One Primer To Rule Them All: Universal Primer That Adds BBa_B0034 Ribosomal Binding Site to Any Coding Standard 10 BioBrick

Anton V. Bryksin,*† Haylee N. Bachman,‡⁺ Spencer W. Cooper,§ Tilak Balavijayan,§ Rachael M. Blackstone,§ Haoli Du,§ Jackson P. Jenkins,§ Casey L. Haynes,§ Jessica L. Siemer,§ Vincent F. Fiore,† and Thomas H. Barker†

†Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30332, United States
‡School of Chemistry & Biochemistry, School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332, United States
§Georgia Institute of Technology, Atlanta, Georgia 30332, United States

ABSTRACT: Here, we present a universal, simple, efficient, and reliable way to add small BioBrick parts to any BioBrick via PCR that is compatible with BioBrick assembly standard 10. As a proof of principle, we have designed a universal primer, rbs_B0034, that contains a ribosomal binding site (RBS; BBa_B0034) and that can be used in PCR to amplify any coding BioBrick that starts with ATG. We performed test PCRs with rbs_B0034 on 31 different targets and found it to be 93.6% efficient. Moreover, when supplemented with a complementary primer, addition of RBS can be accomplished via whole plasmid site-directed mutagenesis, thus reducing the time required for further assembly of composite parts. The described method brings simplicity to the addition of small parts, such as regulatory elements to existing BioBricks. The final product of the PCR assembly is indistinguishable from the standard or 3A BioBrick assembly.

RESULTS AND DISCUSSION

BioBricks are DNA sequences of defined structure and function.¹ BioBricks share a common interface that allows easy enzymatic manipulation, such as assembly of two or more parts together, creating a composite. Standardization of biological parts facilitates automation and part reuse. The BioBrick collection, available to researchers through the BioBrick Foundation, currently lists more than 3400 BioBricks. A portion of that collection are small parts (<150 bps), such as ribosomal binding sites (RBSs), promoters, operators, and terminators that are used to assemble efficient coding BioBricks and to regulate their expression. While there are several BioBrick assembly standards, standard 10 (RFC10) (originally developed at MIT in 2003²) is the most utilized as it is geared toward enzymatic assembly. As such, it is quite reliable even in novice hands, but relative time-consuming. On the other hand, methods such as Overlap Extension PCR cloning³ are fast, but handicapped by the necessity of designing and synthesizing a new set of primers for each individual BioBrick. While there have been numerous efforts to simplify the BioBrick assembly processes,⁴ they were either too complicated or did not get significant traction within the synthetic biology community. Here, we present a universal method that maintains compatibility with the standard 10 assembly, while delivering the simplicity and convenience of Overlap Extension PCR cloning.

There are many BioBricks that code for the protein parts but do not have any controlling elements such as ribosomal binding sites, promoters, or terminators fused to them. There are several benefits of having BioBricks in such a format. First, it helps to eliminate unwanted selection during the vector maintenance due to the low background expression of the protein. Second, it provides a choice to combine BioBricks with any desirable controlling elements, thus giving the opportunity to express the coded protein at just the right level. Those advantages come with the caveat—it may take a novice researcher days, if not weeks, to combine such BioBricks with small controlling elements before the results of the working BioBrick expression are seen. During the 2013 iGEM competition, we found that the success rate of 3A assembly⁵ for small parts, such as a ribosomal binding site, was significantly lower for the parts with a size of less than 150 bp (data not presented). We have calculated that we have spent approximately 70% of our wet lab time attempting to fuse the RBS site (BBa_B0034) with different coding parts. This was significant motivation to develop a reliable technique for BioBrick assembly of the small regulatory parts with the protein coding parts. Our design parameters were (1) the technique...
should be compatible with the RFC10 standard assembly and (2) the assembled product should be indistinguishable from one obtained through 3A assembly.

A closer look at the BioBrick sequences revealed that a 7 bp CTAGATG region is consistent among all coding BioBricks that start with ATG (Figure 1A; underlined). CTAGATG duplex melting temperature ($T_m$) is $-1.64$ °C, which makes it practically impossible to use it as a sole priming region for the forward primer during the PCR. We have speculated that the addition of the distant complementation region at the 5′ end of the primer should increase the probability for the 3′ end CTAGATG sequence to prime. We have also incorporated the RBS sequence (BBa_B0034) and scar region TACTAG in the final design of the forward primer (Figure 1A). Standard VR primer 5′-GTATTACCGCCTTTGAGTGA-3′ was used as a reverse primer for the PCR reaction (Figure 1C).

