SUMOstar™ Insect Cell Expression and Purification Systems

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

PRODUCT MANUAL

LifeSensors Inc.
271 Great Valley Parkway
Malvern, PA 19355

www.lifesensors.com
techsupport@lifesensors.com
sales@lifesensors.com
610.644.8845 (phone)
610.644.8616 (fax)

©2011 LifeSensors, Inc. All Rights Reserved.

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.

SUMOstar Fusion Technology

Yeast SUMO (Smt3) fused with a protein of interest can dramatically enhance expression and promote solubility and correct folding of the protein. SUMOstar, a version of SUMO that is not recognized nor cleaved by natural desumoylases, has been developed primarily 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 both yield and solubility. Attachment of a highly stable protein (ubiquitin or SUMO) at the N-terminus of a partner protein confers stability to the recombinant fusion protein, subsequently increasing its yield. The enhanced solubility demonstrated by fusing Ub/Ubls to the N-terminus of the protein-of-interest may be explained by improved/more rapid folding of the POI resulting from nucleation by the Ubl.

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 hydrolases, or proteases – the enzymes that release the partner protein from ubiquitin by hydrolyzing the peptide bond. In addition, ubiquitin is not a convenient tag in eukaryotic cells since ubiquitinated proteins are targeted for degradation by the proteasome. Other 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 residual amino acids at the cleavage site.

The establishment of the SUMOpro system is largely due to the nature of SUMO protease 1 (Ulp1), a SUMO equivalent of ubiquitin protease. SUMO Protease 1 is superior to other proteases commonly used in recombinant protein production, as it recognizes the 3-dimensional structure of SUMO 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 has not been observed to cleave indiscriminately within the protein-of-interest. Based on SUMO Protease 1, LifeSensors, Inc. has engineered a cognate protease for the cleavage of the otherwise resistant SUMOstar tag, SUMOstar Protease. SUMOstar protease retains all the advantages of SUMO Protease1, and largely exhibits the same characteristics in terms of activity, robustness, and tolerance of wide ranging conditions.

About the pI-secSUMOstar and pI-SUMOstar vectors

This vector is fully compatible with all existing Bac-to-Bac® BEVS technologies (Invitrogen). Expression is driven through the powerful late-stage *polh* promoter. For pI-secSUMOstar, a modified form of the glycoprotein 67 (gp67) signal sequence directs the secretion of your protein-of-interest as a SUMOstar fusion.
Cloning in *E. coli* is facilitated through the inclusion of a marker for ampicillin resistance.

Advantages

1) SUMOstar fusion dramatically enhances expression levels.

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 immunodetection (Western blotting).

5) SUMOstar cleavage of the fusion construct yields native protein with the 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: 20 µg (0.5 µg/µl)
Buffer: 10mM Tris, pH 8.0

2) SUMOstar Protease 1 (Cat. No. 4110)
Size: 1000 units (10 units/µl)
Buffer: 25 mM Tris-HCl, pH 8.0
150 mM NaCl
2mM DTT
10% glycerol

3) SUMOstar Control Protein (Cat. No. 5010)
Size: 100 µg (Lyophilized)
Buffer: PBS, pH 7.2

4) Affinity purified AntiSUMO/SUMOstar Antibody (Chicken IgY, Cat. No. AB7002)
Size: 50 µg,(1.0 mg/ml)
Buffer: PBS, pH 7.2

Storage

pI-SUMOstar Vector or pI-secSUMOstar Vector
Store vial at -20° C or below.

SUMOstar protease 1
For short-term use, store at +4° C. Long-term storage should be at -80° C. Avoid multiple freeze/thaw cycles.

SUMOstar Control Protein
Store vial at -80° C. Reconstitute with 100 µL of H_20 to yield 1 mg/mL. Avoid cycles of freezing and thawing.

AntiSUMO/SUMOstar Antibody
For short-term use (several weeks), store at 4° C. Long-term storage should be at -80° C.

Cloning

Background of Cloning Strategy

The vector is provided as a circular plasmid. For cloning, the vector must be digested with BsmB1 (a.k.a.Esp3I) restriction endonuclease. This Class IIS restriction enzyme recognizes non-palindromic sequences and cleaves at sites that are removed from their DNA 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 PCR primer, the site is removed from the PCR product when digested, meaning that there will be no additional nucleotides between SUMOstar and your gene-of-interest (GOI). Second, overhangs created by Class IIS enzymes are template-derived and thus unique.

Digestion of the vector with BsmBI results in the release of a small fragment, leaving two **unique** overhangs: 5'-**ACCT**-3' and 5'-**CTAG**-3' (compatible with XbaI) . **Please refer to the polylinker map for a detailed illustration.** This strategy allows for directional insertion of the GOI. Additionally, the **ACCT** at the end of the SUMOstar coding sequence results in the GOI being cloned "in frame" with the SUMOstar tag.

Forward Primer Design (Cloning)

To clone your gene of interest into the vector, it must be amplified by PCR with primers designed to specifically work in the above cloning strategy.

