Lab Log Book

2020年7月17日 星期五

Experimenters: Eric Kim , John Yoon

Experiment title: Biobricks amplification

Description: Today, we tried to amplify 15 different biobricks (Shown in the Table 1.) included plasmids. After resuspending these plasmids found in the 2019 iGEM distribution plates(1~6) using 10ul of autoclaved MQ water, we trasferred 1ul of resuspended DNA (per each biobricks) on the 1.5ul test tube for the bacterial transformation. we followed "Chemical Transformation" protocol. For the competent cells, we used DH5-alpha.

Protocol: Bacterial transformation
Change in Protocol: N/A

Results: We failed the bacterial transformations (almost 95%) when we check the plates on the next day(7/18/2020).

Comments:possible reason for failure: short time taken for resuspending of DNA.

Details:

Table1

	A	B	C	D	E	F	G	H	I
1	Name of the parts(biobricks)	Description	Location in the Depository Kit	Antibiotics resistance gene(plasmids)	Test tube label		Biobrick Size (bp)	Expected Colony PCR Product (bp) with VF2/VR	Source
2	psB4A5	Low copy plasmid backbone (5-10 copies) from the original paper, pSC101 origin	2019 Kit plate 6, Well 2I	AMP(Ampicillin)	1A	o	3395	280	http://parts.igem.org/cgi/partsdb/dna_info.cgi?part=pSB4A5
3	BBa_B0034	Strong RBS, 100% strength	2019 Kit Plate 6, Well 1M	AMP	2A	o	12	326	http://parts.igem.org/Part:BBa_B0034
4	BBa_B0031	Weak RBS (from the original paper)	2019 Kit plate 2,Well 1H	CM(Chloramphenicol)	3C	o	14	328	http://parts.igem.org/Part:BBa_B0031
5	pSB3K3	A plasmid backbone containing p15A ori (10-15 copy)	2019 Kit plate 6,Well 6E	KM (Kanamycin)	4K	o	2750	216	http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB3K3
6	BBA_pSB3C5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 4C	CM	5C	o	2738	280	http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB3C5
7	BBA_pSB4K5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 6G	KM	6K	o	3419	270	http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB4K5
8	BBA_pSB4A5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 2I	AMP	7A	o (1A overlaps)			
9	BBA_pSB4C5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 4E	CM	8C	o	3221	270	http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB4C5
10	BBa_R0010	LacI regulated promoter. In the absence of LacI protein and CAP protein, this part promotes transcription. In the presence of LacI protein and CAP protein, this part inhibits transcription.	2019 Kit plate 3,Well 3G	CM - pSB1C3	9C	o	200	514	http://parts.igem.org/Part:BBa_R0010
11	BBa_C0012	LacI coding sequence	2019 Kit plate 2,Well 1N	CM - pSB1C3	10C	o	1153	1467	http://parts.igem.org/sequencing/part_analysis.cgi?part=BBa_C0012
12	BBa_I0500	Inducible pBad/araC promoter, Used in the original paper.	2019 Kit plate 5,Well 18M	CM - pSB1C3	11C	o	1210	1524	http://parts.igem.org/sequencing/part_analysis.cgi?part=BBa_I0500
13	BBa_R0040	Sequence for pTet inverting regulator. The promoter is constitutively ON and repressed by TetR. TetR repression is inhibited by the addition of tetracycline or its analogue, aTc.	2019 Kit plate 6,Well 21P	CM - pSB1C3	12C	o	54	368	http://parts.igem.org/Part:BBa_R0040
14	BBa_C0040	TetR coding sequence with BBa_B0034 (a strong RBS)	2019 Kit plate 2,Well 22H	CM - pSB1C3	13C	o	685	999	http://parts.igem.org/Part:BBa_C0040
15	BBa_B0015	Double terminator	2019 Kit plate 3,Well 4E	CM - pSB1C3	14C	o	129	443	http://parts.igem.org/Part:BBa_B0015
16	BBa_I718017	lox71 Forward	2019 Kit plate 3, Well 1C	CM - pSB1C3	15C	o	34	348	
17	BBa_J61046	loxP Forward	2019 Kit plate 3, Well 3K	CM - pSB1C3	16C	o	34	348	

Photo:N/A

2020年7月20日 星期一

Experimenters: Eric Kim , John Yoon

Experiment title: Biobricks amplification

Description: Today, we tried to amplify 15 different biobricks (Shown in the Table 1.) included plasmids. After resuspending these plasmids found in the 2019 iGEM distribution plates(1~6) using 10ul of autoclaved MQ water, we trasferred 1ul of resuspended DNA (per each biobricks) on the 1.5ul test tube for the bacterial transformation. we followed "Chemical Transformation" protocol. For the competent cells, we used DH5-alpha.

Protocol: Bacterial transformation
Change in Protocol: N/A

Results: We failed the bacterial transformations again when we check the plates on the next day.

Comments:possible reason for failure: ??, we don't know the reason.

Details:

Table2

	A	B	C	D	E	F
1	**Name of the parts(biobricks)**	**Description**	**Location in the Depository Kit**	**Antibiotics resistance gene(plasmids)**	**Test tube label**	
2	psB4A5	Low copy plasmid backbone (5-10 copies) from the original paper, pSC101 origin	2019 Kit plate 6, Well 2I	AMP(Ampicillin)	1A	o
3	BBa_B0034	Strong RBS, 100% strength	2019 Kit Plate 6, Well 1M	AMP	2A	o
4	BBa_B0031	Weak RBS (from the original paper)	2019 Kit plate 2,Well 1H	CM(Chloramphenicol)	3C	o
5	pSB3K3	A plasmid backbone containing p15A ori (10-15 copy)	2019 Kit plate 6,Well 6E	KM (Kanamycin)	4K	o
6	BBA_pSB3C5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 4C	CM	5C	o
7	BBA_pSB4K5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 6G	KM	6K	o
8	BBA_pSB4A5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 2I	AMP	7A	o (1A overlaps)
9	BBA_pSB4C5	Low to medium copy BioBrick standard vector	2019 Kit plate 6,Well 4E	CM	8C	o
10	BBa_R0010	LacI regulated promoter. In the absence of LacI protein and CAP protein, this part promotes transcription. In the presence of LacI protein and CAP protein, this part inhibits transcription.	2019 Kit plate 3,Well 3G	CM - pSB1C3	9C	o
11	BBa_C0012	LacI coding sequence	2019 Kit plate 2,Well 1N	CM - pSB1C3	10C	o
12	BBa_I0500	Inducible pBad/araC promoter, Used in the original paper.	2019 Kit plate 5,Well 18M	CM - pSB1C3	11C	o
13	BBa_R0040	Sequence for pTet inverting regulator. The promoter is constitutively ON and repressed by TetR. TetR repression is inhibited by the addition of tetracycline or its analogue, aTc.	2019 Kit plate 6,Well 21P	CM - pSB1C3	12C	o
14	BBa_C0040	TetR coding sequence with BBa_B0034 (a strong RBS)	2019 Kit plate 2,Well 22H	CM - pSB1C3	13C	o
15	BBa_B0015	Double terminator	2019 Kit plate 3,Well 4E	CM - pSB1C3	14C	o
16	BBa_I718017	lox71 Forward	2019 Kit plate 3, Well 1C	CM - pSB1C3	15C	o
17	BBa_J61046	loxP Forward	2019 Kit plate 3, Well 3K	CM - pSB1C3	16C	o
18						

Photo:N/A

2020年7月21日 星期二

Experimenters: John Yoon, Eric Kim, Stephanie Lee, Peter Lee

Experiment title: Bacterial transformation
Description:
Protocol:
Change in Protocol: N/A
Results:
Comments:
Details:
Photo:N/A

Experiment title: PCR
Description: Amplification of different Lox sites
Protocol:
Q5 polymerase PCR protocol

- New DNA samples prepared (dilution to 100uM and creating separate working solution)
  - make 50ul of working solution for each tube by dilution
  - All primers (forward and reverse **loxPrimer**, **303gvsdup, R32M, R32V, and Y324F**) diluted to a 50ul working solution at 10uM concentration - 5ul of stock, 45ul autoclaved MQ water.
  - All lox sites diluted into 10nM solution through two rounds of 100x dilution, resulting in a 100ul working solution for each.

