DYS SEE

iGEM Thessaly

Lab Book

1/7/19

Goals of the day:

1. Receive and resuspend DNA fragments assigned
2. Blunt-end cloning (SmaI) of IS6110 into pGADT7-AD
3. Cloning of trigger 32B into pSB1C3
4. Resuspension of gBlocks from IDT (LacZa, 32B-LacZa)
5. Cloning of LacZa and 32B-LacZa fragment into a psb1c3 vector
6. Restriction Digestion and Ligation of the gene fragments EGFP, 32b EGFP
7. Digestion & ligation

Procedure:

1. Receive and resuspend IS6110 fragment(987bp no RFC10).

   gBlocks from come lyophilized at fixed quantities depending on fragment length. IS6110 is in the 750-1000bp range and comes at a quantity of 1000 ng, while trigger 32B is in the <250 bp range and comes at a quantity of 250 ng.

   Following the primer resuspension protocol and instruction from IDT, resuspension was done in a concentration of 50 ng/ul of pure water. Thus, the resuspension volumes for the two gBlocks are:

   20 ul for IS6110

   5 ul for trigger 32B

2. Blunt-end cloning (SmaI) of IS6110 into pGADT7-AD

   a) Proper Dilution of pGADT7-AD

   From 27/6, pGAD concentrations are at 1.42-1.44 ug/ul. Dilution is needed in order to reach normal concentration for digestion.

   Target concentration → 500 ng/ul

   Stock concentration → 1444 ng/ul

   Volume to be diluted: x = 1444 ng / (500 ng/ul) = 2.9 total volume

   Thus, add 1 ul of 1.44 ug/ul pGAD into 1.9 ul pure water/

   Final concentration should be 500 ng/ul.

   b) Digestion of pGAD with SmaI

   Because dephosphorylation is required and thus gel DNA extraction, 1 ug of DNA will be digested.

DNA	2 ul diluted pGAD
10X cutsmart buffer	3 ul
SmaI	1 ul
ddH2O	24 ul to

   Total volume 30 *ul*

   - Incubate at 25 degrees C / 1h (pulse spin 2-3s before)
   - Heat inactivate 65 degrees C /20 min

   c) Dephosphorylation of digested pGAD w/ CIP

500 ng DNA	15 ul from

	digestion rxn
10X cutsmart buffer	2 ul
CIP	1 ul
ddH2O	2ul to

Total volume 20 ul

- Incubate 37 degrees C / 30 min
- Heat inactivate at 65 degrees / 15 min

d) Gel purification

- Band excision of cut dephosphorylated vector at 8kb
- Gel extraction using MN columns and protocol (went wrong, hadn't added ethanol to NT3)
  (Elution was done in 15 ul ddH2O)
- Qubit → Concentrations: 1.10 and 1.6 ng/ul respectively for 2 rxns (ponaeipsyxhmou)

e) Ligation of pGADT7-AD w/ IS6110
Ratio will be 3:1 (insert:vector)

Vector 12
DNA ng
Insert 4.5
DNA ng

IS6110: Dilute 1 ul of 50 ng /ul into 10.1 ul ddH2O to reach final concentration of 4.5 ng/ul
Due to problems with DNA extraction from gel, I messed up the final part of the experiment. 3 reaction were done: Negative control (no insert), 1h incubation at RT, overnight incubation at 4 degrees C
Reaction prototype:

1 ul buffer
1 ul Ligase
1 ul diluted IS6110
4.5 ng
7 ul vector (12 ng)
Total 10 ul
1 ul buffer

f) Transformation of ligation rxn 2.5 on *E. coli* DH5-alpha competent cells
2 ul of ligation → 900 ul LB recovery, 1h incubation.
Streaked 100 ul after spin-down at 9,000 x g for 1 and resuspension on 100 ul
Streaking was done on LB Agar A100 plates

3. Restriction Digestion of pSB1C3 / trigger 32B
Following iGEM's protocol for Linearized Plasmid Backbone:

Master Mix for Digestion (5 rxns)	
10X cutsmart buffer	5 ul
EcoRI-HF	0.5 ul
PstI-HF	0.5 ul
ddH2O	19 ul

Total volume 25 ul

Digestion Reaction → total volume 8 ul

pSB1C3:	
plasmid DNA (100ng)	4 ul
Master Mix	4 ul

Trigger 32B:	
DNA fragment (100 ng)	2 ul
Master Mix	4 ul
ddH2O	2 ul

- Incubate at 37 degrees C / 30 min
- Heat kill at 80 degrees C / 20 min

Ligation of pSB1C3 and trigger 32B

Again, iGEM protocol was followed

Digested plasmid (25 ng)	2ul
Digested insert (2 ng DNA for equimolar conc)	1 ul properly diluted
T4 DNA Ligase buffer	1 ul
T4 DNA ligase	0.5 ul
ddH2O	5.5 ul

Total volume 10 ul

4 reactions took place: Negative control (no insert, 4 degrees overnight, RT 2h, RT 20 min w/ 1.2 ul of plasmid)
Heat kill at 80C for 20 min

Master Mix for 5 rxns (4+1):	
1 ul T4 DNA ligase buffer X5	5 ul
0.5 ul T4 DNA ligase X5	2.5 ul
5.5 ul ddH2O X5	27.5 ul

Add 7 ul MM + 2 ul plasmid + 1 ul Insert (or ddH2O on Negative Control)

Transformation of pSB1C3-t32B into competent cells

- 2 ul per ligation reaction
- 900 ul LB on recovery + 1h incubation
- Spin down and resuspend at 100 ul, streak them on A100 LB Agar Plates and incubate o/n at 37 degrees C.

4. Our gBlocks from IDT arrived lyophilized in stocks with different amounts if DNA, so they needed resuspension according to the company's instructions. These 2 gBlocks were in a stock of 500ng, so they were suspended in 10ul of ddH2O to create a stock with 50 ng/ul concentration. LacZa is 396 bp and 32B-LacZa is 532 bp long.

5. Cloning of LacZa and 32B-LacZa fragment into a psb1c3 vector
Digestion of Plasmid and inserts

According to the iGEM's protocol for linearized plasmid backbones.

25 ul MasterMix (5 reactions) :	
10X Cutsmart buffer	5 ul
EcoRI-HF	0.5 ul
PstI-HF	5 ul
ddH2O	19 ul

8ul Digestion Reactions (100 ng of total DNA):

1. psb1c3
4 ul of psb1c3 (25 ng/ul)
4 ul of MasterMix (MM)

2. LacZa
2 ul of LacZa (50 ng/ul)
2 ul ddH2O
4 ul MM

3. 32B- LacZa
2 ul of 32B- LacZa (50 ng/ul)
2 ul ddH2O
4 ul MM

Incubation at 37oC for 1 hour
Heat kill at 80oC for 20 min

Ligation of digested inserts to digested psb1c3 plasmid vector

According to iGEM's protocol for ligation with T4 DNA ligase and NEB's calculator for the equimolar concentrations calculation.
4 reactions :

psb1c3+LacZa (1)
psb1c3+LacZa (2) *
psb1c3+32B-LacZa (3)
psb1c3 only (4) for negative control

*The LacZagBlock was not of very high purity according to the company so I did 2 ligation reactions

10 ul Ligation Reaction (for 1,2,3):

>2 ul of digested plasmid (25 ng)
>1ul of digested and diluted 1:2 LacZa (according to equimolar calculation I need 4.9 ng)

10 ul Ligation Reaction (for 1,2,3):

>2 ul of digested plasmid (25 ng)
>1ul of digested and diluted 1:2 LacZa (according to equimolar calculation I need 4.9 ng)
OR >1ul of digested and diluted 1:2 32B-LacZa (according to equimolar calculation I need 6.6 ng)
>1ul of T4 DNA ligase Buffer
>0.5ul of T4 DNA ligase enzyme
>5.5ul of ddH2O

Incubation at 4°C O/N

For the 4th ligation reaction (Negative Control) the reaction was the same, but without any insert. Just added 1ul more ddH_2O

6. Enzyme master mix :

Components	Volume(μl)
Cutsmart	2,5
EcoRI-HF	0,5
PstI-HF	0,5
DdH2O	19

Ligation (4°C overnight):
1:1 ratio.

Components	Volume(μl)
Digested plasmid backbone	2
Digested fragment	2,9
T4 ligase buffer	1
T4 ligase	0,5
DdH2O	3,6

7. Restriction Enzymes: EcoRI & PstI
PSB1C3 linearized plasmid 100ng
4μl plasmid DNA 4μl MasteMix
Ligation reactions for the digested products with the iGEM protocols :
• #1 tube : PSB1C3 plasmid with beta-lactamase

2 repetitions
• #2 tube : PSB1C3 plasmid with 32Blactamase

(-) control with no DNA template
• #3 tube : PSB1C3 plasmid with EGFP

2 repetitions
#4 tube : PSB1C3 plasmid with 32BeGFP
(-) control with no DNA template
To calculate for the equimolar amount of digested fragments and digested plasmid DNA, we used the NEBbiocalculator.
The ligation reactions were incubating overnight at 4°C

2/7/19

Today's goals:

1. **Transformation of the IS6110**
2. **liquid cultures**
3. **Transformation of ligated products into DH5alpha chemi- competent cells**
4. **Resuspension of arrived primers for the RPA and PCR reactions**
5. **Transformation of DH5a E.coli strains with the products of the ligation.**
6. **Transformation**

Procedure:

1. Transformation of overnight ligation (IS6110, trigger 32B). Same conditions as Monday's transformation.
2. Check plates from overnight incubation at 37 degrees C → good for pGAD, few colonies for t32b

- Inoculation of 8 liquid cultures of 5ml A100 LB w/ pGAD Is6110 single colonies. Incubate at shaking incubator overnight (37 degrees, 210 rpm)
- Inoculation of 8 liquid cultures of 5 ml C50 LB w/ pSB1C3 – t32B single colonies. Same conditions as of 3.→Had almost zero colonies… so didn't do it

3. Transformation of ligated products into DH5alpha chemi- competent cells:

4 transformations:

Trans1: LacZa+psb1c3 (1)
Trans2: LacZa+psb1c3 (2)
Trans3: 32B-LacZa+psb1c3 (3)
Trans4: psb1c3 N.C (4)

Transformation was done with the protocol for the heatshock transformation with the following changes:

- I added1.5 ul to the cells from every ligation reaction
- 900 ul LB was added to the cells upon their recovery and they were incubated for 1 h in 37oC
- The cells were peletted (centrifuged at 9.000g for 1 minute) and resuspended at 100 ul LB
- The streaking was done in LB Agar Plates with 35 mg/ml chloramphenicol
- Then the plates were incubated in 37oC O/N

4. Resuspension of arrived primers for the RPA and PCR reactions
   The resuspention was done according to the company's instructions and they were diluted with a specific amount of ddH2O so that they can be in 100 pmol/ul stocks. After that we diluted some amount of each of them to a final concentration of 10 pmol/ul (Working Solution).
5.
   - 1.5 ul plasmid (PSB1C3)
   - 35μg/ml Chloramphenicol
   - Incubation at 37°C overnight.
6. Transformation of e.Coli DH5A competent cells with the ligated products
   - PSB1C3 + beta-lactamase 1,5 μl in 100μl C.C.
   - PSB1C3 + 32Blactamase 1,5 μl in 100μl C.C.

   Plating at LB agar Chl35 (2 plates total)
   Overnight incubation at 37°C

3/7/2019

Today's goals:

1. **Minipreps from Tuesday's 3 and insert identification**
2. **Liquid LB cultures for pSB1C3-trigger32B**
3. **The transformed cells in the agar plates were not many, but I inoculated some colonies in 5ml C35 liquid LB cultures.**
4. **Inoculation**
5. **Liquid cultures**

Procedure:

1. **Minipreps from Tuesday's 3**
   Use 4 out of 5ml from culture, keep 1 ml for back up and glycerol stock.

Elution in 30 ul ddH2O

Qubit to quantify the isolated DNA after the miniprep

Use 1 ul sample → too high (500-600 ng /ul) pGADT7 – IS6110 (1-8)

Restriction Digestion and gel electrophoresis to identify unique bands at expected size.

IS6110 → Cut with EcoRI-HF and NotI-HF, expect 5.5 kb + 3.5 kb (1 % agarose gel, 60ml)

Results were that digestion didn't happen and the insert was most likely not present. I changed route, to amplify the gBlock w/ primers (overhangs for RFC10) suitable.

PCR w/ RFC10 overhangs on primers on IS6110 template to create RFC10 compatible part

Q5 High-fidelity Master Mix

For 50 ul reaction (2rxns)

		Mix (X2.2)
Q5 MM	25 ul	55 ul
IS6110 987 FWR Primer	2.5 ul	5.5 ul
IS6110 987 RVS Primer	2.5	5.5 ul

- Add 30 ul to each reaction
- Add 1 pg -10 ng DNA (we used 4.5 ng, 1 ul)
- Add 19 ul ddH2O to reach 50 ul total volume

PCR Program (1kb, 72 and 78 degrees were the primer Tm's)

Initial Denature	98 for 30s
Denature	98 for 10 s
Annealing	55 + 72 for 15 s (2 rxns)
Extension	72 for 15 s (1 kb/10s, + 50 %)
Final Extension	2 muin
Hold at 4 degrees	0 0 (forever on cycler)

2. Inoculation of 8 cultures (5ml C50 LB) w/ pSB1C3 trigger 32B cells
3. The transformed cells in the agar plates were not many, but I inoculated some colonies in 5ml C35 liquid LB cultures.
   - 6 liquid cultures with single colonies from the "32B-LacZa" agar plate
   - 9 liquid cultures with single colonies form the "LacZa 1" agar plate

   O/N incubation in a shaking incubation of 37oC and 210 rpm
4. Picked 6 colonies

   6 liquid cultures to grow overnight at 37°C(chloramphenicol).

5. We picked 6 colonies from each plate and inoculated them at 50ml falcons containing LB medium (5ml, Chl35) -> Overnight incubation at 37°C

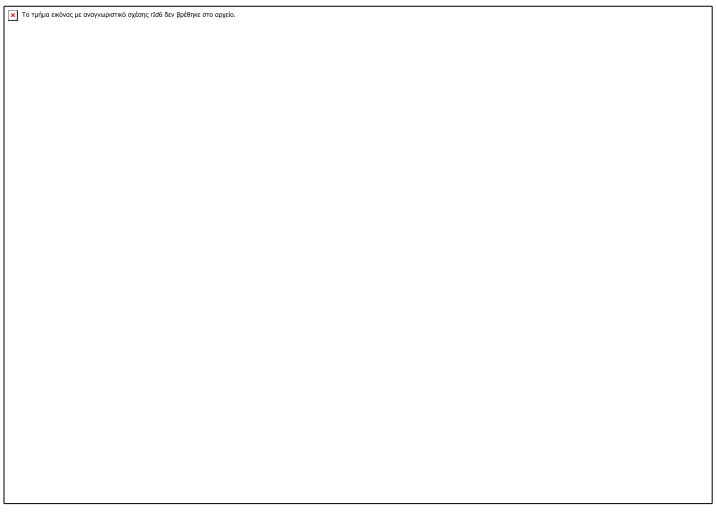
4/7/2019

Today's goals:

1. Isolation of pGAD
2. Minipreps and insert identification for trigger 32B
3. Minipreps for yesterday's liquid cultures (with the NucleoSpin® Plasmid (NoLid) kit from Macherey Nagel)
4. Restriction Digestion
5. Check if the cloning was successful.
6. Minipreps – digestion – electrophoresis

Procedure:

1. Gel electrophoresis: PCR, pGAD insert + no insert
   Results → didn't go well for pGAD, PCR had right band at 1000 bp, + band at 400 bp due to mispriming (wrong annealing for the 55oC reaction)
   PCR Cleanup → (all wrong, hadn't added ethanol to NT3, no yield)
   I had to run gel again and cut band, didn't know and didn't do
   Gel electrophoresis to cut band → all wrong, almost no DNA due to mistake on cleanup
   So, after I burned all the material from PCR, I had to do it again.
   This time, 2 reactions took place. Annealing was at 72 degrees C for both rxns (had better specificity, less 400bp by product)
   50 ul reactions, no master mix
   Added 0.7 ul template instead of 1 ul.
   Gel electrophoresis of samples in 4 wells and excision at 1kb.
   PCR cleanup (right this time)
   Elution at 20 ul (IS6110 RFC10 tube name)
   All 4 wells went into one column to have the DNA packed up.
   Qubit → 132 ng / ul, used 1 ul
   Next step is cloning on pSB1C3.
2. Minipreps for pSB1C3-t32B (30 ul elution) → tubes pSB1C3 – t32B (1-8) + date on side
   Digestion to check if insert was there w/ gel electrophoresis
   Cut w/ EcoRI-HF + PstI-HF, incubate more than 1 h, 37 C
   Gel electrophoresis (1.2 % gel, 150ml, 1.8 g agarose)
   Run at 110 V for 30 min +



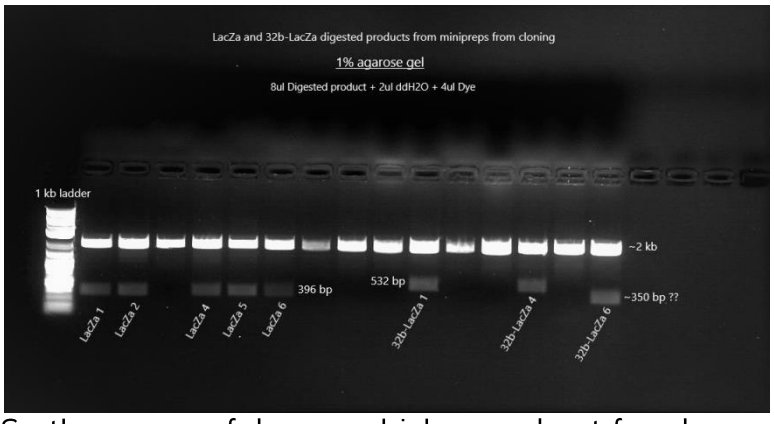
   No bands at 100-200 bp range (will maybe check w/ >2% agarose gel on Friday)

3. Minipreps for yesterday's liquid cultures (with the NucleoSpin® Plasmid (NoLid) kit from Macherey Nagel)
   I used 4ml from each of yesterday's liquid cultures cultures and the rest 1ml was kept for the glycerol stocks.
   The elution was done in 40ul of elution buffer given from the company's kit
4. Restriction Digestion (According to iGEM's protocol for restriction digestion)
   After the minipreps we had ~100 ng/ul, so for the reaction we needed 2.5ul (250 ul)of each minipreped sample (15 samples)

   20ul MasterMix: (The MM was done x17 for the 15 samples)

>14ul of ddH2O
>2.5ul of Cutsmart buffer
>0.5ul of EcorI-HF
>0.5ul of PstI-HF

   Added 17,5ul of the mastermix and 2,5ul of each minipreped sample to each tube.
   Incubation at 37oC for 1,5 h
   Gel electrophoresis
   1% agarose gel, 150ml TAE, run it at 110V for approximately 20 mins



   So the successful ones which were kept for glycerol stocks were LacZa 1,2,4,5,6 and 32B-LacZa 1,4 (the 6th was not the expected size)

5. Minipreps Macherey-Nagel

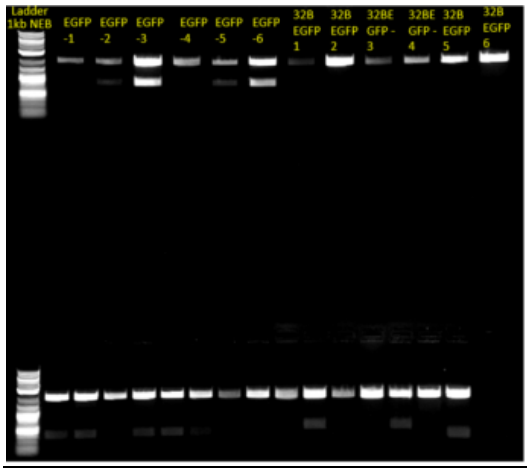
Buffers	Volume(μL)
A1	250
A2	250
A3	300
A4	600
AE	40

   Restriction Digestion

Components	Volume(μL)
DNA	2,5 (250ng)
EcoRI-HF	0,5
PstI-HF	0,5
Cutsmart	2,5
ddH2O	14

- New liquid cultures for 32b EGFP

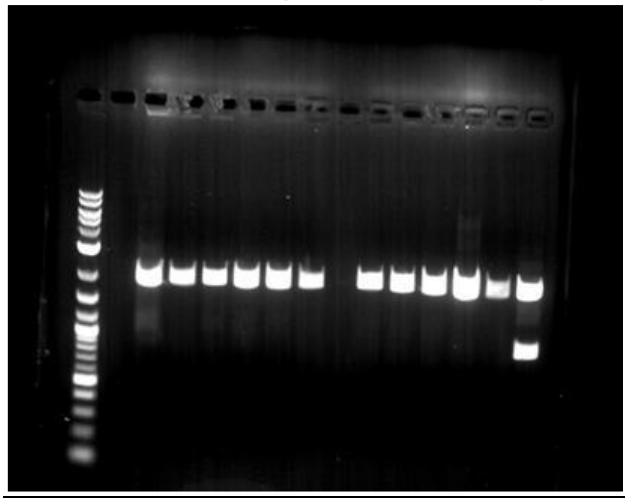
   Results: 1% Gel, 100Volts



6.

- Minipreps of liquid cultures with the MN protocol (NucleSpin Plasmid/ Plasmid NoLid)

• Restriction – Digestion test (1% gel):



• From left to right:
Ladder 1 kb
B-lactamase 1 --> 6
32Blactamase 1 --> 6
• We took the correct pattern band at #6 32Blactamase

Bands at 2kb --> plasmid PSB1C3
1kb --> 32blactamase DNA fragment
• Beta lactamase samples showed no results. Troubleshooting sessions just got started.

Also:
• That day we picked 6 more colonies from the PSB1C3+b-lactamase restored LB agar plates in order to perform the "Liquid culture - Minipreps – digestion – electrophoresis" process again.

• Glycerol stock from the LB medium with the liquid colony #6 that were containing the correct PSB1C3+32Blactamase construct. Store at – 80°C.

5/7/2019

Today's goals:

1. Cloning of IS6110 into pSB1C3
2. Transformation of pSB1C3 into *E. coli DH5a*cells
3. Restriction digestion for the minipreped triggers
4. Glycerol Stocks for yesterday's successful clonings
5. Make glycerol stocks for EGFP construct and repeat the experiment for 32b EGFP construct.
6. Minipreps – digestion – electrophoresis for the PSB1C3+beta-lactamase

Procedure:

1. Restriction Digestion and ligation of IS6110 RFC10 into pSB1C3
   - Usual iGEM Master Mix and reaction for digestion
     For IS6110, used 0.8 ul (around 100ng) to digest properly
   - Ligation was prepared as a Master Mix (4 rxns, X 4.2 as usual)
     Equimolar concentrations were added (12.5 ng) 1:1 ratio
     4 rxns, 10 min RT + NC, 30 min 16oC and overnight at 4 degrees C
     Heat Kill at 80 for 15 min
2. Transformation of pSB1C3 – IS6110 into *E. coli DH5a* competent cells
   - 2 ul ligation used
   - 900 ul LB recovery + 1h incubation
   - Plating on C50 LB Agar

   Incubate over weekend at RT

3. Restriction digestion for the minipreped triggers (Nick's experiment) and gel electrophoresis
   - Again according to iGEM's protocol and the MasterMix was like yesterday's only x6 (5 samples)
   - 17,5ul MM + 2,5ul trigger and incubation at 37oC for 30 mins
   - 2% gel, 60ml TAE, 110V for at least 20 mins we got nothing so there was no insert
4. Glycerol Stocks for yesterday's successful clonings
   25%glycerol stocks for LacZa 1,2,4,6 and 32B-LacZa 1,4 and storage at -80oC (According to protocol)

5. We followed the same procedure that we describe above.->failed
6. No positive results

8/7/2019

Today's goals:

1. Check transformation from 5/7/19 and prepare liquid cultures
2. TA cloning of IS6110 on pGEM-T Easy
3. RPA testing
4. Subcloning of trigger32B into pSB1C3 , 2nd try
5. More RPA testing
6. Cloning of the 32B EGFP fragment
7. 2nd attempt to clone the beta-lactamase construct in PSB1C3 plasmid

Procedure:

1. Inoculate 6-8 5ml LB C50 cultures with pSB1C3-IS6110 single colonies→ didn't do, had no colonies from over weekend transformation.
2. Because of the primer problem discussed above, the next solution is TA cloning of IS6110 RFC10 PCR product into pGEM-T Easy Vector. The basic principle of this is: You have a linearized vector, with T' ends. You create A-tails on your PCR product using a Taq polymerase for a short period of time, and then you ligate them together using the specific kit provided by Promega.

   A-tailing reaction → total volume is 10 ul

   2 ul PCR product (purified)
   (250 ng)
   1 ul 10X Taq Buffer with
   MgCl2
   0.5 ul dATP 10mM
   0.1 ul Taq Polymerase
   6.4 ul ddH2O

   Incubate at 70°C for 30 min.

   - Use 2 ul in a ligation reaction with pGEM-T Easy Vector.

   Ligation Reaction: 10 ul total volume

A-tailed PCR product (25 ng)	1 ul
2X ligation buffer	5 ul
pGEM-T Easy	0.5 ul
T4 DNA ligase (from kit)	1 ul
ddH2O	2.5ul

   Ratio was 3:1, and a positive control was added as well in the experiment

   Incubate at 4°C overnight
3. RPA protocol testing(6 reactions)
   a) Positive Control
   b) Negative Control (no overhangs)
   c) S1F1 – S1R2 rxn
   d) Negative Control (overhangs)
   e) S1F1O – S1R2O
   f) -//-

Typical Reaction:

Primer F	2.4 ul
Primer R	2.4 ul
rehydration buffer	29.5 ul
template + ddH2O (12.2 ul ddH2O + 1 ul DNA (2ng/ul)	13.2 ul

Total 47.5 ul

Vortex and quick spin.
Add to Twist Amp reaction Tube
Add 2.5 ul MgOAc
Quick Spin → Incubate at 37oC for 20min
At 4 min, vortex + spin and put again on 37oC

Didn't do cleanup before running on agarose gel after amplification, result was a smeary gel.

4. Subcloning of trigger 32B (150bp) into pSB1C3 cloning vector (Restriction Digestion, ligation and transformation)
   - Typical digestion of vector w/ iGEM's protocol
     Didn't digest trigger, there was some from last week's digest!!!
     Incubation for plasmid was 1h at 37 degrees C
   - Ligation reaction

     Due to size of fragment, we will try some protocol modifications to resolve the issues occurred on Week 1. Instead of using equimolar amount of insert: vector, we will try 2 different ratios → 3:1 and 5:1. The higher the ratio, the more likely contigs are to be produced. Nevertheless, we can identify them during gel electrophoresis after digestion.

     So, there will be 2 ligation reactions, 3:1 and 5:1 :

     <u>Calculations for 3:1:</u>
     1.625 ng needed. Stock is 12.5 ng/ul from digestion rxn. Dilute 1 ul of digest in 1.2 ul ddH2O. Final concentration is 5.625 ng/ul. Use 1 ul for ligation rxn.

     <u>Calculations for 5:1:</u>
     9.375 needed. Stock is 12.5 ng/ul from digestion rxn. Use 0.75 (0.8) ul from stock without dilutions.
     Typical ligation 10 ul reactions according to iGEM's protocol
     Incubate 2h at 16oC, heat kill 65oC for 15 min

5. Testing our some of our RPA primer sets for PCR to see if they can amplify our product through PCR
   4 Reactions (primer sets used):

   S1F1-S1R1 (a)
   S1F1-S1R1 NTC (a neg)
   S1F1O-S1R1O (b)
   S1F1O-S1R1O NTC (b neg)

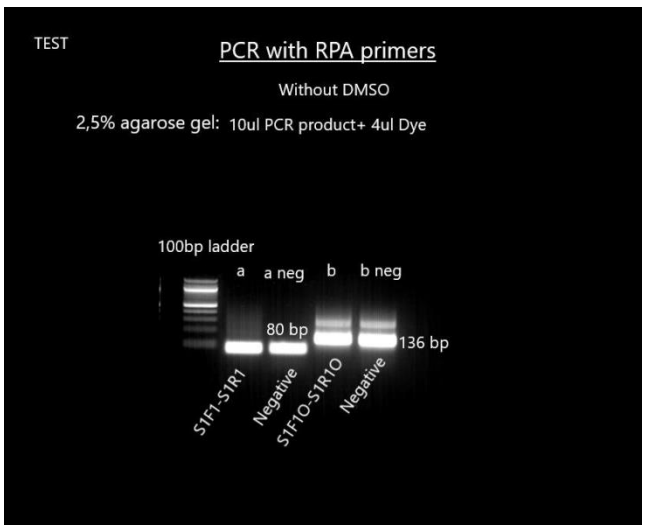
	MasterMix: (the MM was done x5)
PCR water	18,9ul
10X Ktaq Buffer A	2,5ul
10mM DNTPs Mix	0,5ul
5u/ul Ktaq enzyme	0,1ul of

- Added 22ul of MM to each PCR tube
- Added 1ul of template (2ng/ul IS6110 fragment) or PCR water to negatives
- Added 1ul of 10μM forward primer and same for the reverse primer
- Mix and Centrifuge Briefly
- Total Volume of the reaction is 25ul

Cycling Protocol:

95°C for 3 minutes
95°C for 30 seconds
61 and 66 °C for 30 seconds (annealing temp according to NEB's Tm calculator for each primer set)
72 °C for 15 seconds (1kb per min)
Repeat cycles 2-4 34 times
72 °C for 15 seconds

Gel electrophoresis 2,5% agarose gel, 60ml TAE, run at 90V for 30-40 minutes.



- So the NTCs gave us a band which is most likely due to primer-dimers
- So next time we will test the same reaction but with the use of DMSO for more specific amplification.

