invitrogen™

pPIC6 A, B, and C

Pichia expression vectors for selection on blasticidin and purification of recombinant proteins

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User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

pPIC6 vectors are shipped on wet ice. Upon receipt, store vectors at –20°C, and store the X-33 stab at 4°C.

Kit Contents

The kit contents are listed below.

Item	Composition	Amount
pPIC6 A, B and C	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
pPIC6/*lacZ*	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
X-33 *Pichia* strain	1 stab in YPD medium	1 stab

X-33 *Pichia* Strain

The X-33 *Pichia* strain has the following genotype and phenotype:

Genotype: Wild-type
Phenotype: Mut^+

For long term storage of your *Pichia* strain stab, we recommend preparing a glycerol stock immediately upon receipt and storing at –80°C.

Reference Sources

The pPIC6 A, B, and C vectors may be used with the EasySelect™ *Pichia* Expression Kit or the Original *Pichia* Expression Kit available from Invitrogen (see page 34 for ordering). Additional information about recombinant protein expression in *Pichia pastoris* is provided in the manuals for the EasySelect™ *Pichia* Expression Kit and the Original *Pichia* Expression Kit. The manuals can be downloaded from www.invitrogen.com or obtained by contacting Technical Support (see page 35).

More detailed information and protocols dealing with *Pichia pastoris* may also be found in the following general reference (see page 34 for ordering information):

Higgins, D. R., and Cregg, J. M. (1998) *Pichia* Protocols. In *Methods in Molecular Biology*, Vol. 103. (J. M. Walker, ed. Humana Press, Totowa, NJ)

Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

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Materials Supplied by the User

For the procedures described in this manual, you will need the following reagents and equipment. Additional reagents may be required. Check each experiment to ensure you have all the reagents necessary.

Equipment

- Microbiological equipment
- Electroporation device and 0.2 cm cuvettes or reagents for transformation
- 16°C, 37°C, and 65°C water baths or temperature blocks
- 30°C and 37°C shaking and non-shaking incubators
- Hemocytometer or Countess™ Automated Cell Counter (see page 33)
- Microtiter plates (optional)

Reagents

- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation (see page 33).
- Restriction enzymes and appropriate buffers
- Agarose and low-melt agarose
- S.N.A.P.™ Gel Purification Kit or glass milk
- Sterile water
- CIAP (calf intestinal alkaline phosphatase, 1 unit/µL)
- 10X CIAP Buffer
- Phenol/chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/µL)
- 10X Ligation Buffer (with ATP)
- Low Salt LB medium (see page 19 for recipe)
- Blasticidin antibiotic (page 21)
- Low Salt LB plates containing 100 µg/mL blasticidin (see page 19 for recipe)
- YPDS plates containing the appropriate concentration of blasticidin (see page 20 for recipe)
- 50 mL conical centrifuge tubes
- 15 mL polypropylene tubes
- ProBond™ Purification System (optional, see page 33 for ordering)

Introduction

Product Overview

Description of the System

pPIC6 A, B, and C are 3.4 kb vectors used to express recombinant proteins in *Pichia pastoris*. The vector allows high-level, methanol inducible expression of the gene of interest in *Pichia*, and can be used in any *Pichia* strain including the X-33 strain supplied with the kit. pPIC6 contains the following elements:

- 5′ fragment containing the *AOX1* promoter for tightly regulated, methanol-induced expression of the gene of interest (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a)
- Blasticidin resistance gene (Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) for selection in both *E. coli* and *Pichia*
- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant fusion protein (if desired)
- Three reading frames to facilitate in-frame cloning with the C-terminal peptide

The control plasmid, pPIC6/*lacZ*, is included for use as a positive control for expression.

Experimental Overview

The following table describes the basic steps needed to clone and express your gene of interest in pPIC6.

Step	Action	Page
1	Propagate pPIC6 A, B, and C by transformation into a *rec*A, *end*A1 *E. coli* strain such as TOP10, DH5α, or JM109.	2
2	Develop a cloning strategy and ligate your gene into one of the pPIC6 vectors in frame with the C-terminal tag.	3–6
3	Transform into *E. coli* and select transformants on Low Salt LB plates containing 100 µg/mL blasticidin.	7
4	Analyze 10–20 transformants by restriction mapping or sequencing to confirm in-frame fusion of your gene with the C-terminal tag.	7
5	Purify and linearize the recombinant plasmid for transformation into *Pichia pastoris*.	7–10
6	Transform X-33 or your *Pichia* strain and plate onto YPDS plates containing the appropriate concentration of blasticidin.	10–11
7	Select for blasticidin-resistant transformants.	10–11
8	Optimize expression of your gene.	12–13
9	Purify your fusion protein on metal-chelating resin (*e.g.*, ProBond™).	14–15

Methods

Cloning into pPIC6 A, B, and C

General Molecular Biology Techniques

For assistance with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

Many *E. coli* strains are suitable for the propagation of the pPIC6 vectors including TOP10, JM109, and DH5α. We recommend that you propagate the pPIC6 vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen (see page 33).

Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintaining Plasmids

The pPIC6 and pPIC6/*lacZ* vectors contain the blasticidin resistance gene to allow selection of the plasmid using blasticidin. To propagate and maintain the pPIC6 and pPIC6/*lacZ* plasmids, we recommend using the following procedure:

1. Use a small amount of the supplied plasmid stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, JM109, or equivalent.
2. Select transformants on Low Salt LB plates containing 100 µg/mL blasticidin (see page 19 for a recipe).
3. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 7).

Continued on next page

Cloning into pPIC6 A, B, and C, Continued

General Considerations

The following are some general points to consider when using pPIC6 to express your gene of interest in *Pichia*:

- The codon usage in *Pichia* is believed to be similar to *Saccharomyces cerevisiae.*
- Many *Saccharomyces* genes have proven to be functional in *Pichia*.
- The premature termination of transcripts because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret and Sherman, 1984). If you have problems expressing your gene, check for premature termination by northern analysis and check your sequence for AT rich regions. It may be necessary to change the sequence in order to express your gene (Scorer *et al.*, 1993).
- The native 5´ end of the *AOX1* mRNA is noted in the diagram for each multiple cloning site. This information is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.

Cloning Considerations

- Your insert should contain a Kozak translation initiation sequence with an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Note that other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.
    - (**G/A**)NNATG**G**
- pPIC6 is a terminal fusion vector. To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. The vector is supplied in three reading frames to facilitate cloning. Refer to the diagrams on pages 4–6 to develop a cloning strategy.
- If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

Constructing Multimeric Plasmids

pPIC6 A, B, and C contain unique *Bgl* II and *Bam*H I sites to allow construction of plasmids containing multiple copies of your gene. For information on how to construct multimers, refer to the **Appendix**, pages 25–32.

