Chapter 8

Screening for High-Yielding *Saccharomyces cerevisiae* Clones: Using a Green Fluorescent Protein Fusion Strategy in the Production of Membrane Proteins

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Abstract

The overproduction of eukaryotic membrane proteins in milligram quantities is a major bottleneck for their further biochemical and structural investigation. Production trials exploring a range of input factors can be rationalized to improve the likelihood of success. Here we discuss some of these factors in combination with the use of a GFP-based *Saccharomyces cerevisiae* system that enables a quick turnaround time from clone construction to production trials. Since membrane-integrated levels do not necessarily correlate with the amount of functional recombinant protein, we also include the use of fluorescence-detection size exclusion chromatography (FSEC). Using FSEC, the quality of the recombinant material can also be rapidly evaluated as demonstrated for the functional production of the rat vesicular glutamate transporter (VGLUT2) and the human glucose transporter (GLUT1) (5).

Key words: Membrane protein, Overproduction, *S. cerevisiae*, Fluorescence-detection size exclusion chromatography

1. Introduction

Membrane protein overproduction is an empirically based approach where many parameters need to be tested and re-tested. Monitoring by fluorescence enables this process to be carried out quickly, efficiently, and reliably. For this purpose, we use GFP-based fusion technology. As a carboxy-terminal GFP tag will only fold and becomes fluorescent if the upstream membrane protein integrates into the membrane, the resultant fluorescence is a fast and accurate measure of membrane-integrated production (1). Fluorescence is easy to measure directly in liquid culture, standard SDS-gels and

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in detergent-solubilized membranes (2, 3). Detergent-solubilized membranes can also be further subjected to fluorescence size-exclusion chromatography (FSEC) to measure the "monodispersity" of the sample. This is an ideal way to evaluate the quality of the material produced. In short, although the amount of membrane-integrated production is no guarantee that the recombinant protein is functional, the GFP-tag speeds up the empirical process.

We have constructed a reliable protocol for screening the overproduction and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae* (4). This system was adapted from a GFP-based *Escherichia coli* pipeline (3) because yeast possess features absent in *E. coli* that are often essential for producing functional eukaryotic membrane proteins. With similar costs to *E. coli* and the possibility to clone into standard vectors by homologous recombination, *S. cerevisiae* is a convenient and efficient production host. For this reason we prefer to screen and optimize in this yeast rather than *Pichia pastoris*. In addition, because final cell densities are generally in the range of 6.0–8.5 at OD_{600}, rather than around 60–80 for *P. pastoris*, production of 1 mg/L recombinant protein in *S. cerevisiae* represents a larger fraction of total protein that would be the case in *P. pastoris*: a higher fraction of recombinant protein ensures better recovery of it in a purer form. From an analysis of ~150 eukaryotic membrane protein–GFP fusions, we found that around one quarter can be overproduced to >1 mg/L. Of the highly produced eukaryotic membrane proteins in *S. cerevisiae*, more than half of those tested were targeted to the correct organelle and were monodisperse in a mild detergent such as dodecyl-β-D-maltopyranoside (DDM).

Here, we describe in detail the practical steps that constitute our *S. cerevisiae* GFP-based pipeline. This comprises (1) cloning by homologous recombination, (2) whole-cell and in-gel fluorescence for estimating production yields and (3) FSEC for judging the quality of the recombinant material. In Chapter 18, we expand on these methods for large-scale production and purification.

2. Materials

1. Expression vectors (see Chapter 4).
2. Materials for yeast transformation (see Chapter 4).
3. PCR reagents (available from a wide range of suppliers).
4. *Sma*I restriction enzyme (Invitrogen).
5. Growth medium without uracil (for 1 L, 6.7 g yeast nitrogen base without amino acids (BD Difco, cat. No. 291920)), 2 gt yeast synthetic drop-out medium supplement without uracil

(Sigma, cat. No. Y1501), and either 2% glucose (for pre-culture) or 0.1% glucose (for expression culture)). For plates, add 20 g of bacto agar (Sigma, cat No. A5306). D-(+) glucose can be purchased from Sigma (cat. No. G7021).

