2024.06.19 Amplification of p414-TEF-Cas9

Overview

Track: Receptor production, In-Cell Assay, ROSALIND
Objective: Amplification of the p414 backbone for the expression of receptors.

When: 19.06.2024
Who: Mikkel & Reinis
Location: Skylab GMO lab

Methods/protocol

1. Resuspension of IDT primers in PCR grade water.

   Protocol: *Resuspension of primers/DNA*

   - Primer concentration used in the reaction was 5 µM, instead of 0.5 µM

2. PCR of p414-TEF-Cas9 with primers FW_p414_gene[56.3C] and RV_p414_[57.4]

   Protocol: *PCR with Phusion U Hot start polymerase*

3. Electrophoresis with Invitrogen iBase E-Gel system

Conclusions

- The electrophoresis with Invitrogen iBase E-Gel System was unsuccessful
- Future electrophoretic analyses must be performed using different equipment

2024.06.21 Amplification of various DNA templates

Overview

Track: Receptor production, In-Cell Assay, ROSALIND
Objective: Amplification of the fragments required for assemblies in all 3 tracks

When: 2024.06.21
Who: Mikkel & Reinis
Location: B202, R9008

Methods/protocol

1. Resuspension of the primers and G-blocks in PCR grade water.

   Protocol: *Resuspension of primers/DNA*

2. PCR

   Protocol: *PCR with Phusion U Hot start polymerase*

   - The Master Mix was prepared by Reinis beforehand and stored in -20℃.
   - 0.5 µL of 2 µM of each primer and 0.5 µL of the template were added to 23.5 µL of the Master Mix.

   DNA templates & used primers:

DNA Template	FW primer	REV primer
p414-TEF-Cas9	FW_p414_gene[56.3C]	RV_p414_[57.4]
Alfa-6xHis_tags	FW_tag[52.8]	RV_tag_gene[56.1]
HERa	FW_HERa_[55.2]	RV_HERa_[54.5]
HERb	FW_HERb_old_[56.3]	RV_HERb_old_[56]
NR3C1	FV_NR3C1_old_[56.3]	RV_NR3C1_old_[54]

   PCR parameters:

   - 2 min elongation
   - 52℃ annealing temp

   Side notes:

   - Products stored at -20℃

Conclusions

- PCR of samples #3 - #12 were performed
- We can run PCR with two separate annealing Temp. - the first reaction in 50°C and the rest in 55°C

2024.06.21 LB/synthetic media & plates

Overview

Track: Receptor production, In-Cell Assay, ROSALIND
Objective: Preparation of LB broth, LB amp agar, and synthetic YNB media.

When: 2024.06.21
Who: Mikkel, Reinis, Georgios, Sirio
Location: Skylab Multi Lab

Protocols

- *LB amp agar*
- *LB amp broth*
- *YNB - Glc and Gal broth/plates without tryptophan*

Methods

- Prepared a 100 mg/mL solution of LB amp in ethanol
- We did not add glucose, galactose or agar to the synthetic YNB media.
- LB amp100 plate was incubated from friday to monday to check sterility

Conclusion

- Plates of LB amp100, 250 mL LB amp, and 225 mL synthetic media without sugar were prepared and stored in +4℃.

Side notes

- Autoclave program for media preparation takes a couple of hours
- Autoclave in Multi Lab can fit only 3 x 500 mL bottles

2024.06.24 & 25 Incubation of Addgene strains & preparation of LacZ buffer

Overview

Track: In-Cell Assay
Objective: Preparation of AddGene strains and LacZ buffer for future experiments

When: 24.06.2024
Who: Mikkel
Location: Skylab GMO lab

Protocols

- **AddGene strains:** *How do I process my Addgene plasmid (bacterial stab)*
- **LacZ buffer:** *LacZ buffer (for In-Cell Assay)*

Methods

AddGene strains

1. Let 7 LB amp100 plates dry in the BSC for 15 min
2. Dipped an inoculation needle in the bacterial stab and streaked it on the plate. Streaked with new needles.
3. Incubated the plates at 37°C and left the bacterial stabs in the fridge.
4. Restreaked single colonies on the 25th on new LB amp100 plates. Incubated them at 37°C O/N

Side notes

- Prepared freezing tubes and LB amp100 broth for the 26th

LacZ Buffer

- Added 10 mL of a 25 mM $MgCl_2$ solution instead of the powder.
- Divided it into 12 x 20 mL in 50 mL falcon tubes. Stored in a bag in the freezer. Each 20 mL falcon tube is enough for one 96 well plate.

2024.06.26 & 27 Preparation of glycerol stocks & purification of Addgene plasmids

Overview

Track: In-Cell Assay, ROSALIND
Objective: To make glycerol stocks of the Addgene plasmids and to purify them.

When: 26.06.2024
Who: Mikkel, Sirio, Georgios, Lumi
Location: Skylab GMO lab

Plasmids:

- 6x In-Cell Assay plasmids (for each different receptor):
    - pRR-MR-5Z
    - pRR-GR-5Z
    - pRR-PR-5Z
    - pRR-AR-5Z
    - pRR-ERalpha-5Z
    - pRR-ERbeta-5Z
- pUC19-T7-3WJdB-T

Methods/protocol

Glycerol stocks of the plasmids

Protocol: *Preparation of glycerol stocks*

25.06.2024:

- 7 cryo tubes were labeled and 750 µL 50% glycerol was added to each tube and 250 µL 100 mg/mL ampicillin in 50% ethanol was added to 250 mL LB broth

26.06.2024:

- 5 mL LB amp100 broth was inoculated with a colony from each of the 7 plates. Incubated O/N at 37°C with 250 rpm in 15 mL falcon tubes

27.06.2024:

- 750 mL O/N culture was added to the cryotubes & plasmids were purified from the 4 mL O/N culture
- On the 27th we did the following protocol

Plasmid purification

Protocol: *Monarch Plasmid Miniprep Kit (For purification of plasmids from E. coli)*

- 3 mL of culture has been used
- For DNA elution 50 µL of Elution Buffer was used
- Spin at 10'000g (or RCF) for 1 minute

Conclusion

- Both purification and preparation of glycerol stocks were successful

2024.06.28 Test #1 - In-Cell Assay Transformation

Overview

Track: In-Cell Assay
Objective: Transform the yeast cells (*S. cerevisiae CENPK113-3C*) with the previously amplified, purified and stored plasmids.

When: 28.06.2024
Who: Sirio, Georgios, Mikkel
Location: GMO lab skylab

Methods/protocol

Protocol: *Transformation of S. cerevisiae (yeast)*

Conclusion

- After 3 days of incubation (till 01.07.2024), the transformation turned out to be successful.
- There are on average 3~5 colonies for each plate.

2024.07.01 & 07.03: Glycerol Stocks for In-Cell Assay

Overview

Track: In-Cell Assay
Objective: Prepare glycerol stocks of the transformed yeast.

When: 01.07.2024
Who: Sirio, Georgios, Lumi
Location: GMO LAB Skylab

Methods/protocol

Protocol: *Preparation of glycerol stocks*
Strains: The yeast cells that were seeded on 28/06/2024

01.07.2024: Inoculation of Media
03.07.2024: Glycerol solution & Freezing

Conclusion

- Everything went according to the protocol
- Glycerol stocks stored at -80℃

2024.07.01 Test #1 - DNA templates for the Expression System

Overview

Track: Receptor production, In-Cell Assay, ROSALIND
Objective: Begin the preparations for creating of expression systems

When: 01.07.2024
Who: Sirio, Georgios, Mikkel
Location: 202 GMO Lab

Methods/protocol

Preparation of the template that will be used:

Protocol: *gBlocks Resuspension*

Gel Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Samples:

Sample no.	Forward primer	Reverse primer	Plasmid template	Annealing temp.	Date
3	FW_p414_gene	RV_p414	p414-TEF-Cas9	52	21.06.2024
4	FW_HERa	RV_HERa	HERa	52	21.06.2024
5	FW_HERb_old	RV_HERb_old	HERb	52	21.06.2024
6	FW_NR3C1_old	RV_NR3C1_old	NR3C1	52	21.06.2024
7	FW_tag	RV_tag_gene	alfa_6xHIS	52	21.06.2024
8	FW_p414_tag	RV_p414	p414	52	21.06.2024
9	FW_tag	RV_tag_plasmid	alfa_6xHIS	52	21.06.2024
10	FW_pSC1C30	RV_pSC1C30	pSB1C30	52	21.06.2024
11	FW_ERE_minimal	RV_ERE_minimal	ERE minimal Broccoli	52	21.06.2024
12	FW_ERE_minimal	RV_ERE_minimal	HRE minimal Broccoli	52	21.06.2024

Sample placement in the gel:

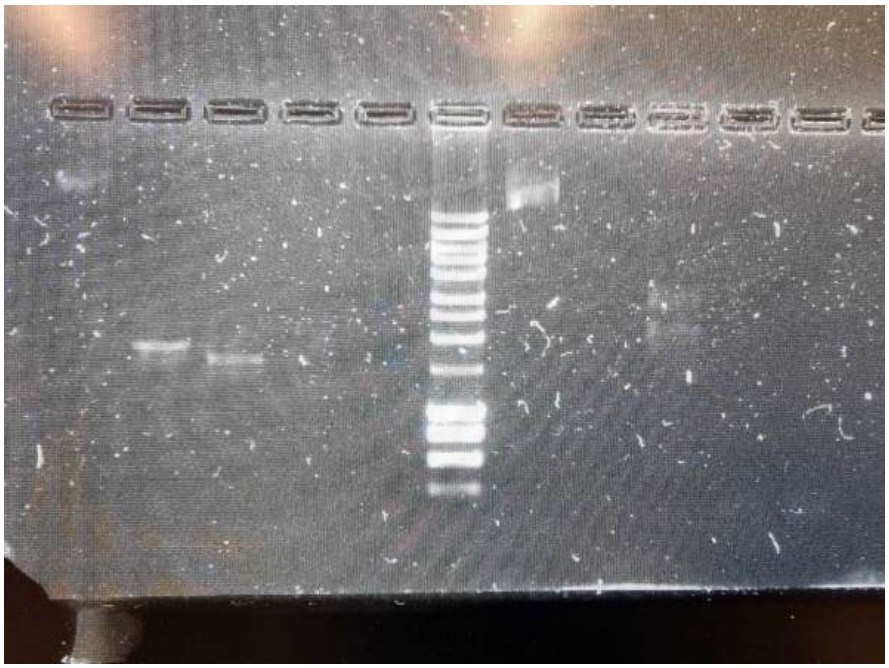
3	4	5	6	7	Ladder	8	9	10	11	12

Electrophoresis parameters:

- 80 V for 20 min --> 130 V for 30 min --> It covered a short distance only
- Time: 45 min

Conclusion

- Test #1 failed



2024.07.03 PCRs and Gel Electrophoresis #1

Overview

Track: Receptor production, ROSALIND
Objective: Re-run all necessary PCRs

When: 03.07.2024
Who: Georgios, Mikkel
Location: 202 GMO Lab

Methods/protocol

PCR

Protocol: *PCR with Phusion U Hot start polymerase*

1. Primer dilutions 100 µM to 10 µM.
2. Preparation of the MasterMix (MM)

Master Mix

H_2O	325 µL
5X HF Buffer	100 µL
10 mM dNTPs	10 µL

3. Aliquot 43.5 µL of MM into 8 PC R tubes.
4. Add 2.5 µL of each 10 µM primer and 1 µL of DNA template to each PCR tubes
5. Add 0.5 µL of Phusion U Hot start polymerase

PCR Reactions 03.07.2024

Tube no.	Primer1	Primer2	Template	Length	Details
13	FW_p414_gene	RV_p414	p414-TEF-Cas9	5350	Anneal 59, 2:15
14	FW_pSC1C30	RV_pSC1C30	pSB1C30 red	2000	Anneal 56, 1:15
15	FW_HERa	RV_HERa	HERa	1794	Anneal 56, 1:15
16	FW_HERb_old	RV_HERb_old	HERb	1593	Anneal 56, 1:15
High Range DNA Ladder Ampliqon					
17	FW_NR3C1_new	RV_NR3C1_new	NR3C1_new	2337	Anneal 56, 1:15
18	FW_pSC1C30	RV_pSC1C30	pSB1C30 black	2000	Anneal 56, 1:15
19	FW_tag	RV_tag_gene	alfa_6xHIS	300	Anneal 55, 0:30
20	FW_ERE_minimal	RV_ERE_minimal	ERE minimal Broccoli	300	Anneal 55, 0:30
21	FW_ERE_minimal	RV_ERE_minimal	HRE minimal Broccoli	300	Anneal 55, 0:30

Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

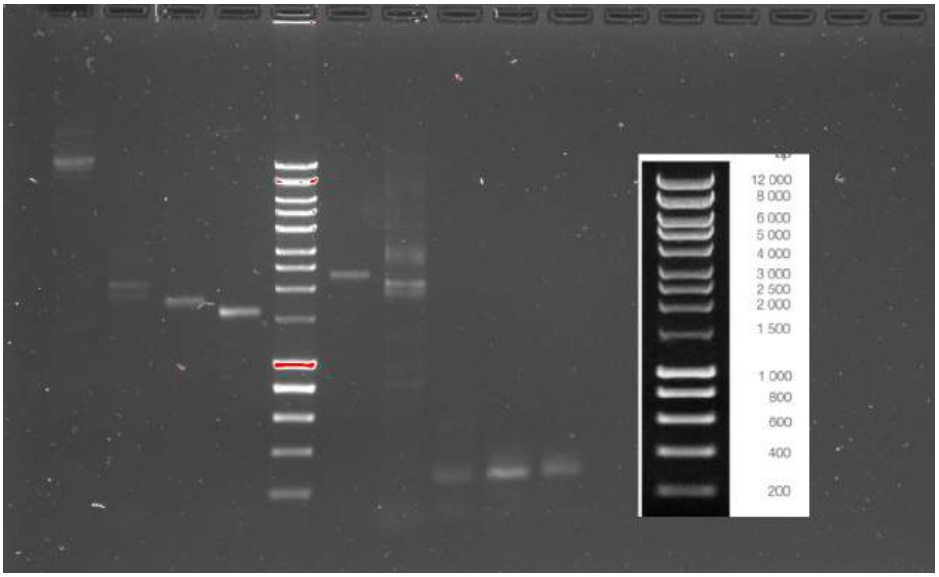
- 1% agarose in TAE electrophoresis gel
- E#1 Start with 80 mA constant rate for 20 minutes, then 200 V constant rate until the length of the gel is over

- E#2 200 V constant rate
    - 3 µL ladder
    - 1.8 µL PCR product

Conclusions

Expected results for wells:

- #3: HERa (tube 15)
- #4: HERb (tube 16)
- #6: NR3C1_new (tube 17)



2024.07.04 PCRs and Gel Electrophoresis #2

Overview

Track: Receptor production, ROSALIND
Objective: Re-run 5 PCR reactions from 03.07.2024

When: 04.07.2024
Who: Mikkel
Location: 202 teaching lab

Methods/protocol

Protocol: *PCR with Phusion U Hot start polymerase*

Changes since yesterday:

- Lower annealing temp
- Templates p414-TEF-Cas9 and pSB1C30 were diluted 100x each
- 34x cycles instead of 30x

PCR

1. Preparation of Master Mix

H_2O	195 µL
5X HF Buffer	60 µL
10 mM dNTPs	6 µL

1. Aliquoted 43.5 µL of Master Mix into 5 PCR tubes.
2. Added 2.5 µL of each 10 µM primer and 1 µL of DNA template to each PCR tube
3. Added 0.5 µL of Phusion U Hot start polymerase

PCR Reactions 03.07.2024

Tube no.	Primer1	Primer2	Template	Length	Details
22	FW_p414_gene	RV_p414	100x p414-TEF-Cas9	5350	Anneal 57, 2:15
23	FW_pSC1C30	RV_pSC1C30	100x pSB1C30 red	2000	Anneal 54, 1:15
24	FW_tag	RV_tag_gene	alfa_6xHIS	300	Anneal 53, 0:30
25	FW_ERE_minimal	RV_ERE_minimal	ERE minimal Broccoli	300	Anneal 53, 0:30
26	FW_ERE_minimal	RV_ERE_minimal	HRE minimal Broccoli	300	Anneal 53, 0:30

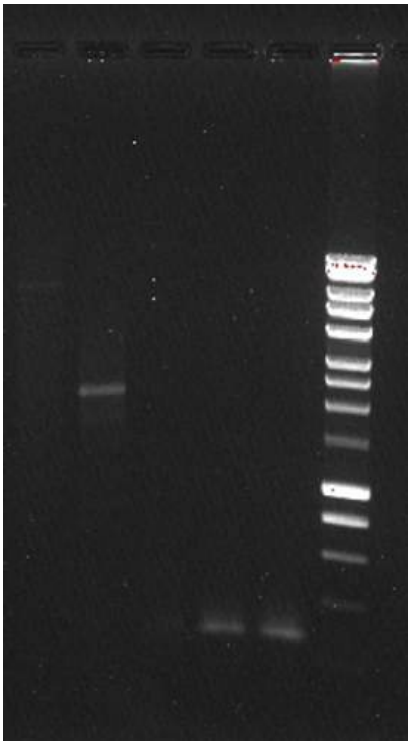
Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Conclusions / Results

Gel layout

1. 100x p414-TEF-Cas9
2. 100x pSB1C30 red
3. alfa_6xHIS
4. ERE minimal Broccoli
5. HRE minimal Broccoli
6. High Range DNA Ladder Ampliqon



2024.07.09 PCRs and Gel Electrophoresis #3

Overview

Track: Receptor production, ROSALIND
Objective: Re-run 4 PCRs for the plasmid and the tag

When: 09.07.2024
Who: Reinis
Location: 202 teaching lab

Methods/protocol

PCR

Protocol: *PCR with Phusion U Hot start polymerase*

Changes to previous attempts:

- higher annealing temperatures from the calculator
- New Master Mixes

Preparation of Master Mixes

HF buffer**:**

	25 µL	10x 25 µL	
H_2O	16.25	81.25	
5X HF Buffer	5	25	1x
10 mM dNTPs	0.5	2.5	200 µM (each)
Primer FWD (10 µM)	1	-	0.4 µM
Primer RV (10 µM)	1	-	0.4 µM
Phusion U Polymerase	0.25	1.25	0.02 U/µL
Template	1	-	1 pg - 10 ng DNA per 50 µL

GC buffer:

		25 µL	10x 25 µL	
H_2O		16.25	81.25	
5X GC Buffer		5	25	1x
10 mM dNTPs		0.5	2.5	200 µM (each)
Primer FWD (10 µM)		1	-	0.4 µM
Primer RV (10 µM)		1	-	0.4 µM
Phusion U Polymerase		0.25	1.25	0.02 U/µL
Template		1	-	1 pg - 10 ng DNA per 50 µL

1. Add 22µL of Master Mix to 5 tubes
2. Add primers and template

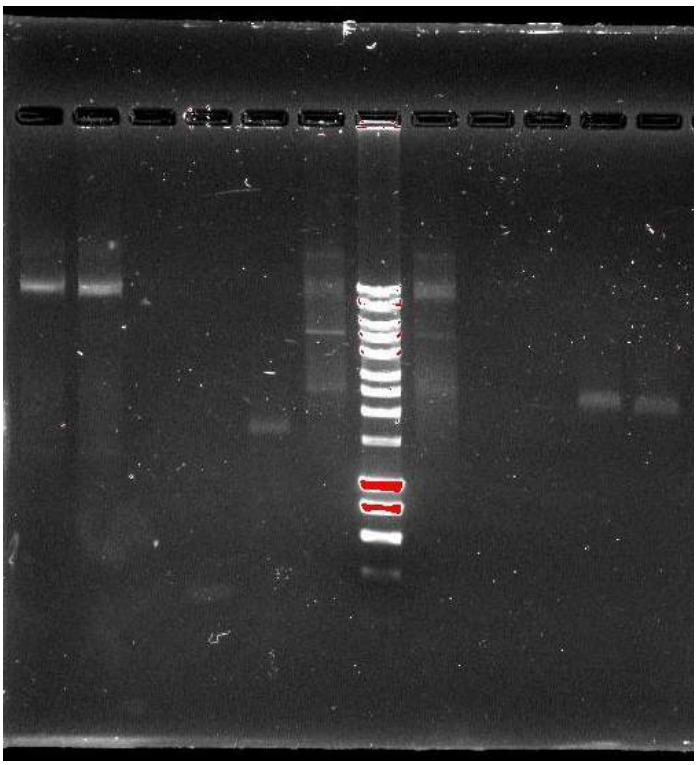
	Primer forward	Primer reverse	template	Buffer	Annealing temp.	Elongation [min]	Length
27	FW_p414_gene	RV_p414	100x p414-TEF-Cas9	HF	65	2:20	5350
28	FW_p414_tag	RV_p414	100x p414-TEF-Cas9	HF	65	2:20	5350
30	FW_tag	RV_tag_gene	alfa_6xHIS	HF	61	0:15	300
29	FW_tag	RV_tag_plasmid	alfa_6xHIS	HF	61	0:30	300
31	FW_HERb_new	RV_HERb_new	HERb_new	HF	61	0:30	
32	FW_p414_gene	RV_p414	100x p414-TEF-Cas9	GC	65	2:20	
33	FW_p414_tag	RV_p414	100x p414-TEF-Cas9	GC	65	2:20	
34	FW_tag	RV_tag_gene	alfa_6xHIS	GC	61	0:30	
35	FW_tag	RV_tag_plasmid	alfa_6xHIS	GC	61	0:30	
NK							

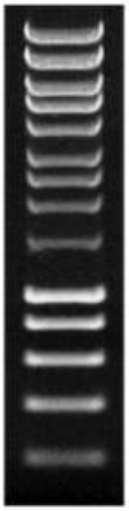
Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Conclusion & Results

Gel layout:
27, 28, NK, 30, 29, 32, High Range DNA Ladder Ampliqon, 33, 34, 35, 31, 31





bp	ng/band		
12 000	60	24	12
8 000	80	32	16
6 000	60	24	12
5 000	50	20	10
4 000	40	16	8
3 000	30	12	6
2 500	25	10	5
2 000	20	8	4
1 500	15	6	3
1 000	100	40	20
800	80	32	16
600	60	24	12
400	40	16	8
200	20	8	4
	5 µl	2 µl	1 µl

2024.07.09 Dilutions of ß-Estradiol

Overview

Track: ROSALIND
Objective: Serial dilutions to safety working concentration

When: 09.07.2024
Who: Georgios
Location: 221 (Noel's lab)

Methods/protocol

Protocol: *Hormone dilution standards*

- Vortex in each mixing step was done
- At the beginning 22.8 mg of ß-estradiol was weighted in the scale. Solution concentration: 83.673mM (**stock #1**)
- Then with 2 serial dilutions 1:10 (100 µL of the stock #1 and 900 µL of 50% EtOH in each) reached concentration 836.76 µM (**stock #2**)
- Then 43.8 µL of the stock #2 and 956.2 µL of the 50 % EtOH were mixed to final concentration of 36.71 µM (**stock #3**)
- Then Dilute 100 µL of **stock #3** in 900 µL 50% ethanol. This yields a 367.13 nM temporary **stock #4**.
- Now, the procedure was the same with the protocol above
- The stocks were stored to fridge (MultiLab, SKylab) since the experiment (up to 2 days), ideally aliquots of the needed volume should be made

Conclusion

All good, as expected.

2024.07.09 USER cloning #1 for enzyme production

Overview

Track: Receptor production
Objective: USER cloning to assemble the production plasmids with the his-tag

When: 09.07.2024
Who: Reinis
Location: 202

Methods/protocol

USER Cloning

Protocol: *USER cloning*

PCR products used:

USER tube name	Backbone tube	Tag tube	Gene tube	Gene name
U3	32	30	15	HERa
U4	32	30	16	HERb
U5	32	30	17	NR3C1_new
U6	32	30	31	HERb_new

Master Mix:

Reagent	Volume µL	MM x 4
MQ water	11	44
Backbone PCR	2	8
Tag PCR	2	8
Gene PCR	2	-
CutSmart buffer	1	4
USER enzyme	1	4
DpnI enzyme	1	4
Total	20 µL	

Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Conclusions / Results

Failed.

2024.07.09 User Cloning of pEREmin & pHREmin: Gels & Transformation

Overview

Track: ROSALIND
Objective: USER cloning of pEREmin & pHREmin

When: 09.07.2024
Who: Sirio, Cristian
Location: 202

Methods/protocol

User Cloning

Protocol: *USER cloning*

USER cloning of the following samples:

- EREmin: *2. ERE minimal Broccoli*
- HREmin: *4. HRE minimal Broccoli*

USER cloning conditions:

Reagents	pEREmin	pHREmin	Size
MQ water	13 µL*	13 µL*	
PCR product #1	2 µL (2. ERE minimal Broccoli)	2 µL (4. HRE minimal Broccoli)	300
PCR product #1	2 µL (E. coli backbone: 100x pSB1C30 red)	2 µL (E. coli backbone: 100x pSB1C30 red)	2000
CutSmart buffer	1 µL	1 µL	
USER enzyme	1 µL	1 µL	
DpnI enzyme	1 µL	1 µL	
Total	**20 µL**	**20 µL**	**2300**

*extra 2 µL of water in the mix (only 2 µL of PCR products were used)

Gel Electrophoresis

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Transformation

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

- Plates with chloramphenicol prepared for the BioBrick Workshop were used

Transformation with the following DNA constructs:

- NC (Negative control): no DNA added
- pEREmin
- pHREmin
- PC (Positive Control): plasmid pSB1C30 (red labeling)

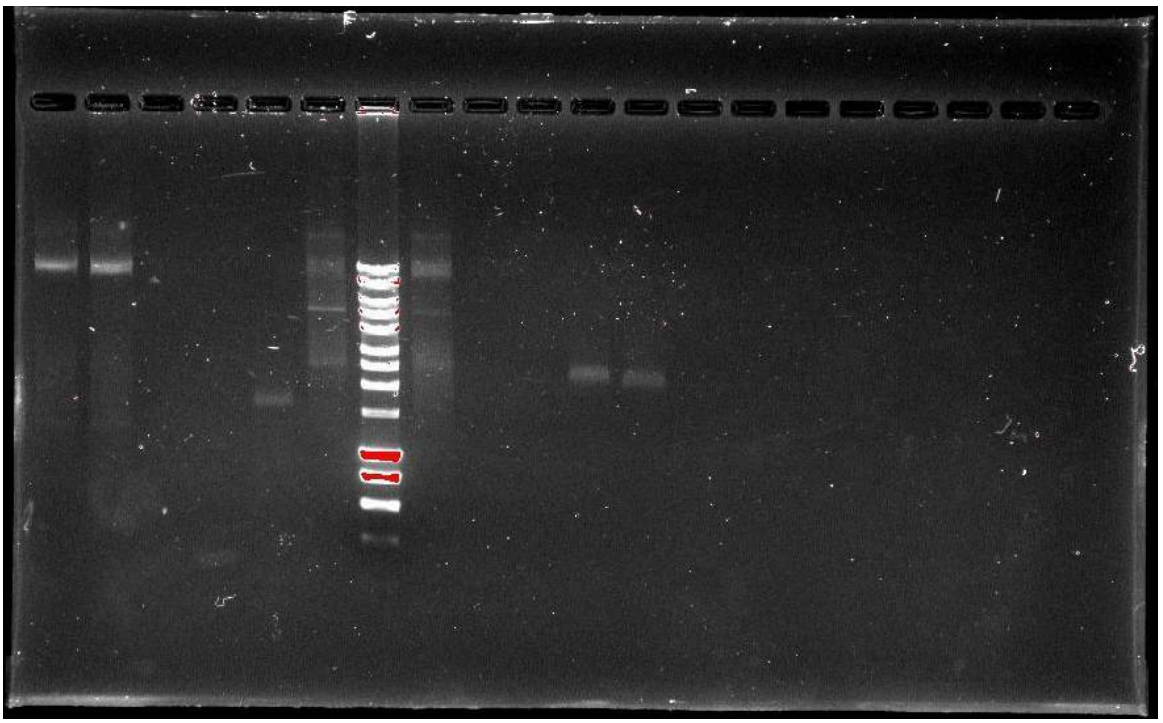
100 µL was plated for each plate.

