Chapter 11

Integral Membrane Protein Expression in *Saccharomyces cerevisiae*

Rebba C. Boswell-Casteel, Jennifer M. Johnson, Robert M. Stroud, and Franklin A. Hays

Abstract

Eukaryotic integral membrane proteins are challenging targets for crystallography or functional characterization in a purified state. Since expression is often a limiting factor when studying this difficult class of biological macromolecules, the intent of this chapter is to focus on the expression of eukaryotic integral membrane proteins (IMPs) using the model organism *Saccharomyces cerevisiae*. *S. cerevisiae* is a prime candidate for the expression of eukaryotic IMPs because it offers the convenience of using episomal expression plasmids, selection of positive transformants, posttranslational modifications, and it can properly fold and target IMPs. Here we present a generalized protocol and insights based on our collective knowledge as an aid to overcoming the challenges faced when expressing eukaryotic IMPs in *S. cerevisiae*.

Key words Integral membrane protein, *Saccharomyces cerevisiae*, Protein expression, Protein overproduction, Yeast

1 Introduction

Saccharomyces cerevisiae is a well-characterized eukaryotic model organism for recombinant protein expression, especially for integral membrane proteins [1–4], because it combines the advantages of unicellular organisms (e.g., rapid growth and genetic material is easily manipulated) with the capacity to perform eukaryotic posttranslational modifications. In contrast to more multifarious eukaryotic organisms, *S. cerevisiae* expression systems are cost-effective, are capable of rapidly reaching high cell densities, can produce high protein yields, and *S. cerevisiae* is generally regarded as safe (GRAS). These advantages position *S. cerevisiae* as a leading expression system for the overproduction of eukaryotic integral membrane proteins (IMPs).

Procuring sufficient quantities of IMPs for downstream studies can be a formidable task. In this chapter we present our approach to

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the over-expression of IMPs using the budding yeast *S. cerevisiae*. However, at almost every step throughout this protocol, an alternative method, vector, buffer, etc. could be substituted to further optimize expression or meet the specific requirements of your IMP of interest. The primary intent of this chapter is to provide a generalized approach to the expression of eukaryotic IMPs and, when possible, provide alternative strategies or important considerations that will aid in the overproduction of target IMPs (Fig. 1).

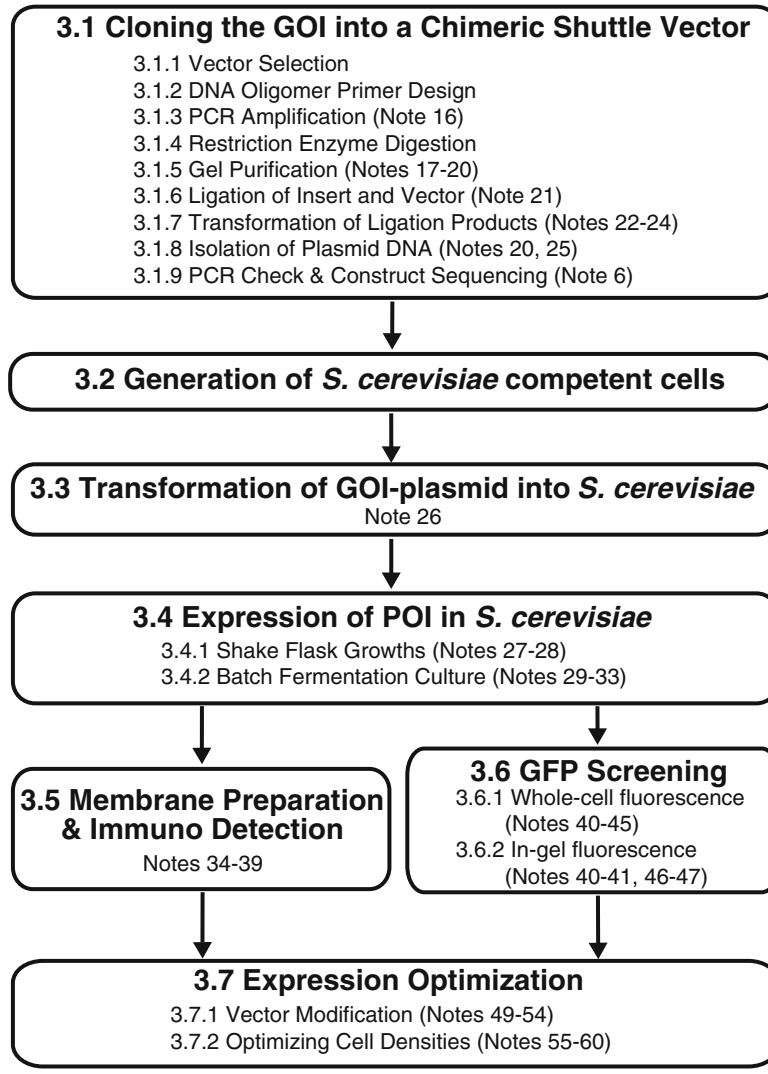

Fig. 1 Expression workflow. General workflow for integral membrane protein expression in *S. cerevisiae* with relevant Notes indicated for each section

2 Materials

Prepare all solutions using ultrapure water (simply referred to as ddH_2O, prepared by purifying deionized water to attain a sensitivity of 18 MΩ at 25 °C). Prepare and store all reagents at room temperature (RT, unless indicated otherwise). All listed pH values were determined at room temperature (unless indicated otherwise).

2.1 Cloning Your Gene of Interest into the "83Xi" Vector

1. DNA oligomer primers of less than 40 nucleotides in length can be ordered as "desalted" quality (*see* **Note 1**).
2. Phusion® High-Fidelity DNA Polymerase (New England BioLabs) kit: 50 mM $MgCl_2$, 5× Phusion Reaction Buffer, DMSO, and Phusion® High-Fidelity DNA Polymerase.
3. dNTP solution mix (New England BioLabs) was obtained separately. Stored –20 °C.
4. QIAquick® PCR purification kit (Qiagen).
5. Ethidium bromide (EtBr, 2 mg/ml). It is highly carcinogenic and light sensitive. Keep wrapped in aluminum foil and store at RT (*see* **Note 2**).
6. 50× TAE (2 M Tris, 1 M acetic acid, 50 mM EDTA).
7. Molecular biology grade ethanol.
8. Molecular biology grade agarose.
9. Restriction enzymes and corresponding 10× buffer stocks (New England BioLabs). Stored at –20 °C. (Specific buffer compositions can be found in the appendix of the NEB catalogue) (*see* **Note 3**).
10. 10× BlueJuice™ DNA gel loading buffer (Invitrogen).
11. Quick-Load® 2-Log DNA Ladder (0.1–10.0 kb, New England BioLabs).
12. QIAquick® gel extraction kit (Qiagen).
13. T4 DNA ligase and ligation buffer.
14. SOC Media: autoclave 1 l of SOB broth and add 10 mM $MgCl_2$ (sterile solution) and 20 mM glucose (sterile solution).
15. *Escherichia coli* strain XL2-Blue. Store at –80 °C.
16. Luria–Bertani (LB) medium and or on LB agar (*see* **Note 4**).
17. Ampicillin (200 mg/ml) (*see* **Note 5**).
18. QIAprep® spin miniprep kit (Qiagen).
19. GAL1 promoter and Cyc terminator sequencing primers (*see* **Note 6**).

