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

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I. Introduction & Protocol Overview

The **In-Fusion™ PCR Cloning Kits** are designed for high-throughput cloning of PCR products without the need for restriction enzymes, ligase, or blunt-end polishing. Using our proprietary In-Fusion Enzyme, this kit rapidly generates precise constructs with inserts in the correct orientation. The In-Fusion™ method is universal—it works with any insert and any vector at any restriction site. If you choose to clone your PCR insert into one of the linearized Creator™ Donor Vectors (Table I) provided with this kit—pDNR-Dual or pDNR-CMV—you will have the additional advantage of obtaining constructs that are immediately ready for expression analysis with our Creator™ Gene Cloning & Expression System.

The In-Fusion™ PCR cloning method

Cloning of PCR products is simple with the In-Fusion method. First, design PCR primers that have at least 15 bases of homology with sequences flanking the desired site of insertion in the cloning vector. For information on PCR primer design, refer to Section IV.A of this manual. Using those primers, amplify the DNA insert by PCR. Then combine the PCR product with the linearized cloning vector in the In-Fusion cloning reaction.

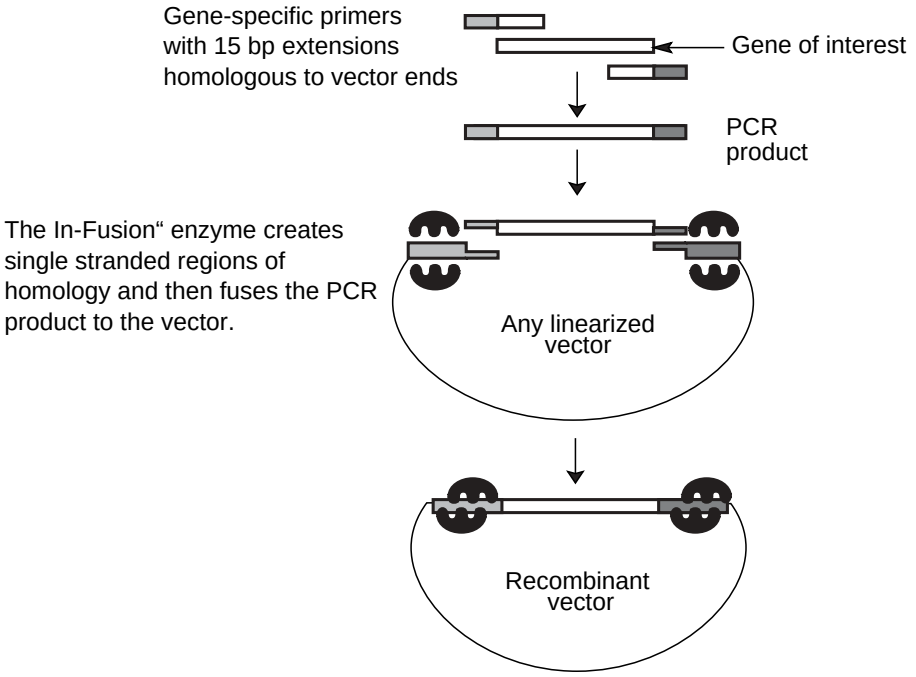


Figure 1. The In-Fusion™ cloning method. During the 30 min benchtop incubation, the In-Fusion enzyme creates single-stranded regions of homology with the vector by strand displacement, and then joins the PCR product to the vector. The resulting clone can be used to transform *E. coli*.

I. Introduction *continued*

In general, the In-Fusion cloning reaction consists of a simple 30 min benchtop incubation* of the PCR product with the linearized cloning vector, followed by transformation of *E. coli* (Figure 1). Each cloning reaction generates precise constructs that have inserts in the correct orientation. With vectors such as our pDNR-Dual, optional blue/white selection on X-Gal plates can be used to screen out rare nonlinearized vector background.

The In-Fusion PCR Cloning method does not require the presence of A-overhangs, so you can use any thermostable polymerase for amplification, including proofreading enzymes such as *Vent* and *Pfu*. We recommend our Advantage™ 2 Polymerase Mix (Cat. No. 639201), a robust enzyme mix that is ideally suited for long-distance (LD) PCR and has been thoroughly tested with the protocols in this User Manual. This 50X mix contains TITANIUM™ *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart™ Antibody to provide automatic hot start PCR (Kellogg *et al*., 1994)—and a minor amount of a proofreading polymerase. Advantage 2 Polymerase Mix is also available in the Advantage™ 2 PCR Kit (Cat. No. 639101).

Applications

The In-Fusion PCR Cloning Kit makes it easy to clone and characterize products. After you obtain a cDNA of interest, clone it into pDNR-Dual or pDNR-CMV for further analysis in one of our many Creator Acceptor Vectors. The linearized pDNR-Dual and pDNR-CMV Donor Vectors (Table I, Appendix A) are manufactured using methods that yield the highest reliability and recombination efficiency. Our Donor Vectors are part of our Creator™ Cloning System, which utilizes Cre recombinase to rapidly transfer any DNA fragment cloned in a Donor Vector (pDNR-Dual or pDNR-CMV) into any Acceptor Vector (available separately) for functional analysis without the need for subcloning or re-ligation. When used with an Acceptor Vector containing a splice acceptor site, pDNR-Dual can also be used to rapidly generate recombinant expression plasmids containing 3' tags.

