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Manual

pTrham - Rhamnose-Inducible Expression Vector

Description

Amid Biosciences designs and produces powerful expression vectors for proteins production in *Escherichia coli*. Our rhamnose-inducible **pTrham** vector provides high expression yield of a desired protein as amino-terminal His-Tag (Histidine Affinity Tag) fusion protein**.** The His-Tag enables purification of the protein with Immobilized Metal Ion Affinity Chromatography (IMAC) resins.

The pTrham expression vector utilizes L-rhamnose-inducible *rhaBAD* promoter of *Escherichia coli* for tight control of expression of cloned genes. P_{rhaBAD} promoter is capable of high expression levels but at same time displays low baseline gene expression in the absence of inducer. Moreover, the baseline gene expression of P_{rhaBAD} promoter even lower than that of the L-arabinose-inducible P_{ara} promoter (*araC-P* $_{BAD}$). This level of control is particularly useful for expression of genes encoding toxic products, where leaky expression could lead to cellular toxicity, difficulties in generating of expression constructs, and selective pressure for mutation of the expressed gene. Expression from *rhaBAD* promoter is linear with respect to inducer concentrations, in contrast to "all or nothing" response of the P_{lac} or P_{araBAD} systems. L-rhamnose, a naturally occurring deoxyhexose, is nontoxic to bacterial cells, unlike some other inducers like IPTG, Tet or, in some cases, arabinose.

E. coli RNA polymerase transcribes genes under the control of the *rhaBAD* promoter, so pTrham vector can be used in most *E. coli* strains.

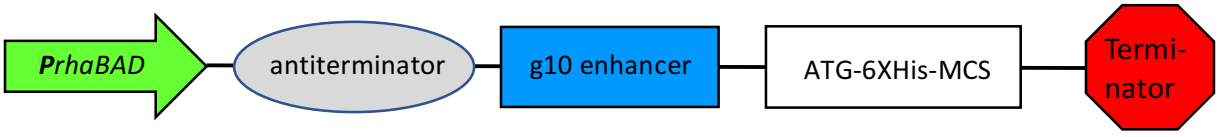
The pTrham vector does not contain the *lac*Z alpha gene fragment and cannot be used for blue/white colony screening. The pTrham vector has low copy number, similar to that of pBR322 plasmid (~20 copies/cell), yielding 0.5–1.0 µg of plasmid DNA per ml of culture.

Features

- L-Rhamnose-inducible E. coli promoter is capable of high expression level and ensures minimal basal expression in the absence of inducer.
- Directional cloning with a choice of restriction enzymes.
- The 6xHis affinity tag is fused to the amino terminus the target protein for efficient purification.
- NdeI site in the multiple cloning site contains an ATG sequence for translation initiation which allows cloning of the target protein without any additional amino acids at its N-terminus.
- Ampicillin resistance.

The pTrham expression vector is designed for providing the highest levels of expression of recombinant proteins. The vector's features and map of the multiple cloning site (MCS) are presented on Figures 1 and 2.

To ensure highly efficient transcription and translation of the cloned gene, the *rhaBAD* promoter (*Egan et al.*, 1993) is complemented with the *rrnG* anti-termination region (*Li et al.*, 1984), the bacteriophage T7 gene 10 translation enhancer, and ribosome binding site (*Olins et al.*, 1989). Small size of the pTrham vector (3.3 kb) facilitates cloning of large inserts and performing DNA manipulations, such as site-directed mutagenesis. The multiple cloning site (MCS) provides directional cloning options using a variety of restriction enzymes.

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                XhoI   SmaI
NdeI            HindIII  EcoRI  KpnI  BglII     NheI  BmtI
|           |   |  |  |  |   |       | |
TCATATGCATCATCATCATCATCACAAGCTTCTCGAGAATTCCCGGGTACCAAGATCTTGAATGAATGAGCTAGCTCGAC
AGTATACGTAGTAGTAGTAGTAGTGTTCGAAGAGCTCTTAAGGGCCCATGGTTCTAGAACTTACTTACTCGATCGAGCTG
  M H H H H H H K L L E N S R V P R S *
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Figure 1. Scheme of the vector's features and map of the MCS. Start codon and sequence encoding 6XHis are underlined.

