CHAPTER TEN

Expression and Purification of Membrane Proteins

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Abstract

Approximately 30% of a genome encodes for membrane proteins. They are one of the most important classes of proteins in that they can receive, differentiate, and transmit intra- and intercellular signals. Some examples of classes of membrane proteins include cell-adhesion molecules, translocases, and receptors in signaling pathways. Defects in membrane proteins may be involved in a number of serious disorders such as neurodegenerative diseases (e.g., Alzheimer's) and diabetes. Furthermore, membrane proteins provide natural entry and anchoring points for the molecular agents of infectious diseases. Thus, membrane proteins constitute ~50% of known and novel drug targets.

Progress in this area is slowed by the requirement to develop methods and procedures for expression and isolation that are tailored to characteristic properties of membrane proteins. A set of standard protocols for the isolation of the targets in quantities that allow for the characterization of their individual properties for further optimization is required. The standard protocols given below represent a workable starting point. If optimization of yields is desired, a variation of conditions as outlined in the theory section is recommended.

1. THEORY

Recombinant membrane proteins can be expressed *in vivo* using eukaryotic cells (e.g., yeast, insect, and mammalian cells), prokaryotic cells (e.g., *E. coli* and *Lactococcus lactis*), or can be synthesized in vitro using cell-free expression systems (e.g., EasyXpress® Kits). *E.coli* is still a popular expression system because the costs are low, no overly specialized equipment or knowledge is required, and the needed labor allocation is well known (see Small-scale Expression of Proteins in E. coli). The C41 and C43 strains (Miroux and Walker; 1996) are well suited for expressing membrane proteins in *E. coli*. The given protocols are focused on membrane protein expression in *E.coli*, but are generally applicable to membrane preparations independent of the source organism. In any case, it is recommended to use codon-optimized genes to avoid adverse effects due to differences in tRNA distribution between organisms.

Proteins can be separated into two general classes according to their location within a cell: proteins that are associated with cellular membranes (membrane proteins) and proteins that are not associated with cellular membranes (soluble proteins). Membrane proteins can be found in various intracellular membranous structures (e.g., the endoplasmic reticulum, mitochondria, and other vesicles) and the plasma membrane. However, the nature of their interaction with these respective biomembranes can be very different. The class of transmembrane proteins – which forms the largest group of membrane proteins – is characterized by the presence of a variable number of transmembrane domains. Such proteins include receptors and channels. Another class of membrane proteins is covalently attached to the lipid bilayer rather than being embedded in the membrane. Yet, other membrane proteins are indirectly attached to the membrane via noncovalent interactions with other proteins. Clearly, these different types of interactions of the proteins with their immediate environment, as well as their individual characteristics such as pH dependency of stability, must be considered if an optimal yield of functional protein is desired.

In general, one can expect to harvest functional membrane proteins only from membranes since these proteins occur as lipid–protein complexes. The yields of overexpressed membrane proteins are severely limited by the capability of the producing organism to import additional protein or replace its own proteins within the membranes. Consequently, cell types (e.g., mammalian cells) that are evolutionarily constrained to exist in a stable environment may not be the best choice to obtain high yields – the capacity of the transport systems and physical limits on the amount of exogenous protein that can be incorporated into a particular subcellular compartment may be overwhelmed. But even in prokaryotic cells, the forced expression of membrane proteins can easily overload the transport capabilities of the host organism, leading to the formation of inclusion bodies. Thus, a general protocol should encompass the isolation of membrane proteins from inclusion bodies. Also, the rate of synthesis of an overexpressed protein can exceed the time required for folding. Refolding the target protein after isolation and purification greatly simplifies the complexity of purification procedures, as the concerns about tailoring the procedures to retain functionality are relegated to the refolding step, where functionality has to be recovered.

In contrast to soluble proteins, the hydrophobic part of membrane proteins makes them difficult to solubilize away from their environment in the cell membrane. Detergents are polar molecules (with hydrophobic and hydrophilic parts) and adhere to the hydrophobic regions of a membrane

protein, thus resolubilizing it from the membrane. This resolubilization results in a water-soluble protein/detergent complex that can be isolated and subsequently purified. The selection of the right detergent can be crucial for the effective solubilization and purification of membrane proteins (see Explanatory Chapter: Choosing the right detergent). Changing the concentrations of protein (2–10 mg ml^{-1}), detergent, or salt (0.1–1 M) may change the aggregation behavior and the solubility of membrane proteins in unexpected ways. It should be noted that prolonged exposure of membrane proteins to excessive amounts of detergent solution can lead to delipidation of the protein, which may be detrimental to protein stability.

Three general cases for solubilizing membrane proteins are to be considered: direct solubilization (consisting almost exclusively of the target protein), solubilization by repeated extraction, and total solubilization (of membranes together with the target protein). In the last case, most of the lipids are solubilized as well, therefore higher amounts of detergent are required. It should be noted that the detergent used to solubilize the protein from the membranes might not be optimal for subsequent chromatographic purification. Ion exchange chromatography does not work well with charged detergents. In such cases, it is recommended that the detergent be exchanged with one of a lower CMC (see Explanatory Chapter: Choosing the right detergent).

Although the hydrophobic interactions of membrane proteins represent the most important complication compared to soluble proteins, the well-known problems encountered in expressing and purifying soluble proteins should not be neglected. The occurrence of fragmentation as a function of handling time and pH must be closely monitored, especially when scaling up a purification protocol. A protocol that works for 2 g of cell pellet is likely to fail when applied to a 50-g cell pellet.

In some cases, high yields of overexpressed membrane proteins may be obtainable only via inclusion bodies. The protein first must be purified and then refolded. Its integrity must be validated by a number of means, including activity or functional assays, circular dichroism spectroscopy (CD), and light scattering.

Chromatography of the solubilized membrane proteins independent of the folded state of the target protein can be quite simple if the protein carries an affinity tag. Ni-NTA purification of proteins containing a 6× His tag uniquely presents the possibility to purify folded protein as well as protein solubilized from inclusion bodies under denaturing conditions (see Purification of His-tagged proteins).

