DTU iGEM15 e-lab notebook: Oligo competent strains (OCS)

Content

ΔmutS::gp35-neo^R and mutS::beta-neo^R

Re do of Amplification of the mutS up and downstream fragments: 05.08.15: 05.08.15

Preformed by Viktor, Author of e-lab notes: Viktor

Purpose:

To amplify the fragments from the B. subtilis genome. The two fragments that will be amplified is mutS upstream and mutS downstream. After PCR was done the product was purified.

Protocol

No specific protocol.

Purification was done using the QIAgen PCR purification kit.

Materials:

- NEB phusion polymerase
- dNTPs
- 5x HF buffer
- Primers for amplification of the upstream fragment
    - priMutS-GP35+beta-N-fwd
    - priMutS-GP35+beta-N-rev
- Primers for amplification of the downstream fragment
    - priMutS-GP35&beta-C-F
    - priMutS-GP35+beta-C-rev
- Bacillus subtilis WT
- Sterile water

Procedure:

1. One colony B. subtilis was inculcated in 10 uL sterile water and boiled for 10 min. at 98 degC. 1 uL was used as template.
2. Duplicates was made
3. Master mix for PCR reaction containing dNTPs and HF buffer

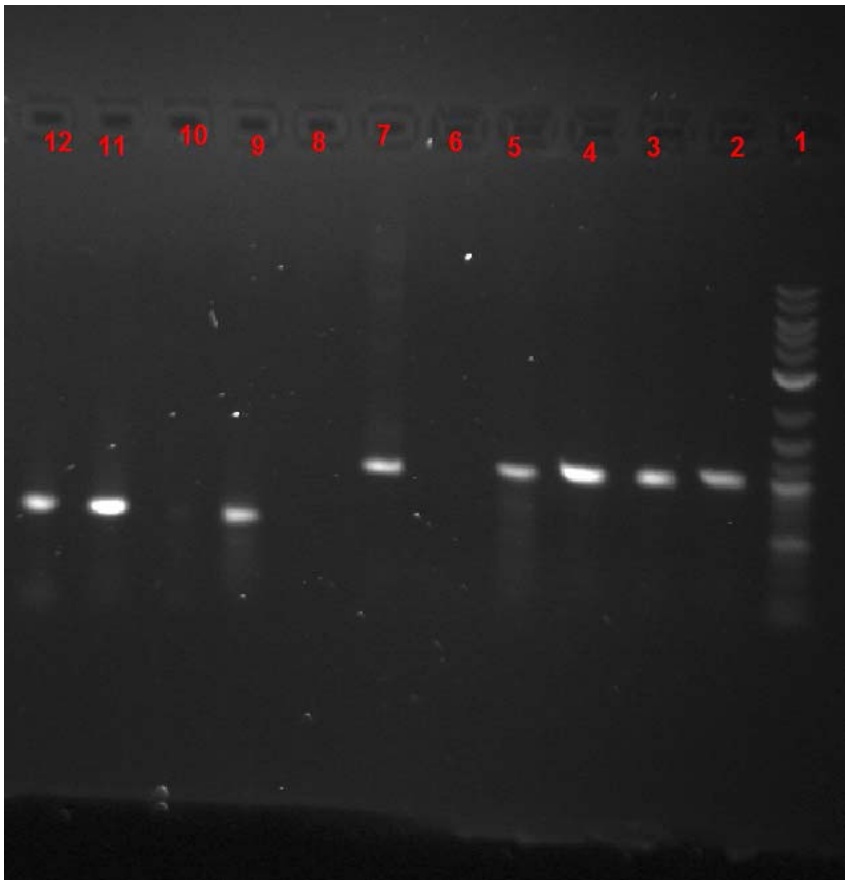
PCR mix			
Sample	**Concentration (uM)**	**Volume (uL)**	**Comments**
NEB phusion		0.5	

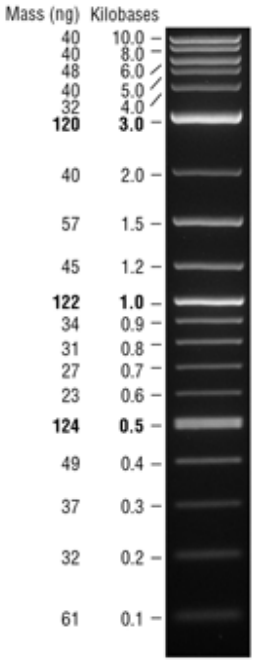
polymerase			
dNTPs	10,000 (10 mM)	1	
5x HF buffer		10	
Fwd primer	25	1	
Rev primer	25	1	
Template (boiled cells)		1	
H_2O		11	
Total		50	

PCR tubes				
Tube	**Template**	**Fwd primer**	**Rev primer**	**Comments**
1	Boiled B. sub. WT	priMutS-GP35+beta-N-fwd	priMutS-GP35+beta-N-rev	Amplifying the upstream fragment
2		priMutS-GP35+beta-N-fwd	priMutS-GP35+beta-N-rev	
3		priMutS-GP35&beta-C-F	priMutS-GP35+beta-C-rev	Amplifying the downstream fragment
4		priMutS-GP35&beta-C-F	priMutS-GP35+beta-C-rev	

PCR program			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	35 cycles
Annealing	59	15	
Elongation	72	30	
Final extension	72	2:00	
Hold	4	Infinite	

Data:





lane 1	Ladder
lane 2	cPCR sample 1
lane 3	cPCR sample 2
lane 4	cPCR sample 3
lane 5	cPCR sample 4
lane 6	cPCR Bacillus WT
lane 7	cPCR plasmid pDG268::GP35
lane 8	No sample loaded
lane 9	mutSL up
lane 10	mutSL up
lane 11	mutSL dw

lane 12	mutSL dw

The purified PCR was nanodroped and following averages was calculated:

- Purified mutS upstream: 31 ng/ul
- Purified mutS downstream 3: 29 ng/ul
- Purified mutS downstream 4: 50 ng/ul
- Purified gp35: 62 ng/ul
- Purified lambda-beta: 38 ng/ul

Results and Conclusion:

Cells were lysed and band on about 500 bp, which is what we expected. There purification was

Re do of Amplification of the gp35-neo and the lambda-beta-neo cassettes: 05.08.15: 05.08.15

Preformed by Viktor, Author of e-lab notes: Viktor

Purpose:

Since the there was no result the first time we will do almost the same. After the PCR was done it was purified.

Protocol

No specific protocol.

Purification: QIAgen PCR purification kit

Materials:

- NEB phusion polymerase
- dNTPs
- 5x HF buffer
- Primers for amplification of the gp35-neo and lambda-beta-neo cassettes
    - priMutS-GP35+beta-mutS-fwd
    - priMutS-GP35&beta-mutS-R
- Template: gp35_pDG268neo and lambda-beta_pDG268neo
- Sterile water

Procedure:

1. Master mix for PCR reaction containing dNTPs and HF buffer

PCR mix			
Sample	**Concentration (uM)**	**Volume (uL)**	**Comments**
NEB phusion polymerase		0.5	
dNTPs	10,000 (10 mM)	1	
5x HF buffer		10	
Fwd primer	25	1	
Rev primer	25	1	
Template		N/A	
H_2O		11	
Total		~~50~~ 52 or 53	

