July

07/04/2018

Preparations

The primers 1-10 (from IDT) were dissolved in dH2O autoclaved, as described by IDT for 100 µM:

Number	Name	Water (µl)
1	gib_pet28a _Tev(LCstart)_r	270 µl
2	gib_pet28a_Tev(LCende)_f	259 µl
3	gib_tev_LC_r	200 µl
4	gib_midBoNTC-LC_doppelMut_r	267 µl
5	gib_midBoNTC-LC_doppelMut_f	272 µl
6	gib_endBoNTC-LC_Mut_r	346 µl
7	gib_endBoNTC-LC_Mut_f	727 µl
8	gib_endBoNTC-LC_tev_r	271 µl
9	gib_BoNTC.HC_tev(LC)_f	198 µl
10	gib_BoNTC.HC_pet28a_r	258 µl

Primer 3 has a concentrarion of 94,5µM.

An over night culture of E.colis with the pet28a vector was prepared:
- 200 ml LB medium + 20 µl Kanamycin
- picking of one colony from the plate
- Incubation over night at 37°C

Gradient-PCR
Template DNA: Plasmid BoNTCs-tromp 2 µl with 100 ng
Dissolved 1:100 → add. 200 µl H_2O (in 1 µl are 0,495 ng)

PCR Approach (x10)

Template DNA	10 µl	-	10 µl	-

Primer	2 µl (P3)	2 µl (P3)	2 µl (P9)	2 µl (P9)
Primer	2 µl (P8)	2 µl (P8)	2 µl (P10)	2 µl (P10)
dNTPs (10mM)	40 µl	40 µl	40 µl	40 µl
Taq/Pfu (10:1)	20 µl	20 µl	20µl	20 µl
Puffer	50 µl	50 µl	50 µl	50 µl
H2O	376 µl	386 µl	376 µl	386 µl

50 µl were pipetted in a tube of the PCR plate and sealed with a PCR Foil, respectively

	1	**3**	**5**	**7**	**°C**
A	**LC**	**LCB**	**HC**	**HCB**	**61,4**
B	**LC**	**LCB**	**HC**	**HCB**	**62,4**
C	**LC**	**LCB**	**HC**	**HCB**	**63,9**
D	**LC**	**LCB**	**HC**	**HCB**	**65,3**
E	**LC**	**LCB**	**HC**	**HCB**	**66,7**
F	**LC**	**LCB**	**HC**	**HCB**	**68,1**
G	**LC**	**LCB**	**HC**	**HCB**	**69,1**
H	**LC**	**LCB**	**HC**	**HCB**	**71,0**

LC: PCR light chain
LCB: blindprobe with light chain primers (3+ 8)
HC: PCR heavy chain
HCB: blindprobe with heavy chain primers (9 +10)

PCR-program:
center: 66,0 °C
gradient+-: 5,0 °C
Time: 45 s

2 min 94 °C

45 s 94 °C
45 s Gradient
1,5 min 72 °C
25 cycles

5 min 72 °C
Infinity 4 °C

07/05/2017

Qualitative agarose gel electrophoresis
1% Gel: 1 g Agarose in 100 ml 1x TAE buffer and 100 µl Peggreen
Loading: 5 µl PCR probe with 1 µl loading buffer
DNA marker: 10 kb from NEB

Refer to: **180705_Gelbilder_erste PCR_LC_HC**

LC-PCR-5-7-17

HC-PCR-5-7-18.JPG

MidiPrep of pet28a plasmid from E.Coli strain
(Performed by EK)

Materials
- Over night culture of pet28a plasmid (see: 7/4, preparation)
- Quiagen Plasmid Midi Kit (Lot No. 11878105)

Contains: buffer 4: neutralisations buffer
Buffer QBT/ QC/ QF
Filter tubes (QUIAGEN tip 100)
- Buffer:
  - E 1 : 5 ml Sol 1 (Gust-Lab) + 5 µl RNAse
  - E 2: 0,5 ml SDS (2%) + 4 ml NaOH (0,5 M) + 5,5 ml H_2O
- Centrifuge

Execution

The Midi Prep was performed as suggested by the Quiagen Plasmid Midi Kit Protocol

- 2 x 50 ml oft he over night culture was centrifuged in 50 ml Falcon Tubes at 6.000 x g, 10 min, 4 °C)
- supernatant was discarded
- resuspension of pellets in 2ml buffer E1, bring together solutions in centrifuge tubes
- addition of 4 ml buffer E2, mix through pipetting, incubation for 5 min at room temperature
- addition of 4 ml H_2O, mixing through pipetting, incubation for 15 min on ice
- centrifugation: 30 min, 4 degrees, 6.000 x g)
- centrifugation of supernatant: 6.000 x g, 30 min, 4°C
- 200 µl of supernatant was used for an analytical gel (1)
- rinsing of filter tubes with 4ml QBT buffer
- Supernatant was given on filter tubes
- Problem: Supernatant was too liquid, Supernatant was put back in centrifuge tubes and treated with 2 ml neutralization buffer
- centrifugation: 6.000 x g, 10 min, 4°C
- Supernatant was then transferred in new, damp filter tube
- 200 µl of throughput were taken for an analytical gel
- remaining troughput was discarded
- filter tube was washed twice with 10 ml buffer QC
- 200 µl of throughput were taken for an analytical gel
- reamaining throughput was discarded
- filter tube was washed with 5 ml buffer QF
- mixing of throughput with 3.5 ml Isopropanol
- centrifugation: 6.000 x g, 30 min 4°C
- discard supernatant
- pellet was reuspended in 2 ml EtOH
- centrifugation: 6.000 x g, 30 min, 4°C
- discard supernatant
- drying of pellet
- resolve pellet in TRIS HCl, 10 mM, pH8

Result
- Nano Drop measurement, calbration with H2O, blanked with TRIS-HCL
- 2 µl DNA were measured
- DNA concentration: 8.5 ng/ µl
- 260/280: 1.02
- 260/280: 0.72

Interpretation
Since the concentration is very low and the product was not pure enough a second Midiprep was performed.

07/06/2018

<u>Gradient-PCR of the heavy chain</u>

HC 10x	Blindprobe 10x
10 µl	-
2 µl (P9)	2 µl (P9)

2 µl (P10)	2 µl (P10)
40 µl	40 µl
20µl	20 µl
50 µl	50 µl
376 µl	386 µl

The same program as in the first gradient PCR, but with a different gradient, was used.

	1	**2**
A	HC	HCB
B	HC	HCB
C	HC	HCB
D	HC	HCB
E	HC	HCB
F	HC	HCB
G	HC	HCB
H	HC	HCB

DpnI digestion of the LC
Sample B1 and C1 were mixed = 90 µl
2 µl DpnI
11 µl Cut Smart buffer
7 µl dH_2O
= 110 µl
Incubation: 1h 30 min at 37 °C

Qualitative agarose gel elektrophoresis
1% Gel with 1 g agarose and 100 ml TAE(x1), 100 µl Peggreeen

IM000222.Tif
Left: HC right: HC blindprobe

HC-PCR-6-7-18.JPG
Freezed: HC-G1-6.7.19; HC-H1-6.7.18

DpnI digest of the HC
Sample A1: 45 µl sample
1 µl DpnI
6 µl Cutsmart Puffer
8 µl dH_2O
= 60 µl reaction
Incubation: 1h 15 min at 37 °C, then stored at -20°C.

MiniPre (pet28a-Plasmid) from E.Coli-Strain
Performed by EK

Materials
Over night culture of pet28a-plasmid E.coli strain (from 04.07.18)
(was stored for 24h at 25°C on the 5.7.)
Plasmid Mini Prep Kit from NEB ((#T1010G)

Execution
(Mini Prep was performed as recommended by the NEB protocol)
- It was worked with 6 samples from one culture to gain more of the product
- All 6 samles were treated the same and were joined together in the end
- Over night culture was transferred in reaction tube and centrifuged (16.000 x g, 4°C, 30 sec.)
- Resuspension of pellet in 200 ul Buffer B1, was mixed well, but not vortexed
- Addition of 200 ul B2, mixing carefully and incubation for 1 min at room temperature
- Addition of 400 ul Buffer B3, mixing carefully, Incubation at room temperature for 2 min
- Centrifugation: 16.000 x g, 4 °C, 5,5 min
- Supernatant was transferred on column, centrifugation (16.000 x g, 4 °C, 1 min)
- Flow through was discarded
- Washing with 200 ul wash buffer 2 and centrifugation (16.000 x g, 4 °C, 1 min)
- Flow through was discarded
- Washing with 400 ul wash buffer
- Column was transferred into new 1.5 ml tube
- Pipetting of 30 ul Elution buffer onto the membrane of the column
- Centrigugation: (16.000 x g, 4 °C, 1 min)
- Flow through was taken and transferred onto the membrane again
- Centrifugation: 16.000 x g, 4 °C, 1 min
- Flow through was stored at -20°C

Result
- Measuring DNA concentration with NanoDrop
- Calibration with RNAse free water and blanking with Elution Buffer
- Measuring of 2 ul DNA Sample

à DNA Concentration= 51,6 ng/ul
- 260/280: 2,09
- 260/230: 1,57

Discussion
- good DNA concentration, however sample is contaminated with RNA and proteins
- this could possibly be explained by the usage of the Buffer B2 at room temp. instead of 30 °C

· DNA Product was still used for further experiments

PCR-Amplifikation oft he pet28a-Plasmid
Performed by EK

Materials
· pet28-Plasmid, DNA-Produkt der MiniPrep
· Primer 1 : gib_pet28a_TEV(LCstart)_r
· Primer 2: gib_pet28a_TEV(LCende)_f

Reaction (1 x)

For the PVCR reaction the DNA Product first had to be 1:100 diluted
à Template DNA Concentration: 0.516 ng/ul

Primers 1&2 were 1:10 diluted.

Reaction:

Template DNA	1 µl
Primer	2 µl (P1)
Primer	2 µl (P2)
dNTPs (10mM)	4 µl
Taq/Pfu (10:1)	2 µl
Puffer	5 µl
H2O	34 µl

2. PCR-Program:
Heat Lid to 110 °C
Temp. 94 °C 2 min
Cycles: 25 x
Temp 94 °C 45 sec.
Grad. 59 °C ± 3,0 °C 45 sec.
Temp 72 °C 1 min 30 sec.
Temp 72 °C 5 min
"Store forever" bei 4 °C

PCR Product was stored at -20 °C.

LC-Gel Purification
1% Gel , 1 g Agarose mixed with 100 ml TAE without dye
110 µl reaction + 10 µl loading dye

6 µl DNA-Ladder
2x 7 µl PCR sample were loaded on gel
Marker and sample band were cut out and stained with ethidium bromide
Sample Band was cut out under UV light, complete band of PCR Product was cut out and distributed on 2 tubes: LC gelex 6.7.18

07/09/2018

Gelelectrophoresis for Purification of HC

1% Gel, 1g Agarose with 100 ml 1x TAE without DNA dye
- 60 ul reaction + 10 ul Loading dye
- 6 ul Marker, 7 ul Sample, HC reaction, 7 ul sample, 6 ul marker, pet28a
- Marker and sample bands were cut and stained with Ethidium bromide
- Sample band was cut out under UV light
- Again placed in gel and whole PCR reaction band was cut out
- Distributed in 2 tubes
  - 1) HCgelex
  - 2) HCgelex
- no band for Pet28a

Gradient PCR
Performed by CS & EK

Materials
- pet28q plasmid, DNA product from MiniPrep
- Primer 1 : gib_pet28a_TEV(LCstart)_r
- Primer 2: gib_pet28a_TEV(LCende)_f

Reaction (x10)

Template DNA	10 µl	-
Primer	2 µl (P1)	2 µl (P1)
Primer	2 µl (P2)	2 µl (P2)
dNTPs (10mM)	40 µl	40 µl
Taq/Pfu (10:1)	20 µl	20 µl
Puffer	50 µl	50 µl
H2O	376 µl	386 µl

50 µl were pipetted in one tube of the PCR plate and then sealed with a PCR foil, respectively.

	1	**2**	**3**	**4**	**5**	**°C**
A	Pet28a				Blind	
B	Pet28a				Blind	
C	Pet28a				Blind	
D	Pet28a				Blind	
E	Pet28a	Pet28a	Pet28a	(old)	Blind	
F	Pet28a				Blind	
G	Pet28a				Blind	
H	Pet28a				Blind	

Pet28a: PCR with gradient from Pet28a
old: alter reaction with new polymerase
Blind: blind probe

PCR-Programm:
Center: 66,0 °C
Gradient+-: 5,0 °C
Time: 45 s

2 min 94 °C

45 s 94 °C
45 s Gradient
1,5 min 72 °C
25 Zyklen

5 min 72 °C
Infinity 4 °C

<u>Agarosegel of Pet28a Vector</u>
1% Gel mit ethidium bromide
Comb above : x M A1 B1 C1 D1 E1 F1 G1 H1 M E2 E3
Comb below: x M A5 B5 C5 D5 E5 F5 G5 H5 M E4

No Bands were visible

<u>Gelextraction from LC and HC</u>

1. LC = 0,69 g = 690 µl
2. LC = 0,52g = 520 µl
3. HC = 0,73 g = 730 µl
4. HC = 0,58 g = 580 µl à yellow binding buffer

· Dissolved for 10 min at 60 °C
· 1.2 750 µl, 2.1 2.2 500 µl, 3.1 3.2 750 µl, 4.1 4.2 500 µl, transferred on column respectively
· Centrifugation: 1 min, 10 000 x g
· Washed twice with wash buffer
· Elution: 40 ul H2O
· Since NanoDrop measurement was bad, another Elution with 6 ul elution buffer (NEB) was performed

Nanodrop Measurement (elution with H2O)

	Sample	Ng/ul	260/280	260/230
1	LC 1.1	1,2	5,87	0,02
2	LC 1.2	2,3	1,03	0,27
3	LC 2.1	0,8	-0,55	0,23
4	LC 2.2	3,3	1,16	0,08
5	HC 3.1	2,7	-7,75	0,17
6	HC 3.2	3,2	2,42	0,03
7	HC 4.1	1,6	3,90	0,05
8	HC 4.2	2,4	1,14	0,24

07/10/2018

PCR LC +HC

10x reaction

	LC	**LC blind sample**	**HC**	**HC blind sample**
Template DNA	10 µl	-	10 µl	-
Primer	2 µl (P3)	2 µl (P3)	2 µl (P9)	2 µl (P9)

Primer	2 µl (P8)	2 µl (P8)	2 µl (P10)	2 µl (P10)
dNTPs (10mM)	40 µl	40 µl	40 µl	40 µl
Taq/Pfu (10:1)	20 µl	20 µl	20µl	20 µl
Puffer	50 µl	50 µl	50 µl	50 µl
H2O	376 µl	386 µl	376 µl	386 µl

50 ul in eine well of the PCR plate:
A1 - A10 : HC 61 °C
B1 - B10 : HCB 61 °C
F1 - F10 : LC 62,4 °C
G1 . G10 : LCB 62,4 °C

Qualitative agarose gel of the PCR

LC: all negative
HC samples: all positive

one positive blind sample (might be caused by pipetting error)
Labeling should be LCB (LC Blindproe)

HC PCR was stored at - 20 °C

Qualitative Agarosegel (Samples from gel extraction from LC; HC and Pet28a from MiniPrep)

Left (1,2,3,4): MiniPrep from 9.7.18, right: MiniPrep from 10.7.18

LC samples visible
only 3.1 3.2 of the HC are visible

DNA Precipitation of the LC

- Combination of LC Samples (1.1, 1.2, 2.1, 2.2), approximately 60 µl
- 40 ul dH20 for 100 ul total sample
- 10 µl NaAct 3M
- 300 µl 100% EtOH
- Incubation: 10 min at -80°C
- Centrifugation: 1 min max rpm
- Discard supernatant
- Washing with 70% EtOH without resuspending the pellet
- 5 min centrifugation
- Discarding of supernatant and drying of sample
- Resuspended ini 40 ul Tris HCL, pH 8

Wednesday

DNA precipitation HC

- Samples 3.1 and 3.2 combinded à appr. 40 µl
- 50 µl toatal reaction volume
- 5 ul NaAct
- 150 ul Eth. 100%
- suspended in 20 ul Tris HCl, pH 8

Gradient PCR of the LC

Refer to first gradient PCR

Qualitative Gel for LC PCR and Precipitation

(Figure missing)

- no PCR product
- no purified HC
- purified LC is visible

DpnI Digest of HC-PCR

110 ul reaction

Purification gel

1 % agaose gel without dye

Gel fell to the ground, was put together again, Band was excised

1. HC = 0,35 g = 350 µl
2. HC = 0,41 g = 410 µl

yellow bindepuffer

Carried out as the first gel, but Elution with 20 ul elution buffer

07/12/2018

PCR-LC (same program as first PCR)

- Different template (different plasmid)
- 300 ng in 2 µl
- diluted to 1 ng/ µl

Restriction digest

Performed by EK

Plasmid:

1. pet28a-Plasmid; MiniPrep von EK
2. pet28a-Plasmid; MidiPrep von EK
3. pet28a-Plasmid; MiniPred von MS

reaction (50 ul):

Component	Amount
Plasmid	1 µg

Puffer (CutSmart)	5 µl
XbaI	1 µl
EcoRV - HF	1 µl
Wasser	23 µl

Reaction at 37°C for 20 min.

