Purpose

This Request for Comments (RFC) describes a novel method for the assembly of standard BioBrick parts. This assembly method for BioBrick parts is an improvement upon the conventional methods of BioBrick part assembly. This assembly method will involve the use of standard BioBrick restriction enzyme systems, PCR purification kit, ligase system, and a PCR polymerase system.

Relation to other BBF RFCs

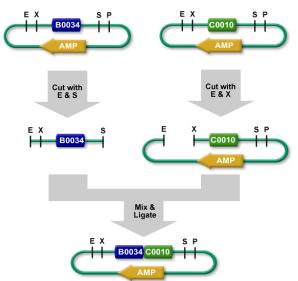
This assembly standard is compatible with BBF RFC 10, BBF RFC 23, BBF RFC 24, and BBF RFC 25.

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Background

Various assembly methods currently exist to aid in the cloning process, with the Standard Assembly, and 3A Assembly being a few of the commonly used methods.



The Standard Assembly [1], developed by Tom Knight, makes use of the EcoRI and XbaI restriction enzyme sites on the left and SpeI and PstI restriction sites on the right of the BioBrick parts for ligation. As shown in Figure 1, digesting one BioBrick part with EcoRI and SpeI restriction enzymes results in sticky ends, and digesting another BioBrick part with EcoRI and XbaI restriction enzymes opens the plasmid and gives it sticky ends to ligate with the first part, generating a plasmid with two BioBrick parts ligated together.

Figure 1: Standard assembly of two BioBrickTM parts

3A Assembly is another conventional method of Biobrick assembly which assembles two BioBrick parts through digesting one BioBrick part with EcoRI and Spel restriction enzymes to generate sticky ends. This digested BioBrick part can be ligated to another BioBrick part digested with XbaI and PstI restriction enzymes and a standard plasmid backbone digested with EcoRI and PstI restriction enzymes (Figure 2). The assembly product is then selected for through antibiotic resistance. [2]

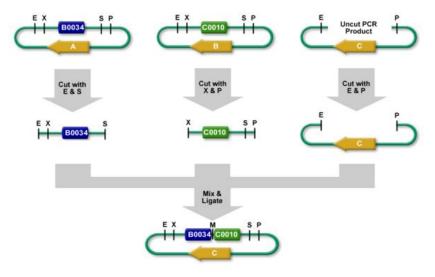


Figure 2: 3A Assembly of two BioBrick[™] parts

Motivation

The conventional methods of BioBrick part assembly described above can be error prone. Both the Standard BioBrick Assembly and the 3A Assembly require the ligation of the desired insert into a plasmid for amplification. During the digestion and ligation process, the plasmid backbones and the desired inserts may ligate in many possible configurations, generating undesired ligation products [Figure 3]. This mixture of desired and undesired ligation products will be amplified through bacterial multiplication, resulting in miniprep products that may contain an unacceptably high error level.

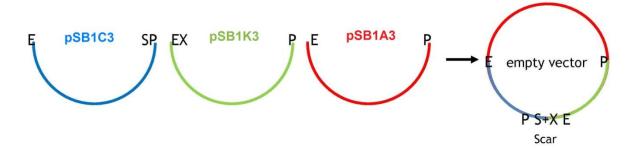


Figure 3: Possible undesired ligation product generated during 3A Assembly

The conventional methods of BioBrick part assembly are time consuming. The Standard BioBrick Assembly Standard and the 3A Assembly allow only the assembly of two BioBrick parts at once. During each step of the assembly process, bacteria containing the desired plasmid must undergo two incubation periods. The first incubation period takes place on antibiotic agar plate; the second incubation period takes place in a liquid culture medium. Consequently, the assembly of a construct containing a promoter, coding sequence, and terminator, will require four incubation periods to complete assuming each step of the assembly is successful on the first attempt.

The conventional methods of assembly may be difficult to debug once errors arise during the assembly. Each step of the conventional assembly methods contains numerous potential errors, including but not limited to unsuccessful ligation of insert into the plasmid vector, unsuccessful heatshock transformation, unsuccessful bacterial amplification, and unsuccessful miniprep procedure. Accounting for all of these potential errors during troubleshooting may be time consuming and difficult.

Novel Method of BioBrick Assembly - PCR Ligation

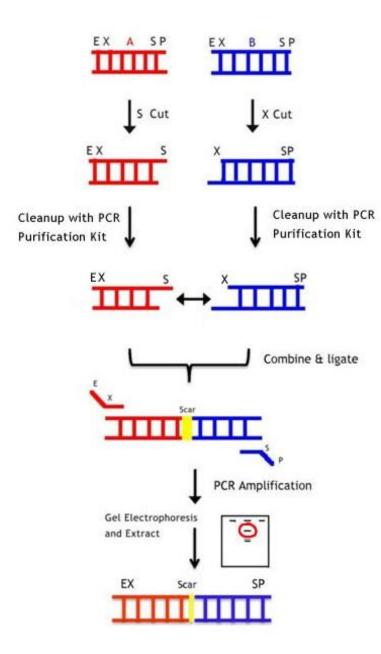


Figure 4: Overview of PCR Ligation Assembly

The PCR-Ligation Method of BioBrick
Assembly allows the ligation of two linear
BioBrick Parts surrounded by the BioBrick
prefix and suffix, hereon referred to as Part
A and Part B [Figure 4]. They can be
permanently ligated to each other using the
Spel sticky end of Part A and the Xbal sticky
end of Part B. This linear ligation product is
then PCR amplified using the Forward
Primer of Part A and the Reverse Primer of
Part B. The PCR product then undergoes gel
electrophoresis, and the DNA product
corresponding to the right length is
extracted and purified for further use.