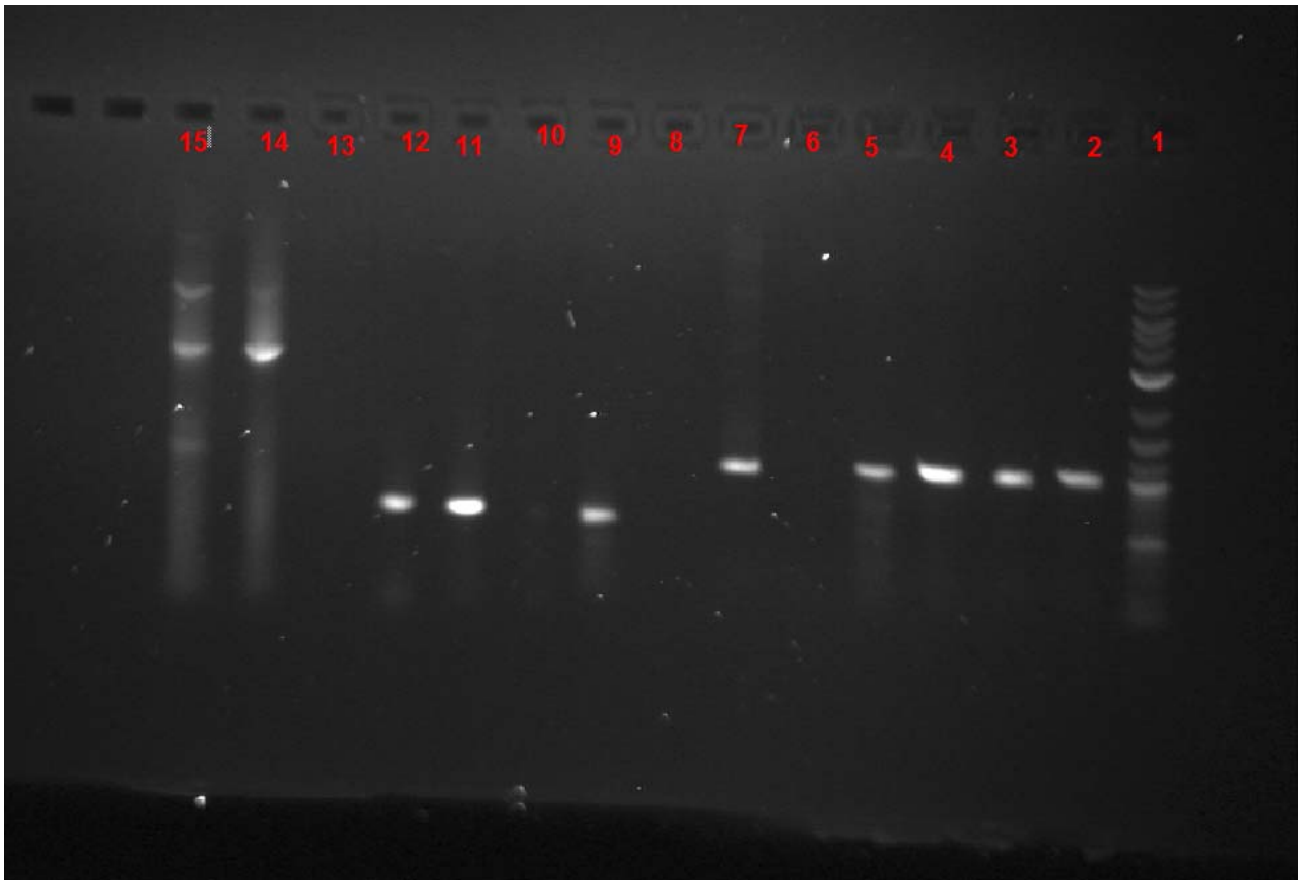
PCR tubes				
Tube	**Template**	**Fwd primer**	**Rev primer**	**Comments**

1	pDG268neo_gp35	priMutS-GP35+beta-mutS-fwd	priMutS-GP35&beta-mutS-R	2 uL of template was used
2	pDG268neo_lambda-beta	priMutS-GP35+beta-mutS-fwd	priMutS-GP35&beta-mutS-R	3 uL of template was used

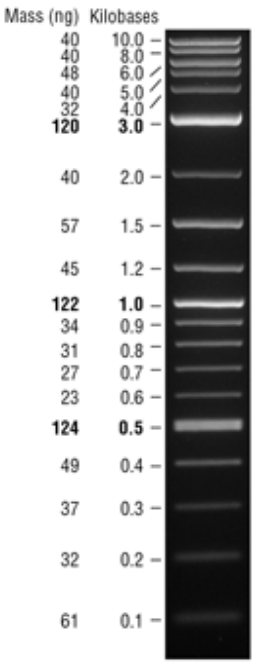
PCR program			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	35 cycles
Annealing	58	15	
Elongation	72	40	
Final extension	72	2:00	
Hold	4	Infinite	

4. On the gel bands at about 6000 bp was observed, this could be plasmid leftovers. To remove this the PCR reaction was digested with DpnI restriction enzyme.
    a. 0.5 ul DpnI was added to each PCR reaction
    b. Tubes were flicked a couple of time and centrifuged for 2 sec.
    c. Then incubated for 50 min at 37 degC
5. The reactions was then purified using QIAgen PCR purification kit.

Data:



Lane 1	Ladder
Lane 2 - 13	Not for this experiment
Lane 14	GP35
Lane 15	lambda-beta



The purified PCR was nanodroped and following averages was calculated:

- Purified gp35: 62 ng/ul
- Purified lambda-beta: 38 ng/ul

Results and Conclusion:

The PCR was successful 2 bands of about 2700 bp was observed at the gel. The PCR product purified.

Gibson Assembly of mutSGP35 neo cassettes and mutS lambda-beta neo cassettes with redbiobrick pSB1C3 (pSB1C3_mutS:: GP35neo^{R}): 19.08.2015

Preformed by Karolina, Aurthor of e-lab notebook: Karolina

Purpose

To assemble the lambda-beta_mutSKO and the gp35_mutSKO into pSB1C3 plasmid. If the experiment is successful the product will look as this:

The gp35 version is the exact same plasmid just the CDS of lambda-beta is changed for the CDS of gp35.

Protocol

NEB Gibson assembly protocol.

Materials

Mut – dw3 (50ng/ul)

GP35 (90ng/ul)

Mut-up (31ng/ul)

pSB1C3 (red biobrick A) (79,3ng/ul)

H2O

Gibson Master Mix

Procedure

Two different gibson reactions was setup:

	Volume (uL)
GP35	1,8
Mut dw3	0,7
Mut up	1,1
pSB1C3	1,5
water	4,9
Master mix	10

	Volume (uL)
Lambda -beta	4,5
Mut dw3	0,7
Mut up	1,1
pSB1C3	1,5
water	2,3
Master mix	10

Gibson Assembly run on Thermocycler for 1h at 50'C.

Chemical transformation, cells with different transformats were plated on Cam 6y and neo 5 y.

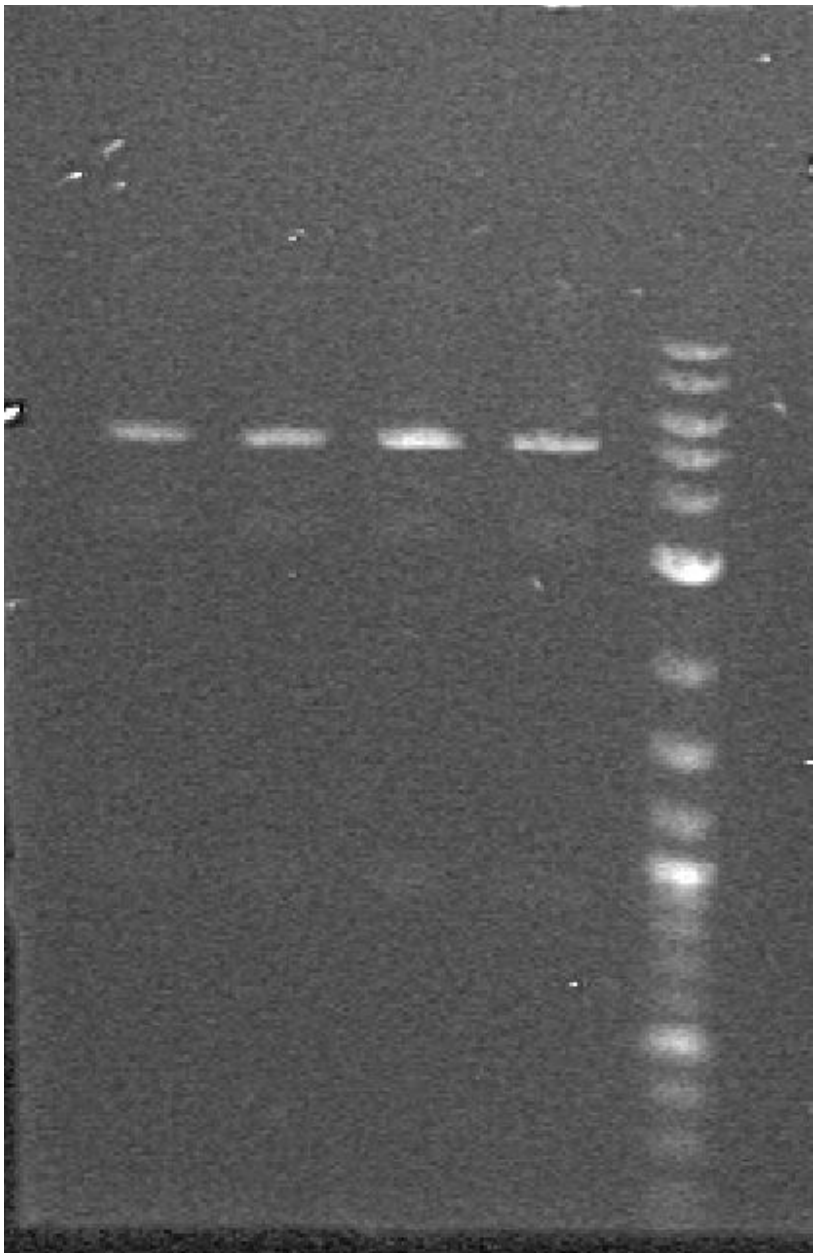
Verification of plasmids

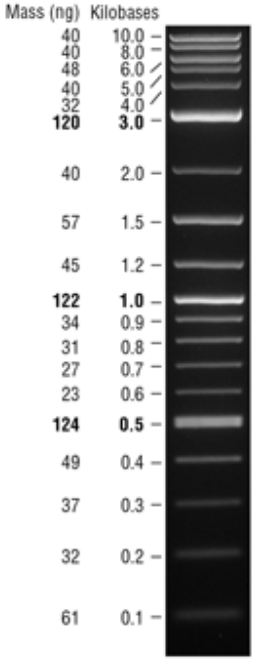
One O/N culture was made from each plate. Samples were miniprept and linearized using XhoI.

