



# EasySelect<sup>TM</sup> *Pichia* Expression Kit

For Expression of Recombinant Proteins Using  
pPICZ and pPICZ $\alpha$  in *Pichia pastoris*

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User Manual



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# Important Information

## Kit Contents

The EasySelect™ *Pichia* Expression Kit contains the following components.

**The *Pichia* EasyComp™ Kit.** This kit contains sufficient reagents for 6 preparations of competent cells. Each competent cell preparation yields enough cells for 20 transformations.

Upon receipt, store Solutions I and III at 4°C. You may store Solution II at 4°C or at room temperature.

Component	Description	Quantity
Solution I	Sorbitol solution containing ethylene glycol and DMSO for the preparation of competent cells	75 ml
Solution II	PEG solution for the transformation of competent cells	150 ml (2 × 75 ml)
Solution III	Salt solution for washing and plating transformed cells	150 ml (2 × 75 ml)

**Stab Vials: *Pichia* and *E. coli* stabs. Store at 4°C.**

Strain	Genotype	Phenotype ( <i>Pichia</i> only)
X-33	wild-type	Mut <sup>+</sup>
GS115	<i>his4</i>	His <sup>-</sup> , Mut <sup>+</sup>
KM71H	<i>arg4 aox1::ARG4</i>	Mut <sup>S</sup> , Arg <sup>+</sup>
GS115/Albumin	<i>HIS4</i>	Mut <sup>S</sup>
GS115/pPICZ/ <i>lacZ</i>	<i>his4</i>	His <sup>-</sup> , Mut <sup>+</sup>
TOP10F' <i>E. coli</i>	F' { <i>proAB</i> , <i>lacI</i> <sup>q</sup> , <i>lacZ</i> ΔM15, Tn10 (Tet <sup>R</sup> )} <i>mcrA</i> , Δ( <i>mrr-hsdRMS-mcrBC</i> ), φ80 <i>lacZ</i> ΔM15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , λ <sup>-</sup> <i>araD139</i> , Δ( <i>ara-leu</i> )7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Str <sup>R</sup> ), <i>endA1</i> , <i>nupG</i>	

**Box 3: Vectors and Zeocin™. Store at -20°C**

Reagent	Amount
pPICZ A, B, and C	20 μg of each vector in TE buffer, pH 8.0* (40 μl at 500 ng/μl)
pPICZα A, B, and C	20 μg of each vector in TE buffer, pH 8.0 (40 μl at 500 ng/μl)
Zeocin™	2 × 1.25 ml, 100 mg/ml

\*TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

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## Important Information, continued

### Kit Contents, continued

Box 4: Primers. Store at -20°C.

Reagent	Sequence	Amount
5' <i>AOX1</i> sequencing primer (5' <i>Pichia</i> primer)	5'-GACTGGTTCCAATTGACAAGC-3'	2 µg, lyophilized 312 pmoles
3' <i>AOX1</i> sequencing primer (3' <i>Pichia</i> primer)	5'-GCAAATGGCATTCTGACATCC-3'	2 µg, lyophilized 314 pmoles
α-Factor sequencing primer	5'-TACTATTGCCAGCATTGCTGC-3'	2 µg, lyophilized 315 pmoles

### Media

The following prepackaged media is included for your convenience. Instructions for use are provided on the package.

Keep the media dry and store at room temperature.

Media	Amount	Yield
YP Base Medium	2 pouches	2 liters of YP medium
YP Base Agar Medium	2 pouches	2 liters of YP agar medium
Yeast Nitrogen Base	1 pouch	500 ml of 10X YNB



### Note

The *Pichia* and TOP10F' *E. coli* stabs supplied with the kit are guaranteed until the expiration date marked on tube when stored at 4°C. We recommend you prepare a set of glycerol master stocks prior to using your *Pichia* (page 9) and TOP10F' *E. coli* cells (page 10).

## Important Information, continued

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### Materials Supplied by the User

For the procedures described in this manual, you will need the following reagents and equipment. Additional reagents may be required. Please check each experiment to ensure you have all the reagents necessary.

- 30°C and 37°C rotary shaking incubator
  - Water baths capable of 16°C, 37°C, and 65°C
  - Centrifuge suitable for 50 ml conical tubes (floor or table-top)
  - Baffled culture flasks with metal covers (50 ml, 250 ml, 500 ml, 1000 ml, and 3 liters)
  - 50 ml sterile, conical tubes
  - 6 ml and 15 ml sterile snap-top tubes (Falcon 2059 or similar)
  - UV Spectrophotometer
  - Restriction enzymes and appropriate buffers
  - Agarose and low-melt agarose
  - Mini agarose gel apparatus and buffers
  - Glass milk
  - Sterile water
  - CIAP (calf intestinal alkaline phosphatase, 1 unit/ $\mu$ l)
  - 10X CIAP Buffer
  - Phenol/chloroform
  - 3 M sodium acetate
  - 100% ethanol
  - 80% ethanol
  - T4 Ligase (2.5 units/ $\mu$ l)
  - 10X Ligation Buffer (with ATP)
  - Low Salt LB medium (see page 53 for recipe)
  - Zeocin™ antibiotic (see page viii for ordering information)
  - Low Salt LB plates containing 25  $\mu$ g/ml Zeocin™
  - YPDS plates containing 100  $\mu$ g/ml Zeocin™ plates
  - Polyacrylamide Gel Electrophoresis apparatus and buffers
  - Media for transformation, growth, screening, and expression (see pages 54–59)
  - Sterile cheesecloth or gauze
  - Breaking Buffer (see **Recipes**, page 59)
  - Acid-washed glass beads (available from Sigma)
  - Electroporator and 0.2 cm cuvettes or reagents for transformation (optional)
  - 1 M sorbitol
  - Replica-plating equipment (optional)
  - Bead Beater™ (optional, Biospec)
  - ProBond™ Purification System (optional)
-

## Accessory Products

### Introduction

The products listed in this section are intended for use with the EasySelect™ *Pichia* Expression Kit. For more information, refer to our website ([www.invitrogen.com](http://www.invitrogen.com)) or call Technical Support (see page 78).

### Accessory Products

Many of the reagents supplied in the EasySelect™ *Pichia* Expression Kit are available separately from Invitrogen. Ordering information is provided below.

Product	Reactions or Amount	Cat. no.
<i>Pichia</i> EasyComp™ Kit	20 transformations	K1730-01
Zeocin™	1 g	R250-01
	5 g	R250-05
pPICZ A, B, and C	20 µg of each vector in TE buffer, pH 8.0 (40 µl at 500 ng/µl)	V190-20
pPICZα A, B, and C	20 µg of each vector in TE buffer, pH 8.0 (40 µl at 500 ng/µl)	V195-20

### Antibodies for Detection of Fusion Protein

A number of antibodies are available from Invitrogen to detect expression of your fusion protein from the pPICZ and pPICZα vectors. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The amount of antibody supplied is sufficient for 25 Westerns.

Antibody	Epitope	Cat. no.
Anti- <i>myc</i>	Detects the 10 amino acid epitope derived from <i>c-myc</i> (Evan <i>et al.</i> , 1985): EQKLISEEDL	R950-25
Anti- <i>myc</i> -HRP		R951-25
Anti-His(C-term)	Detects the C-terminal polyhistidine (6×His) tag (requires the free carboxyl group for detection) (Lindner <i>et al.</i> , 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25

### Purification of Fusion Protein

The polyhistidine (6×His) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™. Ordering information for ProBond™ resin is provided below.

Item	Quantity	Cat. no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti- <i>myc</i> -HRP Antibody	1 kit	K852-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns (10 ml polypropylene columns)	50 columns	R640-50



# Introduction

## Overview

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### Review Articles

The information presented here is designed to give you a concise overview of the *Pichia pastoris* expression system. It is by no means exhaustive. For further information, please read the articles cited in the text along with recent review articles (Buckholz and Gleeson, 1991; Cregg and Higgins, 1995; Cregg *et al.*, 1993; Nico-Farber *et al.*, 1995; Sreekrishna *et al.*, 1988; Wegner, 1990). A general review of foreign gene expression in yeast is also available (Romanos *et al.*, 1992).

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### General Characteristics of *Pichia pastoris*

As a eukaryote, *Pichia pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system.

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### Similarity to *Saccharomyces*

Many of the techniques developed for *Saccharomyces* may be applied to *Pichia*. These include:

- transformation by complementation
- gene disruption
- gene replacement

In addition, the genetic nomenclature used for *Saccharomyces* has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the *HIS4* gene in both *Saccharomyces* and *Pichia*. There is also cross-complementation between gene products in both *Saccharomyces* and *Pichia*. Several wild-type genes from *Saccharomyces* complement comparable mutant genes in *Pichia*. Genes such as *HIS4*, *LEU2*, *ARG4*, *TRP1*, and *URA3* all complement their respective mutant genes in *Pichia*.

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### *Pichia pastoris* as a Methylotrophic Yeast

*Pichia pastoris* is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O<sub>2</sub>, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia*.

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## Overview, continued

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### Two Alcohol Oxidase Proteins

Two genes in *Pichia pastoris* code for alcohol oxidase – *AOX1* and *AOX2*. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* gene. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically  $\geq 30\%$  of the total soluble protein in cells grown with methanol. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a). While *AOX2* is about 97% homologous to *AOX1*, growth on methanol is much slower than with *AOX1*. This slow growth on methanol allows isolation of Mut<sup>S</sup> strains (*aox1*) (Cregg *et al.*, 1989; Koutz *et al.*, 1989).

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### Expression

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-grown cells approximately 5% of the polyA<sup>+</sup> RNA is from the *AOX1* gene. The regulation of the *AOX1* gene is a two step process: a repression/derepression mechanism plus an induction mechanism (e. g. *GAL1* gene in *Saccharomyces* (Johnston, 1987)). Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth on glycerol is recommended for optimal induction with methanol. Note that growth on glycerol alone (derepression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for even detectable levels of *AOX1* expression (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a).

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### Phenotype of *aox1* Mutants

Loss of the *AOX1* gene, and thus a loss of most of the cell's alcohol oxidase activity, results in a strain that is phenotypically Mut<sup>S</sup> (Methanol utilization slow). This has in the past been referred to as Mut<sup>-</sup>. The Mut<sup>S</sup> designation has been chosen to accurately describe the phenotype of these mutants. This results in a reduction in the cells' ability to metabolize methanol. The cells, therefore, exhibit poor growth on methanol medium. Mut<sup>+</sup> (Methanol utilization plus) refers to the wild type ability of strains to metabolize methanol as the sole carbon source. These two phenotypes are used when evaluating *Pichia* transformants for integration of your gene (**Experimental Outline**, page 4).

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## Overview, continued

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### Intracellular and Secretory Protein Expression

Heterologous expression in *Pichia pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the *Saccharomyces cerevisiae* factor prepro peptide has been used with the most success (Cregg *et al.*, 1993; Scorer *et al.*, 1993).

The major advantage of expressing heterologous proteins as secreted proteins is that *Pichia pastoris* secretes very low levels of native proteins. Since there is very low amount of protein in the minimal *Pichia* growth medium, the secreted heterologous protein comprises the vast majority of the total protein in the medium and serves as the first step in purification of the protein (Barr *et al.*, 1992). Note, however, that if there are recognized glycosylation sites (Asn-X-Ser/Thr) in your protein's primary sequence, glycosylation may occur at these sites.

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### Posttranslational Modifications

In comparison to *Saccharomyces cerevisiae*, *Pichia* may have an advantage in the glycosylation of secreted proteins because it may not hyperglycosylate. Both *Saccharomyces cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added posttranslationally to proteins in *Pichia* (average 8–14 mannose residues per side chain) is much shorter than those in *Saccharomyces cerevisiae* (50–150 mannose residues) (Grinna and Tschopp, 1989; Tschopp *et al.*, 1987b). Very little O-linked glycosylation has been observed in *Pichia*.

In addition, *Saccharomyces cerevisiae* core oligosaccharides have terminal  $\alpha$ 1,3 glycan linkages whereas *Pichia pastoris* does not. It is believed that the  $\alpha$ 1,3 glycan linkages in glycosylated proteins produced from *Saccharomyces cerevisiae* are primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for therapeutic use. Although not yet proven, this is predicted to be less of a problem for glycoproteins generated in *Pichia pastoris*, because it may resemble the glycoprotein structure of higher eukaryotes (Cregg *et al.*, 1993).

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## Experimental Outline

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### Selection of Vector and Cloning

To utilize the strong, highly-inducible  $P_{AOX1}$  promoter for expression of your protein, there are two expression vectors included in this kit. One vector, pPICZ, is for intracellular expression while the other vector, pPICZ $\alpha$ , is for secreted expression. Each vector is provided in three reading frames to facilitate cloning in frame with the C-terminal polyhistidine tag. All vectors contain the Zeocin<sup>™</sup> resistance gene for positive selection in *E. coli* and *Pichia*. See pages 11–14 for more information on these vectors.

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### Transformation and Integration

Two different phenotypic classes of recombinant strains can be generated Mut<sup>+</sup> and Mut<sup>S</sup>. Mut<sup>S</sup> refers to the "Methanol utilization slow" phenotype caused by the loss of alcohol oxidase activity encoded by the *AOX1* gene. A strain with a Mut<sup>S</sup> phenotype has a mutant *aox1* locus, but is wild type for *AOX2*. This results in a slow growth phenotype on methanol medium. Both X-33 and GS115 are Mut<sup>+</sup>, and KM71H is Mut<sup>S</sup>. Transformation of X-33 or GS115 with plasmid DNA linearized in the 5' *AOX1* region will yield Mut<sup>+</sup> transformants, while KM71H will yield only Mut<sup>S</sup> transformants. Both Mut<sup>+</sup> and Mut<sup>S</sup> recombinants are useful to have as one phenotype may favor better expression of your protein than the other. You should test between 6–10 recombinants per phenotype because the site of recombination may affect expression. There is no way to predict beforehand which construct or isolate will better express your protein. For more information on recombination in *Pichia*, see page 62.

Once you have successfully cloned your gene behind the *AOX1* promoter, you will then linearize your plasmid to stimulate recombination when the plasmid is transformed into *Pichia*.

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## Experimental Outline, continued

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### Expression and Scale-up

After isolating your *Pichia* recombinants, you will then test expression of both Mut<sup>+</sup> and Mut<sup>S</sup> recombinants. This will involve growing a small culture of each recombinant, inducing with methanol, and taking time points. If looking for intracellular expression, analyze the cell pellet from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). If looking for secreted expression, analyze both the cell pellet and supernatant from each time point. We recommend that you analyze your SDS-PAGE gels by both Coomassie staining and Western blot. We also suggest checking for protein activity by assay if one is available. Not all proteins express to the level of grams per liter, so it is advisable to check by Western blot or activity assay, and not just by Coomassie staining of SDS-PAGE gels for production of your protein.

Choose the *Pichia* recombinant strain which best expresses your protein and optimize induction based on the suggestions on pages 45–46. Once expression is optimized, scale-up your expression protocol to produce more protein for purification.

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### Purification

Both pPICZ and pPICZ $\alpha$  contain a polyhistidine tag that binds divalent cations like Ni<sup>2+</sup> to facilitate purification. Metal-binding resins such as ProBond™ can be used to purify proteins expressed from pPICZ or pPICZ $\alpha$ . We recommend that you use the ProBond™ Purification System (Cat. no. K850-01) to purify fusion proteins expressed using pPICZ or pPICZ $\alpha$ . **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification System Kit manual.** Preliminary preparation steps are described on pages 50–51.

If you are using a metal-chelating resin other than ProBond™, please follow the manufacturer's recommendations for fusion proteins expressed in yeast.

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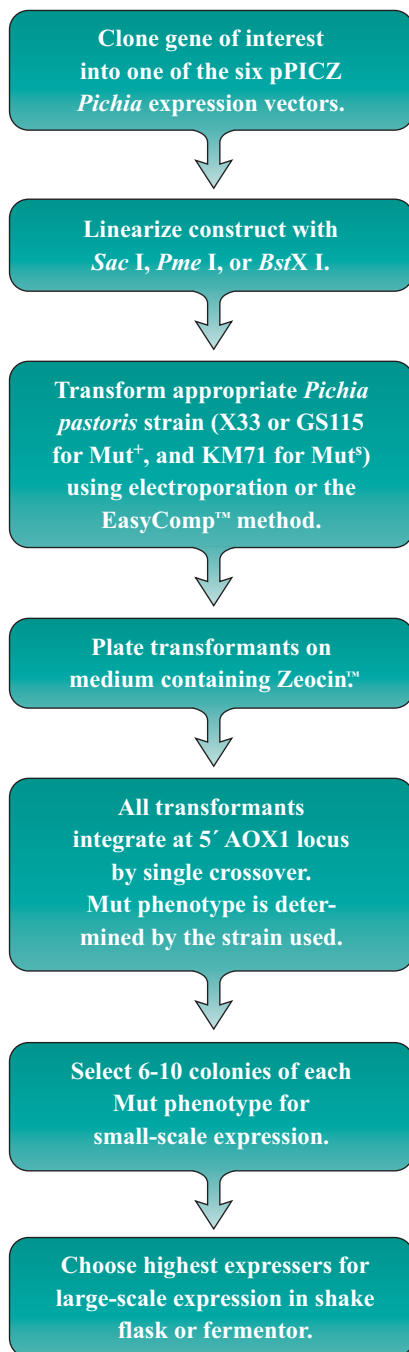
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## Experimental Outline, continued

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### Experimental Process

The overall experimental process is presented below. In addition, there is a discussion about recombination and integration in *Pichia* which will help you choose the right vector (see page 62). More information is provided in a review by Higgins (Higgins, 1995).



## Methods

### *Pichia* Strains

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#### Introduction

*Pichia pastoris* is quite similar to *Saccharomyces cerevisiae* as far as general growth conditions and handling. You should be familiar with basic microbiological and sterile techniques before attempting to grow and manipulate any microorganism. You should also be familiar with basic molecular biology and protein chemistry. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology*, (Guthrie and Fink, 1991), *Current Protocols in Molecular Biology*, (Ausubel *et al.*, 1994), *Molecular Cloning: A Laboratory Manual*, (Sambrook *et al.*, 1989), *Protein Methods*, (Bollag and Edelman, 1991), and *Guide to Protein Purification*, (Deutscher, 1990).

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#### Genotypes of *Pichia* Strains

X-33 is a wild-type *Pichia* strain that is useful for selection on Zeocin™ and large-scale growth. It will grow in YPD and in minimal media.

The *Pichia* host strain GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. GS115 will grow on complex medium such as YPD (also known as YEPD) and on minimal media supplemented with histidine.

The parent strain of KM71H has a mutation in the argininosuccinate lyase gene (*arg4*) that prevents the strain from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt *AOX1*, creating KM71H, a Mut<sup>S</sup>, Arg<sup>+</sup> strain.

