SUMOstar™ Gene Fusion Technology

ENHANCING FUNCTIONAL PROTEIN EXPRESSION AND PURIFICATION IN YEAST

Yeast (*Pichia pastoris*) Secretory SUMOstar Expression System

Product Manual

Catalog Numbers

2160 (Kit)
2161 (Vector)

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Table of Contents

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Background

Ubiquitin, UBLs, and SUMO

In cells, post-translational modification of proteins by the molecule ubiquitin (Ub) is well known to affect protein degradation, compartmentalization, and in some cases even function. In addition, an entire class of proteins known as ubiquitin-like modifiers (UBLs) regulates cellular processes through similar means, and has been the subject of intense research. Examples of UBLs include ISG15, Nedd8, Apg8, Apg12, FAT10, and SUMO. Covalent attachment of SUMO (small ubiquitin-like modifier) modulates protein-protein and protein-DNA interactions, as well as localization and stability of the target protein. This sumoylation occurs in most eukaryotic systems, with SUMO being highly conserved from yeast to humans. Like ubiquitylation, sumoylation is a reversible, dynamic process regulated by enzymes responsible for either the attachment (ligases) or removal (desumoylases) of the molecule. SUMO and ubiquitin share approximately 18% primary sequence homology; however, they share a high degree of structural homology, possessing a common three-dimensional structure, characterized by a tightly packed globular fold consisting of β-sheets wrapped around an α-helix.

SUMOstar Fusion Technology

LifeSensors' SUMOpro system utilizes yeast SUMO (Smt3) fused with a protein of interest for dramatic enhancement of expression and solubility. When coupled with a prokaryotic expression system, this fusion tag is well established as a superior system for expression of heterologous proteins. Attachment of a highly stable protein (ubiquitin or SUMO) at the N-terminus of a partner protein confers stability to the recombinant fusion protein, in addition to exerting chaperoning effects which subsequently increase yield. The enhanced solubility demonstrated by fusing Ub/UBLs to the N-terminus of the protein of interest may be explained by rapid folding kinetics of the protein resulting from nucleation by the UBL. This use of SUMOpro in eukaryotes is prohibited by the presence of native desumoylases that would rapidly cleave the fusion protein, negating the enhancing effect of SUMO. To this end, LifeSensors has developed SUMOstar, a mutant form of Smt3 that is not recognized or cleaved by natural desumoylases.

Recombinant Protein Purification and SUMOstar Protease

SUMO proteases are superior to other proteases commonly used for fusion tag removal with respect to activity and fidelity. SUMO proteases recognize the tertiary structure of SUMO and cleave precisely and efficiently at the N-terminus of the protein of interest, irrespective of the identity of the N-terminal residue (except proline). SUMO proteases have not been observed to cleave indiscriminately within the protein of interest. Other commonly used proteases such as thrombin, enterokinase, rhinovirus proteases, and TEV, do not cleave all fusions efficiently and leave heterogeneous, non-native N-termini. Moreover, some of these enzymes readily cleave within the protein of interest. Based on SUMO Protease 1, LifeSensors has engineered SUMOstar Protease 1, a cognate protease for the cleavage of the otherwise resistant SUMOstar tag. SUMOstar Protease 1 retains all the advantages of SUMO Protease 1, exhibiting the same characteristics in terms of activity, robustness, and tolerance of wide-ranging conditions.

About the SUMOstar Vector

The pY-secSUMOstar vector is a circular 3894 bp shuttle vector for *Pichia pastoris* with a Zeocin™ selection marker. Expression in yeast is driven from an alcohol oxidase 1 promoter (*AOX1*), while transcriptional termination is provided by the *CYC* terminator. As described below, cloning your gene of interest into this vector will result in direct, in-frame N-terminal fusion of your protein to the C-terminus of 6xHis and FLAG-tagged SUMOstar protein.

Advantages

The benefits of the SUMOstar Expression System:

1) SUMOstar fusion enhances expression levels.

2) SUMOstar fusion can enhance protein solubility.

3) SUMOstar can be used for affinity purification and immunodetection.

4) SUMOstar Protease 1 does not cleave within the fused protein of interest.

5) SUMOstar fusion system cleavage yields protein with desired N-termini.

