

**Article Title:** Assembly of BioBrick standard biological parts using three antibiotic assembly

**Authors:** Reshma Shetty<sup>1,2,\*</sup>, Meagan Lizarazo<sup>3</sup>, Randy Rettberg<sup>1,3</sup>, and Thomas F. Knight, Jr.<sup>2,3</sup>

**Running Title:** 3A assembly

<sup>1</sup>Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

<sup>2</sup>Current affiliation: Ginkgo BioWorks, Inc., Boston, MA, USA

<sup>3</sup>Computer Science and Artificial Intelligence Lab, Massachusetts Institute of Technology, Cambridge, MA, USA

\*Corresponding author

**Contact:**

Email: [rshetty@ginkgobioworks.com](mailto:rshetty@ginkgobioworks.com) | Ph: 814-422-5362x106 | Fax: 617-300-8878

Email: [meagan@igem.org](mailto:meagan@igem.org) | Ph: 617-258-5244 | Fax: 617-258-7413

Email: [rettberg@mit.edu](mailto:rettberg@mit.edu) | Ph: 617-715-5186 | Fax: 617-258-7413

Email: [tk@mit.edu](mailto:tk@mit.edu) | Ph: 617-253-7807 | Fax: 617-258-7413

**Abstract**

An underlying goal of synthetic biology is to make the process of engineering biological systems easier and more reliable. In support of this goal, we developed BioBrick

assembly standard 10 to enable the construction of systems from standardized genetic parts. The BioBrick standard underpins the distributed efforts by the synthetic biology research community to develop a collection of more than 6000 standard genetic parts available from the Registry of Standard Biological Parts. Here, we describe the three antibiotic assembly (3A assembly) method for physical composition of BioBrick parts and provide step-by-step protocols. The method relies on a combination of positive and negative selection to eliminate time- and labor-intensive steps such as column cleanup and agarose gel purification of DNA during part assembly.

## **1. Introduction**

A fundamental goal of synthetic biology is to make the process of designing, building and testing biological systems easier and more reliable. Drawing inspiration from other engineering fields, we have been working to develop methods that support the design and construction of multi-component, synthetic biological systems from standardized biological parts. In 2002, Knight proposed and implemented the BioBrick standard for assembly of standard genetic parts (Knight, 2003). Parts that have been refined to adhere to the BioBrick assembly standard are called BioBrick standard biological parts. The key innovation of the BioBrick standard is that assembly of any two BioBrick parts yields a composite object that is itself a BioBrick part that can be further combined with any other BioBrick parts. The idempotent nature of the BioBrick standard has helped to support the distributed development of a collection of over 6000 standard biological parts in the Registry of Standard Biological Parts (<http://partsregistry.org>). Parts in the Registry are catalogued, describing and conveying units of biological function rather than simply

arbitrary DNA sequences. Participants in the international Genetically Engineered Machines competition (iGEM, <http://igem.org>) as well as many academic laboratories have made use of the BioBrick assembly standard in construction of genetic parts, devices and systems (Levskaya *et al.*, 2005; Ajo-Franklin *et al.*, 2007; Canton *et al.*, 2008; Haynes *et al.*, 2008; Shetty *et al.*, 2008; Du *et al.*, 2009; Kelly *et al.*, 2009; Tabor *et al.*, 2009; Agapakis *et al.*, 2010; Afonso *et al.*, 2010; Grünberg *et al.*, 2010; Huang *et al.*, 2010). Several additional physical composition standards have been proposed that extend or build upon the BioBrick assembly standard (Phillips and Silver, 2006; Knight, 2008; Ellison *et al.*, 2009; Grünberg *et al.*, 2009; Peisajovich *et al.*, 2009; Anderson *et al.*, 2010). The BioBricks Foundation (BBF) now manages an open standards setting process for the synthetic biology community via the submission and publication of requests for comment (RFCs). In accordance with BBF RFC 29, the BioBrick assembly standard is now referred to as BioBrick assembly standard 10 in the Registry of Standard Biological Parts and other assembly standards are similarly numbered (Shetty and Rettberg, 2009). For brevity, here we use the term BioBrick parts to refer to parts that adhere to BioBrick assembly standard 10.

In addition to supporting the distributed development of a collection of standard biological parts, the BioBrick standard also enables the iterative and pairwise hierarchical assembly of genetic parts into systems containing many parts. The standard prescribes specific sequences, termed the prefix and suffix sequences, that must flank the 5' and 3' ends of a genetic part, respectively (Figure 1A and 1B). The prefix sequence encodes an EcoRI and XbaI restriction enzyme recognition site and the suffix sequence encodes a

SpeI and PstI recognition site. In addition, the standard prescribes that the sequence of the genetic part itself must not contain recognition sites for EcoRI, XbaI, SpeI or PstI enzymes. During assembly of any two BioBrick parts, the 3' end of the upstream part is digested with SpeI and the 5' end of the downstream part is digested with XbaI. Since SpeI and XbaI produce compatible cohesive ends, ligation of the upstream and downstream parts produces an 8 bp "mixed" or "scar" sequence between the two parts that is not recognized by either enzyme (Figure 1C). Since the resulting composite part is still flanked by the prefix and suffix sequences, it can again be digested with either XbaI or SpeI during a subsequent assembly step. Hence, a biological engineer can use the same, iterative process of restriction digestion and ligation to assemble multiple parts into a synthetic biological system.

