Culture and Induction Protocol to Screen for Protein Expression in *Saccharomyces cerevisiae*

The methods described can be used for testing protein expression with vectors using GAL1, TEF, ADH or GPD promoters. The following sections are included:

I. Competent cell production

II. Transformation

III. Culture conditions and Expression of Recombinant protein

IV. Evaluation of Expressed Protein

V. Troubleshooting

I. Competent cell production

Media & Reagents

Saccharomyces cerevisiae host strain with appropriate auxotrophic marker. ATUM offers vectors with the GAL1, TEF, ADH or GPD promoter in combination with LEU2, URA3, TRP1 or HIS3 to complement these common *S. cerevisiae* auxotrophic markers for selecting transformants.

Electroporation Buffer: 1M sorbitol, 1 mM $CaCl_2$.

Conditioning Buffer: 0.1 M LiAc, 10 mM DTT.

YPD broth: 1% yeast extract, 2% peptone, 2% glucose.

YPD agar plates: 1% yeast extract, 2% peptone, 2% glucose, 2% agar

Equipment

Sterile baffled flasks, 5 X volume of media to be used (for example, use 50 ml of YPD in 250 ml flask).

30°C shaking incubator.

Spectrophotometer and cuvettes.

Centrifuge

Procedure for Preparation of Competent Cells (50 ml culture)[1]

Note: In addition to the electroporation protocol described below, there are many examples[2] of methods that do not require electroporation.

1. Streak your strain for single colonies to a YPD agar plate. Incubate for 1-2 day at 30°C.

2. Grow a 5-25 ml overnight culture of the host strain in YPD broth in a 30°C shaking incubator at 250 rpm.

3. The next day, dilute the overnight culture to an optical density (OD600) of 0.3, in 50 ml of YPD broth in a baffled flask of at least 250 ml capacity. Starting volumes may be scaled up or down.

4. Grow the culture to an OD600 of ~1.6 in a 30°C shaking incubator at 250 rpm.

5. Centrifuge the culture at 500 x g for 15 minutes at room temperature and carefully pour off the supernatant.

6. Wash twice with 50 ml of sterile cold water.

7. Wash once with 50 ml of cold Electroporation Buffer.

8. Shake cells in 20 ml of Conditioning Buffer for 30 min and 225 rpm at 30°C.

9. Wash once with 50 ml of Electroporation Buffer.

10. Suspend cells in enough Electroporation Buffer to reach a volume of 1 ml.

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[1] An improved yeast transformation method for the generation of very large human antibody libraries. Bematiol L, et al. Protein Engineering, Design & Selection, vol. 23 no. 4 pp. 155-159, 2010

2 Frozen competent yeast cells that can be transformed with high efficiency using the LiAc/SS carrier DNA/PEG method. Gietz RD and Schiestl RH. Nat Protoc 2007;(2):1-4.

II. Transformation

Media & Reagents

~0.01-1 µg of expression vector in up to 10 µl of sterile deionized water or 10 mM Tris-Cl, pH 8.3. You may use any method of your choice to prepare purified plasmid DNA for small-scale yeast transformation.

~0.01-1 µg of appropriate control vector in up to 10 µl of sterile deionized water or 10 mM Tris-Cl, pH 8.3. For example, use one of ATUM's IP-Free *Saccharomyces* vectors using the same promoter and complementation marker as your expression vector.

YPD broth (1% yeast extract, 2% peptone, 2% glucose).

1:1 Mix of 1M Sorbitol:YPD Broth

CM glucose agar plates without the auxotrophic requirement complemented by the expression vector. A convenient source of these plates is http://www.teknova.com/: CM glucose-His (C3020), CM glucose-Leu (C3040), CM glucose-Trp (C3060) or CM glucose-Ura (C3080).

Equipment

Electroporation Cuvettes, with 0.2 cm gap

14 ml sterile round bottom culture tubes

Sterile glass beads or sterile spreader

Gene Pulsar II electroporator (Bio-Rad Laboratories, Hercules, CA, USA); cuvette gap, 0.2 cm, charging voltage, 2.5 kV; capacitance, 25 µF.

Procedure for Transformation

1. On ice, mix 0.01-1 µg of expression vector in up to 10 µl of water or buffer with 100 µl of competent cells and transfer to a chilled electroporation cuvette. Repeat for expression control vector. Also, include a 100 µl aliquot of cells with no DNA as negative control.