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#### Construction of KM71H

The *ARG4* gene (~2 kb) was inserted into the cloned, wild-type *AOX1* gene between the *Bam*H I site (codons 15/16 of *AOX1*) and the *Sal* I site (codons 227/228 of *AOX1*). *ARG4* replaces codons 16 through 227 of *AOX1*. This construct was transformed into the parent strain of KM71 (*arg4 his4*) and Arg<sup>+</sup> transformants were isolated and analyzed for the Mut<sup>S</sup> phenotype. Genetic analysis of Arg<sup>+</sup> transformants showed that the wild-type *AOX1* gene was replaced by the *aox1::ARG4* construct. To create KM71H, KM71 was transformed with a gene fragment encoding the *HIS4* gene and a His<sup>+</sup> convertant was isolated.

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#### Important

The advantage of using KM71H is that there is no need to screen for the Mut phenotype on methanol minimal medium. All transformants will be Mut<sup>S</sup>. Secondly, since the *AOX1* locus was completely deleted, it is theoretically possible to replace *aox1::ARG4* with your construct by gene replacement. The phenotype of this strain would be Mut<sup>S</sup> Arg<sup>-</sup>. This means the recombinant strain would require arginine in the medium to grow. Unfortunately, simple inclusion of arginine does not totally alleviate the effects of the *arg4* mutation, and *arg4* strains do not grow well on minimal medium supplemented with arginine. Therefore, we do not recommend that you generate transformants in KM71H by replacing the *aox1::ARG4* construct.

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## Pichia Strains, continued

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### Control Expression Strains

**GS115/His<sup>+</sup> Mut<sup>S</sup> Albumin:** This strain is a control for secreted expression (page 41) and the Mut<sup>S</sup> phenotype when characterizing *Pichia* transformants (page 34). The gene for serum albumin was cloned with its native secretion signal, then integrated into *Pichia* at the *AOX1* locus. This strain secretes albumin (67 kDa) into the medium at levels > 1 gram/liter.

**GS115/pPICZ/*lacZ* Mut<sup>+</sup> β-galactosidase:** The strain GS115/pPICZ/*lacZ* expresses β-galactosidase fused at the C-terminus to the *myc* epitope and the polyhistidine tag. Expression of the 119 kDa fusion protein is driven by the *P<sub>AOX1</sub>* promoter and is inducible by methanol. The fusion protein is visible on a Coomassie-stained SDS-polyacrylamide gel and can be detected antigenically using the Anti-*myc* Antibody (see page viii) or enzymatically using an ONPG assay (β-Gal Assay Kit, Cat. no. K1455-01). GS115/pPICZ/*lacZ* is provided as a positive control for Zeocin™ resistance in *Pichia*, Mut<sup>+</sup> expression (page 33 and 41) and purification.

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### Growth of *Pichia* Strains

The growth temperature of *Pichia pastoris* is 28–30°C for liquid cultures, plates, and slants. Growth above 32°C during induction can be detrimental to protein expression and can even lead to cell death. Other important facts:

- Doubling time of log phase Mut<sup>+</sup> or Mut<sup>S</sup> *Pichia* in YPD is ~2 hours
- Mut<sup>+</sup> and Mut<sup>S</sup> strains do not differ in growth rates unless grown on methanol
- Doubling time of log phase Mut<sup>+</sup> *Pichia* in methanol medium (MM) is 4–6 hours
- Doubling time of log phase Mut<sup>S</sup> *Pichia* in MM is ~18 hours
- One OD<sub>600</sub> = ~5 × 10<sup>7</sup> cells/ml

**Note that growth characteristics may vary depending on the recombinant protein expressed.**

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### Growth on Methanol

When plates or medium containing methanol are used as growth medium, it is advisable to add methanol every day to compensate for loss because of evaporation or consumption.

- For plates add 100 µl of 100% methanol to the lid of the inverted plate.
- For liquid medium add 100% methanol to a final concentration of 0.5%.

Some researchers have had success adding methanol to 1% every day for Mut<sup>S</sup> strains and up to 3% for Mut<sup>+</sup> without any negative effect to their liquid culture.

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## ***Pichia* Strains, continued**

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### **Storing *Pichia* Strains**

To store cells for weeks to months, use YPD medium and YPD agar slants (see page 55).

- Streak each strain for single colonies on YPD.
- Transfer one colony to a YPD stab and grow for 2 days at 30°C.
- The cells can be stored on YPD for several weeks at 4°C.

To store cells for months to years, store frozen at –80°C.

- Culture a single colony of each strain overnight in YPD.
- Harvest the cells and suspend in YPD containing 15% glycerol at a final OD<sub>600</sub> of 50–100 (approximately  $2.5 \times 10^9$ – $5.0 \times 10^9$  cells/ml).
- Cells are frozen in liquid nitrogen or a dry ice/ethanol bath and then stored at –80°C.



### **Note**

After extended storage at 4°C or –80°C, it is recommended that Zeo<sup>R</sup> transformants be checked for correct phenotype and protein expression.

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## ***E. coli* Strains**

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### **Genotype of *E. coli* Strain**

The *E. coli* strain, TOP10F' is provided in case no suitable *E. coli* strain is available. Other strains which may be suitable are TOP10, DH5 $\alpha$ F', JM109, or any other strain which is recombination deficient (*recA*) and deficient in endonuclease A (*endA*).

F' {*proAB*, *lacI*<sup>q</sup>, *lacZ* $\Delta$ M15, Tn10 (Tet<sup>R</sup>)} *mcrA*,  $\Delta$ (*mrr-hsdRMS-mcrBC*),  $\phi$ 80*lacZ* $\Delta$ M15,  $\Delta$ *lacX74*, *deoR*, *recA1*,  $\lambda$ -*araD139*,  $\Delta$ (*ara-leu*)7697, *galU*, *galK*, *rpsL*(Str<sup>R</sup>), *endA1*, *nupG*  $\lambda$ <sup>-</sup>

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### **Important**

Any *E. coli* strain that contains the complete Tn5 transposable element (*i.e.*, DH5 $\alpha$ F'IQ, SURE, SURE2) encodes the *ble* (bleomycin) resistance gene. These strains will confer resistance to Zeocin<sup>TM</sup>. For the most efficient selection it is highly recommended that you choose an *E. coli* strain that does not contain the Tn5 gene (*i.e.*, TOP10, DH5, DH10, etc.).

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We recommend that you make a frozen stock of TOP10F' to keep on hand.

- Culture TOP10F' in 5 ml LB with 10  $\mu$ g/ml tetracycline. Grow overnight.
  - Mix thoroughly 0.85 ml of culture with 0.15 ml sterile glycerol.
  - Transfer to a freezer vial and freeze in liquid nitrogen or a dry ice/ethanol bath.
  - Store at  $-80^{\circ}\text{C}$ .
-

## Selecting a Pichia Expression Vector

**Selecting a Vector** If your protein is cytosolic and non-glycosylated, you may elect to express the protein intracellularly using one of the pPICZ vectors. If your protein is normally secreted, glycosylated, or directed to an intracellular organelle, you may wish to try secreting your protein using one of the pPICZ $\alpha$  vectors. We recommend that you try both the native secretion signal and the  $\alpha$ -factor signal sequence in order to secrete your protein.



### Note

There is no yeast origin of replication in any of the *Pichia* expression vectors included in this kit. Zeo<sup>R</sup> transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome.

**Features of pPICZ A, B, and C** pPICZ A (3329 bp), pPICZ B (3328 bp), and pPICZ C (3329 bp) contain the following elements. All features have been functionally tested.

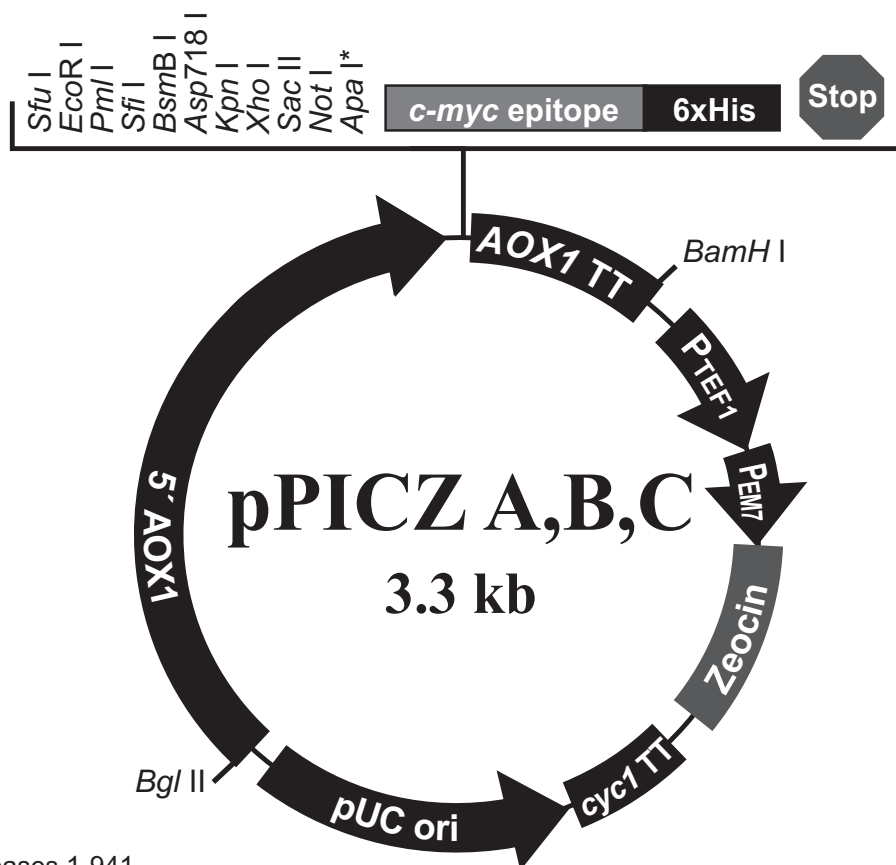
Feature	Benefit
5' AOX1	A 942 bp fragment containing the AOX1 promoter that allows methanol-inducible, high-level expression in <i>Pichia</i> Targets plasmid integration to the AOX1 locus
Multiple cloning site with 10 unique restriction sites	Allows insertion of your gene into the expression vector
C-terminal <i>myc</i> epitope tag	(Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) Permits detection of the fusion protein by the Anti- <i>myc</i> Antibody or Anti- <i>myc</i> -HRP Antibody (see page viii for ordering information) (Evan <i>et al.</i> , 1985)
C-terminal polyhistidine tag	Permits purification of your recombinant fusion protein on metal-chelating resin such as ProBond <sup>™</sup> In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody and the Anti-His (C-term)-HRP Antibody (see page viii) (Lindner <i>et al.</i> , 1997)
AOX1 Transcription Termination (TT)	Native transcription termination and polyadenylation signal from AOX1 gene (~260 bp) that permits efficient 3' mRNA processing, including polyadenylation, for increased mRNA stability
TEF1 promoter	Transcription elongation factor 1 gene promoter from <i>Saccharomyces cerevisiae</i> that drives expression of the <i>Sh ble</i> gene in <i>Pichia</i> , conferring Zeocin <sup>™</sup> resistance (GenBank Acc. no. D12478, D01130).
EM7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the <i>Sh ble</i> gene in <i>E. coli</i> , conferring Zeocin <sup>™</sup> resistance
<i>Sh ble</i> gene ( <i>Streptoalloteichus hindustanus ble</i> gene)	Zeocin <sup>™</sup> resistance gene for selection in <i>E. coli</i>
CYC1 transcription termination region	3' end of the <i>Saccharomyces cerevisiae</i> CYC1 gene that allows efficient 3' mRNA processing of the <i>Sh ble</i> gene for increased stability (GenBank Acc. no. M34014)
pUC origin	Allows replication and maintenance of the plasmid in <i>E. coli</i>
<i>Sac</i> I, <i>Pme</i> I, <i>Bst</i> X I	Unique restriction sites that permit linearization of the vectors at the AOX1 locus for efficient integration into the <i>Pichia</i> genome

Continued on next page

## Selecting a *Pichia* Expression Vector, continued

### Map of pPICZ A, B, and C

The figure below summarizes the features of the pPICZ A, B, and C vectors. The vector sequences for pPICZ A, B, and C are available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78). Details of the multiple cloning sites are shown on page 18 for pPICZ A, page 19 for pPICZ B, and page 20 for pPICZ C.



#### Comments for pPICZ A: 3329 nucleotides

5' AOX1 promoter region: bases 1-941  
 5' end of AOX1 mRNA: base 824  
 5' AOX1 priming site: bases 855-875  
 Multiple cloning site: bases 932-1011  
*c-myc* epitope tag: bases 1012-1044  
 Polyhistidine tag: bases 1057-1077  
 3' AOX priming site: bases 1159-1179  
 3' end of mRNA: base 1250  
 AOX1 transcription termination region: bases 1078-1418  
 Fragment containing TEF1 promoter: bases 1419-1830  
 EM7 promoter: bases 1831-1898  
*Sh ble* ORF: bases 1899-2273  
 CYC1 transcription termination region: bases 2274-2591  
 pUC origin: bases 2602-3275 (complementary strand)

\* The restriction site between *Not* I and the *myc* epitope is different in each version of pPICZ:

*Apa* I in pPICZ A  
*Xba* I in pPICZ B  
*Sna*B I in pPICZ C

*Continued on next page*

## Selecting a *Pichia* Expression Vector, continued

### Features of pPICZ $\alpha$ A, B, and C

pPICZ $\alpha$  A (3593 bp), pPICZ $\alpha$  B (3597 bp), and pPICZ $\alpha$  C (3598 bp) contain the following elements. All features have been functionally tested.

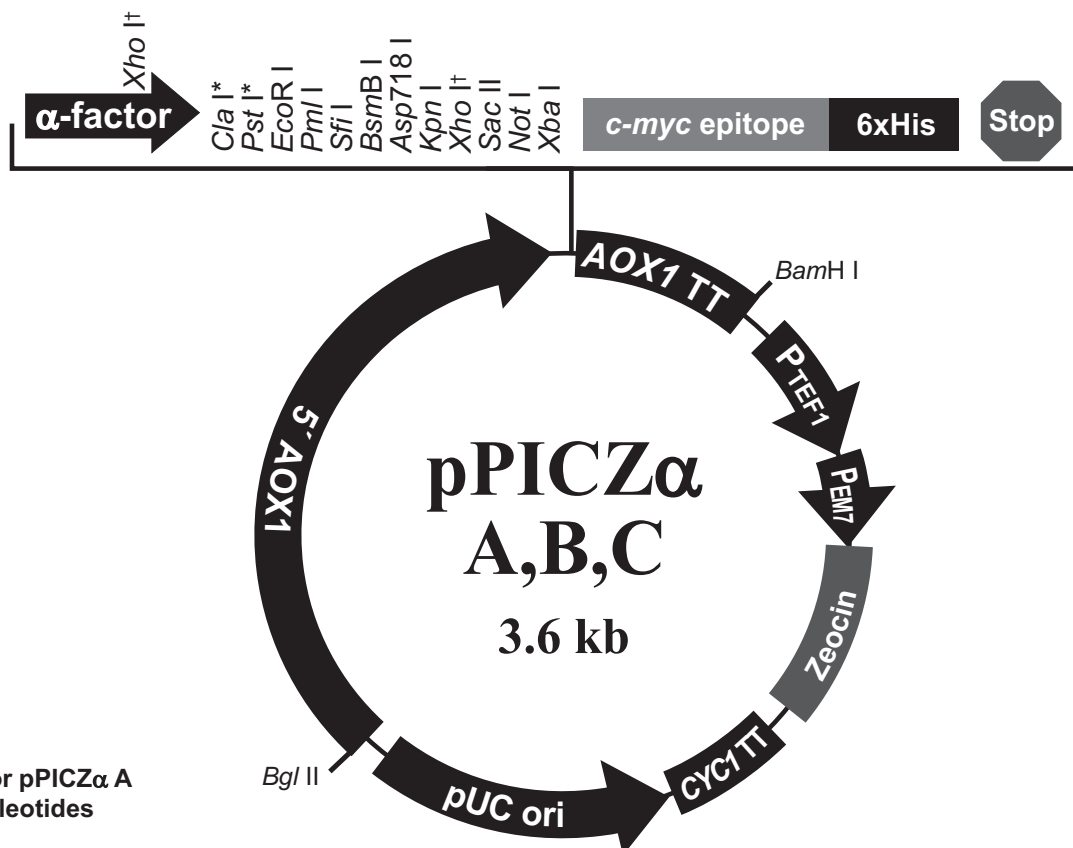
Feature	Benefit
5' AOX1	A 942 bp fragment containing the AOX1 promoter that allows methanol-inducible, high-level expression in <i>Pichia</i> Targets plasmid integration to the AOX1 locus.
Native <i>Saccharomyces cerevisiae</i> $\alpha$ -factor secretion signal	Allows for efficient secretion of most proteins from <i>Pichia</i>
Multiple cloning site with 10 unique restriction sites	Allows insertion of your gene into the expression vector
C-terminal <i>myc</i> epitope tag	(Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) Permits detection of the fusion protein by the Anti- <i>myc</i> Antibody or Anti- <i>myc</i> -HRP Antibody (see page viii for ordering information) (Evan <i>et al.</i> , 1985)
C-terminal polyhistidine tag	Permits purification of your recombinant fusion protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody and the Anti-His(C-term)-HRP Antibody (see page viii for ordering information) (Lindner <i>et al.</i> , 1997)
AOX1 Transcription Termination (TT)	Native transcription termination and polyadenylation signal from AOX1 gene (~260 bp) that permits efficient 3' mRNA processing, including polyadenylation, for increased mRNA stability
TEF1 promoter	Transcription elongation factor 1 gene promoter from <i>Saccharomyces cerevisiae</i> that drives expression of the <i>Sh ble</i> gene in <i>Pichia</i> , conferring Zeocin™ resistance (GenBank Acc. no. D12478, D01130)
EM7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the <i>Sh ble</i> gene in <i>E. coli</i> , conferring Zeocin™ resistance
<i>Sh ble</i> gene ( <i>Streptoalloteichus hindustanus ble</i> gene)	Zeocin™ resistance gene
CYC1 transcription termination region	3' end of the <i>Saccharomyces cerevisiae</i> CYC1 gene that allows efficient 3' mRNA processing of the <i>Sh ble</i> gene for increased stability (GenBank Acc. no. M34014)
pUC origin	Allows replication and maintenance of the plasmid in <i>E. coli</i>
<i>Sac</i> I, <i>Pme</i> I, <i>Bst</i> X I	Unique restriction sites that permit linearization of the vectors at the AOX1 locus for efficient integration into the <i>Pichia</i> genome

Continued on next page

## Selecting a *Pichia* Expression Vector, continued

### Map of pPICZ $\alpha$ A, B, and C

The figure below summarizes the features of the pPICZ $\alpha$  A, B, and C vectors. The vector sequences for pPICZ $\alpha$  A, B, and C are available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78). Details of the multiple cloning sites are shown on page 21 for pPICZ $\alpha$  A, page 22 for pPICZ $\alpha$  B, and page 23 for pPICZ $\alpha$  C.



