

pPICZ α A, B, and C

***Pichia* expression vectors for selection on Zeocin™ and
purification of secreted, recombinant proteins**

Catalog no. V195-20

pPICZ α A, B, and C

Version A

*160410
25-0150*

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Purchaser Notification

Introduction

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.

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4,683,293	4,808,537	4,812,405	4,818,700	4,837,148
4,855,231	4,857,467	4,879,231	4,882,279	4,885,242
4,895,800	4,929,555	5,002,876	5,004,688	5,032,516
5,122,465	5,132,868	5,166,329		

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continued on next page



Purchaser Notification, continued

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Product Serial Number

Below, please record the Product Serial Number found on your User Registration Card for use in future correspondence with Invitrogen.

Product Serial Number _____

Important Information

Contents	20 µg each of lyophilized pPICZα A, B, and C is supplied.
Shipping/Storage	The lyophilized vectors are shipped at room temperature and stored at -20°C.
Materials Supplied by the User	<p>For the procedures described in this manual, you will need the following reagents and equipment. Additional reagents may be required. Please check each experiment to ensure you have all the reagents necessary.</p> <p>Equipment</p> <ul style="list-style-type: none">• <i>Pichia</i> Expression System manual• 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• Hemacytometer• Microtiter plates (optional) <p>Reagents</p> <ul style="list-style-type: none">• <i>Pichia</i> host strain (GS115, KM71, or SMD1168)• Electrocompetent or chemically competent <i>E. coli</i> (must be <i>recA</i>, <i>endA</i>) for transformation. For protocols to prepare competent <i>E. coli</i> and transformation protocols, please see <i>Current Protocols</i> (Ausubel <i>et al.</i>, 1994) or <i>Molecular Biology: A Laboratory Manual</i> (Sambrook <i>et al.</i>, 1989)• Restriction enzymes and appropriate buffers• Agarose and low-melt agarose• Gelzyme™ (Invitrogen, Catalog no. E210-01) or glassmilk• 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 11 for recipe and page 19 for ordering information)• Zeocin™ antibiotic (see page 3 for ordering information)• Low Salt LB plates containing 25 µg/ml Zeocin™• YPDS plates containing 100 µg/ml Zeocin™ plates• 50 ml conical centrifuge tubes• 15 ml polypropylene tubes• Xpress™ Purification System (Catalog no. K850-01, optional)

continued on next page



Important Information, continued



Important

Pichia users should already have the *Pichia* Expression System and the current manual. Procedures for transformation into *E. coli* and *Pichia*, analysis of recombinants, and expression are described in the *Pichia* manual. To ensure that you have the most recent version of this manual, please call Technical Services at 1-800-955-6288 (U.S.) or +31 (0) 594 515 175 (Europe).

Other *Pichia* Products

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

Item	Purpose	Quantity	Catalog no.
<i>Pichia</i> Expression Kit	Complete Kit for Gene Expression in <i>Pichia pastoris</i>	10-50 rxns	K1701-01
5' <i>AOX1</i> Sequencing Primer	Sequencing from the 5' <i>AOX1</i> region into the gene of interest	2 µg	N710-02
3' <i>AOX1</i> Sequencing Primer	Sequencing from the 3' <i>AOX1</i> region into the gene of interest	2 µg	N720-02
5' and 3' <i>AOX1</i> Primers	PCR to confirm <i>Pichia</i> recombinants	2 µg each	N740-02
α-Factor Sequencing Primer	Sequencing from the α-factor leader region into the gene of interest (for pPIC9, pPIC9K, and pPICZα only)	2 µg	N730-02
pPICZ A, B, and C	For easy selection and purification of intracellular proteins	20 µg each	V190-20

Technical Service



For Technical Service, please call, write, fax or E-mail:

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Introduction

Overview

Introduction

pPICZ α A, B, and C are 3.6 kb vectors used to express and secrete recombinant proteins in *Pichia pastoris*. Proteins are expressed as fusions to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α -factor secretion signal. In addition, the gene of interest may be fused to a C-terminal peptide containing the *myc* epitope for detection and a polyhistidine tag for purification on metal-chelating resin. The vector is supplied in three reading frames to facilitate in frame cloning with the N-terminal secretion signal. Selection of these vectors is based on the dominant selectable marker, Zeocin[™], which is bifunctional in both *Pichia* and *E. coli*. pPICZ α can be used in any *Pichia* strain including GS115, KM71, and SMD1168.



Important

Some of the protocols needed to effectively use pPICZ α A, B, and C are located in the *Pichia* Expression Kit manual. To ensure that you have the most recent version of this manual, please call Technical Services at 1-800-955-6288 (U.S.) or +31 (0) 594 515 175 (Europe).

Experimental Process

The following table describes the overall experimental process.

Step	Action	Page
1	Propagate pPICZ 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.	6
2	Develop a cloning strategy and ligate your gene into one of the pPICZ α vectors in frame with the α -factor secretion signal and the C-terminal tag.	6-10
3	Transform into <i>E. coli</i> and select transformants on LB plates containing 25 μ g/ml Zeocin [™] .	11
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.	12
5	Purify and linearize the recombinant plasmid for transformation into <i>Pichia pastoris</i> .	12-13
6	Transform your <i>Pichia</i> strain and plate onto YPDS plates containing 100 μ g/ml Zeocin [™] .	14
7	Select Zeocin [™] -resistant transformants. GS115 and SMD1168 will yield Mut ⁺ transformants and KM71 will yield Mut ^S transformants.	15
8	Optimize expression of your gene as described in the <i>Pichia</i> manual.	16
9	Purify your fusion protein on metal-chelating resin (i.e. ProBond [™]).	17

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Overview, continued

ProBond™ Resin

We recommend that you use the Xpress™ Purification System (Catalog no. K850-01) for purifying fusion proteins expressed using pPICZα A, B, or C. This kit contains six 2 ml precharged, prepacked ProBond™ resin columns, buffers for native and denaturing purification, and an instruction manual. **Please note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the Xpress™ Purification System Kit.**

If you are using a metal-chelating resin other than ProBond™, please follow the manufacturer's recommendations for fusion proteins expressed in yeast.

