invitrogen™

PichiaPink™ Expression System

For High-level and Large-scale Expression and Secretion of Bioactive Recombinant Proteins in *Pichia pastoris*

Catalog nos. A11150, A11151, A11152, A11153, and A11154

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User Manual

Table of Contents

Kit Contents and Storage

Introduction

This manual provides guidelines and instructions for high-level and large-scale expression and secretion of bioactive recombinant proteins using the PichiaPink™ Expression System, and is supplied with the products listed below.

Product	Cat. no.
PichiaPink™ Secretion Optimization Kit	A11150
PichiaPink™ Secreted Protein Expression Kit	A11151
PichiaPink™ Vector Kit	A11152
PichiaPink™ Secreted Protein Vector Kit	A11153
PichiaPink™ Expression Strain Kit	A11154

System Components

Each PichiaPink™ product contains the following components. For a detailed description of the contents of each component, see pages v–vi.

Component	Cat. no.						
	A11150	A11151	A11152	A11153	A11154	A11155	A11156
PichiaPink™ Vector Kit	√		√				
PichiaPink™ Secreted Protein Vector Kit		√		√			
PichiaPink™ Expression Strain Kit	√	√			√		
PichiaPink™ Secretion Signal Kit	√					√	
PichiaPink™ Media Kit	√	√					√
PichiaPink™ Expression System Manual	√	√	√	√	√		

Shipping/Storage

Components of the various PichiaPink™ Kits are shipped as described below. Upon receipt, store the components as detailed.

Item	Shipping	Storage
One Shot® TOP10™ Electrocomp™ *E. coli*	Dry ice	–80°C
PichiaPink™ Expression Strains	Dry ice	–80°C
PichiaPink™ Vectors	Gel ice	–20°C
Sequencing primers	Gel ice	–20°C
PichiaPink™ Secretion Signal Sequences	Gel ice	–20°C
PichiaPink™ Media Pouches	Room Temperature	Room Temperature

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Kit Contents and Storage, continued

PichiaPink™ Vector Kit Components

The PichiaPink™ Vector Kit, included in the PichiaPink™ Secretion Optimization Kit, is also available separately from Invitrogen (Cat. no. A11152). **Upon receipt, store the components as listed below.**

Note: pPink-LC vector is a low copy number vector (LC = low copy number), whereas pPink-HC is a high copy number vector (HC = high copy number).

Item	Composition	Amount	Storage
One Shot® TOP10™ Electrocomp™ *E. coli*	—	21 × 50 µL	–80°C
pPink-LC vector	40 µL of 0.5 µg/µL vector in TE buffer, pH 8.0*	20 µg	–20°C
pPink-HC vector	40 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	20 µg	–20°C
5´ *AOX1* primer	20 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	10 µg	–20°C
3´ *CYC1* primer	20 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	10 µg	–20°C

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

PichiaPink™ Secreted Protein Vector Kit Components

The PichiaPink™ Secreted Protein Vector Kit, included in the PichiaPink™ Secreted Protein Expression Kit, is also available separately from Invitrogen (Cat. no. A11153). **Upon receipt, store the components as listed below.**

Note: pPinkα-HC vector is high copy number vector containing the *Saccharomyces cerevisiae* α-mating factor pre-sequence for secreted protein expression.

Item	Composition	Amount	Storage
One Shot® TOP10™ Electrocomp™ *E. coli*	—	21 × 50 µL	–80°C
pPinkα-HC vector	40 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	20 µg	–20°C
5´ α-factor primer	20 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	10 µg	–20°C
3´ *CYC1* primer	20 µL of 0.5 µg/µL vector in TE buffer, pH 8.0	10 µg	–20°C

PichiaPink™ Expression Strain Kit Components

The PichiaPink™ Expression Strain Kit, included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits, is also available separately from Invitrogen (Cat. no. A11154). **Upon receipt, store the strains at –80°C.**

Note: The *ade2* deletion is a full deletion of the *ADE2* gene and part of its promoter.

Item	Relevant Genotype	Amount	Storage
PichiaPink™ Strain 1	*ade2*	1 mL	–80°C
PichiaPink™ Strain 2	*ade2, pep4*	1 mL	–80°C
PichiaPink™ Strain 3	*ade2, prb1*	1 mL	–80°C
PichiaPink™ Strain 4	*ade2, prb1, pep4*	1 mL	–80°C

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Kit Contents and Storage, continued

PichiaPink™ Secretion Signal Kit Components

The PichiaPink™ Secretion Signal Kit, included in the PichiaPink™ Secretion Optimization Kit, is also available separately from Invitrogen (Cat. no. A11155).

The PichiaPink™ Secretion Signal Kit consists of eight secretion signal sequences supplied as phosphorylated duplexes in 40 pmol lyophilized aliquots. The secretion signals and their sources are listed below. **Upon receipt, store the secretion signal duplexes at –20°C.**

For the list of PichiaPink™ secretion signals, their sources, and sequences, see **PichiaPink™ Secretion Signal Sequences** on pages 54–55in the **Appendix.**

Item	Source	Amount
α-amylase signal sequence	*Aspergillus niger*	40 pmol
Glucoamylase signal sequence	*Aspergillus awamori*	40 pmol
Serum albumin signal sequence	*Homo sapiens*	40 pmol
Inulinase presequence	*Kluyveromyces maxianus*	40 pmol
Invertase signal sequence	*Saccharomyces cerevisiae*	40 pmol
Killer Protein signal sequence	*Saccharomyces cerevisiae*	40 pmol
Lysozyme signal sequence	*Gallus gallus*	40 pmol
α-mating factor pre-sequence	*Saccharomyces cerevisiae*	40 pmol

PichiaPink™ Media Kit Components

The PichiaPink™ Media Kit, included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits, is also available separately from Invitrogen (Cat. no. A11156). It includes the following prepackaged media for your convenience. **Keep the media dry and store at room temperature**.

Media	Amount	Yield
PAD Agar	2 pouches	1 liter/pouch of PAD agar medium
YP	2 pouches	1 liter/pouch of YP base medium
YPS	2 pouches	0.2 liters/pouch of YPS base medium
YP Agar	2 pouches	1 liter/pouch of YP agar medium
Dextrose	1 pouch	1 liter/pouch of 20% dextrose

Accessory Products

PichiaPink™ Kit Reagents

Many of the reagents supplied in the PichiaPink™ Kits are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Cat. no.
PichiaPink™ Vector Kit	1 kit	A11152
PichiaPink™ Secreted Protein Vector Kit	1 kit	A11153
PichiaPink™ Expression Strain Kit	1 kit	A11154
PichiaPink™ Secretion Signal Kit	1 kit	A11155
PichiaPink™ Media Kit	1 kit	A11156

Additional Products

Many of the reagents supplied with the PichiaPink™ kits as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Cat. no.
One Shot® TOP10™ Electrocomp™ *E. coli*	10 reactions 20 reactions	C4040-50 C4040-52
One Shot® TOP10™ Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
Platinum® PCR SuperMix	100 reactions	11306-016
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
PureLink™ PCR Purification Kit	50 preps	K3100–01
PureLink™ Quick Gel Extraction Kit	1 kit	K2100-12
PureLink™ Quick Plasmid Miniprep Kit	50 preps 250 preps	K2100-10 K2100-11
FastTrack® 2.0 mRNA Isolation Kit	1 kit	K1593-02
Micro FastTrack™ 2.0 mRNA Isolation Kit	1 kit	K1520-02
T4 DNA Ligase (5 U/µL)	250 units	15224-041
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
(Miller's LB Broth Base)® Luria Broth Base, powder	500 g	12795-027
Yeast Nitrogen Base	1 pouch (67 g) 500 g (bulk)	Q300-07 Q300-09
E-Shot™ Standard Electroporation Cuvettes, 0.1 cm	50/bag	P510-50
E-Shot™ Standard Electroporation Cuvettes, 0.2 cm	50/bag	P520-50
S.O.C. Medium	10 × 10 mL	15544-034

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Accessory Products, continued

Additional Products, continued

Many of the reagents supplied with the PichiaPink™ kits as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Cat. no.
UltraPure™ 20X SSC	1000 mL	15557-044
Pichia EasyComp™ Kit (20 transformations)	1 kit	K1730-01
UltraPure™ Phenol:chloroform:Isoamyl Alcohol (25:24:1 , v/v)	100 mL	15593-031
PureLink™ Air Porous Tape	50 pieces	12262-010
NuPAGE® LDS Sample Preparation Buffer (4X)	10 mL 250 mL	NP0007 NP0008
NuPAGE® Sample Reducing Agent (10X)	250 µL 10 mL	NP0004 NP0009
NuPAGE® Novex 4–12% Bis-Tris Gels	1 box (10 gels)	NP0321BOX
SimplyBlue™ Safe-Stain	1000 mL	LC6060
SilverXpress™ Silver Staining Kit	1 kit	LC6100
UltraPure™ 10% SDS Solution	4 × 100 mL	15553-027
RNAse A (20 mg/mL)	10 mL 25 mL	12091-021 12091-039
Proteinase K	5 mL	25530-049
TE Buffer (20X), RNAse free	100 mL	T11493
Water, distilled	500 mL	15230-162
Pichia Protocols: Methods in Molecular Biology	1 book	G100-01

Introduction

Overview

Introduction

The PichiaPink™ System is a eukaryotic protein expression system based on the eukaryote *Pichia pastoris*, which can be used for high-level (g/liter) and large-scale (1000+ liter) production of secreted recombinant proteins. This section provides general information on *Pichia pastoris* and the PichiaPink™ System.

PichiaPink™ System

The PichiaPink™ System offers the following advantages over existing *Pichia pastoris* based protein expression systems:

- Easy selection of expression clones using *ADE2* complementation (*i.e.,* complementation of adenine auxotrophy) rather than antibiotic resistance.
- Essentially all transformants in the PichiaPink™ system express the protein of interest.
- Three protease knockout PichiaPink™ strains to help reduce the impact of proteases and the need for heavy protease inhibitor use during expression, as well as a "protease wild-type" strain.
- *ADE2* complementation ensures higher stability of transformants during scale-up of protein expression.
- Choice between the pPinkα-HC vector containing *Saccharomyces cerevisiae* α-mating factor pre-sequence for high-copy number secreted protein expression or the pPink-HC and pPink-LC vectors (for high- and low-copy number expression, respectively) and eight secretion signal sequences for optimization of secreted protein expression.
- Optional intracellular protein expression using the pPink-HC and pPink-LC vectors by omitting the secretion signal sequences at the cloning step.
- Simpler media growth conditions for screening and convenient PichiaPink™ media pouches.

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 *et al.*, 2000; Cregg & Higgins, 1995; Cregg *et al.*, 1993; Daly & Hearn, 2005; Higgins & Cregg, 1998; Li *et al.*, 2007; Macauley-Patrick *et al.*, 2005; Nico-Farber *et al.*, 1995; Sreekrishna *et al.*, 1988; Wegner, 1990). General reviews of foreign gene expression in yeast is also available (Romanos, 1995; Romanos *et al.*, 1992).

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Overview, continued

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 the availability of posttranslational modifications, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems and generally gives higher expression levels. As a yeast, *Pichia* 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 pastoris*. These include:

- transformation by complementation
- gene disruption
- gene replacement

In addition, the genetic nomenclature used for *Saccharomyces* has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the *HIS4* gene in both *Saccharomyces* and *Pichia*. There is also cross-complementation between gene products in both *Saccharomyces* and *Pichia*. Several wild-type genes from *Saccharomyces* complement comparable mutant genes in *Pichia pastoris*. Genes such as *ADE2*, *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 the expression of your heterologous protein of interest in the PichiaPink™ System.

AOX1 gene

AOX1 is one of the two genes in *Pichia pastoris* that code for alcohol oxidase, the other being *AOX2*. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* gene. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically $\geq$ 30% of the total soluble protein in cells grown with methanol. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein in the PichiaPink™ System (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a).

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Overview, continued

Expression

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

ADE2 gene

The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step in the *de novo* biosynthesis of purine nucleotides (Jones & Fink, 1982). In *Saccharomyces cerevisiae*, *ADE2* transcription is regulated by adenine and general amino-acid control, where gene expression is repressed in the presence of adenine and activated in the absence of adenine (Gedvilaite & Sasnauskas, 1994). Expression can also be slightly enhanced under general amino-acid starvation conditions (Gedvilaite & Sasnauskas, 1994; Som *et al.*, 2005; Stotz *et al.*, 1993). Although not experimentally shown for *Pichia pastoris*, *ADE2* expression is thought to be regulated in a similar manner. In *Saccharomyces cerevisiae, Pichia pastoris* and other yeast strains, mutations in *ADE2* lead to the accumulation of purine precursors in the vacuole, which causes the colony to be red in color. The pigmentation phenotype can be used as a tool for selection and screening (Jones & Fink, 1982; Zonneveld & van der Zanden, 1995). In addition, *ade2* mutants are adenine auxotrophs that are unable to grow on medium lacking adenine and have a slow growth phenotype on rich medium.

Selection

The strains in the PichiaPink™ kits are *ade2* auxotrophs that are unable to grow in the absence of adenine due to the full deletion of the *ADE2* gene and part of its promoter. The expression plasmids included in the kit contain the *ADE2* gene (under its own promoter) as the selection marker. Transformation of the PichiaPink™ strains with the expression plasmids enable the strain to grow again on medium lacking adenine (Ade dropout medium or minimal medium) (Jones & Fink, 1982; Zonneveld & van der Zanden, 1995). Further, the color of the transformant colonies indirectly indicates the relative expression levels of your protein of interest (see **Transformation and Integration**, page 7).

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Overview, continued

Posttranslational Modifications

In comparison to *Saccharomyces cerevisiae*, *Pichia* may have an advantage in the glycosylation of secreted proteins because it is not as prone to hyperglycosylation. 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 & 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. This is less of a problem for glycoproteins generated in *Pichia pastoris*, because they resemble the glycoprotein structure of higher eukaryotes (Cregg *et al.*, 1993; Gerngross, 2004; Hamilton *et al.*, 2003; Hamilton & Gerngross, 2007).

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* α-mating factor pre-sequence has been used with the most success (Cereghino *et al.*, 2002; Cregg *et al.*, 1993; Scorer *et al.*, 1993).

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

The PichiaPink™ System offers multiple options for targeting your protein of interest to the secretory pathway. The PichiaPink™ Secreted Protein Expression Kit allows you to clone your gene of interest in frame with the *Saccharomyces cerevisiae* α-mating factor pre-sequence using the pPinkα-HC plasmid for secreted expression. The PichiaPink™ Secretion Optimization Kit enables you to screen multiple signal sequences with your gene of interest in low and high copy vectors (pPink-LC and pPink-HC, respectively) for optimal expression and secretion of your recombinant protein.

The secretion signal sequences are provided as lyophilized duplex oligomers in 40 pmol aliquots with the PichiaPink™ Secretion Optimization Kit. They are also available separately as a stand-alone set from Invitrogen (see page vii for ordering information). For the list of the PichiaPink™ secretion signals, their sources, and sequences, see **PichiaPink™ Secretion Signal Sequences** on pages 54–55 in the **Appendix**.

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Overview, continued

Protease Knock-outs *prb1* and *pep4*

Proteases are known to be secreted into the medium during *Pichia* fermentations, which can result in the degradation of the desired protein product. Although protease inhibitors can be added to the medium during fermentation, the cost to do so can be significant. Some of the proteolytic activities of *Pichia* have been characterized. Proteinase A, encoded by the *PEP4* gene, is a vacuolar, self-activating, aspartyl protease involved in the subsequent activation of additional vacuolar proteases such as proteinase B and carboxypeptidase Y. Although the precursor to proteinase B (encoded by the *PRB1* gene) displays approximately 50% activity compared to its proteinase A-processed mature form, carboxypeptidase Y shows an absolute requirement for proteinase A-mediated proteolytic processing for activity (Gleeson *et al.*, 1998).

To help reduce the impact of proteases and the need for heavy protease inhibitor use, PichiaPink™ System offers three protease knockout strains along with the "protease wild-type" PichiaPink™ Strain 1. The PichiaPink™ Strain 2 is a *pep4* knockout and the PichiaPink™ Strain 3 is a *prb1* knockout, while the PichiaPink™ Strain 4 is double knock-out for both proteases (*i.e., prb1* and *pep4*).

Using the PichiaPink™ System

Choosing the Appropriate PichiaPink™ System for Expressing Secreted Proteins

The PichiaPink™ System provides you with two different kits for secreted expression of your recombinant protein of interest.

