Pichia Expression Kit

PROTEIN EXPRESSION

A Manual of Methods for Expression of Recombinant Proteins in Pichia pastoris

Catalog no. K1710-01

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## Materials

### Kit Contents

**Box 1: Spheroplast Module. Store at room temperature.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS media</td>
<td>20 ml</td>
<td>1 M Sorbitol 0.3X YPD 10 mM CaCl₂</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>2 x 125 ml</td>
<td>Autoclaved, deionized water</td>
</tr>
<tr>
<td>SE</td>
<td>2 x 125 ml</td>
<td>1 M Sorbitol 25 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>SCE</td>
<td>2 x 125 ml</td>
<td>1 M Sorbitol 10 mM Sodium citrate buffer, pH 5.8 1 mM EDTA</td>
</tr>
<tr>
<td>1 M Sorbitol</td>
<td>2 x 125 ml</td>
<td>--</td>
</tr>
<tr>
<td>CaS</td>
<td>2 x 60 ml</td>
<td>1 M Sorbitol 10 mM Tris-HCl, pH 7.5; 10 mM CaCl₂</td>
</tr>
<tr>
<td>40% PEG</td>
<td>25 ml</td>
<td>40% (w/v) PEG 3350 (Reagent grade) in water</td>
</tr>
<tr>
<td>CaT</td>
<td>25 ml</td>
<td>20 mM Tris-HCl, pH 7.5 20 mM CaCl₂</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amount</th>
<th>Genotype</th>
<th>Phenotype (<em>Pichia</em> only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS115</td>
<td>1 stab</td>
<td><em>his4</em></td>
<td>Mut⁺</td>
</tr>
<tr>
<td>KM71</td>
<td>1 stab</td>
<td><em>arg4 his4 aox1::ARG4</em></td>
<td>Mut⁸, Arg⁺</td>
</tr>
<tr>
<td>GS115 Albumin</td>
<td>1 stab</td>
<td><em>HIS4</em></td>
<td>Mut⁸</td>
</tr>
<tr>
<td>GS115 β-Gal</td>
<td>1 stab</td>
<td><em>HIS4</em></td>
<td>Mut⁺</td>
</tr>
<tr>
<td>TOP10F’</td>
<td>1 stab</td>
<td><em>F’</em> {proAB, lacI4, lacZΔM15, Tn10 (TetR)} mcrA, Δ(mrr-bsdRMS-mcrBC), ϕ80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL (StrR), endA1, nupG λ’.</td>
<td></td>
</tr>
</tbody>
</table>

**Box 2: Spheroplast Module. Store at -20°C.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymolyase</td>
<td>10 x 20 µl</td>
<td>3 mg/ml Zymolyase in water (100,000 units/g lytic activity)</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>10 x 1 ml</td>
<td>1 M dithiothreitol in water</td>
</tr>
</tbody>
</table>

*continued on next page*
Kit Contents, continued

Vector Box. Store at -20°C.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHIL-D2</td>
<td>Vector for intracellular expression in <em>Pichia</em></td>
</tr>
<tr>
<td>10 µg, lyophilized in TE, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>pPIC3.5</td>
<td>Vector for intracellular expression in <em>Pichia</em></td>
</tr>
<tr>
<td>10 µg, lyophilized in TE, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>pHIL-S1</td>
<td>Vector for secreted expression in <em>Pichia</em>. Uses the <em>PHO1</em> signal sequence</td>
</tr>
<tr>
<td>10 µg, lyophilized in TE, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>pPIC9</td>
<td>Vector for secreted expression in <em>Pichia</em>. Uses the α-factor signal sequence</td>
</tr>
<tr>
<td>10 µg, lyophilized in TE, pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>

Primer Box. Store at -20°C.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5´ AOX1 sequencing primer 2 µg (312 pmol), lyophilized</td>
<td>5´-GACTGTTCCAATTGACAAGC-3´</td>
</tr>
<tr>
<td>3´ AOX1 sequencing primer 2 µg (314 pmol), lyophilized</td>
<td>5´-GCAAATGGCATTCTGACATCC-3´</td>
</tr>
<tr>
<td>α-Factor sequencing primer 2 µg (315 pmol), lyophilized</td>
<td>5´-TACTATTGCCAGCATGCTGC-3´</td>
</tr>
</tbody>
</table>

Media

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

<table>
<thead>
<tr>
<th>Media</th>
<th>Amount</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>YP Base Medium</td>
<td>2 pouches</td>
<td>2 liters of YP medium</td>
</tr>
<tr>
<td>YP Base Agar Medium</td>
<td>2 pouches</td>
<td>2 liters of YP medium</td>
</tr>
<tr>
<td>Yeast Nitrogen Base</td>
<td>1 pouch</td>
<td>500 ml of 10X YNB</td>
</tr>
</tbody>
</table>

The α-Factor, 5´ AOX1, and 3´ AOX1 primers, and reagents to transform *Pichia* by spheroplasting (*Pichia* Spheroplast Module) are available separately from Invitrogen.

<table>
<thead>
<tr>
<th>Product</th>
<th>Reactions or Amount</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pichia</em> Spheroplast Module</td>
<td>10 spheroplast preparations (50 transformations)</td>
<td>K1720-01</td>
</tr>
<tr>
<td>5´ AOX1 primer</td>
<td>2 µg, lyophilized</td>
<td>N710-02</td>
</tr>
<tr>
<td>3´ AOX1 primer</td>
<td>2 µg, lyophilized</td>
<td>N720-02</td>
</tr>
<tr>
<td>5´ and 3´ AOX1 primers</td>
<td>2 µg, each, lyophilized</td>
<td>N740-02</td>
</tr>
<tr>
<td>α-Factor primer</td>
<td>2 µg, each, lyophilated</td>
<td>N730-02</td>
</tr>
</tbody>
</table>

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Materials, continued

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
- Polyacrylamide Gel Electrophoresis apparatus and buffers
- Media for transformation, growth, screening, and expression (see Recipes, pages 53-58)
- 5% SDS solution (10 ml per transformation)
- Sterile cheesecloth or gauze
- Breaking Buffer (see Recipes, page 58)
- Acid-washed glass beads (available from Sigma)
- Replica-plating equipment (optional)
- Bead Breaker™ (optional)
Purchaser Notification

Introduction

The Pichia Expression Kit is based on the yeast Pichia pastoris. Pichia pastoris was developed into an expression system by scientists at Salk Institute Biotechnology/Industry Associates (SIBIA) and Phillips Petroleum for high-level expression of recombinant proteins. All patents for Pichia pastoris and licenses for its use as an expression system are owned by Research Corporation Technologies (RCT), Inc., Tucson, Arizona. For information on commercial licenses, please see page viii.

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<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4,683,293</td>
<td>4,808,537</td>
<td>4,812,405</td>
<td>4,818,700</td>
<td>4,837,148</td>
</tr>
<tr>
<td>4,855,231</td>
<td>4,857,467</td>
<td>4,879,231</td>
<td>4,882,279</td>
<td>4,885,242</td>
</tr>
<tr>
<td>4,895,800</td>
<td>4,929,555</td>
<td>5,002,876</td>
<td>5,004,688</td>
<td>5,032,516</td>
</tr>
<tr>
<td>5,122,465</td>
<td>5,135,868</td>
<td>5,166,329</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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(b) any use of Expression Products in the manufacture of a Commercial Product
(c) any sale of Expression Products
(d) any use of Expression Products or the Expression Kit to facilitate or advance research or development of a Commercial Product
(e) any use of Expression Products or the Expression Kit to facilitate or advance any research or development program the results of which will be applied to the development of Commercial Products

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continued on next page
Purchaser Notification, continued

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**Contact for Commercial Licensing**

Bennett Cohen, Ph.D.
Research Corporation Technologies
101 North Wilmot Road, Suite 600
Tucson, Arizona 85711-3335
Phone: (520) 748-4400
Fax: (520) 748-0025

**User Registration Card**

Please complete and return the enclosed User Registration Card for each *Pichia* Expression Kit that you purchase. This will serve as a record of your purchase and registration and will allow Invitrogen to provide you with technical support and manual updates. It will also allow Invitrogen to update you on future developments and improvements to the *Pichia* Expression Kit. The agreement outlined above becomes effective upon our receipt of your User Registration Card or 10 days following the sale of the *Pichia* Expression Kit to you. Use of the kit at any time results in immediate obligation to the terms and conditions stated in this license agreement.

**Product Serial Number**

Below, please record the Product Serial Number found on your User Registration Card for use in future correspondence with Invitrogen.

Product Serial Number ______________________
**Product Specifications**

<table>
<thead>
<tr>
<th>Introduction</th>
<th>This section describes the criteria used to qualify the components in the <em>Pichia</em> Expression Kit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectors</td>
<td>All expression vectors are qualified by restriction enzyme digestion. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.</td>
</tr>
<tr>
<td>Spheroplast Reagents</td>
<td>The spheroplast reagents are qualified by spheroplast preparation of GS115 following the protocol provided in the <em>Pichia</em> Expression Kit manual. At least 70% of the <em>Pichia pastoris</em> cells must form spheroplasts in 30 minutes or less.</td>
</tr>
<tr>
<td><em>Pichia</em> Strains</td>
<td>The <em>Pichia</em> strains are by demonstrating viability of the culture. Single colonies should arise within 48 hours after streaking on YPD medium from the stab</td>
</tr>
<tr>
<td>Primers</td>
<td>Sequencing primers are lot tested by automated DNA sequencing experiments.</td>
</tr>
<tr>
<td>Buffers and Solutions</td>
<td>All buffers and solutions are extensively tested for sterility.</td>
</tr>
<tr>
<td>Media</td>
<td>All <em>Pichia</em> growth and expression media are qualified by growing the GS115 <em>Pichia</em> strain.</td>
</tr>
</tbody>
</table>
Introduction

Overview

Review Articles

The information presented here is designed to give you a concise overview of the *Pichia pastoris* expression system. It is by no means exhaustive. For further information, please read the articles cited in the text along with recent review articles (Buckholz and Gleeson, 1991; Cregg et al., 1993; 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 baculo-virus 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 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* including:

- 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. This reaction generates both formaldehyde and hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle called the peroxisome, which sequesters toxic by-products from the rest of the cell. Alcohol oxidase has a poor affinity for O2, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase drives heterologous protein expression in *Pichia*.

Two Alcohol Oxidase Proteins

The *AOX1* and *AOX2* genes code for alcohol oxidase in *Pichia pastoris*. 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 high levels, typically > 30% of the total soluble protein in cells grown with methanol as the carbon source. The *AOX1* gene has been isolated and the *AOX1* promoter is used to drive expression of the gene of interest (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 allows isolation of Mut+ strains (*aox1*) (Cregg et al., 1989; Koutz et al., 1989).
Overview, continued

**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. Please note that growth on glycerol (derepression) is not sufficient to generate even minute levels of expression from the AOX1 gene. The inducer, methanol, is necessary for 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+ (Methanol utilization slow). This has in the past been referred to as Mut. The Mut 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 3).

