Lab Notes
iGEM UI 2018
Institute of Human Virology and Cancer Pathobiology (IHVCB)
Finding Diphthy

Monday, 9 July 2018

Yeay, gBlocks and primers have arrived from IDT!

Tuesday, 10 July 2018

Primer Cloning Optimization

We resuspend the *DiphTox* (DT) gBlocks by TE buffer pH 8.0 after prolonged centrifugation 3000 rpm for 5 minutes, yielding the concentration up to 10 ng/µl. Direct amplification of *DiphTox* was done by PCR using the 'Fwd Cloning' and 'Rev Cloning' primers. Primers were resuspended in TE buffer pH 8.0 as well up to 500 µM. Subsequent dilution of primers into 10 µM of working primer solution using nuclease free water were done to prevent cross-contamination.

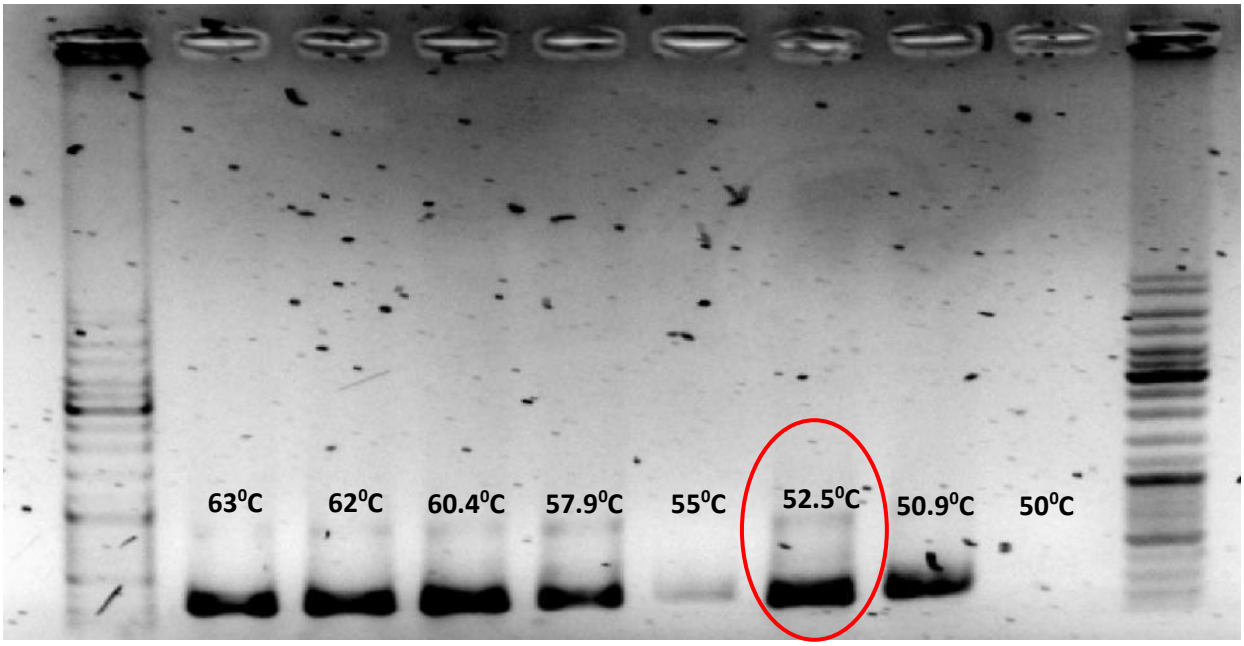
Following tables are the reaction master mix for total of 9 tubes @9 µl.

Materials	Volume
Phusion polymerase 10X	8.1 µl
DMSO 100% v/v	2.43 µl
Fwd Cloning primer 10 µM	4.05 µl
Rev Cloning primer 10 µM	4.05 µl
dNTP mix 800 mM	4.05 µl
5X HF buffer	16.2 µl
Nuclease free water	42.12 µl
Total	**81 µl**

Therefore, the master mix are then aliquoted into 9 PCR tubes @9 µl. One tube contained NFW as DiphTox template substitute as negative control, the others contained 1 µl of *Diph* gBlocks. Following tables represents temperature-time PCR formula for *Phusion* Hi-FI mix.

Process	Temperature	Time
Denaturation (Initial)	98^0C	3 mins
CYCLE 35X		
Denaturation	98^0C	30 s
Annealing	50^0C – 63^0C	30 s
Elongation	72^0C	1 min
Elongation (Last)	72^0C	7 mins

Following electrophoresis is done in agarose with composition of 0.8% agarose and 0.5X TAE. It runs within 50 V, 400 mA for 60 mins. Conclusion: Optimal annealing temperature is 52.5^0C.



Wednesday, 11 July 2018

Primer PCR Colony Optimization

Primer PCR colony optimization was done by PCR using the 'Fwd Uni PCR Colony' and 'Rev Uni PCR Colony' primers. Primers were resuspended in TE buffer pH 8.0 as well up to 500 µM. Subsequent dilution of primers into 10 µM of working primer solution using nuclease free water were done to prevent cross-contamination.

Following tables are the reaction master mix for total of 9 tubes @9 µl.

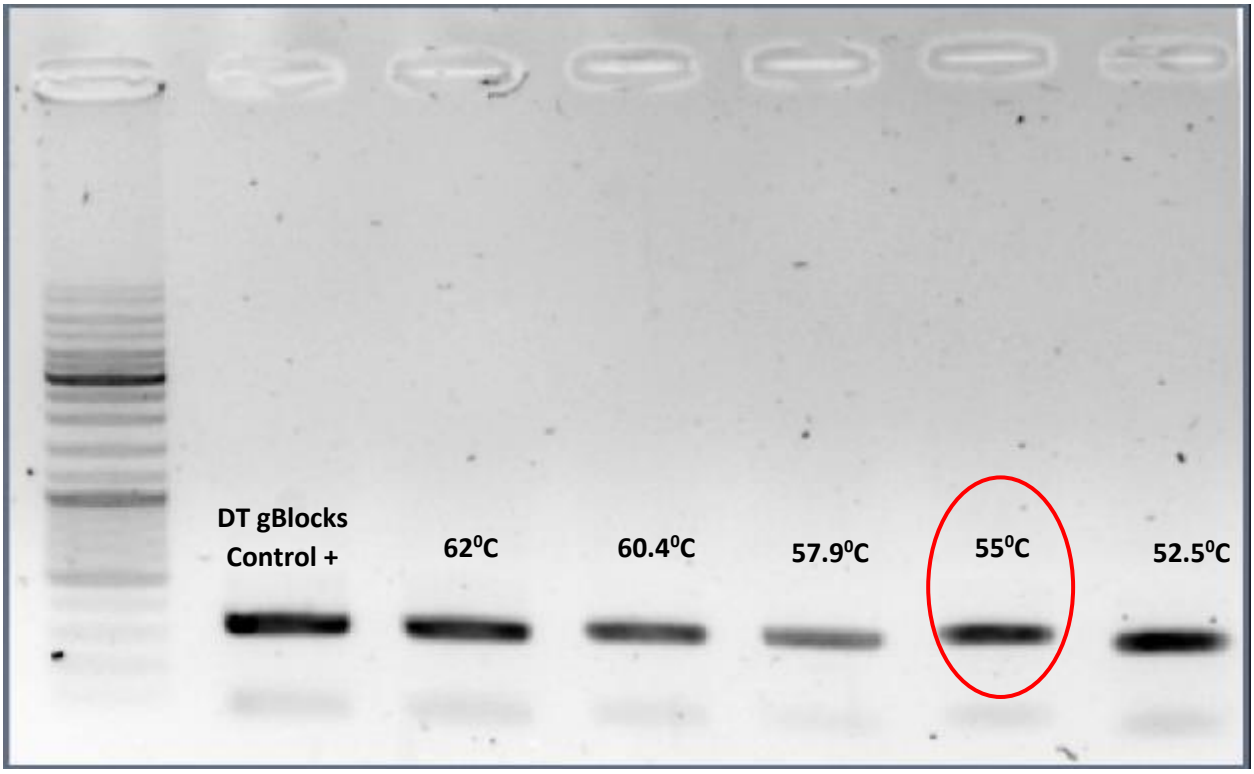
Materials	Volume
Phusion polymerase 10X	8.1 µl
DMSO 100% v/v	2.43 µl
Fwd Cloning primer 10 µM	4.05 µl
Rev Cloning primer 10 µM	4.05 µl
dNTP mix 800 mM	4.05 µl
5X HF buffer	16.2 µl
Nuclease free water	42.12 µl
Total	**81 µl**

Therefore, the master mix are then aliquoted into 9 PCR tubes @9 µl. One tube contained NFW as DiphTox template substitute as negative control, the others contained 1 µl of *DiphTox* gBlocks. Following tables represents temperature-time PCR formula for *Phusion* Hi-FI mix.

Process	Temperature	Time
Denaturation (Initial)	98^{0}C	3 mins
CYCLE 35X		

Denaturation	98^0C	30 s
Annealing	50^0C – 63^0C	30 s
Elongation	72^0C	1 min
Elongation (Last)	72^0C	7 mins

Following electrophoresis is done in agarose with composition of 0.8% agarose and 0.5X TAE. It runs within 100 V, 400 mA for 20 mins. Conclusion: Optimal annealing temperature is 55^0C.



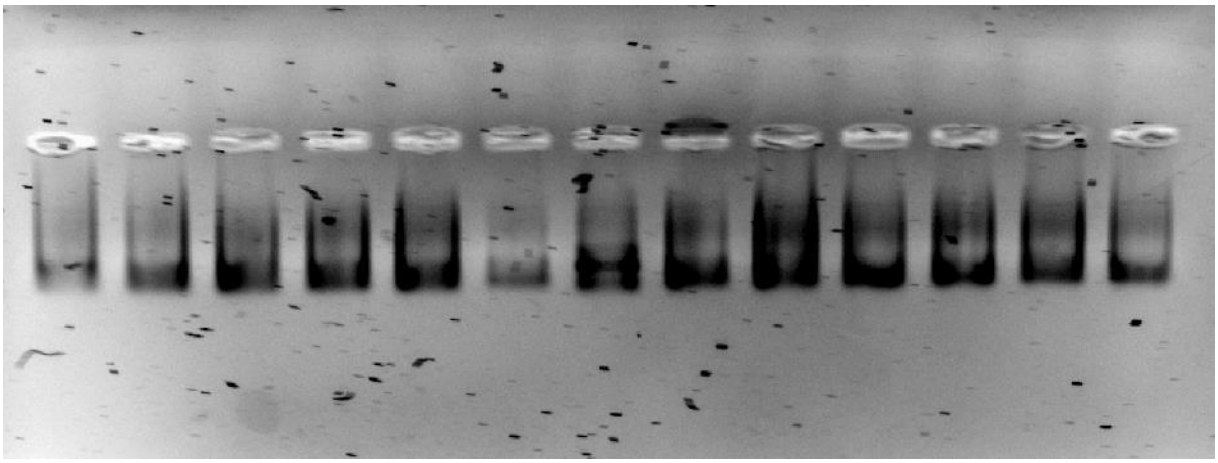
Thursday, 12 July 2018

PCR Amplification of *DiphTox* gBlocks

After optimizing the cloning primers, we directly amplified the template *DiphTox* into total of 15 tubes @9 µL with one negative control tubes. The following tables contains materials delighted for PCR master mix.

Materials	Volume
Phusion polymerase 10X	13.5 µl
DMSO 100% v/v	4.5 µl
Fwd Cloning primer 10 µM	6.75 µl
Rev Cloning primer 10 µM	6.75 µl
dNTP mix 800 mM	6.75 µl
5X HF buffer	27 µl
Nuclease free water	69.75 µl
Total	**135 µl**

The time formula for PCR reaction is the same with previous part for *Phusion polymerase* enzyme. The electrophoresis was done to confirm any amplified gBlocks. Purification of PCR products were done via spin column method. Nanodrop concentration of amplicons are 20.8 ng/µl with total volume of 32 µl. Conclusion: Further PCR could be done to amplify the gBlocks for biobrick digestion. Gel analysis shows that the amplicon bands corresponded with the DT size.



