invitrogen™

EasySelect™ Echo™-Adapted *Pichia* Expression Kit

For expression of the gene of interest in *Pichia pastoris* using pPICZ-E and pPICZα-E with the Echo™ Cloning System

Catalog nos. ET230-xx, ET231-xx, ET232-xx

Version E
07 September 2010
25-0388

User Manual

INDIVIDUAL *PICHIA* EXPRESSION KIT LICENSE AGREEMENT

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

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Table of Contents

Kit Contents and Storage

Types of Kits

Several EasySelect™ Echo™-adapted *Pichia* Expression Kits, pPICZ-E and pPICZα-E Echo™-adapted Expression Vector Kits are available (see table below). This manual is included with all of these kits. Note that not all kits contain the reagents discussed in this manual (see page xviii for more details).

Kit	Reagents Supplied	Catalog nos.
EasySelect™ Echo™-adapted *Pichia* Expression Kit	pPICZ-E Echo™-adapted Expression Vector Kit pPICZα-E Echo™-adapted Expression Vector Kit EasySelect™ *Pichia* Strains Kit *Pichia* EasyComp™ Kit *Pichia* Media Kit	ET230-02
EasySelect™ Echo™-adapted *Pichia* Expression Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page xiii for more information on donor vectors)	pUni/V5-His-TOPO® TA Cloning Kit	ET230-10C
	pUniBlunt/V5-His-TOPO® Cloning Kit	ET230-20C
	pUni/V5-His A, B and C	ET230-30C
	pUniD/V5-His-TOPO® Cloning Kit	ET230-40C
pPICZ-E Echo™-adapted Expression Vector Kit	pPICZ-E Vector Expression Control Vector Cre Recombinase and 10X Buffer 5′ *AOX1* Sequencing Primer Zeocin™	ET231-01
pPICZα-E Echo™-adapted Expression Vector Kit	pPICZα-E Vector Expression Control Vector Cre Recombinase and 10X Buffer α-Factor Sequencing Primer Zeocin™	ET232-01
pPICZ-E Echo™-adapted Expression Vector Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page xiii for more information on donor vectors)	pUni/V5-His-TOPO® TA Cloning Kit	ET231-10C
	pUniBlunt/V5-His-TOPO® Cloning Kit	ET231-20C
	pUni/V5-His A, B and C	ET231-30C
	pUniD/V5-His-TOPO® Cloning Kit	ET231-40C
pPICZα-E Echo™-adapted Expression Vector Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page xiii for more information on donor vectors)	pUni/V5-His-TOPO® TA Cloning Kit	ET232-10C
	pUniBlunt/V5-His-TOPO® Cloning Kit	ET232-20C
	pUni/V5-His A, B and C	ET232-30C
	pUniD/V5-His-TOPO® Cloning Kit	ET232-40C

Continued on next page

Kit Contents and Storage, Continued

Shipping and Storage

The EasySelect™ Echo™-adapted *Pichia* Expression Kit is shipped on dry ice except for the EasySelect™ *Pichia* strains and the *Pichia* Media Kit, which are shipped at room temperature. Each EasySelect™ Echo™-adapted *Pichia* Expression Kit contains two vector kits (1 box for each vector), EasySelect™ *Pichia* Strains (Box 2), EasyComp™ *Pichia* Kit (Box 3), and *Pichia* Media Kit.

The pPICZ-E and pPICZα-E Echo™-adapted Expression Kits are shipped on dry ice. Each of pPICZ-E and pPICZα-E Echo™-Adapted Expression Kit contains the Echo™-adapted expression vector, an expression control vector, a sequencing primer and Zeocin™.

Reagents	Storage
pPICZ-E Echo™-adapted Expression Kit	-20°C
pPICZα-E Echo™-adapted Expression Kit	-20°C
EasySelect™ *Pichia* Strains Kit	+4°C
Pichia EasyComp™ Kit	+4°C
Pichia Media Kit	Room temperature
One Shot® TOP10 Chemically Competent *E. coli* (Optional)	-80°C

pPICZ-E Echo™ Reagents

The following items are supplied in the pPICZ-E Echo™-adapted Expression Kit.

Item	Composition	Amount
pPICZ-E	Supercoiled, lyophilized in TE, pH 8.0	20 µg
pPICZ-E/Uni-*lacZ* expression control	Supercoiled, lyophilized in TE, pH 8.0	20 µg
5′ *AOX*1 sequencing primer	Lyophilized in water	2 µg
Cre Recombinase	Check the label on the tube for exact concentration of the enzyme. Enzyme is supplied in: 50 mM Tris-HCl, pH 8.0 5 mM EDTA 1 mM EGTA 10 mM β-mercaptoethanol 20% Glycerol	15 µl
10X Recombinase Buffer	500 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 300 mM NaCl 1.0 mg/ml BSA	25 µl
Zeocin™	100 mg/ml	2 x 1.25 ml

Continued on next page

Kit Contents and Storage, Continued

pPICZα-E Echo™ Reagents

The following items are supplied in the pPICZα-E Echo™-adapted Expression Kit

Item	Composition	Amount
pPICZα-E	Supercoiled, lyophilized in TE, pH 8.0	20 µg
pPICZα-E/Uni-HSA expression control	Supercoiled, lyophilized in TE, pH 8.0	20 µg
α-Factor sequencing primer	Lyophilized in water	2 µg
Cre Recombinase	Check the label on the tube for exact concentration of the enzyme. Enzyme is supplied in: 50 mM Tris-HCl, pH 8.0 5 mM EDTA 1 mM EGTA 10 mM β-mercaptoethanol 20% Glycerol	15 µl
10X Recombinase Buffer	500 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 300 mM NaCl 1.0 mg/ml BSA	25 µl
Zeocin™	100 mg/ml	2 x 1.25 ml

The *Pichia* EasyComp™ Kit

This kit contains sufficient reagents for 6 preparations of competent cells. Each competent cell preparation yields enough cells for 20 transformations.

Upon receipt, store the kit at +4°C. Solution II can also be stored at room temperature.

Component	Description	Quantity
Solution I	Sorbitol solution containing ethylene glycol and DMSO for the preparation of competent cells	75 ml
Solution II	PEG solution for the transformation of competent cells	150 ml (2 x 75 ml)
Solution III	Salt solution for washing and plating transformed cells	150 ml (2 x 75 ml)

Continued on next page

Kit Contents and Storage, Continued

EasySelect™ *Pichia* Strains Kit

The table below lists the genotype and phenotype of the different *Pichia* stabs. Store the stabs at +4°C.

Strain	Genotype	Phenotype (*Pichia* only)
X-33	wild-type	Mut$^+$
GS115	*his4*	His$^-$, Mut$^+$
KM71H	*arg4 aox1::ARG4*	Mut^S, Arg$^+$
GS115/pPICZ/*lac*Z	*his4*	His$^-$, Mut$^+$
GS115/Albumin	*his4*	His$^-$, Mut^s
TOP10F´	F´ {*pro*AB, *lac*I^q, *lac*ZΔM15, Tn*10* (Tet^R)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, , *rec*A1, λ$^-$*ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*G	

Media

The following prepackaged media is included for your convenience. Instructions for use are provided on the package. Keep the media dry and store at room temperature.

Media	Amount	Yield
YP Base Medium	2 pouches	2 liters of YP medium
YP Base Agar Medium	2 pouches	2 liters of YP agar medium
Yeast Nitrogen Base	1 pouch	500 ml of 10X YNB

Sequence of Primers

The table below lists the sequence and pmoles of the primers included in this kit.

Primer	Sequence	pmoles Supplied
5′ *AOX*1 sequencing primer	5’-GACTGGTTCCAATTGACAAGC-3’	312 pmoles
α-Factor sequencing primer	5’-TACTATTGCCAGCAATTGCTGC-3’	315 pmoles

Continued on next page

Kit Contents and Storage, Continued

One Shot® TOP10 Reagents (Optional)

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit.

Store at -80°C.

Item	Concentration	Amount
SOC Medium (may be stored at room temperature or at +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 *E. coli*	--	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

TOP10: Use this strain for general cloning of your gene of interest. Note that this strain cannot be used for transformation and growth of the donor vectors.

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Product Qualification

Vectors

pPICZ-E, pPICZα-E and the control plasmids are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pPICZ-E	pPICZ-E/Uni-*lacZ*	pPICZα-E	pPICZα-E/Uni-HSA
Bgl I	1777 bp, 1403 bp	Not tested	1403 bp, 2042 bp	Not tested
*Eco*R I (linearizes)	3180 bp	Not tested	3445 bp	Not tested
Hind III	Not tested	3404 bp, 5148 bp	Not tested	3404 bp, 4108 bp
Not I	Not tested	2133 bp, 6419 bp	Not tested	2133 bp, 5379 bp

Primers

The sequencing primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Cre Recombinase

Purity: >95% homogeneity

Endonuclease activity: Negative

Exonuclease activity: Negative

Functional Assay: Cre recombinase is qualified using the assay on page 8 of this manual. The donor vector used is pUni/*lacZ* and the acceptor vector is pcDNA3.1-E. Five microliters of the recombination reaction is transformed into 50 µl One Shot® TOP10 Chemically Competent *E. coli* using the protocol on page 9. Twenty-five µl of the transformation reaction is plated on LB plates containing 50 µg/ml kanamycin (performed in duplicate). One microliter of Cre recombinase should yield > 500 blue, kanamycin-resistant transformants.

Pichia EasyComp™ Kit

The *Pichia* EasyComp™ Kit is qualified by preparation of competent GS115 *Pichia* cells. 50 µl of competent GS115 cells are transformed with 3 µg of linearized pPICZα A DNA.

Transformation efficiency must be greater than 50 cfu/µg DNA.

All buffers and solutions are tested for sterility.

Pichia strains

Each *Pichia* strain is qualified by recovery on YPD medium.

Continued on next page

Product Qualification, Continued

Zeocin™	Zeocin™ is lot-qualified by demonstrating that Low Salt LB medium containing 25 μg/ml Zeocin™ prevents growth of the *E. coli* strain, TOP10.
Growth and Expression Media	*Pichia* growth and expression media are qualified by the ability to support growth of the GS115 *Pichia* strain.
One Shot Competent *E. coli*	All competent cells are qualified as follows: • Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 μg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/μg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells. • To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected. • Untransformed cells are plated on LB plates 100 μg/ml ampicillin, 25 μg/ml streptomycin, 50 μg/ml kanamycin, or 15 μg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Accessory Products

Additional Products

Many of the reagents in the EasySelect™ Echo™-adapted *Pichia* Expression Kit and pPICZ-E or pPICZα-E Echo™-adapted Expression Vector Kits, as well as additional reagents that may be used with these kits, are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
One Shot® PIR1 Chemically Competent *E .coli*	11 x 50 µl	C1010-10
One Shot® PIR2 Chemically Competent *E .coli*	11 x 50 µl	C1111-10
One Shot® TOP10 Chemically Competent *E. coli*	11 x 50 µl	C4040-10
Cre Recombinase	10 reactions	R100-10
Kanamycin	5 g	11815-024
	25 g	11815-032
Zeocin™	1 g	R250-01
	5 g	R250-05
Pichia EasyComp™ Transformation Kit	1 kit	K1730-01
Pichia Protocols	1 book	G100-01
X-33 *Pichia* strain	1 stab	C180-00
KM71H *Pichia* strain	1 stab	C182-00
SMD1168H *Pichia* strain	1 stab	C184-00
GS115 *Pichia* strain	1 stab	C181-00

Donor Vectors

The table below lists a variety of donor vectors currently available from Invitrogen to facilitate cloning of your gene of interest for use with Echo™ Cloning System.

Product	Application	Quantity	Catalog no.
pUniD/V5-His-TOPO® Cloning Kit	Directional cloning of blunt PCR products	10 reactions	ET004-10
pUni/V5-His-TOPO® TA Cloning Kit	Cloning A-tailed PCR products	10 reactions	ET001-10
pUniBlunt/V5-His-TOPO® Cloning Kit	Cloning blunt end products	10 reactions	ET002-10
pUni/V5-His A, B, and C	Cloning DNA fragments using restriction enzymes	10 reactions	ET003-10

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Accessory Products, Continued

Detection of Fusion Protein

A number of antibodies and immunodetection kits are available from Invitrogen to detect expression of your fusion protein from the pPICZ-E or pPICZα-E vectors. Horseradish peroxidase (HRP)-or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection in western blots using colorimetric or chemiluminescent detection methods. Sufficient antibody is provided for 25 westerns. The WesternBreeze™ kit contains enough reagents for 20 blots.

Product	Application	Catalog no.
Anti-V5 Antibody	Detects the 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern et al., 1991) GKPINPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His(C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner et al., 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
WesternBreeze™ Chromogenic kits-αMouse	Chromogenic detection of proteins following Western transfer with an alkaline phosphatase substrate	WB7103
WesternBreeze™ Chemiluminescent kits-αMouse	Chemiluminescent detection of proteins following Western transfer with an alkaline phosphatase substrate	WB7104

Purification of Fusion Protein

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

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns	50 polypropylene columns	R640-50

Purchaser Notification

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

Limited Use Label License No: 22 Vectors and Clones Encoding Histidine Hexamer

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Purchaser Notification, Continued

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Using This Manual

Important

This manual is a comprehensive manual designed to support a variety of different *Pichia* kits. Some reagents discussed in this manual may not be included in the kit you purchased. In addition, you may have your own methods for transformation and expression in *Pichia.* Use the information from this manual according to your needs.

Introduction

Overview of the Echo™ Cloning System

Introduction

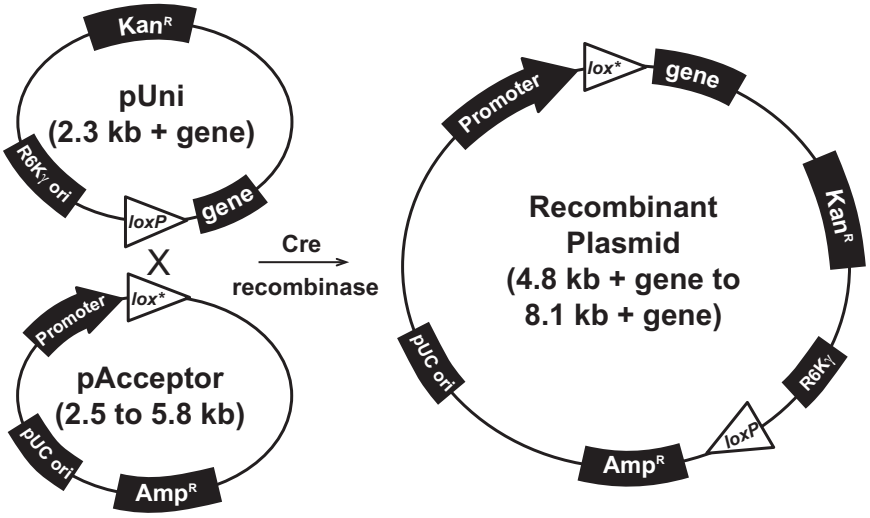
The Echo™ Cloning System allows direct recombination of your gene of interest downstream of an appropriate promoter for expression in the host system of choice. pPICZ-E and pPICZα E are members of the Echo™ Cloning System family of expression vectors and are specifically designed for expression in *Pichia pastoris*. The 5′*AOX*1 promoter controls high-level inducible recombinant protein expression in any *Pichia pastoris* strain of choice.

