BBF RFC 98: GenBrick – A Rapid Multi-Part Assembly Method for BioBricks

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1. Purpose

This BioBricks Foundation Request for Comments (BBF RFC) describes a novel approach, GenBrick, for the rapid assembly of multiple BioBrick RFC10-compatible parts in a single reaction, with completely flexible part order, without recloning or reamplification.

2. Relation to other BBF RFCs

This RFC is not related to any other Request for Comments document.

3. Copyright Notice

4. Acknowledgments:

GenBrick is based on the proprietary Genabler assembly system (www.genabler.com). For commercial uses, please contact Genabler.

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5. Table of Contents

6. Introduction

iGEM is based around the idea of using standard parts from a library to generate new engineered biological systems. Currently iGEM mandates use of the BioBrick RFC10 standard for parts. RFC10 BioBricks are completely composable - that is, any set of parts from the library can be assembled together in any order. However, BioBricks have to be joined together pairwise, two at a time, usually with cloning and sequencing in between, so assembling large constructs can be quite slow.

Many iGEM teams have now turned to using alternative assembly methods such as Gibson assembly (isothermal assembly). Gibson assembly can assemble up to ten parts in a single reaction with high accuracy and efficiency, so saves a great deal of time. However, the order in which the parts assemble together is dictated by overlapping sequences at the ends, so generally parts have to be re-amplified by PCR to add new homology ends for each new assembly. This is opposed to the spirit of flexible composition of BioBricks.

We have attempted to address this problem by developing GenBrick, a new method for rapid assembly of multiple BioBrick RFC10-compatible parts in a single reaction, with completely flexible part order, without recloning or reamplification as required by other multi-part assembly schemes.

7. GenBrick

GenBrick is based on the proprietary Genabler technology developed for Scottish Enterprise by Ginkgo Bioworks. GenBrick parts are fully BioBrick compatible and can be assembled by normal BioBrick assembly, but they can also be assembled using Genabler assembly, which allows rapid 'single pot' assembly of up to ten parts in a single reaction. Like Golden Gate assembly and its derivatives, Genabler assembly relies on the use of Type IIS restriction enzymes (in this case, EarI), which cut outside of their own recognition sequence to generate user-defined sticky ends. However, in the case of Genabler assembly, these ends are not ligated together directly, but are used to attach linkers with single stranded regions (www.genabler.com)

For GenBrick, we wanted to ensure that all parts were RFC10 compatible. We also wanted to facilitate the use of different ribosome binding sites, N and C terminal tags, generation of fusion proteins, etc. We have therefore modified the original Genabler concept to consider two different types of component - 'Bricks' and 'Linkers'. Each assembly consists of alternating bricks and linkers (Figure 1).

Figure 1. Example of GenBrick Assembly

Legend: Genes (bricks) are assembled together with GenBrick. Each brick is flanked by an ‘Eye’ and a ‘hook’ linker.

All components more than 80 or so base pairs in length are made as Bricks; shorter components are made as Linkers. A typical Brick might be either part of a coding sequence, or a promoter sequence, origin of replication, etc. For the kind of assemblies we are interested in, the most important Bricks are Coding Bricks, which each encode a protein domain.

7.1 Coding Bricks and Donor Vectors

Coding Bricks follow these rules:
1. Coding Bricks do not include a start or stop codon. This facilitates the addition of N- or C-terminal tags, and generation of fusion proteins.
2. Coding Bricks are cloned in a Donor Vector flanked by two inward-facing EarI sites. These generate the 5'-overhangs TCG at the upstream end, and GGC (corresponding to GCC on the coding strand) at the downstream end.

```
NNNCTCTTCN     TCGNNN(Brick)NNN      GCCNGAAGAGNNN
NNNGAGAAGNNNN     NNN(Brick)NNNCGG      NCTTCTCNNN
```

3. In order that GenBricks can also be assembled with BioBricks by normal BioBrick assembly, we have defined the following standard prefix and suffix:

Prefix: GAATTCGCGGCCGCTTCTAG **ATG** GCC TCT TCT TCG
Suffix: CTGCAGCGGCCGCTACTAGTAC TCT **TCA** GGC

It will be seen that these are simply the standard RFC10 prefix and suffix for a coding sequence, with the addition of short sequence including the EarI sites (underlined) plus a start and stop codon (bold). When assembled by normal BioBrick assembly, these will result in the generation of a protein with a short N-terminal extension MASSS- and short C-terminal extension A*. When Genabler assembly is used, the EarI sites will be cleaved to generate the overhangs required. We have prepared donor vectors based on pSB1A2 with RFP (BBa_J04450) or *lacZ'*α (BBa_J33207) cassettes between such EarI sites, so that new GenBricks can be made using primers with only EarI sites, and inserted into the donor vectors using these. Note that our preliminary experiments suggest that EarI may require a substantial amount of DNA outside the EarI sites (perhaps 10 bases or more; we are still clarifying the exact amount) for good cleavage.

4. Obviously internal EarI sites must also be removed, as well as EcoRI, XbaI, SpeI and PstI sites if the part is to be deposited in the Registry. To facilitate this, we have also developed a single-step multi-site mutagenesis method which can remove multiple EarI (or other) restriction sites in a single reaction immediately prior to cloning. This procedure is called EMMA (EarI Multisite Mutagenic Assembly) and complements our previous procedure, MABEL (MutAgenesis with Blunt-Ended Ligation). This protocol will be submitted as a separate RFC.

7.2 Linkers

Linkers come in between the Bricks in an assembly. Typical linkers are around 45 base pairs in length, but they can be up to 80 base pairs or so. Each linker consist of 4 oligonucleotides. Following the Genabler terminology, we call these Eye Long, Eye Short, Hook Long, and Hook Short (EL, ES, HL and HS). The complete Linker looks like this:

```
GCCNNN(Hook, Long)NNNNNNNNNNNNN          NN(Eye, Short)NN
   NNN(Hook, short)NNNNN          NNNNNNNNNN(Eye, Long)NNNAGC
```

The long single stranded regions are complementary. The double-stranded regions should have be at least ten bases in length, and the overlap region in the centre should be at around 15 to 18 bases in length with a melting temperature around 40˚C.

Linkers which go between two Coding Bricks can be of different types, eg:

- Stop codon plus RBS plus start codon
- C-terminal tag plus stop codon plus RBS plus start codon

- Stop codon plus RBS plus start codon plus N-terminal tag
- Flexible protein linker to join two domains in a fusion protein
- other - your imagination is the limit!

7.3 Acceptor vectors

The final component needed for assembly is an Acceptor Vector. This is essentially a specialized type of Brick with its own dedicated Linker. Since we want to submit our assemblies to the Registry, we have made acceptor vectors based on pSB1C3, with either an RFP (BBa_J04450) or *lacZ'*α (BBa_J33207) cassette for red-white or blue-white selection (Figure 2), flanked by EarI sites directed outward to generate the necessary three base overhangs. When the Acceptor Vector and its special Linker are used in an assembly, the assembled pieces will form a compliant RFC10 BioBrick in pSB1C3 ready for submission to the Registry.

If a different final vector is required, any BioBrick vector may be converted to a GenBrick Acceptor Vector by inserting the *lacZ'*α or RFP cassette from the existing acceptor vectors, and mutating any internal EarI sites in the plasmid. The only requirement is that the Acceptor Vector must contain at least one antibiotic resistance determinant or other selectable marker which is not present in any of the Donor Vectors used.

Figure 2: Acceptor Vectors (Gasiūnaitė, 2013)

