DATE: 7/8

TRANSFORMATION OF PROMOTER; TERMINATOR AND GFP FROM iGEM HQ:

From iGEM kit:

	Well	Plate #
Promotor	3 O	1
Terminator	1 B	1
GFP	8 G	5

1. Punch a hole through foil cover with pipette tip. DO NOT REMOVE FOIL!
2. Pipette 10µl dH2O into well and by by pipetting. Let sit for 5 min. Suspension should be red.
3. Transfer 2µl of suspension into cells

Store in fridge -20'C

STOCK SOLUTION CHL.AMPH.
We will make 100 µl total and a conc. of 25 mg/ml in EtOH

Need: 25*10^-3 * 5 / 25 = 5* 10^-3 ml

To make 100 µl of stock:
use 2,5 microg Chl.Amph + 100 µl EtOH

Ii was a very small volume! we used 6 mg in 200 µl!

We followed the protocol from Erik and Moa but with some exceptions:

- We put the Chl.Amph in directly with no recovery time
- We did not plate them (since we have liquid Chl.Amph)
- Incubation in liquid culture with 7µl

RESULTS: ALL CELLS DIED :(

DATE: 8/8

Today we have redone the transformation of the iGEM HQ promoter, terminator and GFP.

We used the same procedure as yesterday but this time we had 1h of recovery time for the cells!

OBS!: Unfortunately some GFP- plasmid suspension were lost during preparation => only one more amplification is possible (lets hope this works)

After 1h of recovery we added 7 µl of Chl.Amph to the liquid culture
-> grow overnight 37’C

DATE: 9/8

WHAT WE DID:

Plasmid miniprep- Biosensor from iGEM kit.

The protocol for plasmid prep was followed. The first centrifugation step was done in falcon tubes in the big centrifuge for 4 min 5000g. After transfering the supernatant into spin columns, there was some supernatant left. The column was washed with 500 µl extra supernatant to increase the concentration. Then only one ordinary washing step was performed. Only 35 µl elution buffer was used (tried to increase concentration).

The nanodrop concentrations were:

Promotor: 11,3 ng/µl
Terminator: 30,9 ng/µl
GFP: 28,4 ng/µl

DATE: 12/8

WHAT WE DID:

Dilution of new primers (Biosensor project)

Biobrick/ biosensor primers arrived. Storage stock has a concentrations on 100 pmol/µl and working stoick on 10 pmol/µl.

Tm Primers

Thermofisher Tm calculator

Primer	Temperature C
BB0	67,3
BB1	66,5
BB2	62,3
BB3	65,4
BB4	70,3
BB5	64,3
BB6	66,7
BB7	68,3
BB8	63,5
BB9	67,8
BB10	68,8
BB11	67,1
BB12	68,5
BB13	67,2
BB14	77,5

DATE: 13/8

WHAT WE DID:

PCR amplification: Biosensor parts 1

Primers used for each PCR can be seen below.

Part	Label	PCR1		PCR2	
Promotor	BP	BB0	BB2	BB1	BB2
Termination (GFP)	BTG	BB11	BB12	BB11	BB13 (ev BB14)
GFP	BG	BB9	BB10	-	-
BphR1	BR1	BB7	BB8	-	--
BphR2	BR2	BB3	BB4	-	-
Terminator (BphR2)	BTR2	BB5	BB6	-	-

Part	Label	PCR1		PCR2	
Promotor	BP	BB0	BB2	BB1	BB2
Termination (GFP)	BTG	BB11	BB12	BB11	BB13 (ev BB14)
GFP	BG	BB9	BB10	-	-
BphR1	BR1	BB7	BB8	-	--
BphR2	BR2	BB3	BB4	-	-
Terminator (BphR2)	BTR2	BB5	BB6	-	-

For PCR a mastermix was prepared with 245 µl of water, 70 µl of 5x Primestart buffer and 7 µl dNTP. 46µl of mastermix was added to each tube, where primestar DNA polymerase and primers were added.

The complete recipe for each tube can be seen below.

Buffer 5x Prime star	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25µl
Primer rvs	1,25µl
Prime start polymerase	0,5 µl
Water	32 µl
Template	1 µl
Total	50 µl

For PCR the following protocol was used, step 2-4 was repeated 35 times.

