

BrickClip – rapid assembly of multiple RFC10 BioBricks

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Purpose

This BioBrick Foundation Request for Comments (BBF RFC 104) describes a new approach to multiple part DNA assembly – BrickClip, which does not require use of any restriction enzymes, nor cloning of the parts to specific donor and acceptor vectors. BrickClip allows assembly of up to six parts from existing parts collections, including the Registry of Standard Biological Parts, in a single reaction, in any desired order. The resulting product is exactly the same as would be obtained from normal RFC10 BioBrick assembly. In contrast to other commonly used methods such as Gibson assembly, BrickClip does not require ordering new oligonucleotides for each assembly; while assembly oligonucleotides are required, these are part-specific and may be re-used in any assembly involving a given part. BrickClip is a special case of a more general assembly method – PaperClip¹.

Relation to other BBF RFCs

This Request for Comments allows assembly of multiple BioBricks in one reaction re-creating the original Tom Knight’s standard BBF RFC10.

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Introduction

Assembly of DNA parts into larger systems is an essential procedure in synthetic biology², and many methods are available. For example, the iGEM competition is (at the time of writing) based around the BBF RFC10 'BioBrick' standard proposed by T. Knight, and a large number of RFC10 BioBricks is available through the Registry of Standard Biological Parts. Assembly of BioBricks from the Registry using the RFC10 procedure is completely flexible; that is, any parts can be assembled in any order. This concept has revolutionized synthetic biology. However, RFC10 assembly can be relatively slow, as it is a hierarchical procedure requiring BioBricks to be assembled pairwise, usually with cloning and sequence verification required at each step. For this reason, other procedures such as Gibson assembly³ have become widely used. This procedure allows rapid multiple part assembly in a single reaction taking one hour, with the order of the parts being determined by homology regions at the end of each part. The drawback to this and similar end-homology-based procedures (LIC⁴, SLIC⁵, SLICE⁶, USER⁷, CPEC⁸) is that parts generally need to have end-homology added before assembly, usually by PCR, requiring specific single-use oligonucleotide primers to be acquired for each part for each assembly.

An ideal assembly procedure would allow rapid assembly of multiple parts in a single reaction, while maintaining the flexibility of BioBricks - that is, allowing any parts to be assembled in any order without the necessity for new oligonucleotides or other components to be ordered for each assembly. Ideally it should also allow use of pre-existing parts libraries without the necessity for re-cloning into specific donor vectors, or ensuring the absence of specific restriction sites. We have developed a general procedure fulfilling these requirements: PaperClip assembly¹. In this RFC, we describe a modified form of PaperClip assembly, BrickClip, which has been specially designed so that multiple RFC10 BioBricks from the Registry of Standard Biological Parts or any new Parts can be assembled in a single reaction, giving the same product that would be obtained from standard pairwise RFC10 assembly.

Components required for BrickClip assembly

BrickClip is an example of an oligonucleotide-directed assembly procedure, in which multiple Parts are assembled in a single reaction. The order of Parts is directed by oligonucleotides, which bridge the sequence of adjacent Parts. In contrast to other such procedures, BrickClip does not require new bridging oligonucleotides to be obtained for each assembly. Rather, the bridging double-stranded oligonucleotides ('Clips') are formed by pre-assembly ligation of shorter double-stranded 'half-Clips' corresponding to the ends of each Part. Thus, each component of a BrickClip library consists of (Figure 1, a):

- the Part itself, which may be a linear PCR product, or may be cloned in any vector. For illustrative purposes, in this RFC, we will assume that all Parts are RFC10 BioBricks cloned in pSB1C3. For maximum efficiency of assembly, all Parts should be linearized, by cutting with any restriction enzyme that cuts

outside the Part (e.g., EcoRI, XhoI, SpeI or PstI, in the case of RFC10 BioBricks), though uncut plasmid DNA can also be used with lower efficiency.

