invitrogen™

# *Pichia* Expression Kit

## For Expression of Recombinant Proteins in *Pichia pastoris*

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Manual part no. 25-0043

MAN0000012

**User Manual**

# Table of Contents

# Kit Contents and Storage

## Kit Contents

**The *Pichia* Expression Kit** is shipped at room temperature and contains the following components.

**Spheroplast Module (Box 1). Store at room temperature.**

| Reagent | Amount | Components |
|---|---|---|
| SOS medium | 20 mL | 1 M Sorbitol<br>0.3X YPD<br>10 mM $CaCl_2$ |
| Sterile Water | 2 × 125 mL | Autoclaved, deionized water |
| SE | 2 × 125 mL | 1 M Sorbitol<br>25 mM EDTA, pH 8.0 |
| SCE | 2 × 125 mL | 1 M Sorbitol<br>10 mM Sodium citrate buffer, pH 5.8<br>1 mM EDTA |
| 1 M Sorbitol | 2 × 125 mL | -- |
| CaS | 2 × 60 mL | 1 M Sorbitol<br>10 mM Tris-HCl, pH 7.5;<br>10 mM $CaCl_2$ |
| 40% PEG | 25 mL | 40% (w/v) PEG 3350 (Reagent grade) in water |
| CaT | 25 mL | 20 mM Tris-HCl, pH 7.5<br>20 mM $CaCl_2$ |

**Spheroplast Module (Box 2). Store at –20°C.**

| Reagent | Amount | Components |
|---|---|---|
| Zymolyase | 10 × 20 µL | 3 mg/mL Zymolyase in water<br>(100,000 units/g lytic activity) |
| 1 M DTT | 10 × 1 mL | 1 M dithiothreitol in water |

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

| Strain | Amount | Genotype | Phenotype (*Pichia* only) |
|---|---|---|---|
| GS115 | 1 stab | *his4* | $Mut^+$ |
| KM71 | 1 stab | *arg4 his4 aox1::ARG4* | $Mut^S$, $Arg^+$ |
| GS115 Albumin | 1 stab | *HIS4* | $Mut^S$ |
| GS115 β-Gal | 1 stab | *HIS4* | $Mut^+$ |
| TOP10F´ | 1 stab | F´ {*pro*AB, *lac*$I^q$, *lac*ZΔM15, Tn*10* ($Tet^R$)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, $\lambda^-$ *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L($Str^R$), *end*A1, *nup*G | |

*Continued on next page*

## Kit Contents, continued

**Vectors. Store at –20°C.**

| Reagent | Description |
|---|---|
| **pHIL-D2**<br>10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0* | Vector for intracellular expression in *Pichia.* |
| **pPIC3.5**<br>10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0 | Vector for intracellular expression in *Pichia.* |
| **pHIL-S1**<br>10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0 | Vector for secreted expression in *Pichia*.<br>Uses the *PHO1* signal sequence. |
| **pPIC9**<br>10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0 | Vector for secreted expression in *Pichia*.<br>Uses the α-factor signal sequence. |

*__TE buffer, pH 8.0__: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

**Primers. Store at –20°C.**

| | |
|---|---|
| 5´ *AOX1* sequencing primer<br>2 µg (312 pmoles), lyophilized | 5´-GACTGGTTCCAATTGACAAGC-3´ |
| 3´ *AOX1* sequencing primer<br>2 µg (314 pmoles), lyophilized | 5´-GCAAATGGCATTCTGACATCC-3´ |
| α-Factor sequencing primer<br>2 µg (315 pmoles), lyophilized | 5´-TACTATTGCCAGCATTGCTGC-3´ |

## Media

The following prepackaged media is included for your convenience. Instructions for use are provided on the package. 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 medium |
| Yeast Nitrogen Base | 1 pouch | 500 mL of 10X YNB |

**Note**

The *Pichia* Spheroplast Module for transforming *Pichia* by spheroplasting is available separately from Invitrogen (see page 88 for ordering information).

## Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

# Required Equipment and Supplies

### Required Equipment and Supplies (not provided)

- 30°C rotary shaking incubator
- Water baths capable of 37°C, 45°C, and 100°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 L)
- 50 mL sterile, conical tubes
- 6 mL and 15 mL sterile snap-top tubes (Falcon 2059 or similar)
- UV Spectrophotometer
- Mini agarose gel apparatus and buffers
- Agarose and low-melt agarose
- Polyacrylamide gel electrophoresis apparatus and buffers
- Media for transformation, growth, screening, and expression (see **Recipes**, pages 59–66)
- 5% SDS solution (10 mL per transformation)
- Sterile cheesecloth or gauze
- Breaking Buffer (see **Recipes**, page 66)
- Acid-washed glass beads (available from Sigma)
- Replica-plating equipment (optional)
- Bead Beater™ (optional, available from Biospec)

# Introduction

## *Pichia pastoris* Expression System

### 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, read the articles cited in the text along with the following review articles (Buckholz & Gleeson, 1991; Cregg & Higgins, 1995; Cregg *et al.*, 1993; Nico-Farber *et al.*, 1995; Romanos, 1995; Sreekrishna *et al.*, 1988; Wegner, 1990). A general review of foreign gene expression in yeast is also available (Romanos *et al.*, 1992).

### 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 such as baculovirus or mammalian tissue culture, 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.

### 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, the *HIS4* gene in both Saccharomyces and *Pichia* encodes histidinol dehydrogenase. 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*.

### *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 $O_2$, 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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### Two Alcohol Oxidase Proteins

Two genes in *Pichia pastoris* code for alcohol oxidase–*AOX1* and *AOX2*. The *AOX1* gene product accounts for the majority of alcohol oxidase activity in the cell. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically ≥ 30% of the total soluble protein in cells grown on 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 $Mut^S$ strains (*aox1*) (Cregg *et al.*, 1989; Koutz *et al.*, 1989).

### Expression

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-grown cells approximately 5% of the $polyA^+$ 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 only (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).

### 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 $Mut^S$ (Methanol utilization slow). This has in the past been referred to as $Mut^-$. The $Mut^S$ 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. $Mut^+$ (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).

### Intracellular and Secretory Protein Expression

Heterologous expression in *Pichia pastoris* can be 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, this means that 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) . 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 α1,3 glycan linkages whereas *Pichia pastoris* does not. It is believed that the α1,3 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).

# Experimental Outline

## Selection of Vector and Cloning

To utilize the strong, highly inducible $P_{AOX1}$ promoter for expressing your protein, four expression vectors are included in this kit. pHIL-D2 and pPIC3.5 are used for intracellular expression, and pHIL-S1 and pPIC9 are used for secreted expression (see pages 14–17 for more information). Before cloning your insert, you must:

- decide whether you want intracellular or secreted expression.
- analyze your insert for the following restriction sites: *Sac* I, *Stu* I, *Sal* I, *Not* I, and *Bgl* II. We recommend these sites for linearizing your construct prior to *Pichia* transformation. If your insert has all of these sites, refer to pages 29–30 for alternate sites.

## Transformation and Integration

Two different phenotypic classes of $His^+$ recombinant strains can be generated: $Mut^+$ and $Mut^S$. $Mut^S$ refers to the "Methanol utilization slow" phenotype caused by the loss of alcohol oxidase activity encoded by the *AOX1* gene. A strain with a $Mut^S$ phenotype has a mutant *aox1* locus, but is wild type for *AOX2*. This results in a slow growth phenotype on methanol medium. Transformation of strain GS115 can yield both classes of transformants, $His^+$ $Mut^+$ and $His^+$ $Mut^S$, while KM71 yields only $His^+$ $Mut^S$, because the strain itself is $Mut^S$. Both $Mut^+$ and $Mut^S$ recombinants are useful to have, because one phenotype may favor better expression of your protein than the other. Because of clonal variation, you should test 6–10 recombinants per phenotype. There is no way to predict beforehand which construct or isolate will better express your protein. We strongly recommend that you analyze *Pichia* recombinants by PCR to confirm the integration of your construct (see page 43).

After you have successfully cloned your gene, you will linearize your plasmid to stimulate recombination when the plasmid is transformed into *Pichia*. The table below describes the types of recombinants you will get by selective digestion of your plasmid.

| Restriction Enzyme | Integration Event | GS115 Phenotype | KM71 Phenotype |
|---|---|---|---|
| *Sal* I or *Stu* I | Insertion at *his4* | $His^+$ $Mut^+$ | $His^+$ $Mut^S$ |
| *Sac* I | Insertion at 5´ *AOX1* region | $His^+$ $Mut^+$ | $His^+$ $Mut^S$ |
| *Not* I or *Bgl* II | Replacement at *AOX1* locus | $His^+$ $Mut^S$<br>$His^+$ $Mut^+$ | $His^+$ $Mut^S$ (not recommended, see page 7) |

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# Experimental Outline, continued

## Expression and Scale-up

After confirming your *Pichia* recombinants by PCR, you will test expression of both His$^+$ Mut$^+$ and His$^+$ Mut$^S$ recombinants. This procedure involves growing a small culture of each recombinant, inducing them 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 Coomassie staining and, if you have an antibody to your protein, by western blot. We also suggest checking for protein activity by an activity 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 that best expresses your protein and optimize induction based on the suggestions on pages 52–53. After you optimize expression, scale-up your expression protocol to produce more protein.

## Experimental Process

The overall experimental process is divided into two major sections: **Generating Recombinant Strain** and **Induction (Mut$^+$ and/or Mut$^S$).** Each section contains a table outlining the major steps of the experimental process. Each step is discussed in detail further in the manual. Refer to the indicated pages to read about particular steps of interest. The discussion about recombination and integration in *Pichia* will help you choose the right vector. For more information, refer to the review by Higgins (Higgins, 1995).

## Generating Recombinant Strain

The goal of this section is to create a *Pichia pastoris* strain containing your integrated gene of interest. Before starting your experiments, determine which vector to use.

| Step | Procedure | Page |
|---|---|---|
| 1 | Select the appropriate expression vector (For more information, refer to **Recombination and Integration in *Pichia***, pages 69–72) | 11–17 |
| 2 | Clone your gene of interest into selected vector | 19–24 |
| 3 | Transform *E. coli*, select ampicillin-resistant transformants, and confirm the presence and orientation of your gene of interest | 25 |
| 4 | Linearize the constructs with appropriate restriction enzymes to generate His$^+$ Mut$^S$ and His$^+$ Mut$^+$ recombinant strains | 27–30 |
| 5 | Transform and select His$^+$ transformants (GS115 recombinants, His$^+$ Mut$^+$; KM71 recombinants, His$^+$ Mut$^S$) | 31–37 |
| 6 | Screen His$^+$ transformants for Mut$^+$ and Mut$^S$ strains (6–10 recombinants of each phenotype) | 38–42 |
| 7 | Confirm the integration of your gene of interest in Mut$^+$ and Mut$^S$ recombinants by PCR | 43–44 |

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# Experimental Outline, continued

## Mut+ Induction

The method of induction depends on whether the recombinant is Mut+ or MutS. The differences primarily occur in the culture volumes and the time of induction (see below). Refer to the following pages for more detailed instructions.

| Step | Procedure | Page |
|---|---|---|
| 1 | Guidelines for expression of recombinant proteins in *Pichia* | 45–46 |
| 2 | Grow His+ Mut+ recombinants in 25 mL of buffered glycerol medium to a final $OD_{600}$ = 2–6 | 47 |
| 3 | Harvest the cells and resuspend them to an $OD_{600}$ of 1.0 (~100–200 mL) with methanol medium. Place the cell suspension in a 1 liter baffled flask | 47 |
| 4 | Incubate the culture at 30°C with shaking and take samples for analysis at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hours | 47 |
| 5 | Analyze the medium (if protein of interest is targeted for secretion) and the cell lysates (for intracellular and secreted expression) for protein via PAGE/Coomassie Blue staining, western blot, activity, ELISA, or immunoprecipitation | 49–51 |
| 6 | Optimize expression of your His+ Mut+ recombinant | 52–53 |
| 7 | Scale-up your expression for protein purification | 54–56 |

## MutS Induction

This is very similar to Mut+ induction except that MutS grow very slowly on methanol. To compensate, cells are concentrated to increase cell mass before induction.

| Step | Procedure | Page |
|---|---|---|
| 1 | Guidelines for expression of recombinant proteins in *Pichia* | 45–46 |
| 2 | Grow His+ MutS recombinants in 100–200 mL of buffered glycerol medium to a final $OD_{600}$ = 2–6 | 48 |
| 3 | Harvest the cells and resuspend them to an $OD_{600}$ of 10.0 (~10–20 mL) with methanol medium. Place the cell suspension in a 100 mL or 250 mL baffled flask. | 48 |
| 4 | Incubate the culture at 30°C with shaking and take samples for analysis at 0, 24, 48, 72, 96, 120, and 144 hours | 48 |
| 5 | Analyze the medium (if protein of interest is targeted for secretion) and the cell lysates (for intracellular and secreted expression) for protein via PAGE/Coomassie Blue staining, western blot, activity, ELISA, or immunoprecipitation | 49–51 |
| 6 | Optimize expression of your His+ Mut+ recombinant | 52–53 |
| 7 | Scale-up your expression for protein purification | 54–56 |

# Methods

## *Pichia* Strains

### 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 & Fink, 1991), *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989), *Protein Methods* (Bollag *et al.*, 1996), and *Guide to Protein Purification* (Deutscher, 1990).

### Genotype of *Pichia* Strain

The *Pichia* host strains GS115 and KM71 have a mutation in the histidinol dehydrogenase gene (*his4*) that prevents them from synthesizing histidine. All expression plasmids carry the *HIS4* gene that complements *his4* in the host, so transformants are selected for their ability to grow on histidine-deficient medium. Spontaneous reversion of GS115 and KM71 to $His^+$ prototrophy is less than 1 out of $10^8$.

The parent strain of KM71 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 KM71, a $Mut^S$, $Arg^+$, $His^-$ strain.

Both GS115 and KM71 will grow on complex medium such as YPD (also known as YEPD) and on minimal media supplemented with histidine. Until transformed, neither GS115 nor KM71 will grow on minimal medium alone as they are $His^-$.

**Note:** $Mut^S$ (Methanol utilization slow) phenotype has in the past been referred to as $Mut^-$. The $Mut^S$ designation has been chosen to accurately describe the phenotype of these mutants.

### Construction of KM71

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 $Arg^+$ transformants were isolated and analyzed for the $Mut^S$ phenotype. Genetic analysis of $Arg^+$ transformants showed that the wild-type *AOX1* gene was replaced by the *aox1::ARG4* construct.

### Important

The advantage of using KM71 is that there is no need to screen for the Mut phenotype on methanol minimal medium. All transformants will be $Mut^S$. Secondly, since the *AOX1* locus was not completely deleted, it is theoretically possible to replace *aox1::ARG4* with your construct by gene replacement. The phenotype of this strain would be $His^+$ $Mut^S$ $Arg^-$. 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 $His^+$ transformants in KM71 by replacing the *aox1::ARG4* construct.

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### Control Expression Strains

**GS115/His$^+$ Mut$^S$ Albumin**: This strain is a control for secreted expression and the Mut$^S$ phenotype when screening *Pichia* transformants (page 38). 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/His$^+$ Mut$^+$ β-galactosidase**: This strain is a control for intracellular expression and the Mut$^+$ phenotype when screening *Pichia* transformants (page 38). The gene for β-galactosidase (*lac*Z) was integrated into *Pichia* at the *his4* locus. This strain expresses β-galactosidase (117 kDa) at levels that can be detected on Coomassie-stained SDS-PAGE (see pages 49–51) or assayed using ONPG (see page 86–87).

### 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$^+$ or Mut$^S$ *Pichia* in YPD is ~2 hours
- Mut$^+$ and Mut$^S$ strains do not differ in growth rates unless grown on methanol
- Doubling time of log phase Mut$^+$ *Pichia* in methanol medium (MM) is 4–6 hours
- Doubling time of log phase Mut$^S$ *Pichia* in MM is ~18 hours
- One $OD_{600}$ = ~$5 \times 10^7$ cells/mL

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

### 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 due to 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$^S$ strains and up to 3% for Mut$^+$ without any negative effect to their liquid culture.

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Make frozen stocks for long-term storage of all three *Pichia* strains included in this kit (see below).

### Storing *Pichia* Strains

**To store cells for weeks to months, use YPD medium or YPD agar slants (see page 61).**

1. Streak for single colonies of the desired strain on YPD.
2. Transfer one colony to a YPD stab and grow for 2 days at 30°C.
3. You can store the cells on YPD for several weeks at 4°C.

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

1. Culture a single colony of the desired strain overnight in YPD.
2. Harvest the cells and suspend in YPD containing 15% glycerol at a final $OD_{600}$ of 50–100 (approximately $2.5 \times 10^9$–$5.0 \times 10^9$ cells/mL).
3. Freeze the cells in liquid nitrogen or a dry ice/ethanol bath, and store at –80°C.

**Note**

After extended storage at 4°C or –80°C, we recommend checking the $His^+$ transformants for correct genotype and viability by streaking on MM, MD or MGY plates before using again.

# *E. coli* Strains

## 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, DH5αF´, JM109, or any other strain which is recombination deficient (*rec*A) and deficient in endonuclease A (*end*A).

F´ {*pro*AB, *lac*$I^q$, *lac*ZΔM15, Tn*10* ($Tet^R$)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, $\lambda^-$ *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L($Str^R$), *end*A1, *nup*G

**Note:** If you do not plan to perform single-stranded DNA rescue, *E. coli* strains that do not carry the F´ episome are also suitable for use.