6. Enzyme master mix :

Components	Volume(μl)
Cutsmart	2,5
EcoRI-HF	0,5
PstI-HF	0,5
DdH2O	19

Ligation:

Components	Volume(μl)
Digested plasmid backbone	2
Digested fragment	2,9
T4 ligase buffer	1
T4 ligase	0,5
DdH2O	3,6

7. Restriction – Digestion:
   • Were performed with iGEM protocol
   • Restriction Enzymes: EcoRI&PstI
   • Quantities: 100 ng PSB1C3 plasmid (stock 25ng/μl)

   100 ng beta-lactamase (stock 40ng/μl)
   The reactions were incubating for 1h at 37oC
   Heat kill for 20min at 82oC
   Ligation reaction for the digested products with the iGEM protocol :
   • Quantities: 25ng digested plasmid
   • With the NEBiocalculator we calculated the required insert mass in order to ligate the digested products with **3:1 ratio** (insert/plasmid)
   • For the 3:1 ligation ratio: 35,8 ng insert mass which corresponds to 2,9μl from the digestion reaction

   The ligation reaction was incubating overnight at 4°C.

9/7/2019

Today's goals:

1. **Transformation of TA cloned IS6110**
2. **RPA testing, 2nd try**
3. **Subcloning of trigger 32B on pSB1A3**
4. **Transformation**

Procedure:

All transformations from Monday 8/7 were unsuccessful, no colonies in any of 3 plates (IS6110 RFC10 1:1, 3:1 & 5:1)

Resolution → since competent cells are good and transformation was executed according to protocol, ligation reaction might be the problem. Indeed, I maybe added too little of the enzyme and the rxn didn't go well. (no mastermixes on those rxns, had to pipette 0.5 ul and place it on the edge of the tube to see it, it could have not fallen down during quick spin).

What to do → clone again t32B (got digest) into pSB1A3 (pSB1C3 is running low)

1. Transformation of TA cloned IS6110, 3:1 into competent cells
   2ul ligation
   1h incubation
   400 ul recovery LB
   Streak on LB Agar A100 plates + IPTG + X-Gal
   Plates w/ IPTG + X-Gal
   For each plate, add 10 ul IPTG 0.1 M and 50 ul X-Gal right before streaking the plate w/ bacteria and spread it evenly with them.
2. PCR cleanup of Monday's RPA and gel electrophoresis, along with 2 new RPA rxns

(for 250 bp amplicon, SXF3O, S1R2O and NC) to check if RPA is working → cleanup + gel electrophoresis.
RPA w/ SXF3O – S1R2O

Master Mix (2rxns, NC + working)

	Mix (X2.2)
2.4 ul SXF3O	5.3 ul
2.4 ul S1R2O	5.3 ul
29.5 ul rehydration buffer	64.9 ul
12.2 ul ddH2O	26.9 ul

- Add 1 ul template or ddH2O on working and NC respectively
- Add 46.5 ul MM to each tube
- Then add them on freeze-drier stuff after vortexing-quickspin.
- Protocol is the same after that.

Results from gel electrophoresis (after PCR cleanup):

3. Subcloning of trigger 32B on pSB1A3
   - Typical digestion of plasmid according to iGEM's protocol
   - Ligation reactions: Negative control, 3:1, 5:1
     Master Mix for 3 rxns (X3.2)

plasmid	1.4ul
buffer	1.8ul
ligase	1.2ul
ddH2O	3.6ul

On tubes: Add template + water to reach volume of 10 ul, then add 7.5 ul MM.Add ligase right before distributing MM into tubes, otherwise pSB1A3 ligating is favored.
Tube names: pSB1A3 – t32B ligation NC, 3:1, 5:1 and name/date on side + o/n

4. Transformation of E.coli DH5A competent cells with the ligated product
   - PSB1C3 + beta-lactamase (ratio 3:1) 1,5μl 100ng in 100μl C.C.
   - PSB1C3 + 32beGFP (ratio 3:1) 1,5μl 100ng in 100μl C.C

Plating at LB agar Chl C=35 μg/ml (2 plates total)
Overnight incubation at 37°C

10/7/2019

Today's goals:

1. **Identification of positive colonies from pGEM-IS6110**
2. **Transformation of ligated pSB1A3-t32B**
3. **Perform PCR for IS6110**
4. **Inoculations from LacZa and 32B-LacZa glycerol stocks**
5. **Liquid cultures**

Procedure:

1. Check plates of pGEM -T Easy – IS6110 (Control + 3:1) →We need the whitecolonies!

Results: Due to high efficiency of protocol and of competent cells, there were too many colonies on the respective plates and picking single ones was not an easy task. Nevertheless, the method worked.

Colony PCR to identify the IS6110 genes on pGEM-T Easy colonies (10 colonies)

- Primer used were PCR FWR 987 (forward) and PCR RVS 987 (reverse) w/ overhangs.

  Program on cycler (Taq Pol):

  95 degrees for 3 min
  95 degrees for 30s
  62 degrees for 30s (annealing)
  72 degrees for 90s (1kb per min +50%)
  Cycle 2-4 : 34 times

  72 degrees for 2 min
  Hold at 4 degrees
  Cycles: 35

  Reactions were of 25 ul total volume.

  Typical reaction:

10X buffer A w/ MgCl2	2.5 ul
Primer F	1 ul
Primer R	1 ul
10mM dNTP mix	0.5 ul
Taq Pol	0.1 ul
ddH2O	19.9 ul

  A master mix (MM) for 12 reactions was prepared (10 colonies, 1 NC and 1 extra)

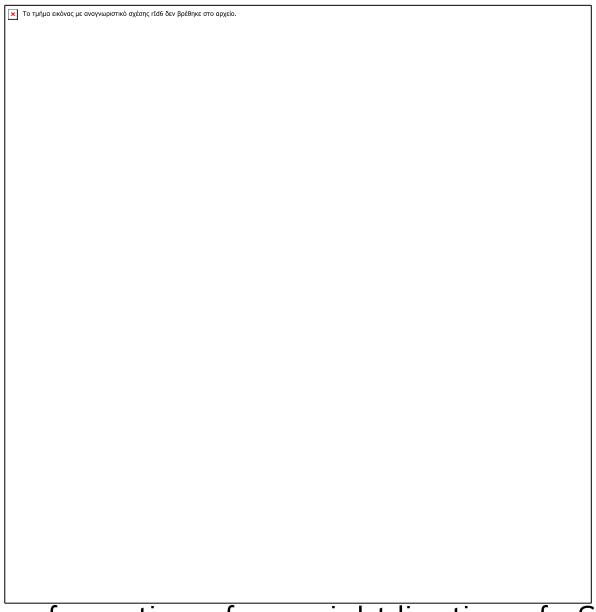
Buffer	30 ul
Primer F	12 ul
Primer R	11 ul
dNTP mix	6 ul
ddH2O	238.8 ul
Taq Pol	1.2 ul

  Add 25 ul of MM to each reaction tube (tubes were named 1-10 for colonies, NC for negative control)

  Inoculate w/ single colonies. Do not inoculate on negative control

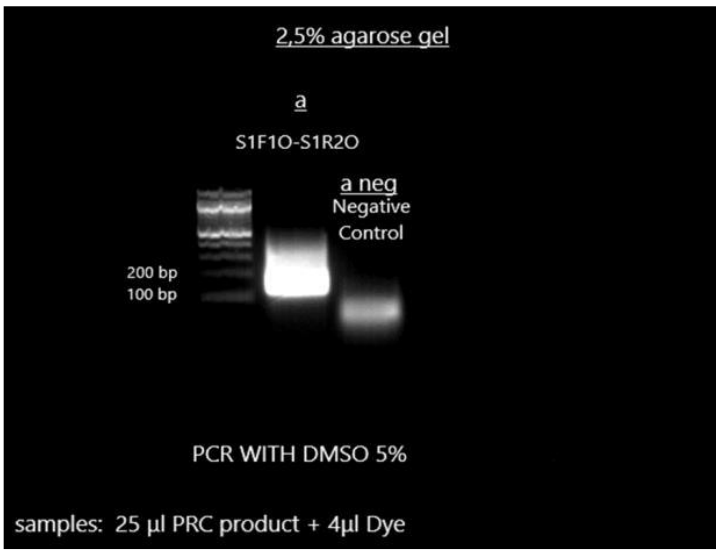
Gel electrophoresis for colony PCR

- Expected band was at 1kb
- Prepared a 1% agarose 60ml TAE gel with 2 rows of 8 wells each.
- Loaded all PCR reaction + 4 ul loading dye
- Result was no band at 1kb.



2. Transformation of overnight ligation of pSB1A3-trigger32B into *E. coli DH5-alpha* competent cells
   - 2 ul ligation transformed
   - 900 ul LB recovery
   - 1h incubation
   - Spin down, resuspend in 150 ul
   - Streak on A100 LB Agar Plates
3. I preformed the same PCR as last time only with slight changes:
   - I used another set of primers (SIF1O-SIR2O) and a NTC
   - Changed the annealing temp to 63oC 'cause last time was incorrect
   - Added DMSO to the PCR MasterMix to a final concentration of 5% (1,25ul)

   The gel electrophoresis ran in the same conditions:



   - Smaller non-specific amplification to the NTC most likely due to the DMSO addition.
   - We see that our primers can amplify our template and also that DMSO can help the reaction for a more specific amplification

4. **Also did some inoculations** on 5ml C35 liquid LB cultures of the samples from my glycerol stocks (LacZa and 32B-LacZa) to test if I did the glycerol stocks correctly
   - I checked only the LacZa 4 and 32B-LacZa 4

- 4 liquid cultures each

5. We picked 8 colonies from each plate (9/7)
   - 8 colonies PSB1C3 + beta-lactamase DH5a
   - 8 colonies PSB1C3 + 32beGFP DH5a

   and inoculated them 5ml LB medium Chl35
   Overnight incubation at 37°C

11/7/2019

Today's goals:

1. **Transformation of pGEM-IS6110, 2nd try**
2. **Prepare liquid colonies for pSB1A3-t32B**
3. **LaZa insert identification**
4. **Inoculation of the rest of the glycerol stocks:LacZa 1,2,5 and 32B-LacZa 1**
5. **Check if the Cloning of 32B EGFP Construct was successful.**
6. **Minipreps – diagnostic digestion – electrophoresis**

Procedure:

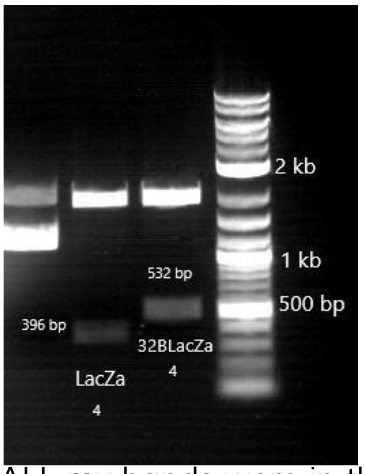
1. Transformation of pGEM-T Easy – IS6110 into DH5-alpha again
   On the last attempt, the colonies were almost indistinguishable, so I tried to dilute a bit before streaking to get good single colonies.
   - 2 ul ligation transformed
   - 900 ul recovery
   - 1h incubation
   - Streak 200 ul and 800 ul on 2 different A100 plates w/ IPTG and X-Gal
2. Check pSB1A3-t32B plates
   Results: Everything good, had colonies both on 3:1 and 5:1, while having less on negative control.
   - Inoculation of 12 LB 5ml A100 cultures w/ single colonies of pSB1A3 – t32B (6 from 3:1 and 6 from 5:1)

3. LaZa insert identification
   Minipreps for yesterday's LacZa 4 liquid cultures
   Same as last time only with the difference that in the bind step I added all the LacZa 4 to the same column and also all the 32B-LacZa to the same column (to concentrate them). Also the elution was done in 50ul of elution buffer provided by the kit.

   Restriction digestion

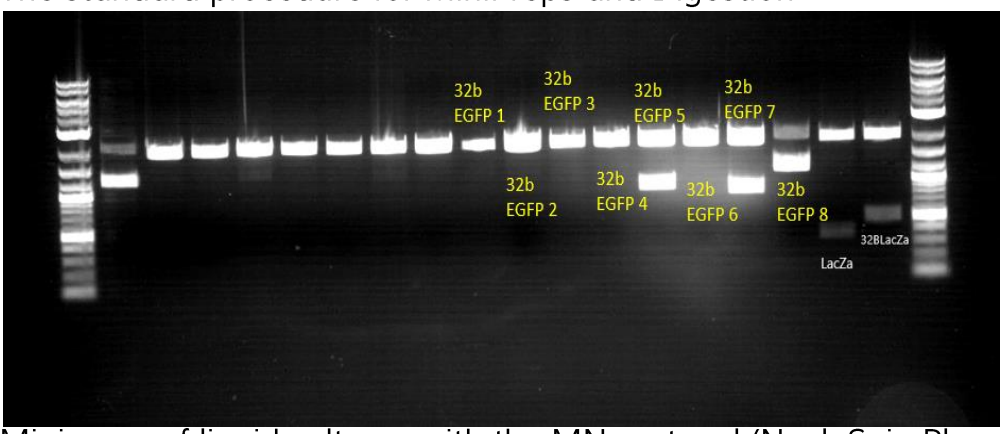
   According to iGEM's protocol again same as last time with the slight change that I added 19ul of MM and 1ul sample to each reaction because I had minipreped samples with a high concentration (put 1,5ul more water to my MM-15,5ul instead of 14ul)

   Gel electrophoresis :1% agarose gel, 60ml TAE, 90V for 15mins



ALL my bands were in the correct size as expected

4. Again inoculation of the rest of the glycerol stocks in 5ml C35 LB liquid cultures to test them on the next day
5. The standard procedure for MiniPreps and Digestion



6. Minipreps of liquid cultures with the MN protocol (NucleSpin Plasmid/ Plasmid NoLid)
   Restriction – Digestion test:

Reagents	Ul per reaction
DNA	2,5
E.coRI HF	0,5
PstI HF	0,5
Cutsmart buffer	2,5
ddH2O	14

12/7/2019

Today's goals:

1. **Identification of positive colonies on pGEM-IS6110**
2. **Minipreps and insert identification of pSB1A3-t32B**
3. **Insert identification for the LacZa 1,2,5 and 32B-LacZa 1 glycerol stock**
4. **Make Glycerol Stocks for 32b EGFP**

Procedure:

1. Identification of positive colonies on pGEM-IS6110

   Check plates for pGEM-T Easy – IS6110.

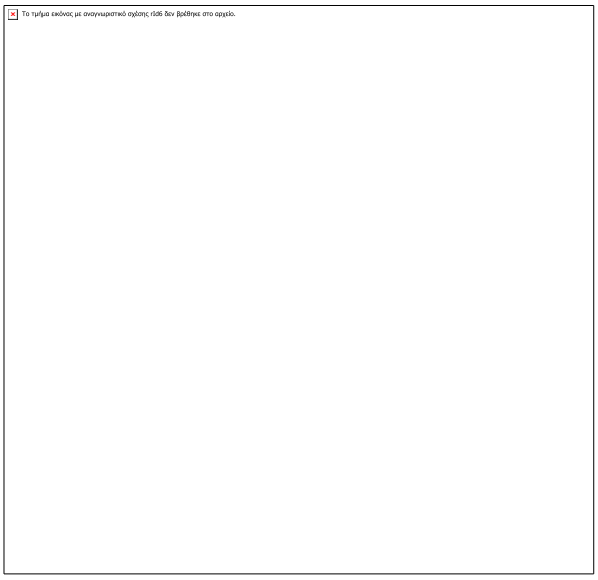
   - 200 ul had some distinguishable colonies!!!

   Colony PCR (19 colonies) from 200 ul plate of pGEM-T Easy – IS6110.

- 25 ul reactions
- Master Mix preparation for 21 reaction (19 colonies, 1 Negative control (NC) and 1 extra)
- Same method used from 10/7/19

Gel electrophoresis of Colony PCR reactions

- Expected band was at 1kb
- Prepared a 1% agarose 60ml TAE gel with 2 rows of 8 wells each.
- Loaded all PCR reaction + 4 ul loading dye
- Ran at 90V for 25 min
- To be able to compare, I added 2 more templates on the gel wells, one was the PCR product that was A-tailed, and the other was the product after the A-tailing process, to see that everything went good on those steps.



- Colonies were kept on a separate A100 LB Agar plate for further utilization.
- Colonies 2 and 10 seemed to have the correct insert expected at 1kb.
- Let the colonies grow a bit and then they were put at 4 degrees C to prevent further growth, so I could use them on Monday.

2. Minipreps from pSB1A3 – t32B (12 minipreps)

- Elution on 40 ul ddH2O
- Tube names → pSB1A3 – t32B 1-12, (Nick 12/7/19 on side)

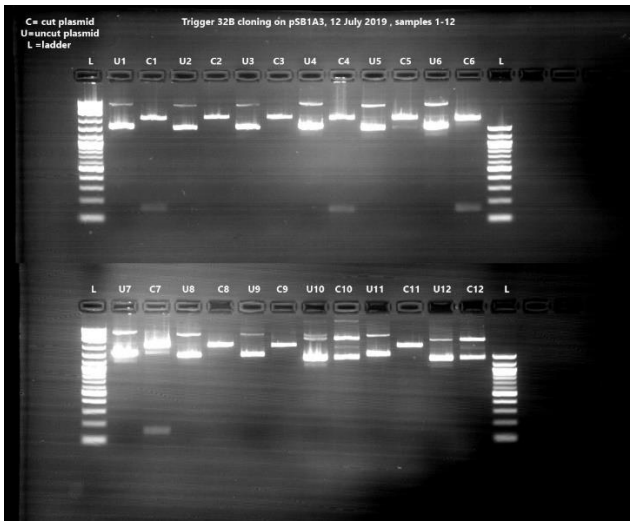
Restriction Digestion w/ EcoRI-HF and PstI -HF for pSB1A3 -t32B

- 20 ul typical reactions
- Incubated 2h30 min at 37 degrees C

Gel electrophoresis of digestion reactions

Expected band was at 150 bp, and I had to run 12 digestion reaction + 12 with uncut plasmid for the control, plus ladders. In order to fit them all together, I prepared a 2% agarose 150ml TAE gel. Ran it on 110V for about 30 min+

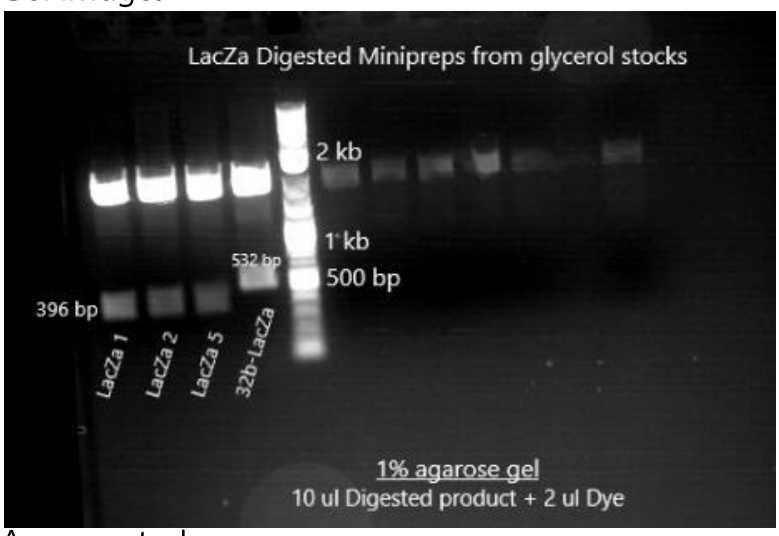
Result:



Samples 1, 4, 6 and 7 had the insert. 1-6 was from 3:1 plate, 7-12 from 5:1 plate. So, 3 clones from the 3:1 ratio and 1 from 5:1 ratio. *Success*!!!

3. **Insert identification for the LacZa 1,2,5 and 32B-LacZa 1 glycerol stock**
   SAME workflow as yesterday but for the rest of the glycerol stocks' liquid cultures:
   - 3 liquid cultures for each glycerol stock (LacZa 1,2,5 and 32B-LacZa 1) 12 liquid cultures
   - in the miniprep step the cultures from the same glycerol stock sample were combined in the same column
   - Had an extra washing step with the AW buffer

   Gel image:



   As expected

4. 500ul Glycerol 50% + 500ul liquid cultures.

15/7/2019

Today's *Goals:*

1. RPA standardization
2. Test RPA efficiency from RPA Experimental Design (segment 1)
3. Prepare Liquid cultures
4. Restriction - digestion & ligation

Procedure:

1. Test RPA efficiency from RPA Experimental Design (Points 1,2,3), segment 2 assigned.

The whole attempt resulted in failure, because there was contamination and there was always a band on the no template control.

- Primer Combinations:

  3S1R2 – S1F1
  4S1R2 – SXF2
  5S1R2 – S7F3
  13 S1R2O – S1F1O
  14 S1R2O – SXF2O
  15 S1R2O – S7F3O

I used 5 strips of TwistAmp Reactions. Each strip holds 8 reactions.

The strips layout was as follows:

First, there were 3 replications of the normal reaction (e.g. 4 4 4 for the primer combination 4) and 3 negative controls, NA (No Amplification, No MgOAc) NP (No primers) and NT (No template)

So, the strip was like:

4 4 4 NA NP NT

The 2 remaining reactions were cut and redistributed for the last combination.

Each reaction received a template of IS6110 at a quantity of 1ng.

- Typical RPA reaction (50ul)

Prepare the following in a separate tube:

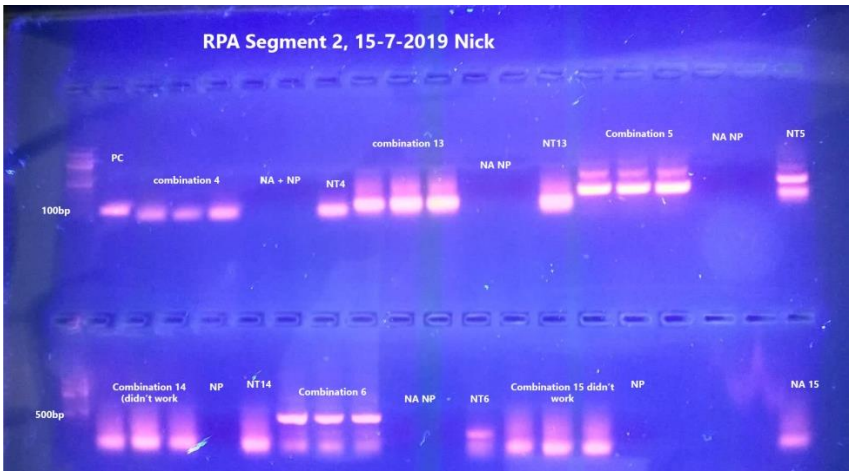
Rehydration Buffer	29.5
Primer F	2.4
Primer R	2.4
ddH2O + template	13.2

Total 47.5 ul

- Vortex + Quick Spin
- Add each mix into its dedicated TwistAmp reaction and pipette up and down.
- Add 2.5 ul MgOAc on the lid of each tube.
- Quick spin the tubes and incubate at 39 for 20 min.

<u>Cleanup of the reaction w/ MN's Nucleospin cleanup kit</u>
Elution in 30 ul ddH2O, eluted twice.

<u>Gel electrophoresis of the samples to check if it worked</u>
1.5 % agarose 150ml TAE gel.
Results:



There were as they seem specific bands at the no template control of every reaction batch (maybe expect combination 6…)

What came to our mind is contamination of our reagents, water, primers, pipettes, equipment with IS6110 amplicon.

2. Test RPA efficiency from RPA Experimental Design (segment 1)

- Primer Combinations:
  1. S1R1 – S1F1
  2. S1R1 – SXF2
  3. S1R1 – S7F3
  10. S1R1O – S1F1O
  11. S1R1O – SXF2O
  12. S1R1O – S7F3O

Used 5 strips of TwistAmp Reactions (8 reactions per strip).
The strips layout was as follows:

- First, there were 3 replications of the normal reaction (e.g. 1 1 1 for the primer combination 1) and 3 negative controls, na (no amplification, no MgOAc) np (no primers) and nt (no template)

So, the first strip was like: (same for the rest of the primer combinations)
1 1 1 na1 np Nt1

- The remaining 2 were used in the last reactions
- Each reaction received a template of IS6110 at a quantity of 1ng.

<u>50ul of RPA reaction</u>
Prepare the following in a separate tube:

Rehydration Buffer	29.5 ul
Forward Primer	2.4 ul
Reverse Primer	2.4 ul
ddH2O + template	13.2 ul

Total 47.5 ul

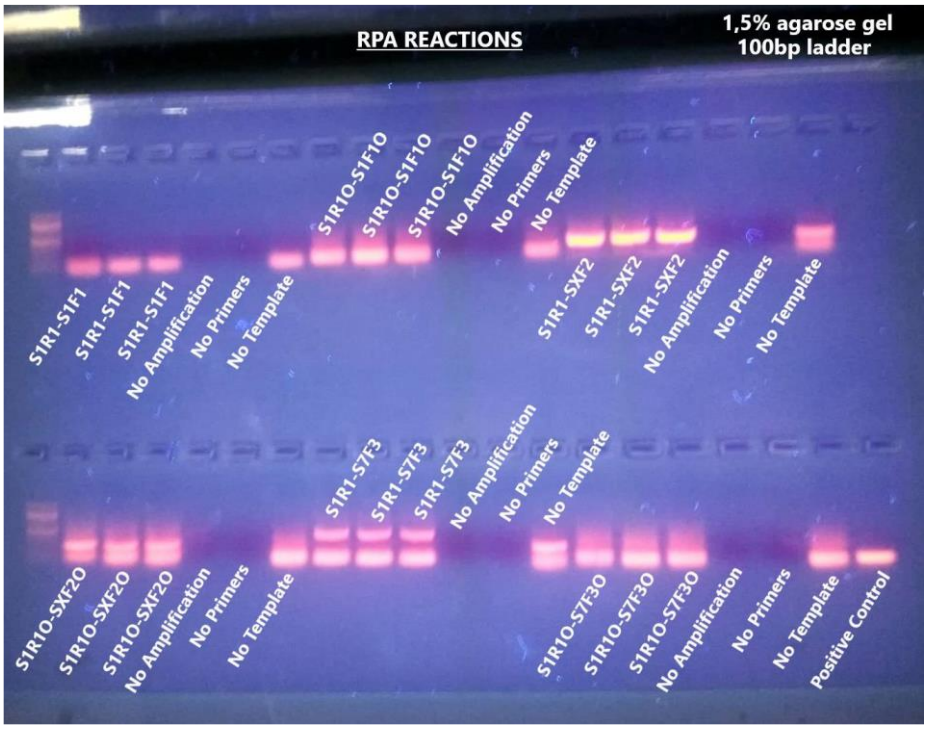
- Vortex + Quick Spin
- Add each mix into its dedicated TwistAmp reaction and pipette up and down.
- Add 2.5 ul MgOAc on the lid of each tube.

- Quick spin the tubes and incubate at 39oC for 20 min with an in-between step of quick spin at 4 minutes of reaction

<u>Cleanup of the reaction w/ MN's Nucleospin cleanup kit</u>

Elution in 30 ul ddH2O, eluted twice.
<u>Gel electrophoresis of the samples to check if it worked</u>

1.5 % agarose gel, 150ml TAE, 110V for at least 20mins.
Gel image:



Although amplified, there was contamination on the NTs and there was always a band present.
We concluded that there was a contamination.

3. We prepared two liquid cultures for each glycerol stock (EGFP, 32B EGFP, 32B Lactamase).
4. This was the third attempt to clone the beta-lactamase construct.
   We used the PSB1A3 this time instead of PSB1C3.
   - Were performed with iGEM protocol
   - Restriction Enzymes: EcoRI&PstI
   - Quantities: 100 ng PSB1A3 plasmid (stock 25ng/μl)

   100 ng beta-lactamase (stock 40ng/μl)
   The reactions were incubating for 1h at 37°C
   Heat kill for 20min at 82°C
   Ligation reaction for the digested products with the iGEM protocol :
   •Quantities: 25ng digested plasmid

   With the NEBiocalculator we calculated the required insert mass in order to ligate the digested products with **5:1 & 3:1 ratio** (insert/plasmid)
   For the 3:1 ligation ratio: 35,8 ng insert mass which corresponds to 2,9μl from the digestion reaction
   For the 5:1 ligation ratio: 59,62 ng insert mass which corresponds to 4,8μl from the digestion reaction
   The ligation reaction was incubating overnight at 4°C.

