Notebook Lab Work

MONDAY, 8/9/2021

Pre culture *C. violaceum*

Activities:

Medium Stock

1. making LB agar (100mL) --> 5 plates, stored in LG fridge 2nd level
2. making LB broth (50mL) --> stored in 100mL duran in LG fridge

Pre Culture

1. pre culture for C. Viola IL and ATCC --> 2 colonies each in 5mL LB broth (marked)
    - shaking at 180rpm adn started at 11.50, room temperature
    - 1 control

Error

1. petry at plate stock got burned (ATCC plate, non master), allows contamination
    - i've been separated and sealed it (marked)
    - i've replace it with new streak plate one (marked)

TUESDAY, 8/10/2021

Genome Isolation

Activities

Genome Isolaton (Geneaid kit)

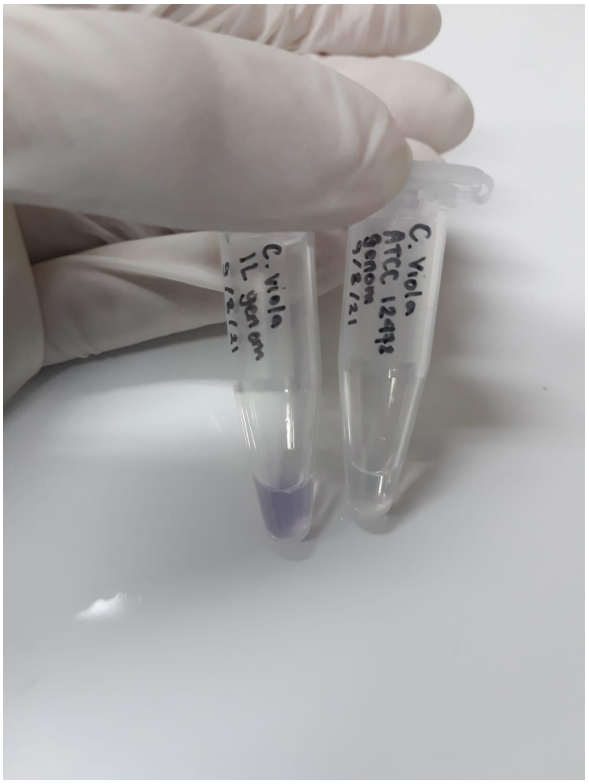
Phenomenons:

1. cell density were too thick due to violacein (perhaps)
2. wash buffer could'nt pass the GD filter --> the pigmen residue should be discarded in advance
3. 12' incubation at 60 and 70 degree celcius

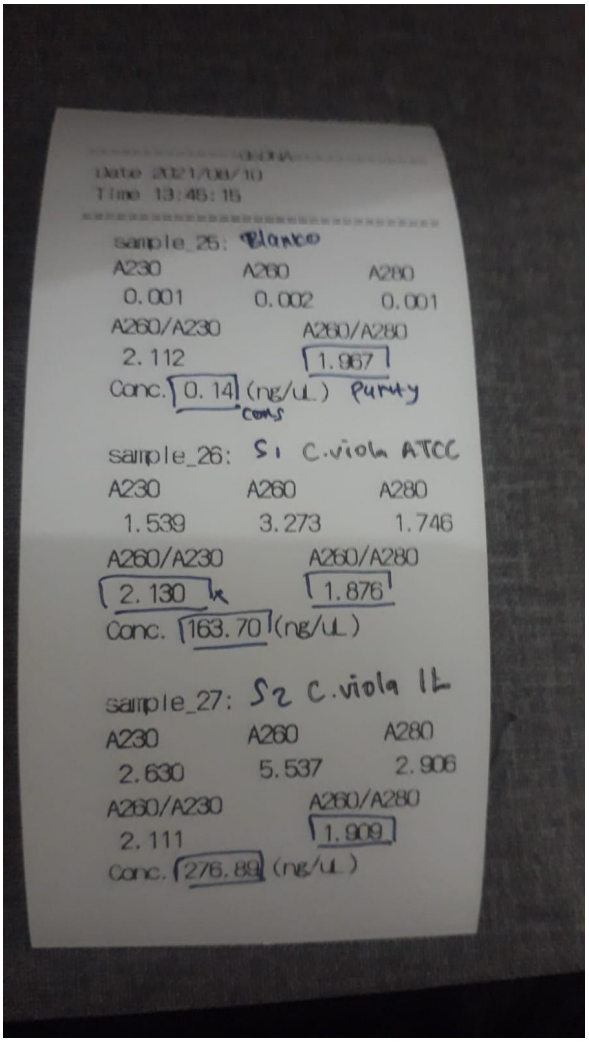
Result:

1. C. violaceum local isolate (IL)
   A260/A230: 2.130
   A260/A280: 1.876
   DNA concentration: 163.70 ng/uL
2. C. violaceum ATCC 14272
   A260/A230: 2.111
   A260/A280: 1.909
   DNA concentration: 276.89 ng/uL

Genome isolation-sample C. violaceum IL (local isolate) and C. violaceum ATCC 14272.jpeg



NanoDrop_result.jpeg



MONDAY, 8/16/2021

Competent Cell Preparation

Acivities:

1. LB medium preparation:
    - 4 x 5mL broth medium for pre-culture
    - 3 x 100 mL broth for 3rd day competent cell
    - 50mL LB agar for refresh cell
2. Refresh cell
    - C. violaceum ATCC, BL21, and Top 10 have been refreshed

DAY 1 Competent Cell

1. Flame the metal inoculating loop until it is red and then cools it down
2. Scrape pick the single colony from culture stock
3. Streak it onto the LB plate
4. Leave the plates place them upside down in the room temperature

THURSDAY, 8/19/2021

things to prepare:

1. LB medium @5mL --> 3 tubes

2. LB agar 50mL --> 3 plates
3. Liquid nitrogen (in confirmation)
4. 0.1 M CaCl2 sollution --> 1.47 CaCl2 + 100mL ddH2O (Autoclaved)
5. 0,1 M CaCl2 + glicerol --> 0.735 CaCl2, 7.5mL glicerol 99%, 42.5mL ddH2O (Autoclaved)
6. falcon 1 pack
7. LB agar with antibiotic (if necessary for testing)

