USER GUIDE

invitrogen™
by life technologies™

His-Patch ThioFusion™ Expression System

Catalog number K360-01

Revision date 19 January 2012
Publication Part number 25-0120

MAN0000029

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

life technologies™

Table of Contents

Kit Contents and Storage

Shipping and Storage

The His-Patch ThioFusion™ Expression System is shipped at room temperature. The components are listed below and should be stored as indicated.

ProBond™ Components

The ProBond™ Purification System includes enough resin, reagents, and columns for six purifications. Upon receipt, store as detailed below.

Item	Composition	Amount	Storage
ProBond™ Resin	50% slurry, 20% ethanol	12 mL	4°C
5X Native Purification Buffer	250 mM $NaPO_4$ 2.5 M NaCl	125 mL	Room Temperature
3 M Imidazole	Imidazole, pH 6.0	8 mL	Room Temperature
Purification Columns	10 mL columns	6 each	Room Temperature

Vectors, Primers, and *E. coli*

The vectors, primers, and *E. coli* stab provided with the His-Path ThioFusion™ Expression System are listed below. Store as detailed below.

Vectors

Name	Amount	Storage
pThioHis A, B, and C	20 µg each, 40 µL at 0.5 µg/µL in TE buffer, pH 8.0*	–20°C

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

Primers

All primers are supplied lyophilized. **Store lyophilized and resuspended primers at –20°C**.

Sequencing Primer	Sequence	Amount	Total Moles
Trx Forward	**5´-TTCCTCGACGCTAACCTG-3´**	2 µg	3.71×10^{-10}
Trx Reverse	**5´-TGTAAAACGACGGCCAGTGC-3´**	2 µg	3.26×10^{-10}

***E. coli* stab**

TOP10 *E. coli* is provided as a stab. **Store the stab at 4°C.** See page v for long term storage.

Genotype: F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

Continued on next page

Long-Term Storage

Store the TOP10 *E. coli* stab supplied with the kit at 4°C. We recommend that you prepare a set of glycerol master stocks within two weeks of receiving the kit. For long-term storage of the TOP10 *E. coli* strain supplied as a stab with this kit, prepare glycerol stocks as follows:

1. Streak a small portion of the TOP10 cells from the stab on an LB plate containing 100 µg/mL ampicillin and incubate at 37°C overnight.
2. Isolate a single colony and inoculate into 5–10 mL of LB medium with 100 µg/mL ampicillin.
3. Grow the culture to stationary phase (OD_{600} = 1–2).
4. Mix 0.8 mL of the culture with 0.2 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C. Use one master stock to create working stocks for regular use.

Resin and Column Specifications

ProBond™ resin is precharged with Ni^{2+} ions and appears blue in color. It is provided as a 50% slurry in 20% ethanol. ProBond™ resin and purification columns have the following specifications:

- Binding capacity of ProBond™ resin: 1–5 mg of protein per mL of resin
- Average bead size: 45–165 microns
- Pore size of purification columns: 30–35 microns
- Recommended flow rate: 0.5 mL/min
- Maximum flow rate: 2 mL/min
- Maximum linear flow rate: 700 cm/h
- pH stability (long term): 3–13
- pH stability (short term): 2–14

Intended Use

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

Required Equipment and Supplies

Required Reagents (not provided)

- LB medium (See **Recipes**, page 29)
- 100 mg/mL ampicillin stock solution
- 100 mM IPTG (see **Recipes**, page 30)
- FSB Solution (see **Recipes**, page 30)
- Dimethyl sulfoxide (DMSO) (See **Recipes**, page 30)
- Restriction enzymes and buffers
- T4 DNA ligase and buffer
- Solutions for SDS-PAGE gels
- Dry Ice
- Technical grade ethanol for dry ice baths
- Reagent grade ethanol for use with DNA

Required Equipment (not provided)

- 1.5-mL microcentrifuge tubes
- 15-mL snap-cap polyethylene tubes (Falcon 2059 or equivalent)
- Microbiological equipment to plate cells and grow cultures
- Shaking incubator, 37°C
- Nonshaking incubators, 37°C
- Centrifuge, refrigerated (4°C), low-speed, 50–500 mL volumes
- Sterile 50 mL centrifuge tubes
- Sterile 250 or 500 mL centrifuge bottles
- UV-Vis spectrophotometer
- Polyacrylamide gel apparatus
- Sonicator with microtip

Introduction

Description of the System

His-Patch ThioFusion™ Expression System

The His-Patch ThioFusion™ Expression System allows expression and purification of soluble heterologous proteins from *E. coli.* Foreign genes inserted into the multiple cloning site of the expression vector, pThioHis, are expressed as fusions to a modified version of the *E. coli* protein thioredoxin (*trx*A) (Bayer, 1968, Holmgren, 1985). Thioredoxin fusions express at high levels and the thioredoxin moiety appears to confer solubility to formerly insoluble heterologous proteins (Holmgren, 1985; LaVallie *et al.*, 1993). The thioredoxin gene in pThioHis has been mutated to create a metal-binding domain in the thioredoxin protein. This allows purification of your thioredoxin fusion on metal-chelating resins such as ProBond™.

Thioredoxin

The 11.7 kDa thioredoxin protein is found in yeast, plants, and mammals, as well as in bacteria. It was originally isolated from *E. coli* as a hydrogen donor for ribonuclease reductase (for a review, see Holmgren, 1985). The gene has been completely sequenced (Wallace and Kushner, 1984). The protein has been crystallized and its three-dimensional structure determined (Katti *et al.*, 1990). When overexpressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein.

Murine interleukin-2, human interleukin-3, murine interleukin-4, murine interleukin-5, human macrophage-colony stimulating factor, murine steel factor, murine leukemia inhibitory factor and human bone morphogenetic protein-2 are some of the proteins that have been produced as soluble C-terminal fusions to the thioredoxin protein in *E. coli* (LaVallie *et al.*, 1993).

To create a metal binding domain in the thioredoxin protein, the glutamate residue at position 31 and the glutamine residue at position 63 were mutated to create histidine residues. When the resulting protein, His-Patch thioredoxin (HP-thioredoxin), folds, the histidines at positions 31 and 63 interact with a native histidine at position 7 to form a "patch". This histidine patch was shown to have high affinity for divalent cations. HP-thioredoxin proteins can therefore be purified on metal-chelating resins (e.g. ProBond™).

Regulating Expression

High level expression of HP-thioredoxin fusion proteins is driven by the *trc* (*trp-lac*) promoter. The *trc* promoter contains the –35 region of the *trp* promoter together with the –10 region of the *lac* promoter. To regulate expression from the *trc* promoter, the gene encoding Lac repressor ($lacI^q$) is provided in the pThioHis vectors.

Isopropyl-β-D-thiogalactopyranoside (IPTG) is used to induce expression of your fusion protein. Translation is enhanced by the bacteriophage T7 gene 10 translation enhancer that provides a highly efficient initiation site for the translation of HP-thioredoxin.

Continued on next page

Description of the System, continued

Purifying with ProBond™ Resin

ProBond™ is a nickel-charged Sepharose resin that can be used for affinity purification of HP-thioredoxin fusions. Proteins bound to the resin may be eluted with either low pH buffer or by competition with imidazole or histidine. See page 19 for more information.

Purifying by Osmotic Shock

Native thioredoxin localizes at particular sites on the cytoplasmic side of the inner membrane known as adhesion zones or Bayer's patches (Bayer, 1968; Lunn and Pigiet, 1982). These sites create an "osmotically sensitive" cellular compartment. Thioredoxin is quantitatively released during osmotic shock into the shock fluid, allowing rapid purification (Lunn *et al.*, 1984). HP-thioredoxin fusion proteins may localize to the adhesion zones, facilitating release into the medium for simplified purification.

Note that the HP-thioredoxin fusions isolated by osmotic shock do **not** bind to ProBond™.

Other Purification Steps

There may be cases when your specific HP-thioredoxin fusion protein may not be purified by any of the methods listed above. Conventional protein purification techniques are then recommended to purify the fusion (Deutscher, 1990).

Anti-Thio™ Antibody

To detect HP-thioredoxin fusion proteins during expression and purification, Life Technologies offers the Anti-Thio™ Antibody (see page 41 for ordering information). Anti-Thio™ is a pure monoclonal mouse IgG_{1K} antibody raised against the thioredoxin protein that recognizes HP-thioredoxin and HP-thioredoxin fusion proteins.

Cleaving Desired Protein from Thioredoxin

There is an enterokinase cleavage site engineered into pThioHis A, B, and C in a 30 bp spacer between the modified *trx*A gene and the multiple cloning site. Enterokinase recognizes the amino acid sequence Asp-Asp-Asp-Asp-Lys and hydrolyzes the peptide bond between lysine and the next amino acid. EnterokinaseMax™ (EKMax™), a recombinant preparation of the catalytic subunit of the enterokinase enzyme with a high specific activity (Collins-Racie *et al.*, 1995; Vozza *et al.*, 1996), is available from Life Technologies (see page 41 for ordering information).

