubes in liquid nitrogen, and store at -80˚C.

CAUTION: Do not re-freezing creatine kinase solution after thawing.

(Previous, L 243-244)

4.2.6. Sonicate the flask using ultrasonic homogenizer until the thin lipid layer is peeled from the bottom and emulsion becomes homogenous completely. Electro microscopic image of biotinylated lipids containing liposomes is shown in Figure 2C.

(Revised, L291-293)

4.2.6. Sonicate the flask with an ultrasonic homogenizer or ultrasonic cleaner. Change the angle of the flask occasionally to allow the solution to touch the film thoroughly. Ensure that thin lipid film is peeled from the bottom and emulsified completely and homogenously.

NOTE: Electron micrograph of biotinylated lipids containing liposomes is shown in Figure 1.

(Previous, L 283-284)

NOTE: The reaction mixture sinks naturally and form a layer on the bottom of the cup. Do not mix the reaction buffer and disturb the bilayer. Cover dialysis cup with a lid to avoid evaporation.

(Revised, L334-335)

NOTE: The reaction mixture sinks naturally to the bottom of the cup and form bilayer. Do not disturb the bilayer by mixing or shaking the cup. Cover dialysis cup with a lid to prevent evaporation.

(Previous, L 330-333)

CAUTION: Membrane proteins are easily aggregated by oxidation and heat denaturation. Once membrane proteins aggregate, they hardly penetrate into acrylamide gel in electrophoresis. To prevent aggregation, add enough amount of reducing agent to SDS-PAGE sample buffer (e.g. 3% 2-mercaptoethanol), and do not heat the sample.

(Revised, L382-384)

CAUTION: Do not boil SDS-PAGE sample, otherwise membrane proteins aggregate, and hardly penetrate into acrylamide gel in electrophoresis. Also, add enough reducing agent to SDS-PAGE sample buffer (e.g. 2-mercaptoethanol at 3% final concentration) to prevent oxidation.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

We checked the protocol section and made sure that all the sentences describing the protocol steps are used in the imperative tense. Here we show two examples of our revision. For other changes, please refer to the revision history.

Example1:

(Previous, L202-209)

4. Preparation of liposomes

4.1. Preparation of asolectin liposomes (Fig. 2B).

4.1.1. An easier way to produce proteoliposome is to use commercial liposome.s Asolectin liposomes purchased from CellFree Science contain 10 mg of lyophilized liposomes in a vial.

Note: Asolectin is a kind of natural lipid extracted from soy bean.

(Revised, L247-256)

4. Preparation of liposomes

NOTE: Here we describe two protocols for preparation of liposomes. One uses ready-to-use lyophilized liposomes (section 4.1), while the other produces liposomes by hydrating a thin lipid film (section 4.2).

4.1. Prepare liposomes using lyophilized liposomes.
Note: An easier way to produce proteoliposome is to use commercially available Asolectin liposome. Asolectin is a kind of natural lipid extracted from soy beans.

Example 2:

(Previous, L224-232)
4.2.1. Liposome can be prepared using purified or synthetic amphiphilic lipids. If a lipid is sold in powder form, dissolve in chloroform or appropriate organic solvent to 10-100 mg / mL concentration.

NOTE: Purification of asolectin is previously described38.

4.2.2. Transfer 50 mg of lipid(s) in an evaporation flask. Functional modified lipids, such as biotinylated lipids, fluorescent lipids, and adjuvant lipids, can be added to the basal lipids to produce functional liposomes.
For other changes, please see the revision history.

(Revised, L272-280)
4.2.1. If a lipid is sold in powder form, dissolve in chloroform or appropriate organic solvent to 10-100 mg/mL concentration.

NOTE: Thin lipid film can be prepared using purified and/or synthesized amphiphilic lipids. Purification method of asolectin is previously described38. Functionally modified lipids, such as biotinylated lipids, fluorescent lipids, and adjuvant lipids, can be added to the basal lipids to produce functional liposomes.

4.2.2. Transfer 50 mg of lipid(s) in an evaporation flask.

8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
We confirmed that each step in the protocol section only contains 2-3 actions sentences.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

We added a more detailed description into the protocol to show how the step is performed.

10. 1.1. How is this done?

We added a description of plasmid construction using seamless cloning, explaining each specific step from PCR to transformation.（Section 1.1 to 1.4, L97-137）

11. 1.3.3: How do you perform phenol/choloroform/iso amyl alcohol purification, do you centrifuge? Speed etc.

