



pPIC9K

***A Pichia* Vector for Multicopy Integration and Secreted Expression**

Catalog no. V175–20

Version G
03 June 2010
25-0106

User Manual

Table of Contents

Kit Contents and Storage.....	v
Accessory Products	vi
Introduction	1
Overview	1
Materials	5
Methods	6
Cloning into pPIC9K.....	6
Analysis of <i>E. coli</i> Transformants	9
Transformation into <i>Pichia</i>	10
<i>In Vivo</i> Screening of Multiple Inserts.....	12
Analyzing Results	16
Troubleshooting.....	17
Appendix	18
Recipes	18
<i>Pichia</i> Genomic DNA Isolation	19
Easy-DNA™ Protocol for Isolation of DNA from <i>Pichia</i>	21
Determination of Copy Number of Multiple Integrants	22
Map and Features of pPIC9K	24
Technical Support.....	26
Purchaser Notification.....	27
References.....	28

Kit Contents and Storage

Shipping and Storage

pPIC9K is shipped on wet ice. Upon receipt, store pPIC9K at -20°C .

Contents

This kit contains:

20 μg of pPIC9K vector, supplied in suspension as 40 μl of 0.5 $\mu\text{g}/\mu\text{l}$ vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Accessory Products

Additional Products

The products listed in this section are intended for use with the pPIC9K vectors. For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (page 26).

Product	Quantity	Catalog no.
Original <i>Pichia</i> Expression Kit	1 kit	K1710-01
EasySelect™ <i>Pichia</i> Expression Kit	1 kit	K1740-01
Easy-DNA™ Kit	15–200 reactions	K1800-01
TA Cloning® Kit	20 reactions	K2000-01
pPIC3.5K	20 µg each	V173-20
pAO815	20 µg each	V180-20
<i>Pichia</i> EasyComp™ Transformation Kit	1 kit	K1730-01
<i>Pichia</i> Protocols	1 book	G100-01
One Shot® TOP10 (chemically competent <i>E. coli</i>)	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent <i>E. Coli</i>	10 reactions	C4040-50
	20 reactions	C4040-52
TOP10 Electrocomp™ Kits	20 reactions	C664-55
	2 × 20 reactions	C664-11
Ampicillin Sodium Salt, irradiated	200 mg	11596-027
Geneticin® Selective Antibiotic (powder)	5 g	11811-031
	25 g	11811-098
Geneticin® Selective Antibiotic (liquid)	20 ml	10131-035
	100 ml	10131-027

Introduction

Overview

Introduction

Multiple copy integration of recombinant genes in *Pichia* has been demonstrated to increase expression of the desired gene in some cases (Brierley, *et al.*, 1994; Clare, *et al.*, 1991a; Cregg, *et al.*, 1993; Romanos, *et al.*, 1991; Scorer, *et al.*, 1993; Scorer, *et al.*, 1994; Thill, *et al.*, 1990; Vedvick, *et al.*, 1991.). The pPIC9K vector included in this kit allows isolation of multicopy inserts by an *in vivo* method, in order to test whether increasing the copy number of your recombinant gene will lead to a subsequent increase in secreted protein expression. This *in vivo* method utilizes resistance to Geneticin® (G418 sulfate) to screen for possible multicopy inserts.

Description

The pPIC9K vector is identical to pPIC9 except for the presence of the kanamycin resistance gene for *in vivo* screening of multiple copy inserts. pPIC9K is functional in *Pichia* strains GS115 and KM71. Additional features of the pPIC9K vector include:

- 9276 bp fusion vector
- Four unique restriction sites for cloning in frame with the α -factor secretion signal: *Sna*B I, *Eco*R I, *Avr* II, *Not* I
- Secreted expression of your gene using the α -factor secretion signal
- For expression, your gene must be cloned in frame with the initiation codon of the signal sequence.
- *HIS4* selection in *Pichia*
- For gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates His⁺ Mut^S)
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates His⁺ Mut⁺ in GS115 and His⁺ Mut^S in KM71)
- For insertion at *HIS4*, linearize with *Sal* I (generates His⁺ Mut⁺ in GS115 and His⁺ Mut^S in KM71)

See page 10 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, or *Sal* I site.



Note

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

Continued on next page

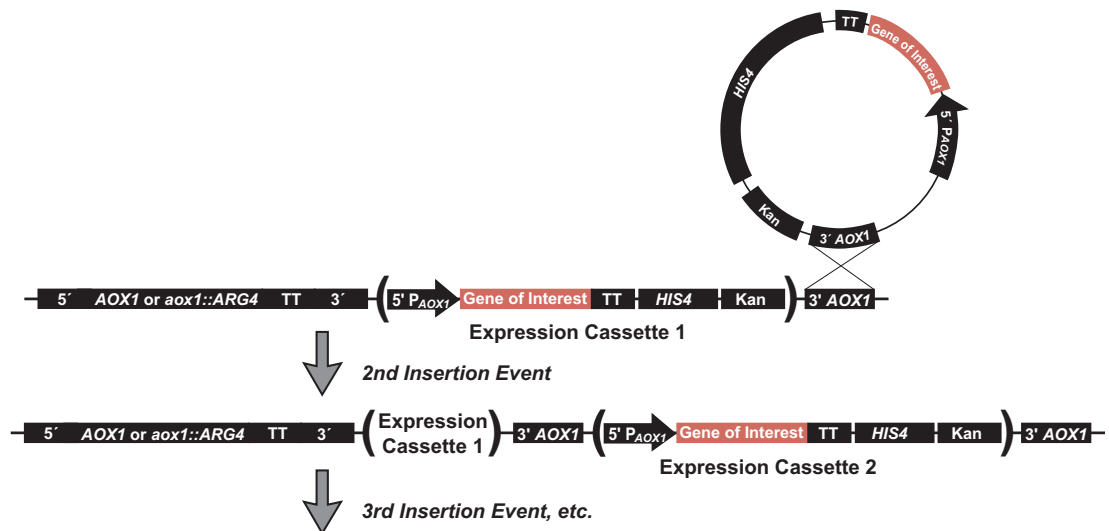
Overview, Continued

Frequency of Multicopy Inserts

Multiple plasmid integration events occur spontaneously in *Pichia* at a frequency between 1 and 10% of all His⁺ transformants. The *in vivo* method allows you to screen for the His⁺ transformants that may have multiple inserts of your gene.