The In-Fusion Dry-Down PCR Cloning Kits provide reaction components in a lyophilized format for enhanced user flexibility. All of the necessary reaction materials except the cloning vector and the PCR insert are supplied in the reaction tube—Simply add 10 µl of distilled water containing the vector and insert to the reaction tube and incubate at room temperature for 30 minutes. For added convenience, the dry-down kits are available in several sizes and the dried reaction tubes can be stored at room temperature. The reaction strips are the most flexible format because they can be used simultaneously or individually, depending on your needs. For high-throughput cloning, a 96-well format is available.

***** Please refer to the Troubleshooting Guide, Section VI.A under low cloning reaction efficiency.

I. Introduction *continued*

The In-Fusion PCR Dry-Down Cloning Kits include Fusion-Blue™ Competent Cells. We also offer selected CF (cell-free) kits for users who wish to supply their own competent cells. The In-Fusion CF Dry-Down PCR Cloning Kits are available in 24 reaction and 96 reaction sizes.

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

TABLE I: CREATOR™ DONOR VECTORS

Vector	Features	Applications
pDNR-Dual	T7 promoter, M13 Forward site, SD site, C-terminal 6xHN tag, *Sac*B selection	• T7 RNA polymerase primer/promoter site upstream of MCS for *in vitro* transcription/translation of gene of interest. • C-terminal tagging by intron splicing in eukaryotes. • Built-in 6xHN tag for expressed protein purification.
pDNR-CMV	CMV promoter, M13 Forward/ Reverse primer sites, T7 promoter, *Sac*B selection	• T7 RNA polymerase primer/promoter site upstream of MCS for *in vitro* transcription/translation of gene of interest. • CMV promoter for expression testing in mammalian cells prior to transfer of sequence into an acceptor vector.

II. List of Components

- Store In-Fusion™ Dry-Down Mix at room temperature, in a sealed bag and/or desiccator.
- Store Fusion-Blue™ Competent Cells, Test Plasmid, and SOC Medium at –70°C.
- Store all other components at –20°C.

Cat. Nos. 639602	Cat. Nos. 639604	
8 rxn	24 rxn	In-Fusion™ Dry-Down Mix
5 µl	5 µl	pDNR-Dual Donor Vector (100 ng/µl, linear)*
5 µl	5 µl	pDNR-CMV Donor Vector (100 ng/µl, linear)*
2 ng	6 ng	Test Plasmid (0.2 ng/µl)
5 µl	5 µl	1.1 kb Control Insert (25 ng/µl)
10	25	Fusion-Blue™ Competent Cells (50 µl/tube)
2	6	SOC Medium (2 ml/tube)

Cat. No. 639606	
24 rxn	In-Fusion™ Dry-Down Mix
5 µl	pDNR-Dual Donor Vector (100 ng/µl, linear)*
5 µl	pDNR-CMV Donor Vector (100 ng/µl, linear)*
5 µl	1.1 kb Control Insert (25 ng/µl)

Cat. No. 639605	
96 rxn	In-Fusion™ Dry-Down Mix
12	Optically Clear PCR Cap Strips
1	Microseal A Film
5 µl	pDNR-Dual Donor Vector (100 ng/µl, linear)*
5 µl	pDNR-CMV Donor Vector (100 ng/µl, linear)*
5 µl	1.1 kb Control Insert (25 ng/µl)

*** efficiency >1.0 x 10^8 cfu/µg**

III. Additional Materials Required

The following materials are required but not supplied:

- **NucleoSpin® Extract II Kit** (Cat. No. 636972) for PCR product purification and purification of your own linearized cloning vectors
- **NucleoTrap® Gel Extraction Kit** (Cat. No. 636018) [Optional]
- **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)
- **LB (Luria-Bertani) medium** (pH 7.0)

		for 1 L
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	$MgCl_2 \cdot 6H_2O$
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 $MgCl_2 \cdot 6H_2O$ in deionized H_2O for a total volume of 100 ml.

III. Additional Materials Required *continued*

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.

- **Fusion-Blue™ Competent Cells (Cat. Nos. 637000 & 636758)**

  As no competent cells are provided for Cat. Nos. 639606 (24 reactions in a dry-down format) & 639605 (96 reactions in a 96-well plate dry-down format), we recommend Fusion-Blue™ Competent Cells or any commercially-available competent cells (e.g., DH10B, DH5α) **with an efficiency of ≥ 1.0 x 10^8 cfu/μg**. The corresponding Fusion-Blue™ Competent Cells are Cat. No. 637000 (24 transformations) and Cat. No. 636758 (96 transformations in a 96-well plate format).

Additional materials required when using pDNR-Dual:

- **X-Gal stock solution**
  (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 40 mg/ml in dimethylformamide)

  Dissolve 400 mg of X-Gal in 10 ml of dimethylformamide. Protect from light by storing in a brown bottle at –20°C.

- **IPTG stock solution** (isopropyl-β-D-thiogalactoside; 100 mM)

  Dissolve 238 mg of IPTG in 10 ml of deionized H_2O. Filter-sterilize and store in 1 ml aliquots at –20°C.

