

PichiaPink™ Expression Strain, Secretion Signal, and Media Kits

Product	Catalog No.	Storage
PichiaPink™ Expression Strain Kit	A11154	-80°C
PichiaPink™ Secretion Signal Kit	A11155	-20°C
PichiaPink™ Media Kit	A11156	Room temperature

Introduction

This product information sheet is supplied with the PichiaPink™ Expression Strain, PichiaPink™ Secretion Signal, and PichiaPink™ Media Kits, and provides guidelines and general instructions for high-level and large-scale expression and secretion of bioactive recombinant proteins using these kits. For detailed experimental protocols, refer to the PichiaPink™ Expression System manual (A10984) available online at www.invitrogen.com or by contacting Technical Support.

PichiaPink™ System Overview

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. 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 three expression vectors (pPink α -HC, pPink-HC, and pPink-LC; available separately from Invitrogen) allowing high- and low-copy number secreted or intracellular expression.
- Eight secretion signal sequences for optimization of secreted protein expression.
- Simpler media growth conditions for screening and convenient PichiaPink™ media pouches.

Selection

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*, *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.

All PichiaPink™ strains 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, and display a slow-growth phenotype on rich medium. Transformation of the PichiaPink™ strains with an expression plasmid containing the *ADE2* gene enable the strains to grow again on medium lacking adenine (Ade dropout medium or minimal medium). Further, the color of the transformant colonies indirectly indicates the relative expression levels of your protein of interest. For more information on PichiaPink™ vector kits available from Invitrogen, visit our website at www.invitrogen.com or contact Technical Support.

Secreted 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 PichiaPink™ Secretion Signal Kit enables you to screen multiple signal sequences with your gene of for optimal expression and secretion of your recombinant protein (see **PichiaPink™ Secretion Signal Kit**, page 3).

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. 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, 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.

PichiaPink™ Expression Strain Kit

The PichiaPink™ Expression Strain Kit (Cat. no. A11154) is also included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits (Cat. nos. A11150 and A11151, respectively). The PichiaPink™ Expression Strain Kit components are described below. The PichiaPink™ Expression Strains are supplied frozen in YPD medium containing 15% glycerol. **Upon receipt, streak the strains on YPD agar and prepare glycerol stocks. 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	<i>ade2</i>	1 mL	–80°C
PichiaPink™ Strain 2	<i>ade2, pep4</i>	1 mL	–80°C
PichiaPink™ Strain 3	<i>ade2, prb1</i>	1 mL	–80°C
PichiaPink™ Strain 4	<i>ade2, prb1, pep4</i>	1 mL	–80°C

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 *Recombinant Protein Expression in Pichia pastoris* (Cregg *et al.*, 2000), and *Pichia Protocols: Methods in Molecular Biology* (Higgins & Cregg, 1998).

For detailed protocols on transforming PichiaPink™ strains, expressing recombinant strains, optimizing protein expression and secretion, and scaling-up protein expression, as well as for guidelines on PichiaPink™ fermentation, refer to the PichiaPink™ Expression System manual (A10984) available at www.invitrogen.com or by contacting Technical Support.

Growth of PichiaPink™ Expression 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.

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

- 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
- 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.
- One OD₆₀₀ = ~5 × 10⁷ cells/mL.
- 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.

Storing PichiaPink™ Expression Strains

We recommend that you prepare frozen stocks of all four PichiaPink™ strains for long term storage. 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.

To store cells for weeks to months, use YPD medium and YPD agar slants (see page 4).

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.

1. **Day 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.
2. **Day 2:** Seed 200 mL of YPD medium with the starter culture to an OD₆₀₀ of 0.2. Grow shaking for 1–2 days at 24–30°C to an OD₆₀₀ of 2–3.
3. **Day 3 or 4:** 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 OD₆₀₀ of 50–100 (approximately 2.5 × 10⁹–5.0 × 10⁹ 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.

