Chapter 5

High-Throughput Production and Optimization of Membrane Proteins After Expression in Mammalian Cells

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Abstract

High-quality protein samples are an essential requirement of any structural biology experiment. However, producing high-quality protein samples, especially for membrane proteins, is iterative and time-consuming. Membrane protein structural biology remains challenging due to low protein yields and high levels of instability especially when membrane proteins are removed from their native environments. Overcoming the twin problems of compositional and conformational instability requires an understanding of protein size, thermostability, and sample heterogeneity, while a parallelized approach enables multiple conditions to be analyzed simultaneously. We present a method that couples the high-throughput cloning of membrane protein constructs with the transient expression of membrane proteins in human embryonic kidney (HEK) cells and rapid identification of the most suitable conditions for subsequent structural biology applications. This rapid screening method is used routinely in the Membrane Protein Laboratory at Diamond Light Source to identify the most successful protein constructs and conditions while excluding those that will not work. The 96-well format is easily adaptable to enable the screening of constructs, pH, salts, encapsulation agents, and other additives such as lipids.

Key words Mammalian cell culture, Transient expression, Membrane proteins, Expression and purification screening, High-throughput

1 Introduction

Membrane proteins (MPs) are found in lipid bilayers that surround cells and organelles. MPs are essential for all forms of life, controlling the interactions of the cell with the environment around it such as cell-cell communication and nutrient uptake [1]. As such, MPs are important pharmaceutical targets. Despite comprising only 30% of the human proteome, MPs currently account for over 50% of all small-molecule drug targets [2]

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Vital to the study of MPs and generation of new therapies is an ability to study MP structure and function in vitro. However, the study of MPs is significantly more challenging than that of soluble proteins. MPs are much less stable than their soluble counterparts and yield lower levels of protein after overexpression. Additionally, MPs must be extracted from their native environment while maintaining their structure and functionality. Since the cell membrane plays an important part in maintaining MP structure, the study of MPs outside their native environment is extremely challenging. Traditionally, MPs have been extracted into detergents, which form a micelle around the transmembrane helices and pull the MP into solution. However, in recent years several detergent-free encapsulation agents such as amphipols, protein-based disc systems (nanodiscs, peptidiscs, and Salipro), and styrene maleic acid lipid particles (SMALPs) have become popular [3–7].

Many different factors can influence the stability of a MP in vitro. These factors include, but are not limited to, solvent pH, salt composition, and the detergent or encapsulation system used. It is therefore important to find the optimum conditions required by each membrane protein to ensure optimal sample quality for downstream analysis such as X-ray crystallography and single-particle electron microscopy. Systematic optimization of these conditions is often iterative and time-consuming, especially given the low yields of MPs. Over the past 15 years, many studies have been reported where GFP-tagged MPs were solubilized with different detergents, clarified using ultra centrifugation and analyzed using fluorescence-detection size exclusion chromatography (F-SEC) to screen for an optimal detergent or additive [8–10]. More recently, a similar study was carried out where a purified protein was incubated with different detergents and then subjected to thermal denaturation. The higher thermal stability of a protein indicates that it is more stable in that detergent [9]. While these screens provide important information about protein stability, they either require a significant amount of sample or only allow for a small number of samples to be screened at a time limiting analysis to only one or two techniques.

To overcome some of the bottlenecks of screening for optimal conditions for MPs, we have developed a high-throughput (HTP) screening system that couples high-throughput cloning with small-scale screens that use the equivalent of 2–3 mL of cell culture to rapidly analyze different conditions such as detergents, buffers, sodium chloride concentration, salts, and lipids using a 96-well format (Fig. 1). Twelve to 24 constructs per MP target are cloned into the pOPIN vector system before a microscale (3 mL) expression in Expi293F cells [11, 12]. Expressed MP constructs are solubilized with β-dodecyl-maltoside (DDM) and Fos-Choline-12 (FC-12) before immobilization by metal affinity using 96-well filter plates as the chromatography column. Eluted proteins are assessed

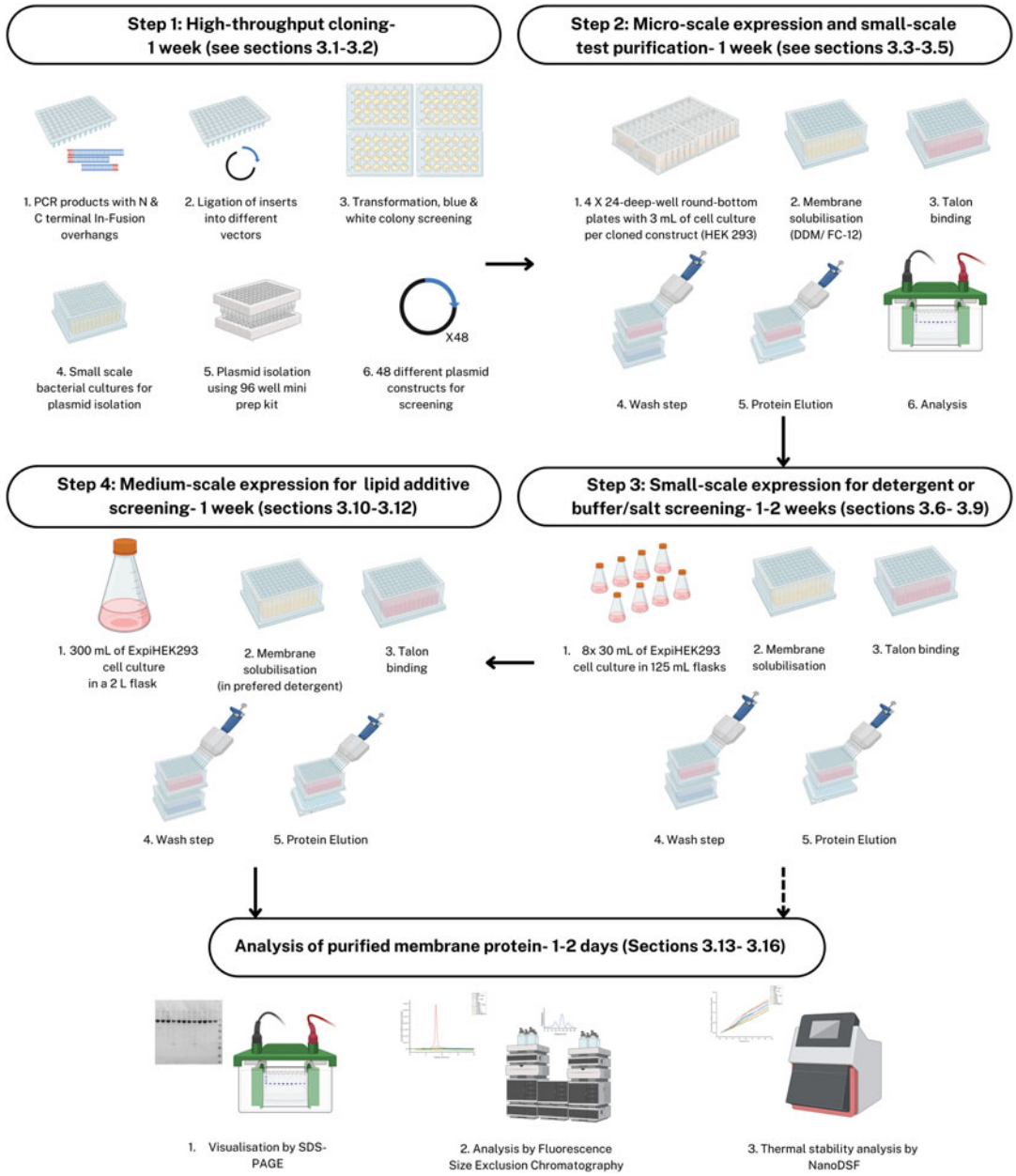

Fig. 1 Flow chart detailing the processes required to optimize the purification of a membrane protein. The whole process should take 4–6 weeks for 48 constructs. It is possible to clone and screen up to 96 constructs in parallel using this process

for purity, stability, and size as well as relative yield between DDM and FC-12 to identify the most suitable constructs and conditions for further study. The 8 most promising constructs are screened against 12 detergent-based conditions before single constructs are scaled up for purification in the most favorable conditions.

Additional screens are used if constructs require further stabilization (buffer/salts, lipids, other encapsulation agents, or specific target-related additives). The screens we describe herein are examples of what can be done, and users of the methodology should consider adapting the components to suit their own needs. The key to the success of our system is obtaining as much information as possible from a small volume of eluate (50 μL). This volume is sufficient for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), F-SEC, and nano-differential scanning fluorimetry (nano-DSF). These analysis techniques collectively provide information on expression levels, extraction levels by different detergents, behavior in different pH conditions, monodispersity, and thermal stability. The results therefore enable MP researchers to focus their efforts on the best constructs, expressions, and purification conditions that will yield protein for subsequent structural or functional experiments.

2 Materials

Unless otherwise stated, all solutions are prepared using ultrapure water prepared by purifying deionized water to a resistivity of 18 MΩ cm at 25 °C. All chemicals and reagents were purchased from standard vendors (unless specifically stated) and used without purification.

2.1 Construct Assembly Using the pOPIN Vector System

1. Template DNA (cDNA, synthetic gene, gene fragments, or other) (*see* **Note 1**).
2. pOPIN vector linearized with relevant restriction enzymes (Table 1) (*see* **Note 2**).
3. Primers: Primers should be designed with 15–21 bp extensions overlapping with in-fusion entry sites. Master stocks of the primers are prepared at 100 μM in nuclease-free water then diluted 1 in 10 to make 10 μM working stocks.
4. Phusion™ Flash PCR Master Mix.
5. DpnI restriction enzyme.
6. QIAquick® gel extraction kit.
7. 1 kb DNA standard ladder.
8. ClonExpress® II One-Step Cloning kit.
9. Competent cells, e.g., TOP10 chemically competent *Escherichia coli*.
10. 37 °C incubator.
11. Sterile 24-well cell culture plates with lids (note these plates do not need to be TC-treated).