The Echo™ Cloning System

The Echo™ Cloning System is based on the univector plasmid-fusion system (UPS) described by Elledge and coworkers to quickly and easily recombine a gene of interest into a series of recipient (acceptor) vectors (Liu et al., 1998; Liu et al., 1999). The system consists of the univector (donor) vector containing the gene of interest and recipient (acceptor) vector containing various regulatory sequences for expression in the host of choice. The Echo™ System utilizes the *cre-lox* site-specific recombination system of bacteriophage P1 (Abremski et al., 1983; Sternberg et al., 1981). The product of the *cre* gene is a site-specific recombinase that catalyzes conservative recombination between two 34 bp *loxP* sequences or, a *loxP* and a *loxH* sequence to resolve P1 dimers generated by replication of circular lysogens. It does not catalyze recombination between two *loxH* sequences.

Plasmid Fusion

The donor (pUni) vector and the acceptor vector (i.e. pPICZ-E or pPICZα-E) each contain a single *lox* site. The donor vector and the acceptor vector each contain a *loxP* site. You may insert your gene of interest into the donor vector via the TOPO® Cloning method or traditional restriction enzyme-mediated cloning. pPICZ-E and pPICZα-E contain the appropriate transcription regulatory sequences to control expression of your gene of interest in *Pichia* and a unique *loxP* site located downstream of these sequences. By mixing the donor vector containing the gene of interest with pPICZ-E or pPICZα-E in the presence of Cre recombinase, a plasmid fusion is created that expresses the gene of interest in *Pichia pastoris*. A generic diagram is shown below.



*lox** = *loxP* or *loxH* depending on acceptor vector

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Overview of Echo™ Cloning System, Continued

loxP or *loxH* Sites

The sequence of the *loxP* site is shown below. The *loxP* site consists of a 34 bp sequence containing two 13 bp inverted repeats (see underlined bases) separated by an 8 bp spacer (Hoess et al., 1982). The inverted repeats may form a stem and loop structure that may reduce expression of the gene of interest in some cases. A variation of the *loxP* site (*loxH*, see below) was created to eliminate the formation of a stem and loop structure and improve expression. We have not observed any differences in expression levels in constructs containing a *loxP* or a *loxH* site. Mutated bases are shown in boldface. Cre-mediated recombination can still occur between a *loxP* and a *loxH* site although the efficiency may be slightly reduced.

- *loxP*: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T
- *loxH*: AT**T** AC**C** TC**A** TAT AGC ATA CAT TAT ACG AAG TTA T

Cre Recombinase

Cre recombinase (MW = 35 kDa) is a site-specific recombinase that binds to specific sequences (*loxP* and *loxH* sites), brings together the target sites, cleaves them, and covalently attaches to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombinant) form. A nucleophilic hydroxylated tyrosine initiates the DNA cleavage event by attack on a specific phosphodiester bond followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid bond (Abremski & Hoess, 1992; Argos et al., 1986). The reaction does not require any host factors or ATP, but does require Mg^{2+} or spermidine for activity (Abremski et al., 1983). Recombination between two supercoiled substrates, each containing a *loxP* or *loxH* site, results in a supercoiled dimer. The extent of the reaction is 10-20% and appears to be stoichiometric (Abremski & Hoess, 1984; Abremski et al., 1983).

Selection of Recombinants

By fusing the two plasmids, kanamycin resistance is now linked to the pUC origin of replication. The recombination reaction is transformed into TOP10 *E. coli* and recombinants are selected by plating the transformation reaction onto plates containing kanamycin. Because the donor plasmid carries the R6Kγ origin of replication, it will not propagate in *E. coli* strains such as TOP10 which do not carry the *pir* gene. In addition, the acceptor vector, which carries the ampicillin resistance gene will not be selected. Therefore every colony that is selected on kanamycin will represent a recombined fusion plasmid.

Continued on next page

Overview of Echo™ Cloning System, Continued

pPICZ-E and pPICZα-E

pPICZ-E and pPICZα-E (~ 3 kb) are derived from pPICZB and pPICZα-B, respectively. They are designed for high-level recombinant protein expression in *Pichia pastoris*. The vectors contain the following elements:

- 5′ fragment containing the *AOX*1 promoter for tightly regulated, methanol-induced expression of the gene of interest (Ellis *et al.*, 1985: Koutz *et al.*, 1989: Tschopp *et al.*, 1987a)
- A *loxP* site for plasmid fusion
- *Saccharomycese cerevisiae* α-factor secretion signal sequence (pPICZα-E) for secretion of recombinant proteins in the medium
- Zeocin™ resistance gene for selection in both *E. coli* and *Pichia* (Baron *et al.*, 1992: Drocourt *et al.*, 1990)
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*

For a map and a description of the features of pPICZ-E or pPICZα-E, refer to the **Appendix**, pages 46-48.

Other Echo™-adapted acceptor vectors are available separately and are provided with their own manuals. For more information on other available acceptor vectors, visit our Web site (www.invitrogen.com) or call Technical Service (see page 61).

Selection of Vector

To utilize the strong, highly-inducible P_{AOX1} promoter (see next page) for expression of your protein, there are two expression vectors included in this kit. One vector, pPICZ-E, is for intracellular expression while the other vector, pPICZα-E, is for secreted expression. All vectors contain the Zeocin™ resistance gene for positive selection in *E. coli* and *Pichia*. See pages 46-48 for more information on these vectors.

Overview of *Pichia pastoris* Expression System

Review Articles

The information presented here is designed to give you a concise overview of the *Pichia pastoris* expression system. It is by no means exhaustive. For further information, read the articles cited in the text along with recent review articles (Higgins & Cregg, 1998), (Buckholz & Gleeson, 1991; Cregg & Higgins, 1995; Cregg et al., 1993; Nico-Farber et al., 1995; Sreekrishna et al., 1988; Wegner, 1990). A general review of foreign gene expression in yeast is also available (Romanos et al., 1992)

General Characteristics of *Pichia pastoris*

As a eukaryote, *Pichia pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces* and has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system.

Similarity to *Saccharomyces*

Many of the techniques developed for *Saccharomyces* may be applied to *Pichia* including transformation by complementation, gene disruption, and gene replacement.

In addition, the genetic nomenclature used for *Saccharomyces* has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the *HIS4* gene in both *Saccharomyces* and *Pichia*. There is also cross-complementation between gene products in both *Saccharomyces* and *Pichia*. Several wild-type genes from *Saccharomyces* complement comparable mutant genes in *Pichia*. Genes such as *HIS4, LEU2, ARG4, TRP1,* and *URA3* all complement their respective mutant genes in *Pichia*.

Pichia pastoris as a Methylotrophic Yeast

Pichia pastoris is a methylotrophic yeast, capable of utlilizing methanol as its sole carbon source. Alcohol oxidase catalyses the oxidation of methanol to formaldehyde and hydrogen peroxide using molecular oxygen. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *Pichia* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is used to drive recombinant protein expression in *Pichia*.

Two Alcohol Oxidase Proteins

Two genes in *Pichia pastoris* code for alcohol oxidase-*AOX1* and *AOX2*. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* gene. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically $\geq$ 30% of the total soluble protein in cells grown with methanol. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest (Ellis et al., 1985; Koutz et al., 1989; Tschopp et al., 1987a). While *AOX2* is about 97% homologous to *AOX1*, growth on methanol is much slower than with *AOX1*. This slow growth on methanol allows isolation of Mut^S strains (see below) (*aox1*) (Cregg *et al.*, 1989; Koutz *et al.*, 1989).

Continued on next page

Overview of *Pichia pastoris* Expression System, Continued

Expression

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-grown cells approximately 5% of the total polyA$^+$ RNA is from the *AOX1* gene. The regulation of the *AOX1* gene is a two step process of repression/derepression and induction mechanism. Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth on glycerol is recommended for optimal induction with methanol. **Note:** Growth on glycerol alone (derepression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for detectable levels of *AOX1* expression (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a).

Phenotype of *aox1* Mutants

Loss of the *AOX1* gene results in a strain that is phenotypically Mut^S (Methanol utilization slow, also referred to as Mut$^-$ in the past). The Mut^S designation is chosen to accurately describe the phenotype of these mutants. This results in a reduction in the cells ability to metabolize methanol and they exhibit poor growth on methanol medium. Mut$^+$ (Methanol utilization plus) refers to the wild type ability of strains to metabolize methanol as the sole carbon source. These two phenotypes are used when evaluating *Pichia* transformants for integration of your gene (**Experimental Outline**, page 7).

Intracellular and Secretory Protein Expression

Heterologous expression in *Pichia pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. The native secretion signal present on some heterologous proteins and several different secretion signal sequences have been used with varied success. *Saccharomyces cerevisiae* α factor secretion signal sequence has been used most successfully (Cregg et al., 1993; Scorer et al., 1993)

The major advantages of expressing recombinant proteins as secreted proteins are:

- *Pichia pastoris* secretes very low levels of native proteins
- Very low amount of protein is present in the minimal *Pichia* growth medium

The secreted recombinant protein usually comprises the vast majority of the total protein in the medium and serves as the first step in purification of the protein (Barr et al., 1992). **Note**: If there are recognized glycosylation sites (Asn-X-Ser/Thr) in your protein's primary sequence, glycosylation may occur at these sites.

Posttranslational Modifications

Unlike *Saccharomyces cerevisiae*, *Pichia* does not hyperglycosylate the secreted proteins. Both *Saccharomyces cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added posttranslationally to proteins in *Pichia* (average 8-14 mannose residues per side chain) is much shorter than those in *Saccharomyces cerevisiae* (50-150 mannose residues) (Grinna & Tschopp, 1989; Tschopp et al., 1987b) Very little O-linked glycosylation has been observed in *Pichia*.

In addition, *Saccharomyces cerevisiae* core oligosaccharides have terminal α1,3 glycan linkages whereas *Pichia pastoris* does not. It is believed that the α1,3 glycan linkages in glycosylated proteins produced from *Saccharomyces cerevisiae* are primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for therapeutic use. Although not yet proven, this is predicted to be less of a problem for glycoproteins generated in *Pichia pastoris*, because it may resemble the glycoprotein structure of higher eukaryotes (Cregg *et al.*, 1993).

Continued on next page

Overview of *Pichia pastoris* Expression System, Continued

Transformation and Integration

Two different phenotypic classes of recombinant strains can be generated: Mut$^+$ and Mut^S. Mut^S refers to the "Methanol utilization slow" phenotype caused by the loss of alcohol oxidase activity encoded by the *AOX1* gene. A strain with a Mut^S phenotype has a mutant *aox1* locus, but is wild type for *AOX2*. This results in a slow growth phenotype on methanol medium. *Pichia* strains X-33 and GS115 are Mut$^+$ while KM71H is Mut^S. Transformation of X-33 or GS115 with plasmid DNA linearized in the 5´ *AOX1* region will yield Mut$^+$ transformants, while KM71H will yield only Mut^S transformants. Both Mut$^+$ and Mut^S recombinants are useful to have as one phenotype may favor better expression of your protein than the other. You should test between 6-10 recombinants per phenotype because the site of recombination may affect expression. There is no way to predict beforehand which construct or isolate will better express your protein. For more information on recombination in *Pichia*, please see page 51.

Once you have successfully cloned your gene behind the *AOX1* promoter, you will then linearize your plasmid to permit recombination when the plasmid is transformed into *Pichia*.

Expression and Scale-up

After isolating your *Pichia* recombinants, you will then test expression of both Mut$^+$ and Mut^S recombinants. This will involve growing a small culture of each recombinant, inducing with methanol, and taking time points. If looking for intracellular expression, analyze the cell pellet from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE). If looking for secreted expression, analyze both the cell pellet and supernatant from each time point. We recommend that you analyze your SDS-PAGE gels by both Coomassie staining and western blot (for proteins expressed at low levels). We also suggest checking for protein by functional assay if one is available.

Choose the *Pichia* recombinant strain that best expresses your protein and optimize induction based on the suggestions on pages 32. Once expression is optimized, scale-up your expression protocol to produce more protein for purification.

Purification

The donor vector contains a C-terminal tag consisting of a V5 epitope and a polyhistidine tag. If the donor vector is recombined with the acceptor vector (pPICZ-E or pPICZα-E) such that the C-terminal tag is maintained, then both pPICZ-E or pPICZα-E will contain the V5 epitope and the polyhistidine tag. The polyhistidine tag facilitates purification by binding to divalent cations like Ni^{2+}usually present in metal-binding resins such as ProBond™. We recommend that you use the ProBond™ Purification System (Catalog no. K850-01) to purify fusion proteins expressed using pPICZ-E or pPICZα-E. Preliminary preparation steps are described on pages 36-37.

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

Continued on next page

Overview of *Pichia pastoris* Expression System, Continued

Experimental Outline

The table below describes the general steps needed to recombine, transform, and express your protein of interest.

Step	Action	Page
1	Perform the recombination reaction using your donor vector and pPICZ-E or pPICZα-E.	8
2	Transform the recombination reaction into competent TOP10 *E. coli*.	9
3	Select transformants on LB plates containing 50 µg/ml kanamycin.	9
4	Analyze transformants by restriction digestion.	10
5	Select the correct clone and linearize the construct with appropriate restriction enzyme.	17
6	Transform your construct into appropriate competent *Pichia* host strain (X-33 or GS115 for Mut$^+$, and KM71H for Mut^S) using your transformation method of choice. Select transformants on medium containing Zeocin™.	18-21
7	Select 6-10 clones for small scale expression and analyze the expression of your recombinant protein by western blot analysis or functional assay.	25
8	Choose the highest expressers for large-scale expression in shake flask or fermenter.	34
9	Purify your protein using metal-chelating resin (Probond™) or any other method of choice.	36

Methods

Recombining Your Gene into pPICZ-E or pPICZα-E

Introduction

You will need a plasmid preparation of your donor vector containing the gene of interest in addition to the pPICZ-E or pPICZα-E vector. Review the information below and on the next page before performing the recombination reaction.

Preparation and Maintenance of pPICZ-E or pPICZα-E

To prepare pPICZ-E or pPICZα-E for use, add 20 µl sterile water to prepare a 1 µg/µl stock solution. You can further dilute a small aliquot of plasmid or use the stock solution as is. Store the stock solution at -20°C when you are finished.

If you wish to propagate the pPICZ-E or pPICZα-E plasmid or prepare plasmid DNA, you may transform the plasmid into One Shot® TOP10 Chemically Competent *E. coli* as described on page 9 or TOP10F′ provided in the kit. Use10-100 ng of plasmid DNA for transformation and select transformants on low salt LB plates containing 25 µg/ml Zeocin™. Be sure to prepare a glycerol stock of your plasmid-containing TOP10 strain for long-term storage (see page 12).

Before Starting

You will need the following reagents and equipment.

- 100 ng of your donor vector construct
- 100 ng of pPICZ-E or pPICZα-E (included in the kit)
- Microcentrifuge tubes
- Heat blocks set at 37°C and 65°C
- Ice bucket with ice
- Cre recombinase (included in the kit)
- 10X Recombinase Buffer (included in the kit)

Recombination Reaction

1. Set up each 20 µl recombination reaction **on ice** as follows:

Donor vector (100 ng)		x µl
pPICZ-E or pPICZα-E (100 ng)		y µl
10X Recombinase Buffer		2 µl
Deionized water	add to a total volume of	19 µl
Cre Recombinase		1 µl
Final Volume		20 µl

2. Incubate at 37°C for 20 minutes.
3. Incubate at 65°C for 5 minutes to inactivate the recombinase.
4. Place the tube on ice and proceed to **Transformation**, next page. If you run out of time, you may store the recombination reaction at +4°C or -20°C overnight. Longer storage times have not been tested.

