Lucigen®
Simplifying Genomics

Expresso™ Biotin Cloning and Expression System

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE

Note: Two different storage temperatures required

Vector Container

Competent Cells

Lucigen Corporation 2905 Parmenter St, Middleton, WI 53562 USA
Toll Free: (888) 575-9695 | (608) 831-9011 | FAX: (608) 831-9012
lucigen@lucigen.com www.lucigen.com

MA141 Rev. C

Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is critical that the reagents supplied by the user, especially the DNA targets to be cloned, are of the highest quality. Please follow the manual carefully or contact our technical service representatives for information on preparation and testing of the target DNA. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Table of Contents

System Designations

The Expresso Biotin Cloning and Expression System contains pre-processed pAviTag N-His and/or pAviTag C-His Vector DNA, Biotin XCell F' 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, D-glucose, arabinose and biotin for small-scale induction of biotinylated protein expression. The System catalog numbers are listed below.

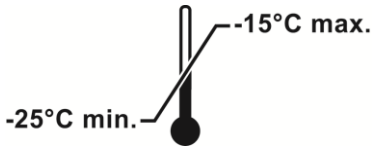
Expresso Biotin Cloning and Expression Kits

	5 Reactions	10 Reactions
Expresso Biotin Cloning and Expression System, N-His	49041-1	49041-2
Expresso Biotin Cloning and Expression System, C-His	49042-1	49042-2

Components & Storage Conditions

The Expresso Biotin 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 pAviTag N-His or pAviTag C-His Expression Vector, Positive Control Insert DNA, DNA primers for screening inserts by PCR and sequencing, 20% L-rhamnose solution, 15% D-glucose solution, 10% Arabinose solution, and 5 mM Biotin solution. This container should be stored at **-20°C**. Container 2 includes Biotin XCell F' 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.

Cloning Kit containers must be stored at -20°C



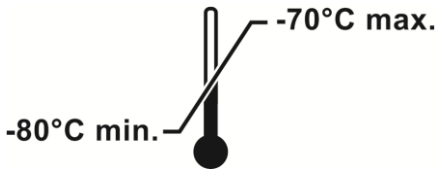
Expresso Biotin Cloning Kit, N-His Container

	Concentration	Volume
pAviTag N-His Kan Vector DNA (5 reactions)	12.5 ng/μL	15 μL
Positive Control D Insert DNA	50 ng/μL	10 μL
Primers for PCR screening and sequencing pRham Forward Primer pETite® Reverse Primer	 50 pmol/μL 50 pmol/μL	 100 μL 100 μL
Rhamnose Solution	20% w/v	1.25 mL
Glucose Solution	15% w/v	1.25 mL
Arabinose Solution	10% w/v	1.0 mL
Biotin Solution	5 mM	1.25 mL

Expresso Biotin Cloning Kit, C-His Container

	Concentration	Volume
pAviTag C-His Kan Vector DNA (5 reactions)	12.5 ng/µL	15 µL
Positive Control E Insert DNA	50 ng/µL	10 µL
Primers for PCR screening and sequencing pRham Forward Primer pETite Reverse Primer	 50 pmol/µL 50 pmol/µL	 100 µL 100 µL
Rhamnose Solution	20% w/v	1.25 mL
Glucose Solution	15% w/v	1.25 mL
Arabinose Solution	10% w/v	1.0 mL
Biotin Solution	5 mM	1.25 mL

Competent Cell containers must be stored at -80°C



Biotin XCell F' Chemically Competent Cells Container

	5 Reaction Kit	10 Reaction Kit
Biotin XCell F' 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)

Sugar and Biotin solutions 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
Arabinose Solution, 10% w/v	5 x 1.0 mL	49023-1
Biotin Solution, 5 mM	5 x 1.25 mL	49051-1

Expresso System Cells available separately

Description	Size	Cat. No.
Biotin XCell F' Chemically Competent Cells (SOLOs)	12 Transformations	60704-1
	24 Transformations	60704-2

System Description

The Expresso Biotin Cloning and Expression Systems use Expressioneering™ Technology to enable rapid cloning of target genes for high efficiency expression of biotinylated 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. The seamless integration of the target gene into the optimized expression elements provides higher yields of perfect protein with no cloning scars or other unwanted artifacts. 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.

