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MANOLIS

01-08-2020

Goals of the day:

1. Primer resuspension
2. Amplify the eGFP sequence, using a kapa taq polymerase.
3. PCR amplification using the NEB's Protocol for Q5® High-Fidelity 2X Master Mix
4. Gel electrophoresis
5. Gel extraction
6. NanoDrop spectrometer
7. Dig-Lig reaction for level 0 assembly
8. Transformation

PROCEDURE

a. Follow instructions from "protocol for Primer Re-suspension dilution", using the information provided in the order leaflet.

When still dry:

• Spin down the pellet.

• Open the tube very carefully

• Add appropriate volume of ddH_2O (creating a concentration of 100pmol/μL)

• Close the lid

• Vortex thoroughly (~20s)

• Spin down

b. Take out the Eppendorf tubes containing the eGFP gene sequence (EGFP 1 & 2) and calculate the concentration using NANODROP, and then create stock working samples:

- EGFP1: 279,5 ng/μL
- EGFP2: 441,6 ng/μL

[EGFP1: CoVo=CfVf => 279,5ng/μL x 1μL = 10ng/μL x Vf => Vf=27,95μL => $V_{H2Oadded}$ = 26,95μL]

[EGFP2: CoVo=CfVf => 441,6ng/μL x 1μL = 10ng/μL x Vf => Vf=44,16μL => $V_{H2Oadded}$ = 43,16μL]

c. PCR Protocol using kappa Taq kit

- Prepare a PCR master mix containing the appropriate volumes of all reaction components common to all or a subset of reactions to be performed. The MasterMix (MM) includes ddH_2O, the primers, the buffer, dNTPs and of course the enzyme.
- Calculate the required volumes of each component based on the following table, solving the dilution equation in each case (C_i x V_i = C_f x V_f)

Component	20 μL reaction	Final concentration
PCR-grade water	15,9μL	-----
10X KAPA Taq Buffer	1μL	1X
10 mM dNTPs mix	0,4μL	0.2 mM (in total)
10 μM Forward Primer	0,8μL	0.4 μM
10 μM Reverse Prime	0,8μL	0.4 μM
5 U/μL KAPPA Taq DNA Polymerase	0,1μL	0.5 U
Template DNA	1μL	-----

MasterMix x 4	Volumes
PCR-grade water	63,6μL
10X KAPA Taq Buffer	4μL
10 mM dNTPs mix	1,6μL
10 μM Forward Primer	3,2μL
10 μM Reverse Prime	3,2μL
5 U/μL KAPPA Taq DNA Polymerase	0,4μL

- Perform PCR with the following cycling protocol:

Step	Temperature	Duration	Cycles
Initial denaturation	95oC	3 min	1
Denaturation	95oC	30 sec	35
Annealing	67oC	30 sec	
Extension	72oC	1 min	
Final extension	72oC	1 min	1
Hold	4-10oC		1

d. Run Gel electrophoresis (Negative Control, EGFP1, EGFP2)

<u>Materials for the gel</u>

For 60ml of 1% w/v agarose we need:

- • 60ml 1X TAE (diluted from 50X TAE stock)
- • 0,6gr agarose
- • 2,16μl EtBr (3.6ul for 100mL gel, 5.4ul for 150mL gel)

- Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 0,6gr of dry

agarose.

- Mix TAE and agarose in a flask
- Microwave for 2-3 mins (until it boils)
- After microwaving, mix it thoroughly under running water (don't smell it) until it cools down.
- When in the right temperature (not too hot), add 2,16μl EtBr. Pipette up and down and mix the
  flask to spread the EtBr everywhere.
- Prepare the gel box, combs.
- Add the liquid gel slowly into the gel box.
- Let the gel solidify for 10-15min
- After 10-15mins, untighten the scaffold and remove the gel box with the gel still on. (If you want
  to cut and store half the gel for another run, cut it and remove it.
- Put the gel in the "electrode box", filled with 1X TAE. If not full, add as TAE needed until it covers
  the gel.
- Load the samples in the gel wells.
- Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press
- RUN (figure than runs) at 110V until it runs the whole gel (depends on gel agarose % and length).
- Put the gel under UV and take a photo to see what you did.

e. Sample Preparation for Gel Run
   - 20μl plasmid in Eppendorf tube
   - Add 4μl loading dye (6X) *(6xVo=1x(20+Vo) => 5Vo=20μL => Vo=4μL)*
   - Final volume will be 25μl
   - For the gel ladder, add 3.0μl

PRECEDURE

a. PCR protocol: Protocol for Q5® High-Fidelity 2X Master Mix

Component	50 μl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	25 μL	1X
10 μM Forward Primer	2.0 μL	0.5 μM
10 μM Reverse Primer	2.0 μL	0.5 μM
1 EGFP (10ng/μL)	2.0 μL	< 1,000 ng
Nuclease-Free Water	19 μL	-----

Thermocycling Conditions for a Routine PCR:

STEPS	TEMP	TIME
Initial Denaturation	98°C	30 seconds
	98°C	5–10 seconds
25–35 Cycles	72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	---

b. Gel electrophoresis: Follow the lab's protocol

(Final volume to be loaded in the gel wells: 60μL from the PCR solution. 30μL in each well.

10μL of loading dye to be added in the PCR small tube: CoVo = CfVf => 6xVo = 1x(50+Vo) => Vo=10μL)

c) DNA extraction from Agarose gel, following the protocol of NucleoSpin® Gel and PCR Clean-up from by Macherey-Nagel

- Excise DNA fragment / solubilize gel slice with a scalpel, cleaned with ethanol and then Determine the weight of the gel slice and transfer it to a clean tube. For each 100 mg of agarose gel < 2 % add 200 μL Buffer NT
- Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 μL sample. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.Load remaining sample if necessary and repeat the centrifugation step.
- Wash silica membrane by adding 700 μL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
- Dry silica membrane by centrifuging for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.
- Elute DNA by placing the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube and adding 15–30 μLBuffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 minute 11,000 x g

d. Spectophotometry using NanoDrop *(98,8ng/μL)*

e. One-pot dig-lig reaction (GoldenBraid)

50-75ng pUPD2	0,5μL
40-70ng of EGFP (post- Gel-extraction)	0,7μL
1μL 10X T4 DNA Ligase buffer	1μL
1μL T4 DNA Ligase	1μL
BsmB1	0,5μL
ddH_20	6,3μL
SUM	10μL

f. Transformation

- Take competent cells out of -80°C and thaw on ice (approximately 20 mins).
- Mix 1μl of DNA (usually 10 pg - 100 ng) into 50-100 μL of competent cells in a microcentrifuge. GENTLY mix by flicking the bottom of the tube with your finger a few times.
- Incubate the competent cell-DNA mixture on ice for 20-30 mins.
- Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath
  for 60 sec

- Put the tubes back on ice for 2-5 min.
- Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h
  (under sterile conditions). (This outgrowth step allows the bacteria time to generate the antibiotic resistance).
- Add Xgal and IPTG on the agar plate.
- Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
- Incubate plates at 37°C overnight.

02-08-2020

Goals of the day

- Plasmid isolation of level 0 pUPD2-inserts (lacI, PromAnderlacO, Terminator, FFAR2x4, eGFP, Anderson promoter)
- Nanodrop
- Diagnostic Restriction-Digestion

PROCEDURE

A) Plasmid isolation of level 0 FFAR2, eGFP and Anderson promoter parts, inserted in pUPD2 vectors.

After inoculation in LB medium and incubation for 20h in 37◦C/210 prm, proceed with plasmid isolation following the protocol, adapted from Marcherey-Nagel. For further instructions see (…)

I. Cultivate and harvest bacterial cells

1. Use 5 mL of a saturated E. coli LB culture, pellet cells in a standard benchtop microcentrifuge for 30s at 11,000 x g. Discard the supernatant and remove as much of the liquid as possible.

II. Cell lysis

2. Add 250 µL Buffer A1. Resuspend the cell pellet completely by vortexing. Make sure no cell clumps remain before addition of Buffer A2!

3. Add 250 µL Buffer A2. Mix gently by inverting the tube 6-8 times. Do not vortex to avoid shearing of genomic DNA. Incubate at room temperature for up to 5 min or until lysate appears clear.

4. Add 300 µL Buffer A3. Mix thoroughly by inverting the tube 6-8 times until blue samples turn colorless.

III. Clarification of lysate

5. Centrifuge for 10 min at 17,000 x g at room temperature. Repeat this step in case the supernatant is not clear

IV. Bind DNA

6. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a maximum of 750 µL of the supernatant onto the column. Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

V. Wash silica membrane

7. Additional washing step with 500 µL Buffer AW, optionally preheated to 50 degrees C, and centrifuge for 1 min at 11,000 x g before proceeding with Buffer A4.

Add 600 µL Buffer A4 (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the empty collection tube.

VI. Dry silica membrane

8. Centrifuge for 5 min at 11,000 x g and discard the collection tube Note: Residual ethanolic wash buffer might inhibit enzymatic reactions.

VII. Elute DNA

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add 40 µL Buffer AE. Incubate for a couple of minutes at room temperature. Centrifuge for 1 min at 11,000 x g.

1 Cultivate and harvest bacterial cells

Use **1–5 mL** of a saturated ***E.coli* LB culture**, pellet cells in a standard benchtop microcentrifuge for **30 s** at **11,000 x *g***. Discard the supernatant and remove as much of the liquid as possible.

11,000 x *g*, 30 s

Note: For isolation of low-copy plasmids refer to section 5.2.

2 Cell lysis

Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing or pipetting up and down. Make sure no cell clumps remain before addition of Buffer A2!

+ 250 µL A1

Resuspend

Attention: Check Buffer A2 for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30–40 °C until precipitate is dissolved completely. Mix thoroughly and cool buffer down to room temperature (18–25 °C).

Add **250 µL Buffer A2**. Mix gently by inverting the tube **6–8 times**. Do not vortex to avoid shearing of genomic DNA. Incubate at **room temperature** for up to **5 min** or until lysate appears clear.

+ 250 µL A2

Mix

RT, 5 min

Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube **6–8 times** until blue samples turn colorless completely! Do not vortex to avoid shearing of genomic DNA!

+ 300 µL A3

Mix

Make sure to neutralize completely to precipitate all protein and chromosomal DNA. LyseControl should turn completely colorless without any traces of blue.

3 Clarification of lysate

Centrifuge for **5 min** at **11,000 x *g*** at room temperature.

Repeat this step in case the supernatant is not clear!

11,000 x *g*, 5–10 min

4 Bind DNA

Place a NucleoSpin® Plasmid/Plasmid (NoLid) Column in a Collection Tube (2 mL) and decant the supernatant from step 3 or pipette a maximum of 700 µL of the supernatant onto the column. Centrifuge for **1 min** at **11,000 x *g***. Discard flowthrough and place the NucleoSpin® Plasmid/Plasmid (NoLid) Column back into the collection tube.

Load supernatant

11,000 x *g*, 1 min

Repeat this step to load the remaining lysate.

5 Wash silica membrane

*Recommended: If plasmid DNA is prepared from host strains containing high levels of nucleases (e.g., HB101 or strains of the JM series), **it is strongly recommended** performing an additional washing step with **500 µL Buffer AW, optionally preheated to 50 °C**, and centrifuge for **1 min** at **11,000 x g** before proceeding with Buffer A4. Additional washing with Buffer AW will also increase the reading length of DNA sequencing reactions and improve the performance of critical enzymatic reactions.*

Optional: + 500 µL AW

11,000 x *g*, 1 min

Add **600 µL Buffer A4** (supplemented with ethanol, see section 3). Centrifuge for **1 min** at **11,000 x *g***. Discard flowthrough and place the NucleoSpin® Plasmid/Plasmid (NoLid) Column back into the **empty** collection tube.

+ 600 µL A4

11,000 x *g*, 1 min

6 Dry silica membrane

Centrifuge for **2 min** at **11,000 x *g*** and discard the collection tube.

Note: Residual ethanolic wash buffer might inhibit enzymatic reactions.

11,000 x *g*, 2 min

7 Elute DNA

Place the NucleoSpin® Plasmid/Plasmid (NoLid) Column in a 1.5 mL microcentrifuge tube (not provided) and add **50 µL Buffer AE**. Incubate for **1 min** at **room temperature**. Centrifuge for 1 min at **11,000 x *g***.

+ 50 µL AE

RT, 1 min

11,000 x *g*, 1 min

Note: For more efficient elution procedures and alternative elution buffer (e.g., TE buffer or water) see section 2.5.

B) Nanodrop quantification

Level 0 parts / pUPD2-inserts	Concentration (ng/µL)	A_{260}/A_{280}

FFAR2	259,5	1,74
FFAR2	287,1	1,79
FFAR2	135	1,82
FFAR2	346,8	1,77
eGFP	203,9	1,78
Anderson promoter	202,2	1,79

C) Double diagnostic Restriction Digestion

1. Select restriction enzymes to digest your plasmid.

2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.

3. In a 1.5mL tube combine the following (our restriction digestions are typically 10 to 20 uL in total volume.

- 1-2μL μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O (to bring total volume to 30μL)

4. Mix gently by pipetting.

5. Incubate tube at appropriate temperature (usually 37 °C) for 1,5-2 hours. For results visualization, conduct gel electrophoresis.

6. Prepare a MasterMix of the common reagents and aliquot 8μL in each Eppendorf tube containing 2μL of DNA

MasterMix for Restriction-Digestion using XhoI for samples	
XhoI	0,5x6 =3μL
Cutsmart buffer	1x6 = 3μL
dH2O	6,5x6 = 39μL
MasterMix for RD using BamHI for FFAR2	
BamHI	0,5x4=2μL
Buffer	1x4=4μL
dH2O	6,5x4=26μL

9. Sample Preparation for Gel Run: add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion, so that final volume shall be 12μl

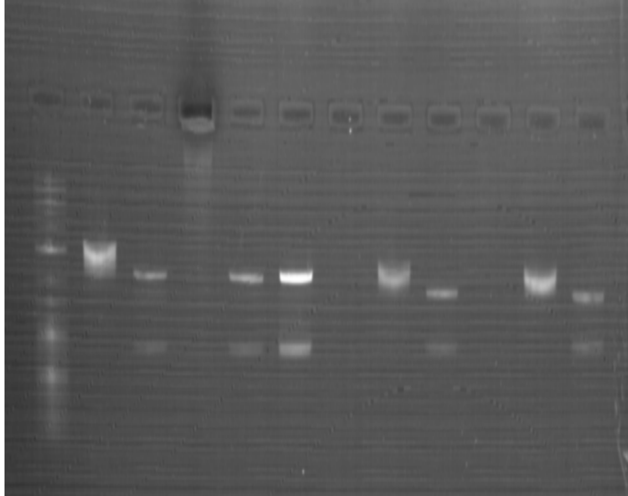
10. For the gel ladder, add 3μl

First Diagnostic Restriction -Digestion

FFAR2 (uncut-3204 bps)	FFAR2 Cut-2312+892 bps	FFAR2	FFAR2	FFAR2	eGFP (uncut-2826 bp)	eGFP (cut-1934+892 bp)	pAnderson (uncut-2156 bp)	pAnderson (cut-1264+ 892 bp)

For a second diagnostic Restriction & Digestion procedure, choose alternative enzymes, pursuant to the protocol. (BamHI for FFAR2, HindIII for eGFP, EcoRI for Anderson promoter)

pUPD2 (cut with EcoRI)	FFAR2 (uncut)	FFAR2 (cut with BamHI)	FFAR2 (cut with BamHI)	FFAR2 (cut with BamHI)	FFAR2 (cut with BamHI	eGFP (uncut)	eGFP (cut with HindIII)	pAnderson (uncut)	pAnderson (cut with EcoRI)



03-08-2020 – 08-08-2020

Goals of the day:

- Isolation and amplification of arabinose promoter sequence from Igem 2019 kit
- Plasmid resuspension from iGEM 2019 Distribution Kit & Transformation (selection on Chloramphenicol)
- Inoculation of single colonies (two to have one backup)
- Minipreps & Diagnostic Digestions + trial KAPA PCR & Q5 PCR + gel extraction + dig-lig overnight to create a level 0 pUPD2-arabinose promoter part.
- Transformation of competent cells, using the dig-lig product.
- Inoculation in LB medium
- Plasmid isolation & Nanodrop + Diagnostic Restriction Digestion & Gel electrophoresis

PROCEDURE

Day 1: DNA Kit Plate Instructions for part resuspension

(The part needed to be used is integrated inside a pSB1C3 vector. "C" stands for CamR gene, that is chloramphenicol-use a plate with chl 35).

1. With a pipette tip, punch a hole through the foil cover into the corresponding well of the part that you want (BBa_K1602055_araCpBAD_Plate_5-18P).
2. Pipette 10μL of dH2O (distilled water) into the well. Pipette up and down a few times and let sit for 5 minutes to make sure the dried DNA is fully resuspended. The resuspension will be **red**, as the dried DNA has cresol red dye.
3. **Transform 1μL** of the resuspended DNA into your desired competent cells, plate your transformation with the appropriate antibiotic **(chloramphenicol)** and grow overnight

(Given that the araBAD promoter is upstream of a GFP gene, adding arabinose to the plate shall serve as an indicator of the functionality of the promoter, by detecting fluorescence)

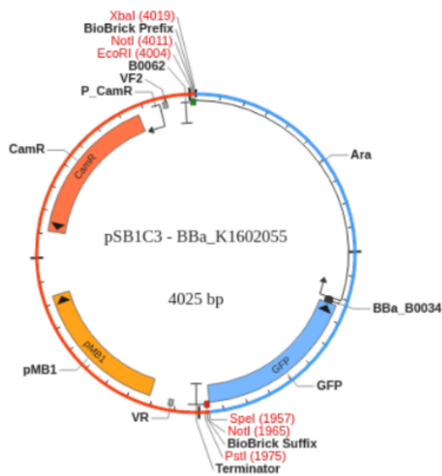
Day 2:

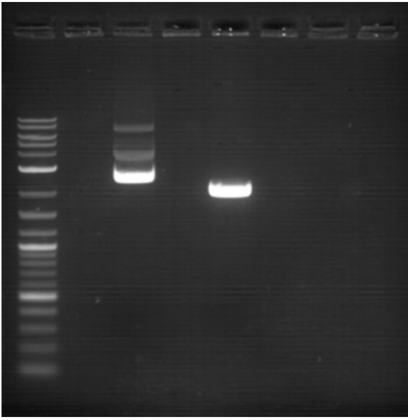
4. Pick a single colony and inoculate in LB medium, containing chloramphenicol and grow for 16 hours.

Day 3:

5. Use the resulting culture to miniprep the DNA **following the relevant protocol.**
6. Diagnostic Restriction Digestion, using EcoRI & PstI

- 1μL miniprep derived pSB1C3-ParaBAD
- 0,5μL EcoRI + o,5μL PstI
- 1μL 10x Cutsmart Buffer
- 7μL dH2O





7. NanoDrop (338,7 ng/μL).
8. Kapa Taq-mediated trial/routine PCR amplification of the araBAD promoter using the customized primers + Gel extraction of the promoter

Primer FOR: ATAACGTCTCGCTCGGGAGttatgacaacttgacgg

Primer REV: TTATCGTCTCGCTCAATGGctagtatttctcctctttctc

9. Calculate the required volumes of each component based on the following table, solving the dilution equation in each case ($C_i \times V_i = C_f \times V_f$) for a 20μL reaction.

Component	20 μL reaction	Final conc.
PCR-grade water	14,9μL	-----
10X KAPA Taq Buffer	2μL	1X
10 mM dNTPs mix	0,4μL	0.2 mM (in total)

10 µM Forward Primer	0,8µL	0.4 µM
10 µM Reverse Prime	0,8µL	0.4 µM
5 U/µL KAPPA Taq DNA Polymerase	0,1µL	0.5 U
Template DNA: plasmid pSB1C3	1µL	≤25 ng for less complex DNA

- **PCR tube containing araBAD:** 2µl buffer+0,4µL+0,8x2µL primers+0,1µL pol + 1µL plasmid +14,9 ddH20
- **PCR tube *negative/No template control (to check DNA impurities or contaminants)l*:** 2µl buffer+0,4µL+0,8x2µL primers+0,1µL pol +15,9 ddH20

10. Perform PCR with the following cycling protocol:

Step	Temperature	Duration	Cycles
Initial denaturation	95oC	3 min	1
Denaturation	95oC	30 sec	35
Annealing	67oC	30 sec	
Extension	72oC	1,5min	
Final extension	72oC	1,5min	1
Hold	25oC		1

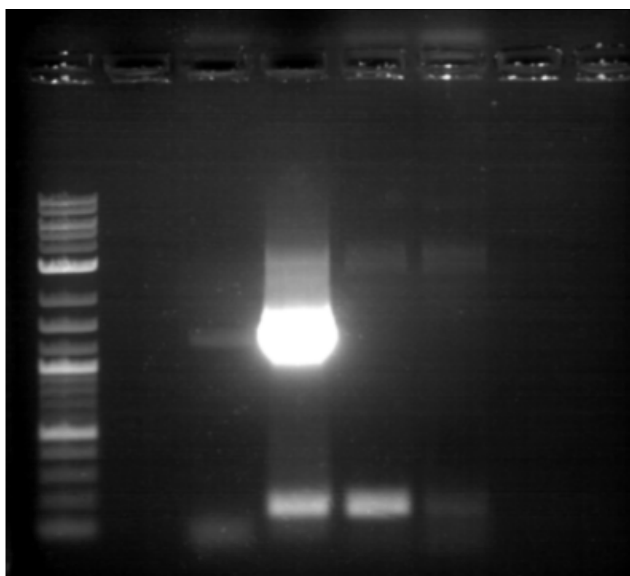


Figure 1 kapa PCR pSB1C3-araBAD_IT/Gradient PCR (NC-67oC-69oC-72oC)

11. <u>Run Gel electrophoresis (Negative Control+araBAD promoter)</u>

Materials for the gel
For 60ml of 1% w/v agarose we need:
- 60ml 1X TAE (diluted from 50X TAE stock)
- 0,6gr agarose
- 2,2μl EtBr (3.6ul for 100mL gel, 5.4ul for 150mL gel)

- Prepare the gel, following lab's protocol and let the gel solidify for 10-15min
- After 10-15mins, untighten the scaffold and remove the gel box with the gel still on.
- Put the gel in the "electrode box", filled with 1X TAE. If not full, add as TAE needed until it covers the gel.
- Load the samples in the gel wells.
- RUN at 100V

Sample Preparation for Gel Run
- 20μl quantity from PCR reaction in Eppendorf tube
- Add 4μl loading dye (6X) *(6xVo=1x(20+Vo) => 5Vo=20μL => Vo=4μL)*
- Final volume will be 24μl
- For the gel ladder, add 3.0μl

11. PCR protocol: Protocol for Q5® High-Fidelity 2X Master Mix

Component	50 μl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	25 μL	1X
10 μM Forward Primer	2.0 μL	0.5 μM
10 μM Reverse Primer	2.0 μL	0.5 μM
araBAD (10ng/μL)	2.0 μL	< 1,000 ng
Nuclease-Free Water	19 μL	-----

Thermocycling Conditions for a Routine PCR:

STEPS	TEMP	TIME
Initial Denaturation	98°C	30 seconds
	98°C	5–10 seconds
25–35 Cycles	72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	---

***12.* Gel electrophoresis: Follow the lab's protocol**

(Final volume to be loaded in the gel wells: 60μL from the PCR solution. 30μL in each well.
10μL of loading dye to be added in the PCR small tube: CoVo = CfVf => 6xVo = 1x(50+Vo) => Vo=10μL)

13. DNA extraction from Agarose gel, following the protocol of NucleoSpin® Gel and PCR Clean-up from by Macherey-Nagel

- Excise DNA fragment / solubilize gel slice with a scalpel, cleaned with ethanol and then Determine the weight of the gel slice and transfer it to a clean tube. For each 100 mg of agarose gel < 2 % add 200 µL Buffer NT
- Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.Load remaining sample if necessary and repeat the centrifugation step.
- Wash silica membrane by adding 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
- Dry silica membrane by centrifuging for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.
- Elute DNA by placing the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube and adding 15–30 µLBuffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 minute 11,000 x g

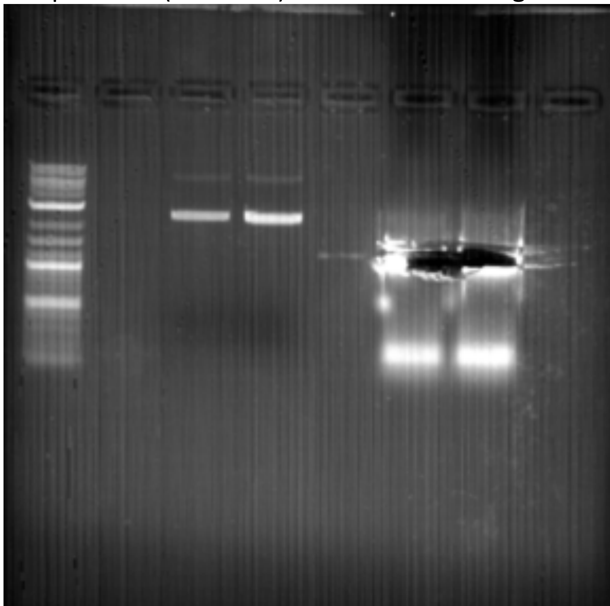


Figure 2 Q5 PCR for araBAD+Gel Extraction

14. <u>Spectophotometry using NanoDrop</u> (175,4 ng/µL)

15. <u>One-pot dig-lig reaction (GoldenBraid)</u>

50-75ng pUPD2	0,5µL
40-70ng of araBAD (post- Gel-extraction)	1µL
1µL 10X T4 DNA Ligase buffer	1µL
1µL T4 DNA Ligase	1µL
BsmB1	0,5µL
ddH_20	6Ml

SUM	10μL

Day 4: Transformation in chemo-competent cells

16. **Transformation, following lab's protocol, using the dig-lig product (pUPD2-araBAD)**

- Take competent cells out of -80°C and thaw on ice (approximately 20 mins).
- Mix 5μl of the dig-lig mixture DNA into 50-100 μL of competent cells in a microcentrifuge. GENTLY mix by flicking the bottom of the tube with your finger a few times.
- Incubate the competent cell-DNA mixture on ice for 20-30 mins.
- Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
- Put the tubes back on ice for 2-5 min.
- Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h
  (under sterile conditions). (This outgrowth step allows the bacteria time to generate the antibiotic resistance).
- Add X-gal on the agar plate or add it inside the cells and spread together.
- Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
- Incubate plates at 37°C overnight.

Day 5: Inoculate in LB medium culture

Day 6: Plasmid isolation and nucleic acid quantification

17. Miniprep
18. NanoDrop

Concentration (ng/μL)	A_{260}/A_{280}
424,6	1,75
423,9	1,46
430,9	1,50
428,8	1,73

19. Diagnostic Restriction Digestion
    - Cut with SlaI
    - In a 1,5 eppendorf tube, add the followings, so as to get a 10μL in total volume

CUT	UNCUT
• 2μL template	2μL template
• 0,5 SlaI (enzymeQuest)	-------
• 1μL buffer	-------
• 6μL dH_2O	8μL dH_2O

20. Incubate for 2 hours at 37oC
21. Conduct Gel electrophoresis, following the lab's protocol
22. Sample preparation for Gel Run
    - 10μL total volume with template

- Loading dye (6Vo=1(10+Vo) =>Vo=2μL

18/08/2020: Creation of a level 0/pUPD2-βarrestin2TEVp construct.

Goal:

- Digestion-Ligation for the purpose of creating a level 0 construct/pUPD2-barrestinTEVp
- Transformation of the subsequent product in chemo-competent cells
- Inoculation of colonies in LB medium
- Plasmid Isolation
- Nanodrop
- Diagnostic Restriction Digestion

PROCEDURE

A. Perform a dig-lig reaction, following the protocols below

- 0,5 μL pUPD2
- 1 μL of β-arrestin-TEVp part (from a working stock of 70ng/μL)
- 1 μL 10X T4 DNA ligase buffer
- 1 μL T4 DNA Ligase
- 0.5 μL restriction enzyme BsmBI for L0
- 6 μL ddH 2 O to reach 10 ul

- 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
- 5 min at 16 degrees C (optimal temp for Ligase)
- Repeat steps 1. and 2. 50 times
- 5 min at 80 degrees C (to kill enzymes)
- Rest at 16 degrees C

B. use 5 μL of dig-lig reaction to transform high-efficiency chemically competent cells, following the protocol. During transformation, I spin down the cells, resuspend at 200 uL volume (I just remove 800) and plate 150 ul.

C. Inoculation of 4 colonies into 4 falcon filled with LB medium and chloramphenicol (35μg/ml)

D. Plasmid isolation using the miniprep Nucleospin kit from Macherey-Nagel

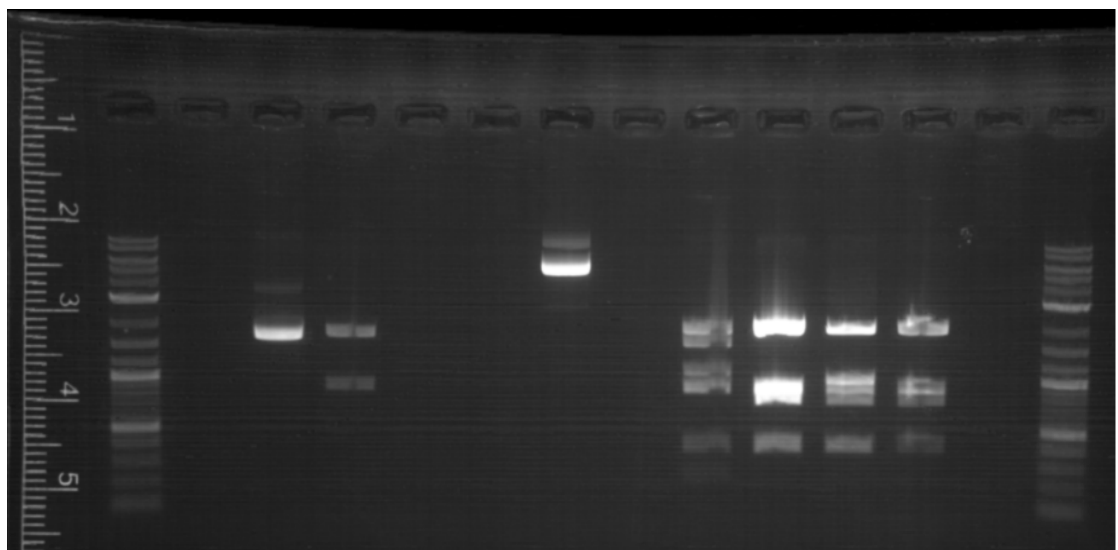
E. Nanodrop quantification of pUPD2-βarrestinTEVp

Concentration (ng/μL)	A_{260}/A_{280}
730,5	1,61
199,7	2,50
488,0	1,71
356,1	1,73

F. Perform a diagnostic Restriction-Digestion test with XhoI, following the aforementioned protocol (SlaI)

1st sample	2nd sample	3rd sample	4th sample
0,5μL template 0,5μL SlaI 1μL buffer 8μL dH_2O	2μL template 0,5μL SlaI 1μL buffer 6,5μL dH_2O	1μL template 0,5μL SlaI 1μL buffer 7,5μL dH_2O	1μL template 0,5μL SlaI 1μL buffer 7,5μL dH_2O

Ladder	**PUPD2 (άκοπο)**	**pUPD2 cut with SlaI**	**Uncut level 0**	**Cut level 0**	**Cut level 0**	**Cut level 0**	**Cut level 0**



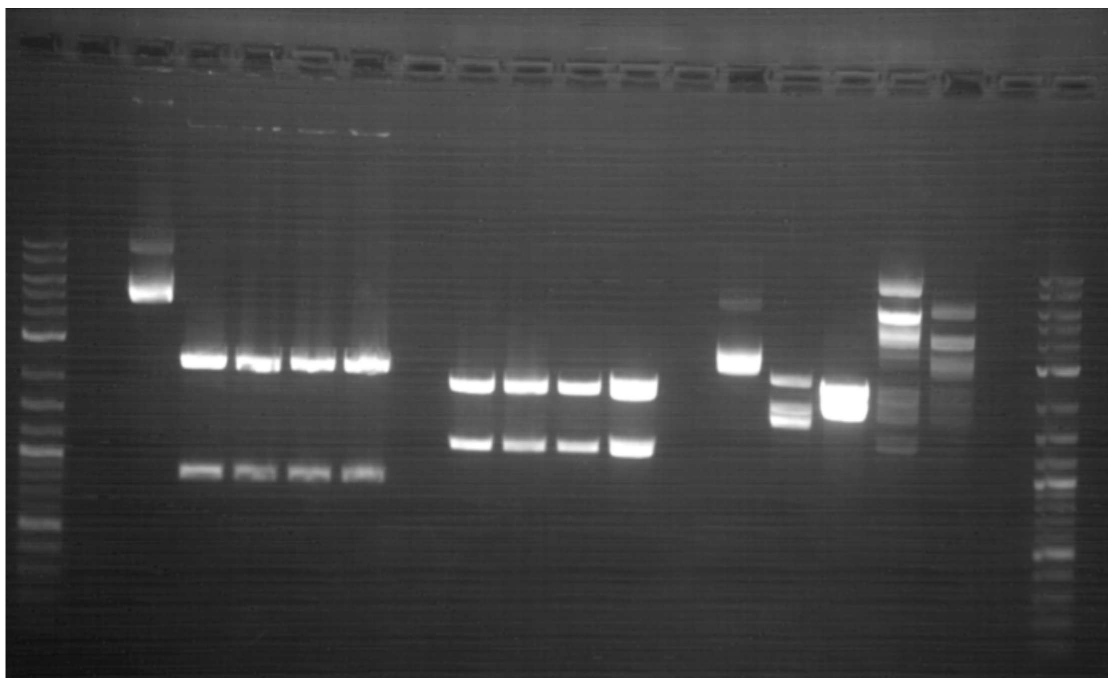
Experiment No: Run a diagnostic Restriction-Digestion for the level 0 pUPD2-araBAD part, as well as a second diagnostic for the level 0 PUPD2-βarrestin2TEVp part

MasterMix1 for PUPD2-araBAD	MasterMix2 for PUPD2-araBAD
0,5μLx 4 = 2μL SlaI 1μLx 4 = 4μL Buffer 8μLx 4 = 32μL dH_2O	0,5μL x 4 = 2μL EcoRV 1μL x 4 = 4μl buffer 7,5μL x 4 = 30μL dH_2O

level 0 araBAD (1)	level 0 araBAD (2)	level 0 araBAD (3)	level 0 araBAD (4)
0,5 μL template 9,5μL from MM	0,5 μL template 9,5μL from MM	0,5 μL template 9,5μL from MM	0,5 μL template 9,5μL from MM

MasterMix for pUPD2-β-arrestin-2-TEVp
0,5μL x 4 = 2μL EcoRV 1μL x 4 = 4μL Buffer

Level 0 β-arrestin-TEVp	**Level 0 β-arrestin-TEVp**	**Level 0 β-arrestin-TEVp**	**Level 0 β-arrestin-TEVp**
0,5μL template 1,5μL from MM 8μL dH_2O	2μL template 1,5μL from MM 6,5μL dH_2O	1μL template 1,5μL from MM 7,5μL dH_2O	1μL template 1,5μL from MM 7,5μL dH_2O



20-08-2020: Creating a level 1 TU: Anderson-lacO-RBSpeGFP-Term

Goals:

- **Dig-Lig using the aforementioned parts**
- **Transformation**
- **Cultivation**
- **Plasmid isolation**
- **Diagnostic Restriction Digestion**

Procedure

A] Digestion-Ligation for the purposes of creating a functional transcriptional unit which will express eGFP, following the respective protocol

• 0,65μL of pEGB-a1R vector (73,45ng) • 1μL pAnderson-lacO-RBS (70ng from a working stock solution of 70ng/μl)

- 0,5μL eGFP (66,9ng)
- 1μL Terminator (70ng from a working stock solution of 70ng/μL)
- 1μL T4 buffer
- 1μL T4 ligase
- 0,5μL BsaI
- 4,35μL ddH_2O

B] Transformation in DH5a chemo-competent cells, using 5μL of the pEGBa1R-egFP dig-lig product, following the aforementioned protocol

C] Selection of colonies with a pipette tip and inoculation in 10mL LB medium for approximately 18-24h

D]Plasmid isolation using the miniprep Nucleospin kit from Macherey-Nagel

E] Plasmid Quantification of the plasmid elution samples using Nanodrop

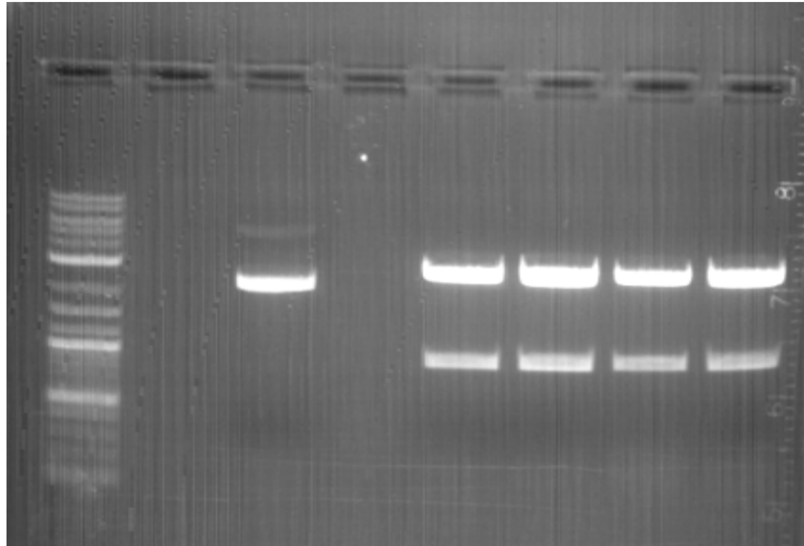
Concentration (ng/μL)	A_{260}/A_{280}
153,4	1,82
209,9	1,83
220,6	1,80
200,9	1,77

F] Perform a diagnostic restriction-digestion reaction cutting with BamHI restriction enzyme, following the respective protocol.

-Prepare the following MasterMix. Use 2μL of each and every of the plasmid elution preparations, aliquot 8μL of the MM in each of the 4 tubes and add 2μL of the loading dye solution.

MasterMix for RD of pEGBa1R-eGFP
0,5μL x 4 = 2,0 μL BamHI 1μL x 4 = 4,0 μL Buffer 6,5μL x 4 = 26 μL dH_2O

Ladder	pEGBa1R-eGFP Uncut plasmid	pEGBa1R-eGFP Cut with BamHI	pEGBa1R-eGFP Cut with BamHI	pEGBa1R-eGFP Cut with BamHI



23-08-2020: Designing level 1 constructs for FFAR2 & β-arrestin-2-TEVp, using the pEGBa1R and pEGBa2 level 1 vectors correspondingly

Goals:

- **Dig-Lig using the destination vectors and the level 0 parts**
- **Transformation**
- **Cultivation in LB medium**
- **Plasmid isolation**
- **Diagnostic Restriction Digestion**

PROCEDURE

A] Perform a dig-lig reaction for level 1 construct assembly, in accordance with the protocol.

pEGBa1R-FFAR2	pEGBa2-β-arrestin-2-TEVp
0,65μL a1R (73,45ng) 0,52μL FFAR2 (70ng) 1μL lacI (70ng) 1μL Terminator (70,49ng) 1μL araBAD promoter (70ng) 1μL buffer 1μL Ligase 1μL BsaI 3,33μL ddH_2O	0,60μL a2 (73,45ng) 1,0 μL β-arrestin-2-TEVp (70ng) 1μL Terminator (70,49ng) 1μL araBAD promoter (70ng) 1μL buffer 1μL Ligase 1μL BsaI 3,90μL ddH_2O

B] Transformation of DH5α chemo-competent cells with 5μL of the dig-lig product and selection of the transformants via the blue-white screening method.

C] Inoculation in LB medium containing kanamycin resistant gene.