Transforming the Recombination Reaction

Introduction

Once you have performed the recombination reaction, you are ready to transform your *E. coli* host. We recommend using TOP10 *E. coli* for transformation, but other strains are suitable. *E. coli* strains should be endonuclease A deficient (*end*A) and recombination deficient (*rec*A) to ensure quality plasmid preparations and reduce the chances of non-specific recombination, respectively.

Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath
- LB plates containing 50 µg/ml kanamycin (see **Important**, below)
- LB medium and SOB medium (see page 45 for recipe)
- 37°C shaking and non-shaking incubator

Important

It is important to select the fusion plasmid using kanamycin. The donor vector contains the R6Kγ origin that can only be maintained in *E. coli* strains containing the *pir* gene. After recombination between the donor vector and acceptor vector, the kanamycin resistance gene (from the donor vector) in the fusion plasmid is linked to the pUC origin (from pPICZ-E or pPICZα-E). The fusion plasmid can be maintained in *E. coli* strains that do not contain the *pir* gene (i.e. TOP10). By selecting for kanamycin resistance, you ensure that only colonies containing the fusion plasmid are selected.

Preparing for Transformation

This transformation protocol is for use with the One Shot® TOP10 Chemically Competent *E. coli* available with the kit. Follow the manufacturer's protocol if you are using other competent cells.

For each transformation, you will need one vial of One Shot® TOP10 Chemically Competent *E. coli* and two selective plates. Perform the following steps before beginning.

1. Equilibrate a water bath to 42°C.
2. Thaw the vial of SOC medium from the kit and bring to room temperature.
3. Warm LB plates containing 50 µg/ml kanamycin at 37°C for 30 minutes.
4. Thaw **on ice** 1 vial of One Shot® cells for each transformation.

One Shot® Transformation Reaction

1. Add 5 µl of the recombination reaction (step 4, page 8) to a vial of One Shot® TOP10 *E. coli* and mix by stirring with a pipette tip. **Do not mix by pipetting up and down.**
2. Heat-shock the cells for 30 seconds at 42°C without shaking.
3. Immediately transfer the tubes to ice.
4. Add 500 µl of room temperature SOC medium.
5. Cap the tube tightly and shake the tube horizontally at 37°C for 45 minutes.
6. Spread 50 µl from each transformation onto a prewarmed LB plate containing 50 µg/ml kanamycin. Pellet the remaining cells, resuspend the cell pellet in 50 µl SOC and plate. Incubate plates overnight at 37°C.
7. An efficient recombination reaction will produce hundreds of colonies. Pick five colonies for analysis.

Continued on next page

Transforming the Recombination Reaction, Continued

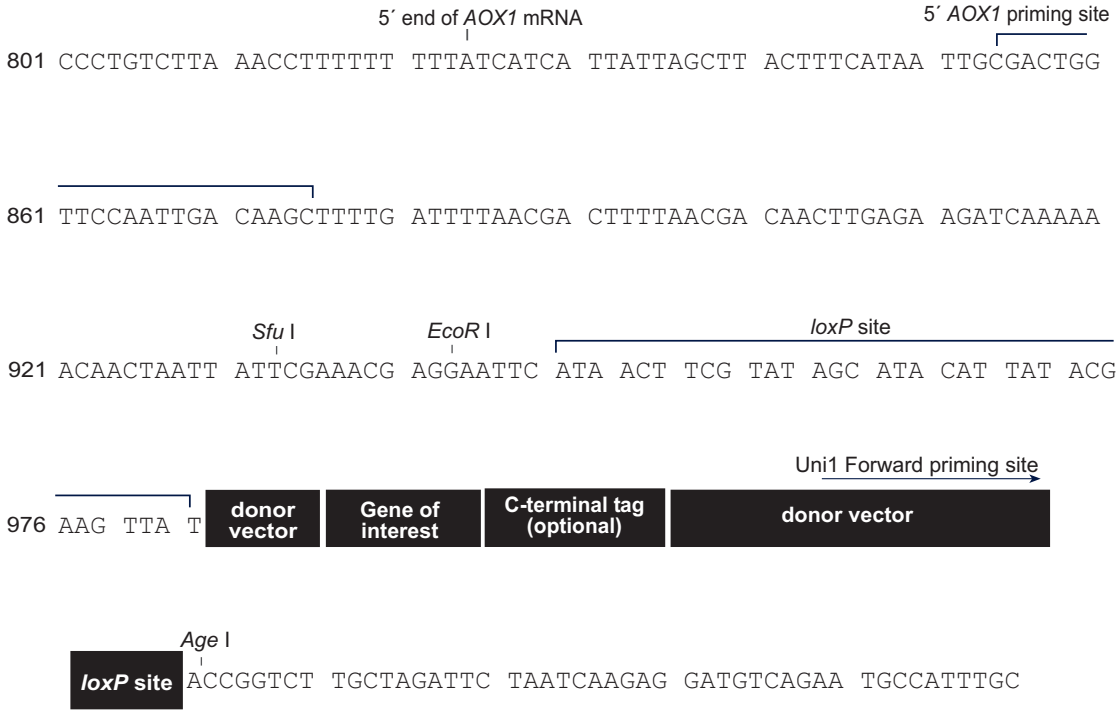
Analyzing Positive Clones

1. Culture the 5 colonies (see previous page) overnight in 2-5 ml LB or SOB medium containing 50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis. Use an enzyme (or enzymes) that cut once in the donor vector and once in the acceptor vector to yield two fragments that are distinguishable from one another. Note that other strategies are possible.
4. (Optional) To sequence the fusion plasmid to confirm the fusion junctions, use the 5′ *AOX1* primer and the Uni1 Forward primer. Refer to the diagrams below and on the next page for the sequence around the pPICZ-E or pPICZα-E *loxP* site. Refer to the donor vector manual for the sequence around the donor vector *loxP* site.

If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology texts (Ausubel et al., 1994; Sambrook et al., 1989)

Sequencing Your Construct in pPICZ-E

The sequence surrounding your insert is shown below. Unique restriction sites are labeled to indicate the cleavage site. Note that the complete sequence of pPICZ-E is available for downloading from our Web site: (www.invitrogen.com) or from Technical Service (see page 61).

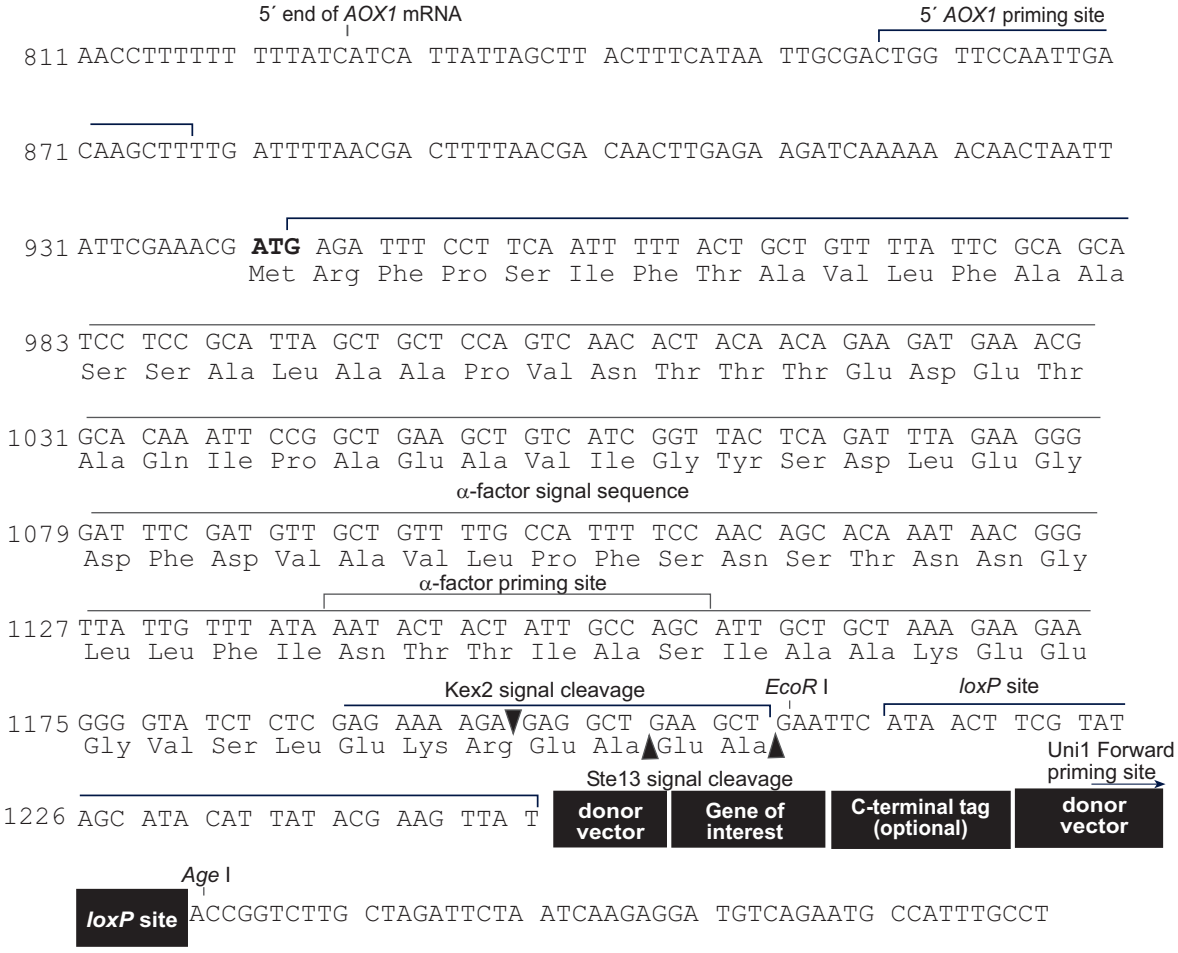


Continued on next page

Transforming the Recombination Reaction, Continued

Sequencing Your Construct in pPICZα-E

The sequence surrounding your insert is shown below. Unique restriction sites are labeled to indicate the cleavage site. Note that the complete sequence of pPICZα-E is available for downloading from our Web site: (www.invitrogen.com) or from Technical Service (see page 61).



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Transforming the Recombination Reaction, Continued

Fusion Plasmid Analysis

It should be clear from restriction analysis that you have a dimer plasmid consisting of the donor vector and pPICZ-E or pPICZα-E. Occasionally, trimers will result. Trimers usually consist of two donor vector molecules and one acceptor molecule, but they usually express as well as the dimer product.

In theory, trimers may result from two sequential fusion events or a single fusion event between a pre-existing monomeric substrate and a dimeric substrate. The production of trimers can be eliminated if gel-purified monomeric supercoiled DNA is used in the recombination reaction.

Preparing Plasmid DNA and Glycerol Stock for Long-Term Storage

Once you have identified the correct clone, isolate plasmid DNA using your method of choice for transformation of your construct into *Pichia* (see page 15-21).

We recommend to make a glycerol stock for long term storage.

1. Streak out the original colony on LB plates containing 50 µg/ml kanamycin to isolate single colonies.
2. Select a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C. (You may also want to store a stock of plasmid DNA at -20°C.)

Pichia Strains

Introduction

After isolating the plasmid DNA of your fusion construct, you are ready to transfom this construct into *Pichia* for expression of the recombinant protein. This section provides information on *Pichia* strains, initiation of *Pichia* culture, growth characteristics and storage of *Pichia pastoris*. You should be familiar with basic sterile microbiological, molecular biology and protein chemistry techniques before attempting to grow and manipulate *Pichia*. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology*, (Guthrie & Fink, 1991), *Current Protocols in Molecular Biology*, (Ausubel *et al.*, 1994), *Molecular Cloning: A Laboratory Manual*, (Sambrook *et al.*, 1989), *Protein Methods*, (Bollag & Edelstein, 1991) and *Guide to Protein Purification*, (Deutscher, 1990).

Genotypes of *Pichia* Strains

X-33 is a wild-type *Pichia* strain that is useful for selection on Zeocin™ and large-scale growth. It will grow in YPD and in minimal media.

The *Pichia* host strain GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. GS115 will grow on complex medium such as YPD (also known as YEPD) and on minimal media supplemented with histidine.

The parent strain of KM71H has a mutation in the argininosuccinate lyase gene (*arg4*) that prevents the strain from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt *AOX1*, creating KM71H, a Mut^S, Arg^+ strain.

Construction of KM71H

The *ARG4* gene (~2 kb) was inserted into the cloned, wild-type *AOX1* gene between the *Bam*H I site (codons 15/16 of *AOX1*) and the *Sal* I site (codons 227/228 of *AOX1*). *ARG4* replaces codons 16 through 227 of *AOX1*. This construct was transformed into the parent strain of KM71 (*arg4 his4*) and Arg^+ transformants were isolated and analyzed for the Mut^S phenotype. Genetic analysis of Arg^+ transformants showed that the wild-type *AOX1* gene was replaced by the *aox1::ARG4* construct. To create KM71H, KM71 was transformed with a gene fragment encoding the *HIS4* gene and a His^+ convertant was isolated.

Important

The advantage of using KM71H is that there is no need to screen for the Mut phenotype on methanol minimal medium. All transformants will be Mut^S. Secondly, since the *AOX1* locus was not completely deleted, it is theoretically possible to replace *aox1::ARG4* with your construct by gene replacement. The phenotype of this strain would be Mut^S Arg^-. This means the recombinant strain would require arginine in the medium to grow. Unfortunately, simple inclusion of arginine does not totally alleviate the effects of the *arg4* mutation, and *arg4* strains do not grow well on minimal medium supplemented with arginine. Therefore, we do not recommend that you generate transformants in KM71H by replacing the *aox1::ARG4* construct.

Continued on next page

Pichia Strains, Continued

Growth of *Pichia* Strains

The growth temperature of *Pichia pastoris* is 28-30°C for liquid cultures, plates, and stabs. Growth above 32°C during induction can be detrimental to protein expression and can even lead to cell death. Other important facts:

- Doubling time of log phase Mut^+ or Mut^S *Pichia* in YPD is ~2 hours
- Mut^+ and Mut^S strains do not differ in growth rates unless grown on methanol
- Doubling time of log phase Mut^+ *Pichia* in methanol medium (MM) is 4-6 hours
- Doubling time of log phase Mut^S *Pichia* in MM is ~18 hours
- One OD_{600} = ~5 x 10^7 cells/ml

Note that growth characteristics may vary depending on the recombinant protein expressed

Growth on Methanol

When plates or medium containing methanol are used as growth medium, it is advisable to add methanol every day to compensate for loss because of evaporation or consumption.

- For plates add 100 µl of 100% methanol to the lid of the inverted plate.
- For liquid medium add 100% methanol to a final concentration of 0.5%.

Some researchers have had success adding methanol to 1% every day for Mut^S strains and up to 3% for Mut^+ without any negative effect to their liquid culture

Initiating *Pichia* culture

To initiate a culture of *Pichia* from the stab provided with the kit, streak a small amount from the stab on YPD plate (see page 41) and incubate at 30°C. Once growth is established, you may pick a single colony and grow it in the appropriate medium for downstream applications. Remember to prepare frozen stocks for long-term storage of all *Pichia* strains included in this kit (see below).

Storage of *Pichia* Strains

To store cells for weeks to months, use YPD medium and YPD agar slants (see page 41).

- Streak each strain for single colonies on YPD.
- Transfer one colony to a YPD stab and grow for 2 days at 30°C.
- The cells can be stored on YPD for several weeks at +4°C.

To store cells for months to years, store frozen at -80°C.