Data

Plate	Number of transfomants
mutS_gp35 (5y neo)	216
mutS_gp35 (6y cam)	210
mutS_Lambda-beta (5y neo)	90
mutS_ Lambda-beta (6y cam)	120

Digestion results





Lane 1	lambda beta
Lane 2	lambda beta
Lane 3	GP35
Lane 4	GP35
Lane 5	ladder

Only a small amount of plasmid got digested. The expected bands was at 892 bp and 4823 bp for pSB1C3_*ΔmutS::beta-neo*^{R}. The difference in intensity of the bands may be because the large DNA fragments binds more ETBr e.i. they are more intense even if they are the same concentration.

Conclusion

Transformation was successful and plasmid was verified and linearized by restriction digestion.

Transformation of pSB1C3_Δ*mutS::gp35-neo*^{R} and pSB1C3_Δ*mutS::beta-neo*^{R} : 21.08.15

Preformed by Pernile and Viktor, Author of e-lab notes: Viktor

Purpose:

A successful transformation of the two casstte will result in two different B. subtilis 168 strains:

1. *ΔmutS::beta-neo*^{R}
2. ΔmutS::gp35_neo^R

Protocol

Cfrench:BacTrans2

Materials:

- Natural competent B. subtilis W168 from Preparing Natural competent B. subtilis W168: 24.07.15
- Plates: LB + neomycin 5y
- Template
    - Linearized pSB1C3_ΔmutS::gp35_neo^R and pSB1C3_ΔmutS::beta-neo^R

Procedure:

1. Protocol was followed

Transformations		
Tube	**DNA**	**Volume (uL)**
1	Lambda-beta (1)	30
2	Lambda-beta (2)	30
3	Gp35 (1)	30
4	Gp35 (2)	30
5 (negative control)	No	No

Plates			
Plate	**Antibiotic**	**Sample (tube)**	**Volume of sample (uL)**
1	Neo 5y	1	100
2		2	100
3		3	100
4		4	100
5 (negative control)		5	100

1. Plates was incubated at 37 degC O/N

Data:

Plate	Number of transformants
1	1
2	1
3	0
4	1
5 (Negative control)	1

Results and Conclusion:

The colonies will be tested using a cPCR, even though there is a colony on the negative control.

Colony PCR of mutS::gp35 and mutS::lambda-beta mutants: 24.08.15

Preformed by Viktor, Author of e-lab notes: Viktor

Purpose:

To verify whether the gp35 or lambda-beta got inserted into mutS in the genome of B. subtilis. These are transformants by linearized pSB1C3_mutS_lambda-beta_KO or pSB1C3_mutS_lambda-beta_KO. Four different transformant are examined (see materials).

Protocol

NEB HF phusion polymerase

Materials:

- NEB HF Phusion polymerase
- NEB HF phusion buffer
- NEB dNTPs
- Primers:
    - pDG268neo-lambda-cPCR-rev
    - priMutS-GP35&beta-mutS-R
- Transformants (from Transformation of pSB1C3_mutS::gp35_neo^R and pSB1C3_mutS::lambda-beta_neoR: 21.08.15)
    - Gp35 (1)
    - Gp35 (2)
    - Lambda-beta (2)
    - Negative control

Procedure:

1. A colony was inoculated in 10 ul ddH2O.
2. Cells were boiled for 10 min. at 95 degC.
3. The PCR tubes was mixed as stated below.

PCR mix			
Sample	**Concentration (uM)**	**Volume (uL)**	**Comments**
NEB phusion polymerase		0.2	Polymerase was added after initial denaturation
HF buffer	5x	4	

dNTPs		0.4	
Fwd primer	25	0.4	
Rev primer	25	0.4	
Template (boiled cells)		1	
H_2O		13.6	
Total		20	

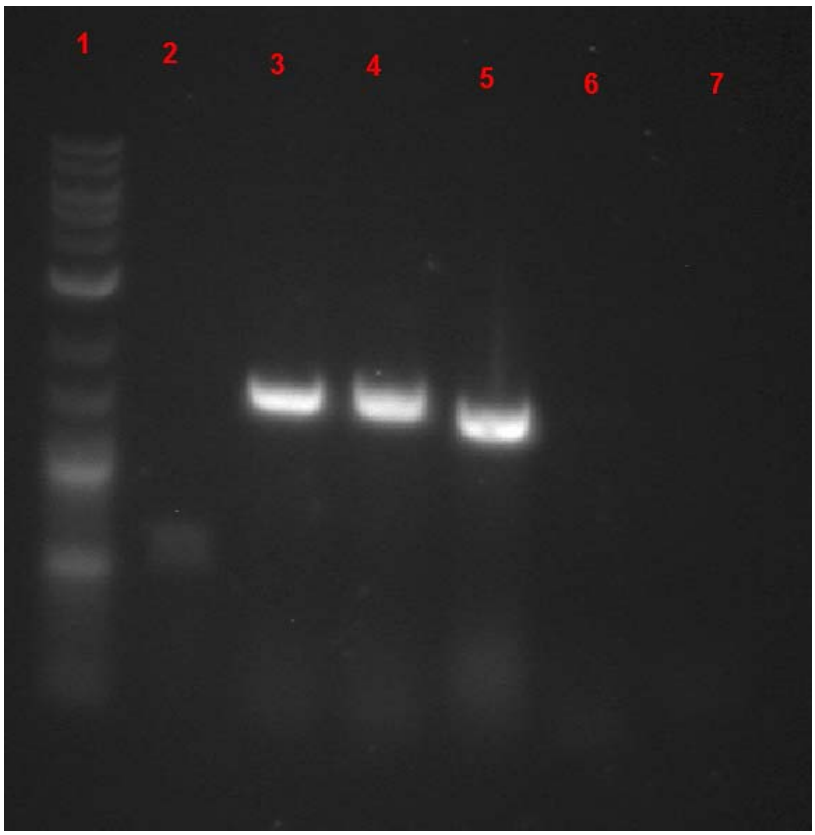
PCR tubes				
Tube	**Template**	**Fwd primer**	**Rev primer**	**Expected band size (bp)**
1 (positive control)	WT B. subtilis 168	priMutS-GP35+beta-N-fwd	priMutS-GP35+beta-N-rev	563
2	gp35 (1)	----||-----	priBiobrick_GP35_beta_r	1510
3	Gp35 (2)	----||-----	----||-----	1510
4	Lambda-beta (2)	----||-----	priBiobrick_Lambda_beta_r	1429
5 (Negative control)**	WT	----||-----	priBiobrick_GP35_beta_r	No band
6 (negative control)**	WT	----||-----	priBiobrick_Lambda_beta_r	No band

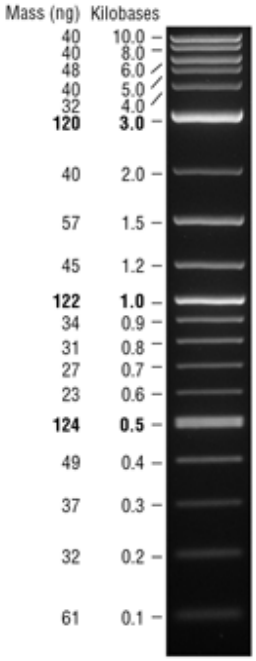
PCR program for tube 1			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	63	20	
Elongation	72	30	
Final extension	72	5:00	
Hold	4	Infinite	

PCR program for tube 2, 3 and 5			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	58	20	
Elongation	72	50	
Final extension	72	5:00	
Hold	4	Infinite	

PCR program for tube 4 and 6			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	60	20	
Elongation	72	50	
Final extension	72	5:00	
Hold	4	Infinite	

Data:





Lane 1	Ladder
Lane 2	Positive control
Lane 3	Gp35 (1)
Lane 4	Gp35 (2)
Lane 5	Lambda-beta (2)

Lane 6	Negative control (gp35)
Lane 7	Negative control (Lambda-beta)

The positive control is really faint, this might be due to that only a small a amount of WT cells was inoculated in the water. The band is though the right size. Lane 6 and 7 are as expected with no bands.

