CHAPTER I

Thursday, 14th of July 2016

We made non-selective LB agar media.

Friday, 15th of July 2016

We made selective LB agar media with Ampicillin. We were also doing Gibson Assembly with Gibson's positive control to make sure the master mix still worked. After the assembly, we transformed the plasmid into already competent *Eschericia coli* TOP10. We used doubly distilled water (ddH_2O) as the negative control of this experiment.

Saturday, 16th of July 2016

One of the researcher saw the results of transformation.

NO.	Agar (25ml)	Antibiotic	Function	Plasmid	Volume transformed	Results
1A	LB	Ampicillin	Positive control	GPC	100µL	Did not grow.
1B					950µL	Many single colonies.
1C		Non Selective media.	Negative Control	ddH_2O	100µL	Contaminated
1D					425µL	
1E						

Table 1.1 Bacterial Transformation Results Variation

We used ddH_2O as the negative control because we thought that the plate would grow no bacteria from the distilled water. Anyhow, the results showed that there were growth of bacteria on the non-selective media (negative control). We assumed that this was prior to the ddH_2O that was not autoclaved before or contaminated Falcon tube.

Monday, 18th of July 2016

Because the ambiguity of the results, we planned to redo the experiment from scratch. That day, we made LB (Lysogeny Broth). We also did the transfer of *E. coli* TOP 10 from the refrigerator stock to the LB using tips.

Tuesday, 19th of July 2016

This day, we made the cell competent. (See protocol "Chemically Competent Cell"). It was such a tiring day.

Wednesday, 20th of July 2016

We transformed the previous plasmid (the Gibson's positive control) to the already competent cells. (See protocol "Transformation").

Thursday, 21st of July 2016

This was the results of the transformation:

NO.	Agar (25ml)	Antibiotic	Function	Plasmid	Volume transformed	Results
2A	LB	Ampicillin	Positive control	GPC	100µL	Did not grow.
2B					950µL	Many single colonies.
2C		Non Selective media.	Negative Control	pQE80L	100µL	Contaminated
2D				ddH$_2$O	425µL	
2E						

Table 1.2 Bacterial Transformation Results Variation

(JELASIN PLIS)

We also made selective agar media with Ampicillin, Chloramphenicol, and Kanamycin.

Friday, 22nd of July 2016

To test our hypotheses in the previous day, we made several ampicillin plate to be transferred with the one plate (the control positive one) to check whether they have the right plasmid or not. We transferred bacteria that had succesfully grown in the agar plate namely 2B into 4 new agar plates with ampicillin. We also transferred the bacteria into plates 1A and 2A.

Saturday, 23rd of July 2016

The result confirmed that our cells had had our plasmid already. The problem with plate 1A and 2A was that we didn't know on what concentration the ampicillin was because it was ready to use; hence the word *stock*.

NO.	Agar (25 ml)	Antibiotic	Volume of antibiotic	Results
3A	LB	Ampicillin	$\frac{1}{2000} * 25ml = 12.5\mu l$	Grow.
3B				Grow.
3C				Grow
3D				Grow.
1A			Stock	Negative.
2A				Negative.

Table 1.3 Results of Agar-to-Agar Bacteria Transfer

We speculated the stock was not working because the excessive amount of Ampicillin given to the agar plate. We concluded that our Gibson's master assembly mix was still working.

Friday, 29th of July 2016

Just to make sure that our Gibson's master assembly mix still worked, we did the test once again while we were waiting our DNA to come.

NO	Agar (25 ml)	Antibiotic	AB volume	Plasmid	Results
4A	LB	Ampicillin	12.5µl	GPC	Grow.
4B				pKS	Grow
4C				pUC19	Grow.
4D				pQE80-L	Grow.

Table 1.4 The results of *E. coli* transformation with several plasmids.

We concluded that we had not to worry about our Gibson's master assembly mix. All was good and we were ready to advance into the next chapter of our lab works.

Thursday, 4th of August 2016

We learned about cell culture methods.

Monday, 8th of August 2016

We resuspended our gBlocks gene fragment, followed by the Gibson's Assembly and transformation afterwards. We transformed 2µL of Gibson's Assembled Plasmid into 50µL already competent *E. coli* TOP-10.

gBlocks	fmoles/ng	Mass	Final concentration	TE added
Fragment 1	1.06	1000ng	100ng/µL	10µL
Fragment 2	0.86			
Fragment 3	1.05			
Fragment 4	0.97			

Table 1.5 DNA Resuspension

gBlocks	Mole		Volume Added
Fragment 1	0.25 pm	1 pm	2.3µL
Fragment 2	0.25 pm		2.9µL
Fragment 3	0.25 pm		2.3µL
Fragment 4	0.25 pm		2.5µL
Gibson Assembly Master Mix			10µL
Total			**20 µL**

Table 1.6 Gibson Assembly

Tuesday, 9th of August 2016

Presented below was the result of Gibson Assembly Transformation. Kindly note that the concentration of Ampicillin and Kanamycin used were 50µg/mL and 100µg/mL respectively. We used the ratio of 1:2000 per volume of agar.

No.	Medium	Plasmid	Selection medium	Resistance gene	Volume transformed	Results
5A	LB	Gibson	Kanamycin	*kan*-R	100µL	Didn't grow
5B					50µL	
5C		pQE80l	Ampicillin			Grow

5D		GPC		*amp*-R		
5E		None	Kanamycin	None		
5F			Ampicillin			

Table 1.7 Results of Transformation Following The Gibson Assembly

We suspected that our cells didn't take the assembled plasmid because of the low amount of DNA transformed. Following the day, we autoligated linearized plasmid backbone pSB1C3 with the T4 ligase.