**Intracellular and Secretory Protein Expression**

Heterologous expression in Pichia can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the Saccharomyces cerevisiae α factor prepro peptide has been used most successfully (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. That, combined with the very low amount of protein in the minimal Pichia growth medium, 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). Note: If there are recognized glycosylation sites (Asn-X-Ser/Thr) in your protein's primary sequence, glycosylation may occur at these sites.

**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 S. 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 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 expression of your protein, four expression vectors are included in this kit. pHIL-D2 and pPIC3.5 are used for intracellular expression while pHIL-S1 and pPIC9 are used for secreted expression (see pages 14-19 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 $Bgl II$. These sites are recommended for linearizing your construct prior to Pichia transformation. If your insert has all of these sites, see pages 28-29 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$^+$ since the strain itself is Mut$^S$. Both Mut$^+$ and Mut$^S$ recombinants are useful to have as one phenotype may favor better expression of your protein than the other. Due to 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 integration of your construct (see page 40).

Once you have successfully cloned your gene, you will then 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.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Integration Event</th>
<th>GS115 Phenotype</th>
<th>KM71 Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Sal I$ or $Stu I$</td>
<td>Insertion at $his4$</td>
<td>His$^+$ Mut$^+$</td>
<td>His$^+$ Mut$^S$</td>
</tr>
<tr>
<td>$Sac I$</td>
<td>Insertion at 5´ $AOX1$ region</td>
<td>His$^+$ Mut$^+$</td>
<td>His$^+$ Mut$^S$</td>
</tr>
<tr>
<td>$Not I$ or $Bgl II$</td>
<td>Replacement at $AOX1$ locus</td>
<td>His$^+$ Mut$^S$ His$^+$ Mut$^+$</td>
<td>His$^+$ Mut$^S$ (not recommended, see page 11)</td>
</tr>
</tbody>
</table>

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 will involve growing a small culture of each recombinant, inducing with methanol, and taking time points. If looking for intracellular expression, analyze the cell pellet from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). If looking for secreted expression, analyze both the cell pellet and supernatant from each time point. We recommend that you analyze your SDS-PAGE gels by both Coomassie staining and western blot, if you have an antibody to your protein. We also suggest checking for protein activity by assay, if one is available. Not all proteins express to the level of grams per liter, so it is advisable to check by western blot or activity assay, and not just by Coomassie staining of SDS-PAGE gels for production of your protein.

Choose the Pichia recombinant strain that best expresses your protein and optimize induction based on the suggestions on pages 47-48. Once expression is optimized, scale-up your expression protocol to produce more protein.

continued on next page
Experimental Outline, continued

**Experimental Process**

The overall experimental process is divided into two major sections: **Recombinant Strain Generation and Induction** (Mut\(^+\) and/or Mut\(^5\)). In each section is a table outlining the major steps needed to accomplish the goal of each section. Each step is discussed in detail further in the manual. Refer to the indicated pages to read about particular steps of interest. A discussion about recombination and integration in *Pichia* is included to help you choose the right vector. More information is provided in a recent review (Higgins, 1995).

**Recombinant Strain Generation**

The goal of this section is to create a *Pichia pastoris* strain containing your integrated gene of interest. You will first need to determine which vector to use.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Select the appropriate expression vector (Please read <em>Recombination and Integration in Pichia</em>, pages 7-10)</td>
<td>14-19</td>
</tr>
<tr>
<td>2</td>
<td>Clone your desired gene into selected vector</td>
<td>21-25</td>
</tr>
<tr>
<td>3</td>
<td>Transform <em>E. coli</em>, select ampicillin-resistant transformants, and confirm presence and orientation of desired gene</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>Linearize constructs with appropriate restriction enzymes to generate His(^+) Mut(^6) and His(^+) Mut(^+) recombinant strains</td>
<td>27-29</td>
</tr>
<tr>
<td>5</td>
<td>Transform and select His(^+) transformants (GS115 recombinants, His(^+) Mut(^-); KM71 recombinants, His(^+) Mut(^5))</td>
<td>30-35</td>
</tr>
<tr>
<td>6</td>
<td>Screen His(^+) transformants for Mut(^+) and Mut(^5) strains (6-10 recombinants of each phenotype)</td>
<td>36-39</td>
</tr>
<tr>
<td>7</td>
<td>Confirm integration of your desired gene in both Mut(^+) and Mut(^5) recombinants by PCR</td>
<td>40-41</td>
</tr>
</tbody>
</table>

**Mut\(^+\) Induction**

The method of induction depends on whether the recombinant is Mut\(^+\) or Mut\(^5\). The differences primarily occur in the culture volumes and the time of induction (see below). Please refer to the following pages for more detailed instructions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Guidelines for expression of recombinant proteins in <em>Pichia</em></td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Grow His(^+) Mut(^+) recombinants in 25 ml each buffered glycerol medium to a final OD(_{600}) = 2-6</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>Harvest cells and resuspend to an OD(_{600}) of 1.0 (~100-200 ml) with methanol medium and place in a 1 liter baffled flask</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Incubate at 30°C with shaking and take samples for analysis at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hours</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>Analyze medium (if desired protein is targeted for secretion) and cell lysates (for both intracellular and secreted expression) for protein via PAGE/Coomassie Blue staining, western blot, activity, ELISA, or immunoprecipitation</td>
<td>45-46</td>
</tr>
<tr>
<td>6</td>
<td>Optimize expression of your His(^+) Mut(^+) recombinant</td>
<td>47-48</td>
</tr>
<tr>
<td>7</td>
<td>Scale-up your expression for protein purification</td>
<td>49</td>
</tr>
</tbody>
</table>

*continued on next page*
**MutS Induction**

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Guidelines for expression of recombinant proteins in <em>Pichia</em></td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Grow His(^+) Mut(^S) recombinants in 100-200 ml each buffered glycerol medium to a final OD(_{600}) = 2-6</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>Harvest cells and resuspend to an OD(_{600}) of 10.0 (~10-20 ml) with methanol medium and place in a 100 ml or 250 ml baffled flask.</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>Incubate at 30°C with shaking and take samples for analysis at 0, 24, 48, 72, 96, 120, and 144 hours</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>Analyze medium (if desired protein is targeted for secretion) and cell lysates (for both intracellular and secreted expression) for protein via SDS/PAGE, western blot, activity, ELISA, or immunoprecipitation</td>
<td>45-46</td>
</tr>
<tr>
<td>6</td>
<td>Optimize expression of your His(^+) Mut(^+) recombinant</td>
<td>47-48</td>
</tr>
<tr>
<td>7</td>
<td>Scale-up your expression for protein purification</td>
<td>49</td>
</tr>
</tbody>
</table>

**Proteins Expressed in *Pichia***

The table below provides a partial list of heterologous proteins that have been successfully expressed in *Pichia pastoris*. Note that both Mut\(^-\) and Mut\(^S\) phenotypes were used successfully as well as secreted and intracellular expression. For reviews, please see these recent references (Cregg and Higgins, 1995; Nico-Farber *et al.*, 1995; Romanos, 1995)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression Levels grams/liter</th>
<th>Where Expressed How Expressed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invertase</td>
<td>2.3</td>
<td>Secreted Mut(^+)</td>
<td>(Tschopp <em>et al.</em>, 1987b)</td>
</tr>
<tr>
<td>Bovine Lysozyme c2</td>
<td>0.55</td>
<td>Secreted Mut(^+)</td>
<td>(Digan <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>Streptokinase (active)</td>
<td>0.08</td>
<td>Intracellular *</td>
<td>(Hagenson <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>Alpha amylase</td>
<td>2.5</td>
<td>Secreted Mut(^S)</td>
<td>(Paifer <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td>Pectate Lyase</td>
<td>0.004</td>
<td>Secreted Mut(^S)</td>
<td>(Guo <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Spinach Phosphoribulokinase</td>
<td>0.1</td>
<td>Intracellular Mut(^S)</td>
<td>(Brandes <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td><strong>Antigens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
<td>0.4</td>
<td>Intracellular Mut(^S)</td>
<td>(Cregg <em>et al.</em>, 1987)</td>
</tr>
<tr>
<td>Pertussis Antigen P69</td>
<td>3.0</td>
<td>Intracellular Mut(^S)</td>
<td>(Romanos <em>et al.</em>, 1991)</td>
</tr>
</tbody>
</table>

*continued on next page*
### Proteins Expressed in *Pichia*, continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression Levels grams/liter</th>
<th>Where Expressed How Expressed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigens, continued</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetanus Toxin Fragment C</td>
<td>12.0</td>
<td>Intracellular Mut'/Mut$^S$</td>
<td>(Clare <em>et al.</em>, 1991a)</td>
</tr>
<tr>
<td>HIV-1 gp120</td>
<td>1.25</td>
<td>Intracellular Mut'$^+$</td>
<td>(Scorer <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Tick Anticoagulant protein</td>
<td>1.7</td>
<td>Secreted Mut$^S$</td>
<td>(Laroche <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td>Bm86 Tick Gut Glycoprotein</td>
<td>1.5</td>
<td>Secreted *</td>
<td>(Rodriguez <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td><strong>Regulatory Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Necrosis Factor (TNF)</td>
<td>10.0</td>
<td>Intracellular Mut$^S$</td>
<td>(Sreekrishna <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>Mouse Epidermal Growth Factor (EGF)</td>
<td>0.45</td>
<td>Secreted Mut$^S$</td>
<td>(Clare <em>et al.</em>, 1991b)</td>
</tr>
<tr>
<td>Human Interferon (IFN) α2b</td>
<td>0.4</td>
<td>Intracellular Mut$^S$</td>
<td>(Garcia <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td><strong>Membrane Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CD38 (soluble portion)</td>
<td>0.05</td>
<td>Secreted Mut$^S$</td>
<td>(Fryxell <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Mouse Serotonin Receptor</td>
<td>0.001</td>
<td>Secreted Mut'$^+$</td>
<td>(Weiss <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td><strong>Proteases and Protease Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>0.8</td>
<td>Secreted Mut'/Mut$^S$</td>
<td>(Despreaux and Manning, 1993)</td>
</tr>
<tr>
<td>Enterokinase</td>
<td>0.021</td>
<td>Secreted Mut'$^+$</td>
<td>(Vozza <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>Ghilanten</td>
<td>0.01</td>
<td>Secreted Mut'$^+$</td>
<td>(Brankamp <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Kunitz protease inhibitor</td>
<td>1.0</td>
<td>Secreted *</td>
<td>(Wagner <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>Human Proteinase Inhibitor 6</td>
<td>0.05</td>
<td>Intracellular Mut'$^+$</td>
<td>(Sun <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Single Chain Antibody</td>
<td>&gt;0.1</td>
<td>Secreted Mut$^S$</td>
<td>(Ridder <em>et al.</em>, 1995)</td>
</tr>
</tbody>
</table>

* 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 page 8). 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\(^\circ\) (KM71). By linearizing the recombinant vector at a restriction enzyme site located in the 5' *AOX1* regions, Mut\(^\circ\) or Mut\(^\circ\) 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 PAOX1, 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.

