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Instruction Manual

Champion™ pET SUMO Protein Expression System

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

Catalog no. K300-01

Version A
27 January 2004
25-0709

Table of Contents

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.

Box	Component	Storage
1	pET SUMO TA Cloning® Reagents	-20°C
2	One Shot® Mach1™-T1^R Chemically Competent *E. coli*	-80°C
3	BL21(DE3) One Shot® Chemically Competent *E. coli*	-80°C
4	SUMO Protease	**Protease:** -80°C **Buffers:** -20°C

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.**

Item	Concentration	Amount
pET SUMO vector, linearized	25 ng/µl in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0	5 x 10 µl
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 in water, pH 8.0	10 µl

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pET SUMO TA Cloning® Reagents, continued

Item	Concentration	Amount
10X Ligation Buffer	60 mM Tris-HCl, pH 7.5 60 mM $MgCl_2$ 50 mM NaCl 1 mg/ml bovine serum albumin 70 mM β-mercaptoethanol 1 mM ATP 20 mM dithiothreitol 10 mM spermidine	100 µl
T4 DNA Ligase	4.0 Weiss units/µl	25 µl
Sterile Water	--	1 ml
SUMO Forward Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8.0	10 µl
T7 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8.0	10 µl
pET SUMO/CAT	0.01 µg/µl in TE buffer, pH 8.0	10 µl

Unit Definition of T4 DNA Ligase

One (Weiss) unit of T4 DNA Ligase catalyzes the exchange of 1 nmol ^{32}P-labeled pyrophosphate into [γ/β-^{32}P]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.

Primer	Sequence	pMoles Supplied
SUMO Forward	5´-AGATTCTTGTACGACGGTATTAG-3´	141
T7 Reverse	5´-TAGTTATTGCTCAGCGGTGG-3´	325

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Kit Contents and Storage, continued

One Shot® Mach1™-T1R Reagents

The table below lists the items included in the One Shot® Mach1™-T1R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg DNA. **Store Box 2 at -80°C.**

Item	Composition	Amount
S.O.C. 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
Mach1™-T1R cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of Mach1™-T1R

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

Genotype: F^- Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_K^- m_K^+) Δ*rec*A1398 *end*A1 *ton*A

One Shot® BL21(DE3) Reagents

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

Item	Composition	Amount
S.O.C. 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
BL21(DE3) Cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

continued on next page

Kit Contents and Storage, continued

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 hsdS*$_B$ ($r_B^- m_B^-$) *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 -80ºC. Store 10X SUMO Protease Buffers at +4ºC or -20ºC.

Item	Composition	Amount
SUMO Protease (1 U/µl)	SUMO Protease in: 25 mM Tris-HCl, pH 8.0 1% Igepal (NP-40) 250 mM NaCl 500 µM DTT 50% (v/v) glycerol	5 x 50 µl
10X SUMO Protease Buffer + Salt	500 mM Tris-HCl, pH 8.0 2% Igepal (NP-40) 1.5 M NaCl 10 mM DTT	500 µl
10X SUMO Protease Buffer – Salt	500 mM Tris-HCl, pH 8.0 2% Igepal (NP-40) 10 mM DTT	500 µl

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.

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

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.* HHHHHHG).

The amount of antibody supplied is sufficient for 25 western blots.

Product	Quantity	Catalog no.
Anti-HisG Antibody	50 µl	R940-25
Anti-HisG-HRP Antibody	50 µl	R941-25
Anti-HisG-AP Antibody	125 µl	R942-25

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Accessory Products, continued

Purifying Recombinant Fusion Protein

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.

Product	Quantity	Catalog no.
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
	100 ml	R901-10
Ni-NTA Purification System	6 purifications	K950-01
Polypropylene Columns (empty)	50	R640-50

Introduction

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.