Applications of Zeocin™

Zeocin™ and the *Sh ble* gene are used for selection in mammalian cells (Mulsant *et al.*, 1989); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992; Wenzel *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection *E. coli* and *Pichia pastoris* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
<i>E. coli</i>	25 µg/ml in Low Salt LB medium*
<i>Pichia pastoris</i>	100 µg/ml in YPDS medium

***Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).**

For more information on Zeocin™ and Zeocin™ resistance, please see page 22.

**Important**

Any *E. coli* strain that contains the complete Tn5 transposable element (i.e. DH5αFIQ, SURE, SURE2) encodes the *ble* (bleomycin) resistance gene. These strains will confer resistance to Zeocin™. For the most efficient selection it is highly recommended that you choose an *E. coli* strain that does not contain the Tn5 gene (i.e. TOP10, DH5, DH10, etc.).

Purchase of Zeocin™

Zeocin™ can be purchased from Invitrogen in 1 gram (Catalog no. R250-01) and 5 gram (Catalog no. R250-05) quantities. For your convenience, the drug is prepared in autoclaved, deionized water and aliquoted into 1.25 ml aliquots at a concentration of 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C.

pPICZ α A, B, and C

Features of pPICZ α A, B, and C

pPICZ α A (3593 bp), pPICZ α B (3597 bp), and pPICZ α C (3598 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
5' <i>AOX1</i>	A 942 bp fragment containing the <i>AOX1</i> promoter that allows methanol-inducible, high-level expression in <i>Pichia</i> Targets plasmid integration to the <i>AOX1</i> locus.
Native <i>Saccharomyces cerevisiae</i> α -factor secretion signal	Allows for efficient secretion of most proteins from <i>Pichia</i>
Multiple cloning site with 10 unique restriction sites	Allows insertion of your gene into the expression vector
C-terminal <i>myc</i> epitope tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn)	Permits detection of the fusion protein by the Anti- <i>myc</i> Antibody (Catalog no R950-25) (Evan <i>et al.</i> , 1985)
C-terminal polyhistidine tag	Encodes six histidine residues that form a metal-binding site for affinity purification of recombinant protein
<i>AOX1</i> Transcription Termination (TT)	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 <i>Sh ble</i> gene in <i>Pichia</i> , conferring Zeocin [™] resistance
EM7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the <i>Sh ble</i> gene in <i>E. coli</i> , conferring Zeocin [™] resistance
<i>Sh ble</i> gene (<i>Streptoalloteichus hindustanus ble</i> gene)	Zeocin [™] resistance gene
<i>CYC1</i> transcription termination region (GenBank accession number M34014)	3' end of the <i>Saccharomyces cerevisiae CYC1</i> gene that allows efficient 3' mRNA processing of the <i>Sh ble</i> gene for increased stability
ColE1 origin (pUC-derived)	Allows replication and maintenance of the plasmid in <i>E. coli</i>
<i>Sac</i> I <i>Pme</i> I <i>Bst</i> X I	Unique restriction sites that permit linearization of the vectors at the <i>AOX1</i> locus for efficient integration into the <i>Pichia</i> genome



NOTE

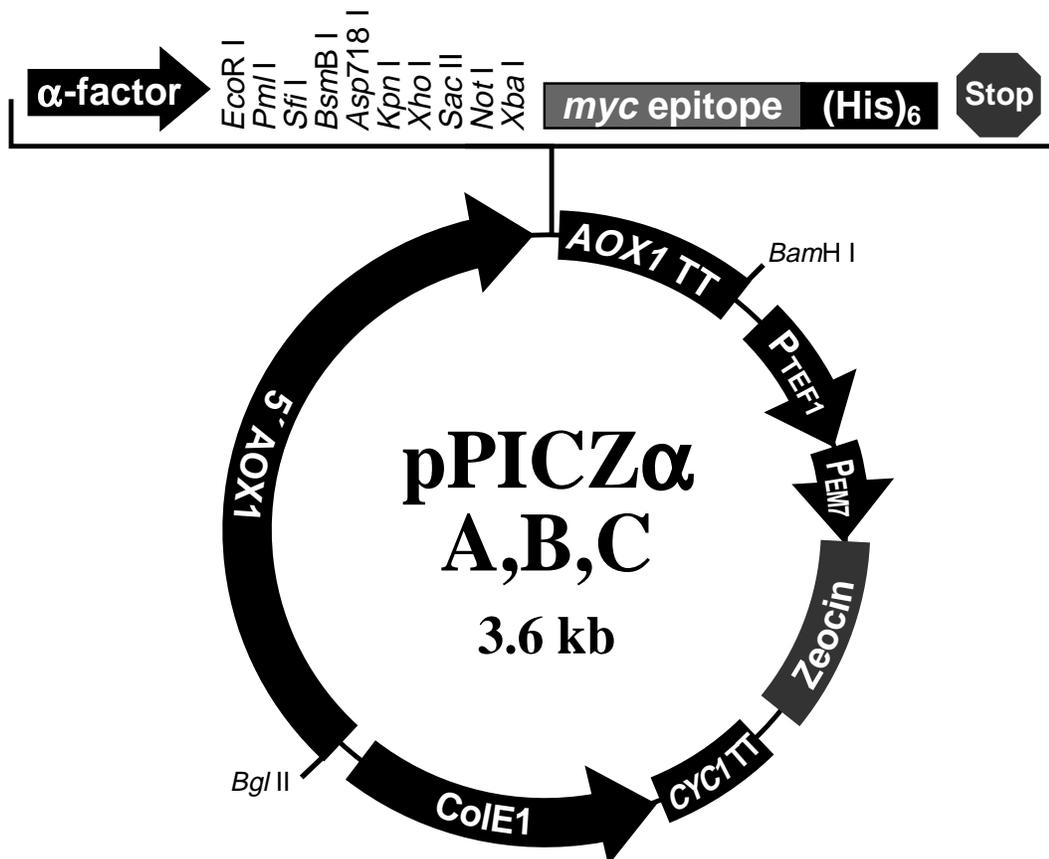
The pPICZ α vectors do not contain a yeast origin of replication. Transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome.

