

## **Culture and Induction Protocol to Screen for Protein Expression in *Pichia pastoris***

The methods described can be used for testing protein expression with vectors using AOX1 or GAP promoters. The following sections are included:

- I. Competent Cell Production
- II. Linearization of Vector
- III. Transformation
- IV. Culture Conditions and Expression of Recombinant Protein
- V. Evaluation of Expressed Protein
- VI. Troubleshooting

### **I. Competent Cell Production**

#### **Media & Reagents**

Host strains: *Pichia pastoris*<sup>1</sup> wild-type strain BG10 (cat. no. PPS-9010), *aox1*Δ (MutS) strain BG11 (cat. No. PPS-9011) or *pep4*Δ *prb1*Δ (protease deficient) strain BG16 (cat. No. PPS-9016) strains. Note that biotechnological strains of *P. pastoris* have recently been reclassified to *Komagatella phaffii*, but are still commonly referred to as *P. pastoris*.

BEDS solution: 10 mM Bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) DMSO (molecular biology grade), 1 M Sorbitol. Filter sterilized and stored at 2-4°C.

1M Dithiothreitol (DTT).

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

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

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<sup>1</sup> J Ind Microbiol Biotechnol.2009; 36(11):1435-8  
[www.atum.bio](http://www.atum.bio)

## **Equipment**

Sterile baffled flasks, 5X 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.

-80°C Freezer (optional).

## **Procedure for Preparation of Competent Cells (50 ml culture)<sup>2</sup>**

1. Streak your strain for single colonies to a YPD agar plate. Incubate for 2 days 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 (OD<sub>600</sub>) of 0.15-0.2, in 50 ml of YPD broth in a baffled flask of at least 250 ml capacity. Starting volumes may be scaled up or down. Each 50 ml culture will produce about 1 ml of competent cells.
4. Grow the culture to an OD<sub>600</sub> of 0.8 to 1.0 in a 30°C shaking incubator at 250 rpm. This will take 4-5 hours.
5. Centrifuge the culture at 500 x g for 15 minutes at room temperature and carefully pour off the supernatant.
6. Gently suspend the pellet in 9 ml of ice-cold BEDS solution. Add 1 ml of 1M DTT. Mix gently.
7. Incubate the cell suspension for 5 minutes at 100 rpm in the 30°C shaking incubator.
8. Centrifuge the cell suspension as in step 5.
9. Suspend the pellet in 1 ml (or less) of ice cold BEDS solution without DTT.
10. The cells are now ready for transformation, or may be stored at -80°C after slowly freezing by placing the 200 µl aliquots in a Styrofoam box placed in a -80°C freezer overnight.

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<sup>2</sup> Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris* BioTechniques 2005; 38:44-48

Competent cells may be stored at least 6 months at this temperature. Note: This is a good spot to pause work.

## **II. Linearization of the Vector**

### **Media & Reagents**

Appropriate restriction enzyme and buffer. For vectors using the AOX1 promoter, use PmeI or SacI-HF from NEB (PmeI is preferred as it cuts more central in the AOX1 promoter). PmeI or SacI digestion directs the integration of the expression vector to the chromosomal AOX1 promoter. For vectors using the GAP promoter use AvrII from NEB. AvrII digestion directs the integration of the expression vector to the chromosomal GAP promoter.

20 ug of expression vector. You may use any method of your choice to prepare purified plasmid DNA for yeast transformation.

20 ug of appropriate control vector, such as Cat. Nos. pJ902-15 or pJ912-19 from ATUM, Inc. which serve as cytoplasmic and secretion controls, respectively.

Sterile 10 mM Tris-HCl, pH 8.0 or deionized water.

0.8% agarose gel prepared with 1 x TBE buffer and 1 ug/ml ethidium bromide (or equivalent).

### **Equipment**

Incubator set at appropriate temperature for DNA digestion, as per NEB protocol.

Microcentrifuge.

