Chapter 6

Assembly of Standardized DNA Parts Using BioBrick Ends in *E. coli*

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Abstract

Synthetic biologists have adopted the engineering principle of standardization of parts and assembly in the construction of a variety of genetic circuits that program living cells to perform useful tasks. In this chapter, we describe the BioBrick standard as a widely used method. We present methods by which new BioBrick parts can be designed and produced, starting with existing clones, naturally occurring DNA, or de novo. We detail the procedures by which BioBrick parts can be assembled into construction intermediates and into biological devices. These protocols are based on our experience in conducting synthetic biology research with undergraduate students in the context of the iGEM competition.

Key words: Synthetic biology, iGEM, BioBrick, Standardized parts, Undergraduate, Standardized assembly

1. Introduction

In 2003, Tom Knight and his colleagues developed the BioBricks method to standardize the assembly of DNA parts into devices and systems (1). The BioBricks method is convenient and cost-effective. More importantly, all BioBrick parts are compatible with each other. As a result, projects compliant with the BioBrick standard build on each other using interchangeable parts (2). The Registry of Standard Biological Parts and its associated online database (http://partsregistry.org) contain thousands of BioBrick parts built by undergraduates participating in the International Genetically Engineered Machines (iGEM) competition (3–6).

You can convert any DNA sequence into a BioBrick part by flanking the DNA with a BioBrick prefix and suffix (Fig. 1). The prefix contains the EcoRI, NotI, and XbaI restriction sites, while

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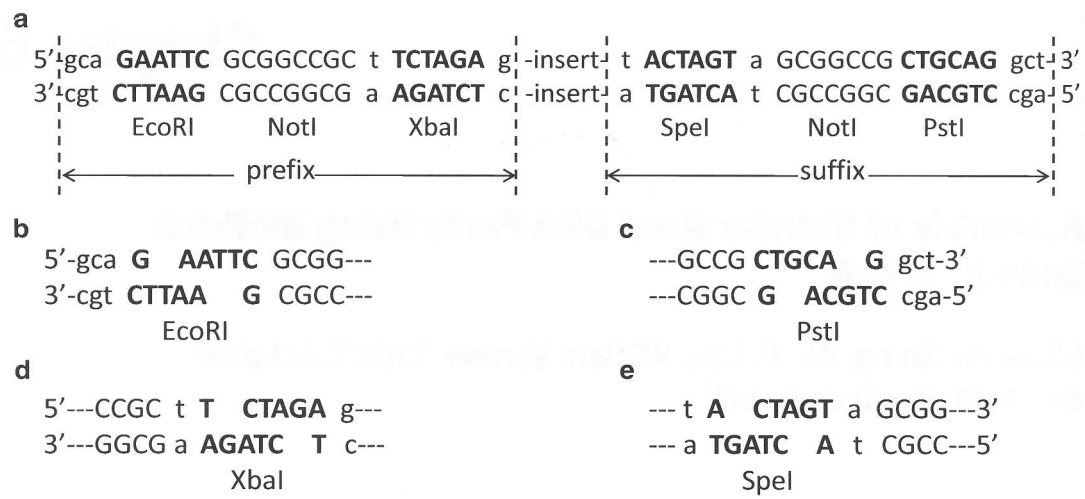

Fig. 1. Sequence of an uncut BioBrick part along with the sequences of cut restriction sites. (**a**) An uncut BioBrick part. (**b**)–(**e**) Cut restriction sites with overhangs for EcoRI, PstI, XbaI, and SpeI. Note that the XbaI and SpeI overhangs are complementary to each other.

the suffix contains the SpeI, NotI, and PstI restriction sites (1). It is important that you remove any sites for these enzymes found within the DNA part so that it will comply with the BioBrick standard. The EcoRI and PstI sites enable the transfer of BioBrick parts from one plasmid backbone to another. The restriction sites for XbaI and SpeI produce complementary sticky ends. Ligation of an XbaI sticky end with an SpeI sticky end produces a mixed site, or scar, that is not recognized by either XbaI or SpeI enzymes.

Synthetic biologists use several key terms for discussing the BioBrick system. A *part* is a basic unit with an indivisible biological function. Common examples are promoters, ribosome binding sites (RBS), coding sequences, and transcription terminators. A *construction intermediate* is formed when two or more parts are ligated together that do not constitute a functional device. A *device* is a combination of parts that carries out a biological function. Examples include reporters, inverters, and cell signal receivers. An *expression cassette* is a device that contains all the parts needed to express a gene. A common example includes a promoter, RBS, protein-coding gene, and a transcription terminator. A *composite part* is a general term for a device with more than one part in it.

Before designing and building a new device, you must answer a few questions. What is the purpose of the design? How many parts are needed? In what order should the parts get put together to make the assembly process most efficient? A simple example is related to the construction of the device shown in Fig. 2. If you followed method A, it would take you 6 days to rebuild the device with a new promoter. Method B would take you only 4 days since you would not have to repeat the ligation of coding + TT.

