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TITLE

Co-translational Insertion of Membrane Proteins into Preformed Nanodiscs

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SUMMARY

Co-translational insertion into pre-formed nanodiscs makes it possible to study cell-free synthesized membrane proteins in defined lipid environments without contact with detergents. This protocol describes the preparation of essential system components and the critical parameters for improving expression efficiency and sample quality.

ABSTRACT

Cell-free expression systems allow the tailored design of reaction environments to support the functional folding of even complex proteins such as membrane proteins. The experimental procedures for the co-translational insertion and folding of membrane proteins into preformed and defined membranes supplied as nanodiscs are demonstrated. The protocol is completely detergent-free and can generate milligrams of purified samples within one day. The resulting membrane protein/nanodisc samples can be used for a variety of functional studies and structural applications such as crystallization, nuclear magnetic resonance, or electron microscopy. The preparation of basic key components such as cell-free lysates, nanodiscs with designed membranes, critical stock solutions as well as the assembly of two-compartment cell-free expression reactions is described. Since folding requirements of membrane proteins can be highly diverse, a major focus of this protocol is the modulation of parameters and reaction steps important for sample quality such as critical basic reaction compounds, membrane composition of nanodiscs, redox and chaperone environment, or DNA template design. The whole process is demonstrated with the synthesis of proteorhodopsin and a G-protein coupled receptor.

INTRODUCTION

Membrane proteins (MPs) are challenging targets in protein production studies due to their insolubility in aqueous environments. Conventional MP production platforms comprise cell-based systems such as *E. coli*, yeast, or eukaryotic cells. The synthesized recombinant MPs are either extracted from cell membranes or refolded from inclusion bodies[1]. After detergent solubilization, MPs can be transferred into suitable membrane environments by established in vitro reconstitution protocols. Besides vesicles and liposomes, MP reconstitution into planar membranes in the form of nanodiscs[2] or salipro[3] particles have become routine techniques in recent times. However, all these strategies imply detergent contact with MPs that can result in destabilization, dissociation of oligomers, and even loss of protein structure and activity[4]. Screening for optimal detergent solubilization and reconstitution conditions can therefore be tedious and time consuming[5].

The open nature of cell-free (CF) systems allows the expression reaction to be directly supplied with preformed membranes with a defined lipid composition. In this lipid-based expression mode (L-CF), the synthesized MPs have the opportunity to co-translationally insert into the provided bilayers [6, 7] (**Figure 1**). Nanodiscs consisting of a membrane scaffold protein (MSP) surrounding a planar lipid bilayer disc[8] appear to be particularly suitable for this strategy[9,10]. Nanodiscs can routinely be assembled in vitro with a variety of different lipids, they are very stable, and stocks can be concentrated up to 1 mM. However, MSP expression in *E. coli* and its purification is necessary. As an alternative strategy, MSP can be co-expressed together with the target MP in CF reactions supplied with liposomes[11–13]. DNA templates for both MSP and MP are added into the reaction and MP/nanodiscs can form upon expression. While MSP production is avoided, the co-expression strategy requires careful fine-tuning of the final DNA template concentrations and higher variations in the efficiency of sample production can be expected.

The co-translational insertion of MPs into membranes of preformed nanodiscs is a non-physiological and still largely unknown mechanism independent from translocon machineries and signal sequences[13–16]. Major determinants of the insertion efficiency are the type of membrane protein as well as the lipid composition of the provided membrane, with a frequent preference for negatively charged lipids[15,17]. As the nanodisc membranes are relatively confined in size, a substantial amount of lipids is released upon MP insertion[18]. Variation of nanodisc size enables insertion and tuning of higher oligomeric MP complexes[15,18]. Among others, the correct assembly of homooligomeric complexes was shown for the ion channel KcsA, for the ion pump proteorhodopsin (PR) and for the multidrug transporter EmrE[15,18]. MPs are likely to enter the symmetric nanodisc membrane from both sides at relatively equal frequency. It should therefore be considered that different monomers or oligomers inserted into one nanodisc may have opposite orientations. However, a bias in orientation could be caused by cooperative insertion mechanisms as reported for the formation of PR hexamers and KcsA tetramers[18]. A further bias in MP orientation might result from orientation switches of inserted MPs probably at the rim of the nanodisc membranes.

The production of CF lysates from *E. coli* strains is a reliable routine technique and can be performed in almost any biochemical laboratory[19,20]. It should be considered that besides

disulfide bridge formation, most other post-translational modifications are absent if a MP is synthesized using *E. coli* lysates. While this might generate more homogenous samples for structural studies, it may be necessary to exclude potential effects on the function of individual MP targets. However, the efficient production of high quality samples of G-protein coupled receptors (GPCR)[14,21,22], eukaryotic transporters[23] or large heteromeric assemblies[24] in *E. coli* CF lysates indicates their suitability for even complex targets. Preparative scale amounts (≈ 1 mg/mL) of a sample can be obtained with the two-compartment continuous exchange cell-free (CECF) configuration, composed of a reaction mixture (RM) and a feeding mixture (FM) compartment. The FM volume exceeds the RM volume 15 to 20-fold and provides a reservoir of low-molecular weight energy compounds and precursors[19]. The expression reaction is thus extended for several hours and the final yield of the MP target is increased. The RM and FM compartments are separated by a dialysis membrane with a 10-14 kDa cutoff. The two compartments require a special design of the CECF reaction container (**Figure 1**). Commercial dialysis cassettes as RM containers in combination with tailored plexiglass containers holding the FM are suitable examples. MP yields can further be manipulated by modifying the RM:FM ratios or by exchanging the FM after a certain period of incubation.

Yield and quality of a MP frequently require intense optimization of reaction parameters. An important advantage of CF expression is the possibility to modify and fine tune almost any compound according to the individual requirements of a MP. A straightforward strategy is to focus first on improving the yield of a MP by establishing a basic production protocol and then to optimize MP quality by fine tuning reaction and folding conditions. The absence of physiological processes in CF lysates and their reduced complexity result in high success rates for the efficient production of MPs[25]. Routine considerations for DNA template design and optimization of Mg^{2+} ion concentration are in most cases sufficient to obtain satisfactory yields[26]. Depending on expression mode, optimization of MP quality can become time consuming, as a larger variety of parameters need to be screened[14, 17, 22].

To establish the described CF expression platform, protocols are necessary for the production of *E. coli* CF lysate (i), T7 RNA polymerase (ii), nanodiscs (iii), and the basic CECF reaction compounds (iv) (**Figure 1**). The *E. coli* K12 strain A19[27] or BL21 derivatives are frequently used for the preparation of efficient S30 (centrifugation at 30,000 x *g*) lysates. Besides S30 lysates, corresponding lysates centrifuged at other *g*-forces (e.g. S12) may be used. The lysates differ in expression efficiency and in proteome complexity. The proteome of the S30 lysate prepared by the described protocol has been studied in detail[28]. The S30 proteome still contains some residual outer membrane proteins which could give background problems upon expression and analysis of ion channels. For such targets, the use of S80-S100 lysates is recommended. A valuable modification during lysate preparation is the induction of the SOS response by combined heat shock and ethanol supply at mid-log growth phase of the cells. The resulting HS30 lysates are enriched in chaperones and can be used in blends with S30 lysates for improved MP folding[22]. CF expression in *E. coli* lysates is operated as a coupled transcription/translation process with DNA templates containing promoters controlled by T7 RNA polymerase (T7RNAP). The enzyme can be efficiently produced in BL21(DE3) Star cells carrying the pAR1219 plasmid[29].

Two copies of MSP1E3D1 assemble into one nanodisc with a diameter of 10-12 nm, but the protocol described below may also work for other MSP derivatives. However, nanodiscs larger than those formed with MSP1E3D1 tend to be less stable while smaller nanodiscs formed with MSP derivatives such as MSP1 may not accommodate larger MPs or MP complexes. MSP1E3D1 nanodiscs can be assembled in vitro with a large variety of different lipids or lipid mixtures. Preformed nanodiscs are usually stable for > 1 year at -80 °C, while stability may vary for different lipid components. For the screening of lipid effects on folding and stability of a MP, it is necessary to prepare a comprehensive set of nanodiscs assembled with a representative variety of lipids/lipid mixtures. The following lipids may give a good starting selection: 1,2-Dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2 dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC).

A protocol for the preparation of a 3 mL CECF reaction is described. Further up or down scaling in a 1:1 ratio is possible. For the two-compartment CECF configuration, a RM containing all compounds and a FM containing only the low-molecular weight compounds have to be prepared. Commercial 3 mL dialysis devices with 10-14 kDa MWCO can be used as RM containers, which are then placed into custom made plexiglass containers holding the FM (**Figure 1D**)[30]. The ratio of RM:FM is 1:20, so 60 mL of FM have to be prepared for 3 mL RM. Quality, concentration, or type of several components can be critical for the final yield and/or quality of the synthesized MP (**Table 1**). DNA templates should be prepared according to published guidelines and codon optimization of the reading frame of the target can further significantly improve product yield[26]. For preparative scale CECF reaction, an established protocol for the production of PR is described. To establish expression protocols for new MPs, it is usually necessary to perform optimization screens of certain compounds (**Table 1**) to improve yield and quality. For Mg^{2+} ions, a well-focused concentration optimum does exist that frequently shows significant variation for different DNA templates. Other CF reaction compounds such as new batches of T7RNAP or S30 lysates may further shift the optimal Mg^{2+} concentration. As an example, a typical Mg^{2+} screen within the range of 14-24 mM concentration and in steps of 2 mM is described. Each concentration is screened in duplicates and in analytical scale CECF reactions. As CECF reaction container, custom-made Mini-CECF Plexiglas containers[30] holding the RM are used in combination with standard 24-well microplates holding the FM (**Figure 1B**). Alternatively, commercial dialysis cartridges in combination with 96-deep well microplates or other commercial dialyzer devices in appropriate setups may be used (**Figure 1C**).

PROTOCOL

1. Preparation of S30 lysate

1.1 **Day 1:** Streak out cells from glycerol stocks on an LB agar plate and incubate at 37 °C overnight.

1.2 **Day 2:** Inoculate 200 mL of LB medium with the cells from the agar plate and incubate at

37 °C for 12-14 h.

1.3 **Day 3:** Inoculate 10 L of sterile YPTG medium (10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl, 19.8 g/L glucose, 4.4 mM KH_2PO_4, 8 mM K_2HPO_4) tempered to 37 °C in a 15 L stirred tank reactor with 100 mL of the pre-culture (1:100). Cultivate at 37 °C, 500 rpm and high aeration (~ 3 air volumes per minute). To prevent excessive foaming, add sterile antifoam.

1.4 Measure optical density (OD) at 600 nm in regular time intervals. After approximately 2 h the culture will enter the log-phase.

1.5 **Modification for HS30 lysate:** When cells are in mid log phase (OD600 ≈ 3.6-4.2) add 300 mL of ethanol to the culture medium and proceed cultivation at 42 °C, 500 rpm, and high aeration (approximately 3 air volumes per minute) for another 45 min. Then proceed with cooling and harvesting of the cell culture as described in step 1.6. For the production of standard S30 lysate, skip this step and proceed with step 1.6.

1.6 When cells are in mid log phase (OD600 ≈ 3.6-4.2), **cool** fermenter below 20 °C within < 30 min. The final OD600 should be around 4.5-5.5. **Harvest** cells at 4,500 x *g* for 20 min at 4 °C and discard the supernatant. Keep cells at 4 °C throughout the following steps.

NOTE: During cooling, excessive foaming can occur. In this case, either add antifoam or reduce stirring speed and/or decrease the air stream in the bioreactor.

1.7 Suspend cells completely in 300 mL of S30-A buffer (10 mM Tris-HCl, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KCl, 6 mM 2-mercaptoethanol) using a spatula followed by pipetting the suspension up and down until homogeneity. Centrifuge at 8,000 x *g* for 10 min at 4 °C. Repeat this step twice.