2. EQUIPMENT

Ultrasonic homogenizer
Refrigerated tabletop centrifuge
Refrigerated high-performance centrifuge (capable of generating ~40 000×g (e.g., Beckman Avanti® J Series or J2 Series centrifuge with a JA-17 rotor))
End-over-end rotator
Water bath
Incubator shaker (equipped with cooling option)
100-ml shake flasks
2-l shake flasks
2-ml microcentrifuge tubes
15-ml polypropylene centrifuge tubes
Spectrophotometer
SDS-PAGE and Western blotting equipment
Disposable gravity flow columns
0.45-μm filters

3. MATERIALS

IMAC resin (e.g., Ni-NTA Superflow, QIAGEN, cat. no. 30430; or Ni Sepharose™ 6 Fast Flow, GE Healthcare, cat. no. 17-5318-06)
Penta·His Antibody, BSA-free (QIAGEN, cat. no. 34660)
Ni-NTA Membrane Protein Kit (QIAGEN, cat. no. 30610)
Contains the following detergents:
- *N*,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)
- Octyl-β-D-glucopyranoside (OG)
- FOS-choline-16 (FOS)
- 6-Cyclohexylhexyl-β-D-maltoside (CYMAL®-6, Cy6)
- Nonyl-β-D-glucopyranoside (NG)
- Decyl-β-D-maltopyranoside (DM)
- *n*-Dodecyl-β-D-maltoside (DDM)

FOS-choline-12 (FC-12) (Affymetrix-Anatrace)
Lysozyme
Benzonase® Nuclease (25 000 units ml^{-1})
Sodium dodecyl sulfate (SDS)
Bromophenol blue

Dithiothreitol (DTT)
Sodium chloride (NaCl)
Imidazole
Glycerol
Tris base
Sucrose
Tryptone
Yeast extract
Hydrochloric acid (HCl)
Deionized water
Potassium phosphate monobasic (KH_2PO_4)
Potassium phosphate dibasic (K_2HPO_4)
Isopropyl-β-D-1 thiogalactopyranoside (IPTG)
Expression construct (sequence-optimized gene recommended, e.g., QIAgenes Expression Kit *E. coli*, cat. no. 39001)
OverExpress™ C41(DE3) competent cells (Lucigen® Corporation)
OverExpress™ C43(DE3) competent cells (Lucigen® Corporation)
BL21(DE3) competent cells (Novagen)
Liquid nitrogen

3.1. Solutions & buffers

Step 1 2× YT

Dissolve in 1 l of deionized water:
16-g tryptone
10-g yeast extract
5-g NaCl

Sterilize by autoclaving and allow media to cool

Step 2 50% Glycerol

Make a 50% (v/v) solution of glycerol in purified water. Filter through a 0.45-μm filter

1 M IPTG

Dissolve 238-mg IPTG in 1-ml water. Filter through a 0.45-μm filter. Store in aliquots at −20°C

Terrific Broth (TB)

Dissolve in 900 ml of deionized water:
- 12-g tryptone
- 24-g yeast extract
- 4-ml glycerol (5 g)

Sterilize by autoclaving and allow media to cool. Add 100 ml of 10×TB salts

10×TB salts

Component	Amount
KH_2PO_4	23.12 g
K_2HPO_4	125.41 g

Dissolve in a final volume of 1 l. Sterilize by autoclaving and allow the solution to cool

5×SDS-PAGE buffer

Component	Final concentration	Stock	Amount
Tris–HCl, pH 6.8	0.225 M	1 M	2.25 ml
Glycerol	50%		5 ml
SDS	5%		0.5 g
Bromophenol blue	0.05%		5 mg
DTT	0.25 M	1 M	2.5 ml

Add water to 10 ml

Step 6 Binding buffer (NTI-10-G)

Component	Final concentration	Amount
Tris base	20 mM	2.5 g
NaCl	500 mM	29.2 g
Imidazole	10 mM	0.7 g

Dissolve in 700-ml purified water. Adjust pH to 7.5 with 25% HCl
Add 126-g glycerol. Adjust volume to 1 l with water and mix for 20 min. Readjust pH to 7.5, if necessary. Filter through a 0.45-μm filter. Store at room temperature

Washing buffer (NTI-25-G)

Component	Final concentration	Amount
Tris base	20 mM	2.5 g
NaCl	500 mM	29.2 g
Imidazole	25 mM	1.7 g

Dissolve in 700-ml purified water. Adjust pH to 7.5 with 25% HCl
Add 126-g glycerol. Adjust volume to 1 l with water and mix for 20 min. Readjust pH to 7.5, if necessary. Filter through a 0.45-μm filter. Store at room temperature

Elution buffer (NTI-500-G)

Component	Final concentration	Amount
Tris base	20 mM	2.5 g
NaCl	500 mM	29.2 g
Imidazole	500 mM	34 g

Dissolve in 700-ml purified water. Adjust pH to 7.5 with 25% HCl
Add 126-g glycerol. Adjust volume to 1 l with water and mix for 20 min. Readjust pH to 7.5, if necessary. Filter through a 0.45-μm filter. Store at room temperature

4. PROTOCOL

4.1. Preparation

Obtain or clone a codon-optimized expression construct containing the gene encoding the membrane protein to be expressed and purified (see Molecular Cloning). The construct should include a 6×His tag for affinity purification and detection by Western blotting. Obtain chemically competent cells of the *E. coli* strains, BL21(DE3), C43(DE3), and C41(DE3), for expressing membrane proteins. Prepare 2× YT and TB media and the various buffers.

4.2. Duration

Preparation	Variable, about 3–7 days
Protocol	About 12–14 days

See Fig. 10.1 for the flowchart of the complete protocol.