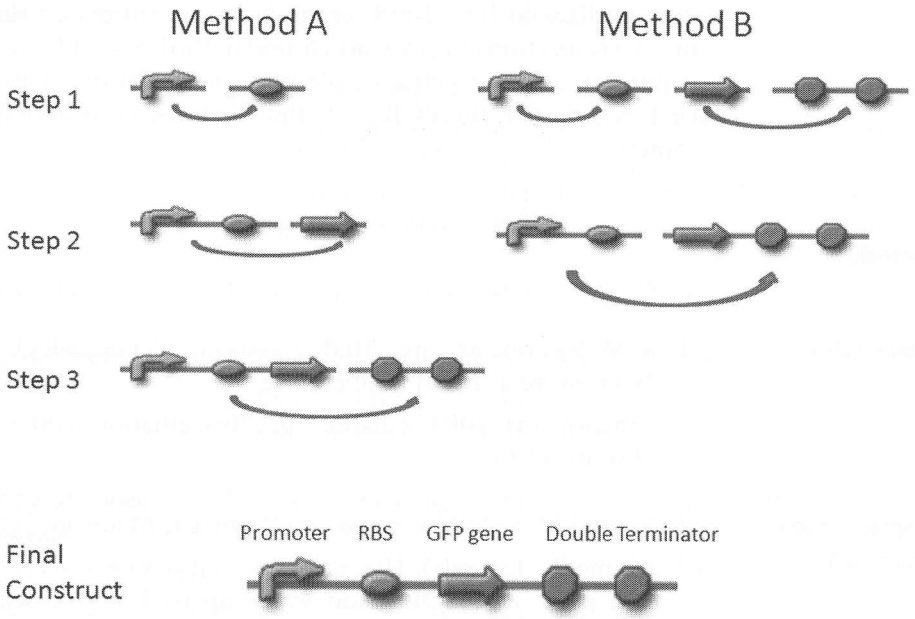

Fig. 2. This figure shows typical BioBrick representations of each of the parts with the *curved arrows* indicating ligation of the two parts together. A and B are two possible ways in which the final construct could be built. Method A shows a linear sequence of ligation in which it takes three sequential ligations to finish, but it produces intermediates that could be used in other devices. Method B shows how some ligations are performed simultaneously to reduce the number of days before the construction is completed.

Therefore, the general strategy illustrated by Method B reduces the time required to rebuild second-generation devices.

Assembly of parts using the BioBrick standard permits you to build from either the 5′ or 3′ end of a desired construct. For example, you can build the construct in Fig. 2 from promoter to terminator or terminator to promoter. The expression cassette shown is for green fluorescent protein (GFP) and consists of a promoter, RBS, GFP coding sequence, and transcriptional terminator (TT). The fastest way to build a GTP expression cassette is to ligate the promoter and RBS together while simultaneously ligating the GFP coding DNA with the terminator. Once the two halves are assembled, they can be ligated to build the final device. Alternatively, the BioBrick standard of assembly allows you to build one intermediate part (e.g., RBS + GFP + TT) and then add to the intermediate each of several different promoters to evaluate the strength of a set of promoters. Once two parts have been put together by the BioBrick assembly method, they cannot be taken back apart.

Because of the flexibility of the BioBrick system, standardized parts can be assembled de novo or from DNA isolated from nature. In order to standardize a new part, you must add BioBrick ends to the 5′ and 3′ ends of the new DNA sequence. If the part is being synthesized de novo, utilizing one of the growing number of companies that will manufacture genes (7), the requested sequence

must be flanked by a BioBrick prefix and a suffix and devoid of internal restriction enzymes sites found in BioBrick ends. Sequences of interest within a genome, plasmid, or an existing part can be used as template for PCR with the BioBrick ends added to the primers.

2. Materials

2.1. Cleaning DNA

1. 3 M Sodium acetate: 40.8 g sodium acetate·3H_2O, 80 mL H_2O. Store at room temperature.
2. Ethanol: (a) 100% ethanol (b) 70% ethanol: 700 mL with 300 mL H_2O.

2.2. Minipreparation of Plasmid DNA

1. Wizard Plus SV Minipreps kit (Promega, Madison, WI).
2. LB media (low salt): 10 g tryptone, 5 g yeast extract, 5 g NaCl, 200 µL 5 M NaOH. Add water up to 1 L. Autoclave (see Note 1).

2.3. Enzyme Digestion of BioBrick Parts

1. Buffer H (Promega): 90 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl.
2. Low buffer: 10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mg/mL BSA, 50 mM NaCl.
3. Medium buffer: same as low buffer, but with 100 mM NaCl.
4. Restriction enzymes: EcoRI, XbaI, SpeI, and PstI (Promega, Madison, WI).

2.4. Gel Electrophoresis

1. Agarose, low EEO (Promega, Madison, WI).
2. TBE buffer: Prepare 5× stock solution with 54 g tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA. Make up to 1 L with H_2O. Dilute 100 mL with 900 mL H_2O for use. Use at 0.5× working concentration.
3. 1% Ethidium bromide (EtBr; Fisher, Pittsburgh, PA). EtBr is mutagenic so handle with care.
4. DNA loading dye (10×): 5 mL glycerol, a 0.2% w/v of bromophenol blue, 0.2% w/v xylene cyanol FF. Make up to 10 mL with H_2O. Store at 4°C. Dilute 100 µL with 900 µL H_2O for use.
5. 1 kb DNA ladder (Invitrogen, Carlsbad, CA).