Generation of Multicopy Inserts *in vivo*

pPIC9K contains the bacterial kanamycin gene (*kan* from Tn903) that confers resistance to Geneticin[®] in *Pichia*. Note that *kan* does not confer resistance to kanamycin in *Pichia*. The level of Geneticin[®] resistance roughly depends on the number of kanamycin genes integrated. A single copy of pPIC9K integrated into the *Pichia* genome confers resistance to Geneticin[®] to a level of ~0.25 mg/ml. Multiple integrated copies of pPIC9K can increase the Geneticin[®] resistance level from 0.5 mg/ml (1–2 copies) up to 4 mg/ml (7–12 copies). Because of the genetic linkage between the kanamycin gene and the "expression cassette" (*PAOX1* and your gene of interest), one can infer that Geneticin[®] resistant clones contain multiple copies of your gene. Secreted protein expression may increase because of a gene dosage effect. Thus, the presence of the *kan* gene on pPIC9K can be used as a tool to detect pPIC9K transformants that harbor multiple copies of your gene. The graphic below shows multiple insertion of your expression cassette linked to the *kan* gene.



Continued on next page

Overview, Continued

Screening on Geneticin®

Direct selection of Geneticin® resistance in yeast does not work well because newly transformed cells need time to express sufficient amounts of the resistance factor. Since yeast grow much more slowly than bacteria, significant numbers of recombinant yeast are killed before they accumulate enough of the resistance factor to survive direct plating on antibiotic.

Do not use Geneticin® resistance as a selectable marker. The procedure to generate Geneticin® resistant clones requires an initial selection of His⁺ transformants followed by a screen for varying levels of Geneticin® resistance. Resistance to Geneticin® conferred by the kanamycin gene present on pPIC9K is used as a screen, **not** as a selection for multicopy integrants.

Alternatives for Generating Multicopy Inserts

In addition to this vector for secreted expression, Invitrogen has available two other vectors, pPIC3.5K and pAO815. These vectors are designed for intracellular expression of recombinant proteins. pPIC3.5K also uses Geneticin® resistance to screen for multicopy inserts and uses the *in vivo* method. pAO815 is used to construct multiple copies of your gene *in vitro* prior to transformation into *Pichia*. Multiple copies are cloned in tandem into pAO815 and then are transformed into *Pichia*. When His⁺ transformants are selected, they will contain multiple copies of your gene.

A summary of each method is presented in the tables provided on the next page. The "best" method is the one that works for your protein; unfortunately, there is no way to predict beforehand which method will work for you.

Continued on next page

Overview, Continued

Methods for Generating Multicopy Inserts

A summary of the advantages and disadvantages of the *in vivo* and *in vitro* method for generating multicopy inserts (previous page) is presented in the tables below. The "best" method is the one that works for your protein; unfortunately, there is no way to predict beforehand which method will work for you.

In vivo Method (pPIC9K and pPIC3.5K vectors)

Advantages	Disadvantages
Easier to initiate experiment because only one copy of your gene is cloned into pPIC3.5K or pPIC9K before transforming into <i>Pichia</i>	Qualitative screen—Geneticin [®] resistance roughly correlates with the number of copies of your gene.
Identifies the 1–10% of spontaneous His ⁺ transformants that have multiple inserts	Screening His ⁺ transformants may involve more work because you will need thousands of His ⁺ transformants to generate enough Geneticin [®] resistant colonies to test
Average size of vector is similar to other <i>Pichia</i> expression vectors	The number of multiple inserts is unknown (although this can be determined through Southern or dot blot analysis)
Multiple inserts are located at a single locus	Screening on Geneticin [®] is sensitive to the density of the cells and may result in the isolation of false positives

In vitro Method (pAO815 vector)

Advantages	Disadvantages
Quantitative—construction of a defined number of multimers	More work up front to clone defined number of multimers
Most of the His ⁺ transformants will contain the proper, defined number of inserts	Size of the vector may become quite large depending on the size of your gene and the number of copies you create
Isolation of recombinants with multiple inserts is easier because most of the His ⁺ transformants will contain multiple copies of your gene	Rearrangements in <i>E. coli</i> may occur
<i>In vitro</i> construction allows step-wise analysis of copy number effects on protein expression	
Multiple inserts are located at a single locus	
No need for a second drug resistance marker in the vector	

Materials

Materials Needed

For the procedures described in this manual, you will need:

- Manual from the *Pichia* Expression System
- Microbiological equipment
- Electrocompetent or chemically competent *E. coli* (must be *recA*, *endA*) for transformation. You will need 3–4 tubes of competent cells per experiment. For protocols to prepare competent *E. coli* and transformation protocols, see *Current Protocols* (Ausubel, *et al.*, 1990) or *Molecular Biology: A Laboratory Manual* (Sambrook, *et al.*, 1989)
- Sterile water
- Phenol/chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/ μ l)
- 10X Ligation Buffer (with ATP)
- LB medium
- LB-ampicillin plates (50–100 μ g/ml ampicillin)
- 16°C, 37°C, and 65°C water baths or temperature blocks
- Geneticin[®]
- YPD- Geneticin[®] plates (see **Recipes**, page 18)
- 50 ml conical centrifuge tubes
- Hemacytometer
- 30°C and 37°C incubator
- Microtiter plates (optional)



Important

Procedures for transformation into *E. coli* and *Pichia*, analysis of recombinants, and expression are described in the *Pichia* manual. This manual is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (page 26).

Continued on next page

Methods

Cloning into pPIC9K

Introduction

It is important to clone your gene in frame with the α -factor signal sequence. Below are some guidelines to consider when developing a cloning strategy for this vector. Refer to page 8 for the multiple cloning site of pPIC9K.



We recommend that you transform pPIC9K into *E. coli*, so that you have a permanent stock and a way to make more plasmid.

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

General Considerations

The following are some general considerations applicable to pPIC9K.

- The codon usage in *Pichia* is believed to be the same as *Saccharomyces cerevisiae*.
- Many *Saccharomyces* genes have proven to be cross-functional in *Pichia*.
- Plasmid constructions should be maintained in a *recA*, *endA* mutant *E. coli* strain such as TOP10. Electrocompetent TOP10 cells are available from Invitrogen (page vi)
- The native 5' end of the *AOX1* mRNA is noted in the multiple cloning site (see page 8). This is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.
- Translation termination is determined by either stop codons in the gene of interest or in the 3' *AOX1* sequence. The stop codons in the 3' *AOX1* sequence are noted in the multiple cloning site (see page 8).
- The premature termination of transcripts because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger, *et al.*, 1991; Scorer, *et al.*, 1993; Zaret and Sherman, 1984). If you have problems expressing your gene, check for premature termination and AT rich regions. It may be necessary to change the sequence in order to express your gene (Scorer, *et al.*, 1993).
- The predicted protease cleavage sites for the α -factor signal sequences are indicated in the multiple cloning site (see page 8).
- The open reading frame (ORF) of the mature gene of interest should be cloned in frame and downstream of the α -factor signal sequence.

