New Lab Book - iGEM 2021

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MONDAY, 12/7/2021

Goals :

1. Prepare Tris- HCl and EDTA for TE buffer.
2. Autoclave the above solutions.

1.Prepare Tris- HCl

We prepared 100 ml of 1.0 M Tris-HCl pH: 8.0 solution according to protocol Tris-HCl (1 M) recipe .

2. Autoclave

We autoclaved the Tris-HCl solution alongside lab equipment (tips and duran bottles).

TUESDAY, 13/7/2021

Goals :

1. Prepare TE buffer.
2. Autoclave the TE buffer.

1.Prepare TE buffer

We prepared 100ml TE buffer according to protocol Preparation of TE Buffer . We will use the TE buffer to resuspend our g-Blocks.

2.Autoclave

We autoclaved the TE buffer.

WEDNESDAY, 14/7/2021

We did not enter the lab due to fundraising business we had to attend to.

THURSDAY, 15/7/2021

Goals :

1. Resuspend our g-Blocks.
2. Quantify our g-Blocks Gene Fragments.

1.Resuspend our g-Blocks

We resuspended our g-Block fragments according to protocol Resuspension of gBlocks .

2.Quantify our g-Blocks Gene Fragments

We used the NanoDrop device in our lab but the concentrations we got from our g-Blocks were no higher than 5ng/ul. Since we expected a value close to 10 ng/ ul we started troubleshooting and realized that our device can not detect such small quantities of DNA.

FRIDAY, 16/7/2021

Goals :

1. Tranformation of competent cells with vector pSB1C3.
2. Autoclave

1.Tranformation of competent cells with vector pSB1C3.

We transformed competent cells we received from NEB and competent cells we prepared on our own according to protocol Transformation of competent cells .

We used 1 chloramphenicol plate for our competent cells and 2 plates for the competent cells from NEB (one of them had 200 ul of the tube and the rest of them were added in the second plate) . We used a pSB1C3 vector we received from our sponsor for the transformation and we incubated the plates overnight at 37 °C.

2.Autoclave

We autoclaved lab equipment, tips and microcentrifuge tubes.

SATURDAY, 17/7/2021

Goals :

1. Prepare liquid cultures of the transformed cells we prepared on Friday 16/7/2021

1.Prepare liquid cultures of the transformed cells we prepared on Friday 16/7/2021

We prepared liquid cultures according to protocol Inoculation of liquid bacterial cultures . Specifically, we used the following quantities of reagents.

Liquid culture of NEB's competent c…

	A	B
1	Reagents	Volume
2	LB media	15 ml
3	Chloramphenicol	15 ul (25ul/ml)
4	Total	15 ml

We incubated our bacterial cultures overnight at a shaking incubator at 37°C.

SUNDAY, 18/7/2021

Goals :

1. Do minipreps of our liquid culture.

1.Do minipreps of our liquid culture.

We used our liquid cultures containing the competent cells and vector to prepare minipreps according to protocol Plasmid DNA Isolation with miniprep .

Since we need a large amount of plasmid DNA we will prepare 10 samples of liquid culture. The 10 samples we prepared were then grouped into 3 tubes for storage. On the following table we present the concentrations for each tube through NanoDrop quantification.

Miniprep concentrations

	A	B	C	D	E	F
1		Concentration (ng/ ul)	A260	A280	260/280	260/230
2	Tube 1	239.3	4.785	2.534	1.89	2.24
3	Tube 2	280.7	5.615	2.984	1.88	2.21
4	Tube 3	235.0	4.70	2.492	1.89	2.09

MONDAY, 19/7/2021

We did not enter the lab because of power outage.

TUESDAY, 20/7/2021

Goals :

1. Prepare the protocol for diagnostic digestion and the purification of the vector

1.Prepare the protocol for the diagnostic digedtion and the purification of the vector

In order to insert the toehold switches sequences to the vector pSB1C3 we will need to digest the vector in order to remove the GFP gene, run an agarose gel and collect only the digested vector. During these procedures, a large amount of the vector is lost. In order to correctly calculate the quantities of the reagents that we will need for the insertion of the 16 toehold switches to the vector, we determined to run a diagnostic digestion with only 600 ng of the vector and estimate the yield.

For the diagnostic digestion of the vector we will use the protocol Diagnostic Restriction Digestions and for the purification we will use the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel. The link for the manual can be found below:
[
https://www.takarabio.com/documents/User%20Manual/NucleoSpin%20Gel%20and%20PCR%20Clean/NucleoSpin%20Gel%20and%20PCR%20Clean-up%20User%20Manual_Rev_04.pdf]

WEDNESDAY, 21/7/2021

Goals:

1. Complete the diagnostic digestion and purification of the vector
2. Calculate the yield of the digestion and purification

1.Complete the diagnostic digestion and purification of the vector

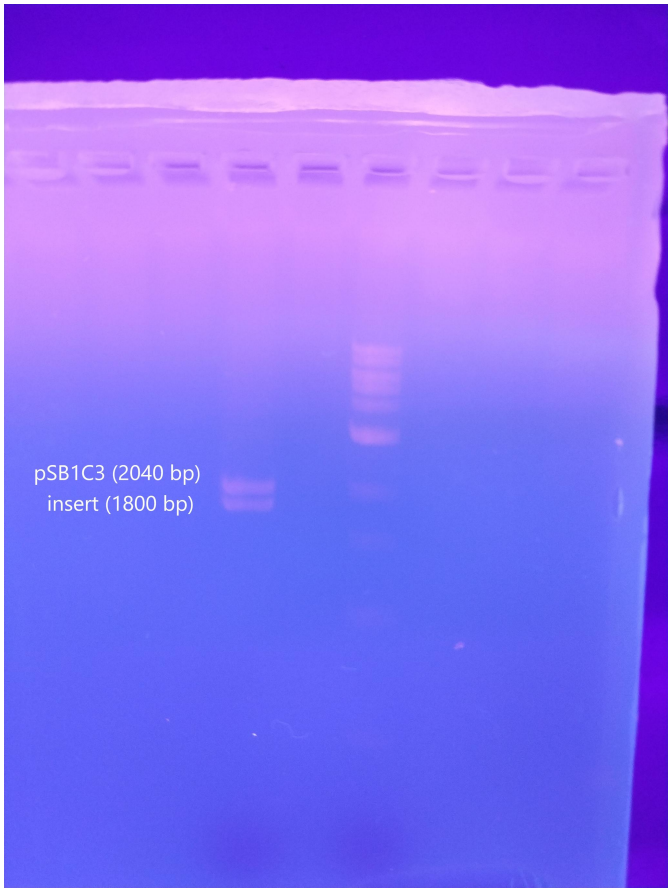
In order to do the diagnostic restriction digestion of our vector we will use the protocol Diagnostic Restriction Digestions, but we will use different quantities of our reagents.

Reagents for diagnostic digestion…

	A	B
1	Reagents	Volume
2	Nuclease free water	23.49 ul
3	Buffer 2:1 (10X)	3 ul
4	DNA	600 ng (2.51ul)
5	EcoRI-HF	0,5 ul
6	PstI	0.5 ul
7	Total	30 ul

We then prepared agarose gel 1% in order to prepare an electrophoresis using the protocol Electrophoresis .
We captured the gel images at different times and after 75 minutes we finally managed to see our insert and our vector seperately.

Electrophoresis of the digested pSB1C3



We then used the protocol Gel Extraction to extract the vector DNA. The mass of the tube was 1.023g and the mass of the gel was 0.17g. So, we used 680 ul of dissolving buffer according to NEB's protocol.

2.Calculate the yield of the digestion and purification

After the extraction was complete we used 1 ul of our DNA to calculate the yield through NanoDrop. We receiced the following results :

Concentration of the linearized vector

	A	B	C	D	E	F
1		Concentration (ng/ ul)	A260	A280	260/280	260/230
2	Vector	39.2	0.785	0.327	2.40	0.05

According to the above, since we used 600 ng of DNA and received a concentration of 39.2 ng / ul and a total of 5 ul (1 out of 6 ul was used to measure the concentration through NanoDrop) we calculated that we have 196 ng of pSB1C3 Vector and that the yield is 39 % .

THURSDAY, 22/7/2021

Goals :

1. Digest our pSB1C3 Vector
2. Calculate the yield of the digestion and purification

1.Digest our pSB1C3 Vector

In order to digest an appropriate amount of vector to use for the ligation of our 16 g-blocks we will use the Tube 2 (280.7 ng/ ul).

Reagents for the digest of the vector

	A	B	C	D
1	Reagents	Tube 1	Tube 2	Tube 3
2	Nuclease free water	15.58 ul	15.58 ul	15.58 ul
3	Buffer 2:1 (10X)	2 ul	2 ul	2 ul
4	DNA	400 ng (1.42 ul)	400 ng (1.42 ul)	400 ng (1.42 ul)
5	EcoRI-HF	0.5 ul	0.5 ul	0.5 ul
6	PstI	0.5 ul	0.5 ul	0.5 ul
7	Total	20 ul	20 ul	20 ul

Incubate all restriction digest reactions at 37°C for 10 minutes and then heat inactivate at 80°C for 20 minutes.

In order to dephosphorylate the vector we used the protocol Dephosphorylation of linearized vector. For this procedure we used the following reagents:

Reagents for the dephosphorylation of linearized vector

	A	B	C
1	DNA	1 pmol of DNA ends	0.4 pmol
2	Antarctic Phosphatase Reaction Buffer	2 ul	0.8 ul
3	Antarctic Phosphatase	1 ul (5 units)	0.4 ul
4	Purified H2O	to 20 ul	6.4 ul
5	Total		8 ul

We then used the protocol Gel Extraction to extract the vector DNA.

2.Calculate the yield of the digestion and purification

After the extraction was complete we used 1 ul of our DNA to calculate the yield through NanoDrop. We receiced the following results :

Concentration of linearized vector-3 samples

	A	B	C	D	E	F
1	Sample	Concentration (ng/ ul)	A260	A280	260/280	260/230
2	Linearized pSB1C3 - 1	20.2	0.404	0.157	2.58	0.03
3	Linearized pSB1C3 -2	43.8	0.877	0.392	2.24	0.55
4	Linearized pSB1C3 -3	36.0	0.720	0.276	2.61	0.10

FRIDAY, 23/7/2021

Goals:

1. Digest our g-blocks
2. Perform the ligation of vector and g-blocks

1.Digest our g-Blocks

In order to digest our g-Blocks we will use the protocol Digestion of g-Blocks but we will scale it down to total volume of 10 ul.

Reagents for the digest of g-Blocks

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Reagents	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10	Tube 11	Tube 12	Tube 13	Tube 14	Tube 15	Tube 16
2	Nuclease free water	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul	10.34 ul
3	Buffer 2:1 (10X)	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul	2 ul
4	DNA	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)	66.6 ng (6.66 ul)
5	EcoRI-HF	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	PstI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
7	Total	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul

2.Ligation of our vector and g-Blocks.

In order to ligate our vector and g-Blocks, we will use the protocol Ligation with T4 ligase

For every reaction we will use the following:

Reagents for ligation reaction

	A	B	C	D
1	Reagents	Volume	Linearized pSB1C3 -1	Linearized pSB1C3 -2
2	Linearized Vector	20 ng	1 ul	0.45 ul
3	g-Blocks	27.5 ng (8.25 ul)	8.25 ul	8.25 ul
4	10X T4 DNA Ligase Buffer:	2 ul	2 ul	2 ul
5	T4 DNA Ligase:	1 ul	1 ul	1 ul
6	Nuclease Free Water:	to 20 ul	7.75 ul	8.3 ul
7	Total	20 ul	20 ul	20 ul

Incubate at room temperature for 10 minutes and then heat inactivate at 80°C for 20 minutes.

(We incubated at 16^{O} C, without heat inactivation.)

SUNDAY, 25/7/2021

Goals:

1. Transform competent cells with the plasmids that contain our toehold switches

1.Transform competent cells with the plasmids that contain our toehold switches

We used the protocol Transformation of competent cells to transform Competent cells from NEB (5-alpha) with our toehold switches.

MONDAY, 26/7/2021

Goals:

1. Prepare LB
2. Prepare Chloramphenicol stock solution
3. Prepare 30 chloramphenicol agar plates
4. Prepare 1L TAE Buffer
5. Troubleshooting to find out why there were no colonies formed from our transformed cells

1.Prepare LB medium

We prepared 700 ml LB medium, by desolving 14g of premixed LB powder according to LENOX in 700 ml dH_2O. We will use this LB medium in order to prepare liquid cultures.

2.Prepare Chloramphenicol stock solution

We prepared a 25mg/ml stock concentration of chloramphenicol in ethanol.

3.Prepare 30 chloramphenicol agar plates

We prepared a 1 L LB-agar medium, autoclaved it and after it had cooled down, we added 1 ml of chloramphenicol. We poured the medium into the plates and let them solidify.

TUESDAY, 27/7/2021

Goals:

1. Transformation of the ligation mixture we prepared on Friday 23/7/2021.
2. Use the digested G-blocks from the freezer and do ligation in room temperature (don't forget heat inactivation) and transformation in NEB cells
3. Digest of G-blocks, ligation in room temperature with a new linearized vector and transformation in NEB cells

1.Transformation of the ligation mixture we prepared on Friday 23/7/2021.

In order to find out if something went wrong with the transformation procedure, we repeated the transformation with the ligated plasmids we prepared on Friday 23/7/2021. We transformed cells with the plasmids containing T-143-3p-2 and T-143-3p-4. We used 5 ul of each ligation mixture to transform 25 ul of competent cells DH5a, that we have ordered from NEB. We, also, prepared a sample containing competent cells and linearized plasmid as a negative control and a sample containing competent cells and the plasmid as a positive control. After the procedure of transformation we streaked chloramphenicol plates and incubate them at 37^o C overnight. The transformation protocol we used is the Bacterial Transformation, from Addgene: https://www.addgene.org/protocols/bacterial-transformation/

2. Ligation of the digested G-blocks and transformation in NEB cells.

The ligation and digestion was done like the protocol used in 23/07/2021. Unfortunately, the next day we didn't recieve any colonies.

3. Digest of G-blocks, ligation in room temperature with a new linearized vector and transformation in NEB cells

This experiment didn't happen because not enough concenatration of the vector was left.

WEDNESDAY, 28/7/2021

Goals:

1. Digest of G-blocks, ligation in room temperature with a new linearized vector and transformation in NEB cells

1.Digest of G-blocks, ligation in room temperature with a new linearized vector and transformation in NEB cells

Digestion of Vector

	Reagents	Volume (ul) / Quantity
1	H2O	23,49
2	Buffer 2.1	3
3	Vector with C=239,3 ng/ul	2,51
4	EcoRI	0,5
5	PstI	0,5

Incubate at 37 C for 15 minutes. Heat inactivate at 80 C for 20 minutes.

Dephosphorylation of linearized v…

	Reagents	Volume (ul) / Quantity (ng)
1	Linearized vector	600 ng
2	Phosphatase Buffer	3 ul
3	Antarctic Phosphatase	1 ul
4	H2O	-
5	Total	30 ul

Incubate at 37 C for 30 minutes and then heat inactivate at 80 C for 2 minutes.

G-block Digestion

	Reagents	Volume (ul) / Quantity
1	H2O	10,34
2	Buffer 2.1	2
3	G-blocks 143-3p-2 & G-blocks 143-3p-4	6,66
4	EcoRI	0,5
5	PstI	0,5

Incubate at 37 C for 10-15 minutes. Heat inactivate at 80 C for 20 minutes

Ligation of G-blocks

	Reagents	Volume (ul) for G-block 143-3p-4, with a ratio of 3:1	Volume (ul) for G-block 143-3p-2, with a ratio of 3:1
1	Linearized vector	0,744	0,744
2	G-blocks	20	8,26
3	T4 Buffer	2	2
4	T4 Ligase	1	1
5	H2O	-	8

Incubate at room temperature for 10 minutes and heat inactivate at 65 C for 10 minutes. Chill on ice.

Transformation:
The transformation protocol we used is the Bacterial Transformation, from Addgene: https://www.addgene.org/protocols/bacterial-transformation/
We transformed 5 ul of DNA to 50 ul of competent cells.

However, due to a mistake during the procedure of ligation, we decided to repeat the same procedure again on Thursday.

THURSDAY, 29/7/2021

Goals:

1. Repeat the transformation protocol

1.Repeat the transformation protocol
We repeated the digestion, ligation and transformation procedures and streaked plates with our samples. Unfortunately, the following day we didn't recieve any colonies.

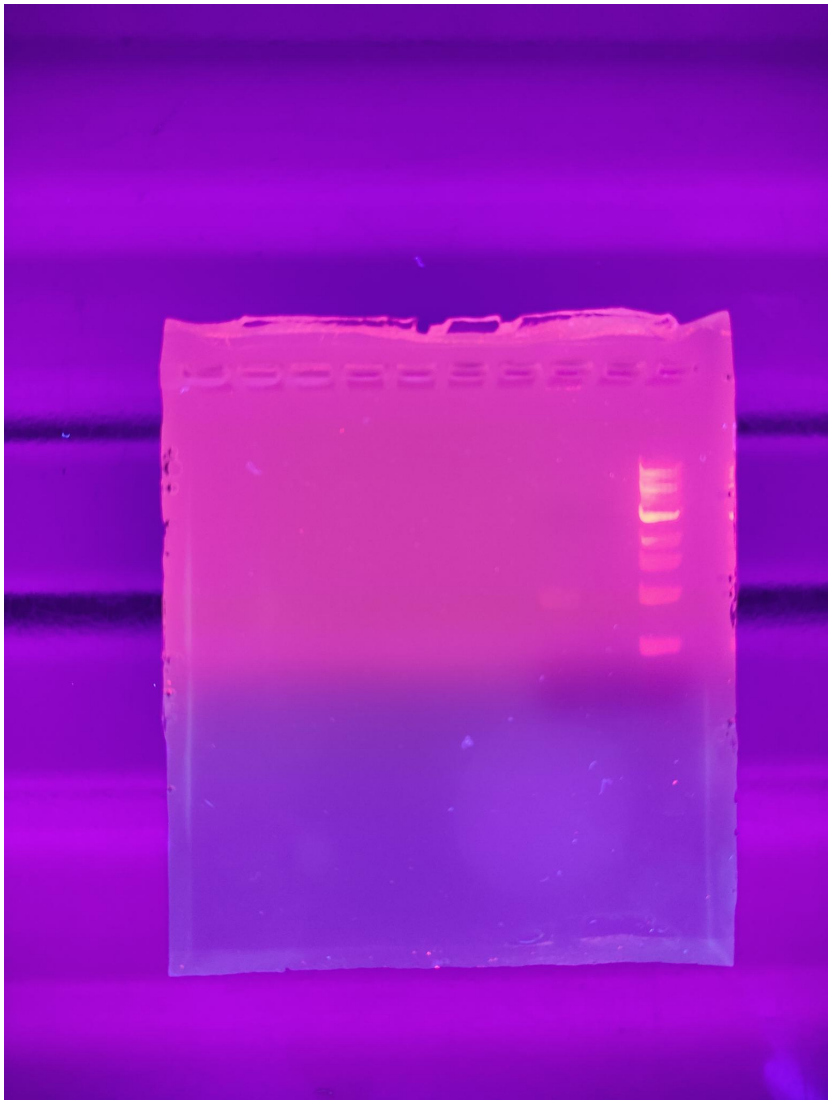
FRIDAY, 30/7/2021

Goals:

1. Run an electrophoresis with g-blocks
2. Preparation of a liquid culture of bacteria with pSB1C3-dsred-polyA (postive control)
3. Calculation of transformation efficiency

1.Run an electrophoresis with g-blocks
In order to determine why there is no bacterial growth after the transformation, we decided to run a gel with samples from our g-blocks. We ran an electrophoresis of two samples of the g-blocks we have ordered. For this procedure, we prepared an 1% agarose gel and loaded the 1kb ladder and 1 ul of T-143-3p-2 and T-143-3p-4. We examined the results after an hour, but there were no bands corresponding to these samples. For this reason, we repeated the same procedure with larger volumes of DNA samples. This time, we detected the two bands, although it was still difficult to distinguish.

Electrophoresis of a g-Block



2. Preparation of a liquid culture of bacteria with pSB1C3-dsred-polyA (postive control)

We prepared a liquid culture of bacteria transformed with the pSB1C3-dsred-polyA plasmid (Thursday 29/7/2021). This will allow us to isolate this vector easier.

3. Calculation of transformation efficiency

We calculated the transformation efficiency of the competent cells we have prepared on Saturday 3/4/2021, using the plate we prepared on Thursday 29/7/2021.

SATURDAY, 31/7/2021

Goals:

1. Perform digestion and ligation of the pSB1C3 vector
2. Preparation of liquid culture with bacteria with pSB1C3

1.Perform digestion and ligation of the pSB1C3 vector

In order to find out if there is a problem with the ligation procedure or with the quantity of the insert, we decided to digest the pSB1C3-dsred-polyA vector and then ligate its parts. For this procedure we digested the vector with PstI and EcoRI and collected both the linearized vector and the insert. The quantity of the reagents needed for the digestion can be found in the following table. We calculated the quantity of the plasmid based on the yield of previous gel extraction procedures (approximately: yield = 30%).

Reagents for the digestion of the pSB1C3

	A	B
1	Reagents	
2	Nuclease free water	7.2 ul
3	Buffer 2:1 (10X)	1 ul
4	DNA	190 ng (0.8 ul)
5	EcoRI-HF	0.5 ul
6	PstI	0.5 ul
7	Total	10 ul

To isolate the backbone and the insert we ran an 1% agarose gel. For the extraction we used the Gel Extraction kit from and NEB and we followed the protocol Gel Extraction . The weight of the gel slice with the linearized vector was 0.156 mg and the weight of the gel with the insert was 0.213 mg. Based on these values we calculated the appropriate quantity of the Gel Dissolving Buffer. After the extraction we measured the concentration of each sample using Nanodrop 2000. We measured the following concentrations:

Concentration of the pSB1C3 vector and the insert after th…

	A	B
1	pSB1C3 vector linearized	2.1 ng/ul
2	insert of the vector	2.5 ng/ul

For the ligation, we calculated the quantity of the insert using the NEBioCalculator. The quantities of the ligation reagents are mentioned in the table below:

Quantities' of the reagents for the ligation

	A	B
1	**Reagent**	**Quantity**
2	Vector	8 ng (3.8 ul)
3	Insert	21.72 ng (8.6 ul)
4	T4 ligase buffer 10x	2 ul
5	T4 ligase	1 ul
6	Nuclease free water	4.5 ul
7	Total	20 ul

The ligation was performed according to protocol Ligation with T4 ligase . After the ligation, we transformed the E.coli competent cells we prepared on Saturday 3/4/2021.

