P. methanolica Expression Kit

A Manual of Methods for Expression of Recombinant Proteins in *Pichia methanolica*

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User Manual

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Kit Contents and Storage

Shipping and Storage

The *P. methanolica* Expression Kit is shipped at room temperature. Upon receipt, store as follows:

- Vectors and primers: –20°C
- BM-Y Medium: Room temperature
- Pichia methanolica stabs: 4°C

Vectors and Primers

Store at –20°C.

Reagent	Amount
pMET A, B, and C	20 µg each (40 µL at 0.5 µg/µL) in TE*, pH 8
pMETα A, B, and C	20 µg each (40 µL at 0.5 µg/µL) in TE*, pH 8
AUG1 Forward sequencing primer	2 µg, lyophilized in TE*, pH 8
AUG1 Reverse sequencing primer	2 µg, lyophilized in TE*, pH 8
α-Factor sequencing primer	2 µg, lyophilized in TE*, pH 8

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

Primer Sequences

The table below provides the sequence and moles supplied of each primer included in the kit.

Primer	Sequence	pMoles Supplied
AUG1 Forward	5´-CAATTTACATCTTTATTTATTAACG-3´	265
AUG1 Reverse	5´-GAAGAGAAAAACATTAGTTGGC-3´	293
α-Factor	5´-TACTATTGCCAGCATTGCTGC-3´	315

Media

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

Media	Amount	Yield
BM-Y	2 pouches	2 liters of BM-Y medium

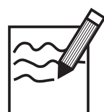
continued on next page

Strains

Store *P. methanolica* stabs at 4°C

P. methanolica	Amount	Genotype	Phenotype
PMAD11	1 stab	*ade2-11*	Ade^- Mut^+
PMAD16	1 stab	*ade2-11 pep4Δ prb1Δ*	Ade^- Mut^+
PMAD16/pMETα B/HSA	1 stab	*ade2-11 pep4Δ prb1Δ ADE2(S.c.) HSA(H.s.)**	Ade^+ Mut^+

*Indicates that the strain contains integrated copies of the *Saccharomyces cerevisae ADE2* gene and the human serum albumin (HSA) gene. For more information, see page 8.



Note

The *Pichia* stabs supplied with the kit are guaranteed until the expiration date marked on the tube when stored at 4°C. We recommend you prepare a set of glycerol master stocks (page 11) prior to using your *Pichia* cells.

Materials Supplied by the User

- 30°C rotary shaking incubator
- 30°C incubator
- Centrifuge suitable for 50 mL conical tubes (floor or table-top)
- Baffled cultured flasks with metal covers (50 mL, 250 mL, 500 mL, 1000 mL, and 3000 mL)
- 50 mL sterile, conical tubes
- 6 mL and 15 mL sterile snap-top tubes
- UV Spectrophotometer
- Restriction enzymes and appropriate buffers
- Agarose gel apparatus and buffers
- Polyacrylamide gel electrophoresis apparatus and buffers
- Media for transformation, growth, screening, and expression (see **Appendix**, pages 36–40)
- Sterile cheesecloth or gauze
- Breaking Buffer (see page 40)
- Acid-washed glass beads (available from Sigma-Aldrich, Cat. no. G8772)
- Replica-plating equipment (optional)
- Bead Beater™ (optional)
- ProBond™ Purification System (optional)

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Vendors for Baffled Flasks

Bellco Glass at www.bellcoglass.com has a wide variety of baffled flasks, ranging from 50 to 4000 mL.

Wheaton Science Products at www.wheatonsci.com only sells side baffled flasks.

Introduction

Description of the System

Before Starting

The information presented here is designed to give you a concise overview of the *Pichia methanolica* expression system. This gene expression technology is not as well-characterized as *Pichia pastoris* or *Saccharomyces cerevisiae*. For a general review of heterologous gene expression in yeast, see Romanos, et al., 1992.

General Characteristics of *Pichia methanolica*

Pichia methanolica is a homothallic haploid organism that offers many of the advantages of a eukaryotic expression system such as protein processing and protein folding, 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 it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *P. methanolica* very useful as a protein expression system.

Similarity to *Saccharomyces*

Many techniques developed for *Saccharomyces* may be applied to *P. methanolica*, including transformation by complementation, gene disruption, and gene replacement. There is cross-complementation between all tested gene products in both *Saccharomyces* and *P. methanolica*. Several wild-type genes from *Saccharomyces* complement comparable mutant genes in *P. methanolica*. Genes such as *ADE2* and *LEU2* complement their respective mutant genes in *P. methanolica* (Hiep *et al.*, 1993b; Raymond *et al.*, 1998).

Differences from *Saccharomyces*

The major difference between *Saccharomyces* and *P. methanolica* is that non-homologous integration is strongly favored over homologous integration during transformation. This is similar to mammalian cell transformation and is not the case in *Saccharomyces* (Hiep *et al.*, 1993b).

Pichia methanolica as a Methylotrophic Yeast

P. methanolica is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. 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 (Kulachkovshy *et al.*, 1997). The promoter regulating the production of alcohol oxidase is the one used to express heterologous genes in *P. methanolica*.

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Two Alcohol Oxidase Proteins

Two genes in *P. methanolica* code for alcohol oxidase-*AUG1* and *AUG2* (alcohol utilizing gene) (Raymond *et al.*, 1998). The *AUG1* gene is responsible for the majority of alcohol oxidase activity in the cell. The *AUG1* gene has been isolated and a plasmid-borne version of the *AUG1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein (Raymond *et al.*, 1998). The nucleotide sequence of *AUG2* is ~83% identical to *AUG1*; strains with an *aug1Δ* genotype grow slowly on methanol. Slow growth on methanol allows isolation of Mut^S strains (*aug1*) (Raymond *et al.*, 1998).

Expression

Expression of the *AUG1* gene is repressed by dextrose (D-glucose) and induced with methanol. Unlike *Pichia pastoris*, *P. methanolica* efficiently shifts from utilizing dextrose as a carbon source to methanol. Grow *P. methanolica* cultures on dextrose and then starve for dextrose prior to induction with methanol.

A Note About Terminology

To differentiate media containing glucose and media containing glycerol, the word 'dextrose' (D) is used to indicate D-glucose.

Phenotype of *AUG1* mutants

Loss of the *AUG1* gene causes a loss of most of the cell's alcohol oxidase activity, resulting in a strain that is phenotypically Mut^S (Methanol utilization slow) and exhibits 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. Identifying the Mut phenotype of your *P. methanolica* transformant may help you optimize growth conditions.

Intracellular and Secretory Protein Expression

Heterologous expression in *P. methanolica* can be intracellular or secreted. Secretion requires an amino-terminal signal sequence for targeting to the secretory pathway. The secretion signal sequence from the *Saccharomyces cerevisiae* α-factor prepro peptide has been used with success (Raymond *et al.*, 1998).

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Posttranslational Modifications

Posttranslational modifications have not been well-characterized in *P. methanolica*, but the α-factor signal sequence will be processed correctly (Raymond *et al.*, 1998). Other types of posttranslational modifications have not been characterized. In general, glycosylated, recombinant proteins produced in yeast systems may not be compatible with mammalian systems because of differences in glycosylation patterns. In particular, proteins bearing yeast glycosyl modifications are probably not suitable as injectable therapeutics. Use *P. methanolica* to express nonglycosylated proteins or remove potential glycosylation sites from the desired protein by site-directed mutagenesis if your desired protein is for use in mammals. Carbohydrate moieties can also be removed after expression by treatment with Endoglycosidase H or Peptide:N-glycosidase F (see page 35).

Proteins Expressed in *P. methanolica*

P. methanolica expression is a relatively new system and few nonproprietary examples of recombinant protein expression exist. Intracellular expression levels of human glutamate decarboxylase at 500 mg/liter have been documented using *P. methanolica* (Raymond *et al.*, 1998). Secreted protein expression levels have also been documented for the extracellular domain of the human cytokine receptor (10 mg/liter) and human leptin (500 mg/liter) (Raymond, 1999). All expressions were performed under fermentation conditions.

Experimental Outline

Experimental Process

The table below outlines the steps necessary to create and test strains for expression of your gene of interest. In addition, each step is discussed in detail elsewhere in the manual. Page numbers are provided so you can read about particular steps of interest. Below the table is a general discussion of the steps needed to express your heterologous protein in *P. methanolica*.

Step	Procedure	Page
1	Decide whether you want to express your protein intracellularly or secrete it.	10
2	Clone your desired gene into pMET or pMETα.	10–18
3	Transform *E. coli*, select ampicillin-resistant transformants, and confirm presence and orientation of desired gene.	19–20
4	Excise the expression cassette with the appropriate restriction enzymes.	21
5	Transform PMAD11 or PMAD16 (*P. methanolica* strains) and select Ade^+ transformants.	22–24
6	Analyze several transformants for expression.	25–30
7	Select the highest expressing clones, optimize expression, and scale-up expression for protein purification.	31–34

Selecting the Vector and Cloning

Two expression vectors are included in this kit. Each vector utilizes the strong, inducible *AUG1* promoter for expression of your gene of interest.

pMET is supplied as three different versions to facilitate in-frame cloning with the C-terminal peptide encoding the V5 epitope and the 6xHis tag. It can be used for intracellular expression or expression of a secreted protein using the protein's own native signal sequence.

pMETα is used for secreted expression and is also supplied as three different versions to facilitate in-frame cloning with the N-terminal α-factor secretion signal. pMETα also contains the C-terminal peptide encoding the V5 epitope and the 6×His tag.

Both vectors utilize the wild-type *ADE2* gene from *Saccharomyces cerevisiae* (*ScADE2*) to select transformants.

Before cloning your insert, first:

- Decide whether you want intracellular or secreted expression.
- **Analyze your insert for the following restriction sites**: *Apa* I, *Fse* I, *Pac* I, *Asc* I, *Kpn* I, and *Pst* I (Note: *Pst* I for pMET only). These sites are recommended for excising the expression cassette prior to *P. methanolica* transformation (see next page) and must **not** be present in your insert.

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Excising the Expression Cassette

After cloning your gene of interest into one of the above expression vectors and confirming the construction, digest the plasmid to release the 'expression cassette'. The expression cassette consists of the *AUG1* promoter, your gene, the *AUG1* transcription termination region, the *ScADE2* gene, and downstream 3´ *AUG1* sequences. Use one or a combination of the enzymes listed above to release the expression cassette. By excising the expression cassette, you separate the expression cassette from the plasmid backbone sequences that decrease transformation efficiency (Raymond *et al.*, 1998).

Transformation

After digestion of your plasmid, transform *P. methanolica* using electroporation or chemical methods and isolate Ade^+ transformants. The kit includes two strains, PMAD11 and PMAD16. Both strains contain the *ade2-11* allele and are phenotypically Ade^-. PMAD16 is a protease-deficient derivative of PMAD11. For more information on these two strains, see page 7.

Integration

In *P. methanolica*, non-homologous recombination events (> 90%) predominate over homologous recombination events (< 10%). Therefore, the expression cassette integrates at a variety of locations, resulting in a Mut^+ phenotype (Hiep *et al.*, 1993b; Raymond *et al.*, 1998).