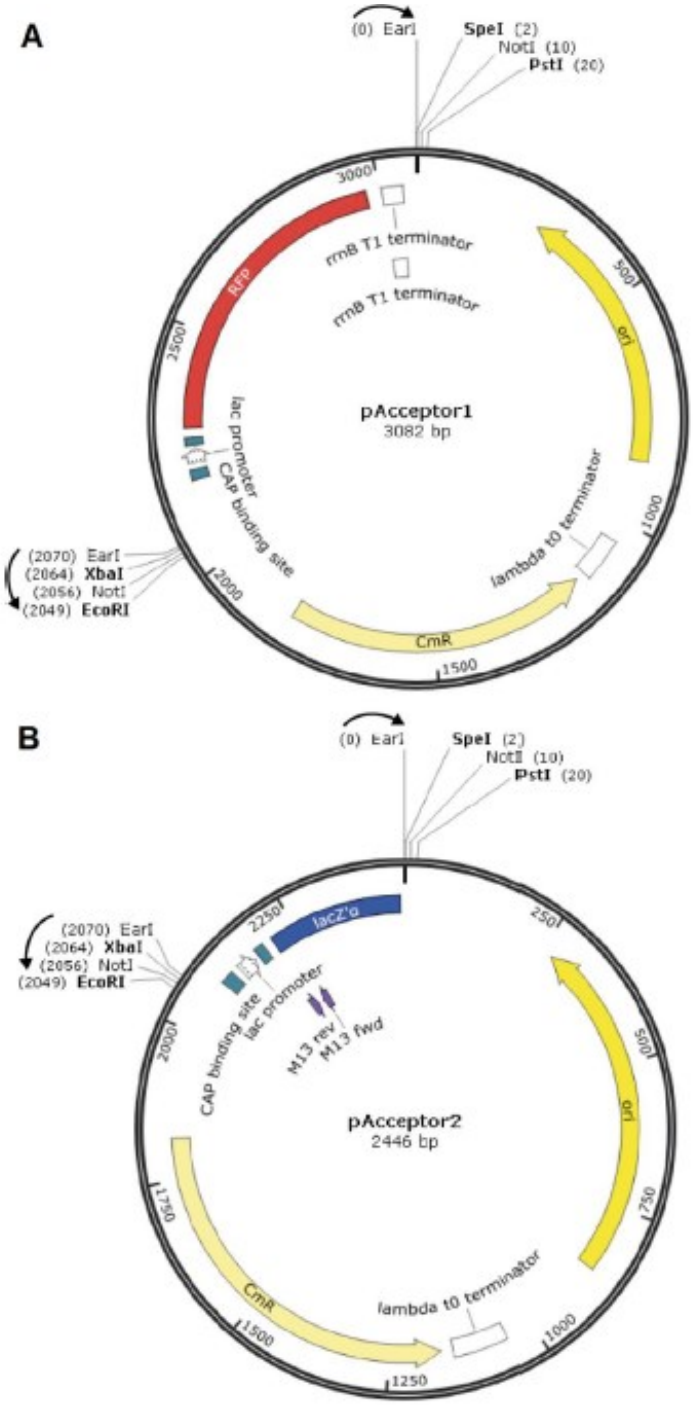


Figure 2: acceptor vector A is an RFP (BBa_J04450) and acceptor vector B is a *lacZ'*α (BBa_J33207) cassette for red-white or blue-white selection.

7.4 Assembly

Once all your Bricks are ready in the Donor Vector (based on pSB1A2), and the Linker oligonucleotides are synthesised, and the Acceptor Vector is ready, assembly can be performed. There are two stages - pre-assembly and final assembly. For example, to assemble a cassette:

[Vector]-Linker1-BrickA-Linker2-BrickB-Linker3-BrickC-vector linker

The following pre-assemblies are required:

V: vector linker eye + vector + linker1 hook
A: linker1 eye + BrickA + linker2 hook
B: linker2 eye + BrickB + linker3 hook
C: linker3 eye + BrickC + vector linker hook

The pre-assemblies each consist of one eye, one Brick and one hook. To make a pre-assembly, just place the Brick (in its Donor Vector) plus the four oligonucleotides (two for the eye, two for the hook) in a tube with some EarI and T4 DNA ligase, and cycle the temperature between 16˚C and 37˚C using a thermal cycler (see Appendix 1 for detailed protocol). The resulting products must then be purified to remove unligated oligonucleotides. Agarose gel electrophoresis and purification of the band from the gel gives best results, but for routine assemblies, simple purification from the reaction using a PCR purification kit should be sufficient. Note that only unreacted oligonucleotides need be removed; unligated insert or vector DNA should not affect the final assembly step.

