User Manual

In-Fusion™ Advantage PCR Cloning Kit User Manual

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I. Introduction

In-Fusion™ Advantage PCR Cloning Kits are designed to join multiple pieces of DNA that have 15 bases of homology at their linear ends. A typical use for this technology would be to clone PCR products into vectors, without the use of restriction enzymes, ligase or phosphatase. In-Fusion cloning kits, which contain our proprietary In-Fusion Enzyme, let you rapidly generate very precise constructs. In-Fusion is high-throughput-compatible and universal—it works with any insert and any vector.

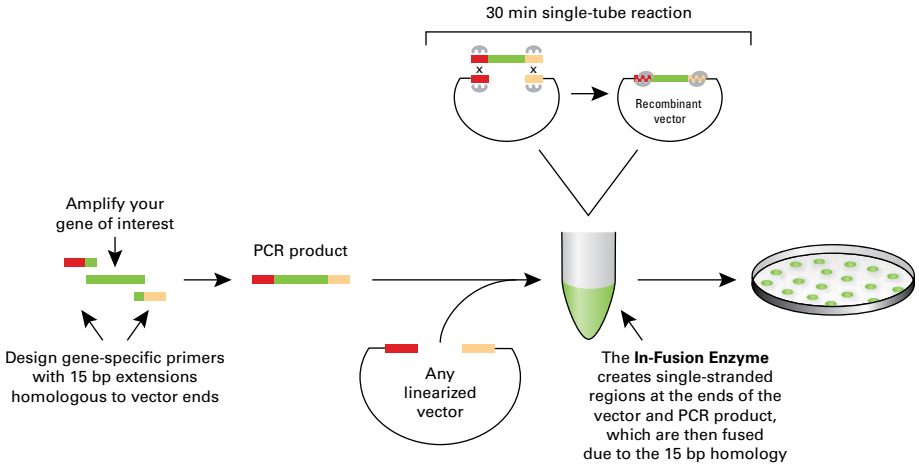


Figure 1. The In-Fusion Cloning Method.

The In-Fusion Advantage PCR Cloning Method

The In-Fusion method is simple and efficient. First, PCR primers are designed that share 15 bases of homology with the sequence at the ends of the linearized cloning vector (i.e., at the desired site of insertion; refer to Section V of this manual). These primers are then used to PCR amplify the insert DNA. The resulting PCR product is treated with our proprietary Cloning Enhancer or spin-column purified, and combined with the linearized vector in the In-Fusion cloning reaction.

In general, the In-Fusion reaction consists of a simple 30 minute incubation of the PCR product with the linearized cloning vector, followed by transformation into *E. coli* (Figure 1). Each reaction generates precise constructs with correctly oriented inserts and no additional nucleotides. The procedure is quite simple, so it is easily automated. With many vectors, such as our pDNR-Dual (and the linearized pUC19 control vector provided with this Kit), blue/white selection on X-Gal plates can be used to screen out rare non-linearized vector background.

Although the highest cloning efficiency is achieved with a high-quality PCR product that appears on an agarose gel as a single, dense band of DNA (with minimal background), PCR products that contain additional non-specific background can also be cloned using In-Fusion. In such cases, the target PCR product is not treated with the Cloning Enhancer. It is, instead, isolated by gel extraction and spin column-purified before cloning. Figure 2 illustrates the differences in the experimental workflow required for Cloning Enhancer treated PCR products versus those that are spin column-purified.

II. In-Fusion Advantage Protocol Overview

The table below is a general outline of the protocol used in the In-Fusion Advantage PCR Cloning Kits. This outline is further illustrated in Figure 2. Please refer to the specified pages for details on performing each step.

