Simplified insertion of a GFP-encoding cassette into a 100-kb plasmid using In-Fusion HD Cloning

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- Restriction digest- and ligation-free cloning method
- Simultaneous, directional cloning of three fragments to form a 4-kb insert
- 100% success rate of tested colonies

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Introduction

Plasmids are semi-autonomous circular DNA molecules and can be transferred from one bacterium to another through a process called conjugation. Fluorescent gene markers such as GFP can be inserted into plasmids to track this movement within complex bacterial communities, using Fluorescence Activated Cell Sorting (FACS) (Klümper, 2015).

The majority of researchers still use traditional restriction-ligation based cloning to insert these fluorescent markers into plasmid vectors at specific sites. However, this cloning method has a major limitation: it requires the presence of unique restriction sites at the desired locus in the plasmid, but which are not present within the insert itself. When working with large plasmids isolated from natural environments, it can be difficult, if not impossible, to find restriction sites that fulfill these requirements within the exact region where the fluorescent marker needs to be cloned.

In the experiment described here, the seamless In-Fusion HD Cloning method was used to assemble a 4-kb GFP genetic cassette within a cloning vector (pKD46), simultaneously including upstream and downstream homology arms to a noncoding site in the final destination vector (a 100-kb wild-type IncK plasmid), without using any restriction enzymes. A wild-type strain of *E. coli* containing the IncK plasmid was then transformed with that cloning vector, at which point the GFP cassette was transferred from the cloning vector into the IncK plasmid via homologous recombination (Figure 1).

Indeed, In-Fusion Cloning enables this independence from restriction sites, accurately and efficiently fusing PCR-generated inserts and linearized vectors through the use of a 15–20 bp overlap at each fragment end. Exonuclease activity chews back one strand of each linear fragment, creating compatible ends for joining and annealing *in vitro*. Cohesive bonds are then formed after gap repair *in vivo* in the bacterial cell, following transformation, thus completing an entire cloning workflow without the use of restriction enzymes or ligase.

Results

PCR amplification was performed to obtain three overlapping fragments: one containing the GFP gene cassette and the kanamycin selection marker (3 kb), one containing 500 bp of homology upstream of the noncoding site of interest in the IncK plasmid, and one containing 500 bp of homology downstream of that same locus. The homology to the IncK plasmid would facilitate downstream homologous recombination between that 100-kb plasmid and a correctly assembled pKD46 cloning vector.

The researchers first attempted to clone these three fragments together using Gene Splicing by Overlap Extension (gene SOEing; Horton, 1990), a PCR-based method for generating recombinant molecules without the use of restriction enzymes. However, due to the GC-rich nature of the region of interest in this case, the T_m for SOE-PCR was higher than 80°C, and despite using adjuvants in the PCR mix, the SOE-PCR was not successful.

The researchers then looked to In-Fusion Cloning to generate this initial construct and designed primer sets for all three aforementioned fragments with ends compatible with In-Fusion technology (Figure 1). The three fragments were PCR-amplified, and the products were then inserted into the pKD46 cloning vector using the In-Fusion HD Cloning Kit. Stellar Competent Cells (included in the kit) were transformed with the cloning reaction mixture, yielding 145 colonies after plating on selective medium. Testing was performed on 30 randomly chosen colonies, all of which showed the correct construct with the 4-kb GFP cassette.

> I tried SOE-PCR for 3 months to stitch together fragments into a large vector but it never worked. I am very happy with the performance of the In-Fusion cloning kit! —Mehreen Anjum, PhD

The cloning vector used here (pKD46) had a temperature-sensitive origin of replication that allowed this plasmid to be maintained in the bacterial cell at 30°C, but if the temperature were raised to 37°C, the bacteria would lose the plasmid. A wild-type strain of bacteria containing the IncK plasmid was transformed with the recombinant cloning vector containing the GFP cassette (with kanamycin resistance marker), and the GFP cassette was transferred from the cloning vector into the IncK plasmid via homologous recombination, facilitated by the homology arms that were cloned above. Transformants were selected on a kanamycin-supplemented agar plate and incubated at 37°C. This increase in temperature resulted in the predicted loss of the cloning vector, and the growth of only the transformants in which the GFP cassette was successfully recombined into the IncK plasmid. PCR followed by Sanger sequencing was then used to confirm the insertion of the GFP cassette into the IncK plasmid at the correct locus. Five out of 15 colonies were randomly tested, and all showed the presence of the correct final clone.

