invitrogen™

Multi-Copy *Pichia* Expression Kit

For the Isolation and Expression of Recombinant Proteins from *Pichia pastoris* Strains Containing Multiple Copies of a Particular Gene

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User Manual

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Kit Contents and Storage

Kit Contents

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

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

Reagent	Amount	Components
SOS medium	20 ml	1 M Sorbitol 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 $CaCl_2$

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

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	

Continued on next page

Materials, continued

Kit Contents, continued

Vectors. Store at –20°C.

Reagent	Description
pAO815 20 µg, lyophilized	Vector for creation of multi-copy genes *in vitro* and intracellular expression in *Pichia*
pPIC3.5K 20 µg, 40 µl at 0.5 µg/µl in TE buffer, pH 8.0	Vector for *in vivo* isolation of multi-copy genes and intracellular expression in *Pichia*
pPIC9K 20 µg, 40 µl at 0.5 µg/µl in TE buffer, pH 8.0	Vector for *in vivo* isolation of multi-copy genes and secreted expression in *Pichia* Uses the α-factor signal sequence

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

Continued on next page

Materials, continued

Required Equipment and Supplies (not provided)

- 30°C and 37°C rotary shaking incubator
- Water baths capable of 16°C, 37°C, 45°C, 65°C, and 100°C
- Centrifuge suitable for 50 ml conical tubes (floor or table-top)
- Baffled culture flasks with metal covers (50 ml, 250 ml, 500 ml, 1000 ml, and 3 L)
- 50 ml sterile, conical tubes
- 6 ml and 15 ml sterile snap-top tubes (Falcon 2059 or similar)
- UV Spectrophotometer
- Mini agarose gel apparatus and buffers
- Agarose and low-melt agarose
- Polyacrylamide gel electrophoresis apparatus and buffers
- Media for transformation, growth, screening, and expression (**Appendix**, pages 64–71)
- 5% SDS solution (10 ml per transformation)
- Sterile cheesecloth or gauze
- *Eco*R I, *Bam*H I, and *Bgl* II restriction enzymes and appropriate buffers
- Glass milk
- Sterile water
- CIP (calf intestinal phosphatase, 1 unit/µl)
- 10X CIP Buffer
- Phenol/chloroform
- 3M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/µl)
- 10X Ligation Buffer (with ATP)
- LB medium
- LB-ampicillin plates (50–100 µg/ml ampicillin)
- Geneticin® antibiotic (see page viii)
- YPD-Geneticin® plates (see **Appendix**, page 67)
- Hemacytometer
- Microtiter plates (optional)
- Breaking Buffer (see **Appendix**, page 71)
- Acid-washed glass beads (available from Sigma)
- Replica-plating equipment (optional)
- Bead Beater™ (optional, available from Biospec)

Accessory Products

Introduction

The products listed in this section are intended for use with the Multi-Copy *Pichia* Expression Kit. For more information, refer to our website (www.invitrogen.com) or call Technical Support (see page 94).

Accessory Products

Many of the reagents supplied in the Multi-Copy *Pichia* Expression Kit, as well as other reagents and kits used in *Pichia* expression experiments are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Cat. no.
Pichia Spheroplast Module (Box 1 and Box 2)	10 preparations (50 transformations)	K1720-01
Geneticin®, powder	1 g 5 g 25 g	11811-023 11811-031 11811-098
Geneticin®, liquid (50mg/ml)	20 ml 100 ml	10131-035 10131-027
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
TA Cloning® Kit (with pCR®2.1 vector)	20 reactions	K2000-01
PureLink™ HiPure Plasmid Miniprep Kit	25 preparations 100 preparations	K2100-02 K2100-03
Platinum® Taq DNA Polymerase	100 reactions 250 reactions 500 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
FastTrack® 2.0 mRNA Isolation Kit	1 kit	K1593-02
Micro-FastTrack™ 2.0 mRNA Isolation Kit	1 kit	K1520-02

Introduction

Overview

Review Articles

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

General Characteristics of *Pichia pastoris*

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

Similarity to *Saccharomyces*

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

- Transformation by complementation
- Gene disruption
- Gene replacement

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

Pichia pastoris as a Methylotrophic Yeast

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

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Overview, continued

Two Alcohol Oxidase Proteins

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

Expression

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

Phenotype of *aox1* mutants

Loss of the *AOX1* gene, and thus a loss of most of the cell's alcohol oxidase activity, results in a strain that is phenotypically Mut^S (Methanol utilization slow). This has in the past been referred to as Mut^-. The Mut^S designation has been chosen to accurately describe the phenotype of these mutants. This results in a reduction in the cells' ability to metabolize methanol. The cells, therefore, exhibit poor growth on methanol medium. Mut^+ (Methanol utilization plus) refers to the wild type ability of strains to metabolize methanol as the sole carbon source. These two phenotypes are used when evaluating *Pichia* transformants for integration of your gene (**Experimental Outline**, page 6).

Intracellular and Secretory Protein Expression

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

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

Continued on next page

Overview, continued

Posttranslational Modifications

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

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

Experimental Outline

Selecting Vector to Generate Multi-Copy Integrants

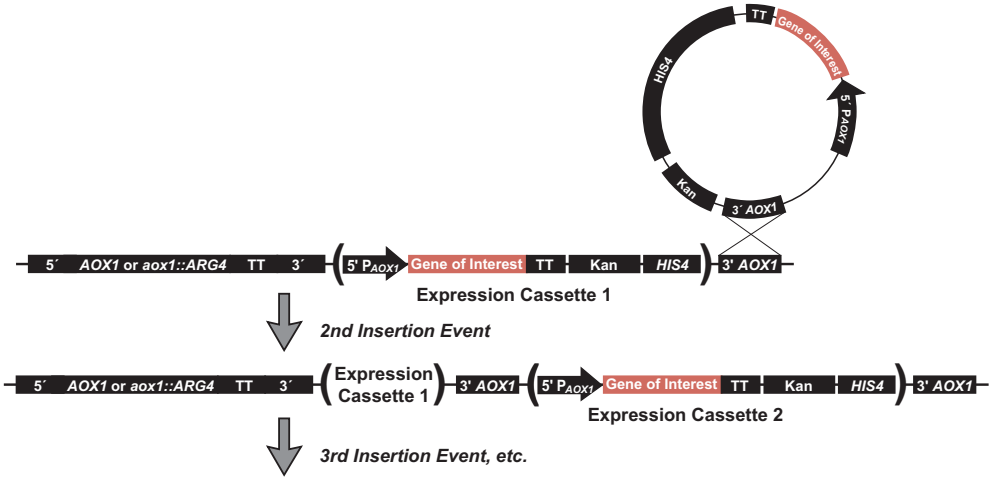
Multiple copy integration of recombinant genes in *Pichia* has been demonstrated to increase expression of the desired protein in some cases (Brierley *et al.*, 1994; Clare *et al.*, 1991a; Cregg *et al.*, 1993; Romanos *et al.*, 1991; Scorer *et al.*, 1993; Scorer *et al.*, 1994; Thill *et al.*, 1990; Vedvick *et al.*, 1991). The three vectors included in this kit allow isolation and generation of multicopy inserts by *in vivo* (pPIC3.5K, pPIC9K) or *in vitro* methods (pAO815), and to test whether increasing the copy number of your recombinant gene will lead to a subsequent increase in protein expression. The *in vivo* method utilizes hyper-resistance to Geneticin® (G-418) to screen for possible multi-copy inserts, while the *in vitro* method produces tandem inserts of your gene by ligation. pAO815 and pPIC3.5K are used for intracellular expression, while pPIC9K is used for secreted expression. All vectors utilize the *AOX1* promoter for inducible, high-level expression. For more information on these vectors, see pages 12–16.

Frequency of Multicopy Inserts

Multiple plasmid integration events occur spontaneously in *Pichia* at a frequency between 1 and 10% of all His^+ transformants. The *in vivo* method allows you to screen for the His^+ transformants that may have multiple inserts of your gene. The *in vitro* method allows you to construct multimers by ligation. When His^+ transformants are selected, they will have a high probability of containing the multimers that you constructed *in vitro*.

Generating Multicopy Inserts *in vivo*

pPIC3.5K and pPIC9K contain the bacterial kanamycin gene (*kan* from Tn*903*) that confers resistance to Geneticin® in *Pichia*. Note that *kan* does not confer resistance to kanamycin in *Pichia*. The level of Geneticin® resistance roughly depends on the number of kanamycin genes integrated. A single copy of pPIC3.5K or pPIC9K integrated into the *Pichia* genome confers resistance to Geneticin® to a level of ~0.25 mg/ml. Multiple integrated copies of either vector can increase the Geneticin® resistance level from 0.5 mg/ml (1–2 copies) up to 4 mg/ml (7–12 copies). Because of the genetic linkage between the kanamycin gene and the "expression cassette" (P_{AOX1} and your gene of interest), one can infer from Geneticin® hyper-resistance that the clone in question contains multiple copies of your gene. Protein expression may increase because of a gene dosage effect. Thus, the presence of the *kan* gene can be used as a tool to detect transformants that harbor multiple copies of your gene. The graphic below shows multiple insertion and linkage of the *kan* gene to your expression cassette.



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Experimental Outline, continued

Screening on Geneticin®

Direct selection of Geneticin® resistance in yeast does not work well because newly transformed cells need time to express sufficient amounts of the resistance factor. Since yeast grows much more slowly than bacteria, significant numbers of recombinant yeast are killed before they accumulate enough of the resistance factor to survive direct plating on antibiotic. The most efficient procedure to generate Geneticin® resistant and hyper-resistant clones requires an initial selection of His^+ transformants followed by screening for varying levels of Geneticin® resistance.

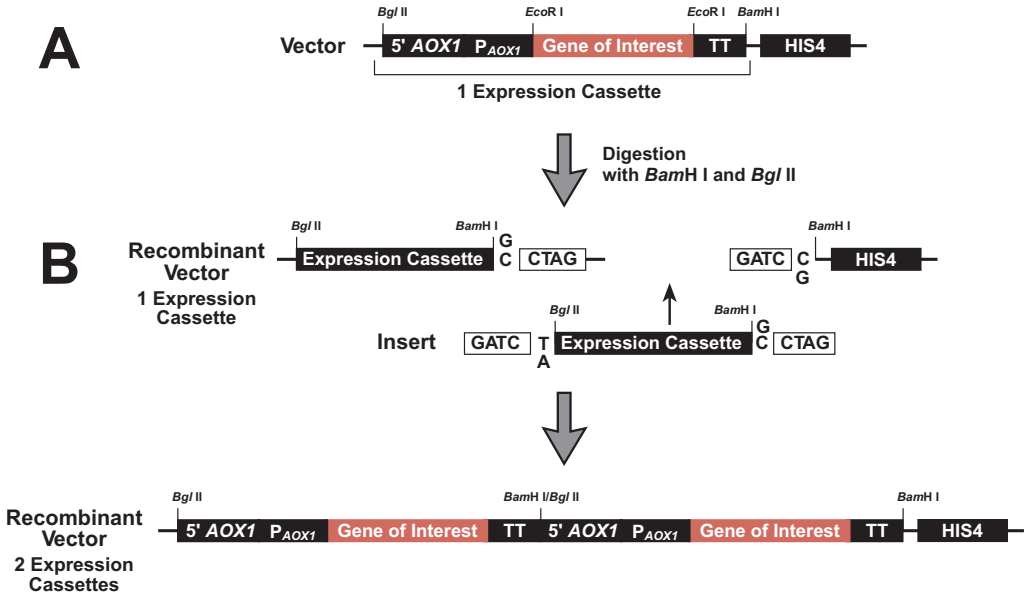
While direct selection of Geneticin® resistant colonies using electroporation is possible (Scorer *et al.*, 1994), secondary selection on Geneticin® results in clones with a greater chance of higher copy number, *i.e.*, secondary selection yields some clones with 5 to 9 copies; direct selection after electroporation yields clones with an average of 1 to 3 copies. Direct selection of Geneticin® colonies is not possible with spheroplast transformation.

Generating Multicopy Inserts *in vitro*

The graphic below shows how pAO815 is used to generate multiple expression cassette copies in a single vector prior to transformation into *Pichia*. The gene of interest is inserted into the vector at a unique *Eco*R I site. The resulting expression cassette (the P_{AOX1} plus your gene) is flanked on the upstream side by a unique *Bgl* II site and on the downstream site by a unique *Bam*H I site (see A below).

pAO815 containing the gene of interest is digested with *Bgl* II and *Bam*H I to excise the expression cassette. The cassette is then reinserted at the *Bam*H I site to create a tandem repeat of the cassette. The reinsertion process can be repeated to generate a series of vectors that contain an increasing number of cassettes linked to a single *HIS4* gene (see B below).

Transformation of *Pichia* with these *in vitro*-formed multimers increases the frequency of multicopy expression cassette recombinants. *Pichia* recombinants may be custom-designed to contain a defined number of multicopy inserts. For more information, see page 24.



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Experimental Outline, continued

Transformation and Integration

Two different phenotypic classes of His$^+$ recombinant strains can be generated: Mut$^+$ and Mut^S (see page 2). Transformation of strain GS115 can yield both classes of transformants, His$^+$ Mut$^+$ and His$^+$ Mut^S, depending on where the plasmid DNA is linearized (see below). KM71 yields only His$^+$ Mut^S since the strain itself is Mut^S. Both Mut$^+$ and Mut^S recombinants are useful to have as one phenotype may favor better expression of your protein than the other may. Ideally, you should test between 6–10 recombinants per phenotype. There is no way to predict beforehand which construct or isolate will better express your protein. It is strongly recommended that *Pichia* recombinants be analyzed by PCR to confirm integration of your construct (see page 83).

Once you have successfully cloned your gene downstream of the *AOX1* promoter, you will linearize your plasmid to stimulate recombination when the plasmid is transformed into *Pichia*. The table below describes the types of recombinants you will get by selective digestion of your expression plasmid.

Restriction Enzyme	Integration Event	GS115 Phenotype	KM71 Phenotype
Sal I or *Stu* I	Insertion at *his4*	His$^+$ Mut$^+$	His$^+$ Mut^S
Sac I (cannot be used with multi-copy constructs in pAO815)	Insertion at 5´ *AOX1* region	His$^+$ Mut$^+$	His$^+$ Mut^S
Bgl II	Replacement at *AOX1* locus	His$^+$ Mut^S	His$^+$ Mut^S (not recommended, see page 8)

Expression and Scale-up

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

Choose the *Pichia* recombinant strain that best expresses your protein and optimize induction based on the suggestions on pages 57–58. Once expression is optimized, scale-up your expression protocol to produce more protein.