6. 20% galactose (w/v) (Sigma).
7. YSB (yeast suspension buffer): 50 mM Tris–HCl (pH 7.6), 5 mM EDTA, 10% glycerol, 1× complete protease inhibitor cocktail tablets.
8. Nunc 96-well black optical-bottom plates (Nunc).
9. SpectraMax M2e microplate reader (Molecular Devices).
10. Acid-washed glass beads, 500 μm (Sigma).
11. TissueLyser mixer (Qiagen).
12. Benchtop ultracentrifuge, Beckman Coulter Optima MAX series with TLA-55 and TLA-120.1 rotors (Beckman).
13. 1.5-mL polyallomer microcentrifuge tubes (Beckman).
14. SB (sample buffer) for in-gel fluorescence: 50 mM Tris–HCl (pH 7.6), 5% glycerol, 5 mM EDTA (pH 8.0), 4% SDS, 50 mM DTT, 0.02% bromophenol blue.
15. Tris-glycine SDS gels.
16. Fluorescent protein standard (Invitrogen).
17. Pre-stained protein standard (Invitrogen).
18. LAS-1000-3000 charge-coupled device (CCD) imaging system (Fujifilm).
19. Coomassie brilliant blue R-250 (Sigma).
20. CRB (cell resuspension buffer): 50 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.6 M sorbitol.
21. Constant Systems TS series cell disruptor (Constant Systems).
22. MRB (membrane resuspension buffer): 20 mM Tris–HCl (pH 7.6), 0.3 M sucrose, 0.1 mM $CaCl_2$.
23. Bicinchoninic acid (BCA) protein assay kit (Pierce).
24. PBS (phosphate buffer saline): For 1 L, 1.44 g $Na_2HPO_4{\cdot}2H_2O$ (8.1 mM phosphate), 0.25 g KH_2HPO_4 (1.9 mM phosphate), 8 g NaCl, 0.2 g KCl, adjust pH to 7.4 using 1 M NaOH or HCl.
25. Dodecylnonaoxyethylene ether (C12E9; Anatrace).
26. *N*,*N*-Dimethyldodecylamine *N*-oxide (LDAO; Anatrace).
27. Cholesteryl hemisuccinate; Tris salt (CHS; Sigma).
28. *n*-Dodecyl-β-D-maltopyranoside (DDM; Anatrace).
29. Superose 6 10/300 GL Tricorn gel filtration column (GE Healthcare).

30. Äkta FPLC system (GE Healthcare).
31. Frac-950 fraction collector with rack C (GE Healthcare).
32. 50 mL aerated capped tubes (Techno Plastic Products (TPP)).

3. Methods

3.1. Rationalizing the Construct Design and Cloning of Membrane Protein-Encoding Gene(s) into a GFP-His_8-Containing Vector

1. Analyze the membrane protein sequence for regions of disorder using the algorithm RONN (http://www.strubi.ox.ac.uk/RONN). Consider designing amino- or carboxy-terminal truncations based on this output. To guide construct design, compare the analysis with the known or predicted topology (see TMHMM (http://www.cbs.dtu.dk/services/TMHMM/)), and also to sequence alignment with close homologues by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/)). See Note 1.
2. As outlined in Chapter 4, create a 2μ vector that codes for yEGFP-His_8 and contains a site for protease cleavage, e.g. TEV protease (see Note 2).
3. Amplify the cDNA clone of interest with primers that contain approximately 35-bp complementary 5′ overhangs to the *Sma*I-linearized GFP-fusion vector (see Chapter 4).
4. Transform *S. cerevisiae* competent cells with 3 μL PCR product and 5 μL linearized vector (see Note 3).