- Culture a single colony of each strain overnight in YPD.
- Harvest the cells and suspend in YPD containing 15% glycerol at a final OD_{600} of 50-100 (approximately 2.5-5.0 x 10^9 cells/ml).
- Cells are frozen in liquid nitrogen or a dry ice/ethanol bath and then stored at -80°C.

Note

After extended storage at +4°C or -80°C, it is recommended that Zeo^R transformants be checked for correct phenotype and protein expression.

Pichia Transformation

Introduction

Once you have created the pPICZ-E or pPICZα-E fusion plasmid and have verified its integrity, you are ready to transform the fusion plasmid into a *Pichia* host strain of your choice.

Zeocin™ Selection

We generally use 100 µg/ml Zeocin™ to select for transformants when using the X-33 *Pichia* strain. If you are transforming the fusion plasmid 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 pPICZ-E or pPICZα-E. 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 *Pichia* EasyComp™ Transformation Kit included in the kit (see page 19). The *Pichia* EasyComp™ Transformation Kit provides reagents to prepare 6 preparations of competent cells. Each preparation will yield enough competent cells for 20 transformations

Important

Since pPICZ-E or pPICZα-E do 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 will be Mut^+ or Mut^S. Use a *Pichia* host that contains the native *AOX1* gene (e.g. X-33, GS115, SMD1168H) to generate a Mut^+ recombinant strain and a *Pichia* host that has a disrupted *AOX1* gene (i.e. KM71H) to generate a Mut^S recombinant strain. Information on *Pichia* recombination is provided on page 51.

His4 Host Strains

Host strains containing the *his4* allele (e.g. GS115) and transformed with the pPICZ-E or pPICZα-E vectors require histidine when grown in minimal media. Add histidine to a final concentration of 0.004% to ensure growth of your transformants.

Continued on next page

Note

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

Pichia Controls

pPICZ-E/Uni-*lacZ* and pPICZα-E/Uni-HSA are control plasmids included in the kit to check for transformation and expression in *Pichia pastoris.* The *lacZ* gene was cloned into a donor vector, pUni/V5-His-Gene-TOPO®. The resulting vector was recombined with pPICZ-E to create pPICZ-E/Uni-*lacZ*, which can be used as an intracellular expression control after transformation into a *Pichia* host.

The human serum albumin (HSA) gene was cloned into a donor vector, pUni/V5-His-Gene-TOPO®. The resulting vector was recombined with pPICZα-E to create pPICZα-E/Uni-HSA/V5-His, which can be used as a secreted expression control. For a map and more details on pPICZ-E/Uni-*lacZ* and pPICZα-E/Uni-HSA controls, see pages 49-50.

Use the control plasmids pPICZ-E/Uni-*lacZ* or pPICZα-E/Uni-HSA to perform a control transformation using any method of choice in a *Pichia* host strain. We have used X-33 as the host strain. Linearize the plasmid using *Pme* I to promote integration (see next page). If you wish to propagate the supercoiled plasmids, you may transform the plasmid into TOP10 *E. coli* (see page 8).

GS115/pPICZ/*lacZ* (Mut^+) and GS115/Albumin (Mut^s) are control strains included in the kit to check the Mut phenotype. Cloning the *lacZ* gene into the pPICZ vector and transforming the resulting construct into GS115 creating the GS115/pPICZ/*lacZ* (Mut^+) strain. This strain expresses β-galactosidase and can also be used as a control for intracellular expression. GS115/Albumin is Mut^s and is also a control for secreted expression. The gene for serum albumin was cloned with its native secretion signal and then transformed into GS115 to create GS115/Albumin. If you decide to use GS115/pPICZ/*lacZ* or GS115/Albumin as a control, then you need to initiate a culture from the stab (see page 14).

We recommend you use pPICZ-E/Uni-*lacZ* and pPICZα-E/Uni-HSA as transformation and expression controls, and GS115/pPICZ/*lacZ* and GS115/Albumin as controls for determining the Mut phenotype (see page 22) and confirming expression conditions.

Before Starting

The following reagents are needed for linearizing your construct, transforming *Pichia* and selecting transformants on Zeocin™.

- 10-50 µg pPICZ-E or pPICZα-E fusion plasmid and appropriate expression control plasmids
- Appropriate restriction enzymes and buffer
- Phenol/chloroform
- 3M sodium acetate
- Agarose gel apparatus and Electroporation device with 0.2 cm cuvettes
- 80% and 100% ethanol
- YPD Medium and YPDS plates containing the appropriate concentration of Zeocin™
- 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)

Continued on next page

Pichia Transformation, Continued

Linearizing Your pPICZ-E or pPICZα-E Construct

To promote integration, we recommend that you linearize your pPICZ-E or pPICZα-E fusion plasmid within the 5′ *AOX1* region. The table below lists unique sites that may be used to linearize pPICZ-E or pPICZα-E prior to transformation. **Other restriction sites are possible**. 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	New England Biolabs
Pme I	414	New England Biolabs
*Bst*X I	707	New England Biolabs

Restriction Digest

1. Digest ~10-50 µg of plasmid DNA with one of the enzymes listed above. We recommend to use more DNA as you will lose some DNA during purification after the restriction digestion and will be helpful if you decide to do multiple transformations with varying concentrations of Zeocin™.
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. You can also use any resin based purification methods to purify the linearized vector.
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.

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 x g for 5 minutes at +4°C. Resuspend the pellet with 500 ml of ice-cold (0°C), sterile water.
4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 ml of ice-cold (0°C), sterile water.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold (0°C) 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 ml of ice-cold (0°C) 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use that day. Do not store cells.

Continued on next page

Pichia Transformation, Continued

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 0.2 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 500 µl of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 ml tube. Rinse the cuvette with additional 500 µl of ice-cold 1 M sorbitol and transfer the contents to the 15 ml tube as above.
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™. **Note**: Inclusion of sorbitol in YPD plates stabilizes electroporated cells as they appear to be somewhat osmotically sensitive
7. Incubate plates for 2 to 7 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™.

Isolation of Multi-copy Recombinants *in vivo*

A quick, direct way to select multi-copy recombinants is to plate the transformation mix on increasing concentrations of Zeocin™.

1. Prepare YPDS plates containing 100, 500, 1000, and 2000 µg/ml Zeocin™
2. Plate 100 to 200 µl of the transformation mix on each plate and incubate at 30°C for 2 to 7 days.
3. Test transformants for the Mut phenotype (page 22) and expression of your protein (page 25)

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 twenty 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, see above.
2. After 1 hour in 1 M sorbitol at 30°C (Step 5, see above), 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™.
5. Incubate plates for 2 to 7 days at 30°C until colonies form.

Analyzing *Pichia* Transformants

Select 6-10 of your Zeocin™-resistant *Pichia* transformants and confirm the Mut phenotype as described on page 22. You can also analyze for the presence of insert using PCR (page 55), or for copy number using Southern analysis (page 60).

EasyComp™ Transformation

Introduction

The *Pichia* EasyComp™ Kit produces chemically competent *Pichia* cells and is included to provide a rapid and convenient alternative to electroporation. However, because of the low transformation efficiency (3 µg plasmid DNA yields about 50 colonies), it is very difficult to isolate multi-copy integrants. If multi-copy integrants are desired, use electroporation (page 16) for best results. **Do not use cells prepared using the EasyComp™ protocol for electroporation as cells are prepared differently for each method.**

Required Reagents and Equipment

- 30°C rotary shaking incubator
- YPD (Yeast Extract Peptone Dextrose) medium (see **Recipes**, page 41)
- 50 ml, sterile conical tubes
- Centrifuge suitable for 50 ml conical tubes (floor or table-top)
- 1.5 ml sterile screw-cap microcentrifuge tubes
- Styrofoam box or paper towels

Before Beginning

Streak a YPD plate with your *Pichia pastoris* strain such that isolated, single colonies will grow. Incubate the plate at 28-30°C for 2 days.

Equilibrate Solution I to room temperature.

Preparing Competent Cells

1. Inoculate 10 ml of YPD with a single colony of your *Pichia* strain. Grow overnight at 28-30°C in a shaking incubator (250-300 rpm).
2. Dilute cells from the overnight culture to an OD_{600} of 0.1-0.2 in 10 ml of YPD. Grow the cells at 28-30°C in a shaking incubator until the OD_{600} reached 0.6-1.0. This will take approximately 4 to 6 hours.
3. Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.
4. Resuspend the cell pellet in 10 ml of Solution I. No incubation time is required.
5. Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.
6. Resuspend the cell pellet in 1 ml of Solution I. The cells are now competent.
7. Aliquot 50 to 200 µl of competent cells into labeled 1.5 ml sterile screw-cap microcentrifuge tubes.

   Note: 50 µl of cells are used for each transformation. Cells can be thawed and refrozen several times without significant loss in transformation efficiency.
8. At this point, the cells may be kept at room temperature, used directly for transformation or frozen for future use. To slowly freeze the cells, place tubes in a Styrofoam box or wrap in several layers of paper towels and place in a -80°C freezer. **Do not snap-freeze the cells in liquid nitrogen.**
9. Proceed to the transformation procedure.

Note

Higher transformation efficiencies are obtained with frozen versus freshly prepared cells. You may choose to use some of the cells immediately following preparation and freeze the remaining cells in small aliquots.

Continued on next page

EasyComp™ Transformation, Continued

Transformation

The following protocol can be used to transform either freshly prepared or frozen competent *Pichia* cells. Transformation efficiency may vary with each strain and vector used.

Required Reagents and Equipment

- 30°C incubator
- Water baths or heat blocks at 30°C and 42°C
- Microcentrifuge at room temperature
- YPDS with 100 µg/ml Zeocin™ plates (see **Recipes**, page 41)

Before Beginning

If you see a precipitate in Solution II, warm the solution at 37°C until the precipitate dissolves. To prevent formation of a precipitate, store Solution II at room temperature.

Equilibrate Solution III to room temperature.

Equilibrate the appropriate number and type of plates to room temperature. You will need one plate for each transformation.

You may want to include controls. We recommend a no DNA and a plasmid only control.

Transformation Protocol

1. For each transformation, thaw one tube of competent cells at room temperature and aliquot 50 µl into a sterile microcentrifuge tube. If transforming fresh cells, use 50 µl of cells from **Preparation of Competent Cells**, Step 7, previous page.
2. Add 3 µg of linearized *Pichia* expression vector DNA to the competent cells.

   Note: Using greater than 3 µg of DNA may increase transformation efficiencies in some cases. The volume of DNA should not exceed 5 µl. Linearized DNA can be used directly from a restriction digest reaction without affecting transformation efficiency. Phenol chloroform extraction and ethanol precipitation are not necessary.
3. Add 1 ml of Solution II to DNA/cell mixture. Mix by vortexing or flicking the tube.
4. Incubate the transformation reactions for 1 hour at 30°C in a water bath or incubator. Mix the transformation reaction every 15 minutes by vortexing or flicking the tube. Failure to mix the transformation reaction every 15 minutes will result in decreased transformation efficiency.
5. Heat shock the cells in a 42°C heat block or water bath for 10 minutes.
6. Split the cells into 2 microcentrifuge tubes (approximately 525 µl per tube) and add 1 ml of YPD medium to each tube.
7. Incubate the cells at 30°C for 1 hour to allow expression of Zeocin™ resistance.
8. Pellet the cells by centrifugation at 3000 x g for 5 minutes at room temperature. Discard the supernatant.
9. Resuspend each tube of cells in 500 µl of Solution III and combine the cells into one tube.
10. Pellet the cells by centrifugation at 3000 x g for 5 minutes at room temperature. Discard the supernatant.
11. Resuspend the cell pellet in 100 to 150 µl of Solution III.
12. Plate the entire transformation on appropriate selection plates using a sterile spreader. Incubate the plates for 2 to 4 days at 30°C. Each transformation should yield approximately 50 colonies.

Continued on next page

EasyComp™ Transformation, Continued

Analyzing *Pichia* Transformants

Select 6-10 of your Zeocin™-resistant *Pichia* transformants and confirm the Mut phenotype as described on page 22. You may also wish to analyze for the presence of insert using PCR (page 55).

Troubleshooting

The table below provides solutions to possible problems you may encounter when preparing and transforming competent *Pichia pastoris* cells.

Problem	Probable Cause	Possible Solution
Low efficiency of transformation	The pH of Solution I or Solution III may have drifted. The pH of both solutions should be 8.0	Check the pH of Solutions I and III. If the pH is low, increase it by adding NaOH. If the pH is high, decrease it by adding HCl. Store solutions at +4°C in order to minimize drift in pH.
	Transformation reaction not mixed during incubation	Be sure to mix the transformation reaction every 15 minutes throughout the 1 hour incubation at 30°C. Vortexing works best.
	Incubation time is too short or temperature is too low	*Pichia pastoris* transformations may be incubated for longer periods of time (up to 3 hours) and at higher temperature (35-37°C). This may, in some instances, result in higher transformation efficiencies.
	Cell density is too low (OD_{600} <0.6)	Resuspend cells from Preparation of Competent Cells, Step 6, page 19, in a smaller volume (i.e. 500 µl).

Determining the Mut Phenotype

Introduction

To verify the Mut phenotype of your Zeocin™-resistant transformants, refer to the guidelines below. **Note**: KM71H recombinants do not need to be screened for their Mut phenotype as they all will be Mut^S.

Screening for Mut$^+$ in X-33 and GS115

Transformation of X-33 or GS115 with linearized constructs favor single crossover recombination at the *AOX1* locus and result in Mut$^+$ transformants. However, with the presence of the *AOX1* sequences in the plasmid, there is a chance that recombination will also occur in the 3´*AOX1* region, disrupting the wild-type *AOX1* gene and creating Mut^S transformants. Testing on MDH and MMH plates will allow you to confirm the Mut$^+$ phenotype (see below).

Mut^S in KM71H

All Zeo^R transformants in KM71H will be Mut^S because of the disruption of the *AOX1* gene (*aox1::ARG4*). There is no need to test recombinants for the Mut phenotype. Transformants need to be purified on minimal plates to ensure pure clonal isolates before either testing for expression (see page 28) or confirming integration by PCR (page 55).

Important

Host strains containing the *his4* allele (e.g. GS115) and transformed with the pPICZ-E or pPICZα-E vectors require histidine when grown in minimal media. Add histidine to a final concentration of 0.004% to ensure growth of your transformant.

We recommend purifying your Zeo^R transformants to ensure isolation of a pure clonal isolates. This is done by streaking for single colonies on YPD or minimal plates with histidine. You may do this before or after testing for the Mut phenotype.

Preparation

The following media (see page 42-43) and materials can be prepared several days in advance and stored at +4°C:

- Minimal Dextrose with histidine (MDH) agar plates, 1 liter
- Minimal Methanol with histidine (MMH) agar plates, 1 liter
- Sterile toothpicks and Scoring Templates (see page 24).

Mut$^+$ in GS115 or X-33

Use the plates containing the Zeo^R transformants and confirm the Mut$^+$ phenotype as described below. **Note**: Instructions are for GS115 strains. These strains require histidine for growth. X-33 **does not** require histidine for growth, so you may leave it out of the medium.

1. Pick one colony using a sterile toothpick and streak/patch one Zeo^R transformant in a regular pattern first on an MMH plate and then on an MDH plate.
3. Use a new toothpick for each transformant and continue until 10 transformants have been patched (1 plate).
4. To differentiate Mut$^+$ from Mut^s, make one patch for each of the controls (GS115/ Albumin Mut^s and GS115/pPICZ/*lacZ* Mut$^+$) onto the MDH and MMH plates.
5. Incubate the plates at 30°C for 2 days.
6. After >2 days at 30°C, score the plates (see page 24). Mut$^+$ strains will grow normally on both plates, while Mut^S strains will grow normally on the MDH plate but show little or no growth on the MMH plate.