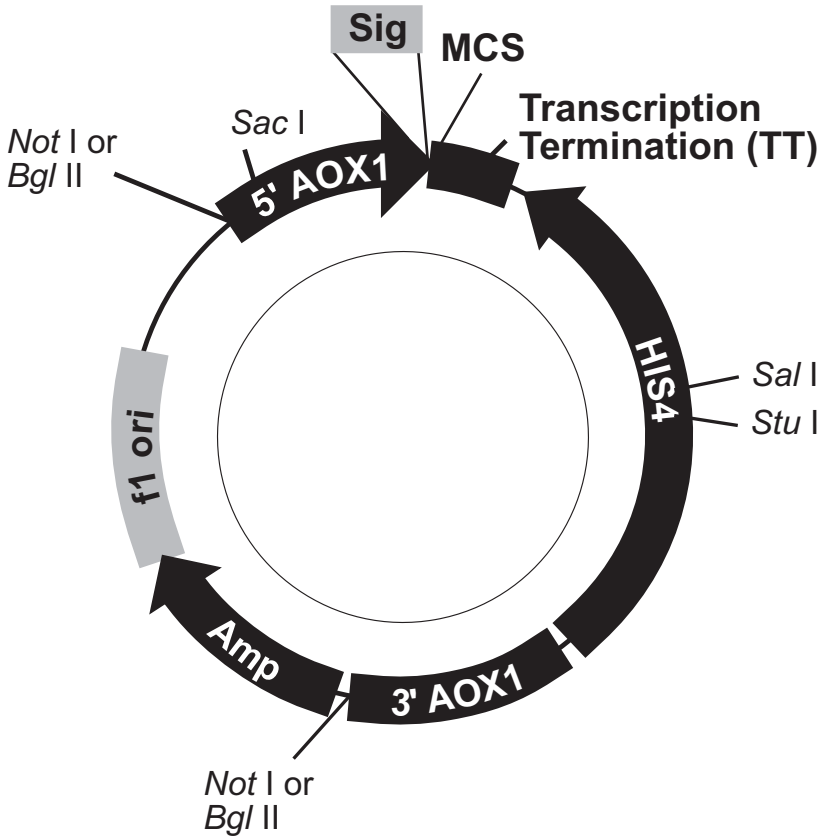

We recommend that you make a frozen stock of TOP10F´ to keep on hand.

1. Culture TOP10F´ in 5 mL LB with 10 µg/mL tetracycline. Grow overnight.
2. Mix thoroughly 0.85 mL of culture with 0.15 mL sterile glycerol.
3. Transfer to a freezer vial and freeze in liquid nitrogen or a dry ice/ethanol bath.
4. Store at –80°C.

# Selecting a *Pichia* Expression Vector

**Generic Structure**

All the vectors included in this kit share several general features shown in black, while some of the vectors also have signal sequences (Sig) and/or an f1 bacteriophage origin. For details of each individual plasmid refer to pages 14–17.




**Note**

There is no yeast origin of replication in any of the *Pichia* expression vectors included in this kit. $His^+$ transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome.

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# Selecting a *Pichia* Expression Vector, continued

## Features

The table below describes the general and optional features of the *Pichia* expression vectors.

| Feature | Description | Benefit |
|---|---|---|
| 5´ *AOX1* | An ~1000 bp fragment containing the *AOX1* promoter | Allows methanol-inducible high level expression in *Pichia*<br>Targets plasmid integration to the *AOX1* locus. |
| Sig | DNA sequence coding for an N-terminal protein secretion signal | Targets desired protein for secretion |
| MCS | Multiple Cloning Site | Allows insertion of your gene into the expression vector |
| TT | Native transcription termination and polyadenylation signal from *AOX1* gene (~260 bp) | Permits efficient transcription termination and polyadenylation of the mRNA |
| *HIS4* | *Pichia* wild-type gene coding for histidinol dehydrogenase (~2.4 kb) and used to complement *Pichia his4* strains | Provides a selectable marker to isolate *Pichia* recombinant strains |
| 3´ *AOX1* | Sequences from the *AOX1* gene that are further 3´ to the TT sequences (~650 bp) | Targets plasmid integration at the *AOX1* gene |
| Amp<br>pBR322 origin | Ampicillin resistance gene<br>*E. coli* origin of replication | Allows selection, replication, and maintenance in *E. coli* |
| f1 origin | Bacteriophage f1 origin of replication (458 bp) | Permits generation of single-stranded DNA for mutagenesis |
| *Not* I<br>*Bgl* II<br>*Sac* I<br>*Sal* I<br>*Stu* I | Unique restriction sites | Permits linearization of vector for efficient integration into the *Pichia* genome |

*Continued on next page*

### Selecting a Vector

If your protein is cytosolic and non-glycosylated, you may elect to express the protein intracellularly. However, there is evidence of a non-glycosylated protein being secreted without extensive modification (Despreaux and Manning, 1993). Note that the protein in question was a secreted, bacterial protein with one N-glycosylation site. Check your protein sequence for possible N-glycosylation sites (Asn-X-Ser/Thr) before cloning a cytosolic protein into a secretion vector.

If your protein is normally secreted, glycosylated, or directed to an intracellular organelle, you may wish to try secreting your protein. We recommend that you try both the native secretion signal and the α-factor signal sequence (in pPIC9) to secrete your protein. There has been better success reported with the α-factor signal sequence than with the *PHO1* signal sequence in pHIL-S1. This may be due to the lack of *KEX2*-like processing signals in the *PHO1* signal sequence (Laroche *et al.*, 1994).

# pHIL-D2

## Description

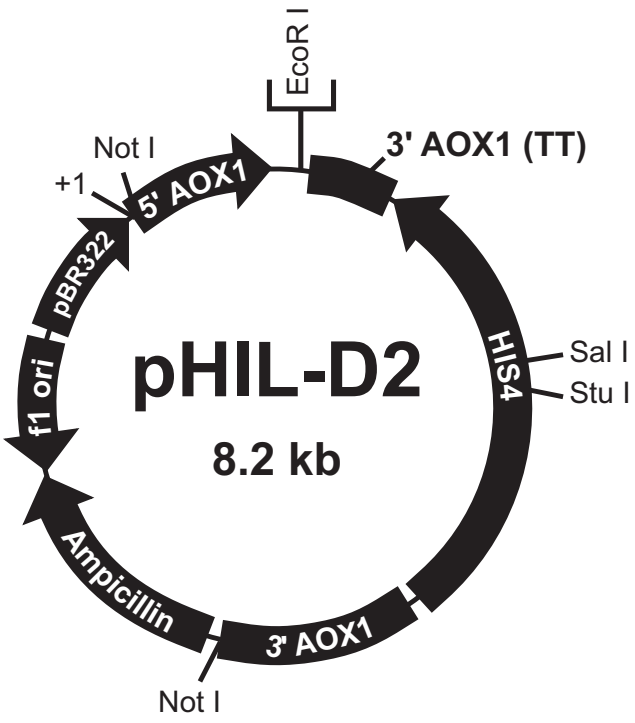
The details of pHIL-D2 are listed below:

- 8,209 bp nonfusion vector
- One unique *Eco*R I site
- For intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990)
- *HIS4* selection in *Pichia*
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates $His^+$ $Mut^+$ in GS115 and His+ $Mut^S$ in KM71)
- For insertion at *HIS4*, linearize with *Sal* I or *Stu* I (generates $His^+$ $Mut^+$ in GS115 and $His^+$ $Mut^S$ in KM71)
- For a gene replacement at *AOX1* in GS115, linearize with *Not* I (generates $His^+$ $Mut^S$)

Refer to page 29 for alternate restriction sites if your insert DNA has a *Not* I, *Sac* I, *Sal* I, or *Stu* I site.

## Map of pHIL-D2

The map below shows the location and size of each feature of pHIL-D2. For the details of the multiple cloning site refer to page 21. The complete sequence of pHIL-D2 is available at www.invitrogen.com or from Technical Support (page 89).




**Comments for pHIL-D2:**
**8209 nucleotides**

5' *AOX1* promoter fragment: bases 14-941
5' *AOX1* primer site: bases 868-888
*Eco*R I Site: bases 956-961
3' *AOX1* primer site: bases 1036-1056
3' *AOX1* transcription
termination (TT) fragment: bases 963-1295
*HIS4* ORF: bases 4223-1689
3' *AOX1* fragment: bases 4578-5334
Ampicillin resistance gene: bases 5686-6546
f1 origin of replication: bases 7043-6588
pBR322 origin: bases 7138-7757

# pPIC3.5

## Description

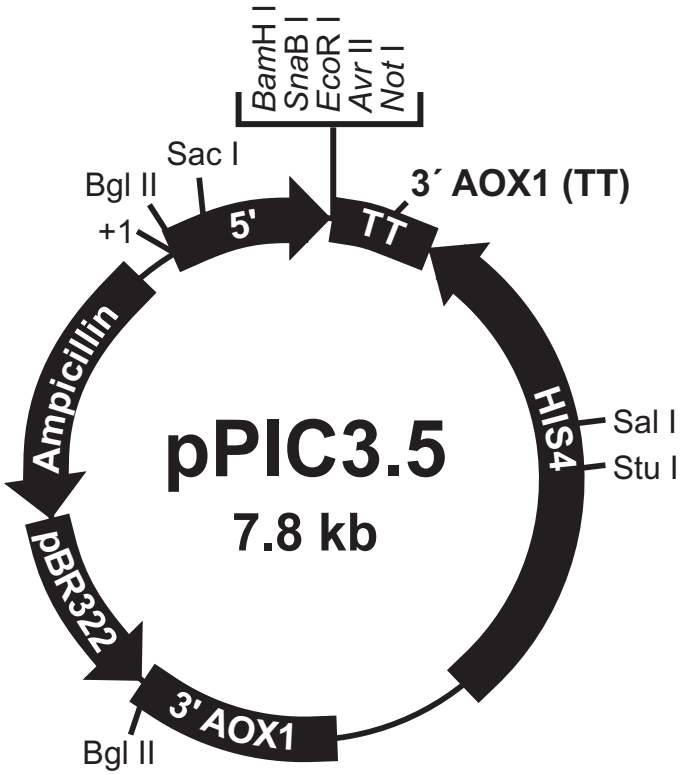
The details of pHIL-D2 are listed below:

- 7,751 bp nonfusion vector
- *Bam*H I, *Sna*B I, *Eco*R I, *Avr* II, *Not* I unique sites
- Intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990)
- *HIS4* selection in *Pichia*
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates $His^+$ $Mut^+$ in GS115 and $His^+$ $Mut^S$ in KM71)
- For insertion at *HIS4*, linearize with *Sal* I or *Stu* I (generates $His^+$ $Mut^+$ in GS115 and $His^+$ $Mut^S$ in KM71)
- For a gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates $His^+$ $Mut^S$)

Refer to page 29 for alternate restriction sites if your insert DNA has a *Not* I, *Sac* I, *Sal* I, or *Stu* I site.

## Map of pPIC3.5

The map below shows the location and size of each feature of pPIC3.5. For the details of the multiple cloning site refer to page 22. The complete sequence of pPIC3.5 is available at www.invitrogen.com or from Technical Support (page 89).




**Comments for pPIC3.5:**
**7751 nucleotides**

5´ *AOX1* promoter fragment: bases 1-937
5´ *AOX1* primer site: bases 855-875
Multiple Cloning Site: bases 938-968
3´ *AOX1* primer site: bases 1055-1075
3´ *AOX1* transcription termination (TT) fragment: bases 981-1314
*HIS4* ORF: bases 4242-1708
3´ *AOX1* fragment: bases 4598-5354
pBR322 origin: bases 6436-5764
Ampicillin resistance gene: bases 7442-6582

# pHIL-S1

## Description

The details of pHIL-S1 are listed below:

- 8,260 bp fusion vector
- *Xho* I, *Eco*R I, *Sma* I, *Bam*H I unique sites
- Secreted expression using the *PHO1* secretion signal
- For expression, your gene must be cloned in frame with the initiation codon of the signal sequence.
- *HIS4* selection in *Pichia*
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates $His^+$ $Mut^+$ in GS115 or $His^+$ $Mut^S$ in KM71)
- For insertion at *HIS4* in GS115 or KM71, linearize with *Sal* I or *Stu* I (generates $His^+$ $Mut^+$ in GS115 or $His^+$ $Mut^S$ in KM71)
- For gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates $His^+$ $Mut^S$)

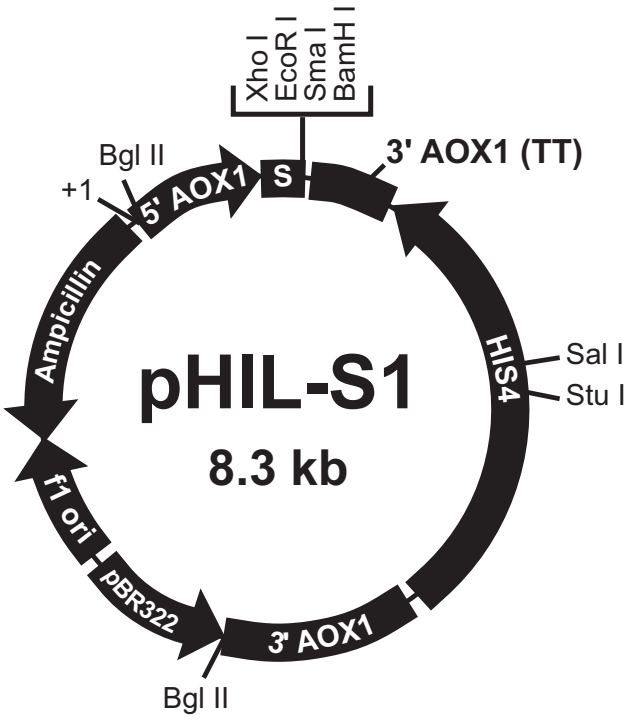
Refer to page 30 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, *Sal* I, or *Stu* I site.

## Map of pHIL-S1

The map below shows the location and size of each feature of pHIL-S1. For the details of the multiple cloning site, refer to page 23. The complete sequence of pHIL-S1 is available at www.invitrogen.com or from Technical Support (page 89).

**Comments for pHIL-S1:**
**8260 nucleotides**

5' *AOX1* promoter fragment: bases 1-941
5' *AOX1* primer site: bases 856-876
PHO1 secretion signal (S): bases 942-1007
Multiple Cloning Site Region: bases 1006-1026
3' *AOX1* primer site: bases 1099-1119
3' *AOX1* transcription termination (TT) fragment: bases 1025-1190
*HIS4* ORF: bases 4286-1753
3' *AOX1* fragment: bases 4641-5397
pBR322 origin: bases 6556-5937
f1 origin of replication: bases 6651-7106
Ampicillin resistance gene: bases 7922-7062



# pPIC9

## Description

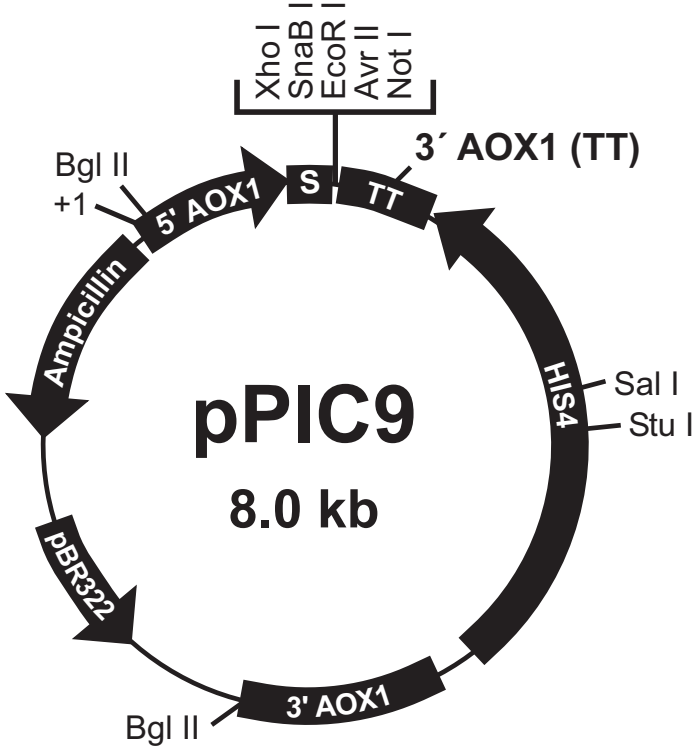
The details of pHIL-S1 are listed below:

- 8,023 bp fusion vector
- *Xho* I, *Sna*B I, *Eco*R I, *Avr* II, *Not* I unique sites
- Secreted expression of your gene using the  -factor secretion signal
- For expression, your gene must be cloned in frame with the initiation codon of the signal sequence.
- *HIS4* selection in *Pichia*
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates $His^+$ $Mut^+$ in GS115 and $His^+$ $Mut^S$ in KM71)
- For insertion at *HIS4*, linearize with *Sal* I or *Stu* I (generates $His^+$ $Mut^+$ in GS115 and $His^+$ $Mut^S$ in KM71)
- For gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates $His^+$ $Mut^S$)

Refer to page 30 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, *Sal* I, or *Stu* I site.

## Map of pPIC9K

The figure below shows the map of pPIC9. Details of the multiple cloning site are provided on page 24. The sequence of pPIC9 is available at www.invitrogen.com or from Technical Support (page 89).