Results and Conclusion:

KO by insert of a recombinase was confirmed in all colonies.

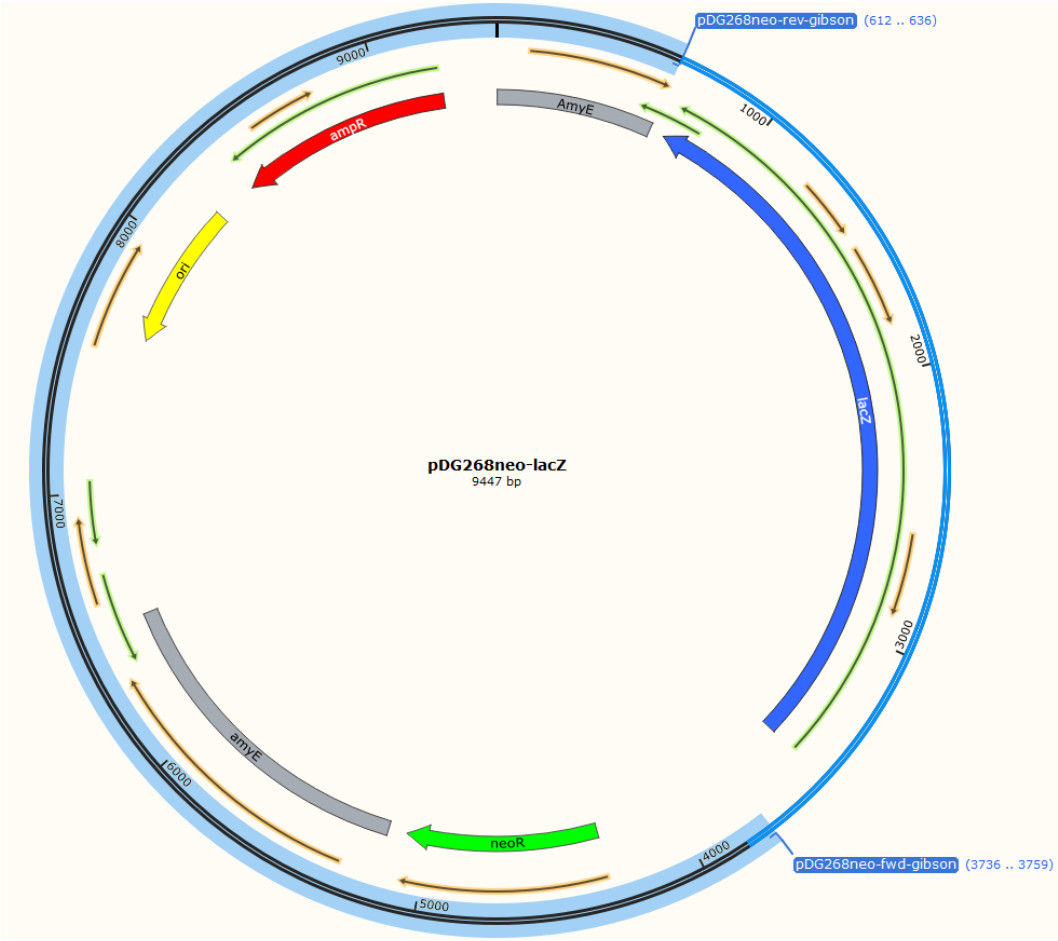
ΔamyE::gp35-neo^R and ΔamyE::beta-neo^R

PCR of pDG268neo backbone for gibson assembly of pDG268neo_lambda-beta: 14.07.15

Preformed by Pernille, Author of e-lab notes: Viktor

Purpose:

Amplify the pDG268neo plasmid using primers that amplify the whole plasmids, but the lacZ gene. In this way we will later be able to insert the recombinases into the plamid. We expect a band on 6348 bp.



Figur 1: pDG268neo. Marked is the amplified part.

Protocol

NEB HF Q5 polymerase 2x master mix protocol

QIAgen PCR purification kit protocol

Materials:

- NEB HF Q5 polymerase 2x master mix
- Primers
    - pDG268neo-rev-gibson
    - pDG268neo-fwd-gibson
- Template: pDG268neo
- dH2O
- QIAgen PCR purification kit

Procedure:

1. Protocol was followed and PCRs was mixed as below:

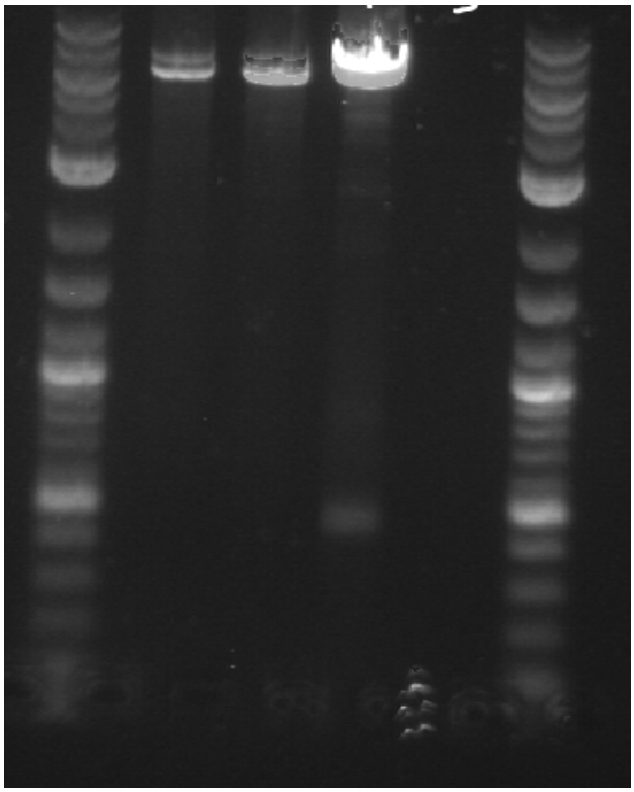
PCR mix			
Sample	**Concentration (uM)**	**Volume (uL)**	**Comments**
Q5 polymerase mastermix		25	
Fwd primer	25	1	
Rev primer	25	1	
Template (pDG268neo)		1	
H_2O		22	
Total		50	

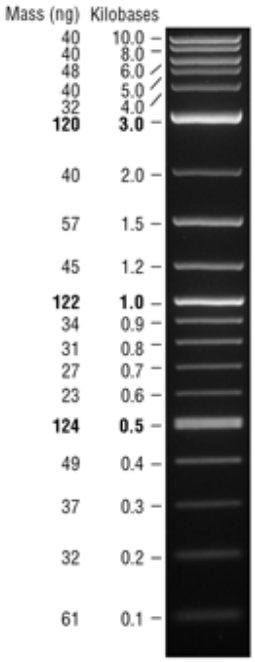
PCR program			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	56.1	15	
Elongation	72	2:10	
Final extension	72	2:00	
Hold	4	Infinite	

PCR purification

2. After the length of the fragments was verified on a agarosegel the PCR was purified using the QIAgen PCR purification kit.

