Gas vesicle all lab work

MÅNDAG, 2018-06-18

Only Our construct Gas Vesicle 2 has arrived so start with only this one

Dilute gblocks and primers

Phusion PCR

Mixing according to protocol, PCR at 64,8

Result:

Nanodrop DNA concentration measurement : 123,3 ng/µL

Nucleic Acid Gel Electrophoresis

Gel preparation:

- 30mL 1% agarose gel
- 1µL gel red

Gel loading:

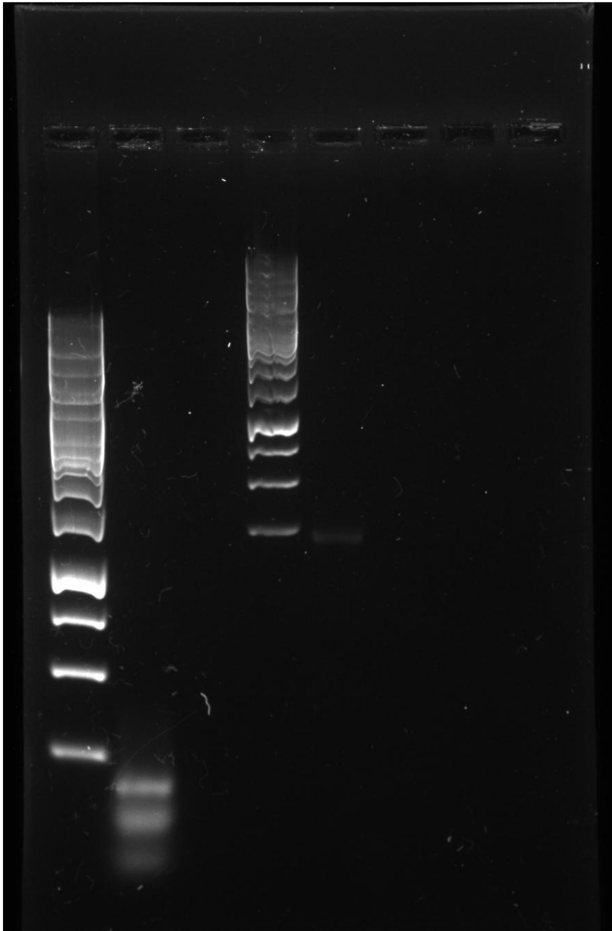
1. ladder 1kb
2. sample 1:
    - 1µL dye
    - 0,5µL DNA amplicon
    - 4,5µL MiliQ

Gel run: 95V for 45 min

First try (lane 1 and 2): no result

Second try (lane 4 and 5): still no result

GelDoc-PC_2018-06-18_17h01m42s.jpg



Result: failed PCR
Cause: elongation time per cycle was set to 0:30 while a fragment of roughly 2kb requires 2 minutes

ONSDAG, 2018-06-20

Digest plasmid (remove MCS)
Cut p413TEF with XbaI and XhoI

- 14µL Nuclease free water
- 2µL 10x fast digest buffer
- 2µL DNA
- 1µL XbaI
- 1µL XhoI

37ºc for 5 minutes, no inactivation afterwards

Purify plasmid
According to protocol
Nanodrop: 16,6 ng/µL

Gradient PCR with Phusion and Dream-Taq

Phusion PCR preparation

Table1

	A	B	C	D	E	F
1	Phusion			Dream-Taq		
2		x1	x9		x1	x9
3	HF buffer	4	36	Dream-Taq buffer	2	18
4	dNTPs	0,4	3,6	dNTPs	0,4	3,6
5	Fw_primer	0,1	0,9	Fw_primer	0,1	0,9
6	Rv_primer	0,1	0,9	Rv_primer	0,1	0,9
7	DNA temp.	0,5	4,5	DNA temp.	0,5	4,5
8	Polymerase	0,2	1,8	Polymerase	0,1	0,9
9	Water	14,7	132,3	Water	18,2	163,8
10	Total	20	180	Total	20	180

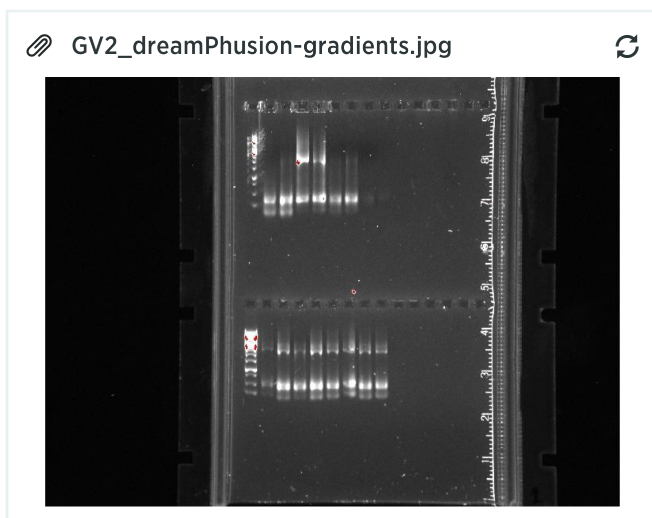
Temperature gradient Phusion and Dream-Taq PCR:

Table2

	A	B	C	D	E	F	G	H	I
1		1	2	3	4	5	6	7	8
2	Dream-Taq	60,0	59,4	58,3	56,3	53,9	52,0	50,7	50,0
3	Phusion	70,0	69,5	68,4	66,4	64,0	62,0	60,7	60,0

Result: top: dream-tag, bottom Phusion, 1kb ladder to the left and decreasing gradient from left to right

GV2_dreamPhusion-gradients.jpg



All Phusion PCRs worked and only 58,3 and 56,3 worked for Dream-Taq

Purify PCR amplicon

To maximise concentrations, all 8 Phusion PCR's are collected in 2 tubes: 1-4 in tube 1 and 5-8 in tube 2
Purification according to protocol
nanodrop tube 1: 230,0 ng/µL
nanodrop tube 2: 242,3 ng/µL

Gibson assembly
P413TEF: 16,6 ng/μL
Gv2_1: 230 ng/μL

Table3

	A	B
1		Volume
2	p413TEF	5μL
3	Gv2_1	1μL
4	Gibson assembly master mix	10μL
5	MiliQ	4μL
6	Total	20μL

Follow protocol

E.coli transformation and growth
Use DH5 alpha cells from -80 freezer

- thaw on ice for 30 min
- add 3μL of assembled plasmid to the cells
- heat shock in 42ºc for 45 sec
- put on ice for 20-30 min
- add LB and restore for 1 hour at 37ºc
- Streak on plates
- Grow overnight

Result: positive control had growth and negative control not.

Note: negative control was just cells without plasmid, this should have been cells that have transfected with plasmids without construct

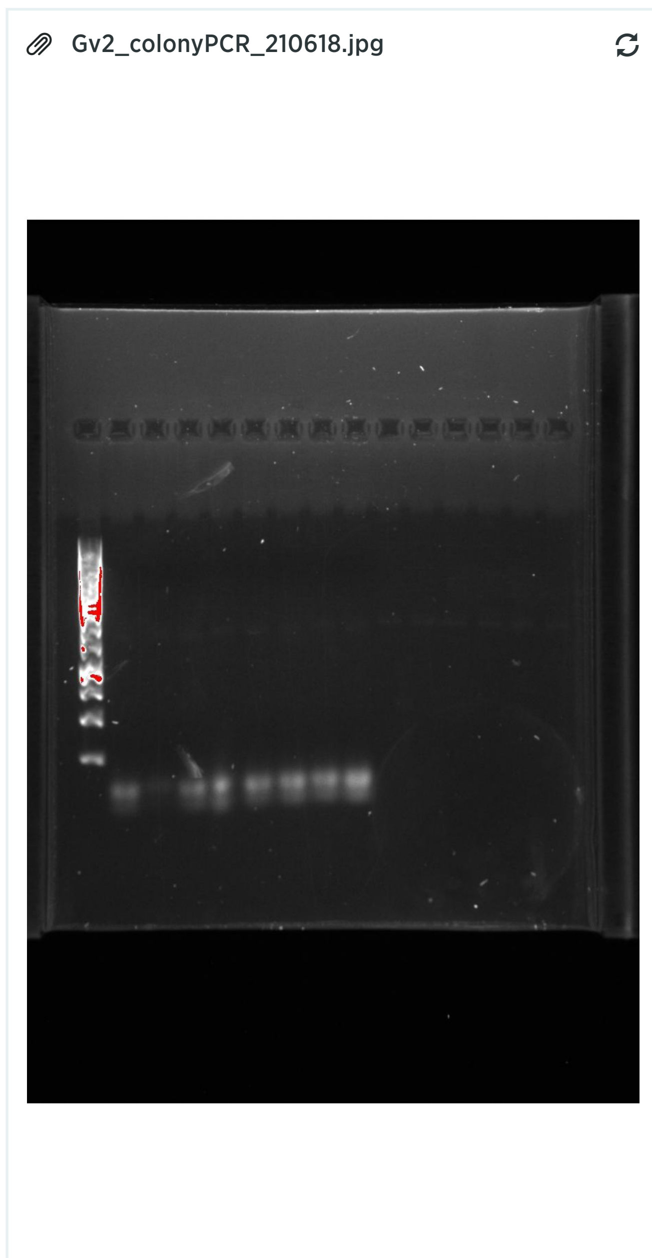
TORSDAG, 2018-06-21

Colony PCR Dream-Taq
8 colonies were arbitrarily selected from the plate

	A	B
1		Volume (in µL)
2	Dream-Taq buffer	5
3	dNTPs	5 (should have been diluted)
4	Fw_primer	5
5	Rv_primer	5
6	DNA temp.	1
7	Polymerase	1
8	Water	33
9	Total	50

PCR with elongation phase of 2 minutes and annealing temp of 58ºc

Gv2_colonyPCR_210618.jpg



Result: No construct observed in the PCR

Repeat all the above

Yields plate with transformed E.coli

MÅNDAG, 2018-07-02

Incubate two different transformed E.coli colonies at 37ºC (t=16:15) in different 10mL tubes

TISDAG, 2018-07-03

Take out at 10:00

Do miniprep in duplo on both according to protocol with low copy number plasmid.

Restriction with NcoI and PstI at 37ºC for 10 minutes according to Fast Digest Protocol

Gel electrophoresis

Table19

	A	B	C	D	E
1	ladder	1.1	1.2	2.1	2.2

Picture missing

TORSDAG, 2018-07-05

Picked 14 colonies of transfromed E.coli

Grow both overnight in LB

FREDAG, 2018-07-06

Cultures removed from shaker at 10:15

Do mini-prep on all accoridng to protocol

Check results:
restriction enzymes NcoI
Gel electrophoresis to check result
No obvious results so restriction and gele electrophoresis is repeated

TISDAG, 2018-07-10

Restriction with NcoI and PstI for 10 minutes accordig to Fast Diggest protocol

Gel Electrophoresis 2nd try:
Ladder, 3, 4, 5, 6, 9, 10, p413TEF
Run for 40 min at 85V

Gel electrophoresis result: GelDoc-PC 2018-07-10_14h49m22s

FREDAG, 2018-07-13

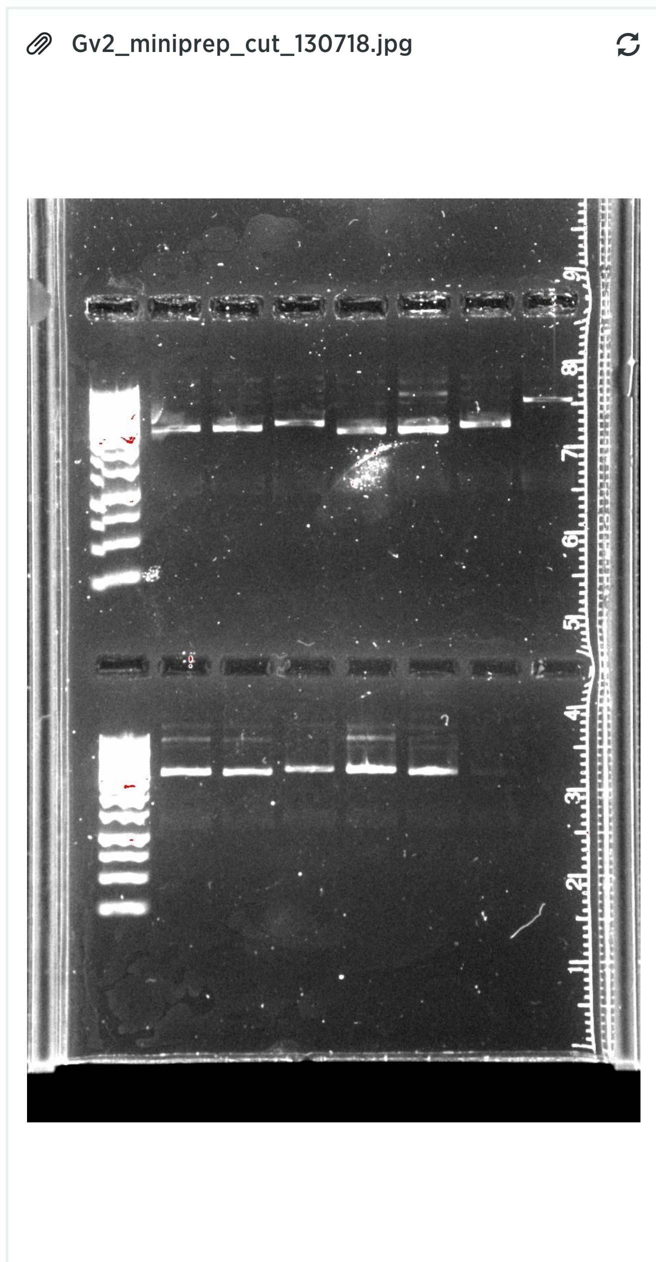
Restriction with PsyI and ScaI for 30 minutes at 37ºC accordig to Fast Diggest protocol

loading:

Table17

	A	B	C	D	E	F	G	H
1	ladder	1	2	3	4	5	6	uncut ctrl
2	ladder	8	7	9	10	11	12	cut ctrl

Gv2_miniprep_cut_130718.jpg



Need a better separation, repeat with longer run time on long gel tray

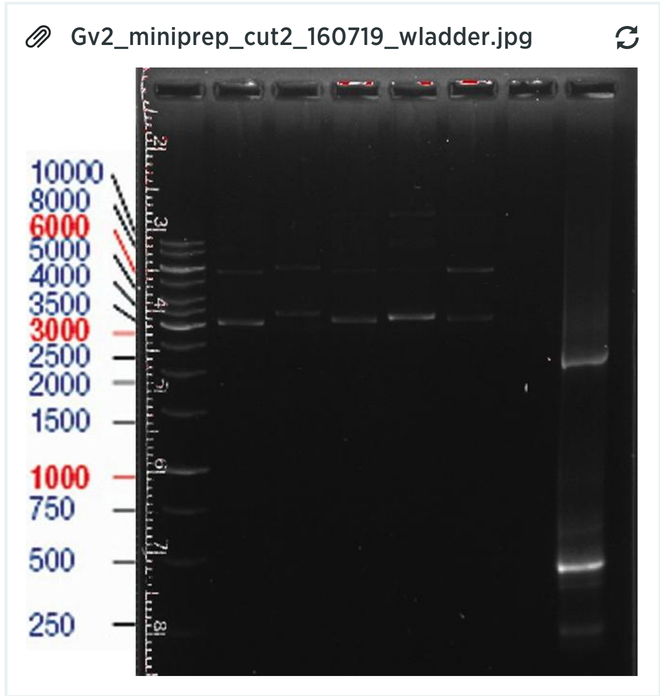
MÅNDAG, 2018-07-16

Gel electrophoresis, longer run

Table18

	A	B	C	D	E	F	G	H
1	ladder	5	6	8	10	11	ctrl cut	ctrl uncut

Gv2_miniprep_cut2_160719_wladder.jpg



Still no good results.

Failed experiment. Repeat from scratch.

TISDAG, 2018-07-24

All primers (listed later) and constructs are diluted to the right concentrations

TORSDAG, 2018-08-02

Acquire plasmids

Get plate with E.coli containing pSP-G2

Get plate with E.coli containing p414TEF

Do mini-prep on both accoridng to protocol

Get purified p413TEF

Get purified p416TEF

Nanodrop DNA concentration measurements:

Table20

	A	B	C	D	E	F	G	H	I	J
1	p413	p416	p414_1	p414_2	p414_3	p414_4	PSPG_1	PSPG_2	PSPG_3	PSPG_4
2	229,8	118,9	27,5	25,4	25,0	6,6	123,4	144,0	120,4	116,5

TORSDAG, 2018-08-09

PCR Amplification:

PTEF/PGK1_1-3b PCR settings:

- Template DNA: pSP-G2
- Primers: PTEF/PGK1-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 1413 + 2*15 tail = 1443 bp
- Annealing temperature: 65.3 ºC
- Extension time: 1 min 30 sec (approx 1 min per 1kb)

T/ADH1_1-3b PCR settings:

- Template DNA: pSP-G2
- Primers: T\ADH1_Fw & -Rv
- Polymerase: Phusion
- Fragment length: 165 + 2*15 tail = 195 bp
- Annealing temperature: 65.1 ºC
- Extension time: 0 min 12 sec (approx 1 min per 1kb, very short, maybe a bit longer to be sure?)

GasVes1 PCR settings:

- Template DNA: gBlock GasVes1
- Primers: Vesic1v2-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 1764 + 2*15 tail = 1794 bp
- Annealing temperature: 65.5 ºC
- Extension time: 1 min 50 sec (approx 1 min per 1kb)

GasVes3b (GvpT) PCR settings:

- Template DNA: gBlock GasVes3b
- Primers: Vesic3b-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 879 + 2*15 tail = 909 bp
- Annealing temperature: 64,8 ºC
- Extension time: 0 min 55 sec (approx 1 min per 1kb)

GasVes2 PCR settings:

- Template DNA: gBlock GasVes2
- Primers: gBlock-Vesci2_Fw & _Rv
- Polymerase: Phusion
- Fragment length: 2304 + 2*15 tail = 2334 bp
- Annealing temperature: 61.1
- Extension time: 2 min 20 sec (approx 1 min per 1kb)

PTEF/PGK1_KJU PCR settings:

- Template DNA: pSP-G2
- Primers: PTEF/PGK1-GvpU/KJ-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 1413 + 2*15 tail = 1443 bp
- Annealing temperature: 60.8
- Extension time: 1 min 30 sec (approx 1 min per 1kb)

T/ADH1_KJU PCR settings:

- Template DNA: pSP-G2
- Primers: T\ADH1-Rv-GvpU & T\ADH1-Fw
- Polymerase: Phusion
- Fragment length: 165 + 2*15 tail = 195 bp
- Annealing temperature: 65.1

- Extension time: 0 min 12 sec (approx 1 min per 1kb, very short, maybe longer to be sure)

GvpKJ PCR settings:

- Template DNA: gBlock GvpKJ
- Primers: GvpKJ-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 645 + 2*15 tail = 675 bp
- Annealing temperature: 64.8
- Extension time: 0 min 41 sec (approx 1 min per 1kb)

GvpU PCR settings:

- Template DNA: gBlock GvpU
- Primers: GvpU-Fw & -Rv
- Polymerase: Phusion
- Fragment length: 417 + 2*15 tail = 447 bp
- Annealing temperature: 64.5
- Extension time: 0 min 25 sec (approx 1 min per 1kb)

First combined PCR at 65,1ºC with 01:50 extension time
Do as a gradient PCR between 60-70ºC

Table5

	A	B	C	D	E	F
1		PTEF/PGK1_1-3b	**GasVes1**	**GasVes3b (GvpT)**	**GvpKJ**	Overall
2	Template DNA	pSP-G2	**gBlock GasVes1**	**gBlock GasVes3b**	**gBlock GvpKJ**	
3	Primers	PTEF/PGK1-Fw & -Rv	**Vesic1v2-Fw & -Rv**	**Vesic3b-Fw & -Rv**	**GvpKJ-Fw & -Rv**	
4	Annealing TºC	65,30	**65,50**	**64,80**	**64,80**	65,10
5	Fragment length	1443	**1794**	**909**	**675**	
6	Extension time	01:30	**01:50**	**00:55**	**00:41**	01:50

Second combined PCR at 64,9ºC with 00:25 extension time
Do as a gradient PCR between 60-70ºC

	A	B	C	D	E
1		T/ADH_1-3b	T/ADH_KJU	GvpU	Overall
2	Template DNA	pSP-G2	pSP-G2	gBlock GvpU	
3	Primers	T\ADH1_Fw & -Rv	T\ADH1-Rv-GvpU & T\ADH1-Fw	GvpU-Fw & -Rv	
4	Annealing TºC	65,10	65,10	64,50	64,90
5	Fragment length	195	195	447	
6	Extension time	00:12	00:12	00:25	00:25

Third combined PCR at 60,95ºC with 02:20 extension time
Do as a gradient PCR between 55-65ºC

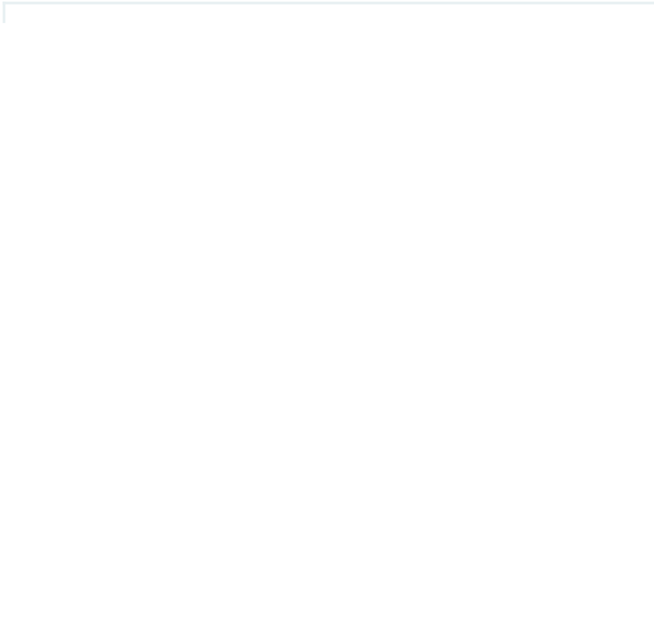
Table7

	A	B	C	D
1		**GasVes2**	PTEF/PGK1_KJU	Overall
2	Template DNA	**gBlock GasVes2**	pSP-G2	
3	Primers	**gBlock-Vesic2_Fw & _Rv**	PTEF/PGK1-GvpU/KJ-Fw & -Rv	
4	Annealing TºC	**61,10**	60,80	60,95
5	Fragment length	**2334**	1443	
6	Extension time	**02:20**	01:30	02:20

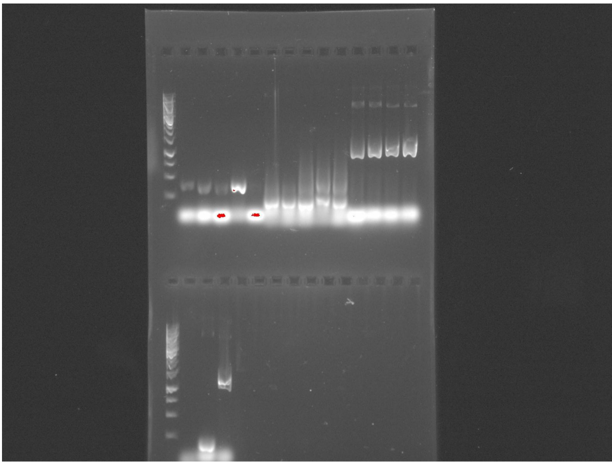
1. Check dilution of PCR primers (10µM)
2. Check dilution of gBlocks (50-250ng needed per tube)
3. Check dilution of plasmid templates (1pg–10ng needed per tube)
4. Fill each PCR tube with:
   - 10µL 5X HF Buffer
   - 1µL dNTPs
   - 2,5µL Fw primer
   - 2,5µL Rv primer
   - 1,5µL DNA template
   - 0,5µL Polymerase
   - fill to 50µL with MiliQ

FREDAG, 2018-08-10

Used 5 tubes/samples. Gel results below.
Loading order:
Gel 1, row 1: Ladder, PTEF/PGK1_1-3b 1-5, GasVes1 1-5, GasVes3b 1-4
Gel 1, row 2: Ladder, GvpKJ 1-5, T/ADH_1-3b 1-5, T/ADH_KJU 1-4
Gel 2, row 1: GvpU 1-5, GasVes2 1-5, PTEF/PGK1_KJU 1-4
Gel 2, row 2: Ladder, GasVes3b 5, T/ADH_KJU 5, PTEF/PGK1_KJU 5



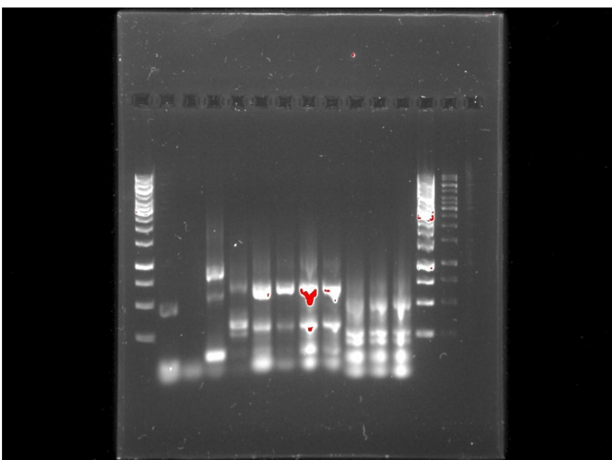
20180810_SuperPcr_gel2.jpg



MÅNDAG, 2018-08-13

Rerun gel for samples GasVes1 3+5, GasVes3b 3-4, GvpKJ 2-5 and GasVes 3-5.

20180813_reruns_superpcr.jpg



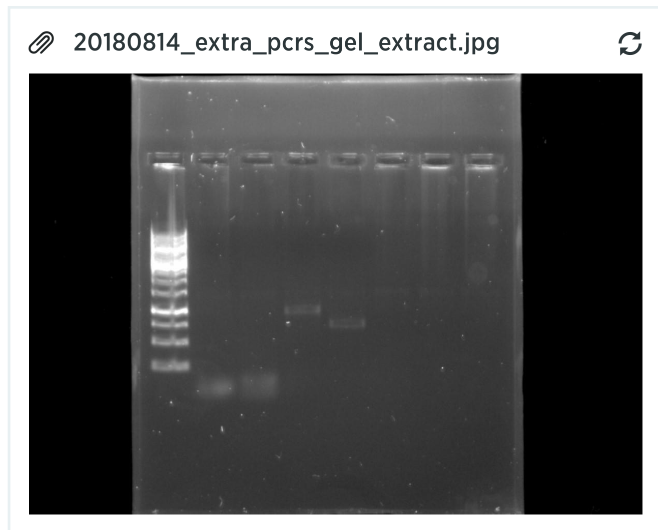
TISDAG, 2018-08-14

Several bands visible for GasVes3b and GvpKJ. 3 µl of the third sample of each of these constructs was run on gel and the correct bands were extracted with ThermoFisher Scientific gel extraction kit according to the protocol of the kit.

PCR for GasVes1 and GasVes2 were remade with an extension time of 45 s and an annealing temperature of 64.8 °C for 10 cycles and 72 °C for the remaining 25.

Gel with GasVes1, GasVes2, GasVes3b, GvpKJ.

20180814_extra_pcrs_gel_extract.jpg



ONSDAG, 2018-08-15

Remade gel extraction of GasVes3b, GvpKJ with 7 * 3 µl of each. Following concentrations were obtained with Nanodrop:
GasVes3b sample 1: 4.3 ng/µl
GasVes3b sample 2: 7.8 ng/µl
GvpKJ sample 1: 14.8 ng/µl
GvpKj sample 2:9.1 ng/µl
Note that some bands that could be primers were visible on the gel.

PCR purification with Thermofisher scientific pcr purification kit of samples from 2018-08-09 (PTEF/PGK1_1-3b sample 4, T/ADH_1-3b sample 3, T/ADH_KJU sample 3, GvpU sample 4, PTEF/PGK1_KJU sample 3 (should double check this)). Final concentrations measured with Nanodrop:
PTEF/PGK1_1-3b: 69.7 ng/µl
T/ADH_1-3b: 248.9 ng/µl
T/ADH_KJU: 199.4 ng/µl
Gvpu: 109.9 ng/µl
PTEF/PGK1_KJU: 73.3 ng/µl

TORSDAG, 2018-08-16

Repeated PCR amplification for GasVes1, GasVes2, GasVes3b and GasVesKJ in gradient PCR from 58-68ºc

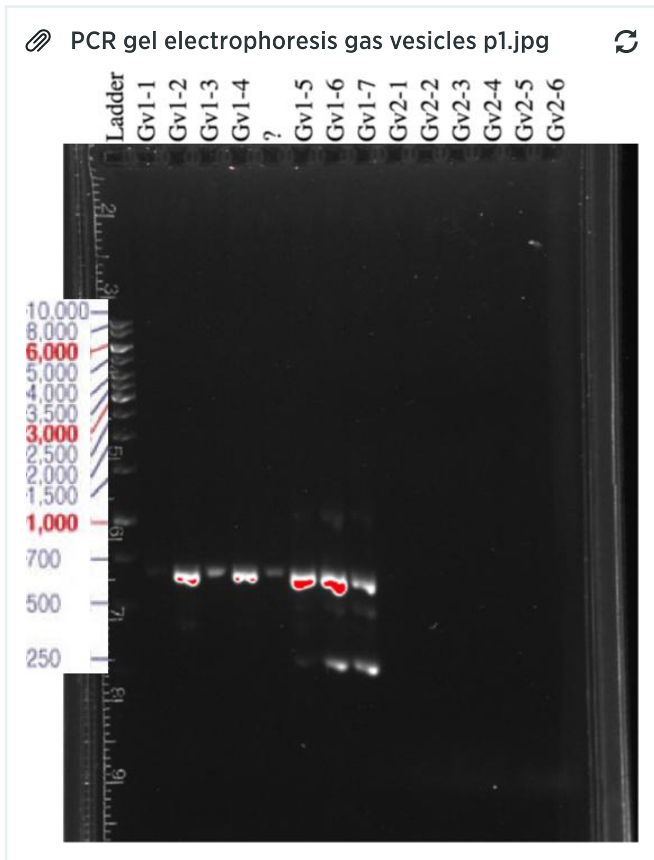
Table11

	A	B	C	D	E	F	G	H
1	1	2	3	4	5	6	7	8
2	58,0	58,7	60,0	62,0	64,4	66,4	67,5	68,0

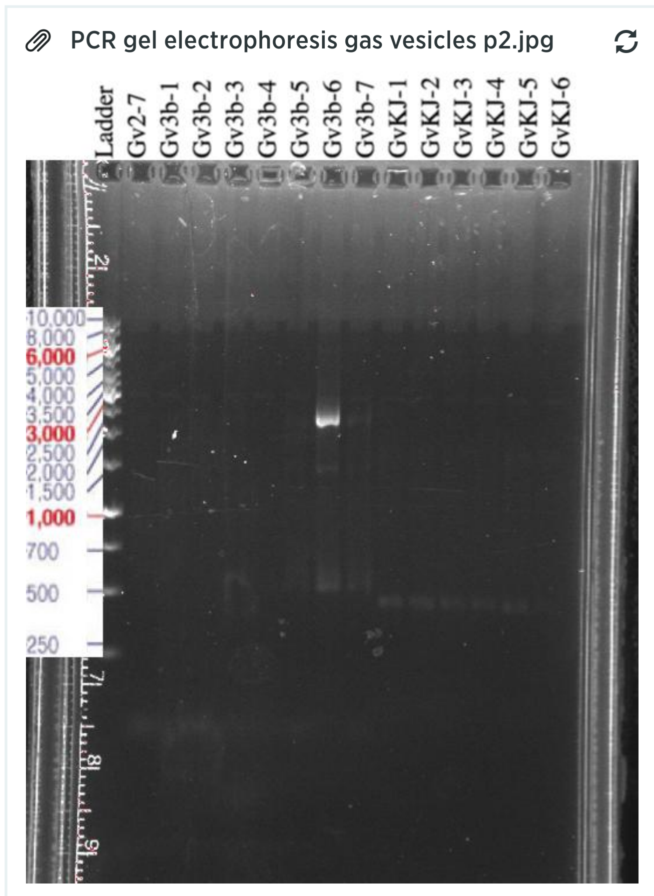
FREDAG, 2018-08-17

Gel electrophoresis of PCR amplicons:

PCR gel electrophoresis gas vesicles p1.jpg



PCR gel electrophoresis gas vesicles p2.jpg



expected bands:

- GasVes1: 1794
- GasVes2: 2334
- GasVes3b: 909
- GasVesKJ: 675

None of the bands match.

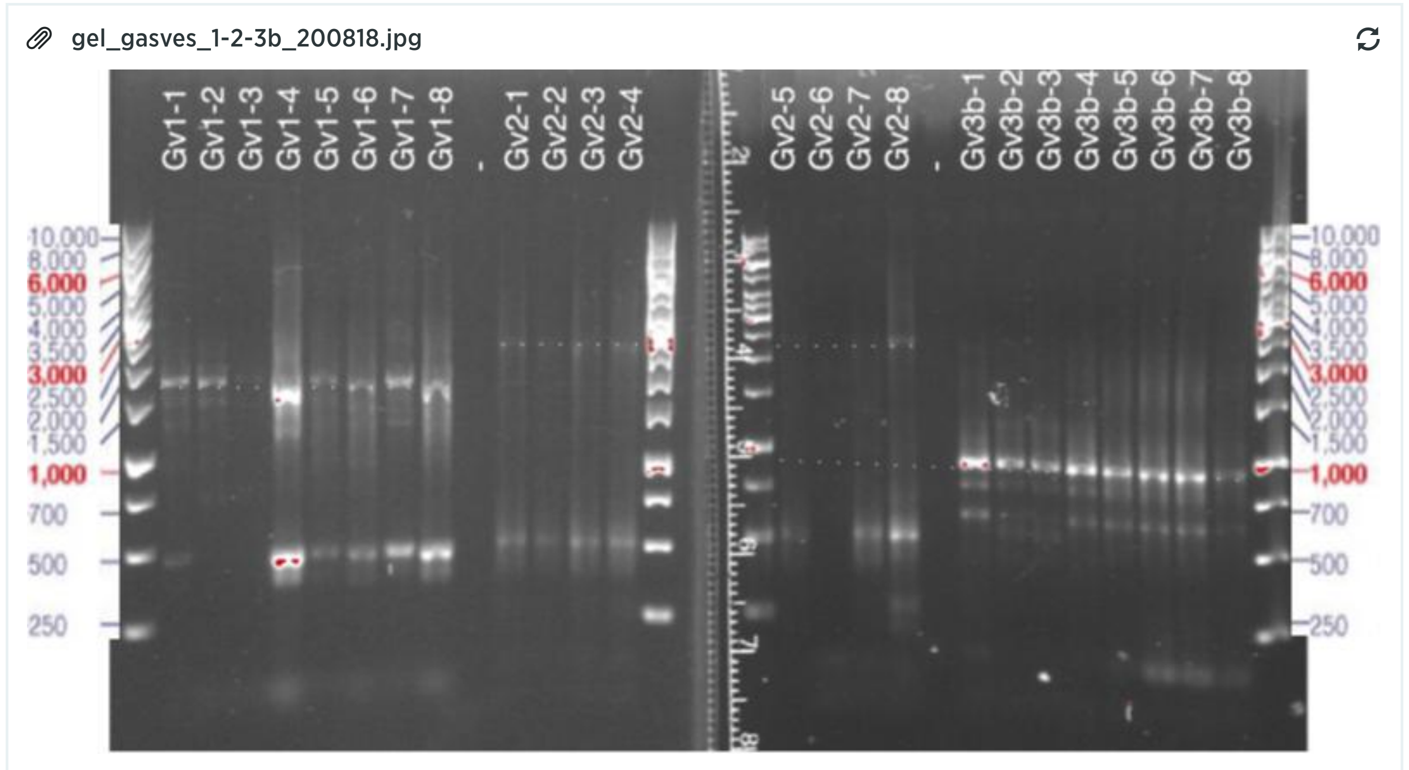
new gradient PCR with only GasVes1, GasVes2 and GasVes3b:

Table12

	A	B	C	D	E	F	G	H	I
1		x1	x9		x1	x9		x1	x9
2	HF buffer	4	36	HF buffer	4	36	HF buffer	4	36
3	dNTPs	0,4	3,6	dNTPs	0,4	3,6	dNTPs	0,4	3,6
4	Vesic1v2-Fw	0,1	0,9	Vesic2-Fw	0,1	0,9	Vesic3b-Fw	0,1	0,9
5	Vesic1v2-Rv	0,1	0,9	Vesic2-Rv	0,1	0,9	Vesic3b-Rv	0,1	0,9
6	GasVes1	0,5	4,5	GasVes2	0,5	4,5	GasVes3b	0,5	4,5
7	Phusion Polymerase	0,2	1,8	Phusion Polymerase	0,2	1,8	Phusion Polymerase	0,2	1,8
8	Water	14,7	132,3	Water	14,7	132,3	Water	14,7	132,3
9	Total	20	180	Total	20	180	Total	20	180

2018-08-20
Gel electrophoresis of PCR amplicons:

gel_gasves_1-2-3b_200818.jpg



Digest plasmid
Remove MCS: cut plasmids p413TEF, p414TEF and p416TEF
Negative Control: linearise plasmids p413TEF, p414TEF and p416TEF with XhoI

	A	B	C	D	E	F	G
1		p413TEF cut	p414TEF cut	p416TEF cut	p413TEF lin	p414TEF lin	p416TEF lin
2	MiliQ	14	14	14	15	15	15
3	10x FD buffer	2	2	2	2	2	2
4	DNA	2	2	2	2	2	2
5	SacI	0	1	1	0	0	0
6	XhoI	1	1	1	1	1	1
7	XbaI	1	0	0	0	0	0

37ºc for 10 minutes (5 min required but 10 to be sure), no inactivation afterwards

Gel Electrophoresis plasmid digestion:

Gel preparation:

- large tray & 8 well comb
- 30mL 1% agarose gel
- 1µL gel red

Gel sample loading (except ladder):

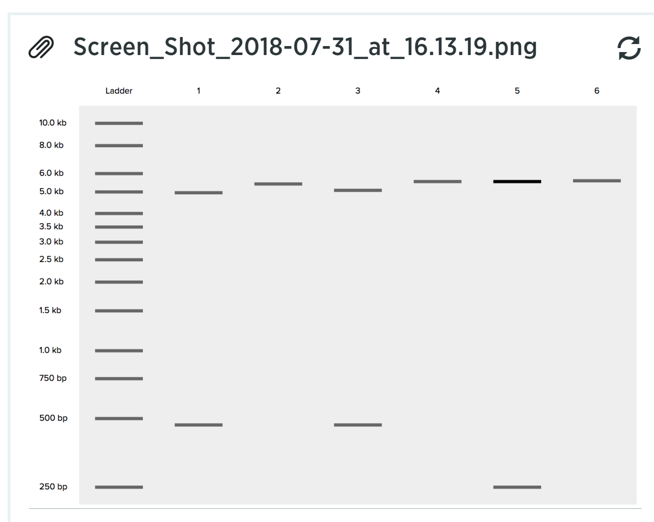
  - 1 µL dye
  - 0,5 µL DNA (according to table below)
  - 4,5 µL MiliQ

Table9

	A	B	C	D	E	F	G	H
1	lane 1	lane 2	lane 3	lane 4	lane 5	lane 6	lane 7	lane 8
2	ladder 1kb	p413TEF cut	p413TEF lin	p414TEF cut	p414TEF lin	p416TEF cut	p416TEF lin	

Gel run: 95V for 45 min

Expected outcome:



Gel Electrophoresis PCR amplification:

Gel preparation:

- large tray & 16 well comb
- 30mL 1% agarose gel
- 1µL gel red

Gel sample loading (except ladder):

- 1 µL dye
- 0,5 µL DNA (according to table below)
- 4,5 µL MiliQ

Gel run: 95V for 45 min

Table10

	A	B	C	D	E	F	G	H	I	J	K
1	lane	lane 1	lane 2	lane 3	lane 4	lane 5	lane 6	lane 7	lane 8	lane 9	lane 10
2	content	ladder 1kb	PTEF/PGK1_1-3b	T/ADH_1-3b	GasVes1	GasVes2	GasVes3b	PTEF/PGK1_KJU	T/ADH_KJU	GvpKJ	GvpU
3	expected bands	ladder 1kb	1 at 1443	1 at 195	1 at 1794	1 at 2334	1 at 909	1 at 1443	1 at 195	1 at 675	1 at 447

DNA Purification Plasmid and PCR amplicons
according to protocol in folder

Concentration measurement by Nanodrop

Table13

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	fragment	p413TEF cut	p414TEF cut	p416TEF cut	PTEF/PGK1_1-3b	T/ADH_1-3b	GasVes1	GasVes2	GasVes3b	PTEF/PGK1_KJU	T/ADH_KJU	GvpKJ	GvpU
2	concentration[ng/µL]												
3	fragment length	5528	4948	5054	1443	195	1794	2334	909	1443	195	675	447
4	pmols	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

ONSDAG, 2018-08-22

Gibson assembly:

For the tables below, the total Volume of DNA has to be below 10µL. If not, scale down proportionally to ng quantities to the left.

Assembly of construct GasVes2 into p413TEF:

To calculate: m/v = pmols * bp * 0,65

Table14

	A	B	C	D
1		pmols	ng	Volume
2	p413TEF cut	0,08	287,46	
3	GasVes2	0,16	242,74	
4	Gibson assembly master mix			10µL
5	MiliQ			10 - V_insert µL
6	Total			20µL

Assembly of constructs GvpU and GvpKJ with double promoter and ADH terminator into p414TEF:

	A	B	C	D
1		pmols	ng	Volume
2	p414TEF cut	0,08	257,30	
3	PTEF/PGK1_KJU	0,16	150,07	
4	T/ADH_KJU	0,42	53,24	
5	GvpKJ	0,16	70,20	
6	GvpU	0,16	46,49	
7	Gibson assembly master mix			10µL
8	MiliQ			10 - V_insert µL
9	Total			20µL

Assembly of constructs GasVes1 and GasVes3b with double promoter and ADH terminator into p416TEF:

Table16

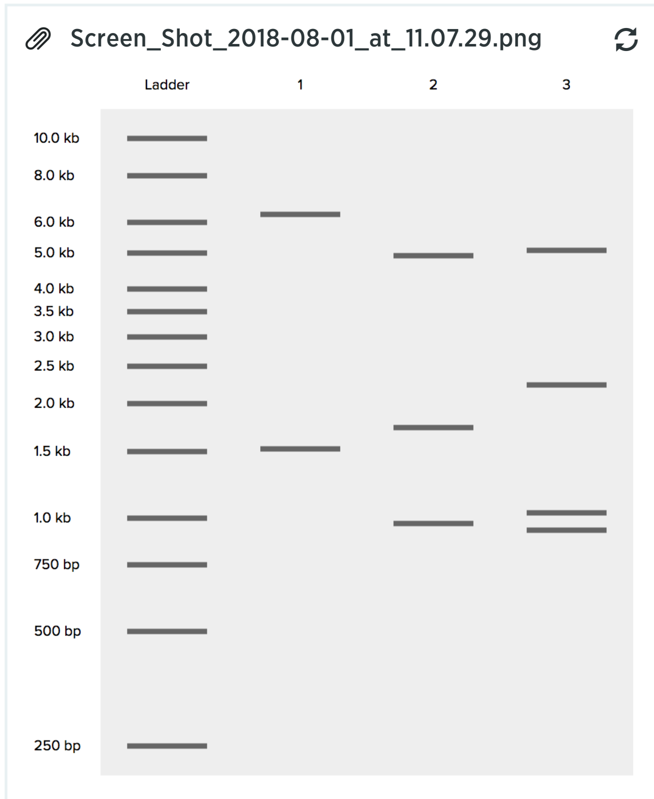
	A	B	C	D
1		pmols	ng	Volume
2	p416TEF cut	0,08	262,8	
3	PTEF/PGK1_1-3b	0,16	150,1	
4	T/ADH_1-3b	0,42	53,2	
5	GasVes1	0,16	186,6	
6	GasVes3b	0,16	94,5	
7	Gibson assembly master mix			10µL
8	MiliQ			10 - V_insert µL
9	Total			20µL

Follow Gibson assembly protocol in folder

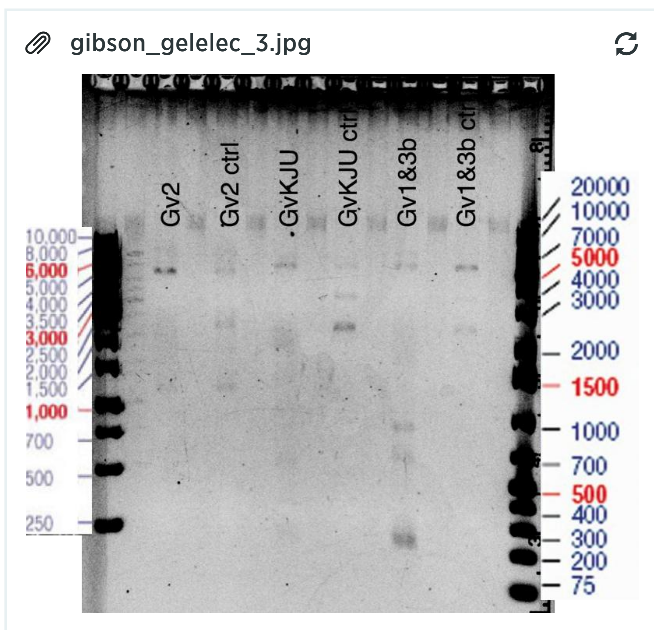
Negative control with all 3 cut plasmids and gibson reagents, no constructs.

Digestion and Gel electrophoresis: to check succes of Gibson assembly
digest p413 at 37ºC with (lane 1): NcoI (in backbone), PstI (in GasVes2)
digest p414 at 37ºC with (lane 2): KpnI (in backbone), Bsu15I (in double promoter), XmaJI (in GvpU)
digest p416 at 37ºC with (lane 3): KpnI (in backbone), Bsu15I (in double promoter, GasVes1 and GasVes3b)

Expected bands:
- 1: p414 with inserts; Gv2
- 2: p414 with inserts; GvpKJU
- 3: p416 with inserts; Gv1&3b



Gel results:
Very faint bands but seem to indicate succes.



E.coli transformation and growth

use DH5 alpha cells from -80 freezer

- thaw on ice for 30 min
- add 3µL of Gibson assembled plasmid to the cells
- heat shock in 42ºc for 45 sec
- put on ice for 20-30 min
- add LB and restore for 1 hour at 37ºc
- Streak on plates
- Grow overnight

TORSDAG, 2018-08-23

No growth on the plates, transformation did not succeed

Decision: drop the gas vesicle project to focus on other parts.