Rapid Multi-fragment DNA Seamless Cloning Premix

Cat. No.: R1522799 | Pack size: 50 reactions | Storage: -20°C

Product Information

Attribute	Value
Pack Size(s)	50 reactions
Specifications & Purity	BioReagent, 2×
Stability And Storage	Store at -20°C long term (24 months).
Storage Conditions	Store at -20°C
Shipped In	Ice chest + Ice pads. This product requires cold chain shipping. Ground and other economy services are not available.
Grade	BioReagent

Product Description

Seamless cloning technology based on recombination principle is a new-generation cloning method. It does not rely on tedious enzyme digestion and ligation procedures, nor end blunting operations. By recombination of 15–25 nt homologous sequences at the ends of DNA fragments and linearized vectors, inserts can be cloned into any site of any vector with extremely low vector self-ligation background. It is a simple, rapid and efficient directional DNA cloning technique.

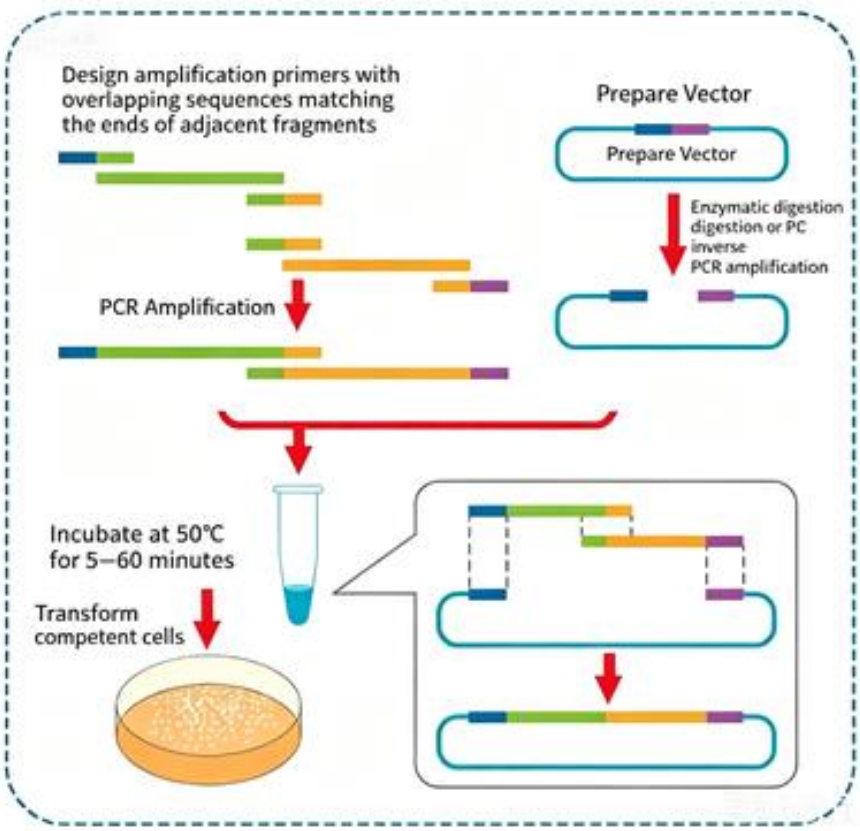
This kit completes recombination of single or multiple DNA fragments in one reaction. Single-fragment recombination can be finished in as little as 5 minutes with a positive rate higher than 95%. HiFi Taq DNA Ligase is adopted in the master mix. Compared with ordinary Taq DNA Ligase, it features higher fidelity and significantly improves seamless cloning success rate. Meanwhile, transformation enhancers are added into the master mix to greatly increase the number of transformants. With guaranteed fidelity and transformation efficiency, the kit components are further optimized for superior stability and tolerance to heat and oxygen environments.

Product Applications

Rapid cloning; multi-fragment DNA assembly; site-directed DNA mutagenesis.

Experimental Procedures

1. Overview of Experimental Workflow



2. Preparation of Linearized Cloning Vector

Select appropriate cloning sites to linearize the vector. Vector linearization can be achieved by enzymatic digestion or inverse PCR amplification.

(1) Enzymatic Digestion Preparation

Some restriction endonucleases cannot efficiently digest supercoiled DNA, which may leave undigested vector DNA and reduce positive rates. Rapid restriction endonucleases are recommended for single or double digestion to ensure complete vector linearization and reduce transformation background (false-positive clones generated by undigested vectors).

Note 1. Vectors linearized by digestion do not require dephosphorylation, and double digestion is preferred.

Note 2. After digestion, inactivate endonucleases or purify vectors before recombination reactions.

Note 3. During vector gel recovery, perform prolonged electrophoresis to distinguish residual circular plasmids before gel cutting to reduce false positives.

(2) Inverse PCR Amplification Preparation

High-fidelity PCR Mix is recommended to avoid amplification mutations. Pre-linearized plasmids are suggested as templates to reduce interference of residual circular plasmids on cloning positive rates.

