Discovery-Oriented Eukaryotic Integral Membrane Protein Overexpression and Purification – DETAILED PROTOCOL

MPEC/STROUD/UCSF

PART ONE

I. Commercial sources of reagents

I.1. Common reagents

Tris free base (Fisher #BP154-1)
Sodium Chloride (Sigma #BP358-212)
β-Mercaptone ethanol (Fisher Science #O3446-100)
EDTA (Sigma #E5134-500G)
Glycerol (Fisher #BP229-4)
PMSF (Sigma #7626-25G)
SDS (Sigma #L3771)
Bromophenol Blue (Sigma #B5525)

I.2. Reagents for high throughput ligase independent cloning

LIC-qualified T4 DNA polymerase (Novagen # 69167)
Phusion DNA polymerase (Finnzymes F-530L)
SmaI restriction enzyme (New England Biolabs #R0141S)
dNTP mix, 10 mM each dNTP (NEB #N0447S)
dATP, Li salt (Roche #11051440001)
dTTP, Li salt (Roche #11051482001)
S. cerevisiae S288C strain genomic DNA (Novagen #69240-3)
QIAvac 96 miniprep kit (Qiagen #27191)
or EZ96 Fastfilter plasmid DNA kit (United Bioinformatica Inc., # D1097-01)
QIAquick 96 PCR purification kit (Qiagen #28181)

I.3. Reagents for protein expression in yeast

Salmon Sperm DNA (Invitrogen #15632-011)
CSM-HIS (Sunrise Science #1023-100)
Yeast nitrogen base w/o amino acids (VWR #9004-146)
Glucose (Sigma #G8270)
Galactose (Sigma #G0625)
Yeast extract (VWR #90004-090)
Bacto Peptone (VWR #90000-382)
DMSO (Sigma #D4540-1L)
Lithium Acetate (Sigma #62393-100G-F)
PEG 3350 (Sigma #P3640-500G)
Tween 20 (Sigma P7949-500ml)
Mouse monoclonal HRP conjugated H-3 probe (Santa Cruz Biotechnology #SC-8036HRP)
Mouse monoclonal HRP conjugated FLAG probe (Sigma #A8592-1MG)
Protein ladder, Precision plus kaleidoscope protein standard (Biorad #161-0375)
SuperSignal west pico chemiluminescent substrate (Pierce #34080)
Glogos II autorad marker (Stratagene #420201)
Novex 4-20% Tris-glycine gels (Invitrogen #EC60255BOX)

I.4. Reagents for protein purification

Imidazole (Sigma #I2399-500G)

n- dodecyl-β-D- maltoside (Anatrace #D310)
Octyl-β-D-glucopyranoside (Anatrace #O311)
Thrombin (Novagen #69671)
PreScission 3C protease (N terminal His-MBP fusion construct, house stock, 10 mg/ml)
Ni-NTA Agarose resin (QIAGEN #1018236)
TALON metal affinity resin (CloneTech #635504)
Benzamidine beads, Sepharose 6B (Amersham #51-5960-00-DD)
Complete EDTA-free protease inhibitor cocktail tablet (Roche, #11873580001)

II. Instrument and useful apparatus

II.1. For HT LIC cloning

PCR machine with 96 well plate capacity: Peltier Thermal Cycler (MJ Research #PTC-200)
8 or 12 Channel multichannel pipette (20 μL, 200 μL, and 1000 μL sizes)
96 well Agarose gel tank: DNA plus (USA Scientific #3431-4000)
Disposable x-tracta agarose gel extraction tool (USA Scientific #5454-0100)
QIAvac 96 manifold (Qiagen #19504)
48 grid vented Q-tray with divider (Genetix Inc., X6029)

II.2. For protein expression test and detergent screening

Bench-top microcentrifuge with PCR tube adapter
Beadbeater (Biospec Inc. # 909M)
Beadbeater canister (Biospec Inc. # 110803-50)
Glass beads 0.5 mm diameter (Biospec Inc. # 11079105)
Traceable Lab controller and timer (Fisher #06-662-7)
High speed microfuge tube for MLA55 rotor (Beckman #357448)
Allegra 25R centrifuge (Bechman Coulter #369436)
Swinging-Bucket Rotor TS-5.1-500 for Allegra centrifuge, (Bechman Coulter #368308)
Allegra centrifuge Microtiter plate holder (Bechman Coulter #368451)
Trans-Blot SD semi-dry electrophoretic transfer cell (Biorad #170-3940)

II.3. For protein purification

Superdex 200 Column, 10/300 GL (GE healthcare #17-5175-01)
NAP-10 Sephadex G-25 desalting column (GE healthcare #17-0854-01)
EconoPac 10DG BioRAD disposable desalting columns (BioRAD #732-2010)
Nutater (ATR #RKUS)

III. Reagent buffers

III.1. Stock reagents

<u>1M TRIS Buffer pH 7.4 RT</u>

TRIS, pH 7.4 RT (1M stock)	1 M	121.1 g

Note: adjust final pH to 7.4RT using ~70 ml concentrated HCl, store at RT.

