Seamless Cloning Master Mix

Cat.:BR1C101

V02

Product Description

Seamless Cloning Master Mix is a simple, fast and efficient universal seamless cloning product, which can quickly and directionally clone inserts to any site of any vector. Enabling highly efficient homologous recombination of 1 - 15 DNA fragments. Seamless Cloning Master excels particularly in rapid cloning, high-throughput cloning, seamless cloning and site-directed DNA mutagenesis.

Components

Components	BR1C101-01 20 T	BR1C101-02 40 T	BR1C101-03 20 T×5
Seamless Cloning Master Mix	0.1 mL	0.2 mL	5×0.5 mL

Storage

Store at-20±5°C.

Notes

1.For Research Use Only. Not for use in diagnostic procedures.

2.Please thaw and mix thoroughly before use. Avoid repeated freezing and thawing.

3.Vector and insert fragment should be linearized by enzyme digestion or PCR amplification before assembly. A homologous sequence (15-25 bp) set at both ends of the vector and the insert fragment, and the insert fragment have the same homologous sequence. The GC content in the homologous region can be 40%-60%.

4.The amount of vector 0.03 pmol=[0.02×vector base pairs]ng, insert single fragment 0.06 pmol=[0.04×insert fragment base pairs]ng, insert multiple fragments, each fragment 0.03 pmol=[0.02× insert fragment base pairs] ng.

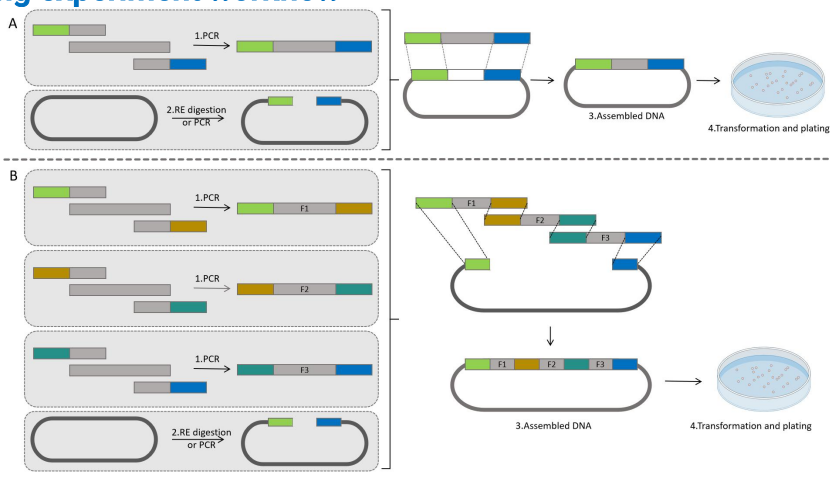
5.In order to ensure the accuracy of sample addition, the linearized vector and insert fragment can be appropriately diluted before the homologous recombination reaction system is configured, and the sample amount of each component is not less than 1 μL.

6.After the reaction, products should be placed on ice and transformed immediately. If it cannot be converted in time, it can be stored at -20°C.

Materials

1. RCR templates, primers, linearized vectors.
2. High-fidelity polymerase.
3. Competent cells: Chemically competent cells prepared by cloning strains.
4. Other materials: ddH_2O, PCR tubes, PCR machine, etc.

Seamless cloning experiment workflow



1.PCR Fragment Amplification: Add a 15-20 bp homologous sequence to the 5' end of the amplification primer, which is shown in color in the figure, to ensure that there is a 15-20 bp homologous sequence at the ends of both the linearized vector and the inserted fragment.

2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or

by Inverse PCR.

