HIFI DNA MULTIPLE ASSEMBLY 2X MASTER MIX FS-02012

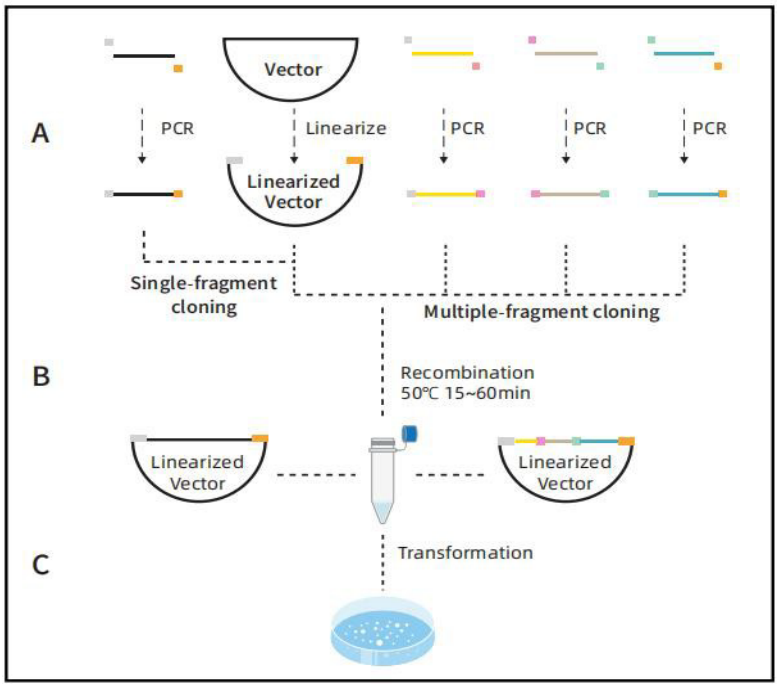
Hi Fidelity DNA Multiple Assembly Master Mix allows for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. It has utility for the synthetic biology community, as well as those interested in one-step cloning of multiple fragments due to its ease of use, flexibility and simple master-mix format.

Hi fi DNA Multiple Assembly 2X Master Mix is a simple, fast, and efficient seamless cloning reagent. It enables targeted cloning of inserts to any site in any vector. Up to 5 inserts can be assembled sequentially at a time, regardless of the digestion site carried by the insert itself. To perform seamless assembly cloning, user needs to linearize the vector, design gene-specific F/R primers with 15-25 homologous bases to vector ends, so that the PCR products 5' and 3 'have the same sequence (15-25 bp) as the two ends of the linearized vector, respectively. Hi fi DNA Multiple Assembly 2X Master Mix works on the inserted fragment(s) and vector DNA, and then they are incubated at 50°C for 15-60 minutes. During incubation, specific enzymatic reactions facilitate a fully assembled DNA construct. ***(Figure 1).***

Hi fi DNA Multiple Assembly 2X Master Mix is the optimization form that significantly improves fragment assembly efficiency and tolerance to impurities. The final product is a fully enclosed, double-stranded DNA that can be directly used for further PCR, RCA, or other molecular biology manipulations ***(e.g., transformation into competent cells).***

Note: Before use, fully thaw the reagents and mix thoroughly. Keep on ice to avoid repeating freeze-thaw cycles.

Figure 1. Hifi DNA Multiple Assembly 2X Master Mix Principle



CAT.#	Description	Size	Storage:
FS-02012-100	Hifi DNA Multiple Assembly 2X Master Mix	100 µL (10 reactions)	-20°C
FS-02012-500		500 µL (50 reactions)	-20°C

Applications

- ✧ Rapid clone: One vector to one insert cloning
- ✧ Multiple clone: Assembly of multiple DNA fragments at same time
- ✧ Site-direct mutagenesis
- ✧ High-throughput library construction

Additional Materials Required, But Not Supplied

- ✧ Templates for fragment amplification, primers, and linearized vectors.
- ✧ High-fidelity DNA polymerase (to amplify fragments): HOT START PROOF PCR 2X MASTER MIX x or other equivalent products.
- ✧ DpnI (Used to remove methylated DNA templates from PCR products)
- ✧ Competent cells: For the plasmid size ≤10 kb, we recommend DH5α chemically competent cells in the transformation. For the plasmid size > 10 kb, XL10 chemically competent cells are recommended.
- ✧ Nuclease-free water, PCR tubes, Thermocyclers, LB plates with selective resistances.