Table 1
pOPIN vectors routinely used by the Membrane Protein Laboratory for the expression of membrane proteins in HEK293 cells

Vector name	Antibiotic resistance marker	Restriction sites for cloning	N or C terminal tag	Tags	Protease	5′ primer extension	3′ primer extension	Notes
pOPINEneo-3C-GFP-2Strep-His	Carbenicillin	NcoI/PmeI	C	GFP, twin-strep, Octa-His	3C	aggagatataccatg	cagaacttccagttt	GFP in this construct is not fluorescent when run on SDS-PAGE gel
pOPINEneo-TEV-eGFP-His	Carbenicillin	NcoI/PmeI	C	GFP, Octa-His	TEV	aggagatataccatg	tacaggttctcgtttcc	
pOPINEneo-3C-eGFP-His	Carbenicillin	NcoI/PmeI	C	GFP, Octa-His	3C	aggagatataccatg	cagaacttccagttt	
pOPINEneo-3C-mCherry-His	Carbenicillin	NcoI/PmeI	C	GFP, Octa-His	3C	aggagatataccatg	cagaacttccagttt	mCherry fusion rather than eGFP
pOPINEneo-link3C-eGFP-His	Carbenicillin	NcoI/PmeI	C	GFP, Octa-His	3C	aggagatataccatg	cagaacttccagttt	An extended linker between the purification tag and target sequence is included to aid access for 3C protease
pOPINEneo-3C-FLAG-His	Carbenicillin	NcoI/PmeI	C	Flag, Octa-His	3C	aggagatataccatg	cagaacttccagttt	
pOPINE-3C-mCherry-His	Carbenicillin	NcoI/PmeI	C	mCherry, Hexa-His	3C	aggagatataccatg	cagaacttccagttt	

(continued)

Table 1
(continued)

Vector name	Antibiotic resistance marker	Restriction sites for cloning	N or C terminal tag	Tags	Protease	5′ primer extension	3′ primer extension	Notes
pOPINEneo-HA-FLAG-TEV-POI-3C-GFP-His	Carbenicillin	BamHI/PmeI	N/C	Signal peptide, FLAG, GFP, Octa-His	TEV/3C	gtacttccagggatcc	cagaacttccagttt	Vector for tagging N and C termini
pOPINF-His	Carbenicillin	KpnI/HindIII	N	Hexa-His	None	aagttctgtttcagggcccg	ctggtctagaaagcttta	Generally N-terminally tagged constructs are less successful but may be required when C-terminal tags disrupt protein-protein interactions
pOPINF-3FLAG	Carbenicillin	KpnI/HindIII	N	Hexa-His, 3 FLAG	None	aagttctgtttcagggcccg	ctggtctagaaagcttta	

12. Autoclaved LB medium.
13. 100 mg/mL sterile-filtered carbenicillin stock.
14. LB-agar plates supplemented with 100 μg/mL carbenicillin, 1 mM IPTG, and 50 μg/mL X-gal (X-gal stock solution is made up at 50 mg/mL in dimethylformamide).
15. Autoclaved 60% glycerol.
16. 96-well PCR plates—clear and colored.
17. Gas-permeable adhesive seals.
18. Adhesive tape pads.
19. Adhesive PCR seals.
20. Multi-channel pipettes and repeat pipettors used to dispense reagents in a 96-well format.
21. 96-well PCR thermocycler with heated lid.
22. E-gel™ Mother Base and E-Gel™ 48 or 96 agarose gels with SYBR™ Safe DNA Gel Stain, 1%.
23. Centrifuge suitable for 96-well PCR plates.
24. Microcentrifuge.
25. Gel imager.
26. Water bath set to 42 °C.

2.2 Verification and Preparation of Plasmids for Transfection

1. Miniprep kit (for low throughput: QIAprep® Spin Miniprep kit (e.g., Qiagen); for HTP: Wizard® SV-Plus 96, e.g., Promega).
2. Suitable vacuum manifold for HTP miniprep kit (e.g., Vac-Man® 96).
3. Primers: forward primer use pOPIN fwd; reverse use the diluted reverse primers used for the cloning PCRs.
4. Phusion™ Flash PCR Master Mix.
5. 1 kb DNA standard ladder.
6. 96-well PCR plates.
7. Adhesive PCR seals.
8. Multichannel pipettes and repeat pipettors used to dispense reagents in a 96-well format.
9. 96-well PCR thermocycler with heated lid.
10. E-gel™ Mother Base and E-Gel™ 48 or 96 agarose gels with SYBR™ Safe DNA Gel Stain, 1%.
11. Centrifuge suitable for 96-well PCR plates.
12. Gel imager.

2.3 Maintenance of Expi293F Cells

1. Expi293F™ cells (cell line catalogue number A14527).
2. Gibco Expi293™ Expression medium.
3. 125 mL sterile non-baffled flasks with vented closure.
4. Humidified CO_2 incubator with a 25 mm orbital throw.
5. Virkon™ tablets.
6. Trypan blue stain (4% solution).
7. Countess™ automated cell counter or inverted light microscope.
8. Countess™ cell counting chamber slides or hemocytometer.

2.4 Transient Transfection and Expression in Expi293F™ Cells (Microscale)

1. Miniprep DNA for transfection (from HTP cloning).
2. 2.0 mL sterile Eppendorf Tubes®.
3. Micro-volume spectrophotometer.
4. Laminar flow hood.
5. Expi293F™ cells in suspension (from Subheading 2.3).
6. Gibco Expi293™ Expression Medium.
7. Sterile 24-well round-bottom blocks.
8. Sterile gas-permeable seals (Air-O-Seal, 4titude).
9. Humidified CO_2 incubator with a 25 mm orbital throw.
10. Tabletop centrifuge suitable for plates and 50 mL Falcon® tubes.
11. Trypan blue stain (4% solution).
12. Countess™ automated cell counter or hemocytometer/microscope.
13. Countess™ cell counting chamber slides.
14. EVOS® FL light microscope with light cubes for GFP (470/525 nm) and Texas Red (585/624 nm).
15. Sterile filtered polyethylenimine (PEI), 1 mg/mL in water, pH 7.0 (PEI MAX 40 K, e.g., Polysciences) (*see* **Note 3**).
16. Gibco™ Opti-MEM™ I reduced serum medium.
17. Sterile filtered stock solutions of enhancers prepared in Opti-MEM™ reduced serum medium: 45% glucose, 0.3 M valproic acid, and 1 M sodium propionate.
18. 50 mL sterile Falcon® tubes.
19. Automatic pipette filler.
20. Sterile serological pipettes (2, 5, 10, 25, and 50 mL).
21. Single- and multichannel pipettes with compatible sterile filter tips.
22. Virkon™ tablets.

2.5 Small-Scale Test Purification

1. Cells containing expressed protein of interest (Subheading 2.4).
2. Lysis buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole pH 8.0. Protease inhibitor cocktail tablet as required (one tablet per 50 mL buffer).
3. Wash buffers: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 25 mM imidazole, 3× CMC DDM or FC-12.
4. Elution buffers: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 300 mM imidazole, 3× CMC DDM or FC-12.
5. 10× purification concentration DDM (0.3%).
6. 10× purification concentration FC-12 (1.4%).
7. Multichannel pipettes.
8. Nonfiltered pipette tips or equivalent.
9. 50 mL pipetting reservoirs.
10. 96-well silicone sealing mat.
11. Shaking platform or rotator (*see* **Note 4**).
12. Centrifuge and rotor capable of taking two deep-well blocks stacked on top of each other.
13. 25 mL of 50% TALON resin pre-equilibrated in lysis buffer.
14. 96-well filter block. 2 mL, square well, long drip, 25 μm polypropylene filter.
15. 2 mL 96-deep-well block.
16. Masking tape.
17. 96-well microtiter plate.
18. Sonicator with 24-probe head to fit 24-well blocks.

2.6 Transient Transfection and Expression in Expi293F™ Cells (Small and Medium Scale)

1. Laminar flow hood.
2. Purified plasmid DNA of interest.
3. Expi293F™ cells.
4. Gibco Expi293™ Expression medium.
5. 125 mL sterile non-baffled flasks with vented closure.
6. Humidified CO_2 incubator with a 25 mm orbital throw.
7. Tabletop centrifuge suitable for 50 mL Falcon® tubes.
8. Trypan blue stain (4% solution).
9. Countess™ automated cell counter or inverted light microscope.
10. Countess™ cell counting chamber slides or hemocytometer.
11. EVOS® FL light microscope with light cubes for GFP (470/525 nm) and Texas Red (585/624 nm).

12. 0.22 μm sterile syringe filters.
13. Sterile filtered polyethylenimine (PEI), 1 mg/mL in water, pH 7.0 (PEI MAX 40 K, e.g., Polysciences).
14. Gibco™ Opti-MEM™ I reduced serum medium.
15. Sterile filtered stock solutions of enhancers prepared in Opti-MEM™ reduced serum medium: 45% glucose, 0.3 M valproic acid, and 1 M sodium propionate.
16. 50 mL sterile Falcon® tubes.
17. Automatic pipette filler.
18. Sterile serological pipettes (2, 5, 10, 25, and 50 mL).
19. Single- and multichannel pipettes with compatible sterile filter tips.

2.7 Harvest and Membrane Preparation

1. Centrifuge and rotor capable of taking two stacked deep-well SBS format plates/blocks (e.g., Beckman J.S. 5.3).
2. Cell disrupter (Constant Systems) or similar.
3. Membrane resuspension buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol protease inhibitor cocktail tablet as required (one tablet per 50 mL buffer).
4. For the buffer/salt screen lysis buffers, see Table 3.
5. Ultracentrifuge, Type 45 Ti rotor, and 70 mL polycarbonate ultracentrifuge tubes—ensure that there is no damage to the tubes, rotor, rotor seals, or ultracentrifuge before use.

2.8 Detergent Screen

1. Cell membranes containing expressed protein of interest (Subheading 2.7).
2. Lysis buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole pH 8.0. Protease inhibitor cocktail tablet as required (one tablet per 50 mL buffer).
3. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 25 mM imidazole, 3 × CMC detergent (added as described in method).
4. Elution buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 300 mM imidazole, 3 × CMC detergent (added as described in method).
5. 2 mL 96-deep-well block containing solubilization detergents (Table 2).
6. 2 mL 96-deep-well block containing purification detergents (Table 2).

Table 2
Detergent screen

	Detergent/ additive	CMC	Extraction block stock concentration (w/v)	Extraction final concentration (w/v)	Purification block stock concentration (w/v)	Purification final concentration (w/v)
1	DDM	0.17 mM (0.0087%)	10%	1%	0.3%	0.03%
2	DDM + CHS	0.17 mM (0.0087%)	10% + 2%	1% + 0.2%	0.3% + 0.06%	0.03% + 0.006%
3	DM	1.7 mM (0.087%)	10%	1%	2.5%	0.25%
4	DM + CHS	1.7 mM (0.087%)	10% + 2%	1% + 0.2%	2.5% + 0.5%	0.25% + 0.05%
5	OG	18–20 mM (0.53%)	15%	1.5%	15%	1.5%
6	LMNG	0.01 mM (0.001%)	10%	1%	0.03%	0.003%
7	OGNG + CHS	1.02 mM (0.058%)	10% + 2%	1% + 0.2%	2% + 0.4%	0.2% + 0.04%
8	LDAO	1–2 mM (0.023%)	10%	1%	0.7%	0.07%
9	C12E8	0.09 mM (0.0048%)	10%	1%	0.15%	0.015%
10	C12E9 + CHS	0.05 mM (0.003%)	10% + 2%	1% + 0.2%	0.09% + 0.018%	0.009% + 0.0018%
11	CYMAL-5	2.4 mM (0.12%)	10%	1%	0.4%	0.04%
12	FC-12	1.5 mM (0.047%)	10%	1%	1.4%	0.14%

2.9 Buffer/Salt Screen

1. Cell membranes containing expressed protein of interest (Subheading 2.7).
2. Lysis, wash, and elution buffers as described in Table 3.
3. 2 mL 96-deep-well block containing solubilization detergents (Table 3).
4. 2 mL 96-deep-well block containing purification detergents (Table 3).