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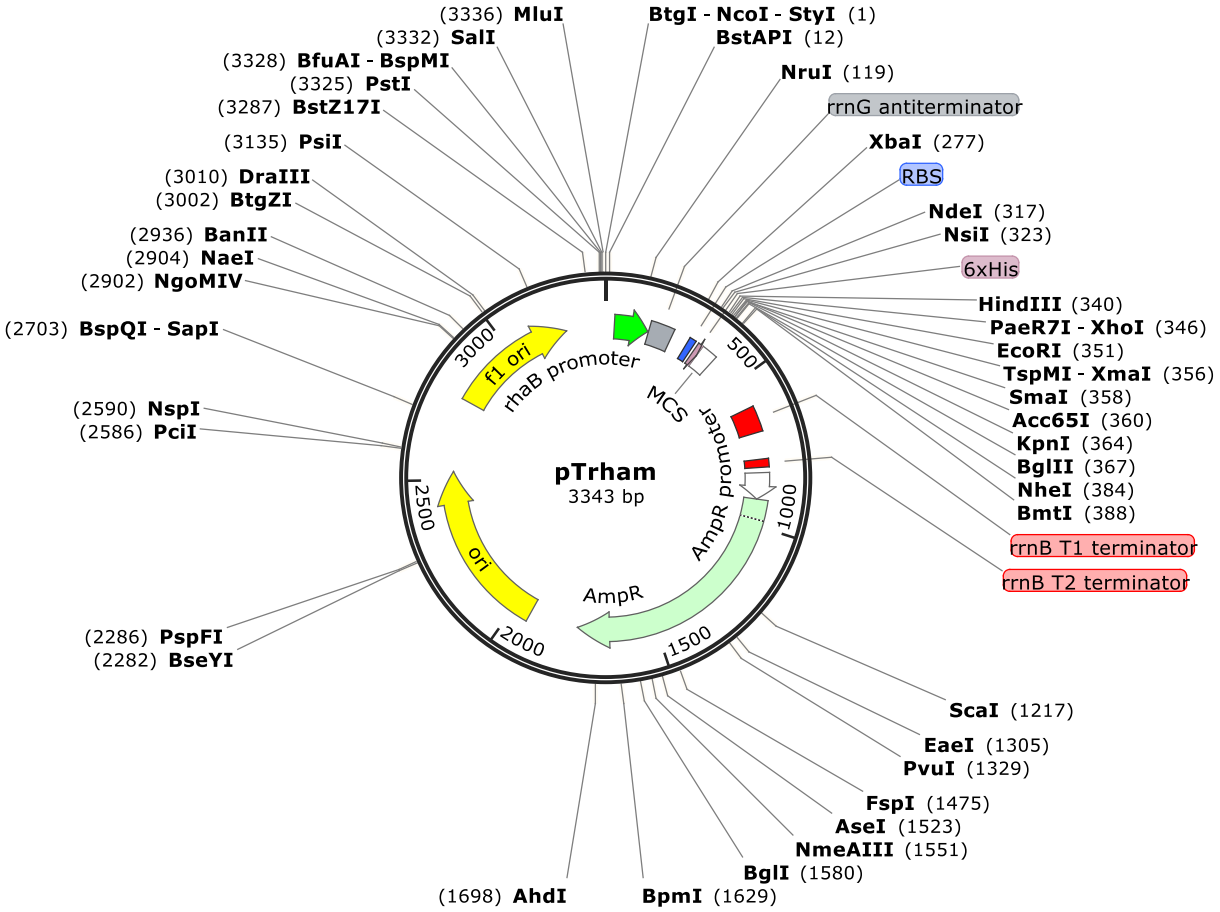
Regulation of Protein Expression in the pTrham System