Conclusion

Gel Electrophoresis

Wells of interest:

Well 7. High Range DNA Ladder Ampliqon (2 µL loaded)
Well 11. pEREmin (2000 + 300 = 2300) --> Perfect! (3µL loaded)
Well 12. pHREmin (2000 + 300 = 2300) --> Perfect! (3µL loaded)



Quantification

The pERE and pHRE are similar to the 400bp band (the last band in the ladder clearly visible starting from the bottom)
The ladder has 16 ng in that band.
Having said that, our samples have (more or less): 16ng / 3µL = **5.3 ng/µL**

Transformation

After 1 O/N only the positive control had colonies (red). We will wait one more day.
After waiting for 3 days:

- PC: Too Many To Count --> 270 ng of DNA
- NC: 0 (as expected)
- pERE: 0
- pHRE: 1

Notes:

- The 3 DNAs are stored in the freezer in the GMO lab in skylab
- User cloning tips: Next time, add more DNA (add no water), so the DNA will be more concentrated

2024.07.10 PCRs and Gels. Preparation for USER reaction in receptor production

Overview

Track: Receptor production
Objective: Re-run PCRs for the plasmid, genes and the his-tag

When: 09.07.2024a
Who: Reinis
Location: 202 teaching lab

Methods/protocol

Protocol: *PCR with Phusion U Hot start polymerase*

Changes since yesterday:

- higher annealing temperatures from the calculator
- DMSO for the his-tag and plasmid

Mixes:

HF buffer:

	25 µL	5x 25 µL	Final concentration
H_2O	16.25	81.25	
5X HF Buffer	5	25	1x
10 mM dNTPs	0.5	2.5	200 µM (each)
Primer FWD (10 µM)	1	-	0.4 µM
Primer RV (10 µM)	1	-	0.4 µM
Phusion U Polymerase	0.25	1.25	0.02 U/µL
Template	1	-	1 pg - 10 ng DNA per 50 µL

GC buffer:

	25 µL
H_2O	16.25
5X GC Buffer	5
10 mM dNTPs	0.5
Primer FWD (10 µM)	1
Primer RV (10 µM)	1
Phusion U Polymerase	0.25
Template	1

22 µL of Master Mix to 5 tubes and add 2 primers + template

Sample no.	Primer forward	Primer reverse	Template	Buffer	DMSO	Annealing temp	Elongation time
36	FW_p414_gene	RV_p414	100x p414-TEF-Cas9	GC	+	65	2:20

37	FW_p414_gene	RV_p415	100x p414-TEF-Cas10	HF	+	65	2:20
38	FW_tag	RV_tag_gene	alfa_6xHIS	HF	+	61	1:15
39	FW_HERa	RV_HERa	HERa	HF	-	61	1:15
41	FW_HERb_new	RV_HERb_new	HERb_new	HF	-	61	1:15
42	FW_NR3C1_new	RV_NR3C1_new	NR3C1_new	HF	-	61	1:15

Results & Conclusions

Failed. Will try again.

2024.07.10 USER cloning #2 for enzyme production

Overview

Track: Receptor production
Objective: Do the user cloning to assemble the production plasmids with the tag in them.

When: 09.07.2024
Who: Reinis
Location: 202

Methods/protocol

User Cloning

Protocol: *USER cloning*

PCR products used:

USER tube name	Backbone tube	Tag tube	Gene tube	Gene name
U7	32	30	39	HERa
U8	32	30	16	HERb
U9	32	30	17	NR3C1_new
U10	32	30	31	HERb_new

Master mix:

Reagent	Volume µL	MM x 4
MQ water	1.5	6
Backbone PCR	2	8
Tag PCR	2	8
Gene PCR	2	-
CutSmart buffer	0.5	2
USER enzyme	1	4
DpnI enzyme	1	4
Total	10	32

Results & Conclusions

Failed. Will try again.

2024.07.10 Yeast ERα inoculation for In-Cell Assay

Overview

Track: In-Cell Assay
Objective: Inoculate ERα yeast cells from glycerol stock

Who: Georgios
Location: GMO Skylab
Yeast strain: S. cerevisiae CENPK113-3C

Methods/protocol

- As we need active yeast for the In-Cell Assay, we inoculate the stock form the -80℃ to YBN broth with 2% glc. (10 mL in total)
- Incubate it in 30 degrees and shake with 220 rpm.

Conclusion

- After two days, there is a great pellet in the bottom of the 15 mL falcon

2024.07.11. PCR for the backbone and HERα

Overview

Track: ROSALIND
Objective: Re-run PCRs for the plasmid, genes and the his-tag, use higher annealing temperatures from the calculator and also DMSO for the tag and plasmid

When: 11.07.2024a
Who: Reinis
Location: 202 teaching lab

Methods/protocol

Protocol: *PCR with Phusion U Hot start polymerase*

Mixes:

HF buffer:

	25 µL	2x 25 µL	Final concentration
H_2O	14.25	28.5	
5X HF Buffer	5	10	1x
10 mM dNTPs	0.5	1	200 µM (each)
Primer FWD (10 µM)	1	-	0.4 µM
Primer RV (10 µM)	1	-	0.4 µM
Phusion U Polymerase diluted 1/10	2	4	0.02 U/µL
Template	1	-	1 pg - 10 ng DNA per 50 µL

GC buffer:

	25 µL
H_2O	14.25
5X GC Buffer	5
10 mM dNTPs	0.5
Primer FWD (10 µM)	1
Primer RV (10 µM)	1
Phusion U Polymerase diluted 1/10	2
Template	1

Add 22 µL of Master Mix to 5 tubes, add 2 primers and template.

Sample no.	Primer forward	Primer reverse	Template	Buffer	Annealing temp	Elongation time
43	FW_p414_ gene	RV_p414	100x p414-TEF-Cas9	GC buffer	64	1:25
44	FW_p414_ gene	RV_p415	100x p414-TEF-Cas10	HF buffer	64	1:25
45	FW_HERa	RV_HERa	HERa	HF buffer	56	0:26

Results & Conclusions

43 and 44 seem to work as previously, HERα didn't get amplified.

2024.07.12 E. coli Transformation pEREmin & pHREmin #2

Overview

Track: ROSALIND
Objective: Re-transformation of:

- 2. *ERE minimal Broccoli*
- 4. *HRE minimal Broccoli* fused with *pSB1C30_BBa_J04450* .

Improving the DNA quantity from the previous experiment: *2024.07.09 User Cloning of pEREmin & pHREmin: Gels & Transformation*

When: 12.07.2024
Who: Sirio
Location: GMO skylab

Methods/protocol

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

The DNA solutions added were done with the rational to increase the concentration of our samples (pERE & pHRE) and to lower the one of the PC.

DNA quantity:

- PC (*pSB1C30_BBa_J04450*): 134 ng/µL
- pERE & pHRE: ~5.3 ng/µL

Calculations:

- PC: 1 µL added --> 134 ng of DNA
- pERE & pHRE: 10µL --> 5.3*10 = 53 ng of DNA added

We are aware that the protocol states that we should add more than 5% of volume (so 2.5 µL) while we are adding 20%, but it's because the DNA quantity is too low.

Plating:

- PC: 60 µL --> Corresponding to 54 ng of DNA (134 ng * 60/150 = 54)
- pHRE & pERE: 150 µL --> Corresponding to 53 ng of DNA (53 ng * 150/150)
- NC: 150 µL

Conclusion & Results

Didn’t work.

2024.07.12 Prepare first test of In-Cell Assay

Overview

Track: In-Cell Assay
Objective: Prepare the final small things for the In-Cell Assay
When: 12.07.2024
Who: Georgios
Location: GMO Skylab

Methods/protocol:

Protocol: *In cell β-galactosidase assay* (for Sunday)

- 6 x 96 well plates were sterilized for 5 min in ethanol and then dried in the BSC. 3 of them will be used as lids for the other 3.
- Sodium carbonate solution was prepared (5.295g in 50 mL Milli-Q)
- The estradiol was diluted all the way to 2.275 μM for the test round.
- Inoculated 0.1 mL of the ERα yeast culture in 10 mL YNB glc media in a 250 mL Erlenmeyer flask.

Conclusion

- Tested out the spectrophotometer
- We're gonna start the assay on Sunday

2024.07.14 PCR p414 & Digestion with restriction enzymes

Overview

Track: ROSALIND
Objective: Digest the PCR products with restriction enzymes to assess their specificity

When: 14.07.2024
Who: Sirio
Location: 202

Methods/protocol

PCR

Protocol: *PCR with Phusion U Hot start polymerase*

GC buffer:

	25 µL	2 x 25µL
H_2O	14.25	28.5
5X GC Buffer	5	10
10 mM dNTPs	0.5	1
Primer FWD (10 µM)	1	-
Primer RV (10 µM)	1	-
Phusion U Polymerase diluted 1/10	2	4
Template	1	-

HF buffer:

	25 µL	2x 25 µL	Final concentration
H_2O	14.25	28.5	
5X HF Buffer	5	10	1x
10 mM dNTPs	0.5	1	200 µM (each)
Primer FWD (10 µM)	1	-	0.4 µM
Primer RV (10 µM)	1	-	0.4 µM
Phusion U Polymerase diluted 1/10	2	4	0.02 U/µL
Template	1	-	1 pg - 10 ng DNA per 50 µL

Samples:

	Primer forward	**Primer reverse**	**Template**	**Buffer**	**Temp. Ann.**	**Elong. time [min:ss]**
46	FW_p414 _gene	RV_p414	100x p414-TEF-Cas9	GC buffer	65	3:00
47	FW_p414 _tag	RV_p414	100x p414-TEF-Cas9	GC buffer	65	3:00
48	FW_ERE _minimal	RV_ERE_ minimal	HRE minimal Broccoli	HF buffer	55	0:30
NC	/	/	/	HF buffer		

DNA Digestion with Restriction Enzymes

Protocol: *DNA Digestion with Restriction Enzyme*

*Samples in purple were not prepared due to the fact that the DNA template was finished or not enough!

DNA sequence	DNA quantity [µL]	Restriction Enzyme 1	Restriction Enzyme 2	Buffer	Recom. Incub. Time	Actual Incub. Time
p414	3	SspI		NEBuffer r2.1	5-15 min	15 min RT + 15 37°
p414	3	SspI	BglI	NEBuffer r3.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 32	5	SspI		NEBuffer r2.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 32	5	SspI	BglI	NEBuffer r3.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 33	5	SspI		NEBuffer r2.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 33	5	SspI	BglI	NEBuffer r3.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 46	5	SspI		NEBuffer r2.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 46	5	SspI	BglI	NEBuffer r3.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 47	5	SspI		NEBuffer r2.1	5-15 min	15 min RT + 15 37°
PCR prod. of p414: Tube 47	5	SspI	BglI	NEBuffer r3.1	5-15 min	15 min RT + 15 37°
PCR prod. HERa: Tube 15	5	HindIII		NEBuffer r2.1	1h	1h

Gel Electrophoresis

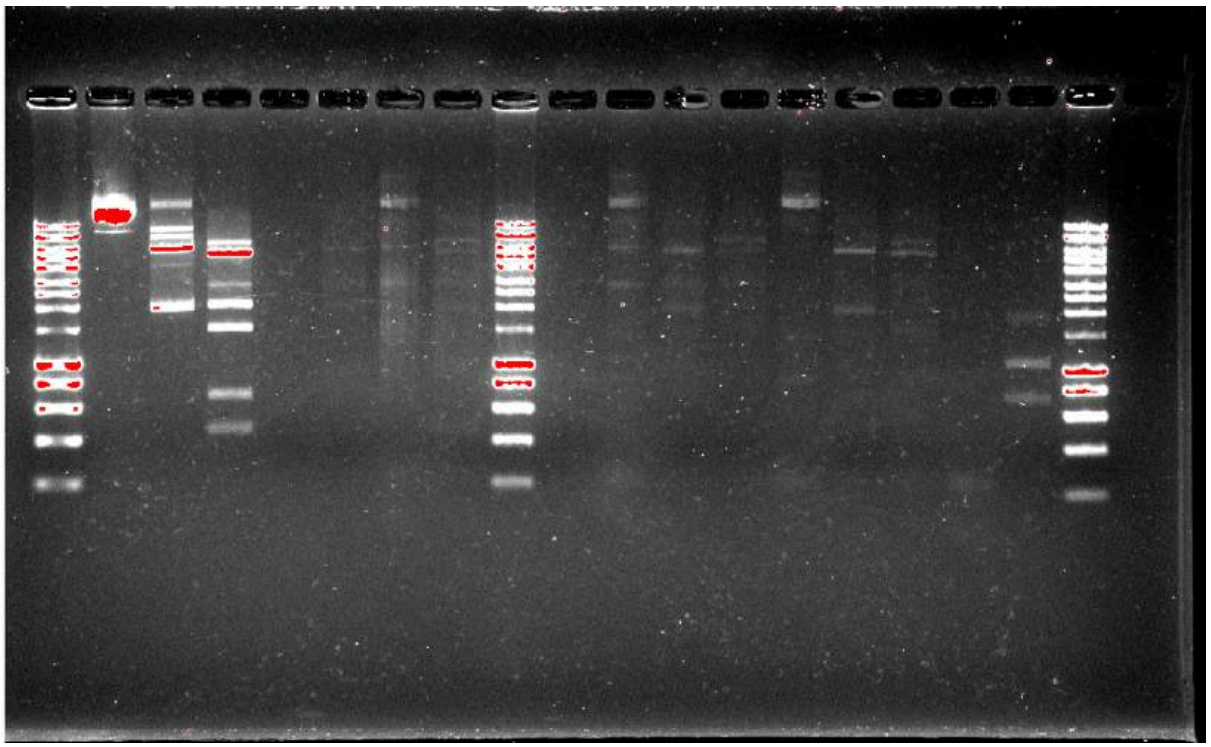
Protocol: *Agarose gel preparation aka Electrophoresis Run*

Samples:

- To the samples from the digestion (25µL) 5 µL of Loading Dye was added
- In the other case the procedure was as usual (1 µL LD, 3 H_2O, and 2µL of DNA)

1	*p414*	*p414 + SspI*	*p414 + SspI & BglI*	*32 (p414 PCR)*	*32 (p414 PCR) + SspI*	*Ladder*	*33 (p414 PCR)*	*33 (p414 PCR) + SspI & BglI*	*NC*	*46 (p414 PCR)*	*46 (p414 PCR) + SspI*	*46 (p414 PCR) + SspI & BglI*	*Ladder*	*47 (p414 PCR)*	*47 (p414 PCR) + SspI*	*47 (p414 PCR) + SspI & BglI*	*48: EREmIn*	*HERa*	*Hera + HindIII*
2	Ladder	**p414**	p414 + SspI	p414 + SspI & BglI	**32 (p414 PCR)**	32 (p414 PCR) + SspI	**33 (p414 PCR)**	33 (p414 PCR) + SspI & BglI	Ladder	NC	**46 (p414 PCR)**	46 (p414 PCR) + SspI	46 (p414 PCR) + SspI & BglI	**47 (p414 PCR)**	47 (p414 PCR) + SspI	47 (p414 PCR) + SspI & BglI	**48: HREmIn**	48 HREmIn + HindIII	Ladder

Conclusion



2024.07.14 In-Cell Assay protocol day 1

Overview

Track: In-Cell Assay
Objective: Fill the 96-sterile plate following the layout and incubate for 17 hrs

When: 14.07
Who: Georgios
Location: GMO Skylab

Methods/protocol

Protocol: *In cell β-galactosidase assay* (pay attention mainly to the entry as some changes were made)

Notes:

- The yeast broth after 2 O/Ns was full of cells.
- The sterilized wells were stored one up the other, as a result some were not totally dry. I used the most dry, the rest were left up side down covered with paper for future use.
- The source cells(with 320 μL) were almost full, so the plates are needed to handing with cautious
- I didn't have enough. I used the same for the dilution of each row (column 1 to column 12).

OD600 measurement for yeast growth:

- Firstly, 1:6 dilution was selected as the main protocol advised. So, 1 mL of the cell broth was mixed with 6 mL of YBN with 2% galactose (galactose yeast medium) and the OD was measured. Blank was cuvette with 1 mL galactose yeast medium. The absorbance was over 1 unit.
- Then, 2 times dilution was selected, adding 6mL of the medium to the final volume of 12 mL. The absorbance was 0.7 compared with the 0.065 that the protocol suggested.
- Then, 1 mL of this was mixed with 9 mL of the medium to final 20 times dilution from the starting point. Then, the absorbance was 0.085(0.065 +- 0.005 in the protocol) and I decided to work with this.

Fill the plate:

The layout below was followed:

- First, I pipette all the cell with the proper amount of yeast suspension, then I dilute the hormone and the EtOH, then then the dilutions and finally the blank (with only medium)
- Each row was uniquely replicated, so changing tips is important.
- I made the dilutions in the first replicate, then the second and then the third(3 minutes gap among them)

	1	2	3	4	5	6	7	8	9	10	11	12
A	320uL yeast 5uL E2	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast
B	320uL yeast 5uL E2	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast
C	320uL yeast 5uL E2	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast
D	320uL yeast 5ul ETOH	120uL gal										
E	320uL yeast 5ul ETOH	120uL gal										
F	320uL yeast 5ul ETOH	120uL gal										
G												
H												

Conclusion

- We need to wait at least 17 hours.

2024.07.15 pERE glycerol stock + plasmid purification

Overview

Track: ROSALIND
Objective: Storage of the E. coli cells with the pERE plasmid in -80℃ and purify the plasmid for further assays

When: 15.07.2024
Who: Georgios
Location: GMO Skylab

Methods/protocol

Protocols:

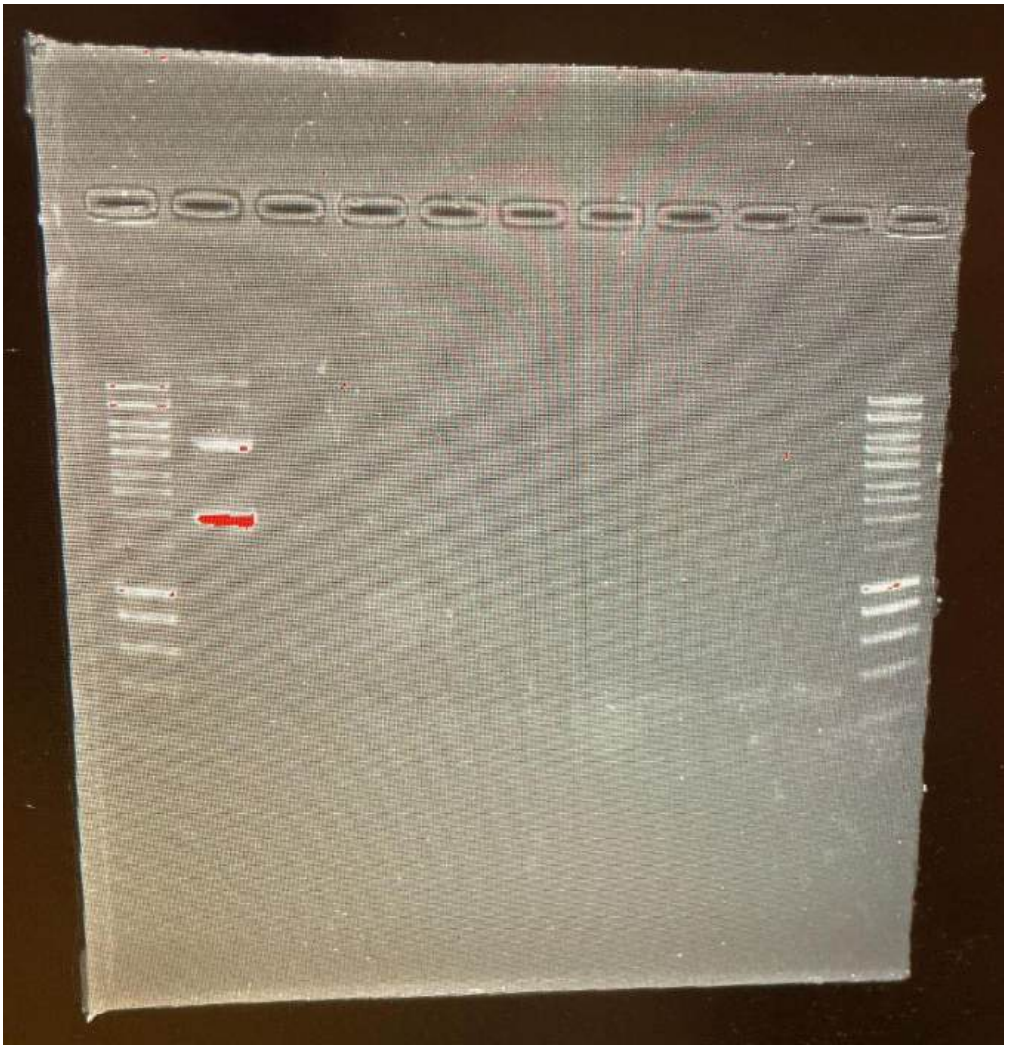
1. Preparation of glycerol stocks
2. Monarch Plasmid Miniprep Kit (For purification of plasmids from E. coli)

Purification protocol:

- 2 X 2 mL of the E. coli broth was used.
- 50 µL of Elution buffer was used and.
- Spin 1 minute at 16.000 RCF.

Conclusion

- The plasmid purification product was loaded in the second well (next of the first ladder). The expected plasmid was 2.4 kb. We can see a bright band a little below this linear ladder, so maybe the plasmid is supercoiled and runs faster. The second bright band is between 4 and 5 kb, maybe related with the 2 X the 2.4 kb of the plasmid but no clue for that and also for the rest two faint bands (as the insert has many complementary nucleotides, see *2. ERE minimal Broccoli* .
- From the ladder brightness, we can see that the two main bands are as bright or more than the 1kb band, so because the 1kb band has 20 ng/µL, we assume that we have loaded around **50 ng in 1 µL from the sample**.
- We can digest with two enzymes. SspI has a unique site inside the backbone and HindIII has an unique site inside the insert part.
- Useful video for gel and digestion interpretation: https://blog.addgene.org/plasmids-101-how-to-verify-your-plasmid



2024.07.17 Restriction enzymes digestion of PCR products

Overview

Track: ROSALIND
Objective: Digest the PCR products with restriction enzymes to assess their specificity

When: 14.07.2024
Who: Sirio
Location: 202

Methods/protocol

PCR

Protocol (Regular Phusion instead of Phusion U): *PCR with Phusion U Hot start polymerase*

	25 µL	2x 25 µL	Final concentration
H_2O	17.25	34.5	
5X HF Buffer	5	10	1x
10 mM dNTPs	0.5	1	200 µM (each)
Primer FWD (10 µM)	0.5	1	0.4 µM
Primer RV (10 µM)	0.5	1	0.4 µM
Phusion Polymerase	0.25	0.5	0.02 U/µL
Template	1	-	1 pg - 10 ng DNA per 50 µL
sum	25		

53 - gBlock ERE minimal broccoli
54 - plasmid

2024.07.17/18 In-Cell Assay #2

Overview

Track: In-Cell Assay
Objective: Complete the In-Cell Assay for the ERα, ERß, MR, GR, AR receptors.

When: 17-18.07.2024
Who: Mikkel
Location: GMO Skylab

Methods/protocol

Protocol: *In cell β-galactosidase assay*

Day 1

- The yeast O/N cultures were very high OD. Diluted them 12 times and checked the OD in the microplate reader (120 µL). The OD for the 5 diluted cultures were 0.078, 0.090, 0.085, 0.095, 0.084, respectively. Went on with this culture.
- Used 96-well plates that had been bathed for 5 min in 70% ethanol and dried upside-down O/N. Used plates as lids as well.
- Added yeast to all the wells according to this plate layout with a single-channel pipette:

	1	2	3	4	5	6	7	8	9	10	11	12		Plate 1
A	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erelpha	
	5uL E2													
B	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erelpha	
	5uL E2													
C	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erelpha	
	5uL E2													
D	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erbeta	
	5uL E2													
E	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erbeta	
	5uL E2													
F	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	Erbeta	
	5uL E2													
G														
	Erelpha	Erelpha	Erelpha	Erbeta	Erbeta	Erbeta								
H	320uL yeast	320uL yeast	320uL yeast	320uL yeast	320uL yeast	320uL yeast	120uL gal	120uL gal	120uL gal					
	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH								

	1	2	3	4	5	6	7	8	9	10	11	12		Plate 2
A	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	MR	
	5uL E2													
B	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	MR	
	5uL E2													
C	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	MR	
	5uL E2													
D	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	GR	
	5uL E2													
E	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	GR	
	5uL E2													
F	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	GR	
	5uL E2													
G														
	MR	MR	MR	GR	GR	GR								
H	320uL yeast	320uL yeast	320uL yeast	320uL yeast	320uL yeast	320uL yeast	120uL gal	120uL gal	120uL gal					
	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH	5ul ETOH								

	1	2	3	4	5	6	7	8	9	10	11	12		Plate 3
A	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	AR	
	5uL E2													
B	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	AR	
	5uL E2													
C	320uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	120uL yeast	AR	
	5uL E2													
D														
E														
F														
G														
	AR	AR	AR											
H	320uL yeast	320uL yeast	320uL yeast				120uL gal	120uL gal	120uL gal					
	5ul ETOH	5ul ETOH	5ul ETOH											

- Then, added the controls and the hormones. Used the following concentrations:
    - 2.275 µM estradiol for ERα #1
    - 9.75 nM estradiol for ERβ #2
    - 1.00 mM aldosterone for MR #3
    - 1.00 mM dexamethasone for GR #4
    - 100 µM testosterone for AR #5
- Finally used a multichannel pipette to make the dilution row. Left out the 12th sample as there wasn't enough culture.

