iGEM Judges,

The Alberta 2009 iGEM team has selected a large-scale cloning and assembly project, with the ultimate goal being to build a complete functional *E. coli* chromosome *in vitro* from a near-minimal set of essential native E. coli genes. In support of this goal, our team has designed a new standardized *in vitro* assembly method, named *Biobytes*. The system is based on a bead platform where constructs with long sticky ends are annealed together sequentially. This system is designed to enable the single day assembly of plasmids composed of up to about half a dozen premade parts. Complementary to existing BioBrick standards for DNA assembly, we expect this system to be useful for rapid generation of large constructs wherever an interpart spacing of 37 bp can be tolerated. A detailed description of our method is attached.

- We are requesting a variance in order to deliver the following DNA from this project, which we believe will be sufficient for future teams to take advantage of our system:Two universal acceptor plasmids (named pAB and pBA) useful for cloning genes to assemble using this strategy
- A number of individual *E. coli* genes cloned into these plasmids
- A small set of useful promoters, reporters and selectable markers cloned into the plasmids

Using these plasmids as well as universal primer sequences for the PCR extraction of bricks from the plasmids anyone will be able to use this system for their own rapid assembly. The design ensures that the existing catalog of BioBrick parts is compatible with the system, and can be easily cloned into the universal plasmids for brick generation. We believe that this new system is ready for both use and formalization, and we are working on a standard document to submit as a BBF RFC. Thank you for your time and consideration.

Sincerely,

David Lloyd

University of Alberta iGEM Team

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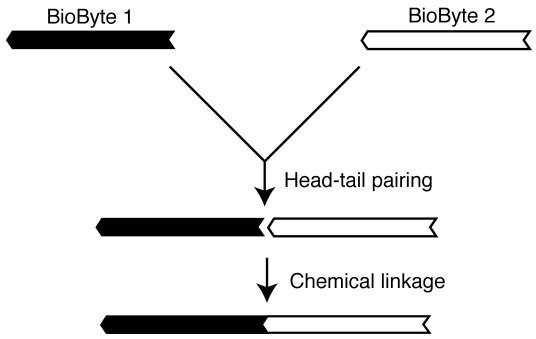
Biobytes

Overview:

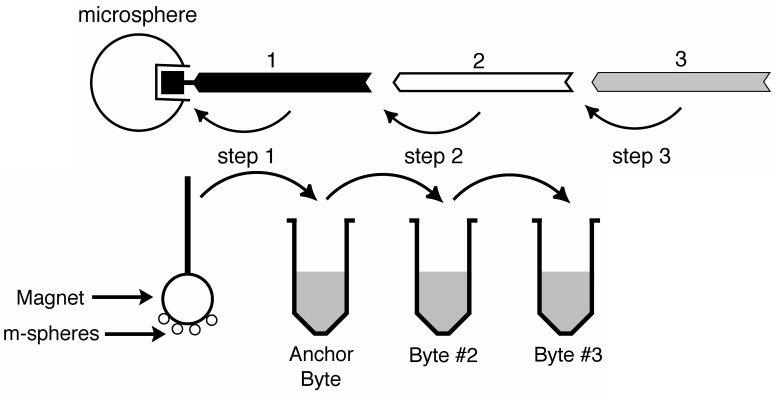
BioBytes constitutes the University of Alberta's contribution to iGEM 2009. Genes or parts produced in the BioByte format can be assembled *in vitro* rapidly, in any desired order with cycle times approaching 15 minutes for the addition of each new gene. BioBytes use non-symetrical 12 bp sticky ends that are joined in a head-to-tail fashion as shown below.

Figure 1.



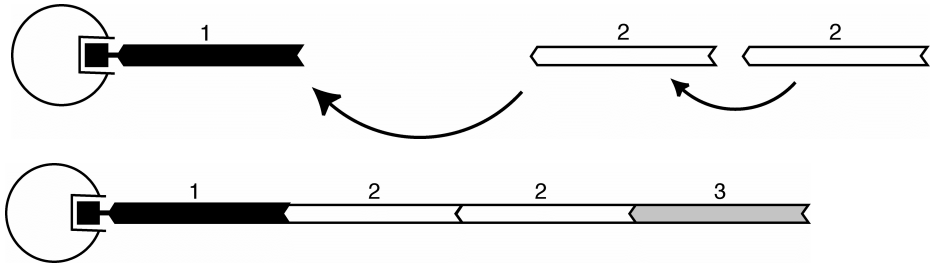
Byte order is determined by using a a biotinylated oligonucleotide linked to inert magnetic microspheres coated with streptavidin. By anchoring the initiator byte to the microsphere via this new end, only its free end is available for interaction. The chain therefore is constrained to grow in only one direction, away from its anchor. The microsphere design also fulfills another important function since anchored chains can be moved out of one reaction mixture leaving unlinked bytes behind, into a new reaction that containing new byte molecules needed for the next round of addition (Figure 3).

Figure 3.



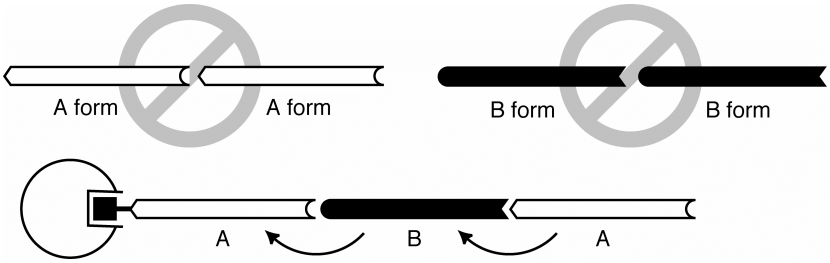
Taken as is, the method described above cannot exclude the possibility that multiple copies of a particular Byte that become ligated prior to chain linkage become incorporated at a given step as shown in Figure 4 below.