2.5. Gel Purification

1. NucleoSpin® Extract II gel extraction kit (Macherey-Nagel, Düren, Germany).

2.6. Ligation

1. 2× Rapid Ligation Buffer (Promega, Madison, WI).
2. T4 DNA ligase (Promega, Madison, WI).

2.7. Transformation

1. Z-Competent™ *E. coli* cells (Zymo Research, Orange, CA), store at ≤70°C.
2. SOC medium: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 200 μL 5 M NaOH, 10 mL 250 mM KCl (1.86 g KCl in 100 mL dH_2O). Add water up to 1 L. Autoclave for about 20 min. After cooling, add 5 mL sterile 2 M $MgCl_2$ (19 g $MgCl_2$ in 100 mL dH_2O).

2.8. Colony PCR

1. GoTaq® 2× Green Master Mix (Promega, Madison, WI).

2.9. Glycerol Stocks of E. coli Cells

1. CryoTube vials (Nunc, Roskilde, Denmark).
2. Sterile glycerol: Autoclave glycerol and store at room temperature.

2.10. Single-Stranded Oligo Assembly

1. 10× Annealing buffer: 1 M NaCl, 100 mM Tris–HCl (pH 7.4).

3. Methods

3.1. Minipreparation of Plasmid DNA

1. Grow 2–4 mL overnight culture for each miniprep. During incubation, culture tubes are shaken at 400 g and slanted to aerate the media.
2. Pour the contents of each culture tube into a microfuge tube.
3. Centrifuge the microfuge tubes for 2 min at 13,000 g.
4. Pour the liquid from the microfuge tubes, leaving the pellets. Gently shake the tubes to remove the remaining liquid. If there is more liquid culture, steps 2–4 can be repeated.
5. Mix each pellet with 250 μL of cell resuspension solution. Fully resuspend the cells by pipetting the solution up and down until the pellet is completely broken up and mixed with the solution.
6. Mix the contents of each microfuge tube with 250 μL cell lysis solution and invert several times.
7. To each microfuge tube, mix in 10 μL alkaline protease solution and invert several times. The tubes are incubated at room temperature for 3 min.
8. Centrifuge the tubes at 13,000 g for 10 min.
9. Insert the spin columns into the 2-mL collection tubes.
10. Use a micropipette to transfer the supernatant from each tube to the spin columns.
11. Centrifuge the spin columns at 13,000 g for 1 min. Discard the flow-through and reinsert the spin columns into the collection tubes.

12. Fill the spin columns with 750 μL wash solution (containing ethanol).
13. Centrifuge the spin columns at 13,000 g for 1 min. Discard the flow-through and reinsert the spin column into the collection tubes.
14. Fill the spin columns with 250 μL wash solution (with ethanol).
15. Centrifuge the spin columns at 13,000 g for 1 min. Discard the flow-through and insert the spin column into a clean 1.5-mL microfuge tube.
16. Fill the spin columns with 50–100 μL of nuclease-free water.
17. Centrifuge the spin columns at 13,000 g for 1 min.
18. Quantitate the plasmid DNA in the microfuge tube and store it at −20°C or use immediately (see Note 1).

3.2. Enzyme Digestion of BioBrick Parts

1. If you are digesting with EcoRI and PstI to verify the size of an insert, use 12 μL of miniprep DNA (see Subheading 3.1), or at least 400 ng. If digesting to make a vector or an insert, the amount of DNA to digest depends how much DNA you need for your ligation (see Notes 2 and 3).
2. Mix the DNA with 2 μL of the appropriate buffer (see Table 1) in a 500-μL microfuge tube. Add 1 μL of each enzyme (total enzyme volume cannot exceed 10% of reaction volume). Increase the volume to 20 μL with dH_2O.
3. For size verification, incubate the reaction at 37°C for at least half an hour. For inserts and vectors, incubate the reaction for at least 3 h. For maximum digestion, incubate the reaction overnight.

Table 1
Salt conditions for double digestion. Useful enzyme combinations for digesting BioBrick parts with their optimal buffer and what the digestion produces. All five reactions are optimal at 37°C

Restriction enzymes		Buffer	Product
EcoRI	XbaI	Low	Front vector
EcoRI	SpeI	Low	Front insert
SpeI	PstI	Medium	Back vector
XbaI	PstI	Low	Back insert
EcoRI	PstI	Buffer H	Whole insert

3.3. Cleaning DNA

1. If the volume of the DNA is less than 200 µL, bring the volume up to 200 µL with sterile dH_2O (see Subheading 3.2).
2. Add one-tenth volume of 3 M sodium acetate to the DNA solution and mix.
3. Add two volumes of 4°C 100% ethanol and vortex for 10 s. Put the tube in a –80°C freezer for 30 min (or overnight in a –20°C freezer).
4. Spin the tube in a microcentrifuge at 13,000 g for 10 min. Pour the ethanol out, keeping the pellet.
5. Wash the pellet with 500 µL of 4°C 70% ethanol. Gently roll the tube. Pour off the ethanol.
6. Dry the pellet in a centrifugal evaporator (SpeedVac).
7. Resuspend the DNA in 20 µL dH_2O (adjust volume as necessary).

