

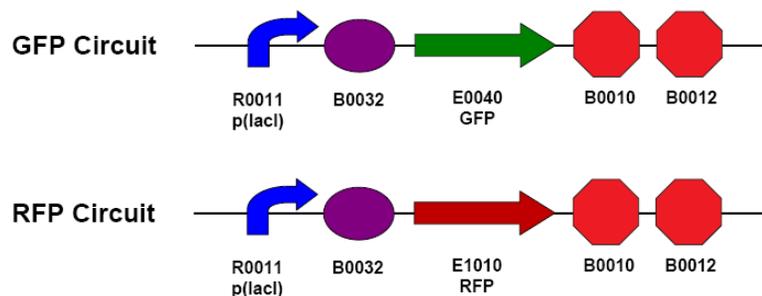
BioE 498 Lab: In-Fusion BioBrick Assembly and Re-engineering

Introduction

The purpose of this lab is to teach you how to assemble and re-engineer standard biological parts called BioBricks into functional genetic circuits using the Clontech In-Fusion PCR Cloning Kit. This is a PCR (Polymerase Chain Reaction) based method and an alternative to Standard BioBrick Assembly. The advantages to the In-Fusion method compared to Standard Assembly are that it is faster and more versatile in the sense that you have more control over the exact DNA sequence of the circuit you want to engineer. This is particularly useful for swapping out different parts or randomizing sequences for directed evolution, for instance. The disadvantage to the In-Fusion method is that you need customized primers and it is more expensive in terms of supply costs.

Additional information about BioBricks and the Registry of Standard Biological Parts that catalogues BioBricks can be found at <http://partsregistry.org>. See the BioE 498 lecture slides for additional information such as PCR and primer design not given in this lab manual. The details of this lab are somewhat complicated, so if you read this introduction and still don't understand how it works, ask the instructor to explain it to you.

Half of the lab teams will construct a green fluorescent protein (GFP) circuit and the other half will construct a red fluorescent protein (RFP) circuit.



Both of these circuits are identical except for the fluorescent protein coding sequence, as each has the same promoter, ribosome binding site (RBS), and double transcriptional terminator. Let's go over each BioBrick in more detail.

R0011 is a *lacI*-regulated promoter since the operator sites allow for the LacI repressor protein to bind and repress transcription when this circuit is transformed into a host strain such as *E. coli* DH5 α *lacI*^q that expresses the *lacI* gene from its chromosome. If the molecule Isopropyl β -D-1-thiogalactopyranoside (IPTG) is added to the media containing DH5 α *lacI*^q cells with the GFP or RFP circuit, IPTG will bind to the LacI protein and induce transcription of the circuit.

B0032 is a medium-strength RBS that allows for translation of the GFP or RFP coding sequence into protein. This RBS has a measured activity of 0.3 relative to a stronger RBS B0034.

E0040 and **E1010** are the GFP and RFP coding sequences, respectively. E0040 comes from jellyfish and there is detectable green fluorescence within 8 minutes after being expressed due to its fast folding time. E1010 comes from coral and also matures quickly to allow for measurable fluorescence after a short time.

B0010 and **B0012** are transcriptional terminators that stop transcription due to a hairpin structure that forms in the RNA during transcription. These two parts are also called B0015 collectively and are the most common transcriptional terminator in the Registry.

pSB1A2 is the plasmid backbone (vector) of these BioBrick parts. This is a high-copy number plasmid (100-300 copies per cell) and has an ampicillin resistance gene. The prefix and suffix sequence are located on either side of the plasmid which contain restriction sites for Standard Assembly.

In-Fusion PCR Cloning Kit

This is the cloning kit we will use for this lab and allows for multiple PCR products to be joined together without the need for digestion and ligation. When you PCR-amplify your gene (or “Insert” in this lab) of interest with at least 15 bp (20 bp in this lab) extensions homologous to the vector ends, these ends will fuse together due to the homology. We will look at how this fusion occurs in more detail later. Fusion of the insert and vector makes a circular plasmid that can then be transformed into cells of interest. In this lab we will PCR-amplify the insert and vector separately, then fuse them together with the In-Fusion kit, transform the circular plasmid containing the genetic circuit into cells expressing *lacI*, then characterize this circuit at the population and single cell levels. Below is a diagram that shows how the In-Fusion reaction works.

