invitrogen™

pPICZα A, B, and C

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

Cat. no. V195-20

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User Manual

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Important Information

Contents

6 µg of each of pPICZα A, B, and C vector in TE buffer, pH 8.0* (40 µl at 150 ng/µl)

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

Shipping/Storage

The vectors are shipped on wet ice and should be stored at –20°C.

Reference Sources

The pPICZα A, B, and C vectors may be used with the Original *Pichia* Expression Kit (Cat. no. K1710-01) and are included in the EasySelect™ *Pichia* Expression Kit (Cat. no. K1740-01) available from Invitrogen. Additional general information about recombinant protein expression in *Pichia pastoris* is provided in the manuals for the Original *Pichia* Expression Kit and the EasySelect™ *Pichia* Expression Kit. The manuals can be downloaded from our Website (www.invitrogen.com) or obtained by calling Technical Support (see page 33). For more information about the Original *Pichia* Expression Kit or the EasySelect™ *Pichia* Expression Kit, refer to our Website or contact Technical Support.

More detailed information and protocols dealing with *Pichia pastoris* may also be found in the following general reference (see page vi for ordering information):

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)

Recommended *Pichia* Host Strain

We recommend using the X-33 *Pichia* strain as the host for expression of recombinant proteins from pPICZα . Other *Pichia* strains are suitable. The X-33 *Pichia* strain is available from Invitrogen (see page vi for ordering information) and has the following genotype and phenotype:

Genotype: Wild-type
Phenotype: Mut^+

Continued on next page

Important Information, continued

Materials Needed

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. See pages vi–vii for ordering information.

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
- Hemacytometer
- Microtiter plates (optional)

Reagents

- *Pichia* host strain (e.g. X-33, SMD1168H, KM71H)
- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation
- Restriction enzymes and appropriate buffers
- Agarose and low-melt agarose
- S.N.A.P.™ Gel Purification Kit 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)
- Zeocin™ selection agent (see page vi for ordering information)
- Low Salt LB plates containing 25 µg/ml Zeocin™ (see page 17 for recipe)
- YPDS plates containing the appropriate concentration of Zeocin™ (see page 18 for recipe)
- 50 ml conical centrifuge tubes
- 15 ml polypropylene tubes
- Optional: ProBond™ Purification System

Accessory Products

Introduction

The products listed in this section are intended for use with the pPICZα vectors. For more information, refer to www.invitrogen.com or call Technical Support (see page 33).

Obtaining Zeocin™

Zeocin™ may be obtained from Invitrogen. For your convenience, the drug is prepared in autoclaved, deionized water and available in 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.

Amount	Catalog no.
1 g	R250-01
5 g	R250-05

Accessory Products

Many reagents that may be used with the pPICZα vectors and for *Pichia* expression are available from Invitrogen. Ordering information is provided below.

Item	Amount	Cat. no.
X-33 *Pichia* strain	1 stab	C180-00
KM71H *Pichia* strain	1 stab	C182-00
SMD1168H *Pichia* strain	1 stab	C184-00
5′ AOX1 *Pichia* Primer	2 µg	N710-02
3′ AOX1 *Pichia* Primer	2 µg	N720-02
pPICZ A, B, and C	20 µg each	V190-20
pPIC6α A,B, and C	20 µg each	V215-20
pPIC6α Starter Kit	1 kit	K215-01
pPIC6 A, B, and C	20 µg each	V210-20
pPIC6 Starter Kit	1 kit	K210-01
Original *Pichia* Expression Kit	1 kit	K1710-01
EasySelect™ *Pichia* Expression Kit	1 kit	K1740-01
Pichia EasyComp™ Transformation Kit	1 kit	K1730-01
Pichia Protocols	1 book	G100-01
One Shot® TOP10 (chemically competent cells)	20 reactions	C4040-03
One Shot® TOP10 Electrocomp™ (electrocompetent cells)	20 reactions	C4040-52
Electrocomp™ TOP10 (electrocompetent cells)	20 reactions	C664-55

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Accessory Products, continued

Plasmid Preparation

Invitrogen offers a number of plasmid DNA purifications systems. For more information, refer to www.invitrogen.com or contact Technical Support (page 33)

Item	Amount	Cat. no.
PureLink™ HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100–02 K2100–03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps 50 preps	K2100–04 K2100–05
S.N.A.P.™ Miniprep Kit	100 reactions	K1900-01
S.N.A.P.™ Midiprep Kit	2 reactions	K1910-01
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25

Detecting Fusion Protein

A number of antibodies are available from Invitrogen to detect expression of your fusion protein from the pPICZα vector. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection in western blots using colorimetric or chemiluminescent detection methods. The amount of antibody supplied is sufficient for 25 Westerns.