The pAviTag N-His and pAviTag C-His Vectors provided in the Expresso Biotin Kits feature the patented AviTag™ motif, a minimal 15-residue biotinylation site that allows efficent target protein biotinylation both *in vivo* and *in vitro* (1). Biotinylated proteins can be captured by avidin or streptavidin, exploiting the extremely high affinity of these proteins for biotin ($K_d = 10^{-15}$ M). The pre-processed vectors facilitate instant cloning of target genes with a choice of amino- or carboxyl-terminal AviTag biotinylation site using Expressioneering Technology. A 6xHis peptide provides additional options for fast and easy affinity purification of proteins under native or denaturing conditions.

In the Expresso Biotin Cloning and Expression System, the gene of interest is expressed under the control of the L-rhamnose-inducible $rhaP_{BAD}$ promoter harbored on the pAviTag 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 systems requiring separate host strains for cloning and protein expression.

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 (2). Transcription is positively controlled by two activators, RhaR and RhaS, which bind rhamnose (3). 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 (4). 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.

Biotin XCell F' Chemically Competent Cells are used both for construction of clones in the pAviTag Vectors and for expression of biotinylated proteins. They contain a copy of the *birA* gene encoding biotin ligase integrated into the genome under the control of the arabinose-inducible $araP_{BAD}$ promoter. Their very high transformation efficiency (≥ 1×10^9 cfu/µg pUC19 DNA) makes these cells ideal for cloning using Expressioneering™ Technology. Biotin XCell F' 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 pAviTag Vectors can be transferred into and expressed in virtually any other host strain containing functional rhaR and rhaS genes. However, efficient *in vivo* biotinylation requires overexpression of biotin ligase.

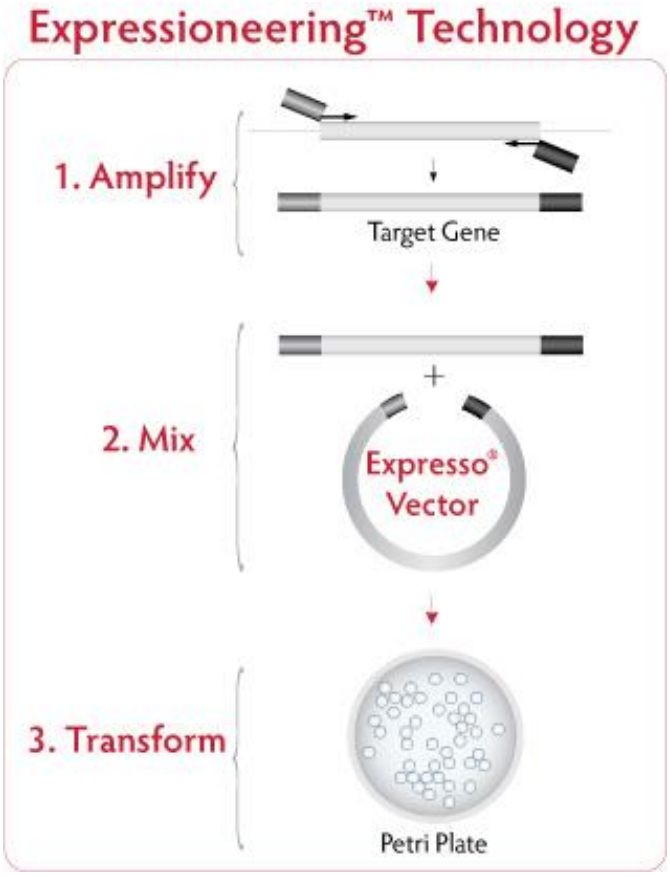


Figure 1. Expressioneering schematic. This homologous recombination system eliminates processing enzymes and cleanup steps while providing directional, 90% efficient, seamless expression constructs in seconds. There are no intervening restriction or recombination sites that can decrease expression efficiency, as found in other systems. A target gene is amplified with primers that contain short homology to the ends of the pAviTag 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 Biotin System, colonies can be inoculated directly from the transformation plate into autoinduction media for expression of the target gene.

pAviTag Vectors

The pAviTag Vectors (Fig. 2) 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 pAviTag Kan Vectors (2.4 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis.