2.Preparation of liquid culture of bacteria with pSB1C3.
We repeated the same procedure as yesterday, but we also add the appropriate quantity of antibiotic.

SUNDAY, 1/8/2021

Goals:

1. Do a glycerol stock with the liquid culture from 31/7/2021
2. Transformation of competent cells with part 1A from Plate 1 (Distribution Kit 2021)
3. Storage of part 1A from Plate 1 (Distribution Kit 2021)

1.Do a glycerol stock with the liquid culture from 31/7/2021
We used 250 ul of 100% Glycerol and 250 ul of dH2O to prepare 500 ul of 50% Glycerol. We then added the 500 ul into a 2 ml microcentrifuge tube and we also added 500 ul of our liquid culture and we stored at -80 °C.

2.Transformation of competent cells with part 1A from Plate 1 (Distribution Kit 2021)
We followed the instructions of iGEM to succesfully pinpoint the part we wanted and punched a hole with a tip on the foil cover. We added 10 ul of dH20 in the well of the specific part and we resuspended by pippetting up and down a few times and we let it sit for 5 minutes. We then transformed 1 ul of the part using 50 ul of competent cells we created, using the protocol Transformation of competent cells .We used 2 plates with Chloramphenicol , one with 50 ul of the total mixture and one with 600 ul and we incubated the plates at 37°C overnight.

3.Storage of part 1A from Plate 1 (Distribution Kit 2021)
We stored the rest of the part from well 1A into a microcentrifuge tube at -20 °C. The tube contains 9 ul of the part.

MONDAY, 2/8/2021

Goals:

1. Digestion and ligation of pSB1C3 and transformation of competent cells
2. Transformation of competent cells with part 1A from Plate 1 (Distribution Kit 2021)

1.Digestion and ligation of pSB1C3 and transformation of competent cells

Since after the cloning performed on Saturday 31/7/2021 there were no colonies, we decided to repeat the same experiment using an 1:7 ratio (vector:insert). For the digestion of the plasmid we used the following reagents:

Reagents for the digestion of pSB1C3

	A	B
1	Reagents	Qunatity
2	Nuclease free water	14.9 ul
3	Buffer 2:1 (10X)	2 ul
4	DNA (235 ng/ul)	2.1 ul
5	EcoRI-HF	0.5 ul
6	PstI	0.5 ul
7	Total	20 ul

We incubated the mixture for 15 minutes at 37^{o} C. The heat inactivation of the enzymes was performed at 80^{o} C for 20 min.

After the digestion, we ran this sample on a gel and then extracted the lanes corresponding to the linearized vector and its insert. For the extraction we used the protocol Gel Extraction. We calculated the quantity of the Gel Dissolving Buffer as follows:

Calculations for the Gel Extraction protocol

	A	B	C	D	E
1		Empty tube weight	Total weight	Gel weight	Buffer volume
2	Linearized vector	1.012 g	1.201 g	189 mg	756 ul
3	Insert	1.018 g	1.165 g	147 mg	588 ul

We measured the concentrations of the linearized vector and the insert using Nanodrop 2000. The concentrations of the two samples are mentioned below:

Concentrations of the linearized vector and the insert after gel extraction

	A	B	C
1		Concentration	Volume after elution
2	Linearized vector	55.1 ng/ul	10 ul
3	Insert	37.7 ng/ul	10 ul

For the ligation reaction we decided to use 20 ng of the vector and we calculated the appropriate amount of the insert with NEBio Calculator. We decided to use an 1:7 ratio. The quantities of the reagents needed for the ligation reaction are listed in the table bellow:

Reagents for the ligation reaction

	A	B
1	**Reagent**	**Quantity**
2	Vector	0.36 ul (20 ng)
3	Insert	3.36 ul (126.7 ng)
4	T4 ligase buffer 10x	2 ul
5	T4 ligase	1 ul
6	Nuclease free water	13.14 ul
7	Total	20 ul

This time we incubated the reaction for a longer period of time. We incubated for 25 minutes at 37^{o} C. For the inactivation of the ligase we heated the mixture for 10 minutes at 65^{o} C.

For the transformation we used 5 ul of this sample and 5 ul of uncut plasmid and transfomed 25 ul of competent cells for each sample. In order to streak plates with these cells, we centrifuged the samples, discarded a part of the supernatant and resuspended the cells in the remaining medium. We used the whole quantity of this mixture to streak plates and incubated at 37^{o} C overnight.

2.Transformation of competent cells with part 1A from Plate 1 (Distribution Kit 2021)
The plates we incubated on 1/8 did not contain any visible colonies, except from plate 1 (50 ul) that contained very small colonies. So, we left the plates in the incubator in hope of a better incubation and we prepared a new transformation with a higher quantity of part 1A. We transformed 5 ul of the part this time using 50 ul of competent cells we created, using the protocol Transformation of competent cells. We used 2 plates with Chloramphenicol ,and we spinned down the tube. One plate contained the supernatant and one contained the pellet with a bit of supenatant. We incubated the plates at 37°C overnight.

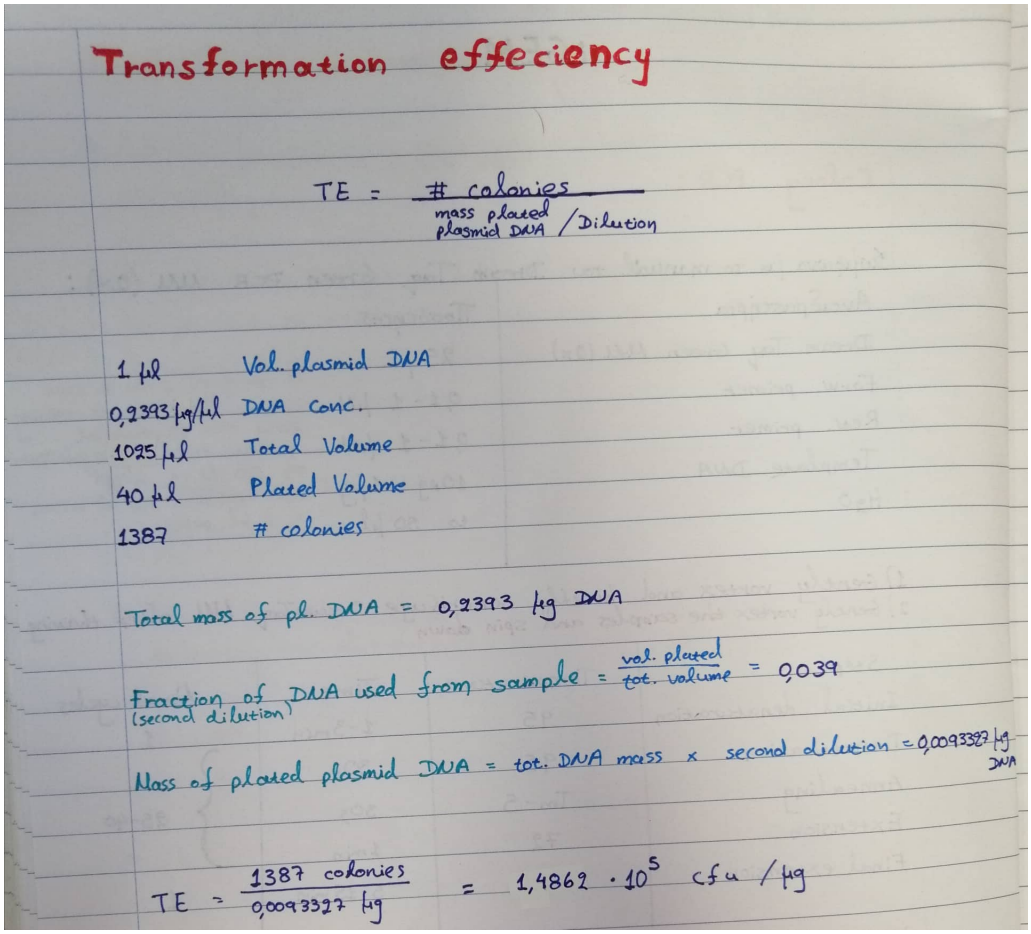
TUESDAY, 3/8/2021

Goals:

1. Calculate competent cell's transformation effeciency
2. Make liquid culture of transformation of A1 iGEM part

1.Calculate competent cell's transformation effeciency

image.png



The transformation effeciency of our competent cells is 1,4862 * 10^5 cfu / µg (Normal range is between 10^6 - 10^8)

2.Make liquid culture of transformation of A1 iGEM part

We prepared a liquid culture of 2 ml LB and 2 ul Chloramphenicol with the transformated competent cells.

WEDNESDAY, 4/8/2021

Goals:

1. Repeat liquid cultures of A1 iGEM part
2. Do liquid culture of colony from diagnostic transformation from 2/08

1.Repeat liquid cultures of A1 iGEM part:

The liquid cultures done at 03/08/2021 did not grow, so no minipreps were done. So the process was repeated with a few changes with the goal to determine what went wrong. The cultures that were made contained 2 ml LB and:

1. 2 ul Chloramphenicol from another Lab
2. 2 ul Chloramphenicol that we made
3. 2 ul Ampicillin
4. just 2 ml LB without antibiotic

This way we will check whether there is something wrong with the Chloramphenicol that we made.

2.Do liquid culture of colony from diagnostic transformation from 2/08

The agar plate with the diagnostic transformation from 2/08 gave a single colony, so a liquid culture was made with 2 ml LB and 2 ul Chloramphenicol. I took care to leave part of the colony on the plate, in case something goes wrong and we need to repeat the process.

Results:

- The liquid culture nr. 1 was a little cloudy
- The liquid culture nr. 2 was a little cloudy (the same as nr. 1)
- The liquid culture nr. 3 was completely clear
- The liquid culture nr. 4 was completely cloudy

Conclusions:

- Our LB works fine because bacteria have grown without antibiotic
- Our Chl works fine because it gave the same results as the Chl from another lab, that has been tested beforehand and it works
- Something is wrong with iGEM part A1, so we will not continue the experiments with it

THURSDAY, 5/8/2021

Goals:

1. New ligation reaction with new T4 ligase and the parts that were digested from pSb1c3-dsred-polya plasmid (try again with its own insert)
2. Do transformation of ligation product from pSb1c3-dsred-polya plasmid (with the vector and the same insert) (1:3 and 1:7) (do 2 reactions one with our plates and one with new plates from another lab)
3. Transformation of competent cells with the ligated plasmid from 2/8/2021

1.New ligation reaction with new T4 ligase and the parts that were digested from pSb1c3-dsred-polya plasmid

Reagents for ligation reaction

	A	B
1		3:1 Ratio
2	Linearized vector	0,7435 ul / 20 ng
3	Insert	1,404 ul / 52,94 ng
4	T4 Buffer	2 ul
5	T4 Ligase	1 ul
6	H20	15,233

Three reactions were made, one with the normal ligation protocol where incubation is done for 15 minutes, one where incubation is done overnight and one where we use the normal protocol but we use the old T4 Ligase and not the new one, like in the above reactions.

- Incubate at room temperature for 15' or overnight
- Heat inactivate at 65 C for 10'
- Chill on ice

2.Transformation of ligation product from pSb1c3-dsred-polya plasmid (with the vector and the same insert) (1:3 and 1:7) (do 2 reactions one with our plates and one with new plate from another lab)

The transformation protocol we used is the Bacterial Transformation, from Addgene: https://www.addgene.org/protocols/bacterial-transformation/
For all the transformation reactions the competent cells from another lab will be used.
For the first the reaction where the ligation was performed normally, after the transformation we streaked in using a new agar plate from another lab. For the other two transformation reactions we streaked them using our own agar plates.

3.Transformation of competent cells with the ligated plasmid from 2/8/2021
We intended to check a variety of possible factors that might affect the efficiency of our cloning procedure. For this reason, we used the ligation mixture from the cloning prepared on 2/8/2021 to transform new competent cells, given to us from another lab. In order to realize, if there is a problem with the chloramphenicol plates we have prepared we streaked two plates: one from our own agar plates and one from another lab. This way we can compare the results on the two plates.

The ligation mixture was prepared and stored on Monday 2/8/2021. This mixture contains:

Components of the ligation reaction prepared on M…

	A	B
1	**Reagent**	**Quantity**
2	Vector	0.36 ul (20 ng)
3	Insert	3.36 ul (126.7 ng)
4	T4 ligase buffer 10x	2 ul
5	T4 ligase	1 ul
6	Nuclease free water	13.14 ul
7	Total	20 ul

For the transformation we prepared two samples. For each one, we used 2.5 ul of the mixture above and we transformed 25 ul of competent cells. We followed the protocol described above:
1st step: We incubated the plates at 37^{o} C.
2nd step: We transfered 2.5 ul of the ligation mixture and 25 ul of cells in each tube and mixed gently by flicking the bottom of the tube.
3rd step: We incubated the samples in ice for 30 minutes.
4th step: We heated the samples at 42^{o} C for 45 seconds.
5th step: We incubated the samples on ice again for 2 minutes.
6th step: We added 1000 ul of LB medium and incubated the samples in a shaking incubator for 60 minutes.
7th step: We centrifuged the samples, discarded a part from the suppernatant and resuspended the sediment in the remaining liquid. We streaked the plates using the whole quantity of the suspension.
8th step: We incubated the plates at 37^{o} C overnight.

SATURDAY, 7/8/2021

Goals:

1. Run a diagnostic PCR with primers we received on 6/8/2021 with our Toehold switches
2. Prepare a new digestion and ligation of our vector with our toehold switches (g-blocks)

1.Run a diagnostic PCR with primers we received on 6/8/2021 with our Toehold switches (Anastasia / Danai)

We run a PCR with the protocol PCR using Q5 High Fidelity .
Specifically we used the following conditions :

PCR conditions-iGEMPCR

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	30 cycles	98 °C	8 s
3		71 °C	15 s
4		72 °C	20 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

And the quantities of our reagents were:

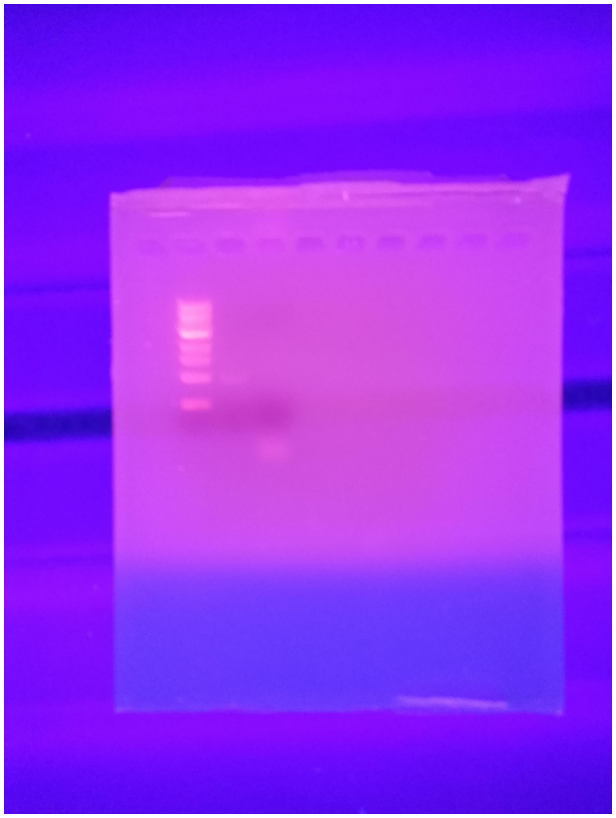
Reagents for PCR protocol-1st try

	A	B	C
1	Q5 High-Fidelity 2X Master Mix	12,5 ul	1x
2	10 μM Forward Primer	1,25 ul	0,5 μM
3	10 μM Reverse Primer	1,25 ul	0,5 μM
4	Template DNA	1 ul	<1000 ng
5	Nuclease-Free Water	9 ul	

We also run an electrophoresis using the protocol Electrophoresis to assess our results. We used an 1% agarose gel and load the samples with the following order (Left to right):
Ladder 1kb - T-miR-143-3p-4 (g-block) - PCR product.

We used 2 ul of Ladder, 2 ul of the g-block and the whole quantity of the PCR product. We added 5 ul of loading buffer in each sample and ran the gel for 35 minutes at 100 V. After the 35 minutes we examined the gel. We got the following results:

Diagnostic PCR product electrophoresis



We can see that the band corresponding to the g-block is near 1kb, as expected, but there is only one band corresponding to the PCR product smaller than 500 kb. We hypothesized that this band corresponds to primer dimers and that we had no amplification product. We decided to test if the annealing temperature is too high.

2.Prepare a new digestion and ligation of our vector with our toehold switches (g-blocks)

Since the cloning performed on Friday 6/8/2021 produced some colonies we decided to repeat the same experiment using an 1:7 ratio (vector:insert). For the digestion of the plasmid we used the following reagents:

Reagents for the digestion of the vector

	A	B
1	Reagents	Quantity
2	Nuclease free water	14.9 ul
3	Buffer 2:1 (10X)	2 ul
4	DNA (235 ng/ul)	2.1 ul
5	EcoRI-HF	0.5 ul
6	PstI	0.5 ul
7	Total	20 ul

For the digestion of the g-blocks we used the following reagents :

Reagents for the digestion of the g-block T-miR-1246-4

	A	B
1	Reagents	Quantity
2	Nuclease free water	10.34 ul
3	Buffer 2:1 (10X)	2 ul
4	DNA (10ng/ul)	62.3 ng (6.23 ul)
5	EcoRI-HF	0.5 ul
6	PstI	0.5 ul
7	Total	20 ul

We incubated the mixture for 20 minutes at 37^{o} C. The heat inactivation of the enzymes was performed at 80^{o} C for 20 min.

After the digestion, we ran this sample on a gel and then extracted the lane corresponding to the linearized vector . For the extraction we used the protocol Gel Extraction. We calculated the quantity of the Gel Dissolving Buffer as follows:

Calculation of buffer for gel extraction protocol

	A	B	C	D	E
1		Empty tube weight	Total weight	Gel weight	Buffer volume
2	Linearized vector	1.007	1.373	0.366	1464 ul

We measured the concentrations of the linearized vector and the insert using Nanodrop 2000. The concentrations of the two samples are mentioned below:

Concentration of linearized vector

	A	B	C
1		Concentration	Volume after elution
2	Linearized vector	34.6 ng/ul	10 ul

For the ligation reaction we decided to use 20 ng of the vector and we calculated the appropriate amount of the insert with NEBio Calculator. We decided to use an 1:7 ratio. The quantities of the reagents needed for the ligation reaction are listed in the table bellow. We also used a different ligase from ThermoFisher:

Reagents for the ligation reaction of pSB1C3 and T-...

	A	B
1	**Reagent**	**Quantity**
2	Vector	0.57 ul (20 ng)
3	Insert	6.23 ul (62.3 ng)
4	T4 ligase buffer 10x	2 ul
5	T4 ligase	1 ul
6	Nuclease free water	10.2 ul
7	Total	20 ul

This time we incubated the reaction for a longer period of time. We incubated overnight at 16^{o} C.

SUNDAY, 8/8/2021

Goals:

1. Prepare liquid cultures for the bacteria we got from transformation on 6/8/2021
2. Transformation with the ligated plasmid (with T-miR-1246-2) from Saturday 7/8/2021

1.Prepare a liquid culture for the bacteria we got from transformation 6/8/2021

We prepared liquid cultures according to protocol Inoculation of liquid bacterial cultures . Specifically, we used the following quantities of reagents.

Reagents for the preparation of a liq...

	A	B
1	Reagents	Volume
2	LB media	15 ml
3	Chloramphenicol	15 ul (25ul/ml)
4	Total	15 ml

We incubated our bacterial cultures overnight at a shaking incubator at 37°C.

2.Transformation with the ligated plasmid (with T-miR-1246-2) from Saturday 7/8/2021

We transformed our competent cells (50 ul) with 5 ul of the ligated plasmid we acquired on 7/8/2021. We spinned the tube and discarded the supetanant to receive the largest quantity possible of competent cells. We incubated overnight.

MONDAY, 9/8/2021

1. Inspect liquid cultures for the bacteria we got from transformation on 6/8/2021
2. Inspect the transformation with the ligated plasmid (with T-miR-1246-2) from Saturday 7/8/2021
3. Prepare a new PCR procedure (2nd try) to amplify our g-Blocks

1.Inspect liquid cultures for the bacteria we got from transformation on 6/8/2021

Our liquid cultures did not grow overnight.

2.Inspect the transformation with the ligated plasmid (with T-miR-1246-2) from Saturday 7/8/2021

Unfortunately, our transformation was not successful.

3.Prepare a new PCR procedure (2nd try) to amplify our g-Blocks

Since we did not get any amplification product from our first PCR, we decided to test differend parameters of our reaction, such as DNA template concentration, annealing temperature and time and extension time. In addition, we wanted to test the Mg^{2+} concentration, since our g-blocks were resuspended in TE buffer, which contains EDTA. We will use the protocol PCR for Toehold Switches (Archived) for our second try using the following conditions using **T-miR-1246-3:**

Temperature and time for PCR diagnostic reaction…

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	30 cycles	98 °C	9 s
3		62 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

The reagents for the 3 different PCR reactions are shown in the table below. We, also, included a negative control reaction that does not contain any DNA template.

