FlyCut™ DNA Assembly Mix Plus

Cat #: C-BSM1202

Size: 50 rxns

Storage: -20°C

Components

Components	Size
FlyCut™ DNA Assembly Mix Plus	250 µl
pUC19 Control Plasmid, Linearized (Ampr, 40 ng/µl)	50 µl
500 bp Control Fragment (20 ng/µl)	50 µl

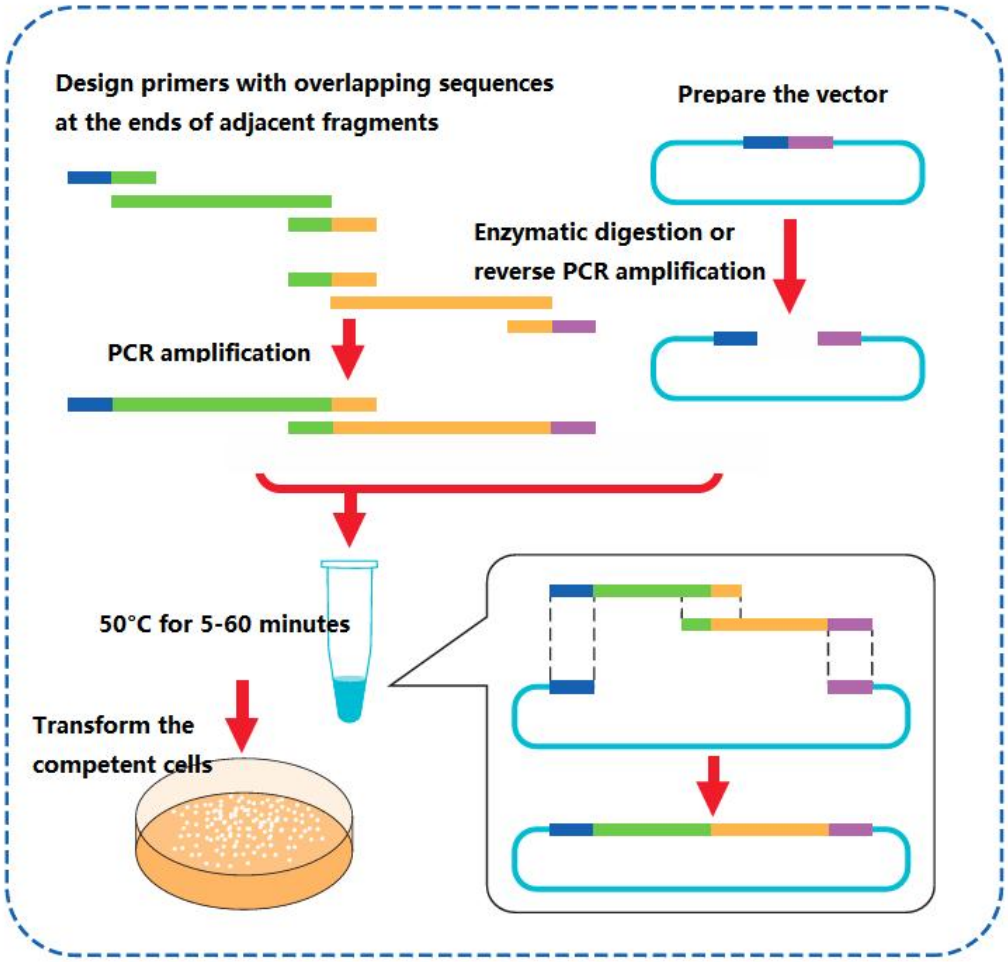
Product Introduction

Seamless cloning technology based on the recombination principle is a new generation cloning method that does not rely on cumbersome enzymatic digestion, ligation steps, or end polishing. It allows the cloning of insert fragments into any position of a linearized vector by recombining the DNA fragment with the 15~25 nt homologous sequence at the end of the linearized vector. The self-ligation background of the vector is extremely low, making it a simple, fast, and efficient DNA directional cloning technique.