2.2 Generation of S. cerevisiae Competent Cells

1. Yeast strain W303-Δpep4 (*leu2-3, 112 trp1-1, can1-100, ade2-1, his3-11,15, Δpep4, MATα*).
2. YPD broth (Research Products International) 0.2 μm sterile filtered.
3. YPD agar. Plates are poured following a 15 min autoclave cycle, and stored at 4 °C.
4. YPD broth plus 15% (v/v) glycerol. 0.2 μm sterile filtered.

2.3 Transformation of S. cerevisiae Competent Cells

1. PLATE solution: 40% PEG 3350 (w/v), 0.1 M lithium acetate, 10 mM Tris–HCl, pH 7, and 1 mM EDTA. Solution is brought to volume with ddH_2O and 0.2 μm sterile filtered (*see* **Note 7**).
2. TE Buffer: 10 mM Tris–HCl, pH 9.0 and 150 mM EDTA.
3. Molecular biology grade sterile DMSO.
4. Sheared salmon sperm DNA (10 mg/ml). Stored at −20 °C.
5. CSM-His plates (500 ml = 20 plates): of 0.77 g/L CSM-His (Sunrise Science Products), 20 g/L low melting agar, 10 g/L glucose, 3.0 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, and this is brought to volume with ddH_2O. Autoclave for 15 min and store plates at 4 °C.

2.4 Protein Expression in S. cerevisiae

1. Glucose solution, 40% (w/v). Autoclave for 15 min and store at room temperature.
2. 20× galactose solution, 40% (w/v). Add galactose to autoclaved, sterile water. Once dissolved and cooled to room temperature filter-sterilize using 0.45 μm filter. Store solution at room temperature (*see* **Note 8**).
3. 10× raffinose solution, 10% (w/v). Filter-sterilize using 0.22 μm filter and store at room temperature (*see* **Note 9**).
4. 10× CSM-His solution, 7.9 g/L. Filter-sterilize using 0.22 μm filter and store at 4 °C (*see* **Note 10**).
5. 20× YNB solution. 13.4% (w/v) yeast nitrogen base without amino acids. Filter-sterilize using 0.22 μm filter and store at 4 °C.
6. 4× YPG solution is used as the inductant. Yeast extract 8% (w/v), peptone 16% (w/v), and galactose 8% (w/v). Add yeast extract and peptone to hot water to dissolve. Once dissolved autoclave for 15 min. After solution cools to room temperature add galactose using the sterile 20× galactose stock, and stir (*see* **Note 11**).
7. Antifoam 204.
8. Resuspension buffer Y, pH 7.5 : 50 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, and 10 mM EDTA, pH 8.0. Store at 4 °C.

2.5 Membrane Preparation and Immunodetection

1. 2× Solubilization Buffer, pH 7.4: 100 mM Tris–HCl, pH 7.4, 800 mM NaCl, and 10% (v/v) glycerol. Store at 4 °C.
2. Coomassie Protein Staining Solution.
3. SDS Polyacrylamide Gel Components.
   (a) Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8
   (b) Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8
   (c) 30% Acrylamide/Bis Solution, 29:1. Store at 4 °C (*see* **Note 12**).
   (d) Ammonium persulfate : 10% (w/v) solution in water. Store at –20 °C
   (e) *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED)
   (f) SDS-PAGE Running Buffer: 25 mM Tris–HCl, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS (*see* **Note 13**).
   (g) 4× Laemmli Sample Buffer: 200 mM Tris–HCl, pH 6.8, 8% (w/v) SDS,) 40% (v/v) glycerol, 50 mM EDTA, pH 8.0, and 0.08% (w/v) bromophenol blue. Mix thoroughly and make 960 µl aliquots in 1.5 ml microfuge tubes. *Prior to use add 40 µl β-mercaptoethanol to the microfuge tube.* Store at –20 °C (*see* **Note 14**).
   (h) Precision Plus Protein ™ Kaleidoscop™ (Bio-Rad).
4. Immunoblotting Components.
   (a) PVDF transfer membrane (0.2 µm, Thermo Scientific)
   (b) Western blot transfer buffer: 25 mM Tris–HCl, 192 mM glycine, and 20% (v/v) methanol.
   (c) Tris buffered saline (TBS, 10× stock): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4
   (d) 1× TBS containing 0.05% (v/v) Tween-20 (TBST)
   (e) Blocking solution: 5% (w/v) milk in TBST (*see* **Note 15**).
   (f) Wash buffer: 1× TBS
   (g) Thick blotting paper (Bio-Rad)
   (h) Thin blotting paper (Bio-Rad)
   (i) Anti-histidine antibody, Penta-His Alexa Fluor® 647 conjugate (Qiagen), or an antibody specific for your POI.

2.6 GFP Detection Components

1. Black Nunc 96 well plates with an optical bottom (Thermo Scientific).

2.7 Instruments and Useful Apparatus

1. PCR machine.
2. Agarose gel tank.
3. Cell disrupter or bead beater.
4. Glass beads 0.5 mm diameter (for use in bead beater).

5. Polyacrylamide gel pouring apparatus.
6. Polyacrylamide gel tank.
7. Various centrifuges (micro, low speed, high speed, etc.) and corresponding centrifuge tubes.
8. Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad).
9. Water bath.
10. UV transilluminator with camera.
11. Shaking incubators.
12. Fermenter.
13. Fluorescent microplate reader.

3 Methods

The following steps are necessary for the functional overexpression of your Protein of Interest (POI) in *S. cerevisiae* (Fig. 1): (1) cloning of the corresponding gene of interest (GOI) into a chimeric shuttle vector, (2) generation of *S. cerevisiae* competent cells, (3) transformation of GOI plasmid into *S. cerevisiae* competent cells, (4) cell growth (shake flasks or batch fermentation), (5) membrane preparations of *S.cerevisiae*, (6) expression confirmation of your POI via western blotting or GFP detection, and (7) expression optimization.