The PichiaPink™ Secreted Protein Expression Kit (Cat. no. A11151) allows you to clone your gene interest in frame with the *Saccharomyces cerevisiae* α-mating factor pre-sequence using the pPinkα-HC plasmid for secreted expression of your recombinant protein. pPinkα-HC is also available separately as the PichiaPink™ Secreted Protein Vector Kit (see page vii for ordering information).

The PichiaPink™ Secretion Optimization Kit (Cat. no. A11150) enables you to screen multiple signal sequences with your gene of interest in both low and high copy vectors (pPink-LC and pPink-HC, respectively) for optimal expression and secretion of your recombinant protein. pPink-LC and pPink-HC vectors are also available separately as the PichiaPink™ Vector Kit (see page vii for ordering information).

Both PichiaPink™ systems allow the selection of transformants containing your gene of interest without using antibiotics by exploiting the adenine auxotrophy of the untransformed PichiaPink™ strains.

PichiaPink™ System for Expressing Intracellular Proteins

If you prefer to express your gene of interest intracellularly, but still wish to utilize the easy selection scheme of the PichiaPink™ System, you may clone your gene in either the pPink-LC or the pPink-HC vector without the secretion signal, provided that you include the yeast consensus sequence and the ATG start codon in your gene of interest. These vectors are available in the PichiaPink™ Secretion Optimization Kit or the PichiaPink™ Vector Kit (see above).

Choosing the Appropriate Vector for Cloning

For any PichiaPink™ strain to grow on minimal media lacking adenine, sufficient *ADE2* gene product must be expressed from the marker gene on the PichiaPink™ vector upon transformation and integration into the *Pichia* genome.

All PichiaPink™ vectors contain the *ADE2* marker for selecting PichiaPink™ transformants without using antibiotics. However, they express the *ADE2* gene product from promoters of different lengths, which dictate the copy number of the integrated plasmids (see **Transformation and Integration**, next page). The pPink-LC vector has an 82 bp promoter for the *ADE2* marker and is considered to be a low copy plasmid (LC), while the pPink-HC vector has a 13 bp promoter for the *ADE2* marker and is considered a high copy plasmid (HC). In addition, the pPink-LC and pPink-HC vectors also allow you to optimize the secretion of your protein of interest by testing multiple secretion signal sequences (see **Choosing the Appropriate Secretion Signal**, next page). The pPinkα-HC vector has the same high copy *ADE2* marker as pPink-HC, but it also contains the *Saccharomyces cerevisiae* α-mating factor pre-sequence for secreted expression of your recombinant protein.

For maps and features of the PichiaPink™ vectors, see pages 56–58.

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Using the PichiaPink™ System, continued

Choosing the Appropriate Secretion Signal

Heterologous expression in *Pichia pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the *Saccharomyces cerevisiae* α-mating factor pre-sequence has been used with the most success (Cereghino *et al.*, 2002; Cregg *et al.*, 1993; Scorer *et al.*, 1993). You may readily clone your gene of interest in frame with this signal sequence using the pPinkα-HC plasmid.

However, in some cases it may be more advantageous to try to express your protein of interest using different signal sequences. The signal sequence can have a significant impact on the yield of your protein. The PichiaPink™ Secretion Optimization Kit allows you to screen multiple signal sequences with your gene of interest in both the low copy vector pPink-LC and the high copy vector pPink-HC. For a list of the PichiaPink™ secretion signals, their sources, and sequences, see **PichiaPink™ Secretion Signal Sequences** on pages 54–55 in the **Appendix**.

Transformation and Integration

The PichiaPink™ System offers four *ade2* strains for easy selection of transformants containing your gene of interest using adenine auxotrophy. These strains differ in their protease knock-out genotypes to help reduce the impact of proteases and the need for heavy use of protease inhibitors during protein expression (see page 5 for more information). We recommend that you perform pilot experiments using all four PichiaPink™ strains to determine which strain produces the desired amount of your recombinant protein before scaling up expression (see **Expression and Scale-Up**, page 8).

Since the host PichiaPink™ strain is an *ade2* knockout (*i.e.,* full deletion of *ADE2* gene), only cells that express sufficient *ADE2* gene product from the marker gene on the plasmid will grow on minimal medium lacking adenine.

Regardless of the host PichiaPink™ strain, you will observe both white and slightly pink colonies on your selection plates upon transformation with the pPink-HC or the pPinkα-HC vector. The color of the colonies indirectly indicates the relative expression levels of your protein of interest as the color of the colony depends on the copy number of the plasmid, which in turn is determined by the promoter strengths of the markers. The pink colonies express very little *ADE2* gene product, while the white colonies express higher amounts of the *ADE2* gene product, suggesting that those colonies have more copies of the integrated construct.

Strains transformed with the low copy plasmid pPink-LC grows faster on medium lacking adenine, generating white colonies due to the stronger promoter on this vector. Since the promoter is stronger, less *ADE2* expression is required to allow the strains to grow on medium lacking adenine. As a result fewer copies of the *ADE2* gene/expression construct are required in the strain.

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Note

When using the pPink-HC vector, the transformation efficiency of the host strain may appear low because colonies with only a few copies of the marker will not produce enough *ADE2* gene product to grow and will be selected against on medium lacking adenine (*i.e.,* adenine dropout medium or minimal medium). Only colonies that have integrated multiple copies of the *ADE2* marker will able to grow without adenine. Since the gene of interest is linked to the selection marker, the white colonies could also result in higher expression of the gene of interest.

Growth in a Fermentor or Bioreactor

The *ADE2* selection system provided by the PichiaPink™ vectors can be advantageous when a transformed PichiaPink™ strain is grown in a bioreactor. Strains that are *ade2* knockouts grow slowly in rich and minimal medium. If a production strain transformed with an *ADE2* vector were to loose copies of the vector/marker, the strain would revert to its slow-growth phenotype and would not be able to outgrow the desired production strain. Consequently, the batch could still be productive and generate the desired protein titer.

It is also important to note that all PichiaPink™ strains are *ade2* full deletion strains, thus will not revert to Ade^+ due to a simple point mutation or through a process called gene conversion, during which only the marker of the plasmid integrates into the genome and the rest of the plasmid is not integrated.

Expression and Scale-up

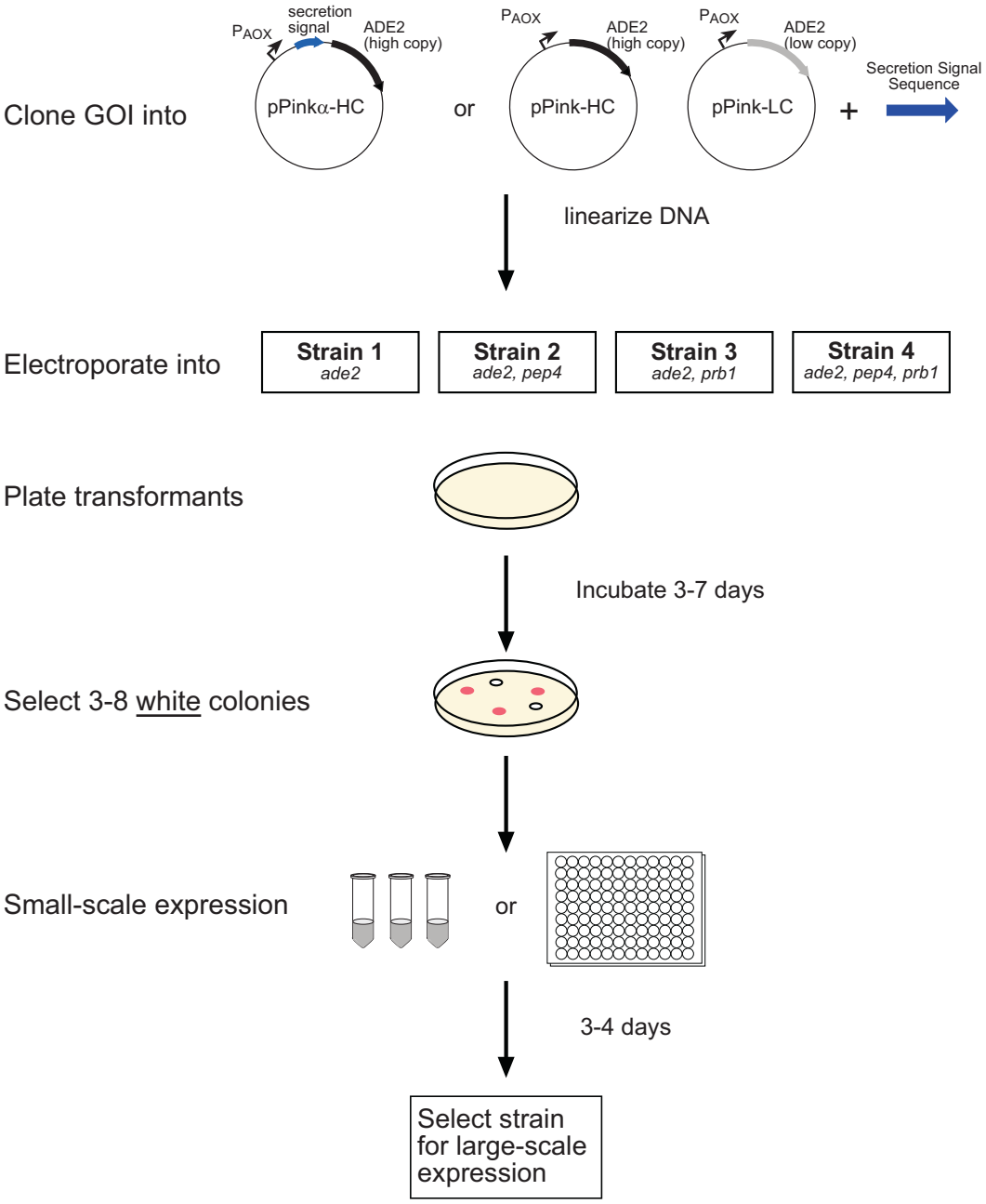
After isolating your *Pichia* recombinants on minimal medium lacking adenine, test the expression and secretion of your protein interest using 3–8 white colonies from each combination of PichiaPink™ strain, vector, and secretion signal. This involves growing a small culture of each recombinant, taking time points, and analyzing the cell pellet and supernatant from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). You may also perform your pilot expression experiments in multi-well plates, which greatly simplifies testing all strain, vector, and secretion signal combinations, especially if you are using the PichiaPink™ Secretion Optimization Kit. We suggest that you analyze your SDS-PAGE gels by Coomassie staining and western blot as expression and secretion are protein dependent, and not all proteins express to the level of grams per liter. We also suggest checking for protein activity if an assay is available.

Choose the recombinant PichiaPink™ strain which best expresses your protein and optimize secreted expression based on the suggestions provided in **Optimizing PichiaPink™ Protein Expression** on pages 44–45. Once expression is optimized, scale-up your expression protocol to produce more protein for purification.

Experimental Outline

Experimental Process

The overall experimental process is presented below. More information about recombination and integration in *Pichia* is provided in a review by Higgins and Cregg (Higgins & Cregg, 1998).



Continued on next page

Experimental Overview, continued

Experimental Steps

The experimental steps necessary to express your protein of interest using the PichiaPink™ System are outlined below. For more details on each step, refer to the indicated pages.

Step	Action	Page
1	Generate recombinant PichiaPink™ vectors containing your gene of interest.	13–25
2	Transform TOP10™ Electrocomp™ *E. coli* and analyze transformants for the correct insert.	26–28
3	Prepare recombinant plasmid DNA to transform PichiaPink™ Strains	29
4	Prepare PichiaPink™ strains for electroporation	30
5	Transform PichiaPink™ strains by electroporation and select transformants	32
	Optional: Transform PichiaPink™ strains using the *Pichia* EasyComp™ Kit	33–37
6	Perform pilot expression experiments using recombinant PichiaPink™ strains	38–40
7	Analyze recombinant protein expression by SDS-PAGE	41–43
8	Optimize protein expression and secretion in recombinant PichiaPink™ strains	44–45
9	Scale-up protein expression in recombinant PichiaPink™ strains	47
10	Express your secreted protein in recombinant PichiaPink™ strains using a fermentor	48–53

Methods

PichiaPink™ Strains

Introduction

PichiaPink™ strains are mutants of *Pichia pastoris* designed for high-level (g/liter) and large-scale (1000+ liter) production of secreted bioactive recombinant proteins. Their general growth conditions and handling requirements are quite similar to *Saccharomyces cerevisiae*; however, we recommend that you familiarize yourself with basic microbiological and sterile techniques, as well as with basic molecular biology and protein chemistry, before attempting to grow and manipulate any microorganism. 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 and Edelstein, 1991), *Guide to Protein Purification* (Deutscher, 1990), *Recombinant Protein Expression in Pichia pastoris* (Cregg *et al.*, 2000), and *Pichia Protocols: Methods in Molecular Biology* (Higgins & Cregg, 1998).

Genotypes of PichiaPink™ Strains

PichiaPink™ Strain 1 is the wild-type *ade2* knockout *Pichia* strain. The *ade2* knockout renders the PichiaPink™ strain an adenine auxotroph (*i.e.,* it requires an external adenine source for growth). These cells are unable to grow on minimal medium or adenine dropout medium, and display a slow-growth phenotype on rich medium. PichiaPink™ Strain 1 is the parental strain from which the rest of the PichiaPink™ strains are derived; therefore, all PichiaPink™ strains carry the *ade2* deletion.

The PichiaPink™ Strain 2 is a *pep4* knockout, which prevents it from synthesizing proteinase A, a vacuolar aspartyl protease capable of self-activation. Since proteinase A also plays a role in the subsequent activation of additional vacuolar proteases, *pep4* knockout strains have a diminished proteinase B activity and lack carboxypeptidase Y activity altogether.

PichiaPink™ Strain 3 is a *prb1* knockout, which prevents it from synthesizing proteinase B, a vacuolar serine protease of the subtilisin family.

PichiaPink™ Strain 4 is double knock-out for both proteinases A and B (*i.e., pep4* and *prb1*), therefore has the lowest protease activity amongst the PichiaPink™ strains.

Continued on next page

PichiaPink™ Strains, continued

Growth of PichiaPink™ Strains

The growth temperature of PichiaPink™ strains is 24–30°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 untransformed PichiaPink™ strains (*i.e., ade2*) in YPD is ~6 to 8 hours
- Untransformed *prb1* PichiaPink™ strains (*i.e.,* PichiaPink™ Strains 3 and 4) grow slightly slower than PichiaPink™ strains expressing functional *PRB1* gene product.
- Doubling time of log phase transformed PichiaPink™ strains (*i.e.,* expressing *ADE2* gene product) in BMGY is ~4 hours
- Doubling time of log phase transformed PichiaPink™ strains in BMMY is ~16 hours
- One OD_{600} = ~5×10^7 cells/mL

Note: Growth characteristics of PichiaPink™ strains may vary depending on the recombinant protein expressed.

Important

The protease deficient *Pichia pastoris* strains (*i.e.,* PichiaPink™ strains 2, 3, and 4) are not as robust as wild-type *Pichia pastoris*, and require greater care in growth and storage, especially during fermentative growth (Gleeson *et al*., 1998).

Growth on Methanol

When using plates or medium containing methanol as growth medium, we recommend that you add methanol every day to compensate for loss because of evaporation or consumption.

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

Note: Some researchers have had success adding methanol to up to 3% for Mut^+ strains similar to PichiaPink™ without any negative effect to their liquid culture.

For long-term storage, prepare frozen stocks of all four PichiaPink™ strains included in this kit (see next page).

Continued on next page

PichiaPink™ Strains, continued

Storing PichiaPink™ Strains

To store cells for weeks to months, use YPD medium and YPD agar slants (see page 60).

1. Streak each strain to obtain single colonies on YPD agar plates. Grow 3–5 days at 24–30°C.
2. Transfer one colony to a YPD stab and grow for 3–5 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.

Day 1:

1. Culture a single colony of each strain in 10 mL of YPD medium for 16–20 hours at 24–30°C, shaking at 300 rpm. This is your starter culture.

   Note: It is important to have adequate aeration for growth. Always use 1:5 ratio of media to flask volume.

Day 2:

2. Seed 200 mL of YPD medium with the starter culture to an OD_{600} of 0.2. Grow shaking for 1–2 days at 24–30°C to an OD_{600} of 2–3.

Day 3 or 4:

3. Harvest the cells by centrifuging at 1,500 × g for 5 minutes. Remove the supernatant and resuspend the cells in YPD medium containing 25% glycerol to a final OD_{600} of 50–100 (approximately 2.5–5.0 × 10^9 cells/mL).
4. Aliquot the cells in cryovials (1 mL aliquots) and freeze in liquid nitrogen or a dry ice/ethanol bath and store at –80°C. Cells will be pink in color.

