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In-Fusion BioBrick assembly and re-engineering

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ABSTRACT

Genetic circuits can be assembled from standardized biological parts called BioBricks. Examples of BioBricks include promoters, ribosome-binding sites, coding sequences and transcriptional terminators. Standard BioBrick assembly normally involves restriction enzyme digestion and ligation of two BioBricks at a time. The method described here is an alternative assembly strategy that allows for two or more PCR-amplified BioBricks to be quickly assembled and re-engineered using the Clontech In-Fusion PCR Cloning Kit. This method allows for a large number of parallel assemblies to be performed and is a flexible way to mix and match BioBricks. In-Fusion assembly can be semi-standardized by the use of simple primer design rules that minimize the time involved in planning assembly reactions. We describe the success rate and mutation rate of In-Fusion assembled genetic circuits using various homology and primer lengths. We also demonstrate the success and flexibility of this method with six specific examples of BioBrick assembly and re-engineering. These examples include assembly of two basic parts, part swapping, a deletion, an insertion, and three-way In-Fusion assemblies.

INTRODUCTION

Synthetic biology is an emerging discipline that aims to design and construct novel biological organisms programmed by genetic circuits. Many synthetic biologists assemble genetic circuits from standardized biological parts called BioBricks. Examples of BioBricks include promoters, ribosome-binding sites (RBS), protein or RNA-coding sequences, and transcriptional terminators. Currently every BioBrick is a physical DNA sequence on a circular plasmid that is stored and distributed by the Registry of Biological Parts (http://www.partsregistry.org) as lyophilized DNA in 384-well plate format. Standardized sequences on BioBricks enable Standard Assembly of two BioBricks via restriction enzyme digestion and ligation in an idempotent fashion (Figure 1a) (1–5). Standard Assembly involves digestion of two plasmids with different restriction enzymes that leave compatible sticky ends which can be ligated together into a new plasmid. This effectively replaces the restriction sites between the assembled parts with a 'scar' sequence, allowing for the new BioBrick to be later combined with other BioBricks. This standardized procedure takes much of the planning out of DNA fragment assembly since the same restriction enzymes can be used for every assembly reaction.

Currently several BioBrick assembly standards (http://openwetware.org/wiki/The_BioBricks_Foundation:RFC) have been proposed to improve upon the original BioBrick standard, largely due to the fact that this original standard produces an 8-bp scar between assembled BioBricks and hence does not allow for the creation of fusion proteins. Nearly all of these current assembly standards involve assembly by restriction enzyme digestion and ligation. There are also several PCR-based methods currently being used for DNA assembly that have the potential for standardization. These methods include In-Fusion (6,7), SLIC (8), T5 exonuclease recombination (9), USER (10), oligonucleotide assembly (11) and SOEing (12). The former four methods generally involve converting overlapping, blunt-end PCR products into fragments with sticky overhangs that can anneal to form circular plasmids, but the method for generating the overhangs differs. For example, the SLIC method (8) uses T4 DNA polymerase while the USER method (10) uses a uracil exonuclease. Unlike restriction digestion, the site at which the overhangs are created is generally not constrained by a specific sequence. The latter two methods involve overlapping oligonucleotides with a PCR-amplified vector (11) or extending overlapping PCR products (12) and do not use subsequent enzymatic treatment.

Here, we describe an alternative BioBrick assembly method that allows for BioBricks to be quickly assembled and re-engineered using the Clontech In-Fusion PCR Cloning Kit (6,7). The proprietary In-Fusion enzyme with exonuclease activity fuses together any PCR

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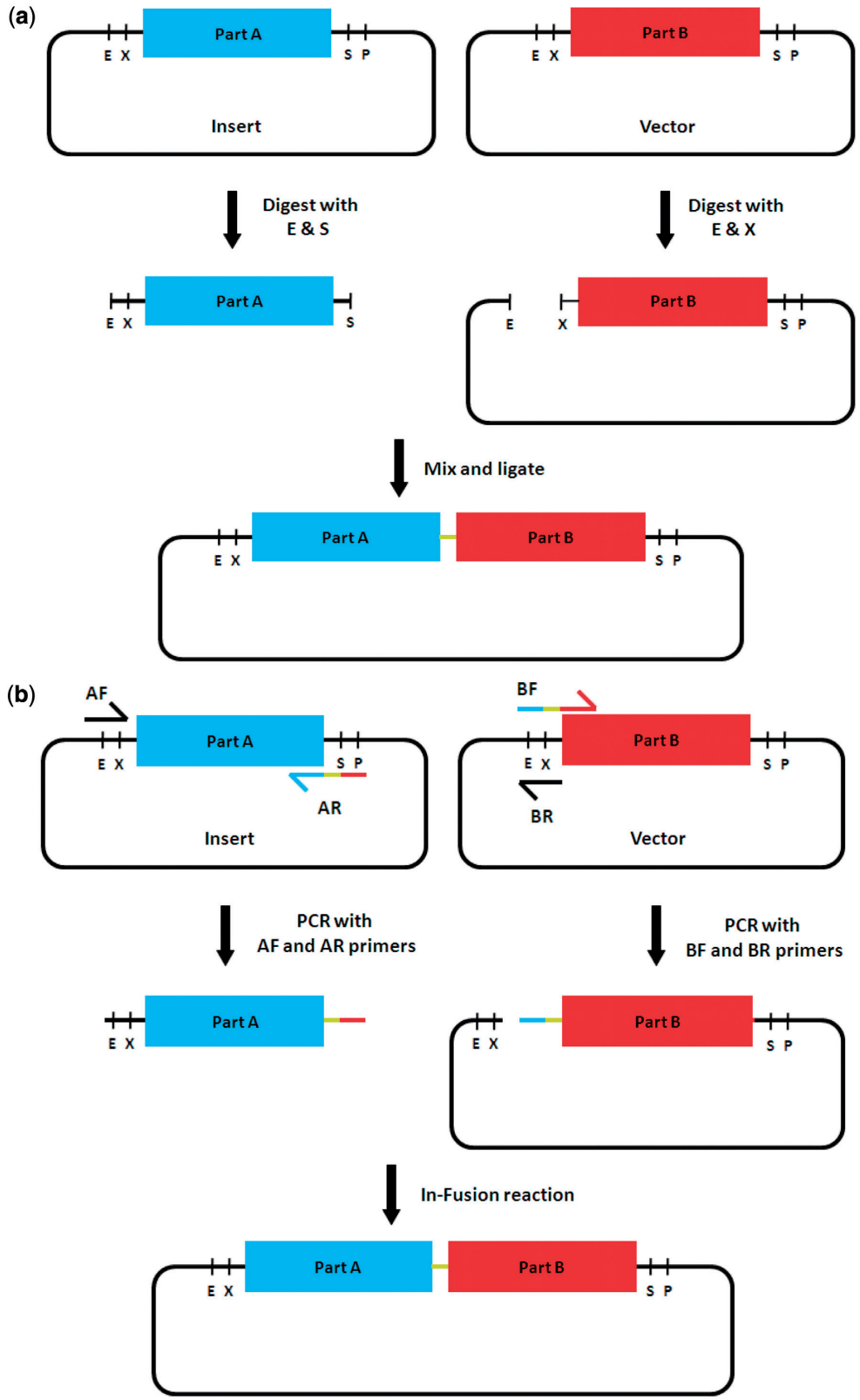