Table I. In-Fusion Advantage 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.	8
2	Design PCR primers for your gene of interest with 15 bp extensions (5′) that are homologous to the ends of the linearized vector.	8-10
3	Amplify your gene of interest with a high-fidelity DNA polymerase.	11
4	Verify on an agarose gel that your target DNA has been amplified and determine the integrity of the PCR product. If a single prominent band of desired size is obtained, you can **EITHER** treat your insert with Cloning Enhancer (follow Protocol I), **OR** treat your insert with DpnI and spin-column purify (follow Protocol II). If a non-specific background smear or multiple bands are visible on your gel, isolate your target fragment by gel extraction and spin-column purify (follow Protocol II).	11-12
5	Treat your target fragment with Cloning Enhancer OR spin-column purify.	Cloning Enhancer Protocol I (p.12) OR Spin-Column Protocol II (p.14)
6	Determine the appropriate amount of Cloning Enhancer-Treated or Spin Column-Purified PCR product (insert) and vector to use in your In-Fusion cloning reaction.	Cloning Enhancer Protocol I (p.12) OR Spin-Column Protocol II (p.14)
7	<u>Set up your In-Fusion cloning reaction:</u> 2 µl of 5X In-Fusion Reaction Buffer 1 µl of In-Fusion Enzyme X µl of Vector X µl of Insert X µl of dH_20 to a Total Reaction Volume of 10 µl. Mix well.	Cloning Enhancer Protocol I (p.13) OR Spin-Column Protocol II (p.14)
8	Incubate the reaction for 15 min at 37°C, followed by 15 min at 50°C, then place on ice.	Cloning Enhancer Protocol I (p.13) OR Spin-Column Protocol II (p.14)
9	Bring the reaction volume up to 50 µl with TE buffer (pH 8), and mix well.	Cloning Enhancer Protocol I (p.13) OR Spin-Column Protocol II (p.14)
10	Transform competent cells with 2.5 µl of the diluted reaction mixture from Step 9.	15

