

### Abstract

Lucigen has developed a new cloning and expression system to simplify recombinant protein expression in *E. coli*. The system uses a recombination-based PCR cloning strategy that is directional, eliminates the need for restriction digestion and ligation, and requires no PCR reaction clean-up or enzyme treatment. Pre-processed pETite™ expression vectors allow one-step cloning of target gene PCR products under the control of a T7-lac promoter, with a choice of N-terminal or C-terminal 6x Histidine tag for purification. PCR product containing 15-18 bp of complementarity to vector sequences at each end is simply mixed with the pETite vector preparation and transformed immediately into high-efficiency chemically-competent cells. There is no requirement for specific sequences at the vector/insert junction, allowing complete freedom of fusion protein design. The pETite vectors are designed for use with new HI-Control™ host strains, which express high levels of lac repressor protein to provide tight control over the T7-lac promoter. HI-Control 10G cells do not express T7 RNA polymerase, and provide the highest transformation efficiency for expression plasmid construction. HI-Control BL21(DE3) cells provide high levels of T7 RNA polymerase for maximal target gene expression, while OverExpress C41(DE3) and C43(DE3) strains produce lower levels of T7 RNAP for optimal expression of toxic proteins, including membrane proteins. We have used these new tools for large-scale cloning and expression trials.

### Summary

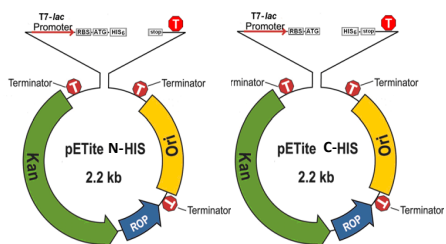
T7 expression systems exploit the high promoter specificity and strong transcriptional activity of bacteriophage T7 RNA polymerase. Despite these advantages, common T7 host strains such as BL21(DE3), and T7-lac promoter vectors such as the pET series are "leaky": significant basal levels of T7 RNA polymerase are typically present in the host strain, leading to expression of target proteins in the absence of induction. Even with target proteins that are only mildly toxic, this leaky expression can lead to instability of the expression vector, potentially compromising yield and integrity of target proteins.

pETite vectors (Fig. 1) are small (2.2 kb) T7-lac promoter vectors built on Lucigen's transcription-free pSMART vector backbone. Their small size enables cloning of larger genes, and facilitates downstream manipulations such as site-directed mutagenesis. The pETite vectors are available with a choice of N-terminal or C-terminal 6x His tag for convenient purification.

New HI-Control host cells (Fig. 2) contain an engineered lacI repressor gene to increase the level of repressor protein by ~200-fold. This level of repressor is sufficient to maintain occupancy of the lacUV5 operator to control basal expression of T7 RNAP within the host strain. The excess repressor is also sufficient to ensure occupancy of the T7-lac promoter on the incoming expression vector. HI-Control BL21(DE3) cells provide enhanced control over leaky target gene expression compared to standard BL21(DE3) (Fig. 4).

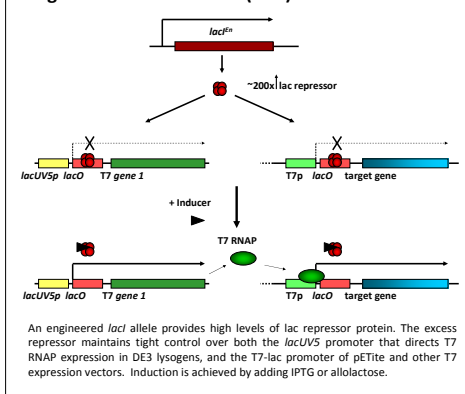
The pETite vectors are provided in a pre-processed format optimized for recombination-based enzyme-free cloning (Fig. 3). Simply amplify the target gene of interest by PCR with primers that append 15-18 base-pairs of homology to vector sequence, verify the PCR reaction product, mix the PCR product directly with the cloning-ready pETite vector, and transform directly into high-efficiency HI-Control 10G cells.

Figure 1. pETite vectors



pETite vectors for enzyme-free cloning and expression of target genes from an inducible T7-lac promoter. Vectors include a choice of N-terminal or C-terminal 6x-His tags. The vectors are built on Lucigen's pSMART backbone, which includes strategically placed terminators to limit transcriptional interference. Kan: kanamycin resistance gene; Ori: origin of replication; ROP: repressor of primer (control of copy #). Ribosome binding site (RBS), translational start (ATG) and stop codons are included in the vector.

Figure 2. HI-Control BL21(DE3) cells

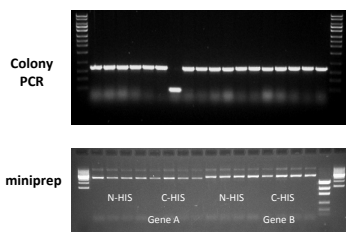
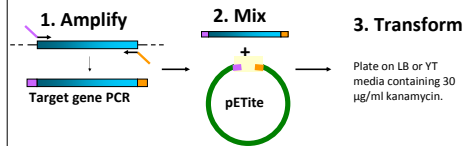


An engineered lacI allele provides high levels of lac repressor protein. The excess repressor maintains tight control over both the lacUV5 promoter that directs T7 RNAP expression in DE3 lysogens, and the T7-lac promoter of pETite and other T7 expression vectors. Induction is achieved by adding IPTG or allolactose.

