

*Expresso*TM T7 SUMO Cloning and Expression System

IMPORTANT!
-80°C and -20°C Storage Required
Immediately Upon Receipt

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Advanced Products for Molecular Biology

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Expresso™ T7 SUMO Cloning and Expression System

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Expresso™ T7 SUMO Cloning and Expression System

System Designations

The Expresso T7 SUMO Cloning and Protein Expression System contains pre-processed pETite™ N-His SUMO Vector DNA, HI-Control™ 10G Chemically Competent Cells for cloning, HI-Control BL21(DE3) Chemically Competent Cells for protein expression, and SUMO Express Protease. The System catalog numbers are listed below.

Expresso T7 SUMOCloning and Expression Kits

	5 Reactions	10 Reactions
Expresso™ T7 SUMO Cloning and Expression System	49003-1	49003-2

Components & Storage Conditions

The Expresso T7 SUMO Cloning and Expression System consists of four separate containers. Container 1 includes the pETite SUMO Expression Vector, SUMO Positive Control Insert DNA, and DNA primers for screening inserts by PCR and sequencing. This container should be stored at **-20°C**. Container 2 contains SUMO Express Protease and SUMO Cleavage Control Protein, and should be stored at **-80°C**. Container 3 includes HI-Control 10G Chemically Competent Cells, which must be stored at **-80°C**. Container 4 contains HI-Control BL21(DE3) Chemically Competent Cells, which must be stored at **-80°C**. The 10-reaction Kits are supplied with two of container 1 and two of container 2.

Expresso T7 SUMO Cloning Kit Container Store at -20°C

	Concentration	Volume
pETite N-His SUMO Kan Vector DNA (5 reactions)	12.5 ng/µl	15 µl
SUMO Positive Control C Insert DNA	50 ng/µl	10 µl
Primers for PCR screening and sequencing		
SUMO Forward Primer	50 pmol/µl	100 µl
pETite Reverse Primer	50 pmol/µl	100 µl

SUMO Express Protease Container Store at -80°C

	Concentration	Amount
SUMO Express Protease	1 unit/µl	50 units
SUMO Cleavage Control Protein	2 µg/µl	50 µg

HI-Control 10G Chemically Competent Cells Container

Store at -80°C

	Cap Color	5 Reaction Kit	10 Reaction Kit
HI-Control 10G Chemically Competent Cells	White	6 x 40 µl	12 x 40 µl
Transformation Control pUC19 DNA (10 pg/µl)		20 µl	20 µl
Recovery Medium (Store at -20°C or -80°C)		1 x 12 ml)	1 x 12 ml)

HI-Control BL21(DE3) Chemically Competent Cells Container

Store at -80°C

	Cap Color	5 Reaction Kit	10 Reaction Kit
HI-Control BL21(DE3) Chemically Competent Cells	Gray	6 x 40 µl	12 x 40 µl
Transformation Control pUC19 DNA (10 pg/µl)		20 µl	20 µl
Recovery Medium (Store at -20°C or -80°C)		1 x 12 ml)	1 x 12 ml)

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Kit components available separately:

Expresso System Cells

Description	Size	Cat. No.
HI-Control™ 10G Chemically Competent Cells (SOLOs)	12 Transformations	60110-1
HI-Control BL21(DE3) Chemically Competent Cells (SOLOs)	12 Transformations	60435-1

SUMO Express Protease and SUMO Cleavage Control Protein

Description	Size	Cat. No.
SUMO Express Protease	200 units	30801-2
SUMO Cleavage Control Protein	50 µg	30805-1

System Description

The Expresso T7 SUMO Cloning and Expression System is a simple method for rapid cloning and expression of proteins in *E. coli*. The system uses an engineered form of the SUMO protein (Small Ubiquitin-like MOdifier) as a fusion partner to aid the expression and purification of difficult target proteins. SUMO is a small protein (100 amino acids), derived from the yeast *SMT3* gene product, that can enhance the expression and solubility of proteins that are otherwise poorly expressed or insoluble (1, 2). A 6xHis motif at the amino terminus of the SUMO tag allows purification of the fusion protein by metal affinity chromatography. If desired, the 6xHis-SUMO tag can then be removed precisely and efficiently by SUMO Express Protease. The protease recognizes the tertiary structure of SUMO and cleaves precisely at its carboxyl terminus, allowing recovery of the intact protein of interest with no extra residues (1-3). The 6xHis-SUMO fragment and the protease (which also has a 6xHis tag) can then be captured by metal affinity, leaving the protein of interest in solution.

The SUMO fusion tag and SUMO Express Protease used in this system have been modified from their native sequences. Mutations in the SUMO tag render it resistant to cleavage by the native SUMO proteases found in eukaryotic cells (4, 5). Compensating mutations have been introduced into the SUMO Express Protease to restore full cleavage activity; only this engineered form of the protease can cleave the SUMO tag used in the Expresso System. Thus, while the Expresso System is designed for expression in *E. coli*, fusion clones constructed using this system can also be transferred into eukaryotic expression vectors for expression of SUMO-tagged proteins in mammalian or insect cells.

The Expresso T7 SUMO Cloning and Expression System incorporates an inducible T7 expression system (7, 8) with improved regulation. The pETite™ N-His SUMO Vector is provided in a pre-processed, linearized format for instant enzyme-free cloning: after amplification of the target gene with appropriate primers, the PCR product is simply mixed with the pETite N-His SUMO vector and transformed directly into chemically competent HI-Control 10G Cells. Recombination within the host cells seamlessly joins the insert to the vector (Figure 1).

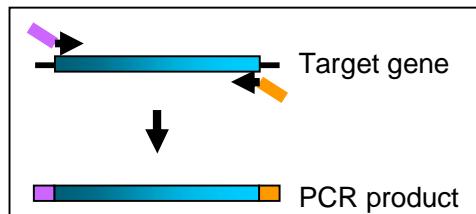
Unlike other ligation-independent cloning systems, no enzymatic treatment or purification of the PCR product is required. No restriction enzymes are used, so there are no limitations on sequence junctions. Open reading frames are directionally cloned into the pETite SUMO Vector, in frame with the SUMO tag. The 6xHis peptide at the amino terminus of the SUMO tag provides for fast and easy affinity purification of proteins under native or denaturing conditions.

HI-Control 10G Cells are used for construction of clones in the pETite vectors. Their recA- endA- genotype allows recovery of high quality plasmid DNA. HI-Control 10G Cells do not express T7 RNA polymerase and therefore are not used for expression from the pETite vectors.