Monday, 16 July 2018

<u>*DiphTox* Prefix-Suffix Digestion</u>

Restriction digestion of *DiphTox* was done with the lab protocol using following mixtures to be inserted into pSB1C3 and pBluescript KS(-)

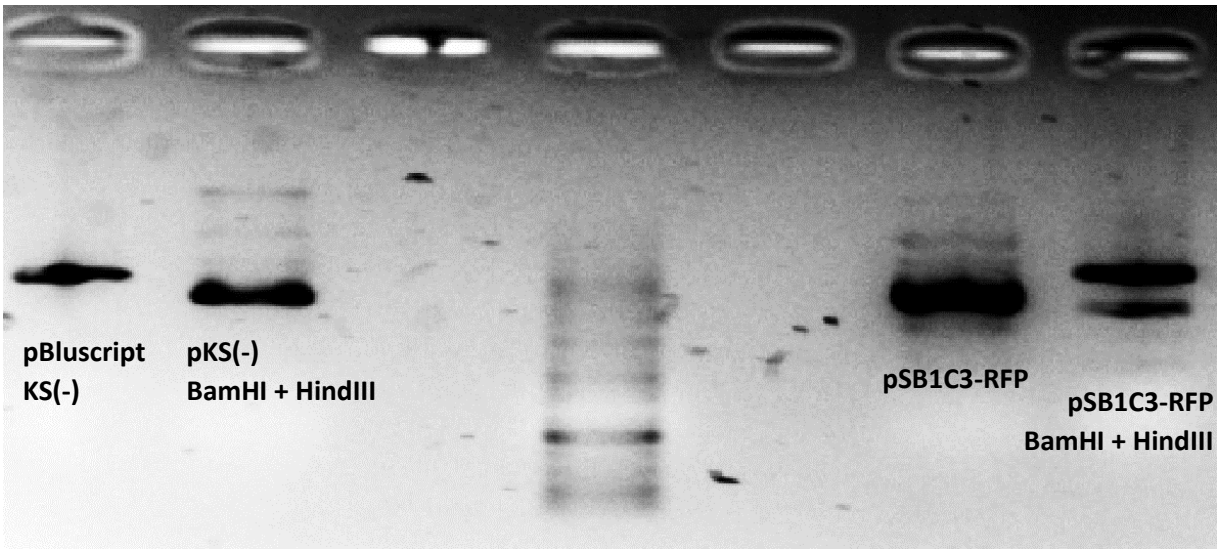
Materials	Volume
EcoRI Buffer 10X & Cutsmart Buffer	@15 µl
EcoRI/PstI & BamHI/HindIII	@*12* µl
BSA 10X	@15 µl
Template >10.000 ng	-
ddH_2O	-
Total	150 µl

<u>TABLE A.</u>

We have isolate pSB1C3-RFP as the main backbone for purpose of *red-white screening* and pBluescript KS(-) at amount of 151.2 ng/ µl and 167.3 ng/ µl. Since then, we purposely do digestion with minimum weight of 10.000 ng to prevent significant loss of result after digestion purification. Digestion are done

sequentially within 8 hours without any desalting in between. We also incubate *E. coli* containing pSB1C3 plasmids for further mini-isolation in 5 mL Luria Britani broth 37^0C, shaken well.

Therefore, we have obtained the result of electrophoresis as the following. Conclusion: the digestion was successful at first try.



Tuesday, 17 July 2018

DiphTox Amplification, Fresh Plasmid Isolation, and Purification of Digested Plasmid

PCR amplification of gBlocks *DiphTox* is obtained within the following results.

- Concentration of nanodrops: 78.1 ng/ µl
- $A_{260/280}$ = 1.85
- Total volume 50 µl

Conclusion: The gBlocks *DiphTox* could not yet be digested. Total weight has not achieved 10.000 ng.

Purification of digested plasmid using *low melting agarose* 1% failed due to lack of visible band during electrophoresis. Crystal violet 2 mg/ml was used as primary DNA staining.

Friday, 20 July 2018

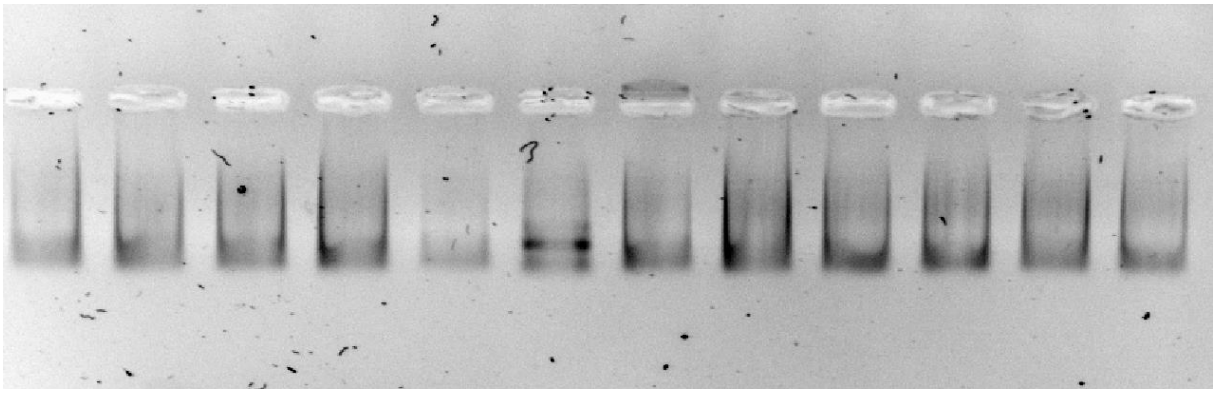
PCR Amplification

We try to further increase the yield of *DiphTox* amplicon up to 20 PCR tubes @9 µl. We obtained the following results.

- Concentration of nanodrops: 344.5 ng/ µl
- $A_{260/280}$ = 1.88
- Total volume 50 µl

Plasmid pSB1C3-RFP and pBluescript KS(-) were isolated from *E.coli* Top10 strain after overnight incubation. We obtain concentration of pSB1C3 and pKS(-) of 405.1 ng/µl and 289.1 ng/µl respectively.

We confirmed the amplicons and plasmids by running in the 0.5% agarose, TAE 0.5X, 100 V for 20 minutes and obtained the following results.



Monday, 23 July 2018

Fresh Plasmid Isolation

We isolated pSB1C3-RFP and pBluescript KS(-) from *E. coli* Top10 strain after overnight incubation to maintain minimum amount of 10.000 ng for digestion (this was done as alternatives after setbacks in the first restriction digestion). The obtained final concentration were 289 ng/ µl of pKS(-) and 441.2 ng/ µl of pSB1C3-RFP. The minimum amount vector requirement had not yet reached!

Tuesday, 24 July 2018

DiphTox amplification

Subsequent PCR amplification of *DiphTox* gBlocks had been done to require minimum concentration of restriction digestion of 10.000 ng. The result of purified PCR products in the end was 573.6 ng/ µl with absorbance 260/280 ratio of 1.47.

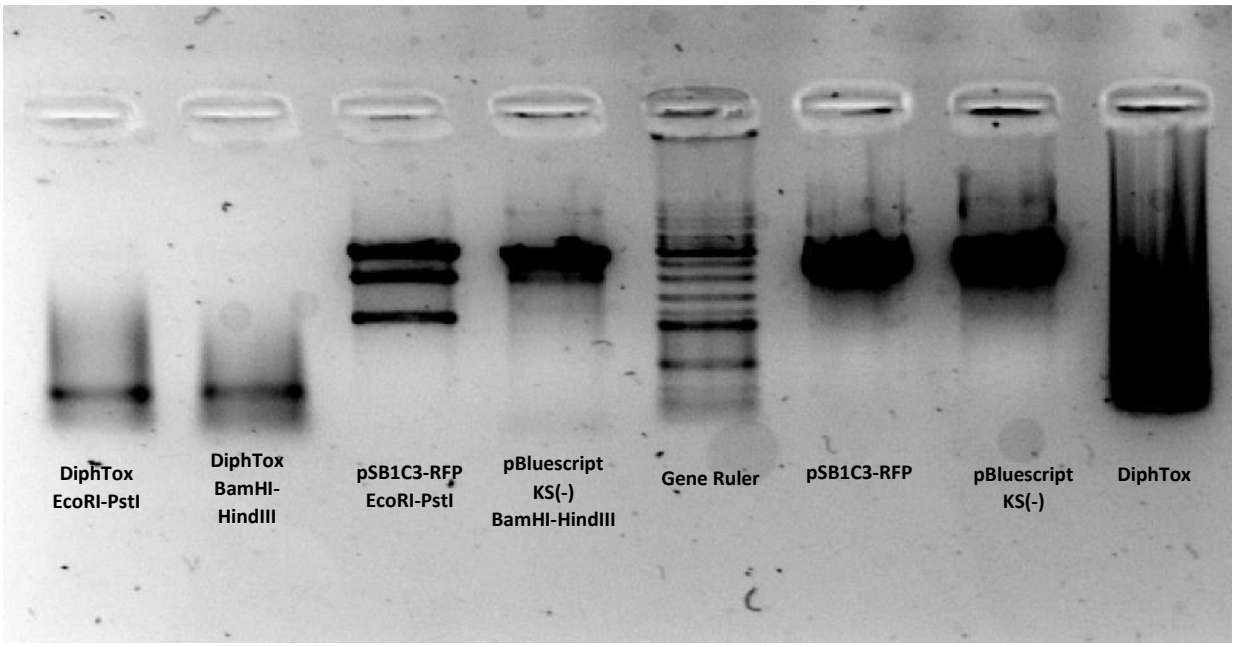
Wednesday, 25 July 2018

Second Vector-Insert Digestion and Purification + Fresh Plasmid Isolation

We isolated the plasmids early in the morning to finally achieve the requirement of plasmids amount using mini-prep methods. The obtained concentration were 117.1 ng/µl of pKS(-) and 151.9 ng/ µl for pSB1C3.

We did sequential digestion within 8 hours by EcoRI/PstI enzyme for pSB1C3 and BamHI/HindIII enzymes for pKS(-). *Low melting agarose* electrophoresis was done by using 1% agarose and 0.5X TAE with *crystal violet* 2 mg/ml. Conclusion: The purification is done by gel cut methods and QiaEX II DNA absorbent resins. The final digestions concentration were summarized in the following tables. Confirmation of gel electrophoresis in agarose 0.5% are documented in the below section.

Samples	**Concentration (ng/ µl)**	**$A_{260/280}$**
DiphTox EcoRI/PstI	19.1	1.83
DiphTox BamHI/HindIII	18.2	1.23
pSB1C3 EcoRI/PstI	37.8	1.34
pKS (-) BamHI/HindIII	22.2	1.29



Ligation was conducted at evening 5 pm for overnight incubation at 16^0C. The formula is stated within below table. The molar ratio between insert and vector is 3:1.