- **LB/X-Gal/IPTG plates**
  1. Warm an LB plate containing the appropriate antibiotic at 37°C for 10 min.
  2. Pipet 40 μl of the X-Gal stock solution and, if necessary, 40 μl of the IPTG stock solution* onto the center of the plate and spread evenly with a sterile spreader.

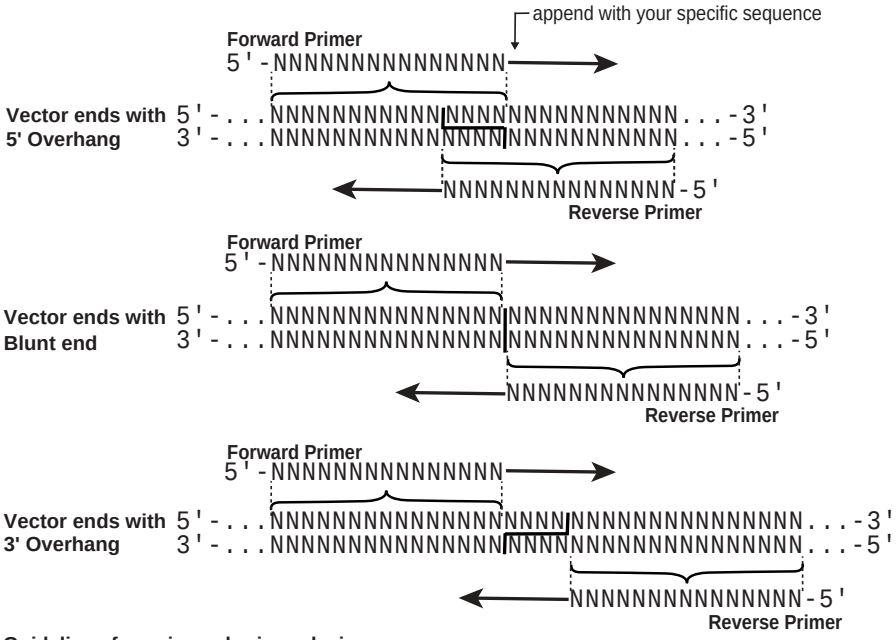
     * Some bacterial strains require IPTG for blue/white screening. Be sure to check the manufacturers instructions for the strain you are using.
  3. Allow the solution to diffuse into the plate by incubating at 37°C for 20–30 min.

IV. PCR & Cloning Preparation

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. PCR Primer Design

Primer design and quality are critical for the success of the In-Fusion cloning reaction. You can clone PCR fragments into any vector using the In-Fusion Dry-Down PCR Cloning Kit as long as the PCR insert shares 15 bases of homology at each end with the linearization site of the vector (Figure 2 outlines the guidelines for primer design. Figure 3 gives specific examples of primers.) Therefore, design PCR primers that will generate the homologous region in the PCR product during the amplification.



Guidelines for universal primer design
Count the homology from the 5' end.

For 5' RE overhangs and blunt ends, count all bases including bases complimentary to the 5' overhang in the homology sequence.

For 3' RE overhangs, do not count the 3' overhang in the homology sequence.

Count these bases in the 15 bp homology

Figure 2. Universal primer design for the In-Fusion™ PCR Cloning System. Successful cloning of a PCR insert requires that the PCR insert share 15 bases of sequence homology with the site of linearization on the vector. If the site of linearization includes restriction enzyme overhangs, bases complimentary to the 5' overhang count towards homology but the 3' overhang does not. See Figure 3 for specific examples.

