USER GUIDE

invitrogen™
by life technologies™

pFLD and pFLDα

Pichia pastoris expression vectors for inducible expression with methylamine and selection on Zeocin™

Catalog number V230-20

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life technologies™

Contents

Kit Contents and Storage

Shipping/Storage

Vectors are shipped at room temperature. Upon receipt, store at -20°C.

Kit Contents

Catalog number V230-20 includes the following vectors, supplied at a concentration of 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL:

- pFLD
- pFLDα
- pFLD/CAT (control plasmid)

Reference Sources

The pFLD vectors may be used with the EasySelect™ *Pichia* Expression Kit (Catalog no. K1740-01) or the Original *Pichia* Expression Kit (Catalog no. K1710-01), available for purchase. Additional general 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.lifetechnologies.com/support or obtained from Technical Support (see page 31). For more information about the EasySelect™ *Pichia* Expression Kit or the Original *Pichia* Expression Kit, refer to www.lifetechnologies.com/support or call Technical Support.

More detailed information and protocols dealing with *Pichia pastoris* may also be found in the following general reference:

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)

Product Use

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

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Recommended *Pichia* Host Strain

We recommend using the X-33 *Pichia* strain as the host for expression of recombinant proteins from pFLD. Other *Pichia* strains including GS115, KM71H, and SMD1168H are suitable. The X-33 *Pichia* strain and other strains are available for purchase (see page 29 for ordering information). The X-33 *Pichia* strain has the following genotype and phenotype:

Genotype: Wild-type

Phenotype: Mut$^+$

Materials Supplied by the User

Equipment

- Microbiological equipment
- Electroporation device and 0.2 cm cuvettes or reagents for transformation
- 16°C and 37°C water baths or temperature blocks
- 30°C and 37°C shaking and non-shaking incubators
- Hemacytometer
- Microtiter plates (optional)

Reagents

- *Pichia* host strain (e.g., X-33, GS115, SMD1168H, KM71H)
- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation
- Restriction enzymes and appropriate buffers
- Agarose and low-melt agarose
- PureLink® HQ Mini Plasmid Purification Kit (see page 29 for ordering) 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)
- Zeocin™ selection agent (see page 29 for ordering information)
- YPDS plates containing the appropriate concentration of Zeocin™ (see page 18 for recipe)
- 50 mL conical centrifuge tubes
- 15 mL polypropylene tubes
- ProBond™ Purification System (optional, see page 30 for ordering)

Introduction

Product Overview

Introduction

pFLD (4.4 kb) and pFLDα (4.7 kb) are vectors used to express recombinant proteins in *Pichia pastoris*. Recombinant proteins are expressed as fusions to a C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag. The vector allows high-level, methanol- and methylamine-inducible expression of the gene of interest in *Pichia*, and can be used in any *Pichia* strain, including X-33, GS115, SMD1168H, and KM71H. The pFLD vectors contain the following elements:

- 5′ fragment containing the *FLD1* promoter for methanol- or methylamine-induced expression of the gene of interest (Shen *et al*, 1998)
- Zeocin™ resistance gene for selection in both *E. coli* and *Pichia* (Baron *et al.*, 1992; Drocourt *et al.*, 1990)
- Ampicillin resistance gene for selection in *E. coli*
- C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant fusion protein (if desired)

Experimental Overview

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

Step	Action	Page
1	Propagate pFLD 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 pFLD vectors in frame with the C-terminal tag.	3–6
3	Transform into *E. coli* and select transformants on select on LB with 50–100 µg/mL ampicillin (LB-Amp).	7
4	Analyze 10–20 transformants by restriction mapping or sequencing to confirm in-frame fusion of your gene with the C-terminal tag.	8
5	Purify and linearize the recombinant plasmid for transformation into *Pichia pastoris*.	7–10
6	Transform your *Pichia* strain and plate onto YPDS plates containing the appropriate concentration of Zeocin™.	9–12
7	Select for Zeocin™-resistant transformants.	9
8	Optimize expression of your gene.	13–14
9	Purify your fusion protein on metal-chelating resin (*i.e.* ProBond™).	16–17

Methods

General Cloning Considerations

Introduction

The multiple cloning site for pFLD is shown on page 4 and the multiple cloning site for pFLDα is shown on page 6. Use these diagrams to design a strategy to clone your gene of interest in frame with the C-terminal peptide. General considerations for cloning and transformation are discussed in this section.

General Molecular Biology Techniques

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

E. coli Strain

Many *E. coli* strains are suitable for the propagation of the pFLD vectors, including TOP10, JM109, and DH5α™. We recommend that you propagate the pFLD 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 (see page 29 for ordering).

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 pFLD vectors contain the ampicillin resistance gene and the Zeocin™ resistance gene to allow selection of the plasmid in *E. coli* using either ampicillin or Zeocin™. The following procedure uses ampicillin to propagate and maintain pFLD plasmids:

1. Use the supplied stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent.
2. Select transformants on LB agar with 50–100 μg/mL ampicillin (LB-Amp).
3. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 7).

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General Considerations

The following are some general guidelines when using pFLD or pFLDα 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).

Special Considerations for pFLD

For pFLD only:

- Your insert should contain an initiation ATG codon as part of a yeast consensus sequence (Romanos *et al.*, 1992). An example of a yeast consensus sequence is provided below. The ATG initiation codon is shown underlined.

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

- To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide containing the V5 epitope and the polyhistidine tag. Refer to the diagram on page 4 to develop a cloning strategy.
- To express your gene of interest **without** the C-terminal peptide, make sure that your gene contains a stop codon.

