User Manual

In-Fusion™ Dry-Down PCR Cloning Kit User Manual

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Cat. Nos. 639602, 639604,
639605, 639606, 639607,
639608 & 639609
PT3941-1 (PR9Z3434)
Published January 2010

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I. Introduction and Protocol Overview

The **In-Fusion™ Dry-Down PCR Cloning Kits** are designed to join multiple pieces of DNA which have 15 base pairs of homology at their linear ends. A typical use for this technology would be cloning of PCR products into vectors without the need for restriction enzyme cleavage of the vector or insert and without the use of ligase or blunt-end polishing. Using our proprietary In-Fusion Enzyme, this kit rapidly generates precise constructs with inserts in the desired orientation. Furthermore, the **In-Fusion Dry-Down PCR Cloning Kits w/Cloning Enhancer** allow direct use of an unpurified PCR product in the cloning reaction. In-Fusion is high-throughput-compatible and universal—it works with any insert and any vector at any restriction site, and allows you to disregard restriction sites within the insert. The linearized vector can be generated using restriction enzymes (single or double cut) or by PCR.

The In-Fusion PCR Cloning Method

The In-Fusion method is simple and efficient. First, design PCR primers that have at least 15 bases of homology with sequences flanking the desired site of insertion in the cloning vector (refer to Section IV.B of this manual). Using those primers, amplify the DNA insert by PCR. Directly treat the PCR product with our proprietary **Cloning Enhancer** (Cat. Nos. 639613, 639614 & 639615), or spin column-purify. Then, combine the PCR product with the linearized vector in the In-Fusion cloning reaction.

In general, the In-Fusion reaction consists of a simple 30 min incubation of the PCR product with the linearized cloning vector, followed by transformation into *E. coli* (Figure 1). Each reaction generates inserts in the correct orientation and precise constructs without incorporation of any additional nucleotides. This procedure can be easily automated. With many vectors, optional blue/white selection on X-Gal plates can be used to screen out rare non-linearized vector background. The protocol works best using high-quality, highly concentrated PCR-generated DNA fragments with minimal background.

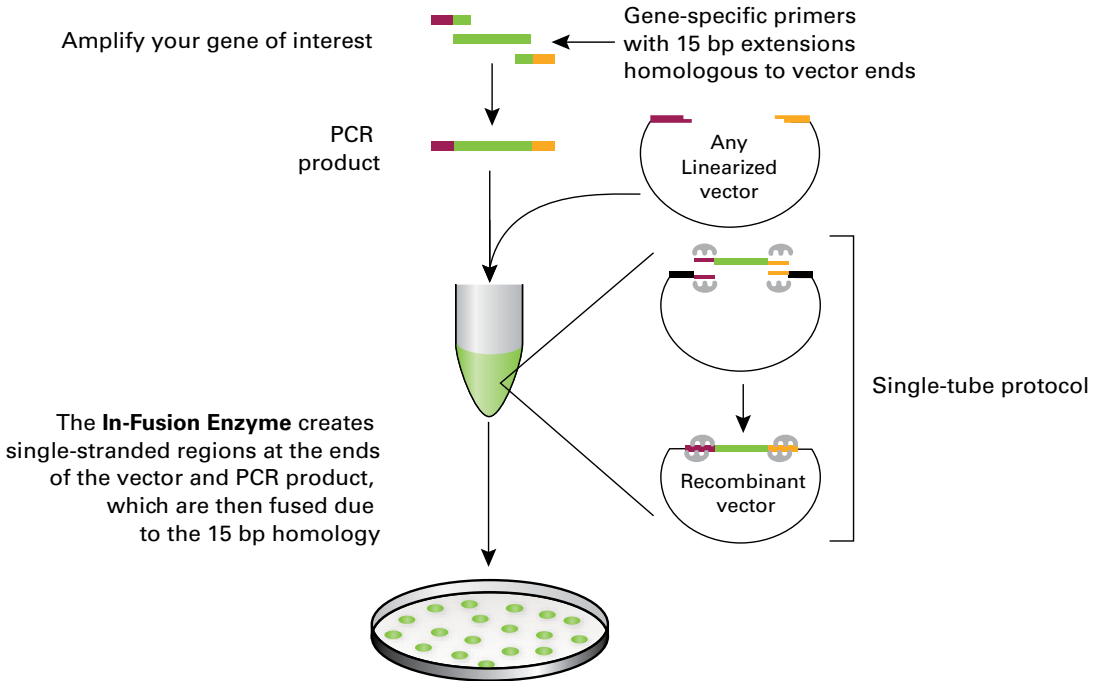


Figure 1. The In-Fusion Cloning Method. During the 30 min incubation, the In-Fusion Enzyme creates single-stranded regions at the ends of the vector and PCR product, which are then fused due to the 15 bp homology. The resulting clone can be used to transform *E. coli.*