Continued on next page

Cloning into pPIC9K, Continued

General Cloning Strategies

Strategies generally fall into three different categories:

1. Ligation of a compatible restriction fragment:
 - a) Forced (directional) insertion involving the use of two different sites in the multiple cloning site.
 - b) Ligation of the fragment with the same restriction end on both ends into a single, compatible site.
 2. PCR amplification of the fragment containing the gene of interest in such a way that compatible restriction ends are generated for ligation into the appropriate vector.
 3. Direct cloning of an amplified fragment containing the gene of interest via the TA Cloning[®] Kit (Catalog no. K2000-01), followed by subcloning of a compatible fragment into pPIC9K.
-

Cloning Procedures

Refer to (Ausubel, *et al.*, 1990), pages 3.16.1 to 3.17.3. or (Sambrook, *et al.*, 1989), pages 5.10 to 5.13. for help with cloning.

Signal Sequence Processing

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

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

Optimization of Signal Cleavage

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

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

Bacterial Transformation

Once you have decided on a cloning strategy, you will need to prepare competent *E. coli* cells for transformation before setting up your ligation reactions. See *Current Protocols in Molecular Biology* (Ausubel, *et al.*, 1990) or *Molecular Biology: A Laboratory Manual* (Sambrook, *et al.*, 1989) for preparation of electrocompetent or chemically competent *E. coli* or use your laboratory's procedure.

Continued on next page

Cloning into pPIC9K, Continued

PAOX1 and Multiple Cloning Site of pPIC9K

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

```

      AOX1 mRNA 5'end (824)                                5' AOX1 primer site (855-875)
      |-----|                                           |-----|
TTATCATCAT TATTAGCTTA CTTTCATAAT TCGGACTGGT TCCAATTGAC
      |-----|
AAGCTTTTGA TTTTAACGAC TTTTAACGAC AACTTGAGAA GATCAAAAAA
      |-----|
CAACTAATTA TTCGAAGGAT CCAAACG ATG AGA TTT CCT TCA ATT
                                Start (949)   α-Factor Signal Sequence
                                Met Arg Phe Pro Ser Ile

TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT
Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala

CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG
Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro

GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe

GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly

                                α-Factor primer site (1152-1172)
TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

GAA GAA GGG GTA TCT CTC GAG AAA AGA ▼ Signal cleavage (1203-1204) SnaBI
Glu Glu Gly Val Ser Leu Glu Lys Arg Glu Ala Glu Ala Tyr
      Eco RI      Ayr II      Not I
      |      |      |
GTA GAA TTC CCT AGG GCG GCC GCG AAT TAA TTCGCCTTAG
Val Glu Phe Pro Arg Ala Ala Ala Asn ***

ACATGACTGT TCCTCAGTTC AAGTTGGGCA CTTACGAGAA GACCGGTCTT

                                3' AOX1 primer site (1327-1347)
GCTAGATTCT AATCAAGAGG ATGTCAGAAT GCCATTTGCC TGAGAGATGC

AGGCTTCATT TTTGATACTT TTTTATTTGT AACCTATATA GTATAGGATT

      ↓ AOX1 mRNA 3' end (1418)
TTTTTTGTCA
  
```

Special Considerations

- The fragment containing the gene of interest must be cloned in frame with the secretion signal open reading frame.
- An initiating ATG is provided by the signal sequence. Translation will initiate at the ATG closest to the 5' end of the mRNA.
- If your insert has a *Bgl* II site, see page 10 for alternate restriction sites to linearize your plasmid for *Pichia* transformation.

Analysis of *E. coli* Transformants

Introduction

At this point you should have ligation reactions that you will transform by chemical means or electroporation into competent *E. coli* cells (TOP10 or equivalent) using your method of choice.

Analysis of Transformants

1. After transformation, plate 10 μ l and 100 μ l of the transformation mix onto LB plates with 50–100 μ g/ml ampicillin (see **Recipes**, page 18) and select ampicillin resistant colonies.
 2. Pick 10 ampicillin resistant transformants and inoculate into 2 ml LB medium with 50–100 μ g/ml ampicillin. Grow overnight at 37°C with shaking.
 3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below). To sequence your construct in pPIC9K, use the α -factor and the 3' *AOX1* primer sequences (see below).
 4. Make a glycerol stock of your desired clone for safekeeping by combining 0.85 ml of an overnight bacterial culture with 0.15 ml of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at –70°C.
-

Sequencing Recombinant Clones

We strongly recommend that you sequence your construct before transforming into *Pichia* to confirm that your gene is in frame with the α -factor secretion signal ATG. We suggest using the α -factor and 3' *AOX1* primer sequences to sequence your construct. Refer to the diagram on the previous page for the sequence and location of these primer binding sites.

For sequencing protocols, refer to Unit 7 in *Current Protocols in Molecular Biology* (Ausubel, *et al.*, 1990) or Chapter 13 in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.*, 1989).

After Sequencing

Once you have cloned and sequenced your insert, proceed to **Transformation into *Pichia***, page 10. You will need to generate enough plasmid DNA to transform *Pichia* (5–10 μ g of each plasmid per each transformation).

Transformation into *Pichia*

Introduction

At this point you will have your gene cloned in pPIC9K. You should also have about 5–10 µg of each construct for each transformation into *Pichia*. For methods to transform *Pichia* and select His⁺ transformants, refer to the *Pichia* Expression System manual. To linearize your construct prior to transformation into *Pichia*, see below.

Linearization of Plasmid DNA

It is recommended that you linearize your vector in such a manner to generate both Mut⁺ and Mut^S recombinants. It is possible that one phenotype will express your multicopy integrant better than the other. To linearize pPIC9K containing one copy of your gene:

- *Bgl* II for replacement at *AOX1* (GS115, Mut^S)
- *Sac* I for insertion at *AOX1* (GS115, Mut⁺ or KM71, Mut^S)
- *Sal* I for insertion at *HIS4* (GS115, Mut⁺ or KM71, Mut^S)

Use strain KM71 if you only want Mut^S recombinants. If your insert contains any of the above restriction sites, see the table below for alternate sites.

