EXPRESSO™ T7 Cloning and Expression System
Rapid enzyme-free recombinant-based cloning and protein expression in E. coli.

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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 directionless, eliminates the need for restriction digestion and ligation, and requires no PCR reaction clean-up or enzyme treatment. Pre-processed pET™ expression vectors allow one-step cloning of target gene PCR products under the control of a T7 promoter, with a choice of Hi-

Terminal or C-terminal His6 tail for purification. PCR product containing 15-18 bp of complementarity to vector sequence at each end is simply mixed with the pETe 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 small vector size (2.2 kb) facilitates downstream manipulation, such as site-directed mutagenesis. The pETe 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 T7lac promoter. Hi-Control BL21 cells do not express T7 RNAP polymerase, and provide the highest transformation efficiency for expression plasmid construction. Hi-Control BL21(DE3)pLysS cells produce 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, concern for host strains such as BL21(DE3) and T7lac promoter vectors such as the pR set 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 integral of target proteins.

The pETe vectors (Fig. 2) are available as Hi-Control BL21(DE3) or T7lac promoter vectors. Their small size enables cloning of larger genes, and facilitates downstream manipulations such as site-directed mutagenesis. The pETe vectors are available with a choice of C-terminal or N-terminal His6 tag for convenient purification.

The Hi-Control host cells (Fig. 3) contain an engineered lacI repressor to increase the level of lac repressor protein by ~200-fold. This level of repression is sufficient to maintain occupancy of the lacO operator to control basal expression of T7 RNAP within the host strain. The excess repressor is also sufficient to ensure tight promoter control by the lacI repressor in the T7 promoter expression vector. Hi-Control BL21(DE3) cells provide enhanced control over leaky target gene expression compared to strain BL21(DE3) (Fig. 4).

The pETe vectors are provided in a pre-processed format optimized for recombination-based enzyme-free cloning (Fig. 5). Simply amplify the target gene of interest with PCR by primers that overlap 15-18 base pairs of homology to the vector sequence, verify the PCR product size, mix the PCR product directly with the closing ready pETe vector, and transform directly into high efficiency Hi-Control DE3 cells.

Figure 1. pETe vectors

The pETe vectors for enzyme-free cloning and expression of target genes from an inducible T7lac promoter. Vectors are comprised of a choice of terminal or C-terminal 5His tags. The vectors are built on Lucigen’s plasmid backbone, which includes strategically placed terminators to limit transcriptional interference. Kan, tetracycline resistance gene, is origin of replication; pET- repressor of primer (control of cap C). Ribosome binding site (RBS), translational start (ATG) and stop codons are included in the vector.

Figure 2. Hi-Control BL21(DE3) cells

The Hi-Control BL21(DE3) cells are a high-efficiency host strain for use with pETe vectors. The cells contain an engineered lacI repressor to increase the level of lac repressor protein by ~200-fold. This level of repression is sufficient to maintain occupancy of the lacO operator to control basal expression of T7 RNAP within the host strain. The excess repressor is also sufficient to ensure tight promoter control by the lacI repressor in the T7 promoter expression vector. Hi-Control BL21(DE3) cells provide enhanced control over leaky target gene expression compared to strain BL21(DE3) (Fig. 4).

Figure 3. Efficient enzyme-free cloning with Hi-Control cells and pETe vectors

The pETe 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 sequence flanking the insert site.
2. **Mix** 1 µl PCR product with 2 µl pre-processed pETe vector. No further enzymatic treatment or cloning is required.
3. **Transform** mixture immediately into high-efficiency, chemically competent Hi-Control DE3 host cells and plate on media containing kanamycin. Typically, ~90% of transformations are recombinant.

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

Simplified example of cloning and expression with Hi-Control BL21(DE3) cells containing the target gene. The target gene was amplified with specially designed primers that contained 15-18 bp of complementarity to the pETe vector backbone, and cloned using Hi-Control BL21(DE3) cells. The target gene was expressed upon induction.

Figure 5. Expression and purification of active soluble fluorescent protein

A DNA Polymerase gene was cloned into pET28a and pETe vectors and the products were transferred into BL21(DE3) and BL21(DE3) Hi-Control cells. Samples selected equal aliquots of samples grown to OD600 0.6 without induction ( ) or after induction for 3 hours with 1 mM IPTG (■). With the pET28-DNAP clone, leaky expression was not detected in BL21(DE3) Hi-Control cells. The Hi-Control cells maintain comparable tight control over the pETe-DNAP clone, despite the absence of the lac gene from the expression vector.

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

An aliquot (1 µl) of each PCR reaction was mixed with 25 ng pETe C-HIS vector and transformed immediately into Hi-Control chemically competent cells. Insertion of pETe C-HIS vector into Hi-Control cells resulted in recombinant plasmids that 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 hydrolase genes.

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

To date we have cloned all 47 genes into the pETe C-HIS vector. Of these, 35 have been subjected to a multiplex activity screen in which lyases are inoculated 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.

Conclusion
The expressing 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 recombining. 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.