I. Introduction and Protocol Overview continued

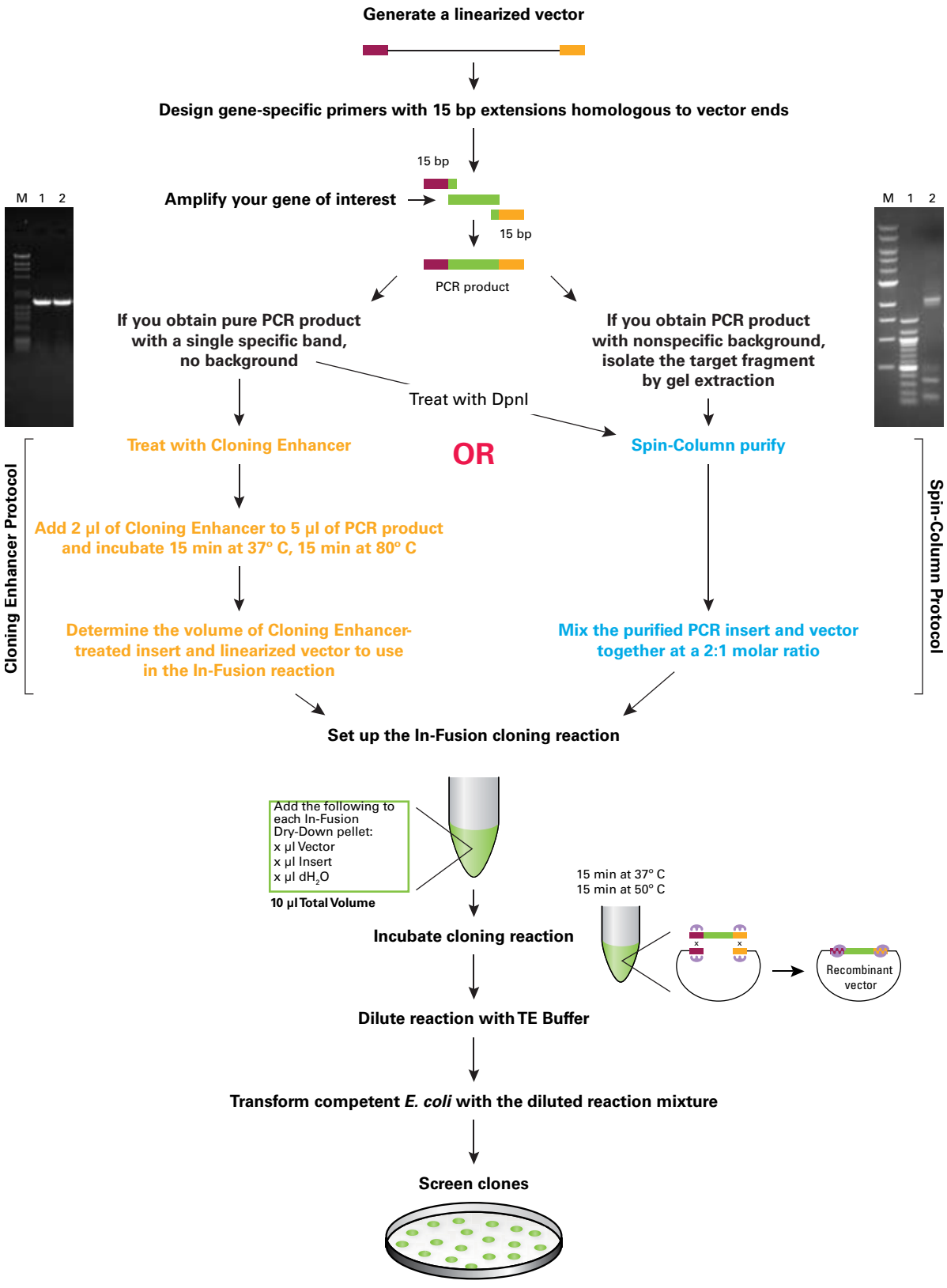


Figure 2. Flow Chart of the In-Fusion™ Dry-Down PCR Cloning Kit Protocols

I. Introduction and Protocol Overview continued

The In-Fusion Enzyme promotes single-strand annealing reactions (SSA), and in this manner can assemble DNA molecules that share short sequence overlaps (or homologies) at their ends, such as a PCR amplified insert and a linearized vector. When the In-Fusion Enzyme is incubated with double stranded inserts and a linearized vector in the provided buffer, the enzyme's exonuclease activity excises nucleotides from the 3' ends of the molecules, exposing the overlapping sequence. The exposed overlapping ends are free to anneal (SSA), forming non-covalently joined molecules that undergo final repair within the target *E.coli* strain. The resulting product is an assembled vector and insert. The reaction is seamless, adding no more bases than you chose to have present.