Although the BioBrick standard constrains the sequence of the genetic parts as well as the overall assembly approach, it does not specify the methods used during the assembly process. As a result, there has been some variation and experimentation on assembly methods by different research groups (Knight, 2003; Sleight *et al.*, 2010). Here, we describe a method for assembly of BioBrick parts called three antibiotic assembly (3A assembly, Figure 2). We designed the 3A assembly method to make use of negative and positive selection to reduce the number of possible incorrect assembly products. The inputs to the process are plasmids propagating an upstream part, a downstream part, and a destination vector into which the two parts will be assembled. The destination vector must have a different antibiotic resistance marker from the plasmids encoding the upstream and downstream parts. (The name 3A assembly derives from the maximum of

three antibiotic resistance markers needed for any assembly step.) The upstream part is digested with EcoRI and SpeI, the downstream part is digested with XbaI and PstI and the destination vector is digested with EcoRI and PstI. The three digested plasmids are then mixed, ligated and transformed with no intervening purification steps. Given the variety of DNA fragments present in the ligation reaction, an assortment of ligation products are possible in 3A assembly (Figure 3). Fortunately, transformation and subsequent colony growth on selective media constitute strong selective pressure for circular ligation products that include the destination vector. In addition, propagation of the uncut or recircularized destination plasmid is selected against by inclusion of the *ccdB* positive selection marker in the BioBrick cloning site of the destination vector (Bernard et al., 1994; Bernard, 1995; Bernard, 1996). Ligation products composed of part concatemers, though possible, occur with significantly lower probability than the desired ligation product. Finally, ligation products with two or more identical replication origins usually fail to propagate in growing *E. coli* cells. Hence, there is significant positive and negative selective pressure for the transformation and propagation of the desired assembly product. As an additional benefit of the 3A assembly method, it is very amenable to scale up and automation because it avoids difficult-to-automate steps such as column cleanup and agarose gel purification of DNA. We have successfully used the method to assemble parts ranging in length from 12 bp to 3-4 kb. Below, we provide detailed protocols for the construction and 3A assembly of BioBrick parts. All protocols are adapted from standard molecular biology techniques and/or the manufacturer's directions, unless otherwise specified (Sambrook and Russell, 2001).

## **2. Construction of new BioBrick standard biological parts**

To adhere to BioBrick assembly standard 10, genetic parts must be designed with specific prefix and suffix sequences (Figure 1A). The suffix sequence for all BioBrick genetic parts is 5' TACTAGTAGCGGCCGCTGCAG 3'. The prefix sequence for genetic parts, with the exception of protein coding sequence parts, is 5' GAATTCGCGGCCGCTTCTAGAG 3'. For protein coding sequences beginning with an ATG start codon, the prefix sequence is truncated by 2 nt at the 3' end (Figure 1B). The 2 nt truncation ensures that, when assembled together, two genetic parts encoding a ribosome binding site and a protein coding sequence have a suitable spacing between the Shine-Dalgarno sequence and the ATG start codon to promote efficient translation in *E. coli* and related organisms (Chen *et al.*, 1994). Hence, the prefix sequence for protein coding sequence parts beginning with an ATG start codon is 5' GAATTCGCGGCCGCTTCTAG 3'. An additional constraint on the sequence of parts conforming to BioBrick assembly standard 10 is that recognition sites for the following restriction enzymes must be absent: EcoRI, XbaI, SpeI, and PstI.

New BioBrick parts can be constructed by either PCR amplification from template DNA or by *de novo* DNA synthesis. For PCR-based construction of parts, we routinely use the primer sequences listed in Table I in which the prefix and suffix sequences are encoded on the forward and reverse primers, respectively. Both the forward and reverse amplification primers are designed to have an extra 8 nt of sequence on the 5' end. The 8 nt spacer sequence serves two purposes. First, it enables efficient cleavage of the linear PCR product by EcoRI or PstI, as restriction enzymes cleave less efficiently near DNA termini (Moreira and Noren, 1995). Second, the selected sequence GTTCTT was found to promote the addition of a 3' A overhang by Taq polymerase thereby facilitating

downstream TA cloning of the PCR amplified part (Brownstein *et al.*, 1996). Optimal PCR conditions will vary based on part length, sequence, and amplification primers. When constructing a new part by PCR amplification, any EcoRI, XbaI, SpeI or PstI recognition sites present in the part sequence must be eliminated by site-directed mutagenesis. For parts that are constructed by *de novo* DNA synthesis, we recommend eliminating the restriction sites listed in Table II when possible to facilitate conversion to other BioBrick assembly standards, part reuse and downstream cloning operations. We also recommend cloning constructed parts into a standard BioBrick vector prior to 3A assembly.

### **3. 3A assembly of BioBrick standard biological parts**

To assemble two BioBrick parts together into a destination vector via 3A assembly (Figure 2), we recommend the following protocol. Enzymes and reagents are available from New England Biolabs, Ipswich, MA in the BioBrick Assembly Kit (Catalog # E0546S).

