CWBIO
Tel : 86-10-56953015
Email : info@cwbio.com

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# Ultra-Universal One Step SeamLess Cloning Mix

**Catalog Number:** CW3036S（20 rxns）
CW3036M（50 rxns）

**Storage Conditions:** -20℃。

**Product Shelf Life:** 1 year

## Components

| Component | CW3036S 20 rxns | CW3036M 50 rxns |
|---|---|---|
| 2×Ultra Cloning MasterMix | 100 μL | 250 μL |
| PUC19 Vector，Linearized（20 ng/μL） | 10 μL | 20 μL |
| 500 bp Control Insert （20 ng/μL） | 10 μL | 20 μL |
| $ddH_2O$ | 1 mL | 1 mL |

This product is for scientific research only, which shall not be used for clinical diagnosis or other purposes.



info@cwbio.com www.cwbiosciences.com Jiangsu CoWin Biotech Co., Ltd.

## Product Introduction

Ultra-Universal One Step SeamLess Cloning Mix is a simple, fast and efficient seamless cloning kit developed based on the principle of homologous recombination. The kit does not rely on tedious enzyme digestion and ligating steps and allows for directional cloning of DNA into any site of any vector without restriction enzyme sites limitations. Linearize the vector completely and introduce 15-25 bp homologous sequences of the ends of the linearized vector to the 5' end of the forward and reverse PCR primers of the insert, mix the PCR products, both ends of which contain sequences identical to the vector ends, with the linearized vector in a certain ratio and perform transformation under the reaction of 2×Ultra Cloning MasterMix at 50 ℃ for 5-30 minutes. This process allows for directional cloning of 1-5 fragments with a positivity rate of over 95%. Unique cofactors and stabilizers have been added to 2×Ultra Cloning MasterMix to effectively improve the efficiency of recombination. The optimized reaction Buffer system has better compatibility and better guarantees the positive cloning rate of the complex system.

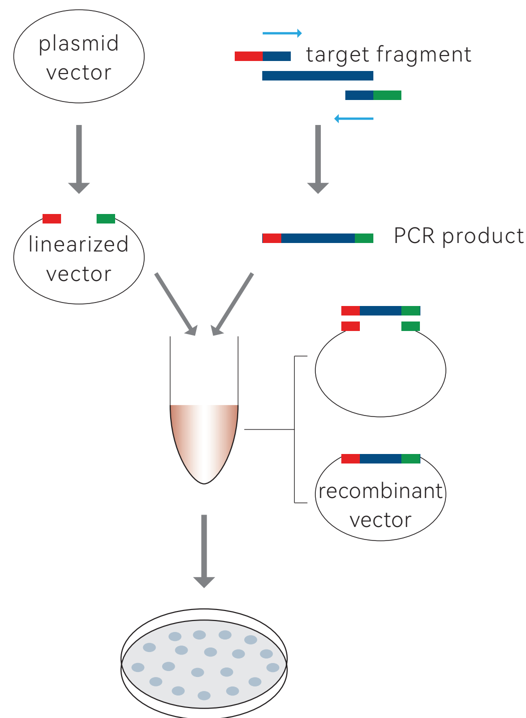



Figure 1. Schematic diagram of the principle of seamless connection of a single fragment



3. Transformation of recombinant products

3.1 Thaw the clone competent on ice, add 10 μL of the reaction product to 100 μL of competent cells, flick the tube wall to mix, and let stand on ice for 30 minutes.

3.2 Heat shock in a 42 ℃ water bath for 90 seconds and immediately place on ice for 2-3 min.

3.3 Add 600 μL of antibiotic-free LB liquid medium and incubate at 220 rpm at 37 °C for 30 minutes

3.4 Centrifuge at 5000 rpm for 5 min, discard the excess medium, resuspend the remaining 100 μL of the bacterial solution, and evenly coat the plate with a sterile cell spreader on the plate with correct resistance.

3.5 Incubate inversely at 37 ℃ for 12-16 h.

4. Identification of positive clones

According to the specific situation, colony PCR identification and plasmid extraction can be performed for restriction enzyme identification or sequencing identification.

## FAQ

1. Low transformation efficiency

1) The primer design is unreasonable. The primer should contain a 15-25 bp overlapping region with a GC content of 40%-60%.

2) Low competent efficiency. Freshly prepared or properly preserved competent cells should be used.

3) The ratio or purity of the vector and the fragment is not good, and the reaction is inhibited. The fragment and the vector can be purified. The purified product should be dissolved in $ddH_2O$, and add in the proportion recommended in the manual.

2. False positives

1) The linearization of the vector is incomplete and contains circular plasmid. It is recommended to prolong the reaction time and use the method of gel extraction when preparing the linearized vector.

