Methylase-assisted subcloning for high throughput BioBrick assembly

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The BioBrick standard makes possible iterated pairwise assembly of cloned parts without any depletion of unique restriction sites. Every part that conforms to the standard is compatible with every other part, thereby fostering a worldwide user community. The assembly methods, however, are labor intensive or inefficient compared to some newer ones so the standard may be falling out of favor. An easier way to assemble BioBricks is described herein. Plasmids encoding BioBrick parts are purified from *Escherichia coli* cells that express a foreign site-specific DNA methyltransferase, so that each is subsequently protected *in vitro* from the activity of a particular restriction endonuclease. Each plasmid is double-digested and all resulting restriction fragments are ligated together without gel purification. The ligation products are subsequently double-digested with another pair of restriction endonucleases so only the desired insert-recipient vector construct retains the capacity to transform *E. coli*. This 4R/2M BioBrick assembly protocol is more efficient and accurate than traditional workflows including 3A assembly. It is also much easier than gel purification to miniaturize, automate and perform at high throughput. As such, it should streamline DNA assembly for the existing community of BioBrick users, and possibly encourage others to join.

Manuscript for submission as an article to PeerJ, April 21, 2020

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Abstract

The BioBrick standard makes possible iterated pairwise assembly of cloned parts without any depletion of unique restriction sites. Every part that conforms to the standard is compatible with every other part, thereby fostering a worldwide user community. The assembly methods, however, are labor intensive or inefficient compared to some newer ones so the standard may be falling out of favor. An easier way to assemble BioBricks is described herein. Plasmids encoding BioBrick parts are purified from *Escherichia coli* cells that express a foreign site-specific DNA methyltransferase, so that each is subsequently protected *in vitro* from the activity of a particular restriction endonuclease. Each plasmid is double-digested and all resulting restriction fragments are ligated together without gel purification. The ligation products are subsequently double-digested with another pair of restriction endonucleases so only the desired insert-recipient vector construct retains the capacity to transform *E. coli*. This 4R/2M BioBrick assembly protocol is more efficient and accurate than traditional workflows including 3A assembly. It is also much easier than gel purification to miniaturize, automate and perform at high throughput. As such, it should streamline DNA assembly for the existing community of BioBrick users, and possibly encourage others to join.

Key words: BioBrick assembly, DNA methyltransferase; methylase-assisted cloning; molecular cloning; laboratory automation; synthetic biology

Introduction

A bottleneck in many synthetic biology projects is the physical linkage of cloned synthetic genes ("parts") to each other to form longer functional assemblies ("devices"). The costs of gene synthesis, cloning and DNA sequencing have decreased significantly, but the reduction in per unit cost has been driven by improvements in miniaturization and automation rather than any change in underlying chemistry. Gene synthesis is still limited in length (≤ 2 kb), nucleotide composition, accuracy and yield (Kosuri & Church 2014). Many DNA assembly methods have been invented (Casini et al. 2015; Chao et al. 2015; Sands & Brent 2016; Vazquez-Vilar et al. 2018; Watson & Garcia-Nafria 2019), which suggests that none work well for every user.

The challenges of assembling cloned parts are not identical to those of ligating PCR products into linearized plasmids so different solutions are demanded. Many synthetic biologists have adopted cloning standards associated with type II or type IIS restriction enzymes. The BioBrick RCF[10] standard (Knight 2003) is most established. All BioBrick-compliant plasmids contain a characteristic pattern of sites recognized by type II restriction endonucleases (EcoRI-NotI-XbaI-insert-SpeI-NotI-PstI). Two such inserts can be combined by digesting one plasmid (recipient) with EcoRI and XbaI, and the other (donor) with EcoRI and SpeI. Alternatively, one plasmid (recipient) can be cut with SpeI and PstI, and the other with XbaI and PstI (donor). The overhangs of XbaI and SpeI digests products are compatible but anneal to form a "scar" not recognized by either restriction endonuclease, so the ligation of the desired insert to the desired recipient plasmid creates a new BioBrick-compatible plasmid. The virtue of this approach compared to *ad hoc* subcloning strategies is that an infinite number of inserts can be combined,

two at a time, without running out of unique restriction sites. The problem, and focus of this study, is that subcloning, particularly the gel purification step, remains labor-intensive and recalcitrant to automation.

Golden Gate assembly (Engler et al. 2009) was invented to solve this problem, though not without some cost. Type IIS restriction endonucleases recognize asymmetric sequences but cut outside of them. BsaI, for example, recognizes the sequence GGTCTC and introduces staggered cuts in both strands downstream regardless of sequence, creating 5' overhangs that are four nucleotides long. This ability to create up to 256 different sticky ends with a single enzyme enables concurrent restriction digests and ligations in a single pot. Golden Gate assembly can also be used to combine multiple parts in a single reaction. It does not leave the characteristic XbaI/SpeI scar of BioBrick assembly so it can be used to fuse open reading frames.

Golden Gate assembly is not, however, without drawbacks. Any BioBrick part can be adjoined to any other part using standard protocols, including those described here. In contrast, the sticky ends produced by BsaI and other type IIS restriction enzymes are only compatible with others designed to be complementary. Cloning standards for type IIS restriction endonucleases, such as MoClo (Weber et al. 2011), Phytobricks (Patron et al. 2015), Golden Braid (Sarrion-Perdigones et al. 2011) or Loop assembly (Pollak et al. 2019), facilitate some repurposing of parts for other devices. The MoClo standard, for example, employs nearly three dozen intermediate vectors, each with a unique pair of restriction sites and overhangs, for every category of parts (e.g. promoters, 5' upstream untranslated regions, open reading frames, terminators etc.) (Weber et al. 2011). In contrast, the original BioBrick standard employs a single

overhang and a single vector (Knight 2003) so it is much easier to comprehend and teach to new students. Moreover, the pairwise assemblies and discrete reaction steps of BioBrick assembly are relatively easy to troubleshoot when the reactions fail to work as designed,

I value the simplicity and universal part compatibility of BioBricks, so I invented a less labor intensive and automation-friendly way to assemble them. The concept that underlies my approach is easy to understand and implement. In nature every restriction endonuclease is paired with a corresponding site specific DNA modifying enzyme, most often a methyltransferase (Loenen & Raleigh 2014). Methyltransferases have previously been employed to protect sites within inserts (Lin & O'Callaghan 2018) or to mark sites at their ends (Chen et al. 2013). Here I describe the cloning of relevant methylases and their expression in a laboratory *E. coli* strains. Cells co-transformed with BioBrick-compatible plasmids thus decorate DNA with methyl groups at specific sites (Figure 1). The methylated plasmids are prepared and double digested in accordance with traditional cloning protocols, except at smaller scales. The restriction fragments are not gel purified but rather combined and reacted with T4 DNA ligase. The undesired ligation products, including the original parental plasmids, are subsequently cut by another pair of restriction enzymes. The desired ligation product (insert-recipient plasmid) is protected from both restriction enzymes, so it alone retains the capacity to transform *E. coli*.

Materials and Methods

Materials

The synthetic methylase genes used in this study (M.EcoRI, M.XbaI, M.Ocy1ORF8430P, M.PstI, M.AvaIII) were purchased from IDT (Coralville, IA) as gBlocks. Seakem LE agarose

was from Lonza Rockland (Rockland, ME) using lambda HindIII, 100 bp (New England BioLabs, Ipswich, MA) and 10 bp (Thermo Fisher) ladders as molecular size markers. Restriction enzymes, T4 DNA ligase and pure bacteriophage lambda DNA were from NEB (Ipswich, MA). TempliPhi rolling circle amplification kits were from Cytiva (Marlborough, MA). MinElute PCR purification and GeneRead Size Selection kits were from Qiagen (Valencia, CA), as was the QIAcube and the custom protocol (vide infra). *E. coli* OmniMax2 cells were from Invitrogen. Ethylenediaminetetraacetic acid (EDTA), L-arabinose and L-rhamnose were from Sigma Chemicals (St. Louis, MO); isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology (St. Louis, MO). LB broth (Miller) was from EMD Millipore (Billerica, MA) and Bacto-agar was from BD Difco (Franklin Lakes, NJ).

Methods

Subcloning via gel purification

Two plasmids were purified in triplicate (from cultures seeded with different colonies) via the Qiagen QIAprep spin miniprep kit. Recipient tagRFP-pUC (1 µg) was digested in 1x NEB CutSmart buffer (80 µL total reaction volume) by EcoRI-HF and XbaI (20 units each), thus releasing a short 15 base pair stuffer fragment ("snippet"); lacI-Ptac-lacO-pUC was similarly digested with EcoRI-HF and SpeI-HF in the same buffer, thereby releasing the lacI-Ptac-lacO insert and pUC donor plasmid. All restriction digests in this study were incubated overnight at 37° C unless otherwise stated. The desired fragments were separated from the undesired ones in 0.8% LE agarose gels; the bands corresponding to the recipient plasmid tagRFP-pUC and insert tagRFP were excised with a razor blade. The desired DNA was purified from the agarose slices via the QiaQuick gel extraction protocol. The fragments (20 fmol ~ 50 ng tagRFP-pUC or 25 ng

lacI-Ptac-lacO), alone or in combination, were reacted to T4 DNA ligase (3 Weiss units) in 1x NEB buffer containing 1 mM ATP (20 µL total reaction volume) overnight in temperature cycled reactions (30° C x 30 sec, 10° C x 30 sec) (Lund et al. 1996). The ligase was heat killed (10 min at 65° C), and the reactions (1 ng) were used to transform chemically competent OmniMax 2 cells (20 µL). All experiments employed the same batch of cells made competent by the classical method of Inoue et al. (Inoue et al. 1990). Transformation efficiency was 3 x 10^7/µg pUC19.

Tip Snip subcloning

The lacI-Ptac-lacO-pUC donor plasmid (1µg) in 1x NEB CutSmart buffer (80 µL total reaction volume) was shortened slightly by an extra restriction enzyme (20 units PstI-HF) that recognizes a site adjacent to those used to release the insert (20 units each of EcoRI-HF and SpeI-HF) (Matsumura 2017). The tagRFP-pUC recipient plasmid (1µg) was cut as usual (20 units each of EcoRI-HF and XbaI in 1x NEB CutSmart buffer, 80 µL total reaction volume). The small restriction fragments ("snippets") in both digests are denatured, annealed to exogenously added anti-snippet oligonucleotides (100 nM BioBrick suffix in the donor digestion, 100 nM BioBrick prefix in the recipient digestion), thereby inactivating their sticky ends, and eliminated via Qiagen GeneRead size selection silica spin column chromatography. The purified restriction fragments were ligated (20 fmol ~ 60 ng tagRFP-pUC, 90 ng lacI-Ptac-lacO + pUC, 50 nM PstI "unlinker") in temperature cycled NEB T4 DNA ligase buffer (20 µL total reaction volume) prior to heat killing and transformation of *E. coli* as described above.

