Fast-Fusion™ Multi Seamless Cloning Kit

For rapid and effective cloning of PCR products

Cat. No. FF006 (20 reactions)
Cat. No. FF007 (60 reactions)

User Manual

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User Manual

Fast-Fusion™ Multi Seamless Cloning Kit

I. Introduction

The GeneCopoeia Fast-Fusion™ Multi Seamless Cloning Kit provides a rapid method for cloning your PCR products. In as little as 5 minutes minutes at 50°C, any PCR fragment can be cloned into your linearized vector. After a simple clean up step, a PCR-generated DNA fragment or other purified DNA fragment can be joined to a vector with overlapping ends (Fig.1).Up to five DNA fragments can be joined together in a single reaction. A single fragment can be efficiently fused and assembled in 5 minutes. Well-prepared vectors generate almost 100% positive clones.

No restriction sites are needed at the junction, enabling insertion of your fragment of interest at any vector position. The linearized vector can be generated by either PCR or restriction enzyme digestion. The PCR products can be produced by either Taq DNA polymerase or other high fidelity DNA polymerase.

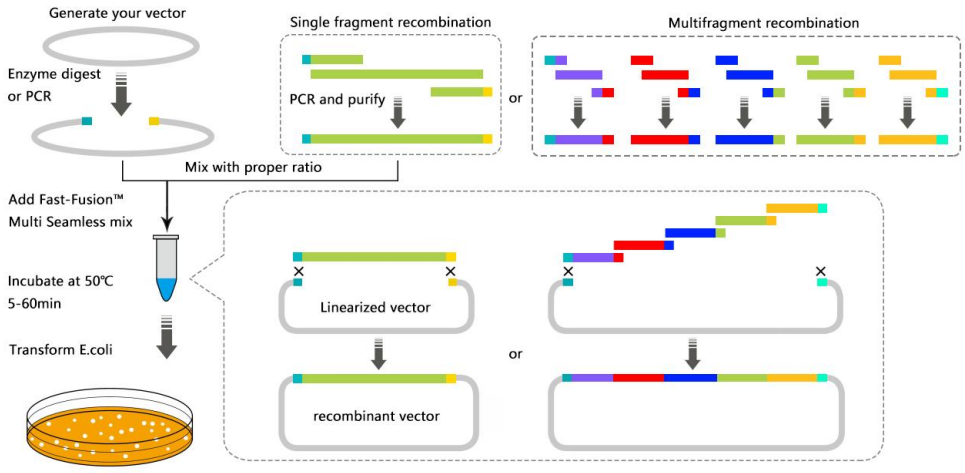


Fig. 1. Experimental workflow of using the Fast-Fusion™ Multi Seamless Cloning Kit.

Working principle

The GeneCopoeia Fast-Fusion™ Multi Cloning Kit enables assembly of multiple DNA fragments with overlapping ends in a single tube reaction under isothermal conditions. The Fast-Fusion™ Multi Seamless Mix includes three different enzymatic activities that perform in one single buffer:

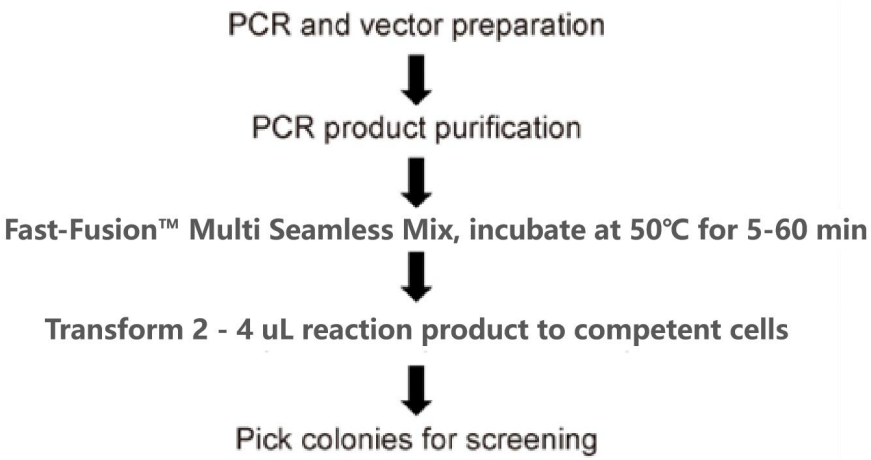
- Exonuclease activity produces single-strand 3' overhangs that facilitate the annealing of fragments with complementarity at the end (overlapping regions).
- Polymerase activity fills the gaps in the annealed fragments.
- DNA ligase activity seals nicks in the assembled DNA.

