

Expresso[®] Rhamnose 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 Cloning and Expression System

Technical Support

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Expresso[®] Rhamnose Cloning and Expression System

System Designations

The Expresso Rhamnose Cloning and Expression System contains pre-processed pRham™ N-His and/or pRham C-His Vector DNA, *E. coli*® 10G Chemically Competent Cells for cloning and protein expression, 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 Cloning and Expression Kits

	5 Reactions	10 Reactions
Expresso Rhamnose Cloning and Expression System, N/C-His Combo	-----	49010-1
Expresso Rhamnose Cloning and Expression System, N-His	49011-1	49011-2
Expresso Rhamnose Cloning and Expression System, C-His	49012-1	49012-1

Components & Storage Conditions

The Expresso Rhamnose Cloning and Expression Kits consist of two or three separate containers. The 5-reaction N-His or C-His Kits are supplied in two containers. Container 1 includes the pRham N-His or pRham C-His Expression Vector, 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 includes *E. coli* 10G Chemically Competent Cells, which must be stored at **-80°C**. The 10-reaction N-His or C-His Kits are supplied with two of container one and one of container two.

The 10-reaction N/C-His Combo Kit is supplied in three containers. One container includes pRham N-His Expression Vector, Positive Control Insert DNA, DNA primers for screening inserts by PCR and sequencing, 20% L-rhamnose solution, and 15% D-glucose solution, and a second includes the pRham C-His Expression Vector, Positive Control Insert DNA, DNA primers, 20% L-rhamnose solution, and 15% D-glucose solution. These containers should be stored at **-20°C**. Container 3 includes *E. coli* 10G Chemically Competent Cells, which must be stored at **-80°C**.

Expresso Rhamnose Cloning Kit, N-His Container Store at -20°C

	Concentration	Volume
pRham N-His Kan Vector DNA (5 reactions)	12.5 ng/μl	15 μl
N-His Positive Control A Insert DNA	50 ng/μl	10 μl
Primers for PCR screening and sequencing		
pRham 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

Expresso Rhamnose Cloning Kit, C-His Container Store at -20°C

	Concentration	Volume
pRham C-His Kan Vector DNA (5 reactions)	12.5 ng/μl	15 μl
C-His Positive Control B Insert DNA	50 ng/μl	10 μl
Primers for PCR screening and sequencing		
pRham 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

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E. cloni[®] 10G Chemically Competent Cells Container

Store at -80°C

	5 Reaction Kit	10 Reaction Kit
<i>E. cloni</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)	6 (1 x 12 ml)	12 (2 x 12 ml)

Rhamnose and Glucose solutions are available separately

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

Expresso System Cells available separately

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

System Description

The Expresso Cloning and Expression Systems use Expressioneering[™] Technology to enable rapid cloning and expression of 6xHis tagged proteins. 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 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 Cloning and Expression System, a PCR product containing the gene of interest is cloned under the control of the L-rhamnose-inducible rhaP_{BAD} promoter harbored on one of the pRham[™] vectors. 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 the Expresso T7 Cloning and Expression System, which uses separate host strains for cloning and protein expression.

The pRham[™] N-His and pRham C-His Vectors provided in the Expresso Rhamnose Kits facilitate instant cloning of target genes with a choice of amino- or carboxyl-terminal 6xHis affinity tags using Expressioneering Technology. The 6xHis peptide provides for fast and easy affinity purification of proteins under native or denaturing conditions. The pRham vectors are similar to the pETite[®] vectors used in the Expresso T7 System, but have the rhaP_{BAD} promoter replacing the T7 promoter. PCR products designed for cloning into the pRham vectors are compatible with the pETite vectors, and vice versa.

The rhaP_{BAD} promoter is a versatile tool for protein expression. In the absence of L-rhamnose, the transcriptional activity of rhaP_{BAD} is very low, allowing stable clone construction even for potentially toxic gene products (1). Transcription is positively controlled by two activators, RhaR and RhaS, which bind rhamnose (2). 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}

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responsive to different concentrations of rhamnose, allowing “tunable” control of the target gene expression level (3). 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, active 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.