**Comments for pPIC9:**
**8023 nucleotides**

5´ *AOX1* promoter fragment: bases 1-948
5´ *AOX1* primer site: bases 855-875
α-Factor secretion signal(s): bases 949-1215
α-Factor primer site: bases 1152-1172
Multiple Cloning Site: bases 1192-1241
3´ *AOX1* primer site: bases 1327-1347
3´ *AOX1* transcription termination (TT): bases 1253-1586
*HIS4* ORF: bases 4514-1980
3´ *AOX1* fragment: bases 4870-5626
pBR322 origin: bases 6708-6034
Ampicillin resistance gene: bases 7713-6853

# Signal Sequence Processing

## Signal Sequence Processing

When cloning into the *Xho* I site of pPIC9, the secretion signal sequence between the *Xho* I site and *Sna*B I may need to be regenerated.

The processing of the α-factor mating signal sequence in pPIC9 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 *STE13* gene product further cleaves the Glu-Ala repeats.

## Optimizing Signal Cleavage

In *Saccharomyces cerevisiae*, the Glu-Ala repeats are not necessary for cleavage by *KEX2*, but the *KEX2* 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, inhibits *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. This is generally dependent on the protein of interest.

**Note**

The *PHO1* signal sequence is atypical of signal sequences even though it is a native *Pichia* secretion signal. If cloning into the *Xho* I site, we recommend regenerating the full signal sequence between the *Xho* I and *Eco*R I sites (see page 23). However, recent evidence suggests that the *PHO1* signal sequence might have to be modified to include *KEX2*-like processing sites for efficient cleavage to occur (Laroche *et al.*, 1994).

# Cloning into the *Pichia* Expression Vectors

## Introduction

After selecting a vector into which to clone your gene of interest (see pages 11–17), develop a cloning strategy. The *AOX1* promoter and the multiple cloning site are presented on the following pages for each vector along with a summary of considerations for each vector to help you decide on a strategy.


We recommend that you transform the three supercoiled *Pichia* expression vectors into *E. coli* to prepare permanent stock.

- Resuspend each vector in 10 µL sterile water to prepare a 1 µg/µL solution. Store the stock solution at –20°C.
- Use the stock solution to transform competent *E. coli* and select transformants on LB agar plates containing 50–100 µg/mL ampicillin (LB-Amp).

## General Considerations

The following are some general considerations applicable to all vectors.

- The codon usage in *Pichia* is believed to be the same as *Saccharomyces cerevisiae* because many genes have proven to be cross-functional.
- Maintain plasmid constructions in a *rec*A mutant *E. coli* strain such as the TOP10F´ strain provided in the kit.
- The native 5´ end of the *AOX1* mRNA is noted in each multiple cloning site. This information is necessary to calculate the size of the expressed mRNA of the gene of interest.
- Translation termination is determined by either stop codons in the gene of interest or in the 3´ *AOX1* sequence. The stop codons in the 3´ *AOX1* sequence are noted in each figure on the following pages.
- The premature termination of transcripts due to "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 are expressing a gene with high AT content, refer to page 53.
- The predicted protease cleavage sites for the *PHO1* and α-factor signal sequences are indicated in each figure.
- If you are attempting to secrete a protein using its native secretion signal, we recommend that you also try pPIC9 in parallel. When using pPIC9, the open reading frame (ORF) of the mature gene of interest is cloned in frame and downstream of the α-factor.

*Continued on next page*

### General Cloning Strategies

Strategies generally fall into three different categories:

1. Ligation of a compatible restriction fragment:
   a. Forced (directional) insertion involving the use of two different sites in the multiple cloning site (for pPIC3.5, pHIL-S1, or pPIC9 vectors).
   b. Ligation of the fragment with the same restriction end on both ends into a single, compatible site (e.g. *Eco*R I cloning in pHIL-D2).
2. PCR amplification of the fragment containing the gene of interest in such a way that compatible restriction ends are generated for ligation into the appropriate vector.
3. Direct cloning of an amplified fragment containing the gene of interest via the TA Cloning® Kit (see page 88 for ordering), followed by subcloning of a compatible fragment into the appropriate *Pichia* expression vector.

### Cloning Procedures

Refer to (Ausubel *et al.*, 1994), pages 3.16.1 to 3.17.3. or (Sambrook *et al.*, 1989), pages 5.10 to 5.13. for help with cloning.

### Bacterial Transformation

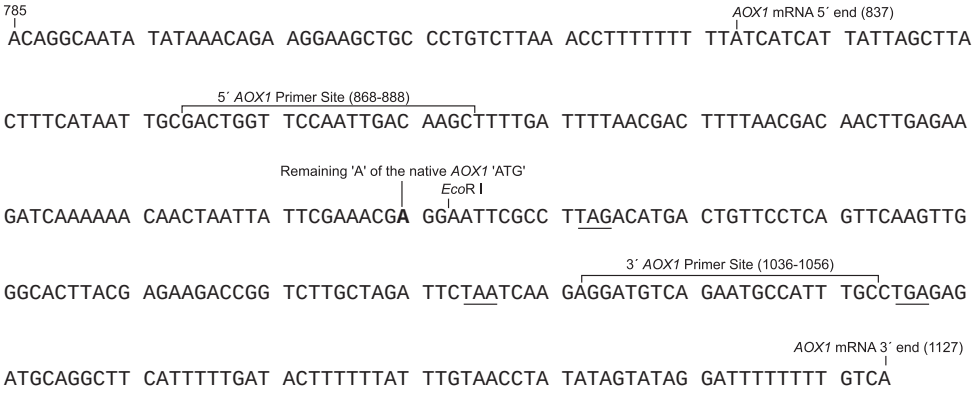
Once you have decided on a cloning strategy, you will need to prepare competent *E. coli* cells for transformation before setting up your ligation reactions. See *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989) for preparation of electrocompetent or chemically competent *E. coli* or use your laboratory's procedure.

For a high-efficiency and convenient transformation, we recommend One Shot® TOP10 Chemically Competent *E. coli* or One Shot® TOP10 Electrocompetent Cells, which are available separately from Invitrogen (see page 88 for ordering information).

*Continued on next page*

### $P_{AOX1}$ and Multiple Cloning Site of pHIL-D2

The figure below shows the detail of the multiple cloning site and surrounding sequences.




### Special Considerations

- For pHIL-D2, the fragment containing the gene of interest should have 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.

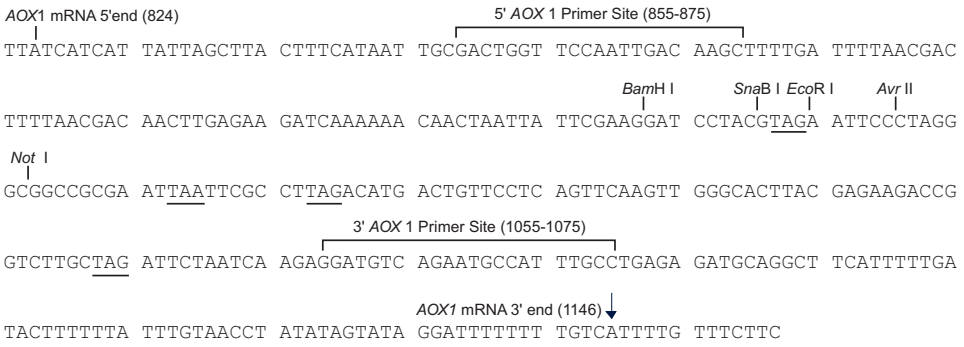
  (A/Y)A(A/T)AATGTCT

  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- to 3-fold effect on the efficiency of translation initiation.
- Shorter, 5´ untranslated leaders reportedly work better in *AOX1* expression. In pHIL-D2, make the untranslated region as short as possible when cloning your gene.
- If your insert has a *Not* I site, refer to page 29 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

*Continued on next page*

### $P_{AOX1}$ and Multiple Cloning Site of pPIC3.5

The sequence below shows the detail of the multiple cloning site and surrounding sequences.




### Special Considerations

- For pPIC3.5, the fragment containing the gene of interest should have 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.

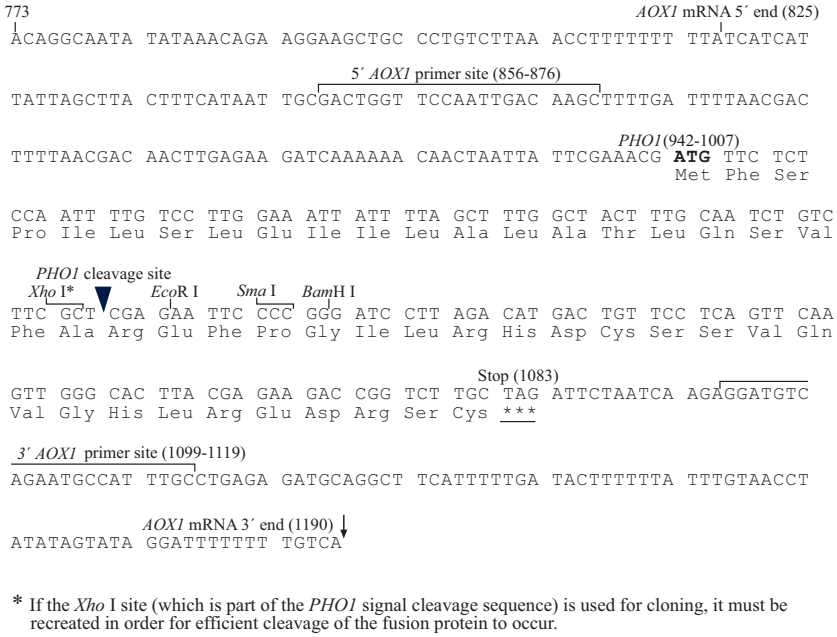
  (A/Y)A(A/T)AATGTCT

  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- to 3-fold effect on the efficiency of translation initiation.
- Shorter, 5´ untranslated leaders reportedly work better in *AOX1* expression. In pPIC3.5, make the untranslated region as short as possible when cloning your gene.
- If you are digesting with *Bam*H I and *Sna*B I or *Sna*B I and *Eco*R I, digest with *Sna*B I first. If you digest with *Bam*H I or *Eco*R I first, the *Sna*B I site will be too close to the end of the DNA and will not digest properly.
- If your insert has a *Bgl* II, *Sac* I, *Sal* I, *or Stu* I site, refer to page 29 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

*Continued on next page*

## $P_{AOX1}$ and Multiple Cloning Site of pHIL-S1

The sequence below shows the detail of the multiple cloning site and surrounding sequences.




* If the *Xho* I site (which is part of the *PHO1* signal cleavage sequence) is used for cloning, it must be recreated in order for efficient cleavage of the fusion protein to occur.

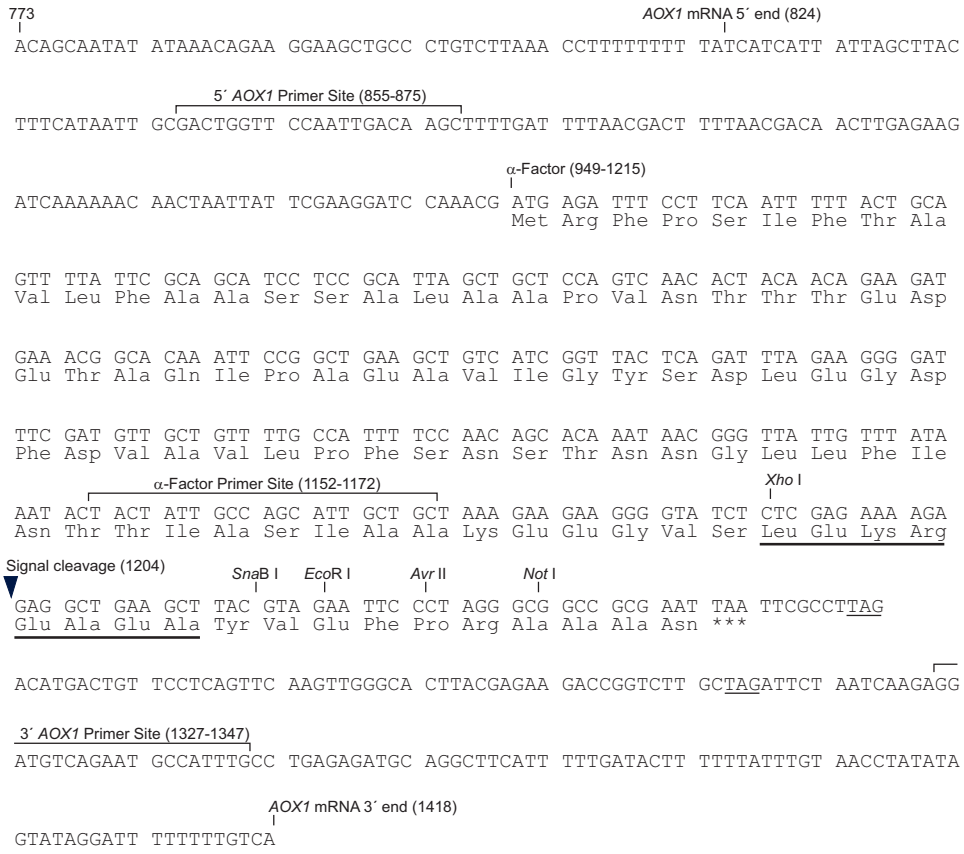
## Special Considerations

- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- If the *Xho* I site is used for cloning, it must be recreated for efficient cleavage of the fusion protein to occur. It is part of the *PHO1* signal peptide sequence.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5′ end of the mRNA.
- If your insert has a *Not* I site, refer to page 29 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.
- The *PHO1* cleavage site has been confirmed for several different fusion proteins by N-terminal peptide sequencing.
- In -factor (pPIC9) or native general, more success has been reported with the secretion signals than with pHIL-S1. This may be due to the lack of *KEX2*-like processing signals (Laroche *et al.*, 1994).

*Continued on next pa*

## $P_{AOX1}$ and Multiple Cloning Site of pPIC9

The sequence below shows the detail of the multiple cloning site and surrounding sequences.




* If cloning into the *Xho* I site, the sequence between the *Xho* I site and *Sna*B I site (underlined) must be recreated in order for efficient cleavage of the fusion protein to occur.

## Special Considerations

- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- If the *Xho* I site is used for cloning, the sequence between the *Xho* I site and the *Sna*B I site encoding the KEX2 site (Glu-Lys-Arg-X) must be recreated for efficient cleavage of the fusion protein to occur. It is part of the α-factor signal peptide sequence. Refer to the discussion on page 18.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5´ end of the mRNA.
- If your insert has a *Bgl* II site, refer to page 30 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

# Transformation into *E. coli*

## Introduction

At this point you have ligation reactions that you will transform by chemical means or electroporation into competent *E. coli* cells (TOP10F´ or equivalent). For procedures to prepare competent cells, refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989).

## Analyzing Transformants

1. After transformation, plate the transformation mix onto LB plates with 50–100 µg/mL ampicillin (see **Appendix**, page 59), and select ampicillin resistant colonies.
2. Pick 10 ampicillin resistant transformants and inoculate into LB medium with 50–100 µg/mL ampicillin. Grow overnight at 37°C with shaking.
3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below). To sequence the *Pichia* expression vectors, use the primers provided.
4. Make a glycerol stock of your desired clone for safekeeping by combining 0.85 mL of an overnight bacterial culture with 0.15 mL of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at –70°C.
5. After you confirm your construct by sequencing, proceed to **Preparing Transforming DNA**, page 27.

## Sequencing Recombinant Clones

We strongly recommend that you sequence your construct before transforming into *Pichia* to confirm the following:

- The correct reading frame (for secretion)
- An ATG in the proper context for eukaryotic translation initiation

Use the primers listed on the next page to sequence your constructs. Resuspend each primer in 20 µL sterile water to prepare a stock solution of 0.1 µg/µL. For the location of the priming sites, see pages 21–24.

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).

*Continued on next page*

| | |
|---|---|
| **5´ *AOX1* Sequencing Primer** | • is 5´ GACTGGTTCCAATTGACAAGC 3´<br>• hybridizes 5´ of the MCS in the *AOX1* promoter region<br>• allows the determination of the 5´ *AOX1*-gene of interest junction<br>• confirms that the ORFs are preserved where necessary |
| **3´ *AOX1* Sequencing Primer** | • is 5´ GCAAATGGCATTCTGACATCC 3´<br>• hybridizes just 3´ of the MCS in the 3´ *AOX1* (TT) region<br>• allows the determination of the 3´ *AOX1*-gene of interest junction |
| **α-Factor Sequencing Primer** | • is 5´-TACTATTGCCAGCATTGCTGC-3´<br>• hybridizes within the -factor leader region in pPIC9<br>• allows the determination of the 5´ end of the gene of interest<br>• confirms that the ORFs are preserved where necessary |

# Preparing Transforming DNA

## Introduction

You should have a *Pichia* multi-copy expression vector with your gene of interest cloned in the correct orientation for expression. The table below describes what you will be doing in the next few sections.

| Step | Action | Pages |
|---|---|---|
| 1 | Prepare your DNA for transformation | 28 |
| 2 | Grow GS115 or KM71 to prepare spheroplasts | 32 |
| 3 | Prepare spheroplasts for transformation | 33 |
| 4 | Transform GS115 or KM71 with your DNA | 35 |
| 5 | Select $His^+$ transformants and characterize for $Mut^+/Mut^S$ phenotype | 38 |
| 6 | Test 10 $His^+$ $Mut^+$ and 10 $His^+$ $Mut^S$ by PCR for integration of your gene | 43 |


We recommend isolating both $His^+$ $Mut^+$ and $His^+$ $Mut^S$ *Pichia* transformants as it is difficult to predict beforehand what construct will best express your protein (see pages 69 and 70). By linearizing your construct DNA in the 5´ *AOX1* region or in the *HIS4* gene and using GS115 ($Mut^+$) and KM71 ($Mut^S$), you can easily isolate $Mut^+$ and $Mut^S$ recombinants. Plan on using ~10 µg digested DNA for each transformation.