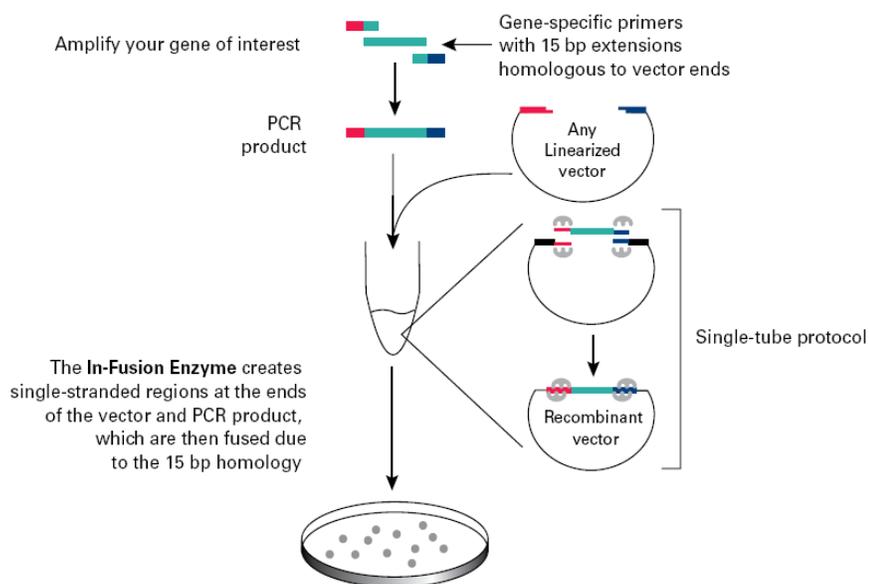
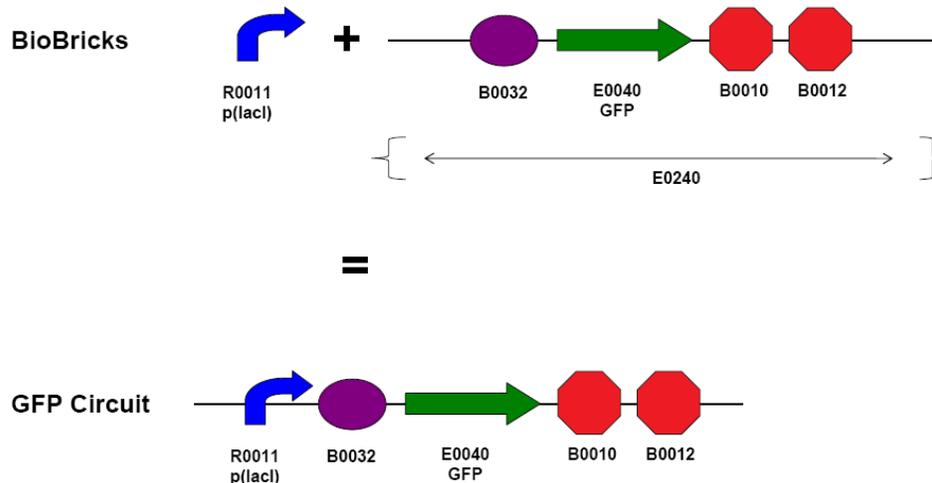


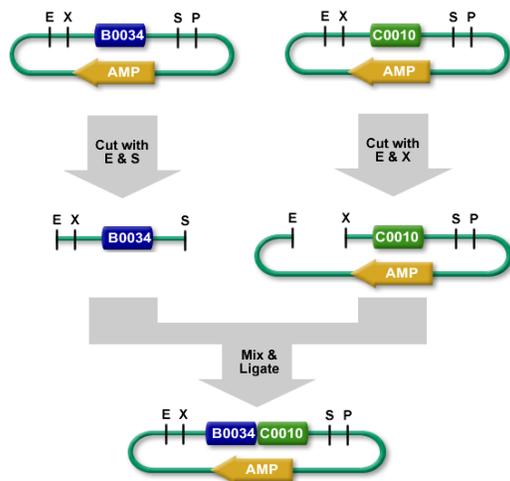
Figure 1. The In-Fusion Method. During the 30 min benchtop incubation, the In-Fusion enzyme creates single-stranded regions at the ends of the vector and PCR product, which are then fused due to the 15 bp homology. The resulting clone can be used to transform *E. coli*.

