Rhamnose Expression and Cloning System: N-His/C-His pTrham Fast Cloning Kits

Manual

Description

Amid Biosciences pTrham Cloning Kits (N-His-pTrham Cloning Kit, catalog # NHPTR-401) and C-His-pTrham Cloning Kit (catalog # CHPTR-401) facilitate rapid cloning and gene expression in *E. coli.* Each Kit consists of linearized N-His or C-His-pTrham Vector that allows cloning of any PCR product amplified with proofreading DNA polymerases, *E. coli* AB 5-alpha Chemically Competent Cells for cloning, control insert DNA, and sequencing primers for clone analysis.

The N-His-pTrham vector, together with C-His-pTrham, represent a unique pair of expression vectors having the same plasmid backbone for production of recombinant proteins fused only to a 6X histidine tag sequence at the N- or C-terminus. Linearized N-His-pTrham and C-His-pTrham (Figure 1) are based on Amid Biosciences pTrham vector (catalog # PTR-401) and have same features as pTrham designed for providing the highest levels of expression of recombinant proteins: the *rhaP*$_{BAD}$ promoter (1-4), the *rrnG* antitermination region (5), the bacteriophage T7 gene 10 translation enhancer, and ribosome binding site (6). The vectors contain an ampicillin resistance marker.

L-rhamnose-inducible *rhaP*$_{BAD}$ promoter is capable of high expression level and ensures minimal basal expression in the absence of inducer. This level of control is particularly useful for expression of genes encoding toxic products. pTrham vector series can be used in any *E. coli* strains.

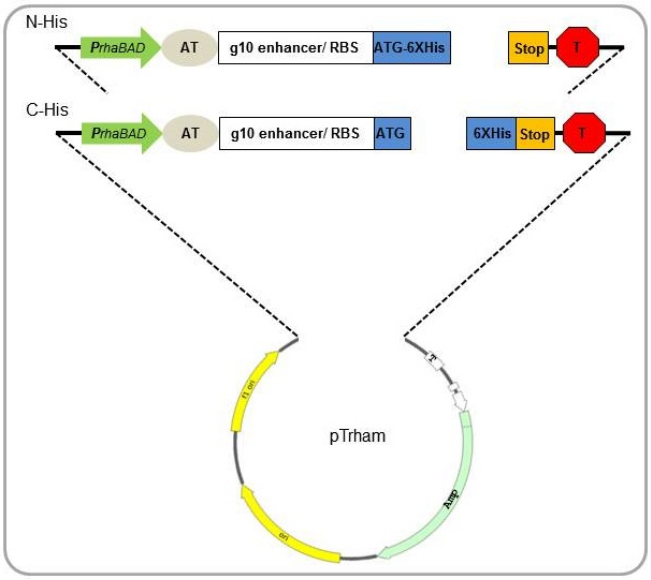

Figure 1. N-His-pTrham and C-His-pTrham vector features. **AT**- antitermination sequence; **RBS**- ribosome binding site; **ATG** and **Stop** - start and stop codons for translation, respectively; **T**- transcription terminator.

Benefits:
Universal - clone any insert
Easy – simply mix and transform
Fast – no further enzymatic step after PCR amplification
Seamless - no extra nucleotides
Efficient > 95% positive colonies
Economical – saving on time and reagents

N-His-pTrham Cloning Kit Components (5 reactions)

Component	Volume
Linearized N-His-pTrham (20 ng/µl)	10 µl
Positive control: a PCR amplicon of GFP, 753 bp (20 ng/µl)	5 µl
Sequencing Primers Forward- pTrham (5 µM) Reverse- pTrham (5 µM)	 10 µl 10 µl
E. coli AB 5-alpha chemically competent cells	5 X 100 µl

C-His-pTrham Cloning Kit Components (5 reactions)

Component	Volume
Linearized C-His-pTrham (20 ng/µl)	10 µl
Positive control: a PCR amplicon of GFP, 753 bp (20 ng/µl)	5 µl
Sequencing Primers Forward- pTrham (5 µM) Reverse- pTrham (5 µM)	 10 µl 10 µl
E. coli AB 5-alpha chemically competent cells	5 X 100 µl

Store all kit components except competent cells at -20 °C
Competent cells must be stored at -80 °C

AB 5-alpha chemically competent *E.coli* cells

AB 5-alpha chemically competent cells are comparable to the DH5✔™ strain of *E. coli*. The transformation efficiency is ≥ $1x10^8$ cfu/µg pUC19 plasmid DNA.