- Plate #1 contained the ERα and ERß - was incubated at 14:51
- Plate #2 contained the MR and GR- was incubated at 15:18
- Plate #3 contained the AR - was incubated at 15:40

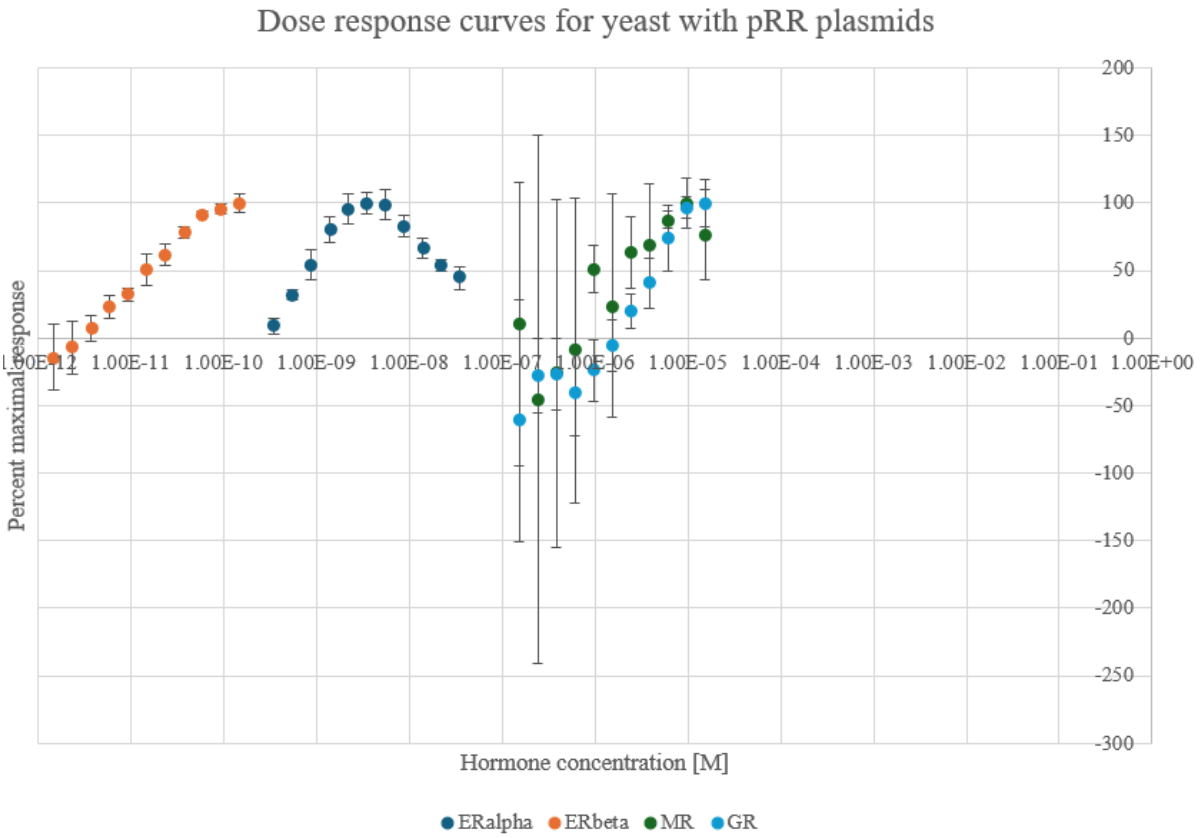
Day 2

- Plan: Start the assays at 07:51, 08:18, and 08:40 (17h incubation)
- Actual starts were very skewed (Remember to thaw LacZ buffer in good time).
- Transferred 50µL from the plates to a new sterile plate using a multi-channel pipette. Same lid.

- Added the LacZ buffer from a multi-channel bath thing
- Incubated until yellow.
    - As they grew too long, this was very quick and I couldn't keep up.

Conclusion

- The first plate contained the ERα and ERß. This looks great.
- The 2nd plate was delayed longer as I couldn't keep up. High OD405 values which can be seen in the results.
    - The concentration range looks correct though!
- The 3rd plate contained the AR receptor and didn't make sense at all. Highest OD405 values were on the controls - which resulted in negative dose response curves.



- I wanna try again. This time:
    - Remember to thaw LacZ buffer in good time. Really important.
    - Only 2 plates. One with ERα + AR and one with MR + GR. Leave out ERß as we have it already. Keep ERα as a control.
    - Grow cells in Erlenmeyer flasks! This could create a more homogenous growth - and more stable OD610 values.

2024.07.23 USER cloning

Overview

Track: Receptor production
Objective: Do the user cloning to assemble the production plasmids with the tag in them.

When: 09.07.2024
Who: Reinis
Location: 202

Methods/protocol

Protocol: *USER cloning*

Mixes:
The following PCR products were used:

USER tube name	Backbone tube	Tag tube	Gene tube	Gene name
U20	61	60	39	HERa
U21	61	60	41	HERb_new
U22	61	60	42	NR3C1_new

2024.07.26-27 In-Cell Assay #3

Overview

Track: In-Cell Assay
Objective: Complete the In-Cell Assay for the ERα, MR, GR, AR receptors.

When: 26-27.07.2024 (prep 22-25.07.2024)
Who: Mikkel + Georgios
Location: GMO Skylab

Methods/protocol

Protocol: *In cell β-galactosidase assay*

Prep:
22.07.24

- Streaked the yeast freezing stocks for the 4 receptors in question + the PR receptor in case the progesterone arrived. All were streaked on YNB + 2% glc plates.

24.07.24

- For each of the 5 plates, I inoculated 10 mL YNB + 2% glc broth with a colony in a 100 mL Erlenmeyer flask (autoclaved the day before)
- Left the plates in the fridge.

25.07.24

- Sterilize 6 x 96-well plates in an ethanol bath for 5 min. Dry overnight.

Day 1

26.07.24

- Prepared the In-Cell Assay plates. Plate #1 with ERα + AR and Plate #2 with MR + GR
    - Messed up a bit in the layout of plate #2, but I think it was fixed.
- Incubated at 16:47 and 17:35

Day 2

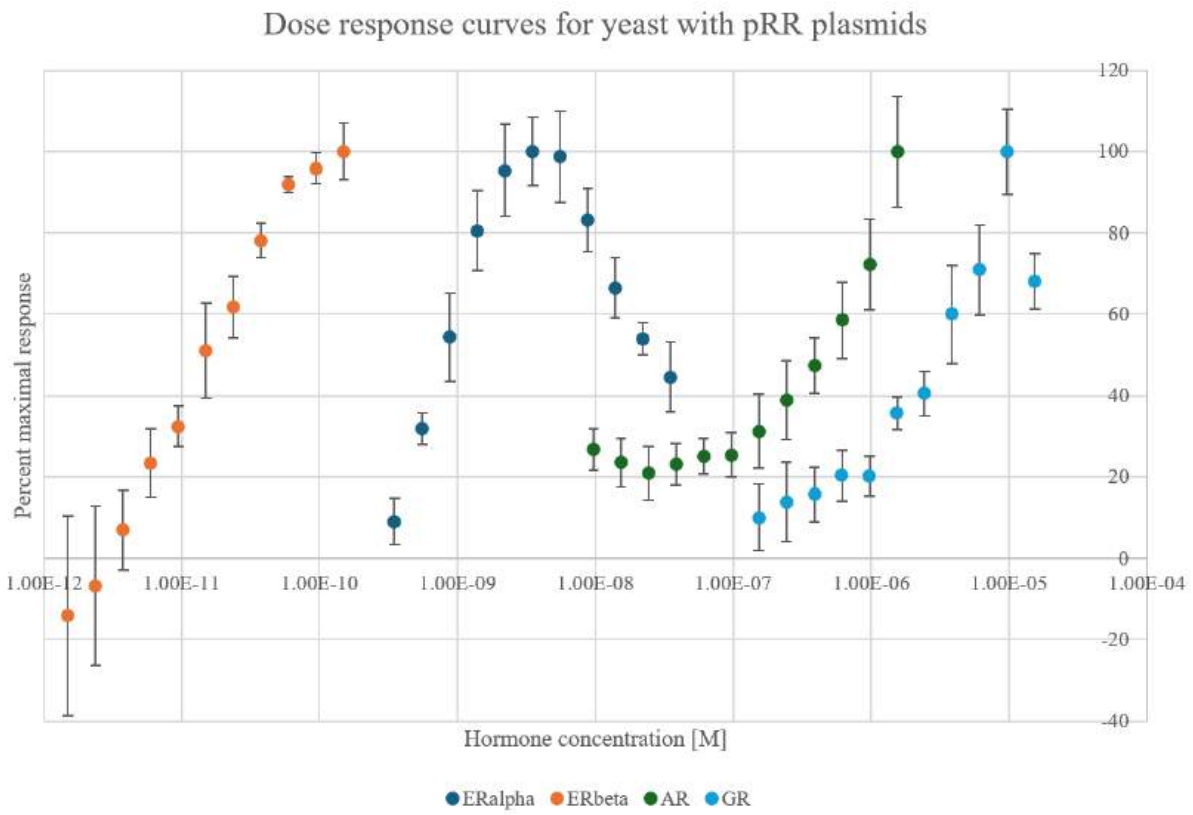
27/7-24

- Took the LacZ buffer out at 9:00
- Measured (610 nm) and incubated the first plate (ERα+AR) at 9:55
- Measured (405 nm) the AR at 10:12
- Measured (610 nm) and incubated the second plate (MR+GR) at 10:18
- Measured (405 nm) the MR at 10:30
- Measured (405 nm) the ERα at 10:33
- Measured (405 nm) the GR at 10:39

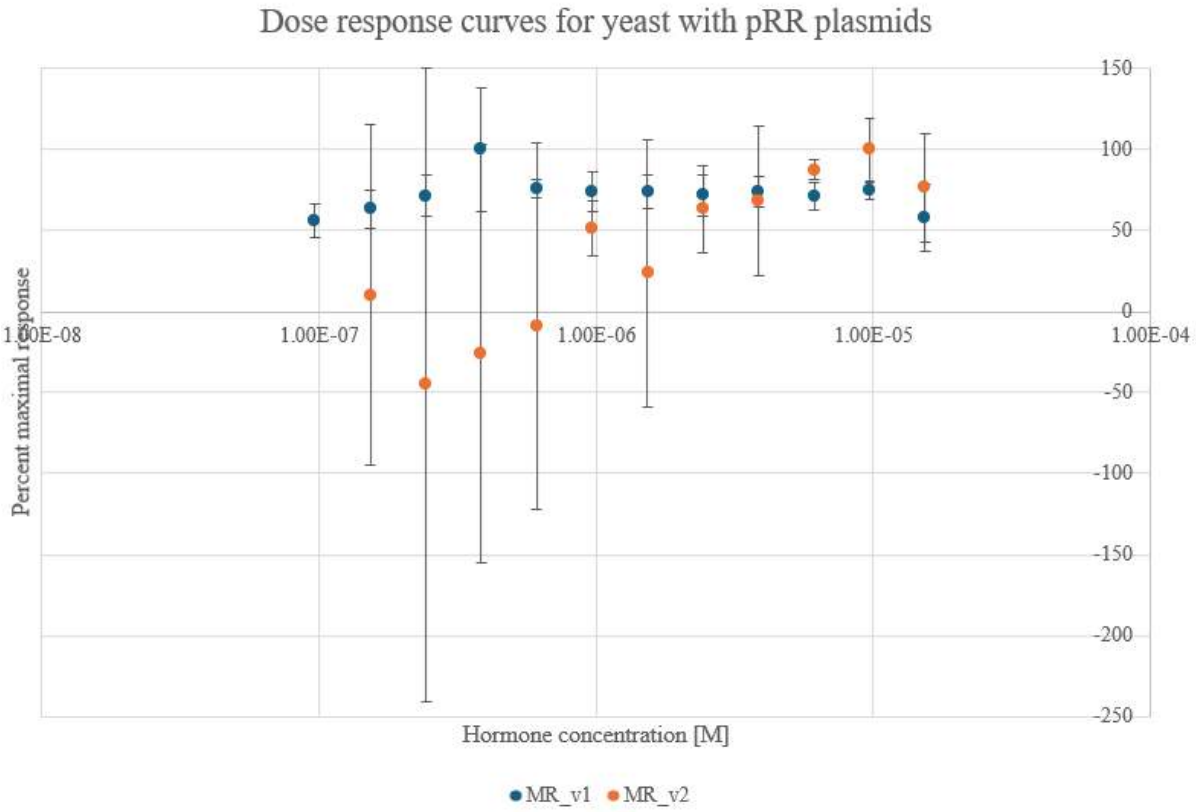
Conclusion

ER, AR, GR look great!
MR looks weird again. Lower standard deviations this time, but no rising tendency of the curve.

These are now our results for the 4 working sensors



These are the two attempts with MR



2024.07.27 Digestions of pEREmin and Gel for the Rosalind gBlocks

Overview

Track: ROSALIND
Objective: Validate the integrity of the plasmid pERE and run again the amplified gBlocks for the cell free

When: 27.07.2024
Who: Georgios
Location: 202

Methods/protocol

Digestions

Protocol: *DNA Digestion with Restriction Enzyme*

- I selected to digest the *2. Rosalind pEREminimal plasmid* with enzymes *SspI* and *HindIII*. The first digest in the backbone *PCR product of the E. coli backbone(pSB1C30)* and the latter in the insert *2. ERE minimal Broccoli* part.
- 2 reactions were conducted - one with the double digestion and one with only HindIII (excess enzyme in the freezer).
- After the incubation, 5 μL of loading buffer was added to stop the reaction.

DNA sequence	DNA quantity [μL]	Restriction Enzyme 1	Restriction Enzyme 2	Buffer	Recom. Incub. Time	Actual Incub. Time	Incub Temp
pERE minimal	5	SspI		**NEBuffer r2.1**	1h	1h	37
pERE minimal	5	SspI	HindIII	**NEBuffer r2.1**	1h	1h	37

Gel Electrophoresis:

Protocol: *Agarose gel preparation aka Electrophoresis Run*

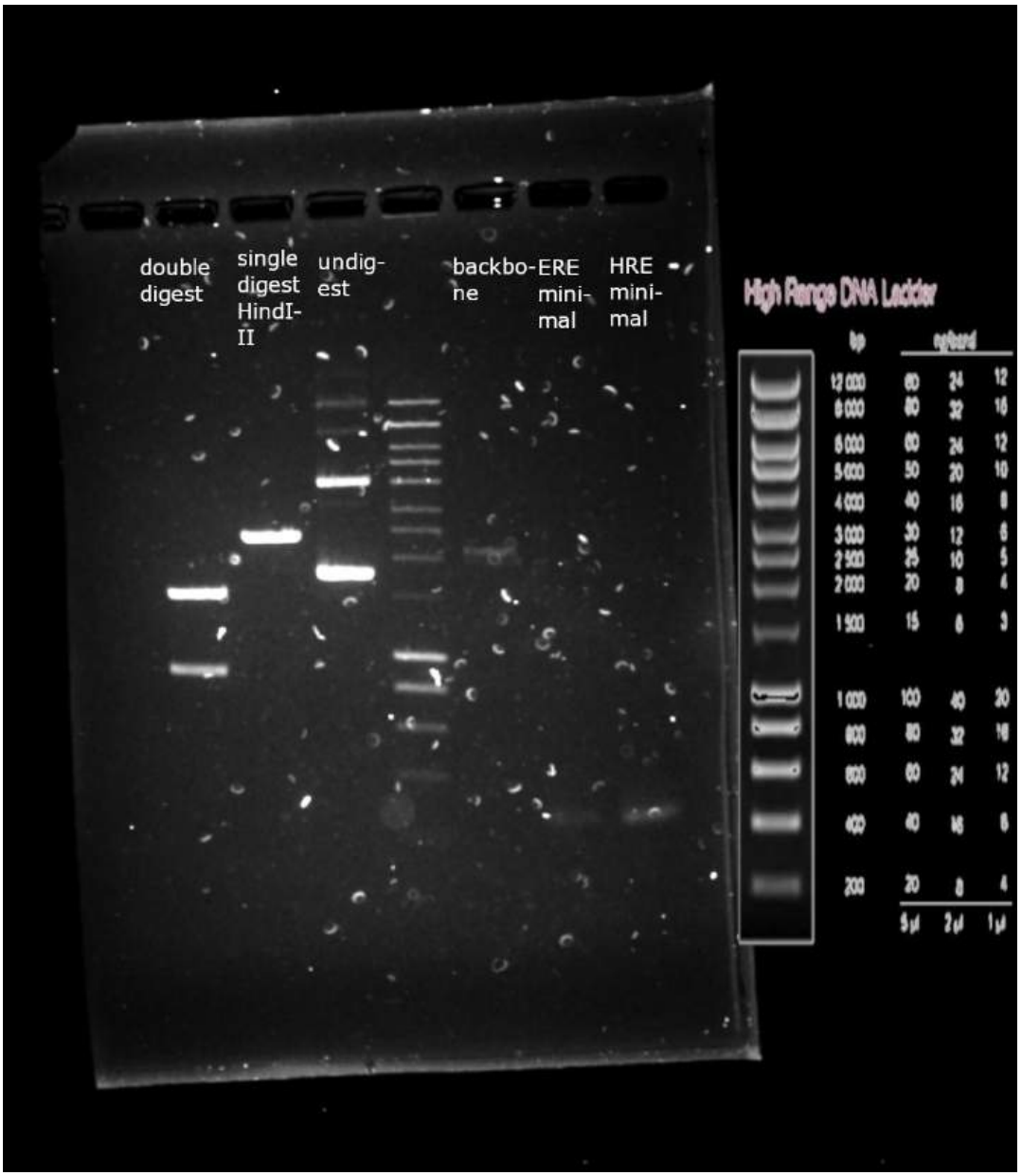
Info for the buffers and temp look here:
NEB restriction enzymes manual - https://nebcloner.neb.com/#!/redigest

- I run it for 31 minutes(4 with 220 constant Volt, 27 with 200 constant Volt)
- The layout of the gel as well the volumes loaded were shown in the table:

Gel Layout

sample	*pERE +double digestion*	*pERE+ HindIII*	*pERE undigest*	*Ladder*	*100X pSB1C30red (reaction #23)*	*ERE minimal (reaction #25)*	*HRE minimal (reaction #26)*
loading buffer vol	None	None	1µL	None	1µL	1µL	1µL
template vol	3µL	3µL	1µL	1µL	2µL	2µL	2µL
H_2O **vol**	3µL	3µL	4µL	5µL	3µL	3µL	3µL
expected bands	1533+862	2395	smear	amplicon high range ladder	2134	279	275

Gel image:



Conclusion

- The purified plasmid looks nice. The bands after the double and the single digestions are exactly where they should be.
- **Tool used to predict the bands:** https://nc3.neb.com/NEBcutter/prj/
- The amplified gBlocks look like they need to be amplified more to conclude, but they are located roughly in the right position (not easy to see ERE and HRE).

Notes:

- The running time was higher than it should be to see the last band of the ladder.

2024.07.29&30 PCRs for USER cloning

Overview

Track: Receptor production, ROSALIND
Objective: PCRs for USER cloning

When: 29-30.07.2024
Who: Reinis
Location: 202

Methods/Protocols:

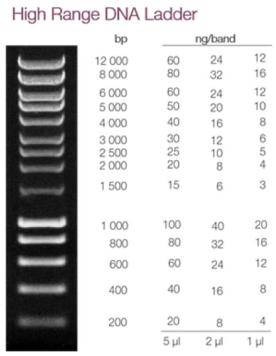
27.07.2024:
Performed PCR for following elements:

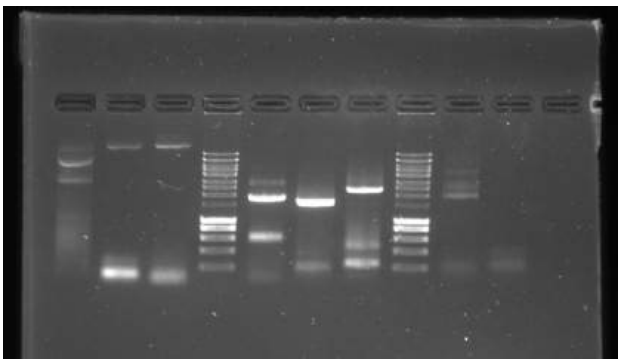
62 - pUC19 with the broccoli - 2919 bases
63 - ERα plasmid from In-Cell Assay - 128 bp
64 - MR plasmid from In-Cell Assay 135 bp
65 - HERα gBlock 1794 bp
66 - HERb_new gBlock 1593 bp
67 - NR3C1_new gBlock 2337 bp
68 - plasmid ERE broccoli (check insertion with rosalind primers) 285 bp
69 - ERE gBlock (positive control for tube 68) 285 bp

Conclusions:

Temperature settings were 65℃ annealing for 20 seconds and 68℃ extension for 45 seconds for all tubes.

All the PCRs worked, 68 and 69 is hard to tell but still seems that the insert gets amplified in tube 68.





30.07.2024:

Yesterday conducted PCR with the uracil primers, everything worked but produced several bands in some cases, however, still going through it

First, purification of the PCR products (60-67)

Quick rundown of the PCR tubes

60 - alfa_his tag
61 - p414 backbone
62 - pUC19 with the broccoli
63 - ERα plasmid from In-Cell Assay - HER5
64 - MR plasmid from In-Cell Assay - ERE5
65 - HERα gBlock
66 - HERb_new gBlock
67 - NR3C1_new gBlock

60 and 61 was eluted with 15 µL (starting volume was ~30 µL), for the rest the elution volume was 10 µL

Tube name	Backbone	Insert 1	Insert 2	Notes
U23	61	60	65	ERα
U24	61	60	66	ERbeta_new
U25	61	60	67	NR3C1 new
U26	62	63	-	ERE5
U27	62	64	-	HRE5

For each part we put 3 µL in the USER reaction, added USER mix and DnpI

Then transformed the plasmids into E. coli, adding 5 µL USER product to 50 µL competent cells. NC - negative control with nothing, PC - the p414 plasmid x100 dilution from original.

2024.07.31-08.01 Colonies pick-up, stock, and DNA extraction

Overview

Track: ROSALIND
Objective: Previously did the transformations into E.coli, on 31.07 there were 1 colony from U25, several colonies from U26 and U27. The colonies were marked, streaked on a new plate (all on one) and also put in overnight cultures.

TO DO:
On 1.08 check if there are new colonies on U23, U24. Check if the overnight cultures grew, if yes - make a -80℃ glycerol stock and purify the plasmids from them. Then we need to figure out if we got what we needed, maybe by restriction digestion or sequencing or PCR.

When: 01.08.2024
Who: Sirio, Cristian, Katerina
Location: GMO lab SkyLab

Methods/protocol

Protocol: *Preparation of glycerol stocks*

Glycerol stocks

- U23: ERα
- Glycerol stocks of U26, U27 were made even though there was no growth.

