Week 27 - Overview

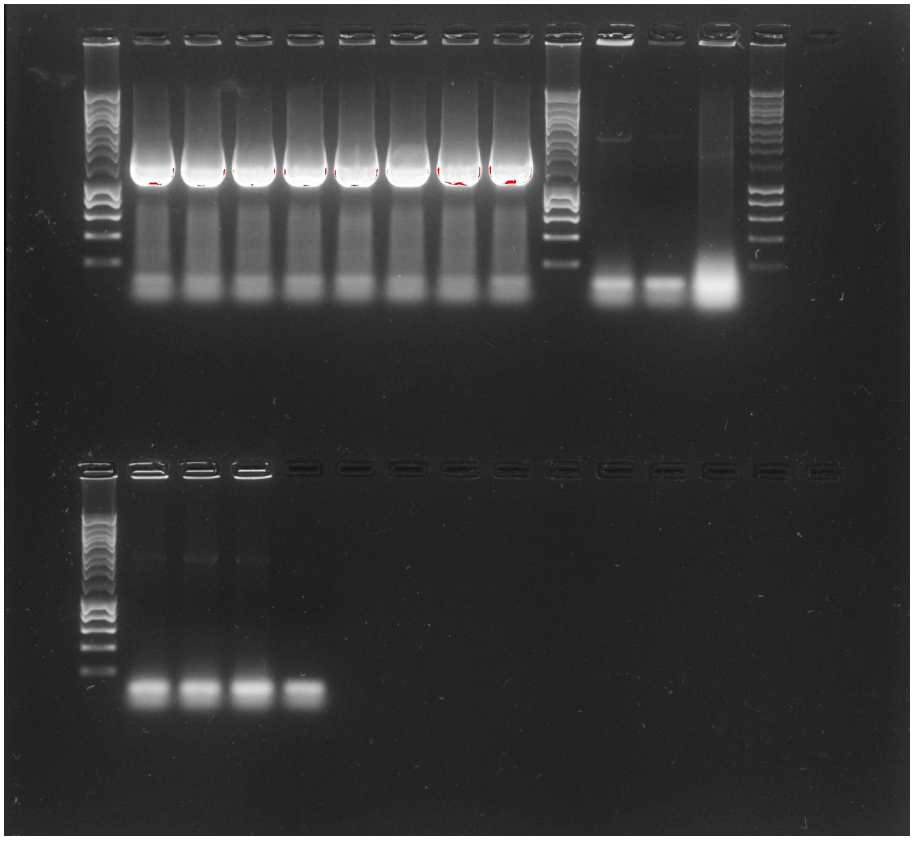
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Weekly summary:

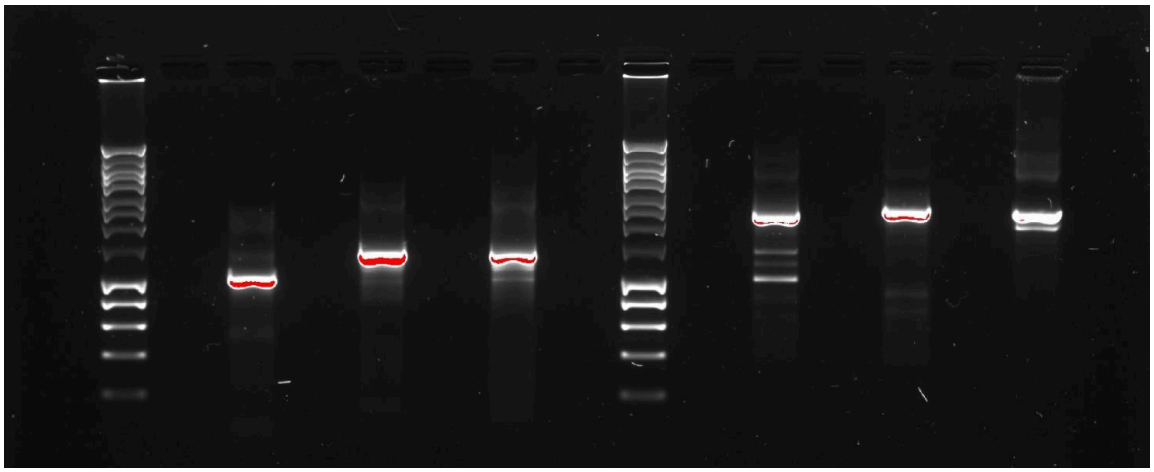
During Week 27, we focused mainly on transforming *E. coli* TG1 with our remaining gene constructs. Multiple rounds of transformations were required, as some attempts were unsuccessful, along with colony PCRs to confirm the presence of our inserts. Positive ci2350-His colonies were sequenced, and one colony with correct sequence was identified and stored for subsequent transformation into *E. coli* BL21.

