Champion™ pET SUMO Protein Expression System

For high-level expression and enhanced solubility of recombinant proteins in *E. coli* and cleavage of native protein

Catalog no. K300-01

Rev. Date: 18 June 2010
Manual part no. 25-0709

MAN0000440
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Kit Contents and Storage

Type of Kit
This manual is supplied with the Champion™ pET SUMO Protein Expression System (Catalog no. K300-01). Sufficient reagents are provided to perform 20 cloning and expression reactions.

Shipping/Storage
The Champion™ pET SUMO Protein Expression System is shipped on dry ice. Each kit contains three boxes as described below. Upon receipt, store the boxes as detailed below.

<table>
<thead>
<tr>
<th>Box</th>
<th>Component</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pET SUMO TA Cloning® Reagents</td>
<td>-20°C</td>
</tr>
<tr>
<td>2</td>
<td>One Shot® Mach1™-T1R Chemically Competent E. coli</td>
<td>-80°C</td>
</tr>
<tr>
<td>3</td>
<td>BL21(DE3) One Shot® Chemically Competent E. coli</td>
<td>-80°C</td>
</tr>
<tr>
<td>4</td>
<td>SUMO Protease</td>
<td>Protease: -80°C Buffers: -20°C</td>
</tr>
</tbody>
</table>

pET SUMO TA Cloning® Reagents
The following reagents are included with the pET SUMO vector (Box 1). Note that the user must supply Taq polymerase. Store Box 1 at -20°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET SUMO vector, linearized</td>
<td>25 ng/μl in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0</td>
<td>5 x 10 μl</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM MgCl₂ 0.01% gelatin</td>
<td>100 μl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8.0</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

continued on next page
Kit Contents and Storage, continued

pET SUMO TA Cloning® Reagents, continued

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Ligation Buffer</td>
<td>60 mM Tris-HCl, pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine</td>
<td>100 μl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>4.0 Weiss units/μl</td>
<td>25 μl</td>
</tr>
<tr>
<td>Sterile Water</td>
<td></td>
<td>1 ml</td>
</tr>
<tr>
<td>SUMO Forward Sequencing Primer</td>
<td>0.1 μg/μl in TE Buffer, pH 8.0</td>
<td>10 μl</td>
</tr>
<tr>
<td>T7 Reverse Sequencing Primer</td>
<td>0.1 μg/μl in TE Buffer, pH 8.0</td>
<td>20 μl</td>
</tr>
<tr>
<td>Control PCR Primers</td>
<td>0.1 μg/μl each in TE Buffer, pH 8.0</td>
<td>10 μl</td>
</tr>
<tr>
<td>Control PCR Template</td>
<td>0.1 μg/μl in TE Buffer, pH 8.0</td>
<td>10 μl</td>
</tr>
<tr>
<td>pET SUMO/CAT</td>
<td>0.01 μg/μl in TE buffer, pH 8.0</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Unit Definition of T4 DNA Ligase

One (Weiss) unit of T4 DNA Ligase catalyzes the exchange of 1 nmol 32P-labeled pyrophosphate into [γ/β-32P]ATP in 20 minutes at 37°C (Weiss et al., 1968). One unit is equal to approximately 300 cohesive-end ligation units.

Sequences of the Primers

The Champion™ pET SUMO Protein Expression System provides a forward and reverse sequencing primer to facilitate sequence analysis of your expression constructs. The sequences of the forward and reverse primers are listed below. Two micrograms of each primer are supplied.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>pMoles Supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO Forward</td>
<td>5’-AGATTCTTTGTAAGTTAGGGATAG-3’</td>
<td>141</td>
</tr>
<tr>
<td>T7 Reverse</td>
<td>5’-TAGTTATTGCTACGGTTGAG-3’</td>
<td>325</td>
</tr>
</tbody>
</table>

continued on next page
The table below lists the items included in the One Shot® Mach1™-T1® Chemically Competent E. coli kit (Box 2). Transformation efficiency is ≥ 1 \times 10^9 \text{ cfu/μg DNA. Store Box 2 at -80°C.}

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.O.C. Medium</td>
<td>2% Tryptone</td>
<td>6 ml</td>
</tr>
<tr>
<td>(may be stored at room</td>
<td>0.5% Yeast Extract</td>
<td></td>
</tr>
<tr>
<td>temperature or +4°C)</td>
<td>10 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 mM KCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM MgSO₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 mM glucose</td>
<td></td>
</tr>
<tr>
<td>Mach1™-T1® cells</td>
<td>--</td>
<td>21 x 50 μl</td>
</tr>
<tr>
<td>pUC19 Control DNA</td>
<td>10 pg/μl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Genotype of Mach1™-T1®

Use this E. coli strain for general cloning purposes. **Do not use these cells for expression.**

Genotype: F⁻ Φ80lacZΔM15 ΔlacX74 hsdR(rK- mK⁺) DarcA1398 endA1 tonA

The table below describes the items included in the BL21(DE3) One Shot® Chemically Competent E. coli kit (Box 3). Transformation efficiency is ≥ 1 \times 10^8 \text{ cfu/μg DNA. Store Box 3 at -80°C.}

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.O.C. Medium</td>
<td>2% Tryptone</td>
<td>6 ml</td>
</tr>
<tr>
<td>(may be stored at room</td>
<td>0.5% Yeast Extract</td>
<td></td>
</tr>
<tr>
<td>temperature or +4°C)</td>
<td>10 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 mM KCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM MgSO₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 mM glucose</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3) Cells</td>
<td>--</td>
<td>21 x 50 μl</td>
</tr>
<tr>
<td>pUC19 Control DNA</td>
<td>10 pg/μl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

continued on next page
**Genotype of BL21(DE3)**

Use this *E. coli* strain for expression only. Do not use these cells to propagate or maintain your construct.

**Genotype:** F ompT hsdSb (rB mB) gal dcm (DE3)

The DE3 designation means this strain contains the lambda DE3 lysogen which carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase.

The strain is an *E. coli* B/r strain and does not contain the *lon* protease. It also has a mutation in the outer membrane protease, OmpT. The lack of these two key proteases reduces degradation of heterologous proteins expressed in the strain.

**SUMO Protease**

The following reagents are supplied with SUMO Protease (Box 4). **Store SUMO Protease at -20°C (after first-time use) or at -80°C for long term storage.** Avoid multiple freeze/thaw cycles at -80°C. Store 10X SUMO Protease Buffers at +4°C or -20°C.

<table>
<thead>
<tr>
<th>Item Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO Protease (1 U/μl)</td>
<td>SUMO Protease in: 25 mM Tris-HCl, pH 8.0 1% Igepal (NP-40) 250 mM NaCl 50 μM DTT 50% (v/v) glycerol</td>
</tr>
<tr>
<td>10X SUMO Protease Buffer + Salt</td>
<td>500 mM Tris-HCl, pH 8.0 2% Igepal (NP-40) 1.5 M NaCl 10 mM DTT</td>
</tr>
<tr>
<td>10X SUMO Protease Buffer – Salt</td>
<td>500 mM Tris-HCl, pH 8.0 2% Igepal (NP-40) 10 mM DTT</td>
</tr>
</tbody>
</table>

**Unit Definition of SUMO Protease**

One unit of SUMO Protease cleaves ≥85% of 2 μg control substrate in 1 h at 30°C.
Accessory Products

**Introduction**

The products listed in this section may be used with the Champion™ pET SUMO Protein Expression System. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 34).