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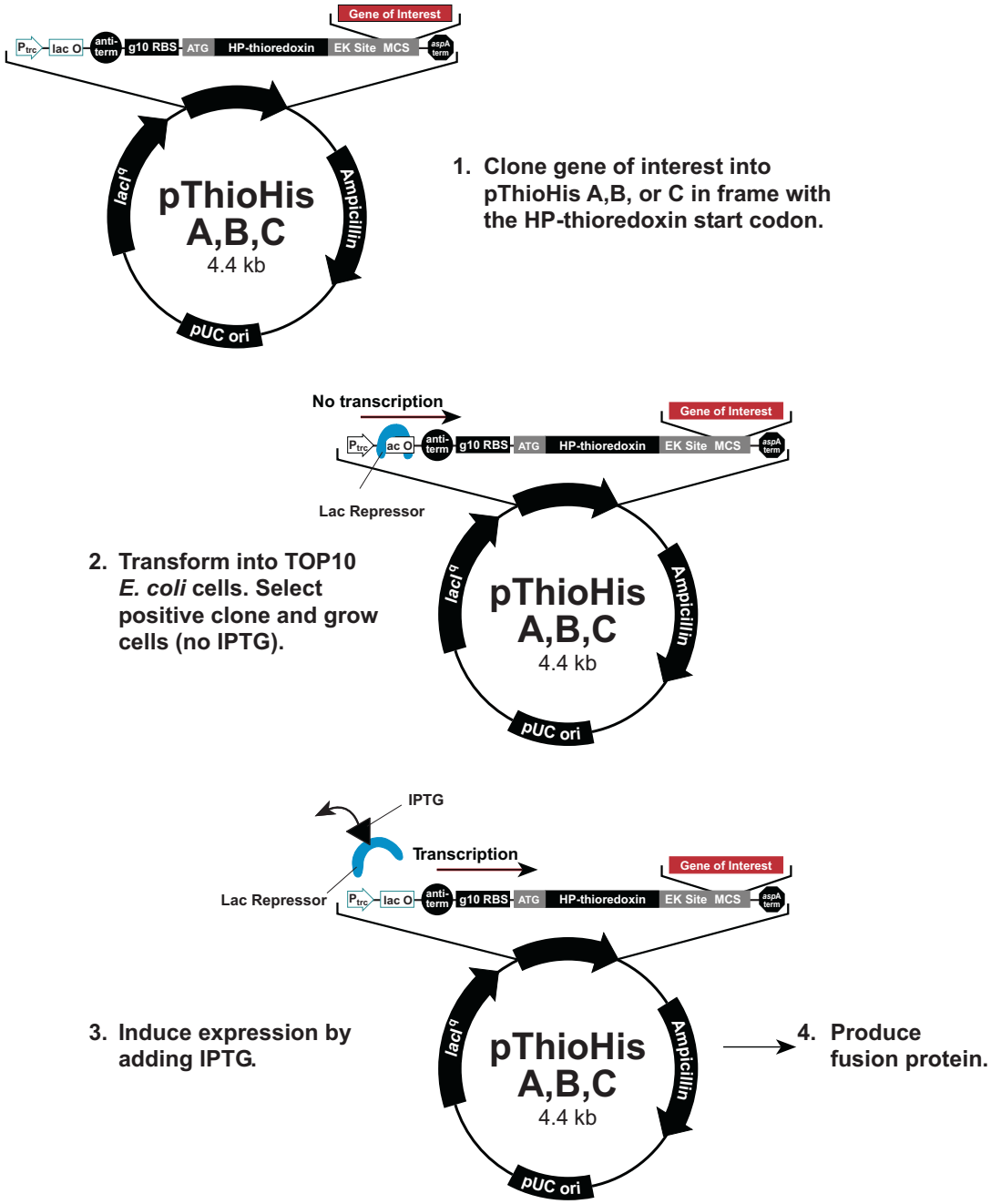
Using the His-Patch ThioFusion™ Expression System

Flow Chart of Expression and Purification

The graphic below summarizes the His-Patch ThioFusion™ Expression System process.



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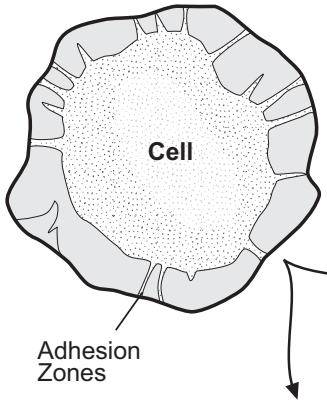
Flow Chart of Expression and Purification, continued

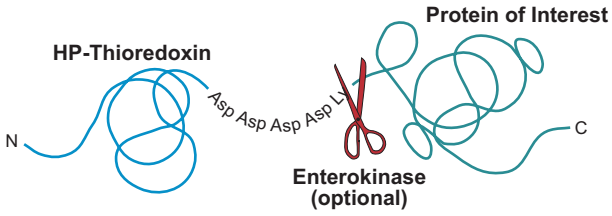
5. Protein may localize to adhesion zones.



6. Partially purify protein by affinity chromatography on ProBond™. An alternative purification scheme is osmotic shock.

Osmotic Shock

ProBond™



7. Optional. Cleave the fusion protein with enterokinase to restore native protein.

Experimental Outline

Introduction

The table below outlines the general steps for expressing and purifying a HP-thioredoxin fusion protein.

Step	Action
1	Ligate the gene of interest in frame with the HP-thioredoxin gene in pThioHis A, B, or C.
2	Transform the ligation product into *E. coli*.
3	Select ampicillin-resistant transformants and analyze the plasmid DNA by restriction mapping or sequencing to confirm the presence, orientation, and reading frame of the gene of interest.
4	Grow the desired clones and induce for expression of the desired fusion protein. Analyze expression of the fusion protein by SDS-PAGE gel or Western blot.
5	Optimize growth and induction conditions to maximize the amount of soluble protein.
6	Purify or partially purify the fusion protein by either affinity chromatography with ProBond™ resin, osmotic shock, or other protein purification methods.
7	Release the protein of interest from HP-thioredoxin by digestion with enterokinase (optional).
8	Scale up purification of fusion protein.

Before You Start

It is important to read through the entire manual to familiarize yourself with the procedures. Some general things you need to check are listed below:

Step	Action
1	Prepare media and plates for expression and transformation based on the amounts required for your particular project.
2	Prepare solutions you need.
3	Have suitable restriction enzymes, T4 DNA ligase, and possibly DNA polymerase on hand to ligate your gene of interest into pThioHis.
4	Prepare competent cells in advance and store at –80°C.

Note

Controls reactions are useful to evaluate your experiment should a problem occur. If a problem does arise, perform the appropriate control reaction before contacting Life Technologies. Refer to **Control Reactions**, page 9, for the suggested control experiments.

Continued on next page

Experimental Outline, continued

General Molecular Biology Techniques

Users should be familiar with sterile technique, molecular biology techniques, and standard microbiological practices. For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

TOP10 is provided for the growth and maintenance of pThioHis plasmids. This strain is provided as a convenience for those who do not have access to other *E. coli* strains. Many *E. coli* strains are suitable for the growth of this vector such as DH5 . We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A). Note that the pThioHis vectors contain the *lac*I^q gene, so it is not necessary to use a strain containing a *lac*I gene.

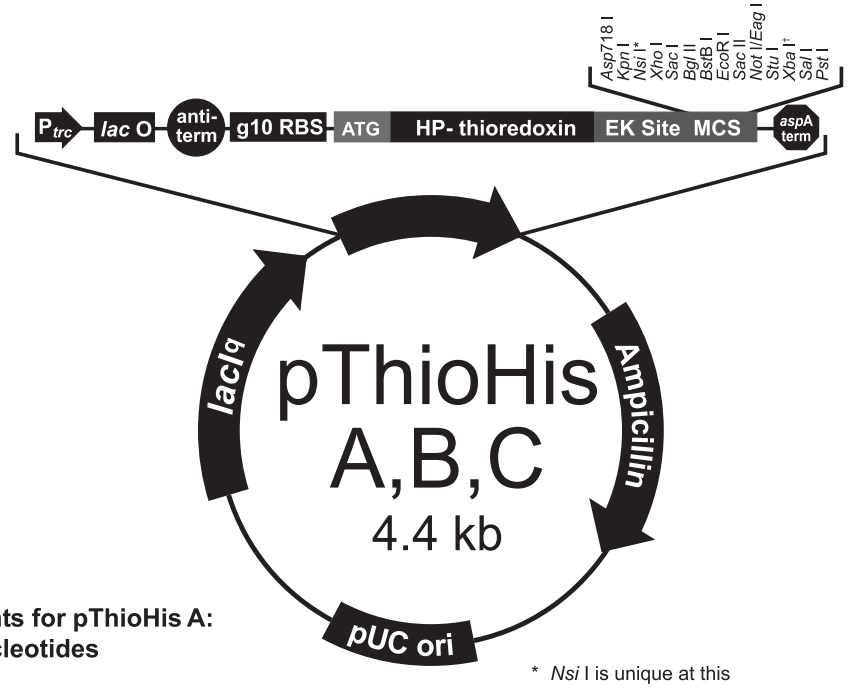
For your convenience, TOP10 is available as chemically competent or electrocompetent cells from Life Technologies (see page 41 for ordering information).

Map and Features of pThioHis A, B, and C

Description of pThioHis

pThioHis A, B, and C are used to create C-terminal fusions to *E. coli* HP-thioredoxin. Each vector has a multiple cloning site (see pages 11–13) that allows in-frame fusion with HP-thioredoxin and an enterokinase cleavage site. The enterokinase site permits the release of HP-thioredoxin from your protein. Sequences of the pThioHis vectors are available for downloading at **www.lifetechnologies.com** or from Technical Support (page 42).

Map of pThioHis



Comments for pThioHis A: 4365 nucleotides

Ampicillin resistance ORF: bases 201-1061
pUC origin: bases 1206-1879
Lac Repressor (*lacI*^{q}) ORF: bases 1967-3049
Trc Promotor Region: bases 3261-3470
  -35 region: bases 3281-3292
  -10 region: bases 3304-3309
  lac operator (*lac*O): bases 3316-3336
  *rrn*B antitermination: bases 3352-3421
  gene 10 region: bases 3434-3442
  Ribosome binding site: bases 3457-3461
Thioredoxin ATG: bases 3471-3473
Trx forward priming site: bases 3777-3794
Enterokinase site: bases 3813-3827
Multiple cloning site: bases 3827-3892
*asp*A Termination: bases 3893-3959
Trx reverse priming site: bases 3967-3986

* *Nsi* I is unique at this location in pThioHis A only.

Nco I is unique at this location in pThioHis B only.

Sph I is unique at this location in pThioHis C only.

† There is a stop codon following the *Xba* I site in pThioHis C

Continued on next page

Map and Features of pThioHis A, B, and C, continued

Features of pThioHis

This table describes the features of pThioHis. This vector is used to make in-frame fusions with HP-thioredoxin as the N-terminal fusion partner.