16/7/2019

Today's goals:

1. RPA standardization
2. Liquid cultures for IS6110
3. Liquid cultures for trigger32B
4. PCR reaction with all primer sets for IS6110
5. Isolate the plasmids in big concentration for our in vitro experiments
6. Transformation

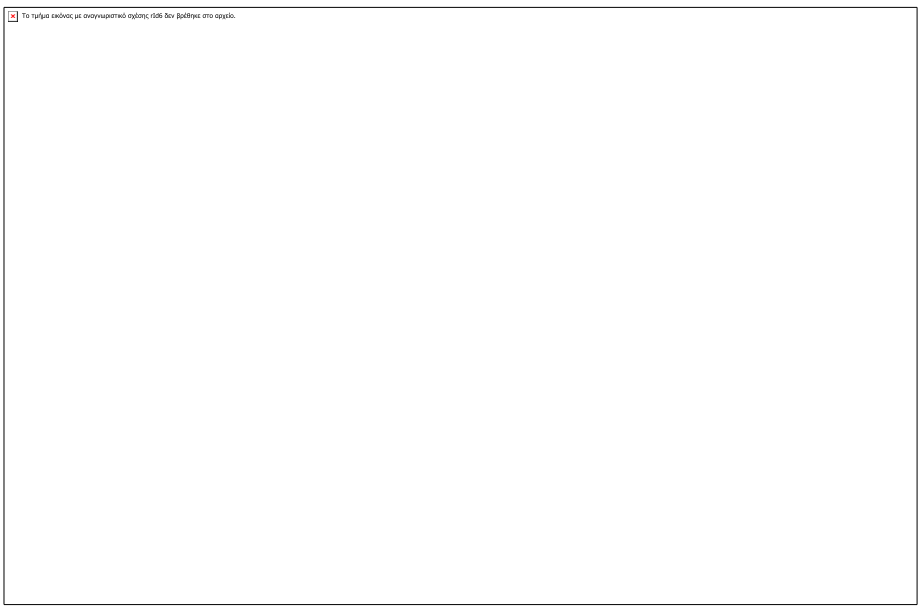
Procedure:

1. Trying to elucidate what could have gone wrong with Monday's results, I repeated the segment 2 in pairs this time, in order to reduce the reaction I was simultaneously doing and prevent human error as much as possible.
   - Combination pairs:

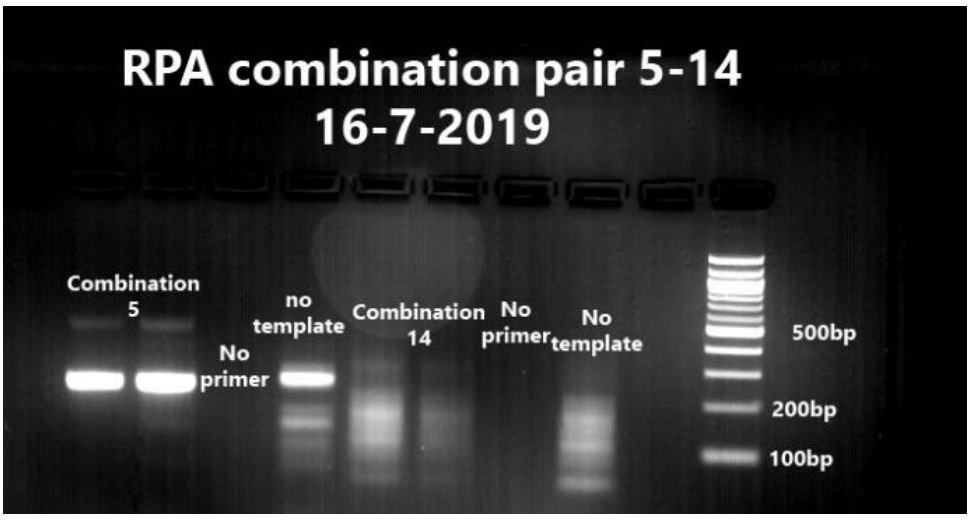
   4-13 (S1R2-S1F1 with and w/o overhangs)
   5-14 (S1R2 -SXF2) w/ and w/o overhangs)
   Procedure was the same as yesterday.

   Results:

   Combination 4-13



   Combination 5-14



- Again, there was a specific band on the no template control.
- Thinking that it was the buffer that was contaminated, we opened a new RPA kit and tested negative controls both with the new and old buffer.
- Results → Contamination on both NT controls.
- New ddH2O, new primer aliquots, new everything…
- Maybe the temperature is the problem, so I will try again tomorrow to check how the reaction perform under different temperatures.

2. Inoculation of 4 LB 5ml A100 cultures for pGEM IS6110
   The goal of this step is to obtain dense miniprepped DNA from the successful clonings from 12/7/19
   Two cultures for each clone, so 4 for IS6110 (2, 10)
3. Inoculation of 8 LB 5ml A100 cultures for pSB1A3 – t32B
   The goal of this step is to obtain dense miniprepped DNA from the successful clonings from 12/7/19
   Two cultures for each clone, so 8 for t32B (1, 4, 6, 7)
4. PCR reaction with all primer sets for IS6110

   To establish if there is a problem with our primers and if there is a contamination I ran a PCR reaction with all the primer sets with overhangs we had designed.

   - Primer sets:
     10. S1F1O
     11. SXF2O
     12. S7F3O
     13. S1F1O
     14. SXF2O
     15. S7F3O
     16. S1F1O
     17. SXF2O
     18. S7F3O

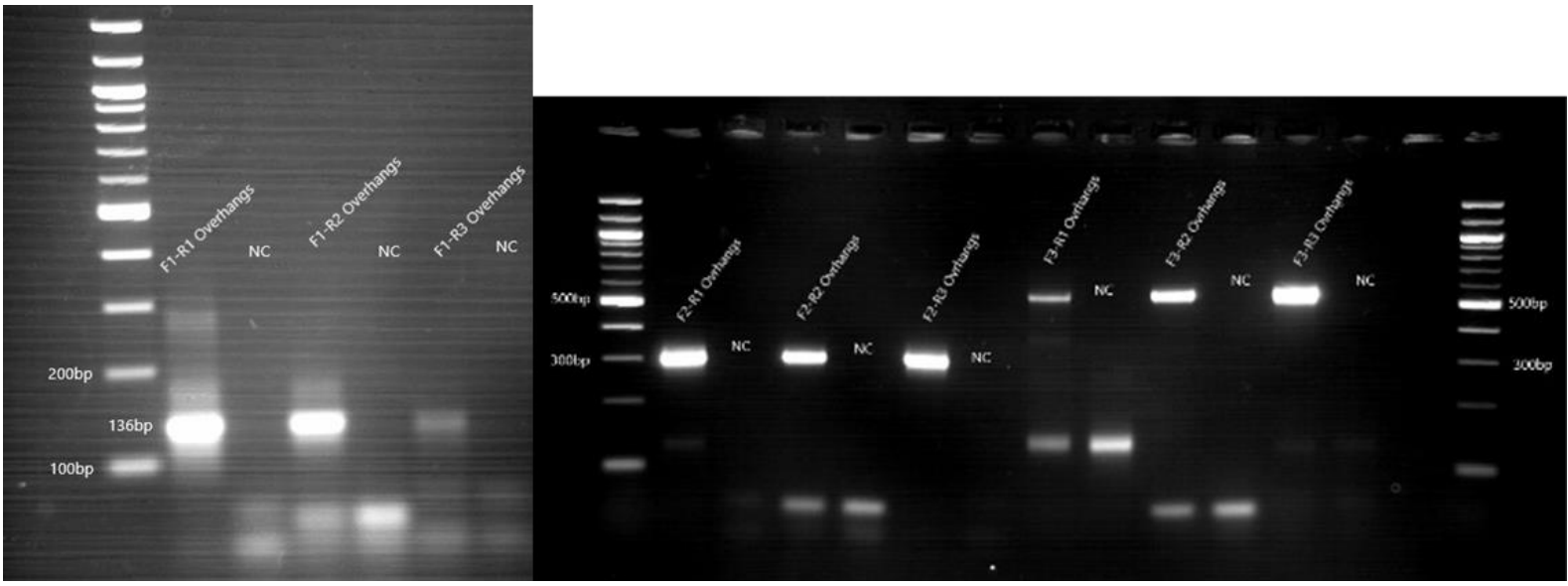
   - ALSO I added in the reaction a non-template control (NTC) FOR EVERY PRIMER SET.
   - So the reaction was in the following order: 10, nt10, 11, nt11, 12, nt12 etc.

   The PCR reaction was the same I used last time (see Monday 8/7/19) only with the addition of DMSO at a final concentration of 5% per reaction.

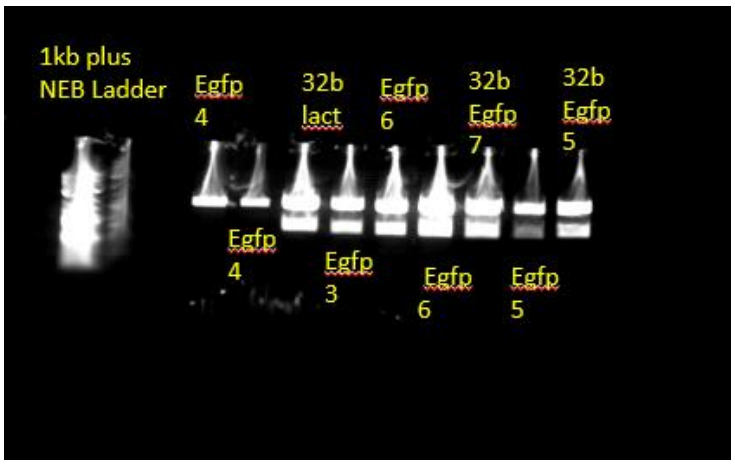
   <u>The cycling protocol was also the same as Monday 8/7/19 with small changes in the primer annealing temps:</u>

   For S1F1O with all reverse primers (reactions 10,13,16) = 66oC
   For SXF2O with all reverse primers (reactions 11,14,17) = 67oC
   For S7F3O with all reverse primers (reactions 12,15,18) = 68oC

Gel electrophoresis:3% agarose gel, 150ml TAE , 2 gels



5. We followed the standard protocols that I describe above for Minipreps and Restriction Digestion



6. Transformation of E.coli DH5A competent cells with the ligated product
   - PSB1A3 + beta-lactamase (ratio 3:1) 1,5μl 100ng in 100μl C.C.
   - PSB1A3 + beta-lactamase (ratio 5:1) 1,5μl 100ng in 100μl C.C

   Plating at LB agar Amp C=100 μg/ml (2 plates total)
   Overnight incubation at 37°C

17/7/2019

Today's goals:

1. Minipreps for IS6110 and t32B
2. RPA testing in different temperatures
3. PCR reaction with all primer sets for IS6110
4. Clone the constructs 32B EGFP and Trigger 32B into the same plasmid(psb1k3) and EGFP construct into psb1k3
5. Liquid cultures with the DH5A containing PSB1A3 + beta-lactamase

Procedure:

1. Minipreps from Tuesday's cultures. Same clones go to the same column. Elution in 40 ul
   pSB1A3-t32B 4 has a concentration of 64.6 ng/ul
   Restriction Digestion w EcoRI and PstI.
   Gel electrophoresis
   Results:

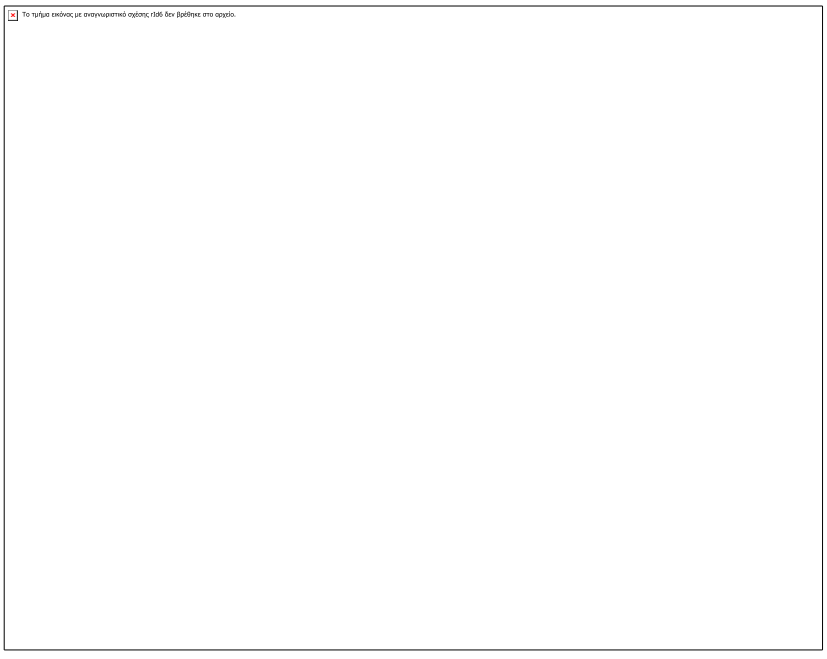
2. RPA test negative controls + 1 working for RT, 37, 39 and 42 degrees C
   - 8 reaction total, 1 working and 1 negative control each
   - Typical reaction, elution after cleanup at 30 ul
   - Run 10 ul + 2 ul dye

   Gel electrophoresis of temp test

   1.5 % gel didn't perform well

After that, I prepared a 3% agarose gel and ran the samples again. Ran 90V 45 min.

Results:

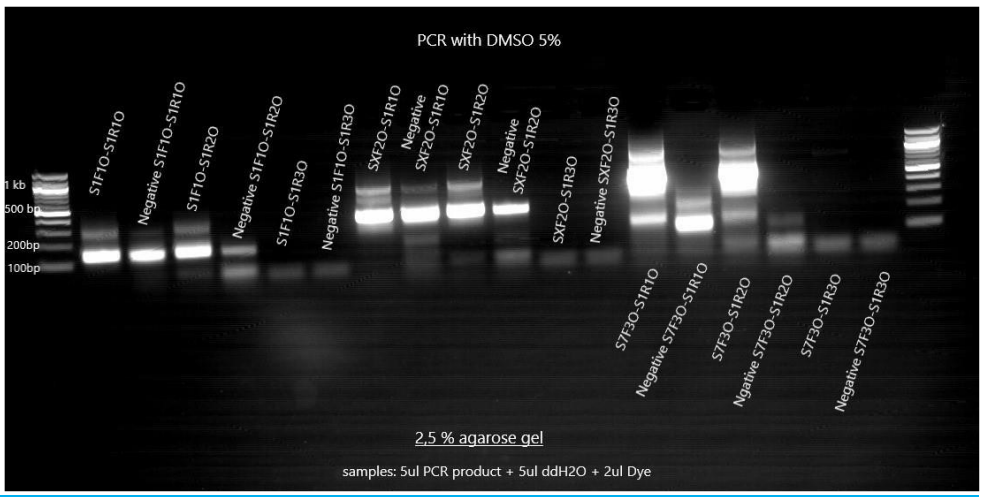


The reaction worked even at RT and showed far better bands at 3% agarose. A band on negative controls persists though.

3. PCR reaction with all primer sets for IS6110

Again PCR reaction with same primer sets, with slight changes in the previous conditions:

- Smaller amount of primers (added 0,88ul and added more water to the reaction accordingly)
- 1ng/ul template instead of 2ng/ul
- ran a 2,5% gel with fewer pcr product and more water. (5ul PCR product + 5ul water + 2ul Dye)



- Again not a big difference BUT We can clearly see that the primer set that we designed for our proof of concept (S1F1O-S1R2O) is clearly the best because not only it amplifies the region we want to but it has a very vague band on the negative control.

4.

Components MixA	Volume(µl)
Cutsmart	5
EcoRI-HF	1
PstI-HF	1
DdH2O	38

Components MixB	Volume(µl)
Cutsmart	2,5
EcoRI-HF	0,5
SpeI-HF	0,5
DdH2O	19

Components	Volume(µl)
Cutsmart	2,5
XbaI	0,5
PstI-HF	0,5
DdH2O	19

The first ligation reaction was performed with 2,5 ul of 100ng/ul digested Psb1k3 and a 3:1 ration of insert EGFP to Psb1k3.

Components	Volume(ul)

Digested Psb1l3	2,5
T4 Buffer	1
T4 ligase	1
Insert EGFP	3
DdH20	3

The ligatio reaction between insert 32B egfp, trigger and the digested vector was done on a ration of 3:3:1.

Components	Volume(ul)
Digested Psb1l3	2,5
T4 Buffer	1
T4 ligase	1
Insert 32b EGFP	3
Trigger	1
DdH20	3

5. We picked 6 colonies from each plate and inoculated them at 50ml falcons containing LB medium (5ml, Amp C=100μg/ml)

Overnight incubation at 37oC
The plates were saved at 4 oC for colony PCR

- In order to improve a part, we had to work in vivo with the M15 E.coli strains that EPFL 2017 had sent to us. These cells have already resistance in Chloramphenicol so we had to change the working plasmid PSB1C3 to a plasmid with resistance to a different antibiotic. This will allow us to choose the right colonies in the future. We chose to move on with the PSB1K3 plasmid (resistance in kanamycin) so we had to start the clonings from the beggining.

Restriction- digestion:
1) 100ng PSB1K3 plasmid
2) 100ng LacZa
3) 100ng 32Btoehold/LacZa and
4) 50ng trigger for 32b toehold

- Were performed with iGEM protocol
- Restriction Enzymes: E.coRI&PstI for PSB1K3 and LacZa

E.coRI&SpeI for 32Btoehold/LacZa
XbaI&PstI for trigger
The reactions were incubating for 1h at 37oC
Heat kill for 20min at 82oC
Ligation reactions:
A) PSB1K3 with LacZa

- Quantities: 100ng digested plasmid corresponds to 2 μl

With the NEBiocalculator we calculated the required insert mass in order to ligate the digested products with 3:1 ratio (insert/plasmid)
For the 3:1 ligation ratio: 15ng insert (LacZa) mass which corresponds to 1,2μl from the digestion reaction.
B) PSB1K3 with 32Btoehold/LacZa and trigger: *3A Assembly (iGEM Protocol)*

- Quantities: 100ng digested plasmid corresponds to 2μl

6,3ng trigger (for 3:1 ratio) corresponds to 1μl
20ng 32Btoehold/LacZa (for 3:1 ratio) corresponds to 1,6μl
Once again, we calculated the required insert mass with the NEBiocalculator.

- Process: We cut them all separatelly and we mix them all together in one reaction for the ligation

The ligation reactions were incubating overnight at 4°C

18/7/2019

Today's goals:

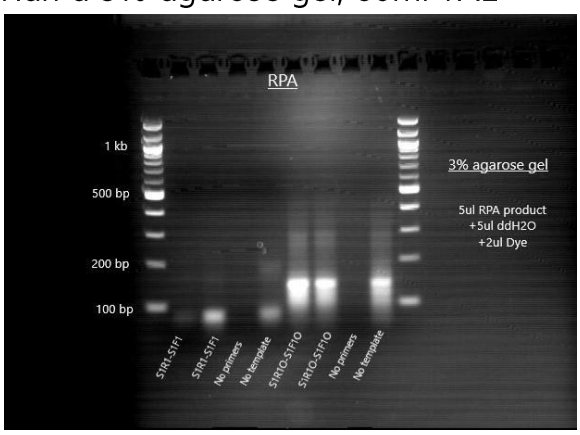
1. Do RPA testing
2. Transformation
3. Minipreps – digestion – electrophoresis for beta-lactamase and Transformation of DH5A competent cells with the ligated products

Procedure:

1. Even though this was the plan, I checked again combination pair 4-13 and after it gave me the same results, so I stopped to save as many reaction we have left as possible.
   - Looking for the contamination source

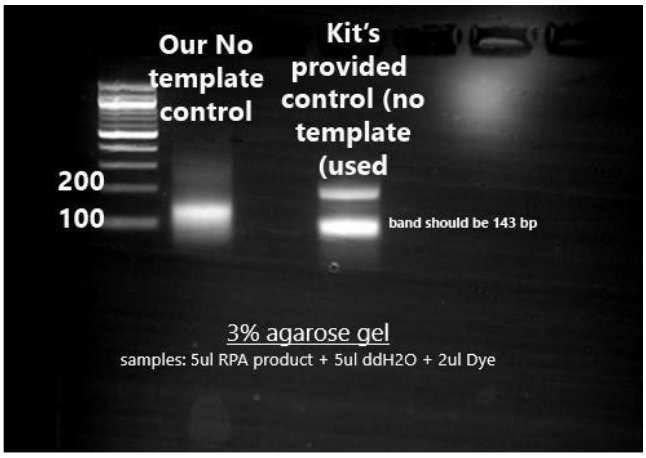
   While trying to find what is the problem with RPA testing, I cleaned all equipment and benches with 10% bleach to disinfect with any DNA that has been contaminating the no template controls.
   - I ran RPA reactions for the following sets:
     1. S1R1-S1F1
     10. S1R1O-S1F1O
     - Each had 2 technical replicates and 2 negative controls (1 no primers control, 1 no template control)
     - The reaction setup was as follows: 1, 1, np1, nt1, 10, 10, np10, nt10
     - The RPA reaction was the same as last time as given by the company's handbook plus the clean-up step

   Ran a 3% agarose gel, 60ml TAE



AGAIN we have bands on our negative control so we need to continue testing the reaction to establish if there is a contamination

- I ran another RPA reaction this time only for the non-template control and also for a non-template control of the company's given positive control



We concluded that there is a contamination so everything in the lab was cleaned as well as all the equipment and benches with 10% bleach to disinfect with any DNA that has been contaminating the non-template controls

2. The standard protocol (50ug/ml kanamycin), 2 plates: one for EGFP, one for 32B EGFP.
3. Minipreps of 12 liquid cultures (MN protocol) (6 for 3:1 ligation ratio and 6 for the 5:1 ligation ratio). -> Restriction – Digestion test : *incorrect pattern of DNA bands*

19/7/2019

Today's Goals:

1. **Extensive lab cleanup**
2. **PCR and RPA reactions for IS6110**
3. **Check if the cloning of EGFP and 32b EGFP/Trigger was successful.**
4. **Colony PCR**

Procedure:

1. Disassemble pipette and UV them for 20 min in Super New Laminar (0.1-2, 0.5-10, 10-100 pipettes). Plastic parts were also washed with 80% ethanol.
2. After extensive cleanup of most of the lab, I will try again the RPA reaction with new ddH2O, as well some reactions with DMSO (to increase specificity)
   The protocol was somehow modified, but PCR first:

10X buffer A w/ MgCl2	2.5 ul
Primer S1R2O	0.8 ul (instead of 1)
Primer S1F1O	0.8 ul (too)
dNTP mix	0.5 ul
Taq Pol	0.1 ul
ddH2O + template	20.3 ul

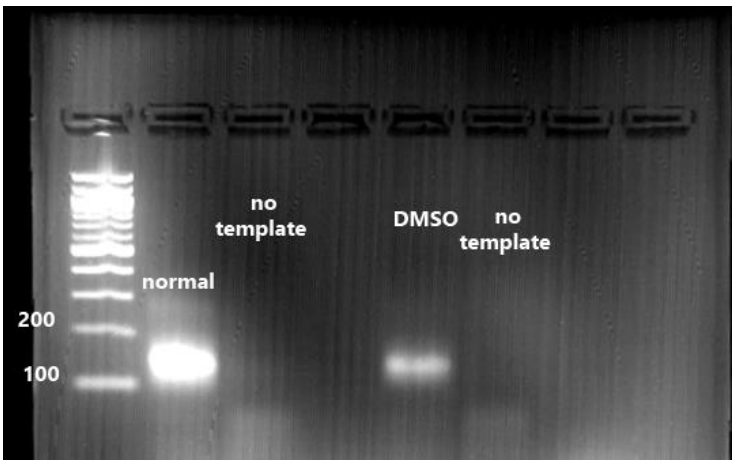
Total reaction volume 25ul

25 ul rxn w/ DMSO:

Same as above, replace 1.25 ul ddH2O with molecular grade DMSO (19 ul ddH2O + template instead of 20.3)
I will add 1 ul of 0.075 ng/ul template, or 1 ul ddH2o in the NC's
MM for 5 reactions (1 extra):

Buffer X 5	12.5 ul
S1R2O X 5	4 ul
S1F1O X 5	4 ul
dNTP mix X 5	2.5 ul
Taq Pol X 5	0.5 ul

Total reaction volume 25ul
Annealing temp is 66 degrees C.
Results after gel electrophoresis at 3% agarose gel (90V run, 45 min)



After so much pain and cleaning, we finally had a clear lane of no template control. Happy as I was, I prompted to try the exactly same procedure with RPA. During this PCR set up, I was extra careful, worked around flame, opened and closed tubes with extra care. The post PCR work was done in a different bench with different pipettes. I did all those things for RPA as well.

RPA modified volumes

Primer S1R2O	1.8 ul
Primer S1F1O	1.8 ul
Reh. Buffer	29.5 ul
ddH2O + template	14.4 ul

Total reaction volume 50ul

- For DMSO reaction, again 1.25 ul of ddH2O was exchange with DMSO.
- Again, 1 ul of 0.075 ng/ul was used for the reactions.

The order of setting up the reaction is:

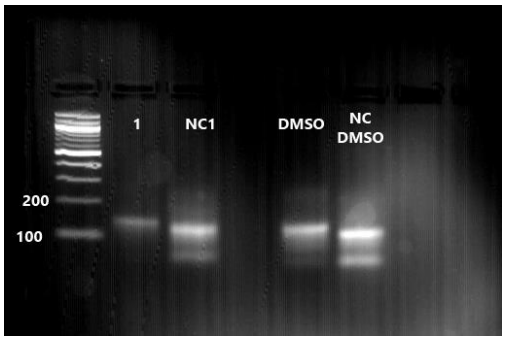
1. Add buffer

2. Add ddH2O (accordingly, negative controls get more)
3. Add primers
4. Add template

Cleanup of the reaction and elution in 30 ul ddH2O.

Gel electrophoresis on 3% agarose gel, at 90V for 45 min

Results:



Opposing to the successful PCR, the no template control had a thicker band than the real sample.

It is maybe the Magnesium Acetate that is contaminated, or the rehydration buffer. We will counsel with as many people as we can before opening the last RPA kit with unopened reagents.

Serial dilutions of template to test sensitivity and RPA problems

One comment from TwistDx is that we should use little amounts of template in order to avoid contamination. According to this, and also to our need for checking the sensitivity of the reaction, I prepared serial dilutions of IS6110. We will maybe try our luck next week.

The dilutions start from 0.01 ng/ul (10^{-2}) and go down one order of magnitude at a time to final dilution of 10^{-9} (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}). 10^{-8} corresponds to around 10 copies per ul. Our smallest attempted concentration so far was 0.075 ng/ul, so maybe this was the problem. Morenext week!

3. Colony PCR:

We picked 13 colonies from each plate.

Components	Volume (ul)
10Mm dNTPs	15
5u/ul Taq Pol	7,5
10 X Buffer	75
Forward and Reverse primer	15 ul each
DdH2O	616

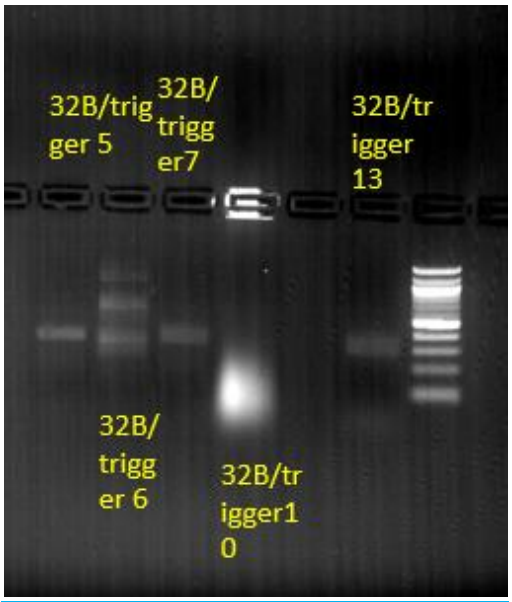
Standard Settings: Every colony pcr was done using the below settings. However the elongation times was changing according to the size of the constructe.g 1320bp x 0.06 = 79

Elongation time = 79 seconds.

Temp (°C)	Time(seconds)	Passes
95	180	1
95	30	34
53	30	34
72	79	34
72	120	1
4	infinity	1

The PCR was running for 35 cycles at 53°C annealing.

Results: Failure



4. 3 batches of CPCR reactions:

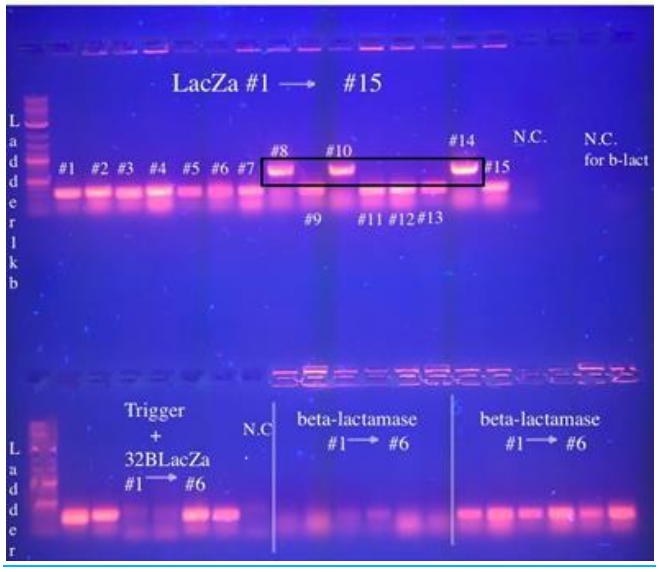
#1: PSB1K3 with LacZa (colonies picked:15)
#2: PSB1K3 with 32Btoehold/LacZa and trigger (colonies picked:6)
#3: PSB1A3 with beta-lactamase colonies (colonies picked: 6 from plate 3:1 and 6 from plate 5:1. Those plates were from 16/7 and have been saved at 4 oC) (L+T)
Preparation of 3 MasterMixes for the 3 colony PCR reactions:

Reagents	Volumes	Mix #1 LacZa	Mix #2 L + T	Mix #3 b-lact
Template (or water)	1µl (20ng)	-	-	-
PCR water	18,9 µl	338,3 µl	159,2 µl	278,6 µl
10x kapa taqbuffer A	2,5 µl	42,5 µl	20 µl	35 µl
10µM dNTPs Mix	0,5 µl	8,5 µl	4 µl	7 µl
10µM Forward Primer	1 µl	17 µl	8 µl	14 µl
10 µM Reverse Primer	1 µl	17 µl	8 µl	14 µl
Kapa taqpolymarase (5u/µl)	0,1 µl	1,7 µl	0,8 µl	1,4 µl

Colony PCR for 35 circles -> Gel electrophoresis 1% run at 150 V
DNA ddH2 dye ladder

O

5 μl 5 μl 2 μl 4μl



20/7/2019

Today's Goals:

1. Prepare liquid cultures and Cloning of the 32B EGFP/Trigger construct

Procedure:

1. Standard procedures

21/7/2019

Today's Goals:

1. pSB1K3 digestion
2. Transformation of the construct 32B EGFP/TRIGGER and check if the cloning of EGFP construct was successful.
3. Inoculation

Procedure:

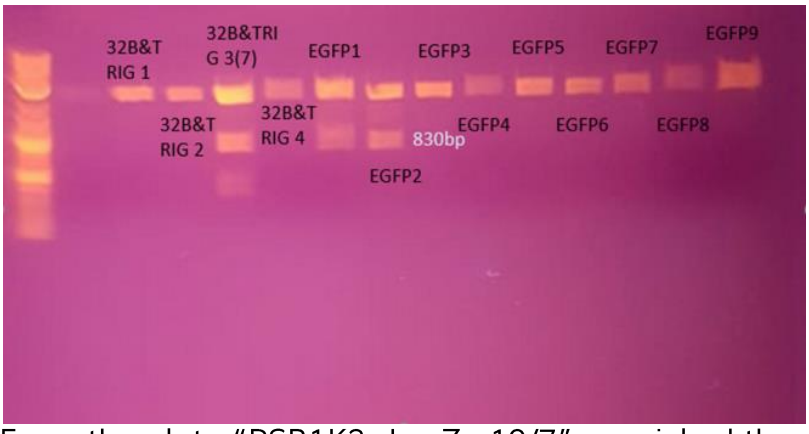
1. Because pSB1K3 is depleted and I need it to clone both beta-lactamase and trigger 32B, I digested a positive clone of pSB1K3-eGFP and isolated it from an Agarose gel.
   Digestion of pSB1K3 from cloned eGFP plasmid
   20 ul reactions → from 2 clones (P1 and P2)
   Typical reaction:

		(Master Mix for 2.2 reactions):
DNA	10 ul	-
Cutsmart	1 ul	4.4 ul
ddH2O	6 ul	13.2 ul
EcoRI	1 ul	2.2 ul
PstI-HF	1 ul	2.2 ul

- Add 10 ul MM + 10 ul DNA, incubate at 37 for 2h+
- Heat kill 80 degrees C/20 min

2. Standard.

   Results: Success for EGFP



3. From the plate "PSB1K3+LacZa 19/7" we picked the colonies #8 and #10 and we inoculated them in 5ml LB Kan50, in order to confirm that these colonies have the right insert in the plasmid via minipreps and diagnostic diagestion.