#### Comments for pPICZ $\alpha$ A 3593 nucleotides

5' AOX1 promoter region: bases 1-941  
 5' AOX1 priming site: bases 855-875  
 $\alpha$ -factor signal sequence: bases 941-1207  
 $\alpha$ -factor priming site: bases 1144-1164  
 Multiple cloning site: bases 1208-1276  
 c-myc epitope: bases 1275-1304  
 Polyhistidine (6xHis) tag: bases 1320-1337  
 3' AOX1 priming site: bases 1423-1443  
 AOX1 transcription termination region: bases 1341-1682  
 TEF1 promoter: bases 1683-2093  
 EM7 promoter: bases 2095-2162  
*Sh ble* ORF: bases 2163-2537  
 CYC1 transcription termination region: bases 2538-2855  
 pUC origin: bases 2866-3539 (complementary strand)

\* *Pst* I is in Version B only  
*Cla* I is in Version C only

†The two *Xho* I sites in the vector allow the user to clone their gene in frame with the Kex2 cleavage site, resulting in expression of their native gene without additional amino acids at the N-terminus.

# General Cloning Information

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## Introduction

Before cloning your gene into one of the pPICZ or pPICZ $\alpha$  vectors, consider some of the general guidelines presented below. If you are cloning into pPICZ $\alpha$ , it is important to clone your gene in frame with the  $\alpha$ -factor signal sequence. The multiple cloning sites for all vectors are presented on pages 18–23 to help you develop a cloning strategy.

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## General Considerations

The following are some general considerations applicable to pPICZ or pPICZ $\alpha$ .

- The codon usage in *Pichia* is believed to be similar to *Saccharomyces cerevisiae*.
- Many *Saccharomyces* genes have proven to be functional in *Pichia*.
- Maintain plasmid constructions in a *recA*, *endA* *E. coli* strain such as TOP10.
- The *BsmB* I site in the multiple cloning site has been specifically engineered to be compatible with inserts that have *Bam*H I and/or *Bgl* II ends. The *Bam*H I and *Bgl* II sites will be destroyed upon ligation, but the insert can be released by digestion with *BsmB* I.
- The premature termination of transcripts because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret and Sherman, 1984). If you have problems expressing your gene, check for premature termination by Northern analysis and check your sequence for AT rich regions. It may be necessary to change the sequence in order to express your gene (Scorer *et al.*, 1993).
- The native 5' end of the *AOX1* mRNA is noted in each multiple cloning site. This is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.

### For pPICZ only:

- For proper initiation of translation, your insert should contain an initiation ATG codon as part of a yeast consensus sequence (Romanos *et al.*, 1992). An example of a yeast consensus sequence is provided below. The ATG initiation codon is shown underlined.

(G/A)NNATGG

Note that other sequences are also possible. Although not as strong as the mammalian Kozak translation initiation sequence, the yeast consensus sequence is thought to have a 2–3-fold effect on the efficiency of translation initiation.

- To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. The vector is supplied in three reading frames to facilitate cloning. Refer to the diagrams on pages 18–20 to develop a cloning strategy.
  - If you wish to express your protein **without** the C-terminal peptide, be sure to include a stop codon.
- 

*Continued on next page*

# General Cloning Information, continued

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## General Considerations

### For pPICZ $\alpha$ only:

- The initiation ATG in the  $\alpha$ -factor signal sequence in pPICZ $\alpha$  corresponds to the native initiation ATG of the *AOX1* gene.
- If you are using pPICZ $\alpha$ , the open reading frame (ORF) of the mature gene of interest should be cloned in frame and downstream of the  $\alpha$ -factor signal sequence and in frame with the C-terminal tag (if desired).

**Note:** Cloning of your gene of interest in frame with the signal sequence does not automatically guarantee that your protein will be in-frame with the C-terminal tag. Please consider both the frame of the signal sequence and the C-terminal fusion tag when designing a cloning strategy.

- If you wish to express your gene of interest without the C-terminal peptide, be sure your gene contains a stop codon.
  - The predicted protease cleavage sites for the  $\alpha$ -factor signal sequence are indicated in the figures on pages 21–23.
- 

## Cloning Procedures

Refer to Ausubel, *et al.*, 1990, pages 3.16.1 to 3.17.3. or Sambrook, *et al.*, 1989, pages 5.10 to 5.13. for help with cloning.

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## Constructing Multimeric Plasmids



pPICZ and pPICZ $\alpha$  contain unique *Bgl* II and *Bam*H I sites to allow construction of plasmids containing multiple copies of your gene. For information on how to construct multimers, please contact Technical Support (see page 78).

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For preparing competent *E. coli* cells for transformation, use your own procedure or refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989). Note that electrocompetent TOP10F' cells are available from Invitrogen.

Item	Amount	Cat. no.
TOP10F' Electrocomp™	6 × 20 reactions	C665-24
TOP10F' Electrocomp™	2 × 20 reactions	C665-11

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*Continued on next page*



## General Cloning Information, continued

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### Important

To propagate pPICZ and pPICZ $\alpha$  or select Zeo<sup>R</sup> transformants in *E. coli*, you will need to prepare Low Salt LB. For Zeocin<sup>™</sup> to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt LB broth and plates using the recipe on page 53.

**Failure to lower the salt content of your LB medium will result in non-selection due to inactivation of the drug.**

To propagate vectors:

- Resuspend the plasmid in 20  $\mu$ l sterile water to make a 1  $\mu$ g/ $\mu$ l solution
  - Dilute 1  $\mu$ l of the plasmid (1  $\mu$ g/ $\mu$ l) to 10–100 pg/ $\mu$ l using sterile water or TE buffer.
  - Transform competent *E. coli* with 1–2  $\mu$ l of the diluted plasmid and select on Low Salt LB with 25  $\mu$ g/ml Zeocin<sup>™</sup>.
- 

### Signal Sequence Processing

The processing of the  $\alpha$ -factor mating signal sequence in pPICZ $\alpha$  occurs in two steps:

1. The preliminary cleavage of the signal sequence by the *KEX2* gene product, with the final Kex2 cleavage occurring between arginine and glutamine in the sequence Glu-Lys-Arg \* Glu-Ala-Glu-Ala, where \* is the site of cleavage.
  2. The Glu-Ala repeats are further cleaved by the *STE13* gene product.
- 

### Optimizing Signal Cleavage

In *Saccharomyces cerevisiae*, the Glu-Ala repeats are not necessary for cleavage by Kex2, but cleavage after Glu-Lys-Arg may be more efficient when followed by Glu-Ala repeats. A number of amino acids are tolerated at site X instead of Glu in the sequence Glu-Lys-Arg-X. These amino acids include the aromatic amino acids, small amino acids, and histidine. Proline, however, will inhibit Kex2 cleavage. For more information on Kex2 cleavage, see (Brake *et al.*, 1984)

There are some cases where Ste13 cleavage of Glu-Ala repeats is not efficient, and Glu-Ala repeats are left on the N-terminus of the expressed protein of interest. This is generally dependent on the protein of interest.

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### Expressing Recombinant Protein with Native N-terminus

To express your protein with a native N-terminus, use the *Xho* I site at bp 1184–1189 to clone your gene flush with the Kex2 cleavage site. Use PCR to rebuild the sequence from the *Xho* I site to the arginine codon at nucleotides 1193–1195. Remember to include the first amino acid(s) of your protein, if necessary, for correct fusion to the Kex2 cleavage site.

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*Continued on next page*

# Cloning into pPICZ

## Multiple Cloning Site of pPICZ A

Below is the multiple cloning site for pPICZ A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPICZ A is available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78).**

```
5' end of AOX1 mRNA                                     5' AOX1 priming site
|
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871 CAAGCTTTTGG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

      Sfu I      EcoR I      Pml I      Sfi I      BsmB I Asp718 I Kpn I Xho I
891 ATTCGAAACG AGGAATTCAC GTGGCCCAGC CGGCCGTCTC GGATCGGTAC CTCGAGCCGC

      Sac II Not I      Apa I      myc epitope
991 GGC|GGCCCGCC AGCTT |GGGCCC| GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG
      |
      |Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu

1042 AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGCCT TAGACATGAC
      Asn Ser Ala Val Asp His His His His His His ***
      Polyhistidine tag

1098 TGTTCCCTCAG TTCAAGTTGG GCACTTACGA GAAGACCGGT CTTGCTAGAT TCTAATCAAG

      3' AOX1 priming site
1158 AGGATGTCAG AATGCCATTT GCCTGAGAGA TGCAGGCTTC ATTTTTGATA CTTTTTTATT

      3' polyadenylation site
1218 TGTAACCTAT ATAGTATAGG ATTTTTTTTTG TCATTTTGTT
```

*Continued on next page*

## Cloning into pPICZ, continued

### Multiple Cloning Site of pPICZ B

Below is the multiple cloning site of pPICZ B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPICZ B is available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78).**

```
5' end of AOX1 mRNA                                     5' AOX1 priming site
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871 CAAGCTTTTG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

      Sfu I      EcoR I      Pml I      Sfi I      BsmB I Asp718 I Kpn I Xho I
891 ATTCGAAACG AGGAATTCAC GTGGCCAGC CGGCCGTCTC GGATCGGTAC CTCGAGCCGC

      Sac II Not I      Xba I      myc epitope
991 GGC GGCCGCC AGCTT TCTA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG
      Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu

1040 AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC TTAGACATGA
      Asn Ser Ala Val Asp His His His His His His ***
      Polyhistidine tag

1096 CTGTTCTCA GTTCAAGTTG GGCACCTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA

      3' AOX1 priming site
1156 GAGGATGTCA GAATGCCATT TGCCGTGAGAG ATGCAGGCTT CATTTTTGAT ACTTTTTTAT

      3' polyadenylation site
1216 TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTC
```

Continued on next page

## Cloning into pPICZ, continued

### Multiple Cloning Site of pPICZ C

Below is the multiple cloning site of pPICZ C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPICZ C is available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78).**

```
5' end of AOX1 mRNA                                     5' AOX1 priming site
811 AACCTTTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871 CAAGCTTTTGG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

      Sfu I      EcoR I      Pml I      Sfi I      BsmB I Asp718 I Kpn I Xho I
931 ATTCGAAACG AGGAATTCAC GTGGCCAGC CGGCCGTCTC GGATCGGTAC CTCGAGCCGC

      Sac II Not I      SnaB I      myc epitope
991 GGC GGCCGCC AGCTT ACGTA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG
      Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu

1041 AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC TTAGACATGA
      Asn Ser Ala Val Asp His His His His His His ***

1097 CTGTTCTCA GTTCAAGTTG GCACTTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA

      3' AOX1 priming site
1157 GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT ACTTTTTTAT

      3' polyadenylation site
1217 TTGTAACCTA TATAGTATAG GATTTTTTTTT GTCATTTTGT TTC
```

Continued on next page





## Cloning into pPICZ $\alpha$ , continued

### Multiple Cloning Site of pPICZ $\alpha$ C

Below is the multiple cloning site of pPICZ $\alpha$  C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPICZ $\alpha$  C is available for downloading from our website ([www.invitrogen.com](http://www.invitrogen.com)) or from Technical Support (see page 78).**

```

5' end of AOX1 mRNA
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA
                                     5' AOX1 priming site
871 CAAGCTTTTGG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT
931 ATTCGAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA
    Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala
983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA
    Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala
                                      $\alpha$ -factor signal sequence
1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
    Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
    Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe
                                      $\alpha$ -factor priming site
1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC
    Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu
                                     Xho I*
1187 GAG AAG AGA GAG GCT GAA GC ATCGAT GAATTCAC GTGGCCAG CCGGCCGTC TCGGA
    Glu Lys Arg Glu Ala Glu Ala
    Kex2 signal cleavage
    Ste13 signal cleavage
    Asp718 | Kpn I | Xho I | Sac II | Not I | Xba I | c-myc epitope
1244 TCGGTACCTC GAGCCGCGGC GCGCCGAGC TTTCTA GAA CAA AAA CTC ATC TCA GAA
    Glu Gln Lys Leu Ile Ser Glu
                                     polyhistidine tag
1301 GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTA
    Glu Asp Leu Asn Ser Ala Val Asp His His His His His His ***
1353 GCCTTAGACA TGACTGTTCC TCAGTTCAAG TTGGGCACTT ACGAGAAGAC CGGTCTTGCT
                                     3' AOX1 priming site
1413 AGATTCTAAT CAAGAGGATG TCAGAATGCC ATTTGCCTGA GAGATGCAGG CTTCATTTTT
                                     3' polyadenylation site
1473 GATACTTTTT TATTTGTAAC CTATATAGTA TAGGATTTTT TTTGTCATTT TGTTTCTTCT

```

\*To express your protein with a native N-terminus, you must clone your gene flush with the Kex2 cleavage site. You will need to use PCR and utilize the Xho I site upstream of the Kex2 cleavage site.

# Transformation into *E. coli*

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## Introduction

Ligation mixtures may be transformed into *E. coli* and selected on **Low Salt LB** medium (see below) with Zeocin™. Transformants are isolated and analyzed for the presence and orientation of insert. There is no blue/white screening for the presence of insert with pPICZ or pPICZα. After obtaining the desired recombinant plasmid, you will be ready to transform into *Pichia*.

---



## Important

For Zeocin™ to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt LB broth and plates using the recipe in the **Appendix**, page 53.

**Failure to lower the salt content of your LB medium will result in non-selection due to inactivation of the drug.**

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## Transformation

Guidelines are as follows:

- Transformation may be performed by either electroporation or chemical methods. Use your preferred method or refer to general molecular biology references (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989)
- Add either Low Salt LB or LB medium to the cells after heat shock or electroporation to allow them to recover.
- Plate on **Low Salt LB medium** with 25 µg/ml Zeocin™.

**Note:** You may also use SOB, 2XYT, or TB medium, but you may have to increase the concentration of Zeocin™ to 50 µg/ml to compensate for differences in the salt concentration.

- Incubate overnight at 37°C.
- 

## Analyzing Transformants

1. After transformation, plate 10 µl and 100 µl of the transformation mix onto Low Salt LB plates with 25 µg/ml Zeocin™ (see above) and select Zeocin™-resistant colonies.
  2. Pick 10 Zeocin™-resistant transformants and inoculate into 2 ml Low Salt LB medium with 25 µg/ml Zeocin™. Grow overnight at 37°C with shaking.
  3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see next page).
  4. Be sure to make a glycerol stock of your purified clone for safekeeping.
- 

*Continued on next page*



## Transformation into *E. coli*, continued

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### Sequencing Recombinant Clones

We strongly recommend that you sequence your construct to confirm that your gene is in frame with the C-terminal peptide before transforming into *Pichia*. Use the sequencing primers included in the kit to sequence your construct.

To sequence your construct in pPICZ, use the 5' AOX1 and the 3' AOX1 Sequencing Primers.

To sequence your construct in pPICZ $\alpha$ , use the  $\alpha$ -factor or the 5' AOX1 and the 3' AOX1 Sequencing Primers.

To use the primers, resuspend each lyophilized primer in 20  $\mu$ l sterile water. This will yield a stock solution of 0.1  $\mu$ g/ $\mu$ l.

For sequencing protocols, Refer to Unit 7 in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or Chapter 13 in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989).

---

### Plasmid Preparation

Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform *Pichia* (5–10  $\mu$ g of each plasmid per each transformation). We recommend the S.N.A.P.<sup>™</sup> Miniprep Kit (Cat. no. K1900-01) or the PureLink<sup>™</sup> HiPure Plasmid DNA Purification Kit (Cat. no. K2100-01) for isolation of pure plasmid DNA. Once you have purified plasmid DNA, proceed to **Preparing Transforming DNA**, next page.

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# Preparing Transforming DNA

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## Introduction

At this point, you should have your gene cloned into one of the pPICZ or pPICZ $\alpha$  vectors. Your construct should contain a yeast consensus sequence (A/YAA/TAATGTCT) and be correctly fused to the secretion signal (pPICZ $\alpha$ ) and/or the C-terminal peptide.

To transform *Pichia*, prepare 5–10  $\mu$ g of plasmid DNA, and linearize the plasmid prior to transformation and selection in *Pichia*. Plate the transformants on YPDS plates containing 100  $\mu$ g/ml Zeocin<sup>™</sup> to isolate Zeocin<sup>™</sup>-resistant (Zeo<sup>R</sup>) clones.

Remember also to isolate two control strains for background protein expression in *Pichia*. Linearize pPICZ or pPICZ $\alpha$  and transform into GS115 to generate a Mut<sup>+</sup> control and KM71H to generate a Mut<sup>S</sup> control.

---

## Method of Transformation

We recommend electroporation or chemical methods for transformation of *Pichia* with pPICZ or pPICZ $\alpha$ . Electroporation yields 10<sup>3</sup> to 10<sup>4</sup> transformants per  $\mu$ g of linearized DNA and does not destroy the cell wall of *Pichia*. If you do not have access to an electroporation device, use the *Pichia* EasyComp<sup>™</sup> procedure on page 29.

**We do not recommend spheroplasting for transformation of *Pichia* with plasmids containing the Zeocin<sup>™</sup> resistance marker.** Spheroplasting involves removal of the cell wall to allow DNA to enter the cell. Cells must first regenerate the cell wall before they are able to express the Zeocin<sup>™</sup> resistance gene. For this reason, plating spheroplasts directly onto selective medium containing Zeocin<sup>™</sup> does not yield any transformants.

---



## Important

Integration can only occur at the *AOX1* locus. Vector linearized within the 5' *AOX1* region will integrate by gene insertion into the host 5' *AOX1* region. Therefore, the *Pichia* host that you use will determine whether the recombinant strain is able to metabolize methanol (Mut<sup>+</sup>) or not (Mut<sup>S</sup>). To generate a Mut<sup>+</sup> recombinant strain, you must use a *Pichia* host that contains the native *AOX1* gene (*i.e.*, X-33, GS115, SMD1168). If you choose to generate a Mut<sup>S</sup> recombinant strain, then use a *Pichia* host that has a disrupted *AOX1* gene (*i.e.*, KM71H). Information on recombination in *Pichia* is available on page 62.