Feature	Benefit
trc promoter	–35 (*trp*B) and –10 (*lac*UV5) hybrid promoter for high-level expression of your fusion protein
lac operator (*lac*O)	Allows binding of the Lac repressor to repress transcription
*rrn*B antitermination region	Reduces the level of premature transcription termination
Bacteriophage gene 10 ribosome binding site	Optimizes translation initiation of HP-thioredoxin
HP-thioredoxin ORF	Allows expression of soluble recombinant protein and purification on ProBond™ resin
Enterokinase cleavage site (replaces the HP-thioredoxin stop codon)	Allows release of desired native protein from HP-thioredoxin
Polylinker region	Allows translational fusions to the 3´ end of the modified *trxA* gene (HP-thioredoxin)
*E. coli asp*A transcription terminator	Efficient transcription termination of fusion protein
Ampicillin resistance ORF	Selection and maintenance in *E. coli*
pUC origin	Maintenance in bacteria and high copy number replication
lacI^q gene	Encodes and overproduces the Lac repressor protein

Methods

Performing Control Reactions

General Guidelines for Control Reactions

The table below gives some suggestions for possible control reactions for the experiments detailed in this manual. It is timely to consider what controls you might want for later experiments so you can transform the appropriate vectors into TOP10 *E. coli*.

Experiment	Control	Reason
Ligation	No DNA	Checks for contamination of ligation reagents by bacteria.
	Linearized pThioHis (may be dephosphorylated)	Checks for vector self-ligation.
	Insert only	Checks for plasmid contamination of insert.
Transformation	Cells only	Checks for the presence of antibiotic in the plates and contamination of competent cells and S.O.C. medium.
	pThioHis A, B, or C, (no insert)	Checks the efficiency of the competent cells. See also Expression and Purification experiments.
Expression	pThioHis A, B, or C, (no insert)	Positive control for expression. Production of HP-thioredoxin in the Pilot Expression experiment shows the cells and the media are working properly.
Purification	pThioHis A, B, or C, (no insert)	Positive control for affinity chromatography and osmotic shock purification steps.

Cloning into pThioHis A, B, and C

Before You Start

The table below outlines the general steps to consider when cloning your gene of interest into pThioHis A, B, and C.

Step	Action
1	Purchase or prepare electrocompetent or chemically competent cells. Store the competent cells at –80°C. See pages 32–36 for procedures for preparing competent cells.
2	Determine a cloning strategy to fuse your protein in frame with HP-thioredoxin using any of the pThioHis vectors.
3	Decide which controls are relevant for your particular application.
4	Prepare LB medium and LB plates containing 100 µg/mL ampicillin. Plan on at least two plates per ligation/transformation reaction, plus extra plates for purification of your clone.

Propagating Vectors

Propagate the vectors in the TOP10 strain provided in the kit. Transform and select on LB plates containing 100 µg/mL ampicillin. Other *E. coli* strains may also be suitable for use (e.g., JM109).

General Molecular Biology Techniques

Consult the general molecular biology references (Ausubel, *et al*., 1994, Sambrook, *et al*., 1989) for recommendations on restriction digests, dephosphorylation of vector, and ligations. Follow the instructions of the manufacturer for restriction enzymes, T4 DNA polymerase, calf intestinal alkaline phosphatase, and T4 DNA ligase in order to clone your gene of interest into pThioHis.

Calculating Molar Ratios

To ligate your gene of interest into pThioHis, you need to know the concentration of each DNA solution to calculate the volume required to achieve a particular molar ratio of vector to insert.

1. Determine the concentration of insert and linearized pThioHis in ng/µL.
2. Use the following formula to calculate the amount of insert needed to give an equimolar (1:1) ratio between insert and linearized pThioHis:

$$\text{x ng insert} = \frac{(\text{bp insert})\ (\text{ng linearized pThioHis})}{(\sim 4{,}400 \text{ bp pThioHis})}$$

   Amount of pThioHis can range from 50–200 ng.
3. Based on the calculation above, compute the volumes needed for the ligation reaction.
4. You may wish to consider other ratios of vector to insert, e.g., 1:2 or 1:3 to increase the chances of obtaining the correct clone.

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Cloning into pThioHis A, B, and C, continued

Cloning into pThioHis

Detailed maps of the multiple cloning sites of pThioHis A, B, and C are provided below and on the following pages to assist you in developing a successful cloning strategy. Choose carefully when selecting a cloning site. Your gene **must** be in frame with the ATG of the HP-thioredoxin gene to obtain proper expression.

Multiple Cloning Site of pThioHis A

Below is the multiple cloning site for pThioHis A. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The histidines that form the metal-binding domain are shown in boldface.

HP-Thioredoxin translational start site

HP-Thioredoxin open reading frame (ORF)

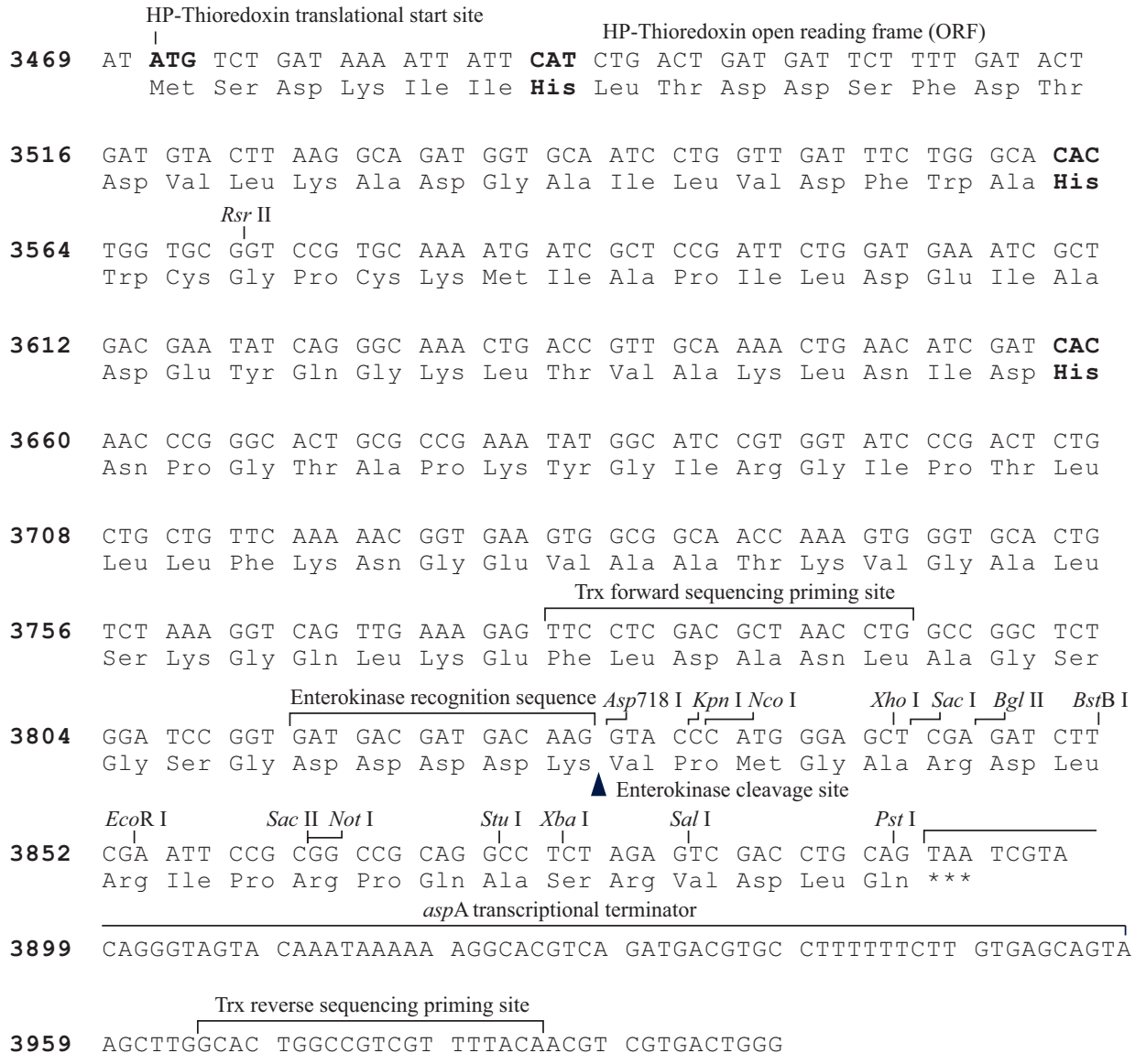
```
3469  AT ATG TCT GAT AAA ATT ATT CAT CTG ACT GAT GAT TCT TTT GAT ACT
         Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr

3516  GAT GTA CTT AAG GCA GAT GGT GCA ATC CTG GTT GAT TTC TGG GCA CAC
      Asp Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala His

              Rsr II
3564  TGG TGC GGT CCG TGC AAA ATG ATC GCT CCG ATT CTG GAT GAA ATC GCT
      Trp Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala

3612  GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAC
      Asp Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp His

3660  AAC CCG GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG
      Asn Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu

3708  CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG
      Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu

                                    Trx forward sequencing priming site
3756  TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGC TCT
      Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly Ser

                    Enterokinase recognition sequence   Asp718 I  Kpn I     Nsi I      Xho I  Sac I  Bgl II
3804  GGA TCC GGT GAT GAC GAT GAC AAG GTA CCT ATG CAT GAG CTC GAG ATC
      Gly Ser Gly Asp Asp Asp Asp Lys Val Pro Met His Glu Leu Glu Ile
                                      ▲ Enterokinase cleavage site
   BstB I  EcoR I      Sac II  Not I          Stu I  Xba I       Sal I              Pst I
3852  TTC GAA TTC CGC GGC CGC AGG CCT CTA GAG TCG ACC TGC AGT AAT CGT
      Phe Glu Phe Arg Gly Arg Arg Pro Leu Glu Ser Thr Cys Ser Asn Arg
                                  aspA transcriptional terminator
3900  ACA GGG TAG TACAAATA AAAAAGGCAC GTCAGATGAC GTGCCTTTTT TCTTGTGAGC
      Thr Gly ***

                  Trx reverse sequencing priming site
3957  AGTAAGCTTG GCACTGGCCGT CGTTTTACAA CGTCGTGACT GGGAAAA
```

