Week 31 - Overview

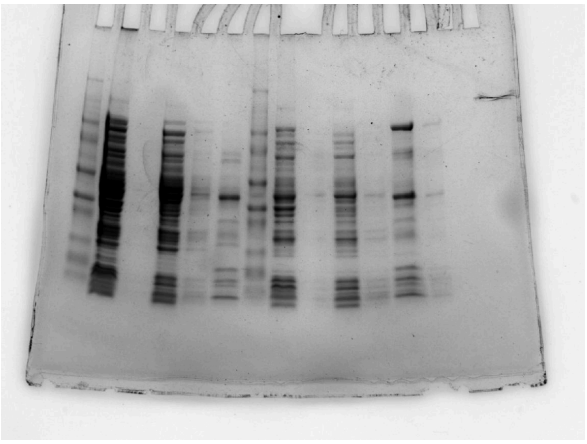
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Weekly summary:

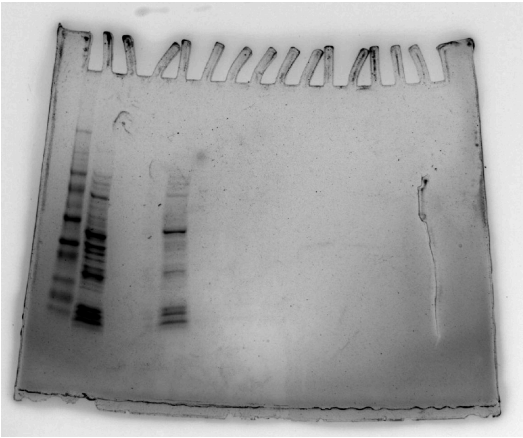
During Week 31, we tested different expression conditions of ci2350-MBP, comparing 37ºC and 25ºC, and testing 3h production and overnight production. We also compared ci2350-His and ci2350-MBP production by SDS-PAGE analysis, and purified ci2350-MBP, again analysed by SDS-PAGE. Transformations using the pET-BLANK vector backbone yielded colonies positive for inserts. We also transformed AKR1D1-His into *E. coli* BL21(DE3), and performed mutagenesis of ci2350-MBP, to introduce the V236I and R215A mutations.

Main results of the week:

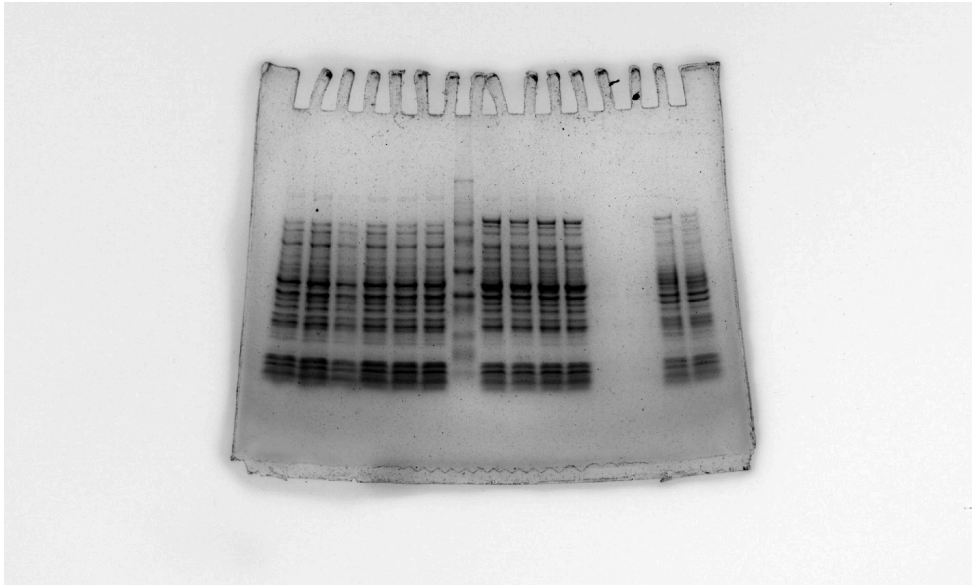
SDS-PAGE analysis of IMAC purification was performed on samples from last Friday's expression, ci2350-MBP cultivated overnight at either 37ºC or 17ºC, and ci2350-His with 3 hour induction time. The ci2350-MBP gel showed successful purification, with our protein visible in the eluted fraction, though additional proteins were also present, possibly due to unspecific binding to the column. The gel also showed an additional band at ~60 kDa, suggesting expression of a truncated ci2350-MBP protein. Our supervisor Camille noted that the corresponding position in the nucleotide sequence contains a repeat of several adenine nucleotides, raising the possibility that the ribosome slipped during translation. This would produce a truncated protein that could still be purified, since the His-tag is located at the N-terminus.



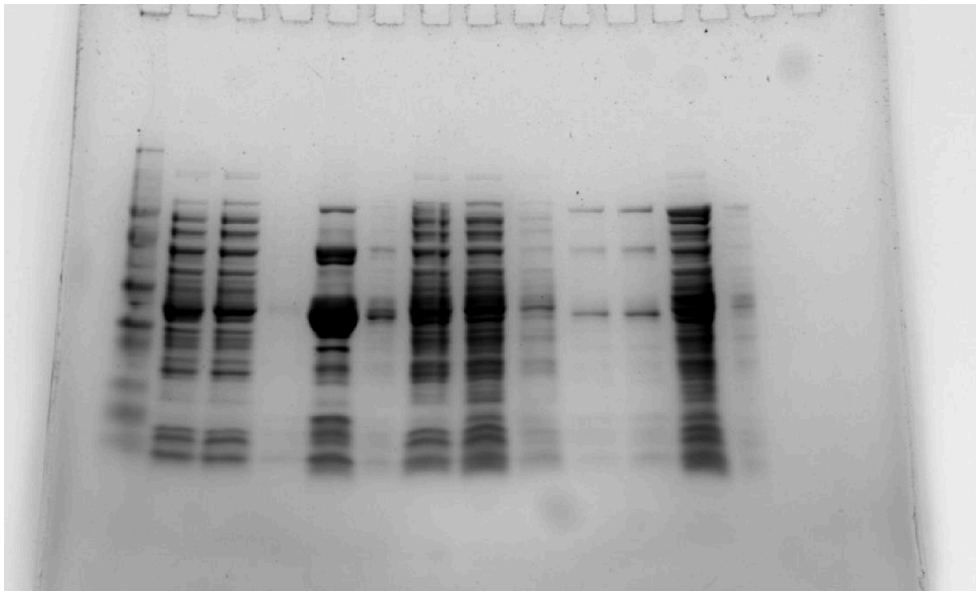
The second gel analysed fractions from the ci2350-His purification. No bands were visible in the eluate, while a band consistent with the expected size of ci2350-His was present in the flowthrough, indicating the overexpressed protein failed to bind to the column. Several explanations could lead to this outcome: insufficiently charged IMAC column, inaccessible His-tag due to misfolding, or the protein may have aggregated together. Given the difficulties encountered with ci2350-His purification, compared to successful purification of ci2350-MBP, we proceeded with the ci2350-MBP for further work. Considering only one successfully purified construct was required to begin steroid reduction assays, further optimisation of ci2350-His purification was not prioritized, due to time constraints.



SDS-PAGE analysis was performed on whole-cell lysates of the ci2350-MBP construct, comparing expression in *E. coli* BL21(DE3) at 37 ºC for 3h with overnight expression at 25 ºC, both induced with 0.4 mM IPTG. A band consistent with the predicted molecular weight of ci2350-MBP was visible at approximately 110 kDa under both conditions, with 37ºC and 3h induction time showing stronger expression than the overnight induction. The same band was faintly visible in pre-induction samples, and more intense after IPTG addition, indicating slightly leaky expression from the T7 promoter alongside successful induction.



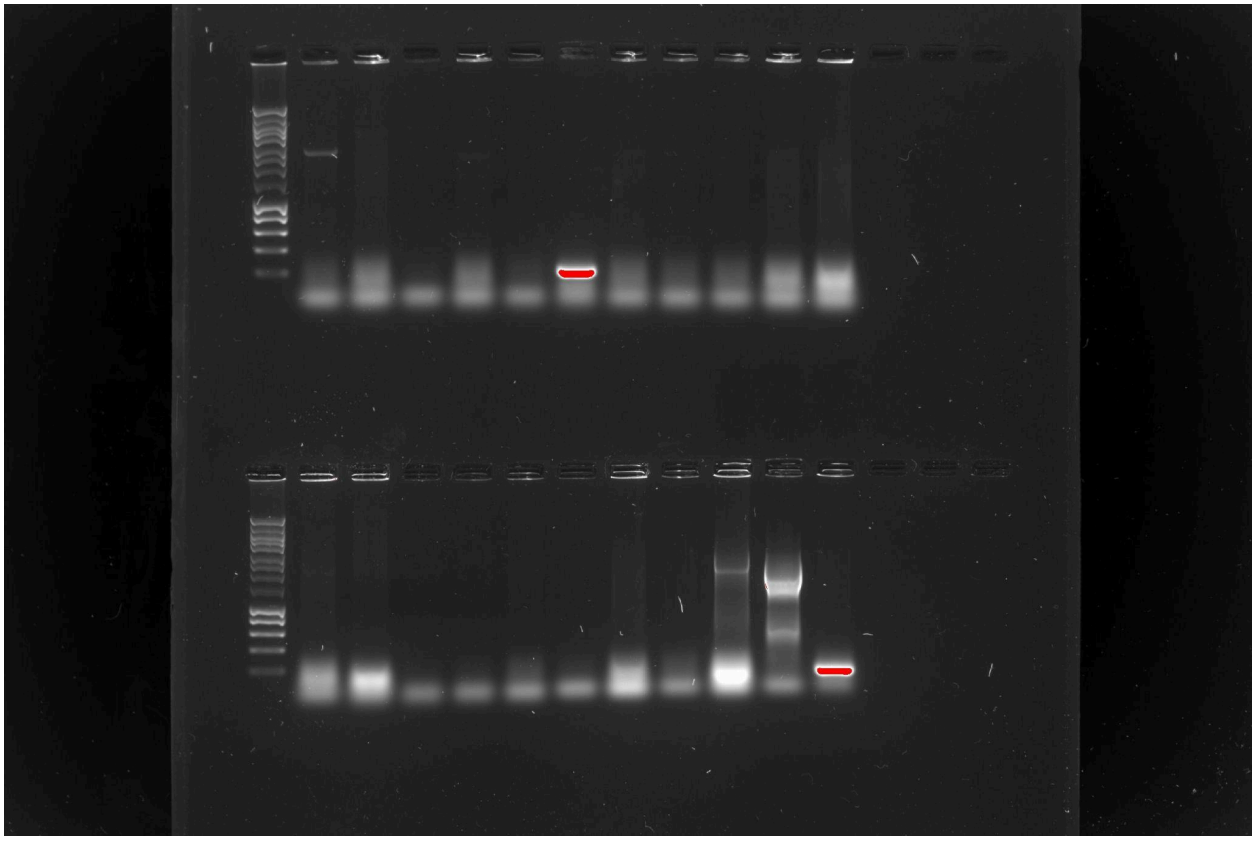
Both ci2350-MBP expression conditions shown above (37ºC for 3h and 25ºC overnight expression) were taken forward for purification, and their resulting fractions have been analysed by SDS-PAGE. The expressed ci2350-MBP protein was present in the eluate from the IMAC column, along with an additional lower-molecular-weight band, most likely a truncated ci2350-MBP. These eluates were stored at 4ºC and used for steroid reduction assays the following week. The presence of additional bands was not considered problematic at this stage, as the steroid reduction assays were intended only to detect whether any reduction activity was present, not to determine enzyme kinetics.