Reagents for diagnostic PCR reactions

	A	B	C	D	E	F
1	Reagent	Reaction 1	Reaction 2	Reaction 3	Negative Control	
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12,5 ul	12.5 ul	12.5 ul	1x
3	10 µM Forward Primer	1,25 ul	1,25 ul	1.25 ul	1.25 ul	0,5 µM
4	10 µM Reverse Primer	1,25 ul	1,25 ul	1.25 ul	1.25 ul	0,5 µM
5	Template DNA	8.19 ul (50 ng)	1.63 ul (10 ng)	1.63 ul (10 ng)	-	<1000 ng
6	Mg 2+	-	-	2 ul	-	
7	Nuclease-Free Water	1.81 ul	8.37 ul	6.37 ul	10 ul	
8	Total	25 ul	25 ul	25 ul	25 ul	

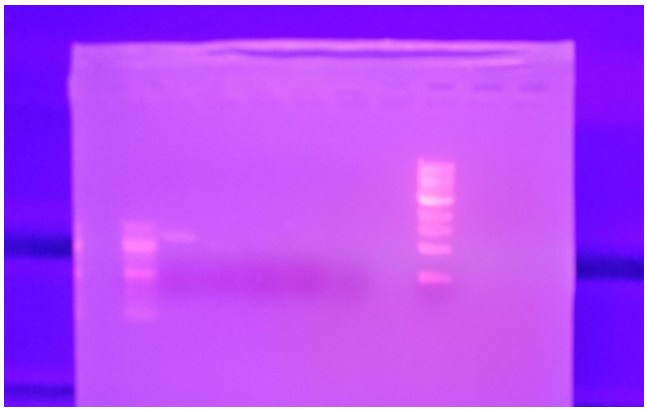
For the PCR reaction we use NEB's Q5 High-fidelity 2x Master Mix. A

In this reaction (reaction 3), we added only 10 ng of DNA template, since we intend to check if for the previous results are due to the lack of Mg^{2+} or due to the low quantity of the g-block (template).

We ran an 1% agarose gel with the products from each PCR reaction. We also, loaded a control sample, that contained 2 ul of the g-block and a 1 kb and a 100 bp ladder. We loaded the samples with the following order:
100 bp ladder - negative- 1 - 2 - 3 - T-1246-3 - 1 kb ladder

We got the following results after 20 minutes.

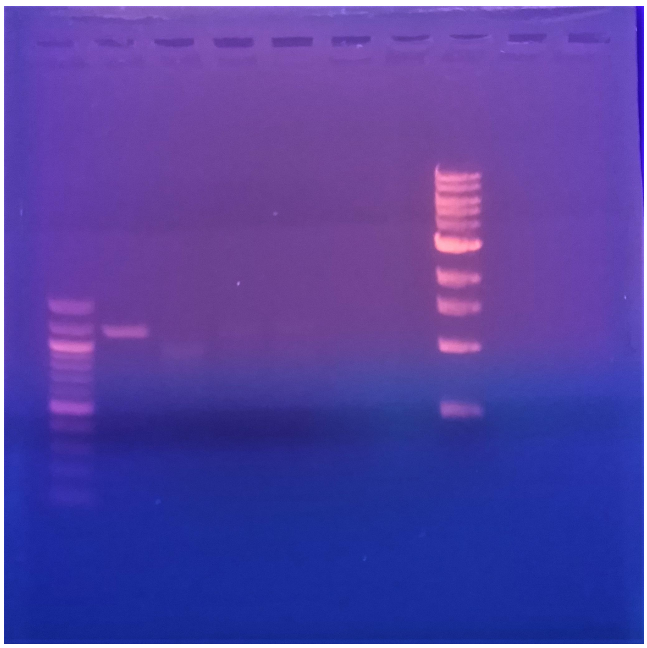
PCR results at 20 min-2nd try



Although it is difficult to distinct in the picture above, there is a band for every sample. However, there is a band for the negative control sample that we are not able to explain.

We got the following results after 35 minutes.

Results from PCR reactions-35 min-2nd try



Here we can dinstinct each band better. The most easily distinct band among sample 1,2 and 3 is the one from sample 1. This is in agreement with the quantities of the template DNA, since sample 1 contains 50 ng of the g-block. However, there is a band corresponding to 1200 bp for the negative control sample.

TUESDAY, 10/8/2021

Goals:

1. Repeat PCR reaction and isolate the product

1.Repeat PCR reaction and isolate the product

From the previous results we realized that we got an amplification product, but we could not tell if there is any DNA contamination or by-products. So, we decided to repeat the same procedure but with 40 cycles, in order to increase the yield and run a gel with TAE with ethidium bromide. This way we believe that we will be able to detect these products. If there is no contamination or by-product we will isolate the toehold with gel extraction.

The temperature and time for every step of PCR reaction is shown in the table below:

Temperature and time for PCR reaction-iGEMPCR2

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	40 cycles	98 °C	9 s
3		62 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

The reagents for each PCR reaction are mentioned in the following table. We also, prepared a negative control sample to check if there is a contamination, that explains the previous results.

Reagents for PCR reaction

	A	B	C
1	Reagent	T-1246-3	Negative Control
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12.5 ul
3	10 µM Forward Primer	1,25 ul	1.25 ul
4	10 µM Reverse Primer	1,25 ul	1.25 ul
5	Template DNA	8.19 ul (50 ng)	-
6	Mg 2+	1 ul	-
7	Nuclease-Free Water	0.81 ul	10 ul
8	Total	25 ul	25 ul

We prepared an 1% agarose gel and loaded the samples with the following order:
Ladder 1 kb - Negative - PCR product - Ladder 100 bp

We examined the gel after 40 minutes, but there was only an hardly distinct band corresponding to our sample.

WEDNESDAY, 11/8/2021

Goals:

1. PCR reaction with the right primers

1.PCR reaction with the right primers

During a brainstorming to find out what is wrong with our reaction, we realized that we were using wrong primers. We decided to repeat the PCR reaction with the correct primers and with 30 cycles.

The temperature and time for each step of the reaction are shown in the table below:

Temperature and time for PCR reaction- iGEMPCR2

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	30 cycles	98 °C	9 s
3		62 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

For the reaction we prepared a sample containing 50 ng of the template and a negative control that did not contain any DNA template. The quantity of each reagent is mentioned in the following table:

Reagents for PCR reaction

	A	B	C
1	Reagent	T-1246-3	Negative Control
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12.5 ul
3	Forward & Reverse primer Master Mix	1,25 ul	1.25 ul
4	Template DNA	8.19 ul (50 ng)	-
5	Nuclease-Free Water	3.06 ul	11.25 ul
6	Total	25 ul	25 ul

We ran the samples on an 1% agarose gel.

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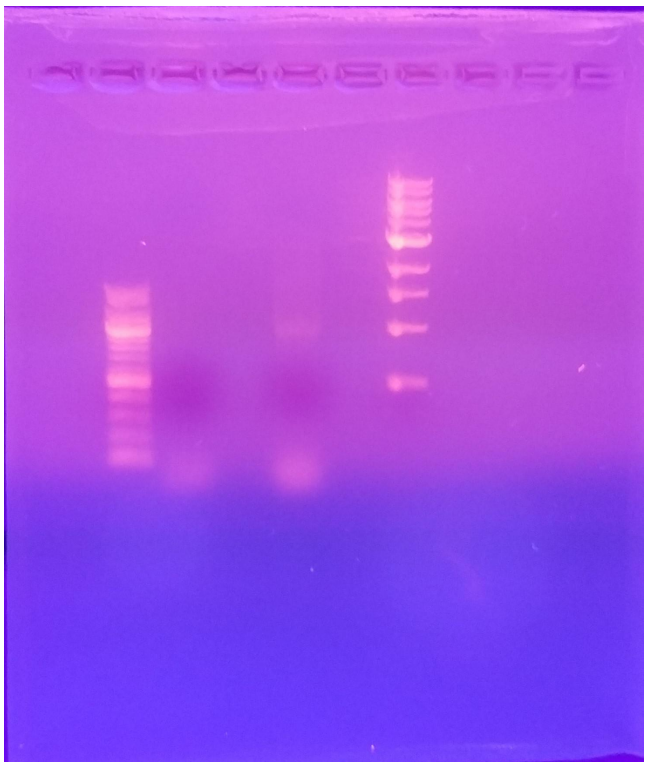


Unfortunately, we did not receive a good PCR band so we decided to troubleshoot.

THURSDAY, 12/8/2021

Goals:

1. Aliquot the primers for PCR
2. Use the NanoDrop 2000 to measure the concentration for Toehold 1246 - (4)
3. PCR reaction for diagnostic purposes

1.Aliquot the primers for PCR

The primers we have in stock have a concentration of 100 pmol/ ul. We want to have a working concentration of 10 pmol/ul. We prepared 5 aliquots for the Reverse Primer and 5 aliquots for the Forward Primer (named R- T primer and F- T primer respectively). Each aliquot has 1 ul of our primer and 9 ul of dH20 (from an aliquoted tube for PCR purposes) . We then created a working box named iGEM PCR, containing aliquots of the Q5 Master Mix (20 tubes of 125 ul each), dH20 aliquots for PCR and the aliquots we created today.

2.Use the NanoDrop 2000 to measure the concentration for Toehold 1246 - (4)

We used the g-Block for Toehold 1246 - (4) (1 ul) to measure its concentration on NanoDrop 2000. We still face the same issue, since the concentration we received is higher than the expected one, specifically, 21.2 ng/ul rather than 10 ng/ul. We brainstormed to troubleshoot the issue and a professor in the lab advised us to proceed with the concentration from the NanoDrop 2000.

3.PCR reaction for diagnostic purposes.

We tried to amplify T-1246-4 g-block using the settings shown in the following table.

Temperature and time for PCR reaction- iGEMPCR3

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	30 cycles	98 °C	9 s
3		71 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

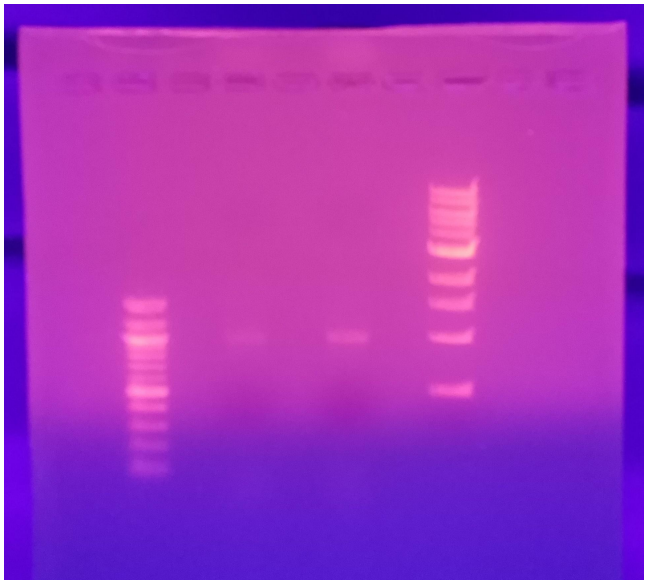
We prepared 2 samples: One with 2 mM Mg^{2+} -this is the standard concentration of the Master Mix we are using- and one with 3 mM Mg^{2+}. The reagents for each reaction are mentioned in the table below:

Reagents for PCR reaction to amplify T-1246- (4)

	A	B	C
1	Reagent	Reaction 1	Reaction 2
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12,5 ul
3	Forward primer	1,25 ul	1,25 ul
4	Reverse primer	1,25 ul	1,25 ul
5	Template DNA	2.35 ul (50 ng)	2.35 ul (50 ng)
6	Mg 2+	-	1 ul
7	Nuclease-Free Water	7.65 ul	6.65 ul
8	Total	25 ul	25 ul

We loaded the samples on an 1% agarose gel with the following order:
100bp Ladder - Reaction 1 - Reaction 2 - 1kb Ladder

Electrophoresis of PCR reaction- T-1246-4



This time we were able to detect and extract the band corresponding to Reaction 2. Tthis band is more clear than the one corresponding to Reaction 1 where the concentration of Mg2+ is 2 mM. We collected the amplified product with gel extraction. We calculated the quantity of the Gel Dissolving Buffer as follows:

Calculations for the Gel Extraction protocol

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	Reaction 1	0.870	1.139	0.269	1076

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of the amplified product - T-1246-4

	A	B	C
1		Concentration	Volume after elution
2	T-1246-4 PCR	7.7 ul	10 ul

Also, we have designed the following reactions as an alternative, in case the reaction described above had not worked. The temperature and time of each reaction step, as well as the quantity of each reagent is mentioned below:

Alternative PCR with lower annealing temperature…

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	30 cycles	98 °C	9 s
3		62 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

Reagents for the alternative diagnostic PCR reaction

	A	B	C	D	E
1	Reagent	Reaction 1	Reaction 2	Negative Control = = to be discussed	
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	8 ul	12.5 ul	
3	Forward primer	1,25 ul	0.8 ul	1.25 ul	
4	Reverse primer	1,25 ul	0.8 ul	1.25 ul	
5	Template DNA	- ul (50 ng)	- ul (32 ng)	-	
6	Nuclease-Free Water	- ul	- ul	10 ul	
7	Total	25 ul	16 ul	25 ul	

In conclusion, we decided to repeat the PCR reaction with the same settings but add more cycles of amplification (35 cycles) in order to increase the yield.

FRIDAY, 13/8/2021

Goals:

1. Measure the concentration of each g-block
2. Amplify T-1246-1 with PCR Reaction

1.Measure the concentration of each g-block (Konstantina)

We found out that the concentration of each samples varies a lot than the indicated by the manufacturer. In some samples the concentration was extremely low (~1 ng/ul)

2.Amplify T-1246-1 with PCR Reaction

We decided to amplify one of the g-blocks (T-1246-1) that corresponds to a toehold switch that seems to work. In order to amplify T-1246-1 we prepared 3 PCR reactions. The first one will be a diagnostic reaction and we will collect the product with gel extraction. The product from the other tworeactions will be isolated with a PCR cleanup kit, to improve the yield.

The settings for the PCR reaction are shown in the table below:

Settings for the PCR reaction-iGEMPCR3

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	35 cycles	98 °C	9 s
3		71 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

Reagents for the three PCR reactions- T-1246-1

	A	B	C	D
1	Reagent	Reaction 1	Reaction 2	Reaction 3
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12.5 ul	12.5 ul
3	Forward primer	1,25 ul	1.25 ul	1.25 ul
4	Reverse primer	1,25 ul	1.25 ul	1.25 ul
5	Template DNA	2.35 ul	2.35 ul	2.35 ul
6	Mg2+	1 ul	1 ul	1 ul
7	Nuclease-Free Water	6.65 ul	6.65 ul	6.65 ul
8	Total	25 ul	25 ul	25 ul

For the first reaction, we prepared an 1% agarose gel and loaded the following samples:
Ladder 1kb - PRC Reaction 1 - Ladder 100 bp

We examined the gel after 40 minutes but there was no visible band for reaction 1.

After that, we used the same gel to run the rest of the samples. We loaded an 1 kb ladder and the two reactions and examined the gel after 20 minutes. There were two blurb and difficult to distinct bands, one for each reaction. As a result we did not proceed with the gel extraction.

During brainstorming to find out what was wrong with this experiment, we found that the aliquots of the same g-block had different concentrations. Although, we could not determine why this happened we decided to measure the concentration of each aliquot before we use it, instead of estimating the volume needed based on previous measurments of a different aliquot.

MONDAY, 16/8/2021

Goals:

1. Amplify T-30e-5p-8 with PCR Reaction
2. Calculate the yield of our PCR

1.Amplify T-30e-5p-8 with PCR Reaction

We decided to amplify one of the g-blocks (T-30e-5p-8) that corresponds to a toehold switch that seems to work. In order to amplify T-30e-5p-8 we prepared 2 PCR reactions. The first one will be a diagnostic reaction and we will collect the product with gel extraction. The product from the other reaction will be isolated with a PCR cleanup kit, to improve the yield. We want to check the quantity of PCR product through gel extraction in order to continue with the PCR clean up.

Unfortunately , after we combined 3 aliquots into one tube (named 8 3in1) and done 12 up down pippete movements we measured only 4 ng/ul in the NanoDrop 2000. So we decided to check the concentration of an aliquot T-1246-2 which has a concentration of 12.4 ng/ul. We calculated accordingly for 2 reactions, as mentioned above.

The settings for the PCR reaction are shown in the table below:

iGEMPCR3

	A	B	C
1	Initial Denaturation	98 °C	30 s
2	35 cycles	98 °C	9 s
3		71 °C	30 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

Reagents

	A	B	C
1	Reagent	Reaction 1	Reaction 2
2	Q5 High-Fidelity 2X Master Mix	12,5 ul	12.5 ul
3	Forward primer	1,25 ul	1.25 ul
4	Reverse primer	1,25 ul	1.25 ul
5	Template DNA	4.03 ul	4.03 ul
6	Mg2+	1 ul	1 ul
7	Nuclease-Free Water	4.97 ul	4.97 ul
8	Total	25 ul	25 ul

We ran an 1% agarose gel according to protocol Electrophoresis and loaded the sample from reaction 1 and a 1 kb and a 100 bp ladder. We loaded the samples with the following order:

1 kb ladder - reaction 1 (T-1246-2) - 100 bp ladder

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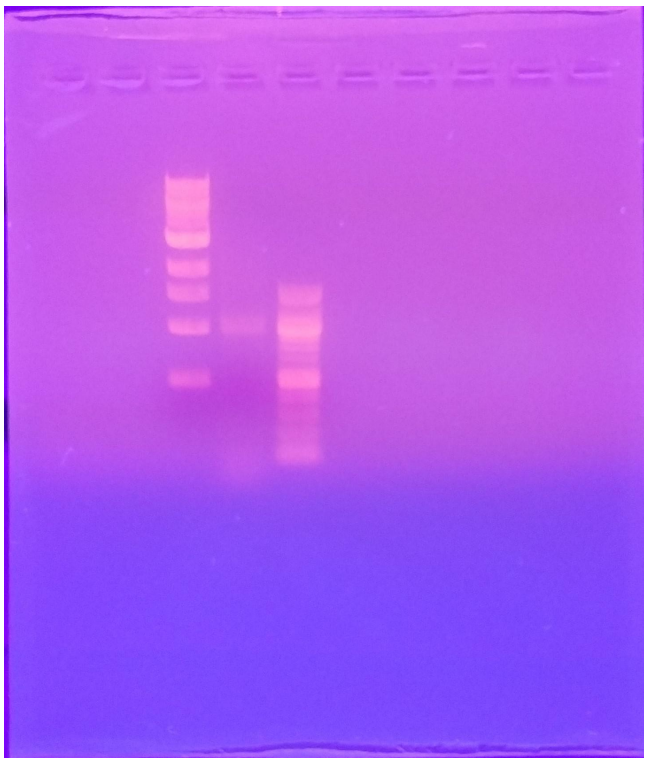


We had PCR product so we proceeded with the gel extraction. We then used the protocol Gel Extraction . The mass of the tube (2 ul eppendorf tube) was 0.892 g.

We decided since the PCR product didn't seem to be pure to use the tube from Reaction 2 as well for gel extraction and not PCR cleanup. We loaded the sample from reaction 2 and a 1 kb ladder. We loaded the samples with the following order:
1 kb ladder - reaction 2 (T-1246-2).

We added the gel from reaction 2 to the same 2 ul eppendorf tube as reaction 1. The total mass of the tube with the gel was 1.291 g so the mass of the gel was 0.399g (approximately 0.4 g). So, we used 1600 ul of dissolving buffer according to NEB's protocol. We also used 10 ul for the elution step.

2.Calculate the yield of our PCR

After the extraction was complete we used 1 ul of our DNA to calculate the yield through NanoDrop. We receiced the following results : 2.9 ng/ul .
According to the above, since we used 100 ng of DNA and received a concentration of 2.9 ng / ul and a total of 10 ul (1 out of 10 ul was used to measure the concentration through NanoDrop) we calculated that we have 29 ng of g- Block. We were not content with the outcome and decided to troubleshoot.

WEDNESDAY, 18/8/2021

Goals:

1. Try different PCR settings
2. Repeat the PCR reaction

1.Try different PCR settings

In order to improve the yield of the PCR reaction we decided to design and run PCR reactions with different settings. We prepared 3 reactions. The settings for the first one are mentioned in the table below indicated as Reaction 1. For the other two we used the same settings, indicated as Reaction 2,3 in the following table. This time we decided to increase the denaturation time, since we expect that the single stranded DNA forms a hairpin and that holds back the annealing of primers.

Different PCR reactions

	A	B	C	D	E
1		Reaction 1		Reaction 2, 3	
2	Initial Denaturation	98 °C	30 s	98 °C	90 s
3	35 cycles	98 °C	12 s	98 °C	12 s
4		71 °C	30 s	71 °C	30 s
5		72 °C	35 s	72 °C	35 s
6	Final Extension	72 °C	2 min	72 °C	2 min
7	Hold	4 - 10 °C		4 - 10 °C	

The reaction 1 and 2 contain the same quantities of each component but they are performed in different settings, while Reaction 2 and 3 are performed in the same settings but contain different quantity of DNA template. Reaction 2 contains 20 ng of DNA template, while Reaction 3 contain 10 ng of DNA template. We decided to scale down the reactions in order to use less quantity of the template. For these three reactions we used T-1246-3 and we measured its concentration using Nanodrop 2000. The concentration measured was 7.2 ng/ul. The reagents needed for each reaction are listed in the table below.

