pEASY®-Uni Seamless Cloning and Assembly Kit

Cat. No. CU101

Version No. Version 1.1

Storage: *Trans*1-T1 Phage Resistant Chemically Competent Cells at -70°C for six months; others at -20°C for one year.

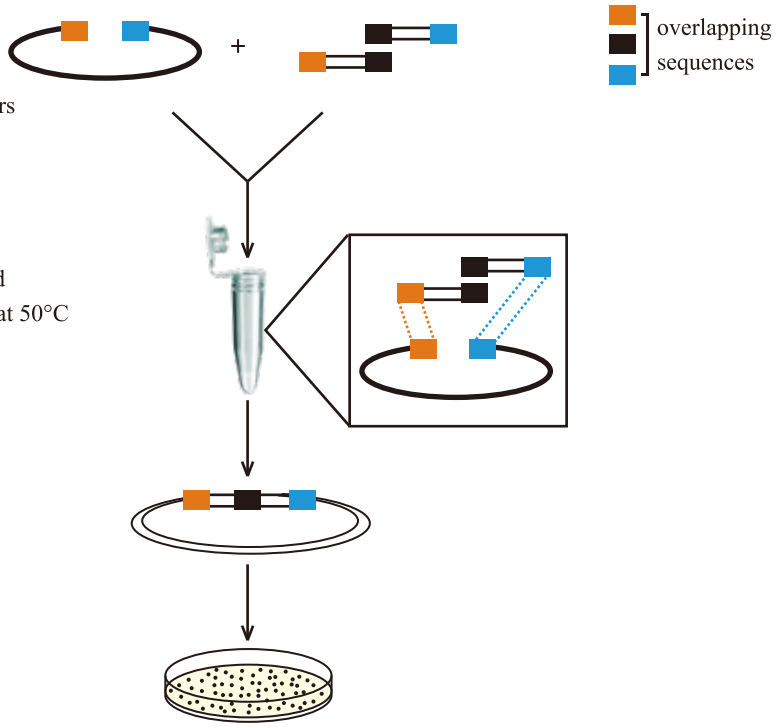
Description

This kit takes advantage of proprietary recombinases and homologous recombination to achieve directional recombination of PCR inserts with 15-25bp overlapping ends into any linearized vector, enabling efficient and seamless assembly of 1-7 fragments.

- Fast: 5~15 minutes.
- Simple: No restriction enzyme digestions.
- High efficiency: > 95% cloning efficiency.
- Seamless: No extra sequences introduced.

Principle

1. Prepare linearized vector by PCR/Enzyme digestion
2. PCR amplify inserts with primers containing overlapping sequences
3. Mix vector, DNA fragments and Assembly Mix together. Incubate at 50°C for 5~15 minutes
4. Transformation



Applications

Single or multi fragments cloning and assembly.

Kit Contents

Component	CU101-01	CU101-02	CU101-03
2×Assembly Mix	50 µl	100 µl	3×100 µl
Linearized pUC19 Control Vector (10 ng/µl)	3 µl	6 µl	18 µl
Control Insert (1 kb，20 ng/µl)	3 µl	6 µl	18 µl
*Trans*1-T1 Phage Resistant Chemically Competent Cell	5×100 µl	10×100 µl	30×100 µl

Cloning

Preparation of Vector and Inserts

A: Preparation of Vector

(1) Enzyme digestion: Digest plasmid vector with restriction enzyme(s) to generate the linearized vector with blunt ends or sticky ends. Purify the digested vector using Gel Extraction Kit (Cat. No. EG101).

(2) PCR amplification: Prepare the linearized vector by Pfu series high-fidelity DNA polymerase. If a single expected band is generated, use PCR Purification Kit (Cat. No. EP101) to purify the product. Otherwise, use Gel Extraction Kit to recover the product.

In order to increase the positive cloning efficiency, we suggest using DMT enzyme to digest plasmid template before PCR purification or gel extraction. Add DMT enzyme (DpnI) (Cat. No. GD111) after PCR amplification (1 μl of DMT enzyme for a 50 μl PCR system), and incubate at 37°C for 30 minutes.

