



SUMOpro[®] Gene Fusion Technology

NEW METHODS FOR ENHANCING FUNCTIONAL PROTEIN
EXPRESSION AND PURIFICATION IN BACTERIA

***E.coli* (T7; Amp or Kan)**

Cat. No. 1000K (Kit, Kan)
1001K (Vector, Kan)
1000A (Kit, Amp)
1001A (Vector, Amp)

Product Manual

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Background**Ubiquitin and SUMO**

In cells, proteins are tagged for degradation by ubiquitin and targeted to the 26S proteasome. In contrast, covalent modification of cellular proteins by the ubiquitin-like modifier SUMO (small ubiquitin-like modifier) regulates various cellular processes, such as nuclear transport, signal transduction, and protein stabilization. Ubiquitin-like proteins fall into two classes: the first class, ubiquitin-like modifiers (UBLs) function as modifiers in a manner analogous to that of ubiquitin. Examples of UBLs are SUMO, Rub1 (also called Nedd8), Apg8, and Apg12. The second class of proteins includes parkin, RAD23, and DSK2 and are designated ubiquitin-domain proteins (UDPs). These proteins contain domains that are related to ubiquitin but are otherwise unrelated to each other. In contrast to UBLs, UDPs are not conjugated to other proteins. Once covalently attached to cellular targets, SUMO regulates protein-protein and protein-DNA interactions, as well as localization and stability of the target protein. Sumoylation occurs in most eukaryotic systems, and SUMO is conserved from yeast to humans. SUMO and ubiquitin only show about 18% homology, but both possess a common three-dimensional structure characterized by a tightly packed globular fold with β -sheets wrapped around an α -helix.

Smt3 Fusions

Yeast SUMO (Smt3) fused with a protein of interest can dramatically enhance expression and promote solubility and correct folding of the protein. It has been known for a long time that ubiquitin exerts chaperoning effects on fused proteins in *E. coli* and yeast, increasing their yield and solubility. Attachment of a highly stable protein (ubiquitin or SUMO) at the N-terminus of a partner protein increases the recombinant fusion protein yield. The enhanced solubility demonstrated by fusing ubiquitin and ubiquitin-like moieties to the N-terminus of the protein-of-interest may be explained by the outer hydrophilicity and inner hydrophobicity of the folded structure of ubiquitin and SUMO, exerting a detergent-like effect on less soluble fusion partner proteins.

Recombinant Protein Purification and Ulp1 Protease

While ubiquitin fusion has been known for many years to enhance protein expression, its utility as a protein purification modality is compromised by the inefficient nature of ubiquitin hydrolase, or protease – the enzyme that releases the partner protein from ubiquitin by hydrolysing the peptide bond. Likewise, commonly used proteases such as thrombin, enterokinase, rhinovirus proteases, and TEV, do not cleave all fusions efficiently and, moreover, can generate unnatural N-termini by leaving intact some amino acids from the cleavage recognition site. In eukaryotic cells ubiquitin is not a convenient tag since ubiquitinated proteins are a target for the degradation machinery.

Recently, Ulp1 core (SUMO Protease 1), a yeast SUMO protease equivalent of a ubiquitin protease, has been evaluated as a tool for purification of recombinant SUMO fusion proteins expressed in *E. coli*. SUMO protease 1 has unique advantages when compared with other proteases commonly used in recombinant protein production. SUMO Protease 1 recognizes the Smt3 structure at the N-terminus of the partner protein and cleaves the junction irrespective of the N-terminal sequence of the protein (except proline). Also, SUMO Protease 1 never cleaves within the protein-of-interest.

Advantages

The advantages of the SUMOpro Expression and Purification System.

- 1) Smt3 fusion may enhance recombinant protein expression.
- 2) Smt3 fusion may enhance solubility.
- 3) No known case of SUMO Protease 1 cleaving within the fused protein of interest.
- 4) SUMO Protease 1 cleavage and subsequent purification yields native protein with a desired N-terminus.

Components

The SUMOpro Expression System provides the reagents to express a protein of interest as a linear chimera with the Smt3 protein tag. The SUMOpro Expression System contains the following four components.

- 1) **pE-SUMO (T7; Amp or Kan)**
Size: 20µg (0.5µg/µl)
Buffer: 10mM Tris
- 2) **SUMO Protease 1 (Ulp1 Core)**
Size: 1000 units (10 units/µl)
Buffer: 10mM Tris-HCl, pH 8.0
75mM NaCl
5mM DTT
1mM EDTA
50% Glycerol
- 3) **Smt3 Control Protein**
Size: 100µg (5.0µg/µl)
Buffer: PBS
- 4) **Smt3 Antibody**
Size: 50µg, (1.0mg/ml)
Buffer: 0.02M Potassium Phosphate
0.15M Sodium Chloride, pH 7.2

Storage

pE-SUMO Vector (T7; Amp or Kan)

Store vial at -20°C. Avoid cycles of freezing and thawing.