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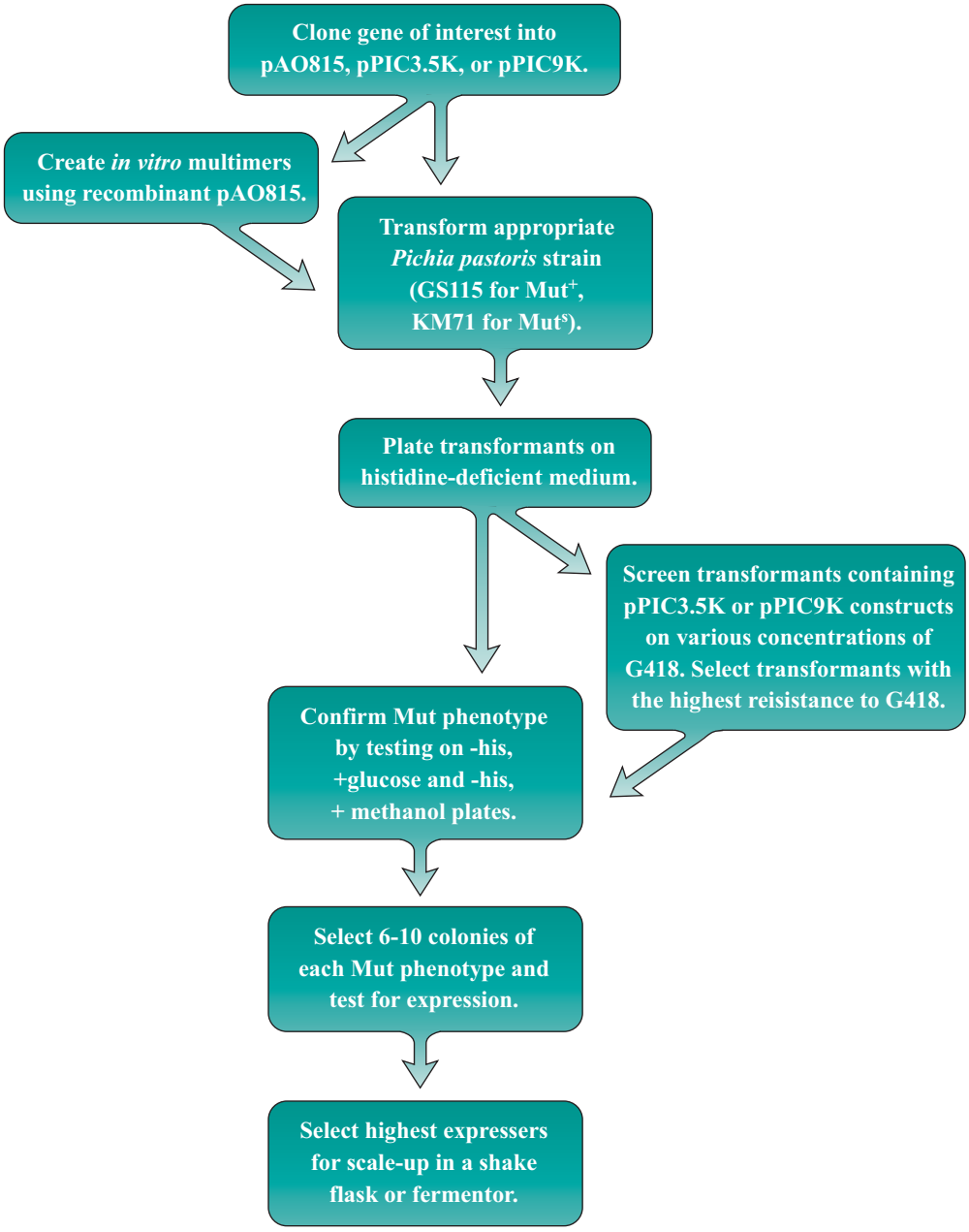
Experimental Outline, continued

Experimental Process

A flow chart is provided below to help you understand the process.



Methods

Pichia Strains

Introduction

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

Genotype of *Pichia* Strain

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

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

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

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

Construction of KM71

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

Important

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

Continued on next page

Pichia Strains, continued

Control Expression Strains

GS115/His$^+$ Mut^S Albumin: This strain is a control for secreted expression and the Mut^S phenotype when screening *Pichia* transformants (page 47). The gene for serum albumin was cloned with its native secretion signal, then integrated into *Pichia* at the *AOX1* locus. This strain secretes albumin (67 kDa) into the medium at levels > 1 gram/liter.

GS115/His$^+$ Mut$^+$ β-galactosidase: This strain is a control for intracellular expression and the Mut$^+$ phenotype when screening *Pichia* transformants (page 47). 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 by visualization on Coomassie-stained SDS-PAGE (see pages 54–56) or assay using ONPG (see page 92–93).

Growth of *Pichia* Strains

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

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

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

Growth on Methanol

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

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

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

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Pichia Strains, continued

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

Storage of *Pichia* Strains

To store cells for weeks to months, use YPD medium or YPD agar slants (see page 57).

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 correct genotype and viability by streaking on MM, MD or MGY plates before using again.

E. coli Strains

Genotype of *E. coli* Strain

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

F´ {*pro*AB, *lac*I^q, *lac*ZΔM15, Tn*10* (Tet^R)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, 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.

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

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

Selecting a *Pichia* Expression Vector

Selecting a Vector

If your protein is cytosolic and non-glycosylated, you may elect to express the protein intracellularly.

If your protein is normally secreted, glycosylated, or directed to an intracellular organelle, you may wish to try secreting your protein. We recommend that you try both the native secretion signal (using pPIC3.5K or pAO815) and the α-factor signal sequence (in pPIC9K) in order to secrete your protein.

We recommend trying *in vivo* and *in vitro* methods to generate or isolate multicopy inserts of your gene. It is difficult to predict beforehand which method will work for your protein. A summary of the advantages and disadvantages of each method is presented in the lists below.

<u>*In vitro*</u> <u>Method</u> (pAO815)

Advantages

- Quantitative--construction of a defined number of multimers
- Most of the His$^+$ transformants will contain the proper, defined number of inserts
- Isolation of recombinants with multiple inserts is easy because most of the His$^+$ transformants will contain multiple copies of your gene
- *In vitro* construction allows step-wise analysis of copy number effects on protein expression
- Multiple inserts are located at a single locus
- No need for a second drug resistance marker in the vector

Disadvantages

- More work up front to clone defined number of multimers
- Size of the vector may become quite large depending on the size of your gene and the number of copies you create
- Rearrangements in *E. coli* may occur

<u>*In vivo*</u> <u>Method</u> (pPIC3.5K and pPIC9K)

Advantages

- Easy to initiate experiment because only one copy of your gene is cloned into the vector before transforming into *Pichia*
- Identifies the 1–10% of spontaneous His$^+$ transformants that have multiple inserts
- Average size of vector is similar to other *Pichia* expression vectors
- Multiple inserts are located at a single locus

Disadvantages

- Qualitative screen–Geneticin® resistance may not necessarily correlate with the number of copies of your gene.
- Screening His$^+$ transformants may involve more work because you will need thousands of His$^+$ transformants to generate enough Geneticin® resistant colonies to test
- The number of multiple inserts is unknown (although this can be determined through Southern or dot blot analysis)
- Screening on Geneticin® is sensitive to the density of the cells and may result in the isolation of false positives

Continued on next page

Selecting a *Pichia* Expression Vector, continued

Features

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

Feature	Description	Benefit
5´ *AOX1*	A ~1000 bp fragment containing the *AOX1* promoter	Allows methanol-inducible high level expression in *Pichia* Targets plasmid integration to the *AOX1* locus.
α-factor signal sequence	269 bp fragment encoding the α-factor signal sequence for secretion in *Pichia* (pPIC9K only)	Allows secretion of desired protein into the medium
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*
*Bam*H I *Bgl* II *Not* I *Sac* I *Sal* I *Stu* I	Unique restriction sites (**Note**: *Stu* I is not unique to pPIC3.5K or pPIC9K.)	Permits linearization of vector for efficient integration into the *Pichia* genome and generation of either Mut$^+$ or Mut^S recombinants
kan	Kanamycin resistance gene from Tn*903* which confers resistance to Geneticin® in *Pichia* and kanamycin resistance in *E. coli* (for pPIC3.5K and pPIC9K)	Allows *in vivo* screening for multicopy inserts by increased resistance to Geneticin® Also allows selection for kanamycin resistance in *E. coli*

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.

pPIC3.5K

Description

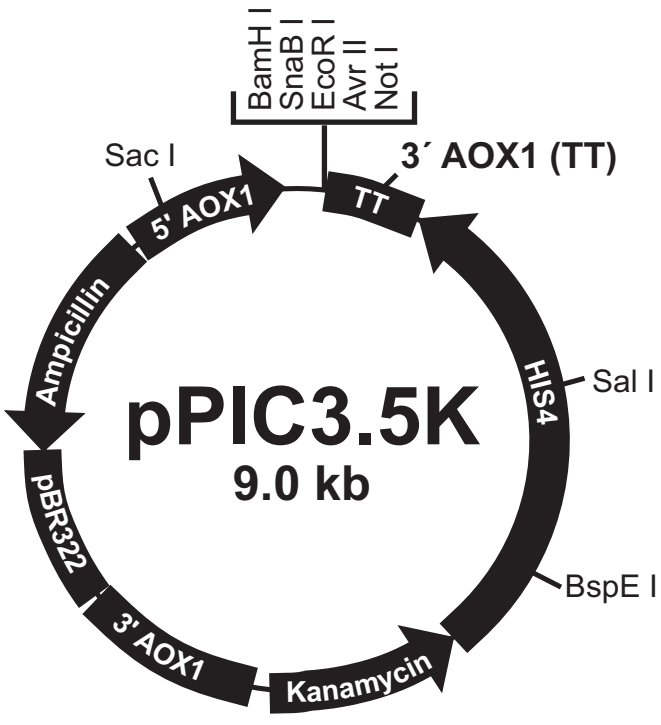
pPIC3.5K is a plasmid designed to allow you to identify *in vivo* multiple integrations of your gene in the *Pichia* genome. Other details about pPIC3.5K are provided below:

- 9004 bp vector
- Five unique restriction sites in the multiple cloning site: *Bam*H I, *Sna*B I, *Eco*R I, *Avr* II, *Not* I
- Intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener & 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 (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)

See page 34 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, or *Sal* I site.

Map of pPIC3.5K

The figure below shows the map of pPIC3.5K. Details of the multiple cloning site are shown on page 20. The sequence of pPIC3.5K is available from our website (www.invitrogen.com) or from Technical Support (page 94).



Comments for pPIC3.5K:
9004 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): bases 981-1314
HIS4 ORF: bases 4242-1708
Kanamycin resistance gene: bases 5471-4656
3´ *AOX1* fragment: bases 5850-6607
pBR322 origin: bases 7689-7016
Ampicillin resistance gene: bases 8694-7834

pPIC9K

Description

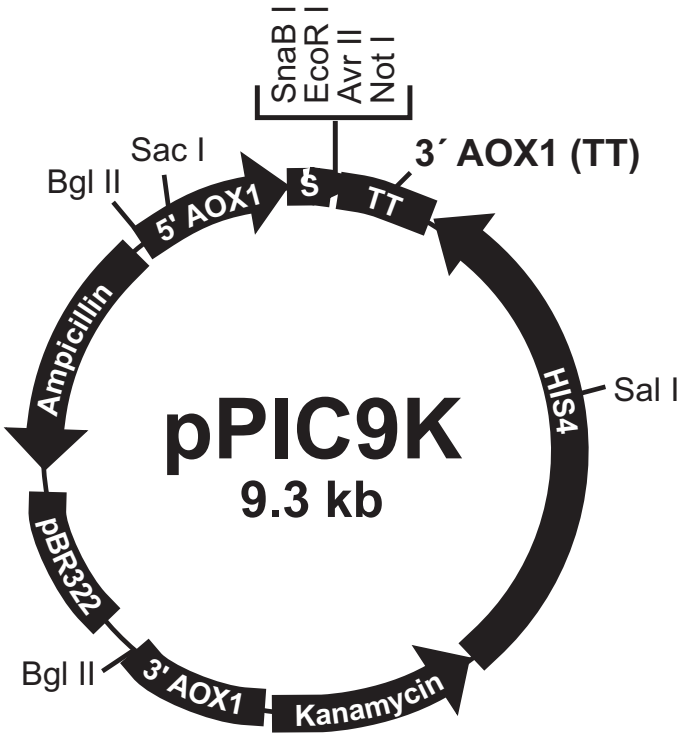
The vector pPIC9K contains the kanamycin resistance gene for *in vivo* screening of multiple copy inserts and secretes recombinant proteins to the medium. pPIC9K is functional in *Pichia* strains GS115 and KM71. Other details are:

- 9276 bp fusion vector
- Four unique restriction sites for cloning in frame with the α-factor secretion signal: *Sna*B I, *Eco*R I, *Avr* II, *Not* I
- 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 (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)

See page 34 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, or *Sal* I site.

Map of pPIC9K

The figure below shows the map of pPIC9K. Details of the multiple cloning site and the α-factor secretion signal are shown on page 21. The sequence of pPIC9K is available on our website (www.invitrogen.com) or from Technical Support (page 94).



Comments for pPIC9K:
9276 nucleotides

5´ *AOX1* promoter fragment: bases 1-948
5´ *AOX1* primer site: bases 855-875
α-Factor secretion signal(s): bases 949-1218
α-Factor primer site: bases 1152-1172
Multiple Cloning Site: bases 1216-1241
3´ *AOX1* primer site: bases 1327-1347
3´ *AOX1* transcription termination (TT): bases 1253-1586
HIS4 ORF: bases 4514-1980
Kanamycin resistance gene: bases 5743-4928
3´ *AOX1* fragment: bases 6122-6879
pBR322 origin: bases 7961-7288
Ampicillin resistance gene: bases 8966-8106

pAO815

Description

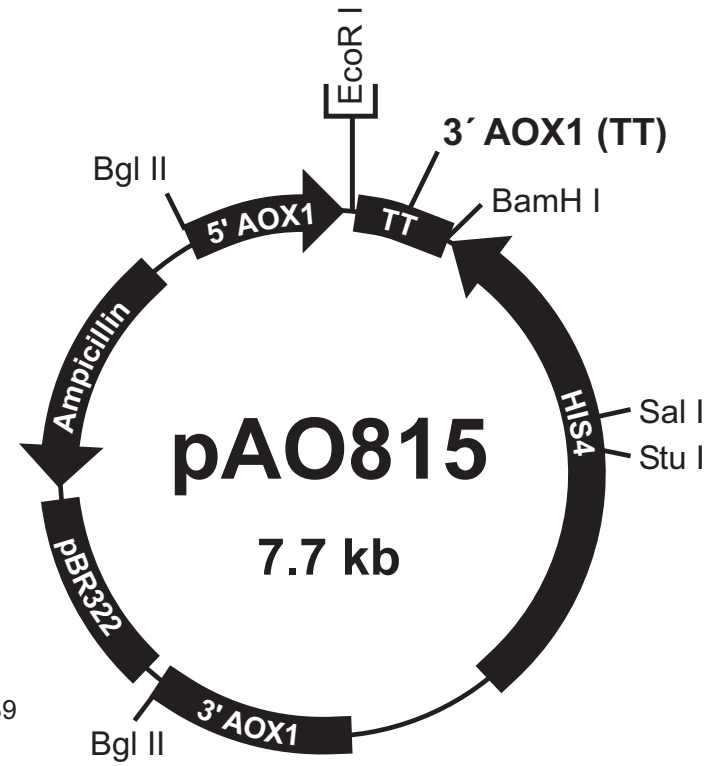
pAO815 is a plasmid designed for *in vitro* generation of multimers of your gene for integration into the *Pichia* genome. Other details about pAO815 are provided below:

- 7709 bp vector
- One unique restriction site: *Eco*R I
- Intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener & Stuart, 1991; Kozak, 1987; Kozak, 1990)
- *HIS4* selection in *Pichia*
- 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)

See page 34 for alternate restriction sites if your insert DNA has a *Bgl* II, *Stu* I, or *Sal* I site.

Map of pAO815

The figure below shows the map of pAO815. Details of the multiple cloning site are shown on page 22. The sequence of pAO815 is available on our website (www.invitrogen.com) or from Technical Support (page 94).



Comments for pAO815:
7709 nucleotides

5´ *AOX1* promoter fragment: bases 1-940
5´ *AOX1* primer site: bases 855-875
*Eco*R I Site: bases 943-948
3´ *AOX1* primer site: bases 1024-1044
3´ *AOX1* transcription termination (TT): bases 950-1277
HIS4 ORF: bases 4199-1665
3´ *AOX1* fragment: bases 4554-5310
pBR322 origin: bases 6394-5740
Ampicillin resistance gene: bases 7399-6539

Cloning into the *Pichia* Multi-Copy Expression Vectors

Introduction

Below are some guidelines to consider when developing a cloning strategy for these vectors. The multiple cloning sites for each vector are presented on the following pages for your convenience. If you are using pPIC9K, it is important to clone your gene in frame with the α-factor signal sequence.

We recommend that you transform the three supercoiled *Pichia* expression vectors into *E. coli*, so that you have a permanent stock.