Picking colonies

Colonies picked and put into a solution: U26 (ERE5) -> 3; U27 (HRE5) -> 2
Empty plates: U24; U25

Conclusion

- U27 grew
- U26 didn't grow in the broth overnight

2024.08.02 Checking plasmids U23, U27 and transformation U24, U25

Overview

Track: Receptor production, ROSALIND
Objective: To check plasmids U23, U27 and transformation U24,U25

When: 08.02.2024
Who: Reinis, Katerina
Location: GMO Lab SkyLab

Methods/Protocols

Purified plasmids U23 (ERα) and U27 (HRE5)

PCR:

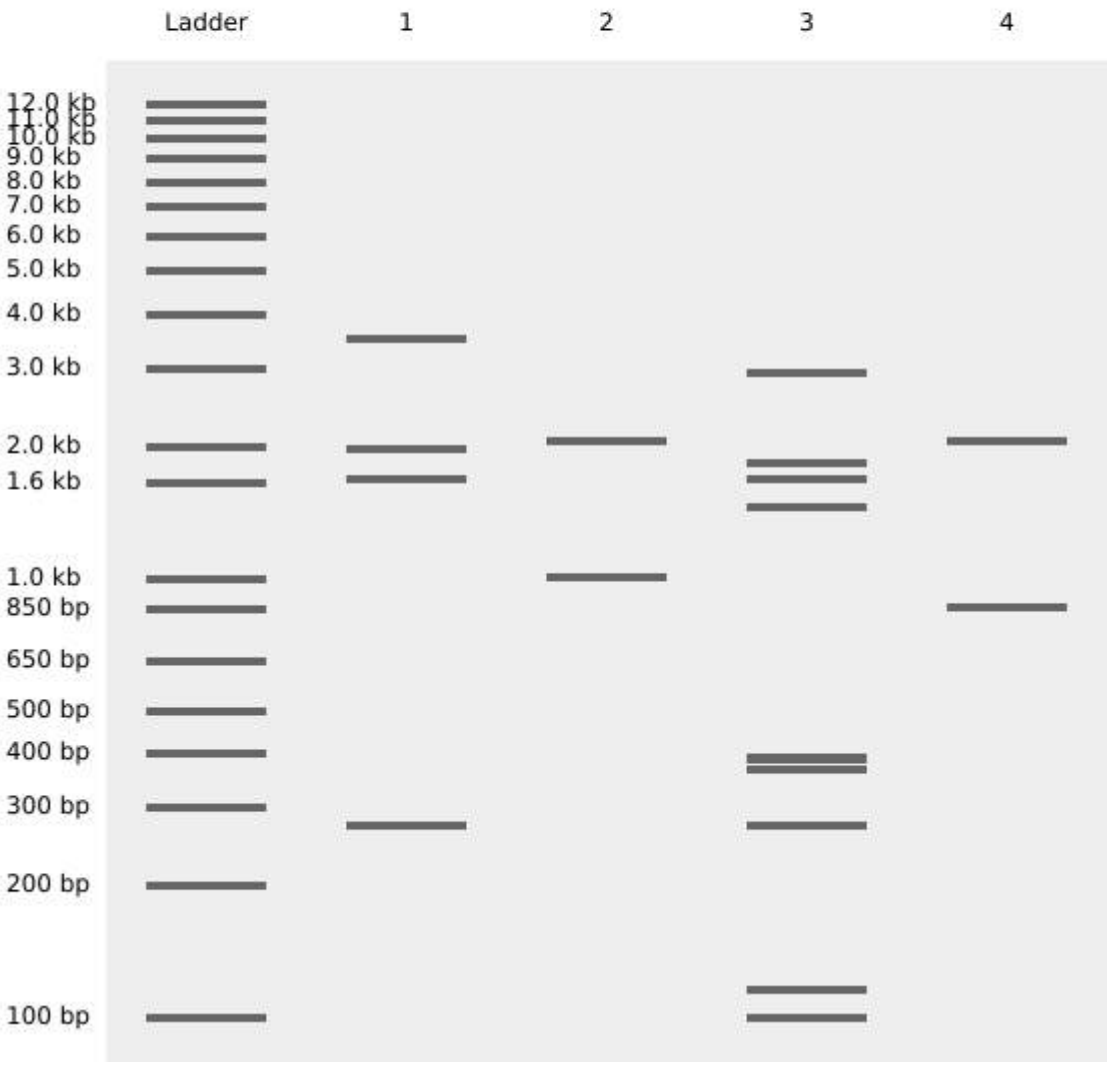
Tube name	Template	FW primer	RV primer	Presumed template
70	U23	FW_p414_gene	RV_tag_gene	p414-TEF-alfa-6xHIS-HERa

Digestions:

Tube name	Template	Restriction enzymes	Presumed template
71	U23	HindIII, SspI	p414-TEF-alfa-6xHIS-HERa
72	U27	HindIII, SspI	pUC19_HRE5
73	p414 x100	HindIII, SspI	p414-TEF-Cas9
74	pUC19	HindIII, SspI	pUC19-T7-3WJdB-T(Addgene)

Expected gel:

Ladder	Life 1 kb Plus
1	p414-TEF-alfa-6xHIS-HERa - HindIII SspI
2	pUC19_HRE5 - HindIII SspI
3	p414-TEF-Cas9 - HindIII SspI
4	pUC19-T7-3WJdB-T(Addgene) - HindIII SspI



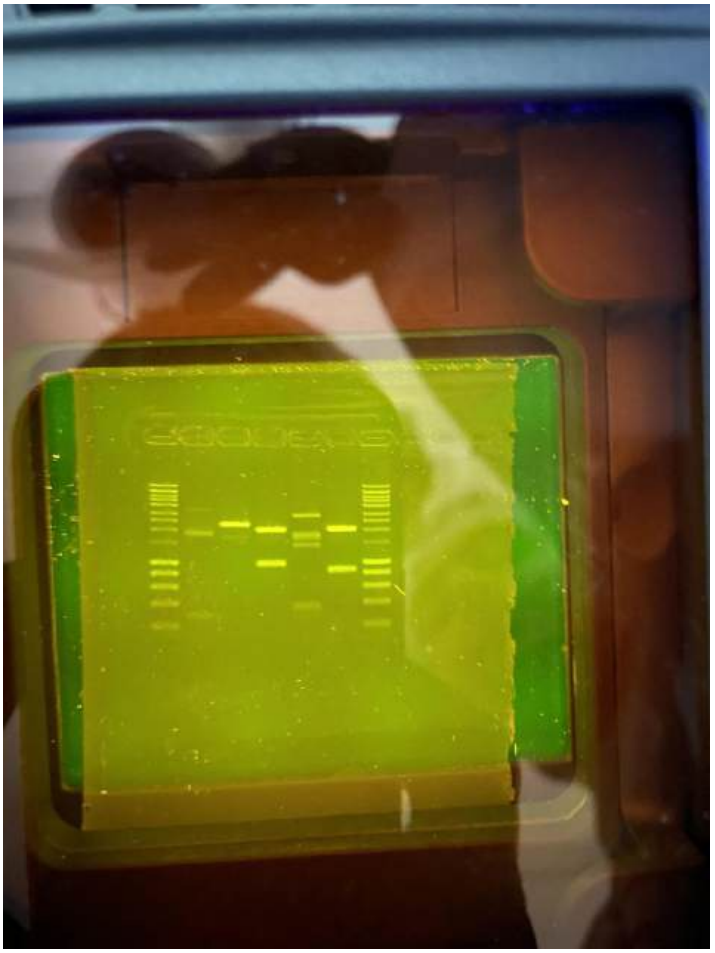
Transformation:

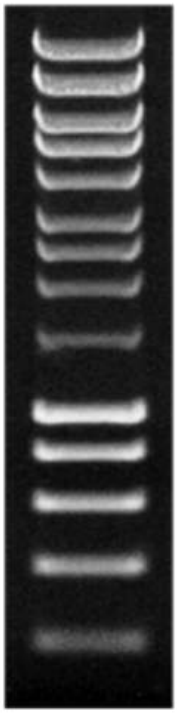
Transformed USER products U24 (ER_beta_new) and U25 (NR3C1_new) again, this time with heat shock after incubating on ice with DNA for 5 min. The heat shock was conducted at 42 degrees for 30 seconds, then put back on ice.

Outgrowth after adding 950 µL LB was done for 1 hour. Then the cells were centrifuged and concentrated 50 µL of all cells was put on plates. PC - p414 100x plasmid

Overnight cultures:
Checked in at 5 pm:

- U24 (ERbeta_new): No colonies
- U25 (NR3C1_new): No colonies
- PC: 5 colonies
- NC. no colonies





High Range DNA Ladder

bp	ng/band		
12 000	60	24	12
8 000	80	32	16
6 000	60	24	12
5 000	50	20	10
4 000	40	16	8
3 000	30	12	6
2 500	25	10	5
2 000	20	8	4
1 500	15	6	3
1 000	100	40	20
800	80	32	16
600	60	24	12
400	40	16	8
200	20	8	4
	5 µl	2 µl	1 µl

Conclusion

- The first well had the pcr reaction (tube #70) with an expected length of roughly 5.6 kb. Apparently something did not work well. We also observed that the tube was melted after the reaction(maybe pressured from the instrument lid).-> **need to repeat**
- The second well (the first of the digestions) is not performed well, given the fourth well (the circular plasmid provided by the supervisor diluted 100x) **the USER reaction need to be repeated**
- The third and fifth wells are correct, **meaning that we have the right HRE5 brick**

2024.08.03 User Cloning ROSALIND

Overview

Track: ROSALIND
Objective: USER cloning of ROSALIND template

When: 03.03.2024
Who: Sirio, Cristian
Location: GMO lab

Methods/protocol

User Cloning

Need to do it for:

- HREmin
- ERE5

Protocol: *USER cloning*

	HREmin U28	ERE5 U29	
MQ water	Enough to get to 20 µL	Enough to get to 20 µL	
PCR product #1	**5 µL (HREminimal) - PCR #26**	**5 µL (ERE5) - PCR #63**	300 (have the same size)
PCR product #2	**5 µL (E. coli backbone: 100x pSB1C30 red) - PCR #23**	**5 µL (pUC19 with the broccoli) - PCR #62**	2000
CutSmart buffer	1µL	1µL	
USER enzyme	1 µL	1 µL	
DpnI enzyme	1 µL	1 µL	
Total	**20 µL**	**20 µL**	**2300**

Add as much backbone as possible, since the DNA concentration of the user reaction is not enough. Then add the insertion to be in excess.

DNA concentration:

- 62: Less concentrate
- 63: More concentrated (more or less 5x)

Conclusion

- Gels run: **NOTHING IN THEM!!!**

2024.08.05 Plasmid purification for pERE5

Overview

Track: ROSALIND
Objective: purified the plasmid (user reaction #26). They are 3 separate colonies

When: 05.08.2024
Who: Georgios
Location: GMO Skylab

Methods/protocol

Protocol: *Monarch Plasmid Miniprep Kit (For purification of plasmids from E. coli)*

- In the purification protocol:
    - 2 X 2 mL of the E, coli broth were used
    - 50 µL of Elution buffer was used and 1 spinned at 16’000 RCF.

Conclusion

TO DO: A digestion to validate the correct constract.

2024.08.05 Stock preparation for IVT buffer

Overview

Track: ROSALIND
Objective: Weight and sterile stocks of the chemicals we need for the in vitro transcription.

When: 05.08.2024
Who: Georgios
Location: Skylab Multilab

Methods/protocol

Protocol: *Rosalind Manual.pdf*

IVT buffer ingredients:

- Tris-HCl
- $MgCl_2$
- DTT
- Nacl
- Spermidine

IVT buffer recipe:

- Each of these solutions was made with 1M except NaCl with 4M.
- All the solutions were made with final volume 1mL (less remaining after filter-sterilization)
- **For 1M Tris-HCl**: 0.1211 g of Trizma in 1 mL MilliQ
- **For 1M $MgCl_2$** (2x aliquot): 0.0952 g of $MgCl_2$ powder to 1 mL MilliQ
- **For the DTT**, the solution is already in 1 M concentration
- **For 4M NaCl**: 0.2337 g if Nacl powder in 1 mL MilliQ
- **For 1M Spermidine**: 0.1562 mL (neat liquid with 6.4M in garage) in 0.843.75 mL MilliQ
- After all, each of them were passed through filters and stored to new 1.5 mL microcentrifuge tubes (room temperature for all except DTT).

2024.08.07 Whole Lotta Stuff

Overview

Track: Receptor production
Objective: PCRs and USER cloning of parts, E. coli and S. cerevisiae transformation

When: 07.08.2024
Who: Georgios
Location: Skylab Multilab

TODO:
1. Transfer the yeast to the other flask
2. Do the E. coli competent cells
3. PCRs
4. USERs
5. E. coli transformation and S. cerevisiae transformations

Methods/protocol

PCRs:

Protocol: *PCR with Phusion U Hot start polymerase*

- First, mixed the following master mix:

Reactions

	50 µL	Notes
H_2O	19 µL	
2X Multiplex Buffer	25 µL	Added last, as contains enzyme
FW primer	2.5 µL	
RV primer	2.5 µL	
Template	1 µL	

- The nucleotide molecules that are used:

PCR reactions 06.08.2024

PCR tube no.	Primer1	Primer2	Template	Length	Details
68	FW_p414_tag[54.6]	RV_p414_[57.4]	p414-TEF-Cas9	5395	Anneal 65, Extension 68 for 3:00
69	FW_tag[52.8]	RV_tag_plasmid[55.4]	Alfa-6xHis_tags	300	Anneal 65, Extension 68 for 3:00

USER reaction:

Components of the reaction

Tube name	Backbone	Insert 1	Notes
U30	68	69	p414-TEF-alfa-6xHIS

USER Reaction

	HREmin U30	
MQ water	7µL(up to 20 µL)	
PCR product #1	5 µL (p414) - PCR #68	5395
PCR product #2	5 µL (His tag) - PCR #69	300
CutSmart buffer	1µL	
USER enzyme	1 µL	
DpnI enzyme	1 µL	
Total	**20 µL**	**5695**

Cycle in the Thermocycler

37 C	20 min
25 C	20 min
4-12 C	hold

S. cerevisiae competent cells and transformations

Protocol: *Transformation of S. cerevisiae (yeast)*
8 S cerevisiae competent cell vials in the -80℃ marked **YEAST COMP**

E. coli competent cells and transformations

Competent cells:

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

- 25 Microcentrifuge tubes of 200 µL aliquots in the -80℃ in the Skylab GMO lab marked DH5a COMP

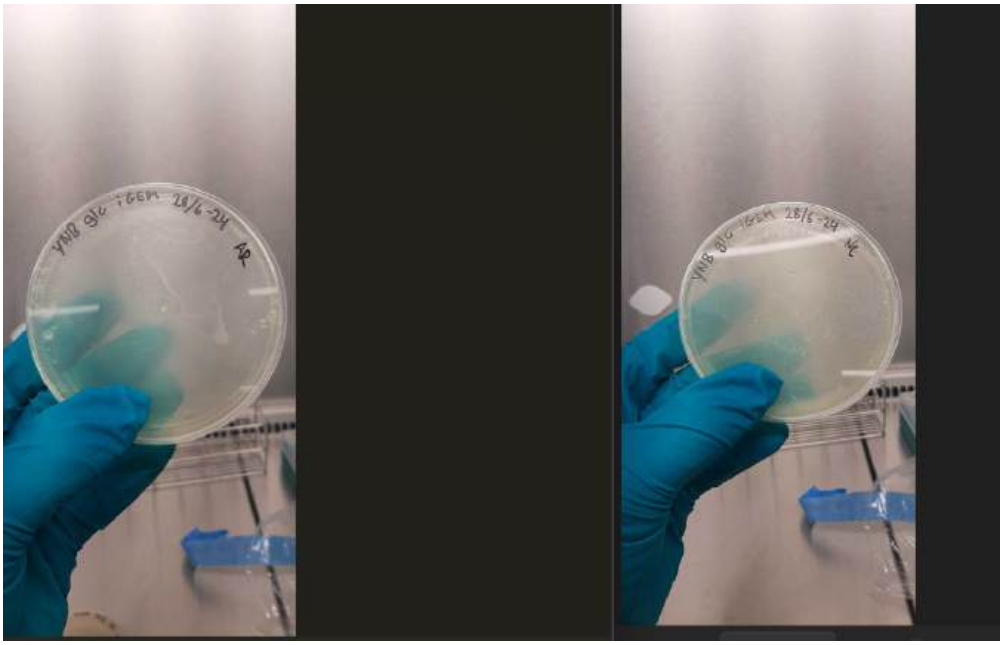
Transformations:

Protocol: T*ransformation mix and go with amp resistance*, paragraph *Fast Transformation of Mix & Go Competent Cells)*

- Two Positive controls were used, pcold (with old cell aliquot) pcnew (with new cell aliquot). Each one had 50µL cells + 5µL of *p414-TEF-Cas9*
- One Negative control with only cells (50µL)
- One U28 plate, with the U28 reactions (10µL) in 100 µL cells.

Conclusion

After ON, all the E. coli plates were empty.
all the Yeast plates had suspicious gray cloud.



After 2 ONs:

- all the E. coli plates were empty → We should try again the protocol *Plasmid transformation of E. coli (Mix&Go)* (including incubation time)
- Yeast plates had colonies, even the Negative Control.

2024.08.09 Digestions and Gel running of pERE5

Overview

Track: ROSALIND
Objective: Validate the integrity of the plasmid pERE5

When: 09.08.2024
Who: Georgios
Location: 202

Methods/protocol

Digestions:

Protocol: *DNA Digestion with Restriction Enzyme*

- I select to digest the *pUC19_ERE5* with enzymes SspI and HindIII. The backbone is *pUC19-T7-3WJdB-T* (Addgene)
- 3 reactions were conducted. One with the double digestion, one with only HindIII (excess enzyme in the freezer) and the last double digestion for the backbone.
- After the incubation, 5 μL of loading buffer was added to stop the reaction.

DNA sequence	DNA quantity [μL]	Restriction Enzyme 1	Restriction Enzyme 2	Buffer	Recom. Incub. Time	Actual Incub. Time	Incub Temp
pERE5	5		HindIII	NEBuffer r2.1	1h	1h	37
pERE5	5	SspI	HindIII	NEBuffer r2.1	1h	1h	37
pUC19-T7-3 WJdB-T(Add gene)	5	SspI	HindIII	NEBuffer r2.1	1h		37
pUC19-T7-3 WJdB-T(Add gene)	5		HindIII	NEBuffer r2.1	1h		37

Gel Electrophoresis:

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Info for the buffers and temp look here: https://nebcloner.neb.com/#!/redigest

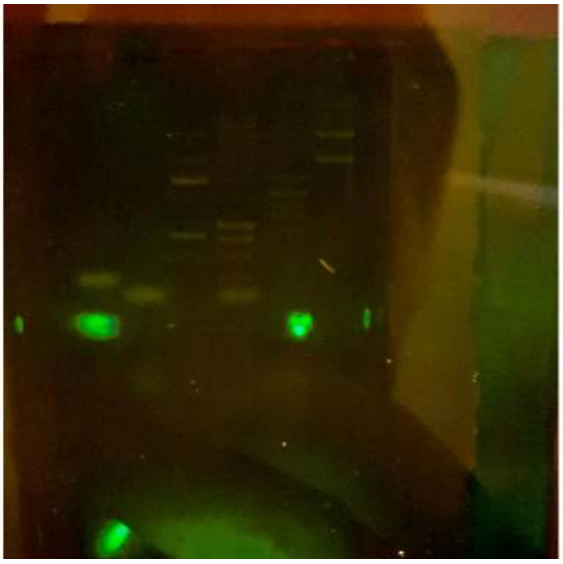
- I run it for 31 minutes (4 with 220 constant Volt, 27 with 200 constant Volt)
- The layout of the gel as well the volumes loaded were shown in the table:

Gel layout

samples	***pERE5 +double digestion***	***pUC19-T7-3WJdB -T (Addgene) + double digestion***	***Ladder***	***pERE5+HindIII***	***pUC19-T7-3 WJdB-T (Addgene)***	***pERE5 undigest***
loading buffer volume	None	None	None	None	None	1µL
template volume	3µL	3µL	1µL	3µL	3µL	1µL
H_2O **volume**	3µL	3µL	5µL	3µL	3µL	4µL
expected bands	2052+981	2052+862	amplicon high range ladder	3033	2914	smear

Conclusion

From the gel, we can conclude: Nothing, it is a totally wrong set up, i will repeat it.



They tool that I used to predict the bands is this: https://nc3.neb.com/NEBcutter/prj/

2024.08.09 E.coli transformation (ERE5, p414-tag)

Overview

Track: ROSALIND
Objective: Transform E.coli with desired plasmids

When: 09.08.2024
Who: Cristian, Georgios, Sirio
Location: Skylab

Methods/protocol

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

Performed 5 transformations in total:

- Positive control (p414) w/old competent cells (5μL DNA)
- Positive control (p414) w/new competent cells (made on 07.08) (5μL DNA)
- Negative control
- U28 (p414-tag) w/new competent cells (10μL DNA)
- U26 (ERE5) w/mix of old and competent cells (80% old, 20% new) (10μL DNA)

Plated on LB-Amp plates.

Conclusion

- After 2 ONs, all the plates were empty (even the positive controls)
- We will try to check the transformation efficiency of our E. coli strains with smaller plasmid (example *pSB1C30_BBa_J04450* that was successful in previous experiments)

2024.08.11 Transformation for ROSALIND templates

Overview

Track: ROSALIND
Objective: Transform with U28, and if it didn't work, also with U28.

When: 11.08.2024
Who: Katerina
Location: GMO lab

Methods/protocol

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

Conclusion

All according to the protocol

2024.08.12 Check transformation efficiency of competent E. coli

Overview

Track: ROSALIND
Objective: Transform the E. coli with smaller plasmid and known concentration to calculate the transformation efficient

When: 12.08.2024
Who: Katerina, Georgios
Location: GMO Skylab

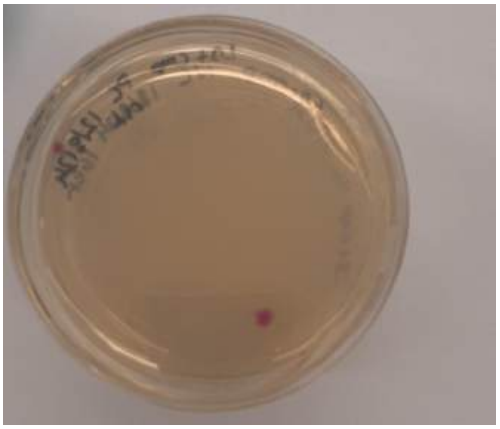
Methods/protocol

Protocol: *Plasmid transformation of E. coli (Mix&Go)*

- AgIN to aliquots, one new and one old, were used.
- Now two different plasmids were used:
    - *pUC19-T7-3WJdB-T* (Addgene) with ampicillin resistance gene
    - *pSB1C30_BBa_J04450* with chloramphenicol resistance gene.
- One positive control with old and one with new cells were selected for the *pSB1C30_BBa_J04450*
- Each plate was poured with **100 µL of SOC-cells-plasmid mix (100µL SOC-50µL cells-2µL plasmid)**

Conclusion

- Need to make new aliquots of competent E.coli cells.
- After 2 ONs, two colonies grow in the positive control of the old competent cells.



2024.08.12 Colony picking & Amplification: U28 & U26

Overview

Track: ROSALIND
Objective: Pick the colony and expand them in LB media + Amp

When: 12.08.2024
Who: Katerina
Location: GMO lab

Methods/protocol

See: 2024.07.31-08.01 Colonies pick-up, stock, and DNA extraction

2024.08.12 Rerun Gel electrophoresis for ERE5 digestions

Overview

Track: ROSALIND
Objective: Rerun Gel electrophoresis for ERE5 digestions

When: 12.08.2024
Who: Georgios, Lumi
Location: GMO lab Skylab

Methods/protocol

Protocol: *Agarose gel preparation aka Electrophoresis Run*

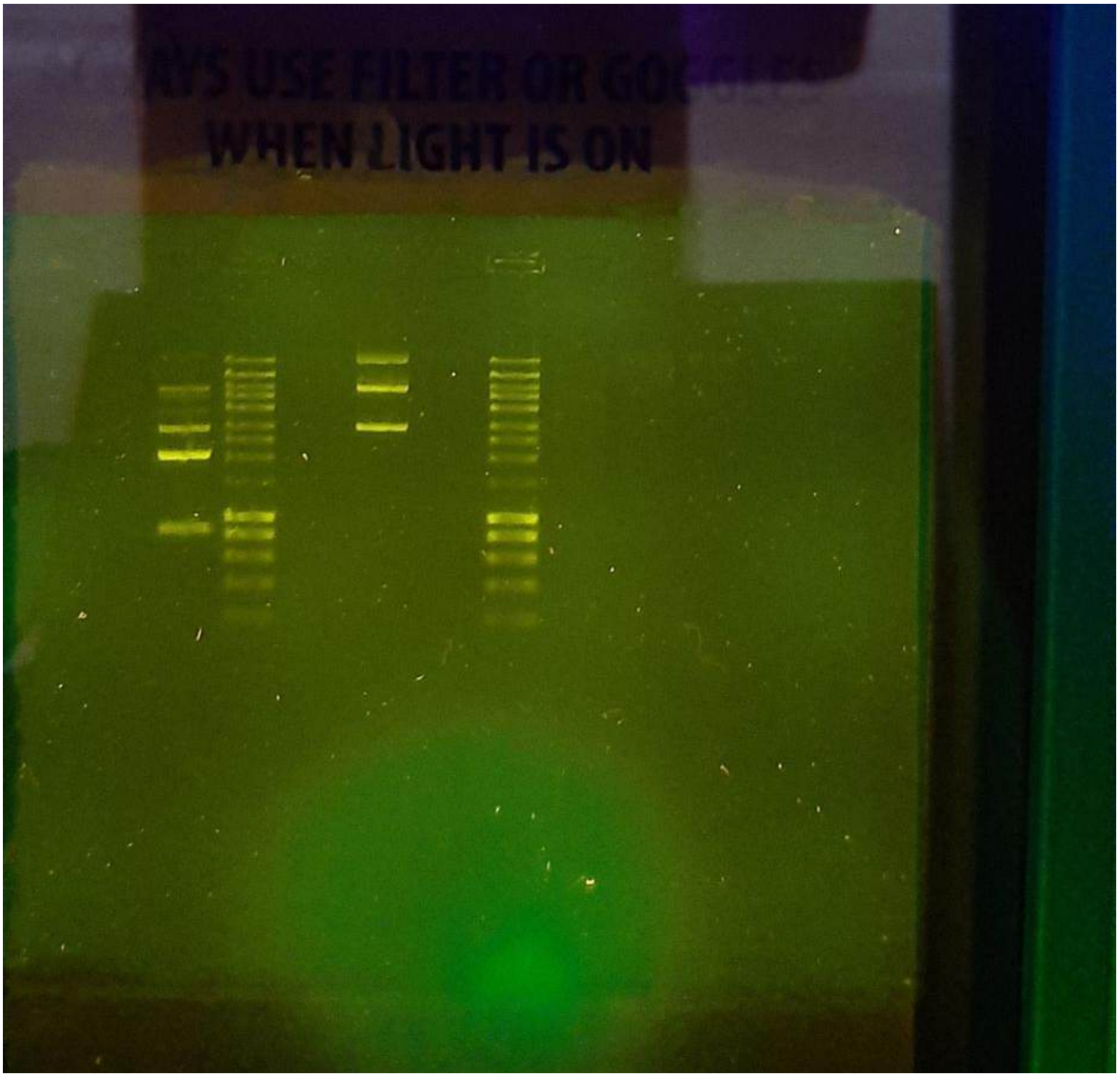
- We prepared 50 mL of TAE with 500 µg of agarose.
- We loaded 1 µL of ladder and 3µL of digestions. 1µL of purified plasmid in 1µL of loading buffer and 4 µL of pcr H20 were mixed and 3µL of this loaded to the gel.

Gel layout

1	samples	pERE5#4+double digestion	pUC19-T7-3WJdB-TIAddgene) + double digestion	Ladder	pERE5#4+HindIII	pUC19-T7-3WJdB-TIAddgene)	pERE5#4 undigest	Ladder	pERE5#5+double digestion	pERE5#5+HindIII	pERE5#5 undigest
2	loading buffer volume	None	None	None	None	None	1ul	None	None	None	1ul
3	template volume	3ul	3ul	1ul	3ul	3ul	1ul	1ul	3ul	3ul	1ul
4	H2O volume	none	none	none	none	none	4ul	none	none	none	4ul
5	expected bands	2052+981	2052+882	amplicon high range ladder	3033	2914	linear	amplicon high range ladder	2052+981	3033	linear

Conclusion

- pERE5 was not present in the gel, probably not purified correct or not present in the colonies


WHEN LIGHT IS ON

2024.08.13 PCR to amplify pHRE5

Overview

Objective: Amplification of the *pUC19-T7-3WJdB-T*(Addgene) integrated with the HRE5 to linearised the template for the in vitro transcription

When: 13.08.2024
Who: Georgios, Katerina
Location: GMO Skylab

Methods/protocol

Protocol: PCR with *Phusion U Hot start polymerase* but instead of Phusion U we used Phusion.