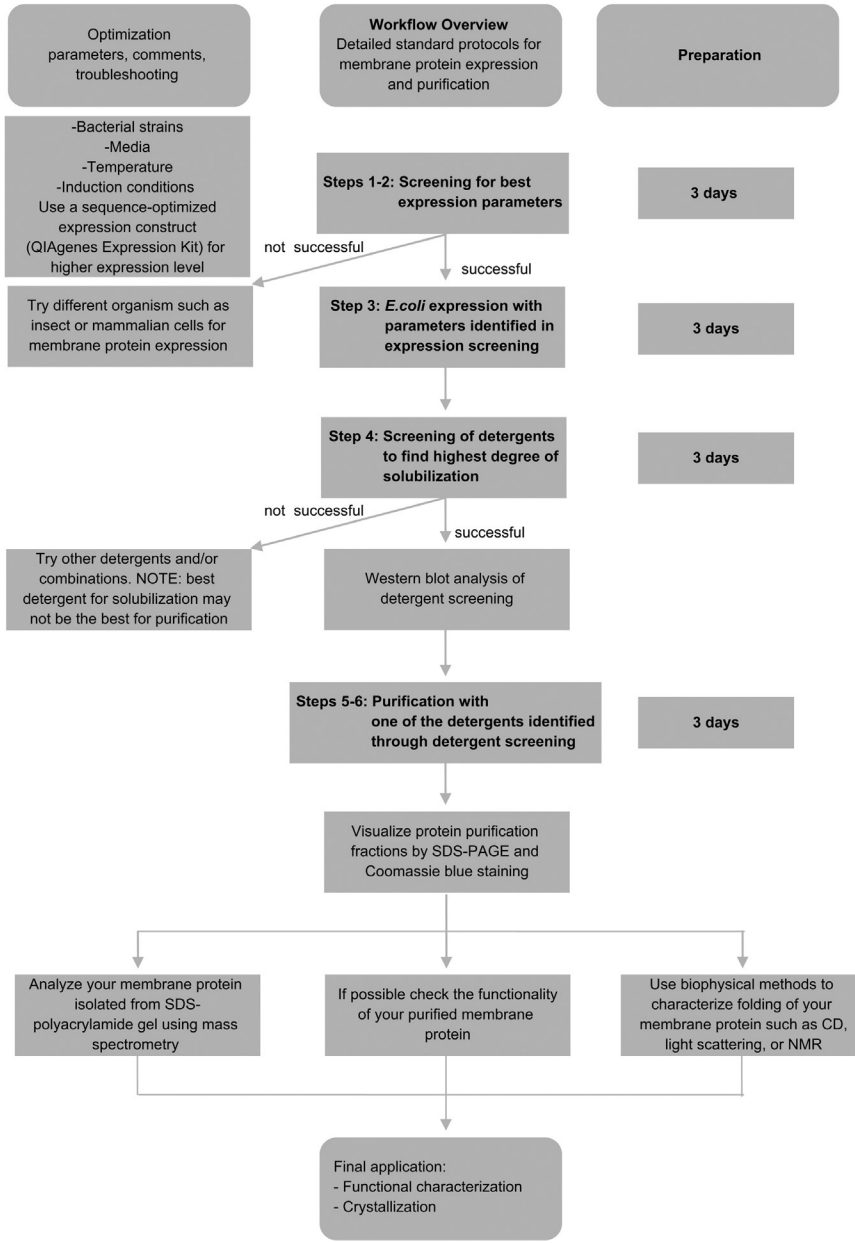

Figure 10.1 Flowchart of the complete protocol.

5. STEP 1 TRANSFORMATION OF *E. COLI*

5.1. Overview

This step describes the transfer of the plasmid DNA encoding the expression constructs into the *E. coli* expression hosts (see also Transformation of Chemically Competent E. coli).

5.2. Duration

2 h + overnight incubation

1.1 Thaw an aliquot of frozen competent *E. coli* cells of strains BL21(DE3), C43 (DE3), and C41(DE3) on ice.

1.2 Transfer 50 μl of the competent *E. coli* cells to prechilled sterile 1.5-ml microcentrifuge tubes.

1.3 Add 0.5 μl of the expression construct plasmid to each of the tubes of competent cells, mix carefully, and keep on ice for 30 min.

1.4 Transfer the tubes to a 42 °C water bath or heating block for 90 s.

1.5 Add 500-μl 2× YT to the cells and incubate for 1 h at 37 °C, shaking at 150 rpm.

1.6 Plate the three transformation mixes on LB agar plates containing the appropriate antibiotic (as determined by the vector. See Pouring Agar Plates and Streaking or Spreading to Isolate Individual Colonies). Incubate the plates at 37 °C overnight.

5.3. Tip

The hour incubation in 2× YT increases the transformation efficiency.

See Fig. 10.2 for the flowchart of Step 1.

6. STEP 2 CULTIVATION OF *E. COLI* – SCREENING FOR THE OPTIMAL EXPRESSION CONDITIONS

6.1. Overview

This step describes the screening of various conditions for the expression of the membrane protein. Samples taken under different conditions will be analyzed by Western blotting (see Western Blotting using Chemiluminescent Substrates).

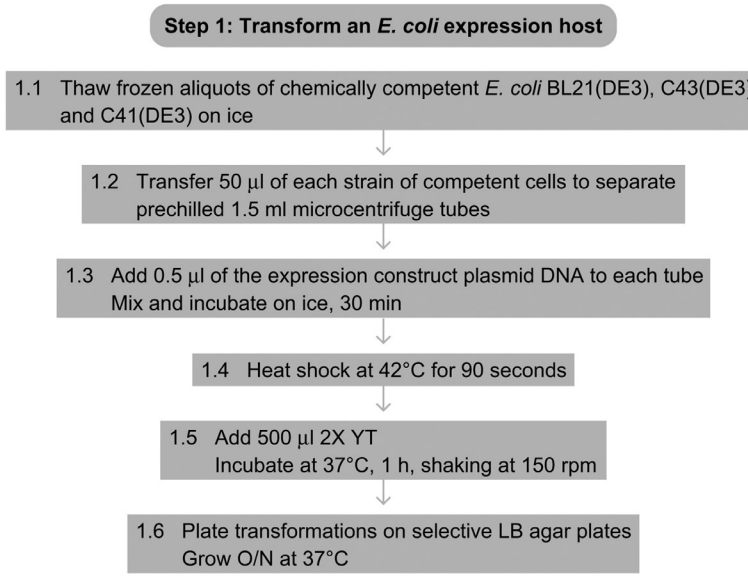

Figure 10.2 Flowchart of Step 1.