Antibody	Epitope	Cat. no.
Anti-*myc*	Detects the 10 amino acid epitope derived from *c-myc* (Evans *et al.*, 1985): EQKLISEEDL	R950-25
Anti-*myc*-HRP		R951-25
Anti-His(C-term)	Detects the C-terminal polyhistidine (6×His) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25

Purifying Fusion Protein

The polyhistidine (6×His) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™. Ordering information for ProBond™ resin is provided below.

Item	Quantity	Cat. no.
ProBond™ Purification System	1 kit	K850-01
ProBond™ Purification System with Anti-*myc*-HRP Antibody	1 kit	K852-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns	50 polypropylene columns	R640-50

Introduction

Overview

Introduction

pPICZα 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 X-33, SMD1168H, and KM71H. pPICZα 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
- Zeocin™ resistance gene for selection in both *E. coli* and *Pichia* (Baron *et al.*, 1992; Drocourt *et al.*, 1990)
- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6×His) 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

Experimental Overview

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

Step	Action	Page
1	Propagate pPICZα A, B, and C by transformation into a *rec*A, *end*A1 *E. coli* strain such as TOP10, DH5α™ , or JM109.	2
2	Develop a cloning strategy and ligate your gene into one of the pPICZα vectors in frame with the α-factor secretion signal and the C-terminal tag.	3–7
3	Transform into *E. coli* and select transformants on Low Salt LB plates containing 25 µg/ml Zeocin™.	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 *Pichia pastoris*.	8–10
6	Transform your *Pichia* strain and plate onto YPDS plates containing the appropriate concentration of Zeocin™.	11
7	Select for Zeocin™-resistant transformants.	11–12
8	Optimize expression of your gene.	13–14
9	Purify your fusion protein on metal-chelating resin (i.e. ProBond™).	15–16

Methods

Cloning into pPICZα A, B, and C

Introduction

The pPICZα vector is supplied with the multiple cloning site in three reading frames (A, B, and C) to facilitate cloning your gene of interest in frame with the C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6×His) tag. Use the diagrams provided on pages 5–7 to help you design a strategy to clone your gene of interest in frame with the α-factor secretion signal and the C-terminal peptide. General considerations for cloning and transformation are discussed in this section.

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 pPICZα vectors including TOP10 (Cat. no. C610-00), JM109, and DH5α™ . We recommend that you propagate the pPICZα vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen. See page vi for ordering information.

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.

Maintenance of Plasmids

The pPICZα vectors contain the Zeocin™ resistance (*Sh ble*) gene to allow selection of the plasmid using Zeocin™. To propagate and maintain the pPICZα plasmids, we recommend using the following procedure:

1. Use the vector in the 0.5 µg/µl stock solution supplied with the kit to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent.
2. Select transformants on Low Salt LB plates containing 25 µg/ml Zeocin™ (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 pPICZα A, B, and C, continued

General Considerations

The following are some general points to consider when using pPICZα 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

pPICZα 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 pPICZα 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.

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.

Continued on next page

Cloning into pPICZα A, B, and C, continued

Expressing 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

pPICZα A, B, and C contain unique *Bgl*II and *Bam*HI sites to allow construction of plasmids containing multiple copies of your gene. For information on how to construct multimers, refer to pages 25–32.

Continued on next page

Cloning into pPICZα A, B, and C, continued

Multiple Cloning Site of pPICZα A

Below is the multiple cloning site for pPICZα 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 complete sequence of pPICZα A is available for downloading at www.invitrogen.com or from Technical Support (see page 33).** For a map and a description of the features of pPICZα, refer to pages 21–22.