Note

Although transformed PichiaPink™ strains are very stable, we recommend that you check your cells for correct phenotype and protein expression after extended storage at 4°C or –80°C.

General Cloning Information

Introduction

Before cloning your gene into one of the PichiaPink™ vectors, review the information and guidelines presented below. If you are cloning into pPinkα-HC, you must clone your gene of interest in frame with the α-mating factor pre-sequence. The multiple cloning sites of PichiaPink™ vectors are presented on page 18 (pPink-HC and pPink-LC) and on page 23 (pPinkα-HC)to help you develop a cloning strategy.

General Molecular Biology Techniques

For assistance with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

We recommend that you propagate and maintain the PichiaPink™ vectors and expression constructs in *E. coli* strains that are recombination (*rec*A) and endonuclease A deficient (*end*A), such as TOP10, DH10B™, DH5α, or JM109.

For your convenience, One Shot® TOP10™ Electrocomp™ *E. coli* are included in PichiaPink™ vector kits. For more information on electrocompetent and chemically competent *E. coli* cells available from Invitrogen, refer to our website at www.invitrogen.com or contact Technical Support (see page 73).

Maintaining Plasmids

The PichiaPink™ vectors contain the ampicillin resistance (*bla*) gene to allow selection of the plasmid using ampicillin. To propagate and maintain the PichiaPink™ vectors, we recommend using the following procedure:

1. Use the vector in the 0.5 µg/µL stock solution supplied with the kit to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent.
2. Select transformants on LB plates containing 100 µg/mL ampicillin.

Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 28).

Continued on next page

General Cloning Information, continued

General Considerations

The following are some general considerations applicable to all PichiaPink™ vectors.

- The codon usage in *Pichia* is believed to be similar to *Saccharomyces cerevisiae.* We highly recommend that you codon optimize your gene of interest during synthesis to match the codon usage in *Pichia* for optimal expression in PichiaPink™ strains.
- Many *Saccharomyces* genes have proven to be functional in *Pichia*.
- Propagate and maintain plasmid constructs in a *rec*A, *end*A *E. coli* strain such as TOP10, DH10B™, DH5α, or JM109.
- "AT rich regions" may cause the premature termination of transcripts in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret and Sherman, 1984). If you have problems expressing your gene, check for premature termination by northern analysis and check your sequence for AT rich regions. It may be necessary to change the sequence in order to express your gene (Scorer *et al.*, 1993).
- The PichiaPink™ Secretion Signal duplexes and the pPinkα-HC vector contain the ATG start codon immediately upstream of secretion signal sequences; therefore, it is not necessary to include the initiation codon when designing inserts containing your gene of interest. However, your insert **may** include an ATG. Since the ribosome generally recognizes the first ATG, unless the internal ATG is in a particularly good context, the ATG in front of the secretion signal will be used for initiation.
- Your insert **must** contain a stop codon.
- The PichiaPink™ Secretion Signal duplexes and the pPinkα-HC vector contain the following yeast consensus sequence at the translation initiation site, where the ATG translation initiation codon is shown underlined. This consensus sequence corresponds to the Kozak sequence taken from the native *AOX1* gene.

  GAAACGATGNN

  Note that other sequences are also possible (Hamilton *et al.*, 1987). Although not as strong as the mammalian Kozak translation initiation sequence, the yeast consensus sequence is thought to have a 2–3-fold effect on the efficiency of translation initiation.
- The native 5´ end of the *AOX1* mRNA is noted in the diagram for each multiple cloning site (pages 18 and 23). This information is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.

Continued on next page

Cloning into pPink-HC and pPink-LC

Introduction

Cloning of the secretion signal sequences and the gene of interest into pPink-HC and pPink-LC vectors is a three-way ligation This section provides detailed protocols for preparing the PichiaPink™ vector and the insert containing your gene of interest, and performing the three-way ligation.

Note

Three-way ligation is used for cloning your gene of interest for secreted expression. If you prefer to express your protein intracellularly, but still wish to utilize the easy selection scheme of the PichiaPink™ System, you may clone your gene in either the pPink-LC or the pPink-HC vector without the secretion signal, provided that you include the yeast consensus sequence and the ATG start codon in your gene of interest.

Considerations for pPink-HC and pPink-LC for Secreted Protein Expression

The following considerations apply only to pPink-HC and pPink-LC. For a detailed protocol to clone your gene of interest and the secretion signal sequence into pPink-HC or pPink-LC, see page 19.

- Cloning of the secretion signal sequences and the insert (*i.e.*, the gene of interest) into pPink-HC and pPink-LC vectors is a three-way ligation. To achieve a high efficiency of cloning, you **must** closely follow the instructions for preparing the vector and the insert (see next page for more information).
- For the three-way ligation, the pPink-LC and pPink-HC vectors must be digested with *Eco*R I and an appropriate restriction enzyme that cuts within the multiple cloning site (MCS) downstream of *Eco*R I (*e.g., Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I or *Swa* I). This double digestion creates compatible ends to the 5′ *Eco*R I end of the signal sequence and the 3′ end of the insert containing your gene of interest.
- The insert containing your gene of interest must have a phosphorylated 5´ blunt end (most easily accomplished by adding an *Mly* I site). After the stop codon, your insert must have 3´ end that is compatible to the restriction enzyme used to cut the vector downstream of the *Eco*R I site. Make sure that your gene of interest does **not** contain internal restriction sites for this enzyme and *Mly* I.

  Note: If your gene of interest contains internal sequences recognized by *Mly* I and the enzyme used to cut your vector downstream of the *Eco*R I site in the MCS, you can use gene synthesis to generate your insert with appropriate ends compatible with this scheme.
- The phosphorylated 5´ end of the PichiaPink™ Secretion Signal duplexes has an *Eco*RI-compatible overhang lacking the G (*i.e.,* AATTC, see the **PichiaPink™ Secretion Signal Sequences** on pages 54–55 in the **Appendix**).
- Refer to the diagrams depicting the multiple cloning sites of pPink-HC and pPink-LC on page 18 to develop a cloning strategy.

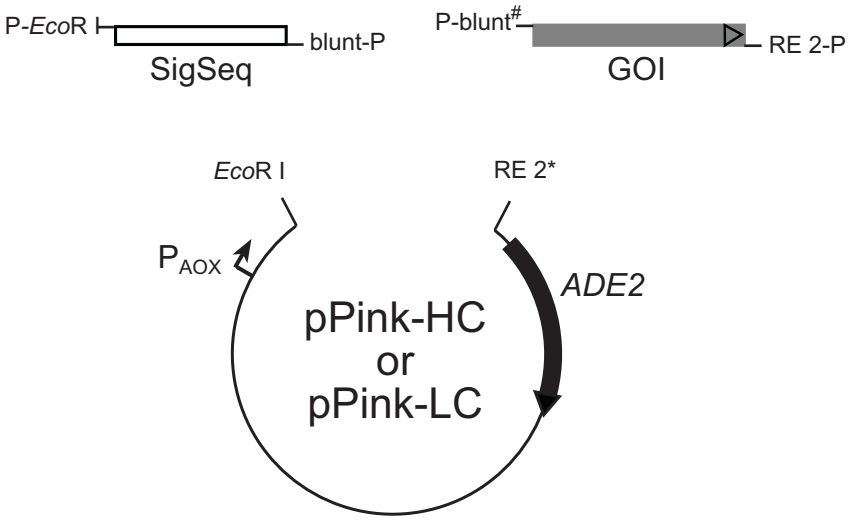
Continued on next page

Cloning into pPink-HC and pPink-LC, continued

Three-way Ligation

The figure below schematically depicts the three-way ligation reaction (see next page for protocol). Prior to performing the three-way ligation, you must digest the pPink-HC and pPink-LC vectors with *Eco*R I and an appropriate restriction enzyme in the multiple cloning site downstream of the *Eco*R I site that does not cut within your gene of interest (*e.g.*, one of *Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I or *Swa* I). This double restriction digest will create compatible ends to the phosphorylated 5´ *Eco*R I end of the signal sequence and the phosphorylated 3´ end of your gene of interest (see diagram below).

Refer to the diagrams depicting the multiple cloning sites of into pPink-HC and pPink-LC vectors on the next page to develop a cloning strategy.



*RE 2: *Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I, or *Swa* I
#blunt: *Mly* I

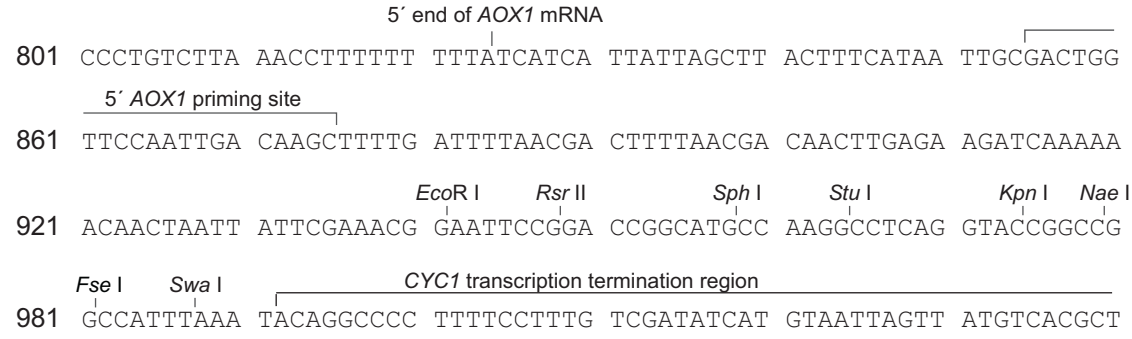
DNA	5´ end	3´ end
pPink-HC and pPink-LC vectors	*Eco*R I	*Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I or *Swa* I
Secretion signal sequence	*Eco*R I, phosphorylated	Blunt, phosphorylated
Insert containing gene of interest	Blunt, phosphorylated (most easily accomplished by adding an *Mly* I site)	*Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I or *Swa* I, phosphorylated

Continued on next page

Cloning into pPink-HC and pPink-LC, continued

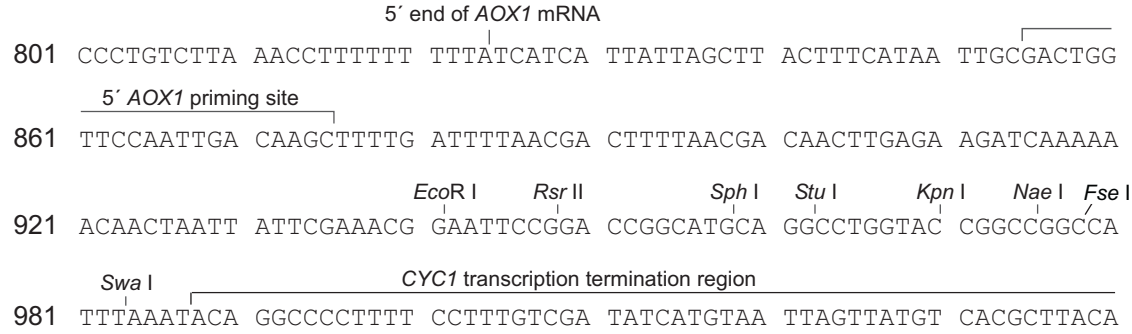
MCS of pPink-HC

Below is the multiple cloning site (MCS) for pPink-HC. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPink-HC is available for downloading at www.invitrogen.com or from Technical Support (see page 73).** For a map of pPink-HC, see page 56.



MCS of pPink-LC

Below is the multiple cloning site (MCS) for pPink-LC. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPink-LC is available for downloading at www.invitrogen.com or from Technical Support (see page 73).** For a map of pPink-LC, see page 57.



Important

The MCSs of pPink-HC and pPink-LC differ at the *Stu* I restriction site. When propagated in *E. coli* cells that are wild-type for Dcm methylation, the pPink-LC vector cannot be digested with *Stu* I due to the methylation of residues adjacent to the *Stu* I recognition sequence. However, pPink-HC vector contains two additional nucleotides on each side of the *Stu* I recognition sequence in the MCS, thus rendering it susceptible to *Stu* I digestion.

Continued on next page

Cloning into pPink-HC and pPink-LC, continued

Restriction Digesting pPink-HC and pPink-LC

Follow the protocol below to digest the pPink-HC and pPink-LC vectors with *Eco*R I and an appropriate restriction enzyme (*i.e., Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I, or *Swa* I) to create compatible ends to the phosphorylated 5´ *Eco*RI end of the signal sequence and the phosphorylated 3´ end of your gene of interest. We recommend dephosphorylating the vector using Calf Intestinal Alkaline Phosphatase (CIAP) prior to three-way ligation to prevent self-ligation of the vector.

Note: Alternatively, you may pre-digest the vectors with any one of the restriction enzymes that cuts between *Eco*R I and RE 2 (see page 17) in the MCS prior to *Eco*R I and RE 2 digestion to help reduce possible background of vector self-ligation due to incomplete digestion.

Materials needed:

*Eco*R I (10 units/µL)

Rsr II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I, or *Swa* I (10 units/µL)

Calf Intestinal Alkaline Phosphatase (CIAP) (1 unit/µL)

PureLink™ Quick Gel Extraction Kit (see page vii).

Procedure:

1. Set up a restriction digest as follows:

pPink-HC or pPink-LC (0.5 µg/µL)	2 µL
10X Restriction Enzyme Buffer	1 µL
Sterile water	5 µL
*Eco*R I (10 units/µL)	1 µL
RE 2* (10 units/µL)	1 µL

2. Incubate for 2 hours to overnight at 37ºC.
3. *Optional*: Add 2 units of CIAP (at 1 unit/µL) to the reaction mix and incubate for 1 hour at 37ºC.
4. Gel purify the digested vectors using the PureLink™ Quick Gel Extraction Kit and proceed to **Three-way Ligation Protocol** (next page).

***RE 2**: *Rsr* II, *Sph* I, *Stu* I, *Kpn* I, *Nae* I, *Fse* I, or *Swa* I

Continued on next page

Cloning into pPink-HC and pPink-LC, continued

Three-way Ligation Protocol

Follow the protocol below to clone your gene of interest in frame with the secretion signal sequence into pPink-HC and pPink-LC vectors.

Materials needed:

T4 DNA Ligase

pPink-HC or pPink-LC vector, linearized (see previous page)

Insert with gene of interest

Secretion signal sequence (see pages 54–55)

Procedure:

1. Resuspend the secretion signal sequence you want to use in 40 µL of TE buffer to prepare 10X stock solution at 1 pmol/µL. Dilute 1 µL of this solution 10-fold to prepare 0.1 pmol/µL working solution for three-way ligation.
2. Set up a three-way ligation reaction in a 0.5 mL microcentrifuge tube as follows:

Component	Amount
5X ligase buffer	2 µL
T4 DNA ligase	0.5 µL
pPink-HC or pPink-LC (~4 fmol)	1 µL (at 20 ng/µL)
Insert with gene of interest (~12–20 fmol)	1 µL (at 10–20 ng/µL)
Secretion signal sequence (1X, ~0.1 pmol)	1 µL (at 10–20 ng/µL)
Sterile water	to 10 µL

3. Mix gently, centrifuge briefly, and incubate at 25ºC for 1–2 hours, or at 16ºC overnight.
4. Proceed to **Transforming *E. coli* Cells**, page 26.

Cloning into pPinkα-HC

Introduction

Cloning of the secretion signal sequences and the gene of interest into pPinkα-HC This section provides detailed protocols for preparing the pPinkα-HC vector and the insert containing your gene of interest.

Considerations for pPinkα-HC

The following considerations apply only to pPinkα-HC. For a detailed protocol on cloning your gene of interest in frame with the α-mating factor pre-sequence into pPinkα-HC, see page 24.

- You **do not need** to include the ATG start codon or the yeast consensus Kozak sequence in your gene of interest as they are already present in the α-mating factor pre-sequence on pPinkα-HC. However, you **must** clone the open reading frame (ORF) of the mature gene of interest in frame and downstream of the α-mating factor pre-sequence.
- The ATG start codon in the α-mating factor pre-sequence in pPinkα-HC corresponds to the native initiation ATG of the *AOX1* gene.
- Prior to performing ligation reaction to clone your insert, you must digest the pPinkα-HC vector with *Stu* I (creates a blunt end) and an appropriate restriction enzyme in the multiple cloning site downstream of the *Stu* I site that does not cut within your gene of interest (*e.g.*, *Kpn* I, *Nae* I, *Fse* I or *Swa* I). This double digestion creates compatible ends to the 5′ *Mly* I end of the signal sequence and the 3′ end of the insert containing your gene of interest.
- The insert containing your gene of interest must have a phosphorylated 5´ blunt end (most easily accomplished by adding an *Mly* I site) and a 3´ overhang after the stop codon that is compatible to the restriction enzyme used to linearize pPinkα-HC (*i.e., Kpn* I, *Nae* I, *Fse* I or *Swa* I). Make sure that your gene of interest does **not** contain internal restriction sites for *Mly* I and the restriction enzyme used to linearize your vector.