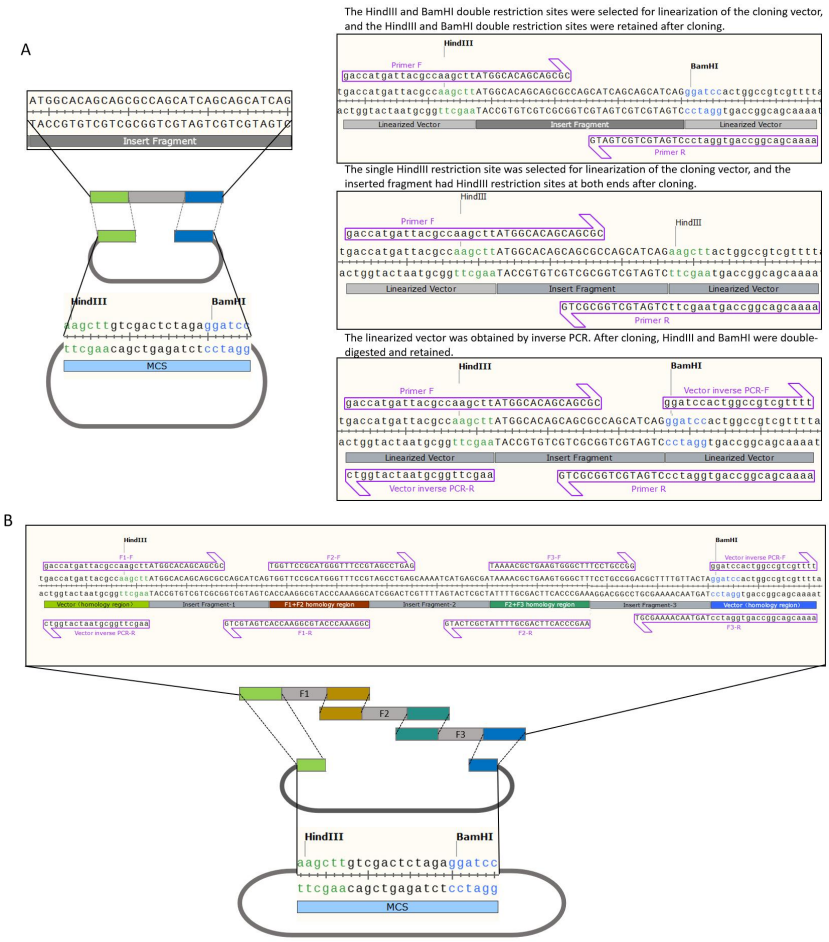
3.Recombination: Mix the linearized vector and all inserts at an appropriate ratio, and then incubate the mixture with Seamless Cloning Master at 50°C for 5-30 min to complete recombination reaction and realize the in vitro circularization of multiple linearized DNA fragments.

4. Transformation: The recombinant product can be directly used for transformation, and then form hundreds of single clones on the plate for later positive screening.

PCR primer design

Introduce two-terminal homologous sequences of the linearized vector at the 5' and 3' ends of the forward and reverse amplification primers of the inserted fragment. Make the 5' and 3' ends of the amplified inserted fragment respectively carry homologous sequences consistent with the two ends of the linearized vector. The length of the homologous part is 15-20 bp, and the homologous part does not contain restriction sites. For multi-fragment homologous recombination, 15-20 bp homologous recombination regions need to be designed both between the inserted fragments and between the inserted fragments and the linearized vector. The recommended Tm value for forward and reverse amplification of inserted fragments is 60-65°C. If the primer length exceeds 40 nt, it is recommended to choose PAGE purification during primer synthesis.

The design of single-fragment and multi-fragment homologous recombination primers is shown in Figures A and B as follows:



Protocol

Linearized Vector Prepare

The upstream and downstream 20 bp of the cloning site of the vector should be free of repetitive sequences, and the GC content should be between 40-60%.

Linearization of the vector can be obtained in several ways:
Single restriction endonuclease digestion: The digestion time can be appropriately extended to ensure thorough digestion and reduce the conversion background.
Double restriction endonuclease digestion: The double restriction method is recommended to allow the vector to be fully digested and to reduce the transformation background.
Inverse PCR: It is recommended to use high-fidelity polymerase to amplify the vector sequence to avoid introducing mutation sites. The use of linearized plasmids as amplification templates is recommended to reduce the residual circular plasmid template and reduce the transformation background.

Insert Fragment Prepare

The primer design can be referred to above. The Insert Fragment can be amplified by using Taq enzyme or high-fidelity enzyme. The presence or absence of the A-tail at the end of the PCR product has no effect on the homologous recombination reaction. The A-tail will be removed during the homologous recombination process and will not appear in the circular vector after homologous recombination. To avoid the introduction of mutations during fragment amplification, the use of high-fidelity polymerase to amplify fragments is recommended.

Homologous Recombination Reaction

Determination of concentration of linearized vectors and inserted fragments: For the recovery and Purification of Gel or PCR products, it is recommended to use high-quality gel recovery kits (Gel DNA Purification Kit, Biori #BR2A103-01/02) for linearized vectors and inserted fragments. The presence of obvious impurity bands can be detected by electrophoresis, or the concentration can be determined using an instrument based on absorbance value. The concentration reliability is relatively high when A260/A280 is between 1.8 and 2.0. When the sample concentration is lower than 10 ng/μL, the concentrations measured by different detection instruments may vary. It is recommended to re-prepare the samples.
Calculation of the amount of linearization carrier and insertion fragment: Generally, the amount of linearization carrier is 0.03 pmol. In the single-fragment homologous recombination system, the amount of the inserted fragment is 0.06 pmol. In the multi-fragment homologous recombination system, the amount of inserted fragments was 0.03 pmol. The specific dosage can be roughly calculated by referring to the formula in the table below.