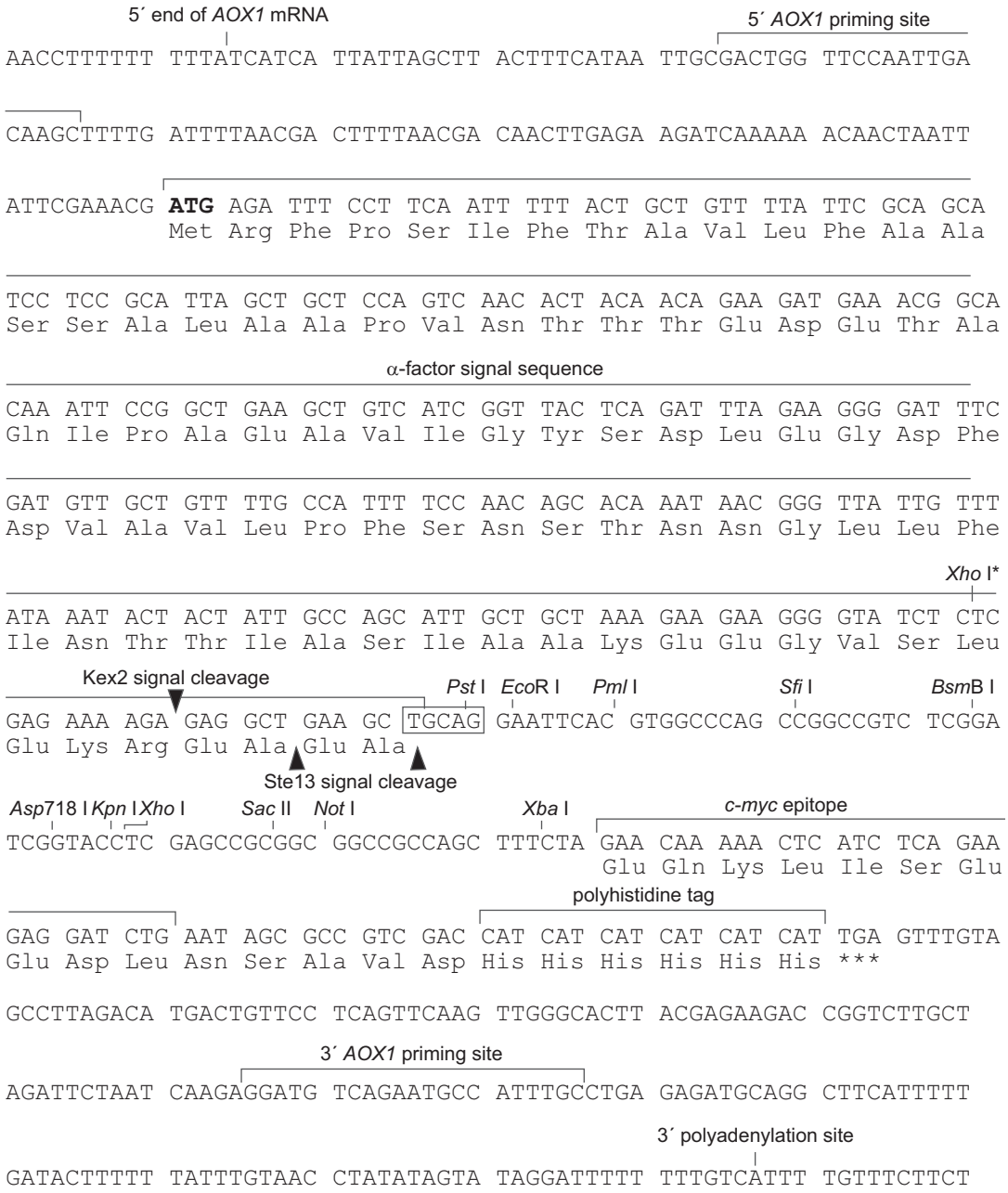
*To express your protein with a native N-terminus, you must use PCR and utilize the *Xho* I site upstream of the Kex2 cleavage site to clone your gene flush with the Kex2 cleavage site (see page 4 for more details).

Continued on next page

Cloning into pPICZα A, B, and C, continued

Multiple Cloning Site of pPICZα B

Below is the multiple cloning site for pPICZα 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 complete sequence of pPICZα B is available for downloading at www.invitrogen.com or from Technical Support (see page 33).** For a map and a description of the features of pPICZα, refer to pages 21–22.



