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Chapter 1

Production of Multi-Subunit Membrane Protein Complexes

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Abstract:

Membrane proteins constitute an important class of proteins for medical, pharmaceutical and biotechnological reasons. Understanding the structure and function of membrane proteins and their complexes is of key importance, but the progress in this area is slow because of the difficulties to produce them in sufficient quality and quantity. Overexpression of membrane proteins is often restricted by the limited capability of translocation systems to integrate proteins into the membrane and to fold them properly. Purification of membrane proteins requires their isolation from the membrane, which is a further challenge. The choice of expression system, detergents and purification tags is therefore an important decision. Here, we present a protocol for the isolation of a seven-subunit membrane protein complex, the bacterial holo-translocon, which can serve as a starting point for the production of other membrane protein complexes for structural and functional studies.

Key words

Membrane protein complex, overexpression in bacteria, endogenous host, complex purification, translocon.

1 Introduction

1.1 Aim of the study

Membrane proteins represent more than 25% of the proteome of all cells. They mediate the cell's interaction with its environment, i.e. transmission of intra- and intercellular signals, cell adhesion and transport across membranes. Membrane proteins are of prime pharmaceutical interest as they constitute ~50% of known and potential new drug targets. In addition to this, membrane proteins are the natural entry and/or anchoring points for infectious agents. Dysfunctional membrane proteins are the basis of many disorders such as cystic fibrosis and Alzheimer's. Understanding the function and molecular structure of this class of proteins is of key interest.

As in the cytosol, the majority of proteins in the membrane occur and function in complexes. For instance, budding yeast membrane proteins were found to interact on average with 2-3 interaction partners [1]. Due to their association with cellular membranes, expression and purification of membrane proteins and their complexes is often a challenging task. Progress is often slowed down by the requirement to optimize protein expression and to subsequently identify conditions to solubilise the membrane proteins without compromising integrity and activity. While the optimal expression and purification conditions needs to be optimised for each new protein and complex, standard protocols as presented here offer a good starting point for subsequent optimisation by variation of conditions.

1.2 Recombinant expression in *Escherichia coli*

E. coli has traditionally been, and still is, the most popular expression system for proteins. This is because of low costs, low expenditure of time and the fact that it is less labour-intense than other expression systems.

Choice of promoter and strain. When expressing membrane proteins, saturation of the *E. coli* membrane protein translocation and folding machinery should be avoided. This would lead to protein misfolding, aggregation and formation of inclusion bodies. Very strong promoters such as the T7 promoter frequently lead to these problems. Weaker promoters synthesize membrane proteins at more moderate levels. Examples include T7/*lac* hybrid promoters or the tightly regulated arabinose promoter, both of which are often used for membrane protein expression [2].

Addressing the same issues, tailor-made strains have been developed for membrane protein production in *E. coli*. The T7 RNA polymerase-based expression strain BL21λ(DE3) and its mutant strains C41λ(DE3) and C43λ(DE3) [3] are the most popular strains for expressing membrane proteins in *E. coli* [2]. C41 and C43 contain mutations in the *lac*UV5 promoter region of the gene encoding for T7 RNA polymerase leading to lower expression of the T7 RNA polymerase [4] and consequently slower transcription and translation rates of the proteins under the control of T7 promoter. The same principle applies to the *E. coli* BL21-AI strain (Invitrogen) which expresses T7 RNA polymerase under the control of a tightly regulated arabinose promoter.

Other considerations. Auto-induction media are commonly used for improved membrane protein production [5]. They can be used for all IPTG-inducible expression systems. Protein production is induced in this media at high cell density when the glucose in the media is depleted, which is during the mid/late log phase of growth. Lactose uptake then leads to production of allolactose which causes the release of the lac repressor and induction of T7 RNA polymerase from the *lac*UV5 promoter.

