invitrogen™

pBAD/TOPO® ThioFusion™ Expression Kit

Five-minute cloning of *Taq* polymerase-amplified PCR products for soluble, regulated expression and purification in *E. coli*

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User Manual

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Kit Contents and Storage

Shipping and Storage

The pBAD/TOPO® ThioFusion™ Expression Kit is shipped on dry ice. Each kit contains a box with pBAD/Thio TOPO® TA Cloning® reagents (Box 1), a box with One Shot® TOP10 Chemically Competent *E. coli* (Box 2), and a stab of LMG194.

Store Box 1 at –20°C and Box 2 at –80°C. Store the LMG194 stab at 4°C.

pBAD/Thio TOPO® TA Cloning® Reagents

pBAD/Thio TOPO® TA Cloning® reagents (Box 1) are listed below. **Note that you must supply the *Taq* polymerase.** Store Box 1 at –20°C.

Item	Concentration	Amount
pBAD/Thio-TOPO® vector, linearized	10 ng/µL plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/mL BSA 30 µM phenol red	20 reactions
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µL
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
20% L-Arabinose	20% in sterile water	1 mL
Trx Forward Sequencing Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL
pBAD Reverse Sequencing Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL
Control PCR Primers	0.1 µg/µL in TE Buffer, pH 8	10 µL
Control PCR Template	0.05 µg/µL in TE Buffer, pH 8	10 µL

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pBAD/Thio TOPO® TA Cloning® Reagents, continued

Item	Concentration	Amount
Sterile Water	--	1 mL
Expression Control Plasmid (pBAD/Thio, supercoiled)	500 ng/µL in TE buffer, pH 8	10 µL

Sequences of the Primers

The table below provides the sequences of the Trx Forward and pBAD Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
Trx Forward	5´-TTCCTCGACGCTAACCTG-3´	371
pBAD Reverse	5´-GATTTAATCTGTATCAGG-3´	363

One Shot® Reagents

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

Store at –80°C.

Item	Composition	Amount
TOP10 cells	--	21 × 50 µL
SOC Medium (may be stored at room temperature or 4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

Genotype of TOP10

TOP10: Use this strain for general cloning and expression of PCR products in pBAD/Thio-TOPO®. This strain cannot be used for single-strand rescue of DNA.

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

Continued on next page

Genotype of LMG194

F- Δ*lac*X74 *gal* E *thi rps*L Δ*pho*A (*Pvu* II) Δ*ara*714 *leu*::Tn*10*.

Note: This strain is deleted for *araBADC*, and is streptomycin and tetracycline resistant.

Storing LMG194 Stab

The LMG194 *E. coli* cells supplied as a stab with the kit are guaranteed until the expiration date marked on tube when stored at 4°C.

Upon receipt, we recommend that you prepare a set of glycerol master stocks within two weeks of receiving the kit.

To prepare 5–10 glycerol master stocks for long-term storage:

1. Streak a small portion of the LMG194 cells that you have received as a stab on an LB plate containing the appropriate antibiotics.
2. Invert the plate and incubate at 37°C overnight.
3. Isolate a single colony and inoculate into 5–10 mL of LB medium containing the appropriate antibiotics.
4. Grow the culture to stationary phase (OD_{600} = 1–2).
5. Mix 0.8 mL of culture with 0.2 mL of sterile glycerol and transfer to a cryovial.

Store at –80°C. Use one master stock to create working stocks for regular use.

Introduction

Description of the System

Product Features

pBAD/TOPO® ThioFusion™ Expression Kit provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for soluble, regulated expression and simplified protein purification in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Expression in *E. coli* is driven by the *ara*BAD promoter (P_{BAD}). The AraC gene product encoded on the pBAD/Thio-TOPO® plasmid positively regulates this promoter. Recombinant proteins are expressed as fusions to His-Patch thioredoxin for high-level expression and simple purification.

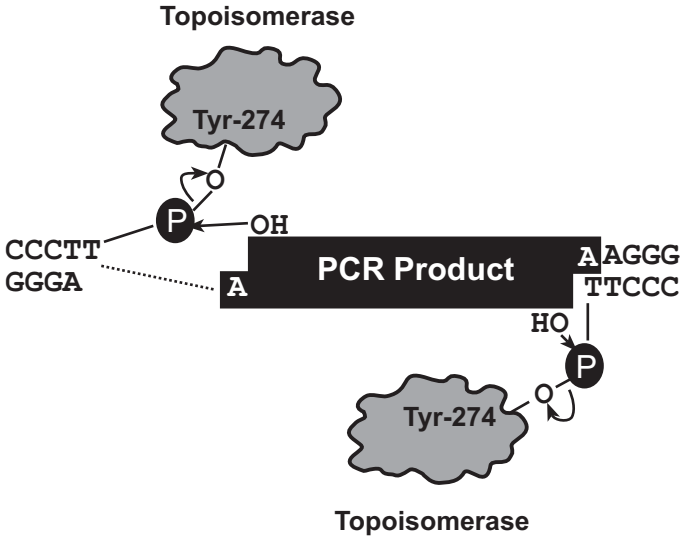
TOPO® Cloning

The PCR expression vector (pBAD/Thio-TOPO®) is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase I bound to the vector (this is referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994).



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Description of the System, Continued

Regulation of Expression by Arabinose

In the presence of arabinose, expression from P_{BAD} is induced while only very low levels of transcription are observed from P_{BAD} in the absence of arabinose (Lee, 1980; Lee *et al.*, 1987). Uninduced levels are repressed even further by growth in the presence of glucose (0.1% to 0.2%). Glucose reduces the levels of 3´, 5´-cyclic AMP, lowering expression from the catabolite-repressed P_{BAD} promoter (Miyada *et al.*, 1984). By varying the concentration of arabinose, protein expression levels can be optimized to ensure maximum expression of protein. In addition, the tight regulation of P_{BAD} by AraC is useful for expression of potentially toxic or essential genes (Carson *et al.*, 1991; Dalbey and Wickner, 1985; Guzman *et al.*, 1992; Kuhn and Wickner, 1985; Russell *et al.*, 1989; San Millan *et al.*, 1989). For more information on the mechanism of expression and repression of the *ara* regulon, see page 30 or refer to Schleif, 1992.