Enzyme inactivation at 80 °C for 20 min

Preparation of Plates for the Interlab Study

36 plates
100 ml LB-media + 100 µl chloramphenicol (25 µg/ml)

Label: LB_CM_120718 EK
Stored at 4°C

07/16/2018

Estimation of DNA amount over Band intensity:

-> Band a litte less intence as marker
-> half intensity

1/2 * (Band at 15 bp with 45 ng) = 22,5 ng in the band ~ 22,5 ng/5µl
=> 4,5 ng/µl LC-DNA

Touch-Down-PCR

Template DNA
- pet28a Plasmid from Jasemi (from Stehle group)
- LC : Binz Plasmid (1ng/µl); Template 5 µl + 29 µl H2O

	Pet28a	**LB**
Template DNA	1 µl	1,5 µl
Primer	2 µl (P1, 1:10)	2 µl (P3, 1:10)
Primer	2 µl (P2, 1:10)	2 µl (P8, 1:10)
dNTPs (10mM)	4 µl	4 µl
Taq/Pfu (10:1)	2 µl	2 µl

Puffer	5 µl	5 µl
H2O	34 µl	33,5 µl

PCR-Program:

1% Agarose gel:

Loading: X – LC - LC Binz - Marker - pet28a x
- 1 µl loading dye + 5 µl sample
- 6 µl Marker

DPN digest:

- 45 µl LC Binz 1 h at 37 °C
- 8 µl H2O
- 1 µl enzyme
- 6 µl buffer

Purification: 1% agarose Gel
Loading: above:- P -LC- P XXX M P -LC Binz- P

below- M P -pet28a- P
LC: 45 µl plasmid + 7 µl loading dye
LC-Binz: 60 µl Plasmid + 10 µl loading dye

Gel extraction:

Gel masses:

sample	Mass (g)	ul
LC	0,44	440
LC-Binz	0,56	560
pet28a	0,59	590

Purified via gel extractions kit. Elution in 40 µl elution-buffer.

Nanodrop-Measurement:

sample	Ng/ul	260/280	260/230
LC	66,5	2,00	0,75
LC Binz	49,3	1,94	0,35

pet28a	81,0	2,05	0,74

Labeling:
- LC PCR 17.7 (66.5 ng/µl)
- LC Binz 17.7 (49.3 ng/µl)
- pet28a 17.7 (81,0 ng/ µl)

20 µl each

07/18/2018

Performed by CS

Mutagenesis of LC

Dilution of primer for mutagenesis: 1:10

PCR of Snap25, 3 reactions for 3 mutations for LC

SNAP25: Amount Delivered 1000 ng -> 100 µl TE ≙ 10 ng/µl
-> Tris was used and heated (20 min, 50 °C)

Template for PCR:
- SNAP25
- LC Binz (for reaction 1,2,3)

Primer
- SNAP25 = gp.r | gr.f
- Reaction 1: P3 – gib_tev_LC f | P4 – gib_midBoNTC-LC_doppelMut_r
- Reaction 2: P5 – gib_midBoNTC-LC_doppelMut_f | P6 – gib_endBoNTC-LC_Mut_r
- Reaction 3: P7 – gib_endBoNTC-LC_Mut_f | P8 – gib_endBoNTC-LC_tev_r

Compartment	**SNAP25**	**Reaction 1**	**Reaction 2**	**Reaction 3**
DNA Template	2 µl	4 µl	4 µl	4 µl
Primer	2 µl	2 µl P3	2 µl P5	2 µl P7
Primer	2 µl	2 µl P4	2 µl P6	2 µl P8
dNTPs	4 µl	4 µl	4 µl	4 µl
Taq/Phu (10:1)	2 µl	2 µl	2 µl	2 µl
Puffer	5 µl	5 µl	5 µl	5 µl

H2O	33 µl	31 µl	31 µl	31 µl

PCR program: Touch-Down-PCR

Agarose gel:

2 % (100 ml 1X Tae-buffer + 2 g agarose) without dye. Control: Ethidium bromide.
Loading: X, A1, A2, A3, Ladder, Snap25

Evaluation
- Band 1 : appr. 700 bp -> ok
- Band 2: appr. 500 bp -> ok
- Band 3: over 1200 bp -> not ok!
- Snap25: no band visibl

07/24/2018

Performed by EK

<u>Repetition: PCR from the 18.7</u>

Tamplate for PCR: -

- SNAP25
- LC Binz (for reaction 1,2,3)

Primer
- SNAP25 = gp.r | gr.f
- Reaction 1: P3 – gib_tev_LC f | P4 – gib_midBoNTC-LC_doppelMut_r
- Reaction 2: P5 – gib_midBoNTC-LC_doppelMut_f | P6 – gib_endBoNTC-LC_Mut_r
- Reaction 3: P7 – gib_endBoNTC-LC_Mut_f | P8 – gib_endBoNTC-LC_tev_r

Compartment	**Snap25**	**Reaction 1**	**Reaction 2**	**Reaction 3**
DNA Template	2 µl	4 µl	4 µl	4 µl
Primer	2 µl	2 µl P3	2 µl P5	2 µl P7
Primer	2 µl	2 µl P4	2 µl P6	2 µl P8
dNTPs	4 µl	4 µl	4 µl	4 µl
Taq/Phu (10:1)	2 µl	2 µl	2 µl	2 µl
Puffer	5 µl	5 µl	5 µl	5 µl
H2O	33 µl	31 µl	31 µl	31 µl

PCR program:
Agarose-Gel:
2 % (100 ml 1X TAE-buffer + 2 g agarose) without dye, control with ethidium bromide.
Loading of gel: X, A1, A2, A3, Marker, Snap25

Evaluation:
· Band 1: no band visible
· Bande 2: appr. 500 bp -> ok
· Bande 3: no band visible
· Snap25: appr.

Purification of SNAP25 via PCR & DNA cleanup kit from NEB

Snap 25: dsDNA < 2 kb
-> buffer ratio: 5:1
45 µl Snap25 PCR-Produkt + 225 µl Puffer

Elution buffer 40 ul, no heating

Labeling: SNAP25_gibson_EK 24.7

Mutation 3 of the LC did not work, Primer 7 is identical to Primer 6, this explains the results (band bigger than 1200 bp)
à new primers were orderd via Bertolt Gust

07/25/2018

Performed by CS and EK

Nanodrop Measurement of SNAP25 purified:

Sample	**Ng/ul**	**260/280**	**260/230**
SNAP25	38,5	1,75	2,02

Gibson-Assembly
Protocoll: as described by Gibson_Kit of NEB

1. SNAP25 + pet28a
2. LC + pet28a

Pet28a: 81 ng/µl
SNAP25: 38,4 ng/ µl
LC (Binz): 49,3 ng/ µl

ratio (Vektor:Insert) 1:3

1. Pet28a + SNAP25

à 1 µl + 5 µl + 10 ul MasterMix = 16 µl – 20 µl= 4 µl H_2O
2. Pet28a + LC (Binz)
à 1 µl + 4 µl + 10 ul MasterMix = 15 µl – 20 µl= 5 µl H_2O

Dissolve Primers:
· g_Phlu2_pet28a_f + 360,2 µl Tris-> for 100 µM
· g_Phlu2_pet28a_r + 361,3 µl Tris -> for 100 µM

Dissolve parts
· Phluorin2 + 72,6 µl Tris
· Syntaxin + 100 µl Tris

PCR for amplification of syntaxin product and phluorin2 product (from IDT)

Templates:
· IDT-Syntaxin
· IDT- Phluorin

Primer:
· gib_part_syntaxin_f | gib_part_syntaxin_r
· g_Phlu2_pet28a_f |g_Phlu2_pet28a_r

Educt	Syntaxin	Pluorin2
DNA Template	1 µl	1 µl
Primer (1:10)	2 µl	2 µl
Primer (1:10)	2 µl	2 µl
dNTPs	4 µl	4 µl
Taq/Phu (10:1)	2 µl	2 µl
Puffer	5 µl	5 µl
H2O	34 µl	31 µl

PCR-Programm:

07/26/2018

Agarose gel
2% agarose + PegGreen

Loading: Syntaxin, Marker, Phluorin2

PCR reaction mix

Part-PCR:

CuZnSOD: 50 µl Tris
Omomyc_NLS_Protease: 50 µl Tris

Primer:

gib_CuZnSOD: 421,1 µl
gib_omomyc: 424,3 µl

Educt	Syntaxin	Phluorin2
DNA Template	1 µl	1 µl
Primer (1:10)	2 µl	2 µl
Primer (1:10)	2 µl	2 µl
dNTPs	4 µl	4 µl
Taq/Phu (10:1)	2 µl	2 µl
Puffer	5 µl	5 µl
H2O	34 µl	31 µl

Agarose gel
Loading: Omomyc, Marker, CuZNSOD

Over night culture of DH5 α
- 2 x 100 ml LB Medium
- + 25 ml DH5α cells each

37 degrees

PCR_clean Up Kit

with Omomyc, CUZNSOD, Syntaxin, Phlourin
-> each one 45 µl reaction with 225 µl binding buffer
-> Elution in 30 µl elution buffer

Nanodrop measurement

Sample	**Ng/ul**	**260/280**	**260/230**
Syntaxin	73,9	1,95	2,28

Phluorin 2	101,5	1,97	2,27
Omomyc	68,8	1,90	2,19
CuZNSOD	89,8	1,88	1,87

Gibson-Assay

1. Syntaxin + pet28a
· 3,3 µl Syntaxin + 1 µl pet28a + 10 µl MasterMix = 14,3 µl - 20 µl = 5,7 µl H2O
2. Phluorin + pet28a
· 2,4 µl Phluorin + 1 µl pet28a + 10 µl MasterMix = 13,4 µl - 20 µl = 6,6 µl H2O

Positive Control: 10 µl control + 10 µl MasterMix
at 50 °C | 1 h

Labeling:
· Syn_pet28a 26.7 CS
· pHL_pet28a 26.7 CS

Back-Up-Box:

diluted in 10 µl

Sample	Purified?	Date	Concentration (ng/ul)
Syntaxin	pure	26.07.18	73,9
Phluorin 2	pure	26.07.18	101,5
SNAP25	pure	26.07.18	38,4
Pet28a	pure	26.07.18	81,0
Omomyc	pure	26.07.18	68,8
CuZnSOD	pure	26.07.18	89,8

07/27/2018

Main Culture: 100 ml LB + 500 ul over night culture
DH5α glycerol stocks: 1 ml over night culture: 500 µl Glycerol 20%
Pouring plates: 8 plates + Kan

Electrocompetent Cells

- 5 ml LB + 50 ul glycerol stock cells (over night culture)
- Main culture: 2x50 ml, 100 ul of over night culture à grow until OD600 0.5-0.6
- centrifugation of 50 ml of the main culture (6 min, 4.000 xg, 4 °C)
- discard supernatant
- 10 ml glycerol (10 %, cold) washing,
- centrifugation (4.000 xg, 4 °C, 6 min), discard supernatant
- 10 ml glycerol (10 %, cold) washing
- resuspend pellet with small (500 ul) rest of supernatant
- aliquot 50 ul

Labeling: DH5a komp. 27.7.18 [1,2,3,4]

PCR reaction:

Primers (new):

- P7- gib_endBONTC_LC_Mut_f
- P8 - gib_endBONTC_LC_tev_r

Educt	**LC (Binz)**
DNA Template	5 µl
Primer (1:10)	2 µl
Primer (1:10)	2 µl
dNTPs	4 µl
Taq/Phu (10:1)	2 µl
Puffer	5 µl
H2O	29 µl

Elektroporation with Plasmid:

-> Gibson reactions of: Synthaxin; SNAP25; Phluorin

- 50 µl culture (bacteria + glycerol) + 1 µl plasmid (Gibson reaction)
- Transfer of bacteria into electroporation cuvettes
- Setting: E2, optimal value range: 5.3 – 5.4 ms
- After Electroporation: immediately add 1 ml cold LB to cells
- Transfer to reaction tube
- 1h incubation at 37 °C
- streak on plates (200 µl, 100 µl, 50 µl)
- incubate at 37 °C over night

Labeling: partx 27.07.18 x µl LB + Kan DH5α CS

07/30/2018

Picking of clones à plates from 27.7

Over night culture:

- Snap25: 200 µl: 1 clone; 100 µl: 1 clone, 2 clone
- Snap25 fail: 100 µl: 1 clone; 200 µl: 1 clone, 2 clone
- Phluorin2: 50 µl: 1 clone; 200 µl: 1 clone, 2 clone
- Syntaxin 200 µl: 1 clone

MiniPrep (NEB Kit)

NanoDrop Measurement

Construct	**Ng/ul**
Syntaxin 1	51,5
Phluorin 50| 1	52,9
Phluorin 50 | 2	6,4
Phluorin 200 | 1	10,0
Phlourin 200 | 2	46,6
Snap fail 100 | 1	44,0
Snap fail 200 | 1	0,6
Snap fail 200 | 2	4,7
Snap 100 | 1	1,1
Snap 100 | 2	14,6
Snap 200 | 1	61,8

DPN-Digest

Compartment	Amount (ul)

Sample (PCR product from the 27.7/ Binz plasmid à Mut 3)	45
H2O	8
Enzyme	1
Buffer	6

2% agarose gel:
Samples: LC Mut 1, LC Mut 2, LC Mut 3

Loading: Marker, Mut1, mut1, mut1, XX, Marker, Mut2, mut2, mut2, x, Marker, Marker, Mut 3, Mut 3, mut3, Marker

Gel masses:
Mut1: 0,38 g
Mut 2: 0,47 g
Mut 3: 0,44 g

Problems with dissolving the gel, Purification hardly possible
à much loss of DNA in the column

NanoDrop Measurement

Sample	**Ng/ul**	**260/280**	**260/230**
Mut 1	38,8	8,85	0,56
Mut 2	20,8	4,21	0,07
Mut 3	5,6	1,60	0,04

<u>Touchdown-PCR der Mutationen</u>

Repetition: PCRs of the mutations LC Mut 1|2|3

Reaction:

Educt	SNAP25	Reaction 1	Reaction 2	Reaction 3
DNA Template	5 µl	4 µl	4 µl	4 µl
Primer	2 µl	2 µl P3	2 µl P5	2 µl P7

Primer	2 µl	2 µl P4	2 µl P6	2 µl P8
dNTPs	4 µl	4 µl	4 µl	4 µl
Taq/Phu (10:1)	2 µl	2 µl	2 µl	2 µl
Puffer	5 µl	5 µl	5 µl	5 µl
H2O	33 µl	31 µl	31 µl	31 µl

Touchdown PCR for LC mut1 and mut3 at 60 °C, Anfangen dann 8 Schritte runter, dann 25 Zyklen bei 57,4°C und LC mut2 dazu stellen

1% Gel:
Loading: Marker, 1, 2, 3

08/06/2018

MiniPrep:

Samples: LC Wt: Wt1 Wt2 Wt3 Wt4 Wt5 Wt6

LC Mut: first Transformation: mut1.1

Second transformation: mut2.1, mut2, mut3, mut4, mut5, mut6, mut7, mut8, mut9, mut10, mut11, mut12, mut13, mut14

Preparation via DNA-precipitation

9/08/2018

Restriction digest of the Plasmid of the MiniPrep (6.8.):

Samples: LC Wt: Wt1 Wt2 Wt3 Wt4 Wt5 Wt6

LC Mut: first Transformation: mut1.1

Second transformation: mut2.1, mut2, mut3, mut4, mut5, mut6, mut7, mut8, mut9, mut10, mut11, mut12, mut13, mut14

Reaction:

Chemical	Volume
MiniPrep- Sample	1 µl
Buffer	2,5 µl
Enzyme	0,5 µl
H_20	21 µl

Condiotions: Reaction 37 °C for 20 min

Inactivation 80 °C for 20 min

Gel:

Overnight culture

Sample:

SNAP25	3.8.18	100 µl
Syntaxin	3.8.18	100 µl
Syntaxin	2.8.18	200 µl
Phluorin	2.8.18	200 µl

Reaction:

Chemical	Volumet
Clone	1 µl
LB	100 ml
Kan	100 µl

MiniPrep of the overnight culture (9.8)

Samples:

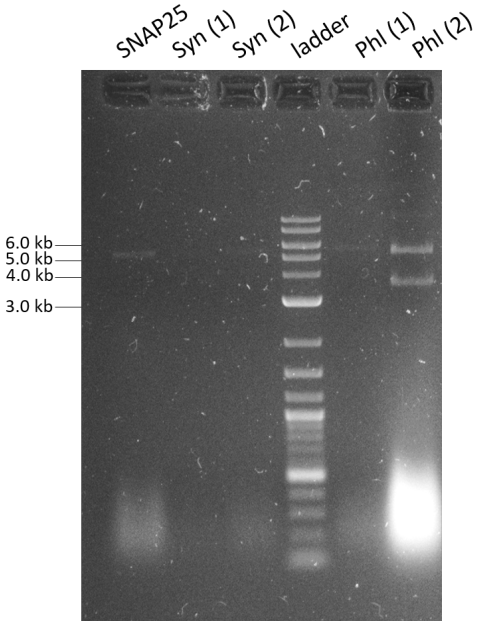
SNAP25	3.8.18	100 µl
Syntaxin (1)	3.8.18	100 µl
Syntaxin (2)	2.8.18	200 µl
Phluorin	2.8.18	200 µl

Miniprep via precipitation of the plasmid

Elution volume: 30 µl with Tris

Agarose-gel (1%):

loading: SNAP Syn (1) Syn (2) Ladder Phl (1) Phl (2)



Overnight culture
Sample: pet28a
Reaction:

Chemical	Volume
Clone	1 µl
LB	100 ml
Kan	100 µl

08/14/2018
MiniPrep of the overnight culture (13.08)
Samples: pet28a
Miniprep via precipitation of the plasmid
Elution volume: 20 µl with Tris

It is important to have a new vectorpet28a. There were no results.