3.1 Cloning of GOI into a Chimeric Shuttle Vector

There are multiple approaches for cloning your GOI into a shuttle vector. These include the high-throughput ligase independent cloning [4] and GAP repair cloning [5, 6], methods previously described with detailed protocols. However, this chapter is organized around the expression of target IMPs and not high-throughput screening methods. For detailed approaches to various high-throughput screening methods for IMPs expressed in *S. cerevisiae*, we refer the readers to previously published detailed protocols [2, 4]. Cloning for our purpose will focus on traditional *E. coli* based approaches utilized for targeted GOIs.

3.1.1 Vector Selection

The vector we selected to use is the "83Xi" designed by the Membrane Protein Expression Center (MPEC.ucsf.edu, Fig. 2). Briefly, this is a GAP compatible expression plasmid that is based on a 2 μ plasmid backbone containing an N-terminal 10× Histidine tag followed by a thrombin protease cleavage site. This plasmid contains a HIS3 selection marker and is driven by a galactose inducible (GAL1) promoter.

3.1.2 DNA Oligomer Primer Design

Primers were designed for the 5′ and 3′ ends of our GOI. These primers included the palindromic sequences for XmaI (N-terminus of GOI) and XhoI (C-terminus of GOI) restriction enzymes, each containing a four basepair overhang (we choose XmaI and XhoI,

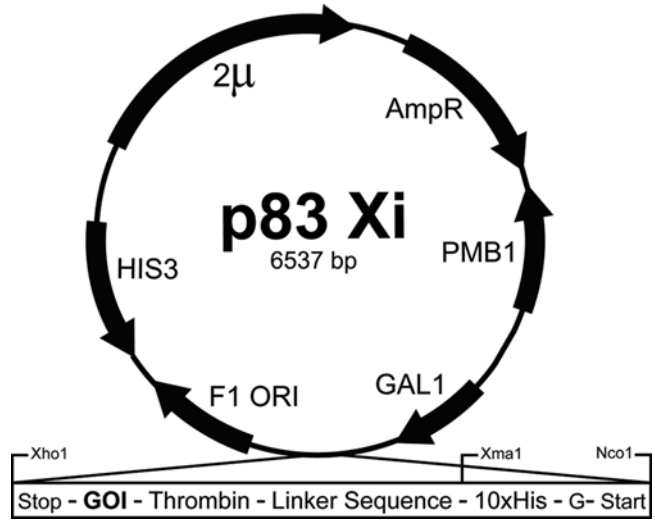

Fig. 2 p83 Xi Plasmid Map: The p83 Xi plasmid is a chimeric shuttle vector based on a 2 µ backbone containing both an ampicillin and HIS3 selection marker. Gene expression is driven by the GAL1 promoter and terminates with the Cyc terminator. Specifically, the p83 Xi plasmid contains an NcoI restriction site with an internal start codon followed by a 10× histidine tag, linker region, thrombin protease, and the multiple cloning site (XmaI, SmaI, and XhoI)

but any unique restriction sites within the multiple cloning site may be used), and the first 12–18 base pairs of either the N- or C-terminal of the GOI. A start codon was not included as it is already designed into the vector. Ensure stop codons (we suggest two) are placed at the end of the gene sequence. Before ordering primers for your GOI, make certain quality checks have been made for melting temperature, self-complementarity, and dimer formation.

3.1.3 PCR Amplifications of GOI from cDNA

1. To amplify your GOI use a high-fidelity proof reading DNA polymerase such as Phusion®.
2. Set up a 50 µl PCR reaction in a 0.5 ml PCR tube by mixing the following components in the given order:

36.5 µl	ddH_2O
10 µl	5× HF buffer
1.0 µl	dNTPs, 10 mM
0.5 µl	100 µM Forward Primer
0.5 µl	100 µM Reverse Primer
1.0 µl	template DNA
0.5 µl	Phusion® polymerase

3. Mix reaction mixture thoroughly. Make sure that the tubes are closed properly and put them into the PCR Machine.

4. Close the lid to the PCR machine and start the following protocol:
   (a) 98 °C for 30 s
   (b) 98 °C for 10 s
   (c) T_m of lowest T_m primer + 3 °C for 30 s
   (d) 72 °C for 30 s per kb
   (e) 72 °C for 10 min
   (f) Hold reaction at 4 °C
   (g) Repeat steps b through d for 35 cycles
5. Run 1–5 μl of the above PCR reaction (plus 1 μl of 10× BlueJuice™ gel loading dye and bring to a 10 μl volume with ddH_2O) on a 0.7% (w/v) agarose gel containing ethidium bromide to check the quality and amount of amplified DNA fragment (*see* **Note 16**).
6. Clean the PCR products using the QIAquick® PCR purification kit. To column purify the remaining PCR products follow the instructions of the kit manufacturer.
7. In short, mix the PCR products with 5 volumes (5 × 49 μl = 245 μl) of PB buffer (binding buffer) and load onto a spin column. The PCR products will bind to the column by spinning the solution through the column at maximum speed in a microfuge.
8. Discard the flow-through.
9. Wash the column with 750 μl PE buffer (contains ethanol), and spin again discarding the flow-through.
10. Spin the column once more to ensure that all residual buffer has been removed.
11. Elute the bound DNA from the spin column into a 1.5 ml microfuge tube by adding 30 μl of EB buffer (elution buffer) to the spin column. Incubate column with buffer for 1–2 min before spinning the column at maximum speed in a microfuge.
12. Purified PCR products will be in the flow-through.

3.1.4 Digestion of GOI and Vector with Restriction Enzymes

1. Digest the purified GOI fragment and the "83 Xi" vector with XmaI and XhoI (or the preferred enzymes you designed within the PCR primers). To do so, add the following items to 25 μl of the purified PCR product and 25 μl of vector (5 μl will be used as an undigested control)

(a) 18 μl	ddH_2O
(b) 5 μl	10× NEB Cut Smart Buffer
(c) 1 μl	XmaI
(d) 1 μl	XhoI

2. Mix the reaction thoroughly and incubate at 37 °C for 2 h.

3.1.5 Gel Purification of DNA Fragments

In order to successfully clone your GOI into the shuttle vector, it is absolutely necessary to gel purify the digested GOI and vector fragments (*see* **Note 17**).

