BBF RFC 26: In-Fusion BioBrick Assembly

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1. Purpose

This BioBricks Foundation Request for Comments (BBF RFC) describes a method to assemble two BioBricks using the Clontech In-Fusion PCR Cloning Kit while maintaining BioBrick standard formats.

2. Relation to other BBF RFCs

BBF RFC 26 does not update or replace any earlier BBF RFC.

3. Copyright Notice

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4. Background

There are currently several BioBrick assembly standards [1] that all involve assembly by restriction enzyme digestion and ligation. This RFC describes an alternative assembly method that allows for BioBricks to be assembled using the Clontech In-Fusion PCR Cloning Kit [2-4]. One PCR-amplified BioBrick has homology on each end with the second PCR-amplified BioBrick (vector amplified with the BioBrick) to allow for the fragments to be fused together in the In-Fusion reaction (see figure below). This method can be adapted to fuse more than two BioBricks together and four fragments have been successfully fused [2,3]. This method can also be used to re-engineer existing BioBricks by using mutagenic primers in the PCR reaction (see detailed protocols in [5] for an example of simultaneous promoter and RBS re-engineering).

The advantages of In-Fusion BioBrick assembly over standard assembly are that it is faster, does not require restriction digestions or ligations or DNA extraction from a gel, and is more flexible in the sense that there is more control over the exact engineered sequence. The disadvantages are that the supplies are more expensive, custom primers are required, and occasionally there are mutations in assembled plasmids. However, sequencing a few miniprepped plasmids from positive colony PCR screened clones

usually results in at least one plasmid without mutations. In general, In-Fusion BioBrick assembly is ideal when re-engineering an existing BioBrick with many parts since there are less assembly reactions to perform. This method is also can be used to introduce mutations into BioBricks [3]. On the other hand, if the BioBricks you want to assemble already exist as digested fragments in the freezer, then standard assembly is ideal for this particular situation. The motivation of this RFC is not to compete with existing assembly methods, but is to provide a faster method for BioBrick assembly that others in the community MAY find useful.

5. Materials

Thermocycler 0.2 mL PCR tubes Standard Desalted Primers Phusion PCR Mastermix (or another high fidelity polymerase mastermix) BioBrick template DNA (miniprepped plasmid diluted 1:1000 in water) Qiagen PCR Purification Kit Nanodrop (not required, but very useful) Clontech In-Fusion PCR Cloning Kit (dry down kit works well) TE Buffer (pH 8.0) Gel box, power supply, and gel supplies LB+Amp (100 µg/mL) plates LB+Amp (100 µg/mL) media Qiagen Miniprep Kit

6. Method



This figure shows the **general** In-Fusion BioBrick assembly scheme for BioBricks on pSB1A2 plasmids. See details below. BioBrick Part A (blue) and Part B (red) are on pSB1A2 plasmids encoding ampicillin resistance. Primers described below are color-coded to show their homology. The thick black line indicates BioBrick prefix homology and the yellow sequence is the scar that is normally between parts after standard BioBrick assembly, if this is desired. If not, leave it off your primer (see below). Here is the general protocol:

1. Read the In-Fusion PCR Cloning Kit manual and understand it completely before starting.

2. Order primers for parts to assemble. Primers can be optimized accordingly for PCR, but this general scheme SHOULD work for most BioBricks. The main rule is that the **forward** primer of the first PCR-amplified fragment needs to have at least 15bp homology to the **reverse** primer of the second fragment, and vice versa, for the In-Fusion reaction to work. 20bp is used in this protocol because in my experience it works better than 15 bp. When designing the primers, it is better to PCR-amplify the vector (plasmid backbone) with the smaller part especially if the part is less than 100 bp since you want the molar ratio between both fragments to be as close as possible. There are two general strategies for assembly depending on which fragment you want to have the vector. The first strategy PCR-amplifies the upstream part by itself and the downstream

part with the vector. The second strategy PCR-amplifies the upstream part with the vector and the downstream part by itself. Each strategy requires four primers, where two are specific to the parts you want to assemble and two are specific to the vector.

