Chapter 2

Membrane Protein Production in the Yeast, *S. cerevisiae*

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Abstract

The first crystal structures of recombinant mammalian membrane proteins were solved in 2005 using protein that had been produced in yeast cells. One of these, the rabbit Ca^{2+}-ATPase SERCA1a, was synthesized in *Saccharomyces cerevisiae*. All host systems have their specific advantages and disadvantages, but yeast has remained a consistently popular choice in the eukaryotic membrane protein field because it is quick, easy and cheap to culture, whilst being able to post-translationally process eukaryotic membrane proteins. Very recent structures of recombinant membrane proteins produced in *S. cerevisiae* include those of the *Arabidopsis thaliana* NRT1.1 nitrate transporter and the fungal plant pathogen lipid scramblase, TMEM16. This chapter provides an overview of the methodological approaches underpinning these successes.

Key words Membrane protein, Recombinant, *S. cerevisiae*, P_{GAL} promoter

Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCD	Charge-coupled device
DoE	Design of experiments
GFP	Green fluorescent protein
GOI	Gene of interest
GPCR	G protein-coupled receptor
h	Hour
LioAc	Lithium acetate
P_{GAL1}	*GAL1* promoter
PEG	Polyethylene glycol
s	Second
T4L	T4 lysozyme

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1 Introduction

Over 1,500 species of yeast are known, but only a small minority of them have been employed as host organisms for the production of recombinant membrane proteins [1]. The two most important are *Saccharomyces cerevisiae* and *Pichia pastoris*; these eukaryotic microbes grow quickly in complex or defined media in a range of convenient formats (from multi-well plates to shake flasks and bioreactors) of various sizes [1].

In 2005, the first crystal structures of mammalian membrane proteins derived from recombinant sources were solved using protein that had been produced in yeast: the rabbit Ca^{2+}-ATPase SERCA1a was produced in *S. cerevisiae* [2] and the rat voltage-dependent potassium ion channel Kv1.2 was produced in *P. pastoris* [3]. Several other host cells have been used since then for eukaryotic membrane protein production [4], all with their own specific advantages and disadvantages. However, yeasts have remained a consistently popular choice [5, 6] because they are quick, easy, and cheap to culture whilst still being able to post-translationally process eukaryotic membrane proteins. Recent structures of recombinant membrane proteins produced in *S. cerevisiae* include those of the *Arabidopsis thaliana* NRT1.1 nitrate transporter and the fungal plant pathogen lipid scramblase, TMEM16.

S. cerevisiae has several advantages over the other commonly-used yeast species, *P. pastoris*: its genetics are better understood (http://www.yeastgenome.org/); it is supported by a more extensive body of literature; and there is a wider range of tools and strains available from both commercial and academic sources. In our laboratory, we often start with *P. pastoris* and, if the production is not straightforward, turn to *S. cerevisiae* to troubleshoot, thereby benefitting from the best attributes of the two hosts [1]. Notably, the structure of the human histamine H_1 receptor was obtained in this way: initial screening to define the best expression construct was performed in *S. cerevisiae* [7] followed by protein production in *P. pastoris* [8].

1.1 Designing a Yeast Expression Plasmid

Yeast expression plasmids used for recombinant protein production typically contain a 2 μ origin of replication and have a copy number of approximately 20 per cell [9]. Critical elements of such expression plasmids are the gene sequence encoding the target membrane protein, the promoter and terminator sequences, and any tags that might aid functional gene expression and protein purification.

In 2013 and 2014, all eight α-helical transmembrane protein structures derived from yeast (structures with PDB codes 4CL4, 4WIS, 4NEF, 4RDQ, 4M1M, 4JCZ, 3WME and 4WFF) were synthesized under the control of a strong, inducible promoter. For

the two structures solved using protein produced in *S. cerevisiae* (4CL4 and 4WIS), the promoter was P_{GAL1}, which is induced with galactose. This promoter is the basis of the commercially-available pYES2 plasmid (Life Technologies V825-20, Fig. 1) as well as the plasmid, pRS426GAL1 [10]; both are suitable plasmids for