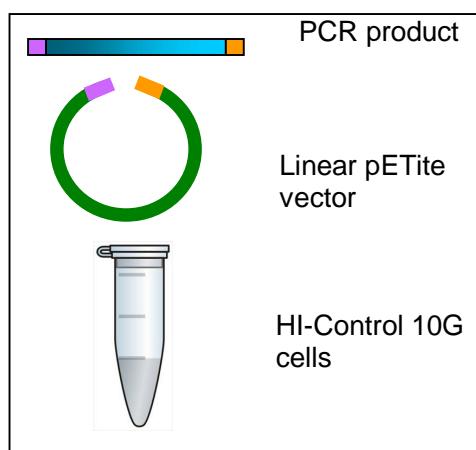
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Hi-Control™ BL21(DE3) Cells are provided for expression of cloned genes from the T7 promoter. These cells produce T7 RNA polymerase from the *lacUV5* promoter. However, unlike the common T7 expression host strain BL21(DE3), Hi-Control BL21(DE3) cells produce high levels of Lac repressor protein from a specially engineered *lacI* gene. The abundant Lac repressor minimizes basal expression of T7 RNA polymerase within the host cells prior to induction. It also prevents premature expression from the T7-lac promoter on the pETite™ vectors. This enhanced control over basal expression allows growth of clones that contain genes whose products might otherwise be toxic to the T7 host strain.

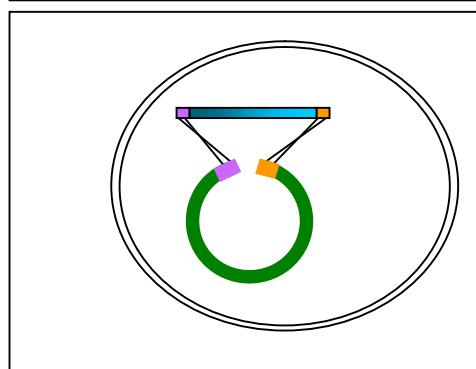
1. **Amplify** target gene, using primers that contain 18-bp overlap with vector ends.



2. **Mix:**
 - unpurified PCR product
 - linear pETite vector preparation
 - HI-Control 10G cells



3. **Transform** by heat shock. Select for recombinants on kanamycin agar plates.



4. **Verify** recombinant clones by PCR or restriction digest and confirm sequence.

5. **Transform** into HI-Control BL21(DE3) cells for expression of the target gene.

Figure 1. Expresso cloning. A target gene is amplified with primers that contain short homology to the ends of the pETite vectors. The PCR product is then mixed with the pre-processed vector and transformed directly into HI-Control 10G cells.

Expresso™ T7 SUMO Cloning and Expression System

pETite™ N-His SUMO Kan Vector

The pETite vectors provide an improved alternative to the most common pET expression vectors (6-8). The small size of the pETite vectors (2.2-2.5 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis. They are based on Lucigen's patented pSMART® vectors, which feature transcriptional terminators to prevent unwanted transcription into or out of the cloned sequence. The pETite vectors do not harbor a gene for the LacI repressor protein. Instead, abundant LacI repressor is provided by the HI-Control™ BL21(DE3) cells.

The pETite N-His SUMO Kan Vector is pre-linearized for instant, directional cloning of inserts (Figures 2 and 3). The vector includes signals for expression, including the T7-lac promoter, ribosome binding site, and translational start and stop codons. The vector is designed for expression of the target protein as a fusion with an amino-terminal 6xHis-SUMO tag, which has been shown to increase the yield and enhance the solubility of a variety of proteins (1, 2). In addition, the SUMO tag is recognized by the highly-specific SUMO Express Protease, allowing precise removal of the tag to produce target protein of native sequence.

The pETite vectors do not contain the *lacZ* alpha gene fragment, so they do not enable blue/white colony screening. However, the background of empty vector is typically <5%, so minimal colony screening is necessary. The pETite vectors have low copy number, similar to that of pBR322 plasmids (~20 copies/cell), yielding 0.5–1.0 µg of plasmid DNA per ml of culture.

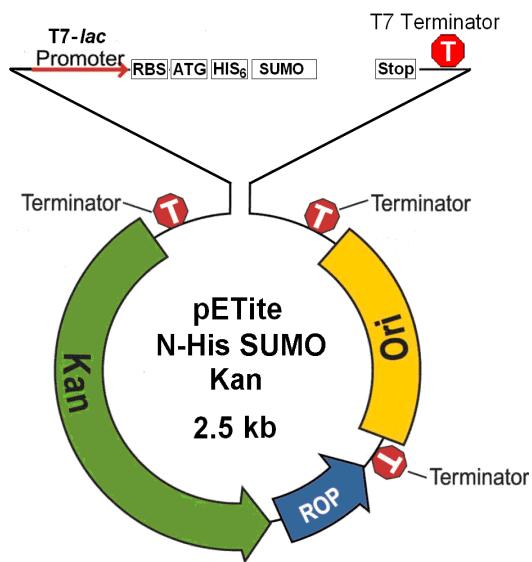


Figure 2. pETite N-His SUMO Kan expression vector. RBS, ribosome binding site; ATG, translation start site; Stop, translation end site; Kan, kanamycin resistance gene; ROP, Repressor of Priming (for low copy number); Ori, origin of replication. CloneSmart® transcription terminators (T) prevent transcription into or out of the insert, and a T7 terminator follows the cloning site. The 6xHis affinity tag is fused to the amino terminus of the SUMO-tagged protein.

HI-Control 10G Chemically Competent Cells

HI-Control 10G Cells are an *E. coli* strain optimized for high efficiency transformation. The HI-Control 10G Cells are ideal for cloning and propagation of plasmid clones. They give high yield and high quality plasmid DNA due to the *endA1* and *recA1* mutations. HI-Control 10G Cells harbor a single-copy BAC plasmid carrying an engineered *lacI^{q1}* repressor allele. The *lacI^{q1}* allele expresses 170x more Lac repressor than does the wild-type *lacI* gene (9). The HI-Control 10G strain does not contain T7 RNA polymerase, so it does not express proteins from the T7 promoter. Excess lac repressor in this strain further minimizes any background transcription by the bacterial polymerase.

Genotype of HI-Control™ 10G Cells:

mcrA Δ(mrr-hsdRMS-mcrBC) *endA1 recA1 φ80d/lacZΔM15 Δ/lacX74 araD139*
Δ(ara,leu)7697 galU galK rpsL nupG λ⁻ tonA /Mini-F lacI^{q1}(Gent^R)

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HI-Control 10G Chemically Competent Cells produce $\geq 1 \times 10^9$ cfu/ μ g supercoiled pUC19 DNA.

As a control for transformation, HI-Control 10G Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/ μ l. Use 1 μ l (10 pg) for transformation. Select pUC19 transformants on plates containing ampicillin or carbenicillin (100 μ g/ml).

HI-Control BL21(DE3) Chemically Competent Cells

The HI-Control BL21(DE3) Cells are a derivative of BL21(DE3) with improved control over target gene expression. BL21(DE3) is the T7 host strain most commonly used for expression of cloned genes from the bacteriophage T7 promoter. This strain is a lysogen of λ DE3, which harbors the T7 bacteriophage RNA polymerase gene under the control of the inducible *lacUV5* promoter. The *lacUV5* promoter is a variant of the *lac* promoter that is inducible to higher levels than its wild-type counterpart, but it also suffers from a higher basal level of activity. This basal expression of T7 RNA polymerase can lead to “leaky” background expression of target genes cloned under a T7 promoter. Such expression can cause difficulty in maintaining clones in the expression host, particularly if the target gene encodes a deleterious protein.

