



pPIC6 α A, B, and C

***Pichia* expression vectors for selection
on blasticidin and purification of
secreted, recombinant protein**

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User Manual

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Kit Contents and Storage

Shipping and Storage

pPIC6 α vectors are shipped on wet ice. Upon receipt, store vectors at -20°C , and store the X-33 stab at 4°C .

Kit Contents

The kit contents are described below.

Item	Composition	Amount
pPIC6 α A, B and C	40 μL of 0.5 $\mu\text{g}/\mu\text{L}$ vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 μg
pPIC6 α /HSA	40 μL of 0.5 $\mu\text{g}/\mu\text{L}$ vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 μg
X-33 <i>Pichia</i> strain	Stab in YPD medium	1 stab

Reference Sources

The pPIC6 α A, B, and C vectors may be used with the EasySelect™ *Pichia* Expression Kit or the Original *Pichia* Expression Kit available from Invitrogen (see page 35). Additional general information about recombinant protein expression in *Pichia pastoris* are described in the manuals for the EasySelect™ *Pichia* Expression Kit and the Original *Pichia* Expression Kit. The manuals can be downloaded from www.invitrogen.com or obtained by calling Technical Support (see page 36). For more information about the EasySelect™ *Pichia* Expression Kit or the Original *Pichia* Expression Kit, refer to www.invitrogen.com or call Technical Support.

More detailed information and protocols dealing with *Pichia pastoris* may also be found in the following general reference:

Higgins, D. R., and Cregg, J. M. (1998) *Pichia* Protocols. In *Methods in Molecular Biology*, Vol. 103. (J. M. Walker, ed. Humana Press, Totowa, NJ)

Intended Use

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

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Kit Contents and Storage, Continued

Materials Supplied by the User

For the procedures described in this manual, you will need the following reagents and equipment. Additional reagents may be required. Check each experiment to ensure you have all the reagents necessary.

Equipment

- Microbiological equipment
- Electroporation device and 0.2 cm cuvettes or reagents for transformation
- 16°C, 37°C, and 65°C water baths or temperature blocks
- 30°C and 37°C shaking and non-shaking incubators
- Hemocytometer or Countess™ Automated Cell Counter (page 34)
- Microtiter plates (optional)

Reagents

- Electrocompetent or chemically competent *E. coli* (must be *recA*, *endA*) for transformation (page 34)
 - Restriction enzymes and appropriate buffers
 - Agarose and low-melt agarose
 - S.N.A.P.™ Gel Purification Kit (see page 34 for ordering) or glass milk
 - Sterile water
 - CIAP (calf intestinal alkaline phosphatase, 1 unit/μL)
 - 10X CIAP Buffer
 - Phenol/chloroform
 - 3 M sodium acetate
 - 100% ethanol
 - 80% ethanol
 - T4 Ligase (2.5 units/μL)
 - 10X Ligation Buffer (with ATP)
 - Low Salt LB medium (see page 17 for recipe)
 - Blasticidin antibiotic (see page 34)
 - Low Salt LB plates containing 100 μg/mL blasticidin (see page 17 for recipe)
 - YPDS plates containing the appropriate concentration of blasticidin (see page 18 for recipe)
 - 50 mL conical centrifuge tubes
 - 15 mL polypropylene tubes
 - ProBond™ Purification System (optional, see page 34 for ordering)
-

Introduction

Product Overview

Description of the System

pPIC6 α A, B, and C are 3.6 kb vectors used to express and secrete recombinant proteins in *Pichia pastoris*. Recombinant proteins are expressed as fusions to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α -factor secretion signal. The vector allows high-level, methanol inducible expression of the gene of interest in *Pichia*, and can be used in any *Pichia* strain including the X-33 strain supplied with the kit. pPIC6 α contains the following elements:

- 5' fragment containing the *AOX1* promoter for tightly regulated, methanol-induced expression of the gene of interest (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a)
- α -factor secretion signal for directing secreted expression of the recombinant protein
- Blastidicin resistance gene (Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) for selection in both *E. coli* and *Pichia*
- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6XHis) tag for detection and purification of a recombinant fusion protein (if desired)
- Three reading frames to facilitate in-frame cloning with the C-terminal peptide

The control plasmid, pPIC6 α /HSA, is included for use as a positive control for expression.

Experimental Overview

The following table describes the basic steps needed to clone and express your gene of interest in pPIC6 α .

Step	Action	Page
1	Propagate pPIC6 α A, B, and C by transformation into a <i>recA</i> , <i>endA1</i> <i>E. coli</i> strain such as TOP10, DH5 α , or JM109.	2
2	Develop a cloning strategy and ligate your gene into one of the pPIC6 α vectors in frame with the α -factor secretion signal and the C-terminal tag.	3–7
3	Transform into <i>E. coli</i> and select transformants on Low Salt LB plates containing 100 μ g/mL blasticidin.	8
4	Analyze 10–20 transformants by restriction mapping or sequencing to confirm in-frame fusion of your gene with the α -factor secretion signal and the C-terminal tag.	8
5	Purify and linearize the recombinant plasmid for transformation into <i>Pichia pastoris</i> .	8–10
6	Transform X-33 or your <i>Pichia</i> strain and plate onto YPDS plates containing the appropriate concentration of blasticidin.	11
7	Select for blasticidin-resistant transformants.	11–12
8	Optimize expression of your gene.	13–14
9	Purify your fusion protein on metal-chelating resin (<i>e.g.</i> , ProBond™).	15–16

Methods

Cloning into pPIC6 α A, B, and C

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

Many *E. coli* strains are suitable for the propagation of the pPIC6 α vectors including TOP10, JM109, and DH5 α . We recommend that you propagate the pPIC6 α vectors in *E. coli* strains that are recombination deficient (*recA*) and endonuclease A deficient (*endA*).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen (see page 34 for ordering).

Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintaining Plasmids

The pPIC6 α and pPIC6 α /HSA vectors contain the blasticidin resistance gene to allow selection of the plasmid using blasticidin. To propagate and maintain the pPIC6 α and pPIC6 α /HSA plasmids, we recommend using the following procedure:

1. Use a small amount of the supplied plasmid stock solution in TE, pH 8.0 to transform a *recA*, *endA* *E. coli* strain like TOP10, DH5 α , JM109, or equivalent.
 2. Select transformants on Low Salt LB plates containing 100 $\mu\text{g}/\text{mL}$ blasticidin (see page 17 for a recipe).
 3. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 8).
-

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Cloning into pPIC6 α A, B, and C, Continued

General Considerations

The following are some general points to consider when using pPIC6 α to express your gene of interest in *Pichia*:

- The codon usage in *Pichia* is believed to be similar to *Saccharomyces cerevisiae*.
 - Many *Saccharomyces* genes have proven to be functional in *Pichia*.
 - 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 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 predicted protease cleavage sites for the α -factor signal sequence are indicated in the diagrams on pages 5–7.
 - The native 5' end of the *AOX1* mRNA is noted in the diagram for each multiple cloning site. 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.
-

Cloning Considerations

pPIC6 α is a terminal fusion vector. To express your gene as a recombinant fusion protein, you must clone your gene in frame with the N-terminal α -factor secretion signal and the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. The vector is supplied in three reading frames to facilitate cloning. Refer to the diagrams on pages 5–7 to develop a cloning strategy. **Note:** The initiation ATG in the α -factor signal sequence corresponds to the native initiation ATG of the *AOX1* gene.

If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

Signal Sequence Processing

The processing of the α -factor signal sequence in pPIC6 α 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.
-

Continued on next page

Cloning into pPIC6 α A, B, and C, Continued

Optimizing Signal Cleavage

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

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

Expressing Your Recombinant Protein with a Native N-terminus

If you wish to have your protein expressed with a native N-terminus, you should clone your gene flush with the Kex2 cleavage site. Use PCR to rebuild the sequence from the *Xho* I site at bp 1184–1189 to the arginine codon at nucleotides 1193–1195. Remember to include the first amino acid(s) of your protein, if necessary, for correct fusion to the Kex2 cleavage site.