3A assembly

A BioBrick-compatible plasmid that encodes chloramphenicol acetyltransferase, RP4 oriT-pUC57-mini-cat (2 µg) in 1x NEB CutSmart buffer (80 µL total reaction volume) by 20 units each of EcoRI-HF, PstI-HF and NotI-HF (so as to eliminate the sticky ends of its stuffer fragment), dephosphorylated in reactions with NEB Calf Intestinal Phosphatase. The lacI-Ptac-lacO-pUC donor plasmid (300 ng) in 1x NEB 2. 1 buffer (15 µL total reaction volume) was digested with 6 units each of EcoRI-HF and SpeI; the tagRFP-pUC donor plasmid was similarly digested with XbaI and PstI. The digests containing pUC-mini-cat recipient vector (60 ng), the lacI-Ptac-lacO and tagRFP-pUC inserts (50 ng each) were reacted in a thermocycler with 3 Weiss units of T4 DNA ligase in 1x NEB T4 DNA ligase buffer (10 µL total reaction volume).

Construction of DNA methyltransferase expression vectors

The methylase expression vectors (Prham-M.EcoRI-p15A-aadA, Prham-M.XbaI-p15A-aadA, Prham-M.Ocy1-p15A-aadA, Prham-M.PstI-p15A-aadA, Prham-M.AvaIII-p15A-aadA) were constructed as follows. BioBrick compatible DNA methyltransferase genes were synthesized without internal BioBrick restriction sites (EcoRI, NotI, XbaI, SpeI or PstI), cloned into IMBB2.4-pUC57-mini using restriction enzymes EcoRI and PstI, and sequenced. The p15A plasmid origin and spectinomycin resistance marker (aadA) were subcloned from pACYC Duet and pCDF Duet (EMD Millipore, Novagen) respectively into a BioBrick compatible plasmid. The intergenic region between rhaS and rhaB, which includes promoters and operators for both genes, was previously described (Matsumura 2017).

The p15A, aadA, Prham and methylase genes were assembled by a combination of traditional and Tip Snip BioBrick assembly. Leaky expression of M.XbaI or M.Ocy1ORF8430P

from BioBricks containing these parts prevented efficient digests of the plasmids with XbaI or SpeI-HF. Those plasmids were amplified *in vitro* by utilizing the TempliPhi rolling circle protocol. The resulting unmethylated amplification product was subsequently digested, and the desired part was gel purified and ligated to other parts. The BioBrick restriction enzymes (EcoRI, XbaI, SpeI and PstI) were eliminated by digesting the plasmids (or amplified versions of them) with XbaI and SpeI-HF, self-ligating the p15A-aadA-Prahm-methylase and using ligation reaction products to transform *E. coli* OmniMax2. All five methylase expression vectors, plus Prham-tagRFP-pUC, which was used to optimize the optimal concentration of glucose for auto-induction, have been deposited in the Addgene repository (ID 149338 – 149343).

2RM assembly

Methylated, uncut lacI-Ptac-lacO-pUC and tagRFP-pUC plasmids (240 ng each) were reacted with XbaI, SpeI (6 units each) and T4 DNA ligase (3 Weiss units) in 1x NEB CutSmart buffer supplemented with 1 mM ATP (25 µL total reaction volume) in a single pot reaction analogous to that of Golden Gate assembly (72 cycles of 5 min. at 37° C, followed by a nested 10 cycles of 30 sec at 10° C and 30 sec at 30° C). The reaction was incubated for another hour at 37° C, then heat killed for 10 min at 65° C; 1 ng of total DNA was used to transform 20 µL competent *E. coli* OmniMax 2 cells.

4R/2M (PstI)

M.EcoRI-protected lacI-Ptac-lacO-pUC (500 ng) was digested overnight at 37° C by 6 units of SpeI and 8 units of PstI in 1x NEB 2.1 buffer (25 µL total reaction volume). M.Ocy1-protected tagRFP-pUC was similarly digested by 8 units of XbaI and 12 units of PstI. Note that

PstI-HF cannot be heat-killed, nor is SpeI-HF fully active in NEB 2.1 buffer, so PstI and SpeI were utilized instead. The restriction enzymes were heat-killed (20 min at 80° C), and the restriction fragments (45 ng tagRFP-pUC, 10 ng tagRFP + pUC) were reacted to T4 DNA ligase (2.4 Weiss units in 1x NEB 2.1 buffer supplemented with 1 mM ATP, 20 µL total reaction volume) overnight in a thermocycler (600 cycles of 30 sec at 30° C, 30 sec at 10°C) . The ligase was heat killed by incubation at 65° C for 10 min. A 2 µL aliquot of each ligation was diluted into a 26 µL 1x NEB 2.1 buffer containing 8 units each of EcoRI-HF and SpeI. The post-ligation digest was incubated for 3 hours at 37° C, and 1 µL of the reaction was used to transform 20 µL of competent *E. coli* OmniMax 2 cells.

Results

Subcloning via gel purification as a gold standard

Established subcloning methods (Matsumura 2015) were initially applied to set quantitative benchmarks for efficiency (number of correctly assembled clones per ng ligated DNA) and accuracy (fraction of correctly assembled clones among total). Two plasmids, lacI-Ptac-lacO-IMBB2.4-pUC57-mini and tagRFP-IMBB2.4-pUC57-mini (hereafter abbreviated lacI-Ptac-lacO-pUC and tagRFP-pUC respectively) were selected as models for this study (Figure 2). Both comply with requirements for established BioBrick RFC[10] assembly protocols. Colonies of cells transformed with the desired assembly product, lacI-Ptac-lacO-tagRFP-pUC, turn pink due to leaky expression of the fluorescent marker protein. Throughout this study, the same *E. coli* strains, DNA purification techniques, restriction enzymes, ligases and reaction buffers were used, generally in accordance with manufacturer's instructions except as noted. Differences in outcome can thus be attributed solely to differences in assembly protocols.

231 Each cloning step was carried out in triplicate, starting with individual isolated bacterial
232 colonies; standard errors are reported as a measure of variation between experimental replicates.
233
234 The most labor-intensive steps of a traditional subcloning experiment are the separation
235 of restriction fragments via agarose gel electrophoresis, excision of bands corresponding the
236 desired fragments and the extraction of DNA from the agarose slice. Overnight incubations of
237 transformed bacteria, restriction digests and temperature cycled ligation reactions were rate-
238 limiting. The aim here was not to accelerate the workflow, but rather to decrease labor input and
239 increase throughput without compromising efficiency or accuracy. After restriction digests, gel
240 purification and ligation, transformation of *E. coli* with the ligation products led to the growth of
241 126 ± 44 pink colonies per ng; a minority of white colonies (11 ± 4 = 8%) grew on those LB-
242 ampicillin plates (Table 1). The background on control plates spread with cells transformed with
243 vector only ligations was low (7 ± 2 cfu/ng), which suggested that restriction digests were nearly
244 complete. The insert only ligation controls produced greater background (61 ± 27 cfu/ng), which
245 suggests that the insert was not effectively separated from the donor plasmid in this experiment.
246
247 Tip Snip subcloning
248 I previously described "Tip Snip" subcloning to circumvent preparative agarose gel
249 electrophoresis and gel extraction (Matsumura 2017). Briefly, the undesired donor plasmid is
250 shortened slightly by the action of an additional restriction enzyme that leaves it with a different
251 sticky end. The short restriction fragments in both digests are inactivated via denaturation and
252 annealing to complementary synthetic oligonucleotides and size selection spin column
253 chromatography. Ligations include a pair of complementary oligonucleotides that prevent self-

ligation of the unwanted donor plasmid. Transformation of chemically competent *E. coli* cells with the ligation reactions produced 384 ± 61 pink cfu/ng, a three-fold improvement over the conventional approach, with less background in insert only ligation controls (9 ± 6 white cfu/ng = 2.3%, three-fold more accurate that of gel purified DNA, Table 1). These results are consistent with the hypothesis that agarose contains inhibitors of T4 DNA ligase, and that restriction enzymes can distinguish desired and undesired restriction fragments more efficiently than does gel electrophoresis.

3A assembly

The 3A BioBrick assembly technique (Shetty et al. 2011) is currently recommended along with Loop Assembly (Pollak et al. 2019) by organizers of the International Genetically Engineered Machine (iGEM) competition. Briefly, both BioBrick inserts are cut from their respective donor plasmids and ligated into a third recipient plasmid that encodes a selectable marker different than those on the donors (Figure 2). This approach circumvents gel purification but has never worked well in my hands. Transformation of competent E. coli with 3A ligation products produced only 4 ± 1 pink colonies per ng, mixed with a similar number (5 ± 2) of white colonies (Table 1). The roughly equal numbers of pink and white colonies indicate that rigorous screening of the generally uncolored colonies produced by 3A assembly would normally be required.

The inefficiency and inaccuracy of 3A assembly is not surprising when all reaction intermediates and products are considered. Cartoons that depict 3A assembly typically only show the desired inserts and recipient plasmid, but the undesired donor plasmids and stuffer fragments

277 are still T4 DNA ligase substrates (Figure 3). The lacI-Ptac-lacO restriction fragment, for
278 example, is supposed to ligate to the pUC-cat fragment because both have EcoRI sticky ends.
279 Yet each of these fragments could also ligate to other copies of themselves, the EcoRI-cut donor
280 plasmid or the EcoRI-cut stuffer. A relatively small fraction of ligation products (2/5 * 2/5 * 2/6
281 = 8/150 = 5.3%) will be those that are desired; any incorrect product that includes the pUC-cat
282 recipient plasmid will enable a transformed cell to form a colony on LB-chloramphenicol plates
283 (Figure 4), as noted by the developers of the 3A assembly method (Shetty et al. 2011).
284
285 Methylase expression vectors
286 The aim of this study is to replace the gel purification step of subcloning by a
287 combination of site-specific DNA methylation and post-ligation restriction digestion (Spear
288 2000; Zeng et al. 1997). To realize this strategy, BioBrick compliant genes encoding the DNA
289 methyltransferases of the EcoRI, XbaI and PstI restriction modification systems were
290 synthesized, cloned into compatible plasmids and sequenced. The complete sequence of SpeI
291 methylase (M.SpeI) is not available on REbase (Roberts et al. 2010), so a putative ortholog
292 M.Ocy1ORF8430P (hereafter abbreviated M.Ocy1) was synthesized instead. Each DNA
293 methyltransferase gene was subcloned via traditional techniques downstream of the T5 (Bujard
294 et al. 1987), tac (de Boer et al. 1983) and rhamnose operon (Egan & Schleif 1993) promoters and
295 a strong ribosome binding site.
296
297 The promoter-methylase expression cassettes were subcloned into a simple plasmid
298 consisting only of the p15A replication origin, which is low in copy number and compatible with
299 more common plasmids that encode the pUC origin, and streptomycin 3"-adenylyltransferase

(aadA) selectable marker. The new expression plasmids (promoter-methylase-p15A-aadA) confer resistance to streptomycin and spectinomycin. They don't contain any of the restriction sites normally used for BioBrick assembly (e.g. EcoRI, XbaI, SpeI or PstI) so they won't release any restriction fragments that would interfere with any downstream subcloning steps.