DiphTox (AT) *inserts*	3.97 µl	*DiphTox* (AT) *inserts*	5.41 µl
pKS (-) *digested* (200 ng)	9 µl	pSB1C3 *digested* (200 ng)	5.29 µl
T4 ligase	1 µl	T4 ligase	1 µl
T4 ligase buffer 10x	2 µl	T4 ligase buffer 10x	2 µl
Nuclease free water	4.03 µl	*Nuclease free water*	6.3 µl
Total	20 µl	**Total**	20 µl

Thursday, 26 July 2018

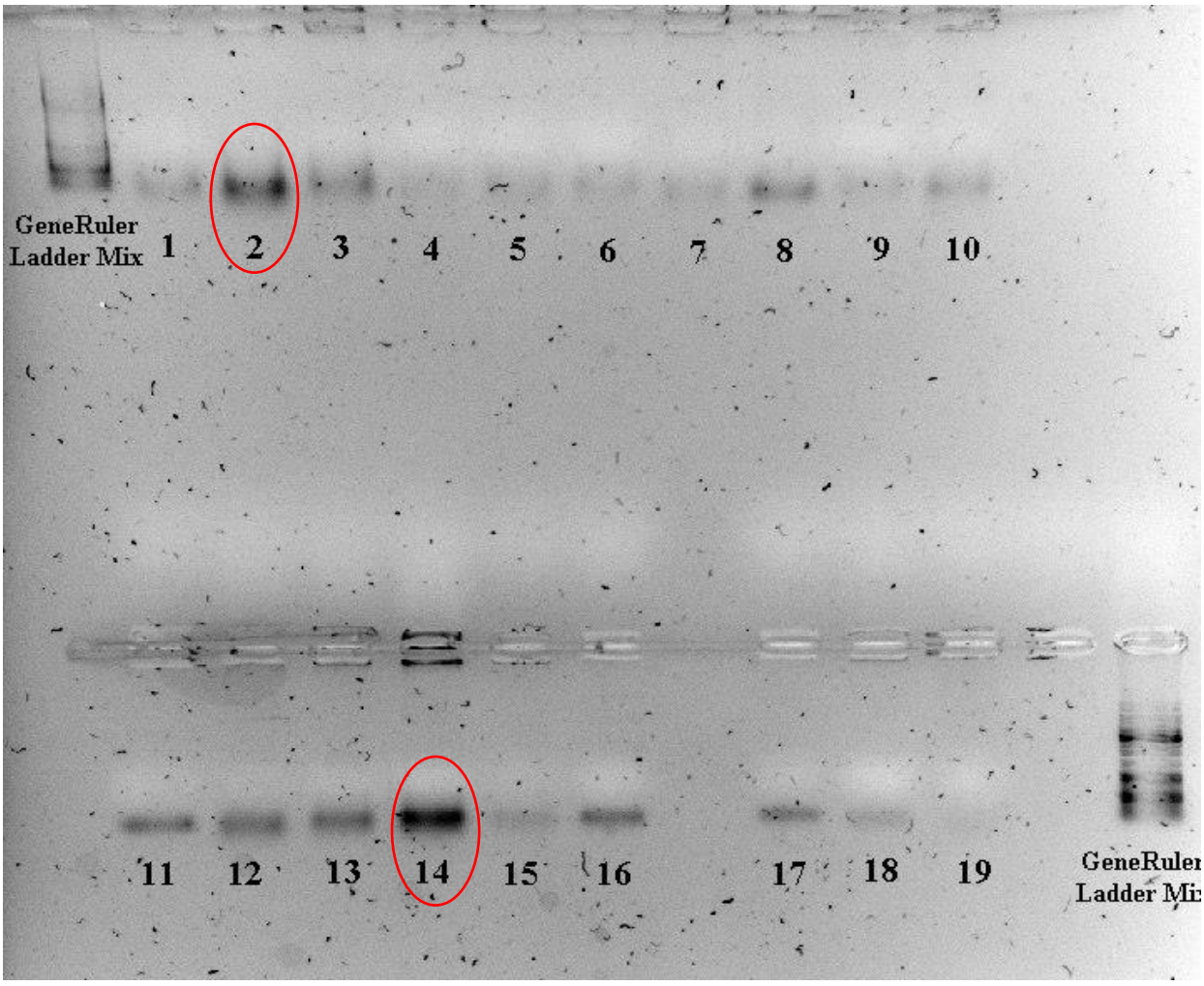
Transformation of Ligated Products

We conducted transformation of *E. coli* WT(-) Top10 by using the ligated products of the predicted pSB1C3-DT and pKS(-)-AT. The results of transformation was done within Luria Broth agar + Chloramphenicol (Cam) antibiotics 25 mg/ml in 1:1000 volume ratio. The transformation protocol could be referred to the protocol page.

Friday, 27 July 2018

PCR colony pSB1C3-DT and pKS(-)-AT

PCR colony could be conducted by picking colonies that were not producing red colours (suspected for pSB1C3 autoligation or pSB1C3-DT), as well as pKS(-)-AT randomly. Picked colonies then were resuspended into aliquoted PCR master mix and replicated by streaking into new LB+Cam agar. PCR tooks for 3 hours and run into gel electrophoresis, as well as overnight incubation of streaked replicas. The results were as following. Conclusion: SUSPECTED for any inserted *DiphTox* inside pSB1C3!



Monday, 30 July 2018

YEAY, the next gBlocks HBEGF-Tar (HT) had arrived in the lab!

Wednesday, 1 August 2018

Primer HT-1 and HT-2 Optimization

We resuspend the HT-1 and HT-2 gBlocks by TE buffer pH 8.0 after prolonged centrifugation 3000 rpm for 5 minutes, yielding the concentration up to 10 ng/µl. Direct amplification of the HT-1 or HT-2 were done by PCR using the 'HT-1' and Fwd Uni PCR colony' or 'HT-2' and 'Rev Uni PCR colony' primers respectively. Primers were resuspended in TE buffer pH 8.0 as well up to 500 µM. Subsequent dilution of primers into 10 µM of working primer solution using nuclease free water were done to prevent cross-contamination.

Following tables are the reaction master mix for total of 9 tubes @9 µl.

Materials	Volume
GoTaq Long PCR mix 2X	40.5 µl
HT-1 primer 10 µM	4.05 µl

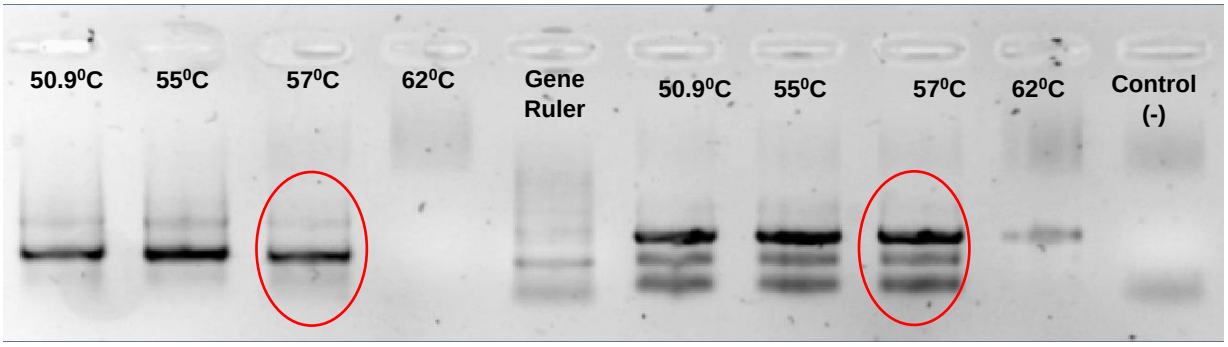
Rev Uni PCR colony primer 10 µM	4.05 µl
Nuclease free water	32.4 µl
Total	**81 µl**

Materials	Volume
GoTaq Long PCR mix 2X	40.5 µl
HT-2 primer 10 µM	4.05 µl
Fwd Uni PCR colony primer 10 µM	4.05 µl
Nuclease free water	32.4 µl
Total	**81 µl**

Therefore, the master mix are then aliquoted into 9 PCR tubes @9 µl. One tube contained NFW as template substitute as negative control, the others contained 1 µl of HT-1 gBlocks. Following tables represents temperature-time PCR formula for GoTaq Long PCR mix.

Process	Temperature	Time
Denaturation (Initial)	95^{0}C	2 mins
CYCLE 35X		
Denaturation	94^{0}C	30 s
Annealing	50^{0}C – 63^{0}C	30 s
Elongation	72^{0}C	1 min
Elongation (Last)	72^{0}C	10 mins

Following electrophoresis is done in agarose with composition of 0.8% agarose and 0.5X TAE. It runs within 50 V, 400 mA for 60 mins. Conclusion: Optimal annealing temperature is 57^{0}C.



Thursday, 2 August 2018

SDS-PAGE and Purification of *DiphTox* in pKS(-)-AT Top10 *E. coli*

Methods for SDS-PAGE could be accessed in the protocol page. We ought to confirm any expression of *DiphTox* proteins, located either intracellularly or extracellularly. In this step, we used Promega Magne-His purification system for *DiphTox* protein. Both lysate and supernatant were purified using Magne-His beads for identification of *DiphTox* location. The analysis was done using polyacrylamide gel 10% with 1X SDS buffer solution, and shaked with PageBlue solution overnight in room temperature.

Conclusion: The SDS-PAGE gel was accidentally spilled and torn apart next morning, yet it was considered as fail results.

Monday, 6 August 2018

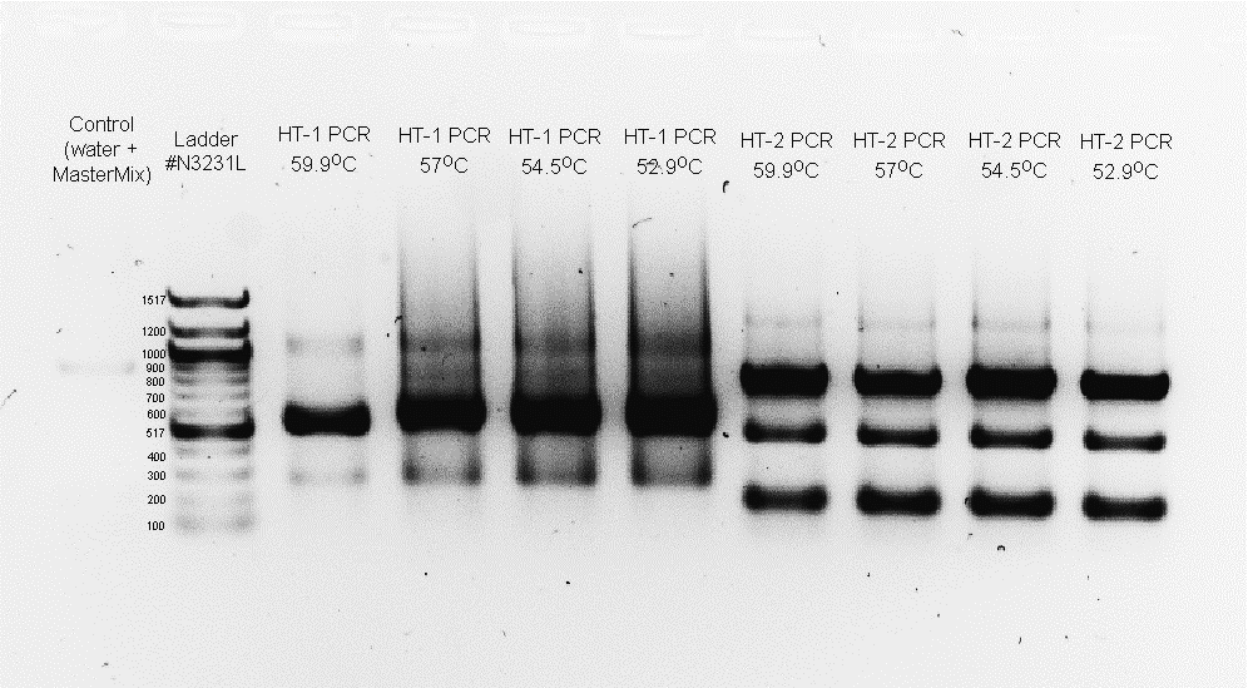
PCR amplification of HT-1 and HT-2

After optimizing the HT-1 and HT-2 primers, we directly amplified the templates HT-1 and HT-2 into total of 9 tubes @9 µl with one negative control tubes. The following tables contains materials delighted for PCR master mix.

Materials	**Volume**
GoTaq Long PCR mix 2X	45.5 µl
Fwd Primer cloning 10 µM	4.55 µl
Rev Primer cloning 10 µM	4.55 µl
Nuclease free water	36.4 µl
Total	**91 µl**

TABLE B.

The time formula for PCR reaction is the same with previous part for *GoTaq polymerase* enzyme with annealing temperature of 52.5^{0}C. The electrophoresis was done to confirm any amplified gBlocks. Purification of PCR products were done via spin column method. Conclusion: Several bands appear in one type of PCR template → suspect any non-specific primer annealing.



