invitrogen™
by life technologies™

pGAPZ A, B, and C
pGAPZα A, B, and C

Pichia expression vectors for constitutive expression and purification of recombinant proteins

Catalog nos. V200–20 and V205–20

Rev. Date: 28 June 2010
Manual part no. 25-0174

MAN0000043

User Manual

Table of Contents

Kit Contents and Storage

Types of Manuals

This manual is included with the following catalog numbers:

Product	Catalog no.
pGAPZ A, B, and C	V200–20
pGAPZα A, B, and C	V205–20

Shipping/Storage

The vectors included with Catalog nos. V200–20 and V205–20 are shipped on wet ice.

Upon receipt, **store vectors at –20°C.**
Store Stabs at 4°C.

For long-term storage of your positive control stab strain, we recommend preparing a glycerol stock (page 15) immediately upon receipt and storing at –80°C.

Component	Shipping	Storage
pPICZ A, B, and C Expression Vectors	Wet ice	Store at –20°C
pPICZα A, B, and C Expression Vectors	Wet ice	Store at –20°C
X-33/pGAPZ B/*lac*Z Positive Control Strain	Wet ice	Store at 4°C

Kit Contents

The following components are included with Catalog nos. V200–20 and V205–20. Note that the vectors are supplied in suspension.

Catalog no.	Component	Quantity	Composition
V200-20	pGAPZ A Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
	pGAPZ B Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
	pGAPZ C Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
	X-33/pGAPZ B/*lac*Z Positive Control Strain	1 Stab	– –
V205-20	pGAPZα A Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
	pGAPZα B Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
	pGAPZα C Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0

Important

Pichia users should already have a *Pichia* Expression System manual. Procedures for analysis of recombinants are described in the *Pichia* manual. For more information, contact **Technical Support** (page 34).

Accessory Products

Additional Products

The following products are available separately from Invitrogen. For more information, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 34).

Item	Quantity	Catalog no.
Pichia Expression Kit	1 kit	K1710–01
EasyComp™ Kit	1 kit	K1730–01
EasySelect™ *Pichia* Expression Kit	1 kit	K1740–01
Multi-Copy *Pichia* Expression Kit	1 kit	K1750–01
pPICZ A, B, and C	20 µg each	V190–20
pPICZα A, B, and C	20 µg each	V195–20
One Shot® TOP10F′	21 × 50 µl (1.0 ml total)	C3030–03
TOP10F´ Ultracomp™	5 × 300 µl (1.5 ml total)	C665–03
TOP10 Electrocomp™ Kits	20 reactions	C664–55
ProBond™ Purification System	6 purification	K850-01
S.N.A.P.™ Miniprep DNA Isolation Kit	100 reactions	K1900-01
PureLink™ Quick Plasmid Miniprep Kit	50 reactions 250 reactions	K2100-10 K2100-11
PureLink™ Gel Extraction Kit	50 preps 250 preps	K2100–12 K2100–25
Zeocin™	1 g 5 g	R250-01 R250-05

Introduction

Overview

Introduction

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme is **constitutively** expressed at high levels in many organisms, including *Pichia pastoris*. The promoter of the gene (*GAP*) encoding the GAPDH protein has recently been characterized and shown to express recombinant proteins to high levels in *Pichia pastoris*, depending on the carbon source used (Waterham *et al*., 1997). The level of expression seen with the *GAP* promoter (P_{GAP}) can be slightly higher than that obtained with the *AOX1* promoter.

The pGAPZ A, B, and C vectors (2.9 kb) and pGAPZα A, B, and C (3.1 kb) vectors use the *GAP* promoter to constitutively express recombinant proteins in *Pichia pastoris*. Proteins can be expressed as fusions to a C-terminal peptide containing the *myc* epitope for detection and a polyhistidine tag for purification on metal-chelating resin (*i.e*. ProBond™). In addition, pGAPZα produces proteins fused to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. Both vectors are supplied in three reading frames to facilitate in frame cloning with the C-terminal tag and/or 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*.

Pichia Strains

The following *Pichia pastoris* strains can be used with the pGAPZ and pGAPZα vectors:

***Pichia* Strain**	**Genotype**	**Purpose**
X-33	wild type	Expression of recombinant proteins from vectors with Zeocin™ resistance as the only selectable marker (*i.e*. pGAPZ and pGAPZα).
GS115	*his4*	Expression of recombinant proteins from vectors containing the *HIS4* or Zeocin™ resistance gene as the selectable marker(s).
KM71	*his4*, *aox1*:*ARG4*	Expression of recombinant proteins from vectors containing the *HIS4* or Zeocin™ resistance gene as the selectable marker(s) in a Mut^S background when using methanol induction for expression.
SMD1168	*his4*, *pepA*	Protease deficient strain for the expression of recombinant proteins from vectors containing the *HIS4* or Zeocin™ resistance gene as the selectable marker(s).

Continued on next page

Experimental Process

The following table describes the overall experimental process.

Step	Action	Page
1	Propagate pGAPZ A, B, and C and/or pGAPZα A, B, and C by transformation into a *rec*A, *end*A1 *E. coli* strain such as TOP10F´, DH5 , or JM109.	6
2	Develop a cloning strategy to ligate your gene into one of the pGAPZα or pGAPZ vectors in frame with the α-factor secretion signal and/or the C-terminal tag.	5–13
3	Transform into *E. coli* and select transformants on low salt LB plates containing 25 µg/ml Zeocin™.	14
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/or the C-terminal tag.	15
5	Purify and linearize the recombinant plasmid for transformation into *Pichia pastoris*.	15
6	Transform your *Pichia* strain and plate onto YPDS plates containing 100 µg/ml Zeocin™.	17
7	Select Zeocin™-resistant transformants.	18
8	Test for expression of the gene of interest.	19
9	Optimize expression of your gene.	21
10	Purify your fusion protein on metal-chelating resin (i.e. ProBond™).	22

ProBond™ Resin

We recommend that you use the ProBond™ Purification System (page vi) for purifying fusion proteins expressed using the pGAPZ or pGAPZα vectors. **Please 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™, please follow the manufacturer's recommendations for fusion proteins expressed in yeast.