The *in vivo* methylase activities produced by these expression vectors was tested as follows. *E. coli* strain OmniMax 2 was co-transformed with each vector and another BioBrick compatible plasmid, propagated to mid-log culture and induced (either with IPTG or L-rhamnose) for three hours. The plasmids were purified and reacted with restriction endonucleases including the one normally associated with each DNA methyltransferase in wild-type bacteria. The degree of protection was assessed by comparing the mobilities in agarose gels of plasmids that were uncut, completely cut by a restriction endonuclease unrelated to the methylase or protected at least in part by *in vivo* methylation. For example, agarose gel electrophoresis showed that lacI-Ptac-lacO-pUC purified from *E. coli* carrying Prham-M.XbaI-p15A-aadA was digested by SpeI but mostly resistant to XbaI. Conversely, tagRFP-pUC protected by Prham-M.Ocy1-p15A-aadA was digested with XbaI but mostly resistant to SpeI (Figure 6).

The rhamnose promoter, reputedly the weakest of the three tested, proved most reliable for consistent and complete *in vivo* methylation. I speculate that DNA methyltransferases that are site-specific at moderate concentrations become toxic to host cells when over-expressed (Bandaru et al. 1996). Extended over-expression could thus favor the accumulation of mutations beneficial to transformed cells but unwanted by human scientists. Induction of transformants at

mid-log phase is itself labor-intensive, as cultures propagated in parallel don't always grow at the same rate, so an auto-induction protocol was developed. The rhamnose promoter is regulated by catabolite repression as well as by L-rhamnose. The plasmid Prham-tagRFP-pUC (Matsumura 2017) was used to transform *E. coli* OmniMax 2. Limiting amounts of glucose were added to saturating concentrations of L-rhamnose (0.1%) in LB medium supplemented with ampicillin. Commercial LB contains varying quantities of glucose, but for the addition of 0.001% glucose to 0.1% L-rhamnose led to maximum tagRFP expression as measured in a microtiter plate spectrofluorimeter. Autoinduction under those growth conditions led complete *in vivo* methylation when the methylase expression vectors were used instead.

The other lesson inferred from the *in vivo* methylation experiments was that M.PstI is rarely able to methylate plasmids within *E. coli* cells as completely as M.EcoRI, M.XbaI or M.Ocy1. Each of these methylases evolved in a different bacterial species so it isn't surprising that one of the four proved less active than the others in the alien environment of the *E. coli* cytoplasm. Most of our plasmids include an NsiI site adjacent to the PstI site. The sequence of M.NsiI was not available on REbase (Roberts et al. 2010) so the gene encoding the M.AvaIII ortholog was synthesized, cloned, sequenced and subcloned downstream of the rhamnose promoter. M.AvaIII proved much more adept at methylating plasmids in the *E. coli* cytoplasm than did M.PstI.

2RM assembly

The potential utility of the methylase expression vectors was demonstrated in a series of assembly experiments. The 3A BioBrick assembly protocol was so named because it employs

three plasmids, each with a distinct antibiotic selection marker. For similar reasons, 2RM assembly utilizes the components of two restriction modification systems: restriction endonucleases XbaI and SpeI-HF, and DNA methyltransferases M.XbaI and M.SpeI homologue M.Ocy1. In this embodiment, the lacI-Ptac-lacO-pUC was purified from triplicate cultures of auto-induced cells containing Prham-M.XbaI-p15A-aadA, while tagRFP-pUC was purified from cultures co-transformed with Prham-M.Ocy1-p15A-aadA. Each plasmid, lacI-Ptac-lacO-pUC and tagRFP-pUC, was cut with one of the two restriction enzymes and protected by methylation from the other. The linearized plasmids (Figure 7) react with T4 DNA ligase to form three sets of products. Most common, presumably, are the two original parental plasmids (Figure 2). Each of the linearized plasmids can also be ligated to other copies of themselves in one of two orientations to form homodimers (Figure 8). All contain unmethylated XbaI or SpeI sites, so they are susceptible to re-digestion by the restriction enymes in the reaction vessel. The linearized plasmids can also ligation to each other to form heterodimers (Figure 9). These products are resistant to both restriction endonucleases so they should accumulate over the course of the digestion/ligation reaction.

When *E. coli* were transformed with one nanogram of each ligation reaction, 118 ± 13 pink cfu/ng and 260 ± 25 white cfu/ng were observed on each plate (Table 1). Colony numbers on plates corresponding to control ligations with only one plasmid (20 ± 4 cfu/ng) or the other (4 ± 1 cfu/ng) were relatively low, suggesting that both methylation and restriction digestion was nearly complete. These results in combination show that restriction digestion of the parental plasmids and homodimeric ligation products was efficient, and that ligation to form heterodimeric products was also efficient. In principle, the ratio of pink to white colonies should

be 1:1, but the 1:2.2 ratio observed here could mean that the ligation product with the undesired orientation conferred greater fitness upon the host cell. The 2RM assembly technique shows how site-specific methylation could potentially enable one pot digestion/ligation reactions. The desired product contains two copies of the selectable marker and origin of replication (Figure 9), which complicates subsequent assembly reactions. Double digests of existing BioBrick-compatible plasmids enable directional cloning, which is more practical.

4R/2M (PstI) assembly

4R/2M assembly utilizes all four BioBrick restriction endonucleases (EcoRI, XbaI, SpeI and PstI). The two parental plasmids are each protected by a different site specific methylase. In 4R/2M (PstI) assembly both plasmids are cut with PstI and the recipient encodes the part that will end up on the 5' end of the desired ligation product. It is protected by M.EcoRI and double digested in NEB 2.1 buffer by SpeI and PstI (Figure 10). The donor plasmid that encodes the insert destined for the 3' end of the desired ligation product; it is protected from SpeI by M.Ocy1 and separately double digested in the same buffer by XbaI and PstI. The restriction endonucleases are subsequently heat-killed (20 min. at 80° C); the four digestion products are combined and reacted with T4 DNA ligase and ATP. The ligase is then heat-killed, and the ligation products (Figures 11 and 12) are diluted and further digested with EcoRI and SpeI.

EcoRI linearizes the donor plasmid and any ligation product that includes it. SpeI linearizes the other parental plasmid, so that the desired insert-recipient plasmid ligation product is the only viable construct that remains intact. Homodimeric constructs are produced in any ligation of fragments produced by type II restriction endonucleases (Figure 11), but none are

viable *in vivo* because plasmids are destabilized by large inverted repeats. Competent E. coli were transformed with the 4R/2M (PstI) assembly reactions, leading to the formation 177 ± 4 pink cfu/ng and only 2 ± 1 white cfu/ng (Table 1). Background colony counts on the control plates representing vector only (1 ± 0.2 cfu/ng) and insert only (1 ± 0.2 cfu/ng) ligations were very low. The 4R/2M (PstI) assembly is thus well suited for routine high throughput BioBrick assembly. I have subsequently used it to assemble 45 more pairs of BioBricks in batches of up to 18.

4R/2M (EcoRI) assembly

In 4R/2M (EcoRI), recipient tagRFP-pUC was methylated *in vivo* by M.PstI while lacI-Ptac-lacO-pUC was protected by M.XbaI prior to purification. The donor tagRFP-pUC (600 ng) was double digested by EcoRI-HF and XbaI (12 units each in 30 µL NEB CutSmart buffer); lacI-Ptac-lacO-pUC (600 ng) was similarly digested with EcoRI-HF and SpeI-HF (Figure 13). The restriction enzymes were heat-killed (20 min. at 80° C) and the restriction fragments (50 ng tagRFP-pUC, 90 ng lacI-Ptac-lacO) were mixed and reacted overnight in a thermocycle with T4 DNA ligase (3 Weiss units in 25 µL NEB CutSmart buffer supplemented with 1 mM ATP). The enzyme was heat-killed (10 min. at 65° C), and the ligation product (1 ng/µL) digested with 8 units each PstI-HF and XbaI in NEB CutSmart buffer (Figures 14 and 15). The transformation of competent *E. coli* cells produced only 19 ± 7 pink colonies, significantly less than the 4R/2M (PstI) experiment with the same plasmids, and 8 ± 6 white colonies per ng (Table 1). As previously noted, M.PstI does not methylate *in vivo* as reliably as our other DNA methyltransferases.

The assembly was repeated, except that the tagRFP-pUC plasmid was reacted *in vivo* with M.AvaIII instead of M.PstI. M.AvaIII catalyzes the methylation of NsiI sites, which exist in most BioBrick compatible plasmids in my lab (Matsumura 2017). NsiI produces sticky ends compatible with those of PstI so it offers a good comparison. This assembly, after digestion with NsiI and XbaI, produced 299 ± 91 pink colonies and only 12 ± 3 white colonies per ng (Table 1). This improved result in consistent with the hypothesis that 4R/2M assembly can be limited by the degree to which the populations of plasmids purified from *E. coli* are methylated.

Discussion

The assembly protocols described here could be further improved in several ways. The 4R/2M (EcoRI) is more efficient when M.AvaIII expression vectors were employed instead of those that produce M.PstI. Not all BioBrick compatible plasmids contain NsiI sites, so *in vivo* M.PstI activity could be enhanced, either by directed evolution (using *in vitro* PstI activity as a selection), co-expression with the PstI restriction endonuclease (as in the wild-type operon) or by identifying an M.PstI ortholog that is more active in the *E. coli* cytoplasm. Another alternative is to clone and express another site-specific DNA methyltransferase that protects some other site that is common in plasmid backbones but very rare within inserts. The tactic of using pairs of methylases to protect desired insert-recipient plasmids from double digests following ligation need not be restricted to BioBrick assembly. It could potentially be generalized to streamline other kinds of subcloning experiments if the relevant DNA methyltransferase expression vectors were available.

The 2RM assembly method is a single pot reaction for the restriction digestion and ligation of BioBrick parts, analogous to Golden Gate assembly except that half of the recombinant plasmids are ligated in the undesired orientation. A directional one pot BioBrick assembly is conceivable, but not with existing plasmids. It's not clear that the development of such a method is warranted. If an assembly standard changed every time assembly techniques improved it would defeat the purpose of creating any standard. Moreover, single pot BioBrick assemblies would require restriction fragments that were multiply methylated or otherwise protected from more than one restriction endonuclease at a time. Such a reaction could probably be made to work, but the combination of multiple restriction endonucleases and T4 DNA ligase in a single reaction vessel would only eliminate mixing two steps (heat killing restriction enzymes, ligation reaction setup) of the 4R/2M protocol. Automation of these steps, and those that precede and follow, would be easier to achieve and effect greater reduction in labor cost.

Conclusions

The existing 4R/2M (PstI) BioBrick assembly is less labor-intensive and easier to scale up than is the traditional gel purification approach. It is more efficient and accurate than is 3A assembly and requires less reagents than does Tip Snip subcloning. The value of the labor savings is proportional to the number of assemblies that can be conducted in parallel. The 4R/2M procedure was not designed to match the convenience of single pot multiplex digestion/ligation Golden Gate assembly reaction, but BioBrick assembly experiments are arguably easier to design and debug. The BioBrick standard thus remains better suited for the high school and undergraduate students who participate in iGEM competitions. The throughput of 4R/2M BioBrick assembly is mostly limited by the numbers of plasmid minipreps that users can perform

in parallel. The quantity of plasmid required is relatively low (≤ 400 ng/digest, as opposed to 1-2 µg for gel purification or Tip Snip) because none is lost during subsequent spin column chromatography. This methodological advance should thus accelerate the work of the BioBricks user community and encourage others to join.

Funding

IM was supported by NSF grants (MCB 1359575, MCB 1413062).