Phenotypes

Two different phenotypic classes of Ade^+ transformants 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 *AUG1* gene. A strain with a Mut^S phenotype has a mutant *AUG1* locus, but is wild type for *AUG2*. This results in a slow growth phenotype on methanol medium.

Multiple Integration Events

P. methanolica is capable of integrating multiple copies of transforming DNA. Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants (1–5%).

Successful expression of the gene of interest to optimal levels **may** depend upon the generation of a recombinant strain that contains multiple copies integrated in the *P. methanolica* genome. We recommend that you directly test several transformants for high-expressing recombinants.

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Expression and Scale-up

After isolating Ade+ transformants, test for expression of your gene of interest:

1. Grow a small culture of each recombinant, induce with methanol, and take time points.
   - If you are looking for intracellular expression, analyze the cell pellet from each time point by SDS polyacrylamide gel electrophoresis (SDS-PAGE).
   - If you are looking for secreted expression, analyze both the cell pellet and medium from each time point.
2. Analyze your SDS-PAGE gels by both Coomassie staining and western blot and check for protein activity by assay, if available. Not all proteins express to high levels, so it is advisable to check by western blot or activity assay, and not just by Coomassie staining of SDS-PAGE gels for production of your protein.
3. Choose the recombinant strain that best expresses your protein and optimize induction based on the suggestions on pages 31–32.
4. Once expression is optimized, scale-up your expression protocol to produce more protein.

Purification

In pMET and pMETα, the gene of interest can be cloned in-frame with the sequence encoding a polyhistidine tag to facilitate purification of the fusion protein. Metal-binding resins, such as ProBond™, can be used to purify proteins expressed from pMET or pMETα. Use the ProBond™ Purification System (see page 56 for ordering information) to purify C-terminal fusion proteins expressed from pMET or pMETα. The ProBond™ kit contains precharged ProBond™ resin, six columns, buffers for native and denaturing purification, and an instruction manual. Preliminary preparation steps are described on pages 33–34. **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification System Kit manual**, which is available at www.invitrogen.com or by contacting Technical Support (see page 57).

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

Methods

P. methanolica Strains

Introduction

P. methanolica is quite similar to *Saccharomyces cerevisiae* in terms of general growth conditions and handling. It is a homothallic haploid organism that can be induced by nutritional starvation to mate and undergo a sexual cycle. Familiarize yourself with basic microbiological and sterile techniques before attempting to grow and manipulate any microorganism. Familiarity with basic molecular biology and protein chemistry is also necessary. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology*, (Guthrie and Fink, 1991), *Current Protocols in Molecular Biology*, (Ausubel *et al.*, 1994), *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Molecular Cloning: A Laboratory Manual*, (Sambrook *et al.*, 1989), *Protein Methods*, (Bollag and Edelstein, 1991), and *Guide to Protein Purification*, (Deutscher, 1990).

Genotype of *P. methanolica* Strain

The *P. methanolica* host strains PMAD11 and PMAD16 are derived from strain CBS6515 (ATCC accession number 58372). The *ade2-11* mutation in PMAD11 was introduced using standard UV mutagenesis and nystatin enrichment (Raymond *et al.*, 1998). PMAD11 was further mutagenized to create PMAD16 which contains deletions in *PEP4* and *PRB1* (Raymond *et al.*, 1998). These deletions eliminate Proteinase A and Proteinase B, respectively, and create a protease-deficient strain for expression of protease-sensitive proteins.

All expression plasmids carry the *ADE2* gene from *Saccharomyces cerevisiae* which complements *ade2-11* in the host, so transformants are selected for their ability to grow on adenine-deficient medium. Spontaneous reversion of PMAD11 and PMAD16 to Ade^+ prototrophy is less than 1 out of 10^8 (C. Raymond, personal communication).

Both PMAD11 and PMAD16 grow on complex medium such as YPD supplemented with adenine (YPAD) and on minimal media supplemented with adenine. YPD needs to be supplemented with adenine for optimal growth at higher cell densities. Until transformed, neither PMAD11 nor PMAD16 will grow on unsupplemented minimal medium alone as they are Ade^-. Note that PMAD16 grows more slowly than PMAD11 on minimal medium.

Which Strain Should I Use?

- For intracellular expression of your protein we recommend PMAD16 to prevent proteolysis. However, if your protein does not appear to be sensitive to proteases, we recommend using PMAD11 because it grows better in minimal medium.
- For secretion of your protein, we recommend PMAD11. Note that it is difficult to predict beforehand which strain will best express your protein. Many researchers elect to test their construct in both strains.

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Control Expression Strain

PMAD16/ pMETα B/HSA (Mut+) is included as a control for both intracellular and secreted expression. The gene for human serum albumin (HSA) was amplified by PCR without its native secretion signal and cloned in-frame with the sequence encoding the α-factor secretion signal and the C-terminal peptide in pMETα B, and then transformed into PMAD16. Ade+ recombinants were selected and characterized for expression. PMAD16/pMETα B/HSA (Mut+) secretes albumin (77 kDa) into the medium. For a map of pMETα B/HSA, see page 44.

Growth of *P. methanolica* Strains

The growth temperature of *P. methanolica* is 28–30°C for liquid cultures, plates, and slants. Growth above 32°C during induction can be detrimental to protein expression. Other important facts:

- Doubling time of log phase *P. methanolica* in YPAD is ~2 hours
- Mut+ and Mut^S strains do not differ in growth rates unless grown on methanol
- Doubling time of log phase Mut+ *P. methanolica* in buffered complex methanol medium (BMMY) is ~6 hours
- Doubling time of log phase Mut^S *P. methanolica* in BMMY is ~18 hours
- One OD_{600} = ~5 × 10^7 cells/mL

Note that growth characteristics may vary depending on the recombinant strain.

Morphology of *P. methanolica*

Cells appear smaller and clump together when viewed under the microscope. This leads to flocculation and fermentation cultures that appear to be more viscous than similar cultures of *Pichia pastoris*. This is normal.

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 methanol to a final concentration of 0.5%.

Make frozen stocks for long-term storage of all three *P. methanolica* strains included in this kit (PMAD11, PMAD16, and PMAD16/pMETα B/HSA).

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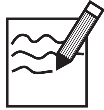
Storage of *P. methanolica* Strains

To store cells for a few weeks, use YPAD medium or YPAD agar slants (see page 38).

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

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

- Culture a single colony overnight in YPAD.
- Harvest the cells and suspend in YPAD containing 15% glycerol at a final OD_{600} of 50–100 (approximately 2.5–5.0 × 10^9 cells/mL).
- Transfer cells to a cryovial and store at –80°C.



Note

After extended storage at 4°C or –80°C, it is recommended that the Ade^+ transformants be checked for correct genotype and viability by streaking on minimal medium with and without adenine. Ade^+ transformants should grow without adenine.

General Cloning Considerations

E. coli Host

Many *rec*A (recombination deficient), *end*A (endonuclease A deficient) *E. coli* strains, such as TOP10 or equivalent (see page 56 for ordering information), are suitable for use with the pMET and pMETα vectors.

Introduction

First decide whether you want to secrete your protein or express it intracellularly. Then develop a cloning strategy. The vectors are supplied in three reading frames to facilitate in-frame cloning. pMET can be used for intracellular expression. pMETα contains the N-terminal α-factor secretion signal coding sequence and can be used for secreted expression. The multiple cloning sites are presented on pages 13–18 for each version of each vector to help you develop a cloning strategy.

Selecting a Vector

Secreting a protein is generally desirable as it facilitates downstream purification. If your protein is cytosolic and non-glycosylated, you can express the protein intracellularly using pMET or secrete it using pMETα. In order to secrete a normally cytosolic protein, check your protein sequence for possible N-glycosylation sites (Asn-X-Ser/Thr) before cloning it into pMETα (see below).

If your protein is normally secreted or directed to an intracellular organelle, try secreting your protein. Try both the native secretion signal (clone into pMET) and the α-factor signal sequence (clone into pMETα without the native secretion signal) to secrete your protein.

Glycosylation Sites

Since glycosylation is not well-characterized in *P. methanolica*, do not express proteins which require specific glycosylation in *P. methanolica*. In addition, any glycosylation may affect the activity of the protein, particularly for use in mammalian cells. In general, we recommend that you minimize glycosylation when expressing heterologous proteins in *P. methanolica*. If you wish to secrete cytosolic proteins, check the sequence for glycosylation sites. You may wish to eliminate the sites through site-directed mutagenesis or treat the purified protein with Endoglycosidase H or Peptide:N-glycosidase F (see page 35).

Propagation and Maintenance

To propagate and maintain the pMET or pMETα vectors, follow the guidelines below.

- Dilute 1 µL of each plasmid (0.5 µg/µL) to 10–100 pg/µL using sterile water or TE buffer.
- Transform competent *E. coli* with 1–2 µL of the diluted plasmid using your method of choice and select on LB medium containing 50–100 µg/mL ampicillin.
- Prepare a glycerol stock for long-term storage (see next page).

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Long-Term Storage

Prepare a glycerol stock of your transformant for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at −20°C.

1. Streak the original colony out on an LB agar plate containing 50–100 µg/mL ampicillin
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow the culture to saturation.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at −80°C.

Applying Selective Pressure

We recommend taking some (if not all) of the following precautions to prevent your clone from being "overrun" by background contaminants:

- **Use carbenicillin instead of ampicillin.** Carbenicillin is more stable than ampicillin, and allows for a longer period of selective pressure, thus preserving your clones longer.
- **Increase the antibiotic concentration.** More antibiotic means that your clones will not be overwhelmed by β-lactamase buildup.
- **Periodically refresh plate media.** If you suspect that tubes/plates may be beginning to fail, spin them down, remove the old media, and replenish the wells with fresh LB media plus glycerol and antibiotic.

Streak clones on selective (preferably carbenicillin) LB agar plates. After about 12 hours, isolate colonies for downstream usage. This will isolate your desired clones from potential background contaminants.

General Considerations

The following are some general considerations:

- The codon usage in *P. methanolica* is believed to be similar to *Saccharomyces cerevisiae* as many genes have proven to be cross-functional. For more information on codon usage, see www.kazusa.or.jp/codon
- Plasmid constructs should be maintained in a *recA*, *endA E. coli* host.
- To secrete a protein using its native secretion signal and pMET, also clone your gene without the native signal sequence in-frame with the α-factor secretion signal sequence in pMETα.
- If you do not have an antibody to your protein, be sure to clone in-frame with the C-terminal peptide coding sequence to allow detection of your protein with either of the Anti-V5 antibodies (see page 56).
- If you do not wish to include the C-terminal peptide, include a stop codon.

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Specific Considerations for pMET

The following are some specific considerations for pMET:

- Shorter 5´ untranslated leaders reportedly work better for expression in yeast. In pMET, make the untranslated region as short as possible (< 100 base pairs) when cloning your gene.
- For pMET, the fragment containing the gene of interest should have a yeast consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **A/Y A A/U A AUGUCU** is a yeast consensus sequence (Romanos *et al.*, 1992). Note that a 'U' at position -3 decreases expression 2-fold.