CAUTION: 2-mercaptoethanol is toxic. Avoid contact with skin or respiratory tract. If possible, work under a hood. Weigh the empty centrifuge beaker before the last washing step. After centrifugation, weigh the wet cell pellet and either proceed with step 1.8 or store the pellet until further use.

NOTE: The weight of the wet cell pellet is 5-7 g per 1 L bioreactor culture. At this point the protocol may be paused and the pellet can be frozen in liquid nitrogen and stored at -80 °C for 4-8 weeks.

1.8 Suspend cells thoroughly in 1.1 volumes (1 g = 1 mL) of S30-B buffer (10 mM Tris-HCl, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT, 1 tablet of complete protease inhibitor). Fill the suspension into a pre-cooled French press pressure cell and disrupt cells at 1,000 psig. Pass cells through the French press once.

1.9 Centrifuge the lysate at 30,000 x *g* for 30 min at 4 °C. Transfer the supernatant into a fresh tube and repeat the centrifugation step.

NOTE: A loose and slimy layer may be present on top of the pellet after the first centrifugation, which should not be transferred with the supernatant.

1.10 Apply a high salt and heat step to remove unstable lysate components and to release mRNA from ribosomes. Adjust the lysate to 0.4 M NaCl and incubate at 42 °C for 45 minutes in a water bath.

NOTE: This step will cause significant precipitate formation. Flip or invert the tube during incubation from time to time.

1.11 Transfer the turbid suspension (usually 50-80 mL) into dialysis tubes with a 10-14 kDa cutoff and dialyze against 5 L S30-C buffer (10 mM Tris-HCl, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 0.5 mM DTT). Exchange the S30-C dialysis buffer after 2-3 h and dialyze for another 12-14 h.

1.12 **Day 4:** Transfer the dialyzed suspension into centrifuge tubes and centrifuge at 30,000 x *g* for 30 min at 4 °C. Transfer supernatant (S30 lysate) into fresh 50 mL tubes and gently mix. The protein concentration of the lysate should be approximately 30-40 mg/mL. Shock freeze aliquots (e.g. 0.5 mL, 1 mL) in liquid nitrogen and store at -80 °C. The aliquots are stable for > 1 year.

2. Expression and purification of T7 RNA polymerase

2.1 **Day 1:** Inoculate 200 mL LB medium containing 100 µg/mL ampicillin with freshly plated BL21(DE3) Star x pAR1219 cells and incubate at 37 °C and 180 rpm for 12-16 h.

2.2 **Day 2:** Inoculate 1 L terrific broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol) in a 2 L baffled flask with 10 mL of the pre-culture and incubate at 37 °C and 180 rpm agitation. The starting OD600 should be around 0.1.

2.3 When the OD600 reaches 0.6-0.9, add IPTG to a final concentration of 1 mM to induce T7RNAP expression and continue cultivation for another 5 h.

2.4 Harvest cells by centrifugation at 4,500 x *g* for 20 min at 4 °C. Freeze cells in liquid nitrogen and store at -80 °C.

NOTE: The protocol may be paused here.

2.5 **Day 3:** Add 30 mL of ice-cold suspension buffer (30 mM Tris-HCl, pH 8.0 with one tablet of complete protease inhibitor) to the frozen cell pellet and thaw the pellet in a water bath at room temperature. Then, suspend the pellet by pipetting up and down until homogeneity.

2.6 Perform cell disruption using a French press as described in the previous section or use a sonication device according to manufacturer's recommendations. Subsequently, centrifuge at

20,000 x *g* for 30 min at 4 °C and transfer supernatant into a fresh tube.

2.7 To precipitate genomic DNA, add streptomycin sulfate slowly and under gentle agitation to the supernatant until a final concentration of 3% (w/v) is reached. Incubate at 4 °C for 5-10 min under gentle agitation. Centrifuge at 20,000 x *g* for 30 min at 4 °C.

2.8 Filter the supernatant with a 0.45 µm filter and load it on a Q-Sepharose column with a column volume (CV) of 40 mL equilibrated with 2 CV equilibration buffer (30 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM NaCl, 5% glycerol and 10 mM 2-mercaptoethanol) using a peristaltic pump at a flow rate of 4 mL/min. Then, connect the column to a UV detector and continue elution with equilibration buffer until the UV signal at 280 nm reaches a stable baseline.

2.9 Elute T7RNAP with a gradient from 50 mM to 500 mM NaCl per CV at a flow rate of 3 mL/min. Collect 1 mL fractions and prepare samples for SDS-PAGE analysis by mixing 10 µL of each fraction with 2 x SDS sample buffer. Run SDS-PAGE and stain the gel with Coomassie Blue R. T7RNAP should appear as a prominent band at approximately 90 kDa along with numerous additional bands of impurities.

2.10 Pool fractions containing T7RNAP and dialyze using membranes with a 12-14 kDa MWCO for 12-16 h in dialysis buffer (10 mM K_2HPO_4/KH_2PO_4, pH 8.0, 10 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5% (w/v) glycerol).

2.11 **Day 4:** Collect T7RNAP solution and concentrate using filter units with 12-14 MWCO to a final concentration of 3-10 mg/mL. T7RNAP may start to precipitate during concentration. Stop concentration instantly as soon as turbidity in the sample occurs. Add glycerol to a final concentration of 50% (w/v) and mix gently. Shock-freeze 0.5-1 mL aliquots and store at -80 °C. Working T7RNAP aliquots can be stored at -20 °C.

NOTE: Approximately 20-40 mL of 5 mg/mL partially purified T7RNAP with 20,000-40,000 units should be obtained from a 1 L fermentation. To test the optimum amount of each T7RNAP batch, CF expression reactions of green fluorescent protein (GFP) containing 0.02 mg/mL-0.1 mg/mL of the prepared T7RNAP sample should be performed.

3. Expression and purification of MSP1E3D1

3.1 **Day 1:** Transform *E. coli* BL21 (DE3) Star cells with the pET28(a)-MSP1E3D1 vector[31] using standard heat shock transformation or electroporation protocols. Streak out or plate transformed cells on LB agar containing 30 µg/mL kanamycin and incubate at 37 °C for 12-16 h.

3.2 **Day 2:** Inoculate 200 mL of LB medium supplemented with 0.5% (w/v) glucose and 30 µg/mL kanamycin with a single colony picked from the agar plate and incubate at 37 °C at 180 rpm for 12-16 h.

3.3 **Day 3:** Inoculate 10 L LB medium supplemented with 0.5% (w/v) glucose and 30 µg/mL

kanamycin with 100 mL of the pre-culture in a stirred-tank bioreactor. Conduct fermentation at 37 °C, 500 rpm and aeration of approximately 3 bioreactor volumes per minute. In the case of excessive foaming, add antifoam.

NOTE: Baffled shaking flasks may be used instead of a fermenter.

3.4 At OD600 of 6.5-7.5, add IPTG to a final concentration of 1 mM and continue incubation at 37 °C for 1 h. Harvest cells by centrifugation at 4,500 x *g* for 20 min at 4 °C. Wash cell pellets with 200 mL of 0.9% (w/v) NaCl and centrifuge again at 8,000 x *g* for 10 min at 4 °C. Discard supernatant and transfer cells into 50 mL tubes using a spatula. The expected wet pellet weight is 8-12 g per L of bioreactor culture. Shock-freeze cell pellets in liquid nitrogen and store at -80 °C until further use.

NOTE: The protocol can be paused here.

3.5 **Day 4:** For purification, thaw cell pellets in a water bath at RT. Suspend the cells in MSP-A buffer (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% (w/v) Triton X-100, 1 tablet of c0mplete protease inhibitor cocktail per 50 mL cell suspension) to yield a 30-40% (w/v) cell suspension.

3.6 Disrupt cells by sonication or using a French press and centrifuge at 30,000 x *g* for 30 min at 4 °C. Transfer the supernatant into a fresh tube and filter through a 0.45 µm filter. Apply the filtrate on a Ni^{2+} loaded nitrilotriacetic acid column (CV > 15 mL) equilibrated with 5 CV of MSP-B buffer (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% (w/v) Triton X-100) by using a peristaltic pump.

NOTE: To facilitate filtration, the supernatant can be sonicated for another minute to break down large DNA fragments.

3.7 After sample loading, wash the column with 5 CV MSP-B, 5 CV MSP-C (40 mM Tris-HCl, pH 8.9, 300 mM NaCl, 50 mM cholic acid), 5 CV MSP-D (40 mM Tris-HCl, pH 8.9, 300 mM NaCl), 5 CV MSP-E (40 mM Tris-HCl, pH 8.0, 300 mM NaCl) and 5 CV MSP-F (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 50 mM imidazole).

NOTE: Cholic acid in the MSP-C buffer is important to remove lipids attached to MSP. Cholic acid is not completely soluble at low pH values. The pH value must therefore be adjusted to 8.9 in MSP-C and MSP-D. It is important to wash the column with MSP-D buffer, as a lower pH might cause residual cholic acid to precipitate and to block or damage the column.

3.8 Elute MSP with MSP-G (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 300 mM imidazole). Collect fractions of 1 mL and monitor UV absorption at 280 nm. Collect and pool the fractions of the elution peak.

3.9 Transfer pooled MSP fractions into 12-14 kDa MWCO dialysis membrane tubes and dialyze against 5 L of MSP-H (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% (w/v) glycerol) for 2-3 h at 4 °C. Change to fresh 5 L MSP-H buffer and dialyze for another 12-16 h at 4 °C.

3.10 **Day 5:** Transfer MSP solution into centrifuge tubes and centrifuge at 18,000 x *g* for 30 min at 4 °C to remove precipitate. Transfer supernatant into a fresh tube and concentrate to 200-800 µM using ultrafiltration devices with 12-14 kDa MWCO at 4 °C. Measure concentration using a UV/VIS measuring device. Use extinction coefficient of 27.31 M^{-1} cm^{-1} and the molecular mass of 31.96 kDa for calculation of concentration.

NOTE: Expression in a bioreactor usually yields 15-30 mg of MSP1E3D1 per L culture.

3.11 Shock-freeze aliquots of the concentrated MSP solution in liquid nitrogen and store at -80 °C.

4. Assembly of MSP1E3D1 nanodiscs

4.1 **Day1:** Prepare 1-2 mL of 50 mM lipid stocks (DMPG, DOPG, POPG DMPC, DOPC or POPC) in 100 mM sodium cholate. Store at -20 °C if not used on the same day.

NOTE: The lipid solution must be clear. If the solution is not clear, sodium cholate concentration may be increased to 150 mM.

4.2 Mix the MSP1E3D1 solution with the lipid solution. Add dodecyl phosphocholine (DPC) at a final concentration of 0.1% (w/v). Assembly reactions may be adjusted to final volumes of 3 mL (**Table 2**) or 12 mL corresponding to typical volumes of dialysis cassettes (10 kDa MWCO). Incubate assembly reactions for 1 h at 21 °C under gentle agitation.

NOTE: For each lipid, a specific MSP1E3D1:lipid ratio has to be used to ensure homogeneous nanodisc preparation (**Table 2**). For new lipids or lipid mixtures, the optimal ratio should be determined with a pilot experiment and size exclusion chromatography analysis[14].

4.3 Pre-wet the membrane of a dialysis cassette with disc formation (DF) buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl). Transfer the assembly mixture into the dialysis cassette with a syringe. Potential air bubbles can be removed by aspiration using the syringe.

4.4 **Days 1-4:** Dialyze against 3 x 5 L DF buffer for 10-20 h at RT under agitation using a stirring bar. Then transfer the assembly mixture from the dialysis cassette into centrifugal filter units with 10 kDa MWCO equilibrated with DF buffer. Concentrate at 4,000 x *g* at 4 °C.