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 and still remains soluble. Thioredoxin is used to increase translation efficiency, and in some cases, solubility, of eukaryotic proteins expressed in *E. coli*. 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).

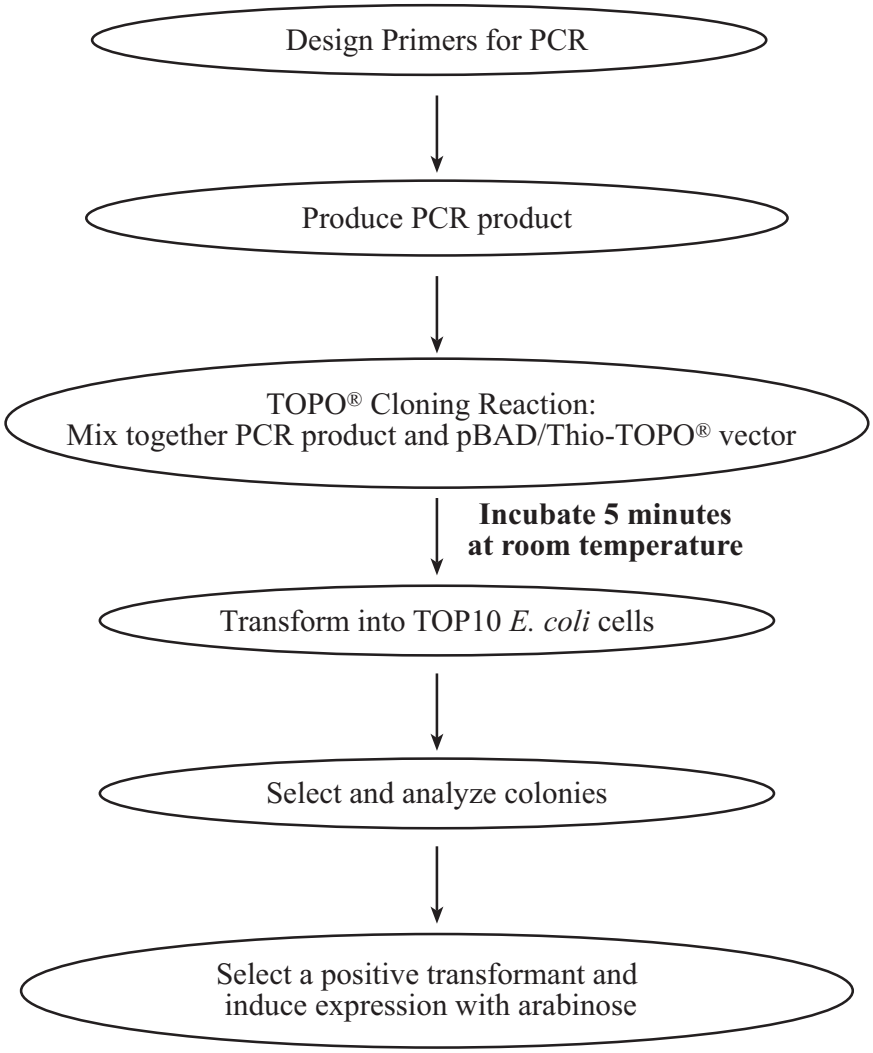
His-Patch Thioredoxin

To create a metal binding domain in the thioredoxin protein, the glutamate residue at position 32 and the glutamine residue at position 64 were mutated to create histidine residues. When His-Patch thioredoxin folds, the histidines at positions 32 and 64 interact with a native histidine at position 8 to form a "patch". This histidine patch was shown to have high affinity for divalent cations (Lu *et al.*, 1996). His-Patch thioredoxin (HP-thioredoxin) proteins can therefore be purified on metal-chelating resins (e.g., ProBond™) See page 33 for ordering information.

Experiment Outline

Experiment Flowchart

The flow chart below describes the general steps needed to amplify, TOPO® Clone, and express your protein of interest.



Methods

Designing PCR Primers

Introduction

It is important to properly design your PCR primers to ensure that you obtain the recombinant protein you need for your studies. Use the information below and the diagram on the next page to design your PCR primers.

Considerations

pBAD/Thio-TOPO® is designed with some specific features to facilitate expression. They are:

- The initiation ATG is correctly spaced from the optimized ribosome binding site to ensure optimal translation
- HP-thioredoxin acts as a translation leader to facilitate high-level expression and in some cases, solubility

  Note: You can remove HP-thioredoxin after protein purification using enterokinase (i.e., EKMax™, see page 19).

Primer Design

Suggestions for primer design are provided in the table below. **Remember that your PCR product will have single 3´ adenine overhangs.**

If you wish to....	Then...
clone in frame with thioredoxin	the forward PCR primer must be designed to ensure that your protein is in frame with the N-terminal leader peptide.
include the V5 epitope and polyhistidine region	the reverse PCR primer must be designed to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag.
NOT include the V5 epitope and polyhistidine region	include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site.
remove the N-terminal leader (for expressing truly native protein) **Note**: Removing the N-terminal leader generally decreases expression levels.	the forward PCR primer can be designed to include a unique *Nco* I site which contains the first ATG of the protein. Example: 5´-ACC ATG G.... The vector can be digested with *Nco* I after cloning and religated, assuming there are no internal *Nco* I sites in your PCR product.