Figure 1. Standard assembly versus In-Fusion assembly. (**a**) Standard Assembly of two BioBricks (Parts A and B) involves restriction digestion and ligation. Both parts are on pSB1A2 vectors encoding ampicillin resistance. The Part A plasmid is digested with EcoRI (E) and SpeI (S), while the second plasmid is digested with EcoRI (E) and XbaI (X). SpeI and XbaI restricted fragments have compatible sticky ends for ligation. The desired digested fragments are gel purified and ligated together to create the assembled plasmid with both parts. A scar sequence is left between both parts that does not have the original restriction site and the restriction sites flanking the parts are maintained. (**b**) In-Fusion assembly of two BioBricks involves PCR, purification, and a subsequent In-Fusion reaction. Parts A and B are PCR-amplified (in this example the vector is amplified with Part B) and purified without gel extraction. Each assembly requires four primers, where two are specific to the junction (parts to assemble) and two are general vector primers. BioBrick Part A (blue) and Part B (red) are on pSB1A2 plasmids encoding ampicillin resistance. Primers described in 'Materials and Methods' section are color-coded to show their homology. The thick black line indicates BioBrick prefix or suffix homology on the pSB1A2 vector. The yellow sequence is the scar that is normally between parts after standard BioBrick assembly, if this is desired, or can be a linker sequence for a fusion protein. The purified PCR products are fused together in the In-Fusion reaction to create a circular plasmid. Restriction sites flanking the parts maintain the standard BioBrick format.

product with a linearized vector into a circular plasmid when the fragments share at least 15 bp of homology on both ends. To assemble two BioBricks, one PCR-amplified BioBrick needs to have homology on each end with the second PCR-amplified BioBrick (vector amplified with the BioBrick) to allow for the fragments to be fused together in the In-Fusion reaction (Figure 1b). More than two BioBricks can be fused together as shown in the examples below and four fragments have been successfully fused (6). This method can also be used to re-engineer BioBricks as shown in the examples below and we have also successfully used this method for vector replacement, site-directed mutagenesis and cumbersome transcriptional terminator re-engineering (results not shown).

In-Fusion Assembly greatly expands the possible circuits to construct since in our experience, the individual parts we want to construct into circuits often do not exist as BioBricks on their own or in the correct order. For this reason In-Fusion provides the flexibility to PCR-amplify any sequence from any part, then assemble these fragments together in one step. In our experience, the advantages of In-Fusion BioBrick assembly over Standard Assembly are that it is faster, more flexible, and has a high success rate (see Supplementary Figure S1 for a timeline and schematic overview for Standard and In-Fusion BioBrick Assembly). In-Fusion Assembly is fast once primers are available since BioBricks can be immediately PCR-amplified from parts extracted from the BioBrick Parts Distribution plate. Standard assembly requires an initial amplification of the BioBrick through transformation, overnight growth, and plasmid extraction. Standard assembly also requires tedious extraction of restricted DNA fragments from a gel, more intermediate enzymatic reactions, and more time to quantitate and optimize these reactions. In-Fusion assembly is more flexible in the sense that there is more control over the exact engineered sequence and mutations can be easily introduced with mutagenic primers (6). We have found this method to have a high success rate in that nearly every assembly reaction yields the desired construct. Standard assembly we have found to be less reliable.

The disadvantages of In-Fusion BioBrick assembly are that the specific assembly supplies are more expensive (~$15/assembly versus ~$5/assembly for Standard Assembly), custom primers are required, and occasionally there are mutations in assembled plasmids. Mutations in the construct may depend on a number of factors, including primer quality and the error rate of the polymerase. However, using reagents specified in the 'Materials and methods' section, we demonstrate here that such mutations are sufficiently rare that typically only a single putative construct needs to be sequenced. In general, In-Fusion assembly is ideal when re-engineering an existing BioBrick with many parts since there are less assembly reactions to perform, or when assembling a circuit with specific parts distributed among several BioBricks. On the other hand, if the BioBricks you want to assemble already exist as digested fragments in the freezer, then Standard Assembly may be ideal for this particular situation. The motivation for optimizing and adapting the In-Fusion method for BioBrick assembly is to improve the success rate, flexibility, and speed for constructing genetic circuits and create a new BioBrick assembly standard (13). Small synthetic biology labs that do not perform high throughput restriction enzyme digests and ligations will especially benefit from using this method.

MATERIALS AND METHODS

Primer design rules

For the In-Fusion reaction to work, the forward primer of the first PCR-amplified fragment must have at least 15-bp homology to the reverse primer of the second fragment, and vice versa. A longer homology length is used in this protocol because in our experience it works better than 15 bp. There are two simple primer design rules that allow for semi-standardized assembly, in the sense that even though the same components cannot be used repeatedly as in a standardized assembly method, following these rules will remove much of the planning required for performing In-Fusion Assembly reactions. The two rules are: (i) the reverse primer for Part A (AR) should be the last 20 bases Part A + scar (if wanted) + first 20 bases Part B (reverse complement of this entire sequence), and (ii) the forward primer for Part B (BF) should be the reverse complement of the AR primer. Since the primers are exactly complementary, this gives at least 40 bp of homology at the junction of Parts A and B in total. In contrast, the forward primer of Part A (AF) and the reverse primer of Part B (BR) do not need customization. These primers are specific to the standard Biobrick 'prefix' (immediately upstream of each part) or 'suffix' (immediately downstream of each part) and may be re-used when assembling different constructs. Of course optimization of the primers may improve chances of a clean PCR product. In the assembly examples described here using these primer design rules, we found that every PCR product amplified with a single amplicon (10/10 PCR products). One particular PCR reaction required a gradient on the primer annealing step during the PCR reaction to generate a single PCR product, but in general a 55°C annealing temperature worked well for all reactions. In general we found these primer design rules to be robust and other examples not described here have used these rules with success.

Primer design software tool

We have designed a software tool for In-Fusion assembly primer design (located at http://sys-bio.org/primerdesign/). This tool allows you to input the DNA sequences for each part to assemble, the scar sequence between the parts, and the length of overlap with the adjoining part (the default length is 20). When 20 bases of homology are added to the 5′-end of each of the primers, along with the 8-base scar, this gives a total junction homology of 48 bp. The program outputs the forward and reverse primers for the Part A and Part B junction (AR and BF primers). The primer sequences generated are based on the rule described above. A future version of this

tool will check if there are repeated sequences (such as a scar sequence) located at the 3′-end of the primer and if so, will extend the primer to two bases past the repeated sequence to ensure specificity. Other future additions will include T_m calculations, GC content, and other relevant primer design specifications. The vector-specific primers (AF and BR) are already standardized and do not need to be designed with this tool.

Maximizing success rates

There are a few additional guidelines to follow to maximize success with this method. First, do not use repeated part sequences (e.g. scar sequences, transcriptional terminators, etc.) on the 3′ end of your primer to avoid multiple PCR products. The primer design tool described above will eventually check for this. Second, it is better to PCR-amplify the vector (plasmid backbone) with the smaller part to assemble especially if the part is less than 100 bp since the molar ratio between both fragments should be as close as possible. Third, dilute the template DNA as much as possible to avoid 'background' plasmids from being transformed later (between 100 and 500 pmol DNA works well for miniprepped plasmids, while plasmids from the 2009 Parts Registry plate differ in concentration).