Recombination and Integration in *Pichia*

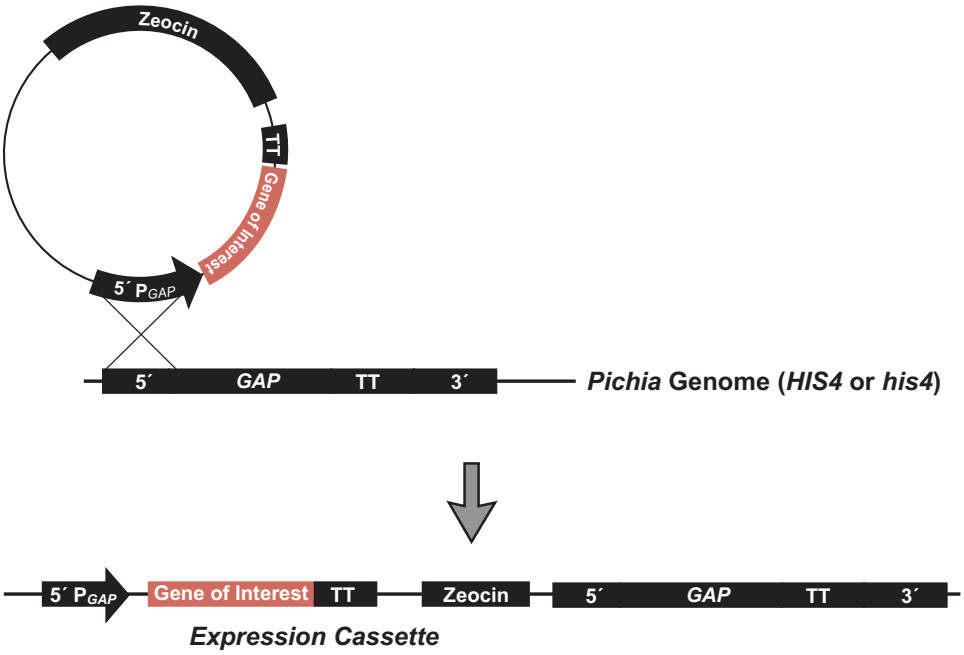
Introduction

Linear DNA can generate stable transformants of *Pichia pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome (Cregg *et al.*, 1985; Cregg *et al.*, 1989). Such integrants show extreme stability in the absence of selective pressure even when present as multiple copies. Note that single crossover events (insertions) are much more likely to happen than double crossover events (replacements). Multiple insertion events occur spontaneously at about 1-10% of the single insertion events.

Gene Insertion at the *GAP* Promoter

Gene insertion events at the *GAP* promoter locus arise from a single crossover event between the locus and the P_{GAP} region on the pGAPZ or pGAPZα vectors. This results in the insertion of one or more copies of the vector upstream or downstream of the *GAP* locus.

The figure below shows the result of an insertion of the plasmid 5´ to the intact *GAP* promoter locus and the gain of P_{GAP}, your gene of interest, and Zeocin™ resistance. This also occurs with non-linearized plasmid and plasmid that religates, although at a lower frequency.



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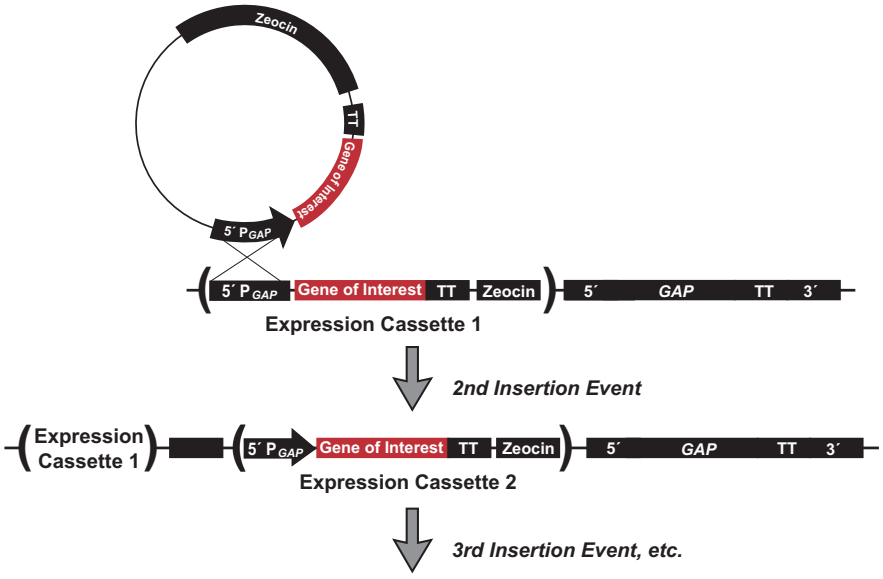
Multiple Gene Insertion Events

Multiple gene insertion events at a single locus in a cell do occur spontaneously with a low, but detectable frequency--between 1 and 10% of all selected Zeo^R transformants. Because of the low frequency of multiple gene insertion events, you will need to screen hundreds to thousands of Zeocin^{TM}-resistant transformants to locate these "jack-pot" clones. We recommend that you use electroporation to generate Zeo^R transformants for screening.

Please note that multiple gene insertion with the pGAPZ and pGAPZα has not been functionally tested by Invitrogen. However, since the backbone of these vectors are identical to pPICZ and pPICZ , a multiple copy event can occur at the *GAP* promoter region locus. Multiple gene insertion events can be detected by quantitative dot blot analysis, Southern blot analysis, and differential hybridization. Please see a *Pichia* Expression Manual for a protocol to screen for multiple inserts.



Methods

General Cloning Considerations

Introduction

Before cloning your gene into one of the pGAPZ or pGAPZα vectors, please consider some of the general guidelines presented below. The multiple cloning sites for pGAPZ A, B, and C are located on pages 8–9 and the multiple cloning sites for pGAPZα A, B, and C are presented on pages 11–13 to help you develop a cloning strategy.

General Considerations

The following are some general considerations applicable to pGAPZ A, B, and C and pGAPZα 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 *rec*A, *end*A *E. coli* strain such as TOP10F´.
- 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 premature termination of transcripts because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff & Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret & 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).

Special Considerations for pGAPZ

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

For expression of a fusion protein containing the myc epitope and the polyhistidine tag, the open reading frame (ORF) of your gene must be cloned in frame with the C-terminal peptide.

Special Considerations for pGAPZα

- The initiation ATG in the α-factor signal sequence corresponds to the native initiation ATG of the *GAP* 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).
- The predicted protease cleavage sites for the α-factor signal sequence are indicated in the figures on pages 11–13.

Continued on next page

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

We recommend that you transform pGAPZ A, B, and C and pGAPZα A, B, and C into a *rec*A, *end*A *E. coli* strain (e.g. TOP10F´) so that you have a permanent stock and are able to propagate the plasmids. Electrocompetent TOP10F´ cells are available from Invitrogen (page vi).

Propagation of pGAPZ and pGAPZα

It is recommended that pGAPZ and pGAPZα be transformed into an *E. coli* cell line for maintenance of the vector.

To propagate the pGAPZ and pGAPZα plasmids:

1. We recommend using 10 ng of the vector to transform competent *E. coli* and select on Low Salt LB with 25 µg/ ml Zeocin™ (see recipe on page 27).
2. Isolate a single colony containing the plasmid.
3. Culture in 5 ml Low Salt LB with 25 µg/ml Zeocin™. Grow overnight at 37°C.
4. Mix thoroughly 0.85 ml of culture with 0.15 ml sterile glycerol.
5. Transfer to a freezer vial and freeze in liquid nitrogen or a dry ice/ethanol bath.
6. Store at –80°C.

Important

For optimal activity of Zeocin™, 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 27.

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

Cloning Procedures

Detailed maps of the multiple cloning sites are provided on the following pages. Please refer to Ausubel, *et al.*, 1994, pages 3.16.1 to 3.17.3. or Sambrook, *et al.*, 1989, pages 5.10 to 5.13. for general help with cloning.

Materials Needed

For the procedures described in this manual, you will need the following reagents and equipment. Additional reagents may be required.