Some common T7 expression vectors harbor a copy of the *lacI* gene, encoding the lac repressor protein (7, 8). The lac repressor protein maintains inducible control over the *lacUV5* promoter as well as the T7-lac promoter on the vector. However, the increased copy number of *lacI* provides only partial protection against leaky expression.

HI-Control BL21(DE3) Cells contain a single-copy BAC plasmid harboring a specially engineered version of the *lacI^{q1}* repressor allele. The *lacI^{q1}* allele expresses ~170-fold more lac repressor protein than the wild-type *lacI* gene (9), or about 10-fold more repressor than expected when *lacI* is harbored on the expression vector. The increased pool of lac repressor in HI-Control BL21(DE3) Cells maintains tight control over the expression of T7 RNA polymerase from the *lacUV5* promoter, reducing leaky expression of genes cloned under a T7 promoter. The excess repressor in this strain is also sufficient to bind to the *lac* operator on the pETite™ vectors, providing an additional level of control over expression from the T7 promoter. The abundant lac repressor does not interfere with the induction of T7 RNA polymerase or target gene expression by IPTG.

Genotype of HI-Control BL21(DE3) Cells:

F⁻ *ompT hsdSB (rB- mB-) gal dcm* (DE3)/Mini-F *lacI^{q1}(Gent^R)*

HI-Control BL21(DE3) Chemically Competent Cells produce $\geq 1 \times 10^8$ cfu/ μ g supercoiled pUC19 DNA.

As a control for transformation, HI-Control BL21(DE3) Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/ μ l. Use 1 μ l (10 pg) for transformation. Select pUC19 transformants on plates containing ampicillin or carbenicillin (100 μ g/ml).

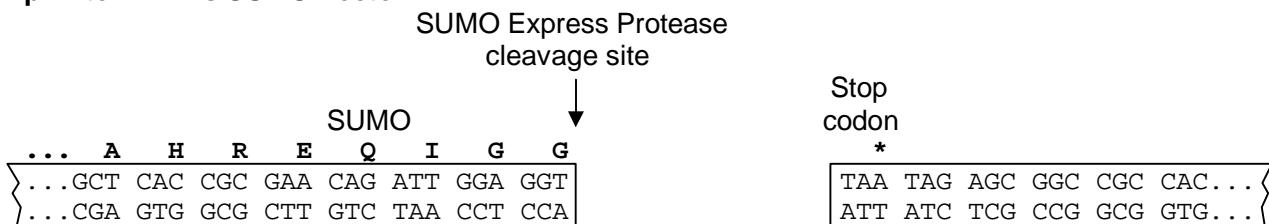
Cloning Strategy

The pETite N-His SUMO Vector preparation enables a simple strategy for precise, directional cloning. The vector is provided in a linearized form, ready for co-transformation with a PCR product containing the gene of interest.

The desired insert is amplified with user-supplied primers that include 18 nucleotides (nt) of overlap with the ends of the vector (Figure 3). The forward primer contains sequence corresponding to the carboxyl terminus of the SUMO fusion partner, and the reverse primer includes a stop codon and vector sequences. Recombination between the vector and insert occurs within the host strain, seamlessly fusing the gene of interest to the vector. No restriction digestion, enzymatic treatment, or ligation is necessary for efficient recombination. The method is similar to cloning by homologous recombination (10), It does not require single-stranded ends on the vector or the insert, as in “PIPE” cloning (11).

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pETite™ N-His SUMO Vector:



PCR Product:

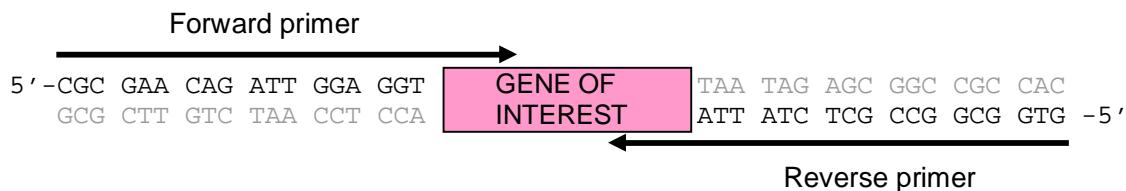


Figure 3. Insertion of a gene into the pETite N-His SUMO Vector for expression. PCR primers add flanking sequences identical to the vector sequence adjoining the insertion site. Recombination within the host cell fuses the blunt PCR product to the vector. See Detailed Protocol.

Positive Control Insert

A SUMO Positive Control C Insert is included with the Kit. It encodes a blue fluorescent protein from *Vibrio vulnificus* (12), flanked by sequences for enzyme-free cloning into the pETite N-His SUMO Vector. It serves as a control both for cloning efficiency and for expression.

BFP enhances the natural fluorescence of NADPH by binding to it. BFP expression leads to rapid development of bright blue fluorescence under long-wavelength UV light that is readily visible in whole cells. The SUMO-BFP fusion protein migrates at ~40 KD on SDS PAGE.

Colony Screening

Background with the pETite N-His SUMO Vector is typically very low (<5%), so minimal screening is necessary. Colony PCR, size analysis of uncut plasmids, or restriction digestion may be used to verify the presence of inserts. Primers included with the kit are suitable for screening by colony PCR and for sequencing of plasmid DNA. We strongly recommend sequence analysis to confirm the junctions of the insert with the vector as well as the predicted coding sequence.

Protein Expression

Recombinant plasmids are constructed in the HI-Control™ 10G host strain and verified by sequencing. They are then transformed into HI-Control BL21(DE3) Cells for expression. Transformants are selected with kanamycin. Individual colonies are grown in liquid culture, and protein expression is induced by addition of IPTG. Expression of SUMO-tagged fusion proteins is evaluated by SDS-PAGE analysis.

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Protein Purification

Materials for purification are not provided with the Expresso T7 System. 6xHis tagged proteins are purified by Immobilized Metal Affinity Chromatography (IMAC). Various IMAC reagents are available, such as: Ni-NTA (Qiagen), TALON® (Clontech), and HIS-Select® (Sigma).

SUMO Express Protease

SUMO Express Protease is derived from the yeast *ULP1* gene product (1). This protease is highly specific for the tertiary structure of SUMO, and cleaves uniquely and precisely at the carboxyl terminus of the SUMO tag (1-3). Because of this extreme specificity, there is no “off-target” cleavage commonly seen with other proteases that recognize short, degenerate amino acid sequences. Like the SUMO tag, SUMO Express Protease bears an amino-terminal 6xHis tag. After cleavage of the fusion protein, both the SUMO tag and the SUMO Express Protease can be removed from the solution by subtractive metal affinity chromatography, leaving only the free target protein in solution.

SUMO Express Protease is robust and active under a variety of buffer conditions, allowing flexibility in cleavage protocols. The protease is tolerant to many common buffer additives, including salt, non-ionic detergents, imidazole, and low concentrations of urea or guanidine (1). The optimal temperature for cleavage is 30°C, but the protease is active from 4° to 37° C. The pH optimum for cleavage is 8.0, but the range 6.0 to 10.0 is tolerated. Since each SUMO fusion protein behaves uniquely, we recommend performing test cleavage reactions on a small scale. For most fusion proteins, 1 unit of SUMO Express Protease will be sufficient to digest 10 -100 µg in 1 hour at 30°C under the recommended conditions.