Table 3
Buffer compositions for buffer/salt screen

Buffer	Buffering agent (mM)	NaCl (mM)	Imidazole (mM)	Glycerol (% v/v)	Detergent
Lysis buffer 6.5	50 MES pH 6.5	50, 100, 200, or 500	10	5	None
Lysis buffer 7.5	50 HEPES pH 7.5	50, 100, 200, or 500	10	5	None
Lysis buffer 8.5	50 Tris–HCl pH 8.5	50, 100, 200, or 500	10	5	None
Wash buffer 6.5	50 MES pH 6.5	50, 100, 200, or 500	25	5	3× CMC
Wash buffer 7.5	50 HEPES pH 7.5	50, 100, 200, or 500	25	5	3× CMC
Wash buffer 8.5	50 Tris–HCl pH 8.5	50, 100, 200, or 500	25	5	3× CMC
Elution buffer 6.5	50 MES pH 6.5	50, 100, 200, or 500	300	5	3× CMC
Elution buffer 7.5	50 HEPES pH 7.5	50, 100, 200, or 500	300	5	3× CMC
Elution buffer 8.5	50 Tris–HCl pH 8.5	50, 100, 200, or 500	300	5	3× CMC
SEC running buffer 6.5	20 MES pH 6.5	50, 100, 200, or 500	0	0	2× CMC
SEC running buffer 7.5	20 HEPES pH 7.5	50, 100, 200, or 500	0	0	2× CMC
SEC running buffer 8.5	20 Tris–HCl pH 8.5	50, 100, 200, or 500	0	0	2× CMC

2.10 Transient Transfection and Expression in Expi293F™ Cells (Medium Scale)

Components are the same as small-scale (Subheading 2.6) except that 2000 mL flasks are used for 300 mL of cell culture.

2.11 Lipid Additive Screen

1. Cell membranes containing expressed protein of interest (Subheading 2.10).
2. Wash buffer containing 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 25 mM imidazole, 3 × CMC detergent (added as described in method).
3. Elution buffer containing 50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 200 mM imidazole, 3 × CMC detergent (added as described in method).
4. Lipid stock plate (Table 4).
5. 2 mL deep 96-well block containing solubilization detergents (Table 2).
6. 2 mL deep 96-well block containing purification detergents (Table 4).

Table 4
Lipid screen

	Detergent/additive	CMC	Detergent in "lipid purification block" stock concentration (w/v)	Lipid in "lipid purification block" stock concentration
1	DDM	0.17 mM (0.0087%)	0.3%	0.0052%
2	DDM + CHS	0.17 mM (0.0087%)	0.3% + 0.06%	0.0052%
3	DM	1.7 mM (0.087%)	2.5%	0.0522%
4	DM + CHS	1.7 mM (0.087%)	2.5% + 0.5%	0.0522%
5	OG	18–20 mM (0.53%)	15%	0.3%
6	LMNG	0.01 mM (0.001%)	0.1%	0.0006%
7	OGNG + CHS	1.02 mM (0.058%)	2% + 0.4%	0.0348%
8	LDAO	1–2 mM (0.023%)	0.7%	0.0138%
9	C12E8	0.09 mM (0.0048%)	0.15%	0.00288%
10	C12E9 + CHS	0.05 mM (0.003%)	0.9% + 0.018%	0.0018%
11	CYMAL-5	2.4 mM (0.12%)	0.4%	0.072%
12	FC-12	1.5 mM (0.047%)	1.4%	0.0282%

2.12 Deep-Well Block-Based Purification

1. Multichannel pipettes (10–50 µL, 100–300 µL, and 1000 µL).
2. Nonfiltered pipette tips or equivalent.
3. 50 mL pipetting reservoirs.
4. 96-well silicone sealing mat.
5. Shaking platform or rotator.
6. Centrifuge and rotor capable of taking two deep-well blocks stacked on top of each other (e.g., Beckman JS 5.3).
7. 25 mL of 50% TALON resin pre-equilibrated in lysis buffer (*see* **Note 5**).
8. 96-well filter block. 2 mL, Square Well, Long Drip, 25 µm polypropylene filter.
9. 2 mL, 96-deep-well block.
10. Masking tape.
11. 96-well microtiter plate.

2.13 SDS-PAGE Analysis

1. Precast Nu-PAGE 26-lane 4–12% Bis-Tris gradient polyacrylamide gel.
2. 10 × MES running buffer.

3. SDS-PAGE loading buffer containing 1:4 ratio of NuPAGE™ Sample Reducing Agent (10×): NuPAGE™ LDS Sample Buffer (4×).
4. Protein ladder (e.g., SeeBlue™).
5. Fluorescent protein ladder (e.g., BenchMark™ Fluorescent).
6. Protein gel electrophoresis apparatus.
7. InstantBlue™ protein stain or equivalent.

2.14 Size Exclusion Chromatography Screening

1. SRT-C-300 HPLC system columns (5 μm, 300 Å 7.8 × 50 mm and 300 mm columns) (*see* **Note 6**).
2. High-performance liquid chromatography system (*see* **Note 7**).
3. 96-well microtiter plate.
4. Pierceable adhesive sealing film.
5. Freshly filtered and relevant degassed SEC running buffers (20 mL for each sample including equilibration and an additional 50 mL for purging the solvent line). SEC buffer (detergent and lipid screens): 20 mM HEPES pH 7.5, 150 mM NaCl, 2 × CMC detergent. For buffer/salt screens, use the relevant SEC running buffer described in Table 3 (*see* **Note 8**).
6. Gel filtration standard made up in 0.5 mL of ultrapure water and diluted 1 in 10 in relevant gel filtration buffer.

2.15 Thermal Stability

1. NanoTemper Prometheus NT.48 (*see* **Note 9**).
2. Prometheus NT.48 Series nanoDSF Grade Standard Capillaries.
3. Microtiter or PCR plate.
4. Lint-free tissues.
5. 100% ethanol.

3 Methods

All procedures should be conducted on ice or at 4 °C unless otherwise stated. Where a protocol states, for example, wash buffer + detergent, the detergent used needs to match that being used in a specific condition.

3.1 Construct Assembly Using the pOPIN Vector System

Organization is key to using a parallelized approach. It is important to keep a record of which primers, templates, and vectors are associated with each well of the 96-well plate. Additionally, it is sensible to group by target and/or vector as well as to order constructs in size where possible. Different colored plates can be used to aid identification.

1. Prepare the Phusion™ Flash master mix (F-548 L) according as per Table 5 depending on the number of reactions (*see* **Note 10**).

Table 5
Phusion flash master mix

Number of reactions (25 μL)	1	28	55	108
2 × Phusion flash master mix (25 μL)	12.5	350	688	1350
Sterile water (25 μL)	8.5	238	468	918
Total volume (25 μL)	21	588	1155	2268

2. Using the multi-dispense pipettor with a 500 μL syringe tip, dispense 21 μL of the mixture into each well of a colored PCR plate.
3. Using a 10 or 20 μL multichannel pipette, transfer (in columns) 1.5 μL of diluted (10 μM) *forward* primer to the appropriate wells of the PCR plate followed by 1.5 μL of diluted (10 μM) *reverse* primer.
4. Using the 10 or 20 μL multichannel pipettes, transfer (in columns) 1 μL of template plasmid (~10–20 ng/μL) to the appropriate wells of the PCR plate (*see* **Note 11**).
5. Ensure that the plate is well mixed and sealed with a clear adhesive PCR seal and load into a thermocycler using the following thermal cycling parameters:

   98 °C 10 s

   (98 °C 1 s, 55 °C 5 s, 72 °C × * min) 1 cycle

   (98 °C 1 s, 60 °C 5 s, 72 °C × * min) × 29 cycles

   72 °C 2 min.

   4 °C hold

   *Extension time dependent on the length of the expected PCR product (allow 15 s/kbp)
6. When thermal cycling is complete, run 5 μL of each sample on an agarose gel (*see* **Note 12**).
7. If using the E-gel™ setup, run following the instructions provided with the gels. An expandable electronic multichannel can be used to dispense first water and then 5 μL of the PCR product including mixing cycles. Load 5 μL of DNA ladder into the marker lanes.
8. Optional: If the DNA bands on the agarose gel look strong and clean, the remaining PCR reaction can be prepared simply with a 1/10 dilution in water (after DpnI treatment if applicable; **steps 12–15**). However, if there are primer artifacts, you should purify. If there are nonspecific bands, it is best to gel-extract the intended product. Ampure XT magnetic bead procedure (Beckman) may be used at this point, but this is not something we routinely do. The benefit is mainly seen when there are <100 bp primer artifacts present which can preferentially insert into the recipient vector.

9. Optional, DpnI treatment (**steps 9–12**): Make a master mix to aliquot 5 μL per well of 5 units DpnI (0.5 μL of 10 U/μL) in 1 × cutting buffer (e.g., NEBuffer™ 4 or CutSmart® buffer, New England Biolabs) (*see* **Note 13**).
10. Optional: Add 5 μL with a repeat pipettor to each reaction. Carefully vortex to mix.
11. Optional: Incubate at 37 °C for 30 min followed by 80 °C for 20 min.
12. Optional: Dilute the reactions ~1/10 with water, i.e., add 175 μL to bring up to ~200 μL. If there are any products which were substantially less intense on the gel, they can be diluted less, e.g., 1/2 or 1/4.
13. Prepare a fresh 96-well clear PCR plate on ice with 10 μL ClonExpress® II One Step fusion reactions according to the list below (the volumes shown are scaled down to half the manufacturer's suggested reaction size; *see* **Note 14**):
    (a) 1 μL 100 ng/μL linearized vector
    (b) 2 μL diluted PCR product
    (c) 2 μL 5× CEII buffer
    (d) 1 μL Exnase II
    (e) 4 μL of water
14. Using a 10 μL pipette, transfer 1 μL (~100 ng) of the appropriate linearized pOPIN vector to each well of a PCR plate.
15. Make a master mix containing the enzyme, buffer, and water. Aliquot 9-X μL of mix per well using a repeat pipettor (n.b. if vector is the same, it can be included in the master mix).
16. Using a 10 μL or 20 μL multichannel pipette, take 2 μL of diluted insert (should equate to 10–250 ng on average—use same volume for all PCRs) and add to the appropriate wells of the PCR plate, pipetting up and down ~5× and stirring to mix.
17. Incubate at 37 °C for 30 min. If proceeding with transformation the same day, take sufficient 20 μL aliquots of in-house competent TOP10 from −80 and allow to thaw on ice. We suggest storing aliquots in 96-tube racks to facilitate HTP processing with multichannel pipettes.
18. Immediately store the reaction on ice or freeze at −20 °C for future use (*see* **Note 15**).
19. Using a 10 or 20 μL multichannel pipette, transfer 2 μL of the cloning reaction per 20 μL aliquot of competent cells in tube rack on ice, ensuring it reaches the cells at the bottom of the tube. Tap the side of rack a few times to mix (*see* **Note 16**).
20. Incubate the cells on ice for 30 min.

