Week 25

** Template → More detail written how to do stuff (for example the first time we do it)

Tuesday 20/6

Date: 20/6	Performed by: Group 1
What was done	**Observations/comments**
Amplifying GPCR:s and STE2/3gRNA	
Primers (Template)	
Dilute primers according to dilution scheme from eurofins → the conc. you want is 100 pmol/µL (µM) look at the volume written in that column to know how much MQ-water you need to add.	Example: Yield (nmol) = 46,5 46,5 nmol / 465 µL = 100 µM In the case shown above, the volume is 465 µl The primers were diluted 1:10 (x10) to achieve a conc. at 10 µM for PCR
IDT blocks (Template)	
→ Dilute in 100 µl MQ-water → final conc. → 100 ng/µl (The start amount is 1000 ng, but double check every time so nothing else is written on the tubes or the ordering sheet)	
IDT-blocks-dilute (Template)	
In PCR --> Ing (recommendation IDT) Dilute 1:10 (x10) *→ 1 µL in PCR	
Marking	
RatI7_1-F---------->R_1_F RatI7_1-R---------->R_1_R Olfr1258_1-F---------->O_1_F Olfr1258_1-R---------->O_1_R Same goes for RatI7_2 and Olf1258_2	

upStr2_ptef1_ratI7--------->R_up2
RatI7_CYCTer_dnSTE2--------->R_dn2
ste3up_olfr1258--------->O_up3
Olfr1258_ste3dwn--------->O_dn3

PCR

We are using 50 µl protocol phusion High-Fidelity DNA polymerase

The annealing temperature used is 52℃ for GPCRs (RatI7 and Olf1258) and 65℃ for gRNA (STE2gRNA and STE3gRNA) according to the temperatures in the primer sheet we've made during primer design.

PCR 50 µl protocol

Medium	Conc. [µL]
Water	31.5
5x phusion buffer	10
dNTP mix	1
Forward primer	3
Reverse primer	3
Phusion	0.5
Template DNA	1*

***** See IDT-blocks-dilute

Always start with the highest volume (usually water), and the Phusion (enzyme) last. **Keep enzyme on cold block!** Once the enzyme is added, either start the reaction directly or keep the sample on ice. (The enzyme will be destroyed if not kept cold.)

Gel electrophoresis (Template)

You need:
Agarose (approx. 30 mL)
GelGreen/GelRed 0.5 µL

How to do it:

1. Put rack into electrophoresis scaffold (opposite nanodrop)
2. Adjust tilt of gel with screws on scaffold
3. Measure ~25 mL of Agarose into a Falcon Tube
4. Add 0.5 µL GelRed in Falcon tube
5. Put inside scaffold and remove bubbles with comb
6. Insert comb and let dry (approx 20-30 min)

The agarose is found in the ~60°C incubator below the table. Should always be kept warm. If there is no agarose, make new.

The Falcon tubes don't need cleaning in between the measuring. Can be left as they are.

To make gels for gel extraction; put tape on the comb to make some of the wells "broader". Keep one or a few wells narrow, use these for ladders.

Mixture of loading dye + water + DNA (Template)

1. 3 µL MQ-water 2. 1 µL loading dye 3. 2 µL DNA Totala: 6 µL Mix water and loading dye on a plastic. Mix in the DNA and add directly to the wells → load 2 µL DNA and 0.5 µL ladder	It's fine to mix in a tube as well, but mixing on a plastic paper saves time. Ask someone who has done it before so they can show you the first time. For the future: use 4 µL MQ-water and 1 µL of DNA instead. It's enough!

Turn on the gel electrophoresis (Template)

1. Remove buffer from scaffold 2. Add gel (cast) to scaffold 3. Look so that the is the correct way 4. Add buffer to maximum volume 5. Add sample → 75 V, 35 min	The gel electrophoresis shown that the PCR-products seems to been amplified correctly and the right size on the band were shown. For the STE2/3gRNAs no other band appeared, and for RatI7 and Olfr1258 some undefined, shorter bands were shown.

Conc. measurement after PCR

The following conc. was measurement after the PCR with nanodrop

Sample	Conc. [ng/µL]
R_1	131
R_2	129
O_1	133
O_2	104
Ste2gRNA	83
Ste3gRNA	115

Make more TAE-buffer (Template)

1. 1960 mL MQ-water
2. 40 mL TAE (50X)
→ TAE-buffer

Date: 20/6	Performed by:
What was done	**Observations**

LB+Amp-medium and O/N-culture (for p413, p416 and Cas9-vector)

Inoculation of *E. coli* O/N-culture	Already made LB-medium exist in the lab, all

• 3 *E. coli* → 3 inoculation
• 100 µg/mL Amp
  ○ 150 mL LB-medium
  ○ 150 µL Amp
→ 5 mL LB in one tube, falcon tube
→ take one colony from each area (p413, p416 and Cas9-vector) and transfer to falcon tube
→ put in 37℃ *E.coli* for 12-16 h
	that needs is to add Amp in them (work in fume hood). Conc. of Amp and LB were not so clear so might need to check with supervisor about that.

Date: 20/6	Performed by: Group 1
What was done	**Observations**

Gel Extraction of Olf and Rat & PCR purification of STE2/3gRNA

GeneJET PCR purification kit was used the purification of PCR product STE2/3gRNA. Step 2 in the protocol was omitted.