GFP circuit assembly

The GFP circuit will be assembled using two different BioBricks, R0011 and E0240.

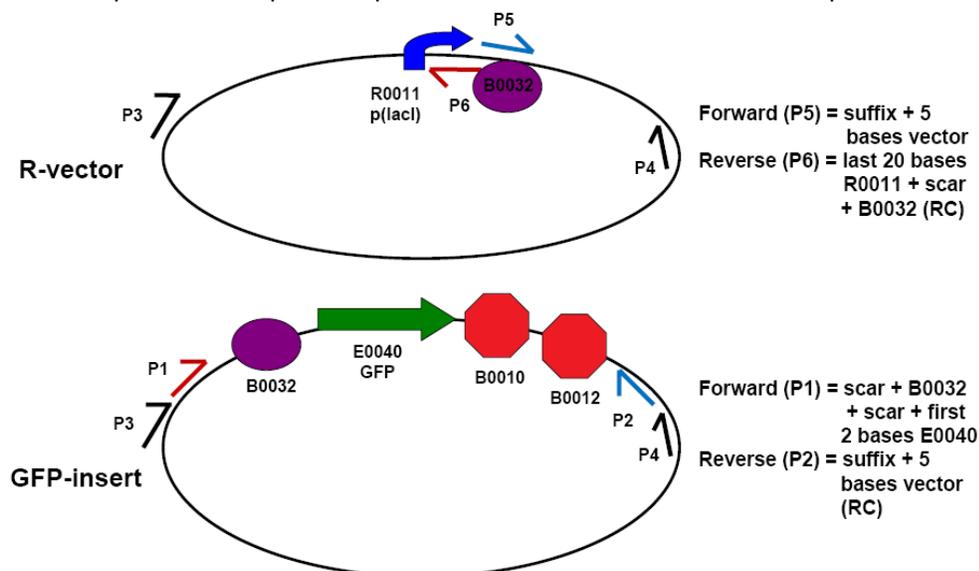


Below is a figure showing how BioBrick Standard Assembly works and this will be important later when we discuss primer design for PCR-amplifying these BioBricks. Each BioBrick part has a prefix and suffix on either side with restriction sites for BioBrick Standard Assembly. The prefix has the EcoRI (E) and XbaI (X) restriction sites. The suffix has the SpeI (S) and PstI (P) sites. When BioBricks are assembled via Standard Assembly a scar results between parts. This is due to digestion and ligation between parts. Plasmids with RNA or protein coding parts have a shorter prefix so that the spacing between the RBS and coding sequence is correct, leading to a 6 bp scar of TACTAG. Non-coding sequence parts have a prefix that is 2 bp longer which leads to a 8 bp scar of TACTAGAG.



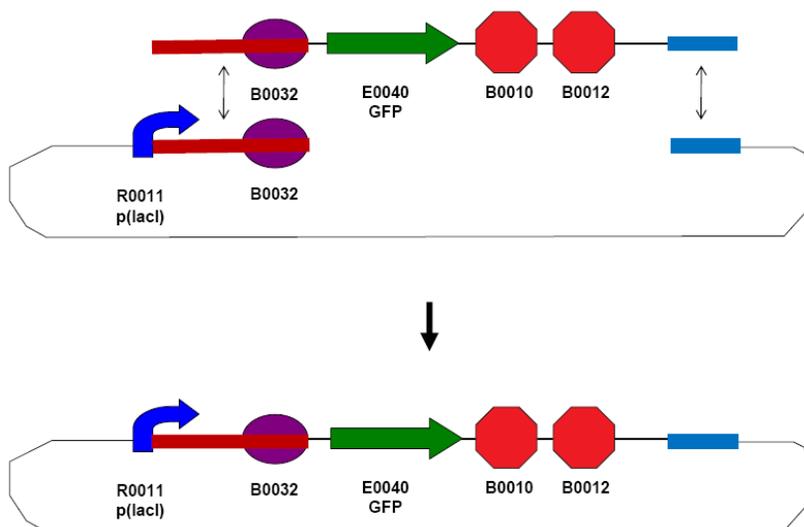
Now to PCR-amplify the insert and vector, we have to design primers that will give us the exact DNA sequences to amplify and fuse together. Since the R0011 part is only 65bp, it is ideal to amplify this with the vector so that there are only two PCR products to fuse together instead of three (two parts + the vector). The figure below shows the R0011 part on the pSB1A2 plasmid and here is called the R-vector. As shown on the right side of the figure, the forward primer for the R-vector (P5) going from the 5' to 3' direction is the BioBrick suffix + adjacent 5 bases downstream on the pSB1A2 vector. This sequence is completely homologous to the bottom strand of the template DNA and will amplify the DNA in a clockwise direction (as indicated by the direction of the arrow). The reverse primer is a little more tricky to