Note

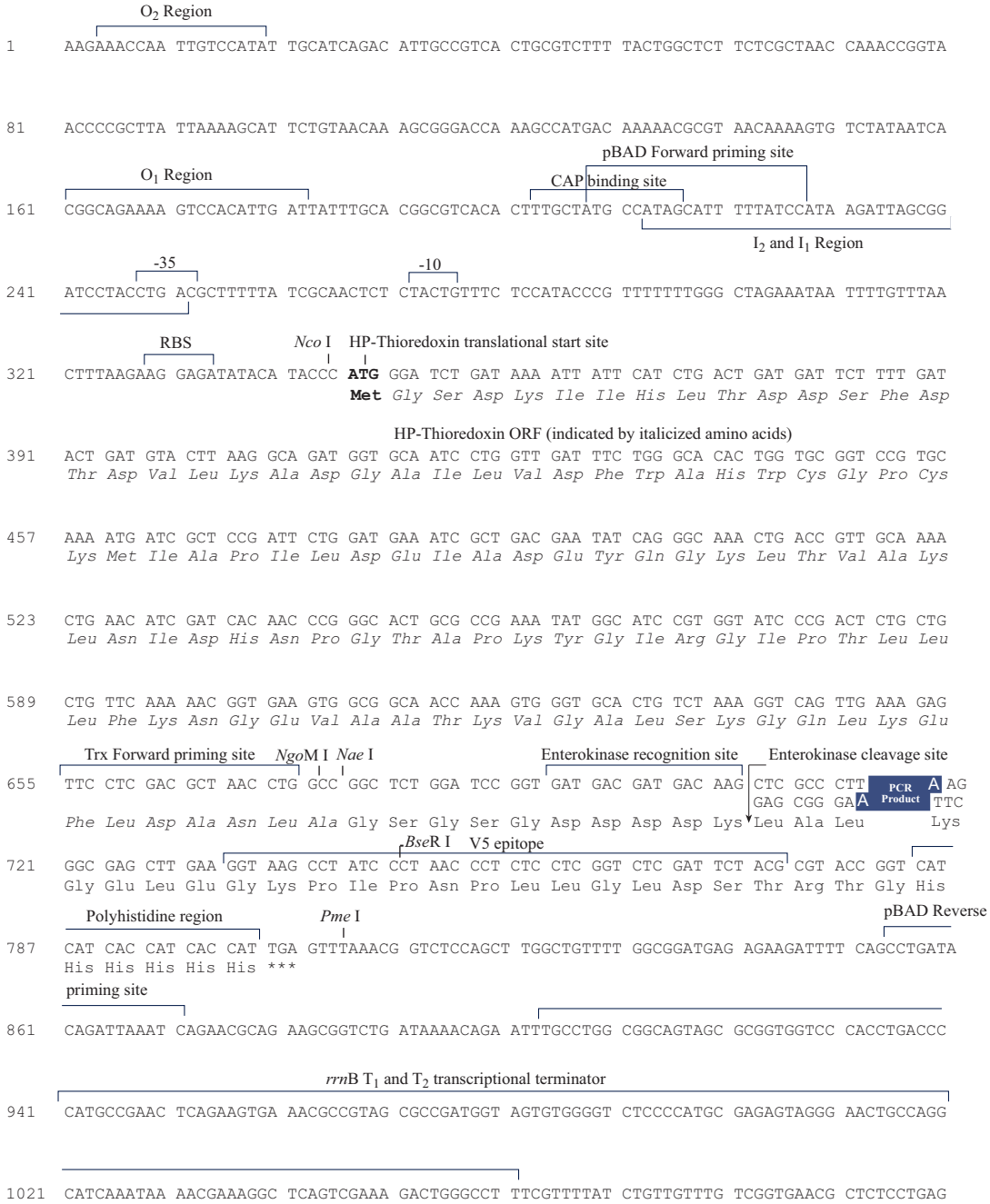
When synthesizing PCR primers, **do not** add 5′ phosphates to the primers, because 5′ phosphates prevent the synthesized PCR product from ligating into the pBAD-TOPO® vector.

Continued on next page

Designing PCR Primers, Continued

TOPO® Cloning Site

The diagram below is supplied to help you design appropriate PCR primers to correctly clone and express your PCR product. Restriction sites are labeled to indicate the actual cleavage site. **The complete sequence of the vector is available for downloading at www.invitrogen.com or from Technical Support (page 34).**



Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

Materials Supplied by the User

- *Taq* polymerase

  Note: For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page 32 for ordering information) to generate your PCR product.
- Thermocycler
- DNA template and primers to produce your PCR product
  Note: dNTPs (adjusted to pH 8) are provided in the kit.

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 24.

Producing PCR Products

1. Set up the following 50 µL PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

DNA Template	10–100 ng
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
Primers	100–200 ng each
Sterile water	add to a final volume of 49 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, gel-purify your fragment before TOPO® Cloning (see page 22). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit from Invitrogen can help you optimize your PCR (see page 32 for ordering information).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into pBAD/Thio-TOPO®, and transform the recombinant vector into *E. coli* in one day. It is important to have everything ready to use to ensure you obtain the best possible results. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 25–26 in parallel with your samples.

Note

Experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time > 5 minutes.

Including salt in the TOPO® Cloning reaction allows for longer incubation times, because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Important

We recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see below).** For this reason, two different TOPO® Cloning reaction protocols are provided to help you obtain the best possible results.

Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding NaCl and $MgCl_2$ to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

Materials Supplied by the User

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50–100 µg/mL ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Continued on next page

TOPO® Cloning Reaction and Transformation, Continued

Preparing for Transformation

For each transformation, you need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g., add 5 µL of the Salt Solution to 15 µL of sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- For each transformation, thaw 1 vial of One Shot® cells on ice.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µL) for eventual transformation into chemically competent TOP10 One Shot® *E. coli* (provided) or electrocompetent *E. coli*. Refer to page 11 for additional information on optimizing the TOPO® Cloning reaction. An Insert:vector molar ratio of 1:1 gives the optimal efficiency in TOPO® Cloning reaction.

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µL	0.5 to 4 µL
Salt Solution	1 µL	--
Dilute Salt Solution	--	1 µL
Sterile Water	add to a total volume of 5 µL	add to a total volume of 5 µL
TOPO® vector	1 µL	1 µL
Final Volume	**6 µL**	**6 µL**

*Store all reagents at –20°C when finished. Salt solutions and water can be stored at room temperature or 4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature.

   Note: For most applications, incubation for 5 minutes yields plenty of colonies for analysis. Depending on your needs, you can vary the length of the TOPO® Cloning reaction from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds is sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time yields more colonies.

2. Place the reaction on ice and proceed to the **One Shot® Chemical Transformation** or **Transformation by Electroporation** (next page).
   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

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TOPO® Cloning Reaction and Transformation, Continued

One Shot® TOP10 Chemical Transformation

1. Add 2 µL of the TOPO® Cloning reaction from Step 2 previous page into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion (see page 12).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µL of room temperature SOC medium.
6. Cap the tube tightly and shake horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 25–200 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, next page).

Transformation by Electroporation

1. Add 2 µL of the TOPO® Cloning reaction into a 0.1 cm cuvette containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using the protocol for your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µL of room temperature SOC medium.
4. Transfer the solution to a 15 mL snap-cap tube and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
5. Spread 10–50 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, next page).

