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Pichia Expression Kit

USER GUIDE

For expression of recombinant proteins in *Pichia pastoris*

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Revision	Date	Description
B.0	15 September 2020	Converted to XML and transferred to CCMS. Corrected the sequence, vector map, multiple cloning site, and coordinates for alternate restriction sites for pHIL-S1. Updated web links.
A.0	10 January 2014	New document.

Contents

For expression of recombinant proteins in *Pichia pastoris*

Kit contents and storage

Kit contents

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

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

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

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

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

Table 3 Stab Vials: *Pichia* and *E. coli* stabs. Store at 4°C.

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

Table 4 Vectors. Store at –20°C.

Reagent	Description
pHIL-D2 10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0[1]	Vector for intracellular expression in *Pichia.*
pPIC3.5 10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0	Vector for intracellular expression in *Pichia.*
pHIL-S1 10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0	Vector for secreted expression in *Pichia*. Uses the *PHO1* signal sequence.
pPIC9 10 µg, 20 µL at 0.5 µg/µL in TE buffer, pH 8.0	Vector for secreted expression in *Pichia*. Uses the α-factor signal sequence.

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

Table 5 Primers. Store at –20°C.

5′ *AOX1* sequencing primer 2 µg (312 pmoles), lyophilized	5′-GACTGGTTCCAATTGACAAGC-3′
3′ *AOX1* sequencing primer 2 µg (314 pmoles), lyophilized	5′-GCAAATGGCATTCTGACATCC-3′
α-Factor sequencing primer 2 µg (315 pmoles), lyophilized	5′-TACTATTGCCAGCATTGCTGC-3′

Media

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

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

Note: The *Pichia* Spheroplast Module for transforming *Pichia* by spheroplasting is available separately from Thermo Fisher Scientific (see “Accessory products” on page 101 for ordering information).

Required materials not included with the kit

Required materials

- 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
- UV Spectrophotometer
- Mini agarose gel apparatus and buffers
- Agarose and low-melt agarose
- Polyacrylamide gel electrophoresis apparatus and buffers
- Media for transformation, growth, screening, and expression (see "E. coli media recipes" on page 71)
- 5% SDS solution (10 mL per transformation)
- Sterile cheesecloth or gauze
- Breaking Buffer (see "Breaking buffer" on page 78)
- Acid-washed glass beads (available from Sigma)
- *(Optional)* Replica-plating equipment
- *(Optional)* BeadBeater™ (available from Biospec)

Introduction

Pichia pastoris expression system

General characteristics of *Pichia pastoris*

As a eukaryote, *Pichia pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. *Pichia* 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* can be applied to *Pichia*. These include:

- Transformation by complementation
- Gene disruption
- Gene replacement

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

Pichia pastoris as a methylotrophic yeast

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia*.

Two alcohol oxidase proteins

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

Expression

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

Phenotype of *aox1* mutants

Loss of the *AOX1* gene, and thus a loss of most of the cell's alcohol oxidase activity, results in a strain that is phenotypically Mut^S (Methanol utilization slow). This has in the past been referred to as Mut^-. The Mut^S designation has been chosen to accurately describe the phenotype of these mutants. This results in a reduction in the cell's 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 the integration of your gene (**"Transformation and integration" on page 16**).

Intracellular and secretory protein expression

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

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

Posttranslational modifications

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

In addition, *Saccharomyces cerevisiae* core oligosaccharides have terminal α1,3 glycan linkages whereas *Pichia pastoris* does not. It is believed that the α1,3 glycan linkages in glycosylated proteins produced from *Saccharomyces cerevisiae* are primarily responsible for the hyper-antigenic nature of these proteins, thus making them particularly unsuitable for therapeutic use. Although not yet proven, this is predicted to be less of a problem for glycoproteins generated in *Pichia pastoris*, because they may resemble the glycoprotein structure of higher eukaryotes (Cregg *et al.*, 1993).

Experiment outline

Vector selection and cloning

To utilize the strong, highly inducible P_{AOX1} promoter for expressing your protein, four expression vectors are included in this kit. pHIL-D2 and pPIC3.5 are used for intracellular expression, and pHIL-S1 and pPIC9 are used for secreted expression (see "pHIL-D2" on page 26 for more information). Before cloning your insert, you must:

- Decide whether you want intracellular or secreted expression.
- Analyze your insert for the following restriction sites: *Sac* I, *Stu* I, *Sal* I, *Not* I, and *Bgl* II. We recommend these sites for linearizing your construct prior to *Pichia* transformation. If your insert has all of these sites, refer to "Alternate restriction sites" on page 40 for alternate sites.

Transformation and integration

Two different phenotypic classes of His^+ recombinant strains can be generated: Mut^+ and Mut^S. Mut^S refers to the Methanol utilization slow phenotype caused by the loss of alcohol oxidase activity encoded by the *AOX1* gene. A strain with a Mut^S phenotype has a mutant *aox1* locus, but is wild type for *AOX2*. This results in a slow growth phenotype on methanol medium. Transformation of strain GS115 can yield both classes of transformants, His^+ Mut^+ and His^+ Mut^S, while KM71 yields only His^+ Mut^S, because the strain itself is Mut^S. Both Mut^+ and Mut^S recombinants are useful to have, because one phenotype may favor better expression of your protein than the other. Because of clonal variation, you should test 6–10 recombinants per phenotype. There is no way to predict beforehand which construct or isolate will better express your protein. We strongly recommend that you analyze *Pichia* recombinants by PCR to confirm the integration of your construct (see "PCR analysis of Pichia integrants" on page 55).

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

Restriction enzyme	Integration event	GS115 phenotype	KM71 phenotype
Sal I or *Stu* I	Insertion at *his4*	His^+ Mut^+	His^+ Mut^S
Sac I	Insertion at 5′ *AOX1* region	His^+ Mut^+	His^+ Mut^S
Not I or *Bgl* II	Replacement at *AOX1* locus	His^+ Mut^S His^+ Mut^+	His^+ Mut^S (not recommended, see "Genotype of Pichia strain" on page 20)

Expression and scale-up

After confirming your *Pichia* recombinants by PCR, test the expression of both His$^+$ Mut$^+$ and His$^+$ Mut^S recombinants. This procedure involves growing a small culture of each recombinant, inducing them with methanol, and taking time points. If looking for intracellular expression, analyze the cell pellet from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). If looking for secreted expression, analyze both the cell pellet and supernatant from each time point. We recommend that you analyze your SDS-PAGE gels by Coomassie™ staining and, if you have an antibody to your protein, by western blot. We also suggest checking for protein activity by an activity assay, if one is available. Not all proteins express to the level of grams per liter, so it is advisable to check by western blot or activity assay, and not just by Coomassie™ staining of SDS-PAGE gels for production of your protein.

Choose the *Pichia* recombinant strain that best expresses your protein and optimize induction based on the suggestions in "Optimize Pichia protein expression" on page 64. After you optimize expression, scale up your expression protocol to produce more protein.

Experiment workflow

The overall experiment workflow is divided into two major sections: **Generate recombinant strain** (page 17) and **Induction (Mut$^+$ and/or Mut^S)** (page 18 and page 19). Each section contains a table outlining the major steps of the experiment workflow. Each step is discussed in detail further in the user guide. Refer to the indicated pages for more information on specific steps. The discussion about recombination and integration in *Pichia* will help you choose the correct vector. For more information, refer to the review by Higgins (Higgins, 1995).

Generate recombinant strain

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

Step	Procedure	Page
1	Select the appropriate expression vector (For more information, see "Recombination and integration in Pichia" on page 97)	"Select a Pichia expression vector" on page 24
2	Clone gene of interest into selected vector	"Clone into Pichia expression vectors" on page 31
3	Transform *E. coli*, select ampicillin-resistant transformants, and confirm the presence and orientation of gene of interest	"Transformation into E. coli" on page 37
4	Linearize the constructs with appropriate restriction enzymes to generate His$^+$ Mut^S and His$^+$ Mut$^+$ recombinant strains	"Prepare transforming DNA" on page 38
5	Transform and select His$^+$ transformants (GS115 recombinants, His$^+$ Mut$^+$; KM71 recombinants, His$^+$ Mut^S)	"Grow Pichia for spheroplasting" on page 43

(continued)

Step	Procedure	Page
6	Screen His$^+$ transformants for Mut$^+$ and Mut^S strains (6–10 recombinants of each phenotype)	"Screen for Mut+ and MutS transformants" on page 50
7	Confirm the integration of your gene of interest in Mut$^+$ and Mut^S recombinants by PCR	"PCR analysis of Pichia integrants" on page 55

Mut$^+$ induction

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

Step	Procedure	Page
1	Guidelines for expression of recombinant proteins in *Pichia*	"Expression in recombinant Pichia strains" on page 58
2	Grow His$^+$ Mut$^+$ recombinants in 25 mL of buffered glycerol medium to a final OD_{600} = 2–6.	"Mut+ intracellular or secreted expression" on page 60
3	Harvest the cells and resuspend them to an OD_{600} of 1.0 (~100–200 mL) with methanol medium. Place the cell suspension in a 1-liter baffled flask.	"Mut+ intracellular or secreted expression" on page 60
4	Incubate the culture at 30°C with shaking and take samples for analysis at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 hours.	"Mut+ intracellular or secreted expression" on page 60
5	Analyze the medium (if protein of interest is targeted for secretion) and the cell lysates (for intracellular and secreted expression) for protein via PAGE/Coomassie™ Blue staining, western blot, activity, ELISA, or immunoprecipitation.	"Analyze samples by SDS-polyacrylamide gel electrophoresis" on page 62
6	Optimize expression of your His$^+$ Mut$^+$ recombinant.	"Optimize Pichia protein expression" on page 64
7	Scale up your expression for protein purification.	"Scale up expression" on page 66

Mut^S induction

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

Step	Procedure	Page
1	Guidelines for expression of recombinant proteins in *Pichia*	"Expression in recombinant Pichia strains" on page 58
2	Grow His$^+$ Mut^S recombinants in 100–200 mL of buffered glycerol medium to a final OD_{600} = 2–6.	"MutS intracellular or secreted" on page 61
3	Harvest the cells and resuspend them to an OD_{600} of 10.0 (~10–20 mL) with methanol medium. Place the cell suspension in a 100-mL or 250-mL baffled flask.	"MutS intracellular or secreted" on page 61
4	Incubate the culture at 30°C with shaking and take samples for analysis at 0, 24, 48, 72, 96, 120, and 144 hours.	"MutS intracellular or secreted" on page 61
5	Analyze the medium (if protein of interest is targeted for secretion) and the cell lysates (for intracellular and secreted expression) for protein via PAGE/Coomassie™ Blue staining, western blot, activity, ELISA, or immunoprecipitation.	"Analyze samples by SDS-polyacrylamide gel electrophoresis" on page 62
6	Optimize expression of your His$^+$ Mut$^+$ recombinant.	"Optimize Pichia protein expression" on page 64
7	Scale up your expression for protein purification.	"Scale up expression" on page 66

Pichia strains

Introduction

Pichia pastoris is quite similar to *Saccharomyces cerevisiae* regarding general growth conditions and handling. You should be familiar with basic microbiological and sterile techniques before attempting to grow and manipulate any microorganism. You should also be familiar with basic molecular biology and protein chemistry. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology* (Guthrie & Fink, 1991), *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989), *Protein Methods* (Bollag *et al.*, 1996), and *Guide to Protein Purification* (Deutscher, 1990).

Genotype of *Pichia* strain

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

The parent strain of KM71 has a mutation in the argininosuccinate lyase gene (*arg4*) that prevents the strain from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt *AOX1*, creating KM71, a Mut^S Arg^+ His^- strain.

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

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

Construction of KM71

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

IMPORTANT! The advantage of using KM71 is that there is no need to screen for the Mut phenotype on methanol minimal medium. All transformants will be Mut^S. Secondly, since the *AOX1* locus was not completely deleted, it is theoretically possible to replace *aox1::ARG4* with your construct by gene replacement. The phenotype of this strain would be His$^+$ Mut^S Arg$^-$. This means the recombinant strain would require arginine in the medium to grow. Unfortunately, simple inclusion of arginine does not totally alleviate the effects of the *arg4* mutation, and *arg4* strains do not grow well on minimal medium supplemented with arginine. Therefore, we do **not** recommend that you generate His$^+$ transformants in KM71 by replacing the *aox1::ARG4* construct.

Control expression strains

GS115/His$^+$ Mut^S Albumin: This strain is a control for secreted expression and the Mut^S phenotype when screening *Pichia* transformants ("His+ MutS in GS115" on page 50). 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 ("His+ MutS in GS115" on page 50). The gene for β-galactosidase (*lacZ*) was integrated into *Pichia* at the *his4* locus. This strain expresses β-galactosidase (117 kDa) at levels that can be detected on Coomassie™-stained SDS-PAGE (see "Analyze samples by SDS-polyacrylamide gel electrophoresis" on page 62) or assayed using ONPG (see "Beta-Galactosidase assay" on page 92).

Growth characteristics of *Pichia* strains

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

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

Note: Growth characteristics can vary depending on the recombinant strain.

Growth on methanol

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

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

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

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

Store *Pichia* strains

To store cells for weeks to months, use YPD medium or YPD agar slants (see page 73).

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

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

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

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

E. coli strains

E. coli strain genotype

The *E. coli* strain TOP10F′ is provided in case no suitable *E. coli* strain is available. Other strains which may be suitable are TOP10, DH5αF′, JM109, or any other strain which is recombination deficient (*rec*A) and deficient in endonuclease A (*end*A).

