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LiClone™ One Step Cloning Kit

Cat. #: M0010 Size: 25 rxns

Introduction

LiClone™ One Step Cloning Kit is simple, fast, and high efficient reagent which enables directional insertion of any amplified DNA product into any linearized vector at any site. Firstly, the vector is linearized at the cloning site. A small sequence overlapped with each end of the cloning site is added onto the insert through PCR. The insert and the linearized vector, with overlapped sequences of 15 bp - 20 bp on both 5'- and 3'-end, respectively, are mixed in an appropriate ratio and incubated with recombinantExnase at 50°C for 5 - 15 min.The kit is applicable for homologous recombination with 1 - 4 fragments. The kit is independent of DNA ligase, significantly reducing the self-ligated colonies. The highly optimized 5× Clone Master Mix significantly improve the recombination efficiency and the tolerance to impurities, enabling the insert to be used directly for recombination without any treatments to simplify the procedure.

Package Information

Component	M0010
5× Clone Master Mix	50 µl

Storage

At -20°C and avoid repeated free-thaw.

Additional Materials Required

1. linearized vector
2. DNA template, primers
3. High-fidelity DNA polymerase (Cat # M0031)
4. Competent cells
5. Other materials: ddH_2O, PCR tubes, PCR instrument, *etc.*

Preparation before the experiment and precautions

1. Preparation of Linearized Vectors: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

2. Acquisition of Inserts: Introducing homologous sequences of 15 bp - 20 bp (highlighted in blue and orange) into 5'-end of Forward (F) & Reverse (R) primer, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.

3. Recombination: Mix the linearized vectors and inserts at an appropriate ratio and incubate with Exnase at 50°C for 5-15 min for recombination reaction and to make two linearized DNA cyclized invitro.

4. Transformation: The recombination products can be used for transformation directly.

Protocol

1. Preparation of Linearized Vectors

1.1. Select an appropriate cloning site on the vector that will be linearized. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region (within 20 bp up and downstream of the site) stays between 40% and 60%.

1.2. Vector linearization: the linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

Double digestion is recommended because it brings complete linearization and low false positive rate. If single digestion is adopted, a longer digestion time is necessary to reduce intact plasmid residues and decrease the false positive rate.

When using reverse PCR amplification to obtain linearized vector, it is highly recommended to use a high-fidelity DNA polymerase (Cat # M0031) for vector amplification to reduce the PCR error rate. It is also recommended to use 0.1 ng - 1 ng circular plasmids or pre-linearized plasmids as PCR templates to reduce the false positive rate caused by residual circular plasmids in a 50 µl PCR reaction system.

Note: When the PCR templates are circular plasmids, digesting the amplification products with Dpn I to reduce the false positive rate caused by residual circular plasmids is recommended.

2. Acquisition of Inserts

2.1. The primer-design principles: Introduce homologous sequences of linearized vector (15 bp -20 bp, excludes restriction enzyme cutting sites) into 5'-end of both Forward (F) & Reverse (R) primer, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.

The primer-design for single-fragment homologous recombination

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

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

Note: Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insert. Tm of 60 - 65°C is recommended.

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

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The primer-design for mutli-fragment homologous recombination

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

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

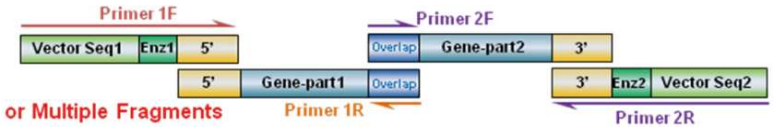
Three types of primer design of middle insert:
A. Introduce homologous sequences (15 bp - 20 bp) from 3'-end of previous fragment into 5'-end of forward primer;

B. Introduce homologous sequences (15 bp - 20 bp) from 5'-end of the latter fragment into 5'-end of reverse primer;

C. Introduce homologous sequences from both fragments (total 15 bp - 20 bp) into 5'-end of forward / reverse primer, respectively.

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

When calculating the Tm of primers, the homologous sequence of vector ends and restriction enzyme cutting site should be excluded and only gene specific amplification sequence should be used.



2.2. PCR of the inserts

Inserts can be amplified by any polymerase (i.e., Taq DNA polymerase or high-fidelity polymerase). It will not interfere with the recombination efficiency whether there is A-tail in the PCR products or not. To prevent possible mutations introduced during PCR, high-fidelity polymerases (Cat # M0031) are highly recommended.

3. The Amount of Linearized Vectors and Inserts

3.1 Determination of DNA concentration:

If the linearized vectors and inserts have been purified by high quality gel DNA recovery kit, and there is no obvious nonspecific band or smear after gel electrophoresis, instruments based on absorbance, i.e., Onedrop, can be used to determine the DNA concentration, but the results of concentration are only reliable when A260/A280 value is between 1.8 and 2.0.

For samples with DNA concentration lower than 10 ng/μl, Qubit® fluorescence-based assay is recommended.

