De novo insertion of small fusion protein tags via In-Fusion Cloning

Data kindly provided by: Fabio Antenucci
Post-doctoral research fellow, Veterinary Clinical Microbiology, University of Copenhagen

Product highlights:

- Overcome the limitations imposed by restriction site availability
- Add small fusion protein tags (<10 aa) without the use of a tag template
- Incorporate multiple inserts and tags into a vector with a single reaction

Introduction

Fusion protein tags are small peptide sequences that can be used to detect, purify, and characterize proteins of interest. In order to generate tagged proteins, these tags need to be inserted into the sequence of targeted proteins via genetic engineering, resulting in the expression of recombinant proteins that harbor the tags in *cis*.

Traditionally, the engineering of tagged recombinant proteins has been performed by restriction-dependent cloning. This method requires a donor template to amplify the selected tag sequence and several cloning steps. The advent of restriction-independent cloning has greatly simplified this process, allowing the engineering of recombinant proteins with small tags (<10 aa) in a single step and without the need for a tag template (*de novo*).

In the experiment described here, In‖Fusion HD Cloning Plus was used for the construction of a recombinant chimera harboring domains from two different template proteins (A and B) and the *de novo* inserted fusion tag A-4 (Zhou et al. 2008; Figure 1).

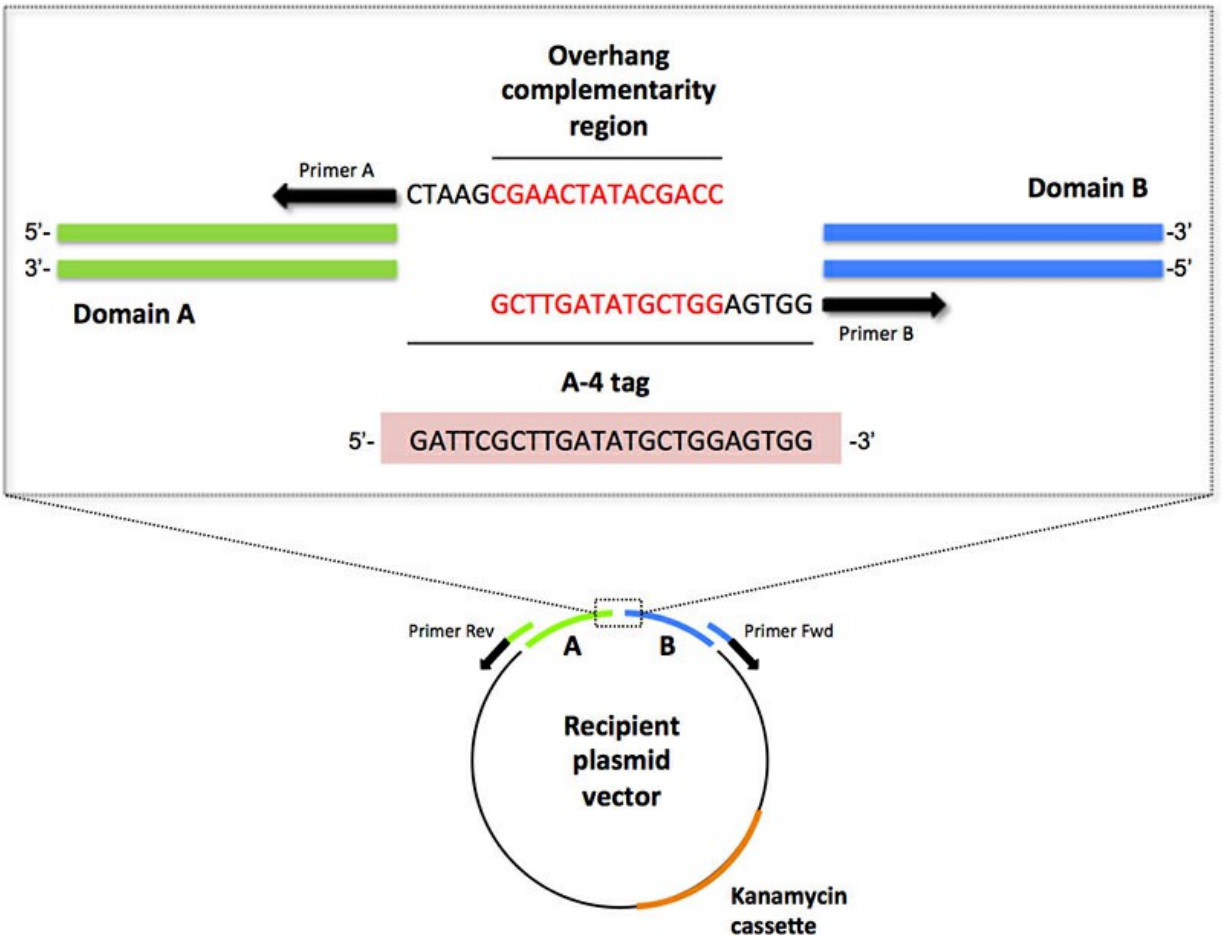

Figure 1. Schematic of the recombinant vector constructed during this experiment. Red letters indicate complementary regions between the 5' overhangs of Primers A and B. The full sequence of the A-4 tag is highlighted in the pink box.