Continued on next page

Cloning into pThioHis A, B, and C, continued

Multiple Cloning Site of pThioHis B

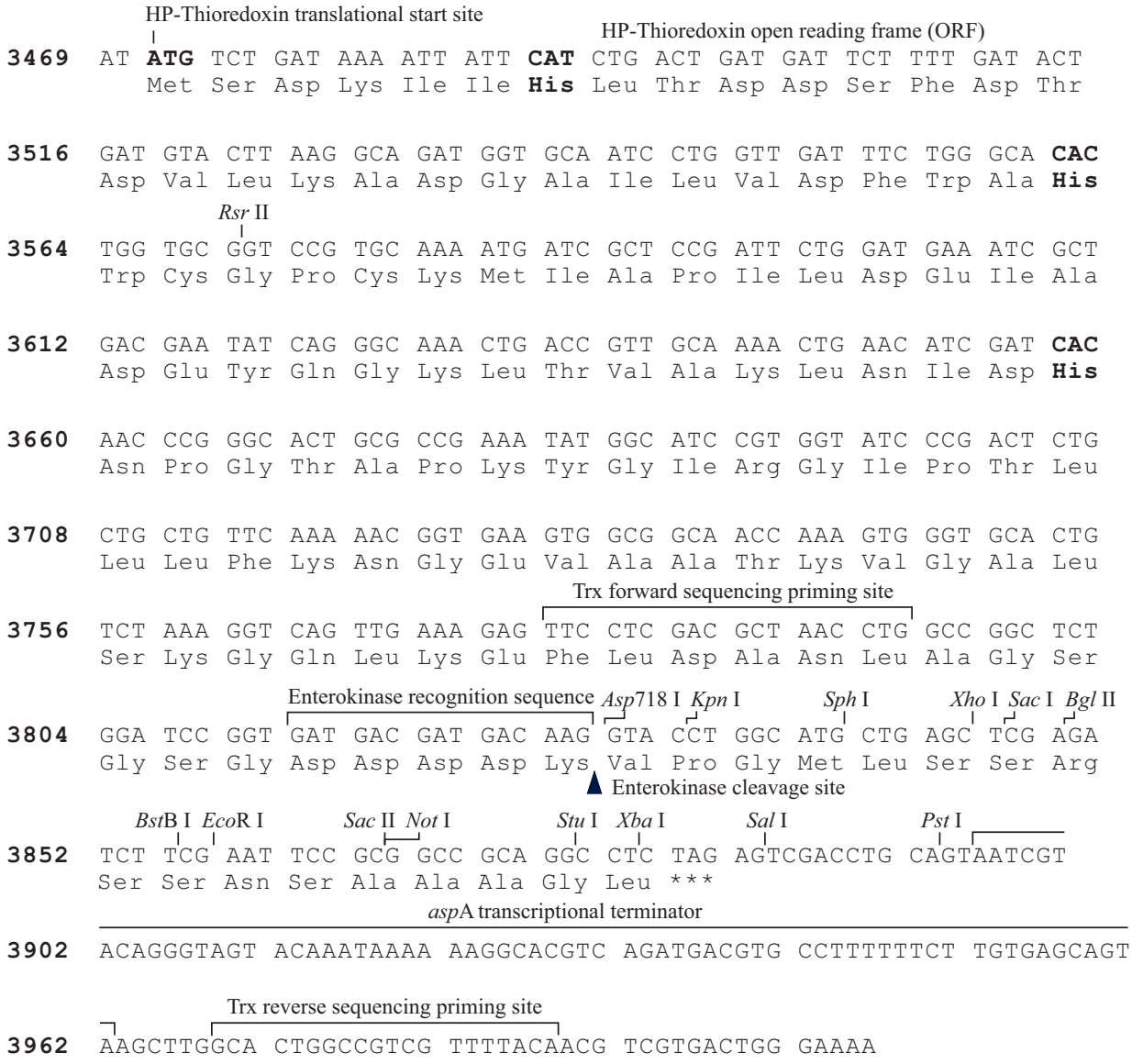
Below is the multiple cloning site for pThioHis B. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The histidines that form the metal-binding domain are shown in boldface.



Cloning into pThioHis A, B, and C, continued

Multiple Cloning Site of pThioHis C

Below is the multiple cloning site for pThioHis C. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The histidines that form the metal-binding domain are shown in boldface. **Note** that there is a TAG stop codon after the *Xba* I site in this vector.



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Cloning into pThioHis A, B, and C, continued

Transforming *E. coli*

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g., TOP10, DH5α) and select on LB plates containing 100 µg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of your insert.

Analyzing Transformants

1. Pick 10 ampicillin resistant transformants and inoculate into LB medium containing 100 µg/mL ampicillin. Grow them overnight at 37°C.
2. Isolate the plasmid DNA by miniprep for restriction analysis and sequencing. To sequence pThioHis, use the Trx Forward and/or Reverse Sequencing Primers.
3. Make sure to purify your positive clones by streaking for single colonies on LB-ampicillin plates. Confirm the identity of your positive clones.
4. Prepare a glycerol stock of your desired clone for safekeeping by combining 0.85 mL of an overnight bacterial culture with 0.15 mL of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at –80°C.
5. Once the desired clone is isolated, proceed to the **Performing a Pilot Expression** section, next page.

Performing a Pilot Expression

Introduction

At this point, you should have TOP10 containing pThioHis with the gene encoding your protein ligated in frame to the HP-thioredoxin gene. In this section, you will analyze expression of your fusion protein and then optimize expression conditions to obtain soluble protein by varying the time of induction.

Step	Action
1	Grow cells containing your construct to mid-log at 37°C in LB Medium.
2	Induce expression by the addition of IPTG.
3	Take time points from cell culture and lyse the cells by sonication and freeze-thaw.
4	Fractionate cell lysate by centrifugation.
5	Analyze pellets and supernatant for your protein by SDS-PAGE or Western blot.
6	Determine the optimal time point for maximum yield of soluble protein.

Important

To analyze expression and eventually purification, it is necessary to use SDS-PAGE. There are many different types of SDS-PAGE systems. Because all fusion proteins are different, it is difficult to recommend a particular system for your individual application. See the section **Analyzing by SDS Protein Gels** on page 40 in the **Appendix** for suggestions.

Detecting Thioredoxin Fusion Proteins

The Anti-Thio™ Antibody, available from Life Technologies, is specific to the thioredoxin protein and is capable of detecting HP-thioredoxin fusion proteins. This is a monoclonal mouse IgG_{1K} that can be used for Western blots and ELISA assays. See page 41 for ordering information.

Controls

See the section **Control Reactions** (page 9) to determine which controls you want to include. Controls are useful to properly evaluate your experiment.

For testing and optimizing expression, we recommend using small volumes (5–50 mL) to determine the optimal conditions for the expression and purification of the fusion protein, and then scaling up for large-scale (1 L). The volume of cell culture depends on how much you need to read the absorbance of the culture and the number of 1 mL time points you will take. The instructions that follow are for 10 mL cultures and may be scaled up or down to meet individual needs.

Continued on next page

Performing a Pilot Expression, continued

Required Media and Plates

- One LB plate containing 100 µg/mL ampicillin
- 1 mL LB Medium with 100 µg/mL ampicillin in a 5 mL sterile culture tube
- 10 mL LB Medium with 100 µg/mL ampicillin in a sterile 25 mL small flask
- 100 mM IPTG
- Five microcentrifuge tubes labeled: "t=0", "t=1", "t=2", "t=3", and "t=4".

Growing Cells: Day 1

Streak out the clone of interest on a LB plate with 100 µg/mL ampicillin and grow at 37°C until single colonies are visible (12–16 hours).

Growing Cells: Day 2

Using a single colony from the plate from Day 1, inoculate 1 mL of LB Medium with 100 µg/mL ampicillin and incubate at 37°C at 200–225 rpm in a shaking incubator overnight.

Growing and Inducing Cells: Day 3

1. Using 500 µL of the overnight culture, inoculate 10 mL of fresh LB Medium containing 100 µg/mL ampicillin in a 25 mL culture flask. Grow this culture at 37°C with shaking (200–225 rpm) to an OD_{550} of 0.5 (approximately 2–3 hours).
2. When an OD_{550} of 0.5 is reached, transfer a 1.0 mL sample to the microcentrifuge tube labeled "t=0", and centrifuge the tube for 2–3 minutes at maximum speed to pellet the cells. Decant the supernatant and freeze the cell pellet at –20°C until ready to assay. This is the zero time sample.
3. To your cell culture, add IPTG to a final concentration of 1 mM using a 100 mM stock solution (0.1 mL of 100 mM IPTG to a final concentration of 1 mM in 10 mL of culture).
4. Return the culture to the 37°C incubator and incubate with shaking (200–225 rpm).
5. At time t=1 hour, read the OD_{550} and record. Then take a 1 mL sample and place in the microcentrifuge tube labeled "t=1". Centrifuge the cells at maximum speed for 2–3 minutes, decant the supernatant, and freeze the cell pellet at –20°C. Repeat at t=2, 3, and 4 hours.
6. The cell pellets can be kept frozen until ready for analysis by SDS-PAGE. See the section **Analyzing by SDS-PAGE Gels**, page 40 in the **Appendix**, for information on SDS-PAGE gels.

Continued on next page

Lysing Cell Samples: Day 4

1. Before preparing whole cell lysates:
   - Prepare an SDS-PAGE gel
   - Prepare a dry ice/ethanol bath
   - Equilibrate a 37°C bath
   - Prepare 20 mM Tris-HCl, pH 8, 2.5 mM EDTA, 5 mM imidazole, and chill it to 4°C. The imidazole keeps the HP-thioredoxin from associating with cell membranes.
2. Remove the five samples from **Growing and Inducing Cells**, Step 6 (previous page), from the freezer. Resuspend each cell pellet in 500 µL of cold (4°C) 20 mM Tris-HCl, pH 8, 2.5 mM EDTA, 5 mM imidazole, and keep on ice.
3. Take a hand-held sonicator with a micro-tip and sonicate each sample one at a time with two or three 10 second bursts. Flash freeze each lysate in a dry ice/ethanol bath.
4. Quickly thaw the lysates at 37°C.
5. Repeat steps 3 and 4 until three sonication-freeze-thaw cycles are completed for all samples.
6. After the last thaw, centrifuge all tubes at maximum speed for 5–10 minutes at 4°C to pellet cell debris and insoluble matter. Decant the supernatants into fresh microcentrifuge tubes. Keep all samples on ice.
7. Resuspend the pellets from Step 6 in 500 µL of 20 mM Tris-HCl, pH 8, 2.5 mM EDTA, 5 mM imidazole. Keep the samples on ice. You now have two samples per time point–a soluble fraction (supernatant) and an insoluble fraction (resuspended pellet).