Note: At first, consider transforming 0.01, 0.1 and 1 ug of vector DNA to help determine competency of your strain by this method.

2. Transfer to an ice-cold 0.2 cm gap electroporation cuvette.

3. Electroporate at 2.5 kV and 25 µF.

4. Immediately add 1 ml of 1M Sorbitol:YPD Broth to the electroporation cuvette, and transfer cells to a 14 ml sterile round bottom culture tube (or similar).

5. Incubate the cells for 1 hour at 30°C and 250 rpm or on a tube roller at 30°C.

6. Pellet cells at 500 x g for 5 minutes, pour off the supernatant. Suspend cells in 1 ml of sterile water to wash away residual Sorbitol:YPD broth. Pellet cells at 500 x g for 5 minutes, pour off the supernatant. Suspend cells in 0.1 ml of sterile water. Plate the cells to the appropriate CM glucose – requirement plate. Consider diluting 1:10 and 1:100 prior to plating.

7. Incubate the plates at 30°C for 2-3 days, until colonies are well formed. No colonies should grow on the negative control.

8. Patch 4-8 isolated colonies to CM glucose– requirement plate. Incubate 1-2 days at 30°C. Store plate at 4°C.

IV. Screening for Expression of Recombinant Protein in 24- Well Format:

Media & Reagents

For expression vectors with constitutive ADH, TEF or GPD promoters, use CM glucose broth without the auxotrophic requirement complemented by the expression vector. A convenient source for CM Glucose Broth is http://www.teknova.com/: CM glucose (2%) broth-His (C8110), CM glucose broth-Leu (C8120), CM glucose-Trp (C8130) or CM glucose-Ura (C8140).

For expression vectors with the galactose-inducible GAL1 promoter, use the protocol for GAL1 expression vectors described below.

For constitutive promoters ADH, TEF or GPD use:

CM glucose (2%) minus His, Leu, Trp, or Ura (as appropriate for selection of vector) for overnight inoculum.

YPD broth for protein production.

Note: although selection if lost in YPD broth, protein yields are generally much better using YPD broth for protein production instead of the selective CM glucose broth.

For GAL1 promoter use:

20% galactose solution for daily dosing.

CM raffinose (2%) minus His, Leu, Trp, or Ura (as appropriate for selection of vector) for overnight inoculum.

Buffered YP Broth (1% yeast extract, 2% peptone), 100 mM potassium phosphate pH 6.7 + raffinose (2%) + galactose (2%) for induction and protein production.

Buffered YP Broth (1% yeast extract, 2% peptone), 100 mM potassium phosphate pH 6.7, + raffinose (2%) can optionally be used as a no induction control.

Note: MP Biomedicals is a good source for *S. cerivisiae* drop out powder and other media components (mp.bio.com).

Equipment

24 deep well x 10 ml plate (e.g., Whatman UNIPLATE 24-well x 10 ml polypropylene, round bottom, cat. no. 7701-5102).

96 deep well x 2 ml plate (e.g., E&K 96 Deep Well Plates, 2.2 ml, Conical Bottom, Clear, Sterile cat. no. EK-2315)

30°C incubator with plate holder.

Breathable membrane tape to seal plates.

Centrifuge with plate holders.

Procedure for Expression Test from the ADH, TEF, or GPD Constitutive Promoters

Note: For 24-well plates use 5 ml overnight inoculum and protein production media. For 96-well plates use 1 ml overnight inoculum and protein production media. The protocol as written is for the 24-well plate method.

1. For each 24-well x 10 ml plate, add 5 ml of appropriate CM glucose broth - requirement per well. Reserve one well to grow the un-transformed host strain as a negative expression control if desired.

2. Using sterile pipette tips, pick a small amount of *S. cerevisiae* from each patch into a well with medium. Place the tip into the well and mix. Pick up to 4 colonies from each transformation. For the negative control, inoculate the *S. cerevisiae* host CM glucose broth + requirement supplement (L-histidine, L-leucine, L-tryptophan or uracil at ~50 µg/ml as needed).

3. Seal the 24-well plate with a breathable membrane tape. Incubate at 30°C with vigorous shaking.

4. Determine the OD600 of the overnight inoculum culture.

5. From this, calculate the volume of the overnight culture required to obtain an OD600 of 0.4 in 5 ml of the protein production medium.