Data:





LANE 1	2-Log DNA Ladder 0.1-10 kb
LANE 2	mastermix 1
LANE 3	mastermix 2
LANE 4	mastermix 3
LANE 5	Blank
LANE 6	2-Log DNA Ladder 0.1-10 kb

Results and conclusion

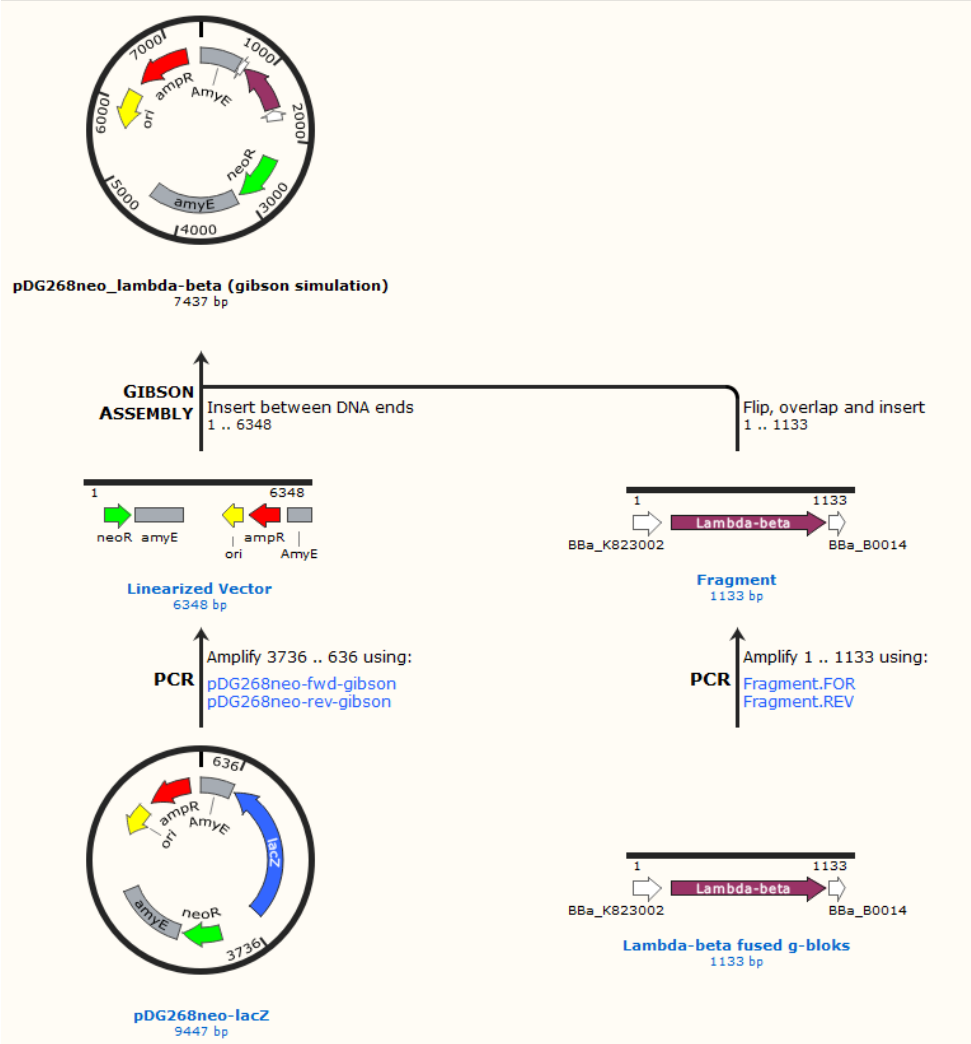
Amplification was successful, because bands at just above 6000 bp was observed on the gel. Product was purified.

Gibson assembly of lambda-beta Gblocks into pDG268neo and transformation into E. coli: 14.07.15

Preformed by Pernille, Author of e-lab notes: Viktor

Purpose:

To insert the two lambda-beta Gblocks into the linearized (by PCR) pDG268neo from [PCR of pDG268neo backbone for gibson assembly of pDG268neo_lambda-beta: 14.07.15]. And amplify the plasmid using E. coli DH5-alpha cells.



Figur 2: The lambda-beta cassette was fused using two Gblocks that was overlapping, but to make the simulation easier the fused version of the Gblocks is used. Fragment.FOR and Fragment.REV primer are imaginary primers only used in the simulation.

Protocol

NEB Gibsom assembly kit protocol.

Materials:

- #3 purified PCR (pDG268neo)
- Gblock lambda-beta-up
- Gblock lambda-beta-dw
- NEB gibsom asssembly kit
- Sterile water
- NEB E. coli heatshock competent cells (c2887)

Procedure:

1. Following was mixed in a PCR tube

Gibson assembly mix			
Sample	**Concentration (uM)**	**Volume (uL)**	
#3PCR (pDG268neo)	80 ng/ul	1	
Gblock lambda-beta-up	10 ng/ul	1.2	
Gblock lambda-beta-dw	10 ng/ul	1.6	
Gibson mastermix		10	
H_2O		6.2	
Total		20	

2. This was incubated for 15 min. at 50 degC in a thermocycler (as protocol states).

Transformation into E. coli

3. 2 uL of assembly reaction was added to 50 uL NEB comp. cells
4. Cooled on ice for 30 min.
5. Heated at 42 degC for 30s
6. On ice for 2 min.
7. 950 uL SOC-medium (from NEB) was added
8. Incubated for 60 min. in a small reaction tube shaking at 210 rpm.
9. 200 and 100 uL was plated on each of following plates: LB, LB + 50y Amp and LB + 100y Amp.
10. Incubated O/N at 37 degC

Data:

Plate	Number of transformants
100y amp (200 ul plated)	26
100y amp (100 ul plated)	8
50y amp (200 ul plated)	21
50y amp (100 ul plated)	0

Results and Conclusion:

The gibson assembly and transformation seems to by successful, but this will be verified later restriction digestion.

Linearization of pDG268neo_lambda-beta: 16.07.15

Preformed by Scot and Verenat, Author of e-lab notes: Viktor

Purpose:

Linearize and verify the length of pDG268neo_lambda-beta, which should later be transformed into B. subtilis. The reason for linearization is that the transformation efficiency in B. subtilis will increase if the plasmid is linearized.

Protocol

iGEM restriction digestion protocol

Materials:

- Miniprep of gp35_pDG268neo from Miniprep of lambda-beta and gp35: 28.07.15
- NEB buffer 2.1
- Sterile water
- Restriction enzymes
    - XcmI
    - PvuII

Procedure:

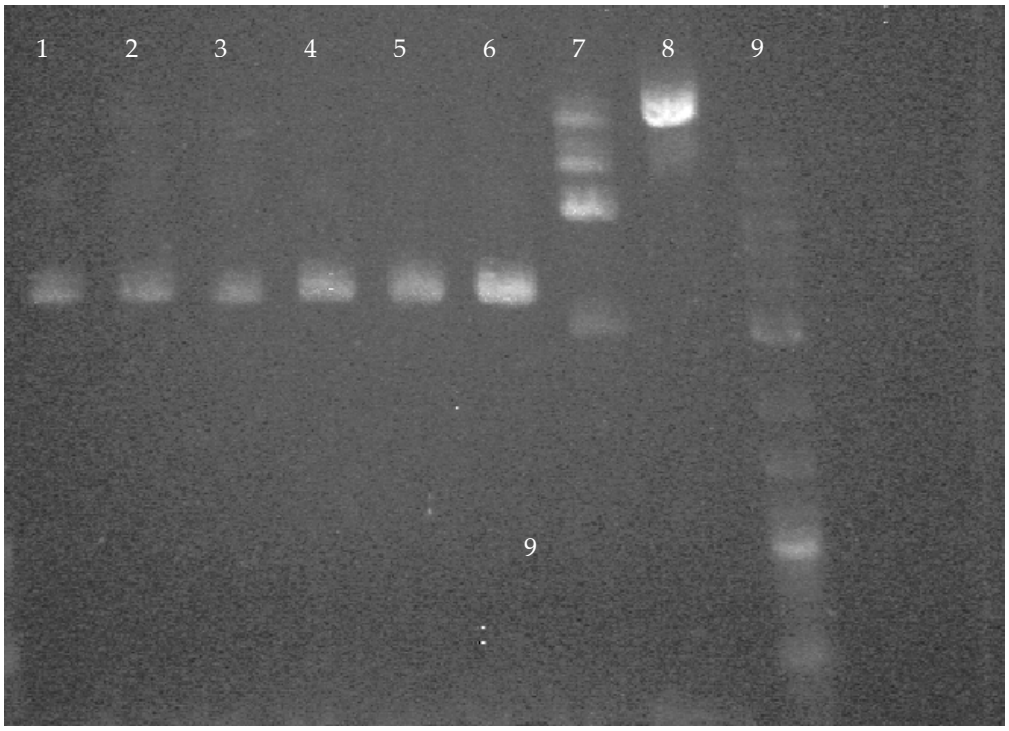
Restriction digestion mix (50 ul reactions)							
Tube	**DNA**	**Concentration of DNA (ng/uL)**	**Volume of DNA (uL)**	**Restriction enzyme**	**Buffer**	**Water (uL)**	**Predicted bands**
1	Lambda-beta #7	45.99	21.7	1 ul XcmI and 1 ul PvuII	5 ul NEB buffer 2.1	21.3	4.1 kb and 3.3 kb
2	Lambda-beta #8	55.84 ng/ul	17.9			25.1	
3	Lambda-beta #9	37.28	26.8			20.2	
4	Lambda-beta #10	47.9	20.9			26.1	
5	Lambda-beta #11	37.6	29.6			20.4	
6	Lambda-beta #12	75.72	13.2			33.8	

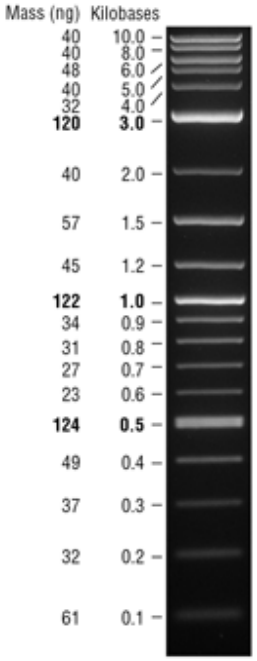
1 uL of each restriction enzyme and 5 uL of buffer was used in each tube.