PichiaPink™ GYWYhcb`G][bU Kit

The secretion signal sequences included in the PichiaPink™ Secretion Signal Kit (Cat. no. A11155) are supplied as phosphorylated duplex oligomers in 40 pmol aliquots lyophilized in TE Buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The sequence underlined in each signal sequence corresponds to the Kozak sequence taken from the native AOX1 gene. For detailed instructions on cloning the secretion signals, refer to the PichiaPink™ Expression System manual (part no. A10984), available on our website at (www.invitrogen.com). **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:

AATTCGAAACGATGAGATTTTCCTTCAATTTTTACTGCTGTTTTATTTCGCAGCATCCTCCGCATTAGCT

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:

AATTCGAAACGATGGTGCTTGGTGGTCTTTGTTTCTGTACGGTCTTCAGGTCGCTGCACCTGCTTTGGCT

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:

AATTCGAAACGATGTCTTTTAGATCCTTGTGGCTTGTCTGGTTGGTTTGTCTGGTTTGGCT

Complement nucleotide sequence of oligo 2:

AGCCAAACCAGAACAAACCAACCAAGCACAAAGCCAACAAGGATCTAAAAGACATCGTTTCG

Serum albumin signal sequence

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

Nucleotide sequence of oligo 1:

AATTCGAAACGATGAAGTGGGTTACCTTTATCTCTTTGTTGTTTCTTTCTCTTCTGCTTACTCT

Complement nucleotide sequence of oligo 2:

AGAGTAAGCAGAAGAGAAAAGAAACAACAAGAGATAAAGGTAACCCACTTCATCGTTTCG

Inulinase signal sequence

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

Nucleotide sequence of oligo 1:

AATTCGAAACGATGAAGTTAGCATACTCCTTGTGCTTCCATTGGCAGGAGTCAGTGCT

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:

AATTCGAAACGATGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCA

Complement nucleotide sequence of oligo 2:

TGCAGATATTTTGGCTGCAAACCAAGCCAAAAGGAAAAGGAAAGCTTGCAAAGCATCGTTTCG

Killer protein signal sequence

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

Nucleotide sequence of oligo 1:

AATTCGAAACGATGACTAAGCCAACCAAGTATTAGTTAGATCCGTCAGTATATTATTTTCATCACATTACTACATCTAGTCGTAGCT

Complement nucleotide sequence of oligo 2:

AGCTACGACTAGATGTAGTAATGTGATGAAAATAATATACTGACGGATCTAACTAATACTGGGTTGGCTTAGTCATCGTTTCG

Lysozyme signal sequence

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

Nucleotide sequence of oligo 1:

AATTCGAAACGATGCTGGGTAAGAACGACCCAATGTGTCTTGTTTTGGTCTTGTGGGATTGACTGCTTTGTTGGGTATCTGTCAAGGT

Complement nucleotide sequence of oligo 2:

ACCTTGACAGATACCCAACAAGCAGTCAATCCCAACAAGACCAAAAACAAGACACATTGGGTCGTTCTTACCCAGCATCGTTTCG



PichiaPink™ Media Kit

The PichiaPink™ Media Kit (Cat. no. A11156) is also included in the PichiaPink™ Secretion Optimization and PichiaPink™ Secreted Protein Expression Kits (Cat. nos. A11150 and A11151, respectively). 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

Preparing Media for PichiaPink™ Experiments

Follow the instructions below to prepare the media for your PichiaPink™ experiments using the PichiaPink™ Media Kit. For additional media and buffers used in PichiaPink™ experiments, refer to the PichiaPink™ Expression System manual (A10984) available online at www.invitrogen.com or by contacting Technical Support.

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 for YPD medium 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.

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 for YPD agar is several months.

YPDS Medium

YPDS medium is used for recovery of cells after electroporation. To prepare 0.2 liters of YPDS, use only one pouch of YPS.

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 above).

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

PAD Agar

PAD (Pichia Adenine Dropout) agar lacks adenine, and is used for selecting transformed PichiaPink™ strains. To prepare 1 liter of PAD agar, use only one pouch of PAD agar 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 above).

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

Purchaser Notification

These products are covered by Limited Use Label License No. 334: PichiaPink™ (see the Invitrogen catalog or our website, www.invitrogen.com). By the use of this product you accept the terms and conditions of all applicable Limited Use Label Licenses.

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