Summary

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

Tips for Seamless Assembly Cloning

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, 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 FS-T-71702 HOT START PROOF PCR 2X MASTER MIX 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.

2. Preparation of Inserts

Although the inserted fragments can be amplified by any polymerases (Taq polymerase or other high-fidelity polymerase), high fidelity polymerases are highly recommended to minimize potential mutations. We recommend high-fidelity polymerase FS-T-71702 HOT START PROOF PCR 2X MASTER MIX in PCR amplification.

2.1 General rules for primer design

Introduce the homologous sequence of linearized vector (15 - 25 bp, excluding restriction endonuclease sites) into 5' ends of both Forward & Reverse primer. Thereby, the ends of amplified inserts and linearized vectors are identical to each other. 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 EcoRI and HindIII restriction enzymes (Figure 2a).

2.2 The design strategy of primers is shown in Figure 2b

- Forward primer of the most upstream fragment

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

- Reverse primer of the most downstream fragment

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

Note1: Gene-specific forward/reverse primer sequence refers to the forward/reverse amplification primer sequence of regular insert fragments. Tm value of 60 - 65°C is recommended.

Note2: Homologous sequences of vector upstream or downstream end refer to the terminal Sequence of the linearized vector (for homologous recombination). GC content of 40% - 60% is recommended.

