Lucigen®
Simplifying Genomics

Expresso™ T7 Cloning and Expression System

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE

Note: Two different storage temperatures required

Vector Container

Competent Cells

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Table of Contents

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.

Email: techsupport@lucigen.com

Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment.

Available Kits

The Expresso T7 Cloning and Protein Expression System contains pre-processed pETite™ N-His or pETite C-His vector DNA, HI-Control™ 10G Chemically Competent Cells for cloning, and HI-Control BL21(DE3) Chemically Competent Cells for protein expression, control insert, primers for clone verification by sequencing or PCR, and recovery medium for transformation.

	5 Reactions	10 Reactions
Expresso™ T7 Cloning and Expression System, N-His	49001-1	49001-2
Expresso™ T7 Cloning and Expression System, C-His	49002-1	49002-2

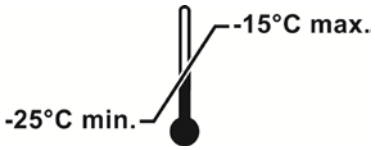
Components & Storage Conditions

The Expresso T7 Cloning Kit consists of three or four separate containers:

- Container 1 contains the pETite N-His or pETite C-His Expression Vector, Positive Control Insert DNA, and DNA primers for screening inserts by PCR and sequencing. Store at **-20ºC**.
- Container 2 contains HI-Control 10G Chemically Competent Cells. Store at **-80°C**.
- Container 3 contains HI-Control BL21(DE3) Chemically Competent Cells. Store at **-80ºC**.

The 10-reaction N-His or C-His Kits are supplied with two of container one.

N-His and/or C-His containers must be stored at -20°C



Expresso T7 Cloning Kit, N-His Container

	Concentration	Volume
pETite 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 pETite T7 Forward Primer pETite Reverse Primer	 50 pmol/µL 50 pmol/µL	 100 µL 100 µL

Expresso T7 Cloning Kit, C-His Container

	Concentration	Volume
pETite 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 pETite T7 Forward Primer pETite Reverse Primer	 50 pmol/µL 50 pmol/µL	 100 µL 100 µL

Competent Cell containers must be stored at -80°C

HI-Control 10G Chemically Competent Cells Container

	5 Reaction Kit	10 Reaction Kit
HI-Control 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)

HI-Control BL21(DE3) Chemically Competent Cells Container

	5 Reaction Kit	10 Reaction Kit
HI-Control BL21(DE3) 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)

Expresso System Cells available separately

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

System Description

The Expresso T7 Cloning and Expression System is a simple method for rapid cloning and expression of 6xHis tagged proteins. The system is based on the inducible T7 expression system (1, 2), but it provides improved control over gene expression.

The pETite vectors facilitate enzyme-free cloning of target genes and inducible expression under control of a bacteriophage T7 promoter. They are provided with HI-Control™ 10G Competent Cells for high-efficiency cloning and HI-Control™ BL21(DE3) cells for tight control of target gene expression prior to induction and enhanced expression following induction.

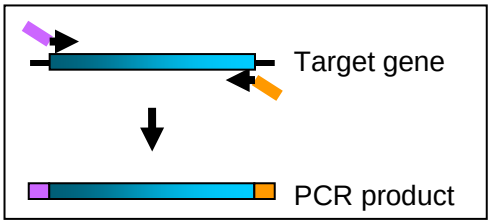
The pETite vectors are provided in a pre-processed, linearized format for rapid enzyme-free cloning. After amplification of the target gene with appropriate primers, the PCR product is simply mixed with the pETite vector and transformed directly into chemically competent HI-Control 10G cells. Recombination within the host cells seamlessly joins the insert to the vector (Figure 1).

