Takara Bio USA, Inc.

In-Fusion® Snap Assembly EcoDry™ User Manual

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I. Introduction

In-Fusion Snap Assembly EcoDry cloning kits are designed for fast, directional cloning of one or more fragments of DNA into any vector. The cornerstone of In-Fusion cloning technology is our proprietary In-Fusion Enzyme, which fuses DNA fragments (e.g., PCR-generated inserts and linearized vectors) efficiently and precisely by recognizing 15-bp overlaps at their ends. These 15-bp overlaps can be engineered by designing primers for amplification of the desired sequences. In-Fusion Snap Assembly kits offer increased cloning efficiency over previous generations of In-Fusion kits, especially for long fragments, short oligonucleotides, and multiple fragments. In-Fusion Snap Assembly EcoDry Master Mixes provide reaction components in a convenient lyophilized format that is stable at room temperature. All of the necessary cloning reaction materials, except the vector and the PCR insert, are supplied in the reaction tube, thereby simplifying reaction setup and reducing the variability between reactions.

- **Clone any insert, into any location, within any vector you choose**
- **Efficiently clone a broad range of fragment sizes**
- **Clone multiple DNA fragments simultaneously into any vector in a single reaction**
- **No restriction digestion, phosphatase treatment, or ligation required**
- **Final constructs are seamless with no extra or unwanted base pairs**

The table below is a general outline of the protocol used for the In-Fusion Snap Assembly EcoDry cloning kits. This outline is further illustrated in Figure 1. Please refer to the specified pages for details on performing each step.

Table 1. In-Fusion Snap Assembly EcoDry protocol outline

Step	Action	Pages
1	Select a base vector and identify the insertion site. Linearize the vector by restriction enzyme digestion or inverse PCR and purify.	4
2	Design PCR primers for your gene of interest with 15-bp extensions (5') that are complementary to the ends of the linearized vector.	5
3	Amplify your gene of interest with PrimeSTAR® Max DNA Polymerase. Verify on an agarose gel that your target DNA has been amplified and determine the integrity of the PCR product.	5
4	Spin-column purify your PCR product.	6
5	Set up your In-Fusion cloning reaction by adding the following to one In-Fusion Snap Assembly EcoDry Master Mix pellet: X µl Linearized vector X µl Insert X µl dH_20 to a total reaction volume of 10 µl. Mix well.	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