6.2. Duration

2–3 days

2.1 For each combination of expression construct and bacterial strain, pick three fresh bacterial colonies and inoculate 3–5-ml cultures of 2× YT containing the appropriate antibiotic. Grow at 37 °C overnight with shaking at 150 rpm. These represent the precultures (see Table 10.1).

2.2 Prepare a glycerol stock of each of the overnight bacterial cultures by thoroughly mixing 100 µl of cell culture with 100 µl of 50% glycerol; snap-freeze the glycerol stocks in liquid nitrogen and store at −80 °C for use in Step 3.

2.3 Determine the optical density of the precultures using a spectrophotometer at a wavelength of 600 nm (OD_{600}).

2.4 Prepare 9 × 9 ml of the media in 100-ml flasks as described in Table 10.1, add the appropriate antibiotic(s), and warm the media to room temperature. These represent the screening cultures.

2.5 Calculate the volume of each preculture needed to inoculate the corresponding 9 × 9-ml screening cultures at a starting OD_{600} of 0.1. These will be used to set up all of the expression conditions as listed in Table 10.1.

2.6 Grow the cultures at 37 °C, shaking at 150 rpm, until the cultures reach an OD_{600} of 0.4.

Table 10.1 Overview of screening conditions for each expression construct

Step/parameter									
Preculture (O/N)	C41(DE3)			C43(DE3)			BL21(DE3)		
Temperature	37 °C			37 °C			37 °C		
Screening cultures									
***E. coli* strain**	C41	C41	C41	C43	C43	C43	BL21	BL21	BL21
Medium	2× YT	2× YT	TB	2× YT	2× YT	TB	2× YT	2× YT	TB
Volume medium	9 ml	9 ml	9 ml	9 ml	9 ml	9 ml	9 ml	9 ml	9 ml
Volume preculture	varies								
Starting OD_{600}	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Temperature	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C
Induction (IPTG, μM)	200	200	200	200	200	200	200	200	200
Induction OD_{600}	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Temperature	37 °C	16 °C	16 °C	37 °C	16 °C	16 °C	37 °C	16 °C	16 °C
Induction length	4 h	10 h	10 h	4 h	10 h	10 h	4 h	10 h	10 h

2.7 Remove 20-µl aliquots from the uninduced cultures immediately before induction. Add 5 µl of 5× SDS-PAGE buffer and store at −20 °C. This serves as the uninduced control.

2.8 Add 1.8 µl of 1-M IPTG (200-µM final concentration) to induce protein expression. Return the flasks to the shaking incubator at the desired temperature (37 °C or 16 °C).

2.9 Induce expression of the protein for the indicated lengths of time (4 or 10 h). For a more complete time course of induction of protein expression, remove 20-µl samples from the induced cells at suitable time points after induction (e.g., every 2 h), mix with 5 µl of 5× SDS-PAGE buffer, and store at −20 °C.

2.10 Thaw the samples taken during the time course of protein expression for SDS-PAGE analysis.

2.11 Heat the samples for 30 min at 46 °C.

2.12 Analyze the samples by SDS-PAGE and Western blotting using an anti-His tag primary antibody and appropriate secondary antibody (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

6.3. Tip

Individual colonies may differ significantly in their expression level even if transformed with the identical plasmid. Picking of three colonies from each transformation can increase the chances of obtaining a highly expressing clone. This will increase the number of screening points threefold (Table 10.1).

6.4. Tip

Be sure to measure the OD_{600} within the linear range. If the OD_{600} exceeds 0.5, dilute the sample and measure the OD_{600} of the dilutions.

6.5. Tip

It is important to grow the 9–10-ml screening cultures in 100-ml flasks in order to maximize protein expression levels in the induced E. coli.

6.6. Tip

It is important not to boil the samples but to incubate them at 46 °C in order to avoid membrane protein aggregation.

6.7. Tip

The strongest signal on the Western blot indicates the best expression conditions.

See Fig. 10.3 for the flowchart of Step 2.

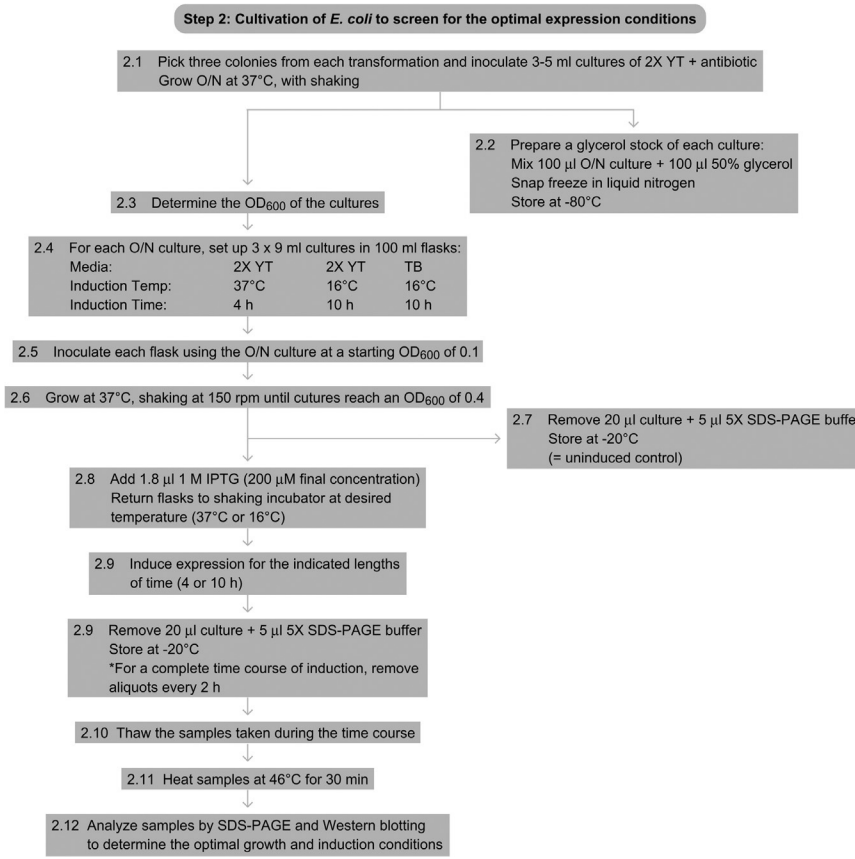

Figure 10.3 Flowchart of Step 2.

