Gibson Assembly® Primer-Bridge End Joining (PBnJ™) Cloning

Gibson Assembly® Cloning without homologous overlap regions

Introduction

Gibson Assembly® cloning has proven to be useful as a molecular biology technique for the seamless assembly of synthetic and natural genes and large-scale genetic pathways. Notably, Gibson Assembly cloning has enabled the synthesis of the first bacterial genome[1], the first synthetic cell[2], and the first minimal cell[3]. Additionally, the Gibson Assembly method has been utilized for genetic recoding (genome-wide codon removal)[4], the engineering of Cre recombinase for improved site-specificity[5], and has been incorporated into a one-step method for cloning gRNA for the CRISPR-Cas9 system[6].

As originally published by Dan Gibson and colleagues, the Gibson Assembly method eliminated the need to have compatible restriction sites through the addition of homologous overlap regions between adjacent fragments prior to assembly. Gibson Assembly joining is initiated by a strand-specific exonuclease that chews back the overlapping regions to create ends that then anneal. Single-stranded gaps are filled in by a proof-reading polymerase and ligated with a thermostable ligase. Here, we describe an additional means of realizing the versatility and utility of the Gibson Assembly method–Primer-Bridge End Joining (PBnJ™).

Primer-Bridge End Joining (PBnJ) is a novel modification of Gibson Assembly cloning that seamlessly joins fragments without homologous overlaps, thereby eliminating steps involved in generating fragment overlap regions by PCR or synthesis prior to assembly. In Gibson Assembly PBnJ cloning, a single primer or primer pair is used to "bridge" together the ends of DNA fragments. This novel application of Gibson Assembly cloning offers increased flexibility and eliminates the inherent limitations of PCR. In this report, we describe an assembly of two DNA fragments using the PBnJ method. This case study serves as an example of reliable assembly using simple primer design with Gibson Assembly PBnJ cloning.

Overview of Gibson Assembly® PBnJ™ Cloning

Gibson Assembly is based on homologous recombination of two sequences via an orchestral enzymatic reaction. PBnJ extends the Gibson Assembly principle by joining two adjacent fragments without homologous sequence using a pair of oligonucleotides (bridge primers) to create junction homology. There are three variations of the PBnJ method described in this note.

Gibson Assembly PBnJ Seamless Joining (Figure 1A) relies on a primer pair to bridge non-homologous overlap regions and the stepwise activities of the Gibson Assembly Ultra Kit followed by the Gibson Assembly HiFi 1-Step Kit. The primers contain phosphorothioate-modified 3' ends, which protect the primers from 3'exonuclease chew-back activity during assembly. After template chew-back, the primers anneal to the single-stranded template sequence and introduce overhang sequence that is homologous to the other DNA fragment, thereby creating a "bridge". This homology is used to assemble the fragments through the activities of the Gibson Assembly HiFi 1-Step Master Mix.

Introducing a single primer with homology to only one of the DNA fragment ends in the Gibson Assembly Ultra procedure will generate DNA ends with 3' overhangs (Gibson Assembly PBnJ 3' Overhang Extension; Figure 1B).

Gibson Assembly PBnJ Sequence Insertion Cloning (Figure 1C) inserts short DNA sequence at the assembly junction by adding these sequences into the "bridge" primers. The inserted sequences are homologous, enabling joining of the DNA ends during the Gibson Assembly Ultra procedure. This technique could be useful for many types of applications such as mutagenesis studies, promoter or enhancer studies, large-scale genome modification studies, as well as simply introducing short sequences (e.g. gRNA templates, watermarks, and restriction sites).

Materials

- DNA fragments were prepared by restriction digestion, purified with gel extraction, and used at concentrations higher than 20 ng/µL.
- Phosphorothioate-modified primers were at 0.1 – 1 µM.
- Gibson Assembly® Ultra kit (SGI-DNA Cat. No. GA 1200-10)
- Gibson Assembly® HiFi 1-step kit (SGI-DNA Cat. No. GA1100-10)
- DNA Clean & Concentrator™-5 (Zymo Research Cat. No. D4003)
- Chemically competent cells (transformation efficiency >10^9 CFU/µg pUC19)

Gibson Assembly PBnJ Cloning: A Case Study with two fragment assembly

To demonstrate the effectiveness of Gibson Assembly PBnJ cloning, we set out to assemble a single 4 kb insert with a 12 kb vector. The assembly strategy and vector and insert details are shown in Figure 2A. Both insert and vector were linearized by restriction digestion since PCR was problematic due to the vector size and presence of inhibitory sequences in the insert. The resulting 5' overhang sequences (denoted by green in the figure) are removed during the assembly procedure. Additionally, we added a DNA barcode (shown in orange) at Junction 1.