Reagents for PCR reactions

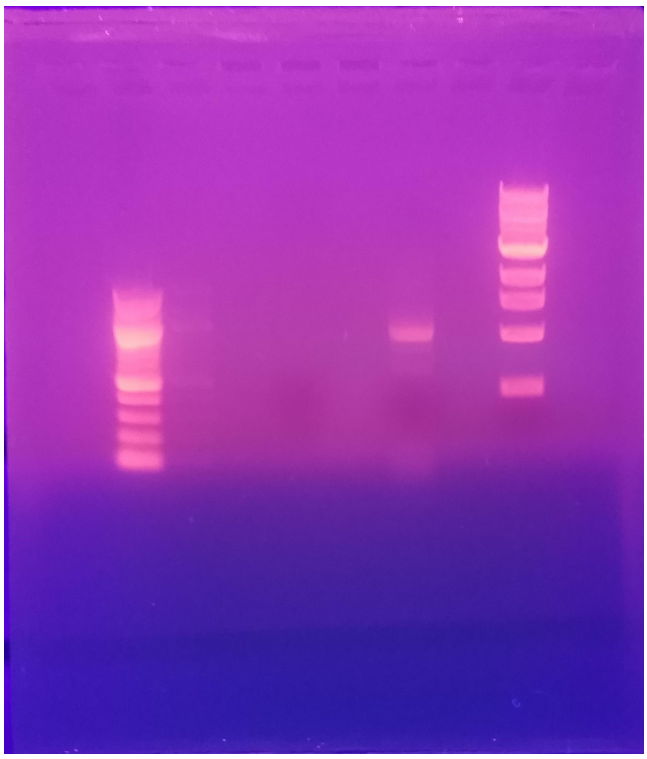
	A	B	C	D
1	Reagent	Reaction 1	Reaction 2	Reaction 3
2	Q5 High-Fidelity 2X Master Mix	5 ul	5 ul	5 ul
3	Forward primer	0.5 ul	0.5 ul	0.5 ul
4	Reverse primer	0.5 ul	0.5 ul	0.5 ul
5	Template DNA	2.77 ul (20 ng)	2.77 ul (20 ng)	1.38 ul (10 ng)
6	Mg2+	0.8 ul	0.8 ul	0.8 ul
7	Nuclease-Free Water	0.43 ul	0.43 ul	1.82 ul
8	Total	10 ul	10 ul	10 ul

For the reaction 1 we used the PCR thermocycler of our lab, while reactions 2 and 3 were performed in the thermocycler of a different lab.

We loaded the samples in an 1% agarose gel with the following order:
Ladder 100 bp - Reaction 3 - Reaction 1 - Reaction 2 - Ladder 1 kb

After 30 minutes we got the following results:

Electrophoresis results from PCR product



The tube from Reaction 3 must have broken because of the high temperatures and we did not expected to see any results. However, we can see that there are some bands similar to the ones of the ladder 100 bp. We supposed that somehow we contaminated this sample with the ladder. There were no bands corresponding to reaction 1, but there was a band corresponding to Reaction 2. The two reactions contain the same components, but they were performed in different settings.

Based on the differences between the two reactions, there could be two reasons for these different results: A longer time for the initial denaturation is needed or a quantity of water or other ingredient is evaporated when using the PCR thermocycler from our lab.

Finally, although it is difficult to distinct in the picture, the amplification product of reaction 2 was not completely pure. There were a band corresponding to primer dimers and an other one between 1kb and 500 bp.

We extracted the amplified T-1246-3 using the protocol Gel Extraction . We estimated the Dissolving Buffer volume as follows:

Calculations for dissolving buffer for gel extraction

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	Reaction 1	0.879 g	0.962 g	83 mg	332 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of T-1246-3 PCR product

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-1246-3 PCR	8.5 ng/ul	10 ul	PCR box, name: T-1246-3 PCR

THURSDAY, 19/8/2021

Goals:

1. Estimate the quantity of PCR product from Wednesday 18/8/2021
2. Repeat the PCR reaction with T-1246-4

1.Estimate the quantity of PCR product from Wednesday 18/8/2021

We used the manufacturer's instructions to calculate the quantity of Ladder 1 kb in the 1 kb lane. We will estimate the quantity of our product by comparing the fluorescence intensity of this lane and the lane corresponding to the amplified g-block. Since we loaded 2 ul of ladder, the total mass of loaded DNA is 1 ug, which is twice as the recommended quantity. When loading 1 ug of ladder (as recommended), the 1 kb lane contains 42 ng of DNA. So, in our electrophoresis this lane is approximately 84 ng. Our lane is more blurb and that means the quantity of the DNA is lower. However, we measured a concentration of 8.5 ng/ul which means that the quantity after the gel extraction was 85 ng.

image.png

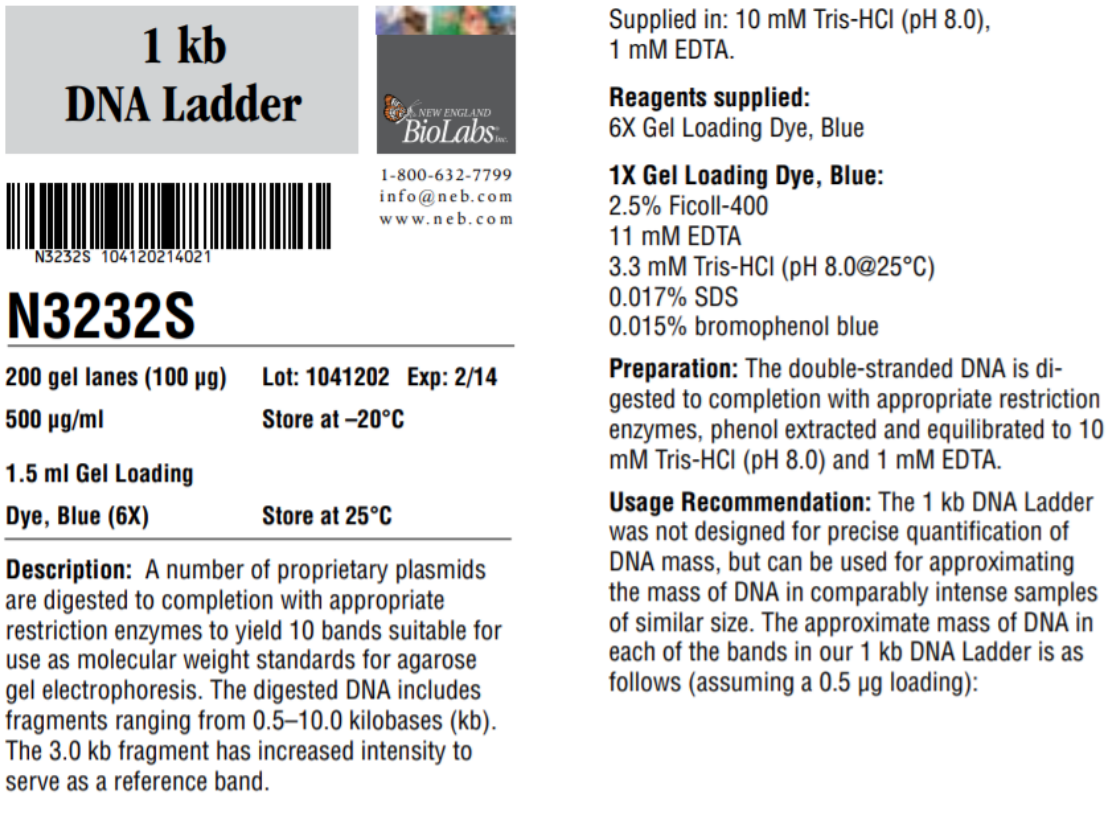


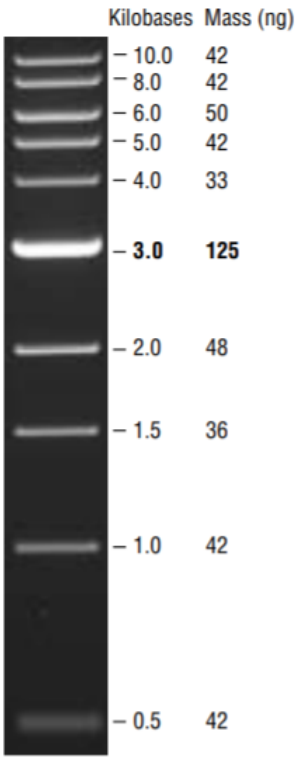
image.png

Fragment	Base Pairs	DNA Mass
1	10,002	42 ng
2	8,001	42 ng
3	6,001	50 ng
4	5,001	42 ng
5	4,001	33 ng
6	**3,001**	**125 ng**
7	2,000	48 ng
8	1,500	36 ng
9	1,000	42 ng
10a	517 >	42 ng
10b	500	

Notes: All fragments have a 4-base, 5´ overhangs that can be end labeled using T4 Polynucleotide Kinase (NEB #M0201) or filled-in using DNA Polymerase I, Klenow Fragment (NEB #M0210) (1). Use α-[^{32}P] dATP or α-[^{32}P] dTTP for the fill-in reaction.

1 kb DNA Ladder is stable for at least 3 months at 4°C.

For long term storage, store at –20°C. If samples need to be diluted, use TE or other buffer of minimal ionic strength. DNA may denature if diluted in dH_2O.



1 kb DNA Ladder visualized by ethidium bromide staining on a 0.8% TAE agarose gel. Mass values are for 0.5 µg/lane.

(see other side)

CERTIFICATE OF ANALYSIS

2.Repeat the PCR reaction with T-1246-4

We decided to repeat the same PCR reaction in our machine with a different g-block, to find out if this protocols works. The settings for the reaction are the same as the settings from Reaction 2 from 18/8/2021 and are shown in the table below:

Settings for PCR reaction with T-1246-4

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	90 s
3	35 cycles	98 °C	12 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

For this reaction we used the toehold T-1246-4. The concentration from this aliquot was measured before and the concentration indicated on the tube was 21,2 ng/ul. However, we measured it again and we found a 11,4 ng/ul concentration. We calculated the necessery volume for the reaction based on this concentration. The reagents are listed in the table below:

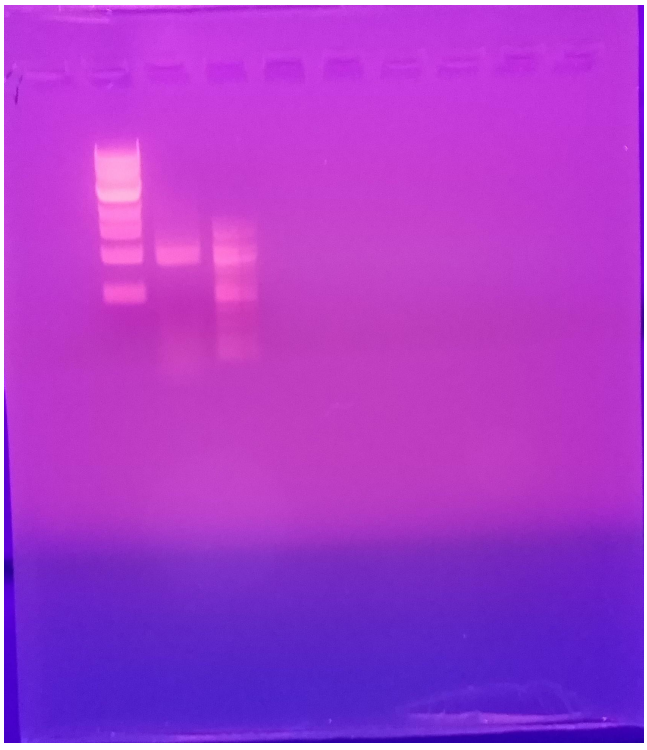
Reagents for PCR reaction with T-1246-4

	A	B
1	Reagent	T-1246-4
2	Q5 High-Fidelity 2X Master Mix	5 ul
3	Forward primer	0.5 ul
4	Reverse primer	0.5 ul
5	Template DNA	1.75 ul (20 ng)
6	Mg2+	0.8 ul
7	Nuclease-Free Water	1.45 ul
8	Total	10 ul

We loaded the samples in the following order in an 1% agarose gel:
Ladder 1 kb- PCR product- Ladder 100 bp
(This time we used 1 ul of each ladder)

We examined the gel after 30 minutes.

Electrophoresis results from PCR reaction (T-1246-4)



We ran the electrophoresis for ten more minutes so that the lanes of ladders become more clear and check if there is only one band corresponding to the PCR product. We examined the gel again and find out that there was indeed only one lane, so we proceeded with gel extraction. We calculated the volume of Dissolving Buffer needed for this protocol as follows:

Calculations for gel extraction protocol

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-1246-4	1.003 g	1.079 g	76 mg	304 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration after Gel Extraction

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-1246-4 PCR	8.8 ng/ul	10 ul	PCR box, name: T-1246-4 PCR

FRIDAY, 20/8/2021

Goals:

1. PCR reaction with increased denaturation time

1.PCR reaction with increased denaturation time

Since the result with the settings from previous days was reproducible, we decided to increase the denaturation time further and test if the yield will be improved. We used the same aliquot as on Thursday 19/8/2021 (T-1246-4, concenration: 11.4 ng/ul). The settings for the PCR reaction are described in the table below:

Settings for PCR reaction-incerased denaturation …

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	13 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

The reagents for the reaction are exactly the same as yesterday and are listed in the following table:

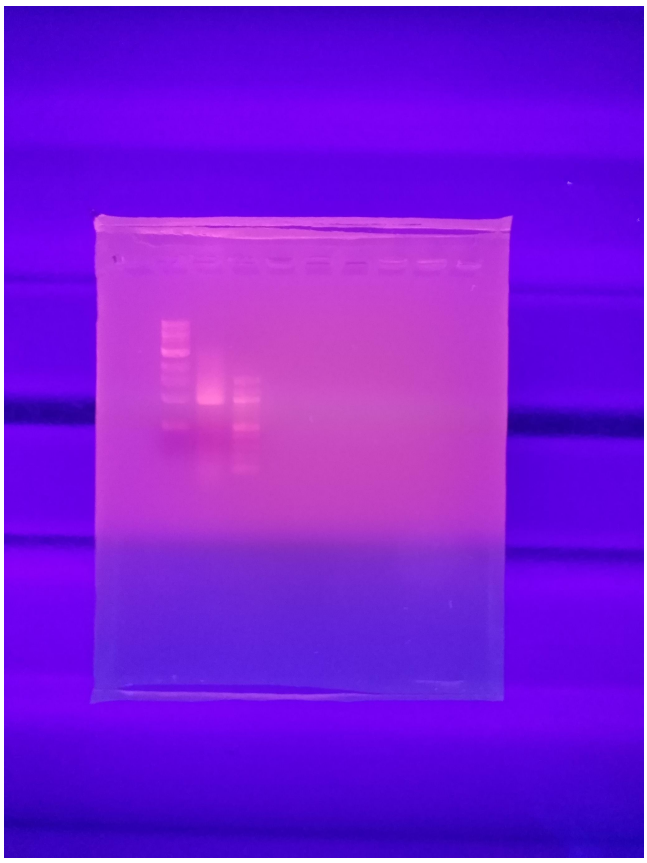
Reagents for PCR reaction with increased denatur…

	A	B
1	Reagent	T-1246-4
2	Q5 High-Fidelity 2X Master Mix	5 ul
3	Forward primer	0.5 ul
4	Reverse primer	0.5 ul
5	Template DNA	1.75 ul (20 ng)
6	Mg2+	0.8 ul
7	Nuclease-Free Water	1.45 ul
8	Total	10 ul

We loaded the samples on an 1% agarose in the following order:
Ladder 1kb - PCR product - Ladder 100 bp
We used 1 ul of each ladder and added 5 ul of loading dye.

We examined the gel after 30 minutes:

Electrophoresis results T-1246-4 increased denaturatio n



This was the most distinct band until now, however there was a smear, which means that the PCR product was not pure. However, we collected the DNA from the part of the band corresponding to 900 bp (the part next to the 1 kb lane from the ladders). For this procedure we used the protocol Gel Extraction. We calculated the volume of Dissolving Buffer as follows:

Calculations for Dissolving Buffer

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-1246-4	0.891 g	1.191 g	301 mg	1200 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of T-1246-4

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-1246-4 PCR	13.8 ng/ul	10 ul	PCR box, name: T-1246-4 PCR

SATURDAY, 21/8/2021

Goals:

1. Repeat the same PCR reaction with less template

1.Repeat the same PCR reaction with less template

We found three main ways to reduce the smear that we got from the previous experiment: Increase the denaturation time within the cycles (15-30 sec), reduce the amount of Mg^{2+} and reduce the amount of template. Sinze we could only test reactions in the same settings today, we decided to try lowering the initial amount of DNA template and lowering the concentration of Mg^{2+} . We used the same g-block as yesterday (T-1246-4, concentration: 11.4 ng/ul) and the same settings. Temperature and time for each PCR step are shown below:

Settigns for PCR reactions

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	13 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

The reagents we used for each PCR reaction are shown in the table below:

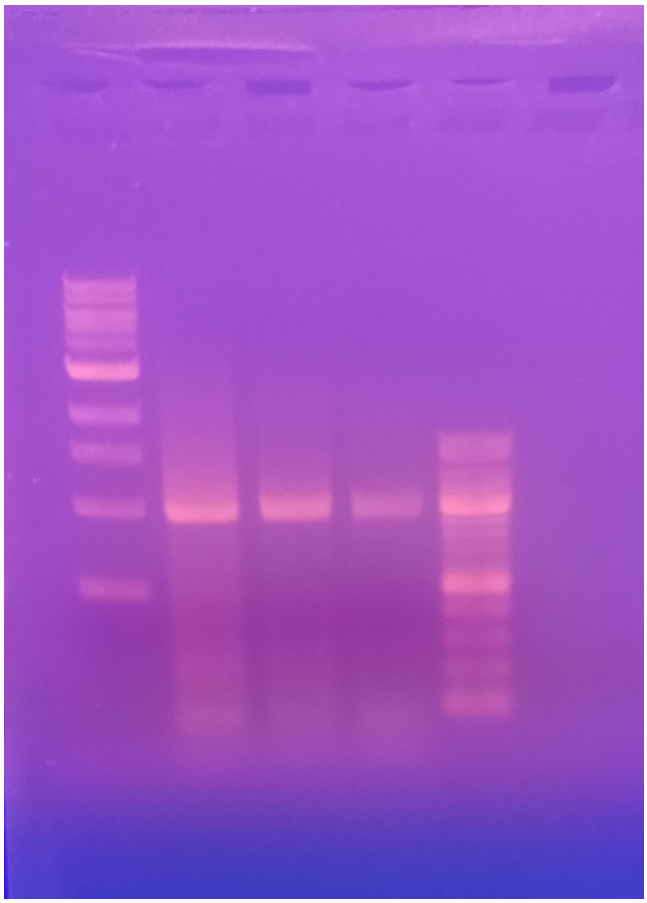
Reagents for PCR reactions

	A	B	C	D
1	Reagent	Reaction 1	Reaction 2	Reaction 3
2	Q5 High-Fidelity 2X Master Mix	5 ul	5 ul	5 ul
3	Forward primer	0.5 ul	0.5 ul	0.5 ul
4	Reverse primer	0.5 ul	0.5 ul	0.5 ul
5	Template DNA	0.87 ul (10 ng)	1.75 ul (20 ng)	1.75 ul (20 ng)
6	Mg2+	0.8 ul	0.6 ul	-
7	Nuclease-Free Water	2.33 ul	1.65 ul	2.25 ul
8	Total	10 ul	10 ul	10 ul

We prepared an 1% agarose gel and loaded the samples in the following order:
1 kb Ladder - Reaction 1 - Reaction 2 - Reaction 3 - 100 bp Ladder
We used 1 ul of each ladder and 5 ul of loading dye

We examined the gel after 40 minutes and we got the following results:

Electrophoresis results T-1246-4 21/8/2021



By compering the brightness of the three lanes, we understand that the yield is higher when the concentration of Mg^{2+} is 3 mM. The first reaction contained half the quantity of DNA template of reaction 2 and it was slightly brighter. However, the smearing of the reaction 1 is higher. We can repeat the reactions 1 and 2, increasing the denaturation time, to test which one is more appropriate.

After the examination of the gel we proceeded with gel extraction. We excised the three lanes and combined them in one tube. We calculated the quantity of the buffer as follows:

Calculations for gel extraction protocol

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-1246-4	0.888 g	1.187 g	299 mg ~ 300 mg	1200 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of T-1246-4 PCR product

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-1246-4 PCR	30.4 ng/ul	10 ul	T-1246-4 PCR 21/8/2021

MONDAY, 23/8/2021

Goals:

1. Repeat the previous reactions with increased denaturation time

As mentioned above, we decided to repeat the reaction 1 and 2 from the previous experiment increasing the denaturation time within the cycles. In conclusion, the settings of the reaction were the following:

Settings for PCR reaction-iGEMPCR3

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	15 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

The reagents we used for each PCR reaction are shown in the table below. This time we combined the remaining aliquot from the previous experiment with another one, since its quantity was too low and measured the concentration of this new sample was measured using nanodrop 2000. The concentration we measured was 11.7 ng/ul. We used this concentration to calculate the appropriate volume of the DNA template.

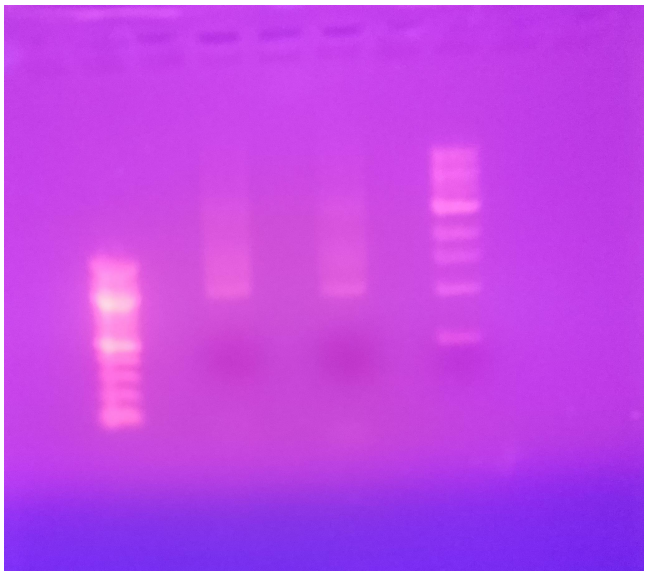
Reagents for the PCR reactions

	A	B	C
1	Reagent	Reaction 1	Reaction 2
2	Q5 High-Fidelity 2X Master Mix	5 ul	5 ul
3	Forward primer	0.5 ul	0.5 ul
4	Reverse primer	0.5 ul	0.5 ul
5	Template DNA	0.85 ul (10 ng)	1.70 ul (20 ng)
6	Mg2+	0.8 ul	0.6 ul
7	Nuclease-Free Water	2.35 ul	1.70 ul
8	Total	10 ul	10 ul

We loaded the samples on an 1% agarose gel in the following order:
Ladder 100 bp - Reaction 1 - Reaction 2 - Ladder 1 kb

We examined the gel after 30 minutes and got the following results:

Electrophoresis 23-8-2021.jpg



This time the bands were not bright enough to proceed with gel extraction. We decided to repeat the same procedure and troubleshoot to find out what was wrong.