continued on next page

pPICZ α A, B, and C, continued

Map of pPICZ α A, B, and C

The figure below summarizes the features of the pPICZ α vectors. The complete nucleotide sequence for pPICZ α A is provided after page 34. The sequences for pPICZ α A, B, and C are available for downloading from our World Wide Web site (<http://www.invitrogen.com>) or from Technical Service, 1-800-955-6288 (U. S.) or +31 (0) 594 515 175 (Europe). Details of the multiple cloning sites are shown on page 8 for pPICZ α A, page 9 for pPICZ α B, and page 10 for pPICZ α C.



Comments for pPICZ α A

3593 nucleotides

5' *AOX1* region: bases 1-940
 5' end of *AOX1* mRNA: base 824
 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 1208-1274
myc epitope tag: bases 1275-1307
 Polyhistidine tag: bases 1320-1340

3' *AOX1* priming site: bases 1423-1443
 3' end of mRNA: base 1514
AOX1 transcription termination region: bases 1341-1682
TEF1 promoter region: bases 1683-2094
 EM7 promoter: bases 2095-2162
Sh ble ORF: bases 2163-2537
CYC1 transcription termination region: bases 2538-2855
 ColE1 origin (pUC-derived): bases 2866-3539

Methods

Cloning into pPICZ α A, B, and C

Introduction

Before cloning your gene into one of the pPICZ α vectors, please consider some of the general guidelines presented below. It is important to clone your gene in frame with the α -factor signal sequence. The multiple cloning sites for pPICZ α A, B, and C are presented on pages 8-10 to help you develop a cloning strategy.

General Considerations

The following are some general considerations applicable to pPICZ α A, B, and C.

- The codon usage in *Pichia* is believed to be similar to *Saccharomyces cerevisiae*.
 - Many *Saccharomyces* genes have proven to be functional in *Pichia*.
 - Plasmid constructions should be maintained in a *recA*, *endA* *E. coli* strain such as TOP10.
 - The *BsmB* I site in the multiple cloning site has been specifically engineered to be compatible with inserts that have *BamH* I and/or *Bgl* II ends. The *BamH* I and *Bgl* II sites will be destroyed upon ligation, but the insert can be released by digestion with *BsmB* I. (Note: This site is not used for construction of *in vitro* multimers, page 25.)
 - The initiation ATG in the α -factor signal sequence corresponds to the native initiation ATG of the *AOX1* gene.
 - The open reading frame (ORF) of the mature gene of interest should be cloned in frame and downstream of the α -factor signal sequence and in frame with the C-terminal tag (if desired).
 - You may wish to express your gene of interest without the C-terminal peptide. In this case, be sure your gene contains a stop codon.
 - The predicted protease cleavage sites for the α -factor signal sequence are indicated in the figures on pages 8-10.
 - 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 native 5' end of the *AOX1* mRNA is noted in each multiple cloning site. This is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.
-



You will need to prepare competent *E. coli* cells for transformation. Please see *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989) for preparation of electrocompetent or chemically competent *E. coli* or use your laboratory's procedure.

We recommend that you transform pPICZ α A, B, and C into a *recA*, *endA* *E. coli* strain (e.g. TOP10) so that you have a permanent stock and are able to propagate the plasmids. Electrocompetent TOP10 cells are available from Invitrogen.

Item	Amount	Catalog no.
TOP10 Electrocomp™	5 x 80 μ l (400 μ l total)	C664-55
TOP10 Electrocomp™	10 x 80 μ l (800 μ l total)	C664-11

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Cloning into pPICZ α A, B, and C, continued



Important

For Zeocin™ to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt LB broth and plates using the recipe on page 11.

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

- 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 Low Salt LB with 25 μ g/ml Zeocin™.
-

Cloning Procedures

Detailed maps of the multiple cloning sites are provided below and on the following pages. Please 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 pPICZ 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.
-

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, please 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.

Cloning Your Gene Flush with the Kex2 Cleavage Site

If you wish to have your protein expressed with a native N-terminus, you may use the *Xho* I site at bp 1184-1189 to clone your gene flush with the Kex2 cleavage site. Use PCR to rebuild the sequence from the *Xho* I site 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.

Construction of Multimeric Plasmids

pPICZ 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, please refer to page 25.

continued on next page

Cloning into pPICZ α A, B, and C, continued

Multiple Cloning Site of pPICZ α A

Below is the multiple cloning site of pPICZ α 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.

```

                    5' end of AOX1 mRNA
                    |
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA
                    |
                    5' AOX1 priming site
                    |
871 CAAGCTTTTGG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT

931 ATTTCGAAACG 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

                     $\alpha$ -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

                     $\alpha$ -factor priming site
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
                    Xho I*

1187 GAG AAA AGA GAG GCT GAA GCT GAATTCAC GTGGCCAG CCGGCCGC TCGGATCGGT
    Glu Lys Arg Glu Ala Glu Ala
                    Kex2 signal cleavage
                    EcoR I Pml I Sfi I BsmB I Asp718 I
                    Ste13 signal cleavage
1244 ACCTCGAGCC GCGGCGGCC GCCAGCTTTC TA GAA CAA AAA CTC ATC TCA GAA GAG
    Kpn I Xho I Sac II Not I Xba I myc epitope
    Glu Gln Lys Leu Ile Ser Glu Glu

                    polyhistidine 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 CTGTTCTCA GTTCAAGTTG GGCACCTACG AGAAGACCGG TCTTGCTAGA

                    3' AOX1 priming site
1411 TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATT TTTTGAT
                    3' polyadenylation site
1471 ACTTTTTTAT TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT

```

* The *Xho* I site upstream of the Kex2 cleavage site is used to clone the gene of interest flush with the Kex2 cleavage site (see page 7).

continued on next page

Cloning into pPICZ α A, B, and C, continued

Multiple Cloning Site of pPICZ α B

Below is the multiple cloning site of pPICZ α 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.

```

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

                     $\alpha$ -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

                     $\alpha$ -factor priming site
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
                    Xho I*
                    |
                    Kex2 signal cleavage
1187 GAG AAA AGA GAG GCT GAA GC TGCAG GAATTCAC GTGGCCCAG CCGGCCGTC TCGGA
    Glu Lys Arg Glu Ala Glu Ala
                    Ste13 signal cleavage
                    EcoR I   Pml I   Sfi I   BsmB I
1243 TCGGTACCTC GAGCCGCGGC GGCCGCCAGC TTTCTA GAA CAA AAA CTC ATC TCA GAA
    Glu Gln Lys Leu Ile Ser Glu
                    Asp718 | Kpn I | Xho I   Sac II   Not I   Xba I   myc epitope
                    |
                    polyhistidine 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 CTTCATTTTT

                    3' polyadenylation site
1472 GATACTTTTT TATTTGTAAC CTATATAGTA TAGGATTTTT TTTGTCATTT TGTTTCTTCT
    
```

* The *Xho* I site upstream of the Kex2 cleavage site is used to clone the gene of interest flush with the Kex2 cleavage site (see page 7).

continued on next page



Cloning into pPICZ α A, B, and C, continued

Multiple Cloning Site of pPICZ α C

Below is the multiple cloning site of pPICZ α 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.

```

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

871 CAAGCTTTTTG 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

       $\alpha$ -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

       $\alpha$ -factor priming site
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
                                     Xho I*

1188 GAG AAG AGA GAG GCT GAA GC ATCGAT GAATTCAC GTGGCCAG CCGGCCGTC TCGGA
      Glu Lys Arg Glu Ala Glu Ala
      Kex2 signal cleavage
      Ste13 signal cleavage
      EcoR I Pml I Sfi I BsmB I

1244 TCGGTACCTC GAGCCGCGGC GGCCGCCAGC TTTCTA GAA CAA AAA CTC ATC TCA GAA
      Asp718 | Kpn I Xho I Sac II Not I Xba I myc epitope
      Glu Gln Lys Leu Ile Ser Glu
      polyhistidine 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

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

1473 GATACTTTTT TATTTGTAAC CTATATAGTA TAGGATTTTT TTTGTCATTT TGTTTCTTCT
      3' polyadenylation site
  
```

* The Xho I site upstream of the Kex2 cleavage site is used to clone the gene of interest flush with the Kex2 cleavage site (see page 7).

Transformation into *E. coli*

Introduction

Ligation mixtures may be transformed into *E. coli* and selected on **Low Salt LB** medium (see below) with Zeocin™. Transformants are isolated and analyzed for the presence and orientation of insert. There is no blue/white screening for the presence of insert with pPICZα A, B, or C. After obtaining the desired recombinant plasmid, proceed to transformation of *Pichia* as described in the *Pichia* Expression Kit manual.

IMPORTANT! Low Salt LB Medium with Zeocin™

For Zeocin™ to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt LB broth and plates using the following recipe.

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

Low Salt LB Medium:

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 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration.
4. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Note: Pre-mixed Low Salt LB Medium and Low Salt LB Agar are available from Invitrogen in convenient pouches or in bulk. Please see page 19 for more information.

Transformation

Guidelines are as follows:

- Transformation may be done by either electroporation or chemical methods. Use your preferred method or refer to general molecular biology references (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).
 - Add either Low Salt LB or LB medium to the cells after heat shock or electroporation to allow them to recover.
 - Plate on **Low Salt LB medium** with 25 µg/ml Zeocin™. **Note:** You may also use SOB, 2XYT, or TB medium, but you may have to increase the concentration of Zeocin™ to 50 µg/ml to compensate for differences in the salt concentration.
 - Incubate overnight at 37°C.
-

continued on next page

Transformation into *E. coli*, continued

Analysis of Transformants

1. After transformation, plate 10 μ l and 100 μ l of the transformation mix onto Low Salt LB plates with 25 μ g/ml ZeocinTM (see **Recipe**, previous page) and select ZeocinTM-resistant colonies.
2. Pick 10 ZeocinTM-resistant transformants and inoculate each colony into 2 ml Low Salt LB medium with 25 μ g/ml ZeocinTM. Grow overnight at 37°C with shaking.
3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below).
4. Be sure to make a glycerol stock of your purified clone for safekeeping.
 - Purify the clone by streaking for single colonies
 - Prepare an overnight bacterial culture from a single colony
 - Combine 0.85 ml of the 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
 - Store at -80°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 and the C-terminal tag. To sequence your construct in pPICZ α , use the α -factor (Catalog no. N730-02) or the 5' *AOX1* (Catalog no. N710-02) and the 3' *AOX1* (Catalog no. N720-02) Sequencing Primers.

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

Plasmid Preparation

Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform *Pichia* (5-10 μ g of each plasmid per each transformation). We recommend the S.N.A.P.TM Miniprep Kit (K1900-01) for quick purification of pure plasmid DNA. Once you have purified plasmid DNA, proceed to ***Pichia* Transformation**, next page.

Introduction

At this point, you should have your gene cloned into one of the pPICZ α vectors. Your construct should be correctly fused to both the N-terminal and C-terminal peptides. You will then prepare 5-10 μg of plasmid DNA and linearize the plasmid prior to transformation and selection in *Pichia*. Transformants are plated on YPDS plates containing 100 $\mu\text{g}/\text{ml}$ Zeocin[™] to isolate Zeocin[™]-resistant clones.