Note

Adding the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µL for 0.1 cm cuvettes or 100 to 200 µL for 0.2 cm cuvettes.

If you experience arcing during transformation, try one of the following:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol-precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

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TOPO® Cloning Reaction and Transformation, Continued

Analyzing Positive Clones

1. Culture 10 colonies overnight in LB or SOB medium with 50–100 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits (see page 32 for ordering information). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.
3. Because the PCR product will clone in either direction, analyze for orientation as well as insertion by restriction analysis or by sequencing. The Trx Forward and pBAD Reverse sequencing primers are included to sequence your insert. Refer to the diagram on page 5 for primer binding sites.

Alternative Method of Analysis

You may directly analyze positive transformants using PCR. You may use the Trx Forward and pBAD Reverse sequencing primers as PCR primers. We recommend performing restriction analysis in parallel to confirm that PCR gives you the correct result. Artifacts can be obtained because of mispriming or contaminating template. The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µL reaction volume. Multiply by the number of colonies to be analyzed (e.g., 10).
2. Pick 10 colonies and resuspend them individually in 20 µL of the PCR cocktail.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at 4°C.
6. Analyze by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, see pages 25–26. Control reactions are described using reagents supplied in the kit.

Continued on next page

Long-Term Storage

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

1. Streak the original colony out for single colony on LB plates containing 50–100 µg/mL ampicillin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store glycerol stock at –80°C, and a stock of plasmid DNA at –20°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamLine the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes. You may not obtain the highest number of colonies, but with the high cloning efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µL of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes. Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies, incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Expressing the PCR Product

Introduction

Since each recombinant protein has different characteristics that may affect optimal expression, it is helpful to vary the arabinose concentration and/or run a time course of expression to optimize the expression of your particular protein. pBAD/Thio is included for use as a positive expression control. TOP10 cells may be used as a general host for expression.

LMG194 Strain

The *E. coli* strain LMG194 (Guzman *et al.*, 1995) is included in the kit to allow additional repression for low basal level expression of toxic genes. This strain is capable of growth on minimal medium (RM medium) which allows repression of P_{BAD} by glucose. **After you have determined that you have the correct construct, transform it into LMG194 prior to performing expression experiments.** Follow the guidelines below for using LMG194:

- Induce the pBAD promoter when cells are growing in LB or RM-Glucose.
- If you are growing your construct under maximal repression, i.e., with D-glucose in RM media, then you must spin down the culture and resuspend it in RM containing 0.2% glycerol and Arabinose (i.e., substitute glycerol for the glucose in the media recipe on page 21).

pBAD/Thio Vector

The positive control vector, pBAD/Thio, is included in the kit as an expression control. Details of this vector are provided on page 28. Transform the vector (10 ng) into TOP10 cells using the procedure on page 9.

Basic Strategy

Once you have some clones that you wish to characterize, we recommend the following strategy to determine the optimal expression level.

1. **Pilot Expression.** Vary the amount of arabinose over a 10,000-fold range (0.00002% to 0.2%) to determine the approximate amount of arabinose needed for maximum expression of your protein. See next page for protocol.
2. To optimize expression of your protein, try arabinose concentrations spanning the amount determined in Step 1, or you may perform a time course.

Note: If your protein is insoluble, analyze the supernatant **and** the pellet of lysed cells for expression of soluble protein (page 16).

Note

Expressing your protein with the N-terminal HP-thioredoxin peptide and the C-terminal tag increases the size of your protein by 13 kDa and 3 kDa, respectively. Be sure to account for any additional amino acids between the tag and your protein.

Continued on next page

Expressing the PCR Product, Continued

Materials Required

- SOB or LB containing 50–100 µg/mL ampicillin (see page 19 for recipe)
- 37°C shaking incubator
- 20% L-arabinose (provided). Additional L-arabinose is available from Sigma (Cat. no. A3256).

Pilot Expression

In addition to testing your transformants, we recommend that you include the pBAD/Thio vector as a positive control and cells without vector as a negative control.

1. For each transformant or control, inoculate 2 mL of SOB or LB containing 50–100 µg/mL ampicillin with a single recombinant *E. coli* colony.

   Note: If you are using LMG194 as a host, use RM medium containing glucose and 100 µg/mL ampicillin for overnight growth (see page 21 for a recipe), and then substitute glycerol for glucose in medium at Step 3 below (see **Using LMG194**, previous page).
2. Grow overnight at 37°C with shaking (225–250 rpm) to OD_{600} = 1–2.
3. The next day, label five tubes 1 through 5 and add 10 mL of SOB or LB containing 50–100 µg/mL ampicillin.
4. Inoculate each tube with 0.1 mL of the overnight culture.
5. Grow the cultures at 37°C with vigorous shaking to an OD_{600} = ~0.5 (the cells should be in mid-log phase).
6. While the cells are growing, prepare four 10-fold serial dilutions of 20% arabinose with sterile water using aseptic technique (e.g., 2%, 0.2%, 0.02%, and 0.002%).
7. Remove a 1 mL aliquot of cells from each tube, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
8. Freeze the cell pellet at –20°C. This is the zero time point sample.

(protocol continued on next page)

Continued on next page

Pilot Expression, continued

9. Use the stock solutions prepared in Step 6 and add arabinose to the five 9 mL cultures as follows.

   Note: For the positive and negative controls, it is not necessary to test all concentrations of arabinose. Use only the highest concentration of arabinose.

Tube	Stock Solution	Volume (mL)	Final Concentration
1	0.002%	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2%	0.09	0.02%
5	20%	0.09	0.2%

10. Grow at 37°C with shaking for 4 hours.
11. Take 1 mL samples at 4 hours and treat as in Step 7 and 8. You will have a total of 10 samples for each transformant and two samples for each control.

Expressing Toxic Proteins

To ensure low levels of expression, you may find it useful to utilize 0.2% glucose to repress the *ara*BAD promoter further. Follow the steps below to express your protein.

1. Transform your construct into LMG194. LMG194 can be grown in RM medium that enables repression of P_{BAD} by glucose or glycerol.
2. Follow the Pilot Expression protocol, using RM medium containing 0.2% glycerol to grow the cells (i.e., substitute glycerol for glucose in the media recipe on page 21).
3. Be sure to monitor the OD_{600} as the cells will grow more slowly in RM medium.
4. Induce with various concentrations of arabinose as described in the Pilot Expression.
5. Monitor OD_{600} over time to be sure cells are growing.