***E. coli* genotype**

F- endA1 recA1 relA1 gyrA96 hsdR17(r_k^-, m_k^+) deoR supE44 phoA Δ(lacZYA-argF) U169 Φ80lacZΔM15 λ^- thi-1

Positive Control Inserts

Included with the kit control inserts for the N-terminal and C-terminal His fusions encode the green fluorescent protein from the jelly fish *Aequorea victoria*. Each GFP insert (753 base pairs) contains flanking sequences for cloning into either the N-His-pTrham or C-His-pTrham vector. The GFP gene is expressed in the presence of L-rhamnose and gives a green glow under UV light if cells produce this type of protein. These can be useful as controls both for cloning efficiency and for expression.

Cloning Strategy

The cloning strategy is based on *in vivo* recombination of the linear vector and insert DNA fragment containing common flanking sequences (7-10). The recombination-mediated approach enables directional cloning of any PCR products into the vector without the need for restriction enzyme digestion or ligation reactions. Inserts designed for cloning into pTrham linearized vectors are generated by PCR with primers having 15 to 20 bases of homology with the ends of the vector. The PCR product is mixed with the corresponding cloning-ready vector and transformed into competent *E. coli* cells (Figures 2 and 3). No further enzymatic step after PCR amplification or purification of the vector and inserts are required. At least two PCR fragments can be assembled simultaneously into a vector with a high efficiency (10). Compared with the conventional restriction-ligation-dependent or other ligase-independent cloning approaches, the recombinational cloning is simplest and fastest method available. In addition to simplicity, the system is very robust and highly efficient. More than 95% positive colonies can be routinely observed upon cloning of the control insert supplied with the kit. Using the recombinational cloning strategy, a target gene derived by PCR with a pair of predesigned primers that include 15-20 nt of overlap with the ends of the vector can be inserted into the N-His-pTrham (Figure 2) to express a recombinant protein fused with an N-terminal sequence MHHHHHH or into that of C-His-pTrham to encode a recombinant protein with a C-terminal sequence HHHHHH (Figure 3).

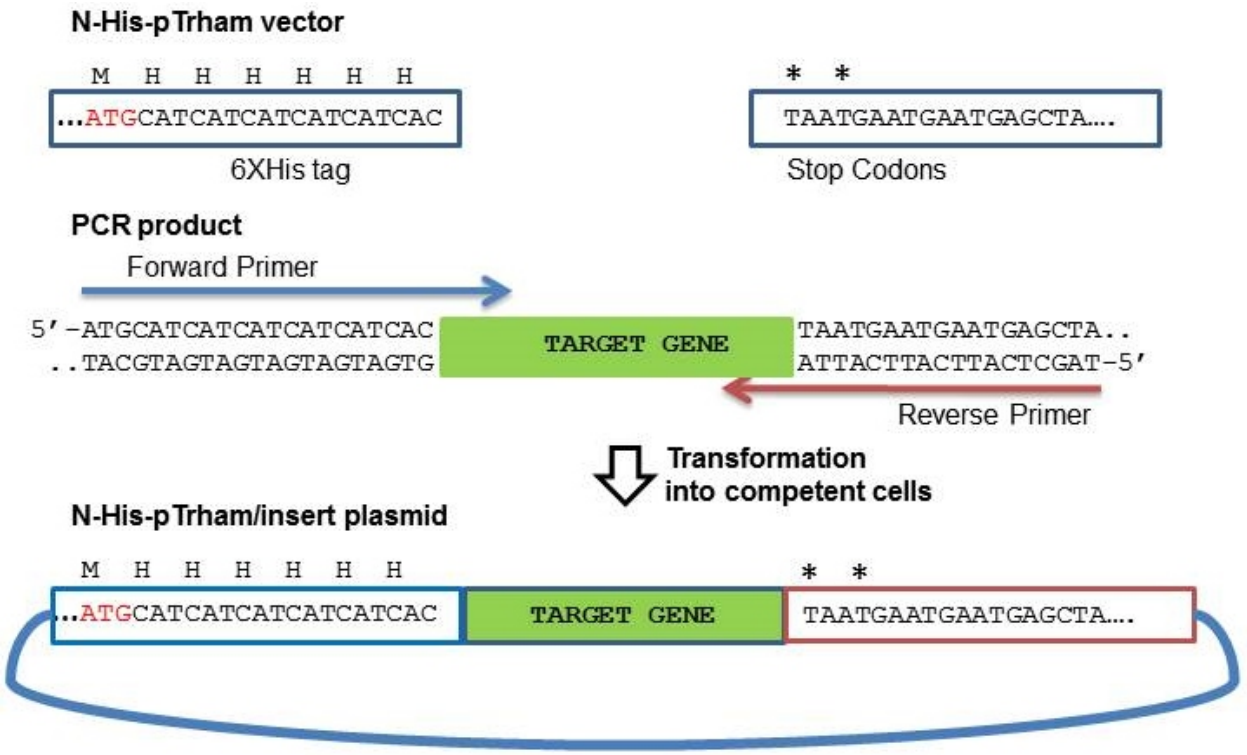

Figure 2. Schematic illustration of cloning of PCR-amplified target gene into N-His-pTrham vector. Sequences for vector ends which are used for PCR primer's design are shown in the boxes. The primer pair for insert amplification has 15-20 base tails overlapping with the vector ends.