Method of Transformation

We do not recommend spheroplasting for transformation of *Pichia* with plasmids containing the Zeocin[™] resistance marker. Spheroplasting involves removal of the cell wall to allow DNA to enter the cell. Cells must first regenerate the cell wall before they are able to express the Zeocin[™] resistance gene. For this reason, plating spheroplasts directly onto selective medium containing Zeocin[™] does not yield any transformants.

We recommend electroporation for transformation of *Pichia* with pPICZ 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.



Important

Since pPICZ 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 (i.e. GS115, SMD1168). If you choose to generate a Mut^S recombinant strain, then use a *Pichia* host that has a disrupted *AOX1* gene (i.e. KM71).

Before Starting

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

- 5-10 μg pure pPICZ 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 100 $\mu\text{g}/\text{ml}$ Zeocin[™] (please see page 21 for recipe)
-

Restriction Digest

1. Digest ~5-10 μg of plasmid DNA with one of the enzymes listed below. Each enzyme cuts one time in the 5' *AOX1* region to linearize the vector. Choose the enzyme that does not cut within your gene: *Sac* I (209 bp); *Pme* I (414 bp); or *Bst*X I (707 bp).
 2. We recommend that you check a small aliquot of your digest by agarose gel electrophoresis for complete linearization.
 3. If the vector is completely linearized, heat inactivate or add EDTA to stop the reaction, 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 in 10 μl sterile, deionized water. Use immediately or store at -20°C.
-

continued on next page

***Pichia* Transformation, continued**

Preparation of *Pichia* for Electroporation

1. Grow 5 ml of your *Pichia pastoris* strain in YPD in a 50 ml conical at 30°C overnight.
 2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1-0.5 ml of the overnight culture. Grow overnight again to an OD₆₀₀ = 1.3-1.5.
 3. Centrifuge the cells at 1500 x 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, then resuspend the pellet with 250 ml of ice-cold (0°C), sterile water.
 5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold (0°C) 1 M sorbitol.
 6. Centrifuge the cells as in Step 3, then 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.
-

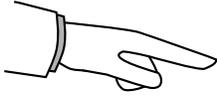
Transformation by Electroporation

1. Mix 80 µl of the cells from Step 6 (above) with 5-10 µg of linearized 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*) suggested by the manufacturer of the specific electroporation device being used. Protocols for two electroporation units are included below.
Using a 0.2 cm cuvette, these protocols generate a pulse length of ~5-10 ms with a field strength of ~7500 V/cm:

Electroporator	Charging voltage (V)	Capacitance (µF)	Resistance (Ω)
Invitrogen Electroporator II Catalog No. S1670-02	1500	50	200
Bio-Rad GenePulser	1500	25	200

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 10, 25, 50, 100, and 200 µl each on separate, labeled YPDS plates containing 100 µg/ml Zeocin™. Plating at low cell densities favors efficient Zeocin™ selection.
 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 100 µg/ml Zeocin™.
-

continued on next page



NOTE

Generally several hundred to several thousand Zeocin[™]-resistant colonies are generated using the above protocol. If more colonies are needed the protocol may be modified as described below. Please note that you will need ~20 150 mm plates with YPDS agar containing 100 µg/ml Zeocin[™].

1. Set up two transformations per construct and follow Steps 1 through 5 of the **Transformation by Electroporation** protocol, previous page.
 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 100 µg/ml Zeocin[™].
 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 100 µg/ml Zeocin[™].
 6. Incubate plates for 2 to 4 days at 30°C until colonies form.
-

Analysis of *Pichia* Transformants

If you wish, you may analyze 6-10 of your Zeocin[™]-resistant *Pichia* transformants for the Mut phenotype, the presence of insert using PCR, or for copy number using Southern analysis. Please refer to the *Pichia* Expression Kit manual for these protocols. If you used GS115 or SMD1168 as your host, the transformants will be Mut⁺. If you used KM71, your transformants will be Mut^S.

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

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

Detection of Recombinant Proteins in *Pichia*

We recommend that you use the following techniques to analyze the expression of your protein. **Remember to analyze BOTH the medium and the cells for the presence of your recombinant protein.** Please note that the α -factor signal sequence will add approximately 9.3 kDa to your protein if it is unprocessed. The C-terminal tag will add 2.5 kDa to 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> Antibody (Catalog no. R950-25)	Can detect as little as 1-10 pg, depending on detection method (alkaline phosphatase, horseradish peroxidase, radiolabeled antibody)
Functional assay	Varies depending on assay	Varies depending on assay Used to compare relative amounts of protein.

Expression Guidelines

Small-scale expression, SDS-PAGE analysis, optimization of secreted expression, and scale-up of expression can be carried out as described in the *Pichia* Expression Kit manual.



Important

Because the pPICZ 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.

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. **Please note that this section only describes sample application onto ProBond™.** For instructions on how to prepare and use ProBond™ resin, please refer to the Xpress™ Purification manual.

ProBond™ Resin

We recommend that you use the Xpress™ Purification System (Catalog no. K850-01) for purifying fusion proteins expressed from pPICZα A, B, or C. This kit contains six 2 ml precharged, prepacked ProBond™ resin columns, buffers for native and denaturing purification, and an instruction manual. **Please note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the Xpress™ Purification Kit.**

If you are using a metal-chelating resin other than ProBond™, please follow the manufacturer's recommendations for fusion proteins expressed in bacteria or yeast.

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.

Expression of 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). Please refer to the *Pichia* Expression Kit manual for details. 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 at +4°C.

continued on next page

Purification, continued

Sample Application (Native Conditions)

The following protocol can 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 medium 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 x 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 medium. Proceed to **Column Washing and Elution Under Native Conditions** in the Xpress™ 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 medium with 7 ml of the Denaturing Binding Buffer.



NOTE

We have observed some *Pichia* proteins may be retained on the ProBond™ column using native purification conditions. Optimization of the purification (see Xpress™ 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. Please refer to the Xpress™ 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 the medium with 1/10 volume of 10X Stock Solution B (Xpress™ 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

Pre-mixed Expression Media

Please see the table below for a wide range of pre-mixed media and media components specifically for *Pichia*. In addition, pre-mixed media for Low Salt LB Medium is also available. Please contact Technical Services at 1-800-955-6288 (U.S.) or +31 (0) 594 515 175 (Europe) for more information.