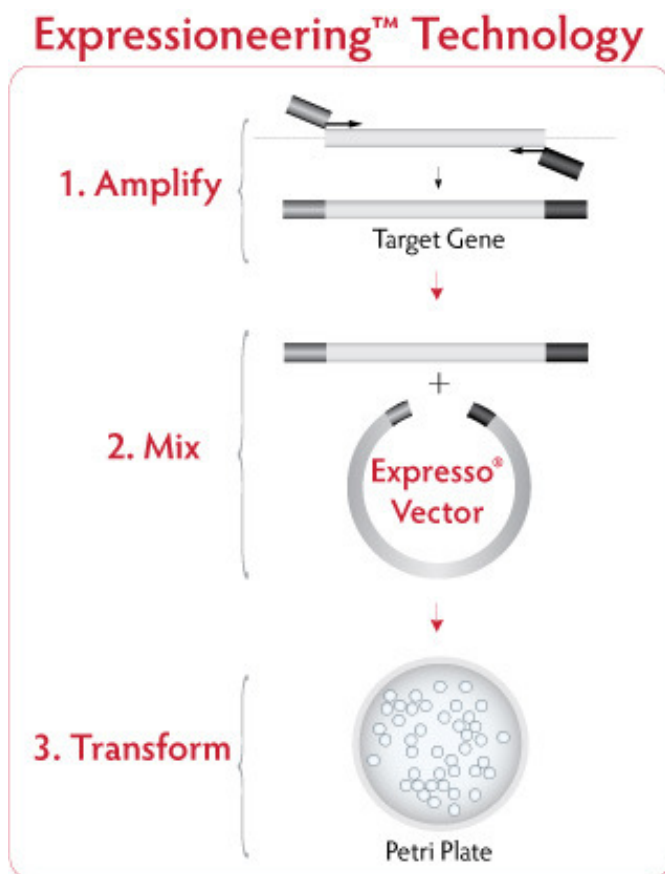


Figure 1. Expressioneering schematic. A target gene is amplified with primers that contain short homology to the ends of the pRham Vectors. The PCR product is then mixed with the pre-processed vector and transformed directly into the high-efficiency chemically competent cells provided. Recombination 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 of the target gene.

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pRham[™] Vectors

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 Vectors (2.3 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis.

The pRham Vectors are supplied in a pre-linearized format for instant, directional insertion of target genes using Expressioneering[™] Technology (Figures 1-3). The vectors include signals for expression, including rhaP_{BAD} promoter, efficient ribosome binding site from the T7 gene 10 leader, and translational start and stop codons. The vectors encode either an N-terminal or C-terminal 6xHis tag. Target gene PCR products designed for cloning into the pRham vectors are compatible with the pETite[®] vectors included in the Expresso T7 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 <10%, 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.

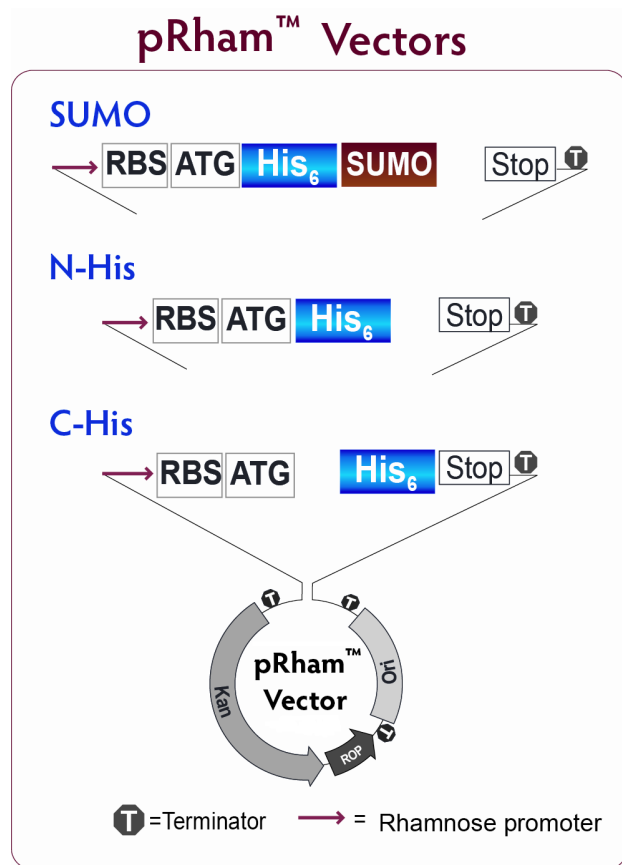


Figure 2. pRham Expression Vectors. Shown are the pRham N-His and pRham C-His Kan Vectors, as well as the pRham N-His SUMO Vector available as part of the Expresso Rhamnose SUMO kit. RBS, ribosome binding site; ATG, translation start site; Stop, translation end site; Kan, kanamycin resistance gene; ROP, 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 (pRham N-His) or at the carboxyl terminus (pRham C-His) of the expressed target 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. They give high yield and high quality plasmid DNA due to the *endA1* mutation.

