BBF RFC 63: DTU Synthetic Promoter Library Standard

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1. Purpose

The purpose of this RFC is to outline a method for generating a BioBrick compatible Synthetic Promoter Library (SPL) within bacteria in order to fine-tune the expression of BioBrick parts and devices.

2. Relation to other BBF RFCs

BBF RFC 63 does not update or replace any earlier BBF RFC.

3. Copyright Notice

4. Introduction to Synthetic Promoter Libraries

Modulation of gene expression of i.e. cellular enzyme activities [1], as well as regulation of transcription are amongst some of the areas where SPLs are currently being used. SPL provides an alternative method for gene regulation compared to older methods, namely those of gene knockouts and strong over expression. These two methods are usually based upon apparent rate limiting steps within metabolic pathways [2].

When working with gene regulation, it is important to elucidate where expression levels are optimal for the given gene being worked on. Under these specifications it is essential to be able to have slight increments in expressional strength when attempting to optimize gene expression. This can be achieved by the usage of an SPL, where the variability in strengths can be achieved by either randomizing the spacer sequences, namely the 17 bases that reside between the -35 and -10 consensus regions, and/or some of the bases within the consensus regions, being the -35 and -10 regions.

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The spacer sequences that surround the consensus regions contribute significantly to the strengths of promoters [3]. In our design, we decided to both randomize the spacer sequences as well as two bases in both consensus regions as seen in **Figure 1** below. N stands for 25% each of A, C, G and T, while S stands for 50% each of C and G, and W stands for 50% A and T.

The point of randomizing both areas is to obtain a promoter library that is not biased towards being strong. This is achieved by giving two bases within each of the consensus regions a 50% chance of being their original bases, ensuring that only 1/16 of all promoters will be strong. This is without taking into consideration the fraction of strong promoters obtainable from the randomized spacer sequences.

As previous studies indicate, consensus regions outside of the -35 and -10 regions seem to contribute very little in terms of altering promoter strengths. Mutating a single nucleotide will not change the promoter strength substantially, however mutating many nucleotides in the spacer sequences surrounding the -35 and -10 regions seem to result in the most significant alterations in promoter strengths [2]. This might be due to the three-dimensional structure that forms from the sequences that are arranged from the randomized spacer sequences [2].

Figure 1

Figure 1 illustrates an SPL designed on the basis of randomizing both the spacer sequences surrounding the consensus regions (-35 and -10 regions) as well as randomizing two bases within each of the consensus regions. N stands for 25% each of A, C, G and T, while S stands for 50% each of C and G, and W stands for 50% A and T.

When wanting to characterize and/or fine tune BioBrick parts and devices, using promoters that are constrained to already set strengths, has the disadvantage that the promoter might induce gene expression that is either too high or too low for the cell to be viable. This problem is nonexistent when using SPL since the SPL will necessarily give you the allowed upper and lower bounds of gene expression for cell viability. Cells with too strong or too weak promoters will simply never grow colonies.

5. Strategy for Integrating SPL into the BioBrick Assembly Standard

There are many different ways to integrate an SPL into the BioBrick Standard, and a lot of ideas were considered when creating this RFC. However, in the end a method was chosen based on the fact that it would be least time consuming for teams looking to use SPL, and at the same time, be easy to do. Instead of relying on ligations to successfully insert the SPL onto the BioBrick plasmid backbone, a Polymerase Chain Reaction (PCR) method MUST be used to not only amplify the backbone but also add the SPL onto the linear BioBrick plasmid backbone at a specific chosen site (see **Figure 2**). Since most teams will probably have to amplify their backbones during the course of a project, this method will only require a small amount of extra work.

Wanting to optimize gene expression and thereafter choosing a promoter that conforms to the strength that efficiently expresses your gene would be best perceivable if the SPL could be easily added and removed from BioBrick parts and/or devices. That is why the SPL will be inserted by PCR in-between the restriction sites EcoRI and XbaI of the BioBrick prefix. This way it is possible to add a part downstream of the SPL by simply ligating a part into the backbone plasmid containing SPL or by using the 3A-assembly standard. Furthermore it is also possible to move the whole insert into another BioBrick plasmid backbone if needed.

