



#### 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 maximal incubation time if the insert is gel purified PCR product.

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  $\mu$ l of *Trans1*-T1 phage resistant chemically competent cells 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  $\mu$ l of room temperature SOC or LB medium. Shake the tube at 37°C (200 rpm) for 1 hour.
6. Pre-warm a selective LB plate at 37°C for 30 minutes.
7. Spread 200  $\mu$ l or all transformant on the pre-warmed plate. Incubate overnight at 37°C.

#### Analysis of positive clones

1. Transfer 5~10 colonies into 10  $\mu$ l Nuclease-free Water.
2. Use 1  $\mu$ l of the mixture as template for 25  $\mu$ l PCR using T7 promoter primer and gene reverse primer, or gene forward primer and T7 terminator primer.
3. PCR

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)
4. Analyze plasmids by restriction enzyme digestion or DNA sequencing.

#### Target gene expression

1. Competent cell  
BL21(DE3) competent cell series are suitable for prokaryotic protein expression.
2. Protein expression
  - Pick single colony and transfer into 5 ml of LB/Amp<sup>+</sup> medium and shake at 37°C (250 rpm) until OD<sub>600</sub> close to 0.5.
  - Add IPTG to a final concentration of 0.5-1 mM and shake at 37°C for 3-5 hours.
  - For optimal result, try different IPTG concentration and inducing time.
3. Check expression  
Aspirate the supernatant and use the pellets for SDS-PAGE.

#### Protein Purification

Refer to the section of *ProteinIso*<sup>®</sup> Ni-NTA Resin in user manual.

#### Notes

- The amount of PCR products used for cloning can be adjusted based on the yield and size of the product.
- Do not use more than 30 minutes for cloning reaction.



- Volume of the whole reaction system should not be more than 5  $\mu$ l.
- Cloning efficiency declines with the increase in the size of cloned fragment (>3 kb).
- PCR purification kit is recommended for use if primer dimmers formed. Gel purification is recommended if multibands are present.

### Troubleshooting

#### (1) Low cloning efficiency

Various factors can affect cloning efficiency, such as primers used in target gene amplification, ratio of insert fragment to vector, etc. If low cloning efficiency is observed, try to use following methods to increase cloning efficiency.

- Purify the PCR product
- Increase the volume of inserts for low concentration of inserts
- Use fresh PCR products

#### (2) Unsuccessful identification of transformants by PCR

When PCR is used for identifying transformants, neither target amplification product nor self-ligation of vector is obtained, it indicates a failure in PCR reaction. Re-optimize PCR reaction condition or extract plasmids, then perform amplification with plasmid as template or identify transformants-containing clones by restriction enzyme digestion.

### PCR for control insert (750 bp)

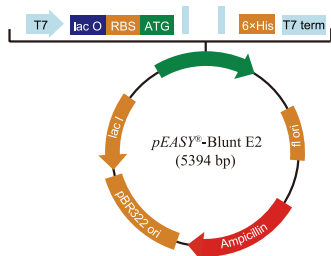
Component	Volume	Final Concentration
EControl Template (5 ng/ $\mu$ l)	1 $\mu$ l	0.1 ng/ $\mu$ l
EControl Forward Primer (10 $\mu$ M)	1 $\mu$ l	0.2 $\mu$ M
EControl Reverse Primer (10 $\mu$ M)	1 $\mu$ l	0.2 $\mu$ M
2 $\times$ TransStart <sup>®</sup> FastPfu PCR SuperMix	25 $\mu$ l	1 $\times$
Nuclease-free Water	Variable	-
Total Volume	50 $\mu$ l	-

### PCR

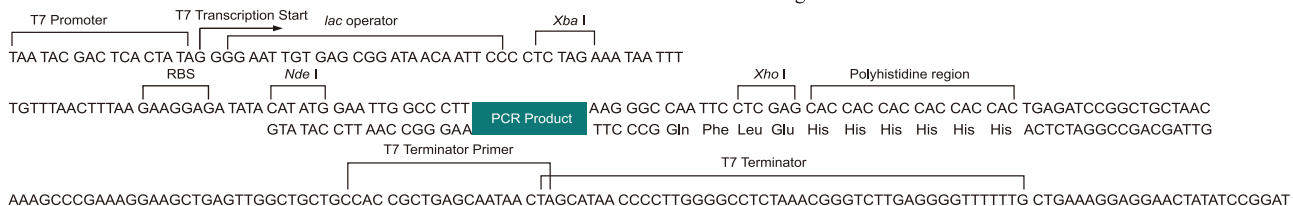
94 $^{\circ}$ C 10 min  
 94 $^{\circ}$ C 30 sec  
 55 $^{\circ}$ C 30 sec  
 72 $^{\circ}$ C 1 min  
 72 $^{\circ}$ C 10 min

} 30 cycles

### pEASY<sup>®</sup>-Blunt E2 Prokaryotic Expression Vector Map



T7 promoter: bases 5,117-5,133  
 T7 transcription start: bases 5,134  
 Lac operator(lacO): bases 5,136-5,160  
 RBS: bases 5,190-5,196  
 His-Tag coding sequence: bases 5,238-5,255  
 T7 terminator: bases 5,323-5,369  
 ROP ORF: bases 2,648-2,839  
 LacI ORF: bases 3,651-4,739  
 pBR origin: bases 1,614-2,233  
 Ampicillin resistance ORF: bases 599-1,459  
 f1 origin: bases 13-450



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