We added a description explain how to perform the phenol-chloroform purification process. (section 1.6.3. and 1.6.4, L204-215)

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the necessary parts required for videography. The highlighted content is no longer than 2.75 pages.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We also highlighted the necessary relevant details in the sub-steps required for videography. The total highlighted content is no longer than 2.75 pages.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc

or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The electro micrograph images of Figures 1 and Figure 2 are taken from an earlier paper that is citable because the original paper was published on Scientific Reports with an open access under a CC BY license (Source: https://www.nature.com/srep/about/open-access-funding-and-payment).

We additionally prepared a docx. file for the copyright permission information (please find JoVE_cppyright permission_ZhouTakeda.docx). The citation information was added and described in the Figure Legends.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Following the editorial comment #15 and the comment from reviewer #2, we revised the Discussion part (Revised, L448-493) and made it fulfill all the mentioned conditions a) ~ e). Please refer to the revision history for each change.

16. Please combine all panels of one figure into a single image file.

We combined all the panels of Figure 2 and 3 into single image files respectively.

17. Please sort the materials table in alphabetical order.

We sorted the materials table in alphabetical order (please find JoVE_Materials_ZhouTakeda.docx).

Reviewer's comments:

Reviewer #1:

Major Concerns:

none

Thank you very much! We furthermore polished the wording in the manuscript. We really hope that this protocol could provide some hints to researchers who are interested in the studies of membrane proteins and help more with their research.

Minor Concerns:

Figure 3A : replace adjubant by adjuvant

Thank you very much for kindly pointing out the error. We corrected the misspelling of adjuvant in Figure 3A.

Reviewer #2:

Minor Concerns:

Lines 49-50

"Membrane proteins have complicated structures with multiple transmembrane domains and play important roles in cell homeostasis."

1. Membrane proteins are not exclusively those that possess multiple transmembrane helices. A membrane protein may possess one or more transmembrane helices. Please correct this.

Thank for very much for kindly pointing out the insufficient explanation of transmambran proteins. We revised the sentence as below:

> (Previous, L49-50)
> Membrane proteins have complicated structures with multiple transmembrane domains and play important roles in cell homeostasis.
> (Revised, L54-55)
> Membrane proteins have complicated structures with one or more transmembrane helices and play important roles in cell homeostasis.

2. A transmembrane domain would refer to the definable region of a membrane protein, as the portion/structure which is integrated into a lipid bilayer; a single transmembrane helix (of a single pass membrane protein) or a collection of multiple transmembrane helices, comprise a domain. The authors seem to use the term "transmembrane domain(s)" to describe single transmembrane helices of a multi-spanning membrane protein. The correct term should be transmembrane helix (singular) or transmembrane helices (plural). Please change all instances throughout the text of "transmembrane domains" to "transmembrane helix" or "transmembrane helices" where appropriate.
(Lines: 49, 359, 361, 363, 364)

We changed “transmembrane domain / domains” to “transmembrane helix / helices” as the reviewer kindly suggested.

Line 89
"….with batch or bilayer method (Fig. 2A) 45."
1. The figure does not describe a lane the batch reaction - please specify the lane for the batch reaction.

Thank you very much. We mistakenly used the word “batch” where it should have been written as “dialysis”. We corrected that and the same mistakes in the Discussion (previous version, L399).

In the Introduction, we removed the words “compared with batch or bilayer method” from the sentence and changed the description as below.

(Previous, L88-89)
which leads to improved translation efficacy compared with batch or bilayer method (Fig. 2A) [45].
(Revised, L93-94)
which leads to excellent translation efficacy (Figure 2A and Figure 2B)

In the Discussion, we corrected the same mistake.

(Previous, L399)
The Bilayer dialysis method improves the productivity 4 to 10 times compared with batch or bilayer method[45].
(Revised, L452-453)

The bilayer-dialysis method improves the productivity 4-10 times compared with bilayer method or dialysis method (Figure 2B)[45].

Lines 119-127

1. It is not clear what extra steps, if any, are required during the phenol/chloroform purification between section 1.3.3 and 1.3.4.
2. Are there any incubation steps and/or centrifugation steps required?
3. Collection/separation of fractions?