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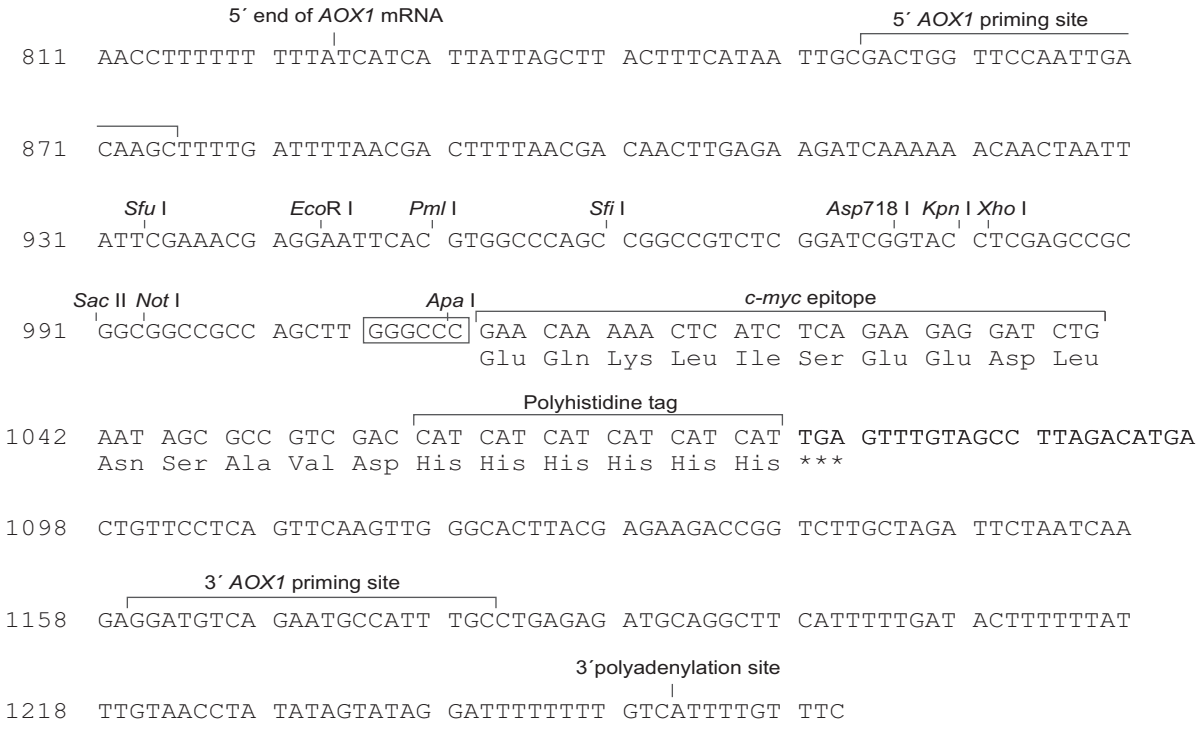
Cloning into pPIC6 A, B, and C, Continued

Multiple Cloning Site of pPIC6 A

Below is the multiple cloning site for pPIC6 A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6 A is available for downloading from www.invitrogen.com or from **Technical Support** (see page 35). For a map and a description of the features of pPIC6, refer to the **Appendix**, pages 16–17.

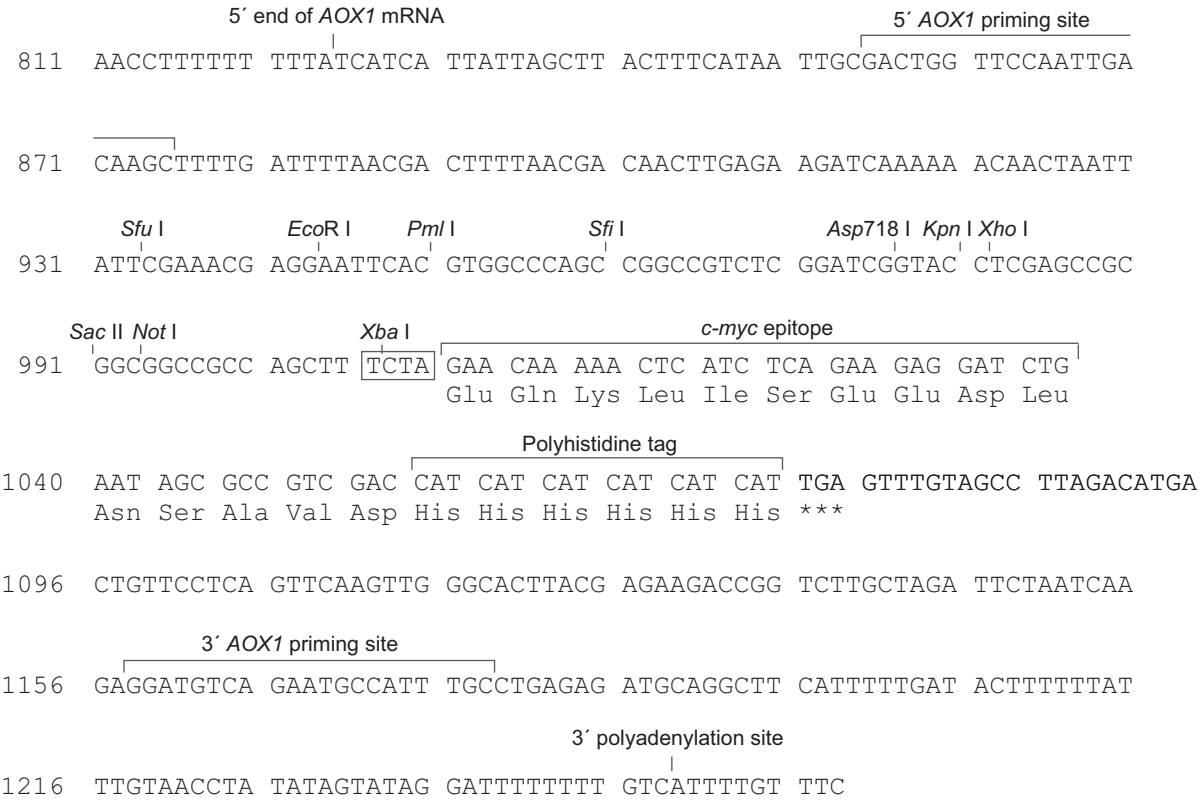


Continued on next page

Cloning into pPIC6 A, B, and C, Continued

Multiple Cloning Site of pPIC6 B

Below is the multiple cloning site for pPIC6 B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6 B is available for downloading from www.invitrogen.com or from **Technical Support** (see page 35). For a map and a description of the features of pPIC6, refer to the **Appendix**, pages 16–17.



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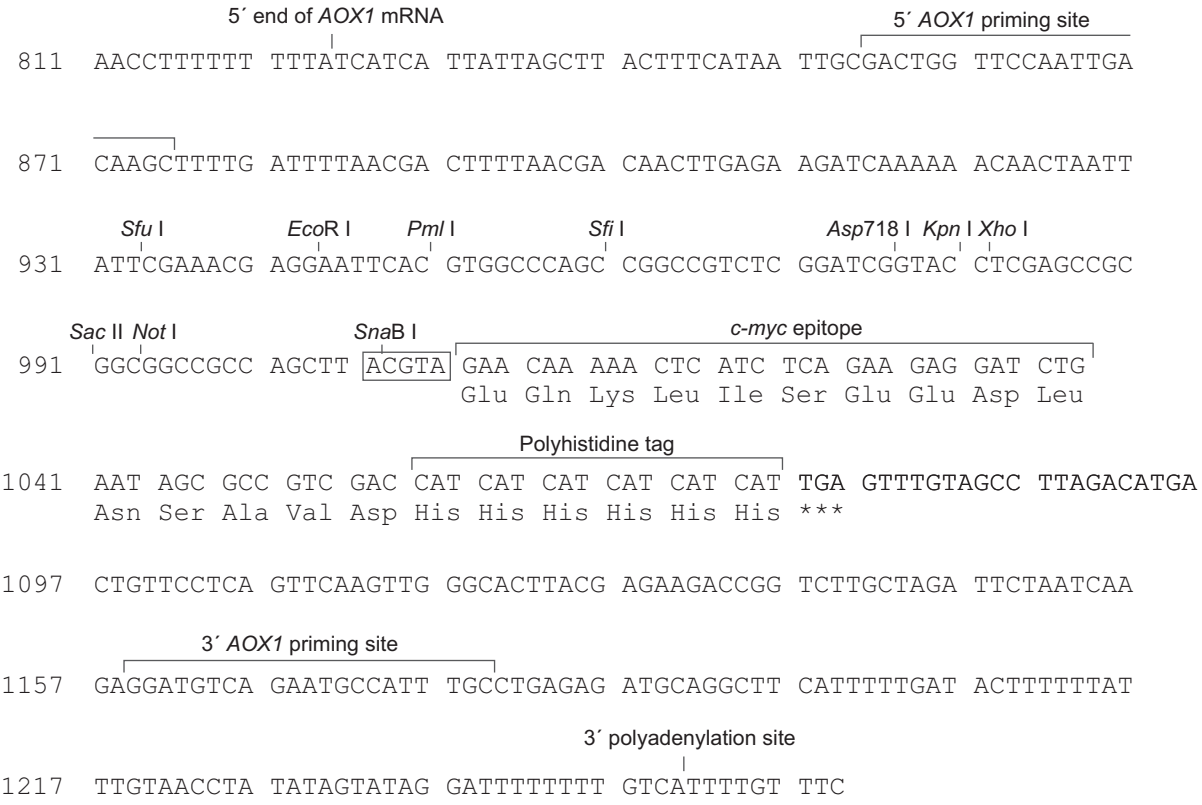
Cloning into pPIC6 A, B, and C, Continued