1. 98 °C 1 min
2. 98 °C 10 sek
3. 64 °C 15 sek
4. 72 °C 1 min
5. 72 °C 4 min
6. 15 forever

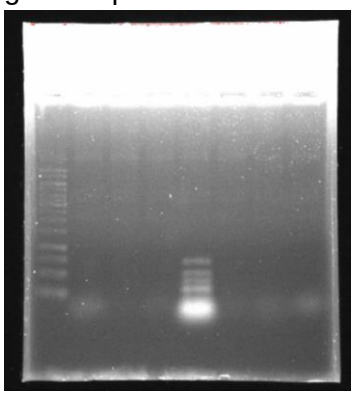
Gel electrophoresis: PCR amplification: Biosensor parts 1

The PCR products were run on a 1% agarose gel, at 90V for 30 min and the gel was post-stained. Expected lengths for the fragments were:

Part	Length PCR1	Length PCR2
BP	78	91
BR2	921	-
BTR2	97	-
BR1	320	-
BG	773	-

BTG	98	112

1µl Loading dye mixed with 2 µl PCR product, 2µl mix was loaded ión the gel. 2 µl ladder of both Low range and 1kb ladder.
The gel electrophoresis didn't work, no bands and very weak primer clouds.
Made a new agarose gel 1% and the PCR products were run for 25 min at 90V, the gel was post-stained.



Maybe forgot template? new PCR!

PCR amplification: Biosensor parts 2

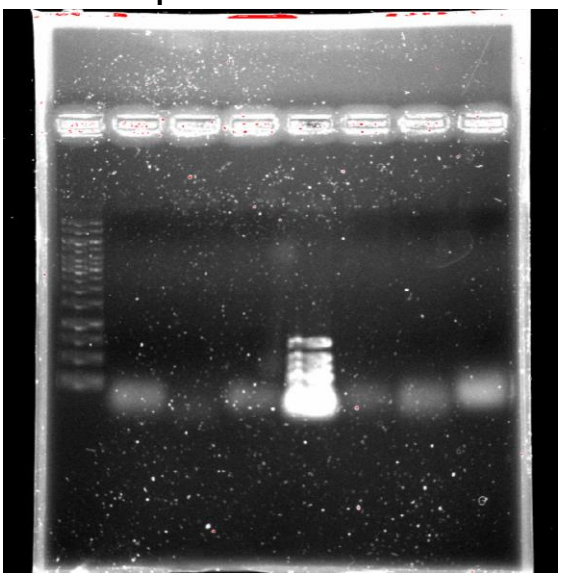
For PCR a mastermix was prepared with 245 µl of water, 70 µl of 5x Primestar buffer and 7 µl dNTP. 46µl of mastermix was added to each tube, where primestar DNA polymerase and primers were added.
The complete recipe for each tube can be seen blow.

Buffer 5x Prime star	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25µl
Primer rvs	1,25µl
Prime start polymerase	0,5 µl
Water	32 µl
Template	1 µl
Total	50 µl

For PCR the following protocol was used, step 2-4 was repeated 35 times.

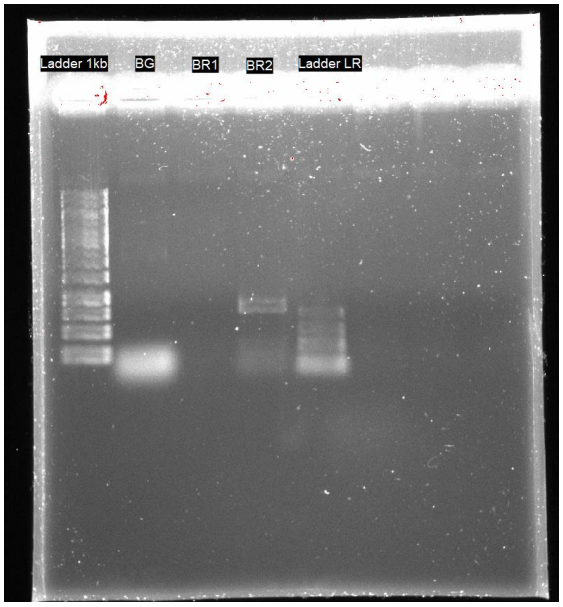
1. 98 °C 1 min
2. 98 °C 10 sek
3. 64 °C 15 sek
4. 72 °C 1 min
5. 72 °C 4 min
6. 15 forever

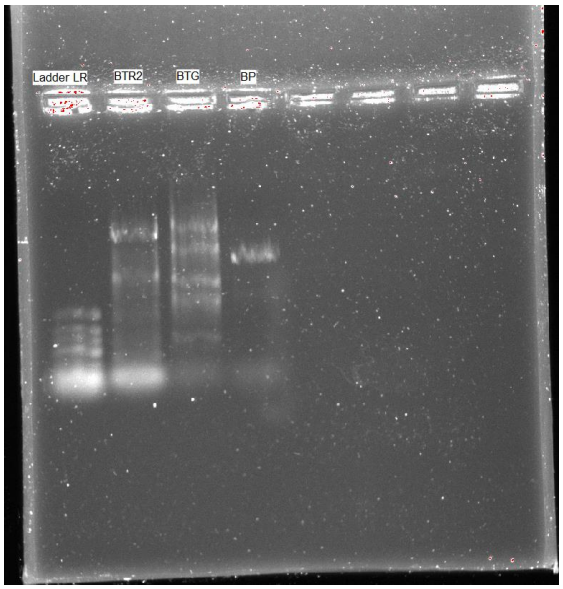
Gel electrophoresis: PCR amplification: Biosensor parts 2



PCR amplification: Biosensor parts 3

Gel electrophoresis, loaded the samples into two different gels because of a mishap while loading the first one.
To get more visible bands 3 µl ladder was added. 3 µl of the samples mixed with loading dye was also added (consisting of 3 µl sample + 1 µl of loading dye). Gels were run on 90 volt for 25 minutes and post-stained with gelred.