Equipment

- *Pichia* Expression Manual
- Microbiological equipment
- Electroporation device and 0.2 cm cuvettes or reagents for LiCl 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 (X-33, GS115, KM71, or SMD1168)
- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation
- Restriction enzymes and appropriate buffers
- Sequencing primers to confirm proper insertion of your gene of interest
- Agarose and low-melt agarose
- 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

Cloning into pGAPZ

Multiple Cloning Site of pGAPZ A

Below is the multiple cloning site of pGAPZ A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Please note that the *Apa* I site is unique to pGAPZ A. The multiple cloning site has been confirmed by sequencing and functional testing.

```
371  AACCACCAGA ATCGAATATA AAAGGCGAAC ACCTTTCCCA ATTTTGGTTT CTCCTGACCC

                                        pGAP forward priming site      Sfu I
431  AAAGACTTTA AATTTAATTT ATTTGTCCCT ATTTCAATCA ATTGAACAAC TATTTCGAAA

       EcoR I     Pml I          Sfl I              Asp718 I  Kpn I Xho I      Sac II  Not I
491  CGAGGAATTC ACGTGGCCCA GCCGGCCGTC TCGGATCGGT ACCTCGAGCC GCGGCGGCCG

                  Apa I                    myc epitope
551  CCAGCTTGGG CCC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC
                    Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala

                Polyhistidine tag
603  GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGCCT TAGACATGAC TGTTCCTCAG
     Val Asp His His His His His His ***

660  TTCAAGTTGG GCACTTACGA GAAGACCGGT CTTGCTAGAT TCTAATCAAG AGGATGTCAG

     3´ AOX1 priming site
720  AATGCCATTT  GCCTGAGAGA TGCAGGCTTC ATTTTTGATA CTTTTTTATT TGTAACCTAT

780  ATAGTATAGG ATTTTTTTTG TCATTTTGTT TCTTCTCGTAC
```

Multiple Cloning Site of pGAPZ B

Below is the multiple cloning site of pGAPZ B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Please note that the *Xba* I site is unique to pGAPZ B. The multiple cloning site has been confirmed by sequencing and functional testing.

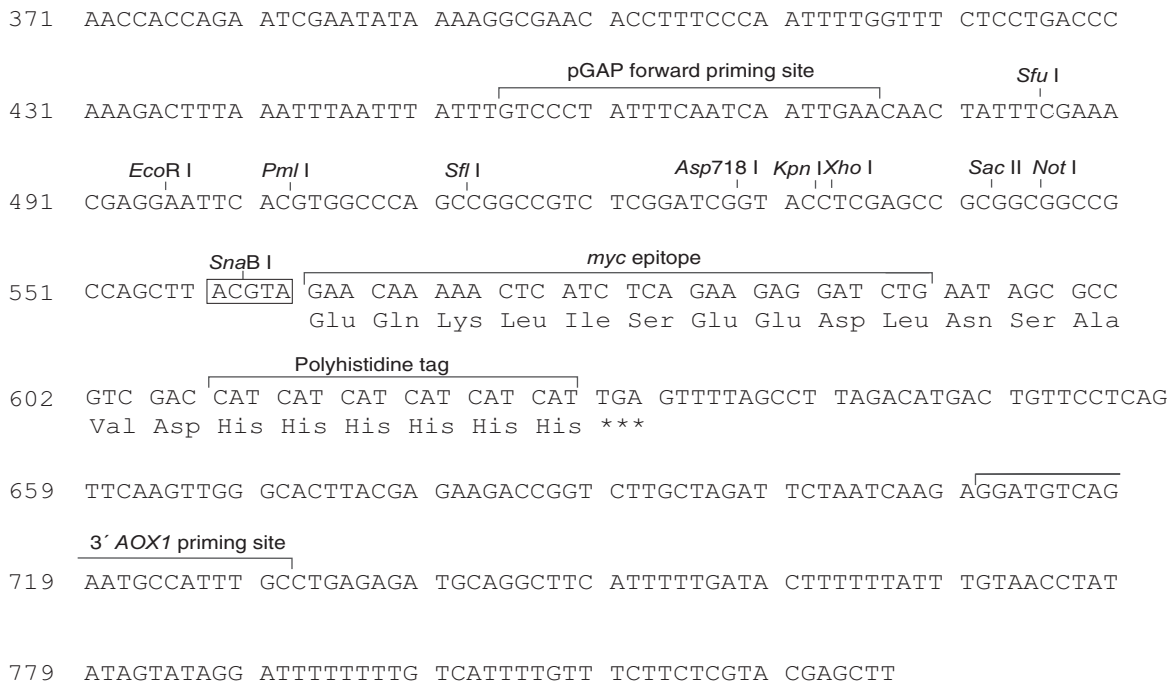
```
371  AACCACCAGA ATCGAATATA AAAGGCGAAC ACCTTTCCCA ATTTTGGTTT CTCCTGACCC

                                        pGAP forward priming site      Sfu I
431  AAAGACTTTA AATTTAATTT ATTTGTCCCT ATTTCAATCA ATTGAACAAC TATTTCGAAA

       EcoR I     Pml I          Sfl I              Asp718 I  Kpn I Xho I      Sac II  Not I
491  CGAGGAATTC ACGTGGCCCA GCCGGCCGTC TCGGATCGGT ACCTCGAGCC GCGGCGGCCG

                Xba I                    myc epitope
551  CCAGCTT TCTA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC
                  Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala

                Polyhistidine tag
601  GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGCCT TAGACATGAC TGTTCCTCAG
     Val Asp His His His His His His ***

658  TTCAAGTTGG GCACTTACGA GAAGACCGGT CTTGCTAGAT TCTAATCAAG AGGATGTCAG

     3´ AOX1 priming site
718   AATGCCATTT GCCTGAGAGA TGCAGGCTTC ATTTTTGATA CTTTTTTATT TGTAACCTAT

778  ATAGTATAGG ATTTTTTTTG TCATTTTGTT TCTTCTCGTA CGAGCTTG
```

Continued on next page

Cloning into pGAPZ, Continued

Multiple Cloning Site of pGAPZ C

Below is the multiple cloning site of pGAPZ C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Please note that the *Sna*B I site is unique to pGAPZ C. The multiple cloning site has been confirmed by sequencing and functional testing.



Cloning into pGAPZα

Introduction

The pGAPZα vectors possess the α-factor mating signal sequence for secretion of your protein. The information in this section is provided to assist you in designing a cloning strategy. Details of the multiple cloning sites of the pGAPZα A, B, and C vectors can be found on the following pages.

Cloning Considerations

The variable region of pGAPZα A, B, and C is located between the α-factor signal sequence and the multiple cloning site. It is designed to facilitate cloning into the vector **in frame with the signal sequence**. Note that if you wish to create a fusion protein with the C-terminal tag, you must consider the 3´ cloning sequence as well. If your insert does not conveniently ligate in frame with both terminal fusion partners, it may be necessary to use PCR to create ends that will result in in-frame fusions. Please consider both the frame of the signal sequence and the C-terminal fusion when designing a cloning strategy.

Signal Sequence Processing

The processing of the α-factor mating signal sequence in pGAPZα 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 × 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 736–741 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 745–747. Remember to include the first amino acid(s) of your protein, if necessary, for correct fusion to the Kex2 cleavage site.