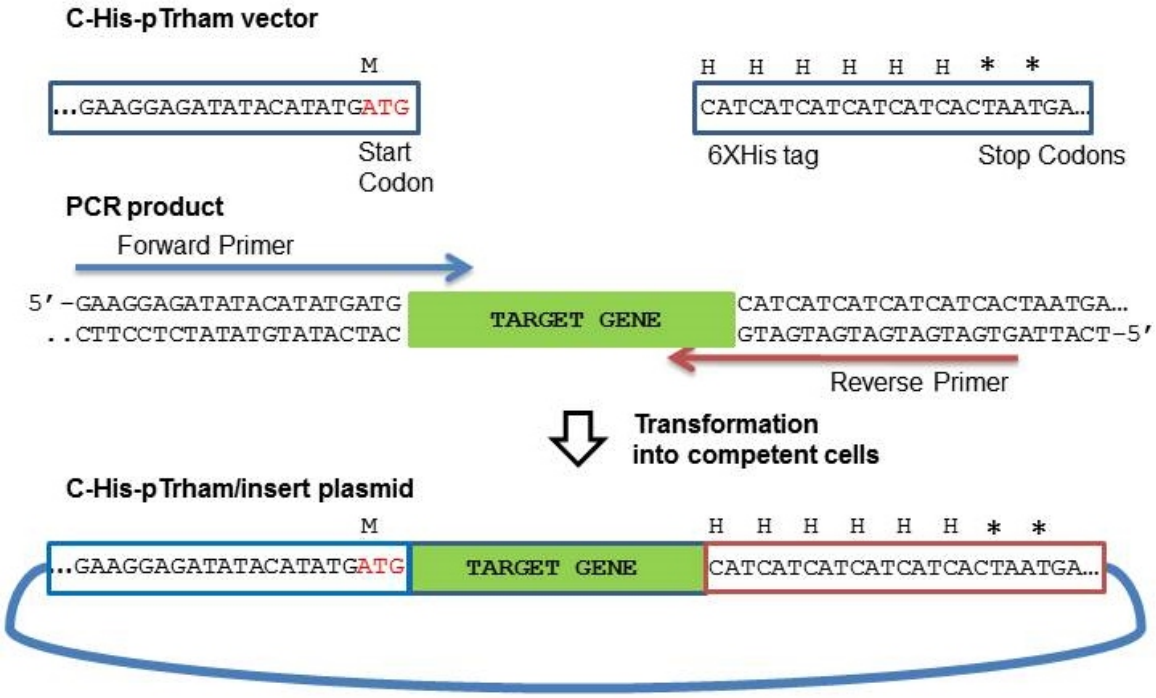

Figure 3. Cloning of PCR-amplified target gene into C-His-pTrham vector. Sequences for vector ends which are used for primer's design are shown in the boxes.

Protocols

Preparation of vector inserts

To clone target genes in the correct orientation and reading frame into N-His-pTrham vector or C-His-pTrham vector, genes of interest must be amplified by PCR using a high fidelity polymerase according to the manufacturer's instructions. Primers should begin with 15-20 bases at the 5'-end homologous with the particular vector ends and have additional 20-24 (depending on the GC content) insert-specific bases at the 3'-end.

Primer design for N-His-pTrham vector fusions

For the N-His-pTrham vector, the forward primer starts with the vector-defined extension 5'-CATCATCATCATCATCAC, followed by the gene sequence starting with the second codon and coding for six or more N-terminal amino acids. The reverse primer uses the vector-specific extension 5'-TAGCTCATTCATTCATTA, followed by the sequence reverse complementary to the sequence encoding six or more C-terminal amino acids of the target gene with omission of the stop codon. Note that two stop codons are encoded by the vector-specific sequence.

Primer design for C-His-pTrham vector fusions

For the C-His-pTrham vector, the forward primer begins with the vector-specific extension 5'-GAAGGAGATATACATATG, followed by the gene sequence starting with the second codon. Start codon (ATG) is included in the vector-specific primer sequence.

The reverse primer uses the vector-specific extension 5'-GTGATGATGATGATGATG, followed by the sequence complementary to the last 20-24 nt of the target gene. Do not include a stop codon in the gene-specific sequence. Stop codons are present in the C-His-pTrham vector sequence immediately after the 6X His tag residues. However, if the C-terminal His tag is not desired include a sequence reverse complementary to the stop codon in the insert-specific portion of the primer.

Note: For PCR conditions and optimal PCR primer annealing temperatures follow the polymerase manufacturer's protocols and oligonucleotide's specific Tm.

