

Cell-Free Membrane Protein Expression for Solid-State NMR

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Abstract

Although cell-free expression is a relative newcomer to the biochemical toolbox, it has already been reviewed extensively, even in the more specialized cases such as membrane protein expression, nanolipo-protein particles, and applications to crystallography and nuclear magnetic resonance (NMR). Solid-state NMR is also a newcomer to the structural biology toolbox, with its own specificities in terms of sample preparation. Cell-free expression and solid-state NMR are a promising combination that has already proven useful for the structural study of membrane proteins in their native environment, the hydrated lipid bilayer. We describe below several protocols for preparing MscL, a mechanosensitive membrane channel, using cell-free expression destined for a solid-state NMR study. These protocols are flexible and can easily be applied to other membrane proteins, with minor adjustments.

Key words: In vitro synthesis, Integral membrane proteins, Membrane protein reconstitution, Liposomes, Solid-state NMR

1. Introduction

Cell-free expression is one of the major new developments in structural biology, both for nuclear magnetic resonance (NMR) and X-ray crystallography, because it allows for overproduction of proteins, both wild-type and mutant, that are often produced with prohibitively low yields using classical biosynthetic methods. Proteins that are expressed cell-free end up in the reaction container and hence in water where membrane proteins precipitate, often irreversibly. Cell-free expression was first thought to be impractical for membrane protein expression because the system did not include a biological apparatus for targeting the protein to the membrane (1). Providing the medium with detergent was also considered risky since detergent could perturb protein expression by interfering with the ribosome, polymerase or any other essential

component of the cell-free expression system. These obstacles were overcome in 2004, when several groups, using optimized cell lysates, developed protocols for membrane protein expression *in vitro*, in the presence of detergents (2–4).

For X-ray crystallography and solution-state NMR, the major techniques used in structural biology today, membrane protein structure determination is still a challenge: first, because the aforementioned necessity of obtaining large quantity of functional proteins is aggravated, *in vivo*, by the limited membrane surface available (5) and second, because membrane proteins are hydrophobic and have to be manipulated in detergents at all times when they are extracted from their native environment, the lipid bilayer (6). Detergents or other surfactants are used for membrane solubilization and during protein purification (which often requires an affinity tag, such as a polyhistidine stretch). They can interfere with protein crystallization or make aggregates that are too large for solution-state NMR. Last but not the least, detergents can interfere with protein folding or function, and the protein structure or dynamics determined in a detergent environment is not necessarily representative of the native structure or dynamics (7, 8). Nevertheless, since 1985, over 200 membrane protein structures have been determined by using X-ray crystallography (9) and, since 1997, about 30 structures by using solution-state NMR (10). In this context, membrane protein cell-free expression has been developed and reviewed, especially for isotope labeling strategies, another issue for NMR, where *in vitro* synthesis offers a very efficient and versatile alternative that greatly minimizes amino acid scrambling (11, 12).

Solid-state NMR is an alternative for membrane protein structure determination *inside* the hydrated lipid bilayer, where the protein is correctly folded and stable for a long time. Sample preparation for solid-state NMR is therefore different than for solution-state NMR. If the protein is expressed cell-free in the presence of detergent, it needs to be purified, renatured and reconstituted in a membrane bilayer. Compared to a protein expressed in a cell, the solubilization step is avoided and the purification step is greatly simplified and accelerated, reducing the chances of spurious proteolysis. Reconstitution of the protein in a lipid membrane is the price to pay to obtain a sample where the membrane protein is almost certainly in its native state, and where the protein function can be checked and monitored (13, 14). All these steps are quite flexible, and they are described below for the expression of the mechanosensitive channel MscL. This protocol is general for membrane proteins, although details such as the nature or the concentration of detergent can vary from one protein to the next.

Cell-free expression in presence of liposomes or nanodiscs is an alternative sample preparation approach that has been developed more recently and that has yet to be proven general for membrane

proteins (15–17). When possible, it presents many advantages for solid-state NMR studies. First and foremost, the protein is already in its final state, saving time and avoiding many biochemical steps where the protein may be partially lost or inactivated. Second, the protein is purified simply by centrifugation, alleviating the necessity of adding an affinity tag. In addition, no detergent is used that can interfere and that needs to be removed. Importantly, the protein is in its native environment at all times, where it is well folded, functional and stable (18). Finally, this approach is quite flexible, allowing for a variety of labeling strategies (19). In our hands, cell-free expression of MscL in the presence of liposomes has proven efficient (3, 14, 18, 19) and we describe it below, as it is the best method so far for providing a solid-state NMR sample.

The protocols presented here make use of commercial kits for cell-free expression, which we found advantageous for its convenience, reliability, for saving the time and manpower to make the lysate, and also for managing the stocks. Cell lysates can also be prepared, following published protocols from various cell types such as bacteria, wheat germ, or others (11, 20). We describe below an optimized procedure for expressing the membrane protein MscL, using the commercial Roche/5Prime continuous exchange vessel and kits, with an *Escherichia coli* extract. Since, for each sample preparation protocol, it is necessary to check for protein integrity, we also describe several tests that should be performed to assess the quality of the sample.

2. Materials

2.1. Cell-Free Expression of the Mechanosensitive Channel MscL in Detergent Micelles

All solutions are prepared with autoclaved nuclease-free Milli-Q water (see Note 1).

1. Thermoregulated shaker for the cell-free reaction vessel.
2. Nuclease-free 50-mL tubes.
3. Nuclease-free 1.5-mL tubes.
4. Nuclease-free glass pipettes.
5. Nuclease-free pipette tips (0–10, 10–200, 200–1,000 μL , and 1–5 mL), autoclaved at 121°C for 20 min.
6. pIVEX-2.3-mscL plasmid (3). Encodes the *E. coli* MscL C-terminally fused to a His₆ tag under the control of the T7 promoter. Dissolve in pure nuclease-free water and store aliquoted at –20°C, at 0.5 $\mu\text{g}/\mu\text{L}$.
7. RTS 9000 cell-free expression kit (Roche/5Prime). Contains lyophilized *E. coli* lysate, reaction mix, feeding mix, reconstitution buffer, continuous exchange reaction vessel, and a syringe (see Fig. 1, Notes 2–5). Store at –80°C.

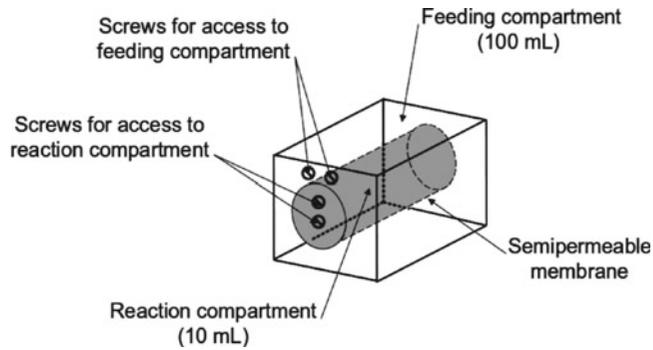


Fig. 1. Roche/5Prime RTS 9000 reaction vessel. The reaction compartment (10 mL) and the feeding compartment (100 mL) are each accessible through two screws. The reaction compartment contains the cell lysate, plasmid DNA, detergent or preformed liposomes and is where the coupled transcription/translation reaction takes place. The separate feeding compartment provides additional ions, energy substrates, nucleotides and amino acids, through a semipermeable membrane (MW cutoff 10 kDa). Simultaneously, by-products that may inhibit the reaction are diluted through the same membrane into the feeding compartment. This continuous exchange allows cell-free expression to last for up to 24 h.