Continued on next page

Cloning into pGAPZα, Continued

Multiple Cloning Site of pGAPZα A

Below is the multiple cloning site of pGAPZα A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. The multiple cloning site has been confirmed by sequencing and functional testing.

```
361  GATTATTGGA AACCACCAGA ATCGAATATA AAAGGCGAAC ACCTTTCCCA ATTTTGGTTT
                                                  pGAP forward priming site
421  CTCCTGACCC AAAGACTTTA AATTTAATTT ATTTGTCCCT ATTTCAATCA ATTGAACAAC

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

532  GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
     Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr
                                  α-factor signal sequence
583  GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT
     Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp

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

685  TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT
     Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser
     Xho I*        Kex2 signal cleavage          EcoR I    Pml I        Sfi I
736  CTC GAG AAA AGA GAG GCT GAA GC[T] GAATTCAC GTGGCCCA GCCGGCCGTC TCGGATC
     Leu Glu Lys Arg Glu Ala▲Glu Ala▲
                          Ste13 signal cleavage
   Asp718 I Kpn I Xho I    Sac II  Not I          Xba I          myc epitope
793  GGTACCTCGA GCCGCGGCGG CCGCCAGCTT TCTA GAA CAA AAA CTC ATC TCA GAA GAG
                                           Glu Gln Lys Leu Ile Ser Glu Glu
                                    polyhistidine tag
851  GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGC CTTA
     Asp Leu Asn Ser Ala Val Asp His His His His His His ***

905  GACATGACTG TTCCTCAGTT CAAGTTGGGC ACTTACGAGA AGACCGGTCT TGCTAGATTC TAAT
                3´ AOX1 priming site
969  CAAGAGGATG TCAGAATGCC ATTTGCCTGA GAGATGCAGG CTTCATTTTT GATACTTTTT TATT

1033 TGTAACCTAT ATAGTATAGG ATTTTTTTTG TCATTTTGTT TCTTCTCG
```

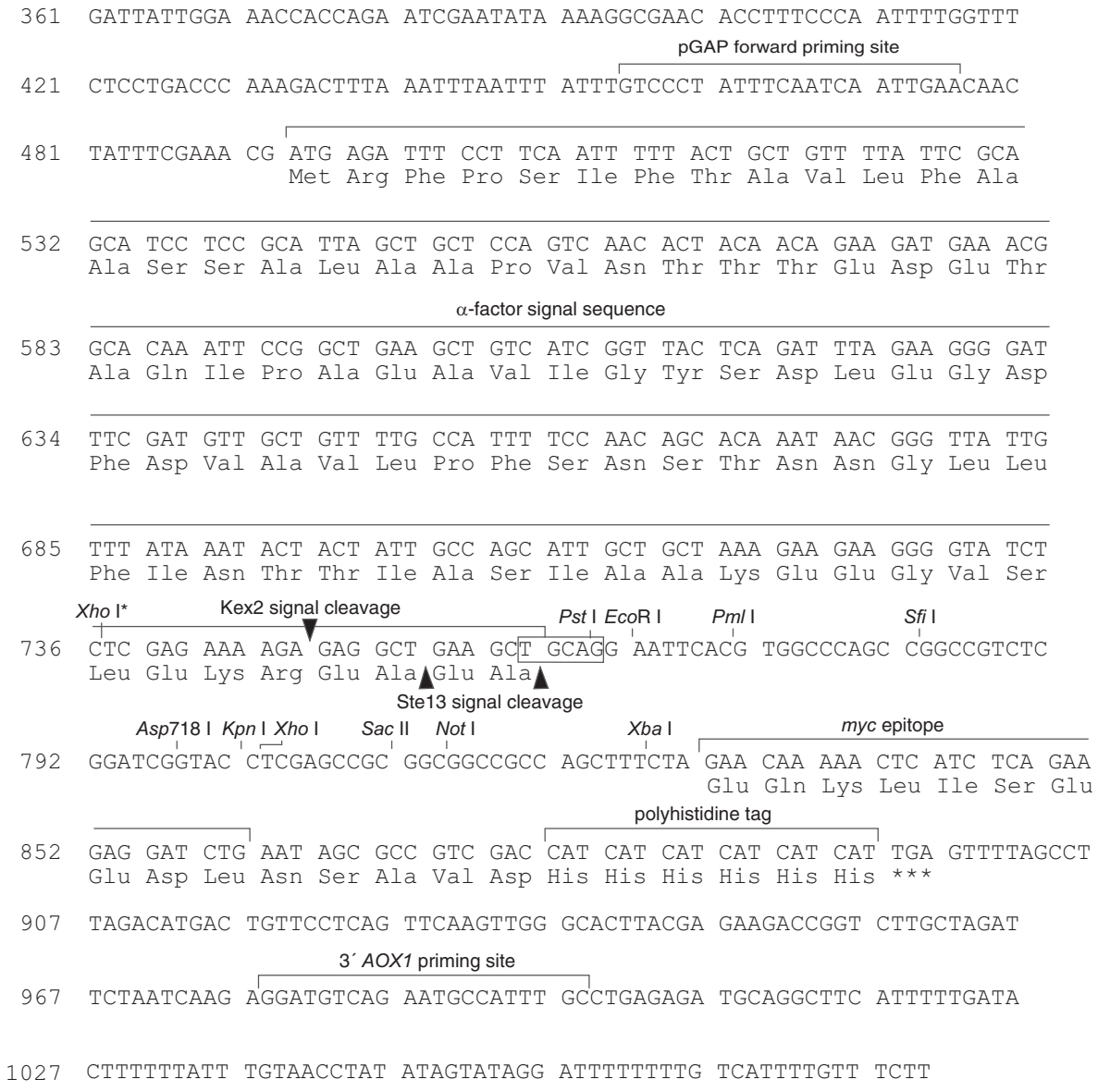
* The *Xho* I site upstream of the Kex2 cleavage site is used to clone the gene of interest flush with the Kex2 cleavage site (see page 14).

Continued on next page

Cloning into pGAPZα, Continued

Multiple Cloning Site of pGAPZα B

Below is the multiple cloning site of pGAPZα B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Please not that the *Pst* I site is unique to pGAPZα B. The multiple cloning site has been confirmed by sequencing and functional testing.