Item	Amount	Catalog no.
YP Base Medium	10 x 30 g pouches*	Q300-01
Requires addition of carbon source (i.e. dextrose)	500 g	Q300-02
	2.5 kg	Q300-03
YP Base Agar Medium	10 x 50 g pouches*	Q300-04
Requires addition of carbon source (i.e. dextrose)	500 g	Q300-05
	2.5 kg	Q300-06
Yeast Nitrogen Base –with ammonium sulfate –without amino acids	67 g pouch Each pouch contains reagents to prepare 500 ml of a 10X YNB solution	Q300-07
	500 g	Q300-09
Low Salt LB Medium	10 x 20 g pouches*	Q100-01
	500 g	Q100-02
	2.5 kg	Q100-03
Low Salt LB Agar Medium	10 x 35 g pouches*	Q100-04
	500 g	Q100-05
	2.5 kg	Q100-06
1 M Potassium Phosphate Buffer, pH 6	70.55 g pouch Each pouch contains reagents to prepare 500 ml of a 1 M potassium phosphate, pH 6 buffer	Q300-14
	500 g	Q300-16
	2.5 kg	Q300-17
Amino Acid Mix (L-leucine, L-lysine, L-glutamic acid, L-isoleucine, and L-methionine, 0.5% each)	5 x 2.5 g pouches Each pouch contains reagents to prepare 100 ml of a 100X amino acid solution	Q300-18
D-Sorbitol	100 g	Q300-20
	500 g	Q300-21
	2.5 kg	Q300-22

*each pouch contains reagents to prepare 1 liter of medium

continued on next page



Recipes, continued

Pre-mixed Expression Media, continued

Item	Amount	Catalog no.
L-Histidine	1 g	Q300-23
D-Biotin	100 mg	Q300-24
	1 g	Q300-25
Dextrose (D-Glucose)	100 g	Q100-36
	500 g	Q100-37
	2.5 kg	Q100-38
Fermentation Basal Salts	1 kg	Q300-10
	5 kg	Q300-11
Fermentation PTM ₁ Trace Salts	500 ml bottle	Q300-12

*each pouch contains reagents to prepare 1 liter of medium

YPD (+ Zeocin™)

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
± 2% agar
± 100 µg/ml Zeocin™

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. Cool solution to ~55°C and add 100 ml of 10X D. Add 1.0 ml of 100 mg/ml Zeocin™, if desired.

Liquid medium without Zeocin™ can be stored at room temperature. Medium containing Zeocin™ should be stored at +4°C in the dark. YPD slants or plates are stored at +4°C. The shelf life of medium is several months. Medium containing Zeocin™ has a shelf life of one to two weeks.

continued on next page

YPDS + Zeocin™ Agar

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
1 M sorbitol
2% agar
100 µg/ml Zeocin™

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 10X D.
5. Cool solution to ~55°C and add 1.0 ml of 100 mg/ml Zeocin™.

Store YPDS slants or plates containing Zeocin™ at +4°C in the dark. The shelf life is one to two weeks.

More on Zeocin™

Resistance to Zeocin™

A Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1988). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13,665 Da protein that binds Zeocin™ in a stoichiometric manner. The binding of Zeocin™ inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™. The nucleic acid and protein sequence is given below:

Nco I

```
ACC ATG GCC AAG TTG ACC AGT GCC GTT CCG GTG CTC
      M  A  K  L  T  S  A  V  P  V  L

ACC GCG CGC GAC GTC GCC GGA GCG GTC GAG TTC TGG
      T  A  R  D  V  A  G  A  V  E  F  W
```

Sma I

```
ACC GAC CGG CTC GGG TTC TCC CGG GAC TTC GTG GAG
      T  D  R  L  G  F  S  R  D  F  V  E
```

SgrA I

```
GAC GAC TTC GCC GGT GTG GTC CGG GAC GAC GTG ACC
      D  D  F  A  G  V  V  R  D  D  V  T

CTG TTC ATC AGC GCG GTC CAG GAC CAG GTG GTG CCG
      L  F  I  S  A  V  Q  D  Q  V  V  P

GAC AAC ACC CTG GCC TGG GTG TGG GTG CGC GGC CTG
      D  N  T  L  A  W  V  W  V  R  G  L

GAC GAG CTG TAC GCC GAG TGG TCG GAG GTC GTG TCC
      D  E  L  Y  A  E  W  S  E  V  V  S

ACG AAC TTC CGG GAC GCC TCC GGG CCG GCC ATG ACC
      T  N  F  R  D  A  S  G  P  A  M  T

GAG ATC GGC GAG CAG CCG TGG GGG CGG GAG TTC GCC
      E  I  G  E  Q  P  W  G  R  E  F  A

CTG CGC GAC CCG GCC GGC AAC TGC GTG CAC TTC GTG
      L  R  D  P  A  G  N  C  V  H  F  V

GCC GAG GAG CAG GAC TGA
      A  E  E  Q  D  STOP
```