TUESDAY, 8/31/2021

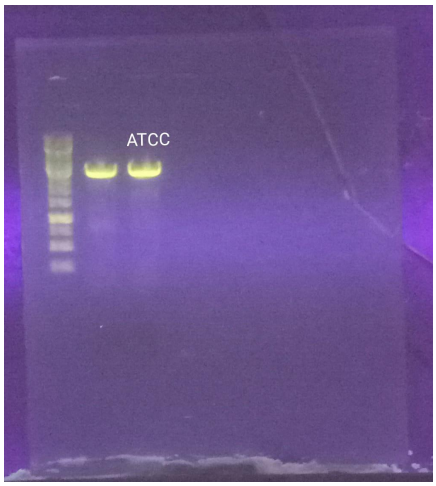
GENE ISOLATION

we did gene issolation for hcnABC in C. violaceum local issolate as well as C. violaceum ATCC 14272.
steps:

1. primer diluted using pH buffer (243ul for F, 269ul for R)
2. further diluted using nuclease free water (5ul of primer into 45uL NFW)

3. PCR Mix
    - master mix 25 ul
    - primer F 1.5ul
    - primer R 1.5ul
    - DNA 1 / 1.5 ul
    - DMSO 1.5ul
    - NFW 17.5ul
4. continue with electrophoresis (100V ~30')
5. result: left for local issolate and right for ATCC

WhatsApp Image 2021-08-31 at 1 7.38.12.jpeg



using 1kb DNA ledder, it is confirmed that the targeted gene (hcnABC ~3000bp) are succesfully isolated. for further process, both band are cut and saved to 1.5 mL microtube for purification.

WEDNESDAY, 9/1/2021

hcnABC Operon Purification

we conducted hcnABC operon purification after we have already done with the operon isolation yesterday. the purification was carried out from electrophoresis gel as well as from the PCR products. in addition, we use a gene purification kit from Geneaid. we follow the steps exactly the same as the protocol with some adjustments in the volume of our samples. here are some of the details:

NB: we purify 2 hcnABC operons, from the C. violaceum ATCC 14272 and local isolate of C. violaceum (IL)

1. total volume of PCR products for IL was 45uL while for the ATCC was 40uL, thus additional 5uL and 10uL of NFW were necessary
2. total volume of disolved electrophoresis gell + gell buffer was ~700uL
3. we use 45uL of ellution buffer at the end of the process

Result:

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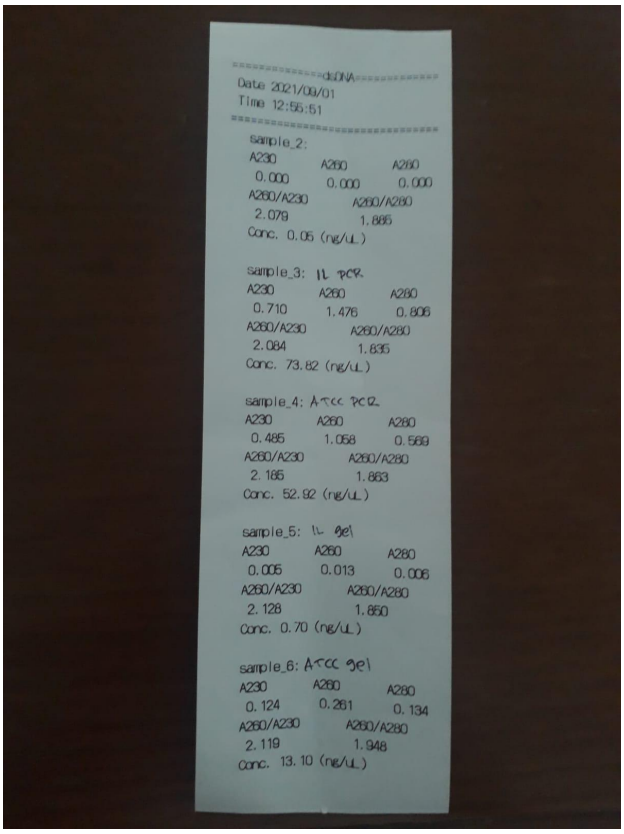

low DNA concentration was observed, however, at least it reaches 50ng/uL. next step, we plan to digest the operon to determine whether or not they possess illegal sites inside the gene. re-PCR is also listed in our next plan to increase the gene concentration.

SATURDAY, 9/4/2021

Competent Cell Preparation

we made 3 competent cells at once: E. coli BL21, E. coli TOP10, and C. violaceum ATCC 14772 with the Sambrook protocol:

1. prepare overnight pre culture taken from streak plate of each strains
2. inoculate 1% of overnight pre-culture to 50mL LB medium
3. incubate in room temperature ~2.5h up to the OD600 reaches 0.5 - 0.6
4. devide the broth culture ito 2 falcons tube
5. centrifuse in 4 degree celcius, 7000rpm in 5 mins
6. discard the supernatant and keep the pelet in ice box
7. add 25mL of pre-chilled 0.1M CaCl2 sollution and resuspend the cell
8. incubate 15 mins in ice box
9. centriifuse 4 degree celcius, 7000rpm in 5 mins. discard the supernatant and keep pelet on ice
10. add 15mL pre-chilled CaCl2 sollution and resuspend the pelet
11. incubate on ice for 15 mins
12. centrifuse in 4 degree celcius, 7000rpm in 5 min, discard the supernatant and kep pelet on ice
13. add 1mL CaCl2 + glycerol solution. resuspend the pelet. better work in LAF/ BSC
14. devide the culture into 1.5 microtube, 60uL each.
15. freeze all tubes using liquid nitrogen

16. keep in -80 degree celcius

from this protocol, we obtained around 30 - 35 tubes of competent cells which stored in Agricultural Genetic Lab

MONDAY, 9/13/2021

TRANSFORMATION

We conducted transformation for BL21, TOP10, and C. violaceum that we've make them as competent cell before:

C. violaceum --> pBBR1-MCS2	
TOP10 --> pBBR1-MCS2 in pSB1C3	Ant: Kanamicin
TOP10 --> BBa_K1602055 in pSB1C3 (18P k.p. 5)	Ant: Kanamicin
TOP10 --> BBa_10500 in pSB1C3 (21E k.p. 4)	Ant: Chloramphenicole
TOP10 --> BBa_K542003 in pSB1C3 (2M k.p1 1)	Ant: Chloramphenicole
TOP10 --> BBa_I13522 in pSB1C3 (5I k.p. 3)	Ant: Chloramphenicole
TOP10 --> BBa_K80800 in pSB1C3 (7E k.p 2)	Ant: Chloramphenicole
BL21 --> pSB1C3	Ant: Chloramphenicole

Steps:

1. thaw the competent cells in the ice box
2. after it thawed, take 50uL competent cell to new microtube
3. add 2uL of plasmid, mix by pippeting
4. incubate in ice cube for at least 30′
5. heat shock at 42oC in EXACTLY 45 seconds
6. after 45″, incubate in the ice box for 5 mins
7. add 950mL LB medium
8. incubate at 37oC in the shaker for 1 hour
9. after incubation, prepare the agar plate with antibiotics
10. spread 100uL of culture to the plate
11. centrifuge the remaining culture
12. discard 750uL of supernatant and resuspend the pelet
13. spread the resuspended culture to the surface agar as 9x concentration
14. incubate at 37oC overnignt

TUESDAY, 9/14/2021

Transformation checking

After overnight incubation, we checked the transformants result of yesterday's work

Good colony growth observed on:

1. TOP10 --> BBa_K1602055 in pSB1C3 9x
2. TOP10 --> BBa_K80800 in pSB1C3 9x
3. TOP10 --> pBBR1-MCS2 in pSB1C3 9x

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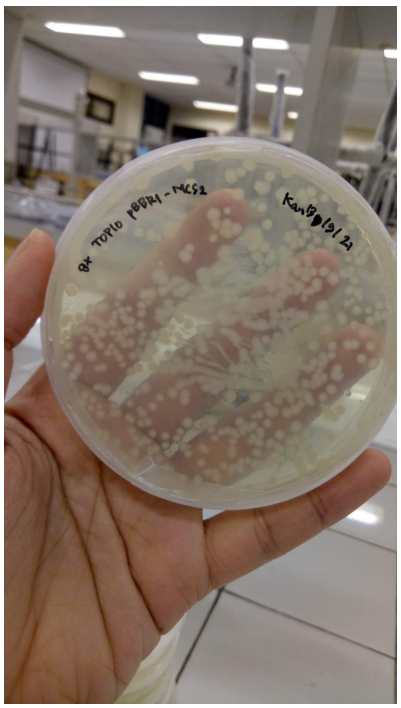
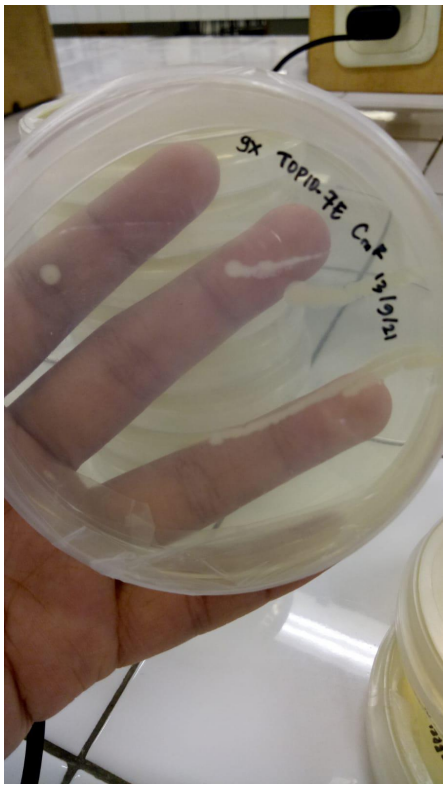


WhatsApp Image 2021-09-14 at 09.17.27.jpeg



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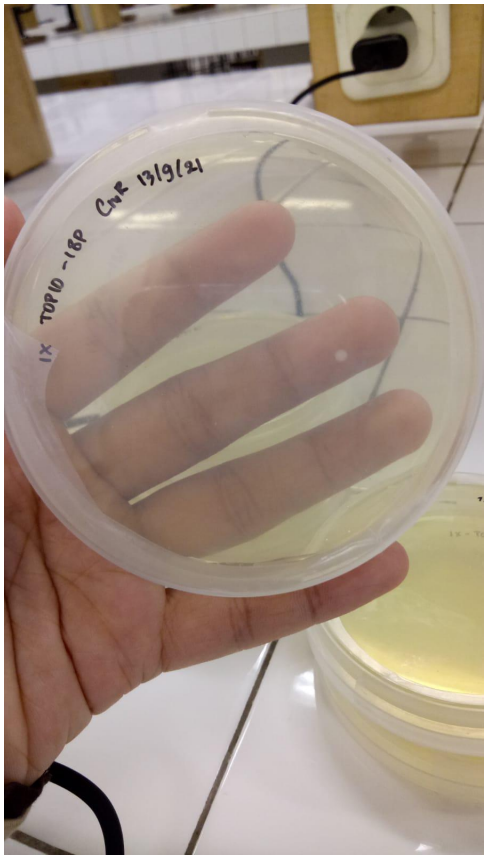
WhatsApp Image 2021-09-14 at 09.16.43.jpeg



those 3 transformants are recultured in broth medium and colony PCR with the following steps,

Colony PCR

PCR Mix:
DNA = Colony dot
VF2 = 1 uL
VR = 1 uL
PCR Mix = 12.5 uL
MFW = 10.5

Total= 25 uL

1. init denaturation 95oC 1 min
2. denaturation 95oC 15 s
3. annealing 54oC 15 s
4. elongation 72oC 30 s
5. final elongation 72oC 5 min
6. store 4oC (~)

we doubt about today's result, therefore we decided to try agin in the following days.

WEDNESDAY, 9/15/2021

Making competent cell E. coli BL21 day 1

we did not use the BL21 from the same source as previous competent cell making, however we use the 'older' E. coli BL21 from our advisor
for the first day, we only inoculate single colony to 5mL LB broth and incubate it overnight in the incubation shaker at 37oC.

THURSDAY, 9/16/2021

Making competent cell E. coli BL21 day 2

we use different protocol for this attempt. here is the protocol:

1. Inoculate 100 ml LB medium with 1 ml of saturated overnight culture
2. Shake at 37C untl OD600= 0.4 (usually 2-3 hours)
3. Place in an ice bath for 10 minutes (After this point the cells should never touch anything that is warm)
4. Pre-cool solution, centrifuge, pipette tips, falcon, eppendorf
5. Transfer the culture into two pre-chilled 50 ml falcon
6. Centrifuge at 2700xg for 10 minutes at 4C
7. Remove the medium, resuspend the cell pellet with 1.6 ml ice cold 100 mM MgCl2-CaCl2 by swirling on ice gently
8. Incubate on ice for 30 minutes
9. Centrifuge at 2700 xg for 10 minutes at 4C
10. Remove the medium, resuspend the cell pellet with 1.6 ml ice-cold 100 mM CaCl2 by swirling on ice gently
11. Incubate on ice for 20 minutes
12. Combine cells to one tube and add 0.5 ml ice-cold 80% glycerol and swirl to mix
13. Freeze 100 ul aliquots in liquid nitrogen
14. Store in -80C.