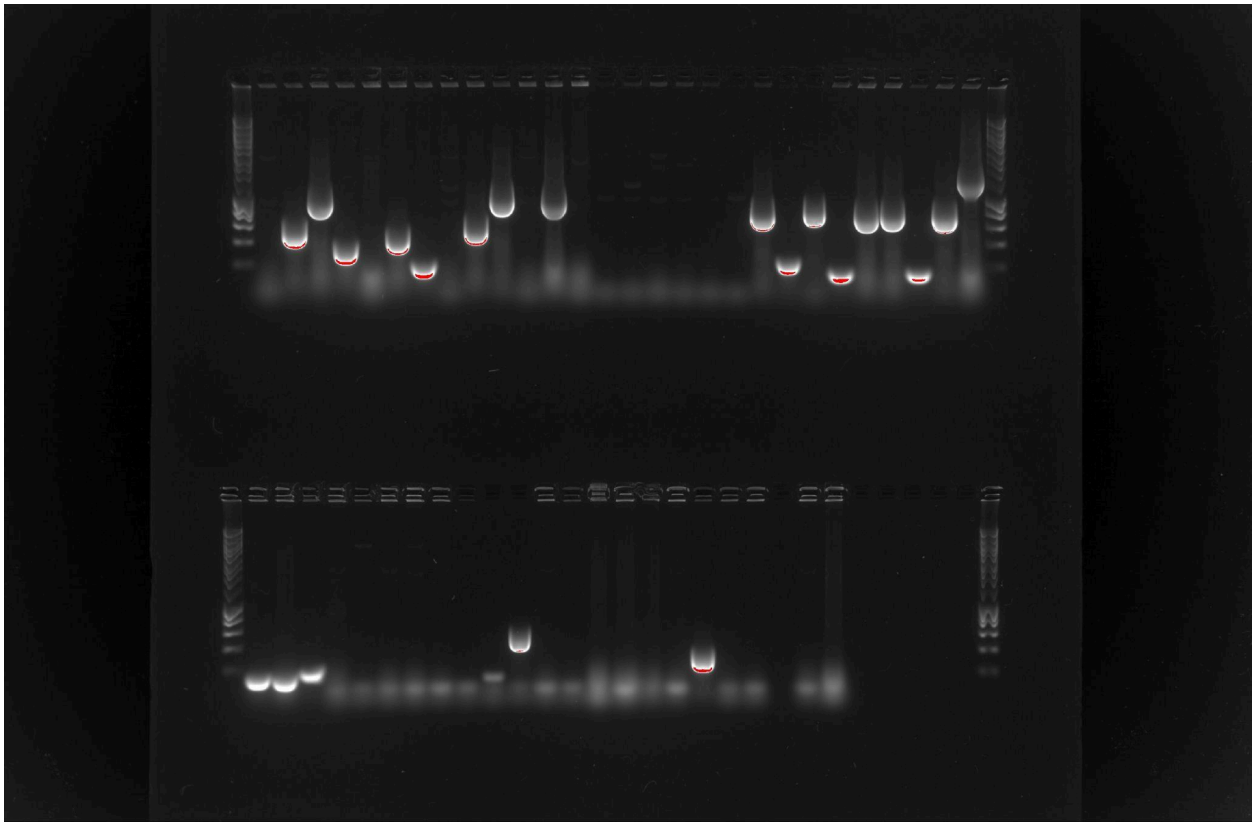


Restriction digestion of pET-BLANK carrying ci2350-MBP with NdeI and BamHI was repeated. In addition to the expected backbone (5.4 kb) and insert (3 kb) bands, a band corresponding to a single digested plasmid (8.4 kb) was present on the gel, indicating incomplete digestion. Despite this, the pET-BLANK backbone was still extracted and used for ligation. We decided to extend the incubation time in subsequent restriction digestions, in order to achieve full digestion.

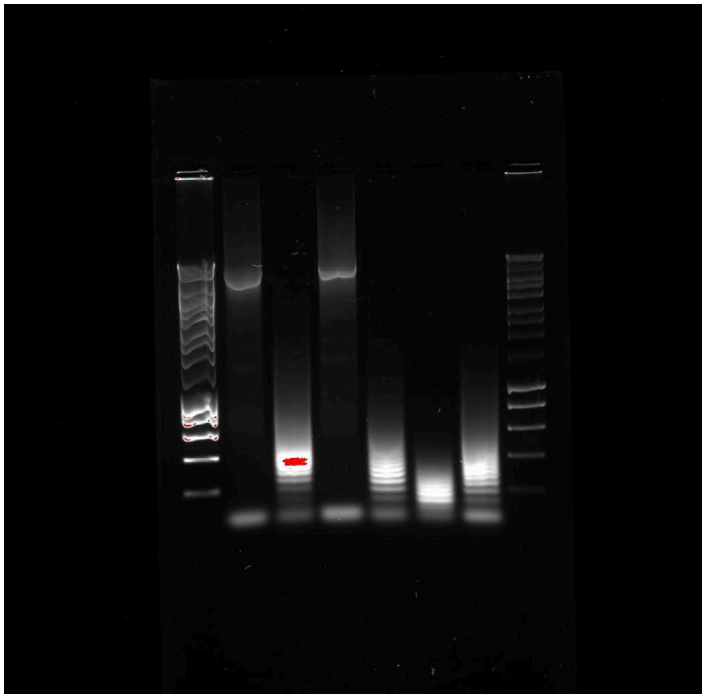


Following ligation of all insert constructs with the digested pET-BLANK backbone and transformation into *E. coli* TG1, very few colonies appeared across all plates. Colony PCR was performed to assess ligation success, and showed no positive colonies, so no samples were sent for sequencing. A second round of ligation and transformation into *E. coli* TG1 was carried out, using an overnight ligation at 16ºC, instead of standard 2 hour incubation. The same digested pET-BLANK samples were used, as they were left over from previous digestion. The second round of transformation yielded positive colonies, from which one AKR1D1-SUMO and two AKR1D1-Trx colonies were sent for sequencing. This suggests that the extended ligation time improved ligation efficiency.

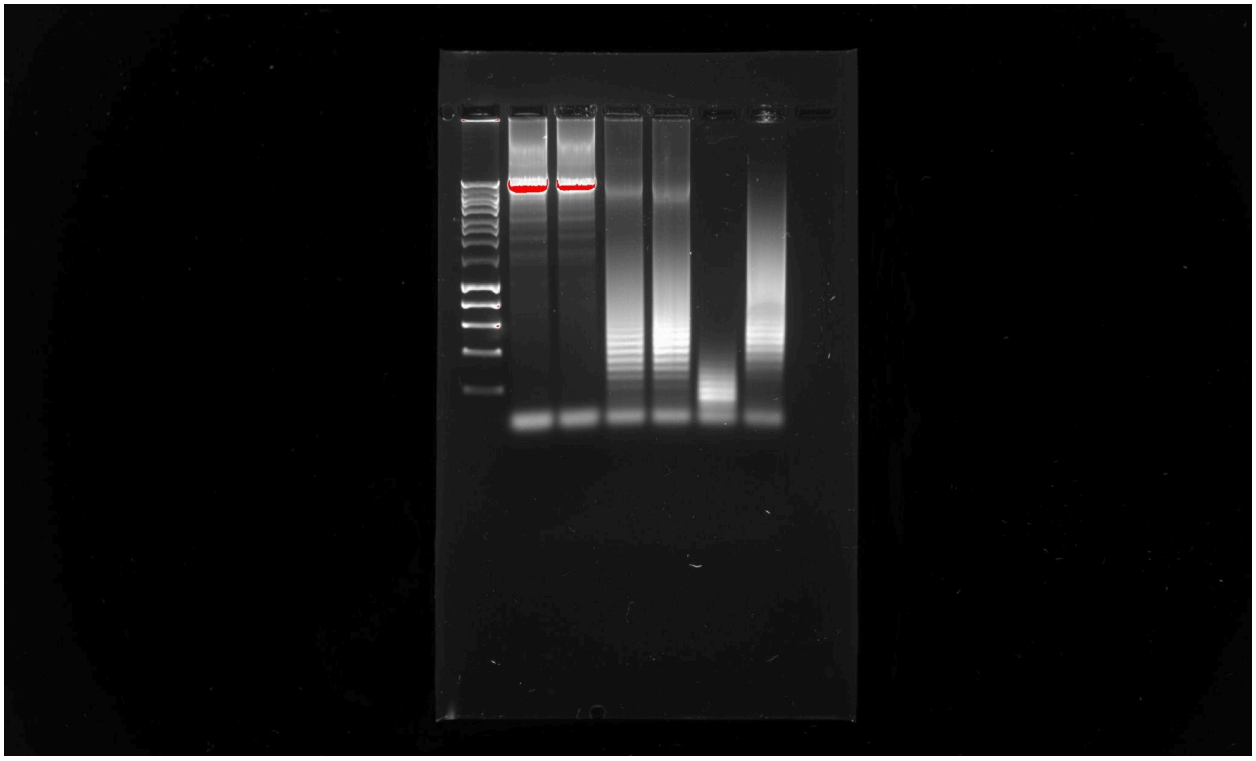




Agarose gel electrophoresis of the mutagenic PCR products generated from pET-BLANK containing ci2350-MBP showed that only the V263I reaction produced an amplified product of the expected size (~8.4 kb), corresponding to the full-length plasmid. The other reaction, R215A, did not produce a product of the correct size, but a smear at low-molecular-weight smear. The V263I PCR product was transformed into *E. coli* TG1, and the R215A mutagenesis will be repeated, with an extended elongation time.



The mutagenic PCR was repeated with an almost doubled elongation time of 17 minutes. As the plasmid is close to the 10 kb limit, improved results were expected applying the 2 min/kb rule typically used for larger constructs. The R215A reaction still produced a low-molecular-weight smear, but a product of the expected size (~8.4 kb) was now also present. This indicated the smear was not a result of low elongation time, but by another factor, most likely non-specific primer binding. If the mutagenesis were to be repeated, increasing the annealing temperature could be tested to improve specificity. All amplified products were used for transformation into *E. coli* TG1.



Day 29: [July 27, 2026], Monday

Weekly responsible: Eleonora
Lab participants: Giulia, Cecilia, Hamsi, Rytis

Daily overview of experiments

Activity	***Protocol File***	***Comments***
Cultivation of ci_MBP at 37 and 25 degrees to start protein expression	Protein Expression a… Protein expression lo…	Six 100mL reactions, four with 0.4 IPTG and 37°C incubation; one with 0.4 IPTG and overnight incubation, one with zero IPTG and overnight incubation
Ligation pET_blank with inserts, transformation		pET_blank with - AKR: Sumo, Trx, MBP and - ci: Sumo, Trx
Sequencing prep		Re-doing the AKR1D1-His sequencing samples from last week
SDS of last week purification		

Cultivation of ci-MBP for Protein Expression

Overnight cultures of ci2350+MBP were taken and each construct was mixed together, for easier calculation of main culture inoculation. LB Medium was taken as a blank for the spectrophotometer, and for dilution of the samples.

OD600 of ci2350+MBP was 0.402 after 10x dilution, meaning OD600 of culture was 4.020.

