pEASY®-Blunt Simple Cloning Kit

Please read the user manual carefully before use.

Cat. No. CB111

Storage

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

Descriptions

pEASY ®-Blunt Simple Cloning Vector eliminates the multi-cloning sites of *pEASY* ® -Blunt Cloning Vector. It is designed for cloning and sequencing *Pfu*-amplified PCR products.

- 5 mimutes fast ligation of *Pfu*-amplified PCR products.
- Kanamycin and Ampicillin resistance genes for selection.
- Easy blue/white selection.
- SR primer and M13 forward primer for sequencing.
- T7 promoter for *in vitro* transcription.
- *Trans*1-T1 Phage Resistant Chemically Competent Cells, high transformation efficiency (>10^9 cfu/μg pUC19 DNA) and fast growing.

Kit Contents

Component	CB111-01 (20 rxns)	CB111-02 (60 rxns)
pEASY ®-Blunt Simple 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
SR 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 yeild, no more than 4 μl)

pEASY ®- Blunt Simple Cloning Vector 1 μl

Gently mix well, incubate at room temperature (20°C-37°C) for 5 minutes. After reaction, 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 101 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.

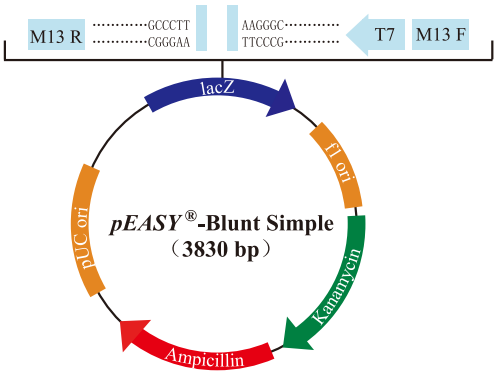
PCR for control insert (700 bp)

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×*EasyPfu* PCR SuperMix	25 µl	1×
Nuclease-free Water	Variable	-
Total volume	50 µl	-

Thermal cycling conditions for control insert

94℃	2~5 min	
94℃	30 sec	30 cycles
50~60℃	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%.



LacZα fragment: bases 1-446
M13 reverse priming site: bases 205-221
T7 promoter priming site: bases 263-282
M13 forward priming site: bases 289-305
f1 origin: bases 447-884
Kanamycin resistance ORF: bases 1,218-2,012
Ampicillin resistance ORF: bases 2,030-2,890
pUC origin: bases 3,035-3,708

SR Primer

CAG GCT TTA CAC TTT ATG CTT CCG GCT CGT ATG TTG TGT GGA ATT GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC TAT GAC CAT GAT TAC GCC AAG CTG
GTC CGA AAT GTG AAA TAC GAA GGC CGA GCA TAC AAC ACA CCT TAA CAC TCG CCT ATT GTT AAA GTG TGT CCT TTG TCG ATA CTG GTA CTA ATG CGG TTC GAC

M13 Reverse Primer

T7 Promoter

M13 Forward Primer

CCC TT PCR Product AA GGG CAG CTT CAA TTC GCC CTA TAG TGA GTC GTA TTAC AA TTC ACT GGC CGT CGT TTT AC A ACG TCG TGA CTG GGA AAA C
GGG AA TT CCC GTC GAA GTT AAG CGG GAT ATC ACT CAG CAT AAT G TT AAG TGA CCG GCA GCA AAA TG T TGC AGC ACT GAC CCT TTT G

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