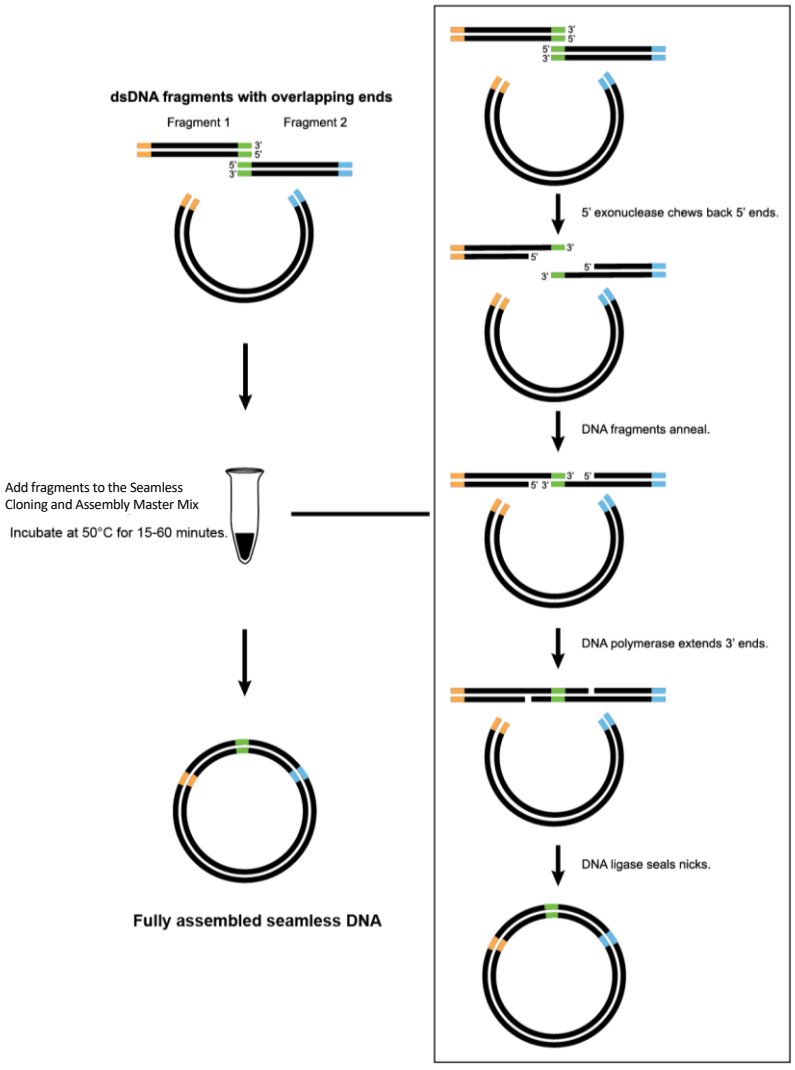
Seamless Cloning & Assembly Master Mix

Catalog No.: NP100054
Size: ：100 ul
Concentration: 2X

1. Introduction

The Seamless Cloning & Assembly Master Mix is a simple, fast, and efficient DNA seamless cloning method that allows connecting multiple overlapping DNA fragments into a joined DNA molecule without the use of restriction enzyme cloning. To perform seamless assembly cloning, primers are designed to generate DNA insert fragment(s) and a linearized vector by PCR with appropriate overlapping (15-25 bp homologous) ends to adjacent fragments. The inserted fragment(s) and vector DNA are combined with OriGene's Seamless Cloning and Assembly Master Mix reagents and incubated at 50°C for 15-60 minutes. During incubation, specific enzymatic reactions facilitate the creation of a fully assembled DNA construct. (Figure 1).

This product can be used for the simultaneous assembly of up to five different inserts with a linearized vector in a single tube reaction, regardless of compatible digestion sites. The final products are fully ligated DNA constructs that are ready to transform into competent cells or used in further PCR or RCA cloning application. The buffer components and enzymes in the 2X Master Mix are optimized to enhance the efficiency and robustness of assembly reaction.



2. Components and Storage

Store all the components at -20°C until ready to use. Before use, fully thaw the reagents and mix thoroughly. Keep on ice to avoid repeating freeze-thaw cycles.

