Large-scale production of functional mammalian membrane proteins using *E. coli*-based cell-free system

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Version 1.0

Summary:

The following is the current protocol for large-scale production of functional mammalian membrane proteins using our cell-free (CF) System. The protocol was adapted from the Invitrogen's *E. coli* CF system. Major improvements include: 1) the design of modular cell-free expression plasmids with various affinity tags at either N- or C- terminus; 2) the use of large scale plasmid preparation (milligrams of DNA), and lysate preparation, to accommodate production of up to 50mg of purified protein per cell-free reaction; 3) the addition of brji-35 detergent in the reaction to prevent precipitation of the synthesized membrane proteins, and 4) the use of various detergents to exchange with brij-35 while maintaining solubility and proper folding of the membrane proteins for functional analysis, x-ray crystallography, or NMR.

All components of the cell-free reaction (plasmid, lysate, and reactions reagents) are made in large batches and tested for efficient protein production. The reaction can be carried out overnight (or in a couple of hours) and the protein extracted/purified the next day. This method allows consistent and quick turn-around production of large quantities of the proteins of interest.

Plasmids and DNA preparation

We have constructed several cell-free expression plasmids (pTAN series), which were based on pIVEX-2.3d vector (Invitrogen, Carlsbad, CA). The new plasmids have various affinity tags that can be placed at the N- or C-terminus. Some plasmids have protease cleavage site for optional tag removal. Mammalian membrane protein genes are obtained from the Mammalian Gene Collection (OpenBioSystems, Huntsville, AL). Although linear DNA fragments could be used in the cell-free reactions, we prefer these plasmids because they can readily be propagated and maintained.

To support large-scale cell free reactions, milligram quantities of DNA were isolated from *E. coli* grown in 100L fermentor. We use either a GenElute HP Plasmid Mega-prep Kit (Sigma) or the QIAfilter Plasmid Giga Kit (Qiagen) using the instructions from the manufacturers.

Production of cell free lysate

- Transform BL21 cells with pAR1219 plasmid (pBR322-based vector for T7 RNA polymerase protein expression, IPTG inducible) and grown in 100L fermentor with appropriate antibiotic.

- Induce with 1mM IPTG at OD 0.7-1.0, and harvesting at OD 3.5-4.0. For a good lysate, it is important that the cells maintain a fast doubling time about 30 mins.

- Harvest the cells via centrifugation (8000xg) and re-suspended in 5L of cold S30 media. DTT was added to 1mM final concentration. The concentrated cells were then re-centrifuged (8000xg) and the pellet kept at -80^{0}C.

To make cell free extract:

- Add 200mL of S30 buffer to 200g of frozen cell and stirred at 4^0C until the cells were completely resuspended.

- Add DTT to 1mM final concentration.

- Disrupt the suspended cells by passing though an M-110L microfluidizer processor (Microfluidics, MA).

- Centrifuged broken cells at 30,000xg for 40 minutes.

- Collect the supernatant (cell-free lysate).

- Snap free with liquid nitrogen and keep at -80^0C.

Each batch of lysate was tested for efficiency by expressing GFP and other known integral membrane proteins, like the membrane protein *Emr*E, in control reactions.

Cell free protein synthesis

Membrane proteins were expressed using a lysate based originally on the *E. coli* Expressway™ Milligram Cell-Free Expression system (Invitrogen, Carlsbad, CA). Modifications include: i) the addition of 1-2% Brij-35 (Brij-58 and Brij-75 can also be used) to solubilize the membrane proteins during the cell free reaction, (ii) the addition of any amino acids for labeling, like seleno-methionine at 2 mM, (iii) in some cases the direct incorporation of lipid or bicelles.