D] Miniprep, following the aforementioned protocol

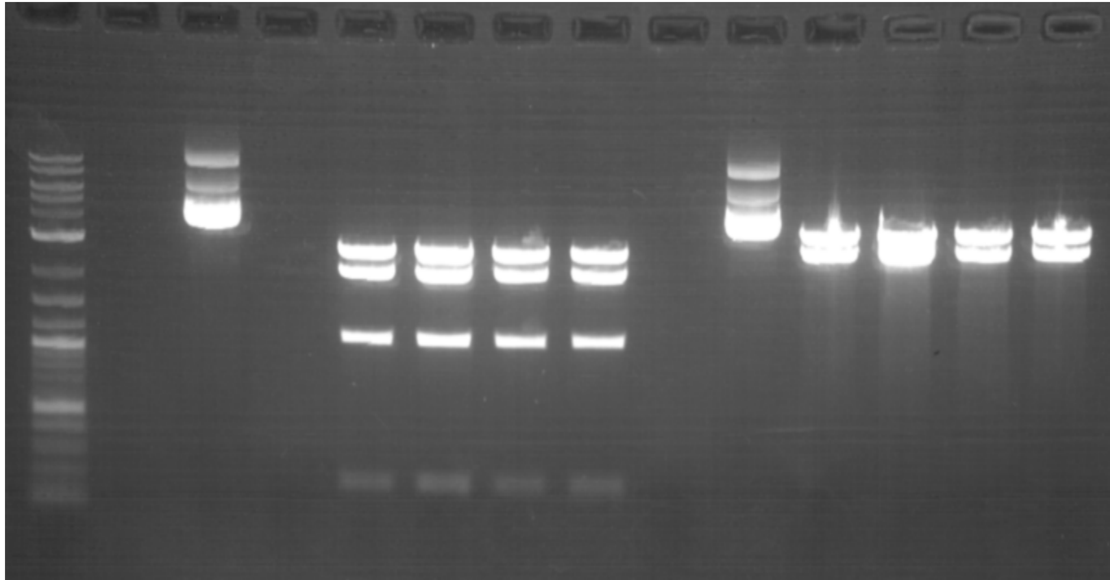
E] Nucleic acid quantification via nanodrop

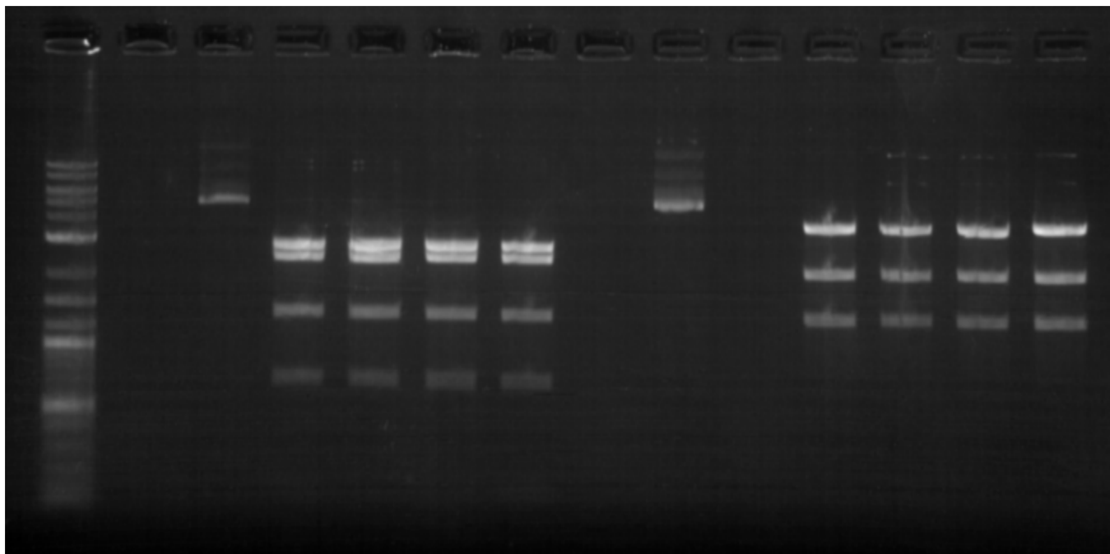
Concentrations (ng/µL)	A260/A280
level 1 / pEGBa1R-FFAR2	
352,9	1,81
239	1,83
358,7	1,83
234,7	1,85
level 1 / pEGBa2-β-arrestin-2-TEVp	
59,6	1,49
44,1	1,83
55,5	1,63
51,0	1,74

F] Double diagnostic Digestions for *a1R-FFAR2 and a2-β-arrestin-2-TEVp* transcription units

1st Diagnostic RD for a1R-FFAR2	2nd Diagnostic RD for a1R-FFAR2
• 2µL DNA template • 0,5µL BamHI • 1µL Buffer • 6,5µL ddH_2O	2µL DNA template 0,5 EcoRV 1µL Buffer 6,5µL ddH_2O

1st Diagnostic RD for a2-β-arrestin-TEVp	2nd Diagnostic RD for a2-β-arrestin-TEVp
• 4µL DNA template • 0,5µL HindIII • 1µL Buffer • 4,5µL ddH_2O	4µL DNA template 0,5 EcoRV 1µL Buffer 4,5µL ddH_2O





27-08-2020: Diagnostic restriction digestion of pUPD2-barrestin & pEGBa2-TU1b (βarrestin-TEVp)

2µL plasmid template	2µL plasmid template
0,5µL HindIII	0,5µL BamHI
1µL Buffer	1µL Buffer
6,5µL dH2O	6,5µL dH2O
MM level 0	**MM level 1**
0,5x5=2,6µL	0,5µLx4=2,0 BamHI
1µLx5=5µL	1µLx4= 4µL Buffer
6,5µLx5 = 32,6µL	6,5x4= 26,0 µL H20

ASTERIA

1/8/2020

Goals of the day

1. Competent Cells

A. Preperation of cells

1. Prepare Inoue transformation buffer (chilled to 0 °C before use).

a. Prepare 0.5 M PIPES (pH 6.7).

   Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H_2O to bring the final volume to 100 ml.

   Sterilize the solution by filtration through a disposable prerinsed Nalgene filter.

   Divide into aliquots and store frozen at -20 °C

b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H_2O.

c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-mm Nalgene filter. Divide into aliquots and store at -20 °C.

2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 h at 37 °C. Transfer the colony into 25 ml of LB broth or LB medium in a 250 ml flask. Incubate the culture for 6-8 h at 37 °C with vigorous shaking (250-300 rpm).

3. At about 7 o'clock in the evening, use this starter culture to inoculate three 1-L flasks, each containing 250 ml of LB. The first flask receives 10 ml of starter culture, the second receives 4 ml, and the third receives 2 ml. Incubate all three flasks overnight at 18 °C with moderate shaking.

2/8/2020

Goals of the day

1. Competent Cells

1. The following morning, read the OD_{600} of all three cultures. Continue to monitor the OD every 45 min.

   Only the flask with 10 ml of starter culture , had the desirable OD_{600} .
2. At about 12 o'clock we had the desirable OD_{600} , one of the cultures (the one with 10ml of starter culture) reaches 0.480, transfer the culture vessel to an ice-water bath for 10 min. Discard the two other cultures.
3. Harvest the cells by centrifugation at 2,500 *x g* (3,900 rpm in a Sorvall GSA rotor) for 10 min at 4 °C.
4. Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 min.
5. Resuspend the cells gently in 80 ml of ice-cold Inoue transformation buffer. The cells are best suspended by swirling rather than pipetting or vortexing.
6. Harvest the cells by centrifugation at 2,500 *x g* for 10 min at 4 °C.
7. Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 min.

B.Freezing of competent cells

1. Resuspend the cells gently in 20 ml of ice-cold Inoue transformation buffer.
2. Add 1.5 ml of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 min.
3. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes.
4. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen.
5. Store the tubes at -80 °C until needed. Freezing in liquid nitrogen enhances transformation efficiency by ~5-fold.Making, 200 ml aliquots of the competent-cell.

3/8/2020

<u>Goals of the day</u>

1. Transformation of Competent Cells

1. Include all of the appropriate positive and negative controls.
2. Add the transforming DNA (up to 25 ng per 50 ml of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 min.

3. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
5. Add 800 ml of LB medium to each tube. Warm the cultures to 37 °C in a water bath, and then transfer the tubes to a shaking incubator set at 37 °C. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
6. Transfer the appropriate volume (up to 200 ml per 90 mm plate) of transformed competent cells onto agar LB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic. When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 sec at room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml of LB medium by tapping the sides of the tube. IMPORTANT Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to RT, spread the transformed cells gently over the surface of the agar plate.
7. The plates should not be incubated for more than 20 h at 37 °C
8. Store the plates at RT until the liquid has been absorbed.
9. Invert the plates and incubate them at 37 °C. Transformed colonies should appear in 12-16 h.

4/8/2020

Goals of the Day

1. Receive and resuspend primers for PCR

1. **Resuspension**

   When still dry:

- Spin down the pellet (5.000-7.000 rpm for 7 sec). You need everything to be on the tip.
- Open the tube very carefully (don't touch the lid from the inside)
- Add appropriate volume of ddH_2O (creating a concentration of 100pmol/μL)
- Close the lid
- Vortex thoroughly (~20s)
- Spin down

This is the STOCK CONCENTRATION (100pmol/μL or 100μM)!!

To create the **working concentration 10pmol/μL or 10μM (1:10 of stock):**

Dilute 10μL of stock concentration into 90μL ddH_2O → total volume is 100μL

5/8/2020

Goals of the Day

1. Digestion-Ligation(DIG-LIG) to make to level0, constructs into pupd2.
2. Transformation the DIG-LIG product to DH5a cells

Procedure

1.One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 μL

- ✓ 50-75 ng acceptor vector

- ✓ 40-70 ng of each part
- ✓ 1 µL 10X T4 DNA ligase **buffer**
- ✓ 1 µL T4 DNA Ligase
- ✓ 0.5 µL restriction enzyme
- ✓ X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C

The protocol lasts 6h 08min.

Pupd2 vector	1 ul
Dig Lig parts	2 ul
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,5ul

2.Transformation

1. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
2. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
8. Incubate plates at 37°C overnight.

6/8/2020

Goals of the Day

1.Take the plates out of the incubator

2.Pick a single colony

Procedure

1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

7/8/2020

Goals of the Day

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Digestion (level 0 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3.

1. Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

I. **Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

II. **Cell lysis**

2. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
3. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
4. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completelyClarification of lysate**
5. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

III. **Bind DNA**

6. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
   Repeat this step to load the remaining lysate.

IV. **Wash silica membrane**

7. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
8. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

V. **Dry silica membrane**

9. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

VI. **Elute DNA**

10. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

11. Centrifuge for **1 min at 11,000 x g**.

2. Quantification

Teto1 —> 128.2 (1.76)

Teto2–> 123.2 (1.75)

Laco1–> 207.5 (1.76)

LacI—>165.7. (1.59)

Term—> 171.7 (1.71)

Tetr1–> 206.7 (1.65)

Tetr2–> 150.5 (1.73)

3.Diagnostic Digestion

1. Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer’s instructions.

Our restriction digestions are 50 uL in total volume.

- 5 µg DNA
- 0.5 µL BsmBI
- 5 µL 10x Buffe
- 39,5 µL dH2O (to bring total volume to 50µL)

sample	DNA	Restriction enzyme	Buffer	ddH_2O
TetO1	2 ul	XhoI , 0,3 ul	1 ul	6,7
TetO2	2ul	XhoI , 0,3 ul	1 ul	6,7
LacO1	1 ul	XhoI , 0,3 ul	1 ul	7,7

LacI1	2 ul	XhoI , 0,3 ul	1 ul	6,7
Terminator	2ul	XhoI , 0,3 ul	1 ul	6,7
TetR1	1 ul	XhoI , 0,3 ul	1 ul	7,7
TetR2	2ul	XhoI , 0,3 ul	1 ul	6,7

4. AGE

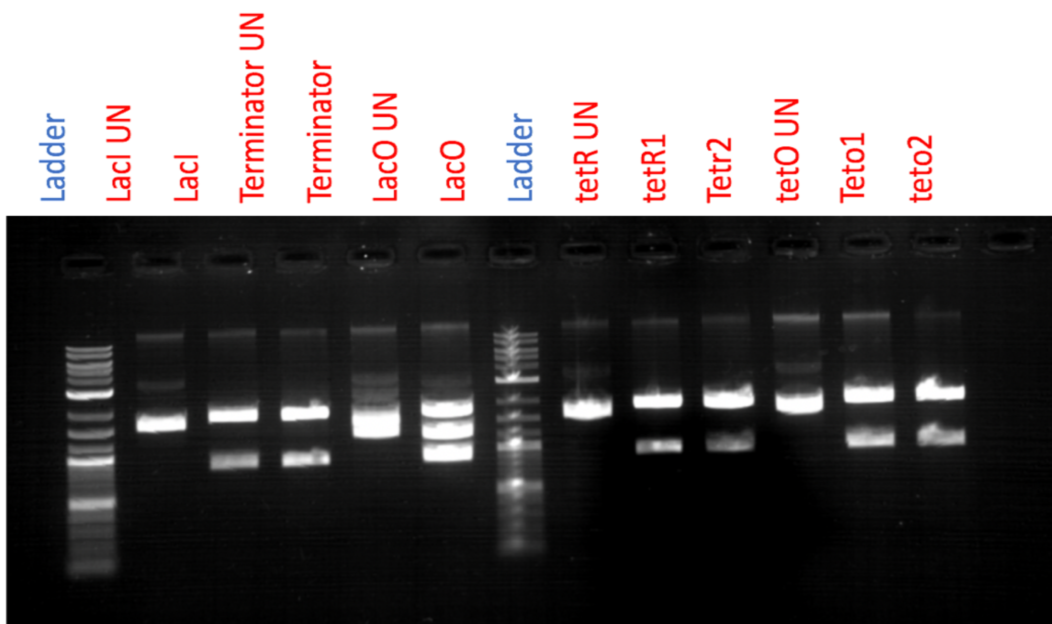
Materials for the gel

For 120ml of 1% w/v agarose we need

- **100ml 1X TAE (diluted from 50X TAE stock)**
- **1,2 gr agarose**
- **7 μl EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1,2 gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs.
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box. Avoid the bubbles.

9. Remove bubbles with a tip (or piece of paper) if needed. If you can't remove them, move them in a place where they won't affect your DNA.
10. Let the gel solidify for 10-15min
11. After 10-15mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.



Expected bands : lacI → 2300 +893 bp

Terminator → 1346 bp

lacO→ 2382 bp

tetR → 1835

tetO→ 1268

8/8/2020

Goals of the Day

1. Digestion (level 0 constructs)
2. Agarose Gel Electrophoresis (AGE)

1. Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:
4. DNA
5. Restriction Enzyme(s)
6. Buffer
7. dH2O up to total volume
8. Mix gently by pipetting.
9. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

Our restriction digestions are 10 uL in total volume.

sample	DNA	Restriction enzyme	Buffer (Cutsmart)	ddH_2O
TetO1	2 ul	EcoRI , 0,3 ul	1ul	6,7
TetO2	2ul	EcoRI , 0,3 ul	1ul	6,7
LacO1	1 ul	EcoRI , 0,5 ul	1 ul	7,5
LacO1	1 ul	XhoI 0,3 ul	1	7,7
LacI1	2 ul	EcoRV 0,3 ul	1 ul	6,7
Terminator	2ul	NotI , 0,5 ul	1 ul	6,5
TetR1	1 ul	EcoRV +HindIII 0,3+0,3 ul	1 ul	7,4
TetR2	2ul	EcoRV +HindIII 0,3+0,3 ul	1 ul	6,7

2. AGE

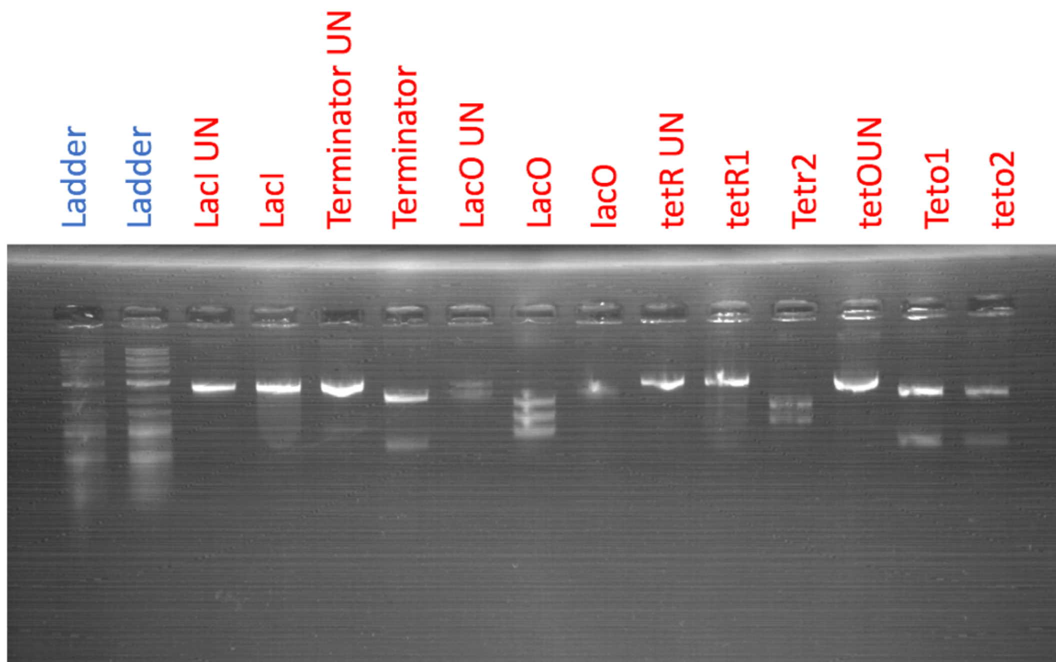
Materials for the gel

For 120ml of 1% w/v agarose we need

- **120 ml 1X TAE (diluted from 50X TAE stock)**
- **1,2 gr agarose**
- **7 μl EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1,2 gr of dry agarose.
2. Mix TAE and agarose in a flask

3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs.
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box. Avoid the bubbles.
9. Remove bubbles with a tip (or piece of paper) if needed. If you can't remove them, move them in a place where they won't affect your DNA.
10. Let the gel solidify for 10-15min
11. After 10-15mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.



Expected bands: LacI → 1737 +1455 bp

Terminator → 2238 bp

LacO (fisrt -with XhoI) → 3192 bp

LacO (second-with EcorI)→ 892 +2382 bp

TetR → 1553 +1174 bp

TetO → 2046 +129 bp

Goals of the day : The expected digestion profile , for the clones with the correct insertions was not found after the digestions, thus I proceeded to transformation with the rest of the dig-lig reaction. I use only the Anderson-promoter.

2.Transformation

1. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
2. Mix 5μl of DNA (dig-lig) into 100 μL of competent cells in a microcentrifuge or falcon tube. Gently mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
5. Put the tubes back on ice for 2-5 min.
6. Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
8. Incubate plates at 37°C overnight.

9/08/2020

Goals of the Day

1. Take the plates out of the incubator
2. Pick a single colony
3. Digestion
4. Agarose Gel Electrophoresis (AGE) (TBE 0,5X)

1.Digestion

1. Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:
4. DNA
5. Restriction Enzyme(s)
6. Buffer
7. dH2O up to total volume
8. Mix gently by pipetting.
9. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
10. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
Terminator	2 ul	EcoRI+ EcoRv 0,5+0,5	2	15
LacI	2ul	EcoRI+ EcoRv 0,5+0,5	2	15
Laco	2 ul	EcoRI+ EcoRv 0,5+0,5	2	15
TetR1	2 ul	EcoRI+ EcoRv 0,5+0,5	2	15
TetR2	2 ul	EcoRI+ EcoRv 0,5+0,5	2	15

2. AGE

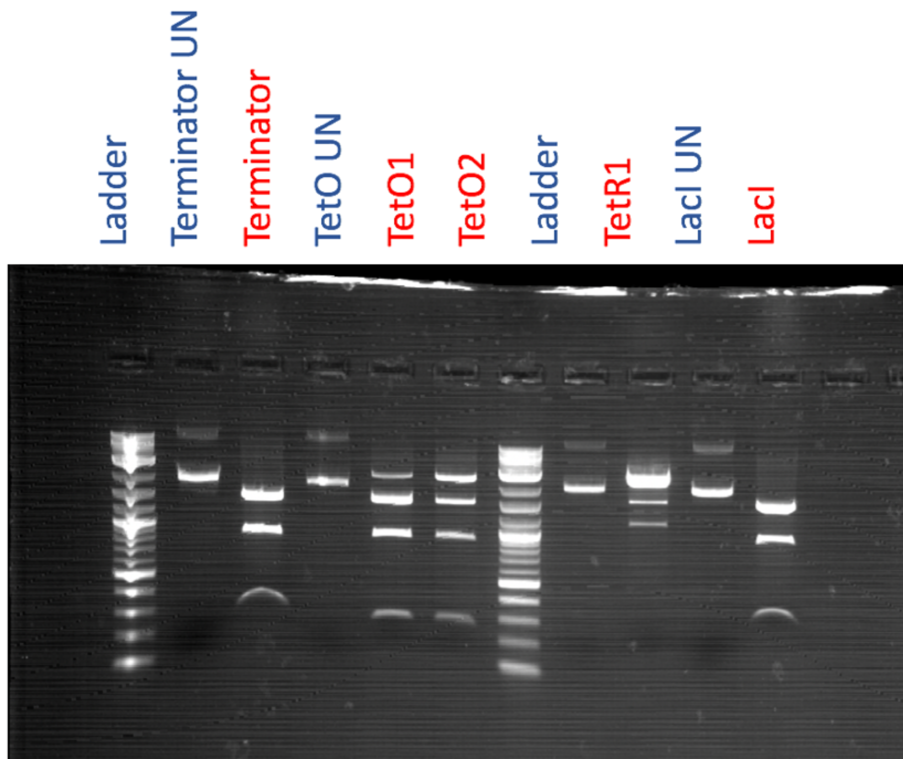
Materials for the gel

For 120ml of 1% w/v agarose we need

- **120ml 1X TAE (diluted from 50X TAE stock)**

- **1,2 gr agarose**
- **7 μl EtBr**

16. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1,2 gr of dry agarose.
17. Mix TAE and agarose in a flask
18. Microwave for 2-3 mins (until it boils)
19. After microwaving, mix it thoroughly under running water until it cools down.
20. When in the right temperature (not too hot), add EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
21. Prepare the gel box, combs.
22. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
23. Add the liquid gel slowly into the gel box. Avoid the bubbles.
24. Remove bubbles with a tip (or piece of paper) if needed. If you can't remove them, move them in a place where they won't affect your DNA.
25. Let the gel solidify for 10-15min
26. After 10-15mins, untighten the scaffold and remove the gel box with the gel still on.



The desirable profile didn’t exist in any of our samples.

3. Take the plates out incubator , put them in fridge so the colonies won’t grow much. Moreover me want to see clearly the blue-white colonies.
4. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

11/08/2020

Goals of the day

1. Miniprep- Plasmid Isolation Protocol – Homemade protocol
2. Quantification of DNA – Nanodrop
3. Digestion (level 0 constructs)
4. Agarose Gel Electrophoresis (AGE) for 3.

1.Miniprep- Plasmid isolation

I. **Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **1 min at 11.000 rpm.** Discard the supernatant and remove as much of the liquid as possiple. Repeat this step with the remaining volume of the LB culture (use as stock 1 ml out of 5ml , just in case.)

II. Cell lysis

2.Add **150 µl Buffer A1** . Resuspend the cell pellet completely by vortexing (or with a pippete). **Make sure no cell clumps remain before addition of Buffer A2!**

3. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

4.Add **300 µL Buffer A3**. Mix thoroughly (and quick) by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!

III. Clarification of lysate

5. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear

III. Bind DNA

6. Decant the supernatant into a new 1,5ml Eppendorf , without agitating the sediment
7. Add 450 µl Isopropanol 100% (1:1 sample : isopropanol), and mix thoroughly.
8. Store the tubes at -80 ^oC or -20 ^oC for 30 min up to 1 hour.
9. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear
10. Discard the supernatant without agitating the sediment
11. Resuspend the sediment with 30-50 µl ddH_2O .
12. Store the tubes at -20 ^oC until needed.

2.Quantification of DNA

Anderson I : 1780.8 (1.73)
Anderson II : 999.4 (1.81)

3.Diagnostic Digestion

1.Digestion

11. Select restriction enzymes to digest your plasmid.
12. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
13. In a 1.5mL tube combine the following:
14. DNA
15. Restriction Enzyme(s)
16. Buffer
17. dH2O up to total volume
18. Mix gently by pipetting.
19. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
20. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
Anderson I	0,3 µl	XhoI 0,5 ul	2 ul	17,2 µl
Anderson I	0,3 µl	EcoRI 0,5ul	2 ul	17,2 µl
Anderson II	0,5 µl	XhoI 0,5 ul	2 ul	17 µl
Anderson II	0,5 µl	EcoRI 0,5ul	2 ul	17 µl

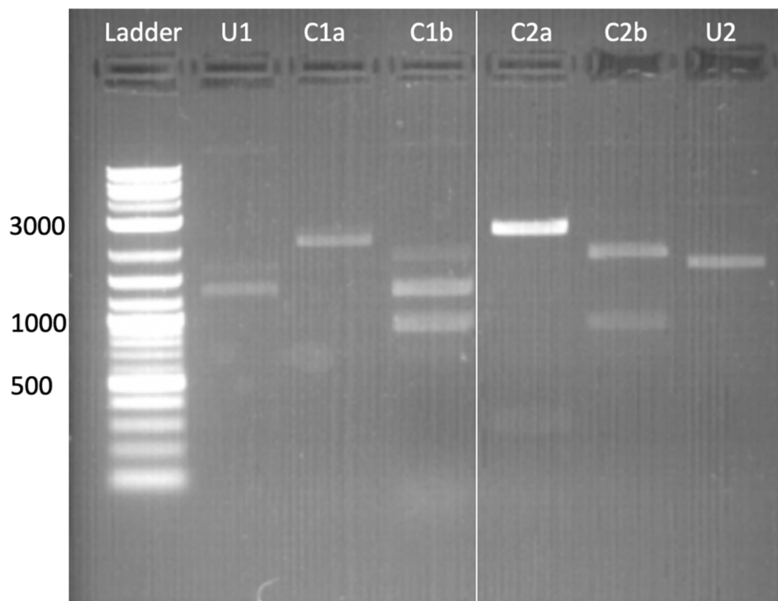


Figure : (U=Uncut , C= Cut) Restriction digestion of **AndersonJ23115** with :
→EcoRI (C1a & C2a) , Expected bands : 2156 bp
→ XhoI (C1b & C2b) , Expected bands : 1264 + 892 bp
→ Positive result: The set of C1a and C1b (same sample)

3.Take the plates out fridge, from previous day, and

we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C

12/08/2020

Goals of the Day

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Digestion (level 0 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3.

- **Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol**

1. **1.Cultivate and harvest bacterial cells**
2. 2.Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.
3. **Cell lysis**
4. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
5. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
6. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!
    a. *Make sure to neutralize completely to precipitate all protein and chromosomal DNA. LyseControl should turn completely colorless without any traces of blue.*
7. **Clarification of lysate**
8. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear
9. **Bind DNA**
10. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 (I do 750 without a problem without needing to do second centrifugation) µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
    a. **Repeat this step to load the remaining lysate.**
11. **Wash silica membrane**
12. *Recommended: If plasmid DNA is prepared from host strains containing high levels of nucleases (e.g., HB101 or strains of the JM series), it is strongly recommended performing an additional washing step with **500 µL Buffer AW**, optionally preheated to 50 degrees C, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4. Additional washing with Buffer AW will also increase the reading length of DNA*

sequencing reactions and improve the performance of critical enzymatic reactions. **Always do this step, it provides nice and clear DNA.**

   a. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

13. **Dry silica membrane**
14. Centrifuge for **5 min at 11,000 x g** and discard the collection tube
    a. *Note: Residual ethanolic wash buffer might inhibit enzymatic reactions.*
15. **Elute DNA**
16. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**. Incubate for 5 min at room temperature. Centrifuge for **1 min at 11,000 x g**. Repeat this step twice (When you centrifuge, pipette the volume from the Eppendorf tube and feed it to the column, let 5 min and then centrifuge again. This increases yield especially for medium to low copy plasmids.

2.Quantification of DNA – Nanodrop

TetR2 : 548.1 (1.74)

LacO1 : 858.8 (1.80)

PrPB1: 783.2 (1.73)

PrPB2 : 1113.5 (1.67)

TetR3 : 704.7 (1.78)

TetR1 : 589.5 (1.79)

LacO2 : 566.8 (1.81)

Terminator: 426.8 (1.79)

1.Digestion

21. Select restriction enzymes to digest your plasmid.
22. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
23. In a 1.5mL tube combine the following:
24. DNA
25. Restriction Enzyme(s)
26. Buffer
27. dH2O up to total volume
28. Mix gently by pipetting.

29. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
30. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
TetR1	1ul	EcoRI 0,5 ul	2 ul	16,5 µl
TetR2	1ul	EcoRI 0,5ul	2 ul	16,5 µl
TetR3	1ul	EcoRI 0,5 ul	2 ul	16,5 µl
LacO1	1ul	EcoRI 0,5ul PstI 0,5 ul	2 ul	16,5 µl
LacO2	1ul	EcoRI 0,5ul PstI 0,5 ul	2ul	16,5 µl
PrPB1	1ul	XhoI 0,5 ul	2ul	16,5 µl
PrPB2	1ul	XhoI 0,5 ul	2ul	16,5 µl
Terminator	1ul	EcoRI 0,5ul PstI 0,5 ul	2ul	16,5 µl

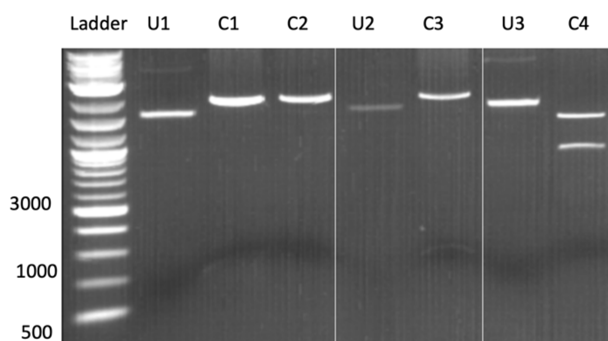


Figure2 : (U=Uncut , C= Cut) Restriction digestion of **lacO** (C1& C2)with :
→EcoRI & PstI (C1a & C2a) , Expected bands : 2029 + 144 bp
→Positive result: C1a
Restriction digestion of **Terminator** (C3)with :
→ EcoRI & PstI (C3), Expected bands : 2029 + 209 bp
→ Positive result : C3
Restriction digestion of **PrpB** (C4) with :
→ XhoI (C4) , Expected bands : 1400+900 bp
→ Positive result : C4

13/08/2020

Goals of the day :

1. Restriction Digestion

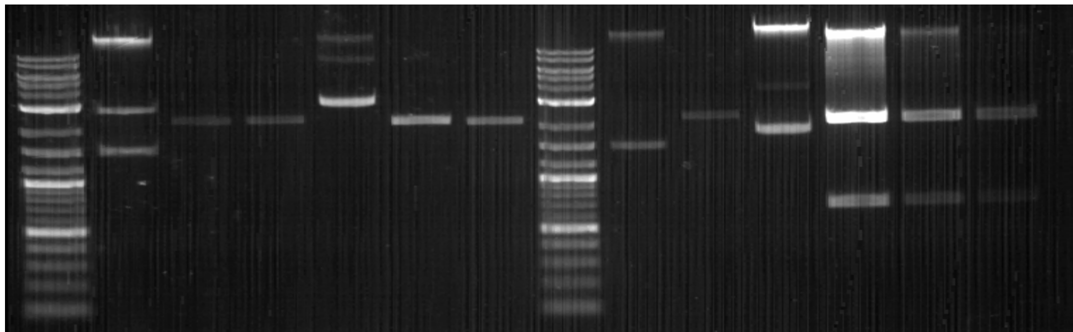
1.Digestion

31. Select restriction enzymes to digest your plasmid.
32. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
33. In a 1.5mL tube combine the following:
34. DNA
35. Restriction Enzyme(s)
36. Buffer
37. dH2O up to total volume
38. Mix gently by pipetting.
39. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
40. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
TetR1	1ul	EcoRI 0,5 ul	2 ul	16,5 µl
TetR2	1ul	EcoRI 0,5ul	2 ul	16,5 µl
TetR3	1ul	EcoRI 0,5 ul	2 ul	16,5 µl
LacO1	1ul	EcoRI 0,5ul PstI 0,5 ul	2 ul	16,5 µl
LacO2	1ul	EcoRI 0,5ul PstI 0,5 ul	2ul	16,5 µl
PrPB1	1ul	XhoI 0,5 ul	2ul	16,5 µl
PrPB2	1ul	XhoI 0,5 ul	2ul	16,5 µl
Terminator	1ul	EcoRI 0,5ul	2ul	16,5 µl

		PstI 0,5 ul		

Ladder prpBUN prpB1 prpB2 lacOUN lacO1 lacO2 ladder termUN term1 tetRUN tetR tetR2. Tetr3



Positive results : prpB1 , prpB2 ,LacO1 , LacO2 , Terminator , TetR2, Tetr3

17/08/2020

Goals of the Day

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Digestion (level 0 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3

5. Transformation stuffer

11.Miniprep- Plasmid isolation

IV. **Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **1 min at 11.000 rpm.** Discard the supernatant and remove as much of the liquid as possiple. Repeat this step with the remaining volume of the LB culture (use as stock 1 ml out of 5ml , just in case.)

V. Cell lysis

2.Add **150 µl Buffer A1** . Resuspend the cell pellet completely by vortexing (or with a pippete). **Make sure no cell clumps remain before addition of Buffer A2!**

3. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

4.Add **300 µL Buffer A3**. Mix thoroughly (and quick) by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!

III. Clarification of lysate

13. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear

VI. Bind DNA

14. Decant the supernatant into a new 1,5ml Eppendorf , without agitating the sediment
15. Add 450 µl Isopropanol 100% (1:1 sample : isopropanol), and mix thoroughly.
16. Store the tubes at -80 ^oC or -20 ^oC for 30 min up to 1 hour.
17. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear
18. Discard the supernatant without agitating the sediment
19. Resuspend the sediment with 30-50 µl ddH_2O .
20. Store the tubes at -20 ^oC until needed.

2.Quantification of DNA

TetR1 → 900,6
TetR2 → 5075,5
TetR3 → 2024,1
TetO1 → 3067,4
TetO2 → 2276
TetO3 → 2748,4
TetO4 → 4496,1

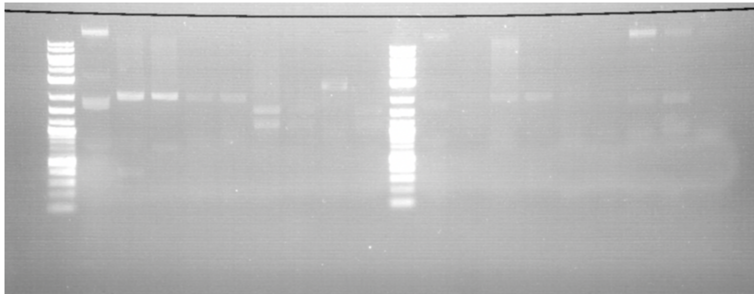
Dilution all samples so as to use 1 ul to digestion (appx. 500 ng)

1.Digestion

41. Select restriction enzymes to digest your plasmid.
42. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
43. In a 1.5mL tube combine the following:
44. DNA

45. Restriction Enzyme(s)
46. Buffer
47. dH2O up to total volume
48. Mix gently by pipetting.
49. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
50. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
TetR	1ul	EcoRI 0,5 ul PstI 0,5 ul	2 ul	16 µl
TetR	1ul	BamHI 0,5 ul EcoRv	2ul	16,µl
TetO	1ul	EcoRI 0,5ul PstI 0,5 ul	2ul	16µl
TetO	1ul	xhoI 0,5 ul	2 ul	16,5 ul



The gel isn't good . Repeat this tomorrow

2.Transformation the stuffer

9. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
10. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
11. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
12. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
13. Put the tubes back on ice for 2-5 min.
14. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

15. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
16. Incubate plates at 37°C overnight.

18/08/2020
Exactly the same procedure as yesterday :

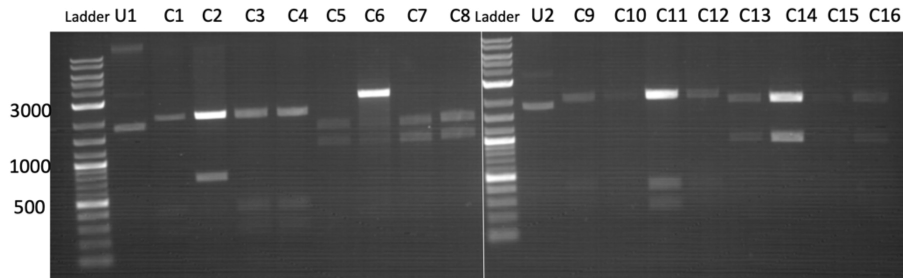


Figure 6: (U=Uncut , C= Cut) Restriction digestion of **TetR** (C1-C8) with :
→EcoRV + PstI (C1-C4) , Expected bands : 2029 + 698 bp
→Positive result: C2/C10 (the same sample)
→ BamHI + EcoRV (C5-C8) , Expected bands :2727 bp
Restriction digestion of **tetO** (C9 –C16) with :
→ PstI + EcoRV (C9 – C12) , Expected bands : 2029 +146 bp
→ XhoI (C13-C16) , Expected Bands : 1283 + 892 bp

2. Restriction digestion Of stuffer

Diagnostic Digestion

6. Select restriction enzymes to digest your plasmid.
7. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
8. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

9. Mix gently by pipetting.
10. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

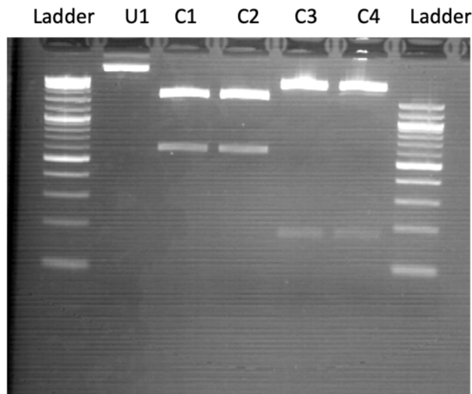


Figure 10: (U=Uncut , C= Cut) Restriction digestion of **Stuffer** with BspHI (C1-C2) and HindIII (C3-C4)
→ Positive result: all
→ Length of stuffer : 180 bp

20/08/2020

Goals of the day :

1.Transformation of TetO (5ul of dig-lig)

2.Transformation

1. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
2. Mix 5μl of DNA (dig-lig) into 100 μL of competent cells in a microcentrifuge or falcon tube. Gently mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
5. Put the tubes back on ice for 2-5 min.
6. Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

21/08/2020

1. 1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

22/08/2020

Goals of the day :

1. Miniprep- Plasmid Isolation Protocol – Homemade protocol
2. Quantification of DNA – Nanodrop
3. Digestion (level 0 constructs)
4. Agarose Gel Electrophoresis (AGE) for 3.

<u>1.Miniprep- Plasmid isolation</u>

VII. **Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **1 min at 11.000 rpm.** Discard the supernatant and remove as much of the liquid as possiple. Repeat this step with the remaining volume of the LB culture (use as stock 1 ml out of 5ml , just in case.)

VIII. Cell lysis

2.Add **150 μl Buffer A1** . Resuspend the cell pellet completely by vortexing (or with a pippete). **Make sure no cell clumps remain before addition of Buffer A2!**

3. Add **250 μL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

4.Add **300 μL Buffer A3**. Mix thoroughly (and quick) by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!

III. Clarification of lysate

21. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear

IX. **Bind DNA**

22. Decant the supernatant into a new 1,5ml Eppendorf , without agitating the sediment
23. Add 450 μl Isopropanol 100% (1:1 sample : isopropanol), and mix thoroughly.
24. Store the tubes at -80 ^{o}C or -20 ^{o}C for 30 min up to 1 hour.
25. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear
26. Discard the supernatant without agitating the sediment

27. Resuspend the sediment with 30-50 μl ddH_2O .
28. Store the tubes at -20 ^oC until needed.

2.Quantification of DNA

TetO 1 → 132.5 (1.74)

TetO2 → 142.7 (1.73)

TetO3 → 125.9 (1.73)

TetO4 → 125.9 (1.73)

3.Diagnostic Digestion

1.Digestion

51. Select restriction enzymes to digest your plasmid.
52. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
53. In a 1.5mL tube combine the following:
54. DNA
55. Restriction Enzyme(s)
56. Buffer
57. dH2O up to total volume
58. Mix gently by pipetting.
59. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.
60. Our restriction digestions are 20 uL in total volume.

sample	DNA	Restriction Enzyme	Buffer	ddH_2O
Tet01	1 ul	Not HF 0,5 ul	2 ul	16,5 ul
TetO2	1 ul	Not HF 0,5 ul	2 ul	16,5 ul
TetO3	1 ul	Not HF 0,5 ul	2 ul	16,5 ul
TetO4	1 ul	Not HF 0,5 ul	2 ul	16,5 ul

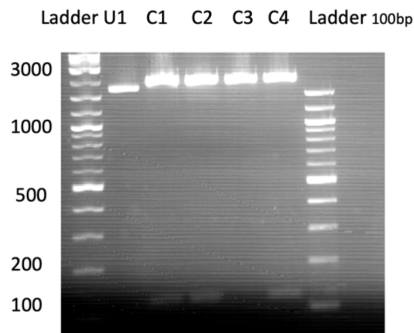


Figure 7: (U=Uncut , C= Cut) Restriction digestion of **TetO** (C1-C4) with :
→Not-HF(C1-C4) , Expected bands : 2046 + 129 bp
→Positive result: C1,C2,C4

23/08/2020

Goals of the day :

1. Digestion-Ligation(DIG-LIG) to make to level0, constructs into pupd2.
2. Transformation the DIG-LIG product to DH5a cells

1.One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- ✓ 50-75 ng acceptor vector
- ✓ 40-70 ng of each part
- ✓ 1 µL 10X T4 DNA ligase **buffer**
- ✓ 1 µL T4 DNA Ligase
- ✓ 0.5 µL restriction enzyme
- ✓ X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

6. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
7. 5 min at 16 degrees C (optimal temp for Ligase)
8. Repeat steps 1. and 2. **50 times**
9. 5 min at 80 degrees C (to kill enzymes)
10. Rest at 16 degrees C

The protocol lasts 6h 08min.

T:andersonJ23115:tetR:terminator

a1R vector	0,4 ul
Terminator	1 ul
TetR	1 ul
AndersonJ23115	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

LE: AndersonJ23115:LacO-ecfp-terminator

a1R vector	0,4 ul
Terminator	1 ul
ecfp	1 ul
AndersonJ23115- LacO	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

TL: AndersonJ23115:TetO-LacI-terminator

a2 vector	0,4 ul
Terminator	1 ul
laci	1 ul
AndersonJ23115- TetO	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

2.Transformation

17. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
18. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
19. Incubate the competent cell-DNA mixture on ice for 20-30 mins.

20. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
21. Put the tubes back on ice for 2-5 min.
22. *Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).*
23. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
24. Incubate plates at 37°C overnight.