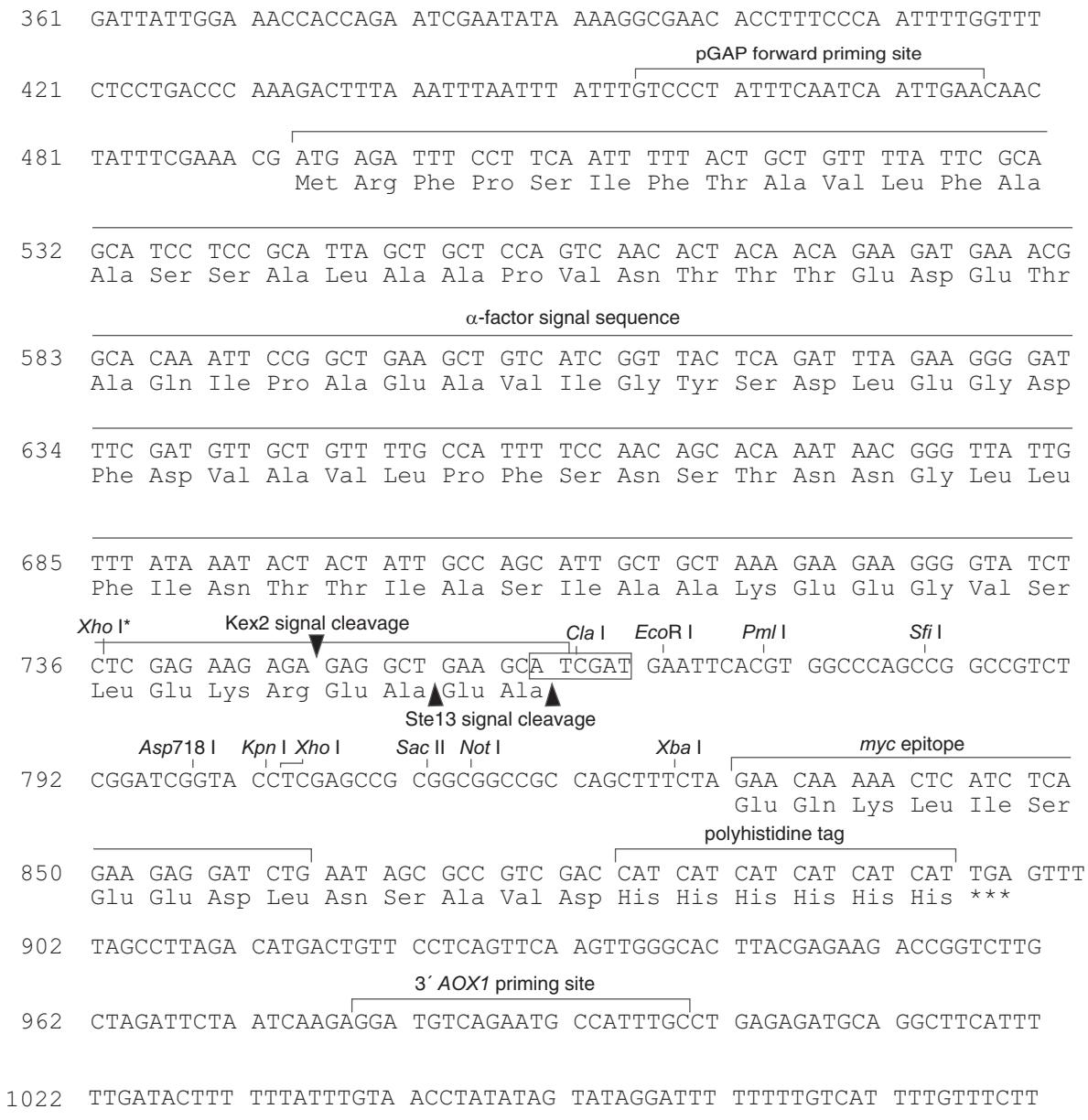
* The *Xho* I site upstream of the Kex2 cleavage site is used to clone the gene of interest flush with the Kex2 cleavage site (see page 14).

Continued on next page

Cloning into pGAPZα, Continued

Multiple Cloning Site of pGAPZα C

Below is the multiple cloning site of pGAPZα C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Please not that the *Cla*I site is unique to pGAPZα C. The multiple cloning site has been confirmed by sequencing and functional testing.



* 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 14).

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 pGAPZ or pGAPZα. After obtaining the desired recombinant plasmid, transform the linearized construct into *Pichia* as described on page 17.

Note

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 27.

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

E. coli 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** (page 27) 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

Analysis of Transformants

1. After transformation, plate 10 µl and 100 µl of the transformation mix onto Low Salt LB plates with 25 µg/ml Zeocin™ (see **Recipe,** page 27) and select Zeocin™-resistant colonies.
2. Pick 10 Zeocin™-resistant transformants and inoculate each colony into 2 ml Low Salt LB medium with 25 µg/ml Zeocin™. Grow overnight at 37°C with shaking.
3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below).

Preparing a Glycerol Stock

Be sure to make a glycerol stock of your purified clone for safe long-term storage.

1. Purify the clone by streaking for single colonies
2. Prepare an overnight bacterial culture from a single colony
3. Combine 0.85 ml of the overnight bacterial culture with 0.15 ml of sterile glycerol
4. Mix by vortexing and transfer to a labeled storage tube
5. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath
6. 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/or the C-terminal tag. To sequence your construct in pGAPZ or pGAPZα, we recommend using the pGAP Forward and the 3´ *AOX1* primer sequences. Please note that these primers are not available separately from Invitrogen. If you would like information on placing custom primer orders, visit our Web site (www.invitrogen.com) and select Custom Primers.

Sequencing Primer	Sequence
pGAP Forward	5´-GTCCCTATTTCAATCAATTGAA-3´
3´ *AOX1*	5´-GCAAATGGCATTCTGACATCC-3´

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 µg of each plasmid per each transformation). We recommend the S.N.A.P.™ Miniprep Kit or the PureLink™ Quick Plasmid Miniprep Kit (see page vi to order) for quick purification of pure plasmid DNA. Once you have purified plasmid DNA, proceed to ***Pichia* Transformation**, next page.

Pichia Transformation

Introduction

At this point, you should have your gene cloned into one of the pGAPZ or pGAPZα vectors. Your construct should be correctly fused to the α-factor secretion signal and/or 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 µg/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. Plating spheroplasts directly onto selective medium containing Zeocin™ will result in complete cell death.

We recommend electroporation for transformation of *Pichia* with the pGAPZ or pGAPZα vectors. 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 33 or the EasyComp™ Kit (Catalog no. K1730-01).

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.

1. 5–10 µg pure pGAPZ or pGAPZα containing your insert
2. YPD Medium
3. 50 ml conical polypropylene tubes
4. 1 liter cold (4°C) sterile water (place on ice the day of the experiment)
5. 25 ml cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
6. 30°C incubator
7. Electroporation device and 0.2 cm cuvettes
8. YPDS plates containing 100 µg/ml Zeocin™ (see page 26 for recipe)

Restriction Digest

1. Digest ~5-10 µg of plasmid DNA with either *Avr* II (191 bp) or *Bsp* HI (356 bp). Each enzyme cuts once in the *GAP* promoter region to linearize the vector. Choose the enzyme that does not cut within your gene.
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 your *Pichia pastoris* strain in 5 ml of 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 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, then resuspend the pellet with 250 ml of ice-cold (0°C), sterile water.
5. Centrifuge the cells, then resuspend the pellet in 20 ml of ice-cold (0°C) 1 M sorbitol.
6. Centrifuge the cells, 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.
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 1–2 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 2–3 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 2–4 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 presence of insert using PCR, or for copy number using Southern analysis. Please refer to a *Pichia* Expression Kit manual for these protocols.

If you choose to analyze for the presence of insert using PCR, please note that the parent plasmids will produce the following sized PCR products when using the pGAP Forward and the 3´ *AOX1* primer sequences:

Vector	PCR Product
pGAPZ	~275 bp
pGAPZα	~540 bp

Remember to add the size of these fragments to the size of your insert to interpret your PCR results.