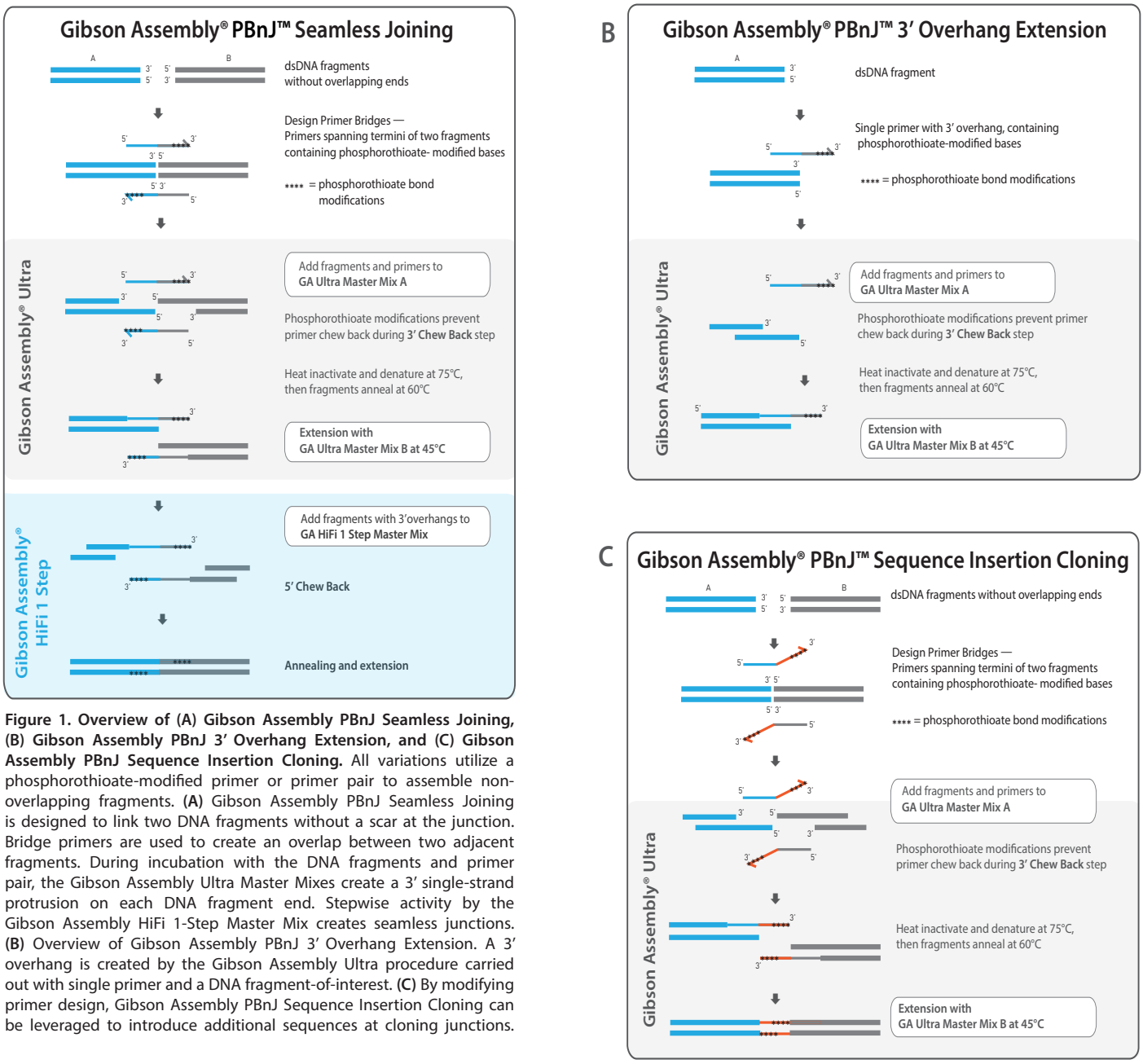
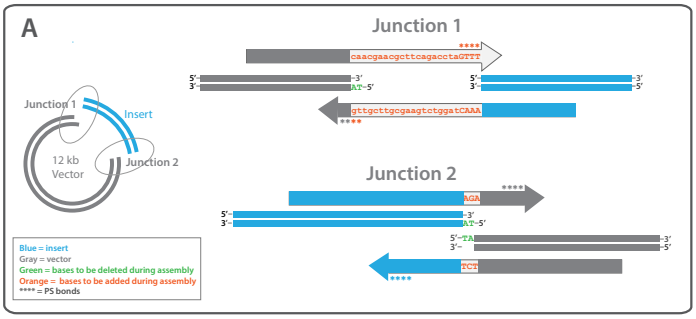


