Universal protocol for the isolation of channel forming membrane proteins with the recombinant expression system of *E. coli*.

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Method Article

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Abstract

1 Abstract

The heterologous expression, isolation and characterization of pore forming protein is one of the most used strategy for understanding permeability properties of the biological membrane into which they are embedded. Some procedures present in literature are so empirical and long to perform, that the user can easily make mistakes. This protocol will teach you how to quantify and therefore optimize the expression of your favorite protein in E. coli using the BL21Gold(de3)ΔABCF strain. Then with a step-by-step approach, it will tell you how to separate the bacterial compartments according their solubility and then how to extract your protein in its native conformation using detergent. Finally, there is a detailed description on how to improve the purity of your protein via anion exchange chromatography. If you want to study bacterial porins reconstituted in their natural environment, here there is even the description on how to drive them into the outer membrane vesicles where they can be copurified. The protocol is easier and less empirical than the ones of most of the other membrane proteins and represents a solid base for soluble proteins as well (like enzyme or pore forming toxins). Expression time and temperature, concentration of the inducer, nature and concentration of the detergent, incubation time and temperature, purification pH, these are all the parameters that you can troubleshoot according to your results. The expression, extraction and purification will not last longer than a full day each and I have not yet found any protein which could not be isolated thanks to a derivation of this protocol.

Introduction

2 Introduction

Pore forming proteins facilitate the solute exchange driven by concentration gradient across any biological membrane (1). Their heterologous expression and characterization is one of the most used strategy for understanding permeability properties of the biological membrane into which they are embedded. For this reason, it is important to obtain porins in good amount and purity and one of the best technique to study them is via their reconstitution into the polarized lipid bilayer (2,3). The struggle of this technique is getting a product active enough for reconstitution experiments but pure enough to show consistent and reproducible results. The biochemical purity of a clean band into a SDS-PAGE is not linked to the electrophysiological purity of the sample, in fact the artificial membrane allows the reconstitution of the pore forming compounds present solution beyond their active concentration: usually much lower than the common staining methods (e.g. Coomassie or silver staining). Because of the high sensitivity for pore forming proteins, the reconstitution of channels into the polarized lipid bilayer is not an assay as specific as the ones used for enzymes (4).

OmpF clusters (drafts) are the main component of the outer membrane in E. coli (5) therefore during the heterologous expression of proteins it is the most common contaminant in reconstitution experiments
and quite often is very hard to remove it from the sample. The customization of the expression strains has always been a key factor for the good outcome of the isolation. Over the years, were produced many expression strains of *E. coli* in which the ORF of OmpF was damaged, decreasing highly the presence of contaminants. Unfortunately, that did not solve the problem because there are other porins that the bacterium expresses in good amount to survive (like OmpC and LamB), which can quite often escape the purification process and get reconstituted into the bilayer. At that time, making knockout mutants was hard, therefore were produced expression strains in which the presence of those proteins was suppressed damaging their promoters (6,7). That unfortunately caused sometimes the reversion of the phenotype under such a high selective pressure and picking the smallest colony into the plate wasn't something durable over many replicas (8). For this reason I came to know (during a private conversation with Dr. Ralf Koebnik) about the BL21Gold(de3)ΔABCF produced by Dr. Meuskens and colleagues (9). In that strain, they genetically removed the ORF of OmpF, OmpC, LamB and OmpA. The latter doesn't make channels in normal conditions but it has a structural importance to the bacterial shape and outer membrane stability. Having a ΔOmpA strain is helpful for the production of the outer membrane vesicles (See 4.6). The performances of this strain pushed me to clone all our ORF in vectors compatible with this technology. For this purpose, I used Gibson assembly because it is fast, cheap and the amount of false positive clones is minimal (10). The Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible system (or similar) guarantees a high protein expression, therefore a clear band into the SDS-PAGE (See Figure 1).

I was involved in the expression, extraction, purification and characterization of membrane proteins via their reconstitution into the polarized lipid bilayer (2,11). My aim wasn't only the characterization of biological channels, but also the development of a potent protocol, as universal as possible, for the isolation of all the channel forming membrane proteins that can be heterologically expressed in *E. coli*. In electrophysiology, the easiest porin you can work with is OmpF. Its isolation protocol involves the treatment of the extract with 2% SDS at 60°C for 1h: it basically destroys everything but OmpF. Then, after an ultracentrifugation, the protein is cleaned with 0.15% octyl-POE and then extracted with 3%. The product is already highly pure and active (12,13) but not all the porins are as easy to isolate. Some protocols present in literature are so empirical and long to perform, that the user can easily make mistakes. The isolation of CymA from *Klebsiella oxytoca*, takes three days from the cell lysis to the pure protein and its band into the SDS-PAGE doesn't show up before the anion exchange chromatography due to poor expression rates (14). Overall the isolation protocols used in the workgroups of Benz and Winterhalter are extremely similar and logical, they represent a solid base for the production of all the proteins I worked with, but the lack of an universal protocol for the isolation of membrane proteins is the driving force behind this paper.

While working with OprO and OprP wild type and mutants (Golla et al., 2022; Piselli et al., 2022; Piselli & Benz, 2021) I tried to compose a protocol that would always work with channel forming membrane proteins and that came to use whenever I had to deal with different proteins. Pore forming toxins are the easiest to extract because they are soluble in a detergent-less buffer and, once you optimize their
expression, they end up in the supernatant after the first ultracentrifugation (18–20). The cell wall channels of the MspA family are for some reasons soluble in a similar manner but not for much time (Piselli et al., 2022). Outer membrane proteins (and membrane proteins in general) are somehow tricky because most of them end up into the inclusion bodies and from there you have to extract and refold them, resuspending the right pellet with the right concentration of the right detergent (See 4.4). Once the protein is present in a clear supernatant, a simple chromatography is enough to have an extremely pure product, biochemically and more important electro-physiologically.

