SUMOstar™ Gene Fusion Technology

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

SUMOstar Expression Systems for *Saccharomyces cerevisia*

Cat. No. 2100 (Kit)
2101 (Vector)

Product Manual

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Background

Ubiquitin and SUMO

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 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, Nedd8, ISG15, Apg8 and Apg12. Proteins of the second class include 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 highly conserved from yeast to humans. SUMO has an apparent molecular weight of ~20kDa. 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.

SUMO Fusions

Adapting its natural function, SUMO recombinantly fused with a protein of interest can dramatically enhance expression and promote solubility and correct folding of the protein. It has been long known that ubiquitin exerts chaperoning effects on fused proteins in E. coli and yeast. 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.

SUMOstar is a Universal SUMO tag for Prokaryotes and Eukaryotes

When expressed as fusions in eukaryotic cells, wild type SUMO tags (e.g., SUMO or SUMO3) are recognized by endogenous SUMO proteases and the tag gets cleaved. Thus, wild type SUMO tags are useful for enhanced protein production and affinity purification exclusively in prokaryotic systems. This critical limitation is completely eliminated with the universal SUMOstar tag. SUMOstar is a SUMO based tag mutated such that it is not recognized or cleaved by wild type SUMO proteases. However, SUMOstar is recognized and is efficiently cleaved by the correspondingly engineered SUMOstar protease1.

Purification and SUMOstar Protease 1

Although ubiquitin fusion can also 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. Similarly, commonly used proteases such as thrombin, enterokinase, rhinovirus proteases, and TEV, also 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.

SUMO Protease 1 has been evaluated for purification of proteins expressed in E. coli and has been found superior to all of the proteases commonly used in recombinant protein production (see above). SUMO Protease 1 recognizes the three-dimensional 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 at P1). The cleavage is extremely efficient, and completely accurate in generating native N-termini from SUMO-fused proteins of interest.

SUMOstar protease cleaves SUMOstar tag as efficiently as SUMO protease 1 cleaves SUMO tag. SUMO proteases 1 and 2 do not cleave the SUMOstar tag.

Benefits

The benefits of the SUMOstar Expression System.

1) SUMOstar fusion may dramatically enhance expression.

2) SUMOstar fusion may dramatically enhance solubility.

3) SUMOstar Protease 1 will not cleave within the fused protein of interest.

Cleavage 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) **pY-SUMOstar (Amp^r, TRP1, pCUP1))**
   Size: 20 µg (1.0 µg/µl)
   Buffer: 10 mM Tris

2) **SUMOstar Protease 1**
   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) **SUMOstar-GFP**
   Size: 100 µg (5.0 µg/µl)
   Buffer: PBS

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

Storage

pY-SUMOstar Vector (Amp^r, TRP1, pCUP1)
Store vial at -80° C or -20° C. Avoid cycles of freezing and thawing.

SUMOstar Protease
For short-term use, store vial at -80° C or +4° C. For long-term use, store vial at -80° C. Avoid cycles of freezing and thawing.

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

SUMO Antibody
Store vial at 4° C prior to restoration. Restore with 0.5 ml of PBS. For extended storage aliquot contents and freeze at -20° C or -80° C. 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. Dilute only prior to immediate use. Expiration date is one (1) year from date of restoration.

Cloning

Background
The pY-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 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 pY-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 extraneous sequence between SUMO 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 BsaI:

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

where **GGTCTC** is the BsaI recognition sequence, N is any nucleotide, and AGGT the overhang that is complementary with the ACCT end of the pY-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**NNNNNNNNAGGTXXXXXXXXXXXXXXXX – 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**NNNNNAGGTXXXXXXXXXXXXXX – 3'

NOTE: on primer design: As a general practice it is recommended that 2 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 pY-SUMOstar, to allow directional cloning of your gene of interest into the vector. It is recommended that XbaI be employed as the restriction site in the reverse primer, since 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, the BsaI restriction site, used in the forward primer, could be added in front of XbaI site. In this case, 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 SacI, SalI, HindIII, NotI, EagI, and XhoI. Below are examples of reverse primers for each of these sites:

BamHI 5' **- GGATCC**TCAxxx… – 3'
SalI: 5' – **GTCGAC**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 pY-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 pY-SUMOstar vector plasmid is provided as a 20 µg 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. The digested plasmid can then be used for ligation.

Ligation
For ligation of the prepared insert into the digested pY-SUMOstar vector, T4 DNA ligase and standard ligation protocols should be employed. 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., Fermentas, New England Biolabs, Roche, Stratagene, Promega).

Transformation
Following incubation of the ligation reactions, plasmids can be transformed into competent E. coli by either chemical transformation or electroporation. Because the ligation is directional, there should be little or no occurrence of no-insert background colonies.

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) 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

Yeast Strains and Growth Media
Expression with the pY-SUMOstar plasmid can be performed in most S. cerevisiae *trp1* strains, except for those that are copper-sensitive (*cup1*^{s}). The following are examples of yeast strains suitable for overexpression of proteins: strain BJ3505 (MATα *pep4 his3PR6-1'Δ1.6R his3 lys2-208 trp1-Δ ura3-52 gal2*) and strain BJ1991 (MATα *prb1-1122 pep4-3 leu2-1 trp1 ura3-52 gal2*).