Acknowledgements

IM thanks Shanthi Hegde, Srikusumanjali Pinnamareddy (both from Lambert High School, Suwanee, GA), Grace Yan and Emily Yang (both from George Walton Comprehensive High School, Marietta, GA) for beta-testing 4R/2M (EcoRI) BioBrick assembly.

Competing Interests Statement

The Author declares no competing interests.

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554
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Table 1(on next page)

Table 1. Colony counts (cfu/ng)

Table 1. Colony counts (cfu/ng)

Assembly protocol	Vector only	Insert only	Vector + insert (red)	Vector + insert (white)
Gel purify (EcoRI)	7 ± 2	61 ± 27	126 ± 44	11 ± 4
Tip Snip (EcoRI)	8 ± 2	9 ± 6	384 ± 61	11 ± 4
3A	0	0	4 ± 1	5 ± 2
2RM	20 ± 4	4 ± 1	118 ± 13	260 ± 25
4R/2M (PstI)	1 ± 0.2	1 ± 0.2	177 ± 4	2 ± 1
4R/2M (EcoRI)	0 ± 0	2 ± 1	19 ± 7	8 ± 6
4R/2M (EcoRI,NsiI)	1 ± 1	10 ± 4	299 ± 91	12 ± 3

Figure 1

Subcloning of a methylated insert into a methylated recipient plasmid.

(Far left) Donor (blue) or recipient (red) plasmids are purified from *Escherichia coli* strains that express foreign DNA methyltransferases that protect restriction sites C or D, respectively. Modified sites are shown in parentheses. (Middle left) Both plasmids are reacted with restriction enzymes A and B, thereby producing four fragments: (top to bottom) insert (blue, methylated), donor plasmid (blue unmethylated), stuffer (red unmethylated), recipient plasmid (red methylated). All the restriction fragments are ligated. Two recapitulate the parental plasmids (mixed blue and red at far left). (Middle right) Four are homodimers (fragments ligating to other copies of themselves, all blue or red). The insert homodimer resists further digestion but lacks any selectable marker or origin of replication. The recipient plasmid homodimer also remains circular, but it is a large inverted repeat, so it is not stable in *E. coli*. Four others are heterodimers (mixed blue and red). Polymeric concatemers (linear trimers, circular tetramers, etc.) also form at low frequency but are not shown. (Far right) Double digestion of the ligation products with restriction enzymes C and D linearizes almost all that are circular, except for the desired double methylated insert-recipient plasmid construct. It alone retains the capacity to transform *E. coli* efficiently.

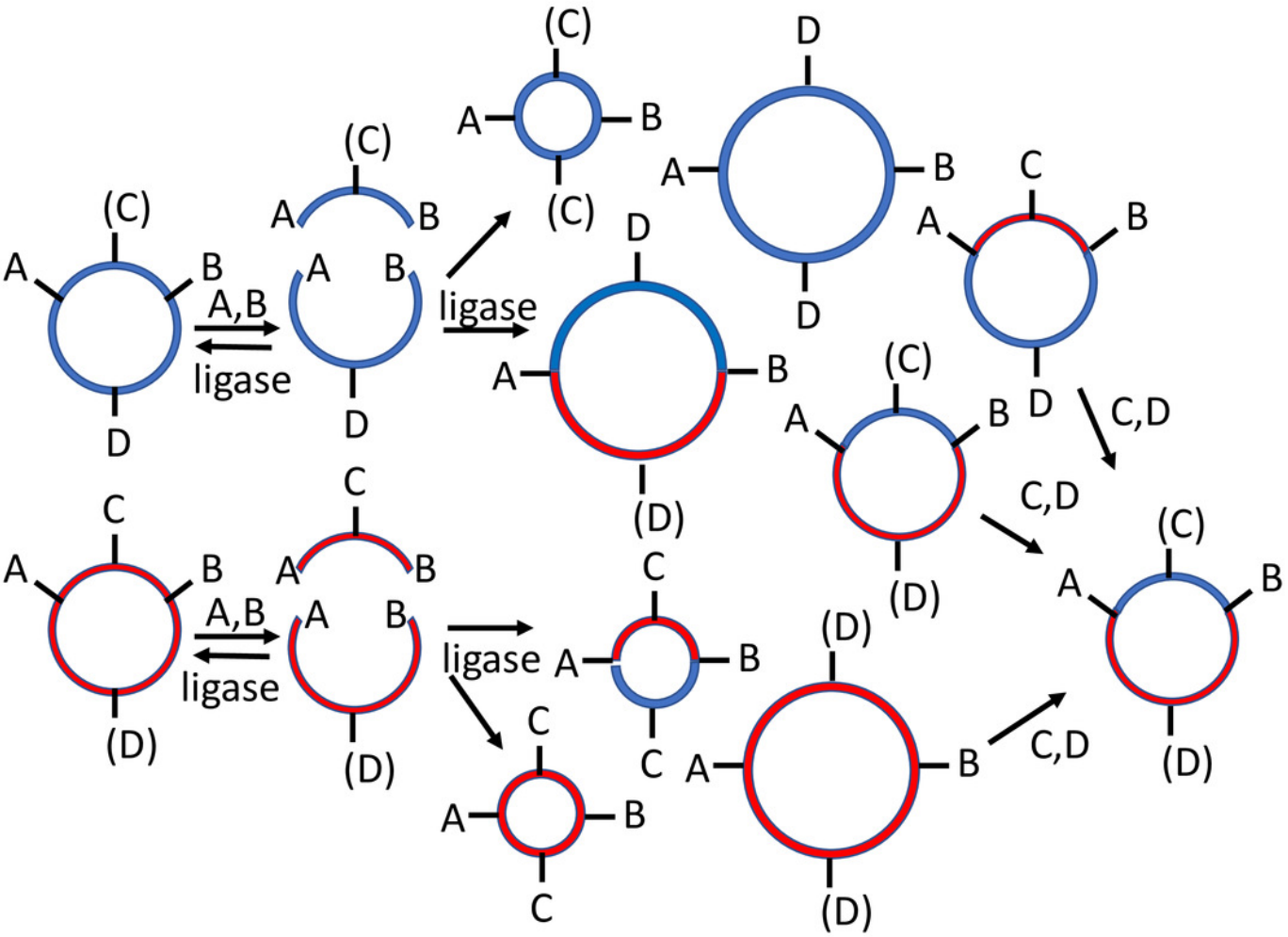
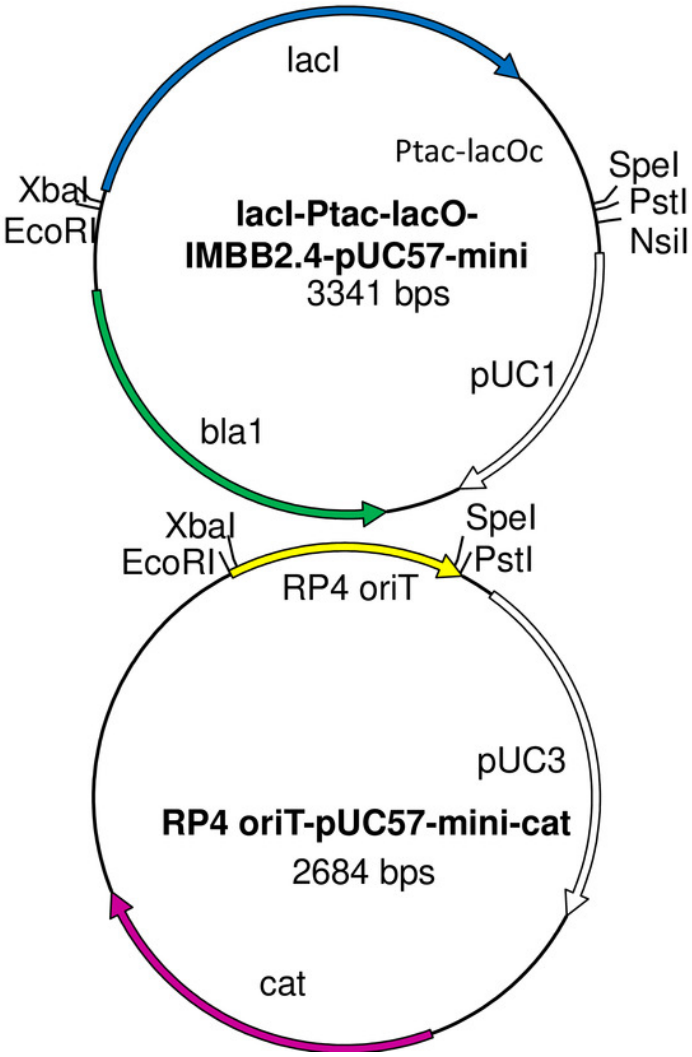

(C)
A
B
(C)
D
A
B
C
A
B
(C)
A
B
A,B
ligase
A
B
ligase
D
D
D
A
B
(D)
C,D
(C)
A
B
D
C,D
C
A
B
A,B
A
B
ligase
C
A
B
(C)
A
B
(D)
(D)
(D)
C
A
B
C
C
A
B
C
(D)
A
B
C,D
(D)

Figure 2

Model plasmids used in this study

(Top left) The lacI-Ptac-lacO insert includes a promoter that is somewhat leaky at high copy number. The IMBB2.4-pUC57-mini backbone, hereafter abbreviated pUC, is BioBrick-compatible and also includes an NsiI site downstream of PstI (Matsumura 2017) . (Top right) The tagRFP reporter protein can cause colonies to turn visibly pink, but only when the gene encoding it is subcloned downstream of a leaky or constitutive promoter. (Bottom) RP4 oriT-pUC-cat is a BioBrick compatible plasmid that confers resistance to chloramphenicol instead of ampicillin. RP4 oriT serves as a small stuffer in these experiments. In this study this latter plasmid is used only as a recipient plasmid (destination vector) for 3A assembly.


lacI
Ptac-lacOc
XbaI
EcoRI
SpeI
PstI
NsiI
lacI-Ptac-lacO-
IMBB2.4-pUC57-mini
3341 bps
pUC1
bla1
XbaI
EcoRI
SpeI
PstI
RP4 oriT
pUC3
RP4 oriT-pUC57-mini-cat
2684 bps
cat

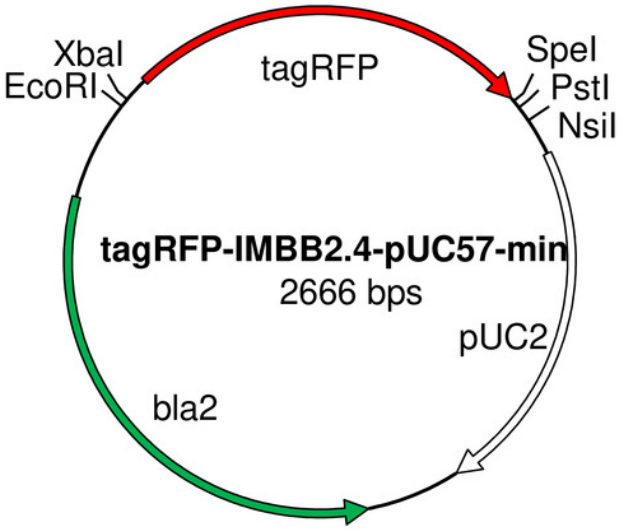

XbaI
EcoRI
tagRFP
SpeI
PstI
NsiI
tagRFP-IMBB2.4-pUC57-min
2666 bps
pUC2
bla2

Figure 3

3A restriction fragments

Double digests of three plasmids (Figure 2) produces six restriction fragments, three of which are desired (inserts 1 and 2, recipient plasmid) and three that are not (donor plasmids 1 and 2, stuffer). Each fragment can ligate to one of four, including another copy of itself (not shown).