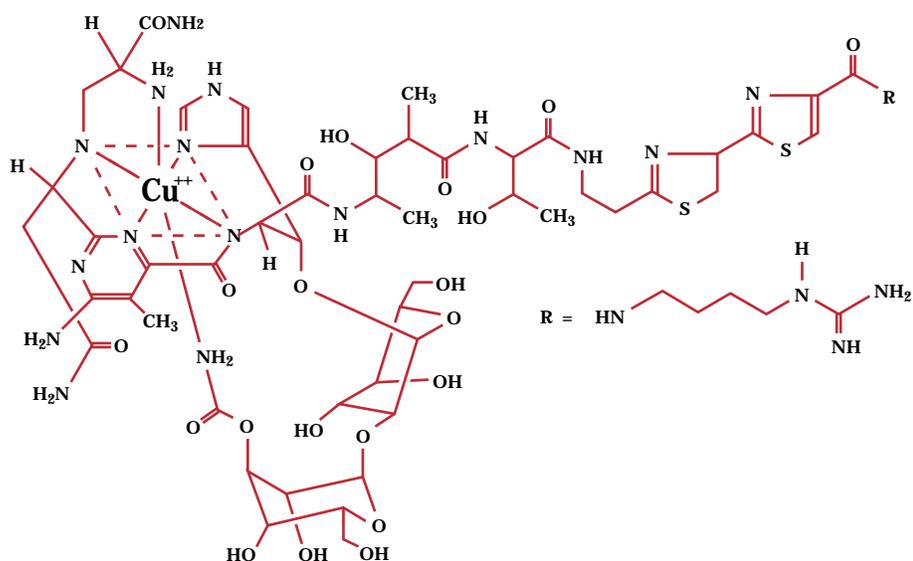
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Description

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. Zeocin™ is not as toxic as bleomycin on fungi.

Chemical Properties

Zeocin™ is a basic, water-soluble compound isolated from *Streptomyces verticillus* as a copper-chelated glycopeptide. The presence of copper gives the solution its blue color. The chemical formula for Zeocin™ is $C_{55}H_{83}N_{19}O_{21}S_2Cu$. It contains several unique amino acids, sugars, and aliphatic amines. For general information about the family of bleomycin antibiotics, please see Berdy, 1980 (**Reference** section). The general structure of Zeocin™ is shown below.



Mechanism of Action

The exact mechanism of action of Zeocin™ is not known; however, it is thought to be the same as bleomycin and phleomycin due to its similarity to these drugs and its inhibition by the *Sh ble* resistance protein (see next section). The copper/glycopeptide complex is selective and involves chelation of copper (Cu^{2+}) by the amino group of the α -carboxamide, single nitrogen atoms of both the pyrimidine chromophore and the imidazole moiety, and the carbamoyl group of mannose. The copper-chelated form is inactive. When the antibiotic enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{1+} and removed by sulfhydryl compounds in the cell. Upon removal of the copper, Zeocin™ is activated to bind DNA and cleave it causing cell death (Berdy, 1980). **High salt concentrations and acidity or basicity inactivate Zeocin™; therefore, it is necessary to reduce the salt in bacterial medium to 90 mM (5 g/liter) or less and adjust the pH to 7.5 to make sure the drug remains active.**

Lithium Chloride Transformation Method

Introduction

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

Preparation of Solutions

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

1 M LiCl in distilled, deionized water. Filter sterilize. Dilute as needed with sterile water.
50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter sterilize. Store in a tightly capped bottle.

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

Preparation of Cells

1. Grow a 50 ml culture of *Pichia pastoris* in YPD at 30°C with shaking to an OD_{600} of 0.8 to 1.0 (approximately 10^8 cells/ml).
 2. Harvest the cells and wash with 25 ml of sterile water. Centrifuge at $1500 \times g$ for 10 minutes at room temperature.
 3. Decant the water and resuspend the cells in 1 ml of 100 mM LiCl.
 4. Transfer the cell suspension to a 1.5 ml microcentrifuge tube.
 5. Pellet the cells at maximum speed for 15 seconds and remove the LiCl with a pipet.
 6. Resuspend the cells in 400 μ l of 100 mM LiCl.
 7. Dispense 50 μ l 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 .**
-

Transformation

1. Boil a 1 ml sample of single-stranded DNA for five minutes, then quickly chill in ice water. Keep on ice. **Note:** It is 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 LiCl-cell solution from Step 7, above, and remove the LiCl with a pipet.
 3. For each transformation sample, add the following reagents IN THE ORDER GIVEN to the cells. PEG shields the cells from the detrimental effects of the high concentration of LiCl.
240 μ l 50% PEG
36 μ l 1 M LiCl
25 μ l 2 mg/ml single-stranded DNA
Plasmid DNA (5-10 μ g) in 50 μ l sterile water
 4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
 5. Incubate the tube at 30°C for 30 minutes without shaking.
 6. Heat shock in a water bath at 42°C for 20-25 minutes.
 7. Centrifuge the tubes at 6000 to 8000 rpm and remove the transformation solution with a pipet.
 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 μ l on YPD plates containing 100 μ g/ml ZeocinTM. Incubate the plates for 2-3 days at 30°C . Proceed to **Analysis of *Pichia* Transformants**, page 15.
-

Construction of *In Vitro* Multimers

Experimental Outline

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

Stage	Description
1	Digest pPICZ α containing your gene of interest with <i>Bgl</i> II and <i>Bam</i> H I to release the expression cassette (P_{AOX1} , the secretion signal, and your gene).
2	To clone multiple copies of the expression cassette, linearize pPICZ 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 pPICZ 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 pPICZ α A, B, or C. For example:

Stage	Description
1	Digest pPICZ 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

Construction of *In Vitro* Multimers, continued

Before Starting

You will need to have on hand the following materials:

- Electrocompetent or chemically competent *E. coli* (must be *recA*, *endA*) for transformation. You will need 3-4 tubes of competent cells per experiment. Use your favorite protocol or refer to *Current Protocols* (Ausubel, *et al.*, 1990) or *Molecular Biology: A Laboratory Manual* (Sambrook, *et al.*, 1989)
- *Bam*H I and *Bgl* II restriction enzymes and appropriate buffers
- Low-melt agarose
- Gelzyme™ (Invitrogen, Catalog no. E210-01) or glassmilk
- Sterile water
- CIAP (calf intestinal alkaline phosphatase, 1 unit/μl, Boehringer Mannheim)
- 10X CIAP Buffer
- Phenol/chloroform
- 3M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/μl)
- 10X Ligation Buffer (with ATP)
- Low Salt LB plates containing 25 μg/ml Zeocin™
- 150 mm plates for plating transformants
- 16°C, 37°C, and 65°C water baths or temperature blocks