2.1 Applications of the method

Over the last few years, I have found that combining the BL21Gold(de3)ΔABCF strain with mainly IPTG inducible systems gives the best results, but it works with Arabinose (Ara) also, as long as the protein shows a clear band into the SDS-PAGE (See Figure 1). This protocol will teach you how to quantify and therefore optimize the expression of your channel forming membrane protein (See 4.3). Then with a step-by-step approach, it will tell you how to separate the bacterial compartments according their solubility (See 4.4). This strain with a fairly abundant protein expression, will give a good product which purity can be refined with chromatography (See 4.5). This expression system drives the outer membrane proteins into the periplasmic space, as long as they are expressed together with a signal peptide compatible with the molecular machine of E. coli. There the BAM complex recognizes and folds them into the outer membrane (22). Whenever the porin concentration gets too high in the outer membrane, the bacterium buds the channels out into the OMVs thus can be selectively centrifuged (See 4.6). The fusion of the porins-enriched OMVs into the planar lipid bilayer, allows the channels characterization into their natural environment (23).

2.2 Limitations

I haven’t yet found any channel forming membrane protein which couldn’t be isolated thanks to a derivation of this protocol.

Reagents

3.1 Reagents Setup (Alphabetic order)

- Acetic acid (Carl Roth). $ \text{CAUTION}$ handle the solution with gloves inside a chemical hood
- Acrylamide/bis acrylamide 37.5/1 30% (Carl Roth). $\text{CAUTION}$ handle the solution with gloves inside a chemical hood
· Agarose (Sigma).
· Ammonium persulfate (APS – Sigma). % solutions are in wt/vol
· Ampicillin (Amp - Sigma)
· Arabinose (Ara - Sigma). % solutions are in wt/vol
· β-mercaptoethanol (Sigma). % solutions are in vol/vol. $\textit{CAUTION}$ handle the solution with gloves inside a chemical hood
· Bromophenol blue (Sigma)
· Coomassie Brilliant blue R-250 (Bachem)
· DNAse I from bovine pancreas (Sigma)
· Ethylenediaminetetraacetic acid (EDTA – Sigma)
· Ethanol (Carl Roth). % solutions are in vol/vol
· Genapol X-080 (Sigma). % solutions are in vol/vol
· Glycerol (Carl Roth). % solutions are in vol/vol
· Glycin (Sigma)
· HEPES (Sigma)
· Hydrochloric acid (HCl - Sigma). $\textit{CAUTION}$ handle the solution with gloves inside a chemical hood
· Isopropanol (Carl Roth)
· Isopropyl β-D-1-thiogalactopyranoside (IPTG - Sigma)
· Lauryldimethylamine oxide (LDAO - Sigma). % solutions are in wt/vol
· Magnesium chloride (MgCl$_2$ - Sigma)
· MilliQ (double distilled) ultrapure water
· n-Octylpolyoxyethylene (Octyl-POE – Bachem). % solutions are in vol/vol
· Page ruler prestained protein ladder (NEB)
· Pepsin (Merk)
· Phenylmethansulfonylfluorid (PMSF – Merk)

· RNAse A (Sigma)

· Sodium Chloride (NaCl – Sigma)

· Sodium dodecyl sulfate (SDS – Sigma). % solutions are in wt/vol. $CAUTION$ handle the solution with gloves inside a chemical hood

· Sodium hydroxide (NaOH - Sigma). $CAUTION$ handle the solution with gloves inside a chemical hood

· Tetramethyl ethylenediamine (TEMED - Carl Roth). $CAUTION$ handle the solution with gloves inside a chemical hood

· Tripton (Sigma)

· Tris (Sigma)

· Triton X-100 (Sigma). % solutions are in vol/vol

· Urea (Sigma)

· Yeast Extract (Sigma)

**Equipment**

### 3.2 Equipment Setup (Alphabetic order)

· Autoclave

· Atomic balance

· Beckers

· BL21Gold(de3)ΔABCF (Addgene) chemicompetent cells (ccc)

· Bottles

· Calculator

· Chemical hood

· Chronometer

· Clean bench

· Eppendorf tubes
· Erlenmeyer Baffled Flasks (1 L and 100 mL)
· Falcon tubes
· Fast Protein Liquid Chromatography (FPLC)
· Filtering apparatus with filters (0.22 and 0.45 μm)
· French Press
· Fridges and freezers
· Gloves
· High speed centrifuge with rotor and tubes
· Homogenizing potter
· Kimtech wipes™
· Precision Balance
· Markers
· Mono Q™ 10/100 GL (GE healthcare)
· Petri dishes for microbiology
· Pipettes and tips
· Rocking table
· SDS-PAGE gel electrophoresis glassware and chamber
· Shaking incubator
· Sonication bath
· Spectrophotometer and cuvettes
· Standing incubator
· Syringe with flat tip needles and 0.22 μm filters
· Tabletop centrifuge
· Thermoblock
· Timer
· Transilluminator
· Ultracentrifuge with rotor and tubes
· Ultrasonicator
· Vacuum pump
· Wheel
· Working bench

Procedure

4 Procedure

4.1 Media and Solutions

4.1.1 SDS-PAGE

· Running Buffer (RB): 1 g/L SDS, 3.03 g/L Tris, 14.41 g/L glycin

· Loading Dye 4x (LD 4x): 200 mM Tris-HCl pH 6.8, SDS 8%, glycerol 40%, β-mercaptoethanol 4%, 50 mM EDTA, 80 mg Bromophenol blue.

· Running Gel 10% (recipe for 2 gels): 3.8 mL MQ water, 2.6 mL Tris-HCl 1.5 M pH 8.8, 100 μL SDS 10%, 3.4 mL Acrylamide/bis acrylamide 37.5/1 30%, 100 μL APS 10%, 10 μL TEMED.

