ClonExpress Ultra One Step Cloning Kit V2

C116

Vazyme

Instruction for Use

Version 23.1

Vazyme

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01/Product Description

The ClonExpress technology is simple, fast, and highly efficient DNA seamless cloning technology. It enables rapid directional cloning of inserts into any site in any vector. Use any method to linearize the vector, and introduce the end sequence of the linearized vector at the 5' end of the insert forward/reverse amplification primer, so that the 5' and 3' ends of the PCR product have the same ends sequence (15 - 20 bp) as the linearized vector, respectively. The PCR product with the same sequence as the end of the vector and the linearized vector are mixed in a certain proportion. Under the catalysis of recombinase, the transformation can be performed at 50°C for 5 - 30 min to complete the directional cloning.

ClonExpress Ultra One Step Cloning Kit V2 is a new generation of recombinant cloning kits, compatible with 1 - 5 fragments homologous recombination. Highly optimized 2 × CE Mix further significantly improves the recombination efficiency of multiple fragments. This product has broader compatibility with the GC content of homology arms, which better guarantees the success rate of cloning in more difficult application scenarios.

02/Components

Components	C116-01 (20 rxns)	C116-02 (40 rxns)
2 × CE Mix	100 μl	2 × 100 μl
500 bp control insert (20 ng/μl)	5 μl	5 μl
pUC19 control vector，linearized (50 ng/μl，Amp^+)	5 μl	5 μl

03/Storage

Store at -30 ~ -15°C and transport at ≤0°C.

04/Applications

◇ Fast Cloning

◇ High-throughput Cloning

◇ Seamless Cloning

◇ DNA Site-directed Mutagenesis

05/Self-prepared Materials

PCR templates, primers, linearized vectors.

High-fidelity polymerase: 2 × Phanta Max Master Mix (Vazyme #P515) or other equivalent Vazyme's products.

Competent cells: Chemically competent cells prepared by cloning strains.

Other materials: ddH_2O, PCR tubes, PCR machine, etc.

06/Notes

For research use only. Not for use in diagnostic procedures.

07/Mechanism & Workflow

07-1/Workflow

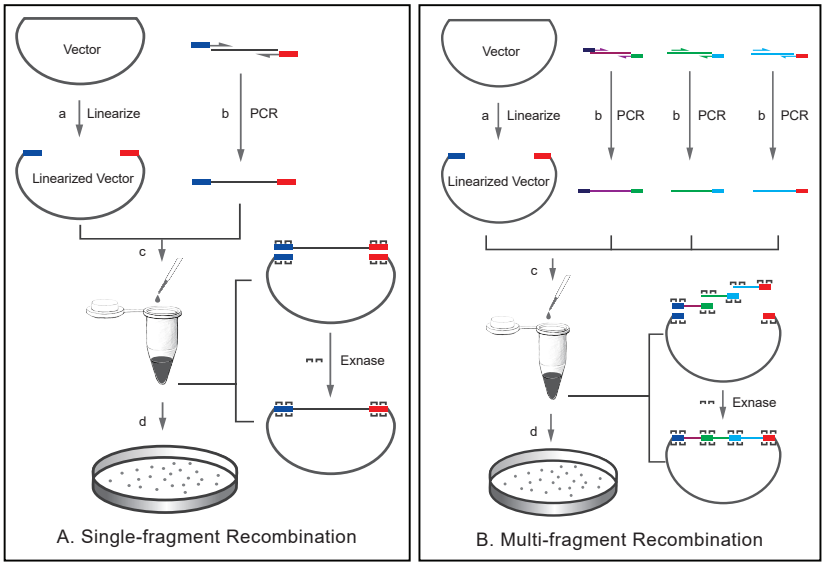


Fig 1. Mechanism of Homologous Recombination (ClonExpress Ultra One Step Cloning Kit V2)

a. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by Inverse PCR.
b. Acquisition of Inserts: Prepared by PCR. The amplification primers used need to add homologous sequences (marked in dark blue, green, light blue and red in the figure) at the 5' end of the primers, so that there are 15 - 20 bp homologous sequences between the amplification products and between the amplification products and the linearized vector, respectively.
c. Recombination: Mix the linearized vector and all inserts at an appropriate ratio, and then incubate the mixture with Exnase (2 × CE Mix) at 50°C for 5 - 30 min to complete recombination reaction and realize the in vitro circularization of multiple linearized DNA fragments.
d. Transformation: The recombinant product can be directly used for transformation, and then form hundreds of single clones on the plate for later positive screening.