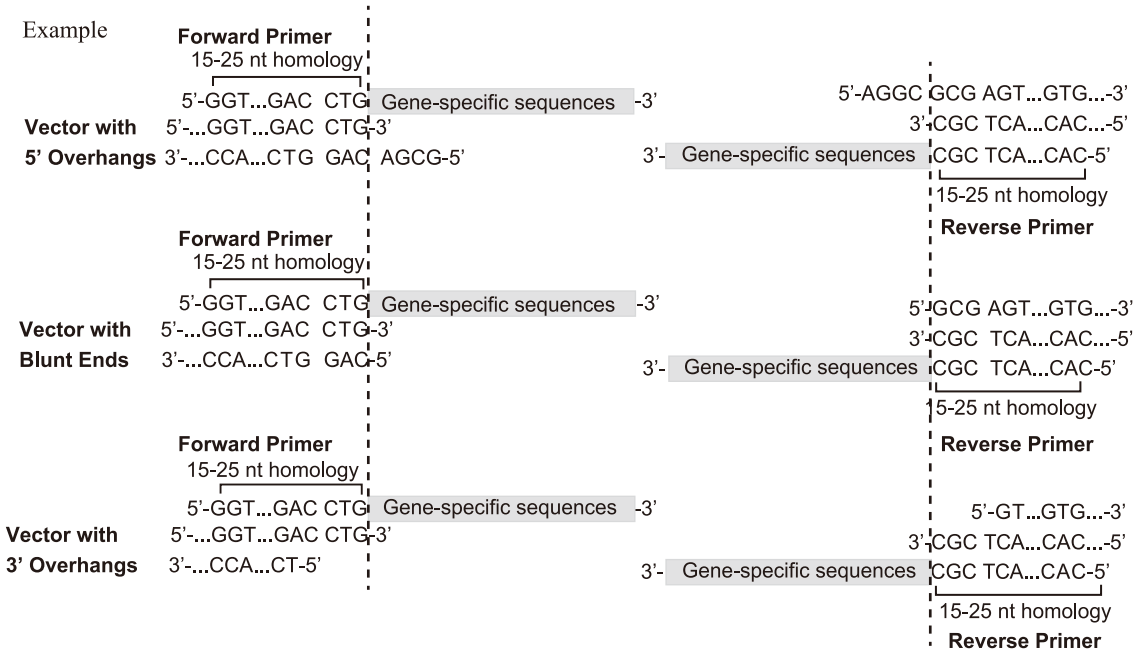
B: Preparation of Target Inserts:

(1)Primer design for single-insert cloning: cloning primers contain specific primer sequence and overlapping sequence of the target insert.

Forward primer (5'-3'): 15-25 nt sequence homologous to linearized vector + 20-25 nt forward primer sequence specific to the target insert.

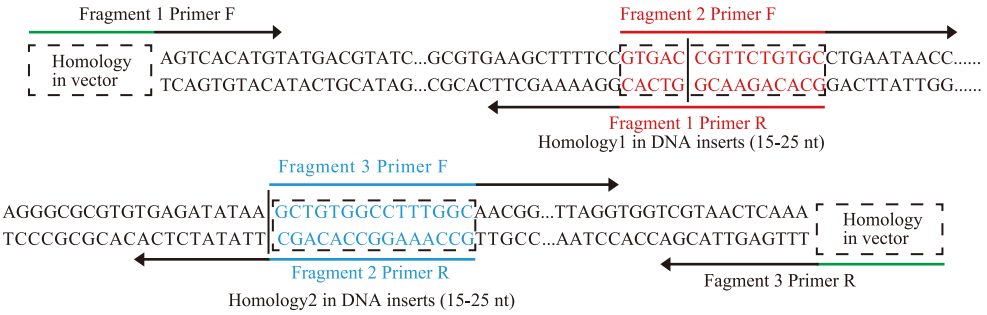
Reverse primer (5'-3'): 15-25 nt sequence homologous to linearized vector + 20-25 nt forward primer sequence specific to the target insert.

According to the structure of the linear vector end (Vector with 5' overhangs, vector with 3' overhangs, and vector with blunt ends), the primers are divided into the following three cases. Its diagram is as follows:



The ends of linearized vectors are different depending on different linearization methods, such as single digestion, double digestion, inverse PCR, etc. Any two of the above three end structures can be grouped randomly. The specific primers for target inserts can be designed following the general principle of regular primers.

(2) Primers for multiple fragments: Primer design for multiple-insert is the same with single-insert. The design method for primers at the junctions of two fragments is shown in the diagram below.



* The above two methods can be selected for designing the homologous region of the target fragments. To design multi-fragment primers, any one or two methods can work.

(3) Polymerase selection: High-fidelity DNA polymerases such as Pfu series.

(4) Reaction conditions

Use 0.2-0.4 μM (final concentration) primers for PCR.

For long cloning primers (around 40 bp) with high Tm, the annealing temperature should be 60-68℃ to amplify the target fragments. Other conditions can be determined based on the selected PCR enzymes.