24/08/2020

Goals of the day :

1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 ºC.

25/08/2020

Goals of the day :

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Digestion (level 0 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3.

1.Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

VII. **Cultivate and harvest bacterial cells**

12. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

VIII. **Cell lysis**

13. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
14. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
15. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completelyClarification of lysate**
16. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

IX. **Bind DNA**

17. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
    Repeat this step to load the remaining lysate.

X. **Wash silica membrane**

18. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
19. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

XI. **Dry silica membrane**

20. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

XII. **Elute DNA**

21. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

22. Centrifuge for **1 min at 11,000 x g**.

2.Quantification

TL1 → 70.3 (1.63)

TL2→ 57.6 (1.70)

TL3→ 57.4 (1.64)

TL4→ 41.0 (1.72)

LE1 → 155.0 (1.73)

LE2→ 119.5 (1.77)

LE3→ 134.8 (1.79)

LE4→320.7 (1.75)

T1→ 154.6(1.76)

T2→ 190.2 (1.79)

T3→ 244.1 (1.77)

T4→ 186.5 (1.77)

3.Diagnostic Digestion

11. Select restriction enzymes to digest your plasmid.
12. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
13. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

14. Mix gently by pipetting.
15. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

Sample	DNA	Restriction enzyme	Buffer	ddH_2O

T1	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
T2	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
T3	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
T4	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
LE1	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
LE2	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
LE3	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
LE4	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
TL1	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
TL2	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
TL3	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul
TL4	1 ul	HindIII 0,5 ul NheI 0,5 ul	2 ul	16 ul

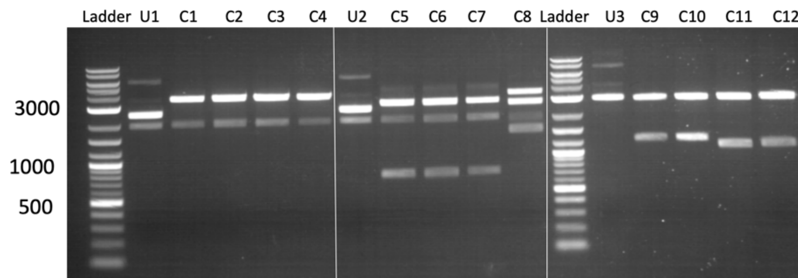


Figure 8: (U=Uncut , C= Cut) Restriction digestion of **T: AndersonJ23115-TetR-double terminator** (C1-C4) with :
→HindIII + NheI (C1-C4) , Expected bands : 3012 + 638 (+23 out of gel) bp
→Positive result: none
Restriction digestion of **LE: AndersonJ23115:Lac0-EGFP-double terminator**(C5-C8) with:
→ HindIII + NheI (C5-C8) , Expected bands : 3020 + 754 bp
→ Positive results : C5,C6,C7
Restriction digestion of **TL: AndersonJ23115:Tet0-LacI-double terminator**(C5-C8) with:
→ HindIII + NheI (C9-C16) , Expected bands : 2573 +1268 bp
→ Positive result : C11,C12

28/08/2020

1.Digestion (level 0 constructs)

2.Agarose Gel Electrophoresis (AGE) for 3.

1.Diagnostic Digestion

16. Select restriction enzymes to digest your plasmid.
17. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
18. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

19. Mix gently by pipetting.
20. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer’s instructions.

Sample	DNA	Restriction enzyme	Buffer	ddH_2O
LE1	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
LE2	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
LE3	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
LE4	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
TL1	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
TL2	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
TL3	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul
TL4	1 ul	HindIII 0,5 ul BtgZI 0,5 ul	2 ul	16 ul

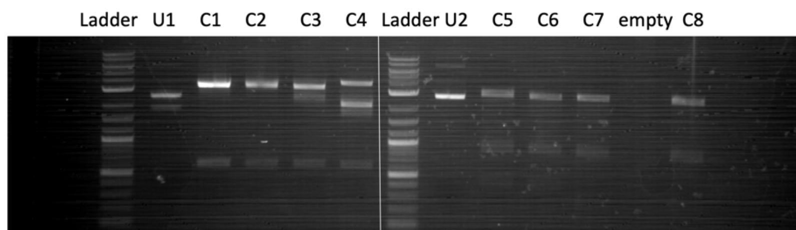


Figure 9: (U=Uncut , C= Cut) Restriction digestion of **LE: AndersonJ23115:Lac0-EGFP-double terminator**(C1-C 4) with :
→HindIII + BtgZI (C1-C4) , Expected bands : 3200+ 597bp
→Positive result: C1,C2
Restriction digestion of **TL: AndersonJ23115:Tet0-LacI-double terminator**(C5-C8) with:
→ HindIII + NheI (C9-C16) , Expected bands : 2573+891+427
→ Positive result : C5,C6

At the end of the day we discuss the digestion profile of the stuffer: the profile was not right. We couldn't see the 3 configurations of plasmid and the band was too high. After that we try to figure what was wrong . We do restriction digestions again of the stuffer :

1. Nanodrop again
   Stuffer 1 : 841.3 (1.80)
   Stuffer 2: 720.9 (1.79)

2. Restriction digestion of stuffer

Diagnostic Digestion

21. Select restriction enzymes to digest your plasmid.
22. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
23. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

24. Mix gently by pipetting.
25. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

Sample	DNA	Restriction enzyme	Buffer	ddH_2O
Stuffer 1	1 ul	BspHI 0,5 ul	2 ul	16,5 ul
Stuffer 1	1 ul	HindIII 0,5 ul	2 ul	16,5 ul
Stuffer2	1 ul	BspHI 0,5 ul	2 ul	16,5 ul
Stuffer2	1 ul	HindIII 0,5 ul	2 ul	16,5 ul

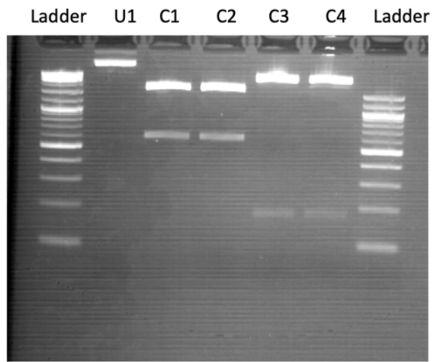
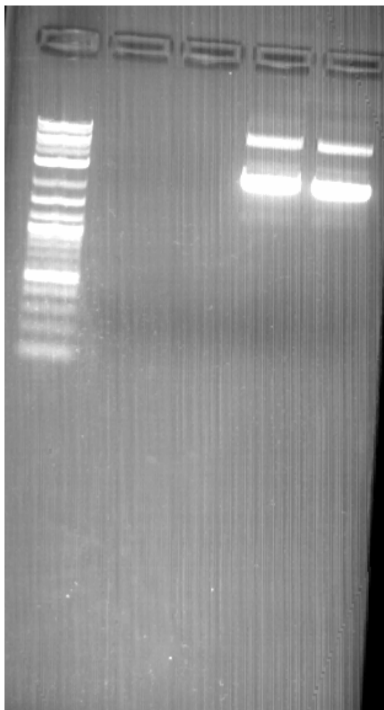


Figure 10: (U=Uncut , C= Cut) Restriction digestion of **Stuffer** with BspHI (C1-C2) and HindIII (C3-C4)

→ Positive result: all

→ Length of stuffer : 180 bp



Here we have a problem with length of the plasmid as we can detect the uncut versions.

03/09/2020

Goals of the day :

1. Transformation of the constructs with ecfp and egfp into BL21 and MC1061

Manolis : egfp
Magda : lacO -egfp
Asteria : lacO-egfp
TetO-egfp
And a negative control : TetR-LacI

2.Transformation

25. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
26. Mix 5μl of DNA (dig-lig) into 100 μL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
27. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
28. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
29. Put the tubes back on ice for 2-5 min.
30. Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
31. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
32. Incubate plates at 37°C overnight.

04/09/2020

Goals of the day :

1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

2.Competente cells MC1061 (see lab protocol)

3. Prepare the M9 medium

Procedure

Preparing the 5X stock:
Add the following reagents to a 2-liter flask:

- 64 g Na_2HPO_4, seven hydrate
- 15 g KH_2PO_4
- 5 g NH_4Cl
- 2.5 g NaCl
- 1 liter of high-quality distilled water

  Once the ingredients are added, heat with stirring until the components are completely dissolved. Pour the solution into smaller bottles with loosened caps and the autoclave at 15 lb/in2 for 15 min. If you wish to add antibiotics or nutritional supplements, do this only after the autoclave cycle is complete, as the high temperature may destroy these components. Wait until the bottle is less than 50°C (it should be warm to touch), and then add the components. After the bottles cool to below 40°C, the caps can be tightened and the concentrated medium stored indefinitely at room temperature.

This one was ready from a day before from a colleague.

1. Preparing the 1X Working Solution:
   To make 1X working solution, the 5X media should be diluted to 1× with high quality sterile

   distilled water.
   Add the following sterile solutions for 1 liter of medium

   - 1 ml 1 M $MgSO_4.7H_2O$
   - 10 ml 20% D-glucose
     Typically, several additional components are also added to make a complete medium.
   - 34 ml 0.5% vitamin B1 (thiamine)
   - 10 ml 20% Casamino Acids
   - 5 µl 1M $CaCl_2$
   - Antibiotic for selection

I made stock for every single reagents for future use. Stock at 4°C.

05/09/2020

Goals of the day :

1. Plate reader assay for ecfp

Settings optimized for EGFP based assays in 96 well plates:

Excitation: 485 nm
Excitation Bandwidth: 9nm
Emission: 515 nm
Emission Bandwidth: 20 nm
Integration time: 20 us
Gain: Optimal
Number of Flashes: 10 (or more ???)
Bottom optics

Controls :

Control 1: Untransformed cells
This gives us the background fluorescence output which is calculated by growing cells without EGFP (EGFP-less) in the same medium and microplate. The measured EGFP-less fluorescence is subtracted from the final fluorescence output of transformed cells (with EGFP).

Control 2 : Empty walls
This gives the absorbance of plate itself . This measure is going to subtract from the final measurement of our construct.

Control 3 : Walls with medium and antibiotic (if it is necessary)
M9 medium with antibiotic will give us the background fluorescence output. M9 mediun it does emit fluorescence and is transparent , but the addition of the antibiotic may give a false absorbance.

1. Previous day : complete the transformation with the desired DNA .
2. Pick 3 colonies from each plate.
3. Put each colony into 5mL of LB pre-cultures and incubate O/N , 37°C and 210 rpm (3 colonies from agar plate→ 3 liquid colonies)
4. Next morning , measure the OD600 of the overnight cultures.
   OD600 : Blank → 0

   + control → 2.046

   -control →1.969

Egfp manolis → 2.008

lacO- egfp → 1.985

lacO-egfp asteria → 2.300

Dilution 1μλ DNA and 194μl ddH_2O each sample.

5. For each one of the colonies : Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the timepoints you are going to choose.
   (In our protocol we are going to measure the OD600 and F1: 0h ,1h,2h, 4h,8h, 10h,24h.
   In total : 7 cultures of 5mL of medium.)
   So for one colony : take 35mL of M9 medium and desired antibiotic and dilute the pre-culture to target OD600=0,1.
   Then take 7 sterilized eppendorfs and deposit 5mL of the initial liquid culture (M9 +antibiotic)

6. Incubate each culture : 37°C and 210 rpm . The duration of incubation varies for each liquid culture.
   (By the end of this step : 1 construct → 3 colonies from the agar plate → each colony into one unique liquid culture of M9 medium and desired antibiotic → **M9 liquid colony: distribute into 7 new cultures (5mL each tube)each one represents a different timepoint for measure.)**

7. In due time : take 600 μl of each liquid culture and split into 3 walls (200μl a walls , technical replicates)
8. Set the optimized parameters and measure.

The measurements show that something is wrong and there is no significant

difference between the – control and the positive control.

The same protocol will be executed the next day again.

06/09/2020

Goals of the day :

1.Plate reader assay for ecfp

Settings optimized for EGFP based assays in 96 well plates:

Excitation: 485 nm
Excitation Bandwidth: 9nm
Emission: 515 nm
Emission Bandwidth: 20 nm
Integration time: 20 us
Gain: Optimal
Number of Flashes: 10 (or more ???)
Bottom optics

Controls :

Control 1: Untransformed cells
This gives us the background fluorescence output which is calculated by growing cells without EGFP (EGFP-less) in the same medium and microplate. The measured EGFP-less fluorescence is subtracted from the final fluorescence output of transformed cells (with EGFP).

Control 2 : Empty walls
This gives the absorbance of plate itself . This measure is going to subtract from the final measurement of our construct.

Control 3 : Walls with medium and antibiotic (if it is necessary)
M9 medium with antibiotic will give us the background fluorescence output. M9 mediun it does emit fluorescence and is transparent , but the addition of the antibiotic may give a false absorbance.

9. Previous day : complete the transformation with the desired DNA .
10. Pick 3 colonies from each plate.
11. Put each colony into 5mL of LB pre-cultures and incubate O/N , 37°C and 210 rpm (3 colonies from agar plate→ 3 liquid colonies)
12. Next morning , measure the OD600 of the overnight cultures.
    OD600 : Blank → 0

    + control → 2.046

    -control →1.969

    Egfp manolis → 2.008

    lacO- egfp → 1.985

    lacO-egfp asteria → 2.300

Dilution 1μλ DNA and 194μl ddH_2O each sample.

13. For each one of the colonies : Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the timepoints you are going to choose.
    (In our protocol we are going to measure the OD600 and F1: 0h ,1h,2h, 4h,8h, 10h,24h.
    In total : 7 cultures of 5mL of medium.)
    So for one colony : take 35mL of M9 medium and desired antibiotic and dilute the pre-culture to target OD600=0,1.
    Then take 7 sterilized eppendorfs and deposit 5mL of the initial liquid culture (M9 +antibiotic)

14. Incubate each culture : 37ºC and 210 rpm . The duration of incubation varies for each liquid culture.
    (By the end of this step : 1 construct → 3 colonies from the agar plate → each colony into one unique liquid culture of M9 medium and desired antibiotic → **M9 liquid colony: distribute into 7 new cultures (5mL each tube)each one represents a different timepoint for measure.)**

15. In due time : take 600 µl of each liquid culture and split into 3 walls (200µl a walls , technical replicates)
16. Set the optimized parameters and measure.

The measurements show that something is wrong and there is no significant difference between the – control and the positive control.

Again the same results as yesterday.

Transformation all the constructs to BL21 to see what will happen with this strain :

2.Transformation

33. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
34. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
35. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
36. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
37. Put the tubes back on ice for 2-5 min.
38. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

39. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.

    Incubate plates at 37°C overnight

07/09/2020

Goals of the day :

1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

08/10/2020

Goals of the day :

1.Plate reader assay for ecfp

Settings optimized for EGFP based assays in 96 well plates:

Excitation: 485 nm
Excitation Bandwidth: 9nm
Emission: 515 nm
Emission Bandwidth: 20 nm
Integration time: 20 us
Gain: Optimal
Number of Flashes: 10 (or more ???)
Bottom optics

Controls :

Control 1: Untransformed cells
This gives us the background fluorescence output which is calculated by growing cells without EGFP (EGFP-less) in the same medium and microplate. The measured EGFP-less fluorescence is subtracted from the final fluorescence output of transformed cells (with EGFP).

Control 2 : Empty walls
This gives the absorbance of plate itself . This measure is going to subtract from the final measurement of our construct.

Control 3 : Walls with medium and antibiotic (if it is necessary)
M9 medium with antibiotic will give us the background fluorescence output. M9

mediun it does emit fluorescence and is transparent , but the addition of the antibiotic may give a false absorbance.

1. Previous day : complete the transformation with the desired DNA .
2. Pick 3 colonies from each plate.
3. Put each colony into 5mL of LB pre-cultures and incubate O/N , 37°C and 210 rpm (3 colonies from agar plate→ 3 liquid colonies)
4. Next morning , measure the OD600 of the overnight cultures.
   OD600 : Blank → 0

   + control → 1.046

   -control →1.667

   Egfp manolis → 1.908

   lacO- egfp → 1.785

   lacO-egfp asteria → 1.990

Dilution 1μλ DNA and 194μl ddH_2O each sample.

5. For each one of the colonies : Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the timepoints you are going to choose.
   (In our protocol we are going to measure the OD600 and F1: 0h ,1h,2h, 4h,8h, 10h,24h.
   In total : 7 cultures of 5mL of medium.)
   So for one colony : take 35mL of M9 medium and desired antibiotic and dilute the pre-culture to target OD600=0,1.
   Then take 7 sterilized eppendorfs and deposit 5mL of the initial liquid culture (M9 +antibiotic)

6. Incubate each culture : 37°C and 210 rpm . The duration of incubation varies for each liquid culture.
   (By the end of this step : 1 construct → 3 colonies from the agar plate → each colony into one unique liquid culture of M9 medium and desired antibiotic → **M9 liquid colony: distribute into 7 new cultures (5mL each tube)each one represents a different timepoint for measure.)**

7. In due time : take 600 μl of each liquid culture and split into 3 walls (200μl a walls , technical replicates)
8. Set the optimized parameters and measure.

Something wrong with measurements

09/09/10

Goals of the day :

1.Dig-lig level 1

2.Transformation to DH5a

1.One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- ✓ 50-75 ng acceptor vector
- ✓ 40-70 ng of each part
- ✓ 1 µL 10X T4 DNA ligase **buffer**
- ✓ 1 µL T4 DNA Ligase
- ✓ 0.5 µL restriction enzyme
- ✓ X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

11. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
12. 5 min at 16 degrees C (optimal temp for Ligase)
13. Repeat steps 1. and 2. **50 times**
14. 5 min at 80 degrees C (to kill enzymes)
15. Rest at 16 degrees C

The protocol lasts 6h 08min.

T:andersonJ23115:tetR:terminator

a1R vector	0,4 ul
Terminator	1 ul
TetR	1 ul
AndersonJ23115	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

TE: AndersonJ23115:TetO-ecfp-terminator

a2 vector	0,4 ul
Terminator	1 ul
ecfp	1 ul
AndersonJ23115- TetO	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

2.Transformation

40. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
41. Mix 5μl of DNA (dig-lig) into 100 μL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
42. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
43. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
44. Put the tubes back on ice for 2-5 min.
45. Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
46. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
47. Incubate plates at 37°C overnight.

10/09/2020

Goals of the day :

3. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
4. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 ^{o}C.

11/09/2020

Goals of the day :

3. Prepare the M9 medium

Procedure

Preparing the 5X stock:

Add the following reagents to a 2-liter flask:

- 64 g Na_2HPO_4, seven hydrate
- 15 g KH_2PO_4
- 5 g NH_4Cl
- 2.5 g NaCl
- 1 liter of high-quality distilled water

  Once the ingredients are added, heat with stirring until the components are completely dissolved. Pour the solution into smaller bottles with loosened caps and the autoclave at 15 lb/in2 for 15 min. If you wish to add antibiotics or nutritional supplements, do this only after the autoclave cycle is complete, as the high temperature may destroy these components. Wait until the bottle is less than 50°C (it should be warm to touch), and then add the components. After the bottles cool to below 40°C, the caps can be tightened and the concentrated medium stored indefinitely at room temperature.

This one was ready from a day before from a colleague.

2. Preparing the 1X Working Solution:
   To make 1X working solution, the 5X media should be diluted to 1× with high quality sterile

   distilled water.
   Add the following sterile solutions for 1 liter of medium

   - 1 ml 1 M $MgSO_4.7H_2O$
   - 10 ml 20% D-glucose
     Typically, several additional components are also added to make a complete medium.
   - 34 ml 0.5% vitamin B1 (thiamine)
   - 10 ml 20% Casamino Acids
   - 5 µl 1M $CaCl_2$
   - Antibiotic for selection

I made stock for every single reagents for future use. Stock at 4°C.

20/09/2020

Goals of the day :

1.Dig-lig level 1 again

2.Transformation the dig lig

1.One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- ✓ 50-75 ng acceptor vector
- ✓ 40-70 ng of each part
- ✓ 1 µL 10X T4 DNA ligase **buffer**
- ✓ 1 µL T4 DNA Ligase
- ✓ 0.5 µL restriction enzyme
- ✓ X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

16. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
17. 5 min at 16 degrees C (optimal temp for Ligase)
18. Repeat steps 1. and 2. **50 times**
19. 5 min at 80 degrees C (to kill enzymes)
20. Rest at 16 degrees C

The protocol lasts 6h 08min.

T:andersonJ23115:tetR:terminator

a1R vector	0,4 ul
Terminator	1 ul
TetR	1 ul
AndersonJ23115	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

TE: AndersonJ23115:TetO-ecfp-terminator

a2 vector	0,4 ul
Terminator	1 ul
ecfp	1 ul
AndersonJ23115- TetO	0,5 UL
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,6ul

2.Transformation of dig-lig

2.Transformation

48. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
49. Mix 5μl of DNA (dig-lig) into 100 μL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
50. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
51. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
52. Put the tubes back on ice for 2-5 min.
53. Add 900 μl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
54. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
55. Incubate plates at 37°C overnight.

21/09/2020

Goals of the day :

5. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
6. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

22/09/2020

Goal of the day :

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Digestion (level 0 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3.

1.Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

XIII. **Cultivate and harvest bacterial cells**

23. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

XIV. **Cell lysis**

24. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

25. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

26. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completelyClarification of lysate**

27. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

XV. **Bind DNA**

28. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.

XVI. **Wash silica membrane**

29. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

30. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

XVII. **Dry silica membrane**

31. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

XVIII. **Elute DNA**

32. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

33. Centrifuge for **1 min at 11,000 x g**.

2.Quantification

TE1→ 220

TE2→ 339

T1→ 540

T2→ 234

3.Diagnostic Digestion

26. Select restriction enzymes to digest your plasmid.
27. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
28. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

29. Mix gently by pipetting.
30. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

Sample	DNA	Restriction enzyme	Buffer	ddH_2O
TE1	1 ul	BamHI 0,5 ul	2 ul	16,5 ul
TE2	1 ul	BamHI 0,5 ul	2 ul	16,5 ul
T1	1 ul	BamHI 0,5 ul	2 ul	16,5 ul
T2	1 ul	BamHI 0,5 ul	2 ul	16 ul

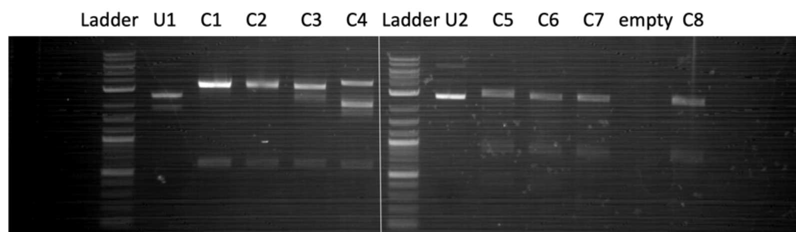


Figure 9: (U=Uncut , C= Cut) Restriction digestion of **LE: AndersonJ23115:Lac0-EGFP-double terminator**(C1-C 4) with :
→HindIII + BtgZI (C1-C4) , Expected bands : 3200+ 597bp
→Positive result: C1,C2
Restriction digestion of **TL: AndersonJ23115:Tet0-LacI-double terminator**(C5-C8) with:
→ HindIII + NheI (C9-C16) , Expected bands : 2573+891+427
→ Positive result : C5,C6

23/09/2020
Goal of the day:

1. Dig lig omega vectors
2. Transformation

1.One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- ✓ 50-75 ng acceptor vector
- ✓ 40-70 ng of each part
- ✓ 1 µL 10X T4 DNA ligase **buffer**
- ✓ 1 µL T4 DNA Ligase
- ✓ 0.5 µL restriction enzyme
- ✓ X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

21. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
22. 5 min at 16 degrees C (optimal temp for Ligase)
23. Repeat steps 1. and 2. **50 times**
24. 5 min at 80 degrees C (to kill enzymes)
25. Rest at 16 degrees C

The protocol lasts 6h 08min.

Omega 1 : a1R-tetR + a2 tet0-lacI

vector	1 ul
Tet0-LacI	1 ul
Anderwson-TetR-terminator	1 ul
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	4,5ul

Omega 2 TE: a1R-LacO:ecfp + a2:stuffer

vector	0,4 ul
LacO-ecfp	0,5 ul
stuffer	0,5 ul
10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul
BsmBI	0,5ul
ddH_2O	5ul

2.Transformation of dig-lig

2.Transformation

56. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
57. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
58. Incubate the competent cell-DNA mixture on ice for 20-30 mins.

59. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
60. Put the tubes back on ice for 2-5 min.
61. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
62. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
63. Incubate plates at 37°C overnight.

24/09/2020

Goals of the day :

1. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.
2. After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

3.Transformations LacO magdas and egfp manoli to BL21

4. Transformation all constructs to MC1061 (with ecfp)

2.Transformation

64. Take competent cells out of -80°C and thraw on ice (approximately 20 mins).
65. Mix 5µl of DNA (dig-lig) into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
66. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
67. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
68. Put the tubes back on ice for 2-5 min.
69. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
70. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
71. Incubate plates at 37°C overnight.

25/09/2020

Goal of the day :

1.Make 1 l of M9 medium (see previous day)

2. Take the plates out incubator , put them in fridge so the colonies won't grow much. Moreover me want to see clearly the blue-white colonies.

3.After 2 hours on the fridge we pick single colonies with a tip of pippete and throw it in 5ml LB without antibiotic. Incubation overnight , at 210 rpm/37 °C.

26/08/2020

Goals of the day:

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Colony PCR (level 2 constructs)

4.Agarose Gel Electrophoresis (AGE) for 3.

1.Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

XIX. **Cultivate and harvest bacterial cells**

34. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

XX. **Cell lysis**

35. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

36. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

37. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completelyClarification of lysate**

38. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

XXI. **Bind DNA**

39. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
    Repeat this step to load the remaining lysate.

XXII. **Wash silica membrane**

40. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
41. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

XXIII. **Dry silica membrane**

42. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

XXIV. **Elute DNA**

43. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
44. Centrifuge for **1 min at 11,000 x g**.

2.Quantification

Ω1.1 → 71.5

Ω1.2→ 33

Ω1.3 → 60

Ω1.4 → 26

Ω2.1 → 17.8

Ω2.2→ 673

Ω2.3 → 754

Ω2.4 → 30

3.Diagnostic Digestion

31. Select restriction enzymes to digest your plasmid.
32. Determine an appropriate reaction buffer by reading the instructions for your enzyme.

33. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

34. Mix gently by pipetting.
35. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

We are going to use : HindIII and EcoRV as restriction enzymes

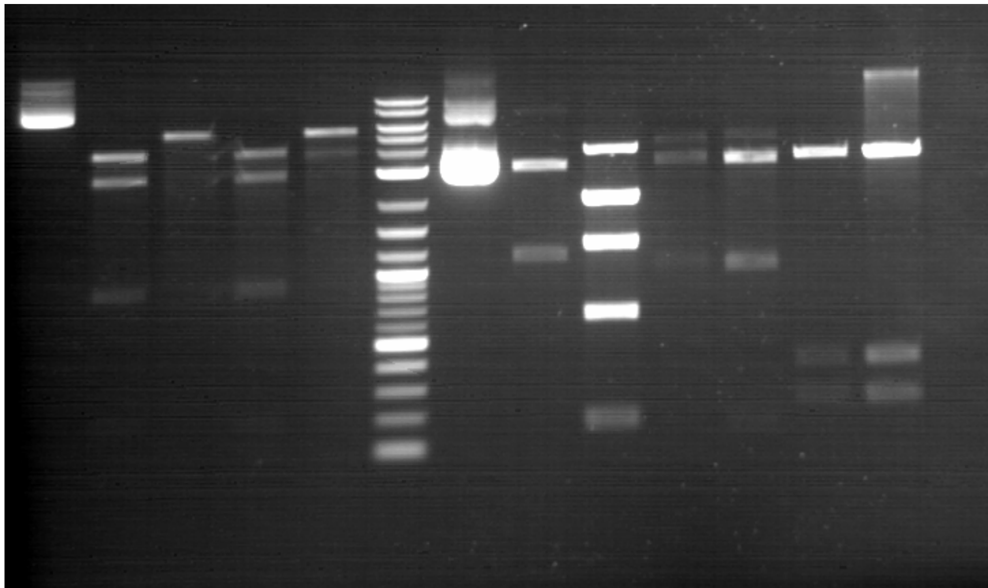
Expected bands : omega 1 → 3734 + 1555

omega 2 → 2900+852 + 152

Sample	DNA	Restriction enzyme	Buffer	ddH$_2$O
Ω1.1	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul
Ω1.2	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul
Ω1.3	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul
Ω1.4	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul
Ω2.1	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul
Ω2.2	1 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	16 ul
Ω2.3	1ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	16 ul

Ω2.4	3 ul	HindIII 0,5 ul EcorV 0,5 ul	2 ul	14 ul

Ladder| ω1 un| ω1.1|ω1.2|ω1.3|ω1.4| Ladder | ω2.1|ω2.2|ω2.3|ω2.4



Didn't have the right profile , repeat this tomorrow and will do a COLONY PCR to see the length insert

28/09/2020

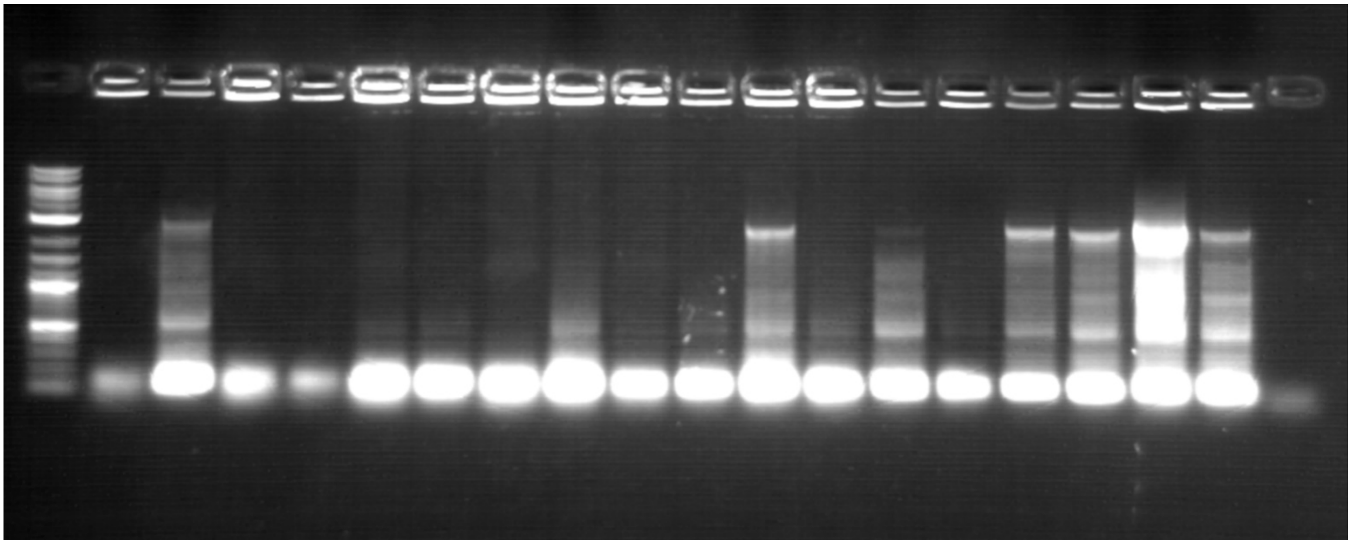
1. **Colony PCR**

Because of the bad plates we couldn't pick colonies so we did Colony PCR

1. Mark the colonies on the plate and label them with numbers If you have a negative control plate, mark 1 to 3 colonies
2. Take out PCR tubes and label them as the colonies
3. Be sure that you have prepared replica plates and draw to the back of them distinct sections; one per colony. Label them as the above
4. Prepare the MasterMix Number of reactions: number of colonies, 1 NTC without colony, 1 to 2 NTC with the colony/ies from the NTC plate, 1extra/10 colonies
5. Transfer 25 ul MM to the PCR tubes
6. Pick a colony with a tip

7. ’’Poke”softlytothereplicaplate
8. DissolvetherestinthePCRtube
9. Repeat for all the colonies

10. Quick spin down



By this we can assume that there is contamination. Tomorrow with fresh and sterilized materials we are going to do it again.

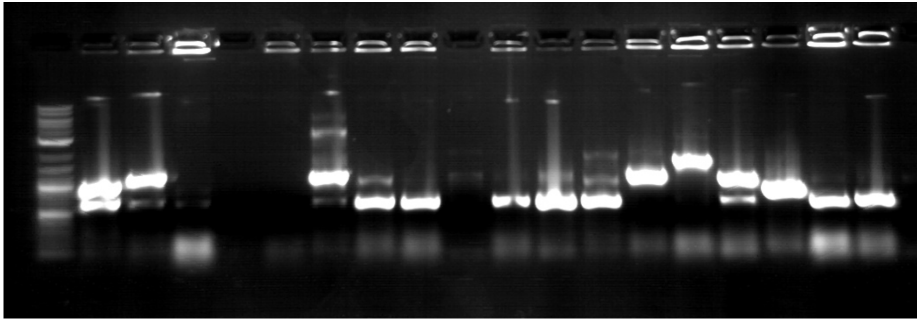
29/09/2020
Goal of the day :
1.COLONY PCR omega 1 and omega 2 constructs

1. Colony PCR

Because of the bad plates we couldn’t pick colonies so we did Colony PCR

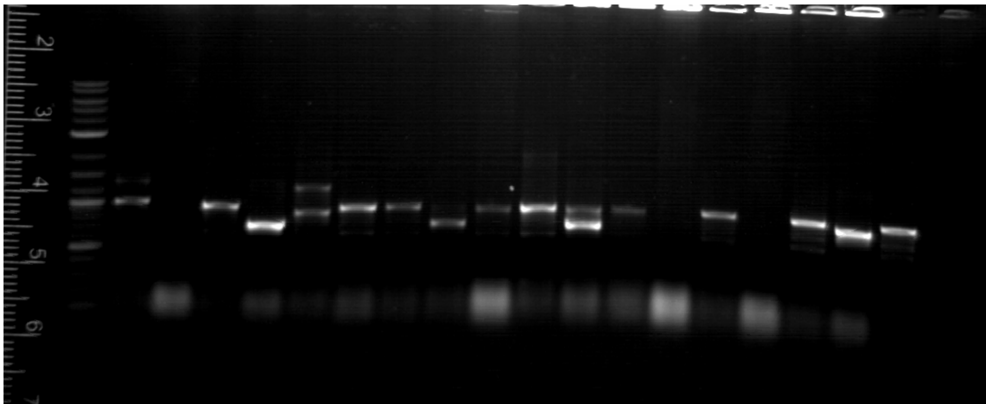
1. Mark the colonies on the plate and label them with numbers If you have a negative control plate, mark 1 to 3 colonies
2. Take out PCR tubes and label them as the colonies
3. Be sure that you have prepared replica plates and draw to the back of them distinct sections; one per colony. Label them as the above
4. Prepare the MasterMix Number of reactions: number of colonies, 1 NTC without colony, 1 to 2 NTC with the colony/ies from the NTC plate, 1extra/10 colonies
5. Transfer 25 ul MM to the PCR tubes
6. Pick a colony with a tip
7. ’’Poke”softlytothereplicaplate
8. DissolvetherestinthePCRtube
9. Repeat for all the colonies

10. Quick spin down



Here: all constructs from Omega 2 TE: a1R-LacO:ecfp + a2:stuffer

We cant have a clear result from this one , and we assume that something is wrong with the dig lig.



Here: all constructs from omega 1: a1R-tetR + a2 tet0-lacI, cant have a clear point of view. Tomorrow a new colony.

30/09/2020

1. Make dNTPS : 100mM of each dNTPs and our stock will be 10µM
So 10µl of each dNTP , (10A+10T+10G+10C=40 µl) and add 60µl of ddH_2O.

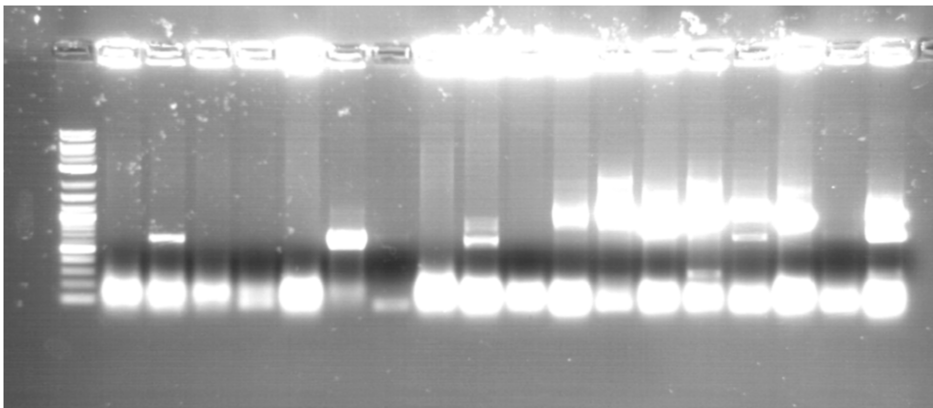
2. Colony PCR

1. Mark the colonies on the plate and label them with numbers If you have a negative control plate, mark 1 to 3 colonies
2. Take out PCR tubes and label them as the colonies

3. Be sure that you have prepared replica plates and draw to the back of them distinct sections; one per colony. Label them as the above
4. Prepare the MasterMix Number of reactions: number of colonies, 1 NTC without colony, 1 to 2 NTC with the colony/ies from the NTC plate, 1extra/10 colonies
5. Transfer 25 ul MM to the PCR tubes
6. Pick a colony with a tip
7. ''Poke"softlytothereplicaplate
8. DissolvetherestinthePCRtube
9. Repeat for all the colonies

10. Quick spin down

Here we have all omega 1 constructs



We have some positives results and tomorrow we are going to continue with minipreps from these and after diagnostic reactions.
We chose from the plates the positive clones and put them into 5mL of LB with the desirable antibiotic

01/10/2020

1.Miniprep- Plasmid Isolation Protocol

2.Quantification of DNA – Nanodrop

3.Restricition Digestion

4.Agarose Gel Electrophoresis (AGE) for 3.

1.Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

XXV. **Cultivate and harvest bacterial cells**

45. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

XXVI. **Cell lysis**

46. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
47. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
48. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completelyClarification of lysate**
49. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

XXVII. **Bind DNA**

50. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
    Repeat this step to load the remaining lysate.

XXVIII. **Wash silica membrane**

51. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
52. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

XXIX. **Dry silica membrane**

53. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

XXX. **Elute DNA**

54. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

55. Centrifuge for **1 min at 11,000 x g**.

2.Quantification

Ω1.1 → 71.5

Ω1.2→ 33

Ω1.3 → 60

Ω1.4 → 26

Ω2.1 → 70

Ω2.2→ 77

Ω2.3 → 71

Ω2.4 → 69

3.Diagnostic Digestion

1. 1.Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:
4. DNA
5. Restriction Enzyme(s)
6. Buffer
7. dH2O up to total volume
8. Mix gently by pipetting.
9. Incubate tube at appropriate temperature for 1,5-2 hours. Always follow the manufacturer's instructions.

Sample	DNA	Restriction enzyme	Buffer	ddH_2O
Ω1.1	3 ul	EcoRI 0,5ul EcorV 0,5 ul	2 ul	14 ul
Ω1.2	3 ul	EcoRI 0,5ul EcorV 0,5 ul	2 ul	14 ul
Ω1.3	3 ul	EcoRI 0,5ul EcorV 0,5 ul	2 ul	14 ul
Ω1.4	3 ul	EcoRI 0,5ul EcorV 0,5 ul	2 ul	14 ul

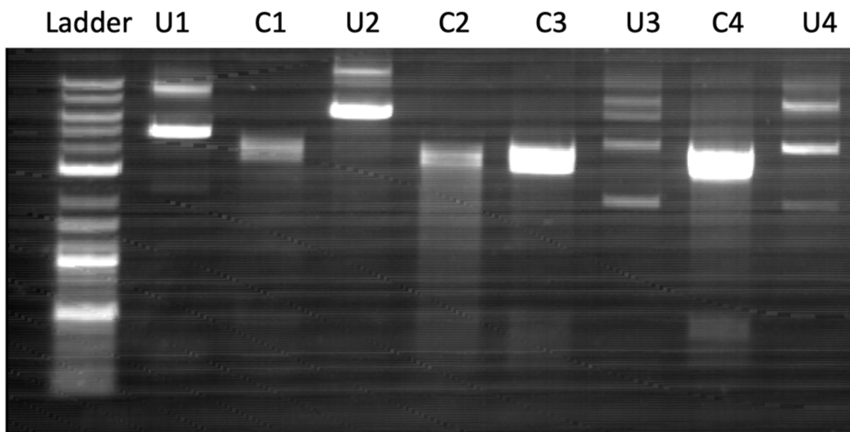


Figure 12: (U=Uncut , C= Cut) Restriction digestion of **omega1R-TetR-LacI** (C1-C 4) with :
→EcoRV +EcoRI(C1-C4) , Expected bands : 4823 + 465bp
→Positive result: C1,C2,C3,C4

VENETIOS

1/8/2020

Goals of the Day

1. Receive and resuspend primers for PCR
2. Conduct PCR for the SEVA backbones
3. Separate digestion of LacZa module from original Golden Braid vectors
4. Agarose Gel Electrophoresis (AGE) for both 2, 3
5. Gel exctraction

Procedure

1. **Resuspension**

   When still dry:

- Spin down the pellet. You need everything to be on the tip.
- Add appropriate volume of ddH_2O (creating a concentration of 100pmol/μL)
- Close the lid
- Vortex thoroughly (~20s)
- Spin down

This is the STOCK CONCENTRATION (100pmol/μL or 100μM)!!

