SUMOstar™ Insect Cell Expression and Purification Systems

Catalogue #3100 (Intracellular Kit)
3101 (Intracellular Vector)
3105 (Secretory Kit)
3106 (Secretory Vector)

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 cleaving 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.

Benefits

Advantages of the SUMOstar Expression System

1) SUMOstar fusion may dramatically enhance expression.

2) SUMOstar fusion may dramatically enhance solubility.

3) No known case of SUMOstar Protease 1 cleaving within the fused protein of interest.

4) The SUMOstar tag can be used for affinity purification and immunofluorescent detection.

5) SUMOstar cleavage of the fusion construct yields native protein with a desired N-terminus.

Components

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

1) pI-SUMOstar Vector (intracellular) or pI-secSUMOstar Vector (secretory)
Size: 10 µl (250 ng/µl, pH 8.5)
Buffer: 10 mM Tris

2) SUMOstar Protease 1 (Runs at approximately 28kDa on SDS-PAGE)
Size: 1000 units (10 units/µl)
Buffer: 10 mM Tris-HCl, pH 8.0
75 mM NaCl
5 mM DTT
1 mM EDTA
50% Glycerol

3) SUMOstar Control Protein (Runs at approximately 48kDa on SDS-PAGE)
Size: 100 µg (5.0 µg/µl)
Buffer: PBS

4) SUMOstar Antibody
Size: 50 µg. aliquot (1µg/µl)
UV absorbance 280 nm
Buffer: 0.02 M Potassium Phosphate
0.15 M Sodium Chloride, pH 7.2

Storage

pI-SUMOstar Vector or pI-secSUMOstar Vector
Store vial at -20° C. Avoid cycles of freezing and thawing.

SUMOstar Protease 1
For short-term use, store vial at -20° C. For long-term use, store vial at -80°C. Avoid cycles of freezing and thawing.

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

SUMOstar Antibody
Avoid cycles of freezing and thawing. Centrifuge product if not completely clear after standing at room temperature. This product is stable for several weeks at 4°C as an undiluted liquid. For extended storage, keep antibody at -20°C. Expiration date is one year from date of restoration.

Cloning

Background
The pI-SUMOstar and pI-secSUMOstar vectors are provided as circular plasmids. For cloning, the vectors have to be digested with BsmbI restriction endonuclease. This will result in dropping out a small fragment and leaving two unique overhangs; ACCT at the 5' end and CTAG (XbaI) at the 3'. Two different overhangs allow directional insertion of the gene of interest (see Multiple Cloning Sit (MCS) map). The ACCT at the end of SUMOstar coding sequence allows a gene of interest to be cloned in frame with the SUMOstar tag. This results SUMOstar fusion protein construct.

Forward Primer Design
To clone your gene of interest into the pI-SUMOstar or pI-secSUMOstar vector, it must be amplified by PCR and digested to produce an overhang complementary to the vector's AGGT. This can be accomplished by way of Class IIS restriction enzymes, which recognize non-palindromic sequences and cleave at sites that are separate from 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 extraneous sequence between SUMOstar and your gene. 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 `BsmBI`: `Gene Target`

`BsmBI: 5' – XXXXXXCGTCTCNAGGT`**`xxxxxxxxxxxxxxx`** `– 3'`

where `CGTCTC` is the `BsmBI` recognition sequence, `N` is any nucleotide, `AGGT` will be the overhang that is complementary with the `TCCA` end of the pI-SUMOstar vector. `GGT` is the last codon of the SUMOstar tag. The XXXXXX (4 to 6 nucleotides) at the 5' of GGT are any nucleotides for direct digestion purpose. The 3' XXX… of GGT are the additional nucleotides (approximately 18 or more) designed from your gene of interest will be required for the primer to anneal specifically with your DNA template during the PCR amplification. If your gene

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

BbsI: 5′ – XXXXXXGAAGACNNAGGT**XXXXXXXXXXXXXXX** – 3′
BbvI: 5′ – XXXXXXGCAGCNNNNNNNNAGGT**XXXXXXXXXXXXXXX** – 3′
BfuAI: 5′ – XXXXXXACCTGCNNNNAGGT**XXXXXXXXXXXXXX** – 3′
BsaI: 5′ – XXXXXXGGTCTCNAGGT**XXXXXXXXXXXXXX** – 3′
BsmAI: 5′ – XXXXXXGTCTCNAGGT**XXXXXXXXXXXXXX** – 3′
BsmFI: 5′ – XXXXXXGGGACNNNNNNNNNNAGGT**XXXXXXXXXXXXXX** – 3′
BtgZI: 5′ – XXXXXXGCGATGNNNNNNNNNNAGGT**XXXXXXXXXXXXXX** – 3′
FokI: 5′ – XXXXXXGGATGNNNNNNNNNAGGT**XXXXXXXXXXXXXX** – 3′
SfaNI: 5′ – XXXXXXGCATCNNNNNAGGT**XXXXXXXXXXXXXX** – 3′

NOTE on primer design: As a general practice it is recommended that four or five additional nucleotides (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 their 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 pI-SUMOstar, to allow directional cloning of your gene of interest into the vector. It is recommended that XbaI is used as the restriction site in the reverse primer. An example of a reverse primer is:

XbaI: 5′ – XXXXXXTCTAGATTA**XXXXXXXXXXXXXXX** – 3′

where TCTAGA is the XbaI recognition sequence, TTA is the reverse complement of a stop codon (TAA), and **XXX** on the right is the reverse complement of the final codon of your gene of interest. Again, it is recommended that extra 4 or 5 additional nucleotides are added to the 5' end for direct digestion, as noted above. If your gene of interest contains a XbaI site, or if another restriction site is desired for any reason, the other cloning sites available for reverse primer design are PstI, XhoI, SphI, and HindIII. Below are examples of reverse primers for each of these sites:

NotI: 5′ – XXXXXXGCGGCCGCTTA**XXXXXXXXXXXXXXX** – 3′
KpnI: 5′ – XXXXXXGGTACCTTA**XXXXXXXXXXXXXXX** – 3′
HindIII: 5′ – XXXXXXAAGCTTTTA**XXXXXXXXXXXXXXX** – 3′

In these cases, digest the pI-SUMOstar vector with the other enzyme before ligation with the digested PCR product. (See Preparation of Vector below.)