SUMO Express Protease is provided at 1 unit/µl in 50% glycerol, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM DTT, 1% Triton X-100. One unit of SUMO Express Protease is sufficient to cleave ≥ 90% of 100 µg of SUMO Cleavage Control Protein in 30 minutes at 4°C. For long-term storage, it should be kept at -80°C. Aliquot into smaller volumes to avoid multiple freeze-thaw cycles. During frequent use, the protease may be kept at -20°C for up to 2 weeks.

Important: The SUMO tag used in the Expresso T7 SUMO System contains amino acid substitutions that render it resistant to cleavage by native desumoylation enzymes present in eukaryotic cells, including SUMO proteases derived from the native *ULP1* gene product (4, 5). The SUMO Express Protease contains compensatory mutations that enable it to recognize and cleave the stabilized SUMO tag used in this system. These modifications allow SUMO fusion clones constructed using the Expresso T7 SUMO System to be transferred to eukaryotic host systems for expression. **Other SUMO proteases lacking these compensatory mutations cannot cleave the SUMO tag used in the Expresso T7 SUMO System.**

SUMO Cleavage Control Protein

A 6xHis-SUMO Cleavage Control Protein is included with the kit. This 6xHis-SUMO fusion protein is efficiently cleaved by SUMO Express protease, allowing confirmation of protease activity. One unit of SUMO Express Protease is sufficient to cleave ≥ 90% of 100 µg of SUMO Cleavage Control protein in 30 minutes at 4°C. The uncleaved control protein migrates at approximately 40 kDa in SDS-PAGE gels. After cleavage, the 6xHis SUMO fragment migrates at ~15-18 kDa, and the released fusion partner migrates at ~25 kDa.

The SUMO Cleavage Control Protein is provided at 2 µg/µl in the recommended cleavage reaction buffer, with the omission of DTT (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol). It should be stored at -80°C.

Materials and Equipment Needed

The Expresso T7 SUMO Cloning and Protein Expression Kit supplies many of the items needed to efficiently generate and express recombinant clones. While simple and convenient, successful use

Expresso™ T7 SUMO Cloning and Expression System

of the Kit requires proper planning for each step. Please read the entire manual and prepare the necessary equipment and materials before starting. The following items are required for this protocol:

- Custom Primers for target gene amplification.
- Microcentrifuge and tubes.
- Water bath at 42°C.
- Sterile 17 x 100 mm culture tubes.
- LB Broth or YT Broth.
- LB or YT agar plates containing kanamycin (see Appendix A for recipes).
- Agarose gel electrophoresis equipment.
- IPTG (100 mM stock).
- Dithiothreitol (DTT).
- Sonicator or cell lysis reagents.
- Resin and columns for immobilized metal affinity chromatography.
- SDS-PAGE equipment.

Detailed Protocol

Preparation of Insert DNA

To perform enzyme-free cloning with the pETite™ Vectors, the target DNA must be amplified with primers that add sequences identical to the ends of the vector adjacent to the cloning site. Rules for designing PCR primers for enzyme-free cloning into the pETite N-His SUMO Vector are presented below.

1) Primer design for target gene amplification

Each PCR primer consists of two segments: 18 nt at its 5' end must match the sequence of one end of the pETite vector, and 18-24 nt at its 3' end anneals to the target gene.

Forward primer (vector sequence includes the last 6 codons of SUMO):

5'- CGC GAA CAG ATT GGA GGT XXX₂ XXX₃ XXX₄ XXX₅ XXX₆ XXX₇ XXX₈
(XXX₂-XXX₈ represents codons 2 through 8 of the target coding region).

IMPORTANT: fusion proteins that have proline immediately following the SUMO tag cannot be cleaved by SUMO Express Protease. If you intend to remove the SUMO tag, ensure that the primer does not encode proline immediately following the GGT codon of SUMO. In addition, the following amino acid residues at the +1 position can impair cleavage: large aliphatic residues, such as valine, leucine, and isoleucine; negatively charged aspartic acid or glutamic acid residues (1); or Lysine. See Appendix E.

Do not include an initiation codon in the forward primer, unless a methionine residue is desired at the amino terminus of the target protein. An ATG codon is contained in the pETite N-His SUMO vector immediately preceding the 6 His codons.

Reverse primer (vector sequence includes Stop anticodon):

5'-GTG GCG GCC GCT CTA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}
XXX_n - XXX_{n-6} represents the **reverse complement** of the sequence of the last 7 codons of the target coding region. XXX_n is the reverse complement of the final codon of the protein. The stop codon of the target gene need not be included, as the vector encodes stop codons.

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Example of reverse primer design:

Consider the following sequence encoding the C-terminal 10 residues of a theoretical protein, ending with a TGA stop codon:

.... **ATC GCT CTA ACA CCG ACC AAG CAG CAG CCA TGA**

The reverse primer should have the following sequence:

5' GTG GCG GCC GCT CTA TTA *TGG CTG CTG CTT GGT CGG TGT* 3'

The required 18 bases corresponding to vector sequence are underlined, and 21 bases corresponding to the reverse complement of the last 7 codons of the gene are *italicized*. The extent of the primer complementary to the target gene may be extended or reduced as necessary to obtain an appropriate T_m for amplification.

2) Amplification of target gene

Amplify the desired coding sequence by PCR, using primers designed as described above. Use of a proofreading PCR polymerase is strongly recommended to minimize sequence errors in the product. The performance of the Expresso T7 System has been verified with PCR products from various proofreading polymerases, including Vent and Phusion® (NEB) and Pfu (Stratagene) polymerases, as well as Taq non-proofreading polymerase. Sequence errors are quite common with Taq polymerase, especially for larger inserts, so complete sequencing of several candidate clones is necessary.

A typical amplification protocol is presented below. Adjustments may be made for the particular polymerase, primers, or template used.

Example amplification protocol:

For a 50 μ l reaction, assemble the following on ice:

5 μ l 10X reaction buffer
4 μ l dNTPs (at 2.5 mM each)
5 μ l 10 μ M Forward primer
5 μ l 10 μ M Reverse primer
X μ l DNA polymerase (follow manufacturer's recommendations)
Y μ l DNA template (~5 ng plasmid DNA, or ~50-200 ng genomic DNA)
Z μ l H ₂ O (bring total volume to 50 ml)
<hr/>
50 μ l

Cycling conditions:

94°C, 2'
94°C, 15"
55°C, 15"
72°C, 1' per kb } 25 cycles
72°C, 10'
4°C, Hold

Analyze the size and amount of amplified DNA by agarose gel electrophoresis. If the reaction yields a single product at a concentration of 10 ng/ μ l or higher, you can proceed directly to **Enzyme-free cloning**. If the desired product is weak or contains spurious bands, it can be purified by agarose gel fractionation prior to use.

IMPORTANT: If the template DNA is an intact circular plasmid encoding kanamycin resistance, it can very efficiently transform the HI-Control™ 10G Cells, creating a high background of parental

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clones on kanamycin agarose plates. Therefore, we strongly recommend restriction digestion of kanamycin-resistant plasmid templates and gel purification of the linearized fragment prior to using it as a template for PCR. Alternatively, the PCR product can be gel purified to isolate it from the circular plasmid DNA.