Results considered failed for all but BR2.
Multiple bands with no clear indication on expected lengths or no bands at all.
BR2 saved for clean up.

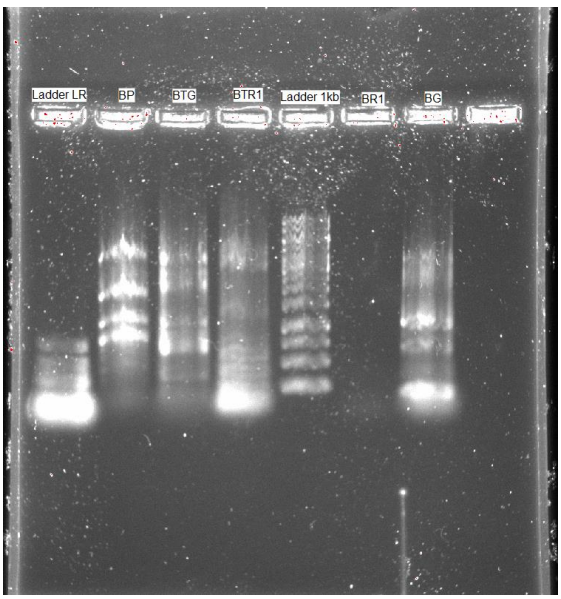
PCR amplification: Biosensor parts 3
Because of low concentration of the template the recipe was change, resulting in 44 µl master mix instead of 46 µl.

Buffer 5x Prime star	circa 9,57 µl
dNTPs (10mM)	circa 0,96 µl
Primer fwd	1,25µl
Primer rvs	1,25µl
Prime start polymerase	0,5 µl
Water	circa 30,61 µl
Template	3 µl
Total	50 µl

For PCR the following protocol was used, step 2-4 was repeated 35 times.
X = 50 °C for BR1 and BP
X = 52 °C for BTR2
X = 56 °C for BG and BTG

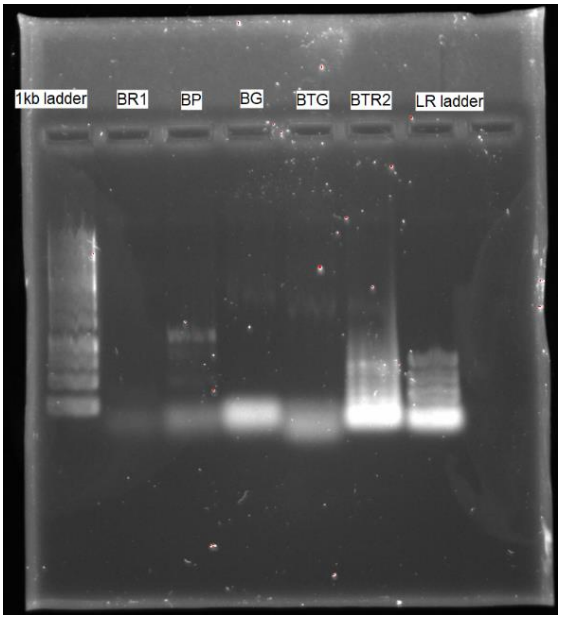
1. 98 °C 1 min
2. 98 °C 10 sek
3. X °C 15 sek
4. 72 °C 1 min
5. 72 °C 4 min
6. 15 forever

Gel run for part 3
This time standard protocol was used. 2 µl of each ladder, 2 µl of sample + loading dye mixture (2 µl sample + 1 µl loading dye). 90 volt for 25 minutes.



BTR1 could possibly have been used but because of the unclear bands and multiple other bands it was deemed unfit for both PCR and gel clean up. BR1 has once more no bands and the other wells showed too many different bands to be usable. In the next PCR the annealing time is lowered as to reduce the amount of unspecific binding and reduce the amount of bands.

DATE: 14/8



PCR amplification: Biosensor parts 4
Because of problems with a pipette the final volumes where 53 µl instead of 50 µl.

