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pEco™ -BAD-nHis, PCR cloning Kit User Manual

(Patent pending)

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

Cat#	Contents	Amounts	Application
IC-1003	**pEco-BAD-nHis** vector built-in Eco™ Cloning cells	**10 tubes x 50ul/ea** (for 10 rxn)	E Coli expression of N-term His-tag protein, best for toxic protein.
	Positive PCR insert	1 x 10ul/ea	
	20% L-arabinose	1ml	
	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 1µl 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%.

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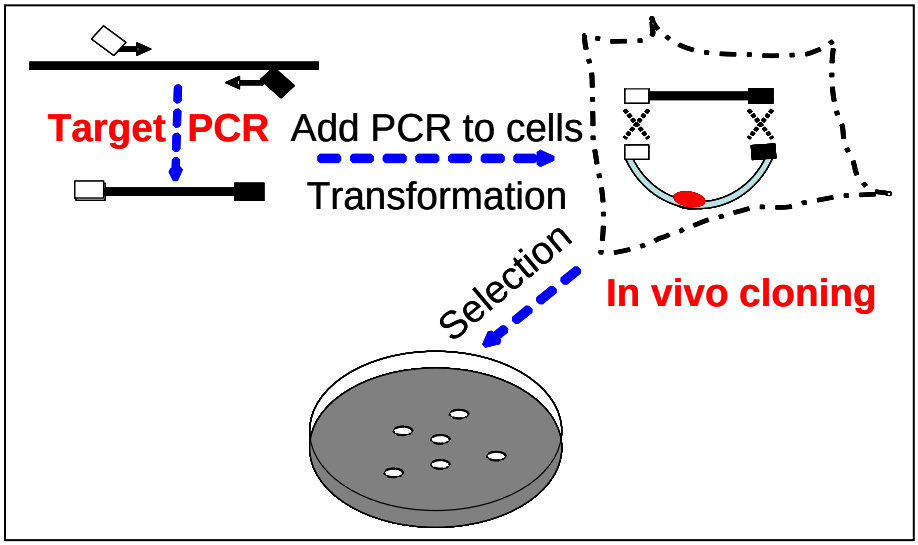

pEco-BAD-nHis cloning cells has a built-in non-T7 based expression vector with an E Coli araBAD promoter. The promoter is tightly inhibited by an AraC regulatory protein expressed from the vector backbone. This basal level self-control mechanism is ideal for the expression of toxic proteins, avoiding the difficulties associated with direct cloning and expression. Target expression is induced by L-arabinose which releases the AraC regulatory protein, and the expression level can be optimized and protein solubility enhanced by varying the concentration of L-arabinose. The PCR insert is cloned in-frame with an N-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
- Precise **directional cloning** of PCR products without any extra amino acids except the affinity tag (N-His)
- Flexibility to allow addition of any cleavage site for removal of N-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)

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- 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* and mammalian 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, immediately proceed to transformation

↓

Pick colonies, 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™ Cloning must contain a 20 - 25bp homologous sequence corresponding to the built-in vector. Design your primer pair as follows:

Fwd: 5'- catggcgcatcaccatcatcatcat + 20bp of (5'end gene specific forward sequence)
Rev: 5'- ttgttagcaggttaacacgcgtcta + 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:

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atggcctctgtgaaggaaaatccactctagtccctacctgcatttctcagccttgct
tacctgttgccaacattgggccaacccgaattcttcccaatctttatcttggctgcca
gcgagatgtcctcaacaaggagctgatgcagcagaatgggattggttatgtgtta
aatgccagcaatacctgtccaaagcctgactttta

The PCR primer for vector **pEco-BAD-nHis** will be:

Fwd: 5'- catggcgcatcaccatcatcatcatatggcctctgtgaaggaaaa
Rev: 5'- ttgttagcaggttaacacgcgtctaaaagtcaggctttggacagg

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

Fwd: 5' catgcatcatcaccatcatcatcatNNNNNNgcctctgtgaaggaaaatcc
(Where the NNNNNN is the in-frame codon sequence of the cleavage site).

- **Notes**:
  1. GenTarget's cloning kits with the same terminal tags share PCR insert sites. The three Eco™ 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**:

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- Thaw Eco™ Cloning cells in ice-water.
- After they are completely thawed, add 1-2 µl purified PCR product (from 50ng 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 **250 µl** aliquot, 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 positives by restriction digestion:
     i. The PCR insert can be cut out at two unique sites: EcoRI + HpaI
     ii. Run a 1.2% agarose gel. You should see two bands: 4 kb backbone + the PCR insert (or multiple bands when the cuts 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.