3.4. Gel Electrophoresis

1. Prepare a gel of appropriate agarose concentration (use the web tool at http://gcat.davidson.edu/iGEM08/gelwebsite/gelwebsite.html).
2. Run DNA samples (see Subheading 3.2) on the gel until there is adequate separation between the desired piece of DNA and the DNA that it was cut from (see Note 4).

3.5. Gel Purification

1. Place the gel (see Subheading 3.4) under UV light at an intensity just high enough to visualize the bands (see Note 5). Cut out the bands containing the insert and the vector to purify the DNA (see Note 6).
2. Place the gel slice in a 1.5-mL microfuge tube and weigh it. Add two volumes of Buffer NT to one volume of gel (100 mg = 200 µL). For gels >2% agarose, double the volume of Buffer NT.
3. Incubate the gel at 50°C for 5–10 min until the gel slice is completely dissolved. Vortex the tube every 2–3 min to speed up the dissolving process.
4. Place a spin column in one of the provided 2-mL collection tubes.
5. Place a NucleoSpin® column into a collection tube. Pipette the DNA solution onto the column. Centrifuge the DNA solution at 13,000 g for 1 min. The maximum volume the column can hold is 800 µL, so repeat this step using the same column if the volume is larger than that.
6. Discard flow-through from the previous step and place the column back in the collection tube.
7. Wash the DNA in the column by applying 600 µL of buffer NT3. Centrifuge the column for 1 min at 13,000 g.

8. Discard the flow-through and spin the column for 2 additional minutes to dry the column.
9. Place the spin column in a clean 1.5-mL microfuge tube.
10. To elute the DNA, add 10–30 μL of buffer EB to the center of the white matrix. Allow the column to sit for 1 min and then centrifuge it at 13,000 g for 1 min.
11. Quantify the DNA, which is eluted in the flow-through.

3.6. Ligation

1. Place 50 ng of digested vector (see Subheadings 3.3 or 3.5), 5 μL of ligation buffer, and 1 μL of T4 ligase into a 500-μL microfuge tube. The amount of insert to add is calculated from the following formula:

$$\text{ng of insert} = (2)(\text{bp insert})(50\,\text{ng linearized plasmid})/(\text{size of plasmid in bp}).$$

   Add water to increase the final volume to 10 μL (see Note 7).
2. Prepare both a positive ligation mixture that contains the digested vector and insert as well as a negative ligation mixture that contains only the digested vector. Add more water to the negative ligation mixture to prepare equal volumes.
3. Leave the ligation mixture at room temperature for 5 min, and then use it directly for transformation of *E. coli* competent cells, or store it by freezing until transformation.

3.7. Transformation

1. Prewarm culture plates to increase the drying rate of plated cells. The culture plates should contain the appropriate antibiotic for the transforming plasmid.
2. Store Z-Competent™ cells at –70°C or colder. Thaw a 100-μL tube of Z-Competent™ cells for 5 min on ice. At the same time, cool the tubes containing the ligation mixtures on ice.
3. Very gently add 25–50 μL of Z-Competent™ cells to each ligation mixture of 10 μL (see Subheading 3.6).
4. Let the mixtures incubate on ice for 5 min.
5. Add SOC media with no antibiotic to a final volume of 60–100 μL/tube. For plasmids using the ampicillin resistance marker, the cells will begin repairing their cell walls immediately and are ready to be plated. For plasmids with other antibiotic resistance markers, incubate without shaking for 20 min before plating.
6. Spread the cells on culture plates containing the appropriate antibiotic. Let the plates incubate overnight until colonies are visible and large enough to pick individually.
7. If transformation with Z-Competent™ cells is unsuccessful, traditional heat-shock transformation or electroporation with a different brand of competent cells may yield higher efficiencies.