SUMO Protease 1 (Ulp1 Core)

Store vial at -80°C. Aliquot into small tubes to avoid cycles of freezing and thawing.

Smt3 Control Protein

Store vial at -80°C. Avoid cycles of freezing and thawing.

Smt3 Antibody

For extended storage, aliquot contents and store at -20°C. This product is stable for several weeks at 4°C.

Components are stable for one year under the specified storage conditions.

Cloning

Background

The pE-SUMO vector is provided as a circular plasmid. For cloning, the vector has to be digested with Bsal (Eco31I) restriction endonuclease. This will result in dropping out a small fragment and leaving two unique overhangs; ACCT at the 5' end and a CTAG (XbaI) overhang at the 3'. Two different overhangs allow directional insertion of the gene of interest (see Multiple Cloning Site (MCS) map). The ACCT at the end of the Smt3 coding sequence, allows a gene of interest to be cloned in frame with the Smt3 tag, resulting in a Smt3 fusion protein construct.

Forward Primer Design

To clone your gene of interest into the pE-SUMO vector, it must be amplified by PCR and digested to produce an overhang complementary to the vector's ACCT. This can be accomplished by way of Class IIS restriction enzymes, which recognize non-palindromic sequences and cleave at sites that are outside their recognition sequences. The latter trait gives Class IIS enzymes two useful properties. First, when a Class IIS enzyme recognition site is engineered at the end of a primer, the site is removed from the PCR product when it is digested, meaning that there will be no additional nucleotide sequence between Smt3 and your cDNA-of-interest. Second, overhangs created by Class IIS enzymes are template-derived and thus unique.

Below is an example of forward primer design incorporating a restriction site for the Class IIS enzyme Bsal:

Gene Target

Bsal: 5' – **GGTCTC**NAGGTXXXXXXXXXXXXXXXXX – 3'

where **GGTCTC** is the Bsal recognition sequence, N is any nucleotide, AGGT will be the overhang that is complementary with the ACCT end of the pE-SUMO vector. XXX is the first codon of your gene of interest and GGT is the last codon of the Smt3 tag. Additional nucleotides will be required for the primer to anneal specifically with your gene of interest during the PCR amplification.

If your gene of interest already contains a Bsal site, then another Class IIS enzyme and site may be used instead. Below are examples of forward primers for some of these enzymes/sites:

AarI: 5' - **CACCTGCNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BbsI: 5' - **GAAGACNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BbvI: 5' - **GCAGCNNNNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BfuAI: 5' - **ACCTGCNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BsaI: 5' - **GGTCTCNAAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BsmAI: 5' - **GTCTCNAAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BsmBI: 5' - **CGTCTCNAAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BsmFI: 5' - **GGGACNNNNNNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 BtgZI: 5' - **CGGATGNNNNNNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 FokI: 5' - **GGATGNNNNNNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'
 SfaNI: 5' - **GCATCNNNNNAGGT**XXXXXXXXXXXXXXXXXX - 3'

NOTE: As a general practice, we recommend that two or more bases (any sequence) be added to the 5' end of each primer to allow more efficient cleavage of the PCR product, since some restriction enzymes cleave poorly when its recognition sequence is at the extreme end of a DNA fragment.

Reverse Primer Design

The reverse primer should contain one of the restriction enzyme sites from the multiple cloning site of pE-SUMO, to allow directional cloning of your gene of interest into the vector. We recommend that XbaI be employed as the restriction site in the reverse primer, because the vector will have the BsaI/XbaI-linearized form that can be used directly for ligations without further treatment. An example of a reverse primer for this purpose is:

XbaI: 5' - **TCTAGATCA**xxx... - 3'

where **TCTAGA** is the XbaI recognition sequence, **TCA** is the reverse complement of a stop codon (TGA), and xxx is the reverse complement of the final codon of your gene of interest. Again, it is recommended that extra bases be added to the 5' end, as noted above.