Primers designed and used in this study

All primers were ordered from Integrated DNA Technologies (IDT) unmodified with standard desalting at the 25 nmol scale. Four primers specific to the pSB1A2 vector (http://partsregistry.org/Part:pSB1A2) were designed and used in this study. In Figure 2a, the Part A forward primer (AF) is: 5′-TTCTGGAATTCGCG GCCGCTTCTAG-3′ (specific to the pSB1A2 non-coding sequence prefix + 5 bases upstream of the prefix). The Part B reverse primer (BR) is: 5′-CTAGAAGCGGCCGCGA ATTCCAGAA-3′ (reverse complement of the AF primer). In Figure 2b, the Part A forward (AF) primer is: 5′-TACT AGTAGCGGCCGCTGCAGGCTTC-3′ (specific to the pSB1A2 suffix + 5 bases downstream of the suffix). The Part B reverse primer (BR) is: 5′-GAAGCCTGCAGCGG CCGCTACTAGTA-3′ (reverse complement of Part A forward primer). AR and BF primers for both figures were customized depending on the parts to assemble or re-engineer. We used 48 bp of homology between parts for assembly reactions as described above except in the example shown in Figure 6 where we used 15 bp.

PCR and template DNA for PCR

Phusion High Fidelity PCR Mastermix was used for PCR in a 25 or 50 µl reaction volume. Reaction steps were used following the Phusion protocol. An amount of 100 pmol forward and reverse primers were added to each reaction. A volume of 1 µl of a 1:1000 diluted miniprepped plasmid or plasmid extracted from the Registry plate (~100–500 pmol total) was added to the PCR reaction for template DNA. PCR was performed in an Eppendorf Mastercycler EP S Gradient thermocycler according to the manufacturer's instructions with the annealing step of 55°C for 30 s. Eppendorf PCR tubes (0.2 ml) were used for all reactions. Digestion with DpnI after the PCR reaction may reduce background plasmids from being transformed later, but in our experience diluting the PCR template reduces background sufficiently that DpnI digestion is unnecessary. In most cases, this eliminates an extra step in the assembly process.

PCR product purification and quantitation

PCR products were purified with the Qiagen PCR Purification Kit and eluted with 30 µl of molecular grade water. PCR products were quantitated with a Nanodrop (Thermo Scientific).

In-Fusion reaction and transformation

In-Fusion reactions were performed using the recommended protocol with some extra details and exceptions noted here. A 2:1 insert:vector molar ratio was normally used with 100 ng of vector for two-way reactions and a 2:2:1 insert:insert:vector molar ratio was normally used with 100 ng of vector for three-way assemblies (exceptions noted below). The 10 µl volume consisting of insert, vector, and molecular grade water was transferred into the In-Fusion dry-down reaction tube and mixed by pipetting up and down several times. This reaction was transferred into a 0.2 ml PCR tube and incubated in a thermocycler for 15 min at 37°C followed by 15 min at 50°C. A volume of 30 µl TE Buffer (pH 8.0) was added to the tube and mixed by pipetting up and down several times. A volume of 2.5 µl of this mixture was added to one 50-µl tube of Fusion-Blue chemically competent cells thawed on ice. Cells were incubated on ice for 30 min, heat shocked at 42°C for 45 s, and put back on ice for 1 min. A volume of 200 µl SOC media was added to the cells and mixed by pipetting up and down a few times. The one hour incubation time was not necessary when transforming plasmids conferring ampicillin resistance, but was required with kanamycin resistance conferring plasmids. A volume of 200 µl of the transformant culture was spread on an LB plate supplemented with 100 µg/ml of ampicillin (without the centrifugation steps in the protocol). This procedure normally gives 30–1000 colonies after overnight incubation at 37°C.

Quantitating success and mutation rates with different homology and primer lengths

The assay described in Figure 3 and results in Tables 1 and 2 quantitated the effect of homology and primer lengths on success and mutation rates. Success rate for each assembly was measured by simply counting the number of fluorescing versus non-fluorescing transformant colonies on LB plates supplemented with 100 µg/ml of ampicillin and 0.1 mM IPTG. Mutation rate for each assembly was determined by sequencing eight plasmids extracted from fluorescing cultures grown from individual clones.

Colony PCR

Screening for the desired construct was generally performed by colony PCR, with the exception of functional