![Gene Insertion Diagram](image)

*continued on next page*
Gene Insertion Events at *his4*

In either GS115 (Mut+) or KM71 (MutS), 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 results in the insertion of one or more copies of the vector at the *his4* locus. Since 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 MutS 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, the other wild type.

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*continued on next page*
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 MutS phenotype in KM71. Quantitative dot blot analysis, Southern blot analysis, and differential hybridization can detect multiple gene insertion events. Please see page 63 for a protocol to screen for multiple inserts.

continued on next page
Recombination and Integration in *Pichia*, continued

**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+ MutS. His+ transformants can be readily and easily screened for their Mut phenotype, with MutS 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 (MutS) 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 diagram](image_url)

*continued on next page*
**Methods**

**Pichia Strains**

**Introduction**

*Pichia pastoris* is similar to *Saccharomyces cerevisiae* as far as general growth conditions and handling. You should be familiar with basic microbiological and sterile techniques, basic molecular biology, and protein chemistry. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology*, (Guthrie and Fink, 1991), *Current Protocols in Molecular Biology*, (Ausubel et al., 1994), *Molecular Cloning: A Laboratory Manual*, (Sambrook et al., 1989), *Protein Methods*, (Bollag and Edelstein, 1991), 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*) which 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 mutated argininosuccinate lyase gene (*arg4*) that prevents it from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt *AOX1*, creating KM71, a Mut\(^5\), 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 areHis\(^-\).

**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\(^5\) phenotype. Genetic analysis of Arg\(^+\) transformants showed that the wild-type *AOX1* gene was replaced by the *aox1::ARG4* construct.

The advantage of using KM71 is that you do not need to screen for the Mut phenotype on methanol minimal medium. All transformants will be Mut\(^5\). 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\(^5\), Arg\(^-\). 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 as *arg4* strains do not grow well on minimal medium containing arginine. We do not recommend generating His\(^+\) transformants in KM71 by replacing the *aox1::ARG4* construct.

*continued on next page*
**Pichia Strains, continued**

### Control Expression Strains

| GS115/His\(^+\) Mut\(^5\) Albumin: | This strain is a control for secreted expression and the Mut\(^5\) phenotype when screening *Pichia* transformants (page 36). 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 36). The *lacZ* gene was integrated into *Pichia* at the *his4* locus. This strain expresses β-galactosidase (117 kDa) at levels that can be visualized by Coomassie-stained SDS-PAGE (see pages 45-46) or assayed using ONPG (see page 65-66). |

### 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\(^5\) *Pichia* in YPD is ~2 hours
- Mut\(^-\) and Mut\(^5\) 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\(^5\) *Pichia* in MM is ~18 hours
- One OD\(_{600}\) = ~5 x 10\(^7\) cells/ml

Note: 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\(^5\) strains and up to 3% for Mut\(^-\) without any negative effect to their liquid culture.

### Storage of Pichia Strains

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

- Streak for single colonies of GS115, KM71, or a His\(^+\) transformant on YPD.
- Transfer one colony to a YPD stab and grow for 2 days at 30°C.
- The cells can be stored on YPD for several weeks at +4°C.

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

- Culture a single colony of GS115, KM71, or a His\(^+\) transformant overnight in YPD.
- Harvest the cells and suspend in YPD containing 15% glycerol at a final OD\(_{600}\) of 50-100 (approximately 2.5-5.0 x 10\(^9\) cells/ml).
- Cells are frozen in liquid nitrogen or a dry ice/ethanol bath and then stored at -80°C.

After long-term storage at +4°C or -80°C, we recommend checking the His\(^+\) transformants for correct genotype and viability. Streak 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 DH5αF’, JM109, or any other strain which is recombination deficient (*recA*), preferably *endA*, and carries a selectable F’ episome. TOP10F’ is suitable for recombinant work and single-stranded DNA rescue.

F’ \{proAB, lacI, lacZΔM15, Tn10 (TetR)\} mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(arA-leu)7697, galU, galK, rpsL(StrR), endA1, nupG λ’.

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

---

**Recommendation**

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

- Culture TOP10F’ in 5 ml LB with 10 µg/ml tetracycline. Grow overnight.
- Mix thoroughly 0.85 ml of culture with 0.15 ml sterile glycerol.
- Transfer to a freezer vial and freeze in liquid nitrogen or a dry ice/ethanol bath.
- Store at -80°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. Details for each individual plasmid are found on pages 16-19.

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.

*continued on next page*
Selecting a *Pichia* Expression Vector, continued

### Features

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

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>5´ AOX1</td>
<td>An ~1000 bp fragment containing the AOX1 promoter</td>
<td>Allows methanol-inducible high level expression in <em>Pichia</em>; Targets plasmid integration to the AOX1 locus.</td>
</tr>
<tr>
<td>Sig</td>
<td>DNA sequence coding for an N-terminal protein secretion signal</td>
<td>Targets desired protein for secretion</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple Cloning Site</td>
<td>Allows insertion of your gene into the expression vector</td>
</tr>
<tr>
<td>TT</td>
<td>Native transcription termination and polyadenylation signal from AOX1 gene (~260 bp)</td>
<td>Permits efficient transcription termination and polyadenylation of the mRNA</td>
</tr>
<tr>
<td>HIS4</td>
<td><em>Pichia</em> wild-type gene coding for histidinol dehydrogenase (~2.4 kb) and used to complement <em>Pichia his4</em> strains</td>
<td>Provides a selectable marker to isolate <em>Pichia</em> recombinant strains</td>
</tr>
<tr>
<td>3´ AOX1</td>
<td>Sequences from the AOX1 gene that are further 3´ to the TT sequences (~650 bp)</td>
<td>Targets plasmid integration at the AOX1 gene</td>
</tr>
<tr>
<td>Amp ColE1 origin</td>
<td>Ampicillin resistance gene; E. coli origin of replication</td>
<td>Allows selection, replication, and maintenance in E. coli</td>
</tr>
<tr>
<td>f1 origin</td>
<td>Bacteriophage f1 origin of replication</td>
<td>Permits generation of single-stranded DNA for mutagenesis</td>
</tr>
<tr>
<td>Not I Bgl II Sac I Sal I Stu I</td>
<td>Unique restriction sites</td>
<td>Permits linearization of vector for efficient integration into the <em>Pichia</em> genome</td>
</tr>
</tbody>
</table>

### 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). Please 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) in order 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

Summary
The details of pHIL-D2 are listed below:
- 8209 bp nonfusion vector
- One unique EcoRI 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+ Mut8 in KM71)
- For insertion at HIS4, linearize with Sal I or Stu I (generates His+ Mut+ in GS115 and His+ Mut8 in KM71)
- For a gene replacement at AOX1 in GS115, linearize with Not I (generates His+ Mut8)
Please see page 28 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. Details of the multiple cloning site are found on page 22. The complete sequence can be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (page 67).

Comments for pHIL-D2:
8209 nucleotides
5' AOX1 promoter fragment: bases 14-941
5' AOX1 primer site: bases 868-888
EcoRI 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
ColE1 origin: bases 7138-7757
Summary
The details of pPIC3.5 are listed below:
- 7751 bp nonfusion vector
- BamHI, SnaBI, EcoRI, AvrII, NotI 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 SacI (generates His+ Mut+ in GS115 and His+ MutS in KM71)
- For insertion at HIS4, linearize with SalI or StuI (generates His+ Mut+ in GS115 and His+ MutS in KM71)
- For a gene replacement at AOX1 in GS115, linearize with BglII (generates His+ MutS)
Please see page 28 for alternate restriction sites if your insert DNA has a NotI, SacI, SalI, or StuI site.

Map of pPIC3.5
The map below shows the location and size of each feature of pPIC3.5. Details of the multiple cloning site are found on page 23. The complete sequence can be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (page 67).

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
ColE1 origin: bases 6436-5764
Ampicillin resistance gene: bases 7442-6582
pHIL-S1

Summary

The details of pHIL-S1 are listed below:

- 8260 bp fusion vector
- Xho I, EcoR I, Sma I, BamH 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\(^5\) 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\(^5\) in KM71)
- For gene replacement at AOX1 in GS115, linearize with Bgl II (generates His\(^+\) Mut\(^5\))

Please see page 29 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. Details of the multiple cloning site are found on page 24. The complete sequence can be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (page 67).

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
ColE1 origin: bases 6556-5937
f1 origin of replication: bases 6651-7106
Ampicillin resistance gene: bases 7922-7062
pPIC9

Summary

The details of pPIC9 are listed below:
- 8023 bp fusion vector
- *Xho I*, *SnaB I*, *EcoR I*, *Avr II*, *Not I* unique sites
- Secreted expression of your gene using the α-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\(^6\) in KM71)
- For insertion at *HIS4*, linearize with *Sal I* or *Stu I* (generates His\(^+\) Mut\(^+\) in GS115 and His\(^+\) Mut\(^6\) in KM71)
- For gene replacement at *AOX1* in GS115, linearize with *Bgl II* (generates His\(^+\) Mut\(^6\))

Please see page 29 for alternate restriction sites if your insert DNA has a *Bgl II*, *Sac I*, *Sal I*, or *Stu I* site.

Map of pPIC9

The map below shows the location and size of each feature of pPIC9. Details of the multiple cloning site are found on page 25. The complete sequence can be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (page 67).

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 949-1218
α-Factor primer site: bases 1152-1172
Multiple Cloning Site Region: bases 1192-1241
3’ *AOX1* primer site: bases 1327-1347
3’ *AOX1* transcription termination (TT) fragment: bases 1253-1586
*HIS4* ORF: bases 4514-1980
3’ *AOX1* fragment: bases 4870-5626
ColE1 origin: bases 6708-6034
Ampicillin resistance gene: bases 7713-6853
Signal Sequence Processing

When cloning into the Xho I site of pPIC9, the secretion signal sequence between the Xho I site and SnaB 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.

Optimization of Signal Cleavage

In Saccharomyces cerevisiae, it has been noted that 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, will inhibit KEX2 cleavage. For more information on KEX2 cleavage, please see (Brake et al., 1984).

There are some cases where STE13 cleavage of Glu-Ala repeats is not efficient, and Glu-Ala repeats are left on the N-terminus of the expressed protein of interest. This is generally dependent on the protein of interest.

NOTE

The PHO1 signal sequence is atypical of signal sequences even though it is a native Pichia secretion signal, and it is wise to regenerate the full signal sequence between the Xho I and EcoR I sites if cloning into the Xho I site (see page 24). 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 16-19), you will have to 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*, so that you have a permanent stock and a way to make more plasmid.

- 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* as many genes have proven to be cross-functional.
- Plasmid constructions should be maintained in a *recA* mutant *E. coli* strain such as the TOP10F’ strain provided.
- The native 5’ end of the *AOX1* mRNA is noted in each multiple cloning site. This is needed to calculate the size of the expressed mRNA of the gene of interest.
- 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 a gene with high AT content is being expressed, please see page 48.
- The predicted protease cleavage sites for the *PHO1* and α-factor signal sequences are indicated in each figure.
- If attempting to secrete a protein using its native secretion signal, it is highly recommended to also try pPIC9 in parallel. In this case, the open reading frame (ORF) of the mature gene of interest would be cloned in frame and downstream of the α-factor.