Specific Considerations for pMETα

- The fragment containing the gene of interest must be cloned in-frame with the secretion signal open reading frame.
- If the *Xho* I site is used for cloning, the sequence between the *Xho* I site and the *EcoR* I site encoding the *KEX2* site (Glu-Lys-Arg-X) must be recreated for efficient cleavage of the fusion protein to occur. It is part of the α-factor signal peptide sequence.
- An initiating ATG is provided by the signal sequence.
- If you wish to clone in-frame with both the N-terminal secretion signal **and** the C-terminal peptide coding sequences, you may have to use PCR to ensure that your gene will be in-frame with both coding sequences.

Bacterial Transformation

Once you have decided on a cloning strategy, prepare competent *E. coli* cells for transformation before setting up ligation reactions. See *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Biology: A Laboratory Manual* (Sambrook *et al.*, 1989) for preparation of electrocompetent or chemically competent *E. coli*.

Invitrogen provides electrocompetent and chemically competent TOP10 *E. coli* strains for your convenience (see page 56 for ordering information).

Cloning into pMET

pMET A Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMET A. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotides indicate the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

*The *Xma* III sites are unique to the polylinker.

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Cloning into pMET, Continued

pMET B Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMET B. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotides indicate the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

```
                                         AUG1 Forward priming site
1081  TAGAAGTTTT ATTTAACATC AGTTTCAATT TACATCTTTA TTTATTAACG AAATCTTTAC

                                                       Ecl136 II Xho I  Sac I
1141  GAATTAACTC AATCAAAACT TTTACGAAAA AAAAATCTTA CTAT TAA GAG CTC GAG
                                                       *** Glu Leu Glu

         EcoR I     Pml I          Sfi I Xma III*  BamH I         Sal I
1197  AGG AAT TCA CGT GGC CCA GCC GGC CGT GGA TCC ACG CGT CGT CGA CCC
      Arg Asn Ser Arg Gly Pro Ala Gly Arg Gly Ser Thr Arg Arg Arg Pro

      Sac II Not I/Xma III*  Spe I              V5 epitope
1245  GCG GCG GCC GCC AGC TT[A CTA GTA] GGT AAG CCT ATC CCT AAC CCT CTC
      Ala Ala Ala Ala Ser Leu Leu Val Gly Lys Pro Ile Pro Asn Pro Leu

                                           Polyhistidine (6xHis) region
1293  CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA
      Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

1341  TCTAGTATAC AATTCTAGGG CTGCCTGTTT GGATATTTTT ATAATTTTTG AGAGTTTGCC

         AUG1 Reverse priming site
1401  AACTAATGTT TTTCTCTTCT ATGATATTTA TCATGTAGTT
```

*The *Xma* III sites are unique to the polylinker.

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pMET C Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMET C. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotides indicate the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

```
                                              AUG1 Forward priming site
1081  TAGAAGTTTT ATTTAACATC AGTTTCAATT TACATCTTTA TTTATTAACG AAATCTTTAC

                                                         Ecl136 II Xho I  Sac I
1141  GAATTAACTC AATCAAAACT TTTACGAAAA AAAAATCTTA CTATT AAG AGC TCG AGA
                                                       Lys Ser Ser Arg

       EcoR I      Pml I          Sfi I Xma III*  BamH I             Sal I
1198  GGA ATT CAC GTG GCC CAG CCG GCC GTG GAT CCA CGC GTC GTC GAC CCG
      Gly Ile His Val Ala Gln Pro Ala Val Asp Pro Arg Val Val Asp Pro

      Sac II  Not I/Xma III*      SnaB I                V5 epitope
1246  CGG CGG CCG CCA GCT TAC GTA GGT AAG CCT ATC CCT AAC CCT CTC CTC
      Arg Arg Pro Pro Ala Tyr Val Gly Lys Pro Ile Pro Asn Pro Leu Leu

                                          Polyhistidine (6xHis) region
1294  GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA TC
      Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

1341  TAGTATACAA TTCTAGGGCT GCCTGTTTGG ATATTTTTAT AATTTTTGAG AGTTTGCCAA

       AUG1 Reverse priming site
1401  CTAATGTTTT TCTCTTCTAT GATATTTATC ATGTAGTTGG
```

*The *Xma* III sites are unique to the polylinker.

Cloning into pMETα

pMETα A Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMETα A. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotide upstream of the *EcoR I* site indicates the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

```
                                         AUG1 Forward priming site
1081  TAGAAGTTTT ATTTAACATC AGTTTCAATT TACATCTTTA TTTATTAACG AAATCTTTAC

                                                                 Sac I
1141  GAATTAACTC AATCAAAACT TTTACGAAAA AAAAATCTTA CTATTAAGAG CTCAAA ATG
                                                                   Met
                                   α-factor signal sequence
1200  AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC GCA
      Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala

1248  TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT
      Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile

1296  CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT
      Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe Asp

1344  GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe
                            α-factor Forward priming site
1392  ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser
      Xho I          Kex2 signal cleavage        EcoR I   Pml I        Sfi I/Xma III*  BamH I
1440  CTC GAG AAA AGA ▼GAG GCT GAA GC[T] GAATTCACGT GGCCCAGCCG GCCGTGGATC
      Leu Glu Lys Arg Glu Ala▲Glu Ala▲
                                  Ste 13 signal cleavage
                Sal I          Sac II  Not I/Xma III*     Spe I              V5 epitope
1494  CACGCGTCGT CGACCCGCGG CGGCCGCCAG CTTACTAGTA GGT AAG CCT ATC CCT
                                                  Gly Lys Pro Ile Pro
                                                          Polyhistidine (6xHis) region
1549  AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT
      Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His

1597  CAC CAT TGA TCTAG TATACAATTC TAGGGCTGCC TGTTTGGATA TTTTTATAAT
      His His ***
                        AUG1 Reverse priming site
1651  TTTTGAGAGT TTGCCAACTA ATGTTTTTCT CTTCTATGAT ATTTATCATG
```

*The *Xma* III sites are unique to the polylinker.

continued on next page

pMETα B Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMETα B. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotides indicate the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

```
                                             AUG1 Forward priming site
1081  TAGAAGTTTT ATTTAACATC AGTTTCAATT TACATCTTTA TTTATTAACG AAATCTTTAC

                                                                      Sac I
1141  GAATTAACTC AATCAAAACT TTTACGAAAA AAAAATCTTA CTATTAAGAG CTCAAA ATG
                                                                     Met
                                 α-factor signal sequence
1200  AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC GCA
      Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala

1248  TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT
      Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile

1296  CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT
      Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe Asp

1344  GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe
                  α-factor Forward priming site
1392  ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser
      Xho I       Kex2 signal cleavage      Pst I EcoR I   Pml I     Sfi I Xma III* BamH I
1440  CTC GAG AAA AGA GAG GCT GAA GC[T GCAG]GAATTC ACGTGGCCCA GCCGGCCGTG
      Leu Glu Lys Arg Glu Ala▲Glu Ala▲
                        Ste 13 signal cleavage
                  Sal I       Sac II  Not I/Xma III*    Spe I          V5 epitope
1494  GATCCACGCG TCGTCGACCC GCGGCGGCCG CCAGCTTACT AGTA GGT AAG CCT ATC
                                                      Gly Lys Pro Ile
                                                     Polyhistidine (6xHis) region
1550  CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC
      Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His

1598  CAT CAC CAT TGA T CTAGTATACA ATTCTAGGGC TGCCTGTTTG GATATTTTTA
      His His His ***
                               AUG1 Reverse priming site
1651  TAATTTTTGA GAGTTTGCCA ACTAATGTTT TTCTCTTCTA TGATATTTAT
```

*The *Xma* III sites are unique to the polylinker.

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pMETα C Multiple Cloning Site

Below is the multiple cloning site and surrounding sequences of pMETα C. Restriction enzymes sites are labeled to indicate the cleavage sites. The boxed nucleotides indicate the variable region. The multiple cloning site has been sequenced and functionally tested. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**

```
                                             AUG1 Forward priming site
1081  TAGAAGTTTT ATTTAACATC AGTTTCAATT TACATCTTTA TTTATTAACG AAATCTTTAC

                                                                  Sac I
1141  GAATTAACTC AATCAAAACT TTTACGAAAA AAAAATCTTA CTATTAAGAG CTCAAA ATG
                                                                     Met
                                   α-factor signal sequence
1200  AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC GCA
      Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala

1248  TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT
      Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile

1296  CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT
      Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe Asp

1344  GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT
      Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe
                         α-factor Forward priming site
1392  ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT
      Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser
      Xho I     Kex2 signal cleavage        Cla I   EcoR I    Pml I      Sfi I Xma III*
1440  CTC GAG AAG AGA GAG GCT GAA GC[A TCGAT]GAATT CACGTGGCCC AGCCGGCCGT
      Leu Glu Lys Arg Glu Ala Glu Ala
                        Ste 13 signal cleavage
      BamH I       Sal I      Sac II  Not I/Xma III*   Spe I        V5 epitope
1494  GGATCCACGC GTCGTCGACC CGCGGCGGCC GCCAGCTTAC TAGTA GGT AAG CCT ATC
                                                       Gly Lys Pro Ile
                                                   Polyhistidine (6xHis) region
1551  CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC
      Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His

1599  CAT CAC CAT TGA TCTAGTATAC AATTCTAGGG CTGCCTGTTT GGATATTTTT
      His His His ***
                              AUG1 Reverse priming site
1651  ATAATTTTTG AGAGTTTGCC AACTAATGTT TTTCTCTTCT ATGATATTTA
```

*The *Xma* III sites are unique to the polylinker.

Transformation into *E. coli*

Bacterial Transformation

Transform your ligation reactions into *E. coli* using your method of choice and select transformants on LB agar containing 50–100 µg/mL ampicillin (see **LB agar plates**, page 36).

Analyzing Transformants

1. Pick 10 ampicillin resistant transformants and inoculate into LB medium with 50–100 µg/mL ampicillin. Grow overnight at 37°C with shaking.
2. Isolate plasmid DNA and analyze for the presence and orientation of insert by restriction analysis.

   Note: We recommend isolating DNA using the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page 56 for ordering information).
3. We recommend that you also sequence your construct to confirm in-frame fusions with the N-terminal secretion signal and/or the C-terminal peptide coding sequences (see below).
4. Make a glycerol stock of your desired clone for long-term storage by combining 0.85 mL of a saturated bacterial culture with 0.15 mL of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at −80°C.
5. Once your construct is confirmed by sequencing, proceed to **Preparing DNA for Transformation**, next page.

Sequencing Recombinant Clones

We strongly recommend that you sequence your construct before transforming into *P. methanolica* to confirm the following:

- The correct reading frame for secretion
- An ATG in the proper context for yeast translation initiation
- Correct fusion to the C-terminal peptide coding sequence, if desired.

Use α-factor or *AUG1* Forward primer and the *AUG1* Reverse primer to sequence your constructs. For the location of the priming sites, refer to the multiple cloning site diagrams on pages 13–18.

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

Preparing DNA for Transformation

Introduction

At this point, you should have a *P. methanolica* expression vector with your gene of interest cloned in the correct orientation for expression. The table below provides page numbers correlating to the next steps for protein expression.