3.8. Colony PCR

1. Compare your positive ligation plate to your negative ligation plate to estimate the number of background negative colonies (see Subheading 3.7). Plan to screen an appropriate amount so that it is highly probable at least one of the positive colonies will have the correct insert size given the amount of background negative colonies. Screen at least one negative colony also.
2. To conduct PCR, you will need a forward and reverse primer specific to the plasmid being screened. The primers should amplify the region where the insert was added (see Note 8).
3. For each colony to be picked, prepare a PCR tube with the following mixture: 12 μL 2× Green Master Mix, 10 μL dH_2O, 1 μL (20 pmol) forward primer, 1 μL (20 pmol) reverse primer.
4. Use a micropipette tip to pick a single colony off of the culture plate. Place the tip into a labeled PCR tube and mix by pipetting up and down.
5. Remove 1 μL of the PCR mixture and place it in a labeled test tube containing 200 μL culture media with antibiotic to reserve some of the cells from the colony to grow later. Incubate these cultures at 37°C.
6. Conduct the following PCR cycle: 95°C for 10 min, followed by 20 cycles of 95°C for 15 s, 46°C (or appropriate annealing temperature for your primers) for 15 s, 72°C for 60 s/kb of DNA of the expected size for a successful DNA ligation.
7. Run the reaction products on an agarose gel (see Subheading 3.4) appropriate for the size of the amplified product. You can use our gel optimization tool to choose the appropriate percent agarose (http://gcat.davidson.edu/iGEM08/gelwebsite/gelwebsite.html). Colonies containing unsuccessful ligations will have the same insert size as the negative control colony (see Notes 9 and 10). Successful ligations will be bigger than the negative control insert size (Fig. 3). For colonies that show the expected insert size, save the corresponding culture and discard all unsuccessful colonies.
8. The BioBrick part can be further verified by miniprepping (see Subheading 3.1) and digesting (see Subheading 3.2) and gel electrophoresis (see Subheading 3.4), or by sequencing (see Note 11). After successful ligation and transformation, the part can be used or manipulated more to construct more new parts.

3.9. Glycerol Stocks of *E. coli* Cells

1. Grow a 2-mL culture of a particular cell type (see Subheading 3.8) overnight for each frozen stock that is needed.
2. Microwave sterile glycerol for 30 s. Do not mix the glycerol so that the top portion remains as hot as possible.
3. Cut a 200-μL pipette tip with a clean razor blade to make a larger opening at the tip.

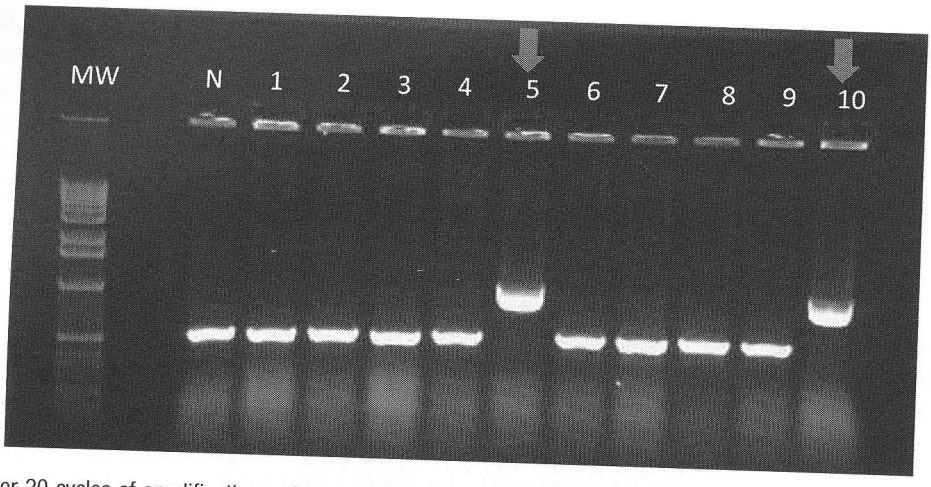

Fig. 3. After 20 cycles of amplification, colony PCR products for each colony were run on a 0.9% agarose gel. Selected colonies were numbered, and the negative colony labeled "N." Colonies #5 and #10 have an insert size bigger than the negative control and roughly the expected insert size for the DNA part (1,100 bp).

4. Label Nunc cryotubes as appropriate and add 150 μL of hot glycerol.
5. Allow the glycerol in the tubes to cool at room temperature for 1 min. Add 850 μL of bacterial culture to each tube, making 15% glycerol mixtures.
6. After putting the caps on, shake the tubes vigorously to ensure that the glycerol mixes evenly with the bacterial culture.
7. Immediately after step 6, put the tubes into a −80°C freezer.

3.10. Connecting Two BioBrick Parts

1. Obtain plasmid DNA for both parts (see Note 1).
2. Digest the plasmid DNA (see Subheading 3.2) for the two parts with the appropriate enzymes for the desired ligation (see Fig. 2, Table 1, and Note 12).
3. Run the digested DNA on a gel (see Subheading 3.4) until there is adequate separation between the desired piece of DNA and the DNA that it was cut from. You can use our online tool to optimize the percent agarose for your gel (http://gcat.davidson.edu/iGEM08/gelwebsite/gelwebsite.html).
4. Place the gel under UV light at an intensity just high enough to visualize the bands (see Note 5). Cut out the bands containing the insert and the vector with a razor and purify the DNA (see Subheading 3.5; see Note 6).
5. Ligate the purified insert and vector together (see Subheading 3.6).
6. Use the ligation mixture to transform into competent *E. coli* cells (see Subheading 3.7).