Figure 3. Efficient enzyme-free cloning with HI-Control cells and pETite vectors

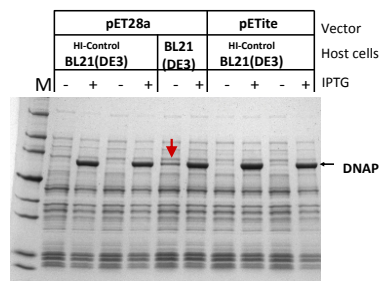
The pETite vector and HI-Control cells have been optimized for cloning and expression of target genes using an extremely simple enzyme-independent cloning strategy:

1. Amplify target gene with primers containing 15-18 base complementarity to vector sequences flanking the insertion site.
2. Mix 1 µl PCR product with 2 µl pre-processed pETite vector. No further enzymatic treatment or cleanup is necessary.
3. Transform mixture immediately into high-efficiency, chemically competent HI-Control 10G host cells and plate on media containing kanamycin. Typically, >90% of transformants are recombinants.



The high efficiency and directionality of the recombination-based cloning strategy means minimal screening is necessary to identify positive clones. In the top panel above, colony PCR was performed on 18 candidate clones of a potentially toxic 0.55 kb gene in the pETite C-HIS vector; all but one contained insert of the correct size. Sequencing verified that the remaining 17 clones were correct. In the lower panel, 2 different genes were cloned into both pETite N-HIS and pETite C-HIS. Plasmid DNA was isolated by miniprep from four randomly-selected colonies of each transformation plate; all contained inserts of correct size.

Figure 4. Improved control with HI-Control BL21(DE3) cells



A DNA Polymerase gene was cloned into pET28a and pETite vectors and the products were transformed into BL21(DE3) and BL21(DE3) HI-Control cells. Samples contained equivalent OD units harvested from cultures grown to OD600 0.6 without induction (-) or after induction for 3 hours with 1 mM IPTG (+). With the pET28-DNAP clone, leaky expression seen in uninduced BL21(DE3) cells (red arrow) is reduced in BL21(DE3) HI-Control cells. The HI-Control cells maintain comparably tight control over the pETite-DNAP clone, despite the absence of the lacI gene from the expression vector.

### Large-Scale Cloning and Expression Case Study

We are currently using the EXPRESSO system to clone and express candidate hydrolase genes from *Fibrobacter succinogenes*. We initially selected 47 genes for analysis. Each of the genes, ranging in size from ~1 kb to > 3 kb, was amplified from genomic DNA using a proofreading DNA polymerase with primers containing 18 base-pair extensions with complementarity to the pETite C-HIS vector (Fig. 6).

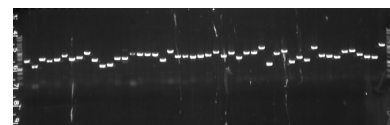


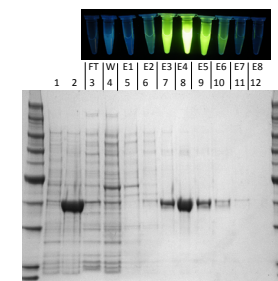
Figure 6. PCR products from 47 *F. succinogenes* target hydrolase genes.

An aliquot (1 µl) of each PCR reaction was mixed with 25 ng pETite C-HIS vector and transformed immediately into HI-Control chemically competent cells. In some cases, the DNA mixture was transformed into 10G HI-Control cells, and recovered plasmids were subsequently shuttled into BL21(DE3) HI-Control cells for expression. In other experiments, the DNA mixture was transformed directly into BL21(DE3) HI-Control cells to facilitate direct screening of candidate clones for expression and enzyme activity. Figure 7 shows examples of uninduced and induced HI-Control BL21(DE3) cells with six different *F. succinogenes* genes

### Conclusion

The Expresso Cloning and Protein Expression System is designed for rapid PCR cloning without restrictions. We have integrated the conversion of PCR amplified genes into expression ready clones with seamless enzyme free recombinering. Column cleanup steps, restriction digestions and primer design are eliminated, speeding up the process greatly. The system produces target proteins with the high yields typical of T7 expression systems, with enhanced control over leaky target gene expression.

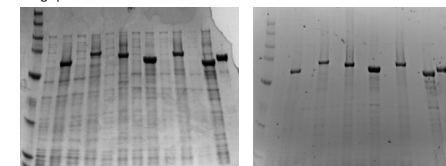
Figure 5. Expression and purification of active soluble fluorescent protein



HI-Control BL21(DE3) cells harboring pETite C-His vector containing a yellow fluorescent protein gene were grown at 37° in LB media to an OD600 0.6 (lane 1), then induced for 4 hours with 1 mM IPTG (lane 2). Cells were harvested and lysed by sonication. Cleared lysate was loaded onto Ni-NTA sepharose. Column flowthrough (lane 3, FT) and wash (lane 4, W) fractions were collected. The bound YFP was eluted with wash buffer containing 300 mM imidazole (lanes 5-12, E1-E8)

Figure 7. Uninduced and induced cultures of HI-Control BL21(DE3) cells containing candidate *F. succinogenes* hydrolase genes in the pETite C-HIS vector.

Left panel: Coomassie blue stained gel; Right panel: the same gel stained with a His-tag specific stain.



To date we have cloned all 47 genes into the pETite C-HIS vector. Of these, 35 have been subjected to a multiplex activity screen in which lysates are incubated with a mixture of fluorescent and colored indicator substrates. Twenty-two of the expression clones showed activity with one or more of the substrates. Several of these enzymes have been purified by IMAC. Additional activity and expression analysis of the remaining genes is ongoing.