Constructing Multimeric Plasmids

pPIC6 α A, B, and C contain unique *Bgl* II and *Bam*H I sites to allow construction of plasmids containing multiple copies of your gene. For information on how to construct multimers, refer to pages 26–33.

Continued on next page

Cloning into pPIC6α A, B, and C, Continued

Multiple Cloning Site of pPIC6α A

Below is the multiple cloning site for pPIC6α A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6α A is available for downloading from www.invitrogen.com or from **Technical Support** (see page 36). For a map and a description of the features of pPIC6α, refer to the **Appendix**, pages 19–20.

```

5' end of AOX1 mRNA                                     5' AOX1 priming site
|                                                       |
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA
|
871 CAAGCTTTTG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT
|
931 ATTCGAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala
|
983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA
      Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala
|
α-factor signal sequence
|
1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
      Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
|
1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe
|
α-factor priming site                                     Xho I*
|
1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu
|
Kex2 signal cleavage                                     EcoR I   Pml I       Sfi I       BsmB I Asp718 I
|
1187 GAG AAA AGA GAG GCT GAA GCT GAATTCAC GTGGCCCAG CCGCCGTC TCGGATCGGT
      Glu Lys Arg Glu Ala Glu Ala
|
Ste13 signal cleavage
|
Kpn I Xho I   Sac II Not I       Xba I       c-myc epitope
|
1244 ACCTCGAGCC GCGGCGGCC GCCAGCTTTC TA GAA CAA AAA CTC ATC TCA GAA GAG
      Glu Gln Lys Leu Ile Ser Glu Glu
|
Polyhistidine (6xHis) tag
|
1299 GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC
      Asp Leu Asn Ser Ala Val Asp His His His His His His ***
|
1351 TTAGACATGA CTGTTCTCTCA GTTCAAGTTG GGAAGACCGG AGAAGACCGG TCTTGCTAGA
|
3' AOX1 priming site
|
1411 TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT
|
3' polyadenylation site
|
1471 ACTTTTTTAT TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT

```

*To express your protein with a native N-terminus, you must clone your gene flush with the Kex2 cleavage site. You will need to use PCR and utilize the *Xho* I site upstream of the Kex2 cleavage site (see page 4 for more details).

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Cloning into pPIC6α A, B, and C, Continued

Multiple Cloning Site of pPIC6α B

Below is the multiple cloning site for pPIC6α B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6α B is available for downloading from www.invitrogen.com or from **Technical Support** (see page 36). For a map and a description of the features of pPIC6α, refer to the **Appendix**, pages 19–20.

```

      5' end of AOX1 mRNA                                     5' AOX1 priming site
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

      CAAGCTTTTG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT
871

931 ATTCGAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala

983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA
      Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala

      α-factor signal sequence
1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
      Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe

1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe

      α-factor priming site                                     Xho I*
1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu

      Kex2 signal cleavage                                     Pst I   EcoR I   Pml I           Sfi I           BsmB I
1187 GAG AAA AGA GAG GCT GAA GC TGCAG GAATTCAC GTGGCCCAG CCGCCGTC TCGGA
      Glu Lys Arg Glu Ala Glu Ala

      Ste13 signal cleavage
      Asp718 | Kpn I Xho I   Sac II   Not I           Xba I           c-myc epitope
1243 TCGGTACCTC GAGCCGCGGC GGCCGCCAGC TTTCTA GAA CAA AAA CTC ATC TCA GAA
      Glu Gln Lys Leu Ile Ser Glu

      Polyhistidine (6xHis) tag
1300 GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTA
      Glu Asp Leu Asn Ser Ala Val Asp His His His His His His ***

1352 GCCTTAGACA TGA CTGTTCC TCAGTTCAAG TTGGGCACTT ACGAGAAGAC CGGTCTTGCT

      3' AOX1 priming site
1412 AGATTCTAAT CAAGAGGATG TCAGAATGCC ATTTGCCTGA GAGATGCAGG CTCATTTTTT

      3' polyadenylation site
1472 GATACTTTTT TATTTGTAAC CTATATAGTA TAGGATTTTT TTTGTCATTT TGTTTCTTCT

```

To express your protein with a native N-terminus, you must clone your gene flush with the Kex2 cleavage site. You will need to use PCR and utilize the *Xho* I site upstream of the Kex2 cleavage site (see page 4 for more details).

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Cloning into pPIC6α A, B, and C, Continued

Multiple Cloning Site of pPIC6α C

Below is the multiple cloning site for pPIC6α C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6α C is available for downloading from our www.invitrogen.com or from **Technical Support** (see page 36). For a map and a description of the features of pPIC6α, refer to the **Appendix**, pages 19–20.

```

5' end of AOX1 mRNA                                     5' AOX1 priming site
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871 CAAGCTTTTG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

931 ATTCGAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA
      Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala

983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA
      Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala

α-factor signal sequence
1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
      Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe

1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe

α-factor priming site                                     Xho I*
1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu

Kex2 signal cleavage                                     Cla I   EcoR I   Pml I   Sfi I   BsmB I
1188 GAG AAG AGA GAG GCT GAA GC ATCGAT GAATTCAC GTGGCCCAG CCGGCCGTC TCGGA
      Glu Lys Arg Glu Ala Glu Ala
      Ste13 signal cleavage
Asp718 I Kpn I Xho I   Sac II   Not I   Xba I   c-myc epitope
1244 TCGGTACCTC GAGCCGCGGC GGCCGCCAGC TTTCTA GAA CAA AAA CTC ATC TCA GAA
      Glu Gln Lys Leu Ile Ser Glu

Polyhistidine (6xHis) tag
1301 GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTA
      Glu Asp Leu Asn Ser Ala Val Asp His His His His His His ***

1353 GCCTTAGACA TGACTGTTCC TCAGTTCAAG TTGGGCACTT ACGAGAAGAC CGGTCTTGCT

3' AOX1 priming site
1413 AGATTCTAAT CAAGAGGATG TCAGAATGCC ATTTGCCTGA GAGATGCAGG CTCATTTTTT

3' polyadenylation site
1473 GATACTTTTT TATTTGTAAC CTATATAGTA TAGGATTTTT TTTGTCATTT TGTTTCTTCT

```

To express your protein with a native N-terminus, you must clone your gene flush with the Kex2 cleavage site. You will need to use PCR and utilize the *Xho* I site upstream of the Kex2 cleavage site (see page 4 for more details).

Cloning into pPIC6α A, B, and C, Continued

E. coli **Transformation**

Transform your ligation mixtures into a competent *recA*, *endA* *E. coli* strain (e.g., TOP10, DH5α, JM109) and select on Low Salt LB agar plates containing 100 µg/mL blasticidin (see below). Note that there is no blue/white screening for the presence of insert with pPIC6α A, B, or C. Once you have obtained blasticidin-resistant colonies, pick 10 transformants and screen for the presence and orientation of your insert.



Important

To facilitate selection of blasticidin-resistant *E. coli*, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.0. Prepare Low Salt LB broth and plates using the recipe in the Appendix, page 17.

Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug.



We recommend that you sequence your construct to confirm that your gene is in the correct orientation for expression and cloned in frame with the α-factor signal sequence and the C-terminal peptide. We suggest using the α-Factor and the 3' *AOX1 Pichia* primer sequences. Refer to the diagrams on pages 5–7 for the sequence and location of the primer binding sites.

For your convenience, Invitrogen offers a custom primer synthesis service (see page 34). Alternatively, the 5' *AOX1 Pichia* Primer can be used if you wish to sequence through the entire α-factor signal sequence.