For the protein expression, multiple cultures were made, with a 0.4 mM IPTG induction concentration and different expression times (3h and overnight). One control culture was made, which will not be induced and will be cultured for the same time as overnight sample.

Regarding the Terrific Broth (TB), 600mL were used in total (540 mL solution A and 60 mL solution B). They were mixed together, and ampicillin was added (600 µL), followed by dividing into 6 Erlenmeyer flasks for cultivation, with 100 mL medium each.

After preparing the medium, cultures were inoculated with 0.370 mL ci2350+MBP (volumes were calculated from Johans instruction, where 0.5 mL of OD600 = 3 overnight culture was needed to inoculate 100 mL main culture). After inoculation, cultures were placed on a shaking incubator at 37C° and 150 rpm at 9:54. Sampling was done at 12:09 (OD600 = 0.235), at 12:52 (OD600 = 0.772), and 13:12 (OD600 = 1.39). Details can be found in this file: Protein expression logging sheets
At 13:15 (first sample to reach OD600 = 1.5) 400µL IPTG was added to five of the reactions and incubated according to the table below (time, temperature).

After 3h cultivation finished, four flasks were taken out, 2x500 uL was taken, centrifuged at max speed for 1 minute. Supernatant was poured out and the pellets were resuspended in enough MilliQ H2O for the OD600 to be 20. They were put into the -80°C freezer until lysis and purification (Wednesday, 29th of July).
The same procedure was done for the two samples that incubated at 25°C overnight.

The exact OD600 measurements for pre- and past IPTG induction are presented in the table below.

Sample	IPTG (mM)	Production time	OD600 Pre IPTG Induction	OD600 Post IPTG Induction
1	0	Overnight, 25°C	1.46	-
2	0.4	Overnight, 25°C	0.78	
3	0.4	3h, 37°C	1.47	6.04
4	0.4	3h, 37°C	0.86	6.54
5	0.4	3h, 37°C	1.22	7.15
6	0.4	3h, 37°C	1.43	7.82

Preparation of samples for SDS and Bradford

2 mL samples at time of induction and after 3 hours were taken for all E. coli BL21 ci2350+MBP cultures. After OD measurement and centrifugation of the remaining volumes, the pellet was resuspended in MQ water to reach 20 OD for later use in SDS and Bradford assay.

Ligation pET blank with inserts followed by transformation.

Nanodrop measurements of the pET blank samples that were extracted from the gel on Friday (24/07/2026) were recorded. Followed by the measurements of the insert that are being used for the ligation .
(the inserts were labelled as the results of 20/07/2026)

Sample	Concentration (ng/µL)	Abs 260/280	Abs 260/230
pET sample 4	20.9	1.750	0.627
pET Pooled (sample 2,3,5)	4.970	2.000	0.294
AKR1D1 SUMO	22.9	2.000	0.561
AKR1D1 TrX	35.8	2.000	0.424
AKR1D1 MBP	35.8	1.895	0.600
Ci2350 SUMO	33.3	1.971	0.318
Ci2350 TrX	34.8	1.944	0.400

We have a total of 12 ligation reactions (70 ng of vector DNA, vector:insert ratio of 3:1). 2 Negative controls (each with one of the vector samples and 5 inserts for both the vectors)

Ligation reaction for pooled vector (Sample 2,3,5)	AKR1D1 SUMO	AKR1D1 TrX	AKR1D1 MBP	Ci2350 SUMO	Ci2350 Trx	Negative control
T4 Buffer	2	2	2	2	2	2
Vector pooled	14	14	14	14	14	14
Insert	2.2	1.47	2.32	2.67	2.58	-
T4 Ligase	0.2	0.2	0.2	0.2	0.2	0.2
MQ	1.52	2.25	1.4	1.05	1.14	3.72

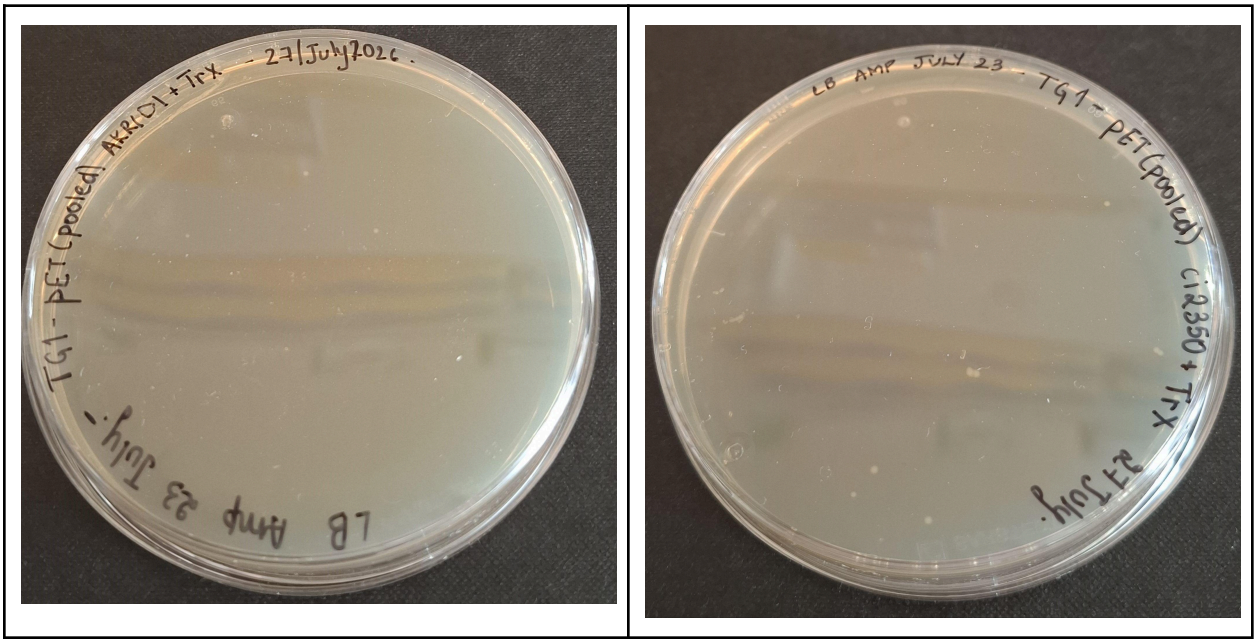
Ligation reaction (vector sample 4)	AKR1D1 SUMO	AKR1D1 TrX	AKR1D1 MBP	Ci2350 SUMO	Ci2350 Trx	Negative control
T4 Buffer	2	2	2	2	2	2
Vector pooled	3.34	3.34	3.34	3.34	3.34	3.34
Insert	2.2	1.47	2.32	2.67	2.58	-
T4 Ligase	0.2	0.2	0.2	0.2	0.2	0.2
MQ	12.26	12.99	12.14	11.79	11.9	14.46

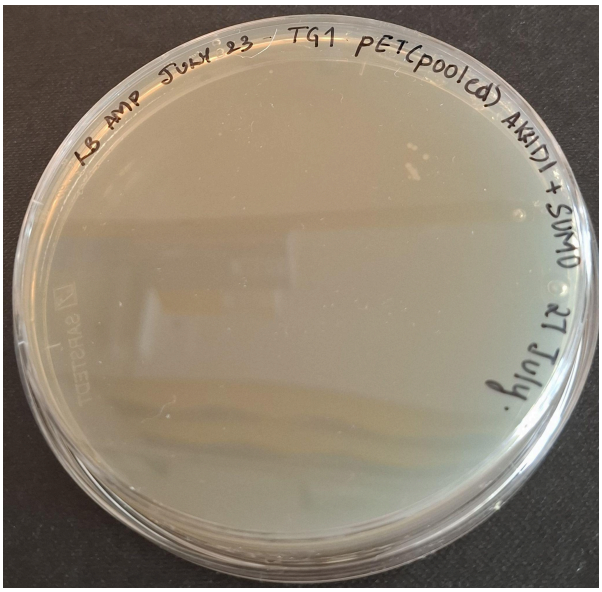
Transformation of pET_blank ligation reactions into TG1

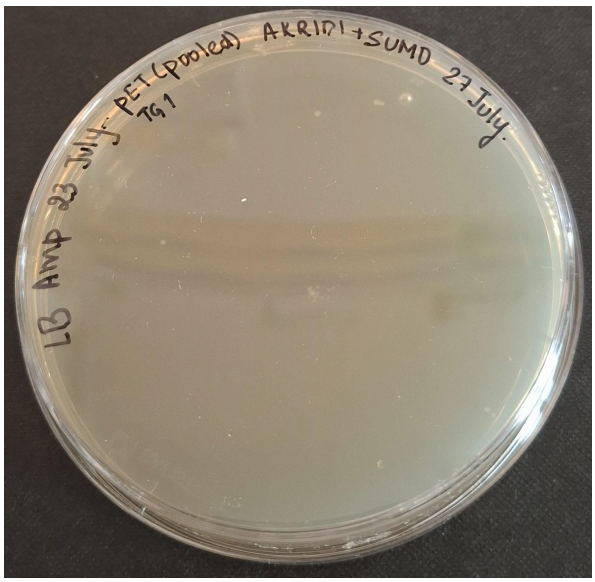
Competent cells TG1 were taken from the -80, thawed on ice. 10 µL of each ligation reaction was added to the thawed TG1 cells. The tubes were incubated on ice for a little above 50 minutes. We used the thermoshaker for incubating the cells at 37 C, 300 RPM for 45 min. After centrifugation, the cell pellet was resuspended in 100 µL, equally divided (50 µL × 2) and plated on separate plates.

Results: The transformation was a **partial success** - there were **colonies on plates** where the **pooled vector** was used, but **not** on the **with vector 4.**

Negative control (only vector in ligation mix) also had some colonies.







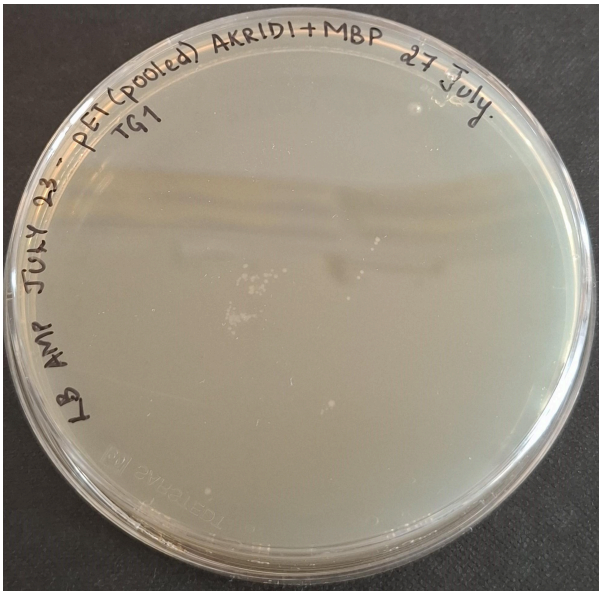


Plate preparation

Since the media was solidified, it was heated up on a hot plate, at first 100℃, then after not liquifying, at 150℃. However, that failed to liquify it sufficiently, so it was autoclaved again.
5 plates without ampicillin and 35 plates with ampicillin were prepared.