	Volume
Linearized plasmid backbone (25ng/µL)	4µL
T4 DNA Ligase	0.75µL
5x T4 DNA Ligase Buffer	4µL
ddH_2O Sigma	11.25µL
Total	20µL

Table 1.8 Autoligation Reaction

Wednesday, 10th of August 2016

We retried the transformation of Gibson's Assembled Plasmid. This time with 5µL of DNA instead of the previous 2µL. We also transformed pSB1C3 in to *E. coli* TOP-10 .

Thursday, 11th of August 2016

No.	**Medium**	**Plasmid**	**Selection medium**	**Resistance gene**	**Volume transformed**	**Results**
6A	LB	Gibson	Kanamycin	*kan*-r	100µL	One colony
6B			Ampicillin		50µL	Grow well
6C			None			
6D		pSB1C3	Chloramphenicol	*cat*	100µL	Seven colonies.
6E			Kanamycin		50µL	Negative
6F			None			Grow well

Table 1.9 Results of Transformation with Gibson's Assembled Plasmid and pSB1C3

In that day we also made the PCR mix to check the Gibson's Assembled Plasmid.

	Concentration	**Volume**
HotStarTaq DNA Buffer	1x	2µL
HotStarTaq DNA Polymerase	15unit/µL	0.125µL

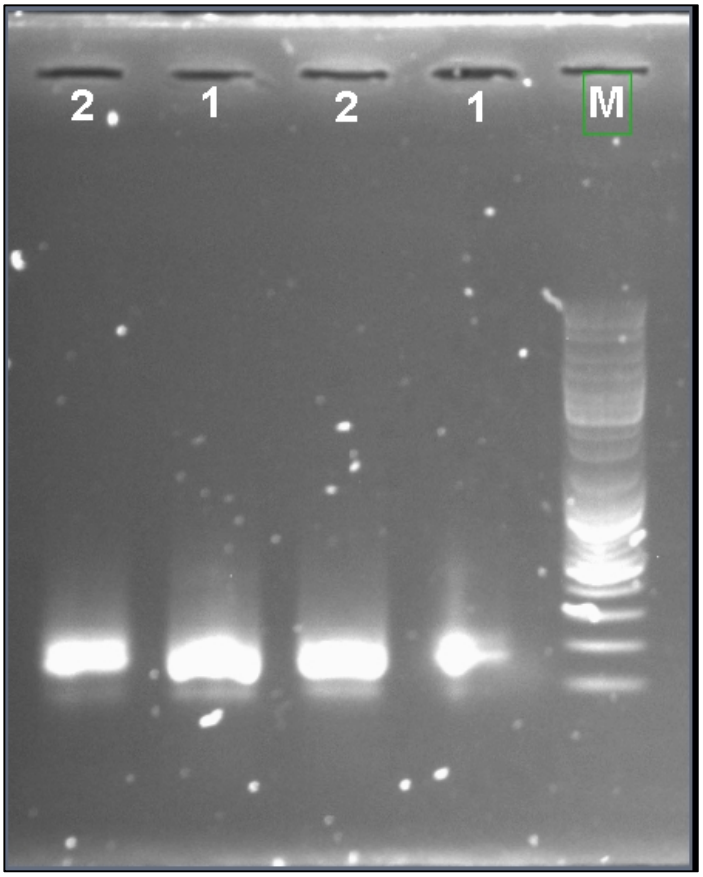
dNTPs	0.3mM	0.6µL
Q solution	1x	4µL
Forward Primer	0.3 mM	0.3µL
Reverse Primer		0.3µL
ddH$_2$O	1x	10.675µL
Template DNA	100ng/µL	2µL
TOTAL		**20µL**

Table 1.10 PCR Reaction Mix

The temperatures and cycles of PCR can be seen at our separated protocols' page.

Friday, 12th of August 2016

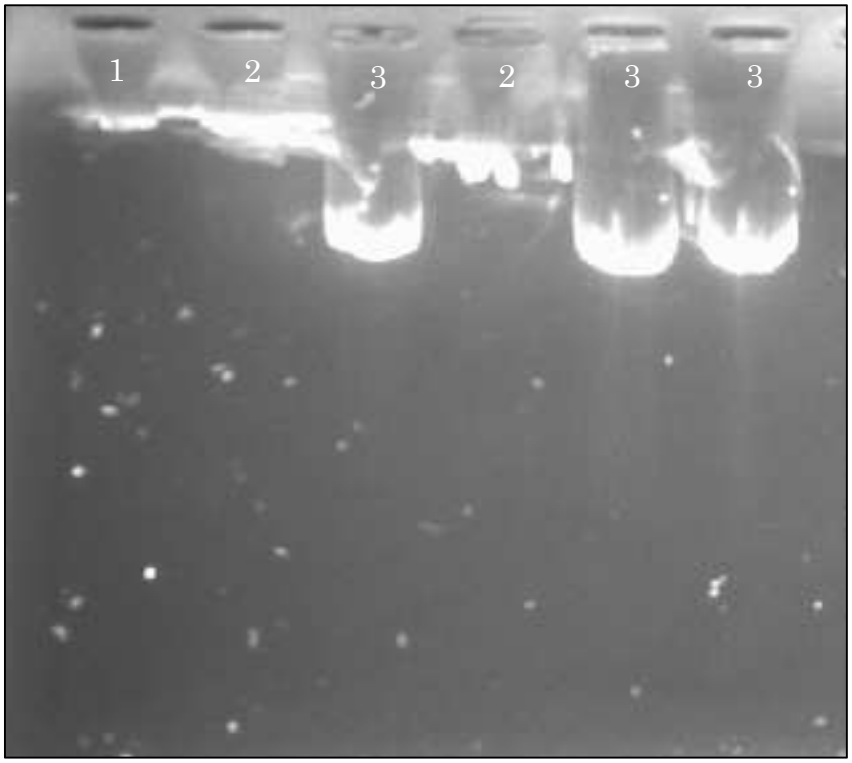
We run the PCR mix with our DNA and followed by gel elecrophoresis on 0.8% agarose.