07-2/Preparation of Linearized Vectors

1. Select appropriate cloning site to linearize the vector. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region is 40% - 60% both in the upstream and the downstream 20 bp regions flanking the cloning site.
2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by Inverse PCR.

◇ When preparing the linearized vector by enzyme digestion, it is recommended to use double enzyme digestion method to make the vector linearized completely, while single enzyme digestion linearization is not the first choice. For restriction endonuclease digestion, please prolong the digestion time appropriately to reduce the circular plasmid residue, thereby decreasing the transformation background (false positive clone).

◇ When preparing the linearized vector by Inverse PCR, it is highly recommended to use high-fidelity DNA polymerase (2 × Phanta Max Master Mix, Vazyme #P515) for vector amplification to reduce the introduction of amplification mutations. It is also recommended to use 0.1 - 1 ng circular plasmids or pre-linearized plasmids as PCR templates to reduce the influence of the residual circular plasmid template on the positive rate of clones in the 50 μl PCR system.

▲ When the template is the circular plasmid, digest amplification products with *Dpn* I and then perform gel extraction and purification to reduce the influence of the residual circular plasmid template on the positive rate of clones.

07-3/Preparation of Inserts

The insert can be amplified with most PCR enzymes (Taq enzyme or high-fidelity enzyme), regardless of whether there is an A tail at the end of the product (it will be removed during recombination and will not appear in the final vector). However, in order to reduce the introduction of amplification mutations, it is recommended to use 2 × Phanta Max Master Mix (high-fidelity polymerase; Vazyme #P515) for amplification.

1. The primer design principles for single-fragment homologous recombination: Introduce the homologous sequence of linearized vector (15 - 20 bp, excluding restriction endonuclease sites) into 5' ends of both Forward & Reverse primer. Thereby, the ends of amplified inserts and linearized vectors are identical to each other.

   Forward primer of insert fragments:

   5' - homologous sequence of upstream vector end + restriction endonuclease site (optional) + sequence of gene specific forward amplification primer - 3'

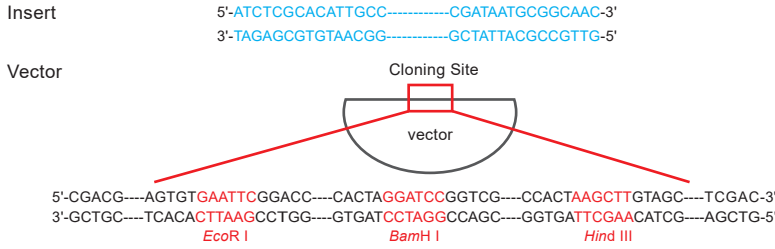
   Reverse primer of insert fragments:

   5'- homologous sequence of downstream vector end + restriction endonuclease site (optional) + sequence of gene specific reverse amplification primer - 3'

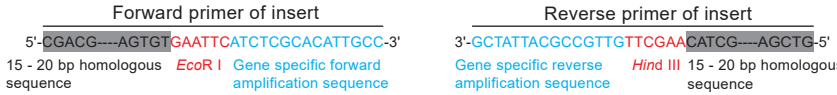
   ▲ Gene-specific forward/reverse amplification primer sequence refers to the forward/reverse amplification primer sequence of regular insert fragments. Tm value of 60 ~ 65°C is recommended.

   ▲ Homologous sequences of vector upstream or downstream end refer to the terminal sequence of the linearized vector (for homologous recombination). GC content of 40% - 60% is recommended.