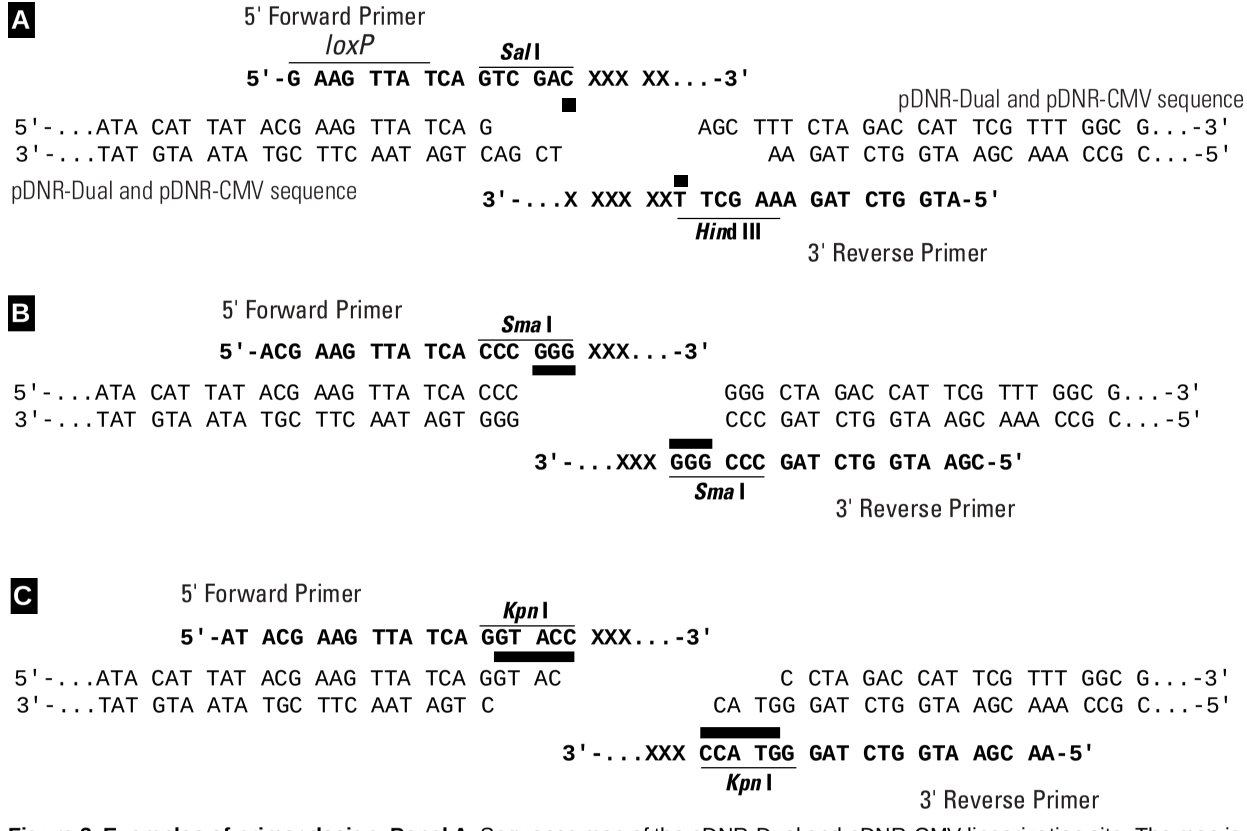


Figure 3. Examples of primer design. Panel A. Sequence map of the pDNR-Dual and pDNR-CMV linearization site. The map is shown in frame with the *loxP* site. **Panel B**. Example of primers designed with a blunt end restriction site. **Panel C**. Example of primers designed with a 3' overhang restriction site. The Primer sequences are shown in bold. The "X" symbols represent bases in the primers corresponding to your gene or sequence of interest. Additional nucleotides (highlighted with a black box) have been added to the primer in order to reconstruct the restriction sites—They are not part of the 15 bases of sequence homology.

IV.PCR & Cloning Preparation *continued*

1. 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 a lot of 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.
2. If you are cloning your fragment into the linearized pDNR-Dual or pDNR-CMV provided in the kits, here are several considerations:

   Primers:

   We have already designed sequences that, when added to your primers, allow your PCR product to be cloned into the linearized pDNR-Dual and pDNR-CMV Vectors. These sequences share the necessary 15 bases of sequence homology with the pDNR cloning vectors on either side of the point of insertion (Figure 3, Panel A), plus one extra base that recreates *Sal* I and *Hin*d III sites flanking the fragment. When you design your primers for cloning your insert into these vectors, add the following sequences to each 5' end of your gene-specific primers:

   5' (Forward) Primer: 5'-G AAG TTA TCA GTC GAC-3'

   3' (Reverse) Primer: 5'-ATG GTC TAG AAA GCT T-3'

   The underlined bases are the *Sal* I (Forward primer) and *Hin*d III (Reverse primer) restriction sites.

   Cloning and addition of tag sequences:

   Ensuring that your first codon is in frame with the triplet frame, shown for the forward primer above, will allow your gene to be shuttled in frame to any Creator™ Acceptor Vector with a 5' tag. Similarly, 3 ' tags can be added if your gene is cloned in frame with the cloning site of the Donor Vector as depicted in Figure 3 (Panel A) downstream of the *Hin*d III site without stop codons or a 3' UTR sequence.

   Note: There is an in-frame ATG site within the MCS of pDNR-Dual and pDNR-CMV. This ATG site is deleted in the provided linearized vectors which are cut with Sal I and Hind III (see pages 22-23).

IV. PCR & Cloning Preparation *continued*

B. Preparation of Linearized Vector

To achieve a successful In-Fusion PCR cloning reaction, you must first generate a very pure preparation of linearized vector (with a 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. In addition, increasing the enzyme digestion time will reduce the background.

The pDNR-Dual and pDNR-CMV vectors included in the kit are ready for cloning. However, if you desire to clone your PCR insert into another vector, 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	Cloning vector
10	µl	10X Enzyme buffer
10–20	U	Restriction enzyme[a]
X	µl	Deionized water (to 100 µl)
100	µl	Total Volume

   [a] We recommend adding half of the enzyme (2.5–5 U/µg) at the beginning of the digest, and the other half about 30 min later.

2. Incubate your restriction digest for at least 2 hr or as long as overnight.
3. After digestion, purify the linearized vector using any PCR purification kit. We recommend using the NucleoSpin Extract II Kit (see Additional Materials Required) and following the instructions for eluting a large vector (>5 kb) DNA.

   We typically cut with 2 enzymes and column purify the linearized vector. This generally gives sufficiently low background. However, if cutting with only one enzyme or if enzymes are of low efficiency (or simply to ensure that background is low in a critical cloning experiment), then gel purify the vector to ensure low background. The pDNR vectors provided with all of our systems are gel purified.
4. [Optional] Check the background of your vector by transforming 5–10 ng of the linearized and purified vector into Fusion-Blue Competent Cells. (See transformation protocol, Section V.C).