**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. EcoRI 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 (Catalog no. K2000-01), followed by subcloning of a compatible fragment into the appropriate *Pichia* expression vector.

continued on next page
Cloning into the *Pichia* Expression Vectors, continued

### Cloning Procedures


### 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. Please 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.

Invitrogen provides both electrocompetent and chemically competent TOP10F’ and chemically competent JM109 *E. coli* strains for your convenience.

<table>
<thead>
<tr>
<th>Description</th>
<th>Efficiency</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Shot® TOP10F’</td>
<td>1 x 10⁸</td>
<td>C3030-03</td>
</tr>
<tr>
<td>Chemically Competent TOP10F’</td>
<td>1 x 10⁸</td>
<td>C665-03</td>
</tr>
<tr>
<td>Electrocompetent TOP10F’</td>
<td>1 x 10⁹</td>
<td>C665-55</td>
</tr>
</tbody>
</table>

### \(P_{AOX1}\) and Multiple Cloning Site of pHIL-D2

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

\[
\begin{align*}
&ACAGGCAATA TATAAAGAG AGAATGCTGC CTTGTTTGGG TTTAACGAC TTTTAAAGAG AACTTGGAGA \\
&CTTCATAAT TGGACTGTT CTCAATTGAC AGACCTTGGG TTTAAGAG AACTTGGAGA \\
&GATCAAAAA CAACTAATTA TTGGAAAGGA GAAATCGCC TTTAGCTGGA CTGTTTGGGA GTTCAAGTTG \\
&GGCATTACGA GAAGACCGGT CTGGCTAGAT TCTAATCAAG AGATATCGAG ATGCCCATTT GCTGAGAGA \\
&TGCAGGCTTC ATATTTGATA CTTTTTATT TGTAACCTAT ATAGATAGG ATTTTTTGG TCA
\end{align*}
\]

### Special Considerations

- For pHIL-D2, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, ACC ATG G is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990).

- 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, please see page 28 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

*continued on next page*
Cloning into the *Pichia* Expression Vectors, continued

**P<sub>AOX1</sub> and Multiple Cloning Site of pPIC3.5**

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

```
TTATCATCAT TATTAGCTTA CTTTCAATA TGCAGCTGGT TCCAATTTGA TTGGTACGAC
        BamH I         SnaB I EcoR I        Avr II
TTTAAACGAC AACTTGAGAA GATCAAAAA CAACTAATTA TGGAGGGAT CCTACGTAGA ATTCCTAGG
        Not I
GGGCGCGCGA ATTATTTCGCT TTAAGCATG ACTGTTCCTG AGTTCAAGTT GGGCACTTAC GAGAAGACC
            3' AOX1 Primer Site (1055-1075)
GTCTGGTCTAG ATTTCTAATCA AGAATGTC AAGATTCATG TGCCCTGAGA GATGCAGGCT TCATTTTTGA
            AOX1 mRNA 3' end (1146)↓
TACTTTTTTA TTTGAAACCT ATATAGATA GAGATTTTT TGTCATTTTG TTCTTC
```

**Special Considerations**

- For pPIC3.5, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC ATG G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990).
- 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*HI and *Sna*BI or *Sna*BI and *Eco*RI, digest with *Sna*BI first. If you digest with *Bam*HI or *Eco*RI first, the *Sna*BI 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, please see page 28 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

*continued on next page*
The sequence below shows the detail of the multiple cloning site and surrounding sequences.

```
773  AOXI mRNA 5' end (825)
ACAGGCAATA TATAAACAGA AGGAAGCTGC CCTGTCTTAA ACCTTTTTTT TTATCATCAT

5' AOXI primer site (856-876)
TATATTACCTA TTTTACTAAT TGGGACTGTT TCCAATTGAC AAGCTTTTGA TTTTAAAGAC

PHO1 (942-1007)
TTTTAAGAC AAATGGAAAG GATCAAAAAA CAATCATTAA TTCGAAACGG ATG TTC TCT
Met Phe Ser

CCA ATT TTG TCC TTG GAA ATT ATT TTA GCT TTG GCT ACT TTG CAA TCT GTC
Pro Ile Leu Ser Leu Glu Ile Ile Leu Ala Leu Ala Thr Leu Gln Ser Val

PHO1 cleavage site

XhoI 1* EcoRI

TTT AACGGAC AACTTGAGAA GATCAAAAAA CAATCATTAA TTCGAAACGG

SmaI 1

ATG TTC TCT

BamHI 1

GTT GGG CAC TTA CGA GAA CGG TCT TGC TAG ATTCTAATCA AGAGGATGTC
Phe Ala Arg Glu Pro Gly Ile Leu Arg His Asp Cys Ser Ser Val Gln

PHO1 cleavage site

Stop (1083)

AGAATGCACT TTGCTGAGA GATCGAGGCT TCAATTTTTA AAGGTAACT
Val Gly His Leu Arg Glu Asp Arg Ser Cys ***

3' AOXI primer site (1099-1119)

ATCGATGCTA AGAACTTTTT TTGTAACC

AOXI mRNA 3' end (1190)
```

* 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, please see 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 general, more success has been reported with the α-factor (pPIC9) or native 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 page*
Cloning into the *Pichia* Expression Vectors, continued

**P*~AOX1~ and Multiple Cloning Site of pPIC9**

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

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>773</td>
<td>AOX1 mRNA 5' end (824)</td>
</tr>
<tr>
<td>ACAGCAATAT ATAAACAGAA GGAAGCTGACC CTGTCTTTAA CCTTTTTTT TATCATCATT ATTAGCTTAC</td>
<td>5' AOX1 Primer Site (855-875)</td>
</tr>
<tr>
<td>TTTCAATAATG GCCGCTGGTT CCAAATGACA AGCTTTGTG TTTAACGACT TTTAACGACA ACTTGAGAAC</td>
<td>α-Factor (949-1218)</td>
</tr>
<tr>
<td>ATCAAAAAAC AACTAATTAT TCGAAGGATC CAAACG</td>
<td></td>
</tr>
<tr>
<td>GT TTA TGC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp</td>
<td></td>
</tr>
<tr>
<td>GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GTG TAC TCA GAT TTA GAA GGG GAT Glu Thr Ala Gin Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp</td>
<td></td>
</tr>
<tr>
<td>TTC GAT GTT GCT GTG TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA Phe Asp Val Val Val Leu Pro Phe Ser Asn Ser Thr Asn Gly Leu Leu Phe Ile</td>
<td>Xho I</td>
</tr>
<tr>
<td>Xho I Primer Site (1152-1172)</td>
<td></td>
</tr>
<tr>
<td>AAT ACT ACT ATT GCC AGC ATT GCT CCT AAA GAA GAA GGG GTA TCT CTC GAG AAA AGA Asn Thr Thr Ile Ala Ser Ile Ala Lys Glu Glu Gly Val Ser Leu Glu Gly Arg</td>
<td></td>
</tr>
<tr>
<td>Signal cleavage (1204)</td>
<td></td>
</tr>
<tr>
<td>SnuB I</td>
<td>EcoR I</td>
</tr>
<tr>
<td>GAG GCT GAA GCT TAC GGA GAA TTC CCT AGG GGC GCC GCG AAT TAA TCGCGCTTAG Glu Ala Glu Ala Tyr Val Glu Phe Pro Arg Ala Ala Ala Asn ***</td>
<td></td>
</tr>
<tr>
<td>ACATGACTGT TCTCTAGTTC AAGTTGGCCA CTTACGAGAA GACCGGTCTT GCTAGATTCT AATCAAGAG</td>
<td>3' AOX1 Primer Site (1327-1347)</td>
</tr>
<tr>
<td>ATGTCAAGAT GCATTTGCCC TGAGAGATGC AGGCCATCATT TTTGATACCT TTTATTTGT AACCTATATA AOX1 mRNA 3' end (1418)</td>
<td></td>
</tr>
<tr>
<td>GTATAGGATT TTTTTTGCTA</td>
<td></td>
</tr>
</tbody>
</table>

* If cloning into the *Xho* I site, the sequence between the *Xho* I site and *SnuB* 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 *SnuB* 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. See discussion on page 20.
- 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, please see page 29 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.
Transformation into *E. coli*

**Introduction**

At this point you should have ligation reactions which you will transform by chemical means or electroporation into competent *E. coli* cells (TOP10F’ or equivalent). To prepare competent cells, see *Current Protocols in Molecular Biology* (Ausubel et al., 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook et al., 1989).

**Analysis of Transformants**

1. After transformation, plate the transformation mix onto LB plates with 50-100 µg/ml ampicillin (see Recipes, page 53) 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 a 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. Once your construct is confirmed by sequencing, proceed to Preparation of Transforming DNA, next page.

**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 below 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 22-25.

For sequencing protocols, please 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). Sequencing primers are described below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
</tr>
</thead>
</table>
| 5´ *AOX1* Sequencing Primer | is 5´ GACTGGTTCCAATTTGACAAGC 3´  
hybridizes 5´ of the MCS in the *AOX1* promoter region  
allows the determination of the 5´ *AOX1*-gene of interest junction  
confirms that the ORFs are preserved where necessary |
| 3´ *AOX1* Sequencing Primer | is 5´ GCAAATGGCATTCTGACATCC 3´  
hybridizes just 3´ of the MCS in the 3´ *AOX1* (TT) region  
allows the determination of the 3´ *AOX1*-gene of interest junction |
| α-Factor Sequencing Primer | is 5´-TACTATTGCCAGCATTGCTGC-3´  
hybridizes within the α-factor leader region in pPIC9  
allows the determination of the 5´ end of the gene of interest  
confirms that the ORFs are preserved where necessary |
Preparation of Transforming DNA

Introduction

You should have a *Pichia* expression vector with your gene of interest cloned in the correct orientation for expression. The table below outlines the next few steps.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prepare your DNA for transformation</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>Grow either GS115 or KM71 to prepare spheroplasts</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Prepare spheroplasts for transformation</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>Transform GS115 or KM71 with your DNA</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Select His&lt;sup&gt;+&lt;/sup&gt; transfectants and characterize for Mut&lt;sup&gt;+&lt;/sup&gt;/Mut&lt;sup&gt;+&lt;/sup&gt; phenotype</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Test 10 His&lt;sup&gt;+&lt;/sup&gt; Mut&lt;sup&gt;+&lt;/sup&gt; and 10 His&lt;sup&gt;+&lt;/sup&gt; Mut&lt;sup&gt;+&lt;/sup&gt; by PCR for integration of your gene</td>
<td>40</td>
</tr>
</tbody>
</table>

We recommend isolating both His<sup>+</sup> Mut<sup>+</sup> and His<sup>+</sup> Mut<sup>+</sup> *Pichia* transformants as it is difficult to predict beforehand what construct will best express your protein (see pages 7 and 8). By linearizing your construct DNA in the 5´ *AOX1* region or in the *HIS4* gene and using GS115 (Mut<sup>+</sup>) and KM71 (Mut<sup>+</sup>), you can easily isolate Mut<sup>+</sup> and Mut<sup>+</sup> recombinants. Plan on using ~10 µg digested DNA for each transformation.