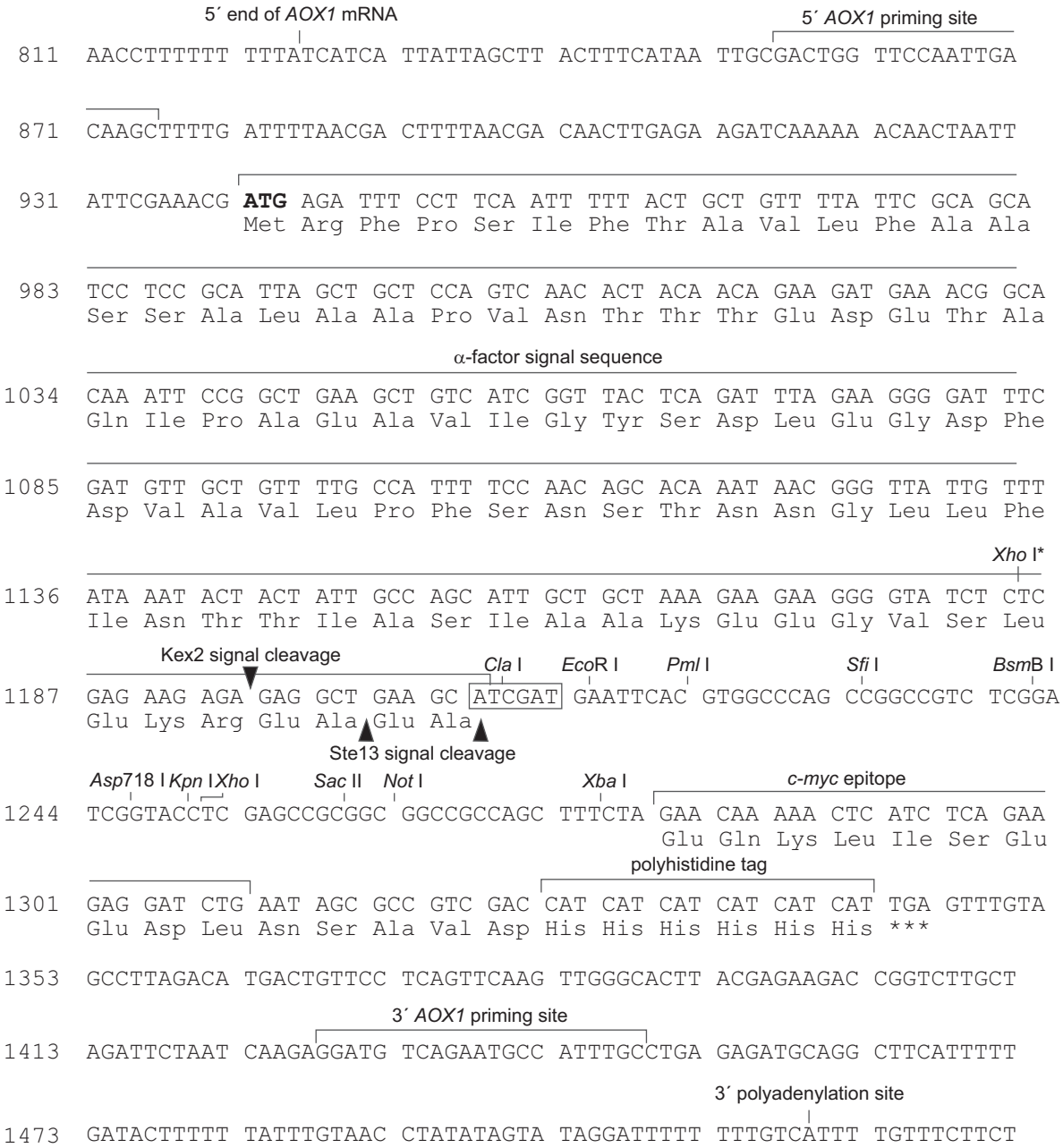
*To express your protein with a native N-terminus, you must use PCR and utilize the *Xho* I site upstream of the Kex2 cleavage site to clone your gene flush with the Kex2 cleavage site (see page 4 for more details).

Continued on next page

Cloning into pPICZα A, B, and C, continued

Multiple Cloning Site of pPICZα C

Below is the multiple cloning site for pPICZα 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 complete sequence of pPICZα C is available for downloading at www.invitrogen.com or from Technical Support (see page 33).** For a map and a description of the features of pPICZα, refer to pages 21–22.



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

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Cloning into pPICZα A, B, and C, continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10, DH5α™ , JM109) and select on Low Salt LB agar plates containing 25 µg/ml Zeocin™ (see below). Note that there is no blue/white screening for the presence of insert with pPICZα A, B, or C. Once you have obtained Zeocin™-resistant colonies, pick 10 transformants and screen for the presence and orientation of your insert.

Important

To facilitate selection of Zeocin™-resistant *E. coli*, 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 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. To facilitate sequencing, the 3′ *AOX1 Pichia* Primer (Cat. no. N720-02) and the 5′ *AOX1* Pichia Primer (Cat. no. N710-02) are available separately from Invitrogen. Refer to the diagrams on pages 5–7 for the sequences and location of the priming sites.

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 a Low Salt LB plate containing 25 µg/ml Zeocin™. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 ml of Low Salt LB containing 25 µg/ml Zeocin™.
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.

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 S.N.A.P.™ Mini- or Midiprep Kits, PureLink™ HiPure Plasmid Mini- or Midiprep Kits, or CsCl gradient centrifugation (see page vii for ordering information). 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 pPICZα 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 Zeocin™-resistant clones.