21. Heat-shock the cells for 30 s at 42 °C by placing the rack in the 42 °C water bath. Make sure that the water level covers the competent cells in the racked tubes.
22. Return the cells to ice for 2 min.
23. Using a multichannel pipette, add 120 μL of SOC or Power Broth (PB) (with no antibiotic) per 20 μL tube of cells.
24. Transfer to 37 °C incubator and incubate for 1 h. at 600 rpm.
25. While incubating, prepare the LB agar plates (two replicates for each experiment to allow plating of two dilutions of cells per reaction; *see* **step 27**). You will need 1 mL of the agar/antibiotic/X-gal/IPTG mix per well in 24-well plates. Dilute the stocks appropriately as follows: carbenicillin (Cb) 100 mg/mL, 1/1000; or kanamycin (Kan) 100 mg/mL, 1/2000; 50 mg/mL X-Gal [in DMF], 1/1000; IPTG 1 M stock, 1/1000; in warm agar in 50 mL Falcon® tubes before pouring (*see* **Note 17**).
26. Pour the plates using a 25/50 mL multi-dispense tip and the repeat pipettor (alternatively, use a standard serological pipette), adding 1 mL of your molten LB agar per well. Avoid using the last 1 mL of agar to prevent introduction of bubbles.
27. Immediately prior to plating, add 20 μL of PB/LB to each well of one set of plates. Using the Matrix IMPACT pipettors for four transformation reactions at a time, aspirate 30 μL of cells per well and transfer 5 and 25 μL to the LB Agar plates supplemented with antibiotic, X-Gal, and IPTG (5 μL of cells onto the plates with PB added, 25 μL onto the empty plates).
28. Tip the plates by hand in an orbital motion to spread the cells and allow at least 10–15 min for the plates to dry uncovered in the laminar flow hood before turning.
29. Incubate overnight at 37 °C.
30. The following day, prepare four 96-deep-well blocks (if using the Wizard® SV96 kit, use the blocks provided) by addition of 1.2 mL of LB supplemented with the appropriate antibiotic.
31. Using a 200/300 μL pipette tip, pick individual white colonies from the overnight plates, into each well. Two colonies should be picked per construct (*see* **Notes 18** and **19**). Replicate the pattern of your transformation plate to fill the deep-well block.
32. When plate is complete, remove tips eight at a time, using a multichannel pipette for convenience, and seal the plates with gas-permeable adhesive seals.
33. Incubate the filled plates at 37 °C overnight (~220 rpm in a normal shaker or 600 rpm in the floor standing incubator).

Table 6
Master mix for construct verification

	Per rxn.	For 1 × 96
Number of (12.5 μL) reactions	1	108
2 × Phusion flash (μL)	6.25	675
Sterile water (μL)	4.68	505
pOPIN forward primer at 100 μM (μL)	0.075	8.1
Total volume (μL)	11	1188

34. *Optional, preparation of glycerol stocks.* Using a multichannel pipette, aliquot out into a sterile microtiter plate 60 μL/well of autoclaved 60% v/v glycerol. Transfer 100 μL from each well of the culture plate and pipette up and down to mix. Store at −80 °C (*see* **Note 20**).
35. Replace the gas-permeable seal on each 96-well culture plate with a tape pad seal and harvest the cells by centrifugation at 1500 × *g* for 15 min (e.g., the Beckman JS5.3 rotor for the Beckman Avanti centrifuge is ideal for this step).
36. Decant the media to waste by inverting the plate over a large beaker, and then rest the plate upside down on a wad of absorbent tissue to remove residual media (take care here as the pellets may not be tightly stuck to the blocks).

3.2 Verification and Preparation of Plasmids for Transfection

1. The plasmid minipreps can be made using the Promega Vac-Man® 96 vacuum manifold according to the manufacturer's protocol (*see* **Note 21**).
2. Prepare the following master mix (Table 6), mixing thoroughly before dispensing.
3. Using a multi-dispense pipette, dispense 11 μL of the master mix into each well of the PCR plate.
4. Using the 10 or 20 μL multichannel pipettes, transfer (in eights/columns) 0.75 μL of diluted (10 μM) *reverse* primer to the appropriate wells of the PCR plate. Remember that you have to repeat this twice, once for each clone.
5. Using the 10 or 20 μL multichannel pipette, transfer (in eights/columns) 0.75 μL of construct plasmid to the appropriate wells of the PCR plate.
6. Seal the plates with a clear adhesive film or foil and load into the thermal cycler.

7. Perform the thermal cycling using these parameters:

   98 °C 10 s

   (98 °C 1 s, 60 °C 5 s, 72 °C × min*) repeat 30 times

   72 °C 1 min

   4 °C Hold

   * Extension time dependent on the length of the expected PCR product (allow 15 s/kbp)

8. Run an aliquot of PCR product (as for the initial PCR) on an agarose gel. Score your clones based upon correct band size and purity of PCR product (*see* **Note 22**).

3.3 Maintenance of Expi293F Cells

1. All manipulations should be carried out in a laminar flow hood. Cells should be transferred between containers using sterile serological pipettes which should be discarded after a single use. Avoid vigorous mixing and pipetting of the cells.
2. Revive a vial of Expi293F following the manufacturer's instructions.
3. Passage cells when cell densities reach 3–5 × 10^6 cells/mL. Although these cells can reach densities >8 × 10^6 cells/mL, the maintenance stock should not be allowed to exceed 5 × 10^6 cells/mL. Record the passage number of the cells and determine cell viability and total cell count during each maintenance (*see* **Note 23**).
4. Count cells using trypan blue exclusion. Add 10 μL cell suspension to 10 μL of trypan blue before applying 10 μL of the mix to a cell chamber slide or hemocytometer.
5. If using the Countess automated cell counter, focus the image and run the "Count" program. Otherwise manually count blue and white cells using a hemocytometer and manual click counter before calculating the proportion of viable cells. Only cell batches showing ≥95% viability are suitable for transfection.
6. When splitting, seed the required culture volume with 0.3 × 10^6 cells/mL using warmed Expi293™ medium. Use a 125 mL flask to maintain 30 mL Expi293F™ cells. Use a 500 mL flask to maintain 100 mL of Expi293F™ cells and a 2000 mL flask to maintain 300 mL of cells. Discarded cells should be decontaminated with 1% Virkon.
7. Ensure that the flask is labeled with your name, cell line, passage number, and seeding date.
8. Incubate the flask at 37 °C, 5–8% CO_2, ~80% humidity at 120 rpm (*see* **Note 24**).
9. Cells seeded in this way will require splitting again 3–4 days later. Maintain the suspension culture of Expi293F™ cells for at least three passages after defrosting before attempting transfection (passage numbers 3–30 can be used in experiments).

3.4 Transient Transfection and Expression in Expi293F™ Cells (Microscale)

A microscale test expression (Subheading 3.4) and test purification (Subheading 3.5) enable a quick assessment of yields and stability before scaling up for other screens or large-scale preparation. Critically, poorly expressed constructs can be discounted before significant investment in cell culture media or research time.

1. One day before transfection, seed a batch of Expi293F™ cells at a cell density of 1–1.2 × 10^6 cells/mL into a suitably sized flask (*see* **Note 25**). Allow 6 mL of cell culture per construct to be tested (assuming duplicate samples will be set up to allow sufficient material to run the test purification with both DDM and FC-12 detergents).
2. 20 h later, cells should have grown to a density of 2–2.5 × 10^6 cells/mL. Prepare the transfection mixtures in 24-deep-well round-bottom plates which will be used for the expression cultures.
3. For 3 mL cultures, aliquot approximately 3 μg DNA into the empty plates. Dilute the polyethylenimine (PEI MAX 40 K) with Opti-MEM™ serum-free medium in a suitable sterile tube, for each sample allowing 16 μL PEI (1 mg/mL stock) and 300 μL Opti-MEM™. Add 316 μL of this mixture to each well (for convenience a repeat pipette or a multichannel can be used), mix by tapping the side of the plate three to four times, and incubate for 10 min at room temperature (*see* **Note 26**).
4. Add 3 mL of the cell suspension to each well and transfer to the shaking incubator and grow at 37 °C, 220–250 rpm, 5–8% CO_2, 80% humidity.
5. Protein expression can be boosted (within 20 h) through the addition of post-transfection supplements. Add the following supplements to a final concentration of 5 mM valproic acid, 6.5 mM sodium propionate, and 0.9% glucose (*see* **Note 27**). The temperature may be dropped to 30 °C at this point, which improves expression yields for some MPs.
6. If a vector that incorporates a fluorescent label is used (e.g., GFP or mCherry), then expression and localization can be tracked using a fluorescence microscope (*see* **Note 28**).
7. Harvest cells between 48 and 72 h post-transfection (*see* **Note 29**). To harvest the cells, centrifuge at 300 × *g* for 5 min, discard supernatant, and proceed to the test purification or flash freeze in liquid nitrogen and store at −80 °C until required.

3.5 Small-Scale Test Purification

1. Equilibrate the TALON resin before starting the experiment. Add the required volume of 50% TALON resin into a Falcon® tube and centrifuge at 300 × *g* for 2 min at 4 °C and discard the ethanol. Wash the resin twice by filling the tube with distilled

water, inverting a few times, and centrifuging at 300 × *g* for 2 min. Wash with lysis buffer (without detergent) three times using the same method. Finally, add an equal volume of lysis buffer and store at 4 °C until use (*see* **Note 30**).

2. Resuspend the cells (grown and isolated from 3 mL culture in 24-deep-well block) in 1.5 mL lysis buffer (add the protease inhibitor tablet to the buffer before resuspension).
3. Disrupt the cell membranes by sonication for 2 min 30 s (5 s on/10 s off)—20% amplitude in an ice + water bath using a 500 W sonicator. We use a 24-head sonicator probe. Ensure the probe head is level and the tips are all submerged in the liquid.
4. Transfer 900 μL of the broken cells into a 96-deep-well block. DDM samples should be placed in row A-D and FC-12 repeats in rows E-H.
5. Using a multichannel add 100 μL of detergent stock (10% DDM or 10% Fos-Choline-12 in a 50 mL reservoir) to each well, seal with a silicone sealing mat, and use the masking tape to attach a second deep-well block over the silicone seal if using the rotator.
6. Mix on the rotator (slow speed setting) or shaking platform (1000 rpm) at 4 °C for 1 h.
7. Centrifuge the block at 3500 × *g* for 30 min at 4 °C.
8. Carefully transfer the clarified supernatant into a new 96-deep-well block using a multichannel pipette. Take care to avoid transferring the cell pellets (*see* **Note 31**).
9. Add the appropriate volume of pre-equilibrated 50% TALON resin to a reagent reservoir. To ensure the resin stays in suspension, keep shaking the reservoir by hand.
10. Add 100 μL of equilibrated 50% TALON into each well. Make sure to cut the ends of the pipette tips (~5 mm) when adding the resin as uncut tips could get clogged and lead to uneven distribution of resin.
11. Cover the block with a silicone sealing mat and allow the target MP to bind to the TALON resin for 1 h at 4 °C using either a platform shaker (1000 rpm) or the rotator (slow speed setting). Use the masking tape to attach a second deep-well block over the silicone seal if using the rotator.
12. Place a 96-well filter plate on top of a waste deep-well block and tape together with masking tape.
13. Transfer the solubilized membrane and resin mixture to the filter plate using 1 mL pipette tips that have had the end of the tip cut off. Collect the flow-through in the waste block.