Preparation of 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 use alkaline lysis, phenol:chloroform extraction, and ethanol precipitation to prepare plasmid DNA for routine *Pichia* transformations.

Procedure

1. To isolate His<sup>+</sup> Mut<sup>+</sup> transformants of GS115, linearize all constructs with *Sal*I, *Stu*I, or *Sac*I.
2. To isolate His<sup>+</sup> Mut<sup>+</sup> transformants of KM71, linearize plasmid constructions with *Sal*I, *Stu*I, or *Sac*I.
   **Note:** If your insert DNA has all three sites, please see the next page.
3. To isolate His<sup>+</sup> Mut<sup>+</sup> transformants of GS115, linearize with *Not*I (pHIL-D2) or *Bgl*II (pPIC3.5, pHIL-S1, and pPIC9)
   **Note:** If you wish to generate recombinants that are Mut<sup>+</sup>, use KM71 as it is much easier and more efficient to generate Mut<sup>+</sup> recombinant strains using single crossover events than double crossover events (e.g. insertions at *AOX1* or *his4* as opposed to gene replacement at *AOX1*).
4. Digest both your construct and the parent vector. The parent vector will be transformed into GS115 and/or KM71 and used as a background control for expression.
5. Use 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.
6. 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.
7. Store at -20°C until ready to transform.

continued on next page
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 will give integration, but at lower efficiencies. Remember to digest the parent vector with the same enzyme when preparing your DNA samples for transformation.

For pHIL-D2

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>5′ AOX1 14-940 bp</th>
<th>3′ AOX1 4577-5333 bp</th>
<th>Vector backbone 5333+ bp</th>
<th>HIS4 gene 1688-4222 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sac I</td>
<td>221</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pme I</td>
<td>424</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bpu 1102 I</td>
<td>599</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Nsi I</td>
<td>689</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Xcm I</td>
<td>711</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Not I</td>
<td>8</td>
<td>5337</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dra I*</td>
<td>424</td>
<td>5169, 5311</td>
<td>5896, 6588</td>
<td>--</td>
</tr>
<tr>
<td>Sal I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2887</td>
</tr>
<tr>
<td>Stu I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2972</td>
</tr>
<tr>
<td>BspE I</td>
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<td>3554</td>
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For pPIC3.5

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<th>Restriction Enzyme</th>
<th>5′ AOX1 1-937 bp</th>
<th>3′ AOX1 4616-5393 bp</th>
<th>Vector backbone 5393+ bp</th>
<th>HIS4 gene 1715-4249 bp</th>
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<tbody>
<tr>
<td>Sac I</td>
<td>209</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pme I</td>
<td>414</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bpu 1102 I</td>
<td>589</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Nsi I</td>
<td>678</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Xcm I</td>
<td>699</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bgl II</td>
<td>2</td>
<td>5363</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dra I*</td>
<td>414</td>
<td>5201, 5343</td>
<td>6534, 6553, 7245</td>
<td>--</td>
</tr>
<tr>
<td>Sal I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2919</td>
</tr>
<tr>
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</tr>
<tr>
<td>BspE I</td>
<td></td>
<td></td>
<td></td>
<td>3586</td>
</tr>
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continued on next page
### Alternate Restriction Sites, continued

**For pHIL-S1**

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>5’AOXI 1-940 bp</th>
<th>3’AOXI 4639-5395 bp</th>
<th>Vector backbone 5395+ bp</th>
<th>HIS4 gene 1750-4284 bp</th>
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</thead>
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<tr>
<td>Sac I</td>
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<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pme I</td>
<td>412</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bpu 1102 I</td>
<td>587</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Nsi I</td>
<td>677</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Xcm I</td>
<td>699</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bgl II</td>
<td>2</td>
<td>5394</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dra I*</td>
<td>412</td>
<td>5232, 5374</td>
<td>7021, 7713</td>
<td>--</td>
</tr>
<tr>
<td>Sal I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2950</td>
</tr>
<tr>
<td>Stu I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>3035</td>
</tr>
<tr>
<td>BspE I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>3617</td>
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**For pPIC9**

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>5’AOXI 1-948 bp</th>
<th>3’AOXI 4881-5638 bp</th>
<th>Vector backbone 5638+ bp</th>
<th>HIS4 gene 1980-4514 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sac I</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pme I</td>
<td>414</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bpu 1102 I</td>
<td>589</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Nsi I</td>
<td>678</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Xcm I</td>
<td>699</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bgl II</td>
<td>2</td>
<td>5622</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dra I*</td>
<td>414</td>
<td>5460, 5602</td>
<td>6793, 6812 7504</td>
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</tr>
<tr>
<td>Sal I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>3178</td>
</tr>
<tr>
<td>Stu I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>3263</td>
</tr>
<tr>
<td>BspE I</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>3845</td>
</tr>
</tbody>
</table>

*Restriction sites are used to generate gene replacements at AOX1 in GS115 only.*
Growth of *Pichia* for Spheroplasting

**Introduction**

In general, spheroplasting and electroporation (page 59) 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 60) or lithium chloride (page 61). These two protocols, particularly lithium chloride, perform less well than spheroplasting or electroporation. If you do not have an electroporation device, try spheroplasting or using PEG 1000. Transformation in *Pichia* is less efficient than for *Saccharomyces*. For references on general yeast transformation, please see (Cregg *et al.*, 1985; Hinnen *et al.*, 1978).

**Explanation of 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 β-1,3 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 lyse. 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, which is monitored by the absorbance (light-scattering), at 800 nm. It has been empirically determined that when 30% of the cells have lysed (70% spheroplasting) that 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.

**Preparation**

Prepare the following media several days in advance and store at +4°C (see Recipes, pages 55-56 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

**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 CaCl2
- **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 CaCl2
- **SOS:** 1 M sorbitol, 0.3X YPD, 10 mM CaCl2

**Prepared fresh for each transformation:**

- **SED:** 19 ml of SE and 1 ml of 1 M DTT (see page 32)
- **PEG/CaT:** 1:1 mixture of 40% PEG and CaT (see page 34)

*continued on next page*
Growth of *Pichia* for Spheroplasting, continued

**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). This culture may be stored 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<sub>600</sub> of each of the three culture flasks. Harvest the cells from the culture which has an OD<sub>600</sub> between 0.2 and 0.3 by centrifuging at room temperature for 5-10 minutes at 1500 x g. Use these cells for preparing the spheroplasts for transformation. Decant the supernatant and discard the other cultures.

**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<sub>600</sub> is between 0.2 and 0.3 (2-4 hours). Harvest the cells and proceed to Preparation of Spheroplasts, page 32.
Preparation of Spheroplasts

Before Starting

You should have a cell pellet from Step 5, page 31.

- Prepare 100 ml of molten RD agarose and keep at 45°C (see Recipes, page 56)
- 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.

Important

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 31 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 1500 x g for 5 minutes at room temperature. Decant and discard the supernatant. Use the cell pellet to prepare spheroplasts.
3. Wash the cell pellet once by resuspending in 20 ml of fresh SED, prepared above and centrifuge at 1500 x 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.

Addition of Zymolyase

You will use one tube of cells prepared above to determine the optimal time of digestion with Zymolyase to make spheroplasts. Once the optimal time is determined, use the other tube of cells to make spheroplasts.

- Zymolyase digests the cell wall and makes the cells extremely fragile. Handle the sample gently. The moment after addition of Zymolyase, digestion of the cell wall begins.
- 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.
1. From one tube of cells (Step 5 above), 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 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. Keep the second tube of cells at room temperature for use in Step 6. Keep the remaining Zymolyase on ice.

continued on next page
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 OD800 for all samples.

4. Determine the percent of spheroplasting for each time point using the equation:
   \[
   \% \text{ Spheroplasting} = 100 - \left(\frac{\text{OD}_{800 \text{ at time } t}}{\text{OD}_{800 \text{ at time } 0}}\right) \times 100
   \]
   
   **For example:**
   
   At time t = 0, the OD800 = 0.256
   
   At time t = 15, the OD800 = 0.032
   
   Calculation:
   \[
   \% \text{ spheroplasting} = 100 - \left(\frac{0.032}{0.256}\right) \times 100
   \]
   \[
   = 100 - (0.125)\times100
   \]
   \[
   = 100 - 12.5
   \]
   \[
   = 87.5\%
   \]

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. 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 x 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 x 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 34). They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations.
Transformation of *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 x 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**, below.

**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, above 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 plates and incubate at 28-30°C. Appearance of colonies after 4-6 days demonstrates that the spheroplasts can regenerate into dividing cells.

*continued on next page*
Transformation of *Pichia*, continued

### Evaluating Your Transformation Experiment

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

### Optional Method

Because of plating in top agarose, transformants can be on top or imbedded in the top agarose making it difficult to pick and patch colonies in the next section. The following protocol allows you to collect the transformants and re-plate them directly onto plates without 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 1500 x g for 5 minutes at room temperature. This will pellet the cells on the bottom of the tube and any remaining agarose will pellet 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 overnight at 30°C. Proceed to Screening for Mut⁺ and Mut⁺S Transformants, next page.

### Chemically Competent *Pichia* Cells

The *Pichia* EasyComp™ Kit (Catalog no. K1730-01) provides reagents to prepare 6 preparations of competent cells. Each preparation will yield enough competent cells for 20 transformation. These cells may be used immediately or frozen and stored for future use. Each 50 µl aliquot of competent *Pichia* cells with 3 µg linearized plasmid DNA will yield 50 colonies on selective medium. Please call Technical Service for more information.
**Screening for Mut\(^+\) and Mut\(^S\) Transformants**

**Introduction**

At this point, you should have plates of His\(^+\) GS115 transformants that you need to score for Mut\(^+\) and Mut\(^S\) phenotype. Included in the kit are two strains that will provide examples of Mut\(^+\) and Mut\(^S\) phenotypes. GS115 Albumin is Mut\(^S\) and GS115 \(\beta\)-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. Again, testing on MD and MM plates will allow you to isolate His\(^+\) Mut\(^+\) transformants (see next page).

**His\(^+\) Mut\(^S\) in KM71**

All His\(^+\) transformants in KM71 will be Mut\(^S\) because of the disruption of the *AOX1* gene (*aox1::ARG4*). There is no need to test recombinants for the Mut phenotype; all recombinants will be Mut\(^S\). Transformation of KM71 with *Sal I* or *Stu I* linearized plasmid constructions favor recombination at the *HIS4* locus while *Sac I*-linearized plasmid constructions favor recombination at the 5' region of the *AOX1* gene. His\(^+\) transformants need to be purified on minimal plates without histidine to ensure pure clonal isolates before either testing for expression (see page 42) or confirming integration by PCR (page 40).

**Screening for His\(^+\) Mut\(^S\) in GS115**

Transformation of GS115 with *Not I*-linearized pHIL-D2 or *Bgl II*-linearized pPIC3.5, pHIL-S1 and pPIC9 constructs favor 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 are not producing alcohol oxidase (the product of the *AOX1* gene), they cannot efficiently metabolize methanol as a carbon source and therefore 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\(^+\)).