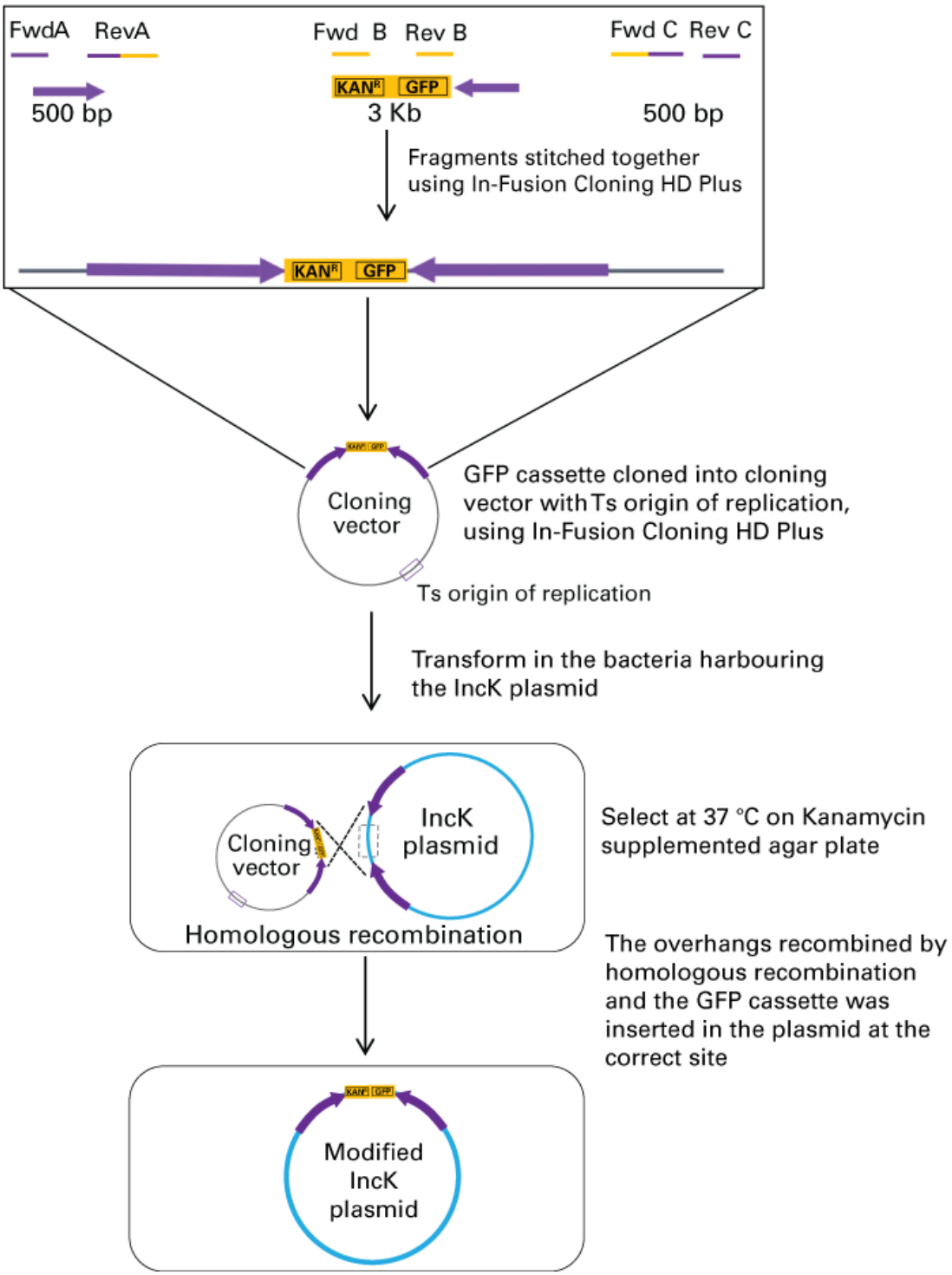

Figure 1. Overall workflow for cloning a 4-kb cassette into a 100-kb plasmid using In-Fusion technology. Three fragments were amplified with ends compatible with In-Fusion Cloning: a GFP coding sequence with kanamycin selection marker (3 kb), a homology arm upstream of the cloning locus on the final plasmid (500 bp), and a homology arm downstream of the same locus (500 bp). In-Fusion HD Cloning Plus was used to simultaneously clone all three fragments into the pKD46 cloning vector, which has a temperature-sensitive (Ts) origin of replication. The resulting successful clone was used to transform a wild-type strain of bacteria harboring the IncK plasmid. The homologous arms (previously cloned into the cloning vector on either side of the GFP cassette) then facilitated the homologous recombination of the GFP cassette with the IncK plasmid, at the desired locus. Incubation at 37°C cured the bacteria of the cloning vector, as the Ts origin of replication could not tolerate the higher temperature. Image kindly provided by Mehreen Anjum, University of Copenhagen.

Conclusions

Modifying large natural plasmids is a tricky process—especially with traditional ligation methods—given the lack of usable restriction enzyme sites at desired loci. With the In**I**Fusion Cloning method, as described above, the researchers were able to obtain correctly modified plasmids with 100% efficiency and precision in a time-efficient manner.

Methods

Primers compatible with In**I**Fusion Cloning were designed for all three fragments, with 15-bp overlaps as recommended in the In**I**Fusion Cloning user manual. Primer set B had overlaps of 15-bp upstream and downstream complementary to the Rev primer A and Fwd primer C, respectively (Figure 1). These primers were used to amplify the fragments, and then all three products were cloned into the pKD46 cloning vector in a single reaction using the In**I**Fusion HD Cloning Kit (Cat. # 638909). The included Stellar Competent Cells were transformed directly with the cloning reaction mixture and plated on selective medium. The cloning reaction and transformation were performed as per the manufacturer's protocol. 30 colonies were randomly chosen for testing, and all showed the correct recombinant construct containing the 4-kb GFP cassette.

The recombinant cloning vector described above was used to transform a wild-type strain of bacteria containing the IncK plasmid. After transformation, the GFP cassette was transferred from the cloning vector into the IncK plasmid via homologous recombination. Transformants were selected on a kanamycin-supplemented agar plate and incubated at 37°C, resulting in the loss of the pKD46 cloning vector from the cells. Random testing of 5 out of 15 resulting colonies was performed via PCR followed by Sanger sequencing. All tested colonies showed recombination of the full GFP cassette into the IncK plasmid at the correct locus.

References

Horton R.M., Cai Z., Ho S.N., Pease L.R. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques* **8,** 528–535 (1990).

Klümper U., *et al.* Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J.* **9,** 934–45 (2015).

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