7. STEP 3 SCALE-UP EXPRESSION OF A MEMBRANE PROTEIN USING THE OPTIMAL EXPRESSION CONDITIONS

7.1. Overview

This step describes the scaled-up expression of a membrane protein using the optimal expression conditions identified above.

7.2. Duration

Overnight + ~8–12 h

3.1 Inoculate 50 ml of 2× YT medium containing the appropriate antibiotic with the bacterial glycerol stock identified as the best expresser (above). Grow at 37 °C overnight with shaking at 150 rpm.

3.2 Prepare a 2-l flask containing 175-ml media (2× YT or TB, identified as being optimal for the expression of the protein).

3.3 Determine the optical density of the overnight culture using a spectrophotometer at a wavelength of 600 nm (OD_{600}).

3.4 Calculate the volume of the overnight culture needed to inoculate the 175-ml cultures at a starting OD_{600} of 0.1 and inoculate the media.

3.5 Grow the cells at 37 °C, with shaking, until the culture reaches an OD_{600} of 0.4.

3.6 Add 35 μl of 1-M IPTG (200-μM final concentration) to induce protein expression. Induce expression of the protein at the temperature and for the length of time identified as being optimal (above).

3.7 Centrifuge the culture at 4000 rpm for 20 min at 4 °C to harvest the cells.

7.3. Tip

It is important to grow the 175-ml culture in a 2-l flask in order to maximize protein expression levels in E. coli.

See Fig. 10.4 for the flowchart of Step 3.

8. STEP 4 SCREENING DETERGENTS TO DETERMINE OPTIMAL SOLUBILIZATION OF MEMBRANE PROTEIN

8.1. Overview

This step describes the screening of seven detergents for their ability to solubilize the membrane protein (find more information on Explanatory Chapter: Choosing the right detergent).

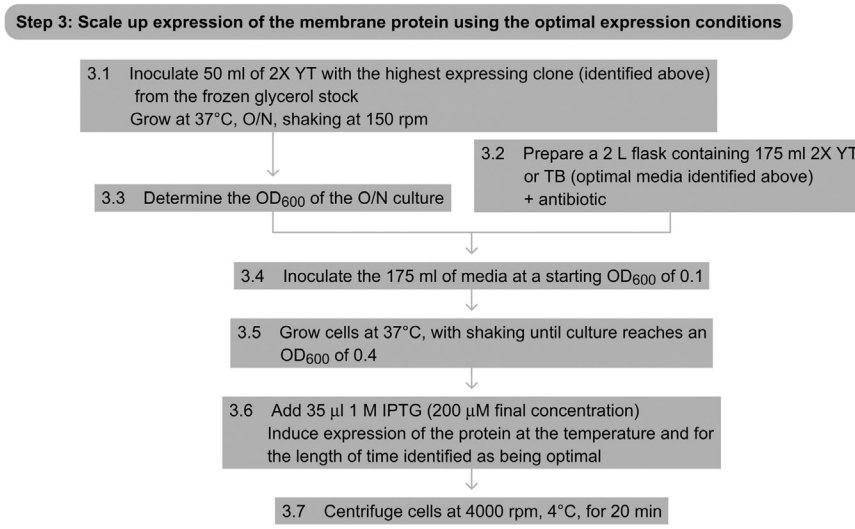

Figure 10.4 Flowchart of Step 3.

8.2. Duration

About 5–6 h (detergent solubilization)
6 h–overnight (SDS-PAGE and Western blotting)

4.1 Suspend the bacterial pellet (from Step 3) in 14 ml of Buffer NTI-10-G.

4.2 Dissolve 14-mg lysozyme in 500 µl of deionized water. Add the lysozyme and 21 µl of Benzonase® Nuclease (525 U) to the resuspended bacteria. Incubate for 30 min at room temperature and then for 30 min on ice.

4.3 Disrupt the cells using an ultrasonic homogenizer. Sonicate the cell lysate for two rounds of 3 min. Keep the cell lysate on ice while sonicating it to prevent it from heating up.

4.4 Centrifuge the lysate at 490 × g for 30 min at 4 °C

4.5 Transfer the supernatant into a fresh tube, mix briefly, and distribute it among seven clean 2-ml microcentrifuge tubes. Centrifuge at 20 000 × g for 1 h at 4 °C.

4.6 Discard the supernatants and label each tube with the name of one of the seven detergents.

4.7 Resuspend each pellet in 500 µl of Buffer NTI-10-G. Weigh out the appropriate amount of each detergent (see Table 10.2) and add it to the labeled tube. For example, add 5.11 mg of DDM to the DDM-labeled tube in order to solubilize the membrane protein using a concentration of 20-mM DDM.

4.8 Incubate samples on an end-over-end rotator for 1 h at room temperature (15–25 °C).

4.9 Remove 20-µl aliquots from each of the seven samples and add 5 µl of 5× SDS-PAGE buffer. Store at −20 °C for Western blot analysis. This is the total protein in detergent.

Table 10.2 Overview of the seven detergents used to determine the optimal solubilization conditions

Detergent	MW	Solubilization concentration (mM)	Amount used (for 0.5-ml NTI-10-G)
OG	292.37	51	7.46 mg
LDAO	229.40	30	3.44 mg
DM	482.56	21	5.07 mg
DDM	510.62	20	5.11 mg
Cy6	508.60	20	5.09 mg
NG	306.40	33	5.06 mg
FC-12	351.50	32	5.62 mg

4.10 Centrifuge the seven samples at 20 000 × g for 1 h at 4 °C.

4.11 Remove 20-µl aliquots from each of the seven samples and add 5 µl of 5× SDS-PAGE buffer. Store at −20 °C for Western blot analysis. This is the soluble membrane fraction.