The rhamnose system is positively regulated by two activators RhaS and RhaR. Both RhaS and RhaR are activated by L-rhamnose. In the absence of L-rhamnose, transcription from *rhaBAD* promoter is extremely low. When L-rhamnose is present, it acts as an inducer which binds to the regulator RhaR which then activates transcription of the *rhaSR* operon leading to induced expression of RhaR and RhaS.
Activated by L-rhamnose RhaS binds to the *rhaBAD* promoter and stimulates the transcription of the gene downstream of the promoter. Usage the rhamnose expression system does not require expression of the regulatory proteins in larger quantities, because the amounts of RhaR and RhaS expressed from the chromosome are sufficient for activation of the transcription even on multi-copy plasmids (*Wilms et al. 2001; Wegerer et al. 2008*).
Additionally, cyclic AMP (cAMP) receptor protein (CRP) functions as coactivator for the transcription of *rhaBAD*. Glucose depletion during cell growth triggers cAMP synthesis which binds to CRP. The active cAMP-CRP complex binds upstream of *rhaBAD* promoter and increases transcription initiation by RNA polymerase. Conversely, transcription from the *rhaBAD* promoter is down-regulated by addition of glucose which keeps cAMP concentration low. As such, addition of 0.5–1% glucose to media will decrease basal expression of genes under the control of the *rhaBAD* promoter and may be beneficial for expression of "toxic" gene products and maintaining plasmid stability.



Map positions of pTrham features
rhaP$_{BAD}$ promoter: 31-149
rrnG antiterminator: 152-232

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Ribosome binding site: 289-311
His tag: 322-339
MCS: 340-394
rrnB T1/T2 terminators: 595-800
beta-lactamase (*Amp*^{R}): 911-1771
pBR322 ori: 1942-2530
f1 ori: 2777-3205

Figure 2. pTrham map and map positions of vector features.

General Considerations

Vector Resuspension and Storage

pTrham vector (5 µg) is shipped as lyophilized product. Spin down the tube with pTrham prior to opening. Resuspend the vector in TE buffer (10mM Tris; 0.1 mM EDTA; pH 8.0) at the desired solution concentration. Alternatively, use nuclease-free water. Take sufficient volume for immediate use from the solution. Store the remaining vector solution at –20°C.

Maintenance of pTrham vector

For propagation and maintaining of pTrham vector, we recommend using 1-10 ng of the vector to transform a *rec*A$^-$, *end*A$^-$ *E. coli* strain like DH5α, DH10B, or equivalent.
Transformants are selected on LB plates containing 100 µg/ml ampicillin.

Cloning into the Expression Vector

Directional ligation-based cloning can be accomplished with unique restriction sites in the multiple cloning site (MCS) of pTrham. To generate recombinant proteins that include the correct N-terminal His-tag fusion peptide, clone the DNA into the MCS in frame with the ATG start codon. Please see the sequence map provided for the vector. Detailed protocols on DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989).

Suitable bacterial hosts for cloning include but not limited to the *E. coli* DH5α, DH10B, and JM109 strains. These strains are convenient hosts for initial cloning of target DNA into pTrham vector and for maintaining plasmids due to high transformation efficiency and high yields of high quality DNA that results from *rec*A and *end*A mutations.

Protein Expression

The rhamnose-inducible promoter is recognized by *E. coli* RNA polymerase, so it is not necessary to use separate host strains for cloning and protein expression of target genes with pTrham system. However, we recommend the *E. coli* B strains, like BL21, BL21(DE3), and etc., as hosts for protein production and purification. These strains are deficient in the *lon* and the *ompT* proteases that can degrade proteins during purification. Thus, at least some target proteins should be more stable in these strains than in host strains containing these proteases. Protein expression is induced by addition of rhamnose, and expression levels of target proteins are evaluated by SDS-PAGE analysis.

Methods and Protocols

A. Vector Preparation

For vector preparation, use the restriction enzyme manufacturer's recommended buffer and incubation conditions for the enzymes you are using. Many combinations of enzymes are compatible when used together in the same buffer.