22/7/2019

Today's Goals:

1. **Isolation of digested pSB1K3**
2. **Ligation of beta-lactamase and trigger**
3. **Prepare Glycerol Stock for EGFP and Liquid cultures for 32BXTrigger EGFP.**
4. **Minipreps – digestion – electrophoresis for lacZa- PSB1K3**

Procedure:

1. Gel electrophoresis of Sunday's digestion
   - 60 mL TAE 1% agarose (0.6 g), 8 well comb
   - Run at 90V for about 25 min
   - Reactions are 20 ul, split them in 2 (10 +10) and add 2 ul loading dye to each.
   - Load them to the gel. Same clone goes side by side, different clones are separated by one empty well
   - Expected band is at 2kb

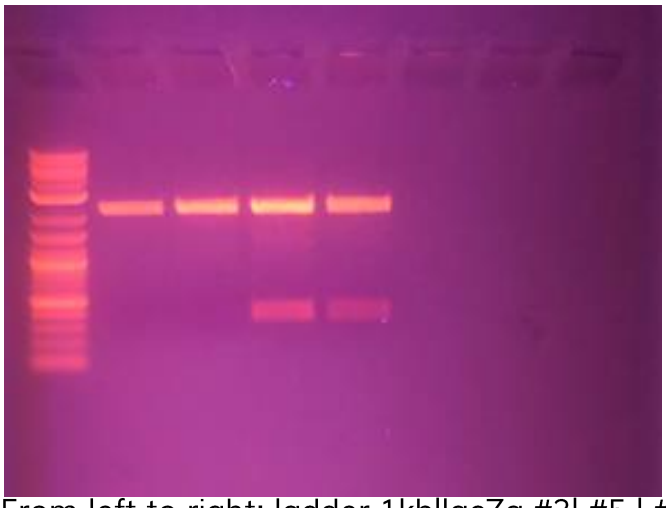
   Gel excision of desired bands (they were there!!!)
   Gel extraction protocol (MN kit) → elution in 20 ul
   Each clone ended up in a different silica column. This means that there were 2 wells per column
   Qubit to quantify the extracted DNA (1 ul per sample used)
   Clone 1 → 55.2 ng/ul
   Clone 2 → 38.6 ng/ul
   In order to have the vector ready to be ligated in terms of concentration, I added a proper dilution step after quantification.
   Sample 1 → 55.2 ng/ul. Desired concentration is 25 ng/ul.
   C1V1 = C2V2 ➔ x = 2.2 ul (diluted 1 ul of DNA into 1.2 ul ddH2O to get desired concentration)
   Multiplied by 19 for the volume of my sample, 19X1.2 = 22.8 ul
   Add 22.8 ul into 19 ul DNA to have a final volume of 41.8 ul DNA of 25 ng/ul/

Sample 2 → 38.6 ng/ul. I made a mistake and the final concentration is 15 ng/ul
For the desired conc of 25 ng/ul, the x = 1.54. This meant that I had to add 1 ul of sample into 0.54 ul ddH2O. I made the mistake of adding 1.54 water, so the result was a more diluted DNA (15 ng/ul to be exact). This is not so bad though, since a ligation reaction typically uses 25-30 ng of vector DNA, so we are covered by that concentration.
1.54 X 19 ul sample = 29.3 ul ddH2O
Add 29.3 ul ddH2O into 19 ul sample DNA for a final concentration of 15 ng/ul in a final volume of 48.3 ul.

2. Digestion of beta-lactamase from IDT stock (total 200 ng, 4 ul, 2 rxns) and ligation of beta-lactamase and trigger32B (separately) into pSB1K3
   Typical iGEM protocols, 8 ul digestion rxn, 10 ul ligation rxns, incubation o/n at 4 degrees C for the ligations after hot-starting them at RT for 10 min.
   MM X 3.2 was prepared for the ligation reaction. Insert:Vector ratio was 3:1 for all inserts.
3. Standard.
4. Minipreps of 4 liquid cultures (MN protocol)

1) PSB1K3 – LacZa #3 #5 #8 #10

- Restriction – Digestion (as previously)
- Electrophoresis



From left to right: ladder 1kb|lacZa #3| #5 | #8 |#10

- Correct pattern of bands: 2kp --> vector

400bp --> LacZa

- Glycerol stocks (500ul liquid culture + 500ul glycerol 50%)

2 tubes: PSB1K3 – LacZa #8
2 tubes: PSB1K3 – LacZa #10

23/7/2019

Today's Goals:

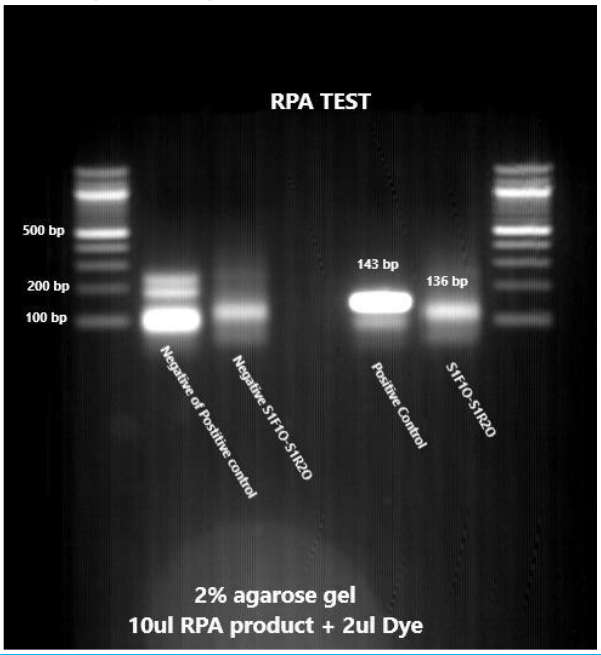
1. **Transformation of trigger32B and beta-lactamase ligated constructs**
2. **RPA testing for the S1F1O-S1R2O set**
3. **Check if the cloning of 32b egfp x trigger was successful.**
4. **Colony PCR**

Procedure:

1. Transformation of pSB1K3 – t32B and pSB1K3 – b-lactamase into DH5-alpha competent cells
   - 2 ul ligation mix transformed
   - 900 ul LB recovery
   - 1h incubation
   - Spin down 9k x g, 1 min
   - Resuspend in 100 ul and streak on LB Agar K50

2. After last week's results I wanted to test the kit's positive control again for contamination, and also to test our proof-of-concept primer set (S1F1O-S1R2O) once more in an RPA reaction
   The RPA reaction was conducted as usually according to the company's manual.
   4 reactions:
   - Positive Control
   - Positive Control's non-template control
   - S1F1O-S1R2O
   - S1F1O-S1R2O non-template control

   The RPA reaction was followed by an clean-up with a 30ul elution step in the end (twice eluted)
   2% agarose gel, 60ml TAE,



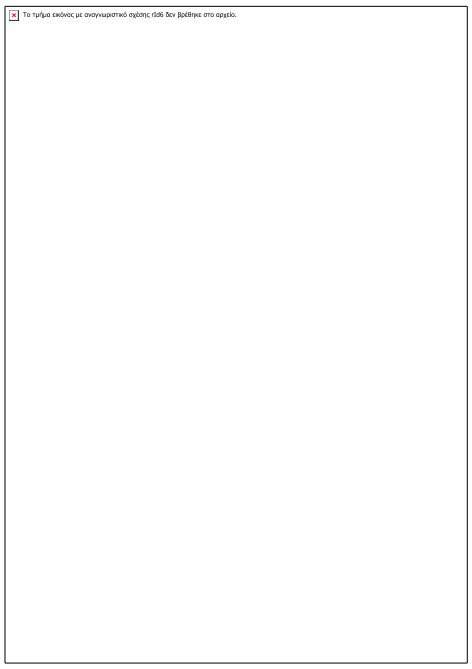
   Again we can see some bands on the negative controls but not as vibrant as before

3.

Reagents	Volume(ul)
Solution 1	104
Solution 2	200
Solution 3	150
EtOH 100%	900
EtOH 70%	500

   Afterwards we performed the digestion that I described previously, but we got no results again.

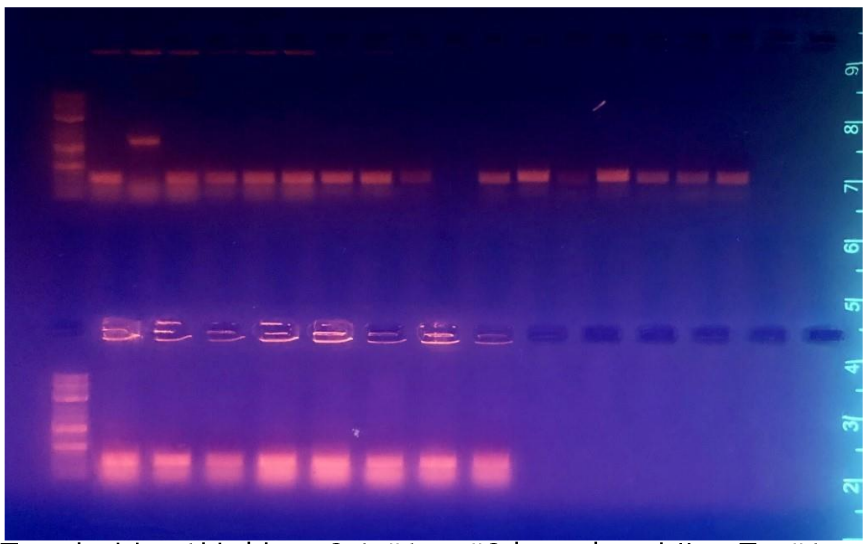
   Results: Failure



4.

1) PSB1K3 – multiLacZa (plate 18/7)
2) PSB1A3 – beta-lactamase 3:1 (plate 16/7)
3) PSB1A3 – beta-lactamase 5:1 (plate 16/7)

- 8 colonies were picked from lactamase plates, 6 from multiLacZa
- MasteMix for 25 reactions



Top: ladder 1kb | lact 3:1 #1-->#8 | n.c. | multiLacZa #1-->6
Down: ladder 1kb | lact 5:1 #1 --> #8
Correct band for beta-lactamase- PSB1A3 #2

24/7/2019

Today's Goals:

1. Identify positive clones for beta-lactamase and trigger32B
2. digestion-ligation for multiLacZa

Procedure:

1. Check plates from Tuesday
   Too many colonies, I streaked too many colonies (or Promega's ligase is too good)

Colony PCR on t32B and b-lact to find positive clones
Typical 25 ul rxns (0.8 ul primers), Master Mix for 40 reactions prepared

- Add 20.3 ul ddH2O to each tube
- Add colony to each tube
- Add 4.7 ul of MM to each tube and start PCR program

PCR Program(beta lactamase, 1.2kb amplicon, 53 degrees annealing of primers):

95 for 3 min

95 for 30 s

53 for 30 s

72 for 1:48 min (1 min/kb + 50%)

Repeat 2-4 34 times

72 for 2 min

4 forever

- Program for trigger 32B (amplicon of 400 but I thought 200 , same primers)
- Same program, step for is 72 C for 18 s (1 min/kb plus 50%)

Gel electrophoresis of PCR reactions
Trigger: 100mL TAE 2% agarose gel, 20 wells (layout below)
LNC 1-18
Run 85 V for 45 min → all wrong genika
Beta-Lactamase: 1% gel, same size and wells-layout
Run 90V for 30 min
Nothing good
Colonies 3 and 4 from trigger seemed promising so I inoculated them anyway to check w/ Minipreps on Thursday

2. Digestion:
   - 32bLacZa 100ng from IDT stock

   Cut with EcoRI and SpeI
   - Trigger32b 100 from stored digested (qubit 64,6 ng/ul)

   Cut with XbaI and PstI
   - PSB1K3 50ng (cut from Nick)

   Cut with EcoRI and PstI
   Ligation:
   - 1 : 3 : 2 ratio (plasmid:trigger:32bLacZa)

dig plasmid 25ng	1ul
Trigger 6,3ng	0,5ul
32blacZa 13,3 ng	1ul
T4 ligase buffer A	1ul
T4 ligase	1ul
DdH2O	5,5 ul

25/7/2019

Today's Goals:

1. Minipreps on yday's cultures → elution in 30 ul
2. Diagnostic digestion (20 ul reactions)
3. RPA testing
4. Check if the cloning of 32b egfp x trigger was successful.
5. minipreps- diegestion- electrophoresis for PSB1A3 - beta-lactamase #2 (23/7 colony's plate)

Procedure:

1. False alarm, there was no band at 150 bp

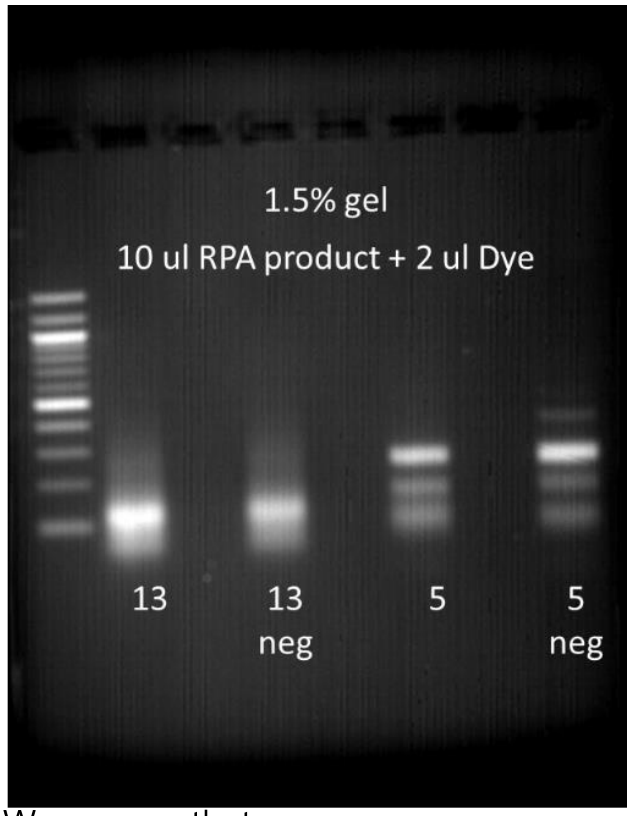
3. After talking to Kostas we wanted to check one more time because we concluded that most likely it is not a contamination but our primers may bind to one another and create some dimers that are amplified. So I ran another test RPA with a lower amount of template (10-9 ng/ul) after Nick created a series of dilutions.

   I checked 2 primer sets that give different size of amplicons to check if the band at the negative control will be specific or not. The primer sets were the following:

   S1F1O-S1R2O (136bp)
   SXF2-S1R2 (250bp)

   4 reactions:
   S1F1O-S1R2O (13)
   S1F1O-S1R2O non-template control (13 neg)
   SXF2-S1R2 (5)
   SXF2-S1R2 non-template control (5 neg)

   The RPA reaction was followed by an clean-up with a 30ul elution step in the end (twice eluted)
   1,5% agarose gel, 100ml TAE



We can see that :
1) the 5th primer set is not good (gives a lot of bands both at positive and at the negative control)
2) It seems like a specific band is amplified at the non-template control so we still are not sure if there is a contamination or if the primers are binding together and are amplified.

So I did a gel extraction to get these bands and send them for sequencing if possible

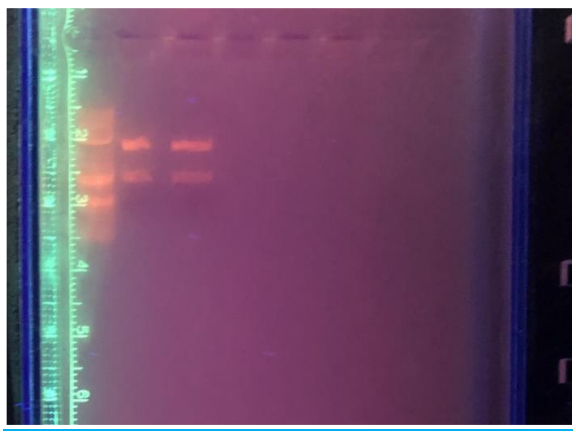
The bands were cut from the gel and stored at 4oC

4. Colony PCR :

Components	Volume (ul)
10Mm dNTPs	15
5u/ul Taq Pol	7,5
10 X Buffer	75
Forward and Reverse primer	15 ul each
DdH2O	616

However we didn't got any results.->Results: Failure

5.



Confirmed right construct for beta-lactamase (1000bps)
Glycerol stock (500ul glycerol 50% + 500ul liquid colony)

26/7/2019

Today's goals:

1. Prepare Liquid Cultures for EGFP construct PSB1K3 to get it in a big concentration.
2. Colony PCR for multiLacZa in DH5a

Procedure:

1. Standard.
2. 26 colonies tested (plates 25/7)
   contamination

27/7/2019

Today's goals:

1. Isolation of the plasmids.
2. Colony PCR for multiLacZa in DH5a

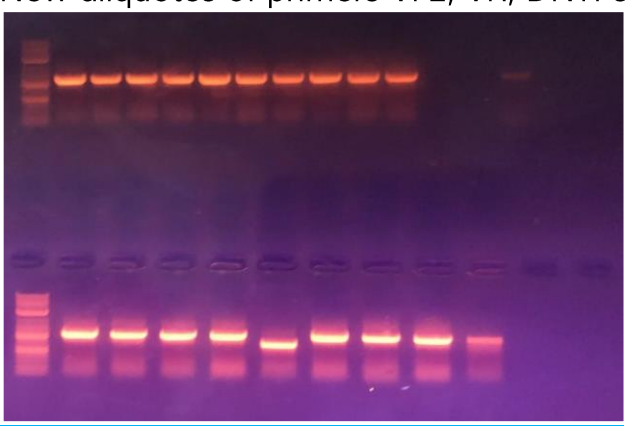
Procedure:

1. Standard Macherey-Nagel Miniprep kit.

   The same day we got some colonies from our glycerol stock 32B % TRIG 3(7) that contained bacteria that may had the plasmid construct with 32B EGFP and trigger and we grew them in agar plates, in order to check with colony pcr if there was at least one colony that the construct. However, the next day we noticed that there were so many colonies in the plate that we could not test the colony pcr.

2. 20 colonies tested (plates 25/7)

   New aliquotes of primers VF2, VR, DNTPs, ddH2O



Top: ladder 1kb| multiLacZa 1->10 |empty|empty| n.c.
Down: ladder 1kb| multiLacZa 11->19
No correct pattern.
#15 gave a band at 700bps that corresponds to 32bLacZa without trigger

29/7/2019

Today's goals:

1. Competent cell preparation
2. DNA quantification
3. Isolation of digested pSB1C3
4. Subcloning of 32BLacZa-t32B
5. Cloning of 32BeGFP-t32B in psb1K3
6. minipreps – diagnostic digestion – electrophoresis for the lacZa #15 and for the random one (27/7) and minipreps – qubit – preperation for sequencing

Procedure:

1. 25ml LB culture w/ M15-T7 *E. coli* cells
   Incubate 37 degrees C /210 rpm until 5pm
   3 x 250 ml LB flasks, no antibiotics

2. Qubit → 20 samples + 2 standards + 1 extra (23rxns)
   - 199 ul x 23 samples = 4577 ul (Add 4 x 915 ul + 917 ul)
   - 1 x 23 ul qubit dye = 23 ul

pSB1A3 t32B 1	208 ng/ul
pSB1A3 t32B 4	188 ng/ul
pSB1A3 t32B 6	98.2 ng/ul
pSB1A3 t32B 7	206 ng/ul
p-GEM-IS6110 1-1	382 ng/ul
2-2	466 ng/ul
P2	238ng/ul
32B 5	332 ng/ul
32B 7	154 ng/ul
eGFP	171 ng/ul
eGFO 5	167 ng/ul
eGFP 6	198 ng/ul
32B Lact 6	202 ng/ul
	260 ng/uls

3. Digestion of pSB1C3- eGFP to isolate pSB1C3 cut with EcoRI/PstI (HF)
   - 20 ul rxns, 10 ul plasmid DNA used
   - 2 rxns, Master Mix for 2.2

Gel excision of band at 2 kb, gel extraction and elution at 30 ul (MN kit)

4. Digestion of pSB1A3 – t32B w/ XbaI/PstI
   - Typical 20 ul rxns, 10 ul DNA used
   - 2 rxns, Master Mix for 2.2
   - Add 10 ul DNA + 10ul MM
   - Incubate 37 degrees C for 2h, heat kill 80 degrees C for 20 min

   Qubit 2

pSB1C3 dig E/P	58.6 ng/ul
pSB1A3 lact	478 ng/ul
pSB1K3 LacZa 8	1140 ng/ul
pSB1K3 LacZa 10	1240 ng/ul
pSB1C3 LacZa 1	336 ng/ul
pSB1C3 LacZa 2	282 ng/ul
pSB1C3 LacZa 5	352 ng/ul
32B LacZa 1	272 ng/ul
32B LacZa 4	494 ng/ul
LacZa 4	396 ng/ul

   pSB1C3 dig E/P dilution to 25 ng/ul

   Total volume → 69 ul

   Ligation of pSB1C3-32BLacZa-t32B
   - 25 ng pSB1C3 (1 ul used)
   - 6 ng t32B (2.5 ul)
   - 2 ul 32B LacZa
   - Incubate o/n at 4 degrees C
5. We had psb1C3 quantified 25ng/ul and 15ng/ul from previous digestion and gel extraction. We aso had t32B digested with XBaI and PstI.

   Gel extraction of t32B 1 and 4 after running them through a 1,2% agarose gel (60ml TAE). The elution was done in 30ul

   Digestion: According again to iGEM's protocol I digested

psb1c3 linear vector with EcoRI-HF and PstI-HF (4ul MM+4ul psb1C3)
32BeGFP with EcoRI-HF and SpeI-HF (4ulMM+2ul 32BeGFP – 80ng +2ul ddH2O)

- Incubation at 37oC for 1,5 h
- Heatkill at 80oC for 20 minutes

Ligation
The ligation was done this time with Minotech's T4 DNA ligase and not NEB's. So the protocol needed to change
3 reactions:

t32BeGFP-t32B
t32BeGFP-t32B (NEB buffer)
Negative control (only the vector)

Each 20ul reaction was as follows (3:3:1):

T4 DNA ligase buffer	2ul
T4 DNA ligase	2,5ul
glycerol	2ul
ATP	2ul
32B-egfp	3,5 ul (35,6ng)
t32B	2,5ul (4,988ng)
ddH2O	3,5ul

- The only difference is that in the second one I used a different buffer (NEB's buffer) and in the 3rd reaction instead of the 2 inserts I used extra water (plus 6ul water)
- Incubation at 4°C O/N

6. We took bands at 2000+ bp that may stood for plasmid+trigger and bands at 500bp that corresponded to LacZa
   1) PSB1A3 – beta lactamase
   2) PSB1K3 – lacZa no8 and no10
   20ul 100ng/ul tubes

30/7/2019

Today's goals:

1. **Transformation of pSB1C3-32BLacZa-t32B, 2nd try**
2. **Effiency check for freshly made competent cells**
3. **Transformation of the 32BeGFP-t32B**
4. **glycerol stock for the PSB1K3- LacZa no15 in order to send them for sequencing**

Procedure:

1. Transformation again → pSB1C3 – 32BLacZa – t32B with 1 ul ligation (had too many cells)
2. Transformation efficiency check with pUC19 for BL21 and M15-T7 (got nothing)
3. Transformation of ligated products (32BeGFP-t32B) into DH5alpha chemi- competent cells:
   3 transformations:

Trans1: 32Begfp-t32b+psb1c3 (1)
Trans2: 32Begfp-t32B+psb1c3 NEB (2)

Trans3: psb1c3 N.C (3)

Transformation was done with the protocol for the heatshock transformation with the following changes:

- I added 5ul to the cells from every ligation reaction
- 900 ul LB was added to the cells upon their recovery and they were incubated for 1 h in 37°C
- The cells were peletted (centrifuged at 9.000g for 1 minute) and resuspended at 100 ul LB
- The streaking was done in LB Agar Plates with 35 mg/ml chloramphenicol
- Then the plates were incubated in 37°C O/N

4. 500ul liquid LB culture + 500ul qlycerol 50%

31/7/2019

Today's goals:

1. **Competent cell preparation**
2. **Transformation of pSB1C3-32BLacZa-t32B**
3. **Transformation of the 32BeGFP-t32B**
4. **Dilution of Nitrocefin and X-gal**
5. **Sunspension of nitrocefin and x-gal (see suspension protocol)**

Procedure:

1. Prepare competent cells for BL21 (DE3) and M15-T7 *E. coli* cell strains
   This whole lot went to ruin, centrifuge was rigged and produced too high g's, the harvested cells were dead.
2. Transformation of pSB1C3 – 32B LacZa – t32B into DH5-alpha cells
   - 2ul ligation
   - 900 ul LB recovery
   - 1h incubation
   - Plate 100 ul and 900 ul (after spin down)
3. No colonies appeared from yesterdays transformation so the transformation as repeated with the same conditions as yesterday
4. Dilution of Nitrocefin (Cayman) 2.58mg of pure powder in 0.5ml DMSO and then diluted with 9,5mL 0f 0.1 M phosphate Buffer, pH=7.0 that we prepared the same day.
   Preparation of 20mg/ml stock solution X-gal by diluting X-gal in DMSO.
5. Nitrocefin 0,5 mM, X-gal 20 mg/ml

1/8/2019

Today's goals:

1. **Identification of positive pSB1C3-32BLacZa-t32B clones and Stock dilutions**
2. **Cloning of 32BeGFP-t32B in psb1K3**
3. **Check if the constructs 32b egfp in psb1c3 and trigger in psb1a3 are functional with the PURE Expression kit.**
4. **PURExpress System 1st test**

Procedure:

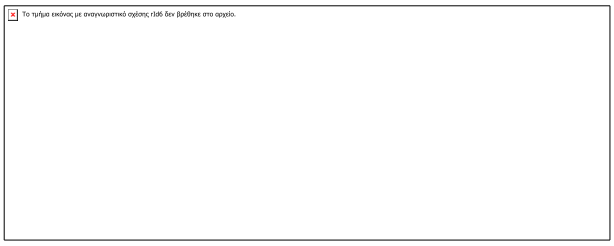
1. Plate check from yday's cloning → all good
   Colony PCR for pSB1C3 – 32BLacZa – t32B construct
   - 25 ul rxns (18 rnxs, 1 NC), Master Mix for 20
   - Add 20.3 ul ddH2O to each tube
   - Add colony
   - Add 4.7 ul of MM

   Stock dilutions to 100 ng/ul

   - pSB1A3 – t32B 1, total volume 60.9 ul
   - pSB1A3 – t32B 4, total volume 36.1 ul
   - pSB1A3 – t32B 7, total volume 66 ul
   - p-GEM-IS6110 10, total volume 119.7 ul

   Gel electrophoresis for Colony PCR

   - 1% agarose 100ml TAE gel, 20-well comb
   - LNC 1-18 colonies
   - Run 90V for 25 min
   - 1 and 2 got bands at 900 bp
   - 3 and 4 and 1000+ bp
   - Inoculation of 1-4 for minipreps



2. Again nothing in my plates. Again the cloning procedure from the beginning just as the last time.
   The only differences:
   - Added a little bit more of each insert in the ligation (t32B : 3,5ul, 32Begfp:4ul) and reduced the vector quantity (1ul). Water was the adjusted accordingly (3 ul)
   - Incubation was done in 16°C O/N

   Also I prepared the previously qubited samples to be sent for sequencing (diluted to a final concentration of 100ng/ul and final volume sent was 20ul)

3. For the egfp characterization, we tested PURE by adding 70ng of 32B EGFP, 70ng EGFP and 70ng of 32B triggerin the plate reader.

	3hours
37°C	EGFP: 70ng 32b egfp : 70ng 32b egfp+ trigger : 70ng

4. Reaction no1: 32B eGFP + trigger #5

Reaction no2: 32B eGFP + trigger #7

Reagents	Ul per reaction
Solution A	2,8
Solution B	2,1
RNAase inhibitor	0,14
DdH2O	0,56
DNA 32b eGFP 70ng	0,7
DNA trigger 70ng	0,7

Reaction no3: eGFP
Reaction no4: 32beGFP

Reagents	Ul per reaction
Solution A	2,8
Solution B	2,1
RNAase inhibitor	0,14
DdH2O	1,26
DNA 70ng	0,7

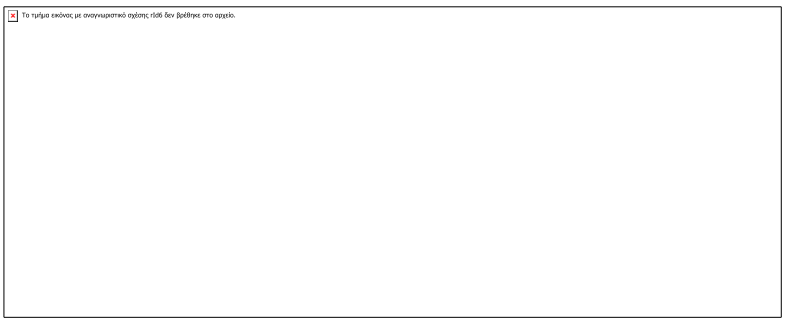
2/8/2019

Today's goals:

1. Minipreps from LB cultures (for 32BLacZa/t32B construct) and diagnostic digestion
2. Transformation of the 32BeGFP-t32B
3. Check if the constructs 32b lactamase in psb1c3 and trigger in psb1a3 are functional with the PURE Expression kit (3 hours).

