Week 32 - Overview

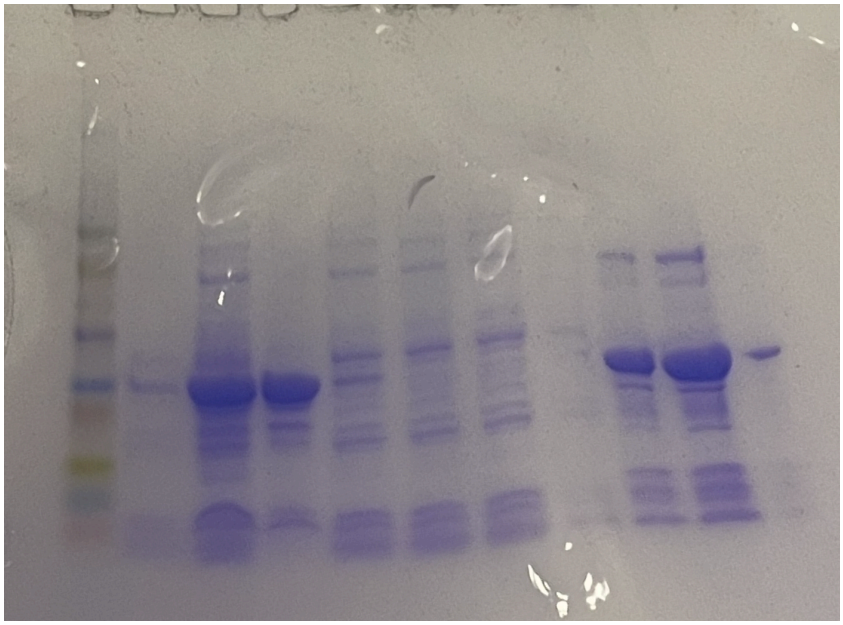
Weekly summary:

During Week 32, we continued with protein expression of our constructs, focusing on AKR1D1-His, AKR1D1-SUMO and ci2350-TrxA. AKR1D1-His was purified and analysed by SDS-PAGE. Mutagenic PCR was performed on the single ci2350-MBP mutants to obtain the double mutants. We started performing testosterone reduction assays, which required troubleshooting after initial problems were encountered.

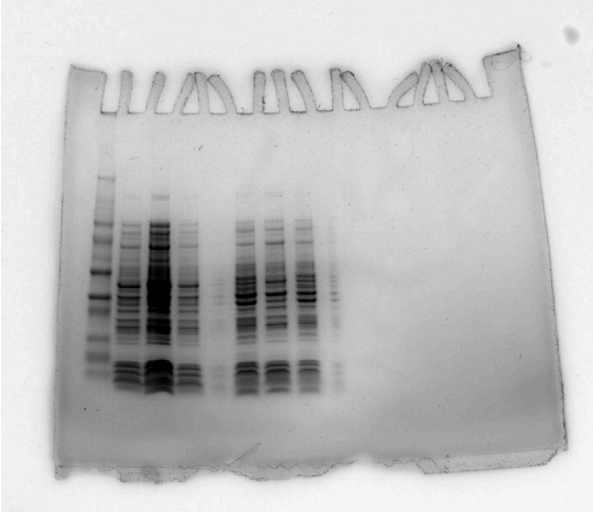
Main results of the week:

After getting sequencing results for constructs AKR1D1-SUMO, AKR1D1-TrxA and both single mutants of ci2350-MBP (V263I and R215A), several correct sequences were identified, making them suitable for further usage, and production. Single ci2350-MBP mutants were subjected to another round of mutagenic PCR, using to insert the other mutation in order to obtain a double mutated construct.

AKR1D1-His was produced by overnight induction with 1 mM IPTG at 17C. Analysis of purification fractions on SDS-PAGE is shown on the figure below. On the figure it is visible that most of the produced AKR1D1-His protein ends up in the insoluble fraction, due to the very large band present in the resuspended insoluble pellet (Lane 4). Despite this, purification was still successful, as there is a large band of the same molecular weight in the eluate (Lane 9).



ci2350-His production, followed by analysis on SDS-PAGE (not purified). Lane details could not be retrieved.



Initial testosterone reduction assays with ci2350-MBP and AKR1D1-His remained inconclusive due to substrate precipitation, cofactor handling errors, and absorbance variability. Troubleshooting improved assay stability, but further optimization is needed to reliably assess enzymatic activity.

Finally, atcABC and moaABCDE were successfully transformed individually into *E. coli*, while co-transformation into BL21(DE3) remained challenging.

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Day 34: [August 3, 2026], Monday

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

Daily overview of experiments

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Activity	***Protocol File***	***Comments***
Plasmid mini prep for sequencing of ligation constructs + mutagenic samples of ci+MBP V236I	QMF28.0029.D6942 …	
Sequencing	File	Samples send: - pET11a-AKR_Trx - pET11a-ci_Trx - Mutants of ci-MBP (V2361; R215a)
Expression: Cultivation of E.coli BL21 with AKR1D1+his and ci+his	Protein expression (f…	Total 1 L of culture Protein expression lo…
Inoculation of new mutant transformants for V236I + R215A		
Picking up testosterone (Camille)		
Preparation for testosterone assay (tuesday)		Concentration via vivaspin and dilution with potassium phosphate buffer

Results and interpretation

Plasmid mini prep for sequencing of positive colony PCR constructs + mutagenic samples of ci_MBP-V236I ; ci_MBP-R215A

Samples of 5 mL LB were inoculated and incubated over the weekend (~64 h) at RT, 150 RPM. Plasmid DNA was extracted using EZNA kit, elution was performed using heated elution buffer (70℃, 2x 25 µL), Nanodrop was used to measure the DNA concentration.

Sample	**Concentration (ng/µL)**	**Abs 260/280**	**Abs 260/230**
AKR1D1-SUMO (S3)	80.5	1.8	2.051
AKR1D1-Trx (S10)	59.0	1.875	2.143
AKR1D1-Trx (S12)	98.9	1.843	2.211
V2631(2) a	84.5	1.875	2.338
V2631(2) c	70.5	1.855	2.364
V2361 a	171	1.820	2.278
V2361 b	151	1.825	2.380
V2361 c	106	1.829	2.229
ci-MBP mutant D	60.1	1.806	2.283
ci-MBP mutant E	93.4	1.808	1.773
ci-MBP mutant F	85.5	1.870	1.773
R215a A	136	1.815	2.210
R215a B	91.0	1.830	1.830
R215a C	159	1.850	2.162
R215a D	121	1.813	2.095

Sample	Concentration (ng/µL)	Abs 260/280	Abs 260/230
R215a E	122	1.788	0.985
V2631 b	68.6	1.840	2.060

Sending ci_MBP-mutants & AKR1D1-SUMO (S3) & AKR1D1-Trx (S10; S12) for Sequencing

pET(Blank)_AKR1D1-SUMO

Sample	Primer	Barcode
AKR1D1-SUMO (S3) - T7 pro fw	T7 pro fw	EF73935155
	T7 ter rev	EF73935156
AKR1D1-Trx (S10)	T7 pro fw	EF73935157
	T7 ter rev	EF73935158
AKR1D1-Trx (S12)	T7 pro fw	EF73935159
	T7 ter rev	EF73935160
V2631(2) a	T7 pro fw	EF73935167
	T7 ter rev	EF73935168
	ci2350_fw	EF73935169
V2631(2) c	T7 pro fw	EF73935170
	T7 ter rev	EF73935171
	ci2350_fw	EF73935172
V2361 a	T7 pro fw	EF73935173
	T7 ter rev	EF73935174
	ci2350_fw	EF73935175
V2361 b	T7 pro fw	EF73935176
	T7 ter rev	EF73935177