2) Contamination due to the same resistance of plasmid. When the plasmid is used as the template for insert PCR amplification, the product should be treated with DpnI enzyme or gel extraction.

3) Amplification of non-specific PCR products. Optimize the PCR reaction system, improve the specificity of amplification, and use gel extraction for PCR products.

## Protocol

1. The amount of linearized vector and insert

1.1 The amount of vector is typically 20-50 ng, with an optimal molar ratio of 2:1-3:1 between inserts and vector, and 1:1 between fragments in the case of multi-fragment ligation.

1.2 The amount for single-fragment cloning

Optimal cloning vector amount = (0.02 × cloning vector base pairs) ng (0.03 pmol).

Optimal insert amount = (0.04 × insert base pairs) ng (0.06 pmol).

1.3 The amount for multi-fragment cloning (2-5 fragments).

Optimal cloning vector amount = (0.02 × cloning vector base pairs) ng (0.03 pmol).

The amount of each insert = (0.02 × base pairs per insert) ng (0.03 pmol).

2. Recombinant reactions

2.1 Prepare the reaction system according to the table below

| Reagent | Reaction System |
|---|---|
| 2×Ultra Cloning MasterMix | 5 μL |
| Linear Vector (20-50 ng) | X μL |
| Insert(s) | Y μL |
| dd $H_2O$ | To 10 μL |

**Note: Negative controls for Linear Vector and Inser(s) can be designed separately to detect whether there is a circular plasmid residue in the linearized vector or circular plasmid template residue in the insert (a negative control is recommended when the insert amplification template has the same resistance as the cloning vector).**

2.2 Reaction system of the control (optional)

| Reagent | Reaction System |
|---|---|
| 2×Ultra Cloning MasterMix | 5 μL |
| PUC19 Vector，Linearized（20 ng/μL） | 3 μL |
| 500 bp Control Insert （20 ng/μL） | 1 μL |
| dd $H_2O$ | To 10 μL |

2.2 Mix gently, react in a 50 ℃ water bath or thermocycler for 5-15 minutes, and the reaction time can be extended to 30 minutes when more than 3 fragments are ligated. At the end of the reaction, the EP tubes should be cooled on ice and transformed directly or stored at -20 ℃.



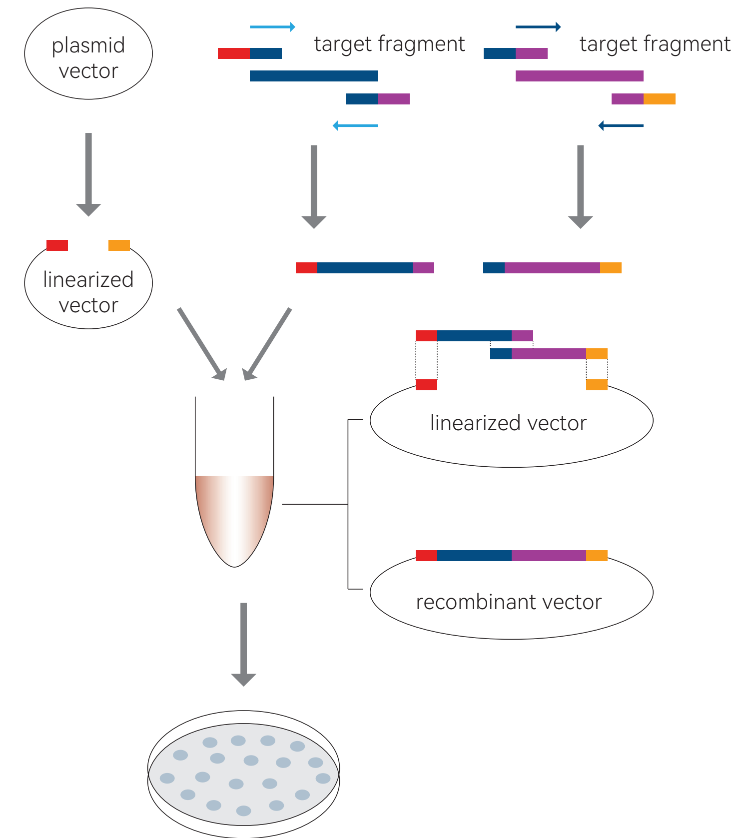



Figure 2. Schematic diagram of the principle of seamless connection of multiple fragments

## Features

1. One or more long/short PCR amplified fragments (blunt/A-end) can be inserted into the vector in 15 minutes.
2. Not limited by the availability of restriction sites on the vector and inserts, as well as the requirement for blunt/sticky ends, cloning can be performed at any site.
3. Seamless cloning with no unwanted base sequences introduced at the insertion point.
4. Efficient and accurate, with a cloning positivity rate of >95%.