NOTE: A turbid dialysis mix could indicate a wrong stoichiometric ratio of MSP:lipid. Precipitate must be removed at 18,000 x *g* for 20 min at 4 °C before concentration of sample. To avoid precipitation during sample concentration, use ultrafiltration units with a large deadstop.

4.5 Concentrate the assembled nanodiscs to a concentration of at least 300 µM. Measure concentration using an UV/VIS reader. Consider that one nanodisc is formed by two MSP1E3D1 molecules and thus, the concentration of MSP must be divided by 2 to determine the correct

amount of nanodiscs.

NOTE: The described setup (**Table 2**) will yield 0.6-1 mL of a 300 µM nanodisc solution.

4.6 Centrifuge the concentrated nanodisc preparation at 18,000 x *g* for 20 min at 4 °C to remove precipitate. Then, aspirate the supernatant and shock-freeze 50 µL aliquots in liquid nitrogen and store at -80 °C.

NOTE: It is advisable to evaluate the success of nanodisc formation using size exclusion chromatography. The sample should be monodisperse and should contain only a low amount of aggregates. Aggregates indicate that the amount of lipid in the setup is too high. As a reference, purified MSP1E3D1 can be used. If the nanodisc preparation shows a peak at the height of the reference MSP1E3D1 peak, the chosen lipid to MSP1E3D1 ratio was too low.

5. Preparative scale 3 mL CECF reaction setup

5.1 **Day 1:** Prepare all necessary stock solutions as listed (**Table 3**). Stocks are stored at -20 °C and thawed at room temperature. Make sure to thoroughly mix all stocks after thawing and before pipetting. Calculate the required volumes for all stocks and make a pipetting scheme (**Table 4**). High-molecular weight compounds will only be added into the RM. For the low-molecular weight compounds, a combined 3 x mastermix for RM and FM can be prepared.

NOTE: Final volumes of compound mixtures might be less than calculated due to volume loss during mixing. This can be avoided by adding an excess volume of 2-5% to compensate for volume loss.

5.2 Equilibrate the membrane of a 3 mL dialysis cassette in 100 mM Tris-acetate, pH 8.0. Make sure to remove excess buffer.

5.3 Using a syringe to transfer the RM into the dialysis cassette. Remove excessive air in the RM compartment by aspiration with the syringe. Place the dialysis cassette into the dialysis chamber.

5.4 Fill up the dialysis chamber with 60 mL of FM. Place the lid on the chamber and tighten the screws to fix it. Incubate the chamber for 12-16 h at 30 °C at 200 rpm.

NOTE: Make sure that the chamber remains in the upright position during agitation, otherwise exchange of low molecular compounds will be hampered. The protocol may be paused here.

5.5 **Day 2:** Using a syringe, aspirate the RM from the dialysis chamber. Transfer the RM to fresh tubes and centrifuge at 16,000 x *g* at 4 °C for 10 min to remove precipitate. Transfer the supernatant into fresh tubes. The protein in the supernatant can now be further analyzed.

NOTE: In some cases, a pellet will be present after centrifugation. This can provide important

information on the suitability of the analyzed nanodiscs or the reaction compounds to keep the synthesized MP soluble. It is therefore advisable to analyze the amount of target potentially present in the precipitate by using SDS-PAGE, Western Blot, or by visual evaluation of the pellet size.

6. Analytical scale 60 µL CECF reaction setup for Mg^{2+} ion screening

6.1 **Day 1:** Mix all components of the respective 3x master mix and distribute 60 µL to the RM and 825 µL to the FM. Fill up FM with H_2O and mix FM by vortexing. Transfer FM into 3 wells of the 24 well microplate (**Table 5**).

NOTE: Since the customized Mini-CECF container might not be accessible, the volumes in **Table 5** are calculated for a reaction carried out in dialysis cartridges (10-14 kDa MWCO, RM: 100 µL) with a FM volume of 1.7 mL in 96 deep-well (2 mL) plates (**Figure 1**).

6.2 Cut regenerated cellulose dialysis tubes with a 10-14 kDa MWCO into approximately 25 x 20 mm sized pieces and store in H_2O with 0.05% (w/v) sodium azide.

CAUTION: Sodium azide is very toxic. Avoid any contact with skin or mucous membrane. Do not use metal containers as sodium azide can react with metals to form explosive metal azides.

6.3 Before Mini-CECF container assembly, remove excessive H_2O from the dialysis membranes with a tissue. Place one membrane piece on each container and fix the membranes with a polytetrafluorethylene ring.

6.4 Transfer the Mini-CECF container into the wells of a 24-well plate with 825 µL of FM. Avoid air bubbles below the dialysis membrane of the Mini-CECF container.

6.5 Add high molecular components to the RM as described (**Table 5**) and mix by pipetting up and down. Add 60 µL to the reaction container. Avoid air bubbles during transfer of the viscous solution.

6.6 Cut 2 8 x 10 cm sheets of a sealing thermoplastic film and wrap them around the 24-well plate with the reaction containers inside. This will reduce evaporation from the reaction mixture. Now place the lid of the cell culture plate on the plate and fix it with tape. Incubate the plate at 30 °C and 200 rpm agitation for 12-16 h.

6.7 **Day 2:** Harvest the reaction mix by piercing through the dialysis membrane with the pipet tip at the Mini-CECF container and aspirate the RM. Transfer RM into fresh tubes and centrifuge at 16,000 x *g* at 4 °C for 10 min to remove precipitates. Transfer the supernatant into fresh tubes. The protein in the supernatant can now be further analyzed.

NOTE: In some cases, after centrifugation a pellet will be present. This can provide important information on the suitability of the analyzed nanodiscs or other reaction compounds to keep

the synthesized MP soluble. It is therefore advisable to analyze the amount of target potentially present in the precipitate by SDS-PAGE, Western Blot, or visual evaluation of the pellet size.

REPRESENTATIVE RESULTS

The impact of fine-tuning reaction compounds on the final yield or quality of synthesized MPs is exemplified. A frequent routine approach is to adjust the optimal Mg^{2+} concentration in CF reactions by expression of green fluorescent protein (GFP) as a convenient monitor of system efficiency. As an example, GFP was synthesized from a pET-21a(+) vector at Mg^{2+} concentrations between 14 and 24 mM (**Figure 2**). SDS-PAGE analysis identified the optimal Mg^{2+} concentration at 20 mM (**Figure 2A**), which is in good accordance with complementary fluorescence measurements (excitation at 485 nm, emission measurement at 535 nm) of the CF reaction supernatant (**Figure 2B**). For the CF expression of the bacterial PR expressed from pIVEX 2.3 vector and of the turkey β_1 adrenergic receptor ($T\beta_1AR$) expressed from pET-21a(+) vector, the optimal Mg^{2+} concentration was identified at 20-22 mM.

As a further example, quality refinement of synthesized MPs with the described strategy is shown. Tunable parameters for the co-translational insertion of MPs into preformed nanodiscs are (i) the lipid composition of the nanodisc membrane and (ii) the final nanodisc concentration in the reaction. It is well known that the hydrophobic environment is an important parameter for correct folding, oligomeric assembly, and stability of MPs. Since lipid composition in nanodiscs can be modulated, the described approach enables a straightforward systematic screening of lipid effects on structure and function of a MP. In initial experiments, it is recommended to screen lipids with phosphatidylcholine (PC) and phosphatidylglycerol (PG) headgroups and to test different chain lengths and saturations. The impact of membrane composition and nanodisc concentration on the solubilization efficiency and quality of two different MPs is shown. PR is a light activated proton pump and a very stable and highly synthesized MP reaching final concentrations of > 100 µM in the RM. Thus, it is recommended to use it as a positive control to ensure correct reaction setup as PR concentration can be easily assessed by measurement of absorption at 530 nm in the RM supernatant. In contrast, $T\beta_1AR$ is an example of the large family of eukaryotic G-protein coupled receptors (GPCRs) and is a complex and unstable MP. For convenient monitoring, a $T\beta_1AR$-GFP fusion construct was synthesized and the total concentration of nanodisc solubilized receptor in the CF reactions was determined via GFP fluorescence (**Figure 3A**). The fraction of functionally folded and ligand-binding active receptor was further determined using a filter binding assay and the labeled ligand [^{3}H]-dihydroalprenolol (**Figure 3B**). The filter binding assay was performed as described previously[14]. Briefly, $T\beta_1AR$-GFP concentration in the RM was determined via its fluorescence. The GPCR was incubated with the radiolabelled ligand for 1 hour at 20 °C, applied to a filter, and unbound ligand was washed off. For determination of unspecific [^{3}H]-dihydroalprenolol binding, the receptor was saturated with unlabeled alprenolol in a control reaction. The counts of the radioactive ligand in the filters were measured in a scintillation counter and the amount of bound ligand was determined via its specific label activity. Percent GPCR binding activity was then calculated from the amount of bound ligand, the $T\beta_1AR$-GFP concentration, and the assay volume. The overall synthesis and nanodisc solubilization of $T\beta_1AR$-GFP is similar with all analyzed membrane compositions and within the range of 8-13 µM (**Figure 3A**). In contrast, a much higher variation is detectable in the

quality of the synthesized GPCR. Lowest activity with less than 10% active fraction is obtained with the lipids DMPC and POPC. With DOPG and POPG, the active fraction of $T\beta_1AR$-GFP could be increased to approximately 30% (**Figure 3B**). The results indicate that charge of the lipid headgroup as well as flexibility of the fatty acid chain are important modulators for folding and activity of this GPCR.

Besides nanodisc composition, their final concentration in the CF reaction can be an important factor for MP quality. Once a suitable membrane composition of a nanodisc has been identified, its concentration during MP synthesis should be screened. It is obvious that the nanodisc concentration needs to be adjusted according to the expression efficiency of a MP. CF synthesis of $T\beta_1AR$-GFP receptor and PR result in final concentrations of approximately 10 µM and 100 µM in the RM, respectively. If the nanodisc concentration is screened within a range of 3.75-60 µM, a complete solubilization of the GPCR is obtained at approximately 30 µM nanodiscs, giving a ratio of $T\beta_1AR$:nanodisc of 1:3 (**Figure 4A**). In contrast, complete solubilization of PR is already achieved with approximately 10 µM nanodiscs and gives a PR:nanodisc ratio of 10:1 (**Figure 4B**). An excess of nanodiscs is therefore necessary to achieve almost complete solubilization of $T\beta_1AR$-GFP, while an inverted ratio in the case of PR indicates the insertion of multiple PR copies into one nanodisc. Subsequent studies of purified PR/nanodisc complexes with native mass spectrometry confirmed this observation and found a prevalence of higher oligomeric forms of PR if synthesized at lower nanodisc concentrations [15, 18]. The titration of CF synthesized MPs with nanodiscs can therefore be used as a tool to trigger oligomeric assemblies and to study their effects on MP function.

Figure 1: CECF expression strategy for the insertion of membrane proteins into nanodiscs. (A) Basic workflow illustrating the major steps of the process. **(B)** Customized analytical scale reaction vessels for CECF expression. **(C)** Commercially available dialysis cartridges for analytical scale CECF setup. **(D)** Preparative scale setup (3 mL RM) including a 3 mL dialysis cassette and a customized plexiglass FM container. **(E)** Co-translational insertion of MPs into preformed nanodiscs and lipid screening in the CECF configuration. The RM contains the required transcription/translation machinery and nanodiscs while low molecular weight compounds are present in both compartments. Biochemical and structural applications of the produced MP/nanodisc samples are further illustrated.

Figure 2: Effect of different Mg^{2+} concentrations on CF GFP synthesis. GFP was synthesized in CF reactions with Mg^{2+} concentrations within a range of 14-24 mM. **(A)** SDS-PAGE analysis shows the strongest band of GFP at 20 mM Mg^{2+}. M: Marker. **(B)** GFP fluorescence after CF expression with different Mg^{2+} concentrations. The maximal GFP fluorescence at 20 mM Mg^{2+} corresponds to 4.6 mg/mL. Error bars represent the standard deviation of a duplicate measurement.