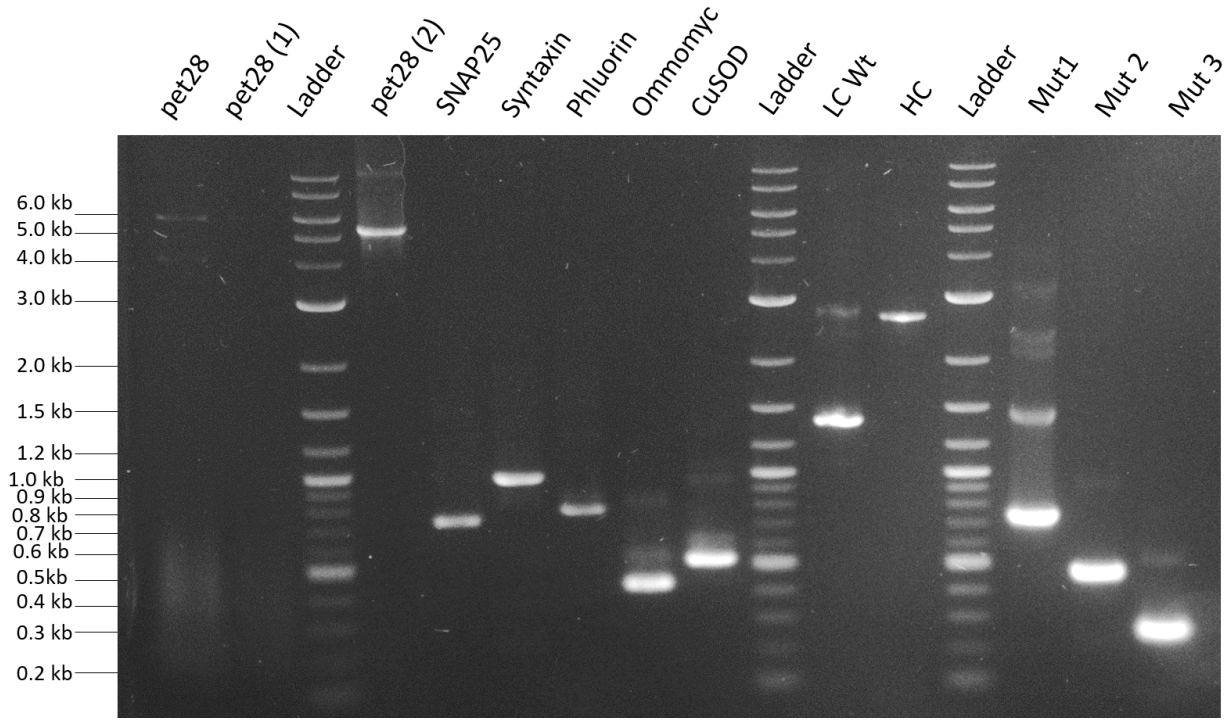
DPn digest:

Chemical	Volume
MiniPrep- Sample	1 µl
Buffer	2,5 µl
Enzyme (DPn1)	0,5 µl
H_20	21 µl

Reaction: 37 °C for 2 h

Control of the gibson-educt via agarose gel (1%)
Loading: pet28 pet28 (1) Marker pet28 (2) SNAP SYntaxin Phluorin Omomyc

CuSOD Marker LCWT HC Marker Mut1 Mut2 Mut3



08/15/2018

Colony PCR of the transformation LC Wt and LC mut (refer to 01/08)

Reaction: MasterMix

Chemical	Volume
Primer (P8)	22 µl
Primer (P14)	22 µl
dNTP	44 µl
Buffer	55 µl
Enzym (Taq-Poly.)	22 µl
H_20	385 µl

The volume of the clone (sample) or the volume (1 µl) of the Miniprep were negligible.

PCR-Program:

	Step	Temperature	Time
	Initial Denaturation	98 °C	5 min.
25 x cycles	Denaturation	98 °C	45 sec.
	Annealing	57.8 °C	45 sec.
	Elongation	72 °C	1 min 45 sec.
	Final Extension	72 °C	10 min

	4	5	6
A	Mut1	Mut 9	WT 2
B	Mut 2	Mut 10	WT 3
C	Mut 3	Mut 11	WT 4
D	Mut 4	Mut 12	WT 5
E	Mut 5	Mut 13	WT 6
F	Mut 6	Mut 14	-
G	Mut 7	Mut 1.1	-
H	Mut 8	WT 1	-

new PCR amplifiaction of pet28

Tamplate: 1. MiniPrep from 14.8 2. Pet28-vektor von AK Feil (Jasemin)

PCR-Ansätze: 1) Primer 12: gib_uni_pet28 Programm: TouchDown mit 2. Pet28-vektor
Primer 13: gib_uni_pet28 Annealing Temp.: for 5 x : 69.1 °C
for 20 x : 68.3 °C

2) Primer 1: gib_pet28_TEV Programm: TouchDown mit MiniPrep
Primer 2: gib_pet28_TEV Annealing Temp.: for 5 x : 61.0 °C
for 20 x : 69.0 °C

Samples: 1.1 Template MiniPrep Primer: 12 + 13
1.2 Template Jasemin Vektor Primer 12 +13
2.1 Template MiniPrep Primer: 1 + 2

Reaction:

Chemical	Volume
DNA-Template	1 µl
Primer (X)	2 µl
Primer (Y)	2 µl
dNTP	4 µl
Buffer	5 µl
Enzym (Taq-Poly.)	2 µl
H_20	34 µl

Program:

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec.
5 x cycles		
20 x cycles		
Denaturation	98 °C	10 sec.
Annealing	variable °C	20 sec.
Elongation	72 °C	45 sec.
Denaturation	98 °C	10 sec.
Annealing	variable °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Agarose Gel (1%):

Loading: 1.1 1.2 Marker 2.1

Controle of the colony PCR via agarose gel (1%):

Loading:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
M	A4	B4	C4	D4	E4	F4	G4	H4	M	A5	B5	C5	D5	E5	F5	G5	H5	M

1	2	3	4	5	6	7	8
M	A6	B6	C6	D6	E6	F6	M

08/16/2018

Sequencing by Mycrosynth

NanoDrop-measuring

Sample	ng/µl	260/280	260/230
Mut 1.1	3533,4	2,05	2,12
WT 6	4182,8	2,01	2,05

Syntaxin 10.08	4020,6	1,97	2,12
Syntaxin 31.7	39,8	1,85	1,99
Mut14	5343,2	1,54	1,56
WT 4	4092,1	1,98	2,01

Reaction for sequencing:

Chemical	Volume
DNA-Template	3 µl
Primer (X) (1:10)	3 µl
Primer (Y)(1:10)	2 µl
H_20	6 µl

Total volume: 12 µl
sequence-code:

Sample	Primer	Code
WT 6	15	4456901
Mut 1.1	14	4456902
Mut 1.1	15	4456903
Mut 4	14	4456904
Syntaxin (10.8)	14	4456905

New PCR-reaction of the MiniPrep-Sample Volume same as 15/08

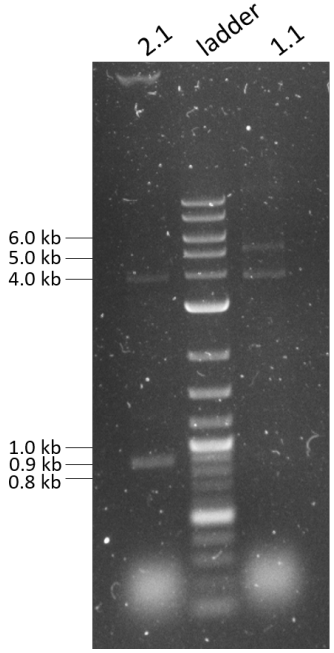
2.1 Reaction with Primer 1 +2 PCR-Programm: TouchDown

8 x cycle of annealing temp. at 64°C
(-1°C/cycle)
25 x cycle of annealing temp. at 69°C

1.1 Reaction with Primer 12 + 13 PCR-Programm: annealing temp. 56,8 °C

Agarose Gel (1%)

Loading: 2.1 Marker 1.1



08/17/2018

New PCR Template: „jasemin pet28a"

Reaction:

Chemical	Volume
DNA-Template	1 µl
Primer (X) (1:10)	2 µl
Primer (Y) (1:10)	2 µl
dNTP	4 µl
Buffer	5 µl
Enzyme (Taq-Poly.)	2 µl
H_20	34 µl

Program:

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec.
8 x cycles		
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	52 °C (-1°C/cycle)	20 sec.
Elongation	72 °C	45 sec.
Denaturation	98 °C	10 sec.
Annealing	63 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Purification vis column with QlAquick PCR Purification Kit (50)

Sample 1.2 (16/08) Jasemin pet28a with primer 12 + 13

Protocoll: 2x wash steps.

Eluate in 30 µl Buffer (Tris)

DPn-Diguest of the Sample 1.2 after purification

Reaction:

Chemical	Volume
Sample 1.2	30 µl
Buffer	5 µl
Enzyme (DPn1)	0,5 µl
H_20	14 µl

Reaction for 2 h at 37 °C

Overnight culture for pet28a-GFP

08/18/2018

MiniPrep of the pet28a-GFP vai precipitation: Sample 1.5 ml

Eluate in 20 µl Tris

Purification via Agarose-Gel: Sample 1.2 (Jasemin-pet28)

Gel-preparation: 45 µl Sample + 9 µl loading dye

Gel weight: 0,45 g -> 450 µl Buffer

Gel-Purification via Monarch DNA Gel Extraction Kit from NEB

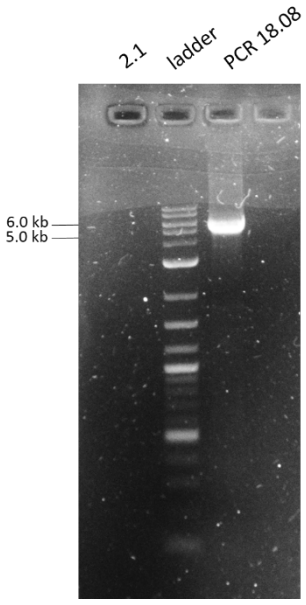
Restriction digest: Sample pet28-GFP after MiniPrep
PCR with template pet28a (jasemin) Primer 1 + 2
Reaction:

Chemical	Volume
DNA-Template	1 µl
Primer (1) (1:10)	2 µl
Primer (2) (1:10)	2 µl
dNTP	4 µl
Buffer	5 µl
Enzyme (Taq-Poly.)	2 µl
H_20	34 µl

Program:

Step	Temperature	Time
25 x cycles		
Initial Denaturation	98 °C	30 sec.
Denaturation	98 °C	10 sec.
Annealing	58 °C	45 sec.
Elongation	72 °C	1 min 30 sec.
Final Extension	72 °C	2 min

08/19/2018
Agarose-Gel (1%)
Loading: Sample 1.2 (after purification) ladder PCR-product (18/08)



DPn-digest: Sample: PCR-product from 18/08 -> pet28a (jasemin)
Reaction:

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

Digest run at 37 °C for 2 h; inactivation at 65 °C for 20 min

Purification after digest via column -> with the QAlquick PCR purification Kit (50)

Elution in 40 µl Buffer

08/20/2018

Gibson-Reaction: Template: pet28a (2.1, Jasemin) , DPn diguested and purificated

Reaction	Vector	Volume	Insert	Volume	MasterMix (Volume)	H_2O (Volume)
1	Pet28a + overhang	2 µl	LC wt	4 µl	10 µl	4 µl
2	Pet28a + overhang	2 µl	LC mut1	2 µl	10 µl	2 µl
			LC mut2	2 µl		
			LC mut3	2 µl		
3	Pet28a + without overhang	2 µl	SNAP25	4 µl	10 µl	4 µl
Blind sample	Pet28a + overhang	2 µl	-	-	10 µl	8 µl

Electroporation of

1. 50 µl E.Coli culture + 2µl Gibson Reaction
2. Mixture in electroporation cuvette
3. Electroporated with the device
4. After electroporation put 1 ml cold LB solution on the cells

08/21/2018

Syntaxin bacteria dilution smear on LB-Kan-plate

Syntaxin overnight with Kan

Clone picking -> pet28 without insert 200 µl clone 1 and clone 2

pet28 without insert 100 µl clone 1 and clone 2

08/22/2018

Overnight culture of LC wt and LC mut

Miniprep of syntaxin and pet28 without insert (samples from 21/08) via precipitation

Eluted with 50 µl Tris HCl pH 8.0

NanoDrop measuring:

Sample	ng/ µl	260/280	260/230
Syntaxin 1	2171.4	2.11	2.22
Syntaxin 2	2212.0	2.10	2.17
pet28 100 µl clone 1	2958.9	2.09	2.20
pet28 100 µl clone 2	2194.4	2.11	2.23
pet28 200 µl clone 1	2519.7	2.11	2.20
pet28 200 µl clone 2	3176.1	2.08	2.18

Sequencing by Eurofins

Sample: Syntaxn 1 2171ng/ µl Syntaxin T7 promotor

Reaction: 2 µl Sample (Syntaxin 1) + 38 µl H_2O Syntaxin T7 terminator

Restriction digest: Enzyme Xba I

Reaction:

Chemical	Volume
MiniPrep- Sample	1 µl
Buffer	2,5 µl

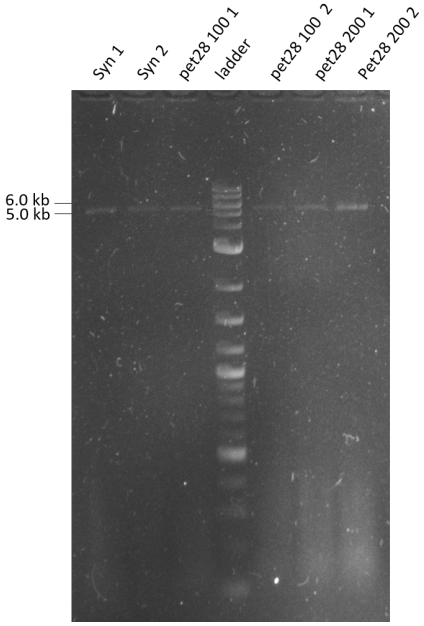
Enzyme (Xba I)	0,5 µl
H_20	21 µl

Samples:

Sample
Syntaxin 1
Syntaxin 2
pet28 100 µl clone 1
pet28 100 µl clone 2
pet28 200 µl clone 1
pet28 200 µl clone 2

Agarose gel:

Loading	syn1	syn2	pet28 100 1	ladder	pet28 100 2	pet28 200 1	pet28 200 2



08/23/2018

Samples

Miniprep of LC Wt and LC mut cells via precipitation

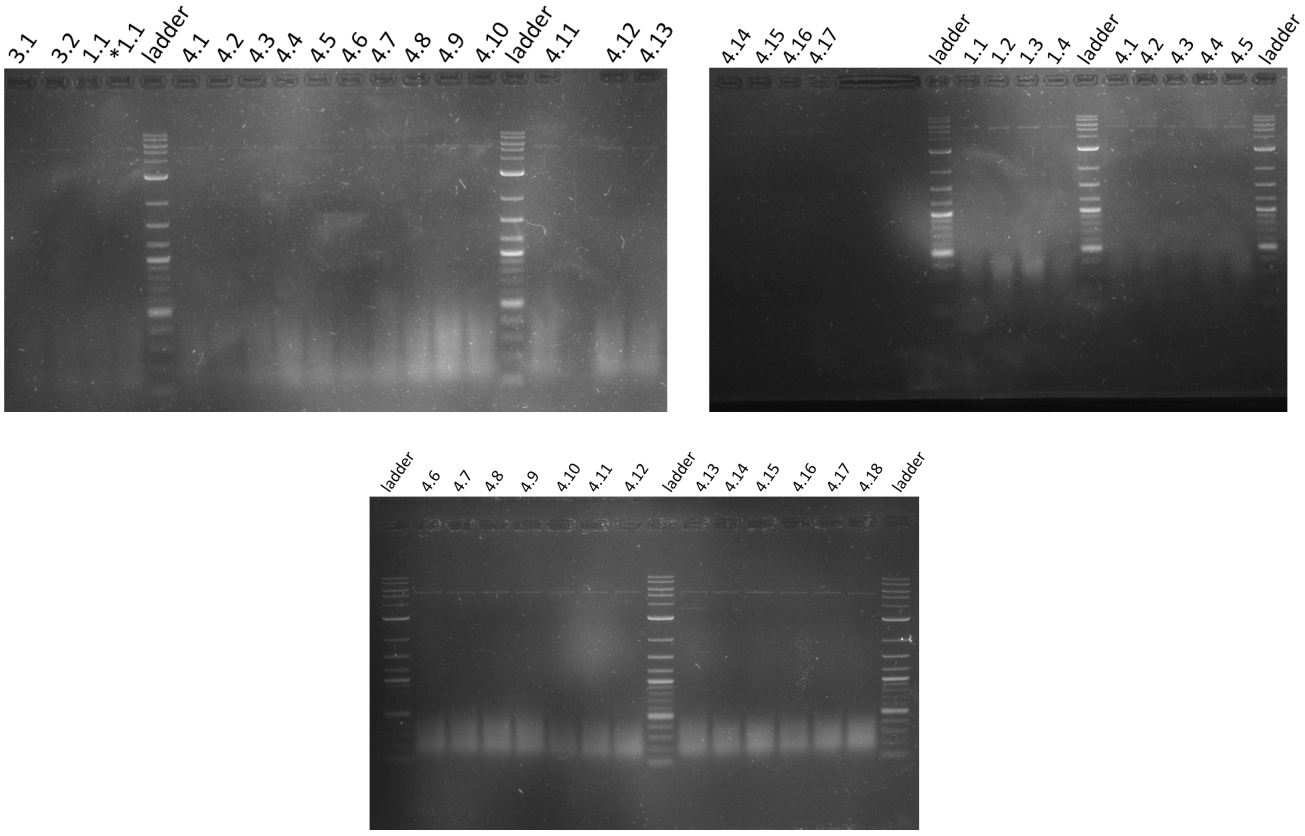
Eluted in 40 µl Tris HCl pH 8.0

Restriction digest

Reaction: Master Mix for 44 reaction

Chemical	Volume
Buffer	100 µl
Enzyme (EcoRV)	22 µl
H_20	924 µl

Agarose gel:



08/24/2018

Sequencing by Mirosynth

Samples:
1. 12 µl Syntaxin (≈ 108 ng/ µl) Code: 4456906
2. 12 µl Syntaxin (≈ 108 ng/ µl) Code: 4456907
3. Syntaxin (1:20) + Primer 15 (1:10) Code: 4456908

Agarose gel of the PCR (23/08) -> all column were empty

08/27/2018

PCR with Q_5 Polymerase with the template of the vector pet28 and the genblock SNAP
For the pet28 amplifiaction we used Primer 12 and Primer 13
SNAP amplification we used Primer 14 and Primer 15

Reaction:

Chemical	Volume
DNA-Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 MasterMix	25 µl

PCR-Programm:

	Step	Temperature	Time
	Initinal Denaturation	98 °C	30 sec.
25 x cycles	Denaturation	98 °C	10 sec.
	Annealing	68 °C	20 sec.
	Elongation	72 °C	2 min 15 sec.
	Final Extension	72 °C	2 min

DPn1-digest:

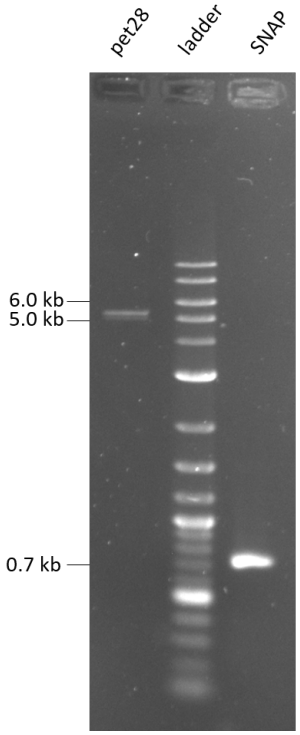
Chemical	Volume
Sample	50 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	3 µl

Reaction for 1 h , 37 °C

Overnight culture for electrocompetent cells : DH5α and LEMO

- 100 µl LB + 50 µl DH5α-glycerol-stock
- 100 µl LB + 100 µl CM + 1 clone

Agarose gel (1%)
Loading: pet28 vector ladder SNAP25



08/28/2018

Electrocompetent cells:
Gibson Assembly: pet28 (Product of the PCR 27/08) with SNAP25 (Product of the PCR 27/08)

Chemical	Volume
Vector (pet28)	3 µl
Insert (SNAP25)	3 µl
Gibson Enzyme	10 µl
H_20	4 µl

Reaction by 45 °C for 90 min.