**Preparation**

The following media (see page 57) and materials can be prepared several days in advance and stored at +4\(^\circ\)C:

- Minimal Dextrose (MD) agar plates, 1 liter
- Minimal Methanol (MM) agar plates, 1 liter
- Sterile toothpicks and Scoring Templates (see page 39)

Streak out the strains GS115 Albumin (His\(^+\) Mut\(^S\)) and GS115 \(\beta\)-Gal (His\(^+\) Mut\(^+\)) on an MD or MGY plate as controls for Mut\(^+\) and Mut\(^S\) growth on MD and MM plates.

*continued on next page*
In contrast to His\(^+\) Mut\(^S\) transformants generated by linearizing with \(\text{Not} \ I\) or \(\text{Bgl} \ II\), most of the His\(^+\) transformants generated by digestion with \(\text{Sac} \ I, \text{Sal} \ I, \text{or Stu} \ I\) should be Mut\(^+\). These will be gene insertion events at either the \(\text{his}4\) or \(\text{AOX1}\) loci, leaving an intact \(\text{AOX1}\) locus.

**NOTE**

**His\(^+\) Mut\(^S\) or His\(^+\) Mut\(^+\) in GS115**

Use the plates containing the His\(^+\) transformants and screen for the Mut\(^+\) and Mut\(^S\) phenotype as described below.

1. Using a sterile toothpick, pick one colony and streak or patch one His\(^+\) transformant in a regular pattern on both an MM plate and an MD plate, making sure to patch the MM plate first.
2. Use a new toothpick for each transformant and continue until 100 transformants have been patched (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\(^+\) \(\beta\)-gal) onto the MD and MM plates.
4. Incubate the plates at 30\(^\circ\)C for 2 days.
5. After 2 days or longer at 30\(^\circ\)C, score the plates. Mut\(^+\) transformants will grow well on both MD and MM plates. Mut\(^S\) transformants will grow well on MD plates, but show little or no growth on the MM plates.

**Important**

We recommend purifying your His\(^+\) transformants to ensure isolation of a pure clonal isolates. You may do this before or after testing for the Mut phenotype.

**Replica-Plating Procedure**

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

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\(^+\) \(\beta\)-gal onto the MD plates.
2. Incubate the plates at 28-30\(^\circ\)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\(^\circ\)C for 2 days.
5. After 2 days at 28-30\(^\circ\)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 \(\text{Pichia}\) 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. This will help you optimize expression of your recombinant clone.

*continued on next page*
Screening for Mut⁺ and Mut⁵ Transformants, continued

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 10 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⁺ Mut⁵ 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⁺ Mut⁵ or His⁺ Mut⁺ recombinant strain.

Please see the Appendix, page 63 for methods to detect multiple integration events.

Vectors for Multiple Integration

Three vectors that can be used to isolate or generate *Pichia* recombinants containing multiple integrations of your desired gene are available separately from Invitrogen. Two of the vectors, pPIC3.5K and pPIC9K, are used *in vivo* to identify possible transformants with multiple copies of your gene inserted. The other vector, pAO815, is used to create 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. This kanamycin resistance gene confers resistance to G418 in *Pichia*. Multiple insertions of the kanamycin gene into the *Pichia* chromosome increases this resistance to G418. Since 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.

For More Information

Please contact our Technical Service (page 67) for more information about these vectors.

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAO815</td>
<td>20 µg, lyophilized</td>
<td>V180-20</td>
</tr>
<tr>
<td>pPIC3.5K</td>
<td>20 µg, lyophilized</td>
<td>V173-20</td>
</tr>
<tr>
<td>pPIC9K</td>
<td>20 µg, lyophilized</td>
<td>V175-20</td>
</tr>
<tr>
<td>Any two of the above <em>Pichia</em> vectors</td>
<td>20 µg each, lyophilized</td>
<td>V177-20</td>
</tr>
</tbody>
</table>
### Screening for Mut$^+$ and Mut$^S$ Transformants, continued

**Scoring**

**Templates**

<table>
<thead>
<tr>
<th>Template</th>
<th>Score</th>
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<td>51</td>
<td>52</td>
</tr>
</tbody>
</table>

![Circular diagram with number labels](image)
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 6-10 Mut⁵ or Mut⁺ Pichia clones using the protocol on page 62 as well as the strain transformed with the parent plasmid. After isolating your DNA, use the procedure below to identify integrants. Amplify the gene of interest using either the α-factor primer (for pPIC9 only) or 5’ AOX1 primer paired with the 3’ AOX1 primer included in the kit. This protocol can confirm integration of the gene of interest but will not provide information on the site of integration.

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
   - *For use, resuspend the lyophilized primer in 20 µl sterile water. The amount of primer may be decreased if desired. For ~20 pmoles primer, use 2 µl of each primer.

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

2. Layer reactions with 50 µl of mineral oil. If PCR machine is equipped with a non-evaporation unit, mineral oil is not required.

3. Load thermocycler and run the following program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start</td>
<td>94°C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>1 minute</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>7 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

4. Analyze 10 µl on a 1X TAE, 0.8 % agarose gel.

5. If screening Mut⁺ integrants, you should see two bands. One will correspond to the size of your gene of interest, the other to the AOX1 gene (approximately 2.2 kb). If screening Mut⁵ 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 will produce the following sized PCR products:

<table>
<thead>
<tr>
<th>Vector</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHIL-D2</td>
<td>188 bp</td>
</tr>
<tr>
<td>pPIC3.5</td>
<td>214 bp</td>
</tr>
<tr>
<td>pHIL-S1</td>
<td>262 bp</td>
</tr>
<tr>
<td>pPIC9 (using the 5’ AOX1 primer)</td>
<td>492 bp</td>
</tr>
<tr>
<td>pPIC9 (using the α-Factor primer)</td>
<td>195 bp</td>
</tr>
</tbody>
</table>

Remember to add these fragments to the size of your insert to interpret your PCR results. See next page for an example.
PCR Analysis of *Pichia* Integrants, continued

**Important**

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

**Note**

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

**Example of PCR Analysis**

The figure below shows the results of a typical PCR analysis using the procedure on page 40. 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.

![PCR Analysis Image](image)

**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). Analyzing the *Pichia* recombinants in lanes 2-4, it can be seen that lanes 2 and 3 contain insert, and that the recombinant in lane 2 may be a Mut<sup>6</sup> as there is no wild-type AOX1. Lane 4, although from a His<sup>+</sup> transformant, does not contain the gene of interest.

**Note**

Invitrogen's Easy-DNA™ Kit (Catalog. no. K1800-01) provides a fast and easy method to isolate genomic DNA from *Pichia pastoris*. Please call Technical Service (see page 67) to inquire about this kit.

The 5´ AOX1, 3´ AOX1, and the α-factor primers are available separately from Invitrogen.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5´ AOX1 Primer, 2 µg, lyophilized</td>
<td>N710-02</td>
</tr>
<tr>
<td>3´ AOX1 Primer, 2 µg, lyophilized</td>
<td>N720-02</td>
</tr>
<tr>
<td>5´ and 3´ AOX1 Primers, 2 µg each, lyophilized</td>
<td>N740-02</td>
</tr>
<tr>
<td>α-Factor Primer, 2 µg, lyophilized</td>
<td>N730-02</td>
</tr>
</tbody>
</table>
Expression of Recombinant *Pichia* Strains

**Introduction**

You should now have several Mut<sup>8</sup> and Mut<sup>+</sup> recombinant strains (using either GS115 or KM71) which have been confirmed by PCR to contain your insert. The purpose of this section is to determine the optimal method and conditions for expression of 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 will need either 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 Recipes, pages 56-58). 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, a wide range of pH values may be used 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, expression in an unbuffered medium may then be tried.

**Aeration**

The most important parameter for efficient expression in *Pichia* is adequate aeration during methanol induction. As a general rule when inducing expression, never allow cultures to be more than 10-30% of your total flask volume. It is strongly recommended that baffled flasks be used. See page 50 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.)

**Kinetics of Growth**

Note that while Mut<sup>+</sup> and Mut<sup>8</sup> strains will grow at essentially the same rate in YPD or glycerol media, Mut<sup>+</sup> will grow faster than Mut<sup>8</sup> when both are grown on methanol because of the presence of the AOX1 gene product.

**Temperature and Shaking**

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

*continued on next page*
Expression of Recombinant *Pichia* Strains, continued

**Before Starting**

You should have verified recombinants in GS115 or KM71 as well as a control recombinant of GS115 or KM71/Vector (no insert). When performing your expression, it is important to include the proper controls so that you will be able to interpret your expression results. The expression controls which should be used are:

- GS115/His⁺ Mut³ albumin Mut³ - Secretion control
- GS115/His⁺ Mut⁺ β−Gal Mut⁺ - Intracellular control
- GS115 or KM71/Vector (no insert) background control

Because recombination can occur in many different ways that effect expression (clonal variation), we recommend that 6-10 verified recombinant clones be screened for expression levels. Start with colonies from the freshest plates available. Colony viability drops over time, so you may want to streak out your strain. You may also start the cultures with a small sample from a frozen glycerol stock generated from a single colony.

**Guidelines for Expression**

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

**Mut⁺ Intracellular or Secreted**

Test the effectiveness of your expression conditions by growing GS115 β-Gal which is Mut⁺ and expresses β-galactosidase intracellularly. Remember to 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 in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD₆₀₀ = 2-6 (~16-18 hours). The cells will be in log-phase growth.

2. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD₆₀₀ of 1.0 in MM, BMM, or BMMY medium to induce expression (approximately 100-200 ml).

3. Place culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.

4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.

5. At each of the times indicated below, transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a microcentrifuge for 2-3 minutes at room temperature.

   Time points (hours): 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96.

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 N₂ or a dry ice/alkohol bath.

   For intracellular expression, decant the supernatant and store just the cell pellets at -80°C until ready to assay. Freeze quickly in liquid N₂ or a dry ice/alkohol bath.

7. Analyze the supernatants and cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot or functional assay (see *Analysis by SDS-Polyacrylamide Gel Electrophoresis*, page 45).
Expression of Recombinant *Pichia* Strains, continued

**MutS Intracellular or Secreted**

You can test the effectiveness of your expression conditions by growing GS115 Albumin that is MutS 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 in a 1 liter 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. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend 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 in a 100 ml baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue to grow.

4. Add 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction.

5. At each of the times indicated below transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature.

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

6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at -80°C until ready to assay. Freeze quickly in liquid N$_2$ 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 **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, next page).
Analysis by SDS-Polyacrylamide Gel Electrophoresis

Introduction

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, please see standard texts such as Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989), Current Protocols in Molecular Biology (Ausubel et al., 1994), Guide to Protein Purification (Deutscher, 1990), or Protein Methods (Bollag and Edelstein, 1991).

Preparation of Samples

You will need to prepare Breaking Buffer (see page 58) and have acid-washed 0.5 mm glass beads on hand.