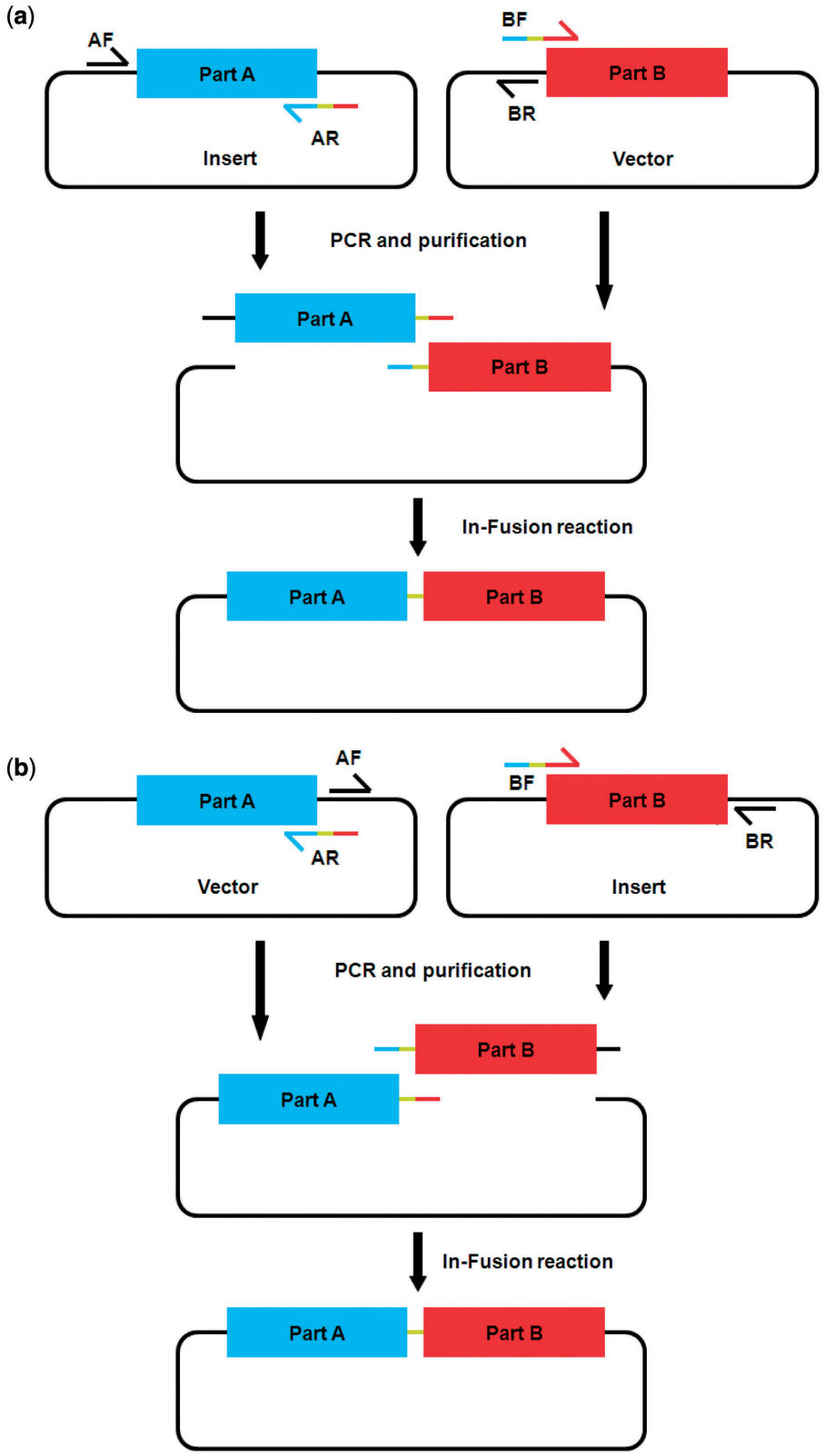

Figure 2. General strategies for In-Fusion BioBrick assembly. There are two general strategies for In-Fusion assembly on pSB1A2 plasmids depending on which BioBrick is PCR-amplified with the vector. The first strategy PCR-amplifies the upstream part by itself and the downstream part with the vector (**a**). The second strategy PCR-amplifies the upstream part with the vector and the downstream part by itself (**b**). Each strategy requires four primers, where two are specific to the parts to assemble and two are general vector primers. The vector primers are described in the 'Materials and Methods' section and can either be specific to the prefix or suffix. When Part B is amplified with the vector, prefix primers are used. When Part A is amplified with the vector, suffix primers are used. All other details are described in Figure 1b.

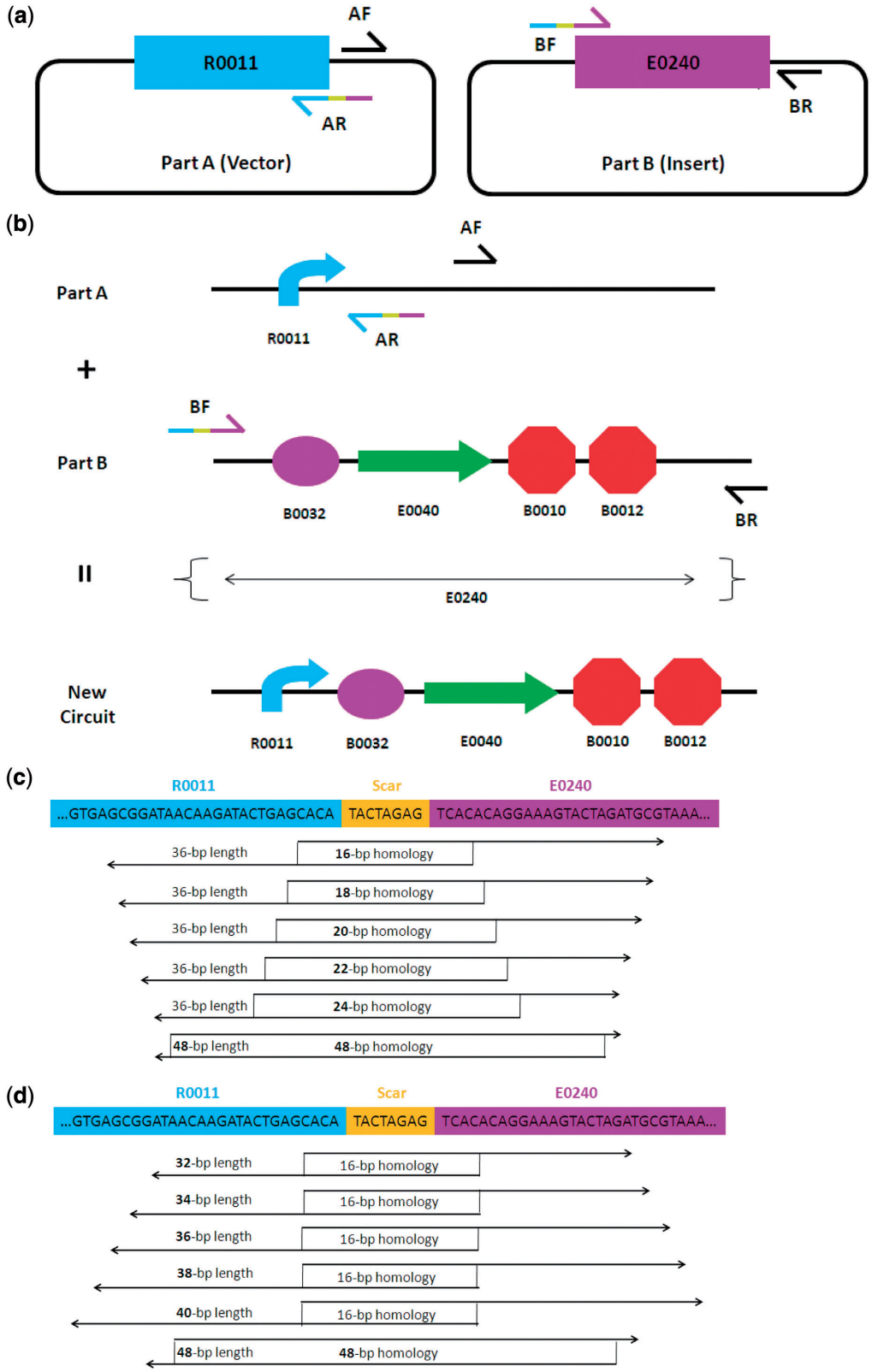

Figure 3. Assembly of two basic parts. (**a**) R0011 and E0240 plasmids are shown with the forward and reverse primers for PCR. R0011 is amplified with the vector and E0240 is amplified as the insert. (**b**) Detailed schematic of the assembly strategy with the forward and reverse primers. The R0011-amplified PCR product has an additional scar sequence and RBS that has homology to the E0240-amplified PCR product. (**c**) Primer design schematic to test the effect of homology length on success and mutation rates. The desired sequence for R0011, scar sequence, and E0240 is shown. For each primer pair, forward primers are shown as right arrows, reverse primers as left arrows, and the region of homology between primers is boxed. Homology and primer lengths are shown where bolded numbers highlight differences between primer pairs. (**d**) Primer design schematic to test the effect of primer length on success and mutation rates. Details are similar to that described in (c).

Table 1. Success and mutation rates of In-Fusion assembly using different homology lengths

Homology level	Success rate	Junction mutation rate
16	61.8% (222/359)	0% (0/7)
18	64.6% (197/305)	0% (0/8)
20	73.1% (196/268)	0% (0/8)
22	74.2% (291/392)	0% (0/6)
24	74.4% (314/422)	0% (0/8)
48[a]	84.8% (495/584)	0% (0/7)

Homology length is indicated with the success and mutation rates. For success rates, numbers in parentheses after the rates indicate the number of successful clones out of the number of total clones tested. For mutation rates, numbers in parentheses after the rates indicate the number of junction mutations out of the number successful clones sequenced.
[a]Primer length is 48 bases (all others are 36 bases).

screening described in the previous section. In these cases, success rate is defined as the percentage of correct constructs out of the total number of colonies screened by colony PCR. Colony PCR allows us to discriminate between the desired construct and construction background (PCR template plasmid that co-transforms with the newly assembled plasmid).