For final assembly, the four pre-assemblies are simply mixed together in a tube, then used to transform *E. coli*. Ligation is not necessary and may even reduce efficiency. If the standard acceptor vectors based on pSB1C3 are used, the transformants are plated on L-agar with chloramphenicol and IPTG (and Xgal if the *lacZ'*α vector is used). Religated vector should be blue or red, correct assemblies should give white colonies (unless chromogenic markers are also present in the assembly). If the Acceptor Vector preassembly is gel-purified, there should be very little background, and almost all colonies should represent the correct assembly. Colonies can then be tested, for example by plasmid DNA miniprep and restriction analysis, or colony PCR using the vector eye long and linker1 hook long oligonucleotides as primers to amplify the entire assembly, if its size permits this.

7.5 Example: Furbox GenBrick Assembly

Using the protocol found in the appendix, a 3-part construct was assembled with an acceptor vector pSB1C3 (BBa_K1122009), a Lac promoter and truncated gene called PLac_LacZ (BBa_J33207) optimised for GenBrick and a fluorescent protein, GFP (BBa_K1122004) (Figure 3). The aim was to test an inverted repeat sequence known as the fur box. When internal concentrations of iron are high within a cell, the Fur protein binds to the fur box and represses the gene upstream from it. The consensus sequence for the fur box is 'TGATAATAATTATCA'. The eye and hook linkers between PLac_LacZ and GFP were engineered to have a fur box, an RBS and a start codon.

Spacer with STOP-Fur box-RBS-START

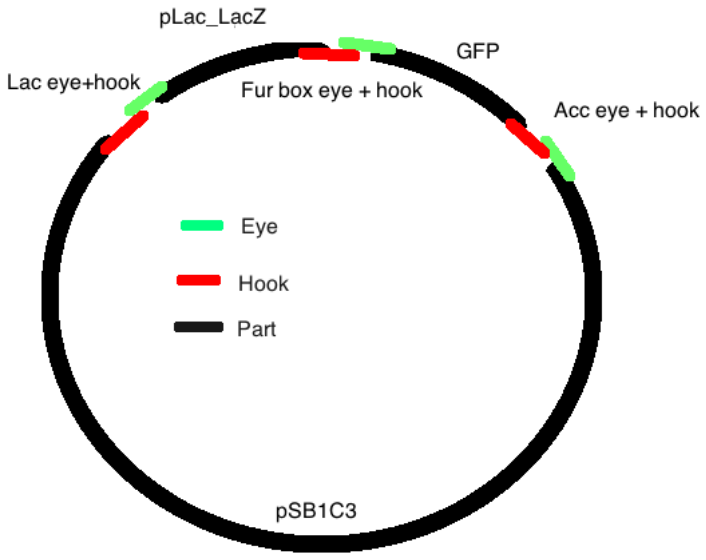
STOP Fur box RBS START

5'GCCCCAAGTTAATCATGATAATAATTATCAAGGAGGTGGAGCGGTATGGGTAGT...3'

Hook Long 6: 5'GCCCCAAGTTAATCATGATAATAATTATCAAGGA3'
Hook Short 6: 5'ATCATGATTAACTTGG3'
Eye Long 6: 5'CGAACTACCCATACCGCTCCACCTCCTTGATAATTATT3'
Eye Short 6: 5'GGTGGAGCGGTATGGGTAGT3'

The other linkers that were used can be found in the database of:
http://2013.igem.org/Team:Edinburgh/GenBrick/Linker_Designer

Figure 3. Fur box Assembly



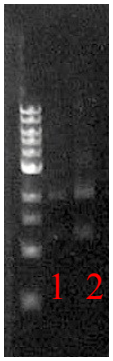
Legend: Parts with their linkers. The acceptor vector is pSB1C3 and has a chloramphenicol resistance and an RFP reporter. 'Acc eye + hook' stands for acceptor vector eyes and hook. The eye and hook linker between PLac_LacZ and GFP was engineered to have a fur box.

The assembly was used as part of the WastED project of EdiGEM 2013. Two transformations were made and plated on IPTG + chloramphenicol plates and left overnight. In one plate, 4 out of 9 colonies were fluorescent; the other five had religated and were red. The other plate had a success rate of 8 out of 20 (the competent cells were concentrated x10 before plating). Figure 4 shows the assembly fluorescing under UV when no iron was present and figure 5 shows the gel of restriction-digestion gel using PstI and XbaI.

