Universal, Seamless, and Directional Integration of DNA Fragments into PCR-Linearized Plasmids using the In-Fusion™ Dry-Down PCR Cloning Kit

The In-Fusion™ PCR Cloning System allows direct cloning of a PCR fragment into any linearized vector. The system works with a broad range of insert sizes, and is available in a convenient lyophilized format that is stable at room temperature. The In-Fusion Cloning System is the most straightforward method for PCR cloning available and is well-suited for automated cloning technologies. The system has been successfully used in various high-throughput cloning projects, including the work at the **Department of Genetics, Stanford University School of Medicine** (January 2005 *Clontechniques*), and many others. The high-throughput capability of In-Fusion has been explored further by the work of the **Novartis Institutes for BioMedical Research in Basel, Switzerland** and is reported in the application note below. These investigators used PCR to generate both vector and insert, and demonstrate the unique ability of the In-Fusion system to successfully carry out seamless, directional, and automated cloning of PCR products. —Editor

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We used the In-Fusion Dry-Down PCR Cloning Kit for the integration of genes of interest into the expression regions of plasmids. In order to achieve fully seamless integration at any desired position of any plasmid, we linearized the vectors by PCR, rather than by digestion with restriction endonucleases. In addition to rendering In-Fusion cloning seamless and universal, plasmid linearization by PCR provided the additional advantage of allowing the template plasmid to be removed by Dpn *I digestion, making preparative gel purification of both linearized plasmid and insert unnecessary. As a result, the protocol only involves PCR and liquid handling steps and thus should theoretically hold great potential for automation.*

Introduction

In the field of structural biology, there are special quality requirements for proteins. Several mg of highly purified, homogeneous protein are generally needed. The protein usually must be stable at high concentrations (~5–20 mg/ml, depending on the protein). A very small change in protein sequence, such as a single amino acid mutation, may have drastic effects on crystallization behavior. The same applies to amino acids that are added to either the N- or C-terminus of the protein domain of interest. Therefore, fully seamless cloning methods are best suited for this field of research. Another important factor is that, in most cases, the ideal constructs for crystallization must be determined empirically. Thus, many constructs must be made, and a method that allows medium- to high-throughput cloning and is suitable for automation is needed.

We have used Clontech's **In-Fusion™ Dry-Down PCR Cloning Kit** (Cat. No. 639604) for the integration of DNA fragments ranging in size from 447 to 1,200 bp into plasmids that we typically use for the *E. coli* expression system. In order to render integration fully seamless, universal, and independent of restriction enzyme recognition sites, we have linearized the plasmids by PCR, rather than by restriction enzyme digestion. With this small modification to the standard protocol, the method fulfilled all the requirements discussed above.

Table I: Primers Used for PCR Linearization of a Modified Novagen pET-28a(+) Plasmid

Primer	Primer Sequence
Linearization primer 1	GGGCCCCTGGAACAGAACTTCCAGGCC
Linearization primer 2	GGATCCGAATTCGAGCTCCGTCGACAA
Forward insert primer	AAGTTCTGTTCCAGGGGCCCTTACAATTTCCATTCGCCATTCAGGCTG
Reverse insert primer	CGGAGCTCGAATTCGGATCCGTTTGACAGCTTATCATCGAATAGC

Note: The plasmid linearization primers were used for PCR linearization of a modified Novagen pET-28a(+) plasmid (containing a PreScission™ protease recognition site instead of the thrombin recognition site) and the corresponding insert amplification primers were used for PCR amplification of the lacZ α-peptide (along with the promoter) from Novagen pETBlue-2. The 20 nt long homology regions are shown in red and blue. The insert amplification primers carry the homology sequences as non-annealing 5' primer tails. The plasmid linearization primers are fully annealing.

Preparation of insert & plasmid

Plasmids were linearized by PCR, using two fully annealing primers. Inserts were amplified by PCR, using primers that carried 15–20 nt long regions that were complementary to the 5' ends of the linearization primers (= homology regions) as non-annealing 5' primer overhangs. A primer design example is shown in Table I, and the homology regions of insert amplification primers and plasmid linearization primers are aligned in Figure 1. The PCR products were examined by analytical agarose gel electrophoresis in order to assure that a single, specific product of the expected size had formed. The PCR products were then incubated with *Dpn* I in order to degrade template plasmids. The *Dpn* I-treated PCR products were spin column-purified and eluted in 50 µl of 10 mM Tris pH 8.5. A schematic representation of the procedure is shown in Figure 2. For additional protocol details, see ref. 1.

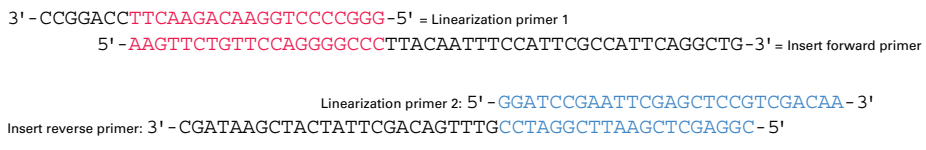

Figure 1. Alignment of the homology regions of plasmid linearization primers and insert amplification primers.