Zeocin™ Selection

We generally use 100 µg/ml Zeocin™ to select for transformants when using the X-33 *Pichia* strain. If you are transforming your pPICZα construct into another *Pichia* strain, note that selection conditions may vary. We recommend performing a dose response curve to determine the appropriate concentration of Zeocin™ to use for selection of transformants in your strain.

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 pPICZα 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 23 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 pPICZα A, B, or C, the *Pichia* EasyComp™ Transformation Kit is available from Invitrogen (see vii 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, refer to www.invitrogen.com or contact Technical Support (page 33).

Important

Since pPICZα 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 (i.e. KM71H).

Note

The pPICZα 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

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 pPICZα 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 Zeocin™ (see page 18 for recipe)

Linearizing Your pPICZα Construct

To promote integration, we recommend that you linearize your pPICZα construct within the 5′ AOX1 region. The table below lists unique sites that may be used to linearize pPICZα 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 pPICZα. Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Supplier
Sac I	209	Many
Pme I	414	New England Biolabs
*Bst*X I	707	Many

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.

   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

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 OD_{600} = 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, 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 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 pPICZα 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 Zeocin™.
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 Zeocin™.

Continued on next page

Pichia Transformation, continued

Note

Generally several hundred Zeocin™-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 ~20 150 mm plates with YPDS agar containing the appropriate concentration of Zeocin™.

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 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 the appropriate concentration of Zeocin™.
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, SMD1168H) as the host for your pPICZα construct, your Zeocin™-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 Zeocin™-resistant transformants, you may 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 (Higgins and Cregg, 1998).

You are now ready to test your transformants for expression of your gene of interest. 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 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.

Important

Note that once you have obtained Zeocin™-resistant transformants, it is not necessary to maintain your recombinant *Pichia* clone in medium containing Zeocin™ for expression studies. Zeocin™ 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-*myc* antibodies (see the next page) Anti-His(C-term) antibodies (see the next page)	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.

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 Novex® 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, refer to our website at www.invitrogen.com or call Technical Support (page 33).

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 vii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Cat. no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine (6×His) tag. WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our website at www.invitrogen.com or call Technical Support (page 33).

Important

Because the pPICZα 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 use X-33, SMD1168H, or KM71H 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 fusion proteins expressed from pPICZα A, B, or C (see page vii for ordering information). **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification System kit.**

If you are using a metal-chelating resin other than ProBond™, follow the manufacturer's recommendations to purify 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.

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 × 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 System) 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

The table below lists the pre-mixed media and media components available from Invitrogen specifically for *Pichia*. Please contact Technical Support (see page 33) for more information.

Item	Amount	Cat. no.
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 with Zeocin™

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 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 Zeocin™ to 25 µg/ml final concentration.

Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for up to 2 weeks.

YPD (+ Zeocin™)

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
± 2% agar
± appropriate concentration of 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. Add 100 ml of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of Zeocin™ from a 100 mg/ml stock solution.

Note: It is necessary to include Zeocin™ in the medium for selection of *Pichia* transformants only. Zeocin™ may be omitted from the medium when performing expression studies. Store YPD slants or plates containing Zeocin™ at 4°C. The shelf life is one to two weeks.

Continued on next page

Recipes, continued

YPDS + Zeocin™ 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 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 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of Zeocin™ from a 100 mg/ml stock solution.

Note: It is necessary to include Zeocin™ in the medium for selection of *Pichia* transformants only. Zeocin™ may be omitted from the medium when performing expression studies. Store YPDS slants or plates containing Zeocin™ at 4°C. The shelf life is one to two weeks.

Zeocin™

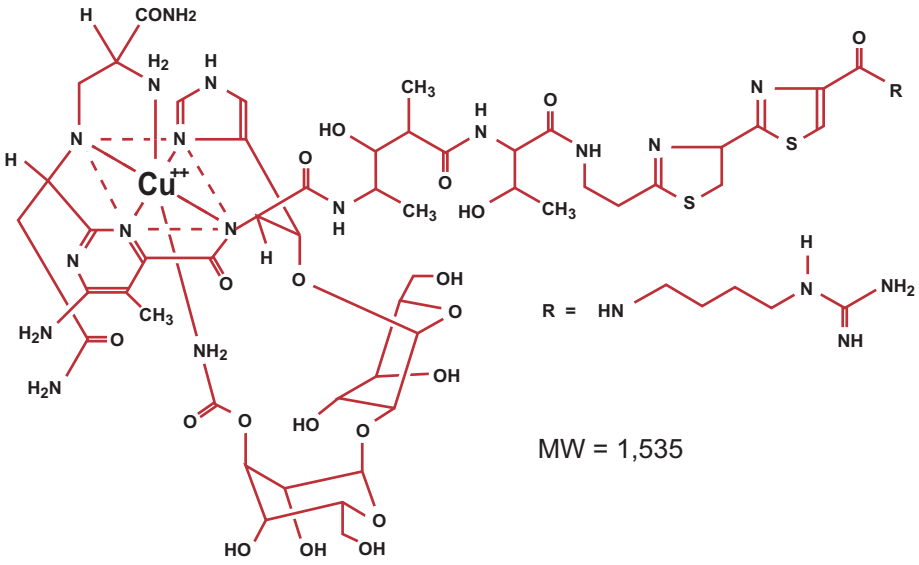
Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in *Pichia* and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/ml in **Low Salt LB** medium* (see page 17 for a recipe)
Pichia	100–1000 µg/ml (varies with strain and medium)