- Dilute 1 µl of each plasmid to 10–100 pg/µl using sterile water or TE buffer.
- Transform competent *E. coli* with 1–2 µl of the diluted plasmid and select on LB with 50–100 µg/ml ampicillin (LB-Amp).

General Considerations

The following are some general considerations applicable to pAO815, pPIC3.5K, and pPIC9K.

- The codon usage in *Pichia* is believed to be the same as *Saccharomyces cerevisiae* and many *Saccharomyces* genes have proven to be cross-functional in *Pichia*.
- Plasmid constructions should be maintained in a *rec*A, *end*A mutant *E. coli* strain such as TOP10F´.
- The native 5´ end of the *AOX1* mRNA is noted in each multiple cloning site. This is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.
- 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 because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff & Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret & Sherman, 1984). If you have problems expressing your gene, check for premature termination and AT rich regions. It may be necessary to change the sequence in order to express your gene (Scorer *et al.*, 1993).
- The predicted protease cleavage sites for the α-factor signal sequence in pPIC9K are indicated in the figure (page 21).
- You must clone the open reading frame (ORF) of the mature gene of interest in frame and downstream of the α-factor signal sequence in pPIC9K.

Continued on next page

Cloning into the *Pichia* Multi-Copy Expression Vectors, continued

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.

   b) Ligation of the fragment with the same restriction end on both ends into a single, compatible site.

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

3. Direct cloning of an amplified fragment containing the gene of interest via the TA Cloning® Kit (see page viii), followed by subcloning of a compatible fragment into the vector of choice.

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.

Note

If your insert has an *Eco*R I site and you are trying to clone into the *Eco*R I site of pAO815, we recommend the following:

1. An enzyme like *Bsa* I has the following restriction recognition site:

   5´-GGTCTCN˅
   3´-CCAGAGNNNNN^

2. An *Eco*R I site may be engineered into the recognition site for *Bsa* I.

   5´-GGTCTCG˅AATTC.....
   3´-CCAGAGCTTAA^G.....

3. You may add this sequence to your DNA fragment by integrating it into your PCR primer or create *in vitro* as an adaptor to another restriction site.

4. Digest your PCR or adapted ligation product with *Bsa* I. This will generate *Eco*R I overhangs on both ends of your fragment without digesting with *Eco*R I.

   5´- AATTC.....
   3´-G.....

5. Ligate into dephosphorylated pAO815. Other enzymes that may be used are *Bsm*A I or *Bsm*B I.

Continued on next page

Cloning into the *Pichia* Multi-Copy Expression Vectors, continued

Signal Sequence Processing

The processing of the α-factor mating signal sequence in pPIC9K occurs in two steps:

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

Optimizing Signal Cleavage

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

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

Bacterial Transformation

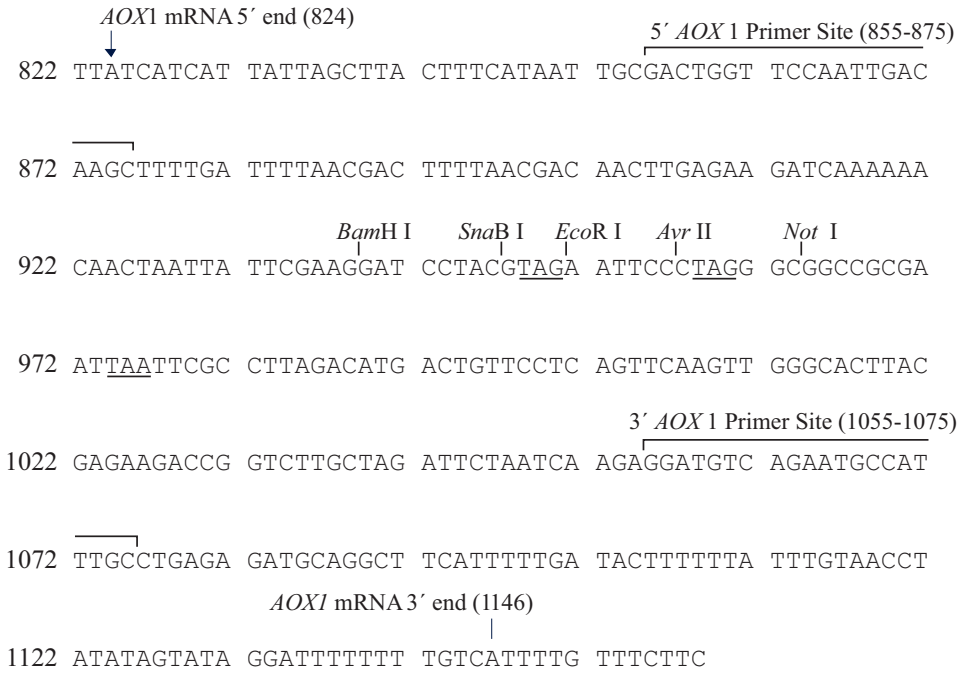
Once you have decided on a cloning strategy, you will need to prepare competent *E. coli* cells for transformation before setting up your ligation reactions. See *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989) for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

Continued on next page

Cloning into the *Pichia* Multi-Copy Expression Vectors, continued

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

The sequence below shows the detail of the multiple cloning site and surrounding sequences. Potential stop codons are underlined.



Special Considerations

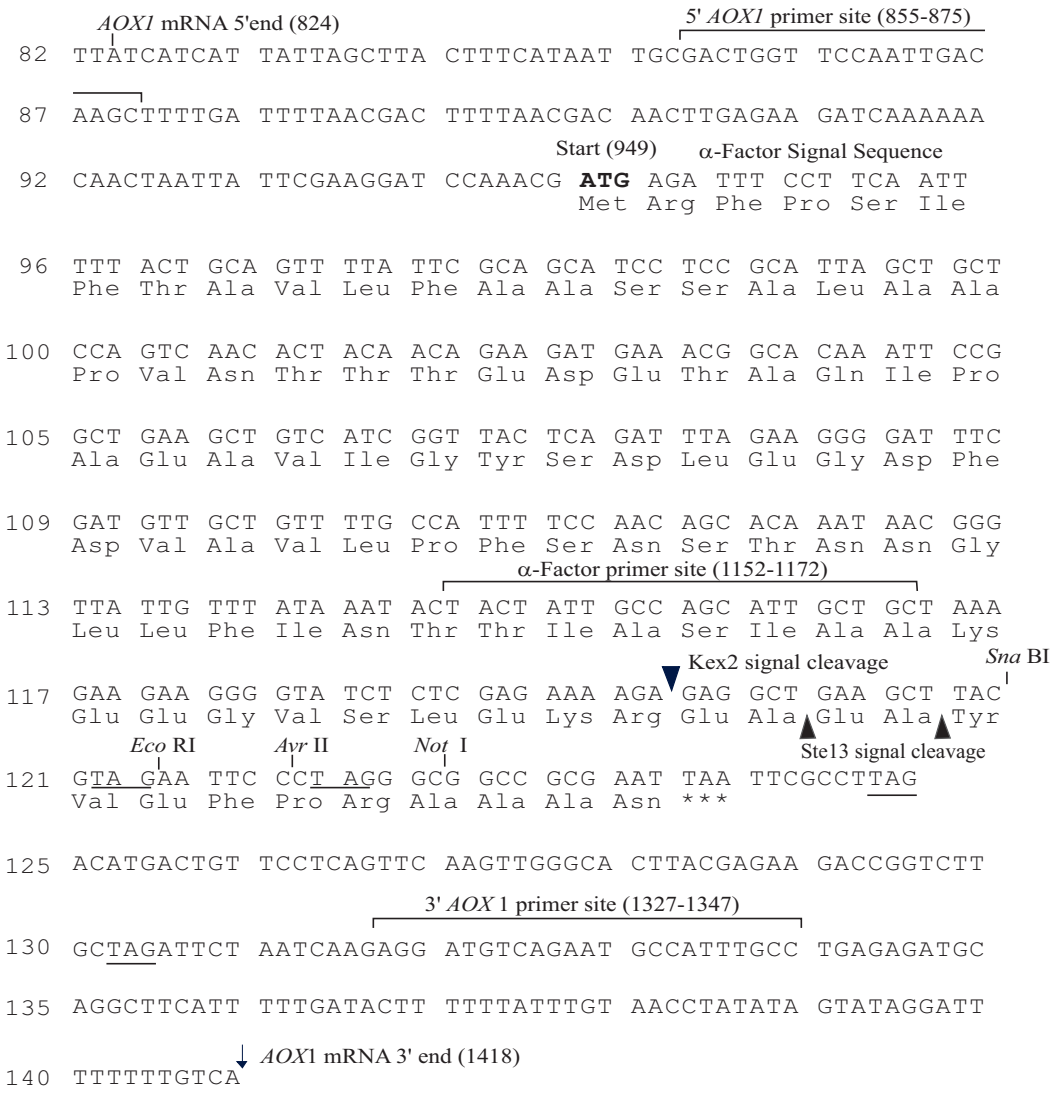
- For pPIC3.5K, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC ATG G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener & Stuart, 1991; Kozak, 1987; Kozak, 1990).
- Be sure to analyze the 5′ untranslated region of the mRNA for secondary structure formation. Secondary structure in the mRNA may have a negative effect on expression of the recombinant protein.
- 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 be digested properly.

Continued on next page

Cloning into the *Pichia* Multi-Copy Expression Vectors, continued

P_{AOX1} and Multiple Cloning Site of pPIC9K

The sequence below shows the detail of the multiple cloning site and surrounding sequences. Potential stop codons are shown underlined.



Special Considerations

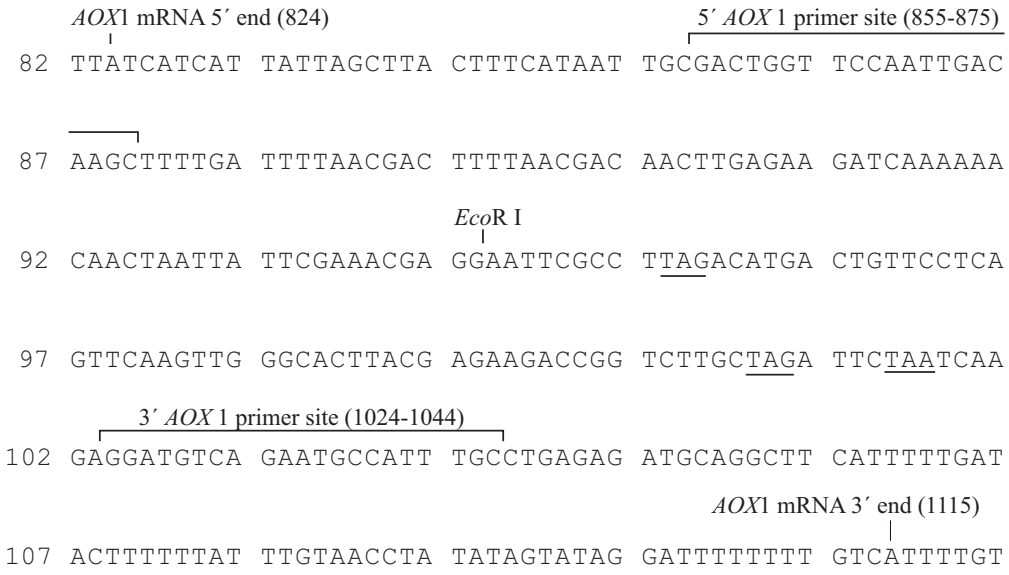
- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5´ end of the mRNA.
- If your insert has a *Bgl* II site, see page 34 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

Continued on next page

Cloning into the *Pichia* Multi-Copy Expression Vectors, continued

P_{AOX1} and Multiple Cloning Site of pAO815

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



Special Considerations

- For *in vitro* multimerization, you need to analyze your insert for *Bam*H I and *Bgl* II restriction sites. If your insert has a *Bam*H I or *Bgl* II site, we recommend that you use the *in vivo* method (pPIC3.5K) to isolate multiple inserts of your gene.
- For pAO815, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC ATG G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener & Stuart, 1991; Kozak, 1987; Kozak, 1990).
- Be sure to analyze the 5′ untranslated region of the mRNA for secondary structure formation. Secondary structure in the mRNA has a negative effect on expression of the recombinant protein.

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, see *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989).

Analysis of Transformants

1. After transformation, plate the transformation mix onto LB plates with 50–100 µg/ml ampicillin (see **Appendix**, page 64) 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 pAO815 or pPIC3.5K, use the 5´ *AOX1* and the 3´ *AOX1* Sequencing primers. To sequence pPIC9K, use the α-Factor primer and the 3´ AOX1 Sequencing primers. Resuspend the primers in 20 µl sterile water to prepare a 0.1 µg/µl solution.
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. Once your construct is confirmed by sequencing, proceed to **Preparing Transforming DNA**, page 32.

Sequencing Recombinant Clones

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

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

Use the primers mentioned above to sequence your constructs. For the location of the priming sites, see pages 20–22.

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

After Cloning Your Gene

After you have cloned your gene into pAO815, you are ready to construct *in vitro* multimers using the protocol on the next page.

If you cloned your gene into pPIC3.5K or pPIC9K, you are ready to prepare plasmid DNA for transformation into *Pichia*. Refer to page 32 for more information.

In Vitro Multimerization Protocol

Introduction

At this point you have your gene cloned into the *Eco*R I site of pAO815 (recombinant pAO815). To create *in vitro* multimers, you will first generate a *Bgl* II-*Bam*H I expression cassette consisting of the *AOX1* promoter and your gene. Second, you will linearize the vector using *Bam*H I to allow cloning of multiple copies of the *Bgl* II-*Bam*H I expression cassette. Note that the linearized vector already contains one copy of your expression cassette. To generate multiple copies of your expression cassette, follow the general steps below. Details are provided on pages 25–31.

1. Treat your *Bgl* II-*Bam*H I expression cassette with ligase *in vitro*. Note that *Bgl* II and *Bam*H I share 4 bases in common between their recognition sites.
2. Generate head-to-tail, head-to-head, and tail-to-tail multimers (Head-to-tail ligation, which is the correct orientation for expression, will destroy both the *Bam*H I and *Bgl* II sites).
3. Treat the ligation mix with *Bam*H I and *Bgl* II to eliminate head-to-head and tail-to-tail multimers.
4. Ligate into *Bam*H I-linearized recombinant pAO815.
5. Transform into *E. coli* and analyze recombinant plasmids for copy number by digesting with *Bgl* II and *Bam*H I.

Alternative Procedure

You may wish to build each desired multimer in increments by ligating each additional expression cassette one (or two) at a time into pAO815. For example:

1 Digest pAO815 with one copy of your gene using *Bam*H I.

2 Ligate a single copy of the *Bgl* II-*Bam*H I expression cassette into the vector.

3 Transform *E. coli* and analyze the transformants for the vector with 2 copies of your insert.

4 Isolate and digest this vector (with 2 copies of your gene) with *Bam*H I and *Bgl* II to isolate a cassette with 2 copies of your gene (optional).

5 Digest the vector with 2 copies of your gene with *Bam*H I and ligate 1 or 2 copies (see Step 4) of the expression cassette into the vector.

6 Transform *E. coli* and analyze the transformants for the vector with 3 or 4 copies of your insert.

7 Repeat until the desired multimer is reached.

Continued on next page

In Vitro Multimerization Protocol, continued

Before Starting

You will need the following materials:

- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation. You will need 3–4 tubes of competent cells per experiment. Use your favorite protocol or refer *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989) for a protocol to prepare competent *E. coli*
- *Eco*R I, *Bam*H I, and *Bgl* II restriction enzymes and appropriate buffers
- Low-melt agarose
- Glass milk
- Sterile water
- CIP (calf intestinal alkaline phosphatase, 1 unit/µl)
- 10X CIP Buffer
- Phenol/chloroform
- 3M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/µl)
- 10X Ligation Buffer (with ATP)
- LB-Amp plates (50–100 µg/ml ampicillin)
- 16°C, 37°C, and 65°C water baths or temperature block

Controls

To evaluate your transformants and expression data later on, we recommend transforming *Pichia* with pAO815 (the parent vector) and pAO815 containing one copy of your expression gene. This allows you to compare expression levels to see if multiple copies significantly increase the amount of protein produced. Also, if you elect to determine how many copies of your gene are in a recombinant by dot or Southern blot, the strain with the parent vector will control for background hybridization and the strain with the single copy gene will provide a signal to normalize your data.