C. PCR Amplification and Purification

In general, 10–100 ng of DNA is sufficient for use 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™ 2 Polymerase Mix

IV. PCR & Cloning Preparation *continued*

(Cat. No. 639201), which offers high-fidelity, efficient amplification of long gene segments (>1 kb), and an automatic hot start that reduces nonspecific products. Hot start PCR is commonly used to enhance the specificity and sensitivity of PCR amplification (D'Aquila *et al.*, 1991; Chou *et al.*, 1992; Faloona *et al.*, 1990). We offer the TaqStart™ Antibody (Cat. No. 639250) for automatic hot start PCR with other *Taq*-based polymerase mixes. Since primers and primer dimers are inhibitory to the In-Fusion PCR cloning reaction, we recommend using hot start PCR with a touchdown protocol to increase the specificity of the resulting PCR products.

Important: If your PCR template is a plasmid DNA, then cut any contaminating linear vector by incubating the 50–100 µl PCR reaction mix with 1 µl of *Dpn* I for 30 min at 37°C before purifying your PCR products.

When 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 1.1 kb Control Insert or the linear vectors, provided in the kit, are useful for this purpose.

The percentage of agarose and the DNA size markers you choose will depend on the expected range of insert sizes. These are general guidelines:

Expected size	% agarose	DNA size markers
0.3–1.5 kb	1.5	ϕX174/*Hae* III
0.5–10 kb	1.2	1 kb DNA ladder
>5 kb	0.8	λ/*Hin*d III

PCR products must be purified for successful In-Fusion cloning. The method of purification required depends on your gel electrophoresis results. If you observe only a single, clear band on the gel corresponding to your product, then removal of unincorporated dNTPs through a simple PCR cleanup is usually sufficient. We recommend the NucleoSpin Extract II Kit (Cat. No. 636972) using the protocol for isolation from PCR.

If, however, multiple bands are observed, indicating the presence of nonspecific contaminants, we recommend that you gel purify your fragment of interest. We have found that either electroelution or silica-based DNA purification systems, such as the NucleoTrap Gel Extraction Kit (Cat. No. 636018) or the NucleoSpin Extract II Kit (Cat. No. 636972) work well.

During purification, be careful of nuclease contamination and avoid exposing the DNA to UV light for long periods of time. All solutions that come in contact with the gel and fragment should be free of nucleases. Avoid communal EtBr baths and use only high-quality agarose.

V. In-Fusion™ PCR Cloning Procedure

A. General Considerations

If you are using the In-Fusion PCR cloning kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your cloning reaction. Performing the control reactions will verify that your system is working properly.

B. Cloning Procedure

In general, maximum cloning efficiency is achieved when using a 2:1 molar ratio of insert:vector. Typically 100 ng of a 4 kb linearized cloning vector plus 50 ng of a 1 kb PCR fragment is found to clone well in a 10 µl In-Fusion reaction. Adjust the amount of your input DNA if the size of your vector or PCR fragment are different from above.

1. Mix your PCR fragment & 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 fragment 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 then suspend the DNA pellet in 10 µl H_2O.

2. Set up the In-Fusion cloning reactions:
   a. Cut one tube off the strip, and peel back the aluminum seal.
   b. Add the 10 µl of vector + insert DNA (from Step 1) and mix well by pipetting up and down.
3. Incubate reactions at 42°C or at room temperature for 30 min, then transfer tubes to ice.

   Note: For some In-Fusion reactions, such as cloning of exceptionally large fragments or the use of cloning vectors larger than 5 kb, simply incubate your reaction at 42°C for 30 min.
4. Proceed with Transformation (Section C). If you cannot transform cells immediately, store cloning reactions at –20°C until you are ready.

TABLE II. RECOMMENDED IN-FUSION™ REACTIONS

Reaction Component	Cloning Reaction	Positive Control Reaction	Negative Control Reaction
PCR insert	50–100 ng	50 ng (2 µl)[a]	–
Linearized vector[b]	100–300 ng	100 ng	100 ng
Deionized water	to 10 µl	to 10 µl	to 10 µl

[a] The 1.1 kb Control Insert is included as a 25 ng/µl solution.
[b] Use 1.0 µl of the 100 ng/µl solution of linearized Donor Vector included with the In-Fusion PCR Cloning Kit (See Table I for vector details).

V. In-Fusion™ PCR Cloning Procedure *continued*

C. Transformation

In addition to the cloning reaction, we recommend that you perform positive and negative control transformations. If desired, transform with the Test Plasmid (provided with In-Fusion Dry-Down PCR Cloning Kits) as a positive control for transformation efficiency of the competent cells. (Competent cells should give >1 x 10^8 cfu/μg.)