Note 1. If PCR products have no non-specific bands, add 1 μL DpnI into 50 μL PCR products, incubate at 37 ℃ for 1 h and 80 ℃ for 20 min to digest plasmid templates for direct recombination. Otherwise, purify PCR products by gel extraction.

Note 2. Purify PCR products for multi-fragment cloning.

3. PCR Primer Design for Insert Fragments

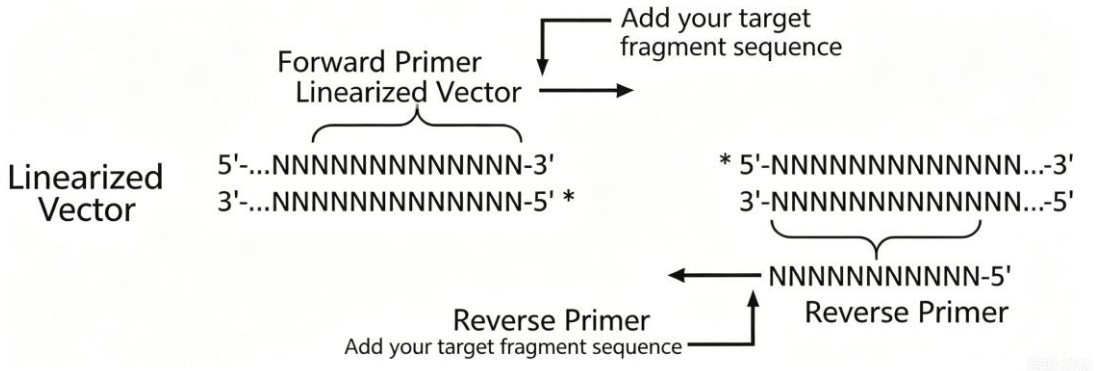
The 5' end of PCR primers must contain 15–25 nt (18 nt recommended) homologous sequences matching the ends of adjacent fragments (inserts or vectors). For sticky-ended vectors with 3' overhangs, primers must include the overhang region. For 5' overhangs, primers may or may not contain the overhang sequence.

Forward primer for insert amplification: 5'-Upstream vector homologous sequence + Restriction site (optional) + Gene-specific forward sequence-3'

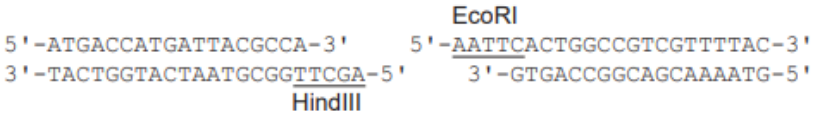
Reverse primer for insert amplification: 3'-Gene-specific reverse sequence + Restriction site (optional) + Downstream vector homologous sequence-5'.

Note 1. Clone regions with few repetitive sequences and uniform GC content. Recombination efficiency peaks when GC content within 25 nt upstream and downstream of vector cloning sites is 40%–60%.

Note 2. For recombinant products larger than 10 kb, design primers with 20–25 bp homologous arms.



Note 3. Ligation terminal sequences of pUC19 vector (Amp$_r$) provided in this kit are as follows.



Note 4. Fragments with multiple repetitive sequences cannot be ligated via seamless cloning.

4. PCR Amplification of Insert Fragments

Use high-fidelity PCR Mix to minimize amplification mutations. Purified PCR products are preferred for seamless cloning. Specific PCR products identified by agarose gel electrophoresis can be used directly, but the loading volume shall not exceed 20% of the total reaction system.

5. Recombination Reaction

(1) Prepare the following reaction system on ice bath:

Component	Reaction System	Negative Control[c]	Positive Control (If Required)[d]
Rapid Multi-fragment DNA Seamless Cloning Premix	5 μL	5 μL	5 μL
Linearized Vector[a]	50~200 ng	50~100 ng	pUC 19 Linearized Control Plasmid, 1 μL
Insert Fragment[b]	10~200 ng	-	500 bp Control Fragment, 1 μL
ddH_2O	To 10 μL	To 10 μL	To 10 μL

a. Optimal vector dosage (ng) = 0.02 × vector base pairs, equivalent to 0.03 pmol.

b. For single insert: optimal fragment dosage (ng) = 0.04 × fragment base pairs. For multiple inserts: optimal dosage per fragment (ng) = 0.02 × fragment base pairs.

c. Negative control verifies residual circular plasmids in linearized vectors and is highly recommended.

d. Positive control eliminates interference from other experimental materials and operations.

Note 1. Swap vector and insert dosages if single insert is longer than the vector.

Note 2. Use 5× vector dosage for inserts shorter than 200 bp.

Note 3. Adopt minimum or maximum dosage when calculated amount exceeds the range limit.

Note 4. Excessively long vectors, inserts or large fragment numbers reduce colony count and positive rate.