Alternate Restriction Sites

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

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

Restriction Enzyme	5' <i>AOX1</i>	3' <i>AOX1</i>	Vector backbone	<i>HIS4</i> gene
<i>Sac</i> I	209	--	--	--
<i>Pme</i> I	414	--	--	--
<i>Bpu</i> 1102 I	589	--	--	--
<i>Xcm</i> I	699	--	--	--
<i>Aat</i> II*	(9102)	--	--	--
<i>Tth</i> III I*	--	(7034)	--	--
<i>Bgl</i> II†	2	6875	--	--
<i>Dra</i> I†	414	6713	6855, 8046, 8065, 8757	--
<i>Sal</i> I	--	--	--	3178
<i>Bsp</i> E I	--	--	--	3845

*Restriction sites are outside the *AOX1* sequences in the vector backbone, but they are close enough for efficient recombination to occur.

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

Continued on next page

Transformation into *Pichia*, Continued

Controls

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

- The parent vector linearized in the same manner as your construct. This will be used as a control to confirm integration via PCR (see the *Pichia* Expression Manual for a protocol) and as control for background in expression analysis and quantitative dot blots or Southern analysis.
- Remember to also isolate a His⁺ transformant with just one copy of your gene inserted. Most of the His⁺ transformants created by transforming with recombinant pPIC9K will only have one copy. Make sure that the transformant you pick is **only** resistant to 0.25 mg/ml Geneticin[®], and that it has the same Mut phenotype as the putative multimeric recombinants you are testing. This recombinant will be used as a control to compare expression levels with multiple copies of your expression cassette and as a control for single copy for quantitative dot blot or Southern analysis. This is a very important control as increasing the copy number of the desired gene does not always lead to increased expression of recombinant protein.

Transformation into *Pichia*

Refer to the *Pichia* Expression Manual for procedures to prepare *Pichia* for transformation, transformation procedures, selection of His⁺ recombinants, and screening for Mut⁺ and Mut^s phenotypes (if desired).

Once you have generated His⁺ transformants using recombinant pPIC9K, proceed to ***In Vivo* Screening of Multiple Inserts**, next page.

In Vivo Screening of Multiple Inserts

Introduction

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

Methods to Screen for Geneticin[®] Resistant Transformants

There are two methods used to screen His⁺ transformants for Geneticin[®] resistance:

Method 1 is technically easier and screens a greater number of clones, but is less reliable. After initial selection of His⁺ transformants, colonies are pooled and plated on YPD- Geneticin[®] plates containing increasing concentrations of Geneticin[®]. **Method 1 is applicable to spheroplast or electroporation transformation methods.**

Method 2 is technically more difficult and screens fewer numbers of clones, but it is more reliable. It involves growing clones in microtiter plates until all clones are at the same density. The cultures are then spotted on the YPD-Geneticin[®] plates and scored for Geneticin[®] resistance.



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



Note

The kanamycin resistance gene was cloned into pPIC9K with its native bacterial promoter. The level of expression is very low. You need to have multicopy integrants before you can begin to see resistance to Geneticin[®].

Continued on next page

***In Vivo* Screening of Multiple Inserts, Continued**

Before Starting

Prepare 4 YPD plates containing each of the following concentrations of Geneticin®:
0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml (see **Recipes**, page 18)

Method 1, (Spheroplasts)

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

1. Using a sterile spreader, remove the top layer of soft agar containing the His⁺ transformants and place into a sterile, 50 ml conical centrifuge tube.
 2. Add 10 to 20 ml of sterile water. There should be a 2X volume of water above the settled agar. Vortex vigorously for 1–2 minutes.
 3. Set centrifuge tube upright on bench and let agar pieces settle (about 1 minute).
 4. Determine the cell density of the supernatant by using a hemacytometer. You need at least 5×10^5 cells/ml so you can plate $\sim 10^5$ cells in 200 μ l or less. (If the cells are too dilute, transfer the liquid to a fresh tube and centrifuge the cells. Resuspend the cell pellet in sterile water in a volume sufficient to give 5×10^5 cells/ml.)
 5. Plate 10^5 cells on YPD-Geneticin® plates containing Geneticin® at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml. Use four plates for each concentration. (You may want to confirm the titer of the cells on YPD plates without Geneticin® in order to calculate the percent of Geneticin® resistant colonies you obtain for each Geneticin® concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5} , 10^{-6} , and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100–200 μ l per plate.)
 6. Incubate plates at 30°C and check daily. Geneticin®-resistant colonies will take 2–5 days to appear while cells plated on YPD without Geneticin® will take 2–3 days. Proceed to **Analysis of Results**, page 16.
-

Continued on next page

In Vivo Screening of Multiple Inserts, Continued

Method 1, (Electroporation)

Use this procedure when electroporation was used to transform *Pichia*.

Transformants will not be plated in top agar. Start with plates containing His⁺ transformants.

1. Pipette 1–2 ml sterile water over the His⁺ transformants on each plate. Use all the plates that have His⁺ transformants.
2. Resuspend the His⁺ transformants into the water by using a sterile spreader and running it across the top of the agar. Be careful not to tear the agar.
3. Transfer and pool the cell suspension into a sterile, 50 ml conical centrifuge tube and vortex briefly (5–10 seconds).
4. Determine cell density using a hemacytometer or spectrophotometer. $1 \text{ OD}_{600} = 5 \times 10^7 \text{ cells/ml}$. Note: any agar present will interfere with a spectrophotometer reading.
5. Plate 10^5 cells on YPD plates containing Geneticin[®] at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml. (You may want to confirm the titer of the cells on the YPD plates without Geneticin[®] in order to calculate the percent of Geneticin[®] resistant colonies you obtain for each Geneticin[®] concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5} , 10^{-6} , and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100–200 μl per plate.)
6. Incubate plates at 30°C and check daily. Geneticin[®]-resistant colonies will take 2–5 days to appear while cells plated on YPD will take 2–3 days. Proceed to **Analysis of Results**, page 16.



Note

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

***In Vivo* Screening of Multiple Inserts, Continued**

Method 2

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

1. Using sterile technique, add 200 µl YPD to each microtiter well.
2. Inoculate each well of the first set of plates with a single His⁺ transformant using a sterile toothpick and stirring to resuspend cells.
3. Cover the microtiter plate and incubate at 30°C for **2 days** (shaking not required).
4. **After 2 days**, take new microtiter plates and add 190 µl of YPD to each well.
5. Inoculate the second set of microtiter plates with 10 µl from the first set of microtiter plates by using a multi-channel pipette. Make sure the second set of plates is marked and oriented in such a way that you can keep track of wells.
6. Cover and incubate the second set of plates overnight at 30°C.
7. The next day, repeat Steps 5–6, creating a third set of microtiter plates.
Note: Successive growth and passage of the clones will bring them all to the same cell density.
8. After incubation, take the third set of plates and resuspend the cells in each well by pipetting up and down with a multi-channel pipette set on 100 µl volume.
9. Spot 10 µl from each well on YPD plates containing Geneticin[®] at a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml. Spot in a regular pattern using the multi-channel pipette or a grid underneath the plate.
10. Let the liquid soak in, then incubate plates at 30°C, and check after 2, 3, 4, or 5 days for Geneticin[®] resistant clones. Proceed to Analysis of Results, next page.

