

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/ μ g pUC19 DNA) and fast growing.

Kit Contents

Component	CT301-01 (20 rxns)	CT301-02 (60 rxns)
<i>pEASY</i> [®] -T3 Cloning Vector (10 ng/ μ l)	20 μ l	3×20 μ l
Control Template (5 ng/ μ l)	5 μ l	5 μ l
Control Primers (10 μ M)	5 μ l	5 μ l
M13 Forward Primer (10 μ M)	50 μ l	150 μ l
M13 Reverse Primer (10 μ M)	50 μ l	150 μ l
<i>Trans</i> 1-T1 Phage Resistant Chemically Competent Cell	10×100 μ l	30×100 μ l

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 μ l (can be increased or reduced based on PCR product yield, not more than 4 μ l)

pEASY[®]- T3 Cloning Vector 1 μ l

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 μ l (10 ng)

3. Optimal reaction volume: 3~5 μ l

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) $\geq$ 3 kb: 20~30 minutes

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



- 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

- Add the ligated products to 50 µl of *Trans1*-T1 Phage Resistant Chemically Competent Cell and mix gently (do not mix by pipetting up and down).
- Incubate on ice for 20~30 minutes.
- Heat-shock the cells at 42°C for 30 seconds.
- Immediately place the tube on ice for 2 minutes.
- Add 250 µl of room temperature SOC or LB medium. Shake the tube at 37°C (200 rpm) for 1 hour.
- 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.
- 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

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

94°C	10 min	} 30 cycles
94°C	30 sec	
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.

- 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)

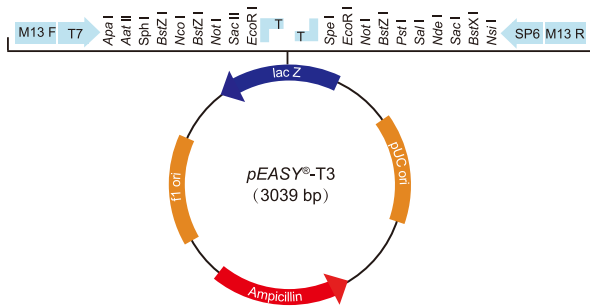
Component	Volume	Final Concentration
Control Template (5 ng/µl)	1 µl	0.1 ng/µl
Control Primers (10 µM)	1 µl	0.2 µM
2× <i>EasyTaq</i> [®] PCR SuperMix	25 µl	1×
Nuclease-free Water	Variable	-
Total volume	50 µl	-

Thermal cycling conditions for control insert

94°C	2-5 min	} 30 cycles
94°C	30 sec	
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

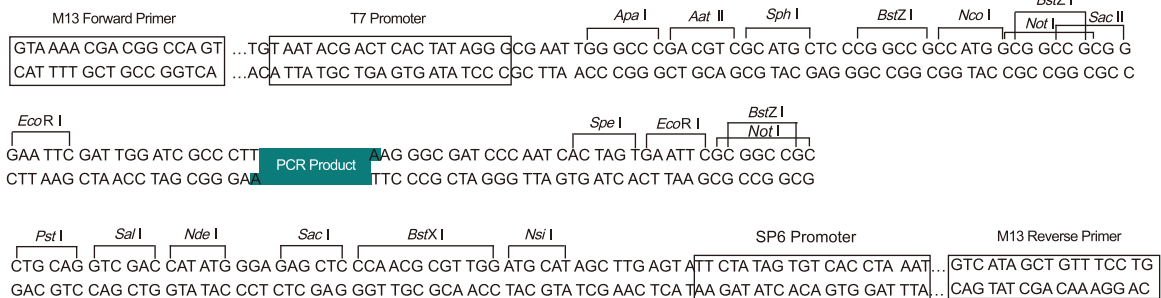
Ampicillin resistance ORF (c): bases 1,361-2,221

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



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