Sample	Primer	Barcode
	ci2350_fw	EF73935178
V2361 c	T7 pro fw	EF73935179
	T7 ter rev	EF73935180
	ci2350_fw	EF73935181
ci-MBP mutant D	T7 pro fw	EF73935182
	T7 ter rev	EF73935183
	ci2350_fw	EF73935184
ci-MBP mutant E	T7 pro fw	EF73935185
	T7 ter rev	EF73935186
	ci2350_fw	EF7393517
ci-MBP mutant F	T7 pro fw	EF73935188
	T7 ter rev	EF73935189
	ci2350_fw	EF73935190
R215a A	T7 pro fw	EF73935191
	T7 ter rev	EF73935192
	ci2350_fw	EF73935193
R215a B	T7 pro fw	EF73935194
	T7 ter rev	EF73935195
	ci2350_fw	EF73935196
R215a C	T7 pro fw	EF73935197
	T7 ter rev	EF73935198
	ci2350_fw	EF73935199
R215a D	T7 pro fw	EF73935200
	T7 ter rev	EF73935201
	ci2350_fw	EF73935202
R215a E	T7 pro fw	EF73935203

Sample	Primer	Barcode
	T7 ter rev	EF73935204
	ci2350_fw	EF73935205
V2631 b	T7 pro fw	EF73935206
	T7 ter rev	EF73935207
	ci2350_fw	EF73935208

Expression: Cultivation of *E.coli* BL21(DE3) with AKR1D1_his and ci_his

Terrific broth solution A & B were mixed together and 1000µL of ampicillin (100 mg/mL) was added.
The starting cultures (ci_his and AKR1D1_his) were measured for OD600 with the spectrophotometer (1:10 dilution):

- Ci_his: 0.411 -> 4.11
- AKR1D1_his: 0.430 -> 4.30

Based on desired concentrations of OD600=3 for 0.5mL culture in 100mL media the following inoculation volumes were calculated:

- ci_his: for 100mL TB media, 0.365 mL of culture needs to be added
- AKR1D1_his: for 100mL TB media, 0.349 mL of culture needs to be added

The following samples were prepared accordingly:

Sample	TB-media volume (mL)	Starter culture added (mL)
ci_his control	50	0.182
ci_his	200	0.548 *
AKR_his control	50	0.174
AKR_his	150	0.524
AKR_his	150	0.524
AKR_his	170	0.593
AKR_his	200	0.698

*for ci_his the TB-media was suppose to be 150mL, not 200mL. Therefore, the starter culture added was based on a volume of 150mL and is incorrect.

Inoculated flasks were put into the 17°C incubator, OD600 was measured and IPTG induction time calculated for when OD600=1.5.

Preparation for Testosterone Assay (on Tuesday)

The samples to prepare are the ci_MBP samples with cultivation at 25°C overnight and 37°C for 3h. IPTG induction was at 0.4 mM for both. Purification was done via column chromatography (see July 29). Purified samples were stored in 50% glycerol at -20°C.
To prepare the sample for the assay, the total sample (4mL of sample diluted in 50% glycerol) was added onto a vivaspin flask (30,000 MWCO). The sample tube was washed with 4 mL of the 100mM potassium phosphate buffer (pH 6.5; see 31 July), and added to the vivaspin flask as well making it contain a total of 8mL.
The flasks were centrifuged for 30 min at 3.000 x g at 4°C.
The following two steps were repeated:

- After the centrifugation step, the flowthrough was discarded and the supernatant mixed with a pipet multiple times, especially close to the membrane to get rid of potential clogging, then the flasks were put back into the centrifuge.
- After this step, the samples were diluted with 5 mL of buffer, mixed and put back into the centrifuge.

These two steps were performed alternately until the sample is concentrated to a volume of ~1 mL, this took in total 4 alternating cycles. The volume was adjusted to ~1.5 mL with a buffer. After the vivaspin, the concentration of the samples was analyzed via Bradford assay.

Day 35: [August 4th, 2026], Tuesday

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

Daily overview of experiments

Cultivation of E.coli BL21 AKR1D1+His, testosterone assay with purified 5 beta reductase from E.coli BL21 ci2350+His cultivated at xx temperature. Colony PCR of missing constructs, analysis of sequencing results. SDS page of E.coli BL21 ci2350+His cultivation overnight at 17°C, pre induction and overnight samples, total protein.

Activity	***Protocol File***	***Comments***
Cultivation of *E.coli* BL21 AKR1D1+His		
IPTG stock 100mM		50 mL IPTG 100mM (1.19g IPTG dissolved in 50mL MQ)
BSA stock		Stock with a concentration of 10 mg/mL in potassium phosphate buffer (100mM, pH 6.5). Dilute to a 100µg/mL concentration.
Bradford for ci_MBP at 37°C and 25°C		Standard curve: Bradfords iGEM26.xlsx
Colony PCR of missing constructs		
analysis of sequencing results		
SDS page of E.coli BL21 ci2350+His cultivation		
LB agar plates preparation		2x Spec (Spectinomycin), 2x Amp, 3x Spec+Amp

Transformation of atcABC, moaABDCE into BL21(DE3) & TG1		

Results and interpretation

Cultivation of E.coli BL21 AKR1D1+His

After unsuccessful cultivation on Monday, a new cultivation batch was started. **ALWAYS CHECK OD AND DO NOT USE THE MIDDLE INCUBATOR AS IT SOMETIMES DOES NOT HOLD THE TEMPERATURE.**

Four cultures were set up of 200 mL each with overnight inoculated E.coli BL21 AKR1D1+His. The cultures will be kept at 37 °C, 170 rpm until induction and subsequently moved to 17°C for overnight incubation. 1 mM of IPTG was used for induction following Penning conditions, so 2 mL per culture. Sheet of the cultures: Protein expression logging sheets

Bradford for ci_MBP at 37°C and 25°C

The standard curve was made with BSA concentrations of 20, 40, 80, 120, 180 µL using the newly prepared BSA solution (100µg/mL, in 100mM potassium phosphate buffer pH 6.5). Buffer was added to each sample to get to a volume of 800µL. 200µL of Bradford reagent (filtered with 0.45µm filter). After Bradford was added, each sample was incubated for 5 minutes before measuring the absorbance at 595nm.