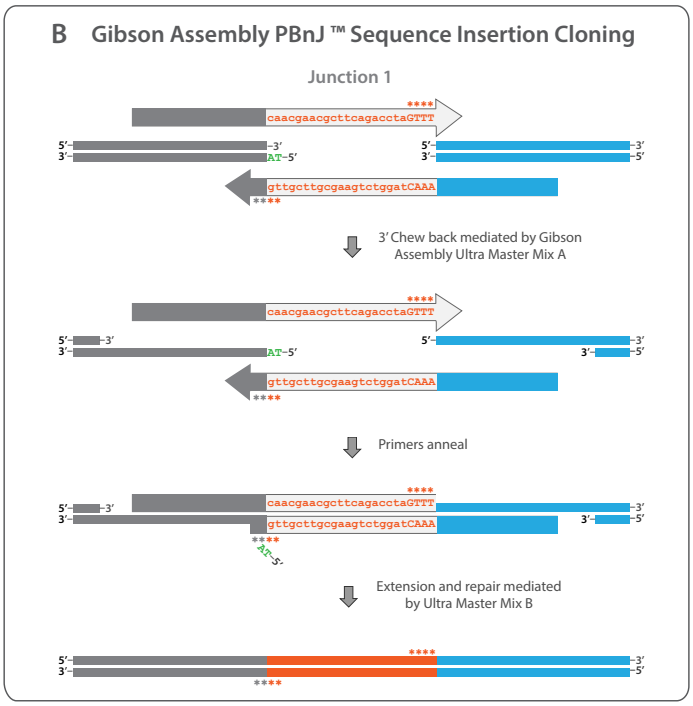
Figure 1. Overview of (A) Gibson Assembly PBnJ Seamless Joining, (B) Gibson Assembly PBnJ 3′ Overhang Extension, and (C) Gibson Assembly PBnJ Sequence Insertion Cloning. All variations utilize a phosphorothioate-modified primer or primer pair to assemble non-overlapping fragments. **(A)** Gibson Assembly PBnJ Seamless Joining is designed to link two DNA fragments without a scar at the junction. Bridge primers are used to create an overlap between two adjacent fragments. During incubation with the DNA fragments and primer pair, the Gibson Assembly Ultra Master Mixes create a 3′ single-strand protrusion on each DNA fragment end. Stepwise activity by the Gibson Assembly HiFi 1-Step Master Mix creates seamless junctions. **(B)** Overview of Gibson Assembly PBnJ 3′ Overhang Extension. A 3′ overhang is created by the Gibson Assembly Ultra procedure carried out with single primer and a DNA fragment-of-interest. **(C)** By modifying primer design, Gibson Assembly PBnJ Sequence Insertion Cloning can be leveraged to introduce additional sequences at cloning junctions.

Assembly at Junction 1 (Figure 2B), represents Gibson Assembly PBnJ Sequence Insertion cloning. A sense primer with homology to the vector and an antisense primer with homology to the insert were designed. Each primer contains a 36 nucleotide overlap and a 24 nucleotide insertion. During assembly, 24 bp sequences are added at Junction 1. At Junction 2 (Figure 2C), Gibson Assembly PBnJ Seamless Joining was used to assemble two non-overlapping fragments. The sense primer contained 44 nucleotides of homology with the insert, a 3-nucleotide insertion, and 13 nucleotides homologous to the vector. The antisense primer was designed with 38 nucleotides of homology with the vector, a 3-nucleotide insertion (depicted in orange), and 19 nucleotides homologous to the insert. These primers are complementary to one another over a span of 35 nucleotides at the 3′ ends.