Procedure:

1. Pellet 4 ml of cells and Elution in 40 ul. Subsequently a 10ul digestion reaction was performed and electrophoresis (uncut + cut)



2. Transformation again like last time.

   Plates:
   Trans1: 32Begfp-t32b+psb1c3 (100ul streaking without centrifuge step)
   Trans2: 32Begfp-t32b+psb1c3 (100ul streaking after centrifugation and resuspension)
   Trans3: Negative control
3. All of our reactions were 7ul and we used Takara's RNase inhibitor.

Solution A	2.8 ul
Solution B	2.1 ul
RNase Inhibitor	0.14ul
DNA template	x
DdH2O	till 7ul

   We used:

   - 70ng 32b lactamase, 70ng trigger
   - 70ng 32b lactamase
   - 70ng Lactamase

   To each well we added 43ul of 0.5mM Nitrocefin, measurement at 490nm.

3/8/2019

Today's goals:

1. PURExpress reaction
2. RPA reaction

3. PURExpress reaction
4. Colony PCR to test that our cloning32BeGFP-t32B
5. Check if the constructs 32b lactamase in psb1c3 and trigger in psb1a3 are functional with the PURE Expression kit (1 hour and 2 hours).

Procedure:

1. PURExpressrxns (7 ul) → lactamase, 32B only toehold, 32B + trigger
   Add in the following order:

0.56 ul ddH2O
2.8 ul Solution A
2.1 Solution B
0.14 ul RNAse inhibitor (Roche)
0.7 ul toehold
0.7 ul trigger

2. RPA to isolate our fragment to induce PURE
   - 6rxns, 2 working and 2 No template Control, 2 without overhangs
   - Primers → S1F1O and S1R2O
   - Typical 50ul rxn for RPA
   - Gel excision, gel extraction etc, elution at 15 ul to get maximum concentration
   - NC → 36.3 ng/ul
   - Overhangs → 48.5
   - NO overhangs → 22 ng/ul
3. Proof of Concept PURE 7 ul rxn
   Lactamase, 32B Lactamase, 32B LAct/Trigger, 32B Lact /NO, 32B Lact O, 32B Lact /NC
   Nothing worked
4. Colony PCR to test that our cloning is successful

   The PCRs were performed with the VR and VF2 primers that anneal 153bp before suffix and 118bp before prefix.
   I used 16 colonies from the 1st transformation-1st plate, 2 colonies from the negative one and 1 sample was the NTC for the PCR (19 total reactions).

   Reaction MasterMix: (x21 for 19 reactions)

10X Ktaq Buffer A	2,5ul
10mM DNTPs Mix	0,5ul
VF2 primer	0,8ul
VR primer	0,8ul
5u/ul Ktaq enzyme	0,1ul

   - Added 20.3 ul ddH2O to each tube
   - Added a single colony to each tube
   - Added 4.7 ul of MM to each tube and start PCR program

   Cycling Protocol:

95°C for 3 minutes
95°C for 30 seconds
53 °C for 30 seconds (annealing temp according to NEB's Tm

calculator for primer set)
72 °C for 1:30 miutes (1kb per min)*
Repeat cycles 2-4 34 times
72 °C for 1:30 minutes
Hold at 4 oC

- 938bp 32Begfp + 155bp t32B + 271bp for the primers = 1.364bp
- Cycles: 35

Electrophoresis: 1% agarose gel (100ml TAE) and got nothing.

5. Standard(70ng trigger).

4/8/2019

Today's goals:

1. **Colony PCR for 32BeGFP-t32B**

Procedure:

1. Exactly as yesterday, only for more colonies (29 colonies). So I ran 30 reactions and the mastermix was done for 33 reactions -> Again nothing

5/8/2019

Today's goals:

1. **Competent cell preparation, 2nd try**
2. **Identification of positive pSB1C3-32BeGFP-t32B colonies**
3. **Colony PCR for 32BeGFP-t32B**
4. **Cloning of 32BeGFP-t32B in psb1K3**
5. **Check if the constructs 32b lactamase in psb1c3 and trigger in psb1a3 are functional with the PURE Expression kit (3 hour).**

Procedure:

1. Precultures (25ml LB) for BL21 (DE3) and M15-T7 and preparation of 4X250 ml LB in 1-L flasks
2. Colony PCR for pSB1C3-32BeGFP-t32B, 35 colonies +1 NC. Typical 25 ul PCR rxn
3. Exactly as yesterday, only for more colonies (35 colonies). So I ran 36 reactions and the mastermix was done for 42 reactions. Again nothing
4. Again Ligation. Same conditions as last time but plus a heatkill, 65°C for 10 mins, step after the O/N incubation at 16°C.
5. Standard. We used 25ng and 7ng of trigger concentration.

6/8/2019

Today's goals:

1. **Competent cell preparation**
2. **Transformation efficiency check for freshly made competent cells**
3. **Transformation of the 32BeGFP-t32B**

Procedure:

1. M15-T7 and BL21 (DE3) competent cells with Inoue protocol
   M15-T7 → 200 ul aliquots
   BL21 (DE3) → 100 ul aliquots
2. Efficiency check w/ 10pg DNA → nothing on so low amounts, works on around 50ng ++ of DNA
3. Transformation with the same conditions as last time but two plates only: 32Begfp-t32B both plates

7/8/2019

Today's goals:

1. **Colony PCR for 32BeGFP-t32B**
2. **liquid cultures.**
3. **Ligation of 32BeGFP-t32B**
4. **Preparation of PCR product and Digest trigger and Check if the RPA product (60ng) would give signal at 42.5 degrees Celcius. Check if a PCR product (77ng) or Digested trigger with EcoRI and PstI(7,4ng) would give signal in the PURE kit(3 hours)**

Procedure:

1. Colony PCR again: 20 colonies taken from both plates. Reaction conditions are the same. ->Nothing again.
2. Inoculation of colonies 1, 11 just to be sure that it what I see in the colony PCR is right, in 5ml LB C35 liquid cultures.
3. Ligation again with NEB's T4 DNA ligase
   Reagents:

1ul T4 DNA ligase buffer
1ul psb1c3
4ul 32Begfp
3,5ul t32B
1ul T4 DNA ligase
Total 10,5ul reaction

   - Incubation at 4oC O/N
   - Also did a ligation reaction with just water instead of inserts to have a negative control
4. Standard

8/8/2019

Today's goals:

1. RPA testing for time and sensitivity
2. Transformation
3. Mini preps from the 32BeGFP-t32B liquid cultures
4. More RPA reactions
5. Check if the constructs 32b lactamase in psb1c3 and trigger in psb1a3 are functional with the PURE Expression kit (3 hours).

Procedure:

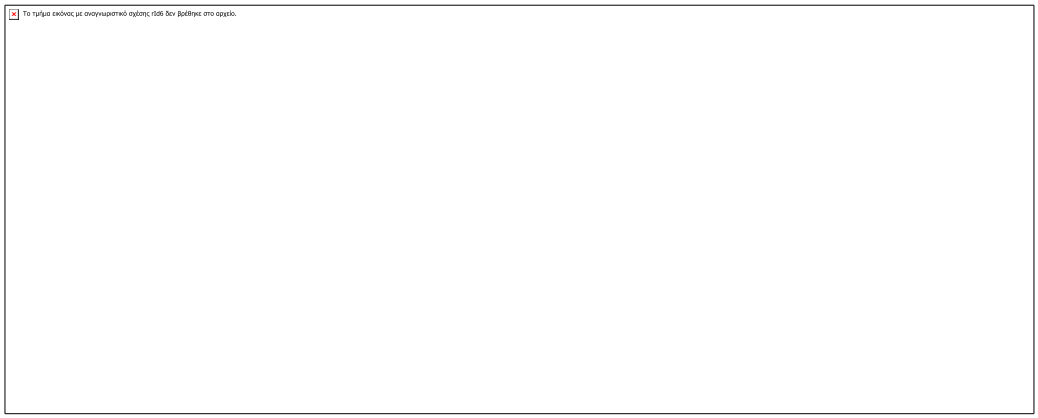
1. RPA testing for time and sensitivity
   - Time → 3, 5, 7, 10, 15, 20 min
   - 6 rxns + 1 NC (at 10 min)
   - Typical RPA rxn at 50 ul, 39 degrees Celsius
   - 10^{-4} ng/ul DNA used
   - Sensitivity → At the lowest time mark that we can see signal signal, run lowest template concentrations for up 10^{-9} ng/ul of IS6110 DNA.

   PCR cleanup of RPA products, elution at 30 ul

   Gel electrophoresis→ load 5 ul DNA + 5 ul ddH2O + 2 ul dye

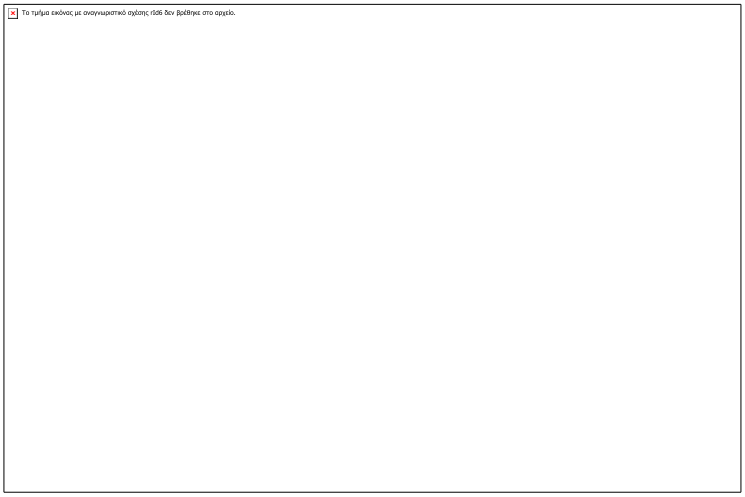
   Well comb layout

   3min 5min 7min 10min 15min 20min NC



Lowest time was 5 min, so I tested all possible concentration at 5mins.

NC -5 -6 -7 -8 -9



2. Transformation

Same conditions as last time with two plates only:

32Begfp-t32B
Negative control (ligation of only the plasmid vector)

The plates was done simply by streaking 100ul and not centrifuging it and then resuspending it in LB.

3. Minipreps from yesterday's liquid cultures
40ul elution in the elution buffer given by the kit (MN)
Restriction digestion

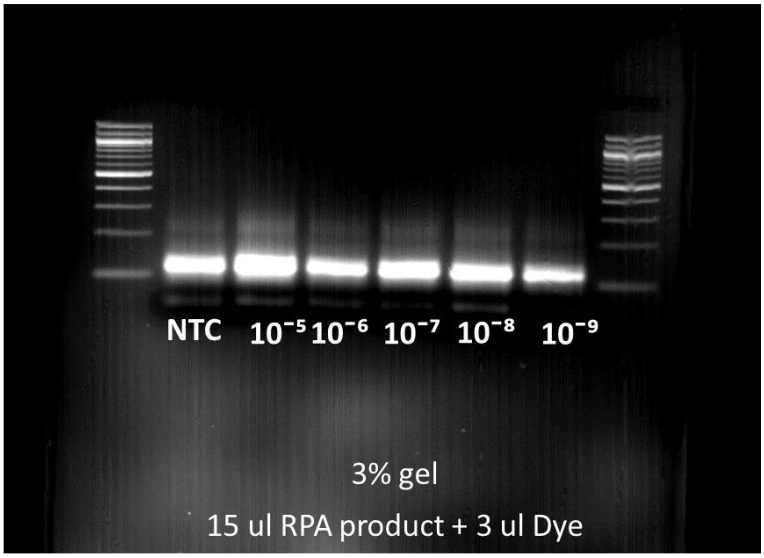
According to iGEM's protocol, as last time
Incubation at 37oC for 1,5h
Gel electrophoresis: 1% agarose gel. I expexted a band at 1093bp but got one at 900bp

4. RPA reaction

10 mins check of diluted concentrations

6 samples:
10-5 ng/ul
10-6 ng/ul
10-7 ng/ul
10-8 ng/ul
10-9 ng/ul
NTC

PRIMER SET: S1F1O-S1R2O
The reaction was conducted as always and the temperature was 39oC
Cleanup to a final elution of 15ul (twice eluted) and afterwards Gel electrophoresis (3% agarose gel, 100ml TAE)



Seems like there is the same amount of amplification to all conditions. Pobably not the case. (Contamination?Primers?)
5. Standard (50 and 3ng of trigger)

9/8/2019

Today's goals:

1. RPA reactions
2. Transformation of Improve parts into protein expression strain
3. Colony PCR for 32BeGFP-t32B

Procedure:

1. Repetition of RPA, didn't really work
2. Transformation of eGFP, 32B eGFP, 32B LacZa, LacZa, 32B LacZa/t32B into M15-T7 cells (resistance to Amp-Kan naturally!)
   - 1.5 ul of plasmid each (~150ng)
   - 800 ul LB recovery
   - 1h incubation

   Spin down + plate at LB Agar plates w/ A100/K50/C35

   Subcloning of 32BeGFP/t32B into pSB1C3

   Ligation (1o ul rxns)→ NC (vector only) and 2 replicates of the working
3. Colony PCR again
   Used 35 colonies + PCR's NTC = 36 reactions. The mastermix was done for 42 reactions. The conditions of the reactions are again the same.
   Got nothing again. Nikoletta was assigned to do it again until successfull. (The vector was the problem,it needed to be cut again)

10/8/2019

Today's goals:

1. Identification of positive clones from last day's transformations

Procedure:

1. Colony PCR for yday's transformations (2colonies per plate) total 10 colonies
   Typical 25 ul PCR rxns , Master Mix for 12(PCRs didn't work because of wrong programs)

12/8/2019

Today's goals:

1. PURExpress system test:

Procedure:

32b – beta lactamase #4 and #6 with:
1) 3ng trigger 32b (from PSB1A3 #6)
2) 50ng trigger 32b (from PSB1A3 #6)
7ul reaction + 43ul nitrocefin
3h at 37 oC , spin down every 1h

13/8/2019

Today's goals:

1. **RPA reaction tests**
2. **Cloning of the construct 32B EGFP x Trigger into PSB1K3**
3. **Digestion- ligation for multieGFP : PSB1C3 + 32beGFP+ Trigger32b**

Procedure:

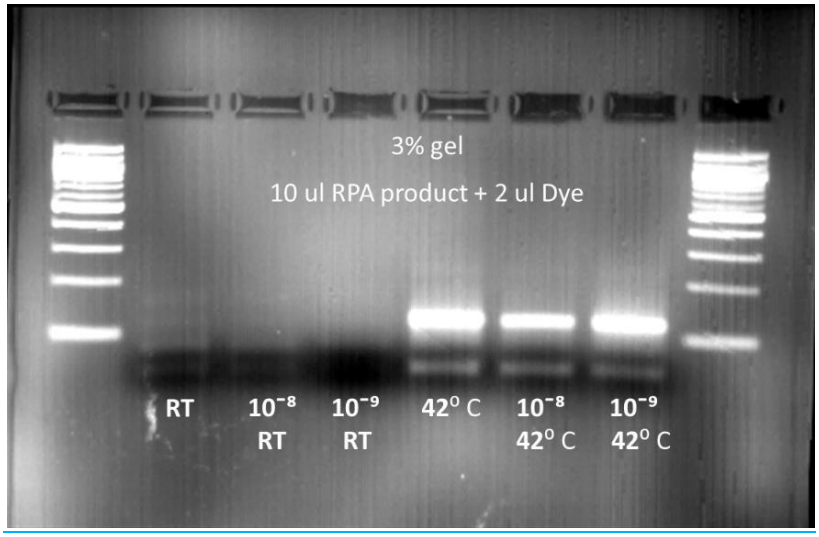
1. Again testing the RPA conditions for the better efficiency of the amplification
   - Testing 2 temperature conditions (RoomTemperature –RT- and 42oC)
   - Testing 2 tempate inputs (10-8ng/ul and 10-9ng/ul)

4 RPA Reactions:

10^{-8}RT
10^{-9}RT
RT negative control
10^{-8} 42
10^{-9} 42
42 negative control

The RPA reaction is the usual one given by the company's handbook, BUT
- The reaction time was 5 minutes instead of 20
- Without the in-between step of Quick spining the tubes

Cleanup of the reaction w/ MN's Nucleospin cleanup kit ->Elution in 15ul ddH2O, eluted twice-> Gel electrophoresis (3% agarose gel, 60ml TAE)



the reaction did not work in Room Temperature in a 5 minute reaction

2. Digestion of the plasmid, toehold egfp and trigger->Gel electrophoresis and Gel extraction
   Qubit for quantification of the plasmid, 32b egfp and trigger

   Plasmid: 11ng/ul
   Trigger: 2.2ng/ul
   Toehold: below limits of detection (we repeated the experiment for the toehold construct)

   Ligation

Components	Volume (ul)
Plasmid	2.28
toehold	1.25
trigger	1.15
Buffer	1
ligase	0.5
DdH2O	3.72

3.

- Digestion of 250ng trigger from “PSB1A3-t32b no4”
- Digestion of 250ng plasmid from “PSB1C3-eGFP no6”
- Digestion of 250ng 32b eGFP from “PSB1C3-32beGFP no7”
- 20ul reaction
- 1,5h at 37 oC

Gel extraction and Clean up
Qubit: trigger 2,2 ng/ul

- PSB1C3 11ng/ul
- 32b eGFP error too low concentration

Again digestion of 100ng PSB1C3 from stock
Ligation: 3 : 3 :1 (trigger:toehold:plasmid)
Trigger 15,7 ng
32b eGFP 2,5 ng
PSB1C3 25 ng 2,28 ul
PSB1C3

Trigger	1,15 ul
32b eGFP	1,25 ul
T4 ligase buffer	1 ul
T4 ligase	0,6 ul
DdH2O	3,72 ul

14/8/2019

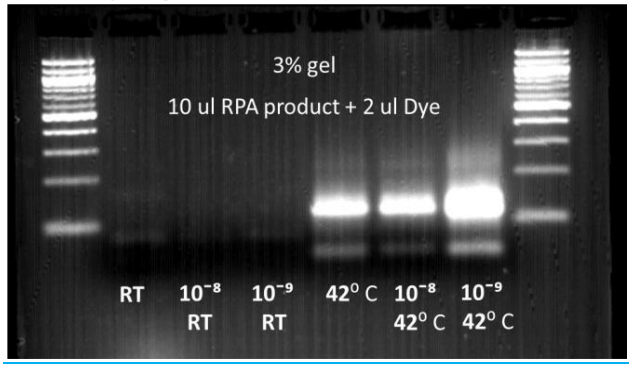
Today's goals:

1. RPA reaction tests
2. Preparation of glycerol stocks of M15 strains with the constructs: EGFP, 32B EGFP, LacZa, 32B LacZa and Transformation of the construct 32B EGFP X Trigger in DH5a strains.
3. Colony PCR for T7M15 cells transformated

Procedure:

1. Again the same RPA reaction. The exact same conditions as yesterday (RT, 42oc, 10-8ng/ul, 10-9ng/ul)

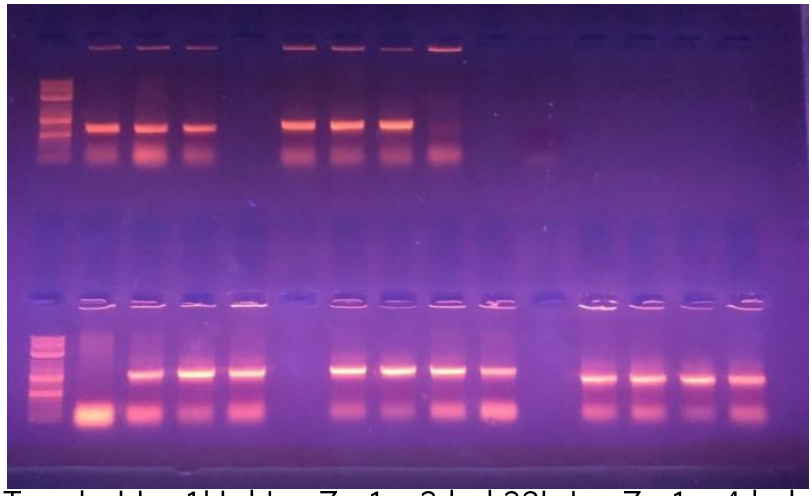
   With the in-between step of Quick spin this time (3mins RPA, quick spin and then another 2mins RPA)
   Cleanup again exactly the same and Gel electrophoresis (3% agarose gel, 60ml TAE)



   Again the outcome was the same as yesterday

2. Standard
3.

   1)PSB1C3 – eGFP (4 colonies), expected band 1151bps
   2) PSB1C3 – 32b eGFP (4 colonies), expected band 1221bps
   3) PSB1C3 – LacZa (3 colonies), expected band 700bps
   4) PSB1C3 – 32b LacZa (4 colonies), expected band 770bps
   5) PSB1C3 - 32b LacZa – trigger (4 colonies), expected band 911bps



Top: ladder 1kb | LacZa 1->3 | - | 32b LacZa 1->4 | - | n.c.
Down: ladder 1kb | eGFP 1->4 | - | 32b eGFP 1->4 | - | multi LacZa 1->4
Almost all the colonies were right

15/8/2019

Today's goals:

1. Repeat the transformation experiment because a mistake was made.
2. Inoculation of liquid cultures T7M15 with the right colonies:

Procedure:

1. Standard
2. 1)PSB1C3 – eGFP no3, no4
   2) PSB1C3 – 32b eGFP no1, no2
   3) PSB1C3 – LacZa no1, no2
   4) PSB1C3 – 32b LacZa no1, no2
   5) PSB1C3 - 32b LacZa – trigger no2, no4
   5ml LB Amp100, Kan50, Chl35
   Also transformation of DH5a with PSB1C3-multi eGFP (thod)

16/8/2019

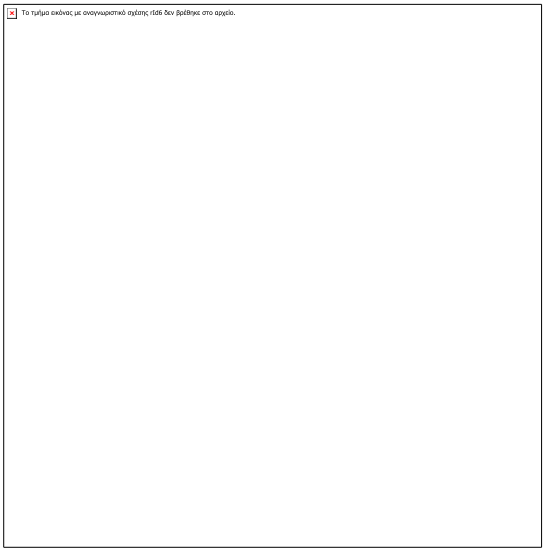
Today's goals:

1. Check if the cloning was successful.
2. Colony PCR for DH5a transformated with PSB1C3-multi eGFP

Procedure:

1. Colony PCR :
   - Picked 17 colonies
   - Annealing 53°C
   - 35 cycles

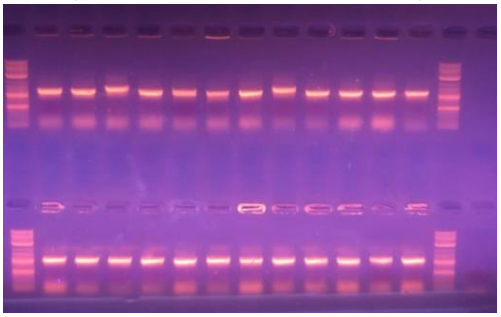
   Sussecc



We prepared liquid cultures for tomorrow.

2.

- 24 colonies, 1 n.c.
- Extension time 84 sec
- Expected band at 1388 bps



Top: ladder 1kb | 1--> 12 |ladder 1kb
Down: ladder 1kb | 13--> 24 |ladder 1kb
Succeed : no3 and no8
Inoculation of no3 and no8 to 5ml LB Chl35, overnight incubation at 37 °C

17/8/2019

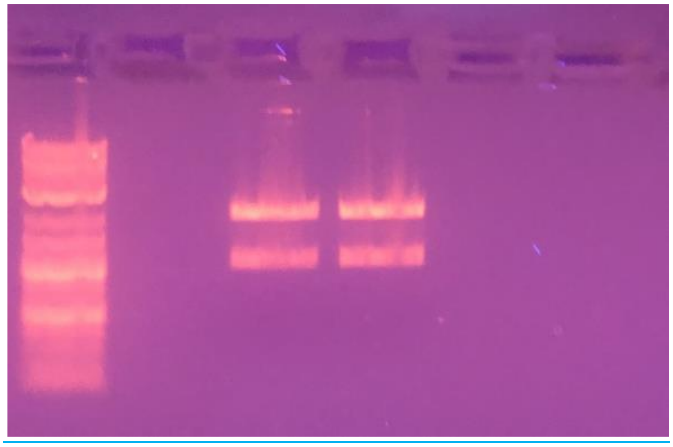
Today's goals:

1. Isolation of the plasmid from DH5a strains and transform M15 strains with it.
2. Minipreps–digestion–electrophoresis to confirm the multi eGFP no3& no8
3. Transformation of the construct (PSB1C3-multieGFP) into T7M17 E.coli strains

Procedure:

1. Standard. The plate had the following antibiotics : 35 ug/ml chloramphenicol, 50ug/ml ampicillin, 50ug/ml kanamycin
2.

- 20ul digestion reaction
- Expected bands at: 2kb (vector), 1117bps (32beGFP-trigger)



3. 100ng DNA (1,2ul) in 100ul cells
   Plating at LB agar Amp100, Kan50, Chl35

18/8/2019

Today's goals:

1. Preparation of Liquid cultures from the plate.

Procedure:

1. Standard.

19/8/2019

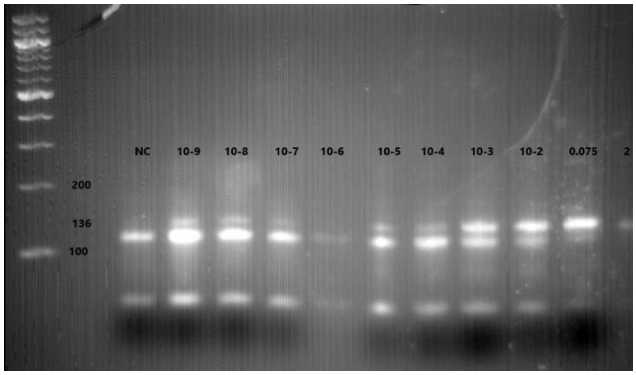
Today's goals:

1. RPA reaction, final conditions
2. Check if the transformation was successful with colony pcr.
3. Preparation of glycerol stocks and liquid cultures LacZa, 32B LacZa, 32B/Trigger LacZa for in vivo experiment.

Procedure:

1. Final RPA → 5 min, 42 degrees C with different template concentrations
   - 2 ngul, 0.075 ng/ul, 10^{-2} to 10^{-9} ng/ul +NC (no template)
   - Typical rxn, 50 ul RPA
   - Cleanup and elution at 15 ul

   Agarose Gel electrophoresis with Nusieve 3:1 Agarose
   150ml TAE, 4% agarose (6g)
   Run at 6V/cm of gel (6x15 cm =90V) for 2h
   15-well comb
   L NC 10-9, 10-8 2

2. 

Picked colonies	17
Annealing 53°C	
35 cycles	

3. Preparation of glycerol stocks of M15 with 32b egfp x trigger using the standard procedure. Success. The M15 strains had our construct.->liquid cultures

20/8/2019

Today's goals:

1. **Subcloning of trigger 13 and trigger 14 into pSB1C3**
2. **In vivo experiments for characterization part**
3. **Cloning of trigger 13 and 14**
4. **Check if the construct 32B LacZa x trigger works as we expected.**

Procedure:

1. Digestion of trigger 13 and trigger 14
   - MM → 25 ul etc from iGEM
   - 2 rxns, 8 ul each
   - Incubate at 37 degrees C for about 1h30min

   Ligation of trigger13 and trigger 14 into pSB1C3 → o/n incubation at 4 degrees C
   25 ng plasmid pSB1C3
   6.25 ng trigger
2. Photometry at 600 nm for yesterday's liquid cultures (pre-inocula), for plate reader assay characterization in vivo

OD600 O/N:
p100:1,865
p105:2,005
p106:2,003
p119:1,925

Each culture was diluted properly to reach OD600:0,05 (125ul Culture + ~5ml LB C50)
Again photometry at 600nm after 1 hour and after 1,5 hours

OD600 1h:

>p100:0,31
>p105:0,32
>p106:0,32
>p119:0,2

OD600
1,5h:

>p100:0,6
>p105:0,63
>p106:0,64
>p119:0,36

Plate reader in vivo characterization assay

1,5 hours post inoculation

- 2hours enzymatic assay (every 30 seconds)
- Shaking 120rpm continuous
- 37oC
- At 490nm
- Used a 96-well plate
- 2 technical replicates for every promoter
- 160ul liquid culture + 40ul nitrocefin
- Plus a blank (water+nitrocefin)

3. Cloning for the new triggers that arrived (t13, t14)

Triggers: 155bp both
Digestion:

According to iGEM's protocol. I digested 50ng (1ul from stock concentration 50ng/ul)
Ligation

Used 25ng of a digested plasmid vector psb1C3 with a concentration of 11ng/ul (nikol+Theo)
Used 5,8ng of the digested triggers (~1ul) for a 3:1 ratio of ligation
3 reactions:

psb1C3-t13
psb1C3-t14
psb1C3 only (negative control)

The ligation was done with NEB's T4 DNA ligase and the reaction was as follows for both triggers:

2,28ul psb1C3
1ul trigger
1ul T4 DNA ligase buffer
0,5ul T4 DNA ligase
water to 10ul->5,22ul

For the 3rd ligation the volumes were the same except intead of insert I supplemented 1ul more water

4. However, the 32B/Trigger LacZa colony didn't grow and we proceeded our experiments with only 32B and LacZa.
   - Dilution of the cell cultures till 0.05
   - We measured the OD600 of the liquid colonies in order to create new cultures with OD600 = 0.05
   - We incubated the diluted 0.05 OD600 cultures in a 37°C shaking incubator until cells reach mid-log growth, about 0.4 - 0.6 OD600

   e.g. OD600 LacZa = 1.763

   C1 x V1=C2 x V2 <=> 1.763 x V1=0.05 x 5ml <=> V1= 140ul

   5ml culture with 140ml from the starter culture and 4.860ml Lb

   - We took out the cultures after 2 hours and measure the OD600 of the cultures.