Special Considerations for pFLDα

For pFLDα only:

- pFLDα is an N-terminal fusion vector. To express your gene, you must clone your gene in frame with the N-terminal α-factor secretion signal. Refer to the diagram on page 6 to develop a cloning strategy.
- To express your gene of interest **without** the C-terminal peptide, make sure that your gene contains a stop codon.
- The predicted protease cleavage sites for the α-factor signal sequence are indicated in the figure on page 6.

Cloning into pFLD

Multiple Cloning Site of pFLD

Below is the multiple cloning site for pFLD. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pFLD is available for downloading from www.lifetechnologies.com or from Technical Support (see page 31). For a map and a description of the features of pFLD, see page 24.

```
            3´ end of FLD1 Mfe I  Pml I      Sfi I         Asp718 I Kpn I Xho I
                       | |         |          |                 |  | |
581  AATTCTTGAT ATTCACACAA TTGTTCACGT GGCCCAGCCG GCCGTCTCGG ATCGGTACCT

           Sac II               Apa I Mfe I            V5 epitope
             |                     | |         ┌─────────────────────┐
641  CGAGCCGCGG CGGCCGCCAG CTTGGGCCCA ATTGGGGT GGT AAG CCT ATC CCT AAC
                                               Gly Lys Pro Ile Pro Asn

     ──────────────────────────────┐            Polyhistidine (6xHis) tag
                                                 ┌─────────────────────┐
697  CCT CTC CTC GGT CTC GAT TCT ACG GGT GTC GAC CAT CAT CAT CAT CAT CAT
     Pro Leu Leu Gly Leu Asp Ser Thr Gly Val Asp His His His His His His

748  TGA GTTTGTAGCC TTAGACATGA CTGTTCCTCA GTTCAAGTTG GGCACTTACG AGAAGACCGG
     ***

                               3´ AOX1 priming site
                             ┌──────────────────────┐
811  TCTTGCTAGA TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT
```

Cloning into pFLDα

Introduction

pFLDα possesses the α-factor mating signal sequence for secretion of your protein. The information in this section is provided to assist you in designing a cloning strategy. Details of the multiple cloning site of the pFLDα can be found on page 6.

Signal Sequence Processing

The processing of the α-factor mating signal sequence in pFLDα occurs in three steps:

1. Signal peptidase cleavage between Ala and Ala in the 19 and 20 positions.
2. Kex2 cleavage between Arg and Glu in the sequence Glu-Lys-Arg * Glu-Ala-Glu-Ala, where * is the site of cleavage.
3. The Glu-Ala repeats are further cleaved by the *STE13* gene product.

Optimization of Signal Cleavage

In *Saccharomyces cerevisiae*, the Glu-Ala repeats are not necessary for cleavage by Kex2, but cleavage after Glu-Lys-Arg may be more efficient when followed by Glu-Ala repeats. A number of amino acids are tolerated at site X instead of Glu in the sequence Glu-Lys-Arg-X. These amino acids include the aromatic amino acids, small amino acids, and histidine. Proline, however, will inhibit Kex2 cleavage. For more information on Kex2 cleavage, see (Brake *et al.*, 1984).

There are some cases where Ste13 cleavage of Glu-Ala repeats is not efficient, and Glu-Ala repeats are left on the N-terminus of the expressed protein of interest. This is generally dependent on the protein of interest.

Expression of Recombinant Protein with Native N-terminus

To have your protein expressed with a native N-terminus, you can use the *Xho* I site at bp 865–870 to clone your gene flush with the Kex2 cleavage site. Use PCR to rebuild the sequence from the *Xho* I site to the arginine codon at nucleotides 744–746. Remember to include the first amino acid(s) of your protein, if necessary, for correct fusion to the Kex2 cleavage site.

Continued on next page

Multiple Cloning Site of pFLDα

Below is the multiple cloning site for pFLDα. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The vector sequence of pFLDα is available for downloading for www.lifetechnologies.com or from Technical Support (see page 31). For a map and a description of the features of pFLDα, see page 25–26.

```
                     3´ end of FLD1
                             |
 581  AATTCTTGAT ATTCACACAA TTCAACAACT ATTTCGAAACG ATG AGA TTT CCT TCA
                                                  Met Arg Phe Pro Ser

                                                       Signal peptidase cleavage
                                                                   ▼
 637  ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC
      Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val

                                α-factor signal sequence

 688  AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC
      Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile

 739  GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC
      Gly Tyr Ser Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser

                                                      α-factor priming site
 790  AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT
      Asn Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile

                                     Xho I     Kex2 signal cleavage      Mfe I
                                       |                 ▼                 |
 841  GCT GCT AAA GAA GAA GGG GTA TCT CTC GAG AAA AGA GAA GCT GAA GCC CA
      Ala Ala Lys Glu Glu Gly Val Ser Leu Glu Lys Arg Glu Ala▲Glu Ala▲
                                                      Ste13 signal cleavage

               Pml I      Sfi I           Asp718 I  Kpn I    Sac II
                 |          |                   |   |          |
 891  ATTGTTCACG TGGCCCAGCC GGCCGTCTCG GATCGGTACC CGCGGCGGCC GCCAGCTTGG

      Apa I Mfe I                           V5 epitope
         | |
 951  GCCCAATTGG GGT GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT
                     Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser

                             Polyhistidine (6xHis) tag
1003  ACG GGT GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC TTAGACATGA
      Thr Gly Val Asp His His His His His His ***

1056  CTGTTCCTCA GTTCAAGTTG GGCACTTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA

               3´ AOX1 priming site
1116  GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT
```

Transforming *E. coli*

Transforming *E. coli*

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10, DH5α™, JM109) and select on LB agar plates containing 50-100 µg/mL ampicillin. Once you have obtained ampicillin-resistant colonies, pick 10 transformants and screen for the presence and orientation of your insert. Note that there is no blue/white screening for the presence of insert with the pFLD vectors.