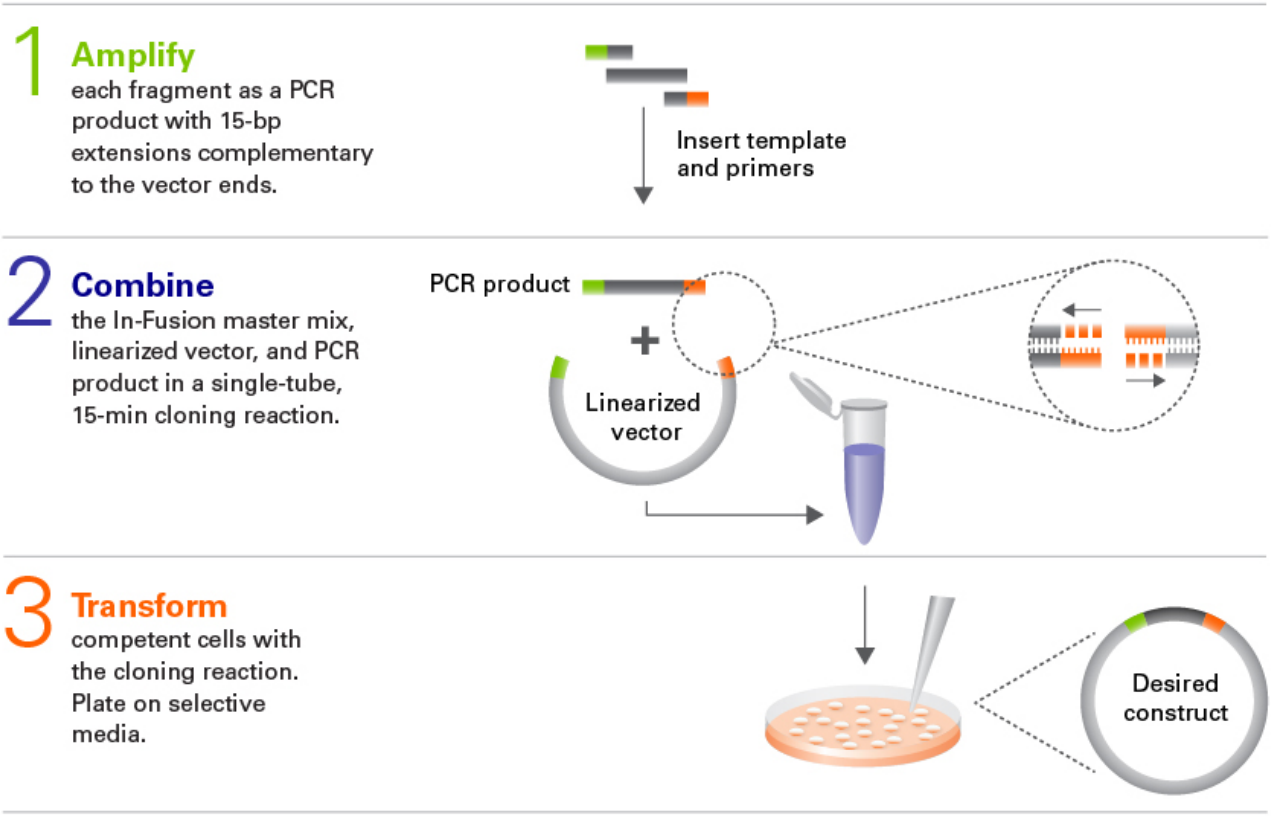


Figure 1. In-Fusion Cloning protocol overview for both liquid and EcoDry formats.

II. List of Components

All In-Fusion Snap Assembly EcoDry cloning kits contain the In-Fusion Snap Assembly Master Mix (in either 8-well strips or 96-well plates), linearized pUC19 Control Vector (50 ng/µl), and 2 kb Control Insert (40 ng/µl).

Store In-Fusion Snap Assembly EcoDry Master Mix at room temperature. Once opened, store in a desiccator at room temperature.

Store all other components at –20°C.

III. Additional Materials Required

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**

IV. PCR Fragment Amplification and Experimental Preparation

A. Preparation of a Linearized Vector

To achieve a successful In-Fusion reaction, you must first generate a linearized vector. The linearized vector can be generated using restriction enzymes (single or double digests) or by inverse PCR.

Due to differences in cutting efficiencies, different restriction enzymes will generate different amounts of 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.) In addition, increasing the enzyme digestion time and the digestion reaction volume will reduce the background.

Recommendations for preparation of a linearized vector by restriction enzyme digestion:

1. Incubate your restriction digest as directed by the restriction enzyme supplier. For many enzymes, incubation for several hours can increase linearization and reduce background.
2. After digestion, purify the linearized vector using any available PCR purification kit. We recommend gel purification using the NucleoSpin Gel and PCR Clean-Up kit, sold separately (Cat. # 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 the background is high, continue digesting the vector for a longer time after the addition of more restriction enzyme(s). Incubate 2 hr to overnight. Gel purify the remainder of the vector and transform again.

B. PCR Primer Design

Our online Primer Design tool can easily design primers for amplification of insert fragments, compatible with either linearization method, as well as vector primers for linearization via inverse PCR: takarabio.com/in-fusion-tools

If you would like more information about primer design, please refer to Appendix A.

