Optimized In-Fusion Cloning System Demonstrates High Efficiency and Accuracy of Multi-Fragment Cloning

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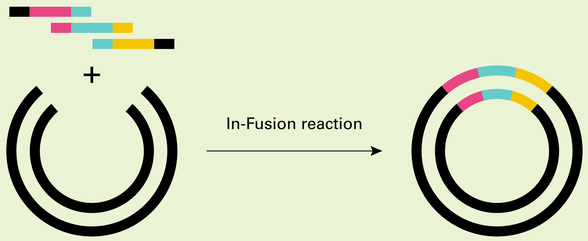
Abstract

In-Fusion Cloning technology enables directional, seamless cloning of any PCR-amplified insert into any linearized vector in a single 15-minute reaction. No additional treatment of the PCR fragment is required (such as restriction digestion, ligation, phosphorylation, or blunt-end polishing). Instead, the In-Fusion enzyme generates short regions of single-stranded overlaps between vector and insert(s), facilitating accurate, directional cloning of the desired fragments. This overlap is designed into the PCR primers used to amplify the desired insert sequences. While cloning single inserts is an extremely efficient process, cloning experiments are becoming increasingly challenging. Multiple inserts must be cloned, and kept in frame, in order to build complicated constructs, to stitch genes together from synthetic building blocks, or to engineer new functionalities through combinatorial protein domain swapping, etc. Here, we present data showing how multiple-fragment cloning efficiency and accuracy can be significantly increased by optimizing various parameters of the In-Fusion Cloning reaction. This further extends the versatility of the In-Fusion Cloning system to meet the coming challenges of innovative research in the synthetic biology field.

Introduction

While In-Fusion Cloning simply and accurately clones single inserts into any vector of choice, cloning applications are becoming increasingly more complex. In traditional cloning systems, increasing the number of fragments can have a detrimental effect on both efficiency and accuracy. However, In-Fusion Cloning maintains the high accuracy typical of single-insert cloning even under more challenging demands, by taking advantage of the host organisms' highly evolved repair machinery. Here, we improved cloning efficiency of multiple-insert cloning by extending the overlapping sequence (see image below), and increasing the stability between fragments.



Increasing the length of overlaps from 15 bp to 20 bp improves the efficiency of multiple-fragment cloning.

Conclusions

- **In-Fusion Cloning is more accurate than Gibson's method when multiple inserts are cloned simultaneously.**
- **When cloning multiple fragments in a single In-Fusion reaction, increasing the overlap between fragments from 15 bp to 20 bp improves cloning efficiency ~5- to 6-fold.**

References

1. Chen, C. G., *et al.* (2014) *Nucleic Acids Research* **42**(4):e26
2. Lestini, R., *et al.* (2013) *Nucleic Acids Research* **41**(22):10358–10370
3. Gibson, D. G.,*et al.* (2009) *Nature Methods* **6**(5):343–345

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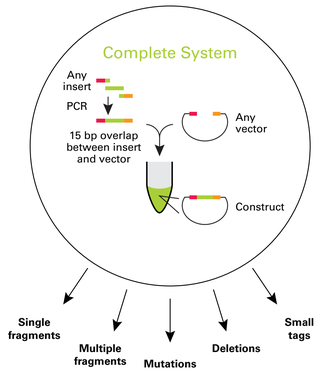
1 In-Fusion Cloning: Seamless, Ligation-Independent Cloning

A



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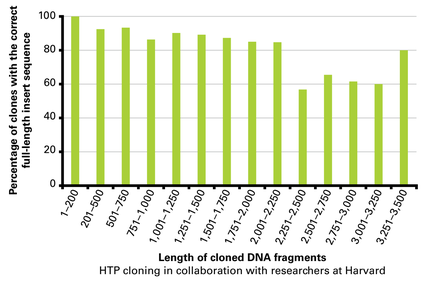

Overall Study Results	Number of Clones
Correct insert	3,081
Incorrect insert	286
Accuracy	91.5%

Fast, easy cloning without ligation. Panel A. In-Fusion® Cloning is a complete, optimized system that allows ligation-independent, directional cloning of PCR products into **any** vector, at **any** site of linearization in just one step. Your linearized vector is combined with your PCR-amplified insert and the In-Fusion enzyme mix. The cloning reaction takes as little as 15 minutes and is immediately ready for transformation into competent cells without further treatment. This powerful cloning technology is seamless, accurate, and adaptable to a wide range of applications, including multiple-fragment cloning, large-fragment cloning, site-directed mutagenesis, the addition of small tags to your gene of interest, direct cloning into large expression vectors, and high-throughput experiments. **Panel B.** The In-Fusion Cloning system has been successfully used by many high-throughput users worldwide. This bar graph shows the percentage of correct clones obtained during generation of an ORF collection by the Harvard Institute of Proteomics. 3,367 individual clones were generated in order to obtain the desired 2,000 ORFs, with cloned inserts ranging in size from 500–3,500 bp. Full-length sequencing of the inserts determined that over 3,000 of these clones contained the correct insert, delivering over 91% cloning accuracy.