Sensitivity of DNA to Short Wavelength UV Light

During gel fractionation, use of a short-wavelength UV light box (e.g., 254, 302, or 312 nm) **must** be avoided. Most UV transilluminators, including those sold for DNA visualization, use shortwave UV light, which can rapidly reduce cloning efficiencies by several orders of magnitude (Figure 4).

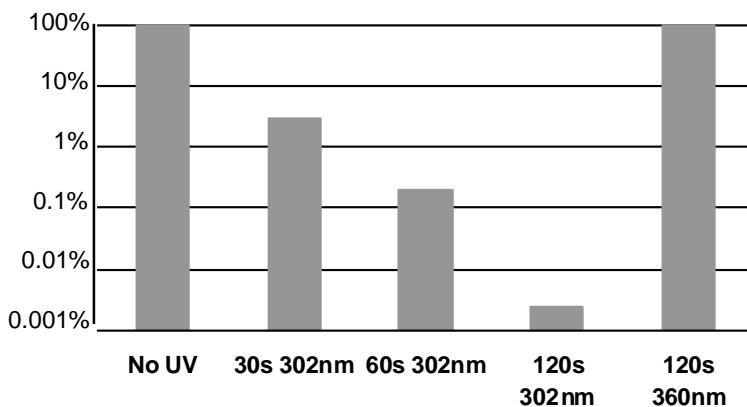


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 120 seconds ("30s 302nm, 60s 302nm, 120s 302nm") or to 360 nm UV light for 120 seconds ("120s 360nm"). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

A hand-held lamp with a wavelength of 360 nm is very strongly recommended. After electrophoresis, DNA may be isolated using your method of choice.

Use a long wavelength (e.g., 360 nm) low intensity UV lamp and short exposure times when isolating DNA fragments from agarose gels.

Enzyme-free cloning with the pETite™ Vectors

In the Expresso T7 enzyme-free cloning strategy, the pre-processed pETite Vector is co-transformed with insert DNA having ends complementary to the vector. After verification of PCR product by agarose gel electrophoresis, the unpurified PCR product (1-3 µl) is mixed with 25 ng of pETite vector and transformed directly into competent HI-Control 10G cells. If desired, the PCR products can be purified before cloning into pETite vectors.

We recommend using 25-100 ng of insert DNA with 25 ng of pETite Vector preparation per transformation.

Optional Control Reactions include the following:

Positive Control Insert DNA	To determine the transformation efficiency with a known insert, use 1 µl (50 ng) of SUMO Positive Control C Insert DNA and 2 µl (25 ng) of pETite™ N-His SUMO Vector.
Vector Background	To determine the background of empty vector, omit insert from the above reaction.

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To ensure optimal cloning results, we strongly recommend the use of Lucigen's HI-Control™ 10G Chemically Competent Cells, which are included with the kit. These cells yield $\geq 1 \times 10^9$ cfu/ μ g of pUC19. The following protocol is provided for transformation.

Transformation of HI-Control 10G Chemically Competent Cells

HI-Control 10G Chemically Competent Cells are provided in 40- μ l aliquots, sufficient for a single transformation. Transformation is performed by incubation on ice followed by heat shock at 42°C. For maximal transformation efficiency, the heat shock is performed in 15-ml disposable polypropylene culture tubes (17 x 100 mm). The use of other types of tubes may dramatically reduce the transformation efficiency. To ensure successful transformation results, the following precautions must be taken:

- All culture tubes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.

Transformation of HI-Control 10G Chemically Competent cells

1. Remove Recovery Medium from the freezer and bring to room temperature.
2. Remove HI-Control 10G cells from the -80°C freezer and thaw completely on wet ice (5-10 minutes).
3. Thaw the tube of pETite vector DNA and microcentrifuge the tube briefly to collect the solution in the bottom of the tube.
4. Add 2 μ l (25 ng) of the pETite vector DNA and 1 to 3 μ l (25 to 100 ng) of insert PCR product to the cells. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
5. Transfer the cells and DNA to a pre-chilled polypropylene culture tube (15-ml; 17 x 100 mm).
6. Incubate culture tube containing cells and DNA on ice for 30 minutes.
7. Heat shock cells by placing the tube in a 42°C water bath for 45 seconds.
8. Return the tube of cells to ice for 2 minutes.
9. Add 960 μ l of room temperature Recovery Medium to the cells in the culture tube.
10. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
11. Plate 100 μ l of transformed cells on YT (LB) agar plates containing 30 μ g/ml kanamycin.
12. Incubate the plates overnight at 37°C.

Transformed clones can be grown in LB, TB, or any other rich culture medium for preparation of plasmid DNA. Growth in TB medium gives the highest culture density and plasmid yield. Use kanamycin (30 μ g/ml) to maintain selection for transformants. Glucose may be added to 0.5% final concentration to ensure complete lack of expression of the recombinant plasmid.

EXPECTED RESULTS USING HI-Control 10G CHEMICALLY COMPETENT CELLS

Plating chemically transformed cells and expected results.

Reaction Plate	μ l/Plate	CFU/Plate	Efficiency
Experimental Insert (~25-100 ng per transformation)	100	variable	NA
Positive Control Insert (50 ng)	100	> 30	> 90% inserts
No-Insert Control (Vector Background)	100	< 5	<10% background
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Ampicillin ^R)	2 (ampicillin plate)	> 200	> 1×10^9 cfu/ μ g plasmid

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The results presented above are expected when transforming 50 ng of intact, purified control insert DNA along with 25 ng of pETite vector using Lucigen's HI-Control 10G Chemically Competent Cells. Cloning AT-rich DNA and other recalcitrant sequences may lead to fewer colonies. With relatively few recombinant clones, the proportion of "empty vector" colonies becomes more significant.

Getting More Recombinants

Certain genes can prove recalcitrant to cloning due to a large size, toxic gene products, secondary structures, extremely biased base composition, or other unknown reasons. For highest transformation efficiencies, we recommend performing the heat-shock transformation in pre-chilled 15 ml culture tubes as specified in the Transformation Protocol. If necessary, the entire 1-ml transformation mix for can be pelleted in a microfuge (10,000 rpm, 30 seconds), resuspended in 100 µl of recovery media, and plated. See Appendix C for troubleshooting suggestions.

Colony PCR Screening for Recombinants

Because the background of empty vector transformants is low, colonies can be picked at random for growth and plasmid purification. If desired, colonies can first be screened for inserts by colony PCR. Lucigen's EconoTaq® PLUS GREEN 2X Master Mix (available separately, Cat. No. 30033-1) is a convenient premix of Taq DNA polymerase, reaction buffer, and dNTPs that provides everything needed for colony PCR, except primers and template DNA. Screening by colony PCR with EconoTaq PLUS GREEN is performed as follows:

Colony PCR with EconoTaq PLUS GREEN 2X Master Mix

Per 25 µl reaction:

12.5 µl EconoTaq PLUS GREEN 2X Master Mix
0.5 µl SUMO Forward primer (50 µM)
0.5 µl pETite Reverse primer (50 µM)
11.5 µl water
<hr/>
25.0 µl

Using a pipet tip, transfer part of a colony to the PCR reaction mix. Disperse the cells by pipetting up and down several times.

