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pEco™ -ENTRY, Eco PCR cloning Kit User Manual

Cloning PCR products for making Gateway Entry clone

Cat#	Contents	Amounts	Application
IC-1005	**pEco-ENTRY** vector built-in Eco™ Cloning cells	**10 tubes x 50ul/ea** (for 10 rxn)	Make Gateway Entry clone without using BP clonase.
	Positive PCR insert	1 x 10ul/ea	
	Sequencing primer pair	Forward and reverse 15ul/each, (25ng/ul)	

Storage:

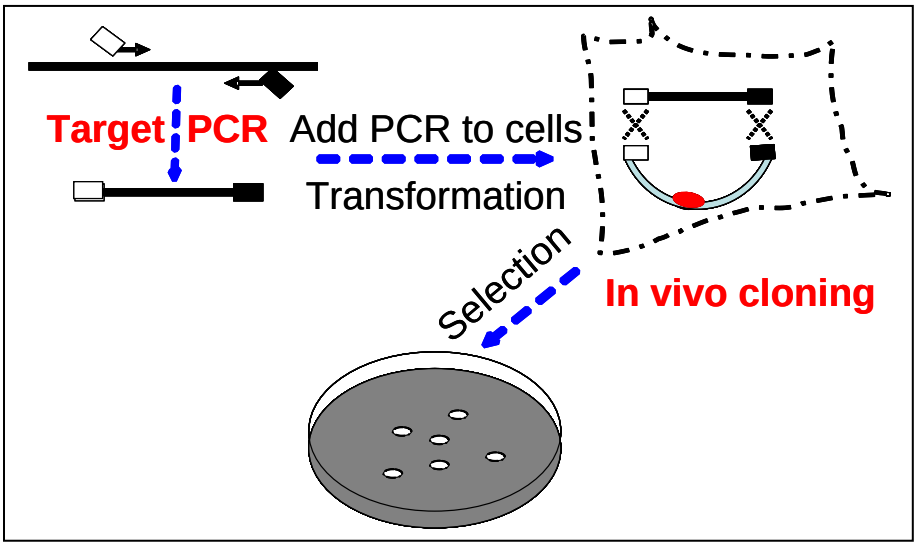
Eco™ Cloning Kit is shipped on dry ice. Upon received, stored at -80°C. Once thawed, must be used, do not re-freeze. Product should be stable for 6 months.

Product Description:

Introduction:

GenTarget's proprietary **fusion *in vivo*** (Patent pending) Eco™ cloning technology is a revolutionized and the easiest PCR cloning method. Simply amplifies your gene of interest with primer pair that flanked with short homologous arm to the expression vector ends, then add 1ul of purified PCR into the engineered, Ready-to-use Cloning cells, and immediately proceed to transformed.

How it works:



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Gentarget's Eco™ PCR Cloning Kit utilizes an engineered E Coli strain with enhanced homologous recombination machinery for an *In Vivo* end-homologous jointing reaction between PCR product and vector. The vector was pre-processed with the cloning cell using a proprietary protocol to obtain high cloning efficiency and low background. It does not need any kinds of *In Vitro* tube reaction, such as ligation, Topo jointing or In-fusion reaction, and so on. **Let the E Coli do the job for you *In Vivo*!**

pEco-ENTRY cloning cells was built-in with a Gateway fully compatible ENTRY clone vector. PCR insert will be cloned to make the ENTRY clone that can be used to make any kinds of Gateway DEST clones via a LR reaction.
(Note: GenTarget provides Eco cloning cells for making DEST clones also without using LR clonase. And the same PCR product is good for make either ENTRY clone or DEST clone.)

Key Features:

1. **The most cost effective and the easiest PCR cloning method**, simply add 1ul of PCR insert into provided cells for transformation regardless of the insert's size and concentration;
2. **No need to buy Gateway vector.** The vector was built-in with cloning cells;
3. **No need to buy cloning competent cells.** The cloning cells is the competent cells;
4. **No need to buy Gateway clonase**. There is no need for any enzymes or any tube reactions;
5. Precisely **directional cloning** of PCR products, making perfect Gateway Entry clones;
6. High efficient (>90% positive rate) and low background;
7. Works fine with any PCR products with or without a 3'-end's -A overhung (the extra –A overhang, if exists, will be removed in cloning step);
8. Good for different PCR sizes, from 200bp to 6 kb.
9. Great for high through-put cloning;

Protocol Outline:

Produce PCR products and clean them

↓

Add 1~2ul of PCR product into provided Cloning cells,
Briefly mixing and immediately proceed to transformation

↓

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

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Detailed protocols:

1. **PCR primer design:**
   - The PCR primers, used for generating inserts for Eco™ Cloning must contains a 20 ~ 25bp homologous sequences 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)

   - Protein cleavage site may be included in forward primer to allow removal 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** for PCR primer design:
     To design the primer pair for the following gene sequence:

     atggcctctgtgaaggaaaatccactctagtccctacctgcatttctcagccttgcttacctgttg
     ccaacattgggccaacccgaattcttcccaatctttatcttggctgccagcgagatgtcctcaac
     aaggagctgatgcagcagaatgggattggttatgtgttaaatgccagcaatacctgtccaaagc
     ctgactttt**tta**

     Its PCR primer for vector **pEco-ENTRY** will be:
     Fwd: 5'- tttgtacaaaaaagcaggcaccatggcctctgtgaaggaaaa
     Rev: 5'- tttgtacaagaaagctgggttaaagtcaggctttggacagg

   - **Note**:
     1. Gentarget's different cloning kits share same PCR Insert. For example, the three Eco™ cloning cells, **Cat# IC-1001, IC-1002 and IC-1003** can use the same PCR to make different expression clones. And other three cloning cells (**Cat# IC-1005, IC-1006 and IC-1007**) can share the same PCR product for making different expression clones.
     2. Stop codon is optional to be included in PCR reverse primer. (**Note**: To express C-term tag protein, do not include a stop codon. So after this ENTR clone is swapped into DEST express vector, the target will be expressed in-frame with C-term tag from that DEST vector.)

2. **Target amplification by PCR**:
   - Using any PCR amplification protocols that work for you to amply your targets. To minimize the PCR errors, we recommend using high fidelity DNA polymerase.
   - Using any PCR purification column to clean your PCR products. If you do not obtain a single, discrete band from your PCR, you need gel-purify your fragment.

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- **Important**: if your PCR template can generate background clones (having Amp resistance), you need treat your PCR product by DPNI or do gel purification of PCR product.

3. **Transformation**:
   - Thaw Eco™ Cloning cells in ice-water. After completely thawed, add 1~2ul purified PCR product (from 20ng to 150ng) into each vial of cells, brief mixing by taping the tube with your finger. For control vials, add 1ul positive PCR-insert (provided) as positive control, and add 1ul water to a a negative control vial cells. Put tubes back on ice, and then proceed for 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 **250u**l of SOC medium, incubated at 37°C, shaking for 1hr.
   - Plating: take **50ul~200ul** aliquot, spread out on pre-warmed LB-agar plates containing 50μg/ml Kanamycin. And grow colonies at 37°C incubator for overnight.
   - **Note:** usually in the absence of PCR-insert, cells force some background colonies; the no-insert negative control generates a few colonies. But in the presence of PCR-insert, greater than 90% colonies are positive. Colony number varies dependent the quality and quantity of PCR products. The concentration of purified PCR product can be from 20ng/ul to 150ng/ul with sizes from 200bp to 10kb. For the simplicity and high through-put cloning purpose, we recommend simply add 1-2ul of PCR into cloning cells regardless of the PCR's concentration and sizes, it will generate enough colonies (5 ~ 100 colonies in general) for downstream works.

4. **Verification of positive clones**:
   - Pick 3-5 colonies, propagate in LB/**Kanamycin**, incubate at 37oC overnight;
   - Isolate the plasmid DNAs using DNA miniprep kit (such as Eco™ Plasmid DNA Miniprep Kit, **Cat# DP-100**).
   - Confirm the positive by restriction digestion:
     PCR inset can be cut out by BsrGI
     Run 1.2% agarose, two bands: 2.55 kb backbone + the PCR insert (or multiple bands when the sites exist within the PCR-insert).
   - Final sequencing verification:
     Use provided sequencing primer pair (**Note**: sequencing primer was provided as ready-to-use dilution, use 1ul for each sequencing reaction with 500ng plasmid in 20ul volume).

Cat #	Vector	Forward primer	Reverse primer
IC-1005	pEco-ENTR	IC-1005-fwd 5'- gtaaaacgacggccag	IC-1005-rev 5'- taatacgactcactataggg