Colony PCR was performed on at least six colonies using the VF2 (5′-TGCCACCTGACGTCTAAGAA-3′) and VR (5′-ATTACCGCCTTTGAGTGAGC-3′) primers specific to the pSB1A2 vector (~100 bp on either side of the part) or primers specific to the desired construct. Negative control reactions were also performed using VF2/VR primers on the plasmid template DNA for the original PCR reaction. Thus, a colony hosting the desired construct will exhibit a mobility shift when compared with the negative control reactions. Colony PCR reactions were performed using 10 µl Fermentas PCR Master Mix per reaction in 0.2 ml PCR tubes. All 10 µl of the colony PCR products were run out on a 1% agarose gel stained with SYBR Safe (Invitrogen) with 1-kb ladder (NEB).

Plasmid extraction and sequencing

At least three correct colonies as identified by colony PCR were grown in test tubes with 5 ml LB supplemented with ampicillin (100 µg/ml) shaking overnight at 250 rpm. We chose three to ensure that one plasmid was correctly constructed without mutations which tend to occur most often at the junctions (regions of homology between fragments). Minipreps were performed with the Qiagen Miniprep Kit using 3 ml culture volume and plasmids were eluted with 30 µl of molecular grade water. Plasmids were submitted for sequencing with VF2 and VR or custom primers to sequence the entire genetic circuit.

General strategies for In-Fusion assembly

We devised two general strategies for assembly depending on which BioBrick is PCR-amplified with the vector (Figure 2). Since both PCR products need to have homology to each other on both ends for the In-Fusion reaction to work, the simplest strategy was amplify each PCR product to have homology to the vector at one end and to have homology at the junction between parts at the other end. As shown in Figure 2a, to amplify the upstream part (Part A) as the insert and the downstream part (Part B) as the vector, the vector specific primers need to be upstream of each part (i.e. in the vector prefix).

Since the forward primer for Part A (AF) and reverse primer for Part B (BR) are complementary sequences, the resulting PCR products share 25 bp of homology at the site where Parts A and B will be joined in the In-Fusion reaction. Likewise, as shown in Figure 2b, to amplify the upstream part (Part A) as the vector and the downstream part (Part B) as the insert, the vector specific primers need to be downstream of each part (i.e. in the vector suffix). Use of these vector-specific primers allows for BioBrick standard formats to be maintained so that new In-Fusion assembled constructs can be submitted to the Registry. These four primers described in the 'Materials and Methods' section can be re-used for every In-Fusion Assembly.

However, custom primers need to be designed in order to provide homology at the junction between parts. The AR and BF primers shown in both Figure 2a and b are complementary, resulting in PCR products with homologous ends that will be fused in the In-Fusion reaction. These primers were designed with simple rules of having a sequence specific to the template DNA of one part at the 3′-end and a 5′ overhang that is homologous to the other part (see 'Materials and Methods' section for details). Following these simple rules will allow for primers to be designed quickly and in a semi-standardized fashion.

RESULTS

We first describe the success rate and mutation rate of In-Fusion assembly using different homology and primer lengths. We then describe additional examples of In-Fusion assembly and re-engineering reactions to demonstrate the versatility and success of this method. These examples include assembly of two basic parts, part swapping (simultaneous promoter and RBS re-engineering), a deletion (conversion of a polycistronic transcribed sequence into a fusion protein-coding sequence), an insertion (of a degradation tag), and three-way assemblies (one to insert an antibiotic resistance gene and swap out a terminator, and another to construct a fusion protein). The insertion and deletion examples are illustrated in Supplementary Figures S2 and S3.

Effect of homology and primer length on success rate and mutation rate of In-Fusion assembly

To understand how homology and primer length affects the success rate and mutation rate of assembly reactions, we devised an assembly assay that would allow for these rates to be determined for a large number of individual clones. The two basic BioBrick parts used for the assembly assay were R0011 (lacI-regulated promoter) and E0240

(consisting of an RBS, GFP-coding sequence and double transcriptional terminator) (Figure 3a). By amplifying the vector with R0011, the PCR product obtained (Part A) starts with the suffix and ends with R0011 plus the E0240 junction sequence (Figure 3b). Part B starts with the R0011 junction sequence and ends with the suffix (Figure 3b). Fusion of these two PCR products occurs at each end and creates a circular plasmid. Transformation of the assembly reaction into competent cells allows for the success rate to be easily determined since colonies can only fluoresce if they have the correctly assembled R0011 and E0240 together in the same plasmid.

We designed primers to vary the homology level from 16–24 bp in increments of 2 bp while keeping the primer length constant at 36 bases (Figure 3c). We chose 16–24 bp because the minimum homology length is 15 bp and this would allow us to determine the relationship between success rates around this minimum at a fine scale. We predicted higher homology levels would increase success rate, but also possibly increase mutation rate at the same time. The 36-base primer length was chosen since it is known that longer primer lengths increases the chances of mutations occurring in the primer. We also used primers that maximized the homology and primer length with 48 bp of homology and a primer length of 48 bases. We predicted that higher homology would increase success rate since another study observed this relationship (8), but at a cost of having a higher mutation rate. We also designed primers to vary the primer length from 32 to 40 bases in increments of 2 bases since we predicted longer primers would increase mutation rate (Figure 3d). Therefore, there were a total of 10 assembly reactions performed in parallel: five to test homology lengths, five to test primer lengths (the 36-base length primers overlapped with the 16 base homology length assembly), and one extreme assembly of 48 bp of homology and a primer length of 48 bases.

These 10 assembly reactions required 20 PCR products, one insert and one vector for each. Remarkably, all 20 PCR products amplified using an annealing temperature of 55°C. Table 1 shows the results of homology length on the success rate and mutation rate of In-Fusion assembly. As homology level increases, the assembly success rate also increases. There appears to be small difference between low homology (16–18 bp) and high homology (20–24 bp) with high homology increasing success by about 10% on average. The extreme case of 48 bp of homology had the highest success rate at nearly 85%. We therefore decided that 48 bp of homology should be used for our primer design rules to maximize success rates. Forty-eight base pairs should not be considered the optimal length, but is a conservative choice since no homology lengths were tested between 24 and 48 bp.

Remarkably, there were no mutations in any of the junctions when we sequenced several successful clones. For all 10 assemblies, we only found one mutation among all of the clones sequenced (73 clean sequences out of 80 attempts) for the entire ~1-kb genetic circuit. Table 2 shows the effect of primer length on the success and mutation rates of In-Fusion assembly. There do not appear to be any remarkable differences between primer lengths of 32–40 bases with respect to either success or mutation rate. Therefore, the optimal homology and primer length is 48 bp because this achieves the highest success rate without the cost of high mutation rate since mutations are rare. The rare mutations in the circuit itself are because Phusion is a very high fidelity polymerase (4.4×10^{-7} according to the product spec sheet), but we can not rule out the possibility that the mutation existed on the template DNA at a low level.