<u>1M TRIS Buffer pH 9.0 RT</u>

TRIS, pH 9.0 RT (1M stock)	1 M	121.1 g

Note: adjust final pH to 9.0 RT using concentrated HCl, store at RT.

<u>500 mM EDTA</u>

EDTA	500 mM	186.1 g
	Final volume	1000 ml

Note: To prepare 1 liter, 0.5M EDTA pH 8.0: Add 186.1 g of disodium EDTA-2H_2O to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of

NaOH pellets). Autoclave for 20 min at 121°C. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH. Store at RT.

100 x PMSF (100 mM)

PMSF (in 100% isopropanol)	100 mM	1.74 g
	Final volume	100 ml

Note: Store at RT.

5M Sodium Chloride

Sodium Chloride (FW 58.44)	5 M	292.2 g
	Final volume	1000 ml

Note: Store at RT.

10 x Glucose (20% w/v)

D-Glucose	20%	400 g
	Final volume	2000 ml

Note: Put ~1000 ml milliQ water into the bottle first, add one large magnetic stir bar. Slowly pour glucose in while water is being stirred. Fill volume to 2000 ml. This is to keep the sugar from crystallizing, not for complete solubilization. As long as no big block of sugar is visible, retrieve the stir bar and autoclave for 20 min at 121°C. Store at RT.

20 x Galactose (40% w/v)

Galactose	40%	800 g
	Final volume	2000 ml

Note: Autoclave ~1400 ml milliQ water with one large magnetic stir bar for 20 min at 121°C. Let the water cool down at RT for ~10 min. Then slowly pour galactose in while water is being stirred. Utilize the heat to fully dissolve Galactose and then fill volume to 2000 ml if necessary. The solution may appear to be light brownish due the impurity in the chemical. Filter the solution using 0.22 μm filter to sterilize and remove impurities. Store at RT.

20 x YNB (13.6% w/v)

YNB	13.6%	136 g
	Final volume	1000 ml

Note: Filter to sterilize. Store at 4°C.

10 x CSM-HIS (8 g/L)

CSM-HIS+Ade 40	8 g/l	16.0 g
	Final volume	2000 ml

Note: Autoclave at 121°C for 20 min to sterilize. Store at 4°C.

III.2. Reagent buffers for HT LIC cloning

Colony resuspension buffer

Tris-HCl, pH 9.0 RT (1 M stock)	10 mM	1 ml
Glycerol	15% (V/V)	18.75 g
MilliQ H_2O		84 ml

Note: Adjust pH to 8.0 using HCl and then adjust final volume to 100 ml. Autoclave at 121°C for 20 min to sterilize. Store in aliquots at RT.

SOC Medium

Bacto-tryptone	20 g/L	20 g
Yeast extract	5 g/L	5 g
NaCl	10 mM	0.584 g

KCl	2.5 mM	0.186 g
MilliQ H_2O		980 ml

Mix components and adjust pH to 7.0 with NaOH and autoclave at 121°C for 20 min to sterilize. After the solution cools down to <50°C, add 10 ml of 2 M Mg^{2+} stock, 10 ml of 2 M Glucose. Aliquot to a number of 15 ml and 50 ml sterile Falcon conical tubes for storage. Store at RT.

<u>2 M Mg^{2+} stock</u>

$MgCl_2$-$6H_2O$	1 M	20.33 g
$MgSO_4$-$7H_2O$	1 M	24.65 g
MilliQ H_2O		90 ml
	Final volume	100 ml

Note: Filter to sterilize. Store at RT.

<u>2 M Glucose</u>

Glucose	2 M	36.04 g

Distilled water to 100 ml. Filter sterilize. Distilled water to 100 ml. Autoclave or filter sterilize

III.3. For protein expression test and detergent screening

<u>Carrier DNA</u>

Commercial Salmon sperm-DNA (10 mg/ml)	2 mg/ml	1.0 ml
MilliQ H_2O		4.0 ml
	Final volume	5.0 ml

Note: Heat water bath to boiling temperature. Boil SS-DNA for 5 min and quickly chill in ice water. Freeze as 250 µl Aliquots and store at -20 ^{o}C. It is not necessary or desirable to boil the carrier DNA every time. Keep a small aliquot in your own freezer box and boil after 3-4 freeze-thaws. But keep on ice when out.

<u>Plate solution :</u>

PEG 3350 (50% stock)	40%	80 ml
LiAc (1 M stock)	0.1M	10 ml
Tris-HCl, pH 7.4 RT (1 M stock)	10 mM	1 ml
EDTA (500 mM stock)	1 mM	0.2 ml
MilliQ H_2O		~8.8 ml
	Final volume	100 ml

Note: Filter to sterilize. Store in aliquots at RT.

<u>3 x Solubilization buffer</u>

Tris-HCl, pH 7.4 RT (1 M stock)	150 mM	15 ml
NaCl (5 M stock)	300 mM	6 ml
MilliQ H_2O		~79 ml
	Final volume	100 ml

Note: Store at 4°C.

<u>2 x SDS loading dye</u>

Tris-HCl, pH 7.4 RT (1 M stock)	100 mM	10 ml
SDS	4 %	4 g
Glycerol	20% (W/V)	20 g
MilliQ H_2O		~65 ml
Adjust pH to 6.8 using HCl then add		
Bromophenol Blue	2 %	0.2 g
	Final volume	100 ml

Note: Store at RT. Add 40 µl BME to 960 µl 2 x SDS loading dye right before use to make working solution.