MONDAY, 9/20/2021

Transformation Preparation

we prepare some equipment for competent cell preparation such as:

1. LB agar with chloramphenicole antibiotic
2. LB agar with kanamicin antibiotic

3. 50mL LB broth for C. violaceum competent cell

TUESDAY, 9/21/2021

Transformation

for the transformation, we use following plasmid and antibiotics,

DH5 --> pBBR1-MCS2	Ant: Kanamicin
DH5 --> BBa_K1602055 in pSB1C3 (18P k.p. 5)	Ant: Kanamicin
BL21 --> BBa_10500 in pSB1C3 (21E k.p. 4)	Ant: Chloramphenicole
BL21 --> BBa_K542003 in pSB1C3 (2M k.p1 1)	Ant: Chloramphenicole
DH5 --> BBa_I13522 in pSB1C3 (5I k.p. 3)	Ant: Chloramphenicole
BL21 --> BBa_K80800 in pSB1C3 (7E k.p 2)	Ant: Chloramphenicole

1. Thaw competent cells on ice.
2. Add 2 µl of the chilled assembly product to the competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 45 seconds. Do not mix.
5. Transfer tubes to ice for 5 minutes.
6. Add 950 µl of room-temperature SOC media to the tube.
7. Incubate the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the selection plates.
10. Centrifuge the remaining 800 µl of the cells --> 6800 xg for 3 minutes
11. Remove 700 µl supernatant, and mix the remaining 100 µl supernatant with the pellet
12. Spread 100 µl of the "9x concentration" cells onto the selection plates
13. Incubate overnight at 37°C.

WEDNESDAY, 9/22/2021

C. violaceum Competent Cell

we make C. violaceum competent cell with the method published by Liow, 2020. here is the protocol:

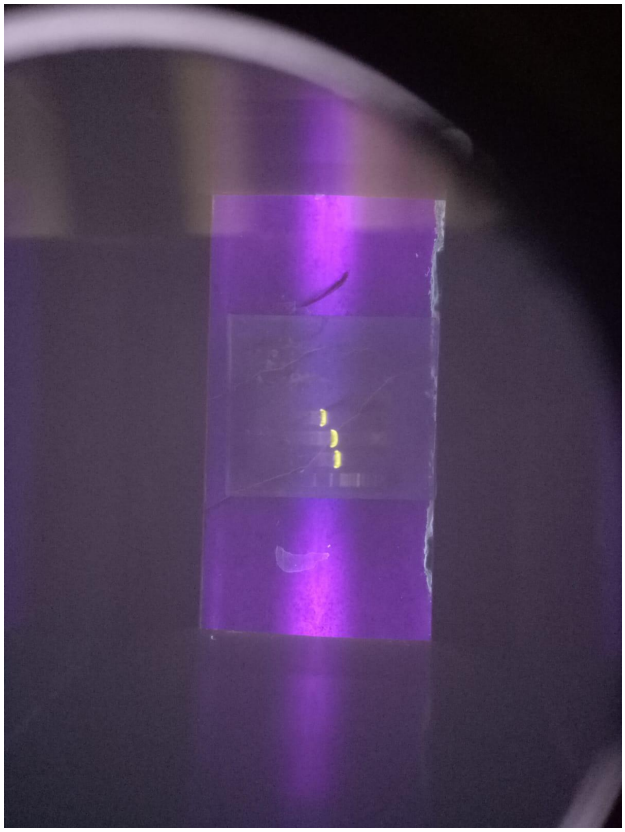
1. inoculate a single colony of C. violaceum in 5mL LB broth. incubate at 26oC overnight
2. Make the main culture by transferring 500 uL pre-culture of C. violaceum to 50 ml LB broth, incubate at 26°C with shaker (200 rpm) until OD600 reached 0.4 - 0.5
3. after reaching the desired cell density, incubate on ice for 15 mins of C. violaceum for 30 min
4. Harvest cell by centrifugation on 4000 rpm for 7 min at 4°C
5. Rinse pellet with 50 ml cold distilled water, and wash pellet by alternate centrifugation-resuspension, then centrifuge on 4000 rpm for 10 min at 4°C (repeat if necessary)
6. re-wash pellet with 5mL 15% glycerol and resuspent. next, centrifuge culture on 4000 rpm fo 10 mins at 4oC
7. add 800uL 15% glycerol and resuspend. store the culture in 60uL aliquotes at -80oC

THURSDAY, 9/23/2021

Colony PCR and Electrophoresis

the colony PCR and electrophoresis aimed to confirm whether the plasmid successfully inserted in the host. some cells that observed to grow are cells which inserted with pBBR1-MCS2, DH5-alpha with BBa_K1602055, BL21 with BBa_K80800, and BL21 with BBa_K542003. here are the result:

WhatsApp Image 2021-09-23 at 12.23.09.jpeg



left to right: DNA ledder, 2M, 7E, 18P, pBBR1

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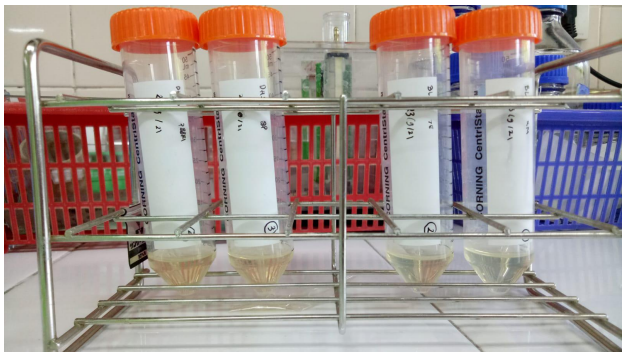