NOTE: the media was darker than usual from heating. If you do not use LB agar the day it is prepared, avoid it solidifying by keeping it in 70℃ (where we dry pipette tips),

Sequencing Preparation

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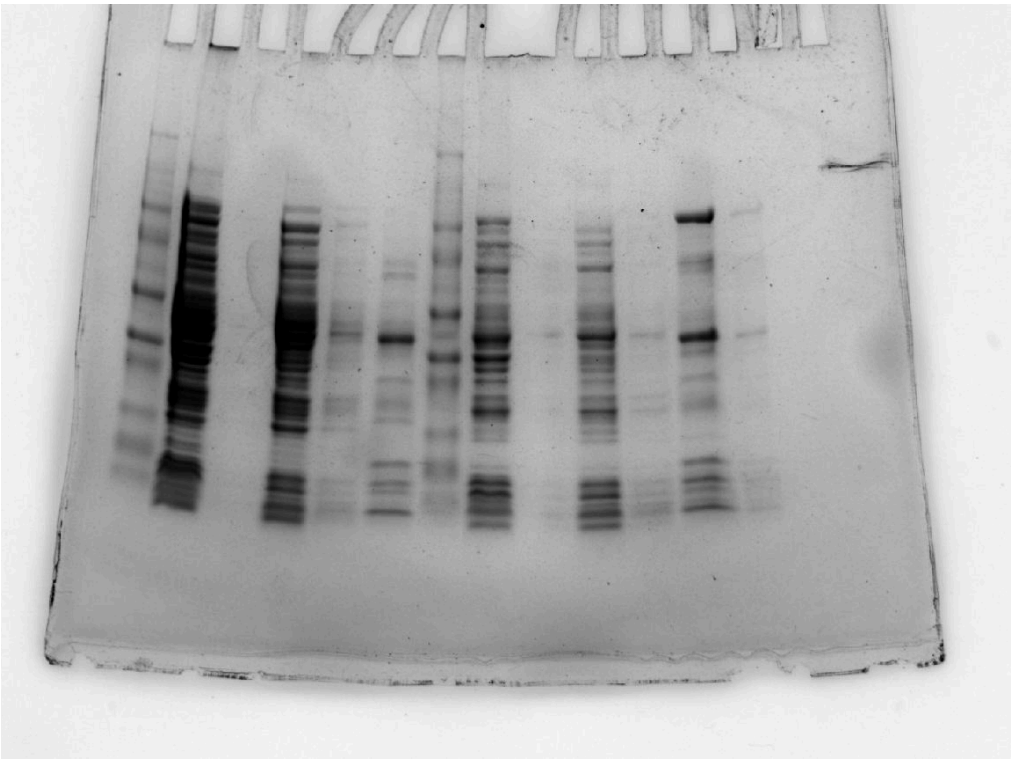
SDS of purification of ci_MBP & ci_His from friday (24/07)

SDS was performed to check the results of previous week's purification. Three samples were checked with purification and tested on the column:

- ciTAG (ciMBP), 0.4 IPTG, kept overnight (from the previous round of cultivation)
- ciTAG (ciMBP), 0.8 IPTG, kept overnight at 17°C (from 22 July 2026)
- Ci2350 wt (ciHIS), 0.8 IPTG, retrieved after 3 hours from induction (from 21 July 2026)

Samples included all the steps of purification for each of the three constructs checked: waste (initial running time of the machine before the sample gets in, to check that nothing is present), flow through, wash and elute (where the overexpressed protein should be). Lysate samples were also added to check total protein expression.

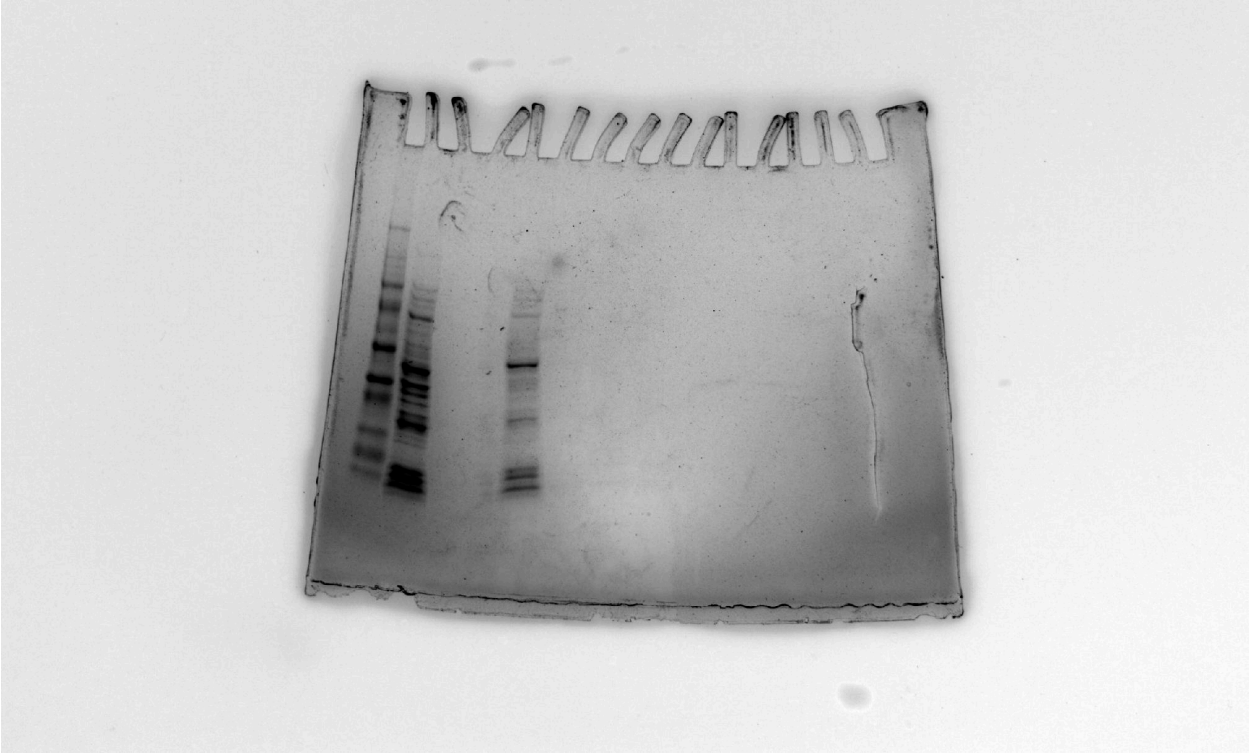
Gel 1: ciTAG samples, in order, ciTAG (ciMBP), 0.4 IPTG and followed by ciTAG (ciMBP), 0.8 IPTG. position 1 and 7 is the ladder, from 2-6 and 8-13 → lysate, purification waste, flow through, wash, elution.



A band was visible in the ciTAG (ciMBP), 0.8 IPTG elution, together with other proteins and another quite overexpressed band at lower kDa. **It was observed by Camille that around the bp corresponding to the weight of our ciMBP construct, there is an abundance of repeated T base pairs. This could naturally be in the sequence of the protein or a result of**

codon harmonization. It may then act as a site where the ribosome slips out and stops translation, therefore cousin the truncation of the protein and the overexpressed band at lower kDA. This may be more reasonable than a wild type E.coli protein, due to the overexpression level.

Gel 2: Ci2350 wt (ciHIS), 0.8 IPTG → ladder, lysate, skipped well, waste, flow through, wash and elute.



No band is visible in the elute in this case, and an over expressed band is visible in the flowthrough, compatible with the one observed for the other construct.

Day 30: [July 28, 2026], Tuesday

Weekly responsible: Eleonora
Lab participants: Giulia, Cecilia, Hamsi, Rytis, Shun

Daily overview of experiments

Activity	***Protocol File***	***Comments***
Buffer preparation for protein purification		Binding buffer, elution buffer, imidazole stock solution
Bradford for ci_MBP		
SDS-gel for ci_MBP		
Colony PCR of transformed ligation reactions (pET_blank + inserts)		
Plasmid prep of pET(Blank)		pET(Blank) was obtained by digesting pET(Blank)-ci235–MBP with BamHI and NdeI, then gel purifying it.

Results and interpretation

Buffer preparation for protein purification

Different buffers were made using already prepared stock solutions (3M NaCL, 1M Sodium Phosphate buffer and 1M Imidazol).

- Imidazole Stock solution: 1M imidazole: 68g for 1L; 34g for 500mL
- Cell Wash buffer 1 (before lysis): 0.5 M NaCl, 20mM sodium phosphate
- Wash buffer 2 (washing the columns): same as binding buffer
- Binding buffer: 500mM NaCl, 20mM sodium phosphate, 30mM imidazole
    - For 500mL: 83 mL of 3M NaCl + 10mL of 1M sodium phosphate + 15mL of 1M Imidazole
- Elution Buffer: 500mM NaCl, 20mM sodium phosphate, 500mM imidazole
    - For 250mL: 42 mL of 3M NaCl + 5mL of 1M sodium phosphate + 125mL of 1M

Imidazole

500 mL of cell binding buffer was made, and 250 mL of elution buffer.

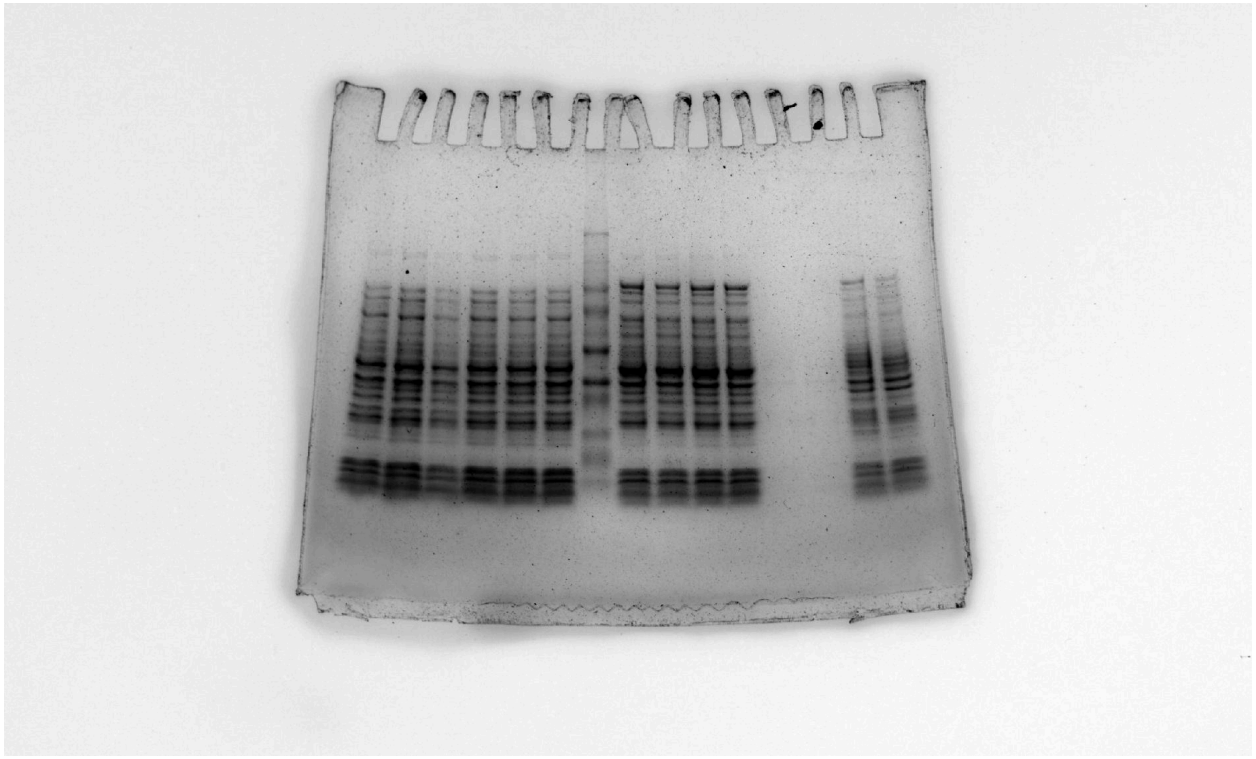
Bradford for ci-MBP

The Bradford standard curve was performed based on the protocol. As a buffer, MQ water was used.
For sample measurement, 400µL sample, 400µL MQ and 200µL Bradford reagent were used.
The samples analysed are the pre- and post inoculation samples.