Gibson Assembly PBnJ cloning was initiated by incubating the insert, vector, and primers with GA Ultra Master Mix A, which mediates 3′ exonuclease chew back. Primers are protected from chew-back by the presence of the phosphorothioate bonds. Following master mix inactivation, primers anneal to the exposed complementary single strand regions of the insert and vector during incubation at 60°C. The reaction is then incubated at 45°C with GA Ultra Master Mix B, which mediates 5′→3′ extension. At this stage, Junction 1 is expected to have assembled. To complete assembly of Junction 2, the reaction was column-purified and incubated with GA HiFi 1-Step Master Mix, which mediated 5′ exonuclease chew back, allowing for annealing of the complementary single-strand regions between the insert and vector. Extension and ligation of the annealed vector and insert is also mediated by GA HiFi 1-Step Master Mix.

The Gibson Assembly PBnJ cloning reaction was set up using 10 fmol of vector and insert and either 10 fmol or 50 fmol of primers. After completion of the Gibson Assembly PBnJ cloning reaction, 5 µL of each reaction was transformed into 50 µL of chemically competent *E. coli*. Ten µL of the 1 mL transformation (recovered in SOC) was plated onto LB plates containing an appropriate antibiotic. Approximately 200 colonies were obtained for each reaction, indicating that the use of excess primers was not necessary. Twelve colonies were screened by colony PCR and the junctions of 8 clones were sequenced.