The end result is a double-stranded fully sealed DNA molecule that can be used as templates for PCR, RCA, or for other molecular biology applications, including direct transformation.

Key Advantages

- Fast and simple—1 minute for operation and 5 minutes for incubation.
- High adaptability—No restriction or recombination sites needed, insert fragments generated by either PCR or restriction enzyme digestion can be used.
- Seamless construction—Final constructs are seamless with no extra or unwanted base pairs.
- Flexibility—Multiple inserts can be assembled in one reaction. Suitable for multi-site mutagenesis.
- High efficiency—Greater than 90% of colonies after transformation contain the correct insert(s).

Protocol overview



II. Contents and Storage

Contents and storage recommendations for the GeneCopoeia Fast-Fusion™ Multi Seamless Cloning Kit (Cat.No.FF006 & FF007) are provided in the following table.

Product	Size	Component	Cat.No.	Specification
Fast-Fusion™ Multi Seamless Cloning Kit (FF006)	20rxns	Fast-Fusion™ Multi Seamless Mix	FF006-01	1× 300μl
		Linear pUC19 (50 ng/μl, Amp +)	FF006-02	1× 5μl
		Multi Control Insert Mix	FF006-03	1× 5μl
Fast-Fusion™ Multi Seamless Cloning Kit (FF007)	60rxns	Fast-Fusion™ Multi Seamless Mix	FF006-01	3× 300μl
		Linear pUC19 (50 ng/μl, Amp +)	FF006-02	3× 5μl
		Multi Control Insert Mix	FF006-03	3× 5μl

Additional materials required but not provided

- Clonable plasmid vector
- PCR amplification reagents, such as UltraHiPF® DNA Polymerase Kit (Cat.No.PC018) and 2× UltraHiPF® PCR Mix (Cat.No.PC033)
- DNA quantitation standard
- Restriction enzymes
- Nucleic acid purification kit or Reagent QP reagent (Cat.No.FF001-03)
- Competent cells for transformation, such as DH5α Competent Cells (Cat.No.CC001), Stbl3 Competent Cells (Cat.No.CC003), 2T1 Competent Cells (Cat.No.CC007)
- S.O.C. medium
- LB plates with antibiotics

III. Key Steps

1. **Vector preparation:** A well-prepared vector can reduce your screening time. Single enzyme-digested vectors will self-ligate resulting in a high background of plasmids lacking inserts following transformation. The best way to avoid this is to digest with two restriction enzymes, followed by gel purification of the vector backbone. For PCR-generated vectors, we recommend digestion with Dpn I which will destroy plasmids that have been Dam methylated by replication in *E. coli*. Transform 50-100 μL of competent cells with 5-10 ng linearized vector as a negative control to determine the transformation background.

2. **Primer design:** Primer design is critical for successful Fast-Fusion™ Multi Seamless cloning. Homology must present at the ends you want to fuse, e.g. vector and insert (or multiple inserts). Check your primers following the guidelines below.

(1) Each Fast-Fusion primer consists of two parts: 1) A sequence at the 5'-end that is homologous to one end of the target vector or another insert, and 2) a gene-specific sequence at the 3'-end that will specifically amplify the target insert (Fig. 3, 4).