- Tube number: **74**

PCR MIX

	20μL	Final conc
H_2O	12.1 μL	
5X HF Buffer	4μL	1x
10 mM dNTPs	0.5μL	200 μM (each)
Primer FWD (10 μM)	1μL	0.5 μM
Primer RV (10 μM)	1μL	0.5 μM
Phusion U Polymerase	0.5μL	0.02 U/μL
Template	1 μL	1 pg - 10 ng DNA per 50 μL

PCR parameters

Initial denaturation	98	30 sec	
Denaturation	98	15 sec	30 cycles
Annealing	64.7	20 sec	
Elongation	72	14 sec	
Final elongation	72	5 min	
Hold	4 or 12	infinite	

Conclusion

- No amplification
- We will try :
    - using the 2X master mix (1st repeat)
    - increase the elongation time (all repeats)

2024.08.15 PCR and USER for Genscript plasmid

Overview

Track: Receptor production
Objective: PCR and USER for Genscript plasmid to make a “switch out” the receptor in the Genscript plasmid

When: 15.08.2024
Who: Reinis
Where: Skylab

Methods/protocol

PCRs:

Protocol: *Phusion U Hot start polymerase*

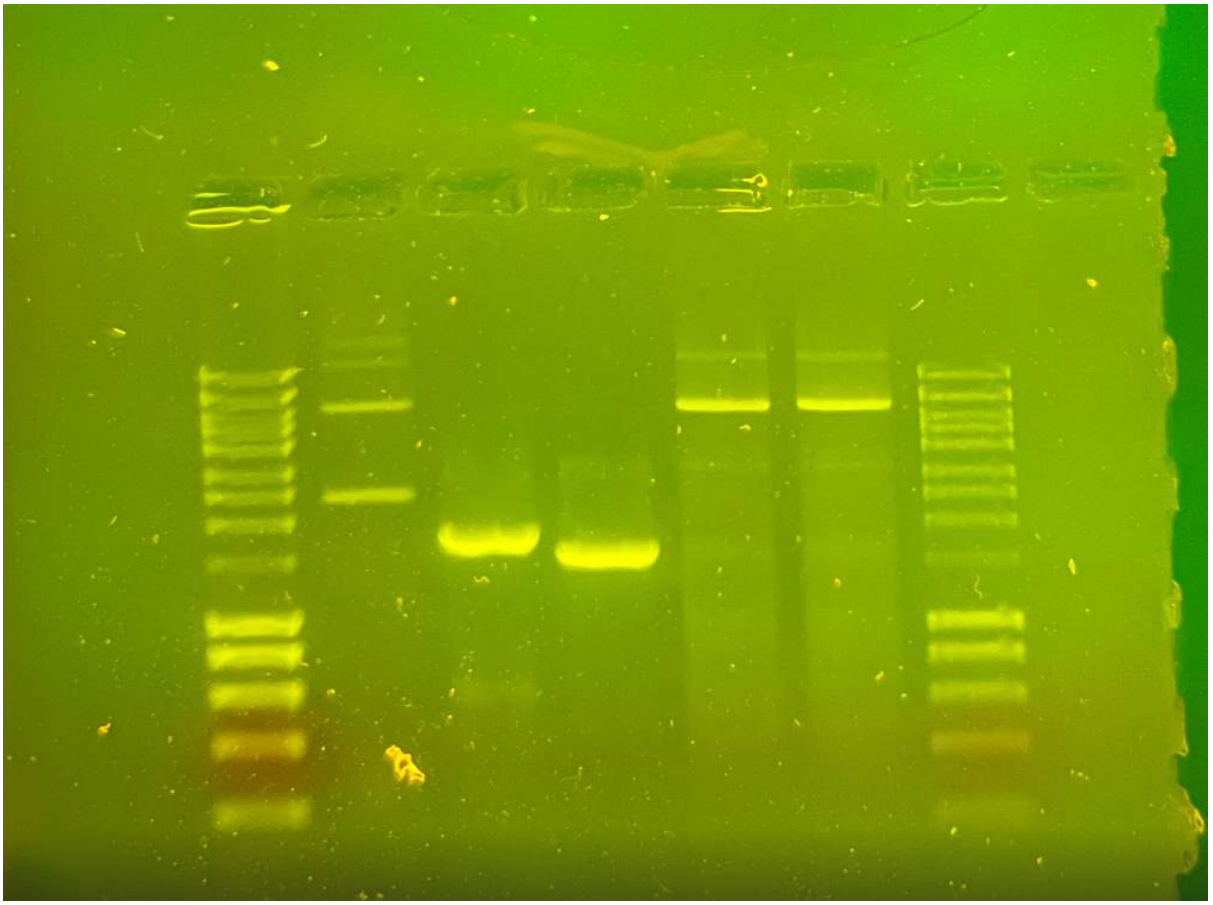
Used Phusion U mastermix, 20 µL volume, 0.5 µL of each primer and template

Tube	FW primer	RV primer	Template	Notes
90	FW_gs_HERα	RV_gs_HERα	HERα gBlock	
91	FW_gs_HERb	RV_gs_HERb	HERb gBlock	
92	FW_gs_bb_HERα	RV_gs_bb	AR-GS plasmid	Only the FW primers are different
93	FW_gs_bb_HERb	RV_gs_bb	AR-GS plasmid	AR genscript plasmid that we ordered as template

PCR Conclusion

The PCRs were successful

Lanes:
74 - amplification from 13.08 of the rosalind part <- FAILED
90
91
92
93



USER cloning:

Performed 2 USER reactions:

Tube name	Insert Tube	Backbone Tube	Notes
U30	90	92	HERα
U31	91	93	HERß

Transformations:
Transformed U30, AR-GS (as PC), NC (nothing)
The plates are in the shaker

O/N cultures of U26
Picked a colony (marked nr7 on the plate) from the U26 cloning plate. Shaking at 30℃ overnight

Conclusion

TODO:

- Make new LB or LB + amp media
- Make new LB+amp plates and make a transformation of U31 as well
- Transformations: Check the plates on 16.08

    - If there are colonies → O/N cultures
- ON culture from U26: If there is growth, purify with miniprep, make restriction digest, check DNA concentration (get in contact with Signe)

2024.08.15 Yeast transformation

Overview

Track: Receptor production
Objective: Made again yeast transformation using yeast competent cells from -80℃

When: 15.08.2024
Who: Reinis
Where: Skylab

Methods/protocols

Put 10 µL of AR-GS plasmid
Protocol: *Transformation of S. cerevisiae (yeast)*

Previous time the top plate dried out - this time wrapped in parafilm and put in a bag to avoid this

Conclusion

TODO:
Check the AR plate for colonies, if there are colonies put an O/N culture in YNB media (I think there should be some in the fridge, if not please make it :)))))

2024.08.16 PCR to amplify pHRE5(vol2) + pEREmin

Overview

Track: ROSALIND
Objective: Amplification of the *pUC19-T7-3WJdB-T*(Addgene) integrated with the HRE5 to linearised template puc_hre5_serial-cloner for the in vitro transcription

When: 16.08.2024
Who: Georgios
Location: GMO Skylab

Methods/protocol

Protocol: *PCR with Phusion U Hot start polymerase* but instead of Phusion U, we use Phusion.

- One reaction with 2X master mix.
- one reaction the same as before
- both of them with higher elongation time.
- Tube number: **71, 72 ,73 (the pEREmin)**

PCR MIX Reaction #71

	20μL	Final conc	Notes
H_2O	12μL		
5X HF Buffer	4μL	1x	
10 mM dNTPs	0.5μL	200 μM (each)	
Primer FWD (10 μM)	1μL	0.5 μM	
Primer RV (10 μM)	1μL	0.5 μM	
Phusion U Polymerase	0.5μL	0.02 U/μL	
Template	1 μL	1 pg - 10 ng DNA per 50 μL	pHRE5

PCR MIX Reaction #72

	20μL	Final conc	Notes
H_2O	7μL		
2X Master Mix Buffer	10μL	1x	
Primer FWD (10 μM)	1μL	0.5 μM	
Primer RV (10 μM)	1μL	0.5 μM	
Template	1 μL	1 pg - 10 ng DNA per 50 μL	pHRE5

PCR MIX Reaction #73

	20µL	Final conc	Notes
H_2O	7µL		
2X Master Mix Buffer	10µL	1x	
Primer FWD (10 µM)	1µL	0.5 µM	
Primer RV (10 µM)	1µL	0.5 µM	
Template	1 µL	1 pg - 10 ng DNA per 50 µL	pEREmin

Initial denaturation	98	2 min	
Denaturation	98	15 sec	30 cycles
Annealing	64	20 sec	
Elongation	72	20 sec	
Final elongation	72	5 min	
Hold	4 or 12	infinite	

Conclusion

- Regardless the result, i realized that the volumes of the DNA templates as well the polymerases activity in the reaction 71 is 5 times more than the recommended in *Phusion manual*

2024.08.17 Gel electrophoresis for pHRE5, pEREmin, AR-GS purified, remaining U26 digestions and U28-U29

Overview

Track: Rosalind
Objective: Validate the last pcr reactions, quantification of the AR-GS plasmid that purified using miniprep kit and check the digestion U26.6

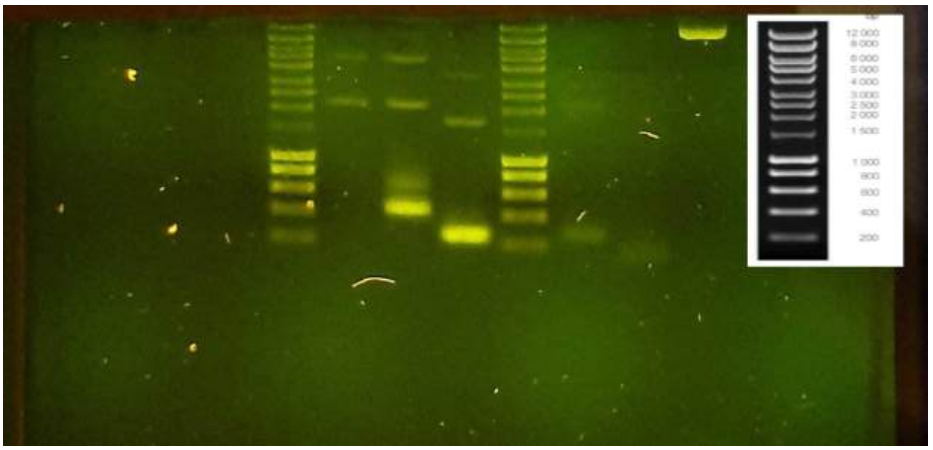
When: 17.08.2024
Who:Georgios
Location: GMO Skylab

Methods/protocol

Protocol: *Agarose gel preparation aka Electrophoresis Run*

Conclusion

- we have the right length for HRE5 and ERE minimal to proceed with the IVT
- the PCRs should only be conducted with the master mix (avoid the dNTS, seems they have expired)
- we don’t have ERE5 purified.
- We don't have successful U28 and U29 reactions.
- The quantification of the AR-GS is: 40ng in 2μL or **20ng/μL**



2024.08.19 ROSALIND only transcription only

Overview

Track: ROSALIND
Objective: Try the transcription with only the DNA template to test the transcription. We want to try to get a good-enough signal with as little DNA as possible. This will in the future allow for a better sensitivity.

When: 19.08.2024
Who: Sirio, Georgios
Location: GMO lab

Methods/protocol

As reference, the DNA quantity needed

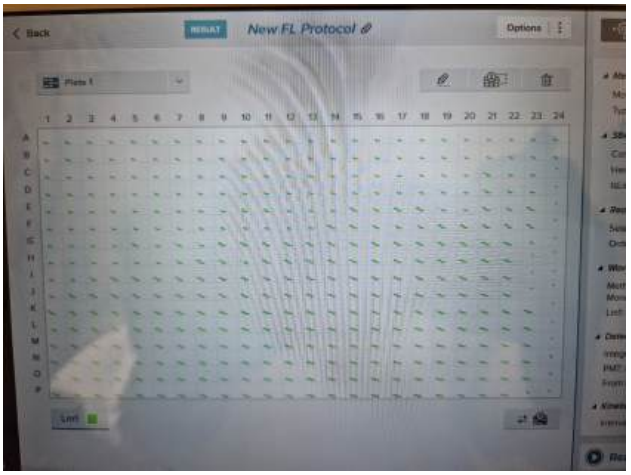
- for HRE5 template of 446 bp → Using the other one, the DNA quantity will change!

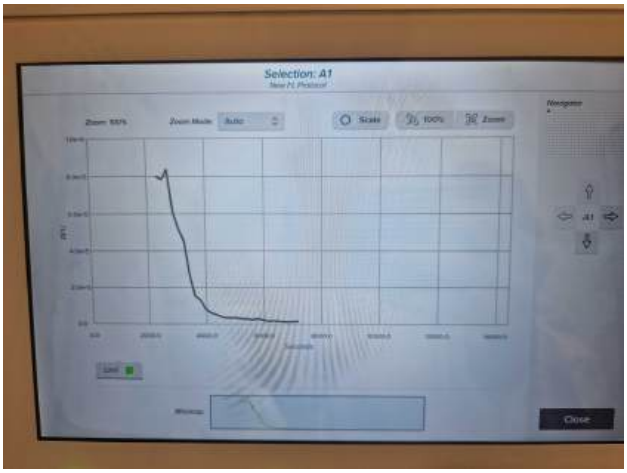
Reaction number	DNA template	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Well ID
R1	HRE5	3	16.48	25	0.6592	A8
R2	HRE5	5	27.47	25	1.0988	A2
R3	HRE5	7.5	41.21	25	1.6484	A3
R4	HRE5	10	54.95	25	2.198	A4
R5	HRE5	15	82.42	25	3.2968	A5
R6	HRE5	20	109.90	25	4.396	A6
NC		0	0			A7
Blank (empty well)						A1
				Total DNA	**13.2972**	

Conclusion

- Failed
- The machine didn't work properly
- Unexpected reads in all the wells


Error: 101
Error: Transport lost more than 2 full steps. Additional information: X Transport stopped => Current Position: 8001 Encoder Position: 7600 Desired Position: 8001 Limit: 30 CheckPosition: 8027 Device error code: 5
OK


Back
New FL Protocol
Options


Selection: A1
Close


Selection: A2
Close
MOLECULAR

2024.08.21-22 Yeast transformation with Genscript plasmid (AR-GS)

Overview

Track: Receptor production
Objective: Cloning Genscript plasmid containing Androgen Receptor (AR) into S. Cerevisiae for protein production.

When: 21.08.2024, continued on 22.08.24 from Step. 12b
Who: Lumi, Georgios
Location: Skylab GMO lab

Methods/protocol

Protocol: *Transformation of S. cerevisiae (yeast)*

2 transformations were performed, one from previously amplified AR-GS (~50ng/μL) *(E. coli or PCR?)*, another from 500 ng/μL stock of a plasmid provided by genscript, together with Negative Control.

Procedure:

21.08.2024

- O/N culture was performed on 20.08.24 with only 90 mL of YPD.
- Step 3. OD660 = 0.06 -> 4.2 mL of O/N culture was added to 50 μL of fresh YPD.
- Step 5.
    - After 3hr OD660=0.45(1mL of the Yeast culture in the cuvette. Blank was 1mL of the YPD)
    - After 5hr OD660=0.7. Enough to continue the protocol.
- Step 7. The pellet was fragile so centrifuge twice (lower volume second time).
- Pay attention in step 11, we should remove the LiAc before continuing.
- We stopped at Step 12a - addition of PEG
    - It is safe to store the aliquots in -80C as PEG has a protective role.

22.08.2024:

- Step 12d. Total amount of 0.5 μg of each DNA used in both cases:
    - amplified AR-GS: 10 μL + 55 μL of MQ H_2O

    - AR-GS stock: 1 µL + 64 µL of MQ H_2O
- 3 yeast tubes are labeled 'Yeast. comp.' (on top), 'NC / ARGS /ARGS Stock' respectively (on side)

Conclusion & notes

- Transformed cells are incubating on a shaker
- On 21.08 2x 10 mL of 20.08.24 O/N YPD Yeast culture was stored in the GMO Lab +4°C fridge for later use.
- There are 7 competent yeast tubes from 21.08.2024 in -80C freezer (green box), labeled 'Yeast comp.'. They are from Step 12a. (PEG was added)

2024.08.22 Purification & Quantification of DNA for ROSALIND

Overview

Track: ROSALIND
Objective: Purify the DNA from previous PCRs: R0, R1, R2, aka: 74, 75, 76.

When: 22.08.2024
Who: Sirio
Location: GMO lab

Methods/protocol

Protocol: *Monarch® DNA Gel Extraction Kit Protocol (NEB #T1020) - DNA purification*

- The protocol was started from step 5
- The samples R0, R1, R2 were transferred all in the same column
- Elution was made with 50 µL of elute buffer

Conclusion

Quantification:

- Blank was taken with water instead with the elution buffer
- Results:
    - 2.4 ng/µL and then 2.3 ng/µL → **2.35 ng/µL**
    - A260: **0.046**
    - A260/280: **1.55**

2024.08.22 ROSALIND reaction

Overview

Objective: Re-do the user cloning for the following templates:

- pERE5
- pHREmin

When: 22.08.2024
Who: Sirio
Location: GMO Lab Skylab

Methods/protocol

Step 1: Prepare the Master Mix

- Simply edit the cell in yellow to have all the other components to be calculated

Preparing the Master Mix (transcription only)

			Volume x1 reaction [µL]
Number of Reactions:	**12**		
Master mix prepared for:	**13**		
IVT buffer	26		2
25 mM Tris-buffered NTPs (each)	7.41		0.57
Total 4x NTP volume:	*29.64*		*2.28*
2U/µL inorganic pyrophosphatase	1.95		0.15
40 mM DFHBI-1T	1.3		0.1
Total MM volume:	**58.89**		**4.53**

After having prepared the MM, calculate the DNA quantity and water respectively.

DNA quantity & Water (transcription only)

Reaction number	DNA template	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial
ERE5	HRE5	3	16.48	11.9	1.3848739496	9.0851260504	C1	
ERE5	HRE5	5	27.47	11.9	2.3084033613	8.1615966387	C2	
ERE5	HRE5	7.5	41.21	11.9	3.4630252101	7.0069747899	C3	
ERE5	HRE5	10	54.95	11.9	4.6176470588	5.8523529412	C4	11.7739495798
EREmin III	**EREmin**	15	49.90	15.1	**3.3046357616**	**7.1653642384**	C5	
NC	/				0	10.47	C6	
Blank	/						C7	
ERE5 III	HRE5	3	16.48	9.7	1.6989690722	8.7710309278	C8	
ERE5 III	HRE5	5	27.47	9.7	2.8319587629	7.6380412371	C9	
ERE5 III	HRE5	7.5	41.21	9.7	4.2484536082	6.2215463918	C10	
ERE5 III	HRE5	10	54.95	9.7	5.6649484536	4.8050515464	C11	14.4443298969
EREmin III	**EREmin**	15	49.90	15.1	**3.3046357616**	**7.1653642384**	C12	
NC					0	10.47	C13	
Blanck (empty well)							C14	
				Total DNA	**32.8275509999**			

Activating reaction:

			Quantity x1 reaction
Number of Reactions:	**12**		
Master mix prepared for:	**13**		
1 mg/mL (aka 50'000U/mL) T7 RNAP	2.6		0.2
H_2O	62.4		4.8
Total MM volume:	**65**		5

Plate:

C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
HRE53nM	HRE55nM	HRE57.5nM	HRE510nM	EREmin15nM	NC	Empty	Empty	Empty	HRE53nM	HRE55nM	HRE57.5nM	HRE510nM	EREmin15nM	NC	Empty	...	
Not sealed								Sealed									

Conclusion

- There are still some issues with the plate reader.
- Results are saved

2024.08.26 in vitro transcription reaction and gel electrophoresis

Overview

Track: ROSALIND
Objective: Re-do the user cloning for the following templates:

Who: Georgios
When: 26.08.2024
Location: GMO skylab

Methods/protocol

Step 1: Prepare the Master Mix

- Simply edit the cell in yellow to have all the other components to be calculated

Preparing the Master Mix (transcription only)

			Volume x1 reaction [µL]
Number of Reactions:	**3**		
Master mix prepared for:	**4**		
IVT buffer	8		2
25 mM Tris-buffered NTPs (each)	2.28		0.57
Total 4x NTP volume:	*9.12*		*2.28*
2U/µL inorganic pyrophosphatase	0.6		0.15
Total MM volume:	**17.72**		**4.43**

After having prepared the MM, calculate the DNA quantity and water respectively.

	Reaction number	DNA template	Molecular Weight of 445bp[g/mol]	Final DNA concentration [nM]	Final DNA concentration [ng/µL]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Notes	Sub-Total DNA per well
1	IVT1	Eremin	274129.34	187.7785579	13.7	274	15.1	18.1456953642	-7.5756953642		adjust the volume of the master mix from above	
2	NC without enzyme	Eremin										
3	NC without template							0	10.57		adjust the volume of the master mix from above	
4	Blanck (empty well)											
5							Total DNA	18.1456953642				

Activating reaction

			Quantity x1 reaction
Number of Reactions:	**2**		
Master mix prepared for:	**3**		
1 mg/mL (aka 50'000U/mL) T7 RNAP	0.6		0.2
H_2O	14.4		4.8
Total MM volume:	**15**		5

2024.08.27 PCR of pUC19 Broccoli & DNA purification (ROSALIND)

Overview

Track: ROSALIND
Objective: PCR for the ROSALIND reaction.

When: 27.08.2024
Who: Sirio
Location: GMO lab

Methods/protocol

PCR

Protocol (PCR using the AQ97 High Fidelity DNA Polymerase 2x Master Mix - DRAFT): *PCR using the AQ97 High Fidelity DNA Polymerase 2x Master Mix - DRAFT*

Master mix prepared:

	Volume	Final conc.	
Total PCR volume (insert here -->)	1000		
H_2O	385		
2X Master Mix Buffer	500	1x	
Primer FWD (10 µM)	50	0.5 µM	
Primer RV (10 µM)	50	0.5 µM	
Template	15	1 pg - 10 ng DNA per 50 µL	pUC19 Broccoli
Total	1000		

- The template for the volume is guessed, as we don't know the DNA concentration
- The 1 mL MM was divided in a strip of 8 PCR tubes (125 µL each)

DNA purification

Protocol: *Monarch® DNA Gel Extraction Kit Protocol (NEB #T1020) - DNA purification*

- 2 columns for each amplification set were used
- Elution was done with nuclease-free water (and used 2 times to increase yield)

DNA quantification: 221 lab

Conclusion

DNA quantification

DNA Template	DNA quantity [ng/μL]	A280/260		
HRE5 1	5.9	1.65	33.6	1.43
HRE5 2	8.3	1.5		
HRE 3	7.2	1.42		
EREmin 1	4.9	1.71	34.8	1.57
EREmin 2	9.1	1.68		
EREmin 3	2.1	1.58		
pUC19 1	9.6	1.76	43.5	1.65
pUC19 2	11.4	1.77		
pUC19 3	5.8	1.43		

NC 451!!! 1.82

These initial concentrations were increased by letting the water evaporate, and then the 3 vials (per template) were united in a single one:

DNA Template	DNA quantity [ng/μL]	A280/260
HRE5 1	33.6	1.43
EREmin 1	34.8	1.57
pUC19 1	43.5	1.65

THE VOLUME FOR EACH VIAL IS 10 μL

2024.08.28 PCR for HREmin, pSB1C30, pUC19, ERE5

Overview

Track: ROSALIND
Objective: Amplification of g blocks to use for the USER

When: 28.08.2024
Who: Georgios
Location: GMO skylab

Methods/protocol

Protocol: *PCR with Phusion U Hot start polymerase*

PCR reaction on 03.07.2024

PCR tube no.	Primer1	Primer2	Template	Length	Details
79	FW_ERE minimal Broccoli	-	4. HRE minimal Broccoli	275	Anneal 60, 0:20
80	FW_pSC1C30_[Tm:56.0]	>RV_pSC1C30_[Tm:55.0]	pSB1C30 red	2134	Anneal 60, 2:00
81	FW_pUC19_ERE5/HRE5[Tm:51.4]	RV_pUC19_ERE5/HRE5[Tm:50.4]	pUC19-T7-3WJdB-T(Addgene)	2919	Anneal 60, 2:00
82	FW_pRR-ERalpha-5Z-ERE5_[Tm:56.3]	RV_pRR-ERalpha-5z_ERE5_[Tm:56.9]	Partial seq. 2 from pRR-ERalpha-5Z	128	Anneal 60, 0:20

PCR MIX reaction #73

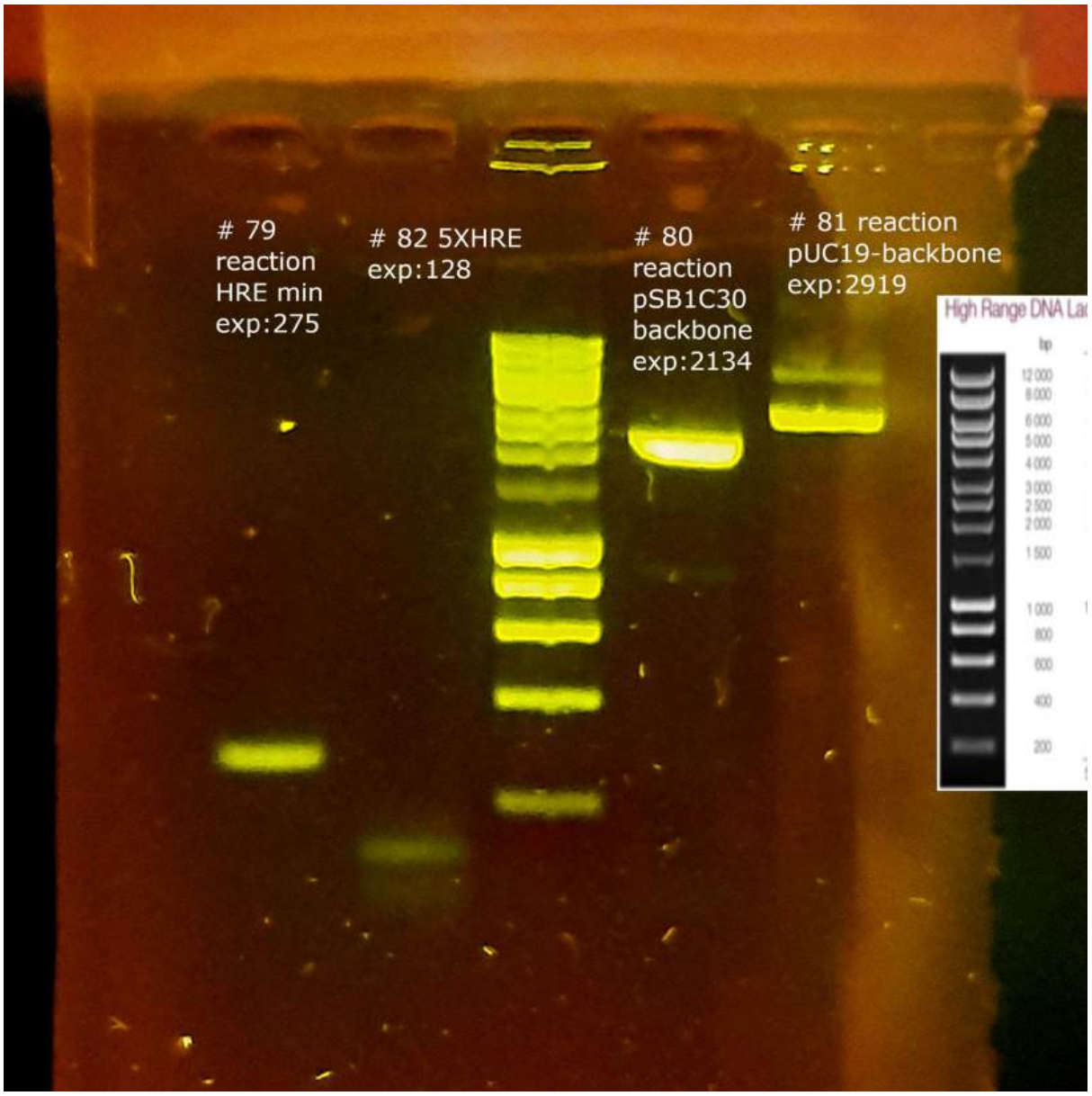
	50µL	Final conc	Notes
H_2O	22		
2X Master Mix Buffer	25	1x	
Primer FWD (10 µM)	1	0.2µM	
Primer RV (10 µM)	1	0.2µM	
Template	1	1 pg - 10 ng DNA per 50 µL	HRE_min,pSB1C30,ERE5,pUC
Total	50		

PCR Conditions

Initial denaturation	98	2 min	
Denaturation	98	15 sec	36x
Annealing	60	20 sec	
Elongation	72	20 sec	
Final elongation	72	5 min	
Hold	4 or 12	infinite	

Conclusion

- Each sample was 2 μL of pcr, 2μL H_2O and 1μL of loading buffer. The ladder was 2 μL without a loading buffer. Everything runs in 1% agarose gel with SYBR Safe.