Preparation 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.
3. Add an equal volume of acid-washed glass beads (size 0.5 mm). Estimate equal volume by displacement.
4. Vortex 30 seconds, then incubate on ice for 30 seconds. Repeat for a total of 8 cycles.
5. Centrifuge at maximum speed for 10 minutes at +4°C. Transfer the clear supernatant to a fresh microcentrifuge tube.
6. Take 50 µl of supernatant (cell lysate) and mix with 50 µl 2X SDS-PAGE Gel Loading buffer (Sample Buffer).
7. Boil for 10 minutes and load 10-20 µl per well. Thickness of the gel and number of wells will determine volume loaded. Remaining sample may be stored at -20°C for western blots, if necessary. Cell lysates may be stored at -80°C for further analysis.

Preparation of supernatant (Secreted Expression only):
1. Thaw supernatants and place on ice.
2. Mix 50 µl of the supernatant with 50 µl of SDS-PAGE Gel Loading buffer.
3. Boil 10 minutes, 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. 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 (Amicon) 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
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 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 will depend on several factors, including its expression level, its solubility, its molecular weight, and whether an abundant cellular protein of the same size will mask 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.

Analysis of 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 either a western blot or a functional assay if you have one.

If you detect low expression of your recombinant protein, see Optimization of *Pichia* Protein Expression, page 47, for guidelines to optimize expression.

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 64 for an RNA isolation protocol.
Optimization of *Pichia* Protein Expression

**Introduction**

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

**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.

**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 50).
- For Mut⁺, induce expression with a higher density culture.

**Low Expression Levels**

- Look for multi-copy recombinants (i.e. jackpot clones) by slot blot (see page 63). There are quite a few examples of increasing the expression levels of a particular protein by increasing the gene dosage. See (Clare *et al.*, 1991a; Clare *et al.*, 1991b; Romanos *et al.*, 1991).
- Check both Mut⁺ and Mut⁸ recombinants for increased expression. Some proteins express better in one type of genetic background than another.
- If secreting your protein, try intracellular expression. The protein may not be processed correctly and fail to secrete. Be sure you check your cell pellets for evidence of expression. If you are having problems with intracellular expression, try secreting your protein. It probably will glycosylate which may be desirable or not. If glycosylation is undesirable, oligosaccharides can be removed with Peptide:N-Glycosidase F (New England BioLabs).
- Scale up to fermentation (page 50). *Pichia* is a yeast and is particularly well suited to fermentation. Please call Invitrogen Technical Service (page 67) for recommendations.

*continued on next page*
Optimization of *Pichia* Protein Expression, continued

**No Expression**  
Be sure to try some of the easier things listed above as no expression can be the same thing as very low expression. If none of these things improve protein expression, 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 64).

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 as your protein will not go through the secretion pathway and therefore, not be modified.
- Try deglycosylating the protein with Peptide:N-Glycosidase For other enzymes (see page 52).
Scale-up of Expression

Guidelines for Expression

Once expression is optimized, you may scale-up your expression protocol. This may be done by increasing the culture volume using larger baffled flasks (below) or fermentation (call Invitrogen Technical Service, see page 50 and page 67). Use the guidelines below to scale-up your expression protocol. To purify your protein, please refer to page 51.

Mut+ Intracellular or Secreted

1. Inoculate a single colony into 25 ml of MGY, BMG, or BMGY in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD₆₀₀ = 2-6 (approximately 16-18 hours).

2. Use this 25 ml culture to inoculate 1 liter of MGY, BMG, 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 (OD₆₀₀ = 2-6)

3. Harvest the cells into sterile centrifuge bottles by centrifuging at 1500-3000 x g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet to an OD₆₀₀ = 1.0 (2-6 liters) in MM, BMM, 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 1500-3000 x g for 5 minutes at room temperature. For intracellular expression, decant the supernatant. Cells can be processed immediately or stored at -80°C until ready for use.

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

MutS Intracellular or Secreted

1. Using a single colony, inoculate 10 ml of MGY, BMG, 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 OD₆₀₀ = 2-6 (approximately 16-18 hours).

2. Use this 10 ml culture to inoculate 1 liter of MGY, BMG, 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 (OD₆₀₀ = 2-6)

3. Harvest the cells by centrifuging at 1500-3000 x 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 MM, BMM, 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. 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 1500-3000 x g for 5 minutes at room temperature. For intracellular expression, decant the supernatant and store the cell pellets at -80°C. For secreted expression, save the supernatant, chill to +4°C, and concentrate, if desired (see next page). Proceed directly to protein purification or store the supernatant at -80°C until ready to process further.

continued on next page
To increase the amount of cells for Mut^8 recombinants, increase the number of flasks, put 200-300 ml in a 3 liter flask, or try fermentation.

**NOTE**

**Concentration of Proteins**

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

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

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

**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. Please call Technical Service (page 67) for more information.

**Vendors for Baffled Flasks**

Bellco (1-800-257-7043) has a wide variety of baffled flasks from 50 to 2000 ml.

Wheaton (1-609-825-1100) only sells side baffle flasks.
Protein Purification and Glycosylation

Introduction
At this point, you have an optimized protocol for expression of 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 Recipes, page 58.

1. Wash cells once in BB by resuspending them and centrifuging 5-10 minutes at 3000 x 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 x 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.

NOTE
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. Recently, some protocols for carbohydrate analysis of proteins have been published to allow the molecular biologist to characterize glycosylated proteins of interest (Ausubel et al., 1994), Unit 17. Further information about glycosylation in eukaryotes is available in a recent review (Varki and Freeze, 1994).
Enzymes for Analysis of 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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Type of enzyme</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglycosidase D</td>
<td>Endo</td>
<td>Cleaves various high mannose glycans</td>
</tr>
<tr>
<td>Endoglycosidase F</td>
<td>Endo</td>
<td>Cleaves various high mannose glycans</td>
</tr>
<tr>
<td>Endoglycosidase H</td>
<td>Endo</td>
<td>Cleaves various high mannose glycans</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Exo</td>
<td>Removes terminal galactosides from Gal-β1,3-GlcNAc, Gal-β1,4-GlcNAc or Gal-β1,3 GalNAc.</td>
</tr>
<tr>
<td>Peptide:N-Glycosidase F</td>
<td>Endo</td>
<td>Glycoproteins between Asn and GlcNAc (removes oligosaccharides)</td>
</tr>
<tr>
<td>Sialidases</td>
<td>Exo</td>
<td>NeuAc-α2,6-Gal, NeuAc-α2,6-GlcNAc or NeuAc-α2,3-Gal</td>
</tr>
<tr>
<td>(Neuraminidases)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobacter ureafaciens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Company</th>
<th>Type of Service</th>
<th>Phone Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyko</td>
<td>Kits for Carbohydrate Analysis</td>
<td>1-800-334-5956</td>
</tr>
<tr>
<td></td>
<td>Reagents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contract Services</td>
<td></td>
</tr>
<tr>
<td>Oxford GlycoSystems</td>
<td>Kits for Carbohydrate Analysis</td>
<td>1-800-722-2597</td>
</tr>
<tr>
<td></td>
<td>Reagents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contract Services</td>
<td></td>
</tr>
<tr>
<td>New England BioLabs</td>
<td>Reagents</td>
<td>1-800-632-5227</td>
</tr>
</tbody>
</table>
Recipes

E. coli Media Recipes

LB (Luria-Bertani) Medium
1% Tryptone
0.5% Yeast Extract
1% NaCl
pH 7.0
1. For 1 liter, dissolve 10 g tryptone
   5 g yeast extract
   10 g NaCl
   in 950 ml deionized water
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave for 20 minutes at 15 lb./sq. in. Let cool to ~55°C and add desired antibiotics at this point.
4. Store at room temperature or at +4°C.

LB agar plates
1. Make LB Medium above and add 15 g/liter agar before autoclaving.
2. Autoclave for 20 minutes at 15 LB/sq. in.
3. Let cool to ~55°C and add desired antibiotics at this point. Pour into 10 cm petri plates. Let the plates harden, then invert, and store at +4°C.
**Pichia Media Recipes**

**Introduction**

The expression of recombinant proteins in *Pichia pastoris* requires the preparation of several different media. Recipes for these media are included on pages 55-58. In addition, pre-mixed media and media components are available from Invitrogen. Please see the table below for ordering information.

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>YP Base Medium</strong></td>
<td>10 x 30 g pouches*</td>
<td>Q300-01</td>
</tr>
<tr>
<td>Requires addition of carbon source (i.e. dextrose)</td>
<td>500 g</td>
<td>Q300-02</td>
</tr>
<tr>
<td></td>
<td>2.5 kg</td>
<td>Q300-03</td>
</tr>
<tr>
<td><strong>YP Base Agar Medium</strong></td>
<td>10 x 50 g pouches*</td>
<td>Q300-04</td>
</tr>
<tr>
<td>Requires addition of carbon source (i.e. dextrose)</td>
<td>500 g</td>
<td>Q300-05</td>
</tr>
<tr>
<td></td>
<td>2.5 kg</td>
<td>Q300-06</td>
</tr>
<tr>
<td><strong>Yeast Nitrogen Base</strong></td>
<td>67 g pouch</td>
<td>Q300-07</td>
</tr>
<tr>
<td>–with ammonium sulfate</td>
<td>Each pouch contains reagents to prepare 500 ml of a 10X YNB solution</td>
<td></td>
</tr>
<tr>
<td>–without amino acids</td>
<td>500 g</td>
<td>Q300-09</td>
</tr>
<tr>
<td><strong>1 M Potassium Phosphate Buffer, pH 6</strong></td>
<td>70.55 g pouch</td>
<td>Q300-14</td>
</tr>
<tr>
<td></td>
<td>Each pouch contains reagents to prepare 500 ml of a 1 M potassium phosphate, pH 6 buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 g</td>
<td>Q300-16</td>
</tr>
<tr>
<td></td>
<td>2.5 kg</td>
<td>Q300-17</td>
</tr>
<tr>
<td><strong>Amino Acid Mix</strong></td>
<td>5 x 2.5 g pouches</td>
<td>Q300-18</td>
</tr>
<tr>
<td>(L-leucine, L-lysine, L-glutamic acid, L-isoleucine, and L-methionine, 0.5% each)</td>
<td>Each pouch contains reagents to prepare 100 ml of a 100X amino acid solution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 g</td>
<td>Q300-20</td>
</tr>
<tr>
<td></td>
<td>500 g</td>
<td>Q300-21</td>
</tr>
<tr>
<td></td>
<td>2.5 kg</td>
<td>Q300-22</td>
</tr>
<tr>
<td><strong>D-Sorbitol</strong></td>
<td>1 g</td>
<td>Q300-23</td>
</tr>
<tr>
<td><strong>L-Histidine</strong></td>
<td>100 mg</td>
<td>Q300-24</td>
</tr>
<tr>
<td></td>
<td>1 g</td>
<td>Q300-25</td>
</tr>
<tr>
<td></td>
<td>1 kg</td>
<td>Q300-10</td>
</tr>
<tr>
<td></td>
<td>5 kg</td>
<td>Q300-11</td>
</tr>
<tr>
<td><strong>Fermentation Basal Salts</strong></td>
<td>500 ml bottle</td>
<td>Q300-12</td>
</tr>
</tbody>
</table>

*each pouch contains reagents to prepare 1 liter of medium

continued on next page
**Stock Solutions**

10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)
Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely. Store at +4°C. Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate. The shelf life of this solution is one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch. **Note:** *Pichia* cells exhibit optimal growth with higher YNB concentrations, therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for *Saccharomyces*.