Main results of the week:

Agarose gel electrophoresis of colony PCR products showed positive results in wells 2-9, corresponding to the expected size of the amplified ci2350-His insert region, confirming successful transformation of *E. coli* TG1 with ci2350-His. Multiple controls were tested, including colonies transformed with empty pET-11a, and colonies from a ligation control (ligation without insert). All controls proved negative, indicating the ligation and transformation were correct. Following our PI Johan's recommendation, in the future transformations we will screen only 1-2 colonies from control plates and prioritize testing more transformant, to increase the chance of identifying a correctly transformed colony. Four ci2350-His positive colonies were sent for sequencing, where one was identified as having a correct construct.

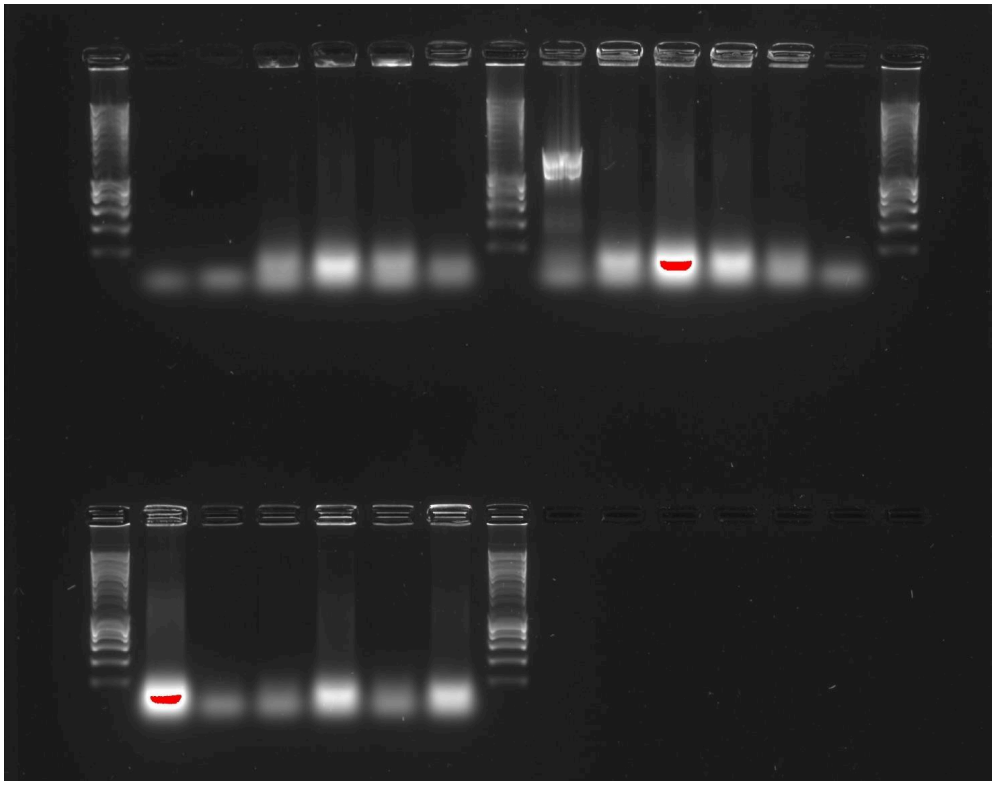


By visualizing restriction digestion products of our ordered inserts and comparing the resulting fragment sizes, we confirmed the inserts were correct. Insert AKR1D1-MBP showed some additional bands, possibly due to too long digestion incubation causing non-specific cutting.



Transformation results were poorer than expected. All plates had very few colonies (<7), suggesting that something went wrong, possibly during digestion or ligation. Plates containing

AKR1D1-Trx and ci2350-Trx had no colonies. Agarose gel electrophoresis of colony PCR products showed only one positive colony (well 9, containing the ci2350-SUMO insert), with all other colonies tested negative. However, the amplified band in well 9 was smeary, which may have resulted from accidentally piercing the well while loading the sample. Since all other colonies were negative, we decided to repeat the transformations using new ligations prepared with the leftover digested plasmid from Week 26.



Because plates with AKR1D1-Trx and ci2350-Trx inserts had no colonies, they were transformed using leftover ligation mix, and resulted in a few colonies, which were tested by colony PCR next week.

Day 10: [June 29, 2026], Monday

Weekly responsible: Patrik, Rytis
Lab participants: Hema, Ruiqi

Daily overview of experiments

Colony PCR was done, along with streaking master plates, inoculating overnight cultivation for sequencing preparation and NucleoSpin Cleanup Kit.

Activity	*Protocol File*	*Comments*
Colony PCR	Colony PCR .docx	Checking if ligation and transformation are successful
Agarose Gel Electrophoresis	Gel electrophoresis.d…	Checking Colony PCR
Streaking Master Plates for backup	Colony PCR .docx	
Inoculating LB Medium tubes with positive transformants for overnight incubation	Colony PCR .docx	For plasmid miniprep and sequencing the next day
~~NucleoSpin Gel and PCR Cleanup kit~~	~~NucleoSpin Gel and PCR Clean-up User ManualTakara Biohttps://www.takarabio.com › documents › Nucleo...~~	~~Tested to see if it can clean up plasmid samples~~

Results and interpretation

Colony PCR

For colony PCR, Jena Bioscience Red Load Taq Master Kit was used. In total 15 reactions were made, with each reaction consisting of 50 µL. One big PCR mix was made containing 800 µL, enough for 16 reactions (including one extra reaction to account for pipetting errors).