The pAviTag 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 Expressioneering system provides for the seamless, directional cloning of genes in an optimal context for high efficiency expression. The 15-residue AviTag™ peptide, GLNDIFEAQKIEWHE, directs efficient biotinylation of the indicated lysine residue (1). A rigid linker peptide, SLSTPPTPSTPPT, situated between the AviTag and the target protein ensures that the AviTag peptide is oriented for efficient biotinylation and for protein capture and display on avidin/streptavidin matrices.

The pAviTag Vectors do not contain the *lac*Z 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 pAviTag 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.

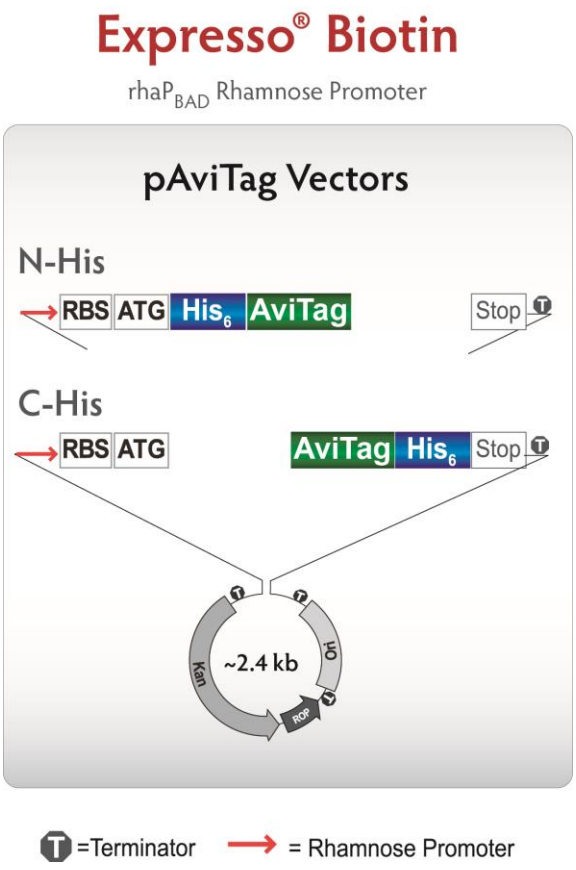


Figure 2. pAviTag Expression Vectors. Shown are the pAviTag N-His and pAviTag C-His Kan Vectors for high efficiency expression of biotinylated proteins. AviTag, biotinylation motif for BirA and affinity tag for streptavidin purification; His6, six consecutive histidines for nickel column affinity purification; 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 AviTag and 6xHis affinity tag are fused to the amino terminus (pAviTag N-His) or at the carboxyl terminus (pAviTag C-His) of the expressed target protein.

Biotin XCell F' Chemically Competent Cells

Biotin XCell F' Chemically Competent Cells are an *E. coli* strain optimized for high efficiency biotinylation of AviTag fusion proteins. They contain a copy of the *bir*A gene encoding biotin ligase

integrated into the genome under the control of the arabinose-inducible araP_{BAD} promoter. The Biotin XCell F' cells are suitable for cloning and propagation of plasmid clones. **Note:**.Biotin XCell F' cells contain wild-type alleles of *rec*A and *end*A. Plasmid minipreps may contain residual endonuclease I activity, leading to instability of isolated plasmids. Classical alkaline lysis procedures for plasmid purification are typically sufficient for most downstream manipulations such as sequencing. Some procedures, such as restriction digests followed by ligation may require additional, more stringent purification procedures.

Biotin XCell F' Genotype: [MC1061 [F´ *pro* A+B+ *lac*IqZΔM15::Tn10 (Tet^R)] *ara*D139 Δ(*ara,leu*)7696 Δ(*lac*)I74 *gal*U *gal*K *hsd*R2($r_{K-}m_{K+}$) *mcr*B1 *rps*L(Str^R)] *ara*P_{BAD}::*bir*A

Biotin XCell F' Chemically Competent Cells produce ≥ 1 x 10^9 cfu/µg supercoiled pUC19 DNA.

As a control for transformation, Biotin XCell F' 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 (100 µg/mL).