(2) For homologies less than 15 bp, the transformation efficiency will vary depending on DNA structure (Fig. 2). GeneCopoeia strongly recommends including more than 15 bp of homology at each end for best results.

(3) Avoid complementarity within each primer to prevent hairpin structures, and between primer pairs to avoid primer dimers.

(4) The melting temperature (T_m) should be calculated based on the 3'-end (gene specific sequence) of the primer, not the entire primer. GeneCopoeia recommends setting the T_m value of the primer between 55°C -65°C by adjusting the length of the gene specific sequence.

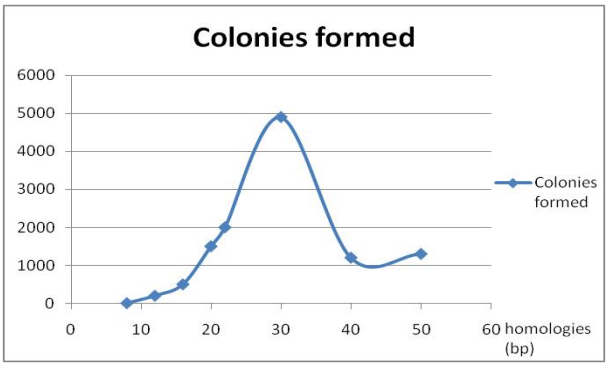


Fig.2. Homologies affect cloning efficiency. The number of colonies formed is calculated from 5 ng of pUC19 vector transformed after standard Fast-Fusion™ reactions with inserts of indicated homologies (Competent cells efficiency: 2×10^9cfu/ug).

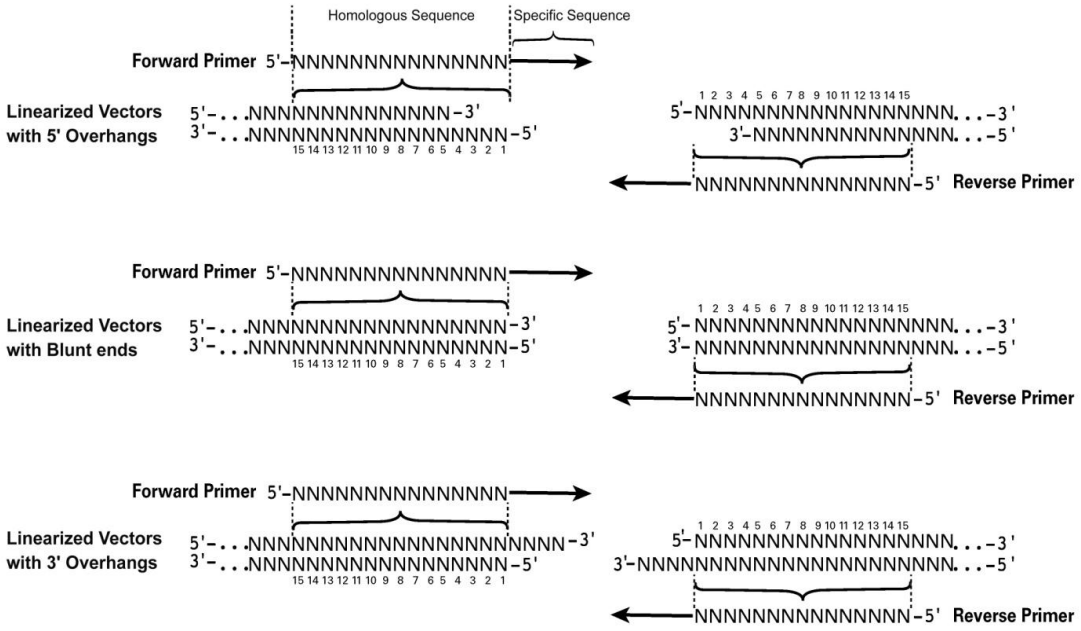


Fig. 3. Primer with 15 bp homology in different vector ends.