Figure 2

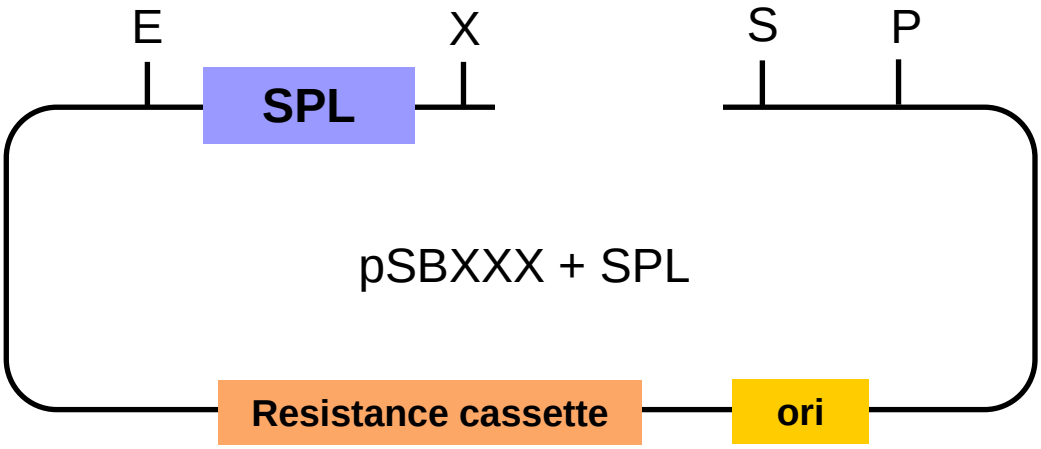


Figure 2 illustrates the linear BioBrick plasmid backbone with SPL inserted between the EcoRI and XbaI sites of the BioBrick prefix.

The design of the SPL leads to the possibility of illegal restriction sites being present within the randomized spacer sequence. If a given promoter is to be used in further ligations it MUST be sequenced first to ensure that it does not contain any recognition sites for EcoRI, XbaI, SpeI or PstI.

6. Primer Design

A PCR MUST be used in order to add the SPL onto the BioBrick plasmid backbone. The following primers for amplification of BioBrick plasmid backbones were used as a starting point for the design of our SPL primers:

i) *Primer Suffix-F*: 5′-ACTAGTAGCGGCCGCTGCAG-3′
ii) *Primer Prefix-R*: 5′-TCTAGAAGCGGCCGCGAATTC-3′

The primers were taken from http://partsregistry.org/Help:Construction_Plasmid. The restriction enzyme recognition sites are marked with the following colors: Blue – EcoRI, Green – XbaI, Red – SpeI, Turquoise – PstI.

In order to amplify and add the SPL successfully, the following modifications have been made to both of the annealing primers, which SHOULD be used:

I) Primer SPL Suffix-F: 5′-GTTTCTTCACTAGTAGCGGCCGCTGCAG-3′

For this primer, a tail with the standard seven extra bases has been added. For more information see http://openwetware.org/wiki/Synthetic_Biology:BioBricks/Part_fabrication.