Components and Storage

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

1) **SUMOstar Vector (pY-secSUMOstar, Zeo, AOX1)**
   Size: 20 µg (0.5 µg/µL)
   Buffer: 10 mM Tris
   Storage: store vial at -20°C

2) **SUMOstar Protease 1**
   Size: 500 units
   Buffer: 25 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 10% glycerol
   Storage: store vial at -80°C; avoid cycles of freezing and thawing

3) **SUMOstar Control Protein (SUMOstar – GFP)**
   Size: 100 µg (5 µg/µL)
   Buffer: PBS
   Storage: store vial at -80°C; avoid cycles of freezing and thawing

4) **SUMOstar Antibody (chicken IgY anti – SUMOpro/SUMOstar)**
   Size: 50 µg
   Buffer: 25 mM Tris-HCl, pH 7.5
   150 mM NaCl
   Storage: For extended storage, aliquot contents and freeze at -20°C or below; 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.

Cloning

Background

The vector is provided as a circular plasmid. For cloning, the vector must be digested with BsmBI restriction endonuclease. This Type IIS restriction enzyme recognizes non-palindromic sequences and cleaves at sites that are outside of its DNA recognition sequence. The latter trait gives Type IIS enzymes two useful properties. First, when a Type 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. Second, overhangs created by Type IIS enzymes are variable instead of fixed, so these sites can be used to design vectors and inserts that will have unique ends upon restriction digest.

Digestion of the vector with BsmBI results in the release of a small fragment, leaving two unique overhangs: 5'– ACCT and 5'– CTAG (compatible with XbaI). Please refer to the polylinker map for a detailed illustration (page 12). This strategy allows for directional insertion of the gene of interest. Additionally, the ACCT at the end of the SUMOstar coding sequence results in the gene of interest being cloned "in frame" with the SUMOstar tag.

Forward Primer Design

To clone your gene of interest into the SUMOstar vector, it must be amplified by PCR and digested to produce a 5' overhang complementary to the vector's ACCT.

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

BsmBI: 5' – N N **C G T C T C** N **A G G T** X X X N N N N N N … – 3'

In this primer, N is any nucleotide, **CGTCTC** is the BsmBI recognition sequence, **AGGT** will be the 5' overhang that is complementary with the ACCT end of the SUMOstar vector, and XXX is the first codon of your gene of interest. 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 BsmBI site, then another Type IIS enzyme and site may be used instead. Below are examples of forward primers for some of these enzymes/sites:

AarI: 5' – N N **C A C C T G C** N N N N **A G G T** X X X N N N N N N … – 3'
BbsI: 5' – N N **G A A G A C** N N **A G G T** X X X N N N N N N … – 3'
BbvI: 5' – N N **G C A G C** N N N N N N N N N **A G G T** X X X N N N N N N … – 3'
BfuAI: 5' – N N **A C C T G C** N N N N N **A G G T** X X X N N N N N N … – 3'
BsaI: 5' – N N **G G T C T C** N **A G G T** X X X N N N N N N … – 3'
BsmAI: 5' – N N **G T C T C** N **A G G T** X X X N N N N N N … – 3'
BsmFI: 5' – N N **G G G A C** N N N N N N N N N **A G G T** X X X N N N N N N … – 3'
BtgZI: 5' – N N **G C G A T G** N N N N N N N N N N **A G G T** X X X N N N N N N … – 3'
FokI: 5' – N N **G G A T G** N N N N N N N N N **A G G T** X X X N N N N N N … – 3'
SfaNI: 5' – N N **G C A T C** N N N N N **A G G T** X X X N N N N N N … – 3'

NOTE: As a general practice it is recommended that two or more 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 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 the SUMOstar Vector (see page 12), 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 BsmBI/XbaI-linearized form that can be used for ligations without further treatment. An example of a reverse primer for this purpose is:

XbaI: 5' – N N **T C T A G A** T C A X X X N N N N N N … – 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. Since XbaI is a Type I enzyme, note that the cut site is within the recognition sequence. 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 you prefer to digest the PCR insert with a single restriction endonuclease, the restriction site used in the forward primer could be added 5' of XbaI site. For example, if BsmBI site is added 5' of XbaI site, the digestion either with XbaI or BsmBI enzyme gives the same 5'-CTAGA overhang:

BsmBI/XbaI: 5' – N N **C G T C T C N C T A G A** T C A X X X N N N N N N … – 3'

If your gene of interest contains an XbaI site, another cloning site available for reverse primer design is SalI, shown below:

SalI: 5' – N N **G T C G A C** T C A X X X N N N N N N … – 3'

Preparation of Insert

After determining the cloning strategy to be employed, 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*, Agilent; DeepVent, New England Biolabs; or *Taq* HiFi, Invitrogen) is recommended. After purification with standard techniques (Sambrook and Russell, 2001), 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.