If you generate nonspecific PCR products that contain additional unwanted background bands, it is still possible to clone using the In-Fusion method. Rather than using the Cloning Enhancer, nonspecific PCR inserts should be gel purified and then used directly in an In-Fusion cloning reaction. When gel-purifying an insert, DO NOT use the Cloning Enhancer treatment. Figure 2 illustrates the differences in the experimental workflow required for cloning pure, clean PCR inserts versus PCR inserts containing nonspecific products. You can choose to spin column-purify PCR products if you would rather not use the Cloning Enhancer.

The In-Fusion method does not require the presence of (nor is it affected by) A-overhangs, so you can use any thermostable polymerase for amplification, including proofreading enzymes. To obtain high yields of small fragments (< 4 kb), we recommend using the Advantage® HF 2 enzyme provided in Clontech's **Advantage HF 2 PCR Kits** (Cat. Nos. 639123 & 639124). For accurate, efficient production of long PCR inserts (>1 kb), we recommend using our **Advantage HD Polymerase Mix** (Cat. No. 639241).

The In-Fusion Dry-Down PCR Cloning Kit Formats

The In-Fusion Dry-Down PCR Cloning Kits provide reaction components in a lyophilized format for maximal convenience and flexibility. All of the necessary cloning reaction materials, except the vector and the PCR insert, are supplied in the reaction tube, thus reducing the variability between reactions. Simply pre-treat your PCR insert with the Cloning Enhancer or gel purify, add 10 µl of distilled water containing the vector and insert to the dry-down reaction tube and incubate for 30 minutes. For added convenience, the dry-down kits are available in multiple sizes. For high-throughput cloning, a 96-well format is available. Always store any unused dry-down reaction tubes in a desiccator at 20°–22°C.

Cloning Enhancer is included with catalog numbers 639607, 639608 & 639609, and is also sold separately in 25, 50 & 100 rxn sizes (Cat. Nos. 639613, 639614 & 639615, respectively). Some of our In-Fusion Dry-Down PCR Cloning Kits include Fusion-Blue™ Competent Cells (Cat. Nos. 639602, 639604 & 639609). We also offer cell-free kits for users who wish to supply their own competent cells. The In-Fusion Dry-Down PCR Cloning Kits are available in 8 reaction, 24 reaction & 96 reaction sizes, with or without Cloning Enhancer or Competent Cells. Please see Section II for a list of components in each of our In-Fusion Dry-Down Kits.

Note:
Cutting wells from the 96-well plate may disturb the seals on remaining wells, thereby damaging the efficacy of the dry-down pellets. If you do not plan on using all 96 pellets in the 96 rxn kit at one time, we recommend that you instead purchase one of the 8 rxn or 24 rxn kits.

II. List of Components

- Always store any unused In-Fusion™ Dry-Down Mix in a desiccator at 20–22°C.
- Store Fusion-Blue™ Competent Cells at –70°C.
- Store all other components at –20°C.

In-Fusion™ Dry-Down PCR Cloning Kits					
Components	Cat. Nos.	639602	639604	639606	649605
	Rxns.	8 rxns	24 rxns	24 rxns	96 rxns
In-Fusion Dry-Down Mix	Component Amounts	8 pellets (1 x strip of 8)	24 pellets (3 x strip of 8)	24 pellets (3 x strip of 8)	96 pellets (96-well plate)
pUC19 Control Vector, linearized* (50 ng/µl)		5 µl	5 µl	5 µl	5 µl
2 kb Control Insert (40 ng/µl)		10 µl	10 µl	10 µl	10 µl
Test Plasmid (0.2 ng/µl)		2 ng	4 ng	Not Included	Not Included
Fusion-Blue Competent Cells** (50 µl/tube)		500 µl (10 tubes)	1250 µl (25 tubes)	Not Included	Not Included
SOC Medium (2 ml/tube)		4 ml (2 tubes)	12 ml (6 tubes)	Not Included	Not Included
Optically Clear PCR Cap Strips		1	3	3	12
Microseal® A Film		Not Included	Not Included	Not Included	1

In-Fusion™ Dry-Down PCR Cloning Kits w/Cloning Enhancer				
Components	Cat. Nos.	639609	639607	639608
	Rxns.	8 rxns	24 rxns	96 rxns
In-Fusion Dry-Down Mix	Component Amounts	8 pellets (1 x strip of 8)	24 pellets (3 x strip of 8)	96 pellets (96-well plate)
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/tube)		50 µl (1 tube)	50 µl (1 tube)	200 µl (4 tubes)
Fusion-Blue Competent Cells** (50 µl/tube)		500 µl (10 tubes)	Not Included	Not Included
SOC Medium (2 ml/tube)		4 ml (2 tubes)	Not Included	Not Included
Optically Clear PCR Cap Strips		1	3	12
Microseal® A Film		Not Included	Not Included	1

*The amount of vector provided in the In-Fusion Dry-Down Kits is sufficient for performing only the control reactions.