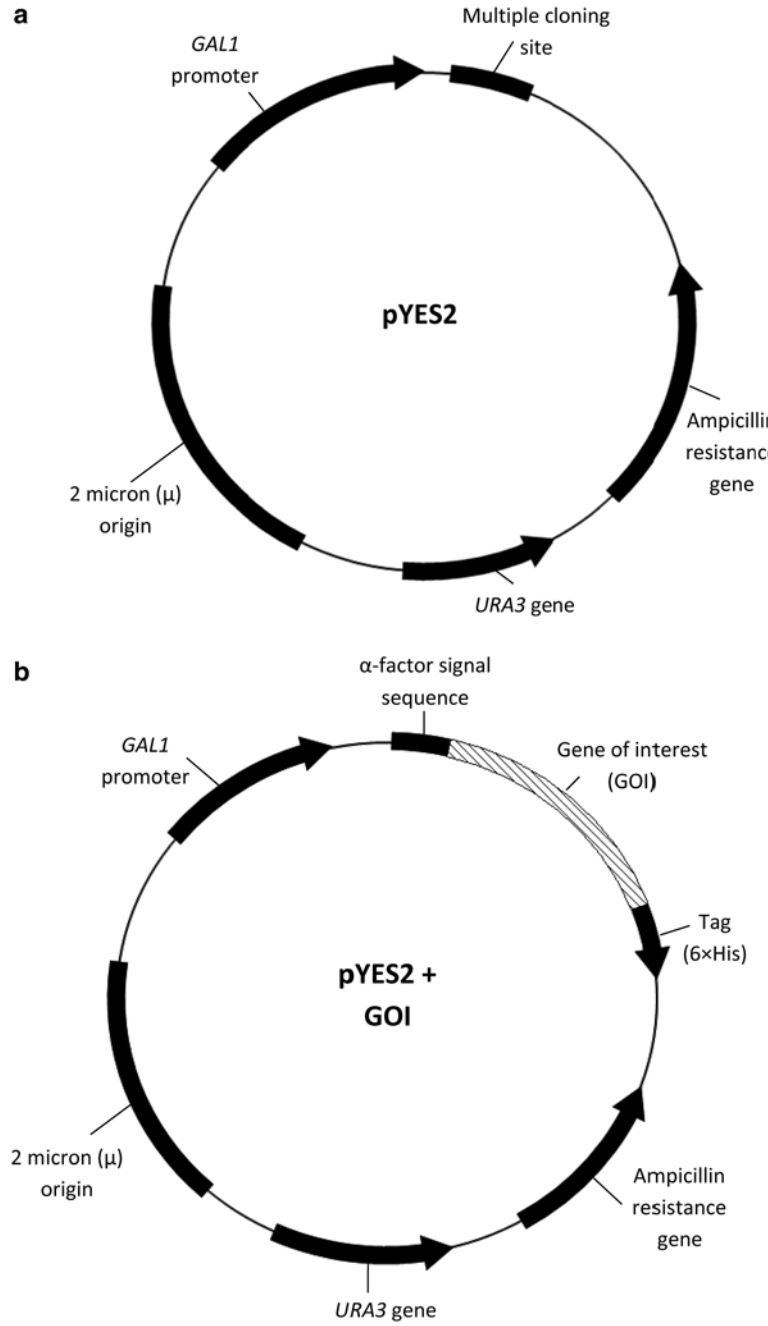

Fig. 1 A schematic representation of (**a**) the pYES2 plasmid backbone and (**b**) the pYES2 plasmid containing the gene of interest (GOI)

initiating expression trials in *S. cerevisiae*. Notably, neither plasmid contains the *S. cerevisiae* α-mating factor sequence signal, which is believed to correctly target recombinant membrane proteins to the yeast membrane. For example, its presence had a positive impact on the yield of the mouse 5-HT_{5A} serotonin receptor [11] but dramatically reduced expression of the human histamine H_1 receptor [8]. The following sequence (containing the Kex2/Ste13 processing sites) may therefore be added by PCR or gene synthesis as an optional element when designing the expression plasmid:
5′ATGAGATTTCCTTCAATTTTTACTGCAGTTT
TATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAAC
ACTACAACAGAAGATGAAACGGCACAAATTCCGGCT
GAAGCTGTCATCGGTTACTTAGATTTAGAAGGGGAT
TTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAAT
AACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTG
CTGCTAAAGAAGAAGGGGTATCTTTGGATAAAAGAGAGG
CTGAAGCT 3′

