# *pEASY*®-T3 Cloning Kit

Please read the user manual carefully before use.

Cat. No. CT301

## Storage

*Trans*1-T1 Phage Resistant Chemically Competent Cell at -70°C for six months; others at -20°C for nine months

## Descriptions

*pEASY*®- T3 Cloning Vector provides dual *Eco*R I and dual *Not* I enzyme sites. It is designed for cloning and sequencing *Taq*-amplified PCR products. The cloned insert can be released from a single enzyme digestion.

- 5 minutes fast ligation of *Taq*-amplified PCR products.
- Ampicillin resistance gene for selection.
- Easy blue/white selection.
- T7 promoter, SP6 promoter, M13 forward and M13 reverse primers for sequencing.
- T7 promoter and SP6 promoter for *in vitro* transcription.
- *Trans*1-T1 Phage Resistant Chemically Competent Cell, high transformation efficiency (>$10^9$ cfu/μg pUC19 DNA) and fast growing.

## Kit Contents

| Component | CT301-01 (20 rxns) | CT301-02 (60 rxns) |
|---|---|---|
| *pEASY*®-T3 Cloning Vector (10 ng/μl) | 20 μl | 3×20 μl |
| Control Template (5 ng/μl) | 5 μl | 5 μl |
| Control Primers (10 μM) | 5 μl | 5 μl |
| M13 Forward Primer (10 μM) | 50 μl | 150 μl |
| M13 Reverse Primer (10 μM) | 50 μl | 150 μl |
| *Trans*1-T1 Phage Resistant Chemically Competent Cell | 10×100 μl | 30×100 μl |

## Preparation of PCR Products

1. Primer requirement: primer cannot be phosphorylated
2. PCR Enzyme: *Taq* DNA polymerases
3. Reaction conditions: in order to ensure the integrity of amplification products, 5-10 minutes of post-extension step is required. After amplification reaction, use agarose gel electrophoresis to verify the quality and quantity of PCR product

## Setting Up the Cloning Reaction System

Add following components into a microcentrifuge tube.

PCR products 0.5-4 μl (can be increased or reduced based on PCR product yield, not more than 4 μl)

*pEASY*®- T3 Cloning Vector 1 μl

Gently mix well, incubate at room temperature (20°C-37°C) for 5 minutes, and then place the tube on the ice.

1. Optimal amount of insert
   Molar ratio of vector to insert = 1:7 (1 kb, ~20 ng; 2 kb, ~40 ng)
2. Optimal volume of vector: 1 μl (10 ng)
3. Optimal reaction volume: 3~5 μl
4. Optimal incubation time

(1) 0.1~1 kb (including 1 kb): 5~10 minutes
(2) 1~2 kb (including 2 kb): 10~15 minutes
(3) 2~3 kb (including 3 kb): 15~20 minutes
(4) ≥3 kb: 20~30 minutes

Use the maximum incubation time if the insert is gel purified.

5. Optimal incubation temperature: for most PCR inserts, the optimal temperature is about 25°C; for some PCR inserts, optimal results can be achieved with higher temperature (up to 37°C).

### Transformation

1. Add the ligated products to 50 µl of *Trans*1-T1 Phage Resistant Chemically Competent Cell and mix gently (do not mix by pipetting up and down).
2. Incubate on ice for 20~30 minutes.
3. Heat-shock the cells at 42°C for 30 seconds.
4. Immediately place the tube on ice for 2 minutes.
5. Add 250 µl of room temperature SOC or LB medium. Shake the tube at 37°C (200 rpm) for 1 hour.
6. In the meantime, mix 8 µl of 500 mM IPTG with 40 µl of 20 mg/ml X-gal. Spread them evenly onto a selective LB plate. Place the plate at 37°C for 30 minutes.
7. Spread 200 µl or all transformants on the pre-warmed plate. Incubate at 37°C overnight.

### Identification of Positive Clones and Sequencing

#### Analysis of positive clones

1. Transfer 5~10 white or light blue colonies into 10 µl Nuclease-free Water and vortex.
2. Use 1 µl of the mixture as template for 25 µl PCR using M13 forward and M13 reverse primers.
3. PCR reaction conditions

| | | |
|---|---|---|
| 94°C | 10 min | |
| 94°C | 30 sec | 30 cycles |
| 55°C | 30 sec | |
| 72°C | x min* | |
| 72°C | 5-10 min | |

* (depends on the insert size and PCR enzymes) the PCR product size from vector self-ligation is 253 bp.