Picture 1 Results of running two PCR product in 0.8% agarose electrophoresis. Whereas number 1 is PCR's first try and number 2 is PCR's second try. M stands for GeneRuler 10kb ladder.

Saturday, 13th of August 2016

We miniprepped the only colony that was suspected to be our plasmid. After the procedure, we run the plasmid on agarose 0.8% via electrophoresis. The results was not so good.



Picture 2 Electrophoresis agarose 0.8% results of: 1.) Miniprepped plasmid, 2.) Gibson's assembled plasmid, 3.) pCDNA-EGFP

We retried the attempt of running our plasmid on 0.8% agarose, this time with BamHI restriction prior to electrophoresis. Below was the mix used to digest our plasmids:

Reagents	Volume
10x NEBuffer 2	7µL
10x BSA	7µL
ddH_2O	26µL
BamHI	2µL
TOTAL	**42µL**

Table 1.11 BamHI Master Mix

After making the master mix we aliquoted 12µL of the master mix into 3 Eppendorf tube to be mixed with each plasmid.

	Miniprepped plasmid	**Gibson's assembled plasmid**	**pCDNA-EGFP**
BamHI Master Mix	12µL	12µL	12µL
DNA	8µL	4µL	2µL
ddH_2O	0µL	4µL	6µL
Total	**20µL**		

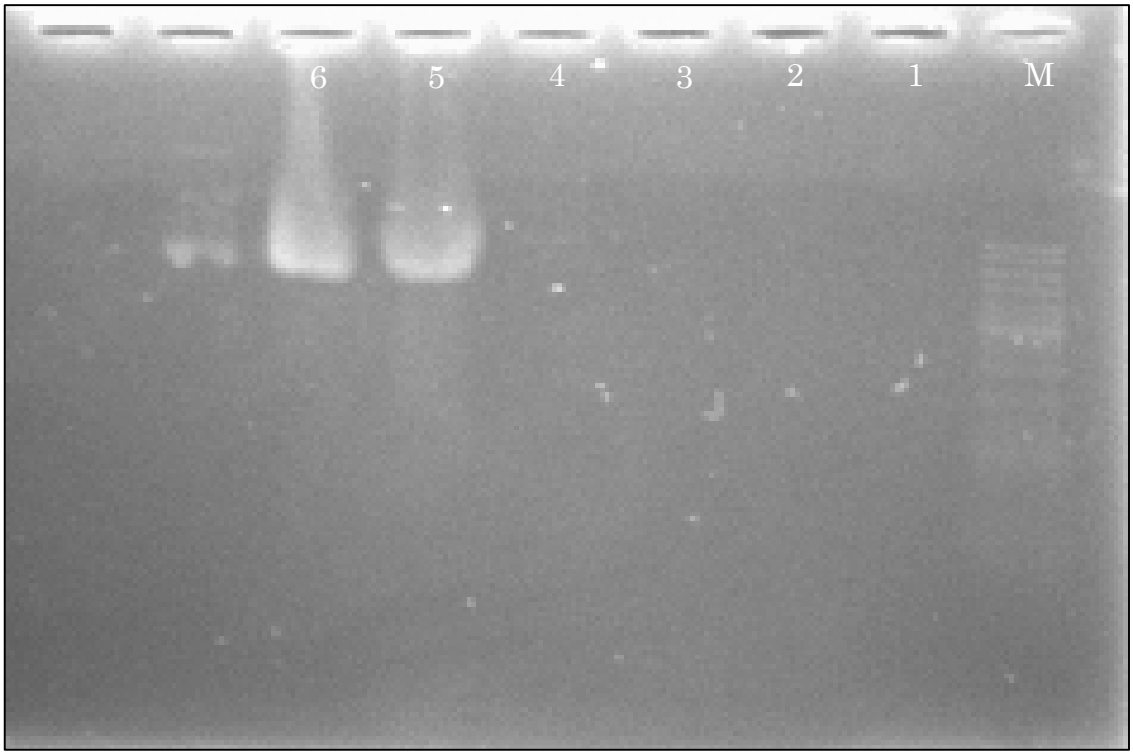
Table 1.12 BamHI Digestion Mix

Following the BamHI digestion, we proceed to do agarose electrophoresis. 5µL of 6x Loading Dye was added to each tube and mixed well. We load 12,5 of each

samples and run the process. Because the day was late already, we didn't have the time to see the results on GelDoc.