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

Isolation of Multi-copy Recombinants *in vivo*

A quick, direct way to select putative multi-copy recombinants is to plate the transformation mix on increasing concentrations of Zeocin™. Please note that multiple gene insertion with the pGAPZ and pGAPZα has not been functionally tested by Invitrogen.

- Prepare YPDS plates containing 500, 1000, and 2000 µg/ml Zeocin™.
- Plate 100–200 µl of the transformation mix on each plate and incubate at 30°C for 2 days.

It is strongly recommended that you purify Zeocin™ resistant transformants at least once. The following steps for expression do not utilize Zeocin™ selection and any mixed colony transformants may lose the expressing clone.

- Streak out the chosen Zeocin™-resistant *Pichia* transformant on YPD plates containing Zeocin™.
- Grow for 2–4 days at 30°C until single colonies form.

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 (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 detection method.

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

Expression Guidelines

Expression can be done in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or Yeast Nitrogen Base with 0.5% glucose.

1. Using a single colony, inoculate 10 ml of YPD. Grow at 28–30°C in a shaking incubator (250–300 rpm) overnight.
2. Use 0.1 ml of the overnight culture to inoculate 50 ml of YPD in a 250 ml flask. Grow at 28–30°C in a shaking incubator (250–300 rpm).
3. At each of the times indicated below, transfer 1 ml of the expression culture into a 1.5-ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.

   Time points (hours): 0,24 (1 day), 48 (2 days), 72 (3 days) 96 (4 days)
4. For secreted expression, **transfer the supernatant to a separate tube.** Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/ethanol bath.
5. For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid nitrogen or a dry ice/ethanol bath.

Continued on next page

Detection of Recombinant Proteins in *Pichia*

We recommend that you use the following techniques to analyze the expression of your protein. **Analyze BOTH the cells and the medium for the presence of your recombinant protein.** Please note that the α-factor signal sequence (in pGAPZα) 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 (in pGAPZα) 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* Antibody Anti-His(C-term) Antibody	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.

Scale-up of Expression

You may want to scale-up your expression protocol to produce more protein. This may be done by increasing the culture volume using larger baffled flasks or fermentation (call Invitrogen Technical Support, see below).

Important

Because the pGAPZ and pGAPZα vectors do not contain the *HIS4* gene, *his4 Pichia* strains containing the integrated plasmid must be grown in complex medium (i.e. YPD) or minimal medium containing 0.004% histidine. **If histidine is not present in the medium, the cells will not grow.**

Optimization of *Pichia* Protein Expression

Introduction

If you obtain no or low protein expression in your initial expression experiment, it may be necessary to optimize expression. Please refer to the following guidelines for assistance.

Remember that *GAP* promoter is a constitutively expressed promoter. If the gene product is toxic to the cells, you may have to try an inducible expression system.

Proteolysis or Degradation

- Do a time course study of expression. Check to see if there is a time point that yields a larger percentage of full-length protein.
- If the protein is being degraded, try expressing in a protease deficient strain like SMD1168 (Invitrogen Catalog no. C175–00).

Low Secreted Expression Levels

- Check the cell pellet to see if overall expression is low or if the protein did not secrete. If it did not secrete, try a different signal sequence (*e.g*. a native signal sequence).
- Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration.
- Try expression with a higher density culture.

Low Expression Levels

- Look for multi-copy recombinants (*i.e*. jackpot clones) by slot blot (please refer to a *Pichia* Expression manual). There are many examples of increasing the expression levels of a particular protein by increasing the gene dosage (Clare *et al.*, 1991a; Clare *et al.*, 1991b; Romanos *et al.*, 1991) .
- Scale up to fermentation. *Pichia* is particularly well suited to fermentation. Contact Invitrogen **Technical Support** for recommendations (page 34).

No Expression

Be sure to try some of the easier things listed above as no expression can be the same thing as very low expression. If none of these things improve protein expression, perform a northern blot analysis to check for transcription of your gene. Refer to a *Pichia* Expression Kit manual for a protocol for RNA isolation.

If you see premature transcriptional termination, check the AT content of your gene. In *Saccharomyces*, there are a few consensus sequences which promote premature termination. One of these, TTTTTATA, resembles a sequence in the HIV-1 gp120 gene, ATTATTTTATAAA, which when expressed in *Pichia* showed premature termination of the mRNA. When this sequence was changed, longer transcripts were found (Scorer *et al.*, 1993).

Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression. Your protein will not go through the secretion pathway and therefore, will not be modified.
- Deglycosylate the protein by treatment with Peptide:N-Glycosidase F or refer to a *Pichia* Expression Kit manual for other enzymes.
- Engineer the gene to remove any N-glycosylation sites (Asn-X-Ser/Thr).

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. **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 ProBond™ Purification manual.

ProBond™ Resin

We recommend that you use the ProBond™ Purification System (page vi) for purifying fusion proteins expressed from pGAPZ or pGAPZα. **Please 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™, 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.

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.

Expression of Secreted Protein

Express your protein using a small-scale culture and the optimal conditions for expression (if determined). 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.

Continued on next page

Preparation of Cell Lysates

Express your protein using a small-scale culture and the optimal conditions for expression (if determined). Once your protein is expressed, follow the protocol below to prepare a cell lysate for chromatography on ProBond™.

Prepare Breaking Buffer (BB) as described in **Recipes**, page 27.

1. Wash cells once in BB by resuspending them and centrifuging 5–10 minutes at 3000 × g at 4°C.
2. Resuspend the cells to an OD_{600} of 50–100 in BB.
3. Add an equal volume of acid-washed glass beads (0.5 mm). Estimate volume by displacement.
4. Vortex the mixture for 30 seconds, then incubate on ice for 30 seconds. Repeat 7 more times. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of your protein.
5. Centrifuge the sample at 4°C for 5–10 minutes at 12,000 × g.
6. Transfer the clear supernatant to a fresh container and analyze for your protein. The total protein concentration should be around 2–3 mg/ml.
7. Save the pellet and extract with 6 M urea or 1% Triton X-100 to check for insoluble protein.

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 × 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 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 medium 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.

Continued on next page

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 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 the medium 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

Yeast Nitrogen Base

Yeast Nitrogen Base is available separately from Invitrogen. Please see the table below for ordering information.

Item	Amount	Catalog no.
Yeast Nitrogen Base (YNB) -with ammonium sulfate -without amino acids	1 × 67 g pouch (each pouch contains reagents to prepare 500 ml of a 10X YNB solution)	Q300–07
	500 g	Q300–09

YPD or YEPD

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (D-glucose)

Note: If you are using the YP Base Medium or the YP Base Agar medium pouches, follow the directions on the pouch.

1. Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. **Note**: Add 20 g of agar if making YPD slants or plates.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 ml of 20% Dextrose.

The liquid medium is stored at room temperature. YPD slants or plates are stored at 4°C. The shelf life is several months.