For membrane protein complexes, a common strategy is the co-expression of the proteins from their natural or artificial operons [6] (see example in Fig. 1a). Co-expression of all subunits of a complex allows complex assembly *in vivo* in the cell. This is particularly important for membrane protein complexes, where reconstitution of the complexes from the purified, detergent-solubilised subunits is often not possible. Co-expression of the subunits of a complex from one poly-cistronic messenger RNA supports a balanced expression. Unexpectedly, *E. coli* operons were shown to be translated with the proper stoichiometry even when the subunit stoichiometry significantly deviates from one copy each per complex [7]. Accordingly, the order of genes in a synthetic operon needs to be experimentally optimised if the protein production is not balanced [8].

Recombinant membrane protein expression. When expressing eukaryotic proteins in *E. coli*, codon-optimization of the corresponding gene is recommended in order to account for the *E. coli* codon usage bias and thus avoid low or unreliable expression. Overexpression of recombinant membrane proteins can be optimised by fusing a green fluorescent protein (GFP) to the membrane protein. Using fluorescence detection, GFP serves as a marker for membrane protein localisation, quantity of folded protein and the efficiency of solubilisation from the membrane by detergents [9,10].

In this chapter we describe the production of the *E. coli* holo-translocon, a seven-subunit membrane protein complex comprising SecY, SecE, SecG, SecD, SecF, YajC and YidC. It is one of the largest recombinant membrane protein complexes produced to date. The holo-translocon complex contains all proteins known to be important for protein translocation into and across the *E. coli* plasma membrane [11,12]. For the balanced expression of the holo-translocon, the ACEMBL system was used [13,14]. ACEMBL comprises acceptor and donor vectors which have multi-integration elements for individual genes or poly-cistrons. Donor vectors differ from acceptor vectors and other commonly used plasmids in that they comprise a conditional origin of replication (oriR6Kγ) and therefore need to be propagated in strains with the phage R6Kγ *pir* gene. All vectors have a LoxP site, but different antibiotic resistances. Donor and acceptor vectors can be fused by Cre recombinase and the donor-acceptor fusion plasmid can be selected in a conventional *pir*$^-$ *E. coli* strain using the appropriate combination of antibiotics [13].

For expression of the holo-translocon, the conserved SecY, SecE and SecG subunits were encoded by a synthetic poly-cistronic operon on a donor vector (Fig. 1a). A second donor vector carried the gene for YajC, which is the only non-essential subunit of the holo-translocon [15]. These two donor vectors were fused to an acceptor vector with a poly-cistron encoding for YidC, SecD and SecF. Hexa-histidine tags were fused to YidC, SecD and SecG and a calmodulin-binding peptide to YajC (Fig. 1a). Double-affinity purification via these tags results in pure holo-translocon complex, comprising one copy of each subunit [15,16].

1.3 Recombinant expression in eukaryotic expression systems

Expression of recombinant eukaryotic membrane protein in *E. coli* frequently results in protein misfolding and aggregation. This can be due to differences in the lipid composition, a different chaperone repertoire, or the lack of post-translational modifications required to obtain folded, active membrane protein. In these cases, eukaryotic expression systems that are more closely related to the natural source of the protein are used. Unfortunately, membrane protein expression in mammalian cells often results in moderate or low protein yields. This is attributed to a limited capability of mammalian transport systems to import additional proteins or to replace its own proteins within the membranes of a particular subcellular compartment. GFP-fusion to the membrane protein of interest is less useful for optimising expression in mammalian cells because in contrast to *E. coli,* the GFP will fold independently from the membrane protein fused to it. Thus, the GFP-fusion method detects folded proteins as well as misfolded proteins which are retained in the endoplasmic reticulum membrane [17].

The majority of membrane proteins produced for structural and functional analysis to date are from baculovirus-infected cells [18]. Baculovirus-insect cell expression has been particularly successful for G protein-coupled receptors (GPCRs), leading to the first crystal structures of this class of membrane proteins [19,20]. The MultiBac system [21] has the same modular architecture of donor/acceptor vectors as described for the ACEMBL system for *E. coli* expression above. This supports the production of membrane protein complexes in insect cells, such as the active γ-secretase complex comprised of four membrane protein subunits [22].