1. Digest the upstream part with EcoRI-HF and SpeI, the downstream part with XbaI and PstI and the destination vector with EcoRI-HF and PstI. The destination vector must have a different antibiotic resistance marker from both the upstream and downstream part. Prepare all three digestion reactions with 500 ng plasmid DNA, 1X NEBuffer 2, 100 µg/ml Bovine Serum Albumin and 1 µL each enzyme in a total volume of 50 µL. Note that issues in the digest step arise if nearly all of the reaction volume consists of plasmid DNA from miniprep. Residual ethanol

- and/or guanidine hydrochloride contamination in miniprep eluant is common and can inhibit subsequent digestion. A significant volume of the digest reaction volume should consist of water to dilute these impurities.
2. Incubate all three reactions at 37°C for 15 minutes to digest the DNA and then at 80°C for 20 minutes to heat inactivate the restriction enzymes. For convenience, we recommend performing this step in a programmable thermocycler. If troubleshooting, confirm digestion by analyzing 20 µL of reaction by agarose gel electrophoresis.
  3. Prepare the ligation reaction with 2 µL from each digest reaction, 1X T4 DNA ligase reaction buffer, and 1 µL T4 DNA ligase in a total volume of 20 µL. It is not necessary to purify the linearized part DNA prior to ligation. Ligation reactions are done at low DNA concentrations to favor production of circular DNA rather than concatemers. Quick ligation buffers are not desirable for the cohesive end ligations used here.
  4. Incubate the ligation reaction at room temperature for 10 minutes. Store the ligation reaction at -20°C or proceed immediately to the chemical transformation step. We prefer chemical transformation to electroporation because it is easier to do a large numbers of transformations in parallel.
  5. Thaw the chemically competent cells on ice. Competent cells can be purchased commercially (e.g., NEB 10-beta competent *E. coli* (High Efficiency)) or can be prepared via the protocol below. Regardless, it is important to verify experimentally that the competent cells used have a high transformation efficiency (>10<sup>8</sup> cfu per µg of plasmid DNA is desirable).



6. Prepare a transformation reaction by adding 1  $\mu\text{L}$  of ligation reaction to 15-50  $\mu\text{L}$  competent cells in a pre-chilled 2 mL eppendorf tube. Increasing the amount of ligation product in the transformation reaction is usually not effective in increasing the number of transformants. Less than 5% of the transformation reaction volume should consist of ligation product. A 2 ml rather than a 1.7 ml eppendorf tube provides better mixing and aeration of the cell culture during outgrowth in a rotator (Step 11).
7. Incubate the transformation reaction on ice for 30 minutes.
8. Immerse the transformation reaction in a 42°C water bath for 60 seconds to heat shock the cells.
9. Incubate the transformation reaction on ice for 2 minutes.
10. Add 200  $\mu\text{L}$  sterile SOC media to the transformation reaction to begin cell outgrowth.
11. Incubate the transformation reaction at 37°C for 2 hours with gentle shaking.  
Although a 1 hour incubation is sufficient for destination vectors with ampicillin or kanamycin resistance markers, we have found that a 2 hour incubation results in an increased number of colonies for destination vectors with chloramphenicol or tetracycline markers.
12. Using sterile 3 mm glass beads, spread 200  $\mu\text{L}$  of the transformation reaction on an LB agar plate supplemented with the antibiotic corresponding to the destination vector. Sterile glass beads are preferable to a glass spreader because multiple transformation reactions can be plated simultaneously.
13. Incubate transformation plates overnight at 37°C for colony growth.

An attractive, automation-friendly alternative to plating the transformation reaction on LB agar plates is to perform serial dilution of the outgrowth cultures. Outgrowth can be done in the first, leftmost column of a 96 deep-well plate in which each well is filled with 316  $\mu\text{L}$  of SOC medium. Fill remaining wells with 684  $\mu\text{L}$  of LB medium supplemented with the appropriate antibiotic and 0.002% cresol red. Serial dilutions, transferring 316  $\mu\text{L}$  each time, result in 10X dilutions every two columns. Incubate plates overnight at 37°C for cell growth. A red to yellow color change in a well indicates cell growth. Pick the last, rightmost (most dilute) yellow well and test for correct assembly as described below. This approach is only viable given the high likelihood and strong selection for correctly assembled parts in 3A assembly.

#### **4. Verification of correct assembly of BioBrick standard biological parts**

Based on our empirical observations, 3A assembly usually yields more than 80% correct clones, assuming that the assembled parts confer no significant growth disadvantage to the host strain. To confirm that two parts have been correctly assembled together via 3A assembly, we routinely screen colonies for sensitivity to the antibiotic(s) corresponding to the two part vectors and verify the length of the assembled part by colony PCR followed by agarose gel electrophoresis as described below (Güssow and Clackson, 1989). Clones that pass these two checks are then analyzed by DNA sequencing.

1. Use a sterile tip to pick, inoculate and mix a colony into 20  $\mu\text{L}$  of sterile  $\text{H}_2\text{O}$ .

We generally pick 4 or 8 colonies from each transformation plate. For this and all

subsequent steps, we use 8-strip PCR tubes and/or 96-well plates to facilitate use of a multi-channel pipettor for the transfer operations.