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Overview, continued

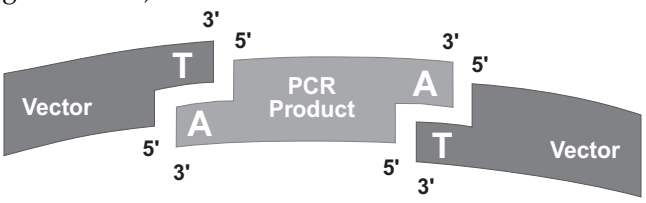
Features of the Champion™ pET SUMO Vector

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

- T7*lac* 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 T7*lac* 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:

- *lac*ZΔM15 for blue/white color screening of recombinants
- *hsd*R mutation for efficient transformation of unmethylated DNA from PCR applications
- Δ*rec*A1398 mutation for reduced occurrence of homologous recombination in cloned DNA
- *end*A1 mutation for increased plasmid yield and quality
- *ton*A 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-thiogalacto-side (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 T7*lac* promoter to drive expression of the gene of interest. The T7*lac* 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).

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.

Using One Shot® Mach1™-T1^R Cells

One Shot® Mach1™-T1^R 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™-T1^R 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™-T1^R 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.

Step	Action	Page
1	Amplify your PCR product using *Taq* polymerase and your own primers and parameters.	8
2	Ligate your PCR product into pET SUMO.	9
3	Transform your ligation into competent Mach1™-T1^R *E. coli*.	10
4	Select colonies and isolate plasmid DNA. Analyze plasmid DNA for the presence and orientation of the PCR product by restriction enzyme digestion or sequencing.	11
5	Select a positive transformant and isolate plasmid DNA. Transform BL21(DE3) and induce expression with IPTG	13-15
6	Purify your recombinant protein.	18-19
7	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.	20-21

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 or 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.

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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.

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 15ºC 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 you 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™-T1^R 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™-T1^R 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).
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, next page.

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 +4ºC 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 OD_{600} = 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 OD_{600} = ~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.

Percentage Substrate Hydrolyzed				
Time	**4°C**	**16°C**	**21°C**	**30°C**
0.5 h	48	73	83	88
1 h	60	87	90	93
2 h	71	94	94	95
3 h	74	95	95	95

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 chromotography 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.

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

continued on next page

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.

Problem	Reason	Solution
No expression of recombinant protein	Gene of interest not in frame with the N-terminal tag	Sequence your construct to verify if the insert is in frame with the N-terminal tag. If not in frame, redesign your PCR primers.
	Incorrect antibody used for detection	Use the Anti-HisG Antibodies or an antibody to your protein
Low expression of recombinant protein	Toxic gene **Note:** Evidence of toxicity includes loss of plasmid or slow growth relative to the control.	• Add 1% glucose to the bacterial culture medium during transformation and expression. • 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).

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Troubleshooting, continued

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.

Problem	Reason	Solution
Large percentage of uncleaved SUMO fusion protein	Protein starts with a proline, lysine, valine, or leucine	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).
	Salt concentration in cleavage reaction too high	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.
	Imidazole concentration in cleavage reaction too high	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.
	Cysteine protease inhibitor used during purification steps	SUMO Protease is a cysteine protease. Do not add cysteine protease inhibitors to any reactions.
	DTT in SUMO Protease Buffer oxidized	Add freshly prepared DTT to the cleavage reaction to a final concentration of 1 mM.
	Recombinant protein denatured	Purify your recombinant protein under native conditions. SUMO Protease may not recognize denatured SUMO fusion protein.
No native protein detected after removal of SUMO Protease	Native protein located in flow-through and not eluted fractions	Be sure to check the flow-through for your native protein. Eluted fractions will only contain the SUMO and SUMO Protease.

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:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Taq polymerase (1 units/µl)	1 µl
Final volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. 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

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

Control Ligation Reaction

Using the control PCR product produced on the previous page and the pET SUMO vector, set up the following ligation reaction.

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.
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 15ºC for 4 hours (preferably overnight). You may also incubate your ligation reaction at room temperature for 30 minutes, if desired.
4. Transform 2 µl of the ligation reaction into one vial of One Shot® Mach1™-T1^R competent cells using the protocol on page 10.

Analysis of Results

Pick 10 colonies and isolate plasmid DNA. Analyze the plasmids for the presence of insert by digesting the DNA with *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® Mach1™-T1^R competent cells. Transform one vial of One Shot® Mach1™-T1^R cells with 10 pg of pUC19 using the protocol on page 10. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥1 x 10^9 cfu/µg 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.