8. Unlabeled amino acids (powder), store at -20°C .
9. $^{13}\text{C}/^{15}\text{N}$ labeled amino acids (powder), store at -20°C .
10. Dithiothreitol (DTT): Prepare 40 mM stock, store at -20°C .
11. 20% (w/v) Triton X-100: Prepare 200 mL, stir until homogeneous, store at 4°C for up to 6 months.
12. Unlabeled amino acid solutions: Prepare 168 mM solutions of each amino acid except for leucine (140 mM) and the labeled amino acids, Ile and Thr, with the appropriate solution (see Note 6). The amount of amino acid to be incorporated is calculated in Subheading 3.1 and is indicated in Table 1. Transfer the appropriate volume of each unlabeled amino acid to a 1.5-mL vial, and sonicate until the solution looks clear. Transfer the individual solutions to a 50-mL tube. Add Tyr and Leu last to avoid precipitation; prepare fresh before using and keep on ice.
13. Labeled amino acid solutions, Ile and Thr: Dissolve 23 mg of Ile and 3.9 mg of Thr in 1.0 and 0.20 mL of reconstitution buffer, respectively (~ 168 mM each), into 1.5-mL tubes; prepare fresh before using and keep on ice (see Subheading 3.1, Table 1 and Note 7).
14. FPLC purification system.
15. 1 M NaOH: To adjust pH, store at room temperature.
16. 100 mM NiSO_4 : Store at room temperature.
17. 20% Ethanol: Store at room temperature for up to a month.
18. 4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF): 100 mM stock solution in water, store at 4°C for up to 6 months.

Table 1
Preparation of amino acid solutions for MscL cell-free expression in a volume $V=110$ mL (see Subheading 3.1 and Fig. 1)

Amino acid	MWi (g/mol)	n	Amount (mg)	Volume (mL)
Ala (A)	89	15	15	0.98
Arg (R)	174	6	11	0.39
Asn (N)	132	6	8.7	0.39
Asp (D)	133	7	10	0.46
Cys (C)	121	0	0	0.10
Gln (Q)	146	4	6.4	0.26
Glu (E)	147	8	13	0.52
Gly (G)	75	13	11	0.85
His (H)	155	7	12	0.46
Ile (I)	131	16	23	1.0
Leu (L)	131	13	19	1.0
Lys (K)	146	9	14	0.59
Met (M)	149	5	8.2	0.33
Phe (F)	165	10	18	0.65
Pro (P)	115	7	8.9	0.46
Ser (S)	105	4	4.6	0.26
Thr (T)	119	3	3.9	0.20
Trp (W)	204	0	0	0.10
Tyr (Y)	181	1	2.0	0.10
Val (V)	117	13	17	0.85

n is the number of each amino acid type, of molecular weight MWi, in the protein sequence. The weight of each amino acid is ($n \times \text{MWi} \times 1.1 \times 10^{-2}$), expressed in mg. Since each amino acid is solubilized at 168 mM, except for leucine, at 140 mM, the corresponding volume of each amino acid solution is ($n \times 11$)/168, except for leucine where it is ($n \times 11$)/140, expressed in mL

19. Chelating column (5 mL): Stored in 20% ethanol at 4°C.
20. FPLC buffer A1: 50 mM NaH_2PO_4 -NaOH (7.1 g/L), pH 8, 300 mM NaCl (17.5 g/L), 10 mM imidazole (680 mg/L), 4.0% Triton X-100 (40 g/L), store at 4°C.
21. FPLC buffer A2: 50 mM NaH_2PO_4 -NaOH (7.1 g/L), pH 8, 300 mM NaCl (17.5 g/L), 10 mM imidazole (680 mg/L), 0.2% Triton X-100 (2 g/L), store at 4°C.

22. FPLC buffer B: 50 mM NaH_2PO_4 -NaOH (7.1 g/L), pH 8, 300 mM NaCl (17.5 g/L), 500 mM imidazole (34 g/L), 0.2% Triton X-100 (2 g/L), store at 4°C.
23. FPLC sample loading buffer: 50 mM NaH_2PO_4 -NaOH (7.1 g/L), pH 8, 300 mM NaCl (17.5 g/L), 10 mM imidazole (680 mg/L), 1% Triton X-100 (10 g/L), store at 4°C.
24. 10× Dialysis buffer: 0.1 M HEPES-KOH, pH 7.5, 1 M KCl, and 2% (*w/v*) Triton X-100, store at 4°C for up to 6 months.
25. 1× Dialysis buffer: Prepare 2 L by diluting 200 mL of 10× buffer in 1,750 mL of water, adjusting the pH to 7.5 using 4 M KOH, and adding water to 2 L final volume. Store at 4°C for up to 6 months.
26. 2 dialysis cassettes with a 10 kDa MW cutoff.
27. Methanol.
28. DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine powder, store at -20°C.
29. 4 M KOH: To adjust pH, store at room temperature.
30. 0.8% Triton X-100: Prepare 10 mL, dissolve and stir until homogeneous, store at 4°C for up to 6 months.
31. Wet polystyrene beads: Wash 5 g of 300–1,200 μm polystyrene beads with 25 mL of pure methanol and then wash four times with 25 mL of Milli-Q water. The beads are kept in water at 4°C and are stable for months, provided a weekly renewal of Milli-Q water.
32. HEPES-KCl buffer: 10 mM HEPES-KOH, pH 7.5, 100 mM KCl, store at 4°C for up to 6 months.
33. HEPES solutions for solubilizing Tyr, Trp, and Phe (Subheading 2.1, see Notes 6 and 7): 60 mM HEPES, pH 13 for Tyr, pH 1 for Trp, and pH 7.5 for Phe. Adjust the pH using KOH or HCl. Keep on ice.

**2.2. Cell-Free
Expression of the
Mechanosensitive
Channel MscL in
Liposomes**

All solutions are prepared with autoclaved nuclease-free Milli-Q water (see Note 1).

1. Thermoregulated shaker for the cell-free reaction vessel.
2. Mini-Extruder with two 1-mL gastight microsyringes and 0.1-μm cutoff polycarbonate membranes.
3. Nuclease-free 50-mL tubes.
4. Nuclease-free 1.5-mL tubes.
5. Nuclease-free glass pipettes.
6. Nuclease-free pipette tips (0–10, 10–200, 200–1000 μL and 1–5 mL). Autoclave at 121°C for 20 min.