- four oligonucleotides, each around 40 bases in length: upstream forward (UF), upstream reverse (UR), downstream forward (DF), and downstream reverse (DR). These oligonucleotides may be re-used in any assembly involving the Part. The oligonucleotides are prepared for use by 5'-phosphorylation (using T4 polynucleotide kinase) and then annealing in pairs to form the Upstream half-Clip, consisting of oligonucleotides UF and UR, and the Downstream half-Clip, consisting of oligonucleotides DF and DR. The Upstream half-Clip has a 5'-overhang CTA at the outer (upstream) end and a part-specific three base 3' overhang at the inner (downstream) end (Figure 1, a, A). The Downstream half-Clip has 5' overhang TAG at the outer (downstream) end and a part-specific three base 5' overhang, which can be anything other than CTA or TAG, at the inner (upstream) end (Figure 1, a, B). Once prepared and annealed, the half-Clips may be stored with the Part and can be used in any assembly involving that Part.

Thus the overall composition of the four oligonucleotides, in relation to the Part, is as follows:

UPSTREAM HALF-CLIP (Figure 1, a, A and E)

```
UF 5' -CTAgagnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-3'
UR   3' -ctcnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-5'
```

DOWNSTREAM HALF-CLIP (Figure 1, a, B and F)

```
DF 5' -xyznnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnta-3'
DR   3' -nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnatGAT-5'
```

Note that the bases, shown as 'xyz' must not be CTA or TAG. The three-base overhangs at the inner ends are present only to prevent these ends from ligating.

The sequence of the Clips for the vector must include Prefix and Suffix:

VECTOR UPSTREAM HALF-CLIP includes Suffix (Figure 1, a, C)

```
UF 5' -CTAgtagcggccgctgcagnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-3'
UR   3' -catcgccggcgacgtcnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-5'
```

VECTOR DOWNSTREAM HALF-CLIP includes Prefix (Figure 1, a, D)

```
DF 5' -xyznnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnngaattcgcgccgctt-3'
DR   3' -nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnncttaagcgccggcgaaGAT-5'
```

The bases, shown as 'xyz' must not be CTA or TAG.