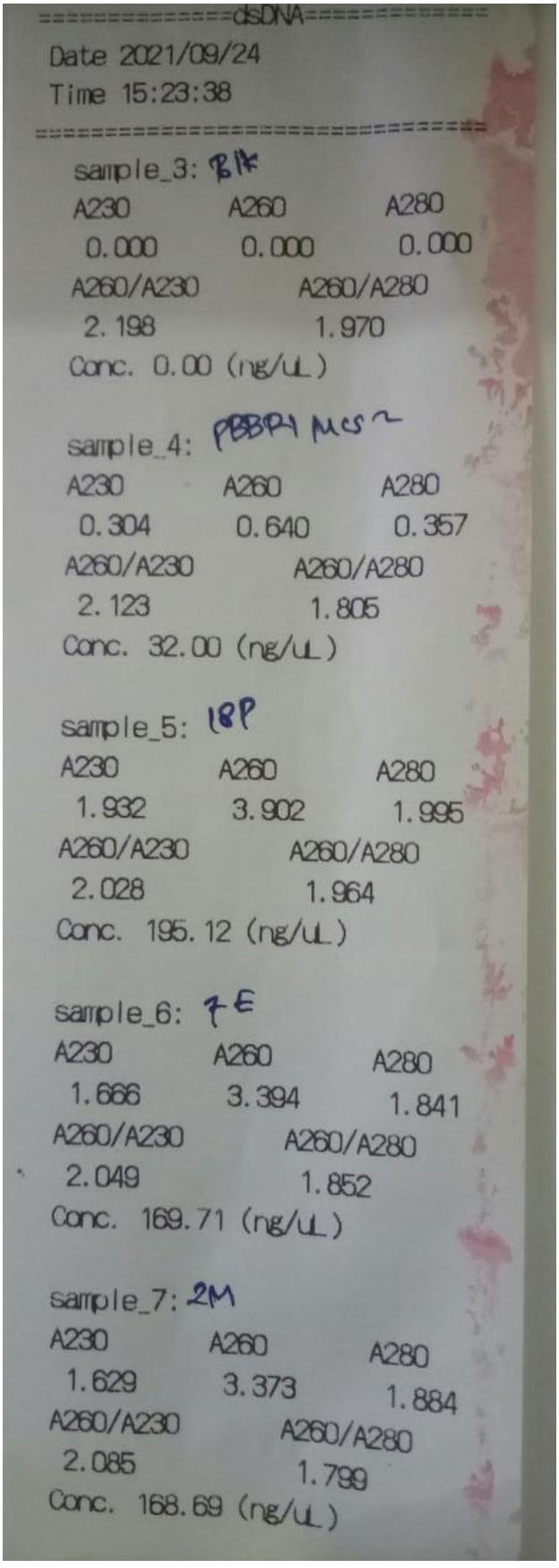
the pBBR1-MCS2 didn't show any band in the electrophoresis. this might be caused by the lack of sequence that match with the primer because we use universal primer for E. coli

FRIDAY, 9/24/2021

Plasmid Isolation

we already subculture the E. coli culture (with 18P, 7E, 2M, and pBBR1-MCS2 plasmids) to LB broth media with overnight incubation. next, we did the plasmid isolation with Geneaid Plasmid Isolation Kit followed with nanodrop to check the DNA conentration. here is the result of the plasmid isolation:

WhatsApp Image 2021-09-25 at 14.22.52.jpeg



BBa_K1602055 in pSB1C3 (18P k.p. 5) = 195.12 ng/uL
BBa_K542003 in pSB1C3 (2M k.p1 1) = 168.69 ng/uL
BBa_K80800 in pSB1C3 (7E k.p 2) = 169.71 ng/uL
pBBR1-MCS2 = 32 ng/uL

besides plasmid isolation, we also did primer elution because our ordered primers have just arrived.

SATURDAY, 9/25/2021

PCR

the PCR today aimed to eliminate the adaptor of the synthesized gene that we ordered from Twist Bioscience. 5 samples were PCR-ed with Phusion PCR method. the detailed protocols, genes, and primers are written in the PCR List bellow:

Detail_1

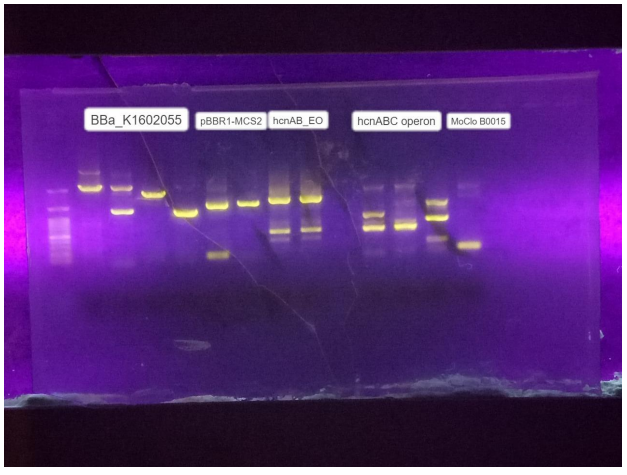
	A	B	C	D	E	F	G	H	I	J	K	L	M
1	DNA Template		18P (BBa_K1602055)					pBBR1-MCS2			hcnAB_EO (sintesis Twist Bio.)		
2	Tube No.		1	2	3	4		5 (high GC)	6 (high GC)		7	8	
3	Primer FWD		Bio-suffix_FWD	Bio-suffix-2_FWD	BB-p1b_FWD	BB-p1b_FWD		BBR1-1_FWD	BBR1-2_FWD		hcnAB_EO-1_FWD	hcnAB_EO-2_FWD	
4	Primer REV		K1602055_REV	K1602055-2_REV	K1602055-2_REV	Bio-prefix_REV		BBR1-1_REV	BBR1-2_REV		hcnAB_EO_REV	hcnAB_EO_REV	
5	Panjang product		+/- 3300	+/- 3300	+/-2485	+/-1225		+/- 1530	+/- 1530		+/- 1750	+/- 1750	
6													
7	Tube No.		1	2	3	4		5 (high GC)	6 (high GC)		7	8	
8	DNA Template		0.5 uL	0.5 uL	0.5 uL	0.5 uL		1 uL	1 uL		1 uL	1 uL	
9	PCR Phusion Green Mix		25 uL	25 uL	25 uL	25 uL		25 uL	25 uL		25 uL	25 uL	
10	Primer FWD		2.5 uL	2.5 uL	2.5 uL	2.5 uL		2.5 uL	2.5 uL		2.5 uL	2.5 uL	
11	Primer REV		2.5 uL	2.5 uL	2.5 uL	2.5 uL		2.5 uL	2.5 uL		2.5 uL	2.5 uL	
12	NFW		19.5 uL	19.5 uL	19.5 uL	19.5 uL		17.5 uL	17.5 uL		19 uL	19 uL	
13	DMSO		-	-	-	-		1.5 uL	1.5 uL		-	-	
14	TOTAL		50 uL	50 uL	50 uL	50 uL		50 uL	50 uL		50 uL	50 uL	
15													

