P3HB Lab Book

18.07

<u>Transformation of BBa_K1149051 into *E. coli* DH5alpha - VP</u>

Goal: Transform the biobrick into *E.coli*

Protocol:

- 2 tubes of 50µL DH5-alpha
- Add 1µL of resuspended DNA into each tube and save the remaining DNA in the freezer (-20°C)
- Close and incubate on ice for 30 minutes
- Heat shocked at 42°C for 20 seconds
- Incubate on ice for 5 minutes
- Pippet 950µL of LB into each transformation
- Incubate at 37°C for 1 hour, shaking at 300 rpm
- Pipette 100µL of each tube on petri plates and spread with glass beads (10%)
- Spin down cells at 6800g for 3 mins
- Discard 800µL of the supernatant
- Resuspend the cells in the remaining 100µL
- Pipette each transformation onto petri plates (90%)
- Incubate overnight at 37°C

Results: (Observation on the 19/07)

Plate 10%.1: 0 colony
Plate 10%.2: 0 colony
Plate 90%.1 (no picking): 10 colonies
Plate 90%.2 (picking): 25 colonies

We observed single colonies growth on both 90% plates but none of the 10%

19.07

<u>Overnight liquid culture of DH5alpha + K1149051 - VP</u>

Goal: Prepare the colony to perform a miniprep

Protocol:
- Picking of 3 single colonies
- Liquid culture in 10mL LB + Chloramphenicol (co) in 15mL Falcon tube
- Streak every toothpick on a plate
- Overnight growth at 37°C, shaking at 130rpm

Results:
We obtained 3 tubes of LB+ chloramphenicol+ DH5alpha+ K1149051

20.07
Extraction of Biobrick K1149051 / Miniprep - VP

Goal: Extract the plasmid of K1149051 of liquid culture tubes A,B,C. Using a Thermoscientific GeneJET Plasmid Miniprep Kit

Protocol:
- Harvest the bacterial culture by centrifugation at 8000rpm (6800g) for 2 minutes at 27°C
- Decant the supernatant and remove all remaining medium (We didn't use the B culture because there was no cells after centrifugation, it was a mistake, we should have)
- Add 250µL of Resuspension solution and mix it by pipetting up and down several times
- Transfer the 250µL of each tubes to 2.0 mL Eppendorf tubes
- Add 250µL of lysis solution and invert the tubes 6 times
- Add 350µL of Neutralization solution and invert the tubes 6 times
- Centrifuge at 8000rpm for 5 minutes
- Bind the DNA by transferring the supernatant to the Thermo Scientific GenJET Spin Column
- Centrifuge at 8000rpm during 2 minutes - Wash the column by adding 500µL of Wash Solution and centrifuge for 1 minutes at 8000rpm
- Discard the flow-through
- Repeat the operation a second time

- Centrifuge the empty column for 1 minutes at 8000rpm
- Elute the purified DNA by transferring the column into a new tube
- Add 50µL of Elution Buffer to the column
- Incubate for 2 minutes
- Centrifuge 2 minutes at 8000rpm
- Collect the flow-through in 2.0 mL labelled Eppendorf tubes
- Tested 1µL of each tube with the NanoDrop machine
- Keep the tubes at -20°C

Results:

Tube	Concentration	A260	A280	260/280	260/230
K1149051 A	427,6	8,553	4,505	1,9	2,22
K1149051 C	654,8	13,095	6,594	1,99	2,30

We noticed there is a high concentration of both A and C colony, and the ratio are close to 1.8 and 2.0 respectively.

27.07

Transformation of BBa_K934001 into *E. coli* DH5alpha - VP

Goal: Transformation of the biobrick into *E.coli*

I did the standard transformation procedure from iGEM. But when I pipet the cell on the petri plate I wasn't working under the hood or next to the flame so there was contamination. I keep the procedure going.

Results: (Observation on the 28/07)

As expected, because of the non sterile environment, nothing grew on my plates.

31.07

Transformation of BBa_934001 into *E.coli* DH5alpha - VP

Goal: Transformation of the biobrick into *E.coli*

I did the standard transformation procedure from iGEM, this time making sure I was working under the hood at all time. This time I used a control, 50µL of competent DH5alpha cells without adding DNA to it.