Multiple Cloning Site of pPIC6 C

Below is the multiple cloning site for pPIC6 C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pPIC6 C is available for downloading from www.invitrogen.com or from **Technical Support** (see page 35). For a map and a description of the features of pPIC6, refer to the **Appendix**, pages 16–17.



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Cloning into pPIC6 A, B, and C, Continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (*e.g.* TOP10, DH5α, JM109) and select on Low Salt LB agar plates containing 100 µg/mL blasticidin (see below). Note that there is no blue/white screening for the presence of insert with pPIC6 A, B, or C. Once you have obtained blasticidin-resistant colonies, pick 10 transformants and screen for the presence and orientation of your insert.

Important

To facilitate selection of blasticidin-resistant *E. coli*, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.0. Prepare Low Salt LB broth and plates using the recipe in the **Appendix**, page 19.

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

We recommend that you sequence your construct to confirm that your gene is in the correct orientation for expression, cloned in frame with the C-terminal peptide, and contains an ATG start codon and a stop codon. We suggest using the 5′ *AOX1 Pichia* and 3′ *AOX1 Pichia* primer sequences. Refer to the diagrams on pages 4–6 for the sequences and location of the priming sites.

Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony on Low Salt LB plate containing 100 µg/mL blasticidin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of Low Salt LB containing 100 µg/mL blasticidin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Plasmid Preparation

Once you have cloned and sequenced your insert, generate enough plasmid DNA to transform *Pichia* (5–10 µg of each plasmid per transformation). We recommend isolating plasmid DNA using the PureLink™ HiPure Plasmid Miniprep Kit or the PureLink™ HiPure Plasmid Midiprep Kit or equivalent (see page 33). Once you have purified plasmid DNA, proceed to ***Pichia* Transformation**, next page.

Pichia Transformation

Introduction

You should now have your gene cloned into one of the pPIC6 vectors. Your construct should contain a Kozak consensus sequence (initiation ATG) and be correctly fused to the C-terminal peptide. This section provides general guidelines to prepare plasmid DNA, transform your *Pichia* strain, and select for blasticidin-resistant clones.

Blasticidin Selection

We generally use 300 µg/mL blasticidin to select for transformants when using the X-33 *Pichia* strain. If you wish to transform your pPIC6 construct into another *Pichia* strain, note that selection conditions may vary. We recommend performing a dose response curve to determine the appropriate concentration of blasticidin to use for selection of transformants in your strain.

Method of Transformation

We recommend electroporation for transformation of *Pichia* with pPIC6 A, B, or C. Electroporation yields 10^3 to 10^4 transformants per µg of linearized DNA and does not destroy the cell wall of *Pichia*. If you do not have access to an electroporation device, use the LiCl protocol on page 23 or the Pichia EasyComp™ Transformation Kit available from Invitrogen (see below).

Pichia EasyComp™ Transformation Kit

If you wish to perform chemical transformation of your *Pichia* strain with pPIC6 A, B, or C, the *Pichia* EasyComp™ Transformation Kit is available from Invitrogen (see page 34 for ordering information). The *Pichia* EasyComp™ Transformation Kit provides reagents to prepare 6 preparations of competent cells. Each preparation yields enough competent cells for 20 transformations. Competent cells may be used immediately or frozen and stored for future use. For more information, visit www.invitrogen.com or contact Technical Support (page 35).

Note

The pPIC6 vectors do not contain a yeast origin of replication. Transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome.

Important

Since pPIC6 does not contain the *HIS4* gene, integration can only occur at the *AOX1* locus. Vector linearized within the 5´ *AOX1* region will integrate by gene insertion into the host 5´ *AOX1* region. Therefore, the *Pichia* host that you use will determine whether the recombinant strain is able to metabolize methanol (Mut^+) or not (Mut^S). To generate a Mut^+ recombinant strain, you must use a *Pichia* host that contains the native *AOX1* gene (*e.g.* X-33, SMD1168H). If you wish to generate a Mut^S recombinant strain, then use a *Pichia* host that has a disrupted *AOX1* gene (*e.g.* KM71H).

Note: The X-33 strain supplied with the pPIC6 vector contains the native *AOX1* gene; therefore, the recombinant strain will be Mut^+.

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Positive Control

The pPIC6/*lacZ* plasmid is provided as a positive control vector for blasticidin resistance in *Pichia* and may be used to optimize expression and purification conditions for your host strain. The pPIC6/*lacZ* plasmid expresses β-galactosidase fused at the C-terminus to the *c-myc* epitope and the polyhistidine tag (see page 18 for more information). Expression of the 119 kDa fusion protein is driven by the P_{AOX1} promoter and is inducible with methanol. The fusion protein is visible on a Coomassie-stained SDS-polyacrylamide gel and can be detected using the Anti-*myc* antibodies (see page 34) or using an ONPG assay (β-Gal Assay Kit, see page 33).

Before Starting

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

- 5–10 µg pure pPIC6 plasmid containing your insert
- YPD Medium
- 50 mL conical polypropylene tubes
- 1 liter cold (4°C) sterile water (place on ice the day of the experiment)
- 25 mL cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
- 30°C incubator
- Electroporation device and 0.2 cm cuvettes
- YPDS plates containing the appropriate concentration of blasticidin (see page 20 for recipe)

Linearizing Your pPIC6 Construct

To promote integration, we recommend that you linearize your pPIC6 construct within the 5′ *AOX1* region. The table below lists unique sites that may be used to linearize pPIC6 prior to transformation. **Other restriction sites are possible.** Note that for the enzymes listed below, the cleavage site is the same for versions A, B, and C of pPIC6. Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.

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

Continued on next page

Restriction Digest

1. Digest ~5–10 µg of plasmid DNA with one of the enzymes listed above.
2. Check a small aliquot of your digest by agarose gel electrophoresis for complete linearization.
3. If the vector is completely linearized, heat inactivate or add EDTA to stop the reaction, phenol/chloroform extract once, and ethanol precipitate using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol.
4. Centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend the DNA in 10 µL sterile, deionized water. Use immediately or store at -20°C.

Preparing *Pichia* for Electroporation

Follow the procedure below to prepare your *Pichia pastoris* strain for electroporation.

