pEASY®-Blunt Cloning Kit

Please read the user manual carefully before use.

Cat. No. CB101

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 Cloning Kit is designed for cloning and sequencing *Pfu*-amplified PCR products.

- 5 minutes fast ligation of *Pfu*-amplified PCR products.
- Kanamycin and Ampicillin resistance genes for selection.
- Easy blue/white selection.
- T7 promoter, M13 forward and M13 reverse primers for sequencing.
- T7 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	CB101-01 (20 rxns)	CB101-02 (60 rxns)
pEASY®- Blunt 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, 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®- Blunt Cloning Vector 1 μl

Gently mix and incubate the mixture 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
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 200 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 plasmids by restriction enzyme digestion with proper restriction endonuclease.

Sequencing

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

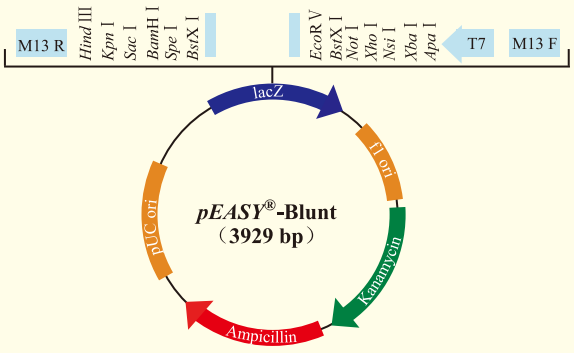
PCR for control insert (700 bp)

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	-
to final 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 1-545
Multiple cloning site: bases 234-355
M13 reverse priming site: bases 205-221
T7 promoter priming site: bases 362-381
M13 forward priming site: bases 388-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1,317-2,111
Ampicillin resistance ORF: bases 2,129-2,989
pUC origin: bases 3,134-3,807

M13 Reverse Primer

Hind III *Kpn* I *Sac* I *BamH* I *Spe* I *BstX* I

CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC GCC AGT GTG CTG GAA TTG
GTC CTT TGT CGA TAC TGG TAC TAA TGC TGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG CGG TCA CAC GAC CTT AAC

EcoR V *BstX* I *Not* I *Xho* I *Nsi* I *Xba* I *Apa* I

CCC TT PCR Product AA GGG CAA TTC TGC AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT
GGG AA TT CCC GTT AAG ACG TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA

T7 Promoter

TCG CCC TAT AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC
AGC GGG ATA TCA CTC AGC ATA ATG TTA AGT GAC CTG CAG CAA AAT GTT GCA GCA CTG ACC CTT TTG

M13 Forward Primer

FOR RESEARCH USE ONLY