1.4 Overexpression in the endogenous host

Isolation of membrane protein complexes from the natural host used to be restricted to membrane protein machines which are highly abundant in the cell, such as complexes for photosynthesis, respiration and oxidative phosphorylation [23]. However, with the advent of miniaturisation and automatisation of biochemical, biophysical and structural analyses, less abundant membrane protein complexes can now be studied.

Purification of endogenous complexes is significantly facilitated if a purification tag is fused to one of the subunits, which is possible if the source can be genetically modified. The tagged subunit is ideally an integral subunit of the complex. The chosen affinity purification tag should be compatible with the presence of detergents in the buffer (frequent choices include His-tags, 3xFLAG-tag and CBP-tag).

The *E. coli* holo-translocon production described below uses the endogenous host, thus providing the required chaperone repertoire and the physiological lipid composition. The purified complex has been shown to be active in co-and post-translational translocation and successfully used for biophysical and structural analyses [14,15,16]. A scheme for the general workflow of holo-translocon production and parameters to be optimised is presented in Fig. 1b.

2 Materials

Prepare all solutions using double-distilled water and analytical grade reagents. Store all stock solutions at 4˚C

2.1 Expression of HTL

1. pACEMBL HTL3 plasmid [15] (Fig.1a).
2. Chemically competent or electrocompetent *E. coli* BL21 Star (DE3) cells (Thermo Fischer) or *E. coli* C43λ(DE3) cells [3] are prepared according to published protocols [24,25].
3. Prepare antibiotic stock solutions: 100 mg/ml ampicillin, 25 mg/ml chloramphenicol (dissolved in EtOH), and 50 mg/ml kanamycin.
4. Prepare LB medium: 10g/L Bacto tryptone, 5g/L yeast extract, 10g/L NaCl (verify pH 7.5, or adjust with NaOH) and autoclave.
5. Prepare LB agar: add 15 g/L Bacto agar to 1L LB medium and autoclave.
6. Incubator 37˚C.
7. Shaker 37˚C.
8. Spectrophotometer
9. Baffled 5 L Erlenmeyer flasks.
10. Prepare *Terrific Broth:* 24 g/L yeast extract, 20 g/L Bacto tryptone, 4ml/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4 and autoclave.
11. Prepare stock solutions of 10% L-Arabinose by dissolving 1 g L-Arabinose in 10 ml of *water*. Filter with 0.22 um syringe filter and store aliquots at 4˚C. 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside) is prepared by dissolving 2.38 g IPTG in 10 mL of water. Filter with a 0.22 um syringe filter.
12. Centrifuge, e.g. Sorvall Lynx 6000 with F9 6x1000 Lex rotor (Thermo Fischer)

2.2 Membrane Preparation

1. French Press (Sim Aminco, Spectronic Instruments) or Cell Disrupter / Microfluidizer (Microfluidics).
2. Centrifuge, e.g. Sorvall RC6 and SS-34 rotor (Sorvall).
3. Prepare 1 L HSGM buffer: 20 mM HEPES-KOH pH 8.0 (20 ml of 1 M stock solution), 130 mM NaCl (26 ml of 5 M stock solution), 5 mM $MgOAc_2$ (5 ml of 1 M stock solution), 10% Glycerol.
4. cOmplete protease inhibitor cocktail tablets, EDTA-free (Roche).
5. Prepare 100 mM PMSF (phenylmethylsulfonyl fluoride) stock solution: add 17.4 mg of *PMSF* per 1 ml of isopropanol and freeze 20 µl aliquots and store at -20˚C.
6. Ultracentrifuge, Ti70 rotor and centrifugation tubes (Beckman Coulter).
7. Dounce homogenizer.

2.3 Detergent Solubilization

1. Prepare 10% w/v DDM (n-Dodecyl-β-D-maltoside, Anatrace) stock solution in water. Dissolve by gently agitating; do not vortex.
2. Head-over-tail rotator or equivalent.
3. Ultracentrifuge, Ti70 rotor and centrifugation tubes (Beckman Coulter).

