In order to construct high and mid copy pelB-TEV-Ag43 constructs,

pet22b-pelB-TEV-Ag43-AmpR, to get TEV protease,

pZa-pBAD-ALP-Cmr, to get mid copy backbone,

pZe-pBAD-ALP-AmpR, to get high copy backbone,

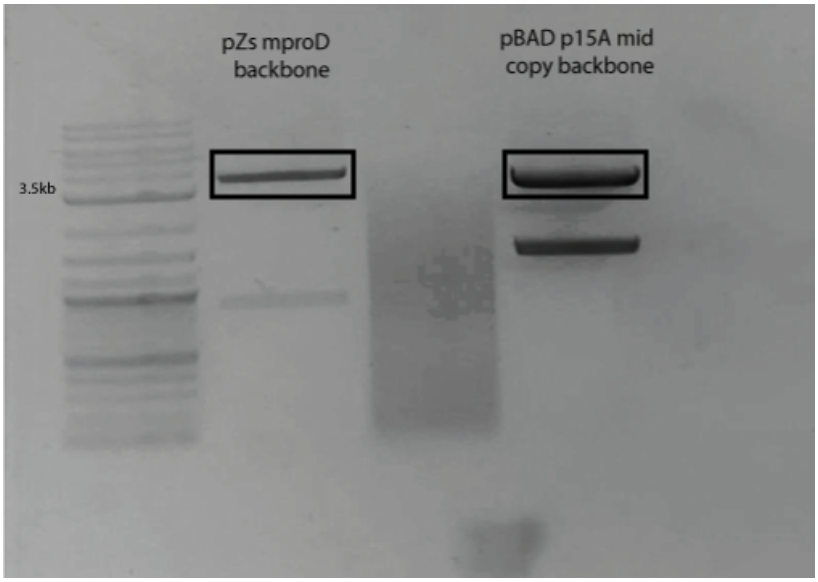
were inoculated, miniprep was done, and nanodrop analysis performed.

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| pZa-pBAD-ALP-Cmr | 167.2 | 1.90 | 1.67 |
| pet22b-pelB-TEV-Ag43-AmpR | 232.2 | 1.91 | 1.73 |
| pZe-pBAD-ALP-AmpR | 600.1 | 1.93 | 2.12 |

Restriction Enzyme Digestions:

pZa-pBAD-ALP-Cmr (KpnI&XhoI)
- 0.5 µl nuclease-free water
- 1 µl Cutsmart
- 8 µl DNA
- 0.25 µl KpnI
- 0.25 µl HindIII

pZe-pBAD-ALP-AmpR (KpnI&XhoI)
- 6.5 µl nuclease-free water
- 1 µl Cutsmart
- 2 µl DNA
- 0.25 µl KpnI
- 0.25 µl HindIII




Sample order: 1) Ladder, 2) pZs-mproD-UreR-KanR, 3) pZe-pBAD-ALP-AmpR , 4) pZa-pBAD-ALP-Cmr

Gel Extraction for pZa-pBAD-ALP-Cmr was done.

Nanodrop Analysis:

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| pZa-pBAD-ALP-Cmr | 32.1 | 2.25 | 0.05 |

pZe-pBAD-ALP-AmpR was inoculated again and miniprep was done.

- Concentration: 704.2 ng/µL

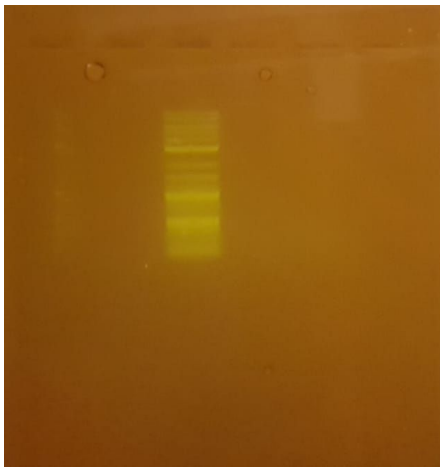
Restriction enzyme digestion for pZe-pBAD-ALP-AmpR:

2 uL DNA
1 uL CutSmart
0.25 uL KpnI
0.25 uL XhoI
6.5 uL dd$H_2O$

Sample was loaded and run on the gel, no bands were observed.

Another restriction digestion was set up to pZe-pBAD-ALP-AmpR for 3 hours:

2 µl DNA
1 µl Cutsmart
0.25 µl KpnI
0.25 µl XhoI
6.5 µl ddH



Failed

To retry restriction digestion, pZe-pBAD-ALP-AmpR was inoculated.

Miniprep and nanodrop analysis were performed.

- Concentration: 564.4 ng/µl

Restriction digestion to pZe-pBAD-ALP-AmpR:

2 µl DNAT
1 µl CutSmart
0.25 µl KpnI
0.25 µl XhoI
6.5 µl dd$H_2O$

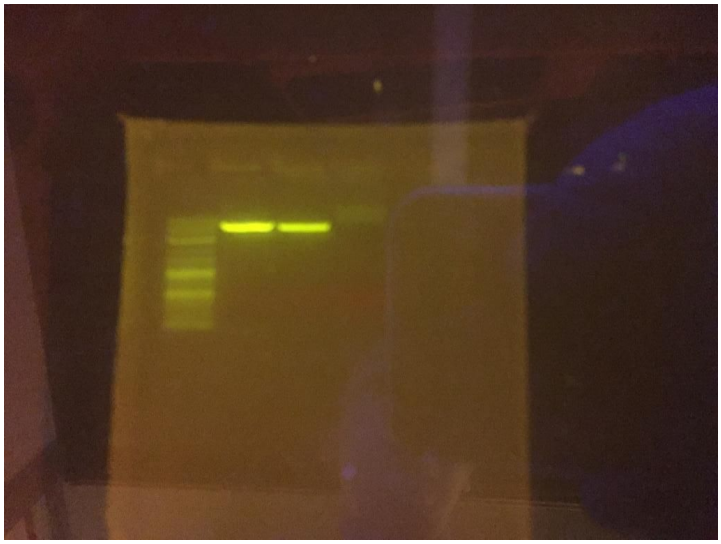
Sample was loaded and run on the gel after 0.5 hour digestion.

There was a single band ⟹ no sign of a successful double digestion

Since digestion was failed, 30 minutes digestion again performed with same concentrations. Only single band was observed → no cut.

Restriction Digestion to pZe-pBAD-ALP-AmpR:

1. Double digestion (KpnI&XhoI)
2. Only with KpnI
3. Only with XhoI



Sample Order: Ladder-1-2-3

Gibson primers were ordered to clone TEV protease into mid and high copy backbones:

| Overhang Backbone | Annealing Backbone | Name | Description | Base Pairs | Tm | Tm w/ Overhang | Anneal | 100 µM stock- µl TE |
|---|---|---|---|---|---|---|---|---|
| pZe-pBAD-ALP-AmpR | pet22b-pelB-TEV-Ag43-AmpR | iGEM2019-P3 | High-Fwd-V1 | 50 | 65 | 79 | 65 | 496 |
| | | iGEM2019-P4 | High-Rev-V1 | 51 | 64 | 79 | | 503 |
| pZa-pBAD-ALP-Cmr, | pet22b-pelB-TEV-Ag43-AmpR | iGEM2019-P5 | Mid-Fwd-V1 | 51 | 60 | 75 | 59 | 718 |
| | | iGEM2019-P6 | Mid-Rev-V1 | 50 | 58 | 78 | | 685 |

PCR reactions:

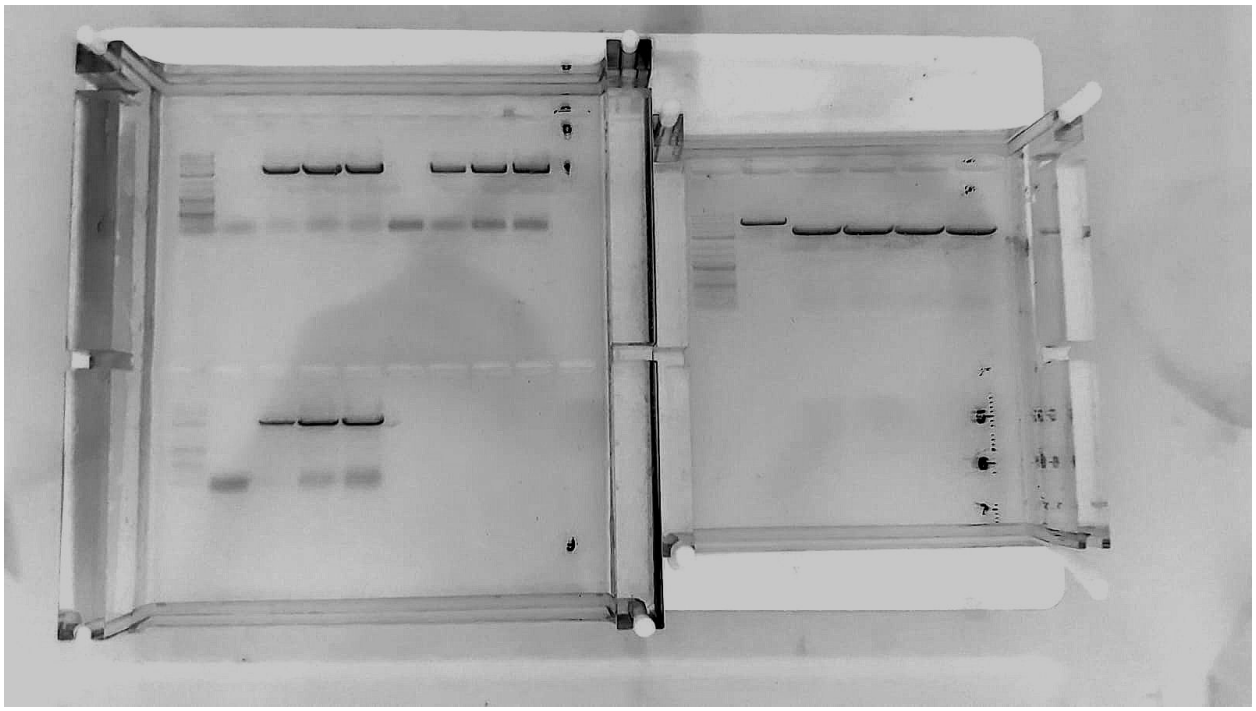
5 µl Forward
5 µl Reverse
50 µl Q5 High-Fidelity 2X Master Mix
40 µl dd$H_2O$

Total: 100 µl

From 100 µl total mix without DNA → 25 µl was transferred to control tube.
75 µl complete mix with DNA was split into 3 PCR tubes.

The steps done for plasmid with Linker TEV + sfGFP were also done for plasmid with AraC + TEV protease with two different tubes (P3+P4 & P5+P6).

12 PCR tubes were prepared (+4 tubes that are wrongly prepared also added ⟹ total 16 tube



Gel extraction was done and nanodrop results are shown below:

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| TEV high | 120.8 | 2.68 | 1.46 |
| TEV mid | 118.5 | 2.09 | 0.25 |

Gibson Assembly:

1. pZA backbone + TEV

52 ng insert ( TEV) + 50 ng plasmid (pZA mid copy)

=> 1.62 µl backbone + 0.44 µl insert + 0.44 µl $ddH_2O$

Pro cells were transformed with the Gibson product, and colony PCR was performed for verification:

A single colony was selected and dissolved in 5 µl $ddH_2O$.

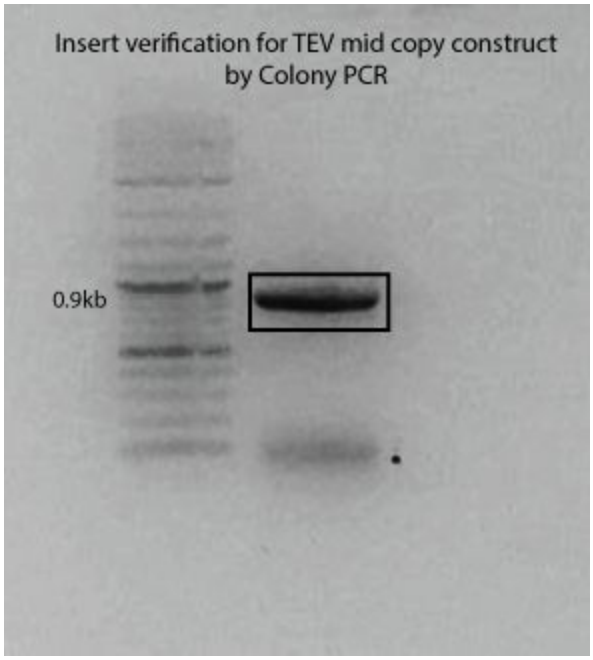
5 µl DNA
2 µl 10X Pfu Reaction Buffer
1 µl forward primer (pREA19)
1 µl reverse primer (pREA115)
10.4 µl $ddH_2O$
0.4 µl dNTP
0.2 µl polymerase

Total: 20 µl

PCR Program:

Initial Denaturation at 95 °C 3 minutes
Denaturation at 95 °C 30 seconds
Annealing 58 °C 30 seconds
Extension 72 °C 1.5 minutes
Final Extension 72 °C 5 minutes

Total 25 Cycles

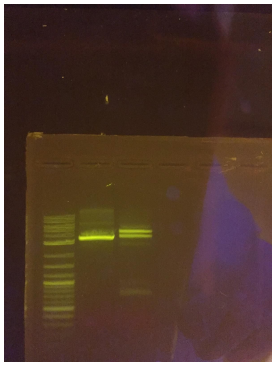


Miniprep was done to TEV mid copy construct from two selected colonies:

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| Col1 | 136.6 | 1.97 | 1.81 |
| Col2 | 113.6 | 1.94 | 2.02 |

The Cmr resistance gene of TEV mid copy construct will be replaced with Amp resistance gene digested from csREA20 plasmid. Thus, digestions was performed on TEV mid construct with SacI & AatII to remove Cmr resistance gene. Also, another digestion was performed on csREA20 plasmid with the same enzymes to remove Amp resistance gene.

gel order: ladder-csrea20-TEV mid construct



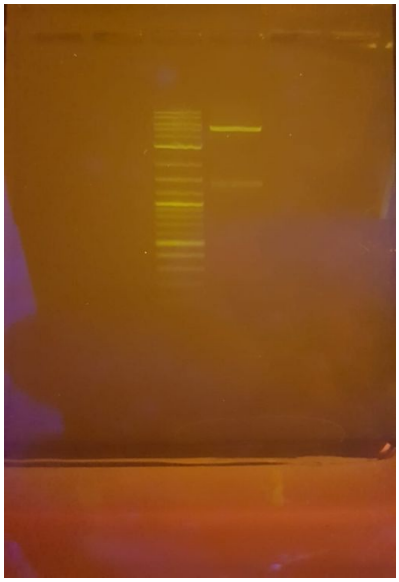
**Digestion I**
TEV mid Col1
concentration: 136.6 ng/µl

8 µl sample
0.25 µl SacI
0.25 µl AatII
1 µl Cut Smart
0.5 µl $ddH_2O$

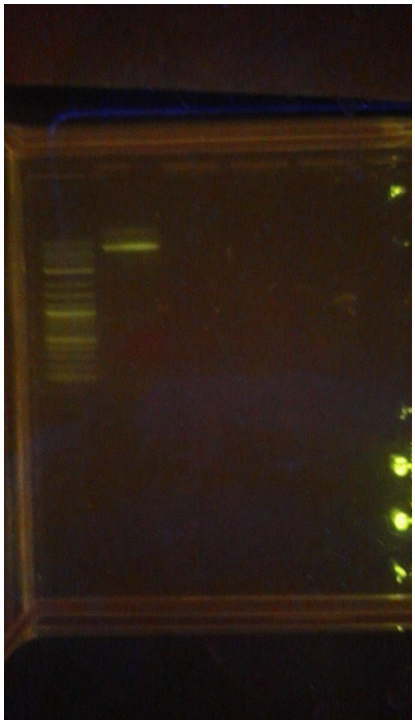
**Digestion II (for verification)**

TEV mid Col2
concentration: 113.6 ng/µl

4.5 µl sample
1 µl Cut Smart
0.25 µl KpnI
4.25 µl $ddH_2O$



**Digestion III**
Tev mid Col2
concentration: 113.6 ng/µl



First, AatII was added and incubated for 1 hour. Then, SacI was added and incubated for 1 hour:

8 µl sample
0.25 µl SacI
0.25 µl AatII
1 µl Cut Smart
0.50 µl $ddH_2O$

Digestion of TEV mid copy:

5 µl sample
3.5 µl $ddH_2O$
1 µl CutSmart
0.25 µl AatII
0.25 µl SpeI-HF

Firstly, AatII was added as an enzyme. Then, without adding SpeI, the reaction mixture was kept at 37°C for 2 hours. Then, to inactivate AatII, the reaction mixture was incubated at 80°C for 20 minutes (in thermocycler). Then, SpeI enzyme was added to the mixture and the mixture was incubated at 37°C for 30 minutes. Then, gel electrophoresis was performed. No bands were observed.

pZE ALP → Miniprep

PCR was done with pZe pureR & p41.

| Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|
| 170.8 | 1.95 | 1.29 |

Cell Inoculation: Col2 (TEV mid)

Miniprep to TEV mid Col2

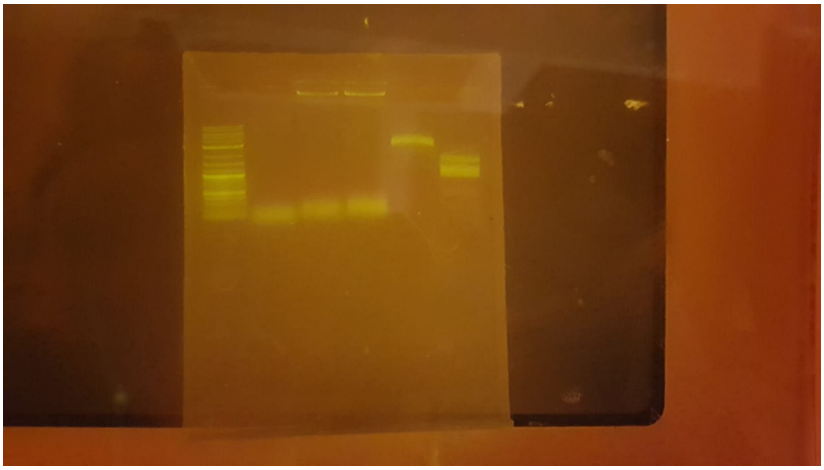
| Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|
| 298.0 | 2.06 | 2.11 |

Colony PCR with csREA20 to obtain AmpR was done.

PCR for TEV mid was done.

Later these two will be cut and ligated, therefore Cmr resistance will be replaced with AmpR.

AmpR and TEV mid backbone were digested with SpeI and AatII for 1.5 hours. Loaded onto gel with pZe ALP.

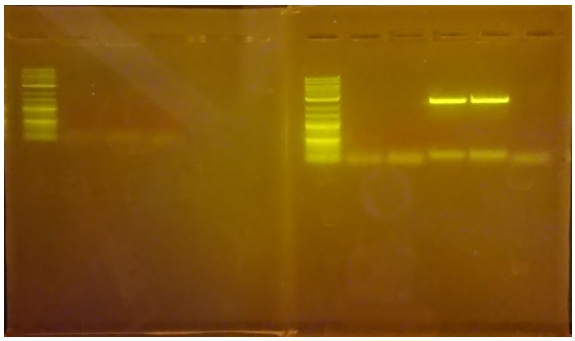


Gel order: pZeALP, TEV mid bb1, TEV mid bb2, ampR1, ampR2

Following PCRs were set up again:

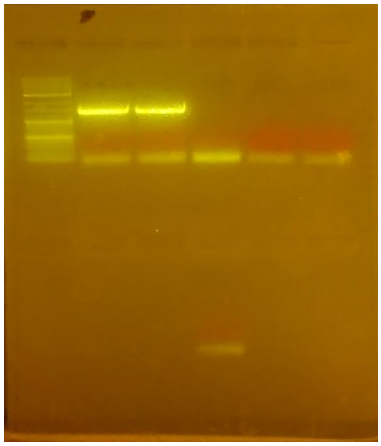
pZE LSS-mKate (high copy) with primers pBadR & pBadF (colony PCR)

Colony 2 TEV mid with primers REA 41 & pZE pure-R

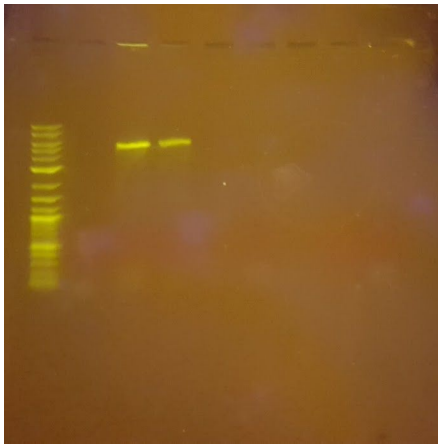


h1-h2-hc t1-tc-a1-a2-ac

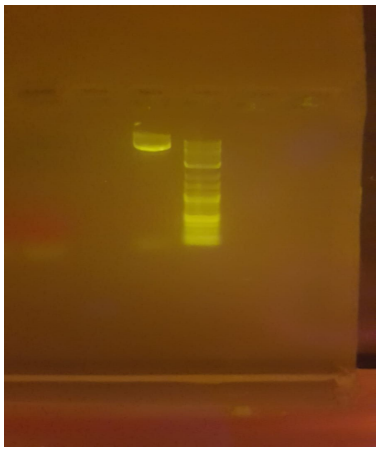
NEW PCR FOR TEV MID COPY & HIGH COPY



h1-h1 (pBAD alp F and R) -hc-t1-t2



h1-h2 (LSS mkate with pBADf and pBADr)
Later, this will be used as high copy backbone.



TEV mid nothing done, for verification.

Gel extraction for high copy backbone:

| Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|
| 20.7 | 3.68 | 0.05 |

Gibson for high copy backbone and TEV protease:

7.5 µl Gibson mix
0.35 µl insert (TEV protease)
2.42 µl backbone (high copy)

Gibson product was transformed to DH5***α*** PRO cells. Transformation was successful.

PCR was done to csREA20 with REA29&30 primers with two replicas, PCR cleanup and nanodrop was performed:

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| 1 | 30.8 | 1.89 | 0.19 |
| 2 | 43.8 | 1.82 | 0.11 |

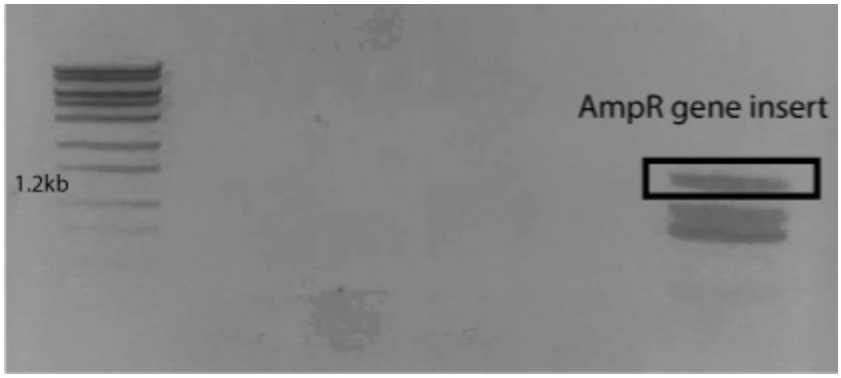
Restriction digestion of AmpR was performed with samples obtained from PCR cleanup. Both of the samples were used for digestion mixture since their concentrations were very low:

18 µl total DNA sample
0.625 µl AatII
0.625 µl SpeI
2.5 µl CutSmart
3.2 µl $ddH_2O$

Total mix: 25 µl

Gel Electrophoresis:

Expected length: 1.1 kb




Gel extraction was performed for AmpR digest

NanoDrop Analysis:

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| AmpR digest | 5.9 | 2.08 | 0.01 |

Ligation with TEV mid and AmpR digest product:

TEV mid+amp (1:5)
2 µl T4 DNA ligase buffer
1 µl T4 DNA ligase
2.27 µl plasmid (TEV mid digest)
8.12 µl insert (AmpR)
6.61 µl $ddH_2O$

20 µl total reaction mix
Reaction time: 80 mins

Transformation of the ligation product to PRO DH5α was done. 3 colonies were selected and inoculated. Miniprep was done:

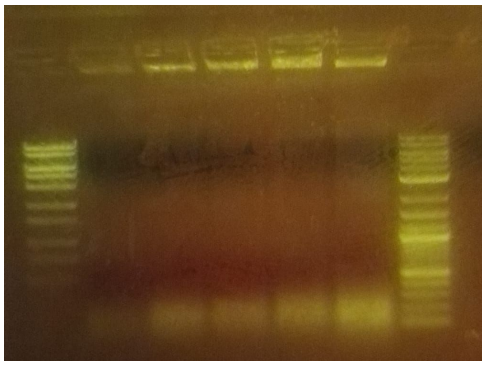
| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| TEV Mid Amp Col1 | 91.1 | 1.96 | 1.79 |
| TEV Mid Amp Col2 | 296.1 | 1.95 | 2.10 |
| TEV Mid Amp Col3 | 235.5 | 2.02 | 1.83 |

Five colonies (6, 7, 8, 9, 10) from pZe-pBAD-TEV-Ag43-AmpR were chosen, seeded and left at 18°C shaker for three days.

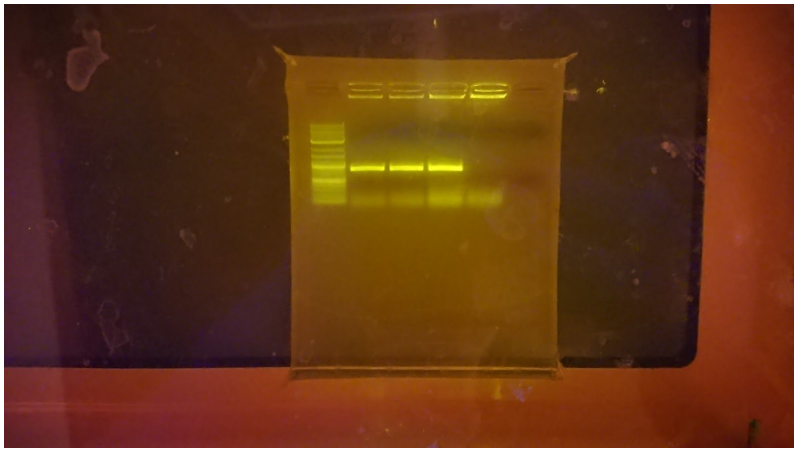
Miniprep was done for colonies 9 & 10:

| Sample | Conc. (ng/µl | 260/280 | 260/230 |
|---|---|---|---|
| Colony 9 | 112.1 | 2.02 | 1.58 |
| Colony 10 | 40.1 | 1.89 | 0.75 |

Colony PCR was done on the 5 selected (6-7-8-9-10) pZa-AmpR-TEV for verification. Annealing temperature was 58°C.



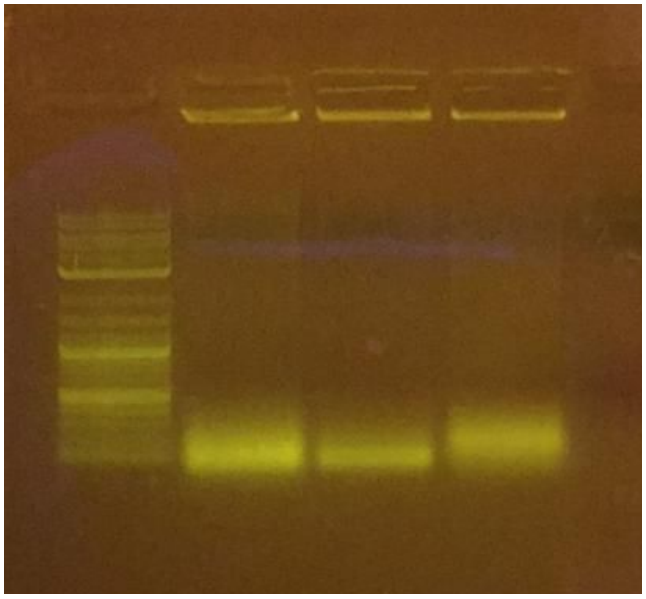
TEV-high copy colony 9 & colony 10 were selected for verification by colony PCR using Q5 Polymerase with two replicas.



Verified colonies were isolated  from the overnight culture. Cell stocks were prepared.

TEV-mid construct PCR was done to col2 with Phusion Polymerase in two replicas. Gradient protocol 61-64°C was used. No bands were observed.

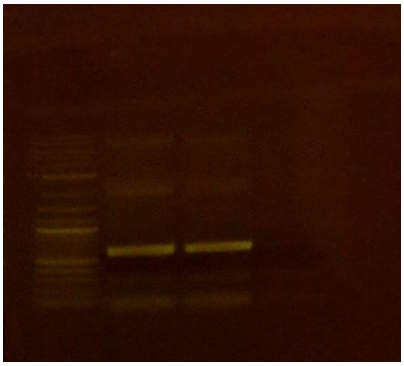
TEV-mid Colony PCR was done on 3 selected colonies



To amplify TEV-mid backbone for exchange of antibiotic resistance, PCR was done:

5 µl BSCat PRO (5 µM concentration)
5 µl PBR_322_R (5 µM concentration)
25 µl Q5 master mix
0.3 µl DNA
14.7 µl $ddH_2O$

Annealing: 63°C
pZA-ALP was used as positive control

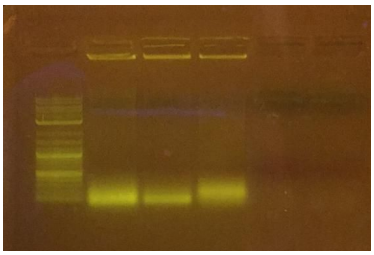


Restriction digestion to Col 2 of TEV mid with KpnI for verification:

0.25 µl KpnI
1 µl CutSmart
1.5 µl DNA sample (concentration: 298 ng/µl)
7.25 µl $ddH_2O$

10 µl total reaction mix
Duration of the digestion: 1.5 hour

PCR was done to TEV-mid col 2 to amplify backbone with different annealing temperature and GC enhancer with 2 replicas. 5 µl GC enhancer for each reaction was added. Annealing temperature was 65-67°C. Control was put on 63°C**.**



PCR to TEV mid col2:

25 µl reactions were prepared
primers used: PureR & SY-CHL-FWD
4 tubes were prepared (3 duplicates and 1 control)

i. The duplicates were placed into the machine with a gradient, respectively 62°C, 64°C, 68°C

Annealing (30 seconds)

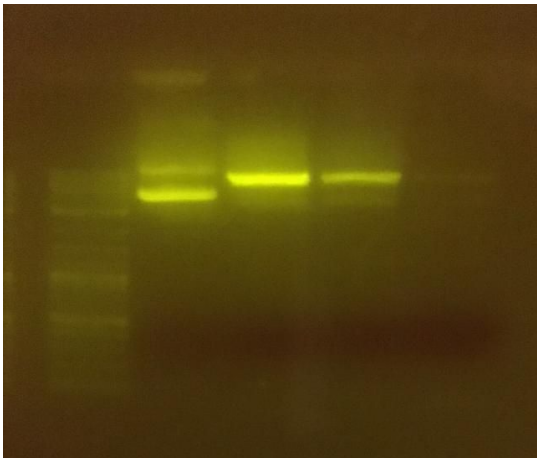
ii. tube 1: 64°C
iii. tube 2: 66°C
iv. tube 3: 68°C
v. pos. cont.: 62°C

Elongation: 4 minutes, Final Elongation: 3 minutes

vi. 26 cycles
vii. total time: 2:30

Gel Electrophoresis:

b. PCR product (expected length: 6000 bp)
c. Tube 1 & tube 2 were cut from the gel and stored at +4°C
d. sample order: ladder-control-tube 1-tube 2-tube 3



Gel extraction was done. Both tube 1 & tube 2 samples were extracted in the same eppendorf.

| Sample | Conc. (ng/µl) | A260/280 | A260/230 |
|---|---|---|---|
| TEV mid backbone | 30.7 | 2.00 | 0.04 |

TEV mid PCR extraction was digested with SpeI & AatII:

25 µl total reaction mixture
18 µl DNA sample
2.5 µl CutSmart
0.5 µl SpeI
0.5 µl AatII
3.5 µl $ddH_2O$

duration: 90 minutes

PCR cleanup for the digestion mix:

mix was completed to 100 µl with $ddH_2O$
200 µl NT1 was added

Nanodrop:

| Sample | Conc. (ng/µl) | A260/280 | A260/230 |
|---|---|---|---|

| | | | |
|---|---|---|---|
| TEV mid digest | 22.0 | 2.00 | 0.05 |

Ligation of TEV Mid & AmpR:

23.30 ng insert (3.95uL)
50 ng plasmid (2.37 uL)
2 uL T4 DNA Ligase Buffer
1 uL T4 DNA Ligase
10.78 uL ddH2O

20 mins waited

Transformation of ligation product to PRO cells.

sfGFP (with mproD), TEV high, TEV mid amp, were prepared for sequencing.

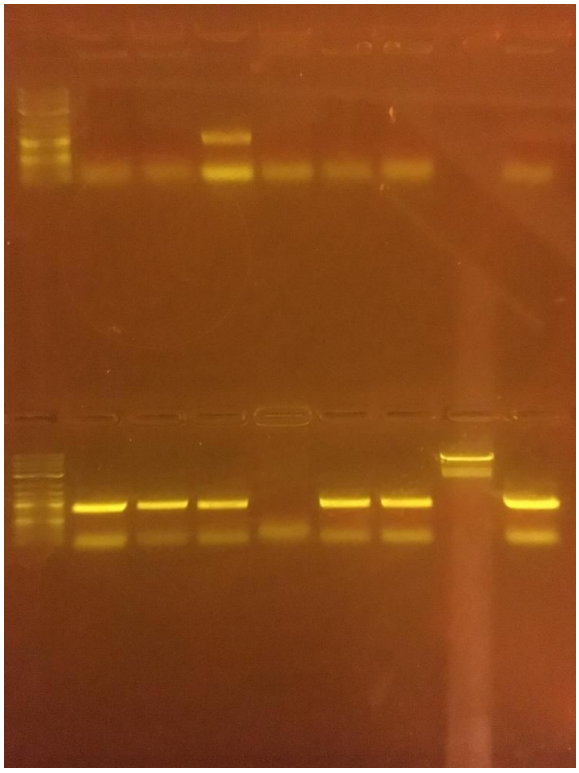
TEV mid digestion for verification
a. cut with SpeI
b. was inconclusive, will be redone

PHOTO

Miniprep
a. TEV mid col 3

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| TEV mid col 3 | 135.9 | 2.06 | 1.94 |

TEV mid col 3 digestion was performed with SpeI.



1. pET22b sfGFP plasmid were inoculated for 12 hours and miniprep was performed
2. 25 µl of digestion was set up on each plasmid
3. 10 µl DNA ≈ 1200 ng for pET22b sfGFP 6
4. 5 µl DNA ≈ 140 ng for pET22b sfGFP 3

Miniprep on sfGFP col 2 & col 4 and two pET22b duplicates

| Sample | Conc. (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| sfGFP col 2 | 254.9 | 2.04 | 2.05 |
| sfGFP col 4 | 552.1 | 2.05 | 2.21 |

pZs-proD-col 2 and pZs-proD-col 4 were transformed to BL21 competent cells.

To create pZs pBAD TEV Ag43, we used a 3 part Gibson Assembly. We used PCR to add overhangs to the AraC TEV Ag43 cassette and the antibiotic resistance gene. We obtained the backbone containing the pSC101 low copy origin via PCR. Then we used gibson assembly to combine all three parts.