Preparation of Vector

The SUMOstar Vector plasmid is provided as a 20 µg aliquot of circular vector that has to be digested with BsmBI alone or in combination with any of the polylinker enzymes, gel-purified and extracted using standard techniques (Sambrook and Russell, 2001). The digested plasmid can then be used for ligation.

Ligation

For ligation of the prepared insert into the digested SUMOstar Vector, T4 DNA ligase and standard ligation protocols should be employed (Sambrook and Russell, 2001). Since the ligation is directional, phosphatase treatment of vector is unnecessary, though it may be beneficial in lowering background of (re-ligated) singly digested plasmid. The T4 DNA Ligase should be used as described by its manufacturer.

Transformation of *E. coli*

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 and TOP10 should be used for transformation because they show a high propensity for foreign DNA uptake and have mutations abolishing the activity of the genes *RecA* and *EndA*. Selection of clones should be in the presence of 100 µg/mL Zeocin™ to ensure construct integrity. Zeocin selection is inhibited by salt concentrations above 5g/L (110mM). Transformed bacteria should be cultured on media made with low salt LB (see recipe under 'Media').

Identification of Positive Clones

With directional cloning, identification of clones can be determined by PCR amplification using priming sites present in the vector:

Forward Primer (AOX1 priming region):
5' – G A C T G G T T C C A A T T G A C A A G C – 3'

Reverse Primer (AOX1 termination region):
5' – G C A A A T G G C A T T C T G A C A T C C – 3'

This may be performed on either purified plasmid DNA or by colony PCR. Plasmid clones can also be checked by restriction endonuclease digestion.

Transformation of *Pichia*

You will need the following reagents for transforming *Pichia* and selecting transformants using Zeocin™ as a selection marker. Note: Inclusion of sorbitol in YPD plates stabilizes electroporated cells, as they are somewhat osmotically sensitive.

- 5 - 10 µg SUMOstar Vector containing your insert
- YPD Medium
- 50 mL conical polypropylene tubes
- 1 L cold (4°C) sterile water (place on ice the day of the experiment)
- 25 mL cold (4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
- 30°C incubator
- Electroporation device and 0.2 cm cuvettes
- YPDS plates containing 100 µg/mL Zeocin™

1) Grow 5 mL of your *Pichia pastoris* strain in YPD in a 50 mL conical tube at 30°C overnight.

2) Inoculate 500 mL of fresh medium in a 2 L flask with 0.1 - 0.5 mL of the overnight culture. Grow to an OD_{600} of 1.3 - 1.5.

3) Centrifuge the cells at 1500 g for 5 minutes at 4°C. Resuspend the pellet with 500 mL of ice-cold (0°C), sterile water.

4) Centrifuge the cells as in Step 3, and then resuspend the pellet with 250 mL of ice-cold, sterile water.

5) Centrifuge the cells as in Step 3, then resuspend the pellet in 20 mL of ice-cold, 1 M sorbitol.

6) Centrifuge the cells as in Step 3, then resuspend the pellet in 1 mL of ice-cold, 1 M sorbitol for a final volume of approximately 1.5 mL. Keep the cells on ice and use the same day. Do not store or freeze cells.

7) Linearize the plasmid DNA by digesting with PmeI, SacI, or BstXI.

8) Mix 80 µL of the cells with 5 - 10 µg of linearized DNA in 5 - 10 µL sterile water and transfer them to an ice-cold 0.2 cm electroporation cuvette.

9) Incubate the cuvette containing the cells on ice for 5 minutes, and then pulse the cells according to the manufacturer's instructions for yeast.

10) Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 mL tube.

11) Let the tube incubate at 30°C without shaking for 1 to 2 hours.

12) Spread 10, 25, 50, 100, and 200 µL each on separate, labeled YPDS plates containing 100 µg/mL Zeocin™. Plating at low cell densities favors efficient Zeocin™ selection.