4.12 Heat the SDS-PAGE samples at 46 °C for 30 min.

4.13 Analyze the samples by SDS-PAGE and Western blotting using anti-His tag primary antibody and an appropriate secondary antibody (see Figs. 10.5 and 10.6, One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE) and Western Blotting using Chemiluminescent Substrates).

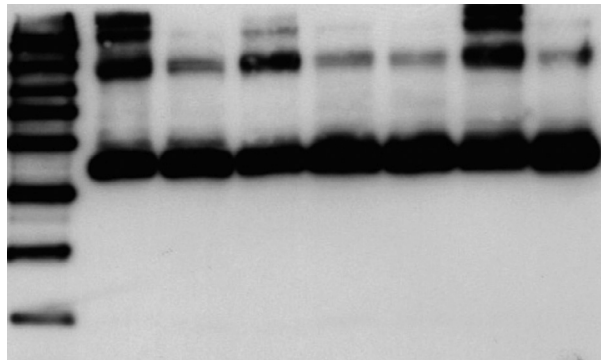

Figure 10.5 Screening for the optimal detergent to solubilize an overexpressed His-tagged membrane protein. *E. coli* cells overexpressing the 35 kDa membrane protein, NhaA, were pelleted and lysed. The cell membranes were resuspended in the indicated detergents (included in the Ni-NTA Membrane Protein Kit). Aliquots of the total protein fraction were taken for SDS-PAGE and Western analysis using anti-His tag antibody. Shown is a Western blot of the total protein fraction.

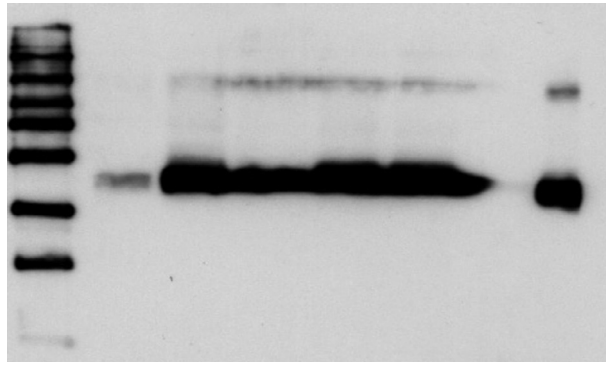

Figure 10.6 The soluble membrane fractions of the lysates were analyzed by Western blotting. After centrifugation of the samples, aliquots of the soluble membrane fraction were taken for SDS-PAGE and Western analysis using anti-His tag antibody. Shown is a Western blot of the soluble membrane fraction. The detergents OG and NG are poor solubilizers of NhaA.

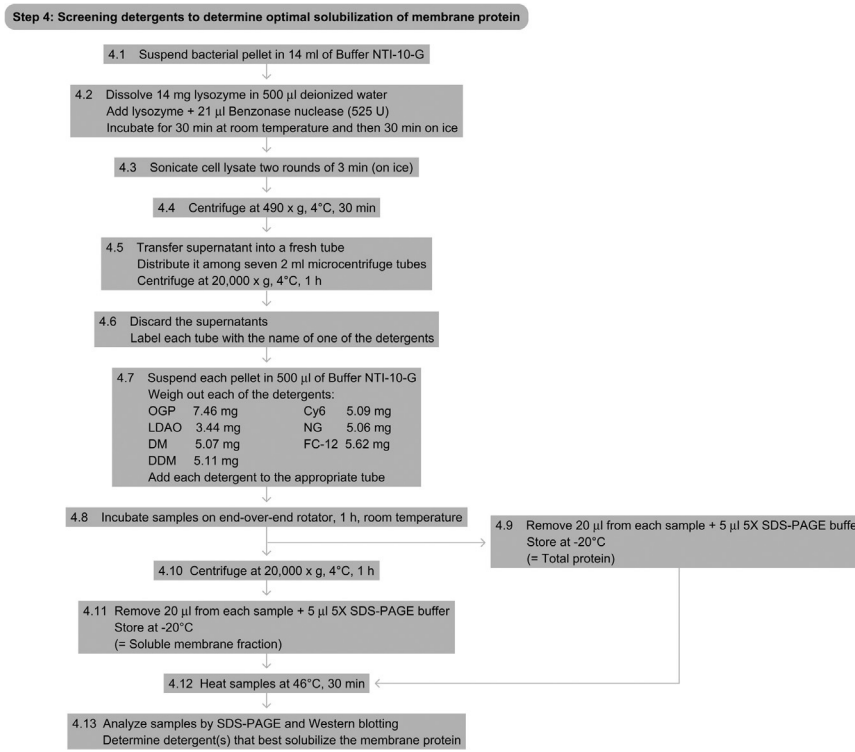

Figure 10.7 Flowchart of Step 4.

See Fig. 10.7 for the flowchart of Step 4.

9. STEP 5 SCALE-UP THE SOLUBILIZATION OF THE MEMBRANE PROTEIN

9.1. Overview

This step describes the large-scale solubilization of the membrane protein using the optimal conditions determined previously.

9.2. Duration

~10 h + overnight incubation (induction and solubilization)
~8 h − overnight (SDS-PAGE and Western blotting)

5.1 Grow and induce two 175-ml cultures of bacteria containing the expression construct as before (Step 3).

5.2 Suspend the bacterial pellet(s) in a total of 28 ml of Buffer NTI-10-G.

5.3 Dissolve 28 mg of lysozyme in 1 ml of deionized water. Add the lysozyme and 72 µl of Benzonase® Nuclease (1800 U) to the resuspended bacteria. Incubate sample on ice for 1 h.

5.4 Divide the lysed bacterial pellet into two equal volumes and disrupt the cells using a sonicator. Sonicate each sample 2 times for 3 min each. Keep the lysate on ice while sonicating to prevent it from heating up. After sonication, pool the two lysates in one tube.