Continued on next page

Analyzing Results

Analysis of Results

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

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

Analyze for the presence of your insert by PCR (see the *Pichia* Expression System manual for a protocol). PCR will only tell you if your gene is present. It will not tell you how many copies of your gene are integrated or at which locus the integration occurred. PCR can reasonably be done on 12–20 transformants. Remember to include the vector only and original construct (one copy) controls in order to analyze your PCR experiment.

Since there is no guarantee that multiple copies will actually increase the amount of protein expressed, most people elect to proceed directly to expression to see if any of these colonies over-express their protein. Be sure to include a single copy insert as a control. Test all your Geneticin[®] resistant colonies for their Mut phenotype so that you induce expression properly. Refer to the *Pichia* Expression System manual for methods to express your protein.



Determination of Copy Number

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

If you find that your Geneticin[®] resistant His⁺ recombinants significantly over-express your protein, you may wish to quantify the copy number of your gene. Copy number may be analyzed by Southern or quantitative dot (slot) blots. See pages 22–23 for information on these techniques. It is very important to include genomic DNA isolated from *Pichia* recombinants transformed with pPIC9K alone, and pPIC9K with a single copy of your gene as controls in order to evaluate your experiment.

Troubleshooting

Introduction

Review the information below to troubleshoot your experiments using pPIC9K.

Problem	Cause	Solution
False positives	Not enough levels of Geneticin [®] resistance	Colonies can appear to be Geneticin [®] resistant, but are actually not. To prevent this, purify your putative Geneticin [®] resistant colonies and confirm the level of Geneticin [®] resistance observed for each colony before proceeding further.
Low number of Geneticin [®] -resistant colonies isolated	Not enough His ⁺ transformants were screened	The frequency of spontaneous, multiple-integration events occurs only at a rate of 1–10%. You will need to screen thousands of His ⁺ transformants to isolate an optimal amount of colonies.
Few recombinants with gene of interest	Not enough His ⁺ transformants were screened	You will need to screen thousands of His ⁺ transformants to isolate an optimal amount of recombinants with the most copies of your gene, as successive multiple insertions are more rare.
Low isolation of His ⁺ transformants	Low transformation efficiency	Using electroporation instead of spheroplasting may increase the transformation efficiency, thereby allowing you to isolate more His ⁺ transformants.

Appendix

Recipes

Introduction

See the current *Pichia* Expression manual for *Pichia* growth and selection media. YPD- Geneticin[®] medium is described below.

YPD-Geneticin[®] Plates

Yeast Extract Peptone Dextrose Medium

- 1% yeast extract
- 2% peptone
- 2% dextrose (glucose)
- 1.5% agar
- Variable amounts of Geneticin[®]

10X D-glucose (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.

100 mg/ml Geneticin[®]

Geneticin[®] is available separately from Invitrogen (Catalog no.11811-031).

Prepare 30-50 ml of 100 mg/ml Geneticin[®] stock solution in sterile water. Filter sterilize and store frozen at -20°C. You will use this solution to make YPD plates containing Geneticin[®] at final concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/ml.

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

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

Final [Geneticin[®]] (mg/ml)	ml Geneticin[®] stock/250 ml YPD
0.25	0.625
0.50	1.25
0.75	1.875
1.00	2.5
1.50	3.75
1.75	4.375
2.00	5.0
3.00	7.5
4.00	10.0

Pichia Genomic DNA Isolation

Introduction

The protocol below allows you to isolate DNA from the desired His⁺ recombinant and untransformed GS115 or KM71. The DNA isolated 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.*, 1990), *Guide to Yeast Genetics and Molecular Biology*, pages 322–323 (Strathern and Higgins, 1991), or Holm, *et al.*, 1986 for other methods to isolate DNA from *Pichia*.

In addition to the protocol listed below, we use our Easy-DNA™ Kit (page vi) to isolate DNA from *Pichia* for PCR and quantitative dot (slot) blots. See page 22 for this protocol.

Lastly, there is a fast DNA isolation protocol for multiple samples (24) which has been reported (Wach, *et al.*, 1994).

Solutions Needed

You will need to prepare the following solutions.

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

Preparation

1. Grow the recombinant strain and the parent strain at 30°C to an OD₆₀₀ of 5–10 in 10 ml of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115).
 2. Collect the cells by centrifugation at 1500 × g for 5–10 minutes at room temperature.
 3. Wash the cells with 10 ml sterile water by centrifugation as in Step 2.
-

Spheroplasting and Lysis

1. Resuspend the cells in 2 ml of SCED buffer, pH 7.5. Make this solution fresh.
 2. Add 0.1–0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve ~80% spheroplasting.
 3. Add 2 ml of 1% SDS, mix gently and set on ice 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

***Pichia* Genomic DNA Isolation, 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 $10,000 \times g$ 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 $10,000 \times g$ 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 $50 \mu\text{l}$ of TE buffer, pH 7.5. Determine the concentration of the DNA sample. The two samples can be stored separately or combined and stored at -20°C until ready for use.
-

Easy-DNA™ Protocol for Isolation of DNA from *Pichia*

Introduction

The method below was developed at Invitrogen to conveniently isolate DNA from *Pichia pastoris*.

Solutions Needed

You will need to prepare the following solutions.