Results: (observation on the 01/08)

Plate 10%: 18 colonies
Plate 10% control: 0 colony
Plate 90% : 104 colonies
Plate 90% control: 0 colony

01.08

Overnight liquid culture of DH5alpha + K934001 - VP

Goal: Prepare the colony to perform a miniprep

Protocol:

- Picking of 3 single colonies from the 90% plate (A,B,C) and 1 colony from the 90% plate from the 18/07 transformation (D) because we threw away one of the tube the last time
- Streak each toothpick on a plate
- Liquid culture in 10mL LB + Chloramphenicol in 15mL Falcon tube

- Overnight growth at 37°C, shaking at 130rpm

Results:
We obtained 3 tubes of LB+ chloramphenicol+ DH5alpha+ K1149051 and 1 tube of LB+ chloramphenicol+ DH5alpha+ K934001

02.08
Static culture + Miniprep of K934001 (3 tubes) + K1149051 (1)

Goal: Make static cultures of both biobrick. Extract the plasmid of K1149051 of liquid culture tube D and K934001 of liquid culture tubes A,B,C. Using a Thermoscientific GeneJET Plasmid Miniprep Kit

Static cultures:
-Make 3 tubes of LB+CAM in 50mL Falcon + 100µL of A,B and C (K934001)
-3 tubes of LB+CAM, 1+ 200µL of D (K1149051) and 2 with the colonies A and C (K1149051) from the plate we streaked them on.
- Incubate them at 37°C (to see if they produce PHA)

Miniprep
I did the standard procedure of the miniprep for all 4 tubes.

Results:
-Tested 1µL of each tube on the NanoDrop machine.

Tube	Concentration	A260	A280	260/280	260/230
A	100.35	2.007	1.058	1.895	1.505
B	143.45	2.869	1.451	1.975	1.955
C	249.45	4.988	2.472	2.315	2.065
K1149051 D	396.85	7.936	4.11	1.93	1.68

There is a higher concentration of the colony from the K1149051 transformation (D) than the K934001 transformation (A,B,C) but the ratios are all close to 1.8 and 2.0 (**ARE THEY REALLY? :/)**

03.08

PCR and electrophoresis gel of 6 samples from both K1149051 and K934001

Goal: Amplify the DNA

I received the forward and reverse primers I designed and ordered, which concentration is respectively 2.35nmol (o.17001) and 22.2 nmol (o.17002).

Protocol:

- Realise Master Stock of 100nM
- Add 235µL of nuclease free water in the o.17001 tube
- Add 222µL of nuclease free water in the o.17002 tube
- Then realise Working Stock of 10nM
- Made 200µL of o.17001 with 20µL of the o.17001 100nM Master Stock + 180µL of nuclease free water
- Made 200µL of o.17002 with 20µL of the o.17002 100nM Master Stock + 180µL of nuclease free water
- Then I planned my tubes for PCR:

Tube 1 (25µL): A (K1149051)

-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 2 (25µL): C (K1149051)

-5µL Buffer
-0.5µL dNTP

-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 3 (25µL): D (K1149051)
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 4 (25µL): A (K934001)
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 5 (25µL): B (K934001)
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 6 (25µL): C (K934001)
-5µL Buffer

-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL DNA
-14.75µL Nuclease free water

Tube 7 (25µL): Biobrick K1149051
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL BioBrick K1149051
-14.75µL Nuclease free water

Tube 8 (25µL): BioBrick K934001
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-2µL BioBrick K934001
-14.75µL Nuclease free water
Tube 9 (25µL): Control
-5µL Buffer
-0.5µL dNTP
-1.25µL Forward primer
-1.25µL Reverse primer
-0.25µL DNA polymerase
-16.75µL Nuclease free water

- Made a master mix with 50µL of Buffer, 5µL of dNTPs, 12.5µL of Forward primers, 12.5µL of Reverse primers
- Pipett 8µL of the master mix in each tube