**Additional Products**

Some of the reagents supplied in the Champion™ pET SUMO Protein Expression System as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO Protease</td>
<td>250 units</td>
<td>12588-018</td>
</tr>
<tr>
<td>One Shot® Mach1™-T1™ Chemically Competent E. coli</td>
<td>20 x 50 μl</td>
<td>C8620-03</td>
</tr>
<tr>
<td>One Shot® BL21(DE3) Chemically Competent E. coli</td>
<td>20 x 50 μl</td>
<td>C6000-03</td>
</tr>
<tr>
<td>One Shot® BL21(DE3)pLysS Chemically Competent E. coli</td>
<td>20 x 50 μl</td>
<td>C6060-03</td>
</tr>
<tr>
<td>Platinum® <em>Taq</em> DNA Polymerase</td>
<td>100 reactions</td>
<td>10966-018</td>
</tr>
<tr>
<td></td>
<td>250 reactions</td>
<td>10966-026</td>
</tr>
<tr>
<td></td>
<td>500 reactions</td>
<td>10966-034</td>
</tr>
<tr>
<td><em>Taq</em> DNA Polymerase, Recombinant</td>
<td>100 units</td>
<td>10342-053</td>
</tr>
<tr>
<td></td>
<td>250 units</td>
<td>10342-012</td>
</tr>
<tr>
<td></td>
<td>500 units</td>
<td>10342-020</td>
</tr>
<tr>
<td>Platinum® <em>Taq</em> DNA Polymerase High Fidelity</td>
<td>100 units</td>
<td>11304-011</td>
</tr>
<tr>
<td></td>
<td>500 units</td>
<td>11304-029</td>
</tr>
<tr>
<td>Kanamycin Sulfate</td>
<td>5 g</td>
<td>11815-024</td>
</tr>
<tr>
<td></td>
<td>25 g</td>
<td>11815-032</td>
</tr>
<tr>
<td>Isopropylthio-β-galactoside (IPTG)</td>
<td>1 g</td>
<td>15529-019</td>
</tr>
<tr>
<td>CAT Antiserum</td>
<td>50 μl</td>
<td>R902-25</td>
</tr>
</tbody>
</table>

**Detecting Recombinant Proteins**

You may detect your recombinant fusion protein using one of the Anti-HisG antibodies available from Invitrogen. The epitope for the Anti-HisG antibodies is an N-terminal polyhistidine (6xHis) tag followed by glycine (*i.e.* HHHHHHHG). The amount of antibody supplied is sufficient for 25 western blots.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HisG Antibody</td>
<td>50 μl</td>
<td>R940-25</td>
</tr>
<tr>
<td>Anti-HisG-HRP Antibody</td>
<td>50 μl</td>
<td>R941-25</td>
</tr>
<tr>
<td>Anti-HisG-AP Antibody</td>
<td>125 μl</td>
<td>R942-25</td>
</tr>
</tbody>
</table>

*continued on next page*
Accessory Products, continued

Once you have cloned your gene of interest in frame with the N-terminal peptide containing the polyhistidine (6xHis) tag and SUMO, you may use Invitrogen’s ProBond™ or Ni-NTA resins to purify your recombinant fusion protein. You may also use ProBond™ or Ni-NTA resins to remove the SUMO fusion protein and SUMO Protease from the cleavage reaction once you have generated native protein. Ordering information for these products is provided below.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProBond™ Nickel-Chelating Resin</td>
<td>50 ml</td>
<td>R801-01</td>
</tr>
<tr>
<td></td>
<td>150 ml</td>
<td>R801-15</td>
</tr>
<tr>
<td>ProBond™ Purification System</td>
<td>6 purifications</td>
<td>K850-01</td>
</tr>
<tr>
<td>Ni-NTA Agarose</td>
<td>10 ml</td>
<td>R901-01</td>
</tr>
<tr>
<td></td>
<td>25 ml</td>
<td>R901-15</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
<td>R901-10</td>
</tr>
<tr>
<td>Ni-NTA Purification System</td>
<td>6 purifications</td>
<td>K950-01</td>
</tr>
<tr>
<td>Polypropylene Columns (empty)</td>
<td>50</td>
<td>R640-50</td>
</tr>
</tbody>
</table>
Overview

Introduction

The Champion™ pET SUMO Protein Expression System utilizes a small ubiquitin-like modifier (SUMO) to allow expression, purification, and generation of native proteins in E. coli. SUMO fusions may increase the expression of recombinant proteins and enhance the solubility of partially insoluble proteins. In addition, the tertiary structure of the SUMO protein is specifically recognized and cleaved by a ubiquitin-like protein-processing enzyme, SUMO Protease. When SUMO is fused to the N-terminus of your protein, cleavage by SUMO Protease results in the production of native protein.

Advantages of the Champion™ pET SUMO System

Use of the Champion™ pET SUMO Protein Expression System offers the following advantages:

• May increase expression of recombinant fusion proteins
• May increase solubility of recombinant fusion proteins
• Allows generation of native protein using SUMO Protease
• Easy removal of the SUMO fusion protein and SUMO Protease after cleavage by affinity chromatography on a nickel-chelating resin

The Champion™ pET Expression System

The Champion™ pET Expression System is based on expression vectors originally developed by Studier and colleagues, and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in E. coli from the T7 promoter (Rosenberg et al., 1987; Studier and Moffatt, 1986; Studier et al., 1990). For more information about the Champion™ pET Expression System, see page 3.

SUMO Fusion Protein and SUMO Protease

In the Champion™ pET SUMO Protein Expression System, you will clone and express your gene of interest as a fusion to SUMO. SUMO is the Saccharomyces cerevisiae Smt3 protein which is an 11 kDa homolog of the mammalian SUMO-1 protein (Saitoh et al., 1997). Smt3, hereby referred to as SUMO, is a member of a ubiquitin-like protein family that regulates several cellular processes including apoptosis, nuclear transport, and cell cycle progression (Muller et al., 2001). Like ubiquitin, SUMO covalently attaches to lysine side chains on cellular target proteins; however, unlike ubiquitin modification, SUMO modification leads to changes in protein function and activity rather than protein degradation. Studies at Invitrogen have shown that fusion of a heterologous protein to SUMO can lead to increased expression levels as well as enhanced solubility of the recombinant protein. The tertiary structure of the SUMO protein is also recognized by a cysteine protease, SUMO Protease (Ulp), which specifically cleaves conjugated SUMO from target proteins (Li and Hochstrasser, 1999; Mossessova and Lima, 2000). For recombinant proteins expressed from pET SUMO, cleavage of SUMO by SUMO Protease results in production of native protein with no extra amino acids added between the cleavage site and the start of your protein.

continued on next page
Overview, continued

Features of the Champion™ pET SUMO Vector

The pET SUMO vector is designed to facilitate cloning of PCR products for regulated expression in *E. coli*. Features of the vector include:

- **T7lac** promoter for high-level, IPTG-inducible expression of the gene of interest in *E. coli* (Dubendorff and Studier, 1991; Studier *et al.*, 1990)
- N-terminal polyhistidine (6xHis) tag for detection and purification of recombinant fusion proteins
- N-terminal SUMO fusion protein for increased expression and solubility of recombinant fusion proteins and generation of native protein following cleavage by SUMO Protease (Li and Hochstrasser, 1999; Mossessova and Lima, 2000; Saitoh *et al.*, 1997)
- **TA Cloning®** site for efficient cloning of Taq-amplified PCR products (see below)
- Kanamycin resistance gene for selection in *E. coli*
- **lacI** gene encoding the lac repressor to reduce basal transcription from the T7lac promoter in the pET SUMO vector and from the lacUV5 promoter in the *E. coli* host chromosome (see page 3 for more information)
- pBR322 origin for low-copy replication and maintenance in *E. coli*

How TA Cloning® Works

The pET SUMO vector provides a quick, one-step cloning strategy for the direct insertion of a PCR product into the vector. *Taq* polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized pET SUMO vector supplied in this kit has single 3′ deoxythymidine (T) residues which allow PCR inserts to ligate efficiently into the vector (see diagram below).