Buffer 5x Prime star	10 µl
dNTPs (10mM)	1 µl
Primer fwd	2 µl
Primer rvs	2 µl
Prime start polymerase	0,5 µl
Water	34,5 µl
Template	3 µl
Total	53 µl

For PCR the following protocol was used, step 2-4 was repeated 35 times.
X = 50 °C for BR1 and BP
X = 52 °C for BTR2
X = 56 °C for BG and BTG

1. 98 °C 1 min
2. 98 °C 10 sec
3. X °C 5 sec
4. 72 °C 1 min
5. 72 °C 4 min
6. 15 forever

Gel run for part 4
Loaded in the gel: 2 µl of each ladder, 2 µl of sample + loading dye mixture (2 µl sample + 1 µl loading dye). 90 volt for 25 minutes.

Another fail….

DATE: 15/8

PCR amplification: Biosensor parts 5

After some speculation of what is wrong with our amplification we think there is a problem with the low concentrations of template.

For example:
iGEM standard backbone: 2070 bp
promoter ~90 bp
=> the template (the promoter) is 4% of total DNA weight

therefore we would need a lot more template to reach the 1 ng of template that is recommended in the recipe

Buffer 5x Prime star	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25 µl
Primer rvs	1,25 µl
Prime start polymerase	0,5 µl
Water	31 µl
Template	5 µl
Total	50 µl

For PCR the same protocol as in part 4 was used, step 2-4 was repeated 35 times.
X = 50 °C for BR1 and BP
X = 52 °C for BTR2
X = 56 °C for BG and BTG

1. 98 °C 30 sec
2. 98 °C 10 sec
3. X °C 5 sec
4. 72 °C 15 sec
5. 72 °C 5 min
6. 15 °C forever

Gel run for part 5

Loaded in the gel: 2 µl of each ladder, 2 µl of sample + loading dye mixture (2 µl sample + 1 µl loading dye). 90 volt for 25 minutes.

PCR amplification: Biosensor parts 6

Ideas:

- use phusion
- use 1 µl template again
- decrease the elongation time (according to phusion)
- 5 sec annealing time

Calculations for dilution of template

Promoter	11,3ng/µl * Xµl = 5µl * 10ng/µl	X = 4,42 µl	H20 = 5,58 µl
Terminator	30,9ng/µl * Xµl = 5µl * 10ng/µl	X = 1,6 µl	H20 = 8,4 µl
GFP	28,4ng/µl * Xµl = 5µl * 10ng/µl	X = 2,0 µl	H20 = 8,0 µl

Recipe

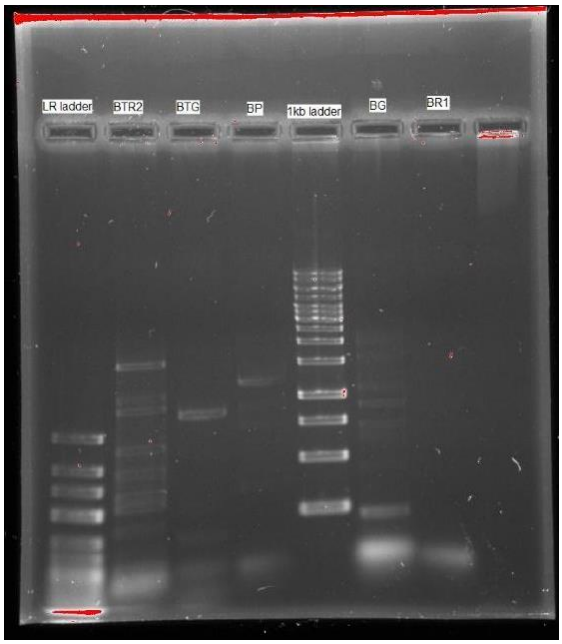
Buffer 5x HF	10 µl

dNTPs (10mM)	1 µl
Primer fwd	1,25 µl
Primer rvs	1,25 µl
Phusion polymerase	0,5 µl
Water	35 µl
Template	1 µl
Total	50 µl

For PCR the same protocol as in part 4 was used, step 2-4 was repeated 20 times.
X = 50 °C for BR1 and BP
X = 52 °C for BTR2
X = 56 °C for BG and BTG

1. 98 °C 30 sec
2. 98 °C 10 sec
3. X °C 5 sec
4. 72 °C 10 sec
5. 72 °C 5 min
6. 15 °C forever

Gel run for #6
A 1,5% agarose was made and run for 25 min at 130 volt to get a better resolution for the small fragments.

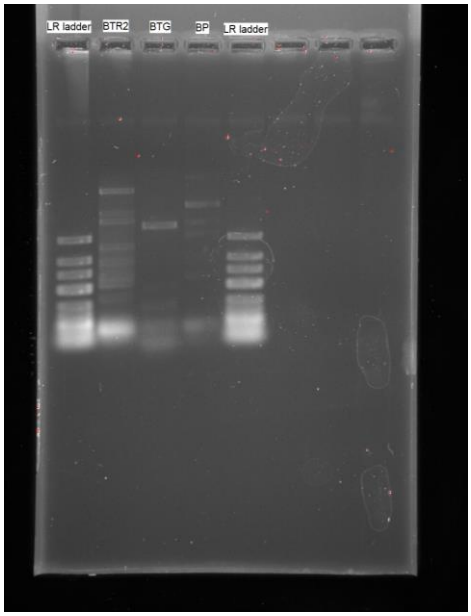


The result was a better resolution, however it wasn't enough and it seems that parts of the LR ladder and potential fragments have migrated out of the gel.