The cell free reaction is composed of four major components: DNA, lysate, reaction buffer and feed buffer. DNA and lysate preparation is described above. The final concentration of circular or linear DNA template in the cell free reaction was 5-10 μg/ml. The lysate, which includes the T7 polymerase, comprises 40% of the reaction volume. The reaction buffer consists of 230 mM Potassium Glutamate, 58 mM Hepes-KOH, pH 7.5, 2% PEG 8000, 80 mM Ammonium Acetate, 13.6 mM Magnesium Acetate, 1.2 mM ATP, 0.88 mM UTP, 0.88 mM CTP, 0.88 mM GTP, 0.65 mM cAMP, 1.7 mM DTT, 0.034 mg/mL Folinic Acid 30 mM 3-phosphoglycerate, 2 mM of all the amino acids except for cysteine and methionine, which have a concentration of 1.5 mM each, 0.17 mg/mL tRNA, 2 mM Sodium Oxalate, and 3.33 units of RNAse Inhibitor. Feed buffer consists of 58 mM Hepes-KOH, pH 8.0, 2 mM Calcium Chloride, 14 mM Magnesium Acetate, 230 mM Potassium Glutamate, 0.35 mM cAMP, 0.3 mM NAD, 1.7 mM DTT, 0.034 mg/mL Folinic Acid, 2 mM sodium oxalate, 30 mM Glucose-6-phosphate, and the same concentration of amino acids as in the reaction buffer.

Large-scale reaction set up:

- 36mL reaction buffer
- 3.6mL amino acid mix
- 1.8mL Cysteine
- 1.8mL Methionine
- 1.8mL tRNA
- 60uL RNA inhibitor (Invitrogen Cat # 10777-019)
- 6-9mL DNA (about 200-500ng/uL)

- 36mL lysate

Incubate 30^{0}C, lightly shaken at 80 rpm for 1.5-2 hr, then add 90mL of feeding mix (total volume of the reaction is 180mL) as follows

- 45mL feeding buffer
- 3.6mL 18aa mix
- 1.8mL Cysteine
- 1.8mL Methionine
- 36mL water

Incubate the reaction overnight at 30^{0}C, 80 rpm. Note that Brij-35 can be added to the reaction buffer and feed buffer at the final concentration of 1-2%.

Protein Extraction and Purification (using His-tag)

- Add buffer A (20mM Tris 8.0, 20mM NaCl) to the overnight reaction to make up a final volume of 250mL.

- In some cases, a second detergent can be introduced to further the extraction yield of protein from the reaction. Although this process is protein specific, the addition of this detergent can increase the yield of soluble membrane protein 20-30 times.

- Centrifuge at 38,000 x g for 20 minutes, and the supernatant used for further purification. Note that detergents at proper concentration were present in all purification steps.

Purification of His-tagged proteins was accomplished using Ni-NTA resin by either gravity flow or FPLC as follows:

- Wash the crude lysate with several volumes of buffer A, followed by a high-salt wash (20mM Tris 8.0, 200mM NaCl) and 30mM imidazole wash (20mM Tris 8.0, 20mM NaCl, 30mM imidazole)

- Elute with 100-300mM imidazole (20mM Tris 8.0, 20mM NaCl, 300mM imidazole).

- Desalt with a desalting column and concentrate to an appropriate concentration (10-30mg/mL) for further biophysical analysis with amicon ultra filters (Millipore, MA).

- Optional: ultracentrifuge. The protein can be ultracentrifuged at 370,000g (95,000 rpm, TLA100.3 rotor, Optima TLX ultracentrifuge, Beckman Coulter, CA) to greatly reduce any aggregates and improve homogeneity.

- Optional: tag removal. The poly His-tag sequence can be removed by overnight thrombin, factor Xa, or enterokinase digestion depending on the template construct used.

The final purified protein can be used for crystallization trials or biophysical analysis. For many of our samples, protein identity can be verified to within 1% of the expected molecular weight by MALDI-TOF mass spectrometry.

Gel filtration

Load 1-2mL of purified protein on a GF column (highload 16/60 with superdex 200 resin, GE Healthcare)

Filtrate with buffer A in the present of the appropriate detergent.

Collect and analyze protein peaks on SDS-PAGE. A mixture of gel filtration standards (Biorad Laboratories, CA) can be used to estimate the apparent molecular weights.

Please contact Professor Geoffrey Chang (gchang@scripps.edu) or Professor Ray Stevens (stevens@scripps.edu) if you have questions or comments regarding this protocol.