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

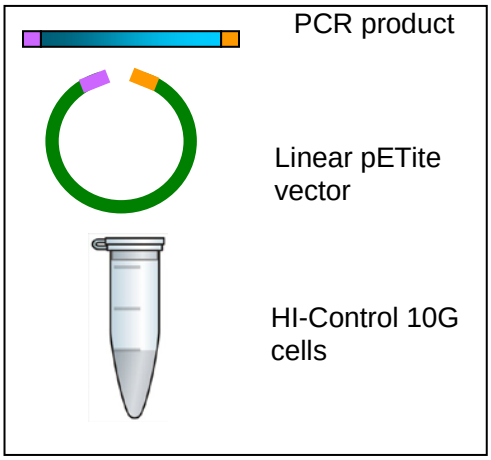
HI-Control 10G cells are used for construction of clones in the pETite vectors. Their recA- endA- genotype allows recovery of high quality plasmid DNA. HI-Control 10G cells do not express T7 RNA polymerase and therefore are not used for expression from the pETite vectors.

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

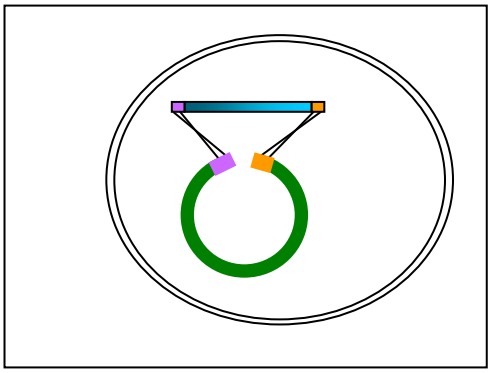
1. Amplify target gene, using primers that contain 18 bp overlap with vector ends.



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



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



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

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

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

pETite™ Vectors

The pETite vectors replace the most common pET expression vectors (1-3). However, the small size of the pETite vectors (2.2 kb) facilitates cloning of larger inserts and performing DNA manipulations, such as site-directed mutagenesis. They are based on Lucigen's patented pSMART® vectors, which feature transcriptional terminators to prevent unwanted transcription into or out of the cloned sequence. The pETite vectors do not harbor a gene for the LacI repressor protein. Instead, abundant LacI repressor is provided by the HI-Control BL21(DE3) cells.

The pETite vectors are pre-linearized for instant, directional cloning of inserts (Figures 2 and 3). The vectors include signals for expression, including T7-lac promoter, ribosome binding site, and translational start and stop codons. The vectors encode either an N-terminal or C-terminal 6xHis tag.

The pETite 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 <5%, so minimal colony screening is necessary. The pETite vectors have low copy number, similar to that of pBR322 plasmids (~20 copies/cell), yielding 0.5–1.0 µg of plasmid DNA per mL of culture.