Analyzing Samples

Materials Needed

- Reagents and apparatus for SDS-PAGE gel
- 1X and 2X SDS-PAGE sample buffer
- Boiling water bath
- Lysis Buffer (see page 21 for recipe)
- Liquid nitrogen, optional

Preparing Samples

Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see next page) to analyze the collected samples.

1. When all the samples have been collected from the pilot expression, resuspend each cell pellet in 100 µL of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5–10 µL of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing them at –20°C.

Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µL of Lysis Buffer.
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times.
   Note: To facilitate lysis, you may add lysozyme to the sample or sonicate the cells.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at 4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µL of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil 5 minutes.
6. Load 10 µL of the supernatant sample and 5 µL of the pellet sample onto an SDS-PAGE gel and electrophorese.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with LaemmLi-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits.

For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to www.invitrogen.com or contact Technical Support (page 34).

Continued on next page

Analyzing Samples, Continued

Analyzing Samples

1. Stain the gel and look for a band of increasing intensity in the expected size range for the recombinant protein.
2. Use the positive control (pBAD/Thio) to confirm that growth and induction was done properly. The positive control should yield a 16 kDa protein when induced with 0.02% arabinose.
3. Determine the approximate arabinose concentration for maximum expression.

Low Expression

If you don't see any expression on a gel, re-run your samples on an SDS-PAGE gel and perform a western blot. Use antibody to your protein or any of the antibodies listed on page 33.

If you still don't see expression of your protein, sequence your construct and make sure it is in frame with the N-terminal and/or C-terminal peptide.

Optimizing Expression

After you have detected expression of your protein of interest, perform some experiments to further optimize expression.

- Use the **Pilot Expression** protocol on pages 14–15, but vary the arabinose concentration over a smaller range. For example, if you obtained the best expression at 0.002% arabinose in the medium, try 0.0004%, 0.0008%, 0.001%, 0.004%, and 0.008%.
- You may also perform a time course of induction to determine if varying the time increases expression. Take time points every hour, over a 5 to 6 hour period.
- If your protein is insoluble, analyze the supernatant and pellet of lysed cells when you vary the arabinose concentration (see **Preparing Samples for Soluble/Insoluble Protein**, previous page).
- Store your cell lysates at –20°C.

Purifying Recombinant Protein

Introduction

After you have expressed your recombinant fusion protein, you are ready to purify your fusion protein using a metal-chelating resin such as ProBond™.

ProBond™

ProBond™ is a nickel-charged Sepharose® resin that can be used for affinity purification of fusion proteins containing the HP-thioredoxin leader peptide and/or a 6×His tag. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole or histidine.

- To scale up your pilot expression for purification, see below.
- To purify your fusion protein using ProBond™, refer to the ProBond™ Purification manual.
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Note

Note that denaturing conditions will destroy the Ni^{2+} binding site created by the histidine 'patch' in HP-thioredoxin.

Additional Purification Steps

There may be cases when your specific HP-thioredoxin fusion protein may not be completely purified by metal affinity chromatography. Other protein purification techniques may be utilized in conjunction with ProBond™ to purify the fusion protein (Deutscher, 1990).

Scaling Up Expression for Purification on ProBond™

The capacity of ProBond™ is about 1 mg of protein per mL. Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein. For a prepacked 2 mL ProBond™ column, start with 50 mL of bacterial culture.

To grow and induce a 50 mL bacterial culture:

1. Inoculate 2 mL of SOB or LB containing 50–100 µg/mL ampicillin with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking (225–250 rpm) to OD_{600} = 1–2.
3. The next day, inoculate 50 mL of SOB or LB containing 50–100 µg/mL ampicillin with 1 mL of the overnight culture.
4. Grow the culture at 37°C with vigorous shaking to an OD_{600} = ~0.5 (the cells should be in mid-log phase).
5. Add the optimal amount of arabinose to induce expression.
6. Grow at 37°C with shaking until the optimal time point is reached. Harvest the cells by centrifugation (3,000 × g for 10 minutes at 4°C).
7. At this point, you may proceed directly to purification (ProBond™ Purification System manual) or store the cells at –80°C for future use.

Continued on next page

Removing the N-terminal Leader

The enterokinase recognition site in the HP-thioredoxin leader may be utilized to remove the leader sequence from your protein after purification. Note that after digestion with enterokinase, there will be three vector-encoded amino acids (Leu-Ala-Leu) remaining at the N-terminus of the protein (see page 5).

To digest your fusion protein with enterokinase, follow the manufacturer's recommendations.

A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available from Invitrogen. Instructions for digestion are included with the product. To remove EKMax™ from the digest, you may use EK-Away™ Resin, also available from Invitrogen (see page 32 for ordering information).

Appendix

Recipes

LB (Luria-Bertani) Medium and LB Agar Plates

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and volume to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed (50–100 µg/mL ampicillin).
4. Store at room temperature or at 4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50–100 µg/mL of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C, in the dark.
5. To add X-gal to the plate, warm the plate to 37°C. Pipette 40 µL of the 40 mg/mL stock solution (see below) onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

X-Gal Stock Solution

1. For a 40 mg/mL stock solution, dissolve 400 mg X-Gal in 10 mL dimethylformamide.
2. Protect from light by storing in a brown bottle at –20°C.

SOB Medium (with Ampicillin)

2% Tryptone

0.5% Yeast Extract

0.05% NaCl

2.5 mM KCl

10 mM $MgCl_2$

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

Continued on next page

Recipes, Continued

RM Medium + Glucose

1X M9 Salts (see recipe below for 10X M9 Salts)
2% Casamino Acids
0.2% glucose
1 mM $MgCl_2$
50–100 µg/mL ampicillin

1. For 1 liter of RM medium, mix 20 g Casamino Acids and 890 mL deionized water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool the autoclaved solution, and add the following sterile solutions:

10X M9 Salts	100 mL
1 M $MgCl_2$	1 mL
20% glucose	10 mL
100 mg/mL ampicillin	0.5 to 1 mL

4. Mix well and store at 4°C for 1 month.

10X M9 Salts

1. Dissolve the following reagents in 900 mL water and adjust the pH to 7.4 with 10 M NaOH.

Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

2. Add water to 1 liter and autoclave for 20 minutes on the liquid cycle.
3. Store at room temperature.

Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 mL, dissolve the following reagents in 90 mL of deionized water:

1 M KH_2PO_4	0.3 mL
1 M K_2HPO_4	4.7 mL
NaCl	2.3 g
KCl	0.75 g
Glycerol	10 mL
Triton X-100	0.5 mL
Imidazole	68 mg

3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 mL.
4. Store at 4°C.