2. Transfer ~2  $\mu\text{L}$  of the colony suspension onto four LB-agar plates each supplemented with one of ampicillin, kanamycin, chloramphenicol and tetracycline. Incubate the plates either at room temperature overnight or at 37°C for 6-8 hours to check for the expected antibiotic resistance and sensitivity.
3. For each colony suspension, prepare 9.5  $\mu\text{L}$  PCR mix using primers BioBricks-f and BioBricks-r that anneal to the BioBrick prefix and suffix, respectively (Table I). Transfer 0.5  $\mu\text{L}$  colony suspension to each reaction tube and run PCR in a programmable thermocycler. Optimal PCR conditions will vary based on part length and sequence but generally an annealing temperature of 56-58°C works well with these primers. We prefer to use colony verification primers BioBricks-f and BioBricks-r rather than sequencing primers VF2 and VR in this step to test for the presence of a BioBrick prefix and suffix in the assembled clones.
4. Analyze the resulting reactions by agarose gel electrophoresis. Confirm that the PCR product has the expected length by comparison to a molecular weight standard (DNA ladder).
5. Sequence plasmid DNA from 1-2 colonies per assembly from those colonies that pass both the antibiotic resistance/sensitivity test and length verification test. We routinely use primers VF2 and VR for sequence verification of parts assembled in BioBrick vectors (Table I).

## **5. Preparation of linearized destination vector by PCR to improve 3A assembly**

Our driving goal in designing the 3A assembly process was to limit the number of possible incorrect assembly products as much as possible by leveraging a combination of positive and negative selection (Figure 3). Hence, incorrect clones arising from 3A assembly are of significant interest because they inform further improvements to the process. In our experience, the three most common failure modes of 3A assembly are the following. First, colonies on the transformation plate are resistant to the antibiotic corresponding to not only the destination vector but also one of the parent vectors. Such a result generally arises from a co-transformation, or possibly a ligation followed by transformation, of the part and destination vectors. Second, a contaminating *E. coli* genomic DNA fragment ligates to the destination vector backbone and is transformed. In this error case, the genomic DNA fragment is often flanked by EcoRI and PstI sites and can ligate directly to the destination vector. Such a two-fragment ligation event competes with the desired three-fragment ligation event needed for correct part assembly. Third, uncut or recircularized destination vector propagates in the host strain as a result of a loss-of-function mutation in the *ccdB* positive selection marker.

While the verification tests described above can generally detect these three error cases prior to DNA sequencing, we prefer to reduce or eliminate these failure cases if possible. In particular, both the second and third failure modes described above stem from issues arising during preparation and digestion of the destination vector. To further reduce the number of possible incorrect assembly products in 3A assembly, we recommend preparing the destination vector by PCR instead of by plasmid DNA purification. Such an approach nearly eliminates contaminating genomic DNA in the destination vector

digestion reaction, thereby reducing competition from EcoRI/PstI linearized genomic DNA fragments in the ligation reaction (second failure mode). Similarly, preparation of the destination vector by PCR also reduces contaminating uncut destination plasmid from the transformation reaction (third failure mode). Since destination vectors can be prepared in batches in advance of any assembly steps, the duration and automation of the 3A assembly process is unaffected by these improvements.

1. Amplify the pSB1A3, pSB1K3, pSB1C3 and pSB1T3 destination vectors by PCR using primers SB-prep-3P and SB-prep-2Eb (Table I). Prepare each amplification reaction with 1X PCR SuperMix High Fidelity (Life Technologies, Carlsbad, CA), 60 pmol each primer and 2-5 ng template destination plasmid in a 100  $\mu$ L total volume. We designed the primers to amplify the destination vector with only EcoRI and PstI recognition sites, leaving only the minimal number of bases 5' to the sites to allow efficient DNA cleavage of the vector by these enzymes. The resulting short fragments produced from digestion of the PCR-amplified destination vector are too short for ligation during assembly, increasing the efficiency of the assembly step by limiting competing ligation products.
2. Thermocycle the reactions using the following conditions.
  1. Initial denaturation at 94°C for 1 minute.
  2. Denaturation step at 94°C for 30 seconds.
  3. Annealing step at 58°C for 30 seconds.
  4. Extension step at 68°C for 3 minutes.
  5. Cycle 40 times to step 2.

6. Final extension at 68°C for 10 minutes.
3. Add 1 ul DpnI to each reaction.
4. Incubate all reactions at 37°C for 1 hour to digest the template DNA and then at 80°C for 20 minutes to heat inactivate the restriction enzyme. For convenience, we recommend performing this step in a programmable thermocycler.
5. Confirm by agarose gel electrophoresis that the full-length destination vector is primary PCR product.
6. Purify the PCR product using the QIAquick PCR Purification Kit from QIAGEN or similar kit to remove enzymes and dNTPs.
7. The resulting PCR-amplified destination vector can be used directly in the EcoRI and PstI digestion of a 3A assembly step. We do not recommend storage of digested linearized plasmid backbones.