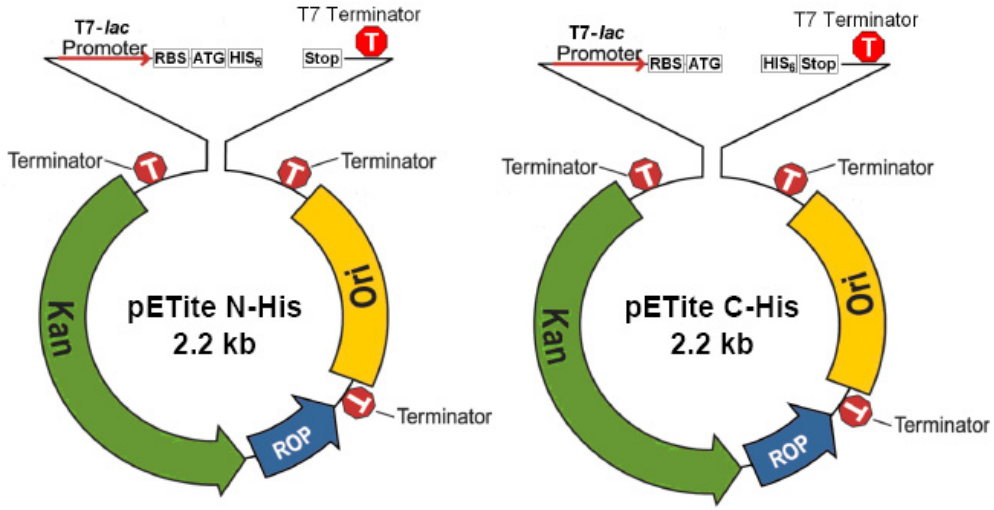


Figure 2. pETite expression vectors. RBS, ribosome binding site; ATG, translation start site; Stop, translation end site; Kan, kanamycin resistance gene; ROP, Repressor of Priming (for low copy number); Ori, origin of replication. CloneSmart® transcription terminators (T) prevent transcription into or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

HI-Control™ 10G Chemically Competent Cells

HI-Control 10G cells are an *E. coli* strain optimized for high efficiency transformation. The HI-Control 10G cells are ideal for cloning and propagation of plasmid clones. They give high yield and high quality plasmid DNA due to the *end*A1 mutation. HI-Control 10G cells harbor a single-copy BAC plasmid carrying an engineered *lacI*^{q1} repressor allele. The *lacI*^{q1} allele expresses 170x more Lac repressor than does the wild-type *lac*I gene (4). The HI-Control 10G strain does not contain T7 RNA polymerase, and the excess lac repressor in this strain further minimizes any background transcription by the bacterial polymerase.

HI-Control 10G Genotype:

*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L (Str^R) *nup*G λ$^-$ *ton*A /Mini-F *lacI*^{q1}(Gent^R)

HI-Control 10G Chemically Competent Cells produce ≥ 1 x 10^9 cfu/µg supercoiled pUC19 DNA.

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

HI-Control BL21(DE3) Chemically Competent Cells

The HI-Control BL21(DE3) cells are a derivative of BL21(DE3) with improved control over target gene expression. BL21(DE3) is the T7 host strain most commonly used for expression of cloned genes from the bacteriophage T7 promoter. This strain is a lysogen of □DE3, which harbors the T7 bacteriophage RNA polymerase gene under the control of the inducible *lacUV5* promoter. The *lacUV5* promoter is a variant of the *lac* promoter that is inducible to higher levels than its wild-type counterpart, but it also suffers from a higher basal level of activity. This basal expression of T7 RNA polymerase can lead to undesired expression of target genes cloned under a T7 promoter prior to induction. Such “leaky” expression can lead to difficulty maintaining clones in the expression host, particularly if the target gene encodes a deleterious protein. Some common T7 expression vectors harbor a copy of the *lacI* gene, encoding the lac repressor protein (2, 3). The lac repressor protein is responsible for maintaining inducible control over the *lacUV5* promoter as well as the T7-lac promoter on the vector. This increased copy number of *lacI* provides only partial protection against leaky expression.

The HI-Control BL21(DE3) cells contain a single-copy BAC plasmid harboring a specially engineered version of the *lacI*^{q1} repressor allele. The *lacI*^{q1} allele expresses ~170-fold more lac repressor protein than the wild-type *lacI* gene (4), or about 10-fold more repressor than expected when *lacI* is harbored on the expression vector. The increased pool of lac repressor in HI-Control BL21(DE3) cells maintains tight control over the expression of T7 RNA polymerase from the *lacUV5* promoter, reducing leaky expression of genes cloned under a T7 promoter. The excess repressor in this strain is also sufficient to bind to the *lac* operator on the pETite vectors, providing an additional level of control over expression from the T7 promoter. The abundant lac repressor does not interfere with the induction of T7 RNA polymerase or target gene expression by IPTG.

HI-Control BL21(DE3) Genotype:

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

HI-Control BL21(DE3) Chemically Competent Cells produce ≥ 1 x 10^7 cfu/µg supercoiled pUC19 DNA.

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

Cloning Strategy

The pETite vector preparation enables a simple 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 15-18 nt of overlap with the ends of the vector (Figure 3). 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).