II. In-Fusion Advantage Protocol Overview, *continued*

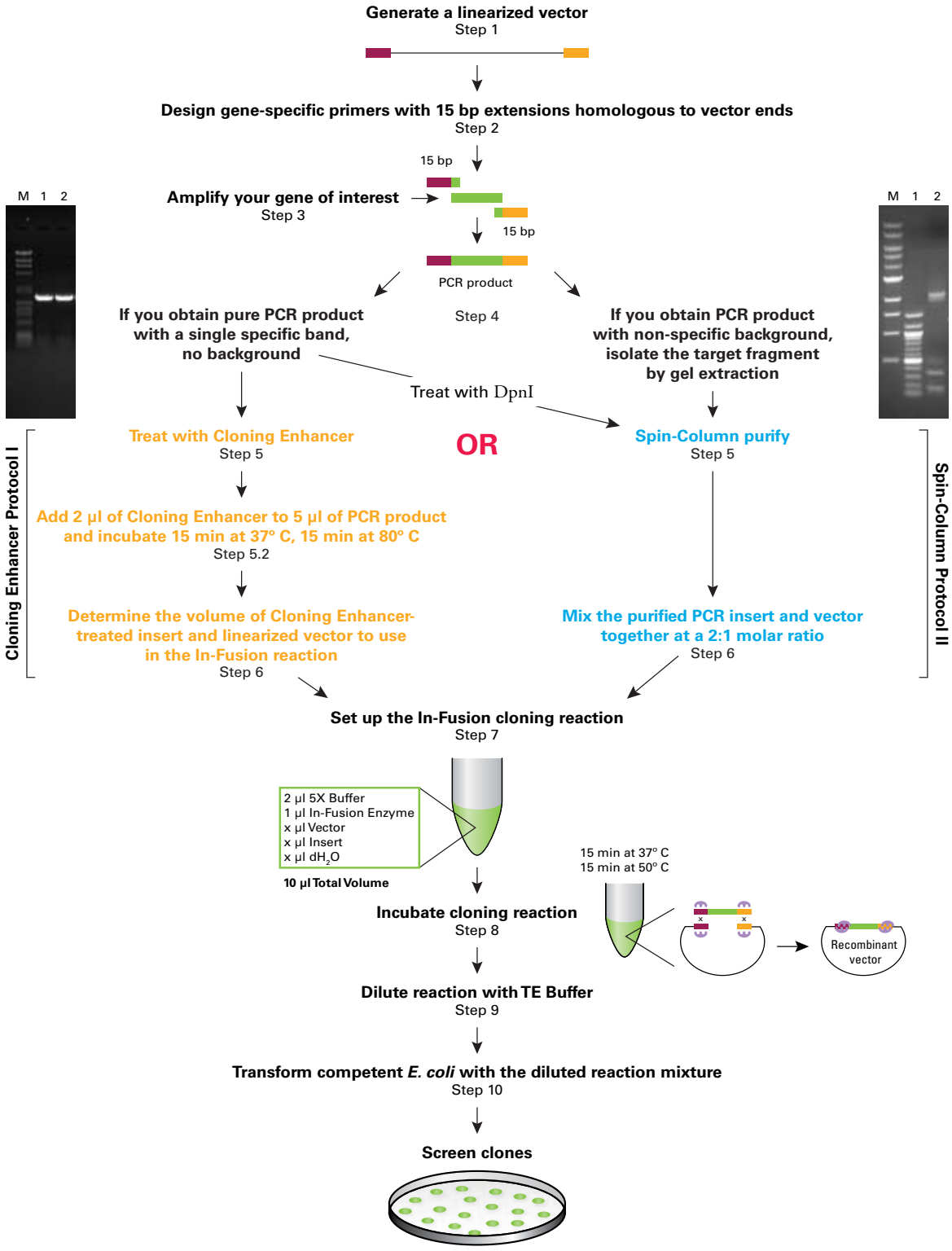


Figure 2. In-Fusion Advantage Protocol Flowchart

III. List of Components

The In-Fusion Advantage PCR Cloning Kits are available in 10, 50 and 100 reaction sizes, with or without Cloning Enhancer or NucleoSpin® Extract II.

Store NucleoSpin Extract II at room temperature.

Store all other components at –20°C.

In-Fusion™ Advantage PCR Cloning Kits				
Components	Cat. Nos.	639619	639620	639621
	Rxns.	10 rxns	50 rxns	100 rxns
In-Fusion Enzyme	Component Amounts	10 µl	50 µl	100 µl
5X In-Fusion Reaction Buffer		20 µl	100 µl	200 µl
pUC19 Control Vector,* linearized (50 ng/µl)		5 µl	5 µl	5 µl
2 kb Control Insert (40 ng/µl)		10 µl	10 µl	10 µl
In-Fusion™ Advantage PCR Cloning Kits w/NucleoSpin®				
Components	Cat. Nos.	639622	639623	639624
	Rxns.	10 rxns	50 rxns	100 rxns
In-Fusion Enzyme	Component Amounts	10 µl	50 µl	100 µl
5X In-Fusion Reaction Buffer		20 µl	100 µl	200 µl
pUC19 Control Vector,* linearized (50 ng/µl)		5 µl	5 µl	5 µl
2 kb Control Insert (40 ng/µl)		10 µl	10 µl	10 µl
NucleoSpin Extract II		10 preps	50 preps	100 preps
In-Fusion™ Advantage PCR Cloning Kits w/Cloning Enhancer				
Components	Cat. Nos.	639616	639617	639618
	Rxns.	10 rxns	50 rxns	100 rxns
In-Fusion Enzyme	Component Amounts	10 µl	50 µl	100 µl
5X In-Fusion Reaction Buffer		20 µl	100 µl	200 µl
pUC19 Control Vector,* linearized (50 ng/µl)		5 µl	5 µl	5 µl
2 kb Control Insert (40 ng/µl)		10 µl	10 µl	10 µl
Cloning Enhancer		50 µl	100 µl	200 µl

*These Kits contain only enough vector for the control reactions.
pUC19 Control Vector Information is available on line at www.clontech.com/support/vectors.asp

IV. Additional Materials Required

The following materials are required but not supplied:

- **TE Buffer** (pH 8.0) required for diluting the In-Fusion reaction prior to transformation

  10 mM Tris-HCl
  1 mM EDTA

- **Sodium Acetate** (3 M) required only if concentrating DNA by precipitation
- **Glycogen** (20 µg/µl) required only if concentrating DNA by precipitation
- **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**

  2% Tryptone
  0.5% Yeast Extract
  10 mM NaCl
  2.5 mM KCl
  10 mM $MgCl_2 \bullet 6H_2O$
  20 mM glucose

  1. For 1 liter, dissolve 20 g of tryptone, 5 g of yeast extract, and 0.5 g of NaCl in 950 ml of deionized H_2O.
  2. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in deionized H_2O for a total volume of 100 ml. Add 10 ml of this stock KCl solution to the solution prepared in Step 1.
  3. Adjust pH to 7.0 with 5 M NaOH, then bring the volume to 980 ml with deionized H_2O.
  4. Prepare a 1 M solution of $MgCl_2$ by dissolving 20.33 g of $MgCl_2 \bullet 6H_2O$ in deionized H_2O for a total volume of 100 ml.
  5. Autoclave both solutions on liquid cycle at 15 lbs/in^2 for 20 min.
  6. Meanwhile, make a 2 M solution of glucose by dissolving 36 g of glucose in deionized H_2O for a total volume of 100 ml. Filter-sterilize this solution.
  7. Let the autoclaved solutions cool to about 55°C, then add 10 ml of the filter-sterilized 2 M glucose solution and 10 ml of 1 M $MgCl_2$. Store at room temperature or 4°C.