7. pIVEX-2.3-mscL plasmid: For protein expression in liposomes, an affinity tag such as a polyhistidine stretch is preferable but not mandatory.
8. RTS 9000 cell-free expression kit (Roche/5Prime). Contains lyophilized *E. coli* lysate, reaction mix, feeding mix, reconstitution buffer, a continuous exchange reaction vessel, and a syringe (see Fig. 1, Notes 2–5), store at -80°C .
9. Unlabeled amino acids (powder), store at -20°C .
10. $^{13}\text{C}/^{15}\text{N}$ labeled amino acids (powder), store at -20°C .
11. DTT (dithiothreitol): Prepare 40 mM stock, store at -20°C .
12. 4 M KOH: To adjust pH, store at room temperature.
13. AEBSF (Subheading 2.1).
14. DOPC.
15. Chloroform.
16. HEPES–KCl buffer (Subheading 2.1).
17. DOPC liposome preparation: Prepare a chloroform solution of 10 mg/mL of DOPC (100 mg of DOPC in 10 mL of dry chloroform). Dry the lipids and then resuspend with RTS reconstitution buffer to obtain a 20 mg/mL aqueous solution. Sonicate 2.5 mL of the solution for 5 min, and then extrude it 13 times with a 0.1- μm filter, to obtain a liposome solution containing 40–50 mg of lipids, and store at 4°C . This is the amount of lipids that is expected in the final sample. DOPC can be replaced by other lipids or lipid mixtures, at a higher or lower concentration.
18. HEPES solutions for solubilizing Tyr, Trp, and Phe (Subheading 2.1).
19. Unlabeled amino acid solutions: Prepare 168 mM solutions of each amino acid except for leucine (140 mM) and the labeled amino acids (item 20), with the appropriate solution (see Note 6). The amount of amino acid to be incorporated is calculated in Subheading 3.1 and is indicated in Table 1. The appropriate volume of each unlabeled amino acid is transferred to a 1.5-mL vial, and sonicated until the solution looks clear. The vials are transferred into a 50-mL tube. Tyr and Leu are added last, to avoid precipitation; prepare fresh before using and keep on ice.
20. Labeled amino acid solutions, Arg, Ile, Pro, Met, and Phe: Weigh 11 mg of Arg, 23 mg of Ile, and 8.9 mg of Pro and dissolve in 0.39, 1.0, and 0.46 mL of reconstitution buffer, respectively (~ 168 mM). Weigh 8.2 mg of Met and dissolve in 0.33 mL of reconstitution buffer plus 50 μL of 40 mM DTT. Weigh 18 mg of Phe and dissolve in 0.65 mL of the appropriate HEPES solution (Subheading 2.1), and sonicate for 1 min. Prepare each amino acid solution fresh before using and keep on ice (see Subheading 3.1, Table 1 and Note 7).

2.3. Sample Characterization

All solutions described should be prepared using Milli-Q water and stored at room temperature unless stated otherwise.

1. FA diluted solution: 37% formaldehyde in water, store at 4°C.
2. Dimethyl sulfoxide (DMSO).
3. 1 M NaOH: To adjust pH.
4. 1 M HCl: To adjust pH.
5. 1 M Tris base, pH 8.
6. SDS running buffer: 100 mM Tris base, 100 mM HEPES, 0.1% SDS, adjust to pH 8 using NaOH or HCl.
7. Staining buffer: 0.5% Coomassie Brilliant Blue R-250 dye, 50% ethanol and 10% acetic acid.
8. Destaining buffer: 20% ethanol and 10% acetic acid.
9. 2× SDS loading buffer: 0.2 M Tris base, pH 8, 8% SDS, 40% glycerol, 0.4% bromophenol blue, 0.4 M DTT, adjust pH 8 using 1 M NaOH or 1 M HCl.
10. 1× SDS loading buffer: dilute 1 mL of 2× SDS loading buffer with 1 mL of water.
11. DSS solution: 50 mM disuccinimidylsuberate in DMSO.
12. HEPES–KCl buffer (see Subheading 2.1).
13. Polyvinylidene fluoride (PVDF) membranes.
14. TBS-Tween buffer 1: 50 mM Tris base, pH 7.4, 150 mM NaCl, 0.05% Tween-20.
15. Monoclonal anti-histidineperoxidase-conjugate antibody solution (for the dot blot): Dilute 2,000× from the stock solution with TBS-Tween buffer 1, prepare fresh.
16. Diaminobenzidine solution: Dissolve one tablet of diaminobenzidine and one tablet of urea hydrogen peroxide in 5 mL of water, prepare fresh.
17. Western-blotting detection kit.
18. Nonfat dry milk (powder).
19. Chemiluminescence reagents (H₂O₂ and luminol), store at 4°C.
20. Towbin buffer: 25 mM Tris base, 192 mM glycine, 20% isopropanol, and 0.1% SDS. Store without isopropanol at 4°C, add isopropanol just before use.
21. Ponceau S solution: 0.1% Ponceau S, 5% acetic acid.
22. TBS-Tween buffer 2: TBS-Tween buffer 1 with 5% (*w/v*) nonfat dry milk, freshly made.
23. Mouse anti-histidine antibody solution (for the Western-blot): Dilute 5,000× from the stock solution with TBS-Tween buffer 1, prepare fresh.
24. Anti-mouseperoxidase-coupled antibody solution (for the Western-blot): Dilute 10,000× from the stock solution with TBS-tween buffer 1, prepare fresh.

25. Micro BCA Protein Assay kit (Pierce).
26. Nuclease-free 1.5-mL tubes.
27. Bovine serum albumine (BSA): 1 mg/mL stock solution.
28. Solubilization buffer: 0.2% Triton X-100, solubilized in HEPES–KCl buffer, store at 4°C for up to 6 months.
29. Acetone, stored at –20°C.
30. Nitric acid solution: 1%, store at room temperature in a safety cabinet. Handle under the hood.
31. Perchloric acid solution: 70%, store at room temperature in a safety cabinet. Handle under the hood.
32. Ammonium molybdate solution: Dissolve 2.5 g in 100 mL of water, store at 4°C.
33. Ascorbic acid solution: Dissolve 10 g in 100 mL of water, prepare fresh, and do not keep for more than 1 week at 4°C.
34. Phosphate stock solution: Dissolve 10 mg of KH_2PO_4 in 100 mL of water and store at 4°C.
35. Nuclease-free 3-mL ultracentrifugation tubes.
36. Sucrose.
37. Sucrose solutions: 10, 20, and 45% (*w/v*) sucrose, solubilized in HEPES–KCl buffer, store at 4°C.
38. Nitrocellulose membrane (for Western blotting).
39. X-ray film (to develop the proteoliposomes must be mixed with sucrose Western blot).
40. Film developer.