DATE: 16/8

Gel for PCR #6

A longer 1,5% gel was made and run on 130 volt for 30 mins. The separation is almost far enough, making the lower bands of the LR ladder visible. Will run 40 min after next PCR.



PCR #7, never fail PCR

Recipe

Buffer 5x HF	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25 µl
Primer rvs	1,25 µl
Phusion polymerase	0,5 µl
Water	35 µl
Template	1 µl
Total	50 µl

Steps 2-4 and 5-7 were repeated 20 times each.

X= 66 °C - 0.8 °C per repeat, down to 50 °C

Program

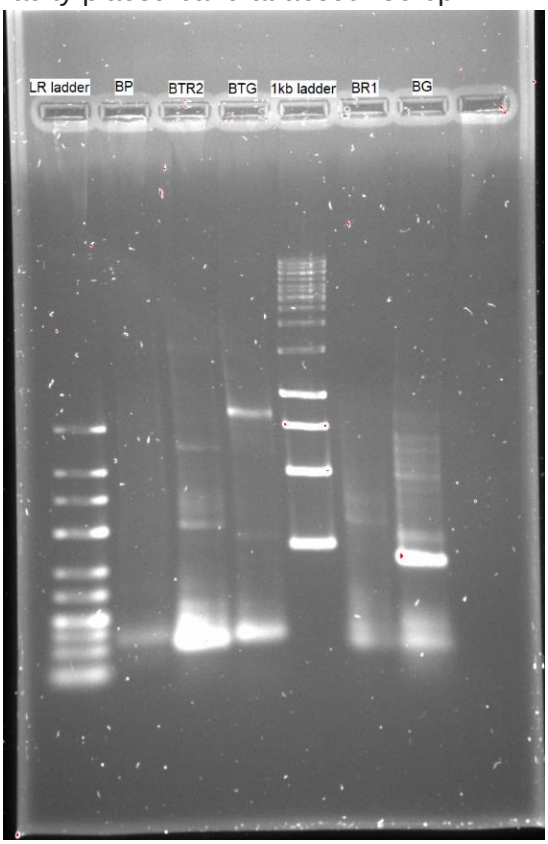
1. 98 °C 30 sec
2. 98 °C 10 sec
3. X °C 5 sec
4. 72 °C 10 sec
5. 98 °C 10 sec
6. 55 °C 5 sec
7. 72 °C 10 sec
8. 72 °C 5 min

Gel for #7

130 volt, 40 min. Long 2% agarose gel

INSERT GEL PIC

Gel methodology worked well, might be able to do a gel clean up on the shorter fragments. Considering digestion of GFP-plasmid to remove sites for unspecific binding with considerations to the strong, faulty placed band at about 250 bp.



DATE: **19/8**

PCR purification of BP and BR2

Followed protocol.
Note: The second step of the protocol, which is optimized, was not performed even though BP < 500bp.

Nanodrop results:
BP: 15,0 ng/µl
BR2: 22,4 ng/µl

Gel with BTG and BTR2, for gel extraction

Nanodrop results:
BTG: 10 ng/µl
BTR2: 3,5 ng/µl

PCR for bphR1, bphR2, GTP

Phusion

Recipe

Buffer 5x HF	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25 µl
Primer rvs	1,25 µl
Phusion polymerase	0,5 µl
Water	35 µl
Template	1 µl
Total	50 µl

Protocol:
“Phusion_bphR1_bphR2_GFP_V1”

95 °C	3:00
95 °C	0:10
50 °C → 55 °C (+5 °C/sek)	0:05
74 °C	0:30
GOTO 2	x 5
95 °C	0:10
65 °C	0:05
74 °C	0:30
15 °C	**2 h (--> don’t leave over night)**

Results: No bands, except ladders.
⟶ We will try again, with a gradient, to see which annealing temperature is better for the different templates.