- **Competent Cells**
  We recommend the use of Fusion-Blue™ Competent Cells or any commercially-available competent cells (e.g., DH10B™, DH5α™) that have a transformation efficiency ≥ 1.0 x 10^8 cfu/µg. Fusion-Blue Competent Cells are available in 24-transformation (Cat. No. 636700) and 96-transformation (Cat. No. 636758) formats.

- **Cloning Enhancer** (Cat. Nos. 639613, 639614 & 639615) [Optional]
  Cloning Enhancer is provided with some of the In-Fusion Advantage PCR Cloning Kits and can also be purchased separately. Cloning Enhancer removes background template DNA and PCR residue, eliminating the need for PCR insert purification prior to cloning when a single PCR product (i.e., no background) is obtained (See Section V.C.).

- **Spin Columns—NucleoSpin® Extract II** (Cat. Nos. 740609.10, 740609.50 & 740609.250) [Optional]
  Spin columns can be used to purify PCR products, eliminating the need for gel extraction when a single PCR product (i.e., no background) is obtained (See Section V.C.). However, Spin Columns can also be used in conjunction with gel purification (e.g., if non-specific background or multiple bands are visible on an agarose gel). When spin columns are needed, we recommend NucleoSpin® Extract II. NucleoSpin® Extract II is provided with some of the In-Fusion Advantage PCR Cloning Kits and can also be purchased separately.

V. PCR and Experimental Preparation

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. Preparation of Linearized Vector by Restriction Digestion

To achieve a successful In-Fusion reaction, you must first generate a linearized vector (with a very low background of uncut vector present). The linearized vector can be generated using restriction enzymes (single or double digests) or by PCR.

Due to differences in cutting efficiencies, different restriction enzymes will generate different amounts of background. Generally speaking, two enzymes cut better than any single enzyme. Efficiency of digestion will always be better if the restriction sites are as far apart as possible. In addition, increasing the enzyme digestion time and the digestion reaction volume will reduce the background.

Prepare a linearized vector by restriction enzyme digestion as follows.

1. We recommend cutting the vector with two different enzymes to reduce background, unless there is only one site available for cloning.
2. Incubate your restriction digest as directed by the restriction enzyme supplier. For many enzymes, incubation from 3 hours to overnight can increase linearization and reduce background.
3. After digestion, purify the linearized vector using any available PCR purification kit. We recommend using the NucleoSpin® Extract II Kit.
4. [Control] Check the background of your vector by transforming 5–10 ng of the linearized and purified vector into competent cells (See Transformation Procedure, Section IX).

   If the background is high, continue digesting the vector for a longer time after the addition of more restriction enzyme(s). Incubate 2 hours to overnight. Gel purify the remainder of the vector and transform again.