Table3

	A	B
1	DNA sequences	
2	**Primers**	(10uM working solution)
3	303gvsdup F	50ul
4	303gvsdup R	50ul
5	R32M F	50ul
6	R32M R	50ul
7	R32V F	50ul
8	R32V R	50ul
9	Y324F F	50ul
10	Y324F R	50ul
11	**Lox sites**	(10 nM working solution)
12	lox66 Reverse	100ul
13	loxP Reverse	100ul
14	Lox2272	100ul
15	LoxN Reverse	100ul
16	lox2272 Reverse	100ul
17	Lox5171	100ul
18	lox5171 reverse	100ul

Change in Protocol:
For mastermix since we are using Q5 polymerase,

1.Pipette the following reagents to form the 25ul master mix:

Table6

	A	B	C	D
1	Reagent(working solution conc.)	Final Concentration	Volume Added	Master mix amount(for 7 samples)
2	5x Q5 Reaction Buffer (?)	1x	5ul	35ul
3	dNTPs (10mM)	200uM	0.5ul	3.5ul
4	Forward loxPrimer (10uM)	0.5uM	1.25ul	8.75ul
5	Reverse loxPrimer (10uM)	0.5uM	1.25ul	8.75ul
6	Template DNA (10nM)		20ng → 14ul	
7	Enhancer		2.75ul	19.25ul
8	Q5 Polymerase	0.02 U/ul	0.25ul	1.75ul
9	Nuclease-free H2O			
10	Total volume		25ul	42 ul

2. Set thermal cycler per directions below:

Temperatures
[Amplification process takes approximately 35 minutes]

Initial denaturation → 98°C for 30 seconds

Phase I - touchdown phase [10 cycles]

- Denaturing → 98°C for 10 seconds
- Annealing → 72°C for 10s, reduce by 0.5C each time for the next 8 cycles
- Extension Phase → 72°C for 10s

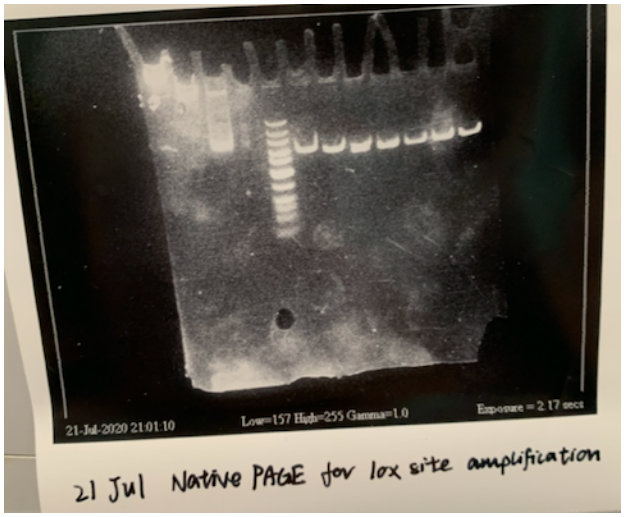
Phase II - generic amplification [15 cycles]

- Denaturing → 98°C for 10s
- Annealing → 68°C for 10s
- Extension → 72°C for 10s

Termination → 72°C for 2 minutes(ensuring complete polymerase chain reaction)

Results: positive through gel electrophoresis
Comments:
Details:
Photo:

Screen Shot 2020-07-23 at 11.50.16 AM.png



Experiment title: Native DNA page
Description: Checking for amplified PCR Products
Protocol: Native PAGE
Change in Protocol: N/A
Results:
Comments:

- Mistakes when setting the gel:
  - Gel should be allowed to set a bit longer. When comb was removed, some remaining un-set solution went in and blocked some wells, interfering with sample loading.
  - We did not properly clean each well before loading the samples.
- To avoid contamination, consider mixing the loading dye on Parafilm instead of directly adding it to the PCR product when preparing it for the gel.

Details:
Photo:N/A

Experiment title: PCR Purification (DNA Clean-up)
Description: to purify PCR product (remove staining product, excess PCR reagents), now verified to contain our desired products, in preparation for upcoming experiments.
Protocol: MEGAquick-spin Purification Kit Protocol B
Change in Protocol: As an elution buffer is unavailable, 50ul of autoclaved MQ water was used as a replacement for the elution part (step 9).
Results:
Comments:

- Due to small size of DNA fragment, 1.5 volume of isopropanol was added to the sample in step 3

Details:
Photo:N/A

2020年7月22日 星期三

Experimenters: John Yoon, Eric Kim, Stephanie Lee, Peter Lee

Experiment title: Ligation Reaction
Description: ligate lox site DNA inserts with plasmid backbone
Protocol: Restriction Digestion of Biobrick Parts,

- Ligation reaction designed with reference to https://international.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

Change in Protocol: N/A

Results:
Comments:
Details:

- We used Nanodrop to check the concentration of DNA in the purified PCR samples.
- 8 tubes prepared for digestion (7 samples + 1 backbone, refer to table below), incubated for approx. 90 minutes.

Table4

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	1 [5171]	11.7	6.4	10.6	2	0.5	0.5	20
3	2 [5171R]	8.2	9.1	7.9	2	0.5	0.5	20
4	3 [2722]	6.2	12.1	4.9	2	0.5	0.5	20
5	4 [2722R]	8	9.4	7.6	2	0.5	0.5	20
6	5 [66R]	8.8	8.5	8.5	2	0.5	0.5	20
7	6 [NR]	8.3	9	8	2	0.5	0.5	20
8	7 [[PR]	9.8	7.7	9.3	2	0.5	0.5	20
9	8 [backbone]	/	7	10	2	0.5	0.5	20

- Due to PstI having no deactivation temperature, DNA cleanup was done on the samples instead.
- Prepare mixture for ligation reaction as follows:
  - Note: less backbone added to NR and PR sample tubes due to insufficient reagent after purification.

Table5

	A	B	C	D
1				
2	Component	20 µl reaction	50ul reaction	
3	T4 DNA Ligase Buffer (10X)*	2 µl	5ul	
4	Vector DNA (4 kb)	50 ng (0.020 pmol)	75 ng (7ul)	
5	Insert DNA (1 kb)	37.5 ng (0.060 pmol)	25 ng (30ul)	
6	T4 DNA Ligase	1 µl	2.5ul	
7	Nuclease-free water	up to 20ul	5.5ul, up to 50ul	
8	Total	20	50	

Photo:N/A

Experiment title: DNA Transformation
Description: transformation of ligated DNA parts into competent cells
Protocol:
Change in Protocol: N/A
Results: FAILED. No colonies observed the next day, most likely due to mistakes during ligation reaction (insufficient ligation time, overly-diluted reagents). To be redone tomorrow.
Comments: N/A
Details:
Photo:N/A

2020年7月23日 星期四

Experiment title: Ligation Reaction
Description:
Protocol:
Change in Protocol: N/A
Results:
Comments: Heat inactivation not required
Details:

- Ligation products from previous experiment were cleaned to redo the reaction. Remaining DNA was collected in 30ul MQ Water (eluent) but the samples were concentrated by drying the samples to approximately 10ul (original target was 5ul but still sufficient).
- Added 2ul ligase buffer, 1ul DNA ligase, then topped up sample with MQ H2O to form the 20ul reaction mixture
- Mixture was incubated overnight at room temperature.

Photo:N/A

2020年7月24日 星期五

Experiment title: Colony PCR
Description:
Protocol:
Change in Protocol: N/A
Results:
Comments: N/A
Details:
Photo:N/A

Experiment title: Miniprep
Description:
Protocol:
Change in Protocol: N/A
Results:
Comments: N/A
Details:
Photo:N/A

2020年7月27日 星期一

Experiment title: Biobrick Amplification
Description:
Team Instructions:

- For inserts longer than 200bp, do restriction digestion on 500ng of plasmid
- For inserts smaller than 200bp, run a PCR
    - (for vector backbones, we decided to also run them in a PCR as the fragments from their restriction digestion would be inappropriate to run on the same gel - e.g. 41bp vs 2.7kb)

Protocol:

0727 Biobrick Amplification Planning

	A	B	C	D	E	F	G	H	I
1	Name of the parts(biobricks)	Description	Antibiotics resistance gene(plasmids)	Test tube label	Biobrick Size (bp)	Expected PCR Product (bp) with VF2/VR	Expected RD Product (bp) with EcoI and PstI	Source	Remarks
2	psB4A5	Low copy plasmid backbone (5-10 copies) from the original paper, pSC101 origin	AMP(Ampicillin)	1A Success	3395	280 (X) --> 1349		http://parts.igem.org/cgi/partsdb/dna_info.cgi?part=pSB4A5	PCR, RD
3	BBa_B0034	Strong RBS, 100% strength	AMP	2A	12	326		http://parts.igem.org/Part:BBa_B0034	PCR
4	BBa_B0031	Weak RBS (from the original paper)	CM(Chloramphenicol)	3C	14	328		http://parts.igem.org/Part:BBa_B0031	PCR
5	pSB3K3	A plasmid backbone containing p15A ori (10-15 copy)	KM (Kanamycin)	4K Success	2750	216 (X) --> 1285		http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB3K3	PCR, RD
6	BBA_pSB3C5	Low to medium copy BioBrick standard vector	CM	5C Success	2738	280 (X) --> 1349		http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB3C5	PCR, RD
7	~~BBA_pSB4K5~~	~~Low to medium copy BioBrick standard vector~~	~~KM~~	~~6K Failed~~	3419	270 (X) --> 1112	1112	~~http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB4K5~~	(no pellet observed during miniprep)
8	~~BBA_pSB4A5~~		~~AMP~~	~~7A~~	~~N/A~~	~~N/A~~		~~N/A~~	(overlap with 1A)
9	BBA_pSB4C5	Low to medium copy BioBrick standard vector	CM	8C Success	3221	270 (X) --> 1349		http://parts.igem.org/sequencing/part_analysis.cgi?part=pSB4C5	PCR, RD
10	BBa_R0010	LacI regulated promoter. In the absence of LacI protein and CAP protein, this part promotes transcription. In the presence of LacI protein and CAP protein, this part inhibits transcription.	CM - pSB1C3	9C Success	200	514		http://parts.igem.org/Part:BBa_R0010	PCR, RD
11	BBa_C0012	LacI coding sequence	CM - pSB1C3	10C Success	1153	1467	1194	http://parts.igem.org/sequencing/part_analysis.cgi?part=BBa_C0012	RD
12	BBa_I0500	Inducible pBad/araC promoter, Used in the original paper.	CM - pSB1C3	11C Success	1210	1524	1251	http://parts.igem.org/sequencing/part_analysis.cgi?part=BBa_I0500	RD
13	BBa_R0040	Sequence for pTet inverting regulator. The promoter is constitutively ON and repressed by TetR. TetR repression is inhibited by the addition of tetracycline or its analogue, aTc.	CM - pSB1C3	12C Success	54	368		http://parts.igem.org/Part:BBa_R0040	PCR
14	BBa_C0040	TetR coding sequence with BBa_B0034 (a strong RBS)	CM - pSB1C3	13C Success	685	999	726	http://parts.igem.org/Part:BBa_C0040	RD