**Transformation efficiency >1.0 x 10^8 cfu/µg. Competent cells are only provided with (Cat. Nos. 639602, 639604, & 639609).

III. Additional Materials Required

The following materials are required but not supplied:

• **TE Buffer** (pH 8.0) required for diluting 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) required for the plating of the In-Fusion control reaction

• **LB (Luria-Bertani) medium** (pH 7.0)

		for 1 liter
1.0%	Bacto-tryptone	10 g
0.5%	Yeast extract	5 g
1.0%	NaCl	10 g

Dissolve ingredients in 950 ml of deionized H_2O. Adjust the pH to 7.0 with 5 M NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Store at room temperature or at 4°C.

• **LB/antibiotic plates**

Prepare LB medium as above, but add 15 g/L of agar before autoclaving. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Let cool to ~55°C, add antibiotic (e.g., 100 µg/ml of ampicillin), and pour into 10 cm plates. After the plates harden, then invert and store at 4°C.

• **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.

• **NucleoSpin® Extract II Kit** (Cat. Nos. 740609.50 & 740609.250) PCR products do not need to be purified for successful In-Fusion cloning. If, however, multiple nonspecific bands are observed (See Section IV.C), we recommend that you first gel-purify your fragment of interest using the NucleoSpin Extract II Kit (Section VI.B). This kit can also be used to purify your linearized vector.

• **Cloning Enhancer** (Cat. Nos. 639613, 639614 & 639615) [Optional]

• **Competent Cells**

Some of the In-Fusion Dry-Down PCR Cloning Kits include **Fusion-Blue™ Competent Cells**. If your In-Fusion Dry-Down Kit does not include competent cells, we strongly recommend the use of competent cells with a transformation efficiency >1 x 10^8 cfu/µg.

Clontech sells Fusion-Blue (chemically) Competent Cells separately in 24-transformation (Cat. No. 636700) and 96-transformation (Cat. No. 636758) formats. Clontech also offers **Stellar™ Electrocompetent Cells** (Cat. No. 636765) for cloning. Both Fusion-Blue and Stellar cell lines offer a transformation efficiency >1 x10^8 cfu/µg.

IV. 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 very pure linearized vector (with very low background of uncut vector present). Restriction enzymes will generate different amounts of background, due to differences in cutting efficiency. Generally speaking, two enzymes cut better than any single enzyme. Efficiency of digestion will always be better if the restriction enzyme sites are as far apart as possible. In addition, increasing the enzyme digestion time and digestion reaction volume will reduce the background.

Prepare a linearized vector 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 µg	Vector
10 µl	10X Enzyme buffer
10–20 U	Restriction enzyme*
X µl	Deionized water (to 100 µl)
100 µl	Total Volume

*We recommend adding half the units of enzyme (2.5–5 U/µg) at the beginning of the reaction. Add the remaining enzyme units approximately 30 min later.

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 (Cat. Nos. 740609.50 & 740609.250).

> **Note:**
> To ensure that background is low in a critical cloning experiment, first gel-purify the vector.

4. **[Control]** Check the background of your vector by transforming 5–10 ng of the linearized and purified vector into Fusion-Blue Competent Cells (See Transformation Procedure, Section VII).
   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. You can 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 (Figure 3 outlines the guidelines for primer design; Figure 4 gives specific examples of primers). Therefore, design PCR primers that will generate the homologous region in the PCR product during the amplification.

Every In-Fusion primer must serve two purposes: it should contain an "In-Fusion-Ready" homologous sequence and be gene specific. The 15 base pairs towards the 5′ end of the primer must match the 15 base pairs at the linear end of the DNA fragment to which it will be joined. The 3′ end of the primer is the gene specific portion of the primer. The 3′ end of the primer must have a melting temperature (Tm) suitable for PCR. If you are using software to design your primers, please note that the Tm should be calculated based upon the 3′ (gene-specific) end of the primer, and NOT the entire primer. If the calculated Tm is too low, increase the length of the gene-specific portion of the primer until you reach a Tm of between 58°–65° C. The Tm difference between the forward and reverse primers should be ≤ 4° C, or you will not get good amplification.