Continued on next page

Determining the Mut Phenotype, Continued

Replica-Plating Procedure

This procedure gives a lower rate of misclassifications, but it increases the overall Mut^+/Mut^S screening procedure by 2 days. You will need equipment to replica-plate.

1. Using sterile toothpicks, patch 10 Zeo^R transformants on an MDH plate.
2. Incubate the plate at 28-30°C for 2 days.
3. After 2 days, replica-plate the patches from the MDH plate onto fresh MMH and MDH plates to screen for Mut^S transformants.
4. Incubate the replica plates at 28-30°C for 2 days.
5. After 2 days at 28-30°C, score the replica plates. Look for patches that grow normally on the MDH replica plate but show little or no growth on the MMH replica plate.

Screening by Functional Assay

Some researchers have used a functional assay to directly screen for high expressing *Pichia* recombinant clones without first screening for Mut^S or Mut^+ phenotypes. If you elect to screen directly for high-expressing recombinants, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

Multiple Integration Events

Pichia pastoris is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology (see page 52 for figure). Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants.

Successful expression of the gene of interest to useful levels may depend upon the generation of a recombinant strain that contains multiple copies integrated at the *AOX1* locus. In addition to simply screening expression levels among several Mut^S or Mut^+ recombinants via SDS-PAGE analysis, it may be desirable to determine the existence of strains that have multiple integrants in the Mut^S or Mut^+ recombinant strain.

See page 58 for methods to determine copy number.

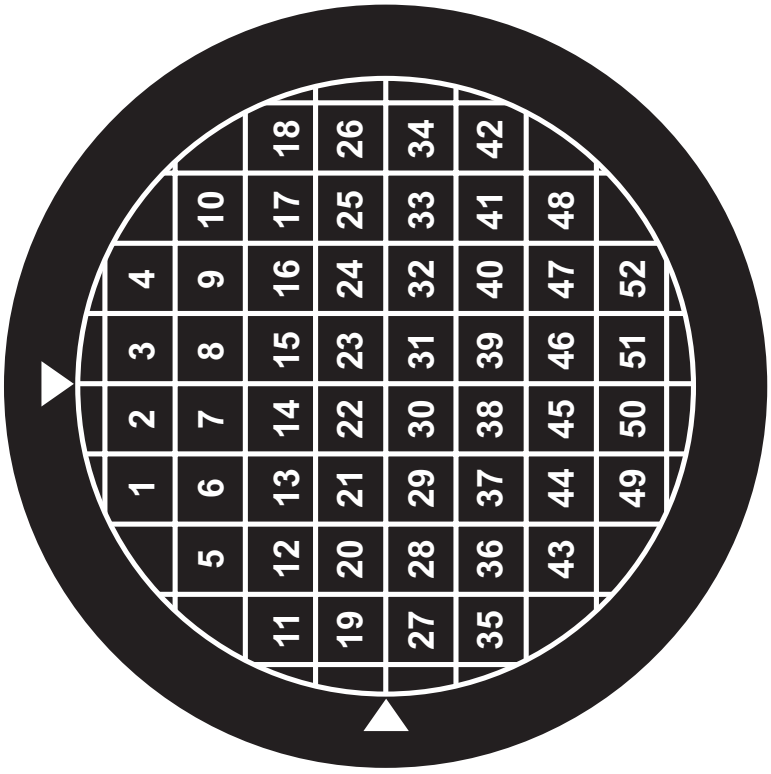
The Next Step

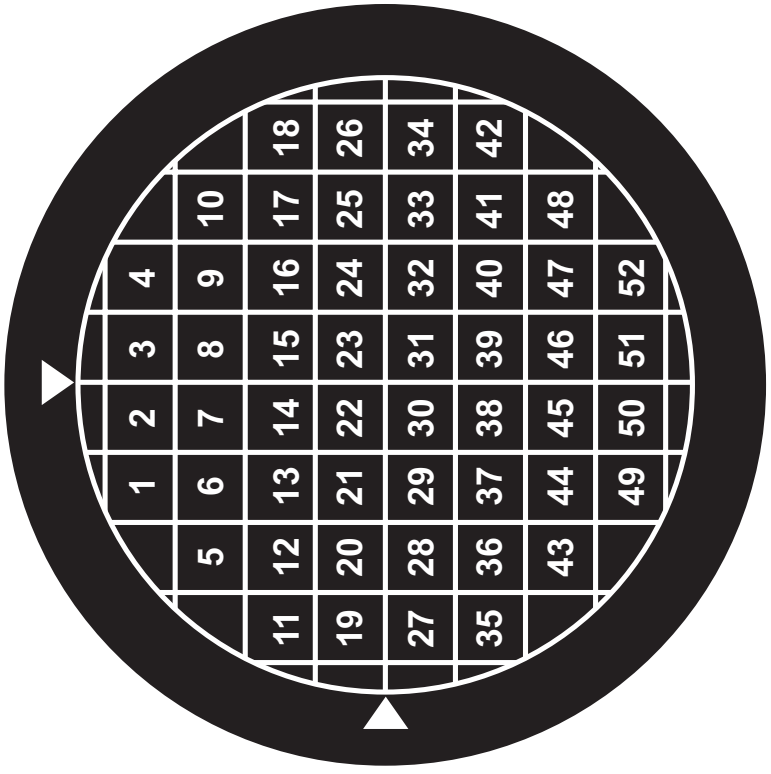
After confirming the Mut phenotype, you may proceed to small-scale expression (page 25) to test for expression of your gene.

Continued on next page

Determining the Mut Phenotype, Continued

Scoring Templates





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.

Control Strains

Use the positive control strains, pPICZ-E/Uni-*lacZ* or pPICZα-E/Uni-HSA transformed into a *Pichia* host, which you made in the previous section (see page 16), to test for expression. Expression in shake flasks is detectable after 48 hours for *lacZ* and reaches a maximum at 96 hours (4 days). β-galactosidase (116 kDa) can be detected by Coomassie Blue staining of the SDS-PAGE gel or performing the β-galactosidase assay (β-Gal Assay Kit, Catalog no. K1455-01). **Note**: β-galactosidase does not contain a C-terminal tag and cannot be detected using either anti-V5 or anti-His(C-term) antibodies. If the expression level is low, then use anti-β-galactosidase antibody to detect expression.

HSA (69 kDa) expression starts as early as 24 hours in the medium and reaches a maximum by 96 hours as determined by Coomassie® Blue staining of the SDS/PAGE gel. If you need to do a western analysis for detection, then use anti-V5 or anti-His(C-term) antibody.

You may also use the strains, GS115/pPICZ/*lacZ* or GS115/Albumin as positive controls for intracellular and secreted expression, respectively. GS115/pPICZ/*lacZ* expresses β-galactosidase (121 kDa) fused at the C-terminus to the *myc* epitope and the polyhistidine tag. GS115/Albumin secretes albumin (67 kDa) into the medium.

Note

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.

Detection

We recommend that you use the following techniques to assay expression of your protein.

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-V5 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

Important

Host strains containing the *his4* allele (e.g. GS115) and transformed with the pPICZ-E or pPICZα-E vectors require histidine when grown in minimal media. Add histidine to a final concentration of 0.004% to ensure growth of your transformant. Complex media such as YPD and BMGY already contain histidine.

Media

You will need either BMGY/BMMY (buffered complex glycerol or methanol medium), BMGH/BMMH (buffered minimal glycerol or methanol medium containing histidine) or MGYH/MMH (minimal glycerol or minimal methanol medium containing histidine) for expression (see **Recipes**, pages 43-44). BMGH, BMMH, BMGY, and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein. Unlike MGYH and MMH, they are all buffered media. Because these media are buffered with phosphate buffer, a wide range of pH values may be used to optimize production of your protein. BMGY/BMMY contain yeast extract and peptone to stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. Inclusion of yeast extract and peptone allow better growth and biomass accumulation.

Proteases

There are some proteins specifically susceptible to proteases that have optimal activity at neutral pH. If this is the case, use MGYH or MMH media for expression. As *Pichia* expression progresses in an unbuffered medium such as MMH, the pH drops to 3 or below, inactivating many neutral pH proteases (Brierley et al., 1994). *Pichia* is resistant to low pH, so the low pH will not affect growth. In contrast, it has been reported that by including 1% Casamino acids (Difco) and buffering the medium at pH 6.0, extracellular proteases were inhibited, increasing the yield of mouse epidermal growth factor (Clare et al., 1991b).

If your secreted protein of interest is susceptible to neutral pH proteases you may prefer to do your expressions in an unbuffered medium (MMH). If your secreted protein of interest is not susceptible to proteases at neutral pH, then you can use BMMY for initial expression. Try expression in an unbuffered medium if the expressed protein is degraded.

Aeration

The most important parameter for efficient expression in *Pichia* is adequate aeration during methanol induction. As a general rule when inducing expression, never allow cultures to be more than 10-30% of your total flask volume. It is strongly recommended that baffled flasks be used. See page 44 for suppliers of baffled flasks. Cover the flasks with cheesecloth (2-3 layers) or another loose fitting cover. Never use tight fitting covers. (Aeration is not as critical when generating biomass before induction.)

Kinetics of Growth

Note that while Mut^+ and Mut^S strains will grow at essentially the same rate in YPD or glycerol media, Mut^+ will grow faster than Mut^S when both are grown on methanol because of the presence of the *AOX1* gene product.

Temperature and Shaking

All expression is done at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. If your incubator temperature fluctuates, set the temperature at 28°C. If using a floor shaking incubator, shake at 225-250 rpm. If using a table-top shaker that sits inside an incubator, shake at 250-300 rpm.

Continued on next page

Signal Sequence Processing

The processing of the α-factor signal sequence in pPICZα–E 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.

Before Starting

When performing your expression, it is important to run the proper controls so that you will be able to interpret your expression results. The expression controls which should be used are:

pPICZα-E/Uni-HSA in *Pichia* strain of your choice	Secretion control
pPICZ-E/Uni-*lac*Z in *Pichia* strain of your choice	Intracellular control
GS115/pPICZ/*lacZ* Mut^+	Mut^+-Intracellular control
GS115/Albumin Mut^s	Mut^s-Secretion control
Appropriate P*ichia* strain /Vector (no insert)	Background control

Since recombination can occur in many different ways that can effect expression (clonal variation), we recommend you screen 6-10 verified recombinant clones for expression levels. Start with fresh colonies as colony viability drops over time. (You may also start the cultures with a small sample from a frozen glycerol stock that was generated from a single colony).

Guidelines for Expression

The following steps should be viewed as guidelines and are presented to get you started with expression. You may have to change the conditions to optimize expression for your particular protein. Use bottom or side baffled flasks whenever possible (available in 50 - 2000 ml sizes). If you are analyzing a number of recombinants, you can try 50 ml conical tubes. Be sure that the medium is well-aerated by increasing the rate of shaking or placing the tubes at an angle in the shaker.

Continued on next page

Expression in *Pichia*, Continued

Mut$^+$ Intracellular or Secreted

To evaluate expression, we recommend including the control strains discussed earlier (see page 16). Include X-33, GS115, or KM71H transformed with the parent vector as a control for background expression.

1. Inoculate a single colony into 25 ml of MGYH, BMGH, or BMGY in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches log phase (OD_{600} = 2-6) (approximately 16-18 hours).
2. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD_{600} of 1.0 in MMH, BMMH, or BMMY medium to induce expression (approximately 100-200 ml).
3. Place culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.
5. At each of the time points (below), transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature.

   Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).
6. 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/alcohol bath.

   For intracellular expression, decant the supernatant and store just the cell pellets at -80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.
7. Analyze the supernatants and cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot or functional assay (see page 30).

Continued on next page

Expression in *Pichia*, Continued

Mut^S Intracellular or Secreted

To evaluate expression, we recommend including the control strains discussed earlier (see page 16). Include X-33, GS115 or KM71H transformed with the parent vector as a control for background expression.

1. Using a single colony, inoculate 100 ml of MGYH, BMGH, or BMGY in a 1 liter baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until the culture reaches an OD_{600} = 2-6 (approximately 16-18 hours.)
2. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. To induce expression, decant the supernatant, and resuspend cell pellet in MMH, BMMH, or BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10-20 ml).
3. Place in a 100 ml baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue to grow.
4. Add 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction.
5. At each of the time points (below) transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction 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), 120 (5 days), and 144 (6 days).
6. 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/alcohol bath.

   For intracellular expression, decant the supernatant, and store just the cell pellets at -80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.
7. Analyze the cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot or functional assay (see **Analysis by SDS-Polyacrylamide Gel Electrophoresis**, next page).

Note

The C-terminal tag will add 2.5 kDa to the size of your protein and the α-factor signal sequence will add 9.3 kDa to the size of the protein, if it is not processed. Be sure to account for any additional amino acids that are in between the signal sequence site and the N-terminus of your protein, and also the end of your protein and the C-terminal tag.

Analysis by SDS-Polyacrylamide Gel Electrophoresis

Introduction

This section provides guidelines to prepare and analyze your samples using SDS polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels are available from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. 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 Web site (www.invitrogen.com) or call Technical Service (see page 61).

Preparing Samples

You will need to prepare Breaking Buffer (see page 44) and have acid-washed 0.5 mm glass beads on hand.

Preparation of cell pellets (Intracellular and Secreted Expression):

1. Thaw cell pellets quickly and place on ice.
2. For each 1 ml sample, add 100 µl Breaking Buffer to the cell pellet and resuspend.
3. Add an equal volume of acid-washed glass beads (size 0.5 mm). Estimate equal volume by displacement.
4. Vortex 30 seconds, then incubate on ice for 30 seconds. Repeat for a total of 8 cycles.
5. Centrifuge at maximum speed for 10 minutes at +4°C. Transfer the clear supernatant to a fresh microcentrifuge tube.
6. Take 50 µl of supernatant (cell lysate) and mix with 50 µl 2X SDS-PAGE Gel Loading buffer (Sample Buffer).
7. Boil for 10 minutes and load 10-20 µl per well. Thickness of the gel and number of wells will determine volume loaded. Remaining sample may be stored at -20°C for western blots, if necessary. Cell lysates may be stored at -80°C for further analysis.

Preparation of supernatant (Secreted Expression only):

1. Thaw supernatants and place on ice.
2. Mix 50 µl of the supernatant with 50 µl of SDS-PAGE Gel Loading buffer.
3. Boil 10 minutes, then load 10-30 µl onto the gel. Remaining sample may be stored at -20°C for western blots, if necessary. Supernatants may be stored at -80°C for further analysis.
4. If no protein is seen by Coomassie or by western blot, then concentrate the supernatant 5-10 fold and analyze samples again by western blot. Centricon and Centriprep filters (Amicon) are very useful for this purpose.

Continued on next page

Analysis by SDS-Polyacrylamide Gel Electrophoresis, Continued

Protein Concentration

Lowry, BCA (Pierce) or Bradford protein determinations can be performed to quantify the amounts of protein in the cell lysates and medium supernatants. In general, *Pichia* cell lysates contain 5-10 µg/µl protein. *Pichia* medium supernatants will vary in protein concentration primarily due to the amount of your secreted protein. *Pichia* secretes very few native proteins. If the protein concentration of the medium is > 50 µg/ml, 10 µl of medium will give a faint band on a Coomassie-stained SDS-PAGE gel.