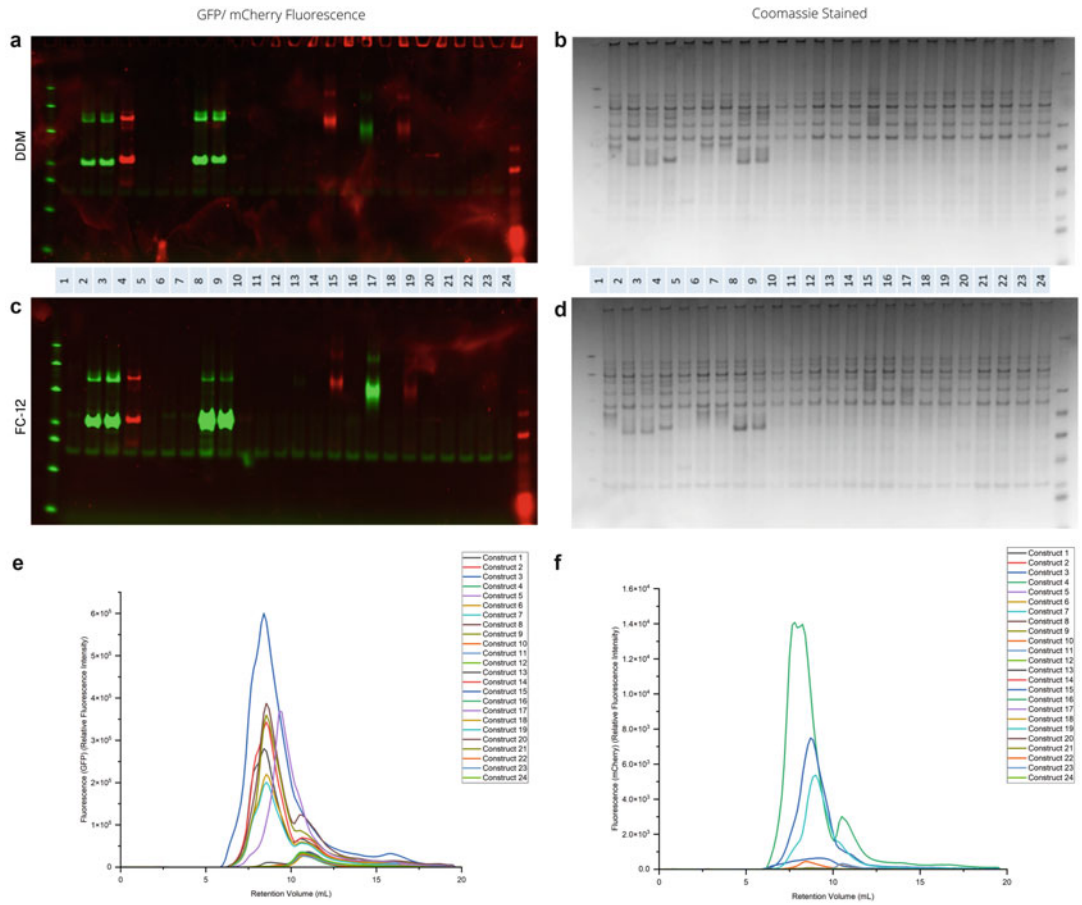

Fig. 2 Analysis of a test purification using DDM and FC-12 to solubilize. (**a**) SDS-PAGE gel of DDM extracted proteins imaged using GFP and mCherry fluorescence. (**b**) Coomassie-stained SDS-PAGE gel of DDM extracted proteins. (**c**) SDS-PAGE gel of DDM extracted proteins imaged using GFP and mCherry fluorescence. (**d**) Coomassie-stained SDS-PAGE gel of FC-12 extracted proteins. (**e**) F-SEC profiles for each construct as measured by GFP fluorescence (**f**) F-SEC profiles of each construct as measured by mCherry florescence

14. Prepare two reagent reservoirs (one for DDM and one for FC-12) with 72 mL of wash buffer and 8 mL of 10× purification concentration detergent.
15. Pipette 500 μL of wash buffer (with the detergent) into the filter plate and centrifuge at 300 × *g* for 1 min at 4 °C. Repeat this wash step two more times.
16. Discard the flow-through and spin the plate at 500 × *g* for 3 min, 4 °C to remove any excess wash buffer (this is important as you do not want eluted protein diluted with wash buffer).
17. Prepare a microtiter plate with 90 μL elution buffer and add 10 μL of the detergent from the purification block in each well (Fig. 2).

18. Place the filter plate on top of a clean microtiter plate and add 50 μL of elution buffer directly to the resin, seal the plate with a foil seal, and incubate for 20 min at 4 °C. Agitation on a platform shaker can help if elution takes longer than expected.
19. Remove the seal and centrifuge at 500 × *g* at 4 °C for 3 min to elute the protein.
20. Continue your experiment from Subheading 3.12 (*see* **Note 32**).
21. Select eight successful constructs for small-scale expression in Expi293F™ cells.

3.6 Transient Transfection and Expression in Expi293F™ Cells (Small Scale)

1. One day before transfection, seed 30 mL Expi293F™ cells at a cell density of 1–1.2 × 10^6 cells/mL in a 125 mL non-baffled flask with porous filter seal. We suggest growing eight constructs in parallel as these fit nicely with the 8 × 12 column/row format of a 96-well plate.
2. On the day of the transfection, prepare the transfection mixture for each construct: for 30 mL scale add 30 μg DNA and 160 μL PEI MAX 40 K to 3 mL Opti-MEM™ serum-free medium. After thorough mixing, incubate the mixture for 10 min at RT and add gently (dropwise) to Expi293F™ cells (*see* **Note 26**). For buffer/salt screens, transfect 50 mL of Expi293F™ cells adjusting using 50 μg DNA and 265 μL PEI MAX 40 K to 5 mL Opti-MEM™ serum-free media.
3. Place cells immediately in shaking incubator and grow at 37 °C, 120–150 rpm, 8% CO_2, 80% humidity.
4. Protein expression can be boosted (within 20 h) through the addition of post-transfection supplements. Add the following supplements to a final concentration: 5 mM valproic acid, 6.5 mM sodium propionate, and 0.9% glucose (*see* **Note 27**). The temperature may be dropped to 30 °C at this point, which improves expression levels of some MPs.
5. If a vector that incorporates a fluorescent label is used (e.g., GFP or mCherry), expression and localization can be tracked using a fluorescence microscope.

3.7 Harvest and Membrane Preparation from Expi293F Cells

1. Harvest cells between 48 and 72 h post-transfection (*see* **Note 28**). Transfer cell cultures into either 50 mL or 500 mL centrifuge tubes depending on culture scale, and centrifuge 700 × *g* for 20 min at 4 °C. Resuspend pellets in membrane resuspension buffer. For the buffer/salt screen, split the 50 mL culture into three equal volumes before centrifugation. Resuspend each of the aliquots of cells into 50 mL of either lysis buffer pH 6.5 50 mM NaCl, lysis buffer pH 7.5 50 mM NaCl, or lysis buffer pH 8.5 50 mM NaCl (Table 3).

2. Disrupt membranes using two passes of the cell disruptor at 15 kpsi. Ensure that lysate is collected in a beaker on ice.
3. Collect membranes by ultracentrifugation at 100,000 × *g* using a Type 45 Ti rotor and 70 mL ultracentrifuge tubes for 1 h at 4 °C.
4. Resuspend membranes for detergent and lipid screens in 12.5 mL of lysis buffer (add the protease inhibitor tablet at this point) or 0.5 mL for each of the three aliquots for the buffer screen and store for up to 6 months at −80 °C. Dounce homogenizers (1 mL or 10 mL) can be helpful when resuspending the membrane pellet.

3.8 Detergent Screen

Each detergent screen analyzes 12 detergents in parallel. Hence, in a 96-well-block, 8 different proteins in 12 different detergents can be analyzed simultaneously. This protocol will assume a full 96-well-block of 8 proteins against 12 detergents.

1. Prepare in advance the "extraction stock block" and the "purification stock block."
2. The "extraction stock block" is used for solubilization of the MPs and contains a stock of different detergents at 10–15% (w/v) (Table 2), such that when added to the membranes the final detergent concentration is 1% (w/v). To prepare the "extraction stock block," prepare 20 mL of each 10% (w/v) detergent stock and 30 mL of each 15% (w/v) detergent stock in distilled water according to Table 2.
3. Aliquot 1.8 mL of 10–15% (w/v) detergent into the appropriate well of a 96-deep-well block as shown by the plate map in Fig. 3.
4. The purification stock block contains detergent at 10x the final working concentration (as described in Table 3). This block is used to prepare the wash and elution buffers. Prepare 20 mL of detergent at 10× CMC in distilled water as described in Table 3.
5. Aliquot 1.8 mL of detergent at 10× CMC into the appropriate well of a 96-deep-well block as shown in the plate map in Fig. 3.
6. Add 900 μL of the resuspended membrane into a 96-deep-well block according to the arrangement shown in Fig. 3.
7. Using a multichannel pipette, add 100 μL of extraction detergent from the extraction stock block to the corresponding well in the block containing the membranes.
8. Shake the block at 450 rpm on a platform shaker or a rotator, 4 °C for 1 h.
9. Continue method from the start of Subheading 3.12.

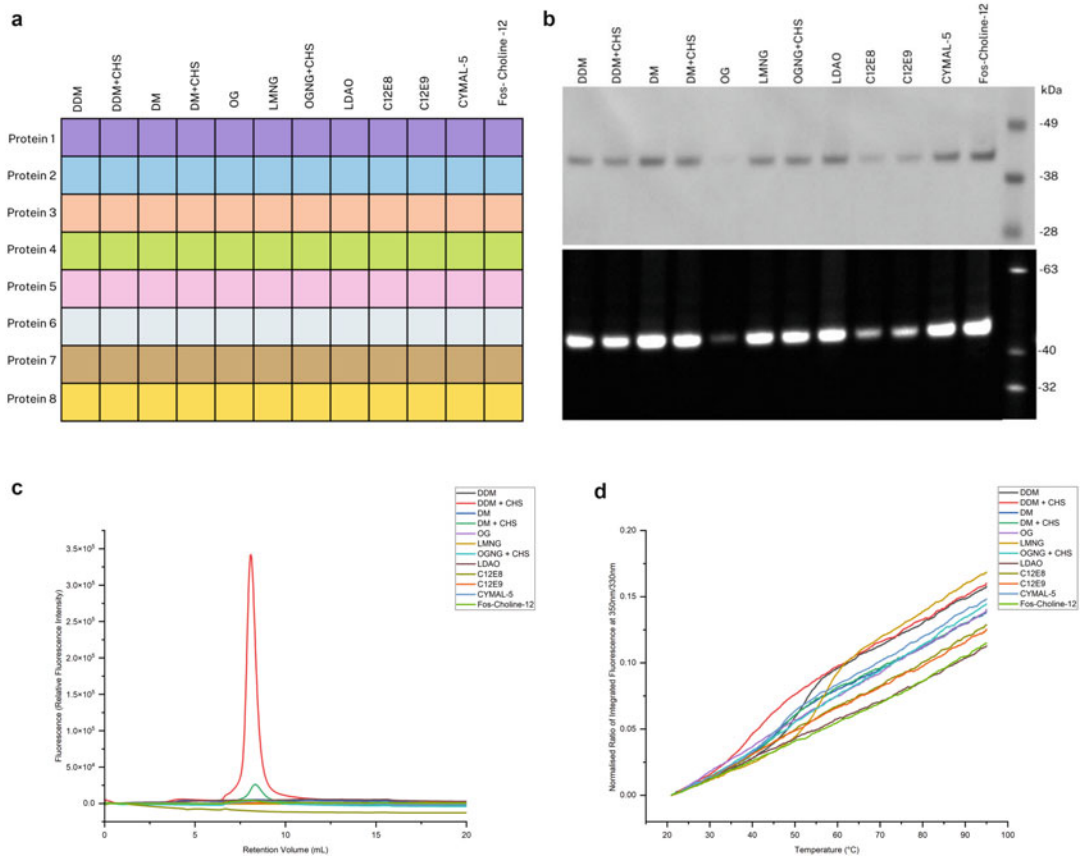

Fig. 3 Analysis of a typical detergent screen. (**a**) Detergent screening plate layout. Detergents were selected to cover major detergent classes used in structural biology. (**b**) Coomassie-stained and equivalent GFP fluorescence imaged protein bands of a target MP transporter purified in 12 different detergent-based conditions. Similar band intensities across each detergent indicate more stable membrane proteins. Comparison of CHS containing and non-containing bands provides information on the usefulness of this additive. (**c**) F-SEC analysis of a MP transporter purified in 12 different detergent-based conditions. (**d**) Thermostability of a MP transporter purified in 12 different detergent-based conditions

10. Select constructs for either further screening with the buffer/salt screen, or if suitable conditions are identified, select at least one construct for expression on a medium scale (Subheading 3.10).