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

Continued on next page

Zeocin™, continued

Handling Zeocin™

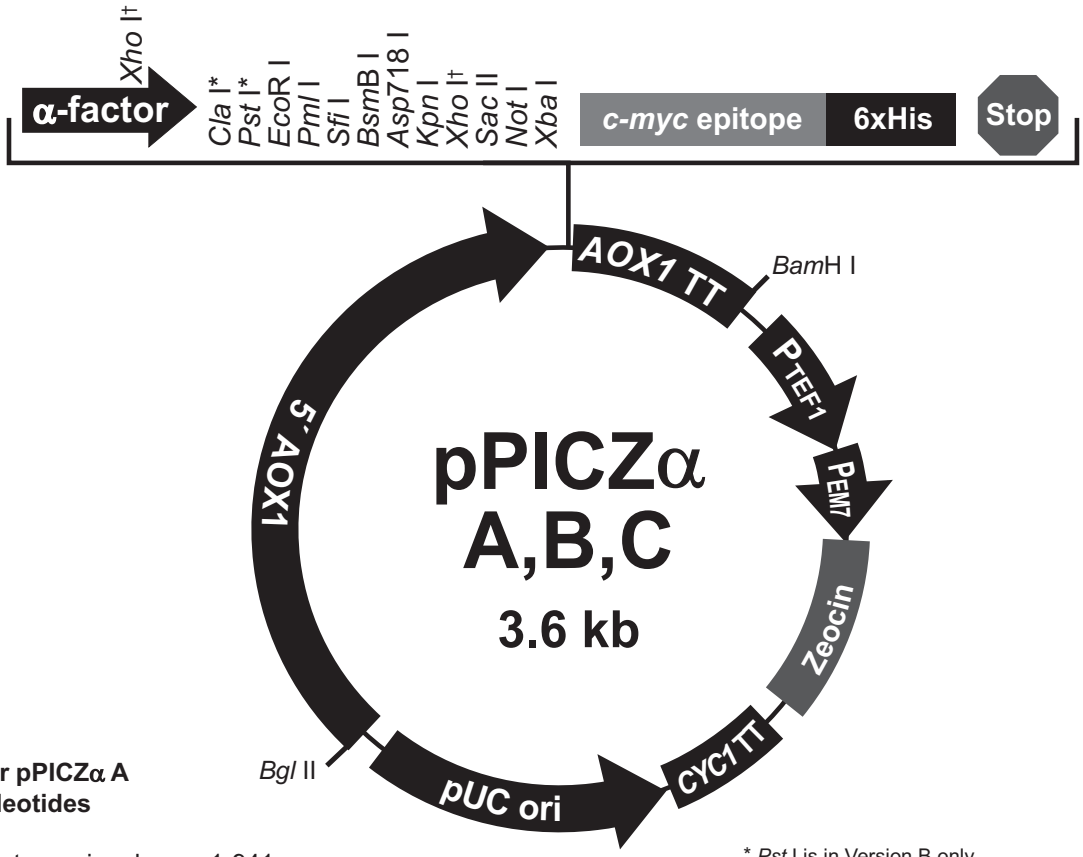
- High salt and acidity or basicity inactivate Zeocin™; therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see **Low Salt LB Medium**, page 17). Note that the salt concentration and pH do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates, and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Store tissue culture medium containing Zeocin™ at 4°C in the dark. Medium containing Zeocin™ is stable for 1–2 months.

pPICZα Vector

Map of pPICZα

The figure below summarizes the features of the pPICZα A, B, and C vectors. **The complete sequences of pPICZα A, B, and C are available for downloading at www.invitrogen.com or from Technical Support (see page 33).** See the next page for a description of the features of the vector.



* *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.