Other commonly-used sequences in *S. cerevisiae* expression plasmids include those that encode polyhistidine (hexa-, octa- (present in pRS426GAL1), and decahistine tags are all common), green fluorescent protein (GFP; present in pRS426GAL1), and T4 lysozyme (T4L). These and others have been reviewed extensively elsewhere [12, 13]. In summary, polyhistidine tags are routinely fused to recombinantly-produced membrane proteins to facilitate rapid purification by metal chelate chromatography using Ni^{2+}-affinity resins. In many cases, the tag is not removed prior to crystallization trials, although protease cleavage sites can be engineered into the expression plasmid if this is desired [6]. GFP tags are used differently, typically to assess functional yield or homogeneity of the purified recombinant protein prior to crystallization trials. However, GFP tags remain fluorescent in yeast (and other eukaryotic) cells irrespective of whether the partner membrane protein is correctly folded in the plasma membrane [14]. GFP is therefore an inappropriate marker to assess the folding status of recombinant membrane proteins produced in yeast, although it is still useful in analyzing the stability of a membrane protein by fluorescence size-exclusion chromatography [15]. Finally, most G protein-coupled receptor (GPCR) crystal structures have been obtained using a fusion protein strategy where the flexible third intracellular loop is replaced by T4L; recently modified T4L variants having been developed to optimize crystal quality or promote alternative packing interactions [16]. Overall, the precise combination and location (at either terminus or within the protein sequence) of any tags needs to be decided based upon their proposed use (for targetting, as an epitope, to promote stability, for purification or as a tool to assess protein quality) and the biochemistry of the target recombinant membrane protein. Once the final, preferred sequence has been designed, it is possible to codon optimize it for expression in *S. cerevisiae*; recent data suggest that the codon sequence around

the translation start site has a bigger influence on membrane protein yields than codon choice in the rest of the open reading frame when recombinant proteins are produced in *E. coli* [17] or *P. pastoris* [18]. The use of degenerate PCR primers to screen for the optimal codon sequence around the start codon may therefore be worth considering [19].

1.2 Choosing an S. cerevisiae Strain

A popular expression strain for structural applications is the *pep4* deletion strain, FGY217 (*MATa, ura3-52, lys2Δ201, pep4Δ*) (34) in which the gene for proteinase A has been deleted to reduce protease-mediated protein degradation. In addition, the yeast deletion collections comprise over 21,000 mutant strains with deletions of the approximately 6,000 *S. cerevisiae* ORFs [20] available as both *MAT*a and *MAT*α mating types. These strains can be obtained from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) or the American Type Culture Collection (http://www.atcc.org/). Complementing this, Dharmacon sells the Yeast Tet-Promoters Hughes Collection (yTHC) with 800 essential yeast genes under the control of a tetracycline-regulated promoter that permits their experimental regulation. These strain resources are supported by a wealth of information in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Use of specific strains from these collections offers the potential to gain mechanistic insight into the molecular bottlenecks that preclude high recombinant protein yields [21].

1.3 A Note on the Yeast Membrane

The yeast membrane has a different composition from that of mammalian membranes which may be important for some membrane protein targets. Yeast strains have therefore been developed that contain cholesterol rather than the native yeast sterol, ergosterol. This was achieved by replacing the *ERG5* and *ERG6* genes of the ergosterol biosynthetic pathway with the higher eukaryotic (e.g., zebrafish and human) genes of the cholesterol biosynthesis pathway, *DHRC24* and *DHRC7* [22–24], respectively. Cell viability does not appear to be impaired in these "humanized" yeast cells, although growth rates and densities are somewhat affected. However, this may be an acceptable trade-off in return for higher yields of functional recombinant membrane protein. Since a relatively small number of heterologous membrane proteins have been produced in cholesterol-producing yeast strains to date, potential exists to optimize recombinant protein production by using them.