Components	Catalog No.
Seamless Cloning and Assembly Master Mix	NP100054

3. Applications

Rapid one vector to one insert cloning
Assembly of multiple DNA fragments
Site-direct mutagenesis
High-throughput library construction

4. Additional Materials Required, But Not Supplied

- Templates for fragment amplification, primers, and linearized vectors.
- High-fidelity DNA polymerase (to amplify fragments).
- *Dpn*I (removal of template plasmid DNA).
- Competent cells. For the plasmid size ≤10 kb, we recommend DH5α chemically competent cells in the transformation (CC100003). For the larger plasmid that are more than 10 kb, XL10 chemically competent cells are recommended.
- Nuclease-free water, PCR tubes, Thermocyclers, LB plates with selective resistances.

5. Summary

- Design primers to amplify DNA fragments (or vector) with appropriate overlapping ends.
- Use high-fidelity DNA polymerase in PCR amplification.
- Linearize a vector by PCR amplification or restriction enzyme digestion.
- Quantify concentration of DNA fragments with agarose gel electrophoresis, Nanodrop™ or other methods.
- Add the appropriate amount of DNA fragments to the 2X Seamless Cloning and Assembly Master Mix and incubate the reaction mixture at 50°C for 15 to 60 minutes. The incubation time depends on the number of fragments in assembly
- Transform directly into *E. coli* competent cells.

6. Tips for Seamless Assembly Cloning

6.1 Vector Linearization

- Generally, a cloning vector can be linearized by inverse PCR amplification or restriction enzyme digestion. The linearization cloning site should locate at the position with an appropriate GC content (40% to 60% in the ±20 bp region), avoiding tandem repeats and high secondary structures.
- When a vector is linearized by restriction enzyme digestion, double digestion is highly recommended to lower false-positive background. In the case of using single enzyme digestion, we recommend extending the digestion time to reduce background from uncut plasmid.
- For a linearized vector generated by inverse PCR, we strongly recommend using Origene's PCR Master Mix (2X) (NP100056) with HF Buffer in the amplification. In a 50 µl PCR reaction, the amount of circular plasmid template should not exceed 1 ng in order to reduce the false positive rate; alternatively, pre-linearized templates are recommended.

6.2 Inserts

- General rules for primer design: Introduce a compatible sequence at the 5' end of each primer in the length of 15-25 bp, the homologous overlap regions created in PCR facilitate the annealing of adjacent fragments in the seamless assembly.
- We use the following case to illustrate how to assemble two inserts, in the length of 0.5kb and 1.0 kb, to a pUC19 vector linearized by *EcoR*I and *Hind*III restriction enzymes (Figure 2a).
- The design strategy of flanking-end primers is shown in Figure 2b. The sequence components (5'-3' direction) in the Fragment B forward primer are overlap regions to the left-arm of linearized pUC19,

restriction enzyme site, and the homologous sequence to the FragB. In the Fragment C reverse primer, from 5' to 3' end, the sequence components are overlaps to the right arm of linearized pUC19, restriction enzyme site, and homologous sequence to the FragC.

- The T_m values of flanking primer is recommended in the range between 60°C and 65°C.
- The GC contents in the overlap regions should be more than 40% and less than 60%.

- Three methods of designing primers for the inserts:

a. Use the 3' end (15 bp – 25 bp) of the upstream fragment as the homologous sequence and add it to the 5' end of the downstream fragment.

b. Use the 5' end (15 bp – 25 bp) of the downstream fragment as the homologous sequence and add it to the 3' end of the upstream fragment (see Figure 2c)

c. Select one part from each of the two neighboring fragments (total of 15 bp – 25 bp) as the homologous sequence and add to the corresponding ends of the other fragments.

- Although insert fragments can be amplified using any polymerases (Taq polymerase or other high-fidelity polymerase), high fidelity polymerases are highly recommended to minimize potential mutations.