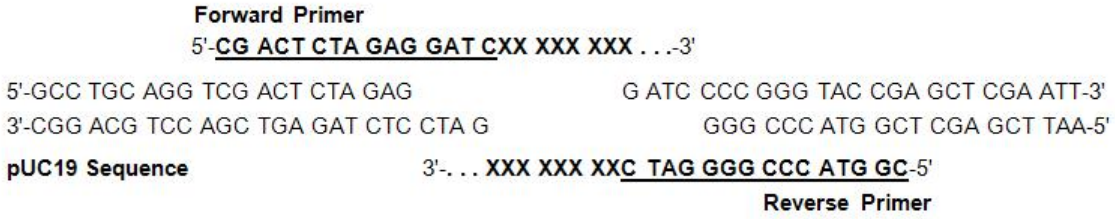


Fig. 4. Example of primer designed for the GeneCopoeia Fast-Fusion Multi system. Primer sequences are shown in bold. Underlined bases are homologous to the end of pUC19 vector digested by restriction enzyme BamH I. X: bases corresponding to the gene or sequence of interest.

3. **PCR amplification and purification:** UltraHiPF® DNA Polymerase Kit (Cat.No.PC018), 2× UltraHiPF® PCR Mix(Cat.No.PC033) and Taq DNA Polymerase Kit (Cat.No.PC003) are all suitable for generating DNA fragments for Fast-Fusion™ Multi Seamless cloning. After PCR, analyze PCR products by electrophoresis on an agarose/EtBr gel. The QP reagent can be used when only a single band is present (Fig. 5). Gel purification is strongly recommended when nonspecific amplification is evident. Quantify the purified fragments by measuring against a known DNA standard running in parallel.

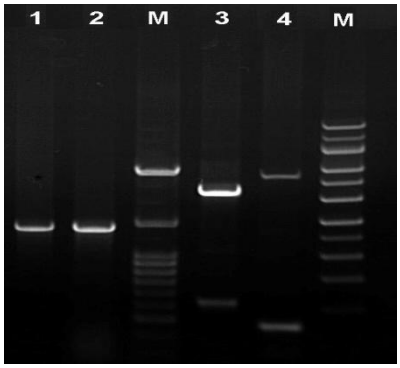


Fig.5. PCR inserts for Fast-Fusion™ Multi Seamless cloning.
Lane 1: Insert PCR without purification.
Lane 2: Insert PCR purified by QP reagent.
Lane3, 4: Nonspecific amplification in PCR reaction.

IV. Cloning Reaction and Transformation Procedure

Fast Fusion™ Multi Seamless Mix must be dissolved thoroughly and mixed gently before use.

1. Cloning Reaction

(1) Vector and insert fragment usage calculation:

The optimal DNA amounts for Fast-Fusion™ Multi Seamless cloning are 0.03 pmol per fragment (including linearized vectors and inserts). The DNA mass of 0.03 pmol of each DNA fragment can be calculated according to the following formula:

Optimal amount per insert fragment = [0.02 x fragment base pairs] ng (0.03 pmol)

For example, when cloning a 0.5kb, 1kb, or 2kb insert into a 2.5kb vector,

The optimal amounts of the cloning vector and each insert fragment are as follows:

The optimal amount of the linearized cloning vector: 0.02 x 2500= 50 ng;

The optimal amount of a 0.5 kb insert: 0.02 x 500= 10 ng;
The optimal amount of a 1 kb insert: 0.02 x 1000= 20 ng;
The optimal amount of a 2 kb insert: 0.02 x 2000= 40 ng;

a. The use of linearized cloning vectors should be between 50-200 ng. When the optimal DNA amount calculated using the above formula is beyond this range, the minimum/maximum amount can be used directly.

b. The amount of each insert fragment used should be at least 10 ng. If the optimal calculated amount is less than 10 ng, then 10 ng can be used directly.