C. PCR Amplification of Target Fragment

The In-Fusion Cloning 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. For the best results, we recommend using our **PrimeSTAR Max DNA Polymerase** (sold separately as Cat. No. R045A), which provides exceptionally accurate and efficient DNA amplification. The amount of time required for the extension step has been standardized, allowing amplification of large amounts of template DNA that would otherwise be difficult to amplify.

1. **Amount of template to use with PrimeSTAR Max DNA Polymerase** (for a 25-µl reaction, with a 5 sec/kb extension time for genomic DNA, λ DNA, and plasmid DNA templates, or a 5–10 sec/kb extension time for cDNA templates)
   - Human genomic DNA 5 ng–100 ng
   - *E. coli* genomic DNA 100 pg–100 ng
   - λ DNA 10 pg–100 ng
   - Plasmid DNA 10 pg–1 ng
   - cDNA ≤ the equivalent of 25–125 ng total RNA*

   *If you are amplifying from a pool of cDNA, the amount of template DNA required depends on the relative abundance of the target message in your mRNA population

2. **PCR product sizes that can be obtained with PrimeSTAR Max DNA Polymerase** (with a 5 sec/kb extension time for genomic DNA and λ DNA templates, or a 5–10 sec/kb extension time for cDNA templates)
   - Human genomic DNA up to 6 kb
   - *E. coli* genomic DNA up to 10 kb
   - cDNA up to 6 kb
   - λ DNA up to 15 kb

3. **Analysis of PCR products:** When PCR cycling is complete, use an agarose gel to confirm that you have obtained a single DNA fragment and to estimate the concentration of your PCR product. Quantify the amount of DNA by measuring against a known standard or DNA mass ladder run on the same gel.

D. Control Reactions

When using an In-Fusion kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your In-Fusion Cloning reaction. The positive control should consist of a circular vector of known concentration (competent cells should give >2 x 10^8 cfu/µg), and the negative control should consist of a known amount of your linearized vector (see Section VII for Expected Results). Performing the control reactions will verify that the system is working properly. The 2-kb Control Insert included in the In-Fusion Snap Assembly EcoDry cloning kits has already been purified, so there is no need for further treatment prior to the cloning reaction.

V. Protocol: In-Fusion Snap Assembly EcoDry Cloning with Spin-Column Purification

A. Guidelines for Spin-Column Purification of PCR-Amplified Fragments

Following PCR, verify by agarose gel electrophoresis that your target fragment has been amplified. If a single band of the desired size is obtained, you can **EITHER** spin-column purify (see protocol below) **OR** treat your PCR product with Cloning Enhancer (**Cat. # 639615, protocol not described in this document. Please refer to the Cloning Enhancer user manual**). However, if non-specific background or multiple bands are visible on your gel, isolate your target fragment by gel extraction, then spin-column purify. If you use PCR to amplify your vector and insert and you obtain both a PCR-amplified vector AND PCR-amplified fragment(s) without nonspecific background, you can use the Cloning Enhancer protocol.

1. If nonspecific background bands are observed on an agarose gel, isolate your target fragment by gel extraction, then spin-column purify.
2. Spin-column purify your PCR product (e.g., insert) by using a silica-based purification system, following the manufacturer's protocol. We recommend the NucleoSpin Gel and PCR Clean-Up kit, sold separately (Cat. # 740609.50). During purification, avoid nuclease contamination and exposure of the DNA to UV light for long periods of time.
3. After purification, proceed with the In-Fusion Cloning Procedure for Spin Column-Purified PCR Fragments (Section V.B).

B. In-Fusion Cloning Procedure

In general, good cloning efficiency is achieved when using 200 ng combined amount of vector and inserts in a 10-µl reaction, regardless of their lengths. More is not better. If the size of the PCR fragment is shorter than 0.5 kb, maximum cloning efficiency may be achieved by using less than 50 ng of fragment.