Strategy 1

Primers for Part A (upstream part = insert) + Part B with the pSB1A2 vector (downstream part = vector), as shown in the figure above:

- Part A Forward primer (**AF**): 5'-TTCTGGAATTCGCGGCCGCTTCTAG-3' (specific to the pSB1A2 non-coding sequence prefix + 5 bases upstream of the prefix)
- Part A Reverse primer (**AR**): last 20 bases Part A + scar (if wanted) + first 20 bases Part B (reverse complement of this entire sequence)
- Part B + vector Forward primer (BF): reverse complement of Part A reverse primer
- Part B + vector Reverse primer (**BR**): 5'-CTAGAAGCGGCCGCGAATTCCAGAA-3' (reverse complement of Part A forward primer)

Strategy 2

Primers for Part A with the vector (upstream part = vector) + Part B (downstream part = insert), not shown in the figure above:

- Part A + vector Forward primer: 5'-TACTAGTAGCGGCCGCTGCAGGCTTC-3' (specific to the pSB1A2 suffix + 5 bases downstream of the suffix)
- Part A + vector Reverse primer: last 20 bases Part A + scar (if wanted) + first 20 bases Part B (reverse complement of this entire sequence)
- Part B Forward primer: reverse complement of Part A reverse primer
- Part B Reverse primer: 5'-GAAGCCTGCAGCGGCCGCTACTAGTA-3' (reverse complement of Part A forward primer)

3. Perform a PCR reaction with a high fidelity polymerase such as Phusion using the RECOMMENDED protocol. For template DNA, dilute miniprepped BioBrick plasmid DNA 1:1000 (or at least 1:100) to minimize these "background" plasmids from getting transformed later. Digestion with DpnI after the PCR reaction MAY reduce background plasmids, but in my experience there is a high success rate (>50%) without the use of DpnI or phosphatase on the vector.

4. Run a gel with 5 μ l of the purified PCR products to determine if the products are of the correct size and there are no secondary bands. If there are secondary bands, run a gradient on the primer annealing step in the PCR reaction to determine an annealing temperature that produces a single PCR product.

5. Purify PCR products with Qiagen PCR Purification Kit and elute with 30 μl water.

6. Measure the concentration of your PCR products with a Nanodrop or by comparing against a ladder on another gel.

7. Perform the In-Fusion reaction using the RECOMMENDED protocol (this includes the transformation step with Fusion-Blue competent cells) with a few extra details and exceptions here. As the protocol recommends, use a 2:1 insert:vector ratio and I have found that 100 ng of vector works well. The In-Fusion reaction ideally SHOULD be performed in 0.2 mL PCR tubes in a thermocycler ($37^{\circ}C$ for 15 minutes, $50^{\circ}C$ for 15 minutes). I transform 2.5 μ l of this reaction into one 50 μ l tube of Fusion-Blue chemically competent cells. The one hour incubation time is not necessary when transforming plasmids conferring ampicillin resistance, but is REQUIRED with kanamycin resistance conferring plasmids. I plate 200 μ l on a LB+Amp plate (without the centrifugation steps in the protocol) and typically get 50-300 colonies.

8. Perform colony PCR on at least 5 colonies (10-12 is ideal) using the VF2 (5'-/ TGCCACCTGACGTCTAAGAA-3') and VR (5'-ATTACCGCCTTTGAGTGAGC-3') primers or primers specific to your construct to find colonies that have the correct size insert.

9. Grow correct colony or colonies in 5 mL LB+Amp overnight. Three is ideal since one SHOULD be correct without mutations which tend to occur most often at the junctions (regions of homology between fragments).

- 10. Perform miniprep(s) with the Qiagen Miniprep Kit.
- 11. Submit plasmid(s) for sequencing with VF2 and VR or custom primers.

7. Discussion

Ideally an assembly standard will use the same laboratory components (e.g. restriction enzymes and ligase) so that the same components can be used to assemble any two (or more) BioBricks together. This standard has one limitation in that custom primers need to be ordered for each individual assembly and hence requires different components. Nonetheless, this In-Fusion BioBrick Assembly method has been used to make several constructs and works well for myself and others in our lab. This method can be adapted to other plasmids in your favorite standard format and to assemble more than two BioBricks together (three is the most we have attempted with success). This protocol hopefully can be expanded upon in the future to fuse >4 fragments together and use the same laboratory components. The method described here is a work-in-progress and the up-to-date method along with detailed laboratory protocols are located in the OpenWetWare link [5] provided in the references section. Please document your success with using this method on the OpenWetWare page!

8. Acknowledgments

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9. Authors' Contact Information

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10. References

[1]

http://openwetware.org/wiki/The_BioBricks_Foundation:Standards/Technical/Format

[2] http://www.clontech.com/products/detail.asp?tabno=2&product_id=162275

[3] Zhu B., et al. In-Fusion assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations. BioTechniques 43:354-359 (September 2007)

[4] Benoit R.M., et al. An improved method for fast, robust, and seamless integration of DNA fragments into multiple plasmids. Protein Expr Purif. 2006 Jan;45(1):66-71.

[5] http://openwetware.org/wiki/In-fusion_biobrick_assembly