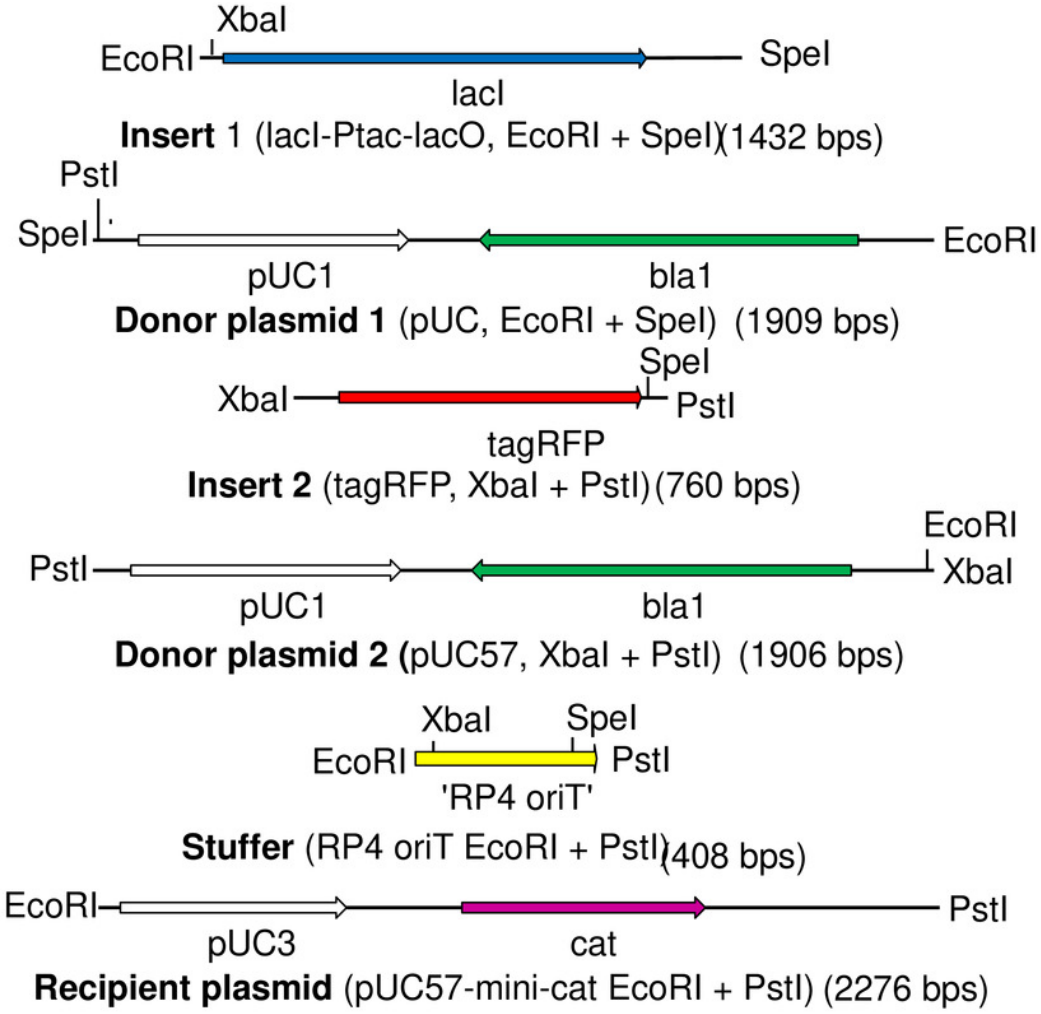


Figure 4

viable trimeric 3A ligation products

The six restriction fragments produced during 3A assembly can ligate to each other to produce a variety of products (not shown). Only those that are circular and contain the right selectable marker (chloramphenicol acetyltransferase in this case) are viable. Still, most *E. coli* colonies will carry undesired ligation products (top and bottom left) so colony screening is required to identify the desired construct (bottom right).

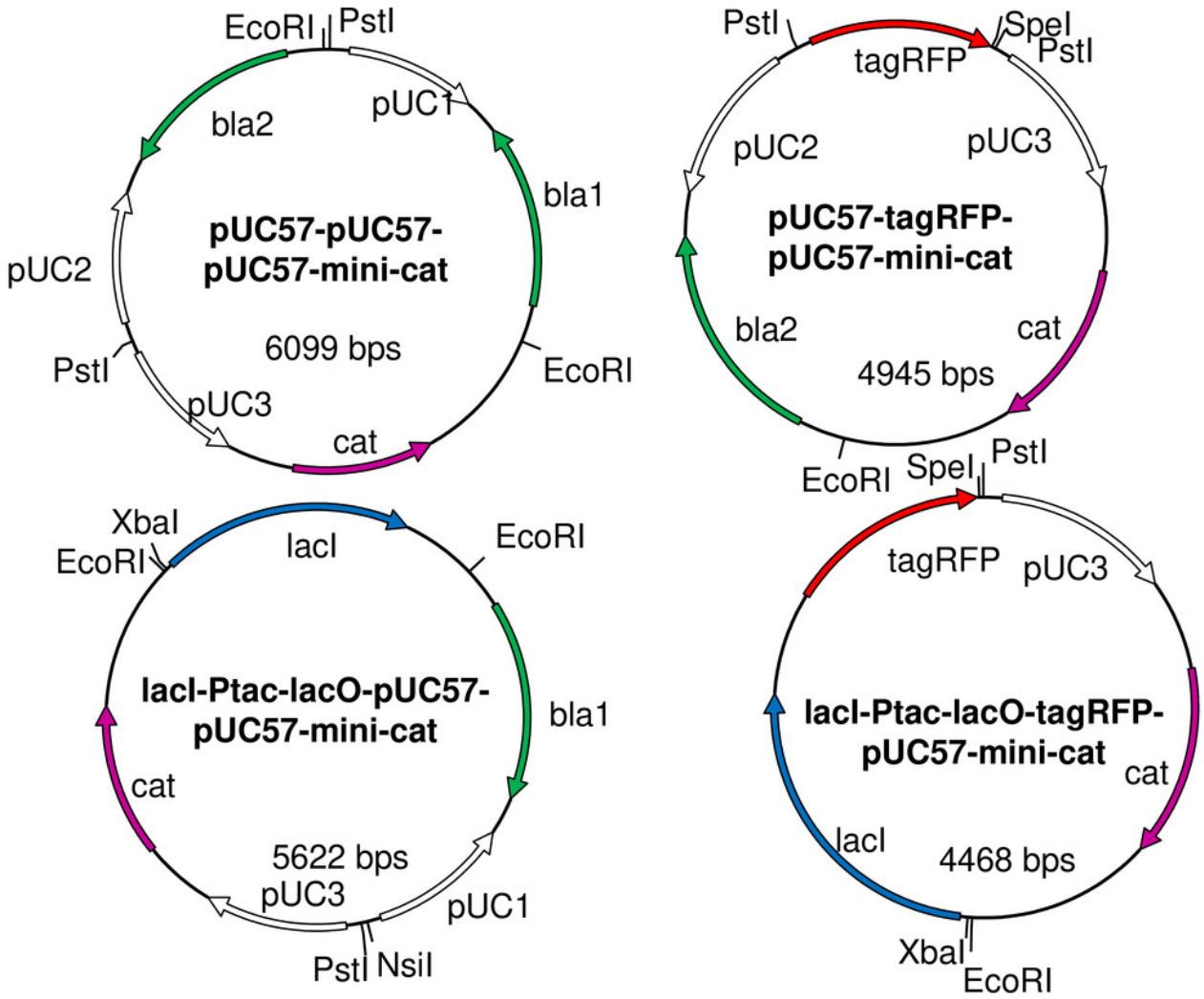


Figure 5(on next page)

DNA methyltransferase expression vector

Five expression vectors for production of recombinant DNA methyltransferases were constructed for this study. The version that expresses M.Ocy1ORF8430P, a putative ortholog of M.SpeI, is shown. The others are similar in design but express M.XbaI, M.EcoRI, M.PstI or M.AvaIII instead. Each plasmid utilizes the low copy number p15A origin (pACYC) and confers resistance to spectinomycin and is thus compatible with pUC plasmids that impart resistance to ampicillin, chloramphenicol or kanamycin. The DNA methyltransferase expression vectors do not contain any of the restriction sites employed in BioBrick assembly protocols (EcoRI, XbaI, SpeI or PstI), so they will not produce restriction fragments that ligate to those that are desired.

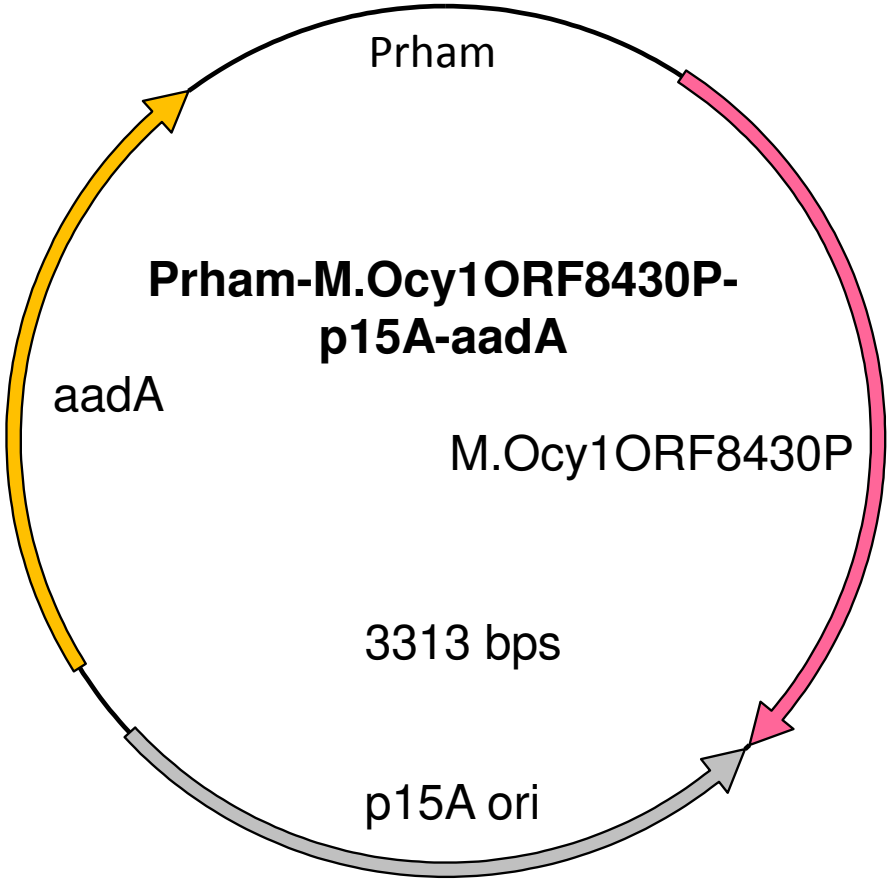

Prham
Prham-M.Ocy1ORF8430P-p15A-aadA
aadA
M.Ocy1ORF8430P
3313 bps
p15A ori

Figure 6

M.XbaI and M.Ocy1ORF8430P protect plasmids from XbaI and SpeI

Model plasmids lacI-Ptac-lacO-pUC and tagRFP-pUC were purified from triplicate cultures of *E. coli* OmniMax 2 co-transformed with Prham-M.XbaI-p15A-aadA or Prham-M.Ocy1ORF8430P-p15A-aadA (Figure 5) respectively. Each purified enzyme was reacted in vitro with XbaI or SpeI-HF, and the extent to which each was cut was assessed by agarose gel electrophoresis. Each of the DNA methyltransferases appears to protect co-transformed plasmid from its corresponding restriction endonuclease, and that protection is sequence specific.