Figure 3: Effect of nanodisc membrane composition on solubilization and quality of a CF synthesized GPCR. Yield and activity of $T\beta_1AR$-GFP synthesized in the presence of 30 µM nanodiscs containing different membrane compositions. The total synthesized protein **(A)** and fraction of ligand-binding active receptor **(B)** are given in µM. Total concentration was determined via fluorescence measurement of the GFP fusion. Activity was measured via filter

binding assay with the radiolabeled ligand [^{3}H]-dihydroalprenolol. Error bars represent standard deviations of independent triplicates. Data taken from previously published manuscript[14].

Figure 4: Solubilization screen of CF synthesized MPs with increasing nanodisc concentrations. The MPs were synthesized in the presence of supplied nanodiscs within a range of 3.75-60 µM. **(A)** Expression of Tβ_1AR-GFP with nanodiscs (DMPC) in the CF reaction. Total concentration was determined via fluorescence measurement of the GFP fusion. Data taken from previously published manuscript[14]. Affinity-tag purified samples were analyzed by SDS-PAGE and Coomassie-Blue staining shows two prominent bands corresponding to Tβ_1AR-GFP at approximately 50 kDa and MSP1E3D1 above 25 kDa **(B)** Expression of PR with nanodiscs (DOPG) in the CF reaction. Total concentration of PR was determined via absorption measurement at 530 nm. Data taken from previously published manuscript[10, 18]. The photos show the red color of the final reactions due to the presence of folded PR. The pictograms illustrate the modulated PR assembly towards lower oligomeric conformations upon increased nanodisc concentrations, as revealed by subsequent native mass spectrometry[18]. Affinity-tag purified samples were analyzed by SDS-PAGE and Coomassie-Blue staining shows two prominent bands corresponding to PR at approximately 20 kDa and MSP1E3D1 above 25 kDa.

Table 1: Critical screening components of CF reactions.

Table 2: Lipid to MSP1E3D1 ratios for 3 mL *in vitro* assembly setups.

Table 3: Preparation of CF stock solutions.

Table 4: Pipetting scheme for a CECF reaction with 3 mL of RM and 60 mL of FM

Table 5: Pipetting scheme for Mg^{2+} concentration screen with 100 µL of RM and 1.7 mL of FM.

DISCUSSION

The setup and strategies to optimize the CF expression and co-translational insertion of functional MPs into nanodiscs are described. The required equipment comprises a bioreactor, a French press device or similar, an UV/VIS and fluorescence reader, CF reaction vessels suitable for a two-compartment configuration setup, and a temperature-controlled incubator. Further standard equipment are centrifuges for harvesting *E. coli* cells as well as tabletop centrifuges reaching at least 30,000 x *g* for preparation of S30 lysates. If S80-S100 lysates should be prepared, ultracentrifuges are necessary. The listed equipment is regularly available in biochemical labs and no larger initial investments are required for getting started with CF expression. Furthermore, fermentation and handling of *E. coli* cells for CF lysate and T7RNAP preparations are common and robust techniques. The most expensive compounds are CF lysate, T7RNAP, and nanodiscs. They can be prepared by reliable and efficient protocols and aliquots are stable for years at -80 °C. The protocols require three days each for CF lysate and T7RNAP preparation and approximately one week for expression and purification of MSP and preforming of nanodiscs. Target template DNA can be supplied using pET-21, pIVEX, or similar vector systems. For the setup and optimization of CF expression systems, the production of GFP variants such as shifted GFP (GFP+) or

superfolderGFP can be used as monitor[20,32]. For MP production conditions, the expression of PR is a good model system or positive control as it folds under many different conditions and can easily be monitored by absorbance at 530 nm[10,15,18]. To establish an efficient CF expression protocol for a new MP, codon optimization of the target reading frame and using fusions with C-terminally attached GFP is recommended[25,26]. Further required materials include chemicals and enzymes that are all commercially available. These components need to be obtained in high quality and an overall cost calculation for the described CF reaction with 3 mL of RM and 60 mL of FM would amount to approximately 150-200 €. A prime application for CF expression systems is therefore the production of proteins difficult to obtain in classical cell-based expression systems such as many MPs or toxins. CF systems are furthermore core platforms in synthetic biology and continuously growing fields of applications make them increasingly indispensable as a tool in molecular research. Among others, routine applications are the labelling of proteins, high-throughput processes or synthetic cell design.

The demonstrated strategy enables detergent-free production of pure MPs already inserted into desired lipid environments of highly soluble nanodiscs. Once the CF expression protocol for a MP is established and optimized, the production is very fast and pure samples can be obtained in less than 24 h even in preparative scale. The MP/nanodisc complexes are purified directly from the CF reaction via streptavidin II or polyhistidine tags attached to the MP. The process allows parallel functional and structural analysis of identical MP samples by an array of different techniques[33]. Speediness and efficiency of the strategy is therefore competitive to conventional approaches employing cell-based expression systems and detergent extraction of MPs from cell membranes followed by routine in vitro reconstitution[5,34]. The open accessibility of CF reactions further facilitates numerous unique mechanistic studies of MP folding and membrane insertion[15,16,18], MP complex assembly[15,18,24] or functional regulation[23].

An important difference of CF expression over cell-based expression is the absence of quality control systems for the synthesized protein product. Any translated polypeptide independent of its functional folding will end up in the final product sample. Furthermore, the insertion process into nanodisc membranes is artificial and translocon independent and may result either in incomplete integration or may not work at all with a number of MP targets. The finally solubilized product fraction in a CF reaction could thus be quite heterogeneous containing fully inserted but also only partially integrated or membrane associated MPs. Consequently, only a small fraction of a purified sample of MP/nanodisc complexes might be functionally folded. Modifying membrane composition and the careful fine-tuning of MP to nanodisc ratios by modulating the concentrations of nanodiscs and DNA templates are suitable tools for optimization. Nevertheless, downstream quality control approaches such as size exclusion profiling and target specific quantitative functional assays are always necessary for optimization of CF expression protocols. Availability of such assays can therefore limit applications, particularly in projects including MPs that require liposome environments for function such as transporters, channels, or pumps. Furthermore, the size limitations of nanodiscs could restrict the insertion of large MP assemblies.

In the documented examples, the variation in the functionally folded fraction of the GPCR $T\beta_1AR$-GFP is within the range of a few percent, up to approximately 30%. Functional folding requires

careful adjustment of a number of parameters[14] and a similar individual and tedious optimization process might be necessary for other GPCRs as well[22]. However, the finally achieved yield of functionally folded GPCR is competitive with other production systems and allows the setup of > 1,000 radioassays for ligand binding studies from a single 60 µL reaction in an analytical scale Mini-CECF reactor. It is worth mentioning that ligand binding studies of GPCR-GFP fusion constructs do not require any purification steps. If necessary, purification can be achieved by taking advantage of affinity-tags attached to the synthesized MP, such as His-tags or Strep-tag II. The RM is then diluted in the desired buffer and loaded on the corresponding affinity chromatography column that has been pre-equilibrated accordingly. The structural evaluation of PR and other CF synthesized MPs by NMR spectroscopy or crystallization have already helped to establish CF expression systems as core platforms in MP research. The described strategy for production of MP/nanodisc complexes could become particularly interesting for future structural studies by electron microscopy.

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DISCLOSURES

The authors have nothing to disclose.

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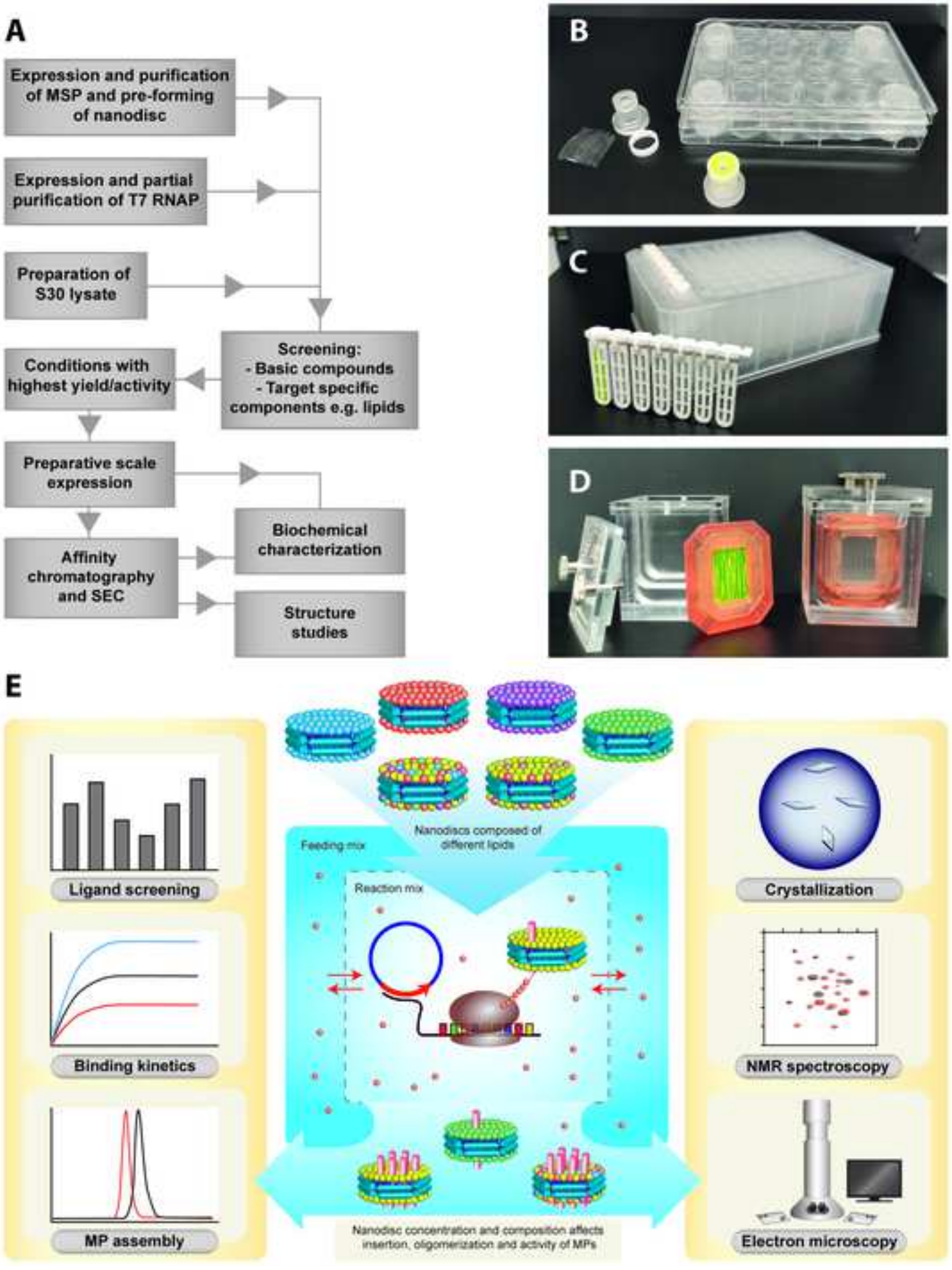
Figure 1



