SUMOpro-3® Gene Fusion Technology

NEW METHODS FOR ENHANCING FUNCTIONAL PROTEIN EXPRESSION AND PURIFICATION IN BACTERIA

***E.coli* (T7; Amp or Kan)**

Cat. No. 1010K (Kit, Kan)
1011 (Vector, Kan)
1010A (Kit, Amp)
1016 (Vector, Amp)

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.

SUMO3 Fusions

Human SUMO3 fused with a protein of interest can dramatically enhance expression and promote solubility and correct folding of the protein. It has long been known 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 (POI) may be explained by improved/more rapid folding of the POI, resulting from nucleation by the Ubl.

Recombinant Protein Purification and Ulp1 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 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 SUMO3 system is largely due to the nature of the protease SENP2 core (SUMO Protease 2), an equivalent of ubiquitin protease. SUMO Protease 2 is superior when compared with other proteases commonly used in recombinant protein production, as it recognizes the hSUMO3 (SUMOpro fusion tag) 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, SUMO Protease 2 has not been observed to cleave indiscriminately within the protein-of-interest.

About the pE-SUMO3 vector

This vector is fully compatible with the pET expression system, allowing for tightly regulated expression of heterologous proteins in BL21(DE3) or related *E. coli* strains. The plasmid contains a selective marker for either resistance to ampicillin (1011A) or kanamycin (1010K).

Advantages

1) Convenient, directional cloning of gene-of-interest (GOI) in frame with SUMO3 fusion tag.
2) SUMO3 fusion can dramatically enhance recombinant protein expression and solubility.
3) No known case of SUMO Protease 2 cleaving within the fused POI.
4) SUMO Protease 2 cleavage yields native protein with a desired N-terminus (except proline).

Components

The SUMOpro-3 Expression System provides the reagents to express a protein of interest as a linear chimera with the SUMO3 protein tag. The SUMOpro-3 Expression System contains the following four components.

1) **pE-SUMO3 (T7; Amp or Kan)**
Size: 20µg (0.5µg/µl)
Buffer: 10mM Tris, pH 8.0

2) **SUMO Protease 2 (Cat. No. 4020)**
Size: 500 Units (10 units/µl)
Buffer: 25 mM HEPES, pH 7.5
150 mM NaCl
2mM EDTA
2mM DTT
10% glycerol

3) **SUMO Protease 2 Control Protein (Cat. No. 5004)**
Size: 100µg (5.0µg/µl)
Buffer: PBS , pH 7.2

4) **Affinity purified AntihSUMO3 Antibody (Chicken IgY, Cat. No. AB7030)**
Size: 50 µg,(1.0 mg/ml)
Buffer: 25mM Tris-HCl, pH 7.5
150mM NaCl

Storage

pE-SUMO3 Vector (T7; Amp or Kan)
Store vial at -20° C or below.

SUMO Protease 2
Store -80° C. Avoid multiple freeze/thaw cycles.

SUMO Control Protein
Store vial at -80° C. Avoid cycles of freezing and thawing.

AntiSUMO3 Antibody
For short-term use (several weeks), store at 4°C. Long-term storage should be at -80°C.

Cloning

Background
The pE-SUMO3 vector is provided as a circular plasmid. For cloning, the vector must be digested with BsaI (a.k.a.Eco31I) 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 SUMO and your gene-of-interest (GOI). Second, overhangs created by Class IIS enzymes are template-derived and thus unique. **In the case of pE-SUMO3, digestion with BsaI generates ACCT (SUMO3 fusion juncture) at the 5’ end and CTAG (XbaI) at the 3’ overhang. Appropriate primer design then allows for convenient directional cloning.**

Forward Primer Design (Cloning)

To clone your gene of interest into the pE-SUMO3 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 BsaI Class IIS restriction site.