## Preparing Plasmid DNA

Plasmid DNA for *Pichia* transformation should be at least pure enough for restriction digestion; however, the cleaner the DNA, the more efficient the transformation. We recommend the PureLink™ HiPure Plasmid Miniprep Kit (see page 88) to prepare plasmid DNA for routine *Pichia* transformations. Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns. You may prepare plasmid DNA using alkaline lysis, phenol:chloroform extraction, and ethanol precipitation.

*Continued on next page*

# Preparing Transforming DNA, continued

## Linearizing Plasmid DNA

We recommend that you linearize your vector in such a manner to generate both Mut$^+$ and Mut$^S$ recombinants. It is possible that one phenotype will express your protein of interest better than the other will.

- To isolate His$^+$ Mut$^+$ transformants of GS115, linearize all constructs with *Sal* I, *Stu* I, or *Sac* I.
- To isolate His$^+$ Mut$^S$ transformants of KM71, linearize plasmid constructs with *Sal* I, *Stu* I, or *Sac* I.

  **Note**: If your insert DNA has all three sites, please see the next page.
- To isolate His$^+$ Mut$^S$ transformants of GS115, linearize plasmid constructs with *Not* I (pHIL-D2) or *Bgl* II (pPIC3.5, pHIL-S1, and pPIC9)

  **Note**: If you wish to generate recombinants that are Mut$^S$, use KM71 because it is much easier and more efficient to generate Mut$^S$ recombinant strains using single crossover events than double crossover events (e.g. insertions at *AOX1* or *his4* as opposed to gene replacement at *AOX1*).

## Procedure

1. Digest both your construct and the parent vector. You will transform GS115 and/or KM71 with the parent vector as a background control for expression.
2. Analyze a small portion of your digest by agarose gel electrophoresis to confirm complete digestion of your fragment. The number of transformants and frequency of targeting will be reduced if digestion is not complete.
3. Extract the digest with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitate the digested DNA. Resuspend DNA pellet in 10–20 µL of TE buffer. It is not necessary to purify the fragment containing your gene away from the rest of the plasmid.
4. Store at –20°C until ready to transform.

*Continued on next page*

### Alternate Restriction Sites

If your insert DNA contains *Sac* I, *Sal* I, and *Stu* I sites, you need to linearize your construct with another enzyme. Use the following table to select another enzyme. A single digestion which linearizes the vector in either of the *AOX1* recombination sequences allows integration, but at lower efficiencies. Remember to digest the parent vector with the same enzyme when preparing your DNA samples for transformation.

**pPHIL-D2**

| Restriction Enzyme | 5´ *AOX1* 14–940 bp | 3´ *AOX1* 4,577–5,333 bp | Vector backbone 5,333+ bp | *HIS4* gene 1,688–4,222 bp |
|---|---|---|---|---|
| *Sac* I | 221 | – | – | – |
| *Pme* I | 424 | – | – | – |
| *Bpu* 1102 I | 599 | – | – | – |
| *Nsi* I | 689 | – | – | – |
| *Xcm* I | 711 | – | – | – |
| *Not* I | 8 | 5,337 | – | – |
| *Dra* I* | 424 | 5,169; 5,311 | 5,896; 6,588 | – |
| *Sal* I | – | – | – | 2,887 |
| *Stu* I | – | – | – | 2,972 |
| *Bsp*E I | – | – | – | 3,554 |

**pPIC3.5**

| Restriction Enzyme | 5´ *AOX1* 1–937 bp | 3´ *AOX1* 4,616–5,393 bp | Vector backbone 5,393+ bp | *HIS4* gene 1,715–4,249 bp |
|---|---|---|---|---|
| *Sac* I | 209 | – | – | – |
| *Pme* I | 414 | – | – | – |
| *Bpu* 1102 I | 589 | – | – | – |
| *Nsi* I | 678 | – | – | – |
| *Xcm* I | 699 | – | – | – |
| *Bgl* II | 2 | 5363 | – | – |
| *Dra* I* | 414 | 5,201; 5,343 | 6,534; 6,553; 7,245 | – |
| *Sal* I | – | – | – | 2,919 |
| *Stu* I | – | – | – | 3,004 |
| *Bsp*E I | – | – | – | 3,586 |

*Continued on next page*

### Alternate Restriction Sites, continued

**pHIL-S1**

| Restriction Enzyme | 5′ *AOX1* 1–940 bp | 3′ *AOX1* 4,639–5,395 bp | Vector backbone 5,395+ bp | *HIS4* gene 1,750–4,284 bp |
|---|---|---|---|---|
| *Sac* I | 209 | – | – | – |
| *Pme* I | 412 | – | – | – |
| *Bpu* 1102 I | 587 | – | – | – |
| *Nsi* I | 677 | – | – | – |
| *Xcm* I | 699 | – | – | – |
| *Bgl* II | 2 | 5,394 | – | – |
| *Dra* I* | 412 | 5,232; 5,374 | 7,021; 7,713 | – |
| *Sal* I | – | – | – | 2,950 |

**pPIC9**

| Restriction Enzyme | 5′ *AOX1* 1–948 bp | 3′ *AOX1* 4,881–5,638 bp | Vector backbone 5,638+ bp | *HIS4* gene 1,980–4,514 bp |
|---|---|---|---|---|
| *Sac* I | 209 | – | – | – |
| *Pme* I | 414 | – | – | – |
| *Bpu* 1102 I | 589 | – | – | – |
| *Nsi* I | 678 | – | – | – |
| *Xcm* I | 699 | – | – | – |
| *Bgl* II | 2 | 5,622 | – | – |
| *Dra* I* | 414 | 5,460; 5,602 | 6,793; 6,812; 7,504 | – |
| *Sal* I | – | – | – | 3,178 |
| *Stu* I | – | – | – | 3,263 |
| *Bsp*E I | – | – | – | 3,845 |

*Restriction sites are used to generate gene replacements at *AOX1* in GS115 only.

# Growing *Pichia* for Spheroplasting

## Introduction

In general, spheroplasting and electroporation (page 73) provide the highest efficiency of transformation for most researchers ($10^3$ to $10^4$ transformants per µg DNA). *Pichia* can also be transformed using PEG 1000 (page 74) or lithium chloride (page 76). These two protocols, particularly lithium chloride, do **not** perform as well as spheroplasting or electroporation. If you do not have an electroporation device, we recommend spheroplasting or using the PEG 1000 method. Transformation in *Pichia* is less efficient than for *Saccharomyces*. For references on general yeast transformation, see (Cregg *et al.*, 1985; Hinnen *et al.*, 1978).

## Spheroplasting

The cell wall of yeast prevents uptake of DNA. To enable yeast to take up DNA, it is necessary to partially remove the cell wall. Zymolyase is a β-glucanase that hydrolyzes the glucose polymers with α1,3 linkages in the cell wall. Addition of Zymolyase partially digests the cell wall. It is critical not to overdigest the cell wall as doing so will cause the cells to die. Zymolyase digestion is monitored by the sensitivity of the cells to SDS. Aliquots of cells are added to SDS, lysing the spheroplasts. This causes a clearing of the solution that is monitored by the absorbance (light-scattering) at 800 nm. It has been empirically determined that when 70% spheroplasting has been achieved, digestion is optimal. Cells are then washed with an isotonic solution to remove the enzyme and incubated with DNA. The cells are resuspended in sorbitol to facilitate cell wall regeneration and plated.

## Preparing Media

Prepare the following media several days in advance and store at 4°C (see **Appendix**, pages 61–64 for details):

YPD (Yeast extract Peptone Dextrose) medium, 1 liter

YPD plates, 1 liter

RDB (Regeneration Dextrose Base) plates, 1 liter

RDHB (Regeneration Dextrose Histidine Base) plates, 1 liter

Prepare the following solution on the day of transformation and maintain at 45°C:

5% SDS solution in water

RD (Regeneration Dextrose), molten agarose, 100 mL

*Continued on next page*

# Growing *Pichia* for Spheroplasting, continued

## Solutions

**Spheroplasting and Transformation Reagents**

**Provided**:

| | |
|---|---|
| 1 M Sorbitol | |
| SE: | 1 M sorbitol, 25 mM EDTA, pH 8.0 |
| DTT: | 1 M DTT in water |
| SCE: | 1 M sorbitol, 1 mM EDTA and 10 mM sodium citrate buffer, pH 5.8 |
| CaS: | 1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$ |
| Zymolyase: | 3 mg/mL in water |
| 40% PEG: | 40% (w/v) PEG 3350 (Reagent grade) in water |
| CaT: | 20 mM Tris, pH 7.5 and 20 mM $CaCl_2$ |
| SOS: | 1 M sorbitol, 0.3X YPD, 10 mM $CaCl_2$ |

**Prepared fresh for each transformation**:

| | |
|---|---|
| SED: | 19 mL of SE and 1 mL of 1 M DTT (see page 33) |
| PEG/CaT: | 1:1 mixture of 40% PEG and CaT (see page 35) |

## Procedure

1. Streak GS115 or KM71 onto a YPD plate such that isolated, single colonies will grow. Incubate the plate at 28–30°C for 2 days.
2. Inoculate 10 mL of YPD in a 50 mL conical tube or 100 mL shake flask with a single colony of GS115 or KM71 from the YPD plate and grow overnight at 28–30°C with vigorous shaking (250–300 rpm). You may store this culture at 4°C for several days.
3. Place 200 mL of YPD in each of three 500 mL culture flasks. Inoculate the flasks with 5, 10, and 20 µL of cells from the culture made in Step 2 and incubate them overnight with vigorous shaking (250–300 rpm) at 28–30°C.
4. The next morning, bring the transformation solutions (SE, SCE, Sterile Water, SOS, PEG, CaS, CaT, 1 M sorbitol) provided in the kit, the RDB plates (for plating transformants), and the RDHB plates (for viability control) to room temperature.
5. Check the $OD_{600}$ of each of the three culture flasks. Harvest the cells from the culture that has an $OD_{600}$ between 0.2 and 0.3. Centrifuge the cells at room temperature for 5–10 minutes at 1,500 × g. Decant the supernatant and discard the other cultures. Proceed to **Preparing Spheroplasts**, page 33.

   **Note**: If the cultures are all over 0.3, choose one of the cultures and dilute (1:4) with fresh medium and incubate at 28–30°C until the $OD_{600}$ is between 0.2 and 0.3 (2–4 hours). Harvest the cells and proceed as in Step 5, above.

# Preparing Spheroplasts

## Before Starting

You should have a cell pellet from Step 5, page 32.

- Prepare 100 mL of molten RD agarose and keep at 45°C (see **Appendix**, page 64)
- Thaw one tube of 1 M DTT (provided in the kit)
- Prepare fresh SED for one batch of spheroplasts as follows:

Using sterile technique, transfer 19 mL of SE (provided) to an appropriate sterile container (e.g., 50 mL conical tube). Add 1 mL of 1 M DTT and mix well. For best results this solution of SED should be made and used immediately.

**Note**

The quality and freshness of DTT is critical for a successful spheroplast preparation. The 1 M DTT provided is analytical reagent grade and must be stored at –20°C.

## Washing the Cells

1. Wash the cells from Step 5, page 32 by resuspending the pellet in 20 mL of sterile water (provided). Resuspend the pellet by swirling the tube. Transfer to a sterile, 50 mL conical tube.
2. Pellet the cells by centrifugation at 1,500 × g for 5 minutes at room temperature. Decant and discard the supernatant. The cell pellet will be used to prepare spheroplasts.
3. Wash the cell pellet once by resuspending in 20 mL of fresh SED, prepared above and centrifuge at 1,500 × g for 5 minutes at room temperature.
4. Wash the cells once with 20 mL of 1 M sorbitol and centrifuge as described in Step 2.
5. Resuspend the cells by swirling in 20 mL of SCE buffer and divide the suspension into two 50 mL conical tubes (~10 mL each).
6. Remove one tube of Zymolyase from –20°C and place it on ice. Mix well by flicking the tube several times. Zymolyase is provided as a slurry and does not go into solution. It is important to mix the slurry thoroughly before each use to ensure addition of a consistent amount of Zymolyase.

## Adding Zymolyase

Use one tube of cells prepared above to determine the optimal time of digestion with Zymolyase to make spheroplasts. After you determine the optimal, use the other tube of to make spheroplasts.

Zymolyase digests the cell wall and makes the cells extremely fragile. **Handle the sample gently**. The moment after adding Zymolyase, the cell wall begins to get digested.

- Prepare at least 20 mL of a 5% SDS solution (not provided) for use below.
- Set your UV-Vis spectrophotometer to 800 nm and blank with 800 µL 5% SDS and 200 µL SCE.
- Set up 17 sterile microcentrifuge tubes and label them 0, 2, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50. Add 800 µL of 5% SDS to each tube.

*Continued on next page*

### Adding Zymolyase, continued

1. From one tube of cells (Step 5, page 33), withdraw 200 µL cells and add to the tube marked "0". This is your zero time point. Set the tube aside on ice.
2. Add 7.5 µL of Zymolyase to the same tube of cells, mix it gently by inversion, and incubate the cells at 30°C. **Do not shake the sample.** This sample will be used to establish the incubation time for optimal spheroplasting as described below. Keep the second tube of cells at room temperature for use in Step 6 below. Keep the remainder of the Zymolyase on ice.
3. Monitor the formation of the spheroplasts as follows: At time 2 minutes, withdraw 200 µL of cells (from the suspension in Step 2) and add to the tube marked "2". Repeat at time t= 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 minutes after adding Zymolyase. Read the $OD_{800}$ for all samples.
4. Determine the percent of spheroplasting for each time point using the equation:

   % Spheroplasting = 100 – [($OD_{800}$ at time t/$OD_{800}$ at time 0) × 100]

   **For example**: At time t = 0, the $OD_{800}$ = 0.256

   At time t = 15, the $OD_{800}$ = 0.032

   **Calculation:** % spheroplasting = 100 – [(0.032/0.256) × 100]

   = 100 – [(0.125) × 100]

   = 100 – 12.5

   = 87.5%
5. Determine the time of incubation that results in approximately 70% spheroplasting. This time of incubation is variable due to differences in lots of Zymolyase. In Invitrogen labs, it takes approximately 15–40 minutes of Zymolyase treatment to achieve optimal spheroplasting.

   **Note**: It is important to establish the minimum time required for the desired amount of spheroplasting. Prolonged incubation with Zymolyase is deleterious to spheroplasts and will result in lower transformation efficiency.
6. Add 7.5 µL Zymolyase to the remaining tube of cells as described in Step 1 above. Incubate the tube at 30°C for the time that was established in Step 5 to obtain the optimal level (70%) of spheroplasting.
7. Harvest the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature. Decant and discard the supernatant.
8. Wash the spheroplasts once with 10 mL of 1 M sorbitol (**gently** disperse the pellet by tapping the tube, **do not vortex**). Collect the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature.
9. Wash the spheroplasts once with 10 mL of CaS and centrifuge as in Step 7. **Gently** resuspend the spheroplasts in 0.6 mL of CaS. The spheroplasts must be used immediately (up to 30 minutes) for transformation (page 35). They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations.

# Transforming *Pichia*

## Before Starting

Make sure your RDB plates are at room temperature and that you have molten RD top agarose available. Thaw your linearized DNA and keep on ice. You should have the following:

- Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of $His^+$ $Mut^+$ recombinants in GS115
- Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of $His^+$ $Mut^S$ recombinants in KM71
- Your construct linearized with *Not* I, *Bgl* II, or equivalent to favor isolation of $His^+$ $Mut^S$ recombinants in GS115
- Parent plasmid linearized with same restriction enzyme

Controls should include no DNA or linearized pBR322 DNA and plasmid only (no cells) to check for contamination.

## Procedure

1. For each transformation, dispense 100 µL of the spheroplast preparation from Step 9 (previous page) into a sterile 15 mL snap-top Falcon 2059 tube (or equivalent).
2. Add 10 µg of DNA and incubate the tube at room temperature for 10 minutes.
3. During the 10 minute incubation, make a fresh PEG/CaT solution. Since each transformation requires 1.0 mL of the PEG/CaT solution, calculate the amount you need and prepare this volume by adding together equal volumes of 40% PEG and CaT (a 1:1 solution).
4. Add 1.0 mL of fresh PEG/CaT solution to the cells and DNA, mix gently, and incubate at room temperature for 10 minutes.
5. Centrifuge the tube at 750 × g for 10 minutes at room temperature and carefully aspirate the PEG/CaT solution. Invert the tube and tap it gently to drain the excess PEG/CaT solution.
6. Resuspend the pellet of transformed cells in 150 µL of SOS medium and incubate it at room temperature for 20 minutes.
7. Add 850 µL of 1 M sorbitol. Proceed to **Plating**, next page.

*Continued on next page*

## Plating

*Pichia* spheroplasts need to be plated in top agarose or agar to protect them from lysis prior to selection.

1. Mix together 100–300 µL of each spheroplast-DNA solution from Step 7, previous page, with 10 mL of molten RD agarose and pour on RDB plates. Allow the top agarose to harden.

   **Note:** There is enough of the spheroplast-DNA solution to plate duplicate and triplicate plates.
2. Invert plates and incubate at 28–30°C. Transformants should appear in 4–6 days.
3. For cell viability: Mix 100 µL of spheroplasts with 900 µL of 1 M sorbitol.
4. Mix 100 µL of this diluted sample with 10 mL of molten RDH and pour on a RDHB plate. Allow top agarose to harden.
5. Invert the plates and incubate at 28–30°C. Appearance of colonies after 4–6 days demonstrates that the spheroplasts can regenerate into dividing cells.