1. After the double restriction enzyme digest is complete add 6 μl of 10× BlueJuice™ gel loading dye (2 μl 10× BlueJuice™ gel loading dye and 13 μl ddH_2O to undigested controls) and mix thoroughly.
2. Load onto a 0.7% (w/v) agarose gel. Run the gel at 100 V for 1–1.5 h for separation.
3. Isolate the fragments of interest by excising them from the gel using a UV transilluminator that visualizes ethidium bromide strained DNA bands. *Warning:* UV light is a potent mutagen—do not over expose DNA (or bare skin) to UV light. To avoid over exposure to the UV light, cut the agarose gel into strips and only expose one lane at a time. Remove the band of interest using a scalpel. Make sure to cut as closely to the band as possible (*see* **Note 18**).
4. Collect the excised bands into pre-weighed 1.5 ml microfuge tubes and calculate weights of the agarose gel slices (*see* **Note 19**).
5. Extract DNA from gel slices using the QIAquick® gel extraction kit, following the manufacturer's instructions.
6. In short, add 3 volumes of QG buffer to one volume of gel slice (0.7 g × 3 volumes = 210 μl) and dissolve the agarose gel by placing the closed microfuge tube at 50 °C for 10 min. Make sure the gel slice is fully dissolved before moving on to the next step.
7. Add one volume of isopropanol to the dissolved gel solution, and mix by inverting the tube.
8. Bind the DNA to the spin column by loading and spinning the solution through the column at maximum speed in a microfuge for 1 min. Repeat until all the DNA solution is bound to the column. Discard the flow-through
9. Wash with 500 μl of QG buffer, spin at maximum speed in a microfuge for 1 min, and discard the flow-through.
10. Wash with 750 μl of PE buffer, spin at maximum speed in a microfuge for 1 min, and discard the flow-through.
11. Spin once more at maximum speed to remove any remaining buffer.
12. Elute the bound DNA from the spin column into a 1.5 ml microfuge tube by adding 30 μl of EB buffer (elution buffer) to the spin column. Incubate column with buffer for 1–2 min before spinning the column at maximum speed in a microfuge (*see* **Note 20**).
13. Purified DNA products will be in the flow-through.

3.1.6 Ligation of DNA Fragments

To ligate the GOI DNA fragment into shuttle vector everything needs to be completely digested with XmaI and XhoI restriction enzymes (or the preferred enzymes you designed within the PCR primers) to prevent the plasmids from self-ligating in the subsequent DNA ligation reaction. For an optimum ligation, choose a ~10× molar excess of insert over plasmid (*see* **Note 21**).

1. Set up the following ligation reactions (20 μl):

(a) $17-(x+y)$ μl	ddH_2O
(b) xμl	Insert (not added to the control reaction)
(c) yμl	Plasmid
(d) 2 μl	10× T4 ligase buffer
(e) 1 μl	T4 Ligase

2. Incubate at room temperature overnight.
3. Store the ligation reactions at 4 °C until needed.

3.1.7 Transformation of Ligation Products into E. coli

The ligation reactions can now be used to transform *E. coli* XL2 Blue competent cells for the propagation of individual ligated plasmids. Once the *E. coli* cells have been plated on a selective medium, only the *E. coli* cells that have been successfully transformed will grow on the selective medium.

1. Remove a tube of ultra or super competent *E. coli* XL2 Blue competent cells from the −80 °C storage (*see* **Note 22**).
2. Aliquot 200 μl of ice-cold competent cells into two separate 1.5 ml microfuge tubes.
3. Add 5 μl of the ligation mixture and 5 μl of ligation control to individual 1.5 ml microfuge tubes (*see* **Note 23**).
4. Gently mix each tube by flicking the tube several times.
5. Incubate the cells on ice for 10 min.
6. Heat-shock the cells for 30 s in a 42 °C water bath.
7. Place on ice for an additional 5 min following the heat-shock.
8. Add 800 μl of SOC medium to the transformed cells and incubate at 37 °C for 1 h in shaking incubator (~250 RPM).
9. Pipette ~25 μl of the transformed cells onto LBAmp plates, spread the cells evenly using a cell spreader or glass beads.
10. Incubate at 37 °C overnight for the transformed *E. coli* colonies to appear.
11. Remove the plates from the incubator and compare the ligation plate (vector plus insert) to the control plate (vector only). The control plate should have significantly fewer colonies (*see* **Note 24**).

3.1.8 Isolation of Plasmid DNA from E. coli Transformants

Plasmid preparations are made from individual *E. coli* colonies to identify those that contain the desired GOI containing plasmid. If significantly more colonies are located on the ligation plate (vector plus insert) when compared to the control plate (vector only), proceed with isolating the plasmid DNA. If the ligation step appears to have failed, determine which of the previous DNA manipulation steps failed and repeat the protocol starting from the failed step (*see* **Note 25**). Isolate plasmid DNA from 5–10 *E. coli* colonies.

1. Pick a single *E. coli* colony, using sterile technique, and inoculate 8 ml portions of LB-Amp broth in sterile plastic tubes.
2. Grow cells at 37 °C in a shaking incubator at 250 RPM overnight.
3. Harvest the cells by centrifugation, 3000 × *g* for 10 min at 4 °C.
4. Use the QIAprep® spin miniprep kit to isolate the plasmid DNA following the manufacturer's instructions.
5. In short, resuspend the cells in 250 µl of buffer P1, transfer the cells to a 1.5 ml microfuge tube.
6. Add 250 µl of buffer P2 to the cells and mix by inverting the tube.
7. Add 350 µl of buffer N3 to the cells and mix my inverting the tube.
8. Spin at maximum speed in a microfuge for 10 min to sediment the cellular debris.
9. Load the supernatant into a spin column and spin at maximum speed in a microfuge for 1 min. Discard flow-through, the DNA will be bound the spin column.
10. Wash the spin column with 500 µl of buffer PB, spin for 1 min and discard the flow-through.
11. Wash the spin column with 750 µl of buffer PE, spin for 1 min, and discard the flow-through.
12. Re-spin the column to remove any buffer.
13. Place the column into a clean 1.5 ml microfuge tube.
14. Add 30 µl of buffer EB to the column and incubate at room temperature for 1–2 min. Spin the column to elute the DNA. DNA is stored at −20 °C (*see* **Note 20**).

3.1.9 PCR Check, Sequencing, and Generation of GOI-Fusion Plasmid Stocks

1. Instead of sending the isolated plasmid DNA to sequencing, perform a PCR check to identify plasmid stocks that contain the GOI. Set up the PCR reactions according to the parameters used in Subheading 3.1.3. The presence of amplification will signify that the plasmid sample has the target GOI sequence.
2. Send plasmids with positive amplification to a sequencing facility for sequence confirmation.

