

Expresso[®] Rhamnose 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[®] Rhamnose SUMO Cloning and Expression System

Technical Support

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SUMO Express Protease is manufactured and supplied by LifeSensors, Inc.

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Expresso[®] Rhamnose SUMO Cloning and Expression System

System Designations

The Expresso Rhamnose SUMO Cloning and Expression System contains pre-processed pRham™ N-His SUMO Vector DNA, *E. coli*® 10G Chemically Competent Cells for cloning and protein expression, SUMO Express Protease, Cleavage Control Substrate, control insert, primers for clone verification by sequencing or PCR, recovery medium for transformation, and solutions of L-rhamnose and D-glucose for small-scale induction of protein expression. The System catalog numbers are listed below.

Expresso Rhamnose SUMO Cloning and Expression Kits

	5 Reactions	10 Reactions
Expresso Rhamnose SUMO Cloning and Expression System	49013-1	49013-2

Components & Storage Conditions

The Expresso Rhamnose SUMO Cloning and Expression System consists of three separate containers. Container 1 includes the pRham N-His SUMO Expression Vector, SUMO Positive Control Insert DNA, DNA primers for screening inserts by PCR and sequencing, 20% L-Rhamnose Solution, and 15% D-Glucose Solution. 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 *E. coli* 10G 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 Rhamnose SUMO Cloning Kit Container Store at -20°C

	Concentration	Volume
pRham 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
Rhamnose Solution	20% w/v	1.25 ml
Glucose Solution	15% w/v	1.25 ml

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

E. coli 10G Chemically Competent Cells Container Store at -80°C

	5 Reaction Kit	10 Reaction Kit
<i>E. coli</i> 10G Chemically Competent Cells	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)

Kit Components Available Separately:

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

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Expresso System Cells

Description	Size	Cat. No.
<i>E. coli</i> [®] 10G Chemically Competent Cells (SOLOs)	12 Transformations	60106-1
	24 Transformations	60106-2
	48 Transformations	60106-3

Rhamnose and Glucose Solutions

Description	Size	Cat. No.
Rhamnose Solution, 20% w/v	5 X 1.25 ml	49021-1
Glucose Solution, 15% w/v	5 X 1.25 ml	49022-1

System Description

The Expresso Rhamnose SUMO Cloning and Expression System uses Expressioneering[™] Technology to enable rapid cloning and expression of SUMO-tagged fusion proteins in *E. coli*. Expressioneering is an *in vivo* recombinational cloning strategy whereby PCR products can be cloned instantly, with no enzymatic treatment (Figure 1). After amplification of the target gene with primers that append 18 bp sequences homologous to the ends of the chosen Expresso System Vector, the PCR product is simply mixed with the pre-processed Vector and transformed directly into the provided high-efficiency chemically competent cells. Recombination within the host cells seamlessly joins the insert to the vector. 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.

In the Expresso Rhamnose SUMO Cloning and Expression System, a PCR product containing the gene of interest is cloned as a fusion to the solubility-enhancing, cleavable SUMO tag under the control of the L-rhamnose-inducible rhaP_{BAD} promoter harbored on the pRham[™] N-His SUMO Vector. Because this promoter is recognized by the bacterial RNA polymerase, a single host strain is used for both clone construction and protein expression. This single-host strategy allows a streamlined workflow compared to systems requiring separate hosts for cloning and expression.

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 rhaP_{BAD} promoter is a versatile tool for protein expression. In the absence of rhamnose, the transcriptional activity of rhaP_{BAD} is very low, allowing stable clone construction even for potentially

Expresso[®] Rhamnose SUMO Cloning and Expression System

toxic gene products (6). Transcription is positively controlled by two activators, RhaR and RhaS, which bind rhamnose (7). RhaR activates its own transcription as well as that of RhaS, which in turn activates transcription from rhaP_{BAD}. This regulatory cascade makes transcription from rhaP_{BAD} responsive to different concentrations of rhamnose, allowing “tunable” control of the target gene expression level (8). For proteins that are potentially toxic to the host cells, or that are difficult to express in soluble form, this tuning capability may enable the adjustment of expression levels for maximal yield of soluble protein.

Transcription from the rhaP_{BAD} promoter is also controlled by the cAMP-dependent transcriptional activator protein CAP, and is therefore subject to catabolite repression. In the presence of glucose, cAMP levels remain low and rhaP_{BAD} remains inactive, even when rhamnose is available. This allows the use of “autoinduction” procedures for protein expression, in which cells are inoculated directly into medium containing rhamnose and a small amount of glucose.