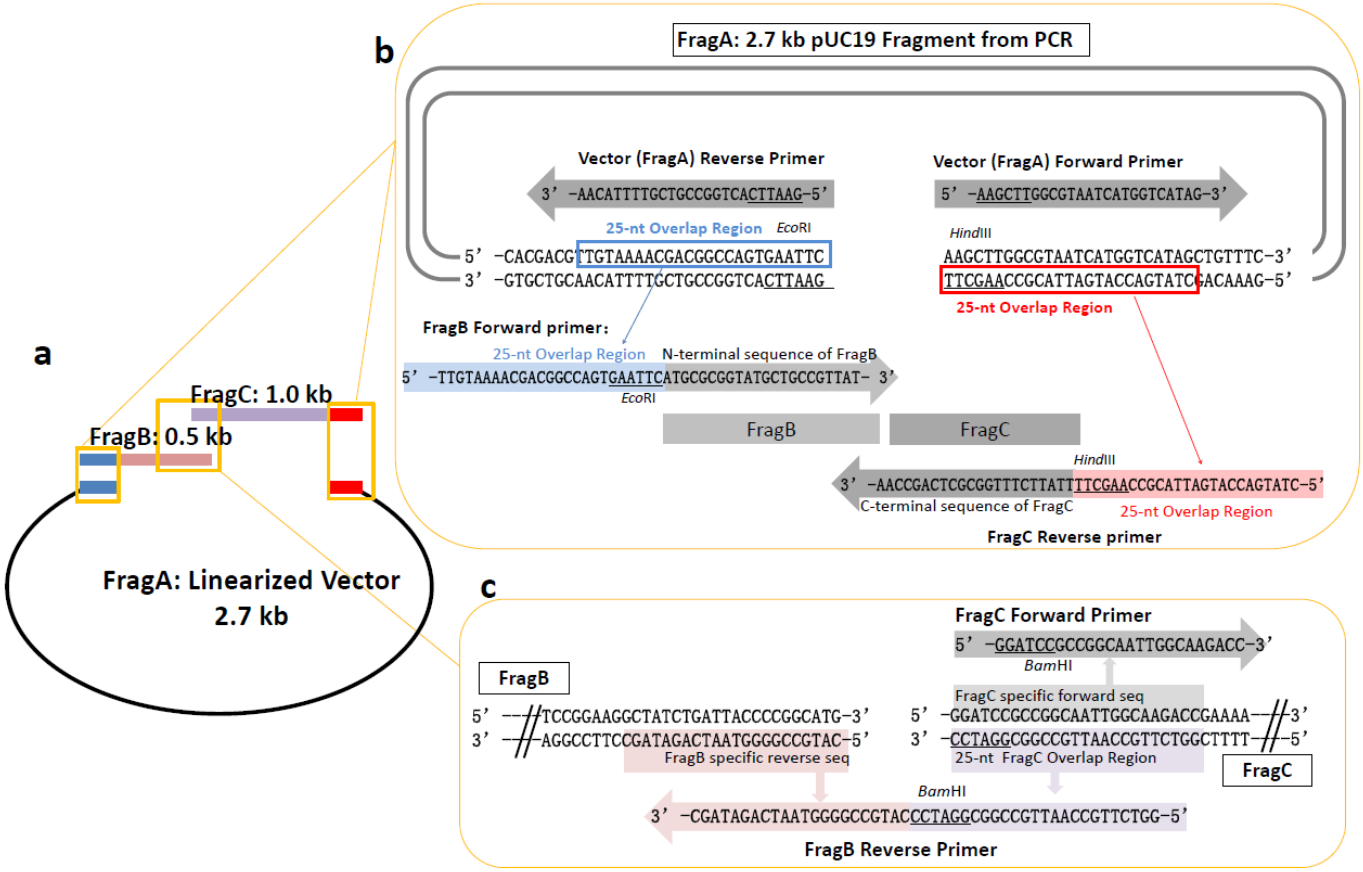


Figure 2：Primer design protocol of 2.7 kb pUC19 vector and 0.5 kb, 1.0 kb inserts.

6.3 *Dpn*I Digestion (optional)

When a large amount of plasmid template is used in the PCR reaction, *Dpn*I digestion is recommended to remove the template, because DpnI only digests plasmid DNA methylated by *E. coli* Dam methylase.

6.4 Calculating the amount DNA fragments in seamless assembly.

In a typical seamless assembly reaction, we recommend using 0.02-0.5 pmols of total DNA for 1-3 fragments and 0.2-1.0 pmols of total DNA for 4-6 fragments.

As the number or length of fragments increases, the assembly efficiency will decrease. The number of pmols per fragment can be calculated by the length and mass, using the following equation:

pmols = (Mass ng) x 1000 / (base pairs x 650 Daltons)

50 ng 5000 bp dsDNA is approximately 0.015 pmols.

50 ng 500 bp dsDNA is approximately 0.15 pmols.