1. The tubes was mixed as listed above (on ice).
2. Tubes was flicked a couple of time and spun for 2 sec.
3. Incubated at 37 degC for 1 hour

4. Samples was purified using the QIAgen PCR purification

Data:





Lane 1	Transformant 7
Lane 2	Transformant 8
Lane 3	Transformant 9
Lane 4	Transformant 10
Lane 5	Transformant 11
Lane 6	Transformant 12
Lane 7	Plasmid pDG268neo
Lane 8	uncut plasmid
Lane 9	Ladder

Results and Conclusion:

The bands were as expected for all digestions. Thereby we have verified that we do have the right insert.

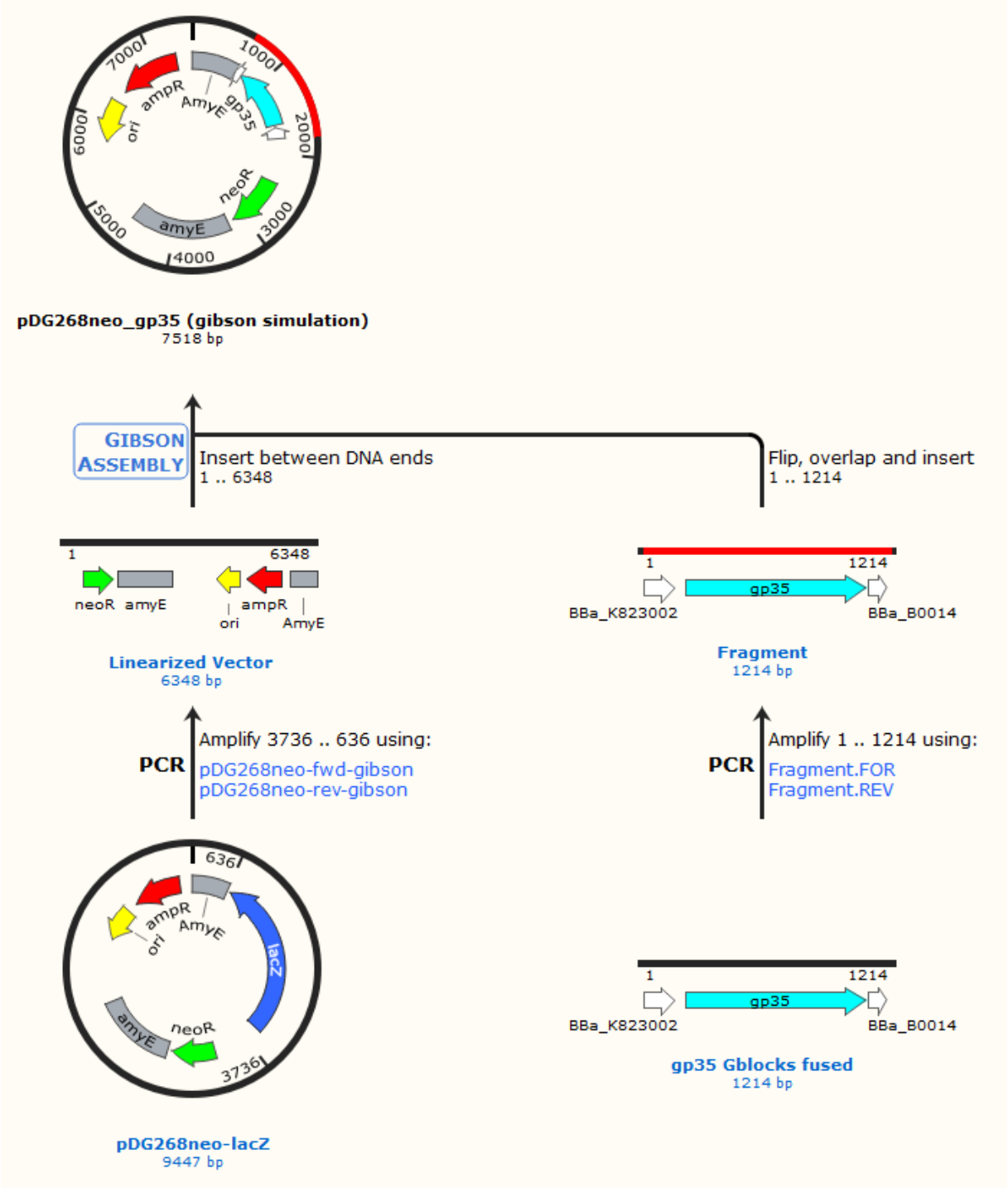
Gibson assembly of gp35 into pDG268neo and transformation into E. coli: 23.07.15

Gibsom assembly of gp35 into pDG268neo

Preformed by Nicolai, Viktor and Vilhelm, Author of e-lab notes: Viktor

Purpose:

Substitute the lacZ gene in pDG268neo with gp35. Thereby make a plasmid gp35_pDG268neo that is able to insert gp35 into the amyE locus in the genome of B. subtilis.



Figur 3: The lambda-beta cassette was fused using two Gblocks that was overlapping, but to make the simulation easier the fused version of the Gblocks is used. Fragment.FOR and Fragment.REV primer are imaginary primers only used in the simulation.

Protocol

NEB Gibsom assembly kit protocol.

Materials:

- #3 purified PCR (pDG268neo)
- Gblock gp35-up
- Gblock gp35-down
- NEB gibsom asssembly kit
- Sterile water
- NEB E. coli heatshock competent cells (c2887)

Procedure:

11. Following was mixed in a PCR tube

Gibson assembly mix			
Sample	**Concentration (uM)**	**Volume (uL)**	
#3PCR (pDG268neo)		1	
Gblock gp35-up	10 ng/ul	1.2	
Gblock gp35-dw	10 ng/ul	1.6	
Gibson mastermix		10	
H_2O		6.2	
Total		20	

12. This was incubated for 15 min. at 50 degC in a thermocycler (as protocol states).

Transformation into E. coli

13. 2 uL of assembly reaction was added to 50 uL NEB comp. cells
14. Error: cells was incubated for 30s at 42 degC
15. Cooled on ice for 30 min.
16. Heated at 42 degC for 30s
17. On ice for 2 min.
18. 950 uL SOC-medium (from NEB) was added
19. Incubated for 60 min. in a small reaction tube shaking at 210 rpm.
20. 100 uL was plated on each of following plates: LB, LB 50y Amp and LB + 100y Amp.
21. Incubated O/N at 37 degC

Miniprep of gp35_pDG268neo (Date 24.07.15)

22. Quick O/N cultures was made inoculating one colony in 3 ml LB + 50y Amp.
23. After 5 hours of incubation the quick O/N was miniprepped, but got low yield
24. New O/N cultures was inoculated in LB + 50y Amp at 37 degC shacking at 200 rpm.

Storage of O/N culture cells (Date 25.07.15)

25. O/N from step 14 was centrifuged for 15 min. at 5000 rpm.
26. Supernatant was discarded and pellet was stores in -20 degC freezer (for later miniprepping).

Data:

Date: 24.07.15

1 transformant on 100y Amp plate and 14 transformants on 50y Amp plate.

Results and Conclusion:

Transformation went OK in spite that the cells was heat-shocked twice.

The poor yield in the first miniprep (step 13) was properly due to the short incubation time of the quick O/N culture. The cells might not have produced many plasmids.

Preparing Natural competent B. subtilis W168: 24.07.15

Preformed by Viktor, Author of e-lab notes: Viktor

Purpose:

Prepare natural competent B. subtilis, to use for transformations.