Continued on next page

In Vitro Multimerization Protocol, continued

Digesting Recombinant pAO815

Set up two separate digestions of recombinant pAO815 containing one copy of your gene:

1. Double digest 1–2 µg recombinant pAO815 with 10 units each of *Bgl* II and *Bam*H I. Use a 20 µl reaction volume and digest for 1–2 hours at 37°C to release your expression cassette. Proceed to **Producing of Expression Cassettes for Multimerization**, below.
2. Digest 2 µg recombinant pAO815 with 10 units of *Bam*H I only. Use a 20 µl reaction volume and digest for 1–2 hours at 37°C to linearize recombinant pAO815. Proceed to **Dephosphorylation of Vector**, below.

Producing Expression Cassettes for Multimerization

1. Load all of the *Bgl* II/*Bam*H I digestion on a 1% low melt agarose gel and electrophorese to separate fragments. Stain the gel with ethidium bromide.
2. Cut out the band containing the expression cassette and place into a microcentrifuge tube. Note that the size of the fragment depends on the size of your gene. The vector backbone will be digested into 2 fragments:
   - 4.0 kb *Bam*H I–*Bgl* II fragment (contains *HIS4* and 3´ *AOX1* sequences)
   - 2.4 *Bgl* II fragment (contains the pBR322 origin and ampicillin resistance marker).
3. Isolate DNA from the agarose using the method of choice.
4. Ethanol precipitate the DNA with 1/10 volume 3 M sodium acetate and 2 volumes 100% ethanol.
5. Resuspend the *Bgl* II–*Bam*H I digested expression cassette in 15 µl sterile water. Store on ice if proceeding immediately to **Ligation and Digestion of Expression Cassette**, next page. Store at –20°C for long-term storage.

Dephosphorylation of Vector

Dephosphorylation is necessary to prevent self-ligation of the vector.

1. Take your digest from **Digestion of Recombinant pAO815**, Step 2, above, and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 µl of sterile water.
2. Set up the dephosphorylation reaction in a microcentrifuge tube as follows:

*Bam*H I digested recombinant pAO815	17 µl
10X CIP Buffer	2 µl
CIP (1 Unit/µl)	1 µl
Total volume	20 µl

3. Incubate at 37°C for 15 minutes.
4. Add 30 µl of sterile water to the reaction to make a final volume of 50 µl.
5. Add 50 µl of phenol/chloroform and extract your DNA solution. Transfer the aqueous solution to a new tube.
6. Precipitate the DNA by adding 5 µl of 3 M sodium acetate and 110 µl 100% ethanol. Incubate on ice for 30 minutes.
7. Resuspend pellet in 8 µl sterile water. Save on ice if you plan to ligate your insert immediately (see **Ligation and Digestion of Expression Cassette**, next page) or store at –20°C.

Continued on next page

In Vitro Multimerization Protocol, continued

Ligation and Digestion of Expression Cassette

Ligation of the expression cassette generates head-to-tail, head-to-head, and tail-to-tail multimers. Creation of head-to-tail multimers will be in the correct orientation for expression and will destroy both the *Bam*H I and *Bgl* II sites between the expression cassettes. Digestion of the multimers with *Bam*H I and *Bgl* II will eliminate those multimers with tail-to-tail and head-to-head orientation. After digestion with these two restriction enzymes, you will have a mixture of multimers containing 1, 2, 3, etc. copies of your gene that can be ligated into *Bam*H I-linearized, recombinant pAO815.

1. Set up a 20 µl ligation reactions as follows:

Bgl II–*Bam*H I digested expression cassette	15 µl
Sterile water	2 µl
10X Ligation Buffer (with ATP)	2 µl
T4 DNA Ligase (2.5 units/µl)	1 µl

2. Incubate at 16°C for 2.5 hours.
3. Heat inactivate the ligase by incubating at 65°C for 20 minutes.
4. Add the following reagents for restriction enzyme digestion (cut-back). Note that *Bam*H I and *Bgl* II may be used with the same restriction buffer:

Sterile water	23 µl
10X restriction enzyme buffer	5 µl
Bgl II (10 units/ml)	1 µl
*Bam*H I (10 units/ml)	1 µl

5. Incubate the reaction at 37°C for 2 hours.
6. Add 50 µl of phenol/chloroform and extract the restriction enzyme digestion to remove the enzymes. Transfer the aqueous solution to a new microcentrifuge tube.
7. Ethanol precipitate the DNA using 5 µl 3 M sodium acetate and 110 µl 100% ethanol.
8. Resuspend pellet in 4 µl sterile water. Save on ice if you plan to ligate your insert immediately or you can store at –20°C. Proceed to **Ligating Multimers into Linearized Vector,** next page.

Continued on next page

In Vitro Multimerization Protocol, continued

Note

You may wish to combine the ligation reaction with the restriction enzyme digestion. T4 ligase will retain most of its activity in all of the four New England BioLabs buffers. Remember to add 1 mM ATP to the reaction in order to ensure ligase activity.

Ligating Multimers into Linearized Vector

You are now ready to ligate the mixture of multimers generated in Step 10, page 27, into dephosphorylated, linearized vector.

1. Set up the following ligation reactions:

Dephosphorylated vector (page 26, Step 7)	4 µl
Expression cassette multimers (page 27, Step 8,)	4 µl
10X Ligation Buffer	1 µl
T4 DNA Ligase (2.5 units/µl)	1 µl
Total volume	10 µl

For the vector only control:

Dephosphorylated vector	4 µl
Sterile water	4 µl
10X Ligation Buffer	1 µl
T4 DNA Ligase (2.5 units/µl)	1 µl
Total volume	10 µl

2. Incubate overnight at 16°C.
3. You may store the ligation reactions at –20°C until ready to use, or transform 1 to 10 µl of each ligation mix into competent *E. coli*. Note that too much of the ligation mixture may cause arcing when transforming electrocompetent cells.

Transformation into *E. coli*

Remember to include the "vector only" and "cells only" controls to evaluate your experiment. The "vector only" control will indicate whether your vector was dephosphorylated. Since the CIP reaction is not 100% and because you often get degradation of the ends, there might be a few colonies on this plate. The "cells only" plate should have no colonies at all.

1. Transform competent *E. coli* by your method of choice.
2. After adding medium to the transformed cells and allowing them to recover, plate 10 µl and 100 µl of each transformation mix onto LB plates with 50–100 µg/ml ampicillin. Save the remainder of your transformation mix at 4°C.
3. Incubate overnight at 37°C. If you do not get transformants or very few transformants, plate out the remainder of the transformation mix onto LB-ampicillin plates.

Continued on next page

In Vitro Multimerization Protocol, continued

Analyzing Transformants

1. Pick 20 transformants and inoculate 2 ml LB containing 50–100 µg/ml ampicillin. Grow overnight at 37°C.
2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pAO815.

   (Be sure to include *Bgl* II–*Bam*H I digested pAO815 as a control. It is possible to get vector rearrangements and deletions with large vectors in *E. coli*. Including *Bgl* II–*Bam*H I digested pAO815 will allow you to detect these rearrangements-deletions in the vector backbone.)
3. Analyze your digests on a 1% agarose gel. You should see bands corresponding to 1 copy, 2 copies, 3 copies, etc. of your expression cassette, plus the vector backbone.

   (The number of copies you obtain may depend on how well a large vector is tolerated by the *E. coli* host strain.)
4. Once you have identified plasmids with multiple copies of your expression cassette, be sure to purify the original colony by streaking for single colonies and confirming your construct. If you have difficulties, see the next page.
5. Prepare frozen glycerol stocks of *E. coli* containing each of your multimeric constructs. Prepare 5–10 µg of each plasmid for transformation into *Pichia*. Proceed to **Preparing Transforming DNA,** page 32.

Continued on next page

In Vitro Multimerization Protocol, continued

Trouble-shooting

The table below will help you optimize formation and isolation of multimers in *Pichia*.

Problem	Possible Reason	Solution
No multimers or low number of multimers in your vector after transformation into *E. coli*	CIP defective	Use fresh CIP
		Add more CIP. Add 1 unit of CIP and incubate 15 more minutes at 37°C. This is somewhat risky as CIP can degrade the ends of your DNA.
	Not enough insert DNA to ligate	Digest more pAO815 containing 1 copy of your expression cassette.
	Construct is unstable in *E. coli*	Use the *in vivo* method to isolate multimers (see page 4).
	Multimers are too long to ligate efficiently	Try ligating each expression cassette separately.
Recombinant vector rearranges and deletions are detected	Construct is unstable in *E. coli*	Use the *in vivo* method to isolate multimers (see page 4).
Pichia His$^+$ transformants do not have multimers	Vector was linearized with the wrong enzyme (Restriction enzymes in the 5´ *AOX1* region are duplicated when multimers are created)	Linearize your construct with *Sal* I or *Stu* I to insert the construct into *his4*.
		Analyze your construct for other unique restriction sites in the vector backbone that are near the 5´ *AOX1* region or the 3´ *AOX1* region. These sites will preserve your multimers and allow recombination with *AOX1*.

Continued on next page

In Vitro Multimerization Protocol, continued

For More Information

There are a number references in the literature you can consult to optimize synthesis of *in vitro* multimers. A partial list is provided below:

Cohen, B. and Carmichael, G. G. (1986) A Method for Constructing Multiple Tandem Repeats of Specific DNA Fragments. *DNA* **5**: 339–343.

Eisenberg, S., Francesconi, S. C., Civalier, C. and Walker, S. S. (1990) Purification of DNA-Binding Proteins by Site-specific DNA Affinity Chromatography. *Methods Enzymol.* **182**: 521–529.

Graham, G. J. and Maio, J. J. (1992) A Rapid and Reliable Method to Create Tandem Arrays of Short DNA Sequences. *BioTechniques* **13**: 780–789.

Rudert, W. A. and Trucco, M. (1990) DNA Polymers of Protein Binding Sequences Generated by Polymerase Chain Reaction. *Nucleic Acids Res.* **18**: 6460.

Simpson, R. T., Thoma, F. and Brubaker, J. M. (1985) Chromatin Reconstituted from Tandemly-repeated Cloned DNA Fragments and Core Histones: A Model System for the Study of Higher-order Structure. *Cell* **42**: 799–808.

Takeshita, S., Tezuka, K.- i., Takahashi, M., Honkawa, H., Matsuo, A., Matsuishi, T. and Hashimoto-Gotoh, T. (1988) Tandem Gene Amplification *in vitro* for Rapid and Efficient Expression in Animal Cells. *Gene* **71**: 9–18.

Taylor, W. H. and Hagerman, P. J. (1987) A General Method for Cloning DNA Fragments in Multiple Copies. *Gene* **53**: 139–144.

Preparing Transforming DNA

Introduction

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

Step	Action	Pages
1	Prepare your DNA for transformation	32–35
2	Grow either GS115 or KM71 and prepare spheroplasts	36–39
3	Transform GS115 or KM71 with your DNA	40–41
4	Select His$^+$ transformants	41
5	If you cloned your gene of interest into pPIC3.5K or pPIC9K, you will screen your His$^+$ transformants for Geneticin® resistance	42–46
6	Confirm the Mut$^+$ Mut^S phenotype of your recombinant strain	47–49
7	Confirm that your gene is present using PCR (optional)	83–84
8	Test for expression of your gene	50–56

We recommend isolating both His$^+$ Mut$^+$ and His$^+$ Mut^S *Pichia* transformants as it is difficult to predict beforehand what construct will best express your protein (see pages 72 and 73 for a partial list of proteins expressed in *Pichia*). By linearizing your construct DNA in the 5´ *AOX1* region or in the *HIS4* gene and using GS115 (Mut$^+$) and KM71 (Mut^S), you can easily isolate Mut$^+$ and Mut^S recombinants. Plan on using ~10 µg digested DNA for each transformation.

Preparing Plasmid DNA

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

Continued on next page

Preparing Transforming DNA, continued

Linearizing Plasmid DNA

We recommend that you linearize your vector in such a manner to generate both Mut$^+$ and Mut^S recombinants. It is possible that one phenotype will express your multicopy integrant better than the other will. Use strain KM71 if you only want Mut^S recombinants. 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*). If your insert contains any of the restriction sites listed below, see page 34 for alternate sites.

1. If you cloned your insert into pPIC3.5K, linearize with:
   - *Sac* I for insertion at *AOX1* (GS115, Mut$^+$ or KM71, Mut^S)
   - *Sal* I for insertion at *HIS4* (GS115, Mut$^+$ or KM71, Mut^S)
2. If you cloned your insert into pAO815, linearize with:
   - *Sal* I or *Stu* I for insertion at *HIS4* (GS115, Mut$^+$ or KM71, Mut^S)

   Note that multiple *Sac* I sites are formed if there are 2 or more multimers in pAO815.
3. If you cloned your insert into pPIC9K, linearize with:
   - *Sac* I for insertion at *AOX1* (GS115, Mut$^+$ or KM71, Mut^S)
   - *Sal* I for insertion at *HIS4* (GS115, Mut$^+$ or KM71, Mut^S)

Procedure

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

Continued on next page

Preparing Transforming DNA, continued

Alternate Restriction Sites

The table below describes alternate restriction sites for linearizing your construct before transformation into *Pichia*.

pPIC3.5K. Note that an additional *Stu* I site was added with the inclusion of the *kan* gene, so that the *Stu* I site in *HIS4* is no longer unique.

Restriction Enzyme	5´ *AOX1*	3´ *AOX1*	Vector backbone	*HIS4* gene
Sac I	209	--	--	--
Pme I	414	--	--	--
Bpu 1102 I	589	--	--	--
Xcm I	699	--	--	--
Bgl II†	2	6616	--	--
Dra I†	414	6454	6596, 7787, 7806	--
Sal I	--	--	--	2919
*Bsp*E I	--	--	--	3580

pAO815. Note that if more than one expression cassette is created in pAO815, the unique sites in the 5´ *AOX1* region are now duplicated and no longer unique.

Restriction Enzyme	5´ *AOX1*	3´ *AOX1*	Vector backbone	*HIS4* gene
Bgl II†	2	5307	--	--
Sal I	--	--	--	2863
Stu I	--	--	--	2948
*Bsp*E I	--	--	--	3580

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

pPIC9K. Note that an additional *Stu* I site was added with the inclusion of the *kan* gene, eliminating the unique *Stu* I site in *HIS4*.

Restriction Enzyme	5´ *AOX1*	3´ *AOX1*	Vector backbone	*HIS4* gene
Sac I	209	--	--	--
Pme I	414	--	--	--
Bpu 1102 I	589	--	--	--
Xcm I	699	--	--	--
Bgl II†	2	6875	--	--
Dra I†	414	6713	6855, 8046, 8065, 8757	--
Sal I	--	--	--	3178
*Bsp*E I	--	--	--	3845

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

Continued on next page

Preparing Transforming DNA, continued

Controls

We recommend that you include the following controls when transforming *Pichia*.

- The parent vector linearized in the same manner as your construct. Use this as a control to confirm integration via PCR (page 83) and as control for background for the expression analysis and the quantitative dot blots or Southern analysis.
- pPIC3.5K, pAO815, or pPIC9K containing one copy of your expression cassette. Be sure to linearize pAO815 in the same manner as your multimer. Most of the His^+ transformants created by transforming with recombinant pPIC3.5K or pPIC9K will only have one copy. Make sure that the transformant you pick is **only** resistant to 0.25 mg/ml Geneticin®. The single copy controls created using pPIC3.5K, pAO815, and pPIC9K should have the same Mut phenotype as the putative multimeric recombinants you are testing. Use these recombinants as a control to compare expression levels with multiple copies of your expression cassette and as a single copy control for quantitative dot blot or Southern analysis. This is a very important control as increasing the copy number of the desired gene does not always lead to increased expression of recombinant protein.