6.5 Assembly

✧ Mix the following reaction on ice.

	Recommended Amount of Fragments Used for Assembly	
	2-3 Fragment Assembly	4-6 Fragment Assembly
Total Amount of Fragments	0.02-0.5 pmols* X µl	0.2-1 pmols X µl
H_2O	10-X µl	10-X µl
Seamless Cloning and Assembly Master Mix (2X)	10 µl	10 µl
Total Volume	20 µl**	20 µl**

* The cloning efficiency is maximized when the molar ratio of inserts to a vector is 3:1. When the insert sizes are smaller than 200 bp, for better results, we recommend using the insert 5-fold molar more than a vector. When using unpurified PCR fragments, the added volume should not exceed 20% of the total volume.

**If a larger amount of fragments is used for assembly, a larger volume of the Seamless Cloning and Assembly Master Mix should be added.

Reaction protocol

	2–3 Fragment Assembly	4–6 Fragment Assembly
Reaction Temp.	50 °C	50 °C
Reaction Time	15 min	60 min

Note: In general, the incubation time should not exceed 60 minutes. After incubation, place the reactions on ice and transform immediately. In some cases, extending the incubation time will enhance the efficiency, but it should not be more than 60 minutes for most cases. If the reaction mixture cannot be transformed in time, keep at -20 °C for long-term storage.

6.6 Transformation

- Thaw the chemically competent cells (such as DH5α competent *E. coli* cells) on ice.
- Add 5 µl of the assembled product to 100 µl of competent cells, mix by gently tapping the tube (do not shake and mix), and place on ice for 30 minutes.
- The volume of the assembled product should not exceed 1/6 of the volume of the competent cells.
- Heat-shock in a 42°C water bath for 45 seconds and place on ice for 2-3 minutes immediately.
- Add 900 µl SOC or LB medium (without antibiotics) to the competent cells and incubate at 37°C for 1 hour (200-250 rpm).
- Warm the LB plates with the selective antibiotic in a 37°C incubator.
- Centrifuge at 5,000 rpm for 5 min and discard the 900 µl of supernatant.
- Resuspend the bacteria using the remaining medium and gently spread it on a LB plate with selective antibiotic.
- Incubate at 37°C for 12-16 hours.

6.7 Validation

- After overnight incubation, hundreds of clones will form on the transformation plate, while the number of clones on the negative control plate should be significantly less.
- Pick several clones from the transformation plate for colony PCR identification and the amplification primer should include one universal sequencing primer from the vector. If the clone is correct, the size of the amplicon should be longer than the insert size. Plasmids can also be validated by restriction digest or sequencing.

7. Precautions

- Competent Cells: The transformation efficiency of various

competent cells may differ by several orders of magnitude; the success rate of cloning is directly related to the efficiency of competent cells.

- Electroporation: Electroporation can increase transformation efficiency by several orders of magnitude. When using the resulting product from Seamless Cloning and Assembly Master Mix for electroporation, the product must be diluted 3-folds with water. 1 µl of the diluted product should be used in transformation with 50 µl of electric competent cells.
- DNA: If the volume of the PCR product used is less than 20% of the total volume of the Seamless Cloning and Assembly reaction, the PCR product can be used without purification. If the volume of the PCR product used is too high, the assembly and transformation efficiency will decrease due to carryover PCR reaction mix and primers. Column purification of PCR products is recommended to increase the efficiency of Seamless Cloning and Assembly by 2-10 folds. Therefore, when the number of fragments is larger than 3, or the fragment length is longer than 5 kb;
  column purification is strongly recommended. The purified DNA product is eluted in ddH2O or other low-EDTA buffers.
- Insert fragments: When the fragments are directly assembled into the cloning vector, the molar concentration of the inserts should be 2-3 times more than that of the vector. When assembling more than 3 fragments, it is recommended to use the similar molar concentration of fragments.
- Biological considerations: Some kinds of DNA with inverted or tandem repeats, are incompatible in *E. coli*. For the inserts carrying toxic genes, less or smaller colonies will exhibit as a result.