understand because it's not completely homologous to the plasmid sequence. The R-vector reverse primer (P6) has the reverse complement (RC) of the following: last 20 bases of R0011 to allow for specific amplification at that location, then the 8 base scar sequence, and B0032. Why the scar and B0032? This is because we want to add a scar sequence and B0032 sequence downstream of the R-vector PCR product so that this non-homologous portion of the primer is the reverse complement sequence of the forward primer of the GFP-insert. This will allow for the fusion to occur during the In-Fusion reaction. Note that the R-vector reverse primer and GFP-insert forward primer are both shown in red. This is to depict that they share homology with each other. P3 and P4 are the VF2 and VR primers, respectively, that are specific to the pSB1A2 plasmid backbone located about 100 bp outside the BioBrick part.

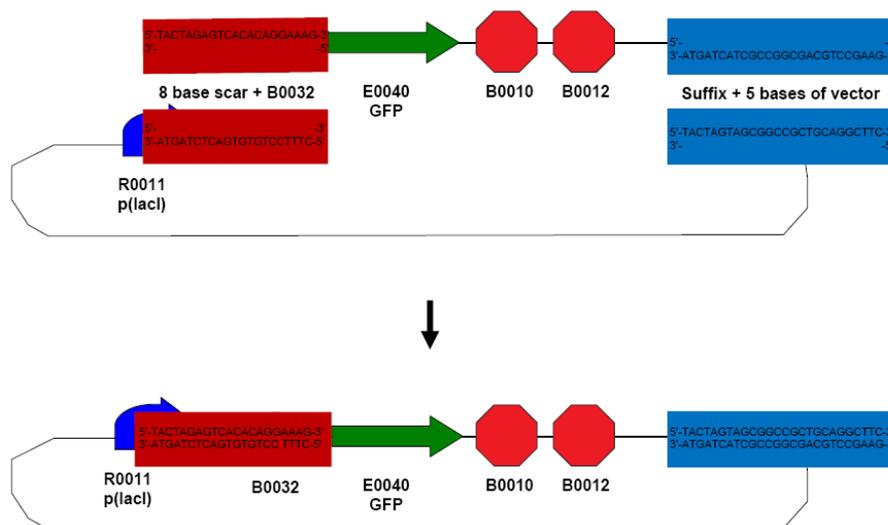


Now let's take a look at the primers for the GFP-insert. The forward primer (P1 GFP) is the 8 base scar + B0032 + 6 base scar + first 2 bases of E0040. It is exactly homologous to the template DNA. Here's the tricky part that will be explained in more detail later if you don't understand now: the non-homologous region of the reverse R-vector primer is the exact reverse complement of the scar and B0032 portion of this forward primer. See why this forward primer shares homology with a portion of the R-vector reverse primer? Now for the reverse primer: it's the reverse complement of the suffix and 5 bases of the vector. This GFP-insert reverse primer (P2 GFP) is the exact reverse complement of the R-vector forward primer, both shown in blue to depict this homology.