Note 5. Apply optimal vector dosage for single-site mutation; apply multi-fragment dosage per site for multi-site mutations.

After system preparation, gently mix components by pipetting to avoid bubbles. Do not vortex.

(2) Incubate the reaction system at 50 ℃ for 5–60 min.

Note 1. Use accurate temperature-controlled instruments such as PCR instruments. Insufficient incubation reduces cloning efficiency.

Note 2. 15 min for 1–2 fragments; 30 min for 3–5 fragments.

Note 3. Extend incubation to 30–60 min for vector backbones >10 kb or inserts >4 kb.

Note 4. Briefly centrifuge to collect reaction liquid at tube bottom after 50 ℃ incubation.

(3) Cool reaction tubes on ice, then proceed to transformation or store at -20 ℃.

Note: Recombination products stored at -20 ℃ should be used within 1 week.

6. Transformation of Recombinant Products

Add 5–10 µL reaction liquid into 100 µL competent cells, mix gently and incubate on ice for 30 min. Heat shock at 42 ℃ for 60 s, then ice bath for 5 min. Add 500 µL SOC or LB medium, incubate at 37 ℃ with shaking (200 rpm) for 50–60 min. Spread bacterial suspension evenly on antibiotic-containing plates, and incubate inverted at 37 ℃ overnight.

Note 1. Positive rates vary with competent cells. Competent cells with transformation efficiency >10^8 CFU/µg are recommended.

Note 2. Colony quantity depends on quantity and purity of PCR products and linearized vectors.

Note 3. A large number of white single colonies grow on positive control plates, while few colonies grow on negative control plates.

7. Positive Clone Identification

Pick single colonies and resuspend in 10 µL ddH_2O. Lyse at 95 ℃ for 10 min, use 1 µL lysate as template for colony PCR. Alternatively, inoculate colonies into resistant medium, culture overnight and extract plasmids for restriction digestion identification.

For positive control clone identification: use universal primers M13F/M13R for colony PCR, and HindIII/EcoRI for digestion assay.

Note 1. Use at least one universal primer in colony PCR to avoid false positives.

Note 2. Sequencing identification is available for further verification if necessary.

Note 3. M13F: TGTAAAACGACGGCCAGT; M13R: CAGGAAACAGCTATGAC.

Troubleshooting

Problem Description	Cause	Solution

Problem Description	Cause	Solution
Low Transformation Efficiency	Low Competent Cell Efficiency	The transformation efficiency of competent cells shall be at least >10^7 CFU/μg. A simple verification can be performed: transform 0.1 ng pUC19 plasmid, and 1000 grown colonies indicate an estimated transformation efficiency of 10^7 CFU/μg. For recombinant products larger than 10 kb, competent cells with 10^8 CFU/μg efficiency, or specialized competent cells for large plasmid DNA and recombinant product transformation, are recommended.
Low Transformation Efficiency	Improper Ratio of DNA Fragments	Prepare the reaction system in accordance with the recommended dosage and ratio in the instruction manual. Concentration detection of linearized vectors and inserts: for purified fragments with single clear bands and no smear residue on electrophoresis, spectrophotometric instruments such as NanoDrop can be used for concentration measurement. Concentration results are reliable only when the A260/A280 ratio is between 1.8~2.0. Agarose gel electrophoresis can be used to determine sample concentration for unpurified linearized vectors and inserts.
Low Transformation Efficiency	Insufficient Purity of DNA Fragments	Perform gel extraction purification on vectors and inserts. Metal ion chelators such as EDTA will inhibit seamless cloning reactions. Dissolve purified products in ddH_2O, and avoid using buffers including Tris-EDTA.
Low Transformation Efficiency	Excessive Reaction Product	In the transformation system, the volume of seamless cloning reaction mixture shall not exceed 10% of the competent cell volume.
Most Clones Have No Insert Fragment	Incomplete Vector Linearization	When preparing linearized vectors via enzymatic digestion, increase the dosage of rapid restriction endonuclease, extend the reaction duration, and purify digested products by gel extraction.

Problem Description	Cause	Solution
Most Clones Have No Insert Fragment	Contamination of Identical Resistant Plasmids	When amplifying inserts via PCR with plasmid templates, use pre-linearized plasmids as templates. Treat PCR products with DpnI methylation-sensitive endonuclease, or purify amplified fragments by gel extraction.
Most Clones Have No Insert Fragment	Insufficient Plate Antibiotic Resistance	Ensure the correct antibiotics are used, and adopt freshly prepared antibiotic agar plates.
Most Clones Contain Incorrect Inserts	Non-specific PCR Amplification Products	Optimize the PCR system to improve amplification specificity, or purify PCR fragments via gel extraction.
Most Clones Contain Incorrect Inserts	Fragments With Multiple Repetitive Sequences	For inserts containing abundant repetitive sequences, use competent cells specialized for repetitive DNA fragment cloning, or adopt restriction ligation or GoldenGate cloning methods.

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