7. After overnight incubation of the transformed cells, pick colonies from the ligation and the control plates for colony PCR to quickly test if the ligation was successful in any of the colonies (see Subheading 3.8).
8. Grow clones with positive results from the colony PCR overnight in tubes of 2 mL LB media containing the appropriate antibiotic.
9. Miniprep the overnight cultures to obtain plasmid DNA. Digest the plasmid DNA with EcoRI and PstI as a conclusive test for whether the ligation was successful.
10. Store cells that have been confirmed to have successful ligations in glycerol stocks at −80°C (see Subheading 3.9).

3.11. Cloning a New Part into BioBrick Ends

There are three main approaches to the fabrication of a new BioBrick part. The easiest but most expensive method is to design the sequence of the part, add the BioBrick prefix and suffix sequences, and have the part synthesized and cloned (see Table 2). Companies that provided this de novo gene synthesis will generate the needed DNA oligonucleotides, assemble them, and provide the part cloned into a plasmid. The second approach is to add BioBrick ends to an existing DNA sequence using custom PCR primers (Fig. 4). The template used can be an existing clone or DNA from a natural source. The third approach is to assemble a part yourself using overlapping single-stranded oligonucleotides. Building parts from oligos work well with relatively small DNA parts, typically less than 300 base pairs long. In the synthesis of all new BioBrick parts, it is important to maintain the integrity of the DNA sequence and the BioBrick ends. Therefore, the DNA sequence of the part itself cannot contain restriction sites for NotI, EcoRI, XbaI, SpeI, or PstI. If a designed sequence contains one or more of these restriction sites, the DNA should be modified to remove the restriction sites. If the sequence is derived from an existing clone or a natural source, the offending restriction sites must be removed by mutagenesis. After the new part has been sequence verified, it can be manipulated through digestion, ligation, and transformation in order to assemble it with other BioBrick parts.

Table 2
BioBrick prefix and suffix to flank de novo part

BioBrick prefix (if insert begins with ATG)	GAATTCGCGGCCGCTTCTAG
BioBrick prefix	GAATTCGCGGCCGCTTCTAGAG
BioBrick suffix	TACTAGTAGCGGCCGCTGCAG

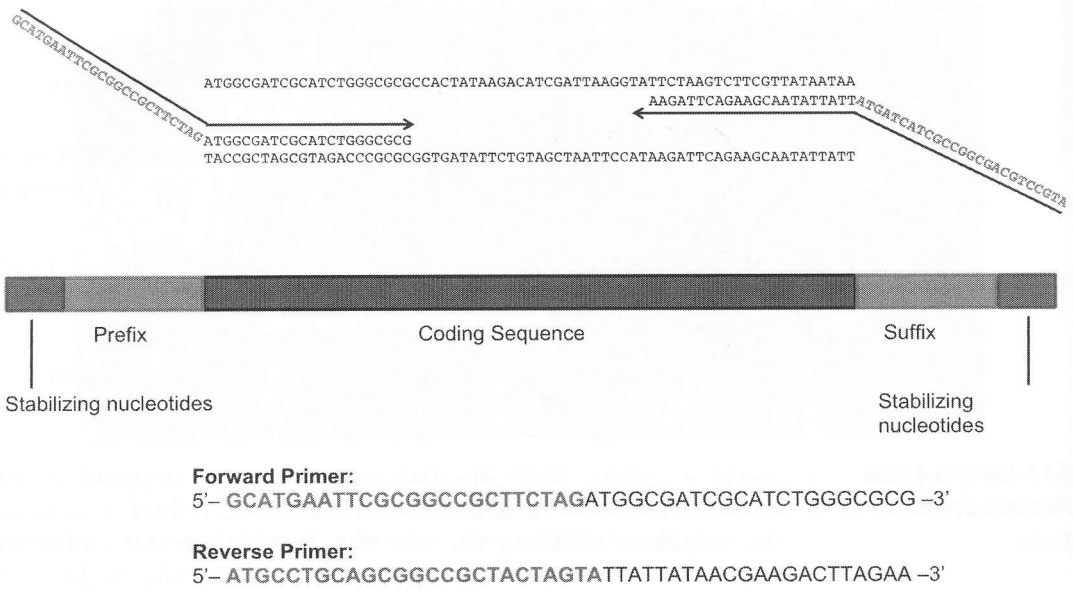

Fig. 4. Designing primers for addition of BioBrick ends by PCR amplification. The given forward and reverse primers would be designed and assembled in order to add the BioBrick prefix and suffix to the given coding sequence. Four stabilizing nucleotides allow restriction enzymes needed to cut the BioBrick ends to bind securely to the target site. We like to use GCAT here, but any sequence that is not a restriction site will suffice.