Cloning Strategy

The pAviTag Vector preparation enables a simple recombinational strategy for precise, directional cloning without the presence of intervening restriction or recombination sites that can decrease expression efficiency. 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 user-supplied 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 AviTag™ 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 (6), and does not require single-stranded ends on the vector or the insert, as in "PIPE" cloning (7). An untagged version of the protein can be engineered using the pAviTag C-His Vector by adding a stop codon at the end of the gene of interest, before the AviTag coding sequence.

Positive Control Inserts

The Positive Control Inserts included with the Kits encode a blue fluorescent protein from *Vibrio vulnificus* (8), flanked by sequences for enzyme-free cloning into the pAviTag N-His or pAviTag 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 AviTag-BFP fusion protein migrates at ~25 kD on SDS-PAGE.

Colony Screening

Background with the pAviTag Vectors 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

The pAviTag vectors have been designed for high efficiency expression of recombinant proteins without intervening restriction or recombination sites that modify proteins and can interfere with transcription and translation efficiency. Recombinant plasmids are constructed in the Biotin XCell F' host strain and expressed in the same host. Parameters for optimal protein expression are provided

in the methods section of the manual. Transformants are selected on plates containing kanamycin. Individual colonies are grown in liquid culture, and expression of the cloned gene is induced by addition of rhamnose. Efficient *in vivo* biotinylation of the AviTag™ peptide requires overexpression of the *birA* gene, which is inducible by arabinose in the Biotin XCell F' host cells. Expression of fusion proteins is evaluated by SDS-PAGE analysis.

Protein Purification

Proteins expressed using the Expresso Biotin System feature two tags that can be used for affinity purification. Biotinylation of AviTag *in vivo* allows capture of the fusion protein on avidin or streptavidin matrices. The very high affinity of avidin/streptavidin for biotin (~10^{-15} M) allows stringent washing of the protein, but makes target protein elution very difficult. The Expresso Biotin SUMO, N-Biotin System (catalog #49043) allows release of target protein from avidin/streptavidin by cleavage with SUMO Express Protease. Alternatively, lower affinity monomeric avidin resins that bind less tightly to biotin allow elution AviTag fusion proteins with biotin. The 6xHis tag enables purification by Immobilized Metal Affinity Chromatography (IMAC). Materials for IMAC purification are not provided with the Expresso Biotin 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 Biotin 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 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 TB 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 pAviTag 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 AviTag (pAviTag N-His Kan Vector) or a carboxyl terminal AviTag (pAviTag 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 pAviTag N-His Vector.

pAviTag N-His Vector:

PCR Product:

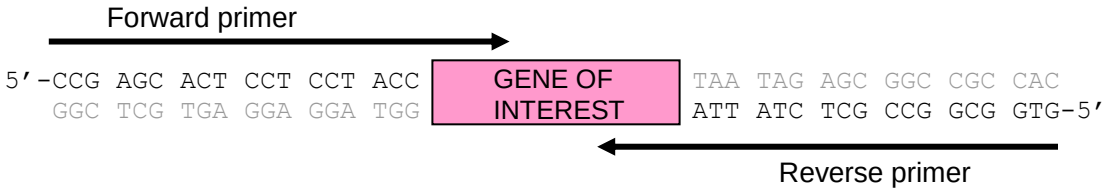


Figure 3. Insertion of a gene into the pAviTag N-His Vector for expression. PCR primers add flanking sequences identical to the vector sequence. Recombination within the host cell fuses the blunt PCR product to the vector. The pAviTag N-His Vector is shown; different flanking sequences are used for the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

1) Primer design for target gene amplification

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

A. Fusion to an N-terminal 6xHis-AviTag (pAviTag N-His Kan Vector):

Forward primer (defined vector sequence includes part of rigid linker):

5'-CCG AGC ACT CCT CCT ACC XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

(XXX_2-XXX_8 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 pAviTag N-His Kan Vector preceding the 6xHis-AviTag codons.

If desired, additional sequences can be introduced between the AviTag and the target protein coding sequence. For example, a sequence encoding a protease cleavage site can be added for removal of the AviTag 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 (pAviTag C-His Kan Vector):

Forward primer (defined vector sequence includes Start codon):

5'-GAA GGA GAT ATA CAT ATG XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

(XXX_2-XXX_8 represents codons 2 through 8 of the target coding region).