2.4 NiNTA Affinity Chromatography

1. Ni-NTA agarose resin (Qiagen).
2. Gravity flow column (e.g. MoBiTec, #10141).
3. Prepare 100 ml Ni-NTA buffer: 20 mM HEPES-KOH pH 8.0 (2 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 5 mM $MgOAc_2$ (0.5 ml of 1 M stock solution), 10% Glycerol, 10 mM imidazole (1 ml of 1 M stock solution, adjusted to pH 8.0), 0.1% DDM (1 ml of 10% stock solution).
4. Prepare 50 ml High salt buffer: 20 mM HEPES-KOH pH 8.0 (1 ml of 1 M stock solution), 500 mM NaCl (5 ml of 5 M stock solution), 5 mM $MgOAc_2$ (250 µl of 1 M stock solution), 10% glycerol, 10 mM imidazole (0.5 ml of 1 M stock solution, adjusted to pH 8.0), 0.1% DDM (0.5 ml of 10% stock solution).
5. Prepare 100 ml Ni-NTA Wash buffer: 20 mM HEPES-KOH pH 8.0 (2 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 5 mM $MgOAc_2$ (0.5 ml of 1 M stock solution), 10% Glycerol, 50 mM imidazole (5 ml of 1 M stock solution, adjusted to pH 8.0), 0.1% DDM (1 ml of 10% stock solution).
6. Prepare 100 ml Ni-NTA Elution buffer: 20 mM HEPES-KOH pH 8.0 (2 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 5 mM $MgOAc_2$ (0.5 ml of 1 M stock solution), 10% Glycerol, 300 mM imidazole (30 ml of 1 M stock solution, adjusted to pH 8.0), 0.1% DDM (1 ml of 10% stock solution).

7. Nanodrop spectrophotometer (Thermo Scientific)

2.5 Desalting Step

1. 5 ml HiTrap Desalting column prepacked with Sephadex G-25 resin (GE Healthcare).
2. AKTA protein purification system or equivalent.
3. Prepare 100 ml CBP-binding buffer: 50 mM HEPES-KOH pH 8.0 (5 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 10% glycerol, 2 mM $CaCl_2$ (200 µl of 1 M stock solution), 0.03% DDM (0.3 ml of 10% stock solution).

2.6 Calmodulin Affinity Chromatography

1. Calmodulin affinity resin (Agilent Technology).

2. Gravity flow column (e.g. MoBiTec, #10141).
3. Prepare 100 ml CBP-binding buffer: 50 mM HEPES-KOH pH 8.0 (5 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 10% glycerol, 2 mM $CaCl_2$ (200 µl of 1 M stock solution), 0.03% DDM (0.3 ml of 10% stock solution).
4. Prepare 100 ml CBP washing buffer: 50 mM HEPES-KOH pH 8.0 (5 ml of 1 M stock solution), 130 mM NaCl (2.6 ml of 5 M stock solution), 10% glycerol, 0.2 mM $CaCl_2$ (20 µl of 1 M stock solution), 0.03% DDM (0.3 ml of 10% stock solution).
5. Prepare 25 ml CBP elution buffer: 50 mM HEPES-KOH pH 8.0 (1.25 ml of 1 M stock solution), 400 mM NaCl (2 ml of 5 M stock solution), 3% glycerol, 2 mM EGTA (100 µl of 0.5 M stock solution), 0.03% DDM (0.3 ml of 10% stock solution).
6. NanoDrop spectrophotometer (Thermo Scientific).

2.7 Sample Concentration

1. Amicon Ultra centrifugal filter units, molecular weight cut-off 100 kDa, Ultra-15 (Amicon).
2. Centrifuge, e.g. Heraeus Megafuge 16R
3. Prepare 10 ml S6 buffer: 20 mM HEPES-KOH pH 8.0 (0.2 ml of 1 M stock solution), 130 mM NaCl (0.26 ml of 5 M stock solution), 5 mM $MgOAc_2$ (50 µl of 1 M stock solution), 0.03% DDM (30 µl of 10% stock solution).