13) Incubate plates from 3 - 10 days at 30°C until colonies form.

14) Pick 10 - 20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100 µg/mL Zeocin™.

Expression

1) Using a single colony, inoculate 25 mL of BMGY in a 250 mL baffled flask. Grow at 30°C in a shaking incubator (250 rpm) until culture reaches an OD_{600} of 2 - 6 (approximately 16 - 18 hours). The cells should be in mid-log phase growth.

2) Harvest the cells by centrifuging at 1500 - 3000 g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD_{600} of 1.0 in BMMY medium to induce expression (approximately 100 - 200 mL).

3) Add 100% methanol to a final concentration of 0.5%; note that you need to add methanol every 24 hours to maintain induction.

4) Place culture in a 1 L baffled flask. Cover the flask with sterile gauze or cheesecloth and return to incubator to continue growth.

5) At each of the times indicated below, transfer 1 mL of the expression culture to a 1.5 mL microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2 - 3 minutes at room temperature. Time points (hours): 0, 12, 24, 48, and 72.

6) Transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at -80°C until ready to assay.

7) Analyze the supernatants and cell pellets for protein expression by Coomassie stained SDS-PAGE, Western blot, or functional assay.

To analyze intracellular protein production, wash cell pellet twice with dH_20, resuspend in 200 µL of 0.1 N NaOH. Incubate 10 minutes at room temperature. Pellet the cells and aspirate the NaOH. Resuspend cells in 200 µL of SDS-PAGE sample buffer and boil 5 minutes. 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 analysis.

Purification

The presence of a secretory signal followed by a FLAG-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). Follow the nickel resin manufacturer's instructions for use. Note that you **must** dialyze the supernatant before beginning chromatography.

Tag removal

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, and as a result, 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. The effects of various chemicals on the activity of SUMOstar Protease 1 are listed in the following table (from Malakhov et al. 2004):

Chemical	Concentration	Activity %
Phosphate-buffered saline		100
Standard buffer*		100
DTT or β-mercaptoethonal	20 mM	100
NaCl	150 mM	100
	500 mM	60
	1 M	30
Urea	1 M	100
	2 M	95
	3 M	5
Gu-HCl	500 mM	60
	1 M	0
Triton X100	1 M	100
Imidazole	300 mM	100
GSH (Glutathione reduced)	20 mM	100
Maltose	20 mM	100
Glycerol	20% v/v	100
Ethylene glycol	20% v/v	100
Sucrose	20% w/v	100
Ethanol	10% v/v	100

Reactions were performed in either PBS or Standard buffer (20 mM Tris, 5 mM β-mercaptoethanol) with or without modificaitons, and were performed at 25°C.

Unit Definition

One unit of SUMOstar Protease 1 cleaves 100 µg of SUMOstar – GFP Control Protein in 1 hour at 30°C

Digestion of SUMOstar

1) Dialyze the purified SUMO fusion proteins for at least 24 hours at 4°C against an appropriate physiological buffer (e.g. 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 10% glycerol) or against PBS. 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 1 (one unit per 100 µg of substrate) and incubate at 30°C for 1 hour 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].

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

   a) 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.

   b) If low cleavage efficiency is observed, consider increasing the time and/or amount of SUMOstar protease. For examples, overnight at 4°C may be convenient.

   c) Consider adding SUMOstar protease incrementally throughout the time course of the reaction.

   d) If a fraction of the fusion protein is misfolded or aggregated, it may be resistant to digestion.

Removal of SUMOstar and SUMOstar Protease 1

SUMOstar and SUMOstar Protease 1 both contain polyhistidine tags at their N-termini; therefore both can be simultaneously removed from the cleavage reaction by IMAC.

The recombinant protein of interest is recovered in the IMAC flow through. Assess the quality of protein product by examination of a small aliquot on an SDS-PAGE. If the protein is in the appropriate buffer it can be used directly 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 and GFP (Green Fluorescent Protein) and can be used to control for SUMOstar Protease 1 activity. Incubation of one unit of SUMOstar Protease 1 with 100 µg of SUMOstar Control Protein should result in > 90% cleavage after 1 hour at 30°C. Please note that the SUMOstar tag runs at 18 kDa on SDS-PAGE following cleavage, with uncleaved SUMOstar Control Protein running at 47 kDa.