Protocol

Natural competent Bacillus subtilis 168

Materials:

- One colony of B. subtilis W168
- Minimal growth medium (MGM) (for recipe see protocol)
- Starvation medium (SM) (for recipe see protocol)
- 50% glycerol (to make glycerol stock)

Procedure:

Duplicate was made of all the following

O/N culture in MGM (Date 23.07.15)

1. One colony B. sub. was inoculated in 10 ml MGM in a 250 ml Erlenmeyer flask. Incubated at 37 degC shaking at 180 rpm

Making the natural comp. cells (Date 24.07.15)

2. 1.4 ml N/O (from step 1) was inoculated in 10 ml in a 250 ml Erlenmeyer flask. Incubated at 37 degC shaking at 180 rpm
3. After 2. 5 hours *(30 min. too early)* 11 ml starvation medium was added.
4. Continue incubation for 2 h 45 min.

Glycerol stock

5. Eppendorf tubes were made containing (enough for 4 transformations):
    a. 1.2 ml comp. cells
    b. 264 uL 50% glycerol
6. These were stored at -80 degC

Data

Cells was not tested yet

Result and conclusion

No conclusion since cells was not tested.

Miniprep of *lambda-beta* and *gp35*: 28.07.15

Preformed by Maja, Thea and Viktor, Author of e-lab notes: Viktor

Purpose:

To miniprep two different plasmids from E. coli. The plasmids which was minipreped was gp35_pDG268neo (for insertion of *gp35* into B. subtilis) and lambda-beta_pSB1C3 (To be submitted as a BioBrick).

Protocol

QIAgen spin miniprep kit

Materials:

- E. coli transformed with gp35_pDG268neo
- E. coli transformed with lambda-beta_pSB1C3
- Buffers
    - Buffer P1
    - Buffer P2
    - Buffer N3
    - Buffer PB
    - Buffer PE
- Spin columns

Procedure:

2. Protocol was followed with following exception
    a. Step 7 (only recommended) was done.
    b. When protocol stated centrifuge for 30 - 60s centrifugation was for 60s
    c. Sterile water was used for elution
3. In total 12 (6 of each plasmid) minipreps was done.

Data:

Concentration data generated by Nanodrop (only showing the once with high enough concentration):

- Lambda 4: 21.5 ug/ul
- Lambda 5: 27.0 ug/ul
- gp35-1: 18.7 ug/ul
- gp35-2: 57.7 ug/ul
- gp35-4: 19.5 ug/ul

- gp35-5: 23.1 ug/ul

Results and Conclusion:

The minipreps was not that efficient, but should good enough for transformation and verification.

Linearization of pDG268neo_gp35: 28.07.15

Preformed by Maja, Nicolai and Viktor, Author of e-lab notes: Viktor

Purpose:

To linearize gp35_pDG268neo, which should later be transformed into B. subtilis. The reason for linearization is that the transformation efficiency in B. subtilis will increase if the plasmid is linearized.

Protocol

No specific protocol used.

Materials:

- Miniprep of gp35_pDG268neo from Miniprep of lambda-beta and gp35: 28.07.15
- NEB buffer 2.1
- Sterile water
- Restriction enzymes
    - XhoI
    - PvuII

Procedure:

Restriction digestion mix						
Tube	**DNA**	**Concentration of DNA (ng/uL)**	**Volume of DNA (uL)**	**Restriction enzyme**	**Buffer**	**Water (uL)**
1	gp35-1	18.72	25	XhoI and PvuII	NEB buffer 2.1	18
2	gp35-2	57.68	17			26
3	gp35-4	19.51	25			18
4	gp35-5	23.18	25			18

1 uL of each restriction enzyme and 5 uL of buffer was used in each tube.

5. The tubes was mixed as listed above (on ice).
6. Tubes was flicked a couple of time and spun for 2 sec.
7. Incubated at 37 degC for 1 hour
8. Samples was purified using the QIAgen PCR purification (date 29.07.15).

Data:

Samples were not nanodropped or run on a gel, since a verification digestion of the same samples was run parallel.

Results and Conclusion:

No results for this experiment.

Transformation of B. subtilis with pDG268neo::lambda-beta: 28.07.15

Preformed by Vilhelm, Maja and Viktor, Author of e-lab notes: Viktor

Purpose:

Tranform the pDG268neo_lambda-beta into B. subtilis this will do double homologous cross-over into *amyE* in the genome and thereby the strain will express the lambda-beta recombinase.

Protocol

Cfrench:BacTrans2

Materials:

- Natural competent cells
- Linearized pDG268neo_lambda-beta
- LB medium
- 5y neomycin + LB plates

Procedure:

1. Protocol was followed with no exceptions and 100 ul was plated on 5y neo.
2. Plates were incubated at 37 degC over night.

Results and Conclusion:

Transformants was observed and is to be verified by a colony PCR later.

Digestion 2 of pDG268neo_gp35: 29.07.15

Preformed by Vilhelm and Viktor, Author of e-lab notes: Viktor

Purpose:

Since the first digestion of pDG268neo_gp35 did not work, we will try again this time using a different restriction enzyme.

Protocol

iGEM protocol: Protocols/restriction digest

Materials:

- EcoRV
- NEB buffer 3.1
- pDG268neo_gp35

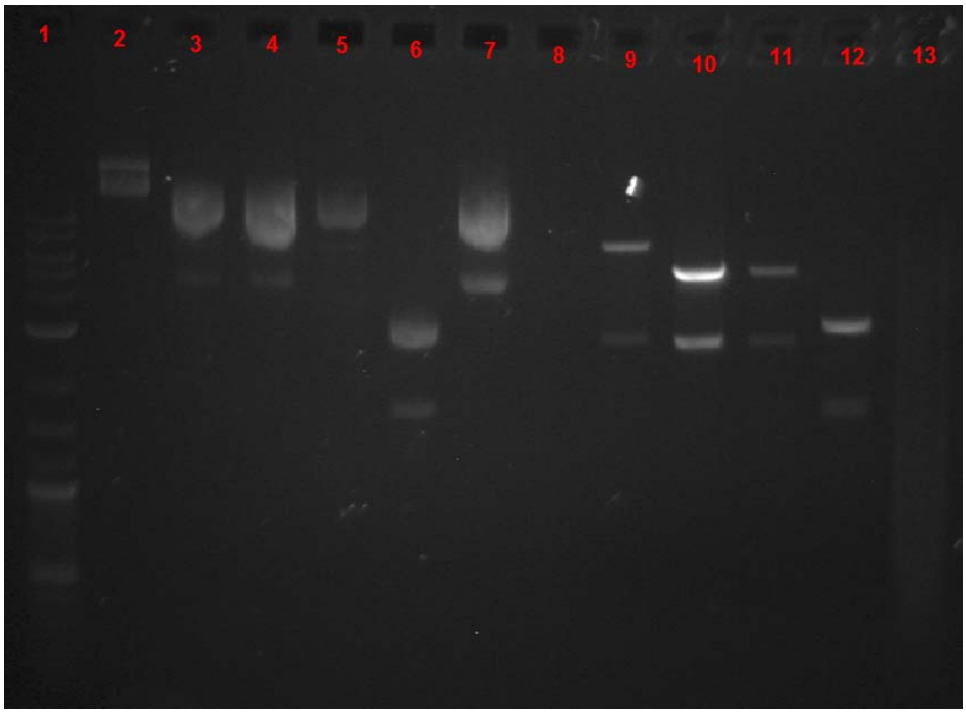
Procedure:

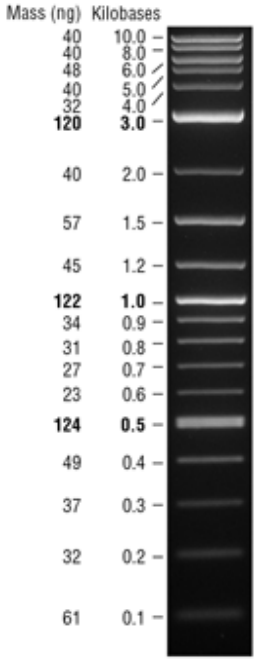
1. Following reaction was set up. All with a total volume of 10 ul.