1. Grow 5 mL of your *Pichia pastoris* strain in YPD in a 50 mL conical tube at 30°C overnight.
2. Inoculate 500 mL of fresh medium in a 2 liter flask with 0.1–0.5 mL of the overnight culture. Grow overnight again to an OD_{600} = 1.3–1.5.
3. Centrifuge the cells at 1,500 × *g* for 5 minutes at 4°C. Resuspend the pellet with 500 mL of ice-cold, sterile water.
4. Centrifuge the cells as in Step 3 and resuspend the pellet with 250 mL of ice-cold, sterile water.
5. Centrifuge the cells as in Step 3 and resuspend the pellet in 20 mL of ice-cold 1 M sorbitol.
6. Centrifuge the cells as in Step 3 and resuspend the pellet in 1 mL of ice-cold 1 M sorbitol for a final volume of approximately 1.5 mL. Keep the cells on ice and use that day. **Do not store cells.**

Transformation by Electroporation

1. Mix 80 µL of the cells from Step 6 (above) with 5–10 µg of linearized pPIC6 DNA (in 5–10 µL sterile water) and transfer them to an ice-cold 0.2 cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) as suggested by the manufacturer of the specific electroporation device being used.
4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 mL tube.
5. Let the tube incubate at 30°C without shaking for 1 to 2 hours.
6. Spread 50–200 µL each on separate, labeled YPDS plates containing the appropriate concentration of blasticidin.
7. Incubate plates for 2 to 3 days at 30°C until colonies form.
8. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing the appropriate concentration of blasticidin.

When selecting for blasticidin-resistant transformants, we often observe colonies of two different sizes (large and small) on YPD plates containing 300 µg/mL blasticidin. Generally, large colonies represent transformants containing pPIC6 integrants, while small colonies represent transformants containing pPIC6 non-integrants. These non-integrants have transduced the pPIC6 plasmid, and therefore, exhibit a low level of blasticidin resistance in the initial selection process. Upon subsequent screening, these non-integrant transformants do not retain blasticidin resistance.

When choosing a blasticidin-resistant transformant for your expression studies, we recommend that you pick blasticidin-resistant colonies from the initial transformation plate and streak them on a second YPD plate containing the appropriate concentration of blasticidin. Select transformants which remain blasticidin-resistant for further studies.

Note

Generally several hundred blasticidin-resistant colonies are generated using the protocol on the previous page. If more colonies are needed, the protocol may be modified as described below. Note that you will need ~20 150 mm plates with YPDS agar containing the appropriate concentration of blasticidin.

1. Set up two transformations per construct and follow Steps 1 through 5 of the **Transformation by Electroporation** protocol, previous page.
2. After 1 hour in 1 M sorbitol at 30°C (Step 5, previous page), add 1 mL YPD medium to each tube.
3. Shake (~200 rpm) the cultures at 30°C.
4. After 1 hour, take one of the tubes and plate out all of the cells by spreading 200 µL on 150 mm plates containing the appropriate concentration of blasticidin.
5. (Optional) Continue incubating the other culture for three more hours (for a total of four hours) and then plate out all of the cells by spreading 200 µL on 150 mm plates containing the appropriate concentration of blasticidin.
6. Incubate plates for 2 to 4 days at 30°C until colonies form.

Mut Phenotype

If you used a *Pichia* strain containing a native *AOX1* gene (*e.g.*, X-33, GS115, SDM1168H) as the host for your pPIC6 construct, your blasticidin-resistant transformants will be Mut$^+$. If you used a strain containing a deletion in the *AOX1* gene (*e.g.*, KM71H), your transformants will be Mut^S.

If you wish to verify the Mut phenotype of your blasticidin-resistant transformants, refer to the general guidelines provided in the EasySelect™ *Pichia* Expression Kit manual or the Original *Pichia* Expression Kit manual or to published reference sources (Higgins and Cregg, 1998).

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

Expression in *Pichia*

Introduction

The primary purpose of small-scale expression is to identify/confirm a recombinant *Pichia* clone that is expressing the correct protein. Small-scale expression conditions may not be optimal for your protein. For this reason, the method you choose for detection (*e.g.* SDS-PAGE, Western, or functional assay) may be an important factor in determining the success of expression. If your method of detection does not reveal any expression, you may want to consider using a more sensitive method.

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

Note

Once you have obtained blasticidin-resistant transformants, it is not necessary to maintain your recombinant *Pichia* clone in medium containing blasticidin for expression studies. Blasticidin is only required for initial screening and selection of recombinant clones.

Detecting Recombinant Proteins in *Pichia*

We recommend that you use the following techniques to assay expression of your protein. Note that the *c-myc* epitope and the polyhistidine (6xHis) tag will contribute 2.5 kDa to the size of your protein. Be sure to account for any additional amino acids that are in between the end of your native protein and the *c-myc* epitope.

Technique	Method of Detection	Sensitivity
SDS-PAGE (Coomassie-stained)	Visualization by eye	Can detect as little as 100 ng in a single band.
SDS-PAGE (Silver-stained)	Visualization by eye	Can detect as little as 2 ng in a single band.
Western Analysis	Antibody to your particular protein Anti-*myc* antibodies (see the next page) Anti-His(C-term) antibodies (see the next page)	Can detect as little as 1-10 pg, depending on detection method (alkaline phosphatase, horseradish peroxidase, radiolabeled antibody).
Functional assay	Varies depending on assay.	Varies depending on assay. Used to compare relative amounts of protein.

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Expression in *Pichia*, Continued

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, visit www.invitrogen.com or call Technical Support (see page 35).

Western Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page 34 for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein is available from Invitrogen for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine (6xHis) tag. WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods (see page 33 for ordering). For more information, visit www.invitrogen.com or call Technical Support (see page 35).

Control Strain

If you have transformed the pPIC6/*lacZ* plasmid into your *Pichia* host strain, you may use this recombinant strain as a positive control for expression. Expression of β-galactosidase in shake flasks is detectable after 48 hours and reaches the maximum at 96 hours. β-galactosidase may be detected using SDS-PAGE and staining the gel with Coomassie Blue or by ONPG assay (β-Gal Assay Kit, see page 33). Cells expressing β-galactosidase can be detected by plating on medium containing methanol and X-gal.

For a small-scale Mut$^+$ expression protocol, refer to the EasySelect™ *Pichia* Expression Kit manual or to general reference texts.

Important

Because the pPIC6 vector does not contain the *HIS4* gene, *his4 Pichia* strains containing the integrated plasmid must be grown in medium containing 0.004% histidine. If histidine is not present in the medium the cells will not grow. If you used X-33 as the host strain, supplementation of the medium with histidine is not required.

Expression Guidelines

General guidelines to perform small-scale expression, optimize expression, and scale-up of expression are provided in the EasySelect™ *Pichia* Expression Kit manual or the Original *Pichia* Expression Kit manual.

Purification

Introduction

In this section, you will grow and induce a 10–200 mL culture of your *Pichia* transformant for trial purification on a metal-chelating resin such as ProBond™ or Ni-NTA. You may harvest the cells and store them at –80°C until you are ready to purify your fusion protein, or you may proceed directly with protein purification. **Note that this section only describes preparation of cell lysates and sample application onto ProBond™.** For instructions on how to prepare and use ProBond™ resin, refer to the ProBond™ Purification manual.

Metal-Chelating Resin

You may use the ProBond™ Purification System or Ni-NTA Purification System, or a similar product to purify your 6xHis-tagged protein (see page 33 for ordering). Both purification systems contain a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ Purification System manual or Ni-NTA Purification System manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, consult the manufacturer's instructions.

Binding Capacity of ProBond™

One milliliter of ProBond™ resin binds from 1–5 mg of recombinant protein. This amount can vary depending on the protein.