BSA (100µg/mL)	Abs. A	Prot. conc. A (µg/mL)	Abs. B	Prot. conc. B	Abs. Average	Prot. conc. Average
0	0	0	0	0	0	0
20	0.17	5.995345764	0.229	19.96265119	0.1995	12.97899848
40	0.3	36.7707645	0.32	41.50544431	0.31	39.1381044
80	0.572	101.1624099	0.472	77.48901083	0.522	89.32571035
120	0.67	124.3623409	0.677	126.0194788	0.6735	125.1909099

180	0.836	163.6601833	0.918	183.0723705	0.877	173.3662769

Based on the average absorbance and BSA concentrations the slope and intercept with the y-axis were calculated. Then, the equation was rearranged for resolving x, the protein concentration.

standard curve equation	y=mx+n
prot. conc. equasion	x=(y-n)/m
Absorbance (y)	
Slope (m)	0.004224150485
Y-intercept (n)	0.1446747573
protein concentration (x)	

Details can be found in the excel file: Bradfords iGEM26.xlsx

For sample analysis (ci_MBP), micro cuvettes were used. The samples were diluted 1:100 with the potassium phosphate buffer (990µL buffer + 10µL sample).
The following volumes were pipette:

- Blank: 56µL buffer + 14µL Bradford
- Sample: 28µL buffer, 28µL sample (1:100 dilution), 14µL Bradford

Duplicates were made. The following absorbance was measured. Based on the BSA curve the protein concentration was calculated.

Sample	Absorbance	Dilution factor	Corrected absorbance	Protein concentration (µg/mL)
ci_MBP 25 A	0.278	100	27.8	6546
ci_MBP 37 A	0.671	100	67.1	15850
ci_MBP 25 B	0.241	100	24.1	5671
ci_MBP 37 B	0.757	100	75.7	17886

LB agar plates preparation

Working concentration of Spec (Spectinomycin) is same as ampicillin (100 µg/mL).
2 plates of Spec (Spectinomycin), 2 plates of Amp, 3 plates of Spec+Amp were prepared.

Transformation of atcABC, moaABDCE into BL21(DE3) & TG1

The dry pET21-atcABC pellet from Twist bioscience was spun down briefly, then resuspended in 25 µL of MQ H_2O, to get a working concentration of ~ 38 ng/µL.

pCDFDuet1-moaABCDE_mobAB from SynBio was also spun down, then resuspended in 50 µL of MQ H_2O, to get a working concentration of ~ 40 ng/µL.

Transformation of atcABC, moaABCDE into E. coli TG1 and double transformation into E. coli BL21 was performed according to the protocol, with a few deviations.
1 µL of each plasmid was mixed with ~200 µL competent cells (TG1). For double transformation into BL21(DE3), it was 1+1 µL of pDNA (~40 ng each). The waiting time after adding DNA was ~25 min, heat shock at 42℃ performed for 45 s.
After centrifugation step (5000 g, 1 min), 100 µL of supernatant was left, cell pellet was resuspended and 50 µL of it was used for one plate (2 plates total).
For double transformation, cells were plated on LB agar with Spec+Amp (both 100 µg/mL).

RESULTS:
Successful transformation into TG1 for both plasmids - atcABC had 300-400 colonies in each plate, while moaABCDE had far less, but still a considerable amount (50-100).
Double transformation into BL21(DE3) failed, there were 0 colonies on each plate.

Day 36: [August 5, 2026], Wednesday

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

Daily overview of experiments

testosterone assay (with adjustment regarding the mastermix), akr1d1-sumo transformation in BL21, stopping the production of AKR1D1-his + sds (to coordinate with camille as she's doing 4 gels at the same time lol), choose the mutants and inoculate them + setting up the reaction for overnight mutagenesis for the second mutation (we are doing two single mutations + the two combined)

Activity	***Protocol File***	***Comments***
Testosterone assay		ci_MBP at different volumes; testosterone at 20mM, NADH at 20mM. Measured absorbance at 350nm.
AKR1D1_SUMO plasmid extraction	8. Plasmid miniprep.pdf	
Transformation of BL21(DE3) with AKR1D1_SUMO, atcABC, moaABCDE, atcABC+moaABCDE		
Stop overnight culture of E.coli BL21 AKR1D1_His + SDS PAGE		
Mutants: Chose mutants, inoculate them, set up overnight reaction for mutagenesis		ci-MBP-D, ci-MBP-F?
Inoculate TG1 with - atcABC - moaABCDE - pET_AKR_MBP #28		

Results and interpretation

Testosterone assay

This assay measures testosterone activity using two enzyme samples: ci_MBP (25°C) and ci_MBP (37°C). Each sample is evaluated across 8 different protein concentrations in triplicate, resulting in 48 total reaction wells on a standard microplate.
Reaction Components:

- Testosterone buffer (20mM)
- Sample mixture (protein + buffer)
- NADH (20mM; added last to start the reaction)

Testosterone mastermix (20mM): 11,050 mL buffer + 520µL testosterone. Mastermix was prepared the day prior.The initial formulation (8mL buffer + 500µL testosterone) formed crystals due to precipitation. Re-adjusted to 11.050mL buffer + 520µL of testosterone, vortexed thoroughly, and incubated at 37°C with shaking until crystals completely dissolved.

NADH solution:

- Target concentration: 20mM (0.02 mol/L)
- Target volume: 800 µL (0.0008 L)
- Calculations:
    - n=c*V -> 0.02 mol/L * 0.0008 L = 1.6*10^-5 mol
    - m=n*M -> 1.6*10^-5 mol * 663.43 g/mol = 0.01061 g = 10.61 mg
- Preparation: Dissolve 10.61 mg NADH in 800µL potassium phosphate buffer (100mM;. pH 6.5)

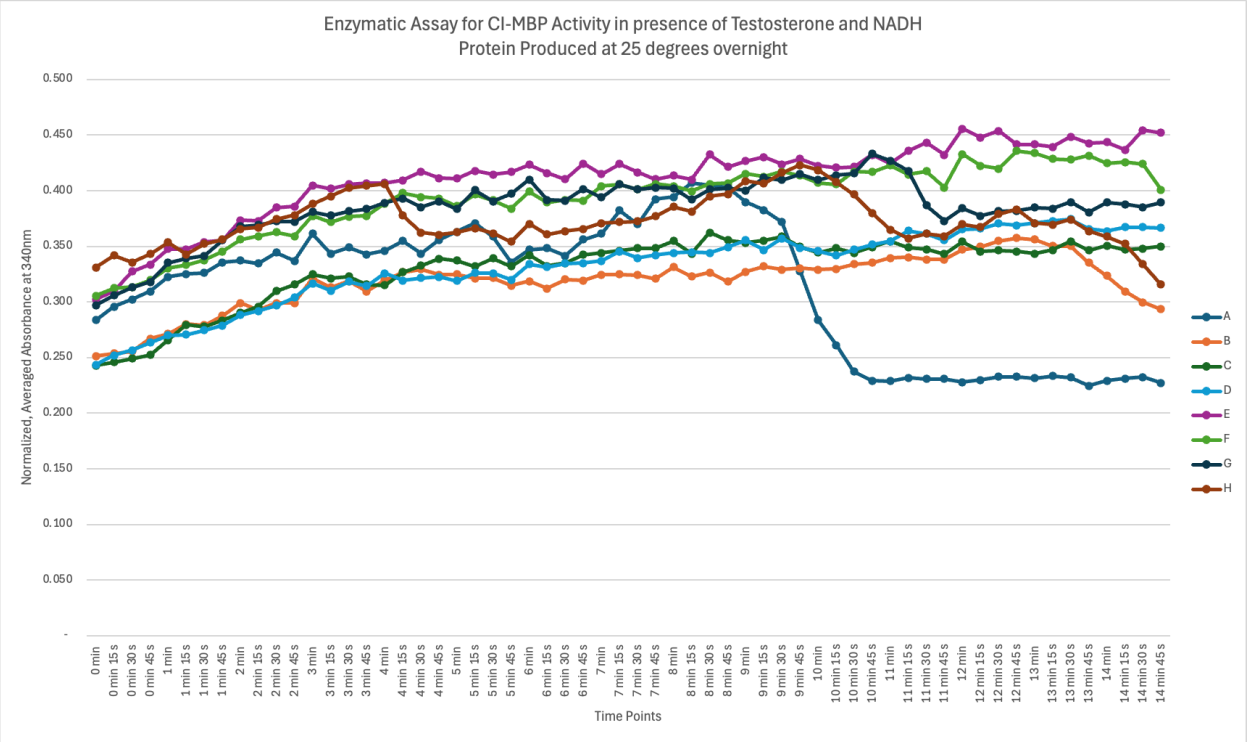
Sample dilution master mixes:
To ensure accuracy, mastermixes for each protein volume condition were prepared for 4 reactions (3 triplicates + 1 extra offset = 80 µL final mastermix volume per condition).