3. Send sequencing primers together with the column-purified plasmids to a DNA sequencing facility for confirmation of sequence (*see* **Note 6**).
4. Keep additional unsequenced plasmids as reserves in case the first plasmid DNA sequences are not 100% correct.
5. Repeat the steps for transformation into *E. coli* cells and the isolation of plasmid DNA using the QIAprep ® spin miniprep kit to generate sequence confirmed stocks of the GOI-fusion construct.

3.2 Generation of S. cerevisiae Competent Cells

1. Inoculate 10 ml of YPD broth and place in a shaking incubator at 30 °C, 220 RPM overnight.
2. Streak a YPD plate for colony isolation and incubate at 30 °C for 48 h.
3. Place 5 ml of YPD broth in five aerated culture tubes (you may increase/decrease this number as desired) and inoculate each tube with a single colony of yeast. Grow at 30 °C, 220 RPM, for 24 h.
4. Spin down growths at 3000 × *g* for 10 min, and discard the supernatant.
5. Resuspend cell pellets in 5 ml of YPD + 15% (v/v) glycerol broth. Aliquot 500 µl into each microfuge tube. Store at −80 °C.

3.3 Transformation of GOI-Fusion Plasmid into S. cerevisiae Competent Cells

1. Remove a vial of competent cells from the −80 °C freezer. Thaw and pellet the cells.
2. Resuspend cells in 150 µl PLATE solution.
3. Add 5 µl of sheared salmon sperm (carrier DNA, 10 µg) plus ~0.1 µg of plasmid DNA of your GOI-fusion construct and vortex well.
4. Add 10 µl if DMSO and vortex briefly.
5. Incubate at room temperature for 15 min.
6. Heat shock at 42 °C for 20 min.
7. Pellet cells in a microfuge for 30 s and remove supernatant.
8. Add 200 µl TE to the cell pellet and gently resuspend cells by aspirating up-and-down with a pipette tip.
9. Pipette 50 and 150 µl of the resuspended cells onto selective CSM-His plates.
10. Incubate at 30 °C for 2 days (*see* **Note 26**).

3.4 Expression the POI in S. cerevisiae

3.4.1 Shake Flask Growths

Preculture outgrowth

1. Inoculate 5 ml SC-His media with a single colony of the transformant from the CSM-His selective plate containing your GOI-fusion plasmid.

2. Incubate 4 h at 30 °C and at 220 RPM.
3. Autoclave 270 ml ddH_2O in a 1 l size baffled flask. Allow flask to cool to room temperature.
4. Add 37.5 ml 10× CSM-His, 18.8 ml of 20× YNB and 40% (w/v) glucose.
5. Inoculate the flask with 5 ml of the starter culture.
6. Incubate for 24 h at 30 °C and at 220 RPM.

Scale up shake flask cultures (7.5 l)

7. Autoclave 15 × 270 ml ddH_2O in 1 l baffled flasks.
8. Add 37.5 ml 10× CSM-His and 10% (w/v) raffinose, 18.8 ml of 20× YNB, and 9.4 ml 40% (w/v) glucose to each flask.
9. Inoculate with 10 ml of preculture.
10. Incubate for 24 h at 30 °C and at 220 RPM (*see* **Note 27**).

Induction of shake flask cultures

11. Following this growth period the optical density at 600 nm ranged between 15 and 20 for most cultures with glucose concentrations generally <0.1%. Induce the cultures using 125 ml of the 4× YPG stock medium to each flask (*see* **Note 28**).
12. Incubate for 16 h at 30 °C and at 220 RPM.

3.4.2 Batch Fermentation Culture

Preparation of the fermenter

1. Add 5.1 l of ddH_2O and 500 µl of antifoam 204 (Sigma) to the fermentation vessel.
2. Calibrate the pH probe using pH standards of 4 and 7.
3. Prepare the DO (dissolved oxygen) probe
   (a) Clean probe
   (b) Pour out old electrolyte solution
   (c) Refill with new electrolyte solution
4. Ensure all tubing is disconnected, clamps are shut, all protective caps are in place (motor, pH probe, and DO probe). Foil all non-sterile connections and place sterile lines in 50 ml conical tubes sealed with foil. Use autoclave tape where necessary (*see* **Note 29**).
5. Autoclave for 1 h (*see* **Note 30**).
6. Allow fermenter to cool to room temperature (*see* **Note 31**).
7. Add 175 ml of 40% (v/v) glucose, 350 ml of 20× YNB, and 700 ml of 10× CSM-His and 10% (w/v) raffinose to the fermentation vessel.
8. Calibrate the DO probe at 0%.
9. Set temperature at 30 °C.

10. Set the agitation rate from 200 to 350 RPM based on a DO scale of 20–90%.
11. Flow air at 2.5 l/min.
12. Calibrate DO probe at 100% (*see* **Note 32**).

Batch fermentation growth

1. Inoculate with 375 ml of overnight growth as previously described in Subheading 3.4.1 (**steps 1–6**).
2. Grow at 30 °C for 24 h

Inoculation of batch fermentation growth

1. Induce the culture by adding 2.5 l of the 4× YPG stock media to the vessel.
2. Increase air flow to 5.0 l/min (*see* **Note 33**).
3. Incubate for 16 h.

3.4.3 Cell Harvest

1. Pellet cells via centrifugation.
2. Resuspend cells in 60 ml of Resuspension Buffer Y per 80 g of cell pellet.
3. Lyse cells immediately or store at −20 °C.

3.5 Membrane Preparation and Immunodetection

1. 12% SDS-PAGE Gels (makes four mini gels): First, prepare and pour the resolving gel using the following components in the order listed (6.6 ml ddH_2O, 8.0 ml 30% acrylamide mix, 5.0 ml 1.5 M Tris–HCl, pH 8.8, 0.2 ml 10% (w/v) SDS, 0.2 ml 10% (w/v) ammonium persulfate, and 0.008 ml TEMED). Once gel has been poured, top gel off with water to prevent drying. Once the resolving gel has set, pour off any remaining water. Next, prepare and pour the stacking gel (5%) using the following components in the order listed (3.4 ml ddH_2O, 0.83 ml 30% acrylamide mix, 0.63 ml 1.5 M Tris–HCl, pH 8.8, 0.05 ml 10% (w/v) SDS, 0.05 ml 10% (w/v) ammonium persulfate, and 0.005 ml TEMED). Insert the comb, taking care not to trap bubbles in the wells. Once the stacking gel has set remove from gel apparatus and store at 4 °C (*see* **Note 12**).
2. If frozen cells are to be used thaw them first.
3. Lyse cells using a C3 Emulisflex (~28,000 psi, 3 passes) or a bead beater (blend at maximum speed for 60 s, stop for 60 s. Repeat for a total of five cycles of beating and cooling.) (*see* **Note 34**).
4. Spin down the crude cell extract at 7500 × *g* for 1 h at 4 °C.
5. Collect the supernatant of the previous low speed spin. Save sample for SDS-PAGE gel (*see* **Note 35**).