Controls

In order to evaluate your transformants and expression data later on, we recommend transforming *Pichia* with pPICZα (the parent vector) and pPICZα containing one copy of your expression gene. 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.

continued on next page

Construction of *In Vitro* Multimers, continued



Important

pPICZ α plasmids containing multimers cannot be linearized because any enzyme that cuts in the 5' *AOXI* region will cut in all of the 5' *AOXI* regions present in the multimer, destroying all your hard work. You can transform with uncut plasmid, but you will need to use 50-100 μg to compensate for the 10 to 100-fold drop in transformation efficiency. However, with selection on Zeocin[™], any transformants you obtain will probably contain you construct. For best results—

- Use electroporation to transform your cells
 - Use at least 50 μg plasmid DNA for each transformation
 - Plate out all of the transformation mix on several YPDS plates containing 100 mg/ml Zeocin[™]. You will need to use the optional outgrowth procedure on page 15.
-

Digestion of Recombinant pPICZ α

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

1. Double digest 1-2 μg of recombinant pPICZ α in 20 μl with 10 units each of *Bgl* II and *Bam*HI. Proceed to **Production of Expression Cassettes for Multimerization**, Step 1.
 2. Digest 2 μg of recombinant pPICZ α in 20 μl with 10 units of *Bam*HI only. Proceed to **Dephosphorylation of Vector**, Step 1.
-

Production of Expression Cassettes for Multimerization

1. Electrophorese your *Bam*HI-*Bgl* II digest from Step 1, above, in a low-melt agarose gel. Note that the size of the fragment depends on the size of your gene. The vector backbone will be approximately 1.9 kb.
 2. Excise the band containing your expression cassette and release the DNA by digestion of agarose with Gelzyme[™] (Invitrogen Catalog no. E210-01). Alternatively, you may use glassmilk. To use Gelzyme[™], follow the steps below:
 - A. Heat the agarose containing your desired DNA fragment at 65°C for 3-5 minutes or until the agarose melts.
 - B. Estimate the volume of the melted agarose and transfer the tube to 40°C.
 - C. Add 1/10 of the volume of 10X Gelzyme[™] buffer (100 mM Tris-HCl, pH 6.5, 10 mM EDTA, pH 8.0) and 10 units of Gelzyme[™].
 - D. Incubate for 60 minutes, then transfer to ice. The solution should remain liquid and not solidify.
 - E. If the agarose solidifies, melt the agarose as in step A, then add 10 more units of Gelzyme[™], and incubate for another 60 minutes until the solution remains liquid on ice.
 - F. Extract the agarose solution with phenol and transfer the aqueous solution to a new tube.
 3. Ethanol precipitate the DNA with 1/10 volume 3 M sodium acetate and 2 volumes 100% ethanol.
 4. Resuspend the *Bgl* II-*Bam*HI digested expression cassette in 15 μl sterile water. Store on ice if proceeding immediately to **Ligation of Expression Cassette**, next page. Store at -20°C for long-term storage.
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continued on next page

Construction of *In Vitro* Multimers, continued

Dephosphorylation of Vector

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

1. Take your *Bam*H I digest from **Digestion of Recombinant pPICZ 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 pPICZ α (page 27, 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. Transfer the aqueous solution to a new tube.
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 **Ligation and Digestion of Expression Cassette**) or store at -20°C.

Ligation and Digestion of 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 pPICZ 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.

continued on next page

Construction of *In Vitro* Multimers, continued

Ligation and Digestion of Expression Cassette, continued

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 **Ligation of 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. 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.

Ligation of 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 28, Step 10)	4 μ l
Expression cassette multimers (Step 10, above)	4 μ l
10X Ligation Buffer	1 μ l
T4 DNA Ligase (2.5 units/ μ l)	1 μ l
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
T4 DNA Ligase (2.5 units/ μ l)	1 μ l
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.

continued on next page

Construction of *In Vitro* Multimers, continued

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 25 μ g/ml ZeocinTM. 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-ZeocinTM plates.
-

Analysis of Transformants

1. Pick 20 transformants and inoculate each colony into 2 ml Low Salt LB containing 25 μ g/ml ZeocinTM. Grow overnight at 37°C.
 2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pPICZ α .
(Be sure to include *Bgl* II-*Bam*H I digested pPICZ α as a control. It is possible to get vector rearrangements and deletions in large recombinant vectors propagated in *E. coli*. Including *Bgl* II-*Bam*H I digested pPICZ α 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 μ g 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 13. Use the outgrowth protocol on page 15 to isolate transformants.
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Construction of *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>Bgl</i> II- <i>Bam</i> HI 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 25)
Recombinant vector rearranges and deletions are detected	Construct is unstable in <i>E. coli</i>	Decrease the number of cassettes in the vector.
No Zeocin™-resistant <i>Pichia</i> transformants	Integration efficiency is low	Transform using more DNA and/or do multiple transformations with more DNA and cells

continued on next page

Construction of *In Vitro* Multimers, continued

For More Information

There are a number references in the literature you can consult in order 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.

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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.

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

Using World Wide Web

Visit the [Invitrogen Web Resource](#) using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe™ Acrobat™ (PDF) format
- Explore our Catalog with full color graphics
- Obtain citations for Invitrogen products

Once connected to Internet, launch your web browser (Netscape 1.1 or newer), then enter the following location (or URL):

<http://www.invitrogen.com>

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

InvitroLink BBS Access

If you don't have Internet access, you may still obtain vector sequences from our electronic bulletin board system (BBS) using your modem and communications program. With your modem set at 14,400 baud or less, 8 bits, 1 stop bit, and parity set to none, dial 1-619-597-6230 to connect.

Electronic Help

If you have trouble accessing any of these services:

- In the U.S. and Canada, send an e-mail to tech_service@invitrogen.com or contact Invitrogen Technical Services at 1-800-955-6288, extension 2.
 - In Europe and Asia, send an e-mail to tech_service@invitrogen.nest.nl or contact our European Headquarters Technical Services at +31 (0) 594 515 175.
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