2.4. NMR Sample Preparation

Four millimeter diameter rotors for high-resolution magic-angle spinning solid-state NMR (see Fig. 2).

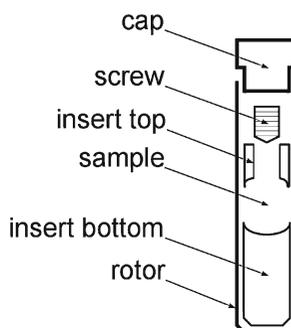


Fig. 2. Four millimeter diameter rotors for high-resolution magic-angle spinning solid-state NMR. The sample (approximately 50 μL) is contained in the *center* of the zirconium rotor, thanks to the *Teflon insert bottom* and *top*, and is kept tightly in place by the *top screw* and the *Kel-F cap*.

3. Methods

The methods outlined below describe the following: (1) calculation of the amount of incorporated amino acids, (2) cell-free expression of the mechanosensitive channel MscL in detergent micelles, purification of protein/detergent micelles, protein reconstitution into liposomes, (3) cell-free expression directly into liposomes, (4) sample characterization, and (5) NMR sample preparation.

3.1. Cell-Free Expression of the Mechanosensitive Channel MscL in Detergent Micelles

The method below describes the production of an MscL sample for solid-state NMR, using a cell-free protein expression system in detergent micelles. Although any labeling scheme could be performed, the sample described below was labeled on isoleucines and threonines, using $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids, and was subsequently used in solid-state NMR experiments (14).

3.1.1. Day 1: Cell-Free Protein Expression in Detergent Micelles

1. Calculate the amount of amino acids to be incorporated during protein expression. The concentration of each amino acid in the protein cell-free expression system ($V = 110$ mL, see Fig. 1) is usually ~ 1 mM. For a given protein, each amino acid concentration depends on its occurrence, n , in the protein sequence. In our case, we have obtained good results with a final concentration of each amino acid equal to $n/10$, expressed in mM. The corresponding weight of each amino acid, of molecular weight MW_i , is therefore $(n \times V \times MW_i / 10)$, expressed in mg. The corresponding volume of each amino acid solution of concentration C_i , where C_i is 168 or 140 μM (Subheading 2.1), is $(n \times V / 10) / C_i$, expressed in mL. The weights and final volumes calculated for this protocol are summarized in Table 1.
2. Thaw the DTT, RTS reconstitution buffer, and MscL plasmid at room temperature.
3. Thaw the other components of the RTS kit (*E. coli* lysate, reaction mix, and feeding mix) on ice.
4. Prepare the unlabeled amino acid solutions (see Subheading 2.1).
5. Prepare the labeled amino acid solutions (see Subheading 2.1).
6. Add the labeled amino acid solution to the unlabeled amino acid solution.
7. Add 3 mL of 40 mM DTT, sonicate (80 W for 5 min or until clear), and store on ice.
8. Reconstitute the lyophilized *E. coli* lysate in 5.2 mL of reconstitution buffer. Shake the bottle gently (see Note 8).
9. Reconstitute the lyophilized reaction mix in 2.2 mL of reconstitution buffer. Shake the bottle gently (see Note 8).

10. Reconstitute the lyophilized feeding mix in 80 mL of reconstitution buffer. Shake the bottle gently (see Note 8).
11. Prepare the feeding solution by adding 26 mL of the reconstituted amino acid solutions (mixture of unlabeled and labeled amino acid solutions) and 3 mL of 40 mM DTT to the feeding mix (80 mL).
12. Prepare the reaction solution by adding the reconstituted reaction mix (2.2 mL), 2.7 mL of the reconstituted amino acid solution and 0.3 mL of 40 mM DTT to the reconstituted *E. coli* lysate (5.2 mL). Remove a 20 μ L aliquot of this solution for later comparison on gel electrophoresis.
13. Add 300 μ L of the MscL plasmid (\sim 150 μ g) to the reaction solution.
14. Add 200 μ L of 20% Triton X-100 solution to the reaction solution.
15. Add 2.2 mL of 20% Triton X-100 solution to the feeding solution.
16. Open both screws of the reaction compartment and fill the reaction compartment with the reaction solution using a nuclease-free pipette (see Fig. 1). Remove air bubbles by tapping the vessel lightly.
17. Open both screws of the feeding compartment and fill the feeding compartment with the feeding solution, using the provided syringe (see Fig. 1). Remove air bubbles by tapping the vessel lightly.
18. Insert the reaction vessel into a thermoregulated shaker.
19. Set the shaking speed to 800 rpm.
20. Set the temperature to 30°C.
21. Incubate the reaction for 22 h.

3.1.2. Day 2: Purification of Detergent/Protein Micelles

1. After 22 h, stop the cell-free protein expression. Remove a 20 μ L aliquot of the reaction run-on solution for gel electrophoresis.
2. Remove the reaction run-on mix using a pipette, add 100 μ L of the AEBSF solution to prevent protease digestion, and stir at room temperature for 15 min (see Note 9).
3. Dilute the sample to a final volume of 50 mL using FPLC buffer A1 and centrifuge at 10,000 $\times g$ for 15 min at 4°C.
4. Connect the chelating column to the FPLC system (see Note 10). Pass 4–5 column volumes (CV) of distilled water through the column, to wash away the ethanol solution in which it has been kept.
5. Degas the FPLC buffers for 10 min in an ultrasonic bath. Wash the pump inlets with the degassed solutions. Wash the system with FPLC buffer A1 until the UV and conductivity baselines are stable.

6. At the end of the sample centrifugation (step 3), dilute the supernatant (reaction run-on mix) with FPLC sample loading buffer to a final volume of 100 mL (final dilution $\sim 10\times$).
7. Filter this solution through a 0.45- μm filter.
8. Resuspend the pellet with 5 mL of FPLC buffer A1 and remove a 20 μL aliquot for gel electrophoresis.
9. Introduce half (50 mL) of the diluted reaction run-on mix into the column (see Note 11). Collect the flow-through in the first 50-mL tube.
10. Wash off the nonspecifically bound material by raising the concentration of imidazole to 100 mM, by mixing 80% of FPLC buffer A1 with 20% of FPLC buffer B (6 CV total). The concentration of detergent remains high (around 3%). Collect the flow-through in the second 50-mL tube.
11. To elute the protein, switch FPLC buffer A1 to buffer A2, to reduce the detergent concentration to 0.2%. Collect the flow-through in the second 50-mL tube.
12. Increase the imidazole concentration to 200 mM by mixing 60% of solution A2 with 40% of solution B (2 CV total), and collect eluant fractions of 2 mL each (see Note 12).
13. Increase the imidazole concentration to 500 mM by switching to 100% FPLC buffer B (6 CV total) and collect eluant fractions of 2 mL each.
14. Wash the column with FPLC buffer A1 (10 CV or until the UV and conductivity baselines are stable), then repeat steps 9–13 with the remaining 50 mL (second half of the diluted reaction run-on mix, see Note 11).
15. At the end of the FPLC protein purification run (see Note 10), the potentially interesting fractions are separated into approximately 40 tubes, whereas the flow-through is contained in two 50-mL tubes. Remove a 20 μL aliquot from each of these 42 tubes, for gel electrophoresis.
16. Determine which fractions contain the histidine-tagged protein by performing a dot blot (see Subheading 3.3.4).
17. Wash the two dialysis cassettes in $1\times$ dialysis buffer.
18. For each half of the solution, pool the fractions containing the protein into a dialysis cassette.
19. Dialyze against 1 L of $1\times$ dialysis buffer, overnight at 4°C .