It is recommended to use Vazyme's online primer design software - CE Design (available at http://www.vazyme.com) for designing primers. The CE Design can automatically generate amplification primers of inserts. For manually design, please refer to the principle below:

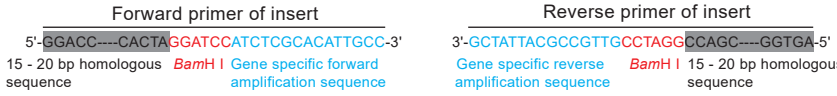


A: Linearized by double digestion (e.g., *Eco*R I + *Hin*d III)



▲ The restriction sites of *Eco*R I and *Hin*d III keep intact after cloning.

B: Linearized by single digestion (e.g., *Bam*H I):



▲ Both ends of the insert keep intact *Bam*H I restriction sites after cloning.

C: Linearized by Inverse PCR

Fig 2. Primer Design for Single-fragment Homologous Recombination

▲ If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency.

2. The general principle of primer design for multi-fragment homologous recombination: Introduce homologous sequences of linearized vector (15 - 20 bp, excludes restriction endonuclease sites) into 5' ends of both Forward & Reverse primers, respectively. Thereby, the ends of amplified inserts and the linearized vector are identical to each other.

   It is recommended to use Vazyme's online primer design software - CE Design (available at http://www.vazyme.com) for designing primers. The CE Design can automatically generate amplification primers of inserts. For manually design, please refer to the principle below:

The specific design program of primers is as follows:

◇ The design of the primers between fragments on both sides and the recombination end of the vector:

Forward primer of the most upstream fragment:

5' - homologous sequence of vector upstream end + restriction enzyme cutting site (optional) + sequence of gene specific forward amplification primer - 3'

Reverse primer of the most downstream fragment:

5' - homologous sequence of vector downstream end + restriction enzyme cutting site (optional) + sequence of gene specific reverse amplification primer - 3'

▲ Gene-specific forward/reverse amplification primer sequence refers to the forward/reverse amplification primer sequence of regular insert fragments. Tm value of 60 ~ 65°C is recommended.

▲ Homologous sequences of vector upstream or downstream end refer to the terminal sequence of the linearized vector (for homologous recombination). GC content of 40% - 60% is recommended.

◇ Three ways to design primers between inserts in the middle are as follows:

a. Introduce homologous sequences (15 - 20 bp) from 3' end of previous fragment into 5' end of the latter fragment;

b. Introduce homologous sequences (15 - 20 bp) from 5' end of the latter fragment into 3' end of the previous fragment (as shown in Fig 3);

c. Take a part of each of the two inserts as a homologous sequence (15 - 20 bp) and add them to the end of the other fragment, respectively.

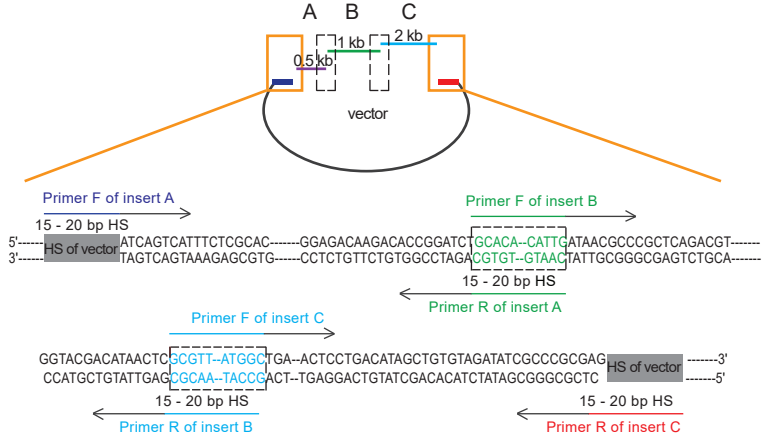


Fig 3. Primer Design for Multi-fragment Homologous Recombination

▲ Primer design of both side fragments and the recombination end of the vector (Refer to Fig 2. Primer design for single-fragment homologous recombination).

▲ If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency.

08/Experiment Process

08-1/Recombination

1. The concentration determination of linearized vectors and inserts

   Suppose the high-quality gel DNA recovery kit has purified the linearized vectors and inserts, and there is no obvious nonspecific band or smear residue after gel electrophoresis. In that case, instruments based on absorbance, such as Onedrop, can be used to determine the DNA concentration, but the results of concentration are only reliable when A260/A280 value is 1.8 - 2.0. It is recommended to determine concentration by Nanodrop, Onedrop, Qubit, PicoGreen, etc. When the sample concentration is lower than 10 ng/μl, the concentration values obtained by different models of instruments based on A_{260} may have large differences.