15	BBa_B0015	Double terminator	CM - pSB1C3	14C Success	129	443		http://parts.igem.org/Part:BBa_B0015	PCR
16	BBa_I718017	lox71 Forward	CM - pSB1C3	15C Success	34	348			PCR
17	BBa_J61046	loxP Forward	CM - pSB1C3	16C Success	34	348			PCR

Touchdown PCR using DreamTaq Polymerase

- According to Thermo calculator, **Tm = 56.8C** (round off to 57C?)
- Reaction Setup (per 20ul reaction):
  - 2ul of 10X DreamTaq Buffer
  - 2ul of 2mM dNTP
  - 0.5ul (later changed to 1ul???) of forward primer [VF2]
  - 0.5ul (later changed to 1ul???)of reverse primer [VR]
  - 1ul (at least 10ng) of Template DNA
  - 0.10ul **(1.25U)** of DreamTaq DNA Polymerase
  - Nuclease-free water **to 20ul**
- 35 Cycles
  - Stage 1 (Initial Denaturing, 1x cycle) --> 98C for 30 seconds, or 95C for 3 minutes [normal protocol]
  - Stage 2 (Touchdown, **10x cycles**) --> 95C for 30s, 62C (Tm + 5C) for 30 sec (reduce by 1C per cycle), 72C for 1 min
  - Stage 3 (Normal, **25x cycles**) --> 95C for 30s, 52C (Tm-5C) for 30s, 72C for 1 minute
  - Stage 4 (Final Extension, 1x cycle) --> 72C for 15 minutes
  - Hold at 4C

Restriction Digestion

- To prepare the reaction mixture, calculations were made for a digestion of 500ng of plasmid

0727 Restriction Digestion Planning

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	10C	147.7	3.4	13.6	2	0.5	0.5	20
3	11C	146.9	3.4	13.6	2	0.5	0.5	20
4	13C	121.6	4.1	12.9	2	0.5	0.5	20

- Reaction mixtures were incubated in a 37C water bath overnight.
  - Feedback the next day --> should not have incubated overnight, 1 hour would be sufficient to complete the reaction.

Change in Protocol: N/A
Results:
Comments: N/A
Details:
Photo:N/A

2020年7月28日 星期二

- Restriction digestion of the parts longer than 200bp (three parts)
- Ligation of LoxP sites
- Agarose gel for RD and PCR Products
- Transformation of LoxP sites

Experiment title: Restriction Digestion
Description: further RD on backbone biobricks as PCR was not appropriate
Protocol: Restriction Digestion of Biobrick Parts

- To prepare the reaction mixture, calculations were made for a digestion of 500ng of plasmid, shown below

- Reaction mixtures incubated for 1 hour in 37C water bath.

0728 Restriction Digestion Planning

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	1A	83	6.0	11.0	2	0.5	0.5	20
3	4K	38	13.2	3.8	2	0.5	0.5	20
4	5C	311	1.6	15.4	2	0.5	0.5	20
5	8C	433.5	1.2	15.8	2	0.5	0.5	20

- 35 Cycles (Tm = 56.8)
    - Stage 1 (Initial Denaturing, 1x cycle) --> 98C for 30 seconds
    - Stage 2 (Touchdown, **10x cycles**) --> 95C for 30s, 67C for 30 sec (reduce by 1C per cycle), 72C for 30s
    - Stage 3 (Normal, **25x cycles**) --> 95C for 30s, 57C (Tm-5C) for 30s, 72C for 30s
    - Stage 4 (Final Extension, 1x cycle) --> 72C for 5 minutes
    - Hold at 4C

Change in Protocol: N/A
Results:
Comments: N/A
Details:
Photo:N/A

2020年7月29日 星期三

2020年7月30日 星期四

To do list:

- [ ] Ligation and transformation of the loxp sites
- [ ] Digest and transform the G Block
- [ ] make competent cells
- [ ] make CM LB agar plates (10x: 300ml)
- [ ] PCR of RBS parts
- [ ] Amplify GFPLAA and transform
- [ ] Ligate and transform inducible promoter (PBAD) with RBS (weak and strong)
- [ ] Digest and transform Cre
- [ ] Come up with the digestion protocols for the experiments (Joe and Munisa)

Digestion of inserts -> ligation -> Transformation

Digested products

	A	B
1	Name of insert	Backbone used
2	Cre Gblock	pSB1C3
3	Rev RFP LAA Gblock	
4	2272 lox	
5	PR lox	
6	66R lox	
7	2722 lox	
8	5171 lox	
9	NR lox	
10	Control(empty)	
11		
12		

Transformed plasmids

	A	B
1	Transformed parts	
2	Cre Gblock	
3	Rev RFP LAA Gblock	
4	2272 lox	
5	PR lox	
6	66R lox	
7	2722 lox	
8	5171 lox	
9	NR lox	
10	Control(empty)	
11		
12	Competent cell efficiency backbone	
13	GFP LAA part	
14		
15		
16		
17		

Restriction Digestion was carried out for remaining biobrick sample 6K (vector backbone), 500ng of plasmid was digested. Expected size of digested products to be 1112bp + 2307bp

0730 Biobrick Restriction Digestion Planning

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	6K	130.2	3.8	13.2	2	0.5	0.5	20

2020年7月31日 星期五

To do list:

- [ ] If there are colonies for the G Block, check the dna with colony pcr, gel and inoculation for overnight
- [ ] If there are colonies for the GFPLAA+RBS, check the dna with colony pcr, gel and inoculation for overnight
- [ ] Miniprep for loxp sites
- [ ] Digestion and transformation G Block + right loxp site
- [ ] Digestion and transformation const. promoter + left loxp site
- [ ] Digestion and cloning of the GFPLAA

0731 Restriction Digestion (Redo from Miniprep)

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	6K	167.5	3.0	14.0	2	0.5	0.5	20

0731 Touchdown PCR for Biobricks 2A, 3C

	A	B	C	D	E	F	G	H	I	J
1	PCR Tube	Template	Template DNA	10x DreamTaq Buffer	DreamTaq Enzyme	10uM dNTP	Forward Primer	Reverse Primer	MQ Water	Total Vol. of Reaction Mixture
2	1	2A (0731 MP)	1	2	0.1	0.4	0.5	0.5	15.5	20
3	2	3C (0731 MP)	1	2	0.1	0.4	0.5	0.5	15.5	20
4	3	2A (redo prev.)	1	2	0.1	0.4	0.5	0.5	15.5	20
5	4	3C (redo prev.)	1	2	0.1	0.4	0.5	0.5	15.5	20
6			1	2	0.1	0.4	0.5	0.5	15.5	20
7			Total for Master Mix	10	0.5	2	2.5	2.5	77.5	

Verification of amplified/digested parts

2A, 3C --> 2% TAE agarose gel (large wells)

6K --> 1% TAE agarose gel (large wells)

- Loading: ladder, 6K (purified and digested , "old 6K" (purified 30/7, re-digested 31/7), "6K purified" (purified and digested 31/7)
- Gel ran for 45-60min at 120V (reduced to 100V for additional 15 minutes to get better separation)

Results:

- 6K digested fragment sizes match, verification successful for both purified samples (30/7 and 31/7)
- PCR failed for 2A, 3C, bands were too large, which suggested that we were observing the template DNA instead of its amplicons.

2020年8月1日 星期六

To do list:

- [ ] Miniprep for the G Block
- [ ] Miniprep for GFPLAA+RBS
- [ ] If there are colonies for the G Block + right lox sites, check the dna with colony pcr and gel and inoculation for overnight
- [ ] If there are colonies for the const. promoter + left loxp site check the dna with colony pcr and gel inoculation for overnight

2020年8月2日 星期日

To do list:

- [ ] Miniprep for the G Block + right lox sites
- [ ] Miniprep for the promoter + left loxP sites

2020年8月5日 星期三

Experiment title: Transformation

Description: Transformation of the constructs

Change in Protocol: N/A

Results: Colonies were observed the next day for every part, Cre was failed to verify

Comments: Heat inactivation not required

Details:

Parts used in the Experiment

	Name	DNA Conc.
1	BBa_B0034 Strong RBS	
2	Reverse RBS RFP LAA G Block	
3	loxN	
4	loxP	
5	lox66	
6	lox71	
7	lox511	
8	lox5171	
9	lox2272	
10	Cre bacteria G Block	

2020年8月6日 星期四

Experiment Title: PCR and miniprep

Description: run PCR on liquid cultures prepared on Wed, check size of amplicons to verify parts.

Change in Protocol:

Details:

- Colonies picked and suspended in 4ul liquid culture overnight (for approximately 16 hours)
- Prepared 20ul PCR reaction mixture:

Reaction Mixture for PCR with Dream...