<u>Lysis buffer</u>

Tris-HCl, pH 7.5 RT (1 M stock)	50 mM	100 ml
Glycerol	20% (W/V)	400 g
MilliQ H_2O		~1500 ml
	Final volume	2000 ml

Note: Store at 4^oC.

<u>TE buffer</u>

TRIS-HCl, pH 9.0 RT (1M stock)	10 mM	10 ml
EDTA (0.5 M stock)	150 mM	30 ml
MilliQ H_2O		~700 ml
	Final volume	1000 ml

Note: Adjust final pH to 8.0RT with concentrated HCl. Store at room temperature.

<u>1 x TBST (1 x TBS with Tween 20)</u>

Tris pH 7.4 RT (1M stock)	25 mM	50 ml
NaCl (5 M stock)	250 mM	100 ml
Tween 20 (100%)	0.05%	1 ml
	Final volume	2000 ml

Note: Store at room temperature.

<u>2 liter of 4x YPG (YP + 8% Galactose)</u>

Yeast extract (BD #288610)	10 g/l	40 g
Peptone (BD #211820)	20 g/l	80 g

Note: Put ~500 ml milliQ water into the bottle, add one large magnetic stir bar. Slowly pour yeast extract and peptone in while water is being stirred. Fill volume to 1600 ml. Autoclave for 20 min at 121^oC. After the solution cool down to <50 ^oC, add 400 ml sterile 40% Galactose solution, stir and store at room temperature. The final volume is 2000 ml.

III. 4. For protein purification

<u>Solubilization Buffer</u>

Membranes		~15 ml
2x Membrane resuspension buffer		30 ml
Imidazole (5M stock)	15mM	0.18 ml
PMSF (100 mM stock)	1mM	0.8 ml
n- dodecyl-β-D- maltoside (Anatrace, FW 510.6)	20mM	1 g
MilliQ H_2O		~14 ml
	Final volume	60 ml

Note: Add one Complete EDTA-free protease inhibitor cocktail tablet (Roche, 11873580001) to the solution. Solubilization with Octyl-β-D-glucopyranoside (FW 292.4) is done in 200 mM OG. Store at 4^oC.

<u>2x Membrane Resuspension Buffer</u>

TRIS-HCl, pH 8.0 RT (1M stock)	50mM	50 ml
Sodium Chloride (5M stock)	1M	200 ml
Sucrose (Sigma #S-0389, FW 342.3)	200mM	68.46 g
MilliQ H_2O		~750 ml
	Final volume	1000 ml

Note: Adjust final pH to 8.0 RT with concentrated HCl. Filter through 0.22 μm membrane and store at 4^oC.

<u>Ni-NTA Buffers</u>

TRIS-HCl, pH 7.4RT (1M stock)	20 mM	20 ml
Sodium Chloride (5M stock)	200 mM	40 ml
Glycerol	10% (w/v)	100 g
n-dodecyl-β-D-maltoside (Anatrace D310, FW 510.6)	1 mM	1 g
MilliQ H_2O		~800 ml
	Final volume	1000 ml

Filter through 0.22 μm membrane to sterilize. Store at 4^oC.
Note: Add desired amount of Imidazole to each wash buffer and adjust final pH to 7.4RT with concentrated HCl. Use Octyl-β-D-glucopyranoside (FW 292.4) 40 mM if solubilization is done in OG.

SEC Buffer

TRIS-HCl, pH 7.4RT (1M stock)	20 mM	20 ml
Sodium Chloride (5M stock)	200 mM	40 ml
Glycerol	10% (w/v)	100 g
n-dodecyl-β-D-maltoside (FW 510.6)	1 mM	1 g
MilliQ H_2O		~800 ml
	Final volume	1000 ml

NB: Adjust final pH to 7.4 RT with concentrated HCl. Store at 4^oC.

PART TWO Detailed protocols

I. High throughput ligase independent cloning

I.1. PCR amplification of target genes

(1) Design LIC primers for amplification of target gene with 83nu LIC-compatible 5' extensions:

Forward primer:
5' CAA GGA CCG AGC AGC CCC TCA* XXX XXX XXX XXX XXX XXX 3'

Note: * This codon has to be TXX for LIC cloning and TCA adds a Serine, if you gene already starts with TXX, ignore this TCA.
XXX are 15-21 bp target gene sequence **without** start codon;

Reverse primer:
5' ACC ACG GGG AAC CAA CCC TCC* XXX XXX XXX XXX XXX XXX 3'

Note: * This sequence has to be TXX for LIC cloning and GGA (reverse complement of TCC) adds a Glycine, if you gene already ends with XXA, ignore this TCC.
XXX are reverse complement of the last 15-21 bp of target gene sequence **WITHOUT** stop codon, this will give you a construct of N-ter FLAG tag with PreScission 3C cleavage site **and** C-ter 10 His tag with Thrombin site;
If you only want N-ter FLAG tag with PreScission cleavage site, include a stop codon in your reverse primer.

