# Week 35 - Overview

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**Weekly summary:**

During Week 35, we focused on comparing AKR1D1 expression with different solubility tags (His, SUMO and TrxA), preparing and expressing ci2350-MBP double mutants, and continuing optimization of testosterone reduction assays. Additional protein purification experiments were performed, including the preparation of empty-vector controls.

**Main results of the week:**

CI2350-MBP double mutant D after sequencing analysis was selected as the most promising candidate for further characterization. The construct was successfully cultivated at 17°C and the cell pellets were collected for subsequent purification.

AKR1D1-His, AKR1D1-SUMO and AKR1D1-TrxA were expressed under comparable conditions at 37°C, with induced and uninduced controls, to evaluate the influence of different solubility tags. Samples were collected for SDS-PAGE and Bradford analysis. AKR1D1-His and atcABC were also subjected to purification.

Further testosterone reduction assays were performed using AKR1D1-SUMO, AKR1D1-TrxA and ci2350-MBP. NADPH concentrations were adjusted to improve absorbance detection, although enzymatic activity remained inconclusive and further assay optimization was required.

# Day 44: [08/26, 2026], Wednesday

**Weekly responsible:** Giulia
**Lab participants:** Ruiqi, Hamsi, Shun

## Daily overview of experiments

Transformed four ci2350-MBP double-mutant candidates into *E. coli* BL21(DE3). Testosterone reduction assays were performed using AKR1D1-SUMO and ci2350-MBP, with increased NADPH concentrations to improve absorbance detection..

| ***Activity*** | ***Protocol File*** | ***Comments*** |
|---|---|---|
| Testosterone assays | | |
| Preparing buffers for sonication and SDS analysis (if there is no more buffer) | | Done on thursday |
| Transformation of mutants into E. coli BL21? | | Could be useful if you have time, so we can just take those which come off good from sequencing, so we don't waste a day waiting for it.<br><br>When sequencing comes, we can inoculate the positive mutants on Thursday for production on Friday. |
| Inoculation of all AKR1D1 constructs for tomorrow's production | | |

# Results and interpretation

## Testosterone assays

Assays were set up for AKR1D1-SUMO and ci-MBP, for measurement in Nanospectre.
The amount of NADPH added was increased compared to the previous assays performed by Eleonora due to absence of the correct peak at lower concentration. A first test was a duplicate of negative (all reagents besides Enzyme), followed by a triplicate of AKR1D1-SUMO enzyme.

The final setup was the following:

| | | |
|---|---|---|
| NADPH (5 mM) | 50 uL | 5 mM |
| Testosterone | 0.25 uL | 40 mM |
| Enzyme (eluted and dissolved in potassium phosphate buffer) | 50 uL (0 in negative control) | NOT known bc no clear quantification |
| Buffer (potassium phosphate 6.5 pH) | Added to 1 mL total | |

## Transformation of double mutants

Four samples of petBlank double ci-MBP mutants were transformed in E.coli BL21 preemptively while waiting for sequencing results.
Samples from plasmid miniprep of E.coli TG1 performed by Camille on 25/08/26 were used and diluted accordingly to reach the final amount of 10-50 ng per transformation reaction.

| Sample | Original concentration | Dilution | Final concentration |
|---|---|---|---|
| D.M. C (double mutant C) | 116 ng/uL | 3x