3.1.3. Day 3: Sample Characterization and Reconstitution into Liposomes

1. Dialyze a second time against 1 L of $1\times$ dialysis buffer for 2 h at 4°C . Remove a 20 μL aliquot for gel electrophoresis.
2. Assess the cell-free expression level, FPLC purification efficiency, and oligomeric state by SDS-PAGE (see Subheadings 3.3.1 and 3.3.3).

3. After SDS-PAGE, wash the gel in water and perform a Western-blot transfer (see Subheading 3.3.5).
4. Estimate the protein concentration. Since MscL does not contain any tryptophan it has to be quantified by using the BCA method (see Subheading 3.3.6 and Note 13).
5. BCA quantification of our MscL preparation indicated that each half of the solution contains approximately 4.8 mg of protein in a 10 mL volume that will be reconstituted in 19.2 mg of DOPC liposomes (protein–lipid ratio of 1:4). DOPC can be replaced by other lipids or lipid mixtures, at a higher or lower concentration (21). Lipids can also be replaced by other mixtures to reconstitute the protein into bicelles or nanodiscs (22, 23).
6. Split the solutions into four fractions of 5 mL, each containing 2.4 mg of protein and 10 mg of Triton X-100. To each fraction, add 9.6 mg of DOPC solubilized in 2.5 mL of the 0.8% Triton X-100 solution, such that the final lipid–detergent ratio is 1:3. Stir slowly for 15 min at room temperature.
7. To each fraction of 7.5 mL add 300 mg of wet polystyrene beads (detergent–bead ratio is 1:10) and put the four tubes on a rotator, to remove the detergent by adsorption onto the beads. Incubate overnight at 4°C. For alternative methods (21, 24), see Note 14.

*3.1.4. Day 4: Preparation
of the Sample for
Solid-State NMR*

1. Add another 300 mg of wet polystyrene beads to each tube. Incubate for another 2 h, at 4°C.
2. After 2 h, set the tubes vertically for 5 min and discard the pellet and the beads.
3. Divide the supernatant into four tubes and centrifuge at $100,000 \times g$ for 30 min at 4°C. Remove a 20 μ L aliquot of one supernatant for gel electrophoresis, discard the rest and resuspend the pellets with 1 mL of HEPES–KCl buffer for each pellet. Split the new resuspended pellets into two tubes and centrifuge at $100,000 \times g$ for 30 min at 4°C. Repeat one more time so that the entire sample fits into a single tube. Remove a final 20 μ L aliquot of the supernatant for gel electrophoresis. Dry the final pellet under argon and store it at -20°C .
4. Assess the reconstitution by SDS-PAGE (see Subheading 3.3.1), and by proteoliposome density characterization on a sucrose gradient (see Subheading 3.3.9). Also, estimate the lipid and water content (see Subheadings 3.3.8 and 3.3.10, and Note 15).
5. The average quantity of protein obtained with the RTS 9000 kit is 10 mg in 10 mL of reaction mix, which is generally sufficient to prepare two or three NMR samples. Transfer ~ 30 mg of pelleted sample to the NMR rotor (see Subheading 3.4).
6. Store the remaining pellet at -20°C .

3.2. Cell-Free Expression of the Mechanosensitive Channel MscL in Liposomes

The method below describes the production of an MscL sample for solid-state NMR, using a cell-free protein expression system directly into lipid vesicles. Although any labeling scheme could be performed, the sample described below was labeled on arginines, isoleucines, methionines, phenylalanines and prolines, using $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids, and was subsequently used in solid-state NMR experiments (19). Lipids used were synthetic DOPC, but other lipids or complex mixtures can also be used, such as asolectin (18) or nanodiscs (17).

3.2.1. Day 1: Cell-Free Protein Expression

1. Thaw the DTT solution, RTS reconstitution buffer and MscL plasmid at room temperature.
2. Thaw the other components of the RTS kit (*E. coli* lysate, reaction mix, and feeding mix) on ice.
3. Prepare the DOPC liposomes (Subheading 2.2).
4. Prepare the HEPES solutions (Subheading 2.1).
5. Prepare the unlabeled amino acid solutions (Subheading 2.1).
6. Prepare the labeled amino acid solutions (Subheading 2.1).
7. Add the labeled amino acid solutions to the unlabeled amino acid solutions.
8. Add 3 mL of 40 mM DTT, sonicate (80 W for 5 min or until clear), and store on ice.
9. Reconstitute the lyophilized *E. coli* lysate in 2.7 mL of reconstitution buffer. Shake the bottle gently (see Note 8).
10. Add the liposome solution (2.5 mL) to the *E. coli* lysate once the latter is completely dissolved.
11. Reconstitute the lyophilized reaction mix in 2.2 mL of reconstitution buffer. Shake the bottle gently (see Note 8).
12. Reconstitute the lyophilized feeding mix in 80 mL of reconstitution buffer. Shake the bottle gently (see Note 8).
13. Prepare the feeding solution by adding 26 mL of the reconstituted amino acid solution (mixture of unlabeled and labeled amino acid solutions) and 3 mL of 40 mM DTT to the feeding mix solution (80 mL).
14. Prepare the reaction solution by adding the reconstituted reaction mix (2.2 mL), 2.7 mL of the reconstituted amino acid solution and 0.3 mL of 40 mM DTT to the reconstituted *E. coli* lysate with the liposomes (5.2 mL). Take a 20 μL aliquot of this reaction solution for later comparison on gel electrophoresis.
15. Add 300 μL of the MscL plasmid ($\sim 150 \mu\text{g}$) to the reaction solution.
16. Open both screws of the reaction compartment and fill the reaction compartment with the reaction solution using a nuclease-free pipette (see Fig. 1). Remove any air bubbles by tapping the vessel lightly.

17. Open both screws of the feeding compartment and fill the feeding compartment with the feeding solution, using the provided syringe (see Fig. 1). Remove any air bubbles by tapping the vessel lightly.
18. Insert the reaction vessel into a thermoregulated shaker.
19. Set the shaking speed to 800 rpm.
20. Set the temperature to 30°C.
21. Incubate the reaction for 22 h.