## **6. Available BioBrick destination vectors**

Several standard BioBrick assembly vectors are available from the Registry of Standard Biological parts including high copy vectors pSB1A3, pSB1K3, pSB1C3, pSB1T3, pSB1AK3, pSB1AC3 and pSB1AT3. In addition, a selection of low and medium copy BioBrick vectors are also available from the Registry including pSB4A5, pSB4K5, pSB4C5, pSB4T5, pSB3K5, pSB3C5 and pSB3T5 (Shetty *et al.*, 2008). All of these vectors contain a BioBrick cloning site and primer binding sites for verification primers VF2 and VR. The VF2 and VR primer binding sites are positioned at a sufficient distance from the cloning site to allow high quality sequence reads of BioBrick parts from Sanger sequencing. Use of part plasmids and destination plasmids with different

replication origins can increase the likelihood that ligation products containing two replication origins will propagate during cell growth (first failure mode described above).

## 7. Preparation of chemically competent cells

A key factor in the success of 3A assembly of BioBrick parts is the transformation efficiency of the competent cells used. Low efficiency competent cells can result in few or no colonies on the transformation plate. We have tested several different protocols for preparation of chemically competent cells and have found that the following protocol yields competent cells with high transformation efficiency. It builds on extensive prior work on bacterial transformation (Hanahan *et al.*, 1991). We have used this protocol successfully with *E. coli* strains TOP10 and Mach1.

1. Detergent residue inhibits competent cell growth and transformation. Remove detergents from all glassware by autoclaving glassware filled  $\frac{3}{4}$  full with DI water prior to use. Prepare all media and buffers in detergent-free glassware. Similarly, grow cultures in detergent-free glassware.
2. Prepare CCMB80 buffer.
  - 10 mM KOAc pH 7.0 (10 mL of a 1M stock solution)
  - 80 mM CaCl<sub>2</sub>·2H<sub>2</sub>O (11.8 g/L)
  - 20 mM MnCl<sub>2</sub>·4H<sub>2</sub>O (4.0 g/L)
  - 10 mM MgCl<sub>2</sub>·6H<sub>2</sub>O (2.0 g/L)
  - 10% glycerol
  - Adjust pH down to 6.4 with 0.1N HCl if necessary.

- Filter sterilize and store at 4°C.
3. Streak the *E. coli* strain on an SOB agar plate and grow at 23°C until single colonies appear.
  4. Prepare a set of seed cultures by picking single colonies into several culture tubes each with 2 mL SOB medium. Shake culture tubes at 23°C overnight.
  5. Prepare frozen seed stocks by adding sterile glycerol to a final concentration of 15% to each seed culture. Aliquot 1 mL seed culture into cryogenic vials. Freeze seed stocks in a dry ice/ethanol bath and store at -80°C.
  6. Inoculate 250 mL SOB medium with a 1 mL vial of seed stock and grow at 20°C to an OD<sub>600nm</sub> of 0.3. This culture may be grown at room temperature if necessary.
  7. Chill glassware, plasticware and reagents prior to use.
  8. Centrifuge the culture at 3000 x g at 4°C for 10 minutes in a flat bottom centrifuge tube. The cell pellet is easier to resuspend in a flat bottom tube.
  9. Gently resuspend cell pellet in 80 mL of ice cold CCMB80 buffer. To make cell resuspension easier, initially add a small volume of buffer, resuspend and then add the rest of the buffer.
  10. Incubate resuspended culture on ice for 20 minutes.
  11. Centrifuge resuspended culture at 3000 x g at 4°C for 10 minutes and gently resuspend cell pellet in 10 mL ice cold CCMB80 buffer.
  12. Measure the OD<sub>600nm</sub> of a mixture of 200 µL SOC and 50 µL resuspended culture. Add sufficient chilled CCMB80 buffer to resuspended culture to yield a final OD<sub>600nm</sub> of 1.0-1.5 in this measurement.



13. Incubate the resuspended culture on ice for 20 minutes.
14. Aliquot competent cells into pre-chilled tubes and store at  $-80^{\circ}\text{C}$ .
15. Measure transformation efficiency of prepared competent cells using plasmid DNA. Target transformation efficiency is  $>10^8$  cfu per  $\mu\text{g}$  of plasmid DNA.

## **8. Conclusions**

3A assembly is a method for the assembly of standard biological parts that conform to BioBrick assembly standard 10. The 3A assembly method relies on a combination of positive and negative selection to achieve a high frequency of correctly assembled clones ( $>80\%$  in our experience). It avoids labor-intensive, difficult-to-automate steps such as column cleanup and agarose gel purification and we have used it successfully with parts ranging in length from 12 bp to 3-4 kb. To use 3A assembly, the destination vector, into which two BioBrick parts will be assembled, must have a different antibiotic resistance marker from the plasmids encoding the two input parts. The 3A assembly method is extensible to other BioBrick assembly standards, assuming that the restriction enzymes used can be heat inactivated.

## **Acknowledgements**

We thank the MIT Synthetic Biology Working Group for valuable discussions and advice as we developed the 3A assembly method. BioBrick is a trademark of the BioBricks Foundation (<http://biobricks.org>).