Growth of *Pichia* for Spheroplasting

Introduction

In general, spheroplasting and electroporation (page 78) provide the highest efficiency of transformation for most researchers (10^3 to 10^4 transformants per µg DNA). *Pichia* can also be transformed using PEG 1000 (page 79) or lithium chloride (page 81). These two protocols, particularly lithium chloride, do not perform as well as spheroplasting or electroporation. If you do not have an electroporation device, we recommend spheroplasting or using the PEG 1000 method. Transformation in *Pichia* is less efficient than for *Saccharomyces*. For references on general yeast transformation, see (Cregg *et al.*, 1985; Hinnen *et al.*, 1978).

Explanation of Spheroplasting

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

Preparing Media

Prepare the following media several days in advance and store at 4°C (see **Appendix**, pages 66–69 for details):

YPD (Yeast extract Peptone Dextrose) medium, 1 liter
YPD plates, 1 liter
RDB (Regeneration Dextrose Base) plates, 1 liter
RDHB (Regeneration Dextrose Histidine Base) plates, 1 liter

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

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

Solutions

Spheroplasting and Transformation Reagents

Provided:

1 M Sorbitol

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

Prepared fresh for each transformation:

SED:	19 ml of SE and 1 ml of 1 M DTT (see page 38)
PEG/CaT:	1:1 mixture of 40% PEG and CaT (see page 40)

Continued on next page

Growth of *Pichia* for Spheroplasting, continued

Procedure

1. Streak GS115 or KM71 onto a YPD plate such that isolated, single colonies will grow. Incubate the plate at 28–30°C for 2 days.
2. Inoculate 10 ml of YPD in a 50 ml conical tube or 100 ml shake flask with a single colony of GS115 or KM71 from the YPD plate and grow overnight at 28–30°C with vigorous shaking (250–300 rpm). You may store this culture at 4°C for several days.
3. Place 200 ml of YPD in each of three 500 ml culture flasks. Inoculate the flasks with 5, 10, and 20 µl of cells from the culture made in Step 2 and incubate them overnight with vigorous shaking (250–300 rpm) at 28–30°C.
4. The next morning, bring the transformation solutions (SE, SCE, Sterile Water, SOS, PEG, CaS, CaT, 1 M sorbitol) provided in the kit, the RDB plates (for plating transformants), and the RDHB plates (for viability control) to room temperature.
5. Check the OD_{600} of each of the three culture flasks.
6. Harvest the cells from the culture that has an OD_{600} between 0.2 and 0.3. Centrifuge the cells at room temperature for 5–10 minutes at 1,500 × g. Decant the supernatant and discard the other cultures. Proceed to **Preparing Spheroplasts**, page 38.

   Note: If the cultures are all over 0.3, choose one of the cultures and dilute (1:4) with fresh medium and incubate at 28–30°C until the OD_{600} is between 0.2 and 0.3 (2–4 hours). Harvest the cells and proceed as in Step 6, above.

Preparing Spheroplasts

Before Starting

You should have a cell pellet from Step 6, page 37.

- Prepare 100 ml of molten RD agarose and keep at 45°C (see **Appendix**, page 69)
- Thaw one tube of 1 M DTT (provided in the kit)
- Prepare fresh SED for one batch of spheroplasts as follows:

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

Note

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

Washing the Cells

1. Wash the cells from Step 6, page 37 by resuspending the pellet in 20 ml of sterile water (provided). Resuspend the pellet by swirling the tube. Transfer to a sterile, 50 ml conical tube.
2. Pellet the cells by centrifugation at 1,500 × g for 5 minutes at room temperature. Decant and discard the supernatant. The cell pellet will be used to prepare spheroplasts.
3. Wash the cell pellet once by resuspending in 20 ml of fresh SED, prepared above and centrifuge at 1,500 × g for 5 minutes at room temperature.
4. Wash the cells once with 20 ml of 1 M sorbitol and centrifuge as described in Step 2.
5. Resuspend the cells by swirling in 20 ml of SCE buffer and divide the suspension into two 50 ml conical tubes (~10 ml each).
6. Remove one tube of Zymolyase from –20°C and place it on ice. Mix well by flicking the tube several times. Zymolyase is provided as a slurry and does not go into solution. It is important to mix the slurry thoroughly before each use to ensure addition of a consistent amount of Zymolyase.

Adding Zymolyase

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

Zymolyase digests the cell wall and makes the cells extremely fragile. Handle the sample **gently**. The moment after addition of Zymolyase, the digestion of the cell wall begins.

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

Continued on next page

Preparing Spheroplasts , continued

Adding Zymolyase, continued

1. From one tube of cells (Step 5, page 38), withdraw 200 µl cells and add to the tube marked "0". This is your zero time point. Set the tube aside on ice.
2. Add 7.5 µl of Zymolyase to the same tube of cells, mix it gently by inversion, and incubate the cells at 30°C. **Do not shake the sample.** This sample will be used to establish the incubation time for optimal spheroplasting as described below. Keep the second tube of cells at room temperature for use in Step 6 below. Keep the remainder of the Zymolyase on ice.
3. Monitor the formation of the spheroplasts as follows: At time 2 minutes, withdraw 200 µl of cells (from the suspension in Step 2) and add to the tube marked "2". Repeat at time t= 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 minutes after adding Zymolyase. Read the OD_{800} for all samples.
4. Determine the percent of spheroplasting for each time point using the equation:

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

   For example: At time t = 0, the OD_{800} = 0.256
   At time t = 15, the OD_{800} = 0.032

   Calculation: % spheroplasting = 100 – [(0.032/0.256) × 100]
   = 100 - [(0.125) × 100]
   = 100 – 12.5
   = 87.5%
5. Determine the time of incubation that results in approximately 70% spheroplasting. This time of incubation is variable due to differences in lots of Zymolyase. In Invitrogen labs, it takes approximately 15–40 minutes of Zymolyase treatment to achieve optimal spheroplasting.

   Note: It is important to establish the minimum time required for the desired amount of spheroplasting. Prolonged incubation with Zymolyase is deleterious to spheroplasts and will result in lower transformation efficiency.
6. Add 7.5 µl Zymolyase to the remaining tube of cells as described in Step 1 above. Incubate the tube at 30°C for the time that was established in Step 5 to obtain the optimal level (70%) of spheroplasting.
7. Harvest the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature. Decant and discard the supernatant.
8. Wash the spheroplasts once with 10 ml of 1 M sorbitol (**gently** disperse the pellet by tapping the tube, **do not vortex**). Collect the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature.
9. Wash the spheroplasts once with 10 ml of CaS and centrifuge as in Step 7. **Gently** resuspend the spheroplasts in 0.6 ml of CaS. The spheroplasts must be used immediately (up to 30 minutes) for transformation (page 40). They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations.

Transformation of *Pichia*

Before Starting

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

- Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of His^+ Mut^+ recombinants in GS115
- Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of His^+ Mut^S recombinants in KM71
- Parent plasmid linearized with same restriction enzyme

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

Procedure

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

Plating

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

1. Mix together 100–300 µl of each spheroplast-DNA solution from Step 7, above, with 10 ml of molten RD agarose and pour on RDB plates. Allow the top agarose to harden. Note there is enough of the spheroplast-DNA solution to plate duplicate and triplicate plates.
2. Invert plates and incubate at 28–30°C. Transformants should appear in 4–6 days.
3. For cell viability: Mix 100 µl of spheroplasts with 900 µl of 1 M sorbitol.
4. Mix 100 µl of this diluted sample with 10 ml of molten RDH and pour on a RDHB plate. Allow top agarose to harden.
5. Invert plates and incubate at 28–30°C. Appearance of colonies after 4–6 days demonstrates that the spheroplasts can regenerate into dividing cells.

Continued on next page

Transformation of *Pichia*, continued

Analyzing His$^+$ Transformants

If you transformed *Pichia* with constructs based on pPIC3.5K or pPIC9K, proceed to **In Vivo Screening of Multiple Inserts**, page 42.

If you transformed *Pichia* with constructs based on pAO815, proceed to **Screening for Mut$^+$ and Mut^S Transformants**, page 47.

Evaluating Your Transformation Experiment

Transformation efficiency is generally 10^3 to 10^4 His$^+$ transformants/µg of DNA using the spheroplast method. There should be no colonies on the "No DNA" or pBR322 plate or the plasmid only (no cells) plate.

Screening by Functional Assay

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

In Vivo Screening of Multiple Inserts

Introduction

You will need as many His+ transformants as you can conveniently generate. Recall that statistically 1–10% of the His+ transformants will have more than one insert. This means that if the frequency of multicopy inserts is 1%, you will have to screen 1000 His+ transformants to get 10 Geneticin® hyper-resistant colonies to test. This may require 1–5 plates containing His+ transformants. It is not unusual to screen thousands of colonies. Once you have Geneticin® resistant colonies, you can then test them for expression of your recombinant protein (page 50) or characterize them for the Mut phenotype (page 47).

Methods to Screen for Geneticin® Resistant Transformants

There are two methods used to screen His+ transformants for Geneticin® resistance. Method 1 is technically easier and screens a greater number of clones, but is less reliable. After initial selection of His+ transformants, they are pooled and plated on YPD-Geneticin® plates containing increasing concentrations of Geneticin®. Method 1 is applicable to spheroplast or electroporation transformation methods. Method 2 is technically more difficult and screens fewer numbers of clones but is more reliable. It involves growing clones in microtiter plates until all clones are at the same density. The cultures are then spotted on the YPD-Geneticin® plates and scored for Geneticin® resistance.

Note

There is a tendency to isolate false positives when screening with Geneticin®. It is very important to purify your putative Geneticin® resistant clones by streaking for single colonies on YPD and then confirming Geneticin® resistance on YPD-Geneticin® plates. We do not recommend replica-plating as a method to screen for Geneticin® resistance. If you do elect to replica-plate, be sure to confirm Geneticin® resistance.

Continued on next page

In Vivo Screening of Multiple Inserts, continued

Before Starting

Prepare 4 YPD plates of each of the following concentrations of Geneticin®: 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml (see **Appendix**, page 67).

Method 1, (Spheroplasts)

Use this procedure if you transformed *Pichia* spheroplasts. Start with plates containing His^+ transformants.

1. Using a sterile spreader, remove the top layer of the soft agar containing the His^+ transformants and place into a sterile, 50 ml conical centrifuge tube.
2. Add 10 to 20 ml of sterile water. There should be a 2X volume of water above the settled agar. Vortex vigorously for 1 to 2 minutes.
3. Set the centrifuge tube upright on the bench and letthe agar pieces settle (about 1 minute).
4. Determine the cell density of the supernatant by using a hemacytometer. You need at least 5×10^5 cells/ml so you can plate ~10^5 cells in 200 µl or less. (If the cells are too dilute, transfer the liquid to a fresh tube and centrifuge the cells. Resuspend the cell pellet in sterile water in a volume sufficient to give 5×10^5 cells/ml.)
5. Plate 10^5 cells YPD-Geneticin® plates containing Geneticin® at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml. Use four plates for each concentration. (You may want to confirm the titer of the cells on the YPD plates without Geneticin® in order to calculate the percent of Geneticin® resistant colonies you obtain for each Geneticin® concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100 to 200 µl per plate.)
6. Incubate plates at 30°C and check daily. Geneticin®-resistant colonies will take 2 to 5 days to appear while cells plated on YPD without Geneticin® will take 2–3 days. Proceed to **Analysis of Results**, page 45.

Continued on next page

In Vivo Screening of Multiple Inserts, continued

Method 1, (Electroporation)

Use this procedure if electroporation was used to transform *Pichia*. Transformants will not be plated in top agar. Start with plates containing His^+ transformants.

1. Pipette 1 to 2 ml sterile water **over** the His^+ transformants on each plate. Use all the plates that have His^+ transformants.
2. Resuspend the His^+ transformants into the water by using a sterile spreader and running it across the top of the agar. Be careful not to tear the agar.
3. Transfer and pool the cell suspension into a sterile, 50 ml conical centrifuge tube and vortex briefly (5 to 10 seconds).
4. Determine cell density using a spectrophotometer (1 OD_{600} = 5×10^7 cells/ml). **Note**: any agar present will interfere with a spectrophotometer reading.
5. Plate 10^5 cells on YPD plates containing Geneticin® at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml.

   (You may want to confirm the titer of the cells on the YPD plates without Geneticin® in order to calculate the percent of Geneticin® resistant colonies you obtain for each Geneticin® concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100 to 200 µl per plate.)
6. Incubate plates at 30°C and check daily. Geneticin®-resistant colonies will take 2 to 5 days to appear while cells plated on YPD will take 2–3 days. Proceed to **Analysis of Results**, page 45.

Note

If you do not plate all of the cell suspension from either method above, add sterile glycerol to 15% and freeze in convenient aliquots at –80°C. You may thaw the aliquots and analyze for Geneticin® resistant colonies at a later date.

Continued on next page

In Vivo Screening of Multiple Inserts, continued

Method 2

You will need three sets of two microtiter plates (6 total) to screen ~180 His^+ recombinants. It is important to grow your clones to approximately the same cell density by successive inoculations to ensure that equivalent numbers of cells are spotted on Geneticin® plates. If you plated your transformants in top agar, it may be necessary to extract them from the agarose and re-plate them on minus histidine plates (see page 48) in order to pick colonies. Remember to include controls for strain background and one copy of your gene. For every 180 colonies, you can expect to isolate 1–10 Geneticin® resistant colonies.

1. Using sterile technique, add 200 µl YPD to each microtiter well.
2. Inoculate each well of the first set of plates with a single His^+ transformant using a sterile toothpick and stirring to resuspend cells.
3. Cover the microtiter plate and incubate at 30°C for 2 days (shaking not required).
4. After 2 days, take new microtiter plates and add 190 µl of YPD to each well.
5. Inoculate the second set of microtiter plates with 10 µl from the first set of microtiter plates by using a multi-channel pipette. Make sure the second set of plates is marked and oriented in such a way that you can keep track of wells.
6. Cover and incubate the second set of plates overnight at 30°C.
7. The next day, repeat Steps 5 and 6, creating a third set of microtiter plates.

   Note: Successive growth and passage of the clones will bring them all to the same cell density.
8. After incubation, take the third set of plates and resuspend the cells in each well by pipetting up and down with a multi-channel pipette set on 100 µl volume.
9. Spot 10 µl from each well on YPD plates containing Geneticin® at a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml. Spot in a regular pattern using the multi-channel pipette or a grid underneath the plate.
10. Let the liquid soak in, then incubate plates at 30°C, and check after 2, 3, 4, or 5 days for Geneticin® resistant clones. Proceed to **Analyzing Results**, below.

Analyzing Results

There may be only a few Geneticin® resistant colonies, and they may be of different sizes, but the colony morphology should be the same. Pick all Geneticin® resistant colonies and purify by streaking for single colonies. Be sure to confirm the observed level of Geneticin® resistance.

You may not find colonies resistant to 2.0, 3.0, or 4.0 mg/ml Geneticin®. "Jackpot" clones resistant to these high levels of Geneticin® are very rare. You may have to screen thousands of His^+ transformants in order to isolate colonies resistant to 2–4 mg/ml Geneticin®.

Since there is no guarantee that multiple copies will actually increase the amount of protein expressed, most people elect to proceed directly to expression to see if any of these colonies overexpress their protein. Be sure to include a single copy insert as a control. Test all your Geneticin® resistant colonies for their Mut phenotype (page 47) so that you induce expression properly.

Continued on next page

In Vivo Screening of Multiple Inserts, continued

Be sure to purify your clones by streaking for single colonies and making frozen, glycerol stocks of all your Geneticin® resistant colonies. Always initiate expression studies from frozen stocks, not old plates.

Determining Copy Number

If you find that your Geneticin®-resistant His^+ recombinants significantly overexpress your protein, you may wish to quantify the copy number of your gene. Copy number may be analyzed by Southern or quantitative dot (slot) blots. See page 88 for information on these techniques. It is very important to include genomic DNA isolated from *Pichia* recombinants transformed with vector alone and recombinant vector with a single copy of your gene as controls in order to evaluate your experiment.