PCR grade water from the Jena Bioscience PCR Kit was used, and the bacterial DNA material was not accounted for in the component volume calculations (meaning that we took 0 µL as the volume for bacterial colonies).

Component	Volume per 1 reaction	Volume for 16 reactions
Red Load Taq Master (5x)	10 µL	160 µL
T7 promoter forward primer (10 µM)	2 µL	32 µL
Ci2350 reverse primer (10 µM)	2 µL	32 µL
PCR Grade Water	36 µL	576 µL
Total	50 µL	800 µL

In total 15 reactions were done:

1-4: Transformants from plate S1

5-8: Transformants from plate S2

9-11: Transformants from plate N

12-14: Transformants from plate P

15: PCR Negative control, without DNA Template (just primers)

Note: Johan said in the future we do only **1-2 control plate samples**, and focus on testing more transformants!

Note: Johan said in the future we make **20 µL PCR reactions,** even though the kit recommends 50 µL, it should work the same.

The thermocycler was programmed according to the table below:

Step	**Duration**	**Temperature**	**Cycles**
Cell Lysis	5 min	95°C	1
Denaturation	30 s	94°C	30
Annealing	30 s	54°C	

Elongation	2 min	72°C	
Final Elongation	5 min	72°C	1
Hold	∞	4°C	

Streaking Master Plates

Four petri dishes containing LB Agar with Ampicillin were streaked, with each petri dish accounting to their respective transformation plates. Each plate was divided into multiple parts, where each part was streaked from one colony tested in the colony PCR.

Master plates:

Master Plate	Number on plate	Colony PCR Sample
S1	1	1
	2	2
	3	3
	4	4
S2	1	5
	2	6
	3	7
	4	8
N	1	9
	2	10
	3	11
P	1	12
	2	12
	3	14

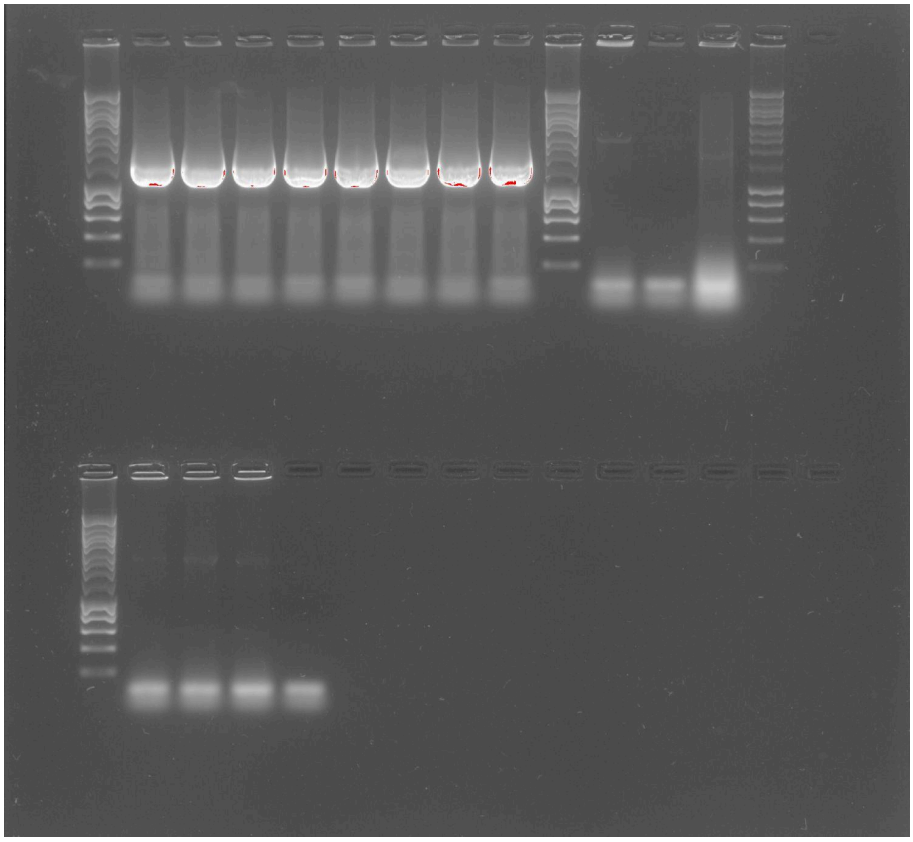
Agarose gel electrophoresis

Big Agarose gel was made (150 mL), with 1% agarose. It was divided in two sections, as all of our samples could not fit into one row.