6. Resuspend the post lysis pellet in ddH_2O to the original volume (pre centrifugation). Save sample for SDS-PAGE gel.
7. Spin supernatant at 101,000 × *g* for 1 h at 4 °C.
8. Separate the supernatant from the high-speed membrane pellet. Save sample of the supernatant for SDS-PAGE gels.
9. Either scrape out the membrane pellet from each tube and store at –20 °C or resuspend each pellet with 1× solubilization buffer (16 ml buffer per g membrane) then store at –20 °C. Save sample for SDS-PAGE gels (*see* **Note 36**).
10. Locate the samples that were saved for SDS-PAGE gels. Normalize the volume of sample added based on the original starting volumes. Bring to 30 µl using Resuspension Buffer Y. Add 10 µl of 4× Laemmli Sample Buffer for a total volume of 40 µl (*see* **Note 37**).
11. For single construct screening: load 5 µl of protein standard in lanes 1 and 6 of a 10 lane 12% SDS-PAGE mini gel. Load 20 µl of the cleared cell lysate, resuspended cell pellet, supernatant from the high-speed spin and solubilized membrane pellet in lanes 2–5 and in lanes 7–10 of the gel.
12. For multiple construct screening: load 5 µl of protein standard in lanes 1 or 6 of a 10 lane 12% SDS-PAGE mini gel. Load 20 µl of the cleared cell lysate, resuspended cell pellet, supernatant from the high-speed spin and solubilized membrane pellet in lanes 2–5 for the first construct and in lanes 7–10 of the gel for the second construct. Prepare two gels loaded identically.
13. Run SDS-PAGE gels in the electrophoresis unit (~140 V for 1 h, or until dye front has reached the base of the gel).
14. Prewet two pieces of thick and thin filter papers (per gel, cut to size) in 1× western transfer buffer, and pre-wet enough 0.2 µm PVDF membrane in 100% (v/v) methanol to adequately cover the gel. Rinse the membrane with 1× western transfer buffer immediately before use.
15. Prepare the semidry transfer blot apparatus. In brief, make a sandwich of thick blot paper, thin blot paper, PVDF membrane, SDS-PAGE gel, thin blot paper, and thick blot paper. (Use ½ the gel for single constructs and one of the full duplicate gels for multiple constructs.)
16. Transfer proteins to the PVDF membrane for 30 min, 25 V, and 0.33 amps (per gel).
17. Stain the remaining SDS-PAGE gels with a Coomassie Protein staining solution.
18. Block the membranes with a solution of 5% (w/v) milk in 1× TBST for 1 h at room temperature (*see* **Note 38**).
19. Make working solutions of the desired antibody. For the Penta-His Alexa Fluor® 647 conjugate antibody use a 1:10,000

dilution in 5% (w/v) milk 1× TBST. *Make sure the antibody solution is protected from light.*

20. Incubate at room temperature for 1 h (*see* **Note 38**).
21. Wash the membrane(s) with TBS for 15 min at room temperature. Repeat for a total of three times (*see* **Note 39**).
22. Image membranes, and evaluate POI localization and expression level.

3.6 GFP-Based Optimization Screen as Described by David Drew et al. [2]

This method is applicable if the expression construct has a GFP fluorescent fusion partner. Expression is determined by using whole-cell and in-gel fluorescence via this method.

1. Follow steps outlined previously for cell growths (Subheading 3.4.1 or 3.4.2).
2. Prepare one culture without galactose addition (protein expression not induced) (*see* **Note 40**).
3. Harvest 10 ml of cells that have been cultured with and without galactose via centrifugation and resuspend in 200 µl Resuspension Buffer Y (*see* **Note 41**).
4. Harvest and resuspend the remaining cells according the steps outlined in Subheading 3.4.3.

3.6.1 Expression Quantification Using Whole-Cell Fluorescence

1. Transfer 200 µl of the resuspended cells to a black Nunc 96-well optical bottom plate (*see* **Note 42**).
2. Measure fluorescence emission in a microplate spectrofluorometer (GFP has an excitation wavelength of 488 nm and emission wavelength of 512 nm) (*see* **Note 43**).
3. Estimate membrane protein expression levels (mg/L) using the following methods:
   (a) Aliquot 200 µl of cell suspension from induced (MP-I, RFU) and non-induced (galactose not added, MP-NI, RFU) cultures.
   (b) Measure fluorescence as described in **steps 1–2** in Subheading 3.6.1
   (c) Measure the fluorescence of a known concentration of the GFP fusion partner (STD, RFU) (*see* **Note 44**).
   (d) Calculate the concentration of the GFP fusion partner in mg/L as follows:

$$\frac{(\mathrm{MP-I})-(\mathrm{MP-NI})}{\mathrm{STD}}\times(conc.\,\mathrm{STD})=conc.\,\mathrm{protein}$$

For example:

$$\frac{(30{,}000\mathrm{RFU}-2000\mathrm{RFU})}{10{,}000\mathrm{RFU}}\times 0.02\mathrm{mg}/\mathrm{ml}=0.056\mathrm{mg}/\mathrm{ml}\ \text{of protein}$$

4. Next divide the calculated GFP concentration from whole cells by 40 (8000 µl cell culture/200 µl resuspended cells) in order to determine the GFP concentration in 200 µl of resuspended cell culture (*see* **Note 45**).
5. According to David Drew et al. "the typical recovery of GFP counts from a 1 l culture into membranes is 60% or 0.6." Multiply the calculated GFP concentration by 0.6.

$$1.4\ \text{mg}/\text{L} \times 0.6 = 0.84\ \text{mg}/\text{L GFP fusion protein}$$

6. Calculate the amount of membrane protein (MP) expression as follows:

$$\frac{\text{Molecular Mass of MP}\,(\text{kDa})}{\text{Molecular Mass of GFP}\,(\text{kDa})} \times \text{amount of GFP}\,(\text{mg}/\text{l}) = \text{Membrane Protein Expression}\,(\text{mg}/\text{l})$$

For Example:

$$\frac{50\ \text{kDa}\,(\text{POI})}{28\ \text{kDa}\,(\text{GFP})} \times 0.84 = 1.5\ \text{mg}/\text{l}$$

3.6.2 Expression Screening Using In-Gel Fluorescence

1. Lyse the resuspended cells obtained in Subheading 3.6 (Subheading 3.5, **step 2**).
2. Follow **steps 1–12** as outlined in Subheading 3.5 to prepare, resuspend the membrane pellet, and run the SDS-PAGE gel.
3. Once the gel has finished running, rinse the gel with ddH_2O and detect the fluorescent bands with a CCD camera system. Gel bands are visualized via exposure to blue light (EPI source) set at 460 nm and cut-off filter of 515 nm (*see* **Note 46**).
4. The gel may then be stained with a Coomassie staining solution and destained if desired (*see* **Note 47**).