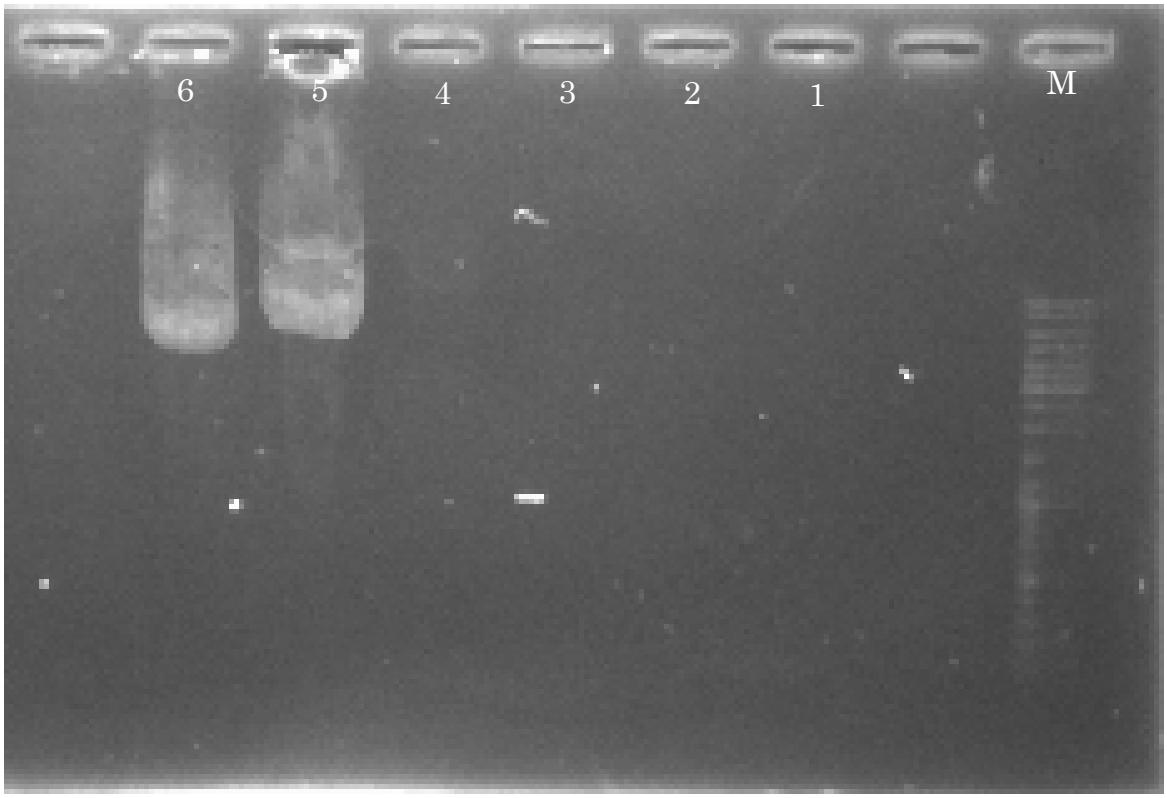
Monday, 15th of August 2016.

This was the result of Saturday's electrophoresis:



Picture 3 Second run of agarose electrophoresis, whereas: 1.) Gibson's assembled plasmid + BamHI, 2.) Gibson's assembled plasmid, 3.) Miniprepped plasmid + BamHI, 4.) Miniprepped plasmid, 5.) pCDNA-EGFP + BamHI, 6.) pCDNA-EGFP. M was GeneRuler 10kb as a ladder.

We concluded that the DNA was degraded or washed because it was in GelRed for two days. Following the day, we rerun electrophoresis with the remaining mix.



Picture 4 Third run of agarose electrophoresis, whereas: 1.) Gbison's assembled plasmid + BamHI, 2.) Gibson's assembled plasmid, 3.) Miniprepped plasmid + BamHI, 4.) Miniprepped plasmid, 5.) pCDNA-EGFP + BamHI, 6.) pCDNA-EGFP. M was GeneRuler 10kb as a ladder.

We didn't know exactly why the samples didn't show up except the pCDNA-EGFP. We speculated that the rest of the sample' concentration were way too small to be run into agarose 0.8%.

Tuesday, 16th of August 2016.

We planned to retry the electrophoresis, this time also with PCR samples. So today, we did the PCR again and miniprepped our supposed to be plasmid. That day we also made competent cells.

Wednesday, 17th of August 2016

Indonesia's Independence Day! We call it a day ☺

Thursday, 18th of August 2016

Preceeding electrophoresis, we use EcoRI and PstI to digest the plasmids instead of BamHI. We made EcoRI master mix to begin with.

Reagents	**Volume**
NEBuffer 2 10x	5µL
ddH_2O	19.5µL
EcoRI-HF	0.5µL
TOTAL	**25µL**

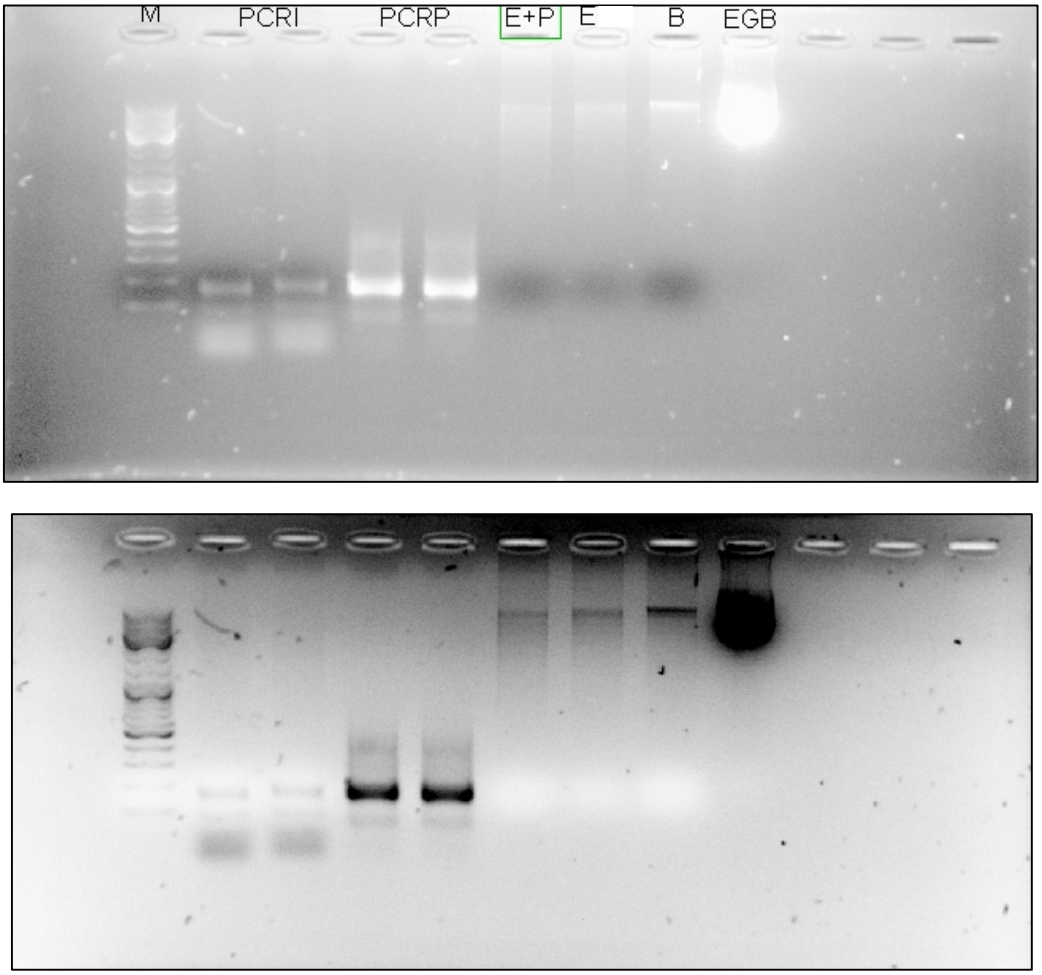
Table 1.13 EcoRI Master Mix.