Table 2. 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 Water	**to 10 µl**	**to 10 µl**	**to 10 µl**

In general, for optimal results under standard conditions use an insert to vector molar ratio of 2:1. When performing a cloning reaction with two or more inserts, the molar ratio of each of the multiple inserts should still be 2:1 with regard 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. For more details, please read the In-Fusion Snap Assembly EcoDry Multiple-Insert Cloning Protocol-At-A-Glance.

For more detailed guidelines on molar ratios please see the "Tips" subsection under "FAQs" on our In-Fusion Cloning tips and FAQs page:
https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-faqs

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

1. Mix your purified PCR fragment and linearized vector together with deionized H_2O for a total volume of 10 µl.
2. **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 1 to each EcoDry pellet. Mix well by pipetting up and down.
3. Incubate the reaction for **15 min at 50 °C**, and then place on ice.

   NOTE: The In-Fusion reaction is completed within the required 15-min incubation. Longer incubation times do NOT increase cloning efficiency, even with multiple-insert cloning reactions.

4. Continue to the Transformation Procedure (Section VI). You can store the cloning reactions at –20°C until you are ready.

VI. Transformation Procedure

Protocol Transformation Using Stellar Competent Cells

The following 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 transformation protocol provided with your cells, but you may need to dilute the In-Fusion reaction mixture prior to transformation to increase cloning efficiency (See Table 4, Troubleshooting Guide). 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 into a 14-ml round-bottom tube (Falcon tube). Do not vortex.
2. Add 2.5 µl of the In-Fusion reaction mixture to the competent cells.

   IMPORTANT: DO NOT add more than 5 µl of the reaction to 50 µl of competent cells. MORE IS NOT BETTER. Using too much of the reaction mixture inhibits the transformation.

3. Place the tubes on ice for 30 min.
4. Heat shock the cells for exactly 45 sec at 42°C.
5. Place 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 by shaking (160–225 rpm) for 1 hr at 37°C.
8. Place 1/100–1/5 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 (i.e., the control vector included with the kit requires 100 µg/ml of ampicillin).

   NOTE: For cloning reactions with more than two fragments, we recommend plating a larger volume (1/5–1/3 of each transformation reaction).

9. Centrifuge the remainder of each transformation reaction at 6,000 rpm for 5 min. Discard the supernatant and resuspend each pellet in 100 µl fresh SOC medium. Spread each sample on a separate LB plate containing the appropriate antibiotic. Incubate all of the 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 an insert, analyze the DNA by restriction digestion or PCR screening.

VII. Expected Results

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 product and linearized vector used for the In-Fusion cloning reaction.

- The presence of a low number of colonies on both plates—typically, a few dozen colonies—is indicative of either transformation with too much of the reaction, or poor DNA/primer quality.
- The presence of many (hundreds) of colonies on the negative control is indicative of incomplete vector linearization.

VIII. Troubleshooting Guide

If you do not obtain the expected results, use the following guide to troubleshoot your experiment. To confirm that your kit is working properly, perform the control reactions.