Comments for pPICZα A
3593 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
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 2095-2162
Sh ble ORF: bases 2163-2537
CYC1 transcription termination region: bases 2538-2855
pUC origin: bases 2866-3539 (complementary strand)

Continued on next page

pPICZα Vector, continued

Features of pPICZa A, B, and C

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

Feature	Benefit
5´ *AOX1* promoter	A 942 bp fragment containing the *AOX1* promoter that allows methanol-inducible, high-level expression of the gene of interest in *Pichia.* Targets plasmid integration to the *AOX1* locus.
α-factor secretion signal (from *Saccharomyces cerevisiae*)	Allows for efficient secretion of most proteins from *Pichia.*
Multiple cloning site	Allows insertion of your gene into the expression vector.
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Permits detection of your recombinant fusion protein with the Anti-*myc* Antibody or Anti-*myc*-HRP Antibody (Evans *et al.*, 1985). See page vii for ordering information.
C-terminal polyhistidine (6×His) tag	Permits 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) Antibody (Lindner *et al.*, 1997) and the Anti-His(C-term)-HRP Antibody. See page vii for ordering information.
AOX1 transcription termination (TT) region	Native transcription termination and polyadenylation signal from *AOX1* gene (~260 bp) that permits efficient 3´ mRNA processing, including polyadenylation, for increased mRNA stability.
TEF1 promoter (GenBank accession nos. D12478, D01130)	Transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives expression of the Zeocin™ resistance gene in *Pichia.*
EM7 promoter	Synthetic prokaryotic promoter that drives constitutive expression of the Zeocin™ resistance gene in *E. coli.*
Zeocin™ resistance gene (*Sh ble*)	Allows selection of transformants in *E. coli* and *Pichia.*
CYC1 transcription termination region (GenBank accession no. M34014)	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the Zeocin™ resistance gene for increased stability.
pUC origin	Allows replication and maintenance of the plasmid in *E. coli.*

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/µg 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 Buffer, pH 8.0.
(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 1500 × 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.

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 neither 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 order given** to the cells. PEG shields the cells from the detrimental effects of the high LiCl concentration.

   240 µl 50% PEG
   36 µl 1 M LiCl
   25 µl 2 mg/ml single-stranded DNA
   Plasmid DNA (5–10 µg) in 50 µl 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 cells at 6000 to 8000 rpm to pellet.
8. Resuspend the pellet in 1 ml of YPD and incubate at 30°C with shaking.

After 1 hour and 4 hours, plate 25 to 100 µl on YPD plates containing the appropriate concentration of Zeocin™. Incubate the plates for 2–3 days at 30°C.

Construction of *In Vitro* Multimers

Experimental Outline

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

Stage	Description
1	Digest pPICZα containing your gene of interest with *Bgl*II and *Bam*HI to release the expression cassette (P_{AOX1} plus your gene).
2	To clone multiple copies of the expression cassette, linearize pPICZα containing your gene of interest using *Bam*HI. Note that the *Bam*HI - linearized vector already contains one copy of your expression cassette.
3	Treat the *Bgl*II - *Bam*HI expression cassette with ligase *in vitro*. Note that *Bgl*II and *Bam*HI 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 *Bam*HI and *Bgl*II sites).
5	Treat the ligation mix with *Bam*HI and *Bgl*II to eliminate head-to-head and tail-to-tail multimers.
6	Ligate into *Bam*HI -linearized recombinant pPICZα .
7	Transform into *E. coli* and analyze recombinant plasmids for copy number by digesting with *Bgl*II and *Bam*HI.

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 pPICZα A, B, or C. For example:

Stage	Description
1	Digest pPICZα containing one copy of your gene with *Bam*HI
2	Ligate a single copy of the *Bgl*II - *Bam*HI expression cassette into *Bam*HI -digested vector
3	Transform *E. coli* 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 *Bam*HI and *Bgl*II to release a cassette with 2 copies of your gene (optional)
5	Digest the vector with 2 copies of your gene with *BamHI* and ligate 1 or 2 copies (see Step 4) of the expression cassette into the vector
6	Transform *E. coli* 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 the following materials on hand:

- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation. You will need 3–4 tubes of competent cells per experiment.
- *Bam*HI and *Bgl*II restriction enzymes and appropriate buffers
- Low-melt agarose
- S.N.A.P.™ Gel Purification Kit (see page vi) or glass milk
- 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 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.

Continued on next page

Construction of *In Vitro* Multimers, continued

Important

Once you have created a pPICZα 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 µg of DNA to compensate for the 10 to 100-fold drop in transformation efficiency. However, with selection on Zeocin™, any transformants you obtain will probably contain your 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 the appropriate concentration of Zeocin™. You may also use increasing concentrations of Zeocin™ to isolate multi-copy transformants. You will need to use the optional overgrowth step in the procedure on page 12.