- It seems that generally the PCRs worked well. The amplicons #79 and #80 are in the right position and brightness.
- The rest two, #82 and #81 seems to be improved.
    - For #82, the ideal Ta is 63 degrees, so the only improvement could be just an increase in the cycles.
    - For #81, the actual Ta is 64, so in 60 degrees maybe unspecific binding occurs.

2024.08.28 PCRs, DNA quantification & IVT ROSALIND reaction

Overview

Track: ROSALIND
Objective: DNA quantification of the previously purified DNAs. IVT ROSALIND reaction transcription only to finally achieve a right measurement.

When: 28.08.2024
Who: Sirio, Lumi
Location: GMO lab

Methods/protocol

- This time, the IVT reaction was run using the following considerations/parameters:
- Using a positive control: the standard fluorescein (using the appropriate wavelength couple)
- Increasing the DNA concentration (30~50 nM instead of 15)
- Using the right amount of T7 RNA pol → **PREVIOUSLY ONLY 1/10 WAS ADDED DUE TO A MISTAKE!**
- Negative control is without DNA template
- Blank is only the IVT buffer (without T7 pol. and w/out fluorophore)
- Plate covered with the sticky membrane

Step 1: Prepare the Master Mix

- Simply edit the cell in yellow to have all the other components to be calculated

Preparing the Master Mix (transcription only)

			VolµMe x1 reaction [µL]
Number of Reactions:	**8**		
Master mix prepared for:	**9**		
IVT buffer	18		2
25 mM Tris-buffered NTPs (each)	5.13		0.57
Total 4x NTP volume:	*20.52*		*2.28*
2U/µL inorganic pyrophosphatase	1.35		0.15
40 mM DFHBI-1T	0.9		0.1
Total MM volume:	**40.77**		**4.53**

After having prepared the MM, calculate the DNA quantity and water respectively.

Molecular weight should be set according to the template:

DNA template	DNA length [bp]	DNA mol. weight [Da]
HRE5 & ERE5:	446	274745.28
HREmin & EREmin:	270	166339.84
pUC19 broccoli:	310	

How does this work?

1. Write the DNA template, and then the correct **DNA length**
2. Type in the "**Final DNA concentration**" desired, then the **DNA concentration** of the DNA sample used (quantified with the nanodrop).
3. The correct DNA volume will be automatically calculated for the 20µL reaction.

DNA quantity & Water (transcription only)

	Reaction name	DNA template	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial
1	pUC broc	pUC19 Broc	310	190947.6	15	57.28428	43.5	1.31688	9.15	F1	
2	pUC broc	pUC19 Broc	310	190947.6	25	95.4738	43.5	2.1948	8.28	F2	
3	pUC broc	pUC19 Broc	310	190947.6	50	190.9476	43.5	4.3896	6.08	F3	7.90128
4	NC			#VALUE!		#VALUE!		0	10.47	F4	
5	Blank			#VALUE!		#VALUE!		0	10.47	F5	
6	HRE5 1		446	274718.16	15	82.415448	33.6	2.4528407143	8.02	F6	
7	HRE5 1		446	274718.16	50	274.71816	33.6	8.1761357143	2.29	F7	10.6289764286
8	EREmin 1		270	166309.2	25	83.1546	34.8	2.3895	8.08	F9	
9	EREmin 1		270	166309.2	50	166.3092	34.8	4.779	5.69	F10	7.1685
10	Blank							0	10.47	F11	
11	Fluorescein 1.1									F12	
12	Fluorescein 1.2									F13	
13	Fluorescein 2.1									F14	
14	Fluorescein 2.2									F15	

Activating reaction

			Quantity x1 reaction
Number of Reactions:	**9**		
Master mix prepared for:	**10**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	20		2
H_2O	30		3
Total MM volume:	**50**		5

Plate:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
2	pUC broc 50 nM	pUC broc 50 nM	pUC broc 50 nM	pUC broc 80 nM	pUC broc 80 nM	pUC broc 80 nM	NC	Blank	HRE5 50 nM	HRE5 50 nM	HRE5 50 nM	Blank	EREmin 50 nM	EREmin 50 nM	EREmin 50 nM

PCR

New CPRs have been run, as the previous ones did not contain much DNA.
The next PCRs will be run with **45 cycles or by increasing the DNA concentration.**

PCR is performed in a 1000 µL volume for the following templates:

- HRE5
- EREmin
- pUC19 Broccoli

PCR table - Only modify the highlighted cells

	Volume	Final conc.	Initial conc.
Total PCR volume (insert here -->)	1000		
H_2O	439.45		
2X Master Mix Buffer	500	1x	
Primer FWD (10 µM)	30	0.3	10
Primer RV (10 µM)	30	0.3	10
Template (insert here -->)	0.55	1 pg - 10 ng DNA per 50 µL	Average amount, does not change much
Total	1000		

DNA quantities for each samples:

1 ng in 50µL --> 20 ng in 1000 µL
10 pg (=0.01 ng) in 50µL --> 0.2 ng in 1000µL

Template	n° of cycles	Desired DNA quantity [ng]	DNA conc. [ng/µL]	Volume required [µL]	Water
HRE5	35	20	33.6	**0.5952380952**	439.4047619048
HRE5	45	2	33.6	**0.0595238095**	439.9404761905
EREmin	35	20	34.8	**0.5747126437**	439.4252873563
EREmin	45	2	34.8	**0.0574712644**	439.9425287356
pUC Broccoli	35	20	43.5	**0.4597701149**	439.5402298851
pUC Broccoli	45	2	43.5	**0.0459770115**	439.9540229885

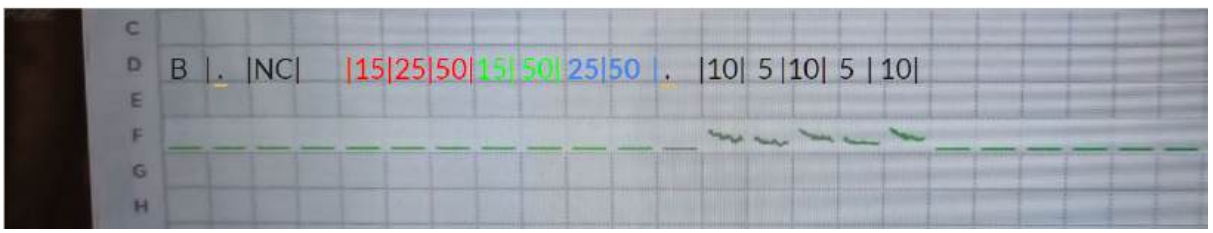
Primer dilutions rosalind:

- 200 µL in total
- 20 µL of primer
- 180 µL of H_2O

RED: pUC19 from IDT
GREEN: HRE5
BLUE: EREmin
BLACK: Fluorescein

Values:
- Fluorescein: 10^6 ~ 10^5
- Others: random peaks to 10^4

Settings: Gain set to "HIGH"



Conclusion

ROSALIND reaction: The reaction was, again, a failure. The detected signal is too low.

PCRs

- Failure. We got some DNA, but the yield was too low. The reason is because I DIDN'T USE THE GEL DISSOLUTION BUFFER with the PCR product. That has also the function to increase the binding affinity of the DNA to the column.
- Experiment needs to be repeated

2024.08.28/29 Sequencing prep & DNA quantification

Overview

Track: ROSALIND, In-Cell Assay, Receptor production
Objective: To prepare chosen plasmids for sequencing and prepare templates for ROSALIND reaction

When: 28.08.2024
Who: Lumi, Sirio
Location: GMO Lab SkyLab

Methods/protocol

Sequencing

Chosen plasmids:

- In-Cell Assay: pRR-AR-5Z, pRR-GR-5Z, pRR-ERa-5Z, pRR-ERb-5Z
- pUC19-T7-HRE5-3WJdB-T
- pSB1C30_EREmin_Broccoli

In-Cell Assay - Plasmids are in stock solutions in -20C freezer (high concentration, more than 20 µL)

ROSALIND
Glycerol stocks of E. coli containing each plasmid were inoculated in 10 mL of the right broth with addition of either Ampicillin/Chloramphenicol
O/N incubation in 37C

Plasmid extraction protocol: *Monarch Plasmid Miniprep Kit (For purification of plasmids from E. coli)*

Plasmids prep for sequencing:

- NanoDrop measurement (see table below)
- all samples were diluted to the concentration 5.6 ng/µL
- 30 µL of each sample was sent for sequencing

DNA quantification:

DNAseq	Quantity #1 [ng/µL]	Quantity #2 [ng/µL]	Quantity #3 [ng/µL]	Average [ng/µL]	A260/280	Volume [µL]	Volume to add after 1/10 dilution
pGR 2nd	227.5	126.0			1.80		
pGR	189.7	119.1	348.8	219.2	1.84		**50**
pERalpha	118.5	112.6	115.1	115.4	1.88		**29.73**
PERbeta	269.8	266.5	328.0	288.1	1.89		**124.34**
pAR	232.1	235.2	265.5	244.2666666667	1.89		**100.85**
pEREmin	118.1	115.3	116.9	116.7666666667	1.79	85	**32.52**
PHRE5	187.0	196.1	192.0	191.7	1.85	85	**72.70**
HRE5 35 l	**9.6**						
HRE5 45 l	**12.2**						
HRE5 35 400	**17.0**						12
HRE 45 400	**44.8**						
EREmin 35 l	**9.6**						
EREmin 45 l	**9.2**						
EREmin 35 400	**18.0**						
EREmin 45 400	**17.6**						
pUC Brocc 35 l	**23.1**						
pUC Brocc 45 l	**17.1**						5
pUC Brocc 35 400	**22.0**						
pUC Brocc 35 400	**18**						

Conclusion:

Results of the sequencing can be found on benchling.

2024.08.29 DNA purification & Quantification - ROSALIND templates: pUC19 Broccoli, HRE5, EREmin

Overview

Track: ROSALIND
Objective: Attempt to get templates in working condition for the ROSALIND reaction

When: 29.08.2024
Who: Sirio, Lumi
Location: GMO lab, 221 for the quantification at the Nanodrop

Methods/protocol

Plasmid extraction protocol: *Monarch Plasmid Miniprep Kit (For purification of plasmids from E. coli)*

Dissolution buffer was used, but a mistake was made during the procedure. Basically the yield is low again, and 400 µL of the flowthough out of 1000 µL is just gone now ._.

Conclusion

- Low yield again. Can be seen here: *2024.08.28/29 Sequencing prep & DNA quantification*

2024.08.30 DNA purification & Quantification from PCRs - ROSALIND pUC19 Broccoli, HRE5, EREmin

Overview

Track: ROSALIND
Objective: Attempt to get templates in working condition for the ROSALIND reaction

When: 30.08.2024
Who: Sirio, Georgios
Location: GMO lab, 221 for quantification

Methods/protocol

Protocol: *NucleoSpin® Gel and PCR Clean-up - Columns from Aafke*

Conclusion

DNA quantification

Sample name	DNA quantity	A260/280
HRE5 35	168.3	1.85
HRE5 45	197.5	1.83
EREmin 35	156.3	1.83
EREmin 45	194.5	1.86
pUC19 Broc 35	146.6	1.84
pUC19 Broc 45	182.6	1.81

2024.08.31 IVT ROSALIND reaction & RNA Gel electrophoresis

Overview

Track: ROSALIND
Objective: IVT ROSALIND reaction

When: 31.08.2024
Who: Sirio, Reinis, Georgios
Location: GMO lab

Methods/protocol

IVT ROSALIND REACTION

Considerations:

- Run the reaction in PCR tubes, and then transfer it to the plate reader after 1~2 hours
- Adding the fluorophore right before measuring

Step 1: Prepare the Master Mix

- Simply edit the cell in yellow to have all the other components to be calculated

Master Mix without the fluorophore:
Preparing the Master Mix (transcription only)

			Volume x1 reaction [µL]
Number of Reactions:	**10**		
Master mix prepared for:	**11**		
T7 reaction buffer	22		2
25 mM Tris-buffered NTPs (each)	6.27		0.57
Total 4x NTP volume:	*25.08*		*2.28*
2U/µL inorganic pyrophosphatase	1.65		0.15
Water	1.1		0.1
Total MM volume:	**49.83**		**4.53**

Master Mix with the Fluorophore

Preparing the Master Mix (transcription only)1

			Volume x1 reaction [µL]
Number of Reactions:	**10**		
Master mix prepared for:	**11**		
T7 reaction buffer	22		2
25 mM Tris-buffered NTPs (each)	6.27		0.57
Total 4x NTP volume:	*25.08*		*2.28*
2U/µL inorganic pyrophosphatase	1.65		0.15
40 mM DFHBI-1T	1.1		0.1
Total MM volume:	**49.83**		**4.53**

Reaction without the fluorophore:

DNA quantity & Water (transcription only)1

	Reaction name	DNA template	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial
1	NC			#VALUE!		#VALUE!		0	10.47	F4	
2	pUC broc	pUC19 Borc.	310	190947.6	50	190.9476	146	1.307860274	9.16	F1	
3	pUC broc	pUC19 Borc.	310	190947.6	150	572.8428	146	3.9235808219	6.55	F2	5.2314410959
4	HRE5 1		446	274718.16	50	274.71816	168	1.6352271429	8.83	F6	
5	HRE5 1		446	274718.16	150	824.15448	168	4.9056814286	5.56	F7	6.5409085714
6	EREmin 1		270	166309.2	50	166.3092	156	1.0660846154	9.40	F9	
7	EREmin 1		270	166309.2	150	498.9276	156	3.1982538462	7.27	F10	4.2643384615

Reactions with the Fluoropore:

DNA quantity & Water (transcription only)

	Reaction name	DNA template	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial
1	NC			#VALUE!		#VALUE!		0	10.47	F4	
2	Blank			#VALUE!		#VALUE!		0	10.47	F5	
3	pUC broc	pUC19 Borc.	310	190947.6	50	190.9476	146	1.307860274	9.16	F1	
4	pUC broc	pUC19 Borc.	310	190947.6	100	381.8952	146	2.6157205479	7.85	F2	
5	pUC broc	pUC19 Borc.	310	190947.6	150	572.8428	146	3.9235808219	6.55	F3	7.8471616438
6	HRE5 35		446	274718.16	50	274.71816	168	1.6352271429	8.83	F6	
7	HRE5 35		446	274718.16	100	549.43632	168	3.2704542857	7.20	F7	
8	HRE5 35		446	274718.16	150	824.15448	168	4.9056814286	5.56	F7	9.8113628571
9	EREmin 35		270	166309.2	50	166.3092	156	1.0660846154	9.40	F9	
10	EREmin 35		270	166309.2	100	332.6184	156	2.1321692308	8.34	F10	
11	EREmin 35		270	166309.2	150	498.9276	156	3.1982538462	7.27	F10	6.3965076923
12	Blank							0	10.47	F11	
13	Fluorescein 1.1									F12	
14	Fluorescein 1.2									F13	
15	Fluorescein 2.1									F14	
16	Fluorescein 2.2									F15	

Activating reaction

			Quantity x1 reaction
Number of Reactions:	**0**		
Master mix prepared for:	**1**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	2		2
H_2O	3		3
Total MM volume:	**5**		5

RNA GEL ELECTROPHORESIS

Gel preparation

- 1.5% agarose gel (so 1.2 g in 80 mL)
- 80 mL: enough to have wells deep enough to host 25 µL

Gel run conditions:

- 2 h 30 min of IVT reaction (run without the fluorophore) in a PCR strip
- Added 5µL of DNA loading buffer to each well of the PCR strip
- Loaded 25 µL (the total amount) in the well
- Run for 20~30 min

Conclusion

ROSALIND IVT FLUORESCENCE

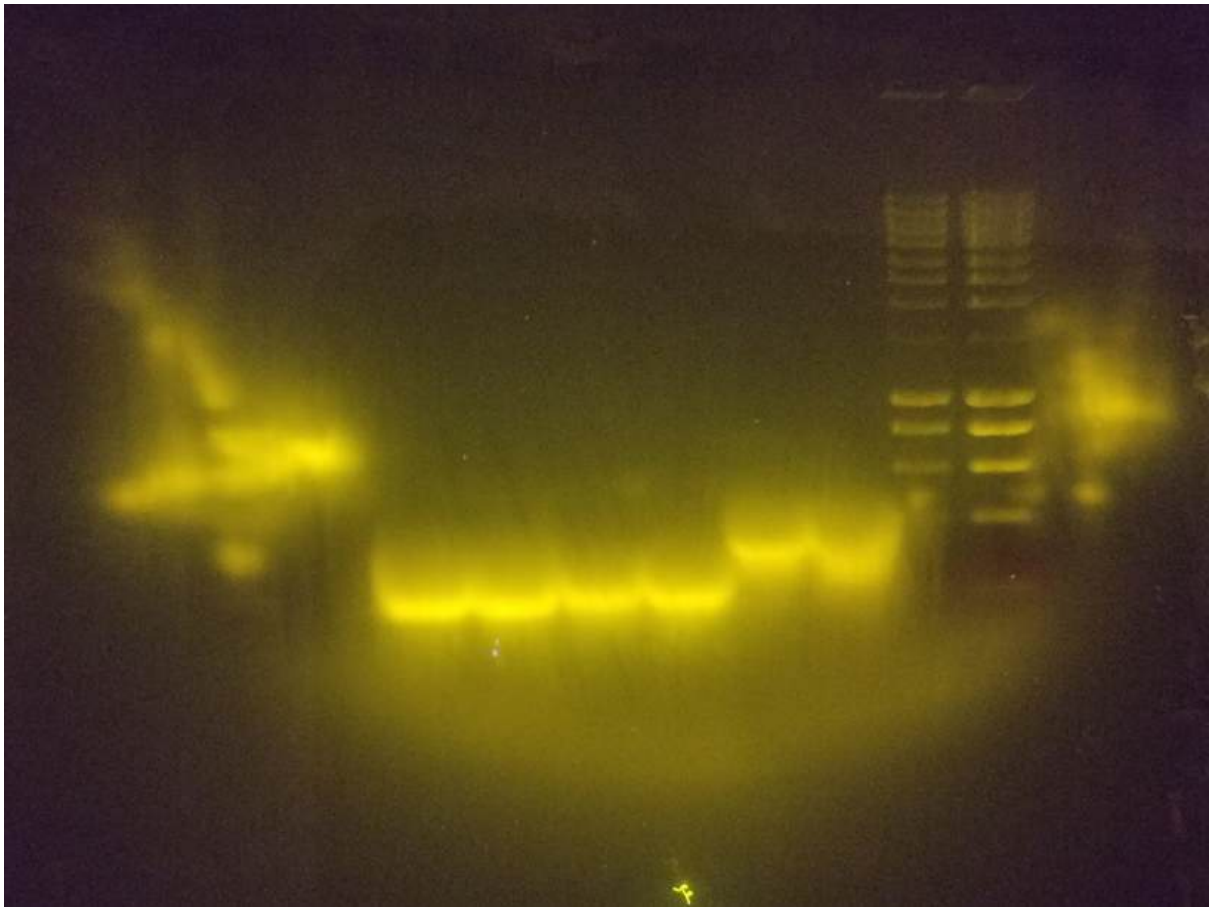
- The reaction was working! Data to be extracted from the machine and analyzed, but the values of each well were comparable to the ones from the fluorescein.
- Samples were loaded as:

Well	Samples	Concentration	Condition
G1	Blank		MM without fluorophore + Activating reaction
G2	NC		Everything but the DNA template
G3	EREmin	50 nM	Fluorophore from the beginning
G4	EREmin	100 nM	Fluorophore from the beginning
G5	EREmin	150 nM	Fluorophore from the beginning
G6	EREmin	100 nM	Fluorophore added before measuring
G7	pUC19 Broc.	50 nM	Fluorophore from the beginning
G8	pUC19 Broc.	100 nM	Fluorophore from the beginning
G9	pUC19 Broc.	150 nM	Fluorophore from the beginning
G10	pUC19 Broc.	100 nM	Fluorophore added before measuring
G11	HRE5	50 nM	Fluorophore from the beginning
G12	HRE5	100 nM	Fluorophore from the beginning
G13	HRE5	150 nM	Fluorophore from the beginning
G14	HRE5	100 nM	Fluorophore added before measuring
G15	Empty		
G16	Fluorescein	10 nM	
G17	Fluorescein	5 nM	
G18	Fluorescein	10 nM	
G19	Fluorescein	5 nM	

RNA GEL

- Also the gel seemed kind of successful
- Samples loaded as follows:

Sample	Ladder	NC	EREmin	EREmin	pUC19 Broc.	pUC19 Broc	HRE5	HRE5	Ladder	Ladder
Quantity [µL]	2	25	25	25	25	25	25	25	2	6
DNA quantity [ng]			166	498	190	573	275	894		
DNA length [bp]			270	270	310	310	446	446		
RNA length [bp]			190	190	336	336	190	190		



The smear around the DNA bands could be RNA, as well as the secondary structure.

2024.09.02 PCR ROSALIND G-block templates & DNA quantification

Overview

Track: ROSALIND
Objective: PCR of the EREmin to have the template for more ROSALIND reactions

When: 02.09.2024
Who: Lumi, Georgios
Location: GMO lab

Methods/protocol

PCR reaction

Templates to amplify:

- **HREmin** (1000 µL PCR reaction - FROM GBLOCK): 1 µL
- **EREmin** (500 µL PCR reaction - FROM GBLOCK): 0.5 µL
- **HRE5** (500 µL PCR reaction - from previous PCR product): 1.5 µL

Since all these samples are quantified, try to calculate the volume so to have 20 ng of DNA every 1000 µL of PCR reaction.

PCR table - Only modify the highlighted cells

	Volume	Final conc.	Initial conc.
Total PCR volume (insert here -->)	1000		
H_2O	439.45		
2X Master Mix Buffer	500	1x	
Primer FWD (10 µM)	30	0.3	10
Primer RV (10 µM)	30	0.3	10
Template (insert here -->)	0.55	1 pg - 10 ng DNA per 50 µL	Average amount, doesn't change much
Total	1000		

Split the 1000 µL in 2 PCR strips of 8 wells each: 16 wells each, so **62.5 µL in each well**.

PCR conditions

	Step	Temp	Time	Cycles
1	Inital denaturation	98	2 min	
2	Denaturation	98	15 sec	37x
3	Annealing	60	20 sec	
4	Elongation	72	20 sec	
5	Final elongation	72	5 min	
6	Hold	4 or 12	Infinite	

PCR purification

Protocol: *NucleoSpin® Gel and PCR Clean-up - Columns from Aafke*
--> CHECK IF THERE IS ENOUGH DNA WASH BUFFER, we should ideally wash twice

Notes:

- Use 1 column for every 200 µL of PCR product, so for 1000µL ---> **5 columns**
- Elute in nuclease-free water.
- 700 * 10 = 7 mL of wash buffer needed

Conclusion

DNA quantification

Template	DNA conc. [ng/µL]	A260/230	Volume estimate [µL]
HREmin 1 - 02/09	114.8	1.85	40
HREmin 2 - 2 02/9	104	1.85	60
HRE5 02/9	92.4	1.78	40
EREmin 02/9	112.3	1.85	40

2024.09.02-05 In-cell assay with samples

Overview

Track: In-Cell Assay
Objective: To test various water samples with the in-cell assay

When: 02/09 till 05/09.2024
Who: Mikkel
Location: GMO Lab SkyLab

Methods/protocol

02/09-2024

- Incubated 5 mL YNB media + 2% glc with yeast carrying ERα, ERß, GR, PR, AR (5 tubes in total) at 30ºC

03/09-2024

- Bathed 6 x 96 well plates in 96% EtOH. Dried in the BSC O/N.
- Prepared 50% EtOH, 2.28E-06M estradiol stock, 1M DTT, LacZ buffer all in the GMO lab
- Found 200µL tips for the assay.
- Made plate layouts

04/09-2024

Protocol: *In cell β-galactosidase assay*

- Prepared sodium carbonate solution (5.3g sodium carbonate in a final volume of 50mL)
- Wanna test S1 (Kildevæld bottled water), S2 (Salling bottled water), S3 (tap water), S4 (Lyngby lake water) with 5 receptors (ERα, ERß, GR, PR, AR)
- Finally I wanna do a dose response curve for ERα with estradiol again as a control.
- Sampled 15 mL water from Lyngby lake.
- Diluted it 10, 100, 1000, and 10000x as sample S4.1, S4.2, S4.3, and S4.4
- Filtered all samples with a 22µM sterile filter
- Followed Day 1 from the following protocol
- Plate 1 was incubated at 15:40 and plate 2 was incubated at 16:45

05/09-2024

- Part 2 of the assay with plate 1 at 8:40 and plate 2 at 9:45
- Placed the LacZ buffer in the shaking chamber at 37degC to thaw it.
- Ran the assay like last time, no issues

Conclusion:

All the data points towards the water being clean. The positive controls are really nice - we were able to replicate the data from the dose response curves nicely. Unfortunately the standard deviations for the tests are quite high, and therefore the conclusion is that all the samples were clean.

2024.09.03 ROSALIND IVT reaction lesser DNA template

Overview

Track: ROSALIND
Objective: Now that the reaction is working, we will try to get some:

- kinetic measurements
- lower DNA concentration.