Type	Component	Optimal mass (ng)	Optimal quantity (pmol)	Proportion (carrier: fragment)
Single-fragment homologous recombination (vector length ＞ insertion fragment length)	Linearized Vector	[0.02×number of vector base pairs] ng	0.03 pmol	1：2
	Insert Fragment	[0.04×number of Insert Fragment base pairs] ng	0.06 pmol	
Single-fragment homologous recombination (vector length ＜ insertion fragment length)	Linearized Vector	[0.04×number of vector base pairs] ng	0.06 pmol	2：1
	Insert Fragment	[0.02×number of Insert Fragment base pairs] ng	0.03 pmol	
Multi-fragment homologous recombination	Linearized Vector	[0.02×number of vector base pairs] ng	0.03 pmol	1：1
	Insert Fragment-1	[0.02×number of Insert Fragment base pairs] ng	0.03 pmol	
	Insert Fragment-2	[0.02×number of Insert Fragment base pairs] ng	0.03 pmol	
	Insert Fragment-3	[0.02×number of Insert Fragment base pairs] ng	0.03 pmol	

For single-fragment homologous recombination reactions, the optimal amount of the inserted fragment should be greater than 20 ng. When the insertion fragment length was less than the linearized vector length, the molar ratio (vector: fragment) was 1:2. When the inserted fragment length is longer than the linearized vector length, the optimal amount of

vector and fragment calculation should be interchanged, that is, the molar ratio (vector: fragment) is 2:1. When the PCR product of the inserted fragment has no non-specific amplification band, it can be used directly without DNA purification. The recommended amount of sample in the homologous recombination system is 2 μL, but the efficiency of homologous recombination will be reduced. Therefore, we recommend recombination after purification.
For multi-fragment homologous recombination, the optimal amount of each insert should be more than 10 ng. If the amount calculated according to the formula in the above table is less than 10 ng, we recommend using 10 ng.
Homologous recombination experiment：It is recommended to configure on ice.

Components	Volume per Reaction
Seamless Cloning Master	5 μL
Linearized Vector	X μL
Insert Fragment	Y μL
ddH_2O	To 10 μL

X/Y is the amount of vector/insert calculated by formula. To ensure that the sample volume is accurate, it is recommended that samples with higher concentrations be diluted appropriately to ensure that the sample volume of each component is not less than 1 μL, thereby reducing operational errors.

Homologous recombination control experiment：

Components	Negative control-1	Negative control-2
Seamless Cloning Master	0 μL	0 μL
Linearized Vector	X μL	0 μL
Insert Fragment	0 μL	Y μL
ddH_2O	To 10 μL	To 10 μL

It is recommended to use negative control-1, which can confirm whether there is the residue of circular plasmids in linearized cloning vectors. If there was a single colony after transformation of the negative control-1 system, it indicated that there was residual circular plasmid, so as to judge whether the double or single enzyme digestion of the vector was complete, or whether the circular plasmid template in the reverse PCR amplification system existed.
It is recommended to use negative control-2, when the templates are circular plasmids and share the same antibiotic resistance with the cloning vector. If there was a single colony after transformation of the negative control-2 system, it indicated that there was a circular plasmid residue.
Reaction conditions for homologous recombination experiments:

The number of fragments	Reaction conditions	Optional optimization scope
1	50°C, 30 min	50°C, 5-30 min
2-3	50°C, 30 min	50°C, 15-30 min
4-5	50°C, 30 min	50°C, 30 min

After the reaction, immediately chill the tube at 4°C or on ice. The recombination product can be stored at -20°Cfor one week. Thaw the product before transformation.
It is recommended to perform the reaction on an instrument with precise temperature control such as a PCR machine.
The reaction time can be appropriately reduced when the number of homologous recombination fragments is small, and the reaction time is 30 min when the number of homologous recombination fragments is large. Take care that the reaction time does not exceed 30 min. With the extension of reaction time from 5-30 min, the number of colonies on the plate increased accordingly.

Transformation of homologous recombination products

1. Thaw the competent cells on ice.
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(for some competent cells, only an ice bath for 25 min is needed). The volume of recombination products should be ≤1/10 of the volume of competent cells.

Heat shock at 42°C water bath for 30 sec (the specific heat shock time varies depending on the type of competent cells,

please refer to the heat shock time provided in the competent cell manual) and then immediately place on ice for 2-3 min.

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

5. 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. Incubate at 37°C for 12-16 h.

Recombinant Product Selection

Colony PCR: Pick several single colonies and mix them with 10 μL ddH2O as the template. Use appropriate forward and reverse primers for colony PCR. Please use at least one primer from the vector for colony PCR.

Restriction enzyme digestion: Pick several single colonies and culture them overnight in a liquid medium with appropriate resistance, then extract the plasmid for restriction enzyme digestion.

Sanger Sequencing: Sanger sequencing with appropriate primers on the vector. Meanwhile, sanger sequencing can check whether mutations are introduced during recombination.

FAQ

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 ddH2O (pH 8.0).

4. The low efficiency of the competent cells: Make sure the transformation efficiency of competent cells is > 107 cfu/μg.

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. 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. Replace other identification methods: for example, plasmid double enzyme digestion identification.

4. 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.