Reverse primer (defined vector sequence includes part of rigid linker):

5'-CGG CGG GGT GGA TAA GCT 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 pAviTag 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 pAviTag C-His Kan Vector can also be used to construct untagged expression clones. Follow the primer design rules for cloning into the pAviTag 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'- CGG CGG GGT GGA TAA GCT 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 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:

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

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

5' CGG CGG GGT GGA TAA GCT *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.

Note: Insert DNA can also be generated by synthesis. If this option is desired, the gene should be synthesized with the 18 nt vector-homologous sequences at each end. Be sure to correctly add at least 18 nt of vector-homologous sequence specific to the particular vector you have chosen to work with. For assistance with this application, please contact Lucigen Technical Support.

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 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 H_20 (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

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 Biotin XCell F' 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).

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.

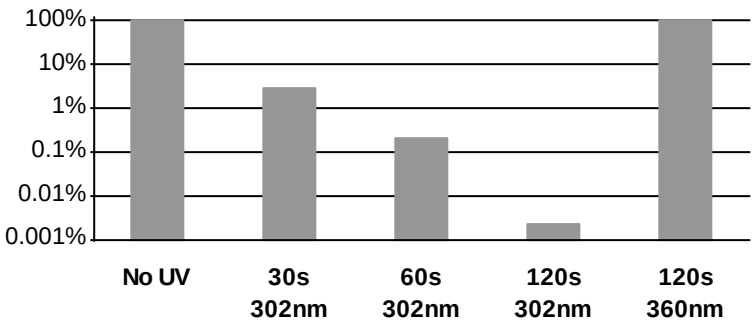


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 120 seconds ("30s 302nm, 60s 302nm,120s 302nm") or to 360 nm UV light for 120 seconds ("120s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

Enzyme-free Cloning with the pAviTag Vectors

With Expressioneering™ Technology, the pre-processed pAviTag 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 pAviTag Vector and transformed directly into competent Biotin XCell F' cells. If desired, the PCR products can be purified before cloning into pAviTag Vectors.

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

Optional Control Reactions include the following:

Positive Control Insert DNA	To determine the transformation efficiency with a known insert, use 1 μl (50 ng) of Positive Control D Insert or Positive Control E Insert DNA and 2 μL (25 ng) of corresponding pAviTag N-His or pAviTag 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 Biotin XCell F' chemically competent cells, which are included with the kit. These cells yield ≥ 1 X 10^9 cfu/μg of pUC19. The following protocol is provided for transformation.

Heat Shock Transformation of Biotin XCell F' Chemically Competent Cells

Biotin XCell F' Chemically Competent Cells are provided in 40-µl aliquots, sufficient for a single transformation. Transformation is performed by incubation on ice followed by heat shock at 42ºC. **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 Biotin XCell F' Chemically Competent cells

1. Remove Recovery Medium from the freezer and bring to room temperature.

2. Remove Biotin XCell F' cells from the -80°C freezer and thaw completely on wet ice (10-15 minutes).
3. Thaw the tube of pAviTag Vector DNA and microcentrifuge the tube briefly to collect the solution in the bottom of the tube.
4. Add 2 μL (25 ng) of the pAviTag Vector DNA and 1 to 3 μL (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 μl 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 μL of transformed cells on LB (or YT) agar plates containing 30 μg/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 μg/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 Biotin XCell F' 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	> 30	> 80% inserts
No-Insert Control (Vector Background)	100	< 10	NA
Supercoiled pUC19 Transformation Control Plasmid (10 pg, Ampicillin^R)	20 (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 pAviTag Vector using Lucigen's Biotin XCell F' Chemically Competent Cells. Cloning AT-rich DNA and other recalcitrant sequences may lead to fewer colonies.