E. coli 10G Genotype:

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

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E. cloni[®]10G Chemically Competent Cells produce $\geq 1 \times 10^9$ cfu/ μ g supercoiled pUC19 DNA.

As a control for transformation, *E. cloni* 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 (100 μ g/ml).

Cloning Strategy

The pRham[™] Vector preparation enables a simple recombinational strategy for precise, directional cloning. The vectors are provided in a linear form, ready for co-transformation with a PCR product containing the gene of interest.

The desired insert is amplified with primers that include 18 nt of overlap with the ends of the vector. Different primer pairs are used for the N-terminal or C-terminal 6xHis tag fusion. 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 (5), and does not require single-stranded ends on the vector or the insert, as in "PIPE" cloning (6). An untagged version of the protein can be engineered using the pRham C-His Vector by adding a stop codon at the end of the gene of interest, before the 6 histidine codons.

Positive Control Inserts

The N-His and C-His Control Inserts included with the Kit encode a blue fluorescent protein from *Vibrio vulnificus* (7), flanked by sequences for enzyme-free cloning into the pRham N-His or pRham C-His Vector. They serve as controls both for cloning efficiency and for expression. The *Vvu* BFP gene product binds to and enhances the natural fluorescence of NADPH. Upon induction of expression, this protein leads to rapid development of bright blue fluorescence that is readily visible in whole cells under long-wavelength UV light. The protein migrates at ~25 kD on SDS-PAGE.

Colony Screening

Background with the pRham Vector is typically very low (<10%), so minimal screening is necessary. Colony PCR, size analysis of uncut plasmid, 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 on plates containing kanamycin. Individual colonies are grown in liquid culture, and protein expression is induced by addition of rhamnose. Expression of His-tagged fusion proteins is evaluated by SDS-PAGE analysis.

Protein Purification

6xHis tagged proteins are purified by Immobilized Metal Affinity Chromatography (IMAC). Materials for purification are not provided with the Expresso Rhamnose System. These reagents may be obtained from any of several suppliers, including: Ni-NTA (Qiagen), Talon (Clontech), and HIS-Select (Sigma).

Materials and Equipment Needed

The *Expresso* Rhamnose Cloning and Protein 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

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necessary equipment and materials before starting. The following items are required for this protocol:

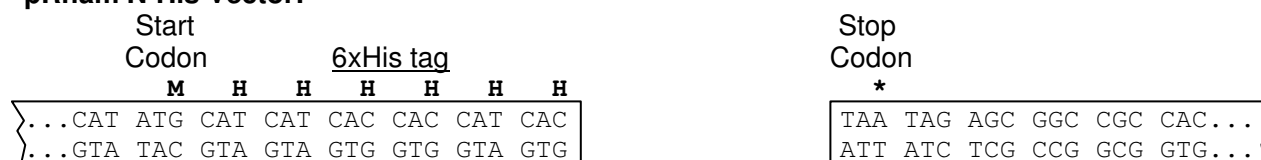
- Custom Primers for target gene amplification.
- Microcentrifuge and tubes.
- Water bath at 42°C.
- Sterile 17 x 100 mm culture tubes.
- LB Broth or YT Broth.
- LB or YT agar plates containing kanamycin (see Appendix A for recipes).
- Agarose gel electrophoresis equipment.
- Sonicator or cell lysis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

Detailed Protocol

Preparation of Insert DNA

To perform enzyme-free cloning with the pRham[™] Vectors, the DNA to be inserted must be amplified with primers that append appropriate flanking sequences to the gene of interest. These flanking sequences must be identical to the vector sequences flanking the cloning site. Different flanking sequences are used for fusing the target protein to an amino-terminal 6xHis tag (pRham N-His Kan Vector) or a carboxyl terminal 6xHis tag (pRham C-His Kan Vector). Rules for correctly designing primer pairs are presented below. Figure 3 presents a schematic illustration of primer design for cloning into the pRham N-His Vector.

pRham N-His Vector:



PCR Product:

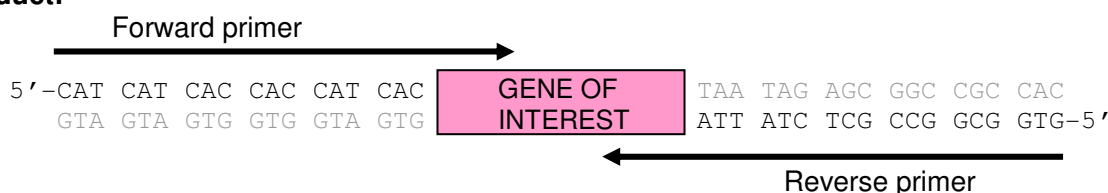


Figure 3. Insertion of a gene into the pRham N-His Kan Vector for expression. PCR primers add flanking sequences identical to the vector sequence. Recombination within the host cell fuses the blunt PCR product to the vector. The pRham N-His Vector is shown; different flanking sequences are used for the pRham C-His Vector.

1) Primer design for target gene amplification

PCR primers for enzyme-free cloning into the pRham Vectors consist of two segments: 18 nt at their 5' ends match the sequences of one of the pRham Vectors, and 18-24 nt at their 3' ends 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.

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A. Fusion to an N-terminal 6xHis tag (pRham[™] N-His Kan Vector):

Forward primer (defined vector sequence includes 6 His codons):

5'-CAT CAT CAC CAC CAT CAC XXX₂ XXX₃ XXX₄ XXX₅ XXX₆ XXX₇ XXX₈

(XXX₂-XXX₈ represents codons 2 through 8 of the target coding region).

Do NOT include an initiation codon in the forward primer. An ATG codon is contained in the pRham N-His Kan Vector immediately preceding the 6 His codons.

If desired, additional sequences can be introduced between the 6 His codons and the target protein coding sequence. For example, additional His codons can be included to increase the length of the His tag to 8-10 residues, or a sequence encoding a protease cleavage site can be added for removal of the N-terminal His tag from the protein following purification.

Reverse primer (defined 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 sequence **complementary** to the last 7 codons of the target coding region).

B. Fusion to a C-terminal 6xHis tag (pRham C-His Kan Vector):

Forward primer (defined vector sequence includes Start codon):

5'-GAA GGA GAT ATA CAT ATG XXX₂ XXX₃ XXX₄ XXX₅ XXX₆ XXX₇ XXX₈

(XXX₂-XXX₈ represents codons 2 through 8 of the target coding region).

Reverse primer (defined vector sequence includes 6 His anticodons):

5'-GTG ATG GTG GTG ATG ATG 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 sequence **complementary** to the last 7 codons of the target coding region).

The defined vector portion of the Forward primer includes an ATG codon for translation initiation. The gene-specific portion of the Forward primer should include sequence beginning from codon 2 (or other desired internal codon) of the protein coding region of interest.

Two in-frame stop codons follow immediately after the 6 His codons in the pRham C-His Kan Vector. A Stop anticodon should NOT be included in the Reverse primer, unless expression of an untagged form of the protein is desired (see below).

C. Primer design for untagged target protein:

The pRham C-His Kan Vector can also be used to construct untagged expression clones. Follow the primer design rules for cloning into the pRham C-His Vector described above, but include sequence complementary to a termination codon between the defined vector-specific sequence and the target gene portion of the reverse primer. This will cause translation to terminate before the 6xHis tag.

Reverse primer for untagged target gene:

5'-GTG ATG GTG GTG ATG ATG TAA 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 sequence complementary to the last 7 codons of the target coding region. Sequence complementary to a TAA termination codon is underlined.)

Examples of reverse primer design:

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

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

For cloning into the pRham[™] N-His Vector, the reverse primer should have the following sequence:

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

For cloning into the pRham C-His Vector, the reverse primer sequence should be:

5' GTG ATG GTG GTG ATG ATG *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 (NEB) and Pfu (Stratagene) DNA polymerases, and Taq non-proofreading polymerase. Sequence errors are quite common with Taq polymerase, especially for larger inserts, so complete sequencing of several candidate clones is strongly recommended.

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 µl)

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

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

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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).

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.