Preparing a **Glycerol Stock**

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on Low Salt LB plate containing 100 µg/mL blasticidin. Incubate the plate at 37°C overnight.
 2. Isolate a single colony and inoculate into 1–2 mL of Low Salt LB containing 100 µg/mL blasticidin.
 3. Grow the culture to mid-log phase ($OD_{600} = 0.5–0.7$).
 4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
 5. Store at –80°C.
-

Plasmid **Preparation**

Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform *Pichia* (5–10 µg of each plasmid per transformation). We recommend isolating plasmid DNA using the PureLink™ HiPure Plasmid Miniprep Kit or the PureLink™ HiPure Plasmid Midiprep Kit or equivalent (see page 34). Once you have purified plasmid DNA, proceed to ***Pichia* Transformation**, next page.

Pichia Transformation

Introduction

You should now have your gene cloned into one of the pPIC6 α vectors. Your construct should be correctly fused to the α -factor signal sequence and the C-terminal peptide. This section provides general guidelines to prepare plasmid DNA, transform your *Pichia* strain, and select for blasticidin-resistant clones.

Blasticidin Selection

We generally use 300 $\mu\text{g}/\text{mL}$ blasticidin to select for transformants when using the X-33 *Pichia* strain. If you wish to transform your pPIC6 α construct into another *Pichia* strain, note that selection conditions may vary. We recommend performing a dose response curve to determine the appropriate concentration of blasticidin to use for selection of transformants in your strain.

Method of Transformation

We recommend electroporation for transformation of *Pichia* with pPIC6 α A, B, or C. 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 LiCl protocol on page 24 or the *Pichia* EasyComp™ Transformation Kit available from Invitrogen (see below).

Pichia EasyComp™ Transformation Kit

If you wish to perform chemical transformation of your *Pichia* strain with pPIC6 α A, B, or C, the *Pichia* EasyComp™ Transformation Kit is available from Invitrogen (see page 35 for ordering information). The *Pichia* EasyComp™ Transformation Kit provides reagents to prepare 6 preparations of competent cells. Each preparation will yield enough competent cells for 20 transformations. Competent cells may be used immediately or frozen and stored for future use. For more information, visit www.invitrogen.com or call Technical Support (see page 36).



Important

Since pPIC6 α does not contain the *HIS4* gene, integration can only occur at the *AOX1* locus. Vector linearized within the 5' *AOX1* region will integrate by gene insertion into the host 5' *AOX1* region. Therefore, the *Pichia* host that you use will determine whether the recombinant strain is able to metabolize methanol (Mut^+) or not (Mut^s). To generate a Mut^+ recombinant strain, you must use a *Pichia* host that contains the native *AOX1* gene (e.g. X-33, SMD1168H). If you wish to generate a Mut^s recombinant strain, then use a *Pichia* host that has a disrupted *AOX1* gene (e.g. KM71H).

Note: The X-33 strain supplied with the pPIC6 α vector contains the native *AOX1* gene; therefore, the recombinant strain will be Mut^+ .



Note

The pPIC6 α vectors do not contain a yeast origin of replication. Transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome.

Continued on next page

Pichia Transformation, Continued

Positive Control

The pPIC6 α /HSA plasmid is provided as a positive control vector for blasticidin resistance in *Pichia* and may be used to optimize expression and purification conditions for your host strain. The pPIC6 α /HSA plasmid expresses human serum albumin (HSA) as a fusion protein with the N-terminal α -factor signal sequence and the C-terminal *c-myc* epitope and polyhistidine tag. Expression of the 79 kDa fusion protein is driven by the P_{AOX1} promoter and is inducible with methanol. The fusion protein is visible on a Coomassie-stained SDS-polyacrylamide gel and can be detected using the Anti-*myc* Antibody (see page 35 for ordering).

Before Starting

You will need the following reagents for transforming *Pichia* and selecting transformants on blasticidin. **Note:** Inclusion of sorbitol in YPD plates stabilizes electroporated cells as they appear to be somewhat osmotically sensitive.

- 5–10 μ g pure pPIC6 α containing your insert
 - YPD Medium
 - 50 mL conical polypropylene tubes
 - 1 liter cold (4°C) sterile water (place on ice the day of the experiment)
 - 25 mL cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
 - 30°C incubator
 - Electroporation device and 0.2 cm cuvettes
 - YPDS plates containing the appropriate concentration of blasticidin (see page 18 for recipe)
-

Linearizing Your pPIC6 α Construct

To promote integration, we recommend that you linearize your pPIC6 α construct within the 5' AOX1 region. The table below lists unique sites that may be used to linearize pPIC6 α prior to transformation. **Other restriction sites are possible.** Note that for the enzymes listed below, the cleavage site is the same for versions A, B, and C of pPIC6 α . Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Supplier
<i>Sac</i> I	209	Many
<i>Pme</i> I	414	New England Biolabs
<i>Bst</i> X I	707	Many

Continued on next page

***Pichia* Transformation, Continued**

Restriction Digest

1. Digest ~5–10 µg of plasmid DNA with one of the enzymes listed above.
 2. 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, phenol/chloroform extract once, and ethanol precipitate using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol.
 4. Centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend the DNA in 10 µL sterile, deionized water. Use immediately or store at –20°C.
-

Preparing *Pichia* for Electroporation

Follow the procedure below to prepare your *Pichia pastoris* strain for electroporation.

1. Grow 5 mL of your *Pichia pastoris* strain in YPD in a 50 mL conical tube at 30°C overnight.
 2. Inoculate 500 mL of fresh medium in a 2 liter flask with 0.1–0.5 mL of the overnight culture. Grow overnight again to an OD₆₀₀ = 1.3–1.5.
 3. Centrifuge the cells at 1500 × g for 5 minutes at 4°C. Resuspend the pellet with 500 mL of ice-cold (0°C), sterile water.
 4. Centrifuge the cells as in Step 3 and resuspend the pellet with 250 mL of ice-cold (0°C), sterile water.
 5. Centrifuge the cells as in Step 3 and resuspend the pellet in 20 mL of ice-cold (0°C) 1 M sorbitol.
 6. Centrifuge the cells as in Step 3 and resuspend the pellet in 1 mL of ice-cold (0°C) 1 M sorbitol for a final volume of approximately 1.5 mL. Keep the cells on ice and use that day. Do not store cells.
-

Transformation by Electroporation

1. Mix 80 µL of the cells from Step 6 (above) with 5–10 µg of linearized pPIC6α DNA (in 5–10 µL sterile water) and transfer them to an ice-cold (0°C) 0.2 cm electroporation cuvette.
 2. Incubate the cuvette with the cells on ice for 5 minutes.
 3. Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) as suggested by the manufacturer of the specific electroporation device being used.
 4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 mL tube.
 5. Let the tube incubate at 30°C without shaking for 1 to 2 hours.
 6. Spread 50–200 µL each on separate, labeled YPDS plates containing the appropriate concentration of blasticidin.
 7. Incubate plates for 2 to 3 days at 30°C until colonies form.
 8. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing the appropriate concentration of blasticidin.
-

Continued on next page

Pichia Transformation, Continued



When selecting for blasticidin-resistant transformants in the X-33 strain, we often observe colonies of two different sizes (large and small) on YPD plates containing 300 $\mu\text{g}/\text{mL}$ blasticidin. Generally, large colonies represent transformants containing pPIC6 α integrants, while small colonies represent transformants containing pPIC6 α non-integrants. These non-integrants have transduced the pPIC6 α plasmid, and therefore, exhibit a low level of blasticidin resistance in the initial selection process. Upon subsequent screening, these non-integrant transformants do not retain blasticidin resistance.