3.2.2. Day 2: Preparation of the Sample for Solid-State NMR

1. After 22 h, stop the cell-free protein expression. Remove a 20 μL aliquot of the reaction run-on solution for gel electrophoresis.
2. Remove the reaction run-on mix using a pipette, add 100 μL of the AEBSF solution to prevent protease digestion, and stir at room temperature for 15 min (see Note 9).
3. Split the sample into six tubes and centrifuge at $100,000\times g$ for 30 min at 4°C. Remove a 20 μL aliquot of one supernatant for gel electrophoresis, discard the rest and resuspend each pellet with 1 mL of HEPES–KCl buffer. Split the resuspended pellets into three tubes and centrifuge at $100,000\times g$ for 30 min at 4°C. Repeat one more time so that the entire sample fits into a single tube. Remove a final 20 μL aliquot of the supernatant for gel electrophoresis. Dry the final pellet under argon and store it at -20°C .
4. Assess cell-free expression efficiency and oligomeric state by SDS-PAGE (see Subheadings 3.3.2 and 3.3.3). At this point, the expressed protein is generally almost pure.
5. If the protein was expressed with a polyhistidine tag, after SDS-PAGE, wash the gel in water and perform a Western-blot transfer (see Subheading 3.3.5).
6. Estimate the protein and lipid concentration using the BCA and Rouser methods respectively (see Subheadings 3.3.7 and 3.3.8, and Note 13).
7. Characterize the proteoliposome density on a sucrose gradient (see Subheading 3.3.9), and estimate the water content (see Subheading 3.3.10 and Note 15).
8. The average quantity of protein obtained with the RTS 9000 kit is 10 mg in 10 mL of reaction mix, which is generally sufficient to prepare two or three NMR samples. About 30 mg of pelleted sample is transferred into the NMR rotor (see Subheading 3.4).
9. Store the remaining pellet at -20°C .

3.3. Sample Characterization

3.3.1. Electrophoresis of Protein Expressed in Detergent Micelles

1. Perform SDS-PAGE on the following samples:
 - (a) Aliquots from the reaction mix before and after the cell-free expression reaction, to check for expression of the target protein.
 - (b) Aliquots from the centrifugation pellet and supernatant, to check whether the protein was totally solubilized in detergent micelles or if there are some aggregates.
 - (c) Aliquots from the purification flow-through and the purified fractions.
 - (d) Analyze the incorporation of the protein by comparing aliquots of the protein-detergent micelles with the proteoliposomes, after the reconstitution step.
 - (e) Aliquots of the supernatant of all the wash steps.
 - (f) Also check the integrity of the purified protein after dialysis.
2. Dilute all SDS PAGE samples 1:1 with 2× SDS loading buffer.
3. Heat the samples to 90°C for 5 min.
4. Cool the samples and load on a gel.
5. Run the gel using SDS running buffer.
6. Wash the gel in water and stain/destain the gel using staining/destaining buffers, respectively.

3.3.2. Electrophoresis of Protein Expressed in Liposomes

1. Perform SDS-PAGE on the following samples:
 - (a) Aliquots from the reaction mix before and after the cell-free expression reaction, to check for expression of the target protein.
 - (b) Also check the centrifugation supernatants.
2. Follow steps 2–6 in Subheading 3.3.1.

3.3.3. Electrophoresis for Oligomeric State Characterization

If the protein is an oligomer (like MscL), assess its integrity by cross-linking the protein with different free primary amine targeting reagents, like formaldehyde (FA) or disuccinimidylsuberate (DSS) (3, 25). NB: the protein buffer must not contain primary amines (such as in Tris).

1. Mix 5 µg of purified MscL, either in detergent or liposomes, in 10 µL of HEPES–KCl buffer and 0.54 µL of the FA diluted solution.
2. Mix 5 µg of purified MscL, either in detergent or liposomes, in 10 µL of HEPES–KCl buffer and 0.2 µL of 50 mM DSS.
3. Incubate both samples, without shaking, for 30 min at room temperature.

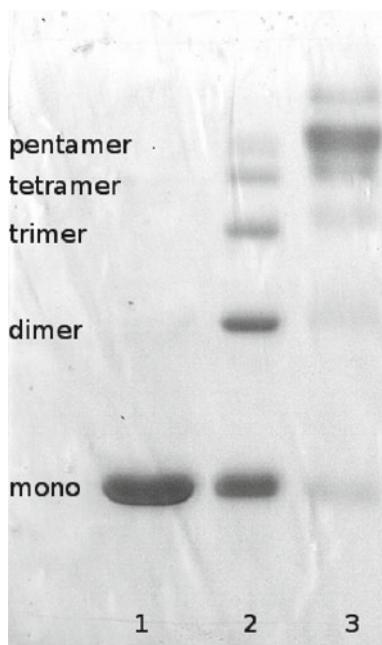


Fig. 3. Coomassie blue stained SDS-PAGE of MscL: (1) purified in detergent, (2) cross-linked with formaldehyde, (3) cross-linked with disuccinimidylsuberate. Formaldehyde generates five protein bands on the gel, corresponding to various oligomeric forms, from monomers to pentamers, while disuccinimidylsuberate generates mostly pentamers, confirming the pentameric nature of *Escherichia coli* MscL (3, 25).

4. Stop the cross-linking reactions by adding 2 μL of 1 M Tris base, pH 8, to each sample. Gently mix and incubate for 10 min at room temperature.
5. Add 3 μL of 2 \times SDS loading buffer to each sample and mix.
6. Heat the FA sample to 60°C and the DSS sample to 90°C, for 5 min in a water bath.
7. Cool the samples and load on a gel.
8. After migration, wash the gel in water and stain/destain the gel using staining/destaining buffers, respectively (see Fig. 3) or perform a Western-blot transfer (see Subheading 3.3.5).

3.3.4. Dot Blot

1. Deposit 2 μL of each fraction of purified protein directly onto a PVDF membrane.
2. Allow the protein solution to air-dry.
3. Prepare the TBS-Tween buffers 1 and 2.
4. Prepare the monoclonal antibody solution.
5. When the protein spots are completely dry, incubate the membrane in the TBS-Tween buffer 2 for 30 min.
6. Wash the membrane twice for 5 min with the TBS-Tween buffer 1.

7. Incubate the membrane with the monoclonal antibody solution for 1 h.
8. Wash the membrane twice for 5 min with the TBS-Tween buffer 1.
9. Prepare the diaminobenzidine solution.
10. Dry the membrane on a tissue.
11. Incubate the membrane with the diaminobenzidine solution for a few seconds, until the spots are colored.
12. Dry the membrane on a tissue. The color remains on the spots containing the histidine-tagged protein, while it vanishes on the other spots.

