



## **pEco™ -T7-nGST, 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-1004</b> | <b>pEco-T7-nGST</b> vector built-in<br>Eco™ Cloning cells | <b>10 tubes x 50ul/ea</b><br>(for 10 rxn)   | <b>E Coli.</b> expression of N-term GST-tag protein. |
|                | Positive PCR insert                                       | 1 x 10ul/ea                                 |  |
|                | Sequencing primer pair                                    | Forward and reverse<br>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:

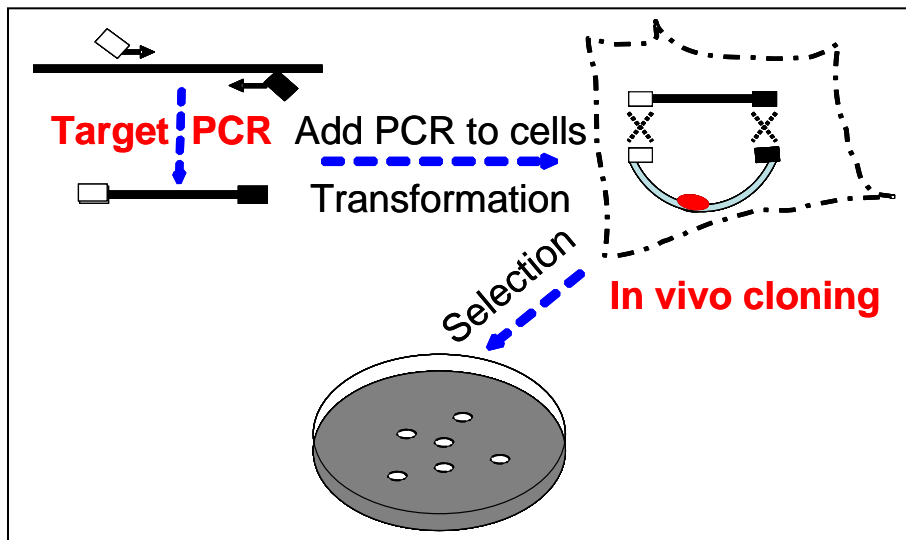
1. Simply amplify your gene of interest with a primer pair that is flanked with short arms homologous to the expression vector
2. Add 2 µl of purified PCR into the engineered, vector build-in cloning cells
3. Immediately proceed to transformation.

#### **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-nGST** cloning cells was built-in with a pET based T7 expression vector. PCR insert will be cloned in-framed with a N-terminal GST-tag.

### 3. Key Features:

- The **easiest and most cost-effective** PCR cloning method available. Simply add 1 $\mu$ l 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 an in frame GST-tag at N-term.
- Flexibility to allow addition of any cleavage site for removal of the nGST 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  $\mu$ l 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'- atcgatctggtccgcgtgaattc + 20bp of (5' end gene specific forward sequence)

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

- ✿ A protein cleavage site may be included in the forward primer to allow excision of the N-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**actctagtcctacctgcatttctcagccttgct  
tacctgttgccaacattgggccaaccgaattcttccaatctttatcttggtgcca  
gcgagatgtcctcaacaaggagctgatgcagcagaatgggattggttatgtgta  
aatgccagcaata**cctgtccaagcctgactttta**

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

**Fwd:** 5'- atcgatctggtccgcgtgaattc**atggcctctgtgaaggaaa**

**Rev:** 5'- ttgtagcaggtaacacgcgtctaa**aagtcaggcttggacagg**

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



Fwd: 5' atcggatctggttccgcgtgaattcNNNNNNgcctctgtgaaggaaatcc

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

## ✿ 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 DPNI or perform gel purification.

## 3. Transformation:

- ✿ Thaw Eco<sup>TM</sup> Cloning cells in ice-water.
- ✿ After they are completely thawed, add 1-2  $\mu$ l 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 1 $\mu$ l positive PCR-insert (provided) as a positive control, and then add  $\mu$ l 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  $\mu$ l** of SOC medium, and incubate at 37 °C, shaking for 1hr.
- ✿ **Plating:** take a **~250  $\mu$ l** competent cells above, spread out on pre-warmed LB-agar plates containing 100  $\mu$ g/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 EcoRI + HpaI: Run 1.2% agarose, the positive clones will show two bands: 3.4 kb backbone + the PCR insert (or multiple bands when the cut exist 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-1004 | pEco-T7-nGST | IC-1004-fwd<br>5'- catggcctttgcagggct | IC-1004-rev<br>5'- tgctagttattgctcagcgg |

#### 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 GST tagged protein by GST-tag affinity column;
- **Purity and function analysis** of the expressed protein using your favorite protocols.