## **References**

Agapakis, C. M., Ducat, D. C., Boyle, P. M., Wintermute, E. H., Way, J.C. and Silver, P.A. (2010). Insulation of a synthetic hydrogen metabolism circuit in bacteria. *J Biol Eng.* **4**, 3.

Ajo-Franklin, C. M., Drubin, D. A., Eskin, J. A., Gee, E. P., Landgraf, D., Phillips, I. and Silver, P.A. (2007) Rational design of memory in eukaryotic cells. *Genes Dev.* **21**, 2271-6.

Afonso, B., Silver, P. A. and Ajo-Franklin, C. M. (2010). A synthetic circuit for selectively arresting daughter cells to create aging populations. *Nucleic Acids Res.* **38**, 2727-35.

Anderson, J. C., Dueber, J. E., Leguia, M., Wu, G. C.; Goler, J. A., Arkin, A. P. and Keasling, J. D. (2010). BglBricks: A flexible standard for biological part assembly. *J Biol Eng.* **4**, 1.

Bernard, P., Gabant, P., Bahassi, E. M. and Couturier, M. (1994). Positive-selection vectors using the F plasmid *ccdB* killer gene. *Gene.* **148**, 71-4.

Bernard, P. (1995). New *ccdB* positive-selection cloning vectors with kanamycin or chloramphenicol selectable markers. *Gene.* **162**, 159-60.

Bernard, P. (1996). Positive selection of recombinant DNA by CcdB. *Biotechniques*. **21**:320-3.

Brownstein, M. J., Carpten, J. D. and Smith, J. R. (1996). Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques*. **20**, 1004-10.

Canton, B., Labno, A. and Endy, D. (2008). Refinement and standardization of synthetic biological parts and devices. *Nat Biotechnol*. **26**, 787-93.

Chen, H., Bjercknes, M., Kumar, R. and Jay, E. (1994). Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. *Nucleic Acids Res*. **22**, 4953-7.

Du, L., Gao, R. and Forster, A. C. (2009). Engineering multigene expression in vitro and in vivo with small terminators for T7 RNA polymerase. *Biotechnol Bioeng*. **104**, 1189-96.

Ellison, M., Ridgway, D., Fedor, J., Garside, E., Robinson, K. and Lloyd, D. (2009). BBF RFC 47: BioBytes Assembly Standard. DOI: 1721.1/49518.

Grünberg, R., Ferrar, T. S., van der Sloot, A. M., Constante, M. and Serrano, L. (2010). Building blocks for protein interaction devices. *Nucleic Acids Res*. **38**, 2645-62.

Grünberg, R., Arndt, K. and Müller, K. (2009). Fusion Protein (Freiburg) Biobrick assembly standard. DOI: 1721.1/45140.

Güssow, D. and Clackson, T. (1989). Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Res.* **17**, 4000.

Hanahan, D., Jessee, J. and Bloom, F. R. (1991). Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol.* **204**, 63-113.

Haynes, K. A., Broderick, M. L., Brown, A. D., Butner, T. L., Dickson, J. O., Harden, W. L., Heard, L. H., Jessen, E. L., Malloy, K. J., Ogden, B. J., Rosemond, S., Simpson, S., Zwack, E., Campbell, A. M., Eckdahl, T. T., Heyer, L. J. and Poet J. L. (2008). Engineering bacteria to solve the Burnt Pancake Problem. *J Biol Eng.* **2**, 8.

Huang, H. H., Camsund, D., Lindblad, P. and Heidorn, T. (2010). Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res.* **38**, 2577-93.

Kelly, J. R., Rubin, A. J., Davis, J. H., Ajo-Franklin, C. M., Cumbers, J., Czar, M. J., de Mora, K., Gliberman, A. L., Monie, D. D. and Endy, D. (2009). Measuring the activity of BioBrick promoters using an in vivo reference standard. *J Biol Eng.* **3**, 4.

Knight, T. F. (2003). Idempotent Vector Design for Standard Assembly of Biobricks.

DOI: 1721.1/21168.

Knight, T. F. (2008). Draft Standard for Biobrick BB-2 Biological Parts. DOI:

1721.1/45139.

Levskaya, A., Chevalier, A. A., Tabor, J. J., Simpson, Z. B., Lavery, L. A., Levy, M., Davidson, E. A., Scouras, A., Ellington, A. D., Marcotte, E. M. and Voigt, C. A. (2005). Synthetic biology: engineering *Escherichia coli* to see light. *Nature*. **438**, 441-2.

Peisajovich, S. G., Horwitz, A., Hoeller, O., Rhau, B. and Lim, W. (2009). BBF RFC 28: A method for combinatorial multi-part assembly based on the Type IIs restriction enzyme AarI. DOI: 1721.1/46721.

Phillips, I. and Silver, P. (2006). A New Biobrick Assembly Strategy Designed for Facile Protein Engineering. DOI: 1721.1/32535.

Moreira, R. F. and Noren, C. J. (1995). Minimum duplex requirements for restriction enzyme cleavage near the termini of linear DNA fragments. *Biotechniques*. **19**, 56-9.

Sambrook, J. and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*.

Shetty, R. P., Endy, D. and Knight, T. F. (2008). Engineering BioBrick vectors from BioBrick parts. *J Biol Eng.* **2**, 5.