IV. PCR and Experimental Preparation continued

Clontech provides an online tool (at **www.clontech.com/ifprimers**) that simplifies the 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.

We generally use desalted oligos in PCR reactions. However, oligo quality can depend on the vendor and varies from lot to lot. If your oligo supply is particularly poor (i.e., has many premature termination products), or your PCR primer is longer than 45 nucleotides, you may need to use PAGE purified oligos, but in general we find that this is unnecessary.

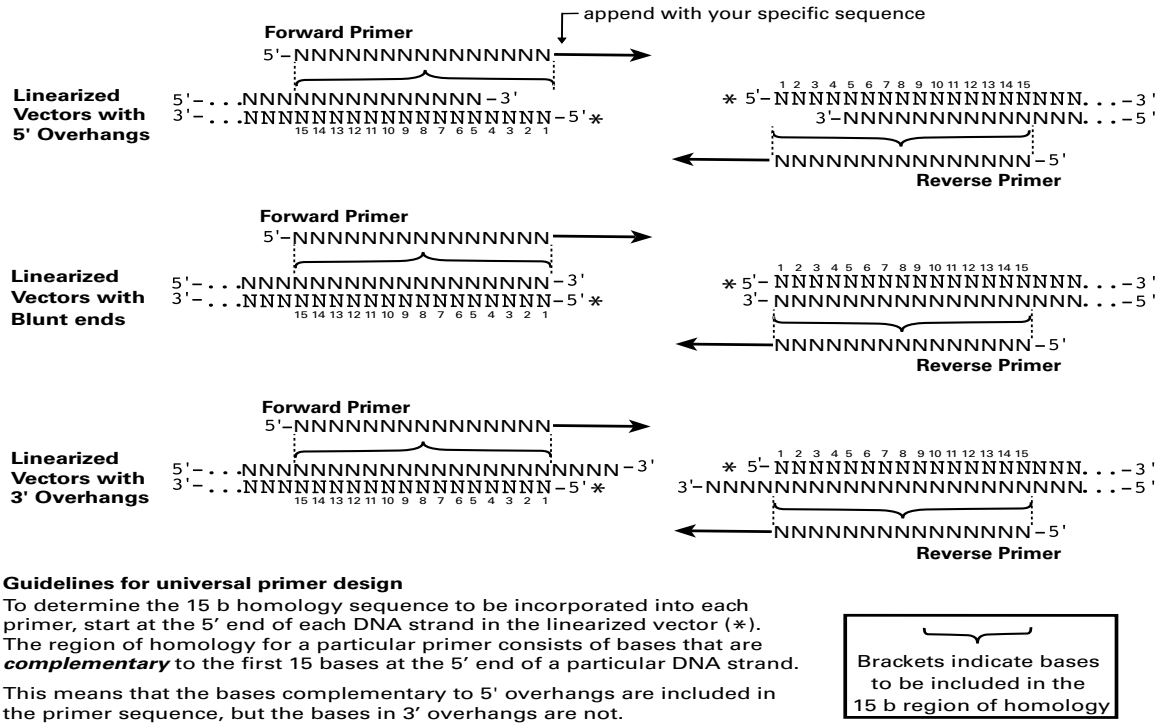


Figure 3. Universal Primer Design for the In-Fusion System. Successful insertion of a PCR fragment requires that the PCR insert share 15 bases of homology with the ends of the linearized vector. This sequence homology is added to the insert through the PCR primers. For vectors with sticky ends, bases 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 **www.clontech.com/ifprimers**