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

Table 3. Troubleshooting guide for In-Fusion cloning experiments

A. No or Few Colonies Obtained from Transformation

Description of 0 problem	Possible explanation	Solution
Low transformation efficiency	Transformed with too much In-Fusion reaction	Do not add more than 5 µl of the In-Fusion reaction to 50 µl of competent cells (see Section VI for details).
	Competent cells are sensitive to the In-Fusion enzyme	If your cloning efficiency is low, you may obtain better results if you dilute the reaction. For some cell strains, it may be better to dilute the In-Fusion reaction with TE buffer 5–10 times prior to transformation (add 40–90 µl of TE buffer to 10 µl of In-Fusion reaction).
	Bacteria were not competent	Check transformation efficiency. You should obtain ≥1 x 10^8 cfu/µg; otherwise use fresh competent cells.
	Regions of homology were not long enough for efficient cloning of >2 fragments at once	Increase homologous region of primers from 15 bp to 20 bp.
Low-quality DNA fragments	Low DNA concentration in reaction	It is imperative to obtain the highest DNA concentration possible in your In-Fusion reaction. Either the amount of vector or the amount of PCR fragment was too low. We recommend always 200 ng combined amount of vector and inserts in a 10 µl reaction (see Table 2).
	Gel purification introduced contaminants	If your fragment was gel purified, it is imperative to obtain the highest DNA concentration possible in your In-Fusion reaction. The total volume of purified vector and insert should not exceed 5 µl. If possible, optimize your PCR amplification reactions such that you generate pure PCR products and use Cloning Enhancer instead (see Section V.A for details).
	Suboptimal PCR product	Repeat PCR amplification and purify product using a different method of purification. Alternatively, perform phenol:chloroform extraction on your original PCR product, followed by ethanol precipitation.
	Primer sequences are incorrect	Check primer sequences to ensure that they provide 15 bases of homology with the region flanking the insertion site (see Section IV).

B. Large Numbers of Colonies Contained No Insert

Description of problem	Possible explanation	Solution
Large numbers of colonies obtained with no insert	Incomplete linearization of your vector	It is important to remove any uncut vector prior to use in the In-Fusion reaction. If necessary, recut your vector and gel purify.

	Contamination of In-Fusion reaction by plasmid with same antibiotic resistance	If your insert was amplified from a plasmid, closed circular DNA may have carried through purification and contaminated the cloning reaction: To ensure the removal of any plasmid contamination, we recommend linearizing the template DNA before performing PCR. If you spin-column purify your insert, treating the PCR product with DpnI before purification will help to remove contaminating template DNA.
	Plates too old or contained incorrect antibiotic	Be sure that your antibiotic plates are fresh (<1 month old). Check the antibiotic resistance of your fragment.
C. Clones Contained Incorrect Insert		
Large number of colonies contain incorrect insert	Your PCR product contained non-specific sequences	If your PCR product is not a single distinct band, then it may be necessary to gel purify the PCR product to ensure cloning of the correct insert. See Section VI.A for more information.

Appendix A. PCR Primer Design

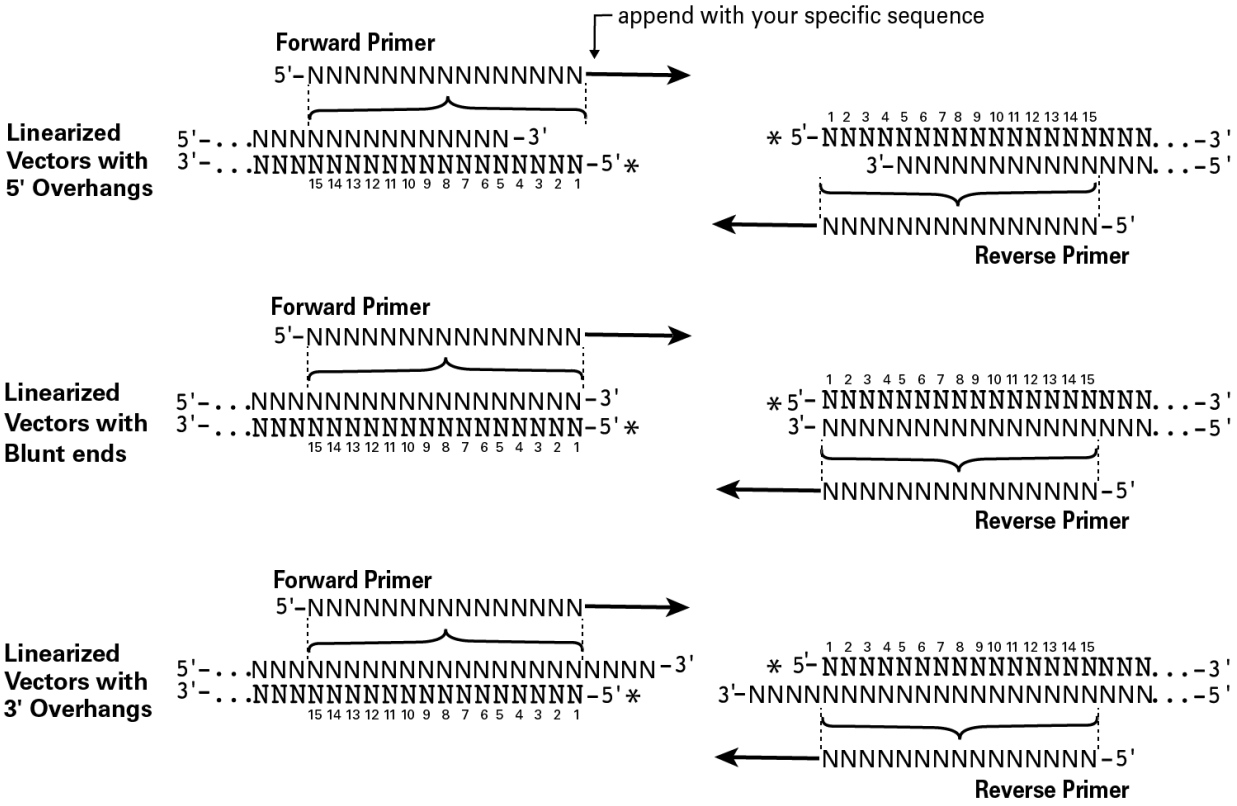
In-Fusion allows you to join two or more fragments (e.g., vector and insert or multiple fragments) as long as they share 15 bases of homology at each end. This homology is achieved through primers designed specifically for your experiment. Figure 2 outlines the guidelines for primer design discussed below and Figure 3 gives specific examples of In-Fusion PCR primers.