Please refer to the table below to adjust the amounts of the vector and insert. For seamless assembly of a single fragment, the recommended molar ratio of the insert to vector is 1-2:1. When assembling multiple fragments, pay close attention to the amount of insert fragments and the vector in the reaction system, as the recommended molar ratio is 1:1 (for example, when fragments A, B, and C and vector V are assembled, the molar ratio A:B:C:V = 1:1:1:1). The total amount of DNA in the whole system should not exceed 200 ng/20 uL, and the amount of small fragments can be appropriately reduced.

Vector		Insert	
Length	**Scale**	**Length**	**Scale**
3k bp	60 ng	200-1000 bp	4-40 ng
5k bp	100 ng	1k-2k bp	40-60 ng
9k bp	180 ng	2k-5k bp	60-100 ng
		>5k bp	＞100 ng

When using the GeneCopoeia Fast-Fusion™ Multi Seamless Cloning Kit for the first time, GeneCopoeia strongly recommends including positive and negative control reactions in parallel with your cloning reactions. The linearized pUC19(50 ng/μl, Amp+) vector and positive insert provided in the kit have already been purified, so there is no treatment needed before use.

(2) Set up the following 20 μL cloning reaction on ice.

Reagents	Cloning reaction	Negative control	Positive control
Linearized Vector	0.03pmol, Xμl (About 50-200 ng)	1 μL Linear pUC19 (50 ng/μl, Amp +)	1 μL Linear pUC19 (50 ng/μl, Amp +)
Target Insert	0.03pmol, Y1-Ynμl（10-200 ng）	-	1 μL Multi Control Insert Mix
Fast-Fusion™ Multi Seamless Mix	15 μL	15 μL	15 μL
ddH_2O	Add ddH_2O to 20 μL	4 μL	3 μL

(3) Homogenize the reaction mix by tapping the tube. Briefly centrifuge 6000-8000rpm/30 seconds to collect the liquid at the bottom of the tube. Incubate at 50°C.

- It is recommended to perform the reaction on an instrument with more accurate temperature control, such as a PCR instrument. Incubation time recommended:

a. When 1~2 fragments are being assembled, it is recommended to incubate at 50°C for 5~30 minutes. The recombination efficiency peaks at about 30 minutes of reaction, and too long reaction time would reduce the cloning efficiency.

b. When 3~3 fragments are being assembled, it is recommended to incubate at 50°C for 60 minutes. If there is a significant length difference between fragments, the reaction time can be appropriately extended to 90 to 120 minutes.

(4) Place the tube on ice until transformation. Store the product directly below -20°C.

- seamless assembly products can be stored at -20°C for one week.

2. Transformation

Transform competent E. coli cells (Cat.No.CC001) with the assembly products following the provided protocol (below) or following the manufacturer's instructions. GeneCopoeia recommends using high-efficiency competent cells (>10^8 cfu/μg).

(1) Transfer 2-4 μL of the seamlessly assembled reaction solution preserved on ice to 100 μL chemically competent cells. Tap the tube gently for 2~3 times to mix them well. Incubate on ice for 30 minutes.
Note: 1 μL is usually sufficient for single-insert cloning. Increase volume for multi-insert assembly is helpful to improve the conversion efficiency.
(2) Heat-shock the cells for exactly 30 seconds at 42°C without shaking, then immediately place the tubes on ice for 2 minutes.
(3) Add 400 μL of room temperature S.O.C. medium to the cells.
(4) Cap the tubes and incubate at 37°C for 1 hour with or without shaking.
(5) Spread 200 to 500 μL cells from each tube on pre-warmed LB plates containing the appropriate antibiotics.
(6) Incubate plates at 37°C overnight.
(7) Pick colonies for analysis.

V. Troubleshooting

The tables below address main problems encountered during Fast-Fusion™ Multi Seamless cloning, along with their possible causes and suggested solutions. Please perform the control reactions to confirm that the kit is working properly before you call us for help.