· Stacking Gel 4% (recipe for 2 gels): 2.975 mL MQ water, 1.25 mL Tris-HCl 0.5 M pH 6.8, 50 μL SDS 10%, 670 μL acrylamide/bis acrylamide 37.5/1 30%, 50 μL APS 10%, 5 μL TEMED

· Coomassie brilliant Blue staining solution: 2.5 g Coomassie brilliant blue R-250, 450 mL ethanol, 100 mL Acetic acid, 450 mL MQ water

· Coomassie brilliant Blue distaining solution: 450 mL ethanol, 100 mL Acetic acid, 450 mL MQ water

4.1.2 Cultivation
· Lysogenic broth Lennox (LBL): 10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl, NaOH to pH 7 (add 15 g/L agarose for the cultivation in plate). Once it is autoclaved, mix it to the proper antibiotic (Amp in my case)

4.1.3 Extraction

· Lysis buffer: 10 mM Tris-HCl pH 8 and 5 mM MgCl$_2$, 10 μg/ml of pancreatic DNAsе I, 100 μg/ml of RNAsе A and 1 mM PMSF

· Cleaning buffer: 10 mM Tris-HCl pH 8, 0.15% octyl-POE (1 mM PMSF is optional)

· Extraction buffer: 10 mM Tris-HCl pH 8, 3% octyl-POE (1 mM PMSF is optional)

4.1.4 Purification

· Binding buffer: 0.5% octyl-POE, 20 mM Tris-HCl pH 8

· Eluting buffer: 0.5% octyl-POE, 20 mM Tris-HCl pH 8, 1 M NaCl

· NaCl 2 M

· NaOH 1 M

· EtOH 20%

· EtOH 100%

· Isopropanol 100%

· Pepsin solution: pepsin 1 mg/mL, 0.5 M NaCl, 0.1 M Acetic acid

4.1.5 Outer membrane vesicles

· Resuspension buffer: 10 mM HEPES pH 7, 150 mM NaCl. $\textbf{CAUTION}$ filter at 0.22 μm or autoclave the buffer before the use.
4.2 Checking the presence of your protein

**TIMING** 5’ per sample collection (See steps 1 to 3) and 25’ for processing them all at once (See steps 4 to 6)

The first and most important skill to master for the success of your experiment is **you must never lose track of your protein**. For an enzyme, you have to develop an assay to detect its activity (4), but for membrane proteins the activity is measured by their reconstitution into the polarized lipid bilayer (2,3). Unfortunately, this assay is not as specific as that for enzymes, especially for crude extracts. For this reason, I run proteins on 10% SDS-PAGE (24) in a non-induced/induced pattern to confirm their presence, a strategy applicable for soluble proteins as well. Membrane proteins don’t run according to their molecular weight (MW) (See Figure 1, Figure 2, Figure 3 and Figure 4) but they show a particular “fingerprint” according to their active conformation (See Figure 3 and Figure 4 - boiled and unboiled samples). The sample for running the SDS-PAGE is collected directly from the bacterial culture during the expression test or the large-scale expression (see 4.3) in the following manner to normalize the load.

1. Measure the optical density of your culture at wavelength of 600 nm (OD$_{600}$) with an optical length of 1 cm. **$ CAUTION$** do not accept values of OD$_{600}$ higher than 1 ± **TROUBLESHOOT** if the OD$_{600}$ is higher than that, just blank the spectrophotometer with 900 μL of clean LBL into the cuvette, then add 100 μL of sample and multiply the OD$_{600}$ by 10.

2. Collect from the medium the amount of volume required for having a 1 mL solution at OD$_{600}$ = 0.5 2

   **EXAMPLE** Measured OD$_{600}$ = $C_1 = 0.874 \rightarrow V_1 = C_2 V_2/C_1 \rightarrow V_1 = 0.5/0.874 \rightarrow V_1 = 0.572$ mL.

3. Centrifuge it in a 1.5 mL Eppendorf tube at 11'000xg for 30’ at room temperature (RT) and discard the supernatant. || **PAUSE POINT** store the pellet at -20°C in order to process all the samples at the same time once the expression, or the expression test, is finished.

4. Resuspend the pellets in 45 μL of RB 1x. Δ **CRITICAL STEP** if the culture is induced with IPTG for less than 4h, the pellets might be very slimy and hard to resuspend due to the presence of mRNA, be careful of not removing any of the bacteria with your tip. ± **TROUBLESHOOT** just add the 45 μL of RB without touching the pellets and go to the step 5, you will carefully resuspend them in step 6.

5. Boil the tubes at 95°C for 10’ and flick the tube every 5’ to break nucleic acids and other large biopolymers, then centrifuge the samples at 11’000xg for 30’ at RT.

6. Add to the sample 15 μL of LD 4x mixing it toughly and repeat step 5.

7. The supernatants are ready for the SDS-PAGE, 8 μL for 15-wells gels and 10 μL for 10-wells gels (see Figure 1). || **PAUSE POINT** you can store the samples at RT for weeks but when you want to use them you have to repeat step 5.
4.3 Expression Test

**TIMING** 1 day for the preparation of the streak, 1 day for the preparation of the inoculum and 1 day for the expression test (See steps 1 to 5)

The expression of the protein is carried out differently according to the induction system. I use mainly IPTG as inducer but Ara works as well. Any other inducible system can also be optimized with this expression test, as long as the protein shows a clear band into the SDS-PAGE (See Figure 1). I strongly recommend to use is BL21Gold(de3)ΔABC (9) because of the electrophysiological purity of the final product. That purity comes with a low price: the strain grows in plate for (TIMING) 24h at 30°C giving extremely small barely visible colonies. In tube it grows even slower for the lack of oxygen therefore I recommend the use of a small flasks even for the inocula (100 mL flask with 25 mL of LBL) and to incubate them for (TIMING) 24h at 30°C with 200 RPM shaking. **CRITICAL STEP** Even though microbiologists are taught to always pick the biggest single colony, here we must drop this good lab practice because big colonies of this strain might be the result of revertants (e.g. bacteria that managed to express some of the knocked-out Omps). I therefore recommend picking an average smaller colony in order to have a purer final product, but not the smallest to avoid having highly defected bacteria. This expression test is optimized for the heterologous expression of membrane proteins, but can be adjusted for any protein expressed in the host of your choice.