---

## Restriction Digest

1. Digest ~5–10  $\mu$ g of plasmid DNA with one of the restriction enzymes listed below. Each enzyme cuts one time in the 5' *AOX1* region to linearize the either pPICZ or pPICZ $\alpha$ .  
**Note:** Choose the enzyme that does not cut within your gene: *Sac* I (209 bp), *Pme* I (414 bp), and *Bst*X I (707 bp).
  2. We recommend that you check a small aliquot of your digest by agarose gel electrophoresis for complete linearization.
  3. If the vector is completely linearized, heat inactivate or add EDTA to stop the reaction, phenol/chloroform extract once, and ethanol precipitate using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol.
  4. Centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend in 10  $\mu$ l sterile, deionized water. Use immediately or store at –20°C.
-

# Electroporation of *Pichia*

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## Introduction

We strongly recommend electroporation if you are specifically interested in isolating multi-copy integrants of your gene in *Pichia*. The frequency of multi-copy insertions ranges from 1 to 10%, requiring hundreds to thousands of transformants to isolate a suitable number of multi-copy clones to test for expression. Electroporation yields some of the highest transformation frequencies in *Pichia* and is the method of choice to isolate multi-copy integrants.

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## Important

Traditionally, spheroplasting has been used to transform *Pichia*, but this method of transformation does not allow direct selection on Zeocin™. Damage to the cell wall leads to increase sensitivity to Zeocin™, causing putative transformants to die before they express the Zeocin™ resistance gene.

---

## Before Starting

You will need the following materials for transforming *Pichia* and selecting transformants on Zeocin™.

**Note:** Inclusion of sorbitol in YPD plates stabilizes electroporated cells as they appear to be somewhat osmotically sensitive.

- 5–10 µg pure pPICZ or pPICZα containing your insert  
**Note:** For transforming with **circular** DNA, you will need 50–100 µg plasmid DNA. If you have constructed multimers in pPICZ or pPICZα, you will not be able to linearize the plasmid.
  - YPD Medium
  - 50-ml conical polypropylene tubes
  - 1 liter cold (4°C) sterile water (place on ice the day of the experiment)
  - 25 ml cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
  - 30°C incubator
  - Electroporation device and 0.2 cm cuvettes
  - YPDS plates containing 100 µg/ml Zeocin™ (See page 56 for recipe)
- 

## Preparing *Pichia* for Electroporation

1. Grow 5 ml of your *Pichia pastoris* strain in YPD in a 50 ml conical at 30°C overnight.
  2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1–0.5 ml of the overnight culture. Grow overnight again to an OD<sub>600</sub> = 1.3–1.5.
  3. Centrifuge the cells at 1,500 × g for 5 minutes at 4°C. Resuspend the pellet with 500 ml of ice-cold, sterile water.
  4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 ml of ice-cold, sterile water.
  5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold 1 M sorbitol.
  6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use that day. Do **not** store cells.
- 

*Continued on next page*

## Electroporation of *Pichia*, continued

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### Transformation by Electroporation

1. Mix 80  $\mu$ l of the cells from Step 6 (previous page) with 5–10  $\mu$ g of linearized DNA (in 5–10  $\mu$ l sterile water) and transfer them to an ice-cold 0.2 cm electroporation cuvette.  
**Note:** For circular DNA, use 50–100  $\mu$ g.
  2. Incubate the cuvette with the cells on ice for 5 minutes.
  3. Pulse the cells using the manufacturer's instructions for *Saccharomyces cerevisiae*.
  4. Immediately add 1 ml of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15-ml tube and incubate at 30°C without shaking for 1 to 2 hours.
  5. Spread 10, 25, 50, 100, and 200  $\mu$ l each on separate, labeled YPDS plates containing 100  $\mu$ g/ml Zeocin™. Plating at low cell densities favors efficient Zeocin™ selection.
  6. Incubate plates from 3–10 days at 30°C until colonies form.
  7. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100  $\mu$ g/ml Zeocin™.
- 

### Isolating Multi-copy Recombinants *in vivo*

A quick, direct way to select putative multi-copy recombinants is to plate the transformation mix on increasing concentrations of Zeocin™.

1. Prepare YPDS plates containing 500, 1000, and 2000  $\mu$ g/ml Zeocin™
  2. Plate 100 to 200  $\mu$ l of the transformation mix on each plate and incubate at 30°C for 2 days
  3. Test transformants for the Mut phenotype (page 33) and expression of your protein (page 37)
- 



### Note

Generally several hundred to several thousand Zeocin™-resistant (Zeo<sup>R</sup>) colonies are generated using the above protocol. For more colonies, you may modify the protocol as described below. Note that you will need ~20 150-mm plates with YPDS agar containing 100  $\mu$ g/ml Zeocin™.

1. Set up two transformations per construct and follow Steps 1 through 5 of the **Transformation by Electroporation** protocol, above.
  2. After 1 hour in 1 M sorbitol at 30°C (Step 4, above), add 1 ml YPD medium to each tube. Shake (~200 rpm) the cultures at 30°C.
  3. After 1 hour, take one of the tubes and plate out all of the cells by spreading 200  $\mu$ l on 150-mm plates containing 100  $\mu$ g/ml Zeocin™.
  4. *Optional:* Continue incubating the other culture for three more hours (for a total of four hours) and then plate out all of the cells by spreading 200  $\mu$ l on 150-mm plates containing 100  $\mu$ g/ml Zeocin™.
  5. Incubate plates for 2 to 4 days at 30°C until colonies form.
- 

### Analyzing *Pichia* Transformants

Select 6–10 of your Zeo<sup>R</sup> *Pichia* transformants and confirm the Mut phenotype as described on page 33. You may also analyze for the presence of insert using PCR (page 68), or for copy number using Southern analysis (page 74).

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# EasyComp™ Transformation

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## Introduction

The *Pichia* EasyComp™ Kit produces chemically competent *Pichia* cells and is included to provide an alternative to electroporation and a rapid, convenient method for transformation. However, because of the low transformation efficiency (3 µg plasmid DNA yields about 50 colonies), it is very difficult to isolate multi-copy integrants. In instances where multi-copy integrants are desired, please use electroporation (page 28) for best results. Note that cells are prepared differently for electroporation. **Do not use cells prepared using the EasyComp™ protocol for electroporation.**

---

## Required Reagents and Equipment

- 30°C rotary shaking incubator
  - YPD (Yeast Extract Peptone Dextrose) medium (see **Recipes**, page 55)
  - 50 ml, sterile conical tubes
  - Centrifuge suitable for 50 ml conical tubes (floor or table-top)
  - 1.5 ml sterile screw-cap microcentrifuge tubes
  - –80°C freezer
  - Styrofoam box or paper towels
- 

## Before Beginning

- Streak a YPD plate with your *Pichia pastoris* strain such that isolated, single colonies will grow. Incubate the plate at 28–30°C for 2 days.
  - Equilibrate Solution I to room temperature.
- 

## Preparing Competent Cells

1. Inoculate 10 ml of YPD with a single colony of your *Pichia* strain. Grow overnight at 28–30°C in a shaking incubator (250–300 rpm).
  2. Dilute cells from the overnight culture to an OD<sub>600</sub> of 0.1–0.2 in 10 ml of YPD. Grow the cells at 28–30°C in a shaking incubator until the OD<sub>600</sub> is 0.6–1.0. This will take approximately 4 to 6 hours.
  3. Pellet the cells by centrifugation at 500 × g for 5 minutes at room temperature. Discard the supernatant.
  4. Resuspend the cell pellet in 10 ml of Solution I. No incubation time is required.
  5. Pellet the cells by centrifugation at 500 × g for 5 minutes at room temperature. Discard the supernatant.
  6. Resuspend the cell pellet in 1 ml of Solution I. The cells are now competent.
  7. Aliquot 50 to 200 µl of competent cells into labeled 1.5 ml sterile screw-cap microcentrifuge tubes.  
**Note:** Use 50 µl of cells for each transformation. You can thaw the cells and refreeze several times without significant loss in transformation efficiency.
  8. At this point, the cells may be kept at room temperature and used directly for transformation or frozen for future use. To freeze cells, place tubes in a Styrofoam box or wrap in several layers of paper towels and place in a –80°C freezer. It is important that you freeze the cells slowly. **Do not snap-freeze the cells in liquid nitrogen.**
  9. Proceed to the transformation procedure.
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# EasyComp™ Transformation, continued

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## Note

We have observed that higher transformation efficiencies are often obtained with frozen versus freshly prepared cells. You may choose to use some of the cells immediately following preparation and freeze the remaining cells in small aliquots.

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## Transformation

You may use the following protocol to transform freshly prepared or frozen competent *Pichia* cells. Transformation efficiency may vary with each strain and vector used.

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## Required Reagents and Equipment

- 30°C incubator
  - Water baths or heat blocks at 30°C and 42°C
  - Microcentrifuge at room temperature
  - YPDS with 100 µg/ml Zeocin™ plates (see **Recipes**, page 56)
- 

## Before Beginning

- The PEG in Solution II may precipitate at temperatures below 27°C. If you see a precipitate, warm the solution at 37°C, swirling occasionally, until the precipitate dissolves. To prevent formation of a precipitate, store Solution II at room temperature.
  - Equilibrate Solution III to room temperature.
  - Equilibrate the appropriate number and type of plates to room temperature. You will need one plate for each transformation.
  - You may want to include controls to check for contamination. We recommend a no DNA and a plasmid only control.
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*Continued on next page*

# EasyComp™ Transformation, continued

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## Transformation Protocol

1. For each transformation, thaw one tube of competent cells at room temperature and aliquot 50 µl into a sterile microcentrifuge tube. If transforming fresh cells, use 50 µl of cells from **Preparing Competent Cells**, Step 7, page 29.
2. Add 3 µg of linearized *Pichia* expression vector DNA to the competent cells.  
**Note:** Using greater than 3 µg of DNA may increase transformation efficiencies in some cases. The volume of DNA should not exceed 5 µl. Linearized DNA can be used directly from a restriction digest reaction without affecting transformation efficiency. Phenol chloroform extraction and ethanol precipitation are not necessary.
3. Add 1 ml of Solution II to the DNA/cell mixture and mix by vortexing or flicking the tube.
4. Incubate the transformation reactions for 1 hour at 30°C in a water bath or incubator. Mix the transformation reaction every 15 minutes by vortexing or flicking the tube. Failure to mix the transformation reaction every 15 minutes will result in decreased transformation efficiency.
5. Heat shock the cells in a 42°C heat block or water bath for 10 minutes.
6. Split the cells into 2 microcentrifuge tubes (approximately 525 µl per tube) and add 1 ml of YPD medium to each tube.
7. Incubate the cells at 30°C for 1 hour to allow expression of Zeocin™ resistance.
8. Pellet the cells by centrifugation at 3,000 × g for 5 minutes at room temperature. Discard the supernatant.
9. Resuspend each tube of cells in 500 µl of Solution III and combine the cells into one tube.
10. Pellet the cells by centrifugation at 3,000 × g for 5 minutes at room temperature. Discard the supernatant.
11. Resuspend the cell pellet in 100 to 150 µl of Solution III.
12. Plate the entire transformation on appropriate selection plates using a sterile spreader. Incubate the plates for 3 to 10 days at 30°C. Each transformation should yield approximately 50 colonies.

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*Continued on next page*

# EasyComp™ Transformation, continued

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## Analyzing *Pichia* Transformants

Select 6–10 of your Zeocin™-resistant *Pichia* transformants and confirm the Mut phenotype as described on page 33. You may also wish to analyze for the presence of insert using PCR (page 68).

**Note:** When selecting Zeocin™-resistant *Pichia* transformants, it is normal to observe a low amount of background (~10–30%).

---

## Troubleshooting

The table below provides solutions to possible problems you may encounter when preparing and transforming competent *Pichia pastoris* cells.

Problem	Probable Cause	Possible Solution
Low efficiency of transformation	The pH of Solution I or Solution III may have drifted. The pH of both solutions should be 8.0	Check the pH of Solutions I and III. If the pH is low, increase it by adding NaOH. If the pH is high, decrease it by adding HCl. Store solutions at 4°C in order to minimize drift in pH.
	Transformation reaction not mixed during incubation	Be sure to mix the transformation reaction every 15 minutes throughout the 1 hour incubation at 30°C. Vortexing works best.
	Incubation time is too short or temperature is too low.	<i>Pichia pastoris</i> transformations may be incubated for longer periods of time (up to 3 hours) and at higher temperature (35–37°C). This may, in some instances, result in higher transformation efficiencies.
	Cell density is too low (OD <sub>600</sub> <0.6)	Resuspend cells from <b>Preparing Competent Cells</b> , Step 6, page 29, in a smaller volume ( <i>i.e.</i> , 500 µl).

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# Determining the Mut Phenotype

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## Introduction

If you used X-33 or GS115 as the host, the transformants should be Mut<sup>+</sup>. To confirm the expected phenotype, two strains are included in the kit that will provide examples of Mut<sup>+</sup> and Mut<sup>s</sup> phenotypes. GS115 Albumin is Mut<sup>s</sup> and GS115/pPICZ/*lacZ* is Mut<sup>+</sup>. Note that KM71H recombinants do not need to be screened for their Mut phenotype as they all will be Mut<sup>s</sup>.

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## Screening for Mut<sup>+</sup> in X-33 and GS115

Transformation of X-33 or GS115 with linearized constructs favor single crossover recombination at the *AOX1* locus. Most of the transformants should be Mut<sup>+</sup>; however, with the presence of the *AOX1* sequences in the plasmid, there is a chance that recombination will occur in the 3' *AOX1* region also, disrupting the wild-type *AOX1* gene and creating Mut<sup>s</sup> transformants. Testing on MDH and MMH plates will allow you to confirm the Mut<sup>+</sup> phenotype (see below).

---

## Mut<sup>s</sup> in KM71H

All Zeo<sup>R</sup> transformants in KM71H will be Mut<sup>s</sup> because of the disruption of the *AOX1* gene (*aox1::ARG4*). There is no need to test recombinants for the Mut phenotype; all recombinants will be Mut<sup>s</sup>. Transformants need to be purified on minimal plates to ensure pure clonal isolates before either testing for expression (see page 37) or confirming integration by PCR (see page 68).

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## Important

Host strains containing the *his4* allele (e.g., GS115) and transformed with the pPICZ or pPICZ $\alpha$  vectors require histidine when grown in minimal media. Add histidine to a final concentration of 0.004% to ensure growth of your transformant.

---

## Preparation

You may prepare the following media (see page 57) and materials several days in advance and store at 4°C:

Minimal Dextrose with histidine (MDH) agar plates, 1 liter

Minimal Methanol with histidine (MMH) agar plates, 1 liter

Sterile toothpicks and Scoring Templates (see page 36)

Streak out the strains GS115 Albumin (Mut<sup>s</sup>) and GS115/pPICZ/*lacZ* (Mut<sup>+</sup>) on an MDH or MGYH plate as controls for Mut<sup>+</sup> and Mut<sup>s</sup> growth.

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*Continued on next page*

## Determining the Mut Phenotype, continued

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### Mut<sup>+</sup> in GS115 or X-33

Use the plates containing the Zeo<sup>R</sup> transformants and confirm the Mut<sup>+</sup> phenotype as described below. See page 36 for scoring templates.

**Note:** Instructions are for GS115 strains. These strains require histidine for growth. X-33 **does not** require histidine for growth, so you may leave it out of the medium.

1. Using a sterile toothpick, pick one colony and streak or patch one Zeo<sup>R</sup> transformant in a regular pattern on both an MMH plate and an MDH plate, making sure to patch the MMH plate first.
  2. Use a new toothpick for each transformant and continue until 10 transformants have been patched (1 plate).
  3. To differentiate Mut<sup>+</sup> from Mut<sup>S</sup>, make one patch for each of the controls (GS115/Mut<sup>S</sup> Albumin and GS115/pPICZ/*lacZ* Mut<sup>+</sup>) onto the MDH and MMH plates.
  4. Incubate the plates at 30°C for 2 days.
  5. After 2 days or longer at 30°C, score the plates. Mut<sup>+</sup> strains will grow normally on both plates, while Mut<sup>S</sup> strains will grow normally on the MDH plate but show little or no growth on the MMH plate.
- 



### Important

We recommend purifying your Zeo<sup>R</sup> transformants to ensure isolation of a pure clonal isolates. This is done by streaking for single colonies on YPD or minimal plates with histidine. You may do this before or after testing for the Mut phenotype.

---

### Replica-Plating Procedure

This procedure gives a lower rate of misclassifications, but it increases the overall Mut<sup>+</sup>/Mut<sup>S</sup> screening procedure by 2 days. You will need equipment to replica-plate.

1. Using sterile toothpicks, patch 10 Zeo<sup>R</sup> transformants on an MDH plate. For controls, make one patch from each of the strains GS115/Mut<sup>S</sup> Albumin and GS115/pPICZ/*lacZ* Mut<sup>+</sup> onto the MDH plate.
  2. Incubate the plate at 28–30°C for 2 days.
  3. After 2 days, replica-plate the patches from the MDH plate onto fresh MMH and MDH plates to screen for Mut<sup>S</sup> transformants.
  4. Incubate the replica plates at 28–30°C for 2 days.
  5. After 2 days at 28–30°C, score the replica plates. Look for patches that grow normally on the MDH replica plate but show little or no growth on the MMH replica plate; these are your Mut<sup>S</sup> transformants. Including Mut<sup>+</sup> and Mut<sup>S</sup> control patches on each plate will provide examples of Mut<sup>+</sup> and Mut<sup>S</sup> phenotypes.
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## Determining the Mut Phenotype, continued

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### Screening by Functional Assay

Some researchers have used a functional assay to directly screen for high expressing *Pichia* recombinant clones without first screening for Mut<sup>s</sup> or Mut<sup>+</sup> phenotypes. If you elect to screen directly for high-expressing recombinants, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

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### Multiple Integration Events

*Pichia pastoris* is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology (see page 63 for figure). Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants.

Successful expression of the gene of interest to useful levels may depend upon the generation of a recombinant strain that contains multiple copies integrated at the *AOX1* locus. In addition to simply screening expression levels among several Mut<sup>s</sup> or Mut<sup>+</sup> recombinants via SDS-PAGE analysis, it may be desirable to determine the existence of strains that have multiple integrants in the Mut<sup>s</sup> or Mut<sup>+</sup> recombinant strain.

See the **Appendix**, page 73 for methods to determine copy number.

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### The Next Step

After confirming the Mut phenotype, you may proceed to small-scale expression (page 37) to test for expression of your gene.

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*Continued on next page*



# Expressing Recombinant *Pichia* Strains

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## Introduction

You should now have several Mut<sup>s</sup> and Mut<sup>+</sup> recombinant strains which have been confirmed by PCR to contain your insert (see page 68 and page 70). The purpose of this section is to determine the optimal method and conditions for expression of your gene. Below are some factors and guidelines which need to be considered before starting expression in *Pichia pastoris*. As with any expression system, optimal expression conditions are dependent on the characteristics of the protein being expressed.