During the ligation process, the digestion mixtures are both purified using a PCR Purification Kit before being ligated. The PCR Purification Kit removes the restriction enzymes, as well as the short DNA fragments resulting from the digestion. This step prevents the re-ligation of these fragments. Furthermore, removal of both Spel restriction enzyme and Xbal restriction enzyme prevents further digestion, leaving only one sticky end on each linear BioBrick part in the ligation mixture. This prevents the generation of undesired ligation product that would result if both restriction enzymes are left in the ligation mixture [Figure 5], and ensures that BioBrick parts will ligate together in the desired configuration.

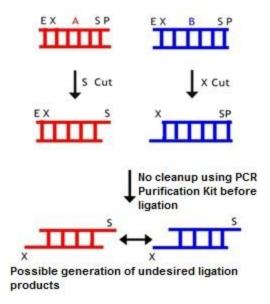


Figure 5: Possible undesired ligation products can be generated if both restriction enzymes are left in the ligation mixture

The PCR Amplification of the ligation product using the forward primer of Part A and the reverse primer of Part B enables control over the ligation product, because only the correct ligation product will be amplified in the PCR amplification using the two primers. Using a high fidelity polymerase system during the PCR amplification improves the fidelity of this assembly method. Each step of the assembly requires only one PCR instead of two bacterial incubation periods. This shortens the amplification time required for the assembly of a construct containing a promoter, a coding sequence, and a terminator from four bacterial incubation periods to two PCR amplifications.

This method of BioBrick assembly is easy to debug once errors arise during the assembly. The PCR amplification product after each step of the assembly undergoes gel electrophoresis. Only the DNA product corresponding to the correct length of the desired ligation product is extracted and purified for further use. In order for the assembly to proceed to the next step, the previous step of the assembly needs to be successful. Since every step of the PCR-ligation assembly is checked before further processing, the number of potential errors that need to be accounted for during troubleshooting is reduced compared to the convention methods of assembly.

Like the conventional methods of BioBrick assembly, the linear product of PCR-ligation also contains the BioBrick prefix at its 5' end and the BioBrick suffix at its 3' end [Figure 4]. Therefore, this method of assembly can be repeated for an infinite number of times to assemble multiple BioBrick parts [Figure 6].

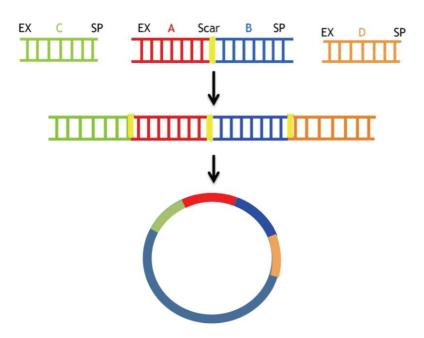


Figure 6: Repeatability of the PCR-Ligation Assembly method

Materials

Thermocycler

0.2 mL PCR Tubes

KAPA HiFi™PCR Hotstart Kit

 $0.2\ \mu g$ of each of the 2 linear BioBrick parts to be assembled, hereafter referred to as parts A and B

 $10\ \mu\text{M}$ solution of forward primer of BioBrick part A

 $10\,\mu\text{M}$ solution of reverse primer of BioBrick part B

Fermentas FastDigest Spel system

Fermnetas FastDigest Xbal system

KAPA T4 Rapid DNA Ligase

Tris/Borate/EDTA Buffer (TBE, pH = 8.0)

3 μl of Ethidium Bromide

Biotechnology Grade Agarose

Gel Box

Power Supply

Gel Comb

Loading Dye

Fermentas 1kb Plus DNA Ladder

Bio Basics EZ-10 PCR Purification Kit

UV Transilluminator

Protocol

1. Digest 0.2 μ g of linear BioBrick Part A with the Spel FastDigestion system; digest 0.2 μ g of linear BioBrick Part B with the Xbal FastDigestion system.

- 2. Purify each digestion mixture separately using Bio Basics EZ-10 PCR Purification kit.
- 3. Mix the products from step 2 in a 1:1 ratio to obtain a 10μ l mixture.
- 4. Use the 10µl mixture and T4 Rapid Ligase System to ligate the two BioBrick parts together
- 5. Use the ligation product from step 4 as the template DNA for PCR amplification. Use 2.5 μ l of the 10 μ M solution of forward primer of Part A and 2.5 μ l of the 10 μ M solution of reverse primer of Part B as primers for the PCR amplification.
- 6. Follow the instructions in the KAPA HiFi™PCR Hotstart Kit to perform the PCR amplification. Only 20 to 25 thermocycles are necessary to amplify the ligation product
- 7. Create a 1% agarose gel for gel electrophoresis of the PCR products
- 8. With loading dye, load the entire PCR product to the 1% agarose gel
- 9. Perform gel electrophoresis at 100V and 400 mA for 60 minutes.
- 10. Extract the visible DNA product under the UV transilluminator that corresponds to the correct length of the desired ligation product
- 11. Purify the extraction products from step 10 using Bio Basics EZ-10 PCR Purification Kit
- 12. The product from step 11 may be used for further assembly and processing

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References

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- [2] Shetty, R. *et al.* 3A Assembly. Accessed on 9-23-2011 http://partsregistry.org/Assembly:3A_Assembly>