Gel extraction according to the protocol was made:

The bands were cut out

The tubes was weighted to following
100 mg → 100 µL binding buffer
700 µL binding buffer to all of them (the weight was approx ~ 700 mg for all)

After the gel extraction the purified DNA samples were labeled to
R_1 Rent, R_2 Rent
O_1 Rent, O_2 Rent

The weight for all sample made on gel

Sample	Tubes [g]	Tubes+Sample [g]
R_1	0.99643	1.61587
R_2	0.99342	1.69815
O_1	1.00133	1.73856
O_2	1.00124	1.69202

The gel with R_1 and R_2 was dropped, but picked up. The gel seems fine and the upper band (out of the two from each) is cut out ~ 1000 bp

For the the O_1 and O_2 gel was the upper bands cut out out ~ 1000 bp

Following conc. was measurment for all the purified DNA (RatI7, Olfr1258 and STE2/3)

Sample	Conc. [ng/µL]
STE2gRNA	49
STE3gRNA	77
R_1	30.4
R_2	12.4

	<table><tr><td>O_1</td><td>14.8</td></tr><tr><td>O_2</td><td>18.0</td></tr></table> Yield approx 60 % for PCR purification and 10-20 % for gel extraction.

Wednesday 21/6

Date: 21/6	Performed by: Group 2+ Isabel & Elin
What was done	**Observations**
Plasmid purification of p413, p416 and Cas9-vector	
A GeneJet plasmid miniprep kit was used. Centrifuge cells in big centrifuge, ~5000rcp, ~17C and 5min.	The kit was new so EtOH (96%) was added to the wash solution. Green buffer is used since it's gonna be on a gel later. Otherwise, use a transparent buffer.

Date: 21/6	Performed by: Group 2+ Isabel & Elin
What was done	**Observations**
PCR of GPCR:s (New try, too low conc. yesterday 21/6)	
New PCR was made with two tubes per GPCR á 50µl for each piece (8 samples in total). PCR run at 52℃ which is the same annealing temp. as was used yesterday.	

Date: 21/6	Performed by: Group 2+ Isabel & Elin
What was done	**Observations**

Digestion of Cas9-vector	
FastDigest restriction enzymes were done according to protocol. Used RE was BsiWI 20 µL Restriction protocol 1 µg vector 2 µL 10x Buffer 1 µL RE (BsiWI)	

Thursday 22/6

Date: 22/6	Performed by: Alex & Angelo
What was done	**Observations**
Concentration measurement for O11 and O12	
Gel for O11/O12 showed clear single band → PCR purification O11/O12 was pcr purified according to the PCR purification protocol and the concentrations measured by the nanodrop.	

	Conc [ng/µL]
O11	38,5
O12	36,9

Date: 22/6	Performed by: Alex & Angelo
What was done	**Observations**
Gibson and transformation (Template)	
Gibson: 50 ng of vector was used and a ratio between insert and vector should be 3:1 Vector (Cas9) = 10 ng/µL STE2 = 49 ng/µL STE3 = 77 ng/µL	Since 50 ng of the vector is necessary (according to the protocol 50-100 ng) 5 µL was used (10 ng/µL x 5 µL = 50 ng) to achieve 50 ng And the ratio is supposed to be 3:1 (3 times more of the insert) → 150 ng (50 ng x 3 = 150 ng)

	STE2	STE3
Vector	5 µL	5 µL
Insert	3 µL	2 µL
Gibson**	5 µL	5 µL

** 10 µL is usually used but this should work fine as well. Perhaps not as optimal. Raphael told us to use this amount. No water was added due to the low concentration of the vector.

Put it on 50°C for 1 h

Calculation approx
STE2: 150 ng / 49 ng/µL = 3 µL
STE3: 150 ng / 77 ng/µL = 2 µL

Transformation:
6 µL Gibson mix
E. coli
Ice for 20-30 min
Heat Shock 42°C for 30-60 sec

2 Samples STE2 and STE3
1 control (cut plasmid (vector cas9))

Add 800 µL LB-medium (after heat shock)

Recovery for 40-60 min

Centrifuge 5000 rfc for 1 min
Discard the flow through
Add 50 µL water and resuspend the pellet (*E. coli*)
Plate on LB-amp-plates
(work steriel)

Keep on ice the whole time

Wrong vector was used; the uncut was used instead of the cut. 5 µL of the vector was added as well due to 5 µL of the vector was added during the gibson step.

Approx 40 colonies for STE2 and STE3 and the negative control (vector) showed colonies as well due to the uncut was used.

Weekend 23-25/6

Date: 23/6	Performed by: Raphael
What was done	**Observations**
Take out transformed cells	
The E. coli plates with transformed cells were taken out from the 37°C incubator and put into the fridge.	“Your STE2 and 3 gibson transformations seem to have worked correctly, I see more than 40 colonies per plates. However, your negative control has countless colonies. Are you 100% sure that you used the digested plasmid and not the undigested original plasmid? The number of colonies on the plate makes me think about a normal plasmid transformation. Moreover, you supposedly used the same volume of vector for both the Gibson and the negative control, so one would have expected to see a similar ratio of colonies in both cases (even though you only transformed a portion of the Gibson, but still). “ - E-mail from R 23/6.

Date: 25/6	Performed by: Raphael
What was done	**Observations**
Inocculations	
The plated, transformed cells were inocculated. 6 colonies of each.	