---

## Detection of Recombinant Proteins in *Pichia*

Small-scale expression conditions may not be optimal for your protein. For this reason, the method you choose for detection (*i.e.*, SDS-PAGE, Western, or functional assay) may be an important factor in determining the success of expression. If your method of detection does not reveal any expression, you may want to consider using a more sensitive method.

We recommend that you use the following techniques to analyze the expression of your protein. Note that the *myc* epitope and the polyhistidine tag will contribute 2.5 kDa to the size of your protein. Be sure to account for any additional amino acids that are in between the end of your native protein and the *myc* epitope.

Technique	Method of Detection	Sensitivity
SDS-PAGE (Coomassie-stained)	Visualization by eye	Can detect as little as 100 ng in a single band
SDS-PAGE (Silver-stained)	Visualization by eye	Can detect as little as 2 ng in a single band
Western Analysis	Antibody to your particular protein Anti- <i>myc</i> antibodies or Anti-His(C-term) antibodies (see page viii for ordering information)	Can detect as little as 1–10 pg, depending on detection method (alkaline phosphatase, horseradish peroxidase, radiolabeled antibody)
Functional assay	Varies depending on assay	Varies depending on assay Used to compare relative amounts of protein.

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## Important

Host strains containing the *his4* allele (*e.g.*, GS115) and transformed with the pPICZ or pPICZ $\alpha$  vectors require histidine when grown in minimal media. Add histidine to a final concentration of 0.004% to ensure growth of your transformant. Complex medium such as YPD and BMGY already contain histidine.

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# Expressing Recombinant *Pichia* Strains, continued

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## Media

For expressing recombinant *Pichia* strains, you need BMGY/BMMY (buffered complex glycerol or methanol medium), BMGH/BMMH (buffered minimal glycerol or methanol medium containing histidine), or MGYH/MMH (minimal glycerol or minimal methanol medium containing histidine) (see **Recipes**, pages 57–58). BMGH, BMMH, BMGY, and BMMY are usually used for expressing secreted proteins, particularly if pH is important for the activity of your protein. Unlike MGYH and MMH, they are all buffered media. Because these media are buffered with phosphate buffer, you may use a wider range of pH values to optimize protein production. BMGY/BMMY contain yeast extract and peptone to stabilize secreted proteins and to prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone allow better growth and biomass accumulation.

---

## Proteases

There are some proteins specifically susceptible to proteases that have optimal activity at neutral pH. If this is the case, expression using MGYH and MMH media may be indicated. As *Pichia* expression progresses in an unbuffered medium such as MMH, the pH drops to 3 or below, inactivating many neutral pH proteases (Brierley *et al.*, 1994). *Pichia* is resistant to low pH, so the low pH will not affect growth. In contrast, it has been reported that by including 1% Casamino acids (Difco) and buffering the medium at pH 6.0, extracellular proteases were inhibited, increasing the yield of mouse epidermal growth factor (Clare *et al.*, 1991b).

If you know your protein of interest is especially susceptible to neutral pH proteases, express your protein in an unbuffered medium (MMH). If there is no evidence that your secreted protein of interest is susceptible to proteases at neutral pH, we recommend you do your initial expressions in BMMY. If the expressed protein is degraded, try expressing your protein in an unbuffered medium.

---

## Aeration

The most important parameter for efficient expression in *Pichia* is adequate aeration during methanol induction. As a general rule when inducing expression, never allow cultures to be more than 10–30% of your total flask volume. It is strongly recommended that baffled flasks be used. See **Recipes**, page 59 for suppliers of baffled flasks. Cover the flasks with cheesecloth (2–3 layers) or another loose fitting cover. Never use tight fitting covers. (Aeration is not as critical when generating biomass before induction.)

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## Kinetics of Growth

Note that while Mut<sup>+</sup> and Mut<sup>s</sup> strains will grow at essentially the same rate in YPD or glycerol media, Mut<sup>+</sup> will grow faster than Mut<sup>s</sup> when both are grown on methanol because of the presence of the AOX1 gene product.

---

## Temperature and Shaking

All expression is done at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. If your incubator temperature fluctuates, set the temperature at 28°C. If using a floor shaking incubator, shake at 225–250 rpm. If using a table-top shaker that sits inside an incubator, shake at 250–300 rpm.

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# Expressing Recombinant *Pichia* Strains, continued

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## Before Starting

When performing your expression, it is important to run the proper controls so that you will be able to interpret your expression results. Use the following expression controls:

GS115 Mut <sup>S</sup> albumin	Mut <sup>S</sup> – Secretion control
GS115/pPICZ/ <i>lacZ</i> Mut <sup>+</sup>	Mut <sup>+</sup> – Intracellular control
GS115 or KM71H/Vector (no insert)	background control

Since recombination can occur in many different ways that can effect expression (clonal variation), we recommend that you screen 6–10 verified recombinant clones for expression levels. Start with colonies from the freshest plates available. Colony viability drops over time, so if you have any doubts, it is better to streak out your strain. (You may also start the cultures with a small sample from a frozen glycerol stock that was generated from a single colony.)

---

## Guidelines for Expression

The following steps are guidelines and are presented to get you started with expression. You may have to change the conditions to optimize expression for your particular protein. Use bottom or side baffled flasks whenever possible. These are available in a variety of sizes (50–2000 ml). If you are analyzing a number of recombinants, you can try 50 ml conical tubes. Be sure that the medium is well-aerated by increasing the rate of shaking or placing the tubes at an angle in the shaker.

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## Expressing Recombinant *Pichia* Strains, continued

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### Mut<sup>+</sup> Intracellular or Secreted

You can test the effectiveness of your expression conditions by growing GS115/pPICZ/*lacZ* which is Mut<sup>+</sup> and expresses  $\beta$ -Galactosidase intracellularly. Remember to include GS115 transformed with the parent vector as a control for background intracellular expression.

1. Using a single colony, inoculate 25 ml of MGYH, BMGH, or BMGY in a 250 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until culture reaches an OD<sub>600</sub> = 2–6 (approximately 16–18 hours). The cells will be in log-phase growth.
2. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD<sub>600</sub> of 1.0 in MMH, BMMH, or BMMY medium to induce expression (approximately 100–200 ml).
3. Place culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.
5. At each of the times indicated below, transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. Use these samples to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.  
Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).
6. For secreted expression, **transfer the supernatant to a separate tube**. Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N<sub>2</sub> or a dry ice/alcohol bath.  
For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N<sub>2</sub> or a dry ice/alcohol bath.
7. Analyze the supernatants and cell pellets for protein expression by Coomassie-stained SDS-PAGE and Western blot or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, page 42).

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*Continued on next page*



# Expressing Recombinant *Pichia* Strains, continued

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## Mut<sup>S</sup> Intracellular or Secreted

You can test the effectiveness of your expression conditions by growing GS115 which is Mut<sup>S</sup> and secretes albumin to the medium. Remember to include X-33, GS115 or KM71H transformed with the parent vector as a control for background intracellular expression.

1. Using a single colony, inoculate 100 ml of MGYH, BMGH, or BMGY in a 1 liter baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD<sub>600</sub> = 2–6 (approximately 16–18 hours).
2. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant, and resuspend cell pellet in MMH, BMMH, or BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10–20 ml).
3. Place in a 100 ml baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue to grow.
4. Add 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction.
5. At each of the times indicated below transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.

Time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days), 120 (5 days), and 144 (6 days).

6. For secreted expression, **transfer the supernatant to a separate tube**. Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N<sub>2</sub> or a dry ice/alcohol bath.

For intracellular expression, decant the supernatant, and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N<sub>2</sub> or a dry ice/alcohol bath.

7. Analyze the cell pellets for protein expression by Coomassie-stained SDS-PAGE and Western blot or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, next page).
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# Analysis by SDS-Polyacrylamide Gel Electrophoresis

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## Introduction

This section provides guidelines to prepare and analyze your samples using SDS-polyacrylamide gel electrophoresis.

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## Polyacrylamide Gel Electrophoresis

If you are pouring your own polyacrylamide gels, please note that any standard SDS-polyacrylamide gel apparatus and protocol will work. For example, a 12% polyacrylamide gel with a 5% stacking gel is recommended for proteins ranging in size from 40–100 kDa. For other recommendations, See standard texts such as *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989), *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Guide to Protein Purification* (Deutscher, 1990), or *Protein Methods* (Bollag and Edelman, 1991).

Alternatively, a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented NuPAGE® Gel System avoids the protein modifications associated with laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use, refer to our website ([www.invitrogen.com](http://www.invitrogen.com)) or call Technical Support (see page 78).

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## Preparing Samples

You will need to prepare Breaking Buffer (see page 59) and have acid-washed 0.5 mm glass beads on hand.

### Preparing cell pellets (Intracellular and Secreted Expression):

1. Thaw cell pellets quickly and place on ice.
  2. For each 1 ml sample, add 100  $\mu$ l Breaking Buffer to the cell pellet and resuspend.
  3. Add an equal volume of acid-washed glass beads (size 0.5 mm). Estimate equal volume by displacement.
  4. Vortex 30 seconds, then incubate on ice for 30 seconds. Repeat for a total of 8 cycles.
  5. Centrifuge at maximum speed for 10 minutes at 4°C. Transfer the clear supernatant to a fresh microcentrifuge tube.
  6. Take 50  $\mu$ l of supernatant (cell lysate) and mix with 50  $\mu$ l 2X SDS-PAGE Gel Loading buffer (Sample Buffer).
  7. Boil for 10 minutes and load 10–20  $\mu$ l per well. Thickness of the gel and number of wells will determine the volume you load. You may store the remaining sample at –20°C for Western blots, if necessary. You may store the cell lysates at –80°C for further analysis.
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*Continued on next page*

# Analysis by SDS-Polyacrylamide Gel Electrophoresis, continued

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## Preparing Samples, continued

### Preparing supernatant (Secreted Expression only):

1. Thaw supernatants and place on ice.
  2. Mix 50  $\mu\text{l}$  of the supernatant with 50  $\mu\text{l}$  of SDS-PAGE Gel Loading buffer.
  3. Boil 10 minutes, then load 10–30  $\mu\text{l}$  onto the gel. You may store the remaining sample at  $-20^{\circ}\text{C}$  for Western blots, if necessary. You may store the supernatants at  $-80^{\circ}\text{C}$  for further analysis.
  4. If you do not see any protein by Coomassie or by Western blot, then concentrate the supernatant 5–10 fold and analyze samples again by Western blot. Centricon and Centriprep filters (Amicon) are very useful for this purpose.
- 

## Concentrating Protein

You may perform Lowry, BCA (Pierce) or Bradford protein assays to quantify the amounts of protein in the cell lysates and medium supernatants. In general, *Pichia* cell lysates contain 5–10  $\mu\text{g}/\mu\text{l}$  protein. *Pichia* medium supernatants will vary in protein concentration primarily due to the amount of your secreted protein. *Pichia* secretes very few native proteins. If the protein concentration of the medium is  $> 50 \mu\text{g}/\text{ml}$ , 10  $\mu\text{l}$  of medium will give a faint band on a Coomassie-stained SDS-PAGE gel.

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## Controls

Include the following samples as controls on your SDS-PAGE:

- Molecular weight standards appropriate for your desired protein
  - A sample of your protein as a standard (if available)
  - A sample of X-33, GS115, or KM71H with the parent plasmid transformed into it. This shows the background of native *Pichia* proteins that are present intracellularly. Inclusion of this sample will help you differentiate your protein from background if you express it intracellularly.
  - Analyze the GS115/pPICZ/*lacZ* and Albumin controls also as they should indicate any problems with the media or expression conditions.
- 



**In addition to Coomassie-stained SDS-PAGE, we strongly recommend performing a Western blot or another more sensitive assay to detect your protein.** Visualization of the expressed protein will depend on several factors including its expression level, its solubility, its molecular weight, and whether it will be masked by an abundant cellular protein of the same size. Western blot analysis, enzymatic activities, or a defined purification profile, if available, may help to identify the expressed protein among the native *Pichia* cellular proteins.

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*Continued on next page*

## Analysis by SDS-Polyacrylamide Gel Electrophoresis, continued

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### Western Blot Analysis

To detect expression of your recombinant fusion protein by Western blot analysis, you may use Anti-myc or Anti-His(C-term) antibodies available from Invitrogen (see page viii) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Cat. no. R900-50) is available for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine tag. The ready-to-use WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our website ([www.invitrogen.com](http://www.invitrogen.com)) or call Technical Support (see page 78).

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### Analyzing Protein Expression

Inspection of your Coomassie-stained SDS-PAGE should reveal the induction over time of your protein co-migrating with your standard. If you are satisfied with the level of expression, try a test purification (page 50) or scale-up expression (page 47).

If there is no recombinant protein visible, then perform either a Western blot or a functional assay if available.

If you detect low expression of your recombinant protein, see **Optimizing *Pichia* Protein Expression**, page 45, for guidelines to optimize expression.

Test your expression conditions with the one of the two control strains included in the kit (GS115/pPICZ/*lacZ* or GS115/Albumin).

If there is no indication of expression at all, use PCR to analyze your recombinants for the correctly sized PCR product (page 69). If you find that you have recombinants, perform a Northern analysis to see if and how much full-length mRNA is induced. See page 75 for an RNA isolation protocol.

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# Optimizing *Pichia* Protein Expression

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## Introduction

Based on available data, there is approximately a 50 to 75% chance of expressing your protein of interest in *Pichia pastoris* at reasonable levels. The biggest hurdle seems to be generating initial success--*i.e.*, expression of your protein at **any** level. While there are relatively few examples of expression of >10 grams/liter, there are many examples of expression in the >1 gram/liter range, making the *Pichia pastoris* expression system one of the most productive eukaryotic expression systems available. Likewise, there are several examples of proteins that have been successfully expressed in *Pichia pastoris* that were completely unsuccessful in baculovirus or *Saccharomyces cerevisiae*, suggesting that the *Pichia pastoris* system is an important alternative to have available. If you obtain no or low protein expression in your initial expression experiment, use the following guidelines to optimize expression.

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## Proteolysis or Degradation

- Do a time course study of expression. Check to see if there is a time point that yields a larger percentage of full-length protein.
  - If secreting your protein, check to see if your protein is susceptible to neutral pH proteases by expressing in unbuffered medium (MMH). In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.
- 

## Low Secreted Expression Levels

- Check cell pellet to see if overall expression is low or if the protein did not secrete. If it did not secrete, try a different signal sequence (*e.g.*, a native or  $\alpha$ -factor signal sequence).
  - Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration (see page 49).
  - For Mut<sup>+</sup>, induce expression with a higher density culture.
- 

## Low Expression Levels

- Look for multi-copy recombinants (*i.e.*, jackpot clones) by dot blot (see page 73). There are quite a few examples of increasing the expression levels of a particular protein by increasing the gene dosage. See (Clare *et al.*, 1991a; Clare *et al.*, 1991b; Romanos *et al.*, 1991).
  - Check both Mut<sup>+</sup> and Mut<sup>S</sup> recombinants for increased expression. Some proteins express better in one type of genetic background than another.
  - If secreting your protein, try intracellular expression. The protein may not be processed correctly and fail to secrete. Be sure you check your cell pellets for evidence of expression. If you are having problems with intracellular expression, try secreting your protein. It probably will glycosylate, which may be desirable or not. If glycosylation is undesirable, oligosaccharides can be removed with Peptide:N-Glycosidase F (New England BioLabs).
  - Scale up to fermentation (page 49). *Pichia* is a yeast, and is particularly well suited to growth in a fermentor.
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*Continued on next page*

## Optimization of *Pichia* Protein Expression, continued

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### No Expression

Be sure to try some of the easier things listed above as no expression can be the same thing as very low expression. If none of these things improve protein expression, use PCR to check for insertion of your gene into the *Pichia* genome (page 68). If your gene is present, perform a Northern blot analysis to check for transcription of your gene. There is a protocol in the **Appendix** for RNA isolation from *Pichia* (see page 75).

If you see premature transcriptional termination, check the AT content of your gene. In *Saccharomyces*, there are a few consensus sequences which promote premature termination. One of these, TTTTATA, resembles a sequence in HIV-1 gp120, ATTATTTTAT AAA, which prematurely terminates mRNA when expressed in *Pichia*. When this sequence was changed, longer transcripts were found (Scorer *et al.*, 1993).

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### Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression as your protein will not go through the secretion pathway and therefore, not be modified.
  - Try deglycosylating the protein with Peptide:N-Glycosidase F or other enzymes (see page 52).
-

## Scale-up of Expression

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### Guidelines for Expression

Once expression is optimized, you will want to scale-up your expression protocol to produce more protein. This may be done by increasing the culture volume using larger baffled flasks (below) or fermentation. Use the guidelines below to scale-up your expression protocol. To purify your protein, see page 50.

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### Mut<sup>+</sup> Intracellular or Secreted

1. Using a single colony, inoculate 25 ml of MGYH, BMGH, or BMGY in a 250 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until culture reaches an  $OD_{600} = 2-6$  (approximately 16–18 hours).
2. Use this 25 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth ( $OD_{600} = 2-6$ ).
3. Harvest the cells using sterile centrifuge bottles by centrifuging at  $1500-3000 \times g$  for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet to an  $OD_{600} = 1.0$  (2–6 liters) in MMH, BMMH, or BMMY medium to start induction.
4. Aliquot the culture between several 3 or 4 liter baffled flask. Cover the flasks with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28–30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached as determined from the time course study.
6. Harvest cells by centrifuging at  $1,500-3,000 \times g$  for 5 minutes at room temperature.

For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.

For secreted expression, **save the supernatant, chill to 4°C, and concentrate it down if desired** (see page 49). Proceed directly to purification (page 50) or store the supernatant at –80°C until ready to process further.

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## Scale-up of Expression, continued

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### Mut<sup>S</sup> Intracellular or Secreted

1. Using a single colony, inoculate 10 ml of MGYH, BMGH, or BMGY in a 100 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD<sub>600</sub> = 2–6 (approximately 16–18 hours).
2. Use this 10 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD<sub>600</sub> = 2–6).
3. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet in 1/5 to 1/10 of the original culture volume of MMH, BMMH, or BMMY medium (approximately 100–200 ml).
4. Place the culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28–30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached.
6. Harvest cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature.

For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.

For secreted expression, **save the supernatant, chill to 4°C, and concentrate it down if desired** (see next page). Proceed directly to purification (page 50) or store the supernatant at –80°C until ready to process further.

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### Note

To increase the amount of cells for Mut<sup>S</sup> recombinants, increase the number of flasks, put 200–300 ml in a 3 liter flask, or try fermentation.

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## Scale-up of Expression, continued

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### Concentration of Proteins

Proteins secreted into the media are usually > 50% homogeneous and will require some additional purification (see page 50 or 52). It is optimal to concentrate the protein if the expression level is not particularly high. There are several general methods to concentrate proteins secreted from *Pichia*. These general methods include:

- Ammonium sulfate precipitation
- Dialysis
- Centrifuge concentrator for small volumes (*e.g.*, Centricon or Centriprep devices available from Amicon)
- Pressurized cell concentrators for large volumes (Amicon ultrafiltration devices)
- Lyophilization

A general guide to protein techniques is *Protein Methods* (Bollag and Edelstein, 1991).