We recommend that you sequence your construct to confirm that your gene is in the correct orientation for expression and cloned in frame with the C-terminal peptide (if desired). Refer to the multiple cloning site diagrams on the previous pages for the sequences and location of the priming sites. To order custom-synthesized primers, visit www.lifetechnologies.com and select Custom Primers.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. We also recommend keeping a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50–100 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50-100 µg/mL ampicillin.
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 We recommend isolating plasmid DNA using the PureLink® HiPure Miniprep Kit (up to 30 µg) or the PureLink® HiPure Midiprep Kit (up to 150 µg), or CsCl gradient centrifugation. Once you have purified plasmid DNA, proceed to ***Pichia* Transformation** on page 9.

Continued on next page

Sequencing Recombinant Clones

We recommend that you sequence your construct before transforming into *Pichia* to confirm that your gene is in frame with the α-factor secretion signal and/or the C-terminal tag. To sequence your construct in pFLD or pFLDα, we recommend using *FLD1* Forward and 3´ *AOX1* primers. The 3´ *AOX1* primer is available separately for purchase (see page 29 for ordering information). The *FLD1* Forward primer can be custom-ordered from us; visit www.lifetechnologies.com and select Custom Primers.

Sequencing Primer	Sequence
α-Factor	5´-TACTATTGCCAGCATTGCTGC-3´
3´ *AOX1*	5´-GCAAATGGCATTCTGACATCC-3´

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).

Pichia Transformation

Introduction

After your gene has been correctly cloned into one of the pFLD vectors, you are ready to transform the vector into your *Pichia* strain. This section provides general guidelines for preparing plasmid DNA, transformation, and selecting for Zeocin™-resistant clones.

Zeocin™ Selection

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

Method of Transformation

We do not recommend spheroplasting for transformation of *Pichia* with plasmids containing the Zeocin™ resistance marker. Spheroplasting involves removal of the cell wall to allow DNA to enter the cell. Cells must first regenerate the cell wall before they are able to express the Zeocin™ resistance gene. For this reason, plating spheroplasts directly onto selective medium containing Zeocin™ does not yield any transformants.

We recommend electroporation for transformation of *Pichia* with pFLD. 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 27 or the *Pichia* EasyComp™ Transformation Kit available for purchase (see below).

Pichia EasyComp™ Transformation Kit

For chemical transformation of *Pichia* strains with pFLD vectors, the *Pichia* EasyComp™ Transformation Kit is available for purchase (see page 29 for ordering information). The *Pichia* EasyComp™ Transformation Kit provides reagents to prepare six preparations of competent cells. Each preparation will yield enough competent cells for 20 transformations. Competent cells may be used immediately or frozen and stored for future use. For more information, refer to www.lifetechnologies.com/support or call Technical Support (see page 31).

Important

Since the pFLD vectors contain DNA sequences from both the *FLD1* and *AOX1* loci (promoter and polyA addition, respectively), homologous recombination in the host can occur at either of these locations. The restriction enzyme site used to linearize the vector will determine the predominant integration locus. Cutting the vector within the *FLD1* promoter sequence (see **Linearizing Your pFLD Construct** on page 10) will target the integration event to the *FLD1* locus. If the vector is completely linearized within the *FLD1* promoter—with no uncut, circular vector remaining—homologous recombination at the *AOX1* locus will not occur.

Continued on next page

Note

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

Before Starting

You will need the following reagents for transforming *Pichia* and selecting transformants on Zeocin™. **Note**: Inclusion of sorbitol in YPD plates stabilizes electroporated cells, which can be osmotically sensitive.

- 5–10 µg pure pFLD or pFLDα containing your insert
- YPD Medium
- 50-mL conical polypropylene tubes
- 1 liter cold (4°C) sterile water (place on ice the day of the experiment)
- 25 mL cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
- 30°C incubator
- Electroporation device and 0.2-cm cuvettes
- YPDS plates containing the appropriate concentration of Zeocin™ (see page 18 for recipe)

Linearizing the pFLD Construct

To promote integration, we recommend that you linearize your pFLD construct within the 5′ *FLD1* region. The table below lists unique sites that may be used to linearize pFLD prior to transformation. **Other restriction sites are possible.** Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)
Nsi I	285
Nde I	406
Cla I	493

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 in 10 µL sterile, deionized water. Use immediately or store at –20°C.

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Preparing *Pichia* for Electroporation

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

1. Grow 5 mL of your *Pichia pastoris* strain in YPD in a 50 mL conical tube at 30°C overnight.
2. Inoculate 500 mL of fresh medium in a 2 liter flask with 0.1–0.5 mL of the overnight culture. Grow overnight again to an $OD_{600} = 1.3–1.5$.
3. Centrifuge the cells at 1500 × *g* for 5 minutes at 4°C. Resuspend the pellet with 500 mL of ice-cold (0°C), sterile water.
4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 mL of ice-cold (0°C), sterile water.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 mL of ice-cold (0°C) 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 mL of ice-cold (0°C) 1 M sorbitol for a final volume of approximately 1.5 mL. Keep the cells on ice and use that day. Do not store cells.

