Tinzyme Co., Limited

Email: sales@tinzyme.com Website: www.tinzyme.com
Tel: +86-755-86134126 WhatsApp/Facebook/Twitter: +86-189-22896756

Multi One-Step Cloning kit

Product Number: CLN1102

Shipping and Storage

-Storage at 20 ℃, transportation at ≤ 0 ℃.

Component

Component	CLN1102 (50 rxn)
2×fast Multi One-Step Cloning kit	500μL
Control (Linearized plasmid + Insert fragment, Amp^r)[a]	40μL

Note: a. The positive control control contains a 4.6 kb linearized vector and a 1 kb insertion fragment with Amp resistance. When using, directly add 10μL of Control to 10μL of 2×fast Multi One-Step Cloning kit, Form a 20μL recombinant reaction system.

Description

The 2× fast Multi CloneMix is a one-step, multi fragment universal seamless cloning kit developed based on the principle of homologous recombination. This series of recombinant cloning kits belongs to a non ligase dependent system and has a very low background for carrier self ligation. The highly optimized 2× fast Multi CloneMix pre mixes enhanced recombinase and the required buffer for recombination reaction, which can significantly improve the recombination efficiency and tolerance to impurities of cloning. Therefore, the prepared high-purity linearized vector and insertion fragment can be directly used for recombinant Kron without purification, greatly simplifying the experimental operation.

This kit can selectively recombine insertion fragments containing overlapping regions at the end of the vector to any site of any vector, without being limited by enzyme cleavage sites (except in special cases such as cloning of toxic genes or instability of the plasmid after insertion of exogenous DNA). It can insert a single fragment or up to 5 sequentially spliced fragments at once. The vector end, insertion fragment end, and adjacent insertion fragment ends require a completely identical sequence of 15-25bp that can be homologous recombined with each other. Under the action of recombinase, the insertion fragment and linearized vector can be recombined by reacting at 37℃ for 5-30 minutes. The recombinant product does not rely on ligase and can be directly transformed into competent cells, with a positive cloning rate of over 95%.

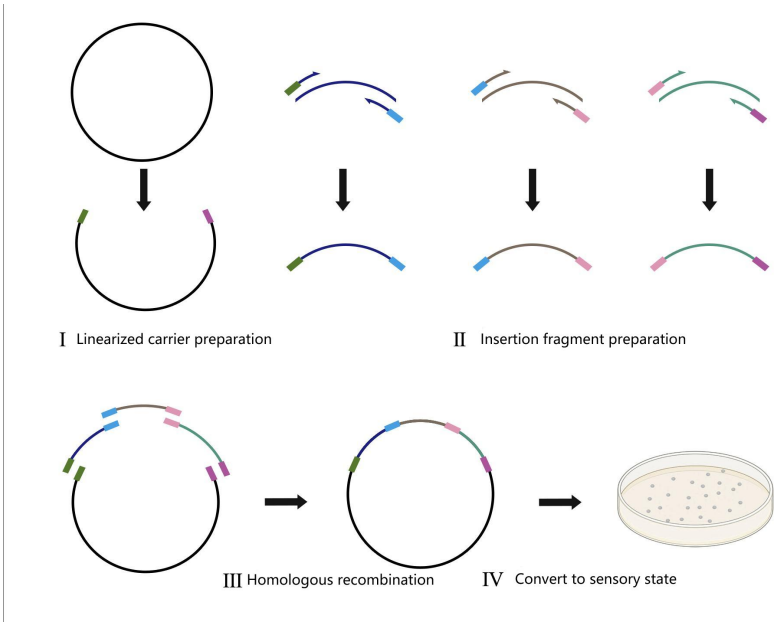
Features

1. Fast: recombination can be completed within 5-30 minutes;
2. High throughput cloning: not limited by enzyme cleavage sites, no need for fragment cleavage;
3. Seamless cloning: does not introduce additional sequences.

Scope of application

Suitable for experiments such as rapid seamless cloning, site directed mutagenesis, and high-throughput cloning of 1-5 fragments.

Principle diagram



Multi fragment homologous recombination

Protocol

1. Preparation of linearized carrier

Select appropriate cloning sites on the target vector and linearize the cloning vector. It is recommended to choose regions with no repetitive sequences, no advanced structures, and GC content between 40% and 65% as much as possible. There are two commonly used methods for linearization of carriers:

1.1. Preparation of linearized carrier by enzymatic digestion method

For larger carriers with suitable enzyme cleavage sites, it is recommended to use the enzyme cleavage method. Among them, double enzyme digestion is more effective than single enzyme digestion. The double enzyme digestion method enables more complete linearization of the vector and reduces the background of transformation (false positive clones); If single enzyme linearization is used, it is necessary to extend the enzyme digestion time appropriately to reduce the residue of circular plasmids and lower the transformation background. It is recommended to take a small amount of enzyme digestion product for electrophoresis after enzyme digestion to ensure that all carriers are cleaved. Both flat and sticky ends are acceptable.