3.2. Measuring Yields by Whole-Cell and In-Gel Fluorescence

1. Inoculate 10 mL growth medium without uracil plus 2% glucose with a single colony in an aerated 50-mL tube (see Note 4).
2. Incubate the culture overnight in an orbital shaker at 30°C, 280 rpm.
3. Spot 10 μL of the overnight culture onto a fresh plate without uracil, allow the spot to dry at room temperature and transfer the plate to a 30°C incubator for 1–2 days.
4. Dilute the overnight culture (from step 2) to OD_{600} 0.12 in two 50-mL aerated tubes, each containing 10 mL growth medium without uracil plus 0.1% glucose (see Note 5).
5. Incubate the cultures in an orbital shaker at 30°C, 280 rpm. At OD_{600} 0.6 (after approximately 7 h), induce production of the membrane protein–GFP fusion by adding 20% (w/v) galactose to achieve a final concentration of 2% (see Note 6).
6. 22 h post-induction, centrifuge the cells at 3,000 × *g* for 5 min, remove the supernatant and resuspend the cell pellet in 200 μL YSB (see Note 7).

Table 1
Membrane protein yield estimates from whole cells

1. Harvest 10 mL yeast cells that have been cultured with and without galactose addition (to estimate background fluorescence), remove supernatant and resuspend in 200 µL YSB
2. Measure the GFP fluorescence *For example*, with no galactose (MP-GFP – GAL) = 3,000 relative fluorescence units (RFU). With galactose (MP-GFP + GAL) = 32,000 RFU
3. Correlate the whole-cell fluorescence with the amount of GFP produced by measuring the fluorescence of a defined concentration of yeast-enhanced green fluorescent protein (yEGFP) in a final volume of 200 µL *For example*, in our plate reader, fluorescence of pure yEGFP at a concentration of 0.03 mg/mL is 11,300 RFU
4. Determine the concentration of GFP in 200 µL cell culture by dividing by 40 (i.e., 8,000 µL (cell culture)/200 µL (resuspension volume)). Note that although the initial cell culture volume was 10 mL, there is an effective 2 mL loss through the transfer of only 200 µL of the resuspended cells (200 µL buffer + cell pellet = 250 µL) to the 96-well plate *Using the above example*: ((32,000 – 3,000)/11,300) × 0.03/40 = 0.001 9 mg/mL, which equates to an expression yield of 1.9 mg/L
5. As the typical recovery of GFP fluorescence from 1 L culture into membranes is 60%, multiply the above number by 0.6: 1.9 mg/L × 0.6 = 1.1 mg/L
6. To calculate the amount of membrane protein, multiply the above number by the molecular weight of the membrane protein/GFP (28 kDa) *For example*: a 56-kDa membrane protein with 32,000 RFU; 1.1 mg/L × (56/28) kDa = 2.2 mg/L

7. Transfer 200 µL of the cell suspension to a black Nunc 96-well optical-bottom plate (see Note 8).
8. Measure the GFP fluorescence emission at 512 nm following excitation at 488 nm in a microplate spectrofluorometer. For plate readers with a bottom-read option, choose this setting. Estimate membrane protein yield (in mg/L) from the yeast whole-cell fluorescence reading by applying the methodology detailed in Table 1.
9. Transfer the cell suspension from the 96-well plate into a 1.5-mL capped tube.

10. Add glass beads so that the final volume including the cell suspension is 500 µL. Add an additional 500 µL YSB.
11. Break the yeast cells with a mixer-mill disruptor set at 30 Hz for 7 min at 4°C. Alternatively, a vortexer can be used, but we recommend using a heavy-duty disruptor, as cell breakage is more efficient, reproducible, and easier to scale up.
12. Remove unbroken cells by centrifugation at 22,000 × *g* in a desktop centrifuge for 5 s at 4°C. Transfer 500 µL supernatant into a new tube. Add 500 µL YSB to the mixture of unbroken cell pellet and glass beads. Repeat step 11 and transfer the supernatant to the 500 µL batch obtained from the first round of cell breakage.
13. To pellet crude membranes, centrifuge the 1 mL supernatant from step 12 at 20,000 × *g* in a desktop centrifuge at 4°C for 1 h. Alternatively, the supernatant can be centrifuged using a desktop ultracentrifuge (120,000 × *g* for 1 h). However, we find the recovery from centrifugation in a desktop centrifuge is sufficient for this analysis and, as the final pellet is less compact, it is easier to resuspend (step 14).
14. Resuspend crude membranes in 50 µL YSB and transfer 15 µL into a tube containing 15 µL SB. Load 10 µL for SDS-PAGE. Include non-fluorescent and fluorescent protein standards, such as Benchmark Fluorescent and SeeBlue Plus Prestained standards (both from Invitrogen), respectively. For this step, we recommend our SB composition with standard SDS denaturing cast gels for in-gel fluorescence. We have also tested pre-cast Criterion (Bio-Rad) and Tris–Gly gels with equal success. We have found that the NuPAGE gels (Invitrogen) are not compatible with in-gel fluorescence (see Note 9).
15. Rinse the SDS gel with de-ionized H_2O and detect the fluorescent bands with a CCD camera system. Expose the gel to blue light (EPI source) set at 460 nm with a cut-off filter of 515 nm. Capture images and increase the exposure time until the fluorescent bands are clearly visible (see Note 10).
16. Analyze the gel and compare the size of the bands to the protein standards. If two closely spaced bands are present, this could indicate that the protein is glycosylated (see Fig. 1). In this case, analyze the sequence for N-linked glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/; see Note 11).
17. Stain the gel with Coomassie Brilliant Blue and transfer to destain (see Note 12).