One Shot® Mach1™-T1R *E. coli*

One Shot® Mach1™-T1R competent *E. coli* are included in the kit to provide a host for stable propagation and maintenance of your recombinant plasmid. The Mach1™-T1R *E. coli* strain is modified from the wild-type W strain (ATCC #9637, S. A. Waksman) and has the following features:

- **lacZΔM15** for blue/white color screening of recombinants
- **hsdR** mutation for efficient transformation of unmethylated DNA from PCR applications
- **ΔrecA1398** mutation for reduced occurrence of homologous recombination in cloned DNA
- **endA1** mutation for increased plasmid yield and quality
- **tonA** mutation to confer resistance to T1 and T5 phage
T7-Regulated Expression

The Basis of T7-Regulated Expression

The Champion™ pET SUMO Protein Expression System uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. In the pET SUMO vector, expression of the gene of interest is controlled by a strong bacteriophage T7 promoter that has been modified to contain a *lac* operator sequence (see below). In bacteriophage T7, the T7 promoter drives expression of gene 10 (φ10). T7 RNA polymerase specifically recognizes this promoter.

To express the gene of interest, it is necessary to deliver T7 RNA polymerase to the cells by inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the Champion™ pET SUMO System, T7 RNA polymerase is supplied by the BL21(DE3) host *E. coli* strain in a regulated manner (see below). When sufficient T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the gene of interest.

Regulating Expression of T7 RNA Polymerase

The BL21(DE3) *E. coli* strain is specifically included in the Champion™ pET SUMO Protein Expression Kit for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This λDE3 lysogen contains a *lac* construct consisting of the following elements:

- the *lacI* gene encoding the lac repressor
- the T7 RNA polymerase gene under control of the *lacUV5* promoter
- a small portion of the *lacZ* gene.

This *lac* construct is inserted into the *int* gene such that it inactivates the *int* gene. Disruption of the *int* gene prevents excision of the phage (i.e. lysis) in the absence of helper phage. The *lac* repressor (encoded by *lacI*) represses expression of T7 RNA polymerase. Addition of the gratuitous inducer, isopropyl β-D-thiogalactoside (IPTG) allows expression of T7 RNA polymerase from the *lacUV5* promoter.

T7/lac Promoter

Studies have shown that there is always some basal expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens even in the absence of inducer (Studier and Moffatt, 1986). In general, this is not a problem, but if the gene of interest is toxic to the *E. coli* host, basal expression of the gene of interest may lead to plasmid instability and/or cell death.

To address this problem, the pET SUMO vector has been designed to contain a T7lac promoter to drive expression of the gene of interest. The T7lac promoter consists of a *lac* operator sequence placed downstream of the T7 promoter. The *lac* operator serves as a binding site for the lac repressor (encoded by the *lacI* gene) and functions to further repress T7 RNA polymerase-induced basal transcription of the gene of interest in BL21(DE3) cells.

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T7-Regulated Expression, continued

**BL21(DE3)pLysS Strain**

If you discover that your gene is toxic to BL21(DE3) cells, you may want to perform your expression experiments in the BL21(DE3)pLysS strain (see page ix for ordering information). The BL21(DE3)pLysS strain contains the pLysS plasmid, which produces T7 lysozyme. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription. This activity results in reduced basal levels of T7 RNA polymerase, leading to reduced basal expression of T7-driven heterologous genes. For more information about BL21(DE3)pLysS, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 34).

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**Note**

Note that while BL21(DE3)pLysS reduces basal expression from the gene of interest when compared to BL21(DE3), it also generally reduces the overall induced level of expression of recombinant protein.

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**Using One Shot® Mach1™-T1R Cells**

One Shot® Mach1™-T1R competent E. coli, which do not contain T7 RNA polymerase, are included in the kit to provide a host for stable propagation and maintenance of your recombinant plasmid. As mentioned on the previous page, the presence of T7 RNA polymerase, even at basal levels, can lead to expression of the desired gene even in the absence of inducer. If the gene of interest is toxic to the E. coli host, plasmid instability and/or cell death may result. **We recommend that you transform your TA Cloning® reaction into Mach1™-T1R cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into BL21(DE3) E. coli.
Experimental Outline

Introduction

To clone your gene of interest into pET SUMO, you must first generate a PCR product. The PCR product is ligated into pET SUMO and transformed into One Shot® Mach1™-T1R™ competent cells. Since the PCR product can ligate into the vector in either orientation, individual recombinant plasmids need to be analyzed to confirm proper orientation.

Experimental Outline

The table below describes the major steps necessary to clone and express your gene of interest and to generate native protein.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amplify your PCR product using <em>Taq</em> polymerase and your own primers and parameters.</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Ligate your PCR product into pET SUMO.</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Transform your ligation into competent Mach1™-T1R E. coli.</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Select colonies and isolate plasmid DNA. Analyze plasmid DNA for the presence and orientation of the PCR product by restriction enzyme digestion or sequencing.</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>Select a positive transformant and isolate plasmid DNA. Transform BL21(DE3) and induce expression with IPTG</td>
<td>13-15</td>
</tr>
<tr>
<td>6</td>
<td>Purify your recombinant protein.</td>
<td>18-19</td>
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<tr>
<td>7</td>
<td>Cleave SUMO from recombinant protein using SUMO Protease. Remove SUMO and SUMO Protease from cleavage reaction using a nickel-chelating resin to obtain native recombinant protein.</td>
<td>20-21</td>
</tr>
</tbody>
</table>
Methods

Cloning Considerations

Introduction

The pET SUMO vector allows expression of a recombinant protein with an N-terminal peptide containing the 6xHis tag and SUMO fusion protein. General guidelines are provided below to help you design PCR primers to amplify your gene of interest for ligation in pET SUMO.

Cloning Considerations

Consider the following when designing your PCR primers:

• A ribosome binding site (RBS) is included upstream of the initiation ATG in the N-terminal tag to ensure optimal spacing for proper translation.

• To fuse the 6xHis tag and SUMO fusion protein to your protein of interest, design your forward primer to ensure that your protein is in frame with the N-terminal peptide.

• If you wish to generate native protein following SUMO Protease cleavage, design your forward primer such that the first 3 bases of the PCR product encode the ATG initiation codon.

• 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.

Refer to the diagram on the next page to help you design your PCR primers.

Important

If the first amino acid in your protein of interest is proline, lysine, valine, or leucine, SUMO Protease may not cleave the SUMO fusion protein. In this case, we recommend designing your forward PCR primer to introduce a serine at the start of your protein. We have found that proteins starting with a serine are cleaved by SUMO Protease with high efficiency. You may also introduce any other amino acid (except proline, lysine, valine, or leucine) to the start of your protein, however, cleavage efficiency may not be optimal.

Note: Any additional amino acids added to the N-terminus of your protein will remain following cleavage of the SUMO fusion protein.