## Evaluating Your Transformation Experiment

After 4–6 days, $His^+$ transformants on your sample plates will become apparent. Transformation efficiency using the spheroplast method is generally $10^3$ to $10^4$ $His^+$ transformants/µg of DNA. There should be no colonies on the "No DNA", pBR322 plate, or the plasmid only (no cells) plate

## Optional Method

Plating in top agarose can cause the transformants to be on top or be imbedded in the top agarose, making it difficult to pick and patch colonies as described in the next section. The following protocol allows you to collect the transformants and re-plate them directly onto plates without using top agarose.

1. Scrape the agarose containing the $His^+$ transformants with a sterile spreader into a sterile, 50 mL, conical centrifuge tube and mix with 20 mL sterile deionized water. Vortex the suspension vigorously to separate the cells from the agarose.
2. Filter the suspension through 4 folds of sterile cheesecloth. Centrifuge the filtrate at 1,500 × g for 5 minutes at room temperature. Centrifugation pellets the cells on the bottom of the tube and any remaining agarose on top of the cells.
3. Remove the agarose pellet carefully from the top of the cells by gently shaking the tube to disperse only the agarose pellet into the water. Decant the supernatant with the agarose pellet.
4. Resuspend the cell pellet in 5 mL of sterile deionized water and sonicate for 10 seconds using a microtip and 20–30% power. Sonicate to get the cells into solution and not to lyse the cells.
5. Dilute cells by $10^4$ and plate 50 µL and 100 µL onto MD plates. Incubate the plates overnight at 30°C. Proceed to **Screening for $Mut^+$ and $Mut^S$ Transformants,** page 38.

### Chemically Competent *Pichia* Cells

The *Pichia* EasyComp™ Kit (see page 88) provides enough reagents to produce 6 preparations of competent cells; each preparation yields enough competent cells for 20 transformations. You may use these cells immediately or store them frozen for future use. Each 50 µL aliquot of competent *Pichia* cells with 3 µg linearized plasmid DNA yields approximately 50 colonies on selective medium. Contact Technical Support for more information (page 89).

### Screening by Functional Assay

Some researchers have used a functional assay to directly test for high expressing *Pichia* recombinant clones without first screening for $Mut^S$ or $Mut^+$ phenotypes. After testing for high expression, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

# Screening for Mut$^+$ and Mut$^S$ Transformants

## Introduction

After colonies appear on plates of His$^+$ GS115 transformants, score for the Mut$^+$ and Mut$^S$ phenotypes. The kit contains two strains that will provide examples of Mut$^+$ and Mut$^S$ phenotypes. GS115 Albumin is Mut$^S$ and GS115 β-Gal is Mut$^+$. His$^+$ KM71 recombinants do not need to be screened for their Mut phenotype as they all will be Mut$^S$.

Remember also to isolate two control strains for background protein expression in *Pichia*. One control is the parent plasmid linearized in such a way to generate His$^+$ Mut$^S$ transformants. The other control is the parent plasmid linearized to generate His$^+$ Mut$^+$ transformants.

## Screening for His$^+$ Mut$^+$ in GS115

Transformation of GS115 with *Sal* I- or *Stu* I-linearized constructs favor recombination at the *HIS4* locus. Most of the transformants should be Mut+; however, with the presence of the *AOX1* sequences in the plasmid, there is a chance that recombination will occur at the *AOX1* locus, disrupting the wild-type *AOX1* gene and creating His$^+$ Mut$^S$ transformants. Testing on Minimal Dextrose (MD) and Minimal Methanol (MM) plates allows you to isolate His$^+$ Mut$^+$ transformants (see next page).

## His$^+$ Mut$^S$ in KM71

There is no need to test recombinants for the Mut phenotype in KM71; all His$^+$ transformants in KM71 will be Mut$^S$ because of the disruption of the *AOX1* gene (*aox1::ARG4*). Transforming KM71 with *Sal* I- or *Stu* I- linearized plasmid constructs favor recombination at the *HIS4* locus while *Sac* I-linearized plasmid constructs favor recombination at the 5´ region of the *AOX1* gene. Purify His$^+$ transformants on minimal plates without histidine to ensure pure clonal isolates before testing for expression (see page 45) or confirming integration by PCR (page 43).

## Screening for His$^+$ Mut$^S$ in GS115

Transforming GS115 with *Not* I-linearized pHIL-D2 or *Bgl* II-linearized pPIC3.5, pHIL-S1, and pPIC9 constructs favors recombination at the *AOX1* locus. Displacement of the alcohol oxidase (*AOX1*) structural gene occurs at a frequency of 5–35% of the His$^+$ transformants. Patching or replica-plating on Minimal Dextrose (MD) versus Minimal Methanol (MM) plates can readily distinguish Mut$^+$ and Mut$^S$ transformants.

Because Mut$^S$ transformants do not produce alcohol oxidase (the product of the *AOX1* gene), they cannot efficiently metabolize methanol as a carbon source; therefore, they grow poorly on minimal methanol (MM) medium. This slow growth on methanol can be used to distinguish His$^+$ transformants, in which the *AOX1* gene has been disrupted (His$^+$ Mut$^S$), from His$^+$ transformants with an intact *AOX1* gene (His$^+$ Mut$^+$).

*Continued on next page*

# Screening for Mut$^+$ and Mut$^S$ Transformants, continued

**Materials Needed**

You can prepare the following media (see page 64) and materials several days in advance, and store at 4°C :

- Minimal Dextrose (MD) agar plates, 1 liter
- Minimal Methanol (MM) agar plates, 1 liter
- Sterile toothpicks and Scoring Templates (see page 42)
- Streak out the strains GS115 Albumin (His$^+$ Mut$^S$) and GS115 β-Gal (His$^+$ Mut$^+$) on an MD or MGY plate as controls for Mut$^+$ and Mut$^S$ growth on MD and MM plates.

**Note**

In contrast to His$^+$ Mut$^S$ transformants generated using a construct linearized with *Not* I or *Bgl* II, most of the His$^+$ transformants generated by the *Sac* I, *Sal* I ,or *Stu* I-digested construct should be Mut$^+$ arising from gene insertion events at the *his4* or *AOX1* loci, leaving an intact *AOX1* locus.

**His$^+$ Mut$^S$ or His$^+$ Mut$^+$ in GS115**

Use the plates containing His$^+$ transformants, and screen for the Mut$^+$ and Mut$^S$ phenotypes as described below.

1. Using a sterile toothpick, pick one His$^+$ colony, and streak or patch it in a regular pattern on an MM plate **and** an MD plate.
   **Note**: Make sure to patch the MM plate first.
2. Use a new toothpick for each transformant, and continue picking transformants until you have patched 100 transformants (2–3 plates).
3. To differentiate Mut$^+$ from Mut$^S$, make one patch for each of the controls (GS115/His$^+$ Mut$^S$ Albumin and GS115/His$^+$ Mut$^+$ β-gal) onto the MD and MM plates.
4. Incubate the plates at 30°C for 2 days.
5. After 2 days or longer at 30°C, score the plates. Mut$^+$ transformants grow well on both MD and MM plates. Mut$^S$ transformants grow well only on MD plates, and show little or no growth on the MM plates.

**Important**

We recommend that you purify your His$^+$ transformants to ensure pure clonal isolates. You may do this before or after testing for the Mut phenotype.

*Continued on next page*

# Screening for Mut$^+$ and Mut$^S$ Transformants, continued

## Replica-Plating Procedure

The following procedure gives a lower rate of misclassifications, but it increases the overall Mut$^+$/Mut$^S$ screening procedure by 2 days. For the procedure, you need replica-plating equipment.

1. Using sterile toothpicks, patch 100 His$^+$ transformant on MD plates (2–3 plates). For controls, make one patch from each of the strains GS115/His$^+$ Mut$^S$ Albumin and GS115/His$^+$ Mut$^+$ β-gal onto the MD plates.
2. Incubate the plates at 28–30°C for 2 days.
3. After 2 days, replica-plate the patches from the MD plates onto fresh MM and MD plates to screen for Mut$^S$ 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 MD replica plates, but show little or no growth on the MM replica plates. Including His$^+$ Mut$^+$ and His$^+$ Mut$^S$ control patches on each plate will provide examples of Mut$^+$ and Mut$^S$ phenotypes.

## 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$^S$ or Mut$^+$ phenotypes. If you elect to screen directly for high-expressing recombinants, be sure to also check the Mut phenotype to help you optimize expression of your recombinant clone.

## 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 72 for figure). Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants (in this case, His$^+$ 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* or *HIS4* loci. In addition to simply screening expression levels among several His$^+$ Mut$^S$ or His$^+$ Mut$^+$ recombinants via SDS-PAGE analysis, it may be desirable to determine the existence of strains that have multiple integrants in the His$^+$ Mut$^S$ or His$^+$ Mut$^+$ recombinant strain.

Refer to the **Appendix**, page 81, for methods to detect multiple integration events.

*Continued on next page*

# Screening for Mut$^+$ and Mut$^S$ Transformants, continued

### Vectors for Multiple Integration

Three vectors for isolating or generating *Pichia* recombinants containing multiple integrations of your desired gene are available separately from Invitrogen (see page 88 for ordering information). Two of the vectors, pPIC3.5K and pPIC9K, are used *in vivo* to identify possible transformants with multiple copies of your gene. The other vector, pAO815, is used for creating tandem copies of your gene *in vitro* before transforming into *Pichia*.

### pPIC3.5K and pPIC9K

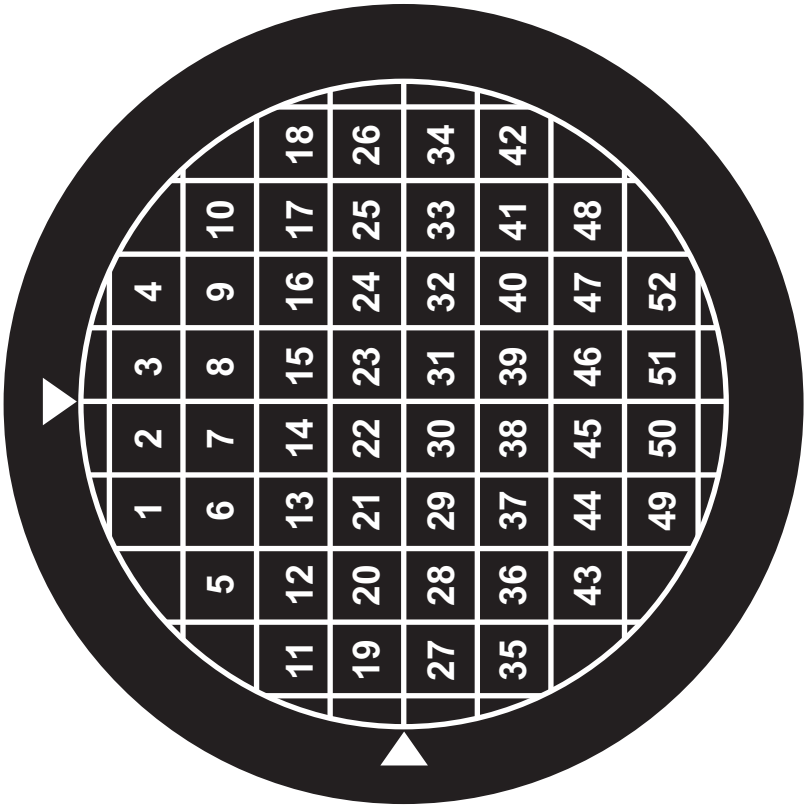
The vectors pPIC3.5K (for intracellular expression) and pPIC9K (for secreted expression) contain the bacterial kanamycin resistance gene cloned between the *HIS4* gene and the 3´ *AOX1* region. The kanamycin resistance gene confers resistance to G418 in *Pichia*. Multiple insertions of the kanamycin gene into the *Pichia* chromosome increase the resistance to G418. Because the kanamycin resistance gene is linked to your gene, isolation of hyper-resistant G418 transformants may also indicate that your gene is present in multiple copies.

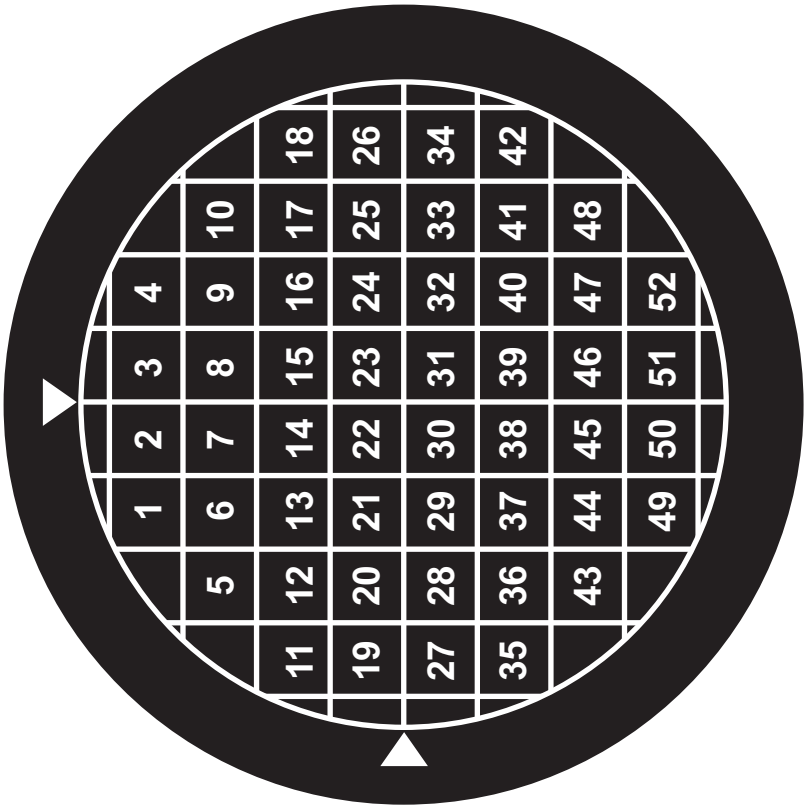
### pAO815

The vector pAO815 (for intracellular expression) allows you to generate multiple copies of your gene *in vitro* by creating an expression cassette (e.g., $P_{AOX1}$–your gene of interest–HIS4), and cloning multiple copies in tandem in the vector. The vector is then transformed into *Pichia* and transformants are selected and tested for increased expression of the desired protein.

## Screening for Mut$^{+}$ and Mut$^{S}$ Transformants, continued

**Scoring Templates**



# PCR Analysis of *Pichia* Integrants

## Introduction

Use the following protocol to analyze *Pichia* integrants to determine if the gene of interest has integrated into the *Pichia* genome. Isolate genomic DNA from 6 to 10 $Mut^S$ or $Mut^+$ *Pichia* clones and the strain transformed with the parent plasmid using the protocol on page 79. After isolating your DNA, use the procedure below to identify integrants. Amplify the gene of interest using the α-factor primer (for pPIC9 only) or the 5´ *AOX1* primer paired with the 3´ *AOX1* primer included in the kit. This protocol can confirm the integration of the gene of interest but does not provide information on the site of integration.

**Note**: A more direct method for PCR screening is available in the **Appendix**, page 78.

## Isolating Genomic DNA from *Pichia*

Invitrogen's Easy-DNA™ Kit provides a fast and easy method to isolate genomic DNA from *Pichia pastoris*. See page 88 for ordering information.

## Analysis by PCR

1. Set up PCR reactions as follows:

| | |
|---|---|
| 10X PCR Buffer | 5 µL |
| Genomic DNA (~1 µg) | 5 µL |
| 100 mM dNTPs (25 mM each) | 1 µL |
| 5´ *AOX1* Primer (0.1 µg/µL) | 5 µL* |
| 3´ *AOX1* Primer (0.1 µg/µL) | 5 µL* |
| Sterile water | to 50 µL |
| *Taq* Polymerase (5 U/µL) | 0.25 µL |

*Resuspend the primers in 20 µL sterile water to prepare a 0.1 µg/µL solution. You may decrease the amount of primer. For ~20 pmoles primer, use 2 µL of each resuspended 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 |
|---|---|---|---|
| Hot Start | 94°C | 2 minutes | 1X |
| Denaturation | 94°C | 1 minute | |
| Annealing | 55°C | 1 minute | 25–35X |
| Extension | 72°C | 1 minute | |
| Final Extension | 72°C | 7 minutes | 1X |

3. Analyze 10 µL on a 1X TAE, 0.8 % agarose gel.

*Continued on next page*

# PCR Analysis of *Pichia* Integrants, continued

## Interpreting PCR

If screening Mut$^+$ 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). If screening Mut$^S$ integrants in GS115, you should see only the band that corresponds to the gene of interest. In KM71, because of the *ARG4* insert in *AOX1*, the PCR product is 3.6 kb. Parent plasmids produce the following sized PCR products. Add these fragments to the size of your insert to interpret your PCR results.

| Vector | PCR Product |
|---|---|
| pHIL-D2 | 188 bp |
| pPIC3.5 | 214 bp |
| pHIL-S1 | 262 bp |
| pPIC9 (using the 5´ *AOX1* primer) | 492 bp |
| pPIC9 (using the α-Factor primer) | 195 bp |

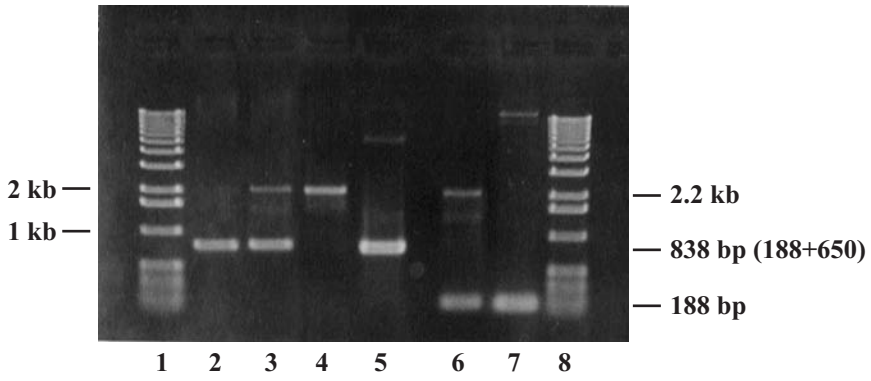
**Important**

If you use the α-factor primer as a PCR primer, you will not see a band with GS115 or KM71. This is because there is no α-factor signal associated with the chromosomal *AOX1* gene.