Thursday, 16 August 2018

Fresh Plasmid Isolation

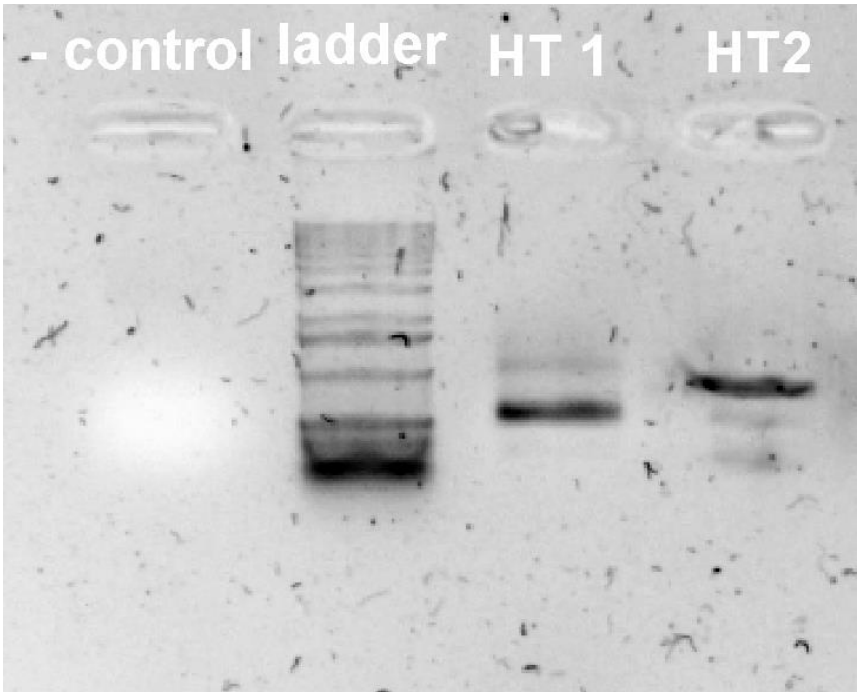
We planned to amplify the amount of pQE80L plasmids inside *E. coli* Top10 strain. Therefore, the *E. coli* containing pQE80L plasmids are incubated within 37^{0}C for 4 hours prior to mini-prep isolation. We

obtained 95 ng/µl with ratio 260/280 of 1.92. Conclusion: The amount of plasmid was enough for EcoRI/PstI restriction digestion.

Monday, 20 August 2018

Fresh Plasmid Isolation, Restriction Digestion, and PCR Amplification

We had managed to further amplify HT-1 and HT-2 fragments by using GoTaq Promega Long PCR mix conformed with the previous formulation (see table B). Gel analysis was done to do final confirmation either the primer was unspecific or that cross-contamination happened. Below are the results of gel agarose 1%, TAE1x, 50 V, 70 mins. Conclusion: The primers was definitely unspecific, causing amplification of HT-1 and HT-2 fragments to be impossible. Ordering primers directly from IDT would consume a month of effective research.



Restriction digestion using the same formula as **TABLE A**. Minimum concentration of templates were 1 µg. After 8 hours incubation in 37^0C, the reactions were then arrested in *heatblock* 80^0C for 15 minutes prior to storage in -20^0C. *Low melting agarose* purification could not be completed on that day since the lab closed early.

Plasmid isolation of pQE80L was finished to provide backups of plasmid stocks. The obtained concentration was 155.2 ng/ µl with ratio of 1.89 purity.

Tuesday, 21 August 2018

Purification of Digested pQE80L and Ligation.

Low melting agarose 1%, TAE 0.5x was conducted to purify the digested samples of plasmids pQE80L. Gel was cut within UV sight after the DNA was submerged into gel red solution for 1 hour. Gel was then purified using QiaEX II beads and obtained the concentration of 21.2 ng/ µl with ratio of 1.88 purity. Conclusion: the digested plasmid (linearized) was successfully purified. Ligation of pQE80L and *DiphTox*

was done at the evening by using vector: insert ratio of 1:3, as following. The reaction mix were incubated in 16^0C overnight.

DiphTox (AT) *inserts*	2.4 µl
pQE80L (-) *digested* (200 ng)	9.5 µl
T4 ligase	1 µl
T4 ligase buffer 10x	2 µl
Nuclease free water	5.1 µl
Total	20 µl

TABLE C.

We managed to ask iGEM-NTU Singapore for assistance in ligating linear fragment of HT-1 and HT-2 into single HT complete gBlocks. We sent the samples to Singapore, and they received the job.

Thursday, 23 August 2018

BFP Construct Transformation and Primer Optimization

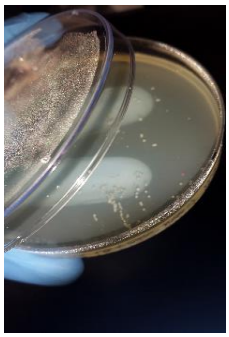
We try to transform the plasmids pSB1C3 in the distribution kit containing *blue fluorescence protein* (BFP) into *E. coli* BL21 strain. The transformed bacteria were then spread into LB agar plate + chloramphenicol antibiotics. After transformation, the spread bacteria were incubated in 37^0C overnight for future selection by PCR colony.

Not only transformation, we also divided our jobs by doing primer optimization of VF2 and VR (i.e. standard biobrick primer in pSB1C3). Specification of PCR formulation were described as the following tables (with total reaction of 9 tubes @9 µl, including one negative control). The time formula for PCR reaction is the same with previous part for *GoTaq polymerase* enzyme with graded annealing temperature of 50^0C-65^0C. Conclusion: The optimal annealing temperature of the primer were 51^0C.

Materials	**Volume**
GoTaq Long PCR mix 2X	45.5 µl
VF2 primer 10 µM	4.55 µl
VR primer 10 µM	4.55 µl
Nuclease free water	36.4 µl
Total	**91 µl**

Friday, 24 August 2018

PCR Colony Confirmation of Transformed BFP



PCR colony was conducted by picking colonies that were not producing red colours (suspected for pSB1C3 autoligation or pSB1C3-BFP). Picked colonies then were resuspended into aliquoted PCR master mix and replicated by streaking into new LB+Cam agar. PCR tooks for 2 hours 50 minutes and run into gel electrophoresis, as well as overnight incubation of streaked replicas. Conclusion: the results could not be observed, since the *GelDoc* machine was broken and need to be serviced at that day.

Tuesday, 28 August 2018

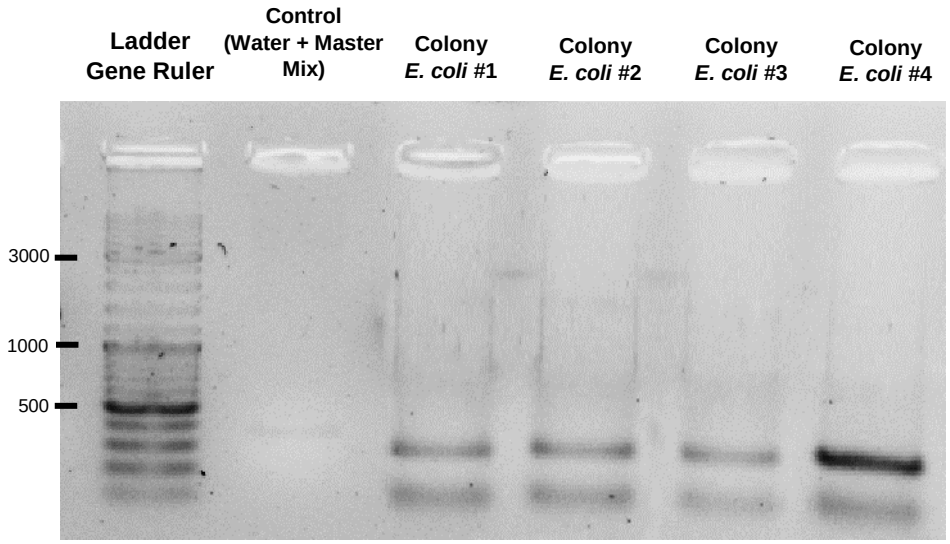
PCR Colony Confirmation of Suspected pSB1C3-DT and Plasmids Digestion

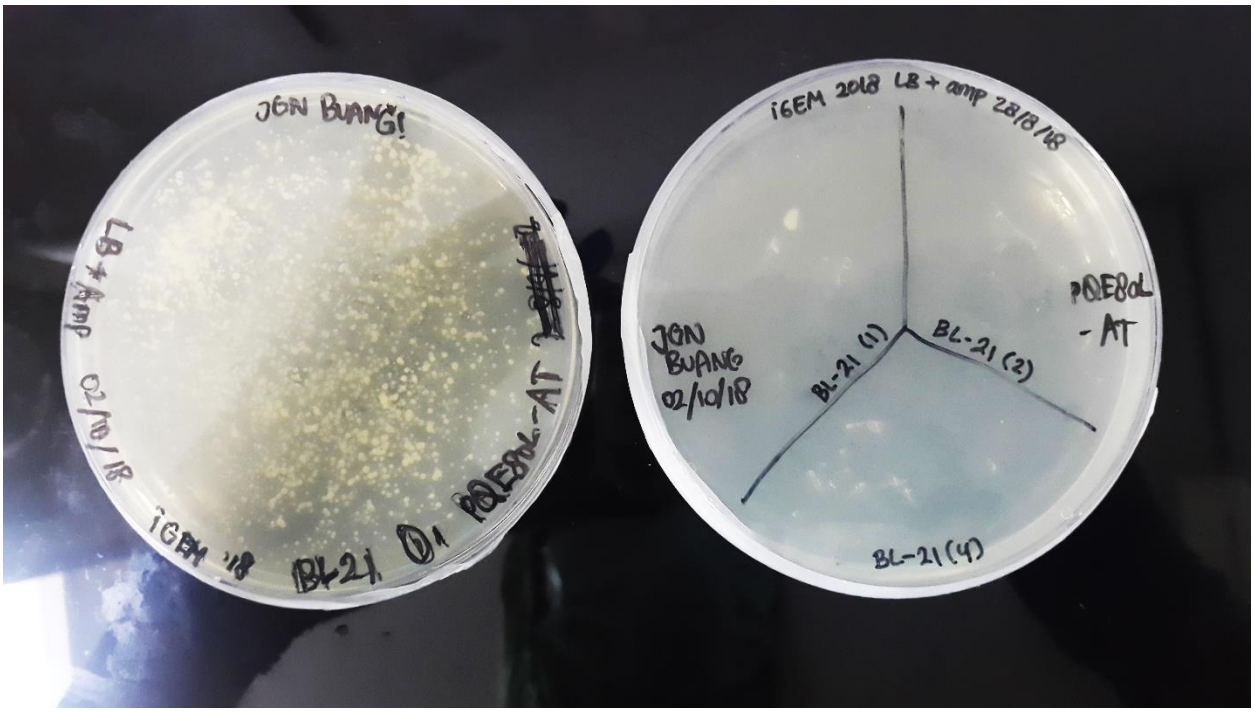
To reconfirm the inserted pSB1C3-DT at the beginning, we do PCR colony based on using VR and VF2 primers, reinforced with Fwd Uni PCR colony and Rev Uni PCR colony results. While waiting for the PCR, we do pSB1C3 and pQE80L isolation to maintain minimum yield for restriction digestion using EcoRI/PstI enzyme couples.

We also modified the time formula for this PCR, in order to increase the expected PCR yield. Check the following tables.

Process	Temperature	Time
Denaturation (Initial)	95^0C	2 mins
CYCLE 35X		
Denaturation	94^0C	30 s
Annealing	50^0C – 63^0C	30 s
Elongation	72^0C	1 min 30 s
Elongation (Last)	72^0C	10 mins

The electrophoresis were then described in the following figures. Conclusion: the pSB1C3-DT was misinterpreted since one month ago, and that described the incorrect size of whole plasmid during electrophoresis, and PCR confirmation shows the size of 300s bp band, instead of 600s.





Wednesday, 29 August 2018

PCR Amplification *DiphTox*, Digested Plasmid Purification, and Ligation

We do PCR amplification from the beginning again with samples stocks from IDT. However, this time, we modified the elongation time from 1 min 30 s to 2 min 10 s. The reason is that longer time would enable polymerase to elongate the template in higher yield. The formula for PCR was the same with **TABLE B** using Fwd and Rev cloning primers. The obtained concentration was 227.6 ng/µl with ratio of 1.88.