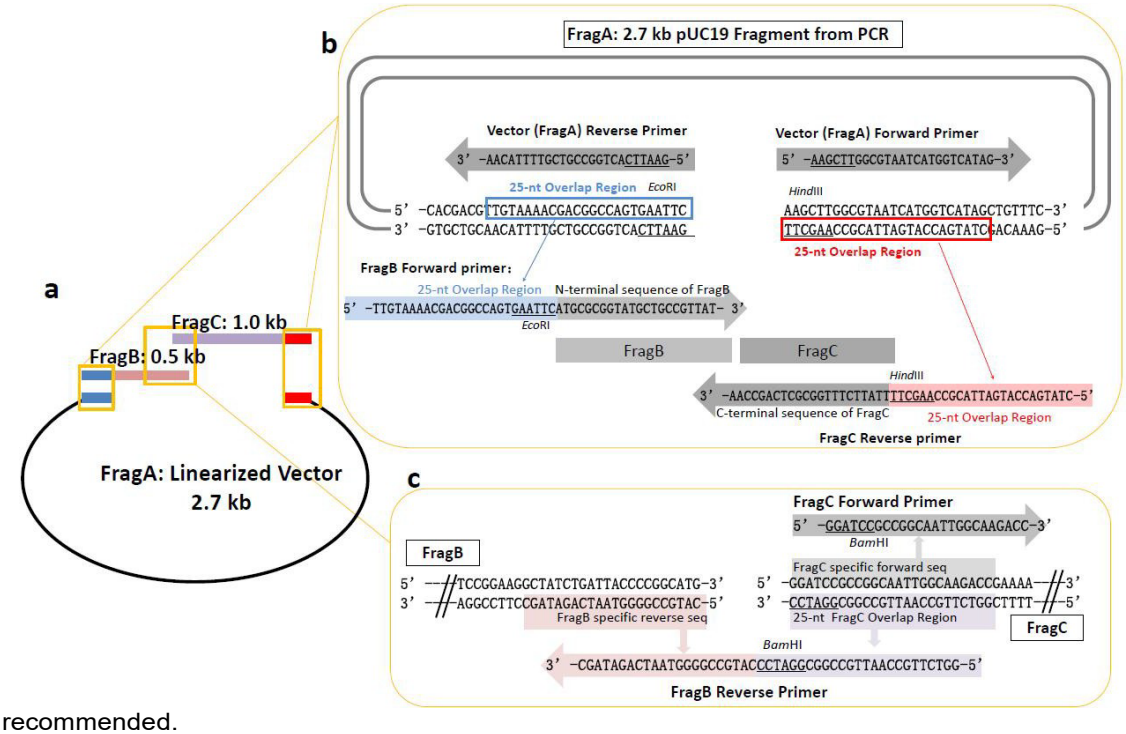


Figure 2. Primer design protocol of 2.7 kb pUC19 vector and 0.5 kb, 1.0 kb inserts

2.3 Three methods of designing primers for the inserts in the middle

- 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.
- 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).
- 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.

3. DpnI Digestion (optional)

In order to reduce the effect of large amount of circular plasmid template, it is necessary to use DpnI to digest the plasmid in the PCR product. DpnI will only digest the Plasmid DNA (methylated by *E.coli* Dam methylase) while it doesn't cleavage unmethylated DNA (PCR product).

4. Calculating the amount DNA fragments in seamless assembly

4.1 For single-fragment homologous recombination, the optimal amount of linearized vector used in the recombinant reaction is 0.03 pmol, and the optimal molar ratio of vector to insert is 1:2 or 1:3, taking 1:2 as an example, the amount of input can be roughly calculated according to the following formula:

The optimal amount of linearized vector required (0.03 pmol) = [0.02 × number of base pairs] ng The optimal amount of insert required (0.06 pmol) = [0.02 × number of base pairs × 2] ng

For example, if a 2 kb fragment is inserted into a 5 kb vector, the optimal amount of vector is 0.02 x 5000 = 100 ng and the maximum amount of insert is 0.02 x 2000 x 2 = 80 ng.

Note1: When the length of the insert is larger than that of the vector, the calculation method of the optimal amount of vector and insert should be inverted.

Note2: 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.

4.2 For multi-fragment homologous recombination, the optimal amount of inserts and linearized vectors are both 0.03 pmol, , the optimal molar ratio of vector to insert is 1:1, the amount of input can be roughly calculated according to the following formula:

The optimal amount of linearized vector required (0.03 pmol) = [0.02 × number of base pairs] ng The optimal amount of of each insert required (0.03 pmol) = [0.02 × number of base pairs] ng

For example, 0.5 kb (fragment 1), 1 kb (fragment 2), and 2 kb (fragment 3) inserts are cloned simultaneously onto a 5 kb vector.

The most suitable amount of linearized vector is 0.02 x 5000=100 ng;

0.5 Kb (fragment 1) is optimal: 0.02 x 500=10 ng;

1 kb (fragment 2) is optimal: 0.02 x 1000=20 ng;

2 Kb (fragment 3) is optimal: 0.02 x 2000=40 ng.

Note1: 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.

5. Assembly

5.1 Mix the following reaction components on ice

Component	Recommended Amount of Fragments Used for Assembly	
	2 Fragment Assembly	**3-6 Fragments**
Linearized Vector	**Assembly** X μL	X μL
Insert	Y μL	$Y_1+Y_2+...Y_n$ μL
Hifi DNA Multiple Assembly 2X Master Mix	10 μL	10 μL
ddH_2O	To 20 μL	To 20 μL

5.2 Reaction protocol

	2 Fragments Assembly	3–6 Fragments Assembly
Reaction Temp.	50°C	50°C
Reaction Time	15 min	30-60 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. Transformation

1) Thaw the chemically competent cells (such as DH5α competent *E. coli* cells) on ice.
2) Add 5 μL of the assembled product to 100 μL of competent cells, mix by gently flicking the tube (do not shake and mix), and place on ice for 30 minutes.
3) The volume of the assembled product should not exceed 1/10 of the volume of the competent cells.
4) Heat-shock in a 42°C water bath for 45 seconds and place on ice for 2-3 minutes immediately.
5) Add 900 μL SOC or LB medium (without antibiotics) to the competent cells and incubate at 37°C for 1 hour (200-250 rpm).
6) Centrifuge at 4,000 rpm for 3 min and discard the 800 μL of supernatant.
7) Resuspend the bacteria using the remaining medium and gently spread it on a LB plate with selective antibiotic.
8) Incubate at 37°C for 12-16 hours.