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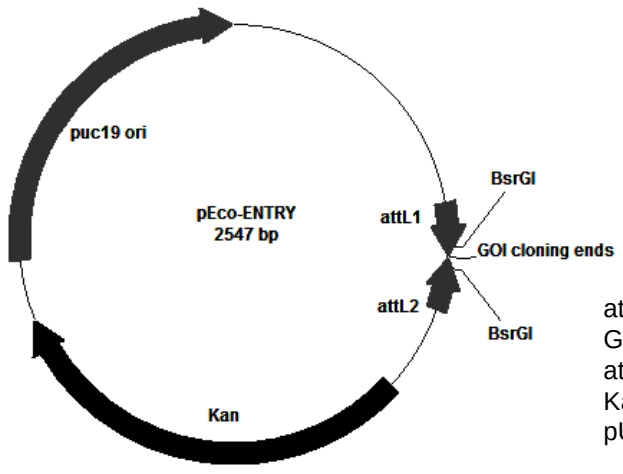
Vector maps:

The figure below summarizes the vector map of pEco-ENTRY. The **complete nucleotide sequence is** available for downloading from our Website at **RESOURCES** page (**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-ENTRY** vector

```
                                                          att L1
 551   AGTCTTAAGC TCGGGCCCCA AATAATGATT TTATTTTGAC TGATAGTGAC
       TCAGAATTCG AGCCCGGGGT TTATTACTAA AATAAAACTG ACTATCACTG

 601   CTGTTCGTTG CAACAAATTG ATGAGCAATG CTTTTTTATA ATGCCAACTT
       GACAAGCAAC GTTGTTTAAC TACTCGTTAC GAAAAAATAT TACGGTTGAA

        BsrGI                  PCR Insert                BsrGI
 651   TGTACAAAAA AGCAGGCACC NNNNNNNNNA ACCCAGCTTT CTTGTACAAA
       ACATGTTTTT TCGTCCGTGG NNNNNNNNNT TGGGTCGAAA GAACATGTTT

                                att L2
       GTTGGCATTA TAAGAAAGCA TTGCTTATCA ATTTGTTGCA ACGAACAGGT
       CAACCGTAAT ATTCTTTCGT AACGAATAGT TAAACAACGT TGCTTGTCCA

       CACTATCAGT CAAAATAAAA TCATTATTTG CCATCCAGCT GATATCCCCT
       GTGATAGTCA GTTTTATTTT AGTAATAAAC GGTAGGTCGA CTATAGGGGA
```

pEco-ENTRY Vector



attL1: 569-668
GOI cloning ends: 670-671
attL2: 771-672
Kanamycin: 941-1750
pUC19 ori: 1871-2544

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Trouble shooting:

Problems	Solution
No colony	✸ Be sure to set up a positive control transformation using provided positive PCR insert1, which should give you 10~100 colonies; ✸ Spread all transformation mixture on plate;
Background colonies	✸ Be sure to set up a background control plate in which no PCR was added into cells, it should generate 0 ~ 5 colonies or less than 10% compared to plates with insert (Noticed: in the absence of PCR insert, cells forces vector self-ligation resulted in a few background colonies). ✸ Make sure that the PCR's template do not cause background colony; If it does, clean PCR products by gel-isolation or treated by DPNI; ✸ Plate less transformation mixture on plate;
Satellite colonies	✸ Be sure to use right amount of antibiotics in LB plate, and make fresh LB plates if necessary; ✸ Do not incubate plates longer than 16 hours; ✸ At colony pick, try to avoid the tiny satellite colonies;

Related Products:

Cat#	Product Name	Amount	Application
DP-100	Eco™ Plasmid DNA Miniprep Kit	100 miniprep	High pure Plamsid DNA isolation
CC03 CC03p	Eco™ E Coli expression Competent Cells	20 rxn/pack	Competent cells for T7 vector protein expression
RM1000	Eco™ Expression RichMedium	1000ml/ea	Auto-induction, High yield protein expression medium
EB-S100 EB-L100	Eco™ Buster E Coli protein extraction reagent	100ml/ea	Protein extraction from cell pellets
IC-1001	PCR cloning kit	kit	PCR cloning kit with a built-in vector (T7 promoter based) in provided cloning cells for E Coli expression of N-term His-tagged protein.
IC-1002	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 mammalian expression of N-term His tagged protein

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IC-1003	PCR cloning kit	kit	PCR cloning kit with a built-in vector (non-T7 promoter based) in provided cloning cells for E Coli expression of N-term His-tagged protein, specially designed for **toxic proteins**.
IC-1004	PCR cloning kit	kit	PCR cloning kit with a built-in vector (T7 promoter based) in provided cloning cells for E Coli expression of N-term GST-tagged protein.
IC-1006	PCR cloning kit	kit	PCR cloning kit with a built-in vector (T7 promoter based) in provided cloning cells, for E Coli expression of C-term His-tagged protein.
IC-1007	PCR cloning kit	kit	PCR cloning kit with a built-in mammalian expression vector (with Neomycin selection marker) in provided cloning cells, for mammalian expression of C-term His-tagged protein.

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