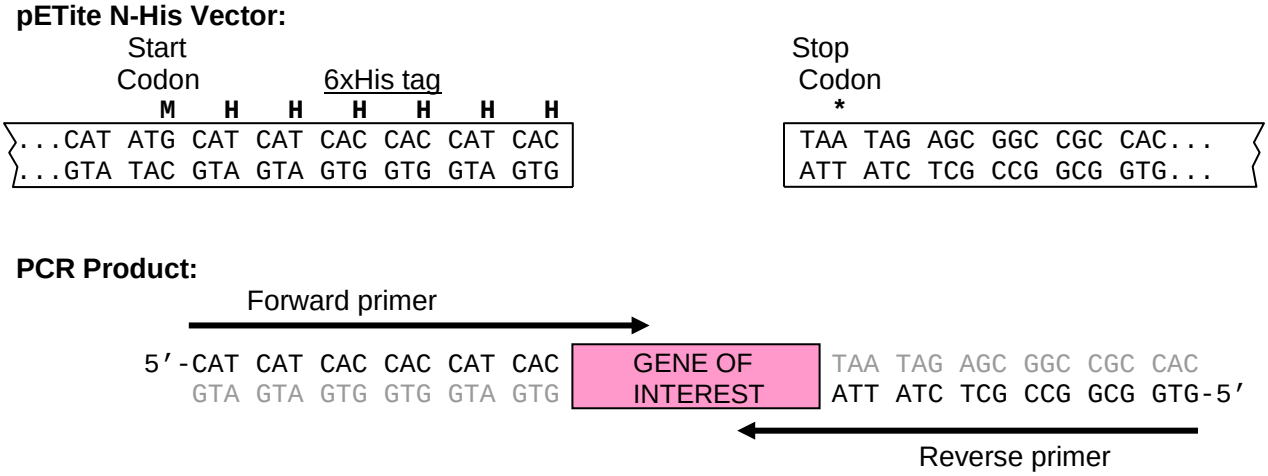


Figure 3. Insertion of a gene into the pETite vectors for expression. PCR primers add flanking sequences identical to the vector sequence. Recombination within the host cell fuses the blunt PCR product to the vector. The pETite N-His vector is shown; different flanking sequences are used for the pETite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

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 pETite N-His or pETite 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 pETite vector is typically very low (<5%), 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 HI-Control 10G host strain, verified, and transformed into HI-Control BL21(DE3) cells for expression. Transformants are selected on plates containing kanamycin. Individual colonies are grown in liquid culture, and protein expression is induced by addition of IPTG. 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 T7 System. These reagents may be obtained from any of several suppliers, including: Ni-NTA (Qiagen), Talon (Clontech), and His-bind (Sigma).

Materials and Equipment Needed

The *Expresso* T7 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 YT Broth.
- YT agar plates containing kanamycin (see Appendix A for recipes).
- Agarose gel electrophoresis equipment.
- IPTG (100 mM stock).
- Sonicator or cell lysis reagents.
- SDS-PAGE equipment.

Detailed Protocol

Preparation of Insert DNA

To perform enzyme-free cloning with the pETite 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 (pETite N-His Kan vector) or a carboxyl terminal 6xHis tag (pETite C-His Kan vector). Rules for correctly designing primer pairs are presented below.

1) Primer design for target gene amplification

PCR primers for enzyme-free cloning into the pETite vectors consist of two segments: 18 nt at their 5' ends match the sequences of one of the pETite 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 tag (pETite N-His Kan vector):

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

5'-CAT CAT CAC CAC CAT CAC 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 pETite 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 (pETite 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 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 pETite 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 pETite C-His Kan vector can also be used to construct untagged expression clones. Follow the primer design rules for cloning into the pETite 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 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.)

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 T7 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.

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 Hi Control 10G cells, creating a high background of parental clones on kanamycin agarose plates. Therefore, we strongly recommend restriction digestion of kanamycin-resistant plasmid templates and gel purification of the linearized fragment prior to using it as a template for PCR. Alternatively, the PCR product can be gel purified to isolate it from the circular plasmid DNA.