Part swapping: simultaneous promoter and RBS replacement

Next we wanted to construct the same R0011 + E0240 circuit, but with RFP (E1010) instead of GFP (E0040). This would have taken three Standard Assembly steps: to first construct R0011 with B0032, E1010 with B0010/12, then to assemble all these parts together. Instead we could simultaneously re-engineer the existing J04450 circuit with a new promoter and RBS in a single step using In-Fusion (Figure 4a). To do this, for Part A, R0011 and B0032 were first amplified with the E1010 junction sequence and vector (Figure 4b). E1010 and B0010/12 were then amplified from J04450 with the B0032 junction sequence to make Part B (Figure 4b). Colony PCR results remarkably showed that all six colonies had the correct construct, but we were able to screen out negative colonies by the colony color for this particular assembly. Two separate colony PCR experiments were performed on the same six slightly red glowing colonies, one to determine the size of the insert (Figure 4c, left) and one to identify colonies that had the correct RBS (Figure 4c, right). Since the negative control J04450 plasmid (#7 in the right gel photo) did not amplify with the B0032-specific primer and the six colonies did amplify, it was assumed that all six colonies (100%) had the correct construct and three of these were verified with sequencing.

Table 2. Success and mutation rates of In-Fusion assembly using different primer lengths

Primer length	Success rate	Junction mutation rate
32	64.3% (198/308)	0% (0/8)
34	66.7% (246/369)	0% (0/8)
36	61.8% (222/359)	0% (0/7)
38	65.9% (245/372)	0% (0/7)
40	58.5% (220/376)	0% (0/6)
48[a]	84.8% (495/584)	0% (0/7)

Primer length is indicated with the success and mutation rates. For success rates, numbers in parentheses after the rates indicate the number of successful clones out of the number of total clones tested. For mutation rates, numbers in parentheses after the rates indicate the number of junction mutations out of the number successful clones sequenced.

[a]Homology level is 48 bases (all others are 16 bases).

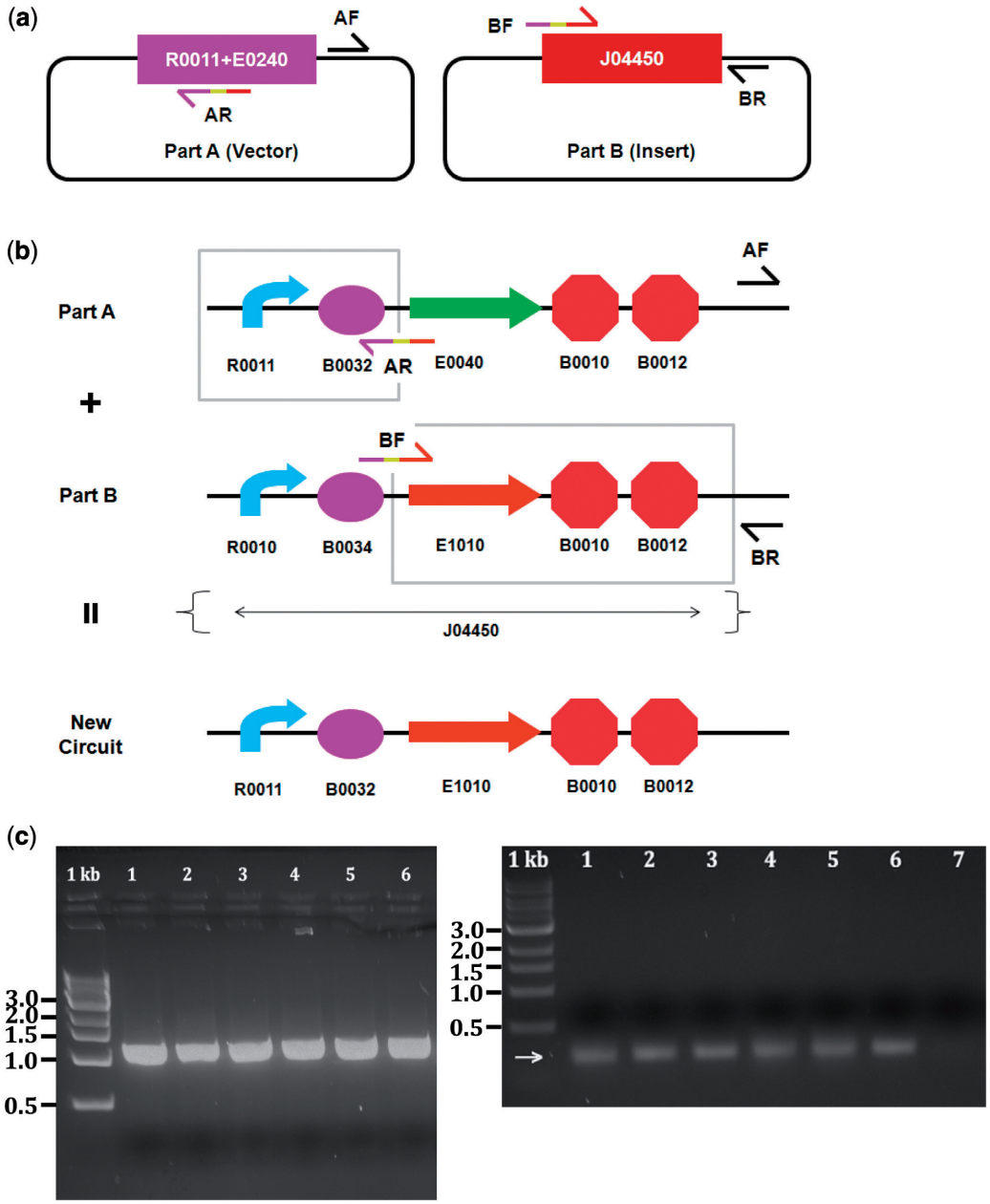

Figure 4. Part swapping: simultaneous promoter and RBS re-engineering. (**a**) R0011+E0240 and J04450 plasmids are shown with the forward and reverse primers for PCR. R0011+E0240 is amplified with the vector and J04450 is amplified as the insert. (**b**) Detailed schematic of the assembly strategy with the forward and reverse primers. Only the promoter (R0011) and RBS (B0032) are PCR-amplified from the R0011+E0240 plasmid. Only E1010 and B0010/12 are PCR-amplified from the J04450 circuit in order to change its promoter and RBS in one assembly step. (**c**) Since both plasmids used as template DNA in the PCR reaction were approximately the same size as the desired construct, two colony PCR reactions were performed on the same six colonies. The gel on the left shows six colonies amplified with VF2/VR primers and the gel on the right shows the same six colonies (#1–6) and negative control J04450 plasmid (#7) amplified with the VF2 and R0011+E0240 AR primer. Correct colonies show a PCR product of about 1.1 kb for the left gel and a PCR product of about 300 bp for the right gel (correct size indicated by arrow).

Three-way assembly: insertion of an antibiotic resistance gene and terminator swapping

Since insertion of DNA into the middle of a multi-part construct takes several Standard Assembly reactions, we demonstrate the efficiency of this construction in a three-way In-Fusion reaction. We wanted to insert the B0032 RBS and kanamycin resistance gene into the T9002 (Lux receiver) circuit, while at the same time change the second transcriptional terminator of T9002 to avoid repeated sequences (3) (Figure 5a). To do this, we first amplified Part A from the prefix of T9002 to upstream of the GFP-coding sequence with the B0032 junction sequence (Figure 5b). Next the B0032 and kanR-coding sequence was amplified for Part B, having the Part A and C junction sequences on either side (Figure 5b). The B0011 terminator was then amplified with the vector back to the prefix as Part C, having Part A and B junction sequences on either side (Figure 5b).