Sample loading order:

Upper Gel: Marker (2 µL), 1-8, Marker, 9-11, Marker (1 µL)

Lower Gel: Marker (2 µL), 12-14, PCR Control



The gel showed POSITIVE RESULTS, meaning we successfully transformed E. coli TG1 with ci2350+His. This is visible in wells 2-9, with the strongest band indicating our PCR product, the amplified ci2350 gene region in the plasmid.

Inoculating LB Medium with positive transformants

Inoculated samples: S1-1, S1-3, S2-3 and S2-4. Put in incubation overnight at 37C and 150 rpm, for plasmid prep and sequencing the next day.

Day 11: [June 30, 2026], Tuesday

Weekly responsible: Rytis, Patrik
Lab participants: Hema, Ruiqi, Giulia visiting

Daily overview of experiments

Plasmid miniprep of overnight cultures of transformed E. coli TG1 containing ci2350+His in pET-11a, along with preparation for sequencing and sending the tubes for sequencing. Digestion of all gene inserts (except ci2350+His, which was previously done) and confirming them on the gel. Finally, gel extraction of inserts.

Activity	***Protocol File***	***Comments***
Plasmid miniprep	Plasmid miniprep - J…	Plasmid miniprep of plasmid containing our insert
Preparation for sequencing	Colony PCR.docx	Using the Mix2Seq sequencing service
Restriction digestion of inserts	Johan's Restriction D…	
Agarose gel electrophoresis	Gel electrophoresis.d…	To confirm digestion of our inserts
Gel extraction	Gel Extraction from A…	EZNA Kit

Results and interpretation

Plasmid MiniPrep

5 mL from overnight transformant cultures were collected by centrifuging multiple times at 10 000 g for 1 minute (3x1.5 mL + 0.5 mL; 4 centrifuges in total). Plasmids were extracted using the EZNA Kit. After extraction, NanoDrop concentration measurements were performed. Plasmid was eluted in 50 µL.

Sample	Concentration (ng/μL)	260/280	260/230
S1-1	102	1.839	2.239
S1-3	120	1.833	2.241
S2-3	125	1.833	2.270
S2-4	134	1.830	2.261

Preparing samples for sequencing
pET11a-ci2350-His constructs (S1-1, S1-3…) were diluted to 75 ng/μL, 5 μL of each added to a separate sequencing tube (8 in total).
T7_pro_fw primer was diluted to 5 μM, 5 μL of each added to 4 separate sequencing tubes.
T7_ter_rev primer was diluted to 5 μM, 5 μL of each added to another 4 separate sequencing tubes.
Tubes were vortexed, then spun down in microcentrifuge to collect in the bottom.

Identification codes:
With T7_pro_fw:
EF73591289 S1-1
EF73591315 S1-3
EF73591327 S2-3
EF73591328 S2-4

With T7_ter_rev:
EF73591329 S1-1
EF73591330 S1-3
EF73591311 S2-3
EF73591332 S2-4

Digestion of inserts

Restriction digestion was done according to Johan's protocol, and phosphatase step was not done, as its not required for inserts (only required for plasmids):

