Development of iGEM Type IIS standard

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Mutagenesis

Estimated time: 3 days

All four pSB3C0#_J04455 vectors in Distribution Kit 2019 contain an Illegal BsaI restriction site (link to sequence Snapgene file old/ Registry). Even though a successful assembly into as well as out of these backbones was performed, BsaI site might interfere with the efficiency with cloning out of these vectors and creates an additional short fragment in the reaction. Illegal BsaI site was removed from pSB3C0# vectors by PCR mutagenesis creating the pSB3C1# series of Level 2 vectors.

pSB3C0# backbones were obtained from the 2019 Distribution Kit as described above for the Level 0 backbone. Then, pSB3C0# vectors were isolated using the GeneJET Plasmid Miniprep Kit and protocol. PCR mutagenesis was then performed with 0.5 - 1 ng of isolated DNA and 5' phosphorylated primers, Mut_L2_Fw and Rv (IDT), with Phusion polymerase (ThermoFisher) according to Phusion polymerase PCR protocol. Product was purified following GeneJET PCR purification kit and protocol and the concentration of DNA was assessed by Nanodrop (ThermoFisher).

500 ng of DNA was digested by 10U DpnI enzyme (#ER1701, ThermoFisher) for 1 hour at 37 ºC and heat inactivated at 80 ºC for 20 min. Digested DNA was purified according to GeneJET PCR purification kit protocol, concentration of DNA was estimated by Nanodrop (ThermoFisher).
20 ng of DNA was ligated using 2 Weiss U of T4 ligase (#EL0011 , ThermoFisher) at 22ºC for 1h and a control reaction without ligase was mixed in order to evaluate the amount of residual non-digested template DNA. After ligation, 5 uL of the cloning reaction were transformed to E.coli DH5α using the Transformation protocol and left growing overnight.

The screening of these backbones was done by restriction of pSB3C0# and pSB3C1# backbones 2h at 37ºC with BsaI (#ER0291, ThermoFisher) and the visualisation in an agarose gel. The backbones were also sequenced with the primers Seq_mRFP_F1 and Seq_L2_R4 (IDT) which would show the illegal BsaI site.

Gibson of new plasmids

Estimated time: 4 days

New plasmids for Type IIS assembly were created using Gibson assembly. These backbones are intended for Level 3 assemblies and consist on adding Level 1 fusion sites (from pSB1K0#) and an mRFP device to medium and low copy number backbones. Details of their construction can be found in Table 1.

Table 1. New backbones with part names, backbone used as base and plasmids from which the mRFP device was amplified.

Part name	Backbone base	mRFP device
pSB3K01 (BBa_K3425005)	pSB3K3	pSB1K01
pSB3K02 (BBa_K3425006)	pSB3K3	pSB1K02
pSB3K03 (BBa_K3425007)	pSB3K3	pSB1K03
pSB3K04 (BBa_K3425008)	pSB3K3	pSB1K04
pSB4K01 (BBa_K3425009)	pSB4K5	pSB1K01
pSB4K02 (BBa_K3425010)	pSB4K5	pSB1K02
pSB4K03 (BBa_K3425011)	pSB4K5	pSB1K03
pSB4K04 (BBa_K3425012)	pSB4K5	pSB1K04
pSB4A01 (BBa_K3425013)	pSB4A5	pSB1K01
pSB4A02 (BBa_K3425014)	pSB4A5	pSB1K02
pSB4A03 (BBa_K3425015)	pSB4A5	pSB1K03
pSB4A04 (BBa_K3425016)	pSB4A5	pSB1K04

Backbone parts were ordered from Twist Bioscience as double-stranded DNA (two per backbone), which means that adapters were added at the ends. The adapters were removed by PCR using oligos ordered from IDT (GIBSON_3K#2_Fw_4A#2_Rv_4K#1_Fw, GIBSON_3K#2_Rv, GIBSON_3K#1_Rv_4A#1_Fw, GIBSON_3K#1_Fw, GIBSON_4A#1_Rv_4K#1_Rv, GIBSON_4A#2_Fw_4K#2_Fw and GIBSON_4K#2_Rv). The mRFP device and fusion sites were amplified from pSB1K0# according to the table, using VF2 and VR primers. Both PCR reactions were done following the **Phusion polymerase PCR protocol**.

The PCR products were purified using the **GeneJET PCR purification kit** and **protocol**. The plasmid from purified RFP device PCRs was removed by 10U DpnI enzyme (#ER1701, ThermoFisher) for 2 hours at 37 ºC

and heat inactivated at 80 ºC for 20 min. It was purified by the **GeneJET PCR purification kit** again. Finally, the plasmids were constructed using the **Gibson assembly protocol**.

After cloning, 5uL of the cloning reaction were transformed to *E.coli* DH5α using the **Transformation protocol** and left growing overnight. The next day, red colonies were picked for screening. Overnight cultures of the positive clones were grown overnight in 12mL + antibiotic, following the **Overnight culture protocol**. These overnight cultures included some controls for copy number experiments, so we detail which *E. coli* DH5α strains were grown and what they were used for in Table 2.

Table 2. Strains grown overnight. Marked with an X are the strains used to evaluate copy number, strains sent for sequencing of their oris or strains used for PCR screening of their mRFP device and full length.