continued on next page
Cloning Considerations, continued

**TA Cloning Site**

Use the diagram below to help you design appropriate PCR primers to ligate your PCR product into pET SUMO.

```
121 ATAGGCGCAA GCAACGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCAG ATCTCGATCC

T7 promoter

-use diagram

201 CGGCAAATTTA ATACGACTCA CTATAGGGGA ATTGGAGCCG GATAACATT CCCCTCTAGA AATAAATTGT TTTAACTTTA

lac operator

281 AGAAGGAGAT ATACAT ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC GCC AGC GCC CTG GTG CCG CGC GCC AGC

Met Gly Ser Ser His His His His His Gly Ser Gly Leu Val Pro Arg Gly Ser

Polyhistidine region

354 GCT AGC ATG TCG ACG TCA GAA GCT AAT CAA GAA GCT AAG CCA GAG GTC AAG CCA GAA GTC AAG CCT GAG

Ala Ser Met Ser Asp Ser Glu Val Asn Glu Ala Lys Pro Glu Val Lys Pro Glu Val Lys Pro Glu Thr

SUMO fusion protein

426 CAC ATC AAT TTA AAG GTG TCC GAT GGA TCT TCA GAG ATC TTC AAG ATC AAA AAG ACC ACT CCT TTA AGA

His Ile Asn Leu Lys Val Ser Asp Gly Ser Ser Glu Ile Phe Phe Lys Ile Lys Thr Thr Pro Leu Arg

SUMO forward priming site

498 AGG CTG ATG GAA GCG TCC GCT AAA AGA CAG GGT AAG GAA ATG GAC TCC TTA AGA TAC TGG TAC GAC GGT ATT

Arg Leu Met Glu Ala Phe Ala Lys Arg Glu Gly Lys Glu Met Ser Asp Ser Leu Arg Phe Leu Tyr Gly Ile

SUMO cleavage site

570 AGA ATT CAA GCT GAT CAG ACC CCT GAA GAT TGG GAC ATG GAG GAT AAC GAT ATT GAT GCT CAC AGA GAA

Arg Ile Glu Ala Asp Glu Thr Pro Glu Asp Asp Leu Asp Met Glu Asp Asp Ile Ile Glu Ala His Arg Glu

642 CAG ATT GGT GGT GAGAAG CTTAGGTAT TATTCGGCGCC AAAGTGCTGC GGGTGATGCT

Gln Ile Gly Gly

PCR product

SUMO cleavage site

701 GCCAACTTAG TCAGGACACCA CCACCACCAC CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGCGTGC

Gln Ile Gly Gly

T7 reverse priming site

781 TGCCACCCGCT GAGCAATAAC TAGCATACCC
```
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. **Remember that your PCR product will have single 3´ adenine overhangs.**

Materials Needed

You will need the following reagents and equipment before beginning. Note that dNTPs (adjusted to pH 8) are provided in the kit.

- Taq polymerase
- Thermocycler
- DNA template and primers for PCR product

Thermostable Polymerases and Polymerase Mixtures

Thermostable polymerases containing extensive 3´ to 5´ exonuclease activity do not leave 3´ A-overhangs. PCR products generated with Taq polymerase clone efficiently in the TA Cloning® System as the 3´ A-overhangs are not removed. 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. We recommend using Platinum® Taq DNA Polymerase High Fidelity (see page ix for ordering information).

If you use a proofreading polymerase mixture that does not have enough Taq polymerase or a proofreading polymerase only, you can add 3´ A-overhangs using the method on page 28.

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) 1 μM each
   Sterile water add to a total volume of 49 μl
   Taq Polymerase (1 unit/μl) 1 μl
   Final 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, you may gel-purify your fragment before proceeding. Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis et al., 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Contact Technical Service (page 34) for more information.
Cloning into pET SUMO

Introduction
For optimal ligation efficiencies, we recommend using fresh (less than 1 day old) PCR products. The single 3’ A-overhangs on the PCR products will be degraded over time, reducing ligation efficiency. If this is the first time you are using this kit, perform the control reactions on pages 25-26 in parallel with your samples.

Amount of PCR Product to Use
A 1:1 molar ratio of vector:insert produces the best ligation efficiency. In general, 0.5 to 1.0 μl of a typical PCR sample with an average insert length (400-700 bp) will give the proper 1:1 molar ratio of vector:insert. You may also perform a second ligation reaction using a 1:3 molar ratio of vector:insert if you are concerned about the accuracy of your DNA concentrations.

Do not use more than 2-3 μl of the PCR sample in the ligation reaction as salts in the PCR sample may inhibit the T4 DNA Ligase.

Materials Needed
You will need the following reagents and equipment before beginning.

- PCR product from Step 2, previous page
- 10X Ligation Buffer (included with the kit)
- pET SUMO vector (included with the kit)
- Sterile water (included with the kit)
- T4 DNA Ligase (included with the kit)

Ligation Reaction
1. Determine the volume of PCR sample needed to reach the required amount of PCR product (see above). Use sterile water to dilute your PCR sample if necessary.

2. Set up the 10 μl ligation reaction as follows:
   
   Fresh PCR product \( X \) μl
   10X Ligation Buffer \( 1 \) μl
   pET SUMO vector (25 ng/μl) \( 2 \) μl
   Sterile water to a total volume of 9 μl
   T4 DNA Ligase (4.0 Weiss units) \( 1 \) μl
   Final volume \( 10 \) μl

3. Incubate the ligation reaction at 15°C for a minimum of 4 hours (preferably overnight). You may also incubate your ligation reaction at room temperature for 30 minutes, if desired. Proceed to Transforming One Shot® Mach1™-T1R Competent Cells, next page.

   Note: You may store your ligation reaction at -20°C until you are ready for transformation.
Transforming One Shot® Mach1™-T1R Competent Cells

Introduction

Once you have ligated your insert into pET SUMO, you will transform your construct into competent E. coli. One Shot® Mach1™-T1R chemically competent E. coli are included with the kit to facilitate transformation. A protocol to transform the competent cells is provided in this section.

We recommend using the One Shot® Mach1™-T1R chemically competent E. coli supplied in the kit for your transformation reactions. Using other E. coli strains may result in higher background levels.

Materials Needed

You will need the following reagents and equipment before beginning.

- Ligation reaction from Step 3, previous page
- One Shot® Mach1™-T1R chemically competent E. coli (Box 2, included with the kit; one vial per transformation)
- S.O.C. medium (Box 2, included with the kit)
- LB plates containing 50 μg/ml kanamycin
- 42°C water bath
- 37°C shaking and non-shaking incubator

Preparing for Transformation

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

- Equilibrate a water bath to 42°C.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 50 μg/ml kanamycin at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

One Shot® Chemical Transformation Protocol

1. Pipette 2 μl of the ligation reaction directly 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.
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 S.O.C. medium.
6. Cap the tube tightly and shake horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 100-200 μl from each transformation on a prewarmed selective plate. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C.
Analyzing Transformants

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in LB or S.O.B. medium containing 50 μg/ml kanamycin.

2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
   **Note:** Since the pET SUMO vector is a low-copy number plasmid, you may need to increase the amount of bacterial culture to obtain enough plasmid DNA for sequencing or analysis purposes.

3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert.

Sequencing

You may sequence your construct to confirm that your gene is in the correct orientation and in frame with the N-terminal tag, if desired. The SUMO Forward and T7 Reverse sequencing primers are included with the kit to help you sequence your insert. Refer to the diagram on page 7 for the primer sequences and the location of the primer binding sites.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use the SUMO Forward primer or the T7 Reverse primer and a primer that binds within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols may be suitable.

**Materials Needed**

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 μM each)

**Procedure**

1. For each sample, aliquot 48 μl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 μl each of the forward and reverse PCR primer.

2. Pick 10 colonies and resuspend them individually in 50 μl of the PCR cocktail from Step 1, above. Don’t forget to make a patch plate to preserve the colonies for further analysis.

3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.

4. Amplify for 20 to 30 cycles.

5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.

6. Visualize by agarose gel electrophoresis.

*continued on next page*
Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 25-26. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50 μg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 μg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.
Expressing the PCR Product

Introduction

BL21(DE3) One Shot® *E. coli* (Box 3) are included with the Champion™ pET SUMO Protein Expression Kit for use as the host for expression. You will need pure plasmid DNA of your pET SUMO construct to transform into BL21(DE3) for expression studies. Since each recombinant protein has different characteristics that may affect optimal expression, we recommend performing a time course of expression to determine the best conditions for expression of your protein. We recommend including the pET SUMO/CAT positive expression control supplied with the kit in your experiments to help you evaluate your results.

BL21(DE3) Strain

The BL21(DE3) *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter. Each time you perform an expression experiment, you will transform your plasmid into BL21(DE3). **Do not use this strain for propagation and maintenance of your plasmid. Use Mach1™-T1R cells instead.** Basal level expression of T7 polymerase, particularly in BL21(DE3) cells, may lead to plasmid instability if your gene of interest is toxic to *E. coli*.

**Note:** If you are expressing a highly toxic gene, the BL21(DE3)pLysS strain is available from Invitrogen for expression purposes. The BL21(DE3)pLysS strain contains the pLysS plasmid to further reduce basal level expression of the gene of interest.

