In-Fusion® Snap Assembly EcoDry™ Multiple-Insert Cloning Protocol-At-A-Glance

Please read the In-Fusion Snap Assembly EcoDry 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 Snap Assembly EcoDry cloning kits. Please refer to the specified User Manual pages for further details on performing each step.

Table I. In-Fusion Snap Assembly EcoDry 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.	4
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).	5
3	Amplify your sequence(s) of interest with PrimeSTAR® Max DNA Polymerase. Verify on an agarose gel that your targets have been amplified and confirm the integrity of the PCR products.	5
4	Spin-column purify your PCR products or treat with Cloning Enhancer.	6
5	Set up your In-Fusion Cloning reaction by adding the linearized vector or inserts, plus deionized H_2O, to an In-Fusion Snap Assembly EcoDry Master Mix pellet.	6–7
6	Incubate the reaction for 15 min at 50°C, then place on ice.	7
7	Transform competent cells with 2.5 µl of the reaction mixture from Step 6.	8

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.

Complete, efficient digestion will reduce the amount of cloning background. Generally speaking, two different cut sites are better than one for cloning. Efficiency of digestion will always be better if the restriction sites do not overlap and have at least 5 bases between them. (This varies with each enzyme, but the majority digest at >90% efficiency in these conditions.)

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, available separately (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, in the absence of In-Fusion cloning master mix. 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

We recommend using our online Primer Design tool to easily design In-Fusion-compatible primers: takarabio.com/in-fusion-tools

For more information, see Appendix A.

C. PCR Amplification of Target Fragments

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 **PrimeSTAR Max DNA Polymerase** (sold separately as Cat. No. R045A). If you are using a different polymerase, please refer to the manufacturer's instructions. If using PrimeSTAR Max DNA Polymerase, please read the User Manual and follow the guidelines below:

Table II. Recommendations for PCR with PrimeSTAR Max DNA Polymerase

Template type	Template amount	Product size	Extension time
Human genomic DNA	5–100 ng	up to 6 kb	5 sec/kb
E. coli 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 V of the User Manual)

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 a NucleoSpin Gel and PCR Clean-Up kit.
2. Plan the In-Fusion cloning reaction. Good cloning efficiency is achieved when using 200 ng combined amount of vector and inserts in a 10µl reaction. More is not better. Use the table below for reaction recommendations.

Table III. Recommended In-Fusion reactions for purified fragments

Reaction component	Cloning reaction	Negative control reaction	Positive control reaction
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
Deionized H_2O	**to 10 µl**	**to 10 µl**	**to 10 µl**

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.**

NOTE: A molar ratio calculator is included in our online cloning tools. The tool currently supports cloning reactions with up to five inserts: https://www.takarabio.com/molar-ratio

3. Mix your purified PCR fragment and linearized vector together with deionized H_2O for a total volume of 10 µl.
4. Set up the In-Fusion cloning reaction(s):
   a. Carefully peel back the aluminum seal(s) from the tube(s) you plan on using—avoid disturbing the seal of any remaining tubes.
   b. Add the 10 µl volume from Step 3 to each EcoDry pellet. Mix well by pipetting up and down.
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 VI of the User Manual)

This transformation protocol has been optimized for transformation using Stellar Competent Cells, sold 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 ≥1 x 10^8 cfu/ug.

For complete information on the handling of Stellar Competent Cells, please see the full Protocol.

1. Thaw Stellar Competent Cells on ice just before use. After thawing, mix gently to ensure even distribution, and then move 50 µl of competent cells to a 14-ml round-bottom tube (Falcon tube). Do not vortex.
2. Add 2.5 µl 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 µl. 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 µl 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 µg/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 µl 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 VII of the User Manual)

The positive control plates typically develop several hundred colonies when using cells with a minimum transformation efficiency of **1 x 10^8 cfu/µg**. 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 VIII 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 tips and FAQs: https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-faqs

Appendix A. 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 ≤4°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 **https://blast.ncbi.nlm.nih.gov/Blast.cgi**)
    - Our online primer design tool simplifies PCR primer design for In-Fusion reactions (at **takarabio.com/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.

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