SUMOstar™ Gene Fusion Technology

NEW METHODS FOR ENHANCING FUNCTIONAL PROTEIN EXPRESSION AND PURIFICATION IN BACTERIA

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

Cat. No. 1100K (Kit, Kan)
1101 (Vector, Kan)
1100A (Kit, Amp)
1106 (Vector, Amp)

Product Manual

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Background

Ubiquitin and SUMO

In eukaryotic cells, proteins are tagged for degradation by ubiquitin and sent 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 stabilization of proteins. Ubiquitin-like modifiers (UBLs) function as modifiers in a manner analogous to that of ubiquitin. Examples of UBLs are SUMO, Rub1 (also called Nedd8), ISG15, Apg8 and Apg12. 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 highly conserved from yeast to humans. SUMO has run at approximately 18kDa on SDS-PAGE. SUMO and ubiquitin exhibit only 18% homology; however, both proteins possess a common three-dimensional structure characterized by a tightly packed globular fold with β-sheets wrapped around an α-helix.

SUMO Fusions

Utilizing its function in nature, SUMO 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 structure (such as that of ubiquitin or SUMO) at the N-terminus of a partner protein increases the yield by increasing stability. The solubilizing effect of ubiquitin and ubiquitin-like proteins may also be explained in part by the outer hydrophilicity and inner hydrophobicity of the core structure of ubiquitin and SUMO, exerting a detergent-like effect on otherwise insoluble proteins.

SUMO Protease and Purification of Untagged Proteins

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. 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. Recently, SUMO Protease I (Ulp1), a yeast SUMO equivalent of ubiquitin protease, has been evaluated as a tool for purification of proteins expressed in E. coli and has been advantages over other proteases commonly used in recombinant protein production (see above). SUMO Protease I recognizes the SUMO structure at the N-terminus of the partner protein and cleaves the junction irrespective of the N-terminal sequence of the protein (with the exception of proline). SUMO Protease 1 is tagged with a hexahistidine moiety, thus following the cleavage reaction the protease can be easily removed, along with the cleaved fusion tag, using an IMAC resin. The resulting flow-through contains the untagged purified protein-of-interest. This expression and purification procedure is only effective in prokaryotic organisms that do not endogenously express SUMO and SUMO proteases.

SUMOstar and SUMOstar Protease

Eukaryotic cells possess endogenous SUMO proteins and SUMO proteases (deSUMOylases). The endogenous deSUMOylases efficiently cleave native SUMO conjugates and likewise SUMO fusion tags from recombinantly expressed constructs. LifeSensors has overcome this obstacle by engineering a novel SUMO tag (SUMOstar) that is not cleaved by endogenous SUMO proteases. A specific SUMOstar protease 1, which specifically recognizes and cleaves the SUMOstar tag, was also designed. SUMOstar Protease 1 has the same robust nature as the original SUMO protease 1, recognizing and specifically cleaving only the tertiary structure of SUMOstar, and never cuts within the protein-of-interest. The SUMOstar system also has similar expression and solubility enhancing characteristics as the original prokaryotic SUMO expression systems, but now expands the unique functional protein production advantages of the SUMO system into eukaryotic expression hosts. The SUMOstar prokaryotic system was developed as a cost effective solution for producing recombinant proteins with desired N-termini and permits the researcher to easily move into yeast, insect, or mammalian systems by virtue of the new LifeSensors SUMOswap^{SM} subcloning system integrated into the SUMOstar expression vectors..

Advantages

The advantages of the SUMOstar Expression and Purification System.

1) SUMOstar fusion may enhance recombinant protein expression.

2) SUMOstar fusion may enhance solubility.

3) No known case of SUMOstar protease cleaving within the fused protein of interest.

4) SUMOstar protease cleavage and subsequent purification yields native protein with a desired N-terminus.

Components

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

1) **pE-SUMOstar (T7; Amp or Kan)**
   Size: 20µg (0.5µg/µl)
   Buffer: 10mM Tris

2) **SUMOstar protease 1**
   Size: 1000 units (10 units/µl)
   Buffer: 10 mM Tris-HCl, pH 8.0
   75mM NaCl
   5mM DTT
   1mM EDTA
   50% Glycerol

3) **SUMOstar Control Protein**
   Size: 100µg (5.0 µg/µl)
   Buffer: PBS

4) **SUMOstar Antibody**
   Size: 50µg,(1.0mg/ml)
   Buffer: 0.02M Potassium Phosphate
   0.15M Sodium Chloride, pH 7.2

Storage

pE-SUMOstar Vector (T7; Amp or Kan)
Store vial at -80°C or -20°C. Avoid cycles of freezing and thawing.