  Note: If your gene of interest contains internal sequences recognized by *Mly* I and the restriction enzyme used to linearize your vector, you can use gene synthesis to generate your insert with appropriate ends compatible with this ligation scheme.
- Refer to the diagram depicting the multiple cloning site of pPinkα-HC on page 23 to develop a cloning strategy.

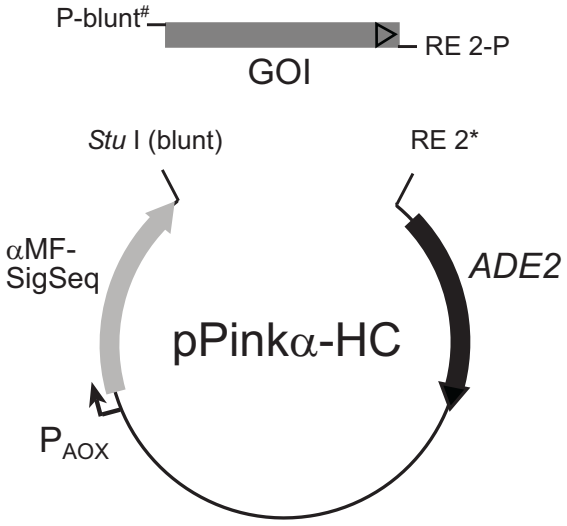
Continued on next page

Cloning into pPinkα-HC, continued

Cloning into pPinkα-HC

The figure below schematically depicts the pPinkα-HC cloning reaction. Prior to performing ligation reaction to clone your insert, you must digest the pPinkα-HC vector with *Stu* I (creates a blunt end) and an appropriate restriction enzyme in the multiple cloning site downstream of the *Stu* I site that does not cut within your gene of interest (*e.g.*, one of *Kpn* I, *Nae* I, *Fse* I, or *Swa* I). This double restriction digest will create compatible ends to the phosphorylated 5´ blunt end (most easily created by adding an *Mly* I site) and the phosphorylated 3´ end of your gene of interest (see diagram below).

Refer to the diagram depicting the multiple cloning site of into pPinkα-HC on the next page to develop a cloning strategy.



*RE 2: *Kpn* I, *Nae* I, *Fse* I, or *Swa* I

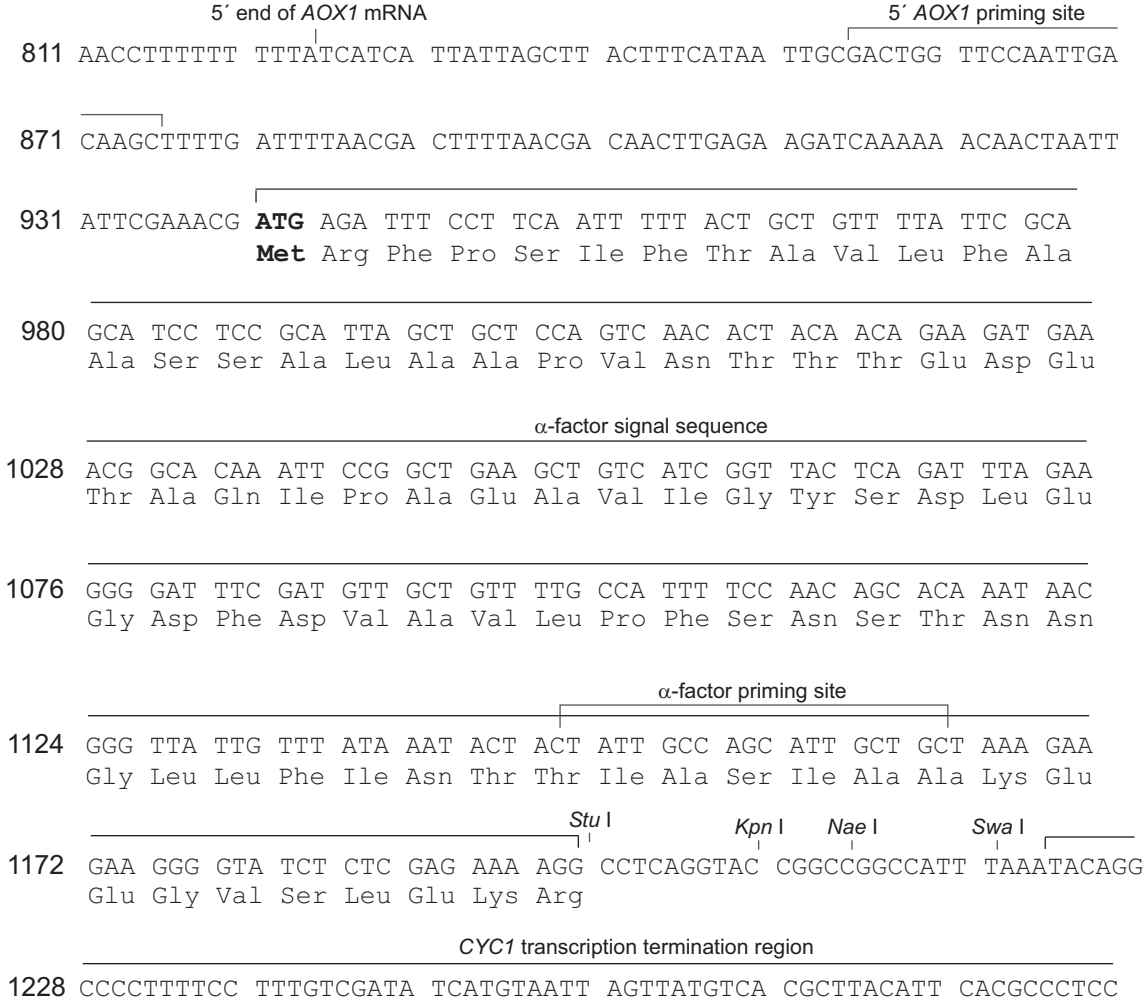
#blunt: *Mly* I

DNA	5´ end	3´ end
pPinkα-HC	*Stu* I (blunt)	*Kpn* I, *Nae* I, *Fse* I, or *Swa* I
Insert containing gene of interest	*Mly* I (blunt), phosphorylated	*Kpn* I, *Nae* I, *Fse* I, or *Swa* I, phosphorylated

Cloning into pPinkα-HC, continued

MCS of pPinkα-HC

Below is the multiple cloning site (MCS) for pPinkα-HC. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The vector sequence of pPinkα-HC is available for downloading at www.invitrogen.com or from Technical Support (see page 73).** For a map of pPinkα-HC, see page 58.



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Cloning into pPinkα-HC, continued

Restriction Digesting pPinkα-HC

Follow the protocol below to digest pPinkα-HC with *Stu* I (creates a blunt end) and an appropriate restriction enzymes to produce compatible ends to the phosphorylated 5´ blunt end (*i.e., Mly* I) and phosphorylated 3´ end compatible to RE 2 (see below) of your gene of interest. We recommend dephosphorylating the vector using Calf Intestinal Alkaline Phosphatase (CIAP) prior to three-way ligation to prevent self-ligation of the vector.

Materials needed:

Stu I (10 units/µL)

Kpn I, *Nae* I, *Fse* I, or *Swa* I (10 units/µL)

Calf Intestinal Alkaline Phosphatase (CIAP) (1 unit/µL)

PureLink™ Quick Gel Extraction Kit (see page vii).

Procedure:

1. Set up a restriction digest as follows:

pPinkα-HC (0.5 µg/µL)	2 µL
10X Restriction Enzyme Buffer	1 µL
Sterile water	5 µL
Stu I (10 units/µL)	1 µL
RE 2* (10 units/µL)	1 µL

2. Incubate for 2 hours to overnight at 37ºC.
3. *Optional*: Add 2 units of CIAP (at 1 unit/µL) to the reaction mix and incubate for 1 hour at 37ºC.
4. Gel purify the digested vectors using the PureLink™ Quick Gel Extraction Kit (see page vii) and continue to **Ligation Procedure** (next page).

***RE 2**: *Kpn* I, *Nae* I, *Fse* I or *Swa* I

Continued on next page

Cloning into pPinkα-HC, continued

Ligation Procedure

Follow the protocol below to clone your gene of interest in frame with α-mating factor pre-sequence into pPinkα-HC.

Materials needed:

T4 DNA Ligase

pPinkα-HC, linearized (see previous page)

Insert with gene of interest

Procedure:

1. Set up a ligation reaction in a 0.5 mL microcentrifuge tube as follows:

Component	Amount
5X ligase buffer	2 µL
T4 DNA ligase	0.5 µL
pPink-HC or pPink-LC (~4 fmol)	1 µL (at 20 ng/µL)
Gene of interest (~12–20 fmol)	1 µL (at 10–20 ng/µL)
Sterile water	to 10 µL

2. Mix gently, centrifuge briefly, and incubate at 25ºC for 1–2 hours, or at 16ºC overnight.
3. Proceed to **Transforming *E. coli* Cells**, next page.

Transforming *E. coli* Cells

Introduction

After you have ligated your insert and the PichiaPink™ Secretion Signal duplex into the appropriate PichiaPink™ vector, you need to transform electrocompetent *E. coli* with your ligation reaction, and analyze the transformants for the presence and orientation of your insert. There is no blue/white screening for the presence of insert with PichiaPink™ vectors. After obtaining the desired recombinant plasmid, you are ready to transform into PichiaPink™ Expression Strains.

Materials Needed

- One Shot® TOP10™ Electrocomp™ *E. coli*
  Note: You may also perform chemical transformation using chemically competent *E. coli* cells. Do not use One Shot® TOP10™ Electrocomp™ *E. coli* for chemical transformation.
- Electroporator and cuvettes (*e.g.*, E-Shot™ Standard Electroporation Cuvettes, 0.1 cm; see page vii)
- S.O.C. medium at room temperature (see page vii)
- 15 mL snap-cap tube (*e.g.*, Falcon)
- LB plates containing 50–100 µg/mL ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Transformation protocol

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

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 4 µL of the ligation reaction from Step 2, page 20 or 25, into a 0.1 cm cuvette (see page vii) containing 50 µl of One Shot® TOP10™ Electrocomp™ *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
   Note: Other electrocompetent *rec*A, *end*A *E. coli* strains are also suitable.
2. Electroporate your samples using your own protocol and your electroporator.
   Note: If you have problems with arcing, see below
3. Immediately add 250 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 mL snap-cap tube and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.

Procedure continued on next page

Continued on next page

Transforming *E. coli* Cells, continued

Transformation protocol, continued

Procedure continued from previous page

5. Spread 50–100 µL from each transformation on a prewarmed LB plate containing 50–100 µg/mL ampicillin and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.

   Note: You may save the remainder of your transformation mix at 4°C. If you do not get transformants or very few transformants, you may then plate out the remainder of the transformation mix onto LB-ampicillin plates.

6. Pick ~8–10 colonies for analysis (see **Analyzing Positive Clones** or **Analyzing Transformants by PCR**, next page).

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the ligation reaction and resuspend in water prior to electroporation.

High-throughput *E. coli* Transformation

We recommend using chemical transformation for high-throughput applications such as cloning multiple secretion signal sequences. You can use 96-well plates and multi-channel pipettors to perform your ligation reactions, and then directly transfer ligation mixtures into a new 96-well plate containing chemically competent cells. You can perform the heat shock step and subsequent incubation in a water bath, heat block, or thermocycler set to the appropriate temperature.

Analyzing Positive Clones

1. Pick 8–10 colonies from each transformation and culture them overnight in LB medium containing 50–100 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ Quick Plasmid Miniprep Kit (see page vii).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.
4. Alternatively, you may colony PCR 8–10 colonies directly from the selection plate for the presence of the insert (see next page).
5. You should isolate plasmid DNA from positive colonies and sequence to confirm proper insertion of the signal sequence and gene of interest.

Continued on next page

Transforming *E. coli* Cells, continued

Analyzing Transformants by PCR

Alternatively, you may analyze positive transformants for the presence of your insert using colony PCR. For pPink-HC and pPink-LC, use a combination of the 5´ *AOX1* or the 3´ *CYC1* primer and a primer that hybridizes within your insert. For pPinkα-HC, use a combination of the 5´ α-factor or the 3´ *CYC1* primer and a primer that hybridizes within your insert. You will need to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are also suitable (*e.g.*, high-throughput, 96-well).

Materials Needed

Platinum® PCR SuperMix (see page vii)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of Platinum® PCR SuperMix into a 0.5 mL microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 8–10 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using the appropriate PCR parameters.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing Recombinant Clones

We strongly recommend that you sequence your construct before transforming into PichiaPink™ to confirm that gene is in frame with secretion signal sequence. Use the sequencing primers included in the kit to sequence your construct.

- To sequence your construct in pPink-HC and pPink-LC, use the 5´ *AOX1* and the 3´ *CYC1* Sequencing Primers.
- To sequence your construct in pPinkα-HC, use the 5´ α-factor or the 5´ *AOX1* and the 3´ *CYC1* Sequencing Primers.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at –20°C.

1. Streak the original colony out for single colony on LB plates containing 50–100 µg/mL ampicillin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Preparing Transforming DNA

Introduction

At this point, you should have your gene cloned into the appropriate PichiaPink™ vectors. Your construct should be correctly fused to the secretion signal sequence and the yeast consensus Kozak sequence. For the next step in your PichiaPink™ experiments, you will purify plasmid DNA and linearize the plasmid DNA prior to transformation and selection in PichiaPink™ Strains. We recommend that you prepare enough plasmid DNA to transform all four PichiaPink™ Strains to enable you choose the best strain for your large scale secreted expression.

Plasmid Preparation

Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform PichiaPink™ Strains (5–10 μg of each plasmid per each transformation). We recommend the PureLink™ Quick Plasmid Miniprep Kit for quick purification of pure plasmid DNA (see page vii). Once you have purified plasmid DNA, proceed to **Preparing PichiaPink™ Strains for Electroporation**, page 30.

Method of Transformation

We recommend electroporation or chemical methods for transforming PichiaPink™ Strains with PichiaPink™ vectors. Electroporation yields 10^3 to 10^4 transformants per μg of linearized DNA and does not destroy the cell wall of *Pichia*. If you do not have access to an electroporation device, use the EasyComp™ procedure on page 33.

In contrast with *Pichia* systems that rely on antibiotic resistance markers for selection, you may also use spheroplasting for transforming PichiaPink™ Strains. Spheroplasting involves removal of the cell wall to allow DNA to enter the cell. When antibiotic resistance markers are used, cells must first regenerate the cell wall before they are able to express the resistance gene. PichiaPink™ transformants, on the other hand, are selected using nutritional markers.

Continued on next page

Preparing Transforming DNA, continued

Restriction Digest

Follow the protocol below to digest your PichiaPink™ construct with the appropriate restriction enzyme. The restriction enzymes listed below are unique cutters within the *TRP2* region of PichiaPink™ vectors, and as such integration of the linearized vector can only occur at the *TRP2* locus of the PichiaPink™ strain.

Materials needed:

Restriction enzyme that does not cut within your gene (*e.g.*, one of *Mam* I, *Eco*N I, *Spe* I, or *Afl* II)

100% and 80% ethanol

3M sodium acetate

Optional: Phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v)

Procedure:

1. Digest ~5–10 µg of plasmid DNA with one of the restriction enzymes listed below. Each enzyme cuts one time in the *TRP2* region to linearize the PichiaPink™ vectors. Choose an enzyme that does not cut within your gene (*e.g.*, one of *Mam* I, *Eco*N I, *Spe* I, or *Afl* II).
2. We recommend that you check a small aliquot of your digest by agarose gel electrophoresis for complete linearization.
3. If the vector is completely linearized, heat inactivate or add EDTA to stop the reaction.

   Optional: You may also phenol/chloroform extract your linearized vector once.
4. Ethanol precipitate the digest using 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol.
5. Centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend in 10 µl sterile, deionized water. Use immediately or store at –20°C.