1. Dilute the inoculum 1:50 (or even 1:25) in 1 L flasks with 200 to 250 mL (to ¼ of the flask total volume) of LBL and place them in the incubator at 30°C with 200 RPM shaking. **CAUTION** the first time measure of the OD$_{600}$, it must be no more than 0.1.

2. Once your culture reaches the optimal OD$_{600}$ of 0.6 (it can be optimized between 0.3 and 1.2 – See below: ‡ TROUBLESHOOT in case of b) keep track of the time and take a sample of it (See 4.2 steps 1 to 3) and induce the expression of your protein and place the flask back into the incubator at 30°C with 200 RPM shaking. **TIMING** 2 to 4 hours to reach the inducible OD$_{600}$.

3. Keep sampling your bacterial culture at regular intervals of 1h and store the pellet at -20°C (See 4.2 steps 1 to 3). **CAUTION** Start one chronometer forward to monitor the overall induction time and one timer reverse for sampling precisely every hour. Keep track of the OD$_{600}$ measured at any time; it will be useful later (See below: ‡ TROUBLESHOOT).

4. Maintain the flasks as much as possible in the incubator at 30°C with 200 RPM shaking and keep sampling for (‡ TIMING) 5 to 6 hours and then even after one O.N. (See 4.2 steps 1 to 3).

5. Process all the pellets simultaneously and (‡ CAUTION) note which of them are slimy (See 4.2 steps 4 to 6), it might be an indication of the protein expression to be confirmed via the SDS-PAGE (See Figure 1 -
6. Once it is clear what is the best expression protocol in terms of initial $\text{OD}_{600}$, amount of inducer, expression time and temperature, it is possible to make the large-scale expression taking the sample of only the non-induced (NI) and the final induced (I) bacterial culture (See Figure 1 - left).

Table 1 - $\text{OD}_{600}$ values of the Figure 1. While the strain harboring the plasmid encoding for the OmpE36 grows constantly without expressing much of the protein until the O.N. sample, the ones encoding for OprO/P show a decrement of the bacterial growth 2h after the induction and then they remain stationary. Eventually they will grow but, for some reason, they reduce the proteins expression.

† **TROUBLESHOOT** the optimization of the expression is given by the comparison between the data present in Figure 1, Table 1 and the slimy pellets. I suggest to starting your protocol inducing at $\text{OD}_{600} = 0.6$, with the standard amount of inducer at 30°C with 200 RPM shaking, then the possible outcomes are two, no one of which is bad news:

a. The $\text{OD}_{600}$ grows constantly (like OmpE36).

b. The $\text{OD}_{600}$ decreases, becomes stationary and then grows again (like OprO/P).

In case of a, the expression of your protein is not harmful for the bacterial growth. With this strain, the expression of a large general diffusion porin together with its signal peptide, brings benefits to the bacterial growth because it facilitate the nutrient permeation inside the bacterial cell. Therefore you can decide when to harvest your bacteria solely depending on the band in the SDS-PAGE, and if it is possible you should always go for the O.N. expression. If the strain does not express your protein, you can resequence the plasmid, retransforming it into the strain, vary the inducer concentration or the expression temperature. The transformation protocol from ccc I use is extremely fast, cheap and sensitive (25) therefore I don't recommend the electroporation method.

In case of b, the expression of your protein is harmful for the strain because of its activity or for the stress caused by the induction. The culture always survives but after some time, when the $\text{OD}_{600}$ grows, the bacteria somehow stop expressing your protein so you will see a decrement in the band size of the SDS-PAGE combined to an increment of the $\text{OD}_{600}$. In this scenario, you can induce the expression of your protein at lower $\text{OD}_{600}$ (0.3 at least) for longer time (one O.N.), or higher $\text{OD}_{600}$ (1.2 at most) for shorter time (2 hours), decrease the amount of inducer and expression temperature to 20°C. Both these parameters can be optimized as shown in Figure 1, setting the sampling time as constant (I recommend NI and O.N.) and varying the inducer concentration or expression temperature. The lowest concentration of inducer that works for the longest expression time at low temperature, usually is the way to go.
· As long as you see the band of the protein into the SDS-PAGE, you are sure that the construct is working and the rest is only optimization.

· The early-induced pellets (1 to 3 hours) should be very slimy because of the mRNA, which should come along with the presence of the protein band into the SDS-PAGE. In that case, if the protein is not present, there must be a problem with the plasmid or the ORF so try to decrease the inducer (with IPTG as low as 0.1 mM) for longer expression time or to sequence the plasmid again. If the ORF is coming from a species phylogenetically distant from E. coli, the optimization of the codon usage will lead to a better expression (https://www.biologicscorp.com/tools/RareCodonAnalyzer). There are plasmids (e.g. pRARE2) that can be cotransformed with yours and they express tRNAs harbouring rare codons of E. coli. If the expression of your protein is highly toxic for the host (e.g. some proteases or pore forming toxins), you might not even see colonies into the transformation plate. If that happens there are plasmids (e.g. pLysS) that express the lysozyme which inactivates the T7 polymerase decreasing the leaky expression of the Lac operon, then try to increase the inducer (with IPTG as high as 2 mM) for shorter expression time (2 hours). If the pellet is not slimy you can try to retransform the plasmid into the strain and repeat the expression test.