Step	Action	Page
1	Prepare plasmid DNA.	20
2	Excise the expression cassette by restriction digest for transformation.	21
3	Grow either PMAD11 or PMAD16 to prepare competent cells using the protocol for electrocompetent cells.	23
4	Transform PMAD11 or PMAD16 with your DNA.	24
5	Select Ade^{+} transformants.	24

Preparing Plasmid DNA

Plasmid DNA for *P. methanolica* transformation should be at least pure enough for restriction digestion; however, the cleaner the DNA, the more efficient the transformation. We recommend isolating DNA using the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page 56) for routine *P. methanolica* transformations. Plan on using 1–3 µg of digested DNA for each transformation.

continued on next page

Excising the Expression Cassette

It is important to excise the expression cassette prior to transformation. When 1 µg of supercoiled plasmid is transformed into *P. methanolica*, only 1 or 2 Ade^+ transformants are isolated. When the plasmid is linearized, the transformation efficiency increases 10-fold. Finally, when sequences derived from bacterial DNA are removed (i.e. the origin and the ampicillin resistance gene), the transformation efficiency increases ~100-fold.

The enzymes below can be used to excise the expression cassette and separate it from the plasmid backbone sequences. The location of each enzyme is summarized in the table or refer to pages 41–42 for vector maps. Be sure to check for restriction sites within your insert.

Enzyme	Recognition Site	*AUG1* promoter	3´ *AUG1* sequences	Vendor
Apa I	GGGCC|C	Yes	--	Invitrogen, Cat. no. 15440-019
Asc I	GG|CGCGCC	Yes	Yes	New England Biolabs
Fse I	GGCCGG|CC	Yes	Yes	New England Biolabs
Pac I	TTAAT|TAA	Yes	Yes	New England Biolabs
Kpn I	GGTAC|C	--	Yes	Invitrogen, Cat. no. 15232-010
Pst I (pMET only)	CTGCA|G	Yes	Yes	Invitrogen, Cat. no. 15215-015

Digestion

Once you have decided on the appropriate restriction enzyme, you are ready to digest your plasmid.

1. Digest 10 µg each of both your construct and the parent vector. The parent vector will be transformed into PMAD11 and/or PMAD16 and used as a background control for expression (true negative control).
2. Analyze a small portion of your digest by agarose gel electrophoresis to confirm complete digestion of your fragment. The number of transformants and frequency of targeting will be reduced if digestion is not complete.
3. Extract the digest with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitate the digested DNA. Resuspend the DNA pellet in 5 µL of TE buffer. It is not necessary to purify the fragment containing your gene away from the rest of the plasmid.
4. Determine the concentration of the DNA and store at −20°C until ready to transform.

Transforming *P. methanolica* by Electroporation

Introduction

Electroporation provides the highest efficiency of transformation (10^2 **stable** transformants per µg DNA) (Raymond *et al.*, 1998). Protocols are provided below to prepare electrocompetent *P. methanolica* and transform them using electroporation. If you do not have access to an electroporator, cells can be transformed using chemical methods (see page 45). Transformation in *P. methanolica* is less efficient than for *Pichia pastoris*.

Selecting Ade$^+$ Transformants

Strains lacking 5-aminoimidazole ribonucleotide-carboxylase (the product of the *ADE2* gene) will become pink or red on limiting adenine. Successful complementation of the mutant allele will result in white colonies on adenine-deficient medium (Hiep *et al.*, 1993a; Hiep *et al.*, 1993b). Pick the white colonies to analyze for expression.

Before Starting

Prepare the following reagents and equipment before starting (see ***Pichia* Media Recipes**, pages 37–40 for details).

- YPAD medium, 1 liter
- Fresh KD Buffer (50 mM potassium phosphate, pH 7.5; 25 mM DTT, sterile)
- Ice-cold STM Buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, sterile)
- 1X YNB
- MD plates (see page 39)
- Sterile centrifuge bottles (large enough to hold 250 mL)
- Sterile pipettes
- Electroporator
- 0.2 cm electroporation cuvettes

continued on next page

Preparing Electrocompetent Cells

1. Streak PMAD11 or PMAD16 onto a YPAD plate to isolate single colonies. Incubate the plate at 28–30°C for 2 days.
2. Inoculate 50 mL of YPAD in a 250 mL shake flask with a single colony of PMAD11 or PMAD16 and grow overnight at 28–30°C with shaking (200–250 rpm).
3. The next morning, measure the OD_{600} of the culture. The OD_{600} can range from 1 to 20, but typically is in the 5–10 range.
4. Inoculate 200 mL of YPAD in a one liter flask to an OD_{600} of 0.3 using the overnight culture. For example, if the OD_{600} of the overnight culture is 5, inoculate with 12 mL of the overnight culture to obtain an OD_{600} of 0.3.
5. Incubate the culture for 4 hours at 28–30°C with shaking (200–250 rpm).
6. Check the OD_{600}. It should be between 0.6 and 1.0. If the OD_{600} is below 0.6, continue to grow the culture until the OD_{600} is at least 0.6. If the OD_{600} is over 1.0, dilute the culture to an OD_{600} of 0.6 with fresh YPAD and grow for at least 1 hour. Cells must be in log-phase growth.
7. Harvest the cells by centrifuging 5 minutes at 1,500 × *g* at room temperature.
8. Decant the supernatant and resuspend the cells in 40 mL sterile, fresh KD Buffer. Fresh, sterile DTT should be added the day of the transformation.
9. Incubate the cell suspension for 15 minutes at 28–30°C (do not shake).
10. Centrifuge the cells 5 minutes at 1,500 × *g* at 4°C. Keep the cells at 0–4°C for all subsequent steps.
11. Carefully decant the supernatant and resuspend the cell pellet in 50 mL of ice-cold, sterile STM buffer.
12. Centrifuge the cells 5 minutes at 1,500 × *g* at 4°C.
13. Repeat Steps 11 and 12 two more times for a total of 3 washes.
14. After pelleting the cells for the third time, decant the supernatant, and resuspend the cells in 1 mL of ice-cold STM buffer. The final volume will be greater than 1 mL because of retention of liquid in the centrifuge bottle.
15. Place the cells on ice and use immediately, or dispense in 100 µL aliquots and freeze at −80°C for later use. **Do not flash-freeze the cells.** Simply place them at −80°C and allow them to freeze slowly. **Note**: The transformation efficiency will drop about 10-fold with frozen cells.

continued on next page

Electroporation

For each transformation, chill a 0.2 cm electroporation cuvette on ice.

We recommend including controls to check for contamination. Include a 'no DNA' and a 'plasmid only' control.

1. For each transformation, transfer 100 µL of electrocompetent cells from Step 14, previous section, to a 1.5 mL microcentrifuge tube.
2. Add 1–3 µg plasmid DNA to the tube and mix gently. Incubate the mixture on ice for 2 minutes. **Note**: No significant increase in the number of transformants has been observed with DNA concentrations > 3 µg.
3. Transfer the cell/DNA mixture to an ice-cold 0.2 cm cuvette.
4. Electroporate using the following parameters:
    1. Voltage: 600 V
    2. Capacitance: 50 µF
    3. Resistance: 8
5. If you have a Bio-Rad GenePulser, try settings of 750 V (375 V/cm), 25 µF, and a resistance setting of ∞ (Raymond *et al.*, 1998).
6. After electroporation, immediately add 1 mL of room temperature YPAD and transfer to a culture tube.
7. Incubate at 28–30°C for 1 hour without shaking.
8. Pellet the cells by centrifuging 3 minutes at 1,500 × *g* at room temperature.
9. Decant the supernatant and resuspend the cell pellet in 100 µL 1X YNB.
10. Plate 50 µL each on two minimal dextrose (MD) plates.
11. Incubate plates at 28–30°C for 3 to 4 days until colonies form.

Selecting Transformants

In general, 2 µg of plasmid DNA yields 50–200 white, stable transformants. Raymond *et al*., 1998 have reported upwards of 10,000 transformants per µg of DNA when unstable transformants are also included in the total.

The Next Step

Once transformants are obtained, most researchers proceed directly to expression (page 25) to identify the transformant that best expresses their protein of interest.

Troubleshooting Electroporation

If you do not obtain any transformants or very few transformants, review the following points.

- Make sure that you use at least 1 µg of DNA and that it is completely digested.
- Vary the voltage from 500 to 700 in 50 volt increments (Raymond *et al.*, 1998).

Small-Scale Expression

Purpose

The purpose of small-scale expression is to quickly identify the highest expressing recombinants for further characterization and optimization.

Media

We recommend BMDY/BMMY (buffered complex dextrose or methanol medium, respectively) for small-scale expression of recombinant proteins from *P. methanolica*. Specifically, high cell densities increase the likelihood that your secreted protein will be detected in the medium. Two pouches of BM-Y medium are included in the kit for your convenience. Follow the directions on the package to prepare BMDY or BMMY. BMDY and BMMY recipes are provided on page 38. Other types of media may also be useful for optimizing expression (see page 31).

Before Starting

Run the proper controls for correct interpretation of expression results:

- Use PMAD16/pMETα B/HSA (Mut^{+}) as a control for intracellular and secreted expression. **Note**: Some unprocessed protein is retained inside the cell and is not secreted. Be sure to assay both the cell pellet and the medium.
- Use PMAD11 or PMAD16 containing the parent vector (no insert) as a negative control.

Important

Purify Ade+ transformants to ensure isolation of pure clonal isolates by streaking for single colonies on minimal plates without adenine.

Clonal Variation

Recombination can occur in many different ways that affect expression (clonal variation). Screen at least 6–10 recombinant clones (or more) for expression levels. Use colonies that are less than 1 week old. Alternatively, start the cultures with a small sample from a frozen glycerol stock that was generated from a single colony.

Hint

Recombinant colonies may also be screened for your protein of interest using an overlay assay. Patched colonies induced with methanol are transferred to a nitrocellulose filter, lysed (for detection of intracellular expression only) and probed with an antibody to the desired protein. Recombinants with the strongest signal are selected for further characterization. The overlay assay protocol is provided on page 47.

continued on next page

Frequency of Expression

Greater than 50% of the transformants will express the protein of interest. Of these, 90% will be single-copy integrants and 10% may be multi-copy. Note that multi-copy does not necessarily mean high expression. In some cases, particularly with secreted proteins, *P. methanolica* recombinants with single-copy integrants express higher levels of recombinant protein than recombinants with multi-copy integrants (C. Raymond, personal communication).

Procedure for Small-Scale Expression

Use 50 mL conical tubes or 250 mL culture flasks for small-scale expression. Be sure that the medium is well-aerated by shaking flasks at least 250 rpm or greater or by placing the flasks at an angle in the shaker. You can use the strain PMAD11/pMETα B/HSA as a positive control for expression to ensure that the induction is working properly.

Note: If you wish to normalize your samples to cell number or amount of cells, record and use the OD_{600} reading for each time point or use wet cell weight.