### **Procedure for Preparation of DNA for Transformation**

1. Digest 20 ug of your vector in a 200 µl reaction volume according to NEBs guidelines. Similarly digest an appropriate control vector. Remove 5 µl of reaction mix before adding the restriction enzyme to act as un-digested control. Follow the recommendation by NEB for temperature and duration of digestion.
2. Check 5 µl of undigested control versus 5 µl of digested reaction by agarose gel electrophoresis. If completely linearized, heat-inactivate according to NEBs recommendations.
3. Ethanol precipitate using 1/10 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol. Centrifuge to pellet DNA, wash pellet with 70% ethanol, air dry, and suspend pellet in 20 µl of deionized sterile water or 10 mM Tris-Cl, pH 8.0.

4. Optional. Measure the eluted DNA concentration and/or check quality and yield by agarose gel electrophoresis.

Note: This is a good place to pause work.

### **III. Transformation**

#### **Media & Reagents**

5-20 ug of linearized expression vector in approximately 10  $\mu$ l of water or 10 mM Tris-Cl, pH 8.0.

Competent *P. pastoris*.

Zeocin, e.g., 100 mg/ml in sterile deionized water. Filter sterilize and store in dark at -20°C.

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

Ice-cold, sterile 1M Sorbitol. Filter sterilize and store at 4°C.

YPDS agar plates with Zeocin (YPDS: 1% yeast extract, 2% peptone, 2% glucose, 1M sorbitol, 2% agar) + Zeocin (we recommend using both YPDS Agar + 250 ug/ml Zeocin and YPDS Agar + 1000 ug/ml Zeocin for each transformation).

YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar)

Higher concentrations of Zeocin tend to select for multi-copy integrants which may produce higher expression levels of your protein, but also reduce the number of resulting colonies. Note that Zeocin is light sensitive; plates should be stored away from light at 4°C and used within 2 weeks of preparation.

#### **Equipment**

Electroporation Cuvettes with 0.2 cm gap.

Shaking incubator.

14 ml sterile round bottom culture tubes.

Sterile glass beads or sterile spreader.

Electroporation apparatus: ECM 630 electroporator (BTX, San Diego, CA USA): cuvette gap, 2.0 mm, charging voltage, 1500 V; resistance, 200 $\Omega$ ; capacitance, 50  $\mu$ F. Or (ii) Gene Pulsar II

electroporator (Bio-Rad Laboratories, Hercules, CA, USA); cuvette gap, 2.0 mm, charging voltage, 1500 V; resistance, 200Ω; capacitance, 25 μF.

### **Procedure for Transformation**

1. On ice, mix 5-20 ug of linearized expression vector in approximately 10 μl of water or buffer with 50 μl of competent cells and transfer to a pre-chilled electroporation cuvette. Repeat for expression control vector. Also, include a 50 μl aliquot of cells with no DNA as negative control. Incubate for 2 minutes on ice.
2. Add 0.5 ml of YPD Broth to a 14 ml round bottom sterile culture tube, and set aside in a rack. Include extra tubes for the expression control and a negative control.
3. Electroporate samples at 1500 V.
4. Immediately add 0.5 ml of ice-cold 1M Sorbitol to the electroporation cuvette, and transfer cells to a round bottom culture tube with YPD broth.
5. Incubate transformations for 1-2 hours at 30°C and 200 rpm.
6. Spread 50, 100, and 200 μl to both YPDS + 250 ug/ml Zeocin and YPDS + 1000 ug/ml Zeocin plates.
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. Optional, but good practice: streak 8 or more isolated colonies to YPD agar plates (no Zeocin) for single colonies. Incubate for 2 days at 30C. Pick and isolated colony from each streak and repeat colony purification.
9. Patch isolated colonies to YPDS plates with either 250 ug/ml or 1000 ug/ml Zeocin as appropriate. Incubate 1 days at 30°C. Store plate wrapped in Parafilm at 4°C until when not in use.

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

### **Media & Reagents**

1M Potassium Phosphate buffer, pH 6.0. Filter sterilize or autoclave at 121°C for 15 minutes and store at 4°C. Note that the pH may need to be adjusted to suit the expressed protein. Use a pH at least 0.5 units away from the Isoelectric point of the secreted recombinant protein. *P. pastoris* will grow at pH 3 to pH 8. A more acidic media will inhibit proteases generated by *P. pastoris*.

134 g/l Yeast Nitrogen Base with ammonium sulfate and without amino acids (10x concentration). Filter sterilize or autoclave at 121°C for 15 minutes and store at 4°C.

0.2 g/l Biotin (500x concentration). Filter sterilize and store at 4°C.