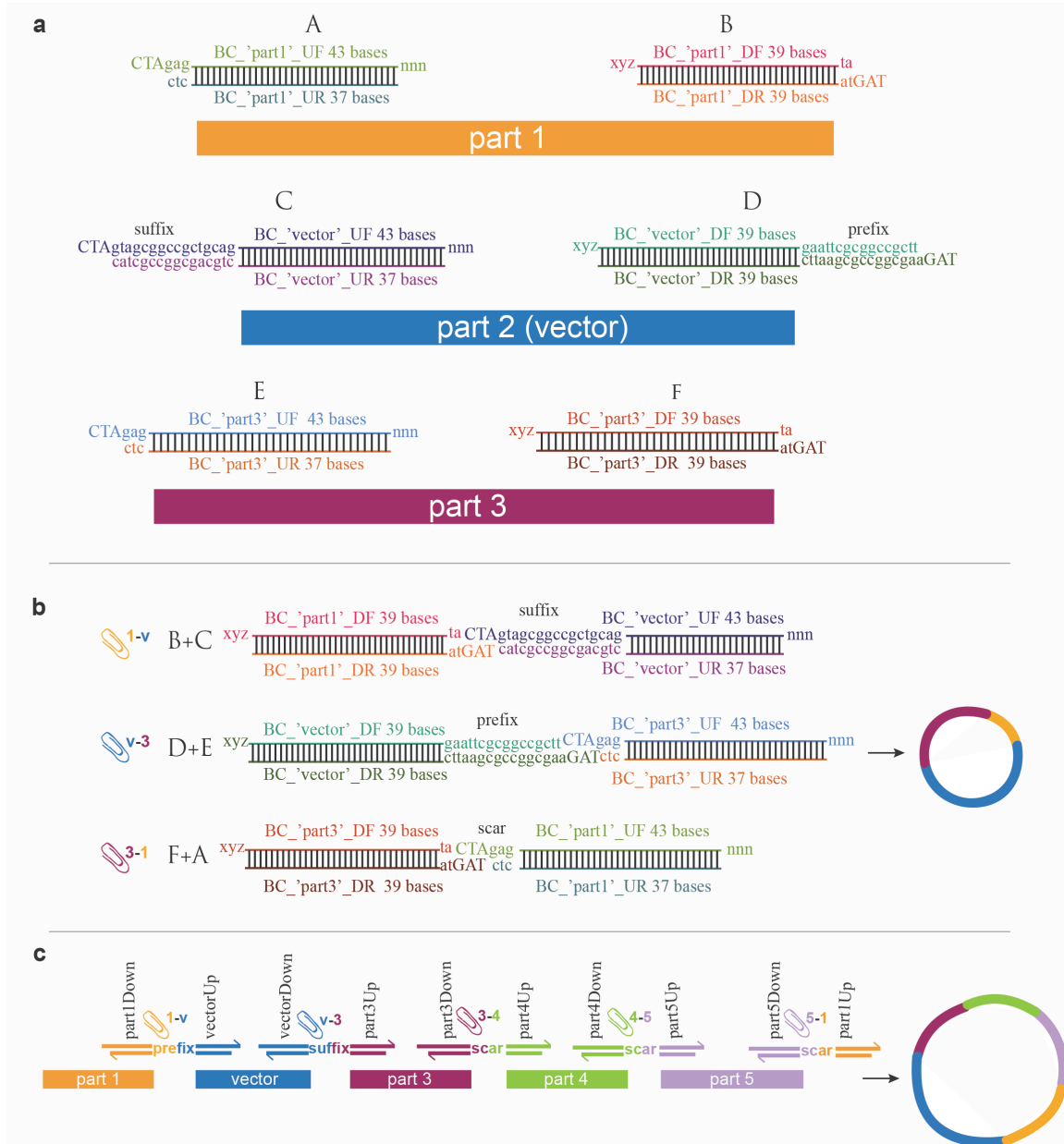


Figure 1. Illustrative scheme for Parts library (a), for Clips preparation for three-part assembly (b) and example of five-part assembly (c).

Outline of assembly procedure

For example, suppose that three parts: 1, 2 (vector) and 3, are to be assembled to make a circular construct 1-2-3 (Figure 1, b). The procedure is as follows:

- 1 – perform the following ligations (one hour):
 - Part 1-Downstream half-Clip (B) + Part 2-Upstream half-Clip (C)
 - Part 2-Downstream half-Clip (D) + Part 3-Upstream half-Clip (E)

- Part 3-Downstream half-Clip (F) + Part 1-Upstream half-Clip (A)

2 – mix the three ligations (no purification required) with the three (preferably linear) Parts to be assembled, and perform a standard PCR reaction (requires approximately one hour depending on the size of the final construct).

3 – transform competent cells with the PCR product (no purification required).

On the other hand, if the parts were to be assembled in the order 1-3-2, it would simply be necessary to do different ligations, as follows:

- Part 1-Downstream half-Clip (B) + Part 3-Upstream half-Clip (E)
- Part 3-Downstream half-Clip (F) + Part 2-Upstream half-Clip (C)
- Part 2-Downstream half-Clip (D) + Part 1-Upstream half-Clip (A)

Using parts of Parts

It should be noted that the actual sequence of the BioBrick Part, which is incorporated into the assembly is determined by the Clip sequences used. Thus, if you have a BioBrick Part from the Registry but do not want to include the entire Part in your assembly, you can just design your Clip oligonucleotides to include the region you want, and exclude unwanted regions at one or both ends. This is particularly useful for constructing fusion proteins, or to add N- or C-terminal tags to proteins, as it is possible to use a coding sequence Part from the Registry, but design the Clip oligonucleotides to leave out the start and/or stop codon. Clips can also add a few extra bases at the start or end of the part simply by including these at the outer ends of the Clip oligonucleotides. To add larger elements, see 'Using Small Parts' below.

Using 'Small Parts'

It should be noted that this procedure will only be effective for parts around 60 bases in length. Smaller parts, such as ribosome binding sites, can conveniently be added in the form of oligonucleotides included in the Clip ligation reactions (as described above). Small Part should be prepared in the form of four oligonucleotides as shown below:

```
UF 5' -CTAgagnnn...nnnn      -3'      DF 5' -hijklnnn...nnnnnta      -3'
UR 3' -   ctcnnn...nnnnvwxyz-5'      DR 3' -   nnn...nnnnnatGAT-5'
```

Note that the bases shown as 'hijkl' should be complementary to those shown as 'vwxyz'. These oligonucleotides should, again, be phosphorylated and annealed in pairs to form an Upstream half-Part and a Downstream half-Part, exactly as for half-Clips.