Order synthetic oligos as salt free format, no need for additional purification or modification. We order from Sigma as 96-well format and they come in as 100 μM normalized concentration.
Using a multichannel pipette, prepare a 5 μM primer mix plate:

100 μM Forward primer	5 μl
100 μM Reverse primer	5 μl

Water	90 µl
Total	100 µl

(2) Set up PCR reaction:

Prepare PCR master mix for one 96 well plate (each contains a 50 µl reaction):

Component	Volume
Yeast genomic DNA (DNA template)	50 µl
5X HF buffer	1050 µl
dNTPs, 10 mM	105 µl
DNAse RNAse free water	3470 µl
Phusion polymerase	50 µl
Total	4725 µl

Mix well and then aliquot 45 µl to each well on a chilled 96-well thin wall PCR plate. Using a 20 µl size multichannel pipette, add 5 µl of the primer mix to each corresponding well. Run PCR reaction using the following program.

PCR program used for yeast high-throughput cloning:

Cycle step	**Temp**	**Time**	**Number of cycles**
Initial denaturation	98°C	30 sec	1
Denaturation Annealing Extension	98°C 55 °C 72 °C	10 sec 15 sec 2 min	35
Final extension	72 °C	10 min	1
Hold	4 °C	forever	

(3) Add 10 µl of 6X Orange-G dye to each well, use a multichannel pipette to load 40 µl PCR product on 1% agarose gel, retrieve gel slices with the disposable gel extraction tool, dissolve each one in 400 µl QG buffer, extract DNA with using QIAquick 96 PCR purification kit and elute with 80 µl ddH_2O.
Note: Gel extraction step is critical for successful cloning, PCR clean-up is NOT enough.

I.2. Blunt-end enzyme *SmaI* digestion of LIC vector 83 nu

(1) Set up a 60 µl digestion in PCR tube or Eppendorf tube:

Component	Volume
Vector miniprep DNA	20 µl
10X NEB buffer4	6 µl
SmaI (20 U/µl)	4 µl
DNAse RNAse free water	30 µl
Total	60 µl

Note: Do not use larger volume than this. Set up digestion in multiple tubes if you want more vector stock.

(2) Incubate digest at 25°C for 4 hours or leave at room temperature (21°C) overnight.
(3) Run entire reaction on 1% agarose gel and extract DNA from gel with Qiagen Gel Extraction Kit. Elute DNA with 30 µl ddH_2O.
(4) Store at -20°C or set up T4 polymerase treatment immediately.

I.3.T4 DNA Polymerase treatment of vector and insert DNA

(1) Setting up T4 polymerase "Chewback" reaction:

For vector:

Volume of miniprep plasmid DNA	5 μl
10 X T4 DNA poly buffer	2 μl
d**T**TP (25mM)	2 μl
DTT (100 mM)	1 μl
LIC qualified T4 DNA polymerase	0.4μl
ddH_2O	9.6μl
Total	20μl

For insert DNA: Prepare master mix to be used for one 96 well plate (each contains a 20 μl reaction):

10 X T4 DNA poly buffer	210 μl
d**A**TP (25mM)	210 μl
DTT (100 mM)	105 μl
LIC qualified T4 DNA polymerase	42 μl
Total	567 μl

Mix well and then aliquot 5.4 μl to each well on a 96-well thin wall PCR plate. Using a 20 μl size multichannel pipette, add 14.6 μl of the gel purified insert DNA to each corresponding well.

(2) Set up PCR machine to incubate reactions at 25°C for 40 min and then heat-inactivate enzyme at 75°C for 20 min.
(3) Store reactions at -20 °C, or use for LIC annealing reactions immediately.

I.4. LIC annealing reaction

(1) Dilute the 20 μl T4 treated vector DNA with 300 μl ddH_2O, aliquot 2.0 μl of the diluted vector DNA into each well of a 96-well thin wall PCR plate. Using a multichannel pipette, add 4.0 μl of the gel purified insert DNA to each corresponding well.
    Note: We highly recommend you test the vector stock with various dilution factors with a known insert DNA before setting up 96-well reaction. Depends on the vector DNA concentration, linearilization efficiency, and T4 "chew-back" efficiency, you can make dilutions from 4 fold to 16 fold accordingly.
(2) Incubate reaction at room temperature for 15 min.
(3) Add 2 μl EDTA (25 mM) and incubate at room temperature for another 10 min.
(4) Aliquot 50 μl of the in-house competent *E. coli* DH5α cells on a prechilled 96-well deep well block. Transform 5 μl LIC reaction mixtures into each well, incubate on ice for 20 min, heat shock at 42°C for 45 sec, incubate on ice for 2 min, add 200 μl SOC medium (prewarmed to 37°C), seal with gas permeable seal and incubate at for 1 hour at 220 rpm agitation. For each sample, plate all 250 μl culture on one LB ampicilin (100 μg/ml) plates. Incubate at 37°C overnight.
    Note: If high efficiency competent cells are available, one can also use PCR plate for transformation and use 100 μl SOC medium.
    The 48 grid vented Q-tray with divider is also an option for plating. But one may expect only 10% colonies comparing with a standard 10 cm Petri dish.

