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

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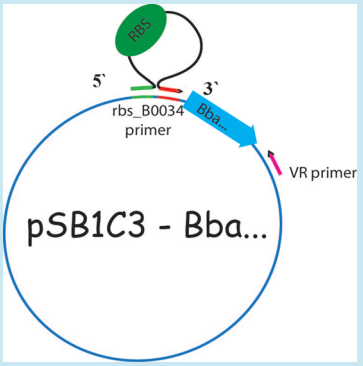
ABSTRACT: Here, we present a universal, simple, efficient, and reliable way to add small BioBrick parts to any BioBrick via PCR that is compatible with BioBrick assembly standard 10. As a proof of principle, we have designed a universal primer, rbs_B0034, that contains a ribosomal binding site (RBS; 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.



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 2003[2]) 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 cloning[3] 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.

RESULTS AND DISCUSSION

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 assembly[5] 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

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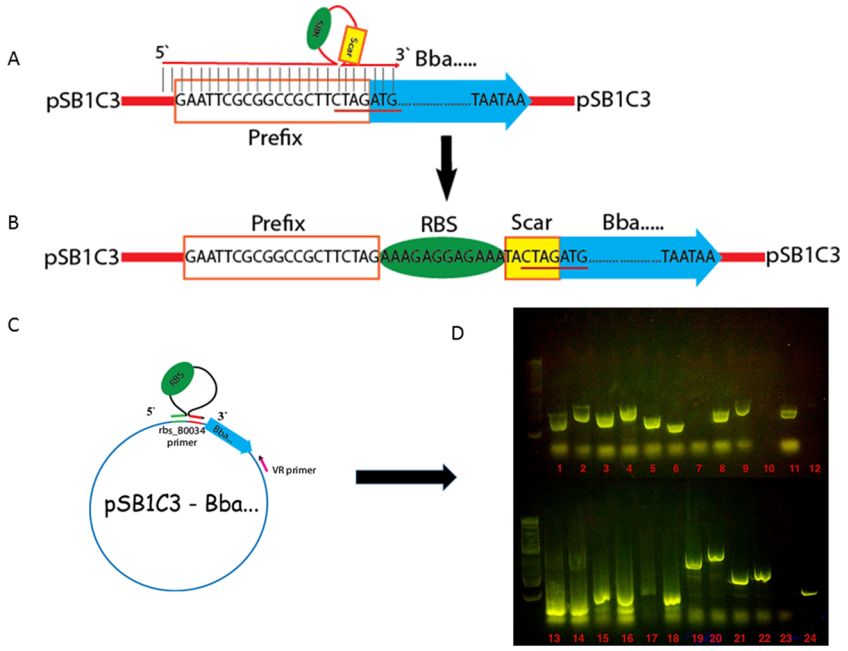

Figure 1. Design and application of the universal primer, rbs_B0034, for the PCR assembly with any coding biobrick. (A) Anatomy of the pSB vector with cloned BioBrick. Alignment of the rbs_B0034 primer to the vector containing BioBrick is demonstrated. (B) Anticipated product of the assembly that is indistinguishable from one obtained via enzymatic assembly. (C) Location of the alignment sites for the forward rbs_B0034 primer and reverse VR primer on pSB1C3-Bba··· vector, where Bba··· is any coding standard 10 BioBrick. (D) Results of the PCR amplification with the primers rbs_B0034 and VR for 24 BioBriks listed in the Table1. 0.8% agarose gel electrophoresis; Unmarked first lane has loaded 1 kb marker (NEB) 1−24 correspond to positions 1−24 in the Table1.

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 μL of competent cells. Transformants were grown at 37 °C in 225 μL of SOC medium for 1 h before spread on Luria−Bertani medium (LB) agar plates supplemented with 34 mg/L of chloramphenicol.