BsaI: 5’ – NN **GGTCTCNAGGT** XXX NNN NNN NNN NNN NNN**– 3’**

In this primer, **GGTCTC** is the BsaI recognition sequence, N is any nucleotide, and **AGGT** will be the overhang generated upon BsaI digestion. This sequence ends with **GGT** (the last codon of the SUMO 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 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' - 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
BsmBI: 5' – NN **CGTCTC** 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 of your PCR product. **If your insert contains an XbaI site or if the digestion of the PCR insert with BsaI alone is preferred for any reason**, **please see Strategy 2 below.** Upon digestion of this PCR product with BsaI (5') and XbaI (3'), a fragment will be generated having overhanging sequence complementary to the expression vector linearized with BsaI alone (recall that BsaI digestion yields an XbaI site at the 3' overhang).

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, 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 BsaI/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 Bsa1 site (or any other Class II Restriction Enzyme listed above) in front of the XbaI sequence allows for digestion with Bsa1 enzyme yielding the same (5') **CTAG** overhang.

An example of a reverse primer for this purpose is:

BsaI/XbaI: 5' – NN **GGTCTC TCTAGA** TTA XXX NNN NNN NNN NNN… – 3'

where **GGTCTC** is the BsaI 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.

A number of alternative restriction sites to XbaI (for either Strategy 1 or 2) are present in the MCS of the vector. Please refer to the polylinker map for more information.

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 pE-SUMOpro. 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 pE-SUMO3 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.

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) single 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:

T7 forward	5' TAATACGACTCACTATAGG 3'
T7 terminator	5' GCTAGTTATTGCTCAGCGG 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.

Expression

Transformation

For expression of the SUMO3 fusion protein, it is necessary to transform the confirmed plasmid clone into an *E. coli* strain containing the DE3 lysogen. The BL21 derived strains are particularly useful for protein expression as they contain mutations in *ompT* and *lon*, which abolish protease activity that could degrade your expressed protein. It is also possible to transfect the bacteria with the helper phage, which can be useful for expressing toxic proteins. The transformation procedure into these cells should be based on your manufacturers' protocols. NOTE: the transformation efficiency of DE3 strains of *E. coli* is normally lower than the cloning strains.

Small scale expression studies and optimization

Prepare LB containing the appropriate antibiotic for your pE-SUMO3 vector, as well as any antibiotic that may be necessary to maintain helper plasmids. In addition, the inclusion of 0.2% dextrose or glucose will help suppress heterologous expression and maintain plasmid integrity. Inoculate 2-3ml of broth with an isolated clone containing your confirmed plasmid. Grow overnight at 37C to a saturated culture at >200 RPM.

Determine optical density (600nm) of overnight culture, and dilute cultures into 2-3ml of fresh LB/antibiotic/dextrose. Shake vigorously at >200RPM. **Proper aeration is crucial for maximal protein expression and culture viability.** Starting density should be 15 to 30-fold lower than desired induction OD (0.015 OD ml-1 for an induction OD ml-1 of 0.5) to ensure maintenance of plasmid integrity. Optimizing expression of SUMO3 fusion protein may require altering induction temperature (e.g. 37, 30, or 20C), time (4 hours to overnight), and/or cell density (0.4 to 0.8 OD ml-1). Titration of the IPTG (0.05 – 1mM) may also be required. It is always advantageous to screen multiple clones for protein production.

Large scale expression

Using an overnight seed culture (see above), inoculate 1L of antibiotic and dextrose containing media. Grow cells with vigorous shaking at 37°C. Induce under optimized parameters (see above) with IPTG when the culture is in mid-log phase (OD between 0.4-0.6 at 600nm). At the desired time, harvest the cells by centrifugation at 5000 rpm. The cell pellet can be resuspended in lysis buffer and immediately processed for protein purification (see below). Alternatively, the pellet can be stored at –80°C indefinitely.

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 PMSF should be included if you fear your protein is susceptible to aberrant protease activity, Complete tablets without EDTA (Roche) offer inhibitors to a broad range of proteases.