When choosing a blasticidin-resistant transformant for your expression studies, we recommend that you pick blasticidin-resistant colonies from the initial transformation plate and streak them on a second YPD plate containing the appropriate concentration of blasticidin. Select transformants which remain blasticidin-resistant for further studies.



Note

Generally several hundred blasticidin-resistant colonies are generated using the protocol on the previous page. If more colonies are needed, the protocol may be modified as described below. Note that you will need ~twenty 150 mm plates with YPDS agar containing the appropriate concentration of blasticidin.

1. Set up two transformations per construct and follow Steps 1 through 5 of the **Transformation by Electroporation** protocol, page 11.
 2. After 1 hour in 1 M sorbitol at 30°C (Step 5, previous page), add 1 mL YPD medium to each tube.
 3. Shake (~200 rpm) the cultures at 30°C.
 4. After 1 hour, take one of the tubes and plate out all of the cells by spreading 200 μL on 150 mm plates containing the appropriate concentration of blasticidin.
 5. (Optional) Continue incubating the other culture for three more hours (for a total of four hours) and then plate out all of the cells by spreading 200 μL on 150 mm plates containing the appropriate concentration of blasticidin.
 6. Incubate plates for 2 to 4 days at 30°C until colonies form.
-

Mut Phenotype

If you used a *Pichia* strain containing a native *AOX1* gene (e.g., X-33, GS115, SDM1168H) as the host for your pPIC6 α construct, your blasticidin-resistant transformants will be Mut⁺. If you used a strain containing a deletion in the *AOX1* gene (e.g., KM71H), your transformants will be Mut^s.

If you wish to verify the Mut phenotype of your blasticidin-resistant transformants, refer to the general guidelines provided in the EasySelect™ *Pichia* Expression Kit manual or the Original *Pichia* Expression Kit manual or to published reference sources (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965).

You are now ready to test your transformants for expression of your gene of interest. Proceed to **Expression in *Pichia***, next page.

Expression in *Pichia*

Introduction

The primary purpose of small-scale expression is to identify/confirm a recombinant *Pichia* clone that is expressing the correct protein. Small-scale expression conditions may not be optimal for your protein. For this reason, the method you choose for detection (e.g. 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.

Once a positive clone has been identified, large-scale expression can be carried out in shake flask or fermentation, and expression conditions can be optimized.



Note

Once you have obtained blasticidin-resistant transformants, it is not necessary to maintain your recombinant *Pichia* clone in medium containing blasticidin for expression studies. Blasticidin is only required for initial screening and selection of recombinant clones.

Detecting Recombinant Proteins in *Pichia*

We recommend that you use the following techniques to assay expression of your protein. **Remember to analyze BOTH the medium and the cells for the presence of your recombinant protein.** Note that the α -factor signal sequence will add approximately 9.3 kDa to the size of your protein if it is unprocessed. The C-terminal tag will add 2.5 kDa to the size of your protein. Be sure to account for any additional amino acids that are in between the signal sequence processing sites and the N-terminus of your protein and also the end of your protein and the C-terminal tag.

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 Anti- <i>myc</i> antibodies (see the next page) Anti-His(C-term) antibodies (see the next page)	Can detect as little as 1 to 10 pg, depending on detection method (alkaline phosphatase, horseradish peroxidase, radiolabeled antibody)
Functional assay	Varies depending on assay.	Varies depending on assay Used to compare relative amounts of protein.

Continued on next page

Expression in *Pichia*, Continued

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, visit www.invitrogen.com or call Technical Support (see page 36).

Western Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page 35 for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (see page 34 for ordering) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine (6xHis) tag. WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods (see page 34 for ordering). For more information, visit www.invitrogen.com or call Technical Support (see page 36).

Control Strain

If you have transformed the pPIC6 α /HSA plasmid into your *Pichia* host strain, you may use this recombinant strain as a positive control for expression. Expression of HSA in shake flasks is detectable after 48 hours of methanol induction and reaches the maximum at 96 hours. HSA may be detected using SDS-PAGE and staining the gel with Coomassie Blue. HSA expression can also be detected using the Anti-*myc* or Anti-His(C-term) antibodies (see page 35 for ordering information).

For a small-scale Mut⁺ expression protocol, refer to the EasySelect™ *Pichia* Expression Kit manual or to general reference texts.



Important

Because the pPIC6 α vector does not contain the *HIS4* gene, *his4* *Pichia* strains containing the integrated plasmid must be grown in medium containing 0.004% histidine. If histidine is not present in the medium the cells will not grow. If you used X-33 as the host strain, supplementation of the medium with histidine is not required.

Expression Guidelines

General guidelines to perform small-scale expression, optimize expression, and scale-up of expression are provided in the EasySelect™ *Pichia* Expression Kit manual or the Original *Pichia* Expression Kit manual.

Purification

Introduction

In this section, you will grow and induce a 10–200 mL culture of your *Pichia* transformant for trial purification on a metal-chelating resin such as ProBond™. You may harvest the cells and store both the supernatant (medium) and the cells at –80°C until you are ready to purify your fusion protein, or you may proceed directly with protein purification. **Note that this section only describes preparation of cell lysates and sample application onto ProBond™.** For instructions on how to prepare and use ProBond™ resin, refer to the ProBond™ Purification manual.

ProBond™ Resin

We recommend that you use the ProBond™ Purification system to purify your 6xHis-tagged protein. The ProBond™ Purification system contains a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, consult the manufacturer's instructions.

Binding Capacity of ProBond™

One milliliter of ProBond™ resin binds at least 1 mg of recombinant protein. This amount can vary depending on the protein.

Expressing Secreted Protein

Express your protein using a small-scale culture (10–20 mL for Mut^S strains; 100–200 mL for Mut⁺) and the optimal conditions for expression (if determined). Details may be found in the *Pichia* Expression Kit manual. Once your protein is expressed, separate the cells from the medium by centrifugation. Store the medium at –80°C or proceed directly to purification. If desired, the cells can be stored at –80°C for future analysis.



Important

Throughout the following protocol, be sure to keep the medium and fractions on ice. Small-scale purifications using the 2 mL ProBond™ columns and buffers can be done at room temperature on the bench top. For large scale purifications, all reagents must be kept at 4°C.

Continued on next page

Purification, Continued

Sample Application (Native Conditions)

The following protocol may be used for chromatography of medium. For sample application onto ProBond™, you will need Native Binding Buffer, pH 7.8 and a 2 mL ProBond™ column, pre-equilibrated using native conditions.

1. Combine 1 mL of medium with 7 mL Native Binding Buffer.
 2. Take a pre-equilibrated ProBond™ column and resuspend the resin in 4 mL of the diluted lysate from Step 1.
 3. Seal the column and batch-bind by rocking gently at room temperature for 10 minutes.
 4. Let the resin settle by gravity or low speed centrifugation ($800 \times g$) and carefully remove the supernatant. Save the supernatant to check for unbound protein.
 5. Repeat Steps 2 through 4 with the remaining 4 mL of diluted lysate. Proceed to **Column Washing and Elution Under Native Conditions** in the ProBond™ Purification manual. Use the recommendations noted for bacterial cell lysates.
-

Sample Application (Denaturing Conditions)

Use the protocol above except pre-equilibrate the ProBond™ column using Denaturing Binding Buffer and combine 1 mL of the *Pichia* cell lysate with 7 mL of the Denaturing Binding Buffer.



Note

We have observed that some *Pichia* proteins may be retained on the ProBond™ column using native purification conditions. Optimization of the purification (see ProBond™ Purification manual) or using denaturing purification may remove these non-specific *Pichia* proteins.

Analysis of Purification

Be sure to save all fractions, washes, and flow-through for analysis by SDS-PAGE. You may need to use western blot analysis to detect your protein if expression is low or not enough protein was loaded onto the column. Refer to the ProBond™ Purification System manual for a guide to troubleshoot chromatography.