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 CloneID™ 1X Colony PCR Master Mix (available separately, Cat. No. 30059-1) 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 CloneID is performed as follows:

Colony PCR with CloneID 1X Colony PCR Master Mix

Per 25 μL reaction:

25 μL Clone ID 1X Master Mix
0.25 μL pRham Forward primer (50 μM)
0.25 μL pETite Reverse primer (50 μM)

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:

98°C 2'

98°C 15"
55°C 15"
72°C 1' per kb
} 25 cycles

72°C 10'
4°C Hold

The CloneID reactions can be loaded directly onto an agarose gel for analysis (e.g. 5 μl directly onto a 0.7 – 2% agarose gel). 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 (9). The pAviTag plasmids contain the low copy number pBR origin of replication and produce DNA yields similar to that of pBR-based plasmids. Biotin XCell F' cells contain a wild-type allele of the *end*A gene encoding endonuclease I. We recommend DNA preparation methods that include steps to remove residual endonuclease activity, which can lead to instability of isolated plasmids and poor sequencing results. pRham™ 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 (final concentration 0.5 to 1%) to the growth medium (10).

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. Vials of 20% (w/v) L-Rhamnose Solution, 10% Arabinose Solution and 5 mM Biotin Solution are provided with the kit. Rhamnose is required to induce target gene expression from the $RhaP_{BAD}$ promoter on the pAviTag vectors. 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). Arabinose is required to induce overexpression of the biotin ligase gene *bir*A for efficient biotinylation in the Biotin XCell F' host cells. Biotin must be added to the culture at a final recommended concentration of 50 µM. 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 or TB medium containing 30 µg/ml kanamycin with a single colony of Biotin XCell F' cells containing a pAviTag expression construct. Shake at 220-250 rpm at 37ºC. When cultures reach an optical density at 600 nm (OD_{600}) 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 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, arabinose and biotin to the remainder of the culture at final concentrations of 0.2%, 0.01% and 50 µM respectively. Continue shaking at 37°C for at least 4-8 hours. For maximal biotinylation efficiency, overnight incubation with good aeration is recommended. Record the OD_{600} 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 or TB 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 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 (5). Arabinose (0.01%) and Biotin (50 µM) must also be added to the media for efficient *in vivo* biotinylation. 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.

	Early autoinduction	Late autoinduction

per mL of culture medium:	10 μL 20% L-rhamnose	10 μL 20% L-rhamnose
	3.3 μL 15% D-glucose	10 μL 15% D-glucose
	1 μL 10% arabinose	1 μL 10% arabinose
	10 μL 5 mM biotin	10 μL 5 mM biotin

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 OD_{600} 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 NaH_2PO_4, 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.

Evaluating target protein biotinylation. To evaluate biotinylation of the target protein, we recommend test-scale capture of the protein from cell lysate using streptavidin agarose in a batch procedure. Follow manufacturer protocols for binding the protein and washing the resin. Be sure to use a sufficient amount of resin to capture all of the biotinylated protein. Evaluate samples of the supernatant and the streptavidin beads by SDS-PAGE. The streptavidin beads must be heated in SDS-PAGE loading buffer (Laemmli buffer) to release the biotinylated protein from streptavidin. Some streptavidin is also released from the resin by heating in SDS-PAGE loading buffer, yielding a a protein band migrating between 10 and 15 kD on a gel.

SDS-PAGE analysis

Add the samples to SDS-PAGE loading buffer and heat to 95ºC for 5 minutes. Centrifuge the samples for 1 minute (12,000 x g). Load volumes of each sample containing equivalent OD_{600} units. Include standards to estimate molecular weight of the recombinant protein. For minigels, 0.05 OD_{600} equivalent per lane usually contains an appropriate amount of protein for Coomassie blue staining. Biotinylated proteins can be detected on western blots using streptavidin-HRP or streptavidin-AP conjugates.

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.

References

1. Beckett, D., Kovaleva, E. and Schatz, P.J. (1999). A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Science* **8,** 921.

2. 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.

3. Egan, S.M. and Schleif, R.F. (1993). A regulatory cascade in the induction of *rha*BAD. *J. Mol. Biol*. **234**, 87.

4. 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.

5. 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.

6. Bubeck, P., Winkler, M. and Bautsch, W. (1993). Rapid cloning by homologous recombination *in vivo*. *Nuc. Acids Res*. **21**, 3601.

7. 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.

8. 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.

9. Sambrook, J. and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual (Third Edition)*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

10. Grossman, T.H., Kawasaki, E.S., Punreddy, S.R. and Osburne, M.S.(1998). Spontaneous cAMP-dependent deprepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene* **209**, 95.