I.5.Colony screening

(1) Aliquot 20 µl colony resuspension buffer to each well of a 96-well PCR plate, pick a colony from the overnight LBA plate with autoclaved flat head toothpick and dipping in the buffer briefly.
*Note:*Pick two colonies and set up two sets of colony PCR reactions for each target to increase success rate.

(2) Prepare master mix for one 96 well plate (each contains a 20 µL reaction):

10 x Taq standard polymerase buffer	210 µl
dNTPs, 10 mM	42 µl
100 µM GalF forward primer	10 µl
100 µM CycR reverse primer	10 µl
DNAse RNAse free water	736 µl
Taq polymerase (5 U/µl)	42 µl
Total	1050 µl

Note: GalF forward primer sequence: 5'-CTT TCA ACA TTT TCG GTT TG-3'
CycR reverse primer sequence: 5'-GGG GGG AGG GCG TGA ATG TAA-3'

Mix well and then aliquot 10 µl to each well on a chilled 96-well thin wall PCR plate. Using a 20 µl size multichannel pipette, add 10 µl of the resuspended colony to each corresponding well. Run PCR reaction using the following program.

PCR program used for yeast colony PCR:

Cycle step	**Temp**	**Time**	**Number of cycles**
Initial denaturation	94°C	5 min	1
Denaturation Annealing Extension	94°C 55 ^{o}C 68 ^{o}C	15 sec 15 sec 2 min	30
Final extension	68 ^{o}C	5 min	1
Hold	4 ^{o}C	forever	

Add 4 µl of 6 x Orange-G dye to each well, use a multichannel pipette to load all 24 µl PCR product on 1% agarose gel using the DNA plus 200 well agarose gel.

(3) For positive clones, inoculate 1.5 mL 2 x LBA medium in 2.0 ml deep well block using the left 10 µl of the resuspended colony. Incubate at 37°C 220 rpm for 24 hours.

(4) Miniprep plasmid DNA following Qiagen's QIAvac 96-well format plasmid miniprep protocol. Elute with 125 µl EB buffer into PCR plate (make sure the plate is elevated so the column tip fit in to each well!).

(5) Restriction mapping to further confirm insert.
Prepare master mix with BamHI and XhoI enzymes

10 x NEB buffer 3	210 µl
BamHI (20 U/µl)	27 µl
XhoI (20 U/µl)	27 µl
DNase RNase free water	1417.5 µl
Total	1681.5 µl

Mix well and then aliquot 16.0 µl to each well on a PCR plate. Using a multichannel pipette, add 4.0 µl of the plasmid DNA to each corresponding well. Incubate at 37°C for 30 min and then add 4 µl of 6 x Orange-G dye to each well, use a multichannel pipette to load all 24 µl PCR product on 1% agarose gel using the DNA plus 200 well agarose gel.

I.6.Backup plasmid DNA of the clones

(1) Prepare 48 grid LBA Q-tray the day before and dry the tray at 37°C overnight. For each Q-tray, ~220 ml LBA liquid medium is needed.

Note: Try your best to keep the volume close to 220 ml, too much or too little will cause surprises you do not want.

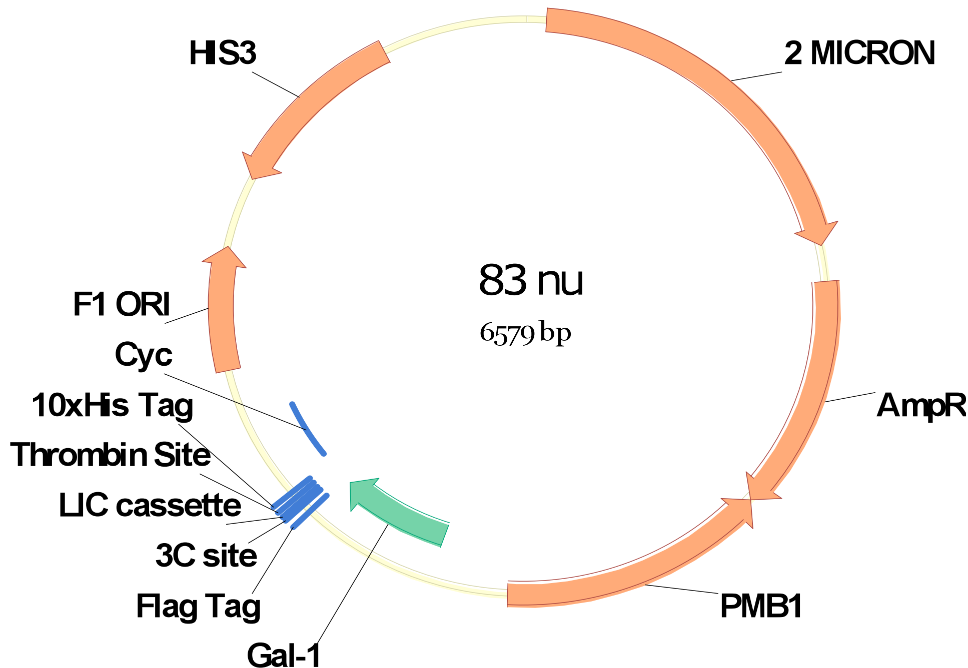
(2) Thaw enough of the in-house competent *E. coli* DH5α cells on ice 30 minutes before transformation. Aliquot 20 μl onto each well of a prechilled 96-well PCR plate. Add 1 μl plasmid DNA into each well, incubate on ice for 5 min, heat shock at 42°C for 30 sec, incubate on ice for 2 min, and add 100 μl SOC medium (prewarmed to 37°C). No cell recovery is needed; plate all 120 μl culture on one grid of the LBA Q-tray. Simply swirl the tray carefully to spread the culture, do not use plating beads or spreader. Incubate at 37°C overnight, do NOT flip the tray.