Scale-up

You may find it necessary to scale-up your purification to obtain sufficient amounts of purified protein. Adjust the pH and NaCl concentration of your lysate with 1/10 volume of 10X Stock Solution B (ProBond™ Purification Kit) before adding it to the column. The pH should be ≥ 7.5 and the NaCl concentration should be ~ 500 mM. Using 10X Stock Solution B to adjust the pH and the ionic strength keeps the total volume small for sample application.

Appendix

Recipes

Low Salt LB Medium with Blasticidin

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.0 with 1N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
 2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
 3. Allow the medium to cool to at least 55°C before adding the blasticidin to 100 µg/mL final concentration.
 4. Store plates at 4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.
-

YPD (+ Blasticidin) Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
± 2% agar
± appropriate concentration of blasticidin

1. Dissolve: 10 g yeast extract
20 g of peptone
in 900 mL of water.
2. Include 20 g of agar if making YPD slants or plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 mL of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of blasticidin from a 10 mg/mL stock solution. **Note:** It is necessary to include blasticidin in the medium for selection of *Pichia* transformants only. Blasticidin may be omitted from the medium when performing expression studies.

Store YPD slants or plates containing blasticidin at 4°C. The shelf life is one to two weeks.

Continued on next page

Recipes, Continued

YPDS + Blasticidin Agar

Yeast Extract Peptone Dextrose Medium with Sorbitol (1 liter)

- 1% yeast extract
- 2% peptone
- 2% dextrose (glucose)
- 1 M sorbitol
- ± 2% agar
- ± appropriate concentration of blasticidin

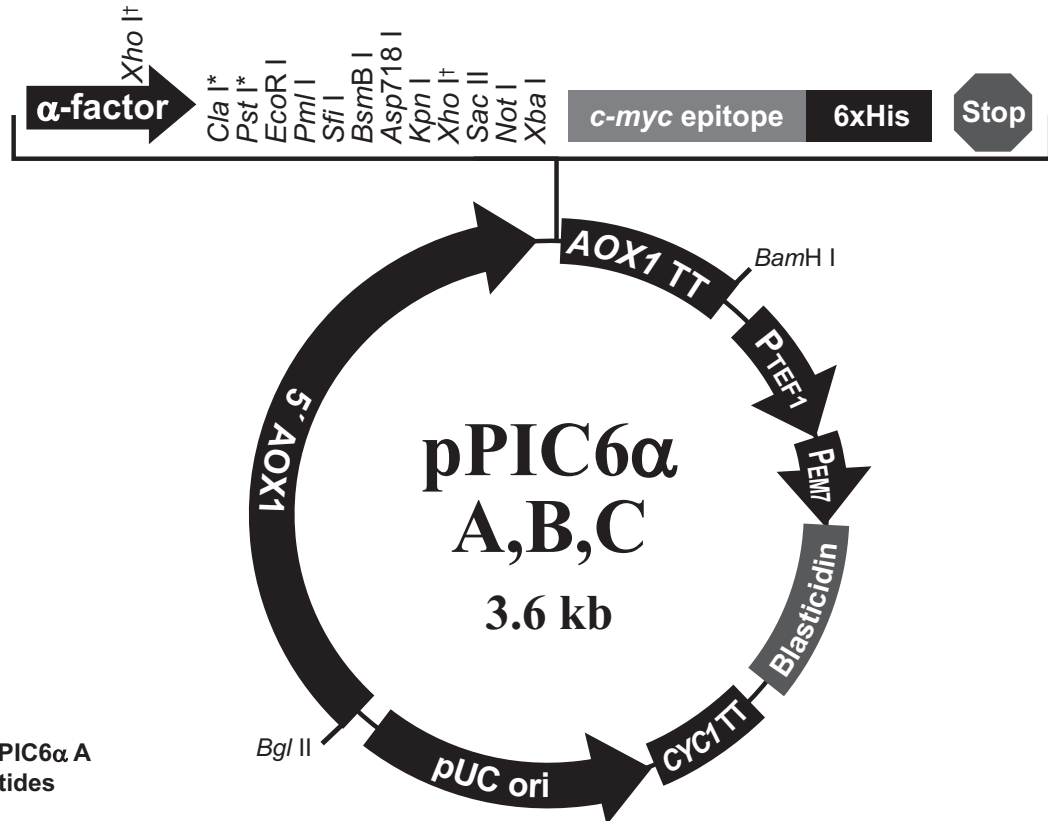
1. Dissolve: 10 g yeast extract
182.2 g sorbitol
20 g of peptone
in 900 mL of water.
2. Add 20 g of agar.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 mL of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of blasticidin from a 10 mg/mL stock solution. **Note:** It is necessary to include blasticidin in the medium for selection of *Pichia* transformants only. Blasticidin may be omitted from the medium when performing expression studies.

Store YPDS slants or plates containing blasticidin at 4°C. The shelf life is one to two weeks.

pPIC6 α Vector

Map of pPIC6 α

The figure below summarizes the features of the pPIC6 α A, B, and C vectors. The vector sequences for pPIC6 α A, B, and C are available for downloading from www.invitrogen.com or from **Technical Support** (see page 36). See the next page for a description of the features of the vector.



Comments for pPIC6 α A 3646 nucleotides

- 5' AOX1 promoter region: bases 1-941
- 5' AOX1 priming site: bases 855-875
- α -factor signal sequence: bases 941-1207
- α -factor priming site: bases 1144-1164
- Multiple cloning site: bases 1208-1276
- c-myc* epitope: bases 1275-1304
- Polyhistidine (6xHis) tag: bases 1320-1337
- 3' AOX1 priming site: bases 1423-1443
- AOX1 transcription termination region: bases 1341-1682
- TEF1 promoter: bases 1683-2093
- EM7 promoter: bases 2097-2163
- Blasticidin resistance gene: bases 2164-2562
- CYC1 transcription termination region: bases 2591-2908
- pUC origin: bases 2919-3592 (complementary strand)

* Pst I is in Version B only
Cla I is in Version C only

†The two Xho I sites in the vector allow the user to clone their gene in frame with the Kex2 cleavage site, resulting in expression of their native gene without additional amino acids at the N-terminus.