If your insert contains an XbaI site or if the digestion of the PCR insert with a single restriction endonuclease is preferred, restriction site, used in the forward primer could be added in front of XbaI site. For example if BsaI site is added in front of XbaI site, the digestion either with XbaI or BsaI enzyme gives the same 5'CTAG-overhang:

BsaI/XbaI: 5' - **GGTCTCTCTAGATCA**xxx... - 3'

Any of the polylinker sites could be used for the reverse primer. If your gene of interest contains an XbaI site, or if another restriction site is desired for any reason, the other cloning sites available for reverse primer design are BamHI, SacI, Sall, HindIII, NotI, EagI, and XhoI. Below are examples of reverse primers for each of these sites:

BamHI 5' - **GGATCCTCA**xxx... - 3'
 SacI: 5' - **GAGCTCTCA**xxx... - 3'
 Sall: 5' - **GTGACTCA**xxx... - 3'
 HindIII: 5' - **AAGCTTCA**xxx... - 3'
 NotI: 5' - **GCGGCCGCTCA**xxx... - 3'
 EagI: 5' - **CGGCCGTC**Axxx... - 3'
 XhoI: 5' - **CTCGAGTC**Axxx... - 3'

Note: Only if XbaI site is used in the reverse oligo of the insert, the vector could be digested with a single BsaI restriction endonuclease. If any other polylinker sites are being utilized the vector needs to be digested with a polylinker site enzyme of your choice in addition to BsaI.

Preparation of Insert

The insert should be amplified by PCR to introduce the restriction sites that will generate the appropriate compatible ends as described above. To maintain the sequence integrity of your clone it is sensible to employ a proof reading enzyme such as Pfu (Stratagene), DeepVent (New England Biolabs) or Taq HIFI (Invitrogen) for your PCR reactions. After purification, the PCR product can be digested with the appropriate restriction enzymes in preparation for directly cloning the insert into the pE-SUMO vector. Alternatively, the PCR product can be first cloned into a high copy number plasmid such as pBlueScript (Stratagene) or pCR4.0 TOPO (Invitrogen) generating a clone that can be readily sequenced. The insert can then be digested out of this plasmid and purified by agarose gel electrophoresis.

Preparation of Vector

The pE-SUMO plasmid is provided as a 20µg aliquot of circular vector that has to be digested with BsaI or BsaII in combination with any of the polylinker enzymes, gel-purified and extracted using standard techniques (Sambrook et al.). The digested plasmid can then be used for ligation.

Ligation

For ligation of the prepared insert into the digested pE-SUMO vector, T4 DNA ligase and standard ligation protocols should be employed (Sambrook et al.). Because the ligation is directional, alkaline phosphatase treatment of vector is unnecessary. The T4 DNA Ligase should be used in the correct buffer and at the appropriate temperature as described by its manufacturer (e.g. MBI Fermentas, New England Biolabs, Roche, Stratagene, Promega).

Transformation

Following ligation, pE-SUMO plasmids can be transformed into competent *E. coli* by either chemical transformation or electroporation. Transformed cells should be grown in LB supplemented with vector specific selection antibiotic (ampicillin or kanamycin).

Standard bacterial strains like DH5α, TOP10 or others, with the wild type gyrase gene must be used for transformations. These strains show a high propensity for transformation of foreign DNA and have mutations abolishing the activity of the products of the genes RecA and EndA.

Identification of Positives Clones

Using one of the primers used for PCR amplification and an external primer, either the T7 forward or reverse (Sequence) individual transformants can be screened for positive clones. Upon amplification and purification of the plasmid DNA, it should be similarly checked by digestion with a number of restriction enzymes to generate a map.

Expression**Transformation**

For expression of the Smt3 fusion protein, it is necessary to transform the confirmed plasmid clone into an *E. coli* strain containing the DE3 lysogen. The BL21 derived strains are particularly useful for protein expression as they contain mutations in ompT and lon, which abolish protease activity that could degrade your expressed protein. It is also possible to transfect the bacteria with the helper phage, which can be useful for expressing toxic proteins. The transformation procedure into these cells should be based on your manufacturers' protocols. NOTE: the transformation efficiency of DE3 strains of *E. coli* is normally lower than the cloning strains.

Culture**(i) Pre culture**

For a large-scale culture, inoculate a small amount of LB containing ampicillin or kanamycin (pE-SUMO vector-specific), and any antibiotic that may be necessary to maintain helper plasmids with a single colony from your transformed plate.

(ii) Large culture

Using the overnight culture, inoculate your expression culture, diluting the seed culture 1/100. Grow cells with vigorous shaking at 37°C. Grow the cells to an Abs 600nm between 0.4-0.6 (mid log phase) and then induce with 0.25–1.0mM IPTG and continue growth for 3 hours. Alternatively, cells can be cooled to 20°C, induced with IPTG, and grown overnight. Then, harvest the cells by centrifugation at 5000 rpm. The cell pellet can be resuspended in lysis buffer and then moved onto the purification step. Alternatively, the pellet can be stored at –20°C indefinitely, either resuspended in lysis buffer or not.