· The temperature of the expression is also a parameter that you can tune, you can go down to 20°C and even cool down the culture on ice for 15’ before the induction. Growing the BL21Gold(de3)ΔABCF at higher temperature (e.g. 37°C) is reported to be linked to the decrement of its performances (9). For some long or highly expressing proteins, it can be useful to decrease the expression temperature (not less than 20°C) to get a better folding and less incorporation into the inclusion bodies in favor of the outer membrane (See Figure 2).

· The very same expression test described above can be performed setting the induction time as constant (I suggest O.N.) and varying the concentration of the inducer in different flasks, or can be repeated in the same manner at a lower temperature: the protocol can be adjusted in all its parameters. In case you need to crystalize the protein, every single mg of protein can make the difference so be sure its expression is well optimized. In case you need your protein for electrophysiological measurements I would not spend much time in optimizing the expression condition because it does not require much product rather than high purity.

4.4 Extraction

**TIMING** 1 to 3 days according to the protein

The extraction of the protein is standard but, as for the expression, requires a few adjustments depending on the protein you are working with. Once the expression of your protein is optimized, upscale the production to 500 mL (or whatever volume you need) and induce at the best OD$_{600}$ for the correct amount
of time skipping all the other steps. Always take a sample from the non-induced and final induced culture for the SDS-PAGE (See 4.3 step 6). $\textbf{CAUTION}$ you must never lose track of your protein; take always samples from each underlying supernatant, resuspension and run them into the SDS-PAGE to check where the protein is (See Figure 2 and Figure 3) and do not discard any fraction before having your protein extracted in a supernatant. Membrane proteins do not run according to their MW in gel electrophoresis (See Figure 1, Figure 2, Figure 3 and Figure 4) so always include the non-induced and induced samples from the expression, and you might even skip the ladder.

1. Once the expression is finished, harvest the cells via centrifugation at 10'000xg for 10' at 4°C and remove the supernatant. $\textbf{PAUSE POINT}$ store the pellet at -20°C for 1h at least. In the supernatant you can find and extract outer membrane vesicles (See 4.6). In that case, centrifuge your bacterial culture for 15'.

2. Thaw and resuspend each gram of bacterial pellet in 10 ml of lysis buffer (See 4.1.3).

3. Disrupt the cells always on ice by 3 times French Press treatment at 13’000 psi, or by 3 cycles of ultrasonication for 1’15” with amp 80% and 2” pulse on 3” pulse off with 30” pause in between every cycle. $\textbf{CAUTION}$ take a sample for the SDS-PAGE (See Figure 2 - Ext1). Other cell lysing methods will do the job with minor adjustments but SDS might ruin the extraction.

4. Centrifuge the lysate at 3’220xg for 30 at 4°C. $\textbf{CAUTION}$ take a sample of the supernatant for the SDS-PAGE (See Figure 2 - Subc).

5. Ultracentrifuge the supernatant at 40’000xg for 15’ at 4°C. $\textbf{CAUTION}$ take a sample of the supernatant for the SDS-PAGE (See Figure 2 - S1).

6. Run an SDS-PAGE with all the samples (See Figure 2). $\textbf{PAUSE POINT}$ you can store pellets and supernatants at 4°C. $\textbf{TIMING}$ this first part of the extraction takes one morning from the resuspension (See step 2) to the SDS-PAGE (See step 6).

$\uparrow$ $\textbf{TROUBLESHOOT}$ the possible scenarios are three:

a. **Your protein is present in the Ext1 but neither in the Subc nor in the S1.** This is the case when your protein is cloned with a signal peptide compatible with the molecular machinery of *E. coli*. It sends the protein towards the periplasm and the outer membrane. You find it therefore in the pellet after the first low-speed centrifugation with periplasmic inclusion bodies, big pieces of outer membrane and peptidoglycan. You are going to extract the protein from that pellet.

b. **Your protein is present in the Ext1 and in the Subc, but not in the S1.** This is the case for most of the membrane proteins and surely the preferable outcome (Figure 2) (26). You can favor this pattern by decreasing the concentration of the inducer and expression temperature (See 4.3). You find your protein
in the pellet after the ultracentrifugation with the cytoplasmic inclusion bodies, small pieces of outer membrane and peptidoglycan. You are going to extract it from that pellet. $\textbf{CAUTION}$ if the band in the Subc line of the SDS-PAGE is much smaller, you might have some of the protein in the first pellet as well, but the latter is the second will most likely yield protein with a better folding. The first time, extract the protein separately from both the pellets and see if you notice any difference in their “fingerprint” (See below). If not, next time just skip the first low-speed centrifugation and proceed with the extraction of the only pellet.

c. Your protein is present in the Ext1, in the Subc and in the S1. This is the case for the bacterial toxins, for some Gram-positive cell wall channels (like MspA homologs) and for soluble proteins in general (27). That means they are soluble in a detergent less solution and they do not precipitate into the cytoplasm. The strain does not store them into the inclusion bodies (unless the expression is extended for a very long time). The next time you will perform the extraction, you can skip the first low-speed centrifugation. You extracted your protein and you are going to purify it from the supernatant of the ultracentrifugation (See 4.5).

Once you understood in which fraction the protein accumulates, you must resuspend that pellet with detergent solutions to clear out the extra membrane proteins and then to extract yours. During these steps, it is useful to know more about your protein. One of the most important information is whether it forms oligomers in its active conformation or stays as monomer. If it is an oligomer, it is helpful to know how many monomers build the quaternary structure. In case of the monomer, it is helpful to know if the channel has a plug or not because it can be visualized in the SDS-PAGE. If your protein is a known one, you will find all these information in the scientific literature. Together they will contribute in understanding what the typical “fingerprint” of your membrane protein into the SDS-PAGE is (See Figure 3 and Figure 4 – boiled and unboiled samples).