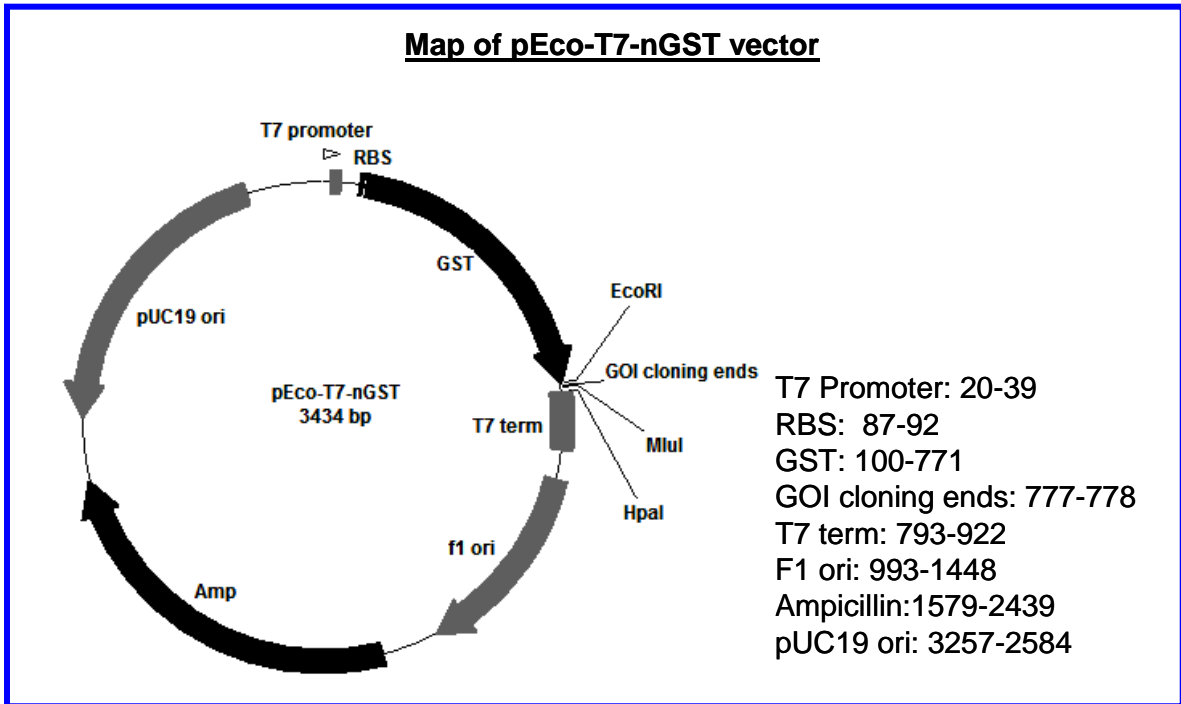
## 6. Vector maps:

The figure below summarizes the vector map of pEco-T7-nGST. 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-nGST** vector

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|     |                       |                                |            |                       |
|-----|-----------------------|--------------------------------|------------|-----------------------|
|     | EcoRI                 |                                | MluI       | HpaI                  |
|     | <b>GST</b>            | <b>PCR insert</b>              |            |                       |
| 751 | AAATCGGATC TGGTTCCGCG | TGAATTCNNN NNNNNN <b>TAG</b> A | CGCGTGTTAA |                       |
|     | TTTAGCCTAG ACCAAGGCGC | ACTTAAGNNN NNNNNN <b>ATC</b> T | GCGCACAATT |                       |
|     | HpaI                  |                                |            |                       |
|     | CCTGCTAACA            | AAGCCCGAAA                     | GGAAGCTGAG | TTGGCTGCTG CCACCGCTGA |
|     | <u>G</u> GACGATTGT    | TTCGGGCTTT                     | CCTTCGACTC | AACCGACGAC GGTGGCGACT |



## 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> <li>☠ Use carbenicillin instead of ampicillin if applicable.</li> <li>☠ Do not incubate plates longer than 16 hours.</li> <li>☠ Try to avoid picking the tiny satellite colonies.</li> </ul>   |



## 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:

| <b>Cat#</b>                    | <b>Product Name</b> | <b>Amount</b> | <b>Application</b>   |
|--------------------------------|---------------------|---------------|--|
| <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-tagged protein</b> .   |
| <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 tagged protein</b> |
| <a href="#"><u>IC-1003</u></a> | PCR cloning kit     | kit           | PCR cloning kit with a built-in vector (non-T7 promoter based) in provided cloning cells for <b>E Coli expression of C-term His-tagged protein</b> , specially designed for toxic proteins.  |
| <a href="#"><u>IC-1006</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 C-term His-tagged protein</b> .  |
| <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-tagged protein</b> .   |