Cycling conditions:

94°C 5'
94°C 15"
55°C 15"
72°C 1' per kb
<hr/>
72°C 10'
4°C Hold

} 25 cycles

The EconoTaq PLUS GREEN reactions can be loaded directly onto an agarose gel for analysis. The Master Mix contains blue and yellow tracking dyes that will separate upon electrophoresis. Empty vector clones will yield a product of ~150 base-pairs.

DNA Purification & Sequencing

Grow transformants in LB or TB medium plus 30 µg/ml kanamycin. Use standard methods to isolate plasmid DNA (13). The pETite™ plasmids contain the low copy number pBR origin of replication and produce DNA yield similar to that of pBR-based plasmids. HI-Control™ 10G Cells are *recA* and *endA* deficient to provide high quality plasmid DNA. SUMO Forward and pETite Reverse Sequencing

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Primers are provided with the Kit at a concentration of 50 µM; they must be diluted before use in sequencing. Their sequences and orientations are shown in Appendix B.

Transformation into HI-Control™ BL21(DE3) cells.

Prior to expression studies, clones should be confirmed by DNA sequencing. Confirmed clones in the pETite™ vectors must be transformed into HI-Control BL21(DE3) Cells for expression from the T7 promoter. The chemically competent HI-Control BL21(DE3) Cells have a transformation efficiency of $\geq 1 \times 10^8$ cfu/µg pUC19 DNA. We recommend using ~0.1 to 10 ng of miniprep DNA. Transformation is performed by heat shock. For maximal transformation efficiency, the heat shock is performed in 15-ml disposable polypropylene culture tubes (17 x 100 mm). Plating several different amounts of the transformed cells onto separate plates will help to ensure the recovery of individual colonies. To ensure successful transformation results, the following precautions must be taken:

- All culture tubes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.

Transformation Protocol for Chemically Competent HI-Control BL21(DE3) Cells

1. Remove Recovery Medium from the freezer and bring to room temperature.
2. Remove HI-Control BL21(DE3) Cells from the -80°C freezer and thaw completely on wet ice (5-10 minutes).
3. Add 1 µl of pETite expression clone miniprep DNA (~0.1 to 10 ng) to thawed cells on ice.
4. Transfer the cells and DNA to a pre-chilled disposable polypropylene culture tube (15-ml, 17 x 100 mm).
5. Incubate the culture tube containing cells and DNA on ice for 30 minutes.
6. Heat shock the cells by placing the culture tubes in a 42°C water bath for 45 seconds.
7. Return the tubes to ice for 2 minutes.
8. Add 960 µl of Recovery Medium to the cells in the culture tube.
9. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37°C.
10. Plate 1 to 100 µl of transformed cells on LB or YT agar plates containing 30 µg/ml kanamycin. For maximal repression of target gene expression, plates should also contain 0.5% glucose (see below).
11. Incubate the plates overnight at 37°C.

Growing Transformed Cultures

Colonies obtained from transformation of pETite expression vectors into HI-Control BL21(DE3) Cells generally should NOT be grown in rich medium, such as Terrific Broth, particularly if you suspect that your protein of interest may be toxic to the cells. Terrific Broth contains high levels of lactose, which can inadvertently induce the expression of the T7 RNA polymerase gene from the *lacUV5* promoter and expression of the target gene from the T7-lac promoter. LB is recommended for routine protein expression experiments.

Controlling leaky expression with glucose: Catabolite repression

Undesired “leaky” expression of target genes during growth prior to induction can lead to slow growth, instability of the expression plasmid, and reduced yield of the target protein, particularly if the protein is toxic to the host strain. A simple way to maintain tight repression of target genes under the control of the T7 promoter is to add glucose to the growth medium (final concentration 0.5 to 1%) (14). In HI-Control™ BL21(DE3) Cells and many other T7 host strains, the T7 RNA polymerase gene

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is expressed from the *lacUV5* promoter. Transcription from this promoter is dependent on the cAMP-dependent transcriptional activator protein, known as CAP or CRP. When glucose is available as a carbon source, cAMP levels remain low and CAP cannot bind to its DNA target upstream of the *lacUV5* promoter. In the absence of glucose, and particularly as cells approach stationary phase, increased cAMP levels lead to significant expression of T7 RNA polymerase and of target genes under the control of T7 promoters, even in the absence of lactose. We recommend the addition of 0.5% glucose to cultures that are not intended for induction. Glucose can also be included in cultures to be induced with IPTG, but may limit the yield of target protein.

Induction of Protein Expression

Small scale expression trials (2 to 50 ml) are recommended to evaluate expression and solubility of the SUMO fusion protein before scaling up for purification.

Inoculate LB medium containing 30 µg/ml kanamycin with a single colony of HI-Control BL21(DE3) cells containing a pETite expression construct. Shake at 220-250 rpm at 37°C.

If cultures will be grown overnight before induction, add glucose to 0.5% to maintain repression of the *lacUV5* and T7-lac promoters. The following morning, dilute 1:100 into LB plus kanamycin.

When cultures reach an optical density at 600 nm (OD₆₀₀) of 0.5-1.0, collect a 1-ml aliquot of uninduced cells by pelleting in a microcentrifuge tube (12,000 x g for 1 minute). Resuspend the cell pellet in 50 µl of SDS-PAGE loading buffer. Store the uninduced sample on ice or at -20°C until SDS-PAGE analysis.

To induce expression, add IPTG to a final concentration of 1 mM. Continue shaking at 37°C for 3 hours or more. Record the OD₆₀₀ of the induced culture and harvest a 1-ml sample by microcentrifugation. Resuspend the cell pellet in 100 µl SDS-PAGE loading buffer and store on ice or at -20°C. Perform SDS-PAGE analysis to evaluate expression.

To evaluate target protein solubility, harvest the remainder of the culture by centrifugation at 4000 Xg for 15 minutes. Pour off growth media and resuspend the cell pellet in 1-5 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). Freeze and thaw the cells to assist lysis, or add lysozyme (1 mg/ml) and incubate 30 minutes on ice. Lyse cells by sonication on ice. Use 6-10 pulses of 10 seconds each with a microtip; allow 1 minute for the samples to cool between pulses. Avoid frothing.

Centrifuge the lysate at 10000 x g for 20 minutes. Collect the supernatant, which contains the soluble protein, and save on ice. Resuspend the pellet, containing insoluble proteins and unlysed cells, in a volume of lysis buffer equivalent to the original lysate.

SDS-PAGE analysis

Add the samples to SDS-PAGE loading buffer and heat to 95°C for 5 minutes. Centrifuge the samples for 1 minute (12,000 x g). Load volumes containing equivalent OD₆₀₀ units. Include standards to estimate molecular weight of the recombinant protein. For minigels, 0.05 OD₆₀₀ equivalent per lane usually contains the appropriate amount of protein for Coomassie® blue staining.

Affinity Purification of 6xHis tagged proteins.