8. Frequently Asked Questions

Why is the transformation efficiency low? (no colonies or a small number of colonies)

- Inappropriate primer design. The primers should include a homologous region (15 bp - 25 bp) with 40-60% GC content.
- The molar ratio between inserts and vector is not optimized. Refer to the molarity ratio suggested in the protocol.
- Carryover inhibition from add-in PCR products. The volume of unpurified DNA should not exceed 20% of the volume of the assembly reaction. It is recommended to purify the linearized vector and PCR product prior to the assembly reaction.
- Low cell competence: The transformation efficiency of the competent cells should be greater than 10^7 cfu/µg. Validation can be performed by transforming 1 ng of pUC19 plasmid and use 1/10 to coat the transformation plate. There should be 1000 colonies, which makes the transformation efficiency to be around 10^7 cfu/µg. The transformation volume of the assembled product should not exceed 1/6 of the competent cell volume. Otherwise, the transformation efficiency will decrease. Select the strains for cloning (such as DH5α/XL10) but not for expression.

Why the inserts are absent from most of colonies or the wrong insert exhibits.

- Non-specific products included in PCR product. Optimize the PCR reaction to increase specificity, or gel recover the PCR product.
- The cloning vector is not fully linearized. Use a negative control to determine whether the vector is linearized. Optimize the enzyme digestion system by increasing the amount of restriction endonuclease, extending the digestion time, or gel recovering the digested vector.
- A plasmid with the same resistance may be in the reaction mix: When a circular plasmid is used as a PCR template, *Dpn*I digestion is recommended when the amplification product is not directly used for assembly. Alternatively, gel purify the amplification product.

Why didn't a band show up after colony PCR validation?

- Wrong primer: A universal primer specific to the vector should be used for the colony PCR.
- Inappropriate PCR setting: If both the target bands and bands for empty plasmids are missing, it is recommended to optimize the PCR reaction program; alternatively, the extracted plasmid can be validated by PCR or restriction enzyme digestion.
- Assembly failure: If only the band for the empty plasmids can be seen, the assembly reaction has failed. We recommend optimizing the enzyme digestion system for the vector.

What is the maximum fragment size that can be assembled?

The Seamless Cloning and Assembly Master Mix can insert 5kb fragments into a 15kb vector.

How many fragments can be assembled in one reaction?
The number of DNA fragments that can be assembled in one reaction depends on the length and sequence of the fragments. The Seamless Cloning and Assembly Master Mix has been shown to successfully insert twelve 0.4 kb fragments into a vector simultaneously. However, we recommend no more than 5 inserts in a single reaction to produce a correct construct. If the fragments fail to assemble in one reaction, we recommend splitting them into sequential assemblies.

What is the shortest overlap length that can be used?
When a 12 bp homologous sequence is used, efficient assembly results can be obtained, but it depends on the GC content of the homologous sequence. We recommend a homologous sequence of 16 bp or longer with T_m > 48 °C (AT pair = 2 °C and GC pair = 4 °C).

What is the longest overlap length that can be used?
The exonuclease activity in the Seamless Cloning & Assembly Master Mix is optimized for homologous sequences ≤100 bp.

Can dsDNA fragments ≤200 bp be assembled?
Yes. When the length of fragments is ≤200 bp, the molarity of fragments used should be 5 times more than that of the vector to obtain optimal results.

Can the reaction be incubated for longer/shorter time?
Yes. When assembling 2-3 fragments, 15 minutes is enough; while assembling 4-6 fragments, 60 minutes is recommended. Usually, it is not recommended to incubate the reaction for less than 15 minutes. Under certain circumstances, longer incubation may increase the assembly efficiency, but the length of incubation should not exceed 4 hours. DO NOT incubate the Seamless Cloning & Assembly reaction overnight.

Can the reaction be incubated at a different temperature?
50°C is the optimal temperature. However, the reaction would also work between 40°C and 50°C.

Can I use a 15 nt homologous sequence made of repeated His-tag condons (e.g. CACCACCACCACCAC)?
No. Three bases different from the repeated His-tag codon must be added after the His-tag. In addition, the repeated sequence must be avoided at the end of the homologous sequence.

Can the Seamless Cloning & Assembly product be used as the template for subsequent PCR?
Yes. Assembled products are complete sequences that can be used for subsequent PCR amplification. If the final product is a closed, circular DNA molecule, the product can be used as a template for rolling-circle amplification (RCA).

Can single-stranded DNA oligonucleotide fragments be combined and assembled with double-stranded DNA fragments?
Yes. But the amount of each oligonucleotide added needs to be optimized. We recommend starting at a concentration of 45 nM per oligonucleotide.

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