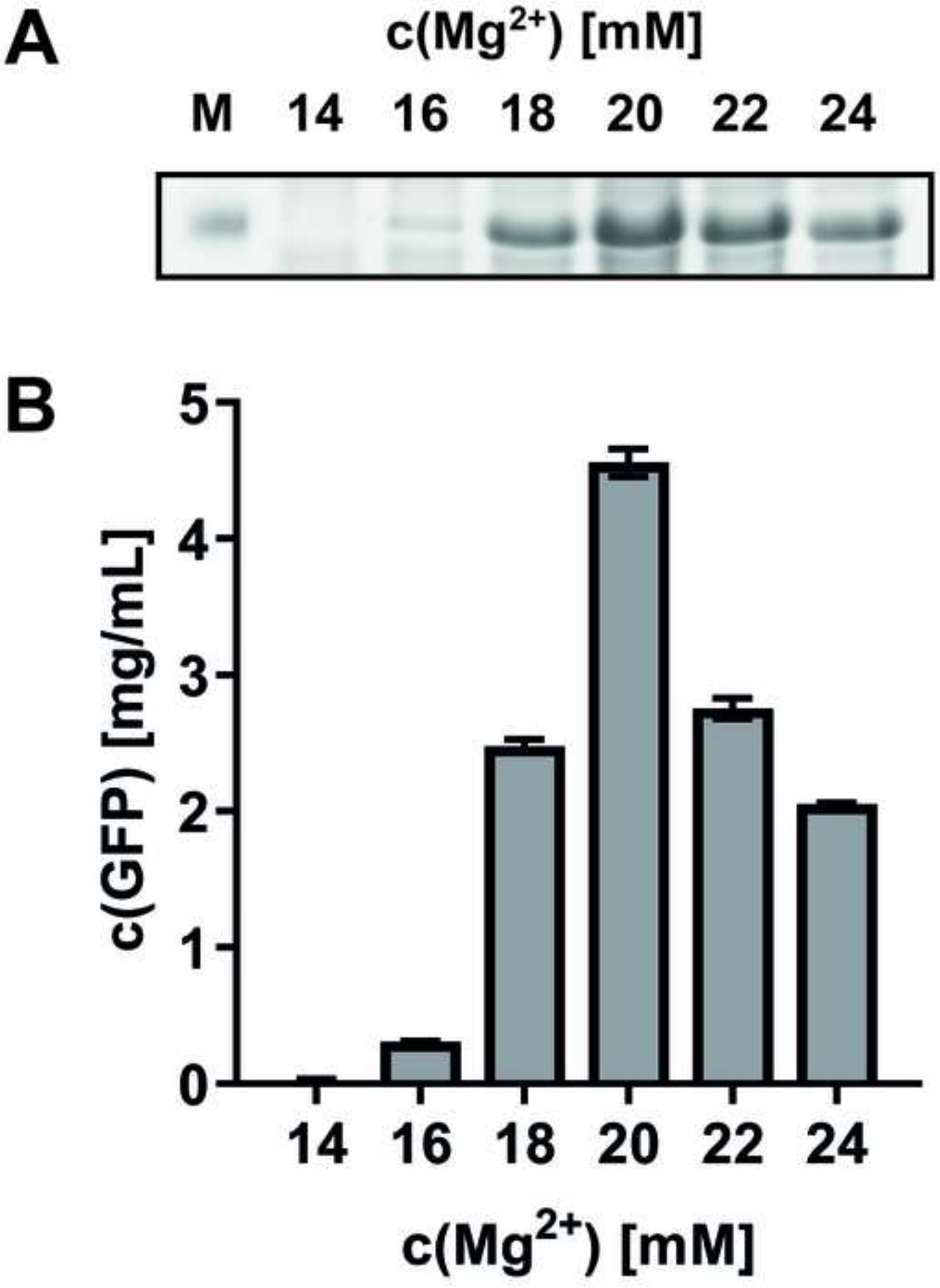


Figure 3

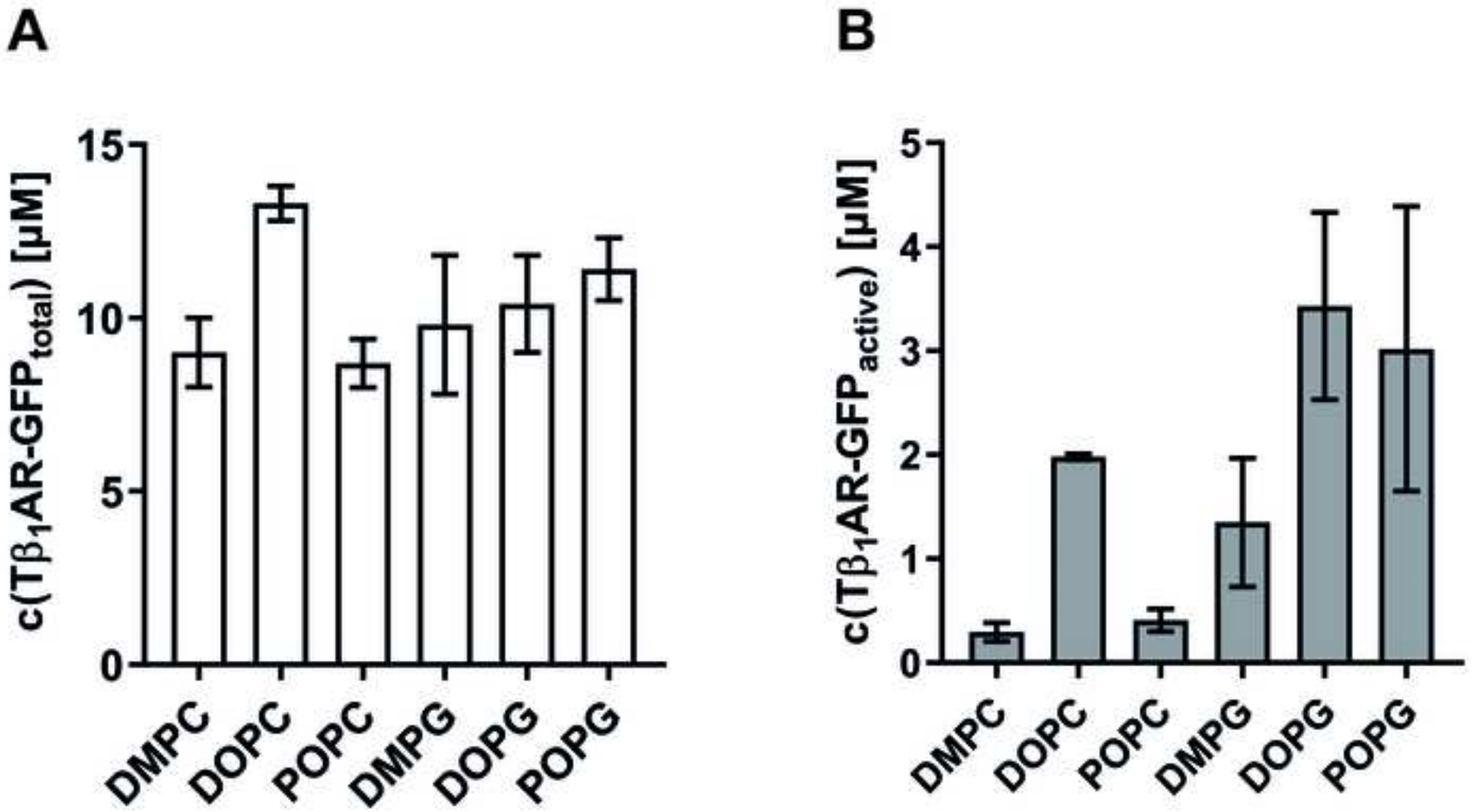


Figure 4

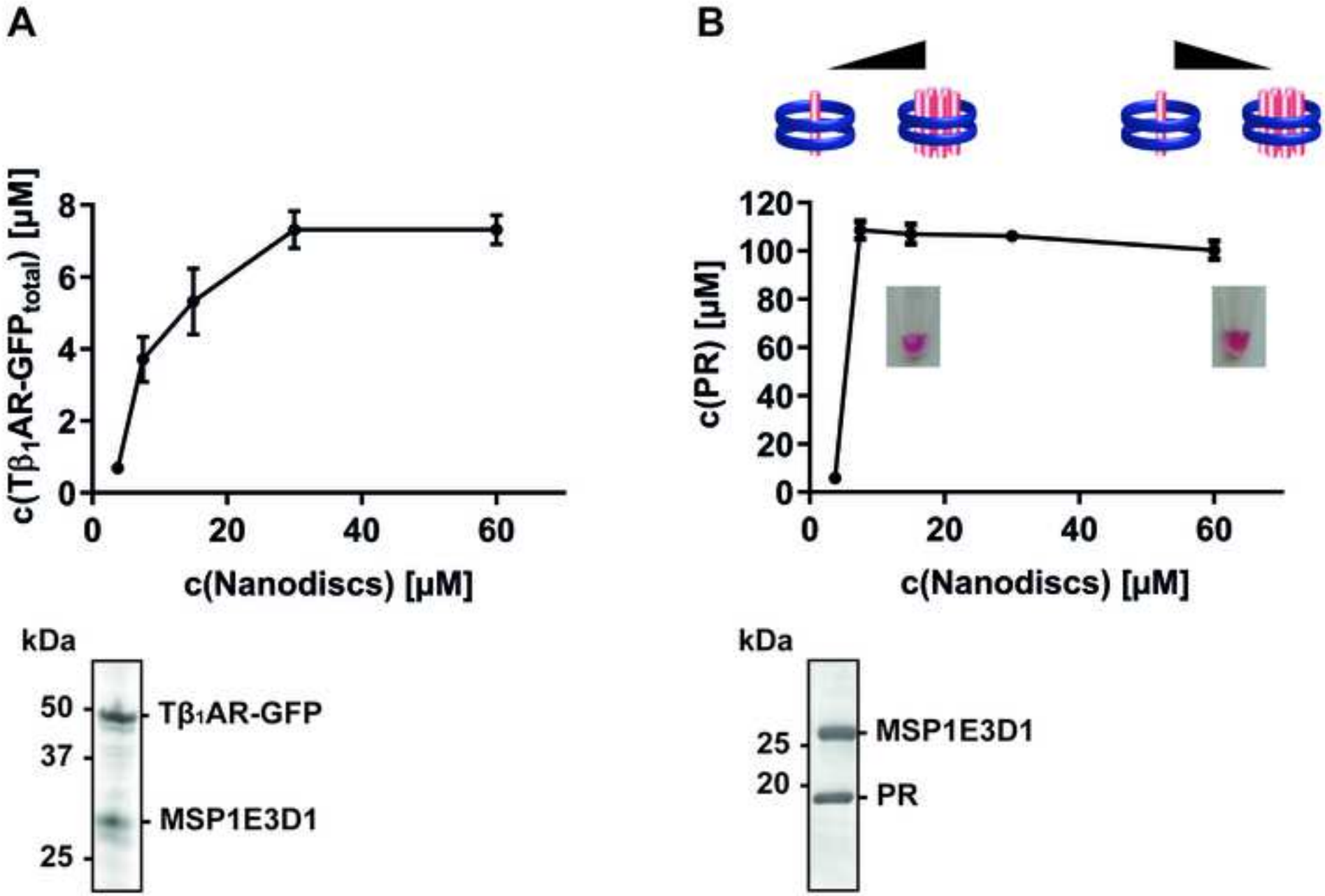


Table 1: Critical screening components of CF reactions.

Compound	Final concentration range	Effect on MP sample
Mg^{2+}	10 - 30 mM	yield
DTT	1 - 20 mM	disulfide bridge formation, folding
20 Amino acid mixture[1]	0.2 – 2 mM	yield,
GSSG : GSH[2]	(1-10 µM) : (1-10 µM)	disulfide bridge formation, folding
Nanodiscs	10 - 100 µM	solubilization, oligomeric assembly, folding
Lipid type	-	solubilization, oligomeric assembly, folding
S30 : HS30 lysate[3]	(50-100 %) : (50-100 %)	folding
S12 - S100	20 – 50 %	yield, MP background in channel assays
DNA template	0.5 – 30 ng/µL RM	yield, oligomeric assembly, folding
DNA template design	-	yield

[1], mixture may be varied according to the individual composition of a MP to e. g. improve yield or optimize NMR labelling schemes.