5.5 Centrifuge the lysate at 490 × g for 1 h at 4 °C

5.6 Carefully transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge it at 40 000 × g for 1 h at 4 °C.

5.7 Discard the supernatant and resuspend the pellet in 5 ml of Buffer NTI-10-G. Determine the protein concentration (see Quantification of Protein Concentration using UV absorbance and Coomassie Dyes) and adjust the volume using Buffer NTI-10-G so that the protein concentration is 5 mg ml^{-1}. Note the final volume of the sample.

5.8 Calculate the amount of detergent needed to solubilize the protein. Use the detergent shown to give optimal results in Step 4. For example, 5.11 mg of DMM was used in 0.5-ml NTI-10-G to solubilize the protein. If the final volume is 7 ml, use 71.54 mg of DMM.

5.9 Transfer the dissolved pellet into a clean 15-ml polypropylene centrifuge tube and incubate on an end-over-end rotator overnight at 4 °C.

5.10 Remove a 20-µl aliquot and mix with 5 µl of 5× SDS-PAGE buffer. Store the sample at −20 °C for analysis by SDS-PAGE. This is the total protein sample.

5.11 Transfer the supernatant to a polycarbonate high-speed centrifuge tube and centrifuge it at ∼40 000 × g for 1 h at 4 °C.

5.12 Transfer the supernatant to a fresh tube. This is the soluble membrane fraction from which the protein will be purified in the next step.

5.13 Remove a 20-µl aliquot and mix with 5 µl of 5× SDS-PAGE buffer. Store the sample at −20 °C for analysis by SDS-PAGE.

9.3. Tip

If possible, centrifuge the sample at 100 000 × g (instead of 40 000 × g) for 1 h at 4 °C.

9.4. Tip

Instead of using an end-over-end rotator, it is possible to stir the sample with the detergent very slowly at 4 °C, overnight.

See Fig. 10.8 for the flowchart of Step 5.

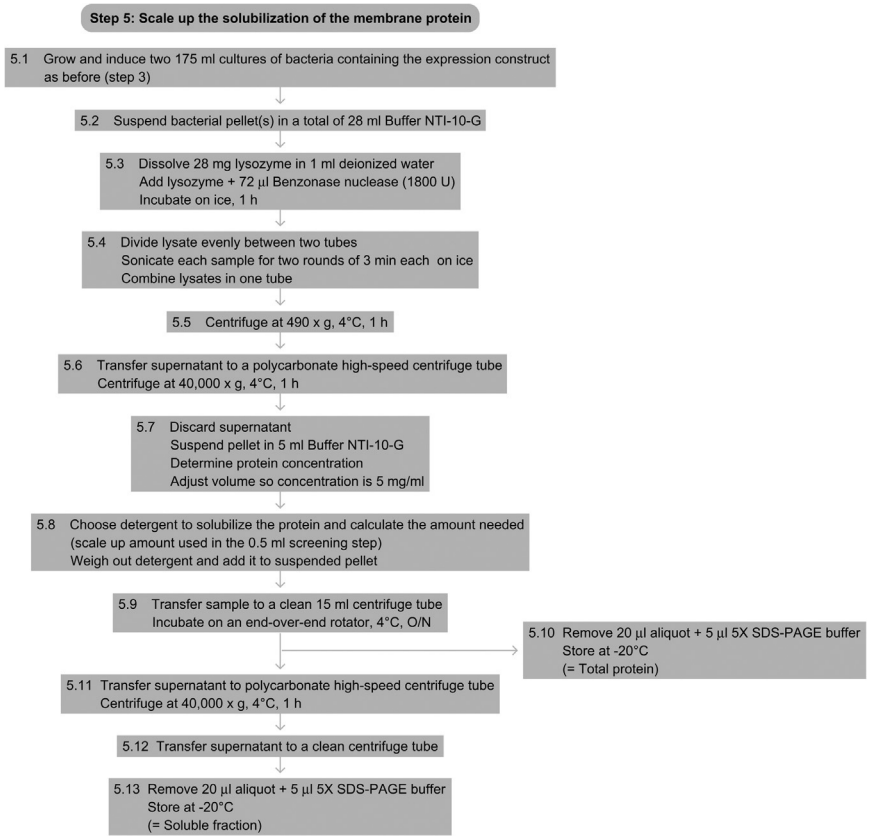

Figure 10.8 Flowchart of Step 5.

10. STEP 6 PURIFICATION OF MEMBRANE PROTEINS USING NI-NTA SUPERFLOW

10.1. Overview

This step describes the purification of the soluble membrane protein by affinity chromatography using Ni-NTA Superflow (see also Purification of His-tagged proteins).

10.2. Duration

~8 h – overnight

6.1 Pipette 1 ml of Ni-NTA Superflow suspension into a disposable gravity flow column. Wash twice with 5 ml of deionized water.

6.2 Equilibrate the column with 4 ml of Buffer NTI-10-G containing the chosen detergent (see Table 10.3).

6.3 Add the appropriate amount of detergent to the wash buffer (NTI-25-G) and elution buffer (NTI-500-G) (see Table 10.4).

6.4 Pipet the soluble membrane protein fraction onto the Ni-NTA Superflow column and allow it to drain by gravity flow. Retain the flow-through fraction. Apply the flow-through fraction to the column and allow it to drain. Reapply the flow-through to the column a second time.

6.5 Remove a 20-μl aliquot of the final flow-through fraction and mix with 5 μl of 5× SDS-PAGE buffer. Store the sample at −20 °C for analysis by SDS-PAGE.