Low melting agarose 1% purification are done within pQE80L and pSB1C3-RFP after 8 hours of sequential EcoRI/PstI digestion. The obtained concentration was 5.9 ng/ µl of pSB1C3 and 37 ng/ µl of pQE80L. The small yield of pSB1C3 could not be used in ligation, causing maximum reaction of ligation could be surpassed. Therefore, the ligation could only be done for pQE80L and *DiphTox* with the same formula as **TABLE A.**

Thursday, 30 August 2018

Transformation of pQE80L-DT Ligation

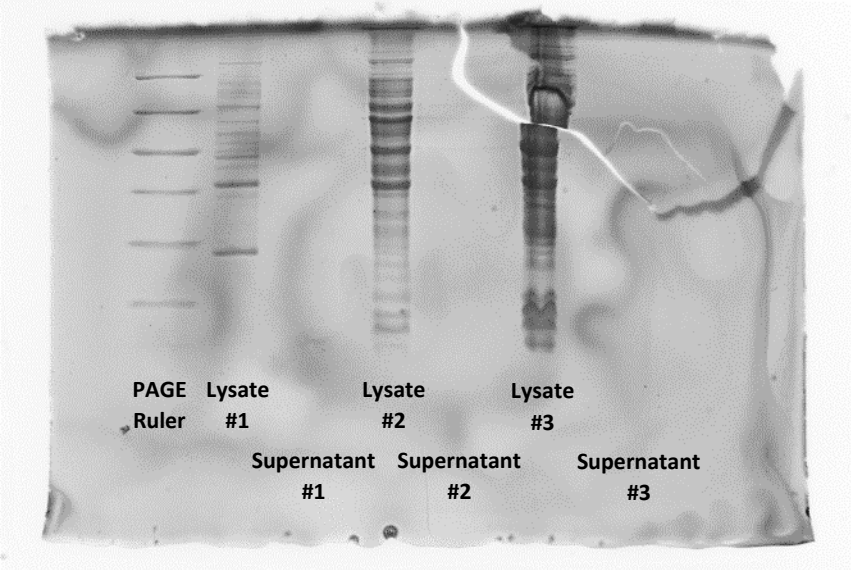
Full day transformation was done complied to the mentioned protocol in other pages. YEAY, iGEM-NTU has already sent back the ligated linear gBlocks of HT-1 and HT-2 inside pcDNA3. The samples were resuspended immediately with 30 µl of 1/3 elution buffer, and obtained 384 ng/µl concentration.

Friday, 31 August 2018

SDS PAGE pQE80L-DT

To fully characterize the DiphTox modified protein, we need to do specific localization of the protein. Localization could be determined by SDS-PAGE of lysate and or supernatant. SDS PAGE method could be accessed via other pages in our website. The lower gel concentration was 10% polyacrylamide, and upper gel of 4%. Fresh poured APS and TEMED are extremely important for rapid polymerization of the gel. Therefore, the SDS page resulted within following figure.

Conclusion: SDS PAGE were torn during process, but we also could not find any specific thickened protein bands in the triplo of pQE80L-DT. Next steps: we need to run it one more time!



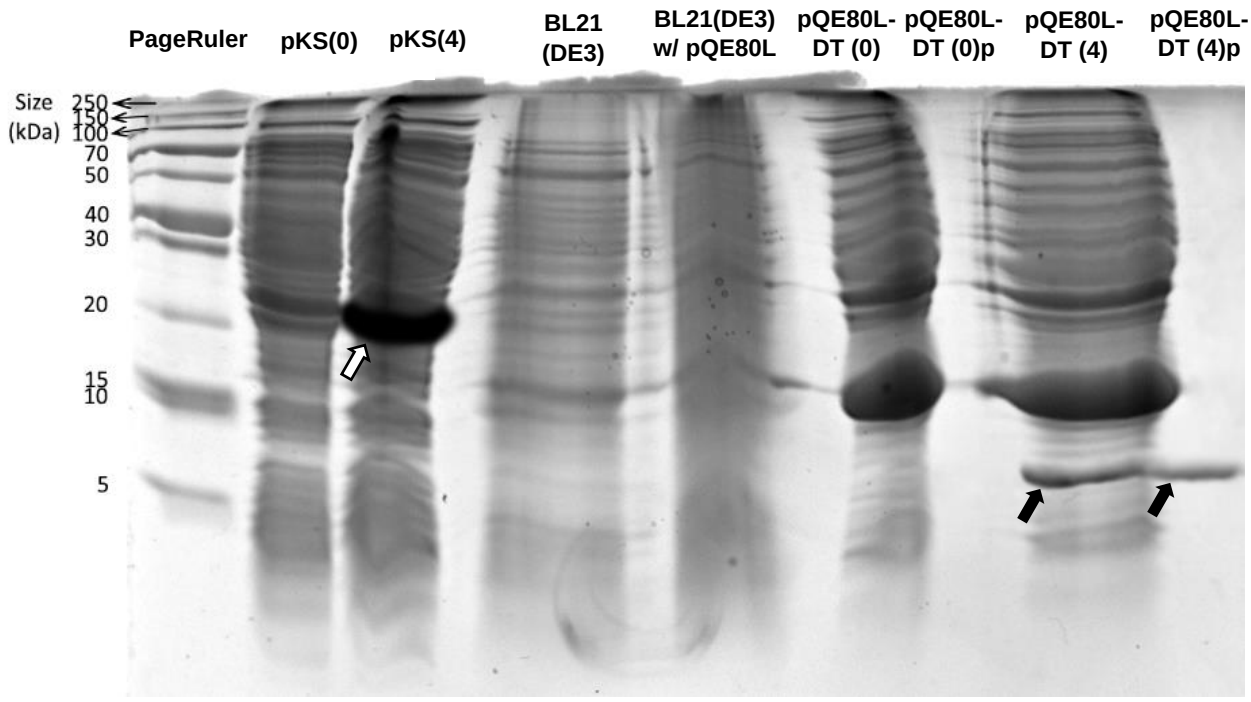
Monday, 03 September 2018

SDS PAGE Cont'd pQE80L-DT

Our lab team try to use fresh IPTG solution from the powder since we suspected that the IPTG has expired. Control positive of the SDS-PAGE were *E. coli* containing pBluescript KS(-) induced with IPTG for 4 hours. Negative controls comprises of BL21 wild type or ampicillin resistant *E. coli*.

SDS-PAGE analysis (photographed via *ImageQuant*) of pQE80L-DT expression in *E. coli* BL21(DE3). White arrow indicates LacZα (size ~20.7 kDa) protein expression due to IPTG induction, indicating our IPTG was in good condition. On the other hand, black arrow shows DT (size ~7 kDa) protein expression as it is induced with IPTG within 4 hours. YEAY, successful expression of DiphTox!

Note*:* pKS(0) = *E. coli* TOP10 transformed with pBluescript KS(-) with no IPTG induction; pKS(4) = *E. coli* TOP10 transformed with pBluescript KS(-) after 4 hours of IPTG induction in 37^0C; BL21(DE3) = wild-type *E. coli* BL21(DE3); BL21(DE3) w/ pQE80L = *E. coli* BL21(DE3) containing empty pQE80L; pQE80L-DT(0) = *E. coli* BL21(DE3) containing recombinant pQE80L-DT with no IPTG induction; pQE80L-DT(0)p = purified protein of *E. coli* BL21(DE3) containing recombinant pQE80L-DT with no IPTG induction; pQE80L-DT(4) = *E. coli* BL21(DE3) containing recombinant pQE80L-DT with 4 hours of IPTG induction; pQE80L-DT(4)p = purified protein of *E. coli* BL21(DE3) containing recombinant pQE80L-DT after 4 hours of IPTG induction.



Tuesday, 11 September 2018

Fresh Plasmid Isolation

Plasmid stocks was getting depleted. We managed to isolate more pSB1C3-RFP for *DiphTox* vector, and finally obtained 251.6 ng/µl from Top10 strain *E. coli* incubated overnight within 50 mL of LB (*Luria-Brittani*) solution. These plasmids are going to be used in restriction digestion process later.

Wednesday, 12 September 2018

Restriction Digestion of DiphTox and pSB1C3

The digestion formula does not differ from the previous **TABLE A.** with 10.000 ng minimum weight. Sequential digestion of EcoRI and PstI into pSB1C3 was done within 8 hours parallel. Subsequent purification of *low melting agarose* 1% was conducted that day to purify the digested fragments. (i.e. LMA 1%, TAE 0.5X, 50 V for 60 mins). DNA purification of LMA could be referred to the method page of DNA purification. Our team obtained the following results, consisting of 13.4 ng/µl of DiphTox fragments and 13.3 ng/ µl of pSB1C3

Therefore, we could proceed to overnight ligation with the same previous method within **TABLE C.** in 16^0C.

Thursday, 13 September 2018

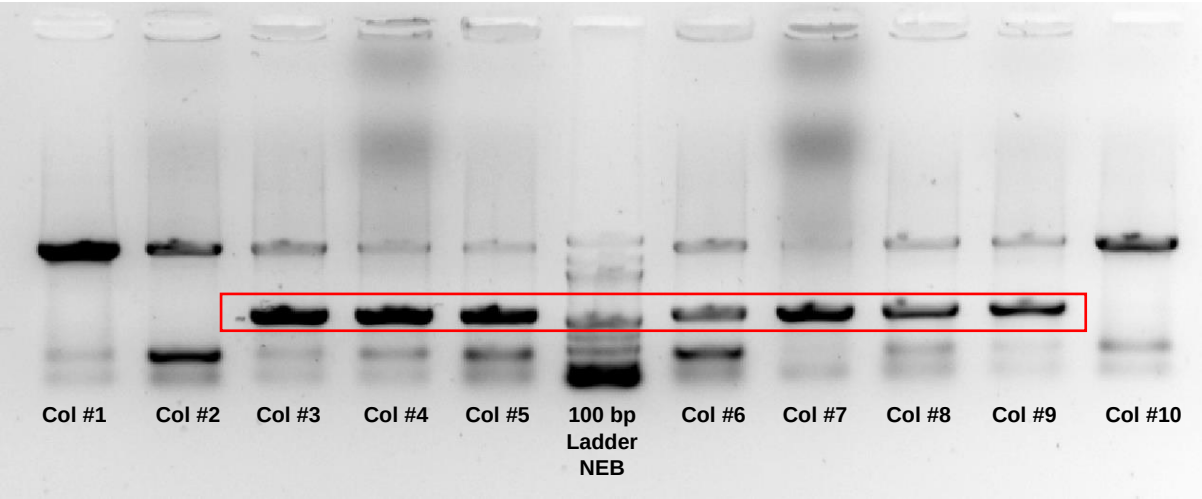
Transformation of pSB1C3-DiphTox (DT) and pcDNA3-HT

Following transformation was done according to the method of Transformation in the other page. We have managed to make negative control on LB agar containing Chloramphenicol antibiotics with wild type *E. coli*. The transformation of pcDNA3-HT into *E. coli* K-12 strain could be conducted for subsequent fragments replication with ampicillin antibiotics as selective substance.