[2], GSH should always be prepared freshly.

[3], total CF lysate concentration in the RM should be at least 35 % with a S30 content of at least 50 % in order to ensure high expression levels.

Table 2: Lipid to MSP1E3D1 ratios for 3 mL total *in vitro* assembly reactions.

Lipid	Ratio Lipid : MSP	MSP1E3D1 (410 µM stock) V(MSP) [µL]	c(MSP_{final}) [µM]	Lipid (50 000 µM stocks) V(lipid) [µL]	c($lipid_{final}$) [µM]	DPC (10 % (w/v) stock) V(DPC) [µL]	c(DPC_{final}) [%]	DF buffer [µL]
DMPC	115 : 1	1500.0	205.0	1415.0	23 575	30.0	0.1	55.5
DOPC	80 :1	1500.0	205.0	984.0	16 400	30.0	0.1	486.0
POPC	85 : 1	1500.0	205.0	1046.0	17 425	30.0	0.1	425.0
DMPG	110 : 1	1500.0	205.0	1353.0	22 550	30.0	0.1	117.0
DOPG	80 : 1	1500.0	205.0	984.0	16 400	30.0	0.1	486.0
POPG	90 : 1	1500.0	205.0	1107.0	18 450	30.0	0.1	363.0

Table 3: Preparation of CF stock solutions.

Compound	Concentration	Preparation
DNA plasmid template[1]	> 400 µg/mL in 10 mM Tris-HCl pH 8.0	Midi/Maxi kit (e.g. Qiagen) preparation[2]
Mixture of 20 amino acids[3]	25 mM each in H_2O	precipitate remains[4]
	c(CTP) 240 mM, c(ATP) 360 mM, c(UTP) 240 mM, c(GTP) 240 mM	
Mixture of 4 NTPs (75 x)	in H_2O. Adjust pH to 7-8 with KOH	A little precipitate remains[4]
Acetyl phosphate	1 M in H_2O, adjust pH 7-8 with KOH	precipitate remains[4]
Phospho(enol)pyruvic acid (K^+)	1 M in H_2O, adjust pH 7-8 with KOH	
Folinic acid	10 mg/mL in H_2O	precipitate remains[4]
DTT	0.5 M in H_2O	
c0mplete protease inhibitor cocktail	1 tablet per 1 mL in H_2O	
Tris-acetate, pH 8.0	2.4 M in H_2O	
$Mg(OAc)_2$	1 M in H_2O	
KOAc	10 M in H_2O	
Ribolock Rnase-Inhibitor	40 U/mL	Thermo Fisher Scientific
tRNA (*E. coli*)	40 mg/mL in H_2O	Roche (Germany)
T7RNAP	3-7 mg/mL[5]	see protocol section 2
Pyruvate kinase	10 mg/mL	Roche (Germany)
Nanodiscs (DMPG)	0.2-1.0 mM[6] in 10 mM Tris-Cl, pH 8.0, 100 mM NaCl	see protocol sections 3 and 4

[1], PCR template could be used at similar concentrations.

[2], the quality of “Mini”-kit prepared DNA is not satisfactory.

[3], cysteine tends to be unstable and may be added separately.

[4], solution is oversaturated, thorough mixing instantly before removing aliquots is necessary.

[5], for each new T7RNAP batch, an initial screen is recommended to identify the best final concentration.

[6], solubility of nanodiscs can depend on their lipid composition.

Table 4: Pipetting scheme for a CECF reaction with a 3 mL RM and 60 mL FM.

Compound For Mastermix (3.0 x)	**c(final)**	**V [µL]**
Mixture of 20 amino acids	1 mM	2520.0
Mixture of 4 NTPs (75 x)	1x	840.0
Acetyl phosphate	20 mM	1260.0
Phospho(enol)pyruvic acid	20 mM	1260.0
Folinic acid	0.1 mg/mL	630.0
Tris-acetate, pH 8.0	100 mM	2625.0
c0mplete 50 x	1x	1260.0
$Mg(OAc)_2$	19.8 (= 20)[1] mM	1260.0
KOAc	180 (= 270)[2] mM	1140.0
DTT	2 mM	252.0
H_2O	-	7953.0
Total		21 000
For RM	**c(final)**	**V [µL]**
3x Mastermix	1 x	1000.0
RNase inhibitor	0.3 U/µL	22.5
tRNA (*E. coli*)	0.5 mg/mL	37.5
Nanodiscs (DMPG)[3]	10 µM	75.0
DNA template[4]	0.015 ng/µL	112.5
Pyruvate kinase	0.04 mg/mL	12.0
T7RNAP[5]	0.03 mg/mL	15.0
S30 lysate	0.35 %	1050.0
All-trans retinal[6]	0.6 mM	9.0
H_2O	-	666.5
Total		3000.0
For FM	**c(final)**	**V [µL]**
Mastermix (3 x)	1 x	20 000
H_2O	-	40 000
Total		60 000

[1], 0.2 mM Mg^{2+} are contributed from the S30 lysate.

[2], 90 mM K^+ are contributed from acetyl phosphate and phospho(enol) pyruvic acid.

[3], calculated stock solution is 400 µM.

[4], calculated stock solution is 400 µg/mL.

[5], calculated stock solution is 6 mg/mL.

[6], specific cofactor for PR, stock solution is 200 mM in DMSO.

Table 5: Pipetting scheme for Mg^{2+} concentration screen with 100 µL RM and 1.7 mL FM.

Compound For Mastermix (3.0 x)	c(final)	V [µL]
Mixture of 20 amino acids	1 mM	864.0
Mixture of 4 NTPs	1x	288.0
Acetyl phosphate	20 mM	432.0
Phospho(enol)pyruvic acid	20 mM	432.0
Folinic acid	0.1 mg/mL	216.0
Tris-acetate, pH 8.0	100 mM	900.0
c0mplete	1x	432.0
$Mg(OAc)_2$	13,8 (= 14) mM[1]	298.0
KOAc	180 (= 270) mM[2]	389.0
DTT	2 mM	86.4
H_2O	-	2862.6
Total [µL][1]	-	7200.0

		Mg^{2+} concentration					
		14 mM	16 mM	18 mM	20 mM	22 mM	24 mM
For RM	c(final)	V [µL]	V [µL]	V [µL]	V [µL]	V [µL]	V [µL]
3x Mastermix	1 x	67.0	67.0	67.0	67.0	67.0	67.0
0.1 M $Mg(OAc)_2$	14-24 mM	0.0	4.0	8.0	12.0	16.0	20.0
RNase inhibitor	0.3 U/µL	1.5	1.5	1.5	1.5	1.5	1.5
tRNA (*E. coli*)	0.5 mg/mL	2.5	2.5	2.5	2.5	2.5	2.5
DNA template[3]	0.015 ng/µL	7.5	7.5	7.5	7.5	7.5	7.5
Pyruvate kinase	0.04 mg/mL	0.8	0.8	0.8	0.8	0.8	0.8
T7RNAP[4]	0.03 mg/mL	1.0	1.0	1.0	1.0	1.0	1.0
S30 lysate	35 %	70.0	70.0	70.0	70.0	70.0	70.0
H_2O	-	49.7	45.7	41.7	37.7	33.7	29.7
Total [µL][5]	-	200.0	200.0	200.0	200.0	200.0	200.0

		Mg^{2+} concentration					
		14 mM	16 mM	18 mM	20 mM	22 mM	24 mM
For FM	c(final)	V [µL]	V [µL]	V [µL]	V [µL]	V [µL]	V [µL]
3x Mastermix	1 x	1133.0	1133.0	1133.0	1133.0	1133.0	1133.0
0.1 M $Mg(OAc)_2$	14-24 mM	0.0	68.0	136.0	204.0	272.0	340.0
H_2O	-	2267.0	2199.0	2131.0	2064.0	1995.0	1927.0
Total [µL][5]	-	3400.0	3400.0	3400.0	3400.0	3400.0	3400.0

[1], 0.2 mM Mg^{2+} are contributed from the S30 lysate. The mastermix is adjusted to the lowest screening concentration.

[2], 90 mM K^+ are contributed from acetly phosphate and phospho(enol) pyruvic acid.

[3], calculated stock solution is 400 µg/mL.

[4], calculated stock solution is 6 mg/mL.

[5], reactions are performed in duplicates.

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xls

Name of Material/ Equipment	Company	Catalog Number
1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DMPG)	Avanti Polar Lipids (USA)	840445P
1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)	Avanti Polar Lipids (USA)	850345C
1,2-dioleoyl-sn-glycero-3-phosphocholine (sodium salt) (DOPC)	Avanti Polar Lipids (USA)	850375C
1,2 dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (sodium salt) (DOPG)	Avanti Polar Lipids (USA)	840475C
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)	Avanti Polar Lipids (USA)	850457C
1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG)	Avanti Polar Lipids (USA)	840034C
2-Amino-2-(hydroxymethyl)-propan-1,3-diol (Tris)	Carl Roth (Germany)	4855
2-Mercaptoethanol	Carl Roth (Germany)	4227
2-Propanol	Carl Roth (Germany)	9781
[^{3}H]-dihydroalprenolol Hydrochloride	American Radiolabeled Chemicals (USA)	ART0134
Acetyl phosphate lithium potassium salt (ACP)	Merck (Germany)	1409
Adenosine 5'-triphosphate (ATP)	Sigma Aldrich (Germany)	A9251
Alprenolol hydrochloride	Merck (Germany)	A0360000
Anion exchange chromatography column material: Q-sepharose®	Sigma-Aldrich (Germany)	Q1126
Autoclave Type GE 446EC-1	Gettinge (Germany)	
Bioreactor Type 884 124/1	B.Braun (Germany)	
Centrifuge	Sorvall RC12BP+; Thermo Scientific (Germany); Sorvall RC-5C; Thermo Scientific (Germany); Mikro 22 R; Hettich (Germany)	
Cholic acid	Carl Roth (Germany)	8137
Coomassie Brilliant Blue R250	Carl Roth (Germany)	3862
Culture flasks 500 ml baffled flasks, 2 l baffled flasks	Schott Duran (Germany)	
Cytidine 5'-triphosphate disodium salt	Sigma-Aldrich (Germany)	C1506
D-glucose monohydrate	Carl Roth (Germany)	6780
Di-potassiumhydrogen phosphate trihydrate	Carl Roth (Germany)	6878
Dialysis tubing Spectrum™ Labs Spectra/Por™ 12-14 kD MWCO Standard RC tubing	Fisher Scientific (Germany)	8700152
Dithiothreit	Carl Roth (Germany)	6908
Ethanol	Carl Roth (Germany)	K928
Folinic acid calcium salt hydrate	Sigma-Aldrich (Germany)	47612
French pressure cell disruptor	SLM; Amico Instruments (USA)	
Glycerol	Carl Roth (Germany)	3783
Guanosine 5'-triphosphate di-sodium salt (GTP)	Sigma-Aldrich (Germany)	G8877
Hydrochloric Acid	Carl Roth (Germany)	K025
IMAC column: HiTrap IMAC HP 5 mL	GE Life Sciences (Germany)	GE17-5248
Imidazole	Carl Roth (Germany)	3899
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Carl Roth (Germany)	2316
Kanamycin	Carl Roth (Germany)	T832
L-Alanine	Carl Roth (Germany)	3076.1
L-Arginine	Carl Roth (Germany)	6908
L-Asparagine	Carl Roth (Germany)	HN23
L-Aspartic Acid	Carl Roth (Germany)	T202
L-Cysteine	Carl Roth (Germany)	T203
L-Glutamic Acid	Carl Roth (Germany)	3774
L-Glutamine	Carl Roth (Germany)	3772
L-Glycine	Carl Roth (Germany)	3187
L-Histidine	Carl Roth (Germany)	3852
L-Isoleucine	Carl Roth (Germany)	3922
L-Leucine	Carl Roth (Germany)	1699
L-Lysine	Carl Roth (Germany)	4207
L-Methionine	Carl Roth (Germany)	9359
L-Proline	Carl Roth (Germany)	1713
L-Phenylalanine	Carl Roth (Germany)	1709
L-Serine	Carl Roth (Germany)	4682
L-Threonine	Carl Roth (Germany)	1738
L-Tryptophane	Carl Roth (Germany)	7700
L-Tyrosine	Carl Roth (Germany)	T207
MD100 dialysis units	Scienova (Germany)	40077
N-2-Hydroxyethylpiperazine-*N*'-2-ethansulfonic acid (HEPES)	Carl Roth (Germany)	6763
n-dodecylphosphocholine	Antrace (USA)	F308S
PAGE chamber: Mini-Protean Tetra Cell	Biorad (Germany)	
PAGE gel casting system: Mini Protean Handcast systems	Biorad (Germany)	
PAGE gel power supply: Power Pac 3000	Biorad (Germany)	
Tryptone/peptone from caseine	Carl Roth (Germany)	6681
Peristaltic pump: ip-12	Ismatec (Germany)	
Phosphoenol pyruvate monopotassium salt	Sigma Aldrich (Germany)	860077
Potassium dihydrogen phosphate	Carl Roth (Germany)	P018
Potassium acetate	Carl Roth (Germany)	4986
Potassium chloride	Carl Roth (Germany)	6781
Pyruvate Kinase	Roche (Germany)	10109045001
Scintillation counter: Hidex 300 SL	Hidex (Finland)	
SDS pellets	Carl Roth (Germany)	8029
Sodium azide	Sigma-Aldrich (Germany)	K305