Trouble-shooting

Since there is a tendency to isolate false positives (colonies that appear to be Geneticin® hyper-resistant, but are not), it is very important to purify your putative Geneticin® resistant colonies and confirm the observed level of Geneticin® resistance before proceeding further.

The other most common problem with the *in vivo* method is that very few Geneticin® resistant colonies are isolated. This usually means that more His^+ transformants need to be screened. Remember that you are isolating spontaneous, multiple integration events. These occur at a frequency of 1–10% that may mean that you need to screen thousands of His^+ transformants as opposed to hundreds. In addition, to isolate recombinants with the most copies of your gene inserted, you may have to screen additional His^+ transformants. Successive multiple insertions are simply more rare.

If you find that your transformation efficiency is low, try electroporation instead of spheroplasting. This may increase the transformation efficiency and help you isolate more His^+ transformants.

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

Introduction

At this point, you may wish to score your His$^+$ transformants for Mut$^+$ and Mut^S phenotype. Included in the kit are two strains that will provide examples of Mut$^+$ and Mut^S phenotypes. GS115 Albumin is Mut^S and GS115 β-Gal is Mut$^+$. His$^+$ KM71 recombinants do not need to be screened for their Mut phenotype as they all will be Mut^S.

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

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

Transformation of GS115 with *Sal* I- or *Stu* I-linearized constructs favor recombination at the *HIS4* locus. Most of the transformants should be Mut$^+$; however, with the presence of *AOX1* sequences in the plasmid, there is a chance that recombination will occur at the *AOX1* locus, disrupting the wild-type *AOX1* gene and creating His$^+$ Mut^S transformants (page 74). Again, testing on MD and MM plates will allow you to confirm His$^+$ Mut$^+$ transformants (see next page).

His$^+$ Mut^S in KM71

All His$^+$ transformants in KM71 will be Mut^S because of the disruption of the *AOX1* gene (*aox1::ARG4*). There is no need to test recombinants for the Mut phenotype; all recombinants will be Mut^S. Transformation of KM71 with *Sal* I- or *Stu* I-linearized plasmid constructions favor recombination at the *HIS4* locus while *Sac* I-linearized plasmid constructions favor recombination at the 5´ region of the *AOX1* gene. His$^+$ transformants need to be purified on minimal plates without histidine to ensure pure clonal isolates before testing for expression (see page 50).

Preparation

The following can be prepared several days in advance and stored at 4°C.

Minimal Dextrose (MD) agar plates, 1 liter (see page 69)
Minimal Methanol (MM) agar plates, 1 liter (see page 69)
Sterile toothpicks and Scoring Templates (see page 49)

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

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

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

1. Using a sterile toothpick, pick one colony and streak or patch one His$^+$ transformant in a regular pattern on both an MM plate and an MD plate, making sure to patch the MM plate first.
2. Use a new toothpick for each transformant, continuing until 100 transformants have been patched (2–3 plates).
3. To differentiate Mut$^+$ from Mut^S, make one patch for each of the controls (GS115/His$^+$ Mut^S Albumin and GS115/His$^+$ Mut$^+$ 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. Look for patches that grow normally on the MD plates but show little or no growth on the MM plates.

Continued on next page

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

Note

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

Replica-Plating Procedure

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

1. Using sterile toothpicks, patch 100 His$^+$ transformant on MD plates (2–3 plates). For controls, make one patch from each of the strains GS115/His$^+$ Mut^S Albumin and GS115/His$^+$ Mut$^+$ 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.

Easier Selection of Transformants

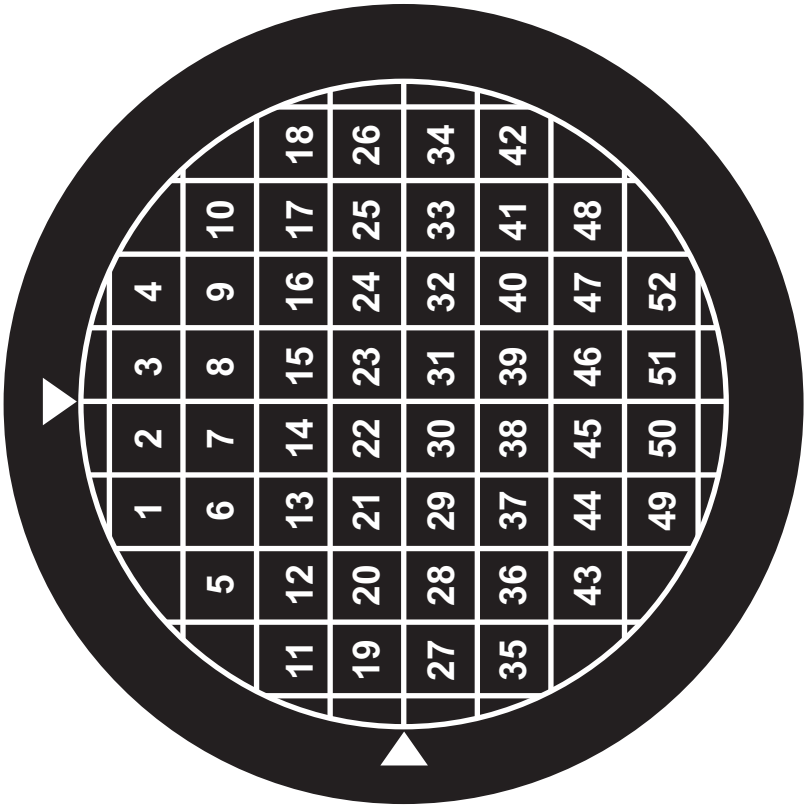
Because of plating in top agarose, transformants can be on top or imbedded in the top agarose making it difficult to pick and patch colonies in the next section. The following protocol allows you to collect the transformants and re-plate them directly onto plates without top agarose.

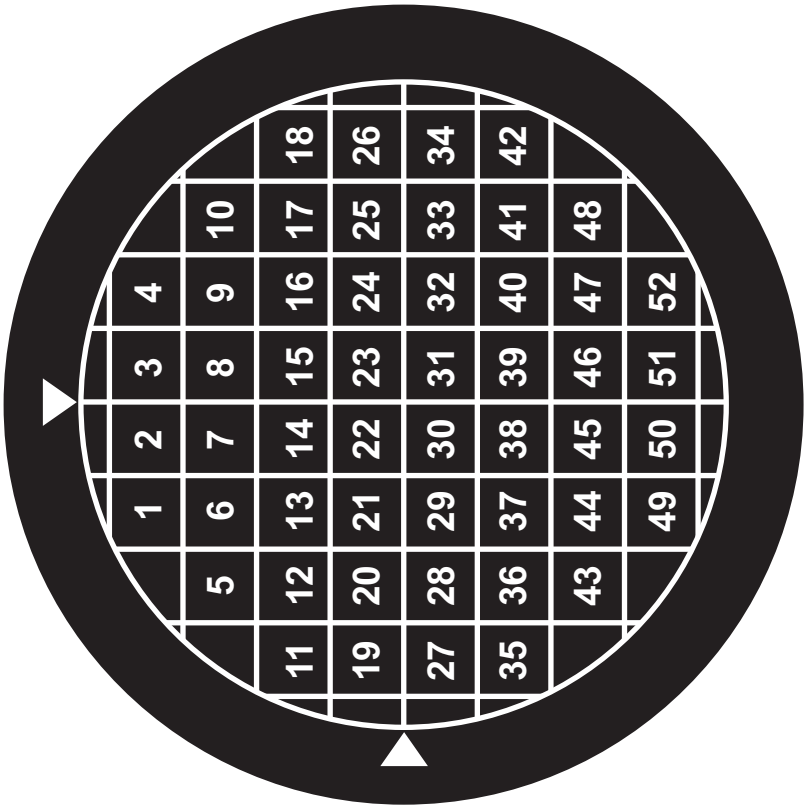
1. Scrape the agarose containing the His$^+$ transformants with a sterile spreader into a sterile, 50 ml, conical centrifuge tube and mix with 20 ml sterile deionized water. Vortex the suspension vigorously to separate the cells from the agarose.
2. Filter the suspension through 4 folds of sterile cheesecloth.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

Continued on next page

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

Scoring Templates





Expression of Recombinant *Pichia* Strains

Introduction

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

Media

You will need BMGY/BMMY (buffered complex glycerol or methanol medium), BMG/BMM (buffered minimal glycerol or methanol medium), or MGY/MM (minimal glycerol or minimal methanol medium) for expression (see **Appendix**, pages 68–70). BMG, BMM, BMGY, and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein. Unlike MGY and MM, they are all buffered media. Because these media are buffered with phosphate buffer, you may use a wide range of pH values to optimize production of your protein. BMGY/BMMY contain yeast extract and peptone which may help stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone act as a "mixed feed" allowing better growth and biomass accumulation.

Proteases

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

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

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

Aeration

The most important parameter for efficient expression in *Pichia* is adequate aeration during methanol induction. As a general rule when inducing expression, never allow cultures to be more than 10–30% of your total flask volume. We strongly recommend that you use baffled flasks. See **Appendix**, page 71 for suppliers of baffled flasks. Cover the flasks with cheesecloth (2–3 layers) or another loose fitting cover. Never use tight fitting covers. (Aeration is not as critical when generating biomass before induction.)

Continued on next page

Expression of Recombinant *Pichia* Strains, continued

Kinetics of Growth

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

Temperature and Shaking

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

Before Starting

You should have verified recombinants in GS115 or KM71 as well as a control recombinant of GS115 or KM71/Vector (no insert and 1 copy). When performing your expression, it is important to run the proper controls so that you will be able to interpret your expression results. You should use the following expression controls:

GS115/His$^+$ Mut^S albumin	Mut^S- Secretion control
GS115/His$^+$ Mut$^+$ β-Gal	Mut$^+$- Intracellular control
GS115 or KM71/Vector (no insert)	Background control
GS115 or KM71/Vector (1 copy)	Expression level due to one copy of gene

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

Guidelines for Expression

The following information is designed to get you started with expression. You may have to change the conditions to optimize expression for your particular protein. Use bottom or side baffled flasks whenever possible (see page 71). If you are analyzing a number of recombinants, you can try 50 ml conical tubes. Be sure that the medium is well-aerated by increasing the rate of shaking or placing the tubes at an angle in the shaker.

Continued on next page

Expression of Recombinant *Pichia* Strains, continued

Mut$^+$ Intracellular or Secreted

You can test the effectiveness of your expression conditions by growing GS115 β-Gal (Mut$^+$) which 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 in a 250 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until culture reaches an OD_{600} = 2–6 (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 supernatant and resuspend cell pellet to an OD_{600} of 1.0 in MM, BMM, or BMMY medium to induce expression (approximately 100–200 ml).
3. Place culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction. Be sure to check the volume of the culture and add methanol accordingly. Evaporation may reduce 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 determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.

   Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).
6. For secreted expression, **transfer the supernatant to a separate tube.** Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.

   For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.
7. Analyze the supernatants and cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, page 54).

Continued on next page

Expression of Recombinant *Pichia* Strains, continued

Mut^S Intracellular or Secreted

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

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

   Time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days), 120 (5 days), and 144 (6 days).
6. For secreted expression, **transfer the supernatant to a separate tube.** Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.

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

Analysis by SDS-Polyacrylamide Gel Electrophoresis

Introduction

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

Polyacrylamide Gel Electrophoresis

Invitrogen offers a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels and electrophoresis apparatus. The patented NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use, refer to our website (www.invitrogen.com) or contact Technical Support (page 94).

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

Preparing Samples

You will need to prepare Breaking Buffer (see page 71) and have acid-washed 0.5 mm glass beads on hand.

Preparing of cell pellets (Intracellular and Secreted Expression):

1. Thaw cell pellets quickly and place on ice.
2. For each 1 ml sample, add 100 µl Breaking Buffer to the cell pellet and resuspend.
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 will determine volume loaded. You may store the remaining sample at –20°C for western blots, if necessary. You may store the cell lysates at –80°C for further analysis.

Continued on next page

Analysis by SDS-Polyacrylamide Gel Electrophoresis, continued

Preparing Samples, continued

Preparing of supernatant (Secreted Expression only):

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

Protein Concentration

Lowry, BCA (Pierce) or Bradford protein determinations can be performed to quantify the amounts of protein in the cell lysates and medium supernatants. In general, *Pichia* cell lysates contain 5–10 µg/µl protein. *Pichia* medium supernatants will vary in protein concentration primarily due to the amount of your secreted protein. *Pichia* secretes very few native proteins. If the protein concentration of the medium is >50 µg/ml, 10 µl of medium will give a faint band on a Coomassie-stained SDS-PAGE gel.

Continued on next page

Analysis by SDS-Polyacrylamide Gel Electrophoresis, continued

Controls

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

- Molecular weight standards appropriate for your desired protein
- A sample of your protein as a standard (if available)
- A sample of GS115 or KM71 with the parent plasmid transformed into it. This shows the background of native *Pichia* proteins that are present intracellularly. Inclusion of this sample will help you differentiate your protein from background if you express it intracellularly.
- Analyze the GS115 β-Gal and Albumin controls also as they should indicate any problems with the media or expression conditions

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

Analyzing Protein Expression

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

If you detect low expression of your recombinant protein, see **Optimizing *Pichia* Protein Expression**, page 57, for guidelines to optimize expression.

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

If there is no indication of expression at all, perform a northern analysis to see if and how much full-length mRNA is induced. See page 91 for an RNA isolation protocol.

Optimizing *Pichia* Protein Expression

Introduction

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

Proteolysis or Degradation

- Do a time course study of expression. Check to see if there is a time point that yields a larger percentage of full-length protein.
- If secreting your protein, check to see if your protein is susceptible to neutral pH proteases by expressing in unbuffered medium (MM). In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.
- Try using SMD1168 (proteinase A-deficient) for expression (see **Proteases**, page 50).

Low Secreted Expression Levels

- Check cell pellet to see if overall expression is low or if the protein did not secrete. If it did not secrete, try a different signal sequence (*e.g.*, a native or α-factor signal sequence).
- Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration (see page 61).
- For Mut$^+$, induce expression with a higher density culture.

Low Expression Levels

- Check both Mut$^+$ and Mut^S recombinants for increased expression. Some proteins express better in one type of genetic background than another.
- If secreting your protein, try intracellular expression. The protein may not be processed correctly and fail to secrete. Be sure you check your cell pellets for evidence of expression. If you are having problems with intracellular expression, try secreting your protein. It probably will glycosylate, which may be desirable or not. If glycosylation is undesirable, oligosaccharides can be removed with Peptide:N-Glycosidase F (New England Biolabs, page 63).
- Scale up to fermentation (page 61). *Pichia* is a yeast and is particularly well suited to fermentation.

Continued on next page

Optimizing *Pichia* Protein Expression, continued

No Expression

Be sure to try some of the easier things listed on the previous page as no expression can be the same thing as very low expression. If none of these things improve protein expression, perform a northern blot analysis to check for transcription of your gene. There is a protocol in the **Appendix** for RNA isolation from *Pichia* (see page 91).

Analyze for the presence of your insert by PCR (see page 83). You can reasonably analyze 12–20 transformants by PCR. Remember to include the vector only and original (one copy) construct controls in order to analyze your PCR experiment.

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

Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression as your protein will not go through the secretion pathway and therefore, not be modified.
- Try deglycosylating the protein with Peptide:N-Glycosidase F or other enzymes (see page 62).
- Engineer gene to remove N-linked glycosylation sites.

Scale-up of Expression

Guidelines for Expression

Once expression is optimized, scale-up your expression protocol to produce more protein by increasing the culture volume using larger baffled flasks (below) or by fermentation. Use the guidelines below to scale-up your expression protocol. To purify your protein, see the references listed on page 62.