THURSDAY, 26/8/2021

Goals:

1. Repeat the same PCR reaction as on 23/8/2021

1.Repeat the same PCR reaction as on 23/8/2021

We decided to repeat the same PCR reaction to confirm the results we got from the previous reaction. The settings for this reaction are shown in the table below:

PCR settings

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	15 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

The reagents we used for each PCR reaction are shown in the table below. We used an aliquot of T-1246-4 g-block and measured its concentration with Nanodrop 2000. The concentration we measured was 6.1 ng/ul. We used this concentration to calculate the appropriate volume of the DNA template.

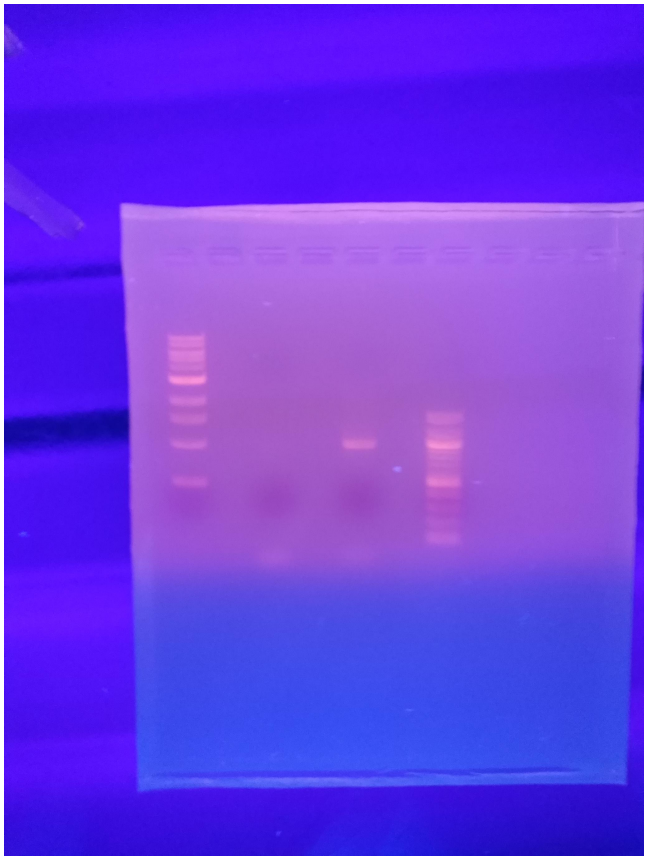
Reagents for PCR

	A	B	C
1	Reagent	Reaction 1	Reaction 2
2	Q5 High-Fidelity 2X Master Mix	5 ul	5 ul
3	Forward primer	0.5 ul	0.5 ul
4	Reverse primer	0.5 ul	0.5 ul
5	Template DNA	1.63 ul (10 ng)	3.27 ul (20 ng)
6	Mg2+	0.8 ul	0.6 ul
7	Nuclease-Free Water	1.57 ul	0.13 ul
8	Total	10 ul	10 ul

We ran an electrophoresis gel with the following samples:
Ladder 1 kb - Reaction 1 - Reaction 2 - Ladder 100 bp

After 30 minutes we examined the results:

Electrophoresis 26-8-2021.jpg



Although the band was not as bright as in other experiments there was no smear. We hypothesized that this was a result of the low quantity of template DNA. We decided to repeat these experiments.

However, we extracted the amplified product using the protocol Gel Extraction and we measured the concentration using Nanodrop 2000. The concentration was 7.1 ng/ul.

FRIDAY, 27/8/2021

Goal:

1. Repeat the same reaction and isolate DNA with gel extraction and PCR cleanup

1.Repeat the same reaction and isolate DNA with gel extraction and PCR cleanup

Settings for PCR reaction

	A	B	C
1		T-1246-4	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	15 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

The reagents we used for each PCR reaction are shown in the table below. We used the g-blockes T-30e-5p-4 and T-30e-5p-6 and measured their concentration with Nanodrop 2000. The concentration we measured was 6.1 ng/ul and 5.0 ng/ul, respectively. We used this concentration to calculate the appropriate volume of the DNA template for each set of reactions. We will run two reactions for the same g-block and isolate the DNA from the first one with gel extraction. If the product is pure, then we will isolate the product from the second reaction with PCR cleanup, hoping that the yield will increase.

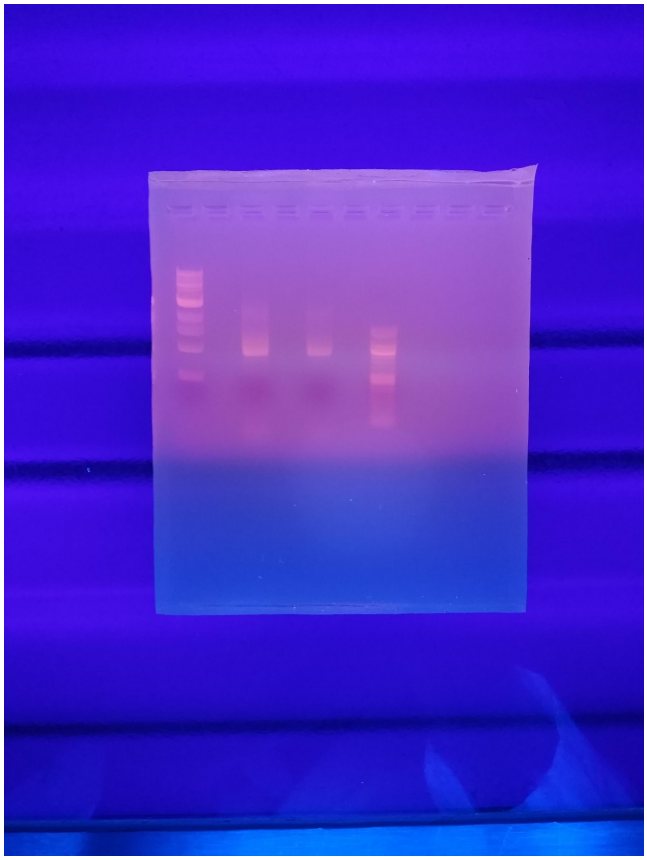
Reagents for PCR reaction

	A	B	C	D	E
1		T-30e-5p-4 (6.1 ng/ul)		T-30e-5p (5.0 ng/ul)	
2	Reagent	Reaction 1	Reaction 2	Reaction 3	Reaction 4
3	Q5 High-Fidelity 2X Master Mix	5 ul	5 ul	5 ul	5 ul
4	Forward primer	0.5 ul	0.5 ul	0.5 ul	0.5 ul
5	Reverse primer	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	Template DNA	2.45 ul (15 ng)	2.45 ul (15 ng)	3.0 ul (15 ng)	3.0 ul (15 ng)
7	Mg2+	0.6 ul	0.6 ul	0.6 ul	0.6 ul
8	Nuclease-Free Water	0.95 ul	0.95 ul	0.4 ul	0.4 ul
9	Total	10 ul	10 ul	10 ul	10 ul

We loaded the first samples (Reaction 1 and Reaction 3) on an 1% agarose gel in the following order:
Ladder 1kb - Reaction 1 - Reaction 3 - Ladder 100 bp

We examined the gel after 30 minutes:

Electrophoresis 27-8-2021 (1st).jpg



We proceeded with gel extraction and calculated the volume of the Dissolving Buffer as follows:

Calculations for gel extraction protocol

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-30e-5p-4 (reaction 1)	0.907 g	1.034 g	0.127 g	508 ul
3	T-30e-5p-6 (reaction 3)	0.908 g	1.088 g	0.180 g	720 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of the samples

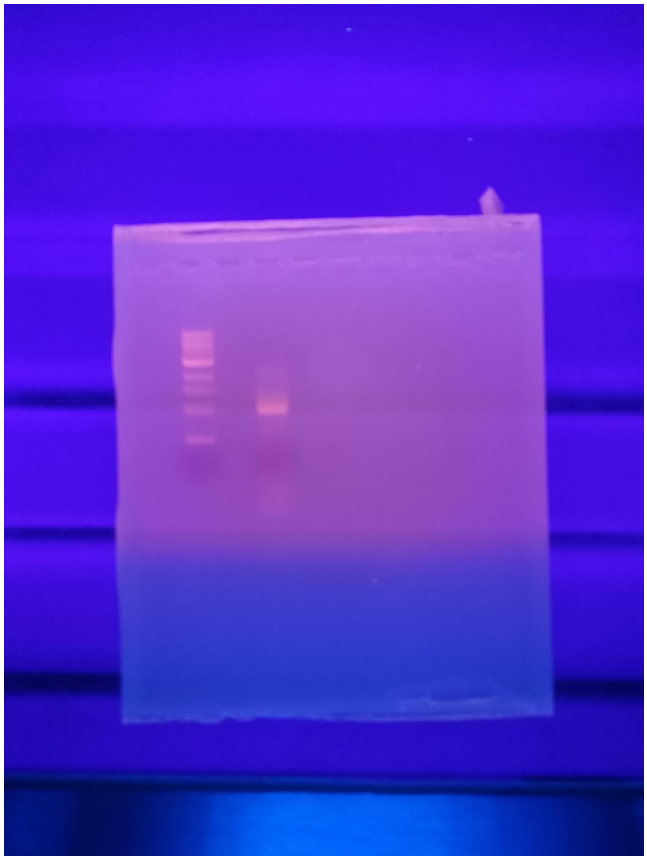
	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-30e-5p-4 (reaction 1)	7.7 ng/ul	10 ul	Gel extraction T-30e-5p (4)
3	T-30e-5p-6 (reaction 3)	7.3 ng/ul	10 ul	Gel extraction T-30e-5p (6)

In reaction 3 there was no smear, so we decided to use PCR cleanup to collect the amplification product from the Reaction 4 and compare the yield when using gel extraction and PCR cleanup. However, the product from reaction 1 was not pure, so we decided to collect the product from Reaction 2 with gel extraction.

The DNA concentration from Reaction 4 was measured using Nanodrop 2000. We found that the concentration was 15.3 ng/ul (Stored: PCR cleanup T-30e-5p-(4))

After that, we prepared another 1% agarose gel and loaded the sample 2 and a ladder in the following order:

Electrophoresis 27-8-2021 (2nd).jpg



We extracted this lane and calculated the quantity of dissolving buffer as follows:

Calculations for dissolving buffer quantity

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-30e-5p-4 (reaction 2)	0.907 g	1.010 g	93 mg	372 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of the sample T-30e-5p-4

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-30e-5p-4 (reaction 2)	8.4 ng/ul	10 ul	T-30e-5p-4 (PCR)

SATURDAY, 28/8/2021

Goals:

1. Try a completely different PCR protocol

1.Try a completely different PCR protocol

We decided to try a different PCR protocol suggested by a member from our lab, that seemed to work with similar sequences. The settings we used are shown in the table below:

PCR reaction settings-SCOANDRI

	A	B	C
1			
2	Initial Denaturation	95 °C	2 min
3	35 cycles	95 °C	1 min
4		57 °C	1 min
5		72 °C	1.30 min
6	Final Extension	72 °C	10 min
7	Hold	15 °C	

We prepared two reactions with the following reagents:

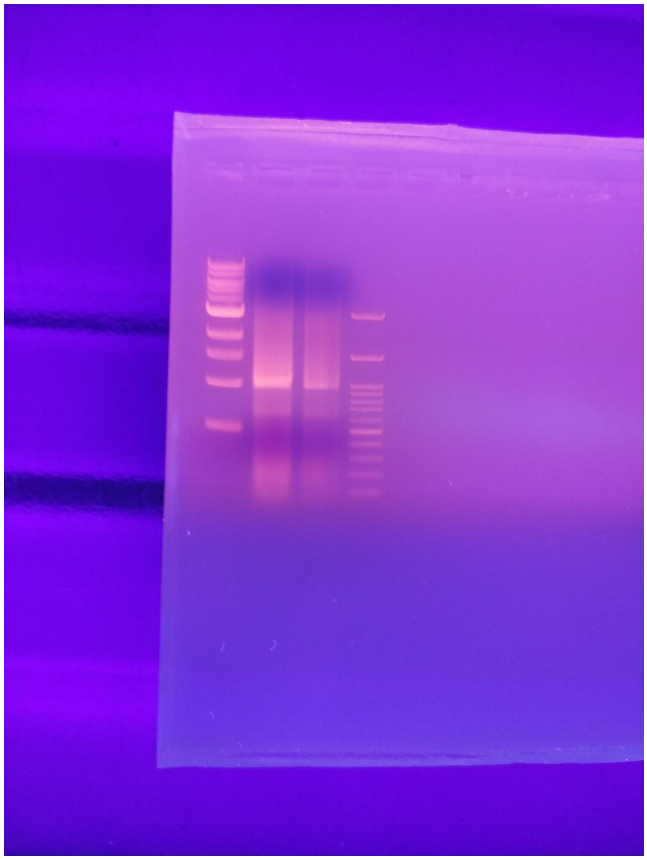
PCR reagents

	A	B	C	D	E
1		T-30e-5p-4 (6.1 ng/ul)			
2	Reagent	Reaction 1	Reaction 2		
3	Q5 High-Fidelity 2X Master Mix	12 ul	5 ul		
4	Forward primer	1.2 ul	0.5 ul		
5	Reverse primer	1.2 ul	0.5 ul		
6	Template DNA	5 ul	3.27 ul		
7	Mg2+	0.9 ul	0.6 ul		
8	Nuclease-Free Water	4.7 ul	0.13 ul		
9	Total	25 ul	10 ul		

We loaded the samples in following order:
Ladder 1 kb - Reaction 1 - Reaction 2 - Ladder 3 kb

We examined the gel after 30 minutes and we got the following results:

Electrophoresis 28-8-2021.jpg



Although the bands are brighter the product was not pure, so we decided that we can test some of the parameters of this protocol, but we can not use the exact same procedure.

MONDAY, 30/8/2021

Goals:

1. Prepare the PCR cloning protocol
2. Prepare ampicillin plates

1.Prepare the PCR cloning protocol

We prepared the protocol for the PCR Cloning and checked if all the necessary reagents were available. We realized that we didn't have ampicillin plates, so we decided to prepare 20 plates, so we can proceed with the cloning the next day.

2.Prepare ampicillin plates

We weighed 17.5 g of LB-agar premixed powder and diluted it in 500 ml of deionized water. We autoclaved the mixture and let it cool down. We added 500 ul of ampicillin and poured it in 18 plates. We let the plates solidify, sealed them with parafilm and stored them at 4^{o} C. Also, we incubated a plate at 37^{o} C overnight to check for bacterial contamination.

TUESDAY, 31/8/2021

Goals:

1. Clone T-30e-5p-5 with PCR cloning

1.Clone T-30e-5p-5 with PCR cloning

We decided to try cloning one of our toeholds with PCR cloning. For this procedure we used the protocol PCR cloning from the NEB's PCR cloning kit. First of all, we prepared two PCR reactions to amplify the toehold T-30e-5p-5. The settings for the reaction are described in the following table:

Settings for PCR for PCR cloning

	A	B	C
1		Reaction 1 & 2	
2	Initial Denaturation	98 °C	2 min
3	35 cycles	98 °C	15 s
4		71 °C	30 s
5		72 °C	35 s
6	Final Extension	72 °C	2 min
7	Hold	4 - 10 °C	

We prepared two 15 ul PCR reactions and combined them before gel extraction , beause we have noticed that this way we get a higher final concentration and a better quality of product. The reagents for these two reactions are shown in the table below:

Reagents for PCR for PCR cloning

	A	B	C	D	E
1		T-30e-5p-5			
2	Reagent	Reaction 1	Reaction 2		
3	Q5 High-Fidelity 2X Master Mix	7.5 ul	7.5 ul		
4	Forward primer	0.75 ul	0.75 ul		
5	Reverse primer	0.75 ul	0.75 ul		
6	Template DNA	3.48 ul (15 ng)	3.48 ul (15 ng)		
7	Mg2+	0.6 ul	0.6 ul		
8	Nuclease-Free Water	1.92 ul	1.92 ul		
9	Total	15 ul	15 ul		

After the end of the PCR reaction, we ran an electrophoresis gel and loaded the following samples:
1 kb ladder - Reaction 1 - Reaction 2 - 100bp ladder

After 30 minutes we examined the gel, excised the two lanes and combined them in a single tube. We calculated the dissolving buffer for the gel extraction protocol as follows:
We proceeded with gel extraction and calculated the volume of the Dissolving Buffer as follows:

Calculations for gel extraction

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-30e-5p-5 (reaction 1 & 2)	0.882 g	1.180 g	289 mg	1132 ul

We measured the concentrations of the PCR product using Nanodrop 2000. The concentration is measured is mentioned below:

Concentration of amplified product

	A	B	C	D
1		Concentration	Volume after elution	Stored
2	T-30e-5p-5 (reaction 1 & 2)	32.9 ng/ul	10 ul	Gel extraction T-30e-5p (5)

We used this concentration to calculate the quantity of the insert for the ligation protocol. Although, it was suggested by the manufacturer to use an 1:3 (vector: insert) ratio, since it we could not clone our g-blocks with BioBrick Assembly method despite the high ratios we used, we decided to use an 1:5 ratio. We calculated the mass of the insert needed for the reaction using NEBio Calculator, using the following parameters:

NEBio Calculator parameters and results

	A	B
1	Vector DNA length	2588 bp
2	Insert DNA length	900 bp
3	Vector DNA mass	25 ng
4	Insert DNA mass (result for 1:5)	43.47 ng

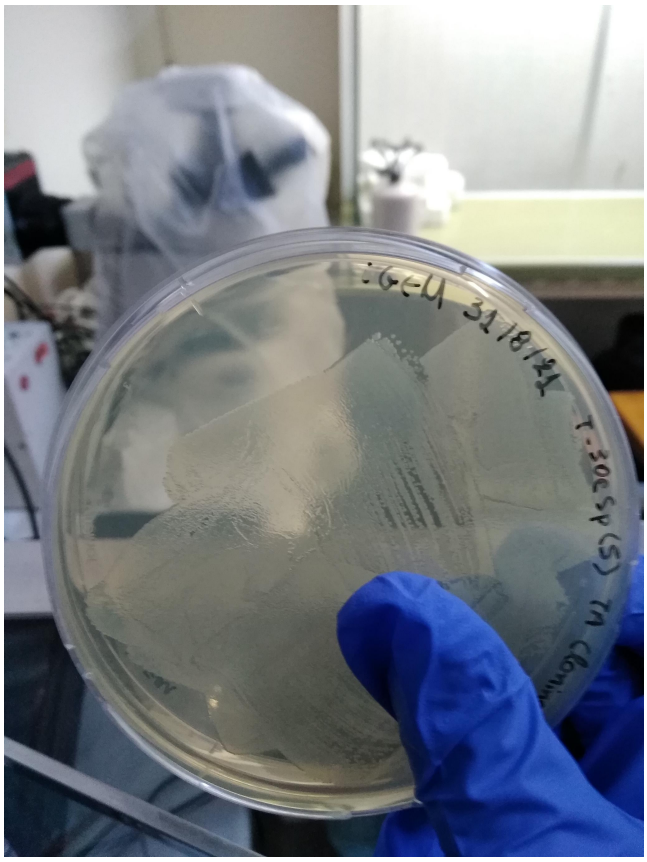
The reagents needed for the ligation reaction are shown in the table below:

Reagents for PCR Cloning Ligation

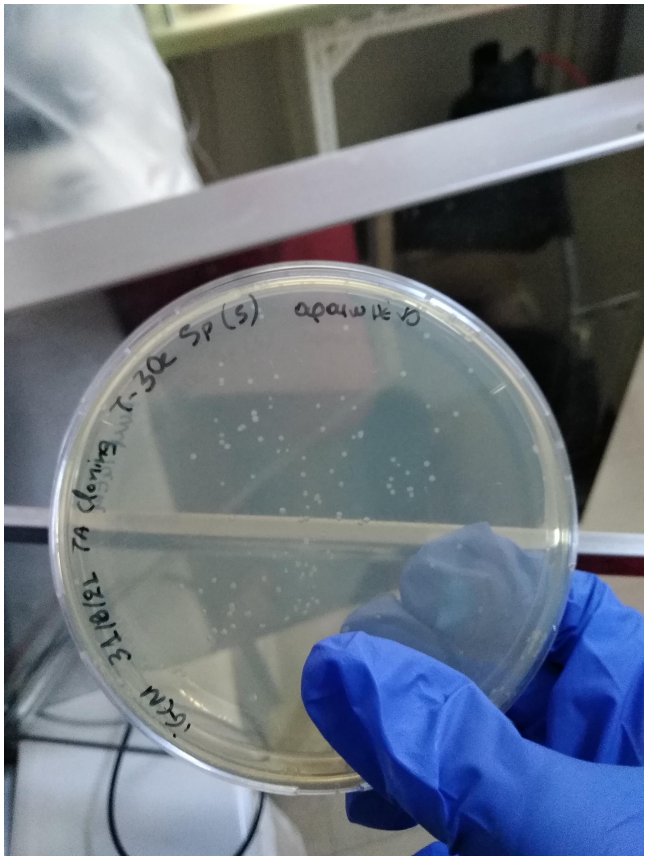
	A	B
1	Linearized pMiniT2.0 (25 ng/ul)	1 ul (25 ng)
2	Insert	1.32 ul (43.47 ng)
3	H2O	2.68 ul
4	Cloning Mix 1	4 ul
5	Cloning Mix 2	1 ul
6	Total	10 ul

We incubated the reaction at room temperature for 15 minutes and then for 2 minutes on ice. We used 2 ul of this ligation reaction to transform 50 ul of competent supplied with the kit. After the transformation we plated 50 ul on an ampicillin plate and 50 ul of an 1:10 dilution on another one. We incubated these plates overnight at 37^{o} C and we got the following results:

IMG_20210901_103815.jpg



IMG_20210901_103841.jpg



WEDNESDAY, 1/9/2021

Goals:

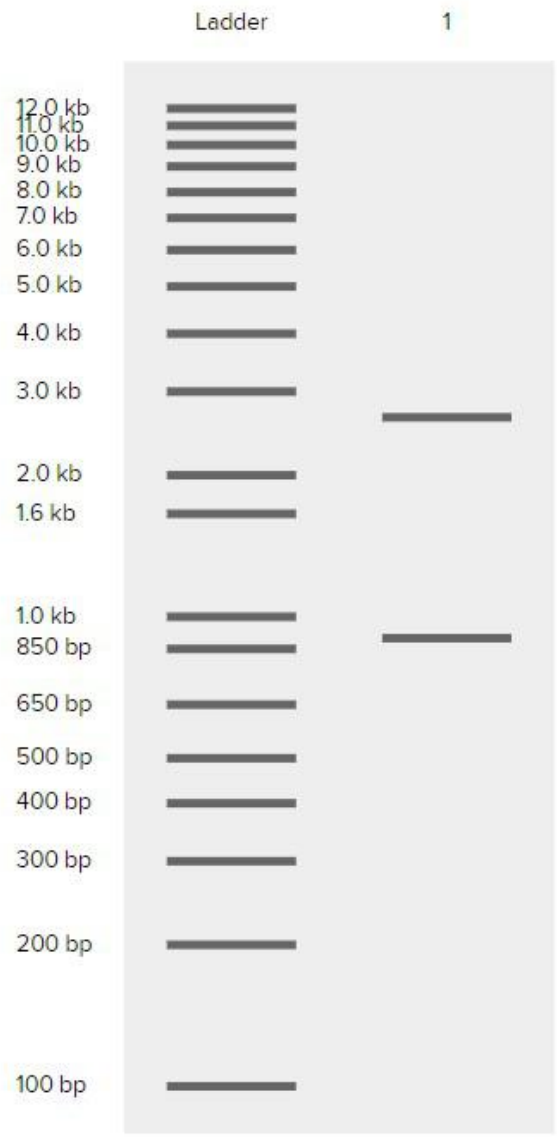
1. Design the experiments for Thursday
2. Prepare liquid cultures of the transformed cells

1.Design the experiments for Thursday

For tomorrow, we have to isolate the DNA plasmids from each liquid culture, in order to perform diagnostic digestions to test if the plasmids have the right insert. For the minipreps we need 1.5 ml of a liquid culture. We will prepare a 3 ml liquid culture of each colony.