2. The calculation of vectors and inserts usage

   For single-fragment homologous recombination, the optimal amount of vector required is 0.03 pmol, the optimal amount of insert required is 0.06 pmol (the molar radio of vector to insert is 1:2). For multi-fragment homologous recombination, the optimal amount of inserts and linearized vectors are both 0.03 pmol (the molar radio of vector to insert is 1:1).

   These mass can be roughly calculated according to the following formula:

   ◇ **Single-fragment homologous recombination**

   The optimal mass of vector required = [0.02 × number of base pairs] ng (0.03 pmol)

   The optimal mass of insert required = [0.04 × number of base pairs] ng (0.06 pmol)

   ◇ **Multi-fragment homologous recombination**

   The optimal mass of vector required = [0.02 × number of base pairs] ng (0.03 pmol)

   The optimal mass of each insert required = [0.02 × number of base pairs] ng (0.03 pmol)

   ▲ For single-fragment homologous recombination: The mass of amplified insert should be more than 20 ng. When the length of the insert is larger than that of the vector, the calculation method of the optimal mass of vector and insert should be inverted.

   ▲ For multi-fragment homologous recombination: The mass of each insert should be more than 10 ng. When the optimal mass calculated by the above formula is below 10 ng, just use 10 ng directly.

   ▲ For single-fragment homologous recombination: If there are no obvious non-specific bands or smear shows in gel electrophoresis, the DNA can be directly used without purification and the total volume of vectors and inserts should be ≤2 μl (1/5 of the total volume of recombination reaction system), which will reduce the recombination efficacy (Purification is recommended before recombination).

3. Prepare the following reaction on ice:

Components	Recombination	Negative control-1[b]	Negative control-2[c]	Positive control[d]
Linearized Vector[a]	X μl	X μl	0 μl	1 μl
Insert[a] (n≤5)	$Y_1+Y_2…+Y_n$ μl	0 μl	$Y_1+Y_2…+Y_n$ μl	1 μl
2 × CE Mix	5 μl	0 μl	0 μl	5 μl
ddH_2O	to 10 μl	to 10 μl	to 10 μl	to 10 μl

a. X/Y is the amount of vector/insert calculated by formula. For ensuring the accuracy of pipetting, dilute the vector and the insert at an appropriate ratio before preparing the recombination reaction system, and the amount of each component is not less than 1 μl.
b. It is recommended to use negative control-1, which can confirm whether there is the residue of circular plasmids in linearized cloning vectors.
c. It is recommended to use negative control-2, when the templates are circular plasmids and share the same antibiotic resistance with the cloning vector.
d. Positive controls can be used to exclude the influence of other materials and operations.

4. Gently pipette up and down for several times to mix thoroughly (DO NOT VORTEX!). Briefly centrifuge to collect the reaction solution to the bottom of the tube.

5. Single-fragment homologous recombination: Incubate at 50°C for 5 min and immediately chill the tube at 4°C or on ice.
   2 - 3 fragments homologous recombination: Incubate at 50°C for 15 min and immediately chill the tube at 4°C or on ice.
   4 - 5 fragments homologous recombination: Incubate at 50°C for 30 min and immediately chill the tube at 4°C or on ice.
   ▲ It is recommended to perform the reaction on an instrument with precise temperature control such as a PCR machine.
   ▲ This product is compatible with the input amount of 0.01 - 0.25 pmol vectors and inserts, so when the total volume of vectors and inserts is greater than 5 μl, the input amount can be appropriately reduced, but the reaction time should not exceed the recommended time.
   ▲ The recombination product can be stored at -20°C for one week. Thaw the product before transformation.

08-2/Transformation

1. Thaw the competent cells on ice (e.g., Fast-T1 Competent Cell).

2. Pipette 5 - 10 μl of the recombination products to 100 μl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX), and then place the tube still on ice for 30 min.
   ▲The volume of recombination products should be ≤1/10 of the volume of competent cells.