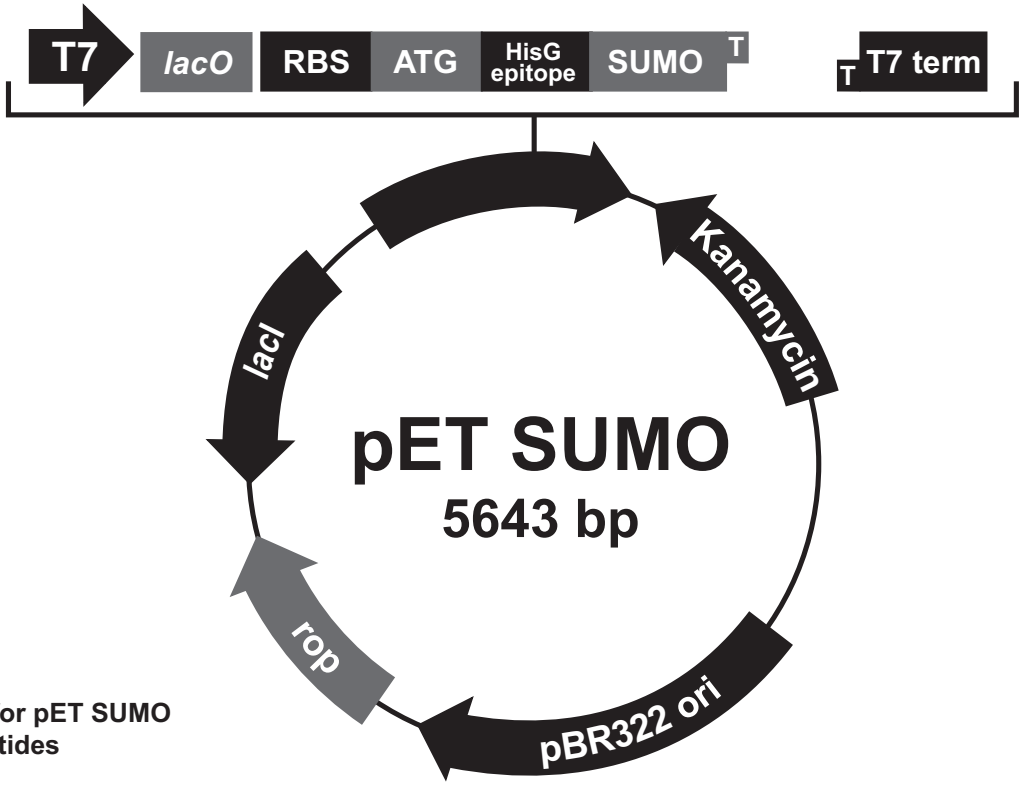
Note

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

Features of pET SUMO

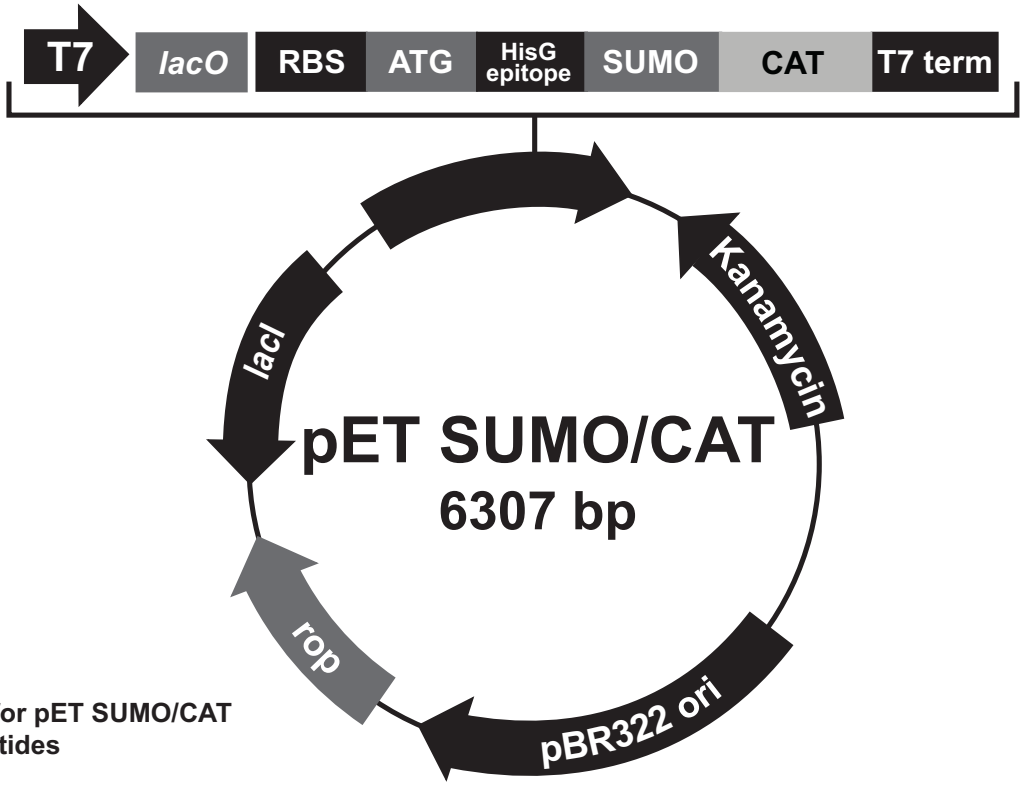
The pET SUMO vector (5643 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TA Cloning® site for efficient translation of PCR product.
N-terminal 6xHis tag	Allows purification of recombinant fusion protein on metal-chelating resin (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.
SUMO ORF	Enhances recombinant protein expression and solubility and allows cleavage by SUMO Protease to produce native protein.
SUMO Forward priming site	Allows sequencing of the insert.
TA Cloning® site (5′ T-overhangs)	Allows ligase-mediated cloning of *Taq*-amplified PCR products.
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which allows efficient transcription termination.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Allows replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor that binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

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 $MgCl_2$

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 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 KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   0.3 ml KH_2PO_4
   4.7 ml K_2HPO_4
   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.

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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™-T1^R: Carries the *lac*ZΔM15 *hsd*R *lac*X74 *rec*A *end*A *ton*A 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™-T1^R *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.

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Product Qualification

Introduction

Criteria used to qualify the components of the Champion™ pET SUMO Protein Expression System are detailed below.

Vectors