2.8 Size Exclusion Chromatography

1. Superose 6 Increase 10/300 GL column (GE Healthcare).
2. AKTA protein purification system or equivalent.
3. Prepare 250 ml S6 buffer: 20 mM HEPES-KOH pH 8.0 (5 ml of 1 M stock solution), 130 mM NaCl (6.5 ml of 5 M stock solution), 5 mM $MgOAc_2$ (1.25 ml of 1 M stock solution), 0.03% DDM (0.75 ml of 10% stock solution).

3 Methods

Once the proteins are solubilized from the isolated membranes it is important to work at 4°C and proceed quickly with the protein purification to avoid protein aggregation and degradation.

3.1 Expression of HTL (SecY-SecE-SecG + YidC-SecD-SecF + YajC)

1. Transform the pACEMBL HTL3 plasmid (Fig. 1a) into chemically competent or electrocompetent *E. coli* BL21 Star (DE3) or *E. coli* C43λ(DE3) cells [3] and select for growth on LB agar plates with ampicillin, chloramphenicol and kanamycin at 37°C overnight.

2. Inoculate a 100 ml preculture in LB medium supplemented with 50 µg/ml Ampicilin, 20 µg/ml Chloramphenicol, 30 µg/ml Kanamycin with one colony from the LB Agar plate and grow at 37°C overnight by shaking at 180 rpm.
3. Inoculate 1 L of *Terrific Broth* with 50 µg/ml Ampicilin, 20 µg/ml Chloramphenicol, 30 µg/ml Kanamycin in a 5 L baffled Erlenmeyer flask with approx. 20 ml preculture to reach an initial optical density (OD_{600nm}) of 0.05 at 600 nm. Shake the *E. coli* cultures at 37°C and 150 rpm. Typical culture volumes for holo-translocon preparations are 6-12 L.
4. When the cell cultures reach an OD_{600} of 1.2-1.5 (mid-log phase) induce protein expression by addition of 21 ml of 10% L-Arabinose (0.2% final concentration) and 0.5 ml of 1 M IPTG.
5. After 3 hours of protein expression (37°C, 150 rpm) the cells are harvested by centrifugation for 20 min at 5000 x g (4°C) (*see* **Note 1**).
6. Discard the supernatant and determine the weight of the cell pellet (*see* **Note 2**).

3.2 Membrane Preparation

1. Thaw ~10 g *E. coli* HTL3 cell pellet on ice.
2. Add 30 ml of HSGM buffer and 1 tablet EDTA-free protease inhibitor cocktail and 0.5 mM PMSF.
3. Resuspend the cells by pipetting.
4. Open the cells by two passages through a French press cell at 1000 psi (*see* **Note 3**).
5. Centrifuge the cell lysate at 16,000 x g for 30 min (at 4°C).
6. Transfer the supernatant into an ultracentrifugation tube, discard the pellet.
7. Centrifuge the cleared lysate for 2 hrs in a Ti70 rotor at 45,000 rpm (149,000 x g) at 4°C.
8. Discard the supernatant and resuspend the brown-yellow pellet (membranes) in ~5 ml HSGM buffer using a Dounce homogenizer. Add cOmplete EDTA-free protease inhibitor cocktail and 0.5 mM PMSF (*see* **Note 4**).
9. Analyse the membrane fraction using a 15% acrylamide gel to confirm the presence of SecD (67.5 kDa), YidC (63 kDa), SecY (50 kDa), SecF (35 kDa), SecE (15 kDa), SecG (14 kDa). The presence of YajC-CBP (14.7 kDa) needs to be confirmed by Western blotting using an anti-CBP antibody (see Fig. 1c, lane 1). If the Coomassie stained gel is difficult to interpret, the presence of SecD, YidC and SecE can be confirmed by Western blotting using an anti-His antibody. SecD and SecE are encoded by poly-cistrons and part of SecYEG and SecDF protein complexes. Therefore, the other complex subunits are likely to be also present in the membrane.