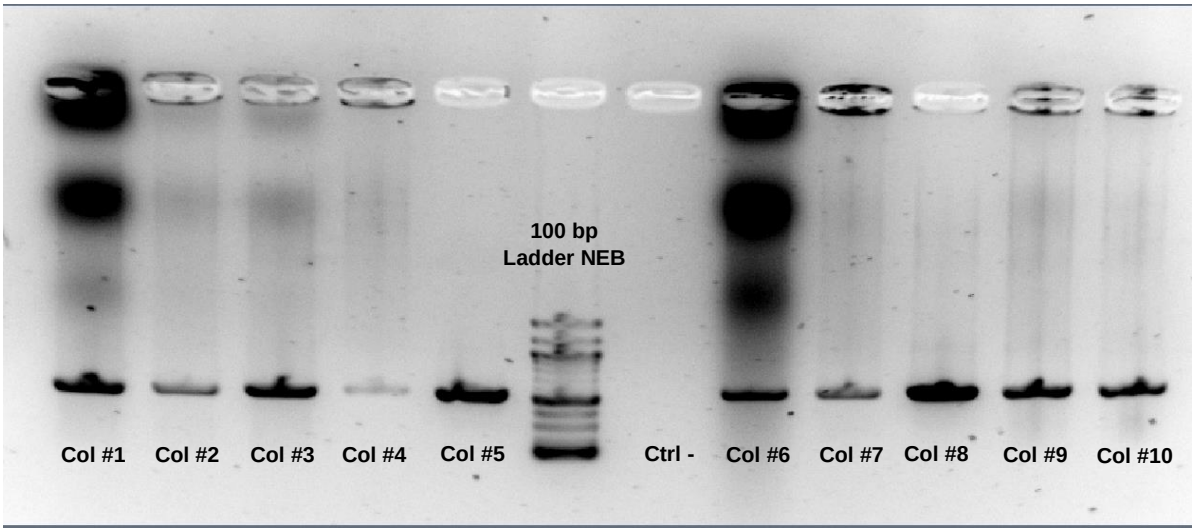
Monday, 17 September 2018

PCR Colony pSB1C3-DT and pcDNA3-HT

From all of the colony formed within LB agar media and antibiotics Chloramphenicol, we screened up to 40 colonies pSB1C3-DT using PCR colony consisting of VR and VF2 universal primer for validation of insertion. The following PCR colony complies to the results. Conclusion: we found several colonies with DiphTox inserts, but the colonies seems to be mixed up with non-complied plasmids pSB1C3. Thus, we need to do spreading methods to specify the colonies into one single type of plasmid.



Additionally, there are only five colonies found in the agar containing ampicillin. Therefore, we do subsequent PCR colony on those colonies with HT-Com primer specially designed for confirmation of HT fragment existence. Conclusion: pcDNA3-HT is confirmed in the *E. coli* Top10.



Tuesday, 18 September 2018

Spreading Selection of Mixed Colonies

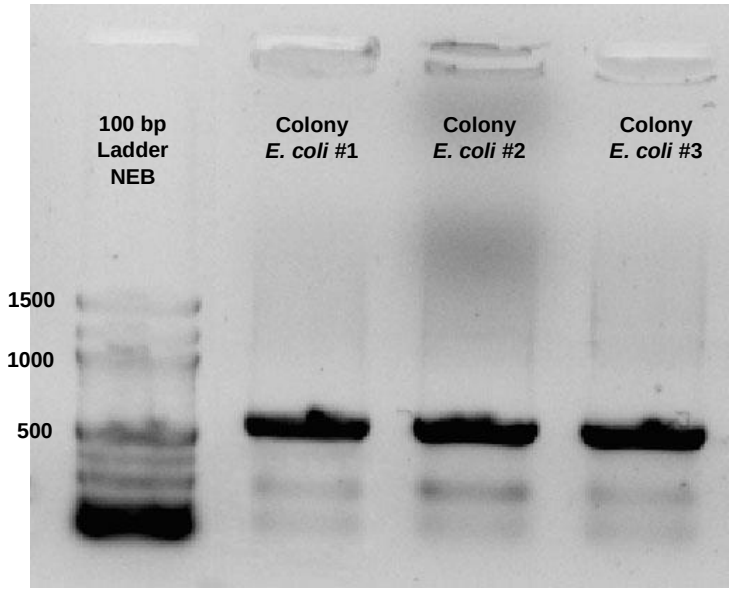
We did subsequent dilution on the suspected colonies containing pSB1C3-DT. The method was complied to the steps elaborated in the InterLab measurement, specifically in handling for the OD600 absorbance fo measuring bacteria concentration in LB solution. The factor of dilution consists of 10^8 and 10^7 of the first

streak from agar plate, dipped into LB + chloramphenicol solution. We did overnight incubation of the culture in 37^0C.

Wednesday, 19 September 2018

PCR Colony of pSB1C3-DT

The method of PCR colony is the same within the previous one. Unfortunately, we only obtain three colonies representative in the whole agar plate. We did not expect that the dilution factor is too high for such initial number of bacteria available from the beginning. Nevertheless, we still do PCR confirmation on that only colony. The following results of PCR colonies was being compared to the negative control containing pSB1C3-RFP. Conclusion: the only colonies contain the expected pSB1C3-DT pure! NICE!



Thursday, 20 September 2018

pcDNA3-HT and Improved BFP gBlocks Restriction Digestion

Both pcDNA-HT and BFP gBlocks are acquired in minimum amount for restriction digestion using EcoRI and PstI. Linearized plasmid backbone of pSB1C3 and pQE80L are adequate for direct ligation. Therefore, the formula for restriction digestion complies within following tables.

Materials	**Volume**
EcoRI Buffer 10X	@15 µl
EcoRI/PstI	*@6* µl each sequential
BSA 10X	@15 µl
Template >10.000 ng	pcDNA3-HT (75 µl) and BFP (84 µl)
ddH_2O	*Rest of the volume*
Total	150 µl

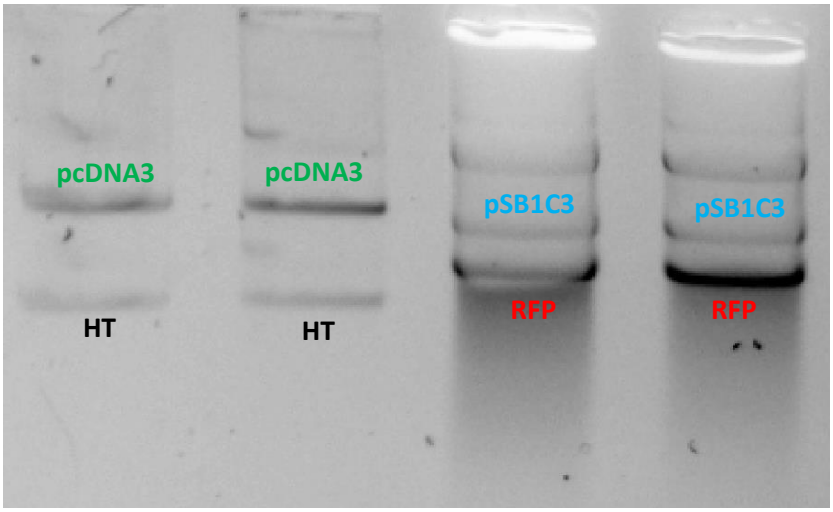
Since the digestion took for 8 hours, we stopped the reaction in that day using *heatblock* 80^0C for 15 mins after each 4 hours sequential digestion.

Friday, 21 September 2018

Low Melting Agarose Purification

We modified several methods in the purification technique to obtain optimum concentration for ligation. Since LMA purification yield such little amount of purified digested DNA, we add *EcoDye* and Loading Dye 6X into the agar and DNA samples respectively, alternatively using *crystal violet* loading dye, prior to well loading. Prior to direct gel purification, we did pre-desalting to purify the DNA from restriction enzyme

or buffer as it would interfere the migration of the DNA in the gel (protein and DNA cooperation would cause smear effect on gel electrophoresis).



The obtained concentration of the digested purifications are 10.3 ng/ µl for HT fragments and 18.3 ng/ µl for BFP. Since then, we could proceed to the next steps of ligation with the following formula.

HB-EGF/Tar (HT) *inserts*	9.3 µl	HB-EGF/Tar (HT) *inserts*	12.7 µl
pQE80L (-) *digested* (200 ng)	8.2 µl	pSB1C3 (-) *digested* (200 ng)	11.3 µl
T4 ligase	1 µl	T4 ligase	1 µl
T4 ligase buffer 10x	2 µl	T4 ligase buffer 10x	2 µl
Nuclease free water	0 µl	*Nuclease free water*	0 µl
Total	20.5 µl	**Total**	27 µl

Improved BFP *inserts*	16.5 µl
pSB1C3 (-) *digested* (200 ng)	11.3 µl
T4 ligase	1 µl
T4 ligase buffer 10x	2 µl
Nuclease free water	0 µl
Total	30.8 µl

The ligation is left overnight until next week in 16^0C to increase chance of successful ligation.

Monday, 24 September 2018

Transformation of pSB1C3-BFP, pSB1C3-HT, and pQE80L-HT into *E. coli*

The transformation procedure could be referred to the method of transformation in other pages.

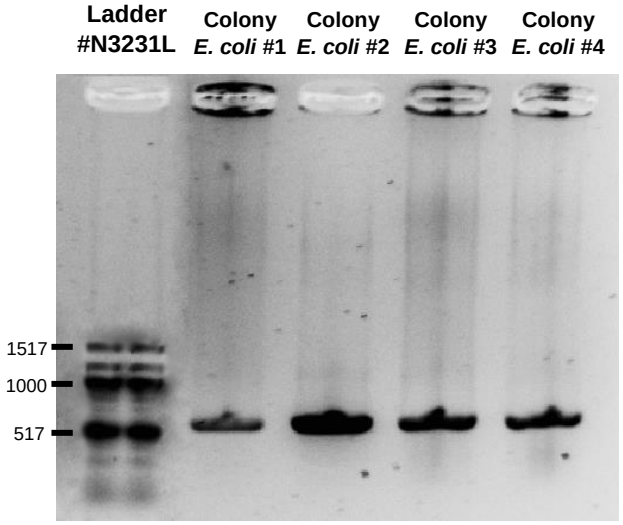
Tuesday, 25 September 2018

PCR Colony Confirmation

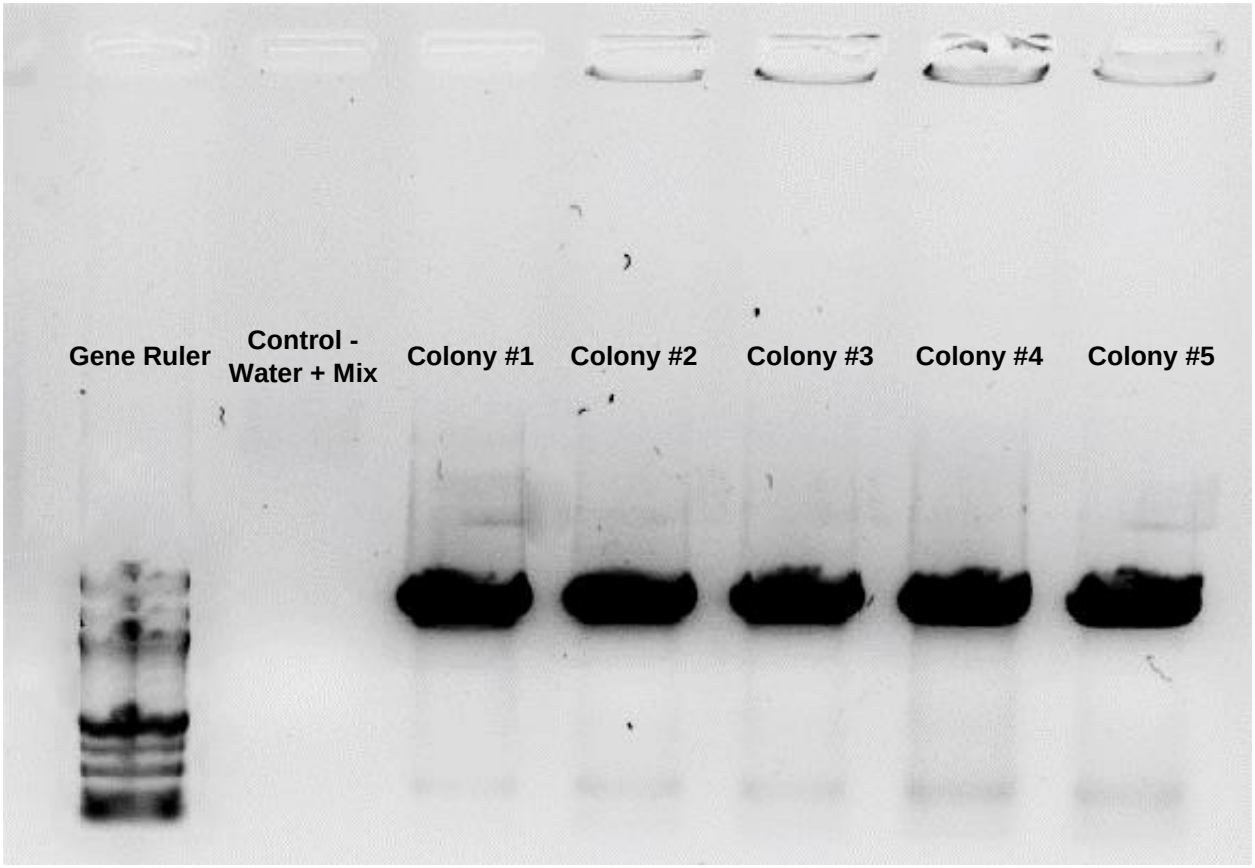
The PCR colony confirmation of pSB1C3-BFP could be done in 5 colonies that appear on the agar plate after being incubated overnight. However, the pSB1C3-HT colonies did not grow at all in plates containing

chloramphenicol; thus, PCR colony could not be conducted for pSB1C3-HT. The PCR colony for pQE80L can be done by screening several colonies by using specific HT-com primer for confirmation.