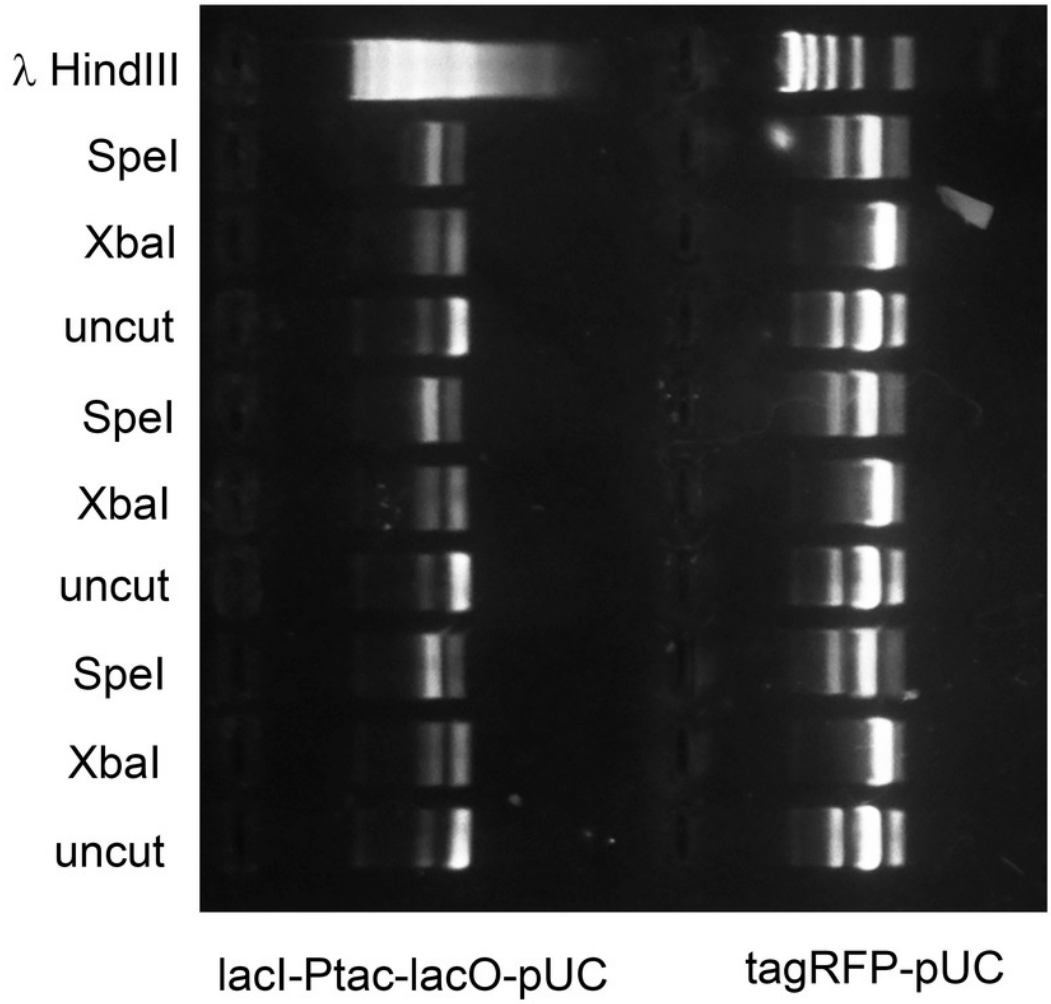

λ HindIII
SpeI
XbaI
uncut
SpeI
XbaI
uncut
SpeI
XbaI
uncut
lacI-Ptac-lacO-pUC
tagRFP-pUC

Figure 7

2RM assembly intermediates

Plasmids 1 (lacI-Ptac-lacO-pUC, methylated at its XbaI site) and 2 (tagRFP-pUC, methylated at its SpeI site) were mixed and digested with both XbaI and SpeI-HF. Each plasmid was protected from the action of one restriction enzyme and susceptible to the other (Figure 6).

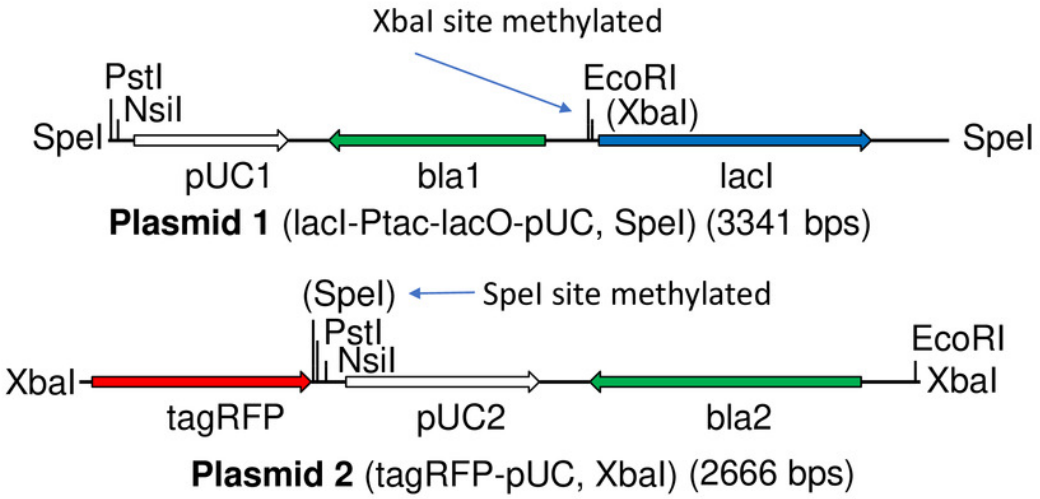


Figure 8

2RM homodimeric ligation products

Linearized plasmids 1 and 2 (Figure 7) can ligate to other copies of themselves in two possible orientations. All these ligation products remain susceptible to digestion by XbaI or SpeI-HF. The head to head dimers form inverted repeats that are unlikely to replicate in *E. coli*.

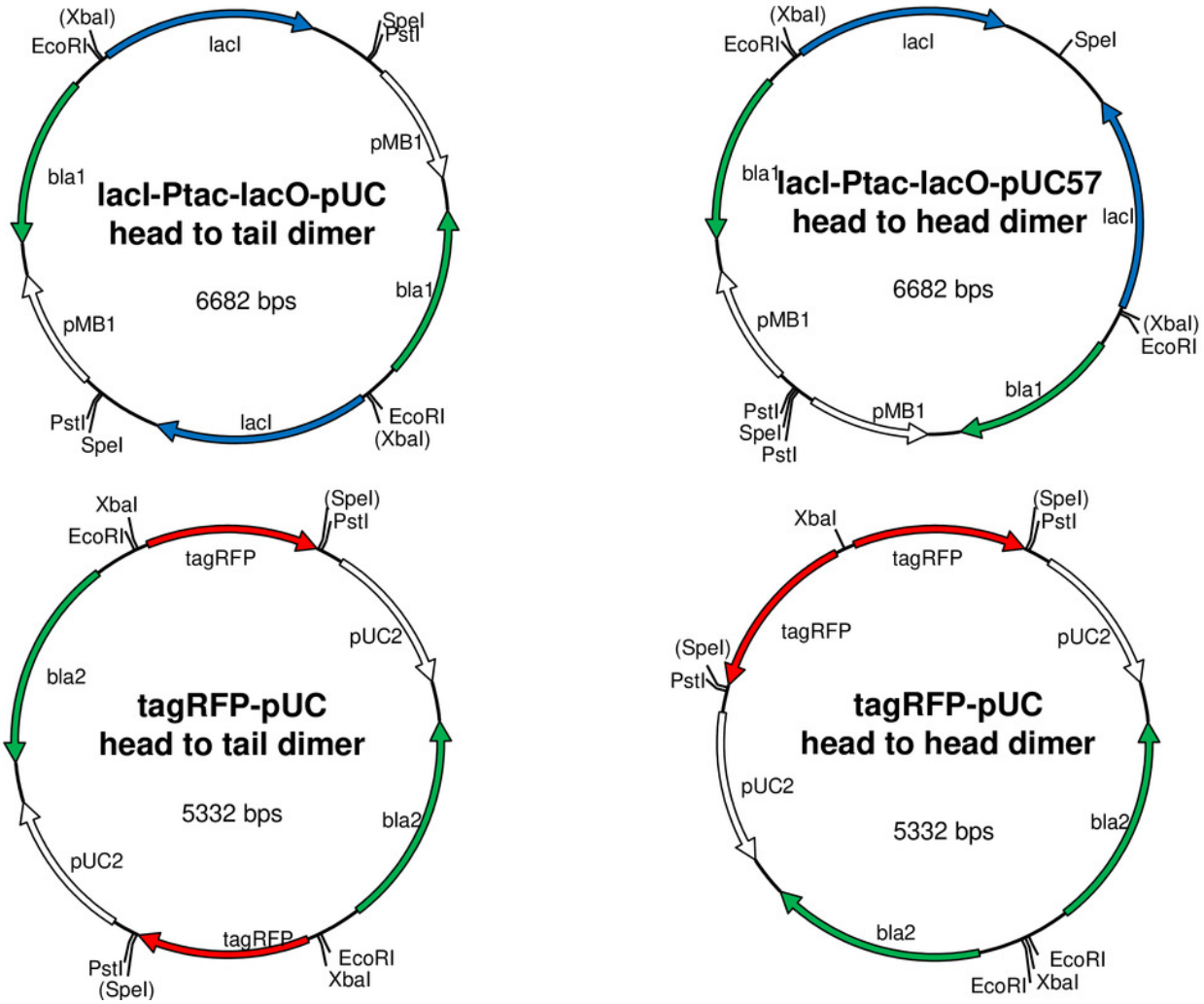
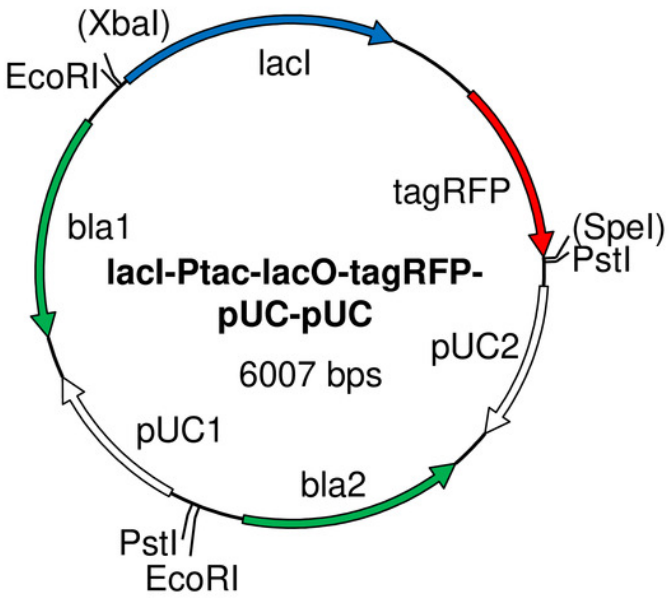


Figure 9

2RM heterodimeric ligation products

Linearized plasmids 1 and 2 (Figure 7) can ligate to each other in two possible orientations, both of which are resistant to XbaI or SpeI-HF. Both products can transform *E. coli* efficiently so colony screening will generally be necessary to distinguish desired clones (left) from those that are not (right). The desired construct (left) is functional but contains a surplus copy of the plasmid origin and selectable marker.