When: 03.09.2024
Who: Reinis
Location: GMO lab

Methods/protocol

Protocol abstract:

1. Prepare the Master Mix in an Eppendorf
2. Prepare the aliquots of water and DNA (see table) in a PCR strip
3. Prepare the Activating Mix (see last table) in an Eppendorf
4. Transfer 4.53 µL of MM in each PCR well
5. Transfer 5µL of Activating Mix in each PCR well
6. Transfer the 20 µL of reaction in the 386 plate (use the P10, 10 µL at a time to avoid bubbles)
7. Cover the 386 well with the membrane (make sure that the row with the reaction has not strange curves or edges and that it's flat)

Master Mix with the Fluorophore

Preparing the Master Mix (Transcription only)1

			Volume x1 reaction [µL]
Number of Reactions:	**14**		
Master mix prepared for:	**15**		
T7 reaction buffer	30		2
25 mM Tris-buffered NTPs (each)	*8.55*		0.57
Total 4x NTP volume:	**34.2**		*2.28*
2U/µL inorganic pyrophosphatase	2.25		0.15
40 mM DFHBI-1T	1.5		0.1
Total MM volume:	**67.95**		**4.53**

KINETIC MEASUREMENTS

- Set reading for 4 hours every 3 min

DNA quantity & Water (transcription only)1

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial	Notes
1	Blank		Use the leftovers from MM and act. reaction						I1		
2	NC (no T7, pUC)	310	190947.6	40	152.75808	146	0	10.47	I2		
3	NC (no DNA)								I3		
4	pUC broc 35	310	190947.6	20	76.37904	146	0.5231441096	9.95	I4		
5	pUC broc 35	310	190947.6	25	95.4738	146	0.653930137	9.82	I5		
6	pUC broc 35	310	190947.6	40	152.75808	146	1.0462882192	9.42	I6		
7	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	I7		
8	HRE5 35	446	274718.16	25	137.35908	168.3	0.8161561497	9.65	I8		
9	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	I9		Messed up
10	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	I10		Messed up
11	HREmin	266	163845.36	25	81.92268	114.8	0.7136121951	9.76	I11		
12	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	I12		
13	EREmin 35	270	166309.2	20	66.52368	156	0.4264338462	10.04	I13	2.8527153096	
14	EREmin 35	270	166309.2	25	83.1546	156	0.5330423077	9.94	I14		
15	EREmin 35	270	166309.2	40	133.04736	156	0.8528676923	9.62	I15		

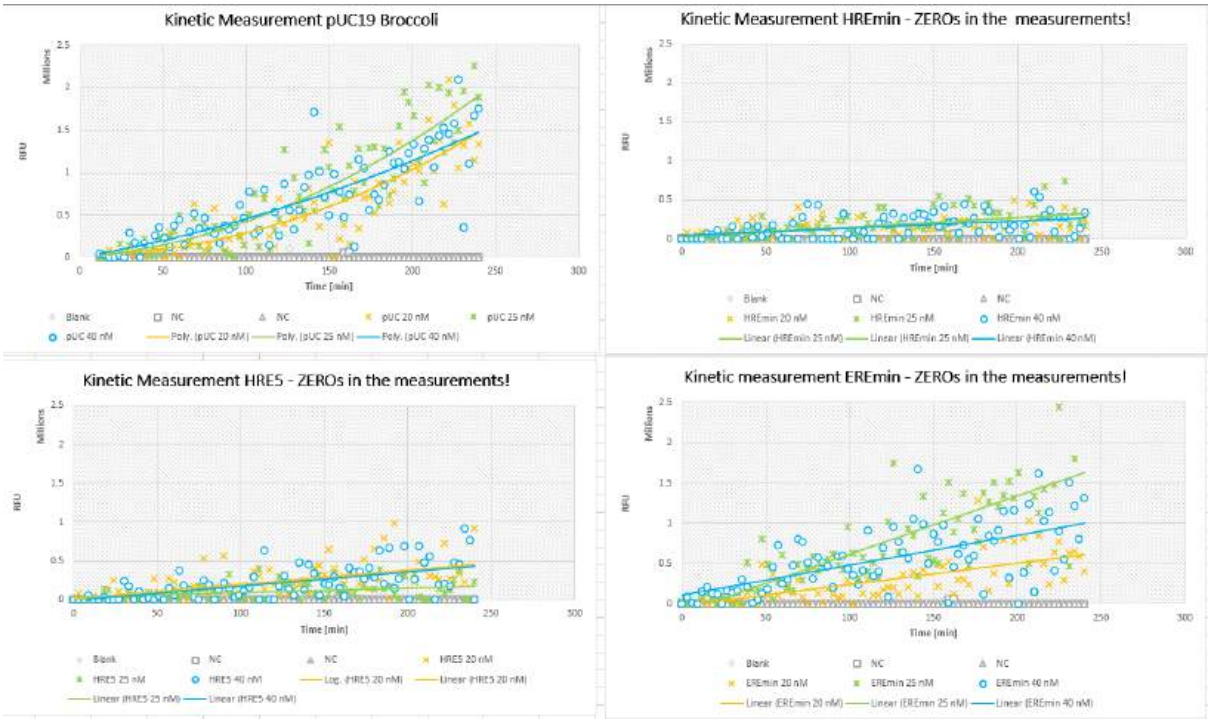
Activating reaction

Activating Reaction (transcription only)

			Quantity x1 reaction
Number of Reactions:	**13**		
Master mix prepared for:	**14**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	28		2
H_2O	42		3
Total MM volume:	**70**		5

Conclusion

- Not too good: after 4h of measurements, basically only pUC19 was above the 1 million threshold (surely above the sensitivity measurement).
- There were many 0s measured in the HREmin, HRE5 and EREmin (while none in the pUC19).
- Reinis said that well 10 (HREmin) was messed up



- Another 4h measurement was started after the first one (with few hours of delay). Data still to be retrieved
- It seems that these DNA concentration was not enough in these samples

Future

- Try again with duplicates: 25 nM, 30 nM, 40 nM, 50 nM

2024.09.04 ROSALIND IVT reaction HRE5,HREmin DNA template with 10~40nM - INCREASED SIGNAL (reading from top)

Overview

Track: ROSALIND
Objective: Now that the reaction is working, we will try to get some:

- kinetic measurements
- lower DNA concentration.

When: 04.09.2024
Who: Georgios, Sirio
Location: GMO lab

Methods/protocol

Protocol abstract:

1. Prepare the Master Mix in an Eppendorf
2. Prepare the aliquots of water and DNA (see table) in a PCR strip
3. Prepare the Activating Mix (see last table) in an Eppendorf
4. Transfer 4.53 µL of MM in each PCR well
5. Transfer 5µL of Activating Mix in each PCR well
6. Transfer the 20 µL of reaction in the 386 plate (use the P10, 10 µL at a time to avoid bubbles)
7. Cover the 386 well with the membrane (make sure that the row with the reaction has not strange curves or edges and that it's flat)

Master Mix with the Fluorophore

Preparing the Masters Mix (transcription only)

			Volume x1 reaction [µL]
Number of Reactions:	**45**		
Master mix prepared for:	**46**		
T7 reaction buffer	92		2
25 mM Tris-buffered NTPs (each)	*26.22*		0.57
Total 4x NTP volume:	**104.88**		*2.28*
2U/µL inorganic pyrophosphatase	6.9		0.15
40 mM DFHBI-1T	4.6		0.1
Total MM volume:	**208.38**		**4.53**

KINETIC MEASUREMENTS

- Set reading for 2+1 hours every 3 min
- The PCRs run in the strip incubate for 2 hrs and then transport in the plate for 1 hr

DNA quantity & Water - (transcription only)1

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial	Notes
1	Blank		Use the leftovers from MM and act. reaction						J1		
2	NC (no T7, pUC)	310	190947.6	40	152.75808	146	0	10.47	J2		
3	NC (no DNA)						0	10.47	J3		
4	pUC broc 35	310	190947.6	10	38.18952	146	0.2615720548	10.21	J4		
5	pUC broc 35	310	190947.6	30	114.56856	146	0.7847161644	9.69	J5		
6	pUC broc 35	310	190947.6	50	190.9476	146	1.307860274	9.16	J6		
7	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	J7		
8	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	J8		
9	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	J9		
10	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	J10		
11	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	J11		
12	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	J12		
13	HRE5 35	446	274718.16	30	164.830896	168.3	0.9793873797	9.49	J13		
14	HRE5 35	446	274718.16	30	164.830896	168.3	0.9793873797	9.49	J14		
15	HRE5 35	446	274718.16	30	164.830896	168.3	0.9793873797	9.49	J15		
16	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	J16		
17	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	J17		
18	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	J18		
19	HRE5 35	446	274718.16	50	274.71816	168.3	1.6323122995	8.84	J19		
20	HRE5 35	446	274718.16	50	274.71816	168.3	1.6323122995	8.84	J20		
21	HRE5 35	446	274718.16	50	274.71816	168.3	1.6323122995	8.84	J21	14.6908106952	
22	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	K1		
23	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	K2		
24	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	K3		
25	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	K4		
26	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	K5		
27	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	K6		
28	HREmin	266	163845.36	30	98.307216	114.8	0.8563346341	9.61	K7		
29	HREmin	266	163845.36	30	98.307216	114.8	0.8563346341	9.61	K8		
30	HREmin	266	163845.36	30	98.307216	114.8	0.8563346341	9.61	K9		
31	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	K10		
32	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	K11		
33	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	K12		
34	HREmin	266	163845.36	50	163.84536	114.8	1.4272243902	9.04	K13		
35	HREmin	266	163845.36	50	163.84536	114.8	1.4272243902	9.04	K14		
36	HREmin	266	163845.36	50	163.84536	114.8	1.4272243902	9.04	K15	12.8450195122	

Reaction in PCR tubes

DNA quantities & Water - Transcription only

	A	B	C	D	E	F	G	H	I
1	pUC broc 35	310	190947.6	20	76.37904	146	0.5231441096	9.95	L1
2	pUC broc 35	310	190947.6	40	152.75808	146	1.0462882192	9.42	L2
3	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	L5
4	HRE5 35	446	274718.16	20	109.887264	168.3	0.6529249198	9.82	L6
5	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	L9
6	HRE5 35	446	274718.16	40	219.774528	168.3	1.3058498396	9.16	L10
7	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	L3
8	HREmin	266	163845.36	20	65.538144	114.8	0.5708897561	9.90	L4
9	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	L7
10	HREmin	266	163845.36	40	131.076288	114.8	1.1417795122	9.33	L8

Activating reaction

			Quantity x1 reaction
Number of Reactions:	**45**		
Master mix prepared for:	**46**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	92		2
H_2O	138		3
Total MM volume:	**230**		5

Conclusion

No results.

2024.09.09-11 ROSALIND Aldosterone Receptor Reaction - Proof of Concept

Overview

Track: ROSALIND
Objective: Using the lower DNA conc. (10 nM), we will get the proof of concept.

When: 11.09.2024
Who: Sirio, Georgios
Location: GMO lab

Methods/protocol

Receptor calculation

Bartosz calculations: **Five binding sites Ver B**. Go for **90 min** incubation.

	one binding site	five binding sites ver A	five binding sites ver B	five binding sites ver C
amount of experiments	4	4	1	1
DNA template	10nM	10nM	10nM	10nM
testosterone	0-0,20uM	0-0,22uM	0-0,44uM	0-0,88uM
receptor	1,1uM	1,2uM	2,4uM	4,8uM
First step (without activation)	1/4/16/90min	1/4/16/90min	90 min	90 min

Receptor Calculations

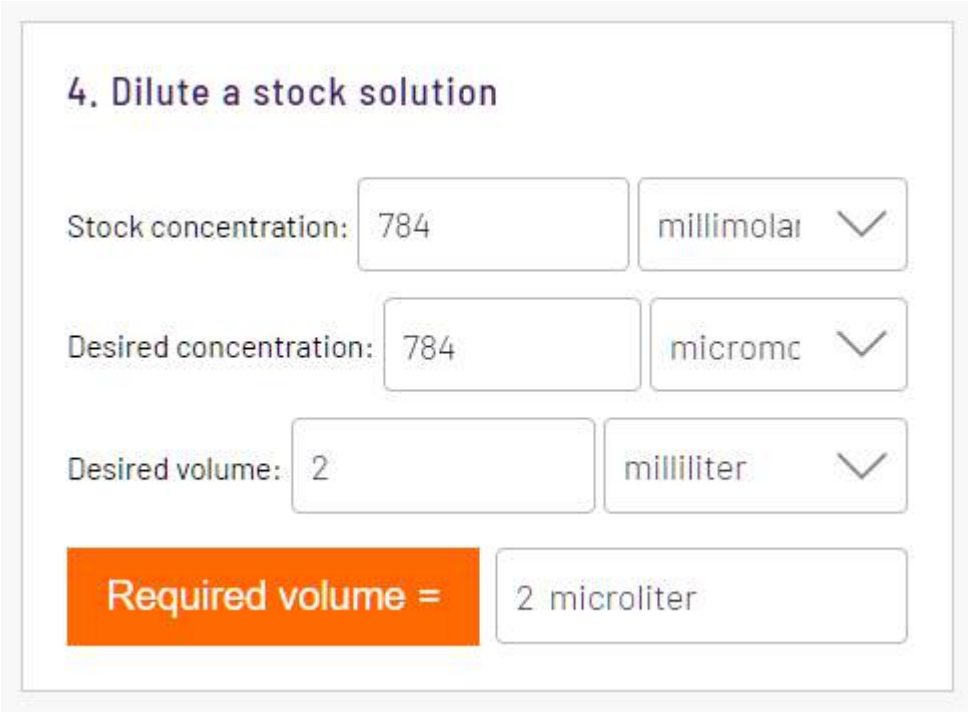
	one binding site	**five binding sites ver A**	**five binding sites ver B**	**five binding sites ver C**
amount of experiments	4	4	1	1
DNA template	10nM	10nM	10nM	10nM
testosterone	0-0,20µM	0-0,22µM	0-0,44µM	0-0,88µM
receptor	1,1µM	1,2µM	2,4µM	4,8µM
First step (without activation)	1/4/16/90min	1/4/16/90min	90 min	90 min

Receptor concentration (using Thermo-Fisher column)

- Initial receptor concentration: 0.14 µg/µL = 1.913 nM
- The volume (357 µL) was reduced to 100 µL → Final concentration = 1.913 * 357/100 = **6.89 µM**
- Buffer contains 10% glycerol → 0.69 µL in 20 µL, it will be 3%

Hormone concentration:

Testosterone



Summary for the reaction:

- Prepare the 2 master mixes
- Prepare the DNA dilutions in the PCR tubes
- Add the Master Mixes to the correspondent PCR tube
- Add the Receptor to the right tubes
- Incubate for 90 min
- Add the activator mix
- Transfer the solutions into the 384-wells plate and read for 4 h

Receptor & Hormone Calculations

	Final Concentration [µM]	Molecular mass [µg]	Actual molec. conc. [µM]	Volume x1 reaction [µL]	Mass per X reactions [µg]	Volume per X reactions
Androgen receptor	2	2.9	6.89	**5.8055152395**	**17.4**	**34.8330914369**
	1.1	1.595	6.89	**3.1930333817**	**9.57**	**19.1582002903**
Testosterone	4	0.0230736	784	**0.1020408163**	**0.1384416**	**0.612244898**
Reaction number:	6					

Master Mix No Hormone

			Volume x1 reaction [µL]
Number of Reactions:	**9**		
Master mix prepared for:	**10**		
IVT buffer	20		2
25 mM Tris-buffered NTPs (each)	5.7		0.57
Total 4x NTP volume:	*22.8*		*2.28*
2U/µL inorganic pyrophosphatase	1.5		0.15
40 mM DFHBI-1T	1		0.1
Total MM volume:	**45.3**		**4.53**

Master Mix + Hormone

		Volume x1 reaction [µL]				
Number of Reactions:	**6**					
Master mix prepared for:	**7**					
IVT buffer	14	2				
25 mM Tris-buffered NTPs (each)	3.99	0.57				
Total 4x NTP volume:	*15.96*	*2.28*				
2U/µL inorganic pyrophosphatase	1.05	0.15				
40 mM DFHBI-1T	0.7	0.1				
Hormone (Testosterone)	0.714	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**32.424**	**4.632**				

DNA quantity & Water (transcription only) 1

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Receptor Volume [µL]	Sub-Total DNA per vial	Notes
1	Blank		Use the leftovers from MM and act. reaction					10.50	M1			
2	NC (no T7)	266	163845.36	10	32.769072	146	0.2244456986	15.25	M2			
3	NC (no DNA)						0	10.47	M3			
4	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	M4			
5	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	M5			
6	HREmin	266	163845.36	10	32.769072	114.8	0.285444878	10.18	M6			
7	HREmin + H	266	163845.36	10	32.769072	114.8	0.285444878	10.08	M4			
8	HREmin + H	266	163845.36	10	32.769072	114.8	0.285444878	10.08	M5			
9	HREmin + H	266	163845.36	10	32.769072	114.8	0.285444878	10.08	M6			
10	HREmin + R	266	163845.36	10	32.769072	114.8	0.285444878	4.38	M7	5.80		
11	HREmin + R	266	163845.36	10	32.769072	114.8	0.285444878	4.38	M8	5.80		
12	HREmin + R	266	163845.36	10	32.769072	114.8	0.285444878	4.38	M9	5.80		
13	HREmin + RH	266	163845.36	10	32.769072	114.8	0.285444878	4.28	M10	5.80		
14	HREmin + RH	266	163845.36	10	32.769072	114.8	0.285444878	4.28	M11	5.80		
15	HREmin + RH	266	163845.36	10	32.769072	114.8	0.285444878	4.28	M12	5.80	34.8	Receptor volume
16	HREmin	266	163845.36	5	16.384536	114.8	0.142722439	10.33	M6	34.8		

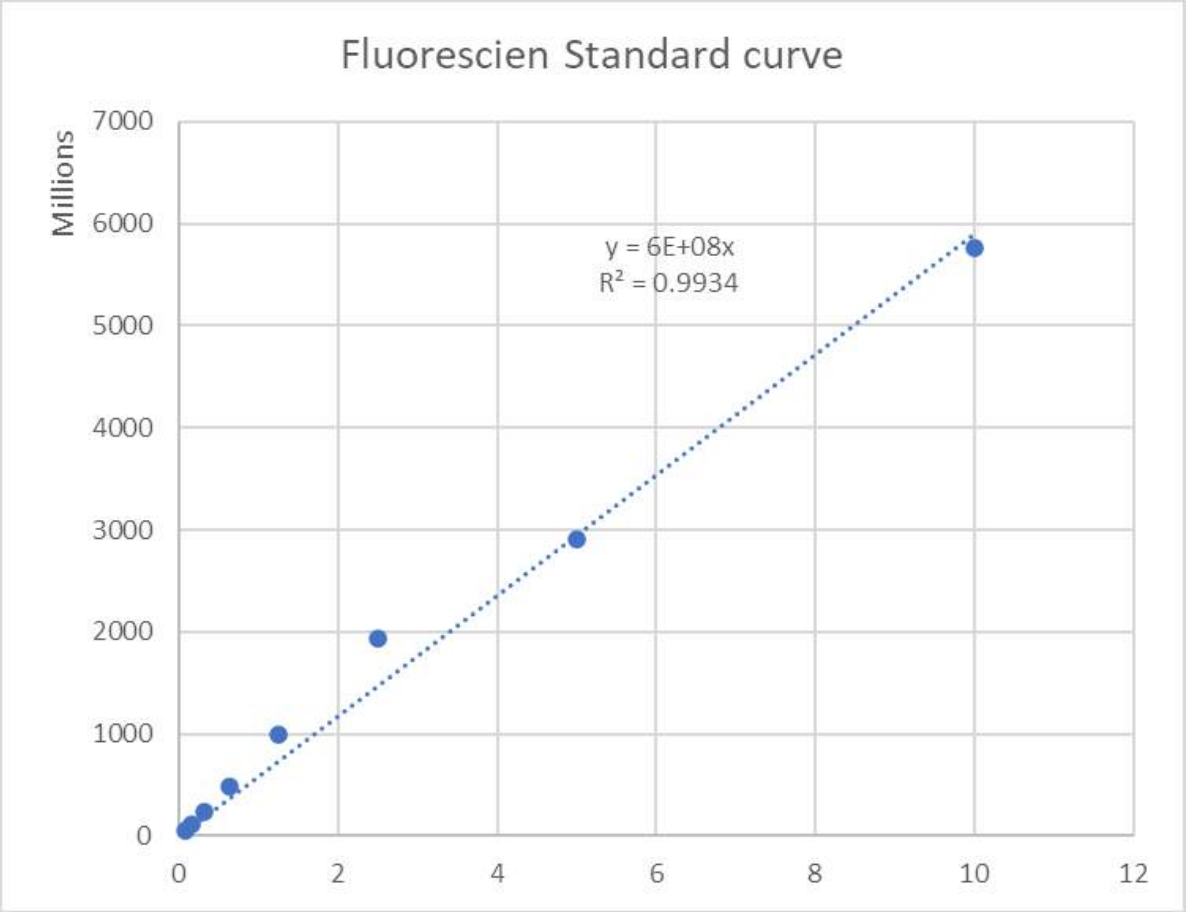
Activation Reaction (transcription only)

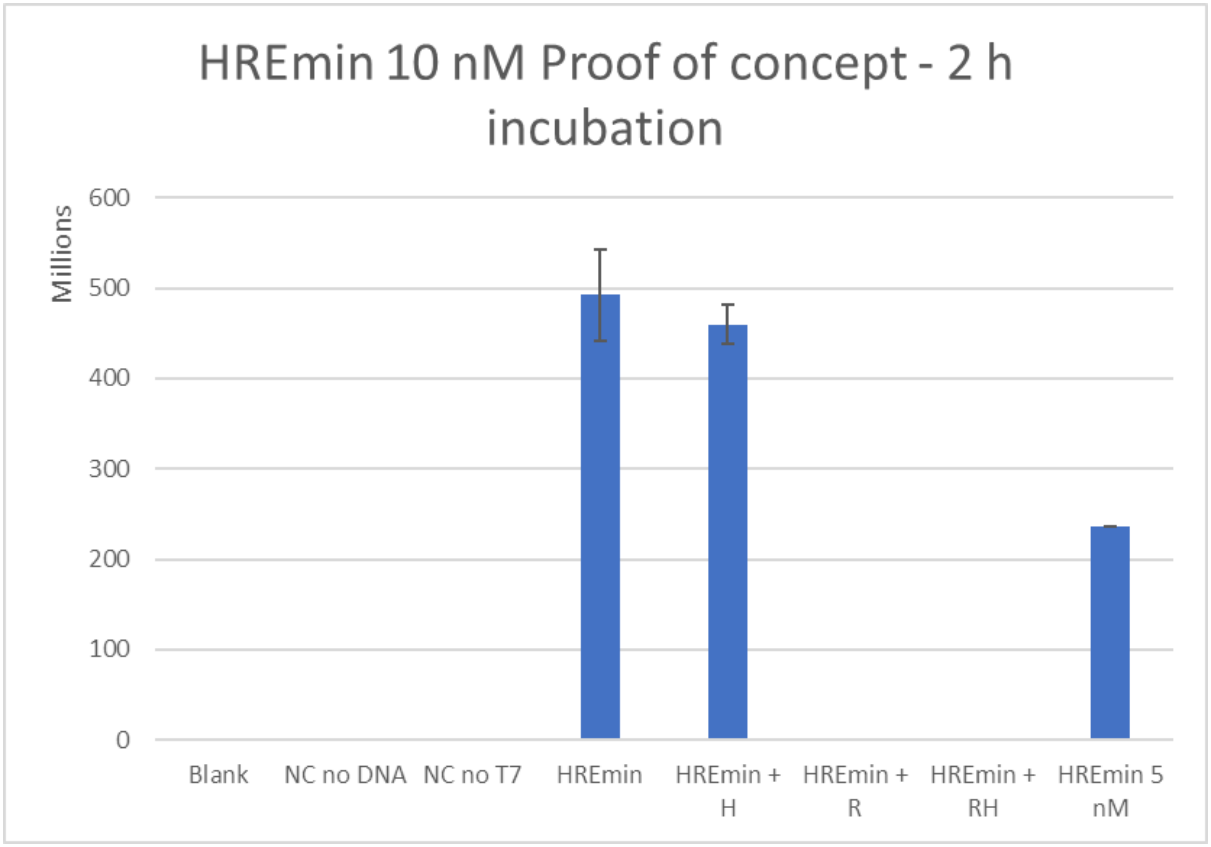
		Quantity x1 reaction
Number of Reactions:	**15**	
Master mix prepared for:	**16**	
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	32	2
H_2O	48	3
Total MM volume:	**80**	5

Conclusion

When adding the receptor, no output is registered. After discussing in a meeting, thanks to Reinis, the dose is the presence of **1% sarkosyl** in the buffer of the receptor.
Even though it was further diluted in the final reaction, a concentration of 0.1% is still sufficient to inhibit completely the T7 polymerase - in our case 0.29%. Thus, we still don't know if our designed part works.

We didn't get a kinetic reading because the plate reader was not working at all for no apparent reason. We had to restart it 5 times before managing to get on reliable reading, after 2 hours of incubation after the T7 pol. was added (45 min at 37°, 30 min at RT and the rest on ice).





Extra table for further experiments (do not use now, HRE5):

DNA quantity & Water (transcription only)2

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA concentration [nM]	DNA quantity [ng]	DNA concentration [ng/µL]	DNA volume	Water	Well ID	Sub-Total DNA per vial	Notes
1	Blank		Use the leftovers from MM and act. reaction						I1		
2	NC (no T7, pUC)	266	163845.36	10	32.769072	146	0	10.47	I2		
3	NC (no DNA)								I3		
4	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I7		
5	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I8		
6	HRE5 35	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I9		
7	HRE5 35 + R	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I7		
8	HRE5 35 + R	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I8		
9	HRE5 35 + R	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I9		
10	HRE5 35 + RH	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I7		
11	HRE5 35 + RH	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I8		
12	HRE5 35 + RH	446	274718.16	10	54.943632	168.3	0.3264624599	10.14	I9	2.938162139	

2024.09.12 ROSALIND test for Proof of Concept

Overview

Track: ROSALIND
Objective: Try which receptor concentration works best, now exchanging the buffer in which the receptor was sold.

When: 12.09.2024
Who: Sirio, Georgios
Location: GMO lab

Methods/protocol

Buffer exchange:

- Using the protein concentrators spin columns with 10 kDa cut-off, we can change the buffer.
- The ideal would be to use the IVT buffer, but we do not have enough, so we're going to use diluted PBS.