Important

We do not recommend using BL21 Star™(DE3) or BL21 Star™(DE3)pLysS *E. coli* strains available from Invitrogen for expression of your pET SUMO construct. These strains may reduce the solubility of your recombinant SUMO protein.

Positive Control

pET SUMO/CAT is provided as a positive control vector for expression. This vector allows expression of an N-terminally tagged chloramphenicol acetyl transferase (CAT) fusion protein that may be detected by western blot or functional assay. To propagate and maintain the plasmid, transform 10 ng of pET SUMO into One Shot® Mach1™-T1R cells using the procedure on page 10.

Basic Strategy

The basic steps needed to induce expression of your gene in BL21(DE3) *E. coli* are outlined below.

1. Isolate plasmid DNA using standard procedures and transform your construct and the positive control separately into BL21(DE3) One Shot® cells.
2. Grow the transformants and induce expression with IPTG over several hours. Take several time points to determine the optimal time of expression.
3. Optimize expression to maximize the yield of protein.

Plasmid Preparation

You may prepare plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) for isolation of pure plasmid DNA. Note that since you are purifying a low-copy number plasmid, you may need to increase the amount of bacterial culture that you use to prepare your plasmid construct.

*continued on next page*
Expressing the PCR Product, continued

Cyclic AMP-mediated derepression of the lacUV5 promoter in λDE3 lysogens can result in an increase in basal expression of T7 RNA polymerase. We recommend adding 1% glucose to the bacterial culture medium to repress basal expression of T7 RNA polymerase for the following conditions:

- To increase the solubility of your protein.
- To stabilize your pET SUMO construct if you are expressing a toxic gene.

### Materials Needed
You will need the following reagents and equipment before beginning.

- Your pET SUMO expression construct (>10 μg/ml)
- pET SUMO/CAT positive control plasmid, optional
- BL21(DE3) One Shot® cells (Box 3, included with the kit)
- S.O.B. or LB containing 50 μg/ml kanamycin (plus 1% glucose, if desired)
- 37°C incubator (shaking and nonshaking)
- 42°C water bath
- 1 M isopropyl β-D-thiogalactoside (IPTG; Invitrogen, Catalog no. 15529-019)
- Liquid nitrogen

### Transforming BL21(DE3) One Shot® Cells
Use the protocol below to transform your construct or the positive control into BL21(DE3) One Shot® cells. You will need one vial of cells per transformation.

**Note**: You will not plate the transformation reaction, but inoculate it into medium for growth and subsequent expression.

1. Thaw on ice, one vial of BL21(DE3) One Shot® cells per transformation.
2. Add 5-10 ng plasmid DNA in a 1 to 5 μl volume into each vial of BL21(DE3) One Shot® cells and mix by stirring gently with the pipette tip. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 μl of room temperature S.O.C. medium.
7. Cap the tube tightly, tape the tube on its side (for better aeration), and incubate at 37°C for 1 hour with shaking (200 rpm).
8. Add the **entire** transformation reaction to 10 ml of LB containing 50 μg/ml kanamycin (and 1% glucose, if desired).

continued on next page
Expressing the PCR Product, continued

Pilot Expression

1. Inoculate 10 ml of LB containing 50 μg/ml kanamycin (and 1% glucose, if desired) with 500 μl of the overnight culture from Step 8, previous page.
2. Grow two hours at 37°C with shaking. OD$_{600}$ should be approximately 0.4-0.6 (mid-log).
3. Split the culture into two 5 ml cultures. Add IPTG to a final concentration of 1 mM to one of the cultures. You will now have two cultures: one induced, one uninduced.
4. Remove a 500 μl aliquot from each culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
5. Freeze the cell pellets at -20°C. These are the zero time point samples.
6. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
7. For each time point, remove 500 μl from the induced and uninduced cultures and process as described in Steps 4 and 5. Proceed to Analyzing Samples, next page.
Analyzing Samples

Materials Needed
You will need the following reagents and equipment before beginning.

- Lysis Buffer (see page 33 for recipe)
- 1X and 2X SDS-PAGE sample buffer (see page 33 for recipes)
- Reagents and apparatus to perform SDS-PAGE electrophoresis
- Boiling water bath

Preparing Samples
Once you have finished your pilot expression, you are ready to analyze the samples you have collected. Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see below).

Note: If you wish to analyze your samples for soluble protein, see below.

1. Thaw the samples (from Pilot Expression, Steps 5 and 7, previous page) and resuspend each cell pellet in 80 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 cell pellet in 500 μl of Lysis Buffer (see Recipes, page 33).
3. 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 need to add lysozyme 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. 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 our Web site (www.invitrogen.com) or contact Technical Service (page 34).

continued on next page
Analyzing Samples, continued

Analyzing Samples

To determine the success of your expression experiment, you may want to perform the following types of analyses:

1. Stain the polyacrylamide gel with Coomassie blue and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control.
2. Perform a western blot to confirm that the overexpressed band is your desired protein (see below).
3. Use the pET SUMO/CAT positive control to confirm that growth and induction were performed properly (see below).

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-HisG Antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein of interest.

In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a HisG epitope. The WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescence methods. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 34).

Assaying for CAT Protein

If you use pET SUMO/CAT as a positive control vector, you may assay for CAT expression using your method of choice. CAT is fused to the N-terminal 6xHis tag, allowing you to detect CAT expression using western blot analysis and an Anti-HisG antibody. CAT Antiserum is also available separately from Invitrogen (see page ix for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 39 kDa.

Note

Expression of your protein with the N-terminal peptide containing the 6xHis tag and SUMO fusion protein will increase the size of your recombinant protein by approximately 13 kDa.

The Next Step

If you are satisfied with expression of your gene of interest, proceed to Purifying the Recombinant Fusion Protein, next page. If you have trouble expressing your protein or wish to optimize expression, refer to the Troubleshooting section, page 22.
Purifying the Recombinant Fusion Protein

Introduction

The presence of the N-terminal polyhistidine (6xHis) tag in pET SUMO allows purification of your recombinant fusion protein with a metal-chelating resin such as ProBond™ or Ni-NTA. Purify your recombinant protein under native conditions.

ProBond™ and Ni-NTA

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of fusion proteins containing the 6xHis 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 the next page.
- To purify your fusion protein using ProBond™ or Ni-NTA, refer to the manual included with each product. You may download the manuals from our Web site (www.invitrogen.com).
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer’s instructions.

Important

SUMO Protease is a highly active cysteine protease. If you purify your recombinant protein in the presence of protease inhibitors, do not use cysteine protease inhibitors (e.g. leupeptin) as they will inhibit the cleavage reactions.

Performing Dialysis

For optimal results, we recommend that the SUMO Protease cleavage reaction be carried out in a buffer containing <300 mM NaCl and <150 mM imidazole (see guidelines on page 20). SUMO Protease is inhibited when the salt and imidazole concentrations exceed these amounts.

If you perform the SUMO Protease cleavage reaction using purified protein directly eluted from the purification column, the salt and imidazole concentrations are likely to exceed the recommended concentrations listed above. We recommend performing dialysis to decrease the salt and imidazole concentrations of your purified protein reaction. Perform the dialysis overnight at +4°C using a suitable dialysis buffer (e.g. 20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM DTT).

Note: The SUMO Protease will contribute a small amount of salt to the final cleavage reaction (see guidelines on page 20).

continued on next page
Purifying the Recombinant Fusion Protein, continued

**Scaling-up Expression for Purification**

We generally scale-up expression to a 50 ml bacterial culture for purification using a 2 ml ProBond™ or Ni-NTA column. 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 to your column.