3.3.5. *Semidry Western-Blot*

1. Prepare the Towbin buffer.
2. Prepare the transfer sandwich by superimposing two filter papers, the gel, the nitrocellulose membrane and another two filter papers in the Towbin buffer.
3. Place the transfer sandwich on the anode plate and clip the cathode plate on top.
4. Clamp the potential at 25 V and blot for 20 min.
5. Prepare TBS-Tween buffers 1 and 2, mouse anti-histidine antibody, and anti-mouse peroxidase-coupled antibody solutions.
6. Assess the sample transfer and relative concentration by staining the membrane with Ponceau S solution. This is a reversible stain that can be removed by washing in TBS-Tween buffer 1.
7. After protein transfer and destaining, saturate the nitrocellulose membrane by incubating in TBS-Tween buffer 2 for 30 min.
8. Rinse the membrane quickly with TBS-Tween buffer 1.
9. Incubate for 45 min at room temperature with the mouse anti-histidine antibodies solution.
10. Wash the membrane for 4 × 5 min with TBS-Tween buffer 1.
11. Incubate for 30 min at room temperature with the anti-mouse peroxidase-coupled antibody solution.
12. Wash the membrane for 5 × 5 min with TBS-Tween buffer 1.
13. Incubate the membrane for 1 min with 1 mL of each of the chemiluminescence reagents (H_2O_2 and luminol).
14. In a light tight box, expose X-ray films to the membrane for varying times, usually between 30 s and 2 min, and develop the films in the photo developer.

3.3.6. Protein Quantification in Detergents

Protein concentration is measured using the BCA method (26, 27), using the test tube procedure of the Micro BCA Protein Assay Kit:

1. Prepare eight BSA standards by diluting the stock solution with the solubilization buffer (the final BSA concentration should fall between 0.5 and 20 $\mu\text{g}/\text{mL}$). Make three replicates of each dilution. Introduce 500 μL of each into 1.5-mL tubes (24 tubes in total) and store at room temperature.
2. Dilute the unknown protein sample with the solubilization buffer to three different concentrations that are expected to be between 1 and 20 $\mu\text{g}/\text{mL}$. Make three replicates of each dilution. Transfer 500 μL of each sample into 1.5-mL tubes (nine tubes in total).
3. Add 500 μL of Micro BCA Working Reagent to each of the 1.5-mL tubes (24 BSA standards and nine unknown proteins) and incubate for 60 min at 60°C. Cool to room temperature.
4. Measure the absorbance at 562 nm.
5. Plot a standard curve based on the absorbance of the BSA samples.
6. Deduce the protein concentration of each unknown protein sample.

3.3.7. Protein Quantification in Proteoliposomes

Lipids need to be removed from the sample by precipitating the proteins using cold acetone, followed by a centrifugation to separate the protein pellet from the supernatant containing the lipids:

1. Prepare the 24 BSA standard tubes (Subheading 3.3.6, step 1).
2. Dilute the unknown protein sample with the solubilization buffer to three different concentrations that are expected to be between 1 and 20 $\mu\text{g}/\text{mL}$. Make three replicates of each dilution. Transfer 500 μL of each sample into 1.5-mL tubes (nine tubes in total).
3. Add 1 mL of cold acetone to each tube of unknown protein. Vortex the tubes and incubate for 60 min at -20°C . Centrifuge at $10,000\times g$ for 10 min at room temperature. Discard the supernatant. Incubate the tubes for 30 min at room temperature to allow for acetone evaporation. Add 500 μL of the solubilization buffer, and vortex the tubes again.
4. Add 500 μL of Micro BCA Working Reagent to each of the 1.5-mL tubes (24 BSA standards and nine unknown proteins) and incubate for 60 min at 60°C. Cool to room temperature.
5. Measure the absorbance at 562 nm.
6. Plot a standard curve based on the absorbance of the BSA samples.
7. Deduce the protein concentration of each unknown protein sample.

3.3.8. Lipid Quantification in Proteoliposomes

The phospholipid content in the proteoliposome sample is assessed by the Rouser method, which measures the phosphate concentration in the sample (28). Full eye, face, and skin protection is required for this method.

1. Set a heating block at 180°C.
2. Wash the glass tubes in nitric acid solution before use and dry them in an oven.
3. Prepare five phosphate standard samples by diluting the phosphate stock solution into five samples containing 1–5 µg of KH_2PO_4 per tube. Classically, 5 µg of KH_2PO_4 give an absorbance of 0.9 at 800 nm. Store at 4°C.
4. Prepare the ascorbic acid solution (Subheading 2.3).
5. Collect three proteoliposomes volumes containing approximately 1–5 µg of lipids. Transfer the samples into clean glass tubes.
6. Add 0.65 mL of the perchloric acid solution and place the tubes in the heated block at 180°C for 30 min of digestion, until the yellow color has disappeared.
7. Add 0.65 mL of the perchloric acid solution per phosphate standard tube (digestion is not necessary).
8. Put all the tubes on ice, and set the heated block at 100°C (alternatively, a boiling water bath can be used).
9. Once cooled, add to the tubes the following: 3.3 mL of water, 0.5 mL of the ammonium molybdate solution, and 0.5 mL of the ascorbic acid solution. Agitate on a vortex after each addition.
10. Put the tubes in the heated block at 100°C for 5 min.
11. Put the tubes on ice for 5 min.
12. Read the absorbance of the cooled samples at 800 nm.
13. Plot a standard curve based on the absorbance of the phosphate standard samples. The exact concentration in the phospholipid samples is calculated based on the standard curve.

3.3.9. Proteoliposome Density Characterization

A discontinuous sucrose flotation gradient analysis (21, 29) can be performed to check that the membrane protein is correctly reconstituted into proteoliposomes. The proteoliposomes can be layered *at the bottom* (see below) or *at the top* (see Note 16) of the sucrose layers.

1. After MscL reconstitution, add 120 mg of sucrose to 0.2 mL of the proteoliposome suspension (containing approximately 96 µg of protein) and mix gently by pipetting, until the sucrose has completely dissolved. The final volume is approximately 0.265 mL and the final sucrose concentration is 45%. Adjust the final volume to 0.4 mL with the buffered 45% sucrose solution.

2. Transfer the resulting suspension to a 3-mL ultracentrifuge tube, and keep it vertical in an appropriate rack for the following steps.
3. Carefully deposit 0.7 mL of the buffered 20% sucrose solution, with minimal flow along the tube wall to avoid layers mixing.
4. Repeat with 0.7 mL of the buffered 10% sucrose solution.
5. Ultracentrifuge at $100,000\times g$ for 1.5 h in a swinging rotor at 18°C .
6. At the end of the centrifugation, carefully remove and transfer the tube into the rack, keeping it vertical. Carefully collect six fractions of 0.3 mL, from top to bottom, into 1.5-mL tubes, and adjust the final volume to 1 mL with water.
7. Wash the bottom of the 3 mL tube with 100 μL of $1\times$ SDS loading buffer and remove a 15 μL aliquot for gel electrophoresis.
8. Centrifuge the 1.5-mL tubes at $16,000\times g$ for 15 min at room temperature, to pellet the proteoliposomes and remove the sucrose.
9. Resuspend each pellet in 100 μL of $1\times$ SDS loading buffer and take a 15 μL aliquot of each for gel electrophoresis.
10. Analyze the aliquots by gel electrophoresis. For an expected ratio of protein to lipid of 1:4 (w/w), proteoliposomes appear at the interface between 20% and 10% sucrose. Protein-free liposomes lie at the top of the sucrose gradient, while lipid-free protein aggregates lie at the bottom of the sucrose gradient (see Note 17).

