Tinzyme Co., Limited

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One Step Seamless Cloning Mix

Product Number: EK0630

Shipping and Storage

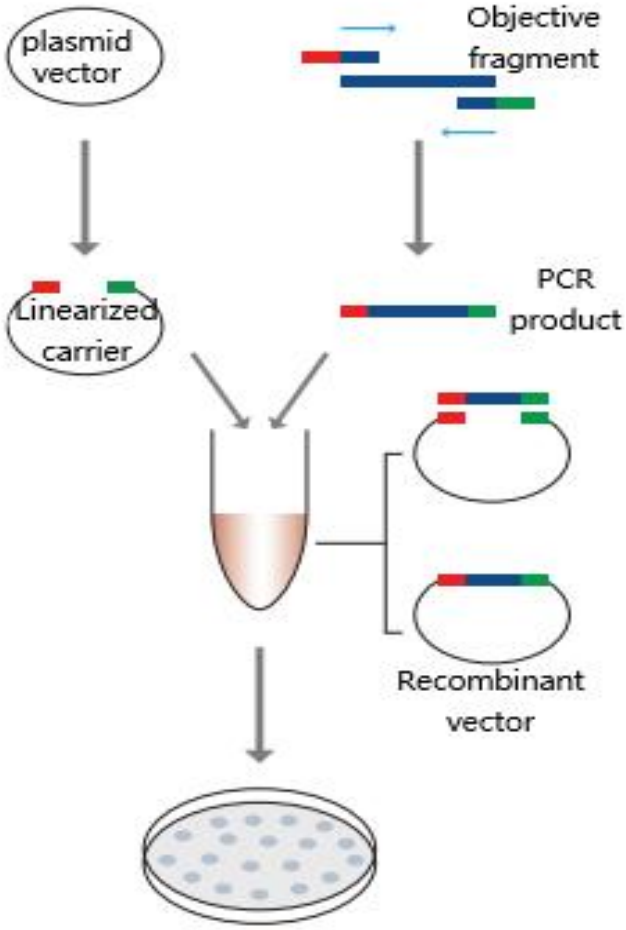
Store at -20℃.

Component

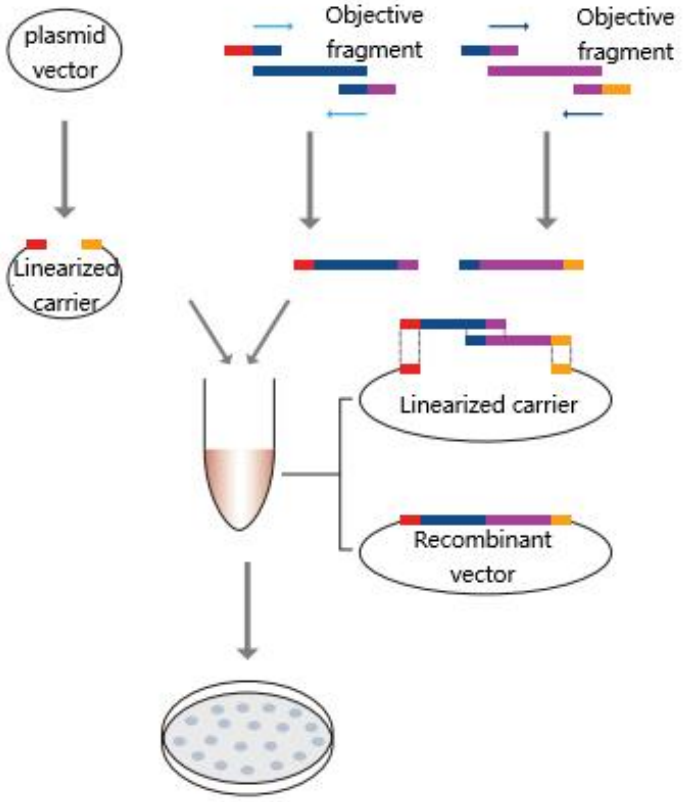
Component	EK0630
2×Cloning MasterMix	100μL
PUC19 Vector, Linearized(20ng/μL)	10μL
500 bp Control Insert (20ng/μL)	10μL
ddH_2O	1mL

Description

Seamless cloning technology is a simple, efficient, and rapid DNA cloning method that enables directional insertion of DNA fragments into any site of a vector. This product does not rely on T4 ligase and is not restricted by restriction enzyme sites of the vector or target fragments. Instead, it directly employs homologous recombination through a specialized enzyme, allowing directional recombination between linearized vectors and PCR fragments with 20-25bp overlapping regions at both ends of the linearized vector. A 5-minute to 15-minute reaction at 50°C can rapidly achieve directional seamless cloning of 1-5 fragments.



Schematic diagram of seamless connection principle for single segment



Schematic diagram of seamless connection principle for multiple segments

Features

1. Within 15 minutes, one or more long or short PCR amplification fragments (flat/A end) can be inserted into the vector.
2. Cloning can be performed at any site without being limited by the availability of the vector and insertion site, as well as the flat/sticky ends.
3. Seamless cloning, insertion points will not introduce unwanted base sequences.
4. Efficient and accurate, with a gram-positive rate of over 95%.

Materials required but not supplied

1. Inserting fragments, specific primers, linearized vectors, and high fidelity PCR reagents
2. competent cells
3. Gel recycling reagent kit
4. PCR instrument, PCR reaction tube, etc.

Preparation and recovery of linearized vectors and inserted DNA fragments

1. Preparation and recovery of linearized carriers

Choose appropriate cloning sites to linearize the vector. There are two ways to linearize the vector: restriction endonuclease digestion and reverse PCR amplification.