The result of the PCR reaction with 24 randomly selected coding BioBricks from the iGEM 2013 kit (Table 1) are presented in Figure 1D. Twenty-one out of twenty-four BioBrick parts were successfully PCR amplified. Attempts to verify the sequences for the three missing PCR amplifications (BBa_K648028; BBa_K538004; and BBa_K530000) revealed that DNA for those clones was either missing on the plate (no clones generated after transformation) or did not contain the standard BioBrick prefix. We have used standard BioBrick assembly with the pSB1C3 backbone provided with the iGEM kit and freshly generated PCR product to obtain the clones. The inclusion of the RBS was verified via sequencing of the constructs with the VR and VF2 primers.

The majority of the BioBrick parts delivered with the iGEM kit are on the pSB1C3 vector backbone. To simplify the addition of the RBS even further, we have attempted to use rbs_B0034 primer duplex in a whole plasmid site directed mutagenesis-like protocol (Figure 2). The list of targets is presented in Table 2. All seven targets were successfully modified. Inclusion of the RBS was verified via sequencing.

Traditional standard BioBrick assembly as well as 3A BioBrick assembly involves multiple enzymatic reactions and purification steps. The overall success for both techniques is relatively high in novice hands when specific attention is paid to the details and parts used in the assembly larger when 150 bp. However, assembly of parts smaller than 150 bp presents a significant challenge even for the experienced researcher and may result in substantial project delays. To address this challenge we have devised a simple, reliable, and universal protocol for the RBS assembly that can potentially also be applied to other small parts assembly.

We have first demonstrated that we can add the RBS sequence to any coding BioBrick through the use of the standard rbs_B0034 primer by PCR. We then simplified this approach one step further demonstrating the incorporation of RBS into the BioBrick containing vector via whole plasmid site directed mutagenesis. The resulting protocol is simple and reliable even in novice hands and is suitable for automation. The final product is indistinguishable from one obtained with either standard or 3A BioBrick assembly.

**METHODS**

**Bacterial Strains, Plasmids, Growth Conditions, and Transformation Procedures.** Chemically competent *E. coli* XL1-Blue cells were used for all the transformations. For each transformation, one microliter of either ligation product or PCR product was used to transform $20 \mu$L of competent cells. Transformants were grown at $37\ ^\circ\text{C}$ in $225 \mu$L of SOC medium for 1 h before spread on Luria–Bertani medium (LB) agar plates supplemented with $34 \text{mg/L}$ of chloramphenicol.

$$dx.doi.org/10.1021/sb500047r$$

ACS Synthetic Biology

957

ACS Synth. Biol. 2014, 3, 956–959
Viewpoint

Table 1. List of the BioBricks Used in the Study with the PCR Amplification/Standard Assembly Approach

| no. | marker | part no. | location in 2013 iGEM kit | backbone |
|-----|--------|----------|--------------------------|----------|
| 1   | FsC: cutinase PET cleaving enzyme | Bba_K808025 | 1 1F | pSB1C3 |
| 2   | tphC: terephthalate periplasmatic binding proteins of the tripartite transporter family | Bba_K808001 | 1 1H | pSB1C3 |
| 3   | tctB_162: small subunit B1 of the tripartite tricarboxylate transporter family | Bba_K808003 | 1 1J | pSB1C3 |
| 4   | tphB: reaction from DCD to protocatechuate | Bba_K808010 | 1 1K | pSB1C3 |
| 5   | toxin + antioxidant-Tse2 + Tsi2 | Bba_K514202 | 1 1M | pSB1C3 |
| 6   | tphA3: catalyses together with tphA2 TPA to DCD | Bba_K808013 | 1 1P | pSB1C3 |
| 7   | Cro J repressor that activates the lytic cycle | Bba_K648028 | 1 1C | pSB1C3 |
| 8   | humanized aequorin | Bba_K548000 | 1 2A | pSB1C3 |
| 9   | Vtc2 | Bba_K530025 | 1 2K | pSB1C3 |
| 10  | CspC (P. igeransis) | Bba_K538004 | 1 3I | pSB1C3 |
| 11  | enhanced lumazine synthase (ELS) | Bba_K542010 | 1 2O | pSB1C3 |
| 12  | K873000:B0015 | Bba_S05060 | 1 3J | pSB1C3 |
| 13  | Cpr10 (O. antarctica) | Bba_K538000 | 1 3K | pSB1C3 |
| 14  | iLOV | Bba_K660004 | 1 3L | pSB1C3 |
| 15  | thioesterase (TanA from E. coli) with 8-His Tag | Bba_K654058 | 1 3M | pSB1C3 |
| 16  | limulus anti-LPS factor (LALF) | Bba_K541505 | 1 3N | pSB1C3 |
| 17  | ReflectinIA from cephalopod | Bba_K541506 | 1 3O | pSB1C3 |
| 18  | LL 37 cathelicidin | Bba_K875009 | 1 3P | pSB1C3 |
| 19  | cry1 (lycopene cyclase) | Bba_K59119 | 1 3Q | pSB1C3 |
| 20  | α-pinene synthase | Bba_K517002 | 1 3R | pSB1C3 |
| 21  | GFP regulated by JOCl2-HSL and LasR | Bba_K649001 | 1 3S | pSB1C3 |
| 22  | Salty_Hcp-CD27 endolysin | Bba_K895004 | 1 3T | pSB1C3 |
| 23  | CRTYB | Bba_K530000 | 1 3U | pSB1C3 |
| 24  | SmtA | Bba_K519010 | 1 3V | pSB1C3 |

Linearized pSB1C3 backbone was used for all cloning, and was obtained as a part of iGEM 2013 DNA Distribution Kit.