500X B (0.02% Biotin)
Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at +4°C. The shelf life of this solution is approximately one year.

100X H (0.4% Histidine)
Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilize and store at +4°C. The shelf life of this solution is approximately one year.

10X D (20% Dextrose)
Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

10X M (5% Methanol)
Mix 5 ml of methanol with 95 ml of water. Filter sterilize and store at +4°C. The shelf life of this solution is approximately two months.

10X GY (10% Glycerol)
Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

100X AA (0.5% of each Amino Acid)
Dissolve 500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine in 100 ml of water. 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:
Combine 132 ml of 1 M K2HPO4, 868 ml of 1 M KH2PO4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Autoclave 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 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.

Store the liquid medium at room temperature. Store YPD slants or plates at +4°C. The shelf life is several months.
**Pichia Media Recipes, continued**

### MGY and MGYH

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

- 1.34% YNB
- 1% glycerol
- 4 x 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 his⁺ strains in this medium, a version can be made that contains histidine (called MGYH) by adding 10 ml of 100X H stock solution.

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

### RD and RDH

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

- 1 M sorbitol
- 2% dextrose
- 1.34% YNB
- 4 x 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 his⁺ 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.

### 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. The plates should be stored at +4°C and should last for several months.

*continued on next page*
**Pichia Media Recipes, continued**

**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.

**MD and MDH**

** Minimal Dextrose Medium + Histidine (1 liter)**

1.34% YNB  
4 x 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 x 10⁻⁵% biotin  
0.5% methanol

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

*continued on next page*
Pichia Media Recipes, continued

**BMG and BMM**

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

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

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

**Breaking Buffer**

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

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

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).

**Preparation of Cells**

1. Grow 5 ml of *Pichia pastoris* in YPD in a 50 ml conical at 30°C overnight.
2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1-0.5 ml of the overnight culture. Grow overnight again to an OD₆₀₀ = 1.3-1.5.
3. Centrifuge the cells at 1500 x g for 5 minutes at +4°C. Resuspend the pellet with 500 ml of ice-cold, sterile water.
4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 ml of ice-cold, sterile water.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml.

   **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 aliquots on MD or RDB plates.
6. Incubate the plates at 30°C until colonies appear. Screen for Mut⁺/Mut⁵ phenotypes as indicated on page 36.
PEG 1000 Transformation Method for *Pichia*

**Introduction**

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

**Preparation of 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 and store at -20°C.
Fresh, reagent grade DMSO from an unopened bottle or made fresh and stored at -70°C until use.

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.

**Preparation of 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 3000 x g at room temperature and wash cells once in 50 ml of Buffer A.
5. Resuspend cells in 4 ml of Buffer A and distribute 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 frozen tubes at -70°C.

**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. Carrier DNA (40 µg of denatured and sonicated salmon sperm DNA) should be included with < 1 µg DNA samples for maximum transformation frequencies.
2. Incubate all sample tubes in a 37°C water bath for five minutes. Mix samples once or twice during this incubation period.
3. Remove tubes from the bath and add 1.5 ml of Buffer B to each. Mix thoroughly.
4. Incubate tubes in a 30°C water bath for 1 hour.
5. Centrifuge sample tubes at 2000 x g for 10 minutes at room temperature. Decant supernatant and resuspend the cells in 1.5 ml Buffer C.
6. Centrifuge samples and resuspend the cell pellet gently in 0.2 ml of Buffer C.
7. Spread 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 activity of Mut$^S$/Mut$^+$ phenotype.
Lithium Chloride Transformation Method

Introduction
This is a modified version of the procedure described for *S. cerevisiae* (Geitz and 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.

Preparation of Solutions
Lithium acetate does not work with *Pichia pastoris*. Use only lithium chloride.
1 M LiCl in distilled, deionized water. Filter sterilize. Dilute as needed with sterile water.
50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter sterilize. Store in a tightly capped bottle.
2 mg/ml denatured, fragmented salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at -20°C.

Preparation of Cells
1. Grow a 50 ml culture of *Pichia pastoris* in YPD at 30°C with shaking to an OD<sub>600</sub> 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 1500 x 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.

Transformation
1. Boil a 1 ml sample of single-stranded DNA for five minutes, then quickly chill in ice water. Keep on ice. Note: It is not necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at -20°C and boil every 3-4 times the DNA is thawed.
2. Centrifuge the LiCl-cell solution from Step 7, above, and remove the LiCl with a pipet.
3. For each transformation sample, add the following reagents IN THE ORDER GIVEN to the cells. PEG shields the cells from the detrimental effects of the high concentration of LiCl.
   - 240 µl 50% PEG
   - 36 µl 1 M LiCl
   - 25 µl 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.
7. Centrifuge the tubes at 6000 to 8000 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. Proceed to Screening Mut<sup>+</sup> and Mut<sup>S</sup> Transformants, page 36.
## Total DNA Isolation from *Pichia*

### Introduction

The protocol below allows you to isolate DNA from the desired His\(^+\) recombinant and the untransformed GS115 or KM71 for use in 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. There is not enough of some of these reagents in the kit to perform this experiment.

**Minimal Medium (MD, MGY)**
- 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% SDS in water
- 5 M potassium acetate, pH 8.9
- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)
- 7.5 M ammonium acetate, pH 7.5
- Phenol:chloroform (1:1 v/v)

### Preparation

1. Grow at 30°C the recombinant strain and the parent strain to an OD\(_{600}\) of 5-10 in 10 ml of minimal media (e.g. MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71)).
2. Collect the cells by centrifugation at 1500 x 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 32-33).
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 x g for 5-10 minutes at +4°C and save the supernatant.

### DNA Precipitation

1. Transfer the supernatant from Step 5 above and add 2 volumes of ethanol. Incubate at room temperature for 15 minutes.
2. Centrifuge at 10,000 x 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.
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 x g for 20 minutes at +4°C and wash the pellets once with 1 ml of 70% ethanol. Resuspend each pellet in 50 µl of TE buffer, pH 7.5. Determine the concentration of the DNA sample. The two samples can be stored separately or combined and stored at -20°C until ready for use.
Detection of 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 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. The DNA can be used 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; Clare et al., 1991b). Following standard procedures for Southern blotting as outlined in Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989), pages 9.31-9.58, generate a blot of digested and gel-separated genomic DNA and probe it with the appropriate fragment from the transforming vector. Be sure to include a control to show the intensity of a single copy gene. You should see a range of intensity with various clones. These can be relatively quantified using densitometry to estimate gene dosage.

Quantitative Dot Blot Analysis: Solutions

You will need the following solutions:

- 50 mM EDTA, 2.5% β-mercaptoethanol pH 9
- 1 mg/ml Zymolyase 100T in water (Seikagaku America, Inc.)
- 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate, pH 7
- 2 x SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH 7)

Quantitative Dot Blot Analysis: Procedure

The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos et al., 1991).

1. Grow Mut+ or Mut- 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.

2. Filter 50 µl of each sample onto nitrocellulose placed into a dot/slot blot apparatus using multi-channel pipette.

3. Air dry filters, then lyse by placing the nitrocellulose filter face down on two sheets of 3 MM paper soaked with the first solution. For each solution, use new 3 MM paper.
   - 15 minutes with 50 mM EDTA, 2.5% β-mercaptoethanol pH 9
   - 4 hours at 37°C with 1 mg/ml Zymolyase 100T
   - 5 minutes in 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate, pH 7
   - two 5 minute incubations in 2 x SSC

4. Bake filters at 80°C, then probe with random primed, 32P-labeled DNA. Use labeled insert DNA to probe the dot blot. Multi-copy integrants can be identified by a strong hybridization signal. Dot blots can then be quantified for copy number by densitometry of the film or blot, or by using a β-scanner (if radiolabeled).
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® or Micro FastTrack® mRNA Isolation Kit. If you wish to use another protocol, you should 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**
You will need the following solutions. Remember to use DEPC-treated water and to use equipment free of RNase.

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

**Growth of 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 x g for 10 minutes at room temperature.
4. Resuspend cell pellet in 400 µl AE buffer and transfer to a microcentrifuge tube.

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

**mRNA Isolation and Northern Analysis**
Please see (Ausubel et al., 1994) for a protocol for mRNA isolation and northern analysis. The FastTrack® mRNA Kit (Catalog no K1593-02; 6 reactions) is designed to isolate mRNA from 0.2 to 1 mg total RNA. The Micro-FastTrack™ Kit (Catalog no. K1520-02; 20 reactions) is designed to isolate mRNA from ~100 µg total RNA. 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 provided below can also be found in (Miller, 1972), page 403 and can be used to evaluate expression of β-galactosidase.

Preparation of Solutions

You will need to prepare the following:

- A fresh crude cell lysate of GS115 β-Gal (see page 51)
- Z buffer
- ONPG solution
- 1 M sodium carbonate solution

Recipes for the solutions are found below.

Z Buffer

60 mM Na₂HPO₄·7H₂O
40 mM NaH₂PO₄·H₂O
10 mM KCl
1 mM MgSO₄·7H₂O
50 mM β-mercaptoethanol
pH 7.0

1. Dissolve 16.1 g Na₂HPO₄·7H₂O
   5.5 g NaH₂PO₄·H₂O
   0.75 g KCl
   0.246 g MgSO₄·7H₂O
   2.7 ml β-mercaptoethanol
in 950 ml deionized water.
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 1.61 g Na₂HPO₄·7H₂O
   0.55 g NaH₂PO₄·H₂O
in 90 ml deionized water.
2. Adjust pH to 7.0 with either NaOH or HCl and add 400 mg of ONPG. Stir to dissolve and bring the volume up to 100 ml with water.
3. Store at +4°C 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 2.
5. Incubate the samples and the control at 28°C until a faint yellow color develops. This should occur at least 10 minutes after the start of the assay to ensure accurate data. Note that the tube with no lysate may not change color.
6. Stop the reaction by adding 0.5 ml of 1 M sodium carbonate to each tube. Record the length of incubation for each sample.
7. Read the OD\textsubscript{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.

**Determination of Specific Activity**

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

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

Remember to take into account the volume of lysate added to the reaction and any dilutions made to the lysate when calculating the amount of protein in the reaction. The number 380 is the constant used to convert the OD\textsubscript{420} reading into units. One unit is defined as the amount of enzyme that will hydrolyze 1 nmole of ONPG per minute at 28°C. The molar extinction coefficient of ONPG under these conditions is 4500. For a sample calculation, please see below.

**Sample Calculation**

Here is a sample calculation:

- Extract concentration = 10 mg/ml
- Assay 10 µl of a 1/100 dilution
- Time = 10 minutes
- OD\textsubscript{420} = 0.4

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

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

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