Mut$^+$ Intracellular or Secreted

1. Using a single colony, inoculate 25 ml of MGYH, BMGH, or BMGY in a 250 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until culture reaches an OD_{600} = 2–6 (approximately 16–18 hours).
2. Use this 25 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD_{600} = 2–6).
3. Harvest the cells using sterile centrifuge bottles by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet to an OD_{600} = 1.0 (2–6 liters) in MMH, BMMH, or BMMY medium to start induction.
4. Aliquot the culture between several 3 or 4 liter baffled flask. Cover the flasks with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28–30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached as determined from the time course study.
6. Harvest cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature.
7. For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.
   For secreted expression, **save the supernatant, chill to 4°C, and concentrate it down if desired** (see page 61). Proceed directly to purification (page 62) or store the supernatant at –80°C until ready to process further.

Continued on next page

Scale-up of Expression, continued

Mut^S Intracellular or Secreted

1. Using a single colony, inoculate 10 ml of MGYH, BMGH, or BMGY in a 100 ml baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} = 2–6 (approximately 16–18 hours).
2. Use this 10 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD_{600} = 2–6).
3. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet in 1/5 to 1/10 of the original culture volume of MMH, BMMH, or BMMY medium (approximately 100–200 ml).
4. Place the culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28–30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached.
6. Harvest cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature.
7. For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.
   For secreted expression, **save the supernatant, chill to 4°C, and concentrate it down if desired** (see next page). Proceed directly to purification (page 62) or store the supernatant at –80°C until ready to process further.

Note

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

Continued on next page

Scale-up of Expression, continued

Concentration of Proteins

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

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

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

Cell Lysis

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

Fermentation

Basic guidelines are available for fermentation of *Pichia* from Invitrogen. We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Contact Technical Support for more information (page 94).

Protein Purification and Glycosylation

Introduction

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

Some Protein Purification Techniques

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

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

Procedure for Cell Lysis

Prepare Breaking Buffer (BB) as described in **Appendix**, page 71.

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

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

Analysis of Glycoproteins

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

Protein Purification and Glycosylation, continued

Enzymes for Analyzing Glycoproteins

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

Enzyme	Type of enzyme	Specificity
Endoglycosidase D	Endo	Cleaves various high mannose glycans
Endoglycosidase F	Endo	Cleaves various high mannose glycans
Endoglycosidase H	Endo	Cleaves various high mannose glycans
β-galactosidase	Exo	Removes terminal galactosides from Gal-β1,3-GlcNAc, Gal-β1,4-GlcNAc or Gal-β1,3-GalNAc.
Peptide:N-Glycosidase F	Endo	Glycoproteins between Asn and GlcNAc (removes oligosaccharides)
Sialidases (Neuraminidases) *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 who will contract to analyze proteins for glycosylation. A number of companies also supply kits and reagents for researchers to do carbohydrate analysis in their own laboratories. A partial list is provided below:

Company	Type of Service	Contact
Glyko	Kits for Carbohydrate Analysis Reagents Contract Services	1-800-334-5956 www.prozyme.com
New England BioLabs	Reagents	1-800-632-5227 www.neb.com

Appendix

E. coli Media Recipes

Introduction

You select transformants containing 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 for 20 minutes at 15 lbs/sq. in. Let cool to ~55°C and add desired antibiotics at this point.
4. Store at room temperature or at 4°C.

LB agar plates

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

Pichia Media Recipes

Introduction

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

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

Stock Solutions

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

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

The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch.

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

500X B (0.02% Biotin)

1. Dissolve 20 mg biotin in 100 ml of water and filter sterilize.
2. Store at 4°C.

The shelf life of this solution is approximately one year.

100X H (0.4% Histidine)

1. Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve.
2. Filter sterilize and store at 4°C.

The shelf life of this solution is approximately one year.

10X D (20% Dextrose)

1. Dissolve 200 g of D-glucose in 1,000 ml of water.
2. Autoclave for 15 minutes or filter sterilize.

The shelf life of this solution is approximately one year.

Continued on next page

Pichia Media Recipes, continued

Stock Solutions, continued

10X M (5% Methanol)

1. Mix 5 ml of methanol with 95 ml of water.
2. Filter sterilize and store at 4°C.

The shelf life of this solution is approximately two months.

10X GY (10% Glycerol)

1. Mix 100 ml of glycerol with 900 ml of water.
2. Sterilize either by filtering or autoclaving. Store at room temperature.

The shelf life of this solution is greater than one year.

100X AA (0.5% of each Amino Acid)

1. Dissolve 500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine in 100 ml of water.
2. Filter sterilize and store at 4°C.

The shelf life of this solution is approximately one year.

1 M potassium phosphate buffer, pH 6.0:

1. Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH).
2. Sterilize by autoclaving and store at room temperature.

The shelf life of this solution is greater than one year.

YPD or YEPD

Yeast Extract Peptone Dextrose Medium (1 liter)

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

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

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

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

Continued on next page

YPD-Geneticin® plates

Yeast Extract Peptone Dextrose Medium

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

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

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

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

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

Continued on next page

Pichia Media Recipes, continued

MGY and MGYH

Minimal Glycerol Medium + Histidine (1 liter)

1.34% YNB
1% glycerol
4×10^{-5}% biotin
+ 0.004% histidine

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

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

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

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

RDB and RDHB Agar Plates

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

Continued on next page

Pichia Media Recipes, continued

RD and RDH Top Agar

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

MD and MDH

Minimal Dextrose Medium + Histidine (1 liter)

1.34% YNB
4 x 10^{-5} % biotin
2% dextrose

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
2. Cool to about 60°C and then add:

   100 ml of 10X YNB

   2 ml of 500X B

   100 ml of 10X D
3. To make MDH, add 10 ml of 100X H stock solution. Mix and store at 4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed.
5. If preparing plates, pour the plates immediately. MD stores well for several months at 4°C.

MM and MMH

Minimal Methanol + Histidine (1 liter)

1.34% YNB
4 x 10^{-5}% biotin
0.5% methanol

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle
2. Cool autoclaved water to 60°C and add:

   100 ml of 10X YNB

   2 ml of 500X B

   100 ml of 10X M
3. To make MMH, add 10 ml of 100X H stock solution. Mix and store at 4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed.
5. After mixing, pour the plates immediately. MM and MMH stores well for several months at 4°C.

Continued on next page

Pichia Media Recipes, continued

BMG and BMM

Buffered Minimal Glycerol
Buffered Minimal Methanol (1 liter)

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

1. Autoclave 700 ml water for 20 minutes on liquid cycle.
2. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0
   100 ml 10X YNB
   2 ml 500X B
   100 ml 10X GY
4. For BMM, add 100 ml 10X M instead of glycerol.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

BMGY and BMMY

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

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

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0
   100 ml 10X YNB
   2 ml 500X B
   100 ml 10X GY
4. For BMMY, add 100 ml 10X M instead of glycerol.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

Continued on next page

Pichia Media Recipes, continued

Breaking Buffer

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

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

Vendors for Baffled Flasks

Bellco (1-800-257-7043) has a wide variety of baffled flasks from 50 to 2,000 ml.

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

Proteins Expressed in *Pichia*

Table

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

Protein	Expression Levels grams/liter	Where Expressed How Expressed	Reference
Enzymes			
Invertase	2.3	Secreted Mut^+	(Tschopp *et al.*, 1987b)
Bovine Lysozyme c2	0.55	Secreted Mut^+	(Digan *et al.*, 1989)
Streptokinase (active)	0.08	Intracellular *	(Hagenson *et al.*, 1989)
Alpha amylase	2.5	Secreted Mut^S	(Paifer *et al.*, 1994)
Pectate Lyase	0.004	Secreted Mut^S	(Guo *et al.*, 1995)
Spinach Phospho-ribulokinase	0.1	Intracellular Mut^S	(Brandes *et al.*, 1996)
Antigens			
Hepatitis B surface antigen	0.4	Intracellular Mut^S	(Cregg *et al.*, 1987)
Pertussis Antigen P69	3.0	Intracellular Mut^S	(Romanos *et al.*, 1991)
Tetanus Toxin Fragment C	12.0	Intracellular Mut^+/Mut^S	(Clare *et al.*, 1991a)
HIV-1 gp120	1.25	Intracellular Mut^+	(Scorer *et al.*, 1993)
Tick Anticoagulant protein	1.7	Secreted Mut^S	(Laroche *et al.*, 1994)
Bm86 Tick Gut Glycoprotein	1.5	Secreted *	(Rodriguez *et al.*, 1994)
Regulatory Proteins			
Tumor Necrosis Factor (TNF)	10.0	Intracellular Mut^S	(Sreekrishna *et al.*, 1989)
Mouse Epidermal Growth Factor (EGF)	0.45	Secreted Mut^S	(Clare *et al.*, 1991b)
2b Human Interferon (IFN)	0.4	Intracellular Mut^S	(Garcia *et al.*, 1995)

Continued on next page

Proteins Expressed in *Pichia*, continued

Table, continued

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

*Mut phenotype was not described in the paper.

Recombination and Integration in *Pichia*

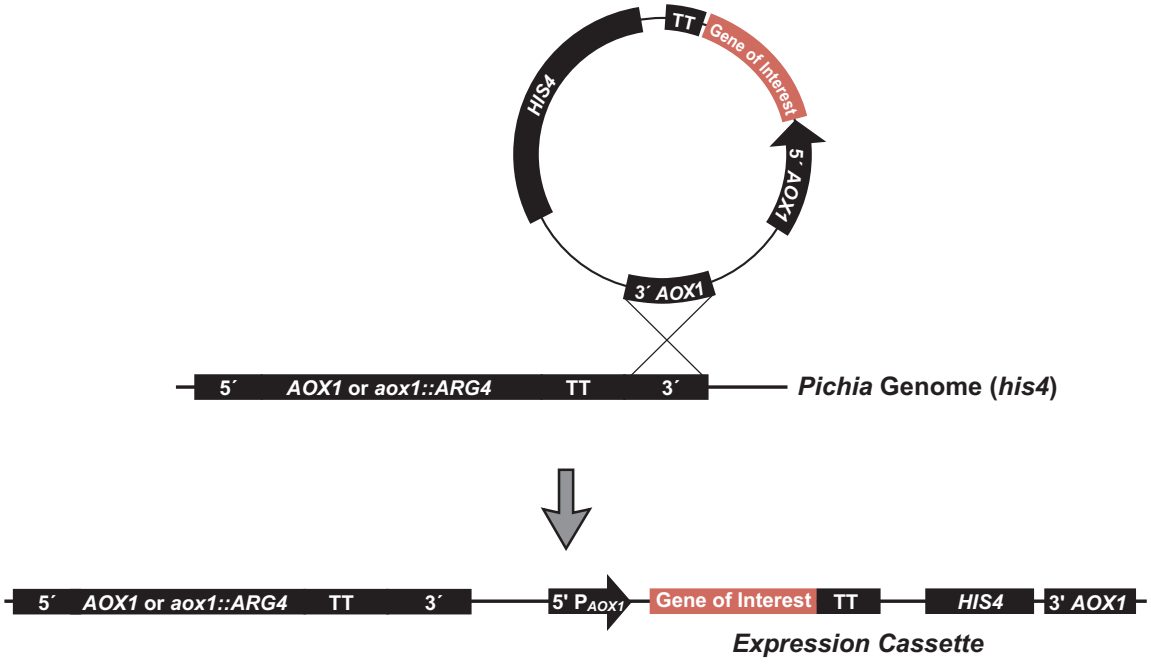
Introduction

Like *Saccharomyces cerevisiae*, linear DNA can generate stable transformants of *Pichia pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome (Cregg *et al.*, 1985; Cregg *et al.*, 1989). Such integrants show extreme stability in the absence of selective pressure even when present as multiple copies. The most commonly used expression vectors carry the *HIS4* gene for selection. These vectors are designed to be linearized with a restriction enzyme such that His^+ recombinants are generated by recombination at the *AOX1* locus (see below) or at the *his4* locus (see next page). Note that single crossover events (insertions) are much more likely to happen than double crossover events (replacements). Multiple insertion events occur spontaneously at about 1–10% of the single insertion events.

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

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

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

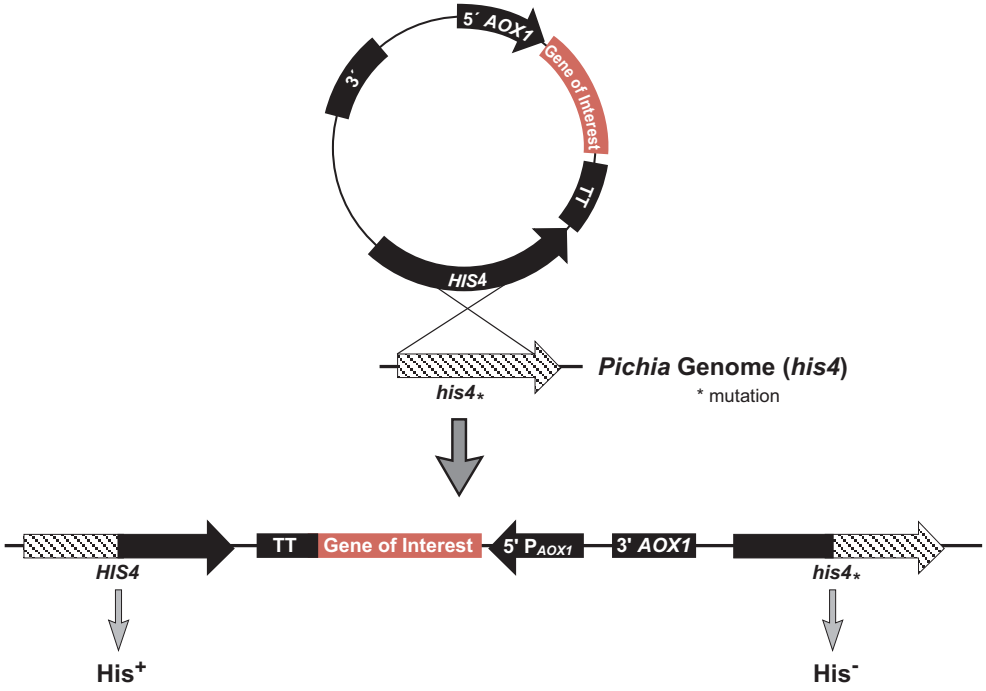


Continued on next page

Recombination and Integration in *Pichia*, continued

Gene Insertion Events at *his4*

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



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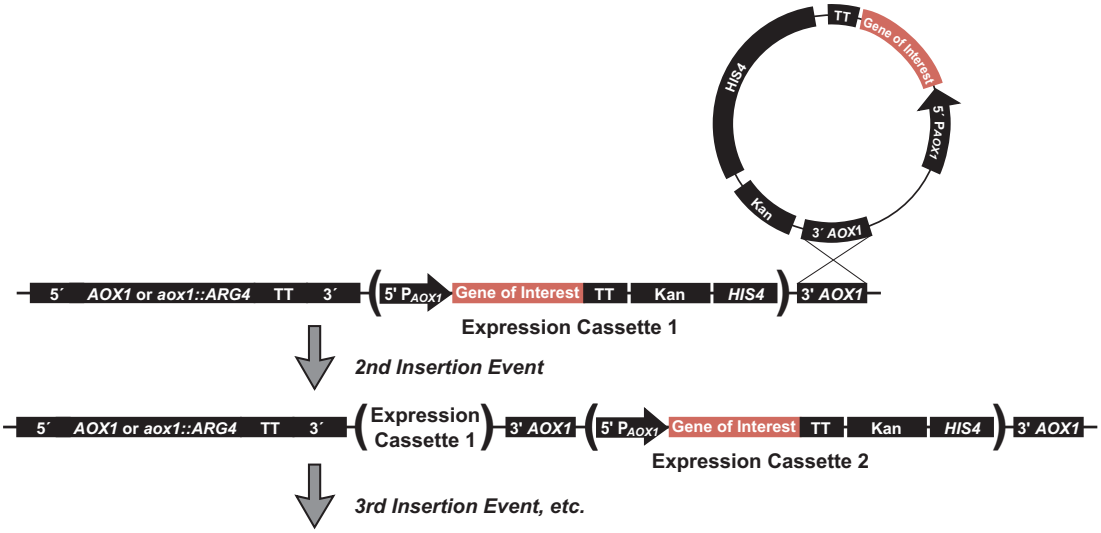
Recombination and Integration in *Pichia*, continued