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.

## Example of PCR Analysis

The figure below shows the results of a typical PCR analysis using the procedure on the previous page. Genomic DNA was isolated from *Pichia* recombinants and from appropriate controls. Ten microliter samples from each PCR were run on a 0.8% agarose gel. Lanes 1 and 8 contain markers for a 1 kb ladder; Lanes 2–4 are *Pichia* recombinants; Lane 5 is pHIL-D2 with the gene of interest; Lane 6 is GS115/pHIL-D2 (no insert); and Lane 7 is pHIL-D2 alone.




## Discussion

Lane 7 shows the 188 bp PCR product made from pHIL-D2 by priming with the 5´ and 3´ *AOX1* primers (see page 26). Lane 6 shows the 188 bp product and the wild-type *AOX1* gene (2.2 kb) from GS115/pHIL-D2. Lane 5 shows the expected size of our gene of interest cloned into pHIL-D2 (650 bp + 188 bp = 838 bp). Analysis of the *Pichia* recombinants in lanes 2–4 reveal that lanes 2 and 3 contain the insert, and that the recombinant in lane 2 may be a Mut$^S$ because there is no wild-type *AOX1*. Lane 4, although from a His$^+$ transformant, does not contain the gene of interest.

# Expression of Recombinant *Pichia* Strains

## Introduction

The purpose of this section is to determine the optimal method and conditions for expressing your gene. Below are some factors and guidelines that 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.

## Media

You need BMGY/BMMY (buffered complex glycerol or methanol medium), BMG/BMM (buffered minimal glycerol or methanol medium), or MGY/MM (minimal glycerol or minimal methanol medium) for expression (see **Appendix**, pages 63–65). BMG, BMM, BMGY, and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein. Unlike MGY and MM, they are all buffered media. Because these media are buffered with phosphate buffer, you may use a wide range of pH values to optimize production of your protein. BMGY/BMMY contain yeast extract and peptone which may help stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone act as a "mixed feed" allowing 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 MGY and MM media may be indicated. As *Pichia* expression progresses in an unbuffered medium such as MM, 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, you may want to do your expressions in an unbuffered medium (MM). 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, you may then try expression in an unbuffered medium.

If the above options fail to protect your protein from degradation, you may wish to transform your gene into SMD1168. This strain genotype is *his4 pep4* and is deficient in proteinase A activity. You can use the same procedures for transforming and expressing GS115 with SMD1168. You can also use this strain in large-scale fermentation.

## 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. We strongly recommend that you use baffled flasks. See **Appendix**, page 66 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.)

*Continued on next page*

### Kinetics of Growth

Note that while Mut$^+$ and Mut$^S$ strains grow at essentially the same rate in YPD or glycerol media, Mut$^+$ grows faster than Mut$^S$ when both are grown on methanol because of the presence of the *AOX1* gene product.

### Temperature and Shaking

Perform expression 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.

### Before Starting

After you have verified recombinants in GS115 or KM71 as well as a control recombinant of GS115 or KM71/Vector (no insert), proceed with expression. When performing your expression, it is important to run the proper controls so that you will be able to interpret your expression results. You should use the following expression controls:

| | |
|---|---|
| GS115/His$^+$ Mut$^S$ albumin | Mut$^S$- Secretion control |
| GS115/His$^+$ Mut$^+$ β-Gal | Mut$^+$- Intracellular control |
| GS115 or KM71/Vector (no insert) | Background control |

Recombination can occur in many different ways that can affect expression. 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 information is designed 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 (see page 66). If you are analyzing a number of recombinants, you can try 50 mL conical tubes. Make sure that the medium is well-aerated by increasing the rate of shaking or placing the tubes at an angle in the shaker.

*Continued on next page*

### Mut$^+$ Intracellular or Secreted

Test the effectiveness of your expression conditions by growing GS115 β-Gal, which is Mut$^+$ and expresses β-galactosidase intracellularly. Include GS115 or KM71 transformed with the parent vector as a control for background intracellular expression.

1. Using a single colony, inoculate 25 mL of MGY, BMG, or BMGY medium in a 250 mL baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an $OD_{600}$ = 2–6 (log-phase growth, approximately 16–18 hours).
2. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. Decant the supernatant and resuspend the cell pellet to an $OD_{600}$ of 1.0 in MM, BMM, or BMMY medium (approximately 100–200 mL) to induce expression.
3. Place the culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth, and return it to the incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction. Be sure to check the volume of the culture, and add methanol accordingly. Evaporation may reduce the culture volume.
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 to determine the optimal time post-induction to harvest. Centrifuge the samples at maximum speed in a tabletop microcentrifuge at room temperature for 2–3 minutes.

   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.** Freeze the supernatant and the cell pellets quickly in liquid $N_2$ or a dry ice/alcohol bath, and store them at –80°C until ready to assay..

   For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid $N_2$ or a dry ice/alcohol bath.
7. Analyze the supernatants and the cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot, or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, page 49).

*Continued on next page*

### Mut$^S$ Intracellular or Secreted

You can test the effectiveness of your expression conditions by growing GS115, which is Mut$^S$ and secretes albumin to the medium. Remember to include GS115 or KM71 transformed with the parent vector as a control for background intracellular expression.

1. Using a single colony, inoculate 100 mL of MGY, BMG, or BMGY medium in a 1 liter baffled flask. Grow the culture at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an $OD_{600}$ = 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 the cell pellet in MM, BMM, or BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10–20 mL).
3. Place the cells in a 100 mL baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth, and return them to the 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 $N_2$ 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 $N_2$ 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 **Analyzing Samples by SDS-Polyacrylamide Gel Electrophoresis**, next page).

# Analyzing Samples by SDS-Polyacrylamide Gel Electrophoresis

## Polyacrylamide Gel Electrophoresis

Invitrogen offers a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels and electrophoresis apparatus. The patented NuPAGE® Gel System avoids the protein modifications associated with LaemmLi-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen 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 www.invitrogen.com or contact Technical Support (page 89).

If you are pouring your own gels, 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 *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989), *Guide to Protein Purification* (Deutscher, 1990), or *Protein Methods* (Bollag *et al.*, 1996).

## Preparing Samples

You will need to prepare Breaking Buffer (see page 66) and have acid-washed 0.5 mm glass beads on hand.

**Preparing of cell pellets (Intracellular and Secreted Expression):**

1. Thaw cell pellets quickly and place on ice.
2. For each 1 mL sample, add 100 µL Breaking Buffer to the cell pellet and resuspend.
1. 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 µL of supernatant (cell lysate) and mix with an appropriate volume of denaturing PAGE Gel Loading buffer (Sample Buffer).
7. Heat the sample as recommended and load 10–20 µL per well. Thickness of the gel and number of wells will determine volume loaded. 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.

*Continued on next page*

# Analyzing Samples by SDS-Polyacrylamide Gel Electrophoresis, continued

## Preparing Samples, continued

**Preparing of supernatant (Secreted Expression only):**

1. Thaw supernatants and place on ice.
2. Mix 50 µL of the supernatant with an appropriate volume of denaturing PAGE Gel Loading buffer.
3. Heat the sample as recommended; then load 10–30 µL onto the gel. Remaining sample may be stored at –20°C for western blots, if necessary. Supernatants may be stored at –80°C for further analysis.
4. Analyze Coomassie-stained gel and western blot (if necessary) for your protein.
5. If no protein is seen by Coomassie or by western blot, then concentrate the supernatant 5–10 fold and analyze samples again by western blot. Centricon and Centriprep filters (Millipore) are very useful for this purpose.

## Protein Concentration

Lowry, BCA (Pierce) or Bradford protein determinations can be performed to quantify the amounts of protein in the cell lysates and medium supernatants. In general, *Pichia* cell lysates contain 5–10 µg/µL 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 µg/mL, 10 µL of medium will give a faint band on a Coomassie-stained SDS-PAGE gel.

*Continued on next page*

# Analyzing Samples by SDS-Polyacrylamide Gel Electrophoresis, continued

## 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 GS115 or KM71 with the parent plasmid transformed into it. This control 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 β-Gal 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 depends on several factors, including its expression level, its solubility, its molecular weight, and whether an abundant cellular protein of the same size masks it. 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.

## 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 there is no recombinant protein visible, then perform a western blot or a functional assay if you have one.

If you detect low expression of your recombinant protein, see **Optimizing *Pichia* Protein Expression**, page 52.

Test your expression conditions with the one of the two control strains included in the kit (GS115 β-Gal or Albumin).

If there is no indication of expression at all, perform a northern analysis to see if and how much full-length mRNA is induced. See page 84 for an RNA isolation protocol.

# Optimizing *Pichia* Protein Expression

## Introduction

Based on available data, there is approximately a 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.

## 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 (MM). In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.
- Try using SMD1168 (proteinase A-deficient) for expression (see **Proteases**, page 45).

## 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 α-factor signal sequence).
- Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration (see page 56).
- For Mut$^+$, induce expression with a higher density culture.

## Low Expression Levels

- Check both Mut$^+$ and Mut$^S$ 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, page 58).
- Scale up to fermentation (page 56). *Pichia* is a yeast and is particularly well suited to fermentation.

*Continued on next page*

### No Expression

Be sure to try some of the easier things solutions on the previous page because very low expression may be perceived as no expression. If none of those improve protein expression, 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 84).

Analyze for the presence of your insert by PCR (see page 43). You can reasonably analyze 12–20 transformants by PCR. Remember to include the vector only and original (one copy) construct controls in order to analyze your PCR experiment.

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

### Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression to bypass the secretion pathway and to avoid protein modification.
- Try deglycosylating the protein with Peptide:N-Glycosidase F or other enzymes (see page 57).
- Engineer your gene to remove N-linked glycosylation sites.

# Scaling Up Expression

## Guidelines for Expression

Once expression is optimized, scale-up your expression protocol to produce more protein by increasing the culture volume using larger baffled flasks (below) or by fermentation. Use the guidelines below to scale-up your expression protocol. To purify your protein, see the references listed on page 57.

## Mut$^+$ 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 1,500–3,000 × 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 × g for 5 minutes at room temperature.
7. 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 it to 4°C, and concentrate it, if desired** (see page 56). Proceed directly to purification (page 57) or store the supernatant at –80°C until ready to process further.

*Continued on next page*

### Mut$^S$ 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 $OD_{600}$ = 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 ($OD_{600}$ = 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.
7. 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 it to 4°C, and concentrate it, if desired** (see next page). Proceed directly to purification (page 57) or store the supernatant at –80°C until ready to process further.

**Note**

To increase the amount of cells for Mut$^S$ recombinants, increase the number of flasks, put 200–300 mL in a 3 liter flask, or try fermentation.

*Continued on next page*

### Concentrating Proteins

Proteins secreted into the media are usually >50% homogeneous and require some additional purification. 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 Millipore)
- Pressurized cell concentrators for large volumes (e.g., Amicon ultrafiltration devices available from Millipore)
- Lyophilization

A general guide to protein techniques is *Protein Methods* (Bollag *et al.*, 1996).

### 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).

### 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 for more information (page 89).

# Protein Purification and Glycosylation

## Introduction

At this point, you have an optimized protocol for expressing your protein and a method to scale-up production of your protein for large-scale purification. You may already have a method to purify your protein. Since every protein is different, it is difficult to recommend specific techniques for purification. For an overview of methods for purification see (Deutscher, 1990) or (Ausubel *et al.*, 1994).

## Some Protein Purification Techniques

Some techniques are listed below and are discussed thoroughly in *Guide to Protein Purification* (Deutscher, 1990). Be sure to perform all steps from cell lysis to purified protein at 4°C.

| | |
|---|---|
| Ion-Exchange Chromatography | Gel Filtration |
| Affinity Chromatography | Chromatofocusing |
| Isoelectric Focusing | Immunoprecipitation |
| Solubilization (Membrane Proteins) | Lectin Affinity Chromatography |

## Procedure for Cell Lysis

Prepare Breaking Buffer (BB) as described in **Appendix**, page 66.

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 $OD_{600}$ 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 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 5–10 mg/mL.
7. Save the pellet and extract with 6 M urea or 1% Triton X-100 to check for insoluble protein.


Biospec (Bartlesville, OK) makes a Bead Beater™ that can handle 5–200 mL volumes of cell suspension.

## Analysis of 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. For carbohydrate analysis of proteins to characterize glycosylated proteins, see (Ausubel *et al.*, 1994), Unit 17. Further information about glycosylation in eukaryotes is available in a review (Varki & 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 |
| β-galactosidase | Exo | Removes terminal galactosides from Gal-β1,3-GlcNAc, Gal-β1,4-GlcNAc or Gal-β1,3-GalNAc. |
| Peptide:N-Glycosidase F | Endo | Glycoproteins between Asn and GlcNAc (removes oligosaccharides) |
| Sialidases (Neuraminidases)<br>*Vibrio cholerae*<br>*Clostridium perfringens*<br>*Arthobacter ureafaciens*<br>Newcastle disease virus | Exo | NeuAc-α2,6-Gal, NeuAc-α2,6-GlcNAc or NeuAc-α2,3-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 | Contact |
|---|---|---|
| Glyko | Kits for Carbohydrate Analysis<br>Reagents<br>Contract Services | 1-800-334-5956<br>www.prozyme.com |
| New England BioLabs | Reagents | 1-800-632-5227<br>www.neb.com |

# Appendix

# *E. coli* Media Recipes

## Introduction

You select transformants with pAO815, pPIC3K, or pPIC9K on LB agar containing 50 to 100 µg/mL ampicillin.

## LB (Luria-Bertani) Medium

1% Tryptone
0.5% Yeast Extract
1% NaCl
pH 7.0

1. For 1 liter, dissolve the following in 950 mL deionized water
   10 g tryptone
   5 g yeast extract
   10 g NaCl
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave the medium for 20 minutes at 15 lbs/sq. in. Let it cool to ~55°C and add desired antibiotics at this point.
4. Store the medium at room temperature or at 4°C.

## LB agar plates

1. Make LB Medium as described above and add to it 15 g/liter agar before autoclaving.
2. Autoclave the medium for 20 minutes at 15 lbs/sq. in.
3. Let the medium cool to ~55°C and add the desired antibiotics. Pour into 10 cm Petri plates. Let the plates harden, invert, and store at 4°C.

# *Pichia* Media Recipes

## Introduction

Expressing 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<br>–with ammonium sulfate<br>–without amino acids | 67 g pouch<br>Each pouch contains reagents to prepare 500 mL of a 10X YNB solution | Q300-07 |
| | 500 g | Q300-09 |

## Stock Solutions

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

1. Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1,000 mL of water. Heat the solution to dissolve YNB completely in water.
2. Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate.
3. Filter sterilize and store at 4°C. 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)**

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

**100X H (0.4% Histidine)**

1. 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.
2. Filter sterilize and store at 4°C. The shelf life of this solution is approximately one year.

**10X D (20% Dextrose)**

1. Dissolve 200 g of D-glucose in 1,000 mL of water.
2. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

*Continued on next page*

### Stock Solutions, continued

**10X M (5% Methanol)**

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

**10X GY (10% Glycerol)**

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

**100X AA (0.5% of each Amino Acid)**

1. Dissolve 500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine in 100 mL of water.
2. Filter sterilize and store at 4°C. The shelf life of this solution is approximately one year.

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

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

### 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 Multi-Copy *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.
4. Store the liquid medium at room temperature. Store the YPD slants or plates at 4°C. The shelf life is several months.

*Continued on next page*

### YPD-Geneticin® plates

**Yeast Extract Peptone Dextrose Medium**

1% yeast extract
2% peptone
2% dextrose (glucose)
2% agar
Variable amounts of Geneticin® (see page 88 for ordering information)

Use 50 mg/mL Geneticin® stock solution to make YPD plates containing Geneticin® at final concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL.

For 250 mL (8 to 10 plates of a single Geneticin® concentration):

1. Combine 2.5 g yeast extract, 5 g peptone, and 5 g agar in 225 mL deionized water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 25 mL of 10X D and mix well.
4. Cool YPD to approximately 55–60°C and add appropriate volume of Geneticin® stock (see chart below). Remember to also make several YPD plates **without** Geneticin®.
5. Mix well by swirling, but take care to minimize bubble formation.
6. Pour agar solution into 10 cm Petri plates. Let plates harden, invert, and store bagged at 4°C. Plates are stable for at least 6 months.

| Final [Geneticin®] (mg/mL) | mL Geneticin® stock per 250 mL YPD |
|---|---|
| 0.25 | 1.25 |
| 0.50 | 2.5 |
| 0.75 | 3.75 |
| 1.00 | 5.0 |
| 1.50 | 7.5 |
| 1.75 | 8.75 |
| 2.00 | 10.0 |
| 3.00 | 15.0 |
| 4.00 | 20.0 |

*Continued on next page*

## MGY and MGYH

**Minimal Glycerol Medium ± Histidine** (1 liter)

1.34% YNB
1% glycerol
$4 \times 10^{-5}$% biotin
± 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.
3. Store at 4°C. The shelf life of this solution is approximately two months.