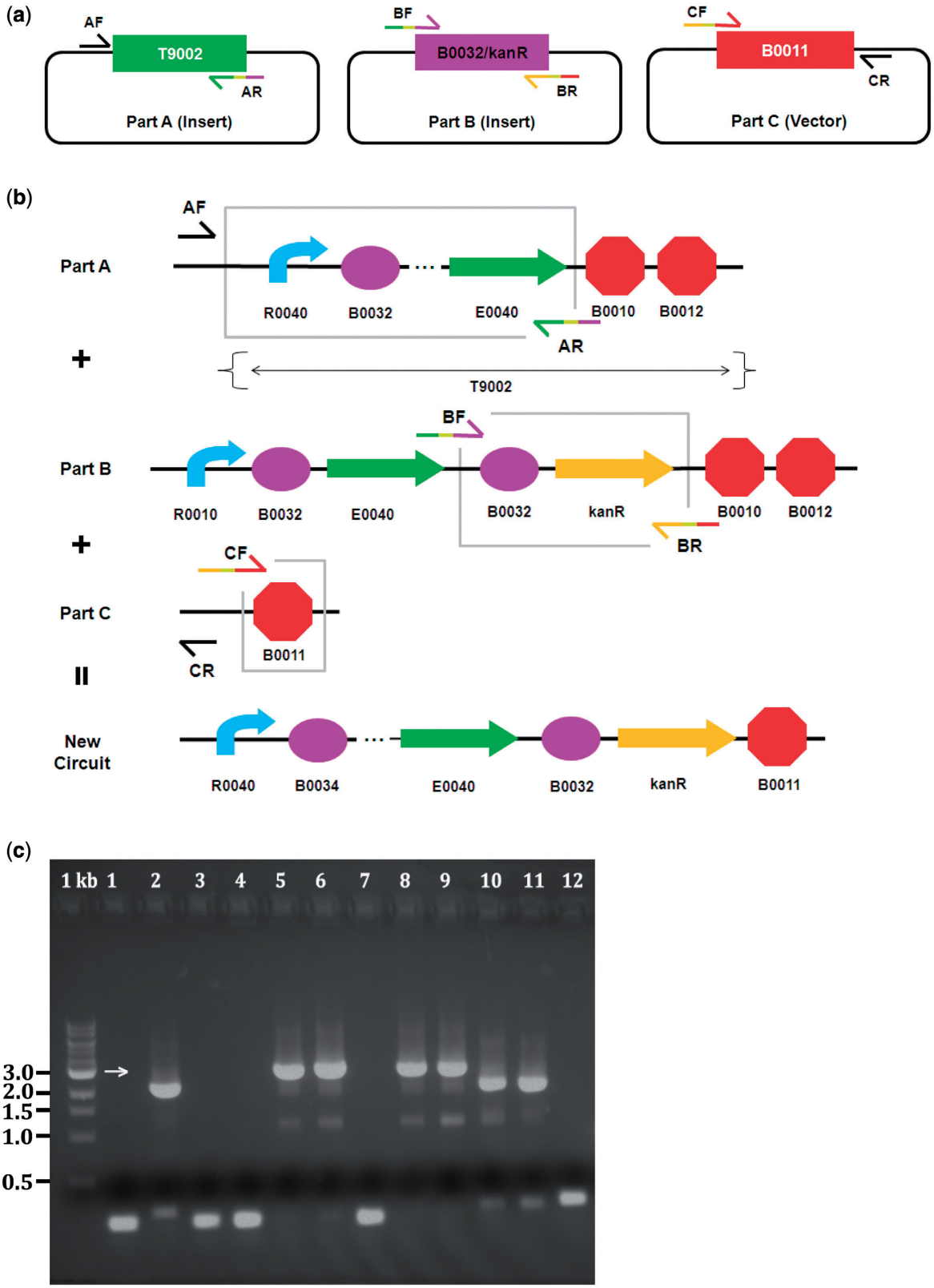

Figure 5. Insertion of an antibiotic resistance gene and terminator swapping through a three-way In-Fusion Assembly. (**a**) T9002, B0032/kanR, and B0011 plasmids are shown with the forward and reverse primers for PCR. B0011 is amplified with the vector and both T9002 and B0032/kanR are amplified as inserts. (**b**) Detailed schematic of the assembly strategy with the forward and reverse primers. The entire T9002 circuit is amplified upstream of B0010 (Part A) with homology to connect an RBS and kanR gene downstream (Part B). To avoid the use of repeated transcriptional terminators (as in T9002 but not shown in the figure), a B0011 terminator (Part C) was placed downstream of kanR. (**c**) Colony PCR results show that 4 out of 12 colonies were positive for the correctly assembled construct (correct size indicated by arrow). A negative control was not necessary since only a ~3-kb fragment would indicate a successful colony.

Colony PCR results showed that 4 out of 12 (33%) colonies had the correct construct and three were verified by sequencing (Figure 5c).

Three-way assembly: creation of a fusion protein

In this assembly, a luxR-GFP fusion protein is incorporated in the middle of the I731014 transcriptional cascade (Figure 6a and b). As in the previous example, an otherwise complex construction using Standard Assembly is efficiently streamlined using the In-Fusion cloning method. The I731014 circuit comprises the backbone assembly component. The GFP-coding sequence and a terminator comprise one of the insert components. The final component consists of a linker sequence amplified from a non-Biobrick vector (pBAD/HisA, Invitrogen).

To determine the effect of the vector:inserts ratio for a three-way assembly, we tested 1:2:2, 1:5:5 and 1:10:10 ratios. A 1:2:2 ratio resulted in 0/24 correct constructs, a 1:5:5 ratio resulted in 4 out of 24 (16.7%) successes, and 1:10:10 ratio resulted in 9 out of 24 (37.5%) correct constructs (Figure 6c). While three-way assemblies generally exhibit a lower success rate compared with two-way assemblies, optimizing the vector:inserts ratio improves the odds of success. Furthermore, this example demonstrates that assembly components representing a wide range of sizes (3.8 kb, 0.8 kb and 0.2 kb) may be simultaneously assembled in a single assembly step. In this example, the minimum 15 bp of homology was used. It is likely that the success rate of three-way assemblies could be further improved by increasing the length of homology at the junctions.

DISCUSSION

There are currently several assembly methods used to construct plasmids for synthetic biology research or other applications. In the synthetic biology community, Standard Assembly is the most widely used method despite competing standards. Custom DNA synthesis is still too expensive for most synthetic biology labs to perform routine constructs, but is ideal when constructing DNA from scratch when there is no template DNA available for PCR or when there are many assembly reactions to perform (14). Overlap extension PCR methods (11,12) are also useful to construct novel DNA sequences without a template, but can be somewhat expensive depending on the length of the construct. For many synthetic biologists, the enormous number of parts available in the Registry allows for diverse circuits to be constructed without the use of DNA synthesis or overlap extension PCR methods.