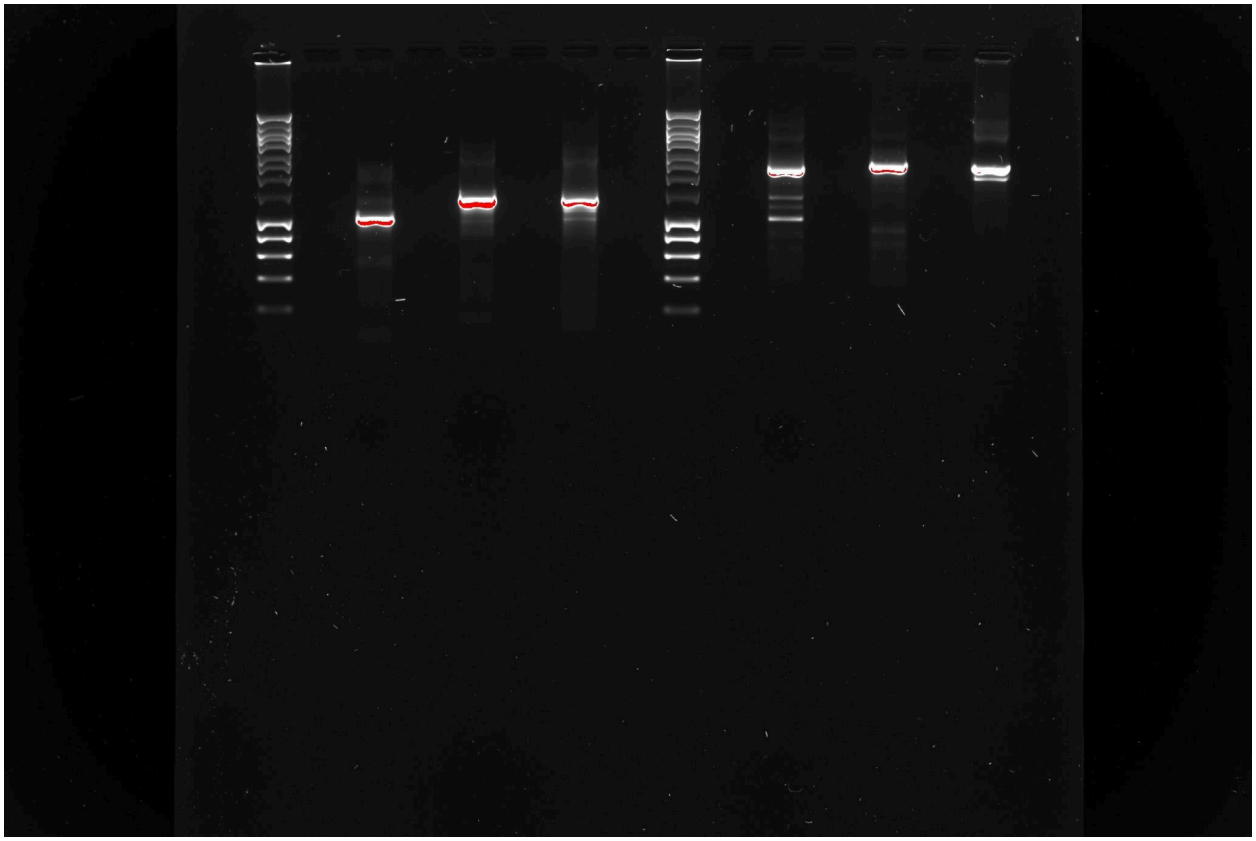
FD Buffer - 2 μL
Gene insert - 10 μL
Water - 6 μL
NdeI - 1 μL
BamHI - 1 μL

Note: Unfortunately, DNA was not put in the BamHI and NdeI controls, so they were not loaded on the gel. The controls could have contained plasmid DNA, to see the integrity of the enzymes, and not DNA (to see the difference between the insert size, as its too small region that was cut).

Agarose gel electrophoresis

Big gel (150 mL) was made, with 1% agarose. 1.5 µL of Ampliqon High Range Ladder was used, as 2 µL was too much last time.

Wells order: Marker, AKR1D1, AKR1D1-TrxA, AKR1D1-SUMO, Marker, AKR1D1-MBP, ci2350-TrxA, ci2350-SUMO



Gel extraction

Gel extraction was done using EZNA Kit, and extracts were measured on NanoDrop. Elutions were done in 30 µL. The elution buffer was preheated to 70C°, and 2 minutes for incubation time of the elution buffer on the column, before centrifuge.

Sample	Concentration (ng/μL)	Abs 260/280	Abs 260/230
AKR1D1	4.970	2	0.625
AKR1D1-TrxA	5.964	1.714	0.667
AKR1D1-SUMO	3.479	1.760	0.226
AKR1D1-MBP	3.976	2	0.276
ci2350-TrxA	7.455	1.875	0.165
ci2350-SUMO	3.479	2.333	0.280

Day 12: [July 1, 2026], Wednesday

Weekly responsible: Rytis, Patrik
Lab participants: Hema, Ruiqi

Daily overview of experiments

Ligation of inserts made the previous day and pET-11a, followed by transformation of competent E. coli TG1.

Activity	***Protocol File***	***Comments***
Ligation of pET-11 with our inserts	Insert calculations E. coli Transformatio…	Ligation done at room temp for 2 hours
Transformation of Competent E. coli TG1 with recombinant plasmids	E. coli Transformatio…	
Autoclaving	Autoclaving.docx	Autoclaved waste and then sterile material (including tips, eppendorf tubes and LB Medium)
LB Medium Preparation for competent cells	Media preparation.docx	3x100 mL of LB medium without amp

Results and interpretation

Ligation of pET-11a with our inserts

For ligation reactions, plasmid extracted on June 26 was used (Sample S8), with 40 ng of plasmid in each ligation sample. Inserts extracted yesterday were used, with a molar ratio of 3:1.

Transformation of competent E. coli TG1 with recombinant plasmid

After transformation protocol, the samples were incubated at 37C at 15:00, and analysed the following day.

Number	Sample	Volume of ligation mixture (µL)
1	Positive Control	1
2	Negative Control	10
3	ci2350+TrxA	10
4	ci2350+SUMO	10
5	AKR1D1	10
6	AKR1D1+TrxA	10
7	AKR1D1+SUMO	10
8	AKR1D1+MBP	10

Day 13: [July 2, 2026], Thursday

Weekly responsible: Rytis, Patrik
Lab participants: Hema, Ruiqi

Daily overview of experiments

Checking if transformations from the previous day were successful, then colony PCR to check for insert presence, followed by agarose gel electrophoresis. Finally, redoing the transformation of E. coli TG1 with ci2350+TrxA and AKR1D1+TrxA, as there were no colonies on these plates.

