Immunoway®
Best Way for Immunology

DNA Assembly Mix Ultra

REF: YS0223

Storage Condition

-20℃

Components

Component	Amount
DNA Assembly Mix Ultra	250 μl
pUC19 Control Plasmid, Linearized (Amp^r, 40 ng/μl)	5 μl
500 bp Control Fragment (20 ng/μl)	5 μl

Description

DNA Assembly Mix Ultra is a kind of seamless assembly method, digest, ligate or end repair are not required. This method has been used to assemble different sizes of DNA fragments with varied overlaps (15~30 bp). And it offers a easy, fast and efficient DNA cloning technique.

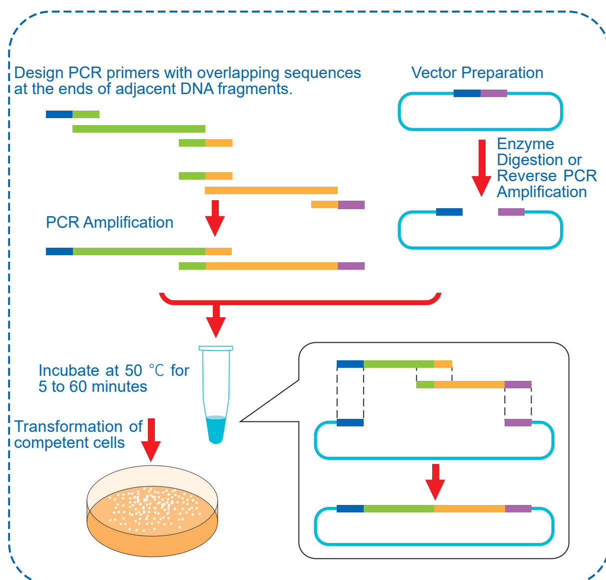
The DNA Assembly Mix Ultra allows for the assembly of one or multiple DNA fragments in a single reaction, with single-fragment insertion completed in as little as 5 minutes and a positive rate exceeding 95%. This Mix uses HiFi Taq DNA Ligase, which has higher fidelity compared to wild-type Taq DNA Ligase, significantly improving the success rate of seamless cloning. Additionally, the Mix includes a transformation enhancer, greatly increasing the number of transformants. While ensuring high fidelity and transformation efficiency, the DNA Assembly Mix Ultra has optimized its components to enhance stability, making it more stable under high temperature or oxidic conditions.

Applications

Rapid cloning; Multi-fragment DNA assembly; Site-directed mutagenesis.

Protocol

1. Summary of Experimental Workflow



2. Prepare Linearized Vector

Select suitable cloning sites and linearize the vector. The vector can be linearized through enzyme digestion or reverse PCR amplification.

① Linearized vector Acquire

Some restriction endonucleases may not digest supercoiled DNA completely, which result in part of vector DNA undigested, leading to decreased cloning efficiency. We recommend LightNing® Restriction Enzymes for digestion (single or double digestion) to ensure complete linearization of the vector and reduce false positive colonies.

Note 1: Linearized vectors obtained through enzyme digestion do not require dephosphorylation, and double digestion is recommended.

Note 2: After digestion, it is suggested to inactivate the restriction enzymes or purify the vector before the recombination reaction.

Note 3: Before gel extraction of the linearized vector, it is recommended to perform electrophoresis for a long time to distinguish it from the residual circular plasmids to reduce the false positive rate.

② PCR-Generated Vector Acquire

Recommend high-fidelity PCR Mix for amplification to reduce mutation. The template DNA should be linearized to reduce false colonies.

Note 1: When preparing linearized vectors using PCR, it is recommended to add 1 μl of LightNing™ DpnI (REF No. EG15585) to 50 μl of PCR products, incubate at 37℃ for 1 hour to remove the circular plasmid template, and then inactivate DpnI by incubating at 80 ℃ for 20 minutes. If no non-specific amplification is detected by electrophoresis of the PCR products, the DpnI-treated PCR products can be directly purified and used for the recombination reaction. Conversely, if non-specific amplification is present, it is recommended to perform gel extraction of the DpnI-treated PCR products to obtain specific PCR products.

Note 2: For multi-fragment cloning, it is advisable to purify the PCR products before use.