1.4 Culturing Yeast Cells to Maximize Functional Recombinant Protein Yields

During a recombinant membrane protein production experiment, understanding the relative importance of the different experimental variables and their influence on protein yield and quality is an essential part of its optimization. Common approaches to increase functional yields are to lower the growth temperature of the expressing culture, alter the pH or composition of the growth

medium, or to change the culture aeration strategy [25]. The addition of molecules such as dimethyl sulfoxide (DMSO), histidine, glycerol, or specific ligands has also been used to increase yields of GPCRs in *P. pastoris* [26] and transporters in *S. cerevisiae* [27]. Often these variables are optimized in a stepwise manner, one factor at a time. A more effective method is to implement a statistical design of experiments (DoE) approach because the influence of numerous factors and their interactions, which may be nonlinear in nature, can be determined [28]. Irrespective of the approach taken, it is important to systematically investigate the effects of all input parameters in order to maximize membrane protein yields from recombinant yeast cultures.

2 Materials

2.1 Yeast Strains and Plasmids

1. Yeast expression strain, e.g., the *pep4* deletion strain, FGY217 (*MATa, ura3-52, lys2Δ201, pep4Δ*) [29].
2. Expression plasmid, e.g., pYES2 (Life Technologies, V825-20) or pRS426GAL1 [10] containing the gene and other sequences of interest.

2.2 Yeast Transformation and Culture Conditions

1. YPD medium (stable at room temperature): 1% yeast extract, 2% bacto-peptone, 2% glucose. For plates, add 2 % bacto-agar.
2. 40% glucose stock solution in water, filter sterilized (0.2 mm pore size).
3. Carrier DNA: 7 mg/mL sonicated salmon testes DNA; store at −20 °C.
4. 100 mM lithium acetate (LiOAc).
5. Polyethylene glycol (PEG) 3350 (50% w/v).
6. 1 M LiOAc.

2.3 Membrane Preparation and Immunoblotting

1. Growth/induction medium lacking uracil with either 2% glucose (for growth) or 2% galactose (for induction). For plates, add 2 % bacto-agar.
2. Breaking buffer: 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 2 mM EDTA pH 7.4, 100 mM NaCl, 5% glycerol.
3. Protease inhibitor cocktail IV (Merck Millipore).
4. Acid-washed glass beads, 500 μm.
5. 2 mL screw-cap breaking tubes.
6. Buffer A: 20 mM HEPES, 50 mM NaCl, 10% glycerol, pH 7.
7. Bicinchoninic acid (BCA) protein assay kit.
8. Tris-glycine SDS gels.
9. Powdered milk or BSA.

10. Primary anti-His_6 monoclonal antibody (Clontech).
11. Secondary antibody peroxidase conjugate.
12. PBS (phosphate buffered saline) (for 1 L): 1.44 g Na_2HPO_4-$2H_2O$ (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.
13. PBS-Tween 20 (PBST): 1 L PBS, 2 mL Tween 20 (0.2%).
14. 5× Laemmli sample buffer: 0.08 M Tris–HCl pH 6.8, 12.5% glycerol, 2.5% SDS, 6.25% β-mercaptoethanol, 0.01% bromophenol blue.
15. Pre-stained protein standard.
16. ECL detection kit.
17. Coomassie brilliant blue R-250.

2.4 Equipment

1. 50 mL, 500 mL, and 2.5 L baffled shake flasks.
2. Water bath or heat block.
3. TissueLyser (Qiagen) for small-scale membrane preparation.
4. Cell disruptor (e.g., Avestin C3) for large-scale membrane preparation.
5. Floor-standing centrifuge such as Beckman Coulter Avanti J-20 and rotors such as JLA 8.1000 and JA 25.50 (Beckman).
6. Benchtop ultracentrifuge, Beckman Coulter Optima MAX series with TLA-55 and TLA-120.1 rotors (Beckman).
7. 1 mL, 10.4 mL and 50 mL polycarbonate ultracentrifuge tubes (Beckman).
8. LAS-1000–3000 charge-coupled device (CCD) imaging system.