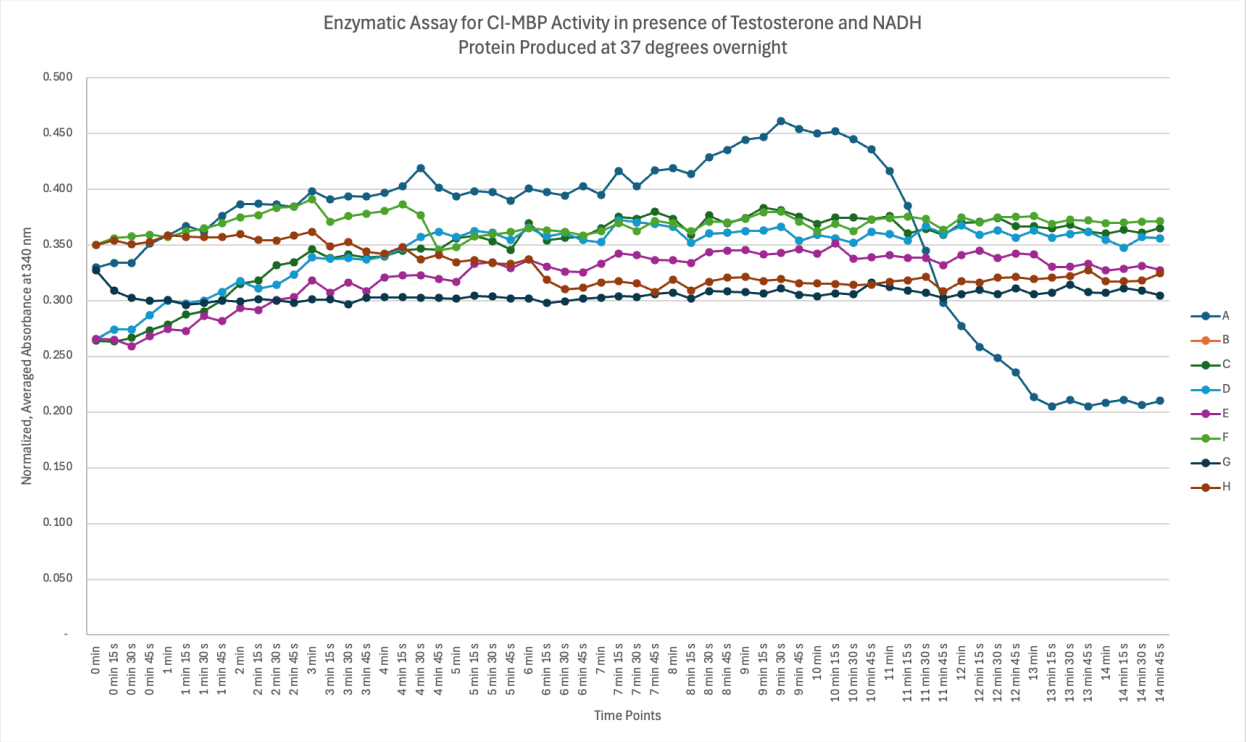
Condition	Protein Vol. / Well (µL)	Protein Vol. for MM (µL)	Buffer Vol. / Well (µL)	Buffer Vol. for MM (µL)	Total MM Vol. (µL)
A	0	-		-	-
B	0.5	2	19.5	78	80
C	1	4	19	76	80
D	2.5	10	17.5	70	80
E	5	20	15	60	80
F	10	40	10	40	80
G	15	60	5	20	80

H	20	-	0	-	-

Load 170µL of testosterone mastermix into each designated well. Add 20µL of the respective sample mastermix. Rapidly add 10µL of NADH using a multichannel pipette to initiate the enzymatic reaction across all wells simultaneously.

The absorbance was measured at a wavelength of 350 nm for 15 minutes (shifted +10 nm from the standard of 340 nm NADH peak to compensate for plate material absorbance). Additionally, a measurement at the wavelength of 600 nm was done as well as negative baseline control; no absorbance expected.





Assays observations: the curves do not provide defensible evidence of CI2350 activity, although they also do not prove that the enzyme is inactive as the assay itself is currently dominated by artifacts. It is expected that NADH oxidation causes an immediate decrease in absorbance, with a steeper decrease at higher enzyme concentration. Instead, most curves initially increase and there is no enzyme-dose response.

- Conditions A do not contain proteins and yet it shows the largest abrupt decrease in absorbance, this could be due to the natural degradation of NADH but the results are not consistent amongst the samples.
- Several samples show sudden jumps or drops and this could be related to bubbles or steroid crystals moving.
- Testosterone solution has precipitated and it was possible to observe white clamps in the wells

The multiwell plate was kept shaking without the lid -> the enzyme might also work in low oxygen/anaerobic conditions

*Note: By mistake instead of NADH, NAD+ was used in this assay which explains the lack of data.

AKR1D1_SUMO plasmid extraction

Performed plasmid miniprep using the E.Z.N.A. Plasmid Miniprep Kit Protocol, followed by NanoDrop analysis. NanoDrop analysis showed that all plasmid preparations had acceptable

DNA purity, with A260/A280 ratios ranging from 1.85 to 1.92. Sample 2 showed a different NanoDrop result from the other samples and was therefore kept separately as a backup. Since samples 1, 3, and 4 showed comparable DNA concentration and purity, they were combined to obtain a larger DNA stock for downstream experiments. The pooled sample was re-measured by NanoDrop, confirming acceptable DNA concentration and purity.