We added descriptions of the specific operations of the phenol-chloroform purification process. (section 1.6.3. and 1.6.4, L158-169)

Line 178

1. What does SUB-AMIX SGC mean? Can this be described please?

SUB-AMIX SGC is a commercial product from CellFree Science Ltd, which is a translation buffer for use in the Wheat cell-free synthesis kit. We changed "SUB-AMIX SGC" to "translation buffer". Details of SUB-AMIX SGC were added in the Table of Materials.

Line 244

"Electro microscopic image…."

1. Please use the term Electron micrograph

We changed "Electro microscopic" into “Electron micrograph” as below. We also moved the electron micrograph from Figure 2C (in previous version) into Figure 1 (in the present version).

(Previous, L244-245)

Electro microscopic image of biotinylated lipids containing liposomes is shown in Figure 2C.

(Revised, L295)

Electron micrograph of biotinylated lipids containing liposomes is shown in Figure 1.

Line 362

"…smoothly; however, they hardly bind to liposome due to the small…"

1. Perhaps the term "integrate into liposomes" instead of "bind to liposome"

Thank you very much. We changed " bind to liposome" (in previous version) into “integrate into liposomes” (in the present version) as below.

(Previous, L380-381)
however, they hardly bind to liposome due to the small hydrophobic region.
(Revised, L413-414)
however, they hardly integrate into liposomes due to the small hydrophobic region.

Line 380 (Figure legend)
"DRD1 was synthesized by each…"
1. Please state the full name of the DRD1 protein, what does it stand for? Then abbreviate with DRD1.
I assume it refers to the Dopamine receptor D1 protein, however, those without knowledge of GPCR biology will not know this.

Thank you very much. Yes, as you kindly pointed out, DRD1 protein refers to the Dopamine receptor D1 protein. We put the full name of DRD1 in the FIGURE LEGENDS of Figure 2 as below.

(Previous, L380-381)
DRD1 was synthesized by each method
(Revised, L437)
Dopamine receptor D1 (DRD1) protein was synthesized by each method

Discussion
The language of the discussion is for the most part, good. However some sections could use a little bit of refinement. Example: lines 400-402 - What does "some kind of membrane protein" refer to?

Thank you very much. We added some more detailed descriptions to make the content easier to understand. Please refer to the revision history for each change.

(Previous, L400-402)
In an extreme case, some kind of membrane protein could not be able to synthesize by bilayer method at all; however, it was successfully synthesized by using bilayer-dialysis method.

(Revised, L453)

In an extreme case, the yield of an ion channel and a transporter increased 30 and 20 times, respectively, with bilayer-dialysis method than that with bilayer method (data not shown).

Other changes

In addition to responding to the editor and the reviewers' valuable comments and correcting grammar and spelling, we made the following revisions to make the paper easier to understand.

Figure 1

Besides the plasmid construction section to the protocol, we also added diagrams to illustrate the seamless cloning process.

Figure 2C in the previous version was removed and integrated into Figure 1.

We revised legend of Figure 1 to reflect the above changes.

Figure 2

We switched the order of the panels in Figure 2, and revised legend of Figure 2 accordingly.

Table of Materials

- In the “Name of Material/Equipment” field, we used common names as described in the manuscript and eliminated trademarks where possible. Specific product names were described in the “Comments/Description” field.
- Along with the addition of the plasmid construction section to the protocol (section 1.1 to 1.4), we added the primers, enzymes, reagents, and kits that we used (primer 1-4, *Dpn*I, PCR purification kit, seamless cloning enzyme mixture).
- The names of the primers were unified, including the sequence primers (Primer 1-6).

Title (Lines 2-3)

We capitalized the first letter of each word in the title.

Affiliation (Lines 8-9)

The name of province and country has been added to the affiliation.

Section 3 title

(Previous, L176)

3. Materials for translation

(Revised, L218)

3. Preparation of materials for translation

Section 4

We have changed wordings in sections 4.1 and 4.2 in order to clarify the difference of their purposes.

(Previous, L204)

4.1. Preparation of asolectin liposomes (Fig. 2B).

(Revised, L250)

4.1. Prepare liposomes using lyophilized liposomes.

(Previous, L222)

4.2. Preparation of liposomes using purified or synthetic lipids.

(Revised, L270)

4.2. Prepare liposomes by hydrating a thin lipid film.

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The electro micrograph images of Figures 1 and Figure 2 in this manuscript are taken from an earlier paper as below.

> Takeda et al. “Production of monoclonal antibodies against GPCR using cell-free synthesized GPCR antigen and biotinylated liposome-based interaction assay.” Scientific reports 5, 11333 (2015).)

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