3. PCR Primer Design for Insert Fragment

The 5' end of PCR primers must contain a 15~25 nt (recommended 18 nt) sequence that is homologous to the adjacent fragment (insert fragment or vector) end. If the vector is digested by restriction enzymes and has a sticky end, the primer design must include the the 3´ end of the overhang, the primer design may or may not include the 5´ end of the overhang.

Forward Amplification Primer for Insert Fragment:

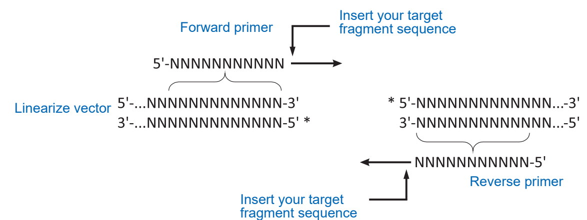
5'—Upstream vector homologous sequence + Restriction enzyme site (optional) + Gene-specific forward amplification sequence— 3'

Reverse Amplification Primer for Insert Fragment:

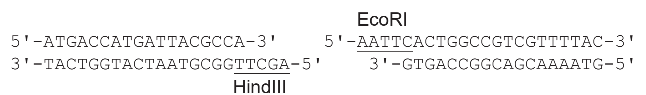
3'—Gene-specific reverse amplification sequence + Restriction enzyme site (optional) + Downstream vector homologous sequence— 5'

Note 1: Choose regions for cloning with minimal repetitive sequences and even GC content. The highest recombination efficiency is achieved when the GC content within the 25 nt region upstream and downstream of the vector cloning site is between 40% and 60%.

Note 2: When the assembled product exceeds 10 kb in length, it is recommended that the primer sequence overlapping with the adjacent fragment should be 20~25 nt in length.



Note 3: The end sequence for the provided pUC19 vector (Ampr) in this kit is as follows:



Note 4: Fragments containing multiple repeat elements cannot be assembled by the homologous recombination strategy.

4. PCR Amplification of Insert Fragment

Recommend high-fidelity PCR Mix for amplification to reduce mutation. Purified PCR products are advised for seamless cloning reactions. If the PCR products are identified as specific amplification products through agarose gel electrophoresis, they can be used directly. However, the volume of the sample added should not exceed 20% of the total reaction volume.

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5. Recombination Reaction

① Prepare the following reaction mixture on ice:

Component	Reaction System	Negative Control [c]	Positive Control (if necessary) [d]
DNA Assembly Mix Ultra	5 µl	5 µl	5 µl
Linearized Vector [a]	50~200 ng	50~100 ng	pUC 19 Control Plasmid, Linearized, 1 µl
Insert Fragment [b]	10~200 ng	-	500 bp Control, Fragment, 1 µl
ddH_2O	To 10 µl		

a. Optimal amount of vector (ng) = 0.02 × number of vector base pairs.

b. Optimal amount of insert fragment (ng) for a single fragment = 0.04 × number of fragment base pairs; for multiple fragments, optimal amount per fragment (ng) = 0.02 × number of fragment base pairs.

c. Negative control can confirm whether there are any residual circular plasmids in the linearized vector, while the positive control can eliminate the influence of other materials and operational factors.

d. Positive control can be used to eliminate the influence of other experimental materials and operational factors.

Note 1: If the length of the insert fragment is greater than that of the vector, exchange the amounts of vector and insert fragment.
Note 2: If the length of the insert fragment is less than 200 bp, use 5 times the amount of vector.
Note 3: If the calculated amounts exceed the minimum/maximum values, it is recommended to directly use the minimum/maximum amounts.
Note 4: The excessively long fragments to be assembled or too many fragments will reduce both the number of colonies and the positive rate.
Note 5: When using this product for single site-directed mutagenesis, the amount of PCR product containing the mutation site should be added according to the optimal vector usage. For discontinuous multiple site-directed mutagenesis, the amount of intermediate fragments added should be referenced to the optimal usage per fragment for multi-fragment assembly.

After preparing the recombination reaction system, gently pipette to mix the components, avoiding bubble formation.

② Incubate the reaction system at 50°C for 5~60 minutes.