The gel analysis result of PCR colony could be explained in the following figure.



Conclusion: The BFP inserts inside pSB1C3 could be interpreted as 1000 bp fragments under VR and VF2 primer amplification. This indicates that BFP could be analyzed further. Additionally, we also obtained the PCR colony confirmation of HT fragments inside pQE80L. NICE news for everyone in the lab that we could continue to the binding and expression assay ☺! However, the HT fragments must be re-digested to be inserted into pSB1C3 for submission.



Wednesday, 26 September 2018

Re-digestion of pcDNA3-HT and pSB1C3

Our team brainstorms that the high chance of HT fragment cloning failure into pSB1C3 is that the biobrick might be incompatible within traditional cloning since both pSB1C3 and HT fragment have the similar length and size. We tried to increase the ratio molar of insert : vector into 4:1, but the available linear plasmid backbone was limited to next ligation, as well as HT fragments, so we tried to re-digest with EcoRI and PstI with minimum amount of 10.000 ng (look at **TABLE A.**).

Thursday, 27 September 2018

Low Melting Agarose Purification

The low melting agarose purification could not be done at that day since the electrophoresis chambers are fully used by other lab staffs for their projects this week. ☹

Monday, 1 October 2018

Low Melting Agarose Purification – Continue

We used the previous modified method of purification that has been proved successfully with pSB1C3-BFP. All details method of this step is quite similar with the previous one. We successfully obtained HT purified concentration of 12.3 ng/ µl and pSB1C3 linear backbone up to 14.5 ng/ µl. The purity ratio 260/280 of both samples are 1.82 and 1.98 respectively. We tried to do ligation (correspond to the **TABLE C.**) in the evening to save time before submission hit at 10th of October.

Tuesday, 2 October 2018

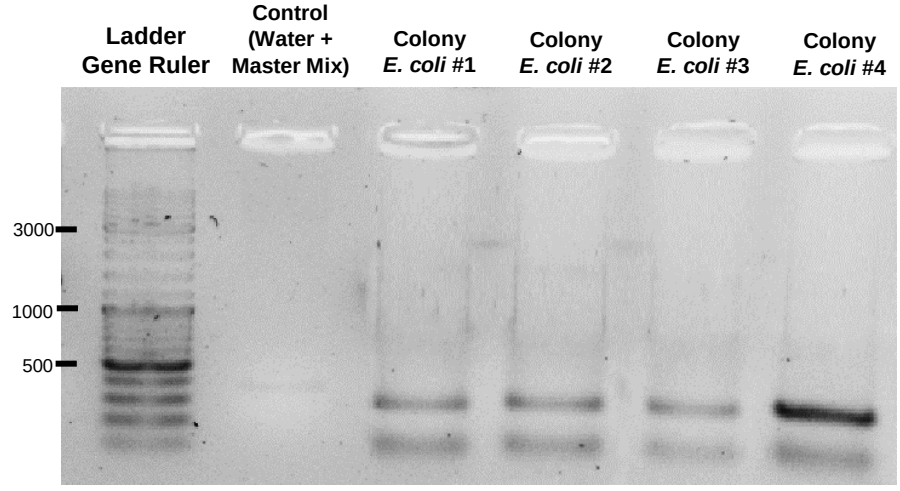
Transformation of pSB1C3-HT into *E. coli*

All terms and conditioned steps follow method of transformation in other pages.

Wednesday, 3 October 2018

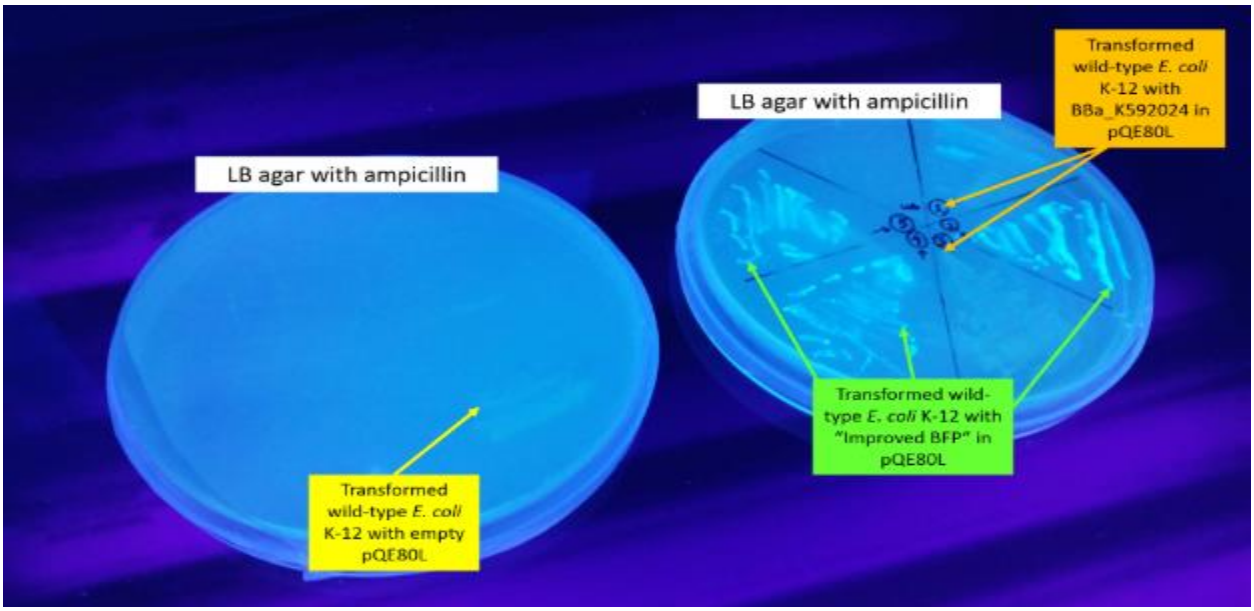
PCR Colony Confirmation

PCR Colony has been done for pSB1C3-HT colonies in *E. coli* Top10 strain. The following results indicated within gel analysis. Conclusion: No inserted HT fragment was found in the colonies. SUMMON REINFORCEMENT!! => iGEM NTU.



Thursday, 4 October 2018

Comparative Assays of BFP and Improved BFP

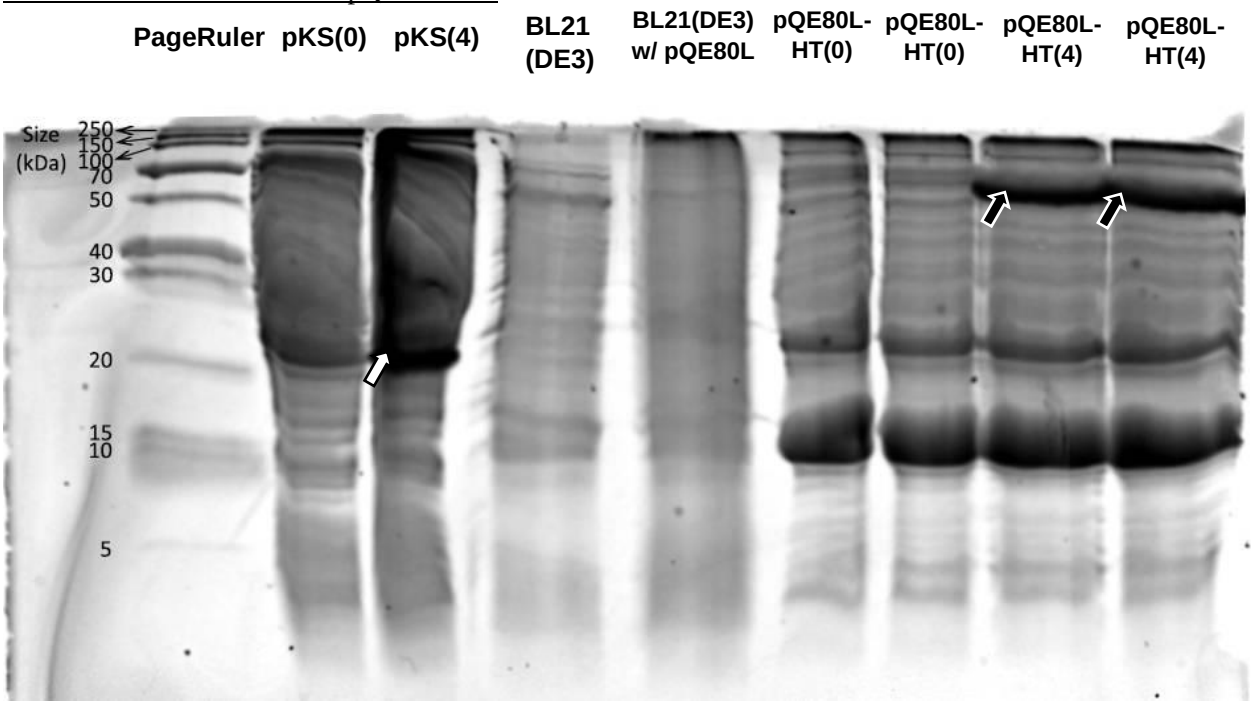


t = 0 hour									
Replicate	Empty pQE80L			pQE80L + "Improved BFP"			pQE80L + BBa_K592024		
	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}
1	921.61	0.030	30720.3	1015.02	0.021	48334.3	882.16	0.020	44108
2	873.76	0.027	32361.5	1021.67	0.018	56759.4	985.20	0.022	44781.8
3	892.78	0.024	37199.2	991.81	0.024	41325.4	1006.52	0.028	35947.1
4	888.09	0.023	38612.6	997.39	0.025	39895.6	1003.99	0.028	35856.8
Mean	894.06	0.026	34723.4	1006.47	0.022	46578.7	969.47	0.025	40173.4
St. Dev.	20.07	0.003	3779.71	14.16	0.003	7724.21	58.98	0.004	4940.07
t = 6 hour									
Replicate	Empty pQE80L			pQE80L + "Improved BFP"			pQE80L + BBa_K592024		
	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}
1	965.12	0.255	3784.8	1318.92	0.235	5612.4	1203.38	0.220	5469.9
2	992.37	0.259	3831.5	1326.06	0.231	5740.5	1198.14	0.226	5301.5
3	1016.78	0.265	3836.9	1387.15	0.223	6220.4	1293.64	0.242	5345.6
4	1010.14	0.270	3741.3	1375.38	0.237	5803.3	1252.74	0.245	5113.2
Mean	996.10	0.262	3798.6	1351.88	0.232	5844.1	1236.98	0.233	5307.6
St. Dev.	23.08	0.007	44.84	34.40	0.006	263.10	45.08	0.012	147.88
t = Overnight (14 hour)									
Replicate	Empty pQE80L			pQE80L + "Improved BFP"			pQE80L + BBa_K592024		
	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}	Fl	OD_{600}	Fl/OD_{600}
1	982.83	0.555	1770.9	3561.14	0.523	6809.1	2997.71	0.736	4073.0
2	911.77	0.605	1507.1	3555.93	0.541	6572.9	2819.19	0.777	3628.3
3	990.52	0.613	1615.9	3601.21	0.578	6230.5	3132.25	0.821	3815.2
4	994.31	0.616	1614.1	3599.75	0.582	6185.1	3162.38	0.820	3856.6
Mean	969.86	0.597	1626.98	3579.51	0.556	6449.39	3027.88	0.789	3843.25
St. Dev.	39.02	0.029	108.59	24.32	0.029	295.73	156.47	0.041	182.52

Complete data of fluorescence assay is shown in the table above, while the overall result is visualized in figure above. At t = 0, 6, and 14 hour, although not shown in Figure 5, fluorescence/OD_{600} of transformed *E. coli* with pQE80L + "Improved BFP" and pQE80L + BBa_K592024 were significantly higher than negative control (transformed *E. coli* with empty pQE80L). At t = 6 hour, fluorescence/OD_{600} of *E. coli* with "Improved BFP" was significantly higher than *E. coli* with BBa_K592024 ($p = 0.005$). Similar result was found after overnight incubation ($p < 0.001$). These results support previous qualitative observation of fluorescence and may be explained by 'double promoter' of "Improved BFP" in pQE80L. In addition, from Figure 5 it is also observed that fluorescence/OD_{600} was decreasing along with time. The probable explanation of this result is higher bacterial concentration in medium over time, resulting in larger denominator and reducing fluorescence/OD_{600}. Moreover, it is also probable that the bacteria underwent cessation, causing decline in BFP production and resulting in lower fluorescence/OD_{600}. Nevertheless, from this experiment, it can be inferred that *E. coli* transformed with "Improved BFP" retains significantly higher fluorescence/OD_{600} than BBa_K592024 under plasmid pQE80L expression system.