3.3. Estimating the Quality of the Recombinant Protein by FSEC

1. Inoculate 10 mL growth medium without uracil with the spotted yeast culture from step 3 of Subheading 3.2 and incubate overnight.
2. The next day, add the overnight culture to a 500-mL shake flask containing 150 mL growth medium without uracil and

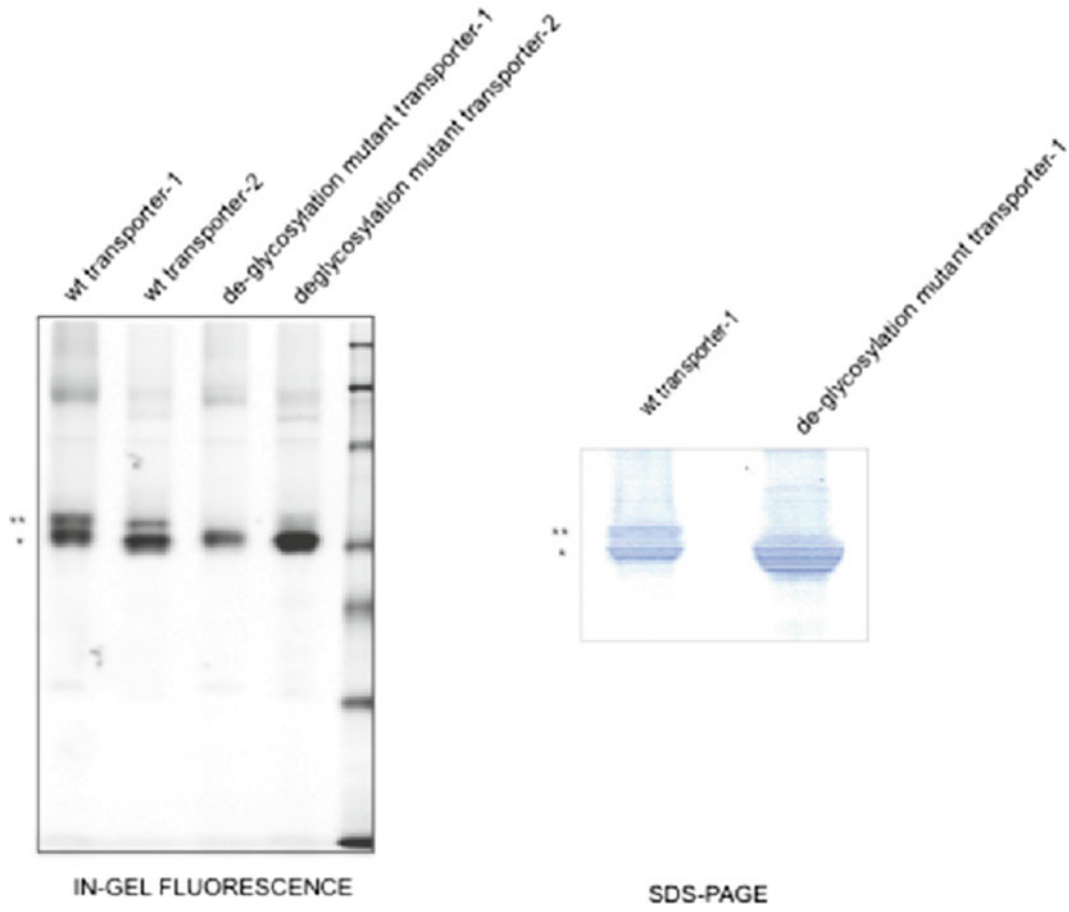

Fig. 1. Example of N-linked glycosylation of recombinant mammalian transporter-GFP fusion from *S. cerevisiae. Left panel*: SDS/PAGE and in-gel fluorescence detection in crude membranes of mammalian transporter-1 and -2 (lanes 1 and 2) and, after mutation of asparagine to alanine in the N-X-S/T motif, of the same transporters (lanes 3 and 4): *single and double asterisks* represent unglycosylated and glycosylated proteins, respectively. *Right panel*: Coomassie staining of the purified transporter before and after asparagine mutation of the mammalian transporter-1, as illustrated in the *left panel.*

2% glucose. Incubate the culture overnight in an orbital shaker at 280 rpm, 30°C.

3. Dilute the 150 mL overnight culture to OD_{600} 0.12 in 1 L growth medium without uracil containing 0.1% glucose in a 2.5-L baffled shake flask. Incubate the culture in an orbital shaker at 280 rpm, 30°C.
4. Harvest the cells after 22 h by centrifugation at 4,000 × *g* at 4°C for 10 min. Decant the supernatant and resuspend the cell pellet in 25 mL CRB/L original cell culture.
5. Disrupt the cells with four passes in a heavy-duty cell disruptor at incremental pressures of 25, 30, 32 and 35 kpsi (approximately $1.7–2.4 \times 10^3$ atm) at 4–15°C. Remove 100 µL cells, transfer into a 96-well plate and measure GFP fluorescence as outlined in step 8 of Subheading 3.2.

6. Remove the unbroken cells and debris by centrifugation at 10,000 × *g* at 4°C for 10 min and collect the supernatant, which contains the membrane fragments. Transfer 100 µL supernatant to a 96-well plate and measure GFP fluorescence as outlined in step 8 of Subheading 3.2. Calculate the yeast cell breakage efficiency by comparing the GFP fluorescence to that measured in step 5 (see Note 13).