3.9 Buffer/Salt Composition Screen

Each buffer/salt composition screen analyzes 12 conditions in parallel. Hence, in a 96-well-block, 8 different constructs in 12 different buffers can be analyzed simultaneously. This protocol will assume a full 96-well-block of 8 proteins against 12 buffers. For this screen, use the detergent in which your protein is most stable (i.e.,

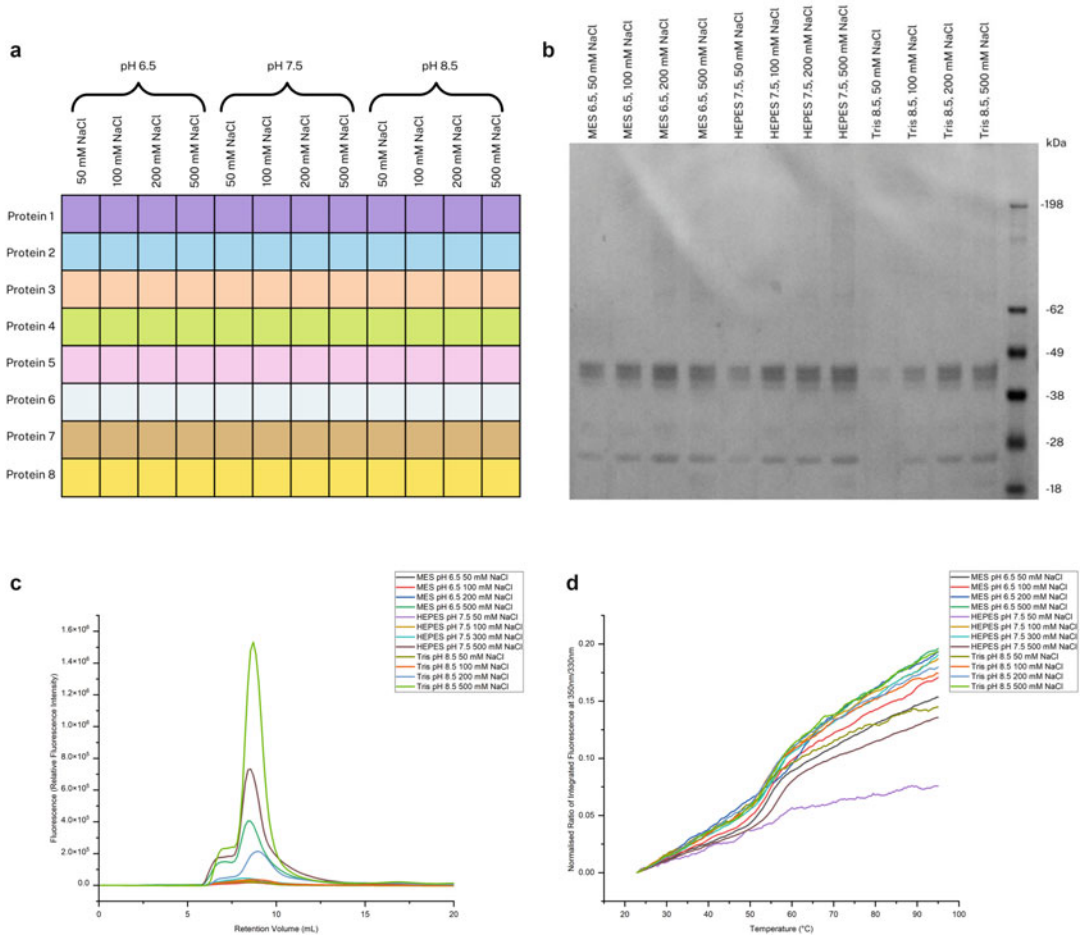

Fig. 4 Analysis of a typical buffer/salt screen. (**a**) Buffer/salt screening plate layout. Three commonly used buffers were selected covering commonly used pHs. (**b**) Coomassie-stained protein bands of a target MP transporter purified in 12 different buffer/salt-based conditions. Band intensities give an indication of success but are inconclusive alone. (**c**) F-SEC analysis of a MP transporter purified in 12 different buffer/salt-based conditions. (**d**) Thermostability of a MP transporter purified in 12 different buffer/salt-based conditions

the hit from the detergent screen). Please note that 50 mL of mammalian cell culture is required for this screen per construct.

1. Three stock blocks are required for the buffer/salt screen; lysis buffer block (no detergent), wash buffer block (including 3× CMC detergent) and an elution block (including 3 × CMC detergent).
2. Prepare 20 mL of each lysis, wash and elution buffers according to Table 3 as well as a 10 mL stock of preferred 10% detergent.
3. Pre-aliquot 1.8 mL of the relevant lysis, wash or elution buffer into the appropriate well of a 96-deep-well block as shown by the plate map in Fig. 4.

4. Stock blocks and detergent should be prepared 1 day in advance and stored at 4 °C overnight. If blocks are prepared further in advance, then store at −20 °C and defrost in the fridge the night before use (make sure individual wells are mixed well before use).
5. Aliquot 800 μL of lysis buffer from the wash buffer block to a 96-deep-well plate.
6. Add 100 μL of the resuspended membranes into the block containing the aliquoted lysis buffer such that membranes resuspended in MES pH 6.5 buffer go into wells corresponding to MES pH 6.5 buffer (regardless of the salt concentration) and so on, according to the arrangement shown in Fig. 4.
7. Add 100 μL of the 10% detergent stock and seal the block with a silicone sealing mat.
8. Shake the block at 450 rpm on a platform shaker or a rotator, 4 °C for 1 h.
9. Continue method from Subheading 3.12.
10. Select the best constructs for scaleup to medium scale (Subheading 3.10).

3.10 Transient Transfection and Expression in Expi293F™ Cells (Medium Scale)

This section can be used to scaleup MP expression to provide material for screens on single constructs against 96 different conditions or as an initial scaleup to test stable expression and purification conditions identified from the small-scale screens described in this chapter.

1. One day before transfection, seed 300 mL Expi293F™ cells at a cell density of 1.2×10^6 cells/mL in a 2000 mL non-baffled flask with porous filter seal.
2. On the day of the transfection, prepare the transfection mixture for each construct: for 300 mL scale, add 300 μg DNA and 1600 μL PEI MAX 40 K with 30 mL Opti-MEM™ serum-free medium. After thorough mixing, incubate the mixture for 10 min at RT and add gently (dropwise) to Expi293F™ cells (*see* **Note 26**).
3. Place cells immediately in shaking incubator and grow at 37 °C, 120–150 rpm, 5–8% CO_2, 80% humidity.
4. Protein expression can be boosted (within 20 h) through the addition of post-transfection supplements. Add the following supplements to a final concentration of 5 mM valproic acid, 6.5 mM sodium propionate and 0.9% glucose (*see* **Note 27**). The temperature may be dropped to 30 °C at this point, which improves expression of some MPs.
5. If a vector that incorporates a fluorescent label is used (e.g., GFP or mCherry), expression and localization can be tracked using a fluorescence microscope.

3.11 Lipid Additive Screen

Each lipid additive screen analyzes a single construct in 8 lipid conditions across 12 detergent conditions. For this screen, select one construct based on hits from the detergent screen.

1. Prepare in advance the "extraction stock block." This is the same block as in the detergent screen (Subheading 3.9).
2. Prepare in advance the "lipid purification block." This block contains detergent at 10× the final working concentration combined with lipids in a w/v ratio of 50:1 detergent-lipid in distilled water (as described in Table 4).
3. Note that the volumes of lipid stock will be very small, we recommend making up enough detergent and lipid for eight to ten screens and dividing them into separate deep-well blocks to be kept at −20 °C and defrosted immediately before use (*see* **Note 33**).
4. Add 900 μL of the resuspended membrane into a 96-deep-well block according to the arrangement shown in Fig. 5.
5. Using a multichannel pipette, add 100 μL of extraction detergent from the extraction stock block to the corresponding well in the block containing the membranes.
6. Shake the block at 450 rpm on a platform shaker or a rotator, 4 °C for 1 h.
7. Continue method from the start of Subheading 3.12. Example results are shown in Fig. 5.
8. Select the best constructs for scaleup to medium scale (Subheading 3.10).

3.12 Block-Based Purification

1. Equilibrate the TALON resin before starting the experiment. Add the required volume of 50% TALON resin into a Falcon® tube and centrifuge at 300 × *g* for 2 min at 4 °C and discard the ethanol. Wash the resin twice by filling the tube with distilled water, inverting a few times, and centrifuging at 300 × *g* for 2 min. Wash with lysis buffer (without detergent) three times using the same method. Finally, add an equal volume of lysis buffer and store at 4 °C until use.
2. Centrifuge the block containing solubilized membrane at 3500 × *g* for 30 min at 4 °C and carefully transfer the supernatant into a new deep-well block.
3. Add 100 μL of equilibrated 50% TALON into each well. Make sure to cut the ends of the pipette tips when adding the resin as uncut tips could get clogged and lead to uneven distribution of resin.