3.7 Expression Optimization

If the initial cloning efforts result in less than optimal expression levels there are several options available to increase protein yields. The two most prevalent options are altering the expression vector or modifying the growth conditions (*see* **Note 48**).

3.7.1 Vector Modifications

There are several options available for modifying the expression vector to improve overall yields. Firstly, the "83Xi" vector could be modified to express a C-terminal tag (*see* **Note 49**). Next, the choice of tag can affect expression levels. While we prefer the general ease of a His tag for downstream applications, an alternative tag may improve protein production (*see* **Note 50**). Another available option is to choose a different promoter. The current vector is driven by the inducible GAL1 promoter. Changing to a differ-

ent tightly regulated promoter with a strong transcriptional start signal, such as ADH2, is a viable option (*see* **Notes 51** and **52**). Additionally, modifying the selection marker could assist in improving protein expression as different media formulations could then be tried (*see* **Note 53**). Finally, your gene of interest can be codon optimized using synthetic gene redesign (*see* **Note 54**).

3.7.2 Optimizing Cell Densities

In addition to optimizing the expression vector, growth conditions can be modified to maximize the amount of biomass being produced and the protein content within each cell. The media conditions may not be optimal to produce your POI, in this case changing to an alternative minimal media (*see* **Note 55**), semidefined medium, or a rich complex medium may be beneficial (*see* **Note 56**). Altering the dissolved oxygen content can also be beneficial (*see* **Note 57**). Oxygen availability can effect growth rates and energy availability [7], which may alter plasmid replication or partitioning. Studies have also shown that reducing growth temperatures can improve yields if toxicity is suspected [8], but it can also be detrimental to the expression of IMPs [1] (*see* **Note 58**). Several reports have shown chemical chaperones can increase protein expression levels by improving the folding of IMPs [9, 10]. These chemical chaperones include: (1) DMSO (2.5% v/v), glycerol (10% v/v), or histidine (0.04 mg/ml) (*see* **Note 59**). You may also alter the media by changing the composition of the carbon sources. We opt to use 1% (w/v) glucose and 1% (w/v) raffinose for a final carbohydrate concentration of 2% (w/v) because it ensures the absence of glucose repression once the galactose inductant has been added. Variations to this include increasing or decreasing the final carbohydrate concentration, using only glucose, or substituting with alternative secondary carbon sources (e.g., lactose, maltose, and raffinose). Finally more mechanical approaches can be taken. These include switching from shake flask growths to a fermentation process, or increasing the complexity of your fermentation scheme (*see* **Note 60**).

4 Notes

1. No further purification is needed if the oligos are ordered in the salt free format. Ensure that the start codon from the target gene has been removed, as it is already incorporated into the "83Xi" plasmid.
2. Ethidium bromide is highly carcinogenic and poses a reproductive hazard. Use proper personal protective equipment (e.g., gloves and lab coat) when handling ethidium bromide and thoroughly rinse with water all equipment that comes into contact with ethidium bromide. Dispose of ethidium bromide waste according to local regulations.

3. The majority of restriction enzymes are stable for at least 1 year and up to 5 years when stored at −20 °C. Use a portable −20 °C freezer box when handling the restriction enzyme or any other enzyme (e.g., DNA polymerase and DNA ligase) used for molecular biology purposes.
4. Autoclaved sterile stocks of LB can be stored at room temperature and will remain sterile for long periods of time if the stocks remain unopened. LB plates made with ampicillin (LBAmp) are stable for 1 month when protected from light at stored at 4 °C.
5. Ampicillin is light sensitive. Ampicillin stocks stored at 4 °C will remain viable for approximately 1 month. Store at −20 °C for time frames extending past 1 month.
6. The sequences for the sequencing primers are as follows: GAL1 forward primer—5′ CTT TCA ACA TTT TCG GTT TG-3′ and Cyc reverse primer—5′ GGG GGG AGG GCG TGA ATG TAA-3′. When ordering oligos, obtain them in the desalted form if possible.
7. This solution will acidify overtime causing transformation efficiency to decline.
8. Do NOT autoclave galactose. Galactose isomerizes at elevated temperature. Galactose stored at room temperature and 0.45 μm sterile filtered is stable for approximately 4 months.
9. Dissolve using warm water. Do NOT autoclave. Raffinose will isomerize if exposed to elevated temperature for extended periods of time.
10. Dissolve using warm water.
11. Dissolve yeast extract and peptone using warm water. Do not autoclave in the presence of galactose. Galactose isomerizes at elevated temperatures and this will result in unacceptable levels of repressive glucose.
12. Caution: unpolymerized acrylamide is a neurotoxin and proper protective equipment should be used.
13. This solution can be made up as a 10× concentrated stock. Add SDS last, as it causes bubbles.
14. Add SDS last as it causes bubbles. SDS precipitates at 4 °C. Warm the Laemmli Sample Buffer prior to use.
15. Make this solution fresh and store at 4 °C. Once hydrated, the milk will spoil if left out at room temperature.
16. To visualize the DNA fragments we add 5 μl of ethidium bromide (2 mg/ml) to melted agarose (70 ml of 0.7% (w/v) agarose in 1× TAE) prior to pouring the gel (*see* **Note 2**).
17. Gel purification is critical for successful cloning, PCR clean-up alone is not sufficient.