Controls

Include the following samples as controls on your SDS-PAGE:

- Molecular weight standards appropriate for your desired protein
- A sample of your protein as a standard (if available)
- A sample of X-33, GS115, or KM71H with the parent plasmid transformed into it. Inclusion of this sample shows the background of native *Pichia* proteins that are present intracellularly and will help you differentiate your protein from background
- Analyze the *lac*Z and HSA controls also as they should indicate any problems with the media or expression conditions

In addition to Coomassie-stained SDS-PAGE, we strongly recommend performing a western blot or another more sensitive assay to detect your protein. Visualization of the expressed protein will depend on several factors, including its expression level, its solubility, its molecular weight, and whether it will be masked by an abundant cellular protein of the same size.

Western Blot Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page xiii for ordering information) or an antibody to your protein of interest. The ready-to-use WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. The Positope™ Control Protein from Invitrogen can be used a s a positive control for detection of fusion proteins containing a V5 epitope and a polyhistidine tag. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 61).

Analysis of Protein Expression

The Coomassie-stained SDS-PAGE gel should reveal the induction over time of your recombinant protein co-migrating with your standard. If you are satisfied with the level of expression, try a test purification (page 36) or scale-up expression (page 34).

If there is no recombinant protein visible, then perform either a western blot or a functional assay if available.

If you detect low expression of your recombinant protein, see **Optimization of *Pichia* Protein Expression**, page 31, for guidelines to optimize expression.

Test your expression conditions with the control strains included in the kit (see page 25).

If there is no indication of expression at all, use PCR to analyze your recombinants for the correctly sized PCR product (page 55). If you find that you have recombinants, perform a northern analysis to see if and how much full-length mRNA is induced (see page 60).

Optimization of *Pichia* Protein Expression

Introduction

Based on available data, there is approximately a 50 to 75% chance of expressing your protein of interest in *Pichia pastoris* at reasonable levels. The biggest hurdle seems to be generating initial success-i.e. expression of your protein at any level. While there are relatively few examples of expression of >10 grams/liter, there are many examples of expression in the >1 gram/liter range, making the *Pichia pastoris* expression system one of the most productive eukaryotic expression systems available. Likewise, there are several examples of proteins that have been successfully expressed in *Pichia pastoris* that were completely unsuccessful in baculovirus or *Saccharomyces cerevisiae*, suggesting that the *Pichia pastoris* system is an important alternative to have available. If you obtain no or low protein expression in your initial expression experiment, use the following guidelines to optimize expression.

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 secreting your protein, check to see if your protein is susceptible to neutral pH proteases by expressing in unbuffered medium (MMH). In addition, try 1% Casamino acids with buffered medium to inhibit extracellular proteases.

Low Secreted Expression Levels

- Check 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 or α-factor signal sequence).
- Concentrate your supernatant by ammonium sulfate precipitation or ultrafiltration (see page 35).
- For Mut^+, induce expression with a higher density culture.

Low Expression Levels

- Look for multi-copy recombinants (i.e. jackpot clones) by dot blot (see page 57). There are quite a few examples of increasing the expression levels of a particular protein by increasing the gene dosage. See Clare, 1991a; Clare *et al.*, 1991b; Romanos *et al.*, 1991).
- Check both Mut^+ and Mut^S recombinants for increased expression. Some proteins express better in one type of genetic background than another.
- If secreting your protein, try intracellular expression. The protein may not be processed correctly and fail to secrete. Be sure you check your cell pellets for evidence of expression. If you are having problems with intracellular expression, try secreting your protein. It probably will glycosylate which may be desirable or not. If glycosylation is undesirable, oligosaccharides can be removed with Peptide:N-Glycosidase F (New England BioLabs).
- Scale up to fermentation (page 35). *Pichia* is a yeast and is particularly well suited to fermentation.

Continued on next page

Optimization of *Pichia* Protein Expression, Continued

No Expression

Be sure to try some of the things listed above as no expression can be the same thing as very low expression. If none of these things improve protein expression, use PCR to check for insertion of your gene into the Pichia genome (page 55). If your gene is present, perform a northern blot analysis to check for transcription of your gene. There is a protocol in the **Appendix** for RNA isolation from *Pichia* (see page 60).

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 HIV-1 gp120, ATTATTTTAT AAA, which when expressed in *Pichia* gave premature termination of the mRNA. When this sequence was changed, longer transcripts were found (Scorer *et al.*, 1993).

Hyperglycosylation

If your protein is hyperglycosylated:

- Try intracellular expression as your protein will not go through the secretion pathway and therefore, not be modified.
- Try deglycosylating the protein with Peptide:N-Glycosidase F or other enzymes.

Scale-up of Expression

Introduction

Once expression is optimized, the expression protocol can be scaled up to produce more protein by increasing the culture volume using larger baffled flasks or fermentation. Use the guidelines below to scale-up your expression protocol.

Mut$^+$ Intracellular or Secreted

1. Using a single colony, inoculate 25 ml of MGYH, BMGH, or BMGY in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD_{600} = 2-6 (approximately 16-18 hours).
2. Use this 25 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28-30°C with vigorous shaking (250-300 rpm) until the culture reaches log phase growth (OD_{600} = 2-6).
3. Harvest the cells using sterile centrifuge bottles by centrifuging at 1500-3000 x g for 5 minutes. To induce expression, decant the supernatant and resuspend cell pellet to an OD_{600} = 1.0 (2-6 liters) in MMH, BMMH, or BMMY medium to start induction.
4. Aliquot the culture between several 3 or 4 liter baffled flask. Cover the flasks with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28-30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal time of induction is reached as determined from the time course study.
6. Harvest cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature.

   For intracellular expression, decant the supernatant. Cells can be processed immediately or stored at -80°C until ready for use.

   For secreted expression, **save the supernatant, chill to +4°C, and concentrate it if desired** (see next page). Proceed directly to purification (page 36) or store the supernatant at -80°C until ready to process further.

Mut^S Intracellular or Secreted

1. Using a single colony, inoculate 10 ml of MGYH, BMGH, or BMGY in a 100 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) to an OD_{600} = 2-6 (approximately 16-18 hours).
2. Use this 10 ml culture to inoculate 1 liter of MGYH, BMGH, or BMGY in a 3 or 4 liter baffled flask and grow at 28-30°C with vigorous shaking (250-300 rpm) until the culture reaches log phase growth (OD_{600} = 2-6).
3. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes. To induce expression, decant the supernatant and resuspend cell pellet in 1/5 to 1/10 of the original culture volume of MMH, BMMH, or BMMY medium (~ 100-200 ml).
4. Place the culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator. Continue to grow at 28-30°C with shaking.
5. Add 100% methanol to 0.5% every 24 hours until the optimal induction time is reached.
6. Harvest cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature.

   For intracellular expression, decant the supernatant and store the cell pellets at -80°C until ready to process.

   For secreted expression, **save the supernatant, chill to +4°C, and concentrate it if desired** (see next page). Proceed directly to purification (page 36) or store the supernatant at -80°C until ready to process further.

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Scale-up of Expression, Continued

Note

To increase the amount of cells for Mut^S recombinants, increase the number of flasks, put 200-300 ml in a 3 liter flask, or try fermentation.

Concentration of Proteins

Proteins secreted into the media are usually > 50% homogeneous and will require some additional purification (see page 35). It is optimal to concentrate the protein if the expression level is not particularly high. There are several general methods to concentrate proteins secreted from *Pichia*. A general guide to protein techniques is *Protein Methods* (Bollag & Edelstein, 1991). Some general methods include:

- Ammonium sulfate precipitation
- Centrifuge concentrator for small volumes (e.g. Centricon or Centriprep devices available from Amicon)
- Pressurized cell concentrators for large volumes (Amicon ultrafiltration devices)
- Lyophilization

Cell Lysis

A general procedure for cell lysis using glass beads is provided on the next page. There is also a cell lysis protocol in *Current Protocols in Molecular Biology*, page 13.13.4. (Ausubel *et al.*, 1994) and in *Guide to Protein Purification* (Deutscher, 1990). We also recommend lysis by French Press (follow the manufacturer's suggestions for yeast).

Fermentation

Basic guidelines are available for fermentation of *Pichia* from Invitrogen. We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Call Technical Service (see page 61) for more information or you may download the manual from our Web site at (www.invitrogen.com).

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 them at -80°C until you are ready to purify your fusion protein, or you may proceed directly with protein purification. **This section only describes the 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 (Catalog no. K850-01) to purify fusion proteins expressed from pPICZ-E or pPICZα-E. N**ote that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification Kit. You may download the manual from our Web site (www.invitrogen.com) or call Technical Service (see page 61).**

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.

Important

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

Ordering Information

ProBond™ resin is available precharged and prepacked in 2 ml columns or in bulk quantities. For ordering information, refer to page xiv.

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Preparation of Cell Lysates

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). 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 the **Recipes**, page 44. **Do not add 1 mM EDTA to the breaking buffer as it will interfere with binding to Probond™.**

1. Wash cells once in BB by resuspending them and centrifuging 5-10 minutes at 3000 x 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 x 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)

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 (2-3 mg/ml total protein) of *Pichia* lysate 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 x g) and carefully remove the supernatant. Save the supernatant to check for unbound protein.
5. Repeat Steps 2 through 4 with the remaining 4 ml of diluted 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.

Continued on next page

Note

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

Analysis of Purification

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

Scale-up

You may find it necessary to scale-up your purification to obtain sufficient amounts of purified protein. Adjust the pH and NaCl concentration of your lysate with 1/10 volume of 10X Stock Solution B (ProBond™ Purification Kit) before adding it to the column. The pH should be greater than or equal to 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.

Pichia Media Recipes

Using *Pichia* Media

The table below is designed to help you decide which *Pichia* medium to use for a particular application.

Medium	Description	Application
YPD or YEPD	Rich, complex broth	General growth and storage
YPDS + Zeocin™	YPD with sorbitol and Zeocin™	Selection of Pichia Zeo^R transformants
MGYH or MGY	Minimal medium containing glycerol and/or histidine	Intracellular Expression: Generation of biomass prior to methanol induction
MDH or MD	Minimal medium containing glucose and/or histidine	Determination of Mut phenotype
MMH or MM	Minimal medium containing methanol and/or histidine	Determination of Mut phenotype Intracellular expression of desired protein
BMGH or BMG	Buffered minimal medium containing glycerol and/or histidine	Secreted Expression: Used to control the pH of the medium and generate biomass
BMMH or BMM	Buffered minimal medium containing methanol and/or histidine	Secreted Expression: Used to control the pH of the medium and induce expression of the desired protein
BMGY	Buffered complex medium containing glycerol	Secreted Expression: Used to control the pH of the medium, decrease protease activity, and generate biomass.
BMMY	Buffered complex medium containing methanol	Secreted Expression: Used to control the pH of the medium, decrease protease activity, and induce expression.

Continued on next page

Pichia Media Recipes, Continued

Stock Solutions

10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at +4°C. Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate. The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch. **Note:** *Pichia* cells exhibit optimal growth with higher YNB concentrations, therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for *Saccharomyces*.

500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at +4°C. The shelf life of this solution is approximately one year.

100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilize and store at +4°C. The shelf life of this solution is approximately one year.

10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

10X M (5% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filter sterilize and store at +4°C. The shelf life of this solution is approximately two months.

10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

Continued on next page

Pichia Media Recipes, Continued

YPD or YEPD

Yeast Extract Peptone Dextrose Medium (1 liter)

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

Note: If you are using the YP Base Medium or the YP Base Agar medium pouches included with the Original Pichia Expression Kit, 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 10X D.

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 (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 ~60°C and add 100 ml of 10X D. Add 1.0 ml of 100 mg/ml Zeocin™, if desired.

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

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YPDS + Zeocin™ Agar

Yeast Extract Peptone Dextrose Medium (1 liter)

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

1. Dissolve 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 10X D.
5. Cool solution to ~60°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.

MGY and MGYH

Minimal Glycerol Medium ± Histidine (1 liter)

1.34% YNB
1% glycerol
4 x 10^{-5}% biotin
± 0.004% histidine

1. Combine aseptically 800 ml autoclaved water with 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X GY.
2. For growth of *his4* strains in this medium, a version can be made that contains histidine (called MGYH) by adding 10 ml of 100X H stock solution.

Store at +4°C. The shelf life of this solution is approximately two months.

MD and MDH

Minimal Dextrose Medium ± Histidine (1 liter)

1.34% YNB
4 x 10^{-5} % biotin
2% dextrose

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
2. Cool to about 60°C and then add:

   100 ml of 10X YNB
   2 ml of 500X B
   100 ml of 10X D
3. To make MDH, add 10 ml of 100X H stock solution. Mix and store at +4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed.
5. If preparing plates, pour the plates immediately. MD stores well for several months at +4°C.

Continued on next page

Pichia Media Recipes, Continued

MM and MMH

Minimal Methanol + Histidine (1 liter)

1.34% YNB
4 x 10^{-5}% biotin
0.5% methanol

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
2. Cool autoclaved water to 60°C and add:

   100 ml of 10X YNB
   2 ml of 500X B
   100 ml of 10X M
3. To make MMH, add 10 ml of 100X H stock solution. Mix and store at +4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed.
5. After mixing, pour the plates immediately. MM and MMH stores well for several months at +4°C.

BMGH and BMMH

Buffered Minimal Glycerol
Buffered Minimal Methanol (1 liter)

100 mM potassium phosphate, pH 6.0
1.34% YNB
4 x 10^{-5}% biotin
1% glycerol or 0.5% methanol

1. Autoclave 690 ml water for 20 minutes on liquid cycle.
2. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0
   100 ml 10X YNB
   2 ml 500X B
   100 ml 10X GY
3. For BMMH, add 100 ml 10X M instead of glycerol.
4. To add histidine, add 10 ml of 100X H stock solution. Mix and store at +4°C.
5. Store media at +4°C. The shelf life of this solution is approximately two months.

Continued on next page

Pichia Media Recipes, Continued

BMGY and BMMY

Buffered Glycerol-complex Medium
Buffered Methanol-complex Medium (1 liter)

1% yeast extract
2% peptone
100 mM potassium phosphate, pH 6.0
1.34% YNB
4 x 10^{-5}% biotin
1% glycerol or 0.5% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0
   100 ml 10X YNB
   2 ml 500X B
   100 ml 10X GY
4. For BMMY, add 100 ml 10X M instead of glycerol.
5. Store media at +4°C. The shelf life of this solution is approximately two months.

Breaking Buffer

50 mM sodium phosphate, pH 7.4
1 mM PMSF (phenylmethylsulfonyl fluoride or other protease inhibitors)
1 mM EDTA (Omit EDTA if using this buffer for purification on metal chelating resins)
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.

Vendors for Baffled Flasks

Bellco (1-800-257-7043) has a wide variety of baffled flasks from 50 to 2000 ml.

Wheaton (1-609-825-1100) only sells side baffle flasks.