Figure 4. Fur box Assembly

Legend: after transforming competent cells with the assembly and leaving overnight on an IPTG + chloramphenicol plate, 2 green fluorescent colonies were restreaked. A gel was performed to show the parts had correctly assembled.

Figure 5. Fur Box Digestion



Legend: On the left is the ladder, the first column is the undigested assembly which shows only one band (the second lower band is an artifact due to leakage). The second column contains the digested assembly. The size of PLac_LacZ + linkers + GFP was correct.

8. Author's Contact Information

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9. References

Gasiūnaitė, Lina (2013) The development of commercial kit of standard biological parts utilizing the Genabler assembly technology. *The University of Edinburgh, School of Biological Sciences*.

10. Appendix – GenBrick Assembly

Design and Preparation - Linker and Segment

1. Overview

- Utilise the linker designer software from the EdiGEM 2013 wiki to design Eye and Hook linker oligo pairs
- Linker and Segment oligos can be custom-made as single-stranded, unphosphorylated DNA
- Forward and reverse oligo pairs are mixed and phosphorylated prior to annealing

2. Preparation

- Re-suspend oligo in nuclease-free water to 100 µM (as per instructions)
- Phosphorylation reaction (not required if oligos with 5' phosphate are ordered)

component	volume (µL)
10X T4 Polynucleotide Kinase buffer	5
T4 Polynucleotide Kinase (NEB M0201)	0.5
10 mM ATP	5
Forward oligo	20
Reverse oligo	20
37°C/30 min	
5 M NaCl	0.5
95°C to RT	

- Annealing is achieved by addition of 5 µL 5 M NaCl (50 mM [final]) prior to heat denaturation at ≤ 95°C and slow cooling to Room temperature.

Pre-assembly

1. Eye-Part-Hook Preparation

component	volume (µL)
50 nM Part plasmid DNA	10
500 nM eye oligo pair	10
500 nM hook oligo pair	10
10 mM ATP	5
10X NEBuffer 4	5
EarI (NEB R0528)	1
T4 DNA Ligase (NEB M0202)	1
Sterile water	8

a) Using 3-part pathway as example:

- Acceptor Vector cassette (+ promoter); Acc_RFP
- LacZ truncated gene (+ PLac); PLac_LacZ
- Green fluorescent protein; GFP

b) Digestion-Ligation reaction of Eye-Part-Hook (E-P-H) (Overnight)

- Acc_RFP-E + Acc_RFP-P + GFP-H
- PLac_LacZ-E + PLac_LacZ-P + Acc_RFP-H
- GFP-E + GFP-P + PLac_LacZ-H

step	1	2	3	4	5	6	7	8	9	10	11	12	13
reaction	digestion	ligation	digestion	ligation	digestion	ligation	digestion	ligation	digestion	ligation	digestion	ligation	hold
temp	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	37°C	16°C	4°C
time	90 min	30 min	30 min	15 min	15 min	15 min	15 min	10 min	15 min	10 min	60 min	30 min	

c) Purification of Digestion-Ligation E-P-H product to remove non-ligated DNA/plasmid

- Run 50 µL E-P-H Digestion-Ligation reaction on 1% agarose gel
- Or: QIAquick PCR Purification kit can be used if Part plasmid does not carry resistance/marker

Assembly

1. Incubation

- Use 5 µL each E-P-H required for assembly
- Add x µl 10X NEBuffer 4 to make 1X final concentration
- Incubate 30-60 minutes at Room Temperature
- 3-part Example:
  - 5 µL Acc_RFP E-P-H ([Acc_RFP -E]+[Acc_RFP -P]+[GFP-H])
  - 5 µL PLac_LacZ E-P-H ([PLac_LacZ-E]+[PLac_LacZ-P]+[Acc_RFP-H)
  - 5 µL GFP E-P-H ([GFP-E]+[GFP-P]+[PLac_LacZ-H])
  - 2 µl NEBuffer 4
  - 3 µL sterile water
- If larger number of E-P-H in assembly, adjust 10X NEBuffer 4 and water accordingly

2. Transformation

- Use 10 µL assembly mix to transform 50 µL NEB 10-beta competent cells C3019H
- Culture above assembly example on LB agar plates with chloramphenicol and IPTG
- Incubate overnight at 37°C (further growth at RT if colonies require)