Multiple Gene Insertion Events

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



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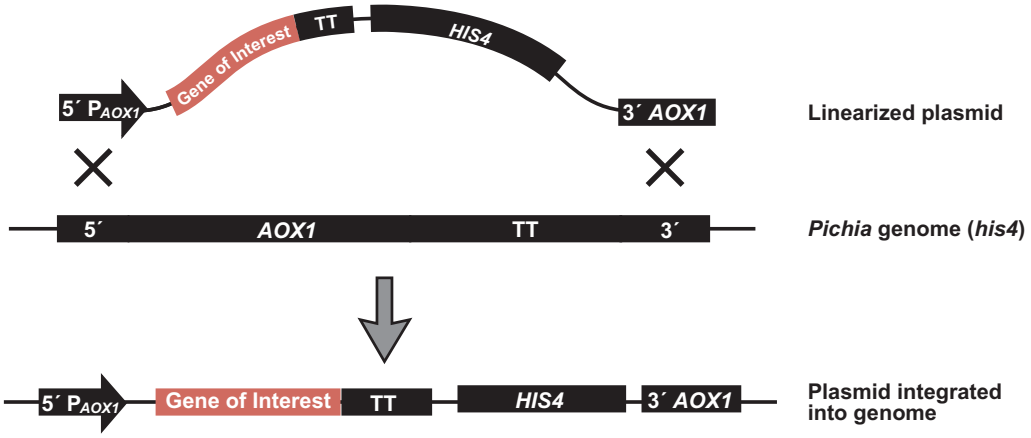
Recombination and Integration in *Pichia*, continued

Gene Replacement at *AOX1* in GS115

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

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



Electroporation of *Pichia*

Introduction

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

Preparing Cells

1. Grow 5 ml of *Pichia pastoris* in YPD (page 66) in a 50 ml conical at 30°C overnight.
2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1–0.5 ml of the overnight culture. Grow overnight again to an OD_{600} = 1.3–1.5.
3. Centrifuge the cells at 1,500 × g for 5 minutes at 4°C. Resuspend the pellet with 500 ml of ice-cold, sterile water.
4. Centrifuge the cells as in Step 3, and resuspend the pellet with 250 ml of ice-cold, sterile water.
5. Centrifuge the cells as in Step 3 and resuspend the pellet in 20 ml of ice-cold 1 M sorbitol.
6. Centrifuge the cells as in Step 3 and resuspend the pellet in 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml.

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

Transformation

1. Mix 80 µl of the cells from Step 6 (above) with 5–20 µg of linearized DNA (in 5–10 µl TE Buffer) and transfer them to an ice-cold 0.2 cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) suggested by the manufacturer of the specific electroporation device being used.
4. Immediately add 1 ml of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile microcentrifuge tube.
5. Spread 200–600 µl aliquots on MD or RDB plates.
6. Incubate the plates at 30°C until colonies appear. Screen for Mut^+/Mut^S phenotypes as indicated on page 47.

PEG 1000 Transformation Method for *Pichia*

Introduction

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

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

Important

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

Preparing Competent Cells

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

Continued on next page

PEG 1000 Transformation Method for *Pichia*, continued

Transformation

1. Use up to 50 µg of each DNA sample in no more than 20 µl total volume. Add the DNA directly to a still-frozen tube of competent cells. Carrier DNA (40 µg of denatured and sonicated salmon sperm DNA) should be included with < 1 µg DNA samples for maximum transformation frequencies.
2. Incubate all sample tubes in a 37°C water bath for five minutes. Mix samples once or twice during this incubation period.
3. Remove tubes from the bath and add 1.5 ml of Buffer B to each. Mix contents thoroughly.
4. Incubate tubes in a 30°C water bath for 1 hour.
5. Centrifuge sample tubes at 2,000 × g for 10 minutes at room temperature. Decant supernatant and resuspend the cells in 1.5 ml Buffer C.
6. Centrifuge samples a second time and resuspend the cell pellet gently in 0.2 ml of Buffer C.
7. Spread entire contents of each tube on an agar plate containing selective growth medium and incubate plates at 30°C for 3 to 4 days. Screen for Mut phenotype (page 47) or select hyper-resistant Geneticin® colonies (page 42).

Lithium Chloride Transformation Method

Introduction

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

Preparing Solutions

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

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

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

2 mg/ml denatured, fragmented salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at –20°C.

Preparing Cells

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

Continued on next page

Lithium Chloride Transformation Method, continued

Transformation

1. Boil a 1 ml sample of single-stranded DNA for five minutes, then quickly chill in ice water. Keep on ice. **Note**: It is neither necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at –20°C and boil every 3–4 times the DNA is thawed.
2. Centrifuge the LiCl-cell solution from Step 7, previous page. Remove the LiCl with a pipet.
3. For each transformation sample, add the following reagents **in the order given** to the cells. PEG shields the cells from the detrimental effects of the high concentration of LiCl.

   240 µl 50% PEG

   36 µl 1 M LiCl

   25 µll 2 mg/ml single-stranded DNA

   Plasmid DNA (5–10 µg) in 50 µl sterile water
4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
5. Incubate the tube at 30°C for 30 minutes without shaking.
6. Heat shock in a water bath at 42°C for 20–25 minutes.
7. Centrifuge the tubes at 6,000 to 8,000 rpm and remove the transformation solution with a pipet.
8. Gently resuspend the pellet in 1 ml of sterile water.
9. Plate 25 to 100 µl on RDB or MD plates. Incubate the plates for 2–4 days at 30°C. Screen for Mut phenotype (page 47) or select hyper-resistant Geneticin® colonies (page 42).

PCR Analysis of *Pichia* Integrants

Introduction

The following protocol is designed to allow you to analyze *Pichia* integrants to determine if the gene of interest has integrated into the *Pichia* genome. Isolate genomic DNA from 6–10 Mut^S or Mut^+ *Pichia* clones using the protocol on page 86. Isolate DNA from the strain transformed with the parent plasmid. After isolating your DNA, use the procedure below to identify integrants. Amplification of the gene of interest is carried out either with the α-factor primer (for pPIC9 only) or 5´ *AOX1* primer paired with the 3´ *AOX1* primer included in the kit. This protocol is useful for confirming integration of the gene of interest but will not provide information on the site of integration.

A more direct method for PCR screening is available on the page 85.

Analysis by PCR

1. Set up PCR reactions as follows:

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

*Resuspend the primers in 20 µl sterile water to prepare a 0.1 µg/µl solution. The amount of primer may be decreased if desired. For ~20 pmoles primer, use 2 µl of each primer.

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

2. Load thermocycler and run the following program:

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

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

Continued on next page

PCR Analysis of *Pichia* Integrants, continued

Interpreting PCR

If screening Mut$^+$ integrants, you should see two bands. One will correspond to the size of your gene of interest, the other to the *AOX1* gene (approximately 2.2 kb). If screening Mut^S integrants in GS115, you should see only the band that corresponds to the gene of interest. In KM71, because of the *ARG4* insert in *AOX1*, the PCR product is 3.6 kb. Parent plasmids will produce the following sized PCR products. Add these fragments to the size of your insert to interpret your PCR results.

Vector	PCR Product
pPIC3.5K	220 bp
pAO815	189 bp
pPIC9K (using the 5´ *AOX1* primer)	492 bp
pPIC9K (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.

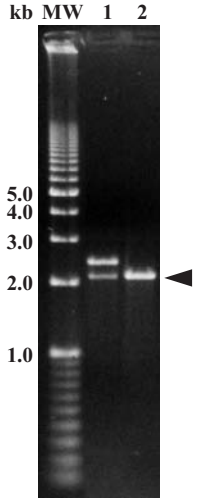
Note

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

Example of PCR Analysis

The figure below shows the results of a typical PCR analysis using the procedure on page 83. Genomic DNA was isolated from *Pichia* recombinants and from appropriate controls. Ten microliter samples from each PCR were run on a 0.8% agarose gel.

Lane 1 contains a 1 kb + 100 bp ladder. Lane 2 shows the wild-type *AOX1* gene (2.2 kb) and a 2.4 kb product containing the gene of interest (GOI, 1.9 kb) and 492 bp of flanking *AOX1* sequence from GS115/pPIC9K/GOI. Lane 3 shows the wild-type *AOX1* gene alone from GS115.



Note

The Easy-DNA™ Kit, available separately from Invitrogen, provides a fast and easy method to isolate genomic DNA from *Pichia pastoris*. See page viii for ordering information.

Direct PCR Screening of *Pichia* Clones

Introduction

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

Before Starting

You will need the following reagents and equipment on hand:

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

Procedure

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

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

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

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

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

Isolating Total DNA from *Pichia*

Introduction

The protocol below allows you to isolate DNA from the desired His^+ recombinant and the untransformed GS115 or KM71 which is suitable for Southern blot analysis, dot/slot blot analysis or genomic PCR. See *Current Protocols in Molecular Biology*, pages 13.11.1 to 13.11.4 (Ausubel *et al.*, 1994), *Guide to Yeast Genetics and Molecular Biology*, pages 322–323 (Strathern and Higgins, 1991), or (Holm *et al.*, 1986).

Solutions

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

Minimal Medium (MD, MGYH)

Sterile water

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

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

1% SDS in water

5 M potassium acetate, pH 8.9

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

7.5 M ammonium acetate, pH 7.5

Phenol:chloroform (1:1 v/v)

Preparing Cells

1. Grow at 30°C the recombinant strain and the parent strain to an OD_{600} of 5–10 in 10 ml of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71).
2. Collect the cells by centrifugation at 1500 × g for 5–10 minutes at room temperature.
3. Wash the cells with 10 ml sterile water by centrifugation as in Step 2.

Spheroplasting and Lysis

1. Resuspend the cells in 2 ml of SCED buffer, pH 7.5. Make this solution fresh.
2. Add 0.1–0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve < 80% spheroplasting (monitor the percent spheroplasting using the procedure on pages 38–39).
3. Add 2 ml of 1% SDS, mix **gently** and set on ice (0 to 4°C) for 5 minutes.
4. Add 1.5 ml of 5 M potassium acetate, pH 8.9, and mix **gently**.
5. Centrifuge at 10,000 × g for 5–10 minutes at 4°C and save the supernatant.

Continued on next page

Isolating Total DNA from *Pichia*, continued

DNA Precipitation

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

Determining the Copy Number of Multiple Integrants

Introduction

You may use quantitative dot blots or Southern hybridization to analyze gene copy number (*i.e.,* the actual number of gene copies in your *Pichia* recombinant) (Brierley, *et al*., 1994; Clare, *et al*., 1991a; Romanos, *et al*., 1991; Scorer, *et al*., 1993; Scorer, *et al*., 1994). This requires isolating genomic DNA from *Pichia* recombinants transformed with the parent vector (0 copies of your gene), pAO815 or pPIC3.5K containing 1 copy of your gene (single copy control), and the *Pichia* recombinants containing multiple copies of your gene. Use the protocol detailed on page 86 to isolate genomic DNA.

Quantitative Dot Blot Solutions

You will need the following solutions, 10–15 ml of each for each dot blot.

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

You will also need 3MM paper.

Quantitative Dot Blot Procedure

The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos, *et al*., 1991). It is very important to spot equivalent numbers of cells onto filters in order to quantify copy number. Alternatively, genomic DNA may be isolated and spotted directly onto nitrocellulose or nylon, fixed, and analyzed.

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

   Alternatively, you may grow individual transformants in culture tubes and normalize the absorbance at 600 nm by adding medium.
2. Filter 50 µl of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multi-channel pipettor. Air dry filters.
3. To lyse the cells on the filter, treat the filter with four solutions as follows: place two sheets of 3 MM paper in a tray and soak with 10–15 ml of 50 mM EDTA, 2.5% β-mercaptoethanol pH 9. Make sure that the paper is uniformly soaked and that there are no puddles. Place the nitrocellulose filter face down on the treated 3MM paper. Incubate for 15 minutes at room temperature.
4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak with 10–15 ml of 1 mg/ml Zymolyase 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate for 4 hours at 37°C.

Continued on next page

Determining the Copy Number of Multiple Integrants, continued

Quantitative Dot Blot Procedure, continued

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

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

Southern Blot Analysis

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

Controls

It is very important to include DNA from the host strain alone (GS115 or KM71), the host strain transformed with the parent vector (pPIC3.5K or pAO815), and the host strain transformed with a vector containing one copy of your gene. It is also a very good idea to make a probe to the *HIS4* gene as an internal control for single copy in addition to a probe to your gene. Note that if your gene inserts into *his4*, two copies of the *HIS4* gene are created, one mutant and the other wild-type (see **Recombination and Integration in *Pichia***, *Pichia* Expression Kit manual).

Continued on next page

Determining the Copy Number of Multiple Integrants, continued

General Guidelines

- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al*., 1989), pages 9.31–9.58.
- Isolate genomic DNA and quantify using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane to accurately determine copy number.
- Probe your Southern blot with probes to both *HIS4* and your gene. Note that the point mutation in the *his4* gene in the host strain will not interfere with hybridization if you make the probe complementary to the wild-type gene.
- If you used pPIC3.5K to generate multimers, use *Bgl* II to digest your DNA (Clare, *et al*., 1991a). Note that if you used pPIC3.5K that all multimers are NOT necessarily in a head-to-tail configuration. Some multimers may be head-to-head and others tail-to-tail. We recommend that you think about what products may be produced. An expression cassette in the opposite orientation may produce a different band. The number of multiple copies will cause one or two bands (depending on orientation) in the Southern blot to increase in intensity once you are >2 copies.
- If you used pAO815 to generate multimers, use *Bgl* II and *Bam*H I to digest the genomic DNA and release the multimer. The molecular weight of the band should allow you to determine the number of multimers. If this multimer is too large, you may wish to digest with an enzyme like *Sac* I. This will collapse the multimer into single fragments containing your gene. These will produce a band that will be quite intense. The relative intensity of this band versus a band containing a single copy of your gene will allow you to determine the copy number.
- *Bgl* II digested DNA from GS115 and GS115 transformed with pPIC3.5K or pAO815 will produce a bands of 2.8 kb (the genomic copy of *HIS4)*, and ~6.7 kb (the vector derived copy of *HIS4)*, respectively, when probed with a complementary fragment to *HIS4*.

Procedure for Total RNA Isolation from *Pichia*

Introduction

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

Solutions

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

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

Growing Cells

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

Lysing Cells

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

mRNA Isolation and Northern Analysis

See (Ausubel *et al.*, 1994) for a protocol for mRNA isolation and Northern analysis. The FastTrack® 2.0 mRNA Kit (Cat. no. K1593-02; 6 reactions) is designed to isolate mRNA from 0.2 to 1 mg total RNA. The Micro-FastTrack™ 2.0 Kit (Cat. no. K1520-02; 20 reactions) is designed to isolate mRNA from ~100 µg total RNA. You will need ~1–5 µg mRNA per time point.

β-Galactosidase Assay

Introduction

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

Preparing Solutions

You will need to prepare the following:

- A fresh crude cell lysate of GS115 β-Gal (see page 54)
- Z buffer
- ONPG solution
- 1 M sodium carbonate solution

Recipes for the solutions are below.

Z Buffer

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

1. Dissolve the following in 950 ml deionized water:

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

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

ONPG Solution

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

1. Dissolve the following in 90 ml deionized water:

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

2. Adjust pH to 7.0 with either NaOH or HCl
3. Add 400 mg ONPG. Stir to dissolve and bring the volume up to 100 ml with water.

4 Store at 4°C away from light.

1 M Sodium Carbonate

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

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β-Galactosidase Assay, continued

Procedure

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

Note

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

Determining Specific Activity

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

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

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

Sample Calculation

Here is a sample calculation:

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

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

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

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

Technical Support

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