Note

You may also linearize your PichiaPink™ vector by cutting it at a unique site within the *AOX1* promoter region to promote integration into the PichiaPink™ genome. We recommend using the *Pme* I restriction enzyme, provided that your gene of interest does not contain the *Pme* I recognition site.

You can **not** linearize your PichiaPink™ vector within the *ADE2* gene and use the *ADE2* locus for integration because the full *ADE2* ORF has been knocked out in the PichiaPink™ strains as it is used as a nutritional selection marker.

Preparing PichiaPink™ Strains for Electroporation

Introduction

This section provides the protocols for generating electrocompetent PichiaPink™ Strains. Use the electrocompetent cells on the same day that you have prepared them. Do **not** store electrocompetent cells as the transformation efficiency decreases dramatically upon storage at –80°C.

Materials Needed

- Working glycerol stock of PichiaPink™ Strains (see page 13)
- YPD media and YPD agar plates (see **Recipes**, page 60)
- Sterile water, ice cold (place on ice the day of the experiment)
- 1 M sorbitol, sterile and ice cold (place on ice the day of the experiment)
- 125 mL and 1 liter baffled culture flasks, sterile
- 300 mL centrifuge bottles, sterile

Protocol

Day 1:

1. Streak each strain from a working glycerol stock or stab for single colonies on YPD agar plates. Grow at 24–30°C for 3–5 days until colonies form.

Day 2:

2. Inoculate 10 mL of YPD media in a sterile 125 mL baffled flask with a single colony from the YPD agar plate.
3. Incubate shaking at 300 rpm at 24–30°C for 1–2 days. This is your starter culture.
   Note: It is important to have adequate aeration for growth. Always use at least a 1:5 ratio of media to flask volume.
4. Use the starter culture to inoculate 100 mL of YPD media in a sterile 1 liter flask to an OD_{600} of 0.2. Grow the culture for 1–2 days at 24–30°C.

Day 3 or 4:

5. Monitor the OD_{600} of the culture until it reaches between 1.3–1.5 (log phase growth). Transfer cells to a 300 mL centrifuge bottle, centrifuge at 1,500 × g at 4°C for 5 minutes. Resuspend the pellet with 250 mL of ice-cold, sterile water.
6. Centrifuge the cells as in previous step, then resuspend the pellet with 50 mL of ice-cold, sterile water.
7. Centrifuge the cells as in previous step, then resuspend the pellet in 10 mL of ice-cold 1 M sorbitol.
8. Centrifuge the cells as in previous step, then resuspend the pellet in 300 µL of ice-cold 1 M sorbitol for a final volume of approximately 500 µL. Keep the cells on ice and use that day. Do **not** store cells.

Transforming PichiaPink™ Strains by Electroporation

Introduction

We recommend electroporation as the method of choice for transforming PichiaPink™ Strains as it yields some of the highest transformation frequencies in *Pichia*. This section provides protocols and guidelines for transforming each of your PichiaPink™ strains by electroporation.

Materials Needed

- Electrocompetent PichiaPink™ Strains (previous page)
- 5–10 µg pure PichiaPink™ plasmid construct (*i.e.*, pPink-HC, pPink, or pPinkα-HC containing your insert) for each transformation
- PichiaPink™ YPDS media (see **Recipes**, page 61)
- PichiaPink™ PAD selection plates (see **Recipes**, page 61)
- Electroporation device and 0.2 cm cuvettes (*e.g.*, E-Shot™ Standard Electroporation Cuvettes, 0.2 cm; see page vii).

Electroporation Protocol

For each transformation (*i.e.*, each strain and plasmid construct):

1. Mix 80 µl of the electrocompetent PichiaPink™ cells (from Step 8, previous page) with 5–10 µg of linearized plasmid DNA (Step 4, page 30) and transfer to an ice-cold 0.2 cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells in the electroporator according to the instrument manufacturer's instructions for yeast. (The time constant should be ~5 milliseconds.)
4. Immediately after pulsing the cells, add 1 mL of ice-cold YPDS media to the cuvette and mix by pipetting up and down.
5. Incubate the cells at 24–30°C for at least 2 hours without shaking. You do not need to remove the cells from the cuvettes.
   Note: You may incubate the cells for up to 12 hours.
6. After incubation, spread 100–300 µL of the cell mixtures on PAD selection plates, and incubate at 24–30°C for 3–10 days until distinct colonies are formed.
7. Pick 3–8 **white** colonies from each plate and restreak on fresh PAD selection plates. Proceed to small-scale expression to test for secreted expression of your gene (see **Expressing Recombinant PichiaPink™ Strains**, page 38).
   Note: You may also wish to analyze the integration of your insert in the PichiaPink™ strain you have transformed. See **PCR Analysis of PichiaPink™ Integrants** in the **Appendix**, page 64.

Note

The color of the colonies indicates the relative expression levels of your protein of interest. The pink colonies express very little *ADE2* gene product, while the white colonies express higher amounts of the *ADE2* gene product, suggesting that those colonies have more copies of the integrated construct. See pages 7–8 for more information.

Continued on next page

Transforming PichiaPink™ Strains by Electroporation, continued

Troubleshooting

The table below provides solutions to possible problems you may encounter when preparing and transforming competent PichiaPink™ cells by electroporation.

Problem	Probable Cause	Possible Solution
Low efficiency of transformation	Too little DNA used in transformation.	Check the concentration of linearized DNA used for transformation. For optimum transformation efficiency, you should use 5–10 µg of linearized plasmid DNA.
	OD_{600} of PichiaPink™ cells not in the optimum range.	Grow cells to OD_{600} 1.3–1.6.
	Incubation time is too short or temperature is too low.	PichiaPink™ transformations may be incubated for longer periods of time (up to 3 hours) and at higher temperature (35–37°C) before plating. This may, in some instances, result in higher transformation efficiencies.
	Plate incubation time too short.	*ade2* strains grow slowly. Transformants can take up to seven days to appear.
	Plasmid linearized in the wrong region.	Linearize the PichiaPink™ vector containing your gene of interest in either the *TRP2* gene or the *AOX1* promoter region for integration in PichiaPink™ strains. You cannot use *ADE2* locus for integration as the PichiaPink™ are *ade2* knockouts.

EasyComp™ Transformation

Introduction

The *Pichia* EasyComp™ Kit produces chemically competent *Pichia* cells and is included to provide an alternative to electroporation and a rapid, convenient method for transforming your PichiaPink™ Strains. However, the low transformation efficiency of chemically competent *Pichia* (3 µg plasmid DNA yields about 50 colonies) makes it more difficult to screen a large number of integrants for the highest secreted expression levels. Note that the cells are prepared differently for electroporation. **Do not use cells prepared using the EasyComp™ protocol for electroporation.**

Materials Needed

- *Pichia* EasyComp™ Kit (see page vii for ordering information)
- 30°C rotary shaking incubator
- PichiaPink™ YPD medium (see **Recipes**, page 60)
- 50 mL, sterile conical tubes
- Centrifuge suitable for 50 mL conical tubes (floor or table-top)
- 1.5 mL sterile screw-cap microcentrifuge tubes
- Styrofoam box or paper towels

Preparing Competent Cells

1. Inoculate 10 mL of YPD with a single colony of your PichiaPink™ strain. Grow for 1–2 days at 24–30°C in a shaking incubator (250–300 rpm).
2. Dilute cells from the overnight culture to an OD_{600} of 0.1–0.2 in 10 mL of YPD. Grow the cells at 24–30°C in a shaking incubator until the OD_{600} reaches 0.6–1.0. This will take approximately 18 to 24 hours.
3. Pellet the cells by centrifugation at 500 × g for 5 minutes at room temperature. Discard the supernatant.
4. Resuspend the cell pellet in 10 mL of Solution I from the EasyComp™ kit (equilibrated to room temperature). No incubation time is required.
5. Pellet the cells by centrifugation at 500 × g for 5 minutes at room temperature. Discard the supernatant.
6. Resuspend the cell pellet in 1 mL of Solution I. The cells are now competent.
7. Aliquot 50 to 200 µl of competent cells into labeled 1.5 mL sterile screw-cap microcentrifuge tubes.

   Note: 50 µl of cells are used for each transformation. Cells can be thawed and refrozen several times without significant loss in transformation efficiency.
8. You may keep the cells at room temperature and use directly for transformation or freeze for future use. To freeze cells, place tubes in a Styrofoam box or wrap in several layers of paper towels and place in a –80°C freezer. It is important that the cells freeze down slowly. **Do not snap-freeze the cells in liquid nitrogen.**
9. Proceed to the transformation procedure, next page.

Continued on next page

EasyComp™ Transformation, continued

Note

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

Transformation

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

Materials Needed

- Chemically competent PichiaPink™ cells (Step 8, previous page)
- Water baths or heat blocks at 30°C and 42°C
- PAD agar plates (see **Recipes**, page 61)

Before Beginning

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

Transformation Protocol

1. For each transformation, thaw one tube of competent cells at room temperature and aliquot 50 µl into a sterile microcentrifuge tube. If transforming fresh cells, use 50 µl of cells from **Preparing Competent Cells**, Step 7, previous page
2. Add 5–10 µg of linearized PichiaPink™ expression vector DNA to the competent cells.

   Note: The volume of DNA should not exceed 5 µl. You may use linearized DNA directly from a restriction digest reaction without affecting transformation efficiency. Phenol chloroform extraction and ethanol precipitation are not necessary.
3. Add 1 mL of Solution II to the DNA/cell mixture and mix by vortexing or flicking the tube.
4. Incubate the transformation reactions for 1 hour at 30°C in a water bath or incubator. Mix the transformation reaction every 15 minutes by vortexing or flicking the tube. Failure to mix the transformation reaction every 15 minutes will result in decreased transformation efficiency.
5. Heat shock the cells in a 42°C heat block or water bath for 10 minutes.
6. Split the cells into 2 microcentrifuge tubes (approximately 525 µl per tube) and add 1 mL of YPD medium to each tube.

Procedure continued on next page

Continued on next page

EasyComp™ Transformation, continued

Transformation Protocol, continued

Procedure continued from previous page

7. Pellet the cells by centrifugation at 3,000 × g for 5 minutes at room temperature. Discard the supernatant.
8. Resuspend each tube of cells in 500 µl of Solution III and combine the cells into one tube.
9. Pellet the cells by centrifugation at 3,000 × g for 5 minutes at room temperature. Discard the supernatant.
10. Resuspend the cell pellet in 100 to 150 µl of Solution III.
11. Plate the entire transformation on PAD selection plates using a sterile spreader. Incubate the plates for 3 to 10 days at 30°C. Each transformation should yield approximately 50 colonies.
12. Pick 3–8 **white** colonies from each plate and restreak on fresh PAD selection plates. Proceed to small-scale expression to test for secreted expression of your gene (see **Expressing Recombinant PichiaPink™ Strains**, next page).
    Note: You may also wish to analyze the integration of your insert in the PichiaPink™ strain you have transformed. See **PCR Analysis of PichiaPink™ Integrants** in the **Appendix**, page 64.

High-throughput Transformation

You can easily adapt the procedure for chemically transforming PichiaPink™ Strains with the *Pichia* EasyComp™ Kit by using 96-well plates and multi-channel pipettors You can perform the heat shock step and subsequent incubation in a water bath, heat block, or thermocycler set to the appropriate temperature, and the centrifugation step by using a 96-well plate swing-out centrifuge insert.

Continued on next page

EasyComp™ Transformation, continued

Troubleshooting

The table below provides solutions to possible problems you may encounter when preparing and transforming competent PichiaPink™ cells using the *Pichia* EasyComp™ Kit.

Problem	Probable Cause	Possible Solution
Low efficiency of transformation	The pH of Solution I or Solution III may have drifted. The pH of both solutions should be 8.0	Check the pH of Solutions I and III. If the pH is low, increase it by adding NaOH. If the pH is high, decrease it by adding HCl. Store solutions at 4°C in order to minimize drift in pH.
	Transformation reaction not mixed during incubation	Be sure to mix the transformation reaction every 15 minutes throughout the 1 hour incubation at 30°C. Vortexing works best.
	Incubation time is too short or temperature is too low.	PichiaPink™ transformations may be incubated for longer periods of time (up to 3 hours) and at higher temperature (35–37°C). This may, in some instances, result in higher transformation efficiencies.
	Cell density is too low (OD_{600} <0.6)	Resuspend cells from Preparing Competent Cells, Step 6, page 34, in a smaller volume (*i.e.*, 500 µL)

Expressing Recombinant PichiaPink™ Strains

Introduction

You should now have several PichiaPink™ recombinant strains which you have confirmed by PCR analysis to contain your insert (see page 65). This section provides guidelines to determine the optimal method and conditions for expression of your gene. We recommend that you consider the factors and guidelines below before starting secreted expression in PichiaPink™ strains. As with any expression system, optimal expression conditions depend on the characteristics of the protein being expressed.

Pilot Expression

The PichiaPink™ System offers four *ade2* strains strains that differ in their protease knockout genotypes to help reduce the impact of proteases and the need for heavy protease inhibitor use during protein expression (see page 5 for more information). In addition, the PichiaPink™ Secretion Optimization Kit provides you with eight separate secretion signal sequences to help you optimize secreted expression of your protein of interest. We recommend that you perform pilot experiments using 3–8 **white** colonies from each combination of PichiaPink™ strain, vector, and secretion signal before scaling up expression.

The pilot experiments involve growing a small culture of each recombinant, taking time points, and analyzing the cell pellet and supernatant from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). You may also perform your pilot expression experiments in tubes (15 mL or 50 mL conical tubes), multi-well plates, or small shake flasks if so desired. Higher density formats such as 96-well plates can greatly simplify the testing of multiple strain, vector, and secretion signal combinations when using the PichiaPink™ Secretion Optimization Kit. Growth in 96-well plates is best done with a 3-mm throw shaker at a high agitation speed. We suggest that you analyze your SDS-PAGE gels by both Coomassie staining and western blot as the expression and secretion is protein dependent, and not all proteins express to the level of grams per liter. We also suggest checking for protein activity if an assay is available.

Detecting Recombinant Proteins in PichiaPink™

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

Technique	Method of Detection	Sensitivity
SDS-PAGE (Coomassie-stained)	Visualization by eye	Can detect as little as 100 ng in a single band
SDS-PAGE (Silver-stained)	Visualization by eye	Can detect as little as 2 ng in a single band
Western Analysis	Antibody to your particular protein	Can detect as little as 1–10 pg depending on detection method (alkaline phosphatase, horseradish peroxidase, radiolabeled antibody)
Functional assay	Varies depending on assay	Varies depending on assay. It is used to compare relative amounts of protein.

Continued on next page

Expressing Recombinant PichiaPink™ Strains, continued

Media

You will need BMGY and BMMY (buffered complex glycerol or methanol medium), for expression of your protein of interest (see **Recipes**, page 62). BMGY and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein. Because these media are buffered with phosphate buffer, a wide range of pH values may be used to optimize production of your protein. BMGY and BMMY contain yeast extract and peptone to stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone allow better growth and biomass accumulation.

Proteases

There are some proteins specifically susceptible to proteases that have optimal activity at neutral pH. If this is the case, expression using MGYA and MMA media may be indicated even if you are using one of the protease knock-out PichiaPink™ strains. As PichiaPink™ expression progresses in an unbuffered medium such as MMA, the pH drops to 3 or below, inactivating many neutral pH proteases (Brierley *et al.*, 1994). PichiaPink™ is resistant to low pH, so the low pH will not affect growth.

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

Aeration

The most important parameter for efficient expression in a PichiaPink™ strain is adequate aeration during methanol induction. As a general rule, **never** allow cultures to be more than 10–30% of your total flask volume when inducing expression. We strongly recommend that you use baffled flasks. Cover the flasks with porous tape such as PureLink™ Air Porous Tape (see page vii) or another loose fitting cover. Never use tight fitting covers. (Aeration is not as critical when generating biomass before induction.)

Kinetics of Growth

While recombinant PichiaPink™ strains expressing *ADE2* gene product grow unhindered in rich and minimal media, untransformed strains (i.*e.*, *ade2* knockouts) grow slowly in rich and minimal medium. This slow growth phenotype ensures that virtually all PichiaPink™ cells in the medium express the protein interest, because revertants cannot compete with and outgrow the desired production strain.

Temperature and Shaking

All expression is done at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. If your incubator temperature fluctuates, set the temperature at 28°C. Shake at 225–250 rpm if using a floor shaking incubator for flasks, or at 250–300 rpm if you are using a table-top shaker for flasks that sits inside an incubator. If you are growing your cells in a well plate, we recommend that you use a shaker with a very small orbital throw (3 mm) and run at a high rpm to ensure adequate mixing and aeration (700–950 rpm).