1. Inoculate 10–50 mL of BMDY with a single colony.
2. Grow overnight at 28–30°C in a shaking incubator (250–300 rpm) until culture reaches an OD_{600} = 2–10 (approximately 16–18 hours). Record the OD_{600}, if desired.
3. Take a 500 µL sample and centrifuge at maximum speed in a microcentrifuge for 2–3 minutes at room temperature.
   - For secreted expression, **transfer the medium to a separate tube.** Store the medium and the cell pellets at −80°C until ready to assay. Freeze quickly in liquid nitrogen or a dry ice/alcohol bath.
   - For intracellular expression, decant the medium and store just the cell pellets at −80°C until ready to assay. Freeze quickly in liquid nitrogen or a dry ice/alcohol bath.
4. Harvest the rest of the culture by centrifuging at 1,500 × *g* for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet in 5 mL of BMMY to induce expression.
5. Grow at 28–30°C with vigorous shaking (at least 250 rpm).
6. After 24 hours of growth, remove a 500 µL aliquot from the culture and process the time point as described in Step 3. Supplement the culture with 500 µL 5% methanol (final concentration 0.5% methanol).
7. Continue to grow the cells and take time points every 24 hours for 3–5 days. At each time point, supplement the culture with additional methanol to bring the culture to a final concentration of 0.5% methanol.
8. Analyze the medium and cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot or functional assay (see **Analyzing Expression**, next page).

Analyzing Expression

Introduction

Note that any standard SDS-polyacrylamide gel apparatus and protocol will work for protein analysis. We use Novex® 4–20% Tris- Glycine gels (see page 56 for ordering information). For other recommendations, see standard texts such as *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989), *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994), *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Guide to Protein Purification* (Deutscher, 1990), or *Protein Methods* (Bollag and Edelstein, 1991).

Preparing Samples

You will need PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and acid-washed 0.5 mm glass beads (Sigma-Aldrich, Cat. no. G8772) on hand.

Preparing cell pellets (Intracellular and Secreted Expression):

1. Thaw cell pellets quickly and place on ice.
2. For each 500 µL sample, add 100–500 µL PBS 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 for 1 minute and then incubate on ice for 1 minute. Repeat for a total of 4 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.

Preparing medium samples (Secreted Expression only):

1. Thaw medium samples 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. Medium samples may be stored at −80°C for further analysis.
4. If no protein is seen by Coomassie or by western blot, then concentrate the medium sample 5–10 fold using a centrifugal filter device, such as a Nanosep® or Centricon® filter, and analyze samples again by western blot.

Protein Concentration

For your convenience, Invitrogen offers Quant-iT™ Protein Assay Kits (see www.invitrogen.com or contact Technical Support, page 57) to quantify the amount of total protein in the cell lysates and medium supernatants.

continued on next page

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 PMAD11 or PMAD16 with the parent plasmid transformed into it. This shows the background of native *P. methanolica* proteins that are present. Inclusion of this sample will help you differentiate your protein from background.
- Analyze the PMAD16/pMETα B/HSA control also as it should indicate any problems with the media or expression conditions.

HSA

HSA is expressed as a fusion to the α-factor signal sequence and the C-terminal peptide encoding the V5 epitope and the 6×His tag. Since HSA is not completely processed, cell pellets contain detectable amounts of unprocessed HSA. Unprocessed HSA can be used as a control for intracellular expression. Approximate molecular weights are listed below. Note that there are glycosylation sites in the α-factor signal peptide, so molecular weights should be considered approximate.

- α-factor signal sequence + HSA + C-terminal peptide: ~86 kDa
- HSA + C-terminal peptide: ~77 kDa

Analyzing Protein Expression

Inspection of your Coomassie-stained SDS-PAGE should reveal the induction of your protein. If recombinant protein is not visible, then perform a western blot or a functional assay if you have one (see next page).

Expression of the HSA fusion protein is detectable on a Coomassie-stained gel 24 hours post-induction, with maximal expression occurring after 48 hours. It is also detectable with either of the Anti-V5 antibodies (see page 56).

If you do not detect expression of your recombinant protein, and you are able to detect expression of HSA, see the next page for guidelines to optimize expression.

Signal Sequence Processing

P. methanolica processes the α-factor signal sequence correctly and appears to be similar to *Saccharomyces*. The *KEX2* gene product has been cloned from *P. methanolica* (C. Raymond, personal communication). In *Saccharomyces*, the processing of the α-factor mating signal sequence occurs in two steps:

1. The preliminary cleavage of the signal sequence by the *KEX2* gene product, with the final *KEX2* cleavage occurring between arginine and glutamine in the sequence Glu-Lys-Arg * Glu-Ala-Glu-Ala, where * is the site of cleavage.
2. The Glu-Ala repeats are further cleaved by the *STE13* gene product.

continued on next page

Optimizing Signal Cleavage

In *Saccharomyces cerevisiae*, it has been noted that the Glu-Ala repeats are not necessary for cleavage by *KEX2*, but the *KEX2* cleavage after Glu-Lys-Arg may be more efficient when followed by Glu-Ala repeats. The inclusion of Val-Pro after Arg inhibits cleavage in both *Saccharomyces* and *P. methanolica* (Raymond, 1999). Proline also inhibits *KEX2* cleavage. For more information on *KEX2* cleavage, see Brake *et al.*, 1984 or Raymond, 1999.

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.

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, enzymatic activities, or a defined purification profile, if available, may help identify the expressed protein among the native *P. methanolica* cellular proteins.

Detecting Fusion Proteins

Cloning your gene in-frame with the C-terminal peptide encoding the V5 epitope and the 6xHis tag enables detection of your protein with the Anti-V5 antibodies. Note that the HSA fusion protein is also detectable with the Anti-V5 antibodies. Antibody ordering information may be found on page 56.

Troubleshooting Low Expression Levels

- For secreted expression, check the cell pellet to see if overall expression is low or if the protein did not secrete. If it did not secrete, try a different signal sequence (e. g. a native or α-factor signal sequence) or express it intracellularly.
- Concentrate your medium by ammonium sulfate precipitation or ultrafiltration (see page 32) and assay for expression of secreted protein.
- Grow the cell cultures to a higher density before inducing expression.
- Aerate the culture by shaking at 250 rpm.
- If you are having problems with intracellular expression, try secreting your protein. It probably will glycosylate, which may not be desirable. If glycosylation is undesirable, oligosaccharides can be removed with Endoglycosidase H or Peptide:N-Glycosidase F (see page 35).

continued on next page

Troubleshooting Hyper-glycosylation

If your protein is hyperglycosylated:

- Try intracellular expression as your protein will not go through the secretion pathway and therefore, will not be modified.
- Try deglycosylating the protein with Endoglycosidase H or Peptide:N-Glycosidase F (see page 35).

Multiple Integration Events

As mentioned earlier, *P. methanolica* is capable of integrating multiple copies of transforming DNA. Although the exact mechanism of multiple integration events is not fully understood, such events are reasonably common among selected transformants (< 10%).

Successful expression of the gene of interest to useful levels **may** depend upon the generation of a recombinant strain that contains multiple copies integrated in the *P. methanolica* genome. Estimating gene copy number is discussed on page 54.

Scale-Up and Optimizing Protein Expression

Guidelines for Expression

Once you have obtained high-expressing recombinant strains, the next step is to identify the best strain for expression of your protein. Use the following guidelines to optimize expression.

Shake Flask or Fermenter

You may optimize expression in shake flasks or small-scale fermenters, depending on your needs and expertise. If you use shake flasks, use bottom or side baffled flasks whenever possible. These are available in a variety of sizes (see page vi for ordering information). If you are experienced with fermentation and wish to ferment *P. methanolica*, refer to Raymond *et al.* 1998 or Raymond, 1999 for guidelines. If you have not performed a fermentation with methylotrophic yeast, we recommend that you use shake flasks or consult with known experts.

Media

The table below describes different media for optimizing expression of recombinant proteins in *P. methanolica*. We recommend BMDY/BMMY for general expression. The other media listed below are those that have been used with *Pichia pastoris* and may be useful for optimizing expression in *P. methanolica*. For recipes, see pages 37–40.

Medium	Definition	Application
BMDY/BMMY	Buffered Complex Dextrose or Methanol	Buffered medium for high cell density growth conditions to optimize expression of secreted proteins. Contains yeast extract and peptone to allow better growth and generation of biomass.
BMD/BMM	Buffered Minimal Dextrose or Methanol	Buffered medium for expression of secreted proteins.
MD/MM	Minimal Dextrose or Methanol	Simplest medium for expression of recombinant proteins.

Proteases

By using the protease-deficient strain, PMAD16, proteolysis can be reduced.

Aeration

The most important parameter for efficient expression in *P. methanolica* at high cell densities is adequate aeration. 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 vi for suppliers of baffled flasks. Cover the flasks with cheesecloth (2–3 layers) or another loose fitting cover. Never use tight fitting covers.

continued on next page

Kinetics of Growth

Note that while Mut^+ and Mut^S strains will grow at essentially the same rate in YPAD or dextrose media, Mut^+ will grow faster than Mut^S when both are grown on methanol because of the presence of the *AUG1* gene product.

Temperature and Shaking

Expression is performed at 30°C in a shaking incubator. Do not allow the temperature to 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.

Concentrating Proteins

Proteins secreted into the media usually require some additional purification. It is optimal to concentrate the protein if the expression level is not particularly high. General methods to concentrate proteins secreted from *P. methanolica*. include:

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

For a general guide to protein techniques, see *Protein Methods* (Bollag and Edelstein, 1991).

Cell Lysis

A general procedure for cell lysis using glass beads is provided on page 34. Alternative protocols may be found in *Current Protocols in Molecular Biology*, page 13.13.4. (Ausubel *et al.*, 1994) and in *Guide to Protein Purification* (Deutscher, 1990).

Fermentation

We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. As a starting point, consult Raymond, et al., 1998 or Raymond, 1999, for details.

Protein Purification

Purpose

For protein purification, grow and induce a 10–200 mL culture of the *P. methanolica* transformant for trial purification on a metal-chelating resin such as ProBond™. Harvest the cells and store at −80°C until you are ready to purify the fusion protein, or you may proceed directly with protein purification. **Note that this section only describes preparation of cell lysates for use with 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 (see page 56 for ordering information) for purifying C-terminal fusion proteins expressed from pMET or pMETα. **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification Kit.**

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

Binding Capacity of ProBond™

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

Important

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

continued on next page

Preparing Cell Lysates

Express the protein using a small-scale culture (10–200 mL) and the optimal conditions for expression (if determined). Once the protein is expressed, follow the protocol below to prepare a cell lysate for chromatography on ProBond™.

Prepare Breaking Buffer (BB) as described on page 40.

1. Wash cells once in BB by resuspending them and centrifuging 5–10 minutes at 1,500 × *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. **Note**: To lyse larger volumes, see the next section.
4. Vortex the mixture for 1 minute, then incubate on ice for 1 minute. Repeat 3 more times. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of your protein.
5. Centrifuge the sample at 4°C for 5–10 minutes at 12,000 × *g*.
6. Transfer the clear supernatant to a fresh container and analyze for your protein. The total protein concentration should be around 2–3 mg/mL.
7. Save the pellet and extract with an equal volume of 6 M urea or 1% Triton X-100 to check for insoluble protein.

Lysing Larger Volumes

Biospec (Bartlesville, OK) makes a Bead Beater™ which can handle 5–200 mL volumes of cell suspension.

Note

See the ProBond™ manual for purification instructions under native and denaturing conditions. We have observed that some contaminating proteins may be retained on the ProBond™ column using native purification conditions. Optimizing the purification (see the ProBond™ Purification manual) or using denaturing purification may remove these non-specific proteins.

Analyzing Purification

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.