1. Dilute the In-Fusion reaction mixture with 40 μl TE buffer.
2. 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.
      - **Do not add more than 5 μl of diluted reaction to 50 μl of competent cells.**
      - Heat shock the cells in a water bath at 42°C for 45 sec, and then place them directly on ice for 1 min.
      - **Competent cells should yield >1 x 10^8 cfu/μg.** If not, replace with a fresh batch of cells.

   b. **If using other competent cells with In-Fusion™ Kits**, follow the transformation protocol provided by the manufacturer **(do not add more than 5 μl of diluted reaction to 50 μl of competent cells)** and proceed to Step 3.
3. After heat shocking, add 450 μl of SOC medium to the cells and then incubate at 37°C for 60 min while shaking at 250 rpm.
4. Take 1/20–1/10 of the cells (25–50 μl) from each transformation, bring the volume to 100 μl with SOC medium, and then spread on separate LB/X-Gal plates containing 100 μg/ml of ampicillin or other appropriate medium for your cloning vector.
5. Spread the remaining cells from each transformation on separate, LB/X-Gal/Amp plates. Incubate all plates at 37°C overnight.
6. The next day, pick white colonies (10 or more) from each experimental plate and 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.

D. Expected Results

The positive control plates should have many white colonies, with a few blue colonies. We typically obtain several hundred white colonies on the positive control reaction plate, when using cells **with a minimum**

V. In-Fusion™ PCR Cloning Procedure *continued*

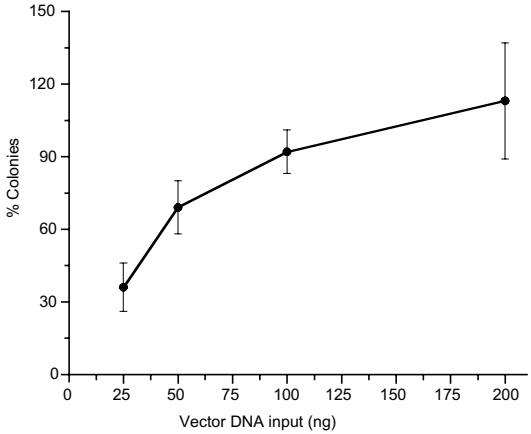


Figure 4. DNA input versus resulting colonies. The optimal amount of vector was found to be 50–100 ng when different amounts of vector were used in the cloning reaction with various amounts of inserts. The resulting number of colonies, represented as a percentage of the number of colonies in the Test Plasmid positive control, was plotted versus the amount of vector DNA used. Several hundred colonies are generally obtained when 1/10 of a transformation with 1 µl Test Plasmid positive control is plated. Error bars represent the standard deviation from the average.

competency of 1 x 10^8 cfu/ug, and less than 5% blue colonies. The negative control plates should have few, if any white colonies.

The number of white colonies on your experimental plates will depend on the amount and purity of the PCR product 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 contamination with a PCR template plasmid carrying ampicillin resistance.

Note on the linearized pDNR-Dual Donor Vector

Linearized pDNR-Dual Donor Vector is derived from a circular vector that contains the bacterial *lacZ* gene, which is removed during linearization (See Appendix A for more detail); linearized pDNR-CMV does not have the blue/white selection. Therefore, when cloning into the linearized pDNR-Dual, any blue colonies that appear on your plates will indicate the presence of rare, contaminating nonlinearized vector.

However, after blue/white screening, the number of blue (background) colonies resulting from nonlinearized pDNR-Dual Donor Vector will not exceed 5% of the number of white colonies observed in the positive control.

VI. 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 (Section V.C).

A. No or few colonies obtained from transformation

Problems with transformation

Inhibitory contaminants in 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.
Transformed with too much	**Do not add more than 1 μl of undiluted reaction to 50 μl of competent cells** (0.5 μl of In-Fusion reaction to 25 μl of competent cells). More is not better. Using too much of the reaction mixture inhibits the transformation. For example, 0.5–1μl of a In-Fusion reaction in 50 μl of cells typically gives over 1,000 colonies, while 2 μl of the same reaction will give less than 100 colonies. As it is difficult for you to pipette 1 μl accurately (e.g., if you are using "yellow tips" with a p20 pipettor), dilute your In-Fusion reaction with TE buffer (pH 8) before performing the transformation—especially if you wish to use a small volume of competent cells (e.g., HTP cloning). See Section V.C for details.

Problems with cloning reaction

Cloning reaction failed	Check the reaction for a shift in vector size by running half of the reaction on a 1% agarose gel (Figure 5). In extremely rare cases, the In-Fusion reaction may fail to clone certain sequences effectively. The In-Fusion cloning reaction depends on sequence homology, so sequences containing extensive or multiple repeats may not clone efficiently. In these cases, an alternative cloning strategy might be needed.
Low DNA concentration in reaction	Either the amount of vector or the amount of PCR fragment was too low to obtain a satisfactory reaction product (Figure 4). Alternatively, the 2:1 ratio of PCR

VI. Troubleshooting Guide *continued*

	fragment to linear vector may not have been optimal.
Primer sequences incorrect	Check primer sequences to ensure that they provide 15 bases of homology with the region flanking the vector cloning site (see Section IV).
Low cloning reaction efficiency	Check the concentration of your PCR fragment. For 100 ng of pDNR-Dual, do not use less than 25 ng or more than 100 ng of your fragment in the cloning reaction; the cloning efficiency may be reduced if you exceed these paramaters. Certain fragments, particularly larger fragments (>4 kb), may also exhibit lower cloning efficiencies. We have found that such fragments can sometimes be successfully cloned by performing the cloning reaction for 30 min at 42°C (instead of 30 min at room temperature).