B. PCR Primer Design

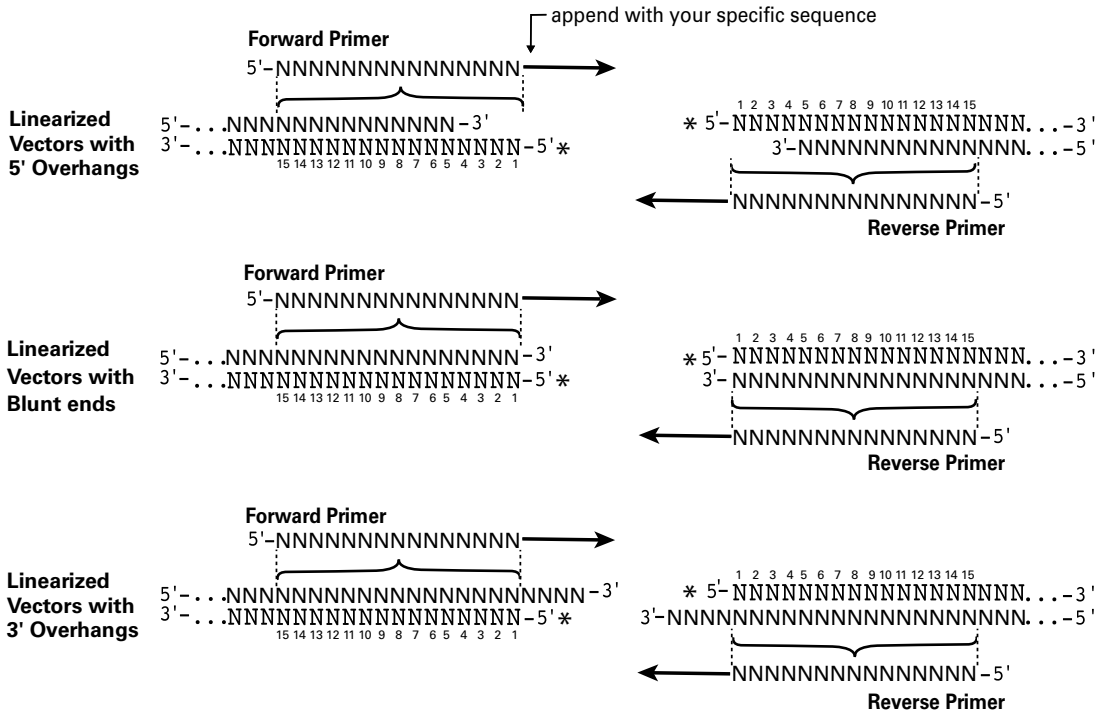
Primer design and quality are critical for the success of the In-Fusion reaction. In-Fusion allows you to join two or more fragments, e.g. vector and insert (or multiple inserts), as long as they share 15 bases of homology at each end. Therefore, In-Fusion PCR primers must be designed in such a way that they generate PCR products containing ends that are homologous to those of the vector. Figure 3 outlines the guidelines for primer design and Figure 4 gives specific examples of In-Fusion PCR primers.

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

1. Every In-Fusion primer must have two characteristics: The 5′ end of the 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). The 3′ end of the primer must contain sequence that is specific to the target gene.
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).

V. PCR and Experimental Preparation, *continued*

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. Clontech provides an online tool (at **http://bioinfo.clontech.com/infusion/**) that simplifies In-Fusion PCR primer design for standard cloning reactions. Simply provide your vector sequence, the restriction enzyme(s) used to linearize the vector (if that is the chosen method for linearization), and the primer sequence required to amplify your region of interest.
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 3. Universal primer design for the In-Fusion System. Successful insertion of a PCR fragment requires that the PCR insert share 15 bases of homology with the ends 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. See Figure 4 for specific examples. An online tool is also provided to assist in primer design and can be found at http://bioinfo.clontech.com/infusion/.