Analyzing Samples

1. In a fresh microcentrifuge tube, take 10 µL from the supernatant fraction and mix it with 10 µL of any 2X SDS-PAGE sample buffer. Likewise, take 10 µL of the resuspended pellet and mix it with 10 µL 2X SDS-PAGE sample buffer.
2. Boil samples for 5 minutes and load onto an SDS-PAGE gel with molecular weight standards. Process and develop the gel as described for the procedure you are using.
3. Compare the pellets with the supernatants for each time point to determine the extent of expression and the solubility of the fusion protein. The HP-thioredoxin fusion protein will be at least 12.8 kDa larger than the native protein (HP-thioredoxin plus the enterokinase site).
4. It may be necessary to perform a Western blot to verify which band is the fusion protein. The Anti-Thio™ Antibody is available for the detection of HP-thioredoxin fusion proteins (see page 2).

Continued on next page

Expected Results

At the zero time point of induction, there should be very little fusion protein expressed, either in the pellet or in the supernatant.

At successive time points postinduction, you should see increasing amounts of fusion protein produced. If your fusion protein is soluble, it will appear in the supernatant fraction of each time point after lysis and reach a maximum. If you do not see the fusion protein in the supernatant fraction, check the pellet and your controls as follows:

The zero time point from the pThioHis control should show no expression of HP-thioredoxin. When induced with IPTG, HP-thioredoxin should appear in the soluble (supernatant) fraction as a ~16 kDa protein (HP-thioredoxin + the enterokinase site + the multiple cloning site). Each pThioHis vector produces an HP-thioredoxin that varies in size from vector to vector because of the frame through the multiple cloning site (see pages 11–13). If you do not obtain expression of HP-thioredoxin, be sure that you are adding IPTG to induce expression.

Once you have determined the optimal time point for expression, proceed to scale-up your culture to 50 mL.

Troubleshooting

If your fusion protein does not express at all, it may be out of frame with HP-thioredoxin. Use the Trx Forward and the Trx Reverse Sequencing Primers to confirm that your insert is in frame.

If your fusion protein is in the pellet, make sure you have 5 mM imidazole in the lysis buffer. HP-thioredoxin has a tendency to associate nonspecifically with cell membranes. You may also try adding 100–250 mM NaCl to the lysis buffer to minimize the interaction of HP-thioredoxin with the membrane.

If you have some fusion protein in the supernatant and some in the pellet, you may try lowering the growth temperature during induction to 25°C or 30°C. Lowering the temperature can help proteins fold properly and solubilize them (LaVallie *et al.*, 1993). Note that you may have to optimize the time of induction as cells grow more slowly at lower temperatures.

Purifying Fusion Proteins

Introduction

At this point, you have determined optimal expression conditions for your particular fusion protein and are ready to scale up to a 50 mL culture and purify the fusion protein. It is necessary to at least partially purify the fusion protein to successfully cleave HP-thioredoxin away from the protein of interest with enterokinase.

Guidelines are provided below to purify your fusion protein by affinity chromatography using ProBond™ resin.

ProBond™ Resin

ProBond™ resin (included in the kit) is a nickel-chelating Sepharose resin. Iminodiacetic acid is crosslinked to the Sepharose resin and chelates Ni^{2+} much like EDTA binds divalent cations. Histidine residues will also chelate Ni^{2+}, particularly if they are in close proximity to each other. Because of the tertiary structure of HP-thioredoxin, the three histidine residues are close enough to form a high affinity binding site for Ni^{2+}. Cell lysates are passed over the column, and HP-thioredoxin fusions will bind to the column. After washing the resin to remove unbound proteins, the fusion is eluted by either lowering the pH or by competition with imidazole.

Note that purification must be done under native conditions. Denaturation of HP-thioredoxin results in the loss of tertiary structure and the metal binding domain.

Binding Capacity of ProBond™

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

Note

ProBond™ resins **cannot be used with osmotic shock fluid**. Thioredoxin fusion proteins in shock fluid do not bind well to the resin. This may be due to the presence of lipids or oxidized amino acid residues.

Controls

Perform the following control experiments along with the fusion protein of interest. The negative control is the TOP10 strain without the plasmid. The positive control is TOP10 containing any one of the pThioHis vectors without an insert. All of the vectors express HP-thioredoxin, which will bind to ProBond™ resin.

Important

When purifying proteins keep all solutions containing the protein and all reagents at 4°C.

Preparing Native Purification Buffers

Introduction

You must dilute and adjust the pH of the 5X Native Purification Buffer to prepare 1X Native Purification Buffer. From this, you can prepare the following buffers:

- Binding Buffer
- Wash Buffer
- Elution Buffer

The recipes on the next page provide enough buffers to complete one native purification using one kit-supplied purification column. Scale up accordingly.

Required Reagents

- 5X Native Purification Buffer, supplied in the kit
- 3M Imidazole, supplied in the kit
- NaOH
- HCl
- Sterile distilled water

Imidazole Concentration in Buffers

Imidazole is included in the wash and elution buffers to minimize the binding of untagged, contaminating proteins and to increase the purity of the target protein with fewer wash steps. If your level of contaminating proteins is high, you can also add imidazole to the binding buffer.

If your protein does not bind well under these conditions, you can experiment with lowering or eliminating the concentration of imidazole in the buffers and increasing the number of wash and elution steps.

Continued on next page

Preparing Native Purification Buffers, continued

1X Native Purification Buffer

For the following recipes, you must dilute and adjust the pH of the 5X Native Purification Buffer (supplied in the kit) to create 1X Native Purification Buffer. To prepare 100 mL of 1X Native Purification Buffer, combine:

- 80 mL of H_2O
- 20 mL of 5X Native Purification Buffer

Adjust the pH to 8.0 with NaOH or HCl.

Binding Buffer

Without Imidazole

Reserve 30 mL of the 1X Native Purification Buffer as the Binding Buffer (used for column preparation, cell lysis, and binding).

With Imidazole (Optional):

You can prepare the Binding Buffer with imidazole to reduce the binding of contaminating proteins. (Note that some His-tagged proteins may not bind under these conditions). To prepare Binding Buffer with 10 mM imidazole, combine:

- 30 mL of 1X Native Purification Buffer
- 100 µL of 3M Imidazole

Adjust the pH to 8.0 with NaOH or HCl.

Wash Buffer

To prepare 50 mL of Wash Buffer with 20 mM imidazole, combine:

- 50 mL of 1X Native Purification Buffer
- 335 µL of 3M Imidazole

Adjust the pH to 8.0 with NaOH or HCl.

Elution Buffer

To prepare 15 mL of Elution Buffer with 250 mM imidazole, combine:

- 13.75 mL of 1X Native Purification Buffer
- 1.25 mL of 3M Imidazole

Adjust the pH to 8.0 with NaOH or HCl.

Growing Cells and Preparing Cell Lysates

Introduction

In this section, you will grow and induce a 50 mL culture of TOP10 cells containing your pThioHis construct using the conditions you developed in the **Pilot Expression** section. You may harvest the cells and store them at –80°C until you are ready to purify your HP-thioredoxin fusion, or you may proceed directly with protein purification using the ProBond™ columns included in the kit.

Required Materials

- 2 mL of LB containing 100 µg/mL ampicillin
- 50 mL of LB containing 100 µg/mL ampicillin in a culture flask
- 100 mM IPTG
- Binding Buffer
- Liquid nitrogen or ethanol/dry ice bath
- 0.8 µm syringe filter
- RNase A (optional)
- DNase (optional)

Growing Cells for Purification

Use the conditions determined in the **Pilot Expression** section to grow and induce a 50 mL culture of TOP10 containing pThioHis with your gene inserted. If you plan to immediately lyse the cells and load the lysate onto a ProBond™ column, prepare a ProBond™ column (page 24) and buffers for chromatography (page 20) while the cells are growing.

1. Using a single recombinant *E. coli* colony inoculate 2 mL of SOB or LB medium containing 100 µg/mL ampicillin.
2. Grow the culture overnight at 37°C with shaking (200–225 rpm).
3. The next day, inoculate 50 mL of SOB or LB medium containing 100 µg/mL ampicillin with 0.2 mL of the overnight culture.
4. Grow the culture at 37°C with vigorous shaking to an OD_{550} = 0.6 (the cells should be in mid-log phase).
5. Add IPTG to the culture to a final concentration of 1 mM (0.5 mL of a 100 mM IPTG stock to 50 mL).
6. Grow the culture with shaking until the optimal time point is reached. (If you found during the pilot expression that lowering the growth temperature during induction increased the yield of soluble protein, then grow the cells at this temperature.)
7. Harvest the cells by centrifugation (3,000 × *g* for 10 minutes at 4°C).
8. At this point, you may proceed directly to purification (next section) or store at –80°C until ready for use.

Continued on next page

Important

Before lysing your cells, prepare a ProBond™ column for chromatography (page 24). Because you need to load the cell lysates onto the column immediately after lysing, we recommend reading the chromatography section entitled **Binding Fusion Proteins to ProBond™**, pages 25–26, before beginning.

Preparing Cell Lysates

1. If the cells are frozen, remove them from the freezer and thaw them quickly at 37°C.
2. Take the cells from Step 1 above or fresh, pelleted cells from Step 7 on the previous page and resuspend the pellet in 8 mL of Binding Buffer (see page 21 for a recipe).
3. Transfer the cell suspension to a 50 mL conical tube.
4. Place the cell suspension on ice and sonicate it with two or three 10-second bursts.
5. Freeze the lysate in liquid nitrogen or an ethanol/dry ice bath, and then quickly thaw the lysate at 37°C.
6. Repeat Steps 4 and 5 three times.
7. **Optional**: You may treat the cell lysate with RNase and/or DNase at a final concentration of 5 µg/mL each. After adding the nucleases, incubate the lysate on ice for 15 minutes.
8. Remove the insoluble debris by centrifugation at 3,000 × *g* for 15 minutes at 4°C.
9. Clarify the lysate by passaging it through a 0.8 µm syringe filter.
10. Keep the cell lysate on ice and proceed immediately to **Binding Fusion Proteins to ProBond™**, page 25.