7. Resuspend each gram of pellet in 9 ml of cleaning buffer (See 4.1.3 – low detergent concentration) using always a homogenizing potter or one cycle of ultrasonication (See above step 3).

8. Incubate the extraction for 1h at RT on the wheel. $\textbf{CAUTION}$ take a sample for the SDS-PAGE after the incubation (See Figure 3 – R1).

9. Ultracentrifuge again the resuspension at 40'000xg for 15 at 4°C. $\textbf{CAUTION}$ take a sample of the supernatant for the SDS-PAGE after the collection (See Figure 3 – S2).

10. Resuspend each gram of pellet in 9 ml of extraction buffer (See 4.1.3 - high detergent concentration) using always a homogenizing potter or one cycle of ultrasonication (See above step 3).

11. Incubate the extraction for 1h at RT on the wheel. $\textbf{CAUTION}$ take a sample for the SDS-PAGE after the incubation (See Figure 3 – R2).

12. Ultracentrifuge again the resuspension at 40’000xg for 15 at 20°C. $\textbf{CAUTION}$ take two samples of the supernatant for the SDS-PAGE after the collection (See Figure 3 – S3 and S3ub).
13. Run an SDS-PAGE with the proper amount of all the samples (See Figure 3). **PAUSE POINT** you can store pellets and supernatants at 4°C. **TIMING** this second part of the extraction takes one afternoon from the resuspension (Step 7) to the SDS-PAGE (Step 13). **CAUTION** the detergents I am using for the extraction are mainly uncharged, therefore they cannot move in an electric field; they instead sit on top of the well and isolate it. **Δ CRITICAL STEP** run each SDS-PAGE at 10 mA constant until the samples enter the stacking gel completely. Then, with a syringe, flow some RB 1x onto the well to remove the detergent, that will assure a better run. Finish to run each gel at 15 mA constant. Once you see the marker well separated into the running gel, you can increase the current to 20 mA constant until the line of the LD wires down of the gel.

‡ **TROUBLESHOOT** if you run always all the resuspensions and all the supernatants, the protein cannot disappear: it will be either into the pellet or into the supernatant. Therefore, the possible scenarios are 3:

a. **The protein is present in the last supernatant**. If this is the case, your extraction was successful and you can either try to use the crude extract as it is, or move on to the purifications step via chromatography (See 4.5).

b. **The protein is present in the last resuspension**. If this is the case, you must re-extract the pellet repeating 4.4 steps 10 to 12. If that does not work, you can elongate the incubation time all the way to one O.N. Increase the concentration of the detergent up to 5%. Change the detergent in favor of more aggressive ones (e.g. Genapol X-080, LDAO, Triton X-100) or use urea 4 to 8 M (26). Increase the extraction temperature up to 37°C (try to avoid that because it might affect the activity of the protein). If nothing works, you should try to express the protein at much lower temperature (e.g. RT or 20°C) of with a lower concentration of the inducer for a longer time (See 4.3). If that doesn't solve the problem, your pellet in the end will be extremely clean, therefore you can extract it with SDS 0.2% at most and try to skip the purification step or go for the cation exchange chromatography.

c. **You manage to extract the protein but it does not fold according to its “fingerprint” or it slowly disappears during the protocol**. If this is the case, your extraction was too harsh, try milder conditions the next time and add 1 mM PMSF. **CAUTION** There is the chance that you are running after the wrong SDS-PAGE band, so check it via Mass Spectrum.

### 4.5 Purification

**TIMING** 3 hours

When you finally found the best conditions for the expression and extraction of your membrane protein, you should have it in a supernatant in its native conformation. Although the protein in this step will hardly
be biochemically pure, it might be electrophysiologically pure enough for the reconstitution into the polarized lipid bilayer. Indeed it is a very sensitive instrument for the detection of membrane active compounds, but is not really specific as any enzymatic activity assay. If your protein is extracted after multiple cleaning steps, most of the pore forming proteins are already gone or present in extremely low concentration. If the sample does not show consistent and reproducible results in reconstitution experiments, the ion exchange chromatography (IEC) will surely remove all the contaminants. You can also use different purification strategies, like the His-Tag or the GST-Tag, but if you cannot remove the tags, they can affect the activity of your channel. On the other hand, size exclusion chromatography is a good technique for the buffer exchange rather than dialysis, but it usually doesn’t work very well for separating different pore forming proteins due to the similarities of their size in a detergent micelle.

Remember, you must never lose track of your protein and for this step you have to check the average isoelectric point of your mature protein entering its primary sequence to an online bioinformatics tool (https://web.expasy.org/compute_pi/). At your buffer pH, your protein has to be negatively charged for the anion exchange chromatography (AEC), or positively charged for the cation exchange chromatography (CEC); therefore I recommend you to work around two points of pH above or below the computed isoelectric point, respectively (never above pH 8 nor below pH 6) (See below step 14). Due to its average value for membrane proteins, the anion exchange chromatography at pH 8 always gave me the best results. In particular, I have always used an FPLC combined to the matrix Mono Q™ 10/100 GL with 1 column volume (CV) = to 2 mL, but any AEC matrix should work with minor optimizations.

The process is the following:

1. All the buffers (See 4.1.4) are filtered through a 0.22 μm filter (sample included) and degassed (TIMING) 5 minutes per 100 mL (vacuum combined to sonication bath). || PAUSE POINT make the buffers in advance, you can store the buffers closed without degassing them for 1 week.

2. Exclude the column from the tubing system and purge EtOH 100% into the pumps at maximum speed until the flow coming out of the tubes is stable. Δ CRITICAL STEP there surely are air bubbles into the pumps and you do not want them into the matrix during the purification.

3. Purge MQ water into the pumps to remove the ethanol, then manually invert the column and flow it at low speed (0.1 to 0.5 mL/min). $ CAUTION the matrix is usually stored in EtOH 20% solution and before using any other buffer you must remove the ethanol from it to avoid precipitation of salts.