Transformation by Electroporation

1. Mix 80 µL of the cells from Step 6 (above) with 5–10 µg of linearized pFLD or pFLDα DNA (in 5–10 µL sterile water) and transfer them to an ice-cold (0°C) 0.2-cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) as suggested by the manufacturer of the specific electroporation device being used.
4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 mL tube.
5. Let the tube incubate at 30°C without shaking for 1 to 2 hours.
6. Spread 50–200 µL each on separate, labeled YPDS plates containing the appropriate concentration of Zeocin™.
7. Incubate plates for 2 to 3 days at 30°C until colonies form.
8. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing the appropriate concentration of Zeocin™.

Continued on next page

Pichia Transformation, Continued

Note

Generally, several hundred Zeocin™-resistant colonies are generated using the protocol on page 11. If more colonies are needed, the protocol may be modified as described below. Note that you will need ~20 150-mm plates with YPDS agar containing the appropriate concentration of Zeocin™.

1. Set up two transformations per construct and follow Steps 1 through 5 of the **Transformation by Electroporation** protocol, page 11.
2. After 1 hour in 1 M sorbitol at 30°C (Step 5, page 11), add 1 mL of YPD medium to each tube.
3. Shake (~200 rpm) the cultures at 30°C.
4. After 1 hour, take one of the tubes and plate out all of the cells by spreading 200 µL on 150-mm plates containing the appropriate concentration of Zeocin™.
5. (Optional) Continue incubating the other culture for three more hours (for a total of four hours) and then plate out all of the cells by spreading 200 µL on 150-mm plates containing the appropriate concentration of Zeocin™.
6. Incubate plates for 2 to 4 days at 30°C until colonies form.

Mut Phenotype

If you used a *Pichia* strain containing a native *AOX1* gene (e.g., X-33, GS115, SMD1168H) as the host for your pFLD construct, your Zeocin™-resistant transformants will be Mut$^+$. If you used a strain containing a deletion in the *AOX1* gene (e.g., KM71H), your transformants will be Mut^S.

To verify the Mut phenotype of your Zeocin™-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. See **Expression in *Pichia***, page 13.

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

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

Detecting Recombinant Proteins in *Pichia*

We recommend that you use the following techniques to assay expression of your protein. Be sure to account for any additional amino acids that are in between the end of your protein and the C-terminal tag.

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

Important

Reminder: Because the pFLD vector does not contain the *HIS4* gene, *his4 Pichia* strains containing the integrated plasmid must be grown in medium containing 0.004% histidine. If histidine is not present in the medium the cells will not grow. If you use X-33, SMD1168H, or KM71H as the host strain, supplementation of the medium with histidine is not required.

Continued on next page

Expression Guidelines

Information and guidelines for performing small-scale expression, optimizing expression, and performing scale-up of expression are provided in the EasySelect™ *Pichia* Expression Kit manual and the Original *Pichia* Expression Kit manual. See below for information on inducing expression of the *FLD1* gene with methanol, methylamine, or methanol plus methylamine.

Inducing with Methanol, Methylamine, or Methanol Plus Methylamine

Expression of the *FLD1* gene may be induced with methanol, methylamine, or methanol plus methylamine. The following guidelines for induction have been developed using the wild-type X-33 strain; other strains may be suitable. For media recipes, see pages 19–21.

1. For all types of induction, grow cultures in MGAs medium at 30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD_{600} of 4.
2. Harvest the cells by centrifuging at 1500–3000 × *g* for 5 minutes at room temperature. Then:
   - For induction with **methanol**, decant supernatant and resuspend cell pellet in 1/5 of the original culture volume of MMAs medium.
   - For induction with **methylamine**, decant supernatant and resuspend cell pellet in 1/5 of the original culture volume of MGMa medium.
   - For induction with **methanol plus methylamine**, decant supernatant and resuspend cell pellet in 1/5 of the original culture volume of MMMa medium.
3. Incubate the resulting culture at 30°C for five days. For media containing methanol or methanol plus methylamine, add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.

   Time points: At each of the times indicated below, transfer 1 mL of the expression culture to a 1.5-mL microcentrifuge tube, and centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days), and 120 (5 days).

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 for purchase. The NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, we also carry a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to www.lifetechnologies.com or call Technical Support (see page 31).

Continued on next page

Western Analysis

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies (see page 30 for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein available for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag (see page **Error! Bookmark not defined.** for ordering). WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available for detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to www.lifetechnologies.com or call Technical Support (see page 31).

Purification

Introduction

In this section, you will grow and induce a 10–200 mL culture of your *Pichia* transformant for trial purification on a metal-chelating resin such as ProBond™. You can harvest the cells and store them at –80°C until you are ready to purify your fusion protein, or you can 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 System manual.

ProBond™ Resin

We recommend the ProBond™ Purification System to purify fusion proteins expressed from pFLD or pFLDα (see page **Error! Bookmark not defined.**). **Note that instructions for equilibration of and chromatography on ProBond™ resin are contained in the ProBond™ Purification Kit.**

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

Binding Capacity of ProBond™

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

Important

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

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). Refer to the *Pichia* Expression Kit manual for details. 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 21.

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

Continued on next page

Sample Application (Native Conditions)

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

1. Combine 1 mL (2–3 mg/mL total protein) of *Pichia* lysate with 7 mL Native Binding Buffer.
2. Prepare a ProBond™ column with resin as described in the ProBond™ manual, and resuspend the resin in the column 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 with column washing and elution under native conditions as described in the ProBond™ 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 the ProBond™ manual) or using denaturing purification may remove these non-specific *Pichia* proteins.