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

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

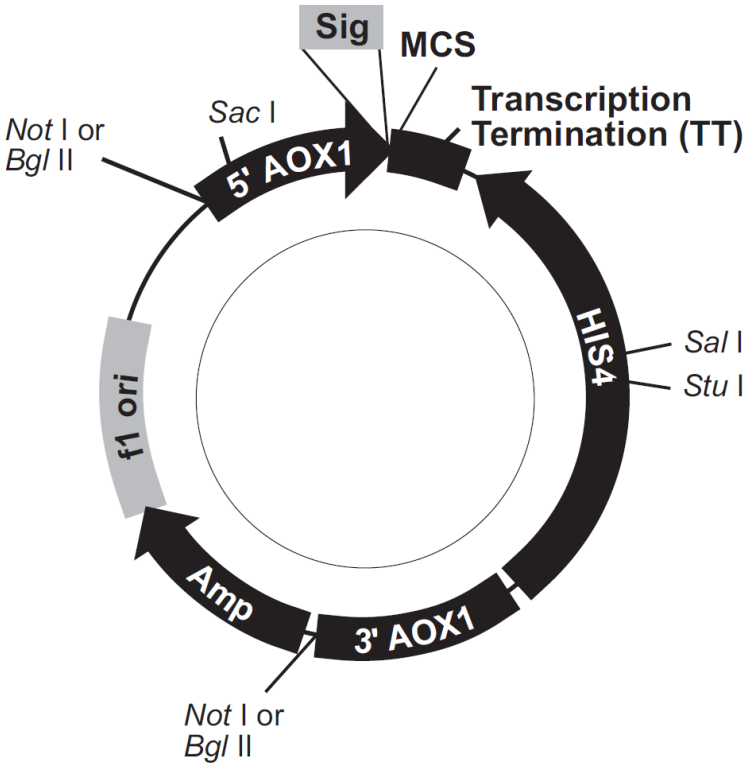
Note: We recommend that you make a frozen stock of TOP10F′.

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

Select a *Pichia* expression vector

Generic structure

All vectors included in this kit share several general features shown in black, while some of the vectors also have signal sequences (Sig) and/or an f1 bacteriophage origin. For details of each individual plasmid, refer to the following pages: "pHIL-D2" on page 26, "pPIC3.5" on page 27, "pHIL-S1" on page 28, "pPIC9" on page 29.



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

Features

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

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

Select a vector

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

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

pHIL-D2

Description

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

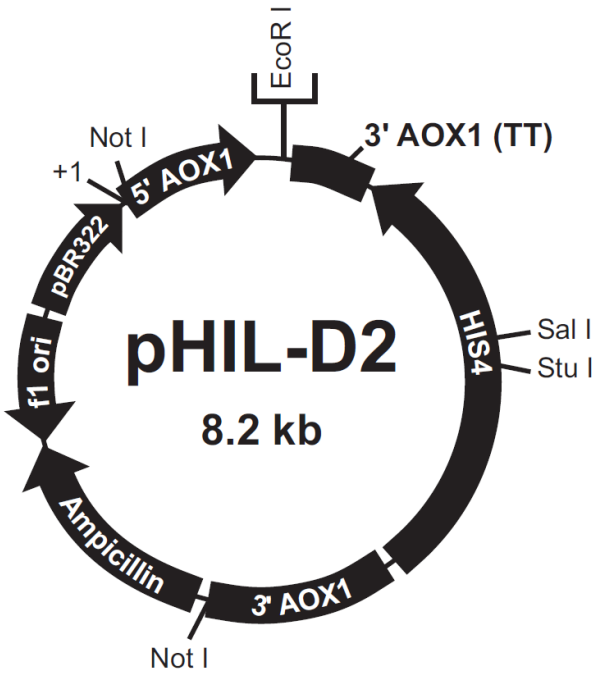
Refer to "Alternate restriction sites" on page 40 for alternate restriction sites if your insert DNA has a *Not* I, *Sac* I, *Sal* I, or *Stu* I site.

Map of pHIL-D2

The following map shows the location and size of each feature of pHIL-D2. For the details of the multiple cloning site, refer to "PAOX1 and multiple cloning site of pHIL-D2" on page 33. The complete sequence of pHIL-D2 is available at **thermofisher.com** or from Technical Support (page 110).

Comments for pHIL-D2:
8209 nucleotides

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



pPIC3.5

Description

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

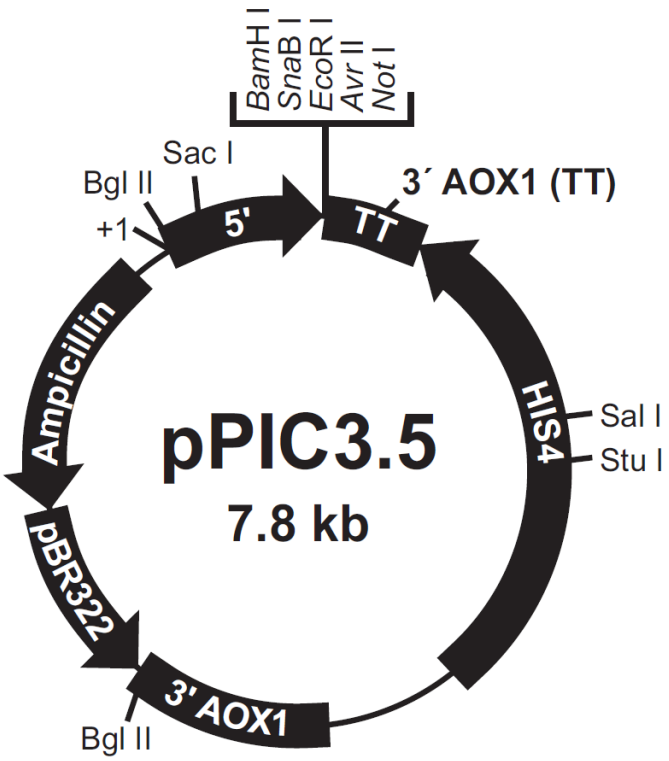
Refer to "Alternate restriction sites" on page 40 for alternate restriction sites if your insert DNA has a *Not* I, *Sac* I, *Sal* I, or *Stu* I site.

Map of pPIC3.5

The following map shows the location and size of each feature of pPIC3.5. For the details of the multiple cloning site, refer to "PAOX1 and multiple cloning site of pPIC3.5" on page 34. The complete sequence of pPIC3.5 is available at **thermofisher.com** or from Technical Support (page 110).

Comments for pPIC3.5:
7751 nucleotides

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



pHIL-S1

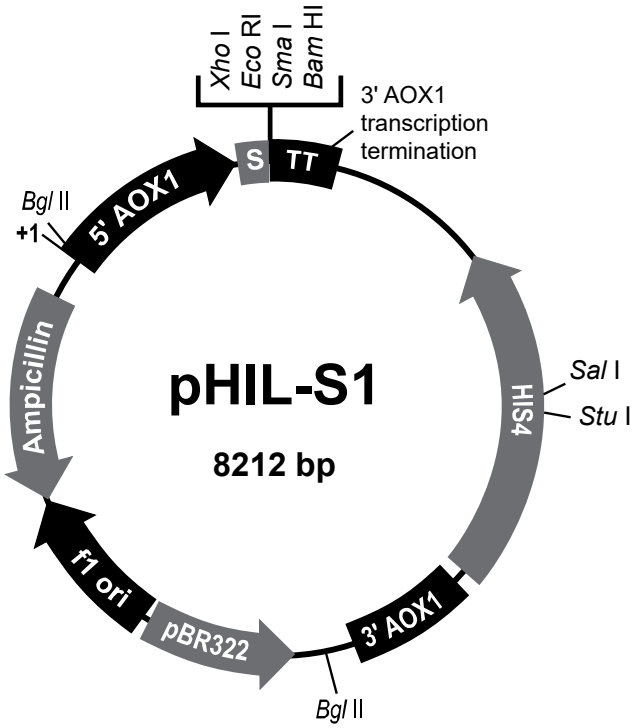
Description

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

Refer to "Alternate restriction sites for pHIL-S1" on page 41 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 following map shows the location and size of each feature of pHIL-S1. For the details of the multiple cloning site, refer to "PAOX1 and multiple cloning site of pHIL-S1" on page 35. The complete sequence of pHIL-S1 is available at **thermofisher.com** or from Technical Support (page 110).



Comments for pHIL-S1:
8212 nucleotides

5' *AOX1* promoter: bases 1-940
5' *AOX1* primer site: bases 855-875
PHO1 secretion signal (S): bases 941-1006
Multiple Cloning Site: bases 1005-1025
3' *AOX1* primer site: bases 1098-1118
3' *AOX1* transcription termination (TT): bases 1024-1189
HIS4 ORF: bases 4273-1740
3' *AOX1* fragment: bases 4628-5376
pBR322 origin: bases 6536-5917
f1 origin of replication: bases 6545-7000
Ampicillin resistance gene: bases 7902-7042

pPIC9

Description

- 8,023 bp fusion vector
- Unique *Xho* I, *Sna*B I, *Eco*R I, *Avr* II, *Not* I sites
- For 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^S in KM71)
- For insertion at *HIS4*, linearize with *Sal* I or *Stu* I (generates His$^+$ Mut$^+$ in GS115 and His$^+$ Mut^S in KM71)
- For gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates His$^+$ Mut^S)

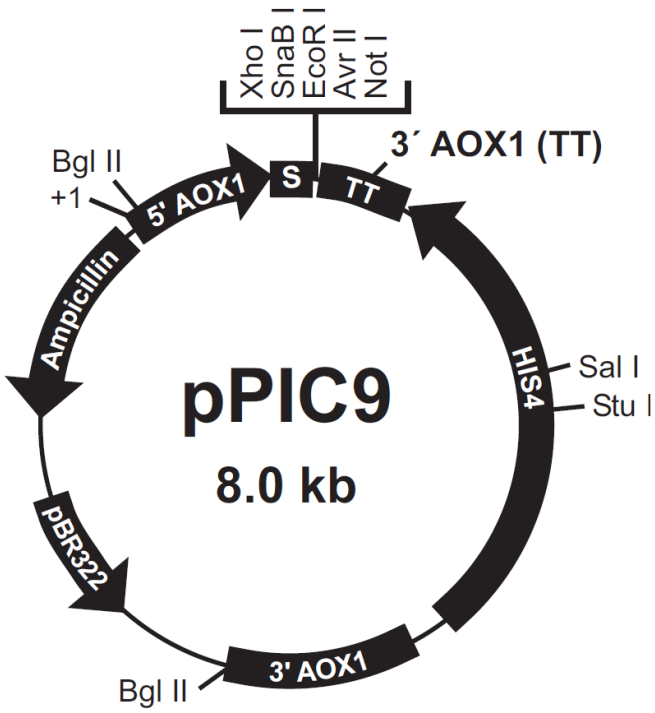
Refer to "Alternate restriction sites for pPIC9" on page 42 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, *Sal* I, or *Stu* I site.

Map of pPIC9

The figure below shows the map of pPIC9. Details of the multiple cloning site are provided in "PAOX1 and multiple cloning site of pPIC9" on page 36. The sequence of pPIC9 is available at **thermofisher.com** or from Technical Support (page 110).

Comments for pPIC9:
8023 nucleotides

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



Signal sequence processing

Signal sequence processing

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

The processing of the α-factor mating signal sequence in pPIC9 occurs in two steps:

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

Optimize signal cleavage

In *Saccharomyces cerevisiae*, the Glu-Ala repeats are not necessary for cleavage by *KEX2*, but the *KEX2* cleavage after Glu-Lys-Arg may be more efficient when followed by Glu-Ala repeats. A number of amino acids are tolerated at site X instead of Glu in the sequence Glu-Lys-Arg-X. These amino acids include the aromatic amino acids, small amino acids, and histidine. Proline, however, inhibits *KEX2* cleavage. For more information on *KEX2* cleavage, see (Brake *et al.*, 1984).

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

Note: The *PHO1* signal sequence is atypical of signal sequences even though it is a native *Pichia* secretion signal. If cloning into the *Xho* I site, we recommend regenerating the full signal sequence between the *Xho* I and *Eco*R I sites (see "PAOX1 and multiple cloning site of pPIC3.5" on page 34). 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).

Clone into *Pichia* expression vectors

Introduction

After selecting a vector into which to clone your gene of interest (see "Select a Pichia expression vector" on page 24), 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.

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

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

General considerations

The following are some general considerations applicable to all vectors.

- The codon usage in *Pichia* is believed to be the same as *Saccharomyces cerevisiae* because many genes have proven to be cross-functional.
- Maintain plasmid constructions in a *rec*A mutant *E. coli* strain such as the TOP10F′ strain provided in the kit.
- The native 5′ end of the *AOX1* mRNA is noted in each multiple cloning site. This information is necessary to calculate the size of the expressed mRNA of the gene of interest.
- Translation termination is determined by either stop codons in the gene of interest or in the 3′ *AOX1* sequence. The stop codons in the 3′ *AOX1* sequence are noted in each figure on the following pages.
- The premature termination of transcripts due to AT rich regions has been observed in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret and Sherman, 1984). If you are expressing a gene with high AT content, refer to "No expression" on page 65.
- The predicted protease cleavage sites for the *PHO1* and α-factor signal sequences are indicated in each figure.
- If you are attempting to secrete a protein using its native secretion signal, we recommend that you also try pPIC9 in parallel. When using pPIC9, the open reading frame (ORF) of the mature gene of interest is cloned in frame and downstream of the α-factor.

General cloning strategies

Strategies generally fall into three different categories:

1. Ligation of a compatible restriction fragment:
   a. Forced (directional) insertion involving the use of two different sites in the multiple cloning site (for pPIC3.5, pHIL-S1, or pPIC9 vectors).

   b. Ligation of the fragment with the same restriction end on both ends into a single, compatible site (e.g. *Eco*R I cloning in pHIL-D2).

2. PCR amplification of the fragment containing the gene of interest in such a way that compatible restriction ends are generated for ligation into the appropriate vector.

3. Direct cloning of an amplified fragment containing the gene of interest via the TA Cloning™ Kit (see "Accessory products" on page 101 for ordering information), followed by subcloning of a compatible fragment into the appropriate *Pichia* expression vector.