SDS-PAGE for ci-MBP

SDS page was performed to check the purified samples from previous day cultivation, with sample ci2350+MBP, once for incubation at 37°C for 3 hours, and once for overnight cultivation at 25°C (IPTG induced and uninduced reaction).

Gel order: samples 1-6 are all repeats before induction, 7 is the ladder, 8-11 are samples 3 hours after induction, 12-13 are medium fractions of culture centrifugation, 14-15 are samples kept at 25°C overnight.



The overexpressed protein is visible in the post induction samples at around 110 mark, and it is still faintly visible in the overnight expression. Further analysis was performed with SDS of

purified samples + soluble fraction after sonication and centrifugation. A lower band is still visible and overexpressed.

Colony PCR of transformed ligation reactions

Twelve ligation reactions were divided on 24 plates where each plate had a final volume of 50uL (TG1+pET+insert). After overnight incubation plates with the highest colonies were chosen. All of the selected plates were of pET pooled samples as the vector.

The plates: Ligation control, AKR1D1+MBP, AKR1D1 + SUMO(x2), AKR1D1+TrXA ci2350+TrXA(x2) {for AKR1D1+SUMO and ci2350+TrxA both the plates showed colonies so we took colonies from both the plates).
In total we took 22 colonies (Labelled as A, B ,C ….V)

Plate / colony name	Labelled as
Ligation control	A,B,C
AKR1D1+MBP	D,E,F,G
AKR1D1+SUMO	H,I,J
AK1D1+SUMO	K,L,M
AKR1D+TrxA	N,O,P
Ci2350 +TrxA	Q,R,S
Ci2350 +TrxA	T,U,V

Master mix :

Component	Volume per 1 reaction (uL)	Mastermix (for 24 reactions, uL)
Red Load Taq Master (5x)	4	96
T7 promoter forward primer (10 µM)	0.8	19.2
reverse primer (10 µM)	0.8	-
MQ H20	14.4	345.6
Total	20	460.8

Reverse primer was not added to the master mix directly since the inserts were different. The reverse primers used were as follows:

- ligation control: T7_ter
- AKR1D1+SUMO, AKR1D1+TrxA, AKR1D1+MBP: AKR1D1_rev

- ci2350+Trx: ci2350_rev

PCR settings are shown in the following table.

Step	Duration	Temperature	Cycles
Cell lysis	5 mins	95 C	1
Denaturation	30 s	95 C	30
Annealing	30 s		
Elongation	2 mins 25 s	72 C	
Final Elongation	5 mins	72 C	1
Hold	Infinite	4 C	

After the PCR samples were stored in the fridge overnight for gel electrophoresis for the next day.

Plasmid pET(blank) prep

10 × 5 mL overnight cultures of TG1 pET(Blank)-ci2350-MBP were spun down in 10 × 1.5 mL Eppendorf tubes, and EZNA plasmid extraction kit was used.
Elution was performed twice (25 µL × 2), using heated Elution buffer (60℃).

Concentration was measured:

Sample	**Concentration (ng/µL)**	**Abs 260/280**	**Abs 260/230**
pET-1	87.5	1.872	2.316
pET-2	99.4	1.835	2.273
pET-3	87	1.842	2.273
pET-4	96.4	1.857	2.321
pET-5	110	1.850	2.312
pET-6	97.4	1.849	2.361
pET-7	108	1.904	2.359
pET-8	98.4	1.869	2.329

Sample	Concentration (ng/μL)	Abs 260/280	Abs 260/230
pET-9	95.4	1.846	2.087
pET-10	99.4	1.835	2.105
pET-Blank-ci-MBP (Pooled)	97.9	1.824	2.118

Then plasmids were pooled together, to get ~480 μL of 97.9 ng/μL stock

Plasmid pET(blank) restriction digestion and gel purification - Rytis

Digestion with NdeI and BamHI was performed in a single, big reaction:

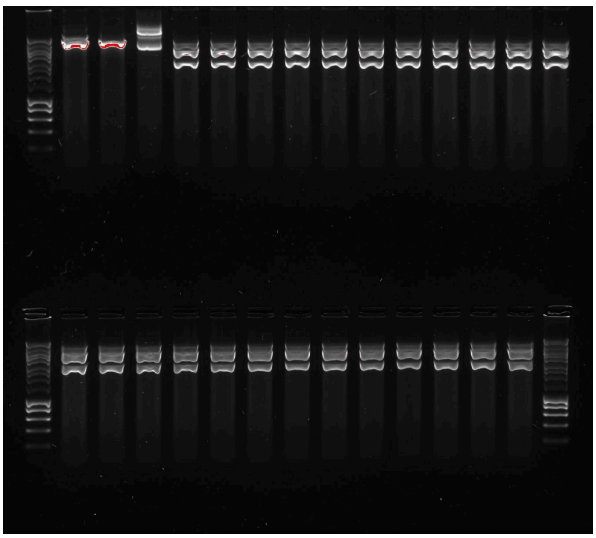
Reagent	Control (NdeI)	Control (BamHI)	Sample (μl)	25 reactions (μl)
FD Green Buffer	2	2	2	50
NdeI	1	-	1	25
BamH1	-	1	1	25
pET-Blank-ci-MBP (Pooled) DNA (70 ng)	5.1	5.1	5.1	127.5
MilliQ H2O	11.9	11.9	10.9	272.5
Total Volume	20	20	20	500

Due to bigger volume, the digestion time was extended:
45 min, 37℃, 300 RPM with NdeI, then BamHI was added, and digested for an additional 20 min.

Enzymes were inactivated at 80℃ for 5 min.

25 μL of Fast AP was added to dephosphorylate the cut vector (37℃, 300 RPM, 10 min).
Then it was inactivated at 80℃ for 5 min.

Gel electrophoresis was performed (1% gel, 10 min 60 V; 1h 5 min 90 V):



First row:
1 Ampliqon High range ladder (1.1 µL)
2 NdeI control
3 BamHI control
4 pET-Blank-ci-MBP (Pooled) (500 ng)
5-15 Digested pET(Blank)-ci-MBP

Second row:
1, 15 Ampliqon High range ladder (1.1 µL)
2-14 Digested pET(Blank)-ci-MBP

Three bands corresponding to 3000 bp (the cut ci-mbp), 5000 bp (pET-Blank) and 8000 bp (undigested pETBlank-ci-MBP) can be seen in the digest, meaning the **digestion** was not total, and **should've been extended more.**

Gel band of 5000 bp was cut carefully to avoid the undigested plasmid.(extraction using EZNA Gel Extraction kit was performed

Elution was done with 2×25 µL Elution buffer, preheated to 60℃.
Concentration was measured:

Sample	**Concentration (ng/µL)**	**Abs 260/280**	**Abs 260/230**
pET(Blank) digest 1	5.964	2.4	0.35
pET(Blank) digest 2	6.41	n/a	n/a
pET(Blank) digest 3	7.952	2.0	1.0
pET(Blank) digest 4	6.461	1.857	1.625
pET(Blank) digest Pooled (1-3)	5.96	1.5	0.632

Day 31: [July 29, 2026], Wednesday

Weekly responsible: Eleonora
Lab participants: Giulia, Cecilia, Hamsi, Rytis, Shun

Daily overview of experiments

Activity	***Protocol File***	***Comments***
Gel electrophoresis for pET_insert ligation reaction from Tuesday	Gel electrophoresis.d…	Results show failed colony PCR/digestion/ligation ? Need to repeat pET ligation? Only ligation?
Ligation of inserts and pET_blank	Ligation with pET-11…	
Transformation of pET-insert into TG1		
Sonification for cell lysis of ci_MBP	E. coli Lysis by Sonic…	Six samples from Monday; sonicator was set to amp=30%, sonication was repeated minimum of 5x per sample.
Column Purification of ci_MBP		
Plasmid prep AKR1D1-His	Followed E.Z.N.A mini prep protocol	All optional steps were followed.
Transformation into BL21 of AKR1D1+His	E. coli Transformatio…	Used 50 uL of competent cells, two transformation reactions + one water control. Left overnight at 37°C
Direct Mutagenesis of ci_MBP	Mutagenic PCR	
Pour agar plates		LB-Agar; LB-Agar + Amp

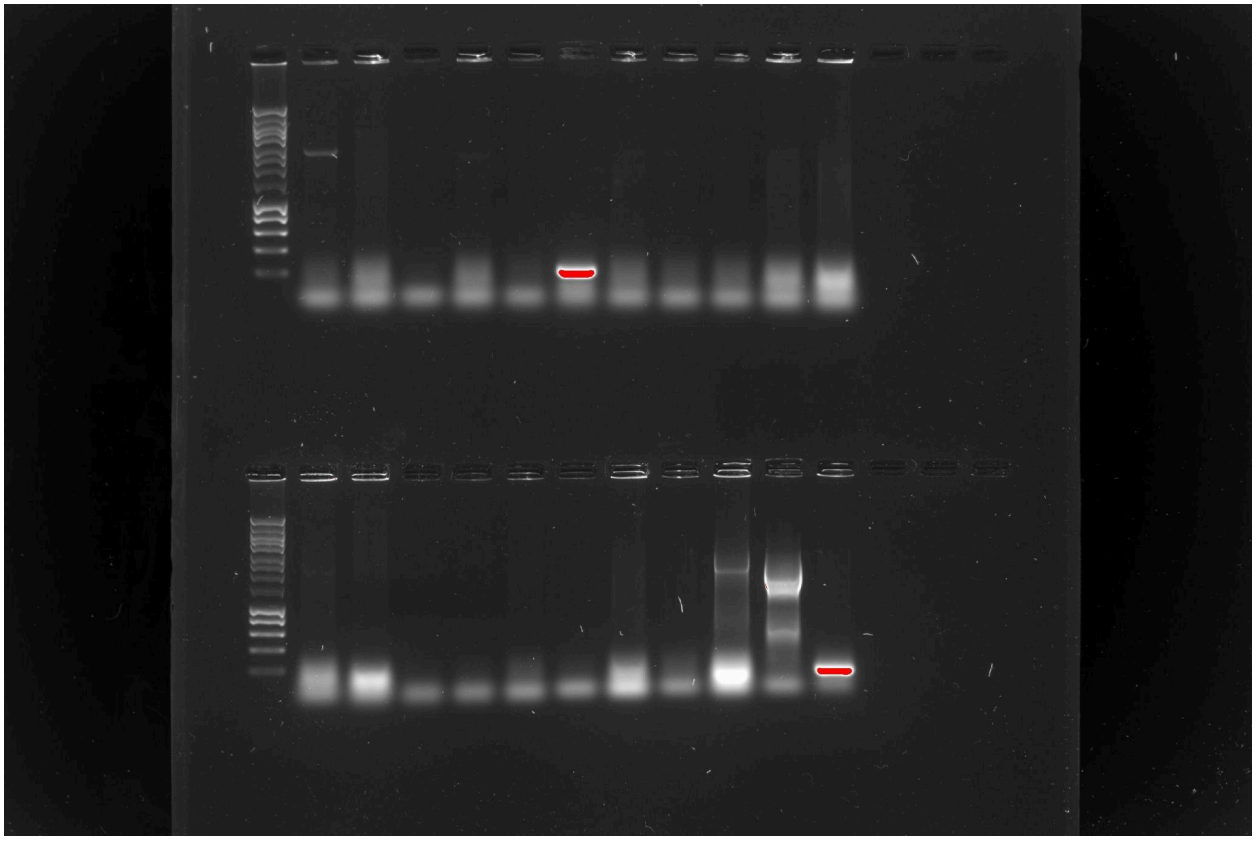
Results and interpretation

Agarose gel electrophoresis for confirming successful Colony PCR and Plasmid Digestion

Big gel (150 mL) was made, with 1% agarose. 1.1 µL of Ampliqon High Range Ladder was used.