Sodium chloride	Carl Roth (Germany)	P029
Spectrophotometer Nanodrop	Peqlab (Germany)	
Spectrophotometer/fluorescence reader Spark®	Tecan (Switzerland)	
tRNA (E. coli)	Roche (Germany)	10109550001
Ultra sonificator	Labsonic U, B. Braun (Germany)	
Uridine 5’-triphosphate tri-sodium salt (UTP)	Sigma-Aldrich (Germany)	U6625
Y-30 antifoam	Sigma-Aldrich (Germany)	A6457
Yeast extract	Carl Roth (Germany)	2904

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the title for conciseness. Is the subtitle necessary?
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. For example: (Merck, Germany), PipetBoy, Falcon, Akta, etc.
4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Representative Results or the Discussion.
5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors comment: *Suggestions have been considered and changes were made.*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is very interesting work. The manuscript itself explains all process and tips necessary for the cell-free expression of membrane protein, which may not be very new. Nevertheless, the author put more emphasis on the preparation process of the samples than the results. This fits very well to aim of JoVE. Sample preparation protocol of the cell free expression is important issue that many researcher are interested in. However, these parts are often poorly regarded, and the results parts are more emphasized in conventional publication. It is often difficult to image the actual procedure by just reading written protocol, if readers are not familiar with that particular field. Therefore, this is great idea to combine this manuscript with a video that demonstrating actual procedure. I believe this will help many researchers to visualize many steps that they had to seek by try and errors former time. I strongly recommend for the publication of this work, but only with the demonstrating video.

Major Concerns:

I think, quality of this work will highly depends on the quality of the video. It can be imagine that the video may become too lengthy if they have to show all procedure. On the other hand, reducing the protocol step may also be a problem.

Authors comment: *We thank the reviewer for his time and thorough reading of the manuscript. We will consider the mentioned concerns.*

Reviewer #2:

Manuscript Summary:

The authors describe the usability of cell-free system coupling with nanodisc for the production and analysis of membrane proteins. This reconstructed in vitro system has highly superiority on controllability of the system that makes possible to tailor design the reaction condition for each membrane protein of interest. This is a protocol type paper and describing clearly at each step that makes be easy to understand and follow the experiments. Although use of liposome membrane for in vitro production and analysis of membrane protein is major so far, this nanodisc technology could be an alternative or complemental technology, that may

be accepted as a general tool in near future.
The reviewer would like to suggest some points as follows for the revision of the paper.

Major Concerns:
Page 2, line 70-72; Authors should describe that the insertion efficiency is also depending on the type of membrane protein, not only the lipid composition.
Authors comment: *We thank the reviewer for his time and thorough reading of the manuscript. The sentence in the introduction has been rephrased accordingly and this issue is further addressed in the results part in fig. 4 and in the discussion.*

Page 2, line 73-78; This part is describing about the oligomeric formation of the cell-free synthesized membrane proteins on the nanodisc membrane. However, membrane insertion of the proteins is not controlled onto the nanodisc. Theoretically, membrane protein can approach to the nanodisc form the both sides of the membrane, which possibly results in a nanodisc with two membrane proteins inserted in opposite orientations. The given examples, KcsA or EmrE, show successful oligomeric formation on the nanodisc membrane. Is these are examples in which the function was observed only for the membrane proteins that are stochastically correctly oriented? Or, is there any mechanism which biases to uniformed orientation? Authors should explain about this point in this part.
Authors comment: *This is an important fundamental question. To our knowledge, the orientation of MPs in nanodiscs containing multiple insertions has not been analyzed yet. It might also be a technical challenge and depending on the MP, the orientation might be not static due to a potential "flip-flop,,at the rim of the membrane disc. We observed with LILBID-MS for KcsA, forming highly stable tetramers, that at certain KcsA:nanodisc stoichiometry of 2.5:1, only tetramers are detectable (Peetz 2017, Anal.Chem). With pure stochastic insertion, also lower oligomers or monomers should have been observed. We assume that certain MPs forming oligomers such as KcsA insert in a cooperative manner, so all protomers of a complex may insert in same orientation. Furthermore, an orientation switch of inserted MPs might be possible at the flexible rim of the nanodiscs. We highlighted this still unsolved question now in the introduction and included the two mentioned possibilities that might bias MP orientation in nanodiscs.*

Page 2-3, Introduction; Authors should describe explain about the examples that do not suit for this nanodisc methods. For example, this method is not available for the cases of i) some transporter or pomp type membrane protein that need a liposome membrane to transport ion or molecules inside, ii) some protein that does not integrate into nanodisc membrane spontaneously, and iii) some membrane protein complex of which consists of hetero-proteins that stoichiometry is properly defined (eg. FoF1-ATPase). This information would be a help for the reader who are considering to try this method.
Authors comment: *We included corresponding considerations into the discussion part where we compiled pros and cons of the technique.*

Minor Concerns:
Page 4, line 140 and 141: "step 5" > "step 1.5"
Page 4, line 150-153: How many time pass the French-press?
Page 5, line 177: "pAR1219", give a reference.
Page 5, line 189-190: "slowly and under …", why this step is needed?

Page 6, line 219: "pET-28(a)-MSP1E3D1", give a reference.
Page 6, line 248: What CV?
Page 6, line 255: what is the composition of MSP-H?
Table of Materials: This table should be classified as chemicals or instruments.
***Author comments**: All concerns have been addressed accordingly.*

Page 6, line 245-249: Way this "step-wise" washing is needed?
***Authors comment:** There is a step where cholic acid is applied. According to the standard protocol by the Sligar group this requires a pH shift to 8.9, as cholic acid has a higher solubility at this pH value. During this step, lipids are stripped off the MSP as indicated in the note. Afterwards MSP (bound to resin) is again equilibrated with the "standard buffer" but at elevated pH. This is necessary to remove residual cholic acid, before decreasing the pH value to 8. We now explained the reason for this step-wise washing in the section.*

Page 8, line 307-308: It is better to add a picture figure for this part to understand visually.
Page 8, line 343-346: It is better to add a picture figure for this part to understand visually.
***Authors comment:** A new figure showing the described reaction containers has been added in figure 1.*

Page 9, line 379-386: Codon optimization of DNA sequence of the interest membrane protein is also effective for protein yield. This should be described in this section.
***Authors comment:** We included a corresponding sentence in paragraph 5 in the CECF reaction setup and also in the discussion.*

Page 10, line 425 and 427: Fig. 4 is missing.
***Authors comment:** That was a mistake, the figure has now been included.*

Table 3: RNase is missing.
***Authors comment:** Ribolock Rnase Inhibitor is included, RNase will not be added.*

Lastly, reviewer thinks that to add a schematic figure explaining flow chart of the experiments would be a help for the readers. For example, Nanodisc Preparation, MSP purification, T7RNAP purification, S30 lysate, Cell-Free Preparation, and so on.
***Authors comment:** A corresponding flow-chart has been added into figure 1.*

Reviewer #3:
Manuscript Summary:
The manuscript by Levin et al. showcases protocols for the generation of an E. coli derived cell-free (CF) expression system supplemented with lipid nanodiscs made using purified MSP to synthesize functional membrane proteins for downstream biochemical and structural studies. Two example proteins are synthesized to demonstrate the efficacy of the reconstitution approach. Protocols for optimizing reaction conditions to obtain high yields of functional membrane proteins in supplemented nanodiscs are discussed with a detailed description of the assembly of the continuous exchange cell-free (CECF) apparatus used. The overall method presented is aimed at reconstituting complex membrane proteins using CF

systems. While there is a lot of redundant information in the current manuscript, the field of membrane protein solubilization using lipid nanodiscs is rapidly growing and the use of CF systems towards this goal is quite promising. Therefore, the ideas presented in this manuscript for optimizing nanodisc to protein ratios and screening for appropriate lipid variants to increase yields are beneficial to the community.

Authors comment: *We thank the reviewer for his time and thorough reading of the manuscript. The manuscript has been revised to reduce redundant information.*

However, the following issues must be resolved before the manuscript can be accepted for publication. My major concerns are listed below:

Major Concerns:
The methods for generating working cell-free expression systems using different bacterial strains have been previously published in both result-based journals and as JoVE articles. The same is true for at least one paper where T7 RNAP purification protocol was detailed in exactly the same way as mentioned in this manuscript. The authors can reference those articles while indicating key variations such as point 1.4 where stress-induced S30 lysates can be made for studying role of chaperones in mediating post translational protein folding in CF systems.
Authors comment: *Most of the protocols has been published before, but the scope of this article is to present a comprehensive guideline of our routine techniques along with the video to instruct the readers. The protocol should serve as kind of a script for the video performance. Other reviewers pointed out that the protocols are very helpful and suggested to present them in even more detail. Furthermore, rather routine and established techniques should be presented and previous publication is therefore expected. We would find it therefore more confusing if essential steps or protocols are omitted and only referred to "as described elsewhere".*

A brief description of the filter binding assay conditions and the method of analysis to generate the plots shown in Figure 3 should be included in the manuscript. For instance, was the GPCR construct tagged with GFP used for ligand binding experiment or was it replaced with a non-tagged native version?
Authors comment: *GFP fusion constructs were used for analysis and this is indicated in figures 3 and 4. We added a brief description of the filter binding assay in the representative results section.*

It will be more informative to include a schematic of the CECF reaction chamber built using plexiglass and adjusted in the 24 well cell culture plates.
Authors comment: *New figures of the containers and the reaction set ups are now included in figure 1. Descriptions containing the exact dimensions of the mentioned homemade plexiglass containers have been referenced.*

Figure 4 is not included in this version of the submitted manuscript.
Authors comment: *We apologize for this mistake, the figure is now included.*

Minor Concerns:
Additionally, I have some comments regarding missing information, typos and a few grammatical corrections:
1. In line 29, "difficult proteins" doesn't make much sense. Consider replacing with "complex".