Cloning procedures

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

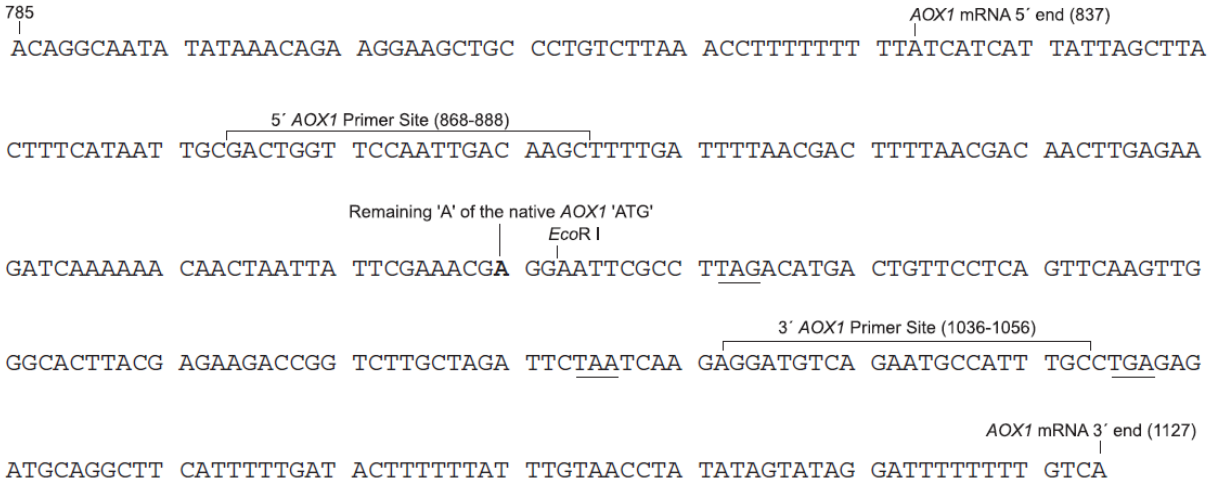
Bacterial transformation

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

For a high-efficiency and convenient transformation, we recommend One Shot™ TOP10 Chemically Competent *E. coli* or One Shot™ TOP10 Electrocompetent Cells, which are available separately from Thermo Fisher Scientific (see "Accessory products" on page 101 for ordering information).

P_{AOX1} and multiple cloning site of pHIL-D2

The following figure shows the multiple cloning site and surrounding sequences.



Special considerations for pHIL-D2

- For pHIL-D2, the fragment containing the gene of interest should have a yeast consensus sequence (Romanos *et al.*, 1992). An example of a yeast consensus sequence is provided below. The ATG initiation codon is shown underlined.

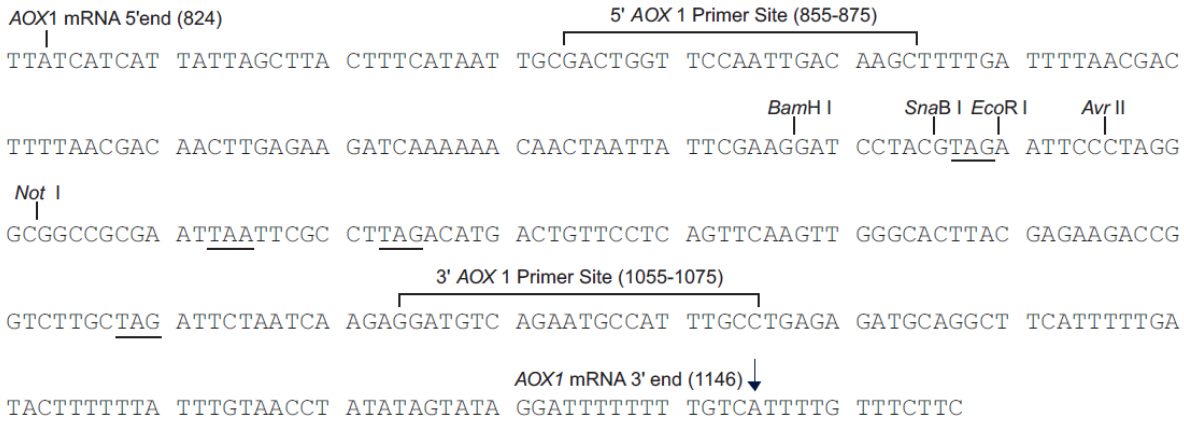
  (A/Y)A(A/T)AATGTCT

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

- Shorter, 5′ untranslated leaders reportedly work better in *AOX1* expression. In pHIL-D2, make the untranslated region as short as possible when cloning your gene.
- If your insert has a *Not* I site, refer to "Alternate restriction sites" on page 40 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

P_{AOX1} and multiple cloning site of pPIC3.5

The following figure shows the multiple cloning site and surrounding sequences.



Special considerations for pPIC3.5

- For pPIC3.5, the fragment containing the gene of interest should have a yeast consensus sequence (Romanos *et al.*, 1992). An example of a yeast consensus sequence is provided below. The ATG initiation codon is shown underlined.

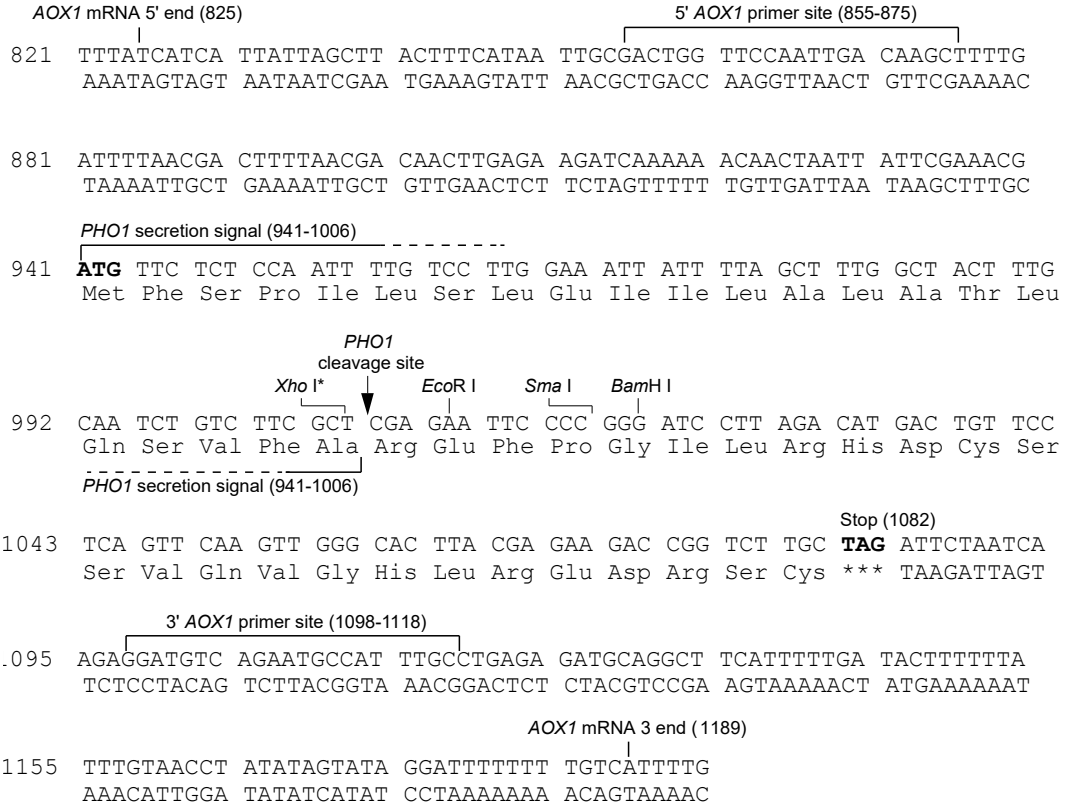
  (A/Y)A(A/T)AATGTCT

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

- Shorter, 5′ untranslated leaders reportedly work better in *AOX1* expression. In pPIC3.5, make the untranslated region as short as possible when cloning your gene.
- If you are digesting with *Bam*H I and *Sna*B I or *Sna*B I and *Eco*R I, digest with *Sna*B I first. If you digest with *Bam*H I or *Eco*R I first, the *Sna*B I site will be too close to the end of the DNA and will not digest properly.
- If your insert has a *Bgl* II, *Sac* I, *Sal* I, *or Stu* I site, refer to "Alternate restriction sites" on page 40 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

P_{AOX1} and multiple cloning site of pHIL-S1

The following figure shows the multiple cloning site and surrounding sequences.



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

Special considerations for pHIL-S1

- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- If the *Xho* I site is used for cloning, it must be recreated for efficient cleavage of the fusion protein to occur. It is part of the *PHO1* signal peptide sequence.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5′ end of the mRNA.
- If your insert has a *Not* I site, refer to "Alternate restriction sites" on page 40 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 α-factor (pPIC9) or native general, more success has been reported with the secretion signals than with pHIL-S1. This may be due to the lack of *KEX2*-like processing signals (Laroche *et al.*, 1994).

P_{AOX1} and multiple cloning site of pPIC9

The following figure shows the multiple cloning site and surrounding sequences.

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

Special considerations for pPIC9

- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- If the *Xho* I site is used for cloning, the sequence between the *Xho* I site and the *Sna*B I site encoding the KEX2 site (Glu-Lys-Arg-X) must be recreated for efficient cleavage of the fusion protein to occur. It is part of the α-factor signal peptide sequence. Refer to the discussion in "Optimize signal cleavage" on page 30.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5′ end of the mRNA.
- If your insert has a *Bgl* II site, refer to "Alternate restriction sites for pPIC9" on page 42 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

Transformation into *E. coli*

Introduction

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

Analyze transformants

1. After transformation, plate the transformation mix onto LB plates with 50–100 µg/mL ampicillin (page 71) and select ampicillin resistant colonies.
2. Pick 10 ampicillin resistant transformants and inoculate into LB medium with 50–100 µg/mL ampicillin. Grow overnight at 37°C with shaking.
3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below). To sequence the *Pichia* expression vectors, use the primers provided.
4. Make a glycerol stock of your desired clone for safekeeping by combining 0.85 mL of an overnight bacterial culture with 0.15 mL of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at –70°C.
5. After you confirm your construct by sequencing, proceed to "Prepare transforming DNA" on page 38.

Sequence 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 the detail of the multiple cloning site and surrounding sequences for each vector that is described in "Clone into Pichia expression vectors" on page 31.

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

5′ *AOX1* sequencing primer

- is `5′-GACTGGTTCCAATTGACAAGC-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

Prepare transforming DNA

Introduction

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

Step	Action	Pages
1	Prepare your DNA for transformation	“Linearize plasmid DNA” on page 39
2	Grow GS115 or KM71 to prepare spheroplasts	“Grow Pichia” on page 44
3	Prepare spheroplasts for transformation	“Prepare spheroplasts for transformation” on page 45
4	Transform GS115 or KM71 with your DNA	“Transform Pichia” on page 47
5	Select His^+ transformants and characterize for Mut^+/Mut^S phenotype	“Screen for Mut+ and MutS transformants” on page 50
6	Test 10 His^+ Mut^+ and 10 His^+ Mut^S by PCR for integration of your gene	“PCR analysis of Pichia integrants” on page 55

Note: We recommend that you isolate both His^+ Mut^+ and His^+ MutS *Pichia* transformants because it is difficult to predict beforehand what construct will best express your protein (see “Recombination and integration in Pichia” on page 97). By linearizing your construct DNA in the 5′ AOX1 region or in the HIS4 gene and using GS115 (Mut^+) and KM71 (Mut^S) cells, you can easily isolate Mut^+ and Mut^S recombinants. Plan on using ~10 µg digested DNA for each transformation.

Prepare plasmid DNA

Plasmid DNA for *Pichia* transformation should be at least pure enough for restriction digestion; however, the cleaner the DNA, the more efficient the transformation. We recommend the PureLink™ HiPure Plasmid Miniprep Kit (see "Accessory products" on page 101) to prepare plasmid DNA for routine *Pichia* transformations. Refer to **thermofisher.com** or contact Technical Support for more information on a large selection of plasmid purification columns. You can also prepare plasmid DNA using alkaline lysis, phenol:chloroform extraction, and ethanol precipitation.

Guidelines for linearizing plasmid DNA

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

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

Note: If your insert DNA has all three sites, see "Alternate restriction sites" on page 40 for alternative restriction enzymes you can use.

- To isolate His$^+$ Mut^S transformants of GS115, linearize plasmid constructs with *Not* I (pHIL-D2) or *Bgl* II (pPIC3.5, pHIL-S1, and pPIC9).

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

Linearize plasmid DNA

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

Alternate restriction sites

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

Alternate restriction sites for pPHIL-D2

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

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

Alternate restriction sites for pPIC3.5

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

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

Alternate restriction sites for pHIL-S1

Restriction enzyme	*5′ AOX1* 1–940 bp	*3′ AOX1* 4,628–5,376 bp	Vector backbone 5,376+ bp	*HIS4* gene 1,740–4,273 bp
Sac I	209	–	–	–
Pme I	414	–	–	–
Bpu 1102 I	589	–	–	–
Nsi I	678	–	–	–
Xcm I	707	–	–	–
Bgl II	2	5,373	–	–
Dra I[1]	414	5,213; 5,353	6,545; 7,693	–
Sal I	–	–	–	2,937

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

Alternate restriction sites for pPIC9

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

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

Grow *Pichia* for spheroplasting

Introduction

In general, spheroplasting and electroporation ("Electroporation of Pichia" on page 79) provide the highest transformation efficiency for most researchers (10^3 to 10^4 transformants per µg DNA). *Pichia* can also be transformed using PEG 1000 ("PEG 1000 transformation of Pichia" on page 80) or lithium chloride ("Lithium chloride transformation of Pichia" on page 82). These two methods, particularly lithium chloride, do **not** perform as well as spheroplasting or electroporation. If you do not have an electroporation device, we recommend spheroplasting or using the PEG 1000 method. Transformation in *Pichia* is less efficient than for *Saccharomyces*. For references on general yeast transformation, see (Cregg *et al.*, 1985; Hinnen *et al.*, 1978).

Spheroplasting

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

Prepare media

Prepare the following media several days in advance and store at 4°C (see "Pichia media recipes" on page 72 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 solutions on the day of transformation and maintain at 45°C:

- 5% SDS solution in water
- RD (Regeneration Dextrose), molten agarose, 100 mL

Prepare spheroplasting and transformation reagents

Provided:

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

Prepare fresh for each transformation:

- SED: 19 mL of SE and 1 mL of 1 M DTT (see "Before starting" on page 45)
- PEG/CaT: 1:1 mixture of 40% PEG and CaT (see "Transform spheroplasts" on page 47)

Grow *Pichia*

1. Streak GS115 or KM71 onto a YPD plate to grow isolated, single colonies. 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, then grow overnight at 28–30°C with vigorous shaking (250–300 rpm). You can store this culture at 4°C for several days.