3.2 The calculation of amount of vectors and inserts:

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)

For example, when cloning an insert of 1 kb to a vector of 5 kb, the optimal mass of vector is 0.02 × 5000 =100 ng, and that of insert is 0.04 × 1000 = 40 ng.

For samples with DNA concentration lower than 10 ng/μl, Qubit® fluorescence-based assay is recommended.

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 example, when cloning inserts of 0.5 kb, 1 kb and 2 kb to a vector of 5 kb, the optimal mass of vector and three inserts are as follows:
The optimal mass of linearized vector required: 0.02 × 5000 = 100 ng;
The optimal mass of insert of 0.5 kb required: 0.02 × 500 = 10 ng;
The optimal mass of insert of 1 kb required: 0.02 × 1000 = 20 ng;
The optimal mass of insert of 2 kb required: 0.02 × 2000 = 40 ng.

Note: a. 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.

b. 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 under 10 ng, just use 10 ng.

c. The amount of linearized vectors should be between 50 ng - 200 ng. When the optimal amount calculated using the above formula is beyond these ranges, just choose the maximum or minimum amount for recombination.

d. For simple or single-fragment homologous recombination: if there are no obvious nonspecific 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).

4. Recombination

4.1 The amount of DNA can be roughly calculated according to the above formula.

Dilute the vector and insert at an appropriate ratio to ensure the accuracy of pipetting before recombination, and the amount of each component is not less than 1 μl.

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4.2 Prepare the following reaction on ice:

Components	Recombination	Negative control-1[b]	Negative control-2[c]
Linearized Vector[a]	X μl	X μl	0 μl
Inserta (n≤4)	Y1-Yn μl	0 μl	Y1-Yn μl
5× Clone Master Mix	2 μl	0 μl	0 μl
ddH_2O	to 10 μl	to 10 μl	to 10 μl

a. X/Y is the amount of vector/insert calculated by formula.

b. It is recommended to use negative control-1, which can confirm the residue of cyclic plasmid template.

c. It is recommended to use negative control-2, when the templates are circular plasmids which share the same antibiotic resistance with the cloning vector.

4.3 Gently pipette up and down for several times to mix thoroughly (**DO NOT VOTEX!**). Spin briefly to bring the sample to the bottom of the tube before reaction.

4.4 Incubate at 50°C for 5-15 min and immediately chill the tube at 4°C or on ice.

Note: a. It is recommended to use a PCR instrument for the reaction.

b. The recombination product can be stored at -20°C for one week. Thaw the product before transformation.

5. Transformation

5.1 Place the competent cells on ice.

5.2 Pipet 5 - 10 μl of the recombination products to 100 μl of competent cells, flip the tube for several times to mix thoroughly (**DO NOT VOTEX!**), and then place the tube still on ice for 30 min.

Note: The volume of transformation products should not be more than 1/10 of the volume of competent cells.

5.3 Heat-shock the tube at 42°C for 45 sec and then immediately chill on ice for 2 - 3 min.

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

5.5 Place the LB plate which contains appropriate selection antibiotic at 37°C.

5.6 Centrifuge the culture at 5,000 rpm for 5 min, discard 900 μl of supernatant. Then, re-suspend the pellet with 100 μl of remaining medium and plate it on an agar plate which contains appropriate selection antibiotic.

5.7 Incubate at 37°C for 12-16 h.

6. Selection of Positive Colonies

6.1 After overnight culture, hundreds of mono-colonies will form on the transformation plate of recombination reaction, whereas fewer of those on the transformation plate of negative control (Fig 1).

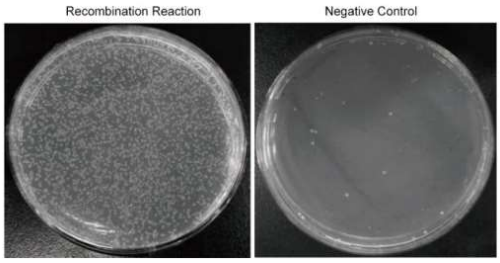

Fig 1. Plate incubated overnight

6.2 Pick several mono-colonies from the plate of recombination reaction for colony PCR with at least one common sequencing primer of the vector. If the colony is positive, there should be a band which length is slightly bigger than that of insert (Fig 2).

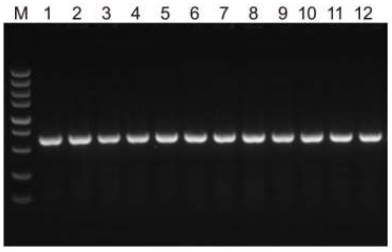

Fig 2. Agarose Gel Electrophoresis of Colony PCR

6.3 Inoculate the remaining medium of positive clones into fresh LB medium and culture overnight. Then, extract the plasmids for further enzyme digestion analysis (Fig 3) or DNA sequencing.