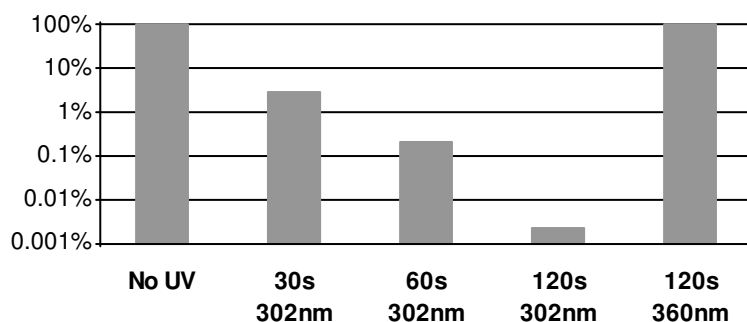


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (“No UV”) or exposure to 302 nm UV light for 30, 60, or 120 seconds (“30s 302nm, 60s 302nm, 120s 302nm”) or to 360 nm UV light for 120 seconds (“120s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

Enzyme-free Cloning with the pRham[™] Vectors

With Expressioneering[™] Technology, the pre-processed pRham 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 μ l) is mixed with 25 ng of pRham 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:

Positive Control Insert DNA	To determine the transformation efficiency with a known insert, use 1 μ l (50 ng) of N-His Positive Control A Insert or C-His Positive Control B Insert DNA and 2 μ l (25 ng) of corresponding pRham N-His or pRham C-His 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.

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Heat Shock 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 (10-15 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 disposable polypropylene 15-ml culture tube (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.

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

Reaction Plate	μ l/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/ μ g 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. cloni*[®] 10G Chemically Competent

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Cells. Cloning AT-rich DNA and other recalcitrant sequences may lead to fewer colonies. With relatively few recombinant clones, the number of “empty vector” colonies becomes more significant. For example, if the Experimental Insert reaction produces only 20 colonies from 100 µl of cells plated, then 5 colonies obtained from 100 µl of the No-Insert Control transformation will represent a background of 25%.

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 pRham[™] 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 pRham Forward primer (50 µM)
0.5 µl pETite Reverse primer (50 µM)
11.5 µl water
<hr/>
25 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	

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 ~180 base-pairs.

DNA Isolation & Sequencing

Grow transformants in LB or TB medium plus 30 µg/ml kanamycin. Use standard methods to isolate plasmid DNA (8). The pRham plasmids contain the low copy number pBR origin of replication and

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produce DNA yields similar to that of pBR-based plasmids. *E. cloni*[®] 10G cells are *recA* and *endA* deficient to provide high quality plasmid DNA. pRham[™] Forward and pETite[®] Reverse Sequencing Primers are provided with the Kit at a concentration of 50 μ M; 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 (final concentration 0.5 to 1%) to the growth medium (9). 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 longer 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 μ g/ml kanamycin with a single colony of *E. cloni* 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 sample. Resuspend the cell pellet in 50 μ l 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 μ l 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 μ g/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 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% rhamnose and a low concentration (0.05 to 0.15%) of D-glucose (4). A vial of 15% (w/v) D-Glucose Solution is included with the kit. Cells will preferentially metabolize glucose during the early stages of

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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. Cells may also be lysed by alternative methods, e.g. using a French pressure cell press.

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.

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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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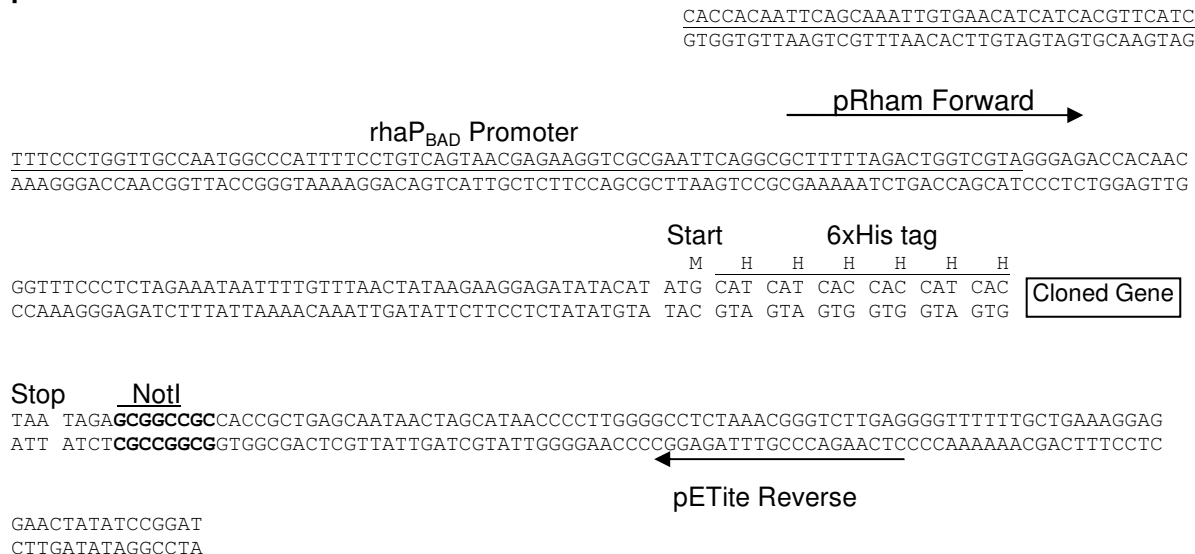
Appendix B: Vector Map and Sequencing Primers

