



## **pEco™ -T7-cHis, PCR cloning Kit User Manual** (Patent pending)

**Cloning PCR products for E Coli. expression of C-term His-tagged protein**

<b>Cat#</b>	<b>Contents</b>	<b>Amounts</b>	<b>Application</b>
<b>IC-1006</b>	<b>pEco-T7-cHis</b> vector built-in Eco™ Cloning cells	<b>10 tubes x 50ul/ea</b> (for 10 rxn)	<b>E Coli.</b> expression of C-term His-tag protein.
	Positive PCR insert	1 x 10ul/ea	
	Sequencing primer pair	Forward and reverse 15ul/each, (25ng/ul)	

### **Storage:**

Eco™ Cloning Kit is shipped on dry ice. Upon received, stored at -80°C. Once thawed, must be used, do not re-freeze. Product should be stable for 6 months.

### **Product Description:**

#### **1. Introduction:**

The revolutionary Eco Fusion *in vivo* cloning method is the easiest PCR cloning method available:

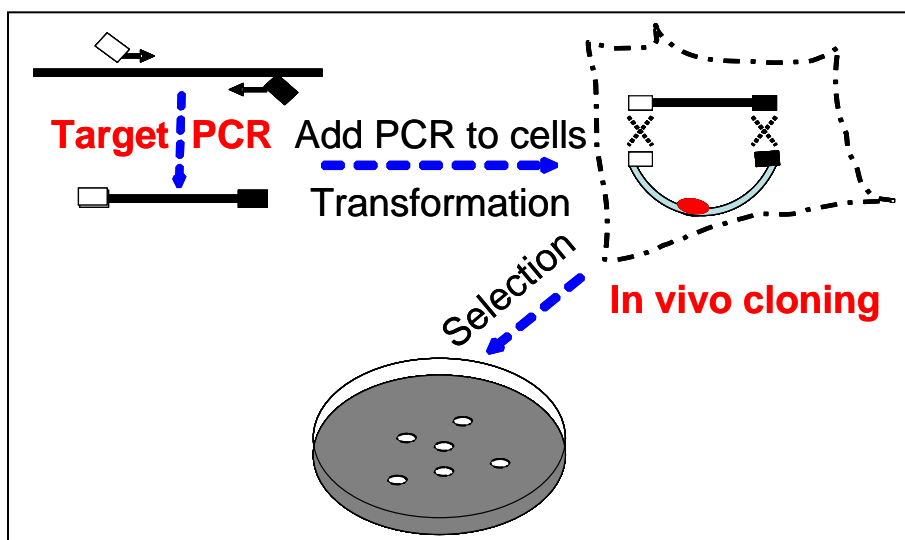
1. Simply amplify your gene of interest with a primer pair that is flanked with short arms homologous to the expression vector
2. Add 2 µl of purified PCR into the engineered, vector build-in cloning cells
3. Immediately proceed to transformation.

#### **2. How it works:**

The engineered *E Coli* strain in **GenTarget's Eco™ PCR Cloning Kit** has an enhanced *E Coli* competent cells enabling an *in vivo* joining reaction for cloning with no tube reactions.

### **Let the E Coli do the job for you *In Vivo*!**

GenTarget provides *E Coli* cloning cells with a selection of built-in vectors for mammalian or *E Coli* expression systems. A proprietary process for making ready-to-use, *E Coli* cells with built-in vectors ensures low background and a positive cloning rate of greater than 90%.



**pEco-T7-cHis** cloning cells has a built-in pET based T7 expression vector. PCR insert will be cloned in-frame with a C-terminal His-tag.

### 3. Key Features:

- The **easiest and most cost-effective** PCR cloning method available. Simply add 1µl of PCR insert into provided cells for transformation regardless of the insert's size and concentration
- No need to buy vectors and no tedious bench work preparing a vector backbone
- No need to buy cloning competent cells
- No need for any enzymes or any tube reactions
- Precisely **directional cloning** of PCR products with a in frame His-tag (C-term 6His)
- Flexibility to allow addition of any cleavage site for removal of C-terminal His-tag if desired
- Compatibility with any PCR product with or without a 3' -A overhang (the extra -A overhang, if it exists, will be removed in the cloning step)
- Can be used with PCR products of varying sizes, from 200 bp to 10 kb. The **same PCR product** can be used to construct multiple different expression vectors
- Engineered *E Coli* expression vectors for high protein yields
- Great for high throughput cloning