Restriction digestion mix						
Tube	**DNA**	**Concentration of DNA (ng/uL)**	**Volume of DNA (uL)**	**Restriction enzyme**	**Buffer**	**Water (uL)**
1	gp35-1	18.72	2.5	0.25 ul EcoRV*	1 ul NEB buffer 3.1	6.25
2	gp35-2	57.68				
3	gp35-4	19.51				
4	gp35-5	23.18				
5	pDG268neo	N/A	2.5	No enzyme	No buffer	6.25

2. Tubes were flicked and centrifuged for 2 sec.
3. Incubated at 37 degC for 25 min.

Data:





Lane 1	Ladder
Lane 2	pDG268neo not digested (control)
Lane 3	pDG268neo_gp35 #1 not digested (control) (Failed loading)
Lane 4	pDG268neo_gp35 #1 not digested (control)
Lane 5	pDG268neo_gp35 #2 not digested (control)
Lane 6	pDG268neo_gp35 #3 not digested (control)
Lane 7	pDG268neo_gp35 #4 not digested (control)
Lane 8	Empty
Lane 9	pDG268neo (control)
Lane 10	pDG268neo_gp35 #1
Lane 11	pDG268neo_gp35 #2
Lane 12	pDG268neo_gp35 #3
Lane 13	pDG268neo_gp35 #4

Results and Conclusion:

Gp35-1 and gp35-2 have the insert correctly. This will be send for sequencing.

Transformation of pDG268 gp35 into natural competent B.subtilis 168: 30.07.

Preformed by Verena, Author of e-lab notes: Verena

Purpose:

The DNA from sample "1" (97.97 ng/µL) and sample "2" (111.88 ng/µL) were transformed into natural competent *B.subtilis* 168 and sent to sequencing at the same time so that transformants with confirmed correct sequence are obtained. The sequence is confirmed by sequencing and the presence of the plasmid in the single colony is confirmed by doing a colony PCR.

Materials:

List out everything you need to perform this experiment including lab equipment, media, enzymes, buffers etc.

Plasmid DNA:
"1": pDG268 gp35 (97.97 ng/µL)
"2": pDG268 gp35 (111.18 ng/µL)

Linearization of Plasmid:
Restriction enzyme XhoI (from Jan's freezer)
Restriction enzyme PvuII (from Jan's freezer)
NEB buffer 2.1.

Sequencing Primers:

priBioBrick Lambda Beta reverse
pDG268 neo Lambda cPCR forward

Procedure:

Transformation:

1. Linearization of plasmid according to the manufacturer's protocol. Incubation for 60 min at 37°C

Sample	Concentration (ug/mL)	Volume (uL)
NEB Buffer 2.1		5 µL
XhoI		1 µL
PvuII		1 µL
Plasmid DNA	final: around 1 µg	10 µL
H_2O		32 µL
Total		50µL

Table 1: Linearization of plasmid by using restriction enzymes.

Transformation into *B.Subtilis* according to the protocol Cfrench:BacTrans2

200 µL from each ube were plated in 5 γ neo plates.

Plates were incubated overnight at 37°C

Results and Conclusion:

84 colonies grew on plate "1".

no colonies grew on plate "2".

Both plates were stored in the freezer at 4°C.

Transformation of pDG268neo_lambda-beta: 29.07.15

Preformed by Viktor, Vilhelm and Maja, Author of e-lab notes: Viktor

Purpose:

Transform the pDG268neo_lambda-beta into B. subtilis 168. mutSL. pDG268neo:lambda-beta is an integrative plasmid that is able to integrate into the *amyE* gene of B. subtilis 168, thereby the strain will express the beta protein from the E. coli phage lambda red.

Protocol

Cfrench:BacTrans2

Materials:

- Natural competent B. subtilis 168 cells (from Preparing Natural competent B. subtilis W168: 24.07.15)
- Three different samples with pDG268neo_lambda-beta
    - Sample #7 (46 ng/ul), #8 (56 ng/ul) and #12 (76 ng/ul)

Procedure:

- Protocol was followed with following exceptions:
    - For pDG268neo_lambda-beta we added about 300 ng of each fragment.
    - Negative control with no DNA added was made
- pDG268neo_lambda-beta transformants were plated on 5y Cam
- incubated O/N at 37 degC

Data

Following transformants was observed:

- #7: 60 transformants
- #8: 20 transformants
- #12: 26 transformants
- Control (on 5y cam): 0 transformants

Results and Conclusion:

Transformation of pDG268neo_lambda-beta was successful and there will be made a glycerol stock of these.

Redo 2: Colony PCR of gp35::amyE: 20.08.15

Preformed by Viktor, Author of e-lab notes: Viktor

Purpose:

To verify whether the gp35 got inserted into *amyE* of B. subtilis' chromosome. This is a 3nd try of Colony PCR of pSB1C3_mutS_lambda-beta_KO of mutS_lambda-beta KO and gp35::amyE: 18.08.15.

Protocol

NEB HF phusion polymerase

Materials:

- NEB HF Phusion polymerase
- NEB HF phusion buffer
- NEB dNTPs
- Primers:
    - priMutS-GP35+beta-C-fwd
    - priMutS-GP35+beta-C-rev
    - pDG268neo-lambda-cPCR-fwd
    - gp35_seq_rev
- Template
    - Transformants #2, #3 and #4 from plate 1 form Transformation of mutS_lambda-beta KO and pDG268neo_gp35: 17.08.15
    - WT B. subtilis 168

Procedure:

4. A colony was inoculated in 10 ul ddH2O. For one colony of #2, #3, #4 and a WT B. subtilis. 168.
5. After inoculation in the PCR tubes the colony was inoculated in about 3 ml LB + 5y neomycin, this was incubated at 37 degC O/N
6. Cells were boiled for 10 min. at 98 degC.
7. The PCR tubes was mixed as stated below.

PCR mix			
Sample	**Concentration (uM)**	**Volume (uL)**	**Comments**
NEB phusion		0.2	Polymerase was added

polymerase			after initial denaturation
HF buffer	5x	4	
dNTPs		0.4	
Fwd primer	25	0.4	
Rev primer	25	0.4	
Template (boiled cells)		1	
H_2O		13.6	
Total		20	

PCR tubes				
Tube	**Template**	**Fwd primer**	**Rev primer**	**Expected band size (bp)**
1 (positive control)	WT B. subtilis 168	priMutS-GP35+beta-C-fwd	priMutS-GP35+beta-C-rev	591
2	*ΔamyE::gp35-neo*^{R} #2	pDG268neo-lambda-cPCR-fwd	gp35_seq_rev	1347
3	*ΔamyE::gp35-neo*^{R} #3	------||-----	------||-----	----||-----
4	*ΔamyE::gp35-neo*^{R} #4	------||-----	------||-----	----||-----
5 (Negative control)	WT B. subtilis 168	------||-----	------||-----	No band

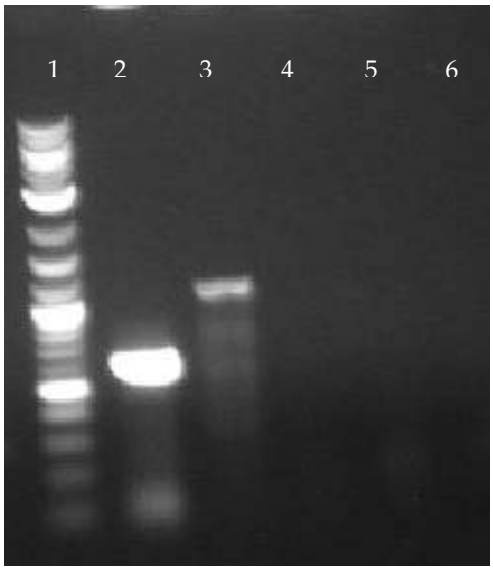
*Primer was named wrong when ordered.

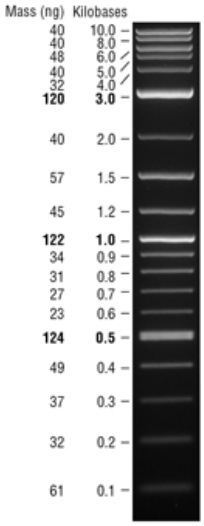
PCR program			
Step	**Temperature (degC)**	**Time (hh:mm:ss)**	**Cycle**
Initial denaturation	98	10:00	
Adding polymerase	4	Hold	
Denaturation	98	10	30 cycles
Annealing	56*	20	
Elongation	72	60**	
Final extension	72	5:00	
Hold	4	Infinite	

*61 degC for positive control

**30s for positive control since the fragment expected is shorter.

Data:





Lane 1	Ladder
Lane 2	Postive control
Lane 3	*ΔamyE::gp35-neo*^{R} #2
Lane 4	*ΔamyE::gp35-neo*^{R} #3
Lane 5	*ΔamyE::gp35-neo*^{R} #4
Lane 6	Negative control

It is seen that the experiment did work, e.i. the cells were lysed (the positive control is as expected). A transformants from #2 have the insert.

Results and Conclusion:

The cPCR confirmed that we have one successful transformant.