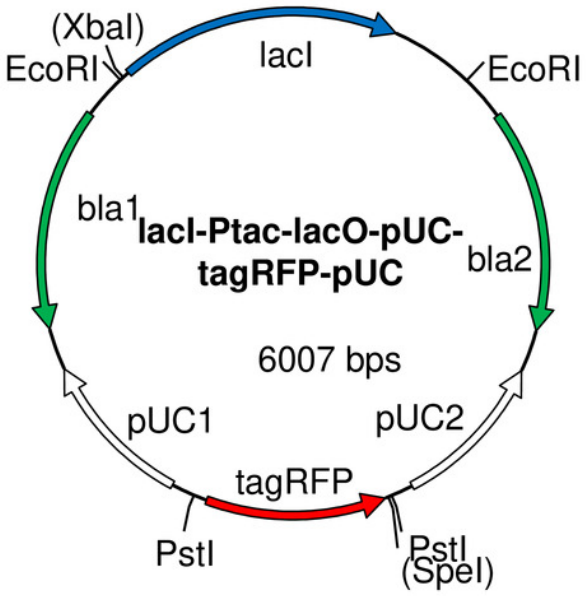


Figure 10

4R/2M (PstI) restriction fragments

In 4R/2M (PstI) BioBrick assembly, the donor plasmid, tagRFP-pUC, is purified from E. coli expressing M.Ocy1ORF8430P and subsequently digested with XbaI and PstI. The recipient plasmid, lacI-Ptac-lacO-pUC, is protected by M.EcoRI and digested with SpeI and PstI.

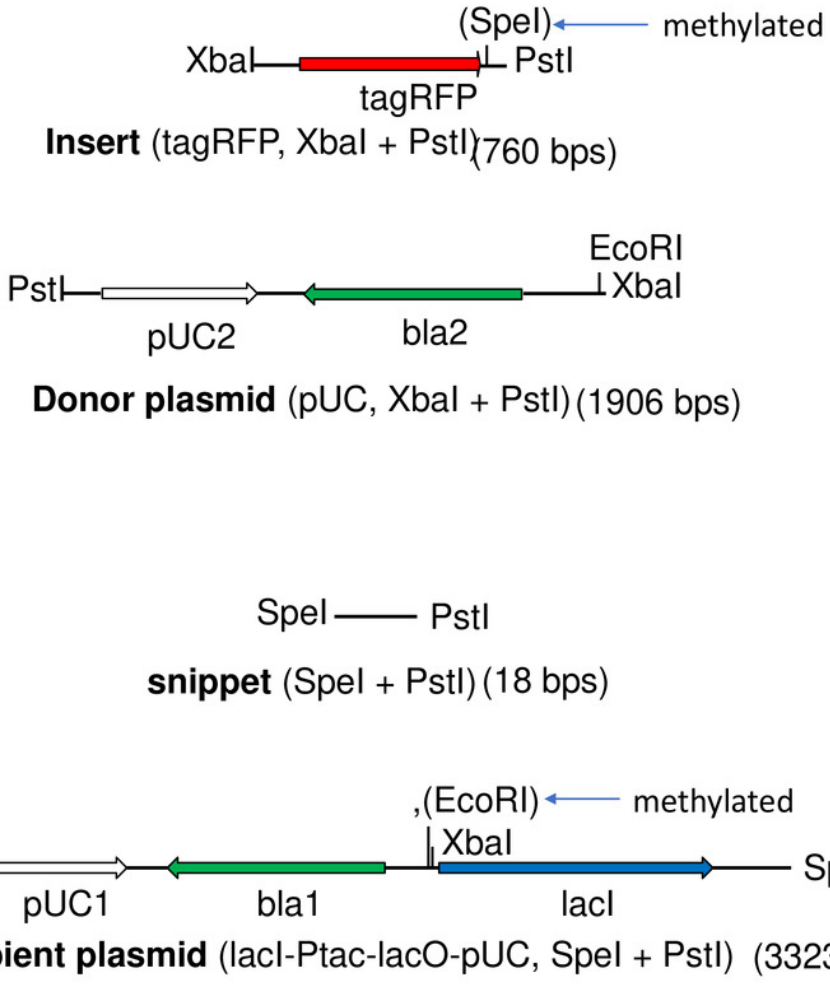


Figure 11

4R/2M homodimeric ligation products

The 4R/2M (PstI) restriction fragments (Figure 10) can ligate to other copies of themselves. None of the resulting homodimeric ligation products, however, replicate stably within *E. coli* because inverted repeats destabilize plasmids.

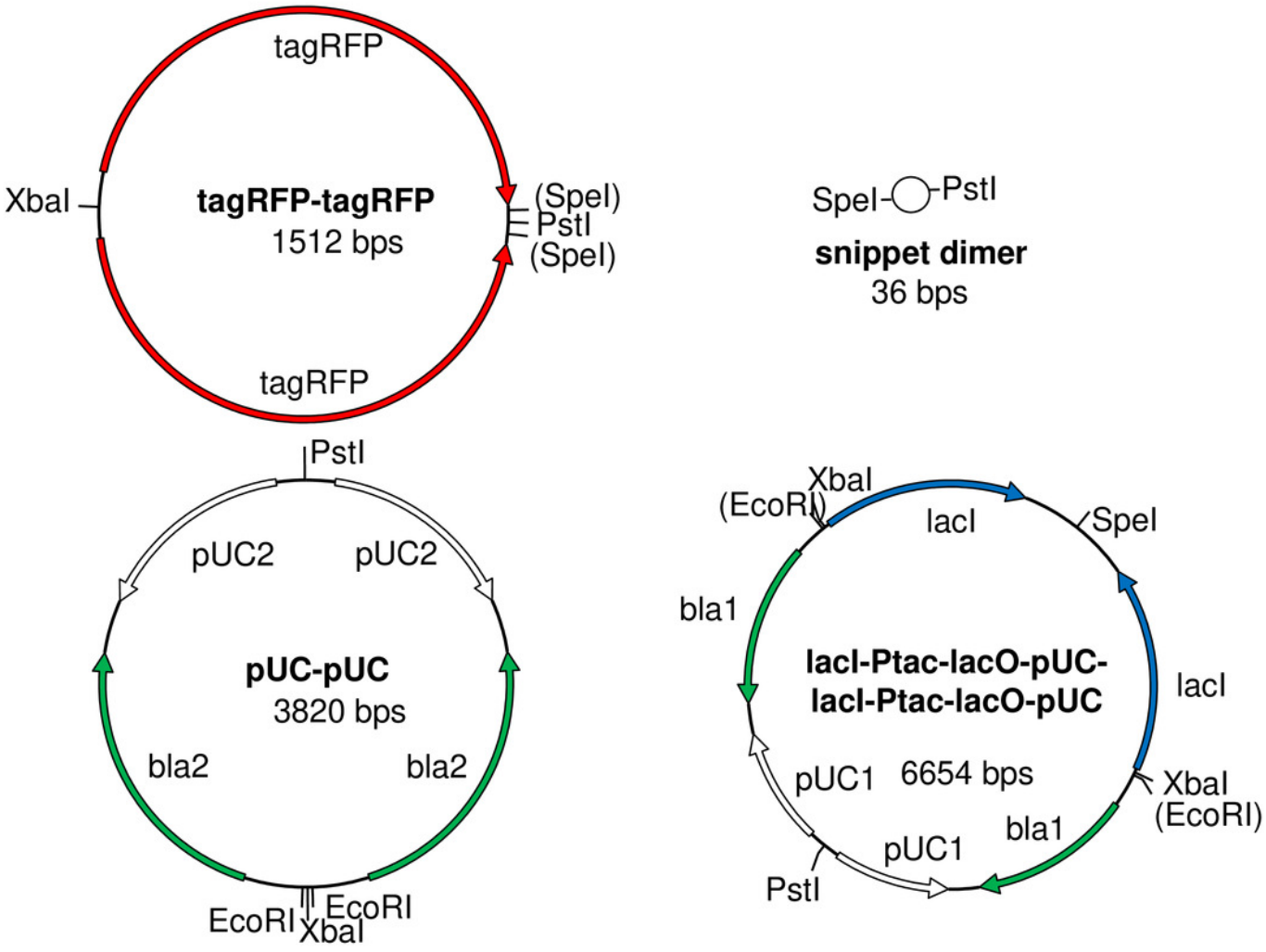
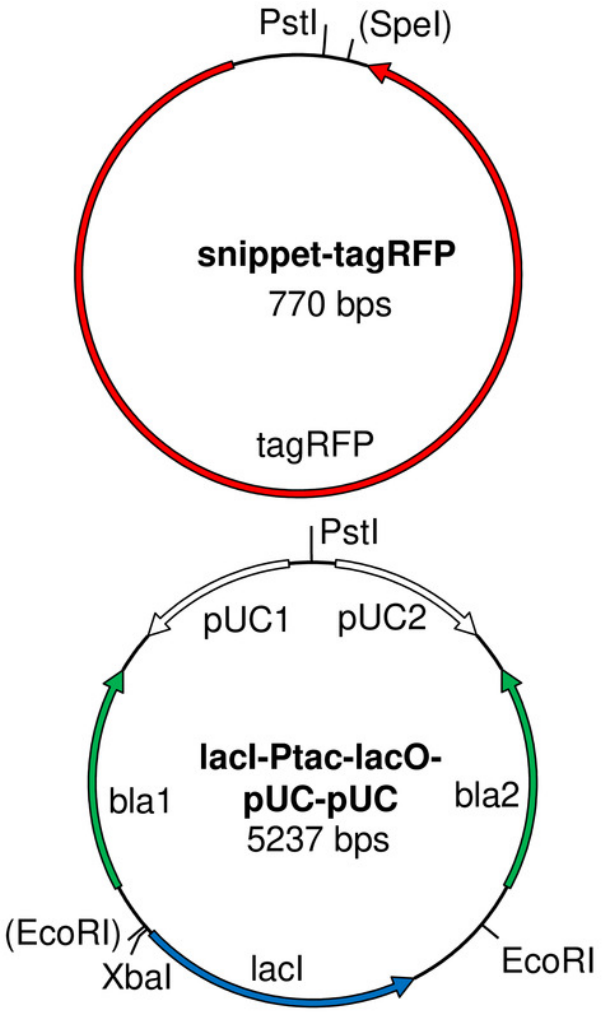


Figure 12

4R/2M P heterodimeric ligation products

Each of the four 4R/2M (PstI) restriction fragments (Figure 10) can ligate to one of two other fragments, creating four distinct ligation products. (Top left) One does not encode a selectable marker or origin of replication. (Top right and Bottom left) Two others remain susceptible to SpeI or EcoRI. (Bottom right) Only the desired ligation product includes a selectable marker and origin of replication and is also resistant to both SpeI and EcoRI. It alone retains its ability to transform E. coli after double digestion of 4R/2M (PstI) ligation products with these enzymes.