E. coli[®] 10G Chemically Competent Cells are used for construction of clones in the pRham[™] Vectors. Their very high transformation efficiency ($\geq 1 \times 10^9$ cfu/ μ g pUC19 DNA) makes these cells ideal for cloning using Expressioneering Technology. Their *recA*⁻, *endA*⁻ genotype allows recovery of high quality plasmid DNA. *E. coli* 10G cells are appropriate for expression and purification of most proteins. Because the rhaP_{BAD} promoter is transcribed by the bacterial RNA polymerase, clones constructed in the pRham vectors can be transferred into and expressed in virtually any other host strain containing functional rhaR and rhaS genes.

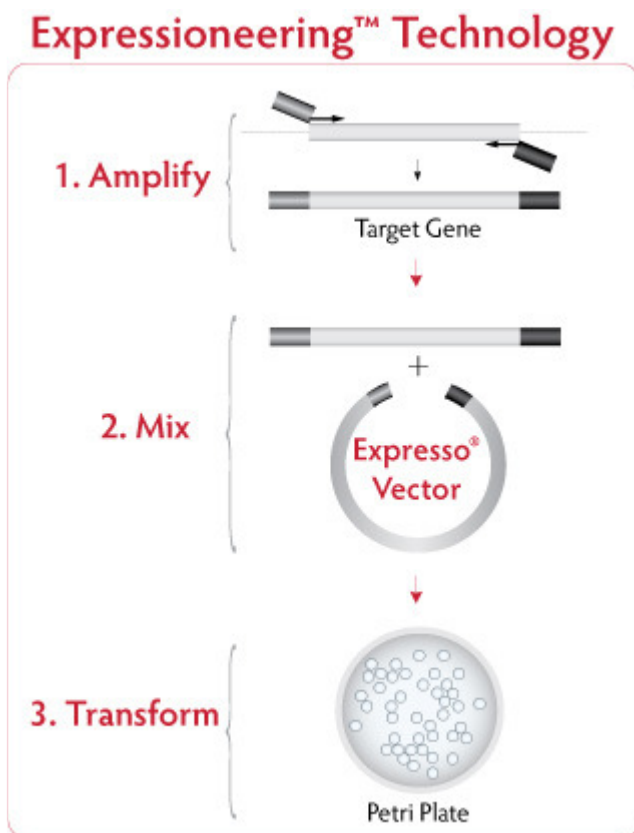


Figure 1. Expressioneering schematic. A target gene is amplified with primers that contain short homology to the ends of the Expresso System vector. The PCR product is then mixed with the pre-processed vector and transformed directly into the high-efficiency chemically competent cells provided. Recombination between the ends of the Vector and PCR product occurs *in vivo*. Clones can be verified by colony PCR or miniprep. With the Expresso Rhamnose System, colonies can be inoculated directly from the transformation plate into autoinduction media for expression.

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pRham[™] N-His SUMO Kan Vector

The pRham Vectors are based on Lucigen's patented pSMART[®] vectors, which feature transcriptional terminators to prevent unwanted transcription into or out of the cloned sequence. The small size of the pRham N-His SUMO Kan Vector (2.6 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis.

The pRham N-His SUMO Kan Vector is supplied in a pre-linearized format for instant, directional insertion of target genes using Expressioneering[™] Technology (Figures 1-3). The vector includes signals for expression, including the rhaP_{BAD} promoter, efficient ribosome binding site from the T7 gene 10 leader, 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. Target gene PCR products designed for cloning into the pRham N-His SUMO vector are compatible with the pETite[®] N-His SUMO vector included in the Expresso T7 SUMO kits, and vice versa.

The pRham 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 pRham 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.