Many protocols are available for purification of 6xHis tagged proteins under native or denaturing conditions. For best results, follow the procedures recommended by the manufacturer of your IMAC resin. SUMO Express Protease is a cysteine protease. Avoid the use of cysteine protease inhibitors, such as leupeptin, during purification.

Removal of SUMO tag using SUMO Express Protease.

Important: The SUMO tag used in the Expresso T7 SUMO System contains amino acid substitutions that render it resistant to cleavage by native desumoylation enzymes present in eukaryotic cells, including commercial SUMO proteases derived from the native *ULP1* gene product.

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The SUMO Express Protease contains compensatory mutations that enable it to recognize and cleave the stabilized SUMO tag used in this system. **Other SUMO proteases lacking these compensatory mutations cannot cleave the SUMO tag used in the Expresso T7 SUMO system.**

After cleavage, the released protein of interest is easily separated from the SUMO tag and SUMO Express Protease by use of the 6xHis tag present on both the protease and the cleaved SUMO tag. The cleavage mixture is simply applied to an IMAC column, and the free target protein recovered in the flow-through; the SUMO tag and SUMO Express Protease remain bound to the IMAC matrix. Although SUMO Express Protease is tolerant to imidazole up to 300 mM, residual imidazole from initial purification of the SUMO fusion protein will interfere with binding of the protease and the cleaved SUMO tag to the IMAC resin. Dialysis is recommended before cleavage to remove imidazole and to exchange the fusion protein into the desired buffer for cleavage.

Dialysis. Dialyze the purified SUMO fusion protein for 24 hours at 4°C to remove imidazole. The recommended buffer for dialysis is (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol). Alternative buffers (e.g. phosphate, HEPES) are also tolerated by the protease. For maximal activity maintain pH in the range 7 to 9. Salt concentrations of 500 mM and higher are detrimental. Non-ionic detergents such as Triton X-100 or Igepal (NP-40) may be used at 1%.

Cleavage. The recommended buffer for cleavage is the same as the dialysis buffer, with the addition of 2 mM fresh dithiothreitol (DTT). We suggest performing small-scale test reactions to evaluate cleavage efficiency with each different fusion protein. Add 1 unit of protease per 10-100 µg of fusion protein. Incubate at 30°C for 1 hour, or at 4°C overnight. To evaluate cleavage, remove a sample of the reaction (5-10 µg of fusion protein). Add an equal volume of 2X SDS-PAGE loading buffer, heat to 95°C for 5 minutes and run on SDS-PAGE along with molecular weight markers. The free 6xHis-SUMO tag has an expected molecular weight of 12 kDa, but migrates at ~15-18 kDa. If partial cleavage is observed, another aliquot of SUMO Express Protease may be added and digestion continued at 4°C overnight.

Recovery of cleaved target protein. After the cleavage reaction is complete, the SUMO tag and SUMO Express protease, as well as any residual uncleaved fusion protein, are removed from the sample by adsorption to a metal affinity chromatography (IMAC) matrix. The sample can be applied directly to an IMAC column. The released protein will be present in the column flowthrough and wash, while the 6xHis tagged SUMO fragment and protease remain bound to the column. **Note:** In some cases the presence of 2 mM DTT in the cleavage reaction may cause reduction of metal ions in the IMAC resin, resulting in leaching of the metal and failure to retain 6xHis-tagged proteins. We recommend testing the IMAC resin with the cleavage buffer before applying the digested sample to the resin. IMAC resins that have been reduced turn brown in color. If necessary, dilute the cleavage reaction to reduce the DTT concentration.

Optional cleavage control reactions. A Cleavage Control Protein (50 µg at 2 µg/µl) is included with the kit to allow verification of protease activity. One unit of SUMO Express Protease is sufficient to cleave 100 µg of the Cleavage Control Protein in 30 minutes at 4°C in the recommended buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM DTT).

To test protease activity, make a fresh 50-fold dilution (0.02 unit/µl) of SUMO Express Protease by mixing 1 µl (1 unit) with 49 µl of recommended dialysis/cleavage buffer containing fresh DTT (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM DTT). Mix thoroughly and assay immediately by mixing 5 µl (0.1 units) of the diluted protease with 5 µl (10 µg) of Cleavage Control Protein. Incubate for 30 minutes at 4°C (or 5 minutes at 30°C). Add 10 µl 2X SDS gel sample buffer and heat to 95°C for 5 minutes. Run 10 µl (5 µg) on SDS-PAGE and stain the gel with Coomassie blue to visualize cleavage products. Undigested Cleavage Control Protein migrates at ~ 40 kDa, while the cleavage products migrate at ~25 kDa and ~15 to 18 kDa.

See Appendix E for Protease digestion troubleshooting recommendations.

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Appendix A: Media Recipes

YT + kan30 Agar Medium for Plating of Transformants

Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Mix components, autoclave and cool to 55°C. To select for pETTite™ transformants, add kanamycin to a final concentration of 30 µg/ml. Pour into petri plates.

LB Culture Medium

Per liter: 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl. Mix components and autoclave.

2X SDS Gel Sample Buffer

100 mM Tris-HCl (pH 6.5), 4% SDS, 0.2% bromophenol blue, 20% glycerol. Add dithiothreitol to a final concentration of 200 mM in the 2X buffer prior to use.

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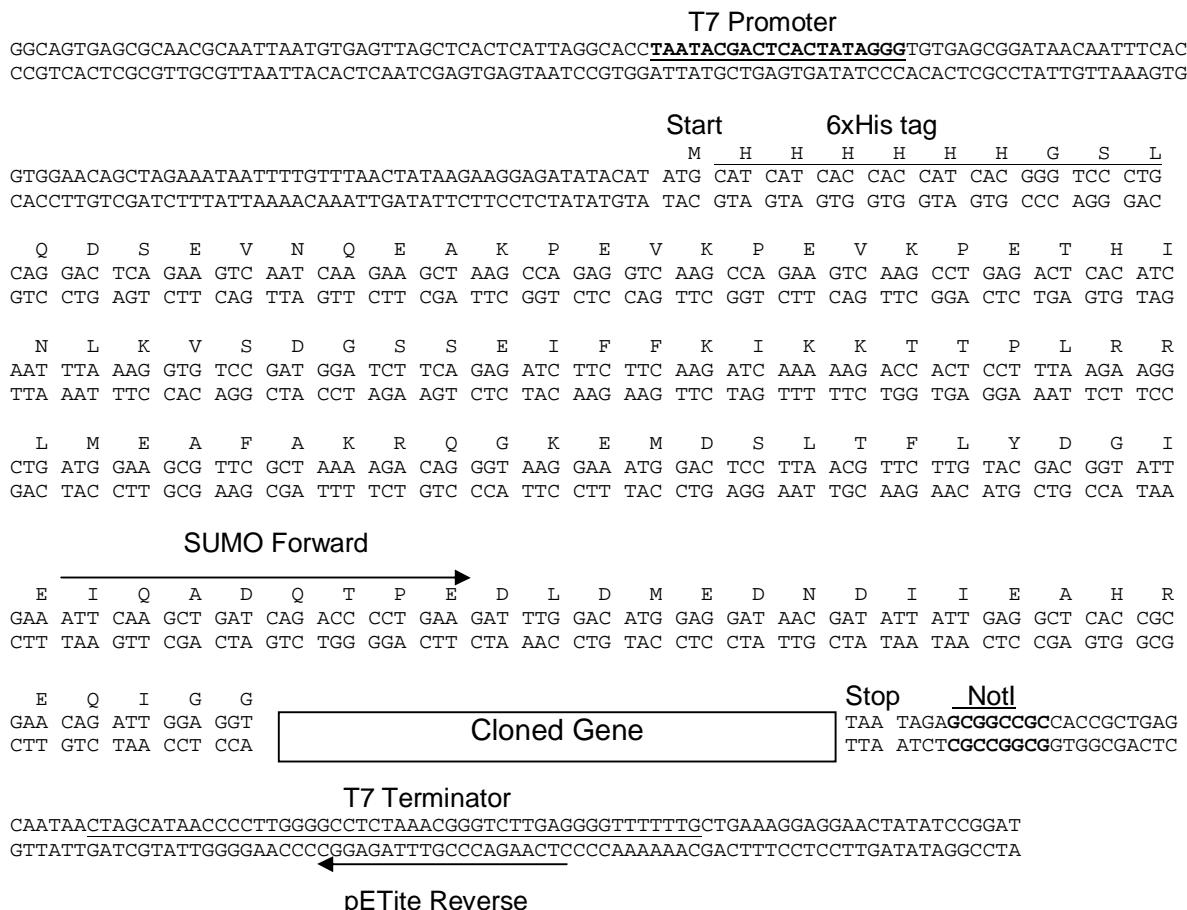
Appendix B: Vector Map and Sequencing Primers