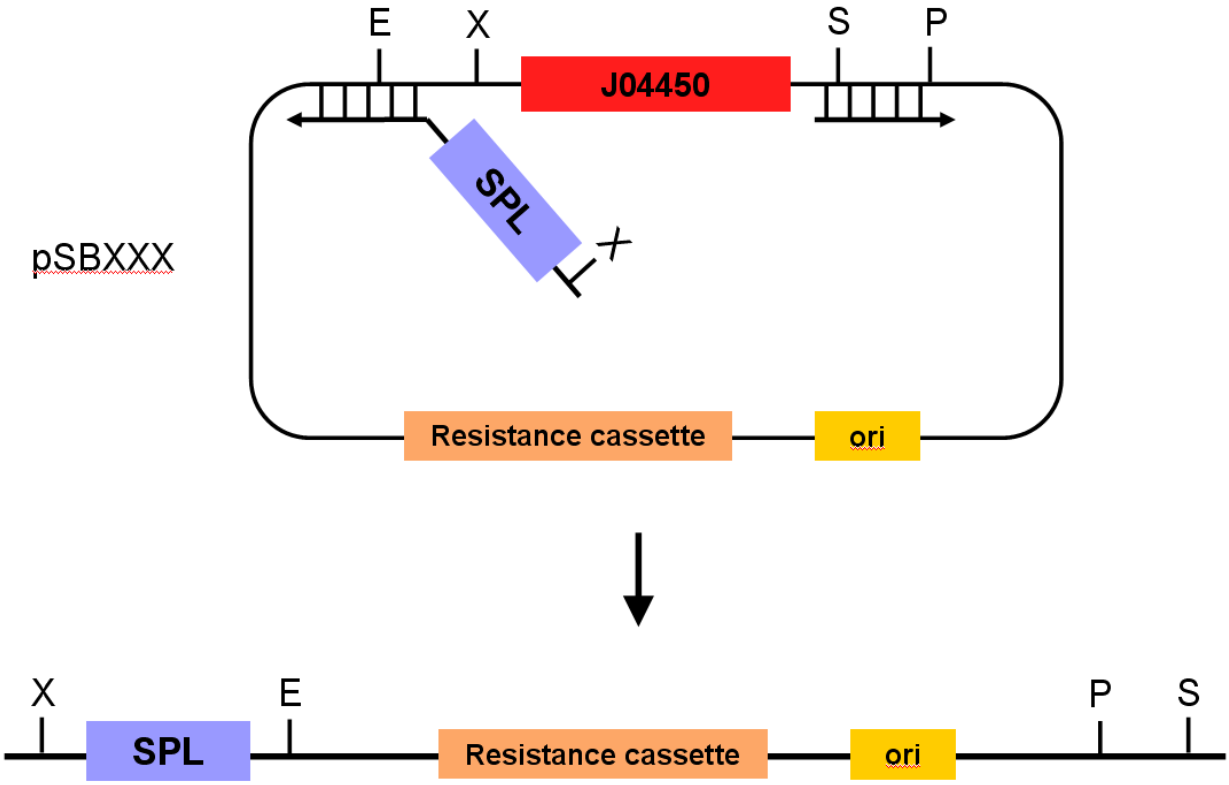
Depending on which backbone needs to be amplified, one of the following SPL primers SHOULD be used:

II) Primer SPL Prefix-R-01:
5′- GTTTCTTCCTCTAGAAGCGGCNNNNATWWTANNNNNNNNNNNNNNNNNNTGTSAWNNNNNNCGC
GAATTCCAGAAATCATCCTTAGCG -3′

III) Primer SPL Prefix-R-02:
5′- GTTTCTTCCTCTAGAAGCGGCNNNNATWWTANNNNNNNNNNNNNNNNNNTGTSAWNNNNNNCGC
GAATTCGAGTCACTAAGGGC -3′

These primers have the SPL sequence inserted between the EcoRI and XbaI sites. Furthermore, 14-18 nt have been added to the 3' end of the primer to ensure that the primers' annealing sequences are long enough. Appendix I contains a list showing which primer to use with regard to which backbone is chosen.

Figure 3



7. **Figure 3** illustrates the primer binding sites on a BioBrick plasmid backbone as well as the final linear plasmid backbone that is generated by the PCR.

In terms of primer annealing specificity, a touch down ramp PCR [4] MAY be used, but as **Table 1** illustrates, the melting temperatures (Tm) are relatively high and therefore a standard PCR can be run instead of a touch down ramp PCR.

Table 1

Primer	Tm - ºC
I) Primer SPL Suffix-F	62,1
II) Primer SPL Prefix-R-01	59,8
III) Primer SPL Prefix-R-02	60

Table 1 illustrates the Tm of the SPL primers.
IDT DNA oligo analyzer was used in order to calculate the Tm.
(http://eu.idtdna.com/analyzer/applications/oligoanalyzer/Default.aspx).

