pEco™ -T7-nGST, Eco cloning Kit User Manual

(Patent pending)

Cloning PCR products for E Coli expression of N-term GST-tagged protein

Cat#	Contents	Amounts	Application
IC-1004	**pEco-T7-nGST** vector built-in Eco™ Cloning cells	**10 tubes x 50ul/ea** (for 10 rxn)	E Coli expression of N-term GST-tag protein. .
	Positive PCR insert	1 x 10ul/ea	
	Sequencing primer pair	Forward and reverse 15ul/each, (25ng/ul)	

Storage:

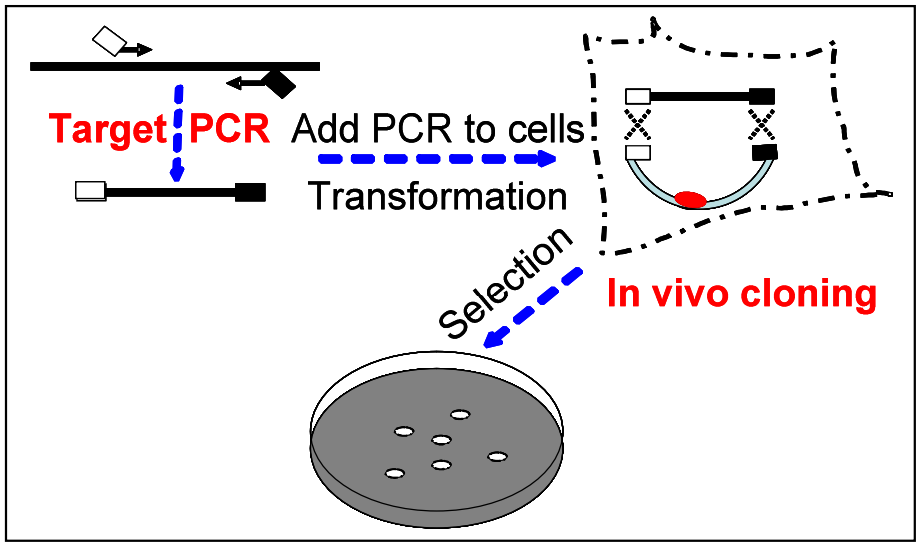
Eco™ Cloning Kit is shipped on dry ice. Upon received, stored at -80°C. Once thawed, must be used, do not re-freeze. Product should be stable for 6 months.

Product Description:

Introduction:

GenTarget's proprietary **fusion *in vivo*** 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-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.

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 expression vectors.** The vector was built-in with cloning cells;
3. **No need to buy competent cells.** The cloning cells is the competent cells;
4. No need for the tedious bench works for preparing vector backbone;
5. No need for any enzymes or any tube reactions;
6. Precisely **directional cloning** of PCR products without any extra amino acids except the affinity tag (His or GST);
7. Flexible to add any cleavage site for removal of N-term His if desired;
8. High efficient (>90% positive rate) and low background;
9. 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);
10. Good for different PCR sizes, from 200bp to 6 kb.
11. Engineered E Coli and mammalian expression vectors for high protein yields;
12. 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 and miniprep plasmids to verify the positive clones

Protein expression from sequencing verified clones

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'- atcggatctggttccgcgtgaattc + 20bp of (5'end gene specific forward sequence)
Rev: 5'- ttgttagcaggttaacacgcgtcta + 20bp of (3'-end gene specific reverse sequence)

   - Protein cleavage site may be included in forward primer to allow excise 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
     ctgactttta

     Its PCR primer for vector **pEco-T7-nGST** will be:
     Fwd: 5'- atcggatctggttccgcgtgaattcatggcctctgtgaaggaaaa
     Rev: 5'- ttgttagcaggttaacacgcgtctaaaagtcaggctttggacagg

     In the case of inserting a protein cleavage site, the Fwd primer will be:
     Fwd: 5' atcggatctggttccgcgtgaattcNNNNNNgcctctgtgaaggaaaatcc
     (where the NNNNNN is the in-framed codon sequence of cleavage site).