- Minimal Medium (MD, MGY)
 - TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)
 - 1 M Sorbitol, 100 mM EDTA, 14 mM β -mercaptoethanol (make fresh)
 - Zymolyase, 3 mg/ml stock solution in water (Seikagaku America, Inc.)
 - SCED (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT, make fresh)
 - Easy-DNA™ Kit (Invitrogen, Catalog no. K1800-01)
 - Chloroform
 - Isopropanol
 - 70 or 80% ethanol
 - RNase A
-

Preparation of Cells

1. Grow the recombinant strain and the parent strain at 30°C to an OD₆₀₀ of 5-10 in 2-5 ml of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71).
 2. Harvest 1.5 ml of the culture by centrifuging at maximum speed in a microcentrifuge for 1-2 minutes at room temperature.
 3. Resuspend cells in 1.5 ml TE and centrifuge as in Step 2.
 4. Resuspend cells in 1 ml fresh 1 M Sorbitol, 100 mM EDTA, 14 mM β -mercaptoethanol. Vortex to resuspend.
 5. Add 1.5 μ l of 3 mg/ml Zymolyase to each tube of cells and incubate at 30°C for 1 hour.
 6. Centrifuge at 2,600 \times g in a microcentrifuge for 8 minutes at room temperature. It is important to centrifuge with less force as the cells are fragile because of digestion with Zymolyase.
 7. **Gently** resuspend cells in 200 μ l fresh SCED and incubate at 37°C for 1 hour.
-

DNA Isolation

1. Add 350 μ l Easy-DNA™ Solution A to the cell suspension from Step 7, above, vortex, and incubate at 65°C for 10 minutes.
 2. Add 150 μ l of Easy-DNA™ Solution B and vortex.
 3. Add 600 μ l chloroform and vortex.
 4. Centrifuge at maximum speed for 20 minutes at room temperature.
 5. Transfer the aqueous layer to a fresh tube, add 600 μ l isopropanol, and mix by inversion. Incubate at room temperature for 10 minutes.
 6. Centrifuge sample at maximum speed for 20 minutes at +4°C.
 7. Wash pellet with cold 70 or 80% ethanol, centrifuge at maximum speed for 2 minutes at +4°C, remove ethanol, and air-dry.
 8. Resuspend the pellet in 50 μ l TE containing 50 μ g/ml RNase A and incubate overnight at room temperature. Quantify the amount of DNA. We generally use 5 μ l of this DNA solution in a 50 μ l PCR reaction.
-

Determination of Copy Number of Multiple Integrants

Introduction

You may wish to determine the actual number of gene copies in your *Pichia* recombinant. You may either use quantitative dot blots or Southern hybridization to analyze gene copy number (Brierley, *et al.*, 1994; Clare, *et al.*, 1991a; Romanos, *et al.*, 1991; Scorer, *et al.*, 1993; Scorer, *et al.*, 1994). This requires isolation of genomic DNA from *Pichia* recombinants transformed with the parent vector (0 copies of your gene), pPIC9K containing 1 copy of your gene (single copy control), and the *Pichia* recombinants containing multiple copies of your gene. Use the protocols detailed on the pages 19 and 21 to isolate genomic DNA.

Quantitative Dot Blot Solutions

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

- 50 mM EDTA, 2.5% β -mercaptoethanol pH 9
 - 1 mg/ml Zymolyase 100T in water (Seikagaku America, Inc.)
 - 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate, pH 7 \times SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH 7)
 - 3MM paper.
-

Quantitative Dot Blot Procedure

The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos, *et al.*, 1991). It is very important to spot equivalent numbers of cells onto filters in order to quantify copy number.

1. Grow Mut⁺ or Mut^S transformants in individual wells of a 96-well microtiter plate in 200 μ l of YPD broth at 30°C until all wells have approximately the same density. This may necessitate several passages; see page 16 for more details. Alternatively, individual transformants may be grown in culture tubes and the absorbance at 600 nm normalized with the addition of medium.
2. Filter 50 μ l of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multi-channel pipette. Air dry filters.
3. To lyse the cells on the filter, you need to treat the filter with four solutions as follows: place two sheets of 3MM paper in a tray and soak with 10–15 ml of 50 mM EDTA, 2.5% β -mercaptoethanol pH 9. Make sure that the paper is uniformly soaked and that there are no puddles. Place the nitrocellulose filter face down on the treated 3MM paper. Incubate for 15 minutes at room temperature.
4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak with 10-15 ml of 1 mg/ml Zymolyase 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate for 4 hours at 37°C.
5. Remove the nitrocellulose filter from the paper and replace the paper with two new sheets. Soak with 10-15 ml of 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate, pH 7. Place the nitrocellulose filter face down on the paper and incubate for 5 minutes at room temperature.
6. Remove the nitrocellulose filter and replace with two new 3MM sheets. Soak with 10-15 ml of 2 \times SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate for 5 minutes at room temperature. **Repeat.**
7. Bake nitrocellulose filters at 80°C or UV-crosslink DNA to nylon. The filters may be probed with a non-radioactive-labeled or random-primed, ³²P-labeled probe complementary to your gene.

Multi-copy integrants can be identified by a strong hybridization signal relative to the single copy control. Dot blots can then be quantified for copy number by densitometry of the film or blot, or by using a scanner (if radiolabeled).

Continued on next page

Determination of Copy Number of Multiple Integrants, Continued

Southern Blot Analysis

For a detailed description of this technique as applied to *Pichia pastoris*, see (Clare, *et al.*, 1991a). It is very important to digest your DNA with the right restriction enzyme(s) to generate a blot of digested and gel-separated genomic DNA. Digestion of DNA from *Pichia* recombinants containing multiple copies will produce a band that will vary in intensity depending on the number of copies of your gene. It is very important to include a control to show the intensity of a single copy gene. The band intensities can be relatively quantified using densitometry to estimate gene dosage.

Controls

It is very important to include DNA from the host strain alone (GS115 or KM71), the host strain transformed with the parent vector (pPIC9K), and the host strain transformed with a vector containing one copy of your gene. It is also a very good idea to make a probe to the *HIS4* gene as an internal control for single copy in addition to a probe to your gene. Note that if your gene inserts into *his4*, two copies of the *HIS4* gene are created (see **Recombination and Integration in *Pichia***, *Pichia* Expression Kit manual).