Continued on next page

pPIC6 α Vector, Continued

Features of pPIC6 α A, B, and C

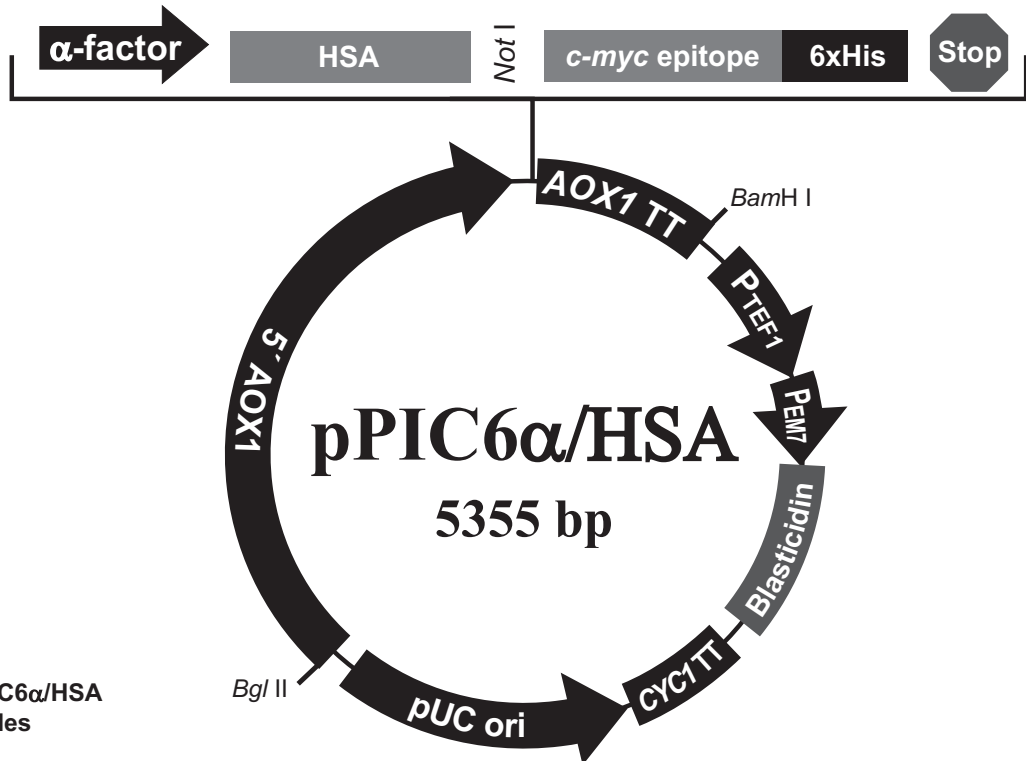
pPIC6 α A (3646 bp), pPIC6 α B (3650 bp), and pPIC6 α C (3651 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
5' <i>AOX1</i> promoter	A 942 bp fragment containing the <i>AOX1</i> promoter that allows methanol-inducible, high-level expression of the gene of interest in <i>Pichia</i> . Targets plasmid integration to the <i>AOX1</i> locus.
α -factor secretion signal (from <i>Saccharomyces cerevisiae</i>)	Allows for efficient secretion of most proteins from <i>Pichia</i> .
Multiple cloning site	Allows insertion of your gene into the expression vector.
<i>c-myc</i> epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant fusion protein with the Anti- <i>myc</i> Antibodies (Evans <i>et al.</i> , 1985).
C-terminal polyhistidine (6xHis) tag	Allows purification of your recombinant fusion protein on metal-chelating resin such as ProBond™. In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibodies.
<i>AOX1</i> transcription termination (TT) region	Native transcription termination and polyadenylation signal from <i>AOX1</i> gene (~260 bp) that permits efficient 3' mRNA processing, including polyadenylation, for increased mRNA stability.
<i>TEF1</i> promoter (GenBank accession numbers D12478, D01130)	Transcription elongation factor 1 gene promoter from <i>Saccharomyces cerevisiae</i> that drives expression of the blasticidin resistance gene in <i>Pichia</i> .
EM7 promoter	Synthetic prokaryotic promoter that drives constitutive expression of the blasticidin resistance gene in <i>E. coli</i> .
Blasticidin resistance gene (<i>bsd</i>)	Allows selection of transformants in <i>E. coli</i> and <i>Pichia</i> .
<i>CYC1</i> transcription termination region (GenBank accession number M34014)	3' end of the <i>Saccharomyces cerevisiae</i> <i>CYC1</i> gene that allows efficient 3' mRNA processing of the blasticidin resistance gene for increased stability.
pUC origin	Allows replication and maintenance of the plasmid in <i>E. coli</i> .

pPIC6α/HSA Vector

Map of pPIC6α/HSA

The figure below summarizes the features of the pPIC6α/HSA vector. The vector sequence for pPIC6α/HSA is available for downloading from www.invitrogen.com or from **Technical Support** (see page 36).



Comments for pPIC6α/HSA 5355 nucleotides

- 5' AOX1 promoter region: bases 1-941
- 5' AOX1 priming site: bases 855-875
- α-factor signal sequence: bases 941-1207
- α-factor priming site: bases 1144-1164
- HSA ORF (no ATG): bases 1211-2965
- c-myc epitope: bases 2984-3013
- Polyhistidine (6xHis) tag: bases 3029-3046
- 3' AOX1 priming site: bases 3132-3152
- AOX1 transcription termination region: bases 3050-3391
- TEF1 promoter: bases 3392-3802
- EM7 promoter: bases 3806-3872
- Blasticidin resistance gene: bases 3873-4271
- CYC1 transcription termination region: bases 4300-4617
- pUC origin: bases 4628-5301 (complementary strand)

Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.*, a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

Blasticidin is soluble in water. Water is generally used to prepare stock solutions of 5 to 10 mg/mL.

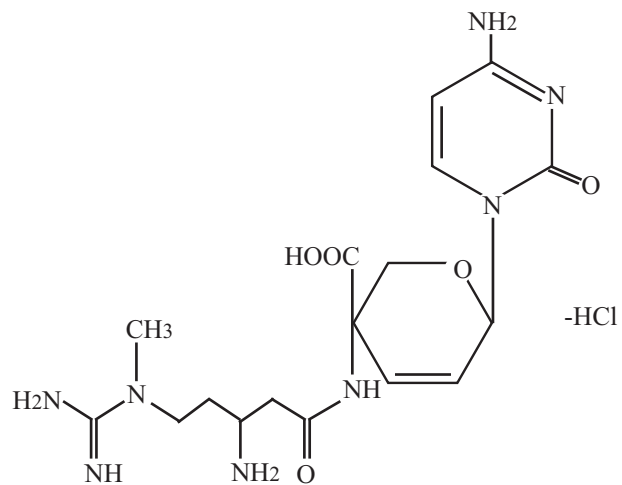
- Dissolve blasticidin in sterile water and filter-sterilize the solution.
 - Aliquot in small volumes suitable for one time use (see last point below) and freeze at -20°C for long-term storage or store at 4°C for short term storage.
 - Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at -20°C .
 - pH of the aqueous solution should not exceed 7 to prevent inactivation of blasticidin.
 - Do not subject stock solutions to freeze/thaw cycles (do not store in a frost-free freezer).
 - Upon thawing, use what you need and discard the unused portion.
-

Continued on next page

Blasticidin, Continued

**Molecular Weight,
Formula, and
Structure**

The formula for blasticidin is $C_{17}H_{26}N_8O_5 \cdot HCl$, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Lithium Chloride Transformation Method

Introduction

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

Preparing Solutions

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

- 1 M LiCl in distilled, deionized water. Filter-sterilize. Dilute as needed with sterile water.
 - 50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter-sterilize. Store in a tightly capped bottle.
 - 2 mg/mL denatured, sheared salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at -20°C .
-

Preparing Cells

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

Continued on next page

Lithium Chloride Transformation Method, Continued

Transformation

1. Boil a 1 mL sample of single-stranded DNA for 5 minutes, then quickly chill on ice. Keep on ice. **Note:** It is not necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at -20°C and boil every 3–4 times the DNA is thawed.
 2. Centrifuge the cells from Step 6, above, and remove the LiCl with a pipet.
 3. For each transformation, add the following reagents IN THE FOLLOWING ORDER to the cells. PEG shields the cells from the detrimental effects of the high LiCl concentration.