3 Methods

3.1 Generation of Yeast Transformants

1. Aseptically pick a single colony from a freshly streaked YPD agar plate (*see* **Note 1**) into 5 mL YPD medium and culture overnight at 30 °C with 220 rpm agitation.
2. Dilute the overnight culture into 5 mL YPD (to an OD_{600} ~ 0.25) and culture to an OD_{600} of 1.0 (*see* **Note 2**).
3. Harvest the cells by centrifugation at 5,300 × *g* for 3 min and remove the supernatant.
4. Wash the cell pellet in 500 µL 100 mM lithium acetate (LiOAc) and transfer to a 1.5 mL microcentrifuge tube.
5. Harvest the cells by centrifugation at 13,000 × *g* for 15 s and remove the supernatant.
6. Repeat **steps 4** and **5**.

7. Boil salmon testes DNA for 5 min and chill on ice for 2 min.
8. Add the individual components of the transformation solution to the yeast pellet in the following order (*see* **Note 3**):
   (a) 240 µL PEG 3350 (50 % w/v).
   (b) 35 µL 1 M LiOAc.
   (c) 25 µL boiled and chilled salmon testes DNA.
   (d) 0.5 µg plasmid DNA made up to 50 µL with water.
9. Vortex the mixture vigorously until the pellet has been completely suspended, which can take up to a minute.
10. Incubate the cells with the transformation mixture at 30 °C for 30 min.
11. Shock the cells at 42 °C for 20 min.
12. Pellet the cells at 6,000 × g for 15 s and remove the transformation mixture.
13. Add 0.5 mL sterile water to the cells and gently suspend them with a pipette (*see* **Note 4**).
14. Plate 100 µL of the cell suspension on a selective agar plate lacking uracil (this is the appropriate selection for use with pYES2 or pRS426GAL1).
15. Incubate plates for 2–3 days at 30 °C; if colonies do not form continue to incubate the plate for up to a week.

3.2 Screening for High-Yielding Transformants

1. Inoculate 10 mL growth medium (lacking uracil plus 2 % glucose) with a single colony in a 50 mL baffled shake flask. Typical screens involve assaying 10–20 single colonies.
2. Incubate the cultures overnight in an orbital shaker at 30 °C, 220 rpm.
3. Spot 10 µL from each overnight culture onto a selective plate and allow the spots to dry. Clearly label each spot on the plate then incubate it at 30 °C for 2–3 days.
4. Measure the OD_{600} of each overnight culture. Dilute the cultures to $OD_{600} = 0.12$ in 10 mL of growth medium (lacking uracil plus 2 % glucose) and culture them to an OD_{600} of 0.6 (*see* **Note 5**).
5. Harvest the cultures by centrifugation in 15 mL Falcon tubes at 1,500 × *g* for 5 min. Remove the supernatants and suspend each pellet in 10 mL of induction medium (lacking uracil plus 2 % galactose). Incubate the cultures for 22 h at 30 °C, 220 rpm.
6. Harvest cells at 5,300 × g, 4 °C for 3 min; remove supernatants and keep the cell pellet on ice.
7. Wash cells once in 2 mL ice-cold breaking buffer then harvest by centrifugation at 5300 × *g*, 4 °C for 3 min.

8. Add 1 mL glass beads to a breaking tube and place on ice; repeat this so there is one breaking tube for each colony being screened.
9. Suspend the cells (from **step 7**) in 1 mL breaking buffer (supplemented with protease inhibitor cocktail IV at a dilution of 1:500) and add to the breaking tube that contains the glass beads. Repeat for all harvested cell samples and keep the tubes on ice.
10. Place a TissueLyser breaking tube holder at −80 °C (or −20 °C) for 10 min.
11. Place the breaking tubes into the chilled TissueLyser breaking tube holder and lyse the cells in a TissueLyser at 50 Hz for 10 min.
12. Remove the supernatants from the glass beads using a pipette and transfer them to clean microcentrifuge tubes.
13. Remove cell debris by centrifugation at 17,000 × *g* for 15 min at 4 °C and transfer the supernatants to clean ultracentrifuge tubes (*see* **Note 6**).
14. Harvest total membrane pellets by centrifugation at 190,000 × *g* for 1 h. Remove the supernatants and suspend each membrane pellet in 100 µL buffer A. Membrane suspensions can be stored at −20 °C.
15. Assay the amount of total membrane protein in each membrane suspension using a BCA assay kit according to the kit instructions.
16. Confirm the presence and relative amount of target protein in each membrane suspension by immunoblot:
    (a) Each sample to be loaded on an SDS-PAGE gel should contain approximately 50 µg total membrane protein, although this will vary according to the expression level of the protein of interest.
    (b) In preparing each sample, mix 4 volumes membrane suspension and 1 volume 5× Laemmli sample buffer. Incubate this mixture for 10 min (*see* **Note 7**).
    (c) Load samples on an SDS-PAGE gel remembering to include both a protein ladder and a standard to allow comparisons between blots. Follow the "Bio-Rad General Protocol for Western Blotting" (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf).
    (d) Depending on the availability of protein-specific antibodies or the presence of tags (such as polyhistidine), incubate the blot with appropriate primary and secondary antibodies.