4. Unscrew the column cap to leave space for the matrix to relax. $ CAUTION if the column is brand new you can skip this step but keep track of the flowrate with MQ water 1 mL/min (40 psi with brand new Mono Q™ 10/100 GL).

5. The FPLC should show a graph with the lines of the percentage of the eluting buffer, the pressure applied to the column and UV absorbance (280 nm) with the conductivity (mSi/cm) of the fluids eluting
out. $CAUTION$ if the pressure is not stable, it might be due to air bubbles into the pumps. $TROUBLESHOOT$ repeat step 2.

6. After 5 CV the lines should be stable, so blank the UV detector. $CAUTION$ with MQ water at 1 mL/min the pressure should be around 40 psi with brand new Mono Q$^\text{TM}$ 10/100 GL. $TROUBLESHOOT$ If the pressure is too high (above 25% more), you can try cleaning it (See steps 16 and 17).

7. Flow 100% of the eluting buffer at 0.5 mL/min for 5 CV (the conductivity should be stable at approximately 100 mSi/cm for NaCl 1 M). $CAUTION$ if the column is brand new, you can skip this step.

8. Invert the column and flow 100% of the binding buffer (See 4.1.4) 0.5 mL/min for 5 CV until the lines are low and stable and blank the detector.

9. Screw the column cap adjusting it onto the matrix. $TROUBLESHOOT$ TIMING steps 2 to 9 takes 30’ with no troubleshooting. $PAUSE POINT$ you can keep flowing the binding buffer at 0.1 mL/min for as long as it does not dry out.

10. Load your sample into the loop and inject it into the column. $CRITICAL STEP$ from this step onwards you can simply program the machine for doing it.

11. Flow the loop 2 to 3 times of its volumes or until the lines are stable (5 CV) at 0.5 mL/min. $CRITICAL STEP$ the first time you purify any protein, collect samples of 1 mL throughout the whole purification.

12. Exclude the loop from the tubing system and apply a step with 15% of the eluting buffer (See 4.1.4) for 5 CV at 0.75 mL/min. $CAUTION$ this step should never be closed than 10% to the attended elution point of your protein (See step 14).

13. Screw the column cap adjusting it onto the matrix.

14. Apply a gradient of eluting buffer from 15% to 50% in 10 to 20 CV at 0.75 mL/min. $CAUTION$ you can have a rough esteem on the elution point of your protein by subtracting its isoelectric point to the working pH and multiply that by 10: that is the percentage of eluting buffer required to elute your protein from the matrix. 2 EXAMPLE protein isoelectric point = 5 $\rightarrow$ working pH = 8 $\rightarrow$ the protein will peak at around 30% of eluting buffer (300 mM NaCl). $TIMING$ steps 10 to 14 take 1h.

15. Run an SDS-PAGE with a sample from all the main peaks in boiled and unboiled conformation to check their “fingerprints” (See Figure 4). $CAUTION$ once you confirmed the presence of your protein via the SDS-PAGE, you can optimize the buffers pH and/or the programming of the machine.

16. Manually, invert the column and clean it with 2 CV of NaCl 2 M, 2 CV of NaOH 1 M and then again with 2 CV of NaCl 2 M at 0.5 mL/min. $CAUTION$ unscrew the column cap to leave space for the matrix to relax. $TIMING$ this cleaning step takes 30’
17. Clean the salts with MQ water at 0.5 mL/min. \( \| \) **PAUSE POINT** you can keep flowing MQ water at 0.1 mL/min for as long as it does not dry out. $ **CAUTION** with MQ water at 1 mL/min the pressure should be around 40 psi with brand new Mono Q™ 10/100 GL. \( \| \) **TROUBLESHOOT** if the pressure is still not in range, you can try to flow it with 2 CV of 100% isopropanol at 0.1 mL/min. \( \Delta \) **TIMING** isopropanol is very viscous so it has to flow slowly to avoid packing the resin too much, therefore this step takes 40’. \( \Delta \) **CRITICAL STEP** every 3 to 5 purification, give your column a deep cleaning storing it in pepsin solution (See 4.1.4) for ( **TIMING**) 1h at 37°C, or for one O.N. at RT. Then repeat step 16.

18. Manually, invert the column and flow EtOH 20% at 0.5 mL/min for 5 CV until the lines are stable → store the column. **TIMING** 5’ otherwise \( \| \) **PAUSE POINT** you can keep flowing EtOH 20% at 0.1 mL/min for as long as it does not dry out.

### 4.6 Isolation of porin-enriched outer membrane vesicles

The fusion of outer membrane vesicles (OMVs) into a planar bilayer allows the characterization of porins reconstituted into their native environment (23). When you need to isolate your protein-enriched OMVs, there are a few important details to assure. First of all you must check that your protein is translated together with a signal peptide compatible with the molecular machine of *E. coli*. That signal peptide must address the protein towards the periplasm, therefore the BAM complex will fold it into the outer membrane (22). My strategy is using the natural signal peptide present in Gram-negative Omps, but cloning the one of OmpF in front of your ORF will do the job (10). Second, the expression of the protein has to be induced for 24h to stimulate the production of the OMVs. I recommend not any longer in order to avoid the contamination of large outer membrane shreds, they will contain anyway Omps reconstituted in their natural environment, but they will not be OMVs. Third, the strain must lack of OmpA, which is involved in the structural stability of the outer membrane, therefore it is already characterized by an overproduction of OMVs. That will grant a higher yield of product.

1. Centrifugue your bacterial culture at 10'000xg for 15’ at 4°C. $ **CAUTION** the slightly longer centrifugation time will make the filtering process easier and faster.

2. Vacuum filter the medium of your culture at 0.45 μm at first and then at 0.22 μm. $ **CAUTION** the filter will block easily so you might need to change it frequently. Do not store the medium, use it freshly made.