Note: There is no DNA ligase in the fast Multi CloneMix recombination reaction system, which does not cause vector self ligation. Therefore, even linearized carriers prepared by single enzyme digestion do not require terminal dephosphorylation treatment. The majority of false positive clones (without inserted fragments) that appear after the transformation of recombinant products are formed by the transformation of non linearized circular vectors. If the proportion of false positive clones is high, it is recommended to prepare a linearized vector again.

After the enzyme digestion is completed, the endonuclease should be quickly inactivated at 65°C for 20 minutes, or the target product should be purified and used for subsequent seamless splicing reactions.

1.2. Preparation of linearized cloning vectors by reverse PCR amplification method

It is recommended to use high fidelity DNA polymerase for vector amplification to reduce the introduction of amplification mutations. This kit is compatible with the reaction system of conventional PCR; Therefore, if the PCR product has a single band, it can be directly used for subsequent recombination reactions. If there are mixed bands in the PCR amplification product, the PCR product should be subjected to electrophoresis and gel recovery purification before being used for seamless splicing to improve product purity and remove some non linearized circular carriers, which is beneficial for improving recombination efficiency.

Note: When using linear DNA sequences as templates, there is no need to remove the original template; When using circular plasmids as templates, they need to be removed. The characteristic of extracting templates with methylation

modification can be utilized, and Dpn I enzyme digestion can be used for removal.

2. Preparation of exogenous DNA insertion fragments

2.1. Design primers for amplifying exogenous DNA fragments

The insertion fragment amplification primer consists of two parts: homologous sequence+specific primer, which introduces homologous sequence at the 5′- end of the forward/reverse primer of the insertion fragment, so that the amplified products and the linearized cloning vector have completely consistent sequences (15-25bp, excluding enzyme cleavage sites) that can be homologous recombined with each other. Usually, the DNA sequence of the linearized cloning vector is introduced at the 5′ - end of the primer

The length of the column is generally selected as 18 bp, and can also be adjusted between 15-25bp. When cloning multiple fragments, it is necessary to maintain homology at the end of adjacent inserted fragments.

Design method of Pimer F insertion fragment forward amplification primer:

5′ - Upstream vector end homologous sequence+enzyme cleavage site (optional)+gene specific forward amplification primer sequence -3′

Primer R insertion fragment reverse amplification primer design method:

5′- Downstream vector end homologous sequence+enzyme cleavage site (optional)+gene specific reverse amplification primer sequence -3′

Note: The gene specific forward/reverse amplification primer sequence refers to the conventional insertion fragment forward/reverse amplification primer sequence, with a Tm value of 60-65 ℃ being optimal;

The upstream/downstream vector end homologous sequence refers to the linearized vector end sequence (used for homologous recombination), with a GC content of 40% to 60% being optimal.

When calculating the annealing temperature of amplification primers, only the Tm value of the gene specific amplification sequence needs to be calculated, and the introduced homologous sequences and enzyme cleavage sites should not be included in the calculation.

When synthesizing primer sequences, it is recommended to choose PAGE purification method, which is beneficial for improving cloning efficiency.

Specific primer design can refer to the following examples:

Example: Primer design for amplifying multiple insertion fragments (using HindIII and EcoRI double enzyme digestion linearized vectors as an example)

Linearization vector sequence (green and purple indicate upstream and downstream homologous arm regions, bold indicates enzyme cleavage site sequence):

The design for inserting fragment amplification primers is (blue and pink indicate overlapping sequences between fragments):

Fragment1 :

Primer F: 5′-gaggcttacgcagcaggt+AAGCTT(optional)+**tgccagtggcgata**-3′

Primer R: 5′-taactca...acacgag-3′

Fragment2 :

Primer F: 5′-ctcgtgt...tgagttattagtttcaacgctg-3′

Primer R: 5′-ttaacta...tgcatag-3′

Fragment3 :

Primer F: 5′-ctatgca...tagttaacggtcaccgtgctggtat-3′

Primer R: 5′-gctcgggtagatcgtcttga+GAATTC(optional)+**ctgcgccttatccggtaa**-3′

2.2. PCR amplification of exogenous DNA fragments

Suggest using high fidelity DNA polymerase for amplification of exogenous DNA fragments; There is no need to consider whether there is an A tail at the end of the product, which does not affect the final splicing accuracy and efficiency. Similar to the process of carrier preparation, if the PCR amplification band of exogenous DNA is single, purification is not necessary and it can be directly used for subsequent splicing reactions. When the purity of PCR amplification products is low, it is recommended to perform gel recovery purification, which is beneficial for improving recombination efficiency.

Note: If the template used for preparing exogenous DNA is a circular plasmid with the same resistance as the subsequent vector to be spliced, the residual exogenous plasmid template will cause a large amount of transformation background, affecting the selection of target clones, and therefore the plasmid template needs to be removed; The target fragment can

be recovered by electrophoresis or the circular plasmid with methylation modification can be removed by Dpn I enzyme digestion.

2.3. Recombination reaction

Prepare the reaction system on ice, react at 37℃ for 5-30 minutes (set 1-2 insertion fragments for 5-15 minutes); 3-5 insertion segments set for 15-30 minutes. After the reaction is complete, place the reaction product on ice for 5 minutes to terminate the reaction. The reaction products can be directly used for conversion experiments or frozen at -20℃ for future use.