Figure 4.



BioBytes solves the problem by constructing each Byte in two alternative forms, The “AB” form and the “BA” form. Each form uses 5’ and 3’ incompatible ends to prevent self-ligation (Figure 6). The 5’ end of the AB form is complementary to the 3’ end of the BA form. Similarly the 3’ end of the AB form is complementary to the 5’ end of the BA form. Thus, each form can be linked in alternating fashion. assuring that only one copy of each is added at each step.

Figure 6.



Upon completion of the desired product, chains are released from the microspheres by enzymatic cleavage of the anchor byte from the bead followed by recircularization and tranformation. Using this approach we have been able to assemble chains composed of 10 Bytes over the course of 4 hours. With further optimization we expect this number to increase. A key advantage to this approach is that it can be multiplexed for the production of many different chains simultaneously, that, can all be linked as SuperBytes by the same method.

By comparison, other published protocols determine fragment order using multiple and variable sticky ends that assemble spontaneously in a single step, a method that necessarily requires unique primer sets for every fragment. With our approach, every byte is produced with a

common set of universal primers with a substantial saving of time, money and the flexibility to alter order at will.

Host Plasmids

The boundary sequences required for sticky end production are acquired by inserting gene sequences destined to become bytes into either of the host plasmids pAB or pBA (full sequences included. Both are derivatives of pUC19 with the following key modifications:

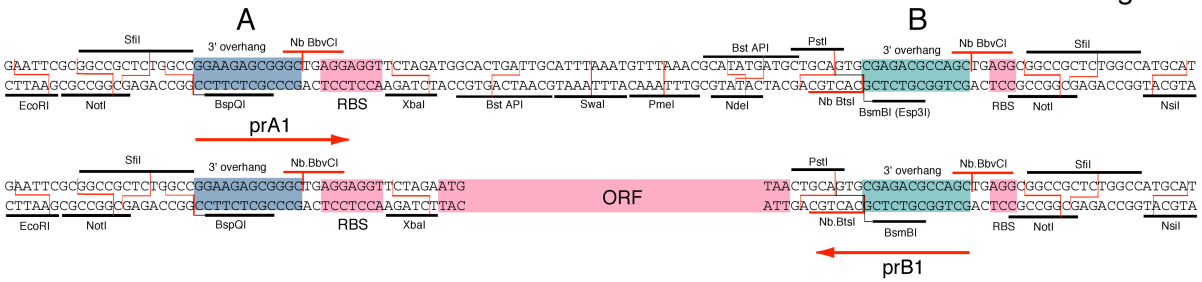
1. Replacement of the pUC19 MCS with the MCS shown in figure 1.
2. Elimination of the Z alpha fragment
3. Elimination of the restriction sites Bts1 and BspQ1

Functional features are indicated in Fig.1 and include:

1. The ability to adapt biobricks to the Byte format using the Xba1 and Pst1 sites contained in the MCS.
2. The ability to adapt the in the ASKA collection (Kitagawa 2005) to the Byte format (4000+ E. coli K12 ORFs). ORFs are cloned into an expression vector, accompanied by a 6xHis tag at the N-terminus and a GFP fusion on the C-terminus. ASKA parts can be freed by digestion with SfiI, leaving noncomplementary 3 bp 3' sticky ends, which can be cloned directionally into the MCS after digestion with BstAPI. Since the ASKA parts are freed from the interior of the CDS, the MCS provides a start codon 10 bp downstream of the RBS, and a stop codon (TGA) downstream of the insertion. ORFs cloned from ASKA plasmids replace the N-terminal methionine of the predicted ORF with Met-Ala-Leu-Arg-Ala, and append Cys-Leu at the C-terminus.
3. Other potentially useful restriction sites have been included in the MCS as well as an RBS consensus positioned 8 bp upstream of the ATG used for ORF's derived from 1. And 2
4. The ability to select for insert-containing constructs through the linearization of parental contaminants by cleavage with the rare- cutters SwaI and PmeI contained in the MCS.
5. Two routes for the production of 12 base overhangs by PCR using universal primers: A) PCR followed by nicking using the engineered enzymes Nb.BbvCI and Nb.BtsI. B) PCR followed by removal of a primer-encoded uracil residue using BioLab's User^{TM} system. Primer positions and sequences are indicated for both.

A/B cassette

Figure 1



B/A cassette

PCR method A: AB is amplified with prA1 and prB1 producing blunt ends that terminate with A sequence (5') and B sequence (3'). that are nicked with Nb.BbvC1 and Nb.BtsI. BA is amplified with prB2 and prA2 and treated as above.

```
prA1 5'-GGAAGAGCGGGCTGAGG     prB1 5'-GCTGGCGTCTCGCACTG
prB2 5'-CGAGACGCCAGCTGAGG     prA2 5'-GCCCGCTCTTCCCACTG
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PCR method B: AB is amplified with prA1u and prB1u. BA is amplified with prB2u and prA2u.
Nicking occurs at uracils using User TM

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prA1u 5'-AGGAAGUACGGUCTGAGGAGGT   prB1u  5'-AGCTGUAGTATUGCACTGCAG
prA2u 5'-AATACUACAGCUGAGGAGGT     prB2u  5'-ACCGUACTTCCUACTGCAG
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pAB /pBA plasmid sequences

> pAB

ctagctgatcttttctacggggtctgacgctcagtggtaccaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcaccta
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