In this case the Clip Ligation for each assembly should be performed in two stages (Figure 2). First – the Downstream half-Clip of the Part 1 should be ligated to the Upstream half-Part of the small Part (X). Second – the Downstream half-Part of the small Part should be ligated to the Upstream half-Clip of the Part 2 (Figure 2, a). Then the products of these two reactions can be ligated together (by placing these two ligation

reactions in one tube) to form the final Clip (1+X+2) to be used in the assembly (Figure 2, b).

This procedure can also be used to add N- or C-terminal tags to a coding sequence part which does not include the start or stop codon (these can simply be left out of the Clip oligonucleotides as described above) or a fusion peptide linker; however, in this case the ends must be modified so as not to include an in-frame stop codon. This is quite straightforward, but a detailed description of the design considerations is beyond the scope of this RFC.

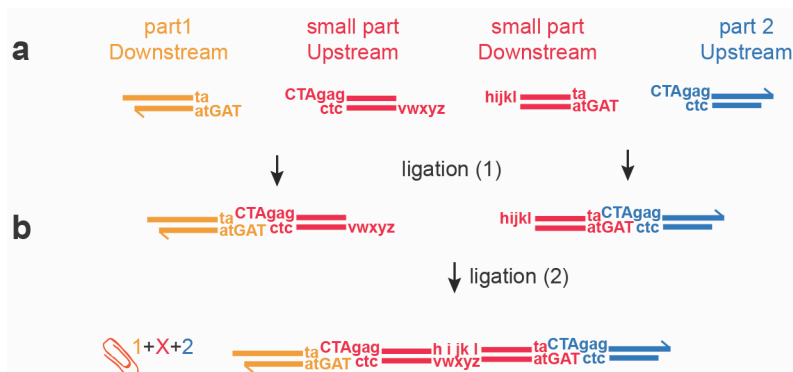


Figure 2. Scheme for addition of desired sequence (in red) between the Parts 1 and 2.

Vector considerations

The majority of Parts in the Registry are cloned in pSB1C3. If the final construct is in pSB1C3 vector as well, recovery of background Parts can be greatly decreased by initially performing PCR using the Part plasmid as a template (< 1 ng), and oligonucleotides UF and DR as primers. The resulting PCR product is then treated with DpnI restriction enzyme to remove residual template DNA, and can then be used as a Part in the assembly.

Alternatively, to avoid recovering Parts after the assembly reaction, it may be advantageous to perform your assembly initially in a different vector such as pSB1K3, using the RFP-tagged version of the vector as a Part (assuming that your assembly does not contain RFP). In this case, all non-red kanamycin-resistant colonies should be the correct product. For submission to the Registry, you can then perform a simple vector swap using, e.g., EcoRI and PstI to transfer the construct back to pSB1C3.

Other caveats and known issues

If assembling by the PCR method described in this RFC, Parts may not contain substantial repetitive sequences (i.e., with T_m above 65°C), and must not contain substantial sequences (>20 bp, T_m above 65°C), which are also present in other parts in the same assembly. If this is the case, deletion between the repeats is likely to occur

during the amplification procedure. For example, we recommend against using the same RBS in more than one place in a single construct. Such constructs are quite likely to be unstable *in vivo* in any case.

Example assembly

We have successfully performed three-part assembly (Figure 1, a and b) of BioBricks – pSB1C3 vector, P_{lac} -lacZ' (BBa_J33207) and KanR (from pSB1K3) and sequence verified the presence of the characteristic Prefix, Scars and Suffix. Using the general PaperClip assembly up to six parts are assembled (example in Figure 1, c) raising more than 1000 resistant colonies (competent cells are CFU=3*10⁷/μg pUC19), with less than 1% error rate as judged by colony phenotype.

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APPENDIX: Detailed protocols

1. Algorithm for design of Clip oligonucleotides

1. Obtain the sequence of the Part. For RFC10 BioBricks this is the sequence as shown in the Registry, i.e. without the RFC10 Prefix or Suffix.