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### Cell Lysis

A general procedure for cell lysis using glass beads is provided on the next page. There is also a cell lysis protocol in *Current Protocols in Molecular Biology*, page 13.13.4. (Ausubel *et al.*, 1994) and in *Guide to Protein Purification* (Deutscher, 1990). We also recommend lysis by French Press (follow the manufacturer's suggestions for yeast).

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### Fermentation

Basic guidelines are available for fermentation of *Pichia* from Invitrogen. We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Contact Technical Support (see page 78) for more information.

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# Purification

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## Introduction

In this section, you will grow and induce a 10–200 ml culture of your *Pichia* transformant for trial purification on a metal-chelating resin such as ProBond™. You may harvest the cells and store them at –80°C until you are ready to purify your fusion protein, or you may proceed directly with protein purification. **Note that this section only describes preparation of cell lysates and sample application onto ProBond™.** For instructions on how to prepare and use ProBond™ resin, refer to the ProBond™ Purification System manual.

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## ProBond™ Resin

We recommend that you use the ProBond™ Purification System (Cat. no. K850-01) for purifying fusion proteins expressed from pPICZ or pPICZα. **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification System.**

If you are using a metal-chelating resin other than ProBond™, follow the manufacturer's recommendations for fusion proteins expressed in bacteria or yeast.

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## Binding Capacity of ProBond™

One milliliter of ProBond™ resin binds at least 1 mg of recombinant protein. This amount can vary depending on the nature of the protein.

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### Important

Throughout the following protocol, be sure to keep the cell lysate and fractions on ice. Small-scale purifications using the 2 ml ProBond™ columns and buffers can be performed at room temperature on the bench top. For large scale purifications, all reagents must be at 4°C.

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## Preparing Cell Lysates

Express your protein using a small-scale culture (10–20 ml for Mut<sup>S</sup> strains; 100–200 ml for Mut<sup>+</sup>) and the optimal conditions for expression (if determined). Refer to the *Pichia* Expression Kit manual for details. Once your protein is expressed, follow the protocol below to prepare a cell lysate for chromatography on ProBond™.

Prepare Breaking Buffer (BB) as described in the **Recipes**, page 59.

1. Wash cells once in BB by resuspending them and centrifuging 5–10 minutes at 3,000 × g at 4°C.
  2. Resuspend the cells to an OD<sub>600</sub> of 50–100 in BB.
  3. Add an equal volume of acid-washed glass beads (0.5 mm). Estimate volume by displacement.
  4. Vortex the mixture for 30 seconds, then incubate on ice for 30 seconds. Repeat 7 more times. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of your protein.
  5. Centrifuge the sample at 4°C for 5–10 minutes at 12,000 × g.
  6. Transfer the clear supernatant to a fresh container and analyze for your protein. The total protein concentration should be around 2–3 mg/ml.
  7. Save the pellet and extract with 6 M urea or 1% Triton® X-100 to check for insoluble protein.
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## Purification, continued

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### Sample Application (Native Conditions)

For sample application onto ProBond™, you will need Native Binding Buffer, pH 7.8 and a 2 ml ProBond™ column, pre-equilibrated using native conditions.

1. Combine 1 ml (2–3 mg/ml total protein) of *Pichia* lysate with 7 ml Native Binding Buffer.
  2. Take a pre-equilibrated ProBond™ column and resuspend the resin in 4 ml of the diluted lysate from Step 1.
  3. Seal the column and batch-bind by rocking gently at room temperature for 10 minutes.
  4. Let the resin settle by gravity or low speed centrifugation (800 × g) and carefully remove the supernatant. Save the supernatant to check for unbound protein.
  5. Repeat Steps 2 through 4 with the remaining 4 ml of diluted lysate. Proceed to **Column Washing and Elution Under Native Conditions** in the ProBond™ Purification manual. Use the recommendations noted for bacterial cell lysates.
- 

### Sample Application (Denaturing Conditions)

Use the protocol above except pre-equilibrate the ProBond™ column using Denaturing Binding Buffer and combine 1 ml of the *Pichia* cell lysate with 7 ml of the Denaturing Binding Buffer.

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#### Note

We have observed some *Pichia* proteins may be retained on the ProBond™ column using native purification conditions. Optimization of the purification or using denaturing purification may remove these non-specific *Pichia* proteins (see ProBond™ Purification System manual).

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### Analysis of Purification

Be sure to save all fractions, washes, and flow-through for analysis by SDS-PAGE. You may need to use Western blot analysis to detect your protein if expression is low or not enough protein was loaded onto the column. Refer to the ProBond™ Purification System manual for a guide to troubleshoot chromatography.

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### Scale-up

You may find it necessary to scale-up your purification to obtain sufficient amounts of purified protein. Adjust the pH and NaCl concentration of your lysate with 1/10 volume of 10X Stock Solution B (ProBond™ Purification System) before adding it to the column. The pH should be greater than or equal to 7.5 and the NaCl concentration should be ~500 mM. Using 10X Stock Solution B to adjust the pH and the ionic strength keeps the total volume small for sample application.

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# Protein Glycosylation

## Analyzing Glycoproteins

When expressing and purifying a glycosylated protein in a heterologous expression system, it is desirable to quickly determine whether the protein is glycosylated properly. There are published protocols for carbohydrate analysis of proteins to allow the molecular biologist to characterize glycosylated proteins of interest (Ausubel *et al.*, 1994), Unit 17. Further information about glycosylation in eukaryotes is available in a review by Varki and Freeze (Varki and Freeze, 1994).

## Enzymes for Analyzing Glycoproteins

These are just a few of the enzymes available for carbohydrate analysis. Abbreviations are as follows: Asn - Asparagine, Gal - Galactose, GlcNAc - N-acetylglucosamine, GalNAc - N-acetylgalactosamine, and NeuAc - N-acetylneuraminic acid.

Enzyme	Type of enzyme	Specificity
Endoglycosidase D	Endo	Cleaves various high mannose glycans
Endoglycosidase F	Endo	Cleaves various high mannose glycans
Endoglycosidase H	Endo	Cleaves various high mannose glycans
$\beta$ -galactosidase	Exo	Removes terminal galactosides from Gal- $\beta$ 1,3-GlcNAc, Gal- $\beta$ 1,4-GlcNAc or Gal- $\beta$ 1,3 GalNAc.
Peptide:N-Glycosidase F	Endo	Glycoproteins between Asn and GlcNAc (removes oligosaccharides)
Sialidases (Neuraminidases) <i>Vibrio cholerae</i> <i>Clostridium perfringens</i> <i>Arthobacter ureafaciens</i> Newcastle disease virus	Exo	NeuAc- $\alpha$ 2,6-Gal, NeuAc- $\alpha$ 2,6-GlcNAc or NeuAc- $\alpha$ 2,3-Gal

## Commercial Carbohydrate Analysis

There are a number of commercial vendors who will contract to analyze proteins for glycosylation. A number of companies also supply kits and reagents for researchers to do carbohydrate analysis in their own laboratories. A partial list is provided below:

Company	Type of Service	Phone Number
Glyko	Kits for Carbohydrate Analysis, Reagents, Contract Services	1-800-334-5956
Oxford GlycoSystems	Kits for Carbohydrate Analysis, Reagents, Contract Services	1-800-722-2597
New England BioLabs	Reagents	1-800-632-5227

## Appendix

### *E. coli* Media Recipes

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#### Low Salt LB (Luria-Bertani) Medium

Low Salt LB medium is needed for use with the Zeocin™ antibiotic. Note that you may substitute Low Salt LB for regular LB for most applications.

1% Tryptone  
0.5% Yeast Extract  
**0.5% NaCl**  
pH 7.5

1. For 1 liter, dissolve the following in 950 ml deionized water:  
10 g tryptone  
5 g yeast extract  
**5 g NaCl**
  2. Adjust the pH of the solution to 7.5 with 1 N NaOH and bring the volume up to 1 liter.
  3. Autoclave for 20 minutes at 15 lb/sq. in and 121°C. Let cool to ~55°C and add desired antibiotics at this point. For Low Salt LB medium with Zeocin™, add Zeocin™ to 25 µg/ml final concentration.
  4. Store at 4°C. If you have added Zeocin™, store medium **in the dark**.
- 

#### Low Salt LB Agar Plates

1. Make Low Salt LB Medium above and add 15 g/liter agar before autoclaving.
  2. Autoclave for 20 minutes at 15 lb/sq. in.
  3. Let cool to ~55°C and add desired antibiotics at this point. For Low Salt LB plates with Zeocin™, add Zeocin™ to 25 µg/ml final concentration.
  4. Pour into 10 cm petri plates. Let the plates harden, then invert, and store at 4°C. If you have added Zeocin™, store plates **in the dark**. Plates containing Zeocin™ are stable for 1–2 weeks.
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# Pichia Media Recipes

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## Introduction

The expression of recombinant proteins in *Pichia pastoris* requires the preparation of several different media. Recipes for these media are included in this section. In addition, Yeast Nitrogen Base is available from Invitrogen (see below for ordering information).

Item	Amount	Cat. no.
Yeast Nitrogen Base	67 g pouch	Q300-07
-with ammonium sulfate	Each pouch contains reagents to prepare 500 ml of a 10X YNB solution	
-without amino acids		
	500 g	Q300-09

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## Stock Solutions

### 10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C. Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate. The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch.

**Note:** *Pichia* cells exhibit optimal growth with higher YNB concentrations; therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for *Saccharomyces*.

### 500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C. The shelf life of this solution is approximately one year.

### 100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilize and store at 4°C. The shelf life of this solution is approximately one year.

### 10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

### 10X M (5% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filter sterilize and store at 4°C. The shelf life of this solution is approximately two months.

### 10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

### 1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1 M  $K_2HPO_4$ , 868 ml of 1 M  $KH_2PO_4$  and confirm that the pH =  $6.0 \pm 0.1$  (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

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## Pichia Media Recipes, continued

### Using *Pichia* Media

The table below is designed to help you decide which *Pichia* medium to use for a particular application.

Medium	Description	Application
YPD or YEPD	Rich, complex broth	General growth and storage
YPDS + Zeocin™	YPD with sorbitol and Zeocin™	Selection of <i>Pichia</i> Zeo <sup>R</sup> transformants
MGYH or MGY	Minimal medium containing glycerol and/or histidine	Intracellular Expression: Generation of biomass prior to methanol induction
MDH or MD	Minimal medium containing glucose and/or histidine	Determination of Mut phenotype
MMH or MM	Minimal medium containing methanol and/or histidine	Determination of Mut phenotype Intracellular expression of desired protein
BMGH or BMG	Buffered minimal medium containing glycerol and/or histidine	Secreted Expression: Used to control the pH of the medium and generate biomass
BMMH or BMM	Buffered minimal medium containing methanol and/or histidine	Secreted Expression: Used to control the pH of the medium and induce expression of the desired protein
BMGY	Buffered complex medium containing glycerol	Secreted Expression: Used to control the pH of the medium, decrease protease activity, and generate biomass.
BMMY	Buffered complex medium containing methanol	Secreted Expression: Used to control the pH of the medium, decrease protease activity, and induce expression.

### YPD or YEPD

#### Yeast Extract Peptone Dextrose Medium (1 liter)

- 1% yeast extract
- 2% peptone
- 2% dextrose (glucose)

**Note:** If you are using the YP Base Medium or the YP Base Agar medium pouches included with the Original *Pichia* Expression Kit, follow the directions on the pouch.

1. Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water.  
**Note:** Add 20 g of agar if making YPD slants or plates.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 ml of 10X D.

The liquid medium is stored at room temperature. Store YPD slants or plates are at 4°C. The shelf life is several months.

*Continued on next page*

## ***Pichia* Media Recipes, continued**

---

### **YPD (+ Zeocin™)**

#### **Yeast Extract Peptone Dextrose Medium (1 liter)**

1% yeast extract  
2% peptone  
2% dextrose (glucose)  
± 2% agar  
± 100 µg/ml Zeocin™

1. Dissolve the following in 900 ml of water:  
10 g yeast extract  
20 g of peptone
2. Include 20 g of agar if making YPD slants or plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Cool solution to ~60°C and add 100 ml of 10X D. Add 1.0 ml of 100 mg/ml Zeocin™, if desired.

You can store liquid medium without Zeocin at room temperature. Store medium containing Zeocin™ at 4°C **in the dark**. Store YPD slants or plates at 4°C. The shelf life of medium is several months. Medium containing Zeocin™ has a shelf life of one to two weeks.

---

### **YPDS + Zeocin™ Agar**

#### **Yeast Extract Peptone Dextrose Medium (1 liter)**

1% yeast extract  
2% peptone  
2% dextrose (glucose)  
1 M sorbitol  
2% agar  
100 µg/ml Zeocin™

1. Dissolve the following in 900 ml of water:  
10 g yeast extract  
182.2 g sorbitol  
20 g of peptone
2. Add 20 g of agar.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 ml of 10X D
5. Cool solution to ~60°C and add 1.0 ml of 100 mg/ml Zeocin™.

Store YPDS slants or plates containing Zeocin™ at 4°C, **in the dark**. The shelf life is one to two weeks.

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*Continued on next page*



## ***Pichia* Media Recipes, continued**

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### **MGY and MGYH**

#### **Minimal Glycerol Medium $\pm$ Histidine (1 liter)**

1.34% YNB  
1% glycerol  
 $4 \times 10^{-5}$ % biotin  
 $\pm$  0.004% histidine

1. Combine aseptically 800 ml autoclaved water with 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X GY.
2. For growth of *his4* strains in this medium, a version can be made that contains histidine (called MGYH) by adding 10 ml of 100X H stock solution.

Store at 4°C. The shelf life of this solution is approximately two months.

---

### **MD and MDH**

#### **Minimal Dextrose Medium $\pm$ Histidine (1 liter)**

1.34% YNB  
 $4 \times 10^{-5}$ % biotin  
2% dextrose

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
  2. Cool to about 60°C and then add:  
100 ml of 10X YNB  
2 ml of 500X B  
100 ml of 10X D
  3. To make MDH, add 10 ml of 100X H stock solution. Mix and store at 4°C.
  4. For plates, add 15 g agar to the water in Step 1 and proceed.
  5. If preparing plates, pour the plates immediately. MD stores well for several months at 4°C.
- 

### **MM and MMH**

#### **Minimal Methanol $\pm$ Histidine (1 liter)**

1.34% YNB  
 $4 \times 10^{-5}$ % biotin  
0.5% methanol

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle
  2. Cool autoclaved water to 60°C and add:  
100 ml of 10X YNB  
2 ml of 500X B  
100 ml of 10X M
  3. To make MMH, add 10 ml of 100X H stock solution. Mix and store at 4°C.
  4. For plates, add 15 g agar to the water in Step 1 and proceed.
  5. After mixing, pour the plates immediately. MM and MMH stores well for several months at 4°C.
- 

*Continued on next page*

## ***Pichia* Media Recipes, continued**

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### **BMGH and BMMH**

#### **Buffered Minimal Glycerol Buffered Minimal Methanol (1 liter)**

100 mM potassium phosphate, pH 6.0  
1.34% YNB  
 $4 \times 10^{-5}$ % biotin  
1% glycerol or 0.5% methanol

1. Autoclave 690 ml water for 20 minutes on liquid cycle.
  2. Cool to room temperature, then add the following and mix well:  
100 ml 1 M potassium phosphate buffer, pH 6.0  
100 ml 10X YNB  
2 ml 500X B  
100 ml 10X GY
  4. For BMMH, add 100 ml 10X M instead of glycerol.
  5. To add histidine, add 10 ml of 100X H stock solution. Mix and store at 4°C.
  6. Store media at 4°C. The shelf life of this solution is approximately two months.
- 

### **BMGY and BMMY**

#### **Buffered Glycerol-complex Medium Buffered Methanol-complex Medium (1 liter)**

1% yeast extract  
2% peptone  
100 mM potassium phosphate, pH 6.0  
1.34% YNB  
 $4 \times 10^{-5}$ % biotin  
1% glycerol or 0.5% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
  2. Autoclave 20 minutes on liquid cycle.
  3. Cool to room temperature, then add the following and mix well:  
100 ml 1 M potassium phosphate buffer, pH 6.0  
100 ml 10X YNB  
2 ml 500X B  
100 ml 10X GY
  4. For BMMY, add 100 ml 10X M instead of glycerol.
  5. Store media at 4°C. The shelf life of this solution is approximately two months.
- 

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## ***Pichia* Media Recipes, continued**

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### **Breaking Buffer**

50 mM sodium phosphate, pH 7.4  
1 mM PMSF (phenylmethylsulfonyl fluoride or other protease inhibitors)  
1 mM EDTA  
5% glycerol

1. Prepare a stock solution of your desired protease inhibitors and store appropriately. Follow manufacturer's recommendations.
  2. For 1 liter, dissolve the following in 900 ml deionized water:  
6 g sodium phosphate (monobasic)  
372 mg EDTA  
50 ml glycerol
  3. Use NaOH to adjust pH and bring up the volume to 1 liter. Store at 4°C.
  4. Right before use, add the protease inhibitors.
- 

### **Vendors for Baffled Flasks**

Bellco (1-800-257-7043) has a wide variety of baffled flasks from 50 to 2000 ml.  
Wheaton (1-609-825-1100) only sells side baffle flasks.

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## Proteins Expressed in *Pichia*

**Table**

The table below provides a partial list of references documenting successful expression of heterologous proteins in *Pichia pastoris*. Note that both Mut<sup>+</sup> and Mut<sup>s</sup> phenotypes were used successfully as well as secreted and intracellular expression.