(5) Purification of target DNA fragments

- If the fragments are derived from plasmid template and the recombinant vector has the same selection marker as the parental plasmid, it is suggested to digest the fragments with DMT enzyme (DpnI) before purification.
- If product is a single band, we recommend using PCR Purification Kit (Cat. No. EP101) to purify your fragments.
- If non-specific amplification occurs, we recommend using Gel Extraction Kit (Cat. No. EG101) to recover your fragments.

Note:

- After cloning by this method, restriction sites used in the linear vector may be eliminated or added. It is recommended to select the restriction sites carefully for those with strict requirements. If necessary, the eliminated base pairs can be added to the cloning forward and reverse primers to restore the original restriction sites.
- If the recombinant plasmid is used for protein expression, the reading frame should be considered carefully for primer design to ensure the intactness of sequences required for protein expression and purification (such as promoter sequence, RBS sequence, start codon, stop codon, protein tag, etc.).

Setting up the cloning reaction

2×Assembly Mix	5 μl
Linearized vector (5-100 ng)	x μl*
Inserts	y μl*
Nuclease-free Water	to 10 μl

*In a 10 μl system, we recommend using 0.01-0.25 pmols of vector and insert respectively. For optimal cloning efficiency, use 1:1~1:2 (vector: insert) molar ratio.

The recommended amounts of vector and insert can be approximately calculated according to the following formula:

Recommended amount of vector: (0.02×bp size of insert) ng, ≈0.03 pmol

Recommended amount of insert: (0.02~0.04×bp size of insert) ng, ≈0.03~0.06 pmol

For more accurate calculation formula, please refer to the notes below.
Gently mix and incubate at 50°C for 5~15 minutes. After reaction, place it on ice for a few seconds. The reaction mixture can be directly used for transformation or stored at -20°C.

Transformation

(1) Thaw a vial of Trans1-T1 Phage Resistant Chemically Competent Cell on ice.
(2)Transfer 2 μl of reaction mixture into 50 μl of Trans1-T1 Phage Resistant Chemically Competent Cell and mix gently by flicking the tube (do not vortex). Incubate on ice for 30 minutes.
(3)Heat-shock in a water bath at 42°C for 30 seconds, and immediately place on ice for 2 minutes.
(4)Add 450 μl of room-temperature SOC/LB medium. Place in a shaking incubator at 37°C for 1 hour at 250 rpm.
(5)Pre-warm LB plate containing the appropriate selection antibiotic at 37°C.
(6)Spread 100 μl of cells evenly on the selection plate and incubate overnight at 37°C

Analysis of Positive Clones

- **Analyzing positive clones by PCR**

(1) Pick a white single colony into 10 μl of sterile water. Mix by vortexing or pipetting up and down.
(2)Add 1 μl of the above mixture into 25 μl of PCR system. Identify the positive clones by appropriate forward and reverse primer.

- **Analyzing positive clones by restriction enzyme digestion**

  Pick several individual colonies and culture them overnight in LB medium containing the appropriate selection antibiotic. Isolate plasmid DNA by EasyPure® Plasmid MiniPrep Kit (Cat. No.EM101). Analyze the plasmids by restriction enzyme digestion.

- **Sequencing**

  Perform sequence analysis using vector universal primers.

Cloning reaction for control insert

2×Assembly Mix	5 μl
Linearized pUC19 Control Vector	1 μl
Control Insert	1 μl
Nuclease-free Water	3 μl

Reaction conditions, transformation and analysis of positive clones are the same as above.

Notes

- Poor quality of purified or recovered vectors or target fragments will significantly reduce the positive clone rate.
- When there are few colonies, it is recommended to increase the amount of recombinants or E. coli cells plated accordingly for transformation.
- Cloning efficiency decreases with as the length of recombinant plasmid increases (> 15 kb). It is suggested to use Trans2-Blue Chemically Competent Cell (Cat. No. CD411) for transformation.
- Cloning efficiency also decreases as the amount of recombinant fragments increases. It is suggested to extend the overlapping sequence of primers or the reaction time.
- The accurate calculation formula for the number of pmols of the vector and the insert is: pmols= weight (ng)/ Insert Length (bp) ×0.65 kDa

For example
For 100 ng of 2000 bp insert, the number of moles is equal to 100/(2000×0.65) ≈ 0.08 pmols.
For 100 ng of 5000 bp insert, the number of moles is equal to 100/(5000×0.65) ≈ 0.03 pmols.

For research use only, not for clinical diagnosis.

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