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 Platinum Hi-Fidelity Tag (Invitrogen), Pfu (Stratagene) or DeepVent (New England Biolabs) 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 pI vector. Alternatively, the PCR product can be first cloned into a high copy number plasmid such as pBlueScript (Stratagene) or pCR Blunt II 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 circular pI-SUMOstar or pI-secSUMOstar vector plasmid should be digested with BsmBI and ligated with your gel purified digested PCR product designed with AGGT/XbaI ends.

Ligation
For ligation of the prepared insert into the digested pI-SUMOstar or pI-secSUMOstar vector, T4 DNA ligase and standard ligation protocols should be employed (Sambrook et al). Since the ligation is directional, 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. New England Biolabs, Roche, Stratagene, Promega, Fermentas).

Transformation
Following incubation of the ligation reactions they can be transformed into competent E. coli by either chemical transformation or electroporation. A cloning strain of E. coli, such as DH5α (Invitrogen) or Novablue (Novagen) or XL10Gold (Stratagene) is optimal for transformation. These strains show a high propensity for transformation of foreign DNA and have mutations abolishing the activity of the products of the RecA and EndA genes. These mutations limit untoward homologous recombination of the plasmid within the cell and prevent degradation of DNA preparations by the non-specific Endonuclease I.

Bacmid Preparation
Following identification of a positive recombinant donor plasmid, transform DH10Bac competent E. coli cells (Invitrogen) by chemical transformation. DH10Bac contains a parent bacmid with a lacZ-mini-attTn7 fusion. Transposition proteins from a helper plasmid facilitate transposition between the insert of the pI-SUMOstar vector and the parent bacmid. Recombinant bacmids appear as white colonies (follow Invitrogen procedure).

Expression

Follow the pFastBac (Invitrogen) protocol for bacmid purification and transfection.

Recombinant Fusion Protein Purification
pI-secSUMOstar contains a gp67 secretion signal upstream of the SUMOstar fusion. Therefore the SUMOstar fusion will be secreted into the media. The transfected insect cells should be pelleted by centrifugation and the media should be used to purify the SUMOstar fusion. You may also harvest the cells to analyze intracellular levels of your recombinant protein (see Invitrogen for cell lysis protocol). When using the pI-SUMOstar vector system (intracellular expression), you must harvest the cells to analyze non-secreted proteins.

To purify 6xHis-tagged recombinant proteins from the culture medium, we recommend that you perform dialysis or ion exchange chromatography prior to affinity chromatography on metal-chelating resins. This step is necessary to remove any media components that strip Ni^{2+} from the metal chelating resin (dialysis and chromatography) and to concentrate the sample for easier manipulation in further purification steps (chromatography). Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to Current Protocols in Protein Science (Coligan et al., 1998), Current Protocols in Molecular Biology, Unit 10 (Ausubel et al.,1994) or the Guide to Protein Purification (Deutscher, 1990).

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

1) Clarified Media pH 7.0-8.0

2) Load onto a Ni^{2+} column or incubate in batch form with the resin for 30 minutes

3) Wash column with 10-20 mM Imidazole

4) Elute bound protein with 250-300 mM Imidazole

Cleavage

Background
SUMOstar Protease 1, a highly active and robust recombinant protease, cleaves the SUMOstar tag 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 1 never cleaves within the fusion protein of interest. SUMOstar Protease 1 cleaves consistently over a broad range of temperature (30°C is optimal), pH [5.5-9.5], and ionic strengths. SUMOstar Protease 1 contains a polyhistidine tag at the N-terminus; therefore, SUMOstar Protease 1 is easily removed following cleavage 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 overnight at 4°C against [20mM Tris-HCl, pH 8.0, 150mM NaCl, 10% glycerol] or PBS. During the dialysis, change the buffer (~1 L) at least 2 times to effectively remove the detergent and imidazole.

2. Add SUMOstar Protease 1 at a concentration of 1 unit protease per 100 µg 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
SUMOstar Protease 1 contains a polyhistidine tag at the N-terminus; therefore, remove SUMOstar Protease 1 from the cleavage reaction by affinity chromatography. Collect protein in flow through. Assess the quality of protein product by 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, Methionine, and a control polypeptide. The SUMOstar control protein should be incubated with SUMOstar Protease 1 as a positive control for SUMOstar protease 1 cleavage of your SUMOstar fusion. Please note: the SUMOstar tag runs on SDS-PAGE at 18kDa following cleavage, while the uncleaved SUMOstar control protein runs at 48kDa. The SUMOstar protease 1 runs at approximately 28kDa on SDS-PAGE.

Western Blots
Anti-SUMOstar is a purified polyclonal IgY antibody generated in hens that also reacts with yeast SUMO by Western blot and ELISA. Immunohistochemistry and immunoprecipitation applications have not yet been tested. The SUMOstar antibody is affinity purified on a SUMOstar protein column.

Recommended Dilution(s) for SUMOstar Antibody
For immunoblotting, a 1:5,000 dilution is recommended. An 18kDa band corresponding to SUMOstar is detected. 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

Contact Us
For additional information or technical support: call, write, fax, or email our corporate headquarters.

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