Note: To prevent overdrying the tray, we recommend using a bench-top incubator without excess vent.

(3) Prepare plasmid DNA and confirm insert by restriction mapping using the method as described before in I.5(5).

I.6.Vector 83 nu sequence

Vector 83 nu (or 83 υ) is based on pRS423-Gal1 shuttle vector with an LIC cloning cassette insert between the BamHI and XhoI restriction enzyme sites. This expression vector has 2 micron origin of replication, HIS3 selection marker, ampicilin antibiotic resistance, and strong Gal1 promoter with Cyc terminator. The LIC region includes N-ter FLAG tag with PreScission 3C cleavage site and C-ter 10 His tag with Thrombin site. TCCATGGCAAGCGACTACAAGGATGACGACGACAAGGGTGCACTTGAAGTCCTCTTTCAAGGACCGAGCAGCCCCGGGTTGGTTCCCCGTGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACGGTGCATAA. The SmaI restriction site in the center of the LIC region (CCCGGG) is where your gene of interest goes in.



<u>The full vector sequence is 6579 bp:</u>

gacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttcttagatgatccaatatcaaaggaaatgatagcattgaaggatgagacta
atccaattgaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcacaggaggtactagact
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II. Membrane protein expression in yeast *S. cerevisiae*

Note: This protocol is for transformation of small number of targets. If you have more than 24 transformations to do, you might want to start with fresh overnight yeast cells. Inoculate 5 ml fresh YPD with a single yeast colony, incubate at 30 ^{o}C at 220 rpm agitation for overnight.

II.1. Day 1: Transformation of *S. cerevisiae* cells, the strain we use is W303-Δpep4 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 Δpep4 MATα*):

For single transformation:

a. Take 0.10 ml of yeast culture (fresh overnight culture or frozen stock or simply colony on a plate) and spin 10 sec in microfuge. Decant the liquid and resuspend the pellet in 0.15 ml PLATE solution.
b. Add 5 µl of carrier DNA (10 µg) plus 0.1 µg plasmid DNA of your target and vortex well.
c. Add 10 µl DMSO and vortex briefly.
d. Leave for 15 min at RT.
e. Heat shock for 20 min at 42°C.
f. Pellet cells in microfuge at 16,000 x g for 30 seconds. Remove supernatant.
g. Add 200 µl TE to the cell pellet and gently resuspend cells by aspirating up-and-down with a pipette tip. Spread all suspended cells onto one selective SC-HIS plates.
h. Incubate at 30°C for 3 days.

For medium to high-throughput transformation (shown is for 96 transformations):

a. Spin the overnight yeast culture, discard the liquid.
b. Prepare master mix solution enough for 96 transformations plus one blank.

Plate solution	14000 µl
Boiled Salmon Sperm DNA (2 mg/ml)	500 µl
DMSO (100%)	1000 µl
Total	15500 µl

c. Mix the solution by vortexing 5 sec and aliquot 150 µl into either 96 well format PCR plate or 8-strip PCR tubes.
d. Add 2 µl plasmid DNA from the 96 well miniprep to each tube and vortex well.
e. Set up PCR machine to incubate reactions at 25°C for 15 min and then heat-shock at 42°C for 20 min.
f. Pellet cells in Allegra centrifuge with Microtiter plate holder at 2000 x g for 2 min. Remove supernatant by multichannel pipette.
g. Add 200 µl TE to the cell pellet and gently resuspend cells by aspirating up-and-down with a pipette tip. Spread suspended cells onto one selective SC-HIS plates.
h. Incubate at 30°C for 3 days.

Note: One can also use the vented 48 grid Q-tray to prepare the selective SC-HIS plates. Expect anywhere from 1 colony to 100 colonies depending on the plasmid DNA concentration and the viability of the transforming cells.

II.2. Day 4 (~5 pm): Preculture outgrowth:

a. Inoculate 5 ml SC-HIS with a single colony of the transformant from the selective plate. Note the blank plate should have no colony. If it looks suspicious, start over from transformation with fresh ingredients.
b. Incubate 24 hours at 30 °C and at 220 rpm.

II.3. Day 5 : Scale up culture.

a. For every target, autoclave 280 ml water in a 1 liter size baffled flask. Also autoclave one 100 ml and one 250 ml size graduated cylinder.
b. ~5 pm of the day, use the sterile 100 ml graduated cylinder to add 95 ml 4xSC-HIS stock medium to each flask.
c. Inoculate the 375 ml 1xSC-HIS medium with the 5 ml preculture.
d. Incubate 24 hours at 30 °C and at 220 rpm.