To show the homology between primers in more detail, the figure below shows how the R-vector and GFP-insert PCR product ends will fuse together. Specifically, the red portion of the GFP-insert PCR product (top) is the scar + B0032. The red portion of the R-vector PCR product (bottom) is the **reverse complement** of the scar + B0032 built into the R-vector reverse primer. The blue portion of the GFP-insert PCR product is the **reverse complement** of the suffix + 5 bases of vector. The blue portion of the R-insert is the the suffix + 5 bases of vector. After the In-Fusion reaction, the ends fuse together to join the circular plasmid. This is shown in the figure below.

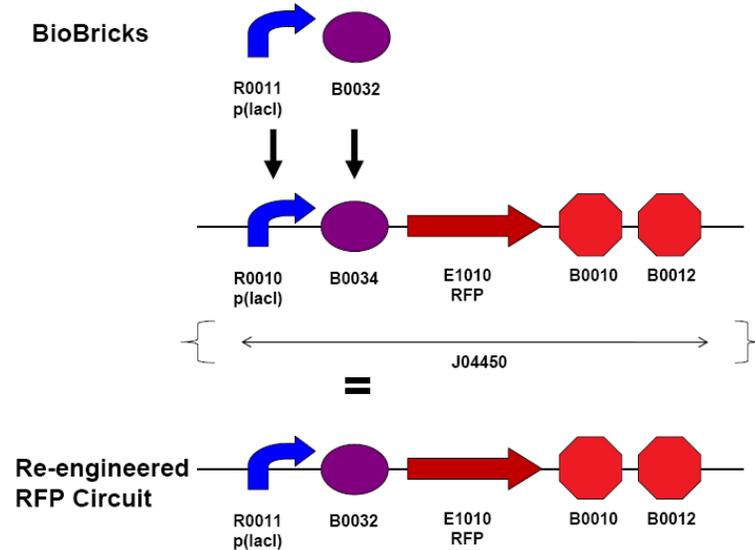


A more detailed look at the DNA sequences of each end is shown in the figure below. These colored regions are zoomed out and obviously not to scale. Since the In-Fusion enzyme is a 3' to 5' exonuclease, the end of each PCR product is chewed up in the 3' to 5' direction, leaving a single stranded region. During the In-Fusion reaction, the complementary single stranded regions fuse together to make the circular plasmid.