Continued on next page

Expressing Recombinant PichiaPink™ Strains, continued

Important

Since recombination can occur in many different ways that can effect protein expression (clonal variation), we recommend that you screen 6–10 verified recombinant clones for expression levels. Start with colonies from the freshest plates available. Colony viability drops over time, so if you have any doubts, it is better to streak out your strain.

Guidelines for Expression

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

Expressing Recombinant PichiaPink™ Strains

Follow the protocol below to test the effectiveness of your expression conditions and choose the best PichiaPink™ strain, secretion signal, and vector combination.

1. Using a single colony, inoculate 10 mL of BMGY medium in a 125 mL baffled flask. Grow the cells for 1–2 days at 24–30°C in a shaking incubator set to 250–300 rpm.
2. Transfer the cells to 50 mL conical tubes and centrifuge at 1,500× g for 5 minutes at room temperature. Decant the BMGY supernatant and resuspend the cell pellet in 1 mL of BMMY medium to induce expression.
   Note: Make sure to replace the caps of the tubes with porous tape to maximize aeration.
3. Return the cells in BMMY medium to the 30°C shaking incubator to continue growth overnight.
4. The next day, remove 100 µL from the sample for gel analysis, and add 100 µL of 40% methanol. Continue to grow the cells overnight in the 30°C shaking incubator set to 300 rpm.
5. Harvest the cells by centrifuging for 10 minutes at 1,500 × g.
6. **Transfer the supernatant to a separate tube** and store both the supernatant and the cell pellet 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- or Silver-stained SDS-PAGE and western blot or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, next page).

Important

If you wish to determine the induction profile of you protein over time, you may collect 100 µL samples of the expression culture at each of the times indicated below, and centrifuging at maximum speed in a microcentrifuge for 2–3 minutes at room temperature. You will use these samples to analyze expression levels and determine the optimal time post-induction to harvest..

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

Analyzing Protein Expression by SDS-PAGE

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 (see page 73).

If you are pouring your own gels, note that any standard SDS-PAGE 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. Refer to standard texts such as *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989), *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Guide to Protein Purification* (Deutscher, 1990), or *Protein Methods* (Bollag and Edelstein, 1991) for further recommendations.

Preparing Cell Pellets (Intracellular and Secreted Expression)

You will need to prepare Breaking Buffer (see page 64) and have acid-washed 0.5 mm glass beads on hand.

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 µm). 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. Mix 50 µl of the supernatant with 12.5 µl of NuPAGE® LDS Sample Preparation Buffer and 7.5 µl of NuPAGE® Sample Reducing Agent (see page viii).
7. Boil for 10 minutes and load 10–20 µL per well. Thickness of the gel and number of wells will determine loading volume. You may store the remaining sample at –20°C for western blots, if necessary. You may store the cell lysates at –80°C for further analysis.

Continued on next page

Analyzing Protein Expression by SDS-PAGE, continued

Preparing Supernatant (Secreted Expression only)

1. Thaw supernatants and place on ice.
2. Mix 50 µl of the supernatant with 12.5 µl of NuPAGE® LDS Sample Preparation Buffer and 7.5 µl of NuPAGE® Sample Reducing Agent (see page vii).
3. Boil 10 minutes, then load 10–30 µl onto the gel. You may store the remaining sample may be stored at –20°C for western blots, if necessary. You may store the supernatants at –80°C for further analysis.
4. If you do not see any protein by Coomassie or by western blot, then concentrate the supernatant 5–10 fold and analyze samples again by western blot. Centricon and Centriprep filters (Amicon) are very useful for this purpose. You may also silver stain your gels for better detection.

Protein Concentration

You may perform Lowry, BCA (Pierce) or Bradford protein assays to quantify the amounts of protein in the cell lysates and medium supernatants. PichiaPink™ medium supernatants will vary in protein concentration primarily due to the amount of your secreted protein. PichiaPink™ secretes very few native proteins. If the protein concentration of the medium is >50 µg/mL, a 10 µl of sample from the medium will give a faint band on a Coomassie-stained SDS-PAGE gel.

Controls

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

- Molecular weight standards appropriate for your desired protein
- A sample of your protein as a standard (if available)
- A sample of your PichiaPink™ strain transformed with the parent plasmid (*i.e.*, without your insert). This shows the background of native PichiaPink™ proteins that are present intracellularly. Inclusion of this sample will help you differentiate your protein from background, especially if you express your protein intracellularly.

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

Continued on next page

Analyzing Protein Expression by SDS-PAGE, continued

Analysis of Protein Expression

Inspection of your Coomassie-stained SDS-PAGE should reveal the secreted expression level of your protein of interest co-migrating with your standard. If you have performed time course experiments, you will also know the induction of your protein over time. If you are satisfied with the level of expression, try a test purification using your method of choice or proceed to scale-up expression (page 47).

If there is no recombinant protein visible, silver stain your gel or perform a western blot. We also recommend that you perform a functional assay if one is available for your protein of interest.

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

If there is no indication of expression at all, use PCR to analyze your recombinants for the correctly sized PCR product (page 64). If you find that you have recombinants, perform a northern analysis to see if and how much full-length mRNA is induced. See page 71 for an RNA isolation protocol.

Optimizing PichiaPink™ Protein Expression

Introduction

Based on available data, there is approximately a 50 to 75% chance of expressing your protein of interest in PichiaPink™ at reasonable levels. The biggest hurdle seems to be generating initial success – *i.e.*, expressing your protein at **any** level. While there are a 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

Although three of the PichiaPink™ strains have protease knockouts and have been shown to exhibit reduced levels of proteolytic degradation of secreted proteins, we recommend that you follow the guidelines below to further increase your chances of achieving high secreted expression levels.

- 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.
- Check to see if your protein is susceptible to neutral pH proteases by expressing in unbuffered medium. In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.
- Induce expression with a higher density culture.

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. The PichiaPink™ Secretion Optimization Kit includes the PichiaPink™ Secretion Signal Kit (also available separately, see page vii), which contains eight separate secretion signal duplexes ready to be cloned with your gene of interest into the PichiaPink™ vectors for optimal secretion.
- Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration (see page 47).
- Induce expression with a higher density culture.

Continued on next page

Optimizing PichiaPink™ Protein Expression, continued

Low Expression Levels

- If you have only used pPink-HC or pPinkα-HC for expression, try pPink-LC. Some proteins express poorly from high copy number plasmids, and benefit from low copy number expression. Always pick white colonies for expression, even if you desire low copy number expression.
- 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.
- Scale up to fermentation (page 48). *Pichia* is a yeast and is particularly well suited for growth in fermentors. Further fermentation guidelines, which provide recommendations for growing *Pichia* in fermentors, are available on our website at www.invitrogen.com.

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, use PCR to check for insertion of your gene into the PichiaPink™ genome (page 64).

If your gene is present, perform a northern blot analysis to check for transcription of your gene. There is a protocol in the **Appendix** for RNA isolation from PichiaPink™ (see page 71).

If you see premature transcriptional termination, check the AT content of your gene. In *Saccharomyces*, there are a few consensus sequences which promote premature termination. One of these, 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).

Hyperglycosylation

If your protein is hyperglycosylated:

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

Protein Glycosylation

Analyzing Glycoproteins

When expressing and purifying a glycosylated protein in a heterologous expression system, it is desirable to quickly determine whether the protein is glycosylated properly. Refer to published protocols for carbohydrate analysis of proteins to characterize glycosylated proteins of interest (Ausubel *et al.*, 1994), unit 17. Further information about glycosylation in eukaryotes is also available in published literature (Varki and Freeze, 1994).

Enzymes for Analyzing Glycoproteins

These are just a few of the enzymes available for carbohydrate analysis.
Abbreviations are as follows:
Asn: Asparagine, GlcNAc: N-acetylglucosamine

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
Peptide:N-Glycosidase F	Endo	Glycoproteins between Asn and GlcNAc (removes oligosaccharides)

Scaling-Up Expression

Guidelines for Expression

Once you have optimized secreted expression of your protein of interest, you are ready to scale-up your expression protocol to produce more protein. You may do this by increasing the culture volume using larger baffled flasks (below) or fermentation. See **PichiaPink™ Fermentation**, next page, for recommendations for growing *Pichia* in fermentors. Use the guidelines below to scale-up your expression protocol. To purify your protein, use your method of choice.

Scale-up Protocol

1. Using a single colony, inoculate 25 mL of BMGY in a 250 mL baffled flask. Grow at 24–30°C in a shaking incubator (250–300 rpm) until culture reaches an OD_{600} of 2–6 (approximately 2–3 days).
2. Use this 25 mL culture to inoculate 1 liter of BMGY in a 3 or 4 liter baffled flask and grow at 24–30°C with vigorous shaking (250–300 rpm) until the culture reaches log phase growth (OD_{600} = 2–6).
3. Harvest the cells by centrifuging in sterile centrifuge bottles at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend the cell pellet in 200 mL of BMMY medium.

   Note: The resuspension step above concentrates the culture 5-fold, thereby reducing the need for concentrating the secreted proteins if the expression level is not particularly high (see below).
4. Aliquot the culture between two 1 liter baffled flasks. Cover the flasks with PureLink™ Air Porous Tape (see page vii), 2 layers of sterile gauze, or cheesecloth and return to incubator. Continue to grow at 24–30°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. You can process the cells immediately or store at –80°C until ready for use.
8. For secreted expression, **save the supernatant, chill to 4°C, and concentrate if desired** (see below). Proceed directly to purification using your method of choice or store the supernatant at –80°C until ready to process further.

Concentrating Proteins

Proteins secreted into the media are usually >50% homogeneous and require some additional purification. If the expression level is not particularly high, we recommend that you concentrate the protein. There are several general methods to concentrate proteins secreted from *Pichia*. These general methods include:

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

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

PichiaPink™ Fermentation

Introduction

Once you have optimized secreted expression of your protein of interest, you are ready to scale-up your expression protocol to produce more protein. *Pichia pastoris*, like *Saccharomyces cerevisiae*, is particularly well-suited for growth in fermentors. *Pichia* has the ability to reach very high cell densities during fermentation which may improve overall protein yields. Use the guidelines below for growing your transformed PichiaPink™ strains in fermentors.

Important

We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Since there are a wide variety of fermentors available, it is difficult to provide exact procedures for your particular case. Read the operator's manual for your particular fermentor before beginning.

Fermentation Parameters

It is important to monitor and control the following parameters throughout the fermentation process. The following table describes the parameters and the reasons for monitoring them.

Parameter	Reason
Temperature (24–30°C)	Growth above 32°C is detrimental to protein expression
Dissolved oxygen (>20%)	*Pichia* needs oxygen to metabolize glycerol and methanol
pH (5.0–6.0)	Important when secreting protein into the medium and for optimal growth
Agitation (500 to 1,500 rpm)	Maximizes oxygen concentration in the medium
Aeration (0.1 to 1.0 vvm* for glass fermentors)	Maximizes oxygen concentration in the medium which depends on the vessel
Antifoam (the minimum needed to eliminate foam)	Excess foam may cause denaturation of your secreted protein and it also reduces headspace
Carbon source (variable rate)	Must be able to add different carbon sources at different rates during the course of fermentation

*volume of oxygen (liters) per volume of fermentation culture (liters) per minute

Monitoring the Growth of PichiaPink™

Monitor cell growth at various time points by using the absorbance at 600 nm (OD_{600}) and the wet cell weight. Monitor the metabolic rate of the culture by observing changes in the concentration of dissolved oxygen in response to carbon availability (see next page).

Continued on next page

PichiaPink™ Fermentation, continued

Maintaining the Dissolved Oxygen Concentration (DO)

The dissolved oxygen concentration is the relative percent of oxygen in the medium where 100% is air-saturated medium. *Pichia* will consume oxygen as it grows, reducing the dissolved oxygen content. However, because oxygen is required for the first step of methanol catabolism, it is important to maintain the dissolved oxygen (DO) concentration at a certain level (>20%) to ensure good growth of your PichiaPink™ strains on methanol. Accurate measurement and observation of the dissolved oxygen concentration of a culture will give you important information about the state and health of the culture. Refer to your operator's manual to accurately calibrate your equipment.

1. Maintaining the dissolved oxygen above 20% may be difficult depending on the oxygen transfer rates (OTR) of the fermentor, especially in small-scale glass vessels. In a glass vessel, oxygen supplementation is needed to keep the DO above 20%, usually ~0.1–0.3 vvm (liters of O_2 per liter of fermentation culture per minute). Oxygen consumption varies and depends on the amount of methanol added and the protein being expressed.
2. You can use oxygen at 0.1 to 0.3 vvm to achieve adequate levels. You can accomplish this in any glass fermentor by mixing with the air feed. For stainless steel vessels, you can use pressure to increase the OTR. Be sure to read the operator's manual for your particular fermentor.
3. If a fermentor cannot supply the necessary levels of oxygen, then you should scale back the methanol feed accordingly. Note that decreasing the amount of methanol may reduce the level of protein expression.
4. To reach maximum expression levels, you may increase the fermentation time to deliver similar levels of methanol at the lower feed rate. For many recombinant proteins, there is a direct correlation between the amount of methanol consumed and the amount of protein produced.

Use of DO Measurements

During growth, the culture consumes oxygen, keeping the DO concentration low. Oxygen is consumed regardless whether you grow the on glycerol or methanol. You can manipulate the DO concentration to evaluate the metabolic rate of the culture and whether the carbon source is limiting. The metabolic rate indicates how healthy the culture is. For example, changes in the DO concentrations allow you to determine whether all the glycerol is consumed from the culture before adding methanol. Secondly, inducing DO spikes ensures that your methanol feed does not exceed the rate of consumption. Excess methanol may be toxic.

Manipulation of DO

If carbon is limiting, shutting off the carbon source should cause the culture to decrease its metabolic rate, and the DO to rise (spike). Terminate the carbon feed and time how long it takes for the DO to rise 10%, after which turn the carbon feed back on. If the lag time is short (< 1 minute), the carbon source is limiting.

Continued on next page

PichiaPink™ Fermentation, continued

Proteases

Proteases are known to be secreted into the medium during *Pichia* fermentations, which can result in the degradation of the desired protein product. Although protease inhibitors can be added to the medium during fermentation, the cost to do so can be significant. To help reduce the impact of proteases and the need for heavy protease inhibitor use, PichiaPink™ System offers three protease knockout strains along with a "protease wild-type" strain.

Recommended Equipment

Below is a checklist for equipment recommendations.

- A jacketed vessel is needed for cooling the yeast during fermentation, especially during methanol induction. You will need a constant source of cold water (5–10°C). This requirement may mean that you need a refrigeration unit to keep the water cold.
- A foam probe is highly recommended as antifoam is required.
- A source of O_2 – either air (stainless steel fermentors at 1–2 vvm) or pure O_2 (0.1–0.3 vvm for glass fermentors).
- Calibrated peristaltic pumps to feed the glycerol and methanol.
- Automatic control of pH.

Inoculum Seed Flask Preparation

Follow the instructions below to prepare PichiaPink™ starter cultures to inoculate the fermentor. Be sure not to put too much medium in the baffled flasks. The volume of the medium should be 10–30% of the total flask volume.

1. Inoculate baffled flasks containing a total of 1–10% of the initial fermentation volume of BMGY with a colony from an MGY plate or from a frozen glycerol stock.
2. Grow the cells at 30°C, 250–300 rpm, 2–3 days until OD_{600} = 2–20. To accurately measure OD_{600} >1.0, dilute a sample of your culture 10 to 100-fold before reading.

Continued on next page

PichiaPink™ Fermentation, continued

Glycerol Batch Phase

Follow the instructions below to prepare your PichiaPink™ strains for glycerol-fed batch phase (page 51).

1. Sterilize the fermentor containing BMGY medium
2. After sterilization and cooling, set the temperature to 30°C, agitation and aeration to operating conditions (usually maximum rpm and 0.1–1.0 vvm air), and adjust the pH of the medium to 6.0.
3. Inoculate the fermentor with approximately 1–10% of the initial fermentation volume from the culture generated in the inoculum shake flasks. Note that the DO will be close to 100% before the culture starts to grow. As the culture grows, it will consume oxygen, causing the DO to decrease. Be sure to keep the DO above 20% by adding oxygen as needed.
4. Grow the batch culture until the glycerol is completely consumed (duration of batch depends on inoculum volume and OD). This is indicated by a sharp increase in the DO to 100%. Perform sampling at the end of each fermentation stage and at least twice daily.