Digesting Recombinant pPICZα

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

1. Double digest 1–2 µg of recombinant pPICZα 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 pPICZα in 20 µl with 10 units of *Bam*HI only.
3. Proceed to **Dephosphorylation of Vector**, Step 1.

Producing Expression Cassettes for Multimerization

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (see page vi) 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 *Bam*HI–*Bgl*II digest from Step1, 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 × g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl 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 µl of sterile water. Store on ice if proceeding immediately to **Ligation of Expression Cassette**, next page. Store at –20ºC for long-term storage.

Continued on next page

Construction of *In Vitro* Multimers, continued

Dephosphorylation of Vector

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

1. Take your *Bam*HI digest from **Digestion of Recombinant pPICZ*α***, Step 2, and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 µl of sterile water.
2. Set up a 20 µl dephosphorylation reaction in a microcentrifuge tube as follows:

*Bam*HI digested recombinant pPICZα (page 27, top, Step 2)	17 µl
10X CIAP Buffer	2 µl
CIAP (1 Unit/µl)	1 µl

3. Incubate at 37°C for 15 minutes.
4. Add 30 µl of sterile water to the reaction for a final volume of 50 µl.
5. Add 50 µl of phenol/chloroform and extract your DNA solution.
6. Precipitate the DNA by adding 5 µl of 3 M sodium acetate and 110 µl 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 µl sterile water. Save on ice if you plan to ligate your insert immediately (see **Ligation and Digestion of Expression Cassette,** next page) or store at –20°C.

Continued on next page

Construction of *In Vitro* Multimers, continued

Ligating and Digesting 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*HI and *Bgl*II sites between the expression cassettes. Digestion of the multimers with *Bam*HI 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 *BamHI*-linearized, recombinant pPICZα..

1. Set up a 20 µl ligation reactions as follows:

*Bgl*II–*Bam*HI digested expression cassette	15 µl
Sterile water	2 µl
10X Ligation Buffer (with ATP)	2 µl
T4 DNA Ligase (2.5 units/µl)	1 µl

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*HI and *Bgl*II may be used with the same reaction buffer:

Sterile water	23 µl
10X restriction enzyme buffer	5 µl
*Bgl*II (10 units/µl)	1 µl
*Bam*HI (10 units/µl)	1 µl

5. Incubate the reaction at 37°C for 2 hours.
6. Add 50 µl 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 µl of 3 M sodium acetate and 110 µl 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 µl 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,** next page.

Continued on next page

Construction of *In Vitro* Multimers, continued

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.

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 28, Step 10)	4 µl
Expression cassette multimers (Step 10, above)	4 µl
10X Ligation Buffer	1 µl
T4 DNA Ligase (2.5 units/µl)	1 µl
Total volume	10 µl

For the vector only control:

Dephosphorylated vector	4 µl
Sterile water	4 µl
10X Ligation Buffer	1 µl
T4 DNA Ligase (2.5 units/µl)	1 µl
Total volume	10 µl

2. Incubate overnight at 16°C.
3. You may store the ligation reactions at –20°C until ready to use, or transform 1–10 µl 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 using the ligation mixture from the previous page.
2. After adding medium to the transformed cells and allowing them to recover, plate 10 µl and 100 µl of each transformation mix onto Low Salt LB plates containing 25 µg/ml Zeocin™. 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-Zeocin™ plates.

Analyzing Transformants

1. Pick 20 transformants and inoculate each colony into 2 ml Low Salt LB containing 25 µg/ml Zeocin™. Grow overnight at 37°C.
2. Isolate plasmid DNA and digest with *Bgl*II and *Bam*HI to release any multimers from pPICZα.

   (Be sure to include *BglII–BamHI* digested pPICZα as a control. It is possible to get vector rearrangements and deletions with large recombinant vectors in *E. coli*. Including *BglII–BamHI* digested pPICZα 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 optional overgrowth step in the procedure on page 12.

Continued on next page

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 *E. coli*	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 *Bam*HI–*Bgl*II expression cassette to your ligation.
	Construct is unstable in *E. coli*	Decrease the number of cassettes in the vector.
	Multimers are too long to ligate efficiently	Try ligating each expression cassette stepwise (see page 29).
Recombinant vector rearranges and deletions are detected	Construct is unstable in *E. coli*	Decrease the number of cassettes in the vector.
No Zeocin™-resistant *Pichia* 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 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.

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.

Technical Support

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