Analysis of 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™ 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 as indicated in the ProBond™ manual before adding it to the column. The pH should be greater than or equal to 7.5 and the NaCl concentration should be ~500 mM.

Appendix

Recipes

YPD (+ Zeocin™)

Yeast Extract **P**eptone **D**extrose Medium (1 liter)

1% yeast extract

2% peptone

2% dextrose (glucose)

±2% agar

± appropriate concentration of Zeocin™

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

Store YPD slants or plates containing Zeocin™ at 4°C. The shelf life is 1–2 weeks.

YPDS (+ Zeocin™)

Yeast Extract **P**eptone **D**extrose Medium with **S**orbitol (1 liter)

1% yeast extract

2% peptone

2% dextrose (glucose)

1 M sorbitol

± 2% agar

± appropriate concentration of Zeocin™

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

Store YPDS slants or plates containing Zeocin™ at 4°C. The shelf life is 1–2 weeks.

Continued on next page

Recipes, Continued

Stock Solutions

10X YNB (1.7% Yeast Nitrogen Base without Ammonium Sulfate)

Dissolve 17 g of yeast nitrogen base (YNB) without 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. The shelf life of this solution is approximately one year.

10X As (5% Ammonium Sulfate)

Dissolve 50 g of ammonium sulfate in 1000 mL of water and filter sterilize.

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 M (5% Methanol)

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

10X G (10% Glycerol)

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

10X Ma (2.5% Methylamine)

Mix 2.5 mL of methylamine HCl with 97.5 mL of water. Filter sterilize and store at 4°C. The shelf life of this solution is approximately two months.

MGAs

Minimal Glycerol with Ammonium sulfate (1 liter)

0.17% YNB
0.5% ammonium sulfate
1% glycerol
4×10^{-5}% biotin
Combine aseptically 700 mL autoclaved water with 100 mL of 10X YNB, 100 mL of As, 2 mL of 500X B, and 100 mL of 10X G.
Store at 4°C. The shelf life of this solution is approximately two months.

Continued on next page

MMAs

Minimal Methanol with Ammonium sulfate Medium (1 liter)

0.17% YNB
0.5% ammonium sulfate
4×10^{-5}% biotin
0.5% methanol

1. For medium, autoclave 700 mL of water for 20 minutes on liquid cycle
2. Cool autoclaved water to 60°C and add:
   100 mL of 10X YNB
   100 mL of As
   2 mL of 500X B
   100 mL of 10X M
3. Mix and store at 4°C.
4. For plates, add 20 g agar to the water in Step 1 and proceed.
5. After mixing, pour the plates immediately. MMAs stores for several months at 4°C.

MGMa

Minimal Glycerol and Methylamine Medium (1 liter)

0.17% YNB
4×10^{-5}% biotin
1% glycerol
0.25% methylamine

1. For medium, autoclave 700 mL of water for 20 minutes on liquid cycle
2. Cool autoclaved water to room temperature and add:
   100 mL of 10X YNB
   2 mL of 500X B
   100 mL 10X G
   100 mL of 10X Ma
3. Mix and store at 4°C.
4. For plates, add 20 g agar to the water in Step 1 and proceed.
5. After mixing, pour the plates immediately. MGMa stores for several months at 4°C.

Continued on next page

MMMa

Minimal Methanol and Methylamine Medium (1 liter)

0.17% YNB
4×10^{-5}% biotin
0.5% methanol
0.25% methylamine

1. For medium, autoclave 700 mL of water for 20 minutes on liquid cycle
2. Cool autoclaved water to 60°C and add:
   100 mL of 10X YNB
   2 mL of 500X B
   100 mL of 10X M
   100 mL of 10X Ma
3. Mix and store at 4°C.
4. For plates, add 20 g agar to the water in Step 1 and proceed.
5. After mixing, pour the plates immediately. MMMa stores for several months at 4°C.

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 and bring up the volume to 1 liter. Store at 4°C.
4. Add protease inhibitors immediately before use.

Zeocin™

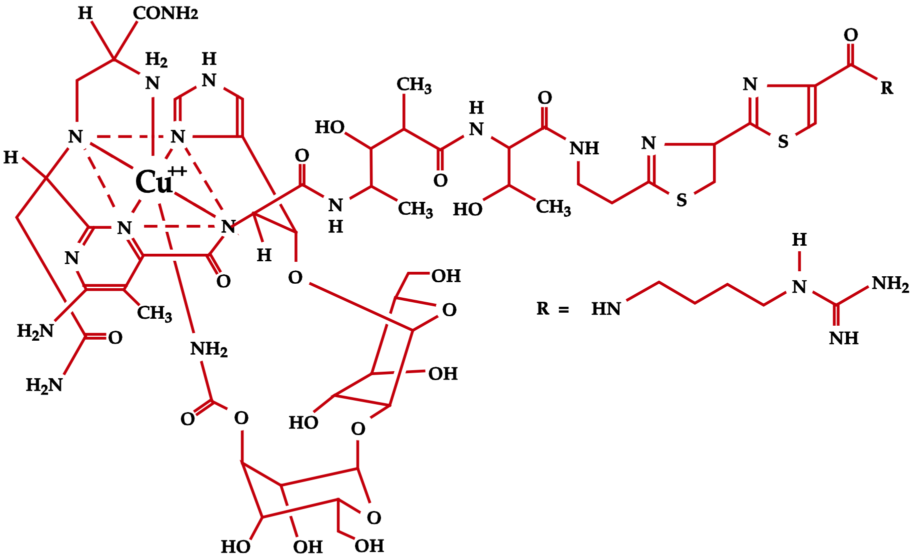
Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. It shows strong toxicity against bacteria, fungi, plants and mammalian cell lines (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1987; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

A Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This 13,665 Da protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), binds stoichiometrically to Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{55}H_{86}O_{21}N_{20}S_2Cu\text{-}HCl$ and the molecular weight is 1527.5. The structure of Zeocin™ is shown below.