The presence of a HIS_6 tag at the N-terminus of the SUMO3 protein sequence allows for initial purification of fusion proteins by IMAC. **All purification steps should be carried out at +4C**. Follow the resin/column manufacturer's instructions for specific details.

Cleavage

Background

SUMO Protease 2, a highly active and robust recombinant protease, cleaves SUMO3 from recombinant fusion proteins. Unlike thrombin, EK, or TEV proteases, whose recognition sites are short, linear sequences, SUMO Protease 2 recognizes the tertiary sequence of SUMO3. As a result, SUMO Protease 2 does not cleave within the protein of interest.

Unit Definition

One unit of SUMO Protease 2 cleaves 90-100 µg of SUMO Protease 2 Control Protein in 1 hour at 30°C.

Digestion of SUMO3 fusion tag

1. Dialyze IMAC purified SUMO3 fusion proteins (4°C) against 20 mM HEPES, 150 mM NaCl, pH 7.5 (or PBS, 7.2). 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 SUMO Protease 2 (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 SUMO Protease 2. Overnight at 4°C, for example, may be convenient during the initial dialysis step.
   iii. Consider adding SUMO Protease 2 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.
   v. Consider reaction conditions or buffer components (e.g. salt, pH) that are known to affect your POI.

Removal of SUMO3 fusion tag and SUMO Protease 2

SUMO3 and SUMO Protease 2 both contain HIS_6 tags at their N-termini; therefore, SUMO3 and SUMO Protease 2 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

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Controls and Validations

SUMO Control Protein 2

SUMO Control Protein 2 is a recombinant fusion protein that contains the SUMO3 tag fused to a polypeptide, and can be used to control for SUMO Protease 2 activity. Incubation of 1 Unit of SUMO protease 2 with 100µg of SUMO Control Protein 2 should result in >90% cleavage after 1hour at 30°C. Please note that the SUMO3 tag migrates at ~15-20kDa by SDS-PAGE following cleavage, with uncleaved SUMO Control Protein 2 running at ~40-50 kDa, depending on acrylamide percentage and reference standards employed.

Digestion of Control Protein

1. Incubate SUMO Protease 2 and SUMO Control Protein 2 (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 SUMO Control Protein 2 (5-10µg) by SDS-PAGE and Coomassie staining.

Western Blots

Anti-SUMO3 is an affinity purified hen polyclonal IgY antibody that reacts with hSUMO3 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 SUMO Control Protein 2 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.

References

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Cat.# 1016
Polylinker Map (pE-SUMO3 Vector, T7, Amp)
Vector Size: 5743 bp

```
               6x His Tag                    SdaI
Met Gly His His His His His His Gly Ser Leu Gln Glu Glu Lys Pro Lys Glu Gly Val Lys Thr
ATG GGT CAT CAC CAT CAT CAT CAC GGG TCC CTG CAG GAG GAG AAG CCC AAG GAG GGT GTG AAG ACA
TAC CCA GTA GTG GTA GTA GTA GTG CCC AGG GAC GTC CTC CTC TTC GGG TTC CTC CCA CAC TTC TGT
```