Table 10.3 Amounts of the various detergents needed to add to Buffer NTI-10-G to equilibrate the column

Detergent	Solubilization concentration (mM)	Amount (for 4-ml NTI-10-G)
OG	51	59.64 mg
LDAO	30	27.53 mg
DM	21	40.54 mg
DDM	20	40.85 mg
Cy6	20	40.69 mg
NG	33	40.44 mg
FC-12	32	44.99 mg

Table 10.4 Amounts of the various detergents needed to add to the wash and elution buffers, NTI-25-G and NTI-500-G, respectively

Detergent	Concentration	Amount (for 10 ml of NTI-25-G)	Amount (for 2 ml of NTI-500-G)
OG	37.5 mM	109.64 mg	21.93 mg
LDAO	3.0 mM	6.88 mg	1.38 mg
DM	2.4 mM	11.58 mg	2.32 mg
DDM	0.225 mM	1.15 mg	0.23 mg
Cy6	0.84 mM	4.27 mg	0.85 mg
NG	9.75 mM	29.87 mg	5.97 mg
FC-12	2.25 mM	7.91 mg	1.58 mg

6.6 Wash the column with 2 × 5-ml volumes of Buffer NTI-25-G containing the chosen detergent.

6.7 Remove 20-µl aliquots of the wash fractions and mix with 5 µl of 5× SDS-PAGE buffer. Store the samples at −20 °C for analysis by SDS-PAGE.

6.8 Elute the protein in 4 × 0.5 ml of Buffer NTI-500-G containing the chosen detergent.

6.9 Remove 20-µl aliquots of the four elution fractions and mix with 5 µl of 5× SDS-PAGE buffer. Store the samples at −20 °C for analysis by SDS-PAGE.

6.10 Heat SDS-PAGE samples at 46 °C for 30 min. There should be the total protein, soluble fraction, flow-through, wash, and elution fractions.

6.11 Analyze samples by SDS-PAGE, followed by Coomassie blue staining (Fig. 10.9) (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE) and Coomassie Blue Staining).

10.3. Tip

It is important not to boil the samples but to incubate them at 46 °C in order to avoid membrane protein aggregation.

See Fig. 10.10 for the flowchart of Step 6.

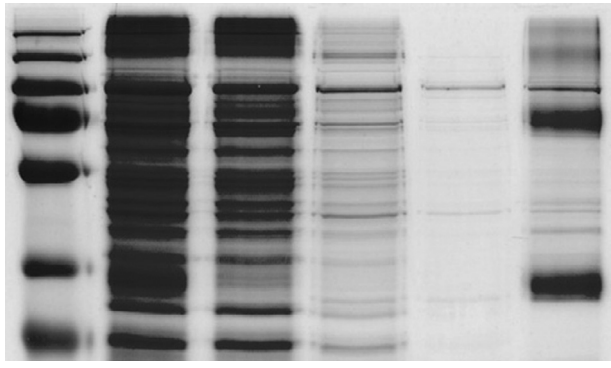

Figure 10.9 NhaA was purified on Ni-NTA Superflow using buffers containing the detergent, DDM. Fractions were separated by SDS-PAGE and proteins were visualized by Coomassie blue staining. NhaA can be seen in both its monomeric and dimeric forms. TP, total protein; SF, soluble fraction; FT, flow-through fraction; W, wash fraction; E, eluate; M, markers.

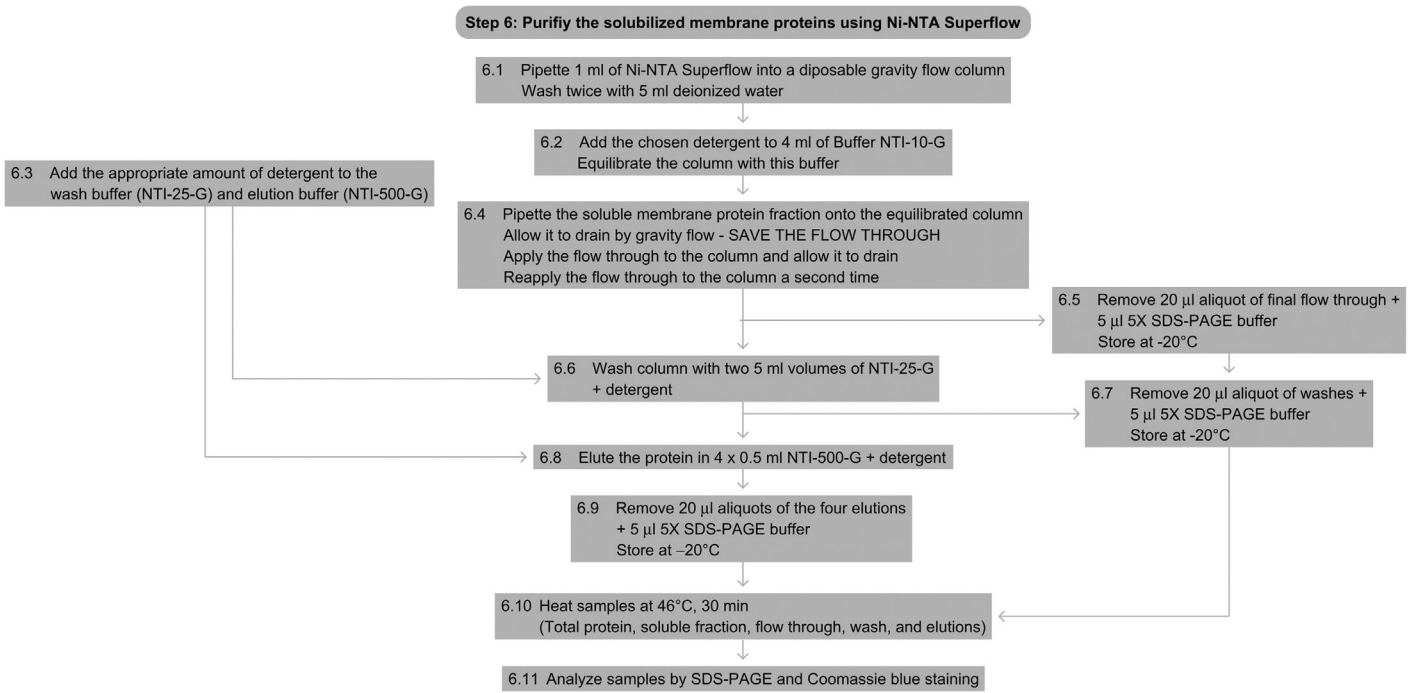

Figure 10.10 Flowchart of Step 6.

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Western Blotting using Chemiluminescent Substrates.
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Coomassie Blue Staining.