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 pAviTag transformants, add kanamycin to a final concentration of 30 μg/ml. Pour into petri plates.

LB-Miller Culture Medium

Per liter: 10 g Bacto-tryptone, 5 g yeast extract, 10 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.

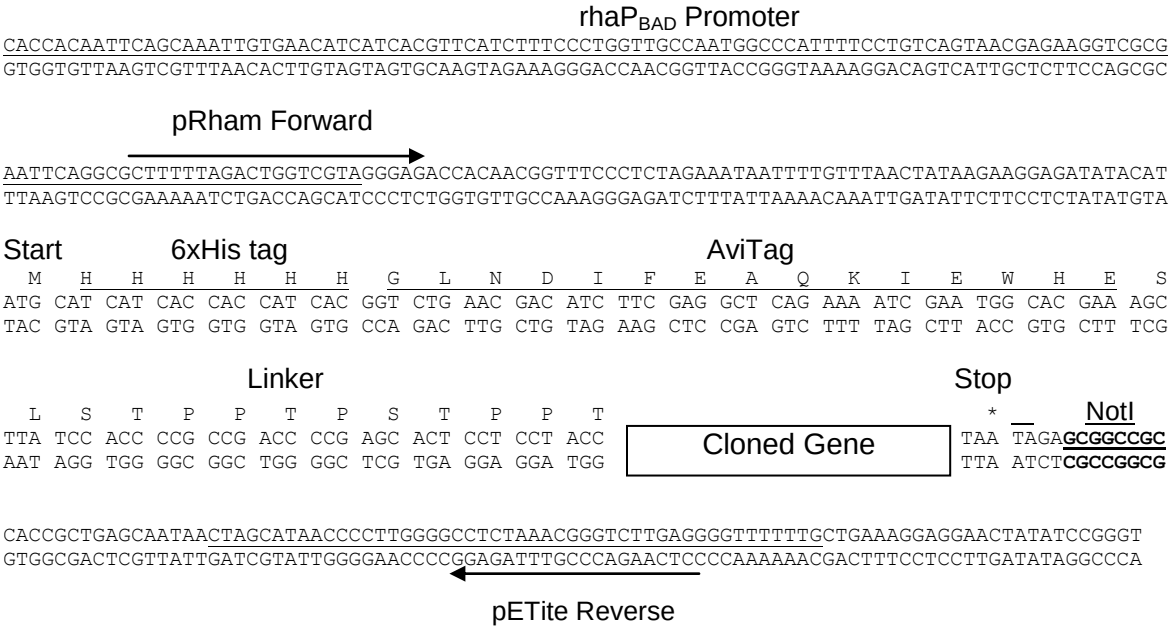
Appendix B: Vector Map and Sequencing Primers

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

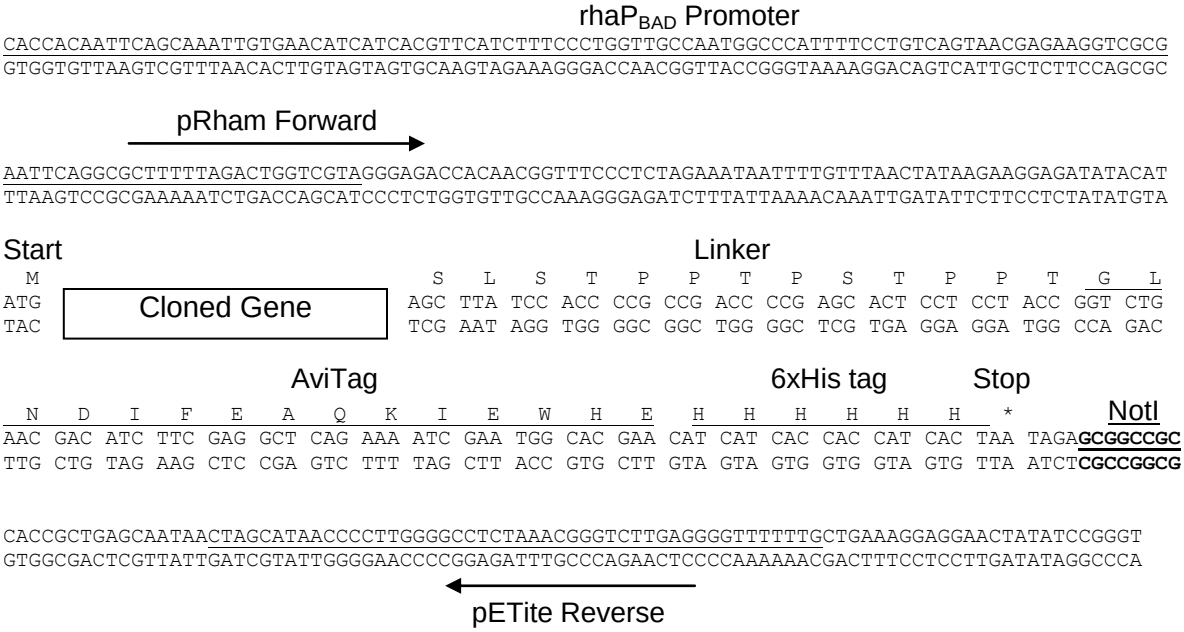
pRham Forward: 5'–GCTTTTTAGACTGGTCGTAGGGAG–3'
pETite Reverse: 5'–CTCAAGACCCGTTTAGAGGC–3'