6. Transfer the calculated amount of culture from the overnight inoculum plate to the protein production plate with 5 ml of protein production medium per well. Seal the plate with a breathable membrane tape. Incubate at 30°C with vigorous shaking.

7. Samples (up to 100 µl) may be taken every 12-24 hours up to 72 hours (freeze samples at -20°C until ready to evaluate).

Note: For secreted samples, centrifuge for 5 min at 1000 x g and collect and save supernatant.

Procedure for Expression Test from the Galactose-Induceble GAL1 Promter

Note: For 24-well plates use 5 ml overnight inoculum and protein induction media. For 96-well plates use 1 ml overnight inoculum and protein induction media. The protocol as written is for the 24-well plate alternative.

1. For each 24-well x 10 ml plate, add 5 ml of appropriate CM raffinose (2%) broth -requirement per well. Reserve one well to grow the un-transformed host strain as a negative expression control if desired.

2. Using sterile pipette tips, pick a small amount of *S. cerevisiae* from each patch into a well with medium. Place the tip into the well and mix. Pick up to 4 colonies from each transformation. For the negative control, inoculate the *S. cerevisiae* host CM glucose broth + requirement supplement (L-histidine, L-leucine, L-tryptophan or uracil at ~50 µg/ml as needed).

3. Seal the 24-well plate with a breathable membrane tape. Incubate at 30°C with vigorous shaking.

4. Determine the OD600 of the overnight culture.

5. From this, calculate the volume of the overnight culture required to obtain an OD600 of 0.4 in 5 ml of protein induction medium.

5. Transfer the calculated amount of culture from the overnight inoculum plate to the protein production plate 5 ml of protein induction medium per well. Seal the plate with a breathable membrane tape. Incubate at 30°C with vigorous shaking.

7. Samples (up to 100 µl) may be taken at 4, 8, 12, 24, and 72 hours (freeze samples at -20°C until ready to evaluate).

8. At 24 and 48 hours add 0.25 ml of 20% galactose (to ~2%) to continue induction.

Note: For secreted samples, centrifuge for 5 min at 1000 x g and collect and save supernatant.

IV. Evaluation of Expressed Protein

Media & Reagents

SDS-PAGE gel, buffers, gel box, molecular weight marker, gel stain, 4 X sample buffer

To prepare Cytoplasmic Yeast Lysis Buffer:

1 ml Novagen Yeast Buster reagent (Novagen 71186)

10 µl 1M stock $MgCl_2$ (10 mM final)

5 µl 200 mM PMSF (1 mM final) (Sigma P7626)

10 µl 100X THP reducing agent (1x final) (Novagen 71190-4FR2)

10 µl Sigma Fungal protease inhibitor (Sigma P8215)

2 µl Epicentre Omnicleave endonuclease (Epicentre OC7850K)

Prepare just before using.

Equipment

PAGE gel box, power supply, staining boxes

Microcentrifuge

Procedure for Checking Expression

1. Pellet 100 µl samples for 5 min at 5000 x g in a micro centrifuge at 4°C.

2. For secreted proteins carefully transfer the supernatant to a new microfuge tube. For cytoplasmic proteins, discard supernatant in an appropriate waste container.

3. For secreted proteins, mix 30 µl of supernatant with 10 µl of 4X SDS-PAGE sample buffer and heat at 95°C for 10 minutes. Load 20 µl/lane onto the SDS-PAGE gel.

4. For cytoplasmic proteins, add 100 µl per sample of freshly prepared Cytoplasmic Yeast Lysis Buffer mix and shake gently for 20-30 minutes at room temperature. Remove 15 µl and add 5 µl of 4X sample buffer. Heat at 95°C for 10 minutes. Load 5 µl/lane onto the SDS-PAGE gel.

V. Troubleshooting

Problem	Probable Cause	Possible Solution
Low efficiency of transformation	Vector concentration too low	Check DNA concentration of vector by measuring A260 or by comparing to standards on an agarose gel.
	Cell density too low	Resuspend cells in a smaller volume of Electroporation Buffer
Expression of protein low or not detectable	Detection method suboptimal.	Concentrate samples and re-run SDS-PAGE. Analyze via western blot if possible.
	Good expression, poor secretion.	Check cell pellet to see if overall expression is low. If good expression, but poor secretion, try a different signal sequence. Try intracellular expression if possible.
	Low expression level	Check codon usage with ATUM. Condon optimizing for *S. cerevisiae* may improve expression levels.