## RD and RDH Liquid Media

**Regeneration Dextrose Medium ± Histidine** (1 liter)

1 M sorbitol
2% dextrose
1.34% YNB
$4 \times 10^{-5}$% biotin
0.005% amino acids
± 0.004% histidine

1. Dissolve 186 g of sorbitol in 700 mL of water and proceed to Step 2.
2. Autoclave 20 minutes on liquid cycle.
3. Cool and maintain the liquid medium in a 45°C water bath.
4. Prepare a prewarmed (45°C) mixture of the following stock solutions:

   100 mL of 10X D
   100 mL of 10X YNB
   2 mL of 500X B
   10 mL of 100X AA
   88 mL of sterile water

   Add to sorbitol solution.
5. For growth of *his4* strains you must add histidine to the media. Add 10 mL of 100X H (histidine) to the prewarmed mixture in Step 4. Store liquid medium at 4°C. Media should last for several months.

## RDB and RDHB Agar Plates

1. Dissolve 186 g of sorbitol in 700 mL of water and add 20 g of agar.
2. Autoclave 20 minutes on liquid cycle.
3. Place the autoclaved solution in a 60°C water bath prior to addition of prewarmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the prewarmed (45°C) mixture from **RD and RDH Liquid Media**, Step 4, above. Add to sorbitol/agar solution. **If you are selecting for $His^+$ transformants, do not add histidine.**
5. Pour the plates immediately after mixing the solutions in Step 4. Store the plates at 4°C. Shelf life is several months.

*Continued on next page*

### RD and RDH Top Agar

1. Dissolve 186 g of sorbitol in 700 mL of water and add 10 g of agar or agarose.
2. Autoclave 20 minutes on liquid cycle.
3. Place the autoclaved solution in a 60°C water bath prior to addition of prewarmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the prewarmed (45°C) mixture from **RD and RDH Liquid Media,** Step 4, previous page. Add to sorbitol/agar solution. **If you are selecting for His$^+$ transformants, do not add histidine.**
5. Place the solution to 45°C after adding the solutions in Step 4. During transformation, use as a molten solution at 45°C.
6. Store top agar at 4°C. Shelf life is several months.

### MD and MDH

**Minimal Dextrose Medium + 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 + 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*

### BMG and BMM

**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 700 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
3. For BMM, add 100 mL 10X M instead of glycerol.
4. 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 the media at 4°C. The shelf life of this solution is approximately two months.

*Continued on next page*

### 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 it appropriately. Follow manufacturer's recommendations.
2. For 1 liter, dissolve in 900 mL deionized water:
   6 g sodium phosphate (monobasic), 372 mg EDTA, and 50 mL glycerol
3. Use NaOH to adjust the pH of the solution and bring up the volume to 1 liter. Store the breaking buffer 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 2,000 mL.

Wheaton (1-609-825-1100) only sells side baffle flasks.

# 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 $Mut^+$ and $Mut^S$ phenotypes were used successfully as well as secreted and intracellular expression.

| Protein | Expression Levels grams/liter | Where Expressed How Expressed | Reference |
|---|---|---|---|
| **Enzymes** | | | |
| Invertase | 2.3 | Secreted $Mut^+$ | (Tschopp *et al.*, 1987b) |
| Bovine Lysozyme c2 | 0.55 | Secreted $Mut^+$ | (Digan *et al.*, 1989) |
| Streptokinase (active) | 0.08 | Intracellular * | (Hagenson *et al.*, 1989) |
| Alpha amylase | 2.5 | Secreted $Mut^S$ | (Paifer *et al.*, 1994) |
| Pectate Lyase | 0.004 | Secreted $Mut^S$ | (Guo *et al.*, 1995) |
| Spinach Phospho-ribulokinase | 0.1 | Intracellular $Mut^S$ | (Brandes *et al.*, 1996) |
| **Antigens** | | | |
| Hepatitis B surface antigen | 0.4 | Intracellular $Mut^S$ | (Cregg *et al.*, 1987) |
| Pertussis Antigen P69 | 3.0 | Intracellular $Mut^S$ | (Romanos *et al.*, 1991) |
| Tetanus Toxin Fragment C | 12.0 | Intracellular $Mut^+/Mut^S$ | (Clare *et al.*, 1991a) |
| HIV-1 gp120 | 1.25 | Intracellular $Mut^+$ | (Scorer *et al.*, 1993) |
| Tick Anticoagulant protein | 1.7 | Secreted $Mut^S$ | (Laroche *et al.*, 1994) |
| Bm86 Tick Gut Glycoprotein | 1.5 | Secreted * | (Rodriguez *et al.*, 1994) |
| **Regulatory Proteins** | | | |
| Tumor Necrosis Factor (TNF) | 10.0 | Intracellular $Mut^S$ | (Sreekrishna *et al.*, 1989) |
| Mouse Epidermal Growth Factor (EGF) | 0.45 | Secreted $Mut^S$ | (Clare *et al.*, 1991b) |
| Human Interferon (IFN) α2b | 0.4 | Intracellular $Mut^S$ | (Garcia *et al.*, 1995) |

*Continued on next page*

# Proteins Expressed in *Pichia*, continued

**Table, continued**

| Protein | Expression Levels grams/liter | Where Expressed How Expressed | Reference |
|---|---|---|---|
| **Membrane Proteins** | | | |
| Human CD38 (soluble portion) | 0.05 | Secreted $Mut^S$ | (Fryxell *et al.*, 1995) |
| Mouse Serotonin Receptor | 0.001 | Secreted $Mut^+$ | (Weiss *et al.*, 1995) |
| **Proteases and Inhibitors Protease** | | | |
| Carboxypeptidase B | 0.8 | Secreted $Mut^+/Mut^S$ | (Despreaux & Manning, 1993) |
| Enterokinase | 0.021 | Secreted $Mut^+$ | (Vozza *et al.*, 1996) |
| Ghilanten | 0.01 | Secreted $Mut^+$ | (Brankamp *et al.*, 1995) |
| Kunitz protease inhibitor | 1.0 | Secreted * | (Wagner *et al.*, 1992) |
| Human Proteinase Inhibitor 6 | 0.05 | Intracellular $Mut^+$ | (Sun *et al.*, 1995) |
| **Antibodies** | | | |
| Rabbit Single Chain Antibody | >0.1 | Secreted $Mut^S$ | (Ridder *et al.*, 1995) |

*Mut phenotype was not described in the paper.

# Recombination and Integration in *Pichia*

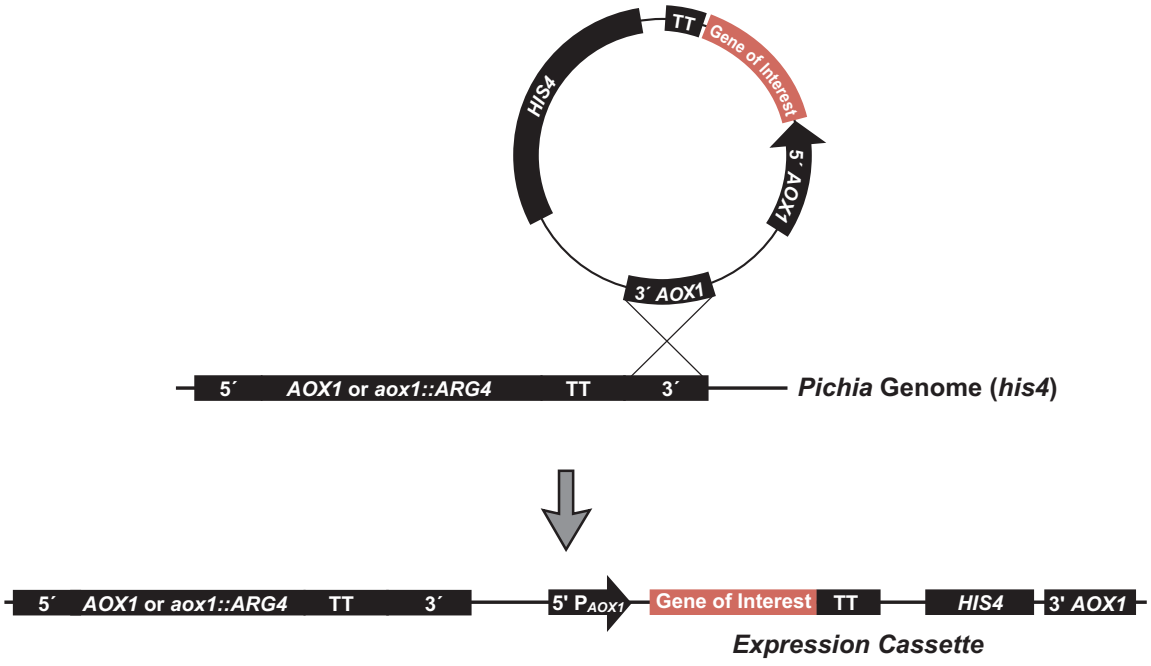
## Introduction

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. The most commonly used expression vectors carry the *HIS4* gene for selection. These vectors are designed to be linearized with a restriction enzyme such that $His^+$ recombinants are generated by recombination at the *AOX1* locus (see below) or at the *his4* locus (see next page). 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* (GS115) or *aox1::ARG4* (KM71) loci arise from a single crossover event between the loci and any of the three *AOX1* regions on the vector: the *AOX1* promoter, the *AOX1* transcription termination region (TT), or sequences even further downstream of *AOX1* (3´ *AOX1*). 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 $His^+$ $Mut^+$ (GS115) or $His^+$ $Mut^S$ (KM71). **By linearizing the recombinant vector at a restriction enzyme site located in the 5´ or 3´ *AOX1* regions, $Mut^+$ or $Mut^S$ recombinants can be conveniently generated depending on the host strain used.**

The figure below shows the result of an insertion of the plasmid 3´ to the intact *AOX1* locus ($Mut^+$) and the gain of $P_{AOX1}$, your gene of interest, and *HIS4* (expression cassette). This event could also happen at the 5´ *AOX1* regions of the plasmid and genome with the resulting insertion positioned 5´ to an intact *AOX1* locus. This also occurs with non-linearized plasmid and plasmid that religates, although at a lower frequency.

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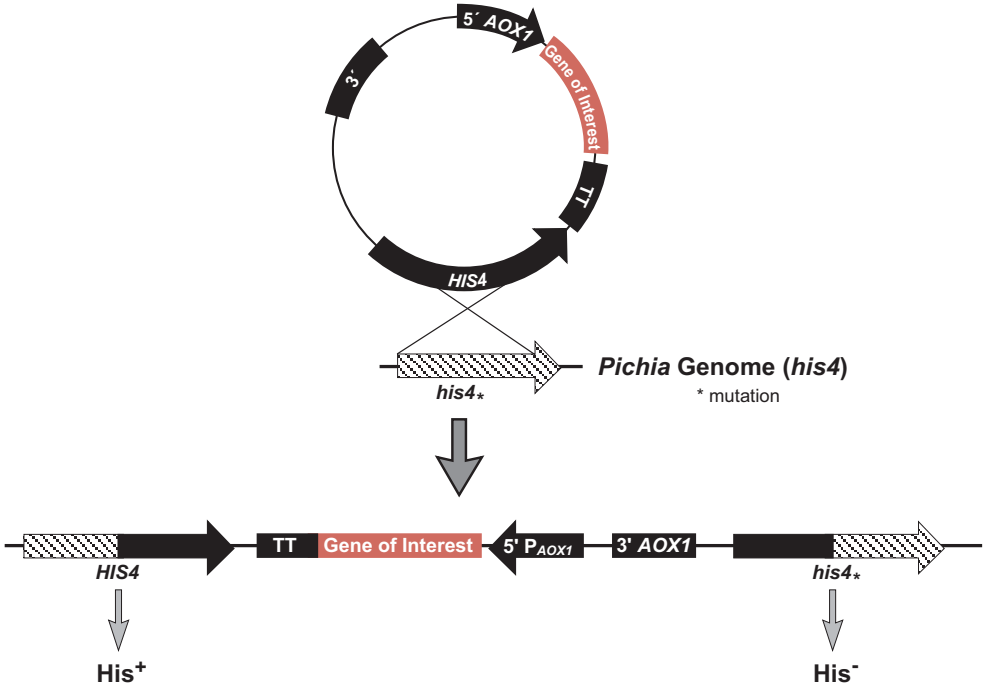
### Gene Insertion Events at *his4*

In GS115 (Mut$^+$) and KM71 (Mut$^S$) gene insertion events at the *his4* locus arise from a single crossover event between the *his4* locus in the chromosome and the *HIS4* gene on the vector. This event results in the insertion of one or more copies of the vector at the *his4* locus. Because the genomic *AOX1* or *aox1::ARG4* loci are not involved in this recombination event, the phenotype of such a His$^+$ transformant has the same Mut phenotype as the parent strain. **By linearizing the recombinant vector at a restriction enzyme site located in *HIS4* gene, Mut$^+$ or Mut$^S$ recombinants can be conveniently generated depending on the host strain used.** The figure below shows the result of an insertion of the plasmid between duplicated copies of the *HIS4*/*his4* genes, one still mutant, and the other wild-type.

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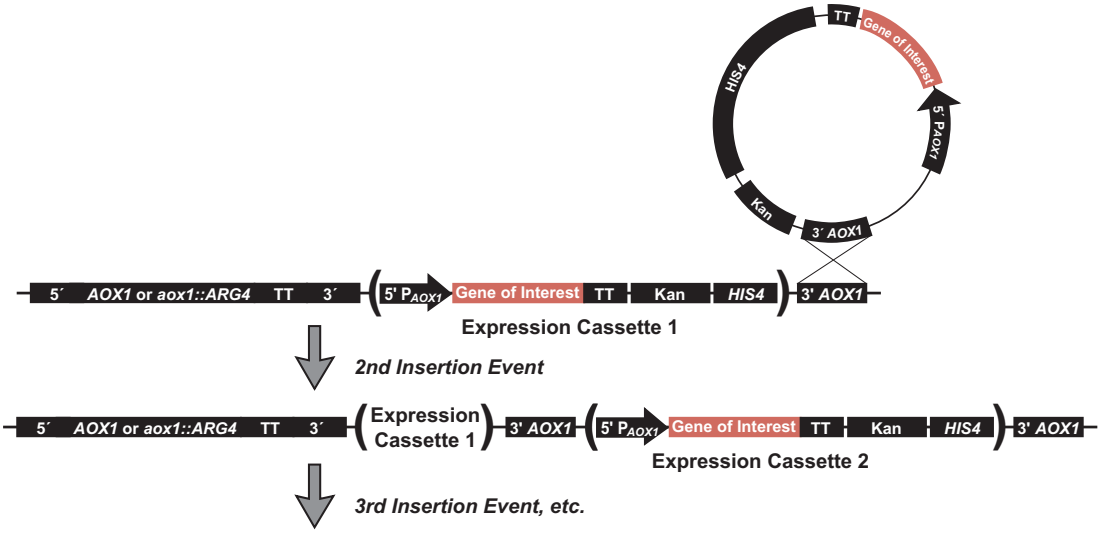
### Multiple Gene Insertion Events

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 $His^+$ transformants. Multi-copy events can occur as gene insertions either at the *AOX1*, *aox1::ARG4*, or *his4* loci. This results in a $Mut^+$ phenotype in GS115 and a $Mut^S$ phenotype in KM71. Quantitative dot blot analysis, Southern blot analysis, and differential hybridization can detect multiple gene insertion events. See page 81 for a protocol to screen for multiple inserts.

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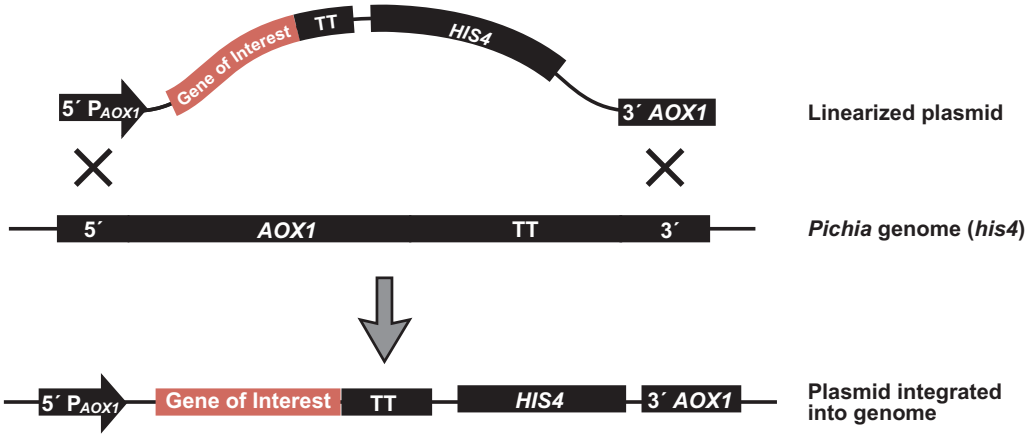
### Gene Replacement at *AOX1* in GS115

In a *his4* strain such as GS115, a gene replacement (omega insertion) event arises from a double crossover event between the *AOX1* promoter and 3´ *AOX1* regions of the vector and genome. This results in the complete removal of the *AOX1* coding region (i.e., gene replacement). The resulting phenotype is $His^+$ $Mut^S$. $His^+$ transformants can be readily and easily screened for their Mut phenotype, with $Mut^S$ serving as a phenotypic indicator of integration via gene replacement at the *AOX1* locus. The net result of this type of gene replacement is a loss of the *AOX1* locus ($Mut^S$) and the gain of an expression cassette containing $P_{AOX1}$, your gene of interest, and *HIS4*. The figure below shows a gene replacement event at the *AOX1* locus.