Component	Volume
2×fast Multi One-Step Cloning kit	10μL
Linearized vector	20ng/kb (minimum 40ng)
Exogenous DNA	Insert fragments at 40ng/kb (minimum 20ng/fragment)
ddH_2O	Up to 20μL

Note: Suggest setting up a negative control, that is, the above system does not add 2×fast Multi CloneMix; If many clones grow from the negative control, it indicates that there are problems such as incomplete linearization of the vector or incomplete removal of the template plasmid for exogenous fragment amplification during the preparation of the vector or exogenous insertion fragment, and it needs to be re prepared or purified.

The optimal molar ratio of the recommended carrier and exogenous insertion fragment is n (carrier): n (insertion fragment)=1:2; It is recommended to calculate the dosage directly based on the fragment length without calculating the molar concentration. Taking a 4kb vector as an example, the dosage is 20ng/kb×4kb=80ng, and the minimum dosage for vectors below 2kb is 40ng;

Taking a 1kb insertion fragment as an example, the usage amount is 40ng/kb×1kb=40ng. For exogenous insertion fragments below 0.5kb, the minimum usage amount is 20ng. It is recommended to use instruments with precise temperature control, such as PCR machines, for the reaction. Differences in reaction temperature or duration can greatly affect the efficiency of cloning.

2.4. Recombinant product conversion

Thawing clones on ice using competent cells (such as DH5 α competent cells).

Take 10μL of the above reaction solution and add it to 100μL of competent cells. Gently mix well and let it stand on ice for 30 minutes.

After being heated in a 42℃ water bath for 90 seconds, immediately place it on ice to cool for 2-5 minutes.

Add 900μL of SOC or LB liquid culture medium (without antibiotics) and shake at 37℃ for 45 minutes to rejuvenate.

Centrifuge the rejuvenated bacterial solution at 5000 rpm for 5 minutes, discard 800μL of supernatant, and suspend the bacterial body with the remaining culture medium. Use a sterile coating rod to coat the bacterial solution on a plate containing the correct resistance, and invert it in a 37℃ incubator for 12-16 hours.

The clones grown on the tablet can be quickly identified using the "colony PCR method". For positive clones, further sequence determination can be carried out according to experimental requirements.

Note: The maximum conversion volume of recombinant products should not exceed 1/10 of the volume of competent cells used.

2.5. Positive clone identification

2.5.1. Colony/bacterial liquid PCR identification: Use a pipette to pick up a single colony and mix it with 10μL of sterile water, then take 1μL as a template, or directly dip the colony into the PCR system for amplification (it is recommended to use at least one universal primer to avoid false positive results);

2.5.2. PCR identification using plasmids as templates: Select monoclonal antibodies into LB medium containing corresponding antibiotics, shake overnight at 37℃ and 220 rpm, extract plasmids as templates, and amplify using vector universal primers or specific primers;

2.5.3. Enzyme digestion identification (if necessary): Select monoclonal antibodies into LB medium containing corresponding antibiotics, shake overnight at 37℃ and 220 rpm, extract plasmids, use corresponding

endonucleases to cleave plasmids, and detect fragment size by electrophoresis;

2.5.4. Sequencing: It is recommended to use vector universal primers for sequencing and identification.

Note

1. When enzyme digestion is used to prepare linearized vectors, in most cases, there is no need to recover the vector. However, for larger exogenous templates, recovering the vector can improve cloning efficiency and positivity rate.
2. Partial restriction endonucleases produce sticky ends, and their linearized carriers can be directly transformed into E. coli cells for repair, resulting in false positive clones. In response to this situation, it is recommended to replace the enzyme, perform double enzyme digestion, or increase the number of validated clones.
3. When preparing linearized vectors or exogenous fragments by reverse PCR amplification, if the PCR product is single, it can be directly used for subsequent recombination reactions. If the PCR product is not single, it should be purified by nucleic acid electrophoresis with gel recovery; When preparing linearized vectors by reverse PCR amplification, circular plasmids are used as templates, and the PCR products need to be treated with Dpn I to reduce the impact of residual circular plasmid templates on the cloning positivity rate.
4. Due to the presence of salt ions in the recombination reaction system, the reaction products cannot be directly electrocuted. If electric shock conversion is required, desalination treatment of the reaction products is necessary. Due to the limited amount of DNA used for the reaction, it is not recommended to use ethanol precipitation or gel to recover pure DNA
5. The desalination process can be carried out using a filter membrane suitable for micro DNA dialysis.
6. When directly using enzyme digestion products or PCR amplification products for recombination reactions, it is recommended to add no more than 1/5 of the total volume. If the exogenous DNA fragment is obtained by PCR amplification and directly used for recombination reaction without recovery and purification, it is also recommended that the reaction volume should not exceed 1/5 of the total volume. If neither the exogenous DNA fragment nor the linear vector is purified, the sum of their volumes should be less than 1/5 of the total volume.
7. In order to achieve higher cloning efficiency and positivity rate during the recombination reaction, it is recommended that the molar ratio of linear vector to exogenous fragment be 1:2.
8. This product is for scientific research use only.