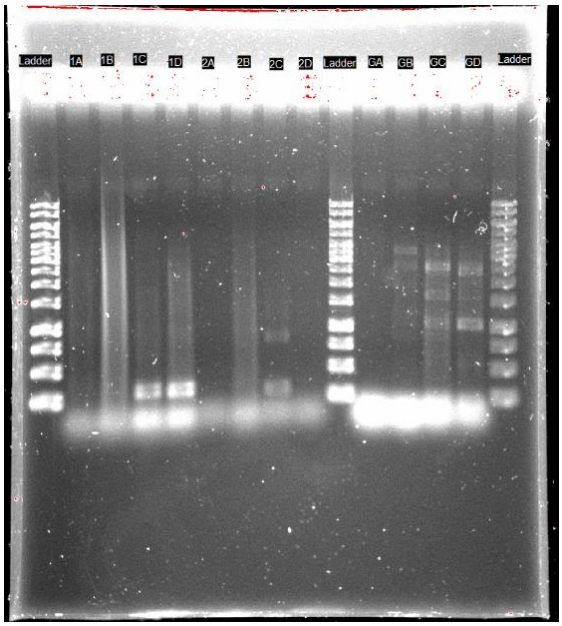
Gradient PCR for bphR1, bphR2, GFP, Phusion

Buffer 5x HF	10 µl
dNTPs (10mM)	1 µl
Primer fwd	1,25 µl
Primer rvs	1,25 µl
Phusion polymerase	0,5 µl
Water	35 µl
Template	1 µl
Total	50 µl

Protocol:

95 °C	3:00
95 °C	0:10
48 °C → 65 °C	0:10
72 °C	0:30
GOTO 2	x 30
72 °C	5:00
12 °C	forever..

The samples are named A, B, C, D after which annealing temperature they have. A is the hottest temperature and D is the coldest one.
A= 63.7 °C
B= 58.3 °C
C= 51.2 °C
D= 48.0 °C



As seen, 1C and 1D yielded good bands that will be extracted through gel clean up. to get rid of the smears and potential bands close to the primer clouds.
For 2 and G, new gradients will be tested, centered around the more successful temperatures (low 50s-high 40s)
For the next PCR we should try to add DMSO.

Gel clean up
Since BphR1 is under 500 bps isopropanol was added to the dissolved gel.
The flowthrough after the last step of 1Cs clean up was used as elution buffer for 1D to increase the concentration. As well, 35 µl elution buffer was used instead of 50 µl. The final concentration was 4.5 ng/µl.

DATE: **20/8**

Gradient PCR for bphR2 & GFP (phusion)

Master mix

dNTP	7 µl
Buffer	70 µl
DMSO	17.5 µl
H2O	32.5 µl

Transfer 46 µl of master mixto each PCR tube. In addition, add to each tube:

Enzyme	0.5 µl
Primer 1	1.25 µl
Primer 2	1.25 µl

The following gradient will be used:
A = 52 °C
B = 49.8 °C
C = 46.0 °C

98 °C	3:00
98 °C	0:10
52 → 46 °C (A → B)	0:10
72 °C	0:30
GOTO 2	x 30
72 °C	10:00
15 °C	forever..

OBS: C-GFP sample has 1 µl of 1 ng/µl GFP, unlike the other two GFP samples (A & B) which each have 5 ng/µl.

PCR products were run on a gel (90V, 30 min)
[INSERT GEL PIC]

As can be seen above, there were no bands on the gel.

Ideas for future PCRs

Since gradient PCR did not yield the wanted results we will go back to the "Never-fail" touchdown protocol. In addition we should try using less template, less dNTPs and more DMSO (up to 10%, note that Tm is lowered by 10 °C). Another option is to try gradient with different temperatures (lower if DMSO is used), or to try a touch-up PCR

DATE: **22/8**

Confirmation of template lengths (R1, R2, GFP)
We suspect that something might be wrong with the template, since the PCRs are not working (though there should be nothing wrong). Therefore we attempt to at least verify the lengths of the fragments.

GFP was cut with RE (EcoRI & PstI).

H2O	14 µl
FD buffer	2 µl
DNA	2 µl
EcoRI	1 µl
PstI	1 µl

Expected lengths:
Cut backbone: 2044 bp
GFP insert: 741 bp
R1: 300 bp
R2: 976 bp

GFP wa

Gel electrophoresis (template confirmation)
[INSERT GEL PICTURE]

Results show that for GFP, template DNA was probably forgotten in the FastDigest step → New digestion of GFP was done (identical protocol to the one shown above)

GFP was run on a gel used to confirm URA marker and backbone (can be found in Team Aqua notes), to save time and resources. The fragment was confirmed there.

DATE: **23/8**

Since there are differences in fragment lengths and primer Tm separate PCR protocols will be attempted for each fragment. All of the notes from previous PCRs were looked over in order to find the best protocol for each fragment, and these protocols were then further tweaked.

PCR: BG(GFP) (Based on PCR3)
Recipe:

Template	1 µl
PS Buffer	10 µl
dNTPs	1 µl
Primer BB9	1,25 µl
Primer BB10	1,25 µl
PS polymerase	0,5 µl
dH2O	35 µl
Total	50 µl

Protocol:

98 °C	3:00
98 °C	0:10
58 °C	0:10
72 °C	1:00
GOTO 2	x 35
72 °C	4:00
15 °C	forever..