2. Take the first 37 bases of the Part sequence and add CTAGAG at the start – this is Upstream Forward (UF).

3. Take the reverse complement of the first 34 bases of the Part sequence, and add CTC at the end – this is Upstream Reverse (UR).

4. Take the last 37 bases of the Part sequence. If the first three bases are not TAG or CTA, then add TA at the end. This is Downstream Forward (DF). On the other hand, if the first three bases are either TAG or CTA, then remove one base from the start of the sequence, and again check that the first three bases are not TAG or CTA, before adding TA at the end and proceeding.

5. Take the reverse complement of UF, add TAG at the start, and delete the last three bases. This is downstream reverse (DF).

6. Using a melting temperature calculator such as that on the IDT system at:

<https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>

check that the T_m of each oligonucleotide is between 55°C and 75°C. If not, repeat the procedure starting with a shorter or longer base length (e.g., 32 or 42 bases rather than 37 bases). Check that the final oligonucleotides, when annealed, will give the correct overhangs as shown below:

UPSTREAM HALF-CLIP

```
UF 5' -CTAgagnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-3'
UR   3' -ctcnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-5'
```

DOWNSTREAM HALF-CLIP

```
DF 5' -xyznnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnta-3'
DR   3' -nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnatGAT-5'
```

Note that the bases, shown as 'xyz' must not be CTA or TAG. The three-base overhangs at the inner ends are present only to prevent these ends from ligating.

7. It is recommended that the oligonucleotides should be named as BC_[PartName]_UF, BC_[PartName]_UR, etc.

8. EXCEPTION: Preparation of Clips for Vector Parts

In the cases of vector Parts, if it is desired that the product should be identical to that formed by normal RFC10 assembly, the Clips must be designed to include the Prefix and Suffix, as shown below:

VECTOR UPSTREAM HALF-CLIP

```
UF 5' -CTAgtagcggcggcgtgcagnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-3'
UR 3' -cttcgccggcgacgtcnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-5'
```

VECTOR DOWNSTREAM HALF-CLIP

```
DF 5' -xyznnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn-3'
DR 3' -nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnncttaagcggcggcgaagAT-5'
```

Again, the bases, shown as 'xyz' must not be CTA or TAG.

2. Preparation of half-Clips

1. Dissolve oligonucleotides at 100 μ M in nuclease-free water.
2. Set up phosphorylation reactions for oligonucleotide pairs UF+UR and DF+DR as follows:
 - 5 μ l 10xT4 PNK buffer (NEB or Thermo Fisher Scientific)
 - 0.5 μ l T4 PNK (NEB or Thermo Fisher Scientific)
 - 5 μ l 10 mM ATP (final 1 mM)
 - 20 μ l oligo 1 (100 μ M) (final 40 μ M)
 - 20 μ l oligo 2 (100 μ M) (final 40 μ M)
3. Incubate 30 min at 37°C
4. Add 5 μ l 5M NaCl (final 500 mM)
5. Place the tubes into a boiling waterbath, and allow to slowly cool down to room temperature (~25°C), then place on ice. Alternatively put the tubes in a PCR machine using a temperature program to cool from 95°C to 4°C at 0.1°C/sec (takes about 20 min).
6. Store the half-Clips at -20°C. For use, defrost them on ice, keep on ice while using.

3a. Preparation of Parts by PCR

If the Parts are not already available as cloned BioBricks in pSB1C3, they can be amplified from a suitable template (e.g., genomic DNA) using oligonucleotides UF and DR as primers, using any standard PCR protocol with a suitable proof-reading DNA

polymerase such as KOD (Merck-Novagen) or Phusion (NEB), according to the manufacturer's protocol. Even if Parts are already cloned, it may be advantageous in some cases to prepare Parts as PCR products prior to assembly in order to reduce the likelihood of recovering non-assembled Parts in the transformed colonies, depending on the Parts and selection used (see 'Vector considerations' in main text).

Following PCR, add 1 μ l of DpnI restriction enzyme (NEB) to the PCR reaction to get rid of the template DNA. Incubate for 1 hour at 37°C. The PCR product should be checked by agarose gel electrophoresis and purified from the reaction mixture using any standard method (e.g., QIAquick PCR purification Kit). If more than one band is observed after the PCR, gel-purification should be performed.