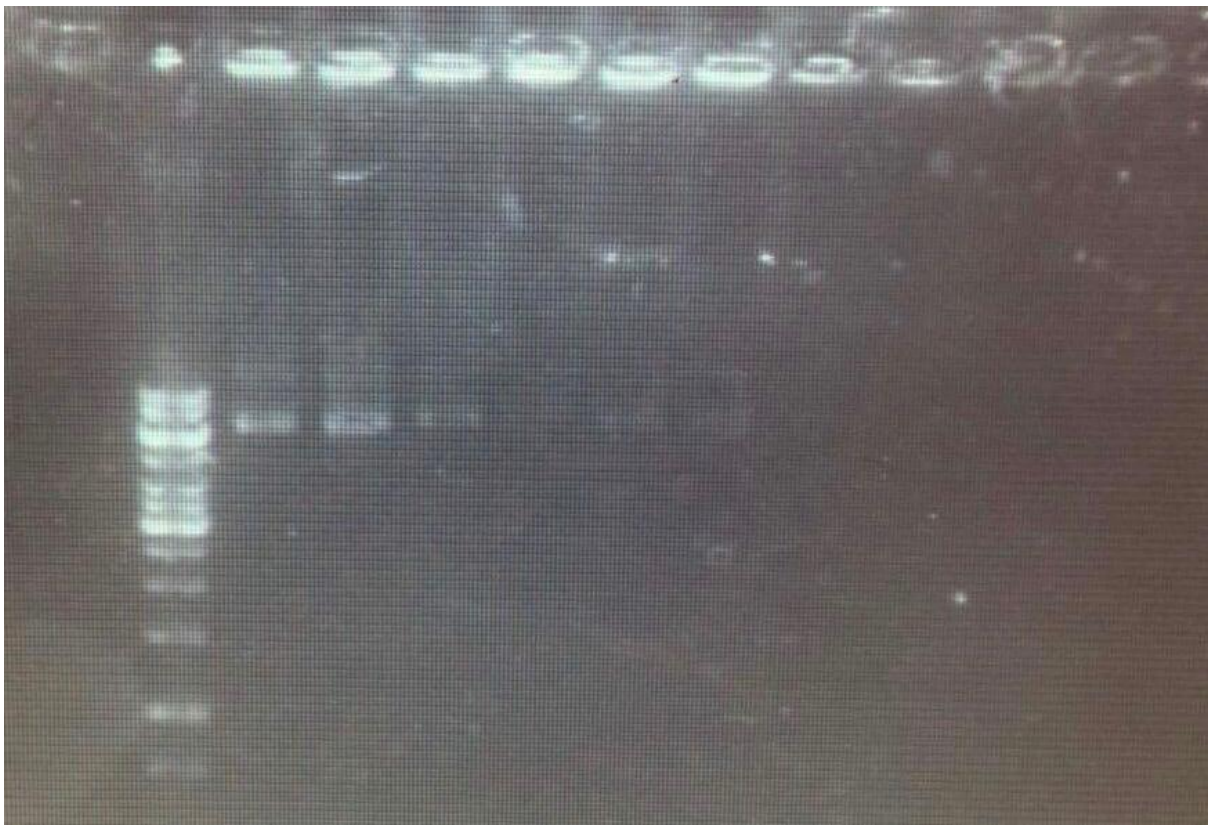
- Add the Nuclease free water necessary in each tube
- At the end add the DNA polymerase and put the tube in the PCR machine
- While the PCR is running, made the gel with 0.5g of Agarose and 50mL of buffer in an 250mL Erlenmeyer
- Heat it for 45 seconds to make it clear then add 5µL of SyberSafe and mix well
- Pour it into the electrophoresis and wait for the gel to harden
- Put 5µL of Ruler in the first well of the gel
- Get the tubes out of the PCR machine
- Mix 5µL of each tube with 1µL of dye and put in the wells
- Run the gel and wait 20 mins

Results:

The 2 positive control didn't work because I used the BioBrick without digesting it first. But we observe bans on all of our sample. But we expect to have a band around 1.4 kb and this band correspond to 6kb which means the DNA wasn't cut.

30.08

I ran an other gel using my samples from the last PCR to check if the problem was the gel or the primers or the PCR. But the gel gave the same results as the last time.



I have to do another PCR.