V. PCR and Experimental Preparation, *continued*

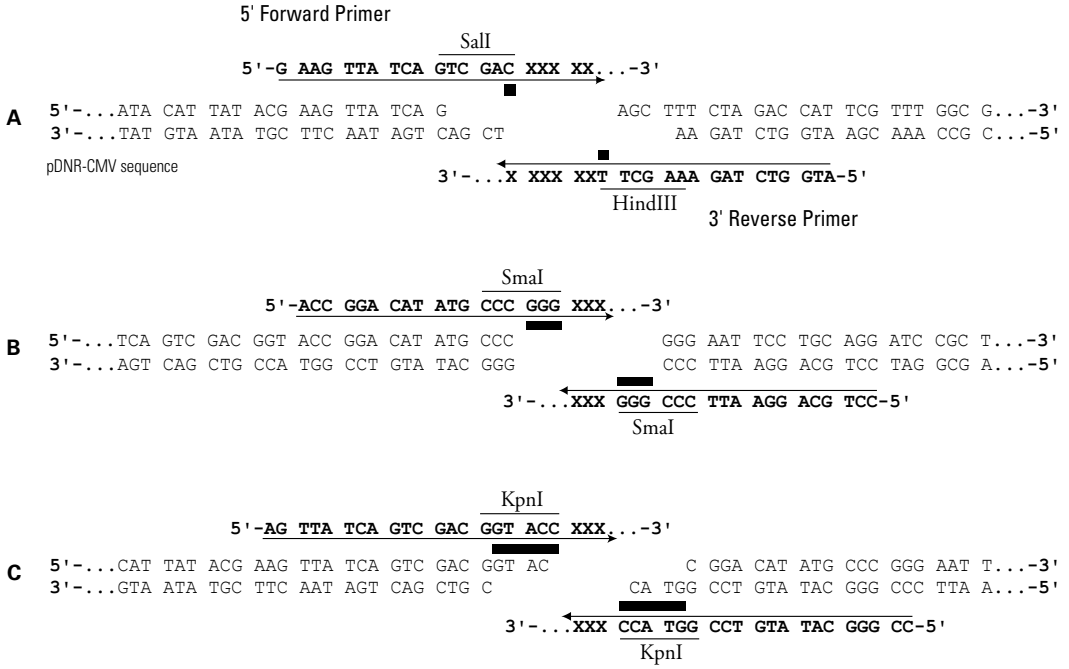


Figure 4. Examples of primers designed for In-Fusion cloning. The above figure shows examples of primers designed with recognition sites for restriction enzymes that generate: 5′ overhangs (**Panel A**), blunt ends (**Panel B**), and 3′ overhangs (**Panel C**). The primer sequences are shown in bold. 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.**

V. PCR and Experimental Preparation, *continued*

C. PCR Amplification of Insert

For most DNA polymerases, 10–100 ng of plasmid DNA is typically enough to use as a PCR template. However, 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.

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. For the best results, we recommend using our **Advantage® HD Polymerase Mix** (Cat. No. 639241), which offers high-fidelity, efficient amplification of long gene segments (>1 kb), and automatic hot start for increased specificity and reduced background. For high yields and error-free amplification of inserts up to 5 kb, we recommend using the Advantage® HF 2 enzyme supplied in our **Advantage HF 2 PCR Kits** (Cat. Nos. 639123 & 639124).

If you will be performing PCR with Advantage HD Polymerase, we recommend using the following amounts of template (for a 50 µl reaction):

Human Genomic DNA	5 ng–200 ng
E. coli Genomic DNA	100 pg–100 ng
λ DNA	10 pg–10 ng
Plasmid DNA	10 pg–1 ng

If you choose not to use Advantage HD, we recommend that you use a robust, high fidelity, thermostable DNA polymerase that is capable of hot start PCR.

When PCR cycling is complete, analyze your PCR product by agarose gel electrophoresis 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 ladder run on the same gel.

IMPORTANT:
Following PCR, verify on an agarose gel that your target fragment has been amplified. If a single band of desired size is obtained, you can **EITHER** treat your PCR product with Cloning Enhancer (**follow Protocol I**) **OR** treat your PCR product with DpnI and spin-column purify (**follow Protocol II**). However, if non-specific background or multiple bands are visible on your gel, we recommend that you isolate your target fragment by gel extraction, then spin-column purify (**follow Protocol II**).

D. Control Reactions

When using the 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. Performing the control reactions will verify that the system is working properly. The 2 kb Control Insert included in the In-Fusion Advantage PCR Cloning Kits has already been purified, so there is no need for further treatment prior to the cloning reaction.

To perform the control reactions, proceed with the In-Fusion Cloning Procedure for Spin Column-Purified PCR Inserts (Section VIII.B).

VI. Which Protocol Should You Follow?

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** treat your PCR product with Cloning Enhancer (**follow Protocol I**), **OR** treat your PCR product with DpnI and spin-column purify (**follow Protocol II**). *However, if non-specific background or multiple bands are visible on your gel*, we recommend that you isolate your target fragment by gel extraction, then spin-column purify (**follow Protocol II**).

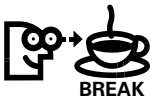
VII. Protocol I: In-Fusion Cloning Procedure w/Cloning Enhancer Treatment

A. Procedure for Treating Unpurified PCR Inserts with Cloning Enhancer

IMPORTANT:
DO NOT treat purified PCR products with the Cloning Enhancer.

Before setting up the In-Fusion cloning reaction, treat unpurified PCR products (e.g. inserts) as follows:

1. Add 2 µl of Cloning Enhancer to 5 µl of the PCR reaction.
2. Incubate at 37°C for 15 minutes, then at 80°C for 15 minutes in a PCR thermal cycler. If you used more than 100 ng of DNA as a template in the PCR reaction, extend the 37°C incubation step to 20 minutes. If you are using a water bath or heat block rather than a thermal cycler, extend each of the incubation steps to 20–25 minutes.



3. Proceed with the In-Fusion Cloning Procedure for Cloning Enhancer-Treated PCR Inserts (Section VII.B). If you cannot proceed immediately, store treated PCR reactions at –20°C until you are ready.

B. In-Fusion Cloning Procedure for Cloning Enhancer-Treated PCR Inserts

IMPORTANT:
Before proceeding to the cloning reaction, be sure your target insert has been pretreated with the Cloning Enhancer, as described in Section A (above). DO NOT follow this procedure if your insert has been purified.