Wells order:

Upper / Lower Gel	Lane	Sample
Upper Gel	1	Ladder (1.1uL)
	2-4	Samples A , B , C - Ligation control
	5-8	Samples D,E,F,G - AKR1D1+MBP
	9-11	Samples H,I,J - AKR1D1+SUMO
	12	Sample K - AKR1D1+SUMO
Lower Gel	1	Marker
	2-3	Samples L,M - AKR1D1+SUMO
	4-6	Samples N,O,P -AKR1D1 + Trx
	7-9	Samples Q,R,S - ci2350 + Trx
	10-12	Samples T,U,V - ci2350 + Trx



Results showed … and therefore the samples were not sent for sequencing.

Ligation of inserts and pET_blank

Ligation reactions were set up for 1:1 and 1: 2 ratios. Two different concentrations of vectors were used (Sample 4 , pooled pET) . The samples are labeled as 1,2,3 .. 10 in the order of the insert added for the overnight ligation. A master mix was made with T4 buffer , vector and MQ water . The vector mass is 70 ng and the concentration of the vector samples : Sample 4 = 6.46 ng/uL and the pooled vector concentration = 5.946 ng/uL . The final volume of the vector that needed to be added to the master mix was calculated to be 10.83 uL and 11.73 uL respectively.
A ligation control was made only with the pooled vector sample .
The ligation was set up overnight at 16°C.

The ligation reaction is for (1:1) reactions

Ligation reaction for vector (Sample 4)	**AKR1D1 SUMO (1)**	**AKR1D1 TrX (2)**	**AKR1D1 MBP (3)**	**Ci2350 SUMO (4)**	**ci+Trx (5)**
T4 Buffer	2	2	2	2	2
Vector Sample 4	10.83	10.83	10.83	10.83	11.73
Insert	1	1	1	1	1
T4 Ligase	0.2	0.2	0.2	0.2	0.2
MQ	5.97	5.97	5.97	5.97	5.07

Ligation reaction is for (1:2).

Ligation reaction for pooled vector	**AKR1D1+ SUMO (6)**	**AKR1D1+ MBP (7)**	**AKR1D1+ Trx (8)**	**ci2350+ SUMO (9)**	**ci2350+ Trx (10)**	**Ligation control**
T4 Buffer	2	2	2	2	2	2
Vector Sample 4	11.73	11.73	11.73	11.73	11.73	11.73
Insert	2	2	2	2	2	-
T4 Ligase	0.2	0.2	0.2	0.2	0.2	0.2
MQ	4.07	4.07	4.07	4.07	4.07	6.07

Sonification of ci_MBP for Cell Lysis

Samples from Monday's cultivation of ci_MBP (0.4 M IPTG) were thawed on ice and resuspended with 10mL of wash buffer (500mM NaCl, 20 mM sodium phosphate). The duplicates were pooled and centrifuged at 10.000 x g for 5 minutes.

The supernatant was discarded and the samples weighed to calculate the volume of the lysis buffer to add (0.4 g = 5 mL of lysis buffer). The lysis buffer is the same as the binding buffer (500mM NaCl, 20 mM sodium phosphate, 30mM imidazole).

Sample	Weight [g]	Lysis Buffer to add [mL]
S1 (no IPTG)	1.81	22.6
S2 (25°C overnight)	2.31	28.8
S3 (37°, 3h)	1.01	12.6
S4 (37°, 3h)	0.79	9.9
S5 (37°, 3h)	1.12	14.0
S6 (37°, 3h)	0.97	12.1

Sonificator was set to an amplitude of 30%, 2 min on time with a 3 seconds on/off cycle. For sample 1 and 2 the sonification was repeated 7 times, for sample 3-6 it was repeated 4 times. Sample 3 and 4 were pooled, as well as 5 and 6.
The samples were kept on ice at all times.

After lysis sample volume was adjusted with the binding buffer and put into the centrifuge for 1.5h (20.000 x g at 4°C).

Purification of ci_MBP via Column Binding

Columns were pre-washed with a binding buffer (500mM NaCl, 20 mM sodium phosphate, 30mM imidazole).

Two samples were used for purification. The supernatants from

- the pooled IPTG induced samples for 37°C cultivation (3h) and
- the one IPTG induced sample for 25°C overnight incubation.

The flowrate was set to 1.5 mL/min (should be higher than 1 and lower than 4). Next, the time for how long it takes a sample to get from the start of the tube until the end of the tube at the flowrate of 1.5 mL/min. In this case the time was 3 minutes and 20 seconds.

For column purification the following steps were followed:

1. Flowthrough: put the start of the tube into your sample and wait 3 min 20 seconds until the end of the tube is moved from a waste flask into the Flowthrough flask. Let the whole sample volume go through the pump.
2. Washing: Put the start of the tube into the binding buffer, wait 3 min 20 seconds before moving the end of the tube from Flowthrough flask into Washing flask. Wait until 10 mL (10x the volume of the column) binding buffer went through the column.

3. Elution: put the start of the tube into the elution buffer, wait 3 min 20 seconds before you move the end of the tube from the Washing flask into the Elution flask. Wait until 5mL (5x the column volume) of the volume went through the column into the Elution flask.
    a. The elutant was concentrated via vivaspin.

Between and after purifying different samples, clean the column with the binding buffer for 10 min.

In total, the following samples were collected:

	Sample 37°C for 3h, 0.4 mM IPTG	Sample 25°C overnight, 0.4 mM IPTG	Sample 25°C overnight, **no** IPTG
Supernatant (after centrifugation; before purification)	2x30 mL	30 mL	30 mL
Flowthrough	Whole sample (2x ~30 mL)	Whole sample (1x ~30 mL	-
Wash	10 mL	10 mL	-
Elution	5 mL	5 mL	-

The elutant was put into a vivaspin (30 kDA cut off) and centrifuged at 4.000 x g for 30 minutes. Then 5 mL of the cell wash buffer was added and the flask centrifuged again. This was repeated a second time.

To the final elutant, glycerol was added in a ratio 1:1.

Plasmid prep AKR1D1-His

Insert experiment discussion and interpretation

Transformation into BL21 of AKR1D1-His

After the AKR1D1+His plasmid from strain B was confirmed by sequencing on July 28, E. coli BL21 was transformed with it. Previously minipreped AKR1D1+His plasmid that was sequenced was used for transformation. 1 µL of the plasmid was used (39.8 ng/µL) for transformation in 50 µL of BL21 competent cells. Negative control was added (only MilliQ H2O).

Amplification primers resuspension

Primers received from IDT were resuspended in MilliQ Water, following the volumes shown in the table. Before resuspension, tubes were spinned down, to allow all of the primers to go down the tube. After resuspension, primers were vortexed, then spun down. All of the stock concentrations in the IDT tubes are 100 µM. Three aliquots of 100 uL of each primer were made, with 10 µM concentration.

Primer Name	Nanomols received	Volume of MilliQ added (µL)	Melting temperature
R215A fw	20	200	63.8 C
R215A rev	21	210	63.8 C
V263I rev	29.5	295	61.6 C
V263I fw	24.4	244	61.6 C

Direct Mutagenesis of ci_MBP

PfuTurbo Hotstart PCR Master Mix (2x) was used for site directed mutagenesis of ci_MBP. Two sets of forward and reverse primers were used for the two point mutations in separate reactions. One control was made for each set of primers (2 controls in total, one for ci2350 set, one for AKR1D1 set). 2 reactions were made for each point mutation, with a total of 4 reactions + 2 controls. Reaction concentrations were corrected and a master mix prepared with water, Pfu Hotstart Master Mix and template vector DNA (ci_MBP previously extracted with miniPrep). Vector DNA originally had a concentration of 97 ng/µL and was diluted 10 times in 20 µL to reach final amount of 10 ng per reaction.

For future PCR experiments: prepare master mix (count 2 reactions more due to volume lost pipetting) and add the different components afterwards (different primer, insert etc…) to avoid errors.

Component	Amount per reaction (µL)	Final concentration
Pfu Hotstart Master Mix (2x)	25	1x
Primer Forward (10 µM)	2.5	0.5 µM
Primer Reverse (10 µM)	2.5	0.5 µM

DNA Template (9.7 ng/µL)	1	10 ng
MilliQ H2O	19	
Total volume	50	

Reaction parameters are based on Agilent Pfu guidelines, annealing temperature is based on primer melting temperature of 62°C - 5°C, 8.5 minutes were chosen for elongation, due to plasmid length of 8427 bp. (According to Agilent guidelines, when the vector is lower than 10 kb the 1 min/1kb rule of thumb can be kept, otherwise it becomes 2 min).

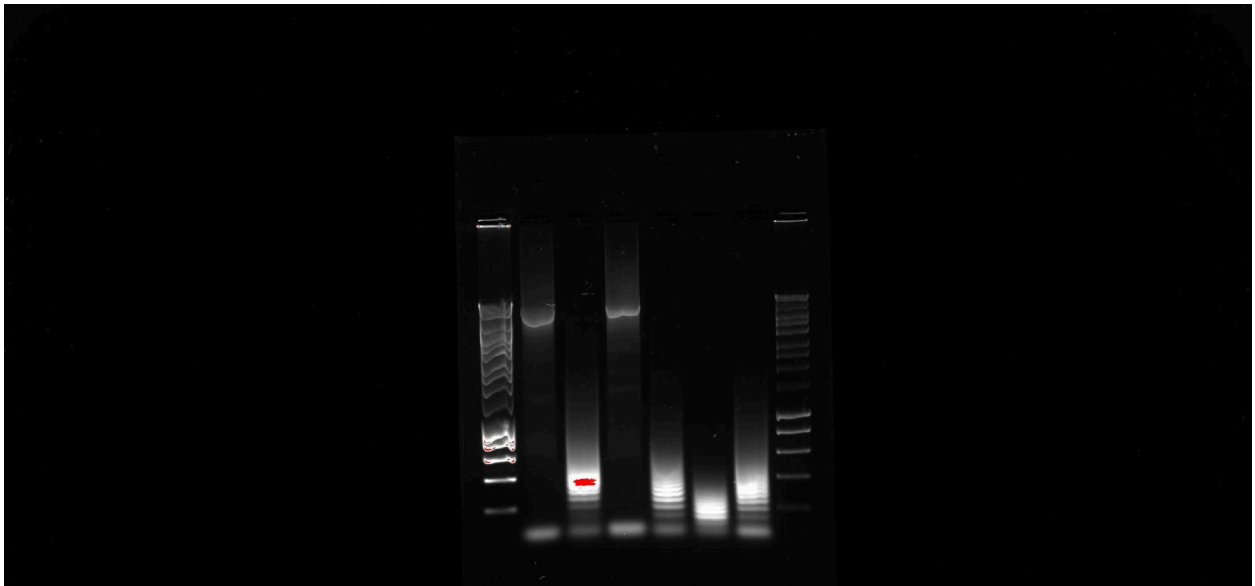
Temperature (C)	Duration	Cycles
95	2 minutes	1
95	30 seconds	30
57	30 seconds	
72	8.5 minutes	
72	10 minutes	1
4	infinite	-

Agarose gel was used to confirm amplification of the plasmid, adding 10 µL of reaction and according to the following scheme:

- 10 µL DNA reaction
- 2 µL MQ H2O
- 3 Gel Red 5X

Tot 15 µL

Amplification was followed by digestion with DpnI, by adding 1 µL directly to the remaining 40 µL of reactions and incubating at 37°C in the thermocycler for 45 min.