Protein Glycosylation

Analyzing Glycoproteins

When expressing and purifying a glycosylated protein in a heterologous expression system, it is desirable to quickly determine whether the protein is glycosylated properly. Protocols for the carbohydrate analysis of proteins have been published (see Unit 17 in Ausubel *et al.*, 1994). Further information about glycosylation in eukaryotes is available in a review (Varki and Freeze, 1994).

Enzymes for Analysis of Glycoproteins

Below are just a few of the enzymes available for carbohydrate analysis. Abbreviations are as follows: Asn - Asparagine, Gal - Galactose, GlcNAc - N-acetylglucosamine, GalNAc - N-acetylgalactosamine, and NeuAc - N-acetylneuraminic acid.

Enzyme	Type of enzyme	Specificity
Endoglycosidase D	Endo	Cleaves various high mannose glycans
Endoglycosidase F	Endo	Cleaves various high mannose glycans
Endoglycosidase H	Endo	Cleaves various high mannose glycans
β-galactosidase	Exo	Removes terminal galactosides from Gal-β1,3-GlcNAc, Gal-β1,4-GlcNAc or Gal-β1,3 GalNAc.
Peptide:N-Glycosidase F	Endo	Glycoproteins between Asn and GlcNAc (removes oligosaccharides)
Sialidases (Neuraminidases) *Vibrio cholerae* *Clostridium perfringens* *Arthobacter ureafaciens* Newcastle disease virus	Exo	NeuAc-α2,6-Gal, NeuAc-α2,6-GlcNAc or NeuAc-α2,3-Gal

Appendix

E. coli Media Recipes

LB (Luria-Bertani) Medium

For your convenience, Invitrogen offers prepackaged LB powder (see page 56 for ordering information). Alternatively, follow the instructions below to prepare your own media.

LB Medium Recipe

1% Tryptone
0.5% Yeast Extract
1% 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 for 20 minutes at 15 lb/sq. in and 121ºC. Cool to ~55°C and add desired antibiotics.
4. Store at room temperature or at 4°C, if antibiotics are added.

LB agar plates

1. Make LB Medium above and add 15 g/liter agar before autoclaving.
2. Autoclave for 20 minutes at 15 lb/sq. in.
3. Cool to ~55°C and add desired antibiotics. Pour into 10 cm petri plates. Let the plates harden, then invert, and store at 4°C.

Pichia Media Recipes

Introduction

The expression of recombinant proteins in *Pichia methanolica* requires the preparation of several different media. Recipes for these media are included in this section. In addition, Yeast Nitrogen Base is available from Invitrogen (see page 56 for ordering information).

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.

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.

10X D (20% Dextrose)

Dissolve 200 g of dextrose (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.

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

YPAD or YPD

Yeast Extract Peptone Adenine Dextrose Medium (1 liter)

1% yeast extract

2% peptone

2% dextrose (glucose)

0.01% adenine

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

Note: Additional adenine is only required to grow *ade2* mutants. Once the host is transformed to Ade$^+$, supplementation with adenine is no longer required.

1. Dissolve 10 g yeast extract, 20 g of peptone, and 0.1 g adenine in 900 mL of water. **Note**: Add 20 g of agar if making YPAD slants or plates.
2. Autoclave for 20 minutes on liquid cycle.
3. Cool to ~55°C and add 100 mL of 10X D. The liquid medium is stored at room temperature. Store YPAD slants or plates at 4°C. The shelf life is several months.

BMDY and BMMY

Buffered Dextrose-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

2% dextrose or 0.5% methanol

Note: 2 pouches of BM-Y medium are included in the kit to help you prepare BMDY and BMMY media. **Follow the directions on the package to prepare BMDY or BMMY.** To prepare BMDY and BMMY from raw materials, see below.

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 D
4. For BMMY, add 100 mL 10X M instead of dextrose.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

continued on next page

BMD and BMM

Buffered Minimal Dextrose

Buffered Minimal Methanol (1 liter)

100 mM potassium phosphate, pH 6.0

1.34% YNB

4 x 10^{-5}% biotin

2% dextrose or 0.5% methanol

1. Autoclave 700 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 D
3. For BMM, add 100 mL 10X M instead of dextrose.
4. Store media at 4°C. The shelf life of this solution is approximately two months.

MD/MM

Minimal Dextrose or Methanol Medium (1 liter)

1.34% YNB

4 x 10^{-5} % biotin

2% dextrose or 0.5% methanol

1. For medium, autoclave 800 mL of water for 20 minutes on liquid cycle. For plates, add 15 g agar to the water.
2. Cool to about 60°C and then add:

   100 mL of 10X YNB

   2 mL of 500X B

   100 mL of 10X D or 100 mL of 10X M
3. Mix and store at 4°C.
4. If preparing plates, pour the plates immediately. MD/MM stores well for several months at 4°C.

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Breaking Buffer

50 mM sodium phosphate, pH 7.4

1 mM PMSF (phenylmethylsulfonyl fluoride or other protease inhibitors)

1 mM EDTA

5% glycerol

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

KD Buffer

50 mM potassium phosphate, pH 7.5

25 mM dithiothreitol (DTT)

Note: Make fresh just before use.

1. Prepare 0.2 M stock solutions of K_2HPO_4 (dibasic; 34.8 g/L) and KH_2PO_4 (monobasic; 27.2 g/L). Prepare a 1 M stock solution of DTT and filter-sterilize. Dispense the 1 M DTT in 1 mL aliquots and store at –20°C.
2. Prepare a 0.2 M solution of potassium phosphate, pH 7.5, by mixing 16 mL of KH_2PO_4 and 84 mL of K_2HPO_4. Check the pH and adjust with KOH or HCl if necessary.
3. Mix 10 mL of 0.2 M potassium phosphate, pH 7.5, 1 mL of 1 M DTT, and 29 mL of sterile water.
4. Filter sterilize and use immediately.

STM Buffer

270 mM sucrose

10 mM Tris, pH 7.6 at 4°C

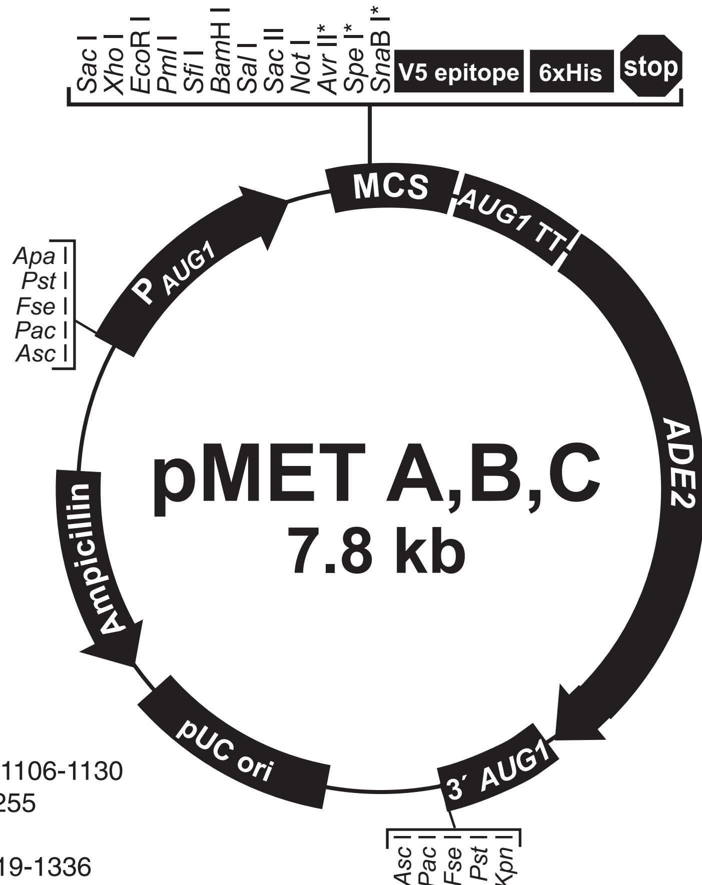
1 mM $MgCl_2$

1. To prepare 1 liter of STM buffer, combine 92.4 g of sucrose, 10 mL of 1 M Tris, and 1 mL of 1 M $MgCl_2$ with 900 mL deionized water.
2. Stir to dissolve and cool to 4°C.
3. Adjust the pH to 7.6 with HCl or NaOH.
4. Filter sterilize and store at 4°C.

Map of pMET

Description

pMET is a 7.8 kb vector designed to express proteins either intracellularly or target them for secretion using the protein's native secretion signal. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**



*Frame-dependent variations.

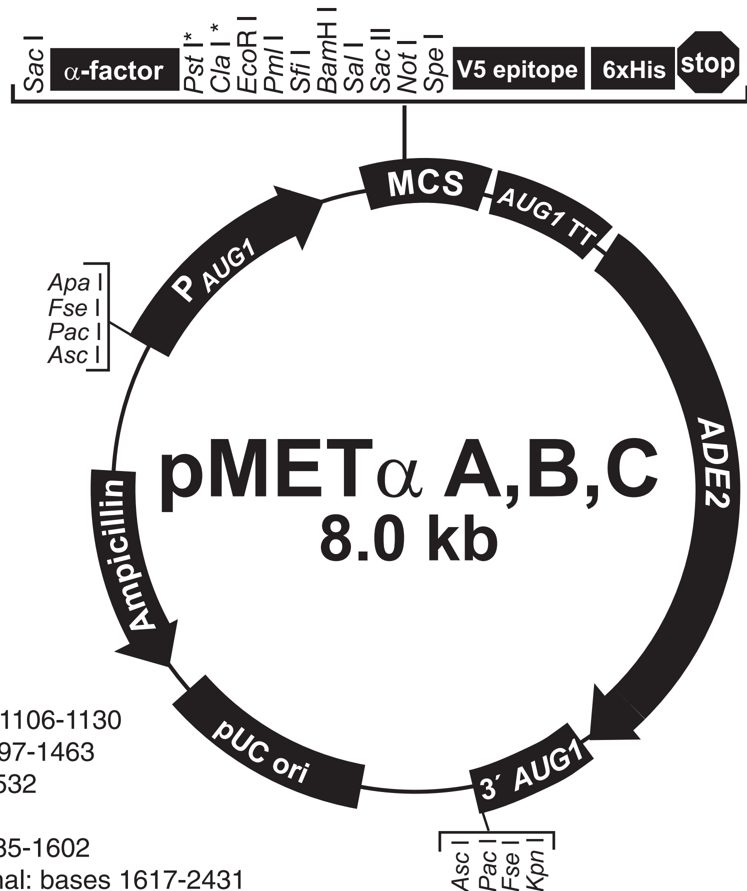
Comments for pMET A
7778 nucleotides

5´ polylinker: bases 1-38
AUG1 promoter: bases 39-1187
AUG1 Forward priming site: bases 1106-1130
Multiple cloning site: bases 1188-1255
V5 epitope: bases 1268-1309
Polyhistidine (6xHis) tag: bases 1319-1336
AUG1 transcription termination signal: bases 1351-2165
AUG1 Reverse priming site: bases 1397-1418
ADE2 promoter: bases 2171-2819
ADE2 gene: bases 2820-4535
3´ *AUG1* genomic sequences: bases 4689-5537
3´ polylinker: bases 5538-5574
pUC origin: bases 5691-6364 (complementary strand)
bla promoter: bases 7370-7468 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6509-7369 (complementary strand)

Map of pMETα

Description

pMETα is an 8.0 kb expression vector designed to secrete expressed proteins into the medium. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**



*Frame-dependent variations.