### 4. Protocol Outline:



Produce and clean PCR products



Add 1-2 µl of PCR product into the cloning cells provided;  
briefly mix and immediately proceed to transformation



Pick colonies, save glycerol stocks, and isolate plasmids by miniprep to verify the  
positive clones



Express protein from the saved glycerol stock

## 5. Detailed Protocol:

### 1. PCR primer design:

- ✱ PCR primers used for generating inserts for Eco<sup>TM</sup> Cloning must contain a 20 - 25bp homologous sequence corresponding to the built-in vector. Design your primer pair as follows:

**Fwd:** 5'- tttgtacaaaaaagcaggcacc + 20bp of (5'end gene specific forward sequence)

**Rev:** 5'- tttgtacaagaaagctgggtt + 20bp of (3'-end gene specific reverse sequence)

- ✱ A protein cleavage site may be included in the forward primer to allow excision of the C-term tag if desired. Its codon sequences must be in frame and set between the homologous leader and the 20bp gene specific sequence.
- ✱ An **example** of PCR primer design:

To design the primer pair for the following gene sequence:

**atggcctctgtgaaggaaaatcc**actctagtccttacctgcatttctcagccttgct  
tacctgttgccaacattgggccaaccgaattcttcccaatctttatcttggctgcca  
gcgagatgtcctcaacaaggagctgatgcagcagaatgggattgggtatgtgtta  
aatgccagcaata**cctgtccaagcctgactttta**

The PCR primer for vector **pEco-T7-cHis** will be:

Fwd: 5'- tttgtacaaaaaagcaggcacc**atggcctctgtgaaggaaa**

Rev: 5'- tttgtacaagaaagctgggtt**aaagtcaggcttggacagg**



If inserting a protein cleavage site, the reverse primer will be:

Rev: 5'- tttgtacaagaaagctggggttNNNNNNaaagtcaggctttggacagg

(where the NNNNNN is the in-framed, cleavage site codon reverse sequence).

## ✿ Notes:

1. GenTarget's cloning kits with the same terminal tags share PCR insert sites. The three Eco<sup>TM</sup> cloning kits with N-terminal tags, **Cat# IC-1001, IC-1002 and IC-1003**, can share the same PCR insert, and the two cloning kits with C-terminal tags, **Cat#: IC-1006 and IC-1007**, can share the same PCR insert.
2. A stop codon does not need to be included in the PCR reverse primer since a stop codon is already built in immediately after the PCR insert.

## 2. Target amplification by PCR:

- ✿ Amplify your target using any PCR amplification protocol that works for you. To minimize PCR errors, we recommend using high fidelity DNA polymerase.
- ✿ Use any PCR purification column to clean your PCR products. If you do not obtain a single, discrete band from PCR, gel purify your fragment.
- ✿ **Important:** if your PCR template can generate background clones having Amp resistance, treat the PCR product with DPN I or perform gel purification.

## 3. Transformation:

- ✿ Thaw Eco<sup>TM</sup> Cloning cells in ice-water.
- ✿ After they are completely thawed, add 1-2 µl purified PCR product (from 20ng to 150ng) into each vial of cells, and mix briefly by tapping the tube with your finger.
- ✿ For control vials, add 1µl positive PCR-insert (provided) as a positive control, and then add µl water to a negative control cells vial.
- ✿ Put tubes back on ice and proceed to heat shock at 42 °C for 40 seconds. (Note: Do not leave DNA-cells mixture on ice for prolonged period, less than 15min are fine). Put tubes back on ice for 1 min, add **250 µl** of SOC medium, and incubate at 37 °C, shaking for 1hr.



- ✱ **Plating:** take a ~250 µl competent cells above, spread out on pre-warmed LB-agar plates containing 100 µg/ml ampicillin. Grow colonies at 37 °C overnight.
- ✱ **Note:** In the absence of a PCR-insert, cells usually form background colonies; the no-insert negative control also generates a few colonies. In the presence of PCR insert, however, > 90% colonies are positive. Colony number varies depending on the quality and quantity of the PCR products. The concentration of purified PCR product can be from 20 ng/µl to 150 ng/µl with sizes ranging from 200 bp to 10 kb. For simplicity (and particularly for high throughput cloning) we recommend adding 1-2 µl of PCR product into the cloning cells. Regardless of the PCR product's concentration and size, it will generate enough colonies (5 ~ 100 colonies in general) for downstream work.