- Note that different enzymes digest with different efficiencies, especially when two sites are close together. In general, enzymes with compatible buffers and whose sites are more than 10 bp apart can be used together in the same reaction. If one of the enzymes is a poor cutter, if the buffers are incompatible, or if the sites are separated by 10 bp or less, the digestions should be performed sequentially. The first digestion should be done with the enzyme that is the poorest cutter and the second enzyme added after digestion has been verified by running a sample of the reaction on agarose gel.
- Note that some restriction enzymes may display "star activity," a less stringent sequence dependence, that results in altered specificity. Conditions that can lead to star activity include high glycerol concentration (> 5%), high pH, and low ionic strength.

- If cloning into a single site, dephosphorylate the vector following restriction digestion to decrease the background of non-recombinants resulting from self-ligation of the vector. Molecular biology grade calf intestinal or shrimp alkaline phosphatase should be used according to the manufacturer's instructions.

- It is also useful to dephosphorylate vectors cut with two enzymes, especially when the sites are close together or if one of the enzymes is a poor cutter. This decreases the non-recombinant background caused by incomplete digestion with one of the enzymes, which is undetectable by gel analysis.
- Following digestion, it is usually worthwhile to gel-purify the vector before insert ligation to remove residual nicked and supercoiled plasmid, which transforms very efficiently relative to the desired ligation products. This step is optional, but usually reduces the effort required for screening the correct construction.

To digest and gel-purify the vector:

1. Assemble the following components in a microcentrifuge tube:

1 µg	pTrham vector
2 µl	10X restriction enzyme buffer
5-10 U	Each restriction enzyme (assuming compatible buffers; the total volume of enzyme added should not exceed 10% of the reaction volume avoid high glycerol concentrations)
2 µl	1 mg/ml acetylated BSA (optional)
x µl	Nuclease-free water to volume
20 µl	Total volume

2. Incubate at appropriate temperature (usually 37°C) for 2–4 h.
3. Run a 3 µl sample together with DNA Markers on agarose gel to evaluate the extent of digestion.
4. When digestion is complete, add calf intestinal alkaline phosphatase directly to the remainder of the digestion. This enzyme works in most restriction buffers under the conditions described here. It is important to use the correct amount of enzyme: too much can cause unwanted deletions and can be difficult to remove for future steps. 1 µg of pTrham vector (3.3 kb) corresponds to about 1.0 pmol DNA ends when linearized. We recommend using 0.05 unit of alkaline phosphatase per pmol ends. Dilute the enzyme in water or 50 mM Tris-HCl, pH 9.0 just before use.
5. Incubate at 37°C for 30 min.
6. Add gel sample buffer to the reaction and load entire sample into a large well (0.5–1.0 cm wide) on a 1% agarose gel containing a proper amount of DNA visualization dye (e.g., ethidium bromide, SYBR, and etc.). Run the gel far enough to separate the linear plasmid from nicked and supercoiled species. It is useful to run uncut vector DNA in an adjacent lane to help distinguish undigested from linearized plasmid DNA.
7. Visualize the DNA band with a long wave UV light source and excise the band from the gel using a clean razor blade. Minimize exposure to the light source, which can cause nicks and double strand breaks in the DNA.
8. Recover the DNA from the gel slice using Gel Extraction DNA Kit.
9. Resuspend the final product in total volume of 30 µl (usually about 50 ng/µl DNA). The DNA can be quantified spectophotometrically. Assume recoveries in the range of 50%.
10. Store the treated vector at –20°C until use.

B. Insert Preparation

Preparation inserts by restriction digestion followed by gel purification is usually straightforward. For subcloning into the pTrham vectors from vectors with the same selective marker (even with PCR as discussed below), it is necessary to remove the original plasmid by gel-purification. As little as 10 pg of contaminating supercoiled plasmid (i.e., less DNA than can be visualized on agarose gel) can typically result in many more colonies containing the original plasmid instead of the desired pTrham subclone.