YPD (+ Zeocin™)

Yeast Extract Peptone Dextrose Medium (1 liter)

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

1. Dissolve the following in 900 ml of water:

   10 g yeast extract
   20 g of peptone
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 20% Dextrose. 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 (D-glucose)
1 M sorbitol
2% agar
100 µg/ml Zeocin™

1. Dissolve the following in 900 ml of water:
   - 10 g yeast extract
   - 182.2 g sorbitol
   - 20 g of peptone
2. Add 20 g of agar.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 ml of 20% Dextrose.
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.

YNB + 0.5% Glucose

1.34 % YNB
0.005% amino acids (if using YNB without amino acids)
0.5% glucose

1. Dissolve 13.4 g YNB in 940 ml sterile water. (It may be necessary to heat the solution in order to dissolve the YNB).
2. Filter the solution. Autoclave 20 minutes on liquid cycle.
3. Add the following:
   - 50 ml of a sterile 10% glucose solution
   - 10 ml of a sterile 100X AA solution (if using YNB without amino acids)
4. Store at 4°C for approximately one year.

Continued on next page

Breaking Buffer

50 mM sodium phosphate, pH 7.4
1 mM PMSF (phenylmethylsulfonyl fluoride. You may use other protease inhibitors)
1 mM EDTA
5% glycerol

1. Prepare a stock solution of your desired protease inhibitors and store appropriately. Follow manufacturer's recommendations.
2. For 1 liter, dissolve the following in 900 ml deionized water:
   - 6 g sodium phosphate (monobasic)
   - 372 mg EDTA
   - 50 ml glycerol
3. Use NaOH to adjust pH and bring up the volume to 1 liter. Store at 4°C.
4. Right before use, add the protease inhibitors.

Low Salt Medium

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 below.

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.

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).

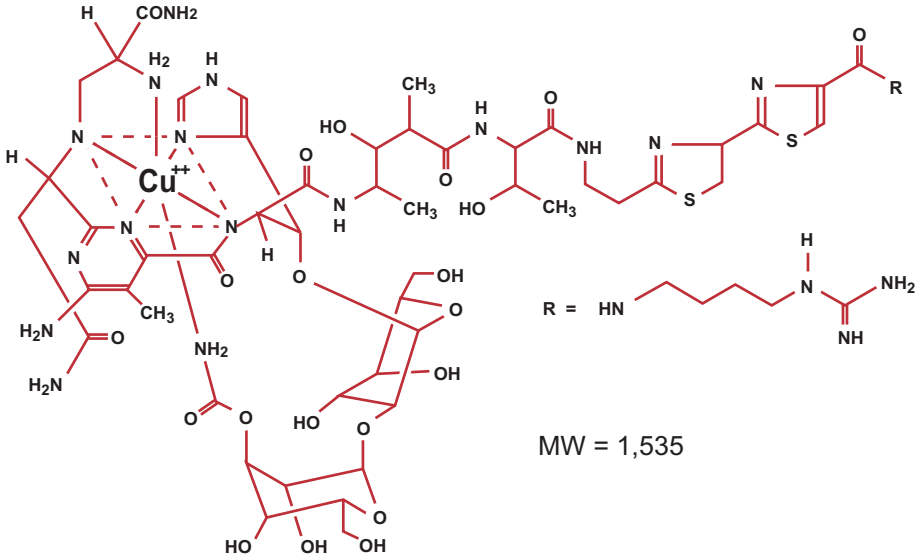
The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity.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

Important

Any *E. coli* strain that contains the complete Tn*5* transposable element (*i.e.* DH5 F´IQ, 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 Tn*5* gene (i.e. TOP10F´, DH5, DH10, etc.).

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™.



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Zeocin™, Continued

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 *E. coli* and mammalian cells are listed below.

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/ml in **Low Salt LB** medium* (see page 27 for recipe)
Pichia	10–1000 µg/ml (varies with strain and medium)

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

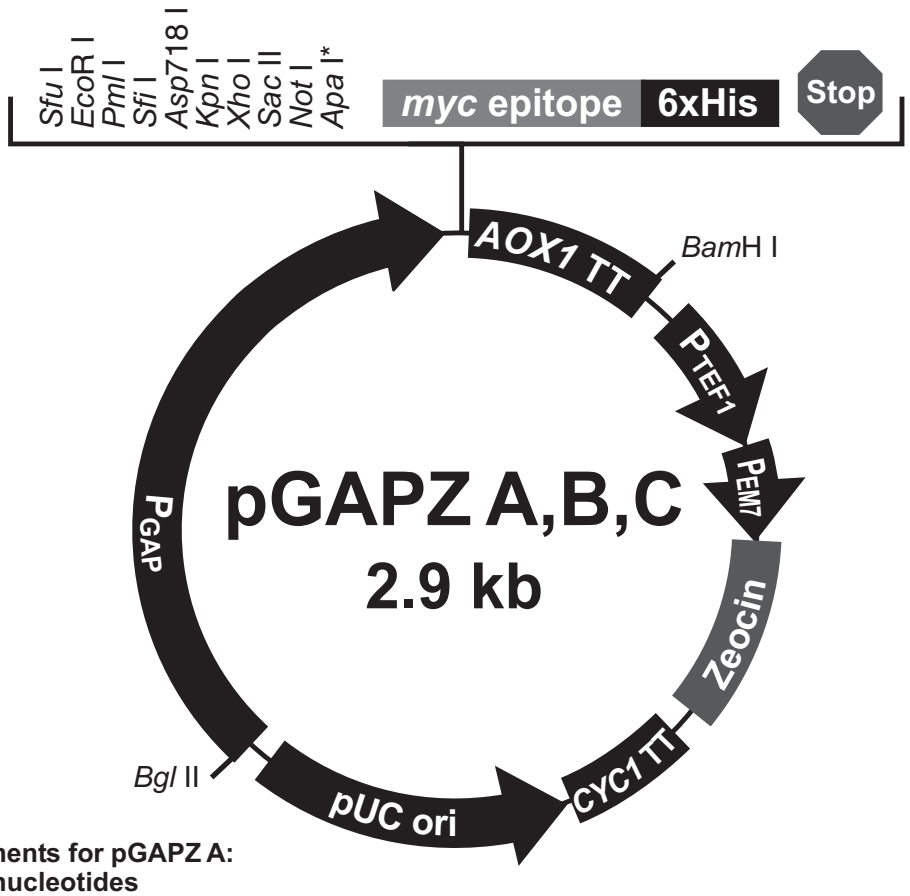
Handling Zeocin™

- 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 27). Please 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 weeks.

Map of pGAPZ A, B, and C

Map of pGAPZ A, B, and C

The figure below summarizes the features of pGAPZ A (2884 bp), pGAPZ B (2882 bp), and pGAPZ C (2883 bp) vectors. The complete nucleotide sequences for pGAPZ A, B, and C are available for downloading from our web site at www.invitrogen.com or from **Technical Support** (page 34).



Comments for pGAPZ A: 2884 nucleotides

GAP promoter region: bases 1-483
Multiple cloning site: bases 484-563
myc epitope tag: bases 564-593
Polyhistidine tag: bases 609-626
AOX1 transcription termination region: bases 630-970
Fragment containing *TEF1* promoter: bases 971-1381
EM7 promoter: bases 1382-1449
Sh ble ORF: bases 1450-1824
CYC1 transcription termination region: bases 1825-2142
pUC origin: bases 2153-2826

* The restriction site between *Not* I and the *myc* epitope is different in each version of pGAPZ:

Apa I in pGAPZ A
Xba I in pGAPZ B
*Sna*B I in pGAPZ C

Features of pGAPZ A, B, and C

Features of pGAPZ A, B, and C

The vectors pGAPZ A, B, and C and pGAPZα A, B, and C contain the following elements. All features have been functionally tested.