Running a Control

1) Incubate SUMOstar Protease 1 and SUMOstar Control Protein (1 unit of protease per 100 µg of Control Protein) for 1 hour at 30°C.

2) Add 5x SDS-PAGE sample preparation buffer to digestion reaction.

3) Heat the sample at 95°C for 5 minutes.

4) Analyze the sample (5 – 10 µg) by SDS-PAGE, and stain the gel with Coomassie blue.

Western and ELISA Applications

Anti-SUMOPro/SUMOstar is an affinity purified, chicken polyclonal IgY antibody that reacts with SUMO and SUMOstar on Western blots and ELISA applications. For immunoblotting, a 1:1000 to 1:5000 dilution is recommended. At these dilutions, 10 - 50 ng of SUMOstar Control Protein should be detectable as a control. For ELISA a 1:5000 to 1:25000 dilution is recommended. Optimal dilutions for other applications should be determined empirically.

Stock Solutions

10x YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 mL of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C. Alternatively, use 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate. The shelf life of this solution is one year. Note: Pichia cells exhibit optimal growth with higher YNB concentrations; therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for Saccharomyces.

500x B (0.02% Biotin)

Dissolve 20 mg biotin in 100 mL of water and filter sterilize. Store at 4°C. The shelf life of this solution is one year.

10x D (20% Dextrose)

Dissolve 200 g of D-glucose in water (1 L final volume), then autoclave for 15 minutes or filter sterilize. The shelf life of this solution is one year.

10x M (5% Methanol)
Mix 5 mL of methanol with 95 mL of water. Filter sterilize and store at 4°C. The shelf life of this solution is two months.

10x GY (10% Glycerol)
Mix 100 mL of glycerol with 900 mL of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is one year.

1 M potassium phosphate buffer, pH 6.0
Combine 132 mL of 1 M K_2HPO_4, 868 mL of 1 M KH_2PO_4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is one year.

Media

Low Salt LB (Luria-Bertani) Medium [1% Tryptone; 0.5% Yeast Extract; 0.5% NaCl; pH 7.0]
1) For 1 L, dissolve the following in 950 mL deionized water: 10 g tryptone, 5 g yeast extract and 5 g NaCl.
2) Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 L.
3) Autoclave for 20 minutes at 15 lb/in^2. Let cool to 55°C and add desired antibiotics.
4) Store at room temperature.

• Low Salt LB medium is needed for use with the Zeocin™ antibiotic. Please note that Low Salt LB can be substituted for regular LB for most applications.

Low Salt LB Agar Plates
1) Make Low Salt LB Medium above and add 15 g/L agarose before autoclaving.
2) Autoclave for 20 minutes at 15 lb/in^2.
3) Let cool to 55°C and add desired antibiotics at this point. Pour into 10 cm Petri plates. Let the plates harden, then invert, and store at 4°C.

YPD [1% yeast extract; 2% peptone; 2% dextrose (glucose)]
1) Dissolve 10 g yeast extract and 20 g of peptone in 900 mL of water. Note: Add 20 g of agarose if making YPD slants or plates.
2) Autoclave for 20 minutes on the liquid cycle.
3) Add 100 L of 10x D.

• The liquid medium is stored at room temperature. YPD slants or plates are stored at 4°C. The shelf life is 3 - 4 months.

YPD + Zeocin™ [1% yeast extract, 2% peptone, 2% dextrose (glucose), 2% agarose, 100 µg/mL Zeocin™]
1) Dissolve 10 g of yeast extract and 20 g of peptone in 900 ml of water.
2) Include 20 g of agarose if making YPD slants or plates.
3) Autoclave for 20 minutes on liquid cycle.
4) Cool solution to 55°C and add 100 mL of 10x D. Add 1 mL of 100 mg/mL Zeocin™, if desired.

• Liquid medium without Zeocin™ can be stored at room temperature. Medium containing Zeocin™ should be stored at 4 °C in the dark. YPD slants or plates are stored at 4 °C. The shelf life of the medium is 3 - 4 months, or 1 - 2 weeks if it contains Zeocin™.