If you make the part using PCR, the forward primer for the part must include the BioBrick prefix, the first 20–25 nucleotides of the part sequence, and four extra base pairs (see Fig. 4) on the 5′ end so that the EcoRI restriction enzyme can bind and cut the restriction site (see Notes 3 and 8). The reverse primer for the part must include the last 20–25 nucleotides of the template, the BioBrick suffix, and four extra base pairs. Reverse primers are the reverse complement of the template strand (see Fig. 4). Try to design the primers so that their melting temperatures in the PCR mix to be used are within 10°C of each other. You can calculate the melting temperatures using the following web tool: http://www.promega.com/biomath/calc11.htm. Compare the salt-adjusted melting temperatures. Lengthening (adding more base pairs that complement the template sequence) or shortening the primers will increase or decrease the melting temperatures, respectively. The desired DNA template can be part of a plasmid or chromosomal DNA. While cell extract containing the desired DNA can be used as template, you might want to purify the DNA so its concentration can be measured before beginning the PCR process. One to five nanograms of template DNA will be used in the PCR amplification. Perform PCR according to a standard amplification protocol. The final amplified product is ready for digestion with EcoRI and PstI to clone the new standardized part into a plasmid vector (see Subheading 3.2).

To produce a part of 300 bp of DNA or less, use oligo assembly:

1. Enter the desired 5′–3′ forward sequence into The Oligator A program at: http://gcat.davidson.edu/igem10/index.html. This tool generates single-stranded DNA oligonucleotide sequences that have very similar melting temperatures in their overlapping sequences. The overlap length for the oligos must be at least 20 base pairs. Limit the length of your oligos to 70 bases or shorter (see Note 13).
2. If you are cloning into EcoRI and PstI sticky ends of a BioBrick plasmid, check the box for adding prefix and suffix, choose EcoRI for the prefix and PstI for the suffix, and the web site will produce sticky ends when the oligos are fully assembled.
3. The output window will show the desired double-stranded DNA sequence, color-coded by the overlapping oligos. The top strand is the sequence submitted, beginning and ending with the BioBrick ends. Save the text from the output in a permanent document.
4. The resulting sequence and individual oligos have sticky ends equivalent to digestion with EcoRI and PstI. Have these individual oligos synthesized at a recommended 100 mM concentration. Store oligos at −20°C until ready to assemble.
5. To a 0.5-mL microcentrifuge tube, add 1 μL of a 100-μM solution of each oligo (the final concentration of each oligo will be 5 μM). Add a one-tenth volume of annealing buffer (2 μL in 20 μL) and water to a final volume of 20 μL.
6. Boil the oligos in 400–500 mL of water for 10 min.
7. Let the mixture cool slowly to room temperature overnight. The product is ready to use for ligation. To determine the concentration of the product, sum the amount of nanograms of each oligo added and divide by the total volume of the mixture. You may need to dilute an aliquot to avoid pipetting volumes below 0.5 μL (see Note 14).

4. Notes

1. When obtaining plasmid DNA for digestion and ligation, a yield of at least 15 ng/μL is desirable. Also, it is helpful to miniprep more than one sample of each type of plasmid DNA every time the miniprep procedure is done. Due to the overnight incubation step, minipreps are a bottleneck process, so minimize the number of times you perform minipreps. Another benefit of having a large volume of DNA is that you have the option of concentrating the DNA if needed (see Subheading 3.3).

2. We have found that digesting 50 times the calculated amount of DNA needed for a ligation generally yields appropriate concentrations. The digestion reaction volume can be increased above 20 µL for large DNA volumes as long as the volume of enzymes does not exceed 10% of the final reaction volume.
3. When preparing a receiving plasmid, you do not need to gel purify the vector. We have found that ethanol precipitation suffices (see Subheading 3.3). The small piece of DNA between the EcoRI and XbaI sites, or the SpeI and PstI sites, does not precipitate efficiently and is excluded from the DNA pellet.
4. We have run gels at the maximum of 120 V for the fastest run time, but that can cause a diffuse band especially with high DNA concentrations. We find a voltage of 80–100 V to be ideal. Secondly, we try to limit each DNA sample to 20 µL in one lane. However, if digestion volume is larger than 20 µL, two or more lanes may be used.
5. Excessive exposure to UV light is harmful. Wear gloves and protective eyewear when working with UV light. UV light also nicks DNA so minimize UV exposure to your DNA of interest.
6. Try to cut off most of the gel around the DNA bands. There is usually gel underneath the band that can be cut off as well. Decreasing the volume of the gel slice will improve yield for the gel purification step. The maximum weight of the gel slice is 400 mg. Higher weights will require more than one spin column during gel purification, and that is not recommended.
7. If the vector or insert DNA solutions is too dilute, the components of the ligation can be scaled up, or the DNA can be dried down to a smaller volume. Although you can perform ligations in as much as 20 µL, you can also vacuum-concentrate the DNA to dryness and resuspend in a small volume of water to keep the total reaction volume at 10 µL. The formula provided calculates the amount of insert for a 2:1 molar ratio of vector to insert, which is ideal. However, we have used ratios slightly lower than 2:1 if there was insufficient insert DNA. Alternatively, you can cut all values in half if purified DNA is limited. Ratios of 3:1 or 5:1 can be used if ligation fails the first time.
8. As a rule of thumb, PCR primer annealing temperatures should be about 5°C lower than the calculated melting temperature. Elongation times should be about 1 min/kb to reduce the number of new mutations.
9. If the insert and the plasmid are the same size, we digest the ampicillin resistance gene with ScaI to cut the plasmid into two pieces.
10. Some DNA sequences are so resistant to cloning as to be called "unclonable" (8). Often, the reason for this is not known, but suspected causes include unusual secondary structures serving