3.3 Detergent Solubilization

1. Add DDM to a final concentration of 1.5% w/v to the membranes (*see* **Note 5**).
2. Gently agitate the sample at 4°C (in the cold room) for 2 hrs by *head-over-tail rotation*.

3. Centrifuge solubilised membranes for 1 hr in a Ti70 rotor at 45,000 rpm (149,000 x g) at 4°C (*see* **Note 6**).

3.4 NiNTA Affinity Chromatography (all steps at 4°C)

1. Equilibrate 5 ml Ni-NTA resin with 10 column volumes of Ni-NTA buffer (*see* **Note 7** for using a prepacked column).
2. Add the Ni-NTA resin to the solubilised membrane supernatant (from step 3.3.3).
3. Gently agitate for 1 hr by *head-over-tail rotation*.
4. Transfer the sample and beads into a gravity flow column. Collect the flow-through.
5. Wash with at least 20 column volumes of Ni-NTA buffer. (*see* **Note 8,** to change the detergent to LMNG at this step).
6. Wash with 3-5 column volumes of High salt buffer.
7. Wash with at least five column volumes of Ni-NTA buffer.
8. Wash with 20 column volumes of Ni-NTA Wash buffer.
9. Verify that there is no A_{280nm} signal in the wash fraction using a NanoDrop spectrophotometer before proceeding.
10. Elute with Ni-NTA Elution buffer and collect the eluate in 1.5-2 ml tubes.
11. Determine the protein concentration of each fraction by A_{280nm} measurements using a NanoDrop spectrophotometer (*see* **Note 9**).
12. Add 0.1 mM PMSF and cOmplete protease inhibitor cocktail to the protein-containing fractions.
13. Analyse the input, flow-through, wash and eluate fractions by SDS PAGE using a 15% acrylamide gel (see Fig. 1c for a typical result).
14. Pool the protein-containing fractions.

3.5 Desalting Step (at 4°C)

1. Equilibrate a 5 ml desalting column with CBP-binding buffer using the AKTA system (*see* **Notes 10, 11**).
2. Inject 3 ml of Ni-NTA eluate (use combined protein-containing fractions from step 3.4.14) onto the desalting column (*see* **Note 12**).
3. Collect 1 ml fractions and pool protein-containing fractions. Repeat this procedure until all holo-translocon-containing eluate fractions from Step 3.4.14 are in CBP-binding buffer.

3.6 Calmodulin Affinity Chromatography (at 4°C)

1. Equilibrate 5 ml Calmodulin affinity resin with CBP-binding buffer.
2. Add the pooled fractions from Step 3.5.3 to the resin.
3. Incubate overnight at 4°C and gently agitate.
4. Transfer the sample into a gravity flow column.
5. Wash the column with at least 10 column volumes of CBP washing buffer.

6. Verify by NanoDrop measurements that there is no A_{280nm} signal in the last wash fraction before proceeding to the next step.
7. Elute with 5 column volumes of CBP elution buffer and collect fractions.
8. Determine the concentration of each fraction by NanoDrop A_{280nm} measurements. (*see* **Note 9**).
9. Analyse the input, flow-through, wash and eluate fractions by SDS PAGE using a 15% acrylamide gel.
10. Pool holo-translocon-containing fractions.

3.7 Sample Concentration

1. Equilibrate a 100 kDa molecular weight cut-off concentrator with S6 Buffer.
2. Load the holo-translocon-containing fractions from Step 3.6.10 into the concentrator.
3. Centrifuge at 3,500 x g, 4°C until the sample volume is reduced to 500 µl (monitor volume regularly).

3.8 Size Exclusion Chromatography (optional)

1. Equilibrate a Superose 6 column with water and then with two column volumes of S6 Buffer (*see* **Note 13**).
2. Load 500 µl of sample from Step 3.7.3 onto the column.
3. Collect 0.5 ml fractions.
4. Analyse the protein-containing fractions on a 15% SDS PAGE gel.

Pool the peak fractions (Fig. 1d), if required concentrate to ~1 mg/ml using a concentrator with 100 kDa molecular weight cut-off. Freeze aliquots in liquid nitrogen and store the purified holo-translocon at -80˚C.