II.4. Day 6 (~5 pm): Induction.

a. Following this growth period the optical density at 600 nm ranged between 15 to 20 for most cultures with glucose concentration generally <0.1%. Induce the culture by adding 125 ml 4xYPG stock medium to each flask.
b. Incubate 16 hours at 30 °C and at 220 rpm.

II.5. Day 7 (~9 am): Cell harvest and membrane preparation.

All samples should be kept at 4 ^{o}C or on ice from now on.

a. Harvest the cells by centrifuging it at 6000 x g for 10 min.
   Note: The cells are very likely to be pink color at this stage from a red pigment formed during biosynthesis of purine nucleotides. This is due to the lack of ADE2 (phosphoribosyl-aminoimidazole-carboxylase) gene in the yeast strain we use.
b. Resuspend the cell pellet in lysis buffer to make 40 ml cell resuspension. You can either proceed to next step for lysis and membrane preparation or store it at -80 °C till later.
c. Add 450 µl 100 mM PMSF stock solution, put all 40 ml into medium size bead beater canister, and fill the volume with prechilled 0.5 mm glass beads. Assemble the unit following the manufacture instructions.
d. Blend at maximum speed for 60 sec, stop for 60 sec. Repeat for a total 5 cycles of beating and cooling. More sets of beadbeater apparatus and an automatic lab controller can facilitate this process nicely.
e. Dissemble the unit and collect ~25 ml homogenate.
   Note: This is for screening process so we do not try to recover all cell lysate here. One can always recover more cell extract by rinsing the beads with lysis buffer. Doing so will increase the crude cell lysate volume and lead to more ultra-centrifugation burden.
f. Spin down the crude cell extract at 6000 x g for 15 min.
   Note: There are two ways to tell the lysis efficiency: (1) visually inspect the cells under microscope; (2) the cell debris pellet typically has 2 layers: bottom pink layer being unlysed cells, top white layer being organelles from lysed cells. The estimate volume ratio of the top lysed cells to the bottom unlysed cells tells you the efficiency. We typically have >90% efficiency at this stage but >70 % is considered acceptable.
g. Collect cell lysate from the supernatant of the previous low speed spin. Be careful not to pour in any cell debris. Use one Ti50.2 ultracentrifuge tube for one sample, fill the tube to maximum volume of 26.3 ml. Spin at 184,000 x g (45,000 rpm using Ti 50.2 rotor) for 1 hour.
h. Discard the supernatant from the high speed spin. To each tube, add 0.5 ml membrane resuspension buffer with 10 µl HALT protease inhibitor cocktail (100 x stock solution). Put a small stir bar and stir for 30 min on ice.
i. After the membrane pellet has been well resuspended, save 20 µl in a high speed microcentrifuge tube for the next step detergent screen. Proceed immediately to next step or freeze the membrane sample at -80°C for later. The total volume of membrane suspension from 0.5 liter culture is typically between 1.5 ml to 3 ml. Freeze the samples at -80°C in 1.5 ml eppendorf tubes or 5 ml

eppendorf tubes for test purification or repeat detergent screen. You can always freeze in smaller aliquots depends on your application next stage.

II.6. Day 8: Detergent solubilization screen and western blot analysis.

Detergent solubilization screen

a. Prepare master mix for samples of DDM detergent solubilization screen.

	For 1	For 24 (+1 spare)
3 x Solubilization buffer	100 µl	2500 µl
DDM (200 mM)	60 µl	1500 µl
MilliQ H_2O	120 µl	3000 µl
Total	280 µl	7000 µl

b. Aliquot 280 µl DDM solubilization master mix to each microfuge tube that already has 20 µl membrane sample. Put one flea size stir bar. Stir at 4 ^{o}C for 1 hour. Make sure the stir bar is moving freely.
c. Align enough PCR tubes (twice the number of samples) or 8 strip PCR tubes on a PCR tube rack. Take out 30 µl from each DDM mixture as “before spin” gel sample and put in every other PCR tube. Leave the tube next to it for the “after spin” gel sample for the same target.
d. Take out the stir bar, spin the mixture at 180,000 x g (50,000 rpm using MLA55) rotor for 20 min.
e. Transfer the supernatant to another eppendorf tube. Pipette up and down to mix. This is important to avoid gradient in solution.
f. Put 30 µl from each as “after spin” into the empty PCR tubes. Proceed to SDS-PAGE western blot analysis.