   Note: We take 10 mL samples for each time point, then take 1 mL aliquots from this 10 mL sample.
5. Analyze samples for cell growth (OD_{600} and wet cell weight), pH, microscopic purity, and protein concentrations or activity. Proceed to **Glycerol Fed-Batch Phase**, below, or freeze the cell pellets and supernatants at –80°C for later analysis.

Note

A cellular yield of 90 to 150 g/liter wet cells is expected for this stage. Recombinant protein will not yet be produced due to the absence of methanol.

Glycerol Fed-Batch Phase

Once all the glycerol is consumed from the batch growth phase, a glycerol feed is initiated to increase the cell biomass under limiting conditions. When you are ready to induce with methanol, you can use DO spikes to make sure the glycerol is limited. Although we recommend that you carry out the methanol feed at 24°C, you may carry out the glycerol fed-batch phase at any temperature between 24–30°C.

1. Initiate a 50% w/v glycerol feed containing 12 mL PTM_1 trace salts per liter of glycerol feed (see page 64 for recipe). Set the feed rate to 5 to 15 mL/hr /liter initial fermentation volume.
2. Carry out glycerol feeding for approximately four hours or longer (see note below). A cellular yield of 150 to 220 g/liter wet cells is typical at the end of this stage while no appreciable recombinant protein is produced.

   Note: The level of expressed protein depends on the cell mass generated during the glycerol fed-batch phase. The length of this feed can be varied to optimize protein yield. We recommend a range of 50 to 300 g/liter wet cells for study. Do not exceed 4% glycerol in the batch phase due to toxicity problems with higher levels of glycerol.

Continued on next page

PichiaPink™ Fermentation, continued

Important

If dissolved oxygen falls below 20%, you should stop the glycerol or methanol feed, and wait until the dissolved oxygen spikes before increasing the oxygen rate. Once you observe a DO spike, you may make adjustments to agitation, aeration, pressure or oxygen feeding.

Guidelines for Methanol Fed-Batch Phase

- Do **not** start the methanol feed until all of the glycerol is consumed to fully induce the *AOX1* promoter on methanol. However, it has been reported that a "mixed feed" of glycerol and methanol has been successful for expressing recombinant proteins (Brierley *et al.*, 1990; Sreekrishna *et al.*, 1989).
- introduce methanol slowly to adapt the culture to growth on methanol. If you add methanol too fast, you will kill the cells.
- Once the culture is adapted to methanol, it is very important to use DO spikes to analyze the state of the culture and to take time points over the course of methanol induction to optimize protein expression.
- Growth on methanol also generates a lot of heat, so temperature control at this stage is very important. Once the culture is fully adapted to methanol, the final feed rate will be limited by the heat and oxygen transfer capacity of the fermentor. The feed rates on the next page are suggested values, but it is critical that you maintain the DO level above 20, and monitor the methanol limitation by observing DO spikes in response to a brief (10 to 30 seconds) shut off of the methanol feed.

Methanol Fed-Batch Phase

1. Terminate glycerol feed and initiate induction by starting a 100% methanol feed containing 12 mL PTM_1 trace salts per liter of methanol. Set the feed rate to 3.6 mL/hr per liter initial fermentation volume. Reduce the temperature set point to 24°C.
2. During the first 2–3 hours, methanol will accumulate in the fermentor and the dissolved oxygen values will be erratic while the culture adapts to methanol. Eventually the DO reading will stabilize and remain constant. If you cannot maintain the DO level above 20%, stop the methanol feed, wait for the DO to spike, and continue on with the same methanol feed rate. Increase agitation, aeration, pressure or oxygen feeding to maintain the DO above 20% only after the accumulated methanol has been consumed as indicated by the DO spike after the feed shut-off.
3. When the culture is fully adapted to methanol utilization (2–4 hours), and is limited on methanol, it will have a steady DO reading and a fast DO spike time (generally under 30 seconds). Maintain the lower methanol feed rate under limited conditions for at least 1 hour after adaptation before doubling the feed. Double the feed rate to ~7.3 mL/hr/liter initial fermentation volume.
4. After 2 hours at the 7.3 mL/hr/liter feed rate, increase the methanol feed rate to ~10.9 mL/hr/liter initial fermentation volume. Maintain this feed rate throughout the remainder of the fermentation.
   Note: The supernatant may appear greenish. This is normal.

Continued on next page

PichiaPink™ Fermentation, continued

Yield

The entire methanol fed-batch phase lasts approximately 40–70 hours with up to a total of approximately 700 mL methanol fed per liter of initial volume. However, this may vary for different proteins.

Harvesting Cells and Supernatant

For small fermentations (1–10 liters), you can collect the culture into centrifuge bottles (500–1000 mL) and centrifuge to separate the cells from the supernatant.

For large fermentations, you can use large membrane filtration units (Millipore) or a Sharples centrifuge to separate cells from the supernatant. The optimal method will depend on whether you need the supernatant (secreted expression) or the cells (intracellular expression) as the source of your protein and what you have available.

You can load the supernatants directly onto purification columns or concentrate using ultrafiltration.

Note: The methods and equipment listed above are by no means complete. The amount of cells or the volume of supernatant will determine what sort of equipment you need.

Cell Lysis

We recommend disrupting the cells using glass beads as described in *Current Protocols in Molecular Biology* (Ausubel et al., 1994), *or Guide to Protein Purification* (Deutscher, 1990). This method may be tedious for large amounts of cells. For larger amounts, we have found that a microfluidizer works very well. French pressing the cells does not seem to work as well as the glass beads or the microfluidizer.

Appendix

PichiaPink™ Secretion Signal Sequences

Introduction

The secretion signal sequences included in the PichiaPink™ Secretion Signal Kit are provided as phosphorylated duplex oligomers in 40 pmol aliquots lyophilized in TE Buffer, pH 8. The sequence underlined in each signal sequence corresponds to the Kozak sequence taken from the native *AOX1* gene.
Resuspend the duplexes in 40 µL TE Buffer, pH 8 before use.

α-mating factor pre-sequence

Source: *Saccharomyces cerevisiae*, **Length**: 19 aa (amino acids), **MW** (Molecular Weight): 2000.3 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**AGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCT

Complement nucleotide sequence of oligo 2:

AGCTAATGCGGAGGATGCTGCGAATAAAACAGCAGTAAAAATTGAAGGAAATCTCATCGTTTCG

α-amylase signal sequence

Source: *Aspergillus niger*, **Length**: 20 aa, **MW**: 2207.6 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**GTCGCTTGGTGGTCTTTGTTTCTGTACGGTCTTCAGGTCGCTGCACCTGCTTTG
GCT

Complement nucleotide sequence of oligo 2:

AGCCAAAGCAGGTGCAGCGACCTGAAGACCGTACAGAAACAAAGACCACCAAGCGACCATCGTTTCG

Glucoamylase signal sequence

Source: *Aspergillus awamori*, **Length**: 18 aa, **MW**: 1825.2 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**TCTTTTAGATCCTTGTTGGCTTTGTCTGGTTTGGTTTGTTCTGGTTTGGCT

Complement nucleotide sequence of oligo 2:

AGCCAAACCAGAACAAACCAAACCAGACAAAGCCAACAAGGATCTAAAAGACATCGTTTCG

Serum albumin signal sequence

Source: *Homo sapiens*, **Length**: 18 aa, **MW**: 2140.5 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**AAGTGGGTTACCTTTATCTCTTTGTTGTTTCTTTTCTCTTCTGCTTACTCT

Complement nucleotide sequence of oligo 2:

AGAGTAAGCAGAAGAGAAAAGAAACAACAAAGAGATAAAGGTAACCCACTTCATCGTTTCG

Continued on next page

PichiaPink™ Secretion Signal Sequences, continued

Inulinase presequence

Source: *Kluyveromyces maxianus*, **Length**: 16 aa, **MW**: 1647.0 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**AAGTTAGCATACTCCTTGTTGCTTCCATTGGCAGGAGTCAGTGCT

Complement nucleotide sequence of oligo 2:

AGCACTGACTCCTGCCAATGGAAGCAACAAGGAGTATGCTAACTTCATCGTTTCG

Invertase signal sequence

Source: *Saccharomyces cerevisiae*, **Length**: 19 aa, **MW**: 2025.5 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**CTTTTGCAAGCTTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCA

Complement nucleotide sequence of oligo 2:

TGCAGATATTTTGGCTGCAAAACCAGCCAAAAGGAAAAGGAAAGCTTGCAAAAGCATCGTTTCG

Killer Protein signal sequence

Source: *Saccharomyces cerevisiae*, **Length**: 26 aa, **MW**: 2926.6 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**ACTAAGCCAACCCAAGTATTAGTTAGATCCGTCAGTATATTATTTTTCATCACA
TTACTACATCTAGTCGTAGCT

Complement nucleotide sequence of oligo 2:

AGCTACGACTAGATGTAGTAATGTGATGAAAAATAATATACTGACGGATCTAACTAATACTTGGGTTG
GCTTAGTCATCGTTTCG

Lysozyme signal sequence

Source: *Gallus gallus*, **Length**: 26 aa, **MW**: 2686.4 Da

Nucleotide sequence of oligo 1:

AATTCGAAACG**ATG**CTGGGTAAGAACGACCCAATGTGTCTTGTTTTGGTCTTGTTGGGATTGACTGCT
TTGTTGGGTATCTGTCAAGGT

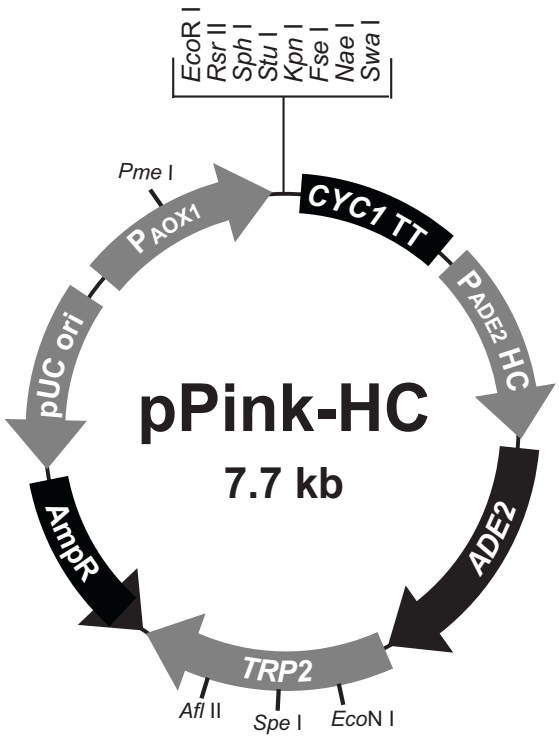
Complement nucleotide sequence of oligo 2:

ACCTTGACAGATACCCAACAAAGCAGTCAATCCCAACAAGACCAAAACAAGACACATTGGGTCGTTCT
TACCCAGCATCGTTTCG

Map of pPink-HC

Map of pPink-HC

pPink-HC (7667 bp) is a PichiaPink™ expression vector containing the full length *Pichia pastoris ADE2* gene product driven by a truncated 13 bp *ADE2* promoter (Nett, 2008), and is considered a high copy plasmid. The backbone of the plasmid is based on pUC19, and contains the Ampicillin (*bla*) resistance gene for selection in *E. coli*. The map below shows the elements of the pPink-HC vector. **The vector sequence of pPink-HC is available at www.invitrogen.com or by contacting Technical Support (page 73).**



Comments for pPink-HC
7667 nucleotides

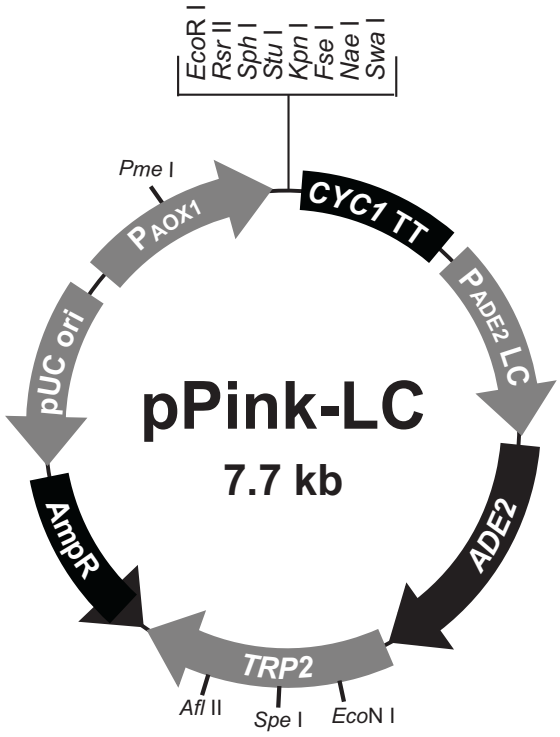
5´ *AOX1* promoter region: bases 1-940
Multiple cloning site: bases 942-988
CYC1 transcription termination region: bases 997-1271
ADE2 promoter region: bases 1286-1298
ADE2 ORF: bases 1299-2984
TRP2 gene: bases 3208-5019
pUC ori: 5388-6061 (c)*
Ampicillin (*bla*) resistance gene: bases 6206-7066 (c)

*(c): complementary strand

Map of pPink-LC

Map of pPink-LC

pPink-LC (7732 bp) is a PichiaPink™ expression vector containing the full length *Pichia pastoris ADE2* gene product driven by the full-length 82 bp *ADE2* promoter (Nett, 2008), and is considered a low copy plasmid. The backbone of the plasmid is based on pUC19, and contains the Ampicillin (*bla*) resistance gene for selection in *E. coli*. The map below shows the elements of the pPink-LC vector. The vector sequence of pPink-LC is available at www.invitrogen.com or by contacting Technical Support (page 73).



Comments for pPink-LC
7732 nucleotides

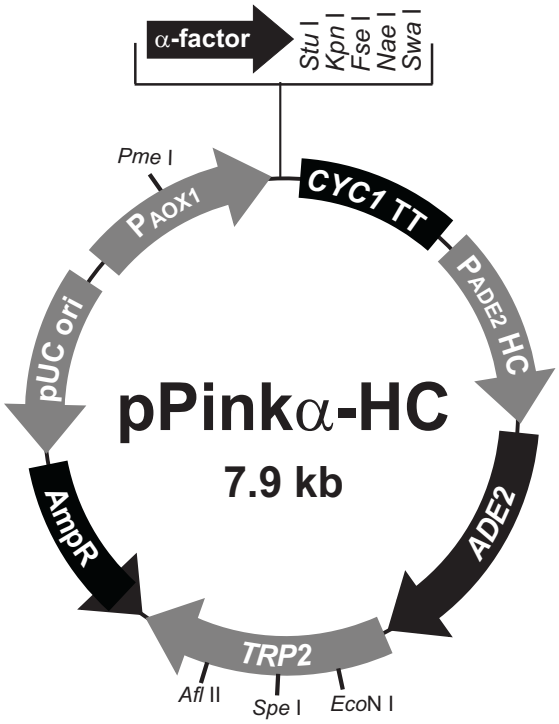
5´ *AOX1* promoter region: bases 1-940
Multiple cloning site: bases 942-988
CYC1 transcription termination region: bases 993-1267
ADE2 promoter region: bases 1282-1363
ADE2 ORF: bases 1364-3049
TRP2 gene: bases 3273-5084
pUC ori: 5453-6126 (c)*
Ampicillin (*bla*) resistance gene: bases 6271-7131 (c)

*(c): complementary strand

Map of pPinkα-HC

Map of pPinkα-HC

pPinkα-HC (7667 bp) is a PichiaPink™ secreted protein expression vector containing the *Saccharomyces cerevisae* α-mating factor pre-sequence for secretion of recombinant proteins. It also expresses the full length *ADE2* gene product from a truncated 13 bp *ADE2* promoter (Nett, 2008), and is considered a high copy plasmid. The backbone of the plasmid is based on pUC19, and contains the Ampicillin (*bla*) resistance gene for selection in *E. coli*. The map below shows the elements of the pPinkα-HC vector. The vector sequence of pPinkα-HC is available at www.invitrogen.com or by contacting Technical Support (page 73).



Comments for pPinkα-HC
7898 nucleotides

5´ *AOX1* promoter region: bases 1-940
α-mating factor secretion signal: 941-1194
Multiple cloning site: bases 1196-1222
CYC1 transcription termination region: bases 1228-1502
ADE2 promoter region: bases 1517-1529
ADE2 ORF: bases 1530-3215
TRP2 gene: bases 3439-5250
pUC ori: 5619-6292 (c)*
Ampicillin (*bla*) resistance gene: bases 6437-7297 (c)

*(c): complementary strand

Recipes

Introduction

The expression of recombinant proteins in *Pichia pastoris* requires the preparation of several different media. However, the PichiaPink™ System uses *ade2* complementation as a selection marker for transformants, and eliminates special media requirements for antibiotic selection or for determining the Mut phenotype (PichiaPink™ strains are Mut$^+$). In addition, the protease knockout strains help reduce the need for heavy protease inhibitor use.