4 Notes

1. For each membrane protein complex, the growth temperature and the optimal time point to harvest the cells have to be determined experimentally. It is recommended to also test different strains (Fig. 1b).

2. It is possible to freeze the cells in liquid nitrogen and store the cells at -80°C at this step.

3. Alternatively, two passages through a microfluidizer or cell disrupter at 25 kPsi can be used to open the cells.

4. It is possible to freeze the dissolved membranes in liquid nitrogen and store at -80°C after this step.

5. For each new membrane protein complex, detergent screens have to be tested to identify a suitable mild, non-denaturing, solubilizing detergent [26], ideally followed by an activity assay (Fig. 1b). Different screens have been reported [27,28], various commercial detergent screens are available (e.g. Qiagen, Jena Bioscience).

6. If the centrifuge tube is not completely filled, it is recommended to either fill the Ti70 centrifugation tubes to the top with buffer or to reduce the centrifugation speed.

7. Alternatively, use a HisTrap FF column (GE Healthcare) coupled to a peristaltic pump for loading and washing the sample. Prior to loading, the supernatant from ultracentrifugation is passed through a 0.22 uM filter to further remove any large particles. During the washing step, the colour of the column will change from brown to almost light blue over the course of about one hour. During this time, detergent exchange (to LMNG for example) or supplementation of any additive (cholesteryl hemisuccinate for example) can be performed. For elution, the column is connected to an AKTA chromatography system.

8. It is possible to exchange the detergent to Lauryl Maltose Neopentyl Glycol (LMNG, Anatrace) at this step. To do this, replace DDM in the Ni-NTA Wash buffer with 0.1% w/v LMNG and in all other buffers with 0.03% w/v LMNG. In the final size exclusion chromatography step, the concentration of LMNG in the S6 buffer can be further lowered to 0.002% w/v LMNG. The critical micelle concentration of LMNG is 0.001% w/v in water.

9. The molecular weight of holo-translocon is 240 kDa and the extinction coefficient is ~500,000 M^{-1} cm^{-1} at 280 nm.

10. Equilibrate the desalting column during the Ni-NTA wash steps.

11. The max pressure of the desalting column is 0.3 MPa.

12. Very important: Quickly proceed from Step 3.4.14 to the desalting step to avoid aggregation of the holo-translocon sample in the Ni-NTA elution buffer due to high imidazole concentration.