3. Heat shock at 42°C water bath for 30 sec and then immediately place on ice for 2 - 3 min.

4. Add 900 μl of SOC or LB liquid medium (without antibiotics). Then, shake at 37°C for 1 h at 200 - 250 rpm.

5. Preheat the corresponding resistant LB solid medium plates in a 37°C incubator.

6. Centrifuge the culture at 5,000 rpm (2,500 × g) for 5 min, discard 900 μl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile spreading rod to gently spread on an agar plate which contains appropriate selection antibiotic.

7. Incubate at 37°C for 12 - 16 h.

08-3/Recombinant Product Identification

① Colony PCR method: Pick several single colonies and mix them with 10 μl ddH_2O as the template; use appropriate forward and reverse primers for colony PCR identification.
▲Please use at least one primer from the vector for Colony PCR.

② Enzyme digestion method: Pick several single colonies and culture them overnight in a liquid medium with appropriate resistance, then extract the plasmid for restriction endonuclease digestion identification.

③ Sequencing identification: Sequencing analysis with appropriate primers on the vector.

09/FAQ & Troubleshooting

◇ How to design primers?

① Primer design: It is recommended to use online primer design software - CE Design, and select the corresponding module for design.

② Three parts of primers: Homology arms (15 - 20 bp, exclude restriction sites and base residues, the content of GC is 40% - 60%) + restriction sites (optional) + specific primers (when calculating the Tm value of primers, the homology arms should be excluded).

◇ Few clones or no clone are formed on the plate

① Incorrect primer design: The primer includes 15 - 20 bp homology arms (exclude restriction sites) and the content of GC is 40% - 60%.

② The amount of linearized vectors and amplified inserts are too low/high in the recombination reaction or the ratio is not appropriate. Please use the amount and ratio according to specification recommended.

③ Contamination in vector and insert inhibits the recombination: The total volume of unpurified DNA should be ≤2 μl (1/5 of the total volume of reaction system). It is recommended that the linearized vector and PCR products are purified by gel extraction. Then, dissolve the purified product in ddH_2O (pH 8.0).

④ The low efficiency of the competent cells: Make sure the transformation efficiency of competent cells is >10^8 cfu/μg. The simple test can be performed. Transform the 0.1 ng of plasmids and take the 1/10 for spreading plates. If 1,000 clones are grown, the estimated transformation efficiency is 10^8 cfu/μg. The transformation volume of the recombinant product should be ≤1/10 of the volume of competent cells, otherwise the transformation efficiency will be reduced. Choose competent cells used for cloning (such as Fast-T1/DH5α/XL10) not those for expressing.

◇ Most clones do not contain inserts or contain incorrect inserts

① Nonspecific amplification is mixed with PCR products: Optimize the PCR reaction system to improve the amplification specificity; purify PCR products for recombination by gel extraction; identify more colonies.

② Incomplete linearization of the vector: The negative control can be used to detect whether the vector is completely linearized. Optimize the digestion system, increase the amount of restriction endonucleases, prolong the time of digestion reaction, and purify the digestied products by gel extraction.

③ Plasmids with the same resistance mixed in reaction system: When the PCR amplification template is a circular plasmid, if the amplification product is directly used in the recombination reaction without purification, it is recommended to digest with *Dpn* I, or perform gel extraction to purify the amplification product.

◇ No target bands in Colony PCR

① Incorrect primer: It is recommended to use the universal primer of the vector for colony detection, or use at least one universal primer.

② Inappropriate PCR system or program: No bands of target or empty plasmid. It is recommended to optimize the PCR reaction system or program; or extract plasmids as templates to perform PCR identification; or perform enzyme digestion identification.

③ Unsuccessful recombination: There is only the band of the empty plasmid, indicating that the recombination was unsuccessful and the linearization of the vector was incomplete. It is recommended to optimize the restriction endonuclease digestion system.

Nanjing Vazyme Biotech Co.,Ltd.
Tel: +86 25-83772625
Email: info.biotech@vazyme.com
Web: www.vazyme.com
Loc: Red Maple Hi-tech Industry Park, Nanjing, PRC
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