Note 1: It is recommended to use a PCR machine or other accurate temperature-controlled instrument for the reaction. Insufficient time may reduce cloning efficiency.
Note 2: For inserting 1~2 fragments, a reaction time of 15 minutes is recommended; for 3~5 fragments, a reaction time of 30 minutes is suggested.
Note 3: If the vector backbone is above 10 kb or the insert fragment is above 4 kb, extend the reaction time to 30~60 minutes.

③ Place the reaction tube on ice for cooling, and then proceed with transformation or store at -20°C.

Note 1: Recombinant products stored at -20℃ are recommended to be used within 1 week.

6. Transformation of Recombinant Products

Take 5~10 µl of the reaction liquid and add it to 100 µl of competent cells. Mix gently and slowly, place on ice for 30 minutes. Heat shock at 42℃ for 60 seconds, then place on ice for 5 minutes. Add 500 µl of SOC or LB medium, shake at 37℃ for 50~60 minutes (200 rpm). Spread the bacterial solution evenly on a plate containing the corresponding antibiotic and incubate it upside down at 37℃ overnight.

Note 1: Different competent cells may yield different cloning efficiencies. It is recommended to use competent cells with transformation efficiency > 10^8 CFU/µg.
Note 2: The number of colonies depends on the quantity and purity of PCR products and linearized vector.
Note 3: Positive control plates usually show abundant white colonies, while negative control plates show very few colonies.

7. Positive Clone Assay

Pick a single colony and mix it in 10 µl of ddH_2O. After a 95℃ incubation for 10 minutes, take 1 µl of the lysate as a template for colony PCR identification. Alternatively, inoculate a single colony in antibiotic medium and incubate it overnight, then extract the plasmid for enzyme digestion identification.

For positive clone detection of the positive control, use primers M13F and M13R for colony PCR, and HindIII, EcoRI for enzyme digestion.

Note 1: If necessary, further sequence verification can be performed.
Note 2: M13F: TGTAAAACGACGGCCAGT
M13R: CAGGAAACAGCTATGAC

F&Q

Descriptipon	Reason	Solution
Low transformation efficiency	Low efficiency of competent cells	The transformation efficiency of competent cells should be at least >10^7 CFU/µg, and can be simply tested as follows: Use 0.1 ng of pUC19 plasmid to transform the competent cells. If 1000 colonies are obtained, the estimated transformation efficiency is 10^7 CFU/µg. When the assembled product is >10 kb, it is recommended to use competent cells with a transformation efficiency >10^8 CFU/µg, or competent cells specifically suited for the transformation of long fragments.
	Unfavorable ratio of DNA fragments	Prepare the reaction system according to the recommended optimal amounts and ratios in the instructions. Determine the concentrations of the vector and insert fragments as follows: If the linearized vector and insert fragment have been purified and show a single band or no remaining smear on agarose gel electrophoresis, their concentrations can be measured using instruments based on spectrophotometric methods. However, the concentration value is reliable only when the A260/A280 ratio is between 1.8 and 2.0. If the linearized vector and insert fragment have not been purified, sample concentrations can be measured using agarose gel electrophoresis.
	Insufficient purity of DNA fragments	Perform gel extraction purification for both the vector and insert fragments. As chelating agents such as EDTA can inhibit seamless cloning reactions, the purified products should be dissolved in ddH_2O. Do not use Tris-EDTA or similar buffer solutions.
	Excessive reaction products	In the transformation system, the volume of seamless cloning reaction products should not exceed 10% of the volume of competent cells.
Large numbers of clones lack the insert fragment	Incomplete linearization of the vector	When preparing linearized vectors using restriction enzymes, increase the amount of the fast restriction enzyme used, extend the reaction time, and perform gel extraction purification of the digestion product.
	Contamination with identical resistant plasmids	When performing PCR amplification of the insert fragment using plasmids as templates, use pre-linearized plasmids as amplification templates, and treat the amplification products with methylation-sensitive restriction enzymes such as DpnI, or perform gel extraction purification of the products.
	Insufficient antibiotic resistance on plates	Ensure the correct use of antibiotics and use freshly prepared antibiotic plates.
Large numbers of clones contain incorrect insert fragments.	Non-specific PCR amplification products	Optimize the PCR system to improve amplification specificity, or perform gel extraction purification of PCR products containing overlapping sequences of primers.
	DNA containing repeat elements	If the fragments contain multiple repeat elements, it is recommended to use the digestion-ligation or Golden Gate method for assembly.