Only V2361 mutation worked and will be used for transformation in E.coli TG1 for colony PCR and sequencing check. The other mutation will be tested again depending on whether the primers are ok or not.

Day 32: [July 30, 2026], Thursday

Weekly responsible: Eleonora
Lab participants: Giulia, Cecilia, Hamsi, Rytis, Shun

Daily overview of experiments

Activity	***Protocol File***	***Comments***
Autoclave waste	Autoclaving.docx	
Glycerol stock of TG1-pET11a-AKR1D1	Glycerol Stock Prepa…	Prepared two stocks, placed in yellow box (-80℃)
SDS-Gel electrophoresis of ci_MBP	SDS-PAGE	Samples for 25C and 37C: Supernatant, Flowthrough, Wash, Elution 1, Elution 2; plus one uninduced control
Transformation of the Overnight Ligation Mixtures into E.coli TG1	E. coli Transformatio…	
Transformation of the site directed mutagenesis into E.coli TG1	E. coli Transformatio…	Both successful amplifications were used for transformation overnight at 37°C. 10 uL of reaction were used for 100 uL of competent cells

Results and interpretation

Glycerol stock of TG1-pET11a-AKR1D1

Protocol was followed to make 2 tubes of stock:
500 µL Overnight culture of *E. coli* TG1 pET11a-AKR1D1-His was mixed with 500 µL 50% glycerol, mixed, and stored in -80℃ (yellow box)

SDS-PAGE of ci_MBP expressed proteins

SDS-PAGE analysis was performed to evaluate and compare the expression levels of ci_MBP across different induction conditions and purification steps.

Cultivation Conditions Analyzed

- 37 °C, 3 h: Induced with 0.4 mM IPTG
- 25 °C, overnight: Induced with 0.4 mM IPTG
- Uninduced Control: 25 °C overnight (0.4 mM IPTG omitted)

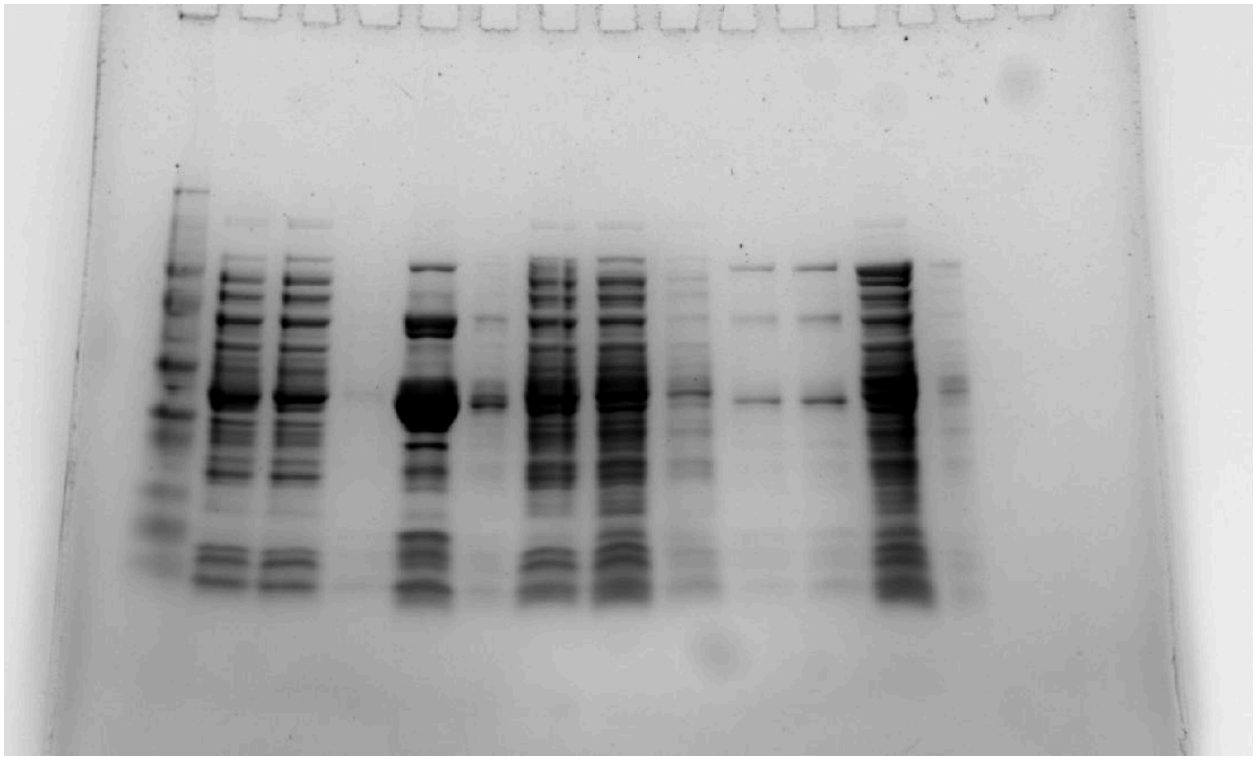
For sample preparation 15 µL of each sample were mixed with 15 µL of SDS loading dye (1:1 ratio) followed by denaturation at 90 °C for 5 minutes. Cooled and briefly spin down using a benchtop centrifuge.

15µL of the dyed samples were loaded onto a pre-cast Bio-Rad gel alongside 5 µL of protein ladder. For each cultivation condition, the following fractions were analyzed:

- Supernatant (S): Soluble fraction following cell lysis via sonication
- Flow-through (FT): Column flow-through following binding
- Wash (W): Column wash fraction
- Eluate 1 (E1): Initial eluate (pre-Vivaspin concentration)
- Eluate 2 (E2): Final eluate (post-Vivaspin concentration)

The loading order is as followed:

1	2	3	4	5	6	7	8	9	10	11	12
Ladder	37°C S	37°C FT	37°C W	37°C E1	37°C E2	25°C S	25°C FT	25°C W	25°C E1	25°C E2	25°C Control (uninduced)



Electrophoresis was initially performed at 200 V for 25 minutes. Upon visual inspection, sample migration had only reached the midpoint of the gel. The run time was therefore extended by an additional 20 minutes, resulting in a total run time of 45 minutes at 200 V.

The gel was stained, destained and visualized following the procedure described in the protocol.

Transformation of the Overnight Ligation Mixtures into E.coli TG1

10 uL of the ligation reaction to the competent cells. Once the ligation mixture was added to the competent cells , the samples were kept on ice for 23-24 minutes . All of the samples were centrifuged at 13,000g for 1 minute except AKR1D1+MBP (1:2) (sample 7) which was centrifuged at 5000 g for 1 minute. Each transformant was plated on 2 plates each so a total of 23 plates were incubated overnight . Each plate had a volume of 50 uL of the TG1 cells with the ligation mixture.

Site directed mutagenesis with modified conditions

PfuTurbo Hotstart PCR Master Mix (2x) was used for site directed mutagenesis of ci_MBP. Two sets of forward and reverse primers were used for the two point mutations in separate reactions. One control was made for each set of primers (2 controls in total, one for mutation V263I one for mutation R215A). 2 reactions were made for each point mutation, with a total of 4 reactions + 2 controls. Reaction concentrations were corrected and a master mix prepared with water, Pfu Hotstart Master Mix and template vector DNA (ci_MBP previously extracted with miniPrep).

Vector DNA originally had a concentration of 97 ng/µL and was diluted 10 times in 20 µL to reach a final amount of 10 ng per reaction.

For future PCR experiments: prepare master mix (count 2 reactions more due to volume lost pipetting) and add the different components afterwards (different primer, insert etc…) to avoid errors.

Component	Amount per reaction (µL)	Final concentration
Pfu Hotstart Master Mix (2x)	25	1x
Primer Forward (10 µM)	2.5	0.5 µM
Primer Reverse (10 µM)	2.5	0.5 µM
DNA Template (9.7 ng/µL)	1	10 ng
MilliQ H2O	19	-
Total volume	50	-

Reaction parameters are based on Agilent Pfu guidelines, annealing temperature is based on primer melting temperature of 62°C - 5°C (based on mt reported on the primers package, which was lower than reported on snapgene). In this case, 17 minutes were chosen for the elongation time. As the amplified plasmid ci+MBP is close to the 10 kb limit, better results can be obtained following the 2 min/1 kb rule that applies to bigger constructs. All other conditions were kept the same. The PCR was left overnight, kept at 4°C and subsequently digested with DpnI in the morning.

Temperature (C)	Duration	Cycles
95	2 minutes	1
95	30 seconds	30
57	30 seconds	
72	17 minutes	
72	10 minutes	1
4	infinite	-

Transformation of the site directed mutagenesis into E.coli TG1

Only V2361 mutagenesis was selected after the gel electrophoresis. A volume of 10 uL the ligation reaction was added to 100 uL of competent cells. Once the ligation mixture was added

to the competent cells , the samples were kept on ice for 23-24 minutes. Each transformant was plated on 2 plates each so a total of 5 plates were incubated overnight . Each plate had a volume of 50 uL of the TG1 cells with the ligation mixture.

Day 33: [July 31, 2026], Friday

Weekly responsible: Eleonora
Lab participants: Giulia, Cecilia, Hamsi, Rytis, Shun

Daily overview of experiments

Activity	***Protocol File***	***Comments***
LB-Agar preparation for plates	Media preparation.docx	Preparation of 1L
Autoclave of LB-Agar and tips	Autoclaving.docx	
Glycerol stock of pET11a-AKR1D1-His in BL21	Glycerol Stock Prepa…	Prepared nine 1mL stocks (500µL cells + 500µL of 50% filtered glycerol), placed in -80°C freezer
Mutant digestion		
Colony PCR of - Ligation reactions of pET_blank + insert constructs - V2361 site directed mutagenesis	Colony PCR.docx	
Prepare 100mM potassium phosphate, pH 6.5	https://www.aatbio.com/resources/buffer-preparations-and-recipes/potassium-phosphate-ph-5-8-to-8-0	For 1L, 100mM, pH 6

Colony PCR of - Ligation reactions of pET_blank + insert constructs

The colony PCR was run for a total of 48 colonies. The PCR Master mix was made for 50 samples.