Preparing ProBond™ Columns

Introduction

Optimal purification parameters will vary with each protein fusion, and some experimentation may be required to obtain the level of purification desired. The procedures on the following pages are designed to provide initial parameters for purification. Further steps or development may be needed to obtain purified protein.

Important

Do **not** use DTT with ProBond™ columns. It will chelate and reduce the Ni^{2+} ion and turn the column reddish-brown. This destroys the resin.

Do **not** use EDTA with ProBond™ as it will chelate Ni^{2+} and remove it from the column.

Preparing Columns

When preparing a column as described below, make sure that the snap-off cap at the bottom of the column remains **intact**. To prepare a column:

1. Resuspend the ProBond™ resin in its bottle by inverting and gently tapping the bottle repeatedly.
2. Pipette or pour 2 mL of the resin into a 10-mL Purification Column. Allow the resin to settle completely by gravity (5–10 minutes) or gently pellet it by low-speed centrifugation (1 minute at 800 × g). Gently aspirate the supernatant.
3. Add 6 mL of sterile, distilled water and resuspend the resin by alternately inverting and gently tapping the column.
4. Repellet the resin using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
5. Add 6 mL of Binding Buffer (see page 21 for a recipe).
6. Resuspend the resin by alternately inverting and gently tapping the column.
7. Repellet the resin using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
8. Repeat Steps 5 through 7.

Storing Prepared Columns

To store a column containing resin, add 0.02% azide or 20% ethanol as a preservative and cap or Parafilm the column. Store at room temperature.

Binding Fusion Proteins to ProBond™

Introduction

At this point you should have a cell lysate from a 50 mL culture of TOP10/pThioHis with your gene inserted (from page 23) and a pre-equilibrated ProBond™ column (see page 24). You are now ready to load the cell lysate onto the column and purify your HP-thioredoxin fusion protein. After loading the cell lysate onto the column, the column is washed to remove unbound proteins and the fusion protein is eluted. We recommend that you save all flow-through, washes, and eluents for troubleshooting purposes.

Binding Capacity of ProBond™

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

Important

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

Protocol

Using the buffers, columns and cell lysate, follow the procedure below to purify your fusion protein:

1. Add 8 mL of lysate to a prepared Purification Column (see page 24).
2. Bind for 30–60 minutes using gentle agitation to keep the resin suspended in the lysate solution.
3. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4ºC for SDS-PAGE analysis.
4. Wash with 8 mL Wash Buffer (see page 21). Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4ºC for SDS-PAGE analysis.
5. Repeat Step 4 three more times.
6. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein with 8–12 mL Elution Buffer (see page 20). Collect 1 mL fractions and analyze with SDS-PAGE.

Note: Store the elution fractions 4ºC. If –20ºC storage is required, add glycerol to the fraction. For long term storage, you may add protease inhibitors.

Continued on next page

Binding Fusion Proteins to ProBond™, continued

Note

If you are experiencing high background levels or if your fusion protein is not binding well using the protocol on the previous page, you may elute your protein with an imidazole step gradient. We recommend using elution buffers that contain increasing concentrations of imidazole (e.g., 50 mM, 200 mM, 350 mM, 500 mM) and pooling fractions that contain your protein. Alternatively, you increase or decrease the pH of the Wash Buffer and Elution Buffer to optimize your purification experiment.

Recharging ProBond™ Resin

You can use the ProBond™ resin for up to three or four purifications of the same protein without recharging. We recommend not recharging the resin more than three times and only reusing it for purifying the same recombinant protein.

To recharge 2 mL of resin in a purification column:

1. Wash the column two times with 8 mL of 50 mM EDTA to strip away the chelated nickel ions.
2. Wash the column two times with 8 mL of 0.5M NaOH.
3. Wash the column two times with 8 mL of sterile, distilled water.
4. Recharge the column with two washes of 8 mL $NiCl_2$ hexahydrate at a concentration of 5 mg/mL prepared in sterile, distilled water.
5. Wash the column two times with 8 mL of distilled water.
6. Add 0.02% azide or 20% ethanol as a preservative and cap or parafilm the column. Store at room temperature.

Enterokinase Digestion

To remove the HP-thioredoxin fusion partner from your protein, you may use enterokinase to cleave the fusion protein. We recommend EnterokinaseMax™, a recombinant preparation of the catalytic subunit of bovine enterokinase (see page 41 for ordering information).

Scaling Up Your Purification

Scaling Up

Once you have a procedure to produce pure or partially pure fusion protein, you are ready to scale up your protein preparation. The scale of your preparation depends on the desired final yield of your fusion protein. Start by simply multiplying the culture volume (i.e., up to 1 liter) and adjusting the purification as needed. Remember to keep all solutions at 4°C. You also need to scale-up your ProBond™ column for larger purifications.

Guidelines

Consider the following when scaling up your purification:

- When scaling up a purification procedure, it is a good idea to take samples along the way to make sure all the steps are working properly. It is sometimes necessary to make some minor changes when increasing the scale of a purification preparation.
- Check to see if you have enough reagents.
- Because of the larger volumes, you need to use a low-speed centrifuge. Cells will take longer to pellet.
- Make sure you have access to a large sonicator to prepare the cell lysates. You may also wish to consider lysing your cells using a French press.
- If your protein requires digestion with enterokinase to be active, we recommend that you digest small samples of your fusion protein as needed for specific experiments.
- You may wish to consider dialysis and concentration of the fusion protein into other buffers for increased stability and higher activity. It may also be desirable to purify your native protein further. Refer to Bollag and Edelstein, 1991 and Deutscher, 1990 for recommendations and procedures.

Troubleshooting

Introduction

The table below provides solutions to possible problems you may encounter when you express and purify your HP-thioredoxin fusion protein.

Problem	Probable Cause	Possible Solution
No fusion protein recovered following elution	Recombinant protein fails to bind because histidine binding site is obscured	Try an alternative purification method such as osmotic shock
	Expression levels too low	Add IPTG to induce expression of your fusion protein. Be sure to optimize expression conditions.
	Not enough sample loaded	Increase the amount of lysate used
	Recombinant protein has very high affinity for ProBond™ resin	Increase the stringency of elution (decrease pH or increase imidazole)
Good fusion protein recovery but contaminated with non-recombinant proteins	Wash conditions not stringent enough	Lower the pH of wash buffer. Wash more extensively.
	Recombinant protein has low affinity for resin and comes off in the wash with many contaminating proteins	Try an imidazole step gradient to elute the protein
Low fusion protein recovery and contaminated with non-recombinant proteins	Fusion protein not binding tightly to resin	Try "reverse-chromatography": bind lysate and allow fusion protein to come off in low stringency washes; collect these fractions; redo chromatography on saved fractions on new or stripped and recharged column.
	Expression levels too low	Consider an additional high stringency wash at a lower pH (e.g., between pH 6 and pH 4) before the elution step
Column turns reddish brown	DTT is present in of the buffers	Use β-mercaptoethanol as a reducing agent

Appendix

Recipes

LB (Luria-Bertani) Medium

Composition (for 1 liter)

Note: Low Salt LB (0.5% NaCl) may be substituted.

1% Tryptone
0.5% Yeast Extract
1% NaCl

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave the solution for 20 minutes at 15 lb/sq. in.
4. Store the solution at room temperature or at 4°C.

LB agar plates

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

SOB Medium

SOB (per liter)

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

1. Dissolve 20 g Tryptone, 5 g Yeast Extract, and 0.5 g NaCl in 950 mL deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 mL of deionized water. Add 10 mL of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.0 with 5 M NaOH, then bring the volume to 1 L with deionized water.
4. Autoclave this solution, cool it to ~55°C, and add 10 mL of sterile 1 M $MgCl_2$.
5. Store the SOB medium at room temperature or 4°C.

S.O.C. Medium

S.O.C. (per liter)

SOB
20 mM glucose

1. After making SOB medium above, add 7.2 mL of 50% glucose.
2. Store the S.O.C. medium at room temperature or 4°C.

Continued on next page

100 mM IPTG

1. Dissolve 238.3 mg of IPTG in 10 mL deionized water.
2. Filter sterilize the solution and store it in 1 mL aliquots at –20°C.

FSB Transformation Solution

Composition (for 100 mL)

10 mM Potassium acetate, pH 7.5
45 mM $MnCl_2$-$4H_2O$
10 mM Ca Cl_2-2 H_2O
100 mM KCl
3 mM Hexamminecobalt(III) chloride (available from Sigma-Aldrich)
10% Glycerol

1. Make 100 mL of 1 M potassium acetate by dissolving 9.82 g in 90 mL deionized water. Adjust pH to 7.5 with HCl. Bring the volume up to 100 mL.
2. For 100 mL of FSB transformation solution combine the following ingredients:

   1 mL 1 M Potassium acetate, pH 7.5
   890 mg $MnCl_2$-$4H_2O$
   150 mg $CaCl_2$-$2H_2O$
   750 mg KCl
   80 mg Hexamminecobalt(III) chloride
   10 mL 100% glycerol
   80 mL deionized water

3. Carefully adjust pH to 6.4 with 0.1 N HCl. If you go past the correct pH, remake the solution. Do not readjust the pH with base.
4. Adjust the final volume to 100 mL with deionized water and filter sterilize. Store the solution at 4°C.

DMSO

It is very important to use fresh, analytical grade DMSO. If you routinely transform cells by chemical means using the method of Hanahan, you probably have frozen aliquots of DMSO in your laboratory. If you do not use this method, then follow this procedure:

1. Order the smallest amount of analytical grade DMSO you can.
2. When the DMSO arrives, take about 5–10 mL and aliquot 200–500 µL per microcentrifuge tube. You can use the rest of the DMSO for other uses or you can aliquot the remainder for competent cells. It depends on whether you plan to use the method described in this manual on a routine basis.
3. Freeze these tubes at –20°C and use one tube per preparation of competent cells. Discard any remaining DMSO in the tube. Use a fresh tube for every preparation of competent cells.