SUMOstar protease 1
For short-term use, store vial at 4°C. For long-term use, store vial at -80°C. Aliquot into small tubes to avoid cycles of freezing and thawing.

SUMOstar Control Protein
Store vial at -80°C or -20°C. Avoid cycles of freezing and thawing.

SUMOstar Antibody
Store vial at 4°C. For extended storage aliquot contents and freeze at -20°C. This product is stable for several weeks at 4°C.

Cloning

Background
The pE-SUMOstar vector is provided as a circular plasmid. For cloning, the vector has to be digested with BsaI (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 SUMOstar coding sequence, allows a gene of interest to be cloned in frame with the SUMOstar tag, resulting in a SUMOstar fusion protein construct.

Forward Primer Design
To clone your gene of interest into the pE-SUMOstar 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 SUMOstar 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 BsaI:

Gene Target
BsaI: 5' – **GGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'

where **GGTCTC** is the BsaI recognition sequence, N is any nucleotide, AGGT will be the overhang that is complementary with the ACCT end of the pE-SUMOstar vector. XXX is the first codon of your gene of interest and GGT is the last codon of the SUMOstar 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 BsaI 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' - **CACCTGC**NNNNAGGTXXXXXXXXXXXXXXX – 3'
BbsI: 5' – **GAAGAC**NNAGGTXXXXXXXXXXXXXXX – 3'
BbvI: 5' – **GCAGC**NNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
BfuAI: 5' – **ACCTGC**NNNNAGGTXXXXXXXXXXXXXXX – 3'
BsaI: 5' – **GGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmAI: 5' – **GTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmBI: 5' – **CGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmFI: 5' – **GGGAC**NNNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
BtgZI: 5' – **GCGATG**NNNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
FokI: 5' – **GGATG**NNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
SfaNI: 5' – **GCATC**NNNNNAGGTXXXXXXXXXXXXXXX – 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-SUMOstar, 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' – **TCTAGA**TCAxxx… – 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' – ***GGTCTC*TCTAGA**TCAxxx… – 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, SalI, HindIII, NotI, EagI, and XhoI. Below are examples of reverse primers for each of these sites:

BamHI 5' **- GGATCC**TCAxxx… – 3'
SacI: 5' – **GAGCTC**TCAxxx… – 3'
SalI: 5' – **GTCGAC**TCAxxx… – 3'
HindIII: 5' – **AAGCTT**TCAxxx… – 3'
NotI: 5' – **GCGGCCGC**TCAxxx… – 3'
EagI: 5' – **CGGCCG**TCAxxx… – 3'
XhoI: 5' – **CTCGAG**TCAxxx… – 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=SUMOstar 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-SUMOstar plasmid is provided as a 20µg aliquot of circular vector that has to be digested with BsaI or BsaI 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-SUMOstar 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-SUMOstar 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, etc. 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 SUMOstar 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-SUMOstar 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 prior to lysis.

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 SUMOstar 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 Ni^{2+} 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

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

Unit Definition

One unit of SUMOstar Protease 1 cleaves 100µg of SUMOstar Control Protein in 1h at 30°C.

Cleavage

1. Dialyze the purified SUMOstar 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 SUMOstar 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 SUMOstar tag and SUMOstar Protease 1 both contain polyhistidine tags at their N-termini; therefore, remove SUMOstar and SUMOstar Protease 1 from the cleavage reaction by affinity chromatography.
Collect cleaved recombinant protein-of-interest form 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

SUMOstar Control Protein

SUMOstar control protein is a recombinant fusion protein that contains the SUMOstar tag, Met (Methionine), followed by a control polypeptide. The SUMOstar control protein is a positive control for SUMOstar protease 1 activity. Please note that the SUMOstar tag runs at 18kDa on SDS-PAGE following cleavage; while uncleaved SUMOstar control protein runs at 48kDa when resolved by SDS-PAGE.

Running a Control Assay with SUMOstar Control Protein

1. Incubate 100µg SUMOstar control protein and 1 unit of SUMOstar Protease 1 (total 100 µl) 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 5min.

4. Load sample (15µl), run SDS-PAGE, stain gel with Coomassie blue.

Western Blots

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

Recommended Dilution(s) for SUMOstar Antibody

For immunoblotting, a 1:5,000 dilution is recommended. For ELISA a 1:5,000 to 1:25,000 dilution is recommended. Researchers should determine optimal titers for other applications.

Technical Support

Web Site

Visit the LifeSensors, Inc. website **http://www.lifesensors.com**, where you can:

- Purchase products and register for discounts and other special product offers
- Download manuals
- Download vector maps and sequences
- Access technical assistance and troubleshooting tips

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