50% PEG	240 μL
1 M LiCl	36 μL
2 mg/mL single-stranded DNA	25 μL
Plasmid DNA in 50 μL sterile water	5–10 μg
 4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
 5. Incubate the tube at 30°C for 30 minutes without shaking.
 6. Heat shock in a water bath at 42°C for 20–25 minutes.
 7. Centrifuge the cells at 3800 to 6800 g to pellet.
 8. Resuspend the pellet in 1 mL of YPD and incubate at 30°C with shaking.
 9. After 1 hour and 4 hours, plate 25 to 100 μL on YPD plates containing the appropriate concentration of blasticidin. Incubate the plates for 2–3 days at 30°C .
-

Constructing *In Vitro* Multimers

Experimental Outline

At this point you should have your gene cloned into the multiple cloning site of either pPIC6 α A, B, or C. To generate multiple copies of your expression cassette:

Step	Description
1	Digest pPIC6 α containing your gene of interest with <i>Bgl</i> II and <i>Bam</i> H I to release the expression cassette (P_{AOX1} plus your gene).
2	To clone multiple copies of the expression cassette, linearize pPIC6 α containing your gene of interest using <i>Bam</i> H I. Note that the <i>Bam</i> H I-linearized vector already contains one copy of your expression cassette.
3	Treat the <i>Bgl</i> II- <i>Bam</i> H I expression cassette with ligase <i>in vitro</i> . Note that <i>Bgl</i> II and <i>Bam</i> H I share 4 bases in common between their recognition sites (GATC).
4	Generate head-to-tail, head-to-head, and tail-to-tail multimers (Head-to-tail ligation, which is the correct orientation for expression, will destroy both the <i>Bam</i> H I and <i>Bgl</i> II sites).
5	Treat the ligation mix with <i>Bam</i> H I and <i>Bgl</i> II to eliminate head-to-head and tail-to-tail multimers.
6	Ligate into <i>Bam</i> H I-linearized recombinant pPIC6 α .
7	Transform into <i>E. coli</i> and analyze recombinant plasmids for copy number by digesting with <i>Bgl</i> II and <i>Bam</i> H I.

Alternative Procedure

You may wish to build each desired multimer in increments by ligating each additional expression cassette one (or two) at a time into pPIC6 α A, B, or C. For example:

Step	Description
1	Digest pPIC6 α containing one copy of your gene with <i>Bam</i> H I.
2	Ligate a single copy of the <i>Bgl</i> II- <i>Bam</i> H I expression cassette into <i>Bam</i> H I-digested vector.
3	Transform <i>E. coli</i> and analyze the transformants for the vector with 2 copies of your insert.
4	Isolate and digest this vector (with 2 copies of your gene) with <i>Bam</i> H I and <i>Bgl</i> II to release a cassette with 2 copies of your gene (optional).
5	Digest the vector with 2 copies of your gene with <i>Bam</i> H I and ligate 1 or 2 copies (see Step 4) of the expression cassette into the vector.
6	Transform <i>E. coli</i> and analyze the transformants for the vector with 3 or 4 copies of your insert.
7	Repeat until the desired multimer is reached.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Materials Needed

- Electrocompetent or chemically competent *E. coli* (must be *recA*, *endA*) for transformation. You will need 3–4 tubes of competent cells per experiment (page 34).
 - *Bam*H I and *Bgl* II restriction enzymes and appropriate buffers
 - Low-melt agarose
 - S.N.A.P.[™] Gel Purification Kit (page 34) or glass milk
 - Sterile water
 - CIAP (calf intestinal alkaline phosphatase, 1 unit/ μ L, Boehringer Mannheim)
 - 10X CIAP Buffer
 - Phenol/chloroform
 - 3M sodium acetate
 - 100% ethanol
 - 80% ethanol
 - T4 Ligase (2.5 units/ μ L)
 - 10X Ligation Buffer (with ATP)
 - Low Salt LB plates containing 100 μ g/mL blasticidin
 - 150 mm plates for plating transformants
 - 16°C, 37°C, and 65°C water baths or temperature blocks
-

Controls

To evaluate your transformants and expression data later on, we recommend transforming *Pichia* with pPIC6 α (the parent vector) and pPIC6 α containing one copy of your gene of interest. This will allow you to compare expression levels to see if multiple copies significantly increase the amount of protein produced. Also, if you elect to determine how many copies of your gene are in a recombinant by dot or Southern blot, the strain with the parent vector will control for background hybridization and the strain with the single copy gene will provide a signal to normalize your data.



Important

Once you have created a pPIC6 α plasmid containing multimers, note that this plasmid cannot be linearized because any enzyme that cuts in the 5' *AOX1* region will cut in all of the 5' *AOX1* regions present in the multimer. You can transform with uncut plasmid, but you will need to use 50–100 μ g of DNA to compensate for the 10 to 100-fold drop in transformation efficiency. However, with selection on blasticidin, any transformants you obtain will probably contain your construct. For best results:

- Use electroporation to transform your cells
 - Use at least 50 μ g plasmid DNA for each transformation
 - Plate out all of the transformation mix on several YPDS plates containing the appropriate concentration of blasticidin. You will need to use the optional outgrowth procedure on page 12.
-

Continued on next page

Constructing *In Vitro* Multimers, Continued

Digesting Recombinant pPIC6 α

Set up two separate digests of recombinant pPIC6 α containing one copy of your gene:

1. Double digest 1–2 μ g of recombinant pPIC6 α in 20 μ L with 10 units each of *Bgl* II and *Bam*H I. Proceed to **Producing Expression Cassettes for Multimerization**, Step 1.
 2. Digest 2 μ g of recombinant pPIC6 α in 20 μ L with 10 units of *Bam*H I only. Proceed to **Dephosphorylating the Vector**, Step 1.
-

Producing Expression Cassettes for Multimerization

The S.N.A.P.[™] Gel Purification Kit available from Invitrogen allows you to rapidly purify DNA fragments from regular agarose gels. Alternatively, you may use glass milk. To use the S.N.A.P.[™] Gel Purification Kit, follow the steps below:

1. Electrophorese your digest from Step 1, above, on a 1 to 5% regular TAE agarose gel. **Note:** Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
 2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
 3. Add 1.5 volumes Binding Buffer.
 4. Load solution (no more than 1 mL at a time) from Step 3 onto a S.N.A.P.[™] column. Centrifuge 1 minute at 3000 \times *g* in a microcentrifuge and discard the supernatant.
 5. If you have solution remaining from Step 3, repeat Step 4.
 6. Add 900 μ L of the Final Wash Buffer.
 7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
 8. Repeat Step 7.
 9. Elute the purified DNA in 15 μ L of sterile water. Store on ice if proceeding immediately to **Ligating the Expression Cassette** (page 30). Store at –20°C for long-term storage.
-

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Constructing *In Vitro* Multimers, Continued

Dephosphorylating the Vector

Dephosphorylation of the *Bam*H I-digested vector is necessary to prevent self-ligation.

1. Take your *Bam*H I digest from **Digesting Recombinant pPIC6 α** , Step 2 and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 μ L of sterile water.
2. Set up a 20 μ L dephosphorylation reaction in a microcentrifuge tube as follows:

<i>Bam</i> H I digested recombinant pPIC6 α (page 28, top, Step 2)	17 μ L
10X CIAP Buffer	2 μ L
CIAP (1 Unit/ μ L)	1 μ L
3. Incubate at 37°C for 15 minutes.
4. Add 30 μ L of sterile water to the reaction for a final volume of 50 μ L.
5. Add 50 μ L of phenol/chloroform and extract your DNA solution.
6. Precipitate the DNA by adding 5 μ L of 3 M sodium acetate and 110 μ L of 100% ethanol. Incubate on ice for 30 minutes.
7. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.
8. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol.
9. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.
10. Resuspend pellet in 8 μ L sterile water. Save on ice if you plan to ligate your insert immediately (see **Ligating and Digesting the Expression Cassette**) or store at -20°C.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Ligating and Digesting the Expression Cassette

Ligation of the expression cassette will generate head-to-tail, head-to-head, and tail-to-tail multimers. Creation of head-to-tail multimers will be in the correct orientation for expression and will destroy both the *Bam*H I and *Bgl* II sites between the expression cassettes. Digestion of the multimers with *Bam*H I and *Bgl* II will eliminate those multimers with tail-to-tail and head-to-head orientation. After digestion with these two restriction enzymes, you will have a mixture of multimers containing 1, 2, 3, etc. copies of your gene that can be ligated into *Bam*H I-linearized, recombinant pPIC6 α .