The pET SUMO and pET SUMO/CAT vectors are qualified by restriction enzyme analysis. The supercoiled pET SUMO vector is qualified prior to linearization. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

Cloning Efficiency

The linearized pET SUMO vector is functionally qualified using the control reagents supplied in the kit. Under conditions described on pages 9, a 750 bp control PCR product is ligated into pET SUMO and transformed into One Shot® Mach1™-T1^R chemically competent *E. coli* using the protocol on page 10. The vector must meet the following criteria:

- >80% cloning efficiency
- >80% transformants (16 out of 20 picked colonies) must contain the appropriate insert

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

T4 DNA Ligase and 10X Ligation Buffer

T4 DNA Ligase is lot-qualified and must pass the following quality control assays:

- Functional absence of endonuclease and exonuclease activities
- Ligation/recut
- Ligation efficiency

10X Ligation Buffer is functionally qualified with the enzyme and meets quality control specifications.

One Shot® Chemically Competent *E. coli*

All competent cells are tested for transformation efficiency using the control plasmid included in the One Shot® kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:

- Greater than 1 x 10^9 cfu/µg plasmid DNA for Mach1™-T1^R
- Greater than 1 x 10^8 cfu/µg plasmid DNA for BL21(DE3)

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and absence of phage contamination.

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Product Qualification, continued

SUMO Protease	SUMO Protease has > 85% single-band purity and must demonstrate functional absence of any non-specific protease activity. The SUMO Protease assay is performed in 1X SUMO Protease Buffer - Salt (50 mM Tris-HCl, pH 8.0, 0.2% Igepal, 1 mM DTT) with 1 unit enzyme and 2 µg of 85% purified control substrate at 30°C for 1 hour in a total volume of 20 µl and must show ≥85% cleavage.

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