Purifying the PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are described below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit allows you to rapidly purify PCR products from regular agarose gels (see page 32 for ordering information).

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment. Weigh the gel slice.
3. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5-mL polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µL Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-mL polypropylene tubes and add 60 µL Gel Solubilization Buffer (GS1) for every 10 mg of gel.
4. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
5. Preheat an aliquot of TE Buffer (TE) to 65–70°C
6. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 4, above onto the column. Use 1 column per 400 mg agarose.
7. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
8. **Optional:** Add 500 µL Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
9. Add 700 µL Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 × g for 1 minute. Discard flow-through.
10. Centrifuge the column at >12,000 × g for 1 minute to remove any residual buffer. Place the column into a 1.5 mL Recovery Tube.
11. Add 50 µL **warm** (65–70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
12. Centrifuge at >12,000 × g for 2 minutes. **The Recovery Tube contains the purified DNA.** Store DNA at –20°C. Discard the column.
13. Use 4 µL of the purified DNA for the TOPO® Cloning reaction.

Continued on next page

Low-Melt Agarose Method

Note that gel purification will dilute your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µL of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 7).
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µL directly into TOP10 One Shot® cells using the method on page 9.

Note

Cloning efficiency may decrease with purification of the PCR product. To produce a single band, optimize your PCR conditions.

Adding 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO® Cloning vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the lack of the terminal transferase activity that adds the 3´ A-overhangs necessary for TOPO® Cloning. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols are also suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7–1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pBAD/Thio-TOPO®.

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see previous page for protocol). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10–15 minutes at 72°C. Use 4 µL in the TOPO® Cloning reaction.

pBAD/TOPO® ThioFusion™ Control Reactions

Introduction

If you have trouble obtaining transformants or vector containing insert, perform the following control reactions to help troubleshoot your experiment. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing antibiotic and X-gal.

Before Starting

Prepare the following reagents before performing the control reaction:

- 40 mg/mL X-gal in dimethylformamide
- LB plates containing 50–100 µg/mL ampicillin and X-gal (see page 20)

Producing Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZα, set up the following 50 µL PCR:

Control DNA Template (50 ng)	1 µL
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
Control PCR Primers (0.1 µg/µL)	1 µL
Sterile Water	41.5 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

Continued on next page

pBAD/TOPO® ThioFusion™ Control Reactions, Continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pBAD/Thio-TOPO® vector, set up two 6 µL TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Control PCR Product	--	1 µL
Salt Solution or Dilute Salt Solution	1 µL	1 µL
Sterile Water	4 µL	3 µL
pBAD/Thio-TOPO®	1 µL	1 µL

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µL of each reaction into separate vials of TOP10 One Shot® cells (page 9).
4. Spread 10–50 µL of each transformation mix onto LB plates containing 50–100 µg/mL ampicillin and X-Gal (see page 20). Plate two different volumes to ensure that at least one plate has well-spaced colonies. To plate small volumes, add 20 µL of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analyzing Results

The vector + PCR insert reaction should produce hundreds of colonies, and greater than 90% of the colonies should be blue.

The "vector only" plate should yield very few colonies (<10% of the vector + PCR insert plate), and these should be all white.

Transformation Control

pUC19 plasmid is included as a control to check the transformation efficiency of One Shot® competent cells. Transform One Shot® competent cells with 10 pg of plasmid per 50 µL of cells using the protocol on page 10. Plate 10 µL of the transformation mixture plus 20 µL of SOC to help ensure even spreading on LB plates containing 100 µg/mL ampicillin. Transformation efficiency should be ~1×10^9 cfu/µg DNA.

Continued on next page

Troubleshooting

Factors Affecting Cloning Efficiency

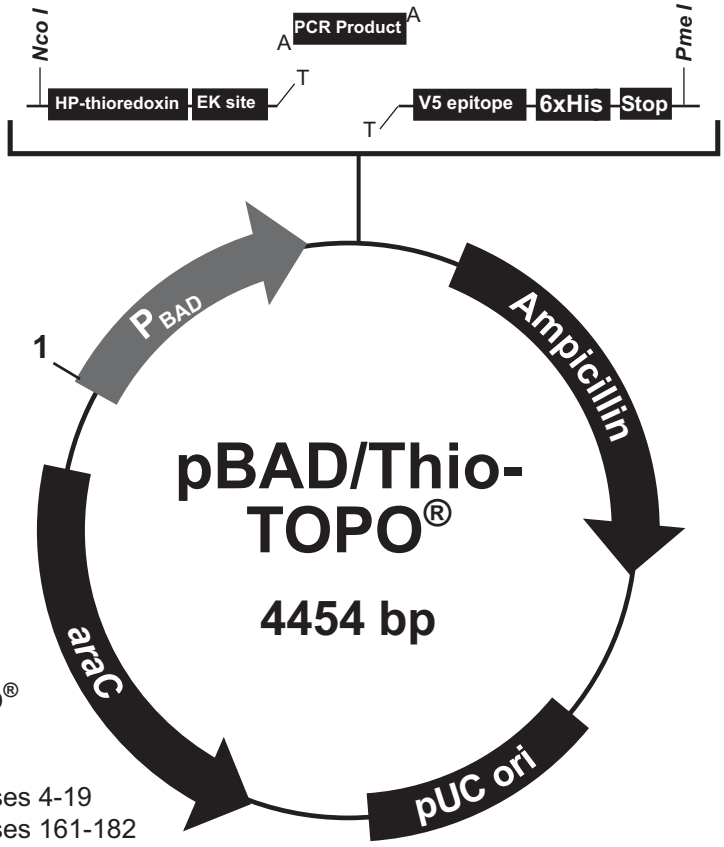
Lower cloning efficiencies may be a result from different variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 90% (or more) cloning efficiency.