Buffer in the purchased Androgen Receptor:

- 25 mM Tris-HCl, pH 8.0,
- 150 mM NaCl,
- 1% sarkosyl, --> To get rid of absolutely!
- 10% glycerol --> To get rid of

PBS composition:

- NaCl: 137 mM
- KCl: 2.7 mM
- Na_2HPO_4: 10 mM
- KH_2PO_4: 1.8 mM

1X Hi-T7® RNA Polymerase Reaction Buffer:

- 40 mM Tris-HCl
- 4 mM $MgCl_2$
- 50 mM NaCl
- 2 mM spermidine
- 1 mM DTT

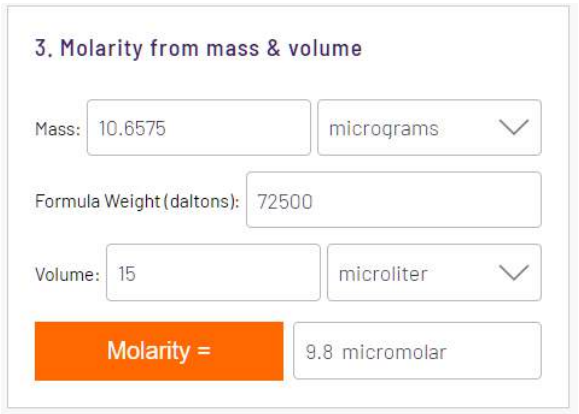
As the concentration of NaCl is too high (even when diluted to the final 20µL reaction volume), we need to dilute it, to have a similar concentration.

Calculations:

- In a sterile 10 mL tube, add 2.9197 mL of PBS.
- Add 10-2.9197 = 7.08 mL of MilliQ (or Nuclease-free Water)
- Sterilize the solution in using a syringe filter

Actual receptor concentration:

- Add the required amount of receptor volume in the column to have **11 µg of receptor --> 22.0209 µL**
- Add diluted PBS until the column is full and spin down
- Repeat the process again for another 2 times
- Finally, spin down until the last mark: **15µL of volume 16.8,**, to reach a concentration of **10.1149 µM 9.08**

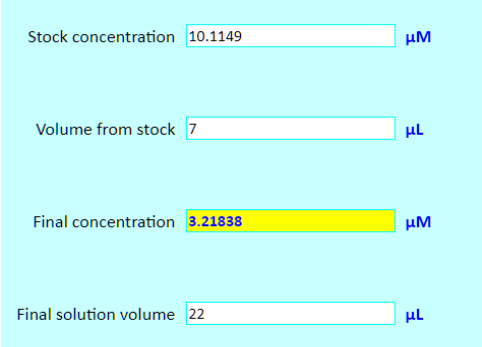


"The dead-stop volume for the 0.5 mL Concentrators is approximately 15 µL." :

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011811_Pierce_Concent_PES_3K_10K_30K_100K_MWCO_0.5mL_UG.pdf)

Pipette the solution in the most concentrated wells (total of 8.16µL) → Remaining volume of: 16.8-(4.43*2) = 7.94 **µL**

Dilute the 7.94 µ with **14.36 µL** (of diluted PBS) to reach a final volume of 7.94+14.3 = **22.3 µL**, and a concentration of **3.21838 µM**



Receptor & Hormone Calculations

	Final Concentration [µM]	Molecular mass [µg]	Actual molec. conc. [µM]	Volume x1 reaction [µL]	Mass per X reactions [µg]	Volume per X reactions
Androgen receptor	0.01	0.0145	1.931	**0.1035732781**	**0.087**	**0.6214396686**
	6.89	9.9905	6.89	**20**	**59.943**	**120**
Testosterone	4	0.0230736	784	**0.1020408163**	**0.1384416**	**0.612244898**
Reaction number:	6					

Master Mix No Hormone

			Volume x1 reaction [µL]
Number of Reactions:	**12**		
Master mix prepared for:	**13**		
IVT buffer	26		2
25 mM Tris-buffered NTPs (each)	7.41		0.57
Total 4x NTP volume:	*29.64*		*2.28*
2U/µL inorganic pyrophosphatase	1.95		0.15
40 mM DFHBI-1T	1.3		0.1
Total MM volume:	**58.89**		**4.53**

Master Mix + Hormone

		Volume x1 reaction [µL]				
Number of Reactions:	**7**					
Master mix prepared for:	**8**					
IVT buffer	16	2				
25 mM Tris-buffered NTPs (each)	4.56	0.57				
Total 4x NTP volume:	*18.24*	*2.28*				
2U/µL inorganic pyrophosphatase	1.2	0.15				
40 mM DFHBI-1T	0.8	0.1				
Hormone (Testosterone)	0.816	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**37.056**	**4.632**				

DNA quantity & Water (transcription only)

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA conc. [nM]	DNA quantity [ng]	DNA conc. [ng/µL]	DNA volume [µL]	Water	Well ID	Receptor Volume [µL]	Receptor Conc. [µM]	Receptor mass [µg]	Actual receptor conc. [µM]	Notes
1	Blank		Use the leftovers from MM and act. reaction					15.50	C1					
2	NC (no T7)	266	163845.36	10	32.77	114.8	0.285	15.18	C2					
3	NC (no DNA)						0.000	10.47	C3					
4	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	C4					
5	HREmin + H	266	163845.36	10	32.77	114.8	0.285	10.08	C5					
6	HREmin + Inhib.	266	163845.36	10	32.77	114.8	0.285	5.18	C6	5				Buffer of the receptor stock
7	HREmin + Inhib.	266	163845.36	10	32.77	114.8	0.285	5.18	C7	5				Buffer of the receptor stock
8	HREmin + Inhib.	266	163845.36	10	32.77	114.8	0.285	5.18	C8	5				Buffer of the receptor stock
9	HREmin + R 0.025	266	163845.36	10	32.77	114.8	0.285	10.03	C9	0.155	0.025	0.03625	3.21838	
10	HREmin + R 0.05	266	163845.36	10	32.77	114.8	0.285	9.87	C10	0.311	0.05	0.0725	3.21838	
11	HREmin + R 0.1	266	163845.36	10	32.77	114.8	0.285	9.56	C11	0.621	0.1	0.145	3.21838	
12	HREmin + R 0.5	266	163845.36	10	32.77	114.8	0.285	7.08	C12	3.107	0.5	0.725	3.21838	
13	HREmin + R 1	266	163845.36	10	32.77	114.8	0.285	3.97	C13	6.214	1	1.45	3.21838	not enough in the column, so addition of 5 ul PBS and load to the reaction
14	HREmin + R 2	266	163845.36	10	32.77	114.8	0.285	6.23	C14	*3.955*	2	2.9	10.1149	
15	HREmin + RH 0.025	266	163845.36	10	32.77	114.8	0.285	9.93	C15	0.155	0.025	0.03625	3.21838	
16	HREmin + RH 0.05	266	163845.36	10	32.77	114.8	0.285	9.77	C16	0.311	0.05	0.0725	3.21838	
17	HREmin + RH 0.1	266	163845.36	10	32.77	114.8	0.285	9.46	C17	0.621	0.1	0.145	3.21838	
18	HREmin + RH 0.5	266	163845.36	10	32.77	114.8	0.285	6.98	C18	3.107	0.5	0.725	3.21838	
19	HREmin + RH 1	266	163845.36	10	32.77	114.8	0.285	3.87	C20	6.214	1	1.45	3.21838	bubles in the C19, transport to C20
20	HREmin + RH 2	266	163845.36	10	32.77	114.8	0.285	5.65	C21	*4.429*	2	2.9	9.0312	
21									*Total vol. 1*	*8.384*		10.6575		
22									Total vol 2:	20.818				
23						Total:	5.138		Total:	29.202	29.20			

Activation Reaction (transcription only)

			Quantity x1 reaction
Number of Reactions:	**18**		
Master mix prepared for:	**19**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	38		2
H_2O	57		3
Total MM volume:	**95**		5

Conclusion

No results.

2024.09.14 ROSALIND receptor ~1 µM

Overview

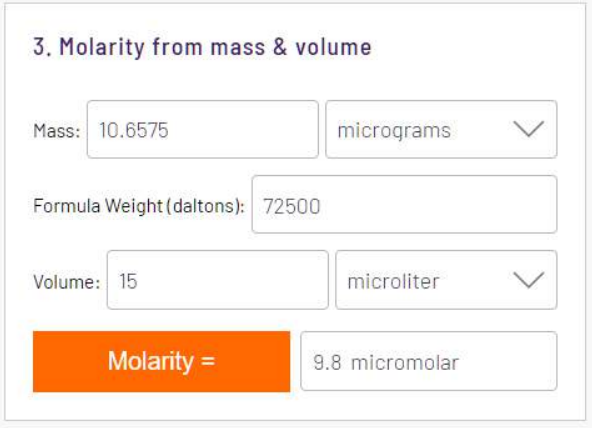
Track: ROSALIND
Objective: Short description of the objective of the lab work

When: 14.09.2024
Who: Sirio
Location: GMO lab Skylab

Methods/protocol

Actual receptor concentration:

- Add the required amount of receptor volume in the column to have **23 µg of receptor** (recovery of 97%: 22.3 µg left) --> Pipette **164.2886 µL from aliquot #2**
- Add diluted PBS until the column is full and spin down
- Repeat the process again for another 2 times
- Finally, spin down until the last mark: **50 µL,**, to reach a concentration of 6.1545 µM
- With the recovery of 97%, the mass in the column will be 22.31 µg, so that's the reason for the above concentration



"The dead-stop volume for the 0.5 mL Concentrators is approximately 15 µL." :

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011811_Pierce_Concent_PES_3K_10K_30K_100K_MWCO_0.5mL_UG.pdf)

Masters Mix No Hormone

		Volume x1 reaction [µL]
Number of Reactions:	**13**	
Master mix prepared for:	**14**	
IVT buffer	28	2
25 mM Tris-buffered NTPs (each)	7.98	0.57
Total 4x NTP volume:	*31.92*	*2.28*
2U/µL inorganic pyrophosphatase	2.1	0.15
40 mM DFHBI-1T	1.4	0.1
Total MM volume:	**63.42**	**4.53**

Master Mix + Hormone

		Volume x1 reaction [µL]				
Number of Reactions:	**9**					
Master mix prepared for:	**10**					
IVT buffer	20	2				
25 mM Tris-buffered NTPs (each)	5.7	0.57				
Total 4x NTP volume:	*22.8*	*2.28*				
2U/µL inorganic pyrophosphatase	1.5	0.15				
40 mM DFHBI-1T	1	0.1				
Hormone (Testosterone)	1.02	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**46.32**	**4.632**				

Activating Reaction (transcription only)

		Quantity x1 reaction
Number of Reactions:	**22**	
Master mix prepared for:	**23**	
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	46	2
H_2O	69	3
Total MM volume:	**115**	5

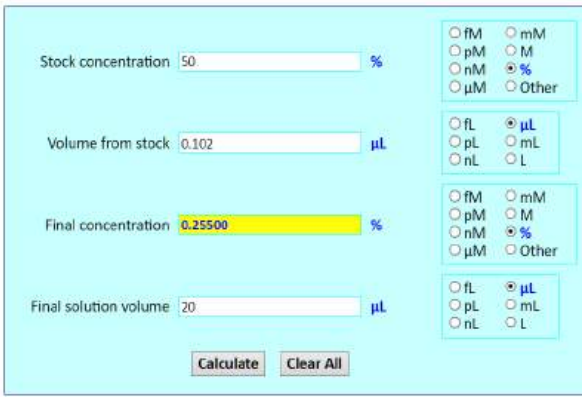
Conclusion

It didn’t work.

2024.09.19 Ethanol effect on ROSALIND transcription

Overview

Track: ROSALIND
Objective: Test the suspected effect on ethanol in the transcription. In the previous reaction, it was **0.255%**. We want to cut-out the effects of ethanol compared to the hormone.
The ethanol was added in the reaction as hormone was dissolved in a 50% ethanol solution, and with a final concentration of:



When: 19.09.2024
Who: Sirio, Georgios
Location: GMO lab

Methods/protocol

- In order to make the reactions with such a small volume, we dilute the 10.4 µL of 96% of EtOH into 189,6 µL of Nuclease-Free Water. Total **[EtOH]= 5%**.
- In this point, we use the **working stock of 784 mM** Testosterone and dilute 1000X to final [Testosterone] = 784 µM.
- For the **Testosterone-DMSO** solution we used 250 µL of Nuclease-Free Water and was mixed with 250 µL of 100% DMSO to final [DMSO]=50%. Then we added 0.5 µL of the working stock of Testosterone, so finally we got **[Testosterone] = 784 µM.**
- For the **Testosterone-H_2O** solution we just used 1000 µL of Nuclease-Free Water and was mixed with 1 µL of the working stock of Testosterone, so finally we got **[Testosterone] = 784 µM.**

Master Mix no additions:

		Volume x1 reaction [µL]
Number of Reactions:	**4**	
Master mix prepared for:	**5**	
IVT buffer	10	2
25 mM Tris-buffered NTPs (each)	2.85	0.57
Total 4x NTP volume:	*11.4*	*2.28*
2U/µL inorganic pyrophosphatase	0.75	0.15
40 mM DFHBI-1T	0.5	0.1
Total MM volume:	**22.65**	**4.53**

Master Mix + EtOH 50% 1

		Volume x1 reaction [µL]				
Number of Reactions:	**5**					
Master mix prepared for:	**6**					
IVT buffer	12	2				
25 mM Tris-buffered NTPs (each)	3.42	0.57				
Total 4x NTP volume:	*13.68*	*2.28*				
2U/µL inorganic pyrophosphatase	0.9	0.15				
40 mM DFHBI-1T	0.6	0.1				
Hormone (Testosterone)	0.612	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**27.792**	**4.632**				

Master Mix + Hormone in EtOH 50%

		Volume x1 reaction [µL]				
Number of Reactions:	**4**					
Master mix prepared for:	**5**					
IVT buffer	10	2				
25 mM Tris-buffered NTPs (each)	2.85	0.57				
Total 4x NTP volume:	*11.4*	*2.28*				
2U/µL inorganic pyrophosphatase	0.75	0.15				
40 mM DFHBI-1T	0.5	0.1				
Hormone (Testosterone)	0.51	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**23.16**	**4.632**				

DNA quantity & Water (transcription only) 1:

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA conc. [nM]	DNA quantity [ng]	DNA conc. [ng/µL]	DNA volume [µL]	Water	Well ID	Volume of EtOH [µL]	EtOH Conc. [%]	Stock EtOH conc. [%]	Notes
1	Blank		Use the leftovers from MM and act. reaction					15.50	E1				
2	NC (no T7)	266	163845.36	10	32.77	114.8	0.285	15.18	E2				
3	NC (no DNA)						0.000	10.47	E3				
4	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E4				
5	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E5				
6	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E6				
7	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	9.88	E9	0.200	0.05	5	
8	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	9.06	E12	1.020	0.255	5	
9	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	9.06	E13	1.020	0.255	5	
10	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	9.06	E14	1.020	0.255	5	
11	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	0.08	E17	10.000	2.5	5	
12	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F6	0.102	0.255	50	
13	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F7	0.102	0.255	50	
14	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F8	0.102	0.255	50	
15	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.08	F13	1.000	2.5	50	
16	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	9.98	E12	0.102	0.255	50	
17	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	9.98	E13	0.102	0.255	50	
18	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	9.98	E14	0.102	0.255	50	
19	HREmin + H (DMSO)	266	163845.36	10	32.77	114.8	0.285	9.98	E12	0.102	0.255	50	DMOS instead of EtOH
20	HREmin + H (DMSO)	266	163845.36	10	32.77	114.8	0.285	9.98	E13	0.102	0.255	50	DMOS instead of EtOH
21	HREmin + H (DMSO)	266	163845.36	10	32.77	114.8	0.285	9.98	E14	0.102	0.255	50	DMOS instead of EtOH

Activating Reaction (transcription only)

			Quantity x1 reaction
Number of Reactions:	**21**		
Master mix prepared for:	**22**		
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	44		2
H_2O	66		3
Total MM volume:	**110**		5

Conclusion

Is didn’t work.

2024.09.20 FINAL ROSALIND REACTION

Overview

Track: ROSALIND
Objective: Final ROSALIND reaction, to:

- Test the influence of interfering agents on the reaction (EtOH, DMSO, hormone)
- Test EDCs on HREmin
- Test HRE5

When: 20.09.2024
Who: Sirio, Georgios
Location: GMO lab

Methods/protocol

Available receptor left:

- From 1st batch: 45 µL --> **22.4786 µg**
- From 2nd batch: 25µL ---> **3.4999 µg**

Total mass: **25.9785 µg**

Summary of the reaction:

- Dilute the hormone (DONE)
- Prepare the MM
- Change the buffer of the hormone
- Prepare the DNA reactions in the PCR strips

- In order to make the reactions with such a small volume, we dilute the 10.4 µL of 96% of EtOH into 189,6 µL of Nuclease-Free Water. Total **[EtOH]= 5%**.
- In this point, we use the **working stock of 784mM** Testosterone and dilute 1000X to final [Testosterone] = 784µM.
- For the **Testosterone-DMSO** solution we used 250 µL of Nuclease-Free Water and was mixed with 250 µL of 100% DMSO to final [DMSO]=50%. Then we added 0.5 µL of the working stock of Testosterone, so finally we got **[Testosterone] = 784µM.**
- For the **Testosterone-H_2O** solution we just used 1000µL of Nuclease-Free Water and was mixed with 1 µL of the working stock of Testosterone, so finally we got **[Testosterone] = 784µM.**

Master Mix no additions

		Volume x1 reaction [µL]
Number of Reactions:	**18**	
Master mix prepared for:	**19**	
IVT buffer	38	2
25 mM Tris-buffered NTPs (each)	10.83	0.57
Total 4x NTP volume:	*43.32*	*2.28*
2U/µL inorganic pyrophosphatase	2.85	0.15
40 mM DFHBI-1T	1.9	0.1
Total MM volume:	**86.07**	**4.53**

Master Mix + EtOH 50%

		Volume x1 reaction [µL]				
Number of Reactions:	**3**					
Master mix prepared for:	**4**					
IVT buffer	8	2				
25 mM Tris-buffered NTPs (each)	2.28	0.57				
Total 4x NTP volume:	*9.12*	*2.28*				
2U/µL inorganic pyrophosphatase	0.6	0.15				
40 mM DFHBI-1T	0.4	0.1				
EtOH	0.408	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**18.528**	**4.632**				

Master Mix + Hormone in EtOH

		Volume x1 reaction [µL]				
Number of Reactions:	**3**					
Master mix prepared for:	**4**					
IVT buffer	8	2				
25 mM Tris-buffered NTPs (each)	2.28	0.57				
Total 4x NTP volume:	*9.12*	*2.28*				
2U/µL inorganic pyrophosphatase	0.6	0.15				
40 mM DFHBI-1T	0.4	0.1				
Hormone (Testosterone) in EtOH	0.408	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**18.528**	**4.632**				

Master Mix + Hormone in H_2O

		Volume x1 reaction [µL]				
Number of Reactions:	**10**					
Master mix prepared for:	**11**					
IVT buffer	22	2				
25 mM Tris-buffered NTPs (each)	6.27	0.57				
Total 4x NTP volume:	*25.08*	*2.28*				
2U/µL inorganic pyrophosphatase	1.65	0.15				
40 mM DFHBI-1T	1.1	0.1				
Hormone (Testosterone) in Water	1.122	0.102	0.44	0.002538096	100	**0.088**
Total MM volume:	**50.952**	**4.632**				

Master Mix + EDC (PCB)

		Volume x1 reaction [µL]				
Number of Reactions:	**3**					
Master mix prepared for:	**4**					
IVT buffer	8	2				
25 mM Tris-buffered NTPs (each)	2.28	0.57				
Total 4x NTP volume:	*9.12*	*2.28*				
2U/µL inorganic pyrophosphatase	0.6	0.15				
40 mM DFHBI-1T	0.4	0.1				
EDC in DMSO	29.568	7.392	0.44	0.002538096	100	**0.088**
Total MM volume:	**47.688**	**11.922**				

DILUTING THE EDCS IN WATER TO HAVE LESS DMSO:

- Dilute **50µL** of the 100 µM in a volume of **462 µL of water** → **Final concentration of 10.82251 µM**
- The volume to be added in the MM per reaction is **7.392 µL**

Master Mix + EDC (benzanthracene)

		Volume x1 reaction [µL]				
Number of Reactions:	**3**					
Master mix prepared for:	**4**					
IVT buffer	8	2				
25 mM Tris-buffered NTPs (each)	2.28	0.57				
Total 4x NTP volume:	*9.12*	*2.28*				
2U/µL inorganic pyrophosphatase	0.6	0.15				
40 mM DFHBI-1T	0.4	0.1				
EDC in DMSO	29.568	7.392	0.44	0.002538096	100	**0.088**
Total MM volume:	**47.688**	**11.922**				

The final

Concentration of DMSO has risen by 0.004% by the addition of the EDC. It was already 0.5% in the buffer (coming from 1µL of the fluorophore).

DNA quantity & water - Test EDCs and HRE5

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA conc. [nM]	DNA quantity [ng]	DNA conc. [ng/µL]	DNA volume [µL]	Water	Well ID	Receptor Volume [µL]	Receptor Conc. [µM]	Receptor mass [µg]	Actual receptor conc. [µM]	Notes
1	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	D4					
2	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	D5					
3	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	D6					
4	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	10.08	D7					
5	HRE5	446	274718.16	10	54.94	92.4	0.595	9.88	D4					
6	HRE5	446	274718.16	10	54.94	92.4	0.595	9.88	D5					
7	HRE5	446	274718.16	10	54.94	92.4	0.595	9.88	D6					
8	HREmin + R	266	163845.36	10	32.77	114.8	0.285	7.39	D11	2.791	1	1.45	7.1663	
9	HREmin + R	266	163845.36	10	32.77	114.8	0.285	7.39	D12	2.791	1	1.45	7.1663	
10	*HREmin + R*	*266*	*163845.36*	*10*	*32.77*	*114.8*	*0.285*	*7.39*	*D13*	*2.791*	*1*	*1.45*	7.1663	Last one!!! (semi)
11	HRE5 + R	446	274718.16	10	54.94	114.8	0.479	7.20	D8	2.791	1	1.45	7.1663	
12	HRE5 + R	446	274718.16	10	54.94	114.8	0.479	7.20	D9	2.791	1	1.45	7.1663	
13	HRE5 + R	446	274718.16	10	54.94	114.8	0.479	7.20	D10	2.791	1	1.45	7.1663	
14	HREmin + RH (H2O)	266	163845.36	10	32.77	114.8	0.285	7.29	D19	2.791	1	1.45	7.1663	
15	HREmin + RH (H2O)	266	163845.36	10	32.77	114.8	0.285	7.29	D20	2.791	1	1.45	7.1663	
16	HREmin + RH (H2O)	*266*	*163845.36*	*10*	*32.77*	*114.8*	*0.285*	*7.29*	*D21*	*2.791*	*1*	*1.45*	7.1663	*Last one!!*
17	HRE5 + RH (H2O)	446	274718.16	10	54.94	92.4	0.595	6.98	D16	2.791	1	1.45	7.1663	
18	HRE5 + RH (H2O)	446	274718.16	10	54.94	92.4	0.595	6.98	D17	2.791	1	1.45	7.1663	
19	HRE5 + RH (H2O)	446	274718.16	10	54.94	92.4	0.595	6.98	D18	2.791	1	1.45	7.1663	
20	HREmin + R + PCB	266	163845.36	10	32.77	114.8	0.285	0.00	D22	2.791	1	1.45	7.1663	
21	HREmin + R + PCB	266	163845.36	10	32.77	114.8	0.285	0.00	D22	2.791	1	1.45	7.1663	
22	HREmin + R + PCB	266	163845.36	10	32.77	114.8	0.285	0.00	D22	2.791	1	1.45	7.1663	
23	HREmin + R + BENZ	266	163845.36	10	32.77	114.8	0.285	0.00	D22	2.791	1	1.45	7.1663	
24	HREmin + R + BENZ	266	163845.36	10	32.77	114.8	0.285	0.00	D22	2.791	1	1.45	7.1663	
25	*HREmin + R + BENZ*	*266*	*163845.36*	*10*	*32.77*	*114.8*	*0.285*	0.00	*D22*	*2.791*	*1*	*1.45*	7.1663	
26						Total	8.429		Total	50.235		26.10		

DNA quantity & water - Test of EtOH, DMSO & Hormone

	Reaction name	DNA length [bp]	DNA mol. weight [Da]	Final DNA conc. [nM]	DNA quantity [ng]	DNA conc. [ng/µL]	DNA volume [µL]	Water	Well ID	Volume of EtOH [µL]	EtOH Conc. [%]	Stock EtOH conc. [%]	Notes
1	Blank		Use the leftovers from MM and act. reaction					15.50	E1				
2	NC (no T7)	266	163845.36	10	32.77	114.8	0.285	15.18	E2				
3	NC (no DNA)						0.000	10.47	E3				
4	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E4				
5	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E5				
6	HREmin	266	163845.36	10	32.77	114.8	0.285	10.18	E6				
7	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	9.98	E9	0.200	0.05	5	PIPETTE EtOH in the PCR TUBE 0.2 µL
8	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	10.08	E12	*0.000*	0.255		
9	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	10.08	E13	*0.000*	0.255		
10	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	10.08	E14	*0.000*	0.255		
11	HREmin + EtOH	266	163845.36	10	32.77	114.8	0.285	0.18	E17	10.000	2.5	5	PIPETTE EtOH in the PCR TUBE 10 µL
12	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F6	*0.102*	0.255	*50*	
13	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F7	*0.102*	0.255	*50*	
14	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.98	F8	*0.102*	0.255	*50*	
15	HREmin + H (EtOH)	266	163845.36	10	32.77	114.8	0.285	9.18	F13	1.000	2.5	50	PIPETTE EtOH in the PCR TUBE 10 µL
16	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	10.08	E12	*0.000*	0.255	*50*	
17	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	10.08	E13	*0.000*	0.255	*50*	
18	HREmin + H (H2O)	266	163845.36	10	32.77	114.8	0.285	10.08	E14	*0.000*	0.255	*50*	
19	FUCK DMSO TEST!												

Activating Reaction (transcription only)

		Quantity x1 reaction
Number of Reactions:	**41**	
Master mix prepared for:	**42**	
0.1 mg/mL (aka 50'000U/mL) T7 RNAP	84	2
H_2O	126	3
Total MM volume:	**210**	5

Conclusion

		Volume x1 reaction [µL]				
Number of Reactions:	**3**					
Master mix prepared for:	**4**					
IVT buffer	8	2				
25 mM Tris-buffered NTPs (each)	2.28	0.57				
Total 4x NTP volume:	*9.12*	*2.28*				
2U/µL inorganic pyrophosphatase	0.6	0.15				
40 mM DFHBI-1T	0.4	0.1				
Hormone (Testosterone) in DMSO	0.408	0.102	0.44	0.002538096	100	**0.088**
DMSO	2.996	0.749				
Total MM volume:	**18.528**	**5.381**				