Applications of Zeocin™

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

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/mL in **Low Salt LB** medium*
Pichia	100–1000 µg/mL (varies with strain and medium)

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

Continued on next page

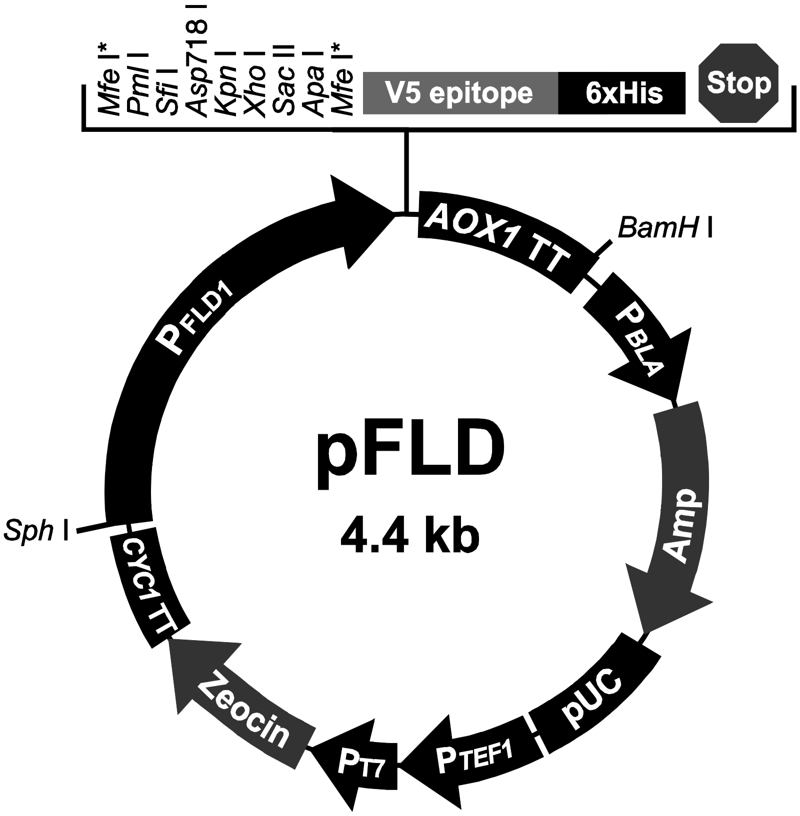
Handling Zeocin™

- High ionic strength and acidity or basicity inhibits the activity of Zeocin™. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see page 18 for a recipe).
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug and plates or medium containing the drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses when handling Zeocin™-containing solutions.
- Do not ingest or inhale solutions containing the drug.

Map of pFLD

Map of pFLD

The figure below summarizes the features of the pFLD vector. The vector sequence for pFLD is available for downloading from www.lifetechnologies.com or from Technical Support (see page 31). See page 26 for a description of the features of the vector.



Comments for pFLD:
4409 nucleotides

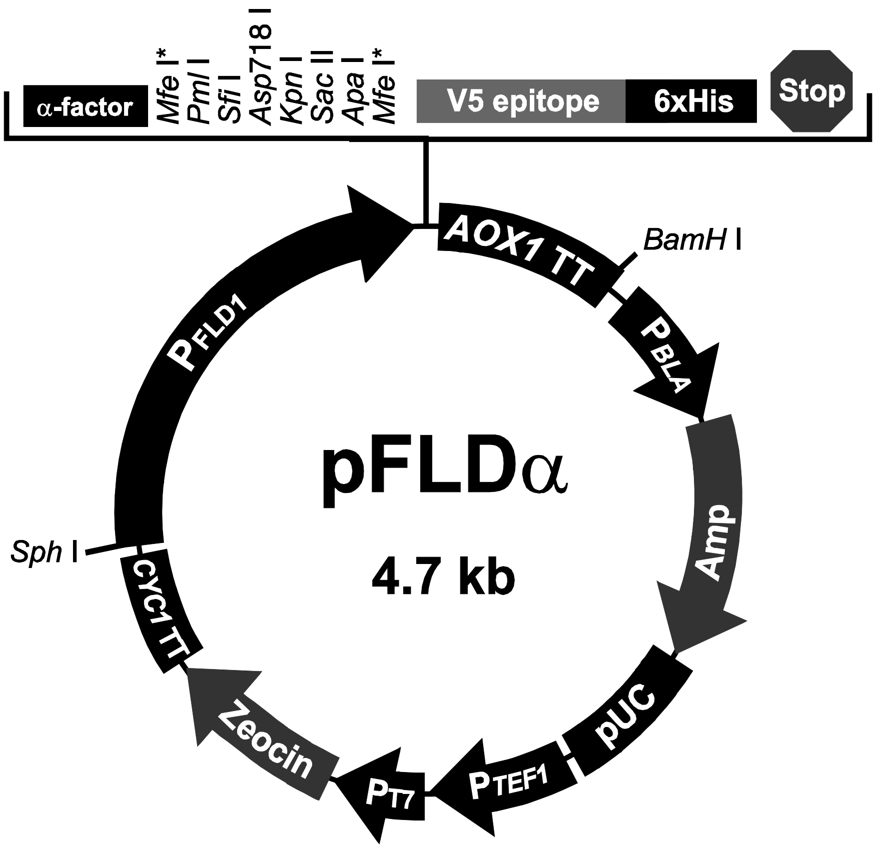
FLD1 promoter region: bases 1-597
Multiple cloning site: bases 598-669
V5 epitope: bases 679-720
Polyhistidine (6xHis) tag: bases 730-747
AOX1 transcription termination region: bases 758-1086
3´ *AOX1* priming site: bases 833-853
bla promoter: bases 1504-1510
Ampicillin resistance gene: bases 1545-2405
pUC origin: bases 2550-3222
TEF1 promoter: bases 3223-3625
T7 promoter: bases 3631-3697
Zeocin resistance gene: bases 3698-4072
CYC1 transcription termination region: bases 4073-4390