PCR: BR1(bphR1) (Based on PCR9)
Recipe:

Template	1 µl
5x HF Buffer	10 µl
dNTPs	1 µl
Primer BB3	1,25 µl
Primer BB4	1,25 µl
Phusion polymerase	0,5 µl
dH2O	35 µl
Total	50 µl

Protocol:

95 °C	3:00
95 °C	0:10
52 °C → 46 °C	0:10
72 °C	1:00
GOTO 2	x 20
95 °C	0:10
52 °C	0:10
72 °C	0:30
GOTO 5	x 35
72 °C	3:00
15 °C	forever..

PCR: BR2(bphR2) (Based on PCR2)
Recipe:

Template	1 µl
5x HF Buffer	10 µl
dNTPs	1 µl
Primer BB7	1,25 µl
Primer BB8	1,25 µl
Phusion polymerase	0,5 µl
dH2O	35 µl
Total	50 µl

Protocol:

95 °C	3:00
95 °C	0:10
55 °C → 49 °C	0:10
72 °C	0:30
GOTO 2	x 20
95 °C	0:10
55 °C	0:10
72 °C	0:30
GOTO 5	x 35
72 °C	3:00
15 °C	forever..

OBS! All PCR machines crashed during runs → Redo all PCR:s (add new polymerase)

Preparation of biosensor promotor + terminators

Due to their short length, the terminators and the promoter was ordered as single stranded oligos. These needed to be mixed and boiled to create the desired double stranded fragments.

98 °C, 5 min boiling to bind complementary strands. Since the nanodrop will not be able to measure concentrations, these were calculated based on the single stranded oligo with the lowest concentration in each pair:

Promoter

	M [g/mol]	m [µg]	n [nmol]	Vol for 100 pmol/µl
FW	28099	539	19,2	192
RV	27381	594	21,7	217

→ Vtot = 409 µl
→ c = 19,2e-9/409e-6 = 46,9 pmol/µl
→ 46,9e-12(28099+27381) = 2602 ng/µl

BTR2

	M [g/mol]	m [µg]	n [nmol]	Vol for 100 pmol/µl
FW	31816	499	15,7	157
RV	31701	617	19,5	195

→ Vtot = 352 µl
→ c = 15,7e-9/352e-6 = 43,75 pmol/µl
→ 43,75e-12(31816+31701) = 2779 ng/µl

BTG

	M [g/mol]	m [µg]	n [nmol]	Vol for 100 pmol/µl
FW	34461	493	14,3	143
RV	34613	538	15,6	156

→ Vtot = 299 µl
→ c = 14,3e-9/299e-6 = 47,8 pmol/µl
→ 47,8e-12(34461+34613) = 3301 ng/µl

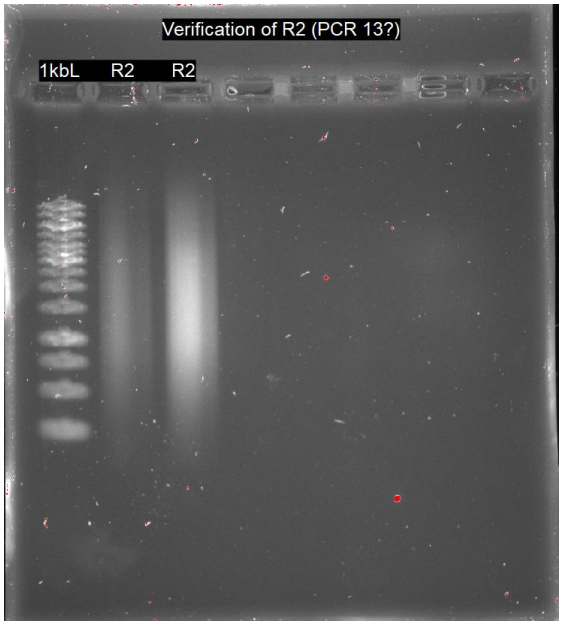
DATE: **26/8**

Rerun of PCRs from 23/8
0,5 µl primestar polymerase added to GFP
0,5 µl phusion polymerase added to R1, R2

All 3 PCRs were rerun using the protocols from 23/8

R2 PCR ran 10 cycles and then stopped...
→ Rerun R2 PCR again, same settings, no new polymerase

Gel electrophoresis
Gel was run 90V, 30 min. R2 was run on a separate gel due to it having to run longer.



GFP had 3 stronger bands, with the wanted band being the strongest → Gel cleanup!
R1 had no bands, R2 (separate gel) was just a big smear

PCRs (R1, R2)
New PCRs were attempted for R1 and R2. Since R2 had stopped and been restarted 2 times, it was decided that the same protocol from earlier would be used again since no conclusions could be drawn. For R1 it was decided to try adding DMSO to improve the efficiency. The touchdown range was also adjusted.