Continued on next page

Osmotic Shock Solutions

Osmotic Shock Solution #1

20 mM Tris-HCl, pH 8
2.5 mM EDTA
20% sucrose

Osmotic Shock Solution #2

20 mM Tris-HCl, pH 8
2.5 mM EDTA

1. Prepare the Osmotic Shock Solution #2 first. For 200 mL, dissolve 0.484 g Tris base, 0.186 g disodium, dihydrate EDTA in 180 mL deionized water.
2. Adjust the pH to 8 with 1 N HCl and bring the volume up to 200 mL with deionized water.
3. In another container, dissolve 20 g of sucrose in 80 mL Osmotic Shock Solution #2.
4. Adjust the volume to 100 mL with more Osmotic Shock Solution #2.
5. Autoclave both solutions for 20 minutes at 121°C and 15 psi.
6. Store the solutions at 4°C.

Protocol for Chemically Competent Cells

Introduction

This protocol is used to make chemically competent cells for transformation with plasmid DNA. These cells will not substitute for electrocompetent cells for electroporation. The cells are grown to mid-log phase, then washed with FSB solution, and treated with DMSO. The cells are frozen in a dry ice/ethanol bath and stored at –80°C.

Yield

This protocol yields enough cells for about 60 transformations. The expected efficiency of chemically competent TOP10 cells is ~1×10^8 cfu per µg of supercoiled DNA.

Important

Sterile technique is absolutely essential to avoid contamination of the competent cells. Remember to use sterile solutions, medium, and supplies.

Preparation

For each cell preparation, prepare the following solutions (see **Recipes**, page 29):

5 mL SOB medium in a sterile culture tube
250 mL SOB in a sterile 500 mL or 1 L culture flask
FSB solution (<10 mL)
Fresh, reagent grade DMSO

Growing Cells: Day 1

Streak TOP10 on an LB plate, invert the plate, and incubate at 37°C overnight.

Growing Cells: Day 2

1. Inoculate 5 mL of SOB medium in a sterile culture tube with one colony from the LB plate.
2. Grow the cells overnight (12–16 hours) in a shaking incubator (200–225 rpm) at 37°C.

Growing Log Phase Cells: Day 3

1. For each cell preparation, place the following items on ice or at 4°C.
   Two 250 mL sterile centrifuge bottles
   Two 50 mL sterile centrifuge tubes
   Two 5 mL sterile pipettes
2. Inoculate 250 mL of fresh SOB medium in a 500 mL or 1 L culture flask with 2.5 mL of the overnight culture.
3. Grow the culture at 37°C at 200–225 rpm in a shaking incubator until the OD_{550} reaches between 0.55–0.65 (2–3 hours).
4. Divide the culture between the two cold (0–4°C), sterile 250 mL centrifuge bottles and place on ice for 30 minutes.

Continued on next page

Protocol for Chemically Competent Cells, continued

Preparing the Cells

1. Centrifuge the 250 mL bottles at 2,000 × *g* for 10–15 minutes at 0–4°C.
2. Decant the medium and resuspend each pellet in 10 mL cold (0–4°C) FSB solution and transfer to two cold, sterile 50 mL centrifuge tubes. Incubate on ice for 15 minutes.
3. Centrifuge the tubes at 2,000 × *g* for 10–15 minutes at 0–4°C.
4. Decant the buffer and resuspend each pellet in 1.3 mL of cold FSB solution using a sterile 5 mL pipette.
5. While gently swirling the tubes, slowly add 65 µL of DMSO drop by drop to each tube. Incubate the tubes on ice for 15 minutes.
6. While gently swirling the tubes, slowly add an additional 65 µL of DMSO drop by drop to each tube.
7. Combine the cell suspensions from both tubes into one and incubate on ice for 15 minutes. Keep the tube on ice.

Aliquoting and Storing Cells

1. Prepare a dry ice/ethanol bath. For each cell preparation, place approximately thirty 1.5 mL microcentrifuge tubes on ice. Keep the cell suspension on ice.
2. Pipette 110 µL of cell suspension into each tube.
3. As soon as all of the cell suspension is aliquoted, quick-freeze the tubes in the dry ice/ethanol bath and store at –80°C.

Chemical Transformation

1. Equilibrate a water bath or heat block to 42°C. Remove the appropriate number of tubes of frozen TOP10 chemically competent cells and thaw them on ice.
2. Add 3–5 µL of each ligation reaction to a separate tube of competent cells. Mix them gently with the pipette tip. **Do not pipette up and down**. Repeat for all ligations.
3. For control reactions, add 1 µL (10 ng in a volume less than 10 µL) of supercoiled plasmid (pThioHis without insert) to a separate tube of cells. Incubate the tubes on ice 30 minutes.
4. Transfer all tubes to 42°C heat block or water bath and incubate them for **exactly** 90 seconds, then place them on ice for 1–2 minutes.
5. Add 800 µL of room temperature S.O.C. medium to each tube and incubate the tubes with shaking (225 rpm) at 37°C for 60 minutes.
6. Plate 25 µL and 100 µL of each transformation mix on the LB-Amp transformation plates. Let all the liquid absorb to the plate, invert the plates, and incubate them at 37°C overnight. Proceed to **Analyzing Transformants**, page 14.

Protocol for Electrocompetent Cells

Introduction

Use this procedure to prepare cells for transformation with plasmid DNA by electroporation. The procedure describes the growth of cells and subsequent washing and concentrating steps. The washing is necessary to ensure that salts are removed to reduce the conductivity of the cell solution. High conductivity may result in arcing during electroporation.

These cells can only to be used for electroporation. Do **not** use them for any other transformation protocol.

Yield

The following procedure yields enough electrocompetent cells for about 30 transformations. Remember to use **sterile** solutions, medium, and supplies.

Note

The expected efficiency of the electrocompetent TOP10 cells is ~1×10^9 cfu/µg supercoiled DNA.

Important

Sterile technique is absolutely essential to avoid contamination of the competent cells. Remember to use sterile solutions, medium, and supplies.

Growing Cells: Day 1

Streak TOP10 on an LB plate, invert the plate, and incubate at 37°C overnight.

Growing Cells: Day 2

1. For each cell preparation, prepare the following a day in advance. See page 29 for **Recipes**.

   50 mL LB medium in a 250 mL sterile culture flask (store at room temperature)
   1 L of LB medium in a 2 L or 4 L sterile culture flask (store at room temperature)
   50 mL of sterile 10% glycerol (store at 4°C)
   1.5 L of sterile water (store at 4°C)

2. Inoculate the 50 mL of LB medium in a 250 mL culture flask with a single colony from the LB plate and incubate at 37°C with shaking (200–225 rpm) for 12–16 hours.

Growing Cells: Day 3

1. For each cell preparation, pre-chill on ice or at 4°C:

   Two sterile 500 mL centrifuge bottles
   Two sterile 50 mL centrifuge tubes
   Two sterile 25 mL pipettes
   One sterile 5 mL pipette

2. Inoculate 1 L of LB medium in a 2 L or 4 L flask with the 50 mL overnight culture. Grow the 1 L culture in shaking incubator (200–225 rpm) at 37°C until the OD_{550} is between 0.5 and 0.6 (approximately 2–3 hours).

3. Transfer the 1 L culture to the two chilled, sterile 500 mL centrifuge bottles and incubate on ice for 30 minutes.

Protocol for Electrocompetent Cells, continued

Harvesting and Washing the Cells

1. Centrifuge the cultures at 2,000 × *g* for 15 minutes at 0–4°C. Keep the cell pellet and decant the broth. Place the bottles back on ice.
2. Resuspend the cell pellet in each bottle in approximately 500 mL of cold (0–4°C) sterile water.
3. Centrifuge the cells at 2,000 × *g* for 15 minutes at 0–4°C. Keep the pellet and decant the water. Place the bottles back on ice.
4. Resuspend the cells in each bottle in approximately 250 mL of cold (0–4°C) sterile water.
5. Centrifuge the cells at 2,000 × *g* for 15 minutes at 0–4°C. Decant the water and place the bottles back on ice.
6. Using a pre-chilled, sterile 25 mL pipette, resuspend the cells in each bottle in 20 mL cold (0–4°C) sterile 10% glycerol and transfer each cell suspension to a chilled, sterile 50 mL centrifuge tube.
7. Centrifuge the cells at 4,000 × *g* for 15 minutes at 0–4°C. Decant the 10% glycerol and place the tubes on ice.
8. Resuspend each cell pellet in 1 mL cold (0–4°C) sterile 10% glycerol. Using a pre-chilled 5 mL pipette, pool the cells into one of the 50 mL tubes. Keep on the cells on ice.

Aliquoting and Storing Cells

1. Prepare a dry ice-ethanol bath.
2. For each cell preparation, place thirty-five 1.5 mL microcentrifuge tubes on ice and pipette 55 µL of the cell suspension into each tube. Keep the cell suspension and tubes at 0–4°C until all of the cell solution is aliquoted.
3. After all of the cell suspension is aliquoted, quick-freeze the tubes in the dry ice/ethanol bath and store them at –80°C until ready for use.

Continued on next page

Protocol for Electrocompetent Cells, continued

Electroporation Transformation

1. Remove the appropriate number of microcentrifuge tubes of TOP10 electrocompetent cells from the –80°C freezer and thaw them on ice. Chill the electroporation cuvettes on ice.
2. Set up your electroporator for electroporation of bacteria using the instructions included with your device.
3. Add 1–2 µL of a ligation reaction to a tube containing 50 µL competent cells. Repeat it for all ligation reactions.
4. For the control reactions, add 1 µL (10 ng) of supercoiled plasmid (pThioHis without the insert) to a separate tube of 50 µL competent cells. Incubate all tubes on ice for 1–2 minutes.
5. Take one sample at a time and transfer the cell/DNA mix to an electroporation cuvette. Place the cuvette in the chamber and discharge the electric pulse.
6. Remove the cuvette and **immediately** add 800 µL of room temperature S.O.C. medium. Using a sterile glass pipette, transfer the sample to a 15 mL snap-cap polypropylene tube (Falcon 2059 or similar). Place the tube on ice. Repeat steps 4–6 until all samples have been transferred to 15 mL tubes.
7. Incubate all tubes with shaking (200–225 rpm) at 37°C for 60 minutes.
8. Plate 25 µL and 100 µL of the transformation mix on the LB-Amp transformation plates. After the liquid is absorbed, invert the plates and incubate them at 37°C overnight.

Purifying by Osmotic Shock

Overview

The method described on the following pages is an alternative purification procedure. Thioredoxin localizes to osmotically sensitive compartments and can be quantitatively released by osmotic shock.