1. Use Table II to determine the amount of linearized vector to use in your In-Fusion reaction.

TABLE II. RECOMMENDED AMOUNT OF VECTOR PER IN-FUSION REACTION

Vector Size	Recommended Nanograms (ng)
<4 kb	100 ng
4 to 6 kb	100 to 150 ng
6 to 10 kb	200 ng
>10 kb	Up to 400 ng

VII. Protocol I: In-Fusion Cloning Procedure w/Cloning Enhancer, *continued*

2. Use Table III to determine the amount of Cloning Enhancer-Treated PCR Insert (from Section VII, Part A) to use in your In-Fusion reaction.

TABLE III. RECOMMENDED AMOUNT OF CLONING ENHANCER-TREATED INSERT PER IN-FUSION REACTION*

InsertSize	Recommended Microliters (µl)
<1 kb	1 µl
1 to 4 kb	1 to 2 µl
4 to 8 kb	4 µl
8 to 12 kb	7 µl

*If you obtain a low product yield from your PCR reaction, we recommend adding more of the Cloning Enhancer treated insert (up to 7 µl).

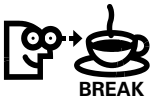
3. **Set up the In-Fusion cloning reaction:**

5X In-Fusion Reaction Buffer	2 µl
In-Fusion Enzyme	1 µl
Vector	x µl*
Cloning Enhancer-Treated PCR Insert	x µl*
dH_2O (as needed)	x µl
Total Volume	10 µl

* *For reactions with larger volumes of vector and insert (> 7 µl of vector + insert), double the amount of reaction buffer and enzyme, and add dH_20 for a total volume of 20 µl.*

4. Adjust the total reaction volume to 10 µl using deionized H_2O and mix the reaction.
5. Incubate the reaction for **15 min at 37°C, followed by 15 min at 50°C**, then place on ice.
6. Bring the reaction volume up to 50 µl* with TE buffer (pH 8), and mix well.

**For some cell strains, it may be better to dilute the reaction with TE buffer to a volume of 100 µl. If your cloning efficiency is low, you may obtain better results if you dilute the reaction further.*



7. Continue to the Transformation Procedure (Section IX). If you cannot transform cells immediately, store the cloning reactions at –20°C until you are ready.

VIII. Protocol II: In-Fusion Cloning Procedure w/Spin-Column Purification

A. Procedure for Spin-Column Purification of PCR Inserts

1. If non-specific background bands are observed on an agarose gel, isolate your target fragment by gel extraction, then spin-column purify. If a single band of the desired size is obtained, add 1 µl of DpnI to 50 µl of the PCR reaction and incubate at 37°C for 60 min, then spin-column purify.
2. Spin-column purify your PCR product (e.g., insert) by using a silica-based purification system, such as NucleoSpin® Extract II. 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 Inserts (Section VIII.B).

B. In-Fusion Cloning Procedure for Spin-Column Purified PCR Inserts

In general, maximum cloning efficiency is achieved when using a 2:1 molar ratio of insert:vector. Typically, 100 ng of a 4–5 kb linearized vector plus 50 ng of a 1 kb PCR product is found to work well in an In-Fusion reaction. If the size of your vector or PCR product is different from this, adjust the amount of your input DNA. Clontech provides an online tool to assist in determining the correct amount of insert and vector to achieve a 2:1 ratio **(http://bioinfo.clontech.com/infusion/)**.

TABLE IV. RECOMMENDED IN-FUSION REACTIONS FOR PURIFIED INSERTS

Rxn Component	Cloning Rxn	Negative Control Rxn	Positive Control Rxn
Purified PCR insert	50–200 ng	–	2 µl**
Linearized vector	100–400 ng	1 µl*	1 µl*
5X In-Fusion Reaction Buffer	2 µl	2 µl	2 µl
In-Fusion Enzyme	1 µl	1 µl	1 µl
Deionized water	to 10 µl	to 10 µl	to 10 µl

*Use 1.0 µl of the linearized pUC19 Control Vector (50 ng/µl) included in the Kit.
**Use 2 µl of the 2 kb Control Insert (40 ng/µl) included in the Kit.