18. If the double digestion appears to have failed, repeat the digestion using a single enzyme at a time.
19. Large pieces of agarose (>400 mg) will result in lower DNA recovery.
20. Minimizing the elution volumes or eluting more than one column (identically prepared) with the same elution buffer will increase the final concentrations of recovered DNA.
21. $$\text{Insert Mass}\,(\text{ng}) = \text{Desired ratio} \times \left[\frac{\text{Insert Length}\,(\text{base pairs})}{\text{Vector Length}\,(\text{base pairs})}\right] \times \text{Vector mass}\,(\text{ng})$$
22. Commercially available high efficiency competent cells work best. However, in-house competent cells will also work but transformation efficiency may be diminished.
23. It is important to include the ligation control containing only the digested vector. This allows you to determine if a successful double digestion and gel purification has occurred—because the vector will remain linear and thus will not impart any antibiotic resistance to the transformed cells.
24. If the control plate does not have significantly fewer colonies, repeat the cloning process starting with the restriction enzyme digestion. Modify by digesting with each enzyme individually. If the enzymes are compatible in the same buffer, heat-inactivate the first enzyme.
25. The cloning process can be considered to have failed if the background number of transformants on the ligation control plate in higher than the number of transformants on the ligation plate containing vector and insert.
26. If colony size is small, incubate for an additional 24 h. If the transformation efficiency is low or absent, remake the PLATE solution—it will acidify over time.
27. After 24 h of growth the glucose concentration should be less than 0.1 % (w/v). This yeast strain will turn pink due to the *ade2-1* mutation.
28. Glucose represses the GAL1 promoter. If the glucose concentrations have not been sufficiently depleted during growth, induction will not occur.
29. Ensure that at least one line of the fermenter is opened to allow for venting, during autoclaving.
30. An hour autoclave time should be sufficient. However, a dummy growth to check for adequate sterility is recommended if the reader is not familiar with the operation of the fermenter. If contamination occurs, increase the time of the autoclave cycle.

31. The fermenter can be left at room temperature to cool or it can be attached to a cooling unit.
32. It is important to calibrate the DO probe at 100% in the exact growth conditions that will be used (e.g., agitation rate, temperature, and airflow), as these conditions can affect the dissolved oxygen content.
33. The addition of the 4× YPG will decrease the dissolved oxygen content because it has not been pre-equilibrated in order to maintain sterility. Increasing the airflow will limit exposure to an anoxic environment.
34. Yeast cells will NOT sufficiently lyse using sonication or freeze fracture methods.
35. There are two ways determine the lysis efficiency: (1) visually inspect cells under a microscope or (2) the cell debris pellet typically has two layers: bottom darker colored layer of unlysed cells, and a top lighter colored layer consisting or organelles and lysed cells. The estimated ratio of the top layer of lysed cells verses the bottom layer of intact cells will give you an estimate of the lysing efficiency.
36. Solubilizing the membrane pellet using a defined ratio of buffer will ease expression comparisons between various constructs.
37. Normalizing the loading volumes for the SDS-PAGE gel is essential for comparing expression levels between various constructs or comparing various growth conditions during optimization.
38. It can be blocked overnight at 4 °C.
39. Increasing the number of washes or the length of each wash can help reduce the amount of background.
40. This culture will be used to estimate the amount of background fluorescence.
41. It is important to remove all of the supernatant, it can affect whole-cell fluorescence measurements by altering the final volume of the resuspended cells.
42. Yeast cells will settle to the bottom of the 96-well plate. Immediately measure fluorescence to ensure accurate readings.
43. Choose the bottom read option on the plate reader if available.
44. This is needed in order to correlate the whole-cell fluorescence with the amount of GFP produced.
45. Although the initial cell volume was 10 ml there is an effective 2 ml loss by only transferring 200 μl of the resuspended cells (approximately 250 μl total volume, 200 μl buffer + cell pellet = 250 μl).
46. Blue light is closer to the excitation wavelength of GFP, therefore it is preferred over UV light. Detection of GFP expression

using western blotting is not recommended because the transfer of GFP-fusion IMPs can be inconsistent between samples.

47. Coomassie staining is a poor indication of the expression level, because some IMPs bind Coomassie better than others.
48. Another option would be to change the cell line being used, but this may require additional changes with the expression vector or growth conditions.
49. Although our suggested plasmid has an N-terminal tag, N-terminal tags can interfere with the processing of signal peptides. If the POI does not contain a signal peptide, then N-terminal tags provide greater flexibility for the development of expression constructs. However, if a signal peptide is suspected or present a C-terminal tag is preferable.
50. There is a wide variety of expression tags available including, but not limited too: GFP, FLAG, galectin, or maltose binding protein (MBP). Our experience has shown that MBP or galectin tags may help improve expression levels and aid in processing properly folded protein.
51. Like the GAL1 promoter, ADH2 is also subject to glucose repression. The ADH2 promoter is induced in the presence of ethanol, and gene expression increases as glucose is consumed. Two advantages of this promoter include: (1) expression is turned on when the culture is in stationary phase (high biomass), and (2) no inductant is required, and therefore, no disruptions to the growth process occur [11, 12].
52. Constitutive promoters are generally not well suited for the expression of IMPs. Toxicity issues can arise from the inability to control onset of expression. Additionally, constitutive promoters usually results in lower expression levels for IMPs [1, 4].
53. The vector we suggest currently has a HIS3 selection marker. However, the yeast strain that we use is compatible with vectors containing at least one of the following selection markers: *leu2-3, 112 trp1-1, can1-100, ura3-1, ade2-1,* and *his3-11.* Since our chosen yeast strain is compatible with numerous selection methods, substituting the current selection markers may provide additional optimal growth conditions.
54. Synthetic gene redesign can either be performed in house based upon your extensive knowledge of your gene of interest, or it can be out-sourced to companies specializing in synthetic gene redesign/production.
55. Changing to alternative minimal medias may require altering the selection marker on the expression vector.
56. Generally, minimal media is preferred as it favors plasmid stability [7]. However this is not the case for plasmids containing an ADH2 promoter [11, 12].

57. Oxygenation can be controlled in shake flask cultures by increasing the speed at which they are shaking and changing flask size (e.g., liter vs. 2.5 l) or shape (e.g., baffled vs. non-baffled). For growth utilizing fermentation, the agitation speed and air supply rate may be altered or the air source can be changed (e.g., nitrogen, air, pure oxygen).
58. The general starting point for temperature reduction screening is 20 °C, but any temperature below 30 °C may be tried.
59. In order to use histidine as a chemical chaperone with the system we describe, the selection marker and current media configuration will need to be altered. Our systems employ a histidine selection marker and use a synthetic complete histidine dropout media. The URA3 marker is common for growths utilizing a histidine chemical chaperone.
60. Increasing the cell biomass is one crude option for increasing the amount of recoverable protein. This can be done through using advanced fermentation techniques (continuous or fed-batch process) to reach higher cell densities. However, these fermentation processes will have to be designed around each particular POI-vector-yeast strain combination used. Feb-batch process are the most commonly used and designing optimized feeding protocols has two major general points of consideration: (1) is the host strain Crabtree positive or negative, and (2) is protein expression constitutive or regulated [13]?

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