BBF RFC 57: Assembly of BioBricks by the Gibson Method

Bill Collins, Hannah Copley, Peter Emmrich, Will Handley, Anja Hohmann, Emily Knott, Paul Masset, Ben Reeve and Theo Sanderson

October 28, 2010

1 Purpose

This Request for Comments (RFC) describes the method for assembly of BioBricks by the Gibson Method, allowing for multiple Bricks to be joined simultaneously without introducing scars.

2 Relation to other BBF RFCs

Whilst BBF RFC 57 offers an alternative assembly method to those described in BBF RFCs 10, 21, 23, 25 and 37, it allows complete backwards compatibility and can be used alongside alternative methods.

3 Copyright Notice

4 Background

The Gibson Method was described by Gibson et al in 2009[1] as a way of assembling long DNA molecules in a single isothermal step. The process requires an overlap of 20 to 150bp of the two genes to be joined. To provide high robustness it is recommended to use an overlap of 40bp. This overlap can be achieved by designing PCR primers which anneal to 20bp on one side of the join and contain an additional 20bp of the other sequence, for a total primer length of around 40bp. Multiple overlapping segments may be assembled in the same step, provided the overlapping regions are distinct. While the Gibson method itself does not require the assembled fragments to be in BioBrick format, you SHOULD observe the rules given in RFC 10 concerning prefixes, suffixes and illegal sites to allow use with other assembly standards. The Gibson method is especially useful to achieve protein fusions, as it does not introduce any codons or scars, allowing seamless cloning of coding regions.

5 Mechanism of assembly

The diagram below depicts the four stages of assembly.

```
Left side of join                        Right side of join
5'-TCTGGAATTCGCGGCCGCTTCTAGAG-3'         5'-TACTAGTAGCGGCCGCTGCAGTCCGG-3'
3'-AGACCTTAAGCGCCGGCGAAGATCTC-5'         3'-ATGATCATCGCCGGCGACGTCAGGCC-5'

Extension...                    -->
5'-TCTGGAATTCGCGGCCGCTTCTAGAGTACTAGTAGCGGCCGC-3'
3'-AGACCTTAAGCGCCGGCGAAGATCTCATGATCATCGCCGGCG-5'
          5'-GCGGCCGCTTCTAGAGTACTAGTAGCGGCCGCTGCAGTCCGG-3'
          3'-CGCCGGCGAAGATCTCATGATCATCGCCGGCGACGTCAGGCC-5'
                         <--
Chewback...
5'-TCTGGAATTCGCGGCCGCTTCTAGAGTACTAGTAGCGGCCGC-3'
3'-AGA-5'<--
                                                 -->5'-CGG-3'
          3'-CGCCGGCGAAGATCTCATGATCATCGCCGGCGACGTCAGGCC-5'

Annealing...
5'-TCTGGAATTCGCGGCCGCTTCTAGAGTACTAGTAGCGGCCGC-3' 5'-CGG-3'
3'-AGA-5' 3'-CGCCGGCGAAGATCTCATGATCATCGCCGGCGACGTCAGGCC-5'

Double-strand repair...                      -->
5'-TCTGGAATTCGCGGCCGCTTCTAGAGTACTAGTAGCGGCCGCTGCAGTCCGG-3'
3'-AGACCTTAAGCGCCGGCGAAGATCTCATGATCATCGCCGGCGACGTCAGGCC-5'
         <--

Ligation...                                     ><
5'-TCTGGAATTCGCGGCCGCTTCTAGAGTACTAGTAGCGGCCGCTGCAGTCCGG-3'
3'-AGACCTTAAGCGCCGGCGAAGATCTCATGATCATCGCCGGCGACGTCAGGCC-5'
     ><
```

6 Method

6.1 PCR Extension

Gibson's method is a way of joining pieces of DNA with a pre-existing overlap. Since typically this overlap does not exist in BioBricks it is necessary to add it. This can be achieved by PCR.

```
Left side of join                        Right side of join
5'-TCTGGAATTCGCGGCCGCTTCTAGAG-3'         5'-TACTAGTAGCGGCCGCTGCAGTCCGG-3'
3'-AGACCTTAAGCGCCGGCGAAGATCTC-5'         3'-ATGATCATCGCCGGCGACGTCAGGCC-5'

5'-TCTGGAATTCGCGGCCGCTTCTAGAG-3'
         < <  3'-GGCGAAGATCTCTACTAGTAGCGGCC-5'

              5'-CCGCTTCTAGAGATGATCATCGCCGG-3' > >
                        3'-ATGATCATCGCCGGCGACGTCAGGCC-5'
```

The authors have developed a tool to simplify the process of designing these primers, accessible at `www.gibthon.org`[2]. The PCR reaction should be run as normal and followed by gel electrophoresis and gel extraction in order to separate the amplified fragments from the template DNA.

Technique for long fragments: Due to the limited processivity of polymerases, you may struggle to reliably produce fragments longer than 2kb. However this can be overcome by designing primers which split these longer fragments into two or more sections with 40bp overlaps. Since no 'flap' is needed, these can be standard 20bp primers.