To grow and induce a 50 ml bacterial culture:

1. Inoculate 10 ml of S.O.B. or LB containing 50 μg/ml kanamycin with a BL21(DE3) transformation reaction.
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD₆₀₀ = 1-2.
3. The next day, inoculate 50 ml of S.O.B. or LB containing 50 μg/ml kanamycin with 1 ml of the overnight culture.  
   **Note:** You can scale up further and inoculate all of the 10 ml overnight culture into 500 ml of medium, but you will need to adjust the bed volume of your ProBond™ or Ni-NTA column accordingly.
4. Grow the culture at 37°C with shaking (225-250 rpm) to an OD₆₀₀ ≈ 0.5 (2-3 hours). The cells should be in mid-log phase.
5. Add IPTG to a final concentration of 0.5-1 mM to induce expression.
6. Grow at 37°C with shaking until the optimal time point determined by the pilot expression is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. Proceed to purification or store the cells at -80°C for future use.

**Additional Purification Steps**

There may be cases when your specific fusion protein may not be completely purified by metal affinity chromatography. Other protein purification techniques may be utilized in conjunction with ProBond™ or Ni-NTA to purify the fusion protein (see Deutscher, 1990 for more information).
Using SUMO Protease

Introduction

Once you have purified your recombinant fusion protein, you may generate native protein by using SUMO Protease to cleave the N-terminal peptide containing the 6xHis tag and SUMO. General guidelines to use SUMO Protease are provided below.

Important

We have found that SUMO Protease may not cleave the SUMO protein when the amino acid immediately following SUMO (i.e. the first amino acid of your protein) is a proline, lysine, valine, or leucine. If your protein starts with one of these four amino acids, we recommend that you add an additional amino acid (preferably serine) to the N-terminus of your protein (see Important note on page 6).

Note: Any additional amino acids added to the N-terminus of your protein will remain following cleavage of the SUMO fusion protein.

General Guidelines

Follow the guidelines below when using SUMO Protease.

• For optimal results, perform the cleavage reaction using partially or fully purified recombinant fusion protein.

• For most fusion proteins, SUMO Protease functions optimally in a reaction mixture containing approximately 150 mM NaCl; however, conditions may be optimized for your particular protein by varying the NaCl concentration from 100 mM to 300 mM. Remember to take into account the contribution of salt from the enzyme (i.e. 12.5 mM in final buffer) and from your substrate. When setting up your cleavage reaction, use the appropriate 10X SUMO Protease Buffer +/- Salt.

• Keep the imidazole concentration less than 150 mM. Concentrations higher than 150 mM can adversely affect the activity of the protease.

Recommended Conditions for Cleavage

An example of a time course experiment with 10 units of SUMO Protease is provided. If the protein of interest is heat-labile, incubate at +4°C with longer incubation times and/or more enzyme (see table on next page).

1. Add the following to a microcentrifuge tube:

   Fusion Protein  20 µg
   10X SUMO Protease Buffer +/- Salt  20 µl
   Water  to a total volume of 190 µl
   SUMO Protease (10 units)  10 µl
   Final volume  200 µl

2. Mix and incubate at 30°C. Remove 20 µl aliquots at 1, 2, 4, and 6 hours.

3. Add 20 µl 2X SDS sample buffer (see page 33 for a recipe). Keep samples at -20°C until experiment is complete.

4. Analyze 30 µl of sample by SDS-PAGE using a suitable gel.

continued on next page
Using SUMO Protease, continued

Analyzing Results

Determine the percent protein cleavage by analyzing the amount of cleaved products formed and amount of uncleaved protein remaining after digestion. After evaluating the initial results, you may optimize the cleavage reaction for your specific protein by optimizing the amount of SUMO Protease, incubation temperature, or reaction time.

Varying Parameters for Cleavage

The percent of 2 μg control substrate hydrolyzed by one unit of SUMO Protease at various temperatures was examined (see table below). More cleaved protein is formed with SUMO Protease by increasing the incubation time. If time is critical, add more SUMO Protease to increase hydrolysis.

<table>
<thead>
<tr>
<th>Time</th>
<th>4°C</th>
<th>16°C</th>
<th>21°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td>48</td>
<td>73</td>
<td>83</td>
<td>88</td>
</tr>
<tr>
<td>1 h</td>
<td>60</td>
<td>87</td>
<td>90</td>
<td>93</td>
</tr>
<tr>
<td>2 h</td>
<td>71</td>
<td>94</td>
<td>94</td>
<td>95</td>
</tr>
<tr>
<td>3 h</td>
<td>74</td>
<td>95</td>
<td>95</td>
<td>95</td>
</tr>
</tbody>
</table>

Removing SUMO and SUMO Protease

Both the SUMO fusion protein and the SUMO Protease contain N-terminal polyhistidine tags allowing their removal from the cleavage reaction using affinity chromatography on a nickel-chelating resin such as ProBond™ Resin (Cat. no. K801-01). Dilute the cleavage reaction in the binding buffer for ProBond™ and perform binding and elution as described in the ProBond™ Purification manual available at www.invitrogen.com.

Note that SUMO and SUMO Protease will remain bound to the resin and the cleaved native protein will be in the flow-through fractions.
# Troubleshooting

## TA Cloning® Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TA Cloning® and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 25-26) in parallel with your samples.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few or no colonies obtained from sample reaction and the transformation control gave colonies</td>
<td>Suboptimal ratio of vector:insert used in ligation reaction</td>
<td>Estimate the concentration of the PCR product. Use a 1:1 or 1:3 molar ratio of vector:insert.</td>
</tr>
<tr>
<td>PCR products stored too long</td>
<td>Use fresh PCR products. Ligation efficiency is reduced after as little as 1 day of storage.</td>
<td></td>
</tr>
<tr>
<td>Too much salt in the ligation reaction</td>
<td>The high salt content of PCR reactions can inhibit ligation. Do not use more than 2-3 μl of the PCR sample in the ligation reaction.</td>
<td></td>
</tr>
<tr>
<td>Used a proofreading polymerase</td>
<td>Do not use proofreading polymerases as they do not add 3’ A-overhangs. Use Taq polymerase.</td>
<td></td>
</tr>
<tr>
<td>Incomplete extension during PCR</td>
<td>Include a final extension step of 7 to 30 minutes. Longer PCR products will need a longer extension time.</td>
<td></td>
</tr>
<tr>
<td>PCR reaction contains artifacts (i.e. does not run as a single, discrete band on an agarose gel)</td>
<td>• Optimize your PCR using Taq polymerase.</td>
<td>• Gel-purify your PCR product using nuclease-free reagents.</td>
</tr>
<tr>
<td>Few or no colonies obtained from sample reaction and the transformation control gave no colonies</td>
<td>One Shot® competent cells stored incorrectly</td>
<td>Store One Shot® competent cells at -80°C.</td>
</tr>
<tr>
<td>One Shot® transformation protocol not followed correctly</td>
<td>Follow the One Shot® transformation protocol provided on page 10.</td>
<td></td>
</tr>
<tr>
<td>Insufficient amount of E. coli plated</td>
<td>Increase the amount of E. coli plated.</td>
<td></td>
</tr>
<tr>
<td>Transformants plated on selective plates containing the wrong antibiotic</td>
<td>• Use LB plates containing 50 μg/ml kanamycin to select for pET SUMO transformants.</td>
<td>• Use LB plates containing 100 μg/ml ampicillin to select for the pUC19 transformation control.</td>
</tr>
<tr>
<td>Large number of background colonies</td>
<td>Used an E. coli strain other than Mach1™-T1® for transformation</td>
<td>For lowest background levels, use the One Shot® Mach1™-T1® cells included with the kit.</td>
</tr>
<tr>
<td>Single 3’ T-overhangs on the vector degraded</td>
<td>Avoid storing the vector for longer than 6 months or subjecting it to repeated freeze/thaw cycles.</td>
<td></td>
</tr>
</tbody>
</table>
## Troubleshooting, continued

### Expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your expression experiment. To help evaluate your results, we recommend including the expression control supplied with kit in your experiment.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No expression of recombinant protein</td>
<td>Gene of interest not in frame with the N-terminal tag</td>
<td>Sequence your construct to verify if the insert is in frame with the N-terminal tag. If not in frame, redesign your PCR primers.</td>
</tr>
<tr>
<td>Incorrect antibody used for detection</td>
<td></td>
<td>Use the Anti-HisG Antibodies or an antibody to your protein</td>
</tr>
</tbody>
</table>
| Low expression of recombinant protein  | Toxic gene | • Add 1% glucose to the bacterial culture medium during transformation and expression.  
|                                         | Note: Evidence of toxicity includes loss of plasmid or slow growth relative to the control. | • Transform BL21(DE3) cells using the protocol on page 14, then perform the expression by growing cells at room temperature rather than 37°C for 24-48 hours.  
|                                         |                       | • Transform your expression construct into a pLysS-containing strain (e.g. BL21(DE3)pLysS).  
|                                         |                       | • Transform your expression construct into an *E. coli* strain in which expression of T7 RNA polymerase is tightly regulated (e.g. BL21-AI™ available from Invitrogen; see our Web site for more information). |
| Recombinant protein is insoluble       | Used BL21 Star™ strain | BL21 Star™ strains may reduce the solubility of your pET SUMO protein. Use the BL21(DE3) strain included with the kit. |
| Protein is unstable                    |                       | • Add 1% glucose to the bacterial culture medium during expression.  
|                                         |                       | • Transform your expression construct into a pLysS-containing strain (e.g. BL21(DE3)pLysS). |

*continued on next page*
**SUMO Cleavage**

The table below lists some potential problems and possible solutions that may help you troubleshoot your SUMO Protease cleavage reaction. To help evaluate your results, we recommend including the expression control supplied with kit in your experiment.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large percentage of uncleaved SUMO fusion protein</td>
<td>Protein starts with a proline, lysine, valine, or leucine</td>
<td>SUMO Protease may not cleave SUMO when your protein starts with one of these amino acids. Add a serine to the N-terminus of your protein to allow cleavage of SUMO (see page 6 for more information).</td>
</tr>
<tr>
<td>Salt concentration in cleavage reaction too high</td>
<td>The optimal salt concentration for the cleavage reaction is approximately 150 mM NaCl. Dialyze eluted fractions of your purified protein to reduce the salt concentration before performing the cleavage reaction.</td>
<td></td>
</tr>
<tr>
<td>Imidazole concentration in cleavage reaction too high</td>
<td>The cleavage reaction should contain a final concentration of less than 150 mM imidazole. Dialyze eluted fractions of your purified protein to reduce the imidazole concentration before performing the cleavage reaction.</td>
<td></td>
</tr>
<tr>
<td>Cysteine protease inhibitor used during purification steps</td>
<td>SUMO Protease is a cysteine protease. Do not add cysteine protease inhibitors to any reactions.</td>
<td></td>
</tr>
<tr>
<td>DTT in SUMO Protease Buffer oxidized</td>
<td>Add freshly prepared DTT to the cleavage reaction to a final concentration of 1 mM.</td>
<td></td>
</tr>
<tr>
<td>Recombinant protein denatured</td>
<td>Purify your recombinant protein under native conditions. SUMO Protease may not recognize denatured SUMO fusion protein.</td>
<td></td>
</tr>
<tr>
<td>No native protein detected after removal of SUMO Protease</td>
<td>Native protein located in flow-through and not eluted fractions</td>
<td>Be sure to check the flow-through for your native protein. Eluted fractions will only contain the SUMO and SUMO Protease.</td>
</tr>
</tbody>
</table>
Appendix

Performing the Control Reactions

Introduction

We recommend performing the following TA Cloning® reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TA Cloning® reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 μg/ml kanamycin.

Producing the Control PCR Product

Use Taq polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer’s recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 μl PCR:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DNA Template (100 ng)</td>
<td>1 μl</td>
</tr>
<tr>
<td>10X PCR Buffer (appropriate for enzyme)</td>
<td>5 μl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Control PCR Primers (0.1 μg/μl each)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>41.5 μl</td>
</tr>
<tr>
<td>Taq polymerase (1 units/μl)</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

Final volume 50 μl

2. Overlay with 70 μl (1 drop) of mineral oil, if required.

3. Amplify using the following cycling parameters:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>2 minutes</td>
<td>94°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 minute</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>1 minute</td>
<td>55°C</td>
<td>25X</td>
</tr>
<tr>
<td>Extension</td>
<td>1 minute</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>7 minutes</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

4. Remove 10 μl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the Control Ligation Reaction, next page.

---

continued on next page
Performing the Control Reactions, continued

<table>
<thead>
<tr>
<th>Control Ligation Reaction</th>
<th>Using the control PCR product produced on the previous page and the pET SUMO vector, set up the following ligation reaction.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. Determine the volume of PCR sample needed to achieve a 1:1 molar ratio of vector:insert. Use sterile water to dilute your PCR sample if necessary.</td>
</tr>
<tr>
<td></td>
<td>2. Set up the 10 ( \mu l ) ligation reaction as follows:</td>
</tr>
<tr>
<td></td>
<td>- Fresh PCR product ( X \mu l )</td>
</tr>
<tr>
<td></td>
<td>- 10X Ligation Buffer ( 1 \mu l )</td>
</tr>
<tr>
<td></td>
<td>- pET SUMO vector (25 ng/( \mu l )) ( 2 \mu l )</td>
</tr>
<tr>
<td></td>
<td>- Sterile water to a total volume of ( 9 \mu l )</td>
</tr>
<tr>
<td></td>
<td>- T4 DNA Ligase (4.0 Weiss units) ( 1 \mu l )</td>
</tr>
<tr>
<td></td>
<td>- Final volume ( 10 \mu l )</td>
</tr>
<tr>
<td></td>
<td>3. Incubate the ligation reaction at 15°C for 4 hours (preferably overnight). You may also incubate your ligation reaction at room temperature for 30 minutes, if desired.</td>
</tr>
<tr>
<td></td>
<td>4. Transform 2 ( \mu l ) of the ligation reaction into one vial of One Shot\textsuperscript{®} Mach1\textsuperscript{™}-T1\textsuperscript{R} competent cells using the protocol on page 10.</td>
</tr>
</tbody>
</table>

| Analysis of Results | Pick 10 colonies and isolate plasmid DNA. Analyze the plasmids for the presence of insert by digesting the DNA with \textit{Bsa I} to release the 750 kb insert. Greater than 80% of the colonies should contain plasmid with the 750 bp insert. |

| Transformation Control | pUC19 plasmid is included to check the transformation efficiency of the One Shot\textsuperscript{®} Mach1\textsuperscript{™}-T1\textsuperscript{R} competent cells. Transform one vial of One Shot\textsuperscript{®} Mach1\textsuperscript{™}-T1\textsuperscript{R} cells with 10 pg of pUC19 using the protocol on page 10. Plate 10 \( \mu l \) of the transformation mixture plus 20 \( \mu l \) of S.O.C. on LB plates containing 100 \( \mu g/ml \) ampicillin. Transformation efficiency should be \( \geq 1 \times 10^9 \) cfu/\( \mu g \) DNA. |
Gel Purifying 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.

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.  
   **Note:** Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.

2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.

3. Add 1.5 volumes Binding Buffer.

4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.

5. If you have solution remaining from Step 3, repeat Step 4.

6. Add 900 µl of the Final Wash Buffer.

7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.

8. Repeat Step 7.

9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 2-3 µl for the ligation reaction and proceed as described on page 9.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the ligation reaction (page 9). Be sure to make the gel slice as small as possible for best results.

Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.
Addition of 3´ A-Overhangs Post-Amplification

Introduction
Direct cloning of DNA amplified by proofreading polymerases into pET SUMO is often difficult because proofreading polymerases remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting
You will need the following items:
- 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 may be suitable.

1. After amplification with a proofreading 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. A sufficient number of PCR products will retain the 3´ A-overhangs.