7. To collect the membranes, centrifuge the cleared supernatant at 150,000 × *g* at 4°C for 120 min. Discard the supernatant and resuspend the pellet to a final volume of 6 mL MRB/L original cell culture using a disposable 10-mL syringe with a 21-gauge needle. Transfer 100 µL membrane suspension to a 96-well plate and measure GFP fluorescence as outlined in step 8 of Subheading 3.2. Calculate the amount of recombinant membrane protein (see Table 1). Calculate the amount of total protein using the BCA protein assay kit following the manufacturer's instructions (see Note 14).
8. Adjust the volume of the membrane suspension to achieve a protein concentration of 3.5 mg/mL in PBS. Transfer 900-µL aliquots of this membrane suspension into 1.5-mL Beckman polyallomer microcentrifuge tubes.
9. Add 100 µL freshly prepared 10% (w/v) stock of $C_{12}E_9$, 12 M, 10 M, 9 M, or LDAO to the 1.5-mL tubes containing 900 µL membrane suspension (achieving a final concentration of 1% detergent and a final protein concentration of 3.2 mg/mL). Incubate the mixtures at 4°C for 1 h with mild agitation. We recommend testing the addition of cholesteryl hemisuccinate (CHS) to the detergent mixture (at a final concentration of 0.2%), as this can be essential for the isolation of monodisperse mammalian membrane proteins.
10. Transfer 100 µL detergent-solubilized membrane protein solution into a 96-well plate and measure GFP fluorescence as outlined in step 8 of Subheading 3.2. To remove the non-solubilized material, centrifuge the remaining 900 µL in a benchtop ultracentrifuge at 100,000 × *g* at 4°C for 45 min.
11. Transfer the clarified supernatant to a new 1.5-mL tube. Transfer 100 µL to a 96-well plate and repeat the GFP fluorescent measurement as outlined in step 8 of Subheading 3.2. Calculate the detergent solubilization efficiency by comparing the GFP fluorescence measurement with that in step 7 (see Note 15).
12. Inject 0.5 mL detergent-solubilized sample onto a Superose 6 10/300 column equilibrated in 20 mM Tris–HCl (pH 7.5), 0.15 M NaCl and 0.03% DDM. After elution of the first 6 mL, collect 0.2-mL fractions row by row into a 96-well plate (see Note 16).