1. Depending on which BioBrick plasmid backbone is chosen (refer to Appendix I for the list), the selected primer pairs being either *I)* & *II)* or *I)* & *III)* SHALL be used for PCR.
2. It is RECOMMENDED that a high fidelity polymerase enzyme i.e. Finnzyme's Phusion enzyme is used to ensure a minimal amount of mutations occur in the BioBrick plasmid backbone during amplification.
3. The following amounts of substrates SHOULD be used if Phusion polymerase enzyme is used:

PCR substrates	Volumes - µL
Total volume	50
Phusion Polymerase (0,02 U/µL)	0,5
x5 Phusion HF buffer	10
dNTP's (5µM)	2
Primer SPL Suffix-F (10µM)	1,25
Reverse primer, either II) or III) (10µM)	1,25
Template - BioBrick plasmid backbone	1
ddH2O	33,5

For other polymerase enzymes, consult the manual for the polymerase for more specific information on PCR mixtures.

4. The following program SHALL be used if Phusion polymerase enzyme is used with SPL primers:

Cycle step	Temperature - ºC	Time	Cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	
Annealing	63*	30 sec	20-25
Extension	72	30 sec / kb	
Final extension	72	10 min	1
Hold	4	forever	1

*) When using Phusion polymerase enzyme for primers that are > 20nt, the annealing temperature SHOULD be 3ºC higher than the actual Tm.

For other polymerase enzymes, consult the manual for the polymerase for more specific information on PCR programs.

5. After PCR is completed, a PCR purification MUST be performed using a PCR clean up kit (e.g. Macherey-Nagel – NucleoSpin Extract II).

6. The BioBrick plasmid backbone should now contain the SPL and is ready to be used as a vector in a BioBrick assembly.

8. Summary of advantages

1. An SPL allows the fine tuning of gene expression by creating a promoter library with a wide variety of strengths.

2. The SPL will necessarily give you the allowed upper and lower bounds of gene expression for cell viability.

3. The method for creating the BioBrick compatible SPL is quick and easy to perform. The method is practically identical to the procedure for generating linear BioBrick plasmid backbones, which many teams will most likely have to do anyway.

4. Once the SPL is in the linear BioBrick plasmid backbone, it can be used in a variety of experiments. A BioBrick part or device can be ligated into the plasmid backbone and the resulting ligation can be transformed and screened for colonies containing the promoter strength needed.

9. Author's Contact Information

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10. References

[1] Solem, C. and Jensen, P. R. (2002). Modulation of Gene Expression Made Easy. Applied and Environmental Microbiology. 68(5): 2397-2403.

[2] Jensen, P. R. and Hammer, K. (1998). The Sequence of Spacers between the Consensus Sequences Modulates the Strength of Prokaryotic Promoters. Applied and Environmental Microbiology. 64(1): 82-87.

[3] Hammer, K., Mijakovic, I. and Jensen, P. R. (2006). Synthetic promoter libraries – tuning of gene expression. Trends in Biotechnology. 24(2): 53-55.

[4] Don, R. H et al. (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research. 19 (14): 4008.

For more information see: http://2010.igem.org/Team:DTU-Denmark/SPL_Section.

Appendix I: BioBrick Plasmid Backbone Lookup Table

BioBrick Plasmid Backbone	Primer II	Primer III	Sizes - bps
pSB1A3	✓	-	2157
pSB1AC3	✓	-	3055
pSB1AK3	✓	-	3189
pSB1AT3	✓	-	3446
pSB1C3	✓	-	2072
pSB1K3	✓	-	2206
pSB1T3	✓	-	2463
pSB2K3	✓	-	4425
pSB3C5	-	✓	2738
pSB3K5	-	✓	2936
pSB3T5	-	✓	3252
pSB4A5	-	✓	3395
pSB4C5	-	✓	3221
pSB4K5	-	✓	3419
pSB4T5	-	✓	3735

Appendix I illustrates which Prefix SPL primers SHOULD be used depending on which BioBrick plamid backbone is selected for amplification.