2. Incubate at 72°C for 8-10 minutes (do not cycle).

3. Place on ice and use immediately in the ligation reaction.

Note: If you plan to store your sample overnight before proceeding with the ligation reaction, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add Taq polymerase buffer, dATP, and 0.5 unit of Taq polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the ligation reaction.
Map and Features of pET SUMO

Map

The figure below shows the features of the pET SUMO vector. The vector is supplied linearized between nucleotides 653 and 654 with a one base pair 5' T-overhang on each strand as indicated. The complete sequence of pET SUMO is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 34).

Comments for pET SUMO
5643 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
T7 terminator: bases 744-872
Kanamycin resistance gene: bases 1431-2246 (C)
pBR322 origin: bases 2342-3015
ROP ORF: bases 3383-3574
lacI ORF: bases 4383-5474 (C)

(C) = complementary strand

continued on next page
## Map and Features of pET SUMO, continued

The pET SUMO vector (5643 bp) contains the following elements. All features have been functionally tested.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 promoter</td>
<td>Allows high-level, IPTG-inducible expression of your recombinant protein in <em>E. coli</em> strains expressing the T7 RNA polymerase.</td>
</tr>
<tr>
<td><em>lac</em> operator (lacO)</td>
<td>Binding site for <em>lac</em> repressor that serves to reduce basal expression of your recombinant protein.</td>
</tr>
<tr>
<td>Ribosome binding site</td>
<td>Optimally spaced from the TA Cloning® site for efficient translation of PCR product.</td>
</tr>
<tr>
<td>N-terminal 6xHis tag</td>
<td>Allows purification of recombinant fusion protein on metal-chelating resin (<em>i.e.</em> ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.</td>
</tr>
<tr>
<td>SUMO ORF</td>
<td>Enhances recombinant protein expression and solubility and allows cleavage by SUMO Protease to produce native protein.</td>
</tr>
<tr>
<td>SUMO Forward priming site</td>
<td>Allows sequencing of the insert.</td>
</tr>
<tr>
<td>TA Cloning® site (5' T-overhangs)</td>
<td>Allows ligase-mediated cloning of <em>Taq</em>-amplified PCR products.</td>
</tr>
<tr>
<td>T7 Reverse priming site</td>
<td>Allows sequencing of the insert.</td>
</tr>
<tr>
<td>T7 transcription termination region</td>
<td>Sequence from bacteriophage T7 which allows efficient transcription termination.</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Allows selection of the plasmid in <em>E. coli</em>.</td>
</tr>
<tr>
<td>pBR322 origin of replication (ori)</td>
<td>Allows replication and maintenance in <em>E. coli</em>.</td>
</tr>
<tr>
<td><em>ROP</em> ORF</td>
<td>Interacts with the pBR322 origin to facilitate low-copy replication in <em>E. coli</em>.</td>
</tr>
<tr>
<td><em>lacI</em> ORF</td>
<td>Encodes <em>lac</em> repressor that binds to the T7lac promoter to block basal transcription of the gene of interest and to the <em>lacUV5</em> promoter in the host chromosome to repress transcription of T7 RNA polymerase.</td>
</tr>
</tbody>
</table>
pET SUMO/CAT is a control vector expressing a SUMO-CAT fusion protein, and was generated by cloning the a Taq-amplified PCR fragment encoding the CAT gene into pET SUMO. The size of the SUMO-CAT fusion protein is approximately 39 kDa. The nucleotide sequence of the pET SUMO/CAT vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (page 34).

Map of pET SUMO/CAT

Description

pET SUMO/CAT is a control vector expressing a SUMO-CAT fusion protein, and was generated by cloning the a Taq-amplified PCR fragment encoding the CAT gene into pET SUMO. The size of the SUMO-CAT fusion protein is approximately 39 kDa. The nucleotide sequence of the pET SUMO/CAT vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Service (page 34).

Comments for pET SUMO/CAT

6307 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
CAT gene: bases 654-1313
T7 reverse priming site: bases 1443-1462 (C)
T7 terminator: bases 1404-1532
Kanamycin resistance gene: bases 2092-2907 (C)
pBR322 origin: bases 3003-3676
ROP ORF: bases 4047-4238
lacI ORF: bases 5047-6138 (C)

(C) = complementary strand
Recipes

**LB (Luria-Bertani) Medium and 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 bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic if needed.
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.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

---

**S.O.B. Medium (with Antibiotic)**

2% Tryptone  
0.5% Yeast Extract  
0.05% NaCl  
2.5 mM KCl  
10 mM MgCl₂

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 MgCl₂. You may also add antibiotic, if needed.
5. Store at +4°C. *Medium is stable for only 1-2 weeks.*

*continued on next page*
Recipes, continued

**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 KH₂PO₄ and K₂HPO₄.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:
   - 0.3 ml KH₂PO₄
   - 4.7 ml K₂HPO₄
   - 2.3 g NaCl
   - 0.75 g KCl
   - 10 ml glycerol
   - 0.5 ml Triton X-100
   - 68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

**2X SDS-PAGE Sample Buffer**

1. Combine the following reagents:
   - 0.5 M Tris-HCl, pH 6.8 2.5 ml
   - Glycerol (100%) 2.0 ml
   - β-mercaptoethanol 0.4 ml
   - Bromophenol Blue 0.02 g
   - SDS 0.4 g
2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

**1X SDS-PAGE Sample Buffer**

1. Combine the following reagents:
   - 0.5 M Tris-HCl, pH 6.8 1.25 ml
   - Glycerol (100%) 1.0 ml
   - β-mercaptoethanol 0.2 ml
   - Bromophenol Blue 0.01 g
   - SDS 0.2 g
2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.
Technical Service

Web Resources

Visit the Invitrogen Web site at www.invitrogen.com for:

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

Contact Us

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

Corporate Headquarters:
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: japinfo@invitrogen.com

European Headquarters:
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

SDS

SDSs (Safety Data Sheets) are available on our web site at www.invitrogen.com/sds.

Certificate of Analysis

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

continued on next page
Technical Service, continued

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Purchaser Notification

Introduction

Use of the Champion™ pET SUMO Protein Expression System is covered under a number of different licenses including those detailed below.

Information for European Customers

The following E. coli strains are genetically modified as described below:

- Mach1™-T1R: Carries the lacZΔM15 hsdR lacX74 recA endA tonA genotype.
- BL21(DE3): Carries the bacteriophage λ DE3 lysogen containing the T7 RNA polymerase gene.

As a condition of sale, use of these products 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.

Information for All Non-U.S. Customers

The parental strain of Mach1™-T1R E. coli is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Limited Use Label License No. 30: T7 Expression System

The composition and/or use of this product may be claimed in U.S. Patent No. 5,693,489 licensed to Life Technologies Corporation by Brookhaven Science Associates, LLC. The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy, and is the subject of patents and patent applications assigned to Brookhaven Science Associates, LLC (BSA). By provisions of the Distribution License Agreement granted to Life Technologies covering said patents and patent applications, Life Technologies grants you a non-exclusive sub-license under patents assigned to BSA for the use of this technology, including the enclosed materials, based upon the following conditions: 1 – these materials are to be used for non-commercial research purposes only. A separate license under patents owned by BSA is required for any commercial use, including the use of these materials for research purposes or production purposes by any commercial entity. Information about commercial license may be obtained from The Office of Technology Transfer, Brookhaven National Laboratory, Bldg. 475D, P.O. Box 5000, Upton, New York 11973-5000. Phone (516) 344-7134. 2 - No materials that contain the cloned copy of the T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this sub-license and agrees to be bound by its terms. This limitation applies to strains BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE, CE6, BL21-SI Competent Cells and any derivatives that are made of them. You may refuse this sub-license by returning this product unused in which case Life Technologies accept return of the product with a full refund. By keeping or using this product, you agree to be bound by the terms of this license.

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Purchaser Notification, continued

Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

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The trademarks mentioned herein are the property of Life Technologies Corporation or their respective owners.
Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech_support@invitrogen.com

For country-specific contact information, visit our web site at www.invitrogen.com