Plate / colony name	Labelled as
pETS4-AKR1D1+SUMO (1:1)	(1-2)
pET pool -AKR1D1+SUMO (2:1)	(3-7)
pET pool -AKR1D1+SUMO (2:1)	8
pET pool -AKR1D1+TrX (2:1)	(9-10)
pET pool -AKR1D1+ TrX (2:1)	(11-13)
pETS4-AKR1D1+ TrX (1:1)	14
pETS4-AKR1D1+ TrX (1:1)	(15-16)
pETS4 - AKR1D1+MBP (2:1)	(17-20)
pET pool - AKR1D1+MBP (2:1)	(21-27)
pET pool - AKR1D1 +MBP (2:1)	(28-31)
pET S4 - AKR1D1 + MBP (1:1)	32
pET pool - ci2350+TrX (2:1)	33
pET pool - ci2350 + TrX (2:1)	(34-37)
pET pool - ci2350+ TrX (1:1)	(38-40)
pET pool - ci2350 + SUMO (2:1)	(41-46)
pET pool - ci2350 + SUMO (2:1)	(47-48)

Master mix :

Component	Volume per 1 reaction (uL)	Mastermix (for 50 reactions, uL)
Red Load Taq Master (5x)	4	200
T7 promoter forward primer (10 µM)	0.8	40
reverse primer (10 µM)	0.8	-
MQ H20	14.4	720

Total	20	960

Reverse primer was not added to the master mix directly since the inserts were different. The reverse primers used were as follows:

- AKR1D1+SUMO, AKR1D1+TrxA, AKR1D1+MBP: AKR1D1_rev
- ci2350+Trx: ci2350_rev

2 master mixes were made with the total volume of the main master mix :
Master mix AKR1D1 :

Component	Volume per 1 reaction (uL)	Volume for 33 reactions , (uL)
Main Master mix	19.2	633.6
AKR1D1 reverse	0.8	26.4
Total	20	660

Master mix ci2350

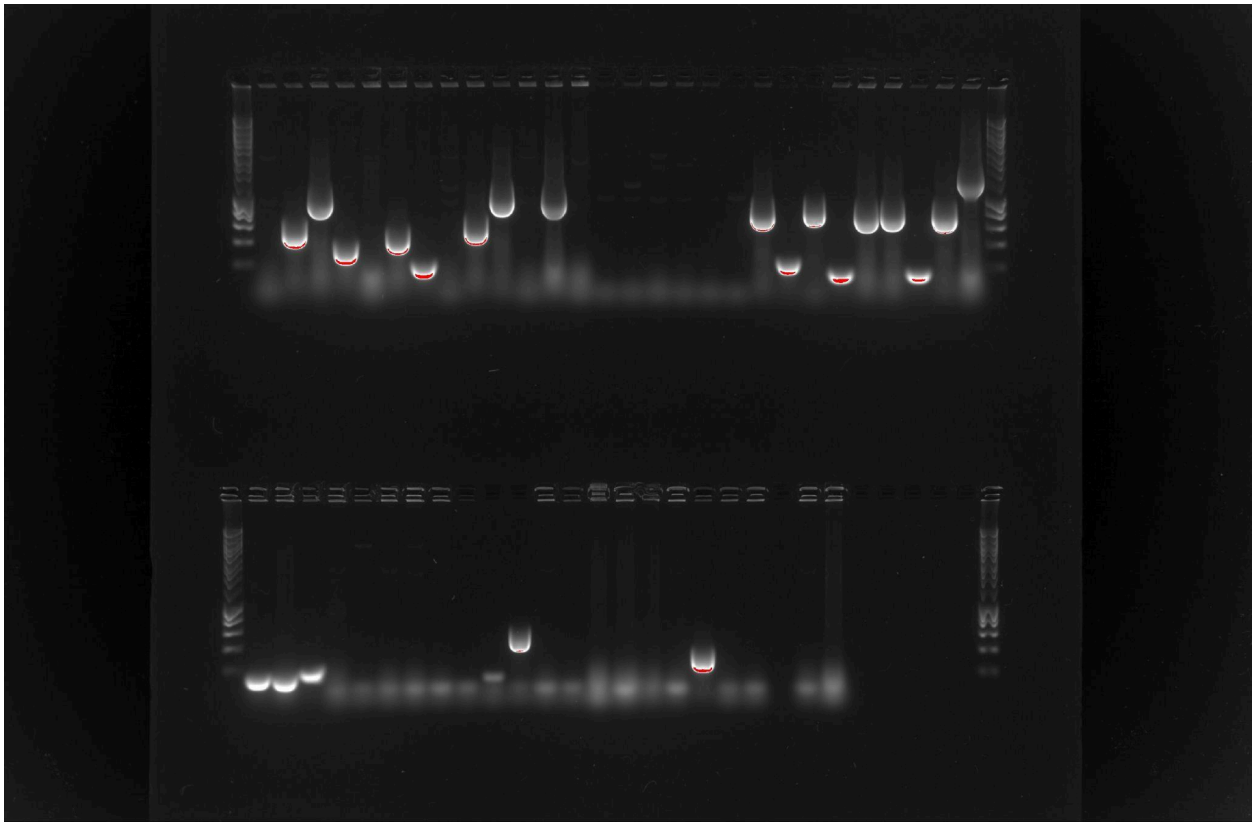
Component	Volume per 1 reaction (uL)	Volume for 17 reactions , (uL)
Main Master mix	19.2	326.4
ci2350 reverse	0.8	13.6
Total	20	340

PCR settings are as shown in the following table.

Step	Duration	Temperature	Cycles
Cell lysis	5 mins	95 C	1
Denaturation	30 s	95 C	30
Annealing	30 s	56 C	
Elongation	2 mins 25 s	72 C	
Final Elongation	5 mins	72 C	1
Hold	Infinite	4 C	

Agarose gel Electrophoresis Loading order

Upper / Lower Gel	Lane	Sample
Upper Gel	1	Ladder (1.1 µL)
	(2-3)	(1-2) - pET S4 - AKR1D1+SUMO - 1:1
	(4-8)	(3-7) -pET Pool - AKR1D1+SUMO - 2:1
	9	8 -pET Pool - AKR1D1+SUMO - 2:1
	(10-11)	(9-10) - pET Pool - AKR1D1+TrX - 2:1
	(12-14)	(11-13) - pET Pool - AKR1D1+TrX - 2:1
	15	14- pET S4- AKR1D1+ TrX- 1:1
	(16-17)	(15-16) - pET S4- AKR1D1+ TrX- 1:1
	(18-21)	(17-20) - pET S4- AKR1D1+ MBP - 1:1
	(22-29)	(21-28) - pET Pool - AKR1D1+ MBP - 2:1
	30	Ladder (1.1 µL)
Lower Gel	1	Ladder (1.1 µL)
	(2-4)	(29-31) - pET Pool - AKR1D1+ MBP - 2:1
	5	32 - pET S4 - AKR1D1+ MBP - 1:1
	6	33 - pET pool - ci2350+ TrX - 2:1
	7-10	(34-37) - pET pool - ci2350+ TrX - 2:1
	(11-13)	(38 - 40)- pET pool - ci2350+ TrX - 1:1
	(14-19)	(41- 46) -pET pool - ci2350 + SUMO - 2:1
	(20-21)	(47-48)-pET pool - ci2350 + SUMO - 2:1
	(23-24)	(43-44) - pool - ci2350 + SUMO - 2:1 (repeated since the wells previously were contaminated.)
	30	Ladder (1.1 µL)



Samples : 3, 10 , 12 were chosen for overnight inoculation at room temperature over the weekend (AKR1D1+SUMO , AKR1D1 + TrX(x2))

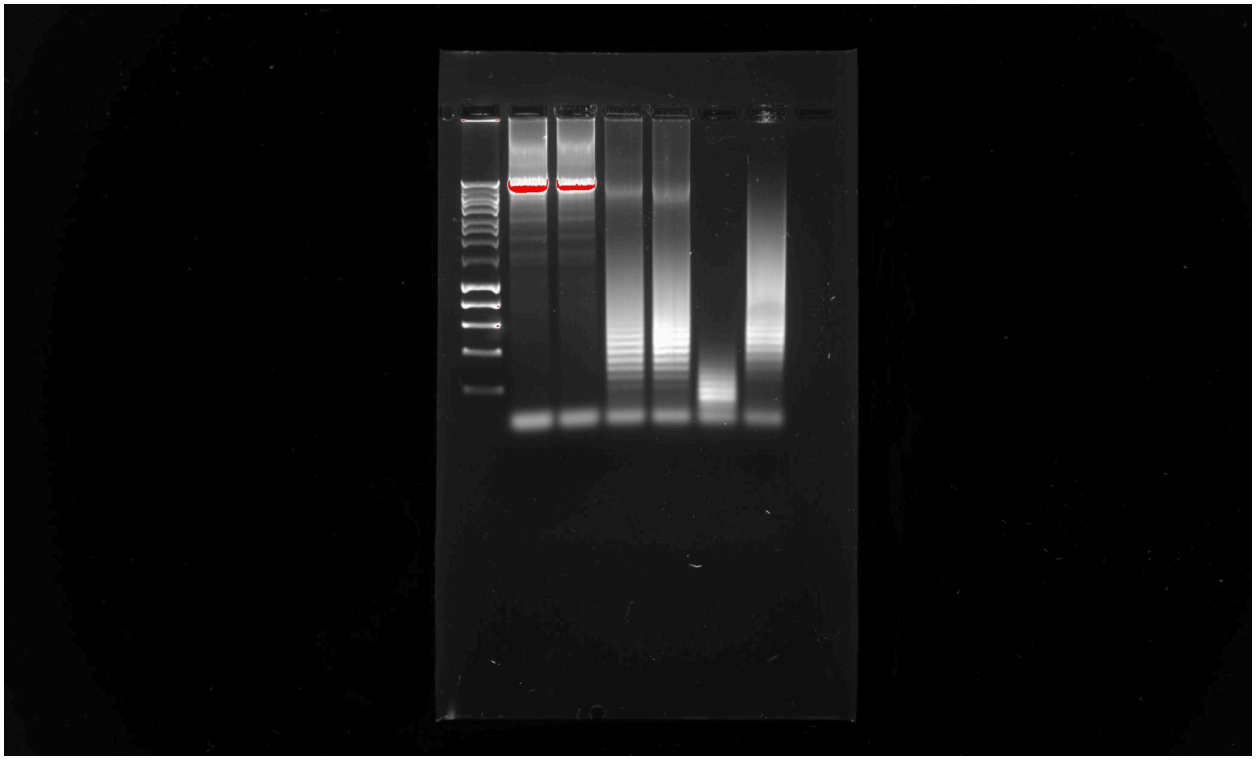
Mutant Digestion

Samples from overnight PCR for both mutations were digested with DpnI for 35 minutes at 37°C. 10 uL were removed from reactions and used for checking amplification with gel electrophoresis following the scheme:

- 10 uL DNA
- 3 uL 5x loading dye
- 2 uL MQ water

Tot loaded: 15 uL

Gel order: ladder, V2361 first, V2361 second, R215a first, R215a second, control V2361, control R215a



Following these results, all amplified sequences were used for transformation in E.coli TG1 to move on with sequencing. 10 uL of reaction was used to transform 100 uL of competent E.coli TG1 cells. Plates were left at room temperature over the weekend, and upon successful transformation will be used for inoculation, plasmid extraction and sequencing check.

Prepare 100mM potassium phosphate, pH 6.5

Prepared 500 mL of 100mM potassium phosphate (pH = 6.5) based on the following link: https://www.aatbio.com/resources/buffer-preparations-and-recipes/potassium-phosphate-ph-5-8-to-8-0

To get 500mL the following was added and adjusted to 500mL with MQ.

- Potassium phosphate dibasic (mw: 174.18 g/mol): 2.937 g
- Potassium phosphate monobasic (mw: 136.09 g/mol): 4.509 g

For control, pH was measured. The pH was 6.53 which was what is expected. Stored at RT.