Results

> “In-Fusion cloning really helped organize the time-frame of my research. I had a nearly 100% success rate by designing primers and performing the cloning according to the recommended specifications.”
>
> *—Fabio Antenucci, UNIVERSITY OF COPENHAGEN*

Following the generation of the recombinant vector (Figure 1), three pairs of PCR primers were generated that produced overlapping amplicons for subsequent sequencing (segments 1–3 in Figure 2). Three clones containing inserts were then selected and screened for target integration, mutations, and/or genetic rearrangements using the outline in Figure 2. Sequencing results demonstrated that all three clones integrated the targeted recombinant open reading frame (ORF) A-A4-B with no detectable mutations or genetic rearrangements.

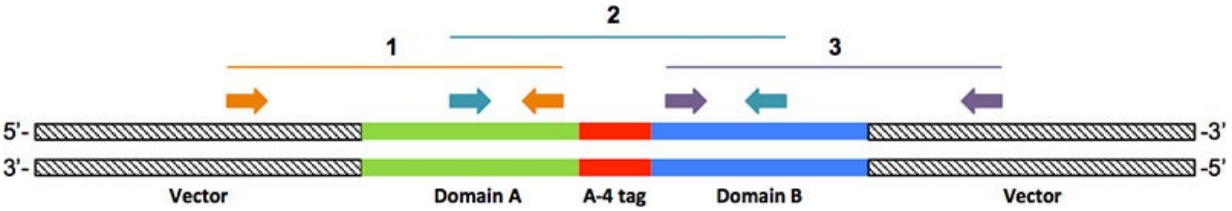

Figure 2. Sequencing strategy used for verifying the presence of the correct ORF in recombinant vector. Three pairs of primers were designed to produce three overlapping amplicons for sequencing (segments 1–3) to assay for the correct order of integration of Domains A and B on either side of the A-4 tag.

Conclusions

Generating tagged fusion proteins can be time-intensive and require multiple subcloning steps for each vector to generate targeted inserts in turn. With a relative efficiency of 100% (3/3 true positive clones), the In-Fusion Cloning protocol demonstrated a fast and reliable method for the *de novo* inclusion of small fusion tags using a single-step cloning reaction.

Methods

A plasmid containing a kanamycin resistance cassette was selected as the expression vector and was linearized by high-fidelity inverse PCR using Phusion Hot Start II DNA Polymerase (2 U/µl; Thermo Fisher Scientific). 15-bp overhangs matching the 5’ region from gene A and the 3’ region from gene B (Primers Rev and Fwd, Figure 1) were introduced in the 5’ end of the designed primers according to In-Fusion requirements for annealing. Selected domains from genes A and B were similarly amplified by high-fidelity PCR using primers that were designed to match the insertion site on the vector (not shown), and included the *de novo* inserted sequence of the A-4 tag in the 5’ overhangs of the primers that connected domain A and B (Primers A and B, Figure 1). PCR amplicons were gel-purified and quantified prior to In-Fusion Cloning. All primers used in this study were designed using CLC Genomics Workbench 7.

In-Fusion Cloning of domains A and B in the recipient vector was performed via a one-step, three-point annealing reaction (Figure 1) using the reagent concentrations and conditions described in Table I below. 1 µl of the cloning reaction was transformed into *E. coli* Stellar Competent Cells according to the In-Fusion protocol. Transformed cells were plated and incubated overnight at 37°C on BHI plates supplemented with 75 µg/ml kanamycin. 13 colonies were obtained, of which 10 putative positive clones were screened for the presence of the insert via colony PCR. Finally, plasmids were extracted from three randomly selected insert-containing clones for further analysis. The effectiveness of the cloning procedure was verified by high-fidelity PCR. Plasmid constructs were then sequenced using primers designed to generate overlapping sequences that enabled the corroboration of the correct order of integration for domains A and B in the recipient vector (Figure 2). Sequencing reads were assembled to verify the presence of the desired chimeric ORF using CLC Genomics Workbench 7.

In-Fusion Cloning reaction	
Components	**Concentration or volume**
Amplicons A and B	50 ng each
Linearized vector	50 ng
5X In-Fusion HD enzyme premix	1 µl
MilliQ water	Up to 5 µl
Total volume	**5 µl**

Table I. In-Fusion Cloning reaction setup.

> I have to thank Dr. Janine T. Bossé from Imperial College London (UK) for teaching me a trick I’d like to share for engineering plasmid constructs. By incorporating reverse amplification of the recipient vector in the standard In-Fusion protocol, one is able to:

- Extend the homologous region of the overhangs to 30 bp (15 bp on each primer), increasing the efficiency of annealing.
- Incorporate digestion of the amplified vector with methylation-sensitive DNases (e.g., DpnI). Because PCR products are not methylated, while the template is, methylation-sensitive digestion removes the template from the reaction mix while leaving your amplified vector untouched. This decreases the incidence of false-positive clones harbouring the template plasmid and increases the rate of true positives."

—Dr. Fabio Antenucci, UNIVERSITY OF COPENHAGEN

References

Zhou, Z., Koglin, A., Wang, Y., McMahon, A.P. & Walsh, C.T. An Eight Residue Fragment of an Acyl Carrier Protein Suffice for Post-Translational Introduction of Fluorescent Pantetheinyl Arms in Protein Modification *in vitro* and *in vivo*. *J. Am. Chem. Soc.* **130,** 9925–9930 (2008).

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