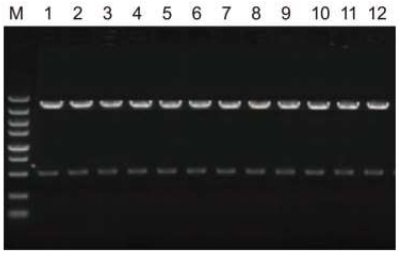

Fig 3. Agarose Gel Electrophoresis of Enzyme Digestion Products

Tips

1. Place the recombination products on ice and transform it to competent cells directly. Commercial super competent cells (transformation efficiency over 10^8 cfu/μg) are highly recommended. The volume of transformation products should not be more than 1/10 of the volumeof competent cells.

2. LiClone™ One Step Cloning Kit is applicable for efficient clone of fragments of 50 bp - 10 kb.

3. Usages of inserts and vectors:

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Cloning fewer or short fragments (< 5 kb)

a. Linearized vectors, prepared by restriction digestion, can be directly used for recombination after inactivating the restriction enzymes. Inactivation is available for most restriction enzymes, please refer to instruction of specific inactivation methods.

b. For linearized vectors prepared by reverse PCR, if the amplification templates are pre-linearized and PCR products show singer band, the PCR products can be used directly for recombination without purification.

c. For inserts, if the yield and amplification specificity of the PCR products is confirmed by agarose electrophoresis and the templates are not circular plasmids which share the same antibiotic resistance with the cloning vector, high specific PCR products can be directly used.

d. For recombination without further purification, please refer to Table 1 / Table 2 for the usages of linearized vectors and inserts prepared in different ways.

Table 1. Usages of Linearized Vectors

Method of Linearization		Template Type	Fast Protocol	Standard Protocol
Digestion		Circular Plasmid	Use directly after inactivating restriction enzymes	Gel Recovery
Reverse PCR	Specific Amplification	Circular Plasmid	Use directly after *Dpn I* digestion (degrade the PCR template)	Gel recovery or gel recovery after *Dpn I* digestion
		Pre-linearized Plasmid, Genomic DNA, cDNA	Use directly	Gel Recovery
	Non-specific Amplification	Gel Recovery		

Table 2. Usages of Ampified Inserts

Amplification Specificity	Template Type	Fast Protocol	Standard Protocol
Specific Amplification	Circular plasmids sharing the same antibiotic resistance with the cloning vector	Use directly after *Dpn I* digestion	Gel recovery or gel recovery after *Dpn I* digestion
Non-specific Amplification	Pre-linearized plasmid, genomic DNA, cDNA	Use directly	Gel Recovery
		Gel Recovery	

e. After Dpn I digestion, the amplified inserts should be incubated at 85°C for 20 min to deactivate Dpn I, so as to prevent cloning vectors from degradation when recombination.

Cloning large fragment (> 5 kb)

It is recommended to purify the linearized vectors and amplified inserts with high quality gel DNA recovery kit before recombination, so as to improve the DNA purity and eliminate residual circular vectors.

Troubleshooting

1. Few clones or no clones formed on the plate

a. Improper primer design: primer includes 15 bp - 20 bp homologous sequences (exclude restriction sites); the content of GC is 40% - 60%.

b. The amount of linearized vectors and amplified inserts are too low/high in the recombination reaction or the ratio of fragments is not appropriate. Please use the amount and radio as specification recommended.

c. Contamination in vector and insert inhibits the recombination: The total volume of unpurified vector and insert digested should be ≤ 2μl (≤1/5 of the total volume of recombination reaction system). Gel extractionpurification is recommended to purify the vector and insert. It isrecommended to dissolve the purified DNA in ddH_2O.

d. The low efficiency of the competent cell: Make sure the transformation efficiency of competent cells is >10^8 cfu/μg. Transform 1 ng vector and pick 1/10 of that to transform, and there are 1000 colonies growing on the plate. Then, the transformation efficiency of competent cells can be estimated as 10^8 cfu/μg. The volume of transformation products should not be more than 1/10 of the volume of competent cells. Choose competent cells used for cloning (such as DH5α/XL10) not those for expressing.

2. Incorrect/none inserts found in the colony plasmids

a. Non-specific amplification is mixed with target inserts: Optimize the PCR reaction system to improve the amplification specificity; purify the PCR products with a gel recovery kit; select more colonies for verification.

b. Incomplete linearization of the vector: Approaches to overcome such situation include using negative controls to confirm the complete linearization of vectors, improving the amount of restriction endonuclease, prolonging the digesting time, and purifying the digesting products before the recombination reaction.

c. Plasmids with the same resistance with vectors mixed in reaction system: When the PCR templates for amplification of vectors or inserts are circular plasmids, digesting the amplification products with Dpn I or purifying them by gel recovery can both effectively reduce or even eliminate the residues of cyclic plasmid templates.

3. No electrophoretic bands in colony PCR

a. Improper primer: it is recommended to use at least one common sequencing primer of the vector.

b. Inappropriate PCR system or program: No bands of targets or empty plasmids. It is recommended to optimize the PCR reaction system or program; extract plasmids as PCR templates or use enzyme digestion for confirmation.

c. Unsuccessful recombination: There is only the band of empty plasmid after colony PCR, which indicates the unsuccessful recombination and incomplete linearization of the vector. One of the approaches to overcome such situation is to optimize the enzyme digestion system.