Protein	Expression Levels (grams/liter)	Where Expressed How Expressed	Reference
<b>Enzymes</b>			
Invertase	2.3	Secreted Mut <sup>+</sup>	(Tschopp <i>et al.</i> , 1987b)
Bovine Lysozyme c2	0.55	Secreted Mut <sup>+</sup>	(Digan <i>et al.</i> , 1989)
Streptokinase (active)	0.08	Intracellular *	(Hagenson <i>et al.</i> , 1989)
Alpha amylase	2.5	Secreted Mut <sup>s</sup>	(Paifer <i>et al.</i> , 1994)
Pectate Lyase	0.004	Secreted Mut <sup>s</sup>	(Guo <i>et al.</i> , 1995)
Spinach Phospho-ribulokinase	0.1	Intracellular Mut <sup>s</sup>	(Brandes <i>et al.</i> , 1996)
<b>Antigens</b>			
Hepatitis B surface antigen	0.4	Intracellular Mut <sup>s</sup>	(Cregg <i>et al.</i> , 1987)
Pertussis Antigen P69	3.0	Intracellular Mut <sup>s</sup>	(Romanos <i>et al.</i> , 1991)
Tetanus Toxin Fragment C	12.0	Intracellular Mut <sup>+</sup> /Mut <sup>s</sup>	(Clare <i>et al.</i> , 1991a)
HIV-1 gp120	1.25	Intracellular Mut <sup>+</sup>	(Scorer <i>et al.</i> , 1993)
Tick Anticoagulant protein	1.7	Secreted Mut <sup>s</sup>	(Laroche <i>et al.</i> , 1994)
Bm86 Tick Gut Glycoprotein	1.5	Secreted *	(Rodriguez <i>et al.</i> , 1994)

*Continued on next page*

## Proteins Expressed in *Pichia*, continued

### Proteins Expressed in *Pichia*, continued

Protein	Expression Levels (grams/liter)	Where Expressed How Expressed	Reference
<b>Regulatory Proteins</b>			
Tumor Necrosis Factor (TNF)	10.0	Intracellular Mut <sup>s</sup>	(Sreekrishna <i>et al.</i> , 1989)
Mouse Epidermal Growth Factor (EGF)	0.45	Secreted Mut <sup>s</sup>	(Clare <i>et al.</i> , 1991b)
Human Interferon (IFN) $\alpha$ 2b	0.4	Intracellular Mut <sup>s</sup>	(Garcia <i>et al.</i> , 1995)
<b>Membrane Proteins</b>			
Human CD38 (soluble portion)	0.05	Secreted Mut <sup>s</sup>	(Fryxell <i>et al.</i> , 1995)
Mouse Serotonin Receptor	0.001	Secreted Mut <sup>+</sup>	(Weiss <i>et al.</i> , 1995)
<b>Proteases and Protease Inhibitors</b>			
Carboxypeptidase B	0.8	Secreted Mut <sup>+</sup> /Mut <sup>s</sup>	(Despreaux and Manning, 1993)
Enterokinase	0.021	Secreted Mut <sup>+</sup>	(Vozza <i>et al.</i> , 1996)
Ghilanten	0.01	Secreted Mut <sup>+</sup>	(Brankamp <i>et al.</i> , 1995)
Kunitz protease inhibitor	1.0	Secreted *	(Wagner <i>et al.</i> , 1992)
Human Proteinase Inhibitor 6	0.05	Intracellular Mut <sup>+</sup>	(Sun <i>et al.</i> , 1995)
<b>Antibodies</b>			
Rabbit Single Chain Antibody	>0.1	Secreted Mut <sup>s</sup>	(Ridder <i>et al.</i> , 1995)

\* Mut phenotype was not described in the paper.

# Recombination and Integration in *Pichia*

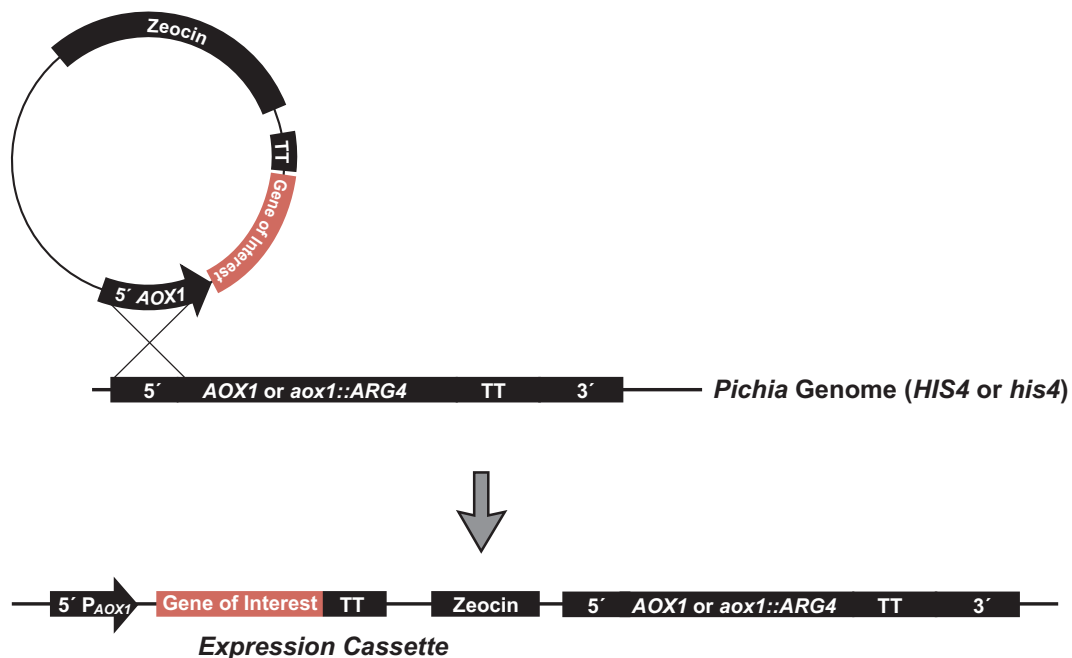
## Introduction

Like *Saccharomyces cerevisiae*, linear DNA can generate stable transformants of *Pichia pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome (Cregg *et al.*, 1985; Cregg *et al.*, 1989). Such integrants show extreme stability in the absence of selective pressure even when present as multiple copies. Note that single crossover events (insertions) are much more likely to happen than double crossover events (replacements). Multiple insertion events occur spontaneously at about 1–10% of the single insertion events.

## Gene Insertion at AOX1 or aox1::ARG4

Gene insertion events at the *AOX1* (X-33 or GS115) or *aox1::ARG4* (KM71H) loci arise from a single crossover event between the loci and either of the two *AOX1* regions on the pPICZ or pPICZ $\alpha$  vectors: the *AOX1* promoter or the *AOX1* transcription termination region (TT). This results in the insertion of one or more copies of the vector upstream or downstream of the *AOX1* or the *aox1::ARG4* genes. The phenotype of such a transformant is Mut<sup>+</sup> (X-33 or GS115) or Mut<sup>S</sup> (KM71H). **By linearizing the recombinant vector at a restriction enzyme site located in the 5' *AOX1* regions, Mut<sup>+</sup> or Mut<sup>S</sup> recombinants can be conveniently generated depending on the host strain used.**

The figure below shows the result of an insertion of the plasmid 5' to the intact *AOX1* locus (Mut<sup>+</sup>) and the gain of P<sub>AOX1</sub>, your gene of interest, and the Zeocin<sup>™</sup> resistance gene. This also occurs with non-linearized plasmid and plasmid that religates, although at a lower frequency.



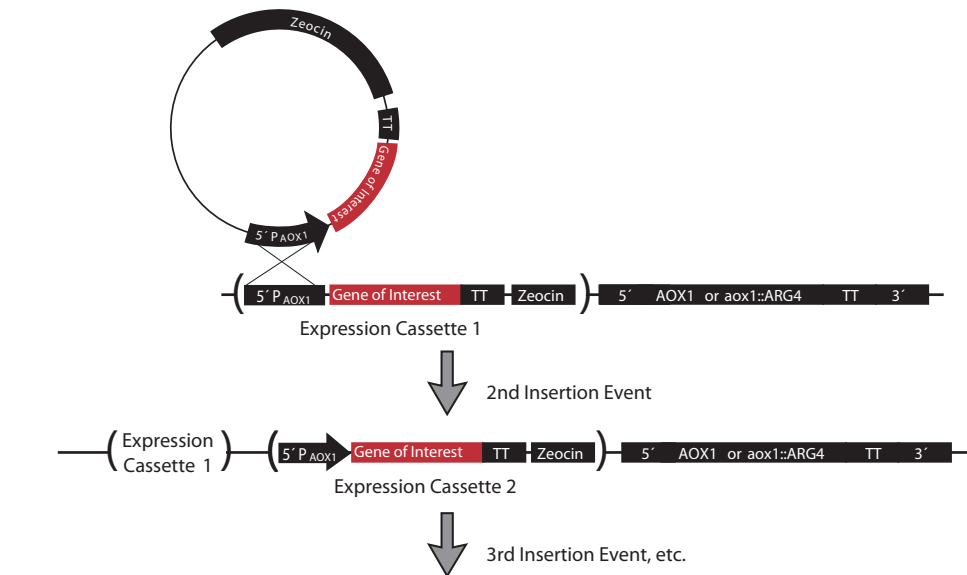
Continued on next page

# Recombination and Integration in *Pichia*, continued

## Multiple Gene Insertion Events

Multiple gene insertion events at a single locus in a cell do occur spontaneously with a low, but detectable frequency--between 1 and 10% of all selected Zeo<sup>R</sup> transformants. Because of the low frequency of multiple gene insertion events, you will need to screen hundreds to thousands of Zeocin<sup>TM</sup>-resistant transformants to locate these "jack-pot" clones. We recommend that you use electroporation to generate Zeo<sup>R</sup> transformants for screening.

Multi-copy events can occur as gene insertions either at the *AOX1* or the *aox1::ARG4* loci. This results in a Mut<sup>+</sup> phenotype in X-33 or GS115 and a Mut<sup>S</sup> phenotype in KM71H. Multiple gene insertion events can be detected by quantitative dot blot analysis, Southern blot analysis, and differential hybridization. See page 73 for a protocol to screen for multiple inserts.



# Lithium Chloride Transformation Method

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## Introduction

This is a modified version of the procedure described for *S. cerevisiae* (Gietz and Schiestl, 1996). This protocol is provided as an alternative to transformation by electroporation. Transformation efficiency is between  $10^2$  to  $10^3$  cfu/ $\mu$ g linearized DNA.

---

## Preparation of Solutions

**Lithium acetate does not work with *Pichia pastoris*. Use only lithium chloride.**

1 M LiCl in distilled, deionized water. Filter sterilize. Dilute as needed with sterile water.

50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter sterilize. Store in a tightly capped bottle.

2 mg/ml denatured, fragmented salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at  $-20^{\circ}\text{C}$ .

---

## Preparing Cells

1. Grow a 50 ml culture of *Pichia pastoris* in YPD at  $30^{\circ}\text{C}$  with shaking to an  $\text{OD}_{600}$  of 0.8 to 1.0 (approximately  $10^8$  cells/ml).
  2. Harvest the cells and wash with 25 ml of sterile water and centrifuge at  $1,500 \times g$  for 10 minutes at room temperature.
  3. Decant the water and resuspend the cells in 1 ml of 100 mM LiCl.
  4. Transfer the cell suspension to a 1.5 ml microcentrifuge tube.
  5. Pellet the cells at maximum speed for 15 seconds and remove the LiCl with a pipet.
  6. Resuspend the cells in 400  $\mu$ l of 100 mM LiCl.
  7. Dispense 50  $\mu$ l of the cell suspension into a 1.5 ml microcentrifuge tube for each transformation and use immediately.  
**Do not store on ice or freeze at  $-20^{\circ}\text{C}$ .**
- 

*Continued on next page*



## Lithium Chloride Transformation Method, continued

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### Transformation

1. Boil a 1 ml sample of single-stranded DNA for five minutes, then quickly chill in ice water. Keep on ice.  
**Note:** It is not necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at  $-20^{\circ}\text{C}$  and boil every 3–4 times the DNA is thawed.
  2. Centrifuge the LiCl-cell solution from Step 7, above, and remove the LiCl with a pipet.
  3. For each transformation sample, add the following reagents **in the order given** to the cells. PEG shields the cells from the detrimental effects of the high concentration of LiCl.  
240  $\mu\text{l}$  50% PEG  
36  $\mu\text{l}$  1 M LiCl  
25  $\mu\text{l}$  2 mg/ml single-stranded DNA  
Plasmid DNA (5–10  $\mu\text{g}$ ) in 50  $\mu\text{l}$  sterile water
  4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
  5. Incubate the tube at  $30^{\circ}\text{C}$  for 30 minutes without shaking.
  6. Heat shock in a water bath at  $42^{\circ}\text{C}$  for 20–25 minutes.
  7. Centrifuge the tubes at 6,000 to 8,000 rpm and remove the transformation solution with a pipet.
  8. Resuspend the pellet in 1 ml of YPD and incubate at  $30^{\circ}\text{C}$  with shaking.
  9. After 1 hour and 4 hours, plate 25  $\mu\text{l}$  to 100  $\mu\text{l}$  on YPD plates containing 100  $\mu\text{g}/\text{ml}$  Zeocin<sup>™</sup>. Incubate the plates for 2–3 days at  $30^{\circ}\text{C}$ . Proceed to **Analysis of *Pichia* Transformants**, page 28.
-

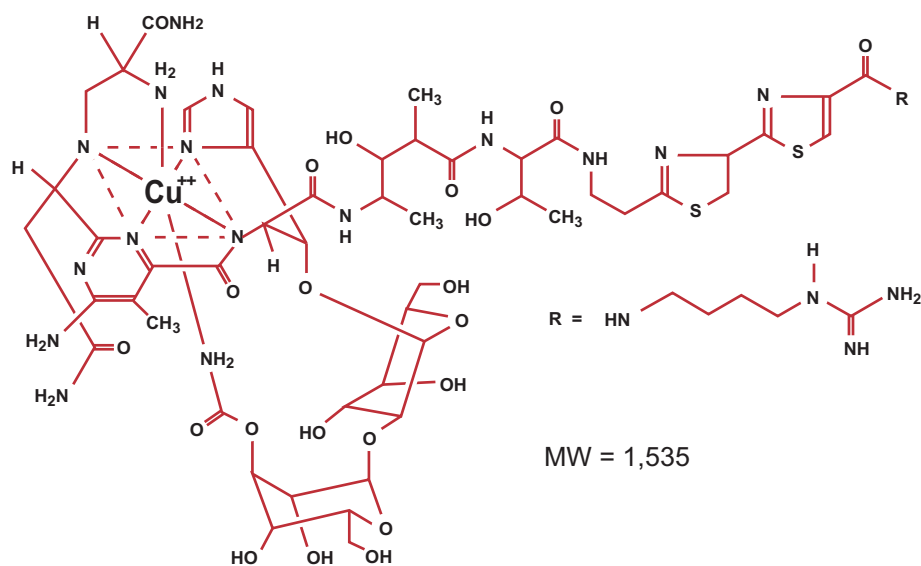
# Zeocin™

## Description

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. Zeocin™ is not as toxic as bleomycin on fungi.

## Chemical Properties

Zeocin™ is a basic, water-soluble compound isolated from *Streptomyces verticillus* as a copper-chelated glycopeptide. The presence of copper gives the solution its blue color. The chemical formula for Zeocin™ is  $C_{55}H_{83}N_{19}O_{21}S_2Cu$ . It contains several unique amino acids, sugars, and aliphatic amines. For general information about the family of bleomycin antibiotics, See Berdy, 1980. The general structure of Zeocin™ is shown below.



## Mechanism of Action

The exact mechanism of action of Zeocin™ is not known; however, it is thought to be the same as bleomycin and phleomycin due to its similarity to these drugs and its inhibition by the *Sh ble* resistance protein (see next section). The copper/glycopeptide complex is selective and involves chelation of copper ( $Cu^{2+}$ ) by the amino group of the  $\alpha$ -carboxamide, single nitrogen atoms of both the pyrimidine chromophore and the imidazole moiety, and the carbamoyl group of mannose. The copper-chelated form is inactive. When the antibiotic enters the cell, the copper cation is reduced from  $Cu^{2+}$  to  $Cu^{1+}$  and removed by sulfhydryl compounds in the cell. Upon removal of the copper, Zeocin™ is activated to bind DNA and cleave it causing cell death (Berdy, 1980). **High salt concentrations and acidity or basicity inactivate Zeocin™; therefore, it is necessary to reduce the salt in bacterial medium to 90 mM (5 g/liter) or less and adjust the pH to 7.5 to make sure the drug remains active.**

*Continued on next page*

## Zeocin™, continued

### Resistance to Zeocin™

A Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1988). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13,665 Da protein that binds Zeocin™ in a stoichiometric manner. The binding of Zeocin™ inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™. The nucleic acid and protein sequence is given below:

Nco I  
ACC ATG GCC AAG TTG ACC AGT GCC GTT CCG GTG CTC  
M A K L T S A V P V L

ACC GCG CGC GAC GTC GCC GGA GCG GTC GAG TTC TGG  
T A R D V A G A V E F W

Sma I  
ACC GAC CGG CTC GGG TTC TCC CGG GAC TTC GTG GAG  
T D R L G F S R D F V E

SgrA I  
GAC GAC TTC GCC GGT GTG GTC CGG GAC GAC GTG ACC  
D D F A G V V R D D V T

CTG TTC ATC AGC GCG GTC CAG GAC CAG GTG GTG CCG  
L F I S A V Q D Q V V P

GAC AAC ACC CTG GCC TGG GTG TGG GTG CGC GGC CTG  
D N T L A W V W V R G L

GAC GAG CTG TAC GCC GAG TGG TCG GAG GTC GTG TCC  
D E L Y A E W S E V V S

ACG AAC TTC CGG GAC GCC TCC GGG CCG GCC ATG ACC  
T N F R D A S G P A M T

GAG ATC GGC GAG CAG CCG TGG GGG CGG GAG TTC GCC  
E I G E Q P W G R E F A

CTG CGC GAC CCG GCC GGC AAC TGC GTG CAC TTC GTG  
L R D P A G N C V H F V

GCC GAG GAG CAG GAC TGA  
A E E Q D \*\*\*

# PCR Analysis of *Pichia* Integrants

---

## Introduction

The following protocol is designed to allow you to analyze *Pichia* integrants to determine if the gene of interest has integrated into the *Pichia* genome. Isolate genomic DNA from 6–10 Mut<sup>S</sup> or Mut<sup>+</sup> *Pichia* clones using the protocol on page 71. Isolate DNA from the strain transformed with the parent plasmid. After isolating your DNA, use the procedure below to identify integrants. Amplify your gene of interest with the  $\alpha$ -factor primer (for pPICZ $\alpha$  only) or 5' the *AOX1* primer paired with the 3' *AOX1* primer included in the kit. This protocol is useful for confirming integration of the gene of interest but will not provide information on the site of integration. A more direct procedure is provided on page 70.

---

## Analysis by PCR

1. Set up PCR reactions as follows:

10X PCR Buffer	5 $\mu$ l
Genomic DNA (~1 $\mu$ g)	5 $\mu$ l
100 mM dNTPs (25 mM each)	1 $\mu$ l
5' <i>AOX1</i> Primer (0.1 $\mu$ g/ $\mu$ l)	5 $\mu$ l*
3' <i>AOX1</i> Primer (0.1 $\mu$ g/ $\mu$ l)	5 $\mu$ l*
Sterile water	29 $\mu$ l
<i>Taq</i> Polymerase (5 U/ $\mu$ l)	0.25 $\mu$ l

\*Resuspend lyophilized primer (2  $\mu$ g) in 20  $\mu$ l sterile water to prepare a 0.1  $\mu$ g/ $\mu$ l solution. The amount of primer may be decreased if desired. For ~20 pmoles primer, use 2  $\mu$ l of each primer.