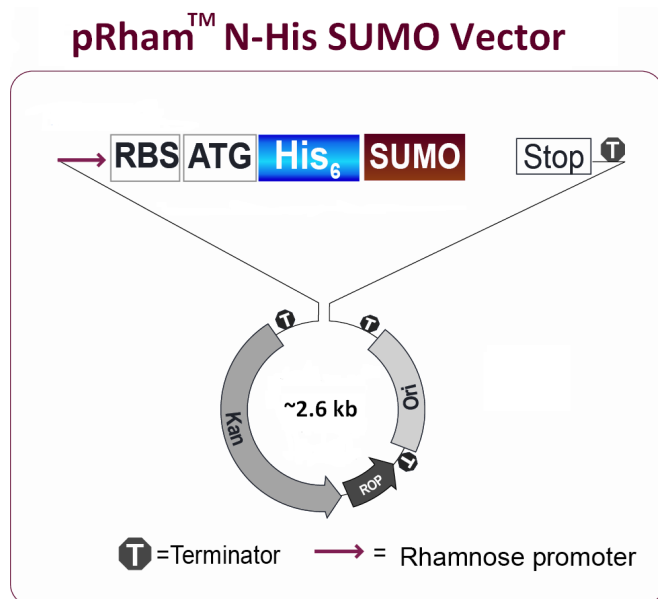


Figure 2. pRham N-His SUMO Kan expression vector. RBS, ribosome binding site; ATG, translation start site; Stop, translation end site; Kan, kanamycin resistance gene; ROP, Repressor of Priming (for low copy number); Ori, origin of replication. CloneSmart[®] transcription terminators (T) prevent transcription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

E. coli[®] 10G Chemically Competent Cells

E. coli 10G Chemically Competent Cells are an *E. coli* strain optimized for high efficiency transformation. The *E. coli* 10G cells are ideal for cloning and propagation of plasmid clones, and give high yield and high quality plasmid DNA due to the *endA1* and *recA1* mutations.

E. coli 10G Genotype:

mcrA Δ (*mrr-hsdRMS-mcrBC*) *endA1* *recA1* ϕ 80d/*lacZ* Δ M15 Δ *lacX74* *araD139* Δ (*ara,leu*)7697 *galU galK rpsL nupG* λ^- *tonA*

*E. coli*10G Chemically Competent Cells produce $\geq 1 \times 10^9$ cfu/µg supercoiled pUC19 DNA.

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As a control for transformation, *E. cloni*[®] 10G Competent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/μl. Use 1 μl (10 pg) for transformation. Select pUC19 transformants on plates containing ampicillin or carbenicillin (100 μg/ml).

Cloning Strategy

The pRham[™] N-His SUMO Vector preparation enables a simple recombinational 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. 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).

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 pRham 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 pRham 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 *E. cloni* 10G host strain and expressed in the same host. Transformants are selected with kanamycin. Individual colonies are grown in liquid culture, and protein expression is induced by addition of rhamnose. Expression of SUMO-tagged fusion proteins is evaluated by SDS-PAGE analysis.

Protein Purification

Materials for purification are not provided with the Expresso Rhamnose 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.

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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 Rhamnose 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 Rhamnose 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 Rhamnose 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* Rhamnose SUMO Cloning and Expression Kit supplies many of the items needed to efficiently generate and express recombinant clones. While simple and convenient, successful use 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.
- Dithiothreitol (DTT).
- Sonicator or cell lysis reagents.
- Resin and columns for immobilized metal affinity chromatography.
- SDS-PAGE equipment.

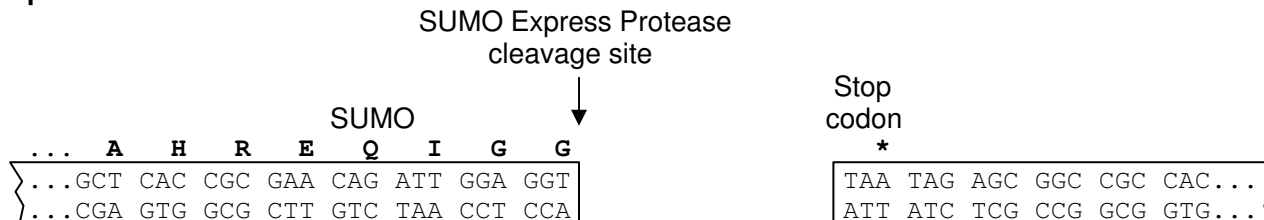
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Detailed Protocol

Preparation of Insert DNA

For cloning with the pRham™ N-His SUMO Vector using Expressioneering™ Technology, 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 pRham N-His SUMO Vector are presented below. Figure 3 presents a schematic illustration of primer design for cloning into the pRham N-His SUMO vector.

pRham N-His SUMO Vector:



PCR Product:

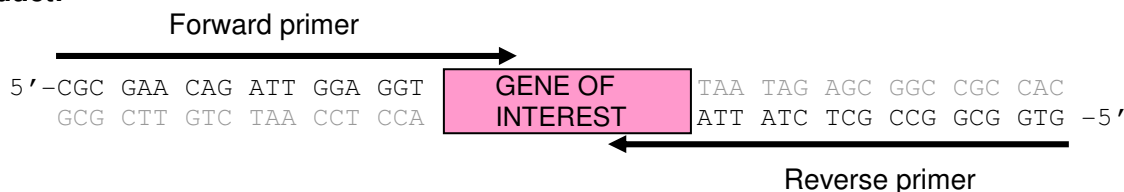


Figure 3. Insertion of a gene into the pRham N-His SUMO Vector for expression. PCR primers add flanking sequences identical to the vector sequence adjoining the insertion site. Recombination within the host cell fuses the blunt PCR product to the vector. See Detailed Protocol.

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 pRham vector, and 18-24 nt at its 3' end anneal to the target gene. Factors affecting the length of the target-specific portion of the primer include GC content, T_m , and potential for formation of hairpins or primer-dimers.

Forward primer (defined 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 pRham N-His SUMO vector immediately preceding the 6 His codons.

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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.

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 Rhamnose 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. Follow the recommendations of the enzyme supplier.

Example amplification protocol:

For a 50 µl reaction, assemble the following on ice:

5 µl 10X reaction buffer
4 µl dNTPs (at 2.5 mM each)
5 µl 10 µM Forward primer
5 µl 10 µM Reverse primer
X µl DNA polymerase (follow manufacturer's recommendations)
Y µl DNA template (~5 ng plasmid DNA, or ~50-200 ng genomic DNA)
Z µl H₂O (bring total volume to 50 ml)

50 µl

Cycling conditions:

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

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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/μl 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 *E. cloni*[®] 10G Cells, creating a high background of parental clones on kanamycin agar 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).

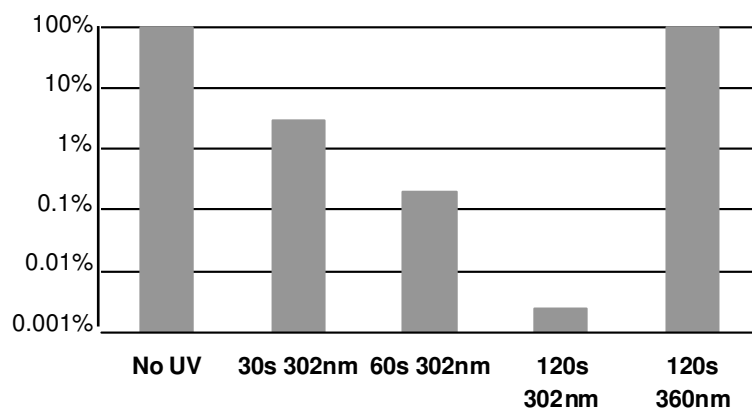


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 120 seconds (“30s 302nm, 60s 302nm, 120s 302nm”) or to 360 nm UV light for 120 seconds (“120s 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 pRham[™] N-His SUMO Vector

With Expressioneering[™] technology, the pre-processed pRham N-His SUMO 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 pRham N-His SUMO Vector and transformed directly into competent *E. cloni* 10G cells. If desired, the PCR products can be purified before cloning into pRham vectors.

We recommend using 25-100 ng of insert DNA with 25 ng of pRham[™] Vector preparation per transformation.

Optional Control Reactions include the following:

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

To ensure optimal cloning results, we strongly recommend the use of Lucigen's *E. cloni* 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 *E. cloni*[®] 10G Chemically Competent Cells

E. cloni 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 *E. cloni* 10G Chemically Competent cells

1. Remove Recovery Medium from the freezer and bring to room temperature.
2. Remove *E. cloni* 10G cells from the -80°C freezer and thaw completely on wet ice (5-10 minutes).
3. Thaw the tube of pRham 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 pRham 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. **Important:** Transfer the mixture of cells and DNA to a pre-chilled polypropylene culture tube (15-ml; 17 x 100 mm). Performing the heat shock in the small tube in which the cells are provided will significantly reduce the transformation efficiency.
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 LB or YT 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.