Comments for pMETα A
8045 nucleotides

5´ polylinker: bases 1-38
AUG1 promoter: bases 39-1187
AUG1 Forward priming site: bases 1106-1130
α-factor signal sequence: bases 1197-1463
Multiple cloning site: bases 1464-1532
V5 epitope: bases 1534-1575
Polyhistidine (6xHis) tag: bases 1585-1602
AUG1 transcription termination signal: bases 1617-2431
AUG1 Reverse priming site: bases 1663-1684
ADE2 promoter: bases 2437-3085
ADE2 gene: bases 3086-4801
3´ *AUG1* genomic sequences: bases 4956-5804
3´ polylinker: bases 5805-5841
pUC origin: bases 5958-6631 (complementary strand)
bla promoter: bases 7637-7735 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6776-7636 (complementary strand)

Features of pMET and pMETα

Features

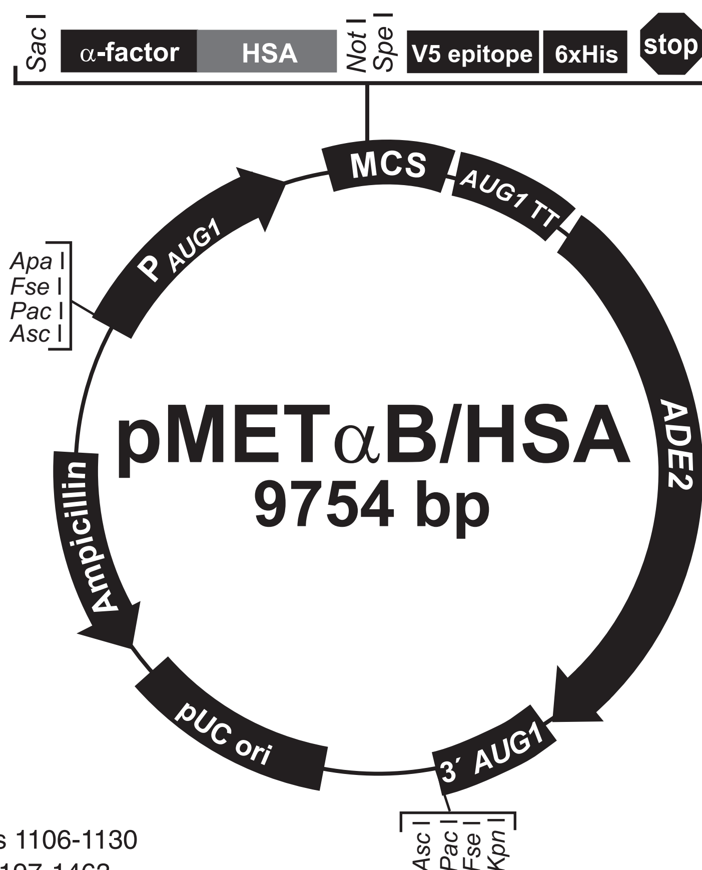
The table below describes the general and optional features of the *P. methanolica* expression vectors.

Feature	Description	Benefit
AUG1 promoter	An ~1100 bp fragment containing the *AUG1* promoter	Allows methanol-inducible high level expression in *P. methanolica* Facilitates homologous recombination at the *AUG1* locus.
α-factor signal sequence (pMETα only)	DNA sequence coding for an N-terminal protein secretion signal	Targets desired protein for secretion.
MCS	Multiple Cloning Site	Allows insertion of your gene into the expression vector.
V5 epitope	Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr	Allows detection of protein.
6xHis tag	Stretch of 6 histidines	Allows purification of expressed protein using metal-chelating resins (i.e. ProBond™).
AUG1 TT	Native transcription termination and polyadenylation signal from *AUG1* gene (~810 bp)	Permits efficient transcription termination and polyadenylation of the mRNA.
ADE2	*Saccharomyces* wild-type gene coding for phosphoribosyl-aminoimidazole carboxylase (~2.4 kb) and used to complement *P. methanolica ade2* strains	Provides a selectable marker to isolate *P. methanolica* recombinant strains.
3´ *AUG1*	Sequences from the *AUG1* gene that are further 3´ to the TT sequences (~850 bp)	Facilitates homologous recombination at the *AUG1* locus.
pUC origin	Origin of replication from pUC	Permits replication and high copy number in *E. coli.*
Ampicillin resistance gene	Confers resistance to ampicillin in bacteria	Allows selection and maintenance in *E. coli.*
Apa I *Fse* I *Pac* I *Asc* I *Kpn* I *Pst* I (pMET)	Rare restriction sites	Permits excision of expression cassette for efficient integration into the *P. methanolica* genome.

Map of pMETα B/HSA

Description

pMETα B/HSA is a 9754 bp vector designed to secrete human serum albumin (HSA). The HSA gene was amplified from pPICZ-HSA and cloned into the *Xho* I and *Not* I sites of pMETα B. The gene was cloned in-frame with the α-factor prepro signal sequence for secretion. **The sequence can be downloaded from www.invitrogen.com or requested from Technical Support (page 57).**



Comments for pMETαB/HSA
9754 nucleotides

5´ polylinker: bases 1-38
AUG1 promoter: bases 39-1187
AUG1 Forward priming site: bases 1106-1130
α-factor signal sequence: bases 1197-1463
Human serum albumin (HSA) mature peptide: bases 1464-3221
V5 epitope: bases 3243-3284
Polyhistidine (6xHis) tag: bases 3294-3311
AUG1 transcription termination signal: bases 3326-4140
AUG1 Reverse priming site: bases 3372-3393
ADE2 promoter: bases 4146-4794
ADE2 gene: bases 4795-6510
3´ *AUG1* genomic sequences: bases 6665-7513
3´ polylinker: bases 7514-7550
pUC origin: bases 7667-8340 (complementary strand)
bla promoter: bases 9346-9444 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 8485-9345 (complementary strand)

LiCl Transformation

Introduction

P. methanolica can be transformed using a chemical method based on LiCl (Tarutina and Tolstorukov, 1994). The transformation efficiency was reported to be 5–20 transformants per microgram of circular YEp13. Note that YEp13 contains the 2 micron origin so it can be maintained episomally. While we have not tested this, it is assumed that if *P. methanolica* is transformed with gene fragments (i.e. the expression cassette), that transformation efficiencies will be higher.

Before Starting

You will need the following reagents and equipment

- YPAD
- 50 mL flasks
- TE Buffer, pH 7.4, sterile (10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA)
- TE Buffer containing 200 mM LiCl, sterile
- Denatured DNA (50 µg per transformation)
- 70% polyethylene glycol (PEG) 4000, sterile
- 42°C water bath

Preparing Competent Cells

The procedure below yields enough competent cells for 5 transformations.

1. Inoculate a single colony of *P. methanolica* into 10–15 mL of YPAD in a 50 mL flask and incubate with vigorous aeration (~250 rpm) at 30°C until the culture reaches a density of 1×10^6 cells/mL. **Note**: High culture density leads to decreased transformation efficiency.
2. Harvest the cells by centrifugation (1,500 × *g*) at room temperature.
3. Wash the cells once with TE buffer, pH 7.4.
4. Resuspend the cells in 10–15 mL of TE buffer containing 200 mM LiCl.
5. Incubate the cell solution at 30°C for 1 hour.
6. Harvest the cells by centrifugation and decant the supernatant.
7. Resuspend the cells in 0.5 mL of TE buffer containing 200 mM LiCl. Cells are ready to transform.

continued on next page

Transformation

1. Use 100 µL of the competent cell solution from Step 7 (page 45) for each transformation.
2. In a 1.5 mL microcentrifuge tube, add transforming DNA (2–10 µg) and 50 µg denatured DNA to 100 µL of competent cells.
3. Incubate the solution at 30°C for 30 minutes.
4. Add 100 µL of 70% PEG 4000 and thoroughly mix.
5. Incubate the transformation solution for 1 hour at 30°C.
6. Heat shock the transformation solution for 5 minutes at 42°C.
7. Incubate the transformation solution overnight (16–20 hours) at 4°C.
8. Flick the bottom of the tube to resuspend the cells, then plate the whole solution onto selective plates.
9. Incubate for 5 to 7 days at 30°C until colonies appear.

Overlay Assay

Introduction

The following procedure can be used to screen recombinant colonies expressing the protein of interest.

Before Starting

You will need the following reagents and equipment.

- MD and YPD plates
- MM plates containing **2% methanol** (the higher concentration of methanol is necessary for successful induction and production of secreted protein)
- Replica plating equipment (Bio101, www.bio101.com)
- Pre-cut nitrocellulose circles sized to fit the plate
- 100% methanol
- PBS
- Lysis Buffer (prepare fresh, 0.1% SDS, 0.2 M NaOH, 35 mM dithiothreitol)
- Antibody to your protein or the Anti-V5 or Anti-V5-HRP antibody (for ordering information, see page 56)
- Reagents for western blot and detection

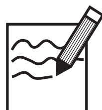
continued on next page

Overlay Assay, Continued

Procedure

Once you have transferred colonies to nitrocellulose and induced expression with methanol, you can detect expression using western blot detection methods.

1. Patch transformants onto MD and YPD plates and incubate at 30°C for 2 days. You should have 25–50 per plate. For a positive control, use PMAD16/pMETα B/HSA.
2. Replica plate from the MD plates onto MM plates (2% methanol).
3. Place a dry, pre-cut nitrocellulose circle onto the plate and mark the circle and the plate for orientation. **Note**: The circle will become sufficiently moist from contact with the plate.
4. Invert the plates and incubate for 2 to 4 days at 30°C.
5. Add 1 mL of 100% methanol to the lid of each plate every day.
6. To assay for expression, peel the nitrocellulose membrane off the plate with tweezers.
7. To detect intracellular expression, lyse the cells by incubating the filter in Lysis Buffer for 30 minutes at room temperature.
8. Remove lysed cells with rapidly flowing distilled water and rinse the filter with PBS.
9. Block the membrane with PBS containing 0.05% Tween-20 and 5% nonfat, dry milk (PBSTM) for 1 hour.
10. Add Anti-V5-HRP (1:2000) and incubate overnight at 4°C.
11. Wash the blot two times with PBS containing 0.05% Tween-20 (PBST).
12. Detect expressing colonies using chemiluminescence (follow the manufacturer's instructions).
13. Circle the high expressing clones and compare with the colonies that were patched onto the YPD plate. Select the appropriate patched colonies for further expression experiments.



Note

As a result of the limited ability of Mut^S cells to metabolize methanol, they may show higher expression levels on a plate because of the presence of methanol. Mut^+ strains metabolize methanol resulting in depletion of methanol and attenuation of induction. Under fermentation conditions where methanol levels are controlled, the relative expression levels may not be different.

Screening for Mut^+ and Mut^S Transformants

Introduction

Determining the Mut phenotype of *P. methanolica* strains may be needed to successfully optimize growth in fermenters. Once you have obtained Ade^+ transformants or a small group of high-expressing recombinants, you can determine the Mut phenotype at any time. In addition, identification of recombinants that have a Mut^S phenotype indicates that your gene has integrated by a homologous recombination event at the *AUG1* locus. A Mut^+ phenotype suggests that your gene has integrated elsewhere in the chromosomal DNA via non-homologous recombination.