40% glycerol. Autoclave at 121°C for 15 minutes and store at room temperature.

Zeocin, e.g., 100 mg/ml in sterile deionized water. Filter sterilize and store in dark at -20°C.

BMGY medium: 1% Yeast extract, 2 % Peptone, 13.4 g/l Yeast Nitrogen Base (without amino acids), 100 mM Potassium Phosphate pH6.0, 0.004 mg/l Biotin, 1% Glycerol

BMMY medium: 1% Yeast extract, 2 % Peptone, 13.4 g/l Yeast Nitrogen Base (without amino acids), 100 mM Potassium Phosphate pH6.0, 0.004 mg/l Biotin, 1% Methanol.

### **Equipment**

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

28°C incubator with plate holder

Breathable membrane tape to seal plates

### **Procedure for Expression**

1. For the **AOX1 Promoter**, add 2.5 ml of BMGY + 250 ug/ml Zeocin per well of the 24-well plate for the expression strains and positive control strain. Do not add Zeocin for the negative control (host) strain. For the constitutive **GAP Promoter**, add 5 ml of BMGY + 250 ug/ml Zeocin per well of the 24-well plate for the expression strains and positive control strain. Do not add Zeocin for the negative control (host) strain.

2. Using a sterile pipette tip, pick a small amount of colony from each patch into a well with growth medium, preferably from the highest Zeocin concentration patch plate. Place the tip into the well and mix. Temporarily, leave the tips in the plate. Pick up to 8 (or more) colonies from each transformation. For the negative control well, inoculate the *P. pastoris* host. Note that it can be advantageous to screen an even larger number of *P. pastoris* transformants, particularly those obtained at higher levels of Zeocin, to identify jackpot clones that bear multiple copies of the expression vector and express at higher levels.
3. Seal the 24-well culture plate with a breathable membrane tape. Incubate for 48-96 hours at 28°C, shaking vigorously. For **AOX1 promoter**, proceed to step 4. For the **GAP promoter**, proceed to step 7.
4. For the **AOX1 promoter**, add 2.5 ml of BMMY to initiate induction. Continue incubation at 28°C for 12 hours.
5. Add 25 ul of methanol/well to continue (boost) induction.
6. Repeat boosting by adding 25 ul of methanol twice per day for up to 96 hours. Samples (100 µl) may be taken before induction and subsequently every 24 hours (freeze samples at -20°C until ready to evaluate).
7. For the **GAP promoter**, Inoculate as per step 2. Seal the 24-well culture plate with a breathable membrane tape. Incubate for 48-96 hours at 28°C, shaking vigorously. Sample (100 µl) every 24 hours up to 96 hours (freeze samples at -20°C until ready to evaluate).

## **V. Evaluation of Expressed Protein**

### **Media & Reagents**

SDS-PAGE gel, buffers, 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<sub>2</sub> (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 microcentrifuge at 4°C.
2. For secreted proteins carefully transfer the supernatant to a new microcentrifuge 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.

## **VI. Troubleshooting**

<b>Problem</b>	<b>Probable Cause</b>	<b>Possible Solution</b>
Low efficiency of transformation	Vector not completely linearized	Check that vectors are completely linearized by comparing to uncut vector on agarose gel.
	Vector concentration too low	Check DNA concentration of linearized vector by measuring A260 or by comparing to standards on an agarose gel.
	The pH of the BEDS solution may have drifted	Check the pH of the BEDS solution. Remake or adjust if higher or lower than pH 8.3
	Incubation time is too short or	Transformations may be



	temperature too low	incubated for up to 3 hours and at higher temperatures (35-37°C) which in some cases may result in higher transformation efficiencies.
	Cell density too low	Resuspend cells in a smaller volume (i.e. 500 µl of BEDS solution)
Expression of protein low or not detectable	Detection method suboptimal.	Concentrate 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 <i>P. pastoris</i> may improve expression levels. Screen a larger number of transformants for jackpot clones.
Random Integration Note: We typically see >80% integration at the AOX1 site	Incomplete linearization	Make sure plasmid is completely linearized, recommend gel purification. Try linearizing with PmeI instead of SacI. Recommend streaking 8-16 transformants for single colony isolation at least 2 times to YPD agar minus selection