Then we aliquoted 4µL of EcoRI Master Mix to an Eppendorf tube, hereafter continued with rest of the EcoRI Master Mix to make EcoRI+PstI master mix.

Reagents	Volume
EcoRI Master Mix	21µL
PstI	0.4µL

Table 1.14 EcoRI+PstI Master Mix

From there on we aliquoted 4µL of EcoRI + PstI to be mixed with our plasmid. We did not purify the DNA before running the agarose electrophoresis. Here was the results:



Picture 5 Fourth run of 0.8% agarose electrophoresis. The two pictures yield the same, only inverted. M was GeneRuler 10kb as a ladder. PCRI was PCR product from isolated plasmid. PCRP was PCR product directly from Gibson's assembled plasmid. E+P was miniprepped plasmid that was treated with EcoRI and PstI. E was miniprepped plasmid treated with EcoRI only. B was the remaining of previous day miniprepped plasmid treated with BamHI. EGB was pCDNA-EGFP treated with BamHI.

We concluded that the plasmid was not our plasmid. Our PCR product should be ~4000bp, which the electrophoresis shown the products were less than 1000 bp. The digested plasmid also did not show the expected results. One band was only showing in the three lanes instead of two. Addition of PstI might not be done properly because of pippetting error, but it didn't explain why BamHI also produce the same result.

We suspected that maybe our plasmid was incorrectly assembled, or might be forming superstrucutre. We planned to restart the entire work from the Gibson's assembly.

Tuesday, 23rd of August 2016

The main reason behind our failure might be on the competency of the cells. It was our fault that we didn't check the competency of our cells in the first attempt of cloning procedure. To prevent future mistake, we included the transformation of Bba_J04450 (Red Fluorescent Protein) as what iGEM had written on its protocol.

Wednesday, 24th of August 2016

As we followed every steps in iGEM's protocol on checking the competency of the cells, turned out that our cells were not competent at all. This was shown with no RFP (10pg/μL) glowing red on our plates.

Thursday, 25th of August 2016

As for note, we didn't have commercially available *E. coli* TOP-10 cells, so in this lab we made it ourselves. We tried making the cells competent again chemically, followed by transformation. In this transformation, we tweaked several things. We use 5μL of DNA instead of 1μL (even though the concentration varies). We also did the heatshock in 38C as long as 90 seconds. This was the protocol that has been used in this lab. Today we transformed our Gibson's assembled plasmid with RFP as the positive control.

Friday, 26th of August 2016

There was still no red light fluoresce on our plates, which means, either our cell was still not competent, or our transformation was failed. We should have used a negative control. In the future, we'll avoiding this mistake and work more precautiosly.

Monday, 3rd of October 2016

It turned out that our plasmid hadn't been assembled yet from Gibson's assembly, proven by previous results of electrophoresis. We found out that the reason behind this issue was the length of the overlaps. We designed 80bp as overlaps of each fragments, when it should be in the range of 30-40bp. That 80bp overlaps was instructed by our supervisors. To get ahead of this obstacle, we ordered primers to shorten the length of overlaps via PCR. Till today the primer hasn't come just yet, so we focus on the parts to send by date.

Formerly we have four new parts to be sent to the iGEM's HQ. These are: optimized enchanced Green Fluoroscent Protein for mammalian cells (EGFP), our synthetic promoter (MAX promoter), modified bGH poly-A tail and H1 promoter (RNA polymerase III promoter). When the gBlocks DNA offered by IDT came, we hadn't noticed an illegal PstI site upon MAX promoter and EGFP. Once again this was generated by our design error. Realizing we hadn't yet our plasmid to work

and two parts unable to send, we focus on preparing our modified bGH poly-A tail (which from now on will be written as poly-A) and H1 promoter.

Later on we also found trouble to generate more copies of our poly-A and H1 promoter because our failures on transforming the subcloned plasmid (pSB1C3-polyA and pSB1C3-H1). Our colonies failed to grow because our chloramphenicol was defective. This was proven by *E. coli* TOP-10 wild type which grows on 1/1000 to 1/100 chloramphenicol at 25µg/ml working concentration. We realized that our chloramphenicol was different than the one that was comercially available in another country. This meant that the powdered form of chloramphenicol succinate needed just to be dissolved in distilled water, not absolute ethanol. We realized that it was way more logic to dissolve an intravenous or intramuscular drugs with water or saline solution, rather then absolute ethanol.

Month ago, our leader was able to orders more primer, to isolate more parts from our designed plasmid. These parts were: another EGFP without illegal restriction site, VA1 RNA, tRNAval, 2A Peptides (P2A) and H1 promoter. This being said, we are now focusing to magnify the amount of these parts and characterize each of them while waiting for our primers to shorten the overlaps and continue our project.

Today we were able to resuspend our primers and PCR all the fragments we needed.

