Higher sequence accuracy for seamless constructs with In-Fusion Cloning

- Seamless cloning enables directional, multiple-insert cloning without scar sequences
- Methods for seamless cloning vary in their enzymatic mechanisms
- Sequence accuracy is improved with ligase-free In-Fusion Cloning compared to Gibson Assembly and NEBuilder kits

Introduction

Researchers have several options when it comes to seamless cloning methods. The mechanism for each varies quite a bit, as does the resulting sequence accuracy, which we will discuss below. The benefits to any seamless method include the lack of unwanted nucleotides—or scar sequences—between vector and insert, as well as the ability to clone multiple inserts in a specific order in just one round of cloning. However, these research tools lose some of their power if accuracy and/or efficiency suffer. High-throughput applications are particularly affected by any pain points in this regard, as problems with potential error rates or background levels will be magnified when projects are scaled up to accommodate larger cloning needs.

Several commercially available seamless cloning kits, including Gibson Assembly and NEBuilder, utilize a three-enzyme mix consisting of an exonuclease, polymerase, and ligase (Gibson et al. 2009). The mechanism for In-Fusion Cloning (Irwin et al. 2012) differs from this process in a simple but significant way. Both mechanisms require that inserts are amplified by PCR such that they include homologous overlaps with a linear vector or other adjacent fragments, and both utilize an exonuclease to chew back the DNA of linear fragments to create cohesive sticky ends. At this point, the workflows part ways. With the three-enzyme methods, a polymerase fills in any nucleotide gaps, and a DNA ligase then seals the nicks in the annealed fragments prior to the transformation step. This process runs the risk of introducing sequence errors and mismatches at the cloning junction. In contrast, In-Fusion technology does not use a polymerase nor DNA ligase in the cloning mechanism, thus eliminating the potential for nucleotide errors. Cohesive bonds are formed *in vivo*, preserving the sequence integrity of the clone. In an effort to understand the repercussions of the differences in these mechanisms, we used commercially available kits for each to perform the demanding task of multiple-insert cloning, specifically looking at sequence accuracy as a measure of success.

Results

In the experiment below, we compared two kits for the three-enzyme method (NEBuilder DNA HiFi Assembly from NEB and the Gibson Assembly Kit from SGI) with the liquid and lyophilized (EcoDry) versions of Takara Bio's In-Fusion Cloning HD Plus kits in order to assess the sequence accuracy of clones made with each method. Each seamless cloning kit was used to clone five inserts (947 bp, 717 bp, 697 bp, 405 bp, and 1,005 bp) into a 2.7-kb vector backbone, with the molar ratio of vector to insert set at 1:2 (Table I).

Table I. Cloning reaction setup to achieve a vector-to-insert molar ratio of 1:2

Linear fragment	Amount
Insert 1 (947 bp)	40 ng
Insert 2 (717 bp)	40 ng
Insert 3 (697 bp)	32 ng
Insert 4 (405 bp)	20 ng
Insert 5 (1,005 bp)	45 ng
Vector (2,671 bp)	37 ng

All clones were designed with 20-bp homologous overlaps between each pair of linear fragments, as per the recommendations for multiple-insert reactions with In-Fusion Cloning. Reactions for each system were carried out per their respective manufacturer's instructions, and 1/10 dilutions were plated on selective medium. 20 clones from each kit were chosen at random in order to check sequence fidelity. Both three-enzyme methods yielded clones with an average of 80% accuracy, while In-Fusion Cloning produced a higher level of accuracy (Table II), with liquid and EcoDry kits yielding 90% and 100%, respectively.

Table II. Sequence accuracy comparison for all four seamless cloning kits	
Commercial kit	**Sequence accuracy (average of 20 clones)**
In-Fusion HD Cloning Plus (liquid format)	90%
In-Fusion HD EcoDry Cloning Plus (lyophilized format)	100%
NEBuilder HiFi DNA Assembly Cloning Kit (NEB)	80%
Gibson Assembly Kit (SGI)	80%

Conclusions

The sequence accuracy of In-Fusion Cloning is consistently higher than both commercial kits for three-enzyme cloning methods, regardless of which format of the In-Fusion enzyme mix is used. It is likely that this difference stems from the lack of ligase and polymerase in the In-Fusion Cloning mix, as both ligase and polymerase performance can be error-prone. Sequence errors can be especially problematic in multiple-insert cloning, as there are more cloning junctions where nucleotide mismatches can occur. The reliable results provided by In-Fusion technology make it especially adaptable to high-throughput workflows. Higher accuracy means less screening and fewer missed clones in large batches, which would then require additional rounds of cloning to recover.

Methods

Each seamless cloning kit was used according to the manufacturer's instructions. Vector-to-insert molar ratios were set at 1:2 for each reaction. Reactions for each kit were run in triplicate, with the exception of the liquid In-Fusion Cloning kit, which was performed once to reproduce previous data. NEB 5-alpha Competent *E. coli* (High Efficiency; NEB) were transformed with the cloning reactions from the NEBuilder HiFi DNA Assembly Cloning Kit (NEB), TransforMax EPI300 Electrocompetent and Chemically Competent *E. coli* were transformed with the cloning reactions from the Gibson Assembly Kit (SGI), and Stellar Competent Cells (included in the In-Fusion Cloning kits) were transformed with the cloning reactions from both In-Fusion HD Cloning Plus and In-Fusion HD EcoDry Cloning Plus. 1/10 dilutions were plated on selective medium for each transformation. 20 clones were chosen at random from transformation plates of each cloning kit and checked for sequence accuracy via Sanger sequencing.

References

Gibson D.G., *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**(5): 343–345 (2009).

Irwin C.R., Farmer A., Willer D.O., Evans D.H. In-Fusion Cloning with Vaccinia Virus DNA Polymerase. Vaccinia Virus and Poxvirology (Methods and Protocols). **890**:23–35 (2012).

Related Products

Cat. #	Product	Size	License
638920	In-Fusion® HD Cloning Plus	96 Rxns	
638911	In-Fusion® HD Cloning Plus	100 Rxns	
638910	In-Fusion® HD Cloning Plus	50 Rxns	
638909	In-Fusion® HD Cloning Plus	10 Rxns	
638919	In-Fusion® HD Cloning Plus CE	96 Rxns	
638918	In-Fusion® HD Cloning Plus CE	100 Rxns	
638917	In-Fusion® HD Cloning Plus CE	50 Rxns	
638916	In-Fusion® HD Cloning Plus CE	10 Rxns	
638915	In-Fusion® HD EcoDry™ Cloning Plus	96 Rxns	
638914	In-Fusion® HD EcoDry™ Cloning Plus	48 Rxns	
638913	In-Fusion® HD EcoDry™ Cloning Plus	24 Rxns	
638912	In-Fusion® HD EcoDry™ Cloning Plus	8 Rxns	

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