Appendix

Recipes

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

LB (Luria-Bertani) Medium

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

SOB Medium (with Kanamycin)

SOB (per liter)

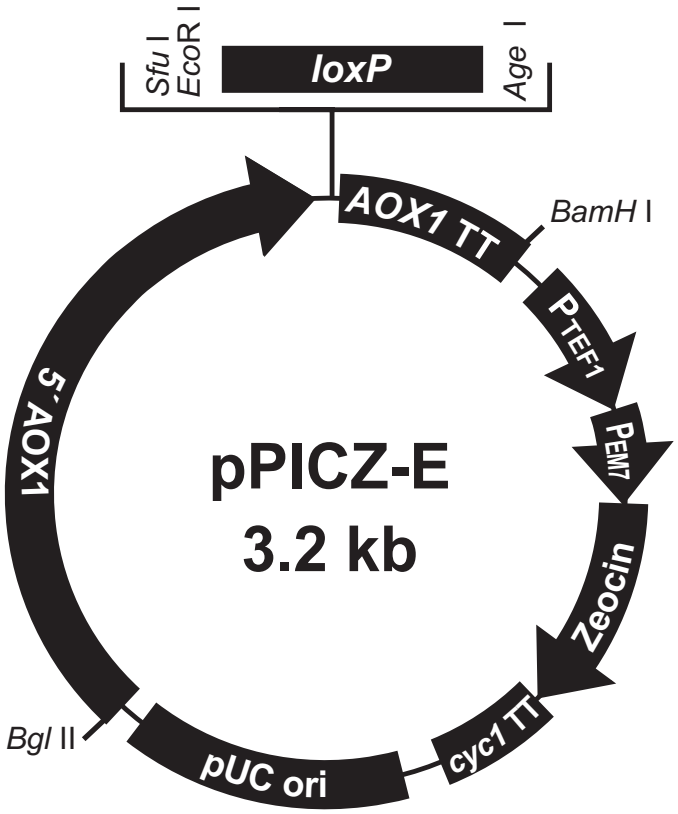
2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add kanamycin to 50 μg/ml.
5. Store at +4°C. Medium is stable for only ~1 month.

Maps of pPICZ-E and pPICZα-E

Map of pPICZ-E

The figure below summarizes the features of the pPICZ-E vector. **The complete sequence for pPICZ-E is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 61)**. Details of the sequences surrounding the *loxP* site in pPICZ-E may be found on page 10.



Comments for pPICZ-E
3180 nucleotides

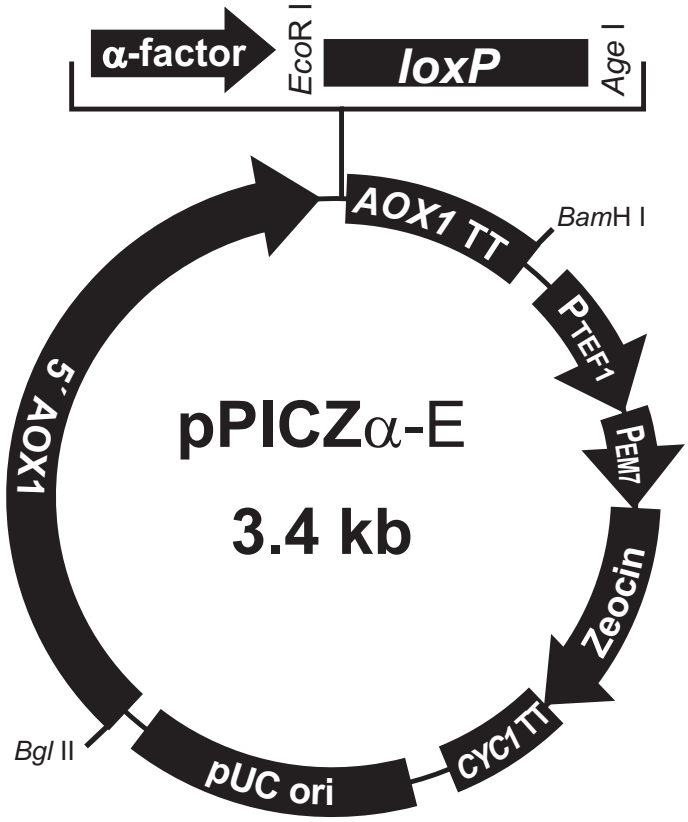
5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
loxP site: bases 949-982
AOX1 transcription termination region: bases 983-1269
TEF1 promoter: bases 1270-1678
EM7 promoter: bases 1683-1749
Zeocin resistance gene: bases 1750-2124
CYC1 transcription termination region: bases 2125-2442
pUC origin: bases 2450-3179

Continued on next page

Maps of pPICZ-E and pPICZα-E, Continued

Map of pPICZα-E

The figure below summarizes the features of the pPICZα-E vector. **The complete sequence for pPICZα-E is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 61).** Details of the sequences surrounding the *loxP* site in pPICZα-E may be found on page 11.



Comments for pPICZα-E
3445 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
α-factor priming site: bases 1144-1164
loxP site: bases 1214-1247
3´ *AOX1* priming site: bases 1275-1295
AOX1 transcription termination region: bases 1248-1534
TEF1 promoter: bases:1535-1943
EM7 promoter: bases: 1948-2014
Zeocin resistance gene: bases: 2015-2389
CYC1 transcription termination region: bases: 2390-2707
pUC origin: bases: 2715-3444

Continued on next page

Features of pPICZ-E and pPICZα-E

Features

The important elements of pPICZ-E (3.2 kb) and pPICZα-E (3.4 kb) are described in the table below. All features have been functionally tested.

Feature	Benefit
5´ *AOX1*	A 942 bp fragment containing the *AOX1* promoter that allows methanol-inducible, high-level expression in *Pichia.* Targets plasmid integration to the *AOX1* locus.
Native *Saccharomyces cerevisiae* α-factor secretion signal in pPICZα-E	Allows for efficient secretion of most proteins from *Pichia.*
loxP site	Allows recombination between the donor vector and pPICZ-E or pPICZα-E acceptor vector ((Hoess et al., 1982).
AOX1 Transcription Termination (TT)	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 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.
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 high copy replication and maintenance of the plasmid in *E. coli.*
Sac I *Pme* I *Bst*X I	Unique restriction sites that permit linearization of the vectors at the *AOX1* locus for efficient integration into the *Pichia* genome.

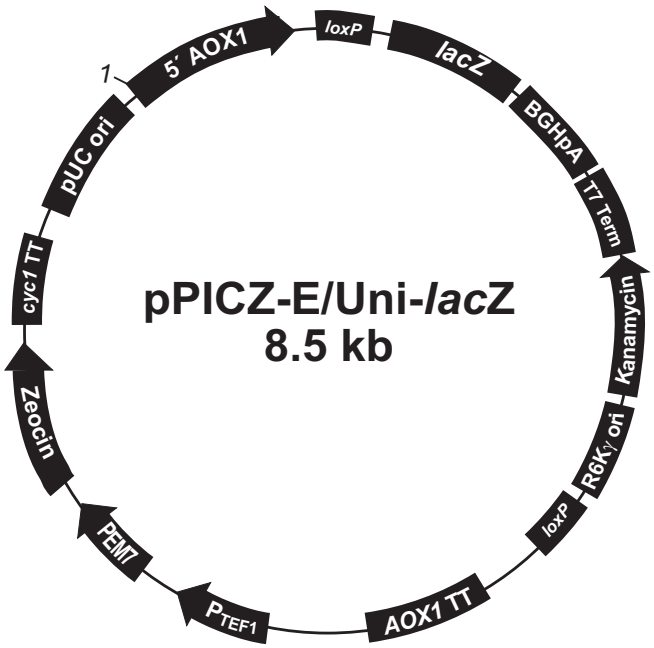
Map of pPICZ-E/Uni-*lacZ*

Description

pPICZ-E/Uni-*lacZ* is a 8.5 kb control vector expressing a β-galactosidase protein (116 kDa). The *lacZ* gene was amplified and cloned into a donor vector, pUni/V5-His-Gene-TOPO®. The resulting vector was recombined with pPICZ-E using Cre recombinase to create pPICZ-E/Uni-*lacZ*. **Note**: pUniV5/Gene-TOPO® is similar to pUni/V5-His-TOPO® TA except that it contains additional DNA between the TOPO® Cloning site and the V5 epitope.

Map of pPICZ-E/Uni-*lacZ*

The figure below summarizes the features of pPICZ-E/Uni-*lacZ* vector. **The complete nucleotide sequence for pPICZ-E/Uni-*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 61).**



Comments for pPICZ-E/Uni-*lacZ*
8550 nucleotides

5´ *AOX1* promoter region: bases 1-942
5´ *AOX1* priming site: bases 855-875
loxP site: bases 949-982
lacZ ORF: bases 1003-4062
BGH polyadenylation region: bases 4222-4430
T7 transcriptional termination region: bases 4445-4573
Kanamycin resistance gene: bases 4752-5546 (C)
kan promoter region: bases 5547-5684 (C)
R6Kγ origin: bases 6018-6290
loxP site: bases 6319-6352
AOX1 transcription termination region: bases 6353-6639
TEF1 promoter: bases 6640-7048
EM7 promoter: bases 7053-7101
Zeocin resistance gene: bases 7120-7494
CYC1 transcription termination region: bases 7495-7812
pUC origin: bases 7822-8496
(C) = complementary strand

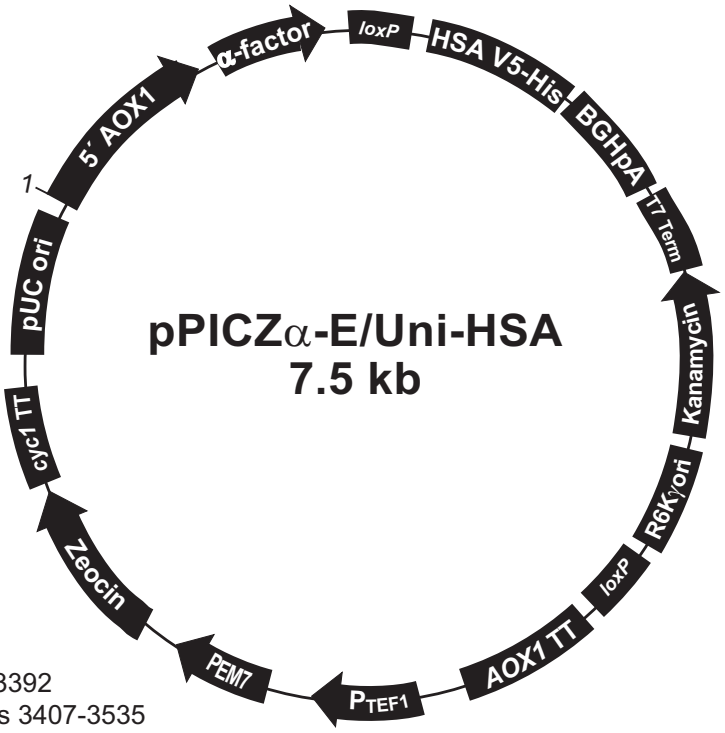
Map of pPICZα-E/Uni-HSA

Description

pPICZα-E/Uni-HSA is a 7.5 kb control vector expressing a human serum albumin (HSA) protein (69 kDa). The HSA gene was amplified and cloned into a donor vector, pUni/V5-His-Gene-TOPO®. The resulting vector was recombined with pPICZα-E using Cre recombinase to create pPICZα-E/Uni-HSA **Note:** pUniV5/Gene-TOPO® is similar to pUni/V5-His-TOPO® TA except that it contains additional DNA between the TOPO® Cloning site and the V5 epitope.

Map of pPICZα-E/Uni-HSA

The figure below summarizes the features of pPICZα-E/Uni-HSA vector. **The complete nucleotide sequence for pPICZα-E/Uni-HSA is available for downloading from our Web Site (www.invitrogen.com) or by contacting Technical Service (see page 61).**



Comments for pPICZα-E/Uni-HSA
7512 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 942-1207
α-factor priming site: bases 1144-1164
loxP site: bases 1214-1247
HSA gene: bases 1268-3024
V5 epitope: bases 3035-3076
6xHis tag: bases 3086-3103
BGH polyadenylation region: bases 3184-3392
T7 transcriptional termination region: bases 3407-3535
Kanamycin resistance gene: bases 3714-4508 (C)
kan promoter region: bases 4509-4646 (C)
R6Kγ origin: bases 4827-5266
loxP site: bases 5281-5314
AOX1 transcription termination region: bases 5315-5601
TEF1 promoter: bases 5602-6010
EM7 promoter: bases 6015-6081
Zeocin resistance gene: bases 6082-6456
CYC1 transcription termination region: bases 6457-6774
pUC origin: bases 6785-7511
(C) =complementary strand

Recombination and Integration in *Pichia*

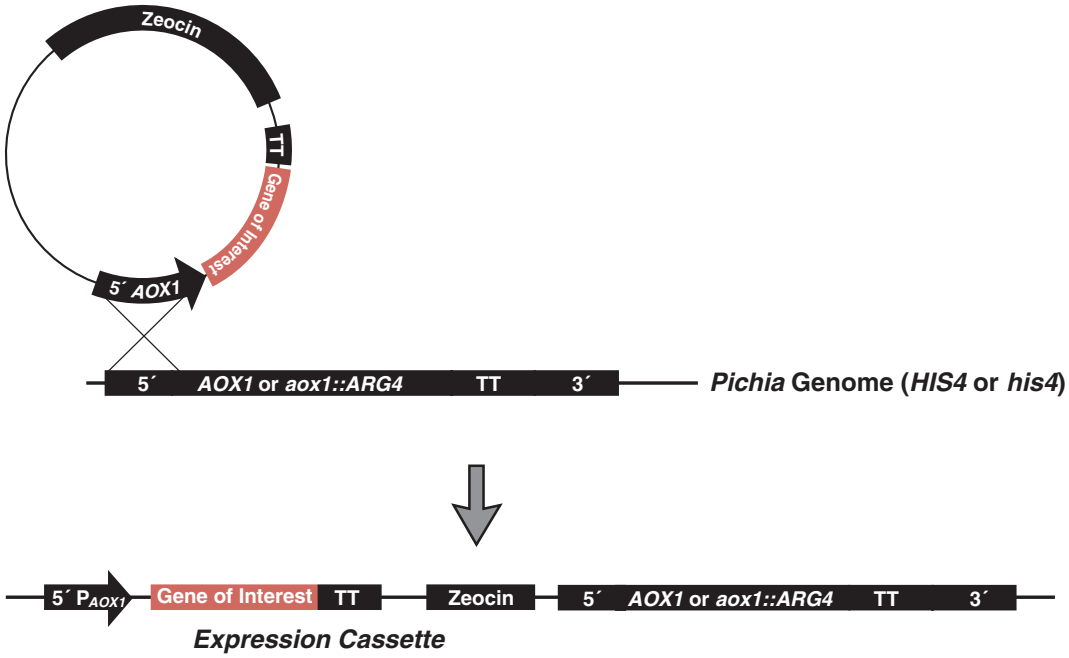
Introduction

Like *Saccharomyces cerevisiae*, 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 *AOX1* or *aox1::ARG4*

Gene insertion events at the *AOX1* (X-33 or GS115) or *aox1::ARG4* (KM71H) loci arise from a single crossover event between the loci and either of the two *AOX1* regions on the pPICZ-E or pPICZα-E vectors: the *AOX1* promoter or the *AOX1* transcription termination region (TT). This results in the insertion of one or more copies of the vector upstream or downstream of the *AOX1* or the *aox1::ARG4* genes. The phenotype of such a transformant is Mut^+ (X-33 or GS115) or Mut^S (KM71H). **By linearizing the recombinant vector at a restriction enzyme site located in the 5´ *AOX1* regions, Mut^+ or Mut^S recombinants can be conveniently generated depending on the host strain used.**

The figure below shows the result of an insertion of the plasmid 5´ to the intact *AOX1* locus (Mut^+) and the gain of P_{AOX1}, your gene of interest, and the Zeocin™ resistance gene. This also occurs with non-linearized plasmid and plasmid that religates, although at a lower frequency.