1.1. The linear vector obtained by enzymatic digestion can be digested with either single or double enzymes. After enzymatic digestion, it is recommended to use PCR product purification kit or gel recovery kit to purify the vector. Due to the absence of DNA ligases in the seamless cloning product system, false positive clones (without inserted fragments) that occur after the transformation of vector self linked recombinant products will not occur. These clones are formed by incomplete enzymatic cleavage of non linearized circular vectors. Therefore, we recommend gel recovery and purification after enzymatic cleavage to minimize the proportion of non linearized vectors.

1.2. Reverse PCR amplification yields linearized vectors, and it is recommended to use high fidelity polymerase amplification to reduce the introduction of amplification mutations. When using circular plasmids as templates, it is recommended to

use the endonuclease DpnI to digest the PCR product to reduce clone false positives caused by residual circular plasmid templates. If using DpnI to digest plasmid templates, heating at 80 ℃ for 20 minutes is required to inactivate DpnI activity to avoid residual Dpnl degradation of the cloning vector during recombination reactions,

2. Preparation and recycling of inserted fragments

2.1. The preparation of inserted fragments can be amplified by any PCR enzyme, and the amplification process is not affected by the flat or sticky ends (A tail) of the amplified product (which will be removed during the recombination process and will not appear in the final cloned product). However, in order to reduce amplification mutations, especially in point mutation experiments, it is recommended to use high fidelity polymerase (CW2965 SuperPfx Master Mix) for amplification. Generally, the PCR product is recommended to be purified by gel recovery to reduce the background ratio. If the inserted fragment comes from a plasmid template and the plasmid has the same resistance as the recombinant fragment, the PCR product needs to be digested with the endoenzyme DpnI to reduce the background and improve the positivity rate.

3. Primer design principles for broadcasting fragments

Design principle of single fragment primers: Introduce homologous sequences at both ends of the linearized vector at the 5 "end of the forward and reverse amplification primers of the inserted fragment, so that the amplified inserted fragment has homologous sequences corresponding to the two ends of the linearized vector at both ends (20-25bp excluding enzyme cleavage sites)

5 '- Upstream vector end homologous sequence (20bp)+enzyme cleavage site (can be deleted)+gene specific forward amplification primer sequence (20bp) -3

5 '- Downstream vector end homologous sequence (20bp)+enzyme cleavage site (can be deleted)+gene specific reverse amplification primer sequence (20bp) -3

Principle of multi fragment primer design: The primer design principles at both ends of the vector are consistent with those for single fragment cloning, and overlapping region primers are designed between fragments.

Principle of multi fragment primer design: The reverse primer of fragment A contains an overlapping region of 20-25bp with the forward primer of fragment B and a specific primer region. The reverse primer of fragment B contains an overlapping region of 20-25bp with the forward primer of fragment C and a specific primer region, and so on. The two end fragment primers contain homologous sequences at both ends of the linear vector.

Protocol

1. The usage of linearized carriers and insertion fragments

1.1. The general dosage of the carrier is around 0.03 pmol, and the optimal molar ratio of the inserted fragment to the carrier is between 2:1-3:1; In the case of multiple fragment connections, the molar ratio between fragments is 1:1.

1.2. Single fragment cloning dosage

1.3. The general dosage of the carrier is around 0.03 pmol, and the optimal molar ratio of the inserted fragment to the carrier is between 2:1-3:1; In the case of multiple fragment connections, the molar ratio between fragments is 1:1.

1.4. Single fragment cloning dosage

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

Optimal insertion fragment usage=(0.04×insertion fragment base pairs) ng (0.06pmol)

1.5. Multi fragment cloning dosage (2-5 fragments)

The optimal amount of cloning vector used is (0.02×cloning vector base pairs) ng (0.03pmol).

The amount of each inserted fragment used is (0.02×base pairs per inserted fragment) ng (0.03pmol)

2. Recombination reaction

Note: 2×Cloning MasterMix contains a connection enhancer PEG, which is very viscous. When taken out of the refrigerator at low temperatures, it becomes even more viscous. It can be quickly thawed in the palm of your hand to reduce viscosity (without affecting quality). Gently flick the mixing hook.

2.1. Establish a reaction system according to the table below (can be prepared using EP tubes at room temperature).

Reagent	Reaction system
2×Cloning MasterMix	5μL
Linear Vector (50-200ng)	XμL
Insert(s)	YμL
dd H_2O	To 10μL

2.2. Comparison reaction system (optional)

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

2.3. Gently mix well and react in a 50℃ water bath or PCR machine for 5-15 minutes. If there are more than 3 fragments connected, the reaction time can be extended to 30 minutes. After the reaction is complete, place the EP tube on ice to cool down and directly convert or store it at -20℃.

3. Recombinant product conversion

3.1. Thaw the cloned state on ice, take 10mL of reaction product and add it to 100μL of state cells. Gently tap the tube wall and mix well. Let it stand on ice 3.1 for 30 minutes.

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

3.3. Add 600μL of LB free liquid medium and incubate at 220rpm on a shaker at 37℃ for 30 minutes,

3.4. Centrifuge at 5000rpm for 5 minutes, discard excess culture medium, resuspend the remaining 100L bacterial solution, and evenly spread it onto a correctly resistant plate using sterile coating.

3.5. Invert the incubator at 37℃ for 12-16 hours.

4. Identification of positive clones

4.1. Depending on the specific situation, colony PCR identification, plasmid extraction for restriction enzyme identification, or sequencing identification can be selected.