10.08

After checking my tubes I left incubating at 37 degree with both K1149051 and K934001, we have a pellet at the bottom of each tube. It also appears that they are reddish so we check to see if there was RFP fluorescence in the colony in the plate and in the tubes. The tube showed some fluorescence but not the plate. I picked 3 colonies (E, F, G) from the K1149051 plate and incubate them overnight at 37 degree shaking at 300 (?) rpm. I want to extract the pellet and test it with Nile red to see if it is PHA.

11.08

I took 1 mL of each tube and miniprep them using the standard procedure, here are the results after using the nanodrop machine

Tube	Concentratio n	A260	A280	260/280	260/280
E	95.8	1.915	0.899	2.135	2.185
F	127.25	2.543	1.238	2.065	1.995
G	156.75	3.134	1.476	2.125	2.2

Then I digested the 3 samples.
Protocol:
Tube E:

- Add 2 µL of FD 10X Buffer and 1 µL of NotI restriction enzyme
- Add V=m/c → 1000/95.8= 10.4µL of the DNA
- Add 20 - (3+ 10.4)= 6.6µL of free ARNase water

Tube E:

- Add 2 µL of FD 10X Buffer and 1 µL of NotI restriction enzyme
- Add V=m/c → 1000/95.8= 10.4µL of the DNA
- Add 20 - (3+ 10.4)= 6.6µL of free ARNase water

Tube E:

- Add 2 µL of FD 10X Buffer and 1 µL of NotI restriction enzyme
- Add V=m/c → 1000/95.8= 10.4µL of the DNA
- Add 20 - (3+ 10.4)= 6.6µL of free ARNase water

Incubate the tubes at 37 degree in the water bath for 30 minutes, then heat them at 80'degree for 5 minutes. Then run a gel using the undigested E, F and G samples as negative control.

On the gel we can see the negative control around 6kb which is the plasmid size and the fragment around 4kb which is the gene we digested.

14.08

I ran a PCR following the same protocol and the same measurements as last time with an annealing temperature of 60 degree instead of 80 this time. I ran a gel and my bands still appear at 6/5kb and not at 4kb as it should be. I don't know the reason because I checked my primers with my advisors and they said they should work fine. I have to send my gene to sequence.

15.08

I made 20 agar + cloramphenicol plate (300µL of Cloramphenicol for 300 mL of LB)and made new LB 1L media. I also made 100 mM magnesium chloride (10.16g Magnesium chloride + 500 mL of molecular water then autoclave) and 100 mM calcium chloride solution (7.35g calcium chloride + 500mL of molecular water then autoclave) for Aya.

I made a TSE buffer for the protocol to exctrat the PHA. I put 20g of sucrose with 100ml of water that I filtrated. Then a solution of Tris-HCL with 1.57g of Tris + 500µL of HCL and add molecular water to have a 200mL solution and filtrate. Another solution of EDTA with 3.72g of EDTA and add water to have a 200mL solution and filtrate. Then I made the buffer with 50mL of sucrose solution, 5mL of EDTA and 10 mL of Tris-HCL.

I made overnight culture with 3 colonies (D,E,F) from the K934001 biobrick in order to use the protocol to stain the HA to see if we have a production of PHA.

16.08

I made 4 bottles of LB + Agar with 9.6g LB Broth Miller for 300mL of molecular water and then autoclave it. Made a 1L bottle of TEA Buffer with 50mL of TEA + 950mL molecular water.

Then I did the protocol to stain the PHA.

- mesure the OD of 1 mL solution diluted 1/10 (100µL of the solution + 900µL of sterile water)
- Then dilute the solution to get the OD at 1:

Tube	1/10 OD	OD of the solution	Dilution to have OD=1
Blank	0	0	0
D	0.436	4.36	1mL solution + 3.36mL sterile water
E	0.391	3.91	1mL solution + 2.91mL stérile water
F	0.484	4.84	1mL solution + 3.84 stérile water

