SUMOstar™ Gene Fusion Technology

NEW METHODS FOR ENHANCING FUNCTIONAL PROTEIN EXPRESSION AND PURIFICATION IN MAMMALIAN CELLS

Cat. No.	
7110	**(Intracellular Kit)**
7111	**(Intracellular Vector)**
7120	**(Secretory Kit)**
7121	**(Secretory Vector)**

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. SUMOstar, an uncleavable version of SUMO, has been developed primarily for eukaryotic systems; and the bacterial version is used primarily for the initial testing and later as a donor vector for eukaryotic systems. 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 SUMOstar 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, a SUMO protease equivalent of ubiquitin protease, has been evaluated as a tool for purification of recombinant SUMO fusion proteins expressed in *E. coli.* Ulp1 is superior when compared with other proteases commonly used in recombinant protein production. Ulp1 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, Ulp1 never cleaves within the protein-of-interest. Based on the ULP1 platform LifeSensors, Inc. have engineered a SUMOstar specific protease, SUMOstar protease.

Advantages

The advantages of the SUMOstar Expression and Purification System.

1) SUMOstar fusion may dramatically 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) **pM-SUMOstar Vector (Intracellular) or pM-secSUMOstar Vector (Secretory)**
   Size: 20 µg (0.5 µg/µl)
   Buffer: 10 mM Tris

2) **SUMOstar Protease1**
   Size: 500 units (10 units/µl)
   Buffer: 10 mM Tris-HCl, pH 8.0
   75 mM NaCl
   5 mM DTT
   1 mM EDTA
   50% Glycerol

3) **SUMO-control protein**
   Size: 100 µg (5.0 µg/µl)
   Buffer: PBS

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

Storage

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

SUMOstar protease1
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 pM-SUMOstar vector is provided as a circular plasmid. For cloning, the vector has to be digested with BsmB1 (Esp3I) 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 pM-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
BsmB1: 5' – **CGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'

where **CGTCTC** is the BsmB1 recognition sequence, N is any nucleotide, AGGT will be the overhang that is complementary with the ACCT end of the pM-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 BsmB1 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'
BsaI: 5' – **GGTCTC**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 pM-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 BsmB1 site is added in front of XbaI site, the digestion either with XbaI or BsmB1 enzyme gives the same 5'CTAG-overhang:

BsmB1/XbaI: 5' – ***CGTCTC*****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 KpnI, SacI, SalI, HindIII, NotI, EagI, and XhoI. Below are examples of reverse primers for each of these sites:

NotI: 5' – **GCGGCCGC**TCAxxx… – 3'
KpnI 5' **- GGTACC**TCAxxx… – 3'
XhoI: 5' – **CTCGAG**TCAxxx… – 3'
ApaI: 5' – **GGGCCC**TCAxxx… – 3'
BstBI: 5' – **TTCGAA**TCAxxx… – 3'

Note: Only if XbaI site is used in the reverse oligo of the insert, the vector could be digested with a single BsmB1 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 BsmB1.

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 pSUMOstar 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 pM-SUMOstar plasmid is provided as a 20 µg aliquot of circular vector that has to be digested with BsmB1 or BsmB1 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 pM-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, pM-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

Transfection in Mammalian Cells
pM-SUMOstar plasmids could be transiently or stably transfected into a number of mammalian cells. Use the Lipofectamine-LTX procedure for transfection (invitrogene) into mammalian cells.

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 10-20 mM Imidazole
4) Elute with 250-300 mM Imidazole

Cleavage

Background
SUMOstar protease, 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 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 contains a polyhistidine tag at the N-terminus; therefore, SUMOstar protease is easily removed from the cleavage reaction by affinity chromatography.

Unit Definition
One unit of SUMOstar protease cleaves 100 µg of SUMOstar-Met-control protein in 1 h at 30°C.

Cleavage
1. Dialyze the purified SUMOstar fusion proteins for at least 24 h at 4°C against [20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 10% glycerol] or [against PBS]. During the dialysis, change the buffer (~1 L) at least 2 times to effectively remove the detergent and imidazole.

2. Add SUMOstar protease at a rate of 1 unit per 100 µg of substrate and incubate at 30°C for 1 h in either Buffer A [20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM dithiothreitol] or Buffer B [PBS pH 7.5, 2 mM dithiothreitol].

Purification
SUMOstar and SUMOstar protease both contain polyhistidine tags at their N-termini; therefore, remove SUMOstar and SUMOstar protease 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 -Met-Control Protein
SUMOstar-Met-control protein is a recombinant fusion protein that contains the SUMOstar tag, Met (Methionine), followed by a control polypeptide. The SUMOstar-Met-control protein is used to control for SUMOstar protease activity. In the control study, SUMOstar-Met-control protein is incubated with SUMOstar protease as a positive control for SUMOstar protease cleavage when cleaving the experimental SUMOstar recombinant fusion protein. Please note:that the SUMOstar tag runs at 18kDa on SDS-PAGE following cleavage; and uncleaved SUMOstar-Met-control protein runs as 47 kDa when resolved by SDS-PAGE.

Running a Control with SUMOstar-Control Protein
1. Incubate 100 µg SUMOstar-Met-control protein and 1 unit of SUMOstar Protease 1 (total 100 µL) at 30°C for 1 h.

2. Take a 12 µL aliquot from the 100 µL reaction mixture and add 3 µL of 6X SDS-PAGE sample buffer.

3. Heat sample (15 µL) at 95°C for 5 min.

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

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

Recommended Dilution(s) for SUMOstar 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 dilutions is recommended. Researchers should determine optimal titers for other applications.

References

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