Detail_2

	A	B	C	D	E	F
1	Tube No.		1	2	3	4
2	DNA Template		genome C. Viola ATCC (high GC)			E8 (MoClo Kit, B0015)
3	Tube No.		1 (high GC)	2 (high GC)	3 (high GC)	4
4	Primer FWD		hcnC4_FWD	hcnC1_FWD	hcnC6_FWD	B0015_FWD
5	Primer REV		hcnC2_REV	hcnC2_REV	hcnC3_REV	B0015_REV
6	Panjang product		+/- 555	+/- 555	727	+/- 175
7						
8	Tube No.		1	2	3	4
9	DNA Template		1 uL	1 uL	1 uL	0.5 uL
10	PCR Phusion Green Mix		25 uL	25 uL	25 uL	25 uL
11	Primer FWD		2.5 uL	2.5 uL	2.5 uL	2.5 uL
12	Primer REV		2.5 uL	2.5 uL	2.5 uL	2.5 uL
13	NFW		17.5 uL	17.5 uL	17.5 uL	19.5 uL
14	DMSO		1.5 uL	1.5 uL	1.5 uL	-
15	TOTAL		50 uL	50 uL	50 uL	50 uL
16						

The PCR products entered the following step, which is electrophoresis. the first electrophoresis was conducted to check the presence of desirable products. we use a 100 bp DNA ladder from geneaid as the marker. here is the documentation of the first electrophoresis:

WhatsApp Image 2021-09-26 at 21.33.43.jpeg



detailed sample per wells are mentioned in PCR list (phusion PCR) page.

all desirable sequences are successfully obtained, thus we continue to purify the specific gene targets based on the length of the fragmen.

SUNDAY, 9/26/2021

Gel and PCR Product Purification

based on yesterday's result, we continue to purify the gel electrophoresis for the desirable genes in the specific band, yet for whell no. 3 and 6 we do PCR product purification because the bands on the electrophoresis are clean with a merely single band. here is the DNA concentration after the purification using Gel/PCR purification Kit from Geneaid:
sample description are writen in the PCR phusion list

Table1

	Sample	DNA Concentration
1	T1	13.154
2	T2	16.277
3	T3	12.196
4	T4	40.619
5	T5	33.277
6	T6	89.711
7	T7	35.234
8	T8	37.004
9	T1(2)	26.598
10	T2(2)	19.461
11	T3(2)	28.206
12	T4(2)	18.477
13		
14		

for the next step, we continue the second step PCR for some samples in order to elongate the overhang sequence for the Gibson Assembly. detailed second PCR are writen in the PCR list Phusion PCR Running 3. unfortunately, we failed to get the sequence because we thought that the DNA template concentrations were too low.

next, we do our 4th step of PCR. actually the goals was the same: to elongate overhang site for certain sequence. we did this differently because the primer Tm is different and our PCR machine unable to do the gradient PCR. here is the PCR protocol:

Running 4

	A	B	C	D	E	F
1	Tube No.		1	2	3	4
2	DNA Template		genome C. Viola ATCC (high GC)	hcnABC operon hasil isolasi dari genome C. Viola ATCC (high GC)	Product Tube 2(2) Running 2	Product Tube 2(1) Running 1
3	Tube No.		1 (high GC)	2 (high GC)	3 (high GC)	4
4	Primer FWD		hcnC7_FWD	hcnC7_FWD	hcnC2_FWD	Bio-suffix-3_FWD
5	Primer REV		hcnC4_REV	hcnC4_REV	hcnC2_REV	K1602055-2_REV
6	Panjang product		+/- 754	+/- 754	+/- 555	+/- 3300
7						
8	Tube No.		1	2	3	no name
9	DNA Template		1 uL	1 uL	7 uL	10 uL
10	PCR Phusion Green Mix		25 uL	25 uL	25 uL	25 uL
11	Primer FWD		2.5 uL	2.5 uL	2.5 uL	2.5 uL
12	Primer REV		2.5 uL	2.5 uL	2.5 uL	2.5 uL
13	NFW		17.5 uL	17.5 uL	11.5 uL	10 uL
14	DMSO		1.5 uL	1.5 uL	1.5 uL	-
15	TOTAL		50 uL	50 uL	50 uL	50 uL
16						

TUESDAY, 9/28/2021

PCR

we did some PCR aimed to add the overhang site for the Gibson assembly and we didn't get the good result due to the low DNA template concentration. however, we still continue to assembly the fragments because the overhand site was just enough ~20 bp.

Gibson Assembly

the Gibson assembly was conducted by mixing 4 inserts which consist of AraC-pBAD promoter (amplified from iGEM kit Plate 4, whel 18P), hcnAB (synthesized by Twist Bioscience and PCR amplified to remove the adaptor and to add the overhand site), hcnC (before the illegal site), and hcnC (after the illegal site). the terminator of this construct was a double terminator which directly attached with pSB1C3 backbone, taken from MoClo Kit wheel no. 8E. bellow is the total mixture of the Gibson Assembly, which later be incubated in 50oC for 1 hour.

the assembly product was stored at -4oC for tomorow's transformation

WEDNESDAY, 9/29/2021

Transformation

we transformed the assembly product as well as the BBa_K1602055 part to both E. coli BL21(DE3) and DH5-alpha. the transformation of BBa_K1602055 was aimed to fulfil the "contribution" section in the judging form. we plan to characterize the expression of this part in the BL21(DE3) and DH5-alpha, because it was only well characterized in TOP10 strain. we made duplo for the transformation which detailed bellow:

BL21(DE3), were transformed with:

- Gibson assembly product (Ga)
- Gibson assembly product, the duplo (Gb)
- BBa_K1602055 (18Pa)
- BBa_K1602055, the duplo (18Pb)

DH5-alpha, were transformed with:

- Gibson assembly product (Ga)
- Gibson assembly product, the duplo (Gb)
- BBa_K1602055 (18Pa)
- BBa_K1602055, the duplo (18Pb)

standard transformatin protocol was used and the transformants was platted onto Chloramphenicole LB agar overnight.

THURSDAY, 9/30/2021

Colony PCR

we do colony PCR and subculture (and overnight incubation) for all of transformed E. coli. we take 2 colonies from each plate. standard colony PCR is used and here is the result of the PCR after electrophoresis:

we get no band from the E. coli which transformed with the construct.
after that for the following days, we decided to do re-transformation from the Gibson assembly result. however, after several try, we still did not get the desirable colony, even, for the second and third transformation, no colonies were grown in our agar plate with Cmr. we suggested that there were some problems related to theconstruct which makes either the CmR resistant gene is not sucessfully inserted or we failed with the wholw construct.

for further investigation, we do colony PCR with several primers which specified to some parts of the inserted gene.