2. Correction in line 49, "..liposomes, MP reconstitution…", in line 50, "..in the form…" and "..particles have become…"
3. First use of abbreviation "L-CF" in line 56. Please expand.
4. Not sure what is meant by "preparative scale amounts.." in line 79.
5. Line 86, "..samples of G-protein.."
6. Please change sentence structure in line 91 "The FM volume several fold exceeds…"
7. Sentence structure in line 106 should be revised, "Much more difficult can be the optimization…".
8. In line 230, point 3.4, is the IPTG induction carried out at OD600 of 6.5-7.5 or is it a typo? Also, it is informative to mention the temperature of incubation after IPTG induction "…continue incubation for 1h at XXC."
10. In line 248, the washing volume for MSP-F buffer is not indicated.
11. In line 255, MSP-H terminology is used without prior definition.
12. Temperature at which MSP centrifugation and concentration are carried out should be mentioned in point 3.10.
13. Is there a citation for the "optimal conditions" in line 312?
15. In Table 4, the label of the right column should be V [ul] but not c(final) for both RM and FM.
16. The plasmid vectors used for the expression of GFP and the two membrane proteins should be mentioned in the discussion or CECF assembly sections.
Authors comment: *All comments have been corrected accordingly. In bioreactor cultivations higher cell densities can be reached. Thus, induction at higher ODs is appropriate..*

9. Cholic acid should be mildly soluble (max around 1 mM) in dilute HCl-based buffer or aqueous solutions. Can the authors comment on the solubility of cholic acid in the MSP-C buffer given that it is 50 mM?
Authors comment: *Cholic acid has a significant higher solubility at the elevated pH value of 8.9 used in buffer MSP-C. The reasons for the described step-wise elution has now been explained in the protocol.*

14. Figure 1 can be better labelled to inform the reader about the process of membrane protein reconstitution in nanodiscs and their subsequent analysis.
Authors comment: *Fig. 1 has been revised and a flow-chart has been added.*

Apart from the above suggested changes, the writing in general can be improved in the discussion and representative results sections.
Authors comment: *The writing has been checked and revised throughout the manuscript..*

Reviewer #4:
Manuscript Summary:
The authors have done an excellent job describing their protocol, which I think will be of interest to a wide community.

I have no major comments, other than a request for the authors to provide clearer guidance on control measurements/experiments/diagnostics to help a reader trying to apply this protocol to a new protein of interest. For example, it would be really handy to give the sequence, or reference (from Addgene or similar) of a suitable GFP plasmid for testing out the cell-free reaction components (i.e. everything except membrane nanodiscs) as well as plasmids of

GFP-tagged proteins that do readily insert and fold (e.g. PR or KcsA or EmrE) since insertion and folding could be an issue when applying the method to a novel protein, and so it would help to have positive controls for this too. In the minor comments section I've requested any little diagnostic measurements a reader could make as they follow the protocol to check everything is going as expected.
Authors comment: *We thank the reviewer for his time and thorough reading of the manuscript. We now highlighted in the discussion to use GFP variants and PR as easy to measure monitors to establish CF expression systems and use them as positive controls.*

Minor Comments :
Preparation of S30 Lysate

Timing : It would help to indicate a reasonable timing for the protocol and also potential pause points.
e.g. Day 1 - Step 1.1 Day 2 - Step 1.2 to Step 1.10? Day 3 - Step 1.11
Potential pause points
- Can the pellets be left on ice overnight after Step 1.5, or is it better to wash and pause after Step 1.6?
Authors comment: *We included a day timing in the protocol. The protocol can be interrupted after harvesting the cells and we included a corresponding note.*

Step 1.7 : It would help to give a typical pellet weight range (e.g. "The wet cell pellet weight is typically between x grams and y grams).
Step 1.7 : How many passages do you typically do? Do you check solution is no longer gooey (i.e. DNA sufficiently sheared)? Is there any chance you mean 10000psi, rather than 1000psi for the French Press?
Step 1.8 : For a novice could it be helpful/useful to measure A280 of supernatant now, and then again after Step 1.9 to get a very rough estimate of protein concentration before and after detrimental lysate removal? What would be the expected values?
Authors comment: *Corresponding information has been added..*

Step 1.11 : For a first time user, could one estimate protein concentration by A280 at this step, or would you recommend other ways to check the final S30 lysate quality?
Authors comment: *We give the average range of protein concentration in the final lysate as an first estimate of lysate quality. However, we think the best way to test extract performance is directly express GFP in a Mg2+ screen. Even at good protein concentrations of an extract, performance might be poor.*

T7 RNA Polymerase

Timing : Again would help to outline plausible timing and pause points
e.g. Day 1 - Step 2.1 Day 2 - 2.2 to 2.4? Day 3 - 2.4 to 2.9? Day 4 - 2.10
Pause Points: - Would you recommend leaving spun down cell pellets (Step 2.4) on ice overnight, or are there better places to pause if needed?
Authors comment: *Timeline and comments have been added.*

Step 2.8 - What is the expected T7RNAP amount at this step? Is it possible to estimate T7RNAP concentration in fractions via A280 or another simple measurement?
Authors comment: *A280 is not accurate enough as there are still too many impurities in the fractions. We collect T7RNAP fractions and load them on an SDS gel. There are several quick*

staining protocols and T7RNAP is visible as a prominent band at ca. 90 kDa and can then be estimated according to band intensity.

Step 2.10 - What is the typical yield of T7RNAP?
Authors note: *We added average yields obtained out of 1 L cultures and also stated an estimate of the corresponding T7RNAP units.*

Expression and Purification of MSP1E3D1
Proposed Timing? Day 1 - 3.1 Day 2 - 3.2 to 3.4 Day 3 - 3.5 to 3.9 Day 4 - 3.10 to 3.11
Step 3.4 - What would be a typical pellet volume?
Step 3.8 - What is typical UV absorption (A280) of fractions (e.g. what is expected yield)?
Authors note: *Timeline and comments have been added.*

Assembly of MSP1E3D1 nanodiscs
Step 4.1 -> Should stock solutions of lipids in sodium cholate be clear?
What method would the authors recommend for assessing success/quality of nanodiscs (e.g. DLS, SEC) for a novice?
Step 4.5/4.6 - What is the expected yield (i.e. what volume of nanodiscs at 300uM is typical?
Authors comment: *We addressed that question in a note in 4.5 referring to the setup volume and MSP concentration described in table 1.*

Line 58 - "Nanodiscs consisting out of a" -> Nanodiscs consisting of a
Line 99 -"High yield and quality of a MP frequently deserves intense optimization of reaction" -> frequently requires intense
Line 178 - What volume flask (4L?) is recommended for 1L growth?
Line 438 - "The scheme indicates the variability of the membrane lipid composition." As written this suggests a lack of control of membrane lipid composition, whereas I think the authors might mean ... "The scheme highlights the ability to control membrane lipid composition".
Table 4 : for RM -> label of third column -> c(final) seems like it should be V_final
Authors comment: *Comments have been addressed.*

Figure 1 : The schematic lacks labels and I'm not sure it illustrates the authors process so well. Since the authors are describing a pipeline, instead of showing a variety of membrane nanodiscs it could help to show ...

Plasmid + Cell-free Expression Mixture + Preformed Nanodisks

all being introduced to the Reaction compartment along with lower molecular weight feeder molecules to the feeder compartment. If this were placed on the left it would neatly illustrate the inputs to the pipeline. The current depiction of protein synthesis and insertion is excellent, but it's less clear what the authors' intend by the 2 membrane nanodiscs containing a single, and multiple proteins below the compartment. One possibility would be to have an arrow from the reaction compartment showing the "output" of the pipeline - a protein-containing nanodisc. If the depiction within the reaction compartment is of a single or two TMs inserted into a nanodisc, and the depiction of the protein-containing nanodisc outside were with multiple TMs, it would then be clear that the final output are proteins that have successfully inserted and folded into the nanodisks. IF the schematic ran left-to-right (e.g. inputs, reaction compartment, output, applications), then the reader might naturally see the pipeline. In the caption it would help to describe the inputs, process, and outputs e.g. "The Reaction Mixture contains the plasmid, cell-free expression mixture and preformed nanodiscs so that proteins

can spontaneously insert and fold into the nanodiscs as they are being synthesized. The resulting MP/nanodiscs can then be purified for further study by a variety of structural and functional approaches."
Authors comment: *The figure has been updated and a flow chart of the process was added.*

Reviewer #5:
Levin and coworkers describe protocols for cell-free expression and lipid nanodisc reconstitution of membrane proteins. Membrane proteins are notoriously difficult to express and characterize and the authors point out that cell-free expression in presence of nanodiscs represents a powerful alternative to conventional expression and detergent based purification due to the stabilizing effect of the nanodiscs. According to the authors, a major advantage of their system is that it allows production, purification and lipid bilayer reconstitution of even difficult to express membrane proteins in a single step, without the need of detergents. The authors acknowledge that the concept of cell free expression in presence of nanodiscs has been described before, but they also say that universally applicable protocols are still lacking. Here, they present detailed procedures for obtaining E. coli cell-free extracts as well as the other required components, including T7 polymerase, membrane scaffold protein, and preformed lipid nanodiscs. They describe how to optimize the overall process in a semi high-throughput manner, and which factors they found are important such as Mg2+ concentration and nature of the lipids used for nanodisc preparation. Overall, the work is of high quality and the protocols are well laid out and sufficiently detailed, so that the procedures can be adopted by laboratories equipped for standard biochemical research. A few suggestions how to improve the article are listed in the following.

(1) While it is acknowledged that this manuscript is focused on the cell-free expression and nanodisc reconstitution part of the "production pipeline", it appears that the actual isolation and purification of the expressed and reconstituted membrane proteins, an integral part of any production pipeline, receives somewhat short shrift. Towards the end of the Discussion, in what appears to be an afterthought, the authors mention that the expressed membrane proteins can be isolated and purified via affinity tags. A few examples of such purifications would give the reader confidence that the method actually delivers what the authors promise, namely sufficient amounts of purified and nanodisc reconstituted membrane proteins for functional studies and structure determination by crystallography, NMR or cryoEM. The Abstract states that the process "is demonstrated with the synthesis of proteorhodopsin" - however, no proteorhodopsin data are shown.
Authors comment: *We thank the reviewer for his time and thorough reading of the manuscript. Due to an erroneous upload, figure 4, that shows proteorhodopsin expression titers was missing and the figure has now been added. The figure also shows an example of the purified PR and ß1AR in nanodiscs. Purification can easily be achieved by affinity chromatography and we added a corresponding comment in the discussion.*

(2) It is recommended that the manuscript be proofread by a native English speaker - there are numerous instances of an unusual syntax and/or choice of words.
- lines 49-51: "also" may not be necessary; has become instead of "became"?
- line 57: "already" may not be necessary
- line 58: "consisting of"
- line 59: "appear to be particularly suitable"
- line 64: "might" necessary?
- line 68: "empty" necessary?
- line 69: "artificial" => non-physiological?

- line 99: "deserves" => requires?
- line 101: "to" may not be necessary
- line 119: "Differences are some variations" - needs to be clarified
- line 158: "detrimental" => contaminating (or contaminations without "lysate components")
- line 192: "Filtrate" => Filter
- line 215: "with less efficacy" => less efficiently (efficacy is commonly used in context with "efficacy of drugs or therapies")
- line 241" filter
- line 369: "stinging" is reserved for bees and wasps - maybe change to "by piercing the dialysis membrane with the pipet tip" or similar?
- line 372: now be
- line 374: it appears that the expression "nanoparticles" is used for the first time. It would be helpful to introduce "nanoparticle" in context of lipid nanodiscs earlier.
- line 489: "must be done" => are required?
- line 491: "compounds CF lysate" => compounds, including CF lysate, …
- line 492: are stable at -80 (or -20 ºC) …
- line 500: skip "more and more" - or use increasingly?
- line 502: high or low "throughput processes"?
- lines 507-509: clarify - e.g. what is "structural analysis of aliquots"?
- lines 527/8: clarify

Authors comment: *The manuscript has been proofread again and the comments have been addressed.*

- line 153: "1,000 psi" => 10,000 psi? (1,000 psi seems low)

Authors comment: *The unit was wrong and was changed to psig.*