To create the **working concentration 10pmol/μL or 10μM (1:10 of stock):**

Dilute 10μL of stock concentration into 90μL ddH_2O total volume is 100μL

2. Q5 high fidelility PCR is conducted creating backbones with BsaI and BsmBI sites.

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	*50–72°C	10–30 seconds
	72°C	20–30 seconds/kb

Final Extension	72°C	2 minutes
Hold	4–10°C	

Using NEB calculator annealing Tm was 71°C

Component		50 µl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix		25 µl	1X
10 µM Forward Primer		2.5 µl	0.5 µM
10 µM Reverse Primer		2.5 µl	0.5 µM
Template DNA		1 µl	0,71 ng
Nuclease-Free Water		to 50 µl	

3. Digestion

1. Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme(s)
- Buffer
- dH2O up to total volume

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature for 1,5-2 hours. Our restriction digestions are 50 uL in total volume.

- 5 µg DNA
- 0.5 µL BsmBI
- 5 µL 10x Buffer
- 39,5 µL dH2O (to bring total volume to 50µL)

I left it 4 hours.

4. AGE

Materials for the gel

For 0ml of 1% w/v agarose we need

- **60ml 1X TAE (diluted from 50X TAE stock)**
- **1,2 gr agarose**
- **4,2 µl EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1,2 gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs.
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box. Avoid the bubbles.
9. Remove bubbles with a tip (or piece of paper) if needed. If you can't remove them, move them in a place where they won't affect your DNA.
10. Let the gel solidify for 10-15min
11. After 10-15mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE. If not full, add as TAE needed until it covers the gel.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 110V until it runs the whole gel (depends on gel agarose % and length).
15. Put the gel under UV and take a photo to see what you did.
16. Analyze the bands on the gel.

Sample Preparation for Gel Run

We had 50ul reaction we need this DNA for a Digestion Ligation tomorrow so we conduct electrophoresis with all our DNA included

50 ul split in half in order to fit in the gel

- Add 25µl plasmid in Eppendorf tube
- Add 5 µl loading dye (6X)
  Final volume will be 30 µl
- After everything is in the tube, spin down (10s run at centrifuge)
- For the gel ladder, add 3 µl

Gel image not available because we didn't want to have mutagenesis from the UV before the gel extraction.

5. Gel exctraction

;

5.2 DNA extraction from agarose gels

Before starting the preparation:

- Check if Wash Buffer NT3 was prepared according to section 3.

1 Excise DNA fragment / solubilize gel slice

Note: Minimize UV exposure time to avoid damaging the DNA. Refer to section 2.5 for more tips on agarose gel extraction.

Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess agarose.

! Determine the weight of the gel slice and transfer it to a clean tube.

For each **100 mg of agarose gel < 2 % add 200 µL Buffer NTI**.

+ 200 µL NTI per 100 mg gel

For gels containing > 2 % agarose, double the volume of Buffer NTI.

Incubate sample for **5–10 min** at **50 °C**. Vortex the sample briefly every 2–3 min until the gel slice is **completely** dissolved!

50 °C 5–10 min

2 Bind DNA

Place a **NucleoSpin® Gel and PCR Clean-up Column** into a Collection Tube (2 mL) and load up to 700 µL sample.

Load sample

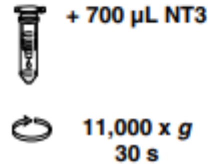
Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

11,000 x *g* 30 s

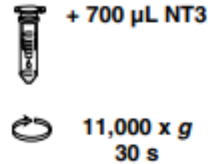
Load remaining sample if necessary and repeat the centrifugation step.

3	**Wash silica membrane**

Add **700 µL Buffer NT3** to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.



Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and low A_{260}/A_{230} (see section 2.7 for detailed information).



4	**Dry silica membrane**

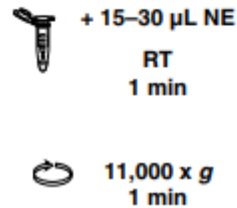
Centrifuge for **1 min** at **11,000 x *g*** to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

5	**Elute DNA**

Place the NucleoSpin® Gel and PCR Clean-up Column into a **new** 1.5 mL microcentrifuge tube (not provided). Add **15–30 µL Buffer NE** and incubate at **room temperature** (18–25 °C) for **1 min**. Centrifuge for **1 min** at 11,000 x *g*.

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. See section 2.6 for detailed information.



We chopped of the gel with the backbone of the SEVA backbones at approximately 3000 bp and the LacZa module at approximately 650 bp.

Weight of agarose extract gr	NT1 ul
0.193	386
0.163	326
0.098	196
0.058	116

2/8/2020

Goals of the Day

1. Quantification of DNA from the gel extract
2. Digestion-Ligation(DIG-LIG) to make to SEVA vectors converted to Level 1 vectors for Golden Braid cloning system.
3. Transformation the DIG-LIG product to DH5a cells

Procedure

1. **Quantification at the quantifier called nanodrop.**

   Nanodrop is high quality photometer. 1ul is measured after cleaning up 3 times with dH2O Blanking with elution buffer.

Samples:

PCR a1R vector 73,2 ng/ul

PCR a2 vector 196,4 ng/ul

Dig a1R LacZa 29,0 ng/ul

Dig a2 LacZa 56,8 ng/ul

2. DIG-LIG

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

We use the same protocol for different level assemblies, while changing the acceptor vectors and the enzyme

Total reaction volume: 10 µL

- 50-75 ng acceptor vector
- 40-70 ng of each part
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme
- X µL ddH_2O to reach 10 ul

You will need a thermocycler (PCR Machine) to run this protocol.

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C

The protocol lasts 6h 08min.

PCR a1R vector	1 ul	PCR a2 vector	0,5ul
Dig a1R LacZa	2 ul	Dig a2 LacZa	1 ul
10X T4 DNA ligase **buffer**	1ul	10X T4 DNA ligase **buffer**	1ul
T4 DNA Ligase	1ul	T4 DNA Ligase	1ul
BsmBI	0,5ul	BsmBI	0,5ul
ddH_2O	4,5ul	ddH_2O	6 ul

3. Transformation

1. Take competent cells out of -80°C and thaw on ice (approximately 20 mins).
2. Mix 5µl of DNA into 100 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.

3. Incubate the competent cell-DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C water bath for 60 sec
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
8. Incubate plates at 37°C overnight.

3/8/2020

Goal of the Day

Take the plates out of the incubator and put them in fridge.

4/8/2020

Goal of the Day

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 5ml LB with antibiotic. Incubation overnight.

5/8/2020

Goals of the Day

1. Minipreps
2. Quantification of isolated plasmids
3. Diagnostic Digestion
4. AGE

Procedure

1. Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

I. **Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible.

II. **Cell lysis**

2. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

3. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

4. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!

Make sure to neutralize completely to precipitate all protein and chromosomal DNA. LyseControl should turn completely colorless without any traces of blue.

III. **Clarification of lysate**

5. Centrifuge for **10 min at 17,000 x g** at room temperature. Repeat this step in case the supernatant is not clear

IV. **Bind DNA**

6. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **maximum of 700 (I do 750 without a problem without needing to do second centrifugation) µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

V. **Wash silica membrane**

7. *Recommended: If plasmid DNA is prepared from host strains containing high levels of nucleases (e.g., HB101 or strains of the JM series), it is strongly recommended performing an additional washing step with **500 µL Buffer AW**, optionally preheated to 50 degrees C, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4. Additional washing with Buffer AW will also increase the reading length of DNA sequencing reactions and improve the performance of critical enzymatic reactions.* **Always do this step, it provides nice and clear DNA.**

Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

VI. **Dry silica membrane**

8. Centrifuge for **5 min at 11,000 x g** and discard the collection tube

Note: Residual ethanolic wash buffer might inhibit enzymatic reactions.

VII. Elute DNA

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**. Incubate for 5 min at room temperature. Centrifuge for **1 min at 11,000 x g**. Repeat this step twice (When you centrifuge, pipette the volume from the Eppendorf tube and feed it to the column, let 5 min and then centrifuge again. This increases yield especially for medium to low copy plasmids.

2. Quantification

For protocol see 2/8/2020

Samples:

a1R colony1 142,7 ng/ul

a1R colony2 112,7 ng/ul

a1R colony3 157,4 ng/ul

a1R colony4 46,8 ng/ul

a2 colony1 46,3 ng/ul

a2 colony2 20,9 ng/ul

3. Diagnostic Digestion

For protocol see 1/8/2020

Master Mix of a1R	Master Mix of a2
BamHI 2 ul	HindIII 1 ul
Cutsmart 8 ul	2.1 buffer 4 ul

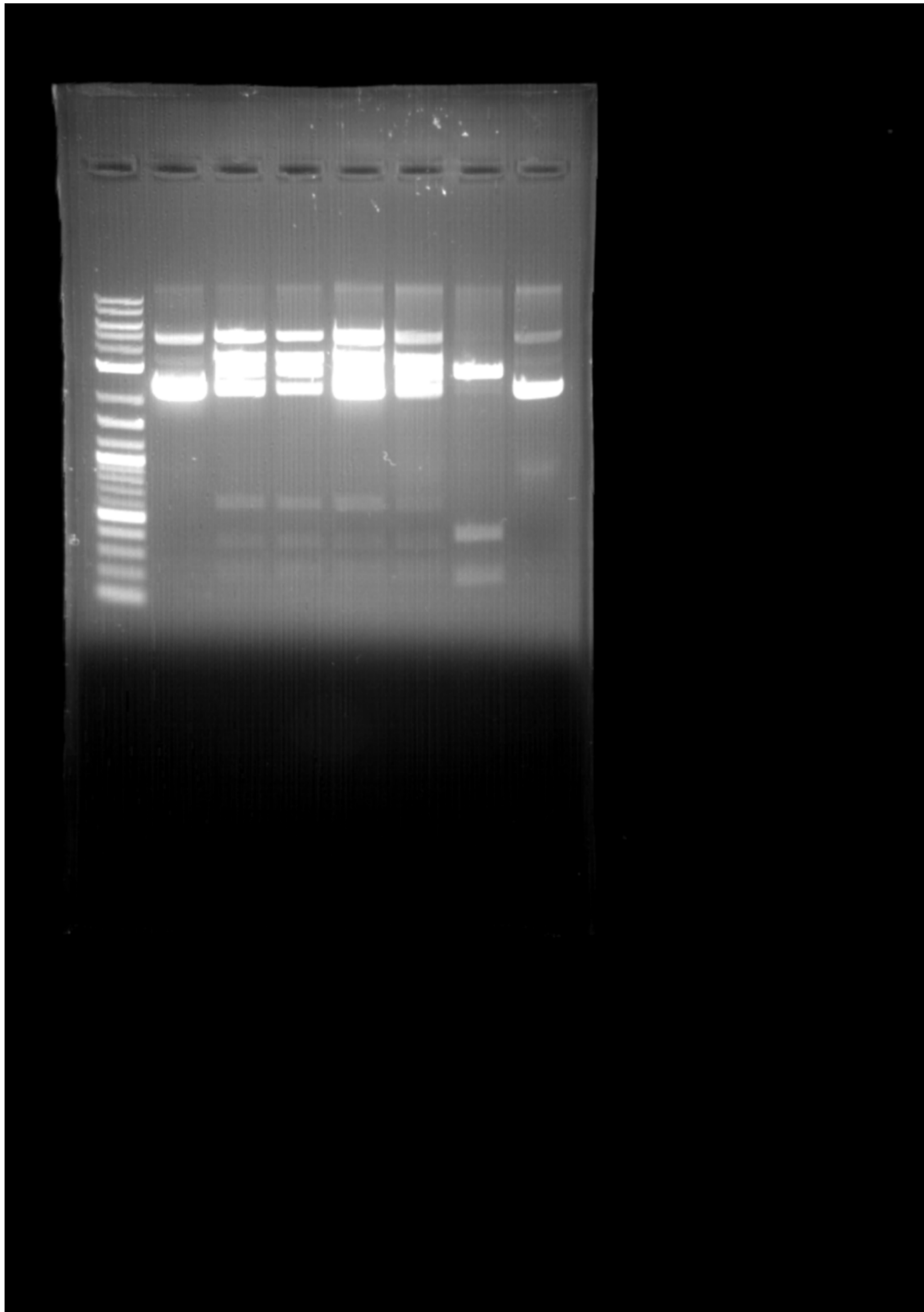
a1R colonies 1,2,3	a1R colony 4 and a2 colonies 1,2
4 ul DNa	10ul DNA
2,5 Master Mix	2,5 Master Mix
13,5 dH2O	7,5 dH2O

4. AGE

For protocol see 1/8/2020
20 ul from rection
4 ul 6x loading dye

Samples	Ladder 3 ul	Uncut plasmid 4 ul
20 ul from rection		Loading dye 6x 2 ul
4 ul 6x loading dye		ddH20 6 ul

Wells from left to right
Ladder|Uncut plasmid|a1R 1|a1R 2|a1R 3|a1R 4|a2 1|a2 2



6/8/2020

Goal of the Day

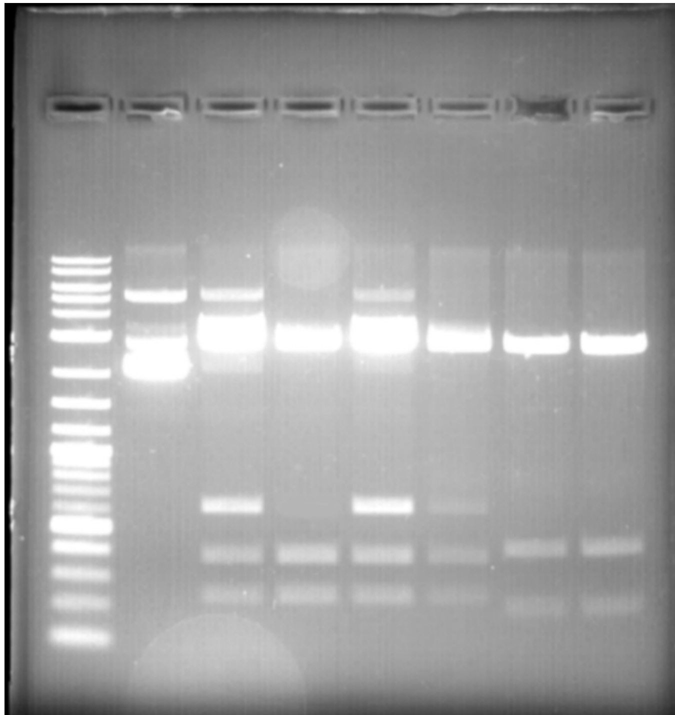
1. Digestion again for better imaging in AGE

Procedure

For protocol see 1/8/2020

Same as yesterday but, half amount of DNA was digested with the same amount of enzyme.

Wells from left to right
Ladder|Uncut plasmid|a1R 1|a1R 2|a1R 3|a1R 4|a2 1|a2 2



a1R 2 and a2 1 sent for Sequencing.

7/8/2020

Goal of the Day

1. Conduct PCR for the SEVA backbones
2. Separate digestion of LacZa module from original Golden Braid vectors
3. Agarose Gel Electrophoresis (AGE) for both 1, 2

4. Gel exctraction
5. Quantification of extracted DNA

Procedure

1. **Conduct PCR for the SEVA backbones**

For protocol see 1/8/2020

pSEVAb43 with initial 194 ng/ul

Dilution 1/200 for 0,97 ng/ul

25 ul	Q5 2x master mix
2,5 ul	Forward primer
2,5 ul	Reverse primer
1 ul	Diluted 1/200 pSEVAb43
19 ul	ddH20

2 samples omega1 omega2

Time (sec)	Temp in Celcius
30	98
5	98
20	71
105	72
120	72
forever	4

Repeat step 2-4 33 times

2. **Separate digestion of LacZa module from original Golden Braid vectors**

For protocol see 1/8/2020

5 ul	Vector including the LacZa needed
0,5 ul	BsaI
5 ul	Cutsmart
39,5 ul	ddH20

3. Agarose Gel Electrophoresis (AGE) for both 1, 2 and Gel extraction

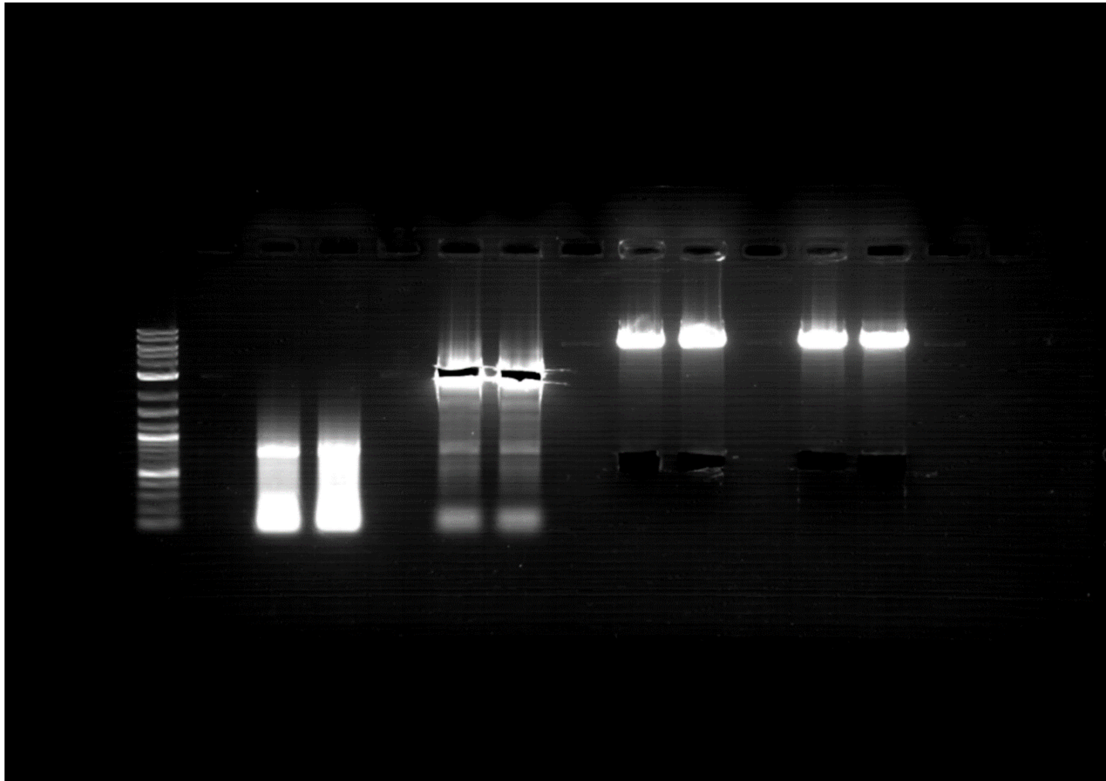
For protocol see 1/8/2020

Sample	Weight (g)	NT1 (ul)
PCR 2	0,121	242
DIG 1	0,189	378

DIG 2	0,197	397

Wells from left to right

Ladder|PCR omega1\PCR omega 1| PCR omega2\PCR omega2 |Dig omega1\DIG omega 1| Dig omega2\Dig omega2



PCR for omega1 backbone was unsuccessful

5. Quantification of gel extractred DNA

PCR 2	102,9 ng/ul
DIG 1	45,2 ng/ul
DIG 2	129,7 ng/ul

9/8/2020

Goals of the Day

1. Conduct PCR for the omega 1 backbone
2. AGE for checking PCR's success

Procedure

1. **PCR**

For protocol see 1/8/2020
Same as yesterday changing the annealing temperature

Time (sec)	Temp in Celcius
30	98

5	98
125	72
120	72
forever	4

Repeat step 3 and 4 33 times

2. AGE

For protocol see 1/8/2020
Wells from left to right Ladder |a2(30ul) |a2(30ul)

10/8/2020

Goals of the Day

1. Conduct KAPA Taq PCR for the omega 1 backbone
2. AGE for checking PCR's success

Procedure

1. **KAPA Taq PCR**

Using low fidelity Polymerase KAPA Taq we check other parameters to see why the backbone is not amplified .

Protocol for 10 ul reaction

Time (sec)	Temp in Celcius
30	95
10	95
10	58, 60, 62
210	72
120	72
Forever	4
Repeat step 2-4 33 times	
Buffer Taq A	1 ul
dNTPs	0,2 ul
Primer FOR	1 ul
Primer REV	1 ul
KAPA Taq	0,04 ul
ddH20	6,96 ul

2. **AGE**

For protocol see 1/8/2020

Wells for left to right Ladder|No template|58|60|62

We see Primer dimer and a non specific byproduct at 58 celcius

11/8/2020

<u>Goals of the Day</u>

1. Conduct KAPA Taq PCR for the omega 1 backbone
2. AGE for checking PCR's success

3. DIG-LIG Omega 2 backbone with the lacZa module
4. Transformation the DIG-LIG product to DH5a cells

Procedure

1. KAPA Taq PCR

For protocol se 28/8/2020

Time (sec)	Temp in Celcius
60	95
20	95
20	50
210	72
20	95
210	72
300	72
Forever	4

Repeat step 2, 3, 4   10 times
Repeat step 5, 6   25 times

2. **AGE**
For protocol see 1/8/2020

Wells for left to right Ladder|No template|Pcr product

This last failure makes us to order a new set of primers

3. DIG-LIG Omega 2 backbone with the lacZa module

For protocol see 2/8/2020

GE PCR omega 2	0,5 ul
GE DIG LacZa	0,5 ul

T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	6,5 ul

4. Transformation the DIG-LIG product to DH5a cells

For protocol see 2/8/2020
Using 5ul of DIG-LIG product instead of 1 ul DNA

12/8/2020

Goals of the Day

1. Picking colonies for liquid cultures
2. Checking a2 with a second digestion because of not good sequencing results
3. Run AGE of the digestion

Procedure

1. Picking colonies for liquid cultures.

   Same as 4/8/2020

   Picked 4 colonies omega 2 and 2 colonies pUPD2

2. Digestion

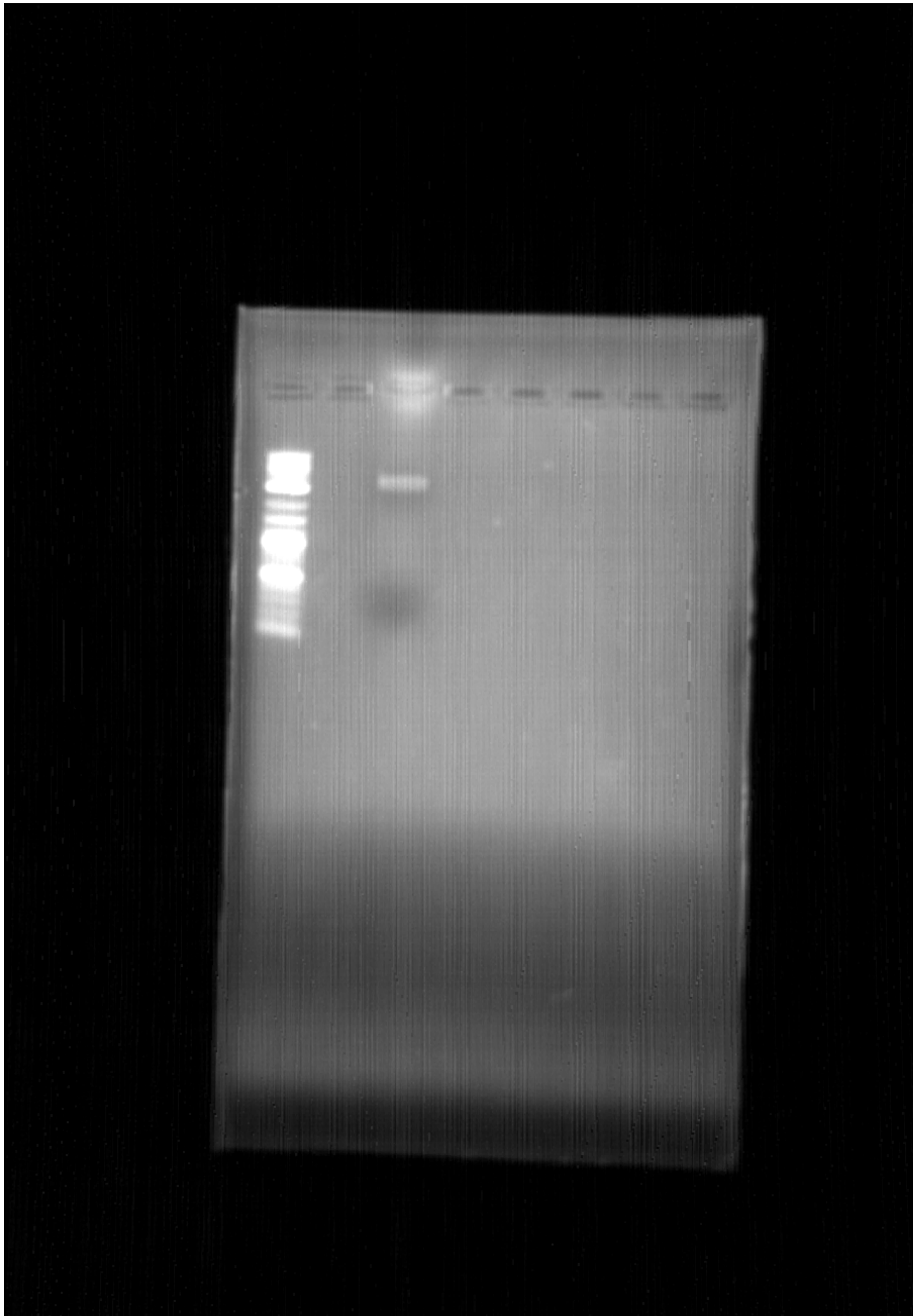
For protocol see 1/8/2020

DNA (a2 plasmid)(46,3 ng/ul)	2ul
HindIII hf	0,2 ul
Cutsmart Buffer	1 ul
dd20	6,8 ul

3. AGE

For protocol see 1/8/2020

Wells from left to right Ladder|a2



13/8/2020

Goals of the Day

1. Minipreps

2. Quantification of isolated plasmids
3. Diagnostic Digestion
4. AGE

Procedure

1. **Minipreps**

   For protocol see 5/8/2020

   5ml cultures

2. Nanodrop

For protocol see 2/8/2020

Omega2 1	50,3 ng/ul
Omega2 2	21,3 ng/ul
Omega2 3	22,0 ng/ul
Omega2 4	257,8 ng/ul
pUPD2 1	183,7 ng/ul
pUPD2 2	193, 8 ng/l

3. Digestion

For protocol see 15/8/2020

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20
Omega2 1	5	1	HindIII 0,3	3,7
Omega2 2	5	1	HindIII 0,3	3,7
Omega2 3	5	1	HindIII 0,3	3,7
Omega2 4	1	1	HindIII 0,3	7,7
pUPD2 1	1	1	EcoRI 0,3	7,7
pUPD2 2	1	1	EcoRI 0,3	7,7

4. AGE

Wells from left to right

Ladder|omega2 1 Uncut|omega2 1 cut|omega2 2 uncut|omega2 2 cut|omega2 3 uncut|omega2 3 cut|omega2 4 uncut|omega2 4 cut|pUPD2 1 uncut| pUPD2 1 cut| pUPD2 2 uncut | pUPD2 2 cut

14/8/2020

Goals of the Day

1. Diagnostic Digestion with different enzyme
2. AGE

Procedure

1. **Diagnostic Digestion with different enzyme**

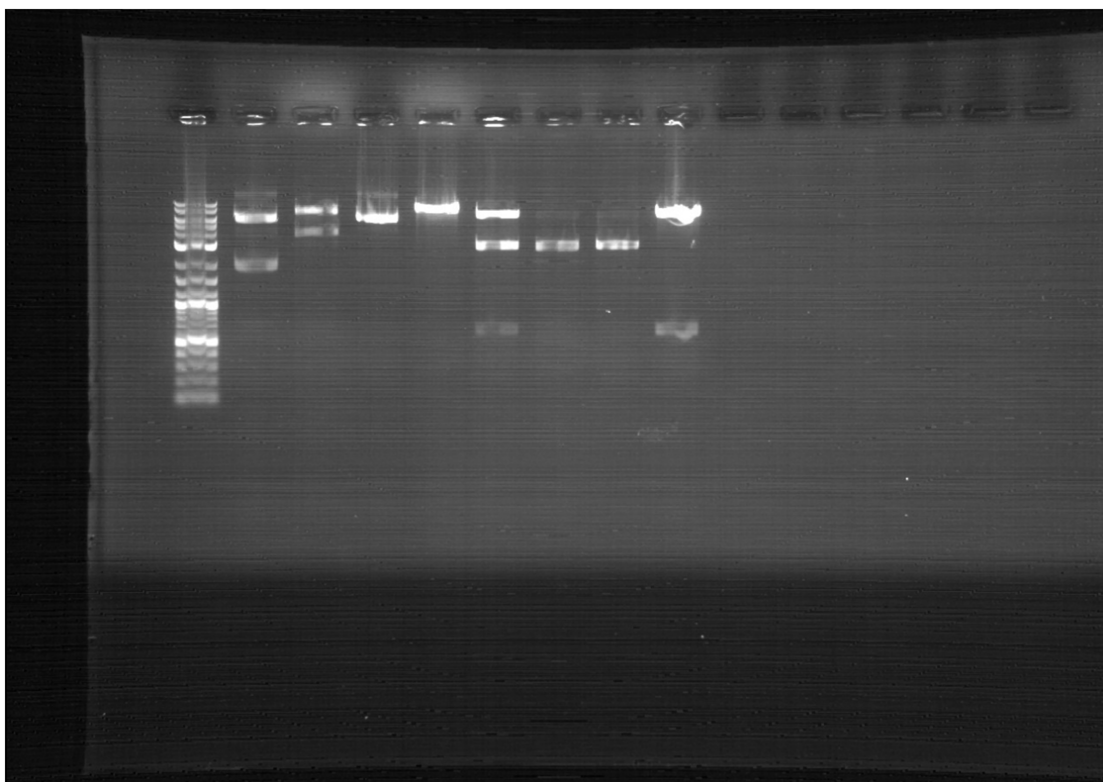
For protocol see 1/8/2020

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
Omega2 1	5	1	HindIII 0,3	3,7
Omega2 4	1	1	HindIII 0,3	7,7
Omega2 1	5	1	EcoRV 0,3	3,7
Omega2 2	5	1	EcoRV 0,3	3,7
Omega2 3	5	1	EcoRV 0,3	3,7
Omega2 4	1	1	EcoRV 0,3	7,7

2. AGE

For protocol see 2/8/2020

Ladder|omega2 1 Uncut|omega2 1 HindIII| omega2 4 uncut |omega2 2 HindIII| omega2 1 EcoRV |omega2 2 EcoRV |omega2 3 EcoRV |omega2 4 EcoRV



15/8/2020

Goal of the Day

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 5ml LB with antibiotic. Incubation overnight.

7/8/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic Dig
4. AGE
5. Dig-Lig

Procedure

1. **Minipreps**

For protocol see 5/8/20

2. **Nanodrop**

For protocol see 1/8/20

SAMPLES	Concentration ng/ul

A2 mini 1	14,0
A2 mini 2	13,1
Omega2	53,3
A2 DigLIG 1	4,9
A2 DigLIG 2	5,7
A2 DigLIG 3	15,2
A2 DigLIG 4	6,3

3. **Dignostic Dig**

For protocol see 1/8/20

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
A2 mini 1	15	2	HindIII	2,5
A2 mini 2	15	2	HindIII	2,5
Omega2	3	2	EcoRV	14,5
A2 DigLIG 3	15	2	HindIII	2,5

4. **AGE**

Because of low concentrations AGE wasn't conducted

5. **DIG-LIG**

For protocol see 1/8/20

pUPD2	1 ul (60ng/ul)
Part CDS1(AIDAc)	1 ul (50 ng/ul)
PartCDS2(Tyr1)	1 ul (50 ng/ul)
T4 buffer	1 ul
T4 Ligase	1 ul
BsmBI	0,5 ul
ddH20	4,5 ul

16/8/2020

Goals of the Day

1. Diagnostic Dig
2. AGE
3. Transformation

Procedure

1. **Diagnostic Dig**

For protocol see 1/8/20

sample	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
Omega2	3	1	EcoRV 0,5	5

2. AGE

For protocol see 1/8/20

3. **Transformation**

For protocol see 1/8/20

Made 3 plates with a2 mini1 , a2 mini2 , LVL 0 AIDA

9/8/2020

Goal of the Day

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert, Wight colonies for AIDA LVL 0. Picking with the tip of the pipette and throw it in 5ml LB with antibiotic. Incubation overnight.

10/8/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic Dig
4. AGE

Procedure

1. **Minipreps**

3. samples AIDA LVL 0

1 sample a2 plate 1

1 sample a2 plate2

2. Nanodrop

AIDA 1	317,0 ng/ul

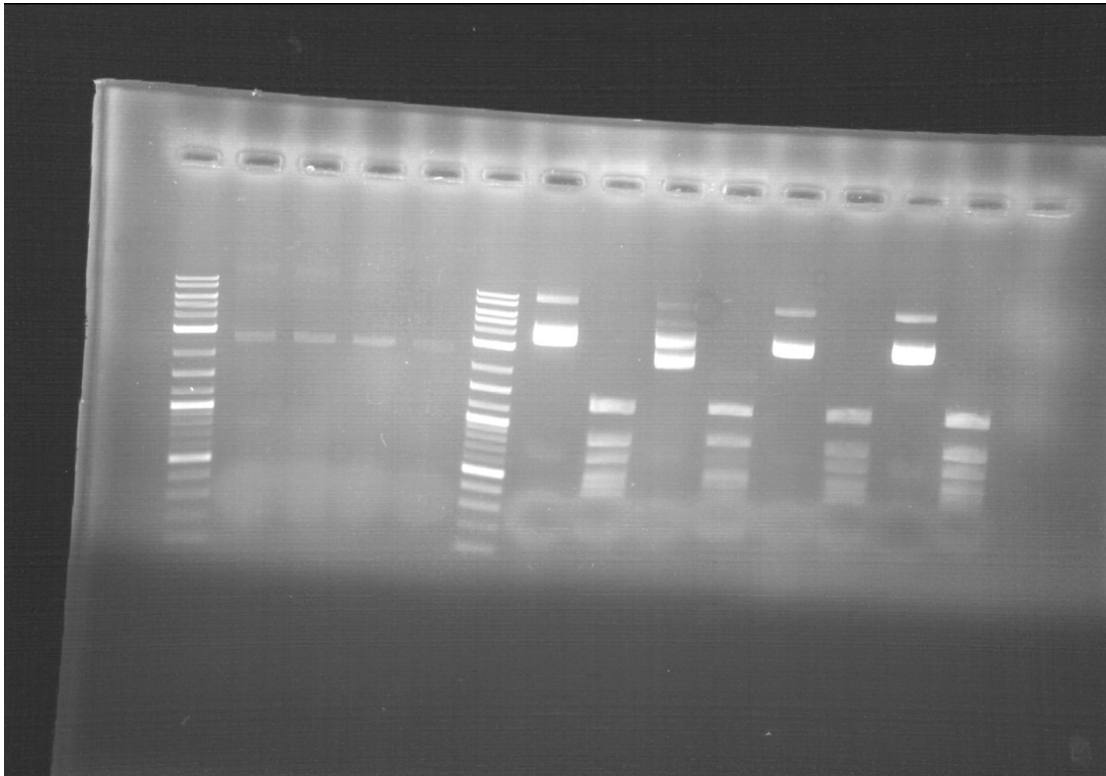
AIDA 2	234,3 ng/ul
AIDA 3	304,4 ng/ul
AIDA 4	257,7 ng/ul
A2 1	217,4 ng/ul
A2 2	147,1 ng/ul

3. Diagnostic Dig

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
AIDA 1	1	1	0,5 EcoRI 0,5 BamHI 0,5 EcoRV	6,5
AIDA 2	1	1	0,5 EcoRI 0,5 BamHI 0,5 EcoRV	6,5
AIDA 3	1	1	0,5 EcoRI 0,5 BamHI 0,5 EcoRV	6,5
AIDA 4	1	1	0,5 EcoRI 0,5 BamHI 0,5 EcoRV	6,5
A2 1	1	1	0,5 HindIII	7,5
A2 2	1	1	0,5 HindIII	7,5

4. **AGE**

Wells from left to right
Ladder|a2 1 uncut|a2 1 cut|a2 2 uncut|a2 2 cut|AIDA 1 uncut|AIDA 1 cut|AIDA 2uncut|AIDA2 cut|AIDA 3 uncut|AIDA3 cut|AIDA4 uncut|AIDA 4 cut|



17/8/2020

Goals of the Day

1. Diagnostic Dig yesterdays minis
2. AGE

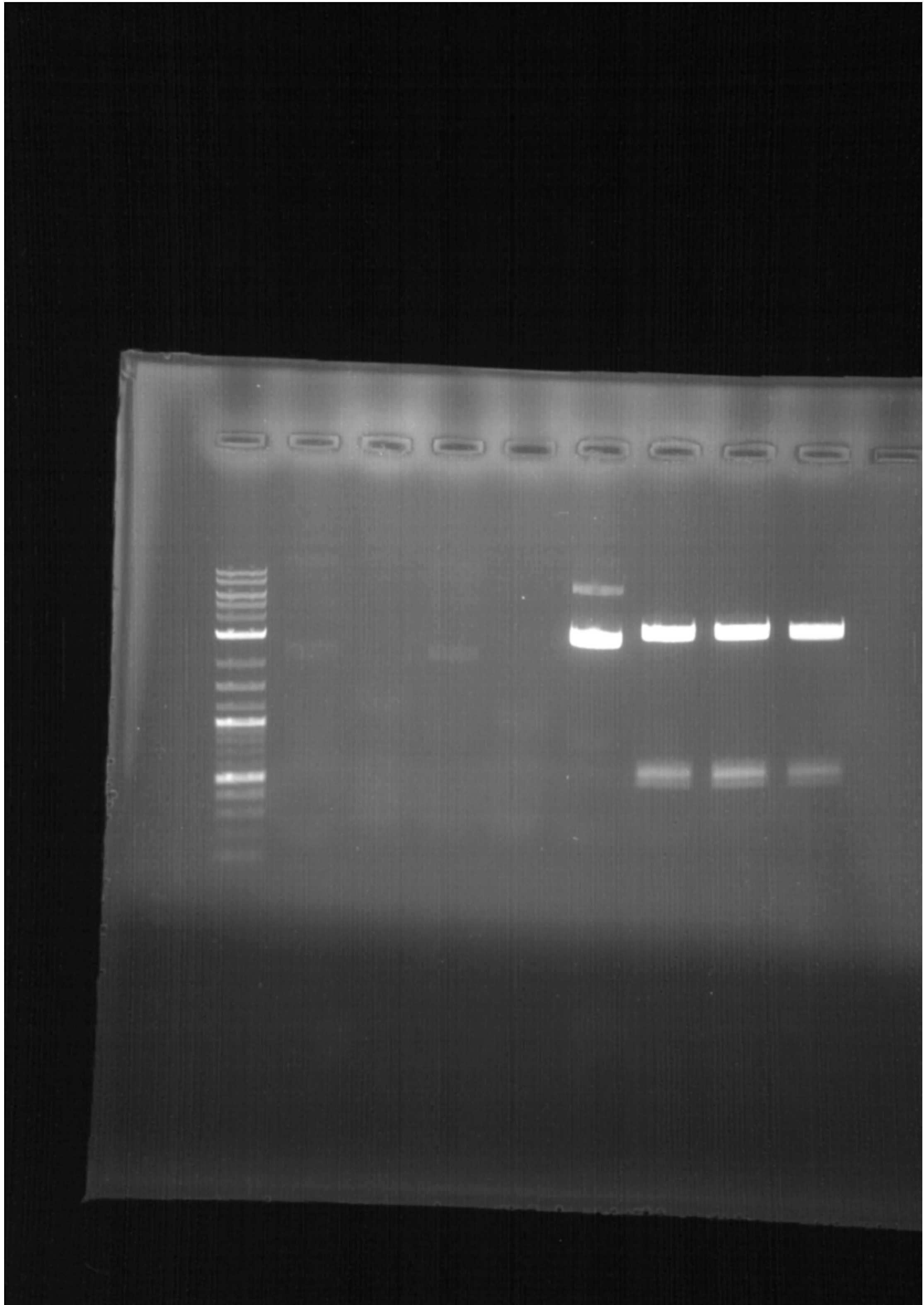
Procedure

1. **Diagnostic Dig**

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
AIDA 1	1	1	0,5 PstI	7,5
AIDA 3	1	1	0,5PstI	7,5
AIDA 4	1	1	0,5PstI	7,5
A2 1	2	1	0,5 HindIII	6,5
A2 2	2,5	1	0,5HindIII	6

2. AGE

Wells from left to right
Ladder|a2 1 uncut|a2 1 cut|a2 2 uncut|a2 2 cut|AIDA 1 uncut|AIDA 1 cut|AIDA2 cut|AIDA3 cut|AIDA 4 cut|



18/8/2020

Goals of the Day

1. Homemade Minipreps

2. Nanodrop
3. Diagnostic Dig
4. AGE
5. Dig LIg a2 and omega 2 again
6. Picking colonies from the old plates for minipreps again

Procedure

1. Homemade Minipreps

I.**Cultivate and harvest bacterial cells**

1. Use **5 mL** of a saturated *E. coli* LB culture, pellet cells in a standard benchtop microcentrifuge for **1 min at 11.000 rpm.** Discard the supernatant and remove as much of the liquid as possiple. Repeat this step with the remaining volume of the LB culture (use as stock 1 ml out of 5ml , just in case.)

II. Cell lysis

2.Add **150 µl Buffer A1** . Resuspend the cell pellet completely by vortexing (or with a pippete). **Make sure no cell clumps remain before addition of Buffer A2!**

3. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. **Do not vortex** to avoid shearing of genomic DNA. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

4.Add **300 µL Buffer A3**. Mix thoroughly (and quick) by inverting the tube 6-8 times **until blue samples turn colorless completely!** Do not vortex to avoid shearing of genomic DNA!