For amplification controls, use 100 ng of recombinant plasmid (positive control) and 100 ng of the appropriate plasmid without insert (negative control).

2. Load thermocycler and run the following program:

Step	Temperature	Time	Cycle
Heat Soak	94°C	2 minutes	1X
Denaturation	94°C	1 minute	25X
Annealing	55°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1X

3. Analyze 10  $\mu$ l on a 1X TAE, 0.8 % agarose gel.
- 

*Continued on next page*

## PCR Analysis of *Pichia* Integrants, continued

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### Interpreting PCR

If screening Mut<sup>+</sup> integrants, you should see two bands, one corresponding to the size of your gene of interest, the other to the *AOX1* gene (approximately 2.2 kb). In KM71H, the PCR product is 3.6 kb because of the *ARG4* insert in *AOX1*. Parent plasmids will produce the following sized PCR products. Add the size of these products to the size of your insert to interpret your PCR results.

Vector	PCR Product
pPICZ	325 bp (A), 323 bp (B), 324 bp (C)
pPICZ $\alpha$ (using the 5' <i>AOX1</i> primer)	588 bp (A), 592 bp (B), 593 bp (C)
pPICZ $\alpha$ (using the $\alpha$ -Factor primer)	299 bp (A), 303 bp (B), 304 bp (C)

---



### Important

If you use the  $\alpha$ -factor primer as a PCR primer, you will not see a band with either GS115 or KM71H. This is because there is no  $\alpha$ -factor signal associated with the chromosomal *AOX1* gene.

---



### Note

Sometimes there will be ghost bands appearing in your PCR. These do not seem to be significant as they have not been shown to be a problem.

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# Direct PCR Screening of *Pichia* Clones

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## Introduction

A simple protocol has been reported in the literature to directly test *Pichia* clones for insertion of your gene by PCR (Linder *et al.*, 1996). Briefly, the cells are lysed by a combined enzyme, freezing, and heating treatment. The genomic DNA can be used directly as a PCR template.

---

## Before Starting

You will need the following reagents and equipment on hand:

- A culture or single colony of a *Pichia* transformant
  - 1.5 ml microcentrifuge tube
  - 5 U/ $\mu$ l solution of Lyticase (Sigma)
  - 30°C water bath or heat block
  - Liquid nitrogen
  - Reagents for PCR
- 

## Procedure

1. Place 10  $\mu$ l of a *Pichia pastoris* culture into a 1.5 ml microcentrifuge tube. For relatively dense cultures, dilute 1  $\mu$ l of the culture into 9  $\mu$ l water. Alternatively, pick a single colony and resuspend in 10  $\mu$ l of water.
2. Add 5  $\mu$ l of a 5 U/ $\mu$ l solution of lyticase and incubate at 30°C for 10 minutes.
3. Freeze the sample at -80°C for 10 minutes or immerse in liquid nitrogen for 1 minute.

4. Set up a 50  $\mu$ l PCR for a hot start:

10X Reaction Buffer	5 $\mu$ l
25 mM MgCl <sub>2</sub>	5 $\mu$ l
25 mM dNTPs	1 $\mu$ l
5' AOX1 primer (10 pmol/ $\mu$ l)	1 $\mu$ l
3' AOX1 primer (10 pmol/ $\mu$ l)	1 $\mu$ l
Sterile water	27 $\mu$ l
Cell lysate	5 $\mu$ l
Total Volume	45 $\mu$ l

5. Place the solution in the thermocycler and incubate at 95°C for 5 minutes.
6. Add 5  $\mu$ l of a 0.16 U/ $\mu$ l solution of *Taq* polymerase (0.8 units).
7. Cycle 30 times using the following parameters:

Step	Temperature	Time	Cycle
Denaturation	95°C	1 minute	30X
Annealing	54°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1X

8. Analyze a 10  $\mu$ l aliquot by agarose gel electrophoresis.
-

# Isolating Total DNA from *Pichia*

---

## Introduction

The protocol below allows you to isolate DNA from the desired recombinant and the untransformed GS115 or KM71H. The purified DNA is suitable for Southern blot analysis, dot/slot blot analysis or genomic PCR. See *Current Protocols in Molecular Biology*, pages 13.11.1 to 13.11.4 (Ausubel *et al.*, 1994), *Guide to Yeast Genetics and Molecular Biology*, pages 322–323 (Strathern and Higgins, 1991), or (Holm *et al.*, 1986).

---

## Solutions

You will need to make the following solutions. There is not enough of some of these reagents in the kit to perform this experiment.

Minimal Medium (MD, MGYH)

Sterile water

SCED (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT)

Zymolyase, 3 mg/ml stock solution in water (Seikagaku America, Inc.,  
1-800-237-4512)

1% SDS in water

5 M potassium acetate, pH 8.9

TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)

7.5 M ammonium acetate, pH 7.5

Phenol:chloroform (1:1 v/v)

---

## Preparation

1. Grow at 30°C the recombinant strain and the parent strain to an OD<sub>600</sub> of 5–10 in 10 ml of minimal media such as MD or MDH.  
**Note:** *his4* strains require histidine for growth.
  2. Collect the cells by centrifugation at 1,500 × g for 5–10 minutes at room temperature.
  3. Wash the cells with 10 ml sterile water by centrifugation as in Step 2.
- 

## Spheroplasting and Lysis

1. Resuspend the cells in 2 ml of SCED buffer, pH 7.5. Make this solution fresh.
  2. Add 0.1–0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve < 80% spheroplasting.
  3. Add 2 ml of 1% SDS, mix **gently** and set on ice (0 to 4°C) for 5 minutes.
  4. Add 1.5 ml of 5 M potassium acetate, pH 8.9, and mix **gently**.
  5. Centrifuge at 10,000 × g for 5–10 minutes at 4°C and save the supernatant.
- 

*Continued on next page*

## Isolating Total DNA from *Pichia*, continued

---

### DNA Precipitation

1. Transfer the supernatant from Step 5, page 71, and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
  2. Centrifuge at  $10,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ .
  3. Resuspend the pellet **gently** in 0.7 ml of TE buffer, pH 7.4 and transfer to a microcentrifuge tube.
  4. **Gently** extract with an equal volume of phenol:chloroform (1:1 v/v) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Split the aqueous layer into two microcentrifuge tubes.
  5. Add 1/2 volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. Place on dry ice for 10 minutes or at  $-20^{\circ}\text{C}$  for 60 minutes.
  6. Centrifuge at  $10,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$  and wash the pellets once with 1 ml of 70% ethanol. Briefly air dry the pellets and resuspend each one in 50  $\mu\text{l}$  of TE buffer, pH 7.5. Determine the concentration of the DNA sample. You may store the samples separately or combined at  $-20^{\circ}\text{C}$  until ready for use.
-



# Determining the Copy Number of Multiple Integrants

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## Introduction

You may wish to determine the actual number of gene copies in your *Pichia* recombinant. You may either use quantitative dot blots or Southern hybridization to analyze gene copy number (Brierley, *et al.*, 1994; Clare, *et al.*, 1991a; Romanos, *et al.*, 1991; Scorer, *et al.*, 1993; Scorer, *et al.*, 1994). This requires you to isolate genomic DNA from *Pichia* recombinants transformed with the parent vector (0 copies of your gene), pPICZ or pPICZ $\alpha$  containing 1 copy of your gene (single copy control), and the *Pichia* recombinants containing multiple copies of your gene. Use the protocol detailed on pages 71–72 to isolate genomic DNA.

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## Quantitative Dot Blot Solutions

For each dot blot, you need 10–15 ml of each of the following solutions.

50 mM EDTA, 2.5%  $\beta$ -mercaptoethanol pH 9  
1 mg/ml Zymolyase 100T in water (Seikagaku America, Inc., 1-800-237-4512)  
0.1 N NaOH, 1.5 M NaCl  
2X SSC

You will also need 3MM paper.

---

## Quantitative Dot Blot Procedure

The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos, *et al.*, 1991). It is very important to spot equivalent numbers of cells onto filters to quantify the copy number. Alternatively, you may isolate genomic DNA, spot it directly onto nitrocellulose or nylon, fix, and analyze for copy number.

1. Grow Mut<sup>+</sup> or Mut<sup>s</sup> transformants in individual wells of a 96-well microtiter plate in 200  $\mu$ l of YPD broth at 30°C until all wells have approximately the same density. This may necessitate several passages. Alternatively, you may grow individual transformants in culture tubes and normalize the absorbance at 600 nm by adding medium.
  2. Filter 50  $\mu$ l of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multi-channel pipettor. Air dry filters.
  3. To lyse the cells on the filter, treat the filter with four solutions as follows: place two sheets of 3 MM paper in a tray and soak with 10–15 ml of 50 mM EDTA, 2.5%  $\beta$ -mercaptoethanol pH 9. Make sure that the paper is uniformly soaked and that there are no puddles. Place the nitrocellulose filter face down on the treated 3MM paper. Incubate for 15 minutes at room temperature.
  4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak with 10–15 ml of 1 mg/ml Zymolyase 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate for 4 hours at 37°C.
- 

*Continued on next page*

## Determining the Copy Number of Multiple Integrants, continued

---

### Quantitative Dot Blot Procedure, continued

5. Remove the nitrocellulose filter from the paper and replace the paper with two new sheets. Soak with 10–15 ml of 0.1 N NaOH, 1.5 M NaCl. Place the nitrocellulose filter face down on the paper and incubate for 5 minutes at room temperature.
6. Remove the nitrocellulose filter and replace with two new 3MM sheets. Soak with 10–15 ml of 2X SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate for 5 minutes at room temperature. Repeat.
7. Bake nitrocellulose filters at 80°C or UV-crosslink DNA to nylon. You may probe the filters with a nonradioactive-labeled or random-primed, <sup>32</sup>P-labeled probe complementary to your gene.

You can identify multi-copy integrants by a strong hybridization signal relative to the single copy control. You can then quantify dot blots for copy number by densitometry of the film or blot, or by using a  $\beta$ -scanner (if radiolabeled).

---

### Southern Blot Analysis

For a detailed description of this technique as applied to *Pichia pastoris*, see (Clare, *et al.*, 1991a). It is very important to digest your DNA with the right restriction enzyme(s) to generate a blot of digested and gel-separated genomic DNA. We recommend that you use a restriction enzyme that cuts **outside** of the expression cassette. This will generate a restriction fragment whose size reflects the number of multimers. For example, if you have cloned a 1.2 kb fragment into pPICZ and transformed into *Pichia*, you can digest the DNA from this recombinant with *Nhe* I (which does not cut within the vector or the insert). The size of the fragment will increase in additions of 4.7 kb over the size of the fragment containing 1 copy of the gene.

Alternatively, digestion of DNA from *Pichia* recombinants containing multiple copies will produce a band that will vary in intensity depending on the number of copies of your gene. It is very important to include a control to show the intensity of a single copy gene. You can quantify the relative band intensities using densitometry to estimate gene dosage.

---

### Controls

It is very important to include DNA from the host strain alone (X-33, GS115 or KM71H), the host strain transformed with the parent vector, and the host strain transformed with a vector containing one copy of your gene.

---

### General Guidelines

- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.*, 1989), pages 9.31–9.58.
  - Isolate genomic DNA and quantify using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane to accurately determine copy number if you are using relative intensities.
  - Probe your Southern blot with a fragment complementary to your gene.
-

# Isolating Total RNA from *Pichia*

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## Introduction

This protocol is designed to isolate 60–300 µg total RNA (Schmitt *et al.*, 1990) from *Pichia*, which is suitable for mRNA isolation using Invitrogen's FastTrack® 2.0 or Micro FastTrack™ 2.0 mRNA Isolation Kit. If you wish to use another protocol, scale-up the reaction to yield about 2 mg of total RNA per time point. The mRNA is for northern blot analysis of *Pichia* recombinants to determine if the gene of interest is being induced and transcribed. You should isolate RNA from induced cultures using an uninduced culture as a negative control.

---

## Solutions

Prepare the following solutions. Remember to use DEPC-treated water and to use equipment free of RNase.

MGYH or BMGY medium	DEPC-treated water
3 M sodium acetate, pH 5.3	Buffered phenol
10% SDS in DEPC treated water	Phenol:chloroform (1:1)
Chloroform:isoamyl alcohol (24:1)	65°C water bath
AE buffer (50 mM sodium acetate, pH 5.3, 1 mM EDTA)	

---

## Growth of Cells

1. Grow two cultures (100–200 ml in MGYH or BMGY), but induce only one of them. Use the same protocol for induction that you used when expressing recombinant *Pichia* strains (pages 37–41).
  2. Take 10 ml time points at 1, 2, 3, 4, and 6 days by centrifuging at 1,500 × g for 10 minutes at room temperature.
  3. Resuspend cell pellet in 400 µl AE buffer and transfer to a microcentrifuge tube.
- 

## Lysing Cells

1. Add 40 µl 10% SDS and vortex for ~ 20 seconds.
  2. Add an equal volume (450–500 µl) of buffer saturated phenol and vortex for ~20 seconds.
  3. Incubate at 65°C for 4 minutes.
  4. Incubate in a dry ice/ethanol bath until crystals show (~1 minute). Centrifuge at maximum speed for 2 minutes at 4°C.
  5. Transfer aqueous phase to new centrifuge tube and add an equal volume of phenol/chloroform and vortex for ~20 seconds. Centrifuge at maximum speed for 2 minutes at 4°C.
  6. Remove upper phase to a new tube and add 40 µl of 3 M sodium acetate, pH 5.3 and 2.5 volumes of 100% ethanol (–20°C). Centrifuge at maximum speed for 15 minutes at 4°C. Remove ethanol.
  7. Wash pellet with 80% ethanol and air dry briefly. Resuspend total RNA in 20 µl DEPC-treated water and store at –80°C. Yield is 60–300 µg total RNA.
- 

## mRNA Isolation and Northern Analysis

See (Ausubel *et al.*, 1994) for a protocol for mRNA isolation and Northern analysis. The FastTrack® 2.0 mRNA Kit (Cat. no. K1593-02) is designed to isolate mRNA from 0.2 to 1 mg total RNA. The Micro-FastTrack™ 2.0 Kit (Cat. no. K1520-02) is designed to isolate mRNA from ~100 µg total RNA. You will need ~1–5 µg mRNA per time point.

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# β-Galactosidase Assay

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## Introduction

The GS115/pPICZ/*lacZ* strain is provided as a His<sup>+</sup> Mut<sup>+</sup> intracellular expression control. Growth of the strain during Mut<sup>+</sup> expression provides a positive control for expression conditions. You may use the cell-free β-galactosidase assay adapted from (Miller, 1972), page 403, to evaluate expression of β-galactosidase.

---

## Preparation of Solutions

Prepare the following:

- A fresh crude cell lysate of GS115/pPICZ/*lacZ* (see page 40)
- Z buffer
- ONPG solution
- 1 M sodium carbonate solution

Recipes for the solutions are found below.

---

## Z Buffer

60 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O  
40 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O  
10 mM KCl  
1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O  
50 mM β-mercaptoethanol  
pH 7.0

1. Dissolve the following in 950 ml deionized water:
    - 16.1 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O
    - 5.5 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O
    - 0.75 g KCl
    - 0.246 g MgSO<sub>4</sub>·7H<sub>2</sub>O
    - 2.7 ml β-mercaptoethanol
  2. Adjust pH to 7.0 with either NaOH or HCl and bring the volume up to 1 liter with water.
  3. **Do not autoclave.** Store at 4°C.
- 

## ONPG Solution

4 mg/ml in 100 mM phosphate buffer, pH 7.0

1. Dissolve the following in 90 ml deionized water:
    - 1.61 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O
    - 0.55 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O
  2. Adjust pH to 7.0 with either NaOH or HCl and add 400 mg of ONPG. Stir to dissolve and bring the volume up to 100 ml with water.
  3. Store at 4°C **in the dark.**
- 

## 1 M Sodium Carbonate

Dissolve 12.4 g sodium carbonate in 100 ml of deionized water. Store at room temperature.

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*Continued on next page*

## $\beta$ -Galactosidase Assay, continued

---

### Procedure

1. Determine protein concentration of your lysate by Lowry, Bradford, or BCA assay.
  2. Equilibrate Z buffer, ONPG solution, and sodium carbonate solution to 28°C.
  3. Add 10–50  $\mu$ l of your crude assay to 1 ml of Z buffer and equilibrate at 28°C. As a control for spontaneous hydrolysis of ONPG, add an aliquot of your lysis buffer to 1 ml of Z buffer.
  4. To initiate the reaction, add 0.2 ml 4 mg/ml ONPG to each of the tubes in Step 2.
  5. Incubate the samples and the control at 28°C until a faint yellow color develops. This should occur at least 10 minutes after the start of the assay to ensure accurate data. Note that the tube with no lysate may not change color.
  6. Stop the reaction by adding 0.5 ml of 1 M sodium carbonate to each tube. Record the length of incubation for each sample.
  7. Read the OD<sub>420</sub> against the control containing buffer alone.
  8. Determine the protein concentration of your lysate in mg/ml.
- 



### Note

If the reaction turns yellow too quickly, you need to dilute your lysate. Try successive 10-fold dilutions of the lysate using your lysis buffer until the reaction starts turning yellow after 10 minutes. This is to ensure that you are measuring a true initial rate.

---

### Determination of Specific Activity

Use the following formula to determine the specific activity of the  $\beta$ -galactosidase in units/mg total protein:

$$\beta\text{-galactosidase units/mg total protein} = \frac{\text{OD}_{420} \times 380}{\text{minutes at } 28^{\circ}\text{C} \times \text{mg protein in reaction}}$$

Remember to take into account the volume of lysate added to the reaction and any dilutions made to the lysate when calculating the amount of protein in the reaction. The number 380 is the constant used to convert the OD<sub>420</sub> reading into units. One unit is defined as the amount of enzyme that will hydrolyze 1 nmole of ONPG per minute at 28°C. The molar extinction coefficient of ONPG under these conditions is 4,500. For a sample calculation, See below.

---

### Sample Calculation

Here is a sample calculation:

Extract concentration = 10 mg/ml  
Assay 10  $\mu$ l of a 1/100 dilution  
Time = 10 minutes  
OD<sub>420</sub> = 0.4

The amount of protein in the reaction = 0.01 ml  $\times$  0.01 (dilution factor)  $\times$  10 mg/ml  
= 0.001 mg protein in the reaction

The specific activity =  $\frac{0.400 \times 380}{10 \times 0.001 \text{ mg}}$  = 15,200 units/mg protein

Pure  $\beta$ -galactosidase has an activity of 300,000 units/mg protein.

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