Important

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

Preparing Cell Lysates

Express your protein using a small-scale culture (10–20 mL for Mut^S strains; 100–200 mL for Mut^+) and the optimal conditions for expression (if determined). Once your protein is expressed, follow the protocol below to prepare a cell lysate for chromatography on ProBond™.

Prepare Breaking Buffer (BB) as described in the **Recipes**, page 20.

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

Continued on next page

Sample Application (Native Conditions)

For sample application onto ProBond™, you need Native Binding Buffer, pH 7.8 and a 2 mL ProBond™ column, pre-equilibrated using native conditions.

1. Combine 1 mL (2–3 mg/mL total protein) of *Pichia* lysate with 7 mL Native Binding Buffer.
2. Take a pre-equilibrated ProBond™ column and resuspend the resin in 4 mL of the diluted lysate from Step 1.
3. Seal the column and batch-bind by rocking gently at room temperature for 10 minutes.
4. Let the resin settle by gravity or low speed centrifugation (800 × *g*) and carefully remove the supernatant. Save the supernatant to check for unbound protein.
5. Repeat Steps 2 through 4 with the remaining 4 mL of diluted lysate. Proceed to **Column Washing and Elution Under Native Conditions** in the ProBond™ Purification manual. Use the recommendations noted for bacterial cell lysates.

Sample Application (Denaturing Conditions)

Use the protocol above except pre-equilibrate the ProBond™ column using Denaturing Binding Buffer and combine 1 mL of the *Pichia* cell lysate with 7 mL of the Denaturing Binding Buffer.

Note

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

Analysis of Purification

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

Scale-up

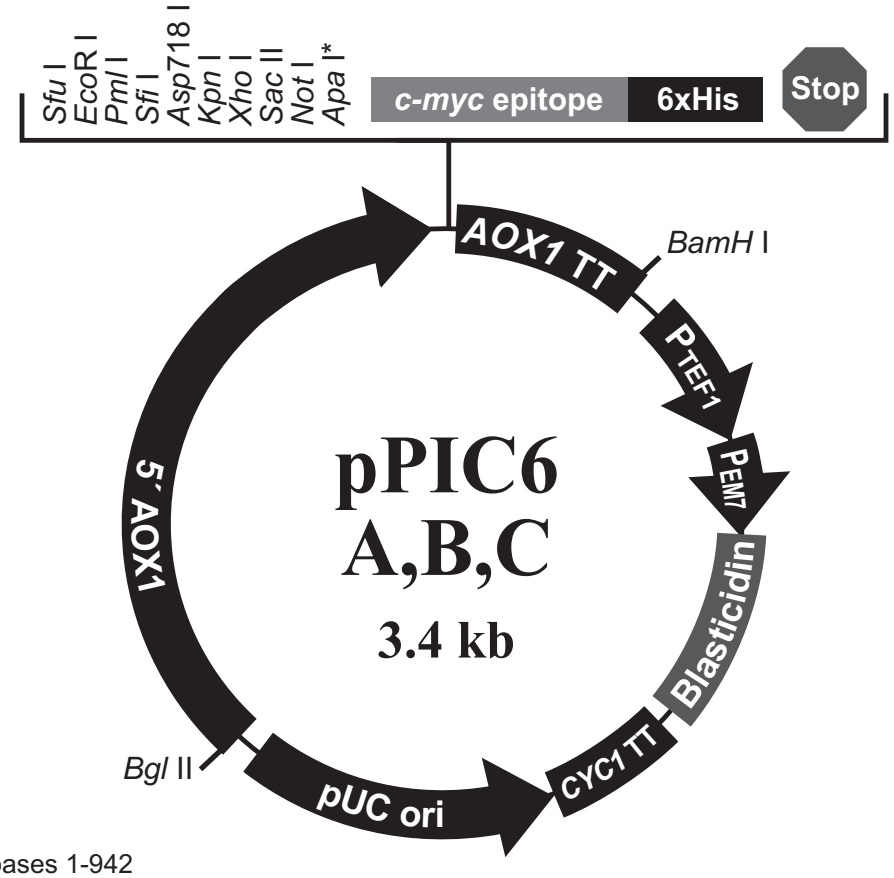
You may find it necessary to scale-up your purification to obtain sufficient amounts of purified protein. Adjust the pH and NaCl concentration of your lysate with 1/10 volume of 10X Stock Solution B (ProBond™ Purification Kit) before adding it to the column. The pH should be ≥ 7.5 and the NaCl concentration should be ~500 mM. Using 10X Stock Solution B to adjust the pH and the ionic strength keeps the total volume small for sample application.

Appendix

pPIC6 Vector

Map of pPIC6

The figure below summarizes the features of the pPIC6 A, B, and C vectors. The vector sequences for pPIC6 A, B, and C are available for downloading from www.invitrogen.com or from **Technical Support** (see page 35). See the next page for a description of the features of the vector.



Comments for pPIC6 A: 3382 nucleotides

5´ *AOX1* promoter region: bases 1-942
5´ *AOX1* priming site: bases 855-875
Multiple cloning site: bases 932-1011
c-myc epitope: bases 1012-1041
Polyhistidine (6xHis) tag: bases 1057-1074
3´ *AOX1* priming site: bases 1160-1180
AOX1 transcription termination region: bases 1078-1419
TEF1 promoter: bases 1420-1828
EM7 promoter: bases 1833-1899
Blasticidin resistance gene: bases 1900-2298
CYC1 transcription termination region: bases 2327-2644
pUC origin: bases 2655-3328 (complementary strand)

* The restriction site between *Not* I and the *c-myc* epitope is different in each version of pPIC6:

Apa I in pPIC6 A
Xba I in pPIC6 B
*Sna*B I in pPIC6 C

Continued on next page

Features of pPIC6 A, B, and C

pPIC6 A (3382 bp), pPIC6 B (3380 bp), and pPIC6 C (3381 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
5´ *AOX1* promoter	A 942 bp fragment containing the *AOX1* promoter that allows methanol-inducible, high-level expression of the gene of interest in *Pichia* Targets plasmid integration to the *AOX1* locus.
Multiple cloning site	Allows insertion of your gene into the expression vector.
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant fusion protein with the Anti-*myc* Antibodies (Evans *et al.*, 1985).
C-terminal polyhistidine (6xHis) tag	Allows purification of your recombinant fusion protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibodies.
AOX1 transcription termination (TT) region	Native transcription termination and polyadenylation signal from *AOX1* gene (~260 bp) that permits efficient 3´ mRNA processing, including polyadenylation, for increased mRNA stability.
TEF1 promoter (GenBank accession numbers D12478, D01130)	Transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives expression of the blasticidin resistance gene in *Pichia.*
EM7 promoter	Synthetic prokaryotic promoter that drives constitutive expression of the blasticidin resistance gene in *E. coli.*
Blasticidin resistance gene (*bsd*)	Allows selection of transformants in *E. coli* and *Pichia.*
CYC1 transcription termination region	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the blasticidin resistance gene for increased stability.
pUC origin	Allows replication and maintenance of the plasmid in *E. coli.*