PCR, Cloning, and Site Directed Mutagenesis Reactions. Phusion DNA polymerase (NEB, Ipswich, MA) and chimeric primer rbs_B0034 5′-TGAATTCGCCGCCCTTTCTAGAGAAGAGGAGAAATACATAGTG-3′ with the reverse standard VR primer 5′-GTATTACCGCCCTTGTAGTGA-3′ were used to PCR amplify different protein coding BioBricks from the iGEM 2013 DNA Distribution Kit plates. Each PCR was subjected to a temperature regimen similar to the following: initial denaturation at 100 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 1.5 min/kb for 30–32 cycles, with a final extension of 68 °C for 10 min. All PCR amplified inserts were cloned into pSB1C3 vector backbone in accordance to the BioBrick standard assembly protocol (see BioBrick Assembly Manual (NEB, Ipswich, MA) for details).

For site-directed mutagenesis RBS insertion procedure acceptor plasmid pSB1C3-“BioBrick” (30 ng) was mixed with 15 μL of 2 μM solution of rbs_B0034 primer and 15 μL of 2 μM solution of rbs_B0034 reverse compliment primer 5′-CATCTAGATTTTCTCCTTTTCTAGAAGCGCCGCGGAATTTCCA-3′ into a total 50 μL volume containing Phusion DNA polymerase reaction mixture containing dNTPs, buffer and the enzyme. The insert and vector were denatured (98 °C for 30 s), annealed (55 °C for 30 s), and polymerase-catalyzed extension (68 °C for 12 min) for 18 cycles.

The DpnI endonuclease works well in Phusion HF buffer. We typically add 10 units of the enzyme directly to the PCR tube right after the final extension is done and incubate the reaction for 1 h at 37 °C. Restriction endonuclease DpnI targets methylated DNA sequences and thus can cleave the DNA template isolated from most E. coli strains but not the PCR product.

Figure 2. Schematics of the one-step RBS (Bba_B0034) BioBrick assembly with the use of rbs_B0034 and rbs_B0034rc primers. The procedure is overall similar to the whole plasmid site directed mutagenesis. The starting material could be any coding BioBrick containing vector and universal rbs_B0034 and rbs_B0034rc primers.
AUTHOR INFORMATION

Corresponding Author
*E-mail: anton.bryksin@gmail.com or anton.bryksin@bme.gatech.edu.

Author Contributions
A.V.B., S.W.C., H.N.B., and T.H.B. conceived and designed the experiments. S.W.C., H.N.B., A.V.B., T.B., R.M.B., H.D., J.P.J., C.L.H., J.L.S., and V.F.F. performed the experiments. S.W.C., A.V.B., H.N.B., V.F.F., T.H.B., and H.D. analyzed the data. H.D., J.L.S., V.F.F., and T.H.B. contributed reagents/materials/analysis tools. A.V.B., S.W.C., H.N.B., and T.H.B. wrote the paper.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded in part by the National Institutes of Health (NIH) (R01NS079691, R01EB011566), the Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, Georgia Tech Petit Institute for Bioengineering and Bioscience and Georgia Tech GT FIRE grant to Thomas H. Barker, and Georgia Tech’s President’s Undergraduate Research Award.

REFERENCES

(1) Endy, D. (2005) Foundations for engineering biology. *Nature* 438, 449–453.
(2) Shetty, R. P., Endy, D., and Knight, T. F., Jr. (2008) Engineering BioBrick vectors from BioBrick parts. *J. Biol. Eng.* 2, 5.
(3) Bryksin, A. V., and Matsumura, I. (2010) Overlap extension PCR cloning: A simple and reliable way to create recombinant plasmids. *Biotechniques* 48, 463–465.
(4) Rokke, G., Korvald, E., Pahr, J., Oyas, O., and Lale, R. (2014) BioBrick assembly standards and techniques and associated software tools. *Methods Mol. Biol.* 1116, 1–24.
(5) Shetty, R., Lizarazo, M., Rettberg, R., and Knight, T. F. (2011) Assembly of BioBrick standard biological parts using three antibiotic assembly. *Methods Enzymol.* 498, 311–326.