pEASY®-Blunt3 Cloning Kit

Please read the user manual carefully before use.

Cat. No. CB301

Storage

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

Descriptions

pEASY ®- Blunt3 Cloning Vector provides dual *Eco*R I and dual *Not* I enzyme digestion sites. It is designed for cloning and sequencing *Pfu*-amplified PCR products. The cloned insert can be released from a single enzyme digestion.

- 5 minutes fast ligation of *Pfu*-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	CB301-01 (20 rxns)	CB301-02 (60 rxns)
pEASY ®-Blunt3 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: *Pfu* 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 ®- Blunt3 Cloning Vector 1 μl

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

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 254 bp.

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

Sequencing

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

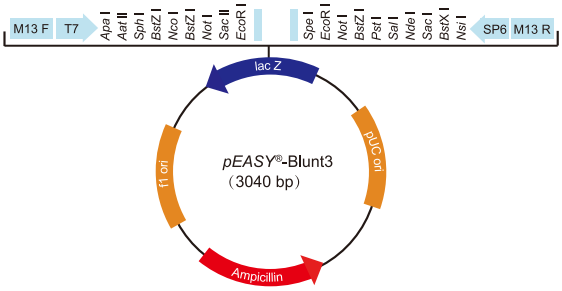
PCR for control insert (700 bp)

Component	Volume	Final Concentration
Control Template	1 µl	0.1 ng/µl
Control Forward Primer (10 µM)	1 µl	0.2 µM
Control Reverse Primer (10 µM)	1 µl	0.2 µM
2×*EasyPfu* PCR SuperMix	25 µl	1×
Nuclease-free Water	Variable	-
Total Volume	50 µl	-

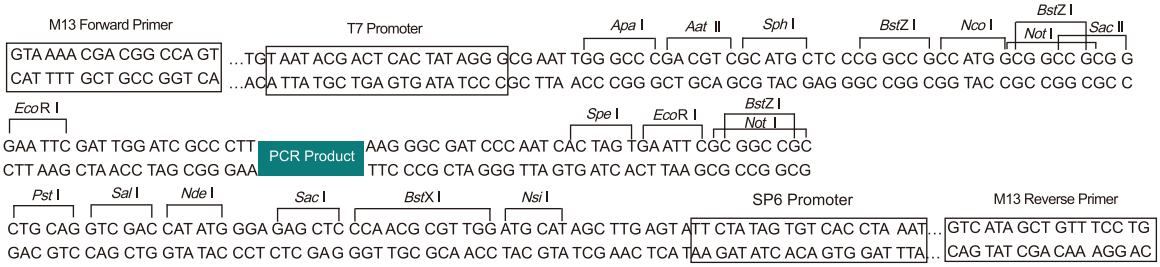
Thermal cycling conditions for control insert

94℃	10 min	
94℃	30 sec	30 cycles
55℃	30 sec	
72℃	1 min	
72℃	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 2,861-3,021, 191-420
Multiple cloning site: bases 10-153
SP6 priming site: bases 164-183
M13 reverse priming site: bases 201-217
*Lac*Z start codon: base 205
Lac operator: bases 225-241
pUC origin: bases 544-1,217
Ampicillin resistance ORF (c): bases 1,362-2,222
f1 origin: bases 2,422-2,859
M13 forward priming site: bases 3,001-3,017
T7 promoter priming site: bases 3,024-3
(c) = complementary strand



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