IV. PCR and Experimental Preparation continued

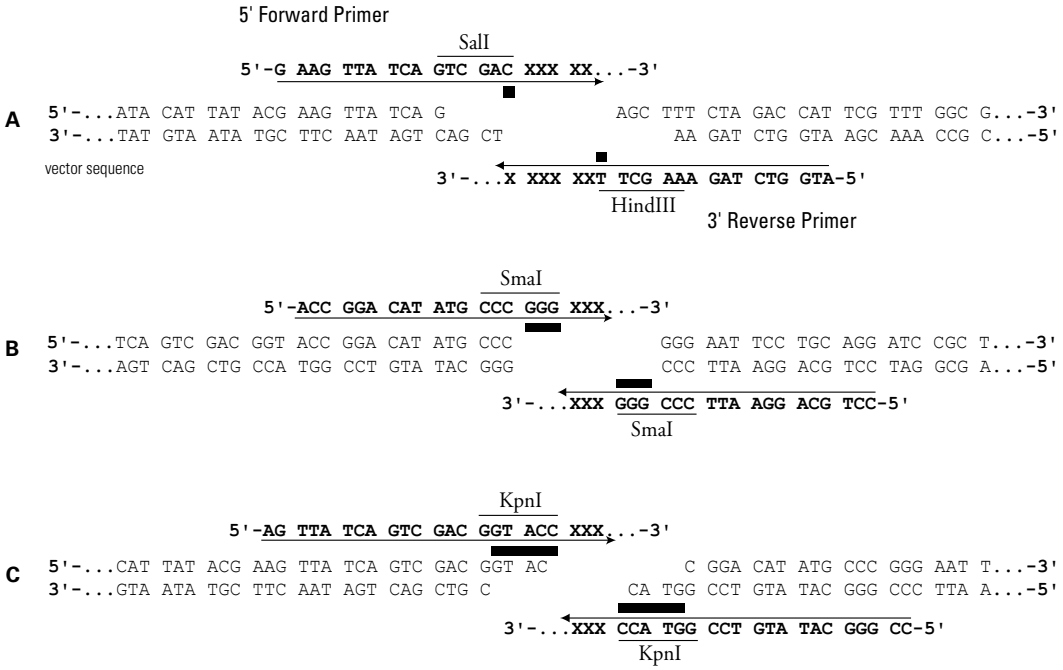


Figure 4. Examples of primers designed for In-Fusion cloning. The above figure shows examples of primers designed with recognition sites for restriction enzymes that generate: 5′ overhangs (**Panel A**), blunt ends (**Panel B**), and 3′ overhangs (**Panel C**). The primer sequences are shown in bold.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

C. PCR Amplification of Insert

It is important to use only 10–100 ng of plasmid DNA as a PCR template. However, if you are amplifying a pool of cDNA, the amount of template DNA depends on the relative abundance of the target message in your mRNA population.

For 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 an automatic hot start that reduces nonspecific products.

When PCR cycling is complete, analyze your PCR product by electrophoresis on an agarose/EtBr 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 molecular weight marker ladder run on the same gel. The linearized vector provided in the kit is useful for this purpose.

IMPORTANT:

- If you generate a single, clean PCR product with little background, you can purify your PCR-amplified insert using spin columns (see Section VI), or simply treat your insert with **Cloning Enhancer** (Cat. Nos. 639613, 639614 & 639615; see Section V).
- If multiple bands are observed, we recommend that you first gel-purify your fragment (see Section VI).
- We recommend **NucleoSpin® Extract II** (Cat. Nos. 740609.50 & 740609.250) for gel purification of PCR-amplified inserts.

IV. PCR and Experimental Preparation continued

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 Dry-Down 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 VI.A).

Note:
The amount of control vector and control insert provided in the In-Fusion Liquid Kits is sufficient for performing only the control reactions.

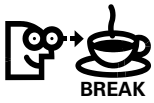
V. In-Fusion Procedure for Cloning Enhancer-Treated Inserts

A. Procedure for Treating Unpurified Single-Band 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) containing a single band of desired size without nonspecific background 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 water baths or heat blocks instead of a thermal cycler, preset them at 37°C and 80°C, respectively, and 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 V.B). If you cannot proceed immediately, store treated PCR reactions at –20°C until you are ready.

V. In-Fusion Procedure for Cloning Enhancer-Treated Inserts continued

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

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

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

TABLE I. RECOMMENDED NANOGRAMS OF VECTOR PER IN-FUSION REACTION

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

2. Use Table II to determine the final amount of Cloning Enhancer-treated PCR insert (from Section V.A) to use in your In-Fusion reaction.

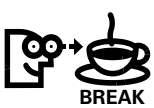
TABLE II. RECOMMENDED MICROLITERS OF CLONING ENHANCER-TREATED INSERT PER IN-FUSION REACTION*

Insert Length	Microliters of Cloning Enhancer-Treated Insert
<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 have a very weak PCR product, we recommended adding more of the Cloning Enhancer treated insert (up to 7 µl).	

3. Mix the insert and vector and adjust the final volume to 10 µl using deionized H_2O. The final volume must not exceed 10 µl.
4. **Set up the In-Fusion reaction:**

Note:
Improved efficiency is observed for In-Fusion reactions with a total volume of vector + insert that is 5 µl or less.

   a. Peel back the aluminum seal(s) from the tube(s) you plan on using and take care to avoid disturbing the seals of the remaining tubes.
   b. Add 10 µl of vector + insert DNA + H_2O (from Step 3) to each In-Fusion Dry-Down pellet. Mix well by pipetting up and down.
5. Incubate the reaction for **15 min at 37°C, followed by 15 min at 50°C**, then place on ice.
6. Dilute the In-Fusion reaction mixture with 40 µl TE buffer (pH 8) and mix well.



7. Proceed with Transformation (Section VII). If you cannot transform cells immediately, store cloning reactions at –20°C until you are ready.