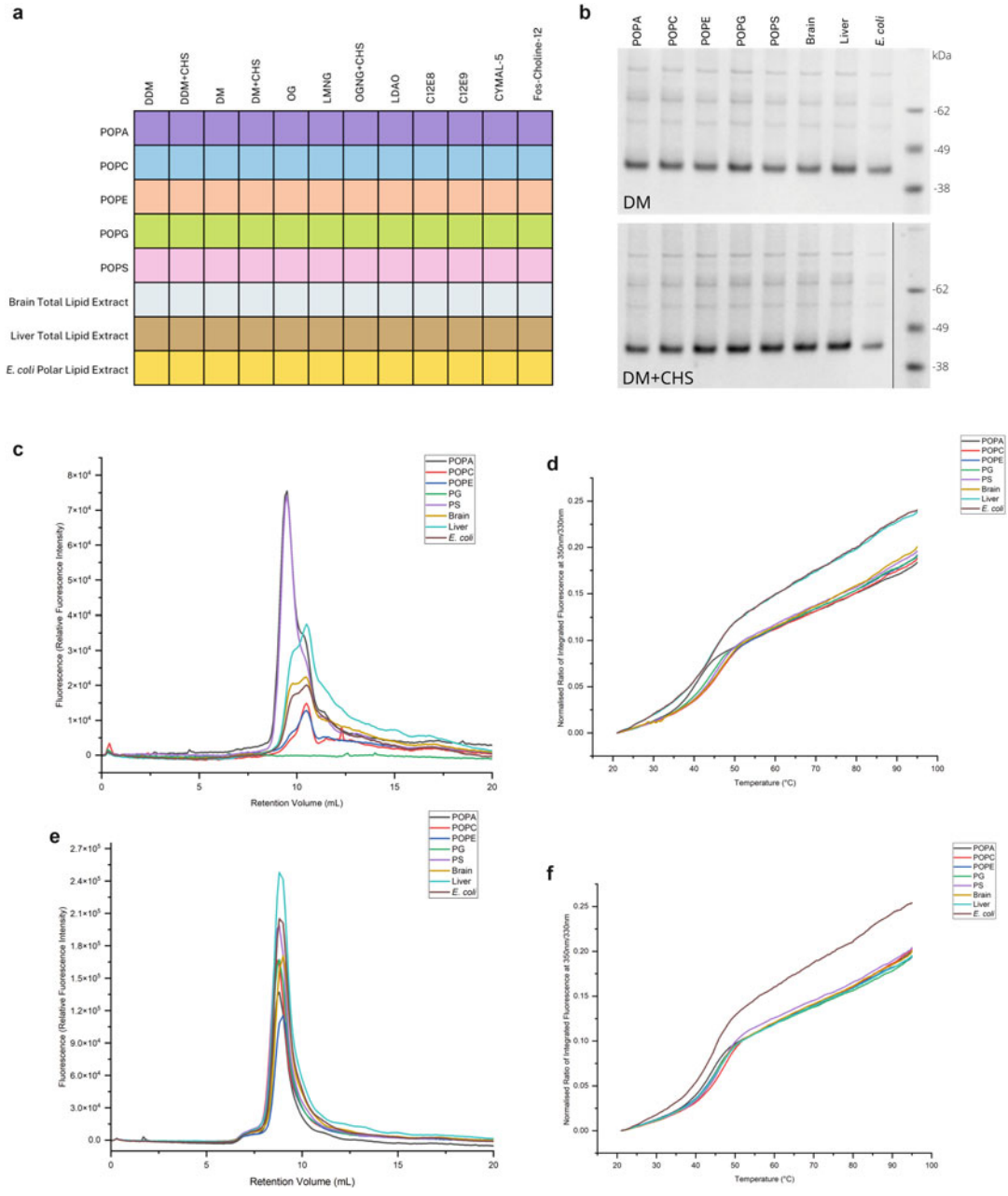

Fig. 5 Analysis of a typical lipid additive screen. (**a**) Detergent screening plate layout. Commonly used individual or mixed lipids were selected for this screen. (**b**) Coomassie-stained protein bands of a target MP transporter purified in the presence of eight different lipid combinations. Band intensities given an indication of success but are inconclusive alone. Comparison of CHS containing and non-containing provide information of usefulness of additive. (**c**) F-SEC analysis of a MP transporter purified in DM in the presence of eight different lipid combinations. (**d**) Thermostability of a MP transporter purified in DM in the presence of eight different lipid combinations. (**e**) F-SEC analysis of a MP transporter purified in DM + CHS in the presence of eight different lipid combinations. (**f**) Thermostability of a MP transporter purified in DM + CHS in the presence of eight different lipid combinations

4. Cover the block with a silicone sealing mat and let the resin bind for 1 h at 4 °C on a rotator at slow speed or shaking at 1000 rpm on a platform shaker.
5. Place a 96-well filter plate on top of a waste deep-well block. Transfer the solubilized membrane plus resin mixture to the filter plate using 1 mL pipette tips, which have had the end of the tip cut off. Collect the flow-through in the waste block.
6. Prepare a deep-well block of wash buffer by adding 1.35 mL of wash buffer and 150 μL of detergent from the purification stock block (or lipid purification block if performing a lipid screen).
7. Place the filter plate onto a new collection block and tape together using masking tape.
8. Pipette 500 μL of wash buffer (with detergent; and detergent + lipid if performing a lipid screen) into the filter plate and centrifuge at 300 × *g* for 1 min at 4 °C. Repeat this wash step two more times.
9. Discard the flow-through and spin the plate at 500 × *g* for 3 min at 4 °C to remove any excess wash buffer.
10. Prepare a microtiter plate with 90 μL elution buffer and add 10 μL of detergent from the purification stock block (or lipid purification block if performing a lipid screen).
11. Place the filter plate on top of a clean microtiter plate and add 50 μL of elution buffer directly to the resin and seal the plate with a foil seal and incubate for 20 min at 4 °C while shaking at 450 rpm on a platform shaker.
12. Remove the seal and centrifuge at 500 × *g* at 4 °C for 3 min to elute the protein.
13. Mix 10 μL of sample with appropriate amount of loading dye and run the samples on an SDS-PAGE (Subheading 3.14).
14. 20 μL of eluted sample should be analyzed by FSEC (Subheading 3.14)
15. 10 μL of eluted sample should be kept for nano-DSF analysis (Subheading 3.15).

3.13 SDS-PAGE Analysis

1. Prepare four SDS-PAGE pre-cast gels (enough for 96 samples) and 1 × MES SDS-PAGE running buffer in the gel tank. Ensure combs are removed and wells rinsed with running buffer.
2. In a 96-well PCR plate, using a multichannel pipette, mix 10 μL of the block eluate with 10 μL of 2x loading dye. Do not heat denature samples.
3. Load 10 μL of each sample onto a 26-well NuPAGE SDS-PAGE gel (*see* **Note 34**).

4. Add 3 μL of SeeBlue™ protein standards (or similar) and 2 μL of the fluorescent protein ladder to appropriate wells.
5. Run the gel at 160 V for 1.5 h or until the dye-front has reached the bottom of the gel (*see* **Note 35**).
6. Break open the cassette and visualize In-Gel GFP fluorescence of target proteins using an imaging system supplied with illumination source and filters compatible with the excitation (485 nm) and emission (525 nm) of GFP (*see* **Note 36**).
7. Carefully rinse the gel in water taking care not to tear the gel staining with ~20 mL of InstantBlue. Stain the gel for around 1 h.
8. Pour off the stain into a fresh bottle and store at 4 °C (the stain can be reused a further one to two times) and rinse the gel in water taking care not to tear the gel.
9. De-stain overnight in water before using a gel imaging system to capture an image of the gel.
10. Check if the protein bands have run as expected based upon expected size and rate the band intensities as either none, low, medium, or high (*see* **Note 37**).

3.14 Size Exclusion Chromatography Analysis

1. Centrifuge elution plate at 4500 × *g* for 15 min at 4 °C and transfer 20 μL into a fresh microtiter plate. Seal with a pierceable seal (*see* **Note 38**).
2. Inject 10 μL of eluate onto an SRT-C-300 HPLC column (20 mL) connected to a 3 mL SRT-C 300 guard column (*see* **Note 39**). This can be done automatically and efficiently using an auto-sampler capable of taking 96-well microtiter plates. Include a 1 in 10 diluted gel filtration standard as the first (after a blank run) and last samples on the run list.
3. Run samples at a flowrate 1.0 mL/min at 4 °C. Ensure that the pressure on the column does not go above 250 bar.
4. Monitor UV absorbance as well as GFP (Ex. 488 nm, em. 507 nm) or tryptophan (ex. 280 nm, em. 350 nm) fluorescence depending on the construct(s) characteristics (*see* **Note 40**).
5. Analyze traces in terms of peak area, elution profile, and volume to get information on (i) expression level, (ii) the degree of monodispersity, and (iii) the approximate molecular mass (*see* **Note 41**).
6. Monodisperse and folded proteins will yield a single symmetrical peak and polydisperse, unstable, or unfolded proteins will yield multiple asymmetric peaks. Give your profile a score, e.g., good (monodisperse, good yield, correct size), acceptable (multiple peaks, acceptable yield, correct size), or poor (no peak or broad profile, low yield) to aid interpretation based upon these parameters.

3.15 Nano-differential Scanning Fluorimetry

1. Measure the concentration of each elution using a multi-well nanodrop or similar.
2. Adjust concentrations so that they are similar for each protein construct by diluting with the relevant detergent containing elution buffer (*see* **Note 42**).
3. Clean the mirror using 100% ethanol and a lint-free tissue.
4. Load ~10 μL (until the capillary is full) into a standard glass capillary and place in position on the Prometheus platform. Be careful not to draw sample up the outside of the capillary (*see* **Note 43**).
5. Depending on the version of the Prometheus, capillaries are supplied as singles or in chips of 24. Up to 48 samples can be run at once using the single capillaries.
6. Establish sensible setting to run samples ensuring the fluorescence intensity at 330/350 nm is above 5000 but below 10,000 counts if possible (*see* **Note 44**).
7. Apply thermal melt parameters of 1 °C per second. Run time should be around 1 h and 15 min (*see* **Note 45**).
8. Under the annotation and result tab, record the conditions for each sample. Additional columns can be added so that construct, buffer components, detergent, and any additives can be added easily to the table.
9. After the run has completed, scan through each trace, ensure that the calculated $Tm_{½}$ makes sense based upon the first derivative profile. Add or remove transition points as required (*see* **Note 46**).

3.16 Data Interpretation

1. Use a spreadsheet to collate all of your data together right from the initial cloning experiments. Include tabs recording protein and DNA sequence, cloning plate maps including vector, primer sequences and domain boundaries, and a tracking sheet detailing whether a construct has been successful at each stage in the process.
2. Tabulating the combined scores from SDS-PAGE, SEC, and nano-DSF enables samples to be ranked from most successful to least successful and therefore which construct or condition to follow up. An ideal construct will have a high-intensity band by SDS-PAGE, a monodisperse profile and $Tm_{½}$ higher than 40 °C. Low-yielding constructs that are monodisperse and thermostable should not be ignored, i.e., do not focus on just the high-yielding conditions.

4 Notes

1. Gene fragments are sequence verified but not clonal; thus, any one copy may contain an error (mutation). It is important to sequence the assembled and cloned gene prior to using it as a template for high-throughput cloning. Commercially available high-fidelity versions reduce the error rate but at increased cost.
2. Vector linearization: it is not necessary to purify the cut vector after restriction digest. If possible, the enzyme(s) can be heat-inactivated (enzyme dependent) and the cut plasmid used directly in the cloning reaction. If heat inactivation is not possible, the DNA can be purified using a PCR purification kit. Gel extraction of the cut vector is not necessary for this type of cloning.
3. We use either a 0.22 μm syringe or cup filter depending on scale.
4. We have used both types of platforms for this screen but find the rotator is better at maintaining the TALON resin in suspension. When using the rotator, the silicone-sealed deep-well block should be taped to a second using masking tape to stop the silicone seal failing. On our instrument we use the slowest speed setting (below 0) to enable gentle mixing.
5. Although other commercial cobalt resins are available on the market, we have found that TALON is superior for our screening and purification purposes.
6. The Sepax SRT-C range of columns are tolerant of pH up to 8.5 (and briefly at pH 9.5) enabling broad pH screens to be carried out. For large complexes the SRT-C SEC-500 may be considered. We use a 7.8 × 50 mm length column as a guard column, helping to maximize the life of the 7.8 × 300 mm column. It is important to follow the recommended cleaning protocol from the manufacturer regularly.
7. We use a modular Shimadzu HPLC with an autosampler adapted for taking microtiter plates, 10 solvent lines, 2 column positions, a UV absorbance detector, fluorescence detector (up to four different wavelengths can be collected in parallel), and fraction collector.
8. We drop the buffer and salt concentration (except for the buffer/salt screen) and remove glycerol at this step to replicate more ideal purification conditions for preparing a MP for crystallization or cryo-EM. However, changing the purification buffer can affect the stability of the MP target, and you may wish to maintain the same buffer conditions used earlier in the purification process. Detergent concentration is also reduced

here, helping to reduce costs as well as minimizing the amount of detergent in the final sample. Do not drop the detergent concentration below 1.5× CMC.