(e) Visualize the blot using an ECL kit and a CCD imaging system.

(f) Analyze the blot to identify high-yielding colonies (using ImageJ for example).

17. Pick high-yielding colonies from the spot plate (**step 3**) and prepare a glycerol stock for long-term storage (*see* **Note 8**) and subsequent scale up to produce large quantities of the target membrane protein, as described in Subheading 3.3.

3.3 Scaling-Up Recombinant Membrane Protein Production

1. Isolate a single colony on a selective plate from a glycerol stock (Subheading 3.2, **step 17**) or use a fresh transformant (isolated on a spot plate as in Subheading 3.2, **step 3**).
2. Inoculate 10 mL growth medium (lacking uracil plus 2% glucose) with the single colony and incubate overnight in an orbital shaker at 220 rpm and 30 °C.
3. The following day, transfer the 10 mL overnight culture to a 500 mL shake flask containing 150 mL growth medium (lacking uracil plus 2% glucose) and incubate overnight as in **step 1**.
4. On the third day, dilute the 150 mL overnight culture to $OD_{600} = 0.12$ in 1 L medium lacking uracil and containing 0.1% glucose and grow in a 2.5 L baffled shake flask at 220 rpm and 30 °C. Induce the culture with 2% galactose (although this can be optimized further) when the OD_{600} has reached 0.6.
5. Harvest the cells (*see* **Note 9**) by centrifugation at 5,300 × *g*, 4 °C for 10 min in a floor-standing centrifuge such as a Beckman Coulter Avanti J-20; the JLA 8.1000 rotor holds bottles of 1 L capacity that can be used for this step. Discard the supernatant. The pellet should ideally weigh between 10 and 20 g for efficient large-scale membrane preparation. If the weight is less than 10 g, the protocol in Subheading 3.2 (from **step 7**) can be used instead.
6. Suspend the cell pellet in 25 mL breaking buffer for every 1 L of original culture. Add protease inhibitor cocktail IV at a 1:500 dilution.
7. Break the cells using a high pressure homogenizer, such as an Avestin C3, according to the manufacturer's instructions. It is important to keep the sample at low temperature (~4 °C) to prevent protein degradation. The low temperature should be maintained from this step onwards.
8. Separate the cell lysate from the cell debris and unbroken cells by centrifugation at 8,000 × *g*, 4 °C for 30 min.
9. Collect the membrane fraction by centrifugation of the supernatant at 100,000 × *g*, 4 °C for 60 min (allow extra time for acceleration and deceleration) in an ultracentrifuge such as a

Beckman Coulter Optima L-80 XP; the 70.1 Ti rotor holds 12 tubes. Discard the supernatant and suspend the pellets in 6 mL per original 1 L culture. To ease the resuspension of membrane pellet, the membranes can be soaked overnight at 4 °C in 1 mL buffer A per tube; pellets should then be homogenized and made up to the required volume with buffer A. Measure total membrane protein concentration using a BCA protein assay kit according to the manufacturer's instructions prior to extraction and purification of the target protein.