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In-Fusion cloning

1–2 µl (100–250 ng) of spin column-purified, linearized plasmid and 1–2 µl (50–200 ng) of spin column-purified insert were combined in a 0.5 ml Eppendorf tube and mixed. The volume of the mixtures was adjusted to 10 µl by adding molecular biology grade H_2O. The diluted mixtures were then mixed with the Clontech's In-Fusion Dry-Down Mix, a component of the In-Fusion Dry-Down PCR Cloning Kit (Cat. No. 639604), and incubated at 42°C for 30 minutes, followed by a 5 minute incubation on ice. 1 µl of each mixture was directly transferred to separate aliquots of Fusion-Blue™ chemically competent cells. The DNA-cell mixtures were incubated on ice for 15 minutes. Heat-shock transformation was carried out at 42°C for 45 seconds. 200 µl of S.O.C. medium were added to each cell aliquot. The cell suspensions were incubated at 37°C, 650 rpm in an Eppendorf Thermomixer for 60 minutes. The completed transformation reactions were plated out onto selective LB agar medium. Plasmids from four colonies of each construct were purified. Analytical PCR was used to identify positive clones. The latter were then sequenced.

This strategy was successfully used for the insertion of DNA fragments ranging in size from 447 to 1,200 bp into Novagen pET plasmids. Hands-on time was minimal, and we usually obtained the desired product in a single attempt.

Conclusion

The In-Fusion cloning strategy is very convenient because the protocol is simple and allows free choice of homology regions. Only short homology regions are needed. Plasmid linearization by PCR renders the method fully independent of restriction enzyme recognition sites. The most important point presented here is the concept that any DNA fragment can be inserted at any position in any plasmid without the introduction of a single unwanted base pair.

E. coli cells transformed with integration products containing PCR-generated loss-of-function mutations in the origin of replication or in the antibiotic resistance gene cannot form colonies on selective agar medium. A risk of introducing mutations in other important plasmid backbone regions, i.e., the *lacI* gene of pET vectors, remains. We have not encountered any problems in this context so far. We strongly recommend the use of a high-fidelity proofreading polymerase for plasmid linearization. Furthermore, the full expression region, including promoter and terminator, should always be sequenced.

Product	Size	Cat. No.	Price
In-Fusion Dry-Down PCR Cloning Kit			
	8 rxns	639602	$160.00
	24 rxns	639604	$407.00
	96 rxns	639605	$886.00
In-Fusion CF Dry-Down PCR Cloning Kit			
	24 rxns	639606	$314.00

Prices are subject to change without notice.

Reference

1. Benoit, R.M., *et al. Protein Expr. Purif.* (2005) **45**(1):66–71.

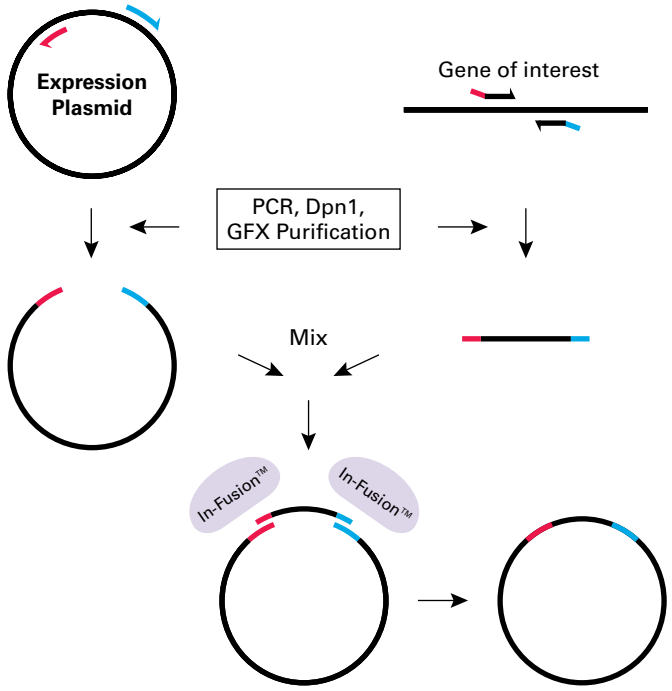

Figure 2. A schematic representation of In-Fusion cloning with PCR-linearized plasmids. Double-stranded DNA is represented as a wide single line. Homology regions are shown in red and blue. Arrows indicate the direction of primer extension. The plasmid is linearized by PCR, using two primers that anneal on different strands and run in opposite directions, resulting in a linear PCR product. The gene of interest is amplified by PCR, using primers that carry 5' primer tails (red and blue) that are homologous to the desired site of insertion in the plasmid. Linearized plasmid and amplified insert are digested with *Dpn* I and spin column-purified. Plasmid and insert are then mixed, the volume adjusted to 10 µl with water, and mixed with In-Fusion Dry-Down Mix. After 30 minutes of incubation at 42°C, 1 µl of the mixture is used to transform Fusion-Blue chemically competent *E. coli* cells.

Figure 2 reprinted from Protein Expression & Purification, Volume **45**(1), R.M. Benoit, R.N. Wilhelm, D. Scherer-Becker, & C. Ostermeier, "An improved method for fast, robust, and seamless integration of DNA fragments into multiple plasmids," pages 66–71, ©2005, with permission from Elsevier.