*Two *Mfe* I sites are designed to allow for the cloning of *Eco*R I fragments.

Map of pFLDα

Map of pFLDα

The figure below summarizes the features of the pFLDα vector. The complete sequence for pFLDα is available for downloading from www.lifetechnologies.com or from Technical Support (see page 31). See page 26 for a description of the features of the vector.



*Two *Mfe* I sites are designed to allow for the cloning of *Eco*R I fragments.

Comments for pFLDα:
4694 nucleotides

FLD1 promoter region: bases 1-597
α-factor signal sequence: bases 622-888
Multiple cloning site: bases 889-954
V5 epitope: bases 964-1005
Polyhistidine (6xHis) tag: bases 1015-1032
AOX1 transcription termination region: bases 1043-1371
3´ *AOX1* priming site: bases 1118-1138
bla promoter: bases 1789-1795
Ampicillin resistance gene: bases 1830-2690
pUC origin: bases 2835-3507
TEF1 promoter: bases 3508-3910
T7 promoter: bases 3916-3982
Zeocin resistance gene: bases 3983-4357
CYC1 transcription termination region: bases 4358-4675

Vector Features

Features

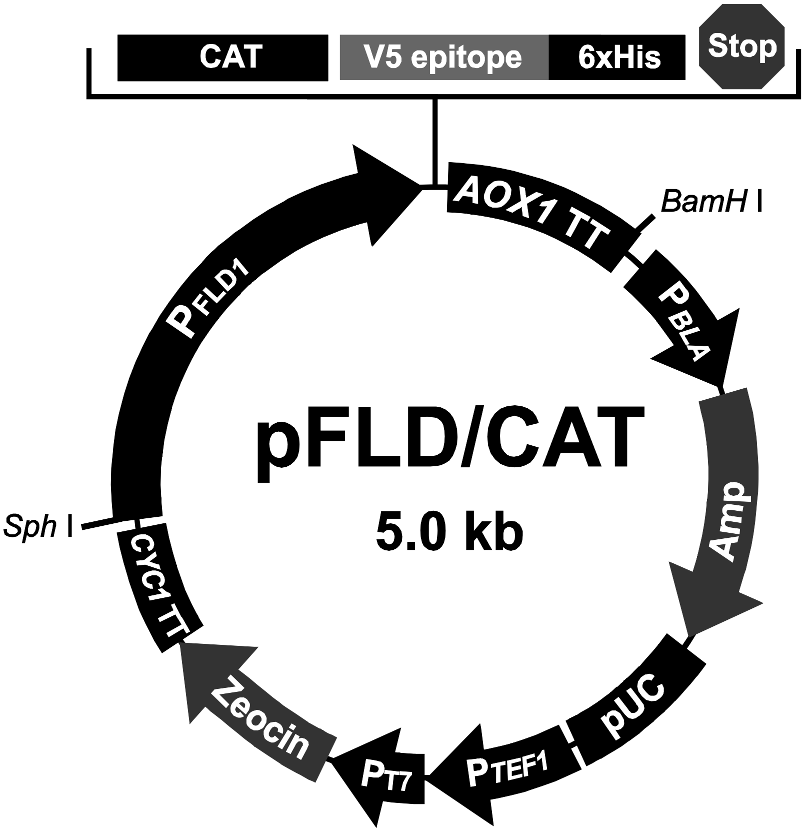
The vectors pFLD and pFLDα contain the following elements. All features have been functionally tested.

Feature	Benefit
FLD1 promoter	A 597-bp fragment containing the *FLD1* promoter that allows methanol- or methylamine-inducible, high-level expression of the gene of interest in *Pichia* Targets plasmid integration to the *FLD1* locus.
α-Factor Secretion Signal (pFLDα only)	Encodes the native *Saccharomyces cerevisiae* α-factor secretion signal that allows for efficient secretion of most proteins from *Pichia* (Cregg *et al.*, 1993)
Multiple cloning site	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)	Permits detection of your recombinant fusion protein with the Anti-V5 Antibody or Anti-V5-HRP Antibody (Southern *et al.*, 1991) (see page 30 for ordering)
C-terminal polyhistidine (6xHis) tag	Permits purification of your recombinant fusion protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Lindner *et al.*, 1997) and the Anti-His(C-term)-HRP Antibody (see page **Error! Bookmark not defined.** for ordering)
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 Zeocin™ resistance gene in *Pichia*
T7 promoter	Synthetic prokaryotic promoter that drives constitutive expression of the Zeocin™ resistance gene in *E. coli*
Zeocin™ resistance gene (*Sh ble*)	Allows selection of transformants in *E. coli* and *Pichia*
CYC1 transcription termination region (GenBank accession number M34014)	3´ end of the *Saccharomyces cerevisiae CYC1* gene that allows efficient 3´ mRNA processing of the Zeocin™ resistance gene for increased stability
pUC origin	Allows replication and maintenance of the plasmid in *E. coli*

Map of pFLD/CAT

Map of pFLD/CAT

pFLD/CAT (5014 bp) is a control vector expressing chloramphenicol acetyltransferase (CAT). It was constructed by cloning the CAT gene into the pFLD vector. The figure below summarizes the features of the pFLD/CAT vector. The vector sequence for pFLD/CAT is available for downloading from www.lifetechnologies.com or from Technical Support (see page 31).