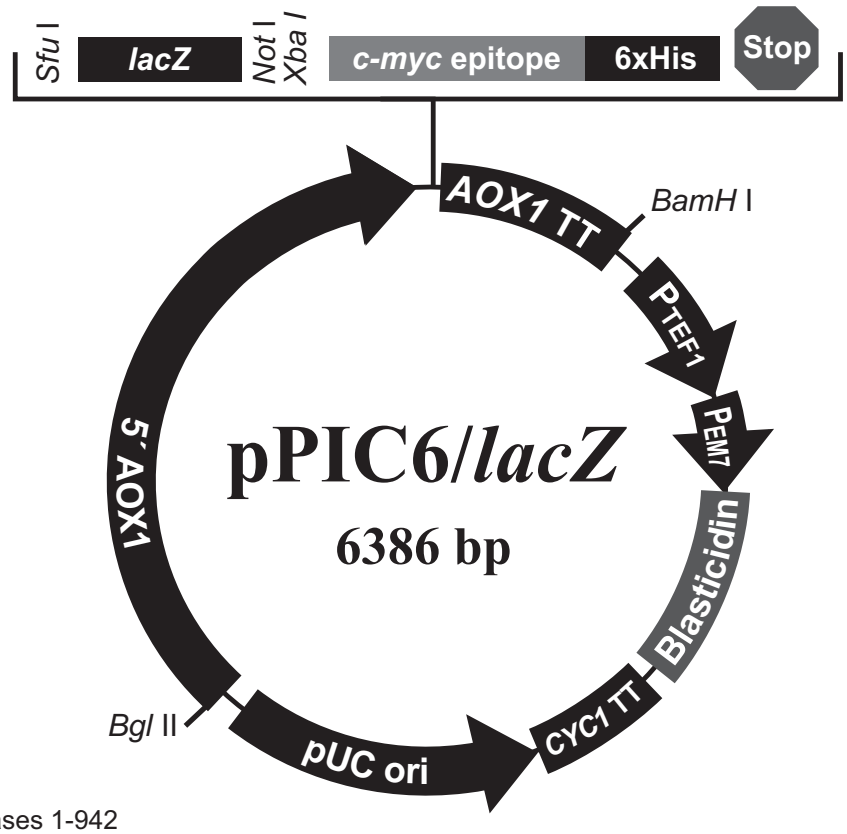
pPIC6/*lacZ* Vector

Description

pPIC6/*lacZ* is a 6386 bp control vector containing the gene for β-galactosidase. The vector was constructed by ligating a 3.1 kb *Bst*B I/*Not* I fragment containing the *lacZ* gene into the pPIC6 B vector.

Map of pPIC6/*lacZ*

The figure below summarizes the features of the pPIC6/*lacZ* vector. The complete sequence for pPIC6/lacZ is available for downloading from www.invitrogen.com or from **Technical Support** (see page 35).



Comments for pPIC6/*lacZ*: 6386 nucleotides

5´ *AOX1* promoter region: bases 1-942
5´ *AOX1* priming site: bases 855-875
LacZ ORF: bases 941-3997
c-myc epitope: bases 4016-4045
Polyhistidine (6xHis) tag: bases 4061-4078
3´ *AOX1* priming site: bases 4164-4184
AOX1 transcription termination region: bases 4082-4423
TEF1 promoter: bases 4424-4832
EM7 promoter: bases 4837-4903
Blasticidin resistance gene: bases 4904-5302
CYC1 transcription termination region: bases 5331-5648
pUC origin: bases 5659-6332 (complementary strand)

Recipes

Low Salt LB Medium with Blasticidin

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.0 with 1N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the blasticidin to 100 µg/mL final concentration.
4. Store plates at 4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.

YPD (+ Blasticidin)

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
± 2% agar
± the appropriate concentration of blasticidin

1. Dissolve: 10 g yeast extract
   20 g of peptone

   in 900 mL of water.
2. Include 20 g of agar if making YPD slants or plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 mL of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of blasticidin from a 10 mg/mL stock solution. **Note:** It is necessary to include blasticidin in the medium for selection of *Pichia* transformants only. Blasticidin may be omitted from the medium when performing expression studies.

Store YPD slants or plates containing blasticidin at 4°C. The shelf life is one to two weeks.

Continued on next page

YPDS + Blasticidin Agar

Yeast Extract Peptone Dextrose Medium with Sorbitol (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
1 M sorbitol
± 2% agar
± the appropriate concentration of blasticidin

1. Dissolve: 10 g yeast extract
   182.2 g sorbitol
   20 g of peptone

   in 900 mL of water.
2. Add 20 g of agar.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 mL of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add the appropriate amount of blasticidin from a 10 mg/mL stock solution. **Note:** It is necessary to include blasticidin in the medium for selection of *Pichia* transformants only. Blasticidin may be omitted from the medium when performing expression studies.

Store YPDS slants or plates containing blasticidin at 4°C. The shelf life is one to two weeks.

Breaking Buffer

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

1. Prepare a stock solution of your desired protease inhibitors and store appropriately. Follow manufacturer's recommendations.
2. For 1 liter, dissolve: 6 g sodium phosphate (monobasic)
   372 mg EDTA
   50 mL glycerol

   in 900 mL deionized water.
3. Use NaOH to adjust pH to 7.4 and bring up the volume to 1 liter. Store at 4°C.
4. Add protease inhibitors immediately before use.

Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g., a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

Blasticidin is soluble in water. Water is generally used to prepare stock solutions of 5 to 10 mg/mL.

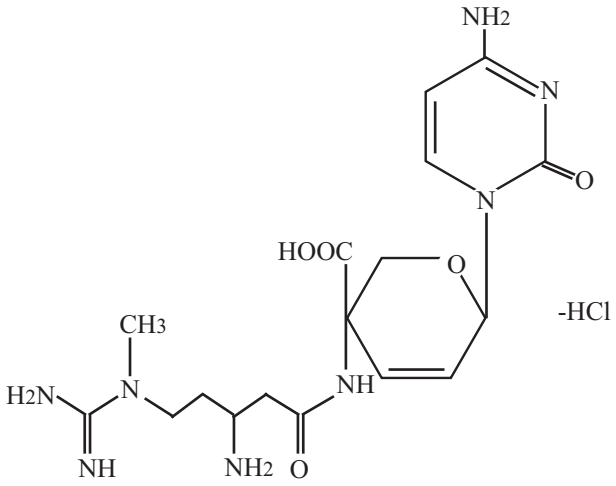
- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see last point below) and freeze at –20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at -20°C.
- pH of the aqueous solution should not exceed 7 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (do not store in a frost-free freezer).
- Upon thawing, use what you need and discard the unused portion.

Continued on next page

Blasticidin , Continued

Molecular Weight, Formula, and Structure

The formula for blasticidin is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Lithium Chloride Transformation Method

Introduction

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

Preparing Solutions

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

- 1 M LiCl in distilled, deionized water. Filter-sterilize. Dilute as needed with sterile water.
- 50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter-sterilize. Store in a tightly capped bottle.
- 2 mg/mL denatured, sheared salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at –20°C.

Preparing Cells

1. Grow a 50 mL culture of *Pichia pastoris* in YPD at 30°C with shaking to an OD_{600} of 0.8 to 1.0 (approximately 10^8 cells/mL).
2. Harvest the cells, wash with 25 mL of sterile water, and centrifuge at 1,500 × *g* for 10 minutes at room temperature.
3. Resuspend the cell pellet in 1 mL of 100 mM LiCl and transfer the suspension to a 1.5 mL microcentrifuge tube.
4. Pellet the cells at maximum speed for 15 seconds and remove the LiCl with a pipet.
5. Resuspend the cells in 400 μL of 100 mM LiCl.
6. Dispense 50 μL of the cell suspension into a 1.5 mL microcentrifuge tube for each transformation and use immediately. **Do not store on ice or freeze at -20°C.**

Continued on next page

Lithium Chloride Transformation Method, Continued

Transformation

1. Boil a 1 mL sample of single-stranded DNA for 5 minutes, then quickly chill on ice. Keep on ice. **Note**: It is not necessary to boil the carrier DNA prior to each use. Store a small aliquot at –20°C and boil every 3–4 times the DNA is thawed.
2. Centrifuge the cells from Step 6, above, and remove the LiCl with a pipette.
3. For each transformation , add the following reagents IN THE FOLLOWING ORDER to the cells. PEG shields the cells from the detrimental effects of the high LiCl concentration.