#### 4. **Save glycerol stocks for later expression and verification of positive clones:**

- ✱ Pick 2-5 colonies; propagate in LB/Amp, and incubate at 37 °C overnight
- ✱ Save an aliquot of each clone in LB-Glycerol medium containing 100 µg/ml ampicillin at a final concentration of 15% Glycerol.
- ✱ Isolate the plasmid DNAs using a DNA miniprep kit
- ✱ Confirm the positive by restriction digestion: PCR inset can be cut out by BsrGI: Run 1.2% agarose, the positive clones will show two bands: 5.79 kb backbone + the PCR insert (or multiple bands if BsrGI has extra cuts within the PCR-insert).
- ✱ Final sequencing verification. Use the provided sequencing primer pair. The sequencing primer comes in a ready-to-use dilution: use 1µl for each sequencing reaction with 500ng plasmid in 20µl volume.

Cat #	Vector	Forward primer	Reverse primer
IC-1006	pEco-T7-cHis	IC-1006-fwd 5'- taatacgactcactataggg	IC-1006-rev 5'- tgctagtattgctcagcgg

#### 5. **Protein expression:**

Once positive clones are confirmed, they can be used directly for protein expression without re-transformation into another strain.

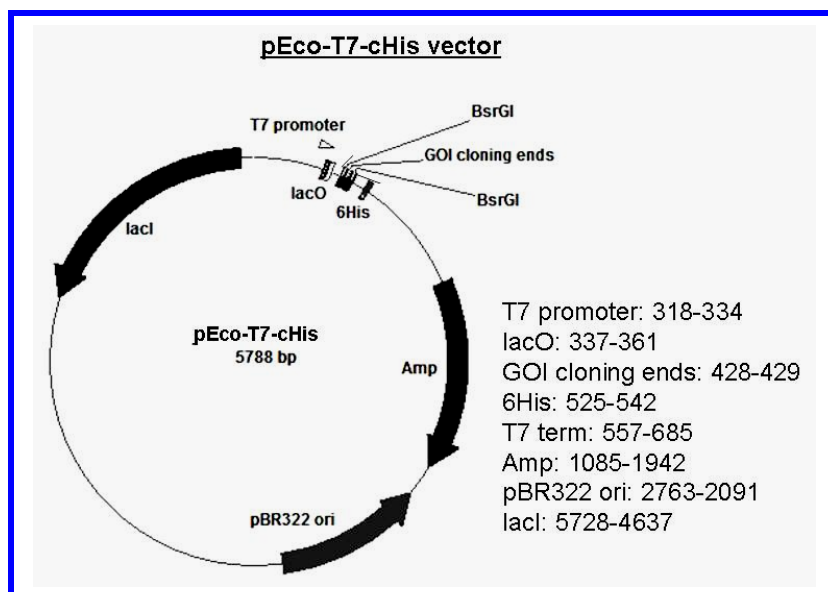
- **Transformation:** transform the sequencing verified plasmid DNA into any strain containing a T7 RNA polymerase, such as BL21(DE3) or BL21(DE3)pLys from which protein are expressed upon IPTG induction. Transformation uses standard heat-shock protocol, such as add 1ul DNA into 50ul competent cell, set ice (5~15min), heat-shock at 42oC



- for 30 seconds, back to ice for 2min, add 250ul SOC, recovery at 37oC, shaking for 1 hour. Plate 10 to 100ul onto LB plates containing 100ug/ml ampicillin. Grow colonies at 37oC incubator for overnight;
- **Propagation:** Pick one clone, grow in LB medium with ampicillin at 37oC, shaking overnight. Add overnight culture into appropriate amount of LB medium containing 100ug/ml of ampicillin by making 1:40 dilution, keep medium volume at 20% of flask volume for better aeration, vigorously shake at 30oC, 300rpm;
  - **Induction:** measure growth OD600, at the time when OD600= ~ 0.5, add an appropriate amount of IPTG, continue grow for 17 ~ 24 hours with vigorously shaking at 30oC, 300rpm;
  - **Harvest** cells by centrifugation.
  - **QC:** Cell pellet was lysed using lysis reagent. Following the lysis protocols, run protein gel for analysis;
  - **Purification:** use your favorite protocols and reagent to purify the expression His tagged protein by His-tag affinity column;
  - **Purity and function analysis** of the expressed protein using your favorite protocols.

## 6. Vector maps:

The figure below summarizes the vector map of pEco-T7-cHis. The **complete nucleotide sequence is** available for downloading from our Website at [Support page \(www.gentarget.com\)](http://www.gentarget.com). To make your clone map, simply paste your gene sequence (**not included the flanking sequences of both ends**) in the Red highlighted position (replacing the **NNNN..NN**). In most case, the pasted sequence is: "ATG...to...last codon".