Number	Target	Primers	Template	Length
1	Fragment 1	Fragment 1 Forward Fragment 1 Reverse	Fragment 1	1527 bp
2	Fragment 2	Fragment 2 Forward Fragment 2 Reverse	Fragment 2	1872 bp
3	Fragment 3	Fragment 3 Forward Fragment 3 Reverse	Fragment 3	1548 bp
4	Fragment 4	Fragment 4 Forward Fragment 4 Reverse	Fragment 4	1677 bp
5	tRNAval	ValForward ValReverse	Fragment 1	94 bp
6	H1	H1Forward H1Reverse	Fragment 1	98 bp
7	VA1	VA1Forward V1Reverse	Fragment 1	194 bp
8	P2A	P2Aforward P2Areverse	Fragment 1	63 bp
9	EGFP	EGFPForward EGFPReverse	Fragment 4	723 bp
10	RFP	VF2 VR	RFP (10pg/µL)	1069 bp

We did the reaction as follow:

	Final Concentration	Volume
DreamTaq DNA Buffer	1x	2µL
DreamTaq HotStart DNA Polymerase	15U/µL	0.15µL
dNTPs	0.2mM	0.4µL
Forward Primer	0.2 µM	0.8µL
Reverse Primer		0.8µL
ddH_2O	1x	15.35µL
Template DNA	10ng/µL	0.5µL
TOTAL		**20µL**

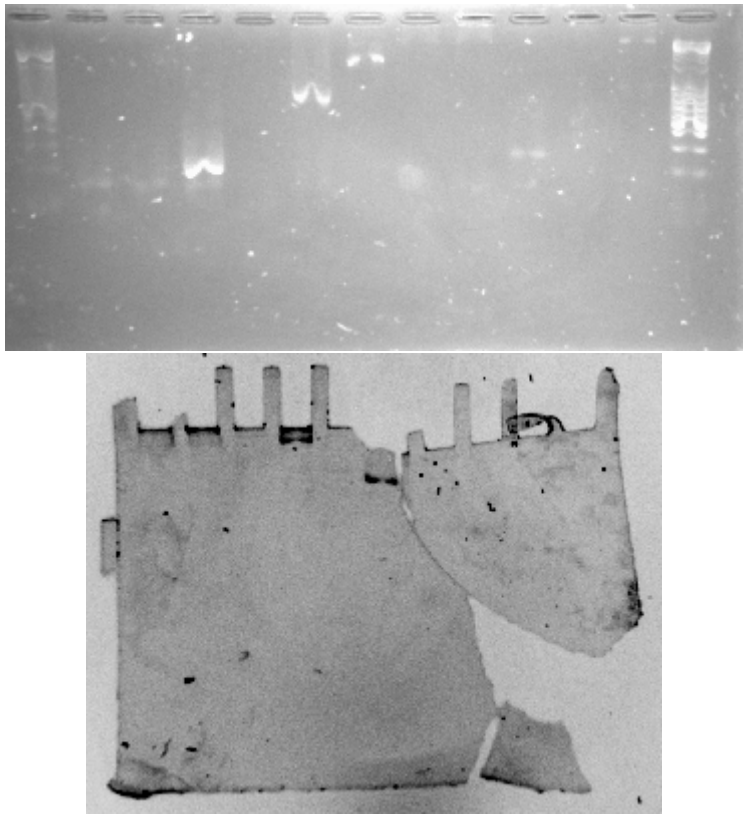
And furthermore set the thermocycler to:

State	Temperature	Time
Pre-PCR	98C	30 seconds
Denaturation	98C	10 seconds
Annealing (Repeat 35x)	53C	30 seconds
Elongation	72C	45 seconds
Post-PCR	72C	10 minutes

Tuesday, 4th of October 2016

We purified the DNA obtained via PCR using Qiaex DNA Purification system. We wanted to make sure that we had the the right product. In order to do this, we need to electrophoresis the DNA to find the length of each product. Because lengths of the products are so small, one even reaching as low as 63 bp, our supervisor suggested to use Polyacrilamide Gel Electrophoresis for DNA (PAGE DNA) with 12% polyacrilamide instead. We did the electrophoresis and leave the polyacrilamide in GelRed overnight. We might want to retry the same motives with 2% agarose the next day. Protocol for PAGE DNA is available in our separated page for protocols.

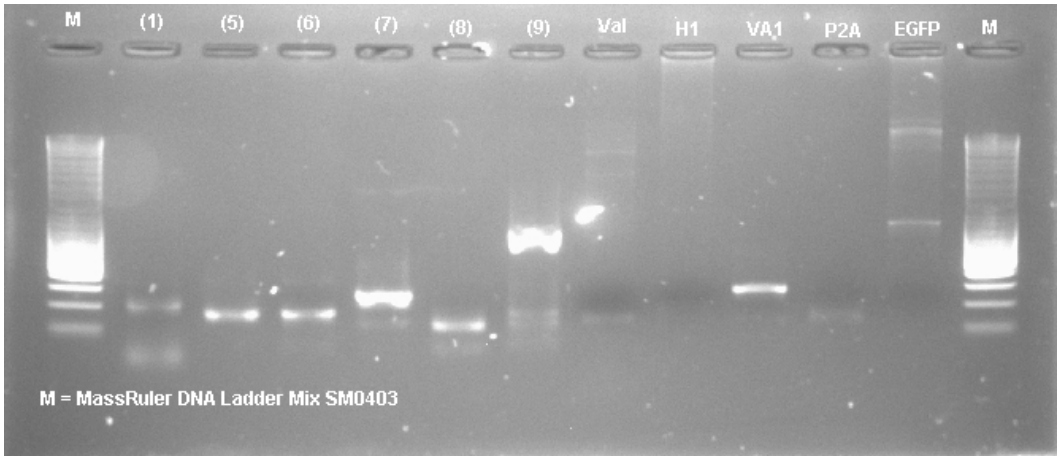
Wednesday, 5th of October 2016



Picture 6 PCR results on Agarose 2.0% and Acrylamide 12%, respectitively.