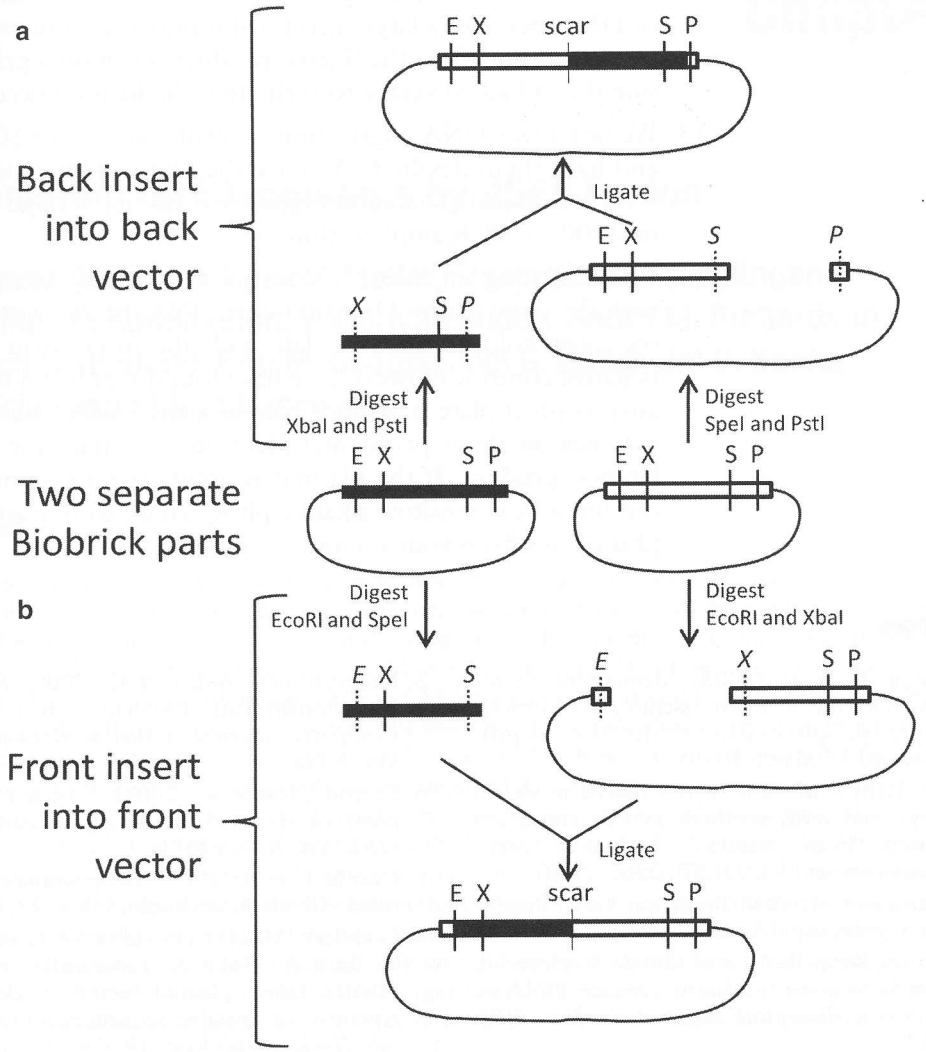

Fig. 5. Inserting a BioBrick part upstream or downstream of another BioBrick part. The letters E, X, S, and P stand for EcoRI, XbaI, SpeI, and PstI restriction sites, respectively. A *dotted line* indicates the restriction site has been digested and has an exposed overhang. (**a**) Inserting a part downstream of another part. (**b**) Inserting a part upstream of another part.

as substrates for recombination and generation of gene products that are toxic to the bacterial cells.

11. Sequence-verify all new constructs and those that contain more than one copy of a part. We have found that parts such as double transcription terminators cause unintended recombination.
12. Consider two hypothetical BioBrick parts, A and B (Fig. 5). Making part A the front insert and part B the front vector produces the same arrangement (part A:part B) as making part A the back vector and part B the back insert. However, the larger part is usually used as the insert for two reasons. First, it is

difficult to get adequate gel purification yields of small pieces of DNA. Second, a large insert will produce a more conspicuous band shift when the ligated product is run on a gel alongside the unligated vector to verify that the ligation occurred.

13. We order our DNA oligos using a small scale (10 or 50 nmol) and have them desalted. We have the oligos shipped in liquid form at 100 μM. This allows us to use 1 μL of the oligo directly in a 100 μL PCR amplification.
14. When ligating an insert, always perform a negative control with the same plasmid but no insert DNA. Ideally, you would see more colonies on the plate with the insert than on the negative control. However, we have found that when the negative control plate has more colonies, the smaller number of colonies on the experimental plate often contain the correct ligation product. If the plasmid recircularizes frequently, you can use a heat-sensitive alkaline phosphatase to remove the 5′ phosphates from your vector.

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