3. Ultracentrifugue the filtered medium at 40’000xg for 2h at 4°C. \( \| \) **CRITICAL STEP** use transparent bottles and you should see a very small transparent pellet at the bottom, mark every single of them before removing the supernatant. \( \| \) **TROUBLESHOOT** if the pellet is white and clearly visible, resuspend them all in one bottle and repeat the 0.22 μm filtration (See step 2). If you do not see the pellet, pipet where it should be for 20 times at least.
4. Keep the bottles open upside down and try to wipe out as much medium as possible with Kimtech wipes™.

5. Resuspend each pellet with 1:500 of the volume that was centrifuged in resuspension buffer (See 4.1.5). 2 EXAMPLE if the bottles contained 65 mL, use ($ CAUTION) gently the 200 μL pipette to resuspend the pellet with 130 μL to resuspension buffer trying to avoid air bubbles. Your sample will have a concentration proportional to its dilution factor (500x).

6. Pool together all the resuspensions into a single tube and filter it with a 0.22 μm syringe filter along with some air to push all the solution. $ CAUTION spread part of the sample with a loop onto a LBL agar plate to test its sterility. The sample has to appear turbid before and after the filtration. ‡ TROUBLESHOOT if you see colonies the following day, repeat the last filtration. If the solution is not turbid, do not dilute it for the SDS-PAGE (See step 7). || PAUSE POINT store the OMVs at 4°C for short periods of time or -20°C for long ones.

7. Analyze a sample from the OMVs to test the presence of your protein with the SDS-PAGE. $ CAUTION dilute it 1:10 in RB 1x and at LD 4x to run it with the NI and I sample in boiled and unboiled form. You will find in there your protein with some background. ‡ TROUBLESHOOT if the SDS-PAGE shows the band into the induced sample but your protein does not appear reconstituted into the OMVs, try to use less inducer, decrease the expression temperature or clone the signal peptide of OmpF in front of the ORF of your protein (10). If the background is missing also, try not to dilute the sample or you might not have any OMVs in there.

Determining the concentration of OMVs is not quite trivial. You cannot use neither turbidimetry nor UV<sub>280</sub> because of the heterogeneous distribution of both their size and protein content. Therefore I choose a pragmatic solution: I set the expression conditions and dilution factor as constant parameters. One μL of the final sample added on top of the planar lipid bilayer, will give to a trained electrophysiologist a reasonable number of fusion events of one or more OMVs (23). This might change according to different preparations, but overall the results are consistent and reproducible. ‡ TROUBLESHOOT If the OMVs don’t fuse you can try to add more volume or incubate them with an equal volume of liposomes in the same buffer with 10 mM MgCl<sub>2</sub> for one O.N. at 4°C.

**Troubleshooting**

**Time Taken**

**Anticipated Results**

5 Concluding remarks
Now you know how to produce membrane proteins in good amount and purity. This is a solid base to start isolating Omps, and every step can be adjusted accordingly. This protocol is definitely longer than that used for OmpF and similar proteins like OmpC, but they are exceptions. The procedure is easier and less empirical than the ones of most of the other membrane proteins and doable for soluble proteins also (like enzyme or pore forming toxins). Expression time and temperature, concentration of the inducer, nature and concentration of the detergent, incubation time, purification pH, these are all the parameters that you have learned how to troubleshoot according to your results with the step-by-step approach I suggested throughout the manuscript. The second time you will perform the isolation, you will go directly to the solution saving time and energy: the expression, extraction and purification will not last longer than a full day each.

References

9 Bibliography


16. Piselli C, Golla VK, Benz R, Kleinekathöfer U. Importance of the lysine cluster in the translocation of anions through the pyrophosphate specific channel OprO. Biochim Biophys Acta Biomembr. 2022 Nov 9;184086.


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Figures
Figure 1

Figure 1 - SDS-PAGE with two examples of protein expression (left) and one expression test (right). On the left, OprO and OprP from Pseudomonas aeruginosa having a MW of approximately 46 KDa per monomer, but in the gel electrophoresis, they run as 52 KDa. On the right, the OmpE36 from Enterobacter cloacae has a MW of approximately 37 KDa per monomer, but in the gel electrophoresis, it runs as 32 KDa protein. The OprO/P are induced at OD600 = 1 with 1 mM IPTG for 4h while the OmpE36 is shown induced at OD600 = 0.6 with 0.02% Ara are sampled every hour and after one O.N (See Table 1).
Figure 2

Figure 2 – SDS-PAGE with the extraction of OprP from P. aeruginosa. The non-induced/induced samples (on the left) assure the expression of the protein together and clearly address what is the corresponding band into the gel. The Ext1, Subc and S1 contain 4 μL of the sample diluted in LD 4x, it is important to normalize the samples for the two supernatants at least. Their pattern indicates that OprP is in the second pellet (after the ultracentrifugation).
Figure 3 - OmpF Extraction from E. coli. From Left to Right the lanes are the non-induced culture (NI), the induced one after 5h, 4 µL of the total extraction (Ext1), 4 µL of the supernatant of the first ultracentrifugation (S1), 10 µL of the resuspension of the pellet with 0.15% octyl-POE (R1) and 10 µL of the supernatant of its ultracentrifugation (S2), 10 µL of the second resuspension in 3% octyl-POE (R2) and 10 µL of the supernatant of its ultracentrifugation boiled (S3) and unboiled (S3ub). The band in S3ub shows the protein in its native conformation (trimer in this case) that is linked to the functionality of the protein.
Figure 4

Figure 4 – SDS-PAGE with the purification fractions of the MspA homolog from Rhodococcus corynebacteroides. The protein has a MW of around 20 KDa but in its native conformation it is folded as an octamer. In fact, in the unboiled wells, it shows a MW of around 180 KDa.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- UniversalPaperTables.docx
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