Shetty, R. and Rettberg, R. (2009). Naming of standards of physical composition of BioBrick parts. DOI: 1721.1/45137.

Sleight, S. C., Bartley, B. A., Lieviant, J. A. and Sauro, H. M. (2010). In-Fusion BioBrick assembly and re-engineering. *Nucleic Acids Res.* **38**, 2624-36.

Tabor, J. J., Salis, H. M., Simpson, Z.B., Chevalier, A. A., Levskaya, A., Marcotte, E. M., Voigt, C. A. and Ellington, A. D. (2009). A synthetic genetic edge detection program. *Cell.* **137**, 1272-81.

Table I: Primer sequences using during construction and 3A assembly of BioBrick parts

<b>Primer description (Name)</b>	<b>Primer sequence</b>
Forward amplification primer for protein coding sequence parts	5' GTTTCTTCGAATTCGCGGCCGCTTCTAG [ATG + 15-21nt coding sequence] 3'
Forward amplification primer for all other parts	5' GTTTCTTCGAATTCGCGGCCGCTTCTAGAG [18-24nt part sequence] 3'
Reverse amplification primer	5' GTTTCTTCCTGCAGCGGCCGCTACTAGTA [18-24nt part sequence (reverse complement)] 3'
Forward colony verification primer (BioBricks-f)	5' GTTTCTTCGAATTCGCGGCCGCTTCTAG 3'
Reverse colony verification primer (BioBricks-r)	5' GTTTCTTCCTGCAGCGGCCGCTACTAGTA 3'
Forward sequencing primer (VF2)	5' TGCCACCTGACGTCTAAGAA 3'
Reverse sequencing primer (VR)	5' ATTACCGCCTTTGAGTGAGC 3'
Vector amplification primer (SB-prep-3P)	5' GCCGCTGCAGTCCGGCAAAAAACG 3'
Vector amplification primer (SB-prep-2Eb)	5' ATGAATTCAGAAATCATCCTTAGCGAA 3'

Table II: Restriction enzymes whose sites are recommended for removal when constructing BioBrick parts by *de novo* DNA synthesis

Restriction enzyme	Explanation
EcoRI, XbaI, SpeI, PstI	Required by BioBrick assembly standard 10
NheI, PstI, PpiI, AscI, PacI, MspI, BglII, BamHI, XhoI, NgoMIV, AgeI, AarI, XmaI, BspEI, BpuEI, BseRI, HindIII	Compatibility with BioBrick assembly standards 12, 15, 21, 25, 28, 37, 44 and 45
ApoI, MfeI, AvrII, NsiI, SbfI	Produce compatible cohesive ends to BioBrick assembly standard 10 enzymes
BbsI, BbvI, BfuAI, BsmAI, BsmBI, BsmFI, BsaI, BspMI, BtgZI, EarI, FokI, HgaI, SapI, SfaNI	Offset cutters that produce arbitrary 3-4 nt overhangs
Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.AlwI	Offset nicking enzymes



## Figure 1

(A) Parts conforming to BioBrick assembly standard 10 are flanked on the 5' end by a prefix sequence encoding EcoRI, NotI and XbaI restriction enzyme recognition sites and on the 3' end by a suffix sequence encoding SpeI, NotI, and PstI recognition sites. (B) BioBrick parts encoding protein coding sequences generally begin with an ATG start codon and end with two TAA stop codons. The prefix sequence is truncated by 2 bp on the 3' end relative to other types of BioBrick parts. The truncation ensures that, when assembled, the Shine-Dalgarno sequence of a ribosome binding site part and the ATG start codon of a protein coding sequence part have correct, intervening spacing for expression in *E. coli* and related hosts. (C) Assembly of two BioBrick parts results in a new BioBrick part with appropriate prefix and suffix sequences. An 8 bp “mixed” or “scar” sequence is formed between the upstream and downstream parts that is not recognized by any of the BioBrick enzymes. Thus, the composite BioBrick part can be reused in another assembly step.

**A**

EcoRI	NotI	XbaI		SpeI	NotI	PstI
5' GAATTC	GCGGCCGC	T TCTAGA	G [part sequence]	T ACTAGT	A GCGGCCGC	CTGCAG 3'
3' CTTAAG	CGCCGGCG	A AGATCT	C [part sequence]	A TGATCA	T CGCCGGCG	GACGTC 5'

BioBrick assembly standard 10 prefix

BioBrick assembly standard 10 suffix

**B**

EcoRI	NotI	XbaI		SpeI	NotI	PstI
5' GAATTC	GCGGCCGC	T TCTAG	[ATG....TAATAA]	T ACTAGT	A GCGGCCGC	CTGCAG 3'
3' CTTAAG	CGCCGGCG	A AGATC	[TAC....ATTATT]	A TGATCA	T CGCCGGCG	GACGTC 5'