## Cloning site for pEco-T7-cHis vector

### T7 promoter

301 TCTCGATCCC GCGAAATTAA TACGACTCAC TATAGGGGAA TTGTGAGCGG  
AGAGCTAGGG CGCTTTAATT ATGCTGAGTG ATATCCCCCTT AACACTCGCC

351 ATAACAATTC CCCTCTAGAA ATAATTTTGT TTAACCTTAA GAAGGAATAT  
TATTGTTAAG GGGAGATCTT TATTAACAACA AATTGAAATT CTTCCTTATA

### BsrGI

### PCR Insert

401 CACAAGTTTG TACAAAAAAG CAGGCACCNN NNNNNNNAAC CCAGCTTTCT  
GTGTTCAAAC ATGTTTTTTC GTCCGTGGNN NNNNNNNTTG GGTGAAAGA

### BsrGI

TGTACAAAGT GGTGATCAAT TCGAAGCTTG AAGGTAAGCC TATCCCTAAC  
ACATGTTTCA CCACTAGTTA AGCTTCGAAC TTCCATTCTG ATAGGGATTG

### 6His

CCTCTCCTCG GTCTCGATTG TACGCGTACC GGTCATCATC ACCATCACCA  
GGAGAGGAGC CAGAGCTAAG ATGCGCATGG CCAGTAGTAG TGGTAGTGGT

T**TGA**GTTTGA TCCGGCTGCT AACAAAGCCC GAAAGGAAGC TGAGTTGGCT  
A**ACT**CAAAC AGGCCGACGA TTGTTTCGGG CTTTCCTTCG ACTCAACCGA

## 7. Troubleshooting:

Problems	Solution
No colony	<ul style="list-style-type: none"><li>Be sure to set up a positive control transformation using the provided positive PCR insert1, which should give you 10~100 colonies.</li><li>Spread all of the transformation mixture onto the plate.</li></ul>
Background colonies	<ul style="list-style-type: none"><li>Be sure to set up a background control plate in which no PCR product was added to the cells. It should generate 0 ~ 5 colonies or less than 10% compared to plates with the insert (Note: in the absence of a PCR insert, cells force vector self-ligation resulting in a few background colonies).</li><li>Make sure that the PCR's template does not cause background colonies; If it does, clean PCR products by gel-isolation or treatment with DPNI.</li><li>Plate less transformation mixture onto the plate.</li></ul>
Satellite colonies	<ul style="list-style-type: none"><li>Be sure to use the right amount of antibiotics in the LB plate, and make fresh LB plates if necessary.</li></ul>



- ☀ Use carbenicillin instead of ampicillin if applicable.
- ☀ Do not incubate plates longer than 16 hours.
- ☀ Try to avoid picking the tiny satellite colonies.

## References:

1. Oliner et al., 1993, Nucleic Acids Res. 1:5192-97
2. Aslanidis et al., 1994, Genome Res. 4 :172-177
3. Kaluz et al. Nucl. Acids Res..1992; 20: 4369-4370

## Related Products:

Cat#	Product Name	Amount	Application
<a href="#"><u>IC-1001</u></a>	PCR cloning kit	kit	PCR cloning kit with a built-in vector (T7 promoter based) in provided cloning cells for <b>E Coli expression of N-term His</b> -tagged protein.
<a href="#"><u>IC-1002</u></a>	PCR cloning kit	kit	PCR cloning kit with a built-in mammalian expression vector (with neomycin selection marker) in provided cloning cells. The vector containing an engineered super CMV promoter for high-yield <b>mammalian expression of N-term His</b> tagged protein
<a href="#"><u>IC-1003</u></a>	PCR cloning kit	kit	PCR cloning kit with a built-in vector ( <b>non-T7 promoter based</b> ) in provided cloning cells for <b>E Coli expression of N-term His</b> -tagged protein, specially designed for <b>toxic proteins</b> .
<a href="#"><u>IC-1004</u></a>	PCR cloning kit	kit	PCR cloning kit with a built-in vector (T7 promoter based) in provided cloning cells for <b>E Coli expression of N-term GST</b> -tagged protein.
<a href="#"><u>IC-1007</u></a>	PCR cloning kit	kit	PCR cloning kit with a built-in mammalian expression vector (with Neomycin selection marker) in provided cloning cells, for <b>mammalian expression of C-term His</b> -tagged protein.