   We dilute again (if needed) to reach the same OD600

   - We created a map for the 96 well plate in our labbooks
   - We transfer 200ul of the culture in the respectable well and right before we place the plate in the plate reader,

   we add : 1) IPTG 0,5ul 0,1M
   2) X – gal 0,4 ul (20mg/ml)
   - Plate reader: 2h, measurement every 2min, 615nm, shaking

   Results:Failure->Afterwards we prepared liquid cultures for the next day again.

LacZa
32b-LacZa
32b-LacZa+trigger

21/8/2019

Today's goals:

1. **Transformation of trigger13 and trigger14**
2. **Transformation of trigger 13 and 14**
3. **Liquid pre-cultures preparation for characterization *in vivo* assay**
4. **Check if the 32B x trigger LacZa works properly.**

Procedure:

1. Transformation of pSB1C3- t13 and pSB1C3 – t14
   - 2 ul ligation
   - 900 ul LB recovery
   - 1h incubation

- 100 ul + 900 ul (after a spin down) plating in C35 LB Agar plates

2. Transformation of ligated products into DH5alpha chemi- competent cells:

3 transformations:
Trans1: t13+psb1c3 (1)
Trans2: t14+psb1c3 NEB (2)
Trans3: psb1c3 N.C (3)

Transformation was done with the protocol for the heatshock transformation with the following changes:

- I added 2ul to the cells from every ligation reaction
- 900 ul LB was added to the cells upon their recovery and they were incubated for 1 h in 37oC
- The streaking was done in LB Agar Plates with 50 mg/ml chloramphenicol (plated 100ul)
- Then the plates were incubated in 37oC O/N

Inoculations of the BL21 and M15 cells with the promoters p100, p105, p106 and p119 in 5ml LB C50 for the BL21 cells and CK50 A100 for the M15 cells.

8 liquid cultures for the characterization of the beta-lactamase part:

4 for the BL21 cells (p100, p105, p106, p119)
4 for the M15 cells (p100, p105, p106, p119)

Incubation O/N at 37oC in a shaking incubator (210rpm)

3. Inoculation of 5ml LB cultures for in vivo characterization
   BL21 (DE3) with C35 selection
   M15-T7 with A100/K50/C50 selection
   P100, p105, p106, p119
4. We follow the same procedure as the day before.
   - However the cells with the construct 32b LacZa didn't grow.

Results: Failure. ->For the next day:

We inoculated 5ml cultures in LB with selectable resistance markers(Amp100, Chl35, Kan50).

Inoculations of:

EGFP
32beGFP
32beGFP – trigger

At 5ml LB from glycerol stocks

22/8/2019

Today's goals:

1. *In vivo* characterization assay
2. Colony PCR for trigger 13 and trigger 14

3. **M9 Preparation and liquid cultures of EGFP, 32B EGFP, 32B EGFP/TRIGGER in LB medium and one liquid culture of EGFP in M9 medium**

Procedure:

1. At 10:00 AM, measure the OD_{600} of the cultures

   BL21 (DE3):

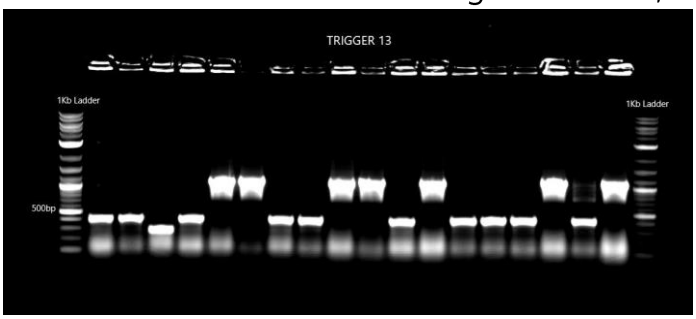
   p-100: 1,765 use 140 ul to in 5 ml LB to reach 0.05 OD
   p-105: 1,912 use 130 ul to in 5 ml LB to reach 0.05 OD
   p-106: 1,748 use 200ul to in 5 ml LB to reach 0.05 OD
   p-119: 1,770 use 197ul to in 5 ml LB to reach 0.05 OD

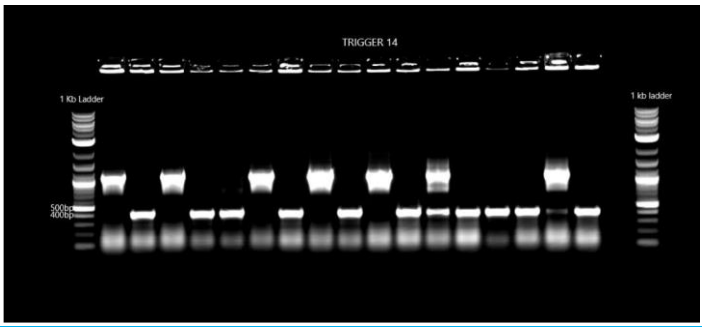
   M15-T7:

   p-100: 1,795 use 194 ul to in 5 ml LB to reach 0.05 OD
   p-105: 1,913 use 130ul to in 5 ml LB to reach 0.05 OD
   p-106: 1,873 use 133 ul to in 5 ml LB to reach 0.05 OD
   p-119: 1,720 use 145ul to in 5 ml LB to reach 0.05 OD
   in vivo characterization→ done (2 technical replicates!)

2. Colony PCR for the triggers

   17 colonies for the t13 trigger and 17 for the t14 trigger plus 2 from the negative plate
   The PCR reaction was the typical reaction for a colony PCR as always
   The cycler conditions also were the same as before for the previous colony PCRs ony the extension step was for 30 seconds (155+271bp- 1minute/kb)
   Gel electrophoresis (1% agarose gel, 150ml TAE)->BANDS expected at 426bp ->SO I inoculated in LB the following colonies: 1,2 (t13) and 20,22(t14)





3. We prepared 1L 1X M9 medium using:

6gr Na2HPO4
3gr KH2PO4
1gr NH4Cl
0.5gr NaCl
0.004gr Cacl2

1lt DH2O
2ml 50 % glycerol
2.5ml 20% casamino acids
0.5 ml 1M MgSO4.7H2O
0.05ml 0.5% thamine

Chloramphenicol 35ug/ml, Kanamycin 50ug/ml and Ampicillin 50ug/ml

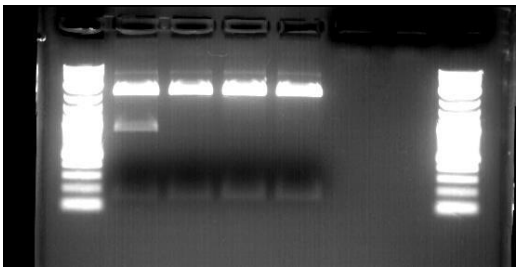
23/8/2019

Today's goals:

1. **PCR for IS6110**
2. **RPA with overhangs, all templates**
3. **Insert identification with digestion**
4. **Check that the construct 32B EGFP X Trigger worked properly. Improve part.**

Procedure:

1. PCR for IS6110 (S1F1O-S1R2O primers) w/ DMSO
   25 ul rxn, 1 working + 1 NC
2. RPA w/ overhangs (all templates, NC + Positive Control)
   Cleanup → elution in 15 ul
   Nusieve 3:1 Agarose Gel electrophoresis
   4% agarose, 150ml TAE gel, 15-well comb
   NCPCR, NC, PCR, PC, 10-9, 10-8 …. 0.075
3. Minipreps from yesterday's inoculations
   T131, t132, t1410, t1422 ->40ul elution
   Restriction digestion
   According to iGEM's protocol used every time (input DNA 250ng)
   Had 4 reactions (t131, t132, t1410, t1422) so the MM was done for 5 reactions
   Incubation for 1,5h at 37°C and electrophoresis with 1,5%agarose gel, 60ml



   Successful Bands expected at 2kb+155bp for both triggers
4.
   - Centrifuge the 5ml LB inoculations of the eGFP constructs: 2000g, 10min, 16°C
   - Remove the supernatant and resuspend the pellet at 5 ml M9 medium
   - Measure the OD600 of:

   1) eGFP1 2)eGFP2 3)32beGFP1 4)32beGFP2 5)multi2 6)multi3 7)blankM9

   - Dilute to OD600=0.08 (M9 medium)
   - Plate reader measurement (excitation : 488nm, emission: 515nm)
     Results: Failure

26/8/2019

Today's goals:

1. M9 minimal medium preparation
2. Qubit and preparation for sequencing
3. Liquid colonies for in vivo characterization part
4. Improve eGFP

Procedure:

1. M9 Preparation (casamino acids, MgSO4, Thiamine)
   For 500 mL M9, we add:
   - 0.5 ml 1M MgSO4 (7H2O)
   - 50 ul 0.5 % Vitamin B1 (Thiamine)
   - 2.5 ml 20% Casamino acids
2. Qubit
   Same reaction conditions

t13(2): 113ng/ul
t14(20):132ng/ul
t14(22):135ng/ul
multi-egfp(3):222ng/ul
milti-egfp(8):256ng/ul

   Also did some qubit reactions for the RPA products just to check with the modeling:

2ng/ul: 38.6ng/ul
0,075ng/ul: 6.24ng/ul
10^{-2}ng/ul: 22ng/ul
10^{-3}ng/ul: 21.4ng/ul
10^{-4}ng/ul: 14.6ng/ul
10^{-5}ng/ul: 11ng/ul
10^{-6}ng/ul: 24.4ng/ul
10^{-7}ng/ul: 18.2ng/ul
10^{-8}ng/ul: 15.6ng/ul
10^{-9}ng/ul: 30.6ng/ul

   Prepare them for sequencing : dilute them properly to 100ng/ul and with them also prepared the PURE and NC PURE samples from the previous qubit
   Inoculation (5ml LB C50) of the t13(2) and t14(22) and incubation O/N at 37oC to prepare tomorrow glycerol stocks
3. Inoculation in M9 of the promoters for the characterization (2 glycerol stocks for each promoter) and incubation O/N at 37°C at shaking incubator 210rpm.
4. We inoculated 5ml cultures in M9 medium + selectable resistance markers (Amp100, Chl35, Kan50). Incubate overnight in a 37°C shaker.
   Inoculation of:
   - EGFP 3,4 (x2)
   - 32beGFP 1,2 (x2)
   - 32beGFP+trigger 1,2,3,4 (x2)

   Total 16 falcons (name e.g. 3a, 3b)
   Also: Inoculation of single colonies from the plate of colonyPCR (22/8) in 5ml Lb
   1) toehold13 – PSB1C3 DH5a no5, no9
   2) toehold14 – PSB1C3 DH5a no4, no13

27/8/2019

Today's goals:

1. *In vivo* characterization assay
2. Qubit and preparation for sequencing
3. Minipreps – digestion (20ul) – electrophoresis for toehold13 & toehold14
4. Measurent at plate reader
5. Preparation of liquid cultures (LacZa, 32B LacZa, 32B LacZa+tigger)

Procedure:

1. Photometry at 600 nm for yesterday's liquid cultures (pre-inocula), for plate reader assay characterization in vivo

5. OD600 22h:
6. >M15 p100 (9) :0,136
7. >M15 p100 (10) :0,207
8. >M15 p105 (1) :0,225
9. >M15 p105 (2) :0,039
10.>M15 p106 (4) :0,1
11.>M15 p106 (6) :0,084
12.>M15 p119 (5) :0,163
13.>M15 p119 (6) :0,163
14.>BL21 p100 (3) :0,335
15.>BL21 p100 (4) :0,450
16.>BL21 p105 (1) :0,460
17.>BL21 p105 (2) :0,278
18.>BL21 p106 (4) :0,507
19.>BL21 p106 (5) :0,318
20.>BL21 p119 (2) :0,285
21.>BL21 p119 (4) :0,136

Left them 3 more hours to incubate and the ran the plate reader assay for the characterization, just as last time

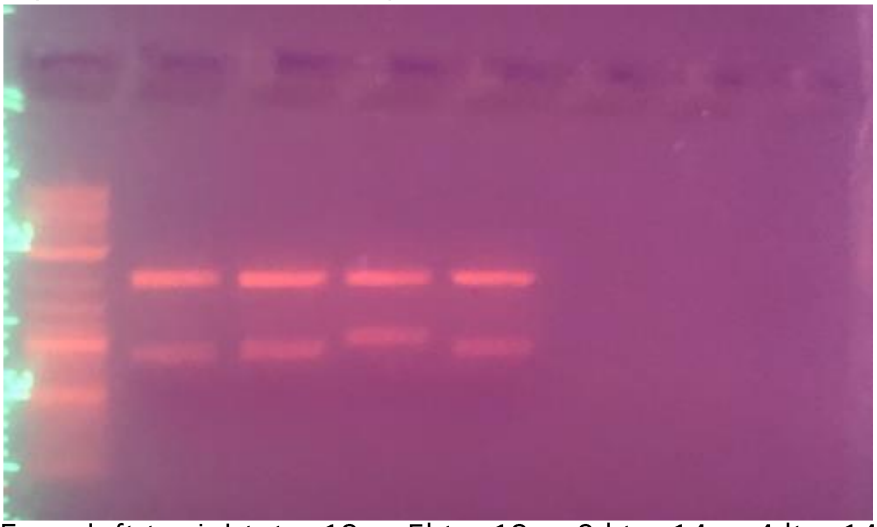
- 2hours enzymatic assay (every 30 seconds)
- Shaking 120rpm continuous
- 37o C

- At 490nm
- Used a 96-well plate
- 2 technical replicates for every glycerol stock for every promoter
- 160ul liquid culture + 40ul nitrocefin
- Plus a blank (water+nitrocefin)

2. Qubit
   Same reaction conditions

p100 (1) :138 ng/ul
p100 (4) :246 ng/ul
p105 (7) :156 ng/ul
p105 (10) :117 ng/ul
p106 (12) :154 ng/ul
p106 (13) :124 ng/ul
p119 (4) :964 ng/ul
p119 (5) :288 ng/ul

   Prepare them for sequencing : dilute them properly to 100ng/ul
3. expected band at 1.025 bps



   From left to right: toe13 no5| toe13 no9 | toe14 no4 |toe14 no13
   (expected band at 1.025 bps)

4.

- Measure the eGFP liquid colonies (200ul) at plate reader (after 16 hours incubation estimated OD600 0.3-0.5 and no further dilution)
- We prepared 2 butches of wells with the same liquid colonies
- We first took one measurement without IPTG
- We took the plate out and added IPTG (0,5ul 0,2M) at the one batch and same wells without IPTG
- 2 hours incubation at plate reader

5. 5ml M9 medium + selectable resistance markers (Amp100, Chl35, Kan50). Incubate overnight in a 37°C shaker.
   Inoculation of:
   - LacZa 1, 2 (x2)
   - 32bLacZa 1, 2 (x2)
   - 32bLacZa+trigger 2,4 (x2)

   Total 12 falcons (name e.g. 1a & 1b)

28/8/2019

Today's goals:

1. Measurent at plate reader of the LacZa constructs
2. ColonyPCR for toehold13
3. Preparation of liquid cultures

Procedure:

1.
   - Some of the liquid colonies didn't grow (all the 32bLacZa and 32bLacZa+trigger no4)
   - 200ul liquid culture + 0,4 xgal 2%
2. 20 colonies tested. ->No positive result
3. 5ml M9 medium + selectable resistance markers (Amp100, Chl35, Kan50). Incubate overnight in a 37°C shaker.
   Inoculation of:
   - LacZa 1a and 2a, 200ul to 5ml M9
   - 32bLacZa 1, 2 from LB agar plate (20/8)
   - 32bLacZa+trigger 2a and 2b, 200ul to 5ml M9

   Total 6 falcons

29/8/2019

Today's goals:

1. ***In vivo* characterization assay**
2. **Measurent at plate reader of the LacZa constructs**
3. **cloning of toehold13**
4. **Preparation of liquid cultures**

Procedure:

1. Measurement of OD600 (for M15-T7 and BL21 (DE3) characterization strains)
   in vivo assay for beta-lactamase
2. Measurement of the OD600 --> the cells didn't grow up so we incubated the liquid colonies overnight in a 37°C shaker
3. Digestion 1)toehold13 100ng (stock 50ng/ul) 2)PSB1C3 from minipreped 11/7 construct PSB1C3-LacZa 500ng
   - 20ul reaction, 2h at 37°C

- Gel extraction
- Clean up
- Qubit --> PSB1C3 5ng/ul
- Ligation 3:1, 25 ng plasmid (5ul) and 38.44ng toehold13 (3ul)

4. 5ml M9 medium + selectable resistance markers (Amp100, Chl35, Kan50). Incubate overnight in a 37°C shaker.
   Inoculation (200ul) from the 26/8 liquid culture to fresh 5ml M9 medium
   - EGFP 1, 2
   - 32beGFP 1a, 2b
   - 32beGFP+trigger 1a, 2a, 3a, 4b

   Total 8 falcons

30/8/2019

Today's goals:

1. **Check if the 32B x trigger LacZa works properly. 2. Prepare Liquid cultures for EGFP measurement (standard procedure)**

Procedure:

1. 200ul liquid culture + 0,4ul xgal20mg/ml.600nm & 615nm

   Standard procedure.

   Liquid cultures:
   5ml M9 medium + selectable resistance markers (Amp100, Chl35, Kan50). Incubate overnight in a 37°C shaker.

   Inoculation (200ul) from the 29/8 liquid culture to fresh 5ml M9 medium

   - EGFP 2a, 2b
   - 32beGFP 1a, 2b
   - 32beGFP+trigger 2a, 3a

   Total 8 falcons

31/8/2019

Today's goals:

1. **EGFP measurement. Improve part.**

Procedure:

1. All the experiment was done in M9 medium

   - Measure the OD600 of:

   EGFP, 32B EGFP, 32B EGFP X Trigger
   Dilute to OD600=0.08 (M9 medium)
   Plate reader measurement (excitation : 488nm, emission: 515nm)->Failure

2/9/2019

Today's goals:

1. **PURExpress reactions for proof of concept**
2. **Preparation of LB liquid cultures for LacZa, 32B LacZa, 32B X Trigger LacZa**
3. **Restriction digestion – ligation for toehold13 into PSB1C3 plasmid**

Procedure:

1. PURExpress aliquoting
   Solution A → 8 tubes, 12 ul each, 4 rxns per tube
   Solution B → 8 tubes, 9 ul each, 4 rxns per tube
   PURExpress reactions for Proof of concept (7 ul rxns)

   Used 1.26 ul RPA product (PURE and NC PURE) at quantities of about 125 ng

2. Standard for M15 cells.
3. Toehold 13
   - Restriction Enzymes : EcoRI&PstI
   - Quantities: 100ng (taken from IDT stock: 50ng/ul)

   PSB1C3 plasmid
   - Restriction Enzymes : EcoRI&PstI
   - Quantities: 50ng (taken from IDT stock: 25ng/ul)

   Incubating for 1h and 30min at 37°C
   Heat kill for 20min at 82°C

   PSB1C3 with toehold13 *(iGEM Protocol)* —>***1:3 ratio***
   - *PSB1K3 plasmid:* 25ng
   - toehold13: 38,11ng

   Once again, we calculated the required insert mass with the NEBiocalculator

Incubation overnight at 4°C Plasmid DNA	4ul
Insert DNA	3ul
T4 ligase buffer	1ul
T4 ligase	1ul
DdH20	1ul

3/9/2019

Today's goals:

1. **Change M9 recipe**
2. **Preparation of new M9 Medium, because the previous M9 didn't work well and Measurement.**

Procedure:

1. Checking again for the M9 medium
   Added some D-glucose instead of glycerol (although ours didn't have glycerol)
   Pre-cultures in LB
   :
   OD600 O/N

p100:1.9
p105:1.85
p106:1.86
p119:1.5
cells-only:0.5

   Inoculate the appropriate quantities in M9 medium liquid cultures to reach 0.05
   Photometry check

OD600 2h:
p100:0.526
p105:0.698
p106:0.721
p119:0.650
cells-only:0.637
OD600 4h:
p100:0.936
p105:1.011
p106:1.087
p119:1.084
cells-only:1.217

   It worked
   Kept them O/N incubated in 37oC , 210rpm to check again in the morning the OD
   New inoculations in LB to repeat the readings the next morning
2. 1lt 1X M9 Medium

Components	Quantity
Na_2HPO_4	6gr
KH_2PO_4	3gr
NH_4Cl	1gr
NaCl	0.5gr
$CaCl_2$	4mg
dH_2O	1lt

   Autoclave and add (for 1lt):

Components	Quantity
20% glucose	20ml
20% Casamino acids	10ml
$MgSO_4$ $7H_2O$ 1M	2ml
thiamine 10mg/ml	34ml
Antibiotics	x

Components	Final Concentration
Casamino Acids	0.2%
Thiamine	1mM

Glucose	0.4%
$MgSO_4$	2mM

The same day we measured the LacZa, 32b lacza and 32b lacza x trigger in plate reader using the standard procedure getting absorbance at 615nm for a total of 10 hours.->Failure

For the next day : Aim: Preparation of liquid cultures

5ml LB medium + selectable resistance markers (Amp100, Chl35, Kan50). 5ml of fresh mediums were inoculated with 200ul liquid culture from 19/8 (LacZa) and from 30/8 (eGFP.
Incubate overnight in a 37°C shaker.
Inoculation of :

LacZa
32bLacZa
32bLacZa+trigger
EGFP
32beGFP
32beGFP+trigger

4/9/2019

Today's goals:

1. RPA for IS6110
2. Testing the new M9 medium
3. Empty psb1C3 vector isolation as control
4. We tested the LacZa construct along with 32B LacZa and 32B + trigger LacZa constructs in the plate reader and the EGFP construct along with 32B EGFP and 32B + trigger EGFP constructs in the plate reader.
5. Colony PCR for toehold13-PSB1C3 DH5a
6. Transformation

Procedure:

1. RPA for IS6110
   - 42 degrees Celsius (2rxns)
   - 42 degrees + DMSO 5% (2 rxns)
   - 50 degrees Celsius (2 rxns)
   - 50 degrees Celsius + DMSO 5% (2 rxns)
   - Typical 50ul rxns
   - 10 copies of template per reaction

   AGE for RPA products after a typical cleanup

2. Testing the OD600 of BL21(DE3) cells after the new M9 prepared yday
   In 4 hours, cells reached an OD600 of 1 (from 0.05)

3. Digestion of the psb1C3 vector

According to iGEM's instructions with small changes:

2,5ul Cutsmart
0,5ul XbaI-HF
0,5ul SpeI-HF
2,5ul ddH2O
+4ul psb1C3 (from t14)

(XbaI and SpeI will cut the vector in such a position, so that he can become circular)
Incubation at 37°C for 1,5h
Gel electrophoresis (1%, 60ml TAE) -> Gel extraction + cleanup

According to the protocol of MN's kit, elution at 15ul (twice)
Ligation
Changing the standard protocol a little bit, 'cause I had no insert:

3ul plasmid
1ul T4 DNA Ligase buffer
0,5ul T4 DNA ligase
5,5ul ddH2O

Incubation O/N at 4°C (first 5mins RT)

4. Standard but we increased the volume of xgal from 0.4ul to 2ul -> Failure

5. I picked 23 colonies from the plate "3ul 3/9" and named them

"1—> 23" Reagents	Volumes
PCR water	19,9 µl
10x kapa taq buffer A	2,5 µl
10µM dNTPs Mix	0,5 µl
10µM Forward Primer VF2	1 µl
10 µM Reverse Primer VR	1 µl
Kapa taqpolymarase (5u/µl)	0,1 µl

6. Transformation of T7M15 cells with:
   a) 32blacZa (no4)
   b) eGFPminipreped
   - 100ul of competent cells transformed with 5ul stock minipreped plasmid
   - 2 plates with Amp100, Kan50, Chl35
   - Plating 100ul cells after precipitation of 900ul and removal of 400ul

5/9/2019

Today's goals:

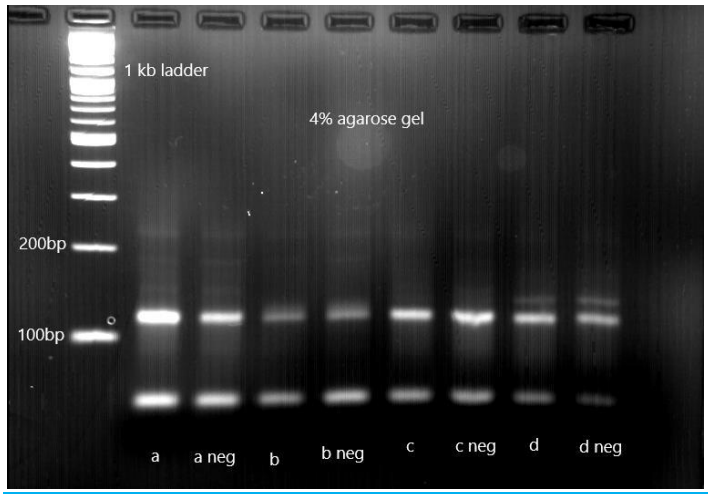
1. Transformation of empty pSB1C3
2. Testing the new M9 medium, day 2
3. RPA test
4. We tested the LacZa construct along with 32B LacZa and 32B + trigger LacZa constructs in the plate reader and the EGFP construct along with 32B EGFP and 32B + trigger EGFP constructs in the plate reader.
5. Colony PCR for toehold13-PSB1C3 DH5a
6. Restriction digestion – ligation for toehold13 into PSB1C3 plasmid

Procedure:

1. Transformation of empty pSB1C3 into DH5-alpha cells
   - 1.5 ul ligation rxn
   - 900 ul LB recovery
   - 1h incubation at 37 degrees C
   - Plate on C50 LB Agar Plates (100 ul)
2. OD600 measurements for BL21 (DE3) cells on M9 minimal medium
   Cells reached around 1.5-1.8 of absorbance, which is the expected value
3. RPA Tests again
   Testing different DMSO concentrations and lower primer concentration
   8 reactions:

S1F1O-S1R2O with 2,5% DMSO (a)
(a negative)
S1F1O-S1R2O with 10% DMSO (b)
(b negative)
S1F1O-S1R2O without DMSO (c)
(c negative)
S1F1O-S1R2O with 1,2ul primers (d)
(d negative)

   Reaction conditions the same but changing the quantity of the DMSO and altering accordingly water quantity OR lowering the primers quantities and accordingly raising water quantity
   Cleanup ->15ul elution in kit's elution buffer (twice) -> electrophoresis in 4% agarose gel, 150ml TAE



4. The same as the day before ->Results: Failure
5. I picked 22 colonies from the plate "4ul 3/9" and named them

"1—> 22" Reagents	Volumes
PCR water	19,9 µl
10x kapa taq buffer A	2,5 µl
10µM dNTPs Mix	0,5 µl
10µM Forward Primer VF2	1 µl

10 μM Reverse Primer VR	1 μl
Kapa taqpolymarase (5u/μl)	0,1 μl

6. Restriction – Digestiona was performed with iGEM protocol

   Toehold 13
   - Restriction Enzymes : EcoRI&PstI
   - Quantities: 100ng (taken from IDT stock: 50ng/ul)

   PSB1C3 plasmid
   - Restriction Enzymes : EcoRI&PstI
   - Quantities: 100ng (taken from IDT stock: 25ng/ul)

   Incubating for 1h and 30min at 37oC
   Heat kill for 20min at 82oC
   Ligation Reaction:
   PSB1C3 with toehold13 *(iGEM Protocol) —>1:3 ratio*
   - *PSB1K3 plasmid:* 25ng
   - toehold13: 38,11ng

   Once again, we calculated the required insert mass with the NEBiocalculator

Incubation at 16°C for 16h Plasmid DNA	2ul
Insert DNA	3ul
T4 ligase buffer	1ul
T4 ligase	1ul
DdH20	3ul

6/9/2019

Today's goals:

1. **Identification of positive empty pSB1C3 colonies**
2. **Liquid cultures for empty pSB1C3 preparation**
3. **We tested the EGFP construct along with 32B EGFP and 32B + trigger EGFP constructs in the plate reader.**
4. **Transformation toehold13-PSB1C3 in DH5a competent cells**

Procedure:

1. Colony PCR for empty pSB1C3
   - 13 colonies + 1 NC
   - Typical 25 ul PCR reactions for colony detection
   - Master Mix preparation for 15 (all except ddH2O)

   Age for colony PCR, indeed got some positive results
2. (Inoculation of 5ml LB cultures for minipreps)
3. Standard->Results: failure
4.
   - PSB1C3 + toehold13 | **4,5ul** ligated product in 100ul C.C

   Centrifugation of 1000ul total. Removal of 600ul and resuspension of the pellet.