3. Place 200 mL of YPD in each of three 500-mL culture flasks. Inoculate the flasks with 5, 10, and 20 µL of cells from the culture made in Step 2, then 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 (to plate transformants), and the RDHB plates (for viability control) to room temperature.

5. Check the OD_{600} of each of the three culture flasks. Harvest the cells from the culture that has an OD_{600} between 0.2 and 0.3. Centrifuge the cells at room temperature for 5–10 minutes at 1,500 × *g*. Decant the supernatant and discard the other cultures. Proceed to "Prepare spheroplasts for transformation" on page 45.

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

Prepare spheroplasts for transformation

Before starting

You should have a cell pellet from Step 5 on page 44.

- Prepare 100 mL of molten RD agarose and keep at 45°C (see "RD and RDH top agar" on page 76)
- Thaw one tube of 1 M DTT (provided in the kit)
- Prepare fresh SED for one batch of spheroplasts as follows:

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

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

Wash the cells

1. Wash the cells from Step 5 on page 44 by resuspending the pellet in 20 mL of sterile water (provided). Resuspend the pellet by swirling the tube. Transfer the resuspended cells to a sterile, 50-mL conical tube.

2. Pellet the cells by centrifugation at 1,500 × *g* for 5 minutes at room temperature. Decant and discard the supernatant. The cell pellet will be used to prepare spheroplasts.

3. Wash the cell pellet once by resuspending the cells in 20 mL of fresh SED (prepared above), then centrifuge at 1,500 × *g* for 5 minutes at room temperature.

4. Wash the cells once with 20 mL of 1 M sorbitol, then centrifuge at 1,500 × *g* for 5 minutes at room temperature.

5. Resuspend the cells by swirling in 20 mL of SCE buffer, then 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 the addition of a consistent amount of Zymolyase™.

Add Zymolyase

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

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

Before you begin:

- Prepare at least 20 mL of a 5% SDS solution (not provided) for use in the procedure.
- 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 on page 45), withdraw 200 µL cells and add to the tube marked 0. This is your zero time point. Set the tube aside on ice.

2. Add 7.5 µL of Zymolyase™ to the same tube of cells, mix it gently by inversion, then incubate at 30°C. **Do not shake the sample.** This sample will be used to establish the incubation time for optimal spheroplasting as described below. Keep the second tube of cells at room temperature for use in Step 6. Keep the remainder of the Zymolyase™ on ice.

3. Monitor the formation of the spheroplasts as follows: At time 2 minutes, withdraw 200 µL of cells (from the suspension in Step 2) and add to the tube marked 2. Repeat at time t = 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 minutes after adding Zymolyase™. Read the OD_{800} for all samples.

4. Determine the percent of spheroplasting for each time point using the equation:

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

   For example:

   At time t = 0, the OD_{800} = 0.256

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

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

   = 100 – [(0.125) × 100]

   = 100 – 12.5

   = 87.5%

5. Determine the time of incubation that results in approximately 70% spheroplasting. This time of incubation is variable due to differences in lots of Zymolyase™. In our 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 results in lower transformation efficiency.

   ---

6. Add 7.5 µL of 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 × *g* for 10 minutes at room temperature. Decant and discard the supernatant.

8. Wash the spheroplasts once with 10 mL of 1 M sorbitol (**gently** disperse the pellet by tapping the tube, **do not vortex**). Collect the spheroplasts by centrifugation at 750 × *g* for 10 minutes at room temperature.

9. Wash the spheroplasts once with 10 mL of CaS, then centrifuge at 750 × *g* for 10 minutes at room temperature. **Gently** resuspend the spheroplasts in 0.6 mL of CaS. The spheroplasts must be used immediately (up to 30 minutes) for transformation ("Transform Pichia" on page 47). They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations.

Transform *Pichia*

Before starting

Ensure that 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 the same restriction enzyme
- No DNA or linearized pBR322 DNA and plasmid only (no cells) as controls for contamination

Transform spheroplasts

1. For each transformation, dispense 100 µL of the spheroplast preparation from Step 9 on page 47 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, prepare 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, then incubate at room temperature for 10 minutes.

5. Centrifuge the tube at 750 × *g* for 10 minutes at room temperature, then 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, then incubate the cells at room temperature for 20 minutes.

7. Add 850 µL of 1 M sorbitol, then proceed to "Plate transformed Pichia spheroplasts" on page 48.

Plate transformed *Pichia* spheroplasts

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

1. Mix 100–300 µL of each spheroplast-DNA solution from Step 7 on page 48 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 the plates and incubate at 28–30°C. Transformants should appear in 4–6 days.

3. For cell viability control, mix 100 µL of spheroplasts with 900 µL of 1 M sorbitol.

4. Mix 100 µL of this diluted sample with 10 mL of molten RDH and pour on a RDHB plate. Allow top agarose to harden.

5. Invert the plates and incubate at 28–30°C. Appearance of colonies after 4–6 days demonstrates that the spheroplasts can regenerate into dividing cells.

Evaluate transformation experiment

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

(Optional) Replate transformants without using top-agarose

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

1. Scrape the agarose containing the His^+ transformants with a sterile spreader into a sterile, 50-mL, conical centrifuge tube, then mix with 20 mL sterile deionized water. Vortex the suspension vigorously to separate the cells from the agarose.

2. Filter the suspension through 4 folds of sterile cheesecloth. Centrifuge the filtrate at 1,500 × *g* for 5 minutes at room temperature. Centrifugation causes the cells to form a pellet at the bottom of the tube with the remaining agarose on top of the cells.

3. Remove the agarose pellet carefully from the top of the cells by gently shaking the tube so that only the agarose pellet is dispersed into the water. Decant the supernatant with the agarose pellet.

4. Resuspend the cell pellet in 5 mL of sterile deionized water, then 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 the cells by 10^4, then plate 50 µL and 100 µL aliquots onto MD plates. Incubate the plates overnight at 30°C. Proceed to "Screen for Mut+ and MutS transformants" on page 50.

Chemically competent *Pichia* cells

The *Pichia* EasyComp™ Kit (see "Accessory products" on page 101) provides enough reagents to produce 6 preparations of competent cells; each preparation yields enough competent cells for 20 transformations. You can use these cells immediately or store them frozen for future use. Each 50 µL aliquot of competent *Pichia* cells with 3 µg linearized plasmid DNA yields approximately 50 colonies on selective medium. Contact Technical Support for more information (page 110).

(Optional) Screen by functional assay

Some researchers have used functional assays to directly test for high expressing *Pichia* recombinant clones without first screening for Mut^S or Mut^+ phenotypes. If you elect to screen directly for high-expressing recombinants, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

Screen for Mut$^+$ and Mut^S transformants

Introduction

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

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 from these constructs should be Mut$^+$. However, with the presence of the *AOX1* sequences in the plasmid, there is a chance of recombination at the *AOX1* locus that disrupts the wild-type *AOX1* gene and creates His$^+$ Mut^S transformants. Testing on Minimal Dextrose (MD) and Minimal Methanol (MM) plates allows you to isolate His$^+$ Mut$^+$ transformants (see "Screen for His+ MutS or His+ Mut+ in GS115" on page 51).

His$^+$ Mut^S in KM71

There is no need to test recombinants for the Mut phenotype in KM71; all His$^+$ transformants in KM71 will be Mut^S because of the disruption of the *AOX1* gene (*aox1::ARG4*). Transforming KM71 with *Sal* I- or *Stu* I- linearized plasmid constructs favor recombination at the *HIS4* locus, while *Sac* I-linearized plasmid constructs favor recombination at the 5′ region of the *AOX1* gene. Purify His$^+$ transformants on minimal plates without histidine to ensure pure clonal isolates before testing for expression (see "Expression in recombinant Pichia strains" on page 58) or confirming integration by PCR ("PCR analysis of Pichia integrants" on page 55).

His$^+$ Mut^S in GS115

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

Because Mut^S transformants do not produce alcohol oxidase (the product of the *AOX1* gene), they cannot efficiently metabolize methanol as a carbon source. Therefore, Mut^S transformants 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$^+$).

Materials needed

You can prepare the following media (see "MD and MDH" on page 76) and materials several days in advance, and store at 4°C :

- Minimal Dextrose (MD) agar plates, 1 liter
- Minimal Methanol (MM) agar plates, 1 liter
- Sterile toothpicks and Scoring templates (see "Score templates" on page 54)
- Streak the strains GS115 Albumin (His$^+$ Mut^S) and GS115 β-Gal (His$^+$ Mut$^+$) on an MD or MGY plate as controls for Mut$^+$ and Mut^S growth on MD and MM plates.

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

Screen for His$^+$ Mut^S or His$^+$ Mut$^+$ in GS115

Use the plates containing His$^+$ transformants and screen for the Mut$^+$ and Mut^S phenotypes as follows:

1. Using a sterile toothpick, pick one His$^+$ colony, then streak or patch it in a regular pattern on an MM plate **and** an MD plate.

   IMPORTANT! Patch the MM plate first.

2. Use a new toothpick for each transformant and continue picking transformants until you have patched 100 transformants (2–3 plates).

3. To differentiate Mut$^+$ from Mut^S, make one patch for each of the controls (GS115/His$^+$ Mut^S Albumin and GS115/His$^+$ Mut$^+$ β-gal) onto the MD and MM plates.

4. Incubate the plates at 30°C for 2 days.

5. After 2 days or longer at 30°C, score the plates. Mut$^+$ transformants grow well on both MD and MM plates. Mut^S transformants grow well only on MD plates and show little or no growth on the MM plates.

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

(Optional) Replica-plating procedure

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

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

(Optional) Screen by functional assay

Some researchers have used functional assays to directly test for high expressing *Pichia* recombinant clones without first screening for Mut^S or Mut^+ phenotypes. If you elect to screen directly for high-expressing recombinants, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

Multiple integration events

Pichia pastoris is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology (for more information, see "Multiple gene insertion events" on page 99). Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants (in this case, His^+ transformants).

Successful expression of the gene of interest to useful levels may depend upon the generation of a recombinant strain that contains multiple copies integrated at the *AOX1* or *HIS4* loci. In addition to simply screening expression levels among several His^+ Mut^S or His^+ Mut^+ recombinants via SDS-PAGE analysis, it may be desirable to determine the existence of strains that have multiple integrants in the His^+ Mut^S or His^+ Mut^+ recombinant strain.

For methods to detect multiple integration events, refer to the "Detect multiple integration events" on page 88.

Vectors for multiple integration

Three vectors for isolating or generating *Pichia* recombinants that contain multiple integrations of your desired gene are available separately from Thermo Fisher Scientific. (See "Accessory products" on page 101 for ordering information). Two of the vectors, pPIC3.5K and pPIC9K, are used *in vivo* to identify possible transformants with multiple copies of your gene. The other vector, pAO815, is used for creating tandem copies of your gene *in vitro* before transforming into *Pichia*.

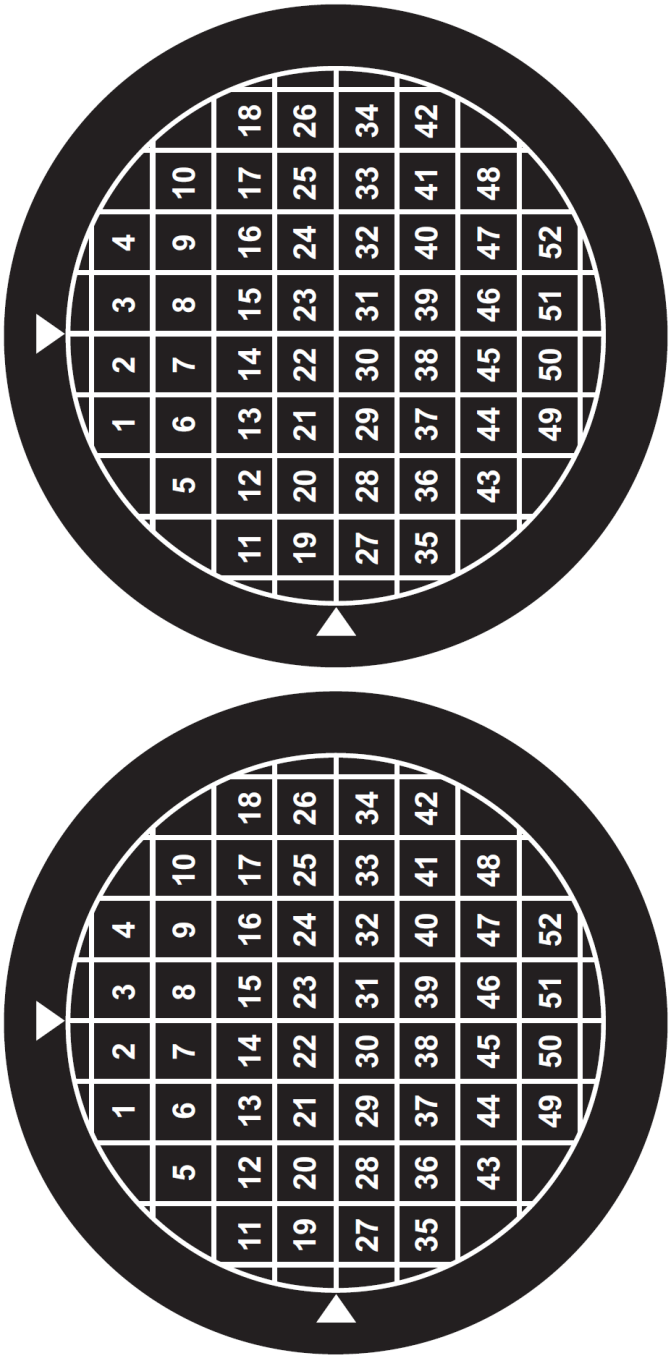
pPIC3.5K and pPIC9K

The vectors pPIC3.5K (for intracellular expression) and pPIC9K (for secreted expression) contain the bacterial kanamycin resistance gene cloned between the *HIS4* gene and the 3′ *AOX1* region. The kanamycin resistance gene confers resistance to G418 in *Pichia*. Multiple insertions of the kanamycin gene into the *Pichia* chromosome increase the resistance to G418. Because the kanamycin resistance gene is linked to your gene, isolation of hyper-resistant G418 transformants can 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 (P_{AOX1}–your gene of interest–HIS4) and cloning multiple copies of the expression cassette 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.