1. Set up a 20 μ L ligation reactions as follows:

<i>Bgl</i> II- <i>Bam</i> H I digested expression cassette	15 μ L
Sterile water	2 μ L
10X Ligation Buffer (with ATP)	2 μ L
T4 DNA Ligase (2.5 units/ μ L)	1 μ L

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

Sterile water	23 μ L
10X restriction enzyme buffer	5 μ L
<i>Bgl</i> II (10 units/ μ L)	1 μ L
<i>Bam</i> H I (10 units/ μ L)	1 μ L

5. Incubate the reaction at 37°C for 2 hours.
6. Add 50 μ L of phenol/chloroform and extract the restriction enzyme digestion to remove the enzymes. Transfer the aqueous solution to a new microcentrifuge tube.
7. To ethanol precipitate the DNA, add 5 μ L of 3 M sodium acetate and 110 μ L of 100% ethanol.
8. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.
9. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.
10. Resuspend pellet in 4 μ L sterile water. Save on ice if you plan to ligate your insert immediately or you can store at -20°C. Proceed to **Ligating Multimers into Linearized Vector**.



Note

You may wish to combine the ligation reaction with the restriction enzyme digestion to enrich for head-to-tail multimers. Use the reaction buffer for the restriction enzymes and add 1 mM ATP to the reaction in order to ensure ligase activity. Perform the reaction at 37°C. T4 ligase will retain most of its activity in the restriction buffer. As head-to-head and tail-to-tail multimers form, they will be digested, increasing the likelihood of obtaining head-to-tail multimers over time.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Ligating Multimers into Linearized Vector

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

1. Set up the following ligation reactions:

Dephosphorylated vector (page 29, Step 10)	4 μ L
Expression cassette multimers (Step 10, above)	4 μ L
10X Ligation Buffer	1 μ L
<u>T4 DNA Ligase (2.5 units/μL)</u>	<u>1 μL</u>
Total volume	10 μ L

For the vector only control:

Dephosphorylated vector	4 μ L
Sterile water	4 μ L
10X Ligation Buffer	1 μ L
<u>T4 DNA Ligase (2.5 units/μL)</u>	<u>1 μL</u>
Total volume	10 μ L

2. Incubate overnight at 16°C.
3. You may store the ligation reactions at -20°C until ready to use, or transform 1–10 μ L of each ligation mix into competent *E. coli*. Note that the amount of the ligation mixture you transform depends on whether you use electrocompetent or chemically competent cells. You may have to decrease the amount you to transform into electrocompetent cells to prevent arcing.

Transformation into *E. coli*

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

1. Transform competent *E. coli* by your method of choice.
2. After adding medium to the transformed cells and allowing them to recover, plate 10 μ L and 100 μ L of each transformation mix onto Low Salt LB plates containing 100 μ g/mL blasticidin. Save the remainder of your transformation mix at 4°C.
3. Incubate overnight at 37°C. If you do not get transformants or very few transformants, plate out the remainder of the transformation mix onto Low Salt LB-blasticidin plates.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Analyzing Transformants

1. Pick 20 transformants and inoculate each colony into 2 mL Low Salt LB containing 100 µg/mL blasticidin. Grow overnight at 37°C.
2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pPIC6α.
(Be sure to include *Bgl* II-*Bam*H I digested pPIC6α as a control. It is possible to get vector rearrangements and deletions with large recombinant vectors in *E. coli*. Including *Bgl* II-*Bam*H I digested pPIC6α will allow you to detect these rearrangements-deletions in the vector backbone.)
3. Analyze your digests on a 1% agarose gel. You should see bands corresponding to 1 copy, 2 copies, 3 copies, etc. of your expression cassette along with the vector backbone.
(The number of copies you obtain may depend on how well a large vector is tolerated by the host strain.)
4. Once you have identified plasmids with multiple copies of your expression cassette, be sure to purify by streaking for single colonies and confirming your construct.
5. Prepare frozen glycerol stocks of *E. coli* containing each of your multimeric constructs.
6. Prepare at least 100 µg of each plasmid for transformation into *Pichia*. You need more DNA because you will be transforming with uncut plasmid DNA. Transformation efficiency is about 1 to 2 orders of magnitude less for uncut versus linearized DNA.
7. Proceed to ***Pichia* Transformation**, page 9. Use the outgrowth protocol on page 12 to isolate transformants.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Troubleshooting

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

Problem	Possible Reason	Solution
No multimers or low number of multimers in your vector after transformation into <i>E. coli</i>	CIAP defective	Use fresh CIAP. Add more CIAP. Add 1 unit of CIAP and incubate 15 more minutes at 37°C. This is somewhat risky as CIAP can degrade the ends of your DNA.
	Not enough insert DNA to ligate	Add more <i>Bam</i> H I- <i>Bgl</i> II expression cassette to your ligation.
	Construct is unstable in <i>E. coli</i>	Decrease the number of cassettes in the vector.
	Multimers are too long to ligate efficiently	Try ligating each expression cassette stepwise (see page 31).
Recombinant vector rearranges and deletions are detected	Construct is unstable in <i>E. coli</i>	Decrease the number of cassettes in the vector.
No blasticidin-resistant <i>Pichia</i> transformants	Integration efficiency is low	Transform using more DNA and/or do multiple transformations with more DNA and cells.

For More Information

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

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

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

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

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

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

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

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

Accessory Products

Introduction

The following products may be used with the pPIC6 α vectors. For details, visit www.invitrogen.com or contact **Technical Support** (see page 36).

Item	Amount	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
One Shot® TOP10F' Chemically Competent <i>E. coli</i>	21 × 50 μ L	C3030-03
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
S.N.A.P.™ Gel Purification Kit	25 preps	K1999-25
Blasticidin	50 mg	R210-01
Positope™ Control Protein	5 μ g	R900-50
WesternBreeze® Chromogenic Kit	Anti-Mouse	WB7103
	Anti-Rabbit	WB7105
	Anti-Goat	WB7107
WesternBreeze® Chemiluminescent Kit	Anti-Mouse	WB7104
	Anti-Rabbit	WB7106
	Anti-Goat	WB7108
Countess™ Automated Cell Counter	1 each	C10227

Primers

For your convenience, Invitrogen offers a custom primer synthesis service. Visit www.invitrogen.com for more details.

Continued on next page

Accessory Products, Continued

Other *Pichia* Products

Other *Pichia* products available from Invitrogen are described below:

Item	Amount	Catalog no.
X-33 <i>Pichia</i> strain	1 stab	C180-00
KM71H <i>Pichia</i> strain	1 stab	C182-00
SMD1168H <i>Pichia</i> strain	1 stab	C184-00
pPIC6 A, B, and C	20 µg each	V210-20
pPICZ A, B, and C	20 µg each	V190-20
pPICZ α A, B, and C	20 µg each	V195-20
Original <i>Pichia</i> Expression Kit	1 kit	K1710-01
EasySelect™ <i>Pichia</i> Expression Kit	1 kit	K1740-01
<i>Pichia</i> EasyComp™ Transformation Kit	1 kit	K1730-01
<i>Pichia</i> Protocols	1 book	G100-01

Antibodies

If you do not have an antibody specific to your protein, Invitrogen offers the Anti-*myc*, or Anti-His(C-term) antibodies to detect your recombinant fusion protein. Horseradish peroxidase (HRP)- and alkaline phosphatase (AP)-conjugated antibodies are available for convenient one-step detection.

Antibody	Epitope	Catalog no.
Anti- <i>myc</i>	Detects a 10 amino acid epitope derived from <i>c-myc</i> (Evan <i>et al.</i> , 1985): EQKLISEEDL	R950-25
Anti- <i>myc</i> -HRP		R951-25
Anti- <i>myc</i> -AP		R952-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner <i>et al.</i> , 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25
Anti-His(C-term)-AP		R932-25

Technical Support

Web Resources



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

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
 - Complete technical support contact information
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-

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