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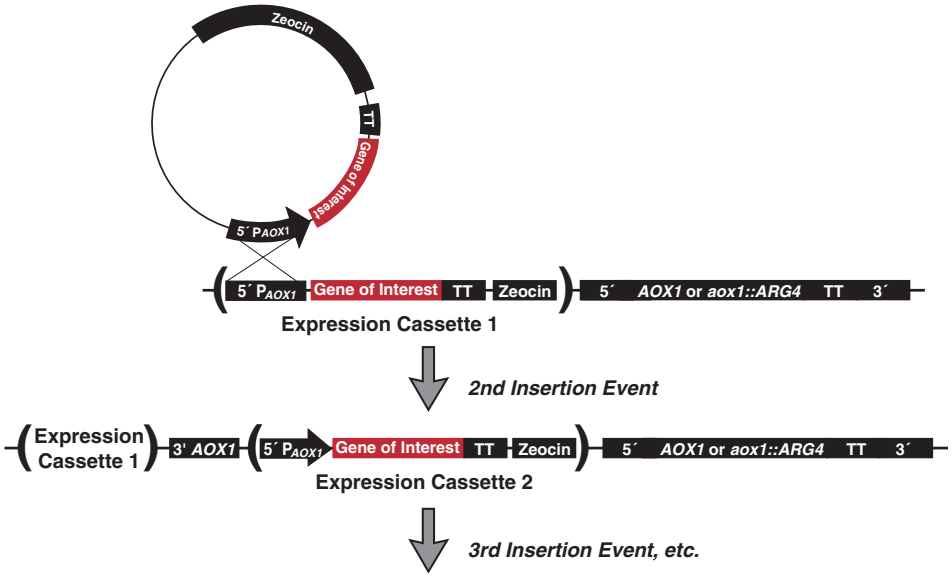
Recombination and Integration in *Pichia*, Continued

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™-resistant transformants to locate these "jack-pot" clones. We recommend that you use electroporation to generate Zeo^R transformants for screening.

Multi-copy events can occur as gene insertions either at the *AOX1* or the *aox1::ARG4* loci. This results in a Mut$^+$ phenotype in X-33 or GS115 and a Mut^S phenotype in KM71H. Multiple gene insertion events can be detected by quantitative dot blot analysis, Southern blot analysis, and differential hybridization. See page 18 for a protocol to screen for multiple inserts.



Zeocin™

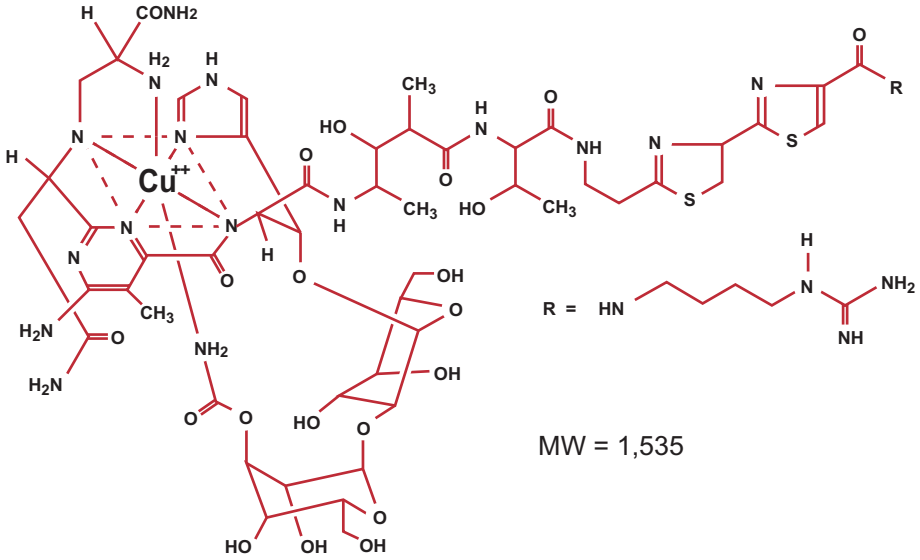
Zeocin™

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 45 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 45). 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.

Direct PCR Screening of *Pichia* Clones

Introduction

A simple protocol has been reported in the literature to directly test *Pichia* clones for insertion of your gene by PCR (Linder et al., 1996) Briefly, the cells are lysed by a combination of enzyme, freezing, and heating treatment. The genomic DNA can be used directly as a PCR template.

Before Starting

You will need the following reagents and equipment on hand:

- A culture or single colony of a *Pichia* transformant
- 1.5 ml microcentrifuge tube
- 5 U/µl solution of Lyticase (Sigma)
- 30°C water bath or heat block
- Liquid nitrogen
- Reagents for PCR

Procedure

1. Place 10 µl of a *Pichia pastoris* culture into a 1.5 ml microcentrifuge tube. For relatively dense cultures, dilute 1 µl of the culture into 9 µl water. Alternatively, pick a single colony and resuspend in 10 µl of water.
2. Add 5 µl of a 5 U/µl solution of lyticase and incubate at 30°C for 10 minutes.
3. Freeze the sample at -80°C for 10 minutes or immerse in liquid nitrogen for 1 minute.
4. Set up a 50 µl PCR for a hot start:

10X Reaction Buffer	5 µl
25 mM $MgCl_2$	5 µl
25 mM dNTPs	1 µl
5´ *AOX1* primer (10 pmol/µl)	1 µl
3´ *AOX1* primer (10 pmol/µl)	1 µl
Sterile water	27 µl
Cell lysate	5 µl
Total Volume	45 µl

5. Mix the solution and overlay with 20 µl of mineral oil.
6. Place the solution in the thermocyler and incubate at 95°C for 5 minutes.
7. Add 5 µl of a 0.16 U/µl solution of *Taq* polymerase (0.8 units).
8. Cycle 30 times using the following parameters:

Step	Temperature	Time
Denaturation	95°C	1 minute
Annealing	54°C	1 minute
Extension	72°C	1 minute

   Include a final extension of 7 minutes.
9. Analyze a 10 µl aliquot by agarose gel electrophoresis.

Total DNA Isolation from *Pichia*

Introduction

The protocol below allows you to isolate DNA from the desired recombinant which is suitable for Southern blot analysis, dot/slot blot analysis or genomic PCR. See *Current Protocols in Molecular Biology*, pages 13.11.1 to 13.11.4 (Ausubel *et al.*, 1994), *Guide to Yeast Genetics and Molecular Biology*, pages 322-323 (Strathern & Higgins, 1991), or (Holm et al., 1986).

Solutions

You will need to make the following solutions. There is not enough of some of these reagents in the kit to perform this experiment

- Minimal Medium (MD, MGYH)
- Sterile water
- SCED (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT)
- Zymolyase, 3 mg/ml stock solution in water (Seikagaku America, Inc., 1-800-237-4512)
- 1% SDS in water
- 5 M potassium acetate, pH 8.9
- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)
- 7.5 M ammonium acetate, pH 7.5
- Phenol:chloroform (1:1 v/v)

Preparation

1. Grow at 30°C the recombinant strain and the parent strain to an OD_{600} of 5-10 in 10 ml of minimal media such as MD or MDH. **Note**: *his4* strains require histidine for growth.
2. Collect the cells by centrifugation at 1500 x g for 5-10 minutes at room temperature.
3. Wash the cells with 10 ml sterile water by centrifugation as in Step 2.

Spheroplasting and Lysis

1. Resuspend the cells in 2 ml of SCED buffer, pH 7.5. Make this solution fresh.
2. Add 0.1-0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve < 80% spheroplasting.
3. Add 2 ml of 1% SDS, mix **gently** and set on ice (0 to +4°C) for 5 minutes.
4. Add 1.5 ml of 5 M potassium acetate, pH 8.9, and mix **gently**.
5. Centrifuge at 10,000 x g for 5-10 minutes at +4°C and save the supernatant.

Continued on next page

Total DNA Isolation from *Pichia*, Continued

DNA Precipitation

1. Transfer the supernatant from Step 5 above and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
2. Centrifuge at 10,000 x g for 20 minutes at +4°C.
3. Resuspend the pellet **gently** in 0.7 ml of TE buffer, pH 7.4 and transfer to a microcentrifuge tube.
4. **Gently** extract with an equal volume of phenol:chloroform (1:1 v/v) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Split the aqueous layer into two microcentrifuge tubes.
5. Add 1/2 volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. Place on dry ice for 10 minutes or at -20°C for 60 minutes.
6. Centrifuge at 10,000 x g for 20 minutes at +4°C and wash the pellets once with 1 ml of 70% ethanol. Air dry the pellets and resuspend each in 50 µl of TE buffer, pH 7.5. Determine the concentration of the DNA sample. The two samples can be stored separately or combined and stored at -20°C until ready for use.

Determination of Copy Number of Multiple Integrants

Introduction

You may wish to determine the actual number of gene copies in your *Pichia* recombinant using either quantitative dot blots or Southern hybridization. ((Brierley et al., 1994; Clare et al., 1991a; Romanos et al., 1991; Scorer et al., 1993; Scorer et al., 1994)). This requires isolation of genomic DNA from *Pichia* recombinants transformed with the parent vector (0 copies of your gene), pPICZ-E or pPICZα-E containing 1 copy of your gene (single copy control), and the *Pichia* recombinants containing multiple copies of your gene. Use the protocol detailed on the previous page to isolate genomic DNA or you may use any method of your choice.

Quantitative Dot Blot Solutions

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

- 50 mM EDTA, 2.5% β-mercaptoethanol pH 9
- 1 mg/ml Zymolyase 100T in water (Seikagaku America, Inc., 1-800-237-4512)
- 0.1N NaOH, 1.5 M NaCl
- 2 x SSC
- 3MM paper

Quantitative Dot Blot Procedure

The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos, *et al*., 1991). It is very important to spot equivalent numbers of cells onto filters in order to quantify copy number. Genomic DNA may also be isolated and spotted directly onto nitrocellulose or nylon, fixed, and analyzed.

1. Grow Mut^+ or Mut^S transformants in individual wells of a 96-well microtiter plate in 200 µl of YPD broth at 30°C until all wells have approximately the same density. Alternatively, individual transformants may be grown in culture tubes and the absorbance at 600 nm normalized with the addition of medium.
2. Filter 50 µl of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multi-channel pipettor. Air dry filters.
3. Lyse the cells on the filter with four solutions as follows: soak two sheets of 3 MM paper in a tray with 10-15 ml of 50 mM EDTA, 2.5% β-mercaptoethanol pH 9. After the paper is uniformly soaked, place the nitrocellulose filter face down on the treated 3MM paper. Incubate for 15 minutes at room temperature.
4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak with 10-15 ml of 1 mg/ml Zymolyase 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate for 4 hours at 37°C.
5. Remove the nitrocellulose filter from the paper and replace the paper with two new sheets. Soak with 10-15 ml of 0.1 N NaOH, 1.5 M NaCl. Place the nitrocellulose filter face down on the paper and incubate for 5 minutes at room temperature.
6. Remove the nitrocellulose filter and replace with two new 3MM sheets. Soak with 10-15 ml of 2 x SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate for 5 minutes at room temperature. Repeat.
7. Bake the nitrocellulose filters at 80°C or UV-crosslink DNA to nylon. The filters may be probed with a nonradioactive-labeled or random-primed, ^{32}P-labeled probe complementary to your gene.

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

Continued on next page

Determination of Copy Number of Multiple Integrants, Continued

Southern Blot Analysis

For a detailed description of this technique as applied to *Pichia pastoris*, see (Clare, *et al*., 1991a). It is very important to digest your DNA with the right restriction enzyme(s) to generate a blot of digested and gel-separated genomic DNA. We recommend that you use a restriction enzyme that cuts **outside** of the expression cassette. This will generate a restriction fragment whose size reflects the number of multimers. For example, if a 1.2 kb fragment is cloned into pichia vector and transformed into *Pichia*, DNA from this recombinant can be digested with *Nhe* I (which does not cut within the vector or the insert). The size of the fragment will increase in additions of 4.7 kb over the size of the fragment containing 1 copy of the gene.

Alternatively, digestion of DNA from *Pichia* recombinants containing multiple copies will produce a band that will vary in intensity depending on the number of copies of your gene. It is very important to include a control to show the intensity of a single copy gene. The band intensities can be relatively quantified using densitometry to estimate gene dosage.

Controls

It is very important to include DNA from the host strain alone (X-33, GS115 or KM71H), the host strain transformed with the parent vector, and the host strain transformed with a vector containing one copy of your gene.

General Guidelines

- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al*., 1989), pages 9.31-9.58.
- Isolate genomic DNA and quantify using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane in order to accurately determine copy number if you are using relative intensities.
- Probe your Southern blot with a fragment complementary to your gene

Procedure for Total RNA Isolation from *Pichia*

Introduction

This protocol is designed to isolate 60-300 µg total RNA (Schmitt et al., 1990) from *Pichia* which is suitable for mRNA isolation using Invitrogen's FastTrack® 2.0 or Micro FastTrack™ 2.0 mRNA Isolation Kit. If you wish to use another protocol, you should scale-up the reaction to yield about 2 mg of total RNA per time point. The mRNA is for northern blot analysis of *Pichia* recombinants to determine if the gene of interest is being induced and transcribed. RNA isolation should be done from induced cultures using an uninduced culture as a negative control.

Solutions

You will need the following solutions. Remember to use DEPC-treated water and to use equipment free of RNase.

MGYH or BMGY medium
AE buffer (50 mM sodium acetate, pH 5.3, 1 mM EDTA)
10% SDS in DEPC treated water
Chloroform:isoamyl alcohol (24:1)
3 M sodium acetate, pH 5.3
DEPC-treated water
Buffered phenol
Phenol:chloroform (1:1)
65°C water bath

Growth of Cells

1. Grow up two cultures (100-200 ml in MGYH or BMGY), but induce only one of them. Use the same protocol for induction that you used in the **Expression** section.
2. Take 10 ml time points at 1, 2, 3, 4, and 6 days.
3. Harvest the cells from each time point by centrifugation at 1500 x g for 10 minutes at room temperature.
4. Resuspend cell pellet in 400 µl AE buffer and transfer to a microcentrifuge tube.

Lysis of Cells

1. Add 40 µl 10% SDS and vortex for ~ 20 seconds.
2. Add an equal volume (450-500 µl) of buffer saturated phenol and vortex for ~20 seconds.
3. Incubate at 65°C for 4 minutes.
4. Incubate in a dry ice/ethanol bath until crystals show (~1 minute). Centrifuge at maximum speed for 2 minutes at +4°C.
5. Transfer aqueous phase to new centrifuge tube and add an equal volume of phenol/chloroform and vortex for ~20 seconds. Centrifuge at maximum speed for 2 minutes at +4°C.
6. Remove upper phase to a new tube and add 40 µl of 3 M sodium acetate, pH 5.3 and 2.5 volumes of 100% ethanol (-20°C). Centrifuge at maximum speed for 15 minutes at +4°C. Remove ethanol.
7. Wash pellet with 80% ethanol and air dry briefly. Resuspend total RNA in 20 µl DEPC-treated water and store at -80°C. Yield is 60-300 µg total RNA.

mRNA Isolation and Northern Analysis

See (Ausubel *et al.*, 1994) for a protocol for mRNA isolation and northern analysis. The FastTrack® 2.0 mRNA Kit (Catalog no K1593-02; 6 reactions) is designed to isolate mRNA from 0.2 to 1 mg total RNA. The Micro-FastTrack™ 2.0 Kit (Catalog no. K1520-02; 20 reactions) is designed to isolate mRNA from ~100 µg total RNA. You will need approximately 1-5 µg mRNA per time point.

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