III. Clarification of lysate

1. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear

***IV.* Bind DNA**

2. Decant the supernatant into a new 1,5ml Eppendorf , without agitating the sediment
3. Add 450 µl Isopropanol 100% (1:1 sample : isopropanol), and mix thoroughly.
4. Store the tubes at -80 ◦C or -20 ◦C for 30 min up to 1 hour.
5. Centrifuge for **20 min at 11,000 x rpm** at 4. Repeat this step in case the supernatant is not clear
6. Discard the supernatant without agitating the sediment
7. Resuspend the sediment with 30-50 µl ddH_2O .
8. Store the tubes at -20 ◦C until needed.

2. Nanodrop

A2 1	873,3 ng/ul
A2 2	1365,9

A2 3	543,8
A2 4	1372,3
SEVA 43 1	1808,1
SEVA 43 2	2123,8

3. Diagnostic Dig

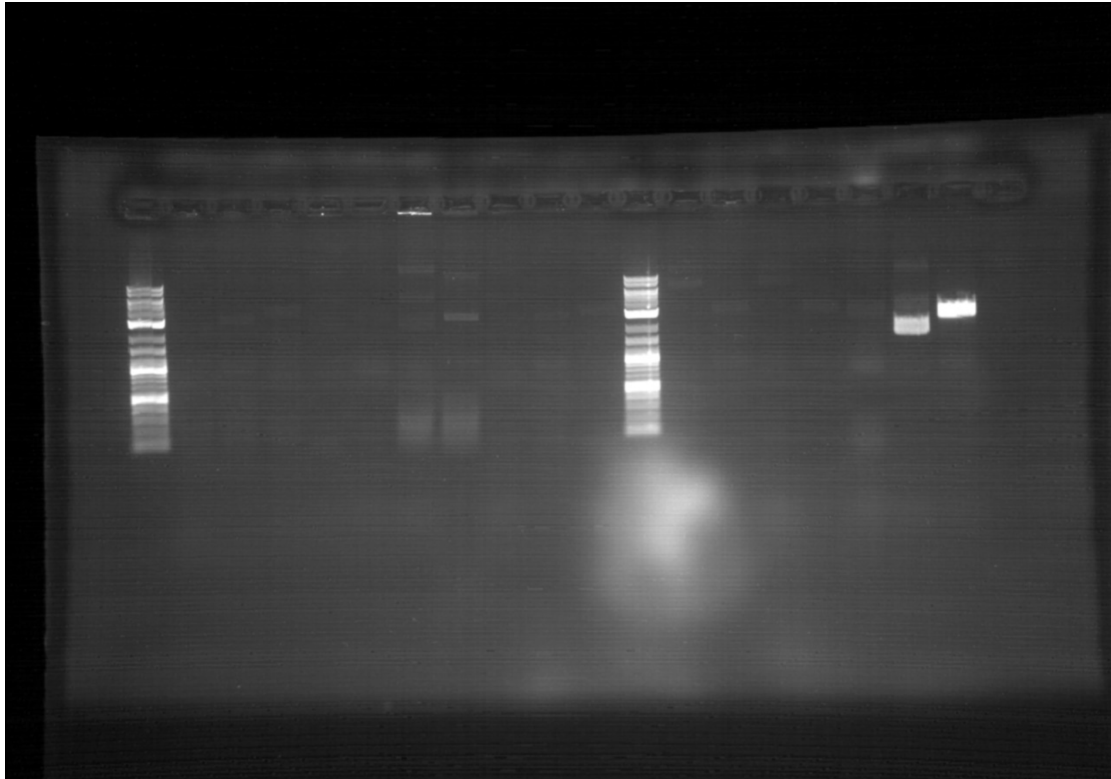
samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
A2 1	1	1	HIndIII 0,5	7,5
A2 2	1	1	HIndIII 0,5	7,5
A2 3	1	1	HIndIII 0,5	7,5
A2 4	1	1	HIndIII 0,5	7,5
SEVA 43 1	1	1	EcoRI 0,5 PstI 0,5	7
SEVA 43 2	1	1	EcoRI 0,5 PstI 0,5	7
A2 1	1	1	EcoRI 0,5	7,5
A2 2	1	1	EcoRI 0,5	7,5
A2 4	1	1	EcoRI 0,5	7,5
TetR (100 ng/ul)	1	1	EcoRI 0,5 PstI 0,5	7

4. AGE

Wells from left to right

Ladder|a2 1 uncut|a2 1 cut|a2 2 uncut|a2 2 cut|a2 3 uncut|a2 3 cut|a2 4 uncut|a2 4 cut|s 43 1 uncut|s 43 1 cut|s 43 2 uncut|s 43 2 cut|

Ladder a2 1 uncut|a2 1 cut|a2 2 uncut|a2 2 cut|a2 4 uncut|a2 4 cut |TetR uncut|TetR cut



5. Dig LIg a2 and omega 2 again

Exactly same as 1/8/20

6. **Picking colonies**

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 5ml LB with antibiotic. Incubation overnight.

19/8/2020

Goals of the Day

1. Transformation DIG-LIG products
2. Run in a gel the whole amount of the blank DNA
3. Homemade minipreps
4. Nanodrop
5. Diagnostic Dig
6. AGE

Procedure

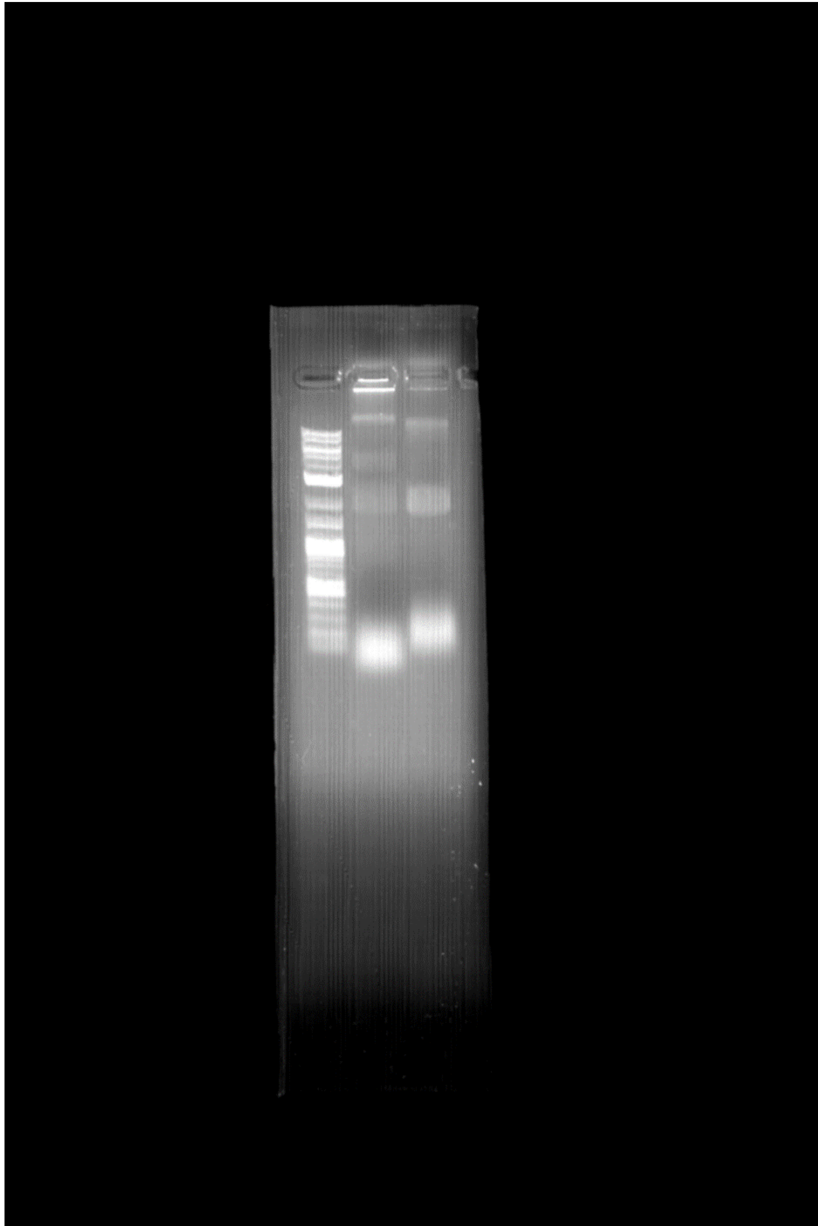
1. Transformation DIG-LIG products

Transformed 2 plates a2 and omega 2 with 5 ul DIG-LIG product each.

2. Run in a gel the whole amount of the blank DNA

Ladder|a2|seva 43

7000 ng DNA each



3. **Homemade minipreps**

Same as Yesterday

4. **Nanodrop**

S 43	804,3 ng/ul
A2 2	502,2ng/ul
A2 3	234,1ng/ul
A2 4	554,5ng/u/

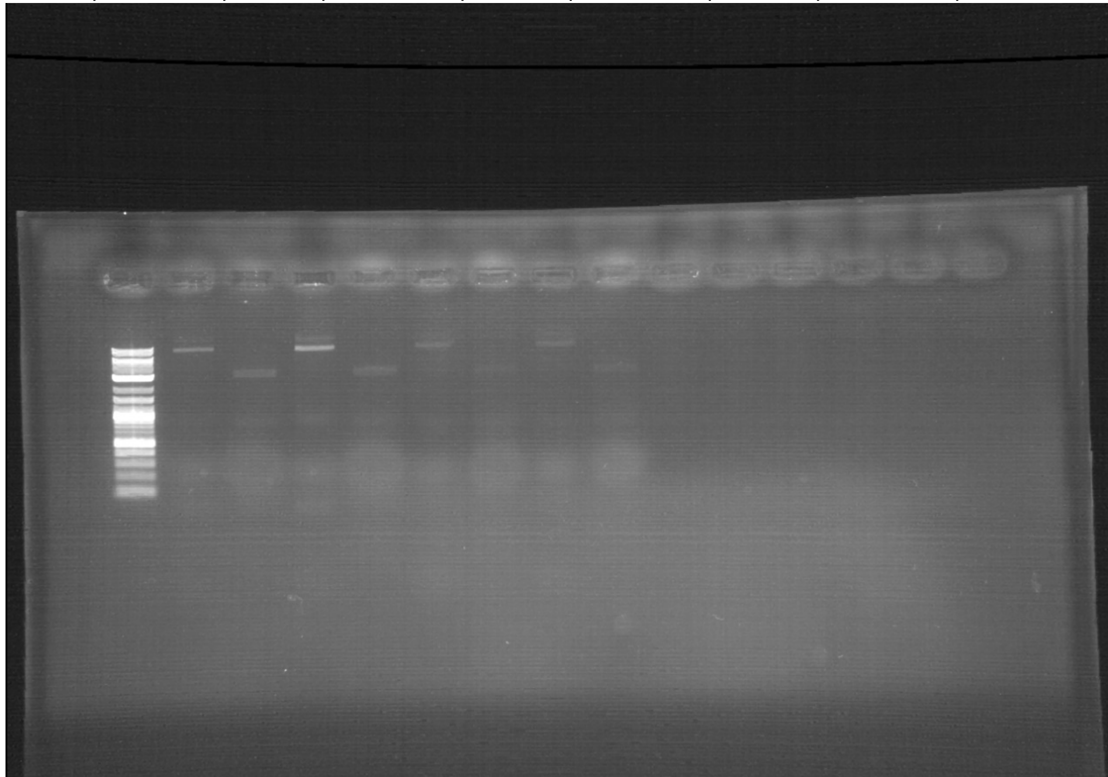
5. **Diagnostic Dig**

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
S 43	1	1	0,5 EcoRI 0,5 PstI	7
A2 2	1	1	0,5 HindIII	7,5
A2 3	1	1	0,5 HindIII	7,5
A2 4	1	1	0,5 HindIII	7,5

6. **AGE**

Wells from left to right

Ladder|s43 uncut|s43 cut|a2 2 uncut|a2 2 cut|a2 3 uncut|a2 3 cut|a2 4 uncut|a2 4 cut



20/8/2020

Goals of the Day

1. Picking colonies
2. DIG-LIG LVL1 AIDA
3. Transformation of DIG-LIG product

Procedure

1. **Picking colonies**

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 10ml LB with antibiotic. Incubation overnight.

2. DIG-LIG LVL1 AIDA

Vector a1R	O,5 ul (56 ng/ul)
Part promoter	0,5 ul (63 ng/ul)
Part CDS (AIDA LVL 0)	1 ul (50 ng/ul)
Part terminator	1 ul (70 ng/ul)
T4 buffer	1ul
T4 ligase	1 ul
BsaI	0,5 ul
ddH20	4,5 ul

3. Transformation

Transformed in a plate 5 ul of the DIG-LIG product

21/8/2020

<u>Goals of the Day</u>

1. Minipreps
2. Nanodrop
3. Diagnostic Dig
4. AGE
5. Picking colonies

<u>Procedure</u>

1. Minipreps

For protocol see 1/8/2020

2. Nanodrop

S43 1	49,5 ng/ul
S43 2	47,4
Omega2 1	21,2
Omega2 2	43,4
A2	54,3

A1R	223,7

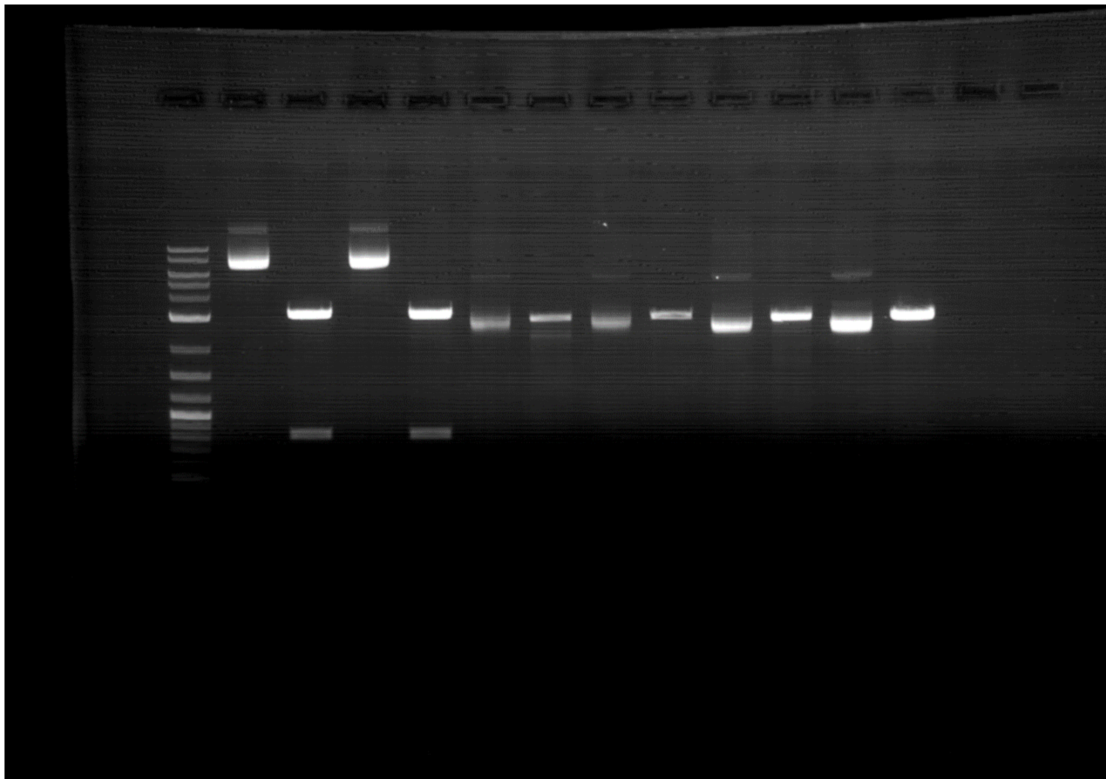
3. Diagnostic Dig

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
S43 1	4	1	0,25 EcoRI 0,25 PstI	4,5
S43 2	4	1	0,25 EcoRI 0,25 PstI	4,5
Omega2 1	4	1	EcoRV 0,5	4,5
Omega2 2	4	1	EcoRV 0,5	4,5
A2	4	1	HindIII 0,5	4,5
A1R	1	1	BamHI 0,5	7,5

4. **AGE**

Wells from left to right

Ladder|s43 1 uncut|s43 1 cut|s 43 2 uncut|s 43 2 cut|omega2 1 uncut|omega2 1 cut|omega2 2 uncut|omega2 2 cut|a2 uncut|a2 cut|a1R uncut|a1R cut



5. **Picking colonies**

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in **50ml** LB with antibiotic. Incubation overnight.

22/8/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic dig
4. K Taq Diagnostic PCR yesterdays bad AGE
5. AGE
6. Picking colonies

Procedure

1. **Minipreps**

For protocol see 1/8/2020

2. Nanodrop

AIDA 1	50,1 ng/ul
AIDA 2	33,6 ng/ul
S 43	100,3 ng/ul
A2	120,9 ng/ul

3. Diagnostic dig

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
AIDA 1	4	1	0,3 EcoRI	4,7
AIDA 2	4	1	0,3 EcoRI	4,7
S 43	2	1	0,3 EcoRI 0,3 PstI	6.4
A2	2	1	0,5 HindIII	6,5

4. K Taq Diagnostic PCR yesterdays bad AGE

Time sec	Temp Celcious
30	95
10	95
10	55
60	72
300	72
forever	4

Repeat step 2,3,4 33 times

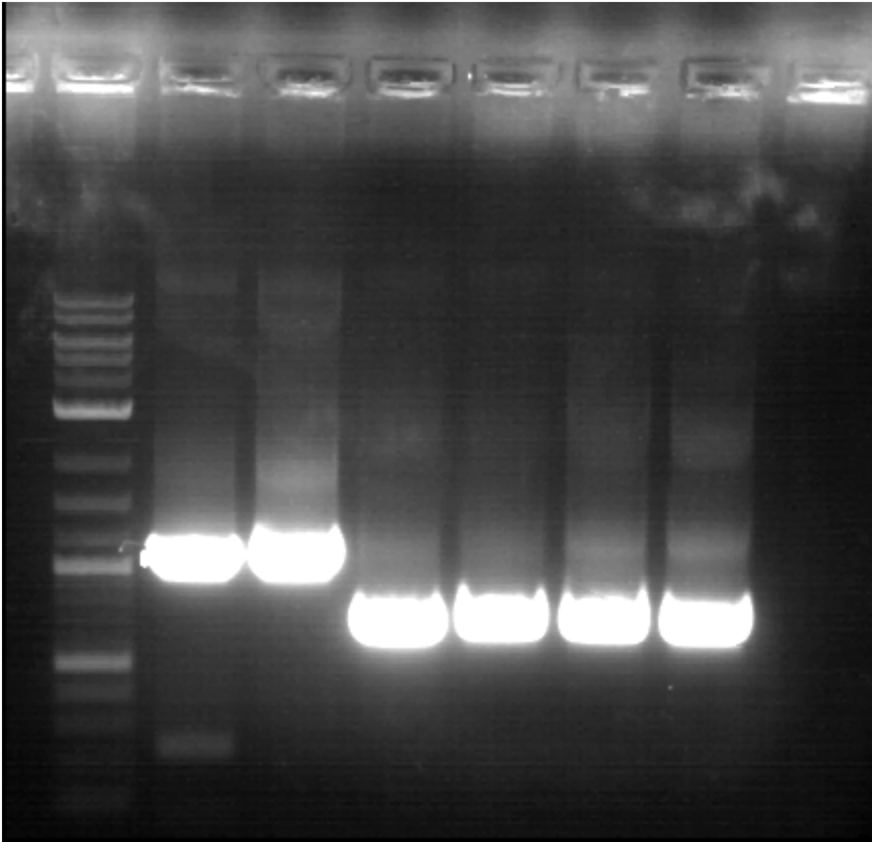
Foe each reaction DNA was diluted at 25 ng/ul

Buffer	1 ul
dNTPs	0,2 ul
Primer f	0,4 ul
Primer r	0,4 ul
K Taq	0,04ul
DNA	1 ul
ddH20	6,96 ul

5. AGE

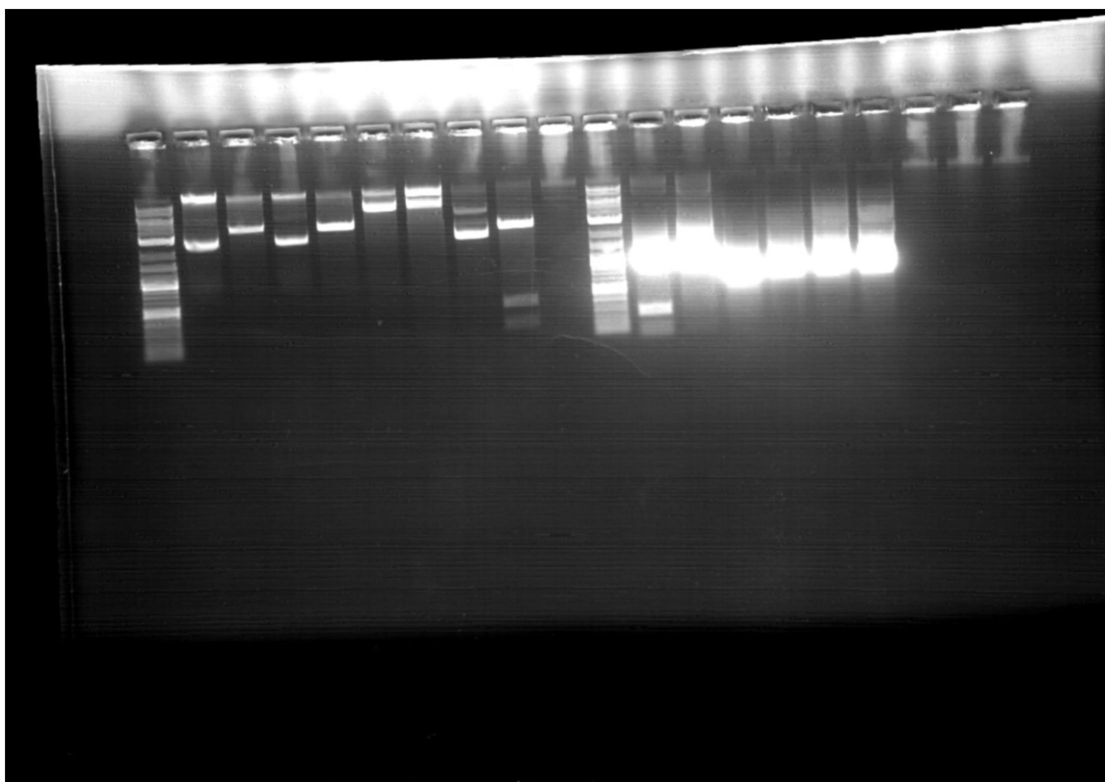
Wells from left to right

Ladder|PCR S43 1|PCR S43 2|PCR omega2 1|PCR omega 2 2|PCR a2|PCR a1R



Wells from left to right

Ladder|AIDA 1 uncut|AIDA 1 cut|AIDA 2 uncut|AIDA 2 cut|s 43 uncut|s43 cut|a2 uncut|a2 cut



6. **Picking colonies**

Picking wight colonies for AIDA blue for omega 2 and green for s43

23/8/2020

<u>Goals of the Day</u>

1. Minipreps
2. Nanodrop
3. Diagnostic dig
4. AGE
5. Transformation a2 and omega2 for stock
6. DIG-LIg AIDA LVL1 again

<u>Procedure</u>

1. **Minipreps**

For protocol see 5/8/2020

2. **Nanodrop**

AIDA 1	29 ng/ul
AIDA 2	27 ng/ul
Omega2	155,7 ng/ul
Seva 43	181,0 ng/ul

3. Diagnostic dig

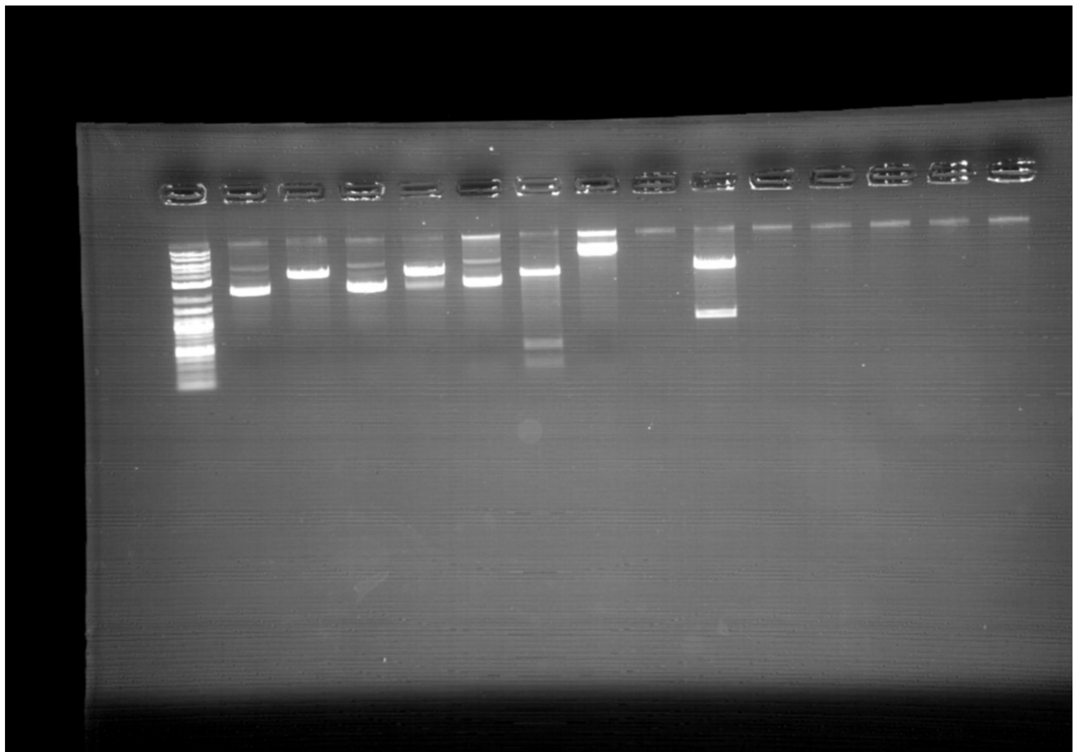
.

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
AIDA 1	5	1	0,3 EcoRI	3,7
AIDA 2	5	1	0,3 EcoRI	3,7
Omega2	1	1	0,5 EcoRV	7
Seva 43	1	1	0,3 EcoRI 0,3 PstI	7,4

4. AGE

Wells form left to right

Ladder| AIDA 1uncut|AIDA1 cut|AIDA 2 uncut|AIDA 2 cut|omega2 uncut|omega2 cut|seva 43 uncut|seva 43 cut



5. Transformation a2 and omega2 for stock

For protocol see 1/8/2020

6. Dig-LIG

Exactly same as 18/8/2020

24/8/2020

Goals of the Day

1. Minipreps for stock a2
2. Nanodrop
3. Diagnostic PCR
4. AGE
5. Picking again colonies

Procedure

1. Minipreps for stock a2

For protocol see 5/8/2020

2. Nanodrop

A2 mini 1	37,3 ng/ul
A2 mini 2	35,9 ng/ul

3. Diagnostic PCR

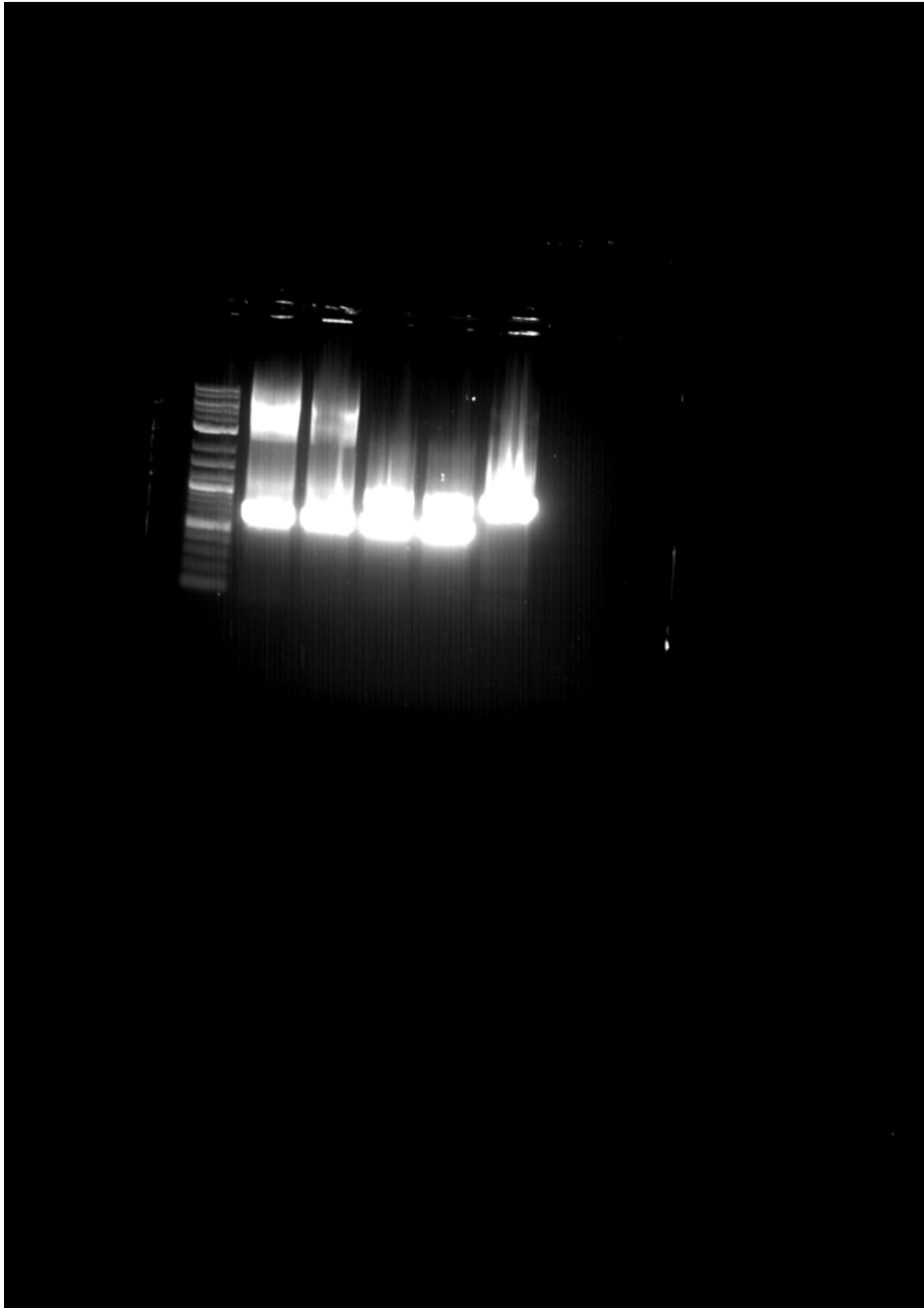
Same procedure and protocol as 20/8/20

Different plasmid for DNA this time

4. AGE

Wells form left to right

Ladder|a2 mini1|a2 mini2|a2 120 ng|omega2 155ng|seva 43 180 ng



5. **Picking again colonies**

A2 and omega2 for stock

Picking single colonies from the plates to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 10ml LB with antibiotic. Incubation overnight.

25/8/2020

Goals of the Day

1. Minipreps for stock a2 and omega2
2. Nanodrop
3. Diagnostic PCR
4. AGE
5. Picking again colonies

Procedure

1. Minipreps for stock a2 and omega2

For protocol see 5/8/20

2. Nanodrop

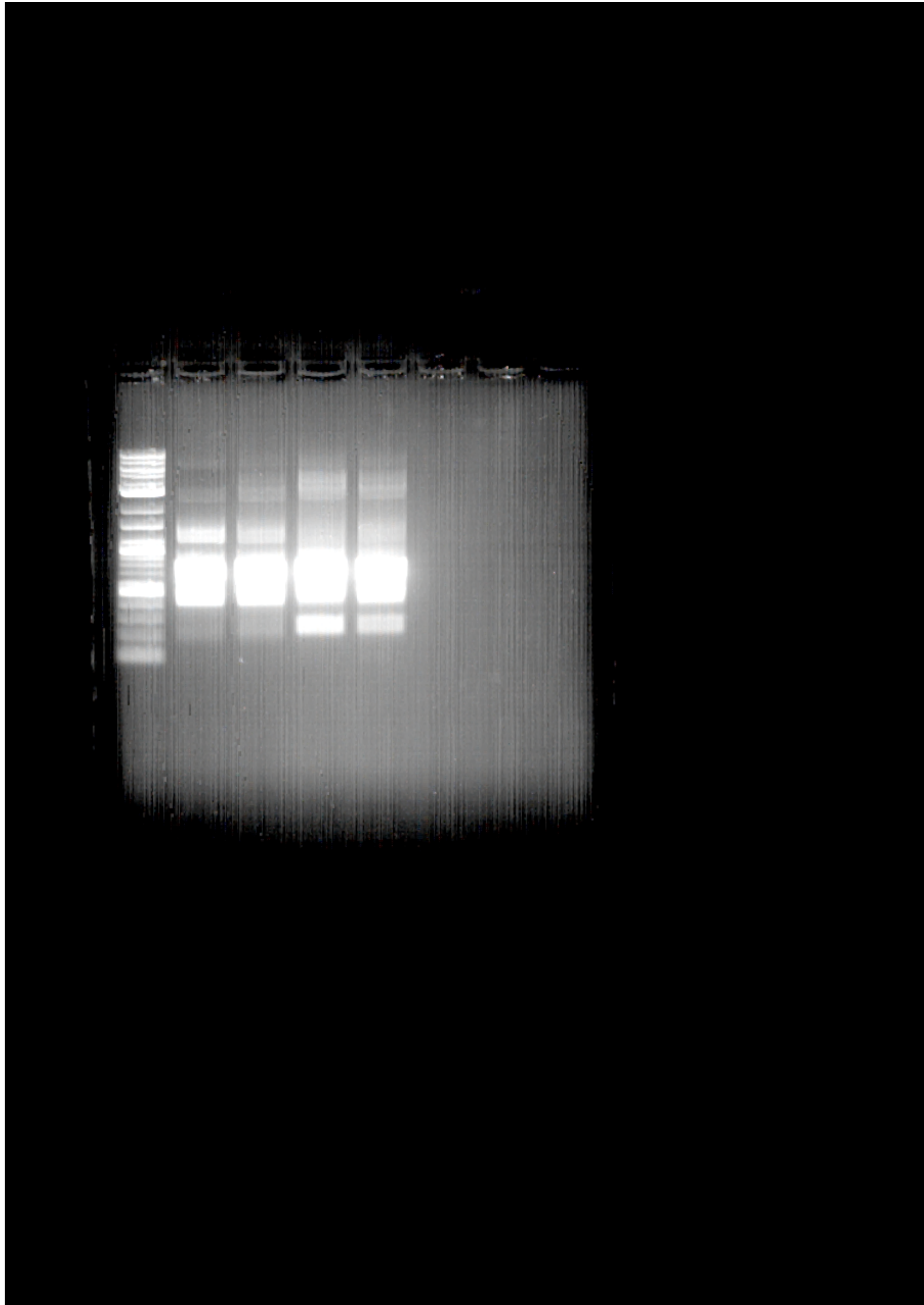
A2 mini 1	31,8 ng/ul
A2 mini 2	30,6 ng/ul
Omega2 mini1	34,9 ng/ul
Omega2 mini2	39,2 ng/ul

3. Diagnostic PCR

Same procedure and protocol as 20/8/20
Different plasmid for DNA this time

4. AGE

Wells from left to right
Ladder|a2 mini1|a2 mini2|omega2 mini1|omega2 mini2



5. Picking colonies

Picking colonies for AIDA LVL 1

26/8/2020

Goals of the Day

1. Minipreps for AIDA LVL1
2. Nanodrop

3. Diagnostic DIG
4. AGE
5. Picking again colonies

Procedure

1. Minipreps for AIDA LVL1

For protocol see 5/8/20

2. Nanodrop

AIDA 1	282,1 ng/ul
AIDA 2	344.1 ng/ul
AIDA 3	269,4 ng/ul

3. Diagnostic DIG

For protocol see 1/8/20

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
AIDA 1	1	1	0,5 BamHI	7,5
AIDA 2	1	1	0,5 BamHI	7,5
AIDA 3	1	1	0,5 BamHI	7,5

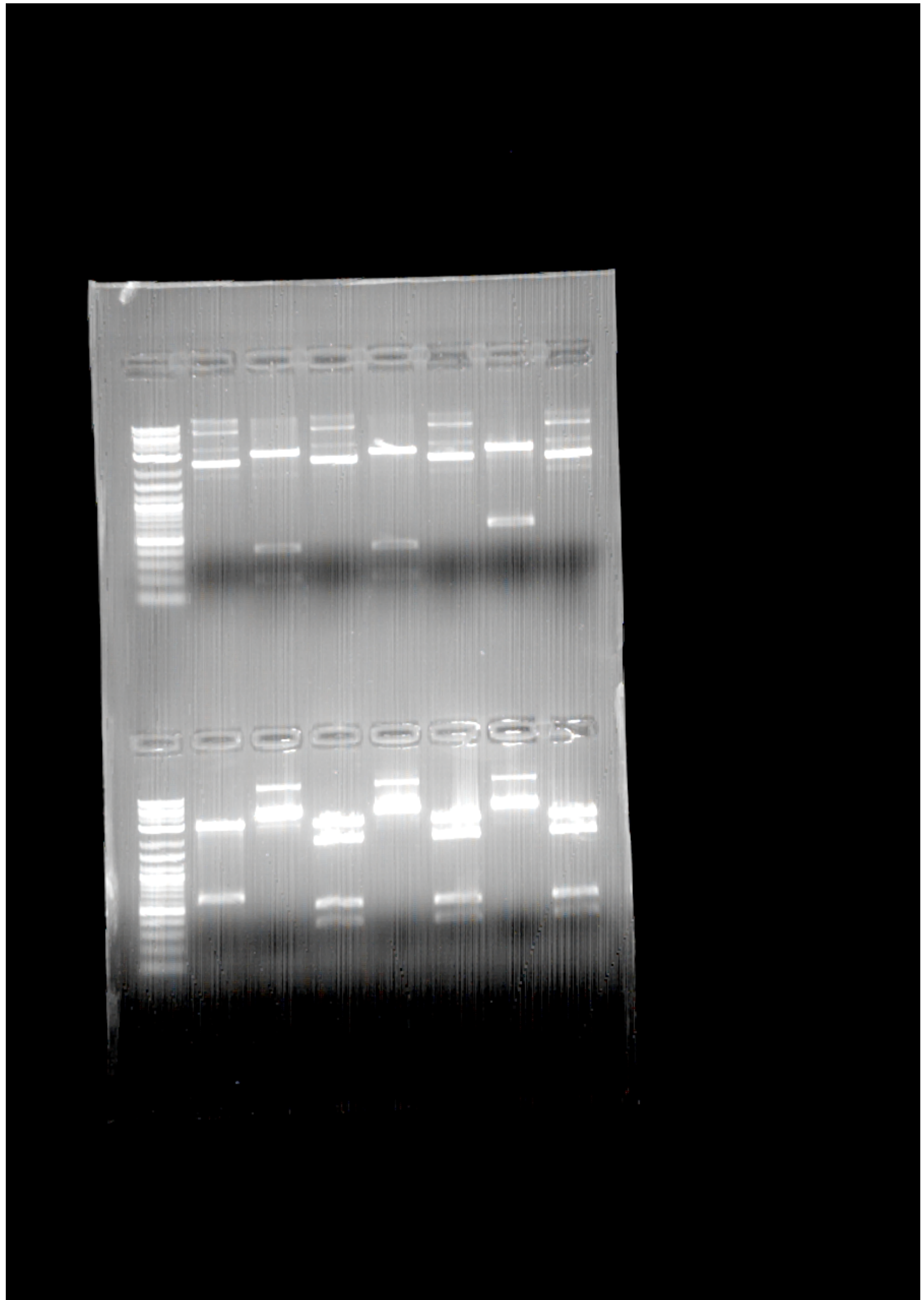
4. AGE

For protocol see 5/8/20

Wells from left to right

Ladder|a2 uncut|a2 cut|a2 uncut|a2 cut|omega2 uncut|omega2 cut|omega2 uncut

Ladder|omega2 cut|AIDA 1 UNCUT|AIDA1 CUT|AIDA2 UNCUT|AIDA2 CUT|AIDA3 UNCUT|AIDA3 CUT|



27/8/2020

Goals of the Day

1. Q5 PCR p3omega1 Lacza
2. DIG the SEVA43 backbone

3. AGE
4. Gel exctarction
5. DIG gel exctracted PCR product
6. Clean up
7. Ovenrnight Ligation

Procedure

For whole day protocols see 1/8/2020

1. Q5 PCR p3omega1 Lacza

Time in sec	Temp in celcius
30	98
5	98
15	58
23	72
120	72
forever	4

Repeat step 2,3,4 33 times

Components for 50 ul reaction	
Q5 2x mix	25 ul
For primer	2,5 ul
Rev primer	2,5 ul
DNA (0.9 ng/ul)	1 ul
ddH20	19 ul

2. DIG the SEVA43 backbone

50 ul reaction

Sample	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
PCR LacZa	10	5	1 NotI	34

3. AGE

Gel not provided because it was destroyed during gel extraction.