R1

Template	1 µl
5x HF Buffer	10 µl
dNTPs	3 µl
Primer BB3	1,25 µl
Primer BB4	1,25 µl
Phusion polymerase	0,5 µl
DMSO	5 µl
dH2O	28 µl
Total	50 µl

Protocol (Never-fail):

98 °C	3:00
98 °C	0:10
66 °C → 50 °C	0:10
72 °C	0:30
GOTO 2	x 20
98 °C	0:10
55 °C	0:05
72 °C	0:30
GOTO 5	x 30
72 °C	5:00
15 °C	forever..

R2

Template	1 µl
5x HF Buffer	10 µl
dNTPs	1 µl

Primer BB7	1,25 µl
Primer BB8	1,25 µl
Phusion polymerase	0,5 µl
dH2O	35 µl
Total	50 µl

Protocol:

95 °C	3:00
95 °C	0:10
55 °C → 49 °C	0:10
72 °C	0:30
GOTO 2	x 20
95 °C	0:10
55 °C	0:10
72 °C	0:30
GOTO 5	x 35
72 °C	3:00
15 °C	forever..

Gel cleanup of GFP

GFP PCR product was loaded on two wells of a gel (~30 µl in each well). Gel was ran for 30 min, 90V and post-stained with GelGreen. An empty 2ml tube was weighed to 1099,83 mg, the desired bands were excised from the gel and transferred into the tube, which was then weighed in at 1424 mg
→ Weight of gel fragments = 328,4 mg
→ Add 324,8 µl binding buffer in step 1 of the gel purification protocol. Afterwards the protocol was followed without deviations.

The achieved concentration was measured to be 38,8 ng/µl, however the purity was strange. 260/280 was fine (2,26), however the value for 260/230 was ridiculously low (0,05). What does this mean?

DATE: **27/8**

Never-fail PCR (higher temp span)
Since the annealing temp. for R1 and R2 is 66,9 °C and 68,7 °C respectively, a never fail with a higher temp. span (70 °C - 60 °C) and higher final annealing temp (67-68 °C) will be attempted.

Recipe:

Template	1 µl
5x HF Buffer	10 µl
dNTPs	1 µl
Primer BB3/BB4	1,25 µl
Primer BB7/BB8	1,25 µl
Phusion polymerase	0,5 µl
dH2O	35 µl
Total	50 µl

Protocol:

98 °C	0:30
98 °C	0:10
70 °C → 60 °C	0:15
72 °C	0:30
GOTO 2	x 20
98 °C	0:10
57 °C	0:15
72 °C	0:30
GOTO 5	x 30
72 °C	3:00
15 °C	forever..

Gel electrophoresis
2% agarose, long gel, 140V, 40 min

→ No bands…

Will remake the primer working stocks and use new buffer and polymerase.

DATE: **28/8**

New primer working stock
10 µl stock solution for BB3, BB4, BB7 and BB8 + 90 µl water
→ 100 µl, 10 pmol/µl

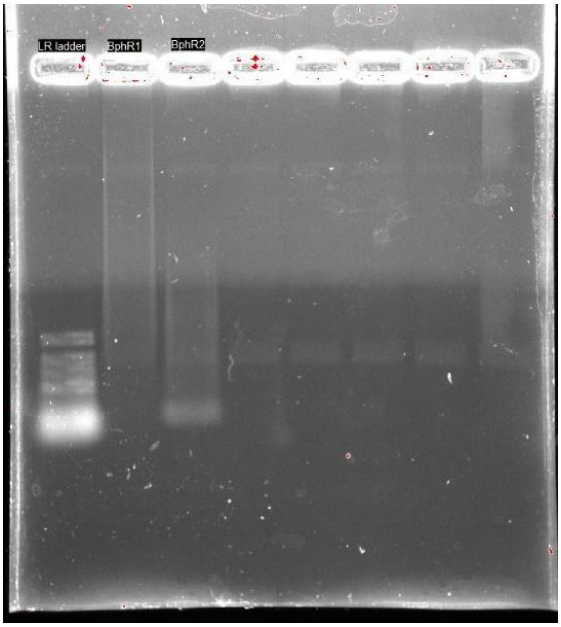
The new primers are marked with bold font, and a blue ring around the tube.

PCRs

Template	1 µl
5x HF Buffer	10 µl
dNTPs	1 µl
Primers	1,25 µl each
Phusion polymerase	0,5 µl
DMSO	5 µl
dH2O	28 µl
Total	50 µl

Protocol (Never-fail):

98 °C	0:30
98 °C	0:10
62 °C → 42 °C	0:10
72 °C	0:30
GOTO 2	x 20
98 °C	0:10
55 °C	0:10
72 °C	0:30
GOTO 5	x 20
72 °C	5:00
15 °C	forever..



As seen above, neither BphR1 nor BphR2 yielded any meaningful results.

DATE: **29/8**

Biosensor PCR

Didn’t work