B. Large numbers of colonies obtained with no insert

Contamination of cloning 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 *Dpn* I to remove the parental vector template after PCR amplification (See Section IV.C; Weiner *et al.,* 1994*;* Fisher *et al.*, 1997).
Incomplete linearization of your cloning vector.	If you are using your own linearized vector for cloning then it must be purified to remove any uncut vector before use in the In-Fusion PCR cloning reaction. Recut your vector. It may be necessary to gel purify your linearized vector as well.
Plates too old or contained incorrect antibiotic.	Be sure that your antibiotic plates are fresh (< 1 month old. Plates should contain 100 μg/ml ampicillin for use with pDNR-Dual and pDNR-CMV.

C. Clones contain incorrect insert

Contamination of PCR product with 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 IV.C for more information.

VII. References

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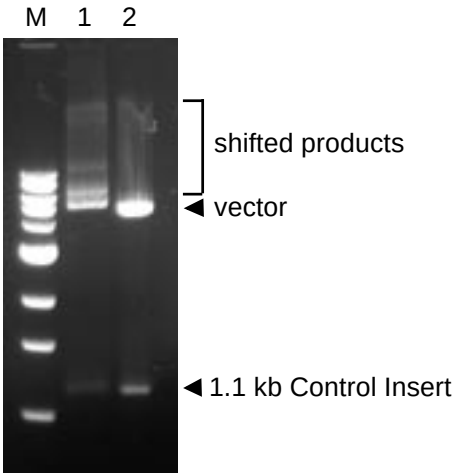

Figure 5. Typical vector shift in successful In-Fusion cloning reaction. A band shift showing an increase in molecular weight indicates a successful reaction (Lane 1). Lane 2 shows a no-enzyme control, containing vector and insert. M = molecular weight standard.

VIII. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

Product	Cat. No.
• In-Fusion™ PCR Cloning Kit	631774 631775
• Fusion-Blue™ Competent Cells 24 transformations 96 transformations	 636700 636758
• Creator™ Acceptor Vector Construction Kit	631618
• Creator™ Acceptor Vectors	many
• NucleoSpin® Extract II Kit	636971 636972 636973
• NucleoTrap® Gel Extraction Kit	636018
• Advantage™ 2 PCR Kit	639206 639207
• Advantage™ 2 Polymerase Mix	639201 639202
• TITANIUM™ *Taq* DNA Polymerase	639208 639209
• TITANIUM™ *Taq* PCR Kit	639210 639211
• Advantage™ GC 2 Polymerase Mix	639114
• Advantage™ GC 2 PCR Kit	639119 639120
• Advantage™ HF 2 PCR Kit	639123 639124
• Advantage™ Genomic PCR Kit	639103 639104
• Advantage™ Genomic Polymerase Mix	639110
• Creator™ pDNR Cloning Kit	631615
• Creator™ SMART™ Premade cDNA Libraries	many
• Creator™ SMART™ cDNA Library Construction Kit	634903

Appendix A: Vector Maps & Multiple Cloning Sites

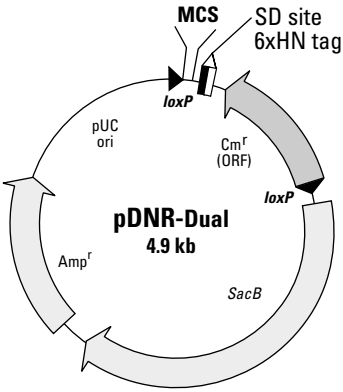

Figure 6. pDNR-Dual Donor Vector Map. pDNR-Dual contains a splice donor (SD) site directly downstream of the Multiple Cloning Site (MCS). When combined with a specialized acceptor vector containing a splice acceptor (SA) site, a recombinant expression construct is generated containing an artificial intron (consisting of the chloramphenicol marker and one *loxP* site), which is spliced out by the eukaryotic host's transcriptional machinery. As a result, a transcript is created that expresses the tag as a 3' fusion to your gene of interest. Sequence and digest information is available, and can be downloaded from our web site at **www.clontech.com**.

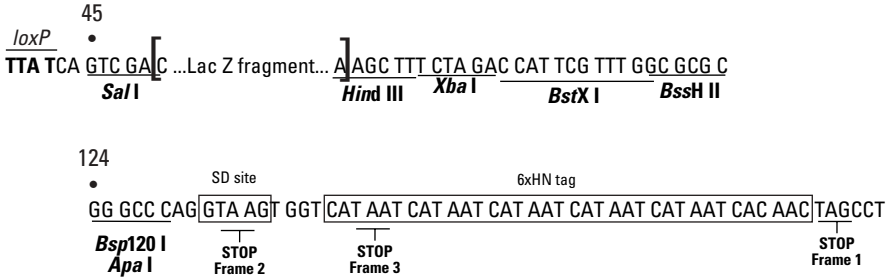

Figure 7. Linearized pDNR-Dual Donor Vector MCS. (Unique restriction sites are shown in bold.) The pDNR-Dual Donor Vector provided in this kit has been linearized, removing the sequence between the *Sal* I and *Hin*d III sites (indicated by the brackets). The MCS is shown in frame with the *loxP* site (Frame 1). The last four nucleotide bases of the *loxP* site can be seen at the left hand side of the diagram in bold. If the coding sequence for the gene of interest is in frame with the upstream *loxP* site in the donor vector, it will automatically be in frame with any 5' peptides in our Acceptor Vectors. If the coding sequence for the gene of interest is in frame with the SD site in the Donor Vector, it will automatically be in frame with any 3' tags in the Acceptor Vector. **Important:** The pDNR-Dual Donor Vector provided in this kit has been linearized, but the vector is a derivative of nonlinearized parental pDNR-Dual Donor Vector, which carries the bacterial *lacZ* gene between the *Sal* I and *Hin*d III sites of the MCS, providing the basis for blue/white screening.