The FlyCut™ DNA Assembly Mix Plus seamless cloning kit enables the recombination of single or multiple DNA fragments in a single reaction. It can achieve single fragment recombination in as fast as 5 minutes with a positivity rate of over 95%. The auxiliary factors added to the Mix effectively enhance the cloning positivity rate, and the optimized reaction system can tolerate impurities in unpurified PCR products to a certain extent. The upgraded version of the seamless cloning kit has higher positivity rates and better compatibility.

Instructions for use:

1. Summary of the Workflow



2. Preparation of Linearized Cloning Vector

Choose suitable cloning sites and linearize the vector. The linearization of the vector can be achieved through enzymatic digestion or reverse PCR amplification.

① Enzymatic Digestion Preparation

Some restriction endonucleases are not effective in digesting supercoiled DNA, which may result in varying amounts of undigested vector DNA, leading to decreased positivity rates. It is recommended to use FlyCutTM rapid restriction enzymes for digestion (single or double digestion) to ensure complete linearization of the vector and reduce transformation background (false-positive clones obtained from undigested vector).

Note 1: Linearized vectors prepared by enzymatic digestion do not require dephosphorylation. Double digestion is

recommended.

Note 2: After digestion, it is recommended to inactivate the restriction enzymes or purify the desired product for use in the recombination reaction.

② Reverse PCR Amplification Preparation

To minimize the introduction of amplification mutations, it is recommended to use a high-fidelity PCR mix for amplification. It is recommended to use pre-linearized plasmids as templates to reduce the impact of residual circular plasmid templates on cloning positivity rates.

Note 1: If the PCR product does not show specific bands, it is recommended to use Template Eliminator to digest the plasmid template for use in the recombination reaction. Otherwise, purification of the PCR product is recommended.

Note 2: For multi-fragment cloning, it is recommended to purify the PCR products before use.

3. Design of PCR Primers for Insert Fragments

The 5' end of the PCR primers must contain a 15~25 nt (recommended 18 nt) sequence homologous to the adjacent fragment (insert fragment or vector) end. If the vector has a sticky end and the 3' end protrudes, the primer design must include the protruding part. If the 5' end protrudes, the primer design can include or exclude the protruding part.

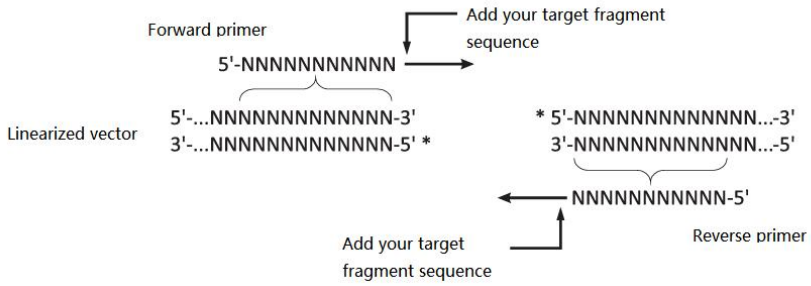
Forward amplification primer for the insert fragment:

5'—Upstream vector homologous sequence + Restriction site (optional) + Gene-specific forward amplification sequence—3'

Reverse amplification primer for the insert fragment:

3'—Gene-specific reverse amplification sequence + Restriction site (optional) + Downstream vector homologous sequence—5'

Note 1: It is recommended to choose regions with no repetitive sequences and uniform GC content for cloning. The highest recombination efficiency is achieved when the GC content in the 25 nt region upstream and downstream of the vector cloning site is between 40% and 60%.



Note 2: The connecting end sequence of the pUC19 vector (Ampr) provided in this kit is as follows: [sequence omitted]

```
                                        EcoRI
5'-ATGACCATGATTACGCCA-3'     5'-AATTCACTGGCCGTCGTTTTAC-3'
3'-TACTGGTACTAATGCGGTTCGA-5'     3'-GTGACCGGCAGCAAAATG-5'
                        HindIII
```

4. PCR Amplification of the Insert Fragment

It is recommended to use a high-fidelity PCR mix to minimize the introduction of amplification mutations. It is advisable to use purified PCR products for seamless cloning reactions. If the PCR product is identified as a specific amplification product by agarose gel electrophoresis, it can be used directly, but the volume added should not exceed 20% of the total reaction volume.