Human SUMO3 Fusion Protein

```
Glu Asn Asp His Ile Asn Leu Lys Val Ala Gly Gln Asp Gly Ser Val Val Gln Phe Lys Ile Lys
GAG AAT GAC CAC ATC AAC CTG AAG GTG GCC GGG CAG GAC GGC TCC GTG GTG CAG TTC AAG ATC AAG
CTC TTA CTG GTG TAG TTG GAC TTC CAC CGG CCC GTC CTG CCG AGG CAC CAC GTC AAG TTC TAG TTC
```

```
Arg His Thr Pro Leu Ser Lys Leu Met Lys Ala Tyr Cys Glu Arg Gln Gly Leu Ser Met Arg Gln
AGG CAC ACG CCG CTG AGC AAG CTG ATG AAG GCC TAC TGC GAG AGG CAG GGC TTG TCA ATG AGG CAG
TCC GTG TGC GGC GAC TCG TTC GAC TAC TTC CGG ATG ACG CTC TCC GTC CCG AAC AGT TAC TCC GTC
```

```
Ile Arg Phe Arg Phe Asp Gly Gln Pro Ile Asn Glu Thr Asp Thr Pro Ala Gln Leu Glu Met Glu
ATC AGA TTC AGG TTC GAC GGG CAG CCA ATC AAT GAA ACT GAC ACT CCA GCA CAG CTG GAG ATG GAG
TAG TCT AAG TCC AAG CTG CCC GTC GGT TAG TTA CTT TGA CTG TGA GGT CGT GTC GAC CTC TAC CTC
```

```
                                                Gly Gly                              BsaI
Asp Glu Asp Thr Ile Asp Val Phe Gln Gln Gln Thr
GAC GAG GAC ACC ATC GAC GTG TTC CAG CAG CAG ACG GG   AGGTTGAGACC ACTAGTGGTACC GGTCTCA
CTG CTC CTG TGG TAG CTG CAC AAG GTC GTC GTC TGC CCTCCA   ACTCTGG TGATCACCATGG CCAGAGTGATC
                                                     ▲
```

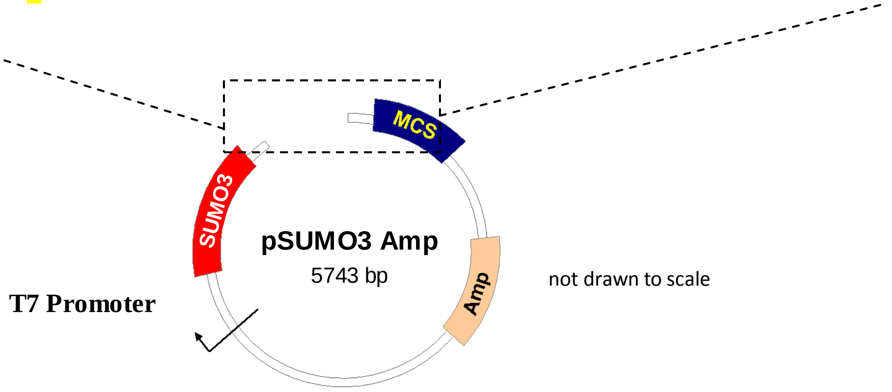
SUMO Protease 2 cleavage site

```
                     MCS
CTAGA GGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG
    T  CCTAGGCTTAAGCTCGAGGCAGCTGTTCGAACGCCGGCGTGAGCTC
```

Multiple Cloning Site (MCS)

pE-SUMO3 vector has to be linearized with BsaI or Eco31I restriction enzyme

```
                                                               NotI
Gly Gly            XbaI[1]  BamHI            SacI     SalI    HindIII    EagI       XhoI
GG                 CTAGA   GGATCC  GAATTC  GAGCTCC  GTCGAC  AAGCTT  GCGGCCGC  ACTCGAG  CACCA
CCTCCA                 T   CCTAGG  CTTAAG  CTCGAGG  CAGCTG  TTCGAA  CGCCGGCG  TGAGCTC  GTGGT
```