1. Problem: Few or no colonies obtained from transformation.

Possibility	Solution
Competent cells' efficiency is insufficient	Check the control reaction. There should be at least 100 colonies from competent cells with efficiencies greater than 10^8 cfu/μg
DNA solution impurity	Purify the DNA by gel purification, etc.
Low DNA concentration in reaction	Check with known concentration DNA standards; concentrate the DNA to greater than 20 ng/μL.
Primer sequences are incorrect	Check your primers to ensure the products provide corresponding bases of homology.
Not enough homology	Homologies longer than 20 bp give the best results. Don't use less than 12 bp if your competent cell efficiency is below 10^9 cfu/μg.

2. Problem: There are many colonies after transformation, but none of the plasmids contain inserts.

Possibility	Solution
Incomplete linearization of vector	Digest vector completely; generate incompatible overhangs; gel-purify your digestion product; transform a no-insert control to verify few background colonies can grow.
Too much DNA transformed	Large amounts of DNA (more than 200 ng/ul) in the reaction will either slow down the reaction or compete with your assembled molecules in transformation. Scale to no more than 50 ng per 100 μL chemically competent cells.
Contamination of PCR template carrying the same antibiotic resistance	1-10 ng of plasmid template is usually sufficient for PCR reaction. Digest the plasmid template with Dpn I, or gel-purify the PCR product.
Low DNA concentration in reaction	Too low insertion fragment concentration will cause the vector to be empty and some vectors will have false positives.
Antibiotics expired or incorrect	Do an empty incubation in 37°C to make sure the antibiotics are not expired.

VI. Accessories:

Composition of Buffers and Solutions

SOC medium (100ml)

2.0g	Bacto-tryptone
0.5g	Bacto-yeast extract
1ml (1M)	EDTA (pH8.0)
0.25 ml (1M)	KCl
1ml (1M)	NaCl
1ml (2M)	glucose, filter-sterilized
1ml (2M)	Mg^{2+}stock, filter-sterilized

Add Bacto-tryptone, Bacto-yeast extract, NaCl and KCl to 97ml of distilled water. Stir to dissolve.Autoclave and cool to room temperature. Add 2M Mg2+ stock and 2M glucose, each to a final concentration of 20mM. Bring the volume to 100ml with sterile, distilled water. The final pH should be 7.0.

Accessorial products

Description	Catalog#	Size
DH5α Chemically Competent E.coli Cells	CC001	(10×100 μl)
Stbl3 Chemically Competent E.coli Cells	CC003	(10×100 μl)
2T1 Chemically Competent E.coli Cells	CC007	(10×100 μl)
UltraHiPF® DNA Polymerase Kit	PC018	100U
	PC019	500U
2× UltraHiPF® PCR Mix	PC033	1 ml
	PC034	5 ml
Taq DNA Polymerase Kit	PC003	1,000U
QP Reagent	FF001-03	500 μl

QP Reagent

1. **Introduction:** QP Reagent is a purification reagent (Cat.No. FF001-03). The QP reagent can precipitate double stranded DNA longer than 100 bp, excluding dNTPs, primers and most of the polymerase.

2. Instructions:

(1) Invert the QP reagent tube several times before use.

(2) For 50 μL of PCR product, add TE buffer to 100 μL, followed by addition of 50 μL QP reagent. Mix thoroughly by vortexing for 5 seconds.

(3) Centrifuge the mixture at 15,000×g for 15 minutes, and discard the supernatant. Re-centrifuge the tube for 10 seconds and remove all the remaining liquid at the bottom.

Note: To obtain better precipitation efficiency for DNA molecules shorter than 200 bp, incubate at 4°C for at least 30 minutes before centrifugation.

(4) Re-suspend the DNA by adding 10-20 μL 0.1×TE buffer (diluted with ddH_2O).

VIII. Limited Use License and Warranty

Limited Use License

Following terms and conditions apply to use of Fast-Fusion™ Multi Seamless Cloning Kit (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

GeneCopoeia is committed to providing our customers with high-quality products. If you should have any questions or concerns about any GeneCopoeia products, please contact us at 301-762-0888.

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