5. Recombination Reaction

① Prepare the following reaction system on ice:

Components	Reaction system	Negative control	Positive control (if necessary)
FlyCut™ DNA Assembly Mix Plus	5 µl	5 µl	5 µl
Linearized vector[a]	50-200 ng	50-100 ng	pUC 19 Control Plasmid, Linearized, 1 µl
Insert fragment[b]	10-200 ng	—	500 bp Control, Fragment, 1 µl
ddH2O	Up to 10 µl		

a. Optimal amount of vector (ng) = 0.02 × number of base pairs in the vector, i.e., 0.03 pmol.

b. For single-fragment insertions, the optimal amount of fragment (ng) = 0.04 × number of base pairs in the fragment; for multi-fragment insertions, the optimal amount per fragment (ng) = 0.02 × number of base pairs in the fragment.

Note 1: If the length of the single-fragment insert is larger than the vector, interchange the amounts of the vector and insert fragment.

Note 2: If the length of the insert fragment is less than 200 bp, use 5 times the amount of vector for the insert fragment.

Note 3: If the calculated amount exceeds the minimum/maximum values, it is recommended to directly use the minimum/maximum amount.

Note 4: Cloning positivity rates will decrease with excessively long vector fragments, insert fragments, or a high number of fragments.

After the recombination reaction system is prepared, gently pipette and mix the components to avoid bubble formation and do not vortex.

② Place the reaction system at 50°C and incubate for 5-60 min.

Note 1: It is recommended to use a PCR instrument or other precise temperature-controlled instrument for the reaction. Insufficient or excessive reaction time will decrease cloning efficiency.

Note 2: For 12 insert fragments, a reaction time of 5-15 min is recommended. For 35 insert fragments, a reaction time of 15-30 min is recommended.

Note 3: When the vector backbone is above 10 kb or the insert fragment is above 4 kb, it is recommended to extend the reaction time to 30-60 min.

Note 4: After the reaction is completed at 50°C, it is recommended to perform a brief centrifugation to collect the reaction mixture at the bottom of the tube.

③ Place the reaction tube on ice for cooling, then perform transformation or store at -20°C.

Note 1: Recombination products stored at -20°C are recommended to be used within 1 week.

6. Transformation of the Recombination Products

Take 5-10 µl of the reaction mixture and add it to 100 µl of competent cells. Gently pipette and mix slowly, then place on ice for 30 min. Heat shock at 42°C for 45-60 sec, followed by incubation on ice for 5 min. Add 500 µl of SOC or LB medium and shake at 37°C for 40-60 min (200 rpm). Spread the bacterial solution evenly on agar plates containing the corresponding antibiotic and incubate overnight at 37°C.

Note 1: Different competent cells may yield different cloning positivity rates. It is recommended to use competent cells with a transformation efficiency > 10^8 CFU/µg.

Note 2: The number of colonies depends on the quantity and purity of the PCR product and linearized vector.

Note 3: Positive control plates typically show abundant white single colonies, while negative control plates show very few colonies.

7. **Positive Clone Detection:**

Pick a single colony and mix it in 10 µl of ddH2O. After 10 min of incubation at 95°C for lysis, take 1 µl of the lysate as a template for colony PCR identification. Alternatively, inoculate a single colony in antibiotic-containing medium and incubate overnight, then extract the plasmid for enzyme digestion identification. For positive control in positive clone detection, use the universal primers M13F and M13R for colony PCR, and HindIII and EcoRI for enzyme digestion identification.

Note 1: It is recommended to use at least one universal primer in colony PCR to avoid false-positive results.

Note 2: If necessary, further sequencing identification can be performed on positive results.

Note 3: M13F: TGTAAAACGACGGCCAGT

M13R: CAGGAAACAGCTATGAC

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes.