

In-Fusion® HD Multiple-Insert Cloning Protocol-At-A-Glance

Please read the [In-Fusion HD Cloning Kit User Manual](#) before using this Protocol-At-A-Glance. **This abbreviated protocol is provided for your convenience, but is not intended for first-time users.**

Cloning more than two fragments at once (e.g. multiple inserts simultaneously into one linearized vector) requires adherence to specific considerations in experimental design and overall cloning protocol. This Protocol-At-A-Glance details these considerations and recommended modifications to ensure cloning success.

Please note the following materials are required but not supplied:

- **Ampicillin** (100 mg/ml stock) **or other antibiotic** required for plating the In-Fusion reaction
- **LB (Luria-Bertani) medium** (pH 7.0)
- **LB/antibiotic plates**
- **SOC medium**

The table below is a general outline of the protocol used for the In-Fusion HD Cloning Kits. Please refer to the specified User Manual pages for further details on performing each step.

Table I. In-Fusion HD Protocol Outline

Step	Action	User Manual Pages
1	Select a base vector and identify the insertion site. Linearize the vector at the insertion site by restriction enzyme digestion or inverse PCR. Isolate and purify the linearized vector.	6
2	Design PCR primers for your sequence(s) of interest with 20-bp extensions (5') that are complementary to the ends of adjacent sequences (the linearized vector or another insert).	6–8
3	Amplify your sequence(s) of interest with CloneAmp™ DNA polymerase. Verify on an agarose gel that your targets have been amplified and confirm the integrity of the PCR products.	8–9
4	Spin-column purify your PCR products OR treat with Cloning Enhancer.	
5	Set up your In-Fusion cloning reaction: 2 µl 5X In-Fusion HD Enzyme Premix X µl Linearized vector X µl Each insert X µl dH ₂ O to a total reaction volume of 10 µl. Mix well.	Spin-Column Protocol I (p. 9–11) OR Cloning Enhancer Protocol II (p. 11)
6	Incubate the reaction for 15 min at 50°C, then place on ice.	
7	Transform competent cells with 2.5 µl of the reaction mixture from Step 6.	12

I. PCR and Experimental Preparation (Section IV of the User Manual)

A. Preparation of a Linearized Vector by Restriction Digestion

For vector linearization via PCR, please see primer design recommendations in the User Manual, Section IV.B.

Complete, efficient digestion will reduce the amount of cloning background. Generally speaking, two enzymes cut better than any single enzyme. Digestion efficiency will always be better if the restriction sites are as far apart as possible.

1. Incubate your restriction digest as directed by the restriction enzyme supplier. Longer reaction times can increase linearization and reduce background.
2. After digestion, purify the linearized vector using a PCR purification kit. We recommend gel purification using the **NucleoSpin Gel and PCR Clean-Up** kit (Cat. No. 740609.50).
3. [Control] Check the background of your vector by transforming competent cells with 5–10 ng of the linearized and purified vector. If background is high, add more restriction enzyme(s) and continue digesting the vector (2 hr to overnight). Gel purify the remainder of the vector and transform again.

B. PCR Primer Design

When designing In-Fusion PCR primers, consider the following:

1. Every PCR primer for multi-insert cloning must be designed in such a way that it generates products containing 5' ends with **20 bp** of homology to the ends of the neighboring cloning fragments (either the linearized vector or other inserts).
2. The 3' portion of each primer should:
 - be specific to your template
 - be between 18–25 bases in length, with GC-content between 40–60%
 - have a T_m between 58–65°C; with the difference between the forward and reverse primers $\leq 4^\circ\text{C}$. T_m should be calculated based upon the 3' (gene-specific) end of the primer, NOT the entire primer.
 - not contain identical runs of nucleotides; the last five nucleotides at the 3' end of each primer should not have more than two guanines (G) or cytosines (C)
3. Avoid complementarity within each primer and between primer pairs
4. Online tools are available to help with primer design:
 - BLAST searches can determine specificity and uniqueness of the 3' end (at www.ncbi.nlm.nih.gov/BLAST/)
 - Our online primer design tool simplifies PCR primer design for In-Fusion reactions (at http://www.takarabio.com/US/Products/Cloning_and_Compentent_Cells/Selection_Guides/Online_In-Fusion_Tools)
5. Desalted oligonucleotide primers are generally recommended for PCR reactions. However, PAGE purification may be needed for primers of poor quality or longer than 45 nucleotides.

C. PCR Amplification of Target Fragment(s)

The In-Fusion method is not affected by the presence or absence of A-overhangs, so you can use any thermostable DNA polymerase for amplification, including proofreading enzymes. We recommend using our **CloneAmp HiFi PCR Premix** (included in every In-Fusion HD Cloning Plus system, and sold separately as Cat. No. 639298). If you are using a different polymerase, please refer to the manufacturer's instructions. If using CloneAmp HiFi PCR Premix, please read the [Protocol-at-a-Glance](#) and follow the guidelines below:

Table II. Recommendations for PCR with CloneAmp HiFi PCR Premix

Template Type	Template Amount	Product Size	Extension Time
Human genomic DNA	5–100 ng	up to 6 kb	5 sec/kb
<i>E. coli</i> genomic DNA	100 pg–100 ng	up to 10 kb	5 sec/kb
λ DNA	10 pg–100 ng	up to 15 kb	5 sec/kb
Plasmid DNA	10 pg–1 ng	up to 15 kb	5 sec/kb
cDNA	≤ the equivalent of 25–125 ng total RNA	up to 6 kb	5–10 sec/kb

When PCR cycling is complete, confirm your product(s) on an agarose gel.

II. In-Fusion Cloning Procedure (Section VI of the User Manual)

Both protocols below are appropriate for PCR that produces a single band of the desired size. If non-specific bands are visible on your gel, use Protocol I.

A. Protocol I: In-Fusion Cloning Procedure w/Spin-Column Purification

1. Isolate each target fragment (insert or linearized vector) by gel extraction followed by spin-column purification using a silica-based purification system, such as the NucleoSpin Gel and PCR Clean-Up kit.
2. Plan the In-Fusion cloning reaction. Good cloning efficiency is achieved when using 50–200 ng of vector and inserts, respectively. More is not better. Use the table below for reaction recommendations.

Table III. Recommended In-Fusion Reactions for Purified Fragments

Rxn Component	Cloning Rxn	Negative Control Rxn	Positive Control Rxn
Purified PCR fragment	10–200 ng*	–	2 µl of 2 kb control insert
Linearized vector	50–200 ng**	1 µl	1 µl of pUC19 control vector
5X In-Fusion HD Enzyme Premix	2 µl	2 µl	2 µl
Deionized Water	to 10 µl	to 10 µl	to 10 µl

* <0.5 kb: 10–50 ng, 0.5 to 10 kb: 50–100 ng, >10 kb: 50–200 ng
 ** <10 kb: 50–100 ng, >10 kb: 50–200 ng

Molar Ratio Recommendations

Generally, the molar ratio of each of the multiple inserts should be 2:1 with regards to the linearized vector, i.e., two moles of each insert for each mole of linearized vector. **The molar ratio of two inserts with one vector should be 2:2:1.** Specific exceptions are listed below:

- If an insert is large with respect to your linearized vector, we recommend a molar ratio of 1:1
- For cloning small DNA fragments (150–350 bp), the suggested insert-to-vector molar ratio is 3–5:1
- For cloning of short synthetic oligos (50–150 bp), the suggested oligo to vector molar ratio is 5–15:1. Depending on the oligo length, the optimal molar ratio must be determined empirically.

3. Set up the In-Fusion cloning reaction:

2 µl	5X In-Fusion HD Enzyme Premix
x µl*	Linearized vector
x µl*	Purified PCR insert
x µl*	Purified PCR insert
x µl	dH ₂ O (as needed)
10 µl Total volume	

* For reactions with larger combined volumes of vector and PCR insert (>7 µl of vector + insert), double the amount of enzyme premix, and add dH₂O for a total volume of 20 µl.

4. Adjust the total reaction volume to 10 µl using deionized H₂O, and mix.
5. Incubate the reaction for **15 min at 50°C**, then place on ice.
6. Continue to the Transformation Procedure (Section III). You can store the cloning reactions at –20°C until you are ready.

B. Protocol II: In-Fusion Cloning Procedure w/Cloning Enhancer Treatment

1. Add 2 µl of Cloning Enhancer to 5 µl of each PCR reaction (insert or linearized vector).
2. Use a thermal cycler to incubate at 37°C for 15 min, then at 80°C for 15 min. If you used more than 100 ng of DNA template, extend the 37°C incubation to 20 min. If you are using a water bath or heat block rather than a thermal cycler, extend each incubation to 20–25 min.

NOTE: If you cannot proceed immediately to the cloning reaction, store Cloning Enhancer-treated PCR reactions at –20°C until you are ready.

3. Set up the In-Fusion cloning reaction:

2 µl	5X In-Fusion HD Enzyme Premix
x µl*	Linearized vector
x µl**	Treated PCR insert
x µl**	Treated PCR insert
x µl	dH ₂ O (as needed)

10 µl Total volume

* Use 50–200 ng of linearized vector.

** Use 1–2 µl of Cloning Enhancer-treated fragments, regardless of their length. The total volume of Cloning Enhancer-treated PCR fragments should be up to 4 µl per 10-µl reaction. If you obtain a low product yield from your PCR reaction, we recommend purification of PCR fragments instead of Cloning Enhancer treatment.

4. Adjust the total reaction volume to 10 µl using deionized H₂O, and mix.
5. Incubate the reaction for **15 min at 50°C**, then place on ice.
6. Continue to the Transformation Procedure (Section III). You can store the cloning reactions at –20°C until you are ready.

III. Transformation Procedure Using Stellar™ Competent Cells

(Section VIII of the User Manual)

This transformation protocol has been optimized for transformation using Stellar Competent Cells, sold in In-Fusion kits and separately in several formats. If you are not using Stellar Competent Cells, follow the protocol provided by the manufacturer. We strongly recommend the use of competent cells with a transformation efficiency $\geq 1 \times 10^8$ cfu/ug.

For complete information on the handling of Stellar Competent Cells, please see the [Protocol](#).

1. Thaw Stellar Competent Cells on ice just before use. After thawing, mix gently to ensure even distribution, and then move 50 μ l of competent cells to a 14-ml round-bottom tube (Falcon tube). Do not vortex.
2. Add 2.5 μ l of the In-Fusion cloning reaction to the competent cells.
3. Place the tubes on ice for 30 min.
4. Heat shock the cells for exactly 45 sec at 42°C.
5. Place the tubes on ice for 1–2 min.
6. Add SOC medium to bring the final volume to 500 μ l. SOC medium should be warmed to 37°C before using.
7. Incubate with shaking (160–225 rpm) for 1 hr at 37°C.
8. Plate 1/5–1/3 of each transformation reaction into separate tubes and bring the volume to 100 μ l with SOC medium. Spread each diluted transformation reaction on a separate LB plate containing an antibiotic appropriate for the cloning vector (e.g., the control vector included with the kit requires 100 μ g/ml of ampicillin.)
9. Centrifuge the remainder of each transformation reaction at 6,000 rpm x 5 min. Discard the supernatant and resuspend each pellet in 100 μ l fresh SOC medium. Spread each sample on a separate antibiotic LB plate. Incubate all plates overnight at 37°C.
10. The next day, pick individual isolated colonies from each experimental plate. Isolate plasmid DNA using a standard method of your choice (e.g., miniprep). To determine the presence of inserts, analyze the DNA by restriction digest or PCR screening.

IV. Expected Results (Section IX of the User Manual)

The positive control plates typically develop several hundred white colonies when using cells with a minimum transformation efficiency of **1 x 10⁸ cfu/ μ g**. The negative control plates should have few colonies.

The number of colonies on your experimental plates will depend on the amount and purity of the PCR products and linearized vector used for the In-Fusion cloning reaction.

- The presence of a low number of colonies on both the experimental plate and positive control plate (typically, a few dozen colonies) is indicative of either low transformation efficiency or low quality DNA fragments.
- The presence of many (hundreds) of colonies on the negative control is indicative of incomplete vector linearization.

If you do not obtain the expected results, use the guide in Section X of the [User Manual](#) to troubleshoot your experiment. To confirm that your kit is working properly, perform the control reactions detailed in Section IV.D of the [User Manual](#).

NOTE: Many troubleshooting topics are covered in our online In-Fusion Cloning [FAQs](#) and [Tips](#).

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This document has been reviewed and approved by the Quality Department.