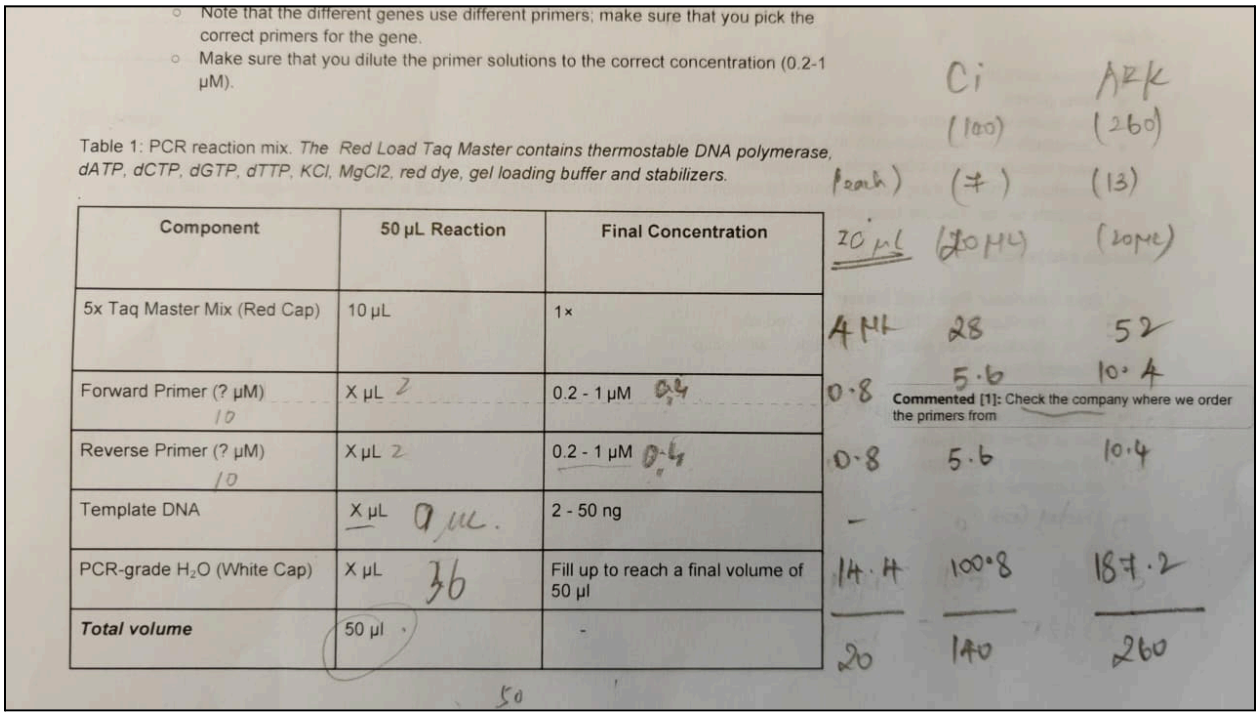
Activity	***Protocol File***	***Comments***
Checking for positive transformations	N/A	We had fewer transformants (max 7) than the ci2350-His (>50). For both genes with TrxA tag, transformation failed
Colony PCR	Colony PCR.docx	Used primers T7 ter_rev & ci2350_fw to check ci2350-SUMO & MBP Used primers AK-R1D1_rev & T7 pro_fw to check AKR1D1-SUMO & MBP
Agarose gel electrophoresis	Gel electrophoresis.d…	Buffer was changed. Initially, gel was inserted backwards, DNA migrated in the wrong direction. This was caught, fixed, then run at 60V, 20 min, then 90V 40 min. The Ampliqon DNA ladder had smearing.
Transformation of competent E. coli TG1	E. coli Transformatio…	Redoing transformation with ci2350+TrxA and AKR1D1+TrxA, with leftover mixture from yesterday (07/01).

Inoculation of TG1-pET11a for plasmid extraction	File	Inoculated 10 x 5 mL tubes for plasmid extraction

Results and interpretation

Colony PCR Master Mix Calculations:

(The calculations were redone and the final amount added is attached as a picture below. Initially the calculations were a bit off before being redone)



- Note that the different genes use different primers; make sure that you pick the correct primers for the gene.
- Make sure that you dilute the primer solutions to the correct concentration (0.2-1 µM).