Sample	**Concentration (ng/µL)**	**Abs 260/280**	**Abs 260/230**
AKR1D1-1	92.9	1.851	2.149
AKR1D1-2	47.7	1.920	2.123
AKR1D1-3	107	1.862	2.160
AKR1D1-4	70.6	1.868	2.000
AKR1D1-(1+3+4)	95.9	1.874	1.856

Stop overnight culture of *E. coli* BL21 AKR1D1_His + SDS PAGE

SDS samples were prepared and measured for the overnight cultures before and after induction of IPTG.
The samples were prepared for a final concentration of OD=10. A master mix of the loading dye and beta mercaptoethanol was made with a total volume of 900 uL
(855uL + 45 uL respectively)

Sample	Concentration	MQ Water	Loading dye
AKR1D1 Bi - 1	1.34	67	67
AKR1D1 Bi - 2	1.80	90	90
AKR1D1 Bi - 3	1.55	77.5	77.5
AKR1D1 Bi - 4	1.66	83	83
AKR1D1 Ai - 1	9.41	117.6	117.6
AKR1D1 Ai - 2	10.02	125.2	125.2
AKR1D1 Ai - 3	12.29	153.6	153.6
AKR1D1 Ai - 4	9.78	122.2	122.2

The samples that were taken from the overnight cultures were resuspended in water and boiled

at 95 C for 10 minutes to increase the lysis of the cells.

Loading order for the gel :
A total volume of 10uL of the sample was loaded in the well

Well no.	Sample
1	Ladder (5uL)
2	AKR1D1 Bi - 1
3	AKR1D1 Ai - 1
4	AKR1D1 Bi - 2
5	AKR1D1 Ai - 2
6	AKR1D1 Bi - 3
7	AKR1D1 Ai - 3
8	AKR1D1 Bi - 4
9	AKR1D1 Ai - 4

The gel ran for a total of 90 minutes at 200 V

Insert the gel picture, alongside any discussion needed

Chose mutants, inoculate them, set up overnight reaction for mutagenesis

Transform atcABC + moaABCDE; AKR1D1-SUMO into BL21(DE3)

The plasmids atcABC, moaABCDE, and AKRD1-SUMO were transformed into *E. coli BL21*.

Because the initial double transformation was unsuccessful, several alternative strategies were evaluated:

- **Sequential transformation:** First transform BL21(DE3) with either atcABC or moaABCDE, prepare competent cells from successful transformants, and then introduce the second plasmid.
- **Reduced antibiotic selection:** Repeat the double transformation while lowering the spectinomycin concentration to 50 µg/mL.

- **Increased plasmid DNA input:** Repeat the double transformation using a higher amount of plasmid DNA

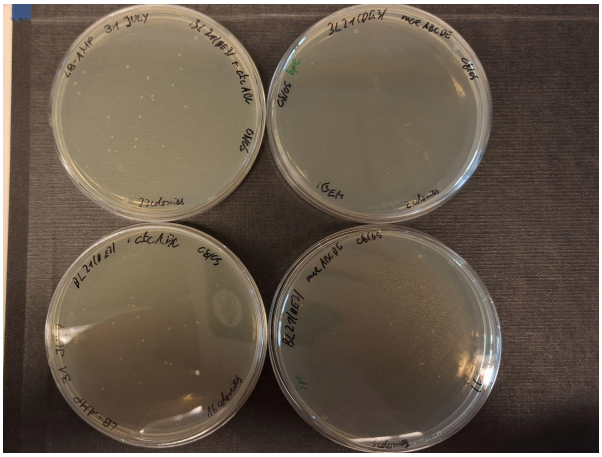
The paper "Optimization of Dual Plasmid Transformation Conditions for Large Plasmids in Escherichia coli" by Xin Qiu *et al*. recommends using approximately 300 ng of plasmid DNA per 100 µL of competent cells and a 60 s heat shock at 42°C for dual-plasmid transformations. Since only a limited amount of plasmid DNA was available, a lower DNA input was used in this experiment (5 µL total plasmid solution, ~200 ng DNA).

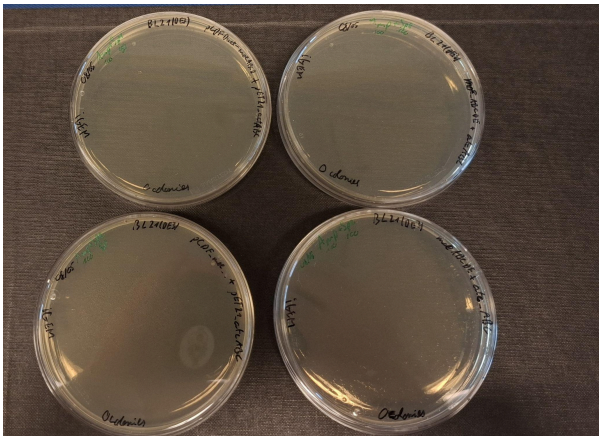
The transformation procedure was as follows:

Aliquots of ~200 µL of competent BL21(DE3) cells were mixed with one of the following:

- 1 µL AKR1D1-SUMO (95.9 ng)
- 1 µL atcABC (~38 ng)
- 1 µL moaABCDE (~40 ng)
- 5 µL atcABC + 5 µL moaABCDE (dual transformation, ~200 ng DNA each)

After DNA addition, the cells were incubated on ice for 30 min. Heat shock was performed at 42°C for 45 s for the single-plasmid transformations and for 60 s for the dual-plasmid transformation.
Plating done same as previously.

	BL21(DE3) transformants: - With only pET21-atcABC 1st plate: 23 colonies 2nd plate: 16 colonies - With only pCDFDuet-1-moaABCDE 1st plate: 2 colonies 2nd plate: 3 colonies

	BL21(DE3) double transformants: - Transformation with both atcABC and moaABCDE - 0 colonies in each

The transformation efficiency of pET21-atcABC is much higher than of pCDFDuet-1 moaABCDE, as confirmed by both today's results, as well as the transformation into TG1 results (on

Inoculate TG1 with atcABC, moaABCDE, pET_AKR_MBP #28

To get more plasmid DNA, 3 × 5 mL LB tubes with corresponding antibiotics (Spec, Amp) were inoculated with TG1-atcABC, TG1-moaABCD. TG1 with pET_AKR_MBP#28 was inoculated into only one tube, for 7 tubes total.
Incubated at 37℃, 150 RPM, overnight.

Day 37: [August 6, 2026], Thursday

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

Daily overview of experiments

Insert day description

Activity	***Protocol File***	***Comments***
Sonification of AKR_His	E. coli Lysis by Sonic…	30% Amp; 3s on/off cycles
Purification of AKR_His	Following cytiva's protocol	
Testosterone assay		
Plasmid extraction of pET21_atcABC, pCDDuet-1_moaABCDE, pET_Blank_AKR_MBP #28		
Prepare fresh competent E.coli BL21(DE3) cells with atcABC; moeABCDE for sequential transformation		All of them were used for transformations
Transformation of BL21(DE3) with atcABC; moaABCDE; atcABC+moaABCDE		Success, all had colonies.
Transformation with mutants		Success

Results and interpretation

Sonification of AKR1D1_His for Cell Lysis

Half of the samples from Wednesday's cultivation of AKR1D1_His (17°C overnight, 1 mM 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 4700 rcf for 10 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	3.010	37.5
S2	2.428	30.35

After resuspending in the binding buffer, sample S1 and S2 were pooled together.
Sonificator was set to an amplitude of 30%, 2 min on time with a 3 seconds on/off cycle for a total number of 7 cycles. The samples were kept on ice at all times.

After lysis samples were split into two 50mL flasks and put into the centrifuge for 1.5h (20.000 x g at 4°C).