4 Notes

1. The plate should be no older than 5 days; if the colonies are older, this can lead to a reduction in the competence of the cells.
2. Transformation is more likely to be successful when cells are growing logarithmically.
3. It is important to add the PEG 3350 first to protect the yeast cells from the high concentration of LiOAc.
4. At this point the cells are fragile and need to be suspended gently with a pipette; do not vortex.
5. This ensures that the cells are in the logarithmic growth phase during induction.
6. An alternative method for separating cell lysates from glass beads is to collect them into 15 mL Falcon tubes. To do that, cut a round hole in the cap of a Falcon tube, pierce the bottom of the breaking tube with a needle, and insert it into the cut cap. Place the cap onto the Falcon tube and collect the lysate by centrifugation at 5,300 × *g* for 3 min; the glass beads and cell debris are retained in the breaking tube. Transfer the supernatants to clean ultracentrifuge tubes.
7. Incubate the mixture between 4 °C (on ice) and 70 °C; the best temperature for the particular protein of interest must be determined empirically by examining the immunoblot to ensure the protein has entered the gel and has not aggregated or degraded. Lower temperatures may require longer incubation times.
8. Transformants can often be stored as glycerol stocks at −80 °C, but their stability should be assessed to confirm this on a case-by-case basis. For unstable transformants, it will be necessary to do a fresh transformation prior to each scale-up experiment.
9. The incubation period may need to be optimized; recombinant protein may be detected 4 h post-induction, but a 22 h culture period is often used for convenience. To optimize the

post-induction incubation period, collect samples at several time intervals and analyze by immunoblot as detailed in Subheading 3.2, **step 16**.

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References

1. Darby RA, Cartwright SP, Dilworth MV, Bill RM (2012) Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review). Methods Mol Biol 866:11–23. doi:10.1007/978-1-61779-770-5_2
2. Jidenko M, Nielsen RC, Sorensen TL, Moller JV, le Maire M, Nissen P, Jaxel C (2005) Crystallization of a mammalian membrane protein overexpressed in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 102(33):11687–11691. doi:10.1073/pnas.0503986102
3. Long SB, Campbell EB, Mackinnon R (2005) Crystal structure of a mammalian voltage-dependent Shaker family K^+ channel. Science 309(5736):897–903. doi:10.1126/science.1116269
4. Bill RM, von der Haar T (2015) Hijacked then lost in translation: the plight of the recombinant host cell in membrane protein structural biology projects. Curr Opin Struct Biol 32:147–155. doi:10.1016/j.sbi.2015.04.003
5. Bill RM (2014) Playing catch-up with *Escherichia coli*: using yeast to increase success rates in recombinant protein production experiments. Front Microbiol 5:85. doi:10.3389/fmicb.2014.00085
6. Byrne B (2015) *Pichia pastoris* as an expression host for membrane protein structural biology. Curr Opin Struct Biol 32:9–17. doi:10.1016/j.sbi.2015.01.005
7. Shiroishi M, Tsujimoto H, Makyio H, Asada H, Yurugi-Kobayashi T, Shimamura T, Murata T, Nomura N, Haga T, Iwata S, Kobayashi T (2012) Platform for the rapid construction and evaluation of GPCRs for crystallography in *Saccharomyces cerevisiae*. Microb Cell Factories 11:78. doi:10.1186/1475-2859-11-78
8. Shiroishi M, Kobayashi T, Ogasawara S, Tsujimoto H, Ikeda-Suno C, Iwata S, Shimamura T (2011) Production of the stable human histamine H(1) receptor in *Pichia pastoris* for structural determination. Methods 55(4):281–286. doi:10.1016/j.ymeth.2011.08.015
9. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110(1):119–122. doi:0378-1119(92)90454-W
10. Drew D, Kim H (2012) Preparation of *Saccharomyces cerevisiae* expression plasmids. Methods Mol Biol 866:41–46. doi:10.1007/978-1-61779-770-5_4
11. Weiss HM, Haase W, Michel H, Reilander H (1995) Expression of functional mouse 5-HT5A serotonin receptor in the methylotrophic yeast *Pichia pastoris*: pharmacological characterization and localization. FEBS Lett 377(3):451–456. doi:10.1016/0014-5793(95)01389-X
12. Terpe K (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 60(5):523–533. doi:10.1007/s00253-002-1158-6
13. Manjasetty BA, Turnbull AP, Panjikar S, Bussow K, Chance MR (2008) Automated technologies and novel techniques to accelerate protein crystallography for structural genomics. Proteomics 8(4):612–625. doi:10.1002/pmic.200700687
14. Thomas J, Tate CG (2014) Quality control in eukaryotic membrane protein overproduction. J Mol Biol 426(24):4139–4154. doi:10.1016/j.jmb.2014.10.012
15. 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(5):784–798. doi:10.1038/nprot.2008.44