- Thaw on ice for 10 minutes
- Take 1mL of solution
- Centrifuge for 5 minutes at 3000g at 4 degree Celsius
- Discard the su<pernatant
- Resuspend the cells with 1 mL of ice-cold TSE Buffer
- Thaw on ice for 10 minutes
- Centrifuge for 5 minutes at 3000g at 4 degree Celsius
- Resuspend the cells with 1mL of ice-cold of sterile water
- Add 1µL of NileRed solution (0.6mg of Nile red in 2mL of DMSO)
- Vortex the tubes
- Place the tubes 5 minutes in the dark
- Centrifuge for 5 minutes at 3000g at 4 degree Celsius
- Resuspend the cells with 1mL of ice cold water and centrifuge (twice)
- Then use a FACS machine to see the results looking at FL2 and FL3

But I didn't have a negative control so I couldn't exploit my results (which in the end were bad there were nothing in the tubes)

I sent my 2 biobricks (K1149051 and K934001) to sequencing by making tubes with 5µL of primers and 5µL of biobrick.

17.08

I was supposed to do an overnight culture of A,C,D from K1149051 and A,B,C from K934001 but I forgot so I couldn't really advance my experiment about PHA. So I spent the day cleaning the lab, making LB solutions, plates, glycerol stocks, autoclaving tips and other instruments.
I made the overnight culture I was supposed to do the night before.

18.08

First I made a glycerol stock of my tubes from overnight culture (A,C,D of K1149051 and A,B,C of K934001) with 500µL of glycerol and 500µL of my solution.
Then I made the protocol of the PHA staining this time using a negative control from DHA5alpha.

For the OD measures:

Tube	1/10 OD	OD of the solution	Dilution to have OD=1
Blank	0	0	0
A (K11)	0.448	4.48	1mL solution + 3.48mL sterile water
C (K11)	0.434	4.34	1mL solution + 3.34mL stérile water
D (K11)	0.33	3.3	1mL solution + 2.3stérile water

A (K93)	0.47	4.7	1mL solution + 3.7stérile water
B (K93)	0.43	4.3	1mL solution + 3.3stérile water
C (K93)	0.44	4.4	1mL solution + 3.3stérile water
Negative control	0.45	4.5	1mL solution + 3.5stérile water

By using the FACS machine I only got good results for A and D from the K1149051 biobrick, so I made overnight culture of those 2 samples, and made 3 tubes for each sample to do the same protocol and to see if it was reproducible. Then I helped my team mates with their experiments and I took care of the lab.

19.09

I took my overnight culture and I started the protocol for staining the PHA. For the OD:

Tube	1/10 OD	OD of the solution	Dilution to have OD=1
Blank	0	0	0
A 1	0.336	3.36	1mL solution + 2.36mL sterile water
A 2	0.311	3.11	1mL solution + 2.11mL stérile water
A 3	0.319	3.19	1mL solution + 2.19 mLstérile water

D 1	0.276	2.76	1mL solution + 1.76 mLstérile water
D 2	0.343	3.43	1mL solution + 2.43 mL stérile water
D 3	0.265	2.65	1mL solution + 1.65 mL stérile water
Negative control	0.672	6.72	1mL solution + 5.72 mL stérile water

then used the FACS machine and got good results all the same basically.

20.07

Did a PCR using less DNA as my advisors believe what we saw on my gel was my DNA template because I put 2µL DNA at 426.7ng/µL so I redid a PCR with DNA diluted 1/1000 times, C1/1000=0.4276ng/µL ⇔ V=m/c=1/0.4276 ⇔ V=2.34µL.

Tube A (50µL): BioBrick K1149051

-10µL Buffer
-0.5µL dNTP
-2.5µL Forward primer
-2.5µL Reverse primer
-0.5µL DNA polymerase
-2.34µL BioBrick K934001
-32.16µL Nuclease free water

Cycle	Temperature (Degree C)	Time
Initial denaturation	98	1 min
Denaturation	98	10 sec

Annealing	58.9	30 sec
Extension	72	3 min
Final elongation	72	10 min
Hold	12	infinity

I didn't have any bands on my PCR, I think my primers are not working so I designed and order new one with more 18 binding sites instead of 14 (without the overhang).

I made Nile Red plate using 300mL LB Agar + 3% glucose, +50µL NileRed, + 150 Chloramphenicol. Spread some of my glycerol stock on the plate, let incubate for the night.

21.08

This is a picture of my plates under red fluoerencse TECAN, it means there is PHA