1. The 5' end of each primer must contain 15 bases that are homologous to 15 bases at one end of the DNA fragment to which it will be joined (i.e., the vector or another insert).

   NOTE: When joining more than two fragments (including the linearized vector), we strongly recommend increasing the homologous region to 20 bp. We have found that this modification yields 5- to 7-fold more transformant colonies, while still maintaining high cloning accuracy. This increase in the length of sequence homology is not detrimental to any standard cloning applications. For more details, please read the In-Fusion Snap Assembly EcoDry Multiple-Insert Cloning Protocol-At-A-Glance.

2. The 3' portion of each primer should:
   - be gene-specific.
   - be between 18–25 bases in length and have a GC-content between 40–60%.
   - have a melting temperature (T_m) between 58–65°C. The T_m difference between the forward and reverse primers should be ≤4°C, or you will not get good amplification.

     NOTE: The T_m should be calculated based upon the 3' (gene-specific) end of the primer, and NOT the entire primer. If the calculated T_m is too low, increase the length of the gene-specific portion of the primer until you reach a T_m of between 58–65°C.
   - not contain identical runs of nucleotides. The last five nucleotides at the 3' end of each primer should contain no more than two guanines (G) or cytosines (C).
3. Avoid complementarity within each primer to prevent hairpin structures, and between primer pairs to avoid primer dimers.
4. You can perform a BLAST search to determine if the 3' portion of each primer is unique and specific (at **www.ncbi.nlm.nih.gov/BLAST/**).
5. We provide an online tool that simplifies In-Fusion PCR primer design for all possible In-Fusion Cloning reactions:
   https://www.takarabio.com/in-fusion-tools
6. We generally use desalted oligonucleotide primers in PCR reactions. However, primer quality can depend on the vendor and varies from lot to lot. If your primer quality is particularly poor (i.e., has many premature termination products), or your primers are longer than 45 nucleotides, they may need to be PAGE purified; however, we usually find this is unnecessary.