In our experience, there are three major advantages to In-Fusion assembly over Standard Assembly. First, In-Fusion provides a flexible method to perform large-scale assemblies by mixing and matching parts from the Registry. Second, this method is faster since gel extraction is unnecessary, there are fewer experimental steps, and fewer reactions to optimize, allowing for many reactions to be performed in parallel. Third, we find the success rate for In-Fusion to be high and consistently are able to engineer the desired construct. Our results indicate that the In-Fusion success rate increases with the homology length without the cost of frequent mutations in assembled plasmids which we found to be rare. More homology between assembly components requires longer primer lengths, increasing the primer cost slightly, but the corresponding increase in success rate may justify the extra cost when performing difficult assemblies. We found that in general two-way In-Fusion assemblies have >60% success rate, so in this case using the minimal homology of 15 bp may be sufficient. However, we found that three-way assemblies have lower and more variable success rates (lower than 40% in the examples shown here) depending on the construct. For these more difficult assembly reactions, having more homology will maximize the chances for success and outweigh the extra primer costs. We also did not find it necessary to purchase expensive purified primers because the mutation rate is low enough for it to not be an issue. Although we didn't perform a systematic analysis, there's no obvious relationship between success rate and PCR product size, consistent with (6), but in some special cases optimizing the insert:vector ratio may be necessary.

In regard to assembly standards, ideally an assembly standard will use the same laboratory components (e.g. restriction enzymes and ligase) so that the same components can be used to assemble any two (or more) BioBricks together. This In-Fusion BioBrick assembly method in its current state cannot be completely standardized because custom primers need to be ordered for each individual assembly and hence different components are required. However, the assembly method described here can be semi-standardized by two simple primer design rules (described in the 'Materials and Methods' section) that allow for much of the planning to be removed from primer design. The primer design tool we built will also decrease the time it takes to order primers and maximize success with In-Fusion assemblies. It would be possible to expand this method to use the same standard vector primers for every assembly reaction, but doing so would introduce large scar sequences between parts due to the minimum amount of homology required between PCR-amplified BioBricks. These large scar sequences would most definitely cause problems for spacing between different parts [e.g. the RBS and coding sequences (15,16), unless these sequences were already together on one part and properly spaced].

In conclusion, we have optimized the In-Fusion assembly protocol and adapted this method for BioBrick Assembly and re-engineering. We used this method to make several diverse constructs and simplified the number of experimental steps as much as possible, as illustrated by the six examples above. The cost per reaction of In-Fusion assemblies is relatively high compared with Standard Assembly, but the consistent success of our diverse assemblies and the elimination of intermediate assembly steps in complex constructions make the cost worthwhile in our experience. We hope this method can be expanded upon in the future to fuse a large number of fragments together (17,18) and be standardized to use the same laboratory components.

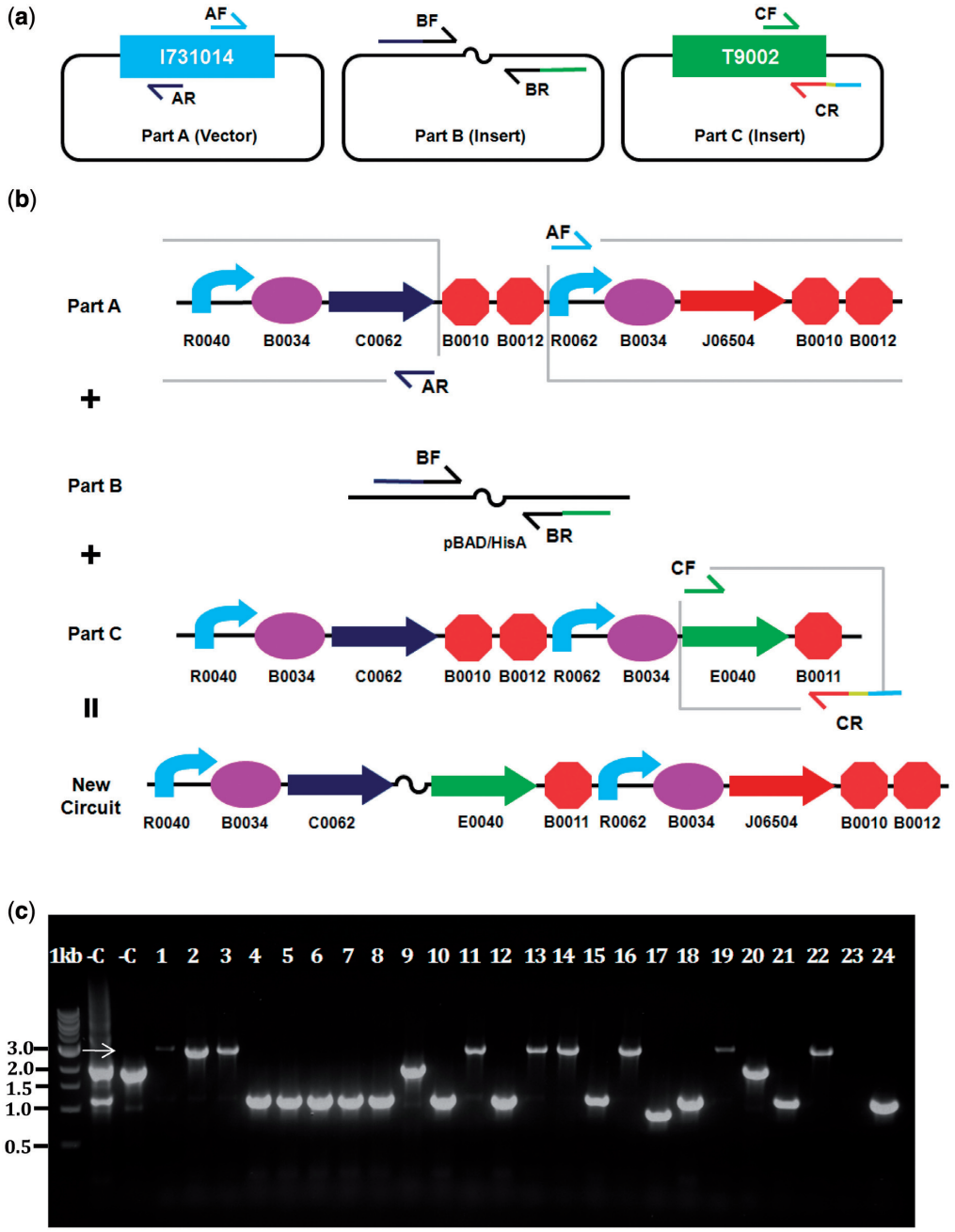

Figure 6. Simultaneous construction of a fusion protein and insertion into a transcriptional cascade via three-way assembly. (**a**) I731014 is analogous to the T9002 transcriptional cascade, but uses the mCherry reporter rather than GFP. GFP was inserted downstream of luxR with a long linker component incorporated between the two coding sequences. (**b**) Detailed schematic showing regions of homology incorporated on 5′ tails of primers. The linker component was amplified from a commercial cloning vector and included several epitope tags. (**c**) A 1:10:10 vector:insert:insert ratio was optimal for this three-way assembly, resulting in a 9 out of 24 success rate. The success rate was evaluated by colony PCR using VF2/VR primers. Negative control reactions were performed on the original I731014 and T9002-F templates. Background from the pBAD/HisA vector was not a factor because this vector has a different selection marker than the final construct. The desired construct exhibits a VF2/VR amplicon of 2.9 kb and a noticeable mobility shift compared to the negative controls (correct size indicated by arrow).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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