Screening for Mut^+ and Mut^S

Because Mut^S transformants are not producing Aug1 protein, they cannot efficiently metabolize methanol as a carbon source and therefore grow poorly on minimal methanol (MM) medium. This slow growth on methanol can be used to distinguish Ade^+ transformants in which the *AUG1* gene has been disrupted (Ade^+ Mut^S) from Ade^+ transformants with an intact *AUG1* gene (Ade^+ Mut^+).

Before Starting

The following media (see page 39) and materials can be prepared several days in advance and stored at 4°C :

- Minimal Dextrose (MD) agar plates, 1 liter
- Minimal Methanol (MM) agar plates, 1 liter
- Sterile toothpicks
- Scoring templates (page 51, optional)
- Streak out the strain PMAD16/pMETα B/HSA on YPAD plates for use as a Mut^+ control

Screening High-Expressing Recombinant Strains or Ade^+ Transformants

Take a single colony of each of your high-expressing recombinant strains or an Ade^+ transformant and test the Mut phenotype as follows. If you wish, you may use the scoring templates on page 51.

1. Using a sterile toothpick, streak or patch one colony onto both an MM plate and an MD plate, making sure to patch the MM plate first.
2. Use a new toothpick for each recombinant strain and continue until all of your recombinant strains have been patched. Remember to patch PMAD16/pMETα B/ HSA as a positive control for Mut+.
3. Incubate the plates at 30°C for 2–5 days.
4. After 2 days or longer at 30°C, score the plates. Look for patches that grow normally on the MD plates but show little or no growth on the MM plates. Compare the patches with the Mut^+ control.

continued on next page

Purify your Ade⁺ transformants to ensure isolation of pure clonal isolates. This is done by streaking for single colonies on minimal plates without adenine and picking single colonies, either before or after testing for the Mut phenotype.

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. **Note**: Equipment for replica plating is available from Bio101 at www.bio101.com

1. Using sterile toothpicks, patch 100 Ade+ transformant on MD plates (2–3 plates). Be sure to include PMAD16/pMETα B/HSA as a Mut+ control.
2. Incubate the plates at 28–30°C for 2 days.
3. After 2 days, replica-plate the patches from the MD plates onto fresh MM and then MD plates to screen for the Mut phenotype.
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 MD replica plates but show little or no growth on the MM replica plates. Compare the patches to the Mut^+ control.

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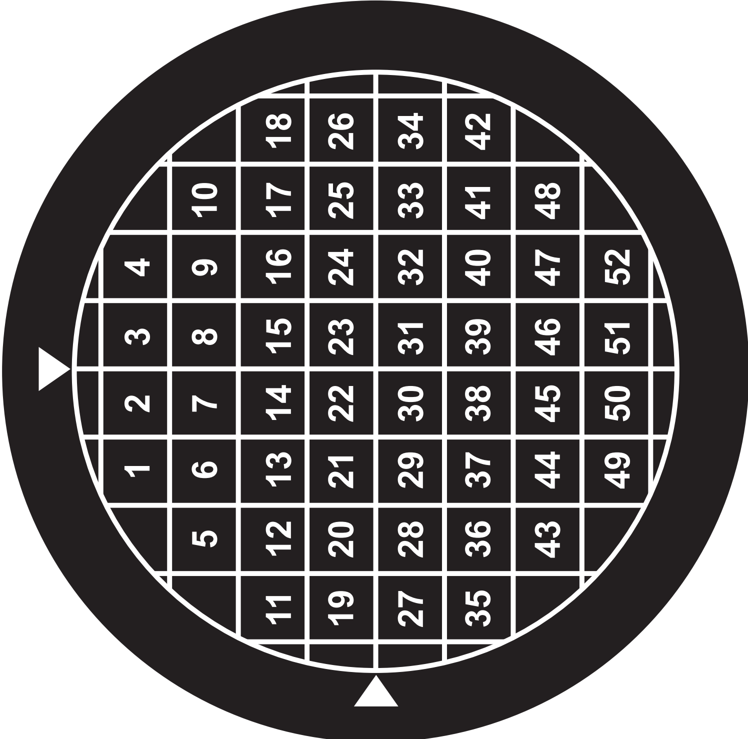
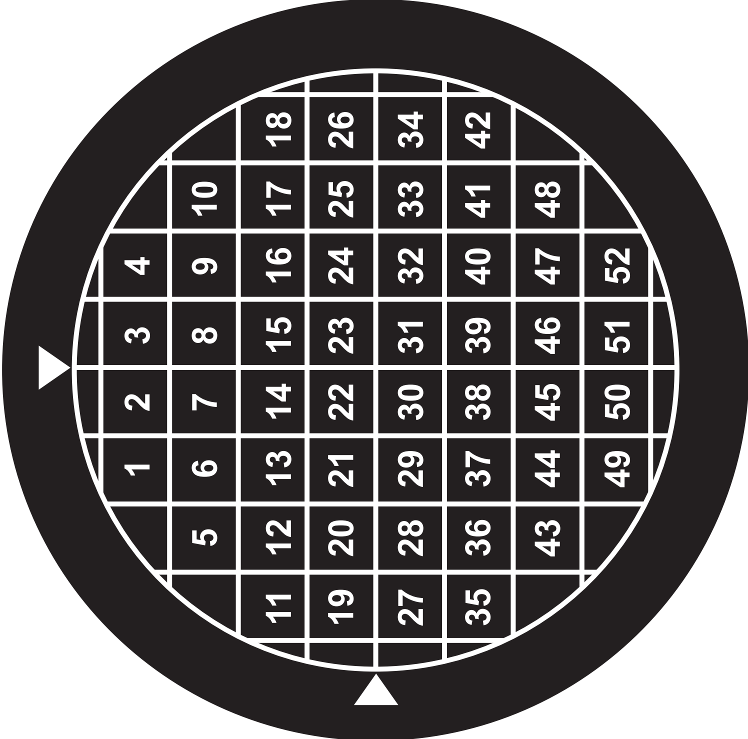
Screening for Mut^+ and Mut^s Transformants, Continued

Scoring Templates





Total DNA Isolation

Introduction

The protocol below allows you to isolate DNA from the desired Ade^+ recombinant and the untransformed PMAD11 or PMAD16. The purified DNA 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 and Higgins, 1991), or (Holm *et al.*, 1986).

Solutions

You will need to make the following solutions:

- Minimal Medium
- 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, see Northstar BioProducts™ at www.acciusa.com/northstar
- 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)

Preparing Cells

1. Grow the recombinant and parental strains at 30°C to an OD_{600} of 5–10 in 10 mL of minimal media such as BMDY. Include adenine for growth of PMAD11 or PMAD16.
2. Collect the cells by centrifugation at 1,500 × *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.

   Note: If you do not know how to check the extent of spheroplasting, see **Preparation of Spheroplasts** in the *Pichia* Expression Kit manual, which is available at www.invitrogen.com or from Technical Support (page 57).
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 × *g* for 5–10 minutes at 4°C and save the supernatant.

continued on next page

DNA Precipitation

1. Transfer the supernatant from Step 5 on page 52 and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.
2. Centrifuge at 10,000 × *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 × *g* for 20 minutes at 4°C and wash the pellets once with 1 mL of 70% ethanol.
7. Briefly air dry the pellets and resuspend in 50 µL of TE buffer, pH 7.5. Determine the concentration of the DNA samples. The samples may be combined or stored separately at −20°C until ready for use.

Detecting Multiple Integration Events

Introduction

You may wish to estimate the number of gene copies in your *P. methanolica* recombinant. Note that because of non-homologous recombination, it is difficult to determine the exact number. You may either use quantitative dot blots or Southern hybridization to analyze gene copy number (Brierley *et al.*, 1994; Clare *et al.*, 1991a; Romanos *et al.*, 1991; Scorer *et al.*, 1993; Scorer *et al.*, 1994). Isolate genomic DNA from recombinants transformed with the parent vector (0 copies of your gene), a recombinant that contains a single copy of your gene (1 copy), and the putative multi-copy recombinants. **Note**: Perform a Southern blot first to identify a recombinant containing a single copy of your gene. Use the protocol on page 52 to isolate genomic DNA.

Southern Blot Analysis

Digestion of DNA from recombinants containing multiple copies will produce bands that may either vary in intensity (depending on the number of copies of your gene) or size (depending on the location of the gene in the genome). Although interpretation of a Southern blot may be difficult, the band intensities can be relatively quantified using densitometry to estimate gene copies.

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.

General Guidelines

- Follow standard procedures 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. 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.

Quantitative Dot Blot Solutions

For (semi) quantitative dot blots, you will need 3MM paper and 10–15 mL of the following solutions for each dot blot:

- 50 mM EDTA, 2.5% β-mercaptoethanol pH 9
- 1 mg/mL Zymolyase 100T in water, available from Northstar BioProducts™ at www.acciusa.com/northstar
- 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate, pH 7
- 2X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH 7)

continued on next page

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. Alternatively, genomic DNA may be isolated and spotted directly onto nitrocellulose or nylon, fixed, and analyzed.

1. Grow recombinants 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. This may necessitate several passages. 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 a multi-channel pipettor. Air dry filters. Mark for orientation.
3. To lyse the cells on the filter, treat the filter with four solutions as follows: place two sheets of 3 MM paper in a tray and soak with 10–15 mL of 50 mM EDTA, 2.5% β-mercaptoethanol pH 9. Make certain that the paper is uniformly soaked and that there are no puddles. 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, 0.015 M sodium citrate, pH 7. 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 2X SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate for 5 minutes at room temperature. Repeat.
7. Bake nitrocellulose filters at 80°C or UV-crosslink DNA to nylon. The filters may be probed with a nonradioactive-labeled or a 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. Dot blots can then be quantified for copy number by densitometry of the film or blot, or by using a β-scanner (if radiolabeled).

Accessory Products

Additional Products

The following additional products may be used with the *P. methanolica* Expression Kit. For more information, visit www.invitrogen.com or contact Technical Support (see page 57).

Item	Amount	Catalog no.
One Shot® TOP10 Chemically Competent	20 reactions	C4040-03
One Shot® TOP10 Electrocomp™	10 reactions	C4040-50
TOP10 Electrocomp™	20 reactions	C664-55
Anti-V5	25 westerns	R960-25
Anti-V5-HRP	25 westerns	R961-25
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50
Ampicillin	200 mg	11593-027
Carbenicillin	5 g	10177-012
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Apa I	6,000 U	15440-019
Kpn I	2,000 U	15232-010
Pst I	3,000 U	15215-015
(Miller's LB Broth Base)® Luria Broth Base, powder	500 g	12795-027
LB Broth	500 mL	10855-021
Yeast nitrogen base (YNB)	1 pouch	Q300-07
	500 g	Q300-09

Gels, Electrophoresis, and Blotting Reagents

A wide variety of NuPAGE® Novex® precast gels, premade buffers, transfer membranes and protein standards are available from Invitrogen. For details, visit www.invitrogen.com or contact Technical Support (see page 57).

Technical Support

Web Resources

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

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