2 Three-Insert Multiple-Fragment Cloning

Single Insert Cloning		
Conditions	**In-Fusion HD Cloning** Incubate at 50°C for 15 min	**Gibson's Method** Incubate at 50°C for 15 min
	Results	**Results**
Vector + insert	635 colonies	401 colonies
Negative control (no insert)	1 colony	39 colonies
Cloning accuracy	100% (26/26 correct colonies)	96% (25/26 correct colonies)

Multiple Insert Cloning			
Conditions	**In-Fusion HD Cloning** Incubate at 50°C for 15 min	**Gibson's Method** Incubate at 50°C for 15 min	Incubate at 50°C for 60 min
	Results	**Results**	**Results**
Vector + insert	89 colonies	111 colonies	392 colonies
Negative control (no insert)	1 colony	39 colonies	78 colonies
Cloning accuracy	100% (26/26 correct colonies)	19% (5/26 colonies)	73% (19/26 correct colonies)

High cloning accuracy is obtained when cloning single and multiple inserts with In-Fusion Cloning. In-Fusion HD Cloning and Gibson's method were each used to clone single and multiple (3) inserts into puC19 (linearized with BamHI). Each cloning system was tested under the recommended conditions of its own cloning protocol. In an effort to make a more direct comparison with In-Fusion cloning, this multiple-insert experiment with Gibson's enzyme mix was also run at the shorter In-Fusion reaction time. All cloning reactions were then transformed into Stellar™ Competent Cells, and 1/10th of each reaction was plated. Colony counts and clone sequence verification were used to evaluate the results of each reaction. The results of single-insert cloning were roughly comparable between the two systems, but the In-Fusion Cloning technology distinguished itself with lower levels of background—an asset seen to even greater extent in the multiple-insert experiment, where it was also found to have much higher cloning accuracy than Gibson's method.

3 Overlaps were Increased from 15 bp to 20 bp for Three-Insert Multiple-Fragment Cloning

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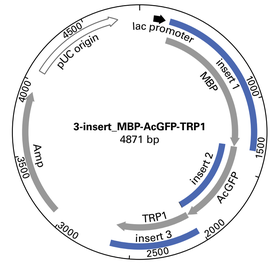

B

Number of Colonies per Plate				
	15-bp overlap		**20-bp overlap**	
Dilution	8.5/10	1/20	8.5/10	1/20
Vector only	242	—	209	—
3-insert, multi-fragment	+++	263	++++	1,592

+++/++++ represent the relative estimation of colonies on plates with too many colonies to count.

Increasing the length of fragment overlaps from 15 bp to 20 bp improves cloning efficiency for three-insert multiple-fragment cloning. Panel A. 1.1 kb, 700 bp, and 600 bp inserts (20 ng each) were cloned into a 2.5 kb linearized vector (100 ng). 1/20th, 1/10th and the remainder of the cloning reactions were plated. **Panel B.** Parallel experiments were performed with either 15-bp or 20-bp fragment overlaps. 15 bp was determined to be the optimal length for overlaps using the original In-Fusion Cloning kit. We tested whether the optimal overlap length differed for the current In-Fusion HD enzyme and if this could improve the efficiency of multiple-fragment cloning. Using 20-bp overlaps improved efficiency by ~6-fold. Plasmids were purified and sequenced. 100% of readable sequences were correct.

4 Five-Insert Multiple-Fragment Cloning

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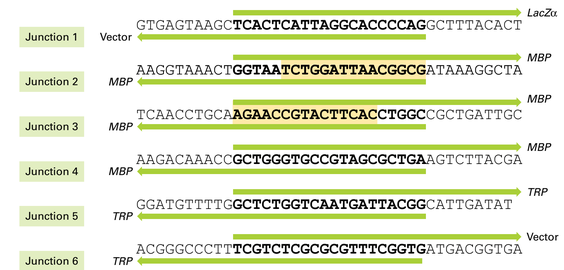
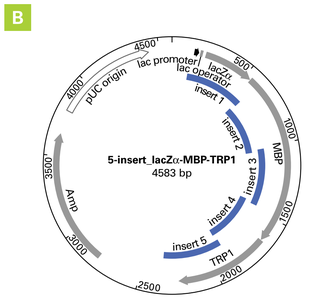


B



C

Number of Colonies per Plate		
	15-bp overlap (blue/white)	**20-bp overlap (blue/white)**
Dilution	1/10	1/10
Vector only	0/5	0/5
5-insert, multi-fragment	21/5	115/12

Increasing the length of overlaps from 15 bp to 20 bp improves cloning efficiency for five-insert multiple-fragment cloning. Panel A. Five dsDNA inserts were designed to contain 20-bp overlaps between fragments. A comparison to 15 bp overlaps was tested by shortening the ends of insert 2 by 5 bp (highlighted in yellow). **Panel B.** 450–480 bp inserts (20 ng each) were cloned into a 2.5 kb linearized vector (33 ng). 1/10th of the cloning reactions were plated. *lacZα* (insert 1), which turns cells blue in our system, was included for rapid identification of positive clones. **Panel C.** Shortening the ends of just one insert decreased the cloning efficiency by ~5-fold. Blue colonies resulting from the 20-bp overlap reactions were further analyzed by colony PCR, and 10/10 were found to contain a band corresponding to the five-insert product (data not shown).