50% PEG	240 µL
1 M LiCl	36 µL
2 mg/mL single-stranded DNA	25 µL
Plasmid DNA in 50 µL sterile water	5–10 µg

4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
5. Incubate the tube at 30°C for 30 minutes without shaking.
6. Heat shock in a water bath at 42°C for 20–25 minutes.
7. Centrifuge the cells at 3,800 to 6,800 × *g* to pellet.
8. Resuspend the pellet in 1 mL of YPD and incubate at 30°C with shaking.
9. After 1 hour and 4 hours, plate 25 to 100 µL on YPD plates containing the appropriate concentration of blasticidin. Incubate the plates for 2–3 days at 30°C.

Constructing *In Vitro* Multimers

Experimental Outline

At this point you should have your gene cloned into the multiple cloning site of pPIC6 A, B, or C. To generate multiple copies of your expression cassette:

Step	Description
1	Digest pPIC6 containing your gene of interest with *Bgl* II and *Bam*H I to release the expression cassette (P_{AOX1} plus your gene).
2	To clone multiple copies of the expression cassette, linearize pPIC6 containing your gene of interest using *Bam*H I. Note that the *Bam*H I-linearized vector already contains one copy of your expression cassette.
3	Treat the *Bgl* II-*Bam*H I expression cassette with ligase *in vitro*. Note that *Bgl* II and *Bam*H I share 4 bases in common between their recognition sites (GATC).
4	Generate head-to-tail, head-to-head, and tail-to-tail multimers (Head-to-tail ligation, which is the correct orientation for expression, will destroy both the *Bam*H I and *Bgl* II sites).
5	Treat the ligation mix with *Bam*H I and *Bgl* II to eliminate head-to-head and tail-to-tail multimers.
6	Ligate into *Bam*H I-linearized recombinant pPIC6.
7	Transform into *E. coli* and analyze recombinant plasmids for copy number by digesting with *Bgl* II and *Bam*H I.

Alternative Procedure

You may wish to build each desired multimer in increments by ligating each additional expression cassette one (or two) at a time into pPIC6 A, B, or C. For example:

Step	Description
1	Digest pPIC6 containing one copy of your gene with *Bam*H I
2	Ligate a single copy of the *Bgl* II-*Bam*H I expression cassette into *Bam*H I-digested vector
3	Transform *E. coli* and analyze the transformants for the vector with 2 copies of your insert
4	Isolate and digest this vector (with 2 copies of your gene) with *Bam*H I and *Bgl* II to release a cassette with 2 copies of your gene (optional)
5	Digest the vector with 2 copies of your gene with *Bam*H I and ligate 1 or 2 copies (see Step 4) of the expression cassette into the vector
6	Transform *E. coli* and analyze the transformants for the vector with 3 or 4 copies of your insert
7	Repeat until the desired multimer is reached

Continued on next page

Constructing *In Vitro* Multimers, Continued

Controls

To evaluate your transformants and expression data later on, we recommend transforming *Pichia* with pPIC6 (the parent vector) and pPIC6 containing one copy of your gene of interest. This will allow you to compare expression levels to see if multiple copies significantly increase the amount of protein produced. Also, if you elect to determine how many copies of your gene are in a recombinant by dot or Southern blot, the strain with the parent vector will control for background hybridization and the strain with the single copy gene will provide a signal to normalize your data.

Important

Once you have created a pPIC6 plasmid containing multimers, note that this plasmid cannot be linearized because any enzyme that cuts in the 5´ *AOX1* region will cut in all of the 5´ *AOX1* regions present in the multimer. You can transform with uncut plasmid, but you will need to use 50–100 μg of DNA to compensate for the 10 to 100-fold drop in transformation efficiency. However, with selection on blasticidin, any transformants you obtain will probably contain your construct. For best results:

- Use electroporation to transform your cells
- Use at least 50 μg plasmid DNA for each transformation
- Plate out all of the transformation mix on several YPDS plates containing the appropriate concentration of blasticidin. You will need to use the optional outgrowth procedure on page 11.

Digestion of Recombinant pPIC6

Set up two separate digests of recombinant pPIC6 containing one copy of your gene:

1. Double digest 1–2 μg of recombinant pPIC6 in 20 μL with 10 units each of *Bgl* II and *Bam*H I. Proceed to **Producing Expression Cassettes for Multimerization**, Step 1.
2. Digest 2 μg of recombinant pPIC6 in 20 μL with 10 units of *Bam*H I only. Proceed to **Dephosphorylating the Vector**, Step 1.

Continued on next page

Producing Expression Cassettes for Multimerization

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (see page 33) allows you to rapidly purify DNA fragments from regular agarose gels. Alternatively, you may use glass milk. To use the S.N.A.P.™ Gel Purification Kit, follow the steps below:

1. Electrophorese your digest from Step 1, above, on a 1 to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 mL at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x *g* in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µL of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified DNA in 15 µL of sterile water. Store on ice if proceeding immediately to **Ligating the Expression Cassette**, next page. Store at –20ºC for long-term storage.

Continued on next page

Constructing *In Vitro* Multimers, Continued

Dephosphorylating the Vector

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

1. Take your *Bam*H I digest from **Digesting Recombinant pPIC6**, Step 2 and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 µL of sterile water.
2. Set up a 20 µL dephosphorylation reaction in a microcentrifuge tube as follows:

*Bam*H I digested recombinant pPIC6 (page 26, Step 2)	17 µL
10X CIAP Buffer	2 µL
CIAP (1 Unit/µL)	1 µL

3. Incubate at 37°C for 15 minutes.
4. Add 30 µL of sterile water to the reaction for a final volume of 50 µL.
5. Add 50 µL of phenol/chloroform and extract your DNA solution.
6. Precipitate the DNA by adding 5 µL of 3 M sodium acetate and 110 µL of 100% ethanol. Incubate on ice for 30 minutes.
7. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.
8. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol.
9. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.
10. Resuspend pellet in 8 µL sterile water. Save on ice if you plan to ligate your insert immediately (see **Ligation and Digestion of Expression Cassette**) or store at –20°C.

Ligating and Digesting the Expression Cassette

Ligation of the expression cassette will generate head-to-tail, head-to-head, and tail-to-tail multimers. Creation of head-to-tail multimers will be in the correct orientation for expression and will destroy both the *Bam*H I and *Bgl* II sites between the expression cassettes. Digestion of the multimers with *Bam*H I and *Bgl* II will eliminate those multimers with tail-to-tail and head-to-head orientation. After digestion with these two restriction enzymes, you will have a mixture of multimers containing 1, 2, 3, etc. copies of your gene that can be ligated into *Bam*H I-linearized, recombinant pPIC6.