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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: terephtalate periplasmatic binding proteine 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 + antitoxin-Tse2 + Tsi2	BBa_K314202	1 1M	pSB1C3
6	tphA3: catalyzes together with tphA2 TPA to DCD	BBa_K808013	1 1P	pSB1C3
7	Cro λ repressor that activates the lytic cycle	BBa_K648028	1 2C	pSB1C3
8	humanized aequorin	BBa_K548000	1 2A	pSB1C3
9	Vtc2	BBa_K530025	1 2K	pSB1C3
10	CspC (*P. irgensii*)	BBa_K538004	1 3I	pSB1C3
11	enhanced lumazine synthase (ELS)	BBa_K542010	1 2O	pSB1C3
12	K873000:B0015	BBa_S05060	1 3J	pSB1C3
13	Cpn10 (*O. antarctica*)	BBa_K538000	1 3K	pSB1C3
14	iLOV	BBa_K660004	1 7M	pSB1C3
15	thioesterase (TesA from *E. coli*) with 8-His Tag	BBa_K654058	1 10E	pSB1C3
16	limulus anti-LPS factor (LALF)	BBa_K541505	1 11K	pSB1C3
17	Reflectin1A from cephalopod	BBa_K541506	1 11M	pSB1C3
18	LL 37 cathelicidin	BBa_K875009	1 11N	pSB1C3
19	crtY (lycopene cyclase)	BBa_K539119	1 12K	pSB1C3
20	α-pinene synthase	BBa_K517002	1 12J	pSB1C3
21	GFP regulated by 3OC12-HSL and LasR	BBa_K649001	1 12N	pSB1C3
22	Salty_Hcp-CD27_endolysin	BBa_K895004	1 13B	pSB1C3
23	CRTYB	BBa_K530000	1 14H	pSB1C3
24	SmtA	BBa_K519010	1 12O	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′-TGGAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATG-3′ with the reverse standard VR primer 5′-GTATTACCGCCTTTGAGTGA-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).

Table 2. List of the BioBricks Used in the Study with the Site Directed Mutagenesis Approach

marker	part	location in 2013 iGEM kit	backbone
GFP	BBa_K895006	1_15L	pSB1C3
SYFP2	BBa_K864100	1_17B	pSB1C3
YFP	BBa_K577006	1_23G	pSB1C3
blue fluorescent protein (mTagBFP)	BBa_K592100	1_19I	pSB1C3
engineered cyan fluorescent protein derived from *A. victoria* GFP	BBa_E0020	3_3M	pSB1C3
engineered mutant of red fluorescent protein from *Discosoma striata* (coral)	BBa_E1010	3_12N	pSB1C3
enhanced yellow fluorescent protein derived from *A. victoria* GFP	BBa_E0030	3_16D	pSB1C3

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′-CATCTAGTATTTCTCCTCTTTCTCTAGAAGCGGCCGCGAATTCCA-3′ in 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.

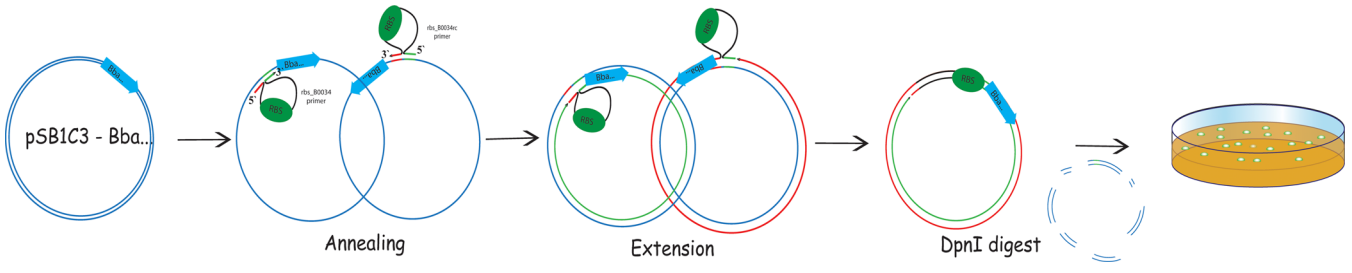

Figure 2. Schematics of the one-step RBS (BBa_B0034) BioBrick assembly with the use of rbs_B0034 and rbs_B0034rc primers. The procedure is overall similar to the whole plasmid site directed mutagenesis. The starting material could be any coding BioBrick containing vector and universal rbs_B0034 and rbs_B0034rc primers.

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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.

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