	A	B
1	DreamTaq (10x)	2ul
2	DreamTaq Polymerase (5U/ul; target 1.25U)	0.1ul
3	dNTP (10mM; target 0.2mM)	0.4ul
4	Forward Primer (VF2)	1ul
5	Reverse Primer (VR)	1ul
6	Template DNA (from overnight liquid media)	2ul
7	Nuclease-free water (MQ basically)	13.5ul
8	**Total**	**20ul**

- Heat-cycling protocol --> saved as "iGEMDreamTaq",
- Separate PCR protocols were run for the smaller fragments (expected size 300-400bp and the larger parts (expected size >1kb)
    - Initial Denaturing: 98C for 30 seconds
    - "Touchdown" for 10 cycles: 95C 30s → 67C 30s (reduce by 1C each cycle) → 72C 30s/1min (depending on amplicon size)
    - "Normal" cycling for 25 cycles: 98C 30s → 57C 30s → 72C 30s/1min
    - Final extension: 72C for 5 minutes
    - Hold at 4C
- PCR products loaded onto 1.5% TAE Gel with 1kb GeneLadde
    - Loading: 2ul loading dye, 2ul PCR product, and 8ul MQ water
    - Run at 120V for 40 minutes

Results: correct bands observed for all samples except Cre. Miniprep was still done for all samples using the TianGen purification kit.
Comments:

- Follow-up action: after Miniprep of Cre sample, colony PCR performed by Eric to check Cre and RFP LAA parts
    - Same reaction mixture, but instead of 2ul template DNa, we re-suspended the selected colony in 15.5ul of MQ water before adding other reagents
- Can probably experiment with electrophoresis voltage and duration later on for higher efficiency.

Experiment title: Cloning of parts
Description:
Change in Protocol: N/A
Results: Colonies were observed the next day for every part, Cre was failed to verify
Comments: Heat inactivation not required
Details:

Restriction Digestion

Digestion Planning for RRFP and loxp sites

	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI-HF added (ul)	XbaI	Total Volume
1	loxP reverse	146.9	3.4	13.6	2	0.5	0.5	20
2	lox2272 reverse	121.6	4.1	12.9	2	0.5	0.5	20
3	lox5171 reverse							
4	lox66 reverse							
5	loxN reverse							

Table7

	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI-HF added (ul)	SpeI-HF added (ul)	Total Volume
1	RFP-gBlock	146.9	3.4	13.6	2	0.5	0.5	20

Digestion Planning for the pBAD promoter with the RBSs

	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	XbaI added (ul)	SpeI added (ul)	Total Volume
1	pBAD promoter	146.9	6.8	10.2	2	0.5	0	0.5	20
2	RBS str	69	4.3	12.7	2	0.5	0.5	0	20
3	RBS we	118.2	2.5	14.5	2	0.5	0.5	0	20

Expected size of Digestion Products

	Tube	Size (bp)
1	pBAD promoter	1233
2	RBS str	2082
3	RBS we	2084

2020年8月7日 星期五

Experiment title: Site-directed mutagenesis
Description: Amplification of the Cre plasmid by TD PCR, digestion by DpnI, blunt-end ligation and transformation
Change in Protocol: TD PCR
Results: Unsuccessful
Comments: Maybe the extension time is too short, annealing temperature is too high or too many cycles
Details:

Samples

	Name	Tm in TD (°C)	Tm after TD (°C)
1	Y324F	70	70
2	R32M	71	71
3	R32V	71	71
4	303gvsup	68	72
5	Control	N/A	
6			

Temperatures
[Amplification process takes approximately 35 minutes]

Initial denaturation → 98°C for 30 seconds

Phase I - touchdown phase [10 cycles]

- Denaturing → 98°C for 10 seconds
- Annealing → 71°C for 10s, reduce by 0.4C each time for the next 8 cycles
- Extension Phase → 72°C for 70 s

Phase II - generic amplification [15 cycles]

- Denaturing → 98°C for 10s
- Annealing → 70°C for 10s
- Extension → 72°C for 70 s

Termination → 72°C for 2 minutes (ensuring complete polymerase chain reaction)

2020年8月10日 星期一

Experiment title: Site-directed mutagenesis
Description: Amplification of the Cre plasmid by TD PCR, digestion by DpnI, blunt-end ligation and transformation
Change in Protocol: TD PCR
Results: Successful
Comments: Maybe the extension time is too short, annealing temperature is too high or too many cycles
Details:

Table8

	Name	Tm in TD (°C)	Tm af-ter TD (°C)
1	Y324F	67	67
2	R32M	67	68
3	R32V	68	68
4	303gvsup	64	69
5	Control	N/A	
6			

Table9

	A	B	C	D	E
1	5X Q5 Reaction Buffer	5	25	1X	y
2	10 mM dNTPs	0.5	2.5	200 µM	y
3	10 µM Forward Primer	1.25		0.5 µM	y
4	10 µM Reverse Primer	1.25		0.5 µM	y
5	Template DNA	10		< 1,000 ng	y
6	Q5 Hot Start High-Fidelity DNA Polymerase	0.25	1.25	0.02 U/µl	y
7	Nuclease-Free Water	6.75	33.75	(1X)	y
8	Total	25	12.5		

Temperatures

[Amplification process takes approximately 35 minutes]

Initial denaturation → 98°C for 30 seconds

Phase I - touchdown phase [10 cycles]

- Denaturing → 98°C for 10 seconds
- Annealing → 72°C for 10s, reduce by 1C each time for the next 8 cycles
- Extension Phase → 72°C for 90 s

Phase II - generic amplification [10 cycles]

- Denaturing → 98°C for 10s
- Annealing → 67°C for 10s
- Extension → 72°C for 90 s

Termination → 72°C for 2 minutes (ensuring complete polymerase chain reaction)
Result:

Experiment title: Colony PCR of RRFP G-Block
Description: Previously purified sample did not work during cloning, re-picking colonies to verify parts.
Protocol Used: Colony PCR (Touchdown)
Change in Protocol: N/A
Results: out of 3 colonies sampled, 2 were shown to have the correctly-sized bands (1.1kb). The remaining seems to be placed at 0.9kb, which is incorrect but we cannot think of a reason for the discrepancy.
Comments: the two successful colonies were cultured overnight (15 hours), for miniprep and cloning the next day.
Details:

- 20ul reaction
- Touchdown PCR with "DreamtaqIGEM" cycling protocol
- 1% TBE agarose gel prepared, reaction products were run for 40 minutes at 130V

2020年8月11日 星期二

Experiment title: Restriction Digestion of Site-directed mutagenesis parts
Description:
Changes in Protocol:
Results:

Separation of digested products through electroporesis in 1% gel.
Lanes (left -> right)

- control - no amplification
- 303gvsup
- 1kb GeneRuler
- R32M
- R32V
- Y324F
- control - no digestion (on separate gel)

image.png

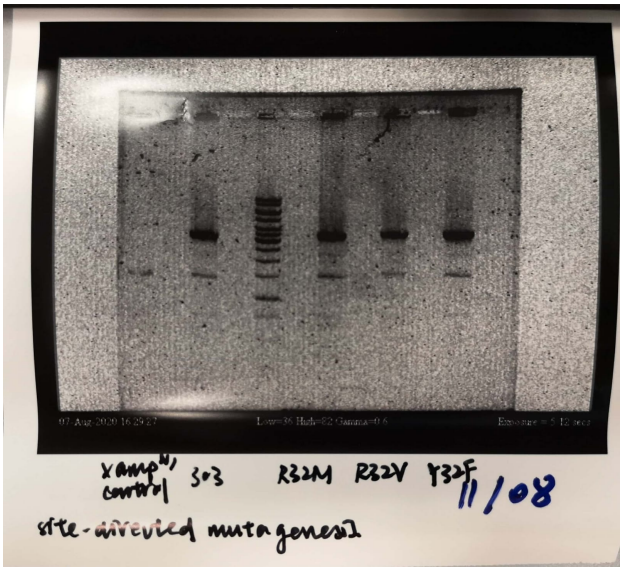
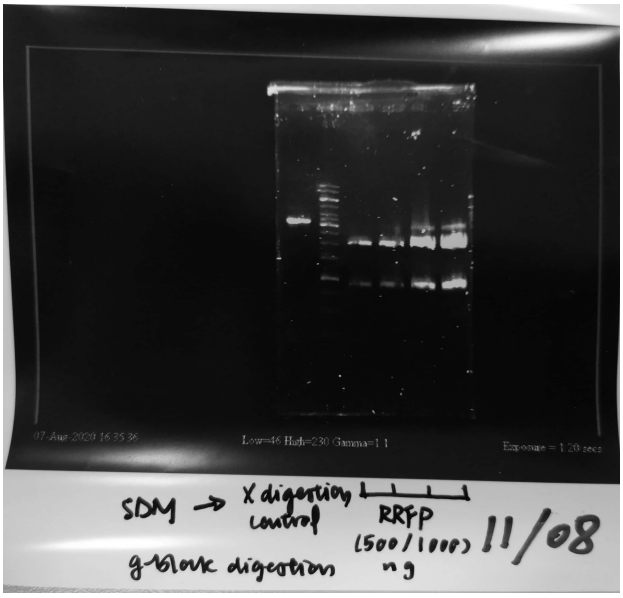


image.png



Left-most lane contains control set-up with no digestion activity.