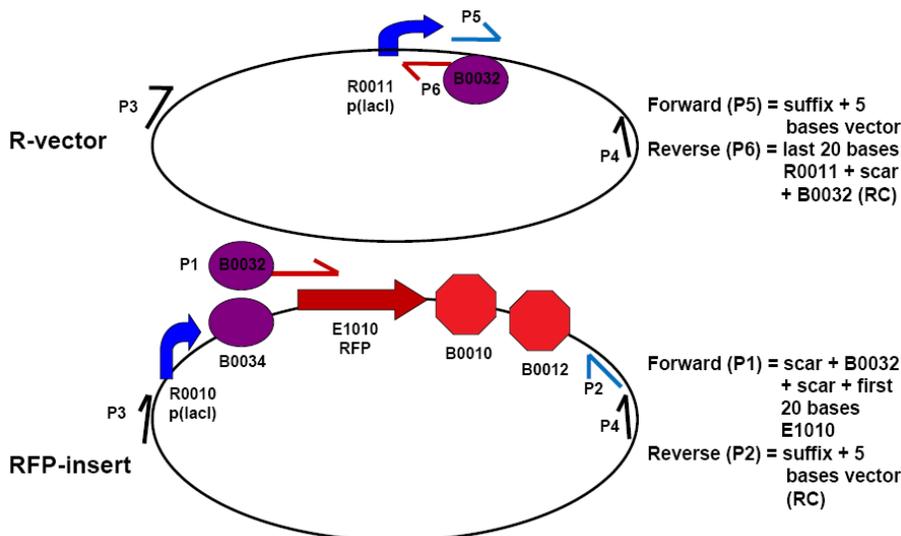


RFP circuit re-engineering

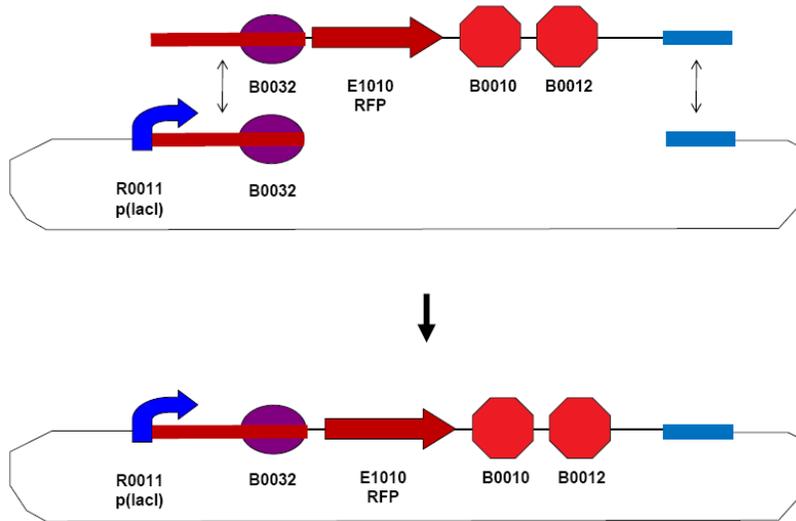
In contrast to the GFP circuit which involves the assembly of two BioBricks together, an existing RFP circuit (J04450) will be re-engineered to have the same promoter and RBS as the GFP circuit. The behavior of these circuits can then be compared at the population and single cell levels. See the figure below for details on individual BioBricks.



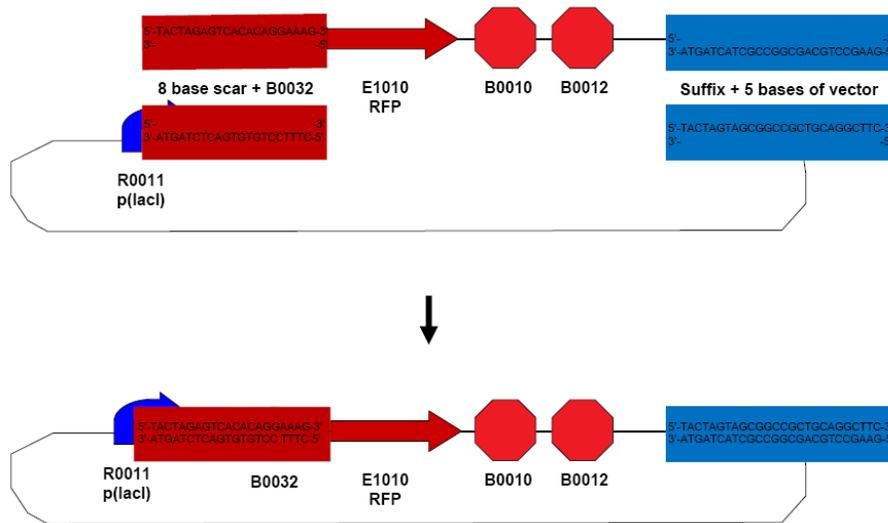
Since we're using the same R0011 promoter for the re-engineered RFP circuit as the GFP circuit, we will use the exact same primers and template DNA for this R-vector PCR reaction. The forward primer for the RFP-insert (P1 RFP) is different than the GFP-insert because here we want to amplify starting from E1010 (RFP coding sequence) and not amplify the upstream scar and RBS. Instead, we want to **add** the 8 base scar and B0032 on to the 5' end of the primer (this will be the reverse complement to the 8 base scar and B0032 on the P6 primer) and have a sequence specific to E1010 on the 3' end. So the forward primer for the RFP-insert is the 8 base scar + B0032 + 6 base scar + first 20 bases of E1010. The reverse primer for the RFP-insert (P2 RFP) is simply the reverse complement of the suffix + 5 bases of vector, the same as for the GFP-insert.



Taking a closer look at the fusion, it works the same way as with the GFP circuit since the RFP-insert PCR product now has the scar and B0032 attached.



Looking at the exact DNA sequence to see the details of the In-Fusion reaction...



Now that we've engineered these circuits on plasmids, we can now transform them into strains expressing *lacI*, induce them with IPTG, and characterize their behavior at the population and single cell levels. Do they behave the same or different?