The sequences of the SUMO Forward and pETite™ Reverse primers are:

SUMO Forward: 5'-ATTCAAGCTGATCAGACCCCTGAA-3'
pETite Reverse: 5'-CTCAAGACCCGTTAGAGGC-3'

Shown below are the regions surrounding the cloning site in the pETite SUMO Vector. For the complete vector sequence, see Appendix F.

pETite N-His SUMO Kan Vector:



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Appendix C: Cloning Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	No DNA, degraded DNA, or insufficient amount of DNA.	Check insert DNA by gel electrophoresis. Determine concentration of insert and add the correct amount. Use the supplied control insert to test the system.
	Incorrect primer sequences.	Be sure the 5' ends of the primer sequences match the version of the pETite™ vector used for transformation.
	Wrong antibiotic used.	Add the correct amount of kanamycin to molten agar at 55°C before pouring plates.
	Incorrect amounts of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain inserts.	Transformants are due to intact plasmid template DNA.	Linearize plasmid DNA used as a template for PCR. Gel-isolate template DNA fragment.
	Inserts are too small to detect.	Analyze colonies by sequencing to confirm the presence of inserts.
	Incorrect amount of antibiotic in agar plates.	Add the correct amount of kanamycin to molten agar at 55°C before pouring plates. DO NOT spread antibiotic onto the surface of agar plates.

Expresso™ T7 SUMO Cloning and Expression System

Appendix D: Expression/Purification Troubleshooting Guide

Problem	Probable Cause	Solution
No colonies when expression clone is transformed into HI-Control BL21(DE3) cells	Wrong antibiotic used.	Add the correct amount of kanamycin to molten agar at 55°C before pouring plates.
	Incorrect amounts of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates.
	Too little DNA used, or too few cells plated.	Verify the concentration of plasmid DNA. Transform with 10 ng of plasmid DNA and plate up to 50 ul of cells.
	Toxic gene product.	Use plates containing 0.5% glucose to prevent leaky expression of T7 RNA polymerase. Incubate plates at room temperature.
Low recovery of recombinant protein	Recombinant protein not overexpressed	Check lysate by SDS-PAGE and/or western blot to confirm overexpression of recombinant protein
	His tag not present	Recombinant proteins may be cleaved during expression or lysate preparation. Use protease inhibitors to prevent cleavage. Check lysate and column flow through by SDS-PAGE and western blot to confirm 6xHis tag is attached to the over expressed protein of the expected molecular weight.
	Recombinant protein expressed in inclusion bodies	Lyse induced bacteria directly in an SDS-PAGE loading buffer and check for expression by SDS-PAGE and/or western blot. Compare these results to SDS-PAGE and/or western blot assays of cleared lysate. During IPTG induction incubate culture at room or lower temperature to obtain more soluble recombinant protein. Try Lucigen's OverExpress™ C41(DE3) and/or C43(DE3) competent cells, which express lower levels of T7 RNA polymerase.

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Appendix E: Protease Digestion Troubleshooting Guide

Problem	Probable Cause	Solution
Incomplete cleavage of SUMO tag.	Inappropriate or suboptimal buffer conditions.	Dialyze purified fusion protein against 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, pH 8.0, or other appropriate buffer. Add fresh DTT (2 mM) to cleavage reaction. Avoid: salt concentrations ≥500 mM, pH>9.0 or <6.5, urea ≥ 2 M, guanidine-HCl ≥ 0.5 M, imidazole concentration
	Aggregation of fusion protein.	Perform cleavage in the presence of urea (up to 2 M) or guanidine-HCl (up to 0.5 M), or up to 1% non-ionic detergent (e.g. Triton X-100, NP-40, Igepal).
	Unfavored amino acid at +1 position following cleavage site.	Fusion proteins with proline immediately following the SUMO tag cannot be cleaved. Other residues at +1, such as large aliphatic residues (Ile, Leu, Val,); negatively charged residues (Asp, Glu) or Lysine may result in slower cleavage rates. Increase protease concentration (up to 1 unit/µg) and/or incubation time, and/or temperature, or reclone target gene with a single glycine codon (e.g., GGA) or glycine + serine codons (e.g. GGA TCC) between the SUMO tag and the target gene. (Added residues will remain attached to the protein of interest after cleavage by SUMO Express Protease.)
	Junction between SUMO tag and amino terminus of target protein inaccessible to protease.	Perform cleavage in the presence of urea (up to 2 M) or guanidine-HCl (up to 0.5 M). Reclone target gene with a single glycine codon (e.g., GGA) or glycine + serine codons (e.g. GGA TCC) between the SUMO tag and the target gene. (Added residues will remain attached to the protein of interest after cleavage by SUMO Express Protease.)
	Inactive protease.	Store SUMO Express Protease at -80°C for up to 1 year, or at -20°C for up to 2 weeks. Avoid multiple freeze-thaw cycles at -80°C. Perform test cleavage reactions with SUMO Cleavage Control Protein.
Uncleaved fusion protein or cleaved SUMO tag present in sample after subtractive IMAC purification.	Residual imidazole in sample.	Dialyze sample to remove imidazole.
	Reduction of metal ions in IMAC resin.	Test compatibility of IMAC resin with cleavage buffer. Dilute cleavage reaction to reduce DTT concentration.

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Appendix F: Sequence of pETite™ N-His SUMO Vector (2535 bp)

The sequence of the pETite N-His SUMO Kan Vector can be found linked to Lucigen's Expresso T7 SUMO Cloning and Expression product page, or linked to the Vector Sequences section of the Technical Information Page.