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

Bgl II-*Bam*H I digested expression cassette	15 µL
Sterile water	2 µL
10X Ligation Buffer (with ATP)	2 µL
T4 DNA Ligase (2.5 units/µL)	1 µL

2. Incubate at 16°C for 2.5 hours.

Continued on next page

Ligation and Digestion of Expression Cassette, Continued

3. Heat inactivate the ligase by incubating at 65°C for 20 minutes.
4. Add the following reagents for restriction enzyme digestion (cut-back). Note that *Bam*H I and *Bgl* II may be used with the same reaction buffer:

Sterile water	23 µL
10X restriction enzyme buffer	5 µL
Bgl II (10 units/µL)	1 µL
*Bam*H I (10 units/µL)	1 µL

5. Incubate the reaction at 37°C for 2 hours.
6. Add 50 µL of phenol/chloroform and extract the restriction enzyme digestion to remove the enzymes. Transfer the aqueous solution to a new microcentrifuge tube.
7. To ethanol precipitate the DNA, add 5 µL of 3 M sodium acetate and 110 µL of 100% ethanol.
8. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.
9. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.
10. Resuspend pellet in 4 µL sterile water. Save on ice if you plan to ligate your insert immediately or you can store at –20°C. Proceed to **Ligating Multimers into Linearized Vector**.

Note

You may wish to combine the ligation reaction with the restriction enzyme digestion to enrich for head-to-tail multimers. Use the reaction buffer for the restriction enzymes and add 1 mM ATP to the reaction in order to ensure ligase activity. Perform the reaction at 37°C. T4 ligase will retain most of its activity in the restriction buffer. As head-to-head and tail-to-tail multimers form, they will be digested, increasing the likelihood of obtaining head-to-tail multimers over time.

Ligating Multimers into Linearized Vector

You are now ready to ligate the mixture of multimers generated in Step 10, above, into dephosphorylated, linearized vector.

1. Set up the following ligation reactions:

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

Continued on next page

Ligating Multimers into Linearized Vector, Continued

For the vector only control:

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

2. Incubate overnight at 16°C.
3. You may store the ligation reactions at –20°C until ready to use, or transform 1–10 µL of each ligation mix into competent *E. coli*. Note that the amount of the ligation mixture you transform depends on whether you use electrocompetent or chemically competent cells. You may have to decrease the amount you to transform into electrocompetent cells to prevent arcing.

Transformation into *E. coli*

Remember to include the "vector only" and "cells only" controls to evaluate your experiment. The "vector only" will indicate whether your vector was dephosphorylated. Since the CIAP reaction is not 100% and because you often get degradation of the ends, there might be a few colonies on this plate. The "cells only" plate should have no colonies at all.

1. Transform competent *E. coli* by your method of choice.
2. After adding medium to the transformed cells and allowing them to recover, plate 10 µL and 100 µL of each transformation mix onto Low Salt LB plates containing 100 µg/mL blasticidin. Save the remainder of your transformation mix at 4°C.
3. Incubate overnight at 37°C. If you do not get transformants or very few transformants, plate out the remainder of the transformation mix onto Low Salt LB-blasticidin plates.

Continued on next page

Analyzing Transformants

1. Pick 20 transformants and inoculate each colony into 2 mL Low Salt LB containing 100 µg/mL blasticidin. Grow overnight at 37°C.
2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pPIC6.

   (Be sure to include *Bgl* II-*Bam*H I digested pPIC6 as a control. It is possible to get vector rearrangements and deletions with large recombinant vectors in *E. coli*. Including *Bgl* II-*Bam*H I digested pPIC6 will allow you to detect these rearrangements-deletions in the vector backbone.)
3. Analyze your digests on a 1% agarose gel. You should see bands corresponding to 1 copy, 2 copies, 3 copies, etc. of your expression cassette along with the vector backbone.

   (The number of copies you obtain may depend on how well a large vector is tolerated by the host strain.)
4. Once you have identified plasmids with multiple copies of your expression cassette, be sure to purify by streaking for single colonies and confirming your construct.
5. Prepare frozen glycerol stocks of *E. coli* containing each of your multimeric constructs.
6. Prepare at least 100 µg of each plasmid for transformation into *Pichia*. You need more DNA because you will be transforming with uncut plasmid DNA. Transformation efficiency is about 1 to 2 orders of magnitude less for uncut versus linearized DNA.
7. Proceed to ***Pichia* Transformation**, page 8. Use the outgrowth protocol on page 11 to isolate transformants.

Continued on next page

Troubleshooting

The table below will help you optimize formation and isolation of multimers in *Pichia*.

Problem	Possible Reason	Solution
No multimers or low number of multimers in your vector after transformation into *E. coli*	CIAP defective	Use fresh CIAP.
		Add more CIAP. Add 1 unit of CIAP and incubate 15 more minutes at 37°C. This is somewhat risky as CIAP can degrade the ends of your DNA.
	Not enough insert DNA to ligate	Add more *Bam*H I-*Bgl* II expression cassette to your ligation.
	Construct is unstable in *E. coli*	Decrease the number of cassettes in the vector.
	Multimers are too long to ligate efficiently	Try ligating each expression cassette stepwise.
Recombinant vector rearranges and deletions are detected	Construct is unstable in *E. coli*	Decrease the number of cassettes in the vector.
No blasticidin-resistant *Pichia* transformants	Integration efficiency is low	Transform using more DNA and/or do multiple transformations with more DNA and cells.

For More Information

There are a number references in the literature you can consult to optimize synthesis of *in vitro* multimers. A partial list is provided below:

Cohen, B. and Carmichael, G. G. (1986) A Method for Constructing Multiple Tandem Repeats of Specific DNA Fragments. *DNA* **5**: 339-343.

Eisenberg, S., Francesconi, S. C., Civalier, C. and Walker, S. S. (1990) Purification of DNA-Binding Proteins by Site-specific DNA Affinity Chromatography. *Methods Enzymol.* **182**: 521-529.

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Accessory Products

Introduction

The following products may be used with the pPIC6 vectors. For details, visit www.invitrogen.com or contact **Technical Support** (see page 35).

Item	Amount	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
One Shot® TOP10F´ Chemically Competent *E. coli*)	21 × 50 µL	C3030-03
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
S.N.A.P.™ Gel Purification Kit	25 preps	K1999-25
β-Gal Assay Kit	80 mL	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
Blasticidin	50 mg	R210-01
Positope™ Control Protein	5 µg	R900-50
WesternBreeze® Chromogenic Kit	Anti-Mouse	WB7103
	Anti-Rabbit	WB7105
	Anti-Goat	WB7107
WesternBreeze® Chemiluminescent Kit	Anti-Mouse	WB7104
	Anti-Rabbit	WB7106
	Anti-Goat	WB7108
Countess™ Automated Cell Counter	1 each	C10227

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Accessory Products, Continued

Other *Pichia* Products

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

Item	Amount	Catalog no.
X-33 *Pichia* strain	1 stab	C180-00
KM71H *Pichia* strain	1 stab	C182-00
SMD1168H *Pichia* strain	1 stab	C184-00
pPIC6α A, B, and C	20 µg each	V215-20
pPICZ A, B, and C	20 µg each	V190-20
pPICZα A, B, and C	20 µg each	V195-20
Original *Pichia* Expression Kit	1 kit	K1710-01
EasySelect™ *Pichia* Expression Kit	1 kit	K1740-01
Pichia EasyComp™ Transformation Kit	1 kit	K1730-01
Pichia Protocols	1 book	G100-01

Antibodies

If you do not have an antibody specific to your protein, Invitrogen offers the Anti-*myc*, or Anti-His(C-term) antibodies to detect your recombinant fusion protein. Horseradish peroxidase (HRP)- and alkaline phosphatase (AP)–conjugated antibodies are available for convenient one-step detection.

Antibody	Epitope	Catalog no.
Anti-*myc*	Detects a 10 amino acid epitope derived from *c-myc* (Evan *et al.*, 1985): EQKLISEEDL	R950-25
Anti-*myc*-HRP		R951-25
Anti-*myc*-AP		R952-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner et al., 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25
Anti-His(C-term)-AP		R932-25

Technical Support

Web Resources

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

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

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/sds.

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