Feature	Benefit
GAP promoter	Allows constitutive, high-level expression in *Pichia* (Waterham *et al.,* 1997). Targets plasmid integration to the *GAP* locus.
α-factor Secretion Signal (pGAPZα A, B, and C only)	Encodes the native *Saccharomyces cerevisiae* α-factor secretion signal that allows for efficient secretion of most proteins from *Pichia* (Cregg *et al*., 1993).
Multiple cloning site with unique restriction sites	Allows insertion of your gene into the expression vector.
C-terminal *myc* epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Permits detection of the fusion protein by the Anti-*myc* Antibody (Evan *et al.*, 1985).
C-terminal polyhistidine tag	Encodes six histidine residues that form a metal-binding site for affinity purification of recombinant protein (i.e. with ProBond™, Catalog no. K850-01) and detection by the Anti-His(C-term) Antibody.
AOX1 Transcription Termination (TT) region	Native transcription termination and polyadenylation signal (~260 bp) from *AOX1* gene that permits efficient 3´ mRNA processing, including polyadenylation, for increased mRNA stability.
TEF1 promoter (GenBank accession numbers D12478, D01130)	Transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives expression of the *Sh ble* gene in *Pichia*, conferring Zeocin™ resistance.
EM7 (synthetic prokaryotic promoter)	Constitutive promoter that drives expression of the *Sh ble* gene in *E. coli*, conferring Zeocin™ resistance.
Sh ble gene (*Streptoalloteichus hindustanus ble* gene)	Zeocin™ resistance gene (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1988).
CYC1 transcription termination region (GenBank accession number M34014)	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the *Sh ble* gene for increased stability.
pUC origin	Allows replication and maintenance of the plasmid in *E. coli.*

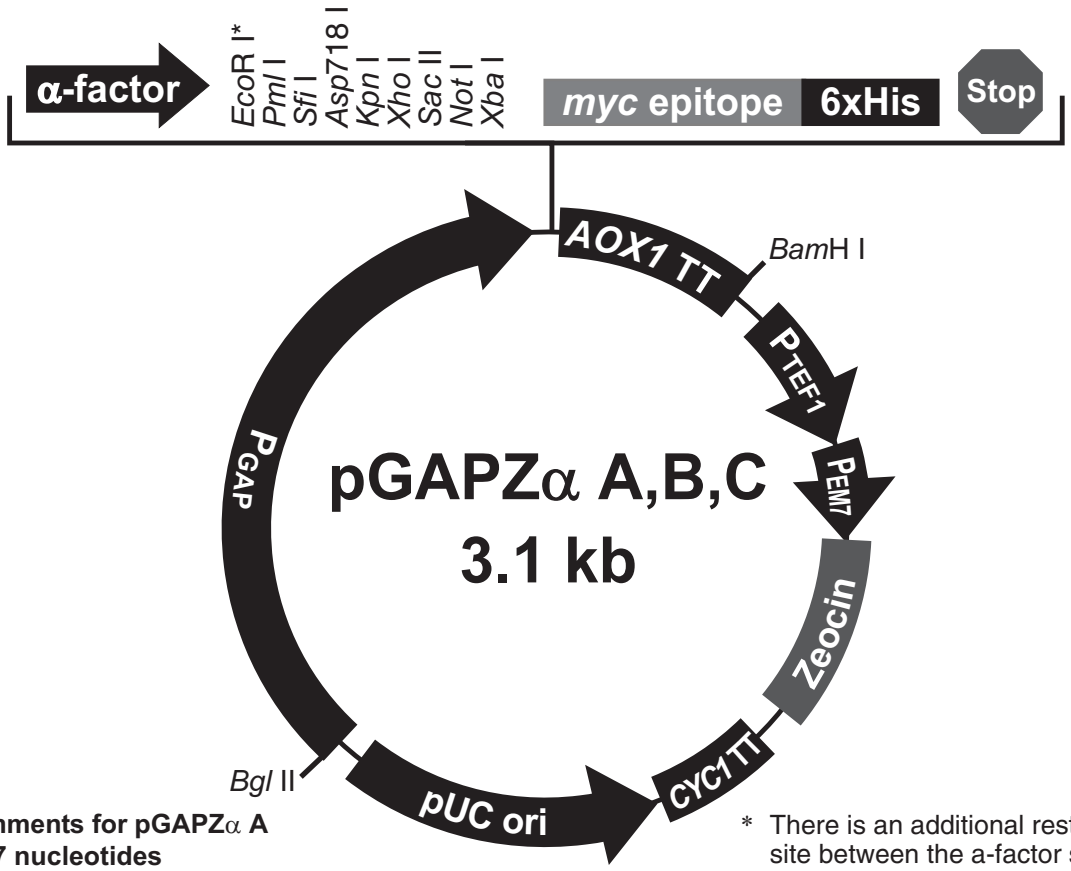
Note

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

Map of pGAPZα A, B, and C

Map of pGAPZα A, B, and C

The figure below summarizes the features of pGAPZα A (3147 bp), pGAPZα B (3151 bp), and pGAPZα C (3152 bp) vectors. The complete nucleotide sequences for pGAPZα A, B, and C are available for downloading from our web site at www.invitrogen.com or from **Technical Support** (page 34).



Comments for pGAPZα A
3147 nucleotides

GAP promoter region: bases 1-483
α-factor signal sequence: bases 493-759
Multiple cloning site: bases 760-828
myc epitope tag: bases 827-856
Polyhistidine tag: bases 872-889
AOX1 transcription termination region: bases 893-1233
TEF1 promoter region: bases 1234-1644
EM7 promoter: bases 1645-1712
Sh ble ORF: bases 1713-2087
CYC1 transcription termination region: bases 2088-2405
pUC origin: bases 2416-3089

* There is an additional restriction site between the a-factor signal sequence and the *Eco*R I site in versions B and C of pGAPZa:

Pst I in pGAPZα B
Cla I in pGAPZα C

Lithium Chloride Transformation Method

Introduction

This is a modified version of the procedure described for *S. cerevisiae* (Gietz & Schiestl, 1996). This protocol is provided as an alternative to transformation by electroporation. Transformation efficiency is between 10^2 to 10^3 cfu/µg 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 × 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 µl of 100 mM LiCl.
7. 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.**

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.
   a. 240 µl 50% PEG
   b. 36 µl 1 M LiCl
   c. 25 µl 2 mg/ml single-stranded DNA
   d. 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 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 25–100 µl on YPD plates containing 100 µg/ml Zeocin™. Incubate the plates for 2–3 days at 30°C. Proceed to **Analysis of *Pichia* Transformants**, page 18.

Technical Support

Web Resources

Visit the Invitrogen web site at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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Information for European Customers

The X-33 cell line is genetically modified and carries the pUC-derived plasmid pGAPZ/*lac*Z. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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