Table 1: PCR reaction mix. *The Red Load Taq Master contains thermostable DNA polymerase, dATP, dCTP, dGTP, dTTP, KCl, MgCl2, red dye, gel loading buffer and stabilizers.*

Component	50 µL Reaction	Final Concentration
5x Taq Master Mix (Red Cap)	10 µL	1×
Forward Primer (? µM)	X µL 2	0.2 - 1 µM 0.4
Reverse Primer (? µM)	X µL 2	0.2 - 1 µM 0.4
Template DNA	X µL 9 µL.	2 - 50 ng
PCR-grade H_2O (White Cap)	X µL 36	Fill up to reach a final volume of 50 µl
Total volume	50 µl	-

Ci (100) (7) 20 µL (2 µL) 4 µL 28 5.6 0.8 5.6 0.8 - 14.4 100.8 20 140

AKR (260) (13) (2 µL) 52 10.4 10.4 187.2 260

Commented [1]: Check the company where we order the primers from

Primer T_m (Primers):
T7 ter_rev: 57 ℃
Ci2350_fw: 60 ℃
AKR1D1_rev: 61 ℃
T7 pro_fw: 60 ℃

PCR parameters were set up as follows:
98 ℃ - 5 min
95 ℃ - 30 s
55 ℃ - 30 s
72 ℃ - 2 min, 15 s

Final elongation: 72 ℃, 5 min
+4 ℃, indefinite hold.

Number	Sample	Number	Sample
1	ci2350 PCR control	11	AKR1D1
2	AKR1D1 PCR control	12	AKR1D1
3	ci2350 Neg. control	13	AKR1D1 SUMO
4	AKR1D1 Neg. control	14	AKR1D1 SUMO
5	ci2350 Pos. control	15	AKR1D1 SUMO
6	AKR1D1 Pos. control	16	AKR1D1 MBP
7	ci2350 SUMO	17	AKR1D1 MBP
8	ci2350 SUMO	18	AKR1D1 MBP
9	ci2350 SUMO		
10	AKR1D1		

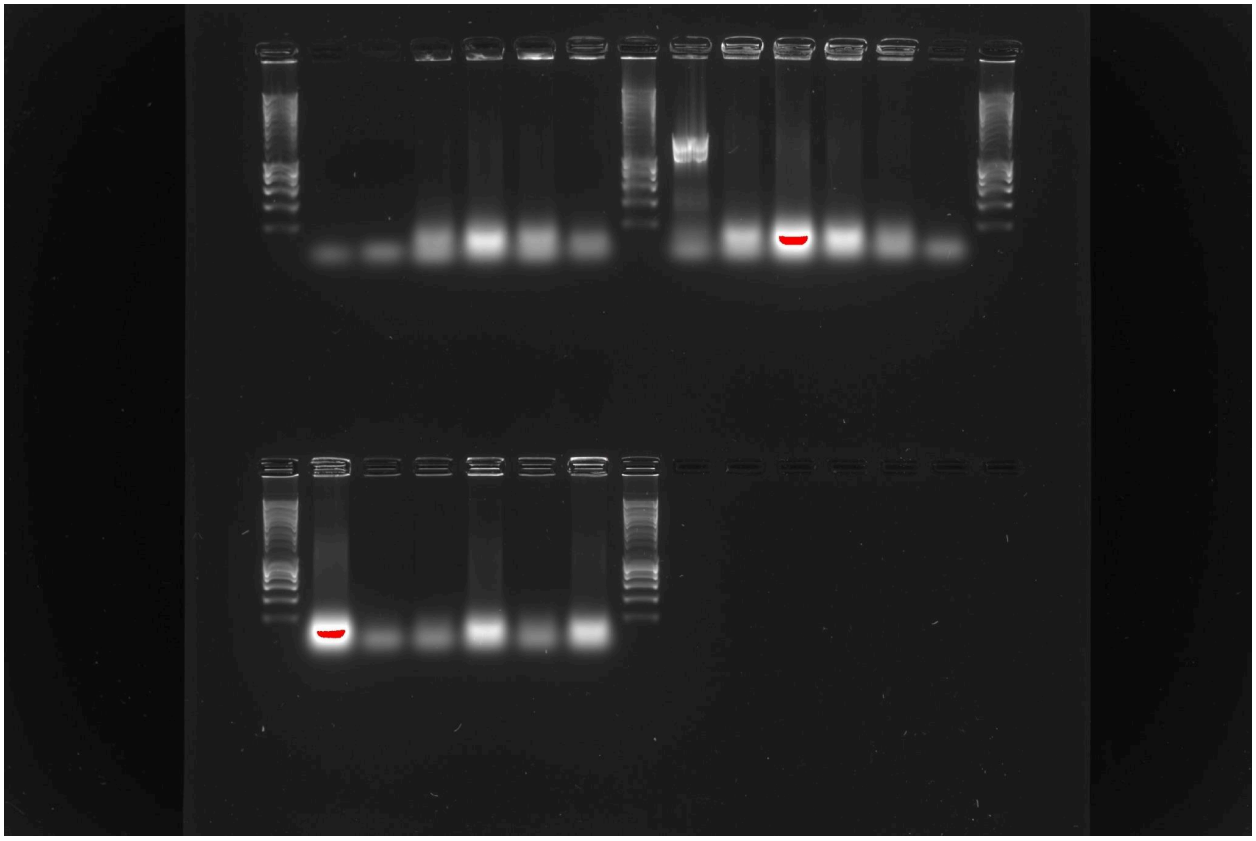
Agarose Gel Electrophoresis

Big Agarose gel was made (150 mL), with 1% agarose. It was divided in two sections, as all of our samples could not fit into one row. Sample volume was 20 µL, and the Ampliqon High Range Ladder was 1.5 µL.