Comments for pFLD/CAT:
5014 nucleotides

FLD1 promoter region: bases 1-597
CATORF: bases 612-1268
V5 epitope: bases 1284-1325
Polyhistidine (6xHis) tag: bases 1335-1352
AOX1 transcription termination region: bases 1363-1691
3´ *AOX1* priming site: bases 1438-1458
bla promoter: bases 2109-2115
Ampicillin resistance gene: bases 2150-3010
pUC origin: bases 3155-3827
TEF1 promoter: bases 3828-4230
T7 promoter: bases 4236-4302
Zeocin resistance gene: bases 4303-4677
CYC1 transcription termination region: bases 4678-4995

Lithium Chloride Transformation Method

Introduction

This modified version of the procedure described for *S. cerevisiae* (Gietz and Schiestl, 1996) is provided as an alternative to transformation by electroporation. Transformation efficiency is between 10^2 and 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 1500 × *g* for 10 minutes at room temperature.
3. Resuspend the cell pellet in 1 mL of 100 mM LiCl and transfer the suspension to a 1.5 mL microcentrifuge tube.
4. Pellet cells at maximum speed for 15 seconds and remove LiCl with a pipette.
5. Resuspend the cells in 400 µL of 100 mM LiCl.
6. Dispense 50 µL of cell suspension into a 1.5-mL microcentrifuge tube for each transformation and use immediately. **Do not store on ice or freeze at -20°C.**

Transformation

1. Boil a 1 mL sample of single-stranded DNA for 5 minutes, then quickly chill on ice. Keep on ice. **Note**: It is neither necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at –20°C and boil every 3–4 times the DNA is thawed.
2. Centrifuge the cells from Step 6, above, and remove the LiCl with a pipette.
3. For each transformation, add the following reagents IN THE ORDER GIVEN to the cells. PEG shields the cells from the detrimental effects of the high LiCl concentration.

   240 µL 50% PEG
   36 µL 1 M LiCl
   25 µL 2 mg/mL single-stranded DNA
   Plasmid DNA (5–10 µg) in 50 µL sterile water
4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
5. Incubate the tube at 30°C for 30 minutes without shaking.
6. Heat shock in a water bath at 42°C for 20–25 minutes.
7. Centrifuge the cells at 6000 to 8000 rpm to pellet.
8. Resuspend the pellet in 1 mL of YPD and incubate at 30°C with shaking.
9. After 1 hour and 4 hours, plate 25 to 100 µL on YPD plates containing the appropriate concentration of Zeocin™. Incubate the plates for 2–3 days at 30°C.

Accessory Products

Introduction

The products listed in this section are intended for use with the pFLD vectors. For more information, refer to www.lifetechnologies.com/support or call Technical Support (see page 31).

Zeocin™

Zeocin™ may be available for purchase. For your convenience, the drug is prepared in autoclaved, deionized water and available in 1.25 mL aliquots at a concentration of 100 mg/mL.

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

Additional Products

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

Item	Amount	Catalog no.
Ampicillin, Sodium Salt, lyophilized	20 mL	11593-019
X-33 *Pichia* strain	1 stab	C180-00
GS115 *Pichia* strain	1 stab	C181-00
KM71H *Pichia* strain	1 stab	C182-00
SMD1168H *Pichia* strain	1 stab	C184-00
3′ AOX1 *Pichia* Primer	2 µg	N720-02
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
CAT Antiserum (supplied amount is sufficient to perform 25 Western blots using 10 mL of working solution per reaction)	50 µL	R902-25
One Shot® TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
One Shot® TOP10 Electrocomp™ (electrocompetent cells)	21 × 50 µL	C4040-52
Electrocomp™ TOP10 (electrocompetent cells)	5 × 80 µL	C664-55
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink® HiPure Miniprep Kit	100 preps	K2100-03
PureLink® HiPure Midiprep Kit	25 preps	K2100-04

Accessory Products, Continued

Detecting Fusion Protein

A number of antibodies are available for purchase to detect expression of your fusion protein from the pFLD vector. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments.
The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibody only).

Antibody	Epitope	Catalog no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern et al., 1991). GKPIPNPLLGLDST	R960-25
Anti-V5-HRP		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25
Anti-His(C-term)	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25
Positope™ Control Protein		R900-50

Purifying Fusion Protein

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

Item	Quantity	Catalog no.
ProBond™ Purification System	Precharged ProBond™ resin and buffers plus 6 × 2 mL columns for native and denaturing purification	K850-01
ProBond ™ Purification System with Anti-V5-HRP Antibody	1 Kit (same as above plus 50 µL of antibody) The amount of antibody supplied is sufficient for 25 Westerns	K854-01
ProBond ™ Purification System with Anti-His(C-term)-HRP Antibody	1 Kit (same as above plus 50 µL of antibody) The amount of antibody supplied is sufficient for 25 Westerns	K853-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
Purification Columns	50 polypropylene columns	R640-50

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com/support.
At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Notes

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