To test if the plasmids contain the right insert we will need to perform diagnostic digestions. Our sequences contain the BioBrick Prefix and Suffix and we can digest each plasmid at these sites. Unfortunatelly, we can not use EcoRI and PstI because they cut in many sites in the plasmid. However, we can use XbaI and SpeI that only cuts within the Biobrick Preffix and Suffix. The results that we expect to get after the digestions are the following.

Simulation of diagnostic digestions with SpeI and XbaI.jpg



The plasmid map can be found here: pMiniT2.0-T-30e-5p-5

Since, we had too many colonies, we hypothesized that there is a lot of background and hence, we have to test many different colonies. We estimate that we have to test for the begining 20 colonies. That means that we should prepare 20 different liquid cultures. We have to store the plate in the fridge to pick new colonies, if we are not able to find plasmids containing the insert.

2.Prepare liquid cultures of the transformed cells

Since we had too many colonies, we decided to pick 20 of them to prepare 20 different liquid cultures. We used 3 ml of LB and added 3 ul of ampicillin. We inoculated each mixture with a single colony and let incubated them at 37^{O} C in a shaking incubator for 18 hours.

THURSDAY, 2/9/2021

Goals:

1. Plasmid isolation with minipreps

2. Diagnostic digestions

1.Plasmid isolation with minipreps

From the 20 liquid cultures we created, only 11 of them had grown. We isolated the plasmid from each bacterial culture using minipreps accordind to protocol Plasmid DNA Isolation with miniprep and we measured the final concentration of the product with Nanodrop 2000. The results are shown in the table below:

Nanodrop concentrations

	A	B
1	Sample	Concentration
2	3	17.7 ng/ul
3	5	9.8 ng/ul
4	7	13.5 ng/ul
5	9	7.8 ng/ul
6	10	16.4 ng/ul
7	12	10.0 ng/ul
8	13	10.7 ng/ul
9	14	20.1 ng/ul
10	15	12.5 ng/ul
11	16	47.9 ng/ul
12	17	8.0 ng/ul

2. Diagnostic digestions

We digested the plasmids we isolated with minipreps with the enzymes XbaI and SpeI. We used the reagents mentioned in the following table:

Reagents for diagnostic digestions

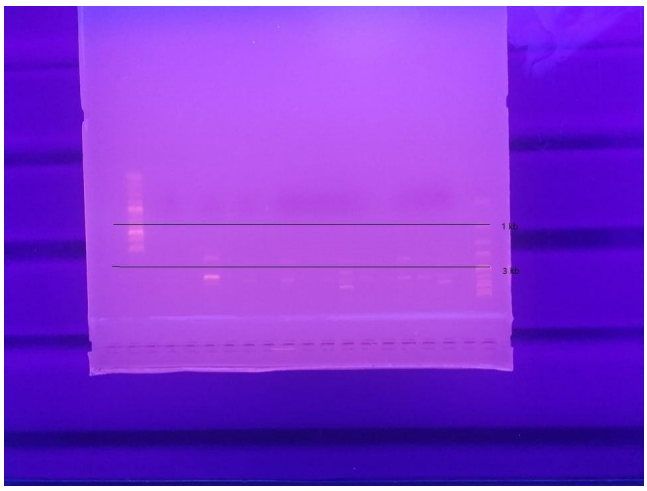
	A	B	C	D	E	F	G	H	I	J	K	L
1	Reagents	3	5	7	9	10	12	13	14	15	16	17
2	Nuclease free water	7.88 ul	-	3.49 ul	-	6.76 ul	-	-	9.57 ul	2 ul	16.79 ul	-
3	Buffer 2:1 (10X)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
4	DNA (250 ng)	14.12 ul	22 ul	18.51 ul	22 ul	15.24 ul	22 ul	22 ul	12.43 ul	20 ul	5.21 ul	22 ul
5	EcoRI-HF	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl
6	PstI	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl
7	Total	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

We incubated each sample at 37^{o} C for 15 minutes and heat inactivated the enzymes at 80^{o} C for 20 minutes. After the digestions we prepared an 1% agarose gel and loaded the samples in the following order:
1 kb - X - 3,5,7-X - 9,10,12,13,14 - X - 15-X - 16- X - 17 - X - 100 kb

X= empty well

We examined the gel after 45 minutes and we got the following results:

2-9-2021.jpg



The length of the vector is 2650 bp and the length of the insert is 893 bp. From this image, we believe that the plasmid was not digested. This could either happen because there is a different insert (not the desired one) or because the toxic minigen did not work.

FRIDAY, 3/9/2021

Goals:

1. Run an electrophoresis with the whole plasmids
2. Prepare new ampicillin plates
3. Prepare new liquid cultures from the transformation we performed on 30/8/2021

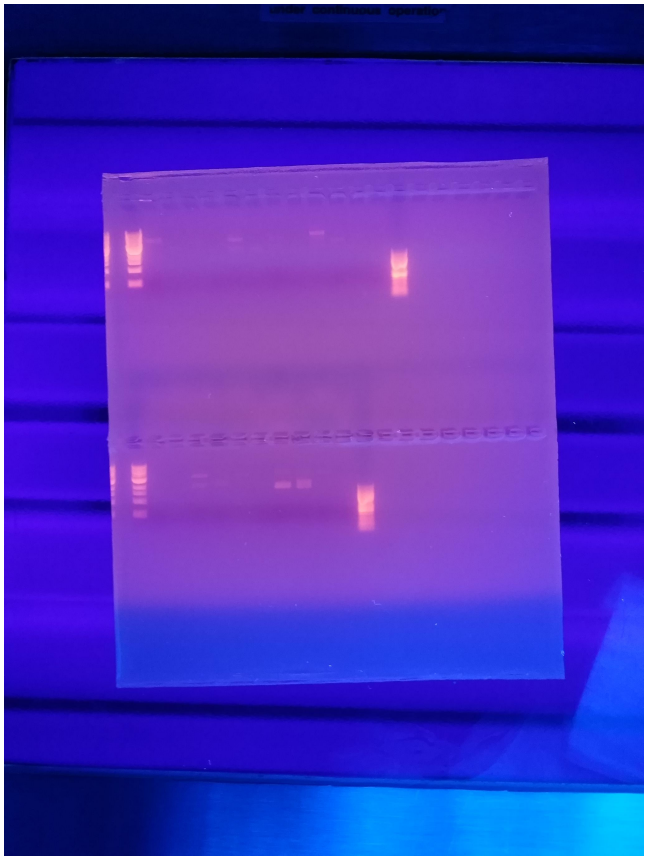
1.Run an elecectrophoresis with the whole plasmids

We decided to run an electrophoresis gel with all the samples. For each plasmid, we will run an uncut plasmid and one digested with XbaI only for 30 minutes at 37^{o} C. This way if there is an insert containing this sequence the plasmid will be linearized and we will be able to compare the sizes of the plasmids.

We prepared an 1% agarose gel and loaded the samples as follows:
Ladder 1kb - 3 - uncut 3 -5 - uncut 5 - 7 - uncut 7- 9 - uncut 9 - 10 - uncut 10 - Ladder 100 bp
Ladder 1kb - 12 - uncut 12 - 13 - uncut 13 - 14 - uncut 14 - 15 - uncut 15 - 16 - uncut 16 - 17 - uncut 17 - Ladder 100 bp

We examined the gel after 40 minutes and we got the following results:

3-9-2021.jpg



Although it is difficult to distinct in the picture, all the plasmids gave similar results: There was only one band corresponding to uncut plasmid at 2 kb and two bands for the digested plasmid. One of them at the same size as uncut plasmid and one between 3-4 kb.

2.Prepare new ampicillin plates

Since 9 of our liquid cultures did not grow, we supposed that something was wrong with the concentration of ampicillin used for the liquid cultures (higher than expected) or for the plates (lower than expected). For this reason we prepared new ampicillin solution 100 mg/ml, by dissolving 200 mg of ampicillin powder in 2 ml H2O and prepared new plates adding this solution.

We prepared 1 L of LB-agar, by dissolving 17.5 g pre-mixed powder in 500 ml water twice and we autoclaved the mixtures. After we cooled them we added 500 ul of ampicillin in each one and poured the mixture into plates. We prepared 31 plates and we left them dry at 37^{o} C overnight. We will seal them with parafilm and store them at 4^{o} C.

3.Prepare new liquid cultures from the transformation we performed on 30/8/2021

We picked twenty colonies from the plate we prepared on Monday 30/8/2021 and inoculated 20 liquid cultures. We incubated these cultures for 18 hours at 37o C in a shaking incubator.

SATURDAY, 4/9/2021

Goals:

1. Isolate DNA from the liquid cultures
2. Diagnostic digestions

1.Isolate DNA from the liquid cultures

Only 13 of our liquid cultures have grown. We collected the plasmids from these 13 cultures with minipreps ad measured the concentration of the plasmid using Nanodrop 2000. The results we got can be found below:

Plasmid concentration

	A	B
1	Sample	Concentration
2	22	7.8 ng/ul
3	27	6.1 ng/ul
4	29	69.6 ng/ul
5	30	37.6 ng/ul
6	31	9.9 ng/ul
7	32	16.8 ng/ul
8	33	38.2 ng/ul
9	34	108.1 ng/ul
10	35	27.5 ng/ul
11	36	32.6 ng/ul
12	37	61.5 ng/ul
13	38	52.1 ng/ul
14	40	6.4 ng/ul

2.Diagnostic digestions

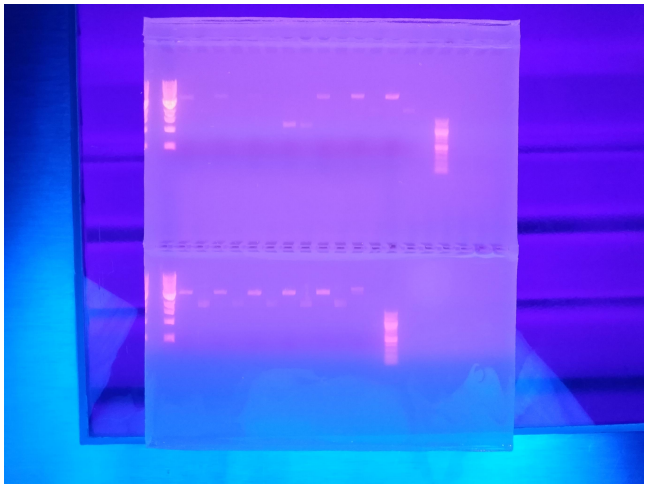
We ran a diagnostic digestion for each plasmid with XbaI only, so that we can estimate the size of the linearized plasmid. The reagents for each reaction can be found below:

Reagents for diagnostic digestions

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Reagents	22	27	29	30	31	32	33	34	35	36	37	38	40
2	Nuclease free water	0.75 ul	0.75 ul	8.96 ul	7.43 ul	0.75 ul	3.31 ul	7.48 ul	9.6 ul	6.21 ul	6.92 ul	8.72 ul	8.36 ul	0.75 ul
3	Buffer rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	DNA (250 ng)	10 ul	10 ul	1.79 ul	3.32 ul	10 ul	7.44 ul	3.27 ul	1.15 ul	4.54 ul	3.83 ul	2.03 ul	2.39 ul	10 ul
5	XbaI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	Total	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul

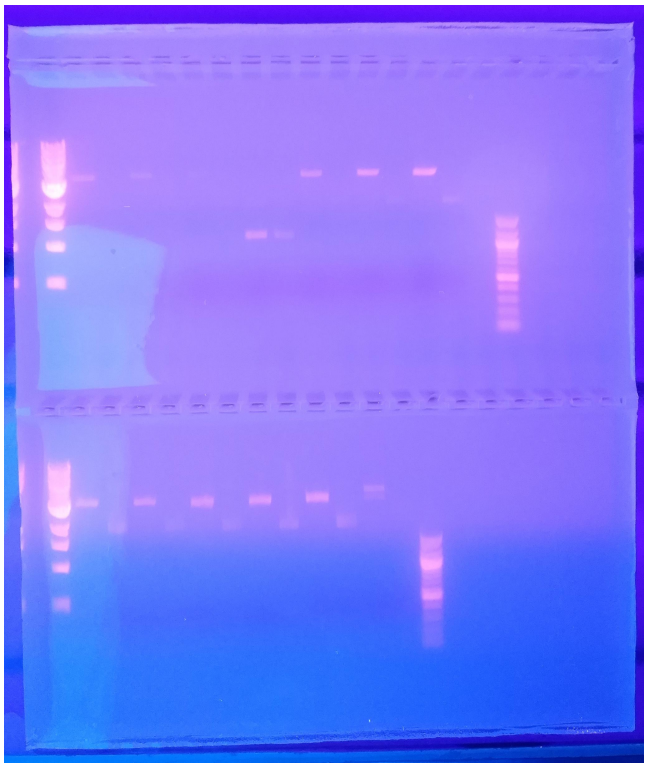
We prepared an 1% agarose gel and loaded the samples in the following order:
Ladder 1 kb - 22 - 22' - 27 - 27' - 29 - 29' - 30 - 30' - 31 - 31' - 32 - 32' - 33 - 33' - Ladder 100 bp
Ladder 1 kb - 34 - 34' - 35 - 35' - 36 - 36' - 37 - 37' - 38 - 38' - 40 - 40' - Ladder 100 bp
x' = uncut plasmid
We examined the gel after 40 minutes and we got the following results:

4-9-2021.jpg



To make it easier to distinct the bands and better estimate the size of each band we ran the gel for 10 more minutes and we got the following image:

4-9-2021 (2nd).jpg



We could see that all the digested plasmids except for 30 and 30' and 29 and 29' gave a band approximately at 3500 bp (between 3-4 kb). All the samples that contained the uncut plasmid (except the samples mentioned above) gave a band at 2 kb. From these images we concluded that probably the plasmids contain the desired insert. To make sure, we will perform again a double diagnostic digestion with the enzymes XbaI and SpeI that cut at the edges of the insert, so we expect to see a band approximately at 1 kb. In order to insure that the whole quantity of the plasmid will be digested and that we will not get bands corresponding to the uncut plasmid we will perform the digestion with the first enzyme (for 30 minutes) and then the second one (for 30 minutes more).

MONDAY, 6/9/2021

Goals:

1. Repeat diagnostic digestions with two enzymes
2. Repeat PCR cloning with new plates
3. PCR for T-1246-2

1.Repeat diagnostic digestions with two enzymes

Since we got some promising results on Saturday 4/9/2021 we decided to repeat the diagnostic digestions, using both enzymes -SpeI and XbaI and see if we will get a band approximately at 1 kb. The reagents used for these reactions are shown in the table below:

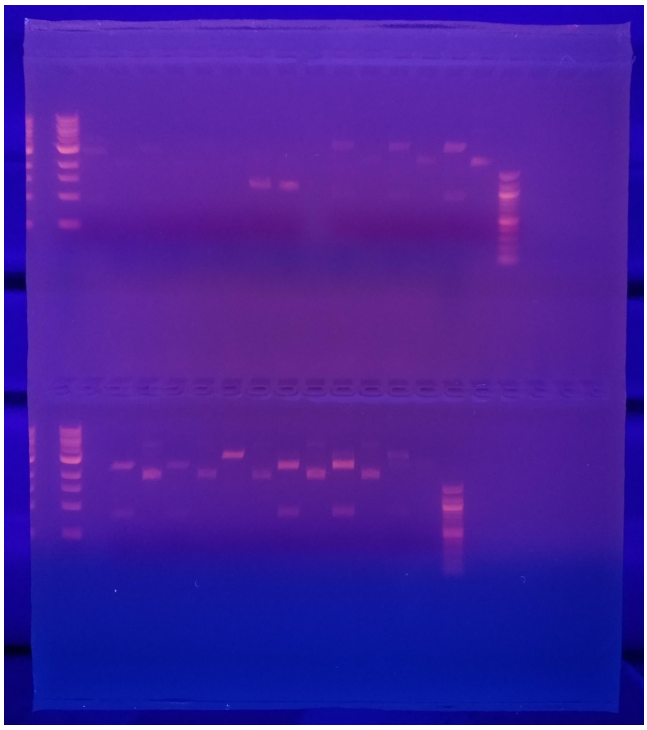
Reagents for double digestions

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Reagent	22	27	29	30	31	32	33	34	35	36	37	38	40
2	H2O	0.75 ul	0.75 ul	8.96 ul	7.43 ul	0.75 ul	3.31 ul	7.48 ul	9.6 ul	6.21 ul	6.92 ul	8.72 ul	8.36 ul	0.75 ul
3	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	DNA	10 ul	10 ul	1.79ul	3.32 ul	10 ul	7.44 ul	3.27 ul	1.15 ul	4.54 ul	3.83 ul	2.03 ul	2.39 ul	10 ul
5	XbaI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul

We prepared an 1% agarose gel and loaded the samples in the following order:
Ladder 1 kb - 22 - 22' - 27 - 27' - 29 - 29' - 30 - 30' - 31 - 31' - 32 - 32' - 33 - 33' - Ladder 100 bp
Ladder 1 kb - 34 - 34' - 35 - 35' - 36 - 36' - 37 - 37' - 38 - 38' - 40 - 40' - Ladder 100 bp

x' = uncut plasmid
We examined the gel after 50 minutes and we got the following results:

6-9-2021.jpg



We can clearly see that for many samples there is a band above 1000 bp that corresponds to the 900 bp insert and a band above 3000 bp that corresponds to the 2.5 kb linearized vector. In this context we believe that the clones 34, 31, 32, 33, 34, 35, 37 and 38 contains the insert and these cultures can be stored as a glycerol stock. We can also, prepare the plasmids for sequencing analysis.

2.Repeat PCR cloning with new plates

We prepared a PCR reaction for 2 of our toehold switches, which we want to amplify them for the need of quantification experiments with the following settings :

IGEMPCR 3

	A	B	C
1	Initial Denaturation	98 °C	2 min
2	35 cycles	98 °C	15 s
3		71 °C	35 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

We prepared the reactions as follows:

Reagents for PCR T-30e-5p-8 and T-1246-2

	A	B	C
1		T-30e-5p-8	T-1246-1
2	Reagent	Reaction 1	Reaction 2
3	Q5 High-Fidelity 2X Master Mix	7.5 ul	7.5 ul
4	Forward primer	0.75 ul	0.75 ul
5	Reverse primer	0.75 ul	0.75 ul
6	Template DNA	2.7 ul (15 ng)	3.94 ul (15 ng)
7	Mg2+	0.6 ul	0.6 ul
8	Nuclease-Free Water	2.7 ul	1.46 ul
9	Total	15 ul	15 ul

3.PCR Cloning for T-1246-2

We prepared 2 PCR reactions for T-1246-2 :

iGEM PCR3 Settings

	A	B	C
1	Initial Denaturation	98 °C	2 min
2	35 cycles	98 °C	15 s
3		71 °C	35 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

And we prepared the reaction as follows:

Reagents for PCR T-1246-2

	A	B	C
1	Reagent	Reaction 1	Reaction 2
2	Q5 High-Fidelity 2X Master Mix	7.5 ul	7.5 ul
3	Forward primer	0.75 ul	0.75 ul
4	Reverse primer	0.75 ul	0.75 ul
5	Template DNA	1.04 ul (15 ng)	1.04 ul (15 ng)
6	Mg2+	0.6 ul	0.6 ul
7	Nuclease-Free Water	4.36 ul	4.36 ul
8	Total	15 ul	15 ul

We then prepared a 1% electrophoresis gel to load our samples and prepared a gel extraction as follows using the protocol Gel Extraction.

Reagents for Gel Extraction

	A	B	C	D	E
1		Empty tube weight	Total weight	Gel weight	Buffer volume
2	Reaction 1+2	0.893 g	1.140 g	247 mg	988 ul

And then we measured the concentation of the product to 5.3ng/ul so we prepared the PCR cloning protocol as follows :

Ligation for T-1246-2

	A	B
1		T-1246-2
2	Linearized pMiniT2.0 (25 ng/ul)	1 ul (25 ng)
3	Insert Ratio 1:3	3.32 ul
4	H2O	0.68 ul
5	Cloning Mix 1	4 ul
6	Cloning Mix 2	1 ul
7	Total	10 ul

We then transformed 2ul of the ligated mixture into 50 ul competent cells and we streaked 2 plates, one with 50 ul of competent of the mixture and one diluted 1:10.