PCR can be used for isolation and/or modification of target genes for expression in pTrham plasmid. With this approach, it is possible to design primers that will:

(1) isolate the translated portion of a cDNA sequence,
(2) add convenient restriction enzyme sites,
(3) place the coding region in the proper reading frame.

In general, primers should contain at least 18–21 nucleotides complementary to the sequence of interest with a GC content of approximately 50%, and restriction sites should be flanked by 3–10 "spacer" nucleotides (depending on the

enzyme) at the 5' end for efficient digestion. One risk in using PCR for insert preparation is the potential to introduce undesired mutations. The error rate of the PCR can be minimized in several ways:

- Use an enzyme with high fidelity, such as *Pfu*, or *Phusion* DNA Polymerases
- Limit the number of PCR cycles.
- Increase the concentration of target DNA.
- Increase the primer concentration.

C. Cloning Inserts in pTrham Vectors

Procedures and recommendations in this section discuss cloning an insert into a pTrham vector. This protocol includes ligation and transformation into a non-expression host, and analyzing your construct. After the construct is verified, plasmid is transformed into an expression host for protein production.

A. Ligation

1. For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.015–0.03 pmol) of pTrham vector with 0.2 pmol insert (e.g., 50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5 ml tube. Add the ligase last.

2 µl	10X T4 Ligase Buffer (200 mM Tris-HCl, 100 mM MgCl2, 250 µg/ml acetylated BSA, pH 7.6, 100 mM DTT, 10 mM ATP)
2 µl	50 ng/µl prepared pTrham vector
x µl	Prepared target gene insert (0.2 pmol)
0.2 µl	T4 DNA Ligase (1 Weiss U/µl)
y µl	Nuclease-free water to volume
20 µl	Total volume

2. Gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up a control reaction in which the insert is omitted to check for non-recombinant background.
Note: For blunt ends, use 10X more ligase (i.e., undiluted enzyme), reduce the ATP concentration to 0.1 mM, and incubate for 6–16 h at 16°C or 2 h at room temperature.

B. Transformation

The *E. coli* DH5α, DH10B, or other similar strains are convenient hosts for initial cloning of target DNA into pTrham vector and for maintaining plasmids due to high transformation efficiency and high yields of plasmid DNA that result from *recA*$^-$*endA*$^-$ mutations. For convenience and consistent performance, Amid Biosciences offers the relevant host strains as prepared competent cells, ready for high-efficiency transformation.

Ligation reactions can be directly added to competent cells. For transformation, 5 µl of the ligation reaction usually yields sufficient numbers of colonies for screening. Inactivation of the ligase could increase transformation efficiency but is not required before transformation.

Plasmid DNA isolated using standard miniprep procedures is usually satisfactory; however, for maximum efficiency, the sample DNA should be free of phenol, ethanol, salts, protein, and detergents, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or in water.

Transformation efficiencies will generally be higher with supercoiled plasmids than with ligation reactions. 1–10 ng plasmid DNA is usually sufficient to produce hundreds of colonies. If BL21 strain and its derivatives will be used as hosts for protein expression with pTrham constructs, expect these strains to be transformed at about 10 to 100-fold lower efficiency of the other strains.

Suggested Transformation Procedure for Chemically Competent Cells

1. Remove appropriate number competent cell tubes from –80°C and let thaw on ice.
2. Gently mix cells by lightly flicking the tube. Aliquot ~50-100µl of cells into chilled, 17 x 100mm polypropylene tube(s). Unused cells may be refrozen, but decrease in efficiency may result. For optimal recovery, refreeze cells in a dry ice/ethanol bath prior to -80°C storage.
3. Add DNA solution (≤5µl per 50µl cells) to cell suspension and gently swirl tube(s) for a few seconds to mix.
4. Incubate on ice for 30 minutes.
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.

Expressing the Target Gene

E. coli 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. Make sure to perform the expression of pTrham vector alone in parallel as a negative control.

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.
9. Analyze 10–20 µl of each time course sample on SDS polyacrylamide
gel. 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.
10. Determine the optimal time post rhamnose-induction to harvest the cells.

References

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