Gene replacement (double-crossover event) is less likely to happen than insertions (single-crossover events). In general, we recommend linearizing your plasmid DNA to create *Pichia* recombinants by single-crossover events. By using GS115 or KM71, the Mut phenotype of the recombinant will be the same as the parent strain.



# Electroporation of *Pichia*

## Introduction

This method does not require the generation and maintenance of spheroplasts, making it a very convenient method for generating *Pichia* transformants. Efficiencies run about the same as spheroplasting (Scorer *et al.*, 1994).

## Preparing Cells

1. Grow 5 mL of *Pichia pastoris* in YPD (page 61) 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 the culture overnight again to an $OD_{600}$ = 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, and resuspend the pellet with 250 mL of ice-cold, sterile water.
5. Centrifuge the cells as in Step 3 and resuspend the pellet in 20 mL of ice-cold 1 M sorbitol.
6. Centrifuge the cells as in Step 3 and resuspend the pellet in 1 mL of ice-cold 1 M sorbitol for a final volume of approximately 1.5 mL.

**Note**: You may freeze the electrocompetent cells in 80 µL aliquots; however the transformation efficiencies will decrease significantly.

## Transformation

1. Mix 80 µL of the cells from Step 6 (above) with 5–20 µg of linearized DNA (in 5–10 µL TE Buffer) and transfer them to an ice-cold 0.2 cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) suggested by the manufacturer of the specific electroporation device being used.
4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile microcentrifuge tube.
5. Spread 200–600 µL of aliquots on MD or RDB plates.
6. Incubate the plates at 30°C until colonies appear. Screen for $Mut^+/Mut^S$ phenotypes as indicated on page 38.

# PEG 1000 Transformation Method for *Pichia*

## Introduction

PEG procedure is usually better than LiCl, but not as good as spheroplasting or electroporation for transformation. However, it is convenient for people who do not have an electroporation device. The transformation efficiency is $10^2$ to $10^3$ transformants per µg of DNA.

## Required Solutions

- Buffer A: 1.0 M Sorbitol (Fisher), 10 mM Bicine, pH 8.35 (Sigma), 3% (v/v) ethylene glycol (Merck)
- Buffer B: 40% (w/v) Polyethylene glycol 1000 (Sigma), 0.2 M Bicine, pH 8.35
- Buffer C: 0.15 M NaCl, 10 mM Bicine, pH 8.35

Filter sterilize the above solutions and store them at –20°C.

- Fresh, reagent grade DMSO that is from an unopened bottle or made fresh and stored at –70°C until use.

**Important**

Cell competence decreases very rapidly after the cells thaw even when held on ice. It is critical to add DNA to frozen cell samples. To perform multiple transformations, it is recommended to process them in groups of six at a time.

## Preparing Competent Cells

1. Streak *Pichia pastoris* strain for single colonies on a YPD plate and incubate the plate at 30°C for two days.
2. Inoculate a 10 mL YPD culture with a single colony from the plate and grow the culture overnight at 30°C with shaking.
3. In the morning, use an aliquot of the overnight culture to inoculate a 100 mL YPD culture to a starting $OD_{600}$ of 0.1 and grow at 30°C to an $OD_{600}$ of 0.5 to 0.8.
4. Harvest the culture by centrifugation at 3,000 × g at room temperature and wash cells once in 50 mL of Buffer A.
5. Resuspend the cells in 4 mL of Buffer A and distribute them in 0.2 mL aliquots to sterile 1.5 mL microcentrifuge tubes. Add 11 µL of DMSO to each tube, mix, and quickly freeze cells in a bath of liquid nitrogen.
6. Store the frozen tubes at –70°C.

*Continued on next page*

### Transformation

1. Use up to 50 µg of each DNA sample in no more than 20 µL total volume. Add the DNA directly to a still-frozen tube of competent cells. Include carrier DNA (40 µg of denatured and sonicated salmon sperm DNA) with < 1 µg DNA samples for maximum transformation frequencies.
2. Incubate all sample tubes in a 37°C water bath for five minutes. Mix the samples once or twice during this incubation period.
3. Remove the tubes from the bath and add 1.5 mL of Buffer B to each. Mix their contents thoroughly.
4. Incubate the tubes in a 30°C water bath for 1 hour.
5. Centrifuge the sample tubes at 2,000 × g for 10 minutes at room temperature. Decant the supernatant and resuspend the cells in 1.5 mL of Buffer C.
6. Centrifuge the samples a second time and resuspend the cell pellet gently in 0.2 mL of Buffer C.
7. Spread the entire contents of each tube on an agar plate containing selective growth medium and incubate plates at 30°C for 3 to 4 days. Screen for Mut phenotype (page 38) or select hyper-resistant Geneticin® colonies (page 41).

# Lithium Chloride Transformation Method

## Introduction

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

## Preparing 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°C.

## Preparing Cells

1. Grow a 50 mL culture of *Pichia pastoris* in YPD at 30°C with shaking to an $OD_{600}$ 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 × 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 µL of 100 mM LiCl.
7. Dispense 50 µL 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°C.**

*Continued on next page*

### 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 neither necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at –20°C and boil every 3–4 times the DNA is thawed.
2. Centrifuge the LiCl-cell solution from Step 7, previous page. 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 µL 50% PEG

   36 µL 1 M LiCl

   25 µLl 2 mg/mL single-stranded DNA

   Plasmid DNA (5–10 µg) in 50 µL sterile water
4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
5. Incubate the tube at 30°C for 30 minutes without shaking.
6. Heat shock in a water bath at 42°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. Gently resuspend the pellet in 1 mL of sterile water.
9. Plate 25 to 100 µL on RDB or MD plates. Incubate the plates for 2–4 days at 30°C. Screen for Mut phenotype (page 38) or select hyper-resistant Geneticin® colonies (page 41).

# Direct PCR Screening of *Pichia* Clones

## 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. You may use the genomic DNA directly as a PCR template.

## Materials Needed

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

## Procedure

1. Place 10 µL of a *Pichia pastoris* culture into a 1.5 mL microcentrifuge tube. For relatively dense cultures, dilute 1 µL of the culture into 9 µL water. Alternatively, pick a single colony and resuspend in 10 µL of water.
2. Add 5 µL of a 5 U/µL 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 µL PCR for a hot start:

| | |
|---|---|
| 10X Reaction Buffer | 5 µL |
| 25 mM MgCl2 | 5 µL |
| 25 mM dNTPs | 1 µL |
| 5´ *AOX1* primer (10 pmol/µL) | 1 µL |
| 3´ *AOX1* primer (10 pmol/µL) | 1 µL |
| Sterile water | 27 µL |
| Cell lysate | 5 µL |
| Total Volume | 45 µL |

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

| Step | Temperature | Time |
|---|---|---|
| Denaturation | 95°C | 1 minute |
| Annealing | 54°C | 1 minute |
| Extension | 72°C | 1 minute |

   Include a final extension of 7 minutes at 72°C.
8. Analyze a 10 µL aliquot by agarose gel electrophoresis.

# Isolating Total DNA from *Pichia*

## Introduction

The protocol below allows you to isolate DNA from the desired $His^+$ recombinant and the untransformed GS115 or KM71 which 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

Prepare the following solutions, because 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)

## Preparing Cells

1. Grow at 30°C the recombinant strain and the parent strain to an $OD_{600}$ of 5–10 in 10 mL of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71).
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 (monitor the percent spheroplasting using the procedure on pages 33–34).
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, the previous page, and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
2. Centrifuge at 10,000 × g for 20 minutes at 4°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°C for 60 minutes.
6. Centrifuge at 10,000 × g for 20 minutes at 4°C and wash the pellets once with 1 mL of 70% ethanol. Briefly air dry the pellets and resuspend each one in 50 µL of TE buffer, pH 7.5.
7. Determine the concentration of the DNA sample. You can store the samples at –20°C separately or combined until ready for use.

# Detecting Multiple Integration Events

## Introduction

It has been demonstrated in a number of papers (Brierley *et al.*, 1994; Clare *et al.*, 1991a; Romanos *et al.*, 1991; Scorer *et al.*, 1993; Scorer *et al.*, 1994) that multiple integration events may increase the levels of protein expressed. If the expression of your protein is low, you may wish to isolate multicopy integrants. Using the protocol detailed on the previous page, isolate genomic DNA from the $His^+$ recombinants that are to be analyzed, as well as from the untransformed GS115 or KM71 to control for any background hybridization. You can use the DNA in either of the following analyses to detect multicopy integration.

## 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. It is also important to understand that your strategy will be different if you use pPIC3.5K versus pAO815 to generate your multiple copies. Digesting DNA from *Pichia* recombinants containing multiple copies produces a band that varies 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 band intensities using densitometry to estimate relative gene dosage.

## Materials Needed for Quantitative Dot Blot

Prepare 10–15 mL of each following of solutions for each dot blot:

- 50 mM EDTA, 2.5% β-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
- 3MM paper.

*Continued on next page*

### 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 and spot it directly onto nitrocellulose or nylon, fix it, and analyze it.

1. Grow $Mut^+$ or $Mut^S$ transformants in individual wells of a 96-well microtiter plate in 200 µL 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 µL 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% β-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 the filter 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 them 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 it for 4 hours at 37°C.
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 from the 3MM paper and replace the 3MM paper with two new sheets. Soak them with 10–15 mL of 2X SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate it for 5 minutes at room temperature. Repeat.
7. Bake the nitrocellulose filters at 80°C or UV-crosslink the DNA to nylon. The filters may be probed with a nonradioactive-labeled or random-primed, $^{32}P$-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 β-scanner (if radiolabeled).

*Continued on next page*

# Determining the Copy Number of Multiple Integrants, continued

## 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 it using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane to accurately determine the copy number.
- Probe your Southern blot with probes to *HIS4* and your gene. The point mutation in the *his4* gene in the host strain will not interfere with hybridization if you make the probe complementary to the wild-type gene.
- If you used pPIC3.5K to generate multimers, use *Bgl* II to digest your DNA (Clare, *et al*., 1991a). If you used pPIC3.5K all multimers are NOT necessarily in a head-to-tail configuration. Some multimers may be head-to-head and others tail-to-tail. We recommend that you think about what products may be produced. An expression cassette in the opposite orientation may produce a different band. The number of multiple copies will cause one or two bands (depending on orientation) in the Southern blot to increase in intensity once you have >2 copies.
- If you used pAO815 to generate multimers, use *Bgl* II and *Bam*H I to digest the genomic DNA and release the multimer. The molecular weight of the band allows you to determine the number of multimers. If this multimer is too large, you may wish to digest with an enzyme like *Sac* I. This collapses the multimer into single fragments containing your gene. These fragments will produce a band that will be quite intense. The relative intensity of this band versus a band containing a single copy of your gene allows you to determine the copy number.
- *Bgl* II digested DNA from GS115 and GS115 transformed with pPIC3.5K or pAO815 produces bands of 2.8 kb (the genomic copy of *HIS4)*, and ~6.7 kb (the vector derived copy of *HIS4)*, respectively, when probed with a complementary fragment to *HIS4*.

# Procedure for Total RNA Isolation from *Pichia*

## 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. RNA isolation should be done from induced cultures using an uninduced culture as a negative control.

## Solutions

Use DEPC-treated water and 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) | |

## Growing Cells

1. Grow up two cultures (100–200 mL in MGY or BMGY), but induce only one of them. Use the same protocol for induction that you used in the **Expression** section.
2. Take 10 mL time points at 1, 2, 3, 4, and 6 days.
3. Harvest the cells from each time point by centrifugation at 1500 × g for 10 minutes at room temperature.
4. Resuspend the cell pellet in 400 µL of AE buffer and transfer it to a microcentrifuge tube.

*Continued on next page*

# Procedure for Total RNA Isolation from *Pichia*, continued

## Lysing Cells

1. Add 40 µL of 10% SDS to the microcentrifuge tube containing the cell suspension and vortex it for ~20 seconds.
2. Add an equal volume (450–500 µL) of buffer saturated phenol to the tube and vortex it for ~20 seconds.
3. Incubate the cell suspension at 65°C for 4 minutes.
4. Incubate the cell suspension in a dry ice/ethanol bath until crystals show (~1 minute). Centrifuge the tube at maximum speed for 2 minutes at 4°C.
5. Transfer the aqueous phase to a new centrifuge tube, add an equal volume of phenol/chloroform, and vortex it for ~20 seconds. Centrifuge the tube at maximum speed for 2 minutes at 4°C.
6. Remove the 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 the tube at maximum speed for 15 minutes at 4°C. Remove the supernatant.
7. Wash the pellet with 80% ethanol and air dry it briefly. Resuspend total RNA in 20 µL of DEPC-treated water and store it at –80°C. The average yield is 60–300 µg of 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 is designed to isolate mRNA from 0.2 to 1 mg total RNA and the Micro-FastTrack™ 2.0 Kit is designed to isolate mRNA from ~100 µg total RNA (see page 88 for ordering information.
You will need ~1–5 µg mRNA per time point.

# β-Galactosidase Assay

## Introduction

The GS115 β-Gal strain is provided as a $His^+$ $Mut^+$ intracellular expression control. Growth of the strain during $Mut^+$ expression provides a positive control for expression conditions. The cell-free β-galactosidase assay described below can also be found in (Miller, 1972), page 403, and can be used to evaluate expression of β-galactosidase.

## Required Solutions

- A fresh crude cell lysate of GS115 β-Gal (see page 57)
- Z buffer
- ONPG solution
- 1 M sodium carbonate solution

Recipes for the solutions are below.

## Z Buffer

60 mM $Na_2HPO_4$-$7H_2O$
40 mM $NaH_2PO_4$-$H_2O$
10 mM KCl
1 mM $MgSO_4$-$7H_2O$
50 mM β-mercaptoethanol
pH 7.0

1. Dissolve the following in 950 mL deionized water:

| | |
|---|---|
| $Na_2HPO_4$-$7H_2O$ | 16.1 g |
| $NaH_2PO_4$-$H_2O$ | 5.5 g |
| KCl | 0.75 g |
| $MgSO_4$-$7H_2O$ | 0.246 g |
| β-mercaptoethanol | 2.7 mL |

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:

| | |
|---|---|
| $Na_2HPO_4$-$7H_2O$ | 1.61 g |
| $NaH_2PO_4$-$H_2O$ | 0.55 g |

2. Adjust pH to 7.0 with either NaOH or HCl
3. Add 400 mg ONPG. Stir to dissolve and bring the volume up to 100 mL with water.
4 Store at 4°C away from light.

## 1 M Sodium Carbonate

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

*Continued on next page*

# β-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 µL 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 3.
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 $OD_{420}$ 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.

## Determining Specific Activity

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

$$\text{β-galactosidase units/mg total protein} = \frac{OD_{420} \times 380}{\text{minutes at } 28°C \times \text{mg protein in reaction}}$$

## Sample Calculation

Here is a sample calculation:

Extract concentration = 10 mg/mL
Assay 10 µL of a 1/100 dilution
Time = 10 minutes
$OD_{420}$ = 0.4

The amount of protein in the reaction = 0.01 mL × 0.01 (dilution factor) × 10 mg/mL
= 0.001 mg protein in the reaction

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

Pure β-galactosidase has an activity of 300,000 units/mg protein.

# Accessory Products

## Accessory Products

Many of the reagents supplied in *Pichia* Expression Kit, as well as other reagents and kits used in *Pichia* expression experiments are available separately from Invitrogen. Ordering information is provided below. For more information, refer to www.invitrogen.com or contact Technical Support (see page 89).

| Product | Amount | Cat. no. |
|---|---|---|
| *Pichia* Spheroplast Module (Box 1 and Box 2) | 10 preparations (50 transformations) | K1720-01 |
| Geneticin®, powder | 1 g<br>5 g<br>25 g | 11811-023<br>11811-031<br>11811-098 |
| Geneticin®, liquid (50mg/mL) | 20 mL<br>100 mL | 10131-035<br>10131-027 |
| Ampicillin Sodium Salt, irradiated | 200 mg | 11593-027 |
| TA Cloning® Kit (with pCR®2.1 vector) | 20 reactions | K2000-01 |
| PureLink™ HiPure Plasmid Miniprep Kit | 25 preparations<br>100 preparations | K2100-02<br>K2100-03 |
| Platinum® Taq DNA Polymerase | 100 reactions<br>250 reactions<br>500 reactions | 10966-018<br>10966-026<br>10966-034 |
| Taq DNA Polymerase, Recombinant | 100 units<br>500 units | 10342-053<br>10342-020 |
| Easy-DNA™ Kit | 1 kit | K1800-01 |
| FastTrack® 2.0 mRNA Isolation Kit | 1 kit | K1593-02 |
| Micro-FastTrack™ 2.0 mRNA Isolation Kit | 1 kit | K1520-02 |
| pAO815 | 20 µg | V180-20 |
| pPIC3.5K | 20 µg | V173-20 |
| pPIC9K | 20 µg | V175-20 |

# Technical Support

## Web Resources

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- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
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### Limited Use Label License No. 74: *Pichia Pastoris* Expression System

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