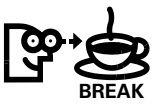
1. Mix your purified PCR insert and vector together in a 2:1 molar ratio.
2. **Set up the In-Fusion cloning reaction**:

5X In-Fusion Reaction Buffer	2 µl
In-Fusion Enzyme	1 µl
Vector	x µl*
Purified PCR Insert	x µl*
dH_2O (as needed)	x µl
Total Volume	10 µl

**For reactions with larger volumes of vector and insert (> 7 µl of vector + insert), double the amount of reaction buffer and enzyme, and add dH_20 for a total volume of 20 µl.*

3. Adjust the total reaction volume to 10 µl using deionized H_2O and mix the reaction
4. Incubate the reaction for **15 min at 37°C, followed by 15 min at 50°C**, then place on ice.
5. Bring the reaction volume up to 50 µl* with TE buffer (pH 8) and mix well.

   **For some cell strains, it may be better to dilute the reaction to 100 µl with TE buffer If your cloning efficiency is low, you may obtain better results if you dilute the reaction further.*



6. Continue to the Transformation Procedure (Section IX). If you cannot transform cells immediately, store the cloning reactions at –20°C until you are ready.

IX. Transformation Procedure

In addition to the cloning reaction, we recommend that you perform positive and negative control transformations. The positive control should consist of a circular vector of known concentration (competent cells should give >1 x 10^8 cfu/µg), and the negative control should consist of a known amount of your linearized vector (see Section X for Expected Results).

1. Transform competent cells with 2.5 µl of the diluted In-Fusion reaction mixture. Follow the transformation protocol provided by your competent cell manufacturer.

IMPORTANT:

DO NOT add more than 5 µl of the diluted reaction to 50 µl of competent cells. **More is not better.** Using too much of the reaction mixture inhibits the transformation. For example, 0.5–1 µl of an undiluted In-Fusion reaction in 50 µl of cells typically yields over 1,000 colonies, while 2 µl of the same reaction will yield fewer than 100 colonies. Since it can be difficult to pipette 1 µl accurately, it is necessary to dilute the In-Fusion reaction with TE buffer (see Section VII.B.6 and Section VIII.B.5) before performing the transformation.

2. Place 1/10^{th} of each transformation reaction (25–50 µl) 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).
3. Centrifuge the remainder of each transformation reaction at 6000 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.
4. 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 insert, analyze the DNA by restriction digestion or PCR screening.

X. Expected Results

The positive control plates typically develop several hundred white 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.

XI. 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.

TABLE V. TROUBLESHOOTING GUIDE FOR IN-FUSION EXPERIMENTS

A. No or Few Colonies Obtained from Transformation

Description of Problem	Explanation	Solution
Low transformation efficiency	Transformed with too much In-Fusion reaction	Do not add more than 5 µl of the diluted In-Fusion reaction to 50 µl of competent cells (see Section IX for details).
	Suboptimal dilution of In-Fusion reaction	For some cell strains, it may be better to dilute the In-Fusion reaction with TE buffer up to a total volume of 100 µl (see Section VII.B, Step 6 OR Section VIII.B, Step 5).
	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.
	Bacteria were not competent	Check transformation efficiency. You should obtain ≥1 x 10^8 cfu/µg; otherwise use fresh competent cells.
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 using between 100 ng and 400 ng of vector, depending on its size (see Table II).
	Wrong molar ratio	The molar ratio of PCR fragment to linear vector used in the In-Fusion protocol may not have been optimal. We recommend using between 100 ng and 400 ng of vector, and 50 to 200 ng of insert. Clontech provides an online tool to assist in determining the correct amount of insert and vector to achieve a 2:1 ratio (**http://bioinfo.clontech.com/infusion/**).
	Gel purification introduced contaminants	If your insert 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. When possible, optimize your PCR amplification reactions such that you generate pure PCR products and use the Cloning Enhancer (see Section VII.A for details).
	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 V.B).
Low cloning efficiency in the experiment, but not in the control	Cloning Enhancer may not have been inactivated properly	Extend the inactivation step of the Cloning Enhancer to 80°C for 20–25 minutes.

XI. Troubleshooting Guide, *continued*

TABLE V. TROUBLESHOOTING GUIDE FOR IN-FUSION EXPERIMENTS

B. Large Numbers of Colonies Contained No Insert

Description of Problem	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: a) To ensure the removal of any plasmid contamination, we recommend linearizing the template DNA before performing PCR. b) If you spin-column purify your insert, be sure to treat the PCR product with DpnI before purification in order to remove contaminating template DNA (see Section VIII.A.1).
	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 for more information.