Comments:
Details:

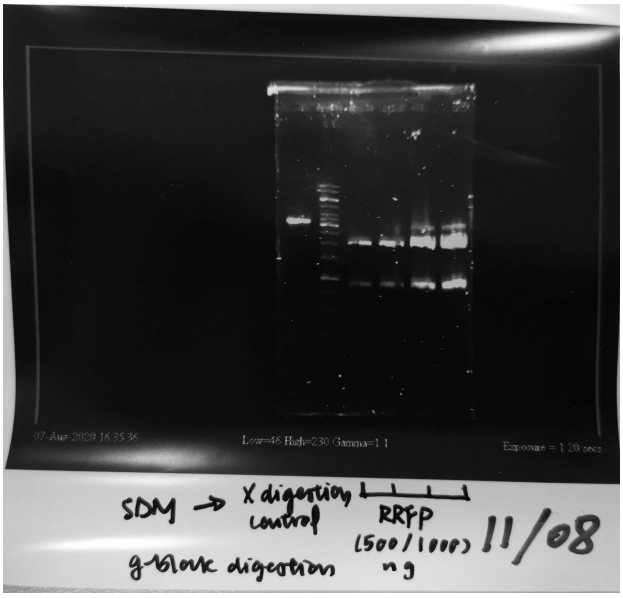
Experiment title: Restriction Digestion of RRFP
Description: purify RRFP samples for cloning, by going through digestion, gel purification and extraction
Protocol Used:
Changes in Protocol:

11 Aug Restriction Digestion Planning

	A	B	C	D	E	F	G	H
1	Tube	Conc. of DNA (ng/ul)	Volume Added (ul)	Top-up with MQ Water (ul)	CutSmart (ul)	EcoI added (ul)	PstI added (ul)	Total Volume
2	RRFP (A)	176.0	2.8	14.2	2	0.5	0.5	20
3	RRFP (B)	166.1	3.0	14.0	2	0.5	0.5	20
4	RRFP (A) [1000ng]	176.0	5.7	11.3	2	0.5	0.5	20
5	RRFP (B) [1000ng]	166.1	6.0	11.0	2	0.5	0.5	20

Results:
Separation of digested products through electrophoresis with 1% agarose gel
([sample from other experiment] - GeneRuler - 500ng RRFP (A) - 500 ng RRFP (B) - 1000ng RRFP (A) - 1000ng RRFP (B))

image.png



Successful digestion, two expected bands were observed for all the RRFP digested products.

Final yield of RRFP was very low after gel extraction, therefore we will re-attempt the procedure (digestion --> gel-extraction) to do troubleshooting.

Comments:
Details:

2020年8月12日 星期三

Experiment title: Restriction Digestion of RRFP
Description:
Protocol Used:
Changes in Protocol: 1000ng of plasmid was added to the reaction mixture instead of 500ng, to increase yield of digested fragments for use in future experiments.

- The same 1000ng reaction mixture was used as 11 Aug ->

Results:

Separation of digested products through electrophoresis with 1% agarose gel
(GeneRuler - [blank] - RRFP sample 1 - [blank] - RRFP sample 2 - [blank])

12 Aug Restriction Digestion of RRFP [EcoRI + Spe I] - Stephanie (1st attempt)

While our fragment of interest was still present, 4 bands is an unusual outcome, especialy since some of the bands were much larger than expected (e.g. around 4.5kb, much larger than the original plasmid's size), and were of equal/higher intensity. Lower band containing RRFP was excised for gel extraction.

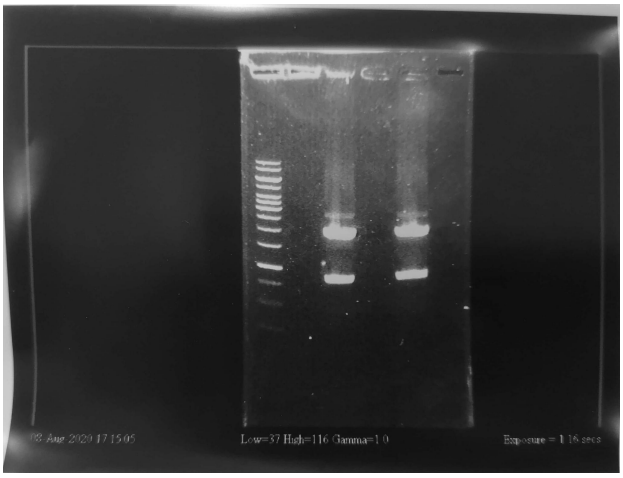
Troubleshooting for first attempt:

- According to troubleshooting guide by NEB, suggested issues include: Star Activity, partial digestion, and binding of enzyme to digested product
    - Star activity and partial digestion seem improbable due to our use of HF enzymes, and the relatively large band-size, which exceeds that of the original plasmid.
    - Therefore, it's most likely the third explanation. If the enzyme failed to separate from the digested fragments, it would increase the mass of the product -> slower movement through the gel.

What I did differently in the second attempt:

- Separate into two 20ul reaction mixtures instead of combining them in one tube.
- Extend incubation time to 1.5 hours

12 Aug Restriction Digestion of RRFP [EcoRI + Spe I] - Stephanie (2nd attempt)



Fairly successful. Bands found in expected size regions, and the band containing the digested RRFP (lowest, 892 bases) band was excised for gel extraction. Third band on top likely represents undigested product, indicating that an even longer incubation time (2 hours?) may be necessary to fully digest 1000ng of plasmid.

Comments:

- Digesting 1000ng of plasmids in a 20ul mixture may require longer incubation time (change to 2 hours?), or a larger reaction mixture volume containing more enzymes (e.g. make it a 30ul reaction mixture).

Details:

2020年8月13日 星期四

2020年8月14日 星期五

Experiment title: Ligation and Transformation of Reverse Lox Sites [5171, 2722]
Description:
Protocol Used: Ligation with T4 Ligase

image.png

Aiming for 1:3 ratio for the backbone:insert

Ligation Reaction Planning

	A	B	C
1	Reagent	5171 R [39.3 ng/ul]	2722 R [24.1 ng/ul]
2	T4 Ligase	0.5	0.5
3	T4 Ligase Buffer [10x]	1.044	1.133
4	Reverse RBS-RFPLAA [8.4 ng/ul]	7.625	7.625
5	Reverse lox Site	1.272	2.075
6	Total Volume	10.441	11.333

Reaction mixture was incubated at room temperature for 2 hours
5ul of reaction mixture from 5171 and 2722 was transformed into 50ul of competent cells.

Change in Protocol:
Results:
Comments:
Details:

2020年8月17日 星期一

Experiment title: SDM of Cre
Description: Site-directed mutagenesis of Cre
Protocol Used: SDM

Reaction Mix

	Reagent	Volume (µl)	Final %	D	E
1	5X Q5 Reaction Buffer	5	1X	30	
2	10 mM dNTPs	0.5	200 µM	3	
3	10 µM Forward Primer	1.25	0.5 µM		
4	10 µM Reverse Primer	1.25	0.5 µM		
5	Template DNA	1	25 ng		
6	Q5 Hot Start High-Fidelity DNA Polymerase	0.25	0.02 U/µl	1.5	
7	Nuclease-Free Water	15.75			
8	Total	25		34.5	5.75

Experiment title: Overnight DpnI digestion of mutated Cre
Description: Overnight DpnI digestion of mutated Cre samples
Protocol Used: SDM

Reaction

	Reagent	Volume (µl)	Final %	D	E
1	PCR product	5			
2	Anza DpnI Buffer (10x)	1	1x	6	
3	Anza DpnI enzyme	1	1x	6	
4	Water	3		18	
5	Total	10		30	5

2020年8月18日 星期二

Experiment title: Blunt-end ligation of mutated Cre PCR products
Description: Overnight blunt-end ligation of linearised mutated Cre PCR products
Protocol Used: SDM

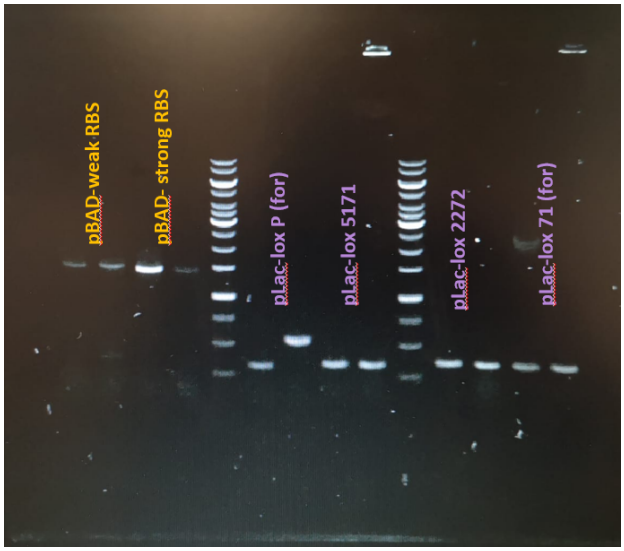
Table10

	Reagent	Volume (µl)	Final %	D	E
1	Anza T4 ligase master mix	2.5	13.75	y	
2	DNA	5			
3	dpnl enzyme	0.2	1.1		
4	dpnl buffer	0.2	1.1		
5	water	2.1	11.55	y	
6	total	10	27.5	5	

2020年8月19日 星期三

Experiment: Colony PCR for pBad-RBS and pLac-forward lox
Description: to verify the success/failure of previous transformation
Protocol Used: Colony PCR
Results:

image.png



PCR for selected colonies, pBad-RBS ligated products verified, but all pLac-lox site products failed to verify except for one colony with the loxP.

Next steps: pLac-loxP (lane 7) all pBAD-RBS constructs were purified through miniprep. All failed samples to be re-done from digestion stage.

Comments:
Details:

2020年8月20日 星期四

Tasks: miniprep, gel extraction of digested products, (overnight) ligation

Experiment:

2020年8月21日 星期五

Experiment: Chemical Transformation

2020年8月25日 星期二

Experiment: Ligation (R.RFPLAA-lox + RBS-GFPLAA+terminator) and Transformation
Description: New attempt to combine the constructs for transformation.
Protocol: Ligation Reaction, Chemical Transformation

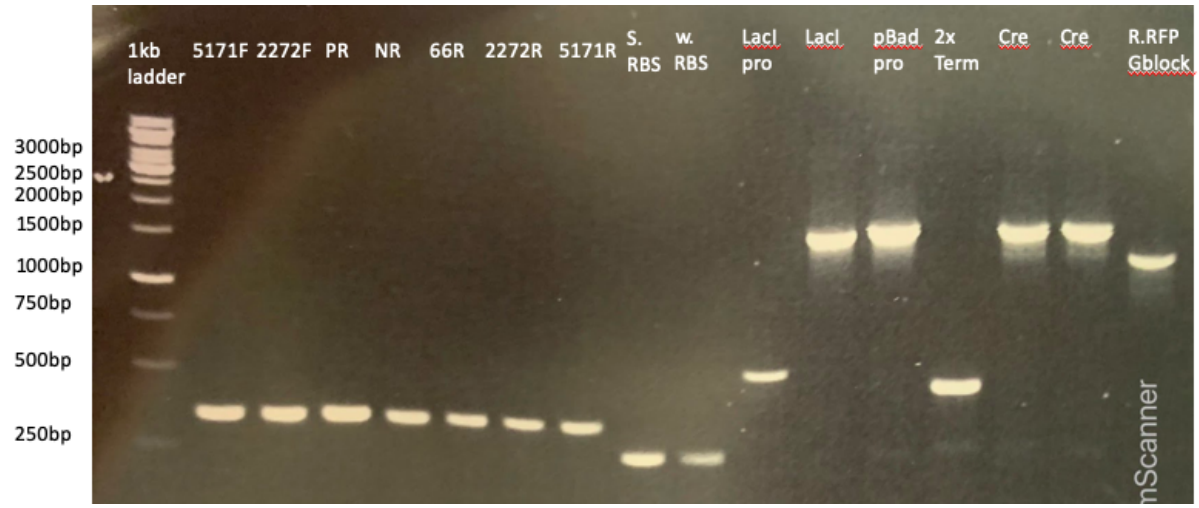
- Parts were digested and purified previously (RRFP backbones digested overnight and cleaned with PCR cleanup kit; GFP insert digested and purified through gel extraction)
- Ligation reaction mixtures were prepared as shown below, and allowed to incubate for 3 hours (25th afternoon)

Table11

	A	B	C	D	E
1	Sample	RFP-5171 [32.6 ng/ul]	RFP-2722 [31.4 ng/ul]	RFP-N [25.9 ng/ul]	RFP-P [33.9 ng/ul]
2	Concentrations ^	32.6	31.4	25.9	33.9
3	T4 Ligase	0.5	0.5	0.5	0.5
4	T4 Ligase Buffer [10x]	0.42	0.43	0.46	0.42
5	Backbone: RRFP-lox	1.5	1.6	1.9	1.5
6	Insert: GFP-Terminator [34.2 ng/ul]	2.2	2.2	2.2	2.2
7	Total Volume	4.649	4.714	5.086	4.585

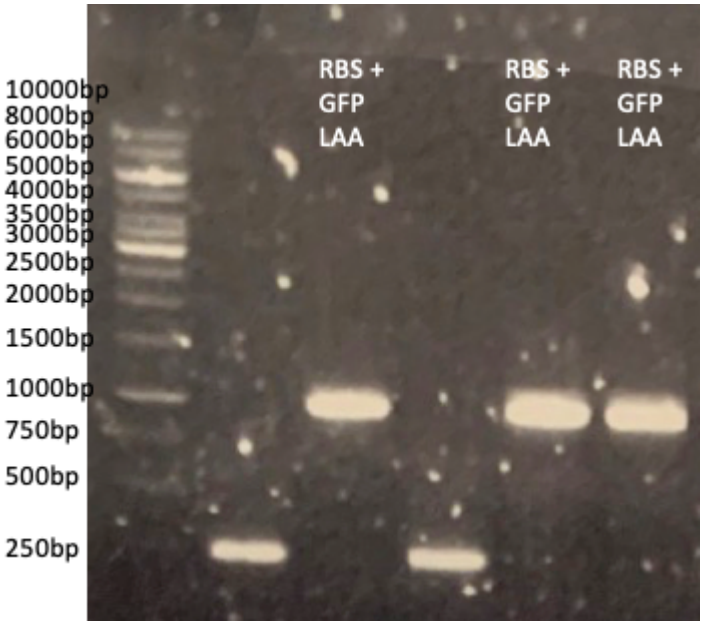
Summary of the step-wise cloning

1. Biobrick amplification



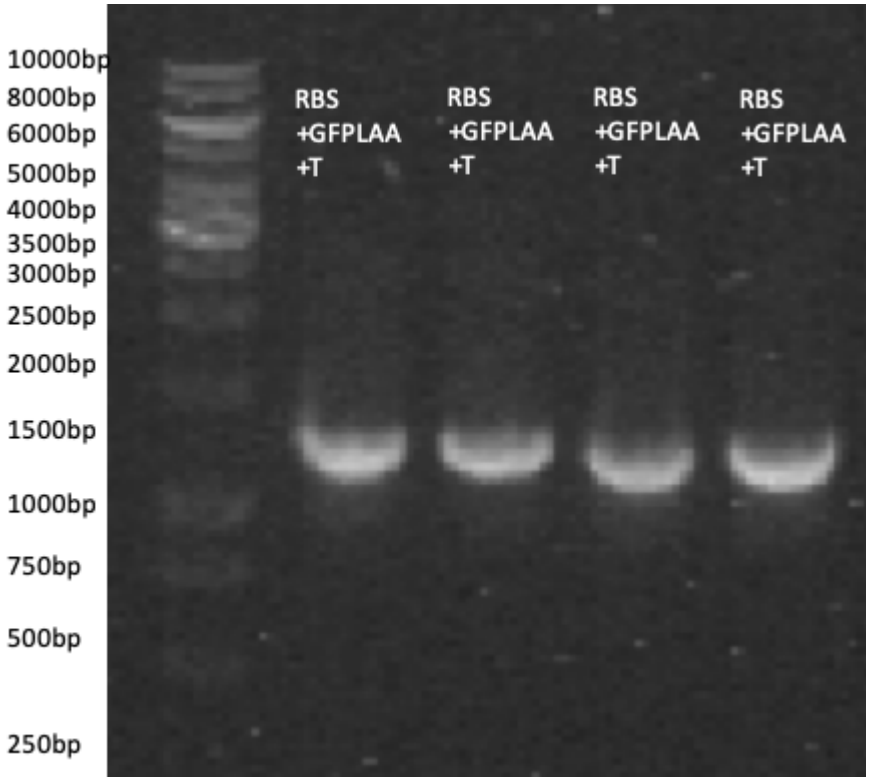
2. RBS with GFP-LAA (Expected size: 1065bp)

Strong RBS: BBa_B0034
GFPLAA:BBa_K1399006
RBS - Backbone (S,P)
GFPLAA- insert (X,P)



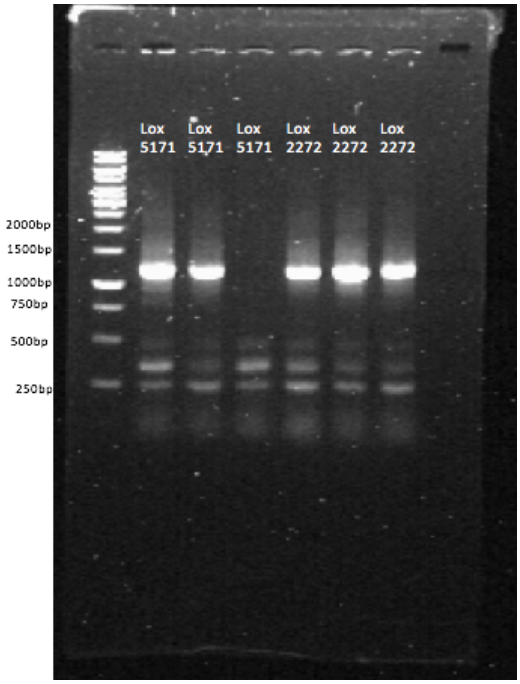
3. RBS with GFP and terminator (Expected size: 1222bp)

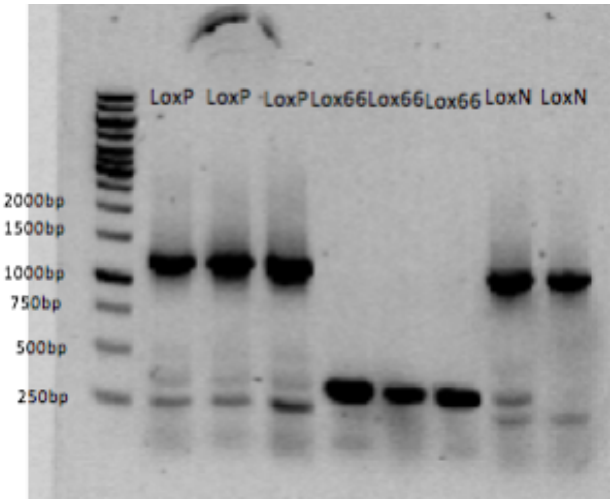
RBS: BBa_B0034
GFPLAA:BBa_K1399006
Terminator: BBa_B0015
[RBS + GFPLAA] - insert (E,S)
Terminator - backbone (E,X)