General Guidelines

- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.*, 1989), pages 9.31–9.58.
 - Isolate genomic DNA and quantify using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane in order to accurately determine copy number.
 - Probe your Southern blot with probes to both *HIS4* and your gene. Note that the point mutation in the *his4* gene in the host strain will not interfere with hybridization if you make the probe complementary to the wild-type gene.
 - Use *Bgl* II to digest your DNA (Clare, *et al.*, 1991a). Note that all multimers are NOT necessarily in a head-to-tail configuration. Some multimers may be head-to-head and others tail-to-tail. We recommend that you think about what products may be produced. An expression cassette in the opposite orientation may produce a different band. The number of multiple copies will cause one or two bands (depending on orientation) in the Southern blot to increase in intensity once you are >2 copies.
 - *Bgl* II digested DNA from GS115 and GS115 transformed with pPIC9K will produce bands of 2.8 kb (from the genomic copy of *HIS4*) and ~6.7 kb (from the vector copy of *HIS4*), respectively, when probed with a complementary fragment to *HIS4*.
-

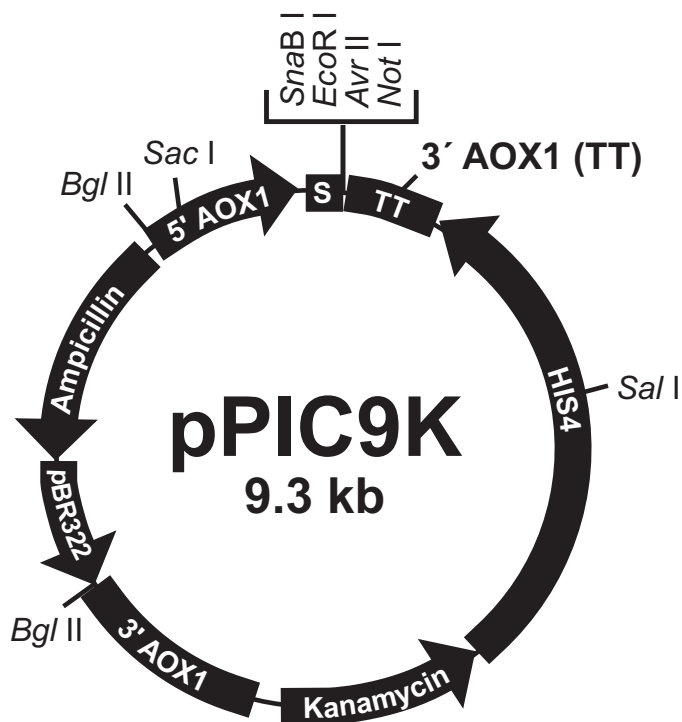
Map and Features of pPIC9K

Map of pPIC9K

The figure below shows the map of pPIC9K. Details of the multiple cloning site and the α -factor secretion signal are shown on page 8. The complete sequence of pPIC9K is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (page 26).

Comments for pPIC9K: 9276 nucleotides

5' AOX1 promoter fragment: bases 1-948
5' AOX1 primer site: bases 855-875
 α -Factor secretion signal(s): bases 949-1218
 α -Factor primer site: bases 1152-1172
Multiple Cloning Site: bases 1192-1241
3' AOX1 primer site: bases 1327-1347
3' AOX1 transcription termination (TT): bases 1253-1586
HIS4 ORF: bases 4514-1980
Kanamycin resistance gene: bases 5743-4928
3' AOX1 fragment: bases 6122-6879
pBR322 origin: bases 7961-7288
Ampicillin resistance gene: bases 8966-8106



Map and Features of pPIC9K, Continued

Features of pPIC9K

The table below describes the features of the pPIC9K expression vector.

Feature	Description	Benefit
5' <i>AOX1</i>	An ~1000 bp fragment containing the <i>AOX1</i> promoter	Allows methanol-inducible high level expression in <i>Pichia</i> Targets plasmid integration to the <i>AOX1</i> locus.
α -factor Signal Sequence	269 bp fragment encoding the α -factor signal sequence for secretion in <i>Pichia</i>	Allows secretion of desired protein into the medium
MCS	Multiple Cloning Site	Allows insertion of your gene into the expression vector
TT	Native transcription termination and polyadenylation signal from <i>AOX1</i> gene (~260 bp)	Permits efficient transcription termination and polyadenylation of the mRNA
<i>HIS4</i>	<i>Pichia</i> wild-type gene coding for histidinol dehydrogenase (~2.4 kb) and used to complement <i>Pichia his4</i> strains	Provides a selectable marker to isolate <i>Pichia</i> recombinant strains
3' <i>AOX1</i>	Sequences from the <i>AOX1</i> gene that are further 3' to the TT sequences (~650 bp)	Targets plasmid integration at the <i>AOX1</i> gene
Amp pBR322 origin	Ampicillin resistance gene <i>E. coli</i> origin of replication	Allows selection, replication, and maintenance in <i>E. coli</i>
<i>Not</i> I <i>Bgl</i> II <i>Sac</i> I <i>Sal</i> I	Unique restriction sites	Permits linearization of vector for efficient integration into the <i>Pichia</i> genome and generation of either Mut ⁺ or Mut ^S recombinants
<i>kan</i>	Kanamycin resistance gene from Tn903 which confers resistance to Geneticin [®] in <i>Pichia</i> and kanamycin resistance in <i>E. coli</i>	Allows <i>in vivo</i> screening for multicopy inserts by increased resistance to Geneticin [®] Also allows selection for kanamycin resistance in <i>E. coli</i>

Technical Support

Web Resources



Visit the Invitrogen web site 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.
-

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our web site (www.invitrogen.com).

Corporate Headquarters:

Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:

Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS

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

Certificate of Analysis

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

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Support Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore, Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Support Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

The *Pichia* Expression System is covered under the licenses detailed below.

Limited Use Label License No. 74: *Pichia* Pastoris Expression System

The *Pichia* Expression System is based on the yeast *Pichia pastoris*. *Pichia pastoris* was developed into an expression system by scientists at Salk Institute Biotechnology/ Industry Associates (SIBIA) and Phillips Petroleum for high-level expression of recombinant proteins. All patents for *Pichia pastoris* and licenses for its use as an expression system are owned by Research Corporation Technologies (RCT), Inc., Tucson, Arizona. Life Technologies has an exclusive license to sell *Pichia* expression kits and vectors to scientists for research purposes only, under the terms described below. Use of *Pichia pastoris* by commercial entities for any commercial purpose requires the user to obtain a commercial license as detailed below. Before using any *Pichia* expression product, please read the following license agreement. If you do not agree to be bound by its terms, contact Life Technologies within 10 days for authorization to return the unused *Pichia* expression products and to receive a full refund. If you do agree to the terms of this license agreement, please complete the User Registration Card and return it to Life Technologies before using the product.

Life Technologies Corporation ("Life Technologies") grants you a non-exclusive license to use the enclosed *Pichia* expression vectors ("Expression Vector") for academic research or for evaluation purposes only. The Expression Vectors are being transferred to you in furtherance of, and reliance on, such license. You may not use the Expression Vectors for any commercial purpose without a license for such purpose from Research Corporation Technologies, Inc., Tucson, Arizona.