Once it is constructed and verified, the pY-SUMOstar plasmid containing your gene should be transformed into a *trp1* strain by standard methods (Ito et al., 1983) and cells should be plated on synthetic media lacking tryptophan (SC-trp media, per liter: 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 2 g amino acid supplement mix minus tryptophan, 20 g glucose) in order to select for transformants. The pY-SUMOstar vector utilizes a copper metallothionein (*CUP1*) promoter of yeast to induce gene synthesis, and 100 µM copper sulfate is added to a synthetic minimal medium culture to overexpress your SUMOstar-tagged protein of interest. NOTE: Because synthetic minimal medium contains traces of copper sulfate, low-level synthesis of the recombinant protein may occur without addition of copper. Because expression of certain proteins in yeast may retard their growth rate, it is advisable to monitor the growth rate of the strain before large-scale expression studies are carried out.

Culture
(i) Small-scale culture
Inoculate several ml of synthetic minimal media with a single colony from your transformed plate and shake overnight at 30°C. In both the small- and large-scale cultures, be sure that the minimal medium is supplemented with any nutrient for which your strain is auxotrophic (but do not add tryptophan).

(ii) Large culture
Inoculate your overnight culture into the desired volume of synthetic minimal media and grow cells with vigorous shaking at 30°C. Grow the cells to an absorbance at 600 nm of about 0.8 (mid-log phase) and then induce with 100 µM copper sulfate and continue growth for 3-6 hours. Harvest the cells by centrifugation for several minutes at 3000 rpm, wash twice by resuspending in lysis buffer and centrifuging again, and you are ready for the purification step. Alternatively, the cell pellet can be stored at –80°C indefinitely.

(iii) Pilot Expression
To analyze protein production on a small scale before preparing a large-scale culture, perform the above induction with just 5 ml culture. Harvest cells, wash twice with dH_20, resuspend in 200 µl of 0.1 N NaOH. Keep 10 min at room temperature. Re-pellet the cells and aspirate the NaOH. Re-suspend cells in 200 µl of SDS-PAGE sample buffer and boil 5 minutes in an Eppendorf tube. Centrifuge the suspension and run 1-10 µl on an SDS-PAGE gel for protein analysis by staining (e.g. Coomassie blue) or Western blot visualized with SUMO Antibody.

Purification

Cells can be lysed in several ways, including breakage with glass beads (such as with a Bead Beater apparatus) or enzymatic lysis (zymolyase or lyticase). Lysis should be performed at a pH optimal for the first step of purification (between 7.0 and 7.4), and PMSF or commercially available protease inhibitor tablets should be included in the lysis buffer if you fear your protein is susceptible to degradation by endogenous yeast proteases. After lysis, centrifuge the suspension at 4°C to remove cell debris and obtain the cell extract.

The presence of a hexahistidine tag at the N-terminus of the SUMOstar protein sequence allows for simple and 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 10-20 mM Imidazole
4) Elute with 250-300 mM 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 1 never cleaves within the fused 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 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 100 µg of SUMOstar-GFP in 1 h at 30°C.

Cleavage
1. Dialyze the purified SUMO 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 1 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 1 contain a polyhistidine tags at the N-terminus; therefore, SUMOstar and SUMOstar Protease 1 are simultaneously removed from the cleavage reaction by IMAC affinity chromatography.

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

Controls and Validations

SUMOstar-Met-GFP
SUMOstar-Met-GFP is a recombinant fusion protein that contains the SUMOstar (Small Ubiquitin-like MOdifier) tag and GFP (Green Fluorescent Protein). A typical application for SUMOstar-GFP is a control study on an SDS-PAGE gel. In the control study, SUMOstar-GFP is loaded with SUMOstar Protease 1 to compare the cleavage of your SUMOstar fusion with that of SUMOstar-GFP. Please note: SUMOstar is 10 kDa but runs on an SDS-PAGE gel as 20 kDa; as a result, SUMOstar-GFP runs as 49 kDa on an SDS-PAGE gel, even though SUMOstar-GFP (353 amino acids) is actually 39 kDa.

Running a Control with SUMOstar-GFP

1. Incubate 100 µg SUMOstar-GFP and 1 unit of SUMOstar Protease 1 (total 100 µL) at 30°C for 1 h.

2. Take 12 µL aliquot from 100 µL sample 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-SUMO/SUMOstar is an affinity purified, Chicken polyclonal IgY antibody that reacts with SUMO and SUMOstar on Western blots.

Recommended Dilution(s) for the anti-SUMO/SUMOstar Ab.

For immunoblotting, a 1:1000 to 1:5,000 dilution is recommended. Most yeast cell lysates can be used as a positive control without induction or stimulation. Researchers should determine optimal dilutions for other applications.

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From the LifeSensors Team