16. Thorsen TS, Matt R, Weis WI, Kobilka BK (2014) Modified T4 lysozyme fusion proteins facilitate G protein-coupled receptor crystallogenesis. Structure 22(11):1657–1664. doi:10.1016/j.str.2014.08.022
17. Norholm MH, Toddo S, Virkki MT, Light S, von Heijne G, Daley DO (2013) Improved production of membrane proteins in *Escherichia coli* by selective codon substitutions. FEBS Lett 587(15):2352–2358. doi:10.1016/j.febslet.2013.05.063
18. Oberg F, Ekvall M, Nyblom M, Backmark A, Neutze R, Hedfalk K (2009) Insight into factors directing high production of eukaryotic membrane proteins; production of 13 human AQPs in *Pichia pastoris*. Mol Membr Biol 26(4):215–227. doi:10.1080/09687680902862085
19. Mirzadeh K, Martinez V, Toddo S, Guntur S, Herrgard MJ, Elofsson A, Norholm MH, Daley DO (2015) Enhanced protein production in *Escherichia coli* by optimization of cloning scars at the vector-coding sequence junction. ACS Synth Biol 4(9):959–965. doi:10.1021/acssynbio.5b00033
20. Giaever G, Nislow C (2014) The yeast deletion collection: a decade of functional genomics. Genetics 197(2):451–465. doi:10.1534/genetics.114.161620
21. Bonander N, Hedfalk K, Larsson C, Mostad P, Chang C, Gustafsson L, Bill RM (2005) Design of improved membrane protein production experiments: quantitation of the host response. Protein Sci 14(7):1729–1740. doi:10.1110/ps.051435705
22. Souza CM, Schwabe TM, Pichler H, Ploier B, Leitner E, Guan XL, Wenk MR, Riezman I, Riezman H (2011) A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan uptake, but not weak organic acid resistance. Metab Eng 13(5):555–569. doi:10.1016/j.ymben.2011.06.006
23. Morioka S, Shigemori T, Hara K, Morisaka H, Kuroda K, Ueda M (2013) Effect of sterol composition on the activity of the yeast G-protein-coupled receptor Ste2. Appl Microbiol Biotechnol 97(9):4013–4020. doi:10.1007/s00253-012-4470-9
24. Hirz M, Richter G, Leitner E, Wriessnegger T, Pichler H (2013) A novel cholesterol-producing Pichia pastoris strain is an ideal host for functional expression of human Na, K-ATPase alpha3beta1 isoform. Appl Microbiol Biotechnol 97(21):9465–9478. doi:10.1007/s00253-013-5156-7
25. Holmes WJ, Darby RA, Wilks MD, Smith R, Bill RM (2009) Developing a scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime. Microb Cell Factories 8:35. doi:10.1186/1475-2859-8-35
26. Andre N, Cherouati N, Prual C, Steffan T, Zeder-Lutz G, Magnin T, Pattus F, Michel H, Wagner R, Reinhart C (2006) Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. Protein Sci 15(5):1115–1126. doi:10.1110/ps.062098206, ps.062098206
27. Figler RA, Omote H, Nakamoto RK, Al-Shawi MK (2000) Use of chemical chaperones in the yeast *Saccharomyces cerevisiae* to enhance heterologous membrane protein expression: high-yield expression and purification of human P-glycoprotein. Arch Biochem Biophys 376(1):34–46. doi:10.1006/abbi.2000.1712, S0003-9861(00)91712-0
28. Bora N, Bawa Z, Bill RM, Wilks MD (2012) The implementation of a design of experiments strategy to increase recombinant protein yields in yeast (review). Methods Mol Biol866:115–127.doi:10.1007/978-1-61779-770-5_11
29. Kota J, Gilstring CF, Ljungdahl PO (2007) Membrane chaperone Shr3 assists in folding amino acid permeases preventing precocious ERAD. J Cell Biol 176(5):617–628. doi:10.1083/jcb.200612100