YPDS + Zeocin™ Agar [1% yeast extract, 2% peptone, 2% dextrose (glucose), 1 M sorbitol, 2% agarose, 100 µg/mL Zeocin™]
1) Dissolve 10 g of yeast extract, 182.2 g of sorbitol, and 20 g of peptone in 900ml of water.
2) Add 20 g of agarose.
3) Autoclave for 20 minutes on liquid cycle.
4) Add 100 mL of 10x D
5) Cool solution to ~60°C and add 1.0 mL of 100 mg/mL Zeocin™.

• Store YPDS slants or plates containing Zeocin™ at 4 °C and in the dark. The shelf life is one to two weeks.

BMGY (Buffered Glycerol-complex Medium) and BMMY (Buffered Methanol-complex Medium) [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4x 10-5% biotin, 1% glycerol or 0.5% methanol]

1) Dissolve 10 g of yeast extract and 20 g peptone in 700ml water.
2) Autoclave 20 minutes on liquid cycle.
3) Cool to room temperature, then add the following and mix well:
    a. 100 mL 1 M potassium phosphate buffer (pH 6.0)
    b. 100 mL 10x YNB
    c. 2 mL 500x B
4) 100 mL 10x GY
5) For BMMY, add 100 mL 10x M instead of glycerol.
6) Store media at 4°C. The shelf life of this solution is approximately two months.

Polylinker Map

ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTA
TACTCTAAAGGAAGTTAAAAATGACGACAAAATAAGCGTCGTAGGAGGCGTAATCGACGAGGTCAGTTGTGAT
Alpha Secretory Sequence

CAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGA
GTTGTCTTCTACTTTGCCGTGTTTAAGGCCGACTTCGACAGTAGCCAATGAGTCTAAATCTTCCCCTAAAGCT

TGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATT
ACAACGACAAAACGGTAAAAGGTTGTCGTGTTTATTGCCCAATAACAAATATTTATGATGATAACGGTCGTAA

GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGACTACAAAGACGATGACGACA
CGACGATTTCTTCTTCCCCATAGAGAGCTCTTTTCTCTCCGACTTCGACGTCTGATGTTTCTGCTACTGCTGT
Flag Tag

AGGGACATCACCATCATCATCACGGAGGTTCGGACTCAGAAGTCAATCAAGAGGCTAAGCCAGAGGTCAAGCC
TCCCTGTAGTGGTAGTAGTAGTGCCTCCAAGCCTGAGTCTTCAGTTAGTTCTCCGATTCGGTCTCCAGTTCGG
6x His Tag **SUMOstar Fusion Protein**

AGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAA
TCTTCAGTTCGGACTCTGAGTGTAGTTAAATTTCCACAGGCTACCTAGAAGTCTCTAGAAGAAGTTCTAGTTT

AAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAACGT
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TCTTGTACGACGGTATTGAAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTAT
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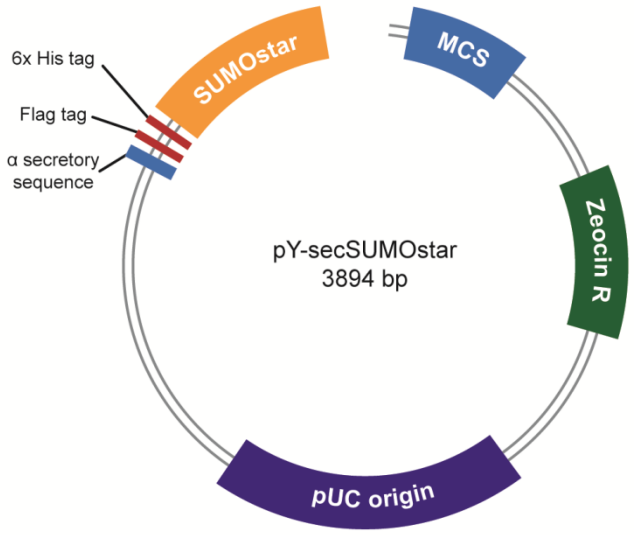
SphI BsmBI **XbaI**

TGAGGCTCACCGCGAACAGATTGG AGGTTGAGACGGCATGCCGTCTCT CTAGAACAAAAACTCA
ACTCCGAGTGGCGCTTGTCTAACCTCCA ACTCTGCCGTACGGCAGAGAGATC TTGTTTTTGAGT
BsmBI **Multi-Cloning Site**

SalI

TCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCA
AGAGTCTTCTCCTAGACTTATCGCGGCAGCTGGTAGTAGTAGT



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