not drawn to scale

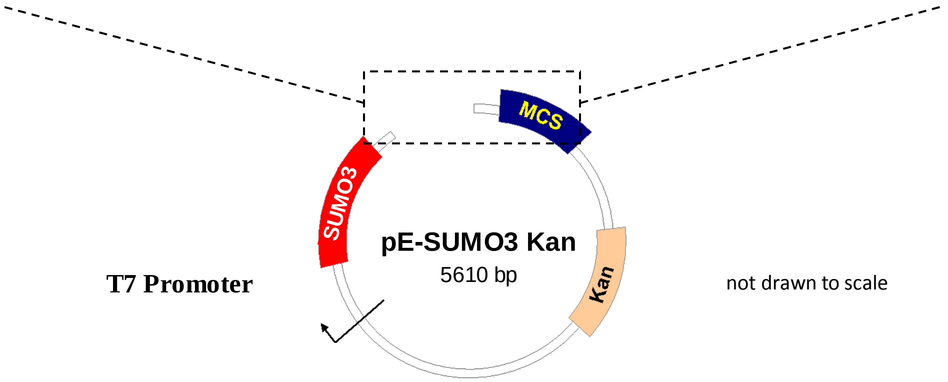
Cat.# 1101
Polylinker Map (pE-SUMO3 Vector, T7, Kan)
Vector Size: 5610bp

```
                6x His Tag                  SdaI
Met Gly His His His His His His Gly Ser Leu Gln Glu Glu Lys Pro Lys Glu Gly Val Lys Thr
ATG GGT CAT CAC CAT CAT CAT CAC GGG TCC CTG CAG GAG GAG AAG CCC AAG GAG GGT GTG AAG ACA
TAC CCA GTA GTG GTA GTA GTA GTG CCC AGG GAC GTC CTC CTC TTC GGG TTC CTC CCA CAC TTC TGT
Human SUMO3 Fusion Protein

Glu Asn Asp His Ile Asn Leu Lys Val Ala Gly Gln Asp Gly Ser Val Val Gln Phe Lys Ile Lys
GAG AAT GAC CAC ATC AAC CTG AAG GTG GCC GGG CAG GAC GGC TCC GTG GTG CAG TTC AAG ATC AAG
CTC TTA CTG GTG TAG TTG GAC TTC CAC CGG CCC GTC CTG CCG AGG CAC CAC GTC AAG TTC TAG TTC

Arg His Thr Pro Leu Ser Lys Leu Met Lys Ala Tyr Cys Glu Arg Gln Gly Leu Ser Met Arg Gln
AGG CAC ACG CCG CTG AGC AAG CTG ATG AAG GCC TAC TGC GAG AGG CAG GGC TTG TCA ATG AGG CAG
TCC GTG TGC GGC GAC TCG TTC GAC TAC TTC CGG ATG ACG CTC TCC GTC CCG AAC AGT TAC TCC GTC

Ile Arg Phe Arg Phe Asp Gly Gln Pro Ile Asn Glu Thr Asp Thr Pro Ala Gln Leu Glu Met Glu
ATC AGA TTC AGG TTC GAC GGG CAG CCA ATC AAT GAA ACT GAC ACT CCA GCA CAG CTG GAG ATG GAG
TAG TCT AAG TCC AAG CTG CCC GTC GGT TAG TTA CTT TGA CTG TGA GGT CGT GTC GAC CTC TAC CTC

                                                Gly Gly                         BsaI
Asp Glu Asp Thr Ile Asp Val Phe Gln Gln Gln Thr
GAC GAG GAC ACC ATC GAC GTG TTC CAG CAG CAG ACG GG   AGGTTGAGACC ACTAGTGGTACC GGTCTCA
CTG CTC CTG TGG TAG CTG CAC AAG GTC GTC GTC TGC CCTCCA   ACTCTGG TGATCACCATGG CCAGAGTGATC
                                                      ▲
                            SUMO Protease 2 cleavage site

                    MCS
CTAGA GGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAG
    TCCTAGGCTTAAGCTCGAGGCAGCTGTTCGAACGCCGGCGTGAGCTC
```

Multiple Cloning Site (MCS)

pE-SUMO3 vector has to be linearized with BsaI or Eco31I restriction enzyme

```
                                                          NotI
Gly  Gly      XbaI1  BamHI         SacI     SalI   HindIII  EagI      XhoI
GG            CTAGA GGATCC GAATTC GAGCTCC GTCGAC AAGCTT GCGGCCGC ACTCGAG CACCA
CCTCCA            T CCTAGG CTTAAG CTCGAGG CAGCTG TTCGAA CGCCGGCG TGAGCTC GTGGT
```



not drawn to scale