4. Gel extraction

For protocol see 1/8/2020

Extraction the backbone of the dig and the LacZa from the PCR

Dig backbone 0,186 g	372 ul NT1
PCR LacZa 0,161 g	322 ul NT1

5. Nanodrop

Dig backbone	29,4 ng/ul
PCR LacZa	113,0 ng/ul

6. DIG gel exctracted PCR product

Sample	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
PCR LacZa	14	5	1 NotI	30

7. Clean up

For protocol see 1/8/2020

Same protocol as Gel extraction. The only difference, we put volume 2/1 as NT1/ul of uncleaned product

8. Nanodrop

PCR LacZa CLEANED	90 ng/ul

9. Ovenrnight Ligation

Overnight incubation at 16 celcius 20 ul reaection

Vector	1,7 ul (50 ng)
Insert	0,85 ul (80 ng)
T4 Buffer	2 ul
Ligase	2 ul
ddH20	13,45 ul

28/8/2020

Goal of the Day

Transformation

Procedure

Plate 5 ul of Ligated product in Spect plates

31/8/2020

Goal of the Day

Picking colonies

Procedure

Picking single colonies from the plate to make liquid cultures. Picking blue colonies from the blue white screening because we want LacZa insert. Picking with the tip of the pipette and throw it in 10ml LB with antibiotic. Incubation overnight.

1/9/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic DIG
4. AGE
5. Re inoculation for 50 ml liquid culture
6. Transformation

Procedure

1. Minipreps

For protocol see 5/8/2020

2. Nanodrop

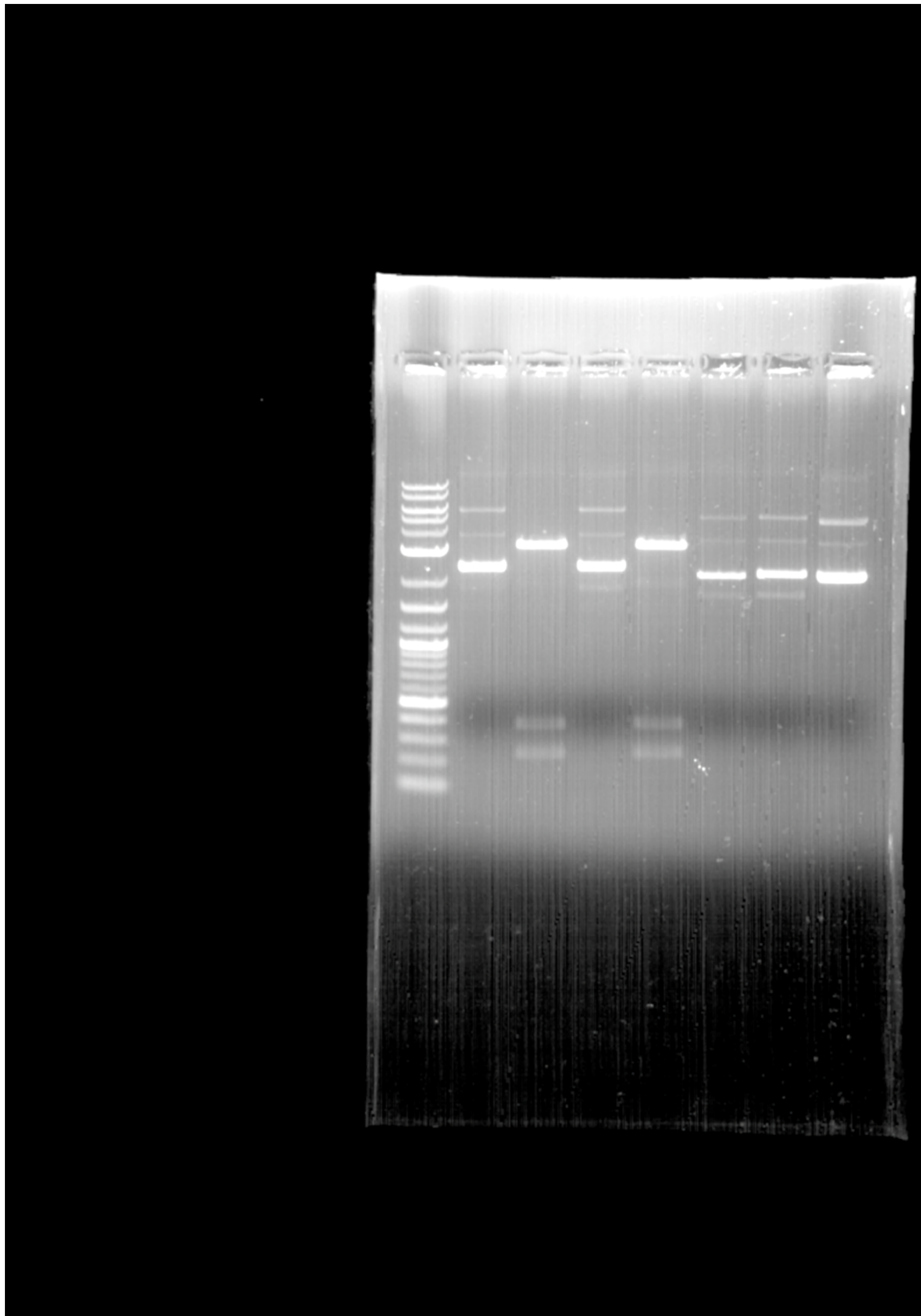
Omega1 mini1	34,4 ng/ul
Omega1 mini 2	36,5 ng/ul

3. Diagnostic DIG

samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
Omega1 mini1	5	1	0,5 BamHi	3,5
Omega1 mini 2	5	1	0,5 BamHI	3,5

4. AGE

Wells from left to right Ladder|Omega1 mini1 Uncut|Omega1 mini1 Cut|Omega1 mini2 uncut|Omega1 mini2 Cut



5. **Re inoculation for 50 ml liquid culture**

   From the omega1 mini1

6. **Transformation**

For protocol see 2/8/20

2/9/2020

<u>Goals of the Day</u>

1. Minipreps
2. Nanodrop

3. Diagnostic DIG
4. AGE
5. Picking colonies

<u>Procedure</u>

1. Minipreps

For protocol see 5/8/2020

2. Nanodrop

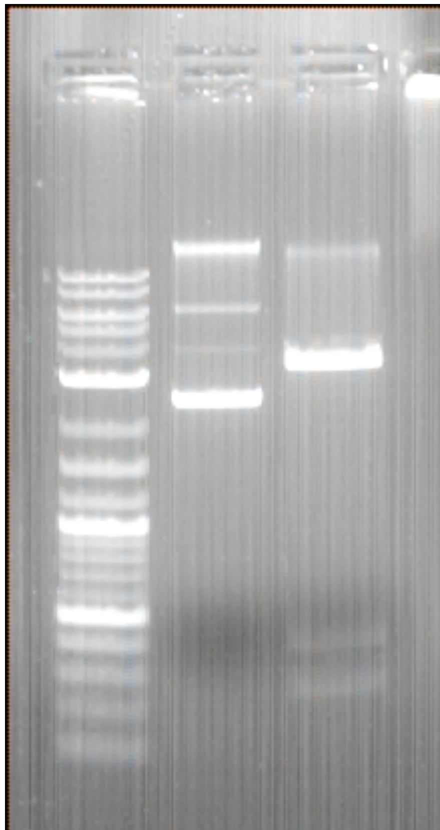
Omega1 mini 50 ml	152,4 ng/ul

3. Diagnostic DIG

Samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
Omega1 mini 50 ml	1	1	0,5 BamHI	7,5

4. AGE

Wells from left to right Ladder|omega 1 Uncut|omega1 cut



5. Picking colonies

Pure plasmid was plated so no blue white screening needed.

3/9/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic DIG
4. AGE
5. Q5 PCR

Procedure

1. Minipreps

For protocol see 5/8/2020

2. Nanodrop

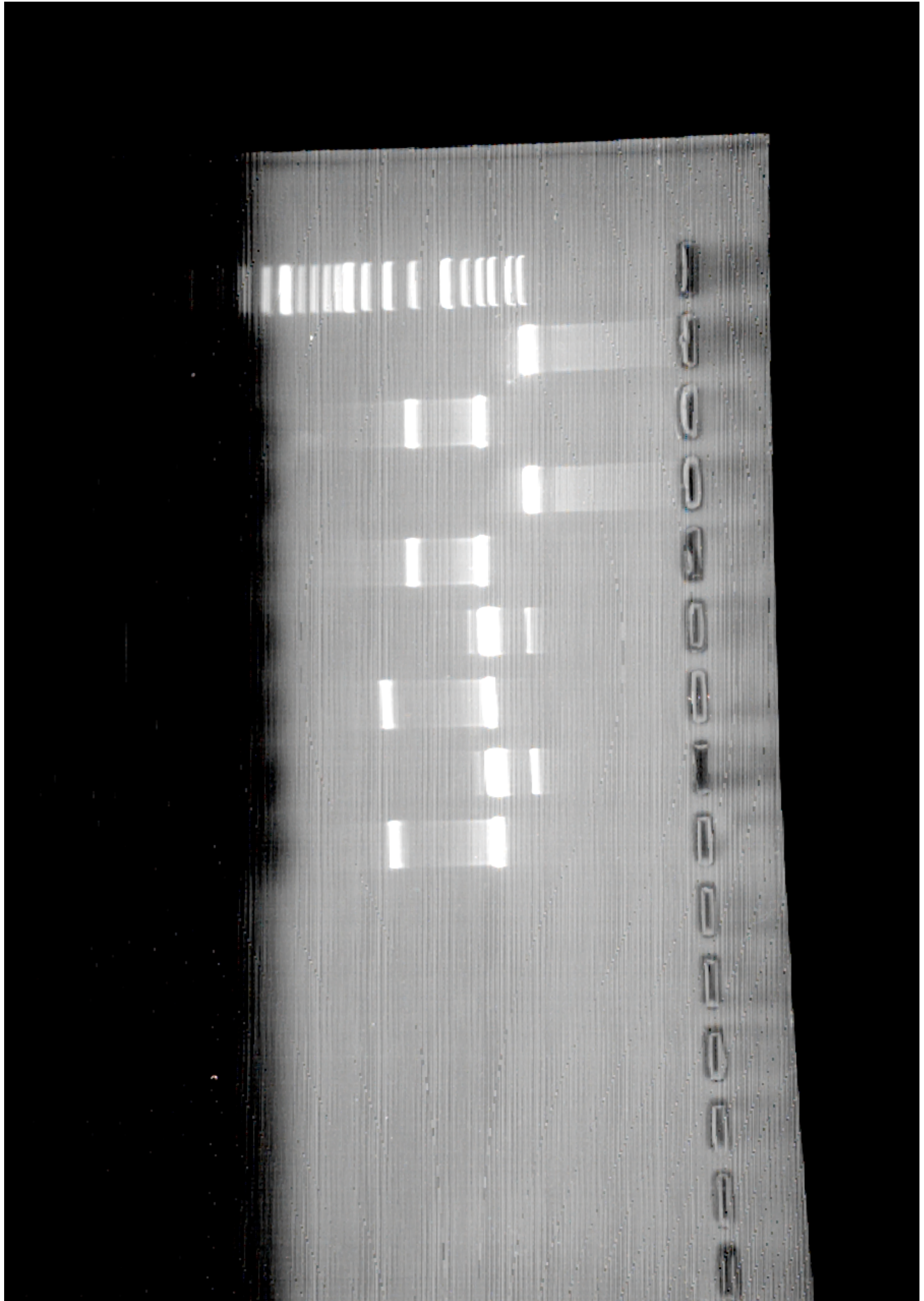
SuptoxD 1	87,2 ng/ul
SuptoxD 2	100,6 ng/ul
SuptoxR 1	60,4 ng/ul
SuptoxR 2	63,0 ng/ul

3. Diagnostic DIG

Samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
SuptoxD 1	3	1	0,5 PstI, 0,5 EcoRI	5
SuptoxD 2	2,5	1	0,5 PstI, 0,5 EcoRI	5,5
SuptoxR 1	4	1	0,5 EcoRI	4,5
SuptoxR 2	4	1	0,5 EcoRI	4,5

4. ACE

Wells from left to right Ladder| SuptoxD 1 uncut| SuptoxD 1 cut| SuptoxD 2 uncut| SuptoxD 2 cut| SuptoxR 1 uncut| SuptoxR 1 cut| SuptoxR 2 uncut| SuptoxR 2 cut|



5. **Q5 PCR SuptoxD, SuptoxR**

For whole day protocols see 1/8/2020

Time in sec	Temp in celcius

30	98
5	98
15	58
23	72
120	72
forever	4

Repeat step 2,3,4 33 times

Components for 50 ul reaction	
Q5 2x mix	25 ul
For primer	2,5 ul
Rev primer	2,5 ul
DNA (0.9 ng/ul)	1 ul
ddH20	19 ul

5/9/2020

Goals of the Day

1. DIG-LIG
2. Transformation the DIG-LIG product

Procedure

1. DIG-LIG

For protocol see 2/8/2020

pUPD2	0,5 ul
SuptoxD amplifeid	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsmBI	0,5 ul
ddH20	6,5 ul

pUPD2	0,5 ul
SuptoxR amplifeid	0,5 ul
T4 ligase	1 ul

T4 Buffer	1 ul
BsmBI	0,5 ul
ddH20	6,5 ul

2. Transformation

Fro protocol see 3/8/2020

6/9/2020

Goal of the Day

1. Picking colonies

Procedure

Pick white colonies from the plate and throw it in 10ml LB for overnight incubation with CamR.

7/9/2020

Goals of the Day

1. Minipreps
2. Nanodrop
3. Diagnostic DIG
4. AGE
5. DIG-LIG

Procedure

1. Minipreps

For protocol see 5/8/2020

2. Nanodrop

RraA=R

DjlA=D

araC=C

sfGFP=sf

sample	Concertation dna ng/ul
R mini1	196,9
R mini2	206,3

D mini 1	214,3
D mini 2	248,0
C mini 1	564,1
C mini 2	289,9
Sf mini 1	247,4
Sf mini 2	385,9

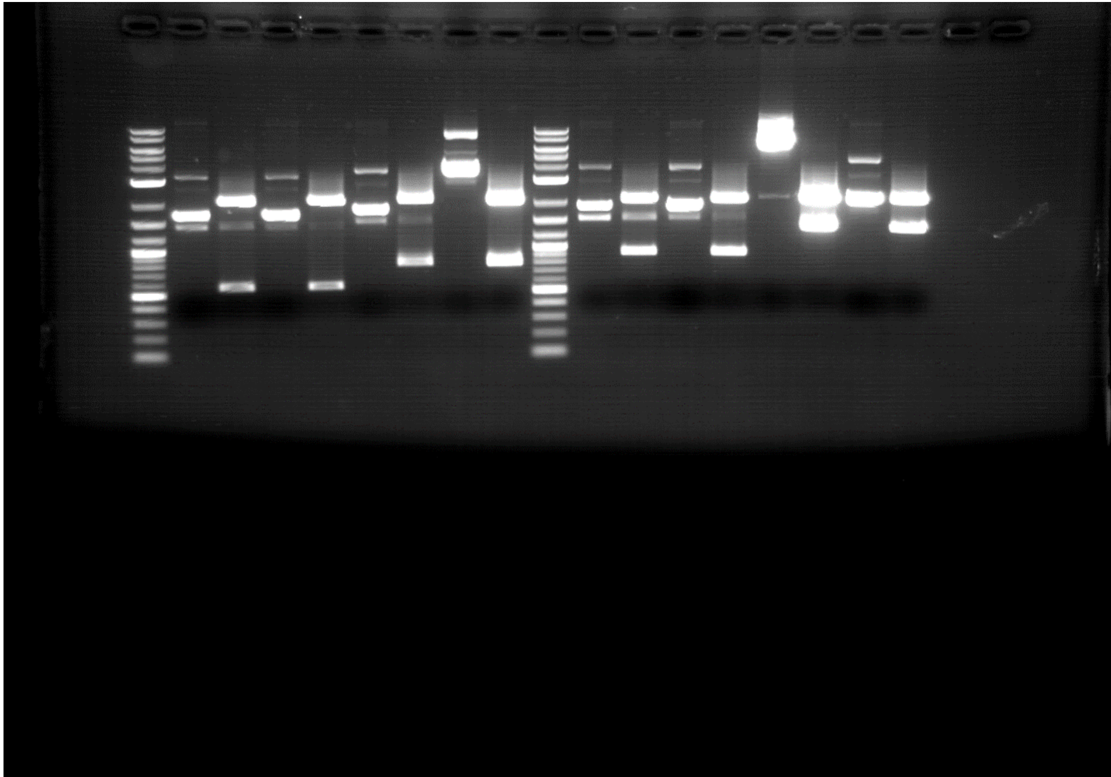
3. Diagnostic DIG

Samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)		ddH20(ul)
R mini1	1	1	0,5 EcoRI	0,5 PstI	7
R mini2	1	1	0,5 EcoRI	0,5 PstI	7
D mini 1	1	1	0,5 EcoRI	0,5 PstI	7
D mini 2	1	1	0,5 EcoRI	0,5 PstI	7
C mini 1	1	1	0,5 EcoRI	0,5 PstI	7
C mini 2	1	1	0,5 EcoRI	0,5 PstI	7
Sf mini 1	1	1	0,5 EcoRI	0,5 PstI	7
Sf mini 2	1	1	0,5 EcoRI	0,5 PstI	7

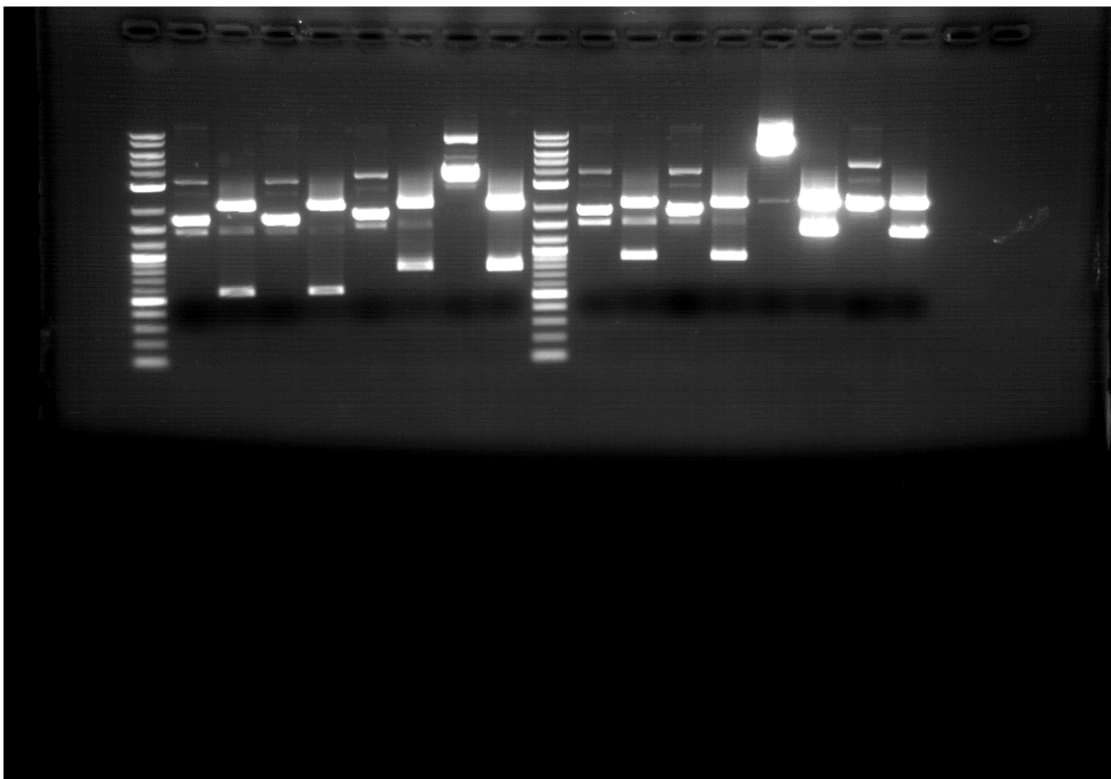
4. AGE

U=uncut C=cut

Wells from left to right Ladder|RU1|RC1|RU2|RC2|DU1|DC1|DU2|DC2|



Wells from left to right Ladder|CU1|CC1|CU2|CC2|SFU1|SFC1|SFU2|SFC2



5. DIG-LIG

For protocol see 2/8/2020

1) arac-R-term

A2 vector	0,5 ul
araC	0,5 ul
R	0,5 ul
Terminator	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

2) araC-D Term

A2 vector	0,5 ul
araC	0,5 ul
D	0,5 ul
Terminator	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

3) araC-AIDA-Term

A1R vector	0,5 ul
araC	0,5 ul
AIDA	0,5 ul
Terminator	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

4)pflic-sfGFP-term

A1R vector	0,5 ul
pflic	0,5 ul
sf	0,5 ul
Terminator	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

8/9/2020

Goal of the Day

1. Transformation

Procedure

1), 2), 3), 4) in four plates of KanR

10/9/2020

Goals of the Day

1. Colony RCR
2. AGE
3. Picking colonies

Procedure

1. Colony PCR

Because of the bad plates we couldn’t pick colonies so we did Colony PCR

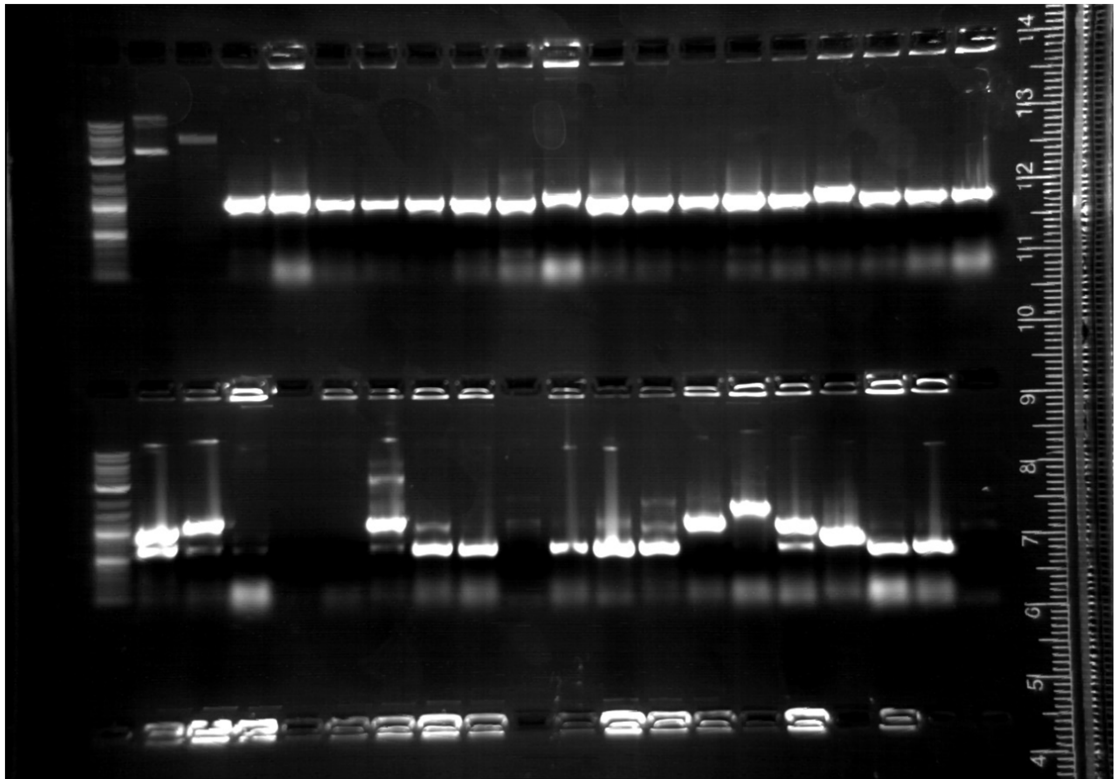
1. Mark the colonies on the plate and label them with numbers If you have a negative control plate, mark 1 to 3 colonies
2. Take out PCR tubes and label them as the colonies
3. Be sure that you have prepared replica plates and draw to the back of them distinct sections; one per colony. Label them as the above
4. Prepare the MasterMix Number of reactions: number of colonies, 1 NTC without colony, 1 to 2 NTC with the colony/ies from the NTC plate, 1extra/10 colonies
5. Transfer 25 ul MM to the PCR tubes
6. Pick a colony with a tip
7. ‘’Poke”softlytothereplicaplate
8. DissolvetherestinthePCRtube
9. Repeat for all the colonies
10. Quick spin down

For the rest see 10/8/2020

2. AGE

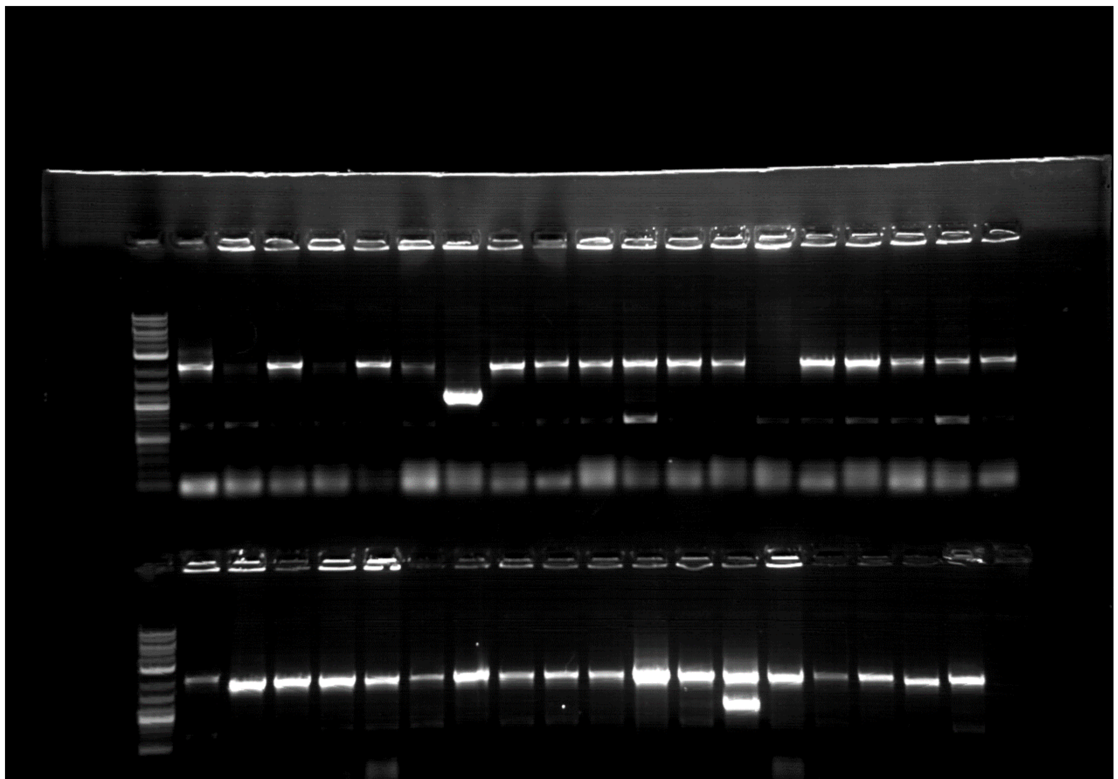
First Line are colonies from the 1) plate

Second Line are colonies from the 2) plate



First line are colonies from the 3) plate

Second Line are colonies from 4) plate



3. Picking colonies

From plate 1) Number 3, 8

From plate 2) Number 11, 12

3/10/20

Goals of the Day

1. Miniprep
2. Nanodrop
3. Diagnostic dig
4. AGE

Procedure

1. Miniprep

DjlA=D

RraA=R

2. Nanodrop

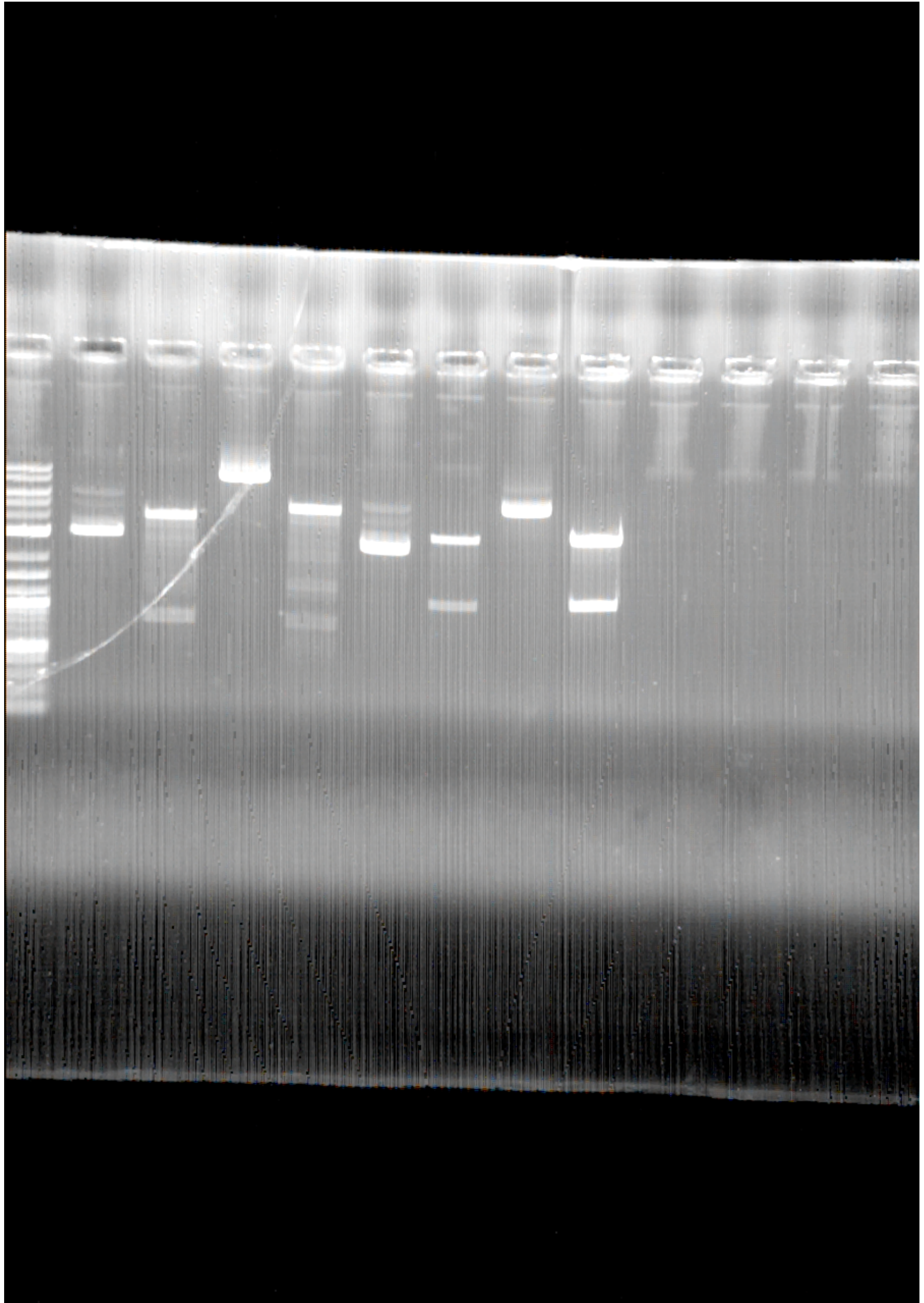
sample	Concertation dna ng/ul
D mini1	54,1
D mini2	70,7
R mini 1	69,2
R mini 2	68,0

3. Diagnostic dig

Samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
D mini1	3	1	0,5 BamHI 0,5 EcoRV	5
D mini2	2	1	0,5 BamHi 0,5 EcoRV	6
R mini 1	2	1	0,5 EcoRV	6,5
R mini 2	2	1	0,5 EcoRV	6,5

4. AGE

Wells from left to right|Ladder|DU1|DC1|DU2|DC2|RU1|RC1|RU2|RC2



4/10/20

Goals of the Day

1. DIG-LIG
2. Transformation

Procedure

1. **DIG-LIG**

3 Reactions

1)omega1r rraa-ecfp

Omega 1R vector	1 ul
Rraa	0,5 ul
eCFP	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsmBI	0,5 ul
ddH20	5,5 ul

2) omega1r pflic:lacI-eCFP

Omega1R vector	1 ul
Pflic:LacI	0,5 ul
eCFP	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

3) omega2 pflic:lacI-eCFP

omega2 vector	1 ul
Pflic:LacI	0,5 ul
eCFP	0,5 ul
T4 ligase	1 ul
T4 Buffer	1 ul
BsaI	0,5 ul
ddH20	5,5 ul

2. **Transformation**

3 plates fo Spectinomycin

5/10/20

Goal of the Day

Picking colonies

6/10/20

Goals of the Day

1. Miniprep

2. Nanodrop
3. Diagnostic dig
4. AGE

Procedure

1. Miniprep

Omega 1R rraa

Omega 1R pflic

Omega 2 pflic

2. Nanodrop

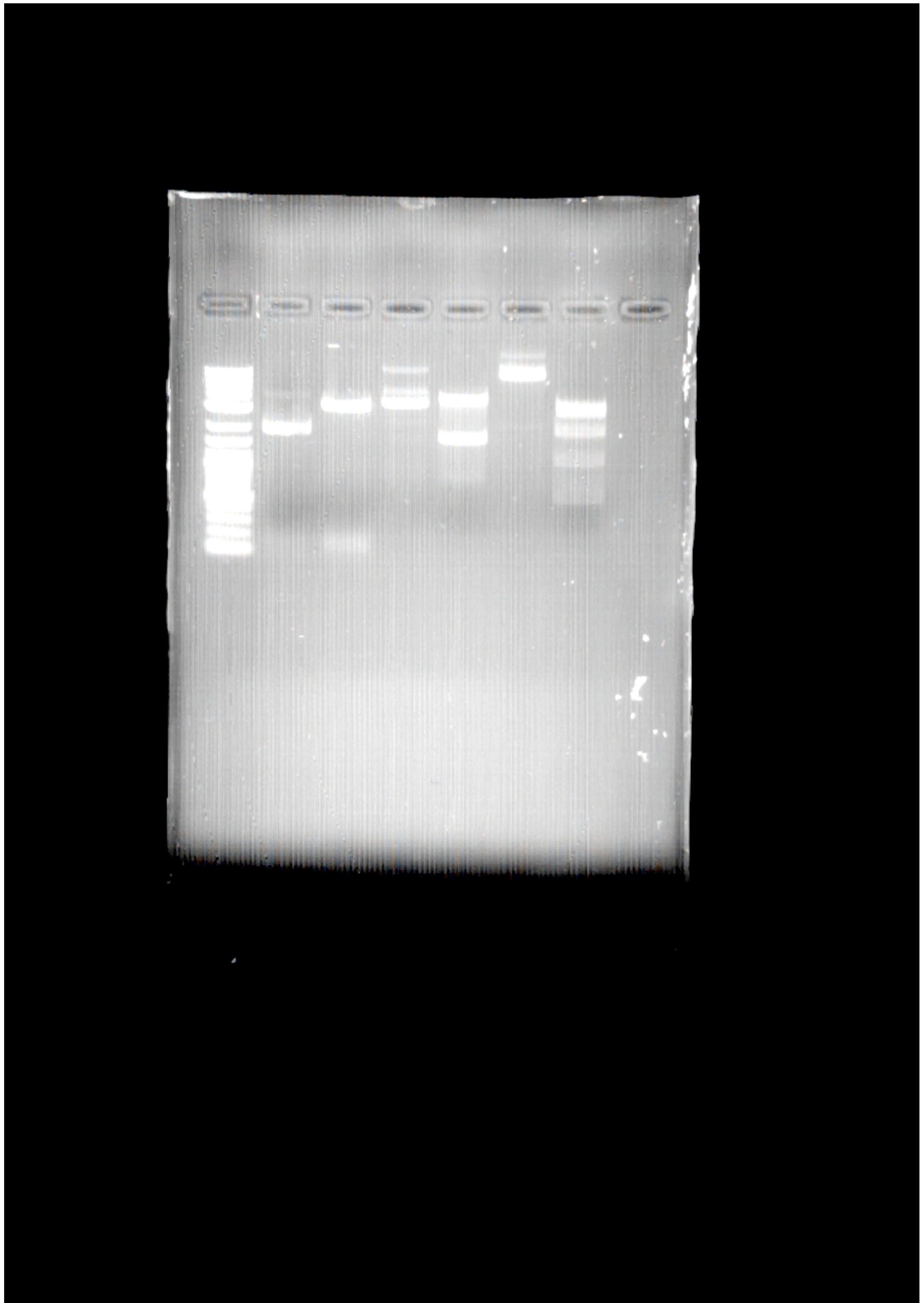
Sample	Concertation dna ng/ul
Omega 1R rraa mini 1	42,8
Omega 1R rraa mini 2	65,8
Omega 1R pflic mini 1	61,0
Omega 1R pflic mini 2	64,0
Omega 2 pflic miini 1	43,9
Omega 2 pflic mini 2	45,6

3. Diagnostic dig

Samples	Amount of DNA for DIG(ul)	Buffer (ul)	Enzyme(ul)	ddH20(ul)
Omega 1R rraa mini 1	4	1	0,5 BamHI	4,5
Omega 1R rraa mini 2	4	1	0,5 BamHI	4,5
Omega 1R pflic mini 1	4	1	0,5 BamHI	4,5
Omega 1R pflic mini 2	4	1	0,5 BamHI	4,5
Omega 2 pflic miini 1	4	1	0,5 EcoRV	4,5
Omega 2 pflic mini 2	4	1	0,5 EcoRV	4,5

4. AGE

Wells from left to right RU|RC|1RFU|1RFC|2FU|2FC



11/10/20

Stock solutions

Made Tyrosinase assay buffer

12/10/20

The previous day you have done a transformation to have fresh bacteria 2 plates: one plate BL21 with tyrosinase and one BL21 without plasmid

Next day in the morning you pick 4 colonies and make liquid cultures LB 10 ml each. 3 colonies from the Tyrosinase an 1 colony from the NO plasmid

Incubate at 210 rpm 37◦C (approximately 4 hours) In order to get OD600=0,8

Negative Controls:

- Tyrosinase Buffer without Bacteria
- Tyrosinase Buffer and BL21 bacteria with no insert
- Bacteria with Tris HCl $CuSO_4$

Each Negative control for the 3 colonies

Make 1 ml aliquotes in 1,5 ml tubes

Centrifuge 3000 g 15 mins take with a pipette the supernatant and resuspend in 1 ml Tyrosinase Buffer(50mM Tris HCl, 10μM $CuSO_4$, 1g/L L-Tyrosine)

Incubate at 30◦C static conditions for 6 hours.

After 6 Hours 100ul from each aliquote culture is taken to check if OD600 is still 0,8

Centrifuge 2500g for 15 mins Then take from each aliquote 3 x 100 ul (3 wells) from the supernatant to measure at 400nm

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

4 different colours are 4 biological replicates

4th Biological doesn't contain proper insert

A line= Bacteria without TYROSINE

B line= Bacteria WITH TYROSINE 1 g/L

C1, C2, C3, C4= Bacteria form each biological replicate measuring OD600

C5=Tyrosinase assay buffer

13/10/20

Same as yesterday

15/10/20

Same as 11/10/2020

MAGDA & FOTEINI

01/08/2020

<u>Goals of the day</u>

1. Competent Cells *E.Coli* DH5a

Inoue Chemi Competent Cells

Procedure

A. Preperation of cells

1. Prepare Inoue transformation buffer (chilled to 0 °C before use).

a. Prepare 0.5 M PIPES (pH 6.7).
Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H_2O to bring the final volume to 100 ml.
Sterilize the solution by filtration through a disposable prerinsed Nalgene filter. Divide into aliquots and store frozen at -20 °C
b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H_2O.
c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-mm Nalgene filter. Divide into aliquots and store at -20 °C.

2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 h at 37 °C. Transfer the colony into 25 ml of LB broth or LB medium in a 250 ml flask. Incubate the culture for 6-8 h at 37 °C with vigorous shaking (250-300 rpm).

3. At about 7 o’clock in the evening, use this starter culture to inoculate three 1-L flasks, each containing 250 ml of LB. The first flask receives 10 ml of starter culture, the second receives 4 ml, and the third receives 2 ml. Incubate all three flasks overnight at 18 °C with moderate shaking.

02/08/2020

<u>Goals of the day</u>

1. Transformation of Competent Cells

1. Include all of the appropriate positive and negative controls.
2. Add the transforming DNA (up to 25 ng per 50 ml of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 min.
3. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
5. Add 800 ml of LB medium to each tube. Warm the cultures to 37 °C in a water bath, and then transfer the tubes to a shaking incubator set at 37 °C. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
6. Transfer the appropriate volume (up to 200 ml per 90 mm plate) of transformed competent cells onto agar LB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic. When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 sec at

room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml of LB medium by tapping the sides of the tube. IMPORTANT Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to RT, spread the transformed cells gently over the surface of the agar plate.

7. The plates should not be incubated for more than 20 h at 37 °C
8. Store the plates at RT until the liquid has been absorbed.
9. Invert the plates and incubate them at 37 °C. Transformed colonies should appear in 12-16 h.

03/08/2020

Goals of the day

- Dig-Lig: pUPD2 – prpBCDE (prpB) – pFliC-LacI –LacO- Terminator

Procedure

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector (pUPD2): 1µL
- 50 ng of each part
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI)

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed “GBLong”:

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

04/08/2020

Goals of the day

- Transformation: prpB, pFlic, LacI, LacO, Terminator

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (prpB L1) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).

   · 150 µL transformation (prpB L1) on one plate.