7. Identification of Recombinant Products

- 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.

Precautions

- Competent Cells: The transformation efficiency of various competent cells may vary 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 obtained product from Hifi DNA Multiple Assembly 2X Master Mix for electroporation, the product must be diluted with water i n a 3-folds dilution. Transfer 1 μL of the diluted product in 50 μL of electric competent cells for transformation .
- DNA: If the volume of the PCR product used is less than 20% of the total volume of the Hifi DNA Multiple Assembly 2X Master Mix 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 for increasing the efficiency of Hifi DNA Multiple Assembly 2X Master Mix by 2-10 folds. Therefore, when the fragments are more 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 DNA with repeat sequences such as reverse or tandem can be selectively rejected by E. coli. Some recombinant proteins may have certain cytotoxicity, and E. coli cannot tolerate these proteins well. These reasons can lead to a significant decrease of clones or colonies.

Frequently Asked Questions

1. **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.
- Inhibition of reaction due to impure vector and insert fragments. 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 competent cell transformation: The transformation efficiency of the competent cells should be greater than 107 cfu/μg. Validation can be performed by transforming 1 ng of pUC19 plasmid and use 1/10 to spread the transformation plate. There should be 1000 colonies, which makes the transformation efficiency to be around 107 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.

2. **Why do most clones not contain insertion fragments or contain incorrect insertion fragments?**

- Non-specific products included in PCR product. Optimize the PCR reaction to increase specificity, or purify the PCR product by collum purification.
- The cloning vector is not fully linearized.Negative control can be used to test whether the vector is completely linearized, then optimize the enzyme digestion system, such as increasing the amount of restriction endonuclease, prolonging the digestion time, and purifing the enzyme products by gel recovery..
- Plasmid carrying the same resistance may be mixed in the reaction system: When a circular plasmid is used as a PCR template, DpnI digestion is recommended when the amplification product is not directly used for assembly. Alternatively, gel purify the amplification product.

3. **Why no band showed up after colony PCR validation?**

- Wrong primer: A universal primer specific to the vector should be used for the colony PCR.
- Inappropriate PCR reaaction system and/or program setting: If both the target bands and bands for empty plasmids are missing, it is recommended to optimize the PCR reaction system and 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.

4. **What is the maximum fragment size that can be assembled?**

- Hifi DNA Multiple Assembly 2X Master Mix can insert 5 kb fragments into a 15 kb vector.

5. **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. Hifi DNA Multiple Assembly 2X 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.

6. **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 Tm > 48 °C (AT pair = 2 °C and GC pair = 4 °C).

7. **What is the longest overlap length that can be used?**

- The exonuclease activity in the Hifi DNA Multiple Assembly 2X Master Mix is optimized for homologous sequences ≤100 bp.

8. **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.

9. **Can the reaction be incubated for longer/shorter time?**

- Yes. When assembling 2 fragments, 15 minutes is enough; while assembling 3-6 fragments, 60 minutes is recommended. Usually, it is not recommended to incubate the reaction for less than 15 minutes. In some cases, longer incubation may increase the assembly efficiency, but such incubation should not exceed 4 hours. DO NOT incubate the Hifi DNA Multiple Assembly 2X Master Mix reaction overnight.

10. **Can the reaction be incubated at a different temperature?**

- 50°C is the optimal working temperature. However, the reaction would also work between 40°C and 50°C.

11. **Can I use a 15 nt homologous sequence composed of repeated His-tag condons (e.g. CACCACCACCAC CAC)?**

- 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.

12. **Can the Hifi DNA Multiple Assembly 2X Master Mix 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).

13. **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.