Commercial purposes include: any use of Expression Products or Expression Vectors in a Commercial Product; any use of Expression Products or Expression Vectors in the manufacture of a Commercial Product; any sale of Expression Products; any use of Expression Products or the Expression Kit to facilitate or advance research or development directed to a Commercial Product; and any use of Expression Products or the Expression Kit to facilitate or advance any research or development program the results of which will be directly applied to the development or manufacture of a Commercial Product. "Expression Products" means products expressed with the Expression Kit, or with the use of any *Pichia* expression vectors (including the Expression Vector) or host strains. "Commercial Product" means any product intended for sale or commercial use.

Commercial entities may conduct their evaluation for one year at which time this license automatically terminates. Commercial entities will be contacted by Research Corporation Technologies during the evaluation period regarding their desire for a commercial license.

Access to the Expression Kit and Vector must be limited solely to those officers, employees and students of your institution who need access to perform the above-described research or evaluation. You must inform each such officer, employee and student of the provisions of this license agreement and require them to agree, in writing, to be bound by the provisions of this license agreement. You may not distribute any Expression Vector or host strain contained herein or in the Expression Kit to others, even those within your own institution. You may only transfer modified, altered, or original material from the Expression Kit or Vector to a third party following written notification of, and written approval from, Life Technologies so that the recipient can be licensed. You may not assign, sub-license, rent, lease or otherwise transfer this license agreement or any of the rights or obligation there under, except as expressly permitted by Life Technologies and RCT.

This license agreement is effective until terminated. You may terminate it at any time by destroying all *Pichia* Expression products in your control. It will also terminate automatically if you fail to comply with the terms and conditions of the license agreement. You shall, upon termination of the license agreement, destroy all *Pichia* Expression products in your control, and so notify Life Technologies in writing.

You may contact Research Corporation Technologies at the following address: Bennett Cohen, Ph.D., Research Corporation Technologies, 101 North Wilmot Road, Suite 600, Tucson, Arizona 85711-3335. Tel: 520-748-4443, Fax: 520-748-0025.

References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1990) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.
- Brierley, R. A., Davis, G. R. and Holtz, G. C. (1994) Production of Insulin-Like Growth Factor-1 in Methylophilic Yeast Cells. *United States Patent* 5,324,639.
- Cavener, D. R. and Stuart, C. R. (1991) Eukaryotic Start and Stop Translation Sites. *Nucleic Acids Res.* 19: 3185-3192.
- Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K. and Romanos, M. A. (1991a) High-level Expression of Tetanus Toxin Fragment c in *Pichia pastoris* Strains Containing Multiple Tandem Integrations of the Gene. *Bio/Technology* 9: 455-460.
- Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K. and Henwood, C. A. (1991b) Production of Epidermal Growth Factor in Yeast: High-level Secretion Using *Pichia pastoris* Strains Containing Multiple Gene Copies. *Gene* 105: 205-212.
- Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993) Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. *Bio/Technology* 11: 905-910.
- Henikoff, S. and Cohen, E. H. (1984) Sequences Responsible for Transcription Termination on a Gene Segment in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4: 1515-1520.
- Holm, C., Meeks-Wagner, D. W., Fangman, W. L. and Botstein, D. (1986) A Rapid, Efficient Method for Isolating DNA from Yeast. *Gene* 42: 169-173.
- Irniger, S., Egli, C. M. and Braus, G. H. (1991) Different Classes of Polyadenylation Sites in the Yeast *Saccharomyces cerevisiae*. *Mol. Cell. Bio.* 11: 3060-3069.
- Kozak, M. (1987) An Analysis of 5'-Noncoding Sequences from 699 Vertebrate Messenger RNAs. *Nucleic Acids Res.* 15: 8125-8148.
- Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. *Proc. Natl. Acad. Sci. USA* 87: 8301-8305.
- Romanos, M. A., Clare, J. J., Beesley, K. M., Rayment, F. B., Ballantine, S. P., Makoff, A. J., Dougan, G., Fairweather, N. F. and Charles, I. G. (1991) Recombinant *Bordetella pertussis* Pertactin p69 from the Yeast *Pichia pastoris* High Level Production and Immunological Properties. *Vaccine* 9: 901-906.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, New York.
- Scorer, C. A., Buckholz, R. G., Clare, J. J. and Romanos, M. A. (1993) The Intracellular Production and Secretion of HIV-1 Envelope Protein in the Methylophilic Yeast *Pichia pastoris*. *Gene* 136: 111-119.
- Scorer, C. A., Clare, J. J., McCombie, W. R., Romanos, M. A. and Sreekrishna, K. (1994) Rapid Selection Using G418 of High Copy Number Transformants of *Pichia pastoris* for High-level Foreign Gene Expression. *Bio/Technology* 12: 181-184.
- Strathern, J. N. and Higgins, D. R. (1991) Recovery of Plasmids from Yeast into *Escherichia coli*: Shuttle Vectors. In: *Guide to Yeast Genetics and Molecular Biology* (C. Guthrie and G. R. Fink, eds), *Methods in Enzymology*, (J. N. Abelson and M. I. Simon, eds). Volume 194. Academic Press, San Diego, CA.
- Thill, G. P., Davis, G. R., Stillman, C., Holtz, G., Brierley, R., Engel, M., Buckholz, R., Kinney, J., Provow, S., Vedvick, T. and Siegel, R. S. (1990) Positive and Negative Effects of Multi-Copy Integrated Expression in *Pichia pastoris*. *International Symposium on the Genetics of Microorganisms*, 2: 477-490.
- Vedvick, T., Buckholz, R. G., Engel, M., Urcan, M., Kinney, J., Provow, S., Siegel, R. S. and Thill, G. P. (1991) High-level Secretion of Biologically Active Aprotinin from the Yeast *Pichia pastoris*. *J. Ind. Microbiol.* 7: 197-201.
- Wach, A., Pick, H. and Phillippsen, P. (1994) Procedures for Isolating Yeast DNA for Different Purposes: Protocol 3. In: J. R. Johnston (ed) *Molecular Genetics of Yeast: A Practical Approach*. IRL Press at Oxford University Press. New York, NY. pp. 10-12.
- Zaret, K. S. and Sherman, F. (1984) Mutationally Altered 3' Ends of Yeast *CYC1* mRNA Affect Transcript Stability and Translational Efficiency. *J. Mol. Biol.* 177: 107-136.

©2002–2008 Invitrogen Corporation. All rights reserved.

For research use only. Not intended for any animal or human therapeutic or diagnostic use.



Corporate Headquarters

Invitrogen Corporation

5791 Van Allen Way

Carlsbad, CA 92008

T: 1 760 603 7200

F: 1 760 602 6500

E: tech_support@invitrogen.com

For country-specific contact information, visit our web site at www.invitrogen.com