Purification of AKR1D1_His via Column Binding

Columns were pre-washed with the binding buffer (500mM NaCl, 20 mM sodium phosphate, 30mM imidazole). The flowrate was set to 1.5 mL/min (should be higher than 1 and lower than 4). The flow through the tube is around ~3 minutes.

For column purification the following steps were made:

1. Flowthrough: put the start of the tube into your sample and wait 3 min 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 before moving the end of the tube from Flowthrough flask into Washing flask. Wait until the 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 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.

The elutant was concentrated via vivaspin. 3mL of the elutant was added onto the flask and put in the centrifuge for 30 min at 4000 x g. Then 5mL of cell wash buffer was added and the sample centrifuged again.

After the purification, the column was washed with the binding buffer (10 min), MQ (10 min) and then 20% EtOH (10 min). Then de-attach the column (make sure no air bubbles are inside) and empty the tube.

Testosterone Assay

Calculations for Testosterone and NADH were performed as if the stock solution concentration was 2mM, but it was 20mM -> this explains testosterone precipitation/crystallization inside the wells during the assay.
Assay Layout
Rows:
A - Potassium Phosphate buffer (100mM) + Absolut Ethanol
B - Potassium Phosphate buffer (100mM) + Absolut Ethanol + NADH
C - Potassium Phosphate buffer (100mM) + Testosterone (20 mM)
D - Potassium Phosphate buffer (100mM) + Testosterone (20 mM) + NADH
E - Potassium Phosphate buffer (100mM) + Testosterone (20 mM) + NADH + 20ul Protein 25 deg
F - Potassium Phosphate buffer (100mM) + Testosterone (20 mM) + NADH + 20ul Protein 37 deg

Column:7 to 9 – Not Sealed
10 to 12 – Sealed
Wells Compositions:

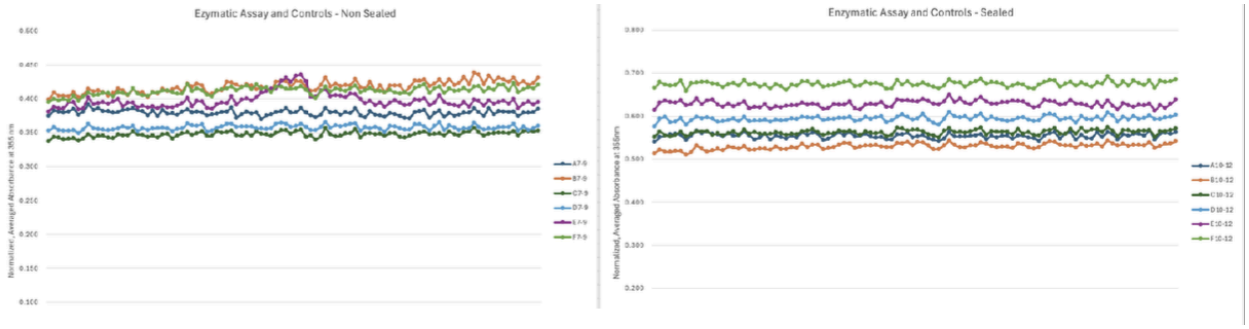
Component	w/out Testosterone	With testosterone
Potassium phosphate buffer	169,5 µL	169,5 µL
Absolute Ethanol	0,5 µL	—
Testosterone 2 mM in absolute ethanol	—	0,5 µL
Protein or phosphate buffer	20 µL	20 µL
NADH 2 mM or phosphate buffer	10 µL	10 µL
Total	**200 µL**	**200 µL**

20mM Testosterone was diluted to 2mM Testosterone and 0,5ul were added in the master mix. The master mix comprised potassium phosphate buffer and either 0,5ul testosterone or absolute ethanol, each well contained further 20ul of either protein or potassium phosphate buffer and 10ul of either NADH or potassium phosphate buffer. Final Volume was 200ul.

Final Testosterone concentration was 5uM (0.25% v/v)

Final NADH concentration was 100uM

Data Analysis
Graphs below represent Data points from Time 0 to Time 24 mins 45 sec



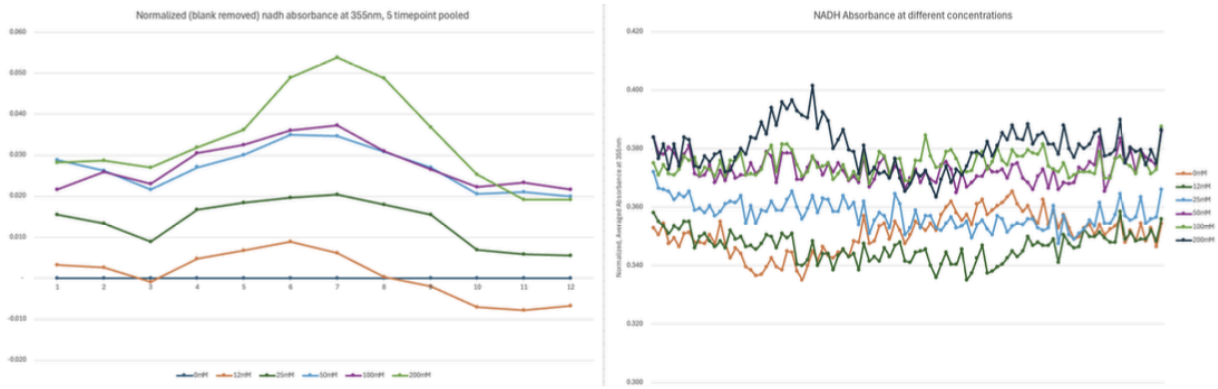
Based on the results of the day before we introduced controls without protein, testosterone or cofactor and started monitoring absorbance at both 355 and 600 nm.
Observations
Critical Points/observation while performing the assay:

- NADH from previous day
- Buffer was kept warm around 37 degrees before putting testosterone in it (to decrease possibility of precipitation)
- Plate wells didn't present bubbles or crystals at the moment of the read
- We run a first 25 min assay and then a 1h assay
- Delay in loading NADH and start the read
- Plate covered with the lead
- NADH looks stables in controls and samples
- Testosterone do not create artifacts anymore
- Sealed Samples are stable over time but create an offset

EXPERIMENT #2 – NADH Standard Curve
Because only a small difference was observed between the NADH-containing and NADH-free controls, an additional diagnostic experiment was performed to evaluate the absorbance response
of NADH. NADH standards ranging from 0 to 200 µM were prepared in potassium phosphate buffer in a final volume of 200 µL and analyzed in duplicate.
Assay Layout
Samples were run in duplicates according to the following scheme, final volume of 200uL:
7 – 200ul buffer, 0ul NADH (0 mM)
8 – 198.75ul buffer, 1.25ul NADH (12.5 mM)
9 – 197.5ul buffer, 2.5ul NADH (25 mM)
10 – 195ul buffer, 5ul NADH (50 mM)
11 – 190ul buffer, 10ul NADH (100 mM)
12 – 180ul buffer, 20ul NADH (200 mM)

Data Analysis
The graphs represent the results. According to this, NADH solution calculations were double

checked. Graphs below represent Data points from Time 0 to Time 24 mins 45 sec



The absorbance traces remained somehow stable over time, as expected in the absence of an NADH-consuming enzyme. The mean absorbance generally increased with NADH concentration, particularly between 25 and 200 µM. However, the response was weak and not clearly linear, while the 0 and 12.5 µM conditions were not reliably distinguishable. Therefore, the experiment indicated that NADH was detectable but that the sensitivity and linearity of the measurement were insufficiently robust under the tested conditions.