3.3.10. Water Quantification in the Sample

Water content is estimated by weighing 10 mg of sample prior to and after 16 h in vacuum.

3.4. NMR Sample Preparation

1. Weigh the empty rotor, cap, insert bottom, top, and screw (see Fig. 2).
2. Introduce the insert bottom into the rotor.
3. Introduce a couple of mg of the pelleted sample into the rotor on the tip of a spatula. Place the rotor into a 1.5-mL tube and centrifuge it at $10,000\times g$ for 1 min at room temperature. Repeat this until approximately 30 mg of sample has been introduced into the rotor.
4. Place the insert top into the rotor, without the top screw. Clean the upper part the rotor with a precision wiper before capping tightly. At this point, the rotor containing sample should be stored at -20°C until the NMR experiment is performed.
5. Introduce the rotor into the magic-angle spinning NMR probe and into the magnet of the NMR spectrometer. Spin the rotor at ~ 10 kHz for 15 min.

6. Extract the rotor and open it. Clean the upper part of the rotor again, in case a drop of water has come out. Place the top screw, tighten it, and then cap the rotor tightly. Weigh the full rotor to deduce the final sample mass. A typical sample consists of 3 mg proteins, 12 mg lipids and 15 mg water.

4. Notes

1. All solutions require Milli-Q water, but only solutions used inside the cell-free expression vessel need to be autoclaved and nuclease-free, to make sure that no ribonucleases are present that would damage the RNAs in the lysate.
2. Continuous-exchange cell-free protein expression with an RTS commercial kit from Roche/5Prime is advantageous for its convenience, reliability, for saving time and manpower in preparing the lysate, and for managing the stocks. The thermo-regulated shaker, on the contrary, is not necessarily from Roche/5Prime.
3. Cell-free reactions should be performed on a small scale (RTS 100 or analog kits) for optimization studies, including detergents or lipids. Protein yield should be evaluated in a cell-free kit equipped with a continuous-exchange system (e.g., RTS 500), where the yield is usually higher. Once the protocol is optimized, the proteins can then be expressed on larger scales (e.g., RTS 9000).
4. Cell-free kits were stored at -80°C rather than -20°C , which increases the lifespan of the kits to over a year. Freeze–thaw cycles should be avoided.
5. Circular DNA was used for cell-free expression of MscL. Linear PCR templates can also be used, but they require larger amounts of DNA. DNA quality is important for cell-free expression. A plasmid purification procedure with a good yield (typically 100 μg of plasmid DNA per standard MIDI-prep), including an anion-exchange step, was found to be necessary. DNA should be dissolved in either nuclease-free water (our case) or Tris solution (typically 10 mM, at a pH between 8 and 8.5), but not in a buffer containing EDTA, as it would change the free magnesium ion concentration and reduce the protein expression yield.
6. Some amino acids are difficult to solubilize. Sonication or heating at 60°C can improve solubility. Met (M) and Cys (C) have to be supplemented with DTT (4 and 8 mM respectively). Tyr (Y), Trp (W), and Phe (F) have to be solubilized in 60 mM HEPES in nuclease-free water, adjusting the pH to 13, 1, or

7.5 respectively, using KOH or HCl. However, Trp (W), Asp (D), Asn (N), Cys (C), or Tyr (Y) will not dissolve completely and have to be used as suspensions. Alternatively, the Roche/5Prime RTS Amino acid Sampler provides appropriate stock solutions of each individual *unlabeled* amino acid.

7. Commercial mixtures of labeled amino-acids are also available (30). All labeled amino-acids can be specifically incorporated into the expressed proteins, except for Gln (Q) and Glu (E) when using the Roche/5Prime buffer, which contains large quantities of unlabeled glutamate. Some scrambling is observed with Ser (S), Asp (D), Asn (N), Gln (Q), and Glu (E). Some metabolic degradation can occur with Arg (R), Cys (C), Trp (W), Met (M), Asp (D), and Glu (E) during prolonged incubation. In such a case, an increased amount of amino acids can enhance the protein production.
8. Too vigorous mixing should be avoided while preparing the different lysates, as it may denature proteins or ribosomes in the extracts.
9. AEBSF is added after the expression is complete to prevent protease degradation of the target product. Proteases inhibitors can also be added to the reaction chamber, but they should be tested on a small scale beforehand.
10. If the chelating column is not precharged with Ni ions, before connecting it to the FPLC system, open it at both ends and wash it with 2 CV of distilled water, 1 CV of 100 mM EDTA, pH 8, 3 CV of distilled water, 2 CV of 0.1 M NiSO₄ solution, and 5 CV of distilled water. Once the column is charged, an additional washing step with 5 CV of 20% ethanol is necessary before storage at 4°C. At the end of the protein purification run, wash the column with water (5 CV), 0.5 M NaOH (5 CV), water (5 CV) and store in 20% ethanol.
11. The total reaction run-on is expected to contain approximately 10 mg of protein but also detergent and impurities, which may bind to the column as well. Since the binding saturation of the column surface is on the order of 10 mg/mL, the reaction run-on is split in two so as to always remain below the saturation limit.
12. For MscL, the fractions containing the protein are eluted between 200 and 500 mM of imidazole, but this is highly protein dependent. Depending on the histidine-tag accessibility, the protein may elute at lower or higher imidazole concentrations.
13. Protein concentration is usually estimated using UV fluorescence of aromatic amino acids. If the protein is not sufficiently fluorescent, quantification can be performed using the commercial Micro BCA bicinchoninic acid protein assay kit (Pierce). Care should be taken if lipids are present in the sample.

14. Instead of using polystyrene beads, detergent extraction can be performed using cyclodextrin inclusion compounds, as described by DeGrip et al. (24), or by dialysis (21).
15. Proteoliposomes can be dehydrated and rehydrated, but care should be taken, since the process may affect protein activity.
16. The discontinuous sucrose flotation gradient analysis to check that the membrane protein is correctly reconstituted can be performed with the proteoliposomes layered either *at the top* or *at the bottom* of the sucrose layers, or both (21, 29). If at the top, the proteoliposomes must be mixed with sucrose for a final sucrose concentration of 10%. The rest of the protocol is the same: After layering 0.7 mL of 45% sucrose, then 0.7 mL of 20% sucrose and then 0.7 mL of the proteoliposomes in 10% sucrose, the proteoliposomes usually appear at the interface between 20 and 10% sucrose.
17. If the amount of lipid-free protein aggregates is not negligible, the discontinuous sucrose flotation gradient can also be used as a purification technique by layering the entire reconstituted proteoliposome suspension.

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