13. Superose 6 max pressure 1.5 MPa; recommended flow rate 0.4 ml/min.

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Figures

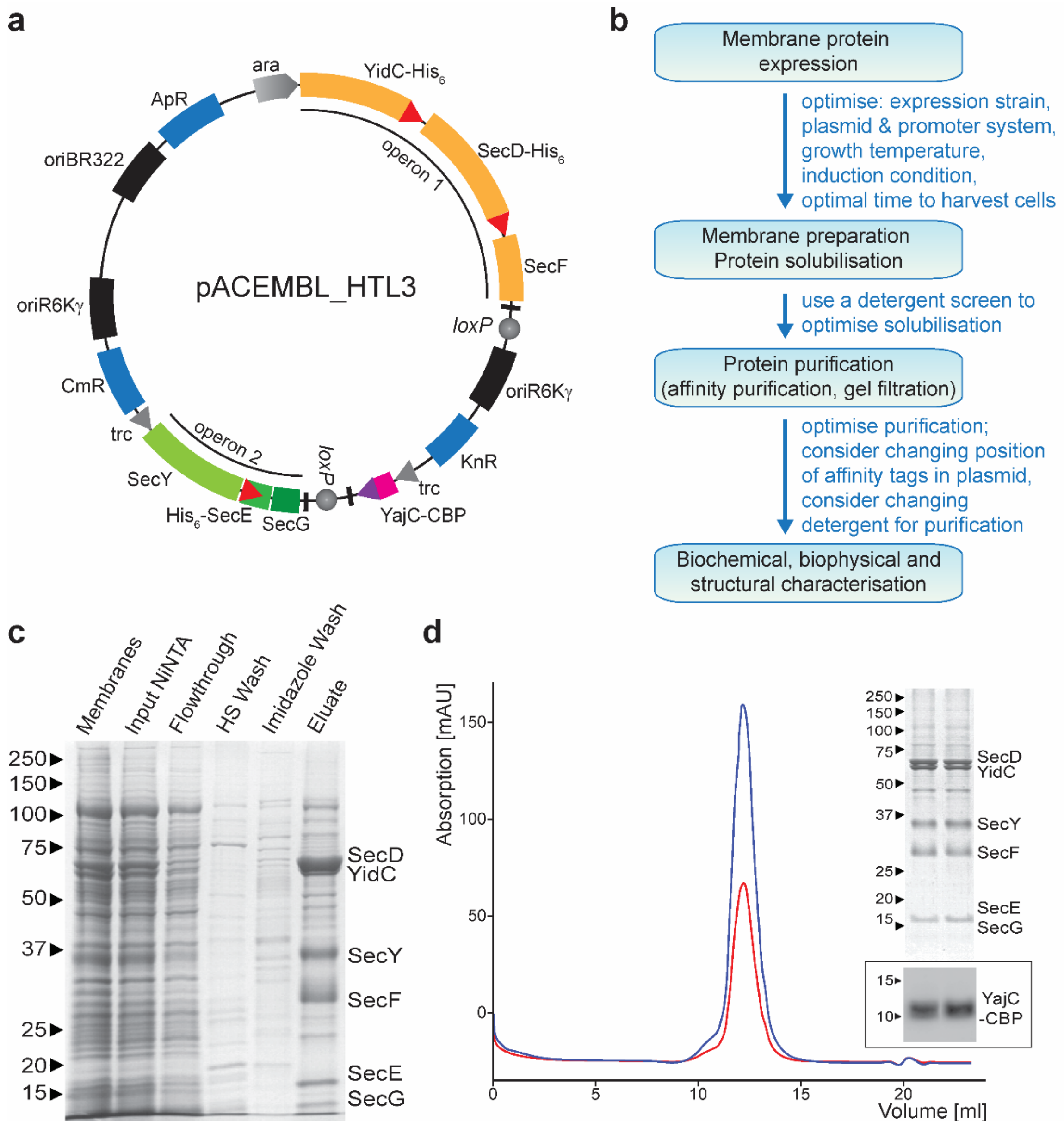

Figure 1. Figure summarizing the production of the holo-translocon complex, comprising seven membrane proteins.

a) The expression vector for the holo-translocon was generated using the ACEMBL system [13]. It comprises two artificial operons encoding for YidC, SecD and SecF (operon 1) and for SecY, SecE and SecG (operon 2), as well as the gene encoding for YajC with a C-terminal CBP-tag. The hexa-histidine tags on SecD, YidC and SecE are indicated as red triangles. The position of the CBP-tag is indicated with a purple triangle. Origins of replication of donor (oriR6Kγ) and acceptor (oriBR322) vectors are indicated in black. Arabinose (ara) and trc promoters are shown as grey arrows and terminators as black rectangles. Antibiotic resistance genes are coloured blue (Ap = ampicillin, Kn = kanamycin, Cm = chloramphenicol).

b) Scheme showing the general workflow for holo-translocon production and parameters that require optimization when a new membrane protein complex is produced.

c) Coomassie-stained SDS PAGE gel of holo-translocon, analysing the membrane fraction, the detergent-solubilized protein fraction (Ni-NTA input), the flow-through from Ni-NTA, wash fractions and the eluate peak fraction (HS = high salt). The bands for holo-translocon subunits are marked.

d) Size exclusion chromatogram of holo-translocon and SDS PAGE of the peak fractions. The blue and red curves show the absorption at 280 nm and 260 nm, respectively. Upper inset: Coomassie-stained SDS PAGE showing the peak fractions; below: Western blot using an antibody directed against the CBP-tag to detect YajC-CBP.