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

pAviTag N-His Kan Vector:



pAviTag C-His Kan Vector:



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 pAviTag 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.

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.

Appendix E: Sequences of pAviTag N-His and C-His Vectors (2359 bp)

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

Notice of Limited Label License, Copyright, Patents, Warranties, Disclaimers and Trademarks

Lucigen's products are sold for research use only and are not to be used in humans or for medical diagnostics. Lucigen's liability with respect to any Expresso product is limited to the replacement of the product. No other warranties of any kind, expressed or implied, including without limitation, any implied fitness for any particular use, are provided by Lucigen. Lucigen is not liable for any direct, indirect, incidental or consequential damages arising out of or in connection with the use or inability to use any of its Expresso products.

Limited Label License

This product is the subject of U.S. Patent #6,709,861 and additional patent applications owned by Lucigen Corporation are pending. The consideration paid for this product grants a Limited License to use the product pursuant to the terms set forth in this Limited Label License. Academic, Not-for-Profit and For-Profit institutions acquire certain limited nontransferable rights with the purchase of this product (see below).

The purchase price of this product includes limited, nontransferable rights to use only the purchased amount of the product. This limited license specifically excludes manufacture of the pAviTag Vectors or derivatives thereof. Lucigen Corporation reserves all other rights; in particular, the purchaser of this product may not transfer or otherwise sell this product or its components or derivatives to a third party, and no rights are conveyed to the purchaser to use the product or its components or derivatives for commercial purposes. "Commercial purposes" includes any activity for which a party receives consideration and may include, but is not limited to, (1) use of the product or its components or derivatives in manufacturing, (2) use of the product or its components or derivatives for diagnostic purposes, (3) transfer or sale of vectors made with the product or components or derivatives of the product, (4) use of this product or materials made therefrom to provide a service, information, or data (e.g., DNA sequence) to a third party in return for a fee or other consideration, or (5) resale of the product or its components or derivatives, whether or not such product or its components or derivatives are resold for use in research. Academic, Not-for-Profit, and For-Profit institutions must obtain a separate license from Lucigen Corporation to use this product for any purpose other than those permitted above. It is the sole responsibility of the buyer to ensure that use of the product does not infringe that patent rights of third parties.

AviTag technology is protected by patents 5,723,584, 5,874,239, 5,932,433 and 6,265,552

The 6xHis tag is licensed from Hoffmann-La Roche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for the use in research. Information about licenses for commercial use is available from QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden, Germany. Purification of 6xHis tagged proteins with Ni-NTA manufactured by QIAGEN is highly recommended for best performances and to avoid poor purification results.

If the purchaser is not willing to accept these use limitations, Lucigen Corporation is willing to accept return of the product for a full refund. For information on obtaining a license, contact Lucigen Corporation, 2905 Parmenter St., Middleton, WI 53562. Email: Lucigen@lucigen.com. Phone: 608-831-9011. Fax 608-831-9012.