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Cat #	Vector	Forward primer	Reverse primer
IC-1003	pEco-BAD-nHis	IC-1003-fwd 5'- gaaataattttgtttaactt	IC-1003-rev 5'- gatttaatctgtatcagg

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

- Add 10 µl of your positive clones in 4ml of LB medium with 100µg/ml ampicillin, grow at 37 °C overnight, shaking (225–250 rpm).
- The next day, measured OD should be approximately 1-2. Inoculate a large volume by making a 1:100 dilution of overnight culture in LB or SOB medium containing 100 µg/ml ampicillin. Grow the cultures at 37 °C with shaking to an OD600 = ~0.5.
- Induce expression by adding L-arabinose in a range of final concentrations (from 0.2% to 0.00005%) to find the concentration for optimal expression.
- Remove a 1 ml aliquot of cells from each tube for analysis of protein expression. Be sure to save aliquots of uninduced control samples.
- Analyze protein expression by SDS-PAGE or other methods.
- **Harves**t cells by centrifugation.
- Lyse the cell pellet using lysis reagent. Following lysis, run a gel protein analysis.
- **Purification**: use your favorite protocol and reagent to purify the expressed His-tagged protein by affinity chromatography.

6. Vector maps:

The figure below summarizes the vector map of pEco-BAD-nHis. The **complete nucleotide sequence is** available for download from our Support page (http://www.gentarget.com/support/vector-sequences/). 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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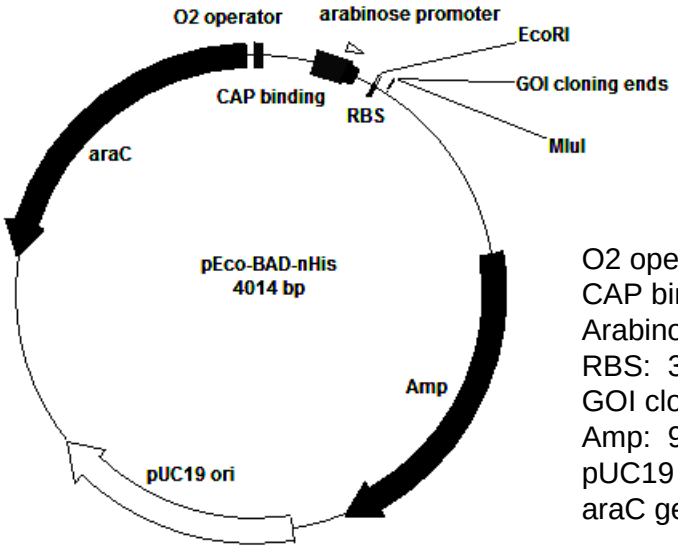
Cloning site for pEco-BAD-nHis vector

```
                                                EcoRI
                                                ~~~~~~
301   CTAGAAATAA TTTTGTTTAA CTTTAAGAAG GAGAATTCAC CATGGCG CAT
      GATCTTTATT AAAACAAATT GAAATTCTTC CTCTTAAGTG GTACCGC GTA
                                                      HpaI
        His-tag              PCR insert               ~~~~~~
351   CAC CAT CAT CAT CATNNNNNN NNNNTAGACG CGTGTTAACC TGCTAACAAG
      GTG GTA GTA GTA GTANNNNNN NNNNATCTGC GCACAATTGG ACGATTGTTC
```

Map of pEco-BAD-nHis vector



O2 operator: 4-19
CAP binding with I1/I2: 161-251
Arabinose promoter: 248-276
RBS: 329-334
GOI cloning ends: 365-366
Amp: 901-1761
pUC19 pro: 1906-2579
araC gene: 3988-3110

7. Troubleshooting:

Problems	Solution
No colony	✸ Be sure to set up a positive control transformation using the provided positive PCR insert1, which should give you 10~100 colonies. ✸ Spread all of the transformation mixture onto the plate.

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Background colonies	✸ 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). ✸ 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. ✸ Plate less transformation mixture onto the plate.
Satellite colonies	✸ Be sure to use the right amount of antibiotics in the LB plate, and make fresh LB plates if necessary. ✸ Use carbenicillin instead of ampicillin if applicable. ✸ Do not incubate plates longer than 16 hours. ✸ Try to avoid picking the tiny satellite colonies.

Related Products:

Cat#	Product Name	Amount	Application
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-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