Score templates



PCR analysis of *Pichia* integrants

Introduction

To determine if your gene of interest has integrated into the *Pichia* genome, isolate genomic DNA from 6 to 10 Mut^S or Mut^+ *Pichia* clones and the strain transformed with the parent plasmid using the protocol described in "Isolate total DNA from Pichia" on page 86. After isolating your DNA, use the following PCR procedure to identify the integrants. Amplify the gene of interest using the α-factor primer (for pPIC9 only) or the 5′ *AOX1* primer paired with the 3′ *AOX1* primer included in the kit. This protocol can confirm the integration of the gene of interest, but it does not provide information on the site of integration.

Note: A more direct method for PCR screening is described in the "Direct PCR screening of Pichia clones" on page 84.

Isolate genomic DNA from *Pichia*

Easy-DNA™ Kit (available separately from Thermo Fisher Scientific) provides a fast and easy method to isolate genomic DNA from *Pichia pastoris*, which can be used instead of the protocol described in "Isolate total DNA from Pichia" on page 86. See "Accessory products" on page 101 for ordering information.

PCR analysis

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[1]
3′ *AOX1* Primer (0.1 µg/µL)	5 µL[1]
Sterile water to	50 µL
Taq Polymerase (5 U/µL)	0.25 µL

[1] Resuspend the primers in 20 µL sterile water to prepare a 0.1 µg/µL solution. You can decrease the amount of primer. For ~20 pmoles of primer, use 2 µL of each resuspended primer.

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

2. Load thermocycler and run the following program:

Step	Temperature	Time	Cycle
Hot Start	94°C	2 minutes	1X
Denaturation	94°C	1 minute	25–35X
Annealing	55°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1X

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

Interpret PCR

If screening Mut$^+$ integrants, you should see two bands, one corresponding to the size of your gene of interest, the other to the *AOX1* gene (approximately 2.2 kb).

If screening Mut^S integrants in GS115, you should see only the band that corresponds to the gene of interest. In KM71, because of the *ARG4* insert in *AOX1*, the PCR product is 3.6 kb. The size of the PCR products generated from parent plasmids are listed in the following table. Add these fragments to the size of your insert to interpret your PCR results.

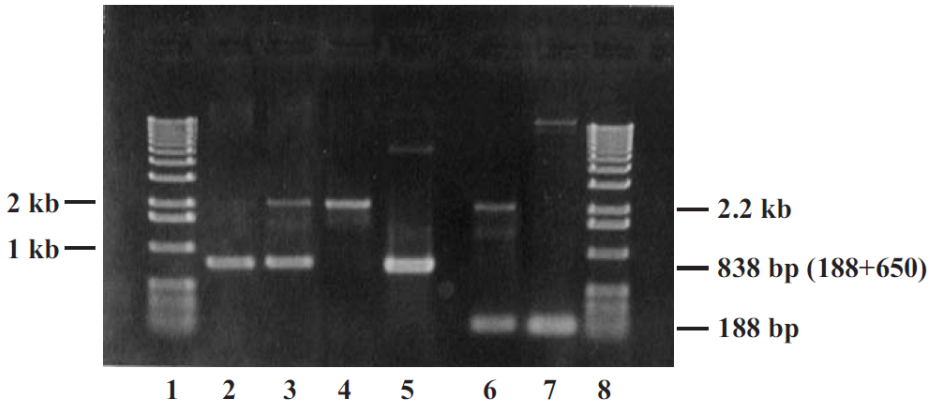
Vector	PCR Product
pHIL-D2	188 bp
pPIC3.5	214 bp
pHIL-S1	263 bp
pPIC9 (using the 5′ *AOX1* primer)	492 bp
pPIC9 (using the α-Factor primer)	195 bp

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

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

Example PCR analysis

The following figure shows the results of a typical PCR analysis using the procedure as described in "PCR analysis" on page 55. Genomic DNA was isolated from *Pichia* recombinants and from appropriate controls. 10 µL samples from each PCR were run on a 0.8% agarose gel. Lanes 1 and 8 contain markers for a 1 kb ladder; Lanes 2–4 are *Pichia* recombinants; Lane 5 is pHIL-D2 with the gene of interest; Lane 6 is GS115/pHIL-D2 (no insert); and Lane 7 is pHIL-D2 alone.



Discussion

Lane 7 shows the 188 bp PCR product made from pHIL-D2 by priming with the 5′ and 3′ *AOX1* primers (see "5′ AOX1 sequencing primer" on page 37). Lane 6 shows the 188 bp product and the wild-type *AOX1* gene (2.2 kb) from GS115/pHIL-D2. Lane 5 shows the expected size of our gene of interest cloned into pHIL-D2 (650 bp + 188 bp = 838 bp). Analysis of the *Pichia* recombinants in lanes 2–4 reveal that lanes 2 and 3 contain the insert, and that the recombinant in lane 2 may be a Mut^S because there is no wild-type *AOX1*. Lane 4, although from a His^+ transformant, does not contain the gene of interest.

Expression in recombinant *Pichia* strains

Introduction

The purpose of this section is to determine the optimal method and conditions for expressing your gene. Consider the following factors and guidelines before starting expression in *Pichia pastoris*. As with any expression system, optimal expression conditions are dependent on the characteristics of the protein being expressed.

Media

You need BMGY/BMMY (buffered complex glycerol or methanol medium), BMG/BMM (buffered minimal glycerol or methanol medium), or MGY/MM (minimal glycerol or minimal methanol medium) for expression (see "MGY and MGYH" on page 75).

BMG, BMM, BMGY, and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein. Unlike MGY and MM, they are all buffered media. Because these media are buffered with phosphate buffer, you can use a wide range of pH values to optimize production of your protein.

BMGY/BMMY contain yeast extract and peptone, which help stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone act as a "mixed feed", which allows 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, which inactivates many neutral pH proteases (Brierley *et al.*, 1994). *Pichia* is resistant to low pH, so the low pH does 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 are 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 perform 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 that you perform your initial expressions in BMMY. If the expressed protein is degraded, you can then try expression in an unbuffered medium.

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

Aeration

The most important parameter for efficient expression in *Pichia* is adequate aeration during methanol induction. As a general rule when inducing expression, never allow cultures to be more than 10–30% of your total flask volume. We strongly recommend that you use baffled flasks. 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.)

Growth kinetics

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

Temperature and shaking

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

Before starting

After you have verified recombinants in GS115 or KM71 and a control recombinant of GS115 or KM71/Vector (no insert), proceed with expression. When performing your expression, run the proper controls so that you can interpret your expression results. Use the following expression controls:

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

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

Expression guidelines

The following information is designed to get you started with expression. You may have to change the conditions to optimize expression for your particular protein. Use bottom or side baffled flasks whenever possible. If you are analyzing a number of recombinants, you can try 50-mL conical tubes. Ensure 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 expression

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

1. Using a single colony, inoculate 25 mL of MGY, BMG, or BMGY medium in a 250-mL baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} = 2–6 (log-phase growth, approximately 16–18 hours).

2. Harvest the cells by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature. Decant the supernatant and resuspend the cell pellet to an OD_{600} of 1.0 in MM, BMM, or BMMY medium (approximately 100–200 mL) to induce expression.

3. Transfer the culture to a 1-liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth, then return it to the incubator to continue growth.

4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction. Be sure to check the volume of the culture and add methanol accordingly. Evaporation can reduce the culture volume.

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

   Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).

6. For secreted expression, **transfer the supernatant to a separate tube.** Freeze the supernatant and the cell pellets quickly in liquid N_2 or in a dry ice/alcohol bath, then store them at –80°C until ready to assay.

   For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or in a dry ice/alcohol bath.

7. Analyze the supernatants and the cell pellets for protein expression by Coomassie™-stained SDS-PAGE and western blot, or functional assay (see "Analyze samples by SDS-polyacrylamide gel electrophoresis" on page 62).

Mut^S intracellular or secreted

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

1. Using a single colony, inoculate 100 mL of MGY, BMG, or BMGY medium in a 1-liter baffled flask. Grow the culture at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} = 2–6 (approximately 16–18 hours.).

2. Harvest the cells by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend the cell pellet in MM, BMM, or BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10–20 mL).

3. Transfer the cells to a 100-mL baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth, then return them to the incubator to continue to grow.

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

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

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

6. For secreted expression, **transfer the supernatant to a separate tube.** Freeze the supernatant and the cell pellets quickly in liquid N_2 or in a dry ice/alcohol bath, then store them at –80°C until ready to assay.

   For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or in a dry ice/alcohol bath.

7. Analyze the cell pellets for protein expression by Coomassie™-stained SDS-PAGE and western blot or functional assay.

Analyze samples by SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis

Thermo Fisher Scientific offers a wide range of pre-cast NuPAGE™ and Tris-Glycine polyacrylamide gels and electrophoresis devices. The NuPAGE™ Gel System avoids the protein modifications associated with LaemmLi-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Thermo Fisher Scientific carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use, refer to **thermofisher.com** or contact Technical Support (page 110).

If you are pouring your own gels, note that any standard SDS-polyacrylamide gel apparatus and protocol will work; for example, a 12% polyacrylamide gel with a 5% stacking gel is recommended for proteins ranging in size from 40–100 kDa. For other recommendations, see standard texts such as *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989), *Guide to Protein Purification* (Deutscher, 1990), or *Protein Methods* (Bollag *et al.*, 1996).

Prepare samples

Before starting, prepare Breaking Buffer (see "Breaking buffer" on page 78) and have acid-washed 0.5-mm glass beads on hand.

Prepare 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 an appropriate volume of denaturing PAGE Gel Loading buffer (Sample Buffer).
7. Heat the sample as recommended and load 10–20 µL per well. Thickness of the gel and number of wells determines the volume loaded. You can store the remaining sample at –20°C for western blots, if necessary. You can store the cell lysates at –80°C for further analysis.

Prepare supernatant (secreted expression only)

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

Protein concentration

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

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 transformed with the parent plasmid. This control shows the background of native *Pichia* proteins that are present intracellularly. Inclusion of this control will help you differentiate your protein from background, if you express it intracellularly.
- Analyze the GS115 β-Gal and Albumin controls, which will indicate any problems with the media or expression conditions

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Note: In addition to Coomassie™-stained SDS-PAGE, we strongly recommend that you perform a western blot or another more sensitive assay to detect your protein. Visualization of the expressed protein depends on several factors, including its expression level, its solubility, its molecular weight, and whether an abundant cellular protein of the same size masks it. Western blot analysis, enzymatic activities, or a defined purification profile, if available, can help to identify the expressed protein among the native *Pichia* cellular proteins.

Analyze protein expression

Inspection of your Coomassie™-stained SDS-PAGE reveals the induction over time of your protein co-migrating with your standard. If there is no recombinant protein visible, then perform a western blot or a functional assay, if you have one.

If you detect low expression of your recombinant protein, see "Optimize Pichia protein expression" on page 64.

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 "Isolate total RNA from Pichia" on page 90 for an RNA isolation protocol.

Optimize *Pichia* protein expression

Introduction

Based on available data, there is approximately a 75% chance of expressing your protein of interest in *Pichia pastoris* at reasonable levels. The biggest hurdle seems to be generating initial success, that is expressing 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 in *Saccharomyces cerevisiae*, suggesting that the *Pichia pastoris* system is an important alternative. If you obtain no or low protein expression in your initial expression experiment, use the following guidelines to optimize expression.

Proteolysis or degradation

- Do an expression time course study. 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 it in unbuffered medium (MM). In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.
- Try using SMD1168 (proteinase A-deficient) for expression (see "Proteases" on page 58).

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 "Concentrate proteins" on page 67).
- For Mut$^+$, induce expression with a higher density culture.

Low expression levels

- Check both Mut$^+$ and Mut^S recombinants for increased expression. Some proteins express better in one type of genetic background than another.
- If secreting your protein, try intracellular expression. The protein may not be processed correctly and fail to secrete. 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 can be desirable or not. If glycosylation is undesirable, you can remove oligosaccharides with Peptide:N-Glycosidase F (New England Biolabs, see "Glycoprotein analysis enzymes" on page 69).
- Scale up to fermentation ("Fermentation" on page 68). *Pichia* is a yeast and is particularly well suited to fermentation.

No expression

Very low expression can be perceived as no expression. See "Low expression levels" on page 65. If protein expression is not improved, perform a northern blot analysis to check for transcription of your gene.

Analyze for the presence of your insert by PCR (see "PCR analysis of Pichia integrants" on page 55). You can reasonably analyze 12–20 transformants by PCR. Include the vector only and original (one copy) construct controls to analyze your PCR experiment.

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

Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression to bypass the secretion pathway and to avoid protein modification.
- Try deglycosylating the protein with Peptide:N-Glycosidase F or other enzymes (see "Glycoprotein analysis" on page 69).
- Engineer your gene to remove N-linked glycosylation sites.

Scale up expression

Guidelines for expression

After you have optimized expression, scale up your expression protocol to produce more protein by increasing the culture volume using larger baffled flasks (below) or by fermentation. Use the following guidelines to scale up your expression protocol. To purify your protein, see the references listed in "Introduction" on page 68.

Mut$^+$ (intracellular or secreted)

1. Using a single colony, inoculate 25 mL of MGYH, BMGH, or BMGY in a 250-mL baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} = 2–6 (approximately 16–18 hours).

2. Use this 25-mL culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3- or 4-liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD_{600} = 2–6).

3. Harvest the cells using sterile centrifuge bottles by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend the cell pellet to an OD_{600} = 1.0 (2–6 liters) in MMH, BMMH, or BMMY medium to start induction.

4. Aliquot the culture between several 3- or 4-liter baffled flask. Cover the flasks with 2 layers of sterile gauze or cheesecloth, then return to the incubator. Continue to grow at 28–30°C with shaking.

5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached as determined from the time course study.

6. Harvest cells by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature.

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

   For secreted expression, **save the supernatant, chill it to 4°C, and concentrate it, if desired** (see "Concentrate proteins" on page 67). Proceed directly to purification ("Introduction" on page 68) or store the supernatant at –80°C until ready to process further.