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EXPECTED RESULTS USING *E. coli*[®] 10G CHEMICALLY COMPETENT CELLS

Reaction Plate	μl/Plate	CFU/Plate	Efficiency
Experimental Insert (~25-100 ng per transformation)	100	variable	NA
Positive Control Insert (50 ng)	100	> 50	> 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 x 10 ⁹ cfu/μg plasmid

The results presented above are expected when transforming 50 ng of intact, purified control insert DNA along with 25 ng of pRham[™] vector using Lucigen's *E. coli* 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 using the SUMO Forward and pETite[®] Reverse primers included with the kit. 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'	} 25 cycles
94 °C 15"	
55 °C 15"	
72 °C 1' per kb	
72 °C 10'	
4 °C Hold	

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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 pRham[™] plasmids contain the low copy number pBR origin of replication and produce DNA yield similar to that of pBR-based plasmids. *E. coli* 10G Cells are *recA* and *endA* deficient to provide high quality plasmid DNA. SUMO Forward and pETite[®] Reverse Sequencing 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.

Controlling leaky expression with glucose: Catabolite repression

Undesired “leaky” expression of target genes 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 rhaP_{BAD} promoter is to add glucose to the growth medium (final concentration 0.5 to 1%) (14). 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 rhaP_{BAD} promoter. In the absence of glucose, and particularly as cells approach stationary phase, increased cAMP levels may lead to significant expression of target genes under the control of the rhaP_{BAD} promoter, even in the absence of rhamnose. For maximal control, we recommend the addition of 0.5% glucose to cultures that are not intended for induction.

Induction of Protein Expression

Small scale expression trials (2 to 50 ml) are recommended to allow evaluation of expression and solubility of the target protein before scaling up for purification. A vial of 20% (w/v) L-rhamnose Solution is provided with the kit. For maximal induction, the recommended final concentration of rhamnose is 0.2%. Lower amounts in the range of 0.001% to 0.1% can be used for lower levels of expression, which may improve solubility of proteins that tend to be insoluble when overexpressed. Induction for up to 8 hours may be required for maximal target protein expression (or up to 24 hours or more for cultures grown at 20° to 30°C). A standard protocol and a convenient autoinduction protocol are outlined below. Optimal conditions for expression of soluble protein, including growth temperature, length of induction, and concentration of rhamnose should be determined empirically for each target protein.

Standard induction. Inoculate LB medium containing 30 µg/ml kanamycin with a single colony of *E. coli* 10G cells containing a pRham expression construct. Shake at 220-250 rpm at 37°C. When cultures reach an optical density at 600 nm (OD₆₀₀) of 0.2 to 0.8, collect a 1-ml aliquot of uninduced cells by pelleting in a microcentrifuge tube (12,000 x g for 1 minute). This will serve as the uninduced control. 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 rhamnose to the remainder of the culture at a final concentration of 0.2%. Continue shaking at 37°C for 4-8 hours (or overnight). 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. Samples of uninduced and induced cells containing equivalent OD units should be loaded to allow evaluation of expression levels.

Alternatively, cultures for induction may be inoculated from an uninduced overnight culture grown in LB plus 30 µg/ml kanamycin, with the addition of 0.5% glucose. We recommend the addition of glucose to cultures not intended for induction to maintain tight repression of the rhaP_{BAD} promoter as

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the culture approaches saturation. The following morning, dilute the overnight culture 1:100 into LB plus kanamycin without glucose, and induce with rhamnose as described above.

Autoinduction. A convenient method for induction requiring minimal user intervention involves inoculating cells directly from a plate or from an overnight culture into media containing both 0.2% L-rhamnose and a low concentration (0.05 to 0.15%) of D-glucose (9). A vial of 15% (w/v) D-glucose Solution is included with the kit. Cells will preferentially metabolize glucose during the early stages of growth, and only when glucose is depleted will the rhaP_{BAD} promoter become active. The timing of induction by rhamnose can be controlled by varying the concentration of glucose between 0.05% and 0.15%. Later onset of induction may be beneficial for protein yield in cases where the expressed protein is toxic to the host cells.

	<u>Early autoinduction</u>	<u>Late autoinduction</u>
per ml of LB medium:	10 µl 20% L-rhamnose	10 µl 20% L-rhamnose
	3.3 µl 15% D-glucose	10 µl 15% D-glucose

The actual timing of the onset of induction will depend on the number of cells in the inoculum as well as the growth rate. For cultures inoculated to an initial OD₆₀₀ of 0.4 and grown at 37 °C, induction of expression may begin at 2 to 4 hours and peak by 8 hours with 0.05% glucose (early autoinduction), or begin after 8 hours and peak by 24 hours with 0.15% glucose (late autoinduction). For cultures inoculated from a single colony, glucose depletion will generally occur later than these estimates.