The sequences of the pRham[™] Forward and pETite[®] Reverse primers are:

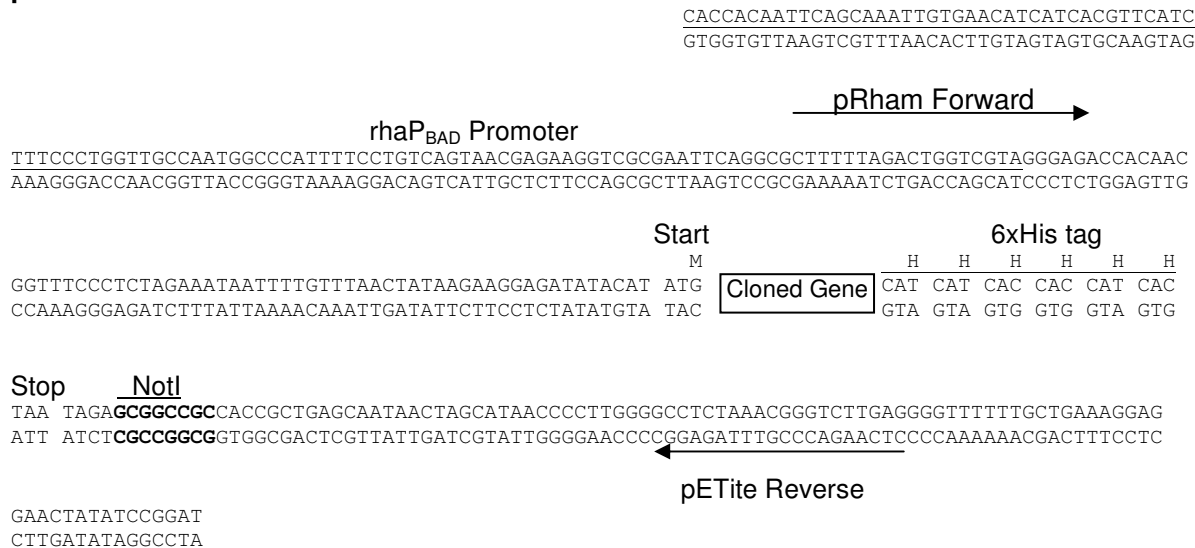
pRham Forward: 5'–GCTTTTTAGACTGGTCGTAGGGAG–3'
pETite Reverse: 5'–CTCAAGACCCGTTTAGAGGC–3'

Shown below are the regions surrounding the cloning sites in the pRham Vectors. For the complete vector sequences, see Appendix E..

pRham N-His Kan Vector:



pRham C-His 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 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.
	Toxic gene product.	Use plates containing 0.5% glucose to prevent leaky expression. Incubate plates at room temperature.
	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 whole cells or 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 overexpressed 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 induction, incubate culture at a lower temperature (e.g. 20° to 30°C) to obtain more soluble recombinant protein. Test induction with lower concentrations of rhamnose. Clone and express target gene as a fusion to SUMO solubility tag using Expresso SUMO Cloning and Expression Systems.

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Appendix E: Sequences of pRham[™] N-His and C-His Vectors (2275 bp)

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

The pRham N-His Kan and pRham C-His Kan Vectors have identical sequences. They differ only in the point of linearization for target gene insertion. The pRham N-His Kan Vector is linearized after the 6xHis tag coding sequence and before the stop codon, for fusion of a 6xHis tag to the amino terminus of the target protein. The pRham C-His Kan Vector is linearized after the start codon and immediately before the 6xHis tag coding sequence, for fusion of a 6xHis tag to the carboxyl terminus of the target protein.