The results were not good, so we planned to do it again the next day.

Thursday, 6th of October 2016



Picture 7 PCR results on 1.5% agarose

The results turned good as we expected. We then proceeded to do small scale PCR using Hi-Fi Taq Polymerase (total volume of 20 µL).

10x Taq Hi-Fi Buffer = 2µL

10 mM dNTPs = 0,4µL

5µM primer forward = 0,8µL

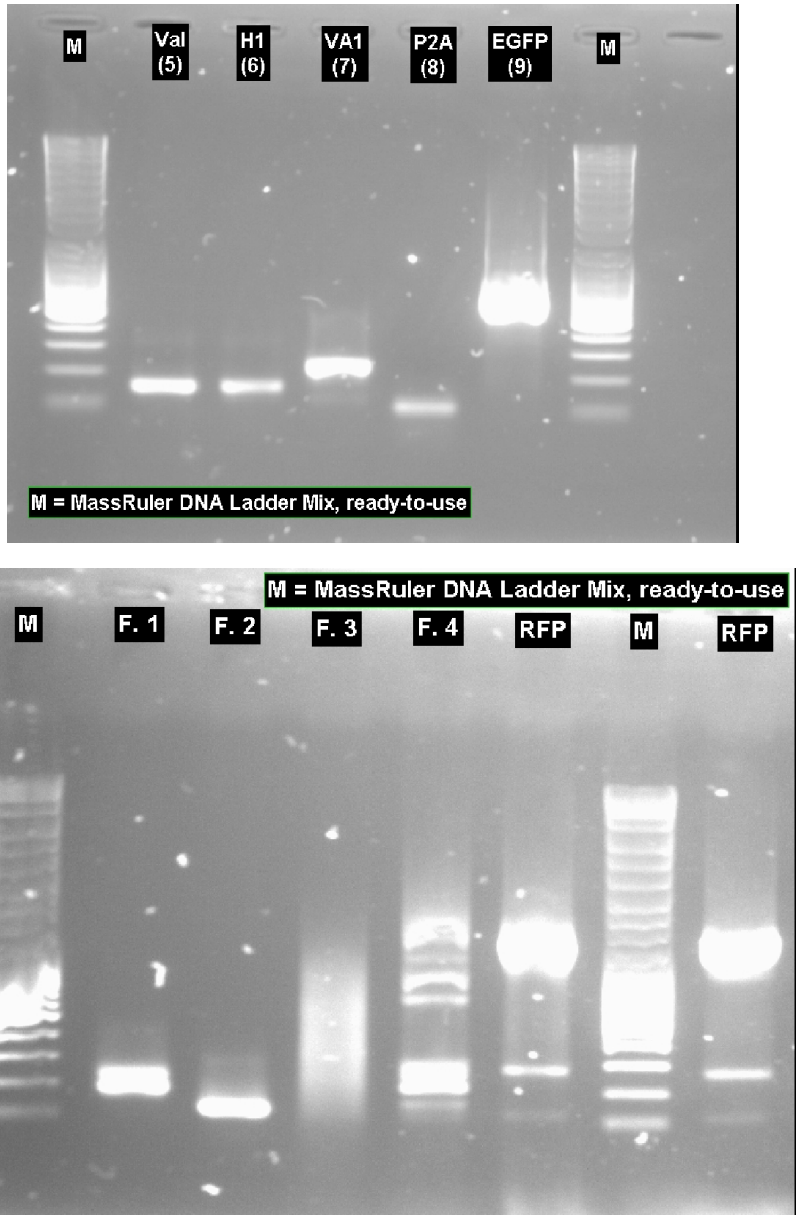
5µM primer reverse = 0,8µL

Taq Hi-Fi Polymerase = 0,15µL

Template DNA (10ng/µL) = 0,5 µL

$MgCl_2$ 50mM = 0,8µL

ddH_2O = 11,45µL



Picture 8 Small scale PCR results on 1,5% and 1% agarose respectitively

Friday, 7th of October 2016

Large scale PCR:

10x Taq Hi-Fi Buffer = 20µL

10 mM dNTPs = 4µL

5µM primer forward = 8µL

5µM primer reverse = 8µL

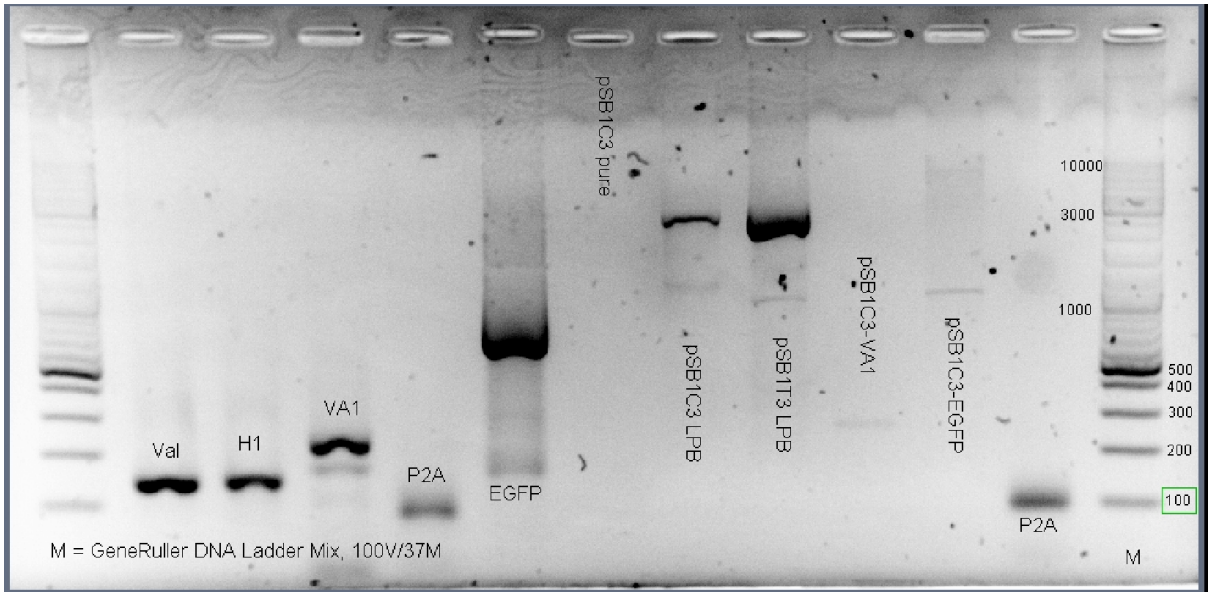
Taq Hi-Fi Polymerase = 1,5 µL

Template DNA (10ng/µL) = 0,5 µL

$MgCl_2$ 50mM = 8µL

ddH_2O = 150 µL

Saturday, 8 October 2016



Picture 9 Large scale PCR results on 1,3% agarose

The result of large scale PCR in 1.3% agarose was as expected. Therefore, we proceeded to purification and EcoRI digest.

10x BSA : 15

DNA template : 100

ddH20 :16

EcoRI : 4

EcoRI buffer 10x:15

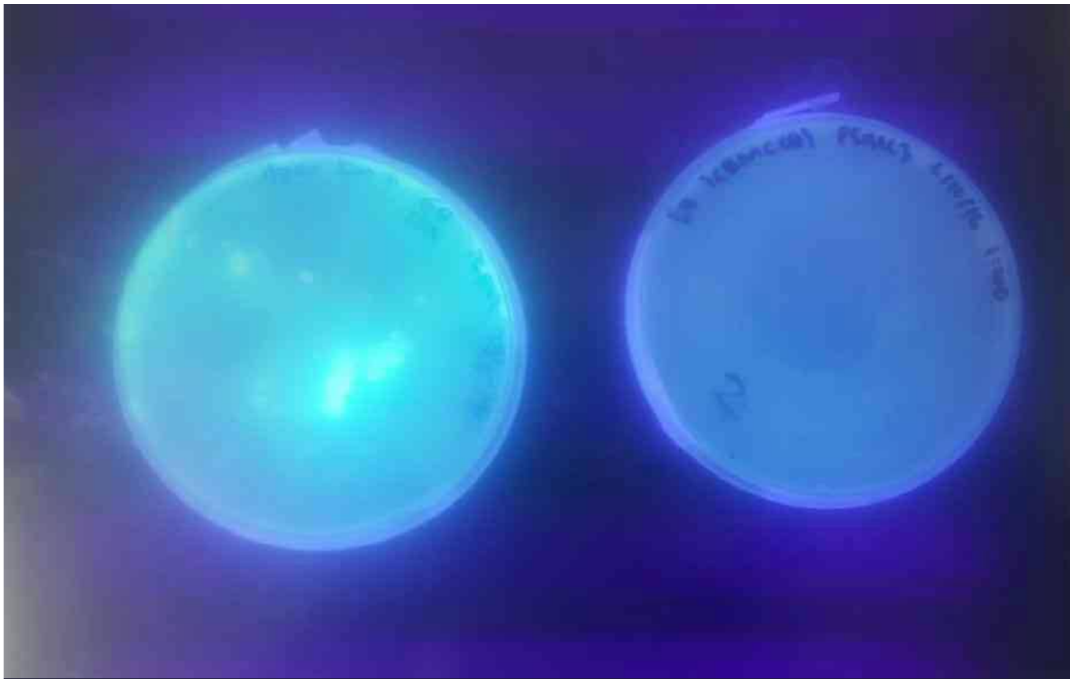
Total: 150

Wednesday, 19th of October 2016

A lot of things happened until this very day. Because there were only two labworkers working since the beginning, it was hard for us to report what we had done when the works that we did intensifies greatly.

In short, we successfully assemble our parts in pSB1C3, confirmed by PCR followed by electrophoresis gel agarose.

Under our lab-developed promoter, MAX promoter, we have succesfully validated our part BBa_K2050420 EGFP. We tested this on E. coli TOP-10 within 18 hours of incubation in 37 degree celcius incubation. The same plasmid which carried different insert was used as a control.



We had yet the time to test the expression of our EGFP on Rhabdomyosarcoma or HeLa.

We also tested BBa_J04450 expression in a rather different chassis, *E. coli* TOP-10. What we found out might be counted as contribution/improvement to this part. iGEM wiki page about this part said the red colour would show at 16 hours. When we did this with all concentration of the competen cell test kit (0,5pg/ul, 5pg/ul, 10pg/ul, 20pg/ul, 50pg/ul), after 16 hours of incubation time on 37 degree of celcius, there was not any red colony. Only after we prolong the incubation time into 24 hours, the only red colony on the plate with 0,5pg/ul transformed BBa_J04450 can be seen red without UV aid. This is important as when people tried to test the competency of the cells and found out there were no red colonies after 16 hours incubation, they would think that their competent cells or their transformation had failed, when actually BBa_J04450 in TOP-10 needed to have a longer incubation time. We haven't tested any further and have not discovered the mechanism behind this.

To conclude our work on this iGEM season, first, we haven't done our main project because lengthy overlaps on our fragment preventing the assemble. We have four parts that's ready to send to iGEM headquarters: tRNA(Val), H1 promoter, VA1 RNA and EGFP. P2A was omitted from the list because technical reason.We have tested that our EGFP worked as expected by showing it with control group under

UV light. We also contributed to the improvement of previous parts, RFP (BBa_J04450). We found out that it takes longer incubation time to see the full red colour on all colonies (~24h).