TUESDAY, 7/9/2021

Goals:

1. Prepare 50% glycerol solution
2. Prepare liquid cultures from the plates streaked on 6/9/2021
3. Cloning for the 8 samples of toehold switches

1.Prepare 50% glycerol solution

Since the cloning procedure from yesterday was succesful, we will have to prepare many glycerol stocks, so we prepared 80 ml of 50% glycerol by mixing 40 ml of 99% glycerol with 40 ml Milli Q water. We autoclaved the solution.

2.Prepare liquid cultures from the plates streaked on 6/9/2021

We examined the plates striked on Monday 6/9/2021 and we got some colonies. (Less than with the first plates, so we concluded that there was something wrong with the ampicillin concentration.) We will prepare 20 liquid cultures picking single colonies from the plates and inoculating 3.5 ml LB medium. We will incubate the cultures at 37^{o} C overnight in a shaking incubator.

3.Cloning for the 8 samples of toehold switches

We prepared a PCR reaction for 8 of our toehold switches with the following settings :

IGEMPCR 3 Settings

	A	B	C
1	Initial Denaturation	98 °C	2 min
2	35 cycles	98 °C	20 s
3		71 °C	35 s
4		72 °C	35 s
5	Final Extension	72 °C	2 min
6	Hold	4 - 10 °C	

We prepared the reactions as follows:

Reagents for PCR Cloning

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1		T-143-3p-1		T-143-3p-2		T-143-3p-3		T-143-3p-4		T-30e-5p-1		T-30e-5p-2	T-30e-5p-3		T-30e-5p-4		
2	Reagent	Reaction 1	Reaction 2	Reaction 1	Reaction 2	Reaction 1	Reaction 2	Reaction 1	Reaction 2	Reaction 1	Reaction 2	Reaction 1	Reaction 1	Reaction 2	Reaction 1		
3	Q5 High-Fidelity 2X Master Mix	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	10 ul		
4	Forward primer	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	1 ul		
5	Reverse primer	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	1 ul		
6	Template DNA	3.65 ul (15 ng)	3.65 ul (15 ng)	4.68 ul (15 ng)	4.68 ul (15 ng)	2.17 ul (15 ng)	2.17 ul (15 ng)	2 ul (15 ng)	2 ul (15 ng)	1.51 ul (15 ng)	1.51 ul (15 ng)	8.3 ul (15 ng)	0.61 ul (15 ng)	0.61 ul (15 ng)	6.25 ul (15 ng)		
7	Mg2+	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul		
8	Nuclease-Free Water	1.75 ul	1.75 ul	0.72 ul	0.72 ul	3.23 ul	3.23 ul	3.4 ul	3.4 ul	3.89 ul	3.89 ul	1.1 ul	4.8 ul	4.8 ul	1.15 ul		
9	Total	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul	25 ul	15 ul	15 ul	20 ul		

We then prepared a 1% electrophoresis gel and loaded our samples in the following way :

Ladder 1 kb - 1 - 1' - 2 - 2' - 3 - 3' - 6 - 6'- Ladder 100 bp
Ladder 1 kb - 4 - 4' - 5 - 5' - 7 - 7' - 8 - 8'- Ladder 100 bp
x' = uncut plasmid

And after 35 minutes we proceeded to extract the DNA using the Gel Extraction protocol.

Gel Extraction Quantities

	A	B	C	D	E
1	Sample	Empty tube weight	Total weight	Gel weight	Buffer volume
2	T-143-3p-1 (reaction 1 & 2)	0.876 g	1.121 g	245 mg	980 ul
3	T-143-3p-2 (reaction 1 & 2)	0.904 g	1.100 g	196 mg	784 ul
4	T-143-3p-3 (reaction 1 & 2)	0.909 g	1.214 g	305 mg	1220 ul
5	T-143-3p-4 (reaction 1 & 2)	0.904 g	1.132 g	228 mg	912 ul
6	T-30e-5p-1 (reaction 1 & 2)	0.890 g	1.097 g	207 mg	828 ul
7	T-30e-5p-2 (reaction 1)	0.910 g	1.100 g	190 mg	760 ul
8	T-30e-5p-3 (reaction 1 & 2)	0.908 g	1.108 g	200 mg	800 ul
9	T-30e-5p-4 (reaction 1)	0.874 g	0.963 g	89 mg	356 ul

We then measured the concentration of each tube on NanoDrop 2000 and got the following results :

Concentrations using Nanodrop

	A	B
1	Sample	Concentration
2	T-143-3p 1	19.7 ng/ul
3	T-143-3p 2	11.2 ng/ul
4	T-143-3p 3	5.8 ng/ul
5	T-143-3p 4	16 ng/ul
6	T-30-5p 1	22.5 ng/ul
7	T-30-5p 2	11 ng/ul
8	T-30-5p 3	19.7 ng/ul
9	T-30-5p 4	14.6 ng/ul

Unfortunately, our lab had to close down around 21.00 so we were forced to end our experiment at this stage and store our plasmids at -20°C.

WEDNESDAY, 8/9/2021

Goals:

1. Isolate plasmids from liquid cultures prepared on 7/9/2021 with minipreps
2. Diagnostic digestions with XbaI and SpeI
3. Complete ligation and transformation

1.Isolate plasmids from liquid cultures prepared on 7/9/2021 with minipreps

17 out of 20 liquid cultures we prepared on 7/9/2021 had grown. We concluded that the ampicillin concentration in the plates and the cultures was correct this time. We decided to isolate the plasmids from 5 of these liquid cultures and perform diagnostic digestions. We measured the concentration of these samples using Nanodrop 2000. The results are shown in the table below:

Concentration of the samples usi…

	A	B
1	Sample	Concentration
2	T-1246-2 1	57.9 ng/ul
3	T-1246-2 3	7.8 ng/ul
4	T-1246-2 4	44.9 ng/ul
5	T-1246-2 10	50.9 ng/ul
6	T-1246-2 11	34.8 ng/ul
7	T-1246-2 18	66.2 ng/ul

2.Diagnostic digestions with XbaI and SpeI

We performed the diagnostic digestions according to the table below:

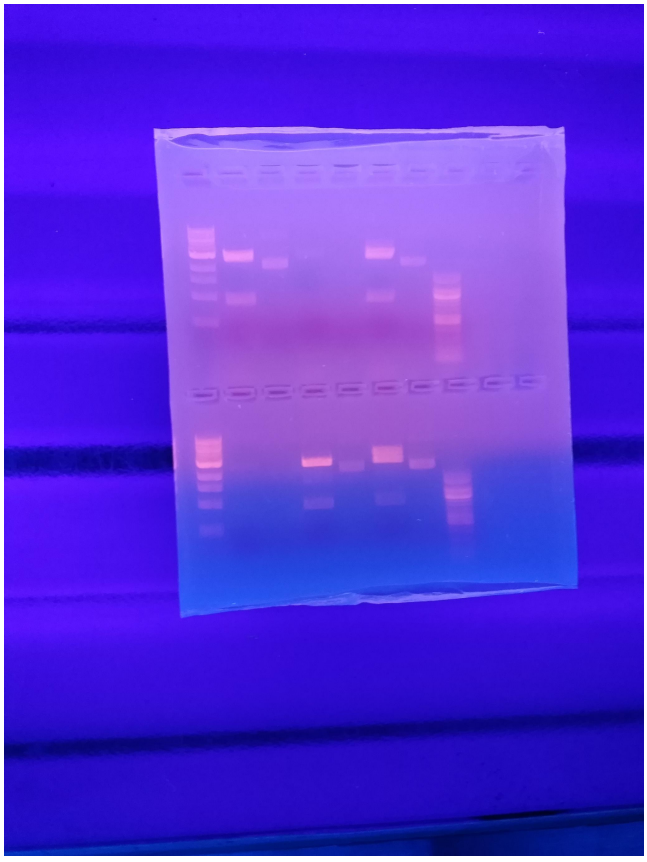
Reagents for diagnostic digestions

	A	B	C	D	E	F	G
1	Reagent	T-1246-2-1	T-1246-2-3	T-1246-2-4	T-1246-2-10	T-1246-2-11	T-1246-2-18
2	H2O	6.44 ul	-	5.19 ul	5.84 ul	2.94 ul	6.98 ul
3	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	DNA	4.31 ul	10.75 ul	5.56 ul	4.91 ul	7.81 ul	3.77 ul
5	XbaI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul

Before we add the second enzyme we incubated the reactions at 37o C for 30 minutes only with XbaI and heat-inactivated at 80o C for 20 minutes. After that, we added SpeI and incubated again at 37o C for 30 minutes and heat-inactivated at 80o C for 20 minutes.

We loaded the samples on an 1% agarose gel in the following order:
Ladder 1 kb - 1 - 1' - 3 - 3' - 4 - 4' - Ladder 100 bp
Ladder 1 kb - 10 - 10' - 11 - 11' - 18 -18' - Ladder 100 bp
x'= uncut plasmid
We examined the gel after 40 minutes and we got the following results:

8-9-2021.jpg



From these results we concluded that the samples that contain the insert are 1, 4, 11.

3.Complete ligation and transformation

Following the experiments we run on 7/9/2021 we completed the ligation and transformation of our plasmids. Specifically these are the reagents and the quantities for our ligation process :

Reagents for PCR Cloning Ligation 8/9/2021

	A	B	C	D	E	F	G	H	I
1		T-143-3p-1	T-143-3p-2	T-143-3p-3	T-143-3p-4	T-30e-5p-1	T-30e-5p-2	T-30e-5p-3	T-30e-5p-4
2	Linearized pMiniT2.0 (25 ng/ul)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)
3	Insert Ratio 1:3	1.46 ul	2.49 ul	4 ul	1.74 ul	1.24 ul	2.54 ul	1.46 ul	1.91 ul
4	H2O	2.54 ul	1.51 ul	-	2.26 ul	2.76 ul	1.46 ul	2.54 ul	2.09 ul
5	Cloning Mix 1	4 ul	4 ul	4 ul	4 ul	4 ul	4 ul	4 ul	4 ul
6	Cloning Mix 2	1 ul	1 ul	1 ul	1 ul	1 ul	1 ul	1 ul	1 ul
7	Total	10 ul	10 ul	10 ul	10 ul	10 ul	10 ul	10 ul	10 ul

We then used 2 ul of each reaction to transform 50 ul. After the end of the transformation reaction we plated 50 ul of each reaction in one plate and 50 ul of an 1:10 ul dilution in another plate. We incubated the plates at 37^{o} C overnight. Also, me must note that during this procedure there was an experimental error, so we are not confident for the result

THURSDAY, 9/9/2021

Goals:

1. Prepare liquid cultures from the plates we streaked on 8/9/2021

1.Prepare liquid cultures from the plates we streaked on 8/9/2021

As we mentioned above, there was an experimental error , so only 6 of our plates contained colonies. So we prepared, 6 liquid cultures. The first 2 contained the T-143-3p-1 , the next 2, T-143-3p-2 and the last 2 contained the T-143-3p-4 . There was no colonies for toehold 30e-5p, so we will repeat the ligation and transformation for every toehold that we did not transform.

FRIDAY, 10/9/2021

Goals:

1. Prepare Glycerol stock from the liquid cultures prepared on 9/9/2021
2. Isolate plasmids from liquid cultures prepared on 9/9/2021 with minipreps
3. Prepare 20 LB Agar plates with ampicillin
4. Run diagnostic digestions of our plasmids

1.Prepare Glycerol stock from the liquid cultures prepared on 9/9/2021

Only 5 of our 6 liquid cultures had visible bacterial growth and specifically 1/2 from T-143-3p-1.

2.Isolate plasmids from liquid cultures prepared on 9/9/2021 with minipreps

We isolated the plasmids from the liquid cultures using the Plasmid DNA Isolation with miniprep protocol. We used 50ul of elution buffer and got the following results using NanoDrop:

Concentration of plasmids after m…

	A	B
1	Sample	Concentration
2	T-143-3p 1	129.4 ng/ul
3	T-143-3p 2 (1)	158 ng/ul
4	T-143-3p 2 (2)	163.6 ng/ul
5	T-143-3p 4 (1)	144.7 ng/ul
6	T-143-3p 4 (2)	40.9 ng/ul

3.Prepare 20LB Agar plates with ampicillin

We prepared 20 LB agar plates with ampicillin with 19.25g LB following the protocol Preparing LB-agar plates .

4.Run diagnostic digestions of our plasmids

We used our plasmids to run diagnostic digestions to verify the results of the transformation procedure:

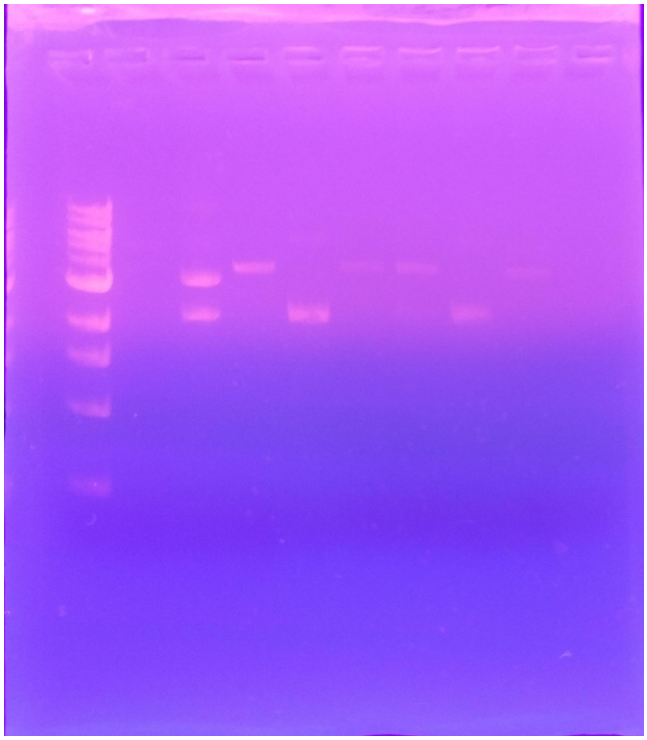
Diagnostic digestions using Spe

	A	B	C	D	E	F
1	Reagent	1) T-143-3p-1	2) T-143-3p-2 (1)	3) T-143-3p-2(2)	4) T-143-3p-4 (1)	5) T-143-3p-4 (2)
2	H2O	9.79 ul	9.96 ul	9.99 ul	9.89 ul	7.70 ul
3	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	DNA (125 ng)	0.96 ul	0.79 ul	0.76 ul	0.86 ul	3.05 ul
5	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	Total	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul

We run an electrophoresis gel and loaded the samples in the following order :

Ladder 1 kb - 1 - 1' - 2 - 2' - 3 -4- 4' -5
x'= uncut plasmid
After 30 minutes we got the following results :

IMG_20210910_153431.jpg



MONDAY, 13/9/2021

Goals:

1. Complete ligation and transformation for 6 toeholds
2. Amplification of 6 last toeholds for PCR cloning

1.Complete ligation and transformation for 6 toeholds

PCR Cloning 13/9/2021

	A	B	C	D	E	F	G
1	Reagent	Quantity					
2		T-143-3p-1	T-143-3p-3	T-30e-5p-1	T-30e-5p-2	T-30e-5p-3	T-30e-5p-4
3	Linearized pMiniT 2.0 Vector (25 ng/ µl)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)	1 ul (25 ng)
4	Insert (27.90 ng)	1.46 ul	4 ul	1.24 ul	2.54 ul	1.46 ul	1.91 ul
5	H20	2.54 ul	-	2.76 ul	1.46 ul	2.54 ul	2.09 ul
6	Cloning Mix 1	4 ul	4 ul	4 ul	4 ul	4 ul	4 ul
7	Cloning Mix 2	1 ul	1 ul	1 ul	1 ul	1 ul	1 ul
8	Total	10 ul	10 ul	10 ul	10 ul	10 ul	10 ul

We then used 2 ul of ligation mixture of each reaction to transform 50 ul of competent cells.

2.Amplification of 6 last toeholds for PCR cloning

We prepared two PCR reactions for each of the following toeholds to amplify them and use them for the PCR cloning. The reagents we used are listed in the following table. For the sample 6 we prepared three reactions since one the two tubes we had initialy prepared fell and only a small amount of the reaction left in the tube.

Reagents for the PCR amplification reaction

	A	B	C	D	E	F	G
1		T-30e-5p 6	T-30e-5p 7	T-30e-5p 8	T-1246 1	T-1246 3	T-1246 4
2	Q5 Master Mix	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul	7.5 ul
3	F. Primer	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul
4	R. Primer	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul	0.75 ul
5	DNA template	3.3 ul	0.83 ul	3.75 ul	4.54 ul	3.75 ul	2.02 ul
6	Mg+	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul	0.6 ul
7	H2O	2.1 ul	4.57 ul	1.65 ul	0.86 ul	1.65 ul	3.38 ul
8	Total	15 ul	15 ul	15 ul	15 ul	15 ul	15 ul

After the end of the reaction we loaded the samples on an 1% agarose gel in the following order:
Ladder 1 kb - 6 - 6 - 6 - 7 - 7 - 8 - 8 - Ladder 100 bp
Ladder 1 kb - 1 - 1 - 3 - 3 -4 - 4 - Ladder 100 bp

We examined the gel after 30 minutes and we excised the slices of each sample and combined the lanes from the same samples in one tube to increase the yield. For the gel extraction we used the protocol Gel Extraction and calculated the amount of dissolve buffer as follows:

Calculations for the dissolving buffer

	A	B	C	D	E
1	Sample	Tube weight	Total weight	Gel weight	Buffer volume
2	6	0.907 g	1.224 g	0.317 g	1268 ul
3	7	0.873 g	1.149 g	0.276 g	1104 ul
4	8	0.907 g	1.141 g	0.234 g	936 ul
5	1	0.906 g	1.090 g	0.184 g	736 ul
6	2	0.893 g	1.059 g	0.166 g	664 ul
7	3	0.894 g	1.045 g	0.151 g	604 ul

After the extraction we measured the concentration of the DNA using Nanodrop 2000. We got the following results:

Concentration of the toehold from…

	A	B
1	T-303-5p 6	51.1 ng/ul
2	T-30e-5p 7	59.8 ng/ul
3	T-30e-5p 8	28.4 ng/ul
4	T-1246 1	27.9 ng/ul
5	T-1246 3	36.5 ng/ul
6	T-1246 4	21.7 ng/ul

TUESDAY, 14/9/2021

Goals:

1. Liquid cultures of the six first toeholds
2. PCR cloning for T-30e-5p-8 and T-1246-1

1.Liquid cultures of the six first toeholds

We prepared 5 liquid culture for each of our 6 toeholds

2.PCR cloning for T-30e-5p-8 and T-1246-1

We used the protocol PCR cloning to clone the amplified toeholds T-30e-5p-8 and T-1246-1. The reagents used for the ligation reaction are mentioned in the table below:

Reagents for ligation for PCR cloning

	A	B	C
1	Reagent	Quantity	
2		T-30e-5p-8	T-1246-1
3	Linearized pMiniT 2.0 Vector (25 ng/ µl)	1 ul (25 ng)	1 ul (25 ng)
4	Insert (27.90 ng)	1 ul	1 ul
5	H20	3 ul	3 ul
6	Cloning Mix 1	4 ul	4 ul
7	Cloning Mix 2	1 ul	1 ul
8	Total	10 ul	10 ul

We used 2 ul of each reaction to transform 50 ul. After the end of the transformation reaction we plated 50 ul of each reaction in one plate and 50 ul of an 1:10 ul dilution in another plate. We incubated the plates at 37^{o} C overnight.

WEDNESDAY, 15/9/2021

Goals:

1. Prepare liquid cultures from the plates streaked on 14/9/2021
2. Prepare glycerol stock from the liquid cultures we prepared on 14/9/2021
3. Prepare Miniprep reactions
4. Run diagnostic restriction digestions

1.Prepare liquid cultures from the plates streaked on 14/9/2021

We wanted to grow liquid cultures from samples T-1246-1 and T-30e-5p-8, isolate the plasmids from these cultures with minipreps and see if these clones contain the insert. We prepared 10 liquid cultures from each sample. We added 3 ul of ampicillin in 3 ml of LB and inoculated the medium with a single colony. We incubated the liquid cultures at 37^{o} C for 18 hours.

2. Prepare glycerol stock from the liquid cultures we prepared on 14/9/2021

We used 500ul from 9 of our liquid cultures, the ones that were successful, to store them using the protocol Preparation of glycerol stock .