Sensitivity of DNA to Short Wavelength UV Light

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

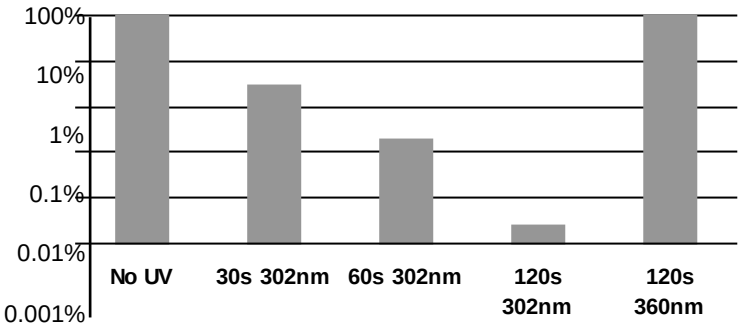


Figure 4. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for

30, 60, or 120 seconds (“30s 302nm, 60s 302nm,120s 302nm”) or to 360 nm UV light for 120 seconds (“120s 360nm”). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

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

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

Enzyme-free Cloning with the pETite Vectors

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

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

Optional Control Reactions include the following:

Positive Control Insert DNA	To determine the transformation efficiency with a known insert, use 1 µL(50 ng) of N-His Positive Control A Insert or C-His Positive Control B Insert DNA and 2 µL (25 ng) of corresponding pETite N-His or pETite 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 HI-Control 10G 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 HI-Control 10G Chemically Competent Cells

HI-Control 10G 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 HI-Control 10G Chemically Competent cells

1. Remove Recovery Medium from the freezer and bring to room temperature.
2. Remove HI-Control 10G cells from the -80°C freezer and thaw completely on wet ice (10-15 minutes).
3. Thaw the tube of pETite vector DNA and microcentrifuge the tube briefly to collect the solution in the bottom of the tube.

4. Add 2 µL (25 ng) of the pETite 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. Transfer the cells and DNA to a pre-chilled disposable polypropylene 15-mL culture tube (17 x 100 mm).
6. Incubate culture tube containing cells and DNA on ice for 30 minutes.
7. Heat shock cells by placing the tube in a 42ºC 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 cuture 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 YT (LB) 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 E. cloni® 10G Chemically Competent Cells

Rxn	Reaction Plate	µL/Plate	CFU/Plate	Efficiency
1	Experimental Insert (~25-100 ng per transformation)	100	variable	NA
2	Positive Control Insert (50 ng)	100	> 50	> 95% inserts as determined by colony PCR
3	No-Insert Control (Vector Background)	100	< 15	Ratio of resulting colonies from Rxn2:Rxn3 is >5:1
4	Supercoiled pUC19 Transformation Control Plasmid (10 pg, Ampicillin^R)	20 (ampicillin plate)	Approx. 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 pETite vector using Lucigen's HI-Control 10G Chemically Competent Cells. The background number of empty vector is constant (< 15 colonies per 100 µL of cells plated). Cloning AT-rich DNA and other recalcitrant sequences may also 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. 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 pETite T7 Forward primer (50 □M)
0.5 µL l pETite Reverse primer (50 □M)
11.5 µL water
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'	
94°C 15"	25 cycles
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 pETite plasmids contain the low copy number pBR origin of replication and produce DNA yields similar to that of pBR-based plasmids. HI-Control 10G cells are *recA* and *endA* deficient to provide high quality plasmid DNA. pETite T7 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.