Sample loading order:

Upper / Lower Gel	**Lane**	**Sample**
Upper Gel	1	Marker
	2	1 ci2350 PCR control
	3	2 AKR1D1 PCR control
	4	3 ci2350 Neg. control
	5	4 AKR1D1 Neg. control
	6	5 ci2350 Pos. control

	7	6 AKR1D1 Pos. control
	8	Marker (1.5)
	9	7 ci2350 SUMO
	10	8 ci2350 SUMO
	11	9 ci2350 SUMO
	12	10 AKR1D1
	13	11AKR1D1
	14	12 AKR1D1
	15	Marker (1.5)
Lower Gel	16	Marker (1.5)
	17	13 AKR1D1 SUMO
	18	14 AKR1D1 SUMO
	19	15 AKR1D1 SUMO
	20	16 AKR1D1 MBP
	21	17 AKR1D1 MBP
	22	18 AKR1D1 MBP
	23	Marker (1.5)



Heat shock transformation using left-over ligation mix

Using left-over ligation mix from July 1st, ci2350-TrxA and AKR1D1-TrxA were transformed by following the transformation protocol. Ligation mix had less than the expected 10 µL, likely due to pipetting errors while preparing the mixture, and/or from when it was used on July 1st.

Overnight ligation of ci2350-Trx & AKR1D1-Trx

For ligation reactions, plasmid extracted on June 26 was used (Sample S9 & Sample S10, Sample S7), with 40 ng of plasmid in each ligation sample. Inserts from June 30th were used, with insert:vector molar ratio of 3:1.

7 reactions were set up:

Label	Sample	Insert amount (ng)	Vector amount (ng)	Volume of ligation mixture (µL)
1	Negative Control (vector S9)	-	40	20
2	Negative Control (vector S10)	-	40	20

3	ci2350_TrxA + vector S9	48.98	40	20
4	AKR1D1_TrxA + vector S9	28.93	40	20
5	ci2350_TrxA + vector S10	48.98	40	20
6	AKR1D1_TrxA + vector S10	28.93	40	20
8	AKR1D1_TrxA + vector S7	28.93	40	20

Due to running out of T4 ligase, for samples 1, 2, 5, 6 - old T4 ligase was used (expired 2025.01).
An order for new T4 ligase was placed.
Ligation mixtures were placed in the fridge at ~16:30, for overnight ligation.

Day 14: [July 3, 2026], Friday

Weekly responsible: Rytis, Patrik
Lab participants: Hema, Ruiqi

Daily overview of experiments

Checking transformations from yesterday. We also did pET-11a miniprep. Transformation of *E. coli* TG1 using an overnight ligation mix, to increase the chances of successful transformation.

Activity	***Protocol File***	***Comments***
Checking ci2350/AKR1D1-TrxA transformants		Some colonies were found. They were not tested on colony PCR, as it is too much work for only 2 plates. They will be checked later, with other transformants.
pET-11a miniprep from overnight cultures		
Transformation of E. coli TG1 using overnight ligation mix	W E. coli Transformatio…	
Preparation of LB Media and LB Agar		Around 40 LB agar + amp plates were made, along with ~20 LB medium tubes + amp
Autoclaving		Autoclaving LB agar+medium and pipette tips Autoclaving waste

Results and interpretation

Checking ci2350+TrxA and AKR1D1+TrxA transformants

Some colonies were found. They were not tested on colony PCR, as it is too time intensive for only 2 plates. They will be checked later, with other transformants.

Plasmid miniprep of pET-11a

EZNA kit was followed, with additional elution step. After the normal elution step (50 µL), another elution was done of 50 µL, to test if there was leftover plasmid on the column. The nanodrop results proved positive, with additional plasmid eluted, however with less purity and concentration.

Sample	Concentration (ng/µL)	260/280	260/230
1	88	1.806	2.058
2	85	1.879	2.192
3	71.6	1.846	2.182
4	74.6	1.829	2.206
5	86	1.840	2.085
6	81.5	1.840	2.050
7	86.5	1.851	2.175
1b	22.4	1.957	2.250
2b	28.8	1.871	2.071
3b	20.4	1.783	2.158
4b	25.8	1.793	1.625
5b	24.4	1.885	1.885
6b	32.8	1.833	1.015
6b (2nd reading)	20.4	1.788	2.158

7b	23.4	1.880	2.350

Note: 6b reading was done twice, as first reading was suspicious. The second reading was completely different, making us question the reliability of all other readings.

Transformation of *E. coli* TG1 with overnight ligation mix

E. coli TG1 was transformed with overnight ligation mix.