Below is an example of forward primer design incorporating a BsmBI Class IIS restriction site.

BsmB1: **5'–**NN **CGTCTCNAGGT** XXX NNN NNN NNN NNN NNN**–3'**

In this primer, **CGTCTC** is the BsmB1 recognition sequence, N is any nucleotide, and **AGGT** will be the overhang generated upon BsmB1 digestion. This sequence ends with **GGT** (the last codon of the SUMOstar tag), followed by XXX, the first codon of your GOI. Additional nucleotides may be required for the primer to anneal specifically to your GOI during PCR amplification.

If your GOI 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' - NN **CACCTGC**NNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3'
BbsI: 5' – NN **GAAGAC**NN**AGGT** XXX NNN NNN NNN NNN NNN– 3'
BbvI: 5' – NN **GCAGC**NNNNNNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3'
BfuAI: 5' – NN **ACCTGC**NNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3
BsaI: 5' – NN **GGTCTC**N**AGGT** XXX NNN NNN NNN NNN NNN– 3
BsmAI: 5' – NN **GTCTC**N**AGGT** XXX NNN NNN NNN NNN NNN– 3
BsaI: 5' – NN **GGTCTC**N**AGGT** XXX NNN NNN NNN NNN NNN– 3
BsmFI: 5' – NN **GGGAC**NNNNNNNNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3
BtgZI: 5' – NN **GCGATG**NNNNNNNNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3
FokI: 5' – NN **GGATG**NNNNNNNNN**AGGT** XXX NNN NNN NNN NNN NNN– 3
SfaNI: 5' – NN **GCATC**NNNNN**AGGT** XXX NNN NNN NNN NNN NNN– 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 (Cloning Strategy 1)

The reverse primer should contain one of the restriction enzyme sites from the vector MCS, allowing directional cloning of your GOI. We recommend that XbaI be employed as the restriction site in the reverse primer in your PCR product. **If your insert contains an XbaI site or if the digestion of the PCR insert with BsmBI alone is preferred for any reason**, **please see Strategy 2 below.** Upon digestion of this PCR product with BsmBI (5') and XbaI (3'), a fragment will be generated having overhanging sequence complementary to the expression vector linearized with BsmBI alone (remember that BsmBI digestion of the vector yields these two unique ends).

An example of a reverse primer for this purpose is:

XbaI: 5' – NN **TCTAGA** TTA XXX NNN NNN NNN NNN – 3'

where **TCTAGA** is the XbaI recognition sequence, TTA is the reverse complement of the stop codon TAA (thought to be preferred in BEVS), XXX is the reverse complement of the final codon, followed by the remainder of your GOI. Again, it is recommended that extra bases be added to the 5' end, as noted above.

Reverse Primer Design (Cloning Strategy 2)

If for any reason BsmB1/XbaI is not a viable option, some flexibility exists in reverse primer design. For example, **an XbaI overhang can be generated in your PCR product without XbaI digestion**, avoiding problems stemming from having an XbaI site within your GOI. Incorporation of a BsmB1 site (or any other Class II Restriction Enzyme listed above) in front of the XbaI sequence allows for digestion with BsmB1 enzyme yielding the same 5'-**CTAG** overhang.

An example of a reverse primer for this purpose is:

BsmB1/XbaI: 5' – NN **CGTCTC TCTAGA** TTA XXX NNN NNN NNN NNN – 3'

where **CGTCTC** is the BsmB1 recognition site that directs cleavage and generation of the overhang **CTAG**. Again, TTA is the reverse complement of the stop codon TAA, XXX is the reverse complement of the final codon, followed by the remainder of your GOI.

Preparation of Insert

After determining the cloning strategy to be employed from those outlined above, generate your PCR product with a thermostable polymerase according to the manufacturer's instructions. For maximal sequence integrity during PCR, the use of thermostable polymerases capable of proof reading activity (e.g. *Pfu*, Stratagene; DeepVent, New England Biolabs; or *Taq* HIFi, Invitrogen) is recommended. After purification with standard techniques (Sambrook, et. al.), digest the PCR product with the desired restriction enzymes (according to the manufacturer's instructions). The PCR product is now ready for direct cloning into the expression vector. Alternatively, the PCR product can be ligated into a sub-cloning vector (e.g. pBluescript) and sequenced prior to this step.

Preparation of Vector

The expression vector is provided as a 20µg aliquot. It can be digested directly with restriction enzymes according to the desired strategy outlined above. Using standard techniques, (Sambrook, et. al.) purify the digested plasmid for ligation.

©2011 LifeSensors, Inc. All Rights Reserved.

DNA Ligation

For ligation of the prepared insert into the digested vector, T4 DNA ligase and standard ligation protocols should be employed (Sambrook et al). **Because cloning is directional, alkaline phosphatase treatment of vector should be unnecessary, but may be beneficial in lowering background of (re-ligated) singly digested plasmid.** The T4 DNA Ligase should be used as described by the manufacturer (e.g. MBI Fermentas, New England Biolabs, Roche, Stratagene, Promega).