Mut^S (intracellular or secreted)

1. Using a single colony, inoculate 10 mL of MGYH, BMGH, or BMGY in a 100-mL baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} = 2–6 (approximately 16–18 hours).
2. Use this 10-mL culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3- or 4-liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD_{600} = 2–6).
3. Harvest the cells by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend the cell pellet in 1/5 to 1/10 of the original culture volume of MMH, BMMH, or BMMY medium (approximately 100–200 mL).
4. Place the culture in a 1-liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth, then return to the incubator. Continue to grow at 28–30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached.
6. Harvest cells by centrifuging at 1,500–3,000 × *g* for 5 minutes at room temperature.
7. For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.

   For secreted expression, **save the supernatant, chill it to 4°C, and concentrate it, if desired** (see). Proceed directly to purification ("Introduction" on page 68) or store the supernatant at –80°C until ready to process further.

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

Concentrate proteins

Proteins secreted into the media are usually >50% homogeneous and require some additional purification. There are several general methods to concentrate proteins secreted from *Pichia*. These general methods include:

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

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

Cell lysis

A general procedure for cell lysis using glass beads is provided in "Cell lysis procedure" on page 69. 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 Thermo Fisher Scientific. We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Contact Technical Support for more information (page 110).

Protein purification and glycosylation

Introduction

At this point, you have an optimized protocol for expressing your protein and a method to scale up production of your protein for large-scale purification. You may already have a method to purify your protein. Since every protein is different, it is difficult to recommend specific techniques for purification. For an overview of purification methods, 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

Cell lysis procedure

Prepare Breaking Buffer (BB) as described in "Breaking buffer" on page 78.

1. Wash the cells once in BB by resuspending them, then centrifuging them for 5–10 minutes at 3,000 × *g* at 4°C.
2. Resuspend the cells to an OD_{600} of 50–100 in BB.
3. Add an equal volume of acid-washed glass beads (0.5-mm size). Estimate the volume by displacement.
4. Vortex the mixture 30 seconds, then incubate on ice for 30 seconds. Repeat 7 more times. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of your protein.
5. Centrifuge the sample at 4°C for 5–10 minutes at 12,000 × *g*.
6. Transfer the clear supernatant to a fresh container and analyze for your protein. Expect the total protein concentration to 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 Products (Bartlesville, OK) makes a BeadBeater™ that can handle 5–200 mL volumes of cell suspension.

Glycoprotein analysis

When expressing and purifying a glycosylated protein in a heterologous expression system, it is desirable to quickly determine whether the protein is glycosylated properly. For carbohydrate analysis of proteins to characterize glycosylated proteins, see (Ausubel *et al.*, 1994), Unit 17. Further information about glycosylation in eukaryotes is available in a review (Varki & Freeze, 1994).

Glycoprotein analysis enzymes

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

Enzyme	Type of enzyme	Specificity
Endoglycosidase D	Endo	Cleaves various high mannose glycans
Endoglycosidase F	Endo	Cleaves various high mannose glycans
Endoglycosidase H	Endo	Cleaves various high mannose glycans
β-galactosidase	Exo	Removes terminal galactosides from Gal-β1,3-GlcNAc, Gal-β1,4-GlcNAc or Gal-β1,3-GalNAc.

(continued)

Enzyme	Type of enzyme	Specificity
Peptide:N-Glycosidase F	Endo	Glycoproteins between Asn and GlcNAc (removes oligosaccharides)
Sialidases (Neuraminidases) *Vibrio cholerae* *Clostridium perfringens* *Arthobacter ureafaciens* Newcastle disease virus	Exo	NeuAc-α2,6-Gal, NeuAc-α2,6-GlcNAc or NeuAc-α2,3-Gal

Commercial carbohydrate analysis

There are a number of commercial vendors that 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.

Recipes and support protocols

E. coli media recipes

Introduction

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

LB (Luria-Bertani) medium

1% Tryptone

0.5% Yeast Extract

1% NaCl

pH 7.0

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

LB agar plates

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

Pichia media recipes

Introduction

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

Item	Amount	Cat. No.
Yeast Nitrogen Base • with ammonium sulfate • without amino acids	67 g pouch[1]	Q300-07
	500 g	Q300-09

[1] Each pouch contains reagents to prepare 500 mL of a 10X YNB solution.

Stock solutions

10X YNB (13.4% yeast nitrogen base with ammonium sulfate without amino acids)

1. Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1,000 mL of water. Heat the solution to dissolve YNB completely in water.

   Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate.

2. Filter sterilize and store at 4°C. The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch.

Note: *Pichia* cells exhibit optimal growth with higher YNB concentrations; therefore, the YNB used in this kit is 2X as concentrated as YNB formulations for *Saccharomyces.*

500X B (0.02% Biotin)

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

100X H (0.4% Histidine)

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

10X D (20% Dextrose)

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

10X M (5% methanol)

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

10X GY (10% glycerol)

1. Mix 100 mL of glycerol with 900 mL of water.
2. Filter sterilize or autoclave. Store at room temperature. The shelf life of this solution is greater than one year.

100X AA (0.5% of each amino acid)

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

1 M potassium phosphate buffer, pH 6.0

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

YPD or YEPD

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract

2% peptone

2% dextrose (glucose)

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

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1. Dissolve 10 g yeast extract and 20 g of peptone in 900 mL of water.

   Note: If making YPD slants or plates, add 20 g of agar.

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2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 mL of 10X D.
4. Store the liquid medium at room temperature. Store the YPD slants or plates at 4°C. The shelf life is several months.

YPD-Geneticin™ plates

Yeast Extract Peptone Dextrose Medium

1% yeast extract

2% peptone

2% dextrose (glucose)

2% agar

Variable amounts of Geneticin™ (see "Accessory products" on page 101 for ordering information)

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

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

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

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

MGY and MGYH

Minimal Glycerol Medium + Histidine (1 liter)

1.34% YNB

1% glycerol

4×10^{-5} % biotin

± 0.004% histidine

1. Aseptically combine 800 mL autoclaved water with 100 mL of 10X YNB, 2 mL of 500X B, and 100 mL of 10X GY.
2. For growth of *his4* strains in this medium, you can prepare a version that contains histidine (called MGYH) by adding 10 mL of 100X H (histidine) stock solution.
3. Store at 4°C. The shelf life of this solution is approximately two months.

RD and RDH liquid media

Regeneration Dextrose Medium + Histidine (1 liter)

1 M sorbitol

2% dextrose

1.34% YNB

4×10^{-5} % biotin

0.005% amino acids

± 0.004% histidine

1. Dissolve 186 g of sorbitol in 700 mL of water, then autoclave the solution for 20 minutes on liquid cycle.
2. Cool and maintain the liquid medium in a 45°C water bath.
3. Prepare a pre-warmed (45°C) mixture of the following stock solutions (see "Stock solutions" on page 72), then add to the sorbitol solution.
   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
4. For growth of *his4* strains you must add histidine to the media. Add 10 mL of 100X H (histidine) stock solution to the pre-warmed mixture in Step 3. Store liquid medium at 4°C. When stored properly, media last for several months.

RDB and RDHB agar plates

1. Dissolve 186 g of sorbitol in 700 mL of water and add 20 g of agar.
2. Autoclave for 20 minutes on liquid cycle.
3. Place the autoclaved solution in a 60°C water bath before the addition of pre-warmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the pre-warmed (45°C) mixture from "RD and RDH liquid media" on page 75, Step 3, then add to the sorbitol/agar solution. **If you are selecting for His^+ transformants, do not add histidine.**
5. Pour the plates immediately after mixing the solutions in Step 4. Store the plates at 4°C. Shelf life is several months.

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 before the addition of pre-warmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the pre-warmed (45°C) mixture from "RD and RDH liquid media" on page 75, Step 3, then add to sorbitol/agar solution. **If you are selecting for His^+ transformants, do not add histidine.**
5. Place the solution to 45°C after adding the solutions in Step 4. During transformation, use as a molten solution at 45°C.
6. Store the top agar at 4°C. Shelf life is several months.

MD and MDH

Minimal Dextrose Medium + Histidine (1 liter)

1.34% YNB

4×10^{-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 with the rest of the protocol.
5. If preparing plates, pour the plates immediately. You can store the MD for several months at 4°C.

MM and MMH

Minimal Methanol + Histidine (1 liter)

1.34% YNB

4×10^{-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, then 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 (histidine) stock solution. Mix and store at 4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed with the rest of the protocol.
5. After mixing, pour the plates immediately. You can store the MM and MMH for several months at 4°C.

BMG and BMM

Buffered Minimal Glycerol

Buffered Minimal Methanol (1 liter)

100 mM potassium phosphate, pH 6.0

1.34% YNB

4×10^{-5} % biotin

1% glycerol or 0.5% methanol

1. Autoclave 700 mL of water for 20 minutes on liquid cycle.
2. Cool autoclaved water 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 (methanol) instead of GY (glycerol).
4. Store the 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

4×10^{-5} % biotin

1% glycerol or 0.5% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 mL of water.
2. Autoclave the solution for 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 (methanol) instead of GY (glycerol).
5. Store the 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 it appropriately. Follow manufacturer's recommendations.
2. For 1 liter, dissolve the following in 900 mL deionized water:
   6 g sodium phosphate (monobasic)
   372 mg EDTA
   50 mL glycerol
3. Use NaOH to adjust the pH of the solution and bring up the volume to 1 liter. Store the breaking buffer at 4°C.
4. Immediately before use, add the protease inhibitors.

Electroporation of *Pichia*

Introduction

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

Prepare electrocompetent cells

1. Grow 5 mL of *Pichia pastoris* in YPD ("YPD or YEPD" on page 73) in a 50-mL conical tube at 30°C overnight.
2. Inoculate 500 mL of fresh medium in a 2-liter flask with 0.1–0.5 mL of the overnight culture. Grow the culture overnight again to an OD_{600} = 1.3–1.5.
3. Centrifuge the cells at 1,500 × *g* for 5 minutes at 4°C, then resuspend the pellet with 500 mL of ice-cold, sterile water.
4. Centrifuge the cells at 1,500 × *g* for 5 minutes at 4°C, then resuspend the pellet with 250 mL of ice-cold, sterile water.
5. Centrifuge the cells at 1,500 × *g* for 5 minutes at 4°C, then resuspend the pellet in 20 mL of ice-cold 1 M sorbitol.
6. Centrifuge the cells at 1,500 × *g* for 5 minutes at 4°C, then resuspend the pellet in 1 mL of ice-cold 1 M sorbitol for a final volume of approximately 1.5 mL.

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

Electroporate cells

1. Mix 80 µL of electrocompetent cells (from Step 6, "Prepare electrocompetent cells" on page 79) with 5–20 µg of linearized DNA (in 5–10 µL TE Buffer), then transfer 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, then transfer the cuvette contents to a sterile microcentrifuge tube.
5. Spread 200–600 µL aliquots of the electroporated cells on MD or RDB plates.
6. Incubate the plates at 30°C until colonies appear. Screen for Mut^+/Mut^S phenotypes as indicated in "Screen for Mut+ and MutS transformants" on page 50.

PEG 1000 transformation of *Pichia*

Introduction

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

Required solutions

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

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

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

Prepare competent cells

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

Transform cells

1. Use up to 50 µg of each DNA sample in no more than 20 µL total volume. Add the DNA directly to a still-frozen tube of competent cells. Include carrier DNA (40 µg of denatured and sonicated salmon sperm DNA) with <1 µg of DNA samples for maximum transformation efficiency.

   IMPORTANT! Cell competence decreases rapidly after the cells thaw even when they are held on ice. **It is critical to add DNA to frozen cells**. To perform multiple transformations, process the cells in groups of six at a time.

2. Incubate all sample tubes in a 37°C water bath for five minutes. Mix the samples once or twice during this incubation period.

3. Remove the tubes from the bath, then add 1.5 mL of Buffer B to each. Mix their contents thoroughly.

4. Incubate the tubes in a 30°C water bath for 1 hour.

5. Centrifuge the sample tubes at 2,000 × *g* for 10 minutes at room temperature. Decant the supernatant, then resuspend the cells in 1.5 mL of Buffer C.

6. Centrifuge the samples a second time, then resuspend the cell pellet gently in 0.2 mL of Buffer C.

7. Spread the entire contents of each tube on agar plates containing selective growth medium, then incubate the plates at 30°C for 3 to 4 days. Screen for Mut phenotype ("Screen for Mut+ and MutS transformants" on page 50) or select hyper-resistant Geneticin™ colonies ("pPIC3.5K and pPIC9K" on page 53).

Lithium chloride transformation of *Pichia*

Introduction

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

Required solutions

IMPORTANT! 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.

Prepare competent cells

1. Grow a 50-mL culture of *Pichia pastoris* in YPD at 30°C with shaking to an OD_{600} of 0.8 to 1.0 (approximately 10^8 cells/mL).
2. Harvest the cells and wash with 25 mL of sterile water, then centrifuge at 1,500 × *g* for 10 minutes at room temperature.
3. Decant the water, then 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 by centrifugation at maximum speed for 15 seconds, then 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.

   IMPORTANT! Do not store the competent cells on ice or freeze at –20°C.

Transform cells

1. Boil a 1 mL sample of single-stranded DNA for five minutes, then quickly chill in ice water. Keep on ice.

   Note: It is neither necessary nor desirable to boil the carrier DNA before each use. Store a small aliquot of the DNA at –20°C and boil every 3–4 times it is thawed.

2. Centrifuge the LiCl-cell solution from "Prepare competent cells" on page 82, Step 7. Remove the LiCl with a pipet.

3. For each transformation sample, add the following reagents to the cells **in the order given**. PEG shields the cells from the detrimental effects of the high concentration of LiCl.

   240 µL 50% PEG

   36 µL 1 M LiCl

   25 µLl 2 mg/mL single-stranded DNA

   Plasmid DNA (5–10 µg) in 50 µL sterile water

4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).

5. Incubate the cells at 30°C for 30 minutes without shaking.

6. Heat-shock the cells in a water bath at 42°C for 20–25 minutes.

7. Centrifuge the tubes at 2,000 × *g* for 10 minutes at room temperature, then remove the transformation solution with a pipet.

8. Gently resuspend the pellet in 1 mL of sterile water.

9. Plate 25 to 100 µL of the cells on RDB or MD plates, then incubate the plates for 2–4 days at 30°C. Screen for Mut phenotype ("Screen for Mut+ and MutS transformants" on page 50) or select hyper-resistant Geneticin™ colonies ("pPIC3.5K and pPIC9K" on page 53).