The table below describes the steps necessary to perform a trial purification of your fusion protein by osmotic shock.

Step	Action
1	Grow the strain containing your fusion construct in a small culture volume (10–25 mL) at 37°C.
2	Induce the expression of your fusion protein using the procedure you developed in the **Pilot Expression** section (page 15).
3	Take two 1 mL samples, one at t=0 induction time and one at the optimal time of induction (t=n).
4	Osmotically shock the cells by transferring them from a high ionic strength buffer (Osmotic Shock Solution #1) to a low ionic strength buffer (Osmotic Shock Solution #2).
5	Fractionate the cell "shockate" into cells and shock fluid by centrifugation.
6	Analyze the cells and the shock fluid by SDS-PAGE. If the fusion is released from the cell, it will be in the shock fluid (supernatant).

Controls

To determine if the osmotic shock procedure is working properly, we recommend that you also perform the same procedure with the TOP10 strain containing one of the pThioHis vectors without an insert. These plasmids overproduce HP-thioredoxin. When a cell containing the pThioHis plasmid is osmotically shocked, HP-thioredoxin is quantitatively released into the shock fluid (Holmgren, 1985). This serves as a positive control.

Materials Needed

The following are for one osmotic shock sample preparation. Increase your reagents for additional samples or controls. See page 31 for recipes.

1. One LB plate containing 100 µg/mL ampicillin
2. 1–2 mL of LB Medium containing 100 µg/mL ampicillin in a 5 mL culture tube.
3. 10–25 mL of LB Medium containing 100 µg/mL ampicillin in a 50 mL culture flask.
4. An SDS-PAGE gel to analyze your samples.
5. Osmotic Shock Solutions #1 and #2 (see page 31).

Note

Osmotic shock works best on fresh cells. Do not freeze cells before shocking. Do not try to shock frozen cells.

Continued on next page

Purifying by Osmotic Shock, continued

Growing Cells: Day 1

Streak the strain containing your fusion construct on an LB plate containing 100 µg/mL ampicillin. Invert the plate and grow it overnight at 37°C until single colonies appear (12–16 hours).

Growing Cells: Day 2

Take one colony and inoculate 1–2 mL of LB Medium containing 100 µg/mL ampicillin. Grow the cells with shaking (200–225 rpm) overnight at 30°C.

Growing and Inducing Cells: Day 3

1. Use 0.5–1.5 mL of the overnight culture to inoculate 10–25 mL of LB Medium containing 100 µg/mL ampicillin to an OD_{550} of about 0.1. Grow the cells at 37°C with shaking (200–225 rpm) until cells reach mid-log phase (OD_{550} = 0.5).
2. When the cells reach the mid-log phase of growth (3–4 hours), read and record the OD_{550} (should be around 0.5). Take a 1 mL sample, centrifuge it for 2–3 minutes at maximum speed to pellet the cells, decant the supernatant, and store the cells on ice. This is the zero time point.
3. Record the volume of the culture. Add IPTG to a final concentration of 1 mM to induce the fusion construct. Grow the cells until the optimal time point is reached as previously determined in the **Pilot Expression** section, page 15.
4. **Read and record the OD_{550} at the optimal time point**. Take a 1 mL sample, centrifuge it for 2–3 minutes at maximum speed to pellet the cells, decant the supernatant, and store the cells on ice.
5. Take the rest of the culture and discard. Do not save the cells.

Osmotic Shocking Your Samples

1. Resuspend the cell pellets from **Growing and Inducing Cells**, Steps 2 and 4 above, in Osmotic Shock Solution #1 to an OD_{550} of 5.0. Use the OD_{550} value you recorded for each time point to determine in what volume you should resuspend the cells.

   Formula: $V_R = (OD_{550} \text{ of sample}/5.0) \times V_S$
   Where V_R is the volume to resuspend the cell pellet and V_S is the original sample volume of the cell suspension

   Example: If a 1 mL sample of your cells has an OD_{550} of 0.5 for the zero time point, then:

   $V_R = (0.5/5.0) \times 1$ mL = 0.1 mL or 100 µL. This is the volume in which to resuspend your cells. Note that each pellet may need to be resuspended in a different volume.
2. Incubate the cells on ice for 10 minutes. Centrifuge the cells for 1 minute at 4°C and decant the buffer.
3. Resuspend the cell pellets in Osmotic Shock Solution #2, using the same volumes from Step 1. Incubate them on ice for 10 minutes. See the next page for steps 4–7.

Continued on next page

Osmotic Shocking Your Samples, continued

4. Centrifuge the resuspended cells for 10 minutes at 4°C. Transfer the supernatant (shock fluid) to a clean tube and keep it on ice.
5. Resuspend the pellets from Step 4 in the same volume of Osmotic Shock Buffer #2 as was used in Step 1. Note that each pellet may be resuspended in a different volume.
6. You now have four samples–a supernatant (shock fluid) and a pellet sample (cells) for the zero time point, and a supernatant and pellet sample for the optimal time point. If you included the positive control, you will have four more samples for a total of eight.
7. You may freeze these samples may be frozen at –20°C if you do not want to run a gel the same day you prepare samples. Proceed to **Analyzing Osmotic Shock Samples**, below.

Analyzing Osmotic Shock Samples

1. Analyze the samples from **Osmotic Shock**, step 6 (above) on an SDS-PAGE gel. Use 10 µL aliquots for each sample. Prepare and load the samples onto the gel to compare the shock fluid with the cells for each time point. Run the gel and process it.

   Remember that the fusion protein will be about 12.8 kDa larger than your native protein.
2. Use the following table to evaluate your experiment:

IF the control sample (containing HP-thioredoxin only...)	AND the sample containing the fusion protein ...	THEN...
is in the supernatant fraction	is also in the supernatant fraction	the fusion protein is released by osmotic shock and can be purified by this method. Proceed to **Scaling Up Your Purification**, page 27.
is in the supernatant fraction	is in the pellet fraction	the fusion protein is **not** releasable by osmotic shock. Try affinity chromatography with ProBond™ resin, page 19.
is in the pellet fraction	is also in the pellet fraction	review the osmotic shock procedure and make sure that you use the correct buffers in the correct order. If the osmotic shock step was done properly, HP-thioredoxin should be in the supernatant.

Activity Assay

If your fusion protein was successfully purified by osmotic shock, you may assay for the activity of your protein. If the fusion protein retains significant levels of activity, you may to defer the enterokinase cleavage step and scale-up your purification to produce more fusion protein.

Analysis by SDS-PAGE Gels

Introduction

This section provides references and suggestions for analysis of protein expression by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). SDS-PAGE allows the analysis of the solubility, purity, and yield of the fusion protein. We recommend that you use 10% Tricine gels to analyze the cell lysates if the fusion protein is less than 20 kDa. This gel system resolves differences between low molecular weight proteins.

Types of SDS-PAGE Gels

There are many types of SDS-PAGE gels. *Protein Methods* by Bollag, *et al*., 1991 describes the basic types. The Tricine gels used at Life Technologies are described by Schägger, *et al*., 1987. Citations for these publications are found in the **References** section, page 44. A large variety of pre-cast gels are available from Life Technologies. These include gels for analysis of proteins (Tris-Glycine, Tricine, Zymogram, IEF, and ZOOM® Gels) and nucleic acids (TBE, TBE-Urea, and DNA Retardation). For more information, visit our website at **www.lifetechnologies.com** or contact Technical Support (see page 42).

Novex® Tricine Gels

Novex® Tricine Gels are ideal for peptides and low molecular weight proteins (less than 10 kDa). The Tricine Gels are based on the Tricine system developed by (Schägger, *et al*., 1987). In this buffer system, tricine substitutes glycine in the running buffer resulting in more efficient stacking and destacking of low molecular weight proteins and higher resolution of smaller peptides. The Novex® Tricine Gels do not contain tricine in the gel; the tricine is supplied by the running buffer. Tricine gels must be used with denatured or reduced proteins only. The separating range of Tricine gels is 2.5–200 kDa (see page 41 for ordering information).

General Procedure for Sample Preparation

1. Before lysing the cells and preparing your samples, assemble the SDS-PAGE gel and equipment.
2. Lyse the cells and fractionate them if necessary. Take 10 µL aliquots from fractionated or unfractionated cell lysates and mix them with 10 µL of 2X SDS-PAGE sample buffer.
3. Boil the samples for 5 minutes and load all 20 µL onto the SDS-PAGE gel. If an aggregate forms after boiling, remake the sample and load it without boiling.
4. Electrophorese the gel and process it according to your protocol of choice.
5. Analyze the extent of solubility, purity, or yield.

Accessory Products

Additional Products

The following products may be used with the His-Patch ThioFusion™ Expression System and can be purchased separately from Life Technologies. Ordering information is provided below.

Product	Amount	Cat. no.
Anti-Thio™ Antibody	50 µL	R920-25
ProBond™ Nickel-Chelating Resin	50 mL 150 mL	R801-01 R801-15
EnterokinaseMax™	250 units 1000 units	E180-01 E180-02
S.O.C. Medium	10 × 10 mL	15544-034
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
PureLink™ HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100-02 K2100-03
Phosphate-Buffered Saline (PBS), pH 7.4	50 mL	10010-023
Trypsin-EDTA (0.05% Trypsin, EDTA•4Na)	100 mL	25300-054
TrypLE™ Express Dissociation Enzyme	100 mL	12604-013
Novex® Tricine SDS Running Buffer (10X)	500 mL	LC1675
Novex® Tricine SDS Sample Buffer (2X)	20 mL	LC1676
NuPAGE® Sample Reducing Agent (10X)	250 µL	NP0004

Competent *E. coli*

The following products may be used with the His-Patch ThioFusion™ Expression System for the growth and maintenance of pThioHis plasmids. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A). Note that the pThioHis vectors contain the *lac*I^q gene, so it is not necessary to use a strain containing a *lac*I gene.

Item	Amount	Cat. no.
One Shot® TOP10 (chemically competent cells)	21 × 50 µL	C4040-03
One Shot® TOP10 Electrocomp™ (electrocompetent cells)	21 × 50 µL	C4040-52
Electrocomp™ TOP10	5 × 80 µL	C664-55

Technical Support

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

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- 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 Sata Sheets (SDS)

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

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