4. The RBS +RFPLAA+ Terminator(Gblock) Rev. Lox sites are ordered from IDT (Expected fragment size:1223bp)

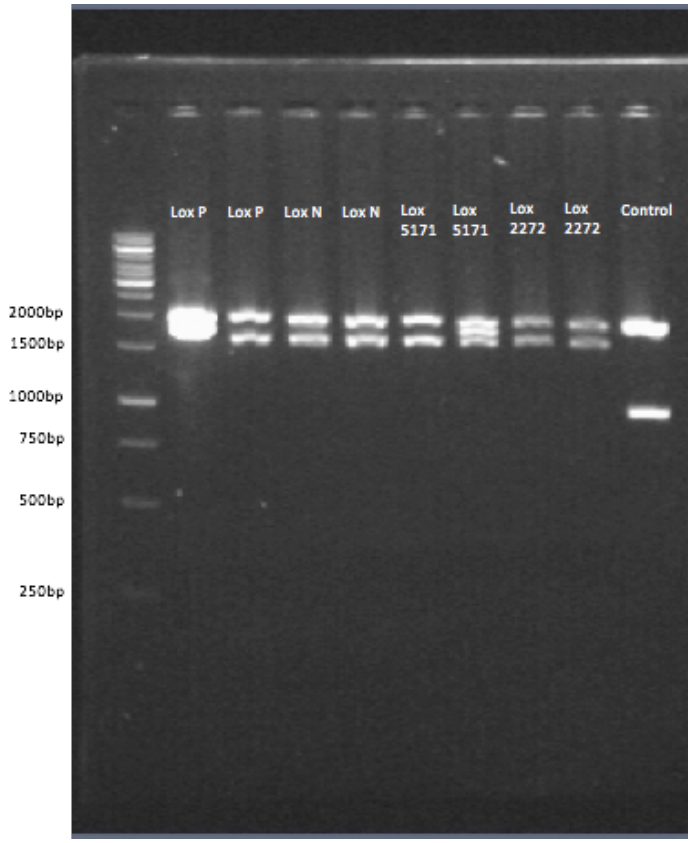
Gblock - insert (E,S)
Rev. Lox sites- backbone (E,X)



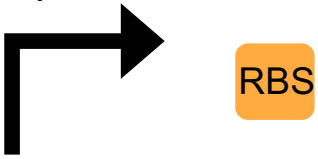


5. Reporter construct (Expected size: 2139bp)

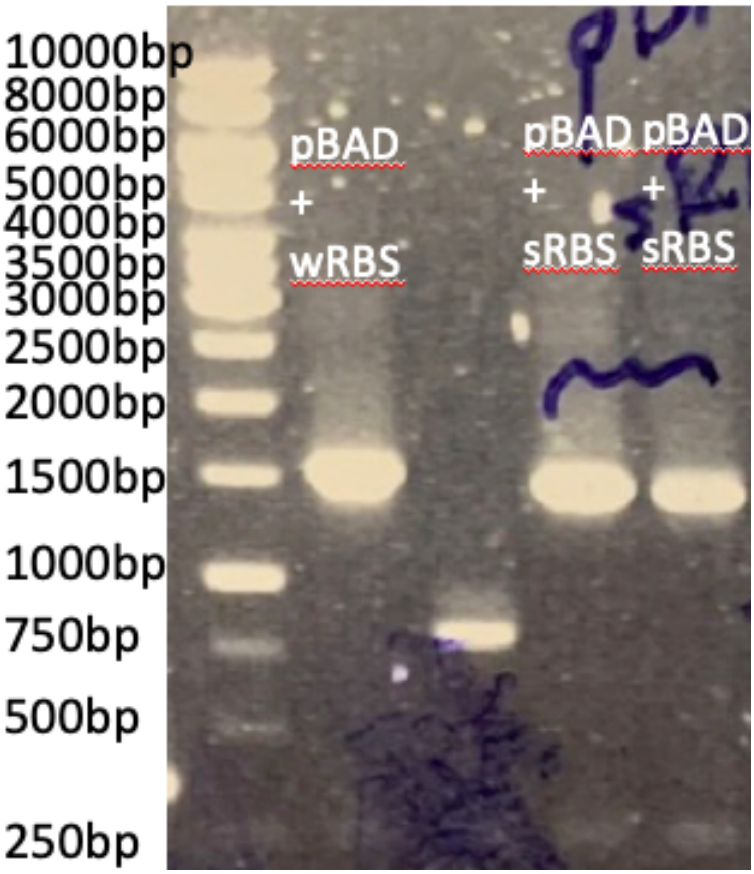
Restriction digested using EcorI and PstI
upper band is the insert



6. pBAD with weak or strong RBS (expected size: 1544bp)



pBAD: BBa_I0500
Strong RBS: BBa_B0034
Weak RBS:BBa_B0031
pBAD - insert
RBS - backbone



21 September 2020

- Restriction enzyme digestion (refer to the protocol)

Backbone digested with S, P	Insert digest with E, S
LacI-loxP	GFP-LAA-RFP-loxP
LacI-lox5571	GFP-LAA-RFP-lox5571
LacI-lox2272	GFP-LAA-RFP-lox2272
LacI-lox71	GFP-LAA-RFP

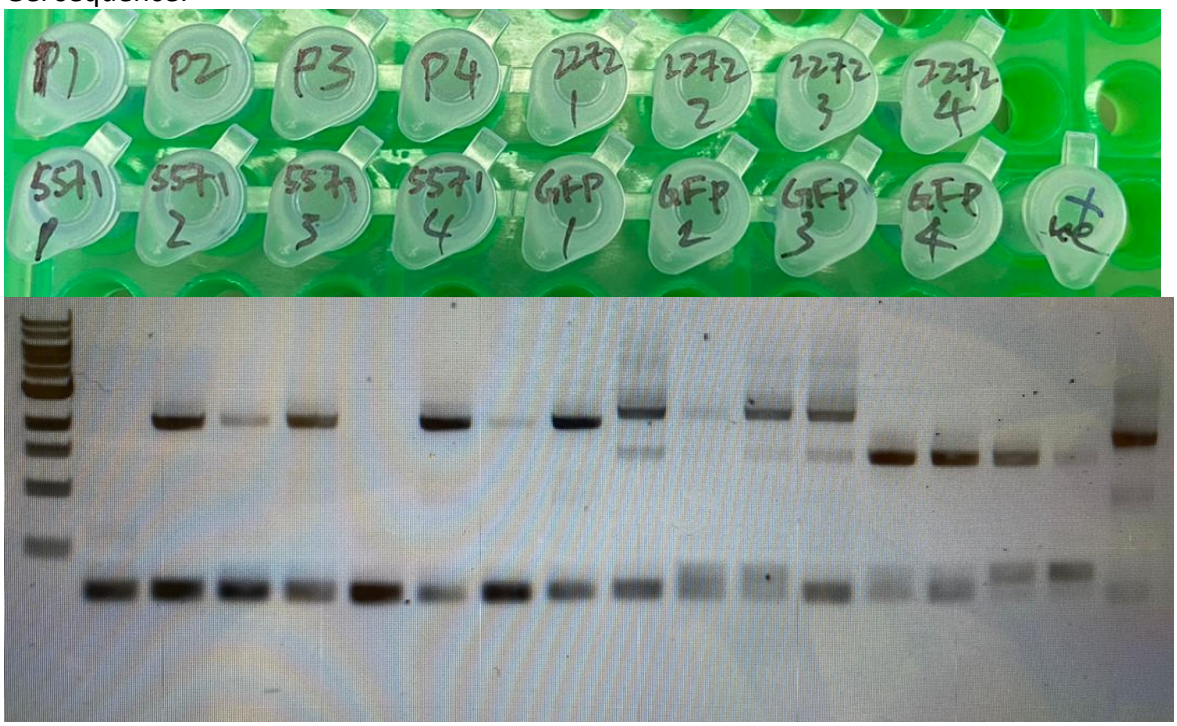
- Gel purification (Result: all completely digested)
- Overnight ligation (refer to the protocol)

22 September 2020

- Transformation to DH5a, recovery in SOB for 1 hour
- Allow growth in agar plate with chloramphenicol

23 September 2020

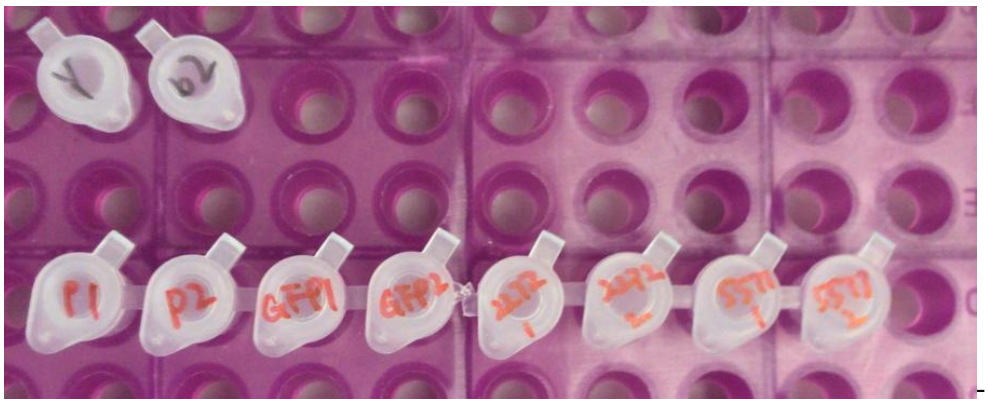
- Pick colony
- Colony PCR: Selected lane 2, 7, 12, 14 for innoculation
  Gel sequence:

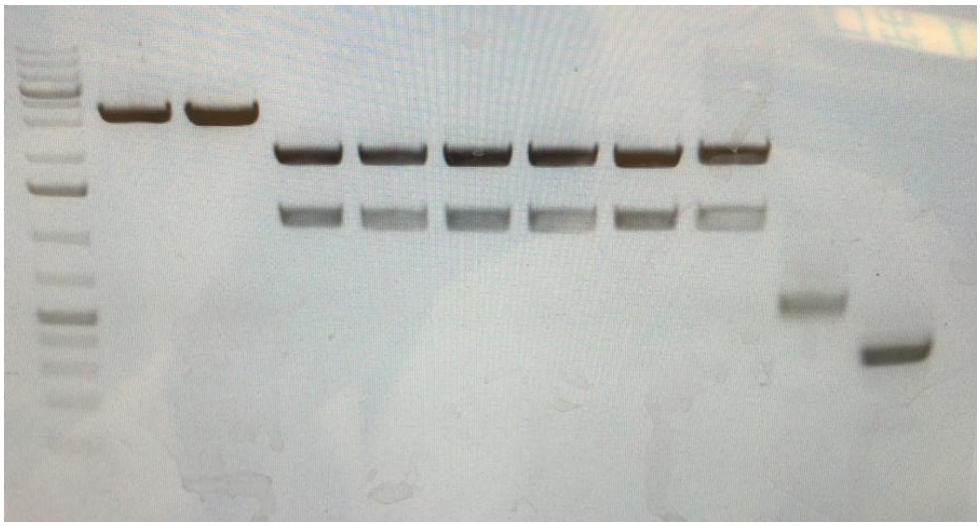


- Inoculate in LB with chloramphenicol
- Sent the cre mutant to CPOS sanger sequencing

24 September 2020

- Miniprep (refer to the protocol)
- Digestion check with ES: All succeeded





25 September 2020

- Sequencing result for Cre: site-directed mutagenesis was successful for all Cre mutant

29 September 2020

- Restriction enzyme digestion (refer to the protocol)

Backbone digested with S, P	Insert digest with X, P
pBAD-sRBS	Cre-Y324F
pBAD-wRBS	Cre-R32M
	Cre-R32V
	Cre-GVSdup
	Cre-WT

Backbone digested with S, P	Insert digest with E, S
LacI-lox71- GFP-LAA-RFP	Lox66

- Gel purification
- Ligation

LacI-lox71- GFP-LAA-RFP	Lox66
pBAD-sRBS	Cre-Y324F

	Cre-R32M
	Cre-R32V
	Cre-303GVSdup
	Cre-WT
pBAD-wRBS	Cre-Y324F
	Cre-R32M
	Cre-R32V
	Cre-303GVSdup
	Cre-WT

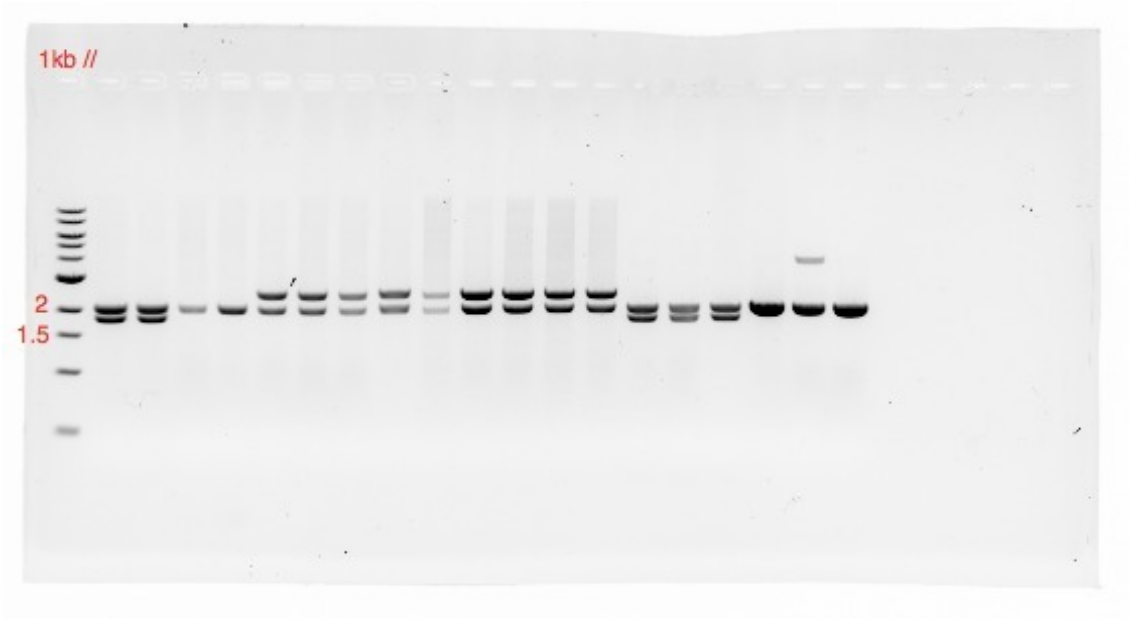
- Transformation to DH5a competent cells, recovery in SOC, grow on agar plate with chloramphenicol

30 September 2020

- Pick colony from the pBAD-RBS-Cre mutant, lox5571 and lox2272
- Inoculate in LB with chloramphenicol

1 October 2020

- Miniprep (refer to the protocol)
- Digestion check



6 October 2020

- Restriction enzyme digestion (refer to the protocol)

Backbone digested with S, P	Insert digest with S, P
psB1A3	sRBS-Cre-Y324F
psB4C5	sRBS-Cre-R32M
	sRBS-Cre-R32V
	sRBS-Cre-303GVSdup
	sRBS-Cre-WT
	Lox5571-reporter
	Lox2272-reporter
	LoxP-reporter

- Gel purification
- Overnight ligation

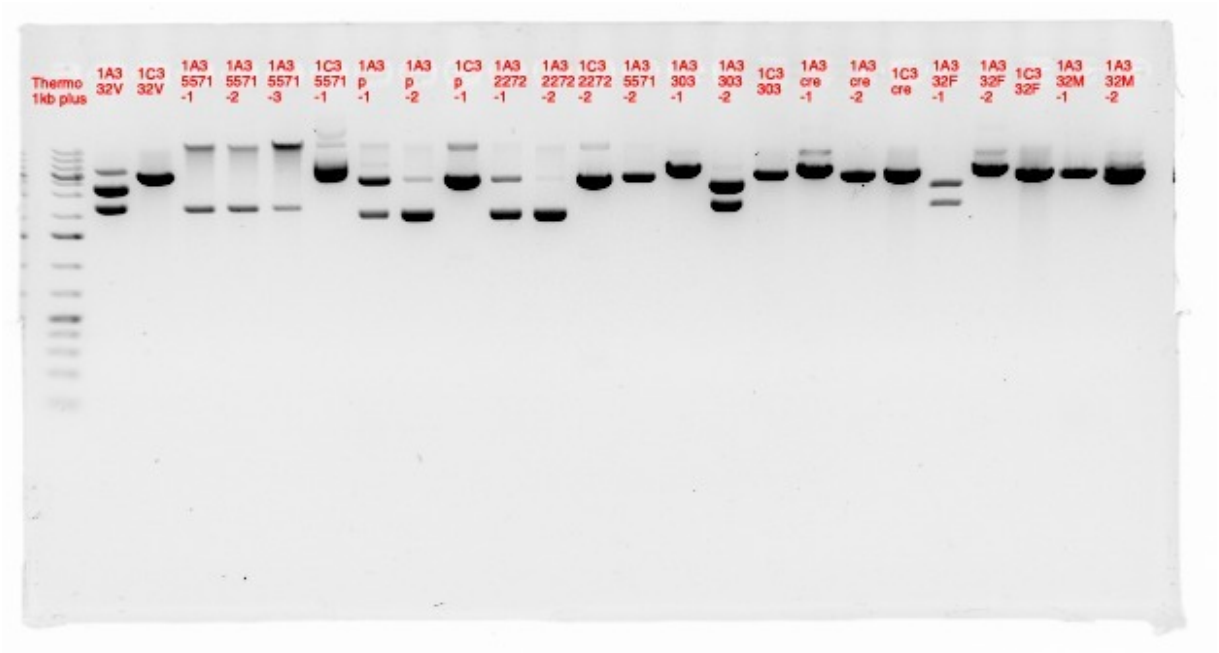
psB1A3	Lox5571-reporter
	Lox2272-reporter
	LoxP-reporter
psB4C5	sRBS-Cre-Y324F
	sRBS-Cre-R32M
	sRBS-Cre-R32V
	sRBS-Cre-303GVSdup
	sRBS-Cre-WT

7 October 2020

- Pick colony
- Inoculate in LB with chloramphenicol

8 October 2020

- Miniprep (refer to the protocol)
- Digestion check (for the Cre mutants, 1A3 is wrongly labelled in the photo, please refer it as psB4C5 instead)



- Not every construct is correctly cloned. Therefore, more cloning might be needed in the future.