VI. In-Fusion Procedure for Gel- & Spin Column-Purified PCR Inserts

A. Procedure for Spin-Column Purification of Multiple- and Single-Band PCR Inserts

Depending on whether your PCR reaction contains a single band of desired size, or yields multiple bands or nonspecific background on a gel, prepare your PCR product as follows (see Figure 2):

1. If nonspecific background or multiple bands are visible on your gel, isolate your target fragment by gel extraction, then spin column-purify (see Step 2). If you obtain a single band of the desired size, add 1 µl of DpnI to 50 µl of the PCR reaction and incubate at 37°C for 60 min.

Note:
Gel-purified inserts do not require DpnI treatment prior to spin column purification.

2. Spin-column purify your PCR product (e.g., insert) by using a silica-based purification system, such as **NucleoSpin® Extract II Kit** (Cat. Nos. 740609.50 & 740609.250). During purification, avoid nuclease contamination and exposure of the DNA to UV light for long periods of time.

3. After spin column purification (following either DpnI treatment or gel extraction), proceed with the In-Fusion Cloning Procedure for Spin Column-Purified PCR Inserts (Section VI.B).

IMPORTANT:
DO NOT treat gel-purified or spin column-purified inserts with the Cloning Enhancer (Figure 2).

B. In-Fusion Cloning Procedure for Gel- & 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 to 5 kb linearized vector plus 50 ng of a 1 kb PCR fragment is found to work well in a 10 µl In-Fusion reaction. Adjust the amount of your input DNA if the size of your vector or PCR fragment is different from above. 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 III. RECOMMENDED IN-FUSION REACTIONS FOR GEL & SPIN COLUMN-PURIFIED INSERTS

Rxn Component	Cloning Rxn	Negative Control Rxn	Positive Control Rxn
PCR insert	50–200 ng	–	50 ng**
Linearized vector	100–300 ng	100 ng*	100 ng*
Deionized water	to 10 µl	to 10 µl	to 10 µl

*Use 1 µl of the 50 ng/µl solution of linearized pUC19 Control Vector included in the kit.
**Use 2 µl of the 40 ng/µl solution of 2 kb Control Insert included in the kit.

1. Mix your purified PCR insert and vector together at a 2:1 molar ratio in 10 µl of deionized H_2O.

   If necessary, co-precipitate your DNA as follows: Mix the vector and PCR insert together at the correct molar ratio in a 50–100 µl volume. Add 1 µl glycogen (20 µg/µl), and 1/10 volume sodium acetate (3 M) and mix. Then add 3 volumes of ethanol (–20°C) to precipitate, and then centrifuge at maximum speed for 10 min at 4°C. Wash the pellet once with 70% ethanol. Air dry the pellet, and suspend the DNA pellet in H_2O to a volume of 10 µl.

VI. In-Fusion Procedure for Gel- & Spin Column-Purified PCR Inserts continued

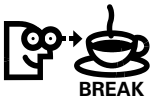
2. **Set up the In-Fusion reactions:**

   a. Peel back the aluminum seal(s) from the tube(s) you plan on using and take care to avoid disturbing the seals of the remaining tubes.

   b. Add 10 µl of vector + insert DNA + H_2O (from Step 1) to each In-Fusion Dry-Down pellet. Mix well by pipetting up and down.

3. Incubate the reaction for **15 min at 37°C, followed by 15 min at 50°C**, then place the tube on ice.

4. Dilute the In-Fusion reaction mixture with 40 µl TE buffer (pH 8) and mix well.



5. Proceed with Transformation (Section VII). If you cannot transform cells immediately, store cloning reactions at –20°C until you are ready.

VII. Transformation Procedure

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

Note:
If your In-Fusion Dry-Down Kit does not include competent cells, we strongly recommend the use of competent cells with a transformation efficiency >1 x 10^8 cfu/µg. Clontech sells Fusion-Blue Competent Cells separately in 24-transformation (Cat. No. 636700) and 96-transformation (Cat. No. 636758) formats. Clontech also offers Stellar™ Electrocompetent Cells (Cat. No. 636765) for cloning. Both Fusion-Blue and Stellar cell lines offer a transformation efficiency >1 x 10^8 cfu/µg.

1. Transform competent cells with 2.5 µl of diluted reaction mixture as follows:

   a. **Using Fusion-Blue™ Competent Cells:**

   - Thaw one vial of frozen Fusion-Blue Competent Cells on ice. Tap tube gently to ensure that the cells are suspended.
   - Add 2.5 µl of the diluted reaction mixture to the cells. Mix gently to ensure even distribution of the DNA solution. Leave the tube on ice for 30 min.