Appendix A: Vector Maps & Multiple Cloning Sites *cont.*

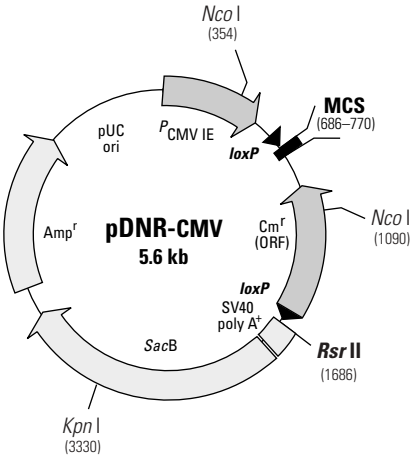

Figure 8. pDNR-CMV Donor Vector Map. pDNR-CMV is designed to verify target gene expression in mammalian cells prior to transfer of the gene of interest to a final expression Acceptor Vector. Cre, a 38-kDa recombinase protein from bacteriophage P1, mediates recombination between DNA sequences at specific locations called *loxP* sites. The pDNR-CMV Donor Vector contains two *loxP* sites, which flank the 5' end of the MCS and the 5' end of the open reading frame encoding the chloramphenicol resistance gene (Cm^r). Expression of the gene of interest is driven by the human cytomegalovirus (CMV) major immediate early promoter/enhancer ($P_{CMV\ IE}$). When the Donor Vector containing your gene of interest is combined with any Acceptor Vector and Cre recombinase, Cre molecules attach to *loxP* sites located on both the Donor and Acceptor vectors. Cre then mediates the transfer of the DNA fragment located between the two *loxP* sites in the Donor Vector, to the Acceptor Vector. As a result, a recombinant plasmid is created that expresses your gene of interest in the desired host system. Sequence and digest information is available, and can be downloaded from our web site at **www.clontech.com**.

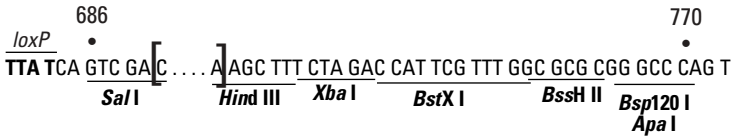

Figure 9. Linearized pDNR-CMV Donor Vector MCS. (Unique restriction sites are shown in bold.) The pDNR-Dual Donor Vector provided in this kit has been linearized, removing the sequence between the *Sal* I and *Hin*d III sites (indicated by the brackets). The MCS is shown in frame with the *loxP* site (Frame 1). The last four nucleotide bases of the *loxP* site can be seen at the left hand side of the map in bold. If the coding sequence for the gene of interest is in frame with the upstream *loxP* site in the donor vector, it will automatically be in frame with any 5' peptides in our Acceptor Vectors.

Appendix B: Vectors & Inserts Tested

TABLE III: VECTORS TESTED

Vector	Size (kb)	Digestion site	Inserts checked[a]	Resistance marker
pAAV-IRES-GFP	6 kb	*Bam*HI-*Sal*I	5/6	Amp
pPD103.5	9.7 kb	*Sma*I-*Xba*I	12/12	Amp
pEGFP-d3-NotI	4.9 kb	*Eco*RI-*Not*I	5/6	Km
pGEM-T Easy	3 kb	*Nco*I-*Sal*I	5/6	Amp
TriEx1	5.8 kb	*Sma*I	12/12	Amp
pIRES-hrGFP-1a	5.0 kb	*Eco*RI-*Sal*I	7/10	Amp
pLITMUS38i	2.8 kb	*Bam*HI	5/12	Amp
pFB	4.8 kb	*Kpn*I	12/12	(pFastbac/Amp)
pCEP4-EBNA	12.0 kb	*Kpn*I-*Bgl*II	4/6	Amp
pTRE2-Hyg	5.3 kb	*Nhe*I-*Eco*RV	4/6	Amp
pSin-Rep5	10.0 kb	*Xba*I-*Apa*I	5/6	Amp

[a]Number of inserts correct/number of inserts checked; all sequences were checked by sequencing.

TABLE IV: INSERTS TESTED

Primer set	Insert size	Inserts checked[a]	Number of Colonies[b]
IF02F2/R2	3.0 kb	12/12	200
IF02F1/R2	4.0 kb	10/12	180
IF02F1/R3	4.9 kb	11/12	140
IF02F2/R5	6.1 kb	9/12	180
IF02F2/R6	7.0 kb	8/12	100
IF02F1/R6	8.0 kb	9/12	60

[a] Number of inserts correct/number of inserts checked. In addition, all sequences were checked by sequencing.

[b] One-tenth of the transformants from a standard transformation (1 µl of the 20 µl cloning reaction) of Fusion-Blue™ competent cells.

Notes

Notes

Notes

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