6.2 Gibson reaction

6.2.1 Gibson Master Mix

A 1.33x Gibson Master Mix should be made up, containing:

	Volume /μl
Volume/l Taq ligase (40u/μl)	50
5x isothermal buffer	100
T5 exonuclease (1u/μl)	2
Phusion polymerase (2u/μl)	6.25
Nuclease-free water	216.75
	375

The Master Mix can be stored at $-20\,^{\circ}\mathrm{C}$ for one year.

6.2.2 5x isothermal buffer

If you do not have access to 5x isothermal buffer, it can be made as follows:

	Volume /μl
25% PEG-8000	0.75g
500 mM Tris-HCl pH 7.5	1500
50mM MgCl_2	75
50mM DTT	150
1mM dATP	30
1mM dTTP	30
1mM dCTP	30
1mM dGTP	30
5mM NAD	300
Nuclease-free water	*remainder*
	3000

6.2.3 Reaction conditions

DNA and Gibson Master Mix should be combined with a volumetric ratio of 1:3 in a PCR tube. The total volume can be from $20 - 50\mu l$. The PCR tube should then be incubated for 1 hour at $50\,^{\circ}\mathrm{C}$.

7 Guidelines specifically for assembly of BioBricks

The previous sections relate to Gibson Assembly as a general means of DNA Assembly. However there are certain issues which must be considered when creating parts for submission to the registry. BBF RFC 10 describes the BioBrick format, all parts created by Gibson Assembly must comply with these specifications.

7.1 Gibson Assembly to place arbitrary sequence of DNA in BioBrick format in a registry plasmid

BBF RFC describes the use of PCR followed by restriction enzymes to place a naturally occuring DNA sequence in Biobrick format. This can also be achieved by Gibson Assembly. PCR extension is used to amplify the plasmid backbone, adding 20bp of homology to the target sequence before the suffix and after the prefix.

```
5’                                        3’
last 20bp of BioBrick---BioBrick Suffix
------------------tactagtagcggccgctgcag
reverse complement of first 20bp of BioBrick----Reverse complement
of appropriate BioBrick prefix
```

7.2 Gibson Assembly to switch backbones using standard primers

This method can be used to put any part which is flanked by a prefix and suffix into any of the standard plasmid backbone detailed below. The primers required to do this are standard, not specific to any particular length of DNA, but simply to anything which is flanked by a prefix and suffix.

Since the main barrier to efficiency in Gibson Assembly is that of ordering primers, simply having the four standard primers detailed below will allow you to quickly and easily switch parts between plasmids.

7.2.1 Compatibility

This method can be used to switch a part in any backbone (or any length of DNA flanked by a prefix and suffix) into one of the following standard plasmids:

- pSB1C3
- pSB1A3
- pSB1AC3
- pSB1AK3
- pSB1AT3
- pSB1C3
- pSB1K3
- pSB1T3
- pSB2K3

More generally, the part being moved can be placed into any Plasmid which has the forward strand beginning and ending with:

```
ttcgctaaggatgatttctggaattcgcggccgcttctaga...

...actagtagcggccgctgcagtccggcaaaaaaacgggcaa
```

7.2.2 Mechanism

There are two pairs of primers involved here, the first pair[1] is designed to amplify the backbone which you want to transplant your part to, and anneals to the suffix (at the beginning of the plasmid) and the prefix (at the end of the plasmid). These will anneal to anything which has the desired backbone and generate a large amount amount of linearised plasmid (for example you could have a recognisable part such as one containing RFP as your template to generate the linear plasmid)

[1] extnd.prt.f ; extnd.prt.r

The second pair[2] extends the part plus prefix and suffix enough so that the plasmid and part now have 40bp of overlap at either end, allowing it to be ligated with the linear plasmid backbone generated above. The final generated part is thus circular. The sequences of the primers are:

extnd.prt.f `ttcgctaaggatgatttctgGAATTCGCGGCCGCTTCTAGA`

extnd.prt.r `ttgcccgtttttttgccggaCTGCAGCGGCCGCTACTAGT`

amplBkBn.f `TACTAGTAGCGGCCGCTGCA`

amplBkBn.r `CTCTAGAAGCGGCCGCGAAT`

and can be ordered from any oligo manufacturer. All T_ms are at 60 °C. Capitals denote the annealing portion of the primer

8 Authors' Contact Information

Bill Collins: `wac26@cam.ac.uk`
Hannah Copley: `hcc39@cam.ac.uk`
Peter Emmrich: `pmfe2@cam.ac.uk`
Will Handley: `wh260@cam.ac.uk`
Anja Hohmann: `afh32@cam.ac.uk`
Emily Knott: `ekk27@cam.ac.uk`
Paul Masset: `pm398@cam.ac.uk`
Ben Reeve: `bdr22@cam.ac.uk`
Theo Sanderson: `trs38@cam.ac.uk`

References

[1] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, and H. O. Smith. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods*, 6:343–345, May 2009.

[2] B. A. Collins. Gibthon Construct Designer, October 2010. Available from: `http://www.gibthon.org`.

[2] ampl.BkBn.f ; ampl.BkBn.r