Transformation into BL21(DE3) HI-Control Cells

Prior to expression studies, clones should be confirmed by DNA sequencing. Confirmed clones in the pETite vectors must be transformed into BL21(DE3) HI-Control cells for expression from the T7 promoter. The chemically competent BL21(DE3) HI-Control cells have a transformation efficiency of ≥1 x 10^7 cfu/µg pUC19 DNA. We recommend using ~0.1 to 10 ng of miniprep DNA. Transformation is performed by heat shock. For maximal transformation efficiency, the heat shock is performed in 15-mL disposable polypropylene culture tubes (17 x 100 mm). Plating several different amounts of the transformed cells onto separate plates will help to ensure the recovery of individual colonies. To ensure successful transformation results, the following precautions must be taken:

- All culture tubes must be thoroughly pre-chilled on ice before use.

- The cells must be completely thawed **on ice** before use.

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

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

Growing Transformed Cultures

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

Catabolite Repression: Controlling Leaky Expression with Glucose

Undesired “leaky” expression of target genes during growth prior to induction can lead to slow growth, instability of the expression plasmid, and reduced yield of the target protein, particularly if the protein is toxic to the host strain. A simple way to maintain tight repression of target genes under the control of the T7 promoter is to add glucose (final concentration 0.5 to 1%) to the growth medium (9). In HI-Control BL21(DE3) cells and many other T7 host strains, the T7 RNA polymerase gene is expressed from the *lacUV5* promoter. Transcription from this promoter is dependent on the cAMP-dependent transcriptional activator protein, known as CAP or CRP. When glucose is available as a carbon source, cAMP levels remain low and CAP cannot bind to its DNA target upstream of the *lacUV5* promoter. In the absence of glucose, and particularly as cells approach stationary phase, increased cAMP levels lead to significant expression of T7 RNA polymerase and of target genes under the control of T7 promoters, even in the absence of lactose. We recommend the addition of 0.5% glucose to cultures that are not intended for induction. Glucose can also be included in cultures to be induced with IPTG, but may limit the yield of target protein.

Induction of Protein Expression

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

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

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

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

To induce expression, add IPTG to a final concentration of 1 mM. Continue shaking at 37°C for 3 hours or more. Record the 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.

To evaluate target protein solubility, harvest the remainder of the culture by centrifugation at 4000 Xg for 15 minutes. Pour off growth media and resuspend the cell pellet in 1-5 mL lysis buffer (50 mM 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.

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

SDS-PAGE analysis

Add the samples to SDS-PAGE loading buffer and heat to 95ºC for 5 minutes. Centrifuge the samples for 1 minute (12,000 x g). Load volumes 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 the appropriate amount of protein for by 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 pETite™ transformants, add kanamycin to a final concentration of 30 µg/mL. Pour into petri plates.

LB-MillerCulture 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 pETite T7 Forward (T7 Promoter) and pETite Reverse primers are:

pETiteT7 Forward (T7 Promoter): 5'–TAATACGACTCACTATAGGG–3'
pETite Reverse: 5'–CTCAAGACCCGTTTAGAGGC–3'

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

pETite N-His Kan Vector:

pETite 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 pETite vector used for transformation.
	Wrong antibiotic used.	Add the correct amount of kanamycin to molten agar at 55ºC before pouring plates.
	Incorrect amounts of antibiotic in agar plates.	DO NOT spread antibiotic onto the surface of agar plates.
High background of transformants that do not contain inserts.	Transformants are due to intact plasmid template DNA.	Linearize plasmid DNA used as a template for PCR. Gel-isolate template DNA fragment.
	Inserts are too small to detect.	Analyze colonies by sequencing to confirm the presence of inserts.
	Incorrect amount of antibiotic in agar plates.	Add the correct amount of kanamycin to molten agar at 55ºC before pouring plates. DO NOT spread antibiotic onto the surface of agar plates.

Appendix D: Expression/Purification Troubleshooting Guide

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

Appendix E: Sequence of pETite™ Vectors

The sequences of the 2235 bp pETite N-His Kan and pETite C-His Kan Vectors can be found on the Vector Sequences Page of the Lucigen website.

The pETite N-His Kan and pETite C-His Kan vectors have identical sequences. They differ only in the point of linearization for target gene insertion. The pETite 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 pETite 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.

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