Direct PCR screening of *Pichia* clones

Introduction

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

Materials needed

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

Perform PCR

1. Place 10 µL of a *Pichia pastoris* culture into a 1.5-mL microcentrifuge tube. For relatively dense cultures, dilute 1 µL of the culture into 9 µL water. Alternatively, pick a single colony and resuspend it in 10 µL of water.
2. Add 5 µL of a 5 U/µL solution of lyticase to the cells, then incubate at 30°C for 10 minutes.
3. Freeze the sample at –80°C for 10 minutes or immerse in liquid nitrogen for 1 minute.
4. Set up a 50-µL PCR for a hot start:

10X Reaction Buffer	5 µL
25 mM $MgCl_2$	5 µL
25 mM dNTPs	1 µL
5′ *AOX1* primer (10 pmol/µL)	1 µL
3′ *AOX1* primer (10 pmol/µL)	1 µL
Sterile water	27 µL
Cell lysate	5 µL
Total volume	45 µL

5. Place the reaction mixture in the thermocycler and incubate at 95°C for 5 minutes.
6. Add 5 µL of a 0.16 U/µL solution of *Taq* polymerase (0.8 units).

7. Cycle 30 times using the following parameters:

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

Include a final extension of 7 minutes at 72°C.

8. Analyze a 10-µL aliquot by agarose gel electrophoresis.

Isolate total DNA from *Pichia*

Introduction

The following protocol allows you to isolate DNA from the desired His^+ recombinants and the untransformed GS115 or KM71, which you can use for Southern blot analysis, dot/slot blot analysis, or genomic PCR. For more information, see *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Guide to Yeast Genetics and Molecular Biology* (Strathern and Higgins, 1991), or (Holm *et al.*, 1986).

Prepare solutions

Prepare the following solutions, because there is not enough of some of these reagents in the kit to perform this experiment.

- Minimal Medium (MD, MGYH)
- Sterile water
- SCED buffer (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT)
- Zymolyase™, 3 mg/mL stock solution in water (Seikagaku America, Inc., 1-800-237-4512)
- 1% SDS in water
- 5 M potassium acetate, pH 8.9
- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)
- 7.5 M ammonium acetate, pH 7.5
- Phenol:chloroform (1:1 v/v)

Prepare cells

1. Grow the recombinant strain and the parent strain in 10 mL of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71) at 30°C to an OD_{600} of 5–10.
2. Collect the cells by centrifugation at 1,500 × *g* for 5–10 minutes at room temperature.
3. Wash the cells with 10 mL of sterile water by centrifugation at 1,500 × *g* for 5–10 minutes at room temperature.

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 in "Prepare spheroplasts for transformation" on page 45–"Add Zymolyase" on page 46).
3. Add 2 mL of 1% SDS, mix **gently**, then incubate on ice (0 to 4°C) for 5 minutes.
4. Add 1.5 mL of 5 M potassium acetate, pH 8.9, and mix **gently**.
5. Centrifuge at 10,000 × *g* for 5–10 minutes at 4°C and save the supernatant.

Precipitate the DNA

1. Transfer the supernatant from "Spheroplasting and lysis" on page 86, Step 5, then add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
2. Centrifuge at 10,000 × *g* for 20 minutes at 4°C.
3. Resuspend the pellet **gently** in 0.7 mL of TE buffer, pH 7.4, then transfer to a microcentrifuge tube.
4. **Gently** extract the DNA with an equal volume of phenol:chloroform (1:1 v/v) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Split the aqueous layer into two microcentrifuge tubes.
5. Add 1/2 volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. Place the tubes on dry ice for 10 minutes or at –20°C for 60 minutes.
6. Centrifuge at 10,000 × *g* for 20 minutes at 4°C, then wash the pellets once with 1 mL of 70% ethanol. Briefly air dry the pellets, then resuspend each pellet in 50 µL of TE buffer, pH 7.5.
7. Determine the concentration of the DNA sample. You can store the samples at –20°C separately or combined until ready for use.

Detect 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 can increase the levels of protein expressed. If the expression of your protein is low, you may wish to isolate multicopy integrants. 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, as described in "Isolate total DNA from Pichia" on page 86. You can use the DNA in Southern blot (page 88) or quantitative dot blot (page 89) analyses to detect multicopy integration.

Southern blot analysis

For a detailed description of this technique as applied to *Pichia pastoris*, see (Clare, *et al*., 1991a). It is very important to digest your DNA with the correct restriction enzymes to generate a blot of digested and gel-separated genomic DNA. It is also important to understand that your strategy will be different if you use pPIC3.5K versus pAO815 to generate your multiple copies. Digesting DNA from *Pichia* recombinants containing multiple copies produces a band that varies in intensity depending on the number of copies of your gene. It is very important to include a control to show the intensity of a single copy gene. You can quantify the band intensities using densitometry to estimate relative gene dosage.

General guidelines for Southern blot analysis

- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al*., 1989).
- Isolate genomic DNA and quantify it using fluorometry. Be sure to eliminate RNA from your sample. It is very important to load the same amount of DNA into each lane to accurately determine the copy number.
- Probe your Southern blot with probes to *HIS4* and your gene. The point mutation in the *his4* gene in the host strain will not interfere with hybridization if you make the probe complementary to the wild-type gene.
- If you have used pPIC3.5K to generate multimers, use *Bgl* II to digest your DNA (Clare, *et al*., 1991a). If you have used pPIC3.5K, all multimers are NOT necessarily in a head-to-tail configuration. Some multimers can be head-to-head and others tail-to-tail. We recommend that you think about what products can be produced. An expression cassette in the opposite orientation can produce a different band. The number of multiple copies causes one or two bands (depending on orientation) in the Southern blot to increase in intensity once you have >2 copies.
- If you have used pAO815 to generate multimers, use *Bgl* II and *Bam*H I to digest the genomic DNA and release the multimer. The molecular weight of the band allows you to determine the number of multimers. If this multimer is too large, you can digest with an enzyme like *Sac* I. This collapses the multimer into single fragments containing your gene. These fragments produce a band that is quite intense. The relative intensity of this band versus a band containing a single copy of your gene allows you to determine the copy number.
- *Bgl* II digested DNA from GS115 and GS115 transformed with pPIC3.5K or pAO815 produces bands of 2.8 kb (the genomic copy of *HIS4)* and ~6.7 kb (the vector derived copy of *HIS4)*, respectively, when probed with a complementary fragment to *HIS4*.

Quantitative dot blot analysis

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

Materials needed for quantitative dot blot

- Whatman™ 3MM paper.

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

- 50 mM EDTA, 2.5% β-mercaptoethanol, pH 9
- 1 mg/mL Zymolyase™ 100T in water
- 0.1 N NaOH, 1.5 M NaCl
- 2X SSC

Quantitative dot blot procedure

1. Grow Mut^+ or Mut^S transformants in individual wells of a 96-well microtiter plate in 200 µL of YPD broth at 30°C until all wells have approximately the same density. This may necessitate several passages.

   Alternatively, you can grow individual transformants in culture tubes and normalize the absorbance at 600 nm by adding medium.

2. Filter 50 µL of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multi-channel pipettor. Air-dry the filters.

3. Place two sheets of 3 MM paper in a tray and soak with 10–15 mL of 50 mM EDTA, 2.5% β-mercaptoethanol, pH 9. Ensure that the paper is uniformly soaked and that there are no puddles. Place the nitrocellulose filter face down on the treated 3MM paper. Incubate the filter for 15 minutes at room temperature.

4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak them with 10–15 mL of 1 mg/mL Zymolyase™ 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate it for 4 hours at 37°C.

5. Remove the nitrocellulose filter from the paper and replace the paper with two new sheets. Soak with 10–15 mL of 0.1 N NaOH, 1.5 M NaCl. Place the nitrocellulose filter face down on the paper and incubate for 5 minutes at room temperature.

6. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak them with 10–15 mL of 2X SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate it for 5 minutes at room temperature. Repeat.

7. Bake the nitrocellulose filters at 80°C or UV-crosslink the DNA to nylon. You can probe the filters with a non-radioactive-labeled or random-primed, ^{32}P-labeled probe complementary to your gene.

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

Isolate total RNA from *Pichia*

Introduction

This protocol is designed to isolate 60–300 µg of total RNA (Schmitt *et al.*, 1990) from *Pichia* that is suitable for mRNA isolation using the Dynabeads™ mRNA Purification kit or the Poly(A)Purist™ MAG Kit. If you wish to use another protocol, scale up the reaction to yield about 2 mg of total RNA per time point. The mRNA is for northern blot analysis of *Pichia* recombinants to determine if the gene of interest is being induced and transcribed. Isolate the RNA from induced cultures using an uninduced culture as a negative control.

Solutions and materials required

Use DEPC-treated water and equipment that is free of RNase.

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

Grow cells

1. Grow two cultures (100–200 mL in MGY or BMGY), but induce only one of them. Use the same protocol for induction that you have used in "Expression in recombinant Pichia strains" on page 58.
2. At each of the times indicated below, transfer 10 mL of the cultures to 50-mL conical tubes.
   Time points: 1, 2, 3, 4, and 6 days.
3. Centrifuge the cells from each time point at 1500 × *g* for 10 minutes at room temperature.
4. Resuspend each cell pellet in 400 µL of AE buffer, then transfer to a microcentrifuge tube. Proceed to "Lyse cells" on page 91.

Lyse cells

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

Isolate mRNA and perform northern analysis

See (Ausubel *et al.*, 1994) for a protocol for mRNA isolation and Northern analysis. See "Accessory products" on page 101 for ordering information for Dynabeads™ mRNA Purification kit and Poly(A)Purist™ MAG Kit.

You will need ~1–5 µg mRNA per time point.

Beta-Galactosidase assay

Introduction

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

Required solutions

- A fresh crude cell lysate of GS115 β-Gal (see "Cell lysis procedure" on page 69)
- Z buffer
- ONPG solution
- 1 M sodium carbonate solution

Recipes for the solutions are below.

Z buffer

60 mM Na_2HPO_4-$7H_2O$

40 mM NaH_2PO_4-H_2O

10 mM KCl

1 mM $MgSO_4$-$7H_2O$

50 mM β-mercaptoethanol

pH 7.0

1. Dissolve the following in 950 mL of deionized water:

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

2. Adjust pH to 7.0 with NaOH or HCl, then bring the volume up to 1 liter with water.
3. **Do not autoclave!** Store at 4°C.

ONPG solution

4 mg/mL ONPG in 100 mM phosphate buffer, pH 7.0

1. Dissolve the following in 90 mL of deionized water:

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

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

1 M sodium carbonate

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

Perform assay

1. Determine the protein concentration of your lysate by Lowry, (Bradford)™, or BCA assay.
2. Equilibrate the 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 of 4 mg/mL ONPG solution to each of the tubes in Step 3.
5. Incubate the samples and the control at 28°C until a faint yellow color develops. This should occur at least 10 minutes after the start of the assay to ensure accurate data. Note that the tube with no lysate may not change color.
6. Stop the reaction by adding 0.5 mL of 1 M sodium carbonate to each tube. Record the length of incubation for each sample.
7. Read the OD_{420} against the control containing buffer alone.
8. Determine the protein concentration of your lysate in mg/mL.

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

Determine specific activity

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

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

Sample calculation

Extract concentration = 10 mg/mL

Assay 10 µL of a 1/100 dilution

Time = 10 minutes

OD_{420} = 0.4

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

= 0.001 mg protein in the reaction

Specific activity = (0.4 × 380)/(10 × 0.001 mg) = 15,200 units/mg protein

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

B

Additional *Pichia* information

Proteins expressed in *Pichia*

The following table provides a partial list of references that document successful expression of heterologous proteins in *Pichia pastoris*. Note that both Mut$^+$ and Mut^S phenotypes as well as secreted and intracellular expression were used successfully.

Protein	Expression (grams/liter)	Where and how expressed	Reference
Enzymes			
Invertase	2.3	Secreted Mut$^+$	(Tschopp *et al.*, 1987b)
Bovine Lysozyme c2	0.55	Secreted Mut$^+$	(Digan *et al.*, 1989)
Streptokinase (active)	0.08	Intracellular [1]	(Hagenson *et al.*, 1989)
Alpha amylase	2.5	Secreted Mut^S	(Paifer *et al.*, 1994)
Pectate Lyase	0.004	Secreted Mut^S	(Guo *et al.*, 1995)
Spinach Phosphoribulokinase	0.1	Intracellular Mut^S	(Brandes *et al.*, 1996)
Antigens			
Hepatitis B surface antigen	0.4	Intracellular Mut^S	(Cregg *et al.*, 1987)
Pertussis Antigen P69	3.0	Intracellular Mut^S	(Romanos *et al.*, 1991)
Tetanus Toxin Fragment C	12.0	Intracellular Mut$^+$/Mut^S	(Clare *et al.*, 1991a)
HIV-1 gp120	1.25	Intracellular Mut$^+$	(Scorer *et al.*, 1993)
Tick Anticoagulant protein	1.7	Secreted Mut^S	(Laroche *et al.*, 1994)
Bm86 Tick Gut Glycoprotein	1.5	Secreted [1]	(Rodriguez *et al.*, 1994)
Regulatory Proteins			
Tumor Necrosis Factor (TNF)	10.0	Intracellular Mut^S	(Sreekrishna *et al.*, 1989)
Mouse Epidermal Growth Factor (EGF)	0.45	Secreted Mut^S	(Clare *et al.*, 1991b)
Human Interferon (IFN) α2b	0.4	Intracellular Mut^S	(Garcia *et al.*, 1995)
Membrane proteins			
Human CD38 (soluble portion)	0.05	Secreted Mut^S	(Fryxell *et al.*, 1995)

(continued)

Protein	Expression (grams/liter)	Where and how expressed	Reference
Mouse Serotonin Receptor	0.001	Secreted Mut$^+$	(Weiss *et al.*, 1995)
Proteases and protease inhibitors			
Carboxypeptidase B	0.8	Secreted Mut$^+$/Mut^S	(Despreaux & Manning, 1993)
Enterokinase	0.021	Secreted Mut$^+$	(Vozza *et al.*, 1996)
Ghilanten	0.01	Secreted Mut$^+$	(Brankamp *et al.*, 1995)
Kunitz protease inhibitor	1.0	Secreted [1]	(Wagner *et al.*, 1992)
Human Proteinase Inhibitor 6	0.05	Intracellular Mut$^+$	(Sun *et al.*, 1995)
Antibodies			
Rabbit Single Chain Antibody	>0.1	Secreted Mut^S	(Ridder *et al.*, 1995)

[1] Mut phenotype was not described in the paper.