PstI
(SpeI)
snippet-tagRFP
770 bps
tagRFP
PstI
pUC1
pUC2
lacI-Ptac-lacO-
pUC-pUC
5237 bps
bla1
bla2
(EcoRI)
XbaI
lacI
EcoRI

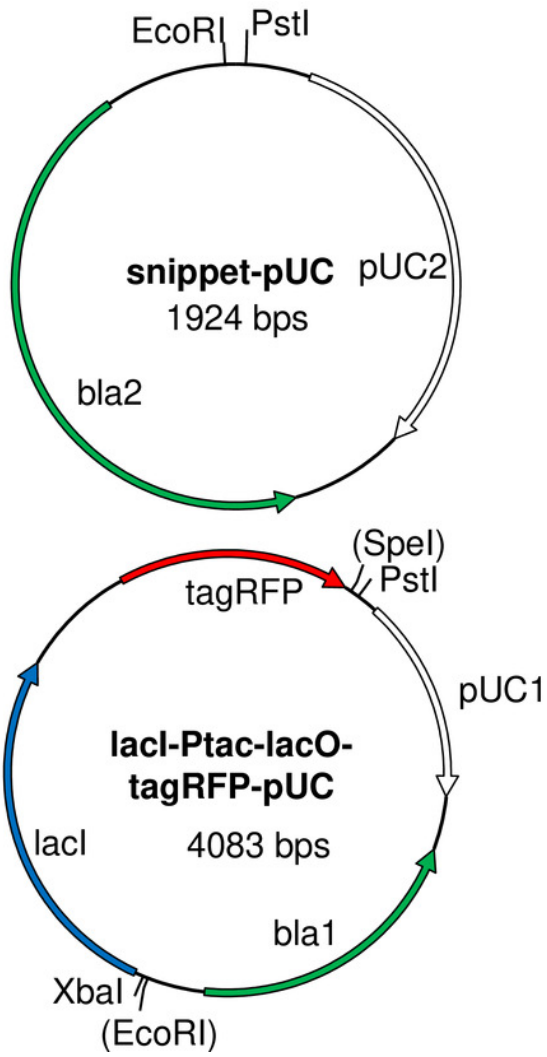

EcoRI
PstI
snippet-pUC
1924 bps
pUC2
bla2
(SpeI)
PstI
tagRFP
pUC1
lacI-Ptac-lacO-
tagRFP-pUC
4083 bps
lacI
bla1
XbaI
(EcoRI)

Figure 13

4R/2M EcoRI restriction fragments

In 4R/2M (EcoRI) BioBrick assembly, the donor plasmid, lacI-Ptac-lacO-pUC, is purified from *E. coli* expressing M.XbaI and subsequently digested with EcoRI-HF and SpeI-HF. The recipient plasmid, tagRFP-pUC, is protected by either M.PstI or M.AvaIII, which recognizes the NsiI site adjacent to PstI, and digested with EcoRI-HF and XbaI.

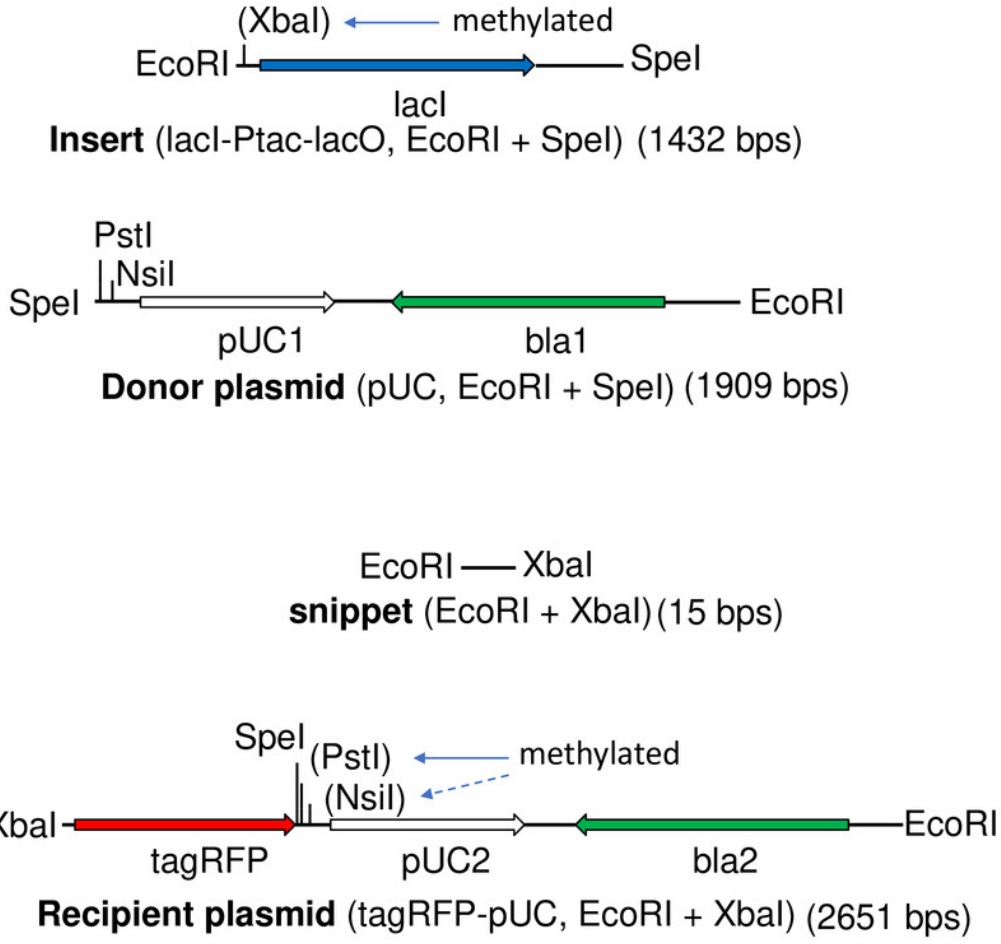


Figure 14

4R/2M EcoRI homodimeric ligation products

4R/2M (EcoRI) restriction fragments (Figure 13) ligate to other copies of themselves, although none of the homodimeric ligation products are viable in *E. coli*.

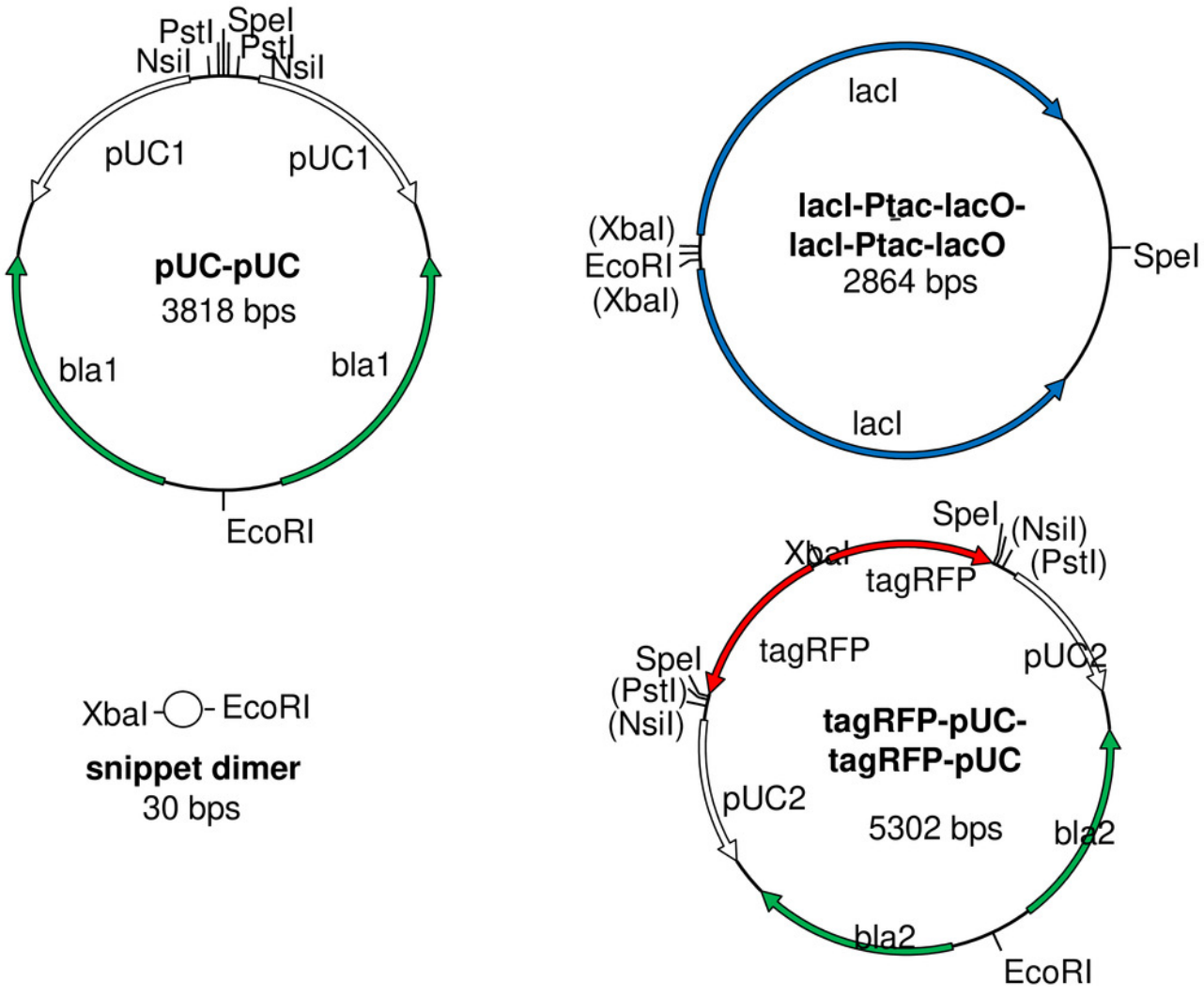


Figure 15

4R/2M heterodimeric ligation products

Each 4R/2M (EcoRI) restriction fragments (Figure 13) can ligate to one of three other fragments, creating six distinct ligation products. Two are the original parental plasmids (Figure 2). (Top left) Another does not include any selectable marker or origin of replication. (Top right and Bottom left) Two others remain susceptible to XbaI, PstI (if M.PstI was used to protect the recipient plasmid) or NsiI (if M.AvaIII was used). (Bottom right) The desired ligation product is the only viable plasmid resists digestion by both XbaI and PstI (or NsiI).