8. Incubate plates at 37°C overnight.

05/08/2020

Goals of the day:

- Create LB cultures: prpB L0, pFliC L0, LacI L0, LacO L0, Terminator L0

LB Cultures

prpB L0:

1. Take the previous day's plate from the incubator (37°C).
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
4. Incubate in a shaker at 37°C overnight.

pFliC L0:

1. Take the plate from the fridge.
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
4. Incubate in a shaker at 37°C overnight.

LacI L0:

5. Take the previous day's plate from the incubator (37°C).
6. Pick 4 white cultures from the plate using a pipette.
7. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
8. Incubate in a shaker at 37°C overnight.

LacO L0:

5. Take the plate from the fridge.
6. Pick 4 white cultures from the plate using a pipette.
7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
8. Incubate in a shaker at 37°C overnight.

Terminator L0:

9. Take the previous day's plate from the incubator (37°C).
10. Pick 4 white cultures from the plate using a pipette.
11. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
12. Incubate in a shaker at 37°C overnight.

06/08/2020

Goals of the day:

- Mini preps: prpB L0, pFliC L0,LacI L0, LacO L0, Terminator L0
- Nano drop : prpB L0, pFliC L0,LacI L0, LacO L0, Terminator L0
- Restriction Digestion: prpB L0, pFliC L0,LacI L0, LacO L0, Terminator L0
- Electrophoresis
- Dig-Lig: pSEVA23 – prpB-LacI – Terminator

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (pFliC L0), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until there is 1ml left in the previously 5ml LB culture.
2. **Cell lysis**
   1. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

3. **Clarification of lysate**
4. Centrifuge for **10 min at 15,000 x g** at room temperature.
4. **Bind DNA**
5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.
5. **Wash silica membrane**
6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.
6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$prpB_1$	150,6ng
$prpB_2$	116,6ng
$pFliC_3$	84,5ng
$pFliC_4$	122,2ng
$LacI_1$	69ng
$LacI_2$	238ng
$LacO_1$	335ng
$LacO_2$	107ng
$Term_1$	165ng
$Term_2$	81ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 3 digestions.
   Restriction enzymes: 1st Digestion: SlaI, 2nd Digestion: EcoRI and 3rd Digestion: EcoRV.
   Enzymes were chosen using SnapGene.
2. Appropriate reaction buffers according to the instructions of every enzyme:
   1st Digestion: R4, 2nd Digestion: Cutsmart, 3rd Digestion: R2
3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$prpB_1$	1µL	0,5µL	1µL	7,5µL
$prpB_2$	1µL	0,5µL	1µL	7,5µL
$pFliC_3$	1µL	0,5µL	1µL	7,5µL
$pFliC_4$	1µL	0,5µL	1µL	7,5µL
$LacI_1$	1µL	0,5µL	1µL	7,5µL
$LacI_2$	0,5µL	0,5µL	1µL	8µL
$LacO_1$	0,5µL	0,5µL	1µL	8µL
$LacO_2$	1µL	0,5µL	1µL	7,5µL
$Term_1$	1µL	0,5µl	1µL	7,5µL
$Term_2$	1µL	0,5µl	1µL	7,5µL

For each digestion:

Uncut:

	DNA	dH_2O
$pFliC_1$	1µL	9µl
$prpB_1$	1µL	9µl
$prpB_2$	1µL	9µl
$pFliC_3$	1µL	9µl
$pFliC_4$	1µL	7µl
$LacI_1$	0,5µL	9,5µl
$LacI_2$	0,5µL	9,5µl
$LacO_1$	1µL	9µl
$LacO_2$	1µL	9µl
$Term_1$	1µL	9µl
$Term_2$	1µL	9µl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2µl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

  Final volume will be 12µl
- For the gel ladder, add 3µl

Agarose Gel Preparation and Electrophoresis

Repeat 2 times to prepare 2 gels:

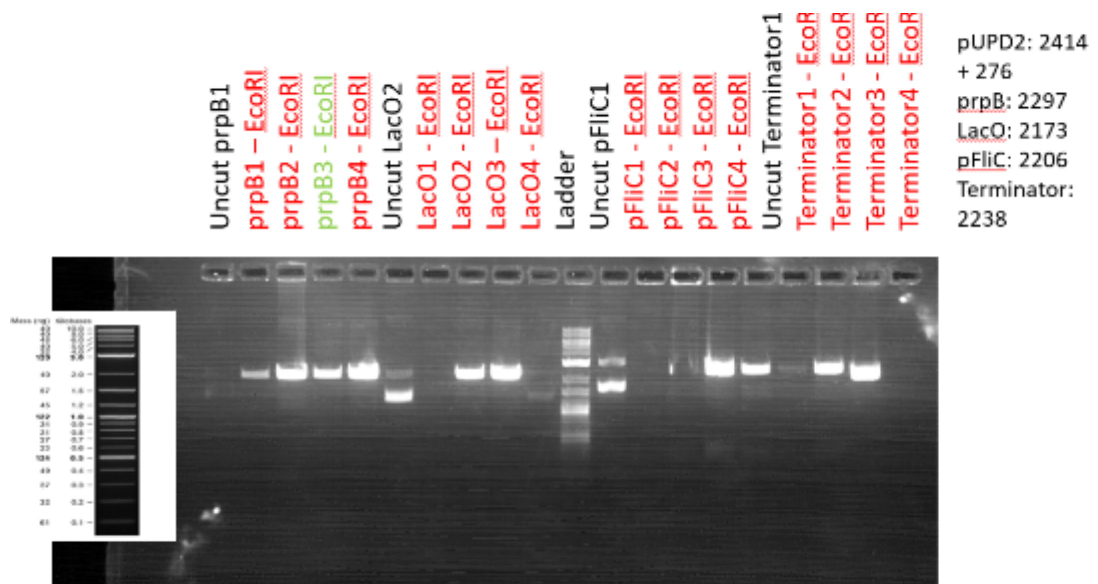
Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5µL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the “electrode box”, filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.

16. Analyze the bands on the gel.



One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector (pSEVA23 a1R): 1µL
- 50 ng of each part:
    - $prpB_3$: 6µL
    - LacI: 1,8µL
    - Terminator: 1,2µL
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsaI to alpha)

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed “GBLong”:

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

10/08/2020

Goals of the day

- Transformation: prpB L1

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (prpB L1) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
    8. 150 µL transformation (prpB L1) on one plate.
9. Incubate plates at 37°C overnight.

11/08/2020

Goals of the day:

- Create LB cultures: prpB L1, pFliC L0

LB Cultures

prpB L1:

1. Take the previous day’s plate from the incubator (37°C).

2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50μL).
4. Incubate in a shaker at 37°C overnight.

pFliC L0:

1. Take the plate from the fridge.
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
4. Incubate in a shaker at 37°C overnight.

12/08/2020

Goals of the day:

- Mini preps: prpB L1, pFliC L0
- Nano drop : : prpB L1, pFliC L0
- Restriction Digestion: prpB L1, pFliC L0
- Electrophoresis
- Create liquid cultures: prpB L1, pFliC L0

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

pFliC:

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (pFliC L0), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until there is 1ml left in the previously 5ml LB culture.
2. **Cell lysis**
   1. Add **250 μL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **250 μL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **300 μL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 μL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 μL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 μL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**
9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
10. Centrifuge for **1 min at 11,000 x g**.

prpB:

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.
2. **Cell lysis**
   1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
3. **Clarification of lysate**
4. Centrifuge for **10 min at 15,000 x g** at room temperature.
4. **Bind DNA**
5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.
5. **Wash silica membrane**
6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.
6. **Dry silica membrane**
8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube
7. **Elute DNA**
9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$pFliC_1$	253ng
$pFliC_2$	240ng
$pFliC_3$	257,2ng
$pFliC_4$	244,7ng
$prpB_1$	69,2ng
$prpB_2$	1051,3ng
$prpB_3$	433,9ng
$prpB_4$	1165,5ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 3 digestions.
   Restriction enzymes: 1st Digestion: SlaI, 2nd Digestion: EcoRI and 3rd Digestion: EcoRV. Enzymes were chosen using SnapGene.
2. Appropriate reaction buffers according to the instructions of every enzyme:
   1st Digestion: R4, 2nd Digestion: Cutsmart, 3rd Digestion: R2
3. In a 1.5mL tube combine the following:
   - DNA
   - Restriction Enzyme
   - Buffer
   - dH2O up to total volume

Our restriction digestions are 10 μL in total volume. So the reaction is :

- y μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$pFliC_1$	1μL	0,5μL	1μL	7,5μL
$pFliC_2$	1μL	0,5μL	1μL	7,5μL
$pFliC_3$	1μL	0,5μL	1μL	7,5μL
$pFliC_4$	1μL	0,5μL	1μL	7,5μL
$prpB_1$	3μL	0,5μL	1μL	5,5μL
$prpB_2$	0,2μL	0,5μL	1μL	8,3μL
$prpB_3$	0,5μL	0,5μL	1μL	8μL
$prpB_4$	0,2μL	0,5μL	1μL	8,3μL

For each digestion:

Uncut:

	DNA	dH_2O
$pFliC_1$	1μl	9μl
$pFliC_2$	1μl	9μl
$pFliC_3$	1μl	9μl
$pFliC_4$	1μl	9μl
$prpB_1$	3μL	7μl
$prpB_2$	0,2μL	9,8μl
$prpB_3$	0,5μL	9,5μl
$prpB_4$	0,2μL	9,8μl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl

- For the gel ladder, add 3µl

Agarose Gel Preparation and Electrophoresis

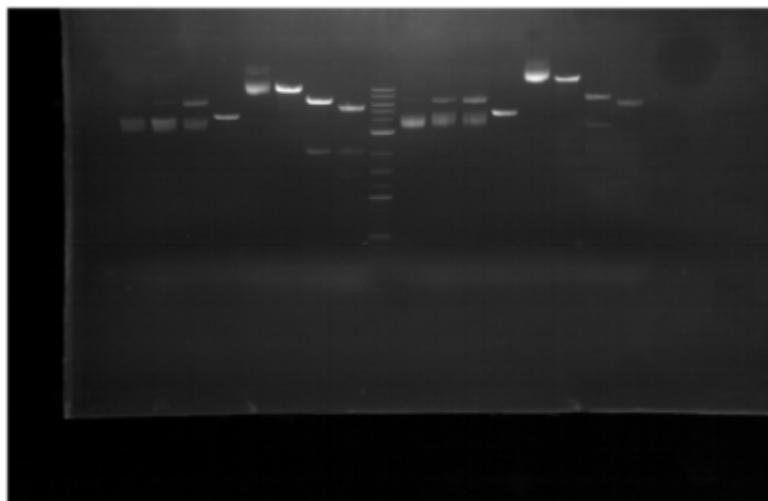
Repeat 2 times to prepare 2 gels:

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5µL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.
17.



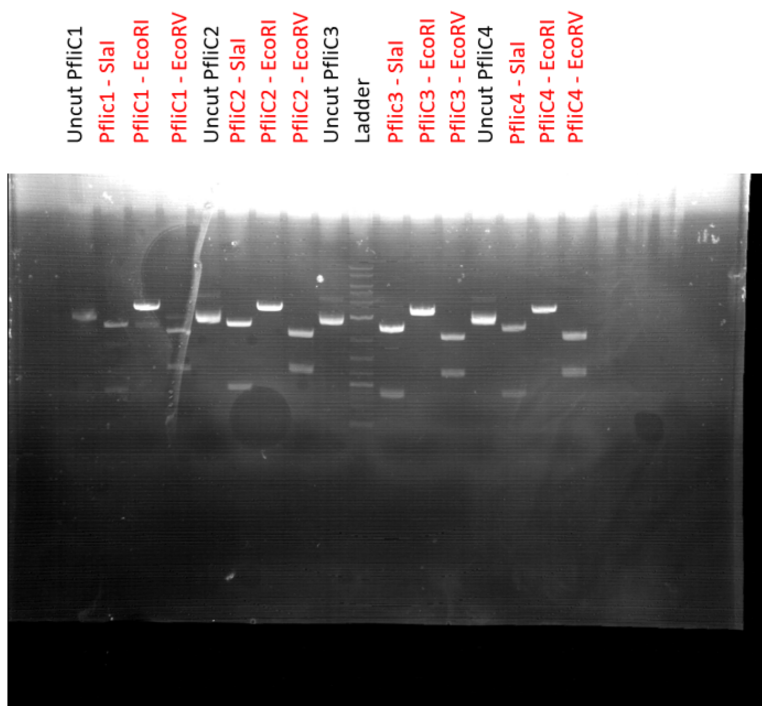
20.8

- pUPD2

SlaI➔ 1798 + 892
EcoRI➔ 214 + 276
EcoRV➔ 2690

- pFlic

SlaI➔ 1314 + 892
EcoRI➔ 2206
EcoRV➔ 2206



LB Cultures

prpB L1:

1. Take the previous day's plate from the incubator (37°C).
2. Pick 3 white cultures from the plate using a pipette.
3. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
4. Incubate in a shaker at 37°C overnight.

pFliC L0:

1. Take the plate from the fridge.
2. Pick 3 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
4. Incubate in a shaker at 37°C overnight.

13/08/2020

Goals of the day:

- Mini preps: prpB L1, pFliC L0
- Nano drop : prpB L1, pFliC L0
- Restriction Digestion: prpB L1, pFliC L0
- Electrophoresis

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

pFliC:

1. **Cultivate and harvest bacterial cells**

Use **2 mL** of each LB culture of the previous day (pFliC L0), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until there is 1ml left in the previously 5ml LB culture.

2. **Cell lysis**
   1. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

prpB:

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.
2. **Cell lysis**
   1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column.

Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. Elute DNA

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$pFliC_1$	289,9ng
$pFliC_2$	235,5ng
$pFliC_3$	168,3ng
$prpB_1$	1051ng
$prpB_2$	541,1ng
$prpB_3$	264,1ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 1 double digestion each.
   pFliC: Restriction enzymes: SlaI + HindIII
   prpB: Restriction enzymes: EcoRV + BamHI.
   Enzymes were chosen using SnapGene.
2. Appropriate reaction buffers according to the instructions of every enzyme:
   pFliC: (SlaI + HindIII) EQ
   prpB: (EcoRV + BamHI) EQ.
3. In a 1.5mL tube combine the following:
   - DNA
   - Restriction Enzyme
   - Buffer
   - dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$pFliC_1$	0,7µL	0,5µL each	1µL	7,3µL

pFliC$_2$	1µL	0,5µL each	1µL	7µL
pFliC$_3$	1µL	0,5µL each	1µL	7µL
prpB$_1$	0,2µL	0,5µL each	1µL	7,8µL
prpB$_2$	0,5µL	0,5µL each	1µL	7,5µL
prpB$_3$	1µL	0,5µL each	1µL	7µL
prpB$_1$ 20.8	3µL	0,5µL each	1µL	5µL
prpB$_3$ 20.8	0,5µL	0,5µL each	1µL	7,5µL

Uncut:

	DNA	dH_2O
pFliC$_1$	0,7µl	9,3µl
prpB3	1µL	9µl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2µl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12µl.
- For the gel ladder, add 3µl.

Agarose Gel Preparation and Electrophoresis

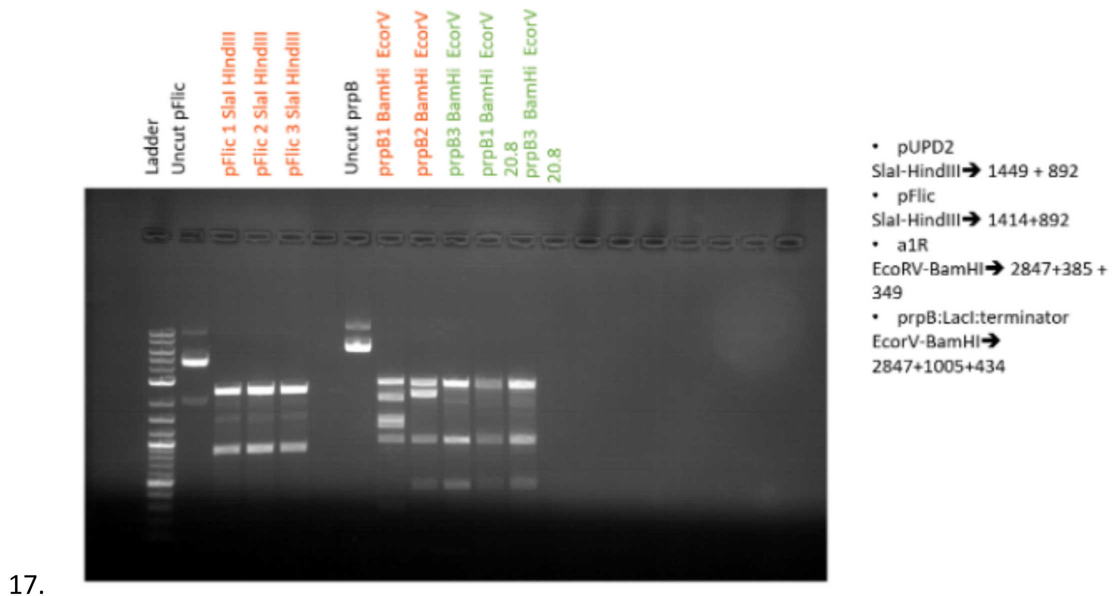
Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5µL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the “electrode box”, filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.

17.



14/08/2020

Goals of the day:

- Mini preps: prpB L1, pFliC L0
- Nano drop : : prpB L1, pFliC L0
- Restriction Digestion: prpB L1, pFliC L0
- Electrophoresis
- Dig – Lig: pSEVA23 a2 – Anderson:LacO – eGFP – Terminator

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 2 digestions.
   pFliC: Restriction enzymes: SacI
   prpB: Restriction enzymes: HpaI
   Enzymes were chosen using SnapGene.
2. Appropriate reaction buffers according to the instructions of every enzyme:
   pFliC: (SacI) Cutsmart
   prpB: (HpaI) Cutsmart
3. In a 1.5mL tube combine the following:
   - DNA
   - Restriction Enzyme
   - Buffer
   - dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$pFliC_1$	0,7µL	0,5µL	1µL	7,8µL
$pFliC_3$	1µL	0,5µL	1µL	7,5µL
$prpB_3$	1µL	0,5µL	1µL	7,5µL
$prpB_3$ 20.8	0,5µL	0,5µL	1µL	8µL

Uncut:

	DNA	dH_2O
$pFliC_1$	0,7µl	9,3µl
prpB3	1µL	9µl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2µl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12µl.
- For the gel ladder, add 3µl.

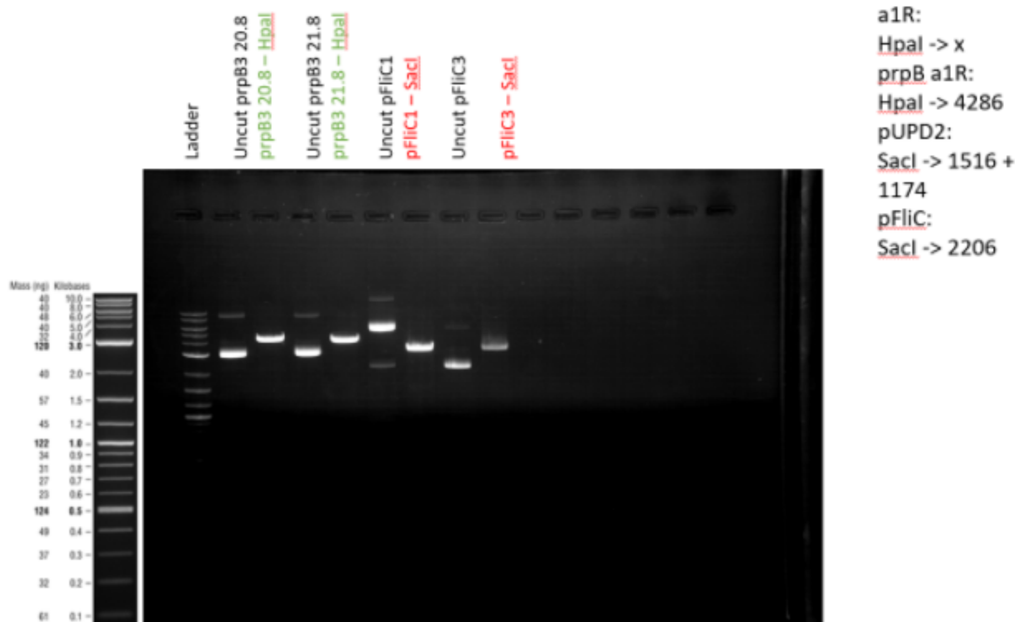
Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5µL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.



15/08/2020

Goals of the day

- Transformation: eCFP L1

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (eGFP L1) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).

10. 150 µL transformation (eGFP L1) on one plate.

8. Incubate plates at 37°C overnight.

24/08/2020

Goals of the day:

- Dig-Lig: pSEVA43 – LacO – eGFP – Terminator (eGFP L1)
- Transformation: prpB L1

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector (pUPD2): 2µL
- 50 ng of pFliC: 1,4µL
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI to pUPD2)
- 4,5µL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (pFliC L0) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 min.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
    11. 150 µL transformation on one plate.

8. Incubate plates at 37°C overnight.

16/08/2020

<u>Goals of the day:</u>

- Mini preps: eCFP L1
- Nano drop: eCFP L1
- Restriction Digestion: eGFP L1
- Electrophoresis
- Dig – Lig: pSEVA43 ω2 – prpB – LacI – Terminator - Anderson:LacO – eGFP – Terminator (prpB L2)

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.

2. **Cell lysis**
1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

3. **Clarification of lysate**
4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**
5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.

5. **Wash silica membrane**
6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**
8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. Elute DNA
9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
10. Centrifuge for **1 min at 11,000 x g**

Nano Drop

eGFP1	71,6ng
eGFP2	52,1ng
eGFP3	64,2ng
eGFP4	45,1ng
eGFP5	880,7ng
eGFP6	44,2ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for digestion.
   Restriction enzyme: HindIII
   Enzymes were chosen using SnapGene.
2. Appropriate reaction buffer according to the instructions: R2
3. In a 1.5mL tube combine the following:
- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 μL in total volume. So the reaction is:

- y μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
eGFP1	3μL	0,5μL	1μL	5,5μL
eGFP2	4μL	0,5μL	1μL	4,5μL
eGFP3	4μL	0,5μL	1μL	4,5μL
eGFP4	4μL	0,5μL	1μL	4,5μL
eGFP5	0,5μL	0,5μL	1μL	8,1μL
eGFP6	4μL	0,5μL	1μL	4,5μL

Uncut:

	DNA	dH_2O
eGFP1	3μL	7μl
eGFP2	4μL	6μl
eGFP3	4μL	6μl
eGFP4	4μL	6μl
eGFP5	0,5μL	9,6μl
eGFP6	4μL	6μl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl.
- For the gel ladder, add 3μl.

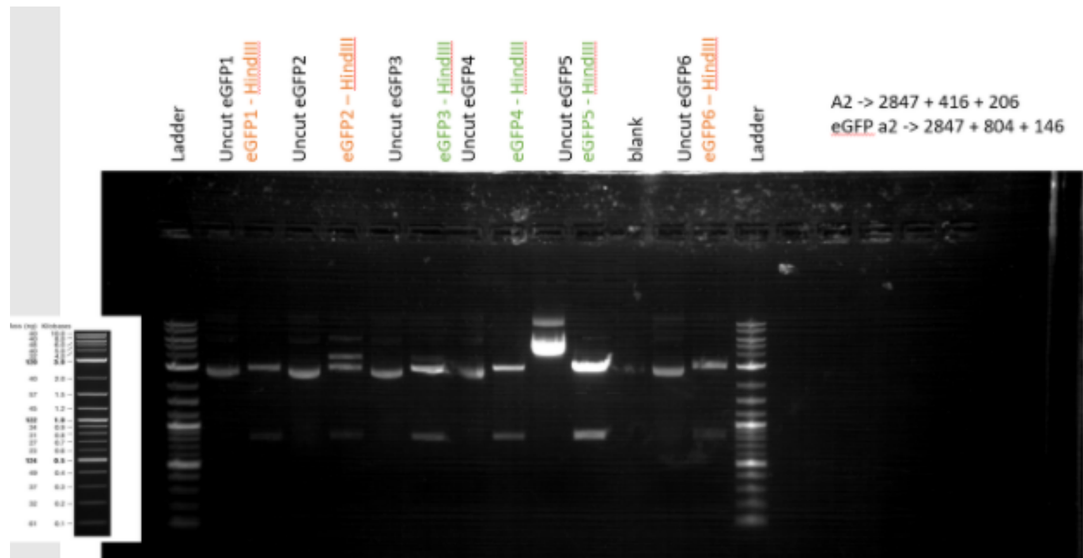
Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)

8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.
17. 

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector
- 50 ng of each part:
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI to omega)
- 5,2µL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

20/08/2020

<u>Goals of the day:</u>

- Mini preps: pFliC L0
- Nano drop: pFliC L0
- Restriction Digestion: pFliC L0
- Electrophoresis
- Dig – Lig: pSEVA23 a1R – pFliC – LacI – Terminator (pFliC L1)

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (pFliC L0), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until there is 1ml left in the previously 5ml LB culture.
2. **Cell lysis**
   1. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
3. **Clarification of lysate**
4. Centrifuge for **10 min at 15,000 x g** at room temperature.
4. **Bind DNA**
5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.
5. **Wash silica membrane**
6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.
6. **Dry silica membrane**
8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube
7. Elute DNA
9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

pFliC1	117,2ng
pFliC2	126,1ng
pFliC3	131,1ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 2 digestions.
   Restriction enzyme: 1st Digestion: SlaI, 2nd Digestion: EcoRI
   Enzymes were chosen using SnapGene.

2. Appropriate reaction buffers according to the instructions of each enzyme: 1st Digestion: R4, 2nd Digestion: Cutsmart

3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 μL in total volume. So the reaction is:

- y μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O

1st Digestion:

	DNA	Restriction Enzyme (SlaI)	Buffer (R4)	dH_2O
pFliC1	2μL	0,5μL	1μL	6,5μL
pFliC2	2μL	0,5μL	1μL	6,5μL
pFliC3	2μL	0,5μL	1μL	6,5μL

2nd Digestion:

	DNA	Restriction Enzyme (EcoRI)	Buffer (Cutsmart)	dH_2O
pFliC1	2μL	0,5μL	1μL	6,5μL
pFliC2	2μL	0,5μL	1μL	6,5μL
pFliC3	2μL	0,5μL	1μL	6,5μL

For each digestion:

Uncut:

	DNA	dH_2O
pFliC1	2μL	8μl
pFliC2	2μL	8μl
pFliC3	2μL	8μl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

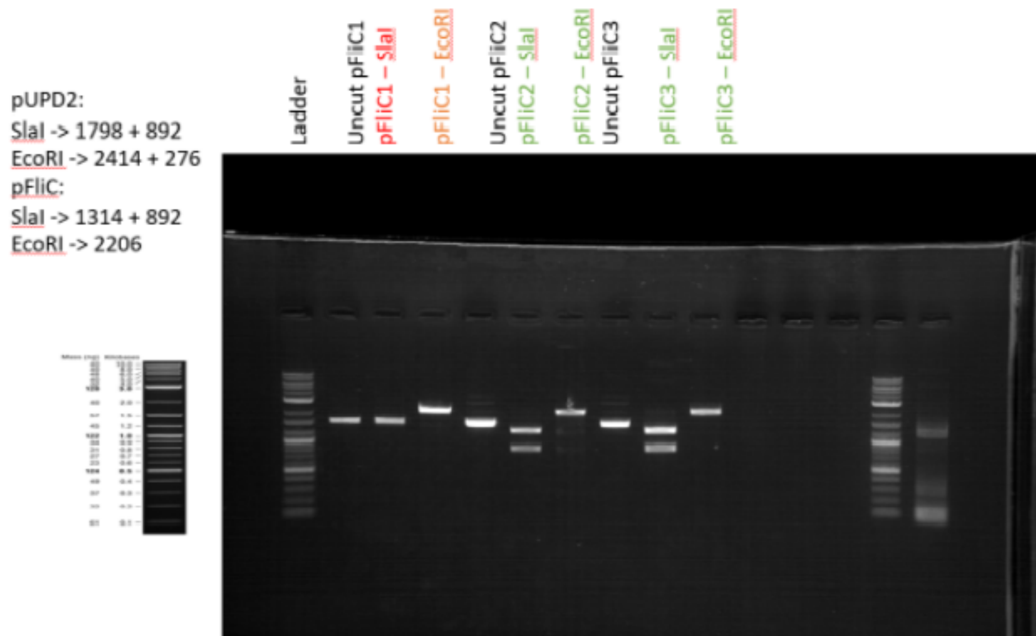
- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl.
- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.
17. 

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 μL

- 50 ng acceptor vector
- 50 ng of each part:
    - pFliC L0: 0,5μL (pFliC3 L0)

        - LacI L0: 0,5µL
        - Terminator L0: 1µL
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsaI to alpha)
- 4,5µL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed “GBLong”:

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

21/08/2020

Goals of the day:

- Transformation: pFliC L1, prpB L2

Transformation Protocol

For each transformation:

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (pFliC L1 and prpB L2) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).

12. 150 µL transformation on one plate.

8. Incubate plates at 37°C overnight.

22/08/2020

Goals of the day:

- Create LB cultures: prpB L2, pFliC L1

LB Cultures

prpB L2:

1. Take the previous day’s plate from the incubator (37°C).
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
4. Incubate in a shaker at 37°C overnight.

pFliC L1:

1. Take the plate from the fridge.
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
4. Incubate in a shaker at 37°C overnight.

23/08/2020

Goals of the day

- Nano drop : a1R-pFliC, omega1-prpB
- Restriction Digestion: pFliC, prpB
- Electrophoresis

Nano Drop

prpB	67,3ng
$pFliC_1$	577,6ng
$pFliC_2$	173,6ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 3 digestions.

   Restriction enzymes: 1st Digestion: BamHI and 2nd Digestion: HindIII , 3nd Digestion: EcorV

   Enzymes were chosen using SnapGene.

2. Appropriate reaction buffers according to the instructions of every enzyme:

   1st Digestion: R4 and 2nd Digestion: R2 , 3nd Digestion: R2

3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme

- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
prpB	2µL	0,5µL	1µL	6,5µL
$pFliC_1$	2µL	0,5µL	1µL	6,5µL
$pFliC_2$	1µL	0,5µL	1µL	7,5µL

Uncut:

	DNA	dH_2O
prpB	2µl	8µl
$pFliC_1$	2µl	8µl
$pFliC_2$	1µl	9µl

4. Mix gently by pipetting.

5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

  Final volume will be 12μl

- For the gel ladder, add 3μl

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5,5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 7μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the “electrode box”, filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.

15. Put the gel under UV.

16. Analyze the bands on the gel.

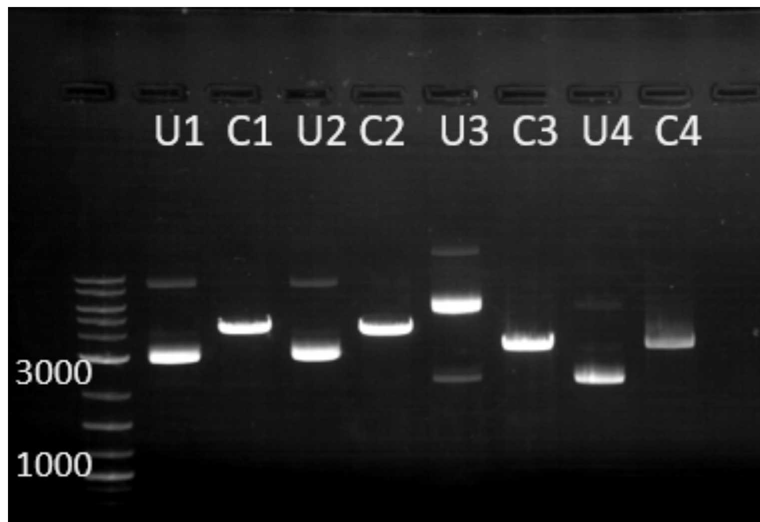


Figure 13. LEVEL 1: prpB-lacI-terminator and LEVEL 0: Promoter pFliC digested with HpaI, SacI. Expected bands: 4286 bp, 2206 bp. Positive result : C1 + C2.

Electrophoresis

25/08/2020

Goals of the day

- Dig-lig: pSEVA44 omega1 – prpB:LacI:Terminator – Anderson:LacO:eGFP
- pSEVA23 a1R-pfliC:lacI-Terimator
- Transformation

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

ü 50 ng acceptor vector (pSEVA23 a1R,SEVA44): 1µL

ü 50 ng of each part:

- pfliC$_3$: 0,5
- prpB$_3$: 6µL
- LacI: 1,8µL
- Terminator: 1,2µL

ü 1 µL 10X T4 DNA ligase **buffer**

ü 1 µL T4 DNA Ligase

ü 0.5 µL restriction enzyme (BsaI to alpha, BsmBI to omega)

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

1. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
2. 5 min at 16 degrees C (optimal temp for Ligase)
3. Repeat steps 1. and 2. **50 times**
4. 5 min at 80 degrees C (to kill enzymes)
5. Rest at 16 degrees C.

26/08/2020

Goals of the day:

- Create LB cultures: prpB L2, pFliC L1

LB Cultures

prpB L2:

1. Pick 4 white cultures from the plate using a pipette.
2. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50µL).
3. Incubate in a shaker at 37°C overnight.

pFliC L1:

1. Take the previous day's plate from the incubator (37°C).
2. Pick 4 white cultures from the plate using a pipette.

3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).

4. Incubate in a shaker at 37°C overnight.

27/08/2020

Goals of the day:

- Mini preps: prpB L2, pFliC L1
- Nano drop : : prpB L2, pFliC L1

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**

   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.

2. **Cell lysis**

1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$pFliC_1$	215ng
$pFliC_2$	38,1ng
$pFliC_3$	612,1ng
prpB	1874,7ng

30/08/2020

Goals of the day:

- Restriction Digestion: pFliC, prpB
- Electrophoresis

Restriction Digestion of DNA Protocol

Procedure

4. Preparation for digestion.
   Restriction enzyme: HindIII
   Enzymes were chosen using SnapGene.
5. Appropriate reaction buffer according to the instructions: R2
6. In a 1.5mL tube combine the following:
- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is:

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$pFliC_1$	1µL	0,5µL	1µL	7,5µL
$pFliC_2$	5µL	0,5µL	1µL	3,5µL
$pFliC_3$	1µL	0,5µL	1µL	7,5µL
prpB	1µL	0,5µL	1µL	7,5µL

For each digestion:

Uncut:

	DNA	dH_2O
$pFliC_1$	1µl	9µl
$pFliC_2$	5µl	5µl

prpB	1μl	9μl

1. Mix gently by pipetting.
2. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

· Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

Final volume will be 12μl

· For the gel ladder, add 3μl

Agarose Gel Preparation and Electrophoresis

Repeat 2 times to prepare 2 gels:

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min

11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.

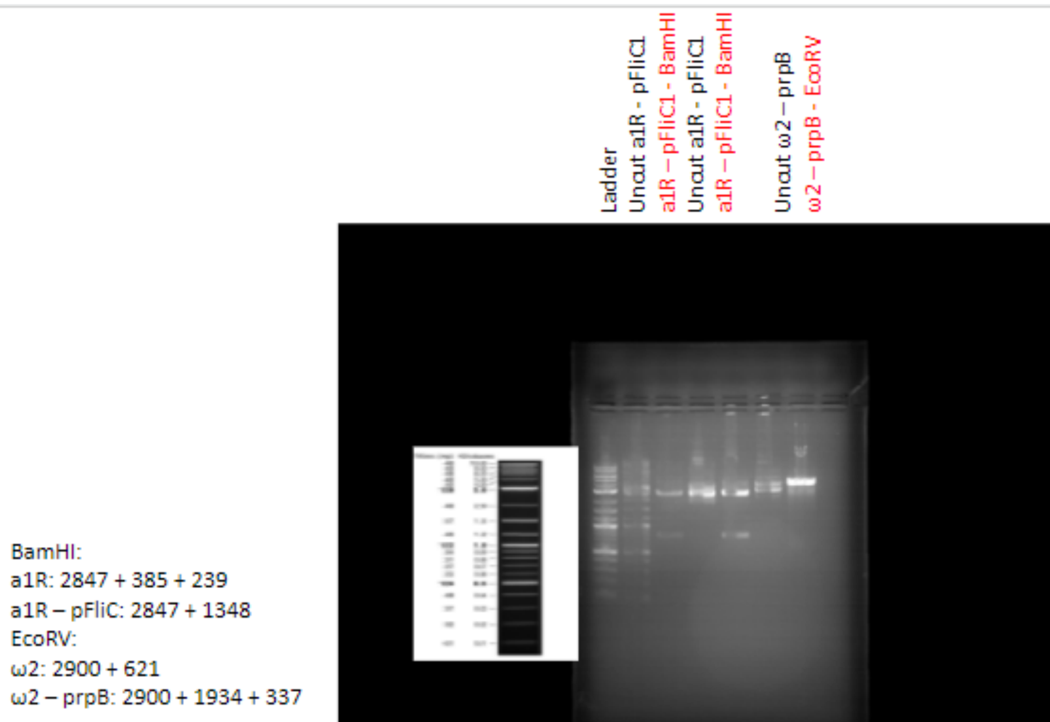
12. Put the gel in the "electrode box", filled with 1X TAE.

13. Load the samples in the gel wells.

14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.

15. Put the gel under UV.

16. Analyze the bands on the gel.



LB Cultures

prpB L1:

1. Take the previous day's plate from the incubator (37°C).

2. Pick 3 white cultures from the plate using a pipette.

3. Move each one in a 10ml liquid culture with antibiotic (kanamycin 50μL).

4. Incubate in a shaker at 37°C overnight.

pFliC L0:

1. Take the plate from the fridge.

2. Pick 3 white cultures from the plate using a pipette.

3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).

4. Incubate in a shaker at 37°C overnight.

31/08/2020

Goals of the day:

- Mini preps: prpB L2, pFliC L1
- Nano drop : : prpB L2, pFliC L1
- Electrophoresis

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**

   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.

2. **Cell lysis**

1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$pFliC_1$	39,6ng
$pFliC_2$	264,2ng
$prpB_1$	155,9ng

Restriction Digestion of DNA Protocol

Procedure

7. Preparation for digestion.
   Restriction enzyme: HindIII
   Enzymes were chosen using SnapGene.
8. Appropriate reaction buffer according to the instructions: R2
9. In a 1.5mL tube combine the following:
   - DNA
   - Restriction Enzyme
   - Buffer
   - dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is:

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
prpB	2µL	0,5µL	1µL	6,5µL
$pFliC_1$	1µL	0,5µL	1µL	7,5µL
$pFliC_2$	2µL	0,5µL	1µL	6,5µL

Uncut:

	DNA	dH_2O
prpB	2µl	8µl
$pFliC_1$	1µl	9µl
$pFliC_2$	2µl	8µl

12. Mix gently by pipetting.

13. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2µl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

Final volume will be 12µl

- For the gel ladder, add 3µl

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5,5µL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.

2. Mix TAE and agarose in a flask

3. Microwave for 2-3 mins (until it boils)

4. After microwaving, mix it thoroughly under running water until it cools down.

5. When in the right temperature (not too hot), add 7µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.

6. Prepare the gel box, combs *etc.*

7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)

8. Add the liquid gel slowly into the gel box.

9. Remove bubbles with a tip.

10. Let the gel solidify for 20min

11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.

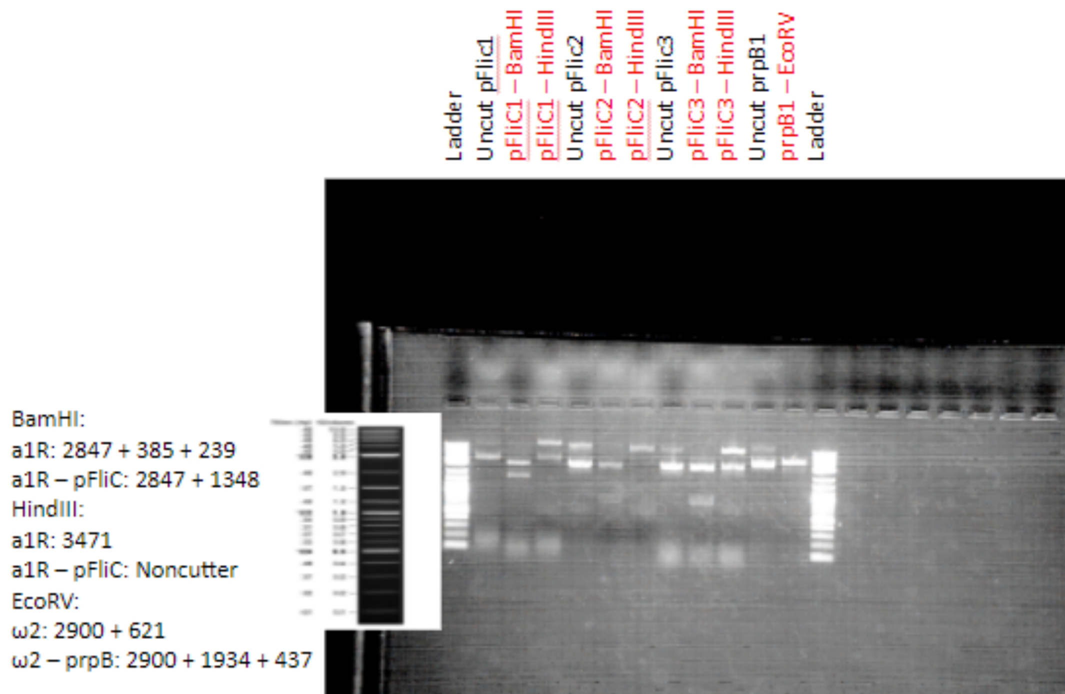
12. Put the gel in the “electrode box”, filled with 1X TAE.

13. Load the samples in the gel wells.

14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.