Variable	Solution
pH > 9 in PCR amplification reaction	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>3 kb)	Increase amount of insert, or gel-purify as described on page 22.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. You may add up to 4 µL of your PCR to the TOPO® Cloning reaction (page 8).
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 24).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 22) or optimize your PCR. If your template DNA carries an ampicillin marker, carryover into the TOPO® Cloning reaction from the PCR may lead to false positives. Linearize the template DNA prior to PCR to eliminate carryover.
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

Map and Features of pBAD/Thio-TOPO®

pBAD/Thio-TOPO® Map

The map below shows the features of pBAD/Thio-TOPO®. **The complete sequence of the vector is available for downloading at www.invitrogen.com or from Technical Support (page 34).**



Comments for pBAD/Thio-TOPO®
4454 nucleotides

Arabinose O_2 operator region: bases 4-19
Arabinose O_1 operator region: bases 161-182
CAP binding site: bases 203-216
Arabinose I_1 and I_2 region: bases 213-251
Arabinose minimal promoter: bases 248-276
Ribosome binding site: bases 329-334
His-Patch Thioredoxin ORF: bases 346-674
Trx Forward priming site: bases 655-672
Enterokinase recognition site: bases 691-705
TOPO® Cloning site: bases 714-715
V5 epitope: bases 730-771
Polyhistidine region: bases 781-801
pBAD Reverse priming site: bases 854-871
*rrn*B transcriptional termination region: bases 904-1061
Ampicillin resistance gene (ORF): bases 1341-2201
pUC origin: bases 2346-3019
AraC ORF: bases 3550-4428 (opposite strand)

Continued on next page

Map and Features of pBAD/Thio-TOPO®, Continued

Features of pBAD/Thio-TOPO®

The important elements of pBAD/Thio-TOPO® (4,454 bp) are described in the following table. All features have been functionally tested. For more information on the regulation of gene expression by arabinose, see page 31.

Feature	Benefit
*ara*BAD promoter (P_{BAD})	Provides tight, dose-dependent regulation of heterologous gene expression (Guzman *et al.*, 1995).
O_2 region	Binding site of AraC that represses transcription from P_{BAD}.
O_1 region	Binding site of AraC that represses transcription of the *ara*C promoter (P_C) (transcribed on the opposite strand).
CAP binding site	Site where CAP (cAMP binding protein) binds to activate transcription from P_{BAD} and P_C.
I_2 and I_1 regions	Binding sites of AraC that activate transcription from P_{BAD}.
–10 and –35 regions	Binding sites of RNA polymerase for transcription from P_{BAD}.
Optimized ribosome binding site	Increases efficiency of recombinant fusion protein expression.
HP-thioredoxin	Provides a highly efficient fusion partner for translation of the fusion protein.
TOPO® Cloning site	Allows quick insertion of your PCR product for expression.
C-terminal V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the fusion protein by the Anti-V5 Antibody or the Anti-V5-HRP Antibody (Southern *et al.*, 1991)
C-terminal polyhistidine region (optional)	Permits purification of recombinant fusion protein on metal-chelating resins (i.e. ProBond™). In addition, it allows detection of the recombinant protein with the Anti-His(C-term) Antibody or the Anti-His(C-term)-HRP Antibody (Lindner *et al.*, 1997)
*rrn*B transcription termination region	Strong transcription termination region.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Replication and growth in *E. coli*.
*ara*C gene	Encodes the regulatory protein for tight regulation of the P_{BAD} promoter (Lee, 1980; Schleif, 1992).

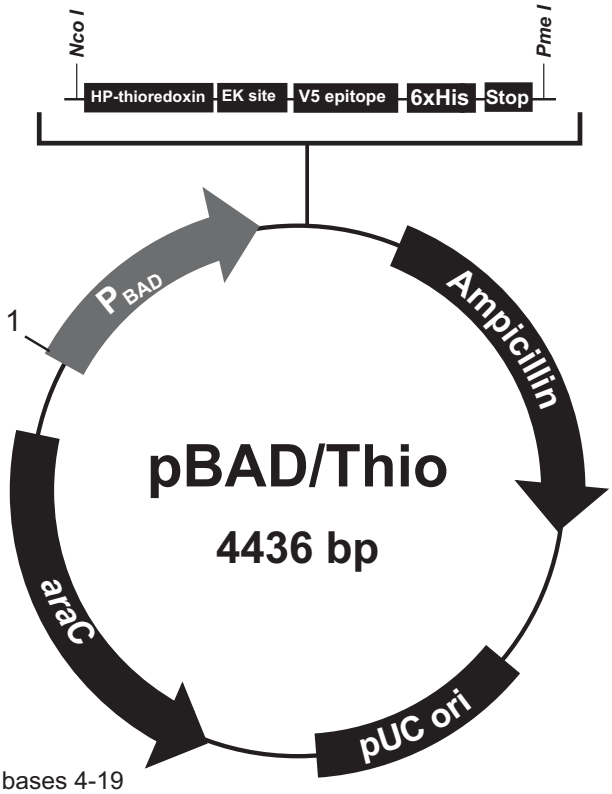
Map of pBAD/Thio

Description

pBAD/Thio is a 4,436 bp control vector expressing a 16 kDa HP-thioredoxin fusion protein. The whole fusion gene may be excised with *Nco* I and *Pme* I.

Map of Control Vector

The figure below summarizes the features of the pBAD/Thio vector. **The complete nucleotide sequence for pBAD/Thio is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 34).**