Transformation

Following the manufacturer's recommendations, the ligation mixture can be transformed into competent *E. coli* by either chemical transformation or electroporation. Standard bacterial strains (e.g. DH5α, TOP10, etc.) should be used as they show a high propensity for DNA uptake, and have abolished *RecA* and *EndA* activity. Selection of clones containing the desired product should be in the presence of ampicillin (50-100µg/ml). For construct integrity, propagation in *E. coli* should always be in the presence of the selective agent.

Identification of Positives Clones

With directional cloning, positive identification can easily be accomplished by PCR screening using external priming sites present in the vector:

polH (forward) 5'-GGA TTA TTC ATA CCG TCC CAC CAT-3'
Tn7 (reverse) 5'-CTG GGT GTA GCG TCG TAA GCT AAT AC-3'

This can be done either on purified plasmid DNA or by so-called "colony PCR." Plasmid clones can also be checked by restriction endonuclease digestion. Prior to generation of baculovirus, the integrity of the PCR generated gene-of-interest should be verified by DNA sequencing.

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, transfection, and viral propagation.

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 medium. The transfected insect cells should be pelleted by centrifugation and the medium 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 His_6-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 medium components that strip Ni^{2+} from the metal chelating resin (dialysis or 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, a highly active and robust recombinant protease, cleaves SUMOstar from recombinant fusion proteins. Unlike thrombin, EK, or TEV proteases, whose recognition sites are short, linear sequences, SUMOstar protease recognizes the tertiary sequence of SUMOstar. As a result, SUMOstar protease does not

©2011 LifeSensors, Inc. All Rights Reserved.

cleave within the 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.

Unit Definition
One unit of SUMOstar protease 1 cleaves 90-100 µg of SUMOstar Control Protein in 1 h at 30°C.

Digestion of SUMOstar fusion tag

1. Dialyze the IMAC purified SUMOstar fusion proteins (4°C) against an appropriate physiological buffer (e.g. 20 mM Tris-HCl, 150 mM NaCl, pH 8.0). If the dialysis volume does not exceed >100-fold sample size, multiple buffer changes (each 4h or greater) should be employed to effectively remove salts or detergents.

2. Add SUMOstar Protease (1U per 100 µg of substrate) and incubate at 30°C for 1 h in the presence of 1-5mM dithiothreitol (DTT).

3. In addition, the following guidelines may be helpful:

   i. If your protein of interest is sensitive to reducing agents, a less aggressive agent (e.g. BME or TCEP) can be used. Longer incubation times may be required.
   ii. If low cleavage efficiency is observed, consider increasing the time and/or amount of SUMOstar protease. Overnight at 4°C, for example, may be convenient.
   iii. Consider adding SUMOstar protease incrementally throughout the time course of the reaction.
   iv. If a fraction of the fusion protein is mis-folded or aggregated, it may be resistant to digestion.

Removal of SUMOstar fusion tag and SUMOstar protease 1
SUMOstar and SUMOstar Protease both contain HIS_6 tags at their N-termini; therefore, SUMOstar and SUMOstar Protease can be easily removed from the cleavage reaction by IMAC. The recombinant protein-of-interest is recovered in the flow through from such an additional IMAC step. 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 fused to a polypeptide, and can be used to control for SUMOstar protease activity. Incubation of 1 Unit of SUMOstar protease with 100µg of SUMOstar Control Protein should result in >90% cleavage after 1hour at 30C. Please note that the SUMOstar tag runs at 18kDa on SDS-PAGE following cleavage, with uncleaved SUMOstar Control Protein running at 47 kDa.

Digestion of Control Protein

1. Incubate SUMOstar Protease 1 and SUMOstar Control Protein (1U per 100µg) for 1h at 30°C.

2. Add 5X SDS-PAGE sample prep buffer to digestion reaction.

3. Heat sample at 95°C for 5 min – This heating step is essential to insure complete denaturation of the control protein.

4. Analyze cleaved SUMOstar Control Protein (5-10µg) by SDS-PAGE and Coomassie staining.

Western Blots
Anti-SUMOstar is an affinity purified hen polyclonal IgY antibody that reacts with SUMOstar in Western blot and ELISA applications. For immunoblotting, a 1:1,000 to 1: 5,000 dilution is recommended. At these dilutions, 10-50ng of SUMOstar Control Protein should be detectable as a control. For ELISA a 1:5,000 to 1:25,000 dilutions is recommended. Optimal dilutions for other applications should be determined empirically.

©2011 LifeSensors, Inc. All Rights Reserved.

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.

LifeSensors Inc.
271 Great Valley Parkway
Malvern, PA 19355
610 644 8845 phone
610 644 8616 fax
sales@lifesensors.com
techsupport@lifesensors.com

To order a LifeSensors product

- **Order Online** at www.lifesensors.com
- **Call** 1 610 644 8845
- **Fax an Order** 1 610 644 8616

Orders are shipped within 24 hours of order receipt for all reagents in stock. Orders placed on Friday will be shipped on the subsequent Monday.

Copyright © 2007 LifeSensors, Inc. All Rights Reserved