Friday, 5 October 2018

SDS-PAGE Confirmation of pQE80L-HT



SDS-PAGE analysis (photographed via *ImageQuant*) of pQE80L-HT expression in *E. coli* BL21(DE3). White arrow indicates LacZα (size ~20.7 kDa) protein expression due to IPTG induction, while black arrow shows increasing HT (size ~57.8 kDa) protein expression as it is induced with IPTG within 4 hours. WOW, HT was successfully expressed!!

Note: pKS(0) = *E. coli* TOP10 transformed with pBluescript KS(-) with no IPTG induction; pKS(4) = *E. coli* TOP10 transformed with pBluescript KS(-) after 4 hours of IPTG induction in 37^0C; BL21(DE3) = wild-type *E. coli* BL21(DE3); BL21(DE3) w/ pQE80L = *E. coli* BL21(DE3) containing empty pQE80L; pQE80L-HT(0) = *E. coli* BL21(DE3) containing recombinant

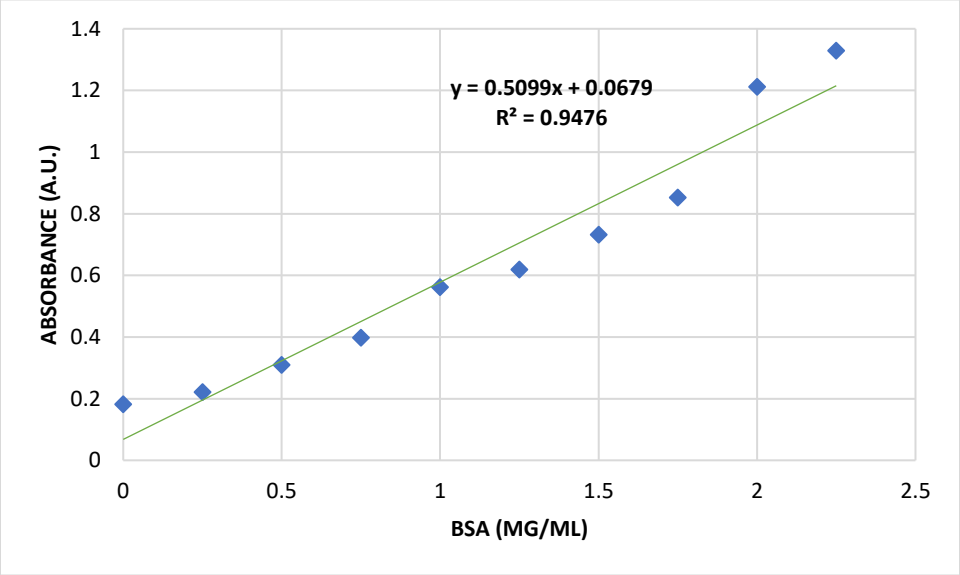
pQE80L-HT with no IPTG induction; pQE80L-HT(4) = *E. coli* BL21(DE3) containing recombinant pQE80L-HT after 4 hours of IPTG induction.

Saturday, 6 October 2018

DNA Submission Preparation and Extraction of DT Protein

We have managed to dry down the subsequent biobrick registered in the iGEM Wiki HQ. Part **BBa_K2607000** (pSB1C3-DT) and **BBa_K2607002** (pSB1C3-BFP improved) are located in well 1A and 1B respectively. We left them overnight to dry in the laminar fume hood.

Not only that, we make standard curve for Bradford assay protein quantification using Coomassie brilliant blue solution. Extraction of the protein DiphTox from recombinant *E. coli* was done by prominent lysis of cells grown that is grown overnight. Native lysis buffer was utilized to maintain specific protein configuration for its function for binding. To measure protein concentration, our team would use Bradford assay using Coomassie Brilliant Blue solution and placed within plate reader for 595 nm absorbance test. Standard curve of the assay was produced using Bovine Serum Albumin (BSA) as concentration standards.



Monday, 8 October 2018

DNA Submission & Binding Assay of DT-HT

One of our members went to FedEx to ship the registered biobricks. YEAY the DNA is submitted!!! Waiting for the NTU to submit our part as well (**BBa_K2607001** / pSB1C3-HT).

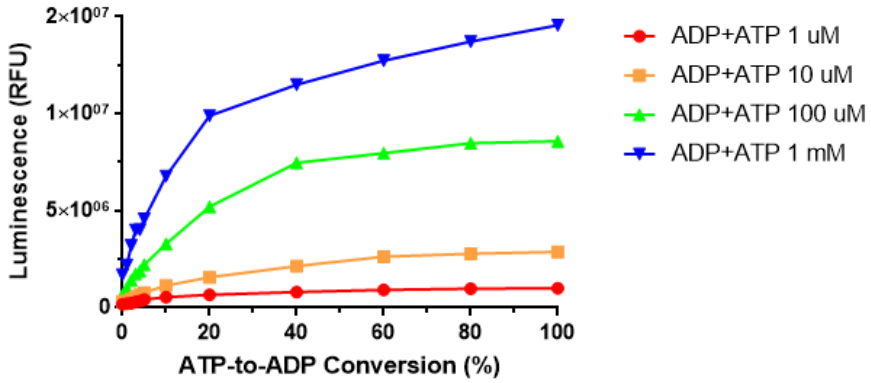
Prior to this step, our team expressed HT in transformed *E. coli* BL21(DE3) with pQE80L-HT by IPTG induction. In addition, we also had to remove outer membrane of the *E. coli*. The membrane removal would enable the HT receptor (in inner membrane) exposed directly towards extracellular environment, and possibly detecting DT. Binding assays of DT and HT was conducted within 96-

well plates by measuring the absorbance of 600 nm. This absorbance index indicates amounts of *E. coli* spheroplasts that successfully bound into DT in various environmental conditions (i.e. pH, temperatures, and DT concentration variables). Incubation was done within 60 minutes and purified magnetically using the available His-Tag. The amount of HT receptor binds to DT correlates positively with the amount of spheroplasts available in the eluents. Therefore, OD_{600} is used as the primary quantification of spheroplasts amounts in the eluents. Specific details regarding methods of binding assays could be accessed via protocol page.

DT Concentration (nM)	OD_{600}									
	4°C					25°C				
	Rep1	*Rep2*	*Rep3*	*Mean*	*St. Dev.*	*Rep1*	*Rep2*	*Rep3*	*Mean*	*St. Dev.*
100	0.07595	0.0765	0.075421	0.075957	0.00054	0.065845	0.066	0.066149	0.065998	0.000152
200	0.09625	0.0942	0.091866	0.094105	0.002194	0.091028	0.0904	0.089112	0.09018	0.000977
300	0.116465	0.119	0.117009	0.117491	0.001335	0.11038	0.112	0.096269	0.106216	0.008653
400	0.134605	0.135	0.129117	0.132907	0.003288	0.119349	0.1258	0.124289	0.123146	0.003374
500	0.142971	0.142	0.13692	0.14063	0.00325	0.092539	0.1339	0.132041	0.119493	0.023362
600	0.131838	0.1308	0.136578	0.133072	0.00308	0.105433	0.108	0.109468	0.107634	0.002042
700	0.140814	0.1398	0.130853	0.137156	0.005482	0.120124	0.1125	0.109777	0.114134	0.005363
800	0.139218	0.1389	0.138472	0.138863	0.000374	0.112397	0.1146	0.104352	0.11045	0.005394
900	0.128478	0.1296	0.124337	0.127472	0.002772	0.113375	0.1076	0.106478	0.109151	0.003701
1000	0.136597	0.1334	0.12997	0.133322	0.003314	0.110781	0.1035	0.102042	0.105441	0.004682
DT Concentration (nM)	OD_{600}									
	37°C					50°C				
	Rep1	*Rep2*	*Rep3*	*Mean*	*St. Dev.*	*Rep1*	*Rep2*	*Rep3*	*Mean*	*St. Dev.*
100	0.052625	0.0523	0.052395	0.05244	0.000167	0.032055	0.0403	0.0403	0.035964	0.004139
200	0.081469	0.0815	0.081521	0.081497	0.000026	0.058493	0.05687	0.05687	0.057857	0.000867
300	0.098739	0.0986	0.098208	0.098516	0.000275	0.053524	0.0535	0.0535	0.053563	0.000089
400	0.121782	0.1212	0.120972	0.121318	0.000418	0.055359	0.0561	0.0561	0.055714	0.000371
500	0.129521	0.1066	0.109124	0.115082	0.012568	0.055447	0.0665	0.0665	0.064436	0.008156
600	0.114763	0.1177	0.118085	0.116849	0.001817	0.065657	0.0682	0.0682	0.067563	0.00168
700	0.111707	0.1142	0.115469	0.113792	0.001914	0.069064	0.0703	0.0703	0.070139	0.001005
800	0.127036	0.1302	0.133242	0.130159	0.003103	0.068916	0.0706	0.0706	0.070327	0.001296
900	0.093508	0.1259	0.13026	0.116556	0.020079	0.073543	0.0749	0.0749	0.074389	0.000738
1000	0.119084	0.1204	0.118508	0.119331	0.00097	0.076607	0.0767	0.0767	0.076858	0.000357

Tuesday, 9 October 2018

ADP Glo Max Assay



The standard curve obtained for percent ATP-to-ADP conversion from luminescence data is shown in the figure above. With this standard curve, we can estimate how much ATP is converted into ADP (or how much ATP is conserved in HT-DT reaction compared to normal) based on given luminescence data later in the experiment.

Hence, our present result suggested that our HT works as expected to inhibit phosphorylation, shown by less luminescence generated compared with control, indicating that less ADP was produced upon HT-DT interaction. The following tables summarize luminescence test upon kinase Tar activity.

Sample		Net OD_{600}	Luminescence (RFU)	Luminescence per OD_{600} (RFU)
Control (*E. coli* BL21(DE3) + empty pQE80L)	Replicate 1	0.297	557028	1875515
	Replicate 2	0.285	637420	2236561
	Replicate 3	0.274	594330	2169088
	Replicate 4	0.292	615446	2107692
	Mean			2097214
	Standard Deviation			156890.3
Experimental (*E. coli* BL21(DE3) + pQE80L + HT)	Replicate 1	0.289	427903	1480633
	Replicate 2	0.301	492191	1635186
	Replicate 3	0.308	459287	1491192
	Replicate 4	0.293	470488	1605761
	Mean			1553193
	Standard Deviation			78730.27