Recombination and integration in *Pichia*

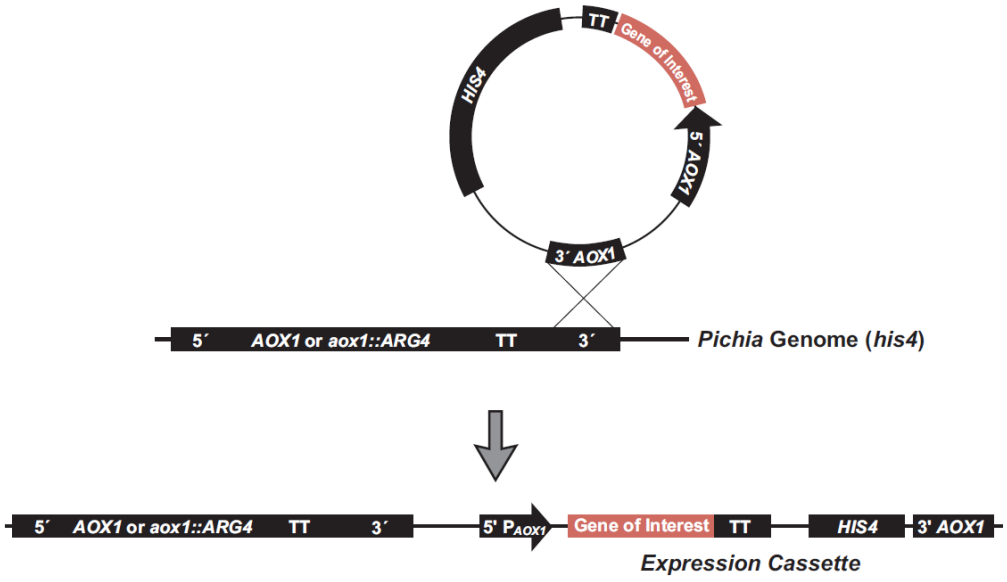
Introduction

Similar to *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 "Gene insertion at AOX1 or aox1::ARG4" on page 97) or at the *his4* locus (see "Gene insertion events at his4" on page 98). Note that single crossover events (insertions) are much more likely to happen than double crossover events (replacements). Multiple insertion events occur spontaneously at about 1–10% of the single insertion events.

Gene insertion at *AOX1* or *aox1::ARG4*

Gene insertion events at the *AOX1* (GS115) or *aox1::ARG4* (KM71) loci arise from a single crossover event between the loci and any of the three *AOX1* regions on the vector: the *AOX1* promoter, the *AOX1* transcription termination region (TT), or sequences even further downstream of *AOX1* (3′ *AOX1*). This results in the insertion of one or more copies of the vector upstream or downstream of the *AOX1* or the *aox1::ARG4* genes. The phenotype of such a transformant is His$^+$ Mut$^+$ (GS115) or His$^+$ Mut^S (KM71). **By linearizing the recombinant vector at a restriction enzyme site located in the 5′ or 3′ *AOX1* regions, Mut$^+$ or Mut^S recombinants can be conveniently generated depending on the host strain used.**

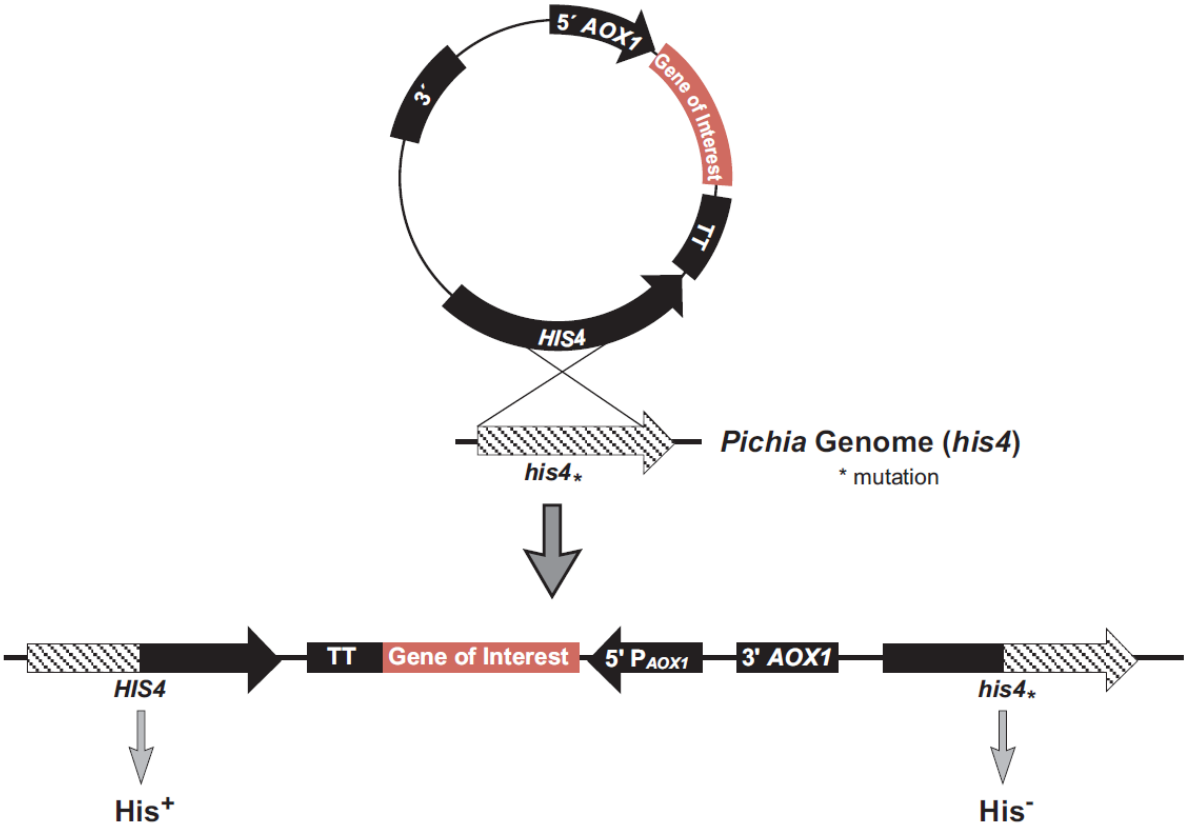
The following figure shows the result of an insertion of the plasmid 3′ to the intact *AOX1* locus (Mut$^+$) and the gain of P_{AOX1}, your gene of interest, and *HIS4* (expression cassette). This event could also happen at the 5′ *AOX1* regions of the plasmid and genome with the resulting insertion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



Gene insertion events at *his4*

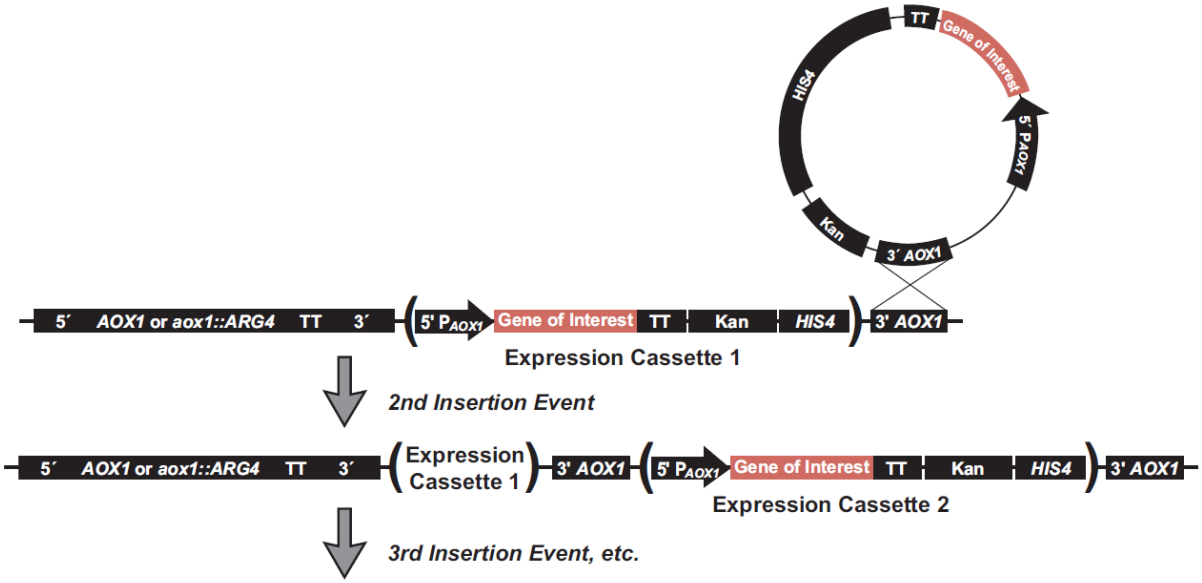
In GS115 (Mut$^+$) and KM71 (Mut^S) gene insertion events at the *his4* locus arise from a single crossover event between the *his4* locus in the chromosome and the *HIS4* gene on the vector. This event results in the insertion of one or more copies of the vector at the *his4* locus. Because the genomic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

The following figure shows the result of an insertion of the plasmid between duplicated copies of the *HIS4*/*his4* genes, one still mutant, and the other wild-type.



Multiple gene insertion events

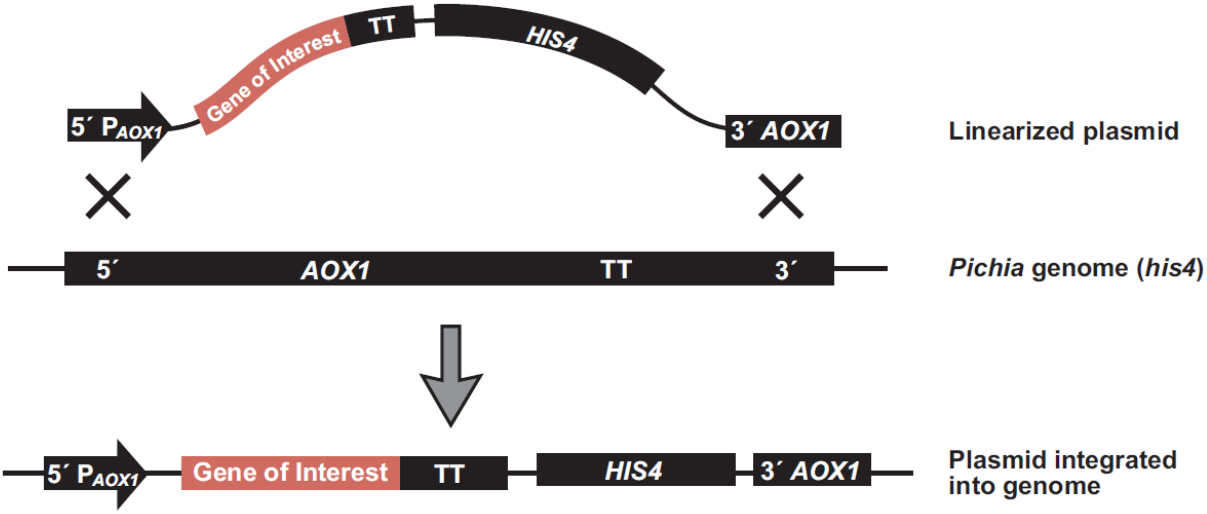
Multiple gene insertion events at a single locus in a cell do occur spontaneously with a low, but detectable frequency–between 1 and 10% of all selected His^+ transformants. Multi-copy events can occur as gene insertions at the *AOX1*, *aox1::ARG4*, or *his4* loci. This results in a Mut^+ phenotype in GS115 and a Mut^S phenotype in KM71. Quantitative dot blot analysis, Southern blot analysis, and differential hybridization can detect multiple gene insertion events. See "Detect multiple integration events" on page 88 for a protocol to screen for multiple inserts.



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 the genome. This results in the complete removal of the *AOX1* coding region (i.e., gene replacement). The resulting phenotype is His^+ Mut^S. His^+ transformants can be readily and easily screened for their Mut phenotype, with Mut^S serving as a phenotypic indicator of integration via gene replacement at the *AOX1* locus. The net result of this type of gene replacement is a loss of the *AOX1* locus (Mut^S) and the gain of an expression cassette containing P_{AOX1}, your gene of interest, and *HIS4*. The following figure shows a gene replacement event at the *AOX1* locus.

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



C

Ordering information

Accessory products

Many of the reagents supplied in *Pichia* Expression Kit, as well as other reagents and kits used in *Pichia* expression experiments are available separately from Thermo Fisher Scientific. Ordering information is provided below. For more information, refer to **thermofisher.com** or contact Technical Support (page 110).

Product	Amount	Cat. No.
Spheroplast Kit for Yeast	1 kit	K1720-01
Geneticin™ Selective Antibiotic (G418 Sulfate), Powder	1 g 5 g 25 g	11811-023 11811-031 11811-098
Geneticin™ Selective Antibiotic (G418 Sulfate), 50 mg/mL Solution	20 mL 100 mL	10131-035 10131-027
Ampicillin, Sodium Salt	200 mg	11593-027
TA Cloning™ Kit with pCR™2.1 Vector and One Shot™ INVαF′ Chemically Competent *E. coli*	20 reactions	K2000-01
PureLink™ HiPure Plasmid Miniprep Kit	25 preparations 100 preparations	K2100-02 K2100-03
Platinum™ *Taq* DNA Polymerase	120 reactions 300 reactions 600 reactions	10966-018 10966-026 10966-034
Taq DNA Polymerase, Recombinant	100 units 500 units	10342-053 10342-020
Easy-DNA™ Kit	1 kit	K1800-01
Pichia EasyComp™ Kit	1 kit	K173001
Dynabeads™ mRNA Purification kit	1 kit	610-06
Poly(A)Purist™ MAG Kit	1 kit	AM1922
pAO815 Vector	20 µg	V180-20

(continued)

Product	Amount	Cat. No.
pPIC3.5K Vector	20 µg	V173-20
pPIC9K Vector	20 µg	V175-20

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E Safety

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- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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