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INSTRUCTIONS FOR USE

Product Name: Universal One-Step Cloning Kit **Catalog # CLN02-K298**

Step 1. Preparation of target vector and insert fragment:

1. Linearized Vector:

Select the appropriate cloning site and linearize the vector by restriction enzyme digestion or reverse PCR amplification.

a. Enzyme digestion preparation of linearized vector:

False-positive clones without inserted fragments may be formed. If their proportion is high, prepare the linearized vectors again and cut the glue for recovery.

- Double enzyme digestion linearization (Recommended): complete linearization, low conversion background.
- Single digestion linearization: the degree of linearization is poor. The time of enzyme digestion can be extended to reduce the conversion background.

b. Linearized vector prepared by reverse PCR amplification

Vector amplification using a high-fidelity polymerase is recommended to reduce the introduction of amplification mutations.

When the purity of products is high, the recombinant reaction can be directly carried out without purification. However, when the purity is low and may contain un-linearized ring plasmids, it is recommended to use a high-quality kit for purification.

2. Primer Design for a single insert fragment:

The 5' and 3' ends of the PCR-amplified insert fragment contain homologous sequences (15–25 bp) matching the corresponding ends of the linearized vector. These homologous regions are introduced via the forward and reverse PCR primers used to amplify the insert.

- Insertion fragment forward amplification primer design: 5 '-upstream vector end homologous sequence + enzyme restriction site (can be retained or deleted) + gene specific forward amplification primer sequence -3'
- Insertion fragment reverse amplification primer design: 3 '- gene-specific reverse amplification primer sequence + cleavage site (can be retained or deleted) + downstream vector terminal homologous sequence -5'

The final primer length is more than 40 bp, and PAGE purification method is recommended for primer synthesis.

When determining the annealing temperature for the amplification primer, calculate the Tm value based solely on the gene-specific sequence of the primer. Do not include the homologous vector-matching sequence in this calculation. For optimal cloning efficiency, ensure the Tm is ≥48 °C**.**

3. Primer design for multiple insertion fragments:

PCR-amplified inserts must have 15-25 bp homologous ends matching the linearized vector. These are added via primer extensions.

Manual Design Example for inserting 3 fragments between EcoRI/HindIII sites in pUC18:

a. Vector-Adjacent Primers (First & Third Fragments):

First Forward: 5'-[Vector homology]-[EcoRI]-[Gene-specific]-3'

Third Reverse: 3'-[Gene-specific]-[HindIII]-[Vector homology]-5'

(Restriction sites are optional)

b. Middle Fragment Primers (First Reverse & Second Forward):

Add fragment-to-fragment homology to either:

First Reverse primer (recommended): 3'-[Gene-specific]-[Fragment 2 homology]-5'

Second Forward primer: 5'-[Gene-specific]-3'

c. Remaining Primers (Second Reverse & Third Forward):

Design same as step b.

4. PCR Amplification of the Insert Fragment

The insert fragment can be amplified using any PCR enzyme. The presence or absence of an A-tail in the PCR product is not critical, as it will be removed during recombination and will not appear in the final vector.

Following PCR amplification, analyze a small aliquot of the product by agarose gel electrophoresis to verify amplification yield and specificity.

Direct use of PCR products in recombination is acceptable when:

- The amplification template isn't a circular plasmid with the same antibiotic resistance as your vector.
- Electrophoresis shows a single, clean band.

Step 2. Concentration Measurement

For DNA quantification, we recommend comparing band intensities via agarose gel electrophoresis. Prepare serial dilutions of both the linearized vector and insert fragment PCR product. Load 1 μL of each dilution (including the undiluted sample) alongside a DNA marker for electrophoresis.

Estimate concentrations by comparing band brightness, particularly useful for unpurified linearized vectors and insert fragments.

Step 3. Recombination reaction

1. Amount of Linearized vector and insert fragments

The recombination reaction system requires a total concentration of 0.02-1 pmol for each fragment and vector. The recommended molar ratio of vector to each inserted fragment is 1:2. The corresponding DNA quantity can be calculated using the following formula:

pmol = (insert segment usage ng) × 1,000 / (insert base pair number × 650 daltons).

Note that when the insert fragment length exceeds that of the vector, the calculation method should be reversed by treating the insert as the vector and the vector as the insert for determination of optimal amounts. Additionally, when the insert fragment is longer than the vector, maintain final reaction concentrations of both linearized vector and insert at ≥1 ng/μL.

For unpurified PCR products, the combined volume of unpurified linearized vector and insert should not exceed 20% of the total reaction volume.

2. Recombination reaction system

The reaction volume can be scaled proportionally; prepare on ice and mix all components before use

10 μL	Clone Universal II Enzyme Premix
X μL	Insert fragment(s) input
Y μL	Target vector input
Up to 20 μL	ddH_2O

3. Recombination Reaction Conditions

After preparing the reaction mixture, gently pipette to mix all components, then briefly centrifuge to collect the liquid at the tube bottom.

The reaction products can be directly converted or stored at -20°C.

Recommended incubation conditions:

- Single fragment insertion: 50°C for 5 minutes
- 2-6 fragment insertion: 50°C for 10-50 minutes (increase time with fragment number; see table below)
- Ultra-long fragment insertion: 50°C for 40-60 minutes

Recombination Reaction Time	Number of Connected Fragments
10 min	2
20 min	3
30 min	4
40 min	5
50 min	6

Step 4. Transformation

1. Warm the screening plate at 37°C for 15 minutes prior to use.
2. Thaw a vial of F-DH5α competent cells on ice. Once thawed, add the target DNA (plasmids or ligation products) and mix gently by stirring. Maintain the mixture on ice for 5 minutes. *Note: The DNA volume should not exceed 10% of the competent cell suspension volume.
3. Transfer the cell-DNA mixture to pre-warmed LB medium using a 200 μL pipette and spread evenly.
4. Incubate plates upside-down at 37°C overnight. For blue-white selection, maintain incubation for at least 15 hours.

Step 5. Clone Verification

Colony PCR offers the most convenient and rapid verification method:

- Pick individual colonies using a sterile tip.
- Resuspend the colony directly into a PCR mix.
- Perform PCR with appropriate primers.