15. Put the gel under UV.

16. Analyze the bands on the gel.



02/09/2020

Goals of the day:

- Restriction Digestion: prpB L2, pFliC L1
- Electrophoresis

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 2 digestions.

pFliC: Restriction enzymes: EcoRI

prpB: Restriction enzymes: HindIII

Enzymes were chosen using SnapGene.

2. Appropriate reaction buffers according to the instructions of every enzyme:

    pFliC: (EcoRI) Cutsmart

    prpB: (HindIII) R2

3. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
$pFliC_2$07.09	1µL	0,5µL	1µL	7,5µL
$prpB_1$07.09	1µL	0,5µL	1µL	7,5µL

Uncut:

	DNA	dH_2O
$pFliC_2$07.09	1µl	9µl
$prpB_1$ 07.09	1µL	9µl

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

  Final volume will be 12μl.

- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*
7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the “electrode box”, filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.

16. Analyze the bands on the gel.

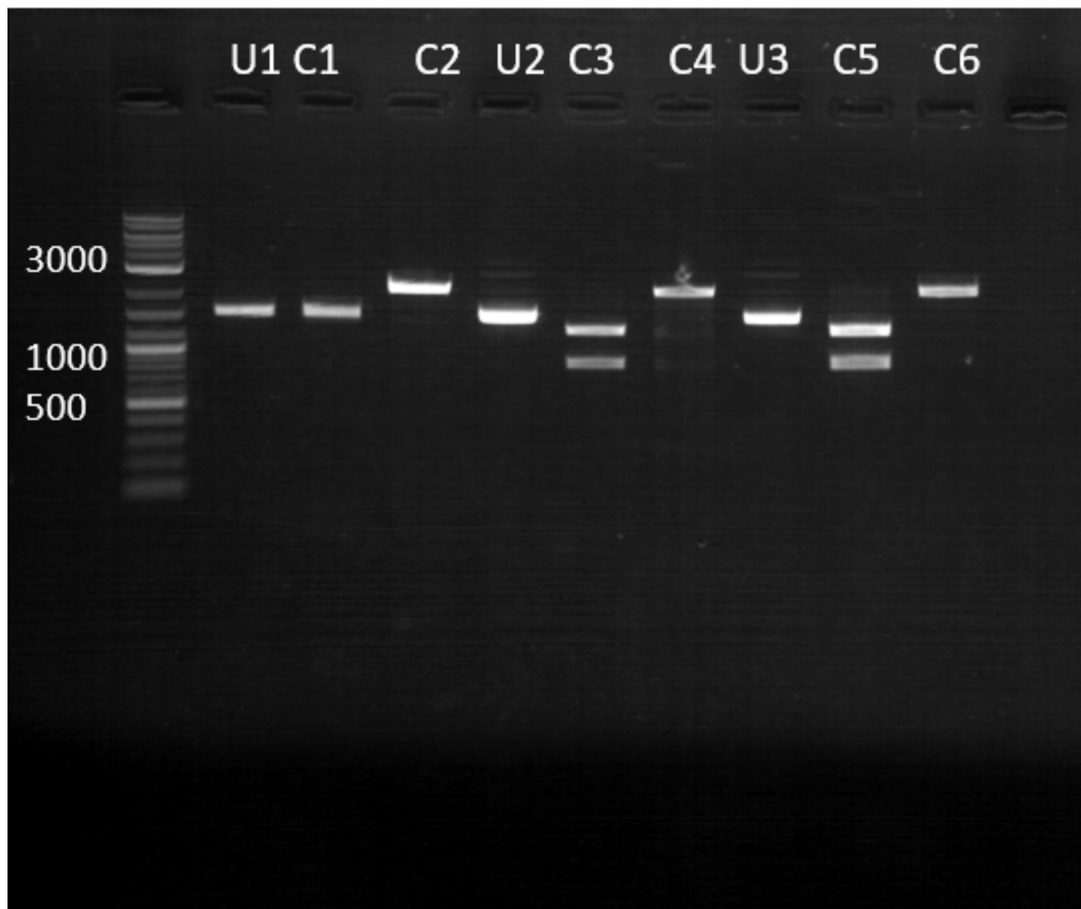


Figure 9. LEVEL 0: Promoter pFliC digested with SlaI, EcoRI. Expected bands: 1314 bp + 892 bp, 2206 bp. Positive result : C5 + C6.

03/09/2020

Goals of the day

· Dig-lig pSEVA22 a1R-pflic-LacI-Terminator

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

ü 50 ng acceptor vector (pSEVA23 a1R,SEVA44): 1µL

ü 50 ng of each part:

- o pfliC$_3$: 0,5
- o LacI: 0,5µL
- o Terminator: 0,6µL

ü 1 µL 10X T4 DNA ligase **buffer**

ü 1 µL T4 DNA Ligase

ü 0.5 µL restriction enzyme (BsaI to alpha)

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed “GBLong”:

6. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)

7. 5 min at 16 degrees C (optimal temp for Ligase)

8. Repeat steps 1. and 2. **50 times**

9. 5 min at 80 degrees C (to kill enzymes)

10. Rest at 16 degrees C.

04/09/2020

Goals of the day

2. Competent Cells *E.Coli* MC1061

Inoue Chemi Competent Cells

Procedure

A. Preperation of cells

2. Prepare Inoue transformation buffer (chilled to 0 °C before use).

a. Prepare 0.5 M PIPES (pH 6.7).
Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H_2O to bring the final volume to 100 ml.
Sterilize the solution by filtration through a disposable prerinsed Nalgene filter. Divide into aliquots and store frozen at -20 °C
b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H_2O.
c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-mm Nalgene filter. Divide into aliquots and store at -20 °C.

2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 h at 37 °C. Transfer the colony into 25 ml of LB broth or LB medium in a 250 ml flask. Incubate the culture for 6-8 h at 37 °C with vigorous shaking (250-300 rpm).
3. At about 7 o'clock in the evening, use this starter culture to inoculate three 1-L flasks, each containing 250 ml of LB. The first flask receives 10 ml of starter culture, the second receives 4 ml, and the third receives 2 ml. Incubate all three flasks overnight at 18 °C with moderate shaking.

05/09/2020

Goals of the day

2. Transformation of Competent Cells

10. Include all of the appropriate positive and negative controls.
11. Add the transforming DNA (up to 25 ng per 50 ml of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 min.
12. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.
13. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
14. Add 800 ml of LB medium to each tube. Warm the cultures to 37 °C in a water bath, and then transfer the tubes to a shaking incubator set at 37 °C. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
15. Transfer the appropriate volume (up to 200 ml per 90 mm plate) of transformed competent cells onto agar LB medium containing 20 mM $MgSO_4$ and the

appropriate antibiotic. When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 sec at room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml of LB medium by tapping the sides of the tube. IMPORTANT Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to RT, spread the transformed cells gently over the surface of the agar plate.

16. The plates should not be incubated for more than 20 h at 37 °C
17. Store the plates at RT until the liquid has been absorbed.
18. Invert the plates and incubate them at 37 °C. Transformed colonies should appear in 12-16 h.

06/09/2020

Goals of the day:

- Create LB cultures: pFliC L1

pFliC L1:

5. Take the previous day's plate from the incubator (37°C).
6. Pick 4 white cultures from the plate using a pipette.
7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
8. Incubate in a shaker at 37°C overnight.

12/09/2020

Goals of the day:

- Transformation pfliC L1

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5μl of DNA into 100 μL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.

4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.

5. Put the tubes back on ice for 2-5 min.

6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate an 150 µL transformation on one plate.

8. Incubate plates at 37°C overnight.

13/09/2020

<u>Goals of the day</u>

Mini preps: pFliC L1

Nano drop : : pFliC L1

Restriction Digestion: pFliC L1

Electrophoresis

M9 5X

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

1. **Cultivate and harvest bacterial cells**
   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.
2. **Cell lysis**
   1. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
   2. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
   3. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

3. **Clarification of lysate**

4. Centrifuge for **10 min at 15,000 x g** at room temperature.

4. **Bind DNA**

5. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

5. **Wash silica membrane**

6. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

7. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

6. **Dry silica membrane**

8. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

7. **Elute DNA**

9. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

10. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

$pFliC_1$	253ng
$pFliC_2$	240ng

Restriction Digestion of DNA Protocol

Procedure

1. Preparation for 2 digestions.
   Restriction enzymes: 1st Digestion: BamHI, 2nd Digestion: HpaI
   Enzymes were chosen using SnapGene.
2. Appropriate reaction buffers according to the instructions of every enzyme:
   1st Digestion: Cutsmart, 2nd Digestion: Cutsmart
   In a 1.5mL tube combine the following:
   - DNA
   - Restriction Enzyme
   - Buffer
   - dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is :

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O

pFliC$_1$	0,6μL	0,5μL	1μL	7,9μL
pFliC$_2$	0,8μL	0,5μL	1μL	7,7μL

For each digestion:

Uncut:

	DNA	dH_2O
pFliC$_1$	0,6μl	9,4μl
pFliC$_2$	0,8μl	9,2μl

4. Mix gently by pipetting.

5. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

  Final volume will be 12μl
- For the gel ladder, add 3μl

Agarose Gel Preparation and Electrophoresis

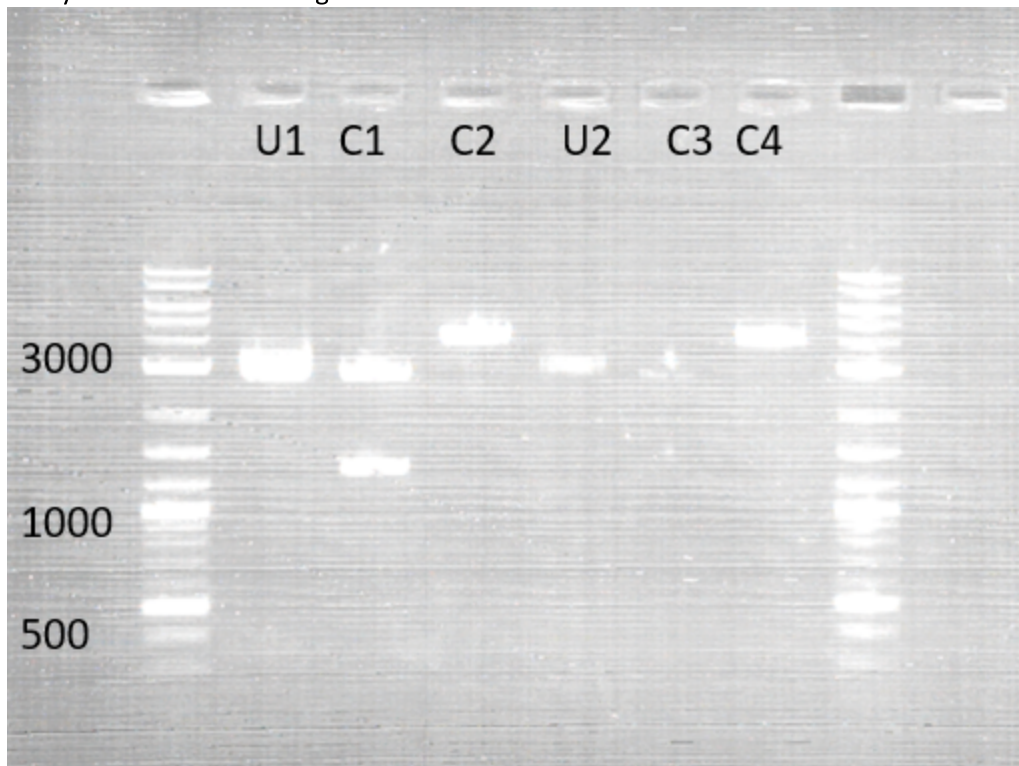
Repeat 2 times to prepare 2 gels:

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

1. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
2. Mix TAE and agarose in a flask
3. Microwave for 2-3 mins (until it boils)
4. After microwaving, mix it thoroughly under running water until it cools down.
5. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
6. Prepare the gel box, combs *etc.*

7. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
8. Add the liquid gel slowly into the gel box.
9. Remove bubbles with a tip.
10. Let the gel solidify for 20min
11. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
12. Put the gel in the "electrode box", filled with 1X TAE.
13. Load the samples in the gel wells.
14. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
15. Put the gel under UV.
16. Analyze the bands on the gel.
17. 

18. **Figure 14. LEVEL 1: pFliC-LacI-terminator digested with BamHI, HpaI. Expected bands: 2847 bp + 1348 bp, 4195 bp. Positive result : C1.**

14/09/2020

<u>Goals of the day</u>

- M9 5X

Procedure

1. Preparing the 5X stock: Add the following reagents to a 2-liter flask:

2. 64 g Na2HPO4, seven hydrate 3

3. 15 g KH_2PO_4

4. 5 g NH_4Cl

6. 2.5 g NaCl

7. 1 liter of high-quality distilled water

8. Once the ingredients are added, heat with stirring until the components are completely dissolved.

9.Pour the solution into smaller bottles with loosened caps and the autoclave at 15 lb/in2 for 15 min.

10.Wait until the bottle is less than 50°C , then add the components.

11.After the bottles cool to below 40°C, the caps can be tightened and the concentrated medium stored indefinitely at room temperature.

15/09/2020

Goals of the day

- Dig – Lig: alpha1R – pFliC – eCFP – Terminator
- Transformation

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector: 0,3µL
- 50 ng of each part:
  - eCFP L1: 1µL
  - pFliC L1: 1µL
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI to omega)
- 5,2µL dH_2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

6. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
7. 5 min at 16 degrees C (optimal temp for Ligase)
8. Repeat steps 1. and 2. **50 times**
9. 5 min at 80 degrees C (to kill enzymes)
10. Rest at 16 degrees C.

Transformation Protocol

For each transformation:

9. Take competent cells out of -80°C and put them on ice.
10. Mix 5µl of DNA (pFliC L1 and prpB L2) into 100 µL of competent cells in an epedorf tube. Gently mix.
11. Incubate the competent cell-DNA mixture on ice for 20 mins.
12. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.

13. Put the tubes back on ice for 2-5 min.

14. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

15. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).

13. 150 µL transformation on plate.

16. Incubate plates at 37°C overnight.

16/09/2020

Goals of the day

Create LB cultures: pFliC:eCFP:Terminator L1

pFliC L1:

5. Take the plate from the fridge.
6. Pick 4 white cultures from the plate using a pipette.
7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
8. Incubate in a shaker at 37°C overnight.

17/09/2020

Goals of the day:

- Mini preps: pFliC:eCFP:Terminator L1
- Nano drop: pFliC:eCFP:Terminator L1
- Restriction Digestion: pFliC;eCFP:Terminator L1
- Electrophoresis
- Dig – Lig: pSEVA23 a1R – pFliC – eGFP – Terminator (pFliC L1)

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

pFliC:

8. **Cultivate and harvest bacterial cells**

   Use **2 mL** of each LB culture of the previous day (pFliC L0), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until there is 1ml left in the previously 5ml LB culture.

9. **Cell lysis**

11. Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

12. Add **250 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

13. Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

10. **Clarification of lysate**

14. Centrifuge for **10 min at 15,000 x g** at room temperature.

11. **Bind DNA**

15. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

12. **Wash silica membrane**

16. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

17. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

13. **Dry silica membrane**

18. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

14. Elute DNA

19. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

20. Centrifuge for **1 min at 11,000 x g**.

Nano Drop

pFliC1	172,6ng
pFliC2	152,1ng
pFliC3	164,2ng

Restriction Digestion of DNA Protocol

Procedure

10. Preparation for digestion.
    Restriction enzyme: HindIII
    Enzymes were chosen using SnapGene.
11. Appropriate reaction buffer according to the instructions: R2
12. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is:

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
pFliC1	1µL	0,5µl	1µl	7,5µL
pFliC2	1µL	0,5µl	1µl	7,5µL
pFliC3	1µL	0,5µl	1µl	7,5µL

Uncut:

	DNA	dH_2O

pFliC1	1μl	9μl
pFliC2	1μl	9μl
pFliC3	1μl	9μl

6. Mix gently by pipetting.
7. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl.
- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

18. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
19. Mix TAE and agarose in a flask
20. Microwave for 2-3 mins (until it boils)
21. After microwaving, mix it thoroughly under running water until it cools down.
22. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
23. Prepare the gel box, combs *etc.*
24. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
25. Add the liquid gel slowly into the gel box.
26. Remove bubbles with a tip.
27. Let the gel solidify for 20min
28. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
29. Put the gel in the "electrode box", filled with 1X TAE.
30. Load the samples in the gel wells.
31. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.

LEVEL 1 pFliC – eCFP-terminator

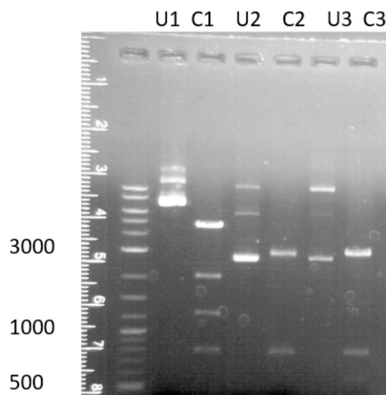


Figure 1a. (U=Uncut C=Cut) Restriction Enzyme with PvuII, Expected bands in bp 3016 + 816

32. Put the gel under UV.
33. Analyze the bands on the gel.

19/09/2020

<u>Goals of the day</u>

1. Transformation pFliC:eCFP:Terminator to MC1061

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA (prpB L1) into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 mins.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).

14. 150 µL transformation on one plate.
8. Incubate plates at 37°C overnight.

20/10/2020

<u>Goals of the day</u>

1. Transformation of Competent Cells

<u>Transformation of Competent Cells</u>

1. Include all of the appropriate positive and negative controls.
2. Add the transforming DNA (up to 25 ng per 50 ml of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two

control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 min.

3. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
5. Add 800 ml of LB medium to each tube. Warm the cultures to 37 °C in a water bath, and then transfer the tubes to a shaking incubator set at 37 °C. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
6. Transfer the appropriate volume (up to 200 ml per 90 mm plate) of transformed competent cells onto agar LB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic. When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 sec at room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml of LB medium by tapping the sides of the tube. IMPORTANT Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to RT, spread the transformed cells gently over the surface of the agar plate.
7. The plates should not be incubated for more than 20 h at 37 °C
8. Store the plates at RT until the liquid has been absorbed.
9. Invert the plates and incubate them at 37 °C. Transformed colonies should appear in 12-16 h.

22/09/2020

<u>Goals of the day</u>

LB Create LB cultures: pFliC:eCFP:Terminator L1

pFliC L1:

9. Take the plate from the fridge.
10. Pick 4 white cultures from the plate using a pipette.
11. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
12. Incubate in a shaker at 37°C overnight.

22/09/20

Goals of the day

- Characterization
- Preparation of SCFAs
- Plate reader for eCFP

Preparation of Sodium Propionate

Prepare 2000mM solution

· 1gr of Sodium Propionate

· 5ml ddH_2O

Prepare 200mM solution

· 1ml 2000 mM

· 9ml ddH_2O

Prepare 20mM solution

· 1ml 200 mM

· 9ml ddH_2O

Prepare 2mM solution

· 1ml 20 mM

· 9ml ddH_2O

Prepare 0,2mM solution

· 1ml 2mM

· 9ml ddH_2O

Prepare 0,02mM solution

· 1ml 0,2 mM

· 9ml ddH_2O

Preparation of Sodium Acetate

Prepare 2000mM solution

- 1gr of Sodium Acetate
- 4ml ddH_2O

Prepare 200mM solution

- 1ml 2000 mM
- 9ml ddH_2O

Prepare 20mM solution

- 1ml 200 mM
- 9ml ddH_2O

Prepare 2mM solution

- 1ml 20 mM
- 9ml ddH_2O

Prepare 0,2mM solution

- 1ml 2mM
- 9ml ddH_2O

Prepare 0,02mM solution

- 1ml 0,2 mM
- 9ml ddH_2O

Preparation of Controls

Control 1: Untransformed cells

This gives us the background fluorescence output which is calculated by growing cells without eCFP, eGFP or sfGFP (eCFP, eGFP or sfGFP -less) in the same medium and microplate. The measured eCFP,eGFP or sfGFP -less fluorescence is subtracted from the final fluorescence output of transformed cells (with eCFP, eGFP or sfGFP).

Control 2 : Empty wells

This gives the absorbance of the plate itself. This measurement is going to be subtracted from the final measurement of our construct.

Control 3 : Wells with medium and antibiotic

M9 medium with antibiotic will give us the background fluorescence output. M9 medium does emit fluorescence and is transparent, but the addition of the antibiotic may give a false absorbance.

The procedure follows:

Plate Reader Assay

1.measure the OD600 of the overnight cultures.
2. For each one of the colonies: Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the time points you are going to choose.
3. Add 0,02mM,0,2mM,2mM,20mM,200mM acetate and propionate to each colony
4. Measure the OD600 and F1: 0h, 4h,8h, 20h
5. Add proper antibiotic (in this case, Kanamicyn)
6. Incubate each culture : 37◦C and 210 rpm . The duration of incubation varies for each liquid culture.
7. In due time : take 600 µl of each liquid culture and split into 3 walls (200µl per walls , technical replicates)
8. Set the optimized parameters and measure.

Preparation of the 96-well plate with all additives

- Beginning of the plate-reader assay
- Timepoints: 0, 4h, 8h, 20h
- During these timepoints the samples are in shaker incubator

eCFP

Measure OD600
Excite at 434
Excite at 477

22/09/20

Goals of the day:

- Plate Reader for pFliC:eCFP:Terminator
- Dig – Lig: pUPD2 – eGFP

Total reaction volume: 10 µL

- 50 ng acceptor vector
- 50 ng of each part:
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI to omega)
- 5,2µL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

11. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
12. 5 min at 16 degrees C (optimal temp for Ligase)
13. Repeat steps 1. and 2. **50 times**
14. 5 min at 80 degrees C (to kill enzymes)
15. Rest at 16 degrees C.

23/09/20

Goals of the day:

- Transformation eGFP

Transformation Protocol

15. Take competent cells out of -80°C and put them on ice.
16. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
17. Incubate the competent cell-DNA mixture on ice for 20 mins.
18. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
19. Put the tubes back on ice for 2-5 min.
20. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
21. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
22. 150 µL transformation on one plate.
23. Incubate plates at 37°C overnight.

24/09/20

Goals of the day

LB Create LB cultures: eGFP

eGFP L0:

13. Take the plate from the fridge.
14. Pick 4 white cultures from the plate using a pipette.
15. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
16. Incubate in a shaker at 37°C overnight.

25/09/20

- Mini preps: eGFP L0
- Nano drop: eGFP L0
- Restriction Digestion: eGFP L0
- Electrophoresis
- Dig-Lig PSEVA23-pFlic-eGFP-Terminator L1

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

8. **Cultivate and harvest bacterial cells**

   Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.

9. **Cell lysis**

11. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

12. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

13. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

10. **Clarification of lysate**

14. Centrifuge for **10 min at 15,000 x g** at room temperature.

11. **Bind DNA**

15. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

12. **Wash silica membrane**

16. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

17. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

13. **Dry silica membrane**

18. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

14. Elute DNA

19. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
20. Centrifuge for **1 min at 11,000 x g**

Nano Drop

eGFP1	206ng
eGFP2	281ng
eGFP3	238ng
eGFP4	255ng

Restriction Digestion of DNA Protocol

Procedure

13. Preparation for digestion.
    Restriction enzyme: HindIII
    Enzymes were chosen using SnapGene.
14. Appropriate reaction buffer according to the instructions: R2
15. In a 1.5mL tube combine the following:
- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 µL in total volume. So the reaction is:

- y µg DNA
- 0.5 µL of each Restriction Enzyme
- 1 µL 10x Buffer
- x µL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
eGFP1	1µL	0,5µL	1µL	7,5µL
eGFP2	1µL	0,5µL	1µL	7,5µL
eGFP3	1µL	0,5µL	1µL	7,5µL
eGFP4	1µL	0,5µL	1µL	7,5µL

Uncut:

	DNA	dH_2O
eGFP1	1µL	9µl
eGFP2	1µL	9µl
eGFP3	1µL	9µl
eGFP4	1µL	9µl

8. Mix gently by pipetting.
9. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2µl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.

Final volume will be 12μl.

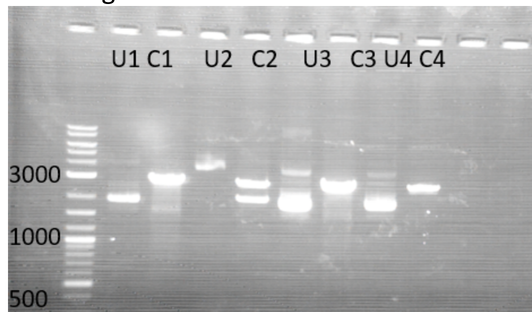
- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

34. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
35. Mix TAE and agarose in a flask
36. Microwave for 2-3 mins (until it boils)
37. After microwaving, mix it thoroughly under running water until it cools down.
38. When in the right temperature (not too hot), add 5μl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
39. Prepare the gel box, combs *etc.*
40. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
41. Add the liquid gel slowly into the gel box.
42. Remove bubbles with a tip.
43. Let the gel solidify for 20min
44. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
45. Put the gel in the "electrode box", filled with 1X TAE.
46. Load the samples in the gel wells.
47. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
48. Put the gel under UV.
49. 

50. Analyze the bands on the gel.
51. **LEVEL 0: CDS eGFP digested with PvuII. Expected bands 2826 bp. Positive result : C3 + C4.**

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 μL

- 50 ng acceptor vector
- 50 ng of each part:
- 1 μL 10X T4 DNA ligase **buffer**
- 1 μL T4 DNA Ligase
- 0.5 μL restriction enzyme (BsmBI to omega)
- 5,2μL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed “GBLong”:

16. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
17. 5 min at 16 degrees C (optimal temp for Ligase)
18. Repeat steps 1. and 2. **50 times**
19. 5 min at 80 degrees C (to kill enzymes)
20. Rest at 16 degrees C.

26.09.20

<u>Goals of the day:</u>

- Transformation eGFP

Transformation Protocol

24. Take competent cells out of -80°C and put them on ice.
25. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
26. Incubate the competent cell-DNA mixture on ice for 20 mins.
27. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
28. Put the tubes back on ice for 2-5 min.
29. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
30. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
31. 150 µL transformation on one plate.
32. Incubate plates at 37°C overnight.

26/09/20

<u>Goals of the day:</u>

· Create LB cultures: pFliC L1

pFliC L1:

5. Take the previous day’s plate from the incubator (37°C).

6. Pick 4 white cultures from the plate using a pipette.

7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).

8. Incubate in a shaker at 37°C overnight.

27/09/20

- Mini preps: eGFP L1
- Nano drop: eGFP L1
- Restriction Digestion: eGFP L1
- Electrophoresis

- Transformation of pFlic-eGFP-Terminator L1 to MC1061

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

15. **Cultivate and harvest bacterial cells**
Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.
16. **Cell lysis**
21. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**
22. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.
23. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**
17. **Clarification of lysate**
24. Centrifuge for **10 min at 15,000 x g** at room temperature.
18. **Bind DNA**
25. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.
Repeat this step to load the remaining lysate.
19. **Wash silica membrane**
26. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.
27. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.
20. **Dry silica membrane**
28. Centrifuge for **3 min at 11,000 x g** and discard the collection tube
21. Elute DNA
29. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.
30. Centrifuge for **1 min at 11,000 x g**

Nano Drop

eGFP1	206ng
eGFP2	281ng
eGFP3	238ng
eGFP4	255ng

Restriction Digestion of DNA Protocol

Procedure

16. Preparation for digestion.

Restriction enzyme: HindIII
Enzymes were chosen using SnapGene.

17. Appropriate reaction buffer according to the instructions: R2
18. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 μL in total volume. So the reaction is:

- y μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
eGFP1	1μL	0,5μL	1μL	7,5μL
eGFP2	1μL	0,5μL	1μL	7,5μL
eGFP3	1μL	0,5μL	1μL	7,5μL
eGFP4	1μL	0,5μL	1μL	7,5μL

Uncut:

	DNA	dH_2O
eGFP1	1μL	9μl
eGFP2	1μL	9μl
eGFP3	1μL	9μl
eGFP4	1μL	9μl

10. Mix gently by pipetting.
11. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl.
- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5μL EtBr**

52. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
53. Mix TAE and agarose in a flask
54. Microwave for 2-3 mins (until it boils)
55. After microwaving, mix it thoroughly under running water until it cools down.

56. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
57. Prepare the gel box, combs *etc.*
58. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
59. Add the liquid gel slowly into the gel box.
60. Remove bubbles with a tip.
61. Let the gel solidify for 20min
62. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
63. Put the gel in the “electrode box”, filled with 1X TAE.
64. Load the samples in the gel wells.
65. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
66. Put the gel under UV.

LEVEL 1 pFliC – eGFP-terminator

U1 C1 U2 C2 U3 C3

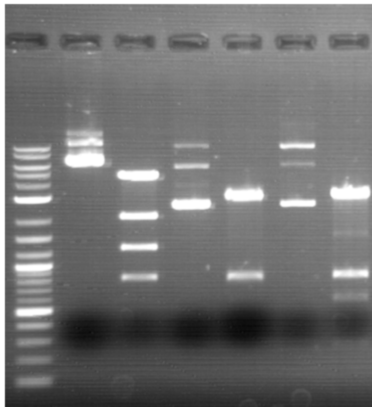


Figure 1b. (U=Uncut C=Cut) Restriction Enzyme with PvuII, Expected bands in bp 3016 + 816

67.

68. Analyze the bands on the gel.

Transformation Protocol

33. Take competent cells out of -80°C and put them on ice.
34. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
35. Incubate the competent cell-DNA mixture on ice for 20 mins.
36. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
37. Put the tubes back on ice for 2-5 min.
38. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
39. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
40. 150 µL transformation on one plate.
41. Incubate plates at 37°C overnight.

28/09/20

<u>Goals of the day:</u>

- Create LB cultures: pFliC L1

pFliC L1:

1. Take the previous day's plate from the incubator (37°C).
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
4. Incubate in a shaker at 37°C overnight.

29/09/20

Goals of the day:

- Plate Reader for eGFP

Plate Reader Assay

1.measure the OD600 of the overnight cultures.
2. For each one of the colonies: Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the time points you are going to choose.
3. Add 0,02mM,0,2mM,2mM,20mM,200mM acetate and propionate to each colony
4. Measure the OD600 and F1: 0h, 4h,8h, 20h
5. Add proper antibiotic (in this case, Kanamicyn)
6. Incubate each culture : 37◦C and 210 rpm . The duration of incubation varies for each liquid culture.
7. In due time : take 600 μl of each liquid culture and split into 3 walls (200μl per walls , technical replicates)
8. Set the optimized parameters and measure.

Preparation of the 96-well plate with all additives

- Beginning of the plate-reader assay
- Timepoints: 0, 4h, 8h, 20h
- During these timepoints the samples are in shaker incubator

eGFP

Measure OD600
Excitation at 488
Emission at 515

30/09/20

Goals of the day:

- Plate Reader for eGFP
- Dig – Lig: pSEVA23 – pFliC-sfGFP-Terminator L1
- Transformation of L1

One-pot dig-lig reaction (Golden Gate/GoldenBraid)

Total reaction volume: 10 µL

- 50 ng acceptor vector
- 50 ng of each part:
- 1 µL 10X T4 DNA ligase **buffer**
- 1 µL T4 DNA Ligase
- 0.5 µL restriction enzyme (BsmBI to omega)
- 5,2µL dH2O

We use a thermocycler (PCR Machine) to run this protocol. The protocol we use in our lab is termed "GBLong":

21. 2 min at 37 degrees C (optimal temp for Restriction Enzymes)
22. 5 min at 16 degrees C (optimal temp for Ligase)
23. Repeat steps 1. and 2. **50 times**
24. 5 min at 80 degrees C (to kill enzymes)
25. Rest at 16 degrees C.

Transformation Protocol

1. Take competent cells out of -80°C and put them on ice.
2. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
3. Incubate the competent cell-DNA mixture on ice for 20 min.
4. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
5. Put the tubes back on ice for 2-5 min.
6. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
7. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
8. 150 µL transformation on one plate.
9. Incubate plates at 37°C overnight.

01/10/20

Goals of the day:

· Create LB cultures: pFliC L1

pFliC L1:

5. Take the previous day's plate from the incubator (37°C).

6. Pick 4 white cultures from the plate using a pipette.

7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).

8. Incubate in a shaker at 37°C overnight.

02/10/20

<u>Goals of the day:</u>

- Mini preps: sfGFP L1
- Nano drop: sfGFP L1
- Restriction Digestion: sfGFP L1
- Electrophoresis
- Transformation into MC1061

Miniprep – Plasmid Isolation (adapted from Macherey-Nagel) Protocol

22. **Cultivate and harvest bacterial cells**
Use **2 mL** of each LB culture of the previous day (prpB L1), pellet cells in a standard benchtop microcentrifuge for **30s at 11,000 x g**. Discard the supernatant and remove as much of the liquid as possible. Repeat this step until the previously 10ml LB culture is empty.

23. **Cell lysis**

31. Add **500 µL Buffer A1**. Resuspend the cell pellet completely by vortexing. **Make sure no cell clumps remain before addition of Buffer A2!**

32. Add **500 µL Buffer A2**. Mix gently by inverting the tube 6-8 times. Incubate at room temperature for **up to 5 min** or until lysate appears clear.

33. Add **600 µL Buffer A3**. Mix thoroughly by inverting the tube 6-8 times **until blue samples turn colorless completely!**

24. **Clarification of lysate**

34. Centrifuge for **10 min at 15,000 x g** at room temperature.

25. **Bind DNA**

35. Place a Nucleospin Plasmid Column in a Collection Tube (2 mL) and decant the supernatant from step III or pipette a **of 750 µL** of the supernatant onto the column. Centrifuge for **1 min at 11,000 x g**. Discard flowthrough and place the Nucleospin Plasmid Column back into the collection tube.

Repeat this step to load the remaining lysate.

26. **Wash silica membrane**

36. Add **500 µL Buffer AW**, and centrifuge for **1 min at 11,000 x g** before proceeding with Buffer A4.

37. Add **600 µL Buffer A4** (supplemented with ethanol) Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the Nucleospin Plasmid Column back into the **empty** collection tube.

27. **Dry silica membrane**

38. Centrifuge for **3 min at 11,000 x g** and discard the collection tube

28. Elute DNA

39. Place the Nucleospin Plasmid Column in a 1.5 mL microcentrifuge tube and add **40 µL Buffer AE**.

40. Centrifuge for **1 min at 11,000 x g**

Nano Drop

eGFP1	206ng
eGFP2	281ng
eGFP3	238ng
eGFP4	255ng

Restriction Digestion of DNA Protocol

Procedure

19. Preparation for digestion.
    Restriction enzyme: HindIII
    Enzymes were chosen using SnapGene.
20. Appropriate reaction buffer according to the instructions: R2
21. In a 1.5mL tube combine the following:

- DNA
- Restriction Enzyme
- Buffer
- dH2O up to total volume

Our restriction digestions are 10 μL in total volume. So the reaction is:

- y μg DNA
- 0.5 μL of each Restriction Enzyme
- 1 μL 10x Buffer
- x μL dH2O

	DNA	Restriction Enzyme	Buffer	dH_2O
eGFP1	1μL	0,5μL	1μL	7,5μL
eGFP2	1μL	0,5μL	1μL	7,5μL
eGFP3	1μL	0,5μL	1μL	7,5μL
eGFP4	1μL	0,5μL	1μL	7,5μL

Uncut:

	DNA	dH_2O
eGFP1	1μL	9μl
eGFP2	1μL	9μl
eGFP3	1μL	9μl
eGFP4	1μL	9μl

12. Mix gently by pipetting.
13. Incubate tube at appropriate temperature (37 °C) for 2 hours.

Sample Preparation for Gel Run

- Add 2μl loading dye (6X) to the uncut plasmids and the plasmids that underwent digestion.
  Final volume will be 12μl.
- For the gel ladder, add 3μl.

Agarose Gel Preparation and Electrophoresis

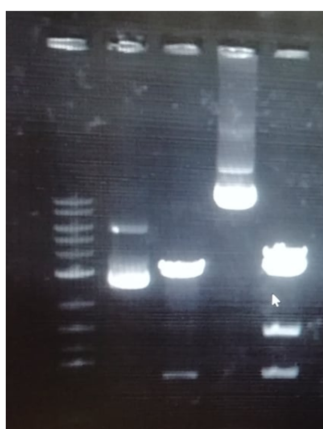
Materials for the gel

For 100ml of 1% w/v agarose we need

- **100ml 1X TAE**
- **1gr agarose**
- **5µL EtBr**

69. Measure the appropriate volume of 1X TAE (falcon tube for the measurement) and 1gr of dry agarose.
70. Mix TAE and agarose in a flask
71. Microwave for 2-3 mins (until it boils)
72. After microwaving, mix it thoroughly under running water until it cools down.
73. When in the right temperature (not too hot), add 5µl EtBr. Pipette up and down and mix the flask to spread the EtBr everywhere.
74. Prepare the gel box, combs *etc.*
75. Calibrate the scaffold until it is completely balanced (adjust the two knobs, place the calibrator at the center of the gel box)
76. Add the liquid gel slowly into the gel box.
77. Remove bubbles with a tip.
78. Let the gel solidify for 20min
79. After 20mins, untighten the scaffold and remove the gel box with the gel still on.
80. Put the gel in the “electrode box”, filled with 1X TAE.
81. Load the samples in the gel wells.
82. Add the lid and electrodes. Connect the other ends of the electrodes on the machine and press RUN at 120V until it runs the whole gel.
83. Put the gel under UV.

LEVEL 1 pFliC – sfGFP-terminator



84. **Figure 1c.** (U=Uncut C=Cut) Restriction Enzyme with PvuII, Expected bands in bp 3016 + 816

85. Analyze the bands on the gel.

Transformation Protocol

10. Take competent cells out of -80°C and put them on ice.
11. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
12. Incubate the competent cell-DNA mixture on ice for 20 mins.
13. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
14. Put the tubes back on ice for 2-5 min.
15. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).

16. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
17. 150 μL transformation on one plate.
18. Incubate plates at 37°C overnight.

03/10/20

Goals of the day:

- Create LB cultures: pFliC L1

pFliC L1:

1. Take the previous day's plate from the incubator (37°C).
2. Pick 4 white cultures from the plate using a pipette.
3. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10μL).
4. Incubate in a shaker at 37°C overnight.

04/10/20

Goals of the day

- Plate reader for sfGFP

Plate Reader Assay

1.measure the OD600 of the overnight cultures.
2. For each one of the colonies: Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the time points you are going to choose.
3. Add 0,02mM,0,2mM,2mM,20mM,200mM acetate and propionate to each colony
4. Measure the OD600 and F1: 0h, 4h,8h, 20h
5. Add proper antibiotic (in this case, Kanamicyn)
6. Incubate each culture : 37°C and 210 rpm . The duration of incubation varies for each liquid culture.
7. In due time : take 600 μl of each liquid culture and split into 3 walls (200μl per walls , technical replicates)
8. Set the optimized parameters and measure.

Preparation of the 96-well plate with all additives

- Beginning of the plate-reader assay
- Timepoints: 0, 4h, 8h, 20h
- During these timepoints the samples are in shaker incubator

sfGFP
_Measure OD600

Excite at 485
Excite at 510

05/10/20

Goals of the day

- Plate reader for sfGFP
- Transformation omega 1R-pFliC:LacI:terminator-Anderson:LacO:eCFP:terminator into MC1061 (Proof of Concept-Prom Module)

Plate Reader Assay

sfGFP
_Measure OD600
Excite at 485
Excite at 510

Transformation Protocol

19. Take competent cells out of -80°C and put them on ice.
20. Mix 5µl of DNA into 100 µL of competent cells in an epedorf tube. Gently mix.
21. Incubate the competent cell-DNA mixture on ice for 20 min.
22. Heat shock each transformation tube by placing the bottom of the tube into a 42°C heat-block for 60 sec.
23. Put the tubes back on ice for 2-5 min.
24. Add 900 µl LB (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1h (under sterile conditions).
25. Plate all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (Xgal).
26. 150 µL transformation on one plate.
27. Incubate plates at 37°C overnight.

06/10/20

Goals of the day:

· Create LB cultures: pFliC L2

pFliC L2:

5. Take the previous day’s plate from the incubator (37°C).
6. Pick 4 white cultures from the plate using a pipette.
7. Move each one in a 5ml liquid culture with antibiotic (chloramphenicol 10µL).
8. Incubate in a shaker at 37°C overnight.

07/10/20

Goals of the day

- Plate reader for Proof of Concept

Plate Reader Assay

1.measure the OD600 of the overnight cultures.
2. For each one of the colonies: Dilute the culture to target OD600=0,1 in 5mL of desired medium and antibiotic. The number of M9 cultures depends on the time points you are going to choose.
3. Add 0,02mM,0,2mM,2mM,20mM,200mM acetate and propionate to each colony
4. Measure the OD600 and F1: 0h, 4h,8h, 20h
5. Add proper antibiotic (in this case, Kanamicyn)
6. Incubate each culture : 37°C and 210 rpm . The duration of incubation varies for each liquid culture.
7. In due time : take 600 μl of each liquid culture and split into 3 walls (200μl per walls , technical replicates)
8. Set the optimized parameters and measure.

Preparation of the 96-well plate with all additives

- Beginning of the plate-reader assay
- Timepoints: 0, 4h, 8h, 20h
- During these timepoints the samples are in shaker incubator

· *eCFP*
Measure OD600
Excite at 434
Excite at 477

08/10/20

Goals of the day

- Plate reader for Proof of Concept

Plate Reader Assay

eCFP
Measure OD600
Excite at 434
Excite at 477

For the analysis of our results regarding our gathered data of characterization and Proof of Concept visit our wiki pages.