3b. Use of linearized plasmids as Parts

If the parts are within a vector backbone such as pSB1C3 and the DNA concentration is high enough, linearize the plasmid with any restriction enzyme, which doesn't cut in the part, or cut out the insert. (Be careful about the selection markers, so that you do not recover your starting plasmid). Use 1 μ g of plasmid in 50 μ l volume, incubate for 1 hour at appropriate temperature (usually +37°C), followed by heat inactivation at a temperature appropriate for the enzyme used (see manufacturer's information).

4. Preparation of Clips for each assembly:

1. Prepare ligation reactions as follows:

4 μ l Downstream half-Clip (already phosphorylated and annealed)
4 μ l Upstream half-Clip (already phosphorylated and annealed)
1 μ l 10xLigase buffer (NEB or Thermo Fisher Scientific)
1 μ l 10 mM ATP
0.5 μ l T4 ligase (NEB or Thermo Fisher Scientific)

2. Incubate for 1 hour at +16°C or overnight.

3. Inactivate ligase for 10 min at 65°C. Spin down, keep on ice, store at -20°C, defrost on ice, keep on ice.

NOTE: if using Small Parts in the form of oligonucleotides, it will be necessary to do two sequential ligations as described in the main text under 'Using Small Parts'. Briefly: first set up two ligations as described above, one with the Downstream half-Clip of the upstream part and the Upstream half-Part of the Small Part; second, with the Downstream half-Part of the Small Part and the Upstream half-Clip of the downstream part. Incubate these for one hour as indicated, then mix the two reactions together and continue the incubation.

Keep the Clips and Parts at -20°C well labelled, as they only need to be prepared once and will be used again in future assemblies.

5. Checking ligation efficiency by PAGE or agarose gel-electrophoresis (optional)

Efficiency of the ligation may be tested by analysing a sample of the ligation mixture on a 12% polyacrylamide gel made in 0.5xTBE buffer, and staining with a suitable DNA stain such as GelGreen™ Nucleic Acid Gel Stain in water (BIOTIUM Inc.) or SafeWhite loading buffer (NBS Biologicals).

6. Assembly reaction using PCR:

1. Prepare the assembly reaction as follows:

x µl	nuclease free water (to make total volume 50 µl)
5 µl	10xPCR buffer for KOD Hot start DNA polymerase (Merck-Novagen)
5 µl	dNTPs (2 mM, final 0.2 mM)
3 µl	MgSO ₄ (25 mM, final 1.5 mM)
200 ng	pSB1C3 backbone
100 ng	part 2
100 ng	part 3 and up to 6 parts works perfect
0.21 µl	Clip1 (pSB1C3Down/part2Up)
0.21 µl	Clip2 (part2Down/part3Up)
0.21 µl	Clip3 (part3Down/... and so on)
1 µl	KOD Hot Start DNA polymerase (Merck-Novagen)

2. Perform two-step PCR as follows:

95°C 2 min

20 cycles:

95°C 20 sec

70°C (10 sec/kb for <500 bp, 15 sec/kb for 500-1000 bp, 20 sec/kb, 1000-3000 bp, 25 sec/kb for >3000 bp total construct size).

4°C hold

7. Booster PCR reaction (optional, improves efficiency for higher numbers of parts; recommended for assemblies with 6 or more parts)

1. Prepare the booster PCR reaction as follows:

x µl	nuclease free water (to make total volume 50 µl)
5 µl	of the first PCR reaction
5 µl	10xPCR buffer for KOD Hot start DNA polymerase (Merck-Novagen)
5 µl	dNTPs (2 mM, final 0.2 mM)
3 µl	MgSO ₄ (25 mM, final 1.5 mM)
1 µl	KOD Hot Start DNA polymerase (Merck-Novagen)

2. Perform PCR using the same program as shown above.

8. Transformation

Transform 100 μ l of chemically competent cells with 5 μ l of the assembly reaction (or 1 μ l if the assembly only involves two parts). Any standard transformation protocol may be used. For chemically competent cells, plating 100 μ l (1/5th) of transformation mixture (or 200 μ l for 5 or 6 part assembly), expect to see 100-2000 colonies.