WEDNESDAY, 10/6/2021

Colony and Construct Investigation with Colony PCR

we tried to do another colony PCR with pair of primers which specified to amplify the hcnABC operon. here is the PCR protocol and primer that we used:

ee.jpeg

DNA Template:

1. 98 oC --> 30 second
2. Cycle (30x):
    - 98 oC --> 10 s
    - 60 oC --> 10 s
    - 72 oC --> 1 minute
3. 72 oC --> 5 minute
4. 4 oC --> unlimited

Table3

	A	B	C
1	DNA Template		GB1-m
2	Tube No.		1
3	Primer FWD		hcnAB_EO2_FWD
4	Primer REV		B0015_REV
5	Panjang product		+/-3100
6			

notes:
-GB1-m : single colony which transformed with the construct

and here is the result after electrophoresis:

222.jpeg

it is confirmed that the hcnABC operon is successfuly assembled, proven by the thin band in the agarose gel of which the possition is slightly above the 300bp marker. here we found out that probably, the antibiotic resistance gene was not sucessfully inserted due to the low DNA concentration when we did the Gibson Asembly.

next, we re-amplify the antbiotic resistance gene from BBa_K1602055. we did the gel purification to get desirable gene for this and did the nanodrop to know the DNA concentration.

THURSDAY, 10/7/2021

Gibson Assembly-2

This day, we did the next Gibson assembly with the assumption that the hcnABC gene have been successfuly constructed from the previous Gibson. thus, for this assembly, we assembled some gene fragments such as hcnABC operon up to the terminator, ORI (from BBR1-MCS2 plasmid), and promoter with antibiotic resistant gene. here is the Gibson mixture fot today's Gibson Assembly:

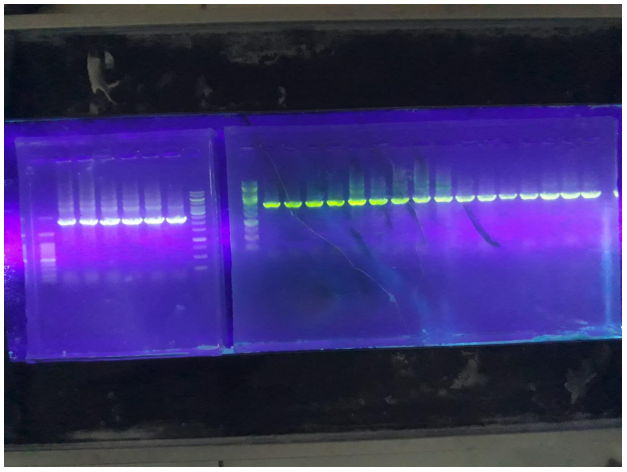
we also directly transform the result to E.coli BL21 and DH5-alpha.

FRIDAY, 10/8/2021

Colony PCR

after overnight incubation, we did the PCR colony for confirmation. we picked ~20 colonies to be amplified using VF2 VR primers. here are the results from the Colony PCR and electrophoresis:

WhatsApp Image 2021-10-09 at 10.27.58.jpeg



left ladder: 100 bp lader
right ladder: 1kb DNA ladder

the band showed strange bands. the correct length for the inserted plasmid should be around 5000bp, however, all of the colonies show that the DNA length was mere around 3000bp. some samples showed multiple band which one of those have bigger size above 4000-ish bp. based on this result, we hypothesized that the PCR mix was not robust enough to amplify large plasmid, thus it merely amplify at some region of the inserted plasmid. due to the similar band result, we choose 2 samples that have the thickest "above 4000-is bp" which are the first and second band from the left of the electrophoresis gel above. thus, we sub-culture them for plasmid isolation.

SATURDAY, 10/9/2021

Plasmid Isolation

The plasmid from overnight subculture of 2 above-mentioned colonies were being isolated using kit and protocol from MinPrep Plasmid DNA Kit from Geneaid. following that, we did the nanodrop to quantify the DNA concentration of the plasmid isolation product. here are the DNA concentration from each result:
Colony 1 : 82.67 ng/uL
Colony 2: 146.2 ng/uL

in order to reconfirmation, we did PCR with the primers that aplify the hcnABC region. here is the PCR protocol and the result (using 1KB DNA ladder)

WhatsApp Image 2021-10-09 at 09.12.24.jpeg

1	DNA Template		
2	Tube No.		1
3	Primer FWD		hcnAB_EO2_FWD
4	Primer REV		B0015_REV
5	Panjang product		+/-3100
6			
7	Tube No.		1
8	DNA Template		1
9	PCR Red Mix		12.5
10	Primer FWD		1
11	Primer REV		1
12	NFW		9.5
13	TOTAL		25
14			

WhatsApp Image 2021-10-09 at 18.48.2133.jpeg

it was thought that the confirmation procedure using PCR (VF2 VR primers with myTaq PCR Mix) was not robust enough to amplify the overall engineered plasmid, due to the limited concentration of dNTP in the PCR Mix. our PI suggested us to use manual mixture from KOD PCR which possesses higher fidelity for amplification, yet the time is not sufficient to order and re-confirmation the insert/ plasmid using this method. therefore, we directly send the plasmid for sequencing.

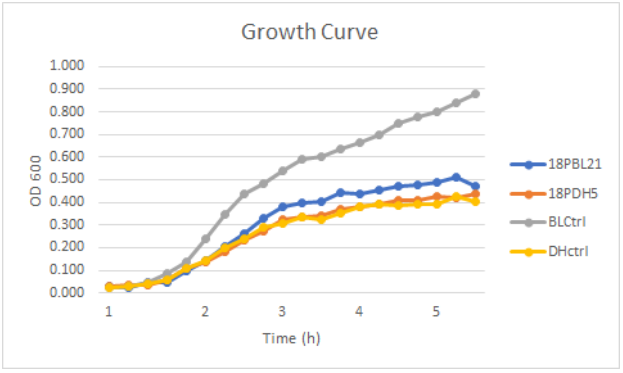
SUNDAY, 10/10/2021

Parts Contribution: Growth Curve for E.coli BL21(DE3) and DH5-alpha

one of the medal criteria for bronze is "Parts Contribution" and we manage to make a contribution for BBA_K1602055, a part which we utilized as the promoter for hcnABC operon in our engineered chasis. in the parts registry page, this part was merely characterized on E. coli TOP10 for the growth curve and fluorescense protein production. thereby, we would further characterize this part on E. coli BL21(DE3) and E.coli DH5-alpha.