Purification

Cells can be lysed by a number of ways such as freeze thaw, sonication, homogenization enzymatic lysis, or a combination of the aforementioned methods. Lysis should be performed at a pH optimal for the first step of purification. The pH should be maintained between pH 7.0 and 8.0. Protease inhibitors such as EDTA or PMSF should be included if you fear your protein is susceptible to aberrant protease activity, Complete tablets (Roche) offer inhibitors to a broad range of proteases.

The presence of a hexahistidine tag at the N-terminus of the Smt3 protein sequence allows for rapid purification of fusions by immobilized metal affinity chromatography (IMAC).

- 1) Clarified cell lysate pH 7.0-8.0
- 2) Load onto Ni²⁺ column or incubate in batch form with resin for 30 minutes
- 3) Wash with 10-20mM imidazole
- 4) Elute with 250-300mM imidazole

Cleavage**Background**

SUMO Protease 1 (Ulp1 Core), a highly active and robust recombinant protease, cleaves Smt3 from recombinant fusion proteins. Unlike thrombin, EK, or TEV proteases, whose recognition sequences are short and degenerate, SUMO Protease 1 recognizes the tertiary sequence of Smt3. As a result, SUMO Protease 1 never cleaves within the fused protein of interest. SUMO Protease 1 cleaves consistently over a broad range of temperature (30°C is optimal), pH [5.5 – 9.5], and ionic strength. SUMO Protease 1 contains a polyhistidine tag at the N-terminus; therefore, SUMO Protease 1 is easily removed from the cleavage reaction by affinity chromatography.

Unit Definition

One unit of SUMO Protease 1 (Ulp1 Coire) cleaves 100µg of Smt3 control protein in 1h at 30°C.

Cleavage

1. Dialyze the purified Smt3 fusion proteins for at least 24h at 4°C against [20mM Tris-HCl, 150mM NaCl, pH 8.0, 10% glycerol] or [against PBS]. During the dialysis, change the buffer (~1L) at least 2 times to effectively remove the detergent and imidazole.
2. Add SUMO Protease 1 at a rate of 1 unit per 100µg of substrate and incubate at 30°C for 1h in either Buffer A [20mM Tris-HCl pH 8.0, 150mM NaCl, 2mM dithiothreitol] or Buffer B [PBS pH 7.5, 2mM dithiothreitol].

Purification

The Smt3 tag and SUMO Protease 1 both contain polyhistidine tags at their N-termini; therefore, remove Smt3 and SUMO Protease 1 from the cleavage reaction by affinity chromatography.

Collect cleaved recombinant protein-of-interest from the column flow through. Assess the quality of protein product by examination of a small aliquot on SDS-PAGE. If the protein is in the appropriate buffer it can be directly used, or further purification steps can be employed.

Controls and Validations**Smt3 Control Protein**

Smt3 control protein is a recombinant fusion protein that contains the Smt3 tag, Met (Methionine), followed by a control polypeptide. The Smt3 control protein is used to control for SUMO Protease 1 activity. In the control study, Smt3 control protein is incubated with SUMO Protease 1 as a positive control for SUMO Protease 1 cleavage when cleaving the experimental Smt3 recombinant fusion protein. Please note that the Smt3 tag runs at 18kDa on SDS-PAGE following cleavage; and uncleaved Smt3 control protein runs as 48kDa when resolved by SDS-PAGE.

Running a Control with Smt3 Control Protein

1. Incubate 100µg Smt3 control protein and 1 unit of SUMO Protease 1 (total 100µl) at 30°C for 1h.
2. Take a 12µl aliquot from the 100µl reaction mixture and add 3µl of 6X SDS-PAGE sample buffer.
3. Heat sample (15µl) at 95°C for 5 min.
4. Load sample (15µl), run SDS-PAGE, stain with Coomassie blue.

Western Blots

Anti-Smt3 is an affinity purified hen polyclonal IgY antibody that reacts with Smt3 in Western blot and ELISA applications. The antibody is a highly-specific for Smt3, purified using a Smt3 protein affinity column.

Recommended Dilution(s) for SUMO Antibody

For immunoblotting, a 1:5,000 dilution is recommended. Yeast cell lysates can be used as a positive control without induction or stimulation. For ELISA a 1:5,000 to 1:25,000 dilution is recommended. Researchers should determine optimal titers for other applications.

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