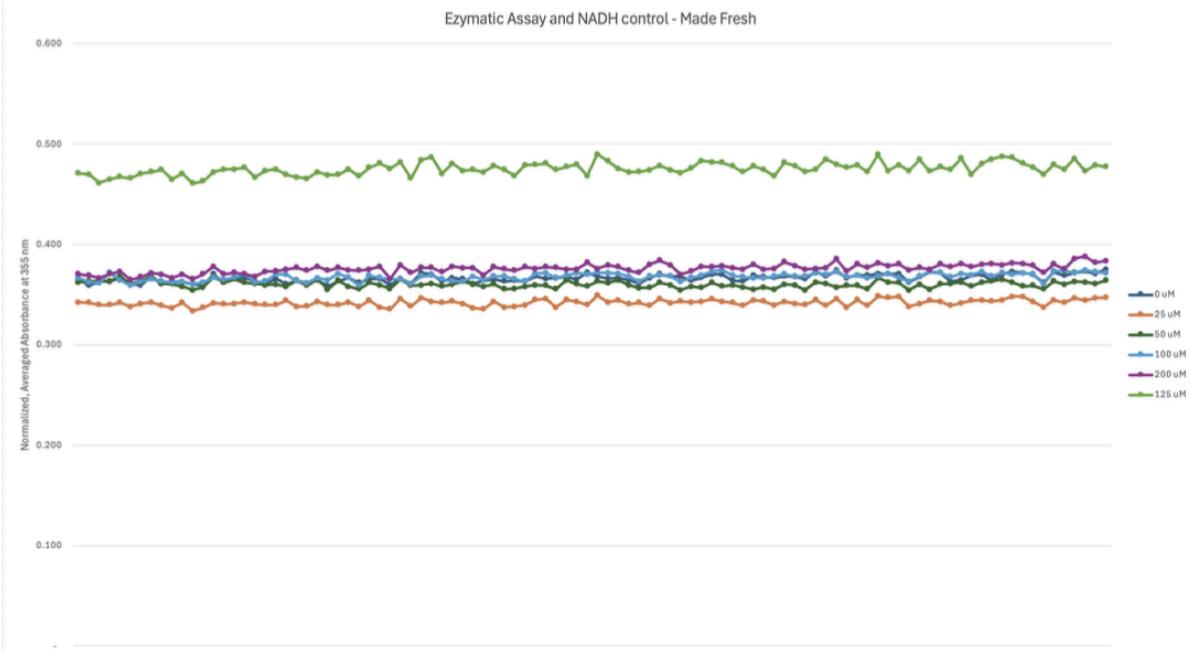
Observations
Samples were rerun at absorbance 340 and 360, not generating useful data and confirming the non-work of NADH, data are not reported.
The calibration will be repeated using fresh NADH and measurements at 355 and 360 nm.
NADH was conserved in 6,5 pH potassium phosphate “Nicotinamide adenine dinucleotide (NADH) is less stable in mildly acidic solutions like pH 6.4 than at alkaline pH. Acidic buffers promote faster degradation, loss of coenzyme activity, and structural modifications such as hydration or cleavage of the nicotinamide ring. Neutral to mildly basic buffers (pH 7.5–8.5) are typically recommended for long-term storage and assays”

EXPERIMENT #2 – NADH Standard Curve, new NADH Stock

We rerun an assay for a calibration curve to check NADH, the NADH solution was made new and used right away. Data is shown below

Assay Layout
Samples were run in duplicates according to the following scheme, final volume of 200uL:
A – 200ul buffer, 0ul NADH (0 uM)
B – 197.5ul buffer, 2.5ul NADH (25 uM)
C – 195ul buffer, 5ul NADH (50 uM)
D – 190ul buffer, 10ul NADH (100 uM)
E – 180ul buffer, 20ul NADH (200 uM)
F – 150ul buffer, 10ul NADH (125 uM)

Data Analysis
Graphs below represent Data points from Time 0 to Time 24 mins 45 sec



NADH appears to remain stable over time, data acquired for 125uM is anomalous as the absorbance is higher than the whole group. Variability intra well remain higher than variability between conditions, standard curve is not possible to build due to the low-quality data

Plasmid extraction of pET21_atcABC, pCDDuet-1_moaABCDE, pET_Blank_AKR_MBP #28

Plasmid extraction was performed, from transformed overnight TG1 cultures, by using the E.Z.N.A. Plasmid Miniprep Kit Protocol followed by NanoDrop analysis.

Sample	Concentration (ng/µL)	Abs 260/280	Abs 260/230
pET21_atcABC #1	123	1.857	1.998
pET21_atcABC #2	110	1.881	2.265
pET21_atcABC #3	130	1.878	2.278
pET21_atcABC (Pooled)	123	1.871	2.205

pCDDuet-1_moaABCDE #1	204	1.851	1.948
pCDDuet-1_moaABCDE #2	213	1.853	2.140
pCDDuet-1_moaABCDE #3	181	1.862	2.212
pCDDuet-1_moaABCDE (pooled_	195	1.867	2.266
pET_Blank_AKR_MBP #28	99.4	1.852	2.198

After confirming the concentrations and purities, separate atcABC and moaABC samples were pooled and measured again. Stored in the yellow box with the other genes.

Prepare competent *E.coli* BL21(DE3) cells for sequential transformation

The transformed *E.coli* BL21(DE3) with the single plasmid (atcABC; moaABCDE), as well as regular wild type *E.coli* BL21(DE3) were made competent for chemical transformation.
Fresh competents cells are usually more efficient than after being frozen, so additional double transformation will be tried also, to see if that'd work.

Inoculated:

- 2 × 5 mL LB+Amp with BL21(DE3)-atcABC
- 2 × 5 mL LB+Spectinomycin with BL21(DE3)-moaABCDE
- 2 × 5 mL LB with BL21(DE3)

And incubated them at 37℃, 160 RPM.
Measured OD_{600} using spectrophotometer.

Sample	**OD_{600}**	**Time when taken**
BL21(DE3)-atcABC #1	0.464	2 h 30 min
BL21(DE3)-atcABC #2	0.439	2 h 30 min
BL21(DE3)-moaABCDE #1	0.166 | 0.310	2h 50 min | 3 h 10 min
BL21(DE3)-moaABCDE #2	0.18 | 0.27	3 h 10 min | 3 h

		20 min
BL21(DE3) #1	0.356	3 h 10 min
BL21(DE3) #2	0.319	3h 10 min

Samples were immediately placed on ice, for at least 10 min, then transferred to pre-chilled 15 mL Falcon tubes, and centrifuged at 4000 g, 5 min, 4C (worked in the cold room/centrifuge room).
Supernatant was discarded, then cells were resuspended in 2 mL ice-cold $CaCl_2$. Cells were incubated on ice for 30 min, then centrifuged again at 4000 g, 5 min, 4C.
Supernatant was discarded, then cells were resuspended in 200 µL ice cold $CaCl_2$, which were then aliquoted into 2 × 100 µL. Since this was done for each culture, this resulted in 12×100 µL of different competent cells.