Strain (*E. coli* DH5α + …)	Copy number experiment	Sequencing	Screening PCRs
pSB3K01		Concentration too low	X
pSB3K02 colony #1	X	X	X
pSB3K02 colony #2	X	X	X
pSB3K04	X	Concentration too low	X
pSB4K02	X	X	X
pSB4K04	X	X	X
pSB4A01		X	X
pSB4A02	X	X	X
pSB4A03		X	X
pSB4A04	X	X	X
pSB1K01 (control)	X		
pSB3C11 (control)	X		
pSB4K5 (control)	X		
pSMART (Cm^R) (control)	X		

Afterwards, plasmid DNA was extracted from the cultures in a controlled way using the **GeneJET Plasmid Miniprep Kit** and protocol. Some resulting plasmids, according to Table X, were sent for sequencing with

the **Eurofins Sequencing Kit** (Seq_L2_F1 for pSB3K0#, and Seq_Rep101 for pSB4K0# and pSB4A0#; which sequenced the ori) and also screened by **PCR**: VF2-VR to see the mRFP1 device and other primers to see the full length (VF2 and Seq_L2_F1 for pSB3K0#; VR and Seq_L2_R3 for pSB4K0#; Mut_L2_Rev and VF2 for pSB4A0#).

Controlled plasmid minipreps were conducted by weighing Eppendorf tubes before and after pelleting the cells to figure out the wet pellet weight. These numbers were used to adjust the amount of DNA that was loaded in an **agarose gel**, so that it corresponded to the same amount of cells. The amount of DNA was assessed by Nanodrop (ThermoFisher) as well and an ANOVA was conducted between the copy numbers (high, medium or low) to see whether they were significantly different or not.

The copy number histogram plots the ng of DNA/mg of pellet * kb of each plasmid. The values for this number can be found on Table 3. The ANOVA was conducted with the following R script on the data that can also be found on Table 3:

```
read.delim("copynumber.csv", sep=",")-> copynumber
boxplot(ng.DNA.mg.pellet...kb ~ Type, data = copynumber, ylab= "ng DNA/kb*mg pellet",
xlab = "Copy number")
anova_cn <- aov(ng.DNA.mg.pellet...kb ~ Type,data = copynumber)
plot(anova_cn)
hist(resid(anova_cn))
summary(anova_cn)
```

The following results were obtained from the ANOVA summary:

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Type	2	1655	827.5	1.251	0.324
Residuals	11	7275	661.4		

Table 3. Data detailing the standardized amount of DNA per plasmid and their copy number.

Name	Type	ng DNA/mg pellet * kb
pSB3K01	Medium	15.156
PSB3K02 #1	Medium	32.808
PSB3K02 #2	Medium	23.557
pSB3K04	Medium	16.883

pSB4K02	Low	17.975
pSB4K04	Low	16.709
pSB4A01	Low	22.304
pSB4A02	Low	11.903
pSB4A03	Low	20.876
pSB4A04	Low	19.595
pSB1K01	High	44.717
pSB3C11	Medium	113.276
pSB4K5	Low	35.203
pSMART	Low	9.048

Cloning of dummy parts

Estimated time: 3 days

Dummy parts were created for Type IIS cloning when not all parts were needed. TU-DY (BBa_K3425017) mimics a transcription unit and MTU-DY (BBa_K3425018) a multi-transcriptional unit or a basic part.

These parts were ordered from IDT as primers (TU_dummy_Fw and Rev; MTU_dummy_Fw and Rev) and annealed with the **Parts Annealing protocol**. They were then cloned to the correct Type IIS backbones by the **Type IIS cloning protocol**, using BsaI for TU-DY and SapI for MTU-DY. The names and backbones for each dummy can be found in Table 4.

Table 4. Names of the dummy parts in backbones, together with which basic dummy part and backbone were used to construct them.

Part name	Dummy	Backbone
pSB1K01-DY	TU-DY	pSB1K01

pSB1K02-DY	TU-DY	pSB1K02
pSB1K03-DY	TU-DY	pSB1K03
pSB1K04-DY	TU-DY	pSB1K04
pSB3C11-DY	MTU-DY	pSB3C11
pSB3C12-DY	MTU-DY	pSB3C12
pSB3C13-DY	MTU-DY	pSB3C13
pSB3C14-DY	MTU-DY	pSB3C14

After cloning, 5uL of the cloning reaction were transformed to *E.coli* DH5α using the **Transformation protocol** and left growing overnight. The next day, white colonies were picked for screening following the **colony PCR and agarose gel protocols**. Overnight cultures of the positive clones were grown overnight, following the **Overnight culture protocol**. Afterwards, plasmid DNA was extracted from the cultures using the **GeneJET Plasmid Miniprep Kit** and protocol. The concentration of DNA was assessed by Nanodrop (ThermoFisher) and the plasmids were sent for sequencing with the **Eurofins Sequencing Kit**.

Sequencing

Estimated time: 1 day (over night)

Sequence confirmation of cloned parts was performed with the use of Mix2Seq Kit OVERNIGHT (Eurofins) according to manufacturer's instructions (found in the **sequencing protocol**). Purified DNA of appropriate concentration was mixed with one of the sequencing primers (VF2, VR or custom primers) and nuclease free water. The results were analyzed with the help of SnapGene 5.1.5. Version and Benchling online tool.