we already transformed the BBa_K1602055 to both chassis with additional chloramphenicole in the LB medium and culture it in 5mL overnight pre-culture. after incubation, we inoculate it to the main culture of which the concentration of the inoculted pre culture was 1% drom the total volume of main-culture. we measure the cell growth using spectrophotometer OD 600 in every 15 minutes of incubation within 5 hours. here is the result of the growth curve for each chassis:

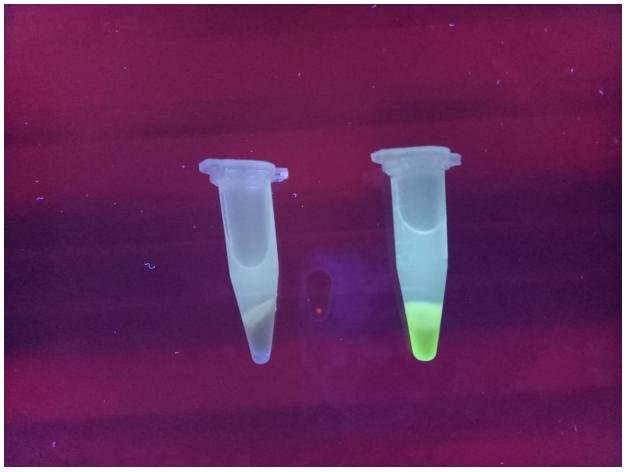
image.png



the BL21 contol showed the highest growth among all of the other engneered and strains, even after 5 hours of incubation, it still showed a significant increment. from the graph, the growth of BL21 control was significantly different with the BL21 which transformed with BBa_K1602055. this condition might happen because the transformed plasmid affected the bacterial metabolic load which makes the growth slightly lessen due to higher energy needed for the replication. however, the growth of DH5-alpha control and BBa_K1602055-transformed plasmid were not too dissimilar. further literature investigation is needed in order to strengthen the data with more background theory.

on the other hand, the remaining cells were centrifugated to know the expressed-GFP-protein which linked to the AraC-pBAD promoter in the BBa_K1602055 after inducted with 2mM L-arabinose. here is the documentation of the cell pelet under the UV light:

WhatsApp Image 2021-10-11 at 13.34.53.jpeg



left: DH5-alpha, Right: BL21(DE3)

the fluorescence was not observed or expressed in DH5-alpha, even though the plasmid is successfully transformed (confirmed by PCR colony and the growth of the cell in the chloramphenicol media). after we consulted it to our PI, he suggested that we should find more literature for this phenomenon. it could be the incompatibility of DH5-alpha strain to express the GFP protein, given that this strain is not specialized for protein expression. by contrast, the BL21(DE3) was completely able to express the GFP protein after inducted with 2mM L-arabinose.

for the next step, we plan to measure the fluorescence in every 15 minutes intervals using spectrofluorophotometer

TUESDAY, 10/12/2021

C. violaceum ATCC 12472 Transformation

this step is the following process after plasmid isolation.

after we obtain the plasmid with exact concentration, we tried to transform it to C. violaceum ATCC 12472 competent cell. we use 2 method for the transformation: heat shock and electroporation. for the heat shock, we addopted the protocol from iGEM Trec Monterey (http://2016.igem.org/Team:Tec-Monterrey/Experiments). however, for the electroporation, we use the protocol which had been optimized by Loke et al (2020). here are the detailed protocol for each method:

heat shock transformation protocol (Trec Monterey):

1. Mix 1 to 5µl of DNA (usually 10pg to 100ng) into 50µL of competent cells in a microcentrifuge tube.
2. Place the competent cell/DNA mixture on ice for 20min.
3. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 seconds (time varies depending on the competent cells you are using).
4. Put the tubes back on ice for 2 min.
5. Add 500µl LB media (without antibiotic) and grow in 37°C shaking incubator for 1 hour.
6. Plate 100 µl of the transformation onto a 10cm LB agar plate containing the appropriate antibiotic.
7. . Incubate plates at 37°C overnight

electroporation protocol (Looke *et al*., 2020):

1. thaw the competent cell on ice and mix with 500 - 800 ng of plasmid DNA
2. Transfer to 0,2 cm cold electroporation cuvette, incubate on ice for 5 min
3. electroporation: use 1 kV pulse for 0.1 cm (10kV/cm) cuvete for ~0.4ms (automatic) ; The capacitor and pulse controller resistance were set to 25 µF and 200 Ω
4. Resuspend cells with 1 ml SOC/LB for recovery, incubate on ice for 3 mins
5. incubate the cell at 220rpm for 4 h in 26oC (room temp)
6. Plate cells on antibiotic-containing LB agar for 48-72 hours

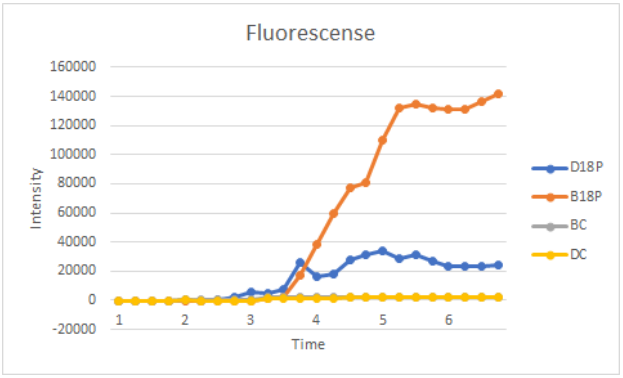
by using these methos, we did not obtain any positive transformants in our agar plate. the main possibility that support the failure might be the cell growth was too low, thus it needs more incubation time.

WEDNESDAY, 10/13/2021

Fluorescence Measurement for Parts Contribution

the fluorescence measure using Spectrofuorophotometer using excitation 504 nm and emission 516 nm. from it, we obtain the following graph:

image.png



the transformed BL21 is observed to have much higher fluorescence intensity rather than DH5-alpha. it is also linear with the previous cell centrifugation where the BL21 shine under the UV light.
in order to re-confirmation, we incubated the remaining cell of BL21 and DH5-alpha for overnight. surprisingly, after an overnight and 48 hours of incubation, the DH5-alpha shines under the UV light. our hypothesis was that the DH5-alpha GFP expression are lower and accumulated after more than 8 hours of incubation.