This section includes information on the media requirements of the PichiaPink™ System, instructions for reconstituting the media in the PichiaPink™ Media Kit, as well as recipes for additional media used when expressing recombinant proteins in PichiaPink™ strains.

Using *Pichia* Media

For your convenience, the PichiaPink™ Media Kit is included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits, and is also available separately from Invitrogen (see page vii for ordering information). It contains the following prepackaged media that can easily be reconstituted.

Medium	Description	Application
YPD	Rich, complex broth	General growth and storage
YPDS	YPD with sorbitol	Recovery of cells after transformation
BMGY	Buffered complex medium containing glycerol	Generating biomass for secreted expression, controlling the pH of the medium, and decreasing protease activity.
BMMY	Buffered complex medium containing methanol	Inducing secreted expression, controlling the pH of the medium, and decreasing protease activity.
MGY or MGYA (optional)	Minimal medium containing glycerol with or without adenine	Generating biomass prior to methanol induction for intracellular expression
MM or MMA (optional)	Minimal medium containing methanol with or without adenine	Intracellular expression of desired protein

Continued on next page

Recipes, continued

PichiaPink™ Media Kit

For your convenience, the PichiaPink™ Media Kit is included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits, and is also available separately from Invitrogen (see page vii for ordering information). It contains the following prepackaged media.

Media	Amount	Yield
PAD Agar	2 pouches	1 liter/pouch of PAD agar medium
YP	2 pouches	1 liter/pouch of YP base medium
YPS	2 pouches	0.2 liters/pouch of YPS base medium
YP Agar	2 pouches	1 liter/pouch of YP agar medium
Dextrose	1 pouch	1 liter/pouch of 20% dextrose

Follow the instructions below to prepare the media for your PichiaPink™ experiments.

20% Dextrose (10X)

To prepare 1 liter of 20% Dextrose (10X) stock solution:

1. Dissolve the contents of the Dextrose pouch from the PichiaPink™ Media Kit in 1000 mL of distilled water.
2. Autoclave for 15 minutes or filter sterilize.

Store at room temperature. The shelf life of this solution is approximately one year.

YPD Medium

YPD medium is used for growing PichiaPink™ strains prior to transformation. To prepare 1 liter of YPD, use only one pouch of YP from the PichiaPink™ Media Kit.

1. Dissolve the contents of the YP pouch from the PichiaPink™ Media Kit in 900 mL of distilled water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 mL of sterile 20% Dextrose (see above).

Store the YPD medium at room temperature. The shelf life is several months.

YPD Agar

YPD agar is used for streaking glycerol stocks of PichiaPink™ strains. To prepare 1 liter, use only one pouch of YP agar from the PichiaPink™ Media Kit.

1. Dissolve the contents of the YP agar pouch from the PichiaPink™ Media Kit in 900 mL of distilled water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 mL of sterile 20% Dextrose (see above).

Store the YPD agar slants or plates at 4°C. The shelf life is several months.

Continued on next page

Recipes, continued

YPDS Medium

YPDS medium is used for the recovery of cells after electroporation. To prepare 0.2 liters of YPDS, use only one pouch of YPS from the PichiaPink™ Media Kit.

1. Dissolve the contents of the YPS pouch from the PichiaPink™ Media Kit in 180 mL of distilled water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 20 mL of sterile 20% Dextrose (see previous page).

Store the YPDS medium at room temperature. The shelf life is several months.

PAD Agar

PAD (Pichia Adenine Dropout) agar lacks adenine, and is used for selecting transformants in the PichiaPink™ system. To prepare 1 liter of PAD agar, use only one pouch of PAD agar pouch from the PichiaPink™ Media Kit.

1. Dissolve the contents of the PAD agar pouch from the PichiaPink™ Media Kit in 900 mL of distilled water.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 mL of sterile 20% Dextrose (see previous page).

Store the PAD agar plates at 4°C. The shelf life is several months.

Stock Solutions

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

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

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

50X B (0.02% Biotin)

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

100X A (0.2% Adenine)

Dissolve 200 mg of L-adenine in 100 mL of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilize and store at 4°C. The shelf life of this solution is approximately one year.

10X D (20% Dextrose)

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

Continued on next page

Recipes, continued

Stock Solutions, continued

10X M (5% Methanol)

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

10X GY (10% Glycerol)

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

1 M potassium phosphate buffer, pH 6.0:

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

BMGY and BMMY

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

1% yeast extract
2% peptone
100 mM potassium phosphate, pH 6.0
1.34% YNB
0.0004% 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
   - 20 mL 50X B
   - 100 mL 10X GY
4. For BMMY, add 100 mL 10X M instead of glycerol.

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

MGY and MGYA

Minimal Glycerol Medium ± Adenine (1 liter)

1.34% YNB
1% glycerol
0.0004% biotin
± 0.002% Adenine

1. Combine aseptically 800 mL autoclaved water with 100 mL of 10X YNB, 20 mL of 50X B, and 100 mL of 10X GY.
2. For growth of *ade2* strains in this medium, a version can be made that contains adenine (called MGYA) by adding 10 mL of 100X A stock solution.

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

Continued on next page

Recipes, continued

MD and MDA

Minimal Dextrose Medium ± Adenine (1 liter)

1.34% YNB
0.0004% biotin
2% dextrose
± 0.002% Adenine

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
   - 20 mL of 50X B
   - 100 mL of 10X D
3. To make MDA, add 10 mL of 100X A 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 MMA

Minimal Methanol ± Adenine (1 liter)

1.34% YNB
0.0004% biotin
0.5% methanol
± 0.002% Adenine

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
   - 20 mL of 50X B
   - 100 mL of 10X M
3. To make MMA, add 10 mL of 100X A 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 MMA stores well for several months at 4°C.

Continued on next page

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 the following in 900 mL deionized water:

   6 g sodium phosphate (monobasic)
   372 mg EDTA
   50 mL glycerol
3. Use NaOH to adjust pH and bring up the volume to 1 liter. Store at 4°C.
4. Right before use, add the protease inhibitors.

PTM_1 Trace Salts

Mix together the following ingredients to prepare PTM_1 Trace Salts for PichiaPink™ fermentation.

Cupric sulfate-$5H_2O$	6.0 g
Sodium iodide	0.08 g
Manganese sulfate-H_2O	3.0 g
Sodium molybdate-$2H_2O$	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-$7H_2O$	65.0 g
Biotin	0.2 g
Sulfuric Acid	5.0 mL
Water	to a final volume of 1 liter

Filter sterilize and store at room temperature.

Note: There may be a cloudy precipitate upon mixing of these ingredients. Filter-sterilize as above and use.

Smash and Grab Buffer

Mix together the following ingredients to prepare Smash and Grab Buffer for easy isolation of PichiaPink™ genomic DNA.

10% SDS	5 mL
20% Triton X-100	5 mL
4 M NaCl	1.25 mL
500 mM EDTA	100 µL
1 M Tris, pH 8.0	500 µL
Water	to a final volume of 50 mL

Filter sterilize and store at room temperature.

PCR Analysis of PichiaPink™ Integrants

Introduction

The following protocol is designed to allow you to analyze PichiaPink™ integrants to determine if the gene of interest has integrated into the genome of your PichiaPink™ strain. Isolate genomic DNA from 6–10 PichiaPink™ clones using the protocol on page 68 or page 70. After isolating your DNA, use the procedure below to identify the integrants. Amplification of the gene of interest is carried out either with the 5´ *AOX1* forward primer (pPink-HC, pPink-LC, and pPinkα-HC) or the α-factor forward primer (pPinkα-HC only) paired with the 3´ *CYC1* reverse primer included in the kit. This protocol is useful for confirming integration of the gene of interest but will not provide information on the site of integration. A more direct procedure is provided on the next page.

PCR Procedure

1. Set up PCR reactions as follows. For amplification controls, use 5–10 ng of recombinant plasmid (positive control) and 5–10 ng of the appropriate plasmid without insert (negative control).

10X PCR Buffer	5 µl
Genomic DNA (~50–300 ng)	5 µl
100 mM dNTPs (25 mM each)	1 µl
5´ *AOX1* Primer or 5´ α-factor primer (500 ng/µl)	5 µl
3´ *CYC1* Primer (500 ng/µl)	5 µl
Sterile water	29 µl
Taq Polymerase (5 U/µl)	0.25 µl

2. Load thermocycler and run the following program:

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

3. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. Parent plasmids will produce the following sized PCR products. Add the size of these products to the size of your insert to interpret your PCR results.

Vector	PCR Product
pPink-HC	355 bp + insert (secretion signal and GOI*)
pPink-LC	351 bp + insert (secretion signal and GOI)
pPinkα-HC (using the 5´ *AOX1* primer)	586 bp + insert (GOI)
pPinkα-HC (using the 5´ α-factor primer)	297 bp + insert (GOI)

***GOI:** Gene of interest

Direct PCR Screening of PichiaPink™ Clones

Introduction

The following high-throughput protocol is designed to directly test PichiaPink™ clones for insertion of your gene by colony PCR. Briefly, the cells in a 96-well plate are lysed by five rounds of heat treatment in a microwave oven, followed by overnight freezing. The genomic DNA is then used directly as a PCR template.

Lysing PichiaPink™

1. Transfer the white colonies on the transformation plates to master plates using a 48 colony grid and incubate at 24–30°C until colonies form.
2. Aliquot 75 µL of TE Buffer into the 96 wells of a PCR plate. Using sterile pipette tips, scrape a small amount of PichiaPink™ cells off the master plates and drop the tips into the wells of the PCR plate
3. Using an eight channel pipette, pick up the tips from the PCR plate eight at a time, pipette up and down several times, and discard the tips. Close the wells of the plate using 8 well strip caps.
4. Place the 96-well plate into the insert of a pipette tip box (so the plate doesn't touch the microwave turntable) and microwave on high for 3.5 minutes. Vortex briefly.

   Note: If overheating occurs because of the power level of the microwave, shorten the microwave times accordingly.
5. Microwave on high for 2 minutes. Vortex briefly.
6. Microwave on high for 1.5 minutes. Vortex briefly.
7. Microwave on high for 1 minutes. Vortex briefly.
8. Microwave on high for 0.5 minutes. Vortex briefly, and freeze at –80°C for 10 minutes or overnight.
9. Thaw at 95°C for 2 minutes in the PCR machine, and centrifuge for 15 minutes at 2,500 rpm using a 96-well plate swing-out centrifuge insert.
10. Use 5 µL of the supernatant as template in the following PCR reaction.

Continued on next page

Direct PCR Screening of PichiaPink™ Clones, continued

PCR Procedure

Using the 5´ *AOX1* forward primer (pPink-HC, pPink-LC, and pPinkα-HC) or the α-factor forward primer (pPinkα-HC only) paired with the 3´ *CYC1* reverse primer (pPink-HC, pPink-LC, and pPinkα-HC) included in the kit, perform the following PCR procedure.

1. Set up a master mix enough for 96 colonies as follows:

10X PCR Buffer	275 µL
dNTPs (2.5 mM)	220 µL
5´ forward primer (100 µM)	4.5 µL
3´ reverse primer (100 µM)	4.5 µL
Sterile water	1685 µL
Taq Polymerase (5 U/µl)	5 µL

2. Mix the solution and aliquot 20 µL of the master mix into the 96 wells of a PCR plate.
3. Using an eight channel pipette, add 5 µL of the PichiaPink™ DNA supernatant to the plate containing the PCR master mix and pipette up and down several times.Close the PCR plate with Microplate Adhesive Film.
4. Load the thermocycler and run the following program:

Step	Temperature	Time	Cycle
Initial Denaturation	95°C	2 minutes	1X
Denaturation	95°C	1 minute	30X
Annealing	54°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1X

5. Analyze a 10 µl aliquot by agarose gel electrophoresis.

Total DNA Isolation from PichiaPink™

Introduction

The protocol below allows you to isolate DNA from untransformed as well as recombinant PichiaPink™ strains. The DNA isolated using this protocol 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.

- YPD medium (see **Recipes**, page 60)
- 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 (see page vii)
- 5 M potassium acetate, pH 8.9
- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)
- 7.5 M ammonium acetate, pH 7.5
- Phenol:chloroform (1:1 v/v)

Preparation

1. Grow at 30°C the recombinant strain and the parent strain to an OD_{600} of 5–10 in 10 mL of YPD medium.
2. Collect the cells by centrifugation at 1,500 × g for 5–10 minutes at room temperature.
3. Wash the cells with 10 mL sterile water by centrifugation as in Step 2.

Spheroplasting and Lysis

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

Continued on next page

Total DNA Isolation from PichiaPink™, continued

DNA Precipitation

1. Transfer the supernatant from Step 5, 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 mL of TE buffer, pH 7.5. Determine the concentration of the DNA sample. You may store the two samples separately or combined at –20°C until ready for use.

Genomic DNA Isolation from PichiaPink™ for PCR Analysis

Introduction

The protocol below provides an alternative procedure for you to isolate genomic DNA from untransformed as well as recombinant PichiaPink™ strains. The DNA isolated using this protocol is suitable genomic PCR.

Materials Needed

- Smash and Grab Buffer (see page 64 for recipe)
- Sterile water
- 0.45 µm glass beads, acid washed
- RNAse A (see page viii)
- Proteinase K (see page viii)
- 10% SDS (see page viii)
- 4 M NaCl
- 3 M sodium acetate
- TE buffer (see page viii)
- 100% and 70% ethanol (ice-cold)
- Phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v)

Procedure

1. Scrape some material off the YPD plate into 1 mL of sterile water in a microcentrifuge tube. Vortex.
2. Centrifuge at full speed for 10 seconds. Remove the supernatant.
3. Resuspend the pellet by adding 200 µL of smash and grab buffer, 200 µL of phenol:chloroform:isoamyl alcohol, and 0.3 g acid washed glass beads (0.45 µL), and vortexing for 3 minutes.
4. Add 200 µL of TE buffer and vortex for 5 seconds to mix. Centrifuge at full speed for 5 minutes and transfer the supernatant to a microcentrifuge tube.
5. Add 1 mL of ice-cold 100% ethanol and centrifuge at full speed for 15 minutes. Carefully remove the supernatant and keep the pellet.
6. Resuspend the pellet in 400 µL of TE buffer and 1 µL of RNAse A. Incubate at 37°C for 5 minutes.
7. Add 1 µL of 4 M NaCl, 10 µL of proteinase K, and 20 µL of 10% SDS. Incubate at 37°C for 5 minutes.
8. Add 400 µL of phenol:chloroform:isoamyl alcohol. Vortex to mix, and centrifuge at full speed for 5 minutes.
9. Transfer the supernatant (~400 µL) to a **new** microcentrifuge tube.
10. Add 40 µL of 3 M sodium acetate and 1 mL of ice-cold 100% ethanol. Vortex to mix, and centrifuge at full speed for 15 minutes. Remove the supernatant and keep the DNA pellet.
11. Add 70% ice-cold ethanol to wash the DNA. Carefully decant the alcohol.
12. Dry the DNA pellet in a 37° C incubator for 10 minutes, and resuspend in 200 µL of TE.

Total RNA Isolation from PichiaPink™

Introduction

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

Solutions

You will need to make the following solutions.

- BMGY medium (see **Recipes**, page 62)
- DEPC-treated water
- AE buffer (50 mM sodium acetate, pH 5.3, 1 mM EDTA)
- Buffered phenol
- 10% SDS in DEPC treated water
- 3 M sodium acetate, pH 5.3
- Chloroform:isoamyl alcohol (24:1 v/v)
- Phenol:chloroform (1:1 v/v)

Growing Cells

1. Grow two cultures (100–200 mL in 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 1,500 × g for 10 minutes at room temperature.
4. Resuspend cell pellet in 400 µl AE buffer and transfer to a microcentrifuge tube.

Continued on next page

Total RNA Isolation from PichiaPink™, continued

Lysis of Cells

1. Add 40 µl 10% SDS to the cells from Step 4, previous page, 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 of 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 is designed to isolate mRNA from 0.2 to 1 mg total RNA. The Micro-FastTrack™ 2.0 Kit is designed to isolate mRNA from ~100 µg total RNA. You will need ~1–5 µg mRNA per time point.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are .available on our website at www.invitrogen.com/support.

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