Guidelines for universal primer design

To determine the 15 b homology sequence to be incorporated into each primer, start at the 5' end of each DNA strand in the linearized vector (*). The region of homology for a particular primer consists of bases that are ***complementary*** to the first 15 bases at the 5' end of a particular DNA strand.

This means that the bases complementary to 5' overhangs are included in the primer sequence, but the bases in 3' overhangs are not.

Brackets indicate bases to be included in the 15 b region of homology

Figure 2. Universal primer design for In-Fusion technology. Successful insertion of a PCR fragment requires that the PCR insert shares 15 bases of homology with each end of the linearized vector. This sequence homology is added to the insert through the PCR primers. For vectors with sticky ends, bases complementary to 5' overhangs are included in the primer sequence; bases in the 3' overhangs are not.

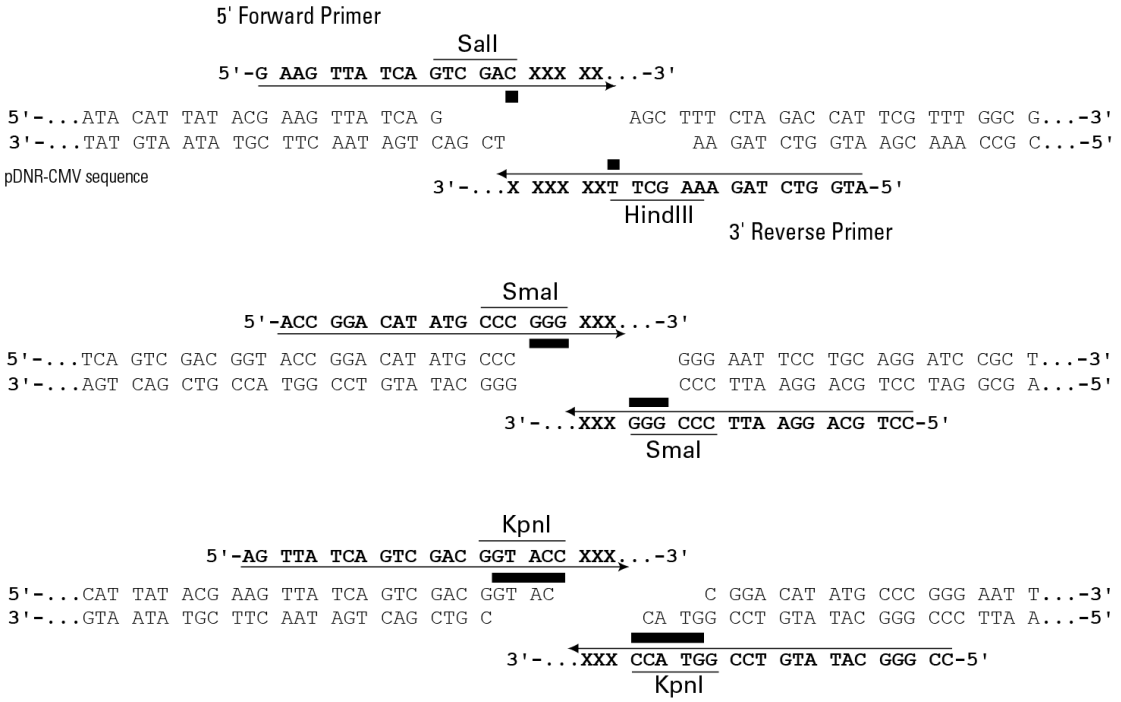


Figure 3. Examples of primers designed for In-Fusion cloning. The above figure shows examples of primers designed with recognition sites for restriction enzymes that generate: 5' overhangs (**Top**), blunt ends (**Middle**), and 3' overhangs (**Bottom**). The primer sequences are shown in bold. The Xs represent bases corresponding to the gene or sequence of interest. Additional nucleotides (indicated with a black box) have been added to each primer in order to reconstruct the restriction sites. **They are not part of the 15 bases of sequence homology.**