BioBrick assembly standard 10 CDS prefix

BioBrick assembly standard 10 suffix

**C**

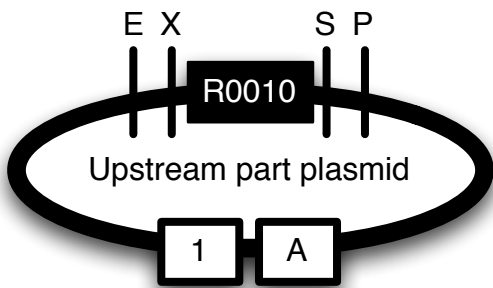
EcoRI	NotI	XbaI		mixed		SpeI	NotI	PstI
5' GAATTC	GCGGCCGC	T TCTAGA	G [upstream part]	T ACTAGA	G [downstream part]	T ACTAGT	A GCGGCCGC	CTGCAG 3'
3' CTTAAG	CGCCGGCG	A AGATCT	C [upstream part]	A TGATCT	C [downstream part]	A TGATCA	T CGCCGGCG	GACGTC 5'

BioBrick assembly standard 10 prefix

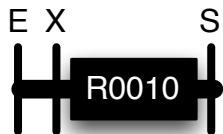
BioBrick assembly standard 10 suffix

## Figure 2

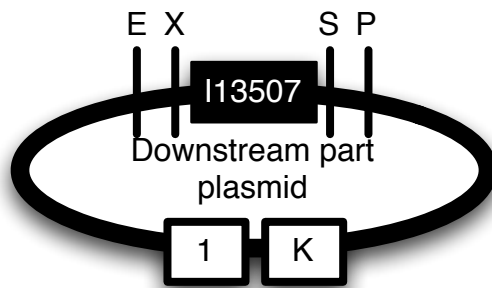
Schematic overview of the 3A assembly process for assembly of BioBrick parts. The destination plasmid must have a different antibiotic resistance marker from both the upstream and downstream part plasmids. To perform a 3A assembly, digest the upstream part plasmid with EcoRI and SpeI. Digest the downstream part plasmid with XbaI and PstI. Digest the destination plasmid with EcoRI and PstI. Ligate the resulting DNA fragments to form a circular plasmid composed of the destination vector and composite part and then transform. Abbreviations are as follows: E=EcoRI site, X=XbaI site, S=SpeI site, P=PstI site, M=mixed XbaI/SpeI site, 1=pUC19-derived high copy replication origin, A=ampicillin resistance marker, K=kanamycin resistance marker, C=chloramphenicol resistance marker, R0010=BBa\_R0010, I13507=BBa\_I13507 and P1010=BBa\_P1010. BBa\_R0010 is a BioBrick genetic part encoding a LacI-repressible promoter. BBa\_I13507 is a composite BioBrick genetic part composed of a ribosome binding site, monomeric red fluorescent protein coding sequence and a transcriptional terminator. BBa\_P1010 encodes the *ccdB* positive selection marker.



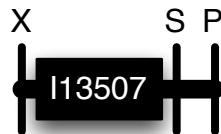
Cut E+S



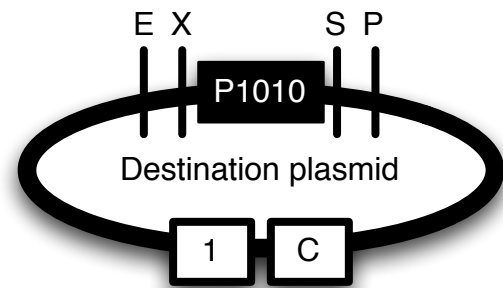
Ligate



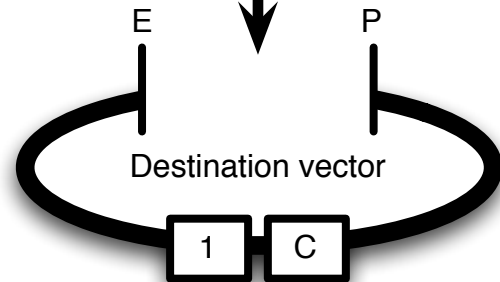
Cut X+P



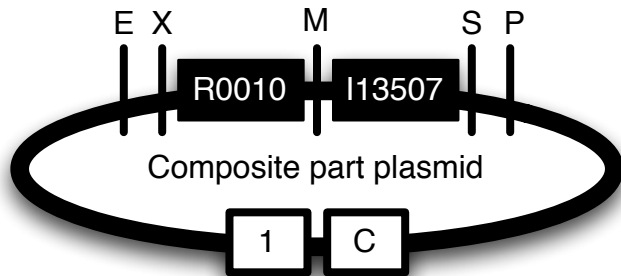
Ligate



Cut E+P



Ligate



### Figure 3

An overview of selective pressures for or against possible ligation products from the 3A assembly process. (A) The desired ligation product is composed of a composite part in the destination vector. (B) Colony growth usually selects against ligation products with two or more identical replication origins. Alternatively, transformed colonies can be screened for resistance to only the antibiotic corresponding to the destination vector. (C) Part concatemers are possible but occur with significantly lower frequency than the desired ligation product. (D) Expression of *ccdB* positive selection marker is toxic to growth of *E. coli* cloning strains preventing propagation of uncut or recircularized destination plasmid. (E) Transformation and subsequent growth selects against both linear ligation products and circular ligation products without a replication origin. (F) Growth on selective media selects for presence of destination vector in the ligation product. There is no selection for propagation of the part plasmid. Abbreviations are identical to Figure 2.