- 2 plates LB agar Chl35 1)plating 100ul and 2)plating 200ul

Overnight incubation at 37oC

9/9/2019

Today's goals:

1. **Q5 PCR for HBV (failed)**
2. **Identification of lacI positive colonies**
3. **Transformation**
4. **Liquid cultures**

Procedure:

1. Failed
2. Colony PCR
   - 8 colonies for DH5a- beta-lactamase (new part from igem's distribution kit)
   - 10 colonies for BL21-psb1C3 only
   - So, I had 18 colonies and a NTC for the PCR reaction = 19 reactions
   - The reaction was set up exactly as a standard colony PCR (like always)
   - The MasterMix was prepared for 21 reactions

   Gel electrophoresis ->1,3% agarose gel the positive colonies which I inoculated in 5ml LB:

psb1C3(1) and (3)
beta-lactamase (1) and (6)

3. Transformation of empty PSB1C3 into T7M15 cells
   - PSB1C3 | 2**ul** minipreped stock in 100ul C.C
   - Plating of 150ul --> LB agar Chl35
4. 5ml LB (some with Chl35, some with Kan50) and inoculation with **DH5a** constructs:
   - LacZa (PSB1C3)
   - 32bLacZa (PSB1C3)
   - 32bLacZa+trigger (PSB1C3)
   - EGFP (PSB1C3)
   - 32beGFP+trigger (PSB1K3)
   - Empty DH5a cells
   - Empty PSB1C3 in DH5a

   No 32beGFP inoculation (no glycerol stock found)

10/9/2019

Today's goals:

1. **Serial dilutions for HBV template**
2. **RPA diluted to 1ml and HBV proof**
3. **Identification of positive empty pSB1C3 and beta lactamase colonies in BL21**
4. **Measurement of the DH5a constucts in the plate reader**

5. Cloning of toehold 13 in psb1a3

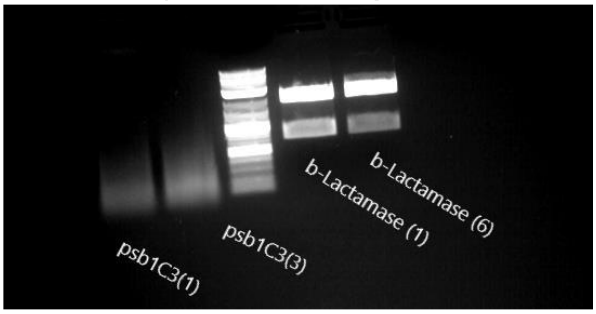
Procedure:

1. Serial dilutions for HBV template
   - Starting concentration → 5ng/ul
   - (Calculations not shown)
   - Concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5},10^{-6},10^{-7},10^{-8},10^{-9},1 copy per ul were created.
2. RPA dilutes to 1ml and HBV proof
   - Both failed miserably
   - Typical 50 ul rxns, 1ml rxn was diluted in 950 ul ddH2O
3. Minipreps from yesterday's liquid cultures
   4 samples:

psb1C3(1)
psb1C3(3)
beta-lactamase (1)
beta-lactamase (6)

   40ul elution
   Restriction Digestion
   - According to iGEM's protocol as always
   - 1,5h incubation at 37°C

   Gel electrophoresis (1% gel, 60ml TAE)



   I could not see the plasmid vectors because they are in BL21 cells which can not be minipreped correctly. But that's ok I knew from the colony PCR they were correct. Beta-Lactamase was also correct.
4.
   - Measure OD600, dilute to 0,0 in fresh M9 supplemented medium
   - For the LacZaconstucts :

   Plate reader: measure for 30min every 1min and 5,5h every 20min (37°C)
   200ul of liquid culture per well + 2ul xgal(C???)
   For the eGFP constructs:
   Plate reader: measure for 6h every 1h
   200ul of liquid culture per well

5. Toehold 13
   - Restriction Enzymes : EcoRI&PstI
   - Quantities: 100ng (taken from IDT stock: 50ng/ul)

   PSB1A3 plasmid

- Restriction Enzymes : EcoRI&PstI
- Quantities: 50ng (taken from IDT stock: 25ng/ul)

Incubating for 1h and 30min at 37°C
Heat kill for 20min at 82°C.

11/9/2019

Today's goals:

1. Transformation of the toehold into DH5a cells.
2. Colony PCRs for the empty psb1c3 vector.

Procedure:

30 colonies tested. Elongation 24seconds ->Success.

12/9/2019

Today's goals:

1. **RPA reactions for HBV**
2. **Colony PCR for toehold13-PSB1A3 DH5a**

Procedure:

1. 4 reactions:

1 copy/ul
10^{-9} ng/ul
10^{-8} ng/ul
NTC

- Used the second primer set
- RPA reaction same as always according to the kit's handbook
- Reactions incubated at 42oC for 10 mins with the in-between quick spin step

Inoculations of BL21 cells with the promoters for the characterization, in 12 (5ml LB C50) liquid cultures.

Used old LB cultures and inoculated from every culture 100ul to 2 new ones for every promoter. (2 biol.l replications)
The same applies for the cells-only cultures.
Incubation at 37°C O/N in shaking incubator 210rpm

2. I picked 20 colonies from the plate "11/9" and named them

"1—> 20" Reagents	Volumes
PCR water	19,9 μl
10x kapa taq buffer A	2,5 μl
10μM dNTPs Mix	0,5 μl
10μM Forward Primer VF2	1 μl
10 μM Reverse Primer VR	1 μl
Kapa taqpolymarase	0,1 μl

(5u/μl)

13/9/2019

Today's goals:

1. Identification of positive LacI and beta-lact clones (for new improve, pSB1K3)
2. *In vivo* characterization assays
3. We made glycerol stocks of the colonies that gave us positive results and tested the sensitivity of our part 32B toehold + Beta-Lactamase, by adding 25ng, 15ng, 7ng and 3ng trigger to a 7ul PURE reaction.

Procedure:

1. Colony PCR for pSB1C3 – lacI/ betalact
   - 6 colonies + 1 NC
   - Typical 25 ul colony PCR reactions
   - Prepared a Master Mix for 8 (all except water)

2. Photometry at 600nm of yesterday's LB pre-cultures
   OD600 O/N:

p100 (1) :1,9
p100 (2) :1,93
p105 (1) :1,85
p105 (2) :1,68
p106 (1) :1,84
p106 (2) :1,87
p119 (1) :1,79
p119 (2) :1,67
cells-only(1):1,97
cells-only(2):2,1

ALSO added the bl21-psb1c3 only control (I do not have these ODs, Nick did it)
Diluted them properly (new inoculations) into new 4ml M9 cultures C50 to reach 0,05

NEW M9:

1X for 50ml
10ml from 5X M9
1,7ml of 10mM Thiamine
1ml of 20% glucose
500ul of 20% casamino acids
100ul of 1M MgSO4
5ul of 1M CaCL2
36,7ul ddH2O

M9 photometry after 1,5h(time of assay) and 3h (just for check)
OD600 1,5h:

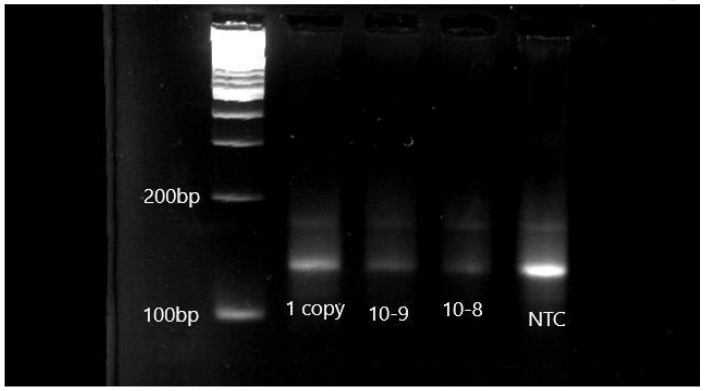
p100 (1) :0,310
p100 (2) :0,27
p105 (1) :0,42
p105 (2) :0,44
p106 (1) :0,34
p106 (2) :0,36
p119 (1) :0,18
p119 (2) :0,20
cells-only(1):0,33
cells-only(2):0,30
psb1c3 only(1): 0,40
psb1c3 only(2): 0,40

Here we put the plate reader assay as last times
(2 technical reps, 2 hours per 30 seconds, 37oC, 490nm, 210rpm)
O nly because we had a more concentrated nitrocefin we used 180ul of culture + 20ul nitrocefin for the assay

OD600 3h:

p100 (1) :1,30
p100 (2) :1,28
p105 (1) :1,43
p105 (2) :1,47
p106 (1) :1,37
p106 (2) :1,36
p119 (1) :0,57
p119 (2) :0,87
cells-only(1):1,30
cells-only(2):1,35
psb1c3 only(1): 1,44
psb1c3 only(2): 1,42

Gel electrophoresis for yesterday's RPA (4% gel, 150ml TAE)



Inconclusive results, the primer set doesn't seem very good.

3.

- The reaction was done at 37 degrees for 1 hour.

- The results shown that even 7ng are enough to change the color of the solution after the addition of 40ul 0.5mM nitrocefin.
- Later, we added 3 and 0.3 ng trigger to the PURE reaction and incubated it for 3 hours at 37 degrees.
- The results shown that even 0.3ng trigger are enough to change the color of the solution.
- In the same day we made agar plates of M15-T7 cell with EGFP , LacZa and Psb1c3 with no insert. + We prepared liquid cultures for toehold 13.

14/9/2019

Today's goals:

1. **PCR reactions for HBV + IS6110**
2. **Check if the cloning was successful and repeat**
3. **Transformation trigger32b-PSB1K3 in DH5a competent cells**
4. **Transformation lacI-lact – in BL21 competent cells**

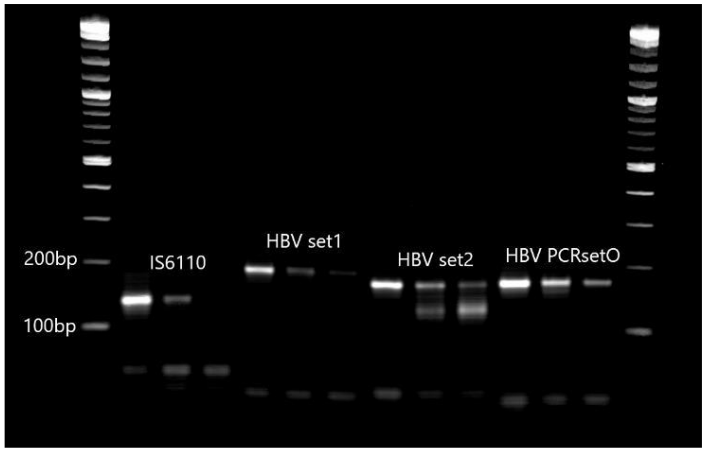
Procedure:

1. 12 reactions:

IS6110 10-8 S1F1O-S1R2O
IS6110 10-9 S1F1O-S1R2O
IS6110 neg
HBV 10-8 set1
HBV 10-9 set1
HBV set1 neg
HBV 10-8 set2
HBV 10-9 set2
HBV set2 neg
HBV 10-8 PCRsetO
HBV 10-9 PCRsetO
HBV PCRsetO neg

- Typical PCR reaction (15s extension) with some different annealing temps:
- PCRset1 HBV :64°C
- S1F1O and HBV set1 : 66°C
- HBV set2 : 68°C

ALSO the reaction MasterMix was created for 14 samples (x14)
The reaction was with 5%DMSO -> electrophoresis in 4% agarose gel, 150ml TAE. The samples ran in the above order of the reactions



We made sure that:
1. IS6110 can be amplified in a PCR reaction without a negative band
2. HBV set 1 primers are the best to use
3. PCR primers witho Overhangs for HBV are not good enough

2. Plasmid isolation, digestion, electrophoresis (Standard) -> Failure

Repeated the cloning experiment of toehold 13, this time by changing the ligation ratio to 7:1.
Toehold 13: 88ng

3.

- PSB1K3 + trigger32b | 5**ul,** 100ng ligated product in 100ul C.C
- Plating of 100ul in LB agar plate Kan50

Overnight incubation at 37oC

4.

- PSB1C3 + lacI-lact | 3ul, 100ng ligated product in 100ul C.C
- Plating of 1)100ul and 2) 100 after precipitation

in LB agar plate Chl35
Overnight incubation at 37oC

15/9/2019

Today's goals:

1. **transformation of DH5a strains with toehold 13**
2. **Colony PCR for trigger32b – PSB1K3 DH5a (20 colonies picked)**

Procedure:

1. Standard

16/9/2019

Today's goals:

1. **New toehold PURE assay**
2. **PCR and isolation for PURE assay**
3. **Testing the RPA for HBV again**

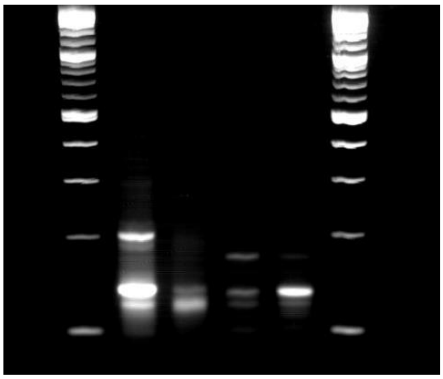
4. PURE expression kit: Test for 32b EGFP trigger 25, 7, 3ng
   Test for 32b Lactamase: trigger 0.03ng & 0.12ng.
5. Check if the cloning of laci was successful
6. Colony PCR for the colony's PCR plate 15/9 trigger32b-PSB1K3 DH5a

Procedure:

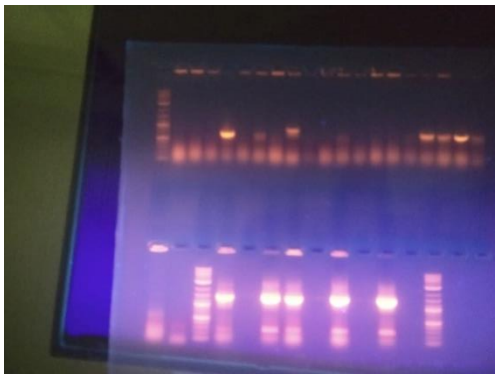
1. PURE toehold 13
   We worked straight from IDT's stock because we hadn't cloned it yet.
2. PCR preparation to load into PURE (10^{-2} template)
   Added DMSO
   Typical 25 ul rxns, even though it was not enough
   PCR for DNAse assay
   Tried both Q5 and Taq, didn't work for some reason, turned to plasmid DNA for the assay
3. This time using set1 primers and PCRsetO primers
   Template was 10^{-8} ng/ul
   4reactions:

set1
set1 neg
PCRsetO
PCRsetO neg

   The RPA reaction was the typical one (5mins, 42oC, with quick-spin step) and the volumes were according to the company's protocol
   Cleanup -> 15ul elution twice -> electrophoresis in 4% agarose gel, 150ml
   Samples ran with the order given above (in the reactions)



   Not much to see, not a good outcome, cannot explain it clearly.
4. Standard procedure for PURE kit (3 hours, measurement with 43ul of 0.05mM nitrocefin at 490nm for 20minutes).
5. Colony PCR. Elongation time 65seconds
   27 colonies picked. Expected bands at 1400bps.



6.

- 18 colonies were picked
- Mix for 20 reactions
- Add 25ul of MM and then transfer individual colony (on ice)
- Run at program “iGEM 53” plants lab.
- Extension time: 150 (t32b) + 270 (primers) = 420 x 0,06sec = 30sec
- Electroforesis gel 100ml, 1%, 110V, ladder 100bps

5ul DNA, 5ul ddH2O, 2ul purple loading dye

- Expected band at 420 bps

17/9/2019

Today’s goals:

1. PCR reaction again for set1 HBV
2. Cloning of trigger 32b to PSB1K3.

Procedure:

1. Testing set1 primers again and running bigger reactions (50ul) for HBV and IS6110 and twice each, so I can elute them together and have a high concentration afterwards whie cleaning it up (want to use it for PURE)

10 reactions:

1.S1F1O-S1R2O (1ng/ul)
2. -//-
3.S1F1O-S1R2O (negative)
4. -//-
5.S1F1-S1R2 (1ng/ul)
6. -//-
7.S1F1-S1R2 (negative)
8. -//-
9.HBV set1 (10-8ng/ul)
10.HBV set1

(negative)

The PCR reaction set up was the same ONLY with doubled volumes for everything. The MasterMix was x12 for 10 reactions
PCR machine: Standard for Ktaq program with 15s of extension (I had max 190bp amplicon length) and annealing temps:
S1F1O and HBV set1 = 66°C
S1F1 = 63°C
Cleanup for 1-8 samples and combined the same ones to 1 column! Elution was done in 15ul twice C. PUREexpress by NICK (not a good result)

2. Digestion withEcoRI and PstI of 500ng trigger 32b and 500ng of psb1k3.
   However there were a limited amount of trigger and plasmids so we decided to start the cloning again the next day.
   We checked the LacZa and 32B/Trigger LacZa with the addition of iptg, to see if they work at a plate reader. After the addition of x-gal ,only the liquid culture with the LacZa construct gave blue color.

18/9/2019

Today's goals:

1. **Isolation of digested pSB1K3/C3 and trigger 32B**
2. **Subcloning of toehold 13 and trigger32B**
3. **DNAse I treatment on IS6110 template**
4. **Electrophoresis for set1 HBV**
5. **Minipreps to isolate a big amount of plasmid containing the 32b trigger, preparation liquid cultures of 32b lactamase and 32B/trigger LacZa, calculate the concentration of the plasmids via Qubit and test again the EGFP and 32B/Trigger LacZa plasmid at a plate reader with the addition of iptg.**

Procedure:

1. Digestion of pSB1C3/pSB1K3, and trigger 32B from miniprepped plasmids for ligation rxns
   - 20 ul rxns, 4 in total
   - 10 ul DNA in each rxn (1000 ng of plasmid with insert)
   - Master Mix for 7 rxns
2. Digestion of Toehold 13 (1.2 ul left on stock → 60ng)
   - Master Mix from iGEM Protocol
   - 1h incubation at 37 degrees Celsius

   AGE for gel excision (1.2% gel to discriminate the bands – maybe that was a mistake --)
   Gel excision

pSB1C3 1	130mg
pSB1C3 2	80mg
pSB1K3 1	146mg
pSB1K3 2	148mg
trigger 32B 1	301mg
trigger	236mg

32B 2

Qubit for samples after gel extraction protocol (typical)

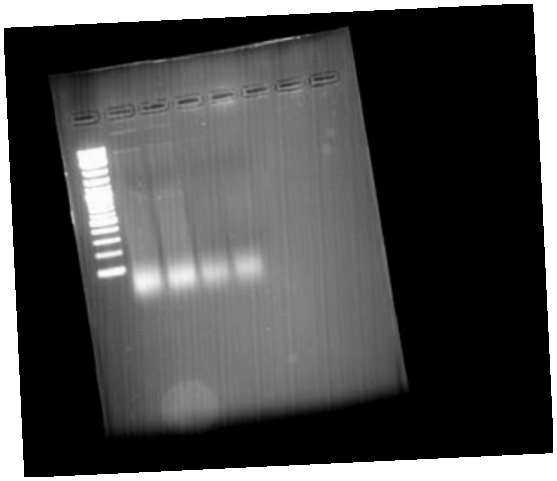
pSB1C3	58.4 ng/ul, 30 ul total
pSB1K3	29.2 ng/ul, 30 ul total
trigger32B	1.4 ng/ul, 30 ul total

Ligation of trigger into pSB1K3 and toehold 13 into pSB1C3

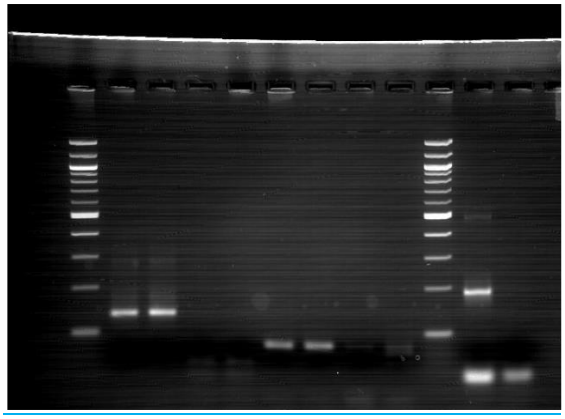
- Typical 10 ul ligation reactions
- 1ul T4 DNA ligase used
- 25-29 ng of plasmid
- 3 to 5:1 ratio in general
- Master Mix prepared for 2.2 rxns

3. DNAse I treatment for IS6110 plasmid template
   10 ul reactions
   - 800 ng of DNA were digested (100ng/ul, total 8 ul per reaction used)
   - 1 ul 10X DNAse reaction buffer used
   - 0.2 ul (1U) DNAse I used
   - 0.8 ul ddH2O
   - Incubate at 37 degrees C
   - To stop the reaction, add 1ul 50mM EDTA and incubate in 75 degrees C for 10 min

Reaction times of 3, 5, 7, and 10 minutes were tested. 3 and 5 minutes were promising, although there was not a good enough smear on the gel.



4. 3% agarose gel for 1,3,5,7,9,10 samples -> The gel was not good, it did not run well and had bands in the negative control



Again the same PCR reaction only this time with normal 25ul volume. All conditions AND reactions (samples) remain the same

Run also a 3% agarose gel and made sure they were correct (don't have the image)
PURE reaction

7ul reactions!
Typical Reaction:
Add in order

2,8ul Solution A
2,1ul Solution B
0,14ul RNAse Inhibitor
0,7ul 32B-beta Lactamase
1,26ul trigger (Here we used the PCR products, and also put 2ul instead of 1,26)

Incubation at 37°C for 3h
Quick Spin every 1h
Plate reader assay

7ul reaction from pure + 43ul Nitrocefin
Plus a blank (water+Nitrocefin)
(30 mins assay, every 30s, 490nm)
Still not so good

5. The standard procedures.

19/9/2019

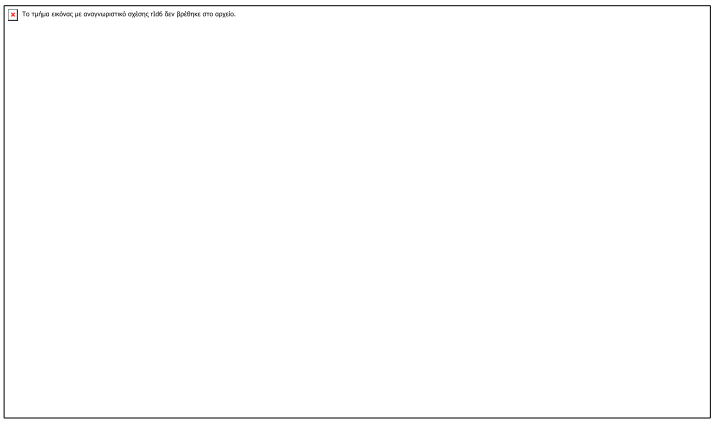
Today's goals:

1. DNAse I treatment on IS6110 template, 2nd try
2. PCR in fragmented IS6110 sample
3. PCR for the IS6110 with T7 term
4. Minipreps to isolate plasmids with 32b lactamase and 32B/Trigger LacZa and qubit to determine their concentration and transformation of DH5a competent cells with plasmid containing our new toehold construct

Procedure:

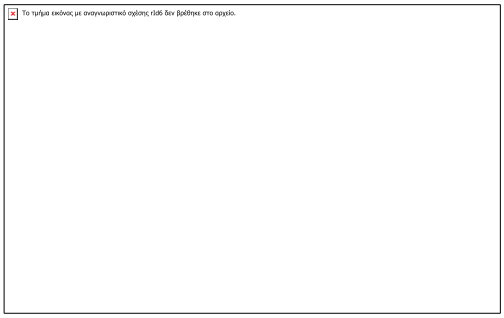
1. DNAse I treatment to IS6110 plasmid again

This time, 20 ul reactions, more DNA (1790ng) but the same enzyme. Reaction times were 3 and 6 min
Results are shown in the gel below (Ladder, 3min, 6min)



For some reason, the 3 min reaction didn't produce a good smear, whereas the 6 min reaction did. However, there was no DNA to be seen at the plasmid band (4kb) as it was on 6 min reaction.

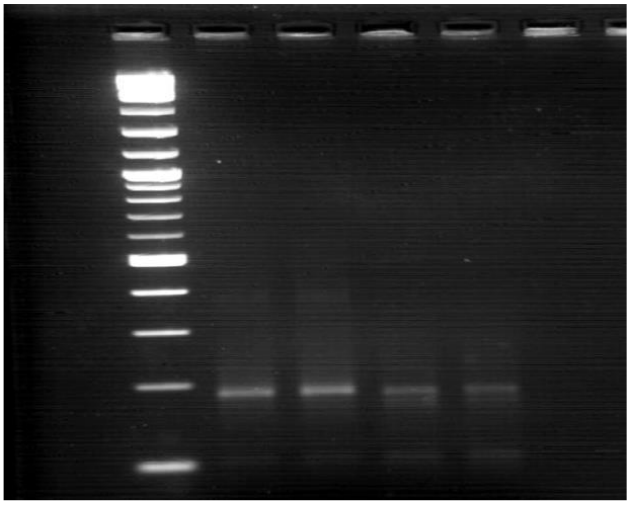
2. PCR in fragmented samples
   To see if we can amplify our biomarker when fragmented in a non-specific manner, a typical PCR reaction (25 ul) was prepared for each condition (3min DNASE treatment, 6 min)
   2 ul of sample was added in the reaction.
   Result in the gel produced a band at the NC (lane 4), due to lack of DMSO in the reaction.



3. PCR reaction again for the reverse primer with the T7 terminator (IS6110)
   Testing a new reverse primer with a T7 term sequence in its 5' overhang plus the pre-existing one.
   Wanted to see if we do not get a signal from RPA in PURE because of the terminator
   4 reactions:

T71 (1ng/ul)
T72 (1ng/ul)
(negative1)
(negative2)

   The PCR reaction set up was the same as a typical reaction. The MasterMix was x12 for 10 reactions
   PCR machine: Standard for Ktaq program with 15s extension (188bp amplicon length) and annealing temp: 66oC)
   Gel electrophoresis ->Reactions ran on the gel in the above order
   Ran a 4% agarrose gel BUT got primers dimers on the negative control again so we did not run a PURE reaction.



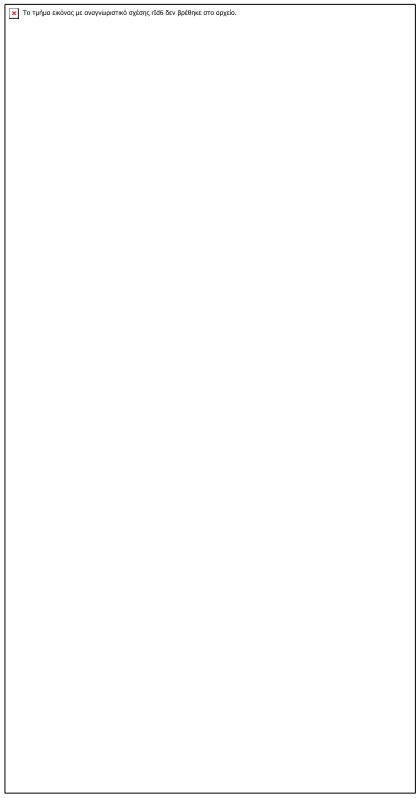
20/9/2019

Today's goals:

1. Kanamycin stock preparation
2. PCR in fragmented samples vol 2 (DMSO addition)
3. PURE with both end digested and linear trigger
4. Colony PCR for toehold13 – PSB1C3 DH5A. Transformation of DH5A with trigger construct in psb1k3.

Procedure:

1. Kanamycin stock preparation (50mg/ml)
   Weigh 0.5 g Kanamycin
   Add 0.5 g Kanamycin into 10ml ddH2O (in volumetric flask)
2. PCR in fragmented samples vol 2 (DMSO addition)
   This time 5% DMSO was added, in hope of eliminating the artifacts in the negative control lane in AGE.
   As expected, the band was not there, indicating that a PCR reaction can detect our biomarker when it non-specifically fragmented.
   From left to right → Ladder, NC, 6 min DNASe, 3 min DNASe
   Expexted bands at 136 bp



3. Digestion of t32B-psb1C3
   Testing a theory if the lack of signal in based on the fact that our amplified product does not have a 5' scar, I digested our t32B trigger-psb1C3 in two different ways (one cut in 2 sites and one cut onlu in the 3' end) and then did a PURExpress reaction for each, to see if they will trigger our toehold.
   Samples:

Dig: Cut with EcorI and PstI
Linear: Cut only with PstI (3')

   Dig Reaction:

Cutsmart Buffer: 1ul
EcorI-HF: 0.5ul
PstI-HF: 0.5ul
Template: 8ul

   Linear Reaction:

Cutsmart Buffer: 1ul
PstI-HF: 0.5ul
ddH2O: 0.5ul
Template: 8ul

   - 2h incubation at 37°C
   - 20 mins Heatkill at 80°C

   Electrophoresis in 1% agarose gel, 60ml TAE
   1. Ladder 2. Uncut 3. Linear 4. Dig
   Saw that they were ok and continued with the PURE reaction
   PURE reaction withLinear, Dig and Only toehold

PURE reaction was the standard one as last time, only I used 0,9ul of template and supplemented the rest 0,36ul with water. ALSO the 3rd reaction is kind of a negative one so instead of sample you add 1,26ul water.
Incubation for 3h at 37°C. ->Quick Spin every hour
Plate reader assay
Conditions as last time, 7ul reaction from pure + 43ul Nitrocefin. Plus a blank (water+Nitrocefin)
(30 mins assay, every 30s, 490nm)

4. 17 colonies tested
   - Mix for 20 reactions
   - Add 25ul of MM and then transfer individual colony (on ice)
   - Run at program "iGEM 53" plants lab.
   - Extension time: 84 sec

   Standard Settings: Every colony pcr was done using the below settings. However the elongation times was changing according to the size of the construct

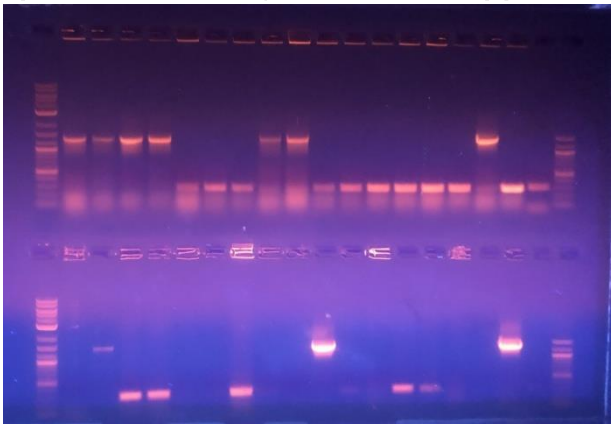
   e.g 1400bp x 0.06 = 84
   Elongation time = 84 seconds.