Evaluating target protein solubility. Harvest cells from 2 to 50 ml of 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.

Collect a sample of the whole lysate for gel analysis. Centrifuge the remainder of the lysate at 12000 x g for 10 minutes. Collect the supernatant (cleared lysate), 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. Analyze samples by SDS-PAGE.

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 of each sample containing equivalent OD₆₀₀ units. Include standards to estimate molecular weight of the recombinant protein. For minigels, 0.05 OD₆₀₀ equivalent per lane usually contains an 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 Rhamnose 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. 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 Rhamnose SUMO system.**

After cleavage, the released protein of interest is easily separated from the SUMO tag and SUMO

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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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References

1. Malakhov, M.P., Mattern, M.R., Malakhova, O.A., Drinker, M., Weeks, S.D. and Butt., T.R. (2004). SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. *J Struct Funct Genomics*. **5**, 75.
2. Marblestone, J.G., Edavettal, S.C., Lim, Y., Lim, P., Zuo, X. and Butt, T.R. (2006). Comparison of SUMO fusion technology with traditional gene fusion systems: Enhanced expression and solubility with SUMO. *Protein Sci*. **15**,182.
3. Mossesso, E. and Lima, C.D. (2000). Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell*. **5**, 865.
4. Liu, L., Spurrier, J., Butt., T.R. and Strickler, J.T. (2008). Enhanced protein expression in the baculovirus/insect cell system using engineered SUMO fusions. *Protein Expr Purif*. **62**, 21.
5. Peroutka, R.J., Elshourbagy, N., Piech, T. and Butt., T.R. (2008), Enhanced protein expression in mammalian cells using engineered SUMO fusions: Secreted phospholipase A₂. *Protein Sci*. **17**, 1586.
6. Haldimann, A., Daniels, L. and Wanner, B. (1998). Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. *J. Bacteriol*. **180**, 1277.
7. Egan, S.M. and Schleif, R.F. (1993). A regulatory cascade in the induction of *rhaBAD*. *J. Mol. Biol*. **234**, 87.
8. Giacalone, M.J., Gentile, A.M., Lovitt, B.T., Berkley, N.L., Gunderson, C.W. and Surber, M.W. (2006). Toxic Protein Expression in *Escherichia coli* using a rhamnose-based tightly regulated and tunable promoter system. *Biotechniques* **40**, 355.
9. Wegerer, A., Sun, T. and Altenbuchner, J. (2008). Optimization of an *E. coli* L-rhamnose-inducible expression vector: test of various genetic module combinations. *BMC Biotechnol*. **8**, 2.
10. Bubeck, P., Winkler, M. and Bautsch, W. (1993). Rapid cloning by homologous recombination *in vivo*. *Nuc. Acids Res*. **21**, 3601.
11. Klock, H.E., Koesema, E.J., Knuth, M.W. and Lesley, S.A. (2008). Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts. *Proteins* **71**, 982.
12. Chang, C.C., Chuang, Y.C. and Chang, M.C. (2004). Fluorescent intensity of a novel NADPH-binding protein of *Vibrio vulnificus* can be improved by directed evolution. *Biochem. Biophys. Res. Comm*. **322**, 303.
13. Sambrook, J. and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual (Third Edition)*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
14. Grossman, T.H., Kawasaki, E.S., Punreddy, S.R. and Osburne, M.S.(1998). Spontaneous cAMP-dependent depression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene* **209**, 95.

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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 pRham[™] 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 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 pRham [™] 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.
	Incorrect tubes used for heat shock.	Use 15 ml disposable polypropylene culture tubes (17 x 100 mm). The use of other types of tubes may dramatically reduce the transformation efficiency.
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.

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Appendix D: Expression/Purification Troubleshooting Guide

Problem	Probable Cause	Solution
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	<p>Recombinant proteins may be cleaved during expression or lysate preparation. Use protease inhibitors to prevent cleavage.</p> <p>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.</p>
	Recombinant protein expressed in inclusion bodies	<p>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.</p> <p>During induction, incubate culture at a lower temperature (e.g. 20° to 30°C) to obtain more soluble recombinant protein.</p> <p>Test induction with lower concentrations of rhamnose.</p>

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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.
	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/ μ g) 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 pRham[™] N-His SUMO Vector (2575 bp)

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