Comments for pBAD/Thio
4436 nucleotides

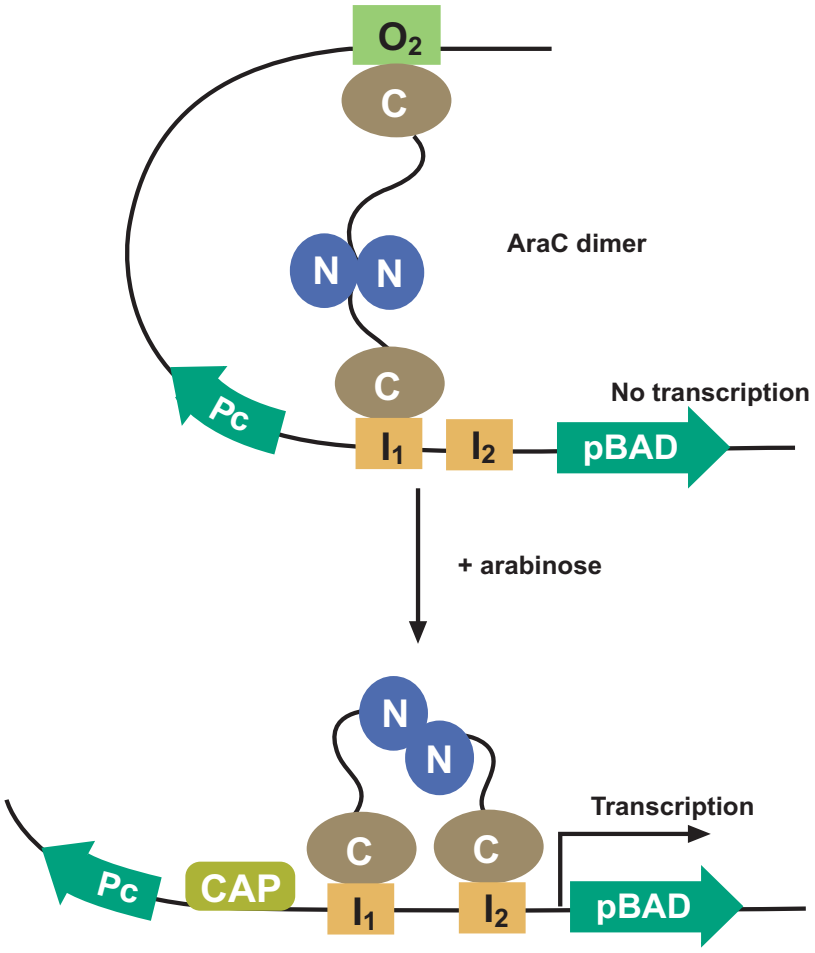
Arabinose O_2 operator region: bases 4-19
Arabinose O_1 operator region: bases 161-182
CAP binding site: bases 203-216
Arabinose I_1 and I_2 region: bases 213-251
Arabinose minimal promoter: bases 248-276
Ribosome binding site: bases 329-332
His-Patch Thioredoxin ORF: bases 346-674
Trx Forward priming site: bases 655-672
Enterokinase recognition site: bases 691-705
V5 epitope: bases 712-753
Polyhistidine region: bases 763-783
pBAD Reverse priming site: bases 836-853
*rrn*B transcriptional termination region: bases 886-1043
Ampicillin resistance gene (ORF): bases 1323-2183
pUC origin: bases 2328-3001
AraC ORF: bases 3532-4410 (opposite strand)

Regulation by Arabinose

Regulation of the P_{BAD} Promoter

The *ara*BAD promoter used in pBAD/Thio-TOPO® is both positively and negatively regulated by the product of the *ara*C gene (Ogden *et al.*, 1980; Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of arabinose the AraC dimer contacts the O_2 and I_1 half sites of the *ara*BAD operon, forming a 210 bp DNA loop (see the figure below). For maximum transcriptional activation two events are required.

- Arabinose binds to AraC and causes the protein to release the O_2 site and bind the I_2 site which is adjacent to the I_1 site. This releases the DNA loop and allows transcription to begin.
- The cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.



Glucose Repression

Basal expression levels can be repressed by introducing glucose to the growth medium. Glucose acts by lowering cAMP levels, which in turn decreases the binding of CAP. As cAMP levels are lowered, transcriptional activation is decreased.

Accessory Products

Additional Products

Many of the reagents supplied with the pBAD/TOPO® ThioFusion™ Expression Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below. For details, visit www.invitrogen.com.

Product	Amount	Cat. no.
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
PCR Optimizer™ Kit	100 reactions	K1220-01
PCR SuperMix High Fidelity	100 reactions	10790-020
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plasmid Miniprep Kit	25 preps	K2100-02
	100 preps	K2100-03
EKMax™ Enterokinase	250 units	E180-01
EK-Away™ Resin	7.5 mL	R180-01
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
β-Gal Antiserum	50 µL	R901-25
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
X-gal	100 mg	15520-034

Electrocompetent Cells

TOP10 cells are also available as electrocompetent cells. See the table below for ordering information.

Kit	Reactions	Cat. no.
One Shot® TOP10 Electrocomp™ *E. coli*	10	C4040-50
	20	C4040-52
TOP10 Electrocomp™ Kits	20	C664-55
	40	C664-11
	120	C664-24

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Detecting Recombinant Protein

Expression of your recombinant fusion protein can be detected using an antibody to the protein itself or to the appropriate epitope. The table below describes the antibodies available for use with pBAD/Thio-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

Epitope	Antibody	Cat. no.
V5 (-GKPIPNPLLGLDST-)	Anti-V5	R960-25
	Anti-V5-HRP	R961-25
C-terminal polyhistidine tag (-HHHHHH-COOH)	Anti-His(C-term)	R930-25
	Anti-His(C-term)-HRP	R931-25
Thioredoxin (non-contiguous epitope)	Anti-Thio™	R920-25

Purifying Recombinant Protein

The metal binding domain encoded by either the His-Patch thioredoxin or the 6×His tag allows simple, easy purification of your recombinant fusion protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed using pBAD/Thio-TOPO®, the ProBond™ Purification System is available separately. Additional ProBond™ resin is available in bulk. See the table below for ordering information.

Product	Amount	Cat. no.
ProBond™ Purification System (includes six 2 mL precharged, prepacked ProBond™ resin columns and buffers for native and denaturing purification)	6 purifications	K850-01
ProBond™ Purification Kit with Anti-V5-HRP Antibody	1 kit	K854-01
ProBond™ Metal-Binding Resin (precharged resin provided as a 50% slurry in 20% ethanol)	50 mL	R801-01
	150 mL	R801-15
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50

Important

Note that under denaturing conditions, the Ni^{2+} binding site encoded by the histidine 'patch' will be destroyed because the HP-thioredoxin protein will be denatured. The binding of nickel ion to the 6×His tag is not affected by denaturing conditions.

Technical Support

Web Resources

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

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

Contact Us

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MSDS

MSDSs (Material Safety Data Sheets) are available at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are .available on our website at www.invitrogen.com/support.

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Introduction

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Information for European Customers

The LMG194 cell line is genetically modified. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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