PCR of the Mutation PCR of LC

Chemical	Reaction 1	Reaction 2	Reaction 3	Reaction 4
Template	Binz Plasmid	Binz Plasmid	Binz Plasmid	Jasemin Plasmid
Primer	3	5	7	1
Primer	4	6	8	2

Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

PCR-Program:

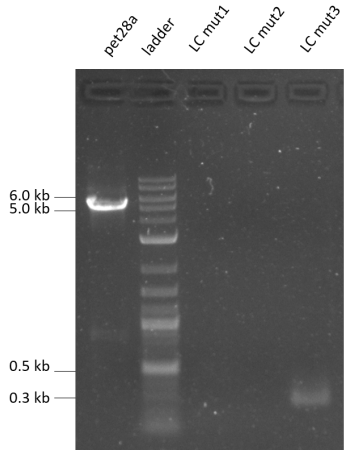
Steps	Reaction 1		Reaction 2		Reaction 3		Reaction 4	
	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time
Initinal Denaturation	98 °C	30 sec.	98 °C	30 sec.	98 °C	30 sec.	98 °C	30 sec.
Denaturation	98 °C	10 sec.	98 °C	10 sec.	98 °C	10 sec.	98 °C	10 sec.
Annealing	64 °C	20 sec.	67 °C	20 sec.	53 °C	20 sec.	56 °C	20 sec.
Elongation	72 °C	30 sec.	72 °C	30 sec.	72 °C	30 sec.	72 °C	2 min 15 sec
Denaturation	98 °C	10 sec.	98 °C	10 sec.	98 °C	10 sec.	98 °C	10 sec.
Annealing	68 °C	20 sec.	67 °C	20 sec.	67 °C	20 sec.	-	-
Elongation	72 °C	30 sec.	72 °C	30 sec.	72 °C	30 sec.	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min	72 °C	2 min	72 °C	2 min	72 °C	2 min

:5 x cycles

20 x cycles

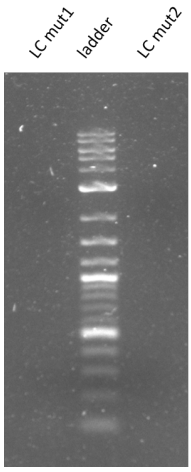
Agarose Gel (1%)

Loading: Reaction 4 ladder Reaction 1 Reaction 2 Reaction 3



New PCR of Reaction 1 and 2. Same reaction and Program.

Agarose Gel:



Gibson Assembly were been plated on LB KAN Plates.

08/29/2018

Negative transformation from yesterday.

Gibson Assembly: pet28 (Product of the PCR 27/08) with SNAP25 (Product of the PCR 27/08)

Chemical	Volume
Vector (pet28)	3 µl
Insert (SNAP25)	3 µl
NEBuilder	10 µl
H_20	4 µl

Reaction by 45 °C for 90 min.

Transformation of Gibson Assembly with DH5α cells. Plated on LB-KAN plates.

08/30/2018

Colony – PCR of pet28a_SNAP25

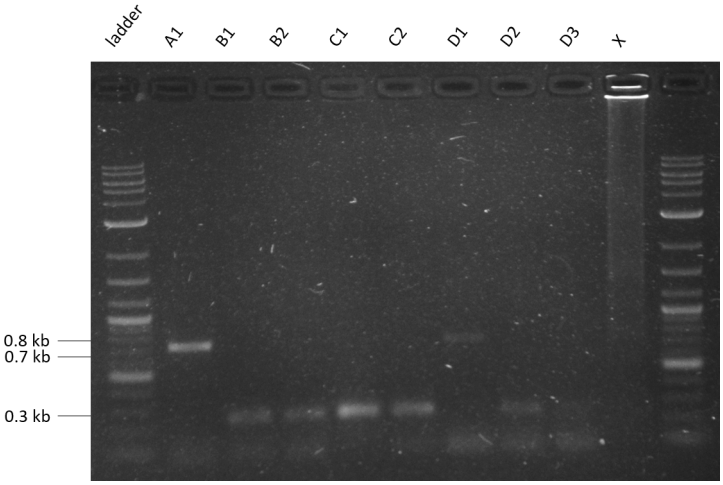
Samples: letter – Plate Number - Clone

A1	B1	C1	C2	D1	D2	D3

Reaction: MasterMiX

Chemical	Volume
Primer 14 (1:10)	2 µl
Primer 15 (1:10)	2 µl
Taq-Polymerase	50 µl
dNTP	40 µl
Buffer	50 µl
H_2O	376 µl

Agarose Gel (1%)



A1and D1 are positive. SNAP = 729 bp.

Overnight cultures were created.

08/31/2018

Sequencing by Eurofins

Sample: D1	Primer: 15	Code:	ALF0090095
D1	14		ALF0090096
A1	15		ALF0090097
A1	14		ALF0090098

Microsnyth: 4456910 T 7 terminator
4456909 T7 promotor

09/03/2018

Purification of the PCR product: Synatxin (01/09)
Eluted in 50 µl elution buffer.
Control with Agarose gel (1%)
Loading: ladder Syntaxin

PCR with Q5 polymerase. Amplification of CuSOD and Ommomyc.
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec.
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	68 °C	20 sec.
Elongation	72 °C	1 min 30 sec.
Final Extension	72 °C	2 min

Agarose Gel (1%)
Loading: CuSOD ladder Ommomyc
Was negative -> the wrong Primer were used! :(
Colony PCR: of LC Wt and LC mut

Reaction: MasterMix:

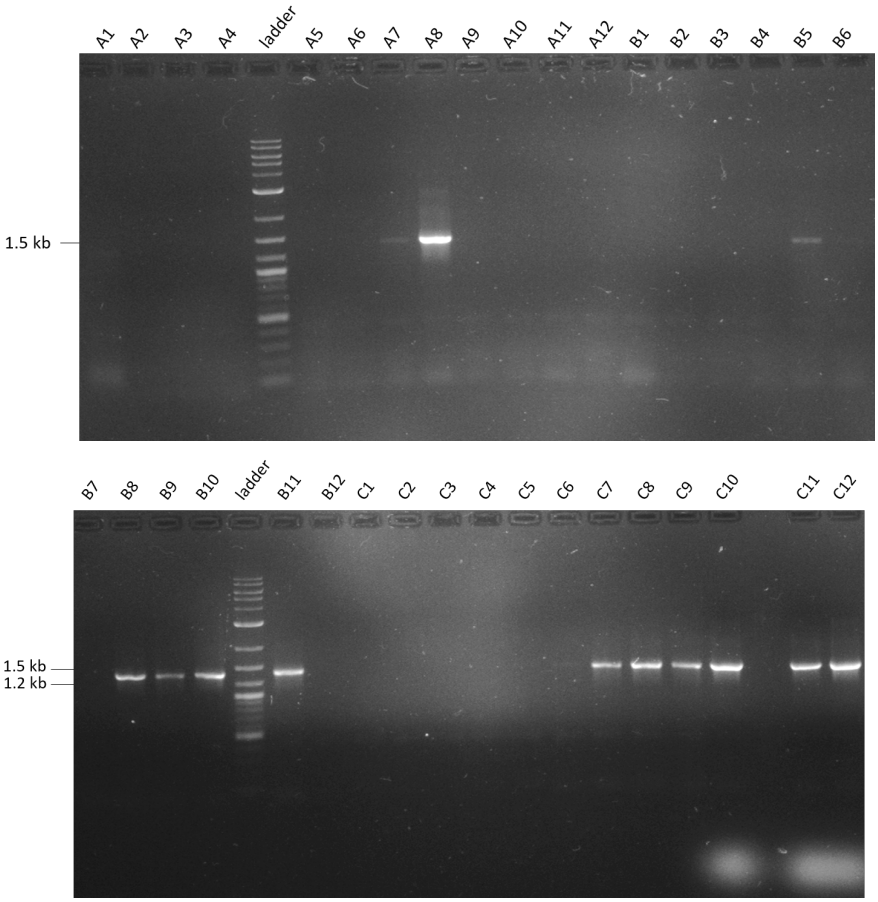
Chemical	Volume
Primer (1:10)	5 µl
Primer (1:10)	5 µl
Taq-Polymerase	50 µl
dNTP	100 µl
Buffer	125 µl
H_2O	915 µl

Program:

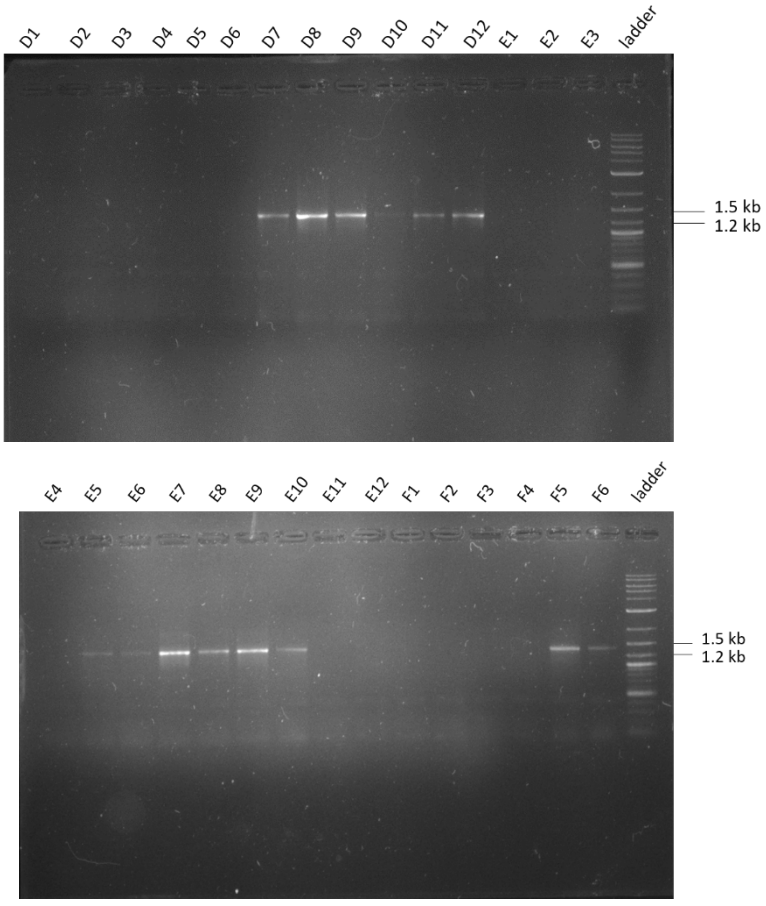
Step	Temperature	Time
Initial Denaturation	95 °C	5 min.
25 x cycles		
Denaturation	95 °C	45 sec.
Annealing	59 °C	45 sec.
Elongation	72 °C	1 min 45 sec.
Final Extension	72 °C	10 min

Agarose Gel (1%):

Gel 1



Gel 2



In summary positive clones are:

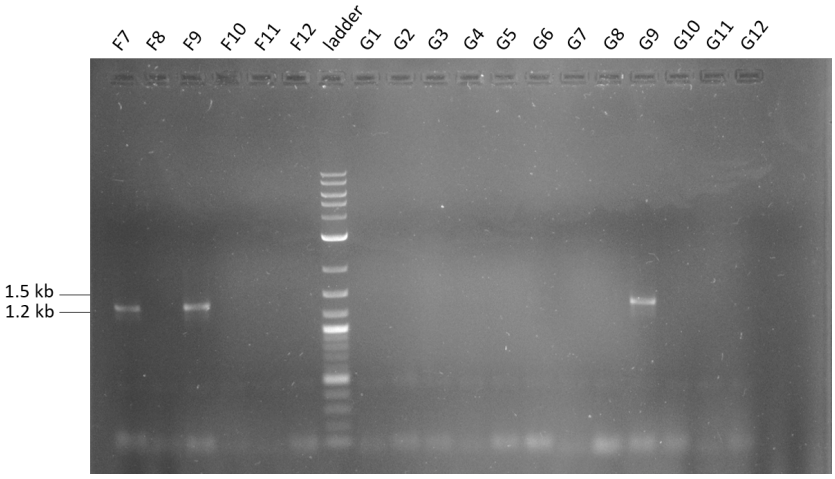
Samples: LC wt = B5; F5; F6 LC mut: A8; B8- B11; C7-C12; D7-D9; D11; D12; E7-E10

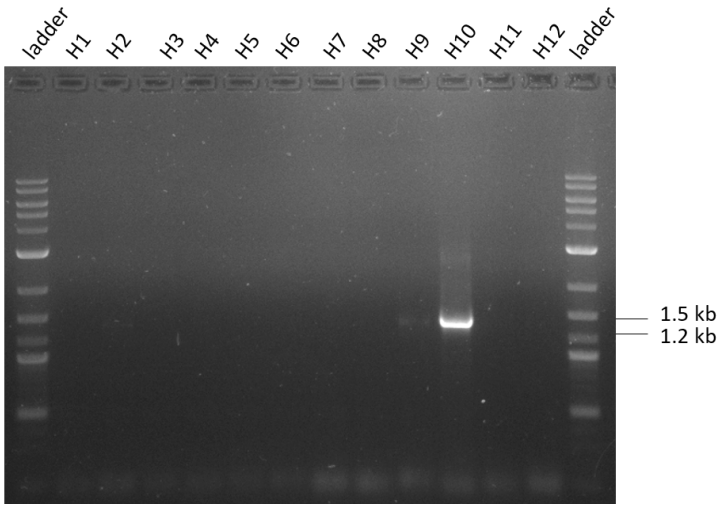
Gibson Assembly: Sample: pet28a with Syntaxin
Reaction:

Chemical	Volume
Vector (pet28)	3 µl
Insert (Syntaxin))	3 µl
NEBuilder	10 µl
H_20	4 µl

Reaction at 50 °C for 1h
Transformation with 2 µl Gibson assembly product in XL-1 blue.

09/04/2018
Agarose gel of the last samples of the colony PCR.





Clone-picking of positive clones.

PCR

PCR of HC with Q_5-Polymerase- The Template is “bontcs-thro-botox” plasmid

Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.
5 x cycles		
20 x cycles		
Denaturation	98 °C	10 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	45 sec.
Denaturation	98 °C	10 sec.
Annealing	71 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Transformation: pet28 from jasemin. 1 µl in XL-1 blue cells.

Purification via column.

Samples: colony PCR product from Sample A8 and F5.

Eluted 20 µl Elution Buffer.

Sequencing by eurofins: 20 µl Product + 2 µl P3

Mut A8: ALF0030094

Wt F5: ALF0030093

09/05/2018

Test expression of our SNAP protein out of LEMO cells. -> see protocol of protein expression.

09/06/2018

Miniprep of plasmid via precipitation

Samples: pet28a -> 30 µl Tris HCl pH 8.0

LC -> 40 µl Tris HCl pH 8.0

Syntaxin -> 40 µl Tris HCl pH 8.0

NanoDrop-Measuring:

Sample	ng/µl	260/280	260/230
A8	3533,4	2,05	2,12
F5	4182,8	2,01	2,05

Sequencing by Microsynth

4456911: 12 µl Sample A8 + Primer T7 terminator

4456912: 12 µl Sample F5 + Primer T7 terminator

4456913: 9 µl Sample A8 + 3 µl Primer P5

4456914: 9 µl Sample F5 + 3 µl Primer P5

Sequencing by Eurofins.

ALF0030092: 20 µl sample A8 + Primer T7 promotor

ALF0030091: 20 µl sample F5 + Primer T7 promotor

Restriction digest:

Reaction: 25 µl for each 16 Samples = 400 µl Samples: LC mut, LC wt, Syntxain

MasterMix:

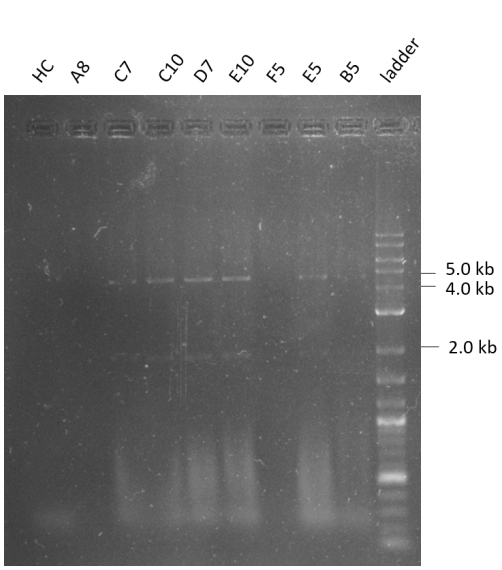
Chemical	Volume
Buffer	40 µl
Enzyme (BamHI HF))	8 µl
Enzyme (EcoRV HF)	8 µl
H_20	328 µl

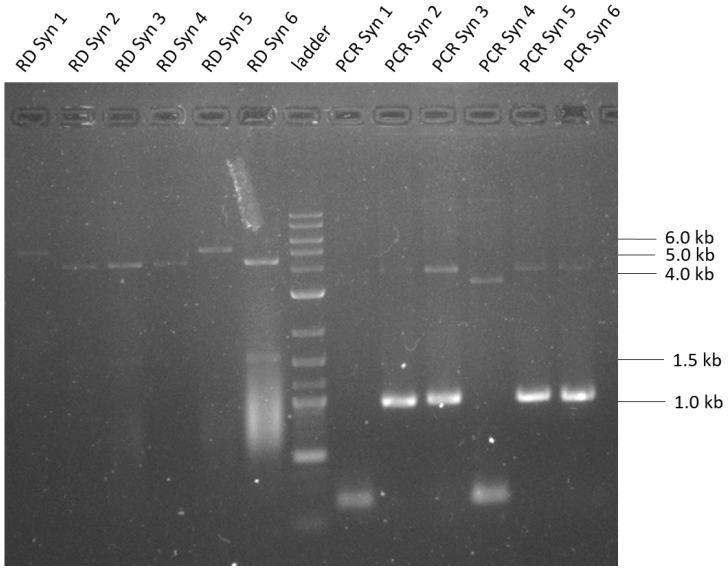
Reaction: at 37 °C for 20 min. Inactivation at 65 °C

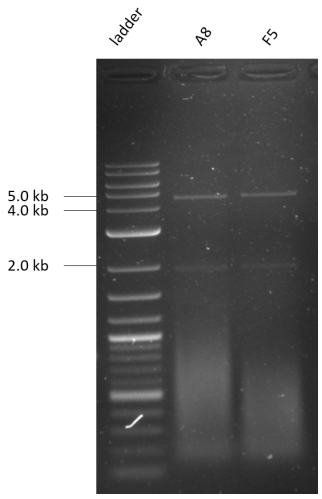
Colony PCR: of Syntaxin 6 clones

Reaction: MasterMix:

Chemical	Volume
Primer (1:10)	5 µl
Primer (1:10)	5 µl
Taq-Polymerase	50 µl
dNTP	100 µl
Buffer	125 µl
H_2O	915 µl






ladder
A8
F5
5.0 kb
4.0 kb
2.0 kb

Start of Cre Cloning

Transformation of E.Colis with
- pcDNA3.1- Vector (100pg/µL)
- pSB1C3-Cre (200-300pg/µL)

Electroporation
- 50 µl competent E.colis + 1µl DNA
- Electroporation
- Immediate addition of 1ml of cold LB
- 1h Incubation at 37°, 250 rpm

Pouring of 10 Plates
- LB + Carbenicillin (1:1000) (100µl for 100 ml LB)

Spreading of the total 50µl onto plates
Incubation o.n. at 37°C

09/07/2018
PCR
PCR of Phluorin2 with Q_5-Polymerase- The Template is a gene block from IDT
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Programm:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

NanoDrop-Measuring:

Sample	ng/µl	260/280	260/230
Syn 2	4164.8	1.94	2.07
Syn 3	4456.7	1.94	2.08

Sequencing by Eurofins.
ALF0030090: 20 µl sample A8 + Primer T7 promotor
ALF0030089: 20 µl sample F5 + Primer T7 terminator
Gel-purification Manu

Gel-weight: 0.4 g

Cre Cloning

no clones
- checking of antibiotic

New transformation
- same as on Wednesday, but with 1µl higher concentrated pcDNA3.1 (0.5µg/µl) , Cre Plasmid: same

09/08/2018
Transformation 1 µl Syntaxin in LEMO cells. 25 µl cell suspension plated on LB-KANN plates.

Cre Cloning

- Bacterial lawn on pcDNA3.1 plate
--> Dilution streak to pick single clones
- 1-2 clones on plate with Cre??
--> incubate again

09/09/2018
Transformation:
1 µl LC mut (A8) in LEMO cells
1 µl LC wt (F5) in LEMO cells

09/10/2018
Last Transforamtion (09/09) was negative. So new Transformation:
1 µl LC mut (A8) in LEMO cells
1 µl LC wt (F5) in LEMO cells
Overnight culture of Syntaxin
Overnight culture of LEMO cells.
Over night culture of pcDNA3.1- clone, 5ml LB

09/11/2018
New glycerol stock of LEMO cells
Syntaxin solution for protein laboratory -> see labbbook protein purification

09/12/2018
New Transforamtion of LC wt and LC mut.
The transformation were prepared on LB-KAN-Ramnose plates.
1.2 mg L-Ramnose were solved in 10 ml H_2O. The solution were steril filtrated.
Glycerol stock of : pet28a_Syntaxin in LEMO cells
Pet28a_SNAP in LEMO cells

09/13/2018
Gibson Assembly: Sample: pet28a with phluorin2

Reaction:

Chemical	Volume
Vector (pet28)	3 µl

Insert (phluorin2)	3 µl
NEBuilder	10 µl
H_20	4 µl

Reaction at 50 °C for 1h
Transformation with 1 µl Gibson assembly product in XL-1 blue. Cell solution after 1 h incubation plated out on LB-KAN plates.
New glycerol stock of XL1-blue cells.

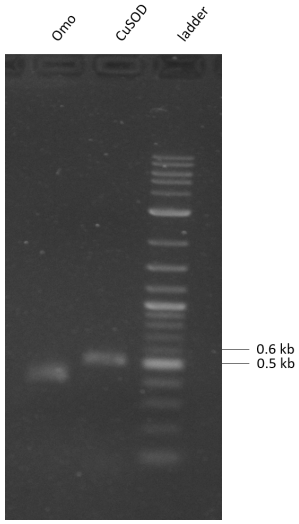
09/14/2018
Transformation worked.
PCR von CuSOD and Ommomyc
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Agarose Gel:



09/15/2018

Colony PCR from pet28a_phluorin2
Reaction: MasterMix:

Chemical	Volume
Primer (1:10)	5 µl
Primer (1:10)	5 µl
Taq-Polymerase	50 µl

dNTP	100 µl
Buffer	125 µl
H_2O	915 µl

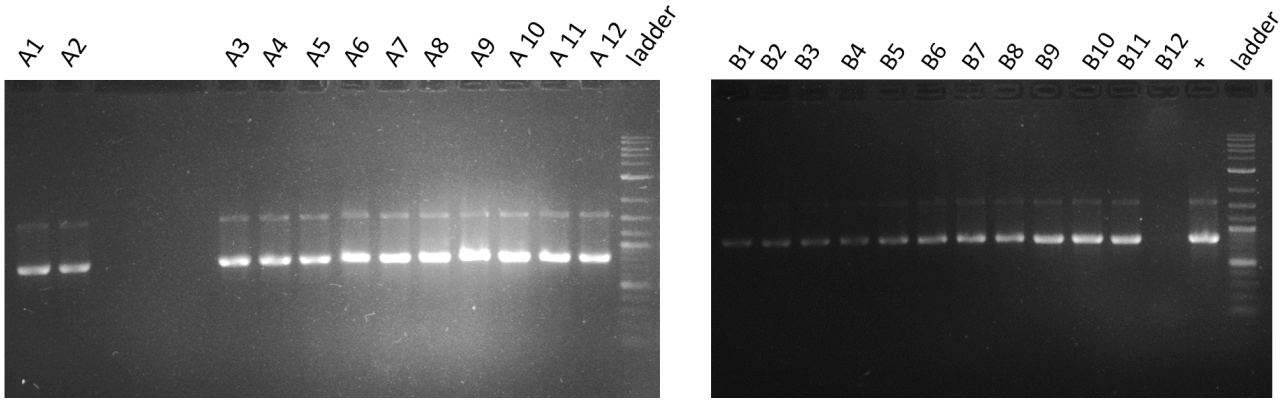
Programm:

Step	Temperature	Time
Initinal Denaturation	95 °C	5 min.

25 x cycles

Denaturation	95 °C	45 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Agarose gel:



09/17/2018

PCR of amp-BoNTC

Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.

25 x cycles

Denaturation	98 °C	10 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

Agarose Gel: negative

Transformation from Phluorin

Cre Cloning

Miniprep of Over night Culture (pcDNA3.1)

Protocol: in lab
resuspended in 40 µL Tris-HCL
Nanodrop: 2583,1 ng/µL,
260/280: 2,06
260/230: 2,19
New Transformation of pB1C3, but with more DNA! (2µL)
+ new LB + Carb plates

09/18/2018
PCR from plasmid pet28a_LC
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10) P12	2 µl
Primer (1:10) P amp_BoNT	2 µl
Taq/Phu-Polymerase	2 µl
dNTP	4 µl
Buffer	5 µl
H_2O	34 µl

Programm:

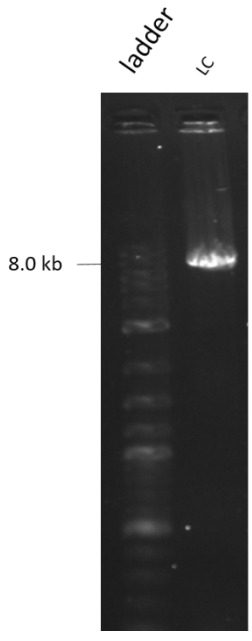
Step	Temperature	Time
Initinal Denaturation	95 °C	30 sec.
25 x cycles		
Denaturation	95 °C	45 sec.
Annealing	65 °C	45 sec.
Elongation	72 °C	6 min
Final Extension	72 °C	2 min

<u>Cre Cloning</u>

Cre: no clones
→ **new Transformation**, 1µL DNA
plating out on CM and Carb plates
Transformation of Reporter plasmid (pLacZ)
plating out on Amp Plate

09/19/2018
PCR from plasmid pet28a_LC for only amplification of LC
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10) P12	2 µl
Primer (1:10) P amp_BoNT	2 µl
Taq/Phu-Polymerase	2 µl
dNTP	4 µl
Buffer	5 µl



H_2O	34 µl

Programm:

Step	Temperature	Time
Initinal Denaturation	95 °C	30 sec.
Denaturation	95 °C	45 sec.
Annealing	65 °C	45 sec.
Elongation	72 °C	6 min
Final Extension	72 °C	2 min

25 x cycles

Agarose Gel: see left

Purification via Column: pet28a sample A8

Sequencing:

Eurofins: A8: ALF0030087 T7
ALF0030088 T7 Terminator
F5: ALF0030085 T7
ALF0030086 T7 terminator

PCR of LC-pet28a with Primer: amp-BoNT and P12 TaRaKa-Polymerase

Reaction:

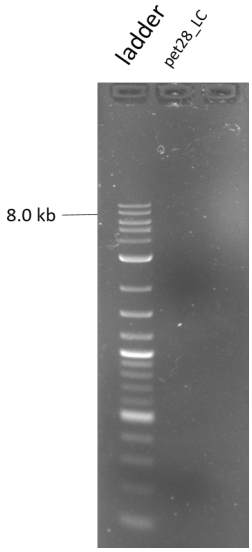
Chemical	Volume
Template (A8)	1 µl
Primer 12 (1:2)	2.5 µl
Primer amp. (1:2)	2.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

Program:

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec.
Denaturation	98 °C	10 sec.
Annealing	58 °C	5 sec.
Elongation	72 °C	5 min
Final Extension	72 °C	2 min

30 x cycles

Agarose Gel:



New PCR of pet28a_LC with TaKaRa.
Same reaction and program – annealing 56 °C for 15 sec.

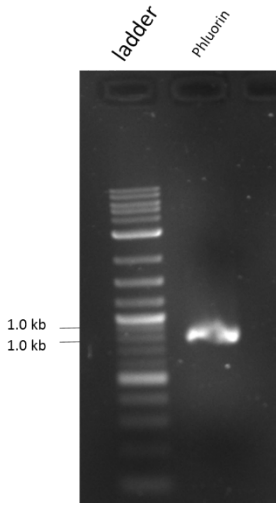
PCR of Phluorin
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	65 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

Agarose Gel:



PCR of Mutagenisis 216 & 423
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10) P12	2 µl
Primer (1:10) P amp_BoNT	2 µl
Taq/Phu-Polymerase	2 µl
dNTP	4 µl
Buffer	5 µl
H_2O	34 µl

Programm:

Step	Temperature	Time
Initinal Denaturation	95 °C	30 sec.
25 x cycles		
Denaturation	95 °C	45 sec.
Annealing	65 °C	45 sec.

Elongation	72 °C	6 min
Final Extension	72 °C	2 min

Agarose gel: all negative

Cre Cloning

no clones on CM or Carb plate
picking of one clone from pLacZ transformation
inoculation of appr. 5ml LB + Carb (10 am)
3. Over night culture: pLacZ transformation, 100 ml

09/21/2018

Maxi Prep of pLacZ

- Centrifugation of 100 ml over night culture form pLacZ transformation, 2 min, 6000 rp at room temperature, Supernatant is discarded
- Resuspension of Pellet in 5 ml Sol I, Incubation for 10 min at room temperature
- Addition of 10 ml Sol II and mix the contents thoroughly by gently inverting the vessel several times, Vessel is stored on ice for 10 min
- Addition of 7.5 ml ice-cold Sol III, and mixing the contents by inverting the vessle several times, Vessel is stored on ice for 10 min
- Centrifugation of the suspension for 10 min, 6000 rpm, 4°C, Supernatant is removed into a new vessel
- Centrifugation for 10 min, 6000 rpm, 4 °C, Supernatant is remoevd into a new vessel
- Centrifugation for 10 min, 6000 rpm, 4°C, Supernatant (15 ml) is moved into a new vessel
- Precipitation of the DNA:
- one tenth of volume of sodium acetate (1.5 ml) and 1 volume of isopropanol (15 ml) is added to the DNA solution
- Contents are mixed by inverting the tube
- 10 min incubation on ice
- Centrifugation at 6000 rpm for 20 min at 4°C
- Supernatant is discarded
- Washing of pellet with 500 ul EtOH by centrifugation for 10 min at 6000 rpm, 4°C
- Pellet was dried at room temperature
- DNA was dissolved in 400 ul TRIS HCL buffer

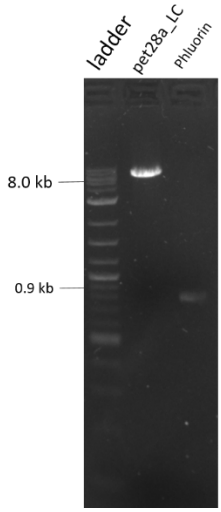
Measurement on Nanodrop

--> 5225 ng/µl
260/280: 1,32
260/230: 1,42

Cre from 2013 was given to Lukas for PCR

09/21/2018

Purification via column. Samples: Phluorin and pet28a_Lc (TaKaRa)



Gel

NanoDrop measuring:

Sample	ng/µl	260/280	260/230
Pet28_LC	78.5	1.91	2.43
Phluorin	33.8	1.82	2.53

DPn1-digest:

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

Reaction for 1 h, 37 °C
Purification via column pet28a_Lc (TaKaRa)

Gibson Assembly:
Reaction pet28a_LC + phluorin

Chemical	Volume
Vector (pet28a_LC)	3 µl
Insert (phluorin)	4 µl
NEBuilder	10 µl
H_20	3 µl

Program: 50 °C for 60 min.
Transformation in E.Coli XL-1 blue with 2 µl or 3 µl Gibson Assembly product. After 1 h incubation, the transformed E.Coli were platted on LB-KAN plates:

PCR: Hibit
Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer 8 (1:2)	2.5 µl
Primer amp. (1:2)	2.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

PCR-Program:

	Steps	Temp.	Time
	Initinal Denaturation	98 °C	30 sec.
5 x cycles	Denaturation	98 °C	10 sec.
	Annealing	53 °C	15 sec.
	Elongation	72 °C	7 min
20 x cycles	Denaturation	98 °C	10 sec.
	Annealing	68 °C	15 sec.
	Elongation	72 °C	7 min
	Final Extension	72 °C	2 min

Agarose gel: PCR was negative.

Cre Cloning

2 new Cres (codon optimized for yeast) from 2015
psb-Cre (20 ng/µl)
psb-CreSN (～190 ng/µl)
Transformation of yeast-Cre (2015),
2 µl of psb Cre
1 µl of psb CreSN
Streaking out on CM and Carb Plates

Lukas Fuhs: PCRs
Amplification of CreI CDS from registry template BBa_J61047

Template: BBa_J61047 in pSB1A2, Origin Distribution 2013, Plate 5, Well 5D
T7 guide RNA Insertion 2 Primer: 5'-ATTACAGAAGACTACTAGCACTGAGCGTCAGACCCCG-3'
VF2: 5'-TGCCACCTGACGTCTAAGAA -3'
VR: 5'-ATTACCGCCTTTGAGTGAGC -3'
BioBrick rev: 5'-AATTCTGCAGCGGCCGCTACTAGTA -3'
BioBrick fwd: 5'-GATCGAATTCGCGGCCGCTTCTAGAG -3'

PCR1:
Composition:
12,5ul 2x Q5 Mastermix
1,25ul 10uM T7 guide RNA insertion 2
1,25ul 10uM VF2
1,0ul Template (300pg/ul)
0,25ul 1M Arginine HCl
8,75ul ddH2O

PCR conditions:
98°C 40s
98°C 10s-
65°C 30s 40x
72°C 60s-
72°C 150s 1

2,5ul of PCR run on 2,0% Agarose gel, 10mM LiAc Buffer, Midori Green Advane, 1V/cm.
Faint band of expected size, significant byproducts observed

PCR2:
Composition:
12,5ul 2x Q5 Mastermix
1,25ul 10uM VR
1,25ul 10uM VF2
12,5ul Template PCR1
0,25ul 1M Arginine HCl
9,75ul ddH2O

PCR conditions:
98°C 40s
98°C 10s-
65°C 30s 25x

72°C 60s-
72°C 150s

PCR competely run on 2,0% Agarose gel, 10mM LiAc Buffer, Midori Green Advane, 1V/cm.
Band of correct size extracted via paperstrip purification, used as template for third PCR.

PCR3:
Composition:
25,0ul 2x Q5 Mastermix
2,50ul 10uM VR
2,50ul 10uM VF2
5ul Template (~15ng)
0,5ul 1M Arginine HCl
14,75ul ddH2O

PCR conditions:
98°C 30s
98°C 10s-
72°C 75s- 40x
72°C 150s

PCR completely run on 2,0% Agarose gel, 10mM LiAc Buffer, Midori Green Advane, 4V/cm.
Band of correct size (~1087bp) extracted via paperstip method, elution in two steps in ~30ul Elution buffer.
Concentration determined using Nanodrop Lite as ~6ng/ul.
Frozen at -20°C until further use. Transfered to main iGEM lab.

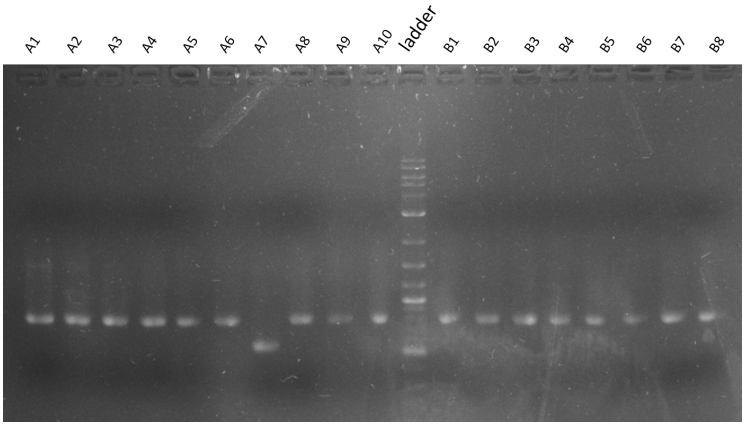
09/22/2018
Colony PCR of pet28a_LC_phluorin
Reaction: MasterMix (50 x)

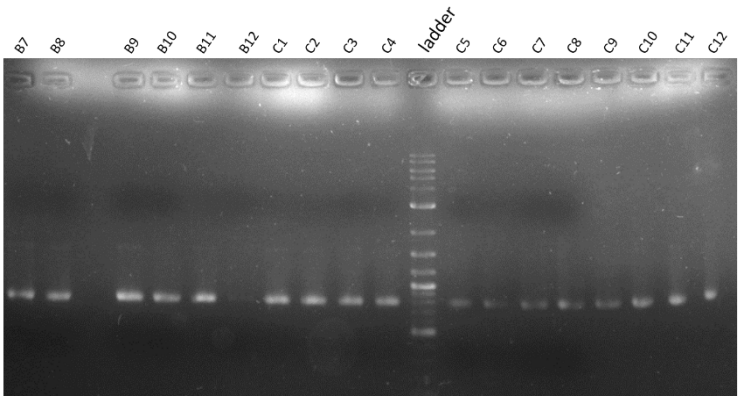
Chemical	Volume
Primer 1	5 µl
Primer 2	5 µl
Taq Polymerase	50 µl
dNTP	100 µl
Buffer	125 µl
H_2O	865 µl

PCR Programm:

Step	Temperature	Time
Initinal Denaturation	94 °C	5 min.
30 x cycles		
Denaturation	94 °C	45 sec.
Annealing	60 °C	45 sec.
Elongation	74 °C	45 sec.
Final Extension	74 °C	5 min

Agarose gel:





PCR of Hibit TaKaRa (seconde):

Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer (1:2)	0.5 µl
Primer (1:2)	0.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

Program:

Step	Temperature	Time
Initial Denaturation	95 °C	30 sec.

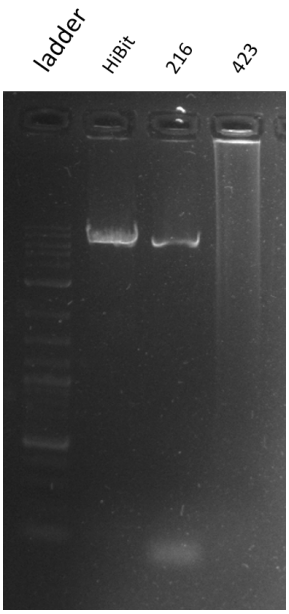
30 x cycles

Denaturation	95 °C	10 sec.
Annealing	58 °C	15 sec.
Elongation	72 °C	7 min
Final Extension	72 °C	2 min

PCR of amber 216 and 423.

Same reaction and program.

Agarose gel:



Miniprep of plasmid via precipitation
Samples: Phluorin 2
Eluted in 40 µl Tris HCl pH 8.0

1. Restriction Digest: pLACZ

1:20 dilution of plasmid: 2µl plasmid DNA + 38 µl H2O
Measurement on Nanodrop: 484 ng/µl
Restriction digest (50µl reaction)

Component	**Amount**
Plasmid DNA	2 µl
10x CutSmart Buffer	5 µl
EcoRI-Hf	1 µl
ddH20	42 µl
	50 µl total volume

Incubation: 60 min at 37°C
Gel

2. Restriction Digest of pcDNA3.1- and CreBiobrick

Insert:
- 10 µl Biobrick DNA (c=10 ng/µl) --> 100 ng
- 5 µl CutSmart Buffer
- 1 µl Xba1
- 1µl Not1-HF
- 33 µl H20

First: 45min digestion with Xba1, then 45 min digestion with added Not1, 37°C

Vector: pcDNA3.1- (c=500 ng/µl)
- 1µl DNA
- 5µl CutSmart Buffer

- 1 µl Xba1-HF
- 1 µl Not1-HF
- 41 µl H2O

1h digestion: 37°

Heat Inactivation 20 min, 80°C

3. Ligation

20µl reaction

2 µl 10X T4 DNA Ligase Buffer
3 µl Vector DNA (30 ng)
14 µl Insert DNA (28 ng)
1 µl T4 Ligase
--> Over night 10°C

09/25/2018

PCR of Cre Product

Taq/Pfu Polymerase

Component	ul	final concentration
10X Standard Taq Reaction Buffer	5	1x
10 mM dNTPs	4	200 µM
Primer f/r	4	0.2 µM
Template DNA	1	200 ug
Taq/Pfu Polymerase	2	1.25 units/50 µl PCR
Nuclease-free water	34	

PCR Program

Step	Temp (°C)	Time (min)	Number of Cycles
Initial Denaturation	94	1-2	1
Denaturation	94	0.5-1	
Annealing	60	0.5	25-35
Extension	72	2	
Final Extension	72	5	1
Hold	4	-	1

1% Agarose Gel for control of the PCR, PegGreen
120 V, 1h

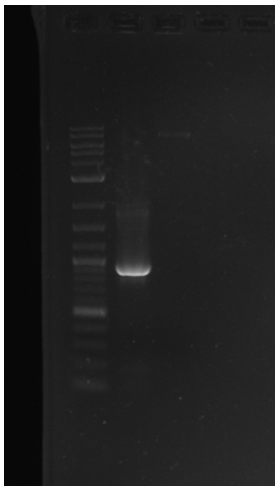
1 ul Loading Dye + 4 ul DNA

Nanodrop Measurement
--> 304,7 ng/ul
260/280: 1.56
260/230: 0.98

Pocket	Construct

1	1 kbMarker
2	PCR of Cre Biobrick
3	Restriction Digest pLacZ
4	

Gel:



Discussion

1. lane: 1 kb Marker
2. lane: Cre-Biobrick PCR Product: is 900 kb, should be at 1000?
3. Lane: pLacZ digested with EcoR1, should have 2 bands??

2. Transformation of Ligation mixture from 9/24
50 ul XL1-Blue transformed with 1 ul of Ligation Mix
2h incubation at 37°C
plated on Carb Plates

09/24/2018

DPn1-digest: Samples: PCR product Hibit (TaKaRa) and amb216

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

Reaction for 1 h, 37 °C
PCR of HC
Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.

25 x cycles

Denaturation	98 °C	10 sec.
Annealing	64 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

NanoDrop measuring: Sample: Miniprep pet28a_LC_phluorin

Sample	ng/µl
A2	12
B9	64

09/25/2018

Gibson Assembly:

Reaction HiBit (pet28a_LC_hibit)

Chemical	Volume
Insert (phluorin)	6 µl
NEBuilder	10 µl
H_20	4 µl

Program: 50 °C for 60 min.

Transformation in E.Coli XL-1 blue with 2 µl or 3 µl Gibson Assembly product. After 1 h incubation, the transformed E.Coli were platted on LB-KAN plates.

PCR of pet28a_LC_Phluorin

Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer 8 (1:2)	0.5 µl
Primer 13 (1:2)	0.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

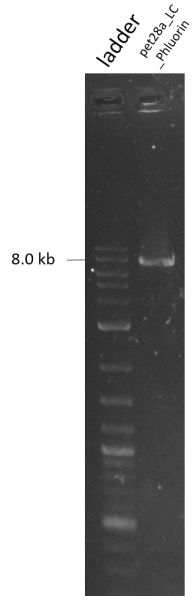
Program:

Step	Temperature	Time
Initial Denaturation	95 °C	30 sec.

30 x cycles

Denaturation	95 °C	10 sec.
Annealing	61 °C	15 sec.
Elongation	72 °C	7 min
Final Extension	72 °C	2 min

Agarose Gel:



09/26/2018

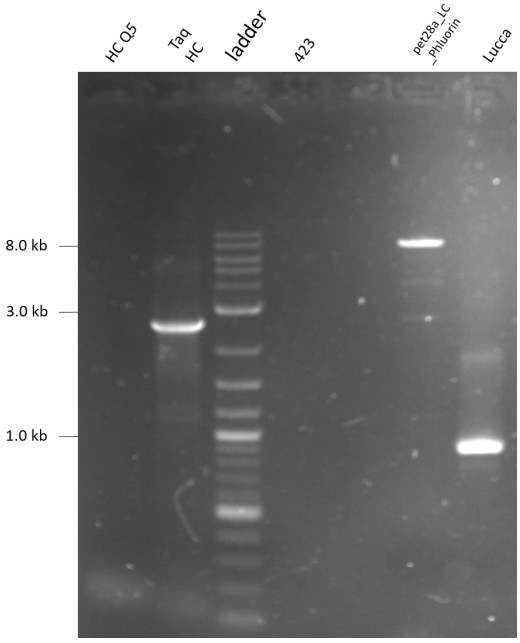
DPn1-digest: Samples: PCR amber 423 and PCR pet28a_LC_Phluorin (TaKaRa)

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

Reaction for 1 h, 37 °C

Purification via column -> elution volume 50 µl

Agarose Gel:



Overnight culture. 5 ml LB+ 5 µl + Klon 1

5 ml LB+ 5 µl + Klon 2

PCR of HC

Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer 9 (1:2)	2.5 µl
Primer 10 (1:2)	2.5 µl

TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

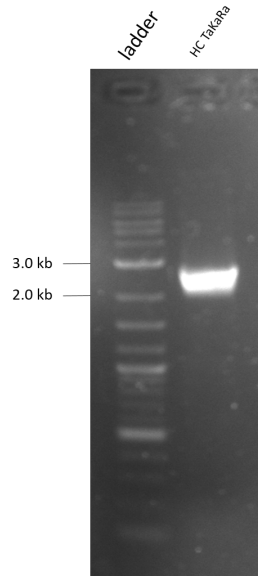
Program:

Step	Temperature	Time
Initial Denaturation	95 °C	30 sec.

25 x cycles

Denaturation	95 °C	10 sec.
Annealing	68 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

Agarose Gel:



Cre Cloning

looks like no clones on Cre + pcDNA3.1-
-> Control Gel

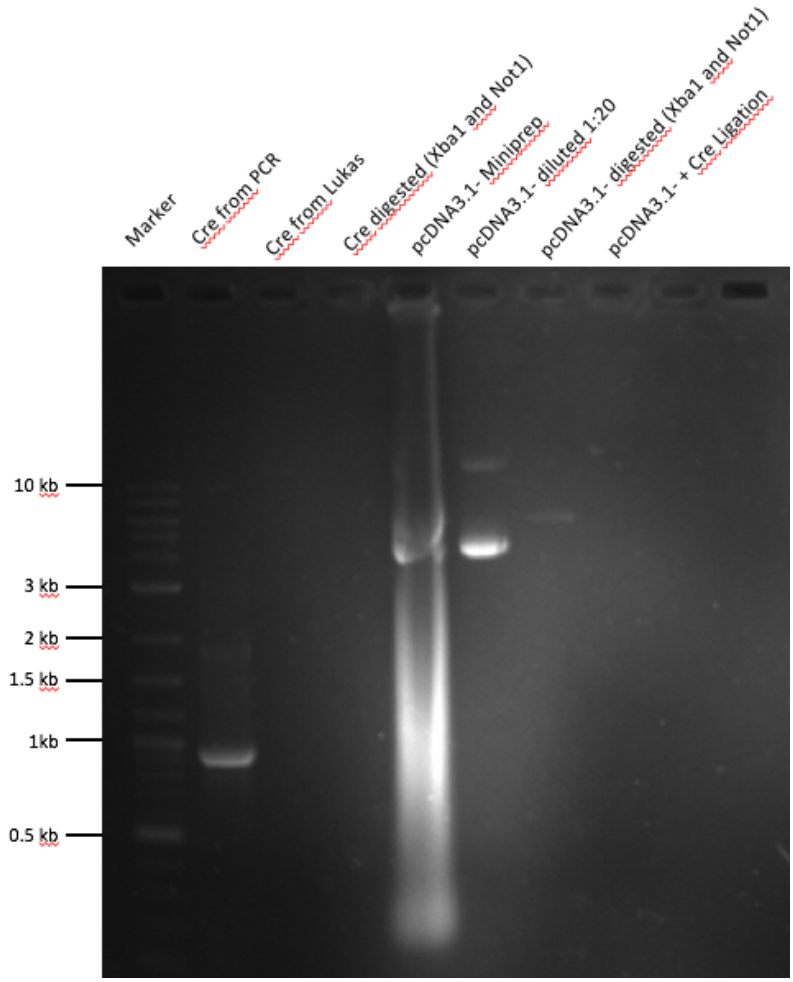
Control Gel

1% Agarose Gel + 100 ul Peggreen

Lane	Construct
1	1 kb Marker
2	Cre from PCR
3	Cre from Lukas Fuhs
4	Cre digested with XBA1 and Not 1
5	pcDNA3.1 Miniprep
6	pcDNA 3.1- 1:20 diluted
7	pcDNA 3.1- digested with XBa1 and Not 1
8	Ligation pcDNA3.1- and Cre

1h at 140 V

Gel:



2. Purification of Cre PCR Product
Using Quiagen PCR
Eluted in 40 ul Elution Buffer

3. Nanodrop Measurement of Cre PCR Product
--> 41,3 ng/ul
260/280: 1,82
260/230: 2,52

4. Sending to Microsynth for Sequencing

needed: 1.5 ng/100 bp
Cre: appr. 1000 bp --> at least 15 ng in 12 ul total needed
250 ng /12

12 ul reaction:

6 ul PCR Product
3 ul Biobric fwd Primer

3 ul H20

No. 4456919

5. Over Night culture: Cre 2015

5ml LB + One clone from Transformed Yeast Cre

09/27/2018

DPn1-digest: Samples: PCR amber 423 and PCR pet28a_LC_Phluorin (TaKaRa)

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

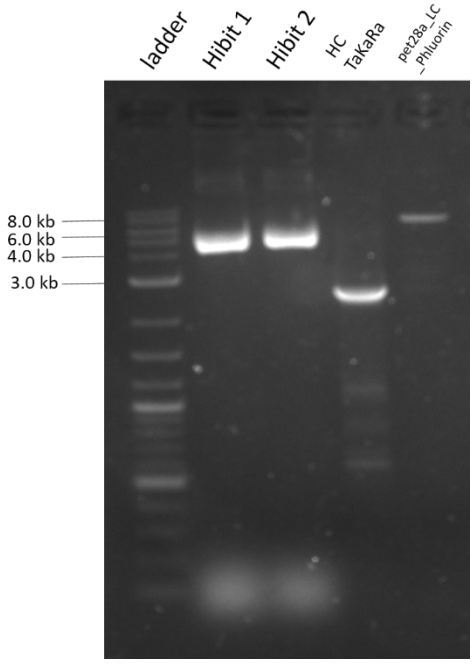
Reaction for 1 h, 37 °C
Purification via column -> elution volume 50 µl
Miniprep of plasmid via precipitation
Samples: Hibit Klone 1 & 2
Eluted in 40 µl Tris HCl pH 8.0

Miniprep of 2015 Cre

--> refer to protocol

09/28/2018

Agarose gel:



PCR of pet28_LC_hibit TaRaKa-Polymerase
Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer 8 (1:2)	2.5 µl

Primer amp. (1:2)	2.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

Program:

Step	Temperature	Time
Initial Denaturation	95 °C	30 sec.

25 x cycles

Denaturation	95 °C	10 sec.
Annealing	68 °C	15 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

PCR of Lc mut

Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

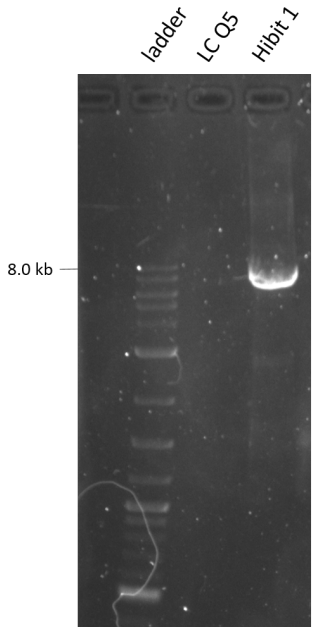
Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.

25 x cycles

Denaturation	98 °C	10 sec.
Annealing	64 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

Agarose Gel:



Cre Cloning

no sequencing result :(

Repetition Cre 2013 PCR

this time with own Biobrick Primers to see if the even bind (since sequencing with these Primers did not work)

Component	ul	final concentration	Master Mix (7 more reactions)
10X Standard Taq Reaction Buffer	5	1x	35
10 mM dNTPs	4		28
Primer f (10mM)	2	0.2 µM	14
Primer r	2	0.2 µM	14
Template DNA	1		7
Taq/Pfu Polymease	2		14
Nuclease-free water	34		238

PCR Program

Step	Temp (°C)	Time (min)	Number of Cycles
Initial Denaturation	94	1-2	1
Denaturation	94	0.5-1	
Annealing	different!	0.5	25-35
Extension	72	2	
Final Extension	72	5	1
Hold	4	-	1

A	54°C
B	
C	
D	
E	
F	
G	
H	58

2. Repetition XBa1 and Not1 Restriction Digest

Insert:
- 5 µl Biobrick DNA (c=41 ng/µl) --> 205 ng/ul
- 5 µl CutSmart Buffer
- 1 µl Xba1

- 1µl Not1-HF
- 38 µl H20

First: 1h digestion with Xba1, then 1h digestion with added Not1, 37°C

Vector: pcDNA3.1- (c=200 ng/µl) (1:10 diltuted??) --> NanoDrop again!
- 1 µl DNA
- 5 µl CutSmart Buffer
- 1 µl Xba1-HF
- 1 µl Not1-HF
- 41 µl H2O

1h with Xba1 then 1h digstion with Not1 added, digestion: 37°

Heat Inactivation 20 min, 80°C

3. Repetition Ligation: Cre2013 and pcDNA3.1-

Biobrick Length: 900-1000 bp
Vector Length: 5.4k bp

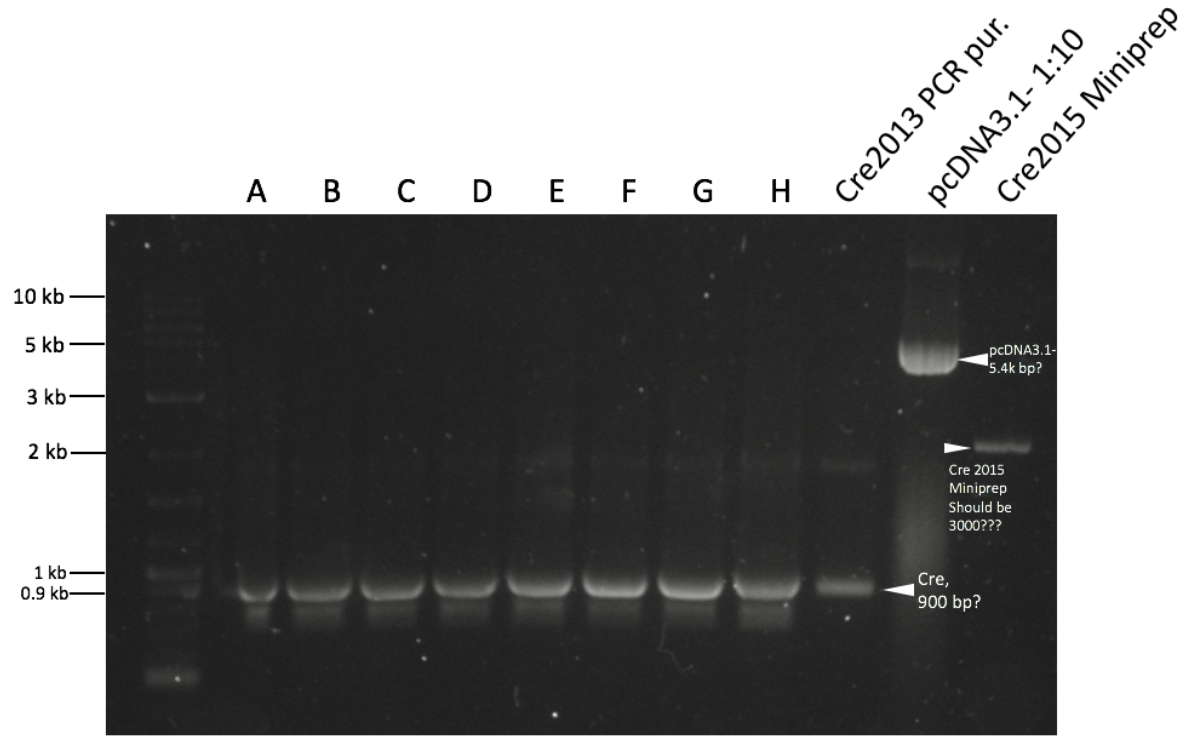
20µl reaction

2 µl 10X T4 DNA Ligase Buffer
8 µl Vector DNA (32 ng)
8 µl Insert DNA (32 ng)
1 µl T4 Ligase
1 ul H20
--> Over night 10°C

4. Gel of Gradient PCR

1h. 140 V

1	2	3	4	5	6	7	8	9	10	11	12
Ladder	A	B	C	D	E	F	G	H	cre PCR purified	pcDNA3.1- 1:10	Mini Prep Cre2015 1:10



Discussion

Cre
iGEM Biobrick Primers from 2017 work
best annealing temp: 58 degrees
still 100 bp to short -> Why?
Sequencing needs to work! --> one more try, perhaps error last time

pcDNA3.1-
pcDNA3.1- seems to be to short --> digest with single cut enzyme and try again

Cre2015
seems to be 1000 bp to short --> digest with single cut enzyme and try again

5. Over night Culture of one more Cre 2015 clone
(in case vector is empty)
5ml LB + CM + clone, 37 degrees over night

09/29/2018
Plasmid purification via gel purification.
Sample: HC TaKaRa

<u>Cre Cloning</u>

1. Transformation of Ligation Mix from 9/28

50 ul XL1Blue + 2 ul (6ng) Ligation Mix
streaking out on CM Plates
incubation 37 degrees over night

2. Digestion of pcDNA3.1-

Vector: pcDNA3.1- (c=200 ng/µl) (1:10 diltuted??) --> NanoDrop again!
- 2 µl DNA
- 5 µl CutSmart Buffer
- 1 µl EcoR1
- 42 µl H2O

3. Digestion of Cre2015

Vector: psB1C2 (c=?)
- 2 µl DNA
- 5 µl CutSmart Buffer
- 1 µl EcoR1
- 42 µl H2O

4. Gel

digested Cre2015 (EcoR1)
digested pcDNA3.1- (EcoR1)
digested Cre2013 (Xba1 and Not1)
digested pcDNA3.1- (Xba1 and Not1)
Ligation Mix (pcDNA3.1- + Cre2013) from 9/28

1h, 140 V

09/30/2018

PCR of LC mut with TaRaKa-Polymerase- The Template is purified pet28_LC_mut Template (Sample A8).
Reaction:

Chemical	Volume
Template (A8)	1 µl
Primer 8 (1:2)	2.5 µl
Primer amp. (1:2)	2.5 µl
TaKaRa	0.5 µl
dNTP	4 µl
Buffer	25 µl
H_2O	18.5 µl

Program:

Step	Temperature	Time
Initial Denaturation	95 °C	30 sec.
25 x cycles		
Denaturation	95 °C	10 sec.
Annealing	68 °C	20 sec.
Elongation	72 °C	2 min 15 sec.
Final Extension	72 °C	2 min

Agarose Gel:

Gibson Assembly:

Reaction 1. Vector: pet28a_LC_mut_Hibit Insert: HC

2. Vector: pet28a_LC_mut_phluorin2 Insert: HC

Chemical	Volume
Vector (x)	3 µl
Insert (HC	4 µl
NEBuilder	10 µl
H_20	3 µl

Program: 50 °C for 60 min.

E.Coli XL-1 blue were transformed with 2 µl or 3 µl Gibson Assembly product. After 1 h incubation, the transformed E.Coli were platted on LB-KAN plates:

Scheme:

Gibson Assembly Product	Volume of Transformation	Volume for Plate
pet28a_HC_LC_mut_Hibit	2 µl	200 µl
pet28a_HC_LC_mut_Hibit	3 µl	200 µl
pet28a_HC_LC_mut_Hibit	2 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_Hibit	3 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl

PCR

PCR of LC mut with Q_5-Polymerase- The Template is purified pet28_LC_mut Template (Sample A8).

Reaction:

Chemical	Volume
Template	1 µl
Primer (1:10)	2,5 µl
Primer (1:10)	2,5 µl
Q_5 Polymerase	25 µl
H_2O	19 µl

Program:

Step	Temperature	Time
Initinal Denaturation	98 °C	30 sec.
25 x cycles		
Denaturation	98 °C	10 sec.
Annealing	60 °C	20 sec.
Elongation	72 °C	45 sec.
Final Extension	72 °C	2 min

Cre Cloning

Colony PCR of Cre Cultures

Component	25 ul reaction	Final Concentration	Master Mix (8x)
10x Standard Taq Reaction buffer	2.5 ul	1x	20
10 mM dNTPs	0.5 ul	200 uM	4
10 uM Forwar Primer (VF)	0.5 ul	0.2 uM	4
10 uM Reverse Primer (VR)	0.5 ul	0.2 uM	4
Template	colony		
Taq DNA Pol	0.5 ul		4
H2O	20,5		164

picked 7 colonies
colony PCR and over night culture

PCR Program

Step	Temp	t
Initial Denaturation	95	5'
Denaturation	95	45''
Annealing	54	45''
Extension	72	45''
		x30
Final Extension	72	10'
Hold	4	-

10/01/2018
Clones of the plates from yesterday.
Colony PCR:
Samples:

Gibson Assembly Product	Volume of Transformation	Volume for Plate	ShortCut
pet28a_HC_LC_mut_Hibit	2 µl	200 µl	H 2 200
pet28a_HC_LC_mut_Hibit	3 µl	200 µl	H 3 200
pet28a_HC_LC_mut_Hibit	2 µl	After centrifugation: 100 µl	H 2 z
pet28a_HC_LC_mut_Hibit	3 µl	After centrifugation: 100 µl	H 3 z
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl	P 2 200
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl	P 3 200
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl	P 2 z
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl	P 3 z

Reaction: MasterMix (50 x)

Chemical	Volume
Primer 1	5 µl
Primer 2	5 µl
Taq Polymerase	50 µl
dNTP	100 µl

Buffer	125 µl
H_2O	865 µl

PCR Programm:

Step	Temperature	Time
Initinal Denaturation	94 °C	5 min.

25 x cycles

Denaturation	94 °C	45 sec.
Annealing	60 °C	45 sec.
Elongation	74 °C	45 sec.
Final Extension	74 °C	5 min

	1	2	3	4	5	6	7	8	9	10	11	12
A	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200
B	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200
C	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z
D	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z
E	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200
F	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200
G	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	-	-	-	-	-
H	P 3 z	+	-	-	-	-	-	-	-	-	-	-

+ = positive control

Second picking of clones.

Same Samples:

Gibson Assembly Product	Volume of Transformation	Volume for Plate	ShortCut
pet28a_HC_LC_mut_Hibit	2 µl	200 µl	H 2 200
pet28a_HC_LC_mut_Hibit	3 µl	200 µl	H 3 200
pet28a_HC_LC_mut_Hibit	2 µl	After centrifugation: 100 µl	H 2 z
pet28a_HC_LC_mut_Hibit	3 µl	After centrifugation: 100 µl	H 3 z
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl	P 2 200
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl	P 3 200
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl	P 2 z
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl	P 3 z

Same PCR Program as the first colon PCR (1/10). But the elongation step was expended to 1 minute and 45 seconds.
PCR Programm:

Step	Temperature	Time
Initinal Denaturation	94 °C	5 min.

25 x cycles

Denaturation	94 °C	45 sec.
Annealing	60 °C	45 sec.
Elongation	74 °C	1 min. 45 sec.
Final Extension	74 °C	5 min

	1	2	3	4	5	6	7	8	9	10	11	12
A	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200
B	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200
C	H 3 200	H 3 200	H 3 200	H 3 200	P 2 200	P 2 200	P 2 200	P 3 200	P 3 200	P 3 200	P 3 200	P 3 200
D	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z
E	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z
F	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z
G	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z
H	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z

PCR did not work --> wrong primers were used :(

Miniprep of 7 colonies
Nanodrop

A) 1126 ng/ul
B) 1401 ng/ul
C) 3847,3 ng/ul
D) 1471 ng/ul
E) 2862 ng/ul
F) 751 ng/ul
G) 1029 ng/ul

3. Restriction Digest

A: 1 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1, 24 ul H2O
B: 1 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1. 24 ul H2O
C: 0.5 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1, 24,5 ul H2O
D: 1 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1, 24 ul H2O
E: 0.5 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1, 24,5 ul H2o
F: 1,5 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1 23,5 ul H20
G: 1 ul DNA, 3 ul Buffer, 1 ul Xba1, 1 ul EcoR1, 24 ul H2O

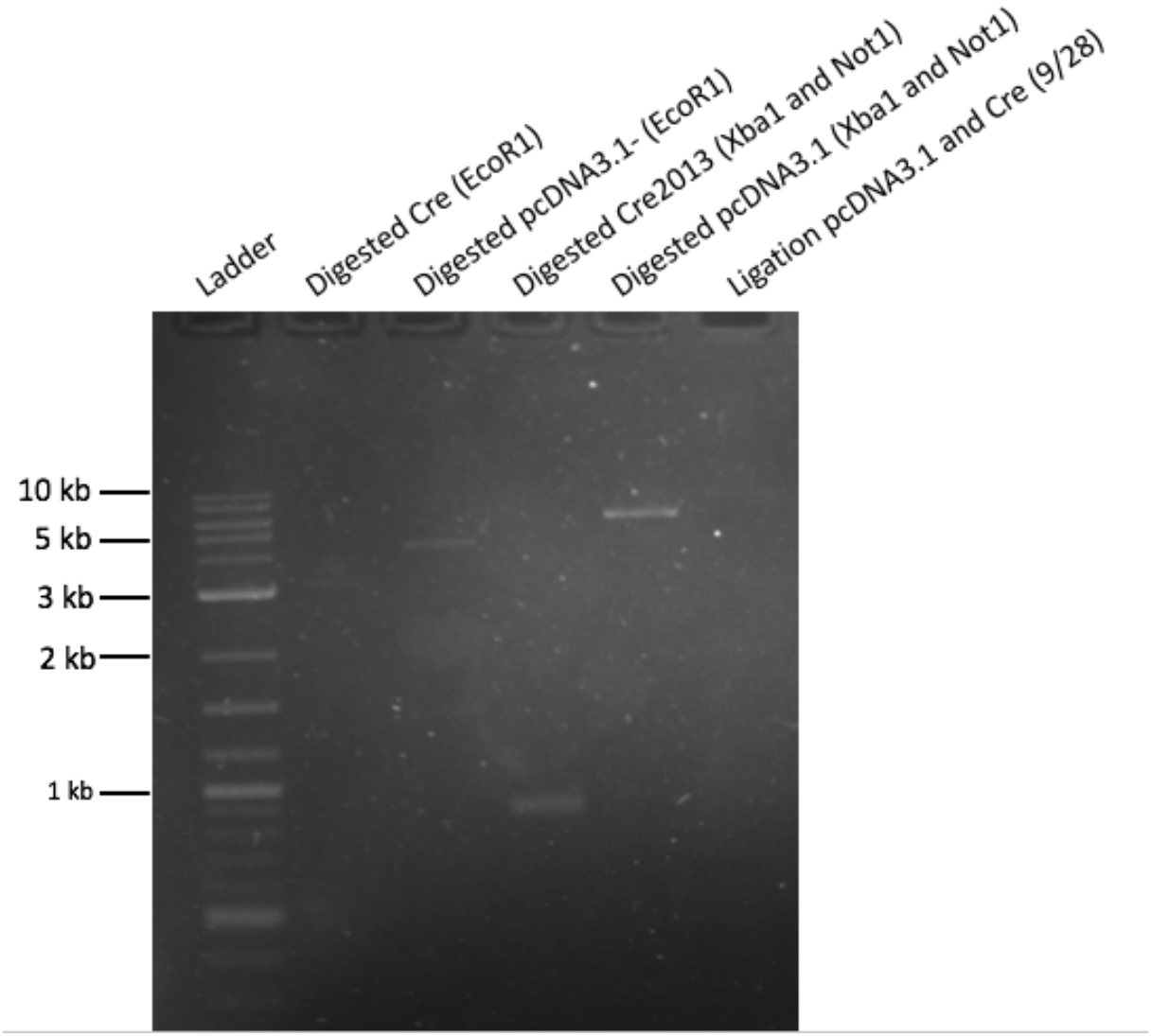
4.Sequencing of Cre Biobrick

12 ul reaction:

5 ul PCR Product
3 ul Biobrick fwd Primer
4 ul H20

5. Gel

Cre_pcDNA3.1-_Digest.png



10/02/2018

DPn1- Digest: Template LC mut after Q5 amplification (01/10)

Reaction:

Chemical	Volume
Sample	45 µl
Buffer	6 µl
Enzyme (DPn1)	1 µl
H_20	8 µl

Program: 37 °C for 2 h

After Digest: Purification via column. Eluted in 50 µl

Gibson Assembly:

Reaction	1. Vector: pet28a_LC_mut_Hibit	Insert: HC
	2. Vector: pet28a_LC_mut_phluorin2	Insert: HC
	3. Vector: pet28	Insert: HC + LC + Ommomyc

Chemical	Reaction1 Volume	Reaction 2 Volume	Reaction 3 Volume
Vector (x)	2 µl	2 µl	1 µl
Insert (each)	5 µl	5 µl	4 µl (for HC 1 µl (for Omo) 1 µl (for LC)
NEBuilder	10 µl	10 µl	10 µl
H_20	3 µl	3 µl	3 µl

Program: 50 °C for 60 min.

E.Coli XL-1 blue were transformed with 2 µl or 3 µl Gibson Assembly product. After 1 h incubation, the transformed E.Coli were platted on LB-KAN plates:

Scheme:

Gibson Assembly Product	Volume of Transformation	Volume for Plate
pet28a_HC_LC_mut_Hibit	2 µl	200 µl
pet28a_HC_LC_mut_Hibit	3 µl	200 µl
pet28a_HC_LC_mut_Hibit	2 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_Hibit	3 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_ommomyc	2 µl	200 µl
pet28a_HC_LC_mut_ommomyc	3 µl	200 µl
pet28a_HC_LC_mut_ommomyc	2 µl	After centrifugation: 100 µl
pet28a_HC_LC_mut_ommomyc	3 µl	After centrifugation: 100 µl

10/03/2018

Colony PCR:

Samples:

Gibson Assembly Product	Volume of Transformation	Volume for Plate	ShortCut
pet28a_HC_LC_mut_Hibit	2 µl	200 µl	H 2 200
pet28a_HC_LC_mut_Hibit	3 µl	200 µl	H 3 200
pet28a_HC_LC_mut_Hibit	2 µl	After centrifugation: 100 µl	H 2 z
pet28a_HC_LC_mut_Hibit	3 µl	After centrifugation: 100 µl	H 3 z
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl	P 2 200
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl	P 3 200
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl	P 2 z
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl	P 3 z
pet28a_HC_LC_mut_ommomyc	2 µl	200 µl	O 2 200
pet28a_HC_LC_mut_ommomyc	3 µl	200 µl	O 3 200
pet28a_HC_LC_mut_ommomyc	2 µl	After centrifugation: 100 µl	O 2 z.
pet28a_HC_LC_mut_ommomyc	3 µl	After centrifugation: 100 µl	O 3 z.

Reaction: MasterMix (50 x)

Chemical	Volume
Primer 1	5 µl
Primer 2	5 µl
Taq Polymerase	50 µl
dNTP	100 µl
Buffer	125 µl
H_2O	865 µl

PCR Programm:

Step	Temperature	Time
Initinal Denaturation	94 °C	5 min.

25 x cycles

Denaturation	94 °C	45 sec.
Annealing	60 °C	45 sec.
Elongation	74 °C	1 min. 45 sec.
Final Extension	74 °C	5 min

First 96er well: only Hibit

	1	2	3	4	5	6	7	8	9	10	11	12
A	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200
B	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200	H 2 200
C	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200
D	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200	H 3 200
E	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z
F	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z	H 2 z
G	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z
H	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z	H 3 z

Second 96er well

	1	2	3	4	5	6	7	8	9	10	11	12
A	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.
B	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.
C	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 3 200	O 3 200	O 3 200	x	x
D	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 3 200	P 3 200	P 3 200	P 3 200	x	x	x
E	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z
F	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z
G	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z	P 2 z
H	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z	P 3 z

Cre Cloning

Discussion Gel

First band: at 4000-5000 --> undigested Vector? or single cut vector?
- but too short

Second Band: at 1500 -->

Third Band: 900 bp --> Cre?

10/03/2018

Transformation of Cre-pcDNA3.1-

10/04/2018

Miniprep of plasmid via precipitation

Samples:
1. pet28a-HC-LCmut-Ommomyc: clone A8
2. pet28a-HC-LCmut-Hibit: clone A1
3. pet28a-HC-LCmut-Hibit: clone A8
4. pet28a-HC-LCmut-Hibit: clone A10

Eluted in 40 µl Tris HCl pH 8.0

Purification of colony PCR product via column for sequencing

Samples:
1. pet28a-HC-LCmut-Ommomyc: clone A8
2. pet28a-HC-LCmut-Hibit: clone A8

Eluted in 30 µl Elution Buffer.

Sequencing:

Restriction digest of plasmid.

Samples:
1. pet28a-HC-LCmut-Ommomyc: clone A8
2. pet28a-HC-LCmut-Hibit: clone A1
3. pet28a-HC-LCmut-Hibit: clone A8
4. pet28a-HC-LCmut-Hibit:: clone A10

Reaction:

Chemical	Volume
Sample	1 µl
Buffer	2.5 µl
Enzyme (EcoRV HF)	0.5 µl
Enzyme (Spe I HF)	0.5 µl
H_20	20.5 µl

Reaction: 37 °C for 20 min.

Control via Agrose gel: doesn't work. So we did the digest again.

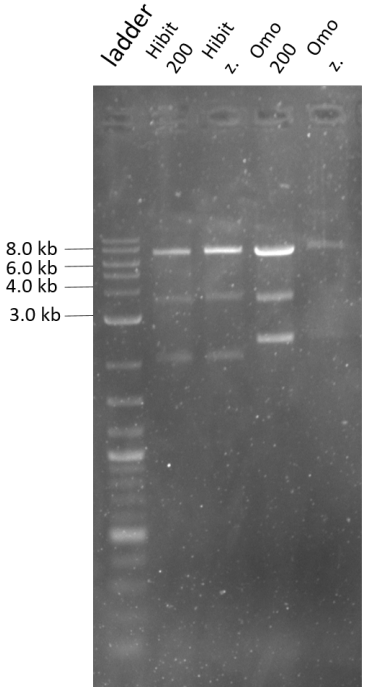
Same sample but another reaction.

Reaction:

Chemical	Volume
Sample	2 µl
Buffer	2.5 µl
Enzyme (EcoRV HF)	0.5 µl
Enzyme (Spe I HF)	0.5 µl
H_20	19.5 µl

Reaction: 37 °C for 30 min.

Agarose gel:



New glycerol stock of XL-1 blue cells.

Cre Cloning

7 clones → Picked and over night culture

10/05/2018

Transformation of LEMO cells
Samples: 1. pet28a-HC-LCmut-Ommomyc: clone A8
2. pet28a-HC-LCmut-Hibit: clone A8
Transformation with 3 µl. The electroporation was "not working". Nevertheless, the cells were platted on LB-KAN plates.
Miniprep of plasmid via precipitation
Samples: 1. pet28a-phluorin2 out of LEMO cells
Eluted in 40 µl Tris HCl pH 8.0

10/06/2018
Colony-PCR
Sample: pet28a_HC_LC_mut_Phluorin2 (XL-1 blue)
Reaction: 1 clone :(

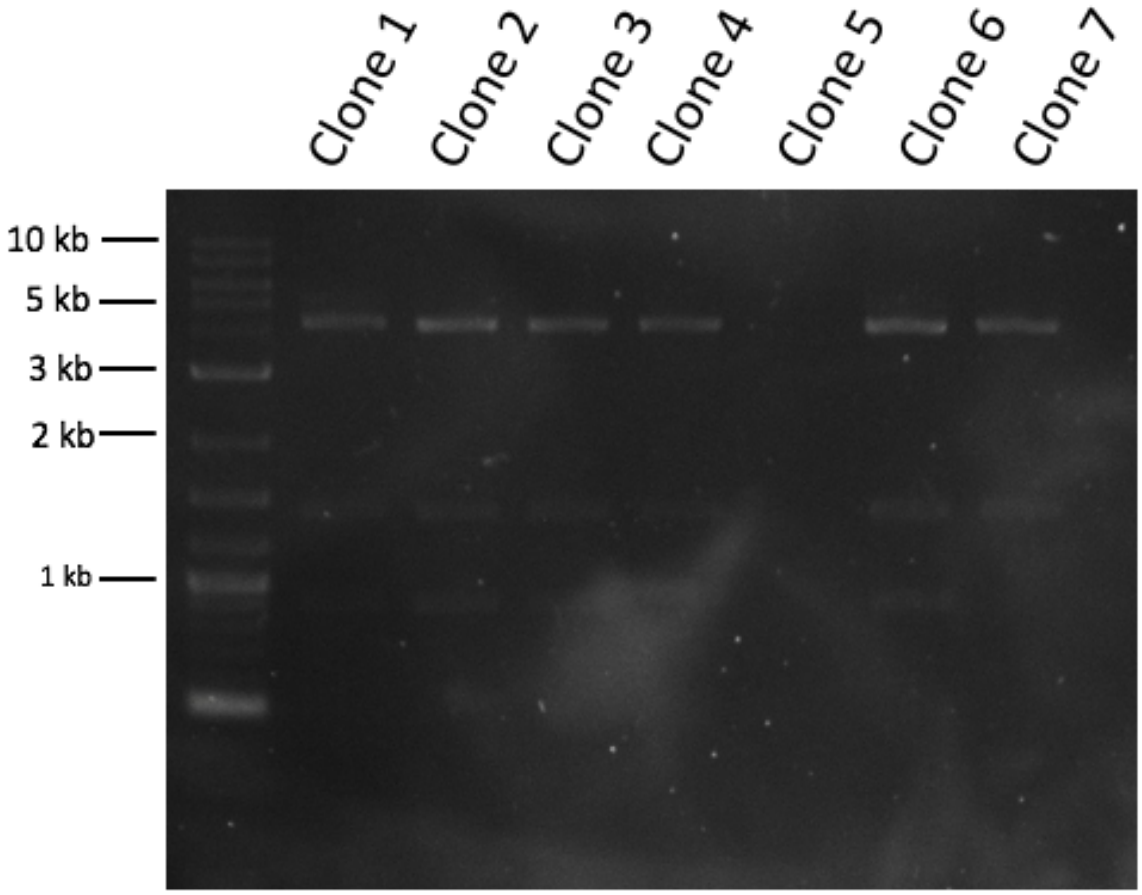
Chemical	Volume
Template	2 µl
Primer 14 (1:10)	1 µl
Primer 15 (1:10)	1 µl
Taq Polymerase	1 µl
dNTP	2 µl
Buffer	2.5 µl
H_2O	18.5 µl

Agarose Gel (1%):
Loading: Ladder clone positive controlle
Was negative

New transformation of pet28a_HC_LC_mut_ommomyc in LEMO-cells.
Transformation volume: 2 µl

Cre

Colony PCR



→ Clone 2 → was sent for sequencing, using the T7 Promotor

Results of Sequencing:
→ Cre is not a Cre, but part of a DH-7 plasmid ??
→ Cre is depraved from project

10/07/2018

Gibson Assembly: Sample: pet28a + HC + LC mut + Phluorin2
Reaction:

Chemical	Volume
Vector (pet28)	2 µl
Insert (HC)	4 µl
Insert (LC mut)	1 µl
Insert (Phluorin2)	1 µl
NEBuilder	10 µl
H_20	2 µl

Reaction at 50 °C for 1h
Transformation with 3 µl Gibson assembly product in XL-1 blue.

Overnight culture for MiniPrep
LEMO cells with pet28a_HC_LC_mut_omomyc
pet28a_HC_LC_mut_Hibit
with LB + KAN

10/08/2018
Miniprep of plasmid via precipitation
Samples: 1. pet28a_HC_LC_mut_omomyc
2. pet28a_HC_LC_mut_Hibit
Eluted in 40 µl Tris HCl pH 8.0

NanoDrop measuring:

Sample	ng/µl	260/280	260/230
Hibit	425.8	2.09	1.76
Omomyc	848.7	2.10	1.83

Sequencing

Eurofins:	ALF0030081	Omomyc	T7 Promotor
	ALF0030080	Omomyc	T7 Terminator
	ALF0030079	Hibit	T7 Promotor
	ALF0030078	Hibit	T7 Terminator
Microsnyth:	4456935	Hibit	Primer 8
	4456936	Hibit	Primer 14
	4456937	Hibit	Primer 15
	4456938	Omomyc	Primer 8
	4456939	Omomyc	Primer 14
	4456940	Omomyc	Primer 15

New Transformation of the “old” gibson assemply.
Gibson Assembly from 10/07 and 10/02. Sample: pet28a_HC_LC_mut with Phlourin2
Transforamtion with XL-1 blue and 2 µl Gibson reaction

10/09/2018
Gibson Assembly:
Reaction 1. Vector: pet28a_LC_mut_phluorin2 Insert: HC
2. Vector: pet28 Insert: HC + LC + Phluorin2

Chemical	Reaction1 Volume	Reaction 2 Volume
Vector (x)	3 µl	3 µl
Insert	3 µl	2.5 µl (HC) 2.5 µl (LC) 2.5 µl (phlu)
NEBuilder	10 µl	10 µl
H_20	4 µl	2.5 µl

Program: 50 °C for 60 min.

E.Coli XL-1 blue were transformed with 2 µl Gibson Assembly product. After 2 h incubation, the transformed E.Coli were platted on LB-KAN plates:

Transformation from yesterday were negative.

10/10/2018

Colony PCR:

Samples:

Gibson Assembly Product	Volume of Transformation	Volume for Plate	ShortCut
pet28a_HC_LC_mut_phluorin2	2 µl	200 µl	P 2 200
pet28a_HC_LC_mut_phluorin2	3 µl	200 µl	P 3 200
pet28a_HC_LC_mut_phluorin2	2 µl	After centrifugation: 100 µl	P 2 z
pet28a_HC_LC_mut_phluorin2	3 µl	After centrifugation: 100 µl	P 3 z

Reaction: MasterMix (50 x)

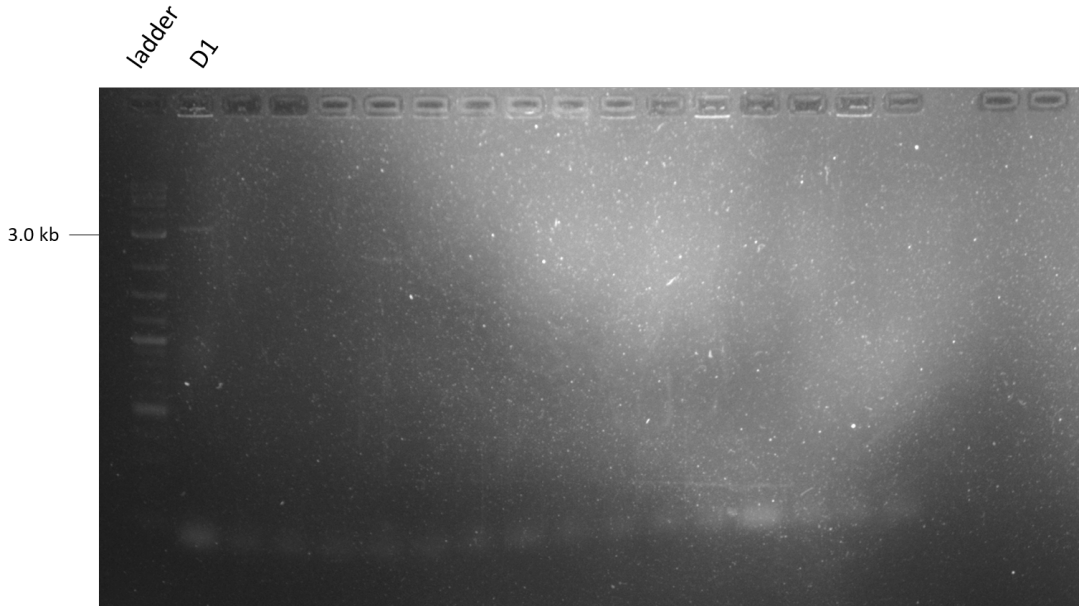
Chemical	Volume
Primer 1	5 µl
Primer 2	5 µl
Taq Polymerase	50 µl
dNTP	100 µl
Buffer	125 µl
H_2O	865 µl

PCR Programm:

Step	Temperature	Time
Initinal Denaturation	94 °C	5 min.
25 x cycles		
Denaturation	94 °C	45 sec.
Annealing	60 °C	45 sec.
Elongation	74 °C	45 sec.
Final Extension	74 °C	5 min

	1	2	3	4	5	6	7	8	9	10	11	12
A	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 200	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.
B	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.
C	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 2 z.	O 3 200	O 3 200	O 3 200	x	x
D	P 2 200	P 2 200	P 2 200	P 2 200	P 2 200	P 3 200	P 3 200	P 3 200	P 3 200	x	x	x
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Agarose Gel:



10/11/2018

NanoDrop measuring:

Sample	ng/µl	260/280	260/230
Hibit	5179.5	1.55	1.55
Omomyc	5166.4	1.51	1.50

Purification via column

After:

NanoDrop measuring:

Sample	ng/µl	260/280	260/230
Hibit	107.8	2.01	2.45
Omomyc	111.4	2.05	2.39

10/12/2018

NanoDrop measuring:

Sample	ng/µl	260/280	260/230
Phluorin	4961.9	1.80	1.87
pet28_LC_HC_Phluorin	4985.6	1.73	1.79

10/13/2018

Overnight Culture of LEMO cells:

Samples: 1. pet28a_HC_LC_mut_omomyc
2. pet28a_HC_LC_mut_Hibit

For Protein Purification