If the plasmid DNA carrying ampicillin resistance marker was used as a template for PCR, the template plasmid can be depleted after PCR by digesting with DpnI enzyme (New England Biolabs, Ipswich, MA, USA) in the same buffer used for PCR in order to avoid a high background from the template on ampicillin plates.

Analyze the PCR products by agarose gel electrophoresis. If products are highly pure, they can be used directly for cloning without cleanup procedure. Alternatively, products can be gel-purified.

Cloning inserts into linearized pTrham vectors

For cloning, the PCR fragments and linear pTrham vector are simply mixed and co-transformed into chemically competent Amid Biosciences *E. coli* AB 5-alpha cells. The optimal ratio of insert to the vector to achieve a high cloning efficiency is 2 to 1, however, 1:1 or 4:1 (insert to vector) ratios work well too. The *E. coli* DH5α, DH10B, or other similar strains are suitable hosts for cloning of target DNA into pTrham vector.

It appears that a *recA*-independent homologous recombination mechanism might be responsible for *in vivo* assembly of molecules containing overlapping ends (10-11).

For convenience and consistent performance, pTrham Cloning Kits come with prepared competent cells, ready for high-efficiency transformation.

Typical control reaction is set up as follows:

Component	Volume
Linearized N-His or C-His-pTrham vector (20 ng/µl)	2 µl
Positive control (20 ng/µl)	1 µl
Sterile ddH_2O	7 µl
Total Volume	10 µl

Transformation Procedure for Chemically Competent Cells

1. Combine components as listed above in single 1.5 ml tube on ice. Incubate on ice for 2-5 minutes prior to adding to competent cells.
2. Remove appropriate number competent cell tubes from –80°C and let thaw on ice.

3. Gently mix cells by lightly flicking the tube. Aliquot ~50-100µl of cells into chilled, 17 x 100mm polypropylene tube(s).
4. Transfer 5 µl of mixture (vector + insert) into 50 µl of thawed *E. coli* AB 5-alpha chemically competent cells and incubate on ice for 20-30 min.
5. Place tube(s) in 42°C water bath for ~30 to 45 seconds without shaking. For 50µl aliquots, 30 seconds is recommended for maximum efficiency.
6. Place tube(s) on ice for ~2 minutes.
7. Dilute transformation reaction(s) to 1ml by addition of 900-950µl SOC. SOC Medium: 2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ & 10mM $MgSO_4$. Other media can be used to grow transformed cells, including standard LB or TB broths. However, SOC is optimal choice for recovery of the cells and obtaining maximum transformation efficiencies.
8. Shake tube(s) ~200 rpm for 1 hour at 37°C.
9. Plate by spreading 50-200µl of cell transformation mixture on LB agar plates containing ampicillin (100 µg/ml) and incubate overnight at 37°C.

Note: Optional control transformation can be performed with vector alone.

Expressing the Target Gene

E. coli AB 5-alpha cells, DH5α, DH10B, BL21, or BL21(DE3) strains can be used as host for protein expression experiments. It is recommended to conduct a time course of expression to determine the optimal expression conditions for your particular protein since each recombinant protein has different characteristics that may influence optimal expression parameters.

Sample Induction Protocol

1. Inoculate 5 ml of SOB or LB + Ampicillin (100 µg/ml) with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking.
3. On next day, inoculate 50 ml of SOB or LB + Ampicillin (100 µg/ml) in a sterile 250 ml flask with 0.5 - 1 ml of the grown overnight culture.
4. Grow the culture at 37°C with shaking approximately at 200 rpm to an OD600 = 0.6.
5. Remove 2 X 0.5 ml aliquots of cells prior to induction with L-rhamnose, centrifuge one sample in a microcentrifuge. Discard the supernatant and freeze the pellet at –20°C; this will be the time zero sample. The other 0.5 ml aliquot transfer to 5 ml tube and leave shaking; this will be “no induction” sample.
6. Add sterile filtered L-rhamnose to a final concentration of 0.2% (1 ml of 10% L-rhamnose stock to 50 ml culture) and grow at 37°C with shaking. Take 0.5 ml samples at one hour intervals for 4 - 6 hours. Longer time course samples (following 8 or 24 hours after rhamnose addition) can be taken too. Centrifuge each sample, aspirate the supernatant and store the pellet at -20°C.
7. When all time points are collected, resuspend each pellet in 100 µl of neutral pH buffer (for example, 20 mM Tris-HCl, pH 7.0 or phosphate buffer) and add an equal volume of 2X Laemmli Buffer.
8. Analyze 10–20 µl of each time course sample on SDS polyacrylamidegel. Stain the gel with Coomassie Blue and look for a band of increasing intensity in the expected size range for the protein. Compare it to time zero and “no induction” controls to distinguish the recombinant proteins from the background proteins.
9. Determine the optimal time post rhamnose-induction to harvest the cells.

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