Note:
DO NOT add more than 5 µl of 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 (e.g. if you are using "yellow tips" with a p20 pipettor), we have suggested (Section V.C.6 and Section VI.C.4) that you dilute the In-Fusion reaction with TE buffer before performing the transformation—especially if you wish to use a small volume of competent cells (e.g. HTP cloning).

   - Heat shock the cells in a water bath at 42°C for 45 sec, and then place them directly on ice for 1 min.
   - After a heat shock, add 450 µl of SOC medium to the cells and then incubate at 37°C for 60 min while shaking at 250 rpm.

   b. If using other competent cells with In-Fusion™ Kits, follow the transformation protocol provided with the cells **(DO NOT add more than 5 µl of diluted reaction to 50 µl of competent cells)** and proceed to Step 3.

2. Take 1/10 of the cells (25–50 µl) from each transformation, bring the volume to 100 µl with SOC medium, and then spread on separate LB plates containing 100 µg/ml of ampicillin or other appropriate antibiotic for your cloning vector.
3. Centrifuge the remaining mix at 6000 rpm for 5 min, resuspend the cells in 100 µl fresh SOC and spread the remainder of the transformation mix on a separate LB plate containing the appropriate antibiotic. Incubate all plates at 37°C overnight.
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 DNA by restriction digest or PCR screening.

VIII. Expected Results

The positive control plates typically develop several hundred white colonies when using cells with a minimum competency 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, poor DNA quality, or poor primer quality.
- The presence of many (hundreds) of colonies on the negative control is indicative of contamination with a PCR template plasmid carrying antibiotic resistance.

IX. 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 IV. 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 mixture	Do not add more than 5 µl of diluted In-Fusion reaction (or 1 µl of undiluted In-Fusion reaction) to 50 µl of competent cells (See Section VII 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.
	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. For Cloning Enhancer-treated inserts, we recommend using between 100 ng and 400 ng of vector, depending on its size (see Table I). In the case of gel-purified inserts, we recommend using between 100 and 300 ng of vector (Table III).
	Wrong molar ratio	The 2:1 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 300 ng of vector for cloning gel purified inserts. 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**).
	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).

IX. Troubleshooting Guide continued

TABLE IV. 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 (vector) may have carried through purification and contaminated the cloning reaction. To ensure the removal of any plasmid contamination, we recommend linearizing the vector template before performing PCR. Alternatively, the PCR product can be treated with DpnI to remove the parental vector template after PCR amplification (Weiner *et al.,* 1994; Fisher *et al.,* 1997).
	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 Contain Incorrect Insert		
Large number of colonies contain incorrect insert	PCR product containing nonspecific 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.

X. References

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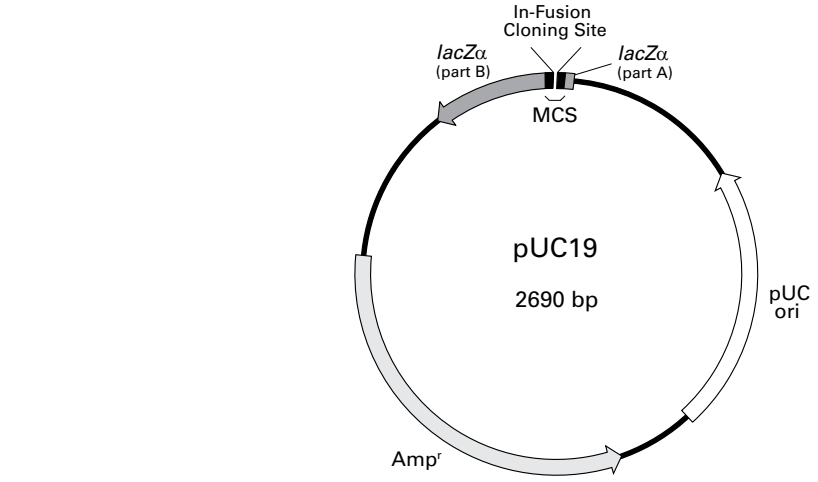
Appendix A: Control Vector Map and In-Fusion Cloning Site



SacI SmaI XmaI AccI HincII SalI SphI
EcoRI KpnI XbaI PstI HindIII

GAATTCGAGC TCGGTACCCG GGGATC **In-Fusion** GATCCTCT AGAGTCGACC TGCAGGCATG CAAGCTTGG
CTTAAGCTCG AGCCATGGGC CCCTAG **Cloning Site** CTAGGAGA TCTCAGCTGG ACGTCCGTAC GTTCGAACC

Partial BamHI Site
Partial BamHI Site

Figure 5. pUC19 Linearized Vector Map & In-Fusion Cloning Site. Sequence and digest information is available in PT4065-5 and can be downloaded from our website at **www.clontech.com/manuals**.

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