Temp (°C)	Time(seconds)	Passes
95	180	1
95	30	34
53	30	34
72	84	34
72	120	1
4	infinity	1

Electroforesis gel 100ml, 1%, 110V, ladder 1kb and ladder 100bps

- 5ul DNA, 5ul ddH2O, 2ul purple loading dye.

Expected band at 1400 bps
The same day, we tried to transform again DH5a competent cells with the product of ligation between psb1k3 and trigger 32b.



Top: 1kb ladder | toehold13 #1 to #17 (nikol's) | n.c. | 100bps ladder

Down: 1kb ladder | toehold13 #1 to #17 (thod's) | n.c.| 100bps ladder

Theoretically, all the bands that were between 1200 –1500bps, corresponds toehold13 construct.
I took the colonies #1 #4 #9 #16 and inoculated them in 5ml LB Chl35
Nikol took the colonies #2 #10 #17 and inoculated them in 5ml LB Chl35.

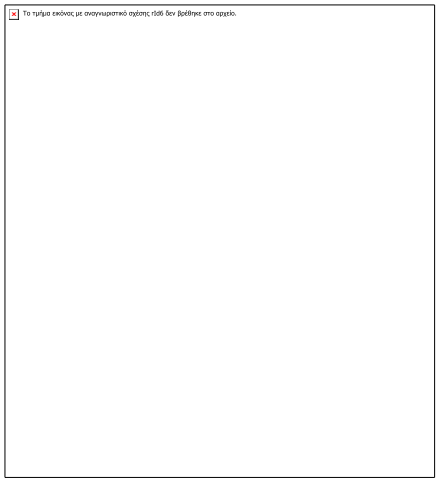
21/9/2019

Today's goals:

1. PCR for IS6110 and HBV
2. Cloning of trigger 32b to psb1k3
3. Minipreps – diagnostic digestion – electrophoresis to confirm toehold13 constructs

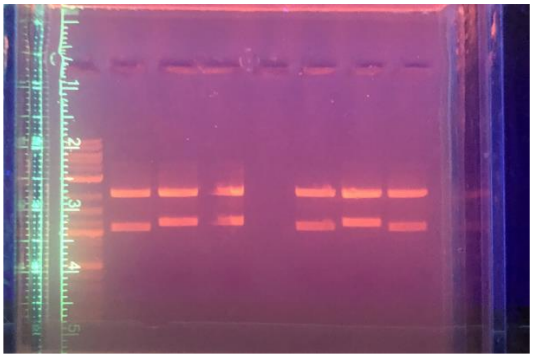
Procedure:

1. PCR for IS6110 (added T7 terminator)
   PCR program is IgemISt7, gradient one
   2 volumes of DMSO were tested, 5 and 10 percent
   PCR for HBV (set 1, with T7 terminator)
   Again, a gradient program named IgemHBVt7
   PCR for IS6110 (trigger 13 reverse primer)
   Program named IgemISt13
   All reactions contain DMSO
   All reaction have a template of 10^{-4}ng/ul at 1ul for each rxn.
   Master Mix prepared for all reaction (buffer, dNTP mix, kTaq, part of the final water volume)
   Gel from right to left
   100 bp ladder, NC HBV, HBV, 3 empty lanes for IS6110 T7, IS6110 t13, IS6110 t13 NC

   IS6110 for T7 for some reason didn't work, maybe it's the gel's fault. I will maybe try again Monday to run the remainder of the reaction!
2.
   - Digestion of 1000ng trigger and 500ng of psb1k3.
   - Gel extraction
   - Qubit : 5,1 ng trigger, 19ng plasmid
   - Ligation 3:1. 5:1 ratios
3.

Quantities per reaction: DNA	1,5 ul
E.coRI HF	0,5 ul



22/9/2019

Today's goals:

1. Transformations of trigger32b – PSB1K3 in DH5a

Procedure

1.
   a) with 3:1 ligation product
   b) with 5:1 ligation product

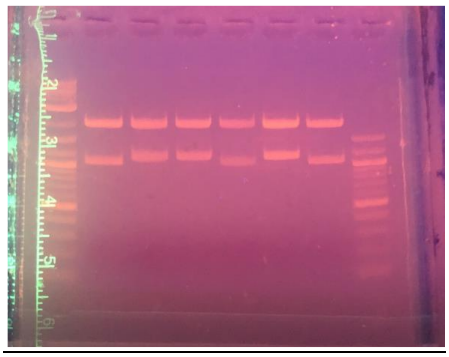
23/9/2019

Today's goals:

1. Extra confirmation about the succesfull cloning of toehold13 in PSB1C3
2. Transformation of 32blactamase – PSB1C3 in Bl21 cells
3. Liquid cultures
4. Digestion of t32B-psb1C3
5. Clean up
6. Qubit reactions for Dig and Linear samples
7. PureExpress

Procedure

1. Run again the same digested products no1,2,9,10,16,17 (from 21/9 stored at -20) with 1,5% agarose gel for 45min



From left to right: ladder 1kb|toehold13 #1 #2 #9 #10 #16 #17 |100bp ladder
We can see from the gel that the colonies #1 #10 #17 have the right construct of toehold13

2.

- PSB1C3 + 32blactamase in Bl21 | 3ul, 100ng ligated product in 100ul C.C

- 1 plates with 100ul reaction without precipitation in LB agar plate Chl35
  Overnight incubation at 37oC

3. 5ml LB and inoculation with:

- Toehold13- PSB1C3 Chl35 no1,10,17
- Bl21 cells no antibiotics (3 colonies picked from nick's plate)
- LacI-lact - PSB1C3 in Bl21 (from stock no2 thod) Chl35
- Bl21 with PSB1C3 empty vector (from plate 11/9 afro)

4. Digestion of t32B-psb1C3
   Testing again if the lack of signal in based on the fact that our amplified product does not have a 5' scar,

   4 reactions:
   >2x Dig: Cut with EcorI and PstI
   >2x Linear: Cut only with PstI (3')

   All Reactions were conducted differently in a total volume of 20ul:
   Dig Reactions:
   >Cutsmart Buffer: 3ul
   >EcorI-HF: 1ul
   >PstI-HF: 1ul
   Template: 15ul
   Linear Reactions:
   >Cutsmart Buffer: 3ul
   >PstI-HF: 1ul
   >ddH_2O: 1ul
   Template: 15ul

   Quick spin step
   2h incubation at 37^oC
   20 mins Heatkill at 80^oC

5. Clean up
   According to the MN's Gel and PCR cleanup kit instructions
   - I combined the same reactions (2x Dig and 2xLinear) into the same columns
   - Elution in 20ul (twice)

6. Qubit reactions for Dig and Linear samples
   The typical reaction according to the kit's manual.
7. PureExpress
   The first reaction of the 2nd Pure kit, so we needed to make aliquots first of the solutions A and B.
   Aliquotes: 4 reactions per tube.

   6 Pure reactions:
   5ng trigger+32B-betaLactamase (~100ng/ul)
   linear trigger
   Dig trigger
   RPA product
   RPA NC product
   Leakage (Only the toehold)

   The reactions were the typical ones for a 7ul reaction

All the templates were used in 1,26ul (maximum) except for the leakage control in which I used water instead of template

24/9/2019

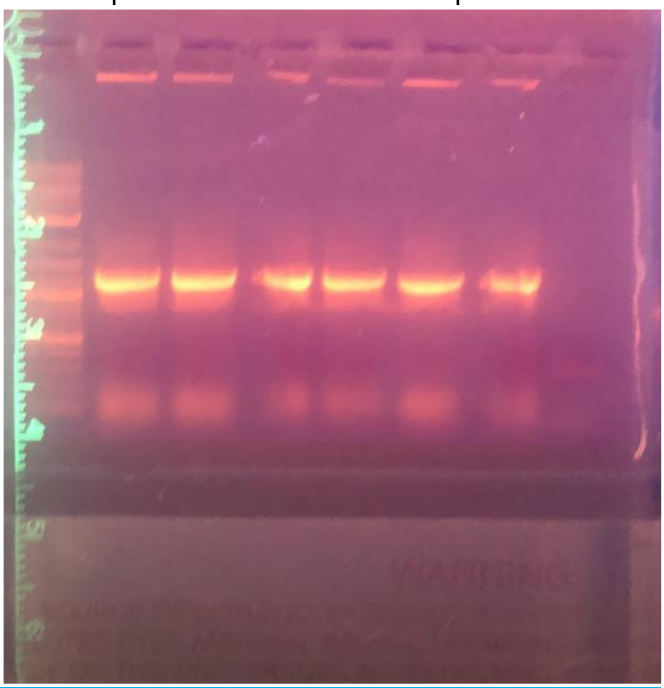
Today's goals:

1. **Yesterday we forgot to add IPTG in the liquid cultures**
2. **Colony PCR for 32blactamase – PSB1C3 BI21**

Procedure

1. We did new liquid cultures (100ul from yesterday's cultures) in 5ml LB, the right antibiotics and IPTG 12ul 0.1M
2. 6 colonies tested +1 N.C.
   - Mix for 8 reactions
   - Add 25ul of MM and then transfer individual colony (on ice)
   - Run at program "iGEM 53" plants lab.
   - Extension time: 80sec (1025+153+118bps x 0,06sec)
   - Electroforesis gel 60ml, 1%, 110V, ladder 1kb

   5ul DNA, 5ul ddH2O, 2ul purple loading dye
   - Expected band at 1300 bps



From left to right: ladder 1kb|32blact #1 #2 #3 #4 #5 #6 |N.C.
Succeed

25/9/2019

Today's goals:

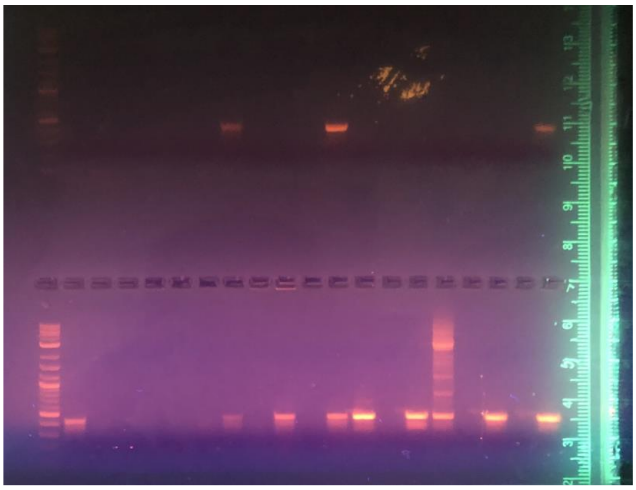
1. Plate reader measurments of the 23/9 liquid cultures
   - BI21 cells (3 biological repeats no1,2,3)
   - BI21 with PSB1C3 empty vector (2 biological repeats no1,2)

- LacI-lact - PSB1C3 in Bl21 (2 biological repeats no1,2)
- 32b beta-lactamase – PSB1C3 (2biological repeats no5,6)

2. Run an electrophoresis gel with nick's ColonyPCR products (trigger32b-PSB1K3 in Bl21)
3. Colony PCR for pSB1K3-t32B

Procedure

1.
   - Firstly, we dilute to 0,1 OD600 in 5ml LB medium. Extra addition of and 12,5ul IPTG 0,1M and Chl35 (except Bl21 empty cells)
   - Incubation at 37°C shaker for about 2 hours untill OD600 0,5
   - Measure the exact OD600 and dilute to 0,5 in LB medium (same as above)
   - 2hours plate reader measurment
2. 36 colonies, 150ml gel 1,5% agarose
3. Typical 25ul Colony PCR reactions, 36 colonies picked (35+1NC)



Top: 1kb ladder | trigger32b #1 to #19
Down: 1kb ladder | trigger32b #20 to #36
Liquid cultures of no7,11,19,20,31,36

26/9/2019

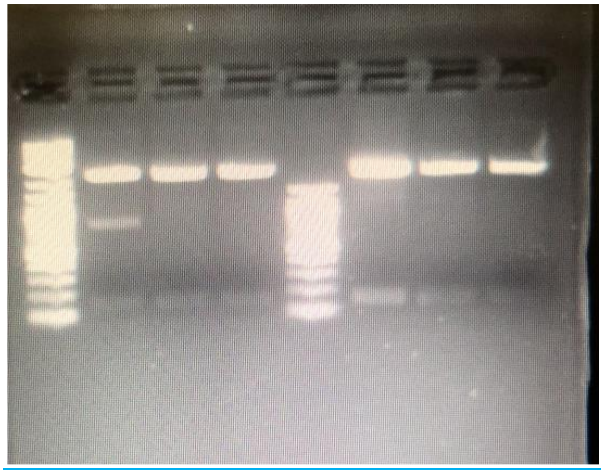
Today's goals:

1. **Minipreps – diagnostic digestion – electrophoresis to confirm trigger32b in PSB1K3 construct**
2. **Co-transformation**

Procedure

1. Quantities per reaction:

DNA	2,5 ul
E.coRI HF	0,5 ul
PstI HF	0,5 ul
Cutsmart buffer	2,5 ul
ddH2O	14 ul



From left to right: ladder 1kb|trigger32b no7|no11|no19|-|no20|no31|no36
All of them succeeded

2. Co-transformation of the plasmids:
   - PSB1C3-32Blact &
   - PSB1K3-trigger32b

   in Bl21 cells (nick,thod)
   2 antibiotics: Chl35, Kan50

   6 plasmid mixes were prepared, to be sure that even one mix gives some colonies:

   We transformed the following concentrations of the plasmid with toehold 32B lactamase and the plasmid with the 32B Trigger:
   - 2ng trigger, 2ng toehold
   - 20ng trigger, 20ng toehold
   - 50ng trigger, 50ng toehold
   - 75ng trigger, 75ng toehold
   - 100ng trigger, 100ng toehold
   - 150ng trigger, 150ng toehold

27/9/2019

Today's goals:

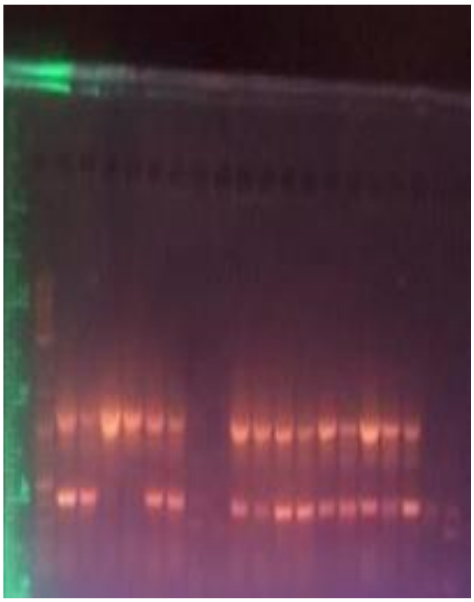
1. **double" ColonyPCR for the co-transformated colonies with PSB1C3-32Blact & PSB1K3-trigger32b DH5a.**
2. **Liquid cultures**

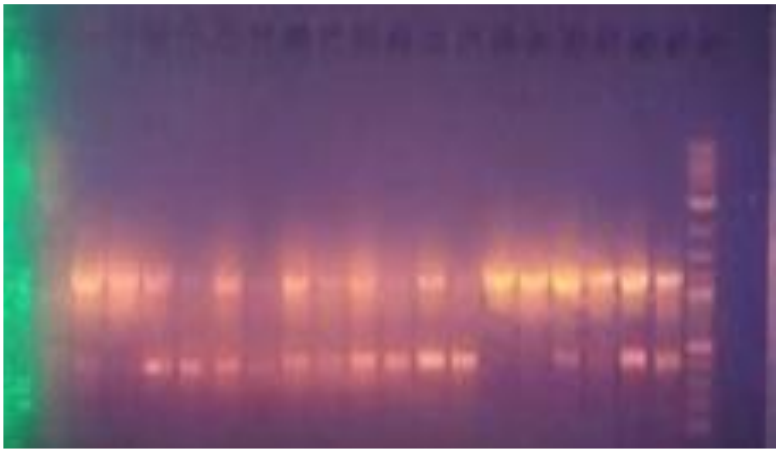
Procedure

1. 6 LB agar plates with different concentrations of 2,20,50,75,100,150 ng/ul
   - From all the plates with took around 50 colonies
   - I tested 6 colonies from the plate "100ng/ul"
   - Thod tested 12 colonies from the plate "150ng/ul"
   - For every colony we had 2 PCR tubes: one for the amplification of the trigger and one for the 32blact amplification

- Classic Colony'sPCR protocol were followed
- Mix for 12 reactions
- The "trigger" tube had 30sec extension time (2min final elongation step)
- The "32blact" tube had 84sec extension time

In the same row, there is one well for the colony after an 84sec extension time and right after that, there is a well with the same colony after an 30sec extension time. 1st row: 31 – 39
2nd row: 40 - 48
The successful colonies are 31, 33,35,36,37,38,41,42,43,44,45,48





2. 5ml LB + 25ul 0,1M IPTG (Chl35 and Kan50 in all cultures except Bl21 empty cells)

- Bl21 cells (2 biological repeats no1,2,)
- Bl21 with PSB1C3 empty vector (2 biological repeats no1,2)
- LacI-lact - PSB1C3 in Bl21 (2 biological repeats no1,2)
- 32b beta-lactamase – PSB1C3 (2 biological repeats no1,2)
- Co-transformated colonies no **31,33,36,41**

Overnight incubationa at 37°C shaker.

28/9/2019

Today's goals:

1. **Whole new impove plate reader test**

Procedure

1.
   - Dilute to 0,05 OD600 in 5ml LB supplemented medium. Extra addition of 0,1 ml glycose 20% and 12,5ul IPTG 0,1M and right antibiotics
   - Incubation at 37°C shaker for about 2 hours untill OD600 0,5
   - Measure the exact OD600 and dilute to 0,5 in LB medium (same as above)
   - 2hours plate reader measurment

   Results: Not the expected

29/9/2019

Today's goals:

1. **Liquid cultures**

Procedure

1. 5ml LB + 25ul IPTG 0,1M (Chl35 and Kan50 in all cultures except Bl21 empty cells)
   - Bl21 cells (2 biological repeats no1,2,)
   - Bl21 with PSB1C3 empty vector (2 biological repeats no1,2)
   - LacI-lact - PSB1C3 in Bl21 (2 biological repeats no1,2)
   - 32b beta-lactamase – PSB1C3 (2 biological repeats no1,2)
   - Co-transformated colonies no 42,43,44,45

   Overnight incubation at 37°C shaker.

30/9/2019

Today's goals:

1. **Plate reader measuments of yesterday's cultures for the new improve**

Procedure

1.
   - Dilute to 0,1 OD600 in 5ml LB supplemented medium. Extra addition of 0,1ml glycose 20% and 12,5ul IPTG 0,1M and right antibiotics
   - Incubation at 37°C shaker for about 2 hours untill OD600 0,5
   - Measure the exact OD600 and dilute to 0,5 in LB medium (same as above)
   - 2hours plate reader measurment

   Results: Not the expected

2/10/2019

Today's goals:

Because of the negative results we took from the colonies'smeasurments at plater reader, we decided to change the vector plasmid in order to avoid the present of two same ORI's in the same colony.

So we borrowed 2ul = 100ng PetM-11 plasmid form MrsSkamnaki's lab
We transformed 100ng plasmid in 100ul DH5a cells
Later that day we realised that PetM-11 plasmid has the same ORI as our PSB's plasmids.

3/10/2019

Today's goals:

1. Qubit for toehold13 in order to send 100ng for sequencing

Procedure

1.

Toehold13 no1: 32 ng/ul
Toehold13 no10: 162 ng/ul
Toehold13 no17: 252 ng/ul

4/10/2019

Today's goals:

1. PURE test of:
   - LacI-beta lactamase (534 ng/ul 14/9 nick)
   - Beta-lactamase (86 ng/ul 29/7 nikol)
   - 32b beta-lactamase (100 ng/ul)
   - 32b beta-lactamase + trigger32b (2 seperate constructs)

   Trigger32b no3 (70 ng/ul 18/9)
   Two repetitions each
2. Liquid cultures
3. PURExpress reaction for the positive control using different volumes of Nitrocefin to check efficiency

Procedure

1. Beta- lactamase 70ng / lacI- beta lactamase 70ng

Solution A	2,8ul
Solution B	2,1 ul
RNAase inhibitor	0,14 ul
DNA template	0,83 ul / 0,13ul
H2O	1,13 ul / 1,83ul

- 32b beta-lactamase 70ng + trigger32b 7ng / 70ng

Solution A	2,8ul
Solution B	2,1 ul
RNAase inhibitor	0,14 ul

DNA toehold	0,7 ul / 0,7 ul
DNA trigger	0,1 ul / 1 ul
H2O	1,16 ul / 0,26ul

Two repetitions each
For the 96-well plate: 7ul PURE reaction + 43 ul nitrocefin 0,5μM
Beta-lactamase had been diluted to 1:10 --> 7ng/7ul pure reaction
490nm
30min total measurement time, measure every 30sec

2. Lb cultures for lacI-beta lactamase and multi plasmids (no31,36,43) with 25ul IPTG 0,1M
3. PUREexpress reaction for the positive control using different volumes of Nitrocefin to check efficiency:

   The Pure reaction was conducted as usual x2
   >2,8ul Solution A
   >2,1ul Solution B
   >0,14ul RNAse inhibitor
   >0,83ul template DNA (beta lactamase gene in 100ng/ul)
   >1,13ul ddH2O

   42oC for 3 hours
   Every hour quick spinand then in the plate reader assay, we used 83 and 93 ul of Nitrocefin for the 2 positive controls.

5/10/2019

Today's goals:

1. **Measurement at plate reader for the impove**

Procedure

1. In the beggining, we wanted to test if our constructs do actually work. So we put 40ul of the Lb liquid culture with 10ul nitrocefin 0,5μM in 1,5ml tube and incubated them for 1h, waiting for the color change. After 50min the cultures have turned red. We were able then to continue for the measurement.

   Same proccess as always: dilution to 0,1 OD600 to LB medium (+25ul IPTG 0,1M +Chl35 and Kan50 when needed) - 2h incubation – dilution to 0,5 – measurement

6/10/2019

Today's goals:

1. **liquid cultures for LacI Lactamase and 32b x trigger**

Procedure

1. 5ml LB medium + 20ul IPTG 0.1mM + 100ul of the colony 31.
   The same steps were followed for the colonies 36, 43 and for LacI Lactamase.
   We added kanamycin 50ug/ml and chloramphenicol 50ug/ml for the colonies containing 32b x trigger and only chloramphenicol 50ug/ml for the colony containing LacI Lactamase.

7/10/2019

Today's goals:

1. Improve test
2. Testing some new forward RPA primers we ordered that are just like the old ones but have different lengths of spacers in their 5' overhangs. We tested the theory that in vitro transcription/translation fails due to the lack of a spacer in the 5' ends of the amplicon.

Procedure:

- Diluted liquid culture 31 and culture LacI Lactamase to OD 0,6 0,8 1 1,2 1,4 and 1.6.
- Added 10ul 0.5mM nitrocefin to 40ul of liquid cultures (OD 0,6 0,8 1 1,2 1,4 and 1.6.) and checked to see after what time each construct changes the color of nitrocefin from yellow to red.
- We continued the experiments by diluting the colonies 31, 43 till OD 0,1 and LacI Lactamase till OD 0,1.
- We waited until OD of 31a was 0.5 and 31b 1, 43 OD= 0,5 and OD of LacI Lactamase was 0.5.
- Plate: 2 technical replicates for 31a , 2 replicates for 31b , 2 replicates for 43 and 2 replicates for LacI.
- We let the plate incubate at 37 degrees at the plate reader for 3 hours and measure its absorbance at 490nm and 600nm.

Results: We got the results that we expected. After around 75minutes, the absorbance of our construct was about the same as the absorbance of LacI Lactamase. In addition as you can the see in the picture below, the color changed from yellow to red.

2. We tested each forward primer with the existing reverse primer S1R2O

Forward Primers used:

>S1F1O (17) → gives 152bp amplicon

>S1F1O (23)→ gives 158bp amplicon

>S1F1O (30)→ gives 165bp amplicon

>S1F1O (36) → gives 171bp amplicon

>S1F1O (42) → gives 177bp amplicon

The primer stocks were diluted appropriately (100μM) and after a 1:10 dilution I prepared the working solutions of the primers in 10μM.

1) PCR reactions:
2) S1F1O (17)
3) S1F1O (17) NTC
4) S1F1O (23)
5) S1F1O (23) NTC
6) S1F1O (30)
7) S1F1O (30) NTC
8) S1F1O (36)
9) S1F1O (36) NTC
10) S1F1O (42)
11) S1F1O (42) NTC

PCR conditions were the same as usual for a standard PCR reaction with 5% DMSO.

Primers were used in 0,8ul each

Template of the reactions were the IS6110 gene in 10^{-3} ng/ul concentration, and 1ul was used for each reaction

PCR cycling protocol was the usual for a standard Ktaq PCR reaction, while the annealing temp of the primers was 68°C and the extension time was 20 seconds.

RESULT: I had a contamination in every reaction, probably due to my water.

Nick ran the next day again the reaction

8/10/2019

Today's goals:

4. Plate reader measurments of the liquid cultures
   - Bl21 cells (2 biological repeats no1,2,)
   - Bl21 with PSB1C3 empty vector (2 biological repeats no1,2)
   - LacI-lact - PSB1C3 in Bl21 (2 biological repeats no1,2)
   - 32b beta-lactamase – PSB1C3 (2biological repeats no1,2)
   - 32b lactamase – trigger (2 biological repeats)

Procedure

- Firstly, we dilute to 0,1 OD600 in 5ml LB medium. Addition of 12,5ul IPTG 0,1M and Chl35 and Kan50 (except Bl21 empty cells)

- Incubation at 37°C shaker for about 2 hours untill OD600 0,5
- Measure the exact OD600 and dilute to 0,5 in LB medium (same as above)
- 2hours plate reader measurement

Results

We got the results that we expected, our improved part is functional in vivo.

10/10/2019

Today's goals:

1. PURE for 32b lactamase and LacI Lactamase
2. PCR with betaine/DMSO

Procedure

1. We followed the same process as always for the PURE reaction: Added 70ng of 32b lactamase and two replicates of LacI Lactamase, each one 70ng.

   Results: We got the results that we expected. The color of nitrocefin didn't change and that means that LacI Lactamase doesn't work in vitro.

11/10/2019

Today's goals:

1. Digestion of trigger t32B again just like 23/9 to be used in a PURExpress reaction
2. Cleanup of digested reactions and combination of the same digested samples in a single column
3. RPA with Betaine and DMSO test
4. Cleanup of RPA reactions

Procedure:

1. Digestion of trigger t32B again just like 23/9 to be used in a PURExpress reaction

   Reactions:
   >2xDig
   >2xLinear
   **I digested 3mg for each reaction

2. Cleanup and combination of the same digested samples in a single column
   The cleanup was conducted according to the kit's instructions
   Elution was done in 20ul

3. RPA with Betaine and DMSO test
   Templates used: IS6110 in 10-4 ng/reaction and HBV gene in 10-8 ng/reaction
   Betaine was used in 8 μL of a 5M stock (for a final concentration of 0.8M)
   DMSO was used in a final concentration of 2,5%

Reactions:

1. S1F1O-S1R2O
2. S1F1O-S1R2O NTC
3. S1F1O-S1R2O Betaine
4. S1F1O-S1R2O Betaine NTC
5. S1F1O-S1R2O DMSO+ Betaine
6. S1F1O-S1R2O DMSO+Betaine NTC
7. HBV set1
8. HBV set1 NTC
9. HBV set1 Betaine
10. HBV set1 Betaine NTC

The RPA reactions were conducted according to the existing protocol with the addition of DMSO and Betaine as explained previously.
5 mins reaction, in 42oC

4. Cleanup

The cleanup was conducted according to the kit's instructions
Elution was done in 15ul (twice)

RESULT:
He gel was ran by Nick next day, and it had probably ethanol, because it was difficult to load it in the gel's wells. So the result was not good and will not be presented here

12/10/2019

Today's goals:

1. **PUREexpress reactions for the proof of concept after ordering and getting in an ultamer oligo the sequence of our amplicon as it should appear after the amplification step.**

Procedure:

1. PUREexpress reactions

Reactions:

1. Ultramer
2. Ultramer
3. Ulramer spacer in 5' end
4. Ulramer spacer in 5' end
5. Digested trigger(from 11/10)
6. Digested trigger
7. Linear trigger
8. Linear trigger
9. PCR product (with 5' spacer)
10. PCR product (with 5' spacer)
11. PCR negative

12. PCR negative
13. HBV
14. HBV
15. HBV neg
16. HBV neg
17. 32B toehold + 70ng trigger
18. 32B toehold + 70ng trigger
19. Only 32B toehold
20. Only 32B toehold

>The PURE reactions were conducted as usual for a typical PURE express reaction of 7ul.
>Toehold 32B was used in 70ng
>Every template was used in ~100ng per reaction except for the leakage control (only toehold) in which It was only water used and no template.

2. Typical PCR reaction were prepared, with the addition of 0.8M betaine final concentration to each reaction, which corresponds to 4 ul of 5M stock in a 25 ul reaction.

15/10/2019

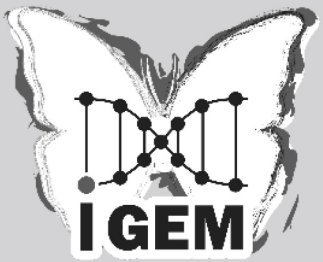
Today's goals:

1. **RPA with betaine for HBV**

Procedures:

A typical RPA reaction was set up, with HBV as the DNA template. 2 rxns were prepared, 1 with 10 copies of template and 1 as a no template control. 0.8M of betaine as final concentration was added, thus 8ul of 5M betaine stock in a 50ul reaction.
Non-specific amplification was present, and the specific band was absent.

Lab Book



Thessaly