9. There are other ways of assessing MP thermostability if a Prometheus is not available. Many laboratories have access to RT-PCR machines that can be used along with MP-compatible dyes [13–15].
10. Making a "master mix" of all the common reagents is convenient and reduces the possibility of pipetting errors. We typically use Phusion Flash polymerase; other proofreading polymerases, e.g., Q5, KOD, CloneAmp, may be used. Taq polymerase is not suitable for this type of cloning. Do not use buffers containing chelating agents such as EDTA as these will inhibit the Mg^{2+}-dependent activity of the DNA polymerases to be used. Scaled-down volumes can be used for the PCRs (12.5 μL).
11. If there is only one template, then add the DNA to the master mix; similarly, if there are two, make up two master mixes, one for each template (in this case you would aliquot 22 μL per well).
12. Agarose gels: we use high-throughput E-gels with SYBR-safe from Invitrogen which are ready-made cassettes allowing loading of up to 48 or 96 samples using a multichannel.
13. Note the DpnI digest requirement if the PCR template vector has the same antibiotic selection as the recipient plasmid. The DpnI can be heat-inactivated if not purifying the PCR before cloning.
14. We use the ClonExpress II as it is cheaper than In-Fusion. After the cloning reaction, there is no need to add stop solution, just put on ice/store frozen and use 2 μL to transform competent *E. coli*. Scaled-down volumes can be used for the cloning reactions (10 μL or 5 μL). These are half or a quarter of the manufacturer's recommended volumes.
15. We do not use EDTA stop solution, so reactions must be kept cold to avoid the reaction proceeding for longer than the optimal 30 min.
16. The cloning is usually so efficient this way that we can use in-house prepared $CaCl_2$ competent *E. coli* (we use TOP10; DH5alpha, etc. would also be suitable). Commercial high-efficiency Stellar or OmniMax require dilution of ~1/100 before plating to ensure single colonies can be picked.
17. If you are using a TOP10 strain, the IPTG can be omitted. 12-well or 6-well plates may be used instead of 24-well plates. You may also have to adapt certain steps according to equipment availability, e.g., you may not have expanding

multichannel pipettes and agarose gel systems which can be loaded with a multichannel.

18. Picking two colonies will normally give positive clones for ~96% of the PCR products. At this point blue colonies should constitute <<10% if the reactions were successful. The blue colonies are derived from inefficiently linearized parental plasmid and are non-"recombinant." Leaving the tips in the "picked" well until the plate is complete is a good memory aid and prevents "double picking."
19. Note regarding small colonies: depending on the cloned insert, sometimes there can be a mixture of colony sizes, e.g., some normal-size blue and white colonies plus some smaller white colonies. If these smaller colonies are well isolated (i.e., not satellite colonies clustered around the bigger colonies), then they are likely to have the insert, the slower growth being caused by leaky expression of the protein from the high-copy plasmid which has the pUC origin of replication. Since the pUC high-copy mutation is temperature-sensitive [16], it is possible to suppress it by lowering the growth temperature to 30 °C which may give more reliable plasmid preps in these cases but yields would be lower than usual.
20. Glycerol stocks are a convenient and stable archive format and can save time if plasmids require re-prepping at a later date.
21. DNA preparation kits are chosen according to scale required. Miniprep for 3 mL scale; maxiprep for 30 and 300 mL scale; gigaprep for up to 10 L large-scale expressions. Choose endotoxin-free kits where possible and try to maintain sterility after the ethanol wash step (filter-sterilize the elution buffer and work in laminar flow hood as far as possible). We previously used enriched medium (Power Broth) for the miniprep cultures, but this often-caused clogging of the SV96 filter plate, so we now use LB to avoid this. Plasmids prepared by this method are suitable for use in *E. coli* transformations, construct verification, and HEK transfections.
22. The pOPIN Forward primer is based on the T7 forward priming sequence and is present in most pOPIN vectors (gac cga aat taa tac gac tca cta tag gg).
23. Avoid growing maintenance stocks to high density ($>5 \times 10^6$ cells/ mL) for routing sub-culturing as it may reduce protein yields.
24. The recommended CO_2 concentration for Expi293F is 8% but it is possible to use 5%. Depending on the model of incubator, humidity may not be controllable but it should be 80% or more.

25. Suitably sized flask: This will depend on the volume of cell required for transfection the following day. For example, 48 different constructs will require almost 300 mL of cell culture for which we would use a 2 L flask. We do not aliquot cell into the block the day before to minimize evaporation in the blocks (even in humid environments).
26. Do not mix DNA and PEI directly as they will immediately precipitate.
27. Valproic acid and sodium propionate are histone deacetylase inhibitors (iHDACs) which can increase gene expression levels through interfering with the regulation of normal cellular acetylation of histones. The combined use of valproic acid, sodium propionate, and glucose substantially enhances gene expression [17, 18].
28. Localization of MPs as they express can be a used as a guide to expected success. If membrane proteins that should be located in the plasma membrane are instead observed accumulating within the cell, then there could be issues with the stability of such proteins.
29. This may not be the optimal harvest point for all MP constructs but is a good time-point at which most MPs will have expressed. Specific optimization of expression conditions is something that should be investigated later if higher protein yields are required [12].
30. Although the protocol described here uses TALON resin, this is not essential. For IMAC purification we recommend TALON over Ni-NTA in order to improve the purity of the target MP [19]. Equally if constructs have FLAG or twin-Strep tags, then using M2 FLAG resin or Strep-Tactin XT works well.
31. To avoid disturbing pelleted material, tilt the plate and insert pipette tips so that they touch the side of the plate. Stop when the tips reach the side of the well where the bottom tapers away. Gently pipette up the liquid and transfer into a new plate. Do not pipette up and down or go back into the same wells. If this happens repeat the centrifugation step.
32. Subheading 3.12 describes how to complete the block-based purification while each analytical tool is described in Subheadings 3.13, 3.14, 3.15 and 3.16. Figure 2 illustrates a set of example results for a small-scale test expression and purification. DDM is a mild detergent that successfully extracts and maintains the stability of many MPs in solution. FC-12 is a harsh detergent that will additionally extract unfolded MPs. Thus, a comparison of the target MP SDS-PAGE band intensities from equivalent purification in FC-12 and DDM or other mild detergents can be used as a crude measure of identifying

stabilizing detergent-based conditions. For example, in instances where SDS-PAGE-stained protein band intensities between the mild detergent and FC-12 purified MPs are similar or the mild detergent is weaker, it is likely that the construct and solvent conditions related to the mild detergent are stabilizing (e.g., construct 4 in Fig. 2). If the FC-12 band is significantly stronger (e.g., construct 3 in Fig. 2), then the construct and solvent conditions related to the mild detergent are likely to be destabilizing. In this case this construct has a broad SEC profile beginning at the void volume of the column supporting this conclusion, and subsequent scaleup resulted in aggregated protein. The extraction and purification stock blocks should be prepared at least 1 day in advance and stored at 4 °C overnight. If the blocks are prepared further in advance, then store at −20 °C and defrost in the fridge the night before use (make sure they are mixed well before use).

33. Phospholipids should not be stored for long periods of time as aqueous suspensions or be repeatedly frozen and thawed. Storage of lipids in an excess of water results in hydrolysis of the sample.
34. A multichannel adjustable spacer pipette is recommended to dispense sample into the wells of the SDS-PAGE gel.
35. GFP and mCherry will continue to fluoresce, even on an SDS-PAGE gel as long as the SDS-PAGE gel does not get too hot. You could consider running your gels at a lower voltage or in the cold room if you have a problem with GFP denaturation.
36. If using a mCherry fusion construct, then the illumination source and filters should be compatible with excitation at 587 nm and emission at 610 nm.
37. Membrane proteins will typically run 10–30% smaller than expected on an SDS-PAGE gel due to differences in ability to bind to SDS compared to soluble proteins [20]. Comparison of band intensities between conditions can provide valuable information of overall protein stability and likely success. It is important that analyses are not overly reliant on SDS-PAGE gels; a band on a gel alone does not indicate a successful construct. Often the best conditions are also not the ones that give the highest yields by SDS-PAGE or SEC. If possible, mass spectrometric analysis of each construct should also be conducted to confirm the molecular weight of the protein (especially after the first test expression) to ensure there are no mass discrepancies indicating posttranslational modifications, mutations, or artifacts from the cloning process. In-gel tryptic digest MSMS analysis can be used to confirm the identity of each protein band [21].

38. If microtiter plates are unavailable, then vials can be used. By using a suitable rack and width-adjustable multichannel pipette, this transfer can be made more straightforward and less time-consuming. Using our HPLC system, up to 10 different solvent conditions can be screened in one run. A second run will be required for more than 10 solvent conditions.
39. The Sepax SRT-300 is an ideal column to use; it has a similar profile to the Superdex 200 10/300 but contains a matrix which is durable between pH 3.5 and 8.5 and occasional use at pH 9.5. Additional surface chemistry makes handling hydrophobic detergents more straightforward.
40. Tryptophan fluorescence can be useful for constructs that do not have fluorescent tags where yields are low but successful results depend on having a clean purification as well as the absence of components that quench fluorescence.
41. We recommend grouping screen components together either by row (construct/ lipid) or by column (detergent/ buffer) to aid interpretation.
42. To enable comparisons between different protein constructs, samples for nano-DSF should analyzed at the same concentration. We recommend samples should be between 1 and 10 μM, but this will be dependent on purification yield.
43. When loading samples into capillaries, we recommend aliquoting your purified MP of interest into a PCR plate. Tip the capillaries into the cap of the capillary container and tilt the PCR plate on its side so that the capillary is inserted horizontally to the well. Capillaries should be mostly filled. Avoid air bubbles as these will disrupt your signal.
44. This range is recommended but not essential. Many proteins will give lower levels of expression or may have a low extinction coefficient. We have successfully obtained data from sample with a signal response of less than 500 fluorescence units.
45. We typically use a 1 °C per min melt but you can also choose to ramp up the temperature faster if short on time. We have obtained successful data from a 7 °C per min melt that correlates well with the parameters described here.
46. The calculated Tm½ should ideally be above 40 °C. Constructs below this will likely require further optimization either through the modification of the buffer or addition of suitable additives such as lipids or know substrates or inhibitors. It is common not to observe a suitable response either because the concentration is too low or the protein is not in an optimal condition for nanoDSF analysis. In these cases, use the SDS-PAGE and F-SEC data to select the best constructs.

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