3.Prepare Miniprep reactions

We used the protocol Plasmid DNA Isolation with miniprep to isolate our plasmids and then we used Nanodrop 2000 to measure the concentration of each reaction:

Concentration of Miniprep 15/9/2021

	A	B
1	Sample	Concentration
2	T-143-3p-1 (1)	16.4 ng/ul
3	T-143-3p-1 (5)	33.7 ng/ul
4	T-143-3p-3 (5)	47.1 ng/ul
5	T-143-3p-3 (5)	101.9 ng/ul
6	T-30e-5p-1-(1)	57.1 ng/ul
7	T-30e-5p-2-(2)	56.3 ng/ul
8	T-30e-5p-2-(4)	219.9 ng/ul
9	T-30e-5p-2-(5)	65.6 ng/ul
10	T-30e-5p-4-(5)	71.6 ng/ul

4.Run diagnostic restriction digestions

Using the above concentrations we run diagnostic restriction digestions to ensure the success of the procedure. We used one enzyme, Spe:

Diagnostic Restriction Digestions 15/9/2021

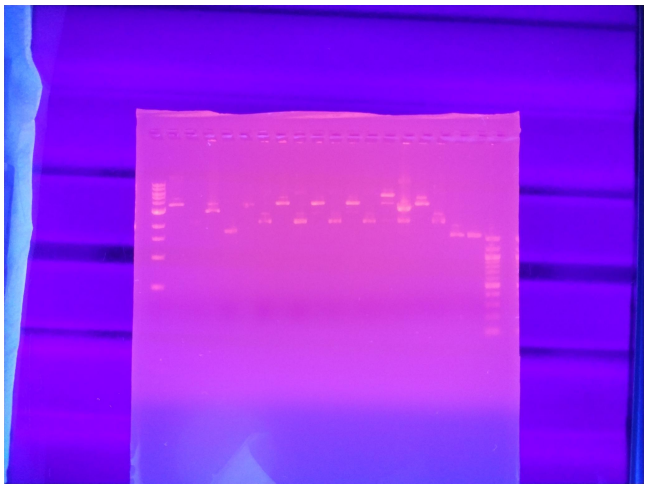
	A	B	C	D	E	F	G	H	I	J
1		1	2	3	4	5	6	7	8	9
2	Reagent	T-143-3p-1 (1)	T-143-3p-1 (5)	T-143-3p-3 (5)	T-143-3p-3 (5)	T-30e-5p-1-(1)	T-30e-5p-2-(2)	T-30e-5p-2-(4)	T-30e-5p-2-(5)	T-30e-5p-4-(5)
3	H2O	3.13 ul	7.05 ul	8.1 ul	9.53 ul	8.57 ul	8.53 ul	10.19 ul	8.85 ul	9.01 ul
4	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
5	DNA	7.62 ul	3.7 ul	2.65 ul	1.22 ul	2.18 ul	2.22 ul	0.56 ul	1.9 ul	1.74 ul
6	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
7	Total	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul

We then used a 1% agarose gel to load our samples on an electrophoresis gel and loaded them as follows :

1kb- 1-1'-2'-2'-3'3'-4-4'-5-5'-6-6'-7-7'-8-8'-9-9'-100bp
x'= uncut plasmid

After 35 minutes we received the following result :

IMG_20210915_180324.jpg



So we our 1,4,5 and 6 reaction were successful.

THURSDAY, 16/9/2021

Goals:

1. Isolate plasmids from the liquid cultures from yesterday
2. Diagnostic digestions
3. Prepare liquid cultures

1.Isolate plasmids from the liquid cultures from yesterday

Only 8 of the liquid cultures we prepared yesterday had grown. We used minipreps to isolate the plasmid from each culture and we measured the concentration of each sample with Nanodrop 2000. We got the following results:

Concentrations of the samples

	A	B	C
1	Sample	Code	Concentration
2	T-1246-1 3	A1	65.7 ng/ul
3	T-1246-1 5	A2	55.8 ng/ul
4	T-30e-5p-8-1	B1	27.6 ng/ul
5	T-30e-5p-8-3	B2	31.1 ng/ul
6	T-30e-5p-8-5	B3	98.6 ng/ul
7	T-30e-5p-8-7	B4	69.9 ng/ul
8	T-30e-5p-8-9	B5	36.1 ng/ul
9	T-30e-5p-8-10	B6	38.6 ng/ul

2.Diagnostic digestions

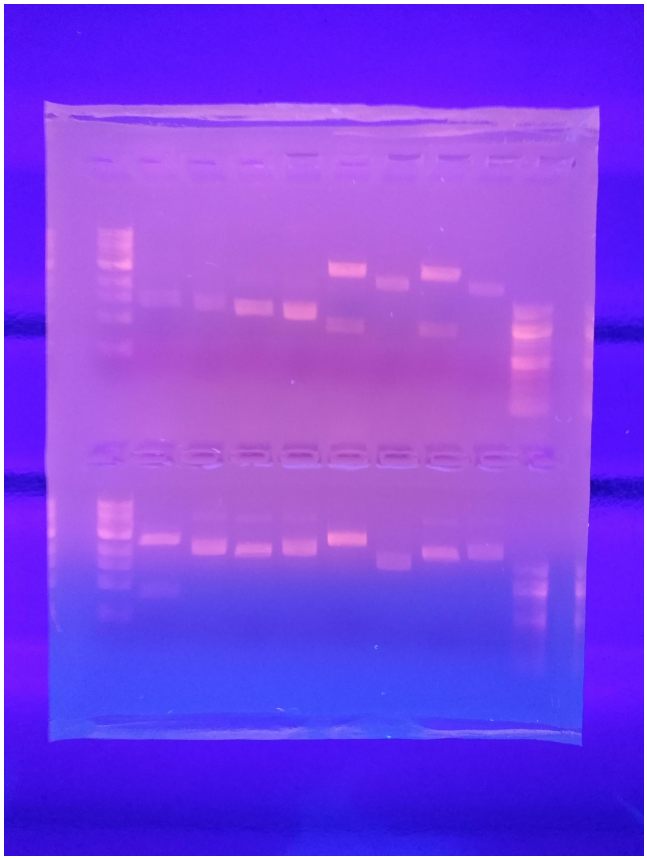
We digested these plasmids with the enzymes XbaI and SpeI and ran an electrophoresis gel to check if they contain the desired insert. The reagents used for these reactions are mentioned in the table below. We incubated the reaction only with the first enzyme at 37o C for 30 minutes and we heat-inactivated at 80o C for 20 minutes and after that, we reapeated the same procedure with the second enzyme.

Diagnostic digestions with XbaI and SpeI

	A	B	C	D	E	F	G	H	I
1	Reagent	A1	A2	B1	B2	B3	B4	B5	B6
2	H2O	8.85 ul	8.51 ul	6.23 ul	6.74 ul	9.49 ul	8.97 ul	7.29 ul	7.52 ul
3	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	DNA	1.9 ul	2.24 ul	4.52 ul	4.01 ul	1.26 ul	1.78 ul	3.46 ul	3.23 ul
5	XbaI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
6	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul

We loaded the samples on an 1% agarose gel in the following order and ran the gel for 30 minutes. We got the following results:
1 kb - A1 - A1' - A2 - A2' - B1 - B1' - B2 - B2' - 100 bp
1 kb - B3 - B3' - B4 - B4' - B5 - B5' - B6 - B6' - 100 bp
x'= uncut plasmid

16-9-2021.jpg



From these results we concluded that A1, A2 had no insert and thus we prepared 10 more liquid cultures from T-1246-1 to repeat the same procedure tomorrow and B1, B2 and B3 had insert, so the cloning of T-30e-5p-8 was succesful.

FRIDAY, 17/9/2021

Goals:

1. Isolate the plasmids from the liquid cultures we prepared yesterday
2. Complete the cloning of T-1246-3, T-1246-4, T-30e-5p-6, T-30e-5p-7
3. Run diagnostic digestions
4. Autoclave equipment and prepare LB medium

1.Isolate the plasmids from the liquid cultures we prepared yesterday

We used NEB's Monarch minipreps kit to isolate the plasmid from the cultures that have grown. We measured the concentration of each sample using Nanodrop 2000 and got the results listed in the following table:

Concentration measured with Nanodrop 2000

	A	B	C
1	Sample	Code	Concentration
2	T-1246-1-1	A1	140.2 ng/ul
3	T-30e-5p-3-7	B1	117.1 ng/ul
4	T-30e-5p-3-8	B2	34.0 ng/ul
5	T-30e-5p-4-3	C1	27.9 ng/ul
6	T-30e-5p-4-6	C2	38.3 ng/ul
7	T-30e-5p-4-7	C3	60.7 ng/ul
8	T-30e-5p-4-10	C4	25.5 ng/ul

2.Complete the cloning of T-1246-3, T-1246-4, T-30e-5p-6, T-30e-5p-7

For the ligation reaction of the samples T-1246-3, T-1246-4, T-30e-5p-6 and T-30e-5p-7 we used the following reagents. This time we decided to use a 5:1 ratio.

PCR cloning: Ligation reaction

	A	B	C	D	E
1	Reagents	T-30e-5p-6	T-30e-5p-7	T-1246-3	T-1246-4
2	Vector (25 ng)	1 ul	1 ul	1 ul	1 ul
3	Insert (43.47 ng)	0.85 ul	0.72 ul	1.19 ul	2.00 ul
4	H2O	3.15 ul	3.28 ul	2.81 ul	2.00 ul
5	Cloning Mix 1	4 ul	4 ul	4 ul	4 ul
6	Cloning Mix 2	1 ul	1 ul	1 ul	1 ul
7	Total	10 ul	10 ul	10 ul	10 ul

After the ligation we transformed 50 ul of competent cells supplied with the kit and streaked two plates for each sample: One with 50 ul of the transformation mixture and one with an 1:10 dilution. We incubated the plates at 37^{o} C overnight.

3. Run diagnostic digestions

We run diagnostic digestions for our samples

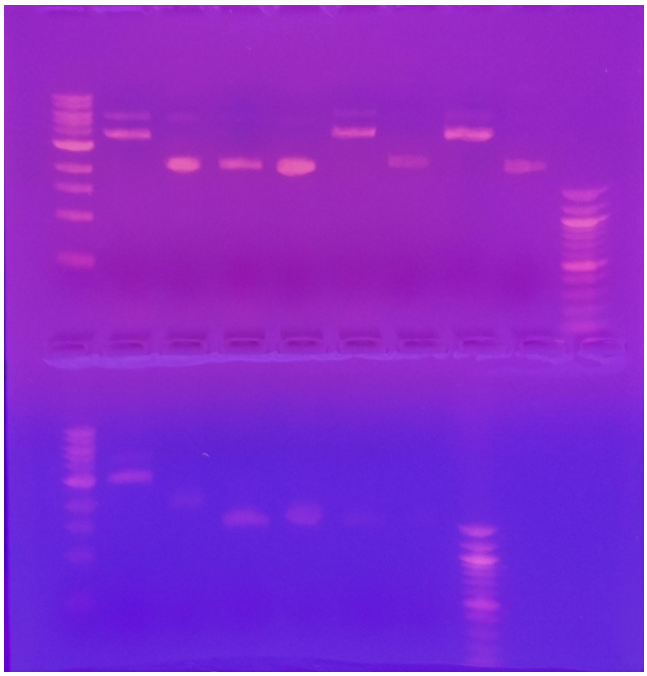
Diagnostic Restriction Digestions 17/9/2021

	A	B	C	D	E	F	G	H	I
1	Reagent	A1	B1	B2	C1	C2	C3	C4	
2	H2O	9.86 ul	9.69 ul	7.08 ul	6.27 ul	7.49 ul	8.69 ul	5.85 ul	
3	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	
4	DNA	0.89 ul	1.06 ul	3.67 ul	4.48 ul	3.26 ul	2.06 ul	4.9 ul	
5	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	
6	Total	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	12.5 ul	

And we loaded our samples as follows:
1kb-A1-A1'-B1-B1'-B2-B2'-C1-C1'-100bp
1kb-C2-C2'-C3-C3'-C4-C4'-100bp
x'= uncut plasmid

After 30 minutes we got the following results:

IMG_20210917_183131 2nd.jpg



4.Autoclave equipment and prepare LB medium
We autoclaved tips, bottles, tubes and 600 ml of LB medium.

SATURDAY, 18/9/2021

Goals:

1. Prepare liquid cultures from the plates streaked yesterday

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We prepared 24 liquid culture picking 6 colonies from each sample (T-1246-3, T-1246-4, T-30e-5p-6, T-30e-5p-5). We used 3 ml of LB medium and 3 ul of ampicillin for each culture and incubated at 37 ^{o}C for 18 hours.

SUNDAY, 19/9/2021

Goals:

1. Minipreps to isolate the plasmids from the liquid cultures
2. Diagnostic digestions
3. Preparation of new liquid cultures

1.Minipreps to isolate the plasmids from the liquid cultures

We noticed that the LB that we used to prepare the liquid cultures yesterday had been contaminated. Thus, we only used four of the samples to isolate the plasmid and run diagnostic digestions, only to check if our suspicion that the samples were contaminated was correct.

We used the NEB's Monarch Minipreps kit and isolated the plasmids from the samples. We measured the concentration of the samples using Nanodrop 2000. We found the following concentrations:

Concentration of the plasmids

	A	B
1	T-30e-5p(6)-1	36.4 ng/ul
2	T-30e-5p(7)-1	45.7 ng/ul
3	T-1246(3)-1	29.6 ng/ul
4	T-1246(4)-1	33.7 ng/ul

2.Diagnostic digestions

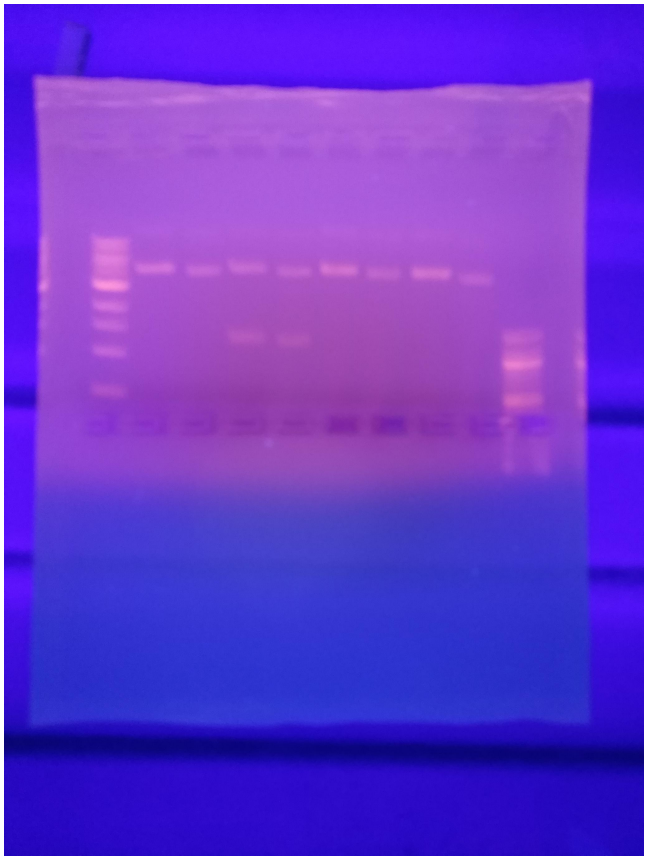
We used the following reagents for the diagnostic digestion of the plasmids. We digested the plasmid using only one enzyme, SpeI.

Reagents for diagnostic digestions

	A	B	C	D	E
1		1	2	3	4
2	Reagent	T-30e-5p(6)-1	T-30e-5p(7)-1	T-1246(3)-1	T-1246(4)-1
3	H2O	7.32 ul	8.02 ul	6.53 ul	7.05 ul
4	rCutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul
5	DNA	3.43 ul	2.73 ul	4.22 ul	3.70 ul
6	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul
7	Total	12.5 ul	12.5 ul	12.5 ul	12.5 ul

We loaded the samples in the following order on an 1% agarose gel and examined the gel after 25 minutes.
1kb-1-1'-2'-2'-3'3'-4-4'-100bp

19-9-2021.jpg



From these results we confirmed that the samples were contaminated and we could not use them.

3.Preparation of new liquid cultures

For this reason, we prepared five new liquid culture from each plate, by mixing 3 ml of LB medium and 3 ul of ampicillin and adding a single colony in each culture.

MONDAY, 20/9/2021

Goals:

1. Minipreps to isolate the plasmid
2. Diagnostic digestions
3. Preparation of liquid cultures

1.Minipreps to isolate the plasmid

Out of the twenty colonies we prepared, only 7 had grown. One from the sample T-1246-4, two from the samples T-30e-5p-6 and four from the samples T-30e-5p-7. We used NEB's Monarch Minipreps kit to isolate the plasmids from each of these cultures and we measured the concentration of the plasmid using Nanodrop 2000. We got the following results:

Concentration of the plasmids

	A	B	C
1	Sample	Code	Concentration
2	T-1246-4-5	A1	122.5 ng/ul
3	T-30e-5p-6-4	B1	132.5 ng/ul
4	T-30e-5p-6-5	B2	48.9 ng/ul
5	T-30e-5p-7-2	C1	68.3 ng/ul
6	T-30e-5p-7-3	C2	53.7 ng/ul
7	T-30e-5p-7-4	C3	66.6 ng/ul
8	T-30e-5p-7-5	C4	167.8 ng/ul

2.Diagnostic digestions

For the diagnostic digestions of these plasmids we used the following reagents. We only digested the plasmids with one enzyme, SpeI.

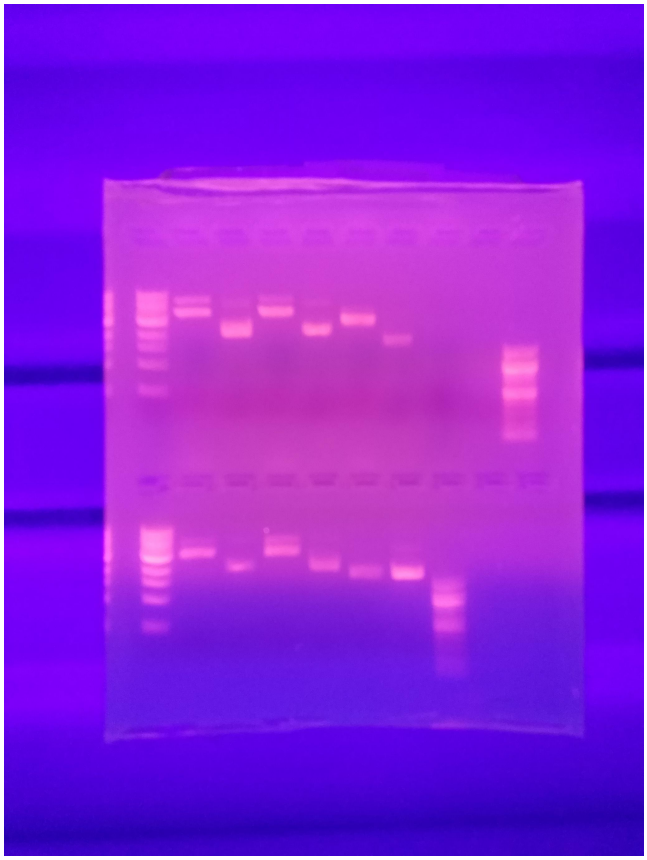
Reagents for diagnostic restrictions

	A	B	C	D	E	F	G	H
1	Reagent	A1	B1	B2	B3	B4	B5	B6
2	H2O	9.73 ul	9.81 ul	8.2 ul	8.92 ul	8.43 ul	8.88 ul	10.0 ul
3	CutSmart	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul	1.25 ul
4	Template	1.02 ul	0.94 ul	2.55 ul	1.83 ul	2.32 ul	1.87 ul	0.74 ul
5	SpeI	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul

We loaded the samples on an 1% agarose gel in the following order:
Ladder 1 kb - A1 - A1' - B1 - B1' - B2 - B2' - C1 - C1' - Ladder 100 bp
Ladder 1 kb - C2 - C2' - C3 - C3' - C4 - C4' - Ladder 100 bp

We examined the gel after 30 minutes and we got the following results:

20-9-2021.jpg



We can see and extra band that in some samples that is probably a quantity of uncut plasmids that remained because of incomplete digestion. We supposed that this was due to the aliquot of the enzyme have been freezed and thawed too many times, so we decided to use a different aliquot in the next enzymes. However, we can see that the length of the linearized plasmids A1, B1, C2 and C3 is approximately 3500 which is the desired length.

This means that we have the plasmids with the correct insert for the toeholds: T-1246-4, T-30e-5p-6, T-30e-5p-7.

3.Preparation of liquid cultures

Hence, we decided to prepare 20 new liquid cultures from the same plate (sample T-1246-3) to isolate a plasmid with the insert T-1246-3. We mixed 3 ml of LB medium and 3 ul of ampicillin and inoculated the culture with a single colony. We incubated the liquid cultures at 37 oC for 18 hours in a shaking incubator.

TUESDAY, 21/9/2021

Goals:

1. Isolate the plasmids using minipreps
2. Run diagnostic digestions
3. Prepare liquid cultures

1.Isolate the plasmids using minipreps

Out of the 20 liquid cultures we had prepare yesterday, only one had grown. We isolated the plasmid from this culture using NEB's Monarch minipreps kit. Its concentration was 108.1 ng/ul.

2.Run diagnostic digestions

For the diagnostic digestions of this plasmid we used both enzymes, SpeI and XbaI. We used the following reagents:

Diagnostic digestions

	A	B
1	H2O	9.6 ul
2	CutSmart	1.25 ul
3	Template	1.15 ul
4	SpeI	0.5 ul
5	XbaI	0.5 ul

Firstly, we incubated the reaction containing only the first enzyme at 37 oC for 30 minutes and heat-inaactivated at 80 ^{o}C for 20 minutes. Then we repeated the same procedure with the second enzyme.

We loaded the samples on an 1% agarose gel in the following order:
Ladder 1 kb - 1 - 1' - Ladder 100 bp

We examined the gel after 30 minutes but the plasmid was uncut.

3.Prepare liquid cultures

We prepared 15 liquid cultures from the plate of T-1246-3 and incubated them at 37 ^{o}C for 18 hours in a shaking incubator.

WEDNESDAY, 22/9/2021

Goals:

1. Isolate the plasmids using minipreps
2. Autoclave the equipment and prepare LB medium and new agar plates
3. Prepare liquid cultures
4. Streak plates with colonies from the plate T-1246-3

1.Isolate the plasmids using minipreps

No liquid cultures had grown. For these reason we decided to run some experiments to test if the bacteria from the colonies of the plate are alive.

2.Autoclave the equipment and prepare LB medium and new agar plates

We prepared 500 ml of LB medium by dissolving 10g of pre-mixed powder according to Lennox in 500 ml H2O. Also, we prepared 300 ml of LB-agar, by dissolving **...g** of LB-agar powder. We autoclaved both the solutions and tips and bottles. After the LB-agar cooled down we added 300 ul of ampicillin and prepared 9 ampicillin plates.

3. Prepare liquid cultures

We prepared 20 liquid cultures and we inoculated each one with a single colony, except for the first one. In the first one, we added many colonies, to use it as a control.

4.Streak plates with colonies from the plate T-1246-3

We used colonies from the two T-1246-3 plates and streaked two plates for each one: one from the old batch and one from the batch we prepared on the same day.

THURSDAY, 23/9/2021

Goals:

1. Check if the cultures had grown and if there are colonies on the plates

1.Check if the cultures had grown and if there are colonies on the plates

The liquid cultures had not grown, but the two of the four plates, the ones with the non-diluted cells, had colonies. The plates that contained the diluted cells had no colonies.

From these results we concluded that the cells are alive, but we could not figure out why the liquid cultures could not grow.