4. Analyze positive clones by restriction enzyme digestion and DNA sequencing.
   Inoculate positive clones on LB/Amp$^+$ or LB/Kan$^+$ liquid medium, grow at 37°C for 6 hours at 200 rpm. Isolate plasmid DNA by plasmid MiniPrep Kit. Analyze colonies by restriction enzyme digestion with proper restriction endonuclease.

#### Sequencing

Analyze the sequence by sequencing with M13 F, M13 R and T7 promoter.

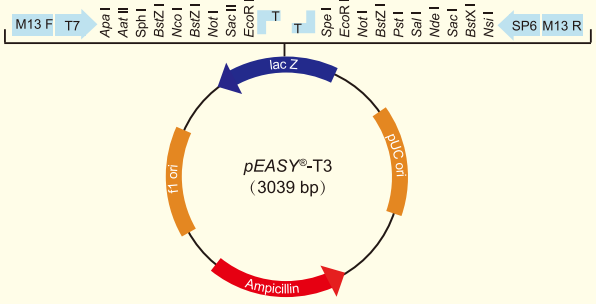
### PCR for control insert (700 bp)

| Component | Volume | Final Concentration |
|---|---|---|
| Control Template (5 ng/µl) | 1 µl | 0.1 ng/µl |
| Control Primers (10 µM) | 1 µl | 0.2 µM |
| 2×*EasyTaq*® PCR SuperMix | 25 µl | 1× |
| Nuclease-free Water | Variable | - |
| Total volume | 50 µl | - |

### Thermal cycling conditions for control insert

| | | |
|---|---|---|
| 94°C | 2-5 min | |
| 94°C | 30 sec | 30 cycles |
| 55°C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 10 min | |

Ligate 1 µl of control PCR insert with 1 µl vector. Hundreds of colonies should be produced with cloning efficiency over 90%.




*Lac* operon sequence: bases 2,860-3,020, 190-419
Multiple cloning site: bases 10-152
SP6 priming site: bases 163-182
M13 reverse priming site: bases 200-216
*LacZ* start codon: base 204
*Lac* operator: bases 224-240
pUC origin: bases 543-1,216
Ampicillin resistance ORF (c): bases 1,361-2,221
f1 origin: bases 2,421-2,858
M13 forward priming site: bases 3,000-3,016
T7 promoter priming site: bases 3,023-3
(c) = complementary strand

M13 Forward Primer
GTA AAA CGA CGG CCA GT
CAT TTT GCT GCC GGTCA

T7 Promoter
…TGT AAT ACG ACT CAC TAT AGG GCG AAT TGG GCC CGA CGT CGC ATG CTC CCG GCC GCC ATG GCG GCC GCG G
…ACA TTA TGC TGA GTG ATA TCC CGC TTA ACC CGG GCT GCA GCG TAC GAG GGC CGG CGG TAC CGC CGG CGC C

*Apa* I *Aat* II *Sph* I *BstZ* I *Nco* I *BstZ* I *Not* I *Sac* II

*Eco*R I

GAA TTC GAT TGG ATC GCC CTT PCR Product AAG GGC GAT CCC AAT CAC TAG TGA ATT CGC GGC CGC
CTT AAG CTA ACC TAG CGG GAA TTC CCG CTA GGG TTA GTG ATC ACT TAA GCG CCG GCG

*Spe* I *Eco*R I *BstZ* I *Not* I

*Pst* I *Sal* I *Nde* I *Sac* I *BstX* I *Nsi* I

CTG CAG GTC GAC CAT ATG GGA GAG CTC CCA ACG CGT TGG ATG CAT AGC TTG AGT ATT CTA TAG TGT CAC CTA AAT…
GAC GTC CAG CTG GTA TAC CCT CTC GAG GGT TGC GCA ACC TAC GTA TCG AAC TCA TAA GAT ATC ACA GTG GAT TTA…

SP6 Promoter

M13 Reverse Primer
GTC ATA GCT GTT TCC TG
CAG TAT CGA CAA AGG AC

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