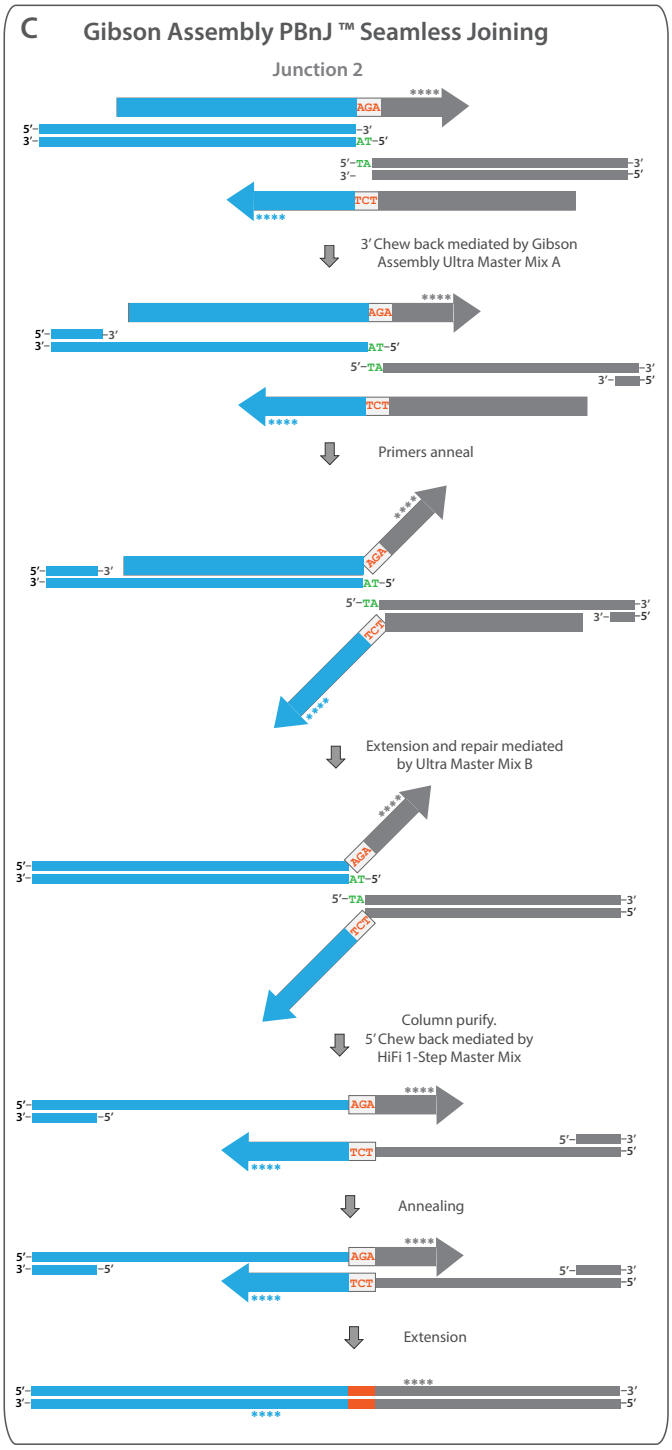


Figure 2. Schematic showing the Gibson Assembly PBnJ Cloning workflow. **(A)** Junctions of a single linearized insert and vector are shown. Blue denotes insert, gray denotes vector, green denotes bases that will be deleted during the assembly reaction, and orange indicates bases that are not present in either the insert or vector but will be added in the primers and subsequently in the final construct. Primers contain 4 phosphorothioate bonds at the 3′ ends and are denoted by ****. **(B)** Stepwise illustration of Gibson Assembly PBnJ Sequence Insertion cloning at Junction 1. In this design, an additional 24 nucleotides were introduced in Junction 1 with bridge primers. **(C)** Stepwise illustration of Gibson assembly PBnJ Seamless Joining at Junction 2. Here, the insert and vector both contain 5′ overhangs (shown in green) from linearization by restriction enzyme digestion. We designed a pair of 60-mer primers that contained homology with the insert, a 3-nucleotide mutation, and homology to the vector. Applying the Gibson Assembly PBnJ workflow to our designed primers, vector, and insert fragments at Junctions 1 and 2 resulted in a completely ligated, circular construct.

Results and Discussion

All twelve colonies were positive by colony PCR screening (data not shown) indicating that all instances tested yielded an assembled construct. Sequence analysis at both junctions demonstrated that 5 of the 8 (63%) clones analyzed were error-free. Three clones contained different single point mutations at Junction 1 and all 8 clones exhibited perfect sequence at Junction 2. These results point to the accuracy and efficiency of this method.

In this study, we demonstrate the effectiveness of Gibson Assembly PBnJ cloning for seamlessly joining fragments without relying on homologous overlap regions between adjacent fragments. Key features of these two methods are shown in Table 1.

Table 1. Key features of Gibson Assembly Ultra kit cloning and Gibson Assembly PBnJ Seamless Joining

Feature	Gibson Assembly Ultra kit	Gibson Assembly PBnJ Seamless Joining
Starting material	ds DNA fragments	ds DNA fragments
Homologous overlap regions	Necessary. Added by PCR or through synthesis (e.g. DNA Tiles™)	No
Primers	Utilized to prepare homologous overlapping ends if preparing fragments by PCR	Contain 3′ phosphorothioate bonds to prevent primer chew back
Methods utilized	Create homologous overlap regions → Gibson Assembly Ultra method	Gibson Assembly Ultra method → Gibson Assembly HiFi 1-Step method
End product	Fully assembled, seamless construct	Fully assembled, seamless construct
Applications	• Simultaneous assembly of up to 15 inserts • Proven, robust method for cloning single or multiple fragments from 100 bp to 100 kb	• Assemble large fragments with non-homologous ends • Assemble fragments when PCR is problematic • Edit (add or delete) sequences at junctions

Experimental Considerations

1. Gibson Assembly cloning efficiently joins DNA fragments at femtomolar concentrations. When using bridge primers in conjunction with the Gibson Assembly method, using the appropriate molar amounts of oligonucleotides is crucial. In general, the optimal concentration of bridge primers is 1- to 5-fold the molar concentration of fragments that will be bridged, not to exceed 10-fold. Excess of bridge primers (i.e. 100-fold) may result in inhibition of the assembly reaction and should be avoided. When bridge primers are used for more than one junction, a titration of primer pairs might be necessary to achieve optimal assembly efficiency.

2. For this study, we investigated Gibson Assembly PBnJ cloning solely with complementary pair of phosphorothioate-modified primers. Alternatively, standard primers can be used with Gibson Assembly PBnJ by adding the primers to the reaction after the GA Ultra Master Mix A 3′ chew-back and heat-inactivation steps.

3. A single "bridge" primer can be used for PBnJ Seamless Joining. However, primer pairs are recommended for more consistent result.

4. The Gibson Assembly PBnJ method can be used in assemblies where other junctions are compatible with the standard Gibson Assembly methods.

5. Additional studies could address using Gibson Assembly PBnJ cloning for multi-fragment assembly and could further define the parameters (i.e. how many fragments can be joined simultaneously, how much editing is possible, and upper and lower size ranges of fragments and final constructs) of this newly described method.

References

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