## Additional Materials and Reagents Required

- Insert fragments, specific primers, linearized vectors
- High-fidelity PCR reagents (CW2965 Super Pfx MasterMix recommended).
- Competent cells (CW0808 DH5α Competent Cell, CW0807 TOP10 Competent Cell).
- Gel extraction kit (CW2302 Gel Extraction Kit).
- Thermocycler, PCR reaction tube, etc.

## Preparation of Linearized Vector and Inserts

1. Preparation of linearized vectors

   Select appropriate cloning site to linearize the vector. There are two ways to linearize the vector: restriction enzyme digestion and inverse PCR.

   (1) The linear vector obtained by enzyme digestion can be single or double enzyme digestion, and it is recommended to purify the vector with PCR product purification kit or gel extraction kit after enzyme digestion. False-positive clones (without inserts) that occur after transformation of recombinant products are formed by incompletely digested and unlinearized vector transformation, so we recommend to perform gel extraction after digestion to minimize the proportion of unlinearized vectors.

   (2) To obtain linearized vector by inverse PCR amplification, high-fidelity polymerase amplification is recommended to reduce the introduction of amplification mutations. When using circular plasmid as template, it is recommended to use endonuclease Dpn I to digest the PCR product to reduce false positives caused by residual circular plasmid template. If using a Dpn I digested plasmid template, heat at 80 ℃ for 20 minutes to inactivate Dpn I activity to avoid degradation of the vector by residual Dpn I during the recombination reaction.

2. Preparation of inserts

   The inserts can be prepared with any PCR enzyme amplification process that is not affected by blunt-end or sticky end (A-tail) of the amplified product (it will be removed during recombination and will not be present in the final cloned product). However, to reduce amplification mutations, especially in point mutation experiments, high-fidelity polymerase (CW2965 Super Pfx Master Mix) is recommended.

   In general, it is recommended that the PCR product should be purified to reduce the background ratio. If the insert is derived from the plasmid template and the plasmid has the same resistance as the recombinant vector, the PCR product needs to be digested with the endonuclease DpnI to reduce the background and increase the positive rate.



3. Primer design principles for inserts

   Single-fragment primer design principle: Introduce homologous sequences of both ends of the linearized vector at the 5' end of the forward and reverse amplification primers insert, so that the amplified insert has homologous sequences corresponding to the two ends of the linearized vector (20-25 bp, enzyme restriction sites not included) at both ends of the insert.

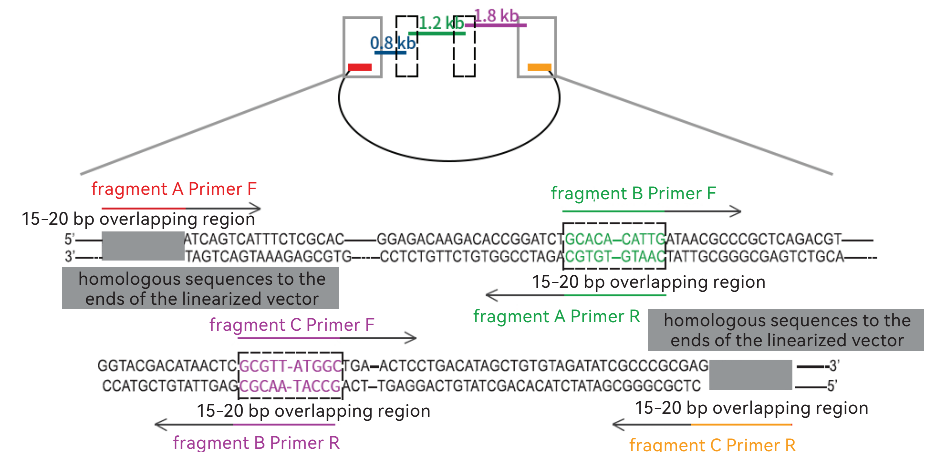
   5'-Upstream vector end homologous sequence (20 bp) + restriction site (deletable) + gene-specific forward amplification primer sequence (20 bp)-3'

   5'-Downstream vector end homologous sequence (20 bp) + restriction site (deletable) + gene-specific reverse amplification primer sequence (20 bp)-3'

   Multi-fragment primer design principles: The design principle of primers at both ends of the vector is consistent with that of single-fragment cloning, and primers in overlapping regions should be designed between fragments

   The reverse primer of fragment A contains a 20-25 bp overlapping region with the forward primer of fragment B and a specific primer region, while the reverse primer of fragment B contains a 20-25 bp overlapping region with the forward primer of fragment C and a specific primer region, and so on. The primers at both ends of the fragments contain homologous sequences to the ends of the linearized vector.

   As shown in the following picture:




Note: To improve cloning efficiency, the overlap between fragments can be increased and the Tm values need to be consistent.