Appendix B. pUC19 Linearized Vector Information

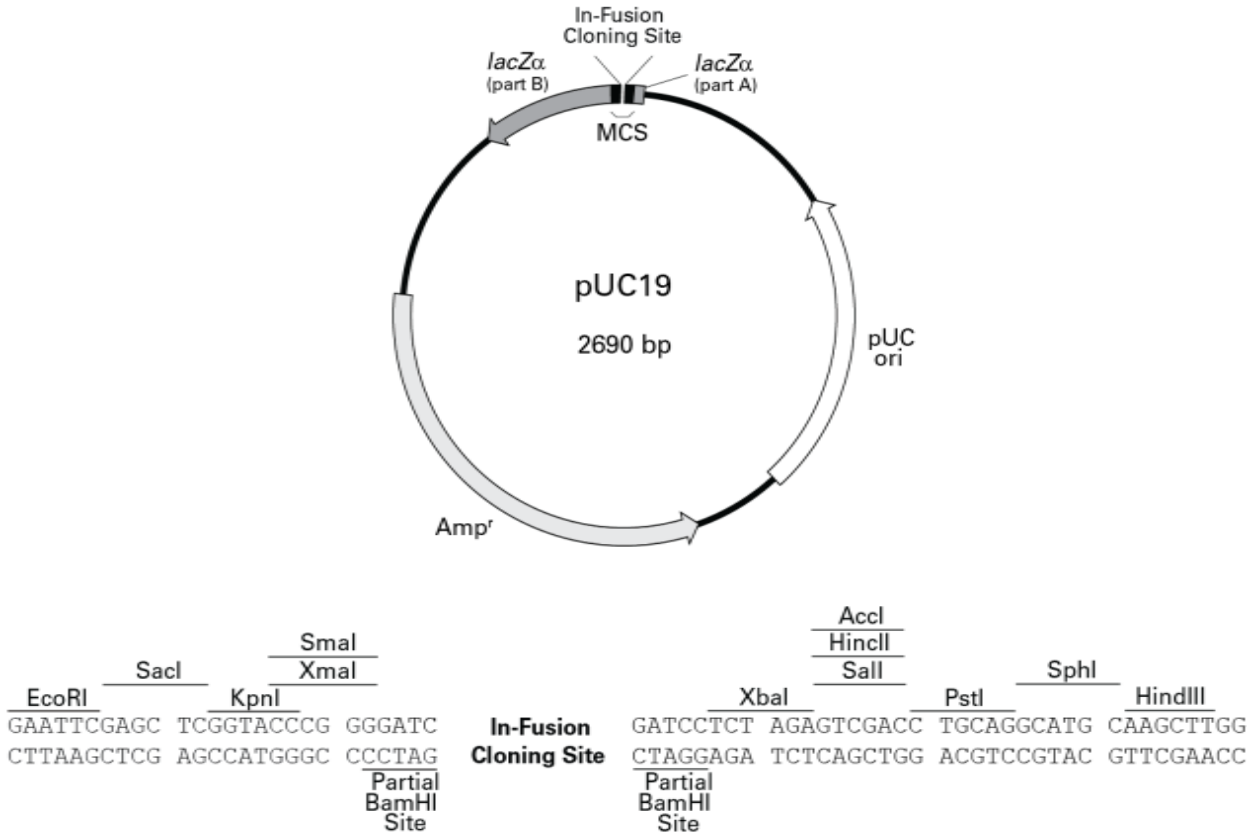


Figure 4. pUC19 Linearized Vector map and multiple cloning sites (MCS). pUC19 is a commonly used, high copy number cloning vector. This linearized version was generated by PCR, and contains the blunt ends shown in the MCS sequence above. The vector encodes the N-terminal fragment of β-galactosidase (lacZα), which allows for blue/white colony screening (i.e., α-complementation), as well as a pUC origin of replication and an ampicillin resistance gene that allow propagation and selection in *E. coli*.

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