Sequential transformation of BL21(DE3) with atcABC; moaABCDE; double transformation of fresh competent BL21(DE3) with atcABC + moaABCDE

Sequential transformation of moaABCDE; atcABC into the fresh competent cells of BL21(DE3)-atcABC; BL21(DE3)-moaABCDE was performed.

Sequential transformation:
Add 0.75 µL pCDFDuet-moaABCDE (143 ng) to BL21(DE)_atcABC competent cells
Add 1 µL of pET21_atcABC (123 ng) to BL21(DE3)_moaABCDE
Double transformation Add 1 µL of pET21_atcABC + 1 µL pCDFDuet to BL21(DE3) competent cells.
Incubate 30 min, heat shock 45-50 s, 5 min on ice, add 500 uL LB, recovery 60 min. Spin down, discard 450 uL, plate 50 uL, on LBA plates with Amp+spec.

Transformation of *E. coli* TG1 with ci2350 mutants

Insert experiment discussion and interpretation

Day 38: [August 7, 2026], Friday

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

Daily overview of experiments

We initiated four AKR1D1-SUMO production cultures and induced them with 1 mM IPTG for overnight incubation at 17°C. Double-mutant candidates were inoculated for plasmid extraction, and AKR1D1-Trx was inoculated for subsequent production. AKR1D1-His purification fractions were analysed by SDS-PAGE. Additional enzyme-assay troubleshooting is listed.

Activity	***Protocol File***	***Comments***
AKR1D1-Sumo Production		
Inoculation of double mutants strains for sequencing		
Inoculation of AKR1D1-TRX for production		
Enzyme Activity Assay and Troubleshooting		
SDS page of AKR1D1-His		

Results and interpretation

AKR1D1_Sumo production

AKR1D1_Sumo was produced in 4 cultures starting from pervious day preculture. Inoculum had OD of 2.31 and was adjusted to have OD of 0.015 in culture of 200 ml. At induction the OD for the cultures was the following:

Culture A	1.32
Culture B	1.41
Culture C	1.53
Culture D	1.9

After induction with 1 mM IPTG the cultures were kept growing overnight at 17 °C and stopped saturday morning

Inoculation of double mutants strains for sequencing

Double mutants were inoculated and kept at room temperature over the weekend for plasmid extraction the following week.

Inoculation of AKR1D1-TRX for production

AKR1D1-TRX was inoculated and kept at room temperature over the weekend for production the following week

Enzyme Activity Assay and Troubleshooting

EXPERIMENT #1 – NADPH Standard Curv NADPH checked for linearity, the package was open on this day Looks ok

EXPERIMENT #2 - AKR1D1 + His Activity Assessment

Problems

- Samples splashed over the lid so its very likely everything is cross contaminated abs values read are not reliable
- Nadph should have been 20ul, I loaded 10ul started the read, stopped and added the extra 10ul and started the read again
- Protein was stored at Cell wash buffer (sodium phosphate, nacl, ph7.4)
- SDS confirmed presence of purified + vivaspin (cut off 30k) akrhis tag (i suspect is very low concentration), eluted samples have way more protein at the expected band

SDS-PAGE Analysis of AKR1D1-His Purification Fractions

To monitor the purification profile of AKR1D1-His, SDS-PAGE analysis was performed on fractions collected throughout the process. A total of nine samples were evaluated: cell wash supernatant, total lysate (post-sonication, pre-centrifugation), insoluble pellet, clarified

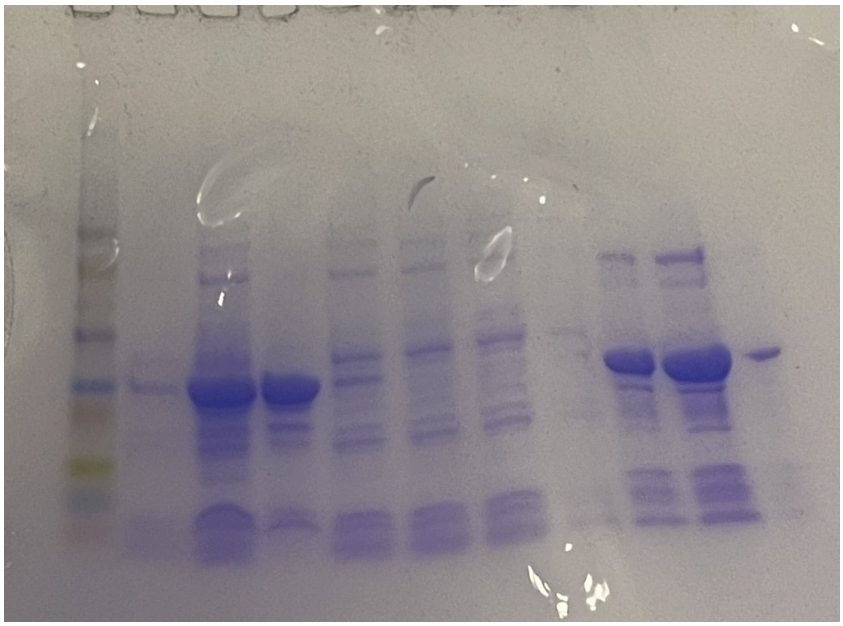
supernatant (soluble fraction), flowthrough (divided across two fractions), column wash, pre-concentrated eluate, and the final concentrated eluate post-Vivaspin processing.

To isolate the insoluble fraction, the total cell lysate was centrifuged at maximum speed; the resulting supernatant was collected as the soluble fraction, and the remaining pellet was resuspended in 15 µL of 1% SDS buffer.

For sample preparation, 30 µL mixtures were generated using 15 µL of each fraction and 15 µL of 2x SDS loading buffer. The loading buffer was freshly supplemented with 10% beta-mercaptoethanol. The mixtures were heated at 95°C for 10 minutes to denature the proteins. Following heating, the samples were centrifuged at 10,000 x g for 5 minutes to collect condensation.

Note: This centrifugation step should not exceed 30 seconds in the future since it could cause sedimentation of the insoluble pellet fraction.

Each well was loaded with 10 µL of the sample mixture, and 5 µL of a full-range rainbow molecular weight marker (Cytiva) was used as a reference. Electrophoresis was initiated at 80V for 15 minutes for sample stacking, after which the voltage was increased to 160V for 1 hour. Following the run, the gel was rinsed in Milli-Q water, heated in a microwave for 30 seconds, and stained with Coomassie blue for 1 hour on an orbital shaker (100 rpm). Destaining was performed in three consecutive steps lasting 10 minutes, 30 minutes, and 1 hour, respectively, prior to gel imaging.



Order: Lane 1: Cytiva Rainbow Ladder; Lane 2: Cell wash supernatant; Lane 3: Total cell lysate; Lane 4: Resuspended insoluble pellet; Lane 5: Clarified supernatant; Lane 6: Flowthrough fraction 1; Lane 7: Flowthrough fraction 2; Lane 8: Column wash; Lane 9: Pre-concentrated eluate; Lane 10: Concentrated post-Vivaspin eluate. Lane 11: Overflow from lane 10.