   - **Note**: Stop codon is optional to be included in PCR reverse primer since a stop codon is already built in immediately after the PCR insert.

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.
   - **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 containing100μg/**ml ampicillin**. 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. **Save glycerol stocks for later expression and verification of positive clones**:
   - Pick 2-5 colonies, propagate in LB/Amp, 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 two unique sites: EcoRI + HpaI
     Run 1.2% agarose, two bands: 3.4 kb backbone + the PCR insert (or multiple bands when the cut 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-1004	pEco-T7-nGST	IC-1004-fwd 5'- catggcctttgcagggct	IC-1004-rev 5'- tgctagttattgctcagcgg

5. **Protein expression**:
   - **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 37^{o}C 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 30^{o}C, 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 30^{o}C, 300rpm; [Note: for best expression results, use Gentarget's auto-induction medium, Eco^{TM} RichMedium **(**Cat# **RM1000)** for propagation, it does not need to add IPTG for induction].
- **Harves**t cells by centrifugation.
- **QC**: Cell pellet was lysed using lysis reagent, [**Note**: we recommend use Gentarget's Eco^{TM} Buster protein extract reagents (**Cat# EB-S100** or **EB-L100**)]. 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.

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 **RESOURCES** page 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".

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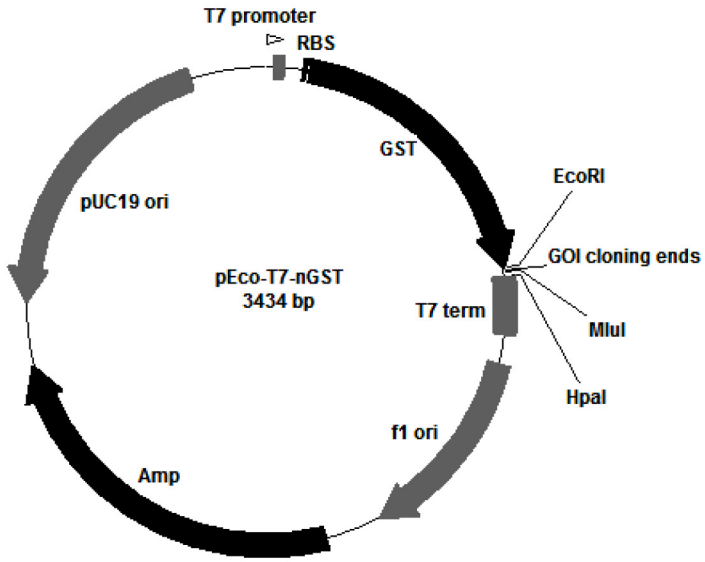
   Cloning site for pEco-T7-nGST vector

                                    EcoRI
                    GST                        PCR insert        MluI   HpaI
751     AAATCGGATC TGGTTCCGCG TGAATTCNNN NNNNNNTAGA CGCGTGTTAA
        TTTAGCCTAG ACCAAGGCGC ACTTAAGNNN NNNNNNATCT GCGCACAATT

        HpaI
        CCTGCTAACA AAGCCCGAAA GGAAGCTGAG TTGGCTGCTG CCACCGCTGA
        GGACGATTGT TTCGGGCTTT CCTTCGACTC AACCGACGAC GGTGGCGACT
```

Map of pEco-T7-nGST vector



T7 Promoter: 20-39
RBS: 87-92
GST: 100-771
GOI cloning ends: 777-778
T7 term: 793-922
F1 ori: 993-1448
Ampicillin:1579-2439
pUC19 ori: 3257-2584

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; ✸ Use carbenicillin instead of ampicillin if applicable; ✸ 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
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 C-term His-tagged protein, specially designed for toxic proteins.
IC-1005	PCR cloning kit	kit	PCR cloning kit with a built-in Gateway Entry vector in provided cloning cells for making your target in Gateway Entry clone without using BP clonase
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