Western blot analysis

a. Prepare working solution of SDS loading dye by adding 40 µl BME to 960 µl of the 2 x SDS loading dye stock. Mix well by inverting the tube a few times.
b. Add 30 µl SDS loading dye to each gel sample, mix well by pippeting up and down.
c. Incubate the sample at 37°C for 5 min.
d. In the meantime, prepare enough 4-20% SDS-PAGE gels and electropherisis apparatus. You need 2 mini gels for every 7 targets. (Each Invitrogen mini gel has 15 wells and every target has two gel samples. Two western blots will be done for each sample using anti-FLAG or anti-HIS polyclonal antibody.)
e. For each gel, start with 5 µl protein ladder. Then load 10 µl “before spin” sample follow by 10 µl “after spin” sample of the same target. Run gel at 200 Volts constant for 1 hour.
f. Wet enough 0.45 µm PVDF membranes using 100% methanol and then presoak them and thick filter papers in western transfer buffer for 5 min at 4°C.
g. Prepare semi-dry transfer blot cassettes following manufacture instructions. Transfer proteins to PVDF membrane for 30 min at 25 V constant.
h. Block the membranes with 2.5% non-fat dry milk in 1 x TBST buffer for 30 min.
i. Make working solutions of the antibodies as follows: 1:1000 dilution of Anti-HIS probe in 3% BSA and 1:5000 dilution of anti-FLAG probe in 2.5% milk. Incubate the membranes in either antibody (separately) at room temperature for 1 hour.
j. Wash the membranes 3 times in TBST, 5 min each time.
k. Prepare working solution of the ECL plus chemiflurescence HRP substrate by mixing 0.5 ml peroxide solution and 0.5 ml luminal/enhancer solution for every membrane. Pat dry the membranes with paper towels and then put them in the Avery sheet protector, apply 1 ml freshly prepared substrate working solution. Wait for 1 min and then blot dry.
l. Develop in dark room using X-ray film. We also include a strip of autorad marker as label.

III. Protein characterization (void check) protocol

Note: Start as early as possible and reserve ultracentrifuge for a 60 minute spin. Also, reserve diode array chromatography station with auto injector in advance, ensure reagents and media are available in advance. Superdex 200 Column is equilibrated overnight with SEC buffer. Typically six samples are done in a day and are left on the autosampler to run overnight. All experiments are performed at 4 °C.

III.1. Ni affinity chromatography purification

a. In a Ti-45 tube on ice, prepare Solubilization Buffer mix containing membranes and a medium stir bar. Transfer to a stir plate at 4 °C for heavy stirring for 60 minutes.
b. After an hour, take a 50µl before spin sample. Remove stir bars from Ti 45 tubes and balance tubes. Spin membranes at 42,000 rpm (138,000 x g) for 60 minutes.
c. After ultracentrifugation, take another 50µL from supernatant as an after spin sample. Split supernatant into two ice cold 50ml Falcon tubes with each containing circa 25 ml. Add 2 ml Ni-NTA slurry (1 ml resin) resuspended in Ni-NTA Buffer plus 15 mM imidazole to each Falcon tube. Nutate batch along the axis parallel to the height of the tube at 4 °C for 90 minutes.
d. At this time take care to preheat the UV lamp that will be used for the Ni elution. Transfer mixture to a gravity SEC column and pass flow-through over the resin bed twice. Wash immediately with 20 resin bed volumes of Ni-NTA Buffer plus 15 mM imidazole. Deliberate attempts should be made to not disturb the resin bed, add buffers slowly at first.
   Note: slow down drip rate to one drop per second when collecting wash sample.
e. Wash with 20 resin bed volumes of Ni-NTA Buffer plus 30 mM imidazole. Collect a wash sample. Do not let all the buffer flow through the column as the final 2~3 ml of this wash is performed in the chromatography station on a detector.
f. At the chromatography station let 30 mM imidazole wash all the way. Carefully add ~5 ml Ni-NTA Buffer plus 300 mM imidazole and slow drip rate down to one drop per two seconds. Follow the absorbance real-time and collect 1ml of the top peak of the elution to get as concentrated sample as feasible.
g. Desalt concentrated 1 ml in NAP-10 (GE healthcare) desalting column equilibrated in SEC buffer. Elute with 1.5 ml SEC buffer.
h. Measure OD_{280} of samples. Setup autosampler to run 500 µl injections overnight.

III. 2. Cleavage of Targets - further purification

Note: This protocol follows III.1. exactly until step f. Again all manipulations are performed at 4 °C.

a. At the chromatography station let 30 mM imidazole wash all the way. Carefully add ~7 ml Ni-NTA Buffer plus 300 mM imidazole and slow drip rate down to one drop per two seconds. Follow the absorbance real-time and collect the entire elution peak; this generally is a volume of 3-7 ml.
b. Immediately desalt on 10DG BioRAD desalting columns equilibrated in SEC buffer. Measure OD_{280} of samples.
c. Add 3C and Thrombin to catalyze tag removal. Thrombin (Novagen) is added at 4 units per OD, 3C (MBP-His fusion, house stock) is added at 0.6 mg per 10 OD. Cleave and nutate along the axis parallel to the height of the tube at 4 °C overnight.
d. The next morning add 100ul of prewashed and resuspended benzamidine beads (Sepharose 6B, GE) per 50 units of Thrombin and 1ml of TALON resin (CloneTech) per 1mg of 3C-MBP-His fusion. Nutate batch along the axis parallel to the height of the tube at 4 °C for 60 minutes.
e. Pass suspension through column and collect flow-through to obtain cleaved membrane protein. Measure OD_{280}.
f. At this stage, it may be necessary to concentrate protein to about 1-2 OD/ml for the ensuing purification on SEC. Use an appropriate spin filter depending on the MW the protein of interest.
g. Perform SEC with manual injections, pool peak fractions and concentrate to taste.
h. Run gels on final samples and perform single western for each batch to verify that tag has cleaved.