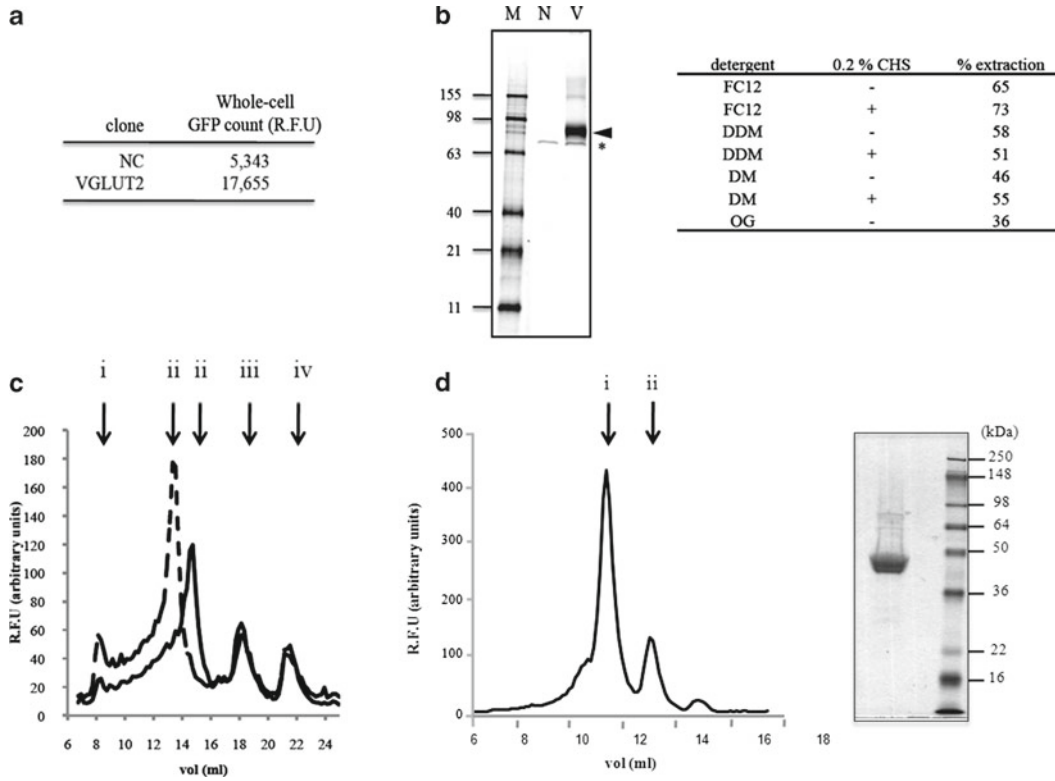

Fig. 2. Example of overproduction screening of rat VGLUT-2 and human GLUT-1. (**a**) Table illustrating the amount of whole-cell fluorescence from cells producing VGLUT-2 and a non-induced control (as outlined in Table 1, this information is used to calculate yields in mg/L). (**b**) *Left panel*: SDS-PAGE and in-gel fluorescence detection of crude membranes; lane M = Benchmark™ fluorescent molecular weight marker, lane N = non-induced cells, lane V = induced cells (the *asterisk* indicates endogenous fluorescent protein and the *arrow* indicates VGLUT2-GFP production) *Right panel*: Solubilization efficiency of membranes containing VGLUT-2 using Fos-choline 12 (FC12), dodecyl-b-D-maltopyranoside (DDM), decyl-β-D-maltopyranoside (DM) in the presence or absence of 0.2% cholesterol hemisuccinate (CHS). (**c**) FSEC trace of DM-solubilized VLGUT2 membranes in the absence (*solid line*) and presence of CHS (*dashed line*); i = aggregation peak, ii = VGLUT2-GFP fusion peak, iii = free GFP peak, iv = endogenous fluorescent protein. *Note*: as outlined in ref. 5, although solubilization was higher in other detergents, DM with CHS gives the best monodisperse profile and was used in the isolation of functional VGLUT-2. (**d**) *Left panel*: FSEC trace of DDM-solubilized human GLUT-1; i = GLUT1-GFP, ii = free GFP peak, *right panel*: SDS-PAGE analysis of purified human GLUT-1 in DDM as detected by Coomassie staining. *Note*: as outlined in ref. 5, recombinant human GLUT-1 transports D-glucose comparably with GLUT-1 purified from its native source.

13. Set the 96-well parameters of the plate reader to read wells row by row. To improve the signal-to-noise ratio, measure the GFP fluorescence emission at 512 nm by excitation at 470 nm (this wavelength is used instead of 488 nm, as it produces a lower background fluorescent signal). Plot the GFP fluorescence in each well against the fraction number (see Note 17).
14. Analyze the FSEC trace. As shown in Fig. 2, a monodisperse protein–detergent complex peak is symmetrical and elutes within a total volume of 2 mL. Larger volumes are not characteristic of stable and/or well-folded proteins in detergent (see Note 18).

4. Notes

1. Eukaryotic membrane proteins typically contain regions that are predicted by their amino acid sequence to be disordered. Most often these regions are in large loops or the amino- or carboxy-terminal tails. If a disordered region is protein-specific it is less likely to be conserved or contain a transmembrane (TM) segment. In many cases, minor amino- or carboxy-terminal truncations have been shown to improve stability (6, 7).
2. Although we recommend a *GAL1* promoter, in some cases, a constitutive promoter gives higher yields. As there are examples where an amino-terminal GFP fusion is more suitable for functional or structural work than a carboxy-terminal GFP fusion, an amino-terminal GFP fusion vector should also be considered. However, an amino-terminal GFP fusion may reduce production for N_{out}-membrane protein topologies as it may interrupt amino-terminal translocation, and as the upstream GFP can be translated more efficiently than the downstream membrane protein, the GFP fluorescence from whole cells may no longer be a reliable reporter of membrane-integrated production.
3. It is recommended that at least ten close homologues are initially tested to maximize the use of this approach, since the typical success rate for the production of mammalian membrane proteins in *S. cerevisiae* is 20%. We use a strain that has the gene encoding the vacuolar Pep4 protease deleted. The genotype should be compatible with the vector selection marker used.
4. Aerated capped tubes should be used, as they allow a more reliable estimate of yields at a large scale than non-aerated tubes.
5. 0.1% glucose, and not 2% glucose, is used in the production medium because high levels of glucose repress the *GAL1* promoter, while the former helps to maintain cell growth.
6. In order to avoid diluting the culture medium, prepare the 20% galactose stock in medium lacking uracil. Induction before OD_{600} 0.6 (typically) causes a reduced biomass that lowers protein yields. Although whole-cell GFP fluorescence can be higher if the cells are induced at a higher OD_{600} than 0.6, we find that there is proportionately greater degradation, so there is no linear gain in the amount of membrane-integrated material produced.
7. It is important to culture for 22 h after galactose addition. Although the OD_{600} is constant after 12 h, membrane protein production is maximal at 12–20 h post-induction (8). Because different final volumes can affect the level of whole-cell fluores-

cence measured, it is important to remove all the supernatant. We recommend removal of supernatant by vacuum suction, or if it is removed by hand, by patting the tubes dry using absorbent paper.

8. Because yeast cells settle to the bottom of the plate, proceed to the next step within 5 min of transfer to obtain accurate measurements.
9. Do not boil samples for SDS-PAGE, as this denatures GFP and often causes membrane protein samples to aggregate.
10. Blue light is recommended over UV light, as it is closer to the excitation wavelength of GFP. In addition, we do not recommend detecting GFP–fusion production by Western blotting, as the transfer of membrane protein–GFP fusions is often inconsistent among membrane protein samples.
11. Asn-X-Ser/Thr N-linked glycosylation acceptor sequences must be on the luminal side of the ER to be glycosylated by oligosaccharyltransferase and a distance of about 12–13 amino acids away from the end of the TM segment.
12. The intensity of Coomassie Brilliant Blue staining is a poor indication of the yield, as some membrane proteins bind the dye better than others.
13. Breakage efficiency should be greater than 80%. If lower than this, consider diluting the sample before breakage to improve efficiency.
14. If the membranes isolated from 1 L of cells are resuspended in 6 mL MRB, the GFP fluorescent counts typically match the original whole-cell fluorescent counts (8). This corresponds to approximately 60% of the amount of GFP measured in whole cells being incorporated into membranes. Note, membrane suspensions can be rapidly frozen in liquid nitrogen and stored at −80°C for up to 6 months. Although this is the routine in our laboratory, some membrane protein crystallographers avoid freezing and storing membranes and continue with purification immediately.
15. The detergent solubilized supernatant can be rapidly frozen in liquid nitrogen and stored at −80°C. Note, however, that some membrane protein crystallographers do not freeze and analyze by FSEC directly.
16. The use of a low percentage of DDM (0.03%) in the buffer used for separation of the detergent-solubilized membranes by SEC does not rescue membrane protein that has aggregated in the original detergent.
17. Ideally, as originally outlined by Kawate and Gouaux, fluorescence can be measured with higher sensitivity using an in-line detector, connected directly to the SEC column (9).

18. It is advisable to compare monodispersity with a membrane protein sample that has previously been purified and solubilized in your selected detergent. If none of the selected homologues are highly produced and monodisperse in a mild detergent, and the addition of cholesterol and/or ligand do not improve monodispersity, consider constructing further mutants or screening more homologues. Using this strategy we and others have purified a number of mammalian GPCRs, transporters, channels, and enzymes (5, 6, 10, 11).

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References

1. Drew DE, von Heijne G, Nordlund P, de Gier JW (2001) Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli*. FEBS Lett 507:220–224
2. Drew D, Slotboom DJ, Friso G, Reda T, Genevaux P, Rapp M, Meindl-Beinker NM, Lambert W, Lerch M, Daley DO, Van Wijk KJ, Hirst J, Kunji E, De Gier JW (2005) A scalable GFP-based pipeline for membrane protein overexpression screening and purification. Protein Sci 14:2011–2017
3. Drew D, Lerch M, Kunji E, Slotboom DJ, de Gier JW (2006) Optimization of membrane protein overexpression and purification using GFP fusions. Nat Methods 3:303–313
4. Drew D, Newstead S, Sonoda Y, Kim H, von Heijne G, Iwata S (2008) GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. Nat Protoc 3:784–798
5. Sonoda Y, Cameron A, Newstead S, Omote H, Moriyama Y, Kasahara M, Iwata S, Drew D (2010) Tricks of the trade used to accelerate high-resolution structure determination of membrane proteins. FEBS Lett 584:2539–2547
6. Warne T, Serrano-Vega MJ, Tate CG, Schertler GF (2009) Development and crystallization of a minimal thermostabilised G protein-coupled receptor. Protein Expr Purif 65:204–213
7. Bowie JU (2001) Stabilizing membrane proteins. Curr Opin Struct Biol 11:397–402
8. Newstead S, Kim H, von Heijne G, Iwata S, Drew D (2007) High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 104:13936–13941
9. Kawate T, Gouaux E (2006) Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. Structure 14:673–681
10. Iizasa E, Mitsutomi M, Nagano Y (2010) Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin *in vitro*. J Biol Chem 285:2996–3004
11. Sugawara T, Ito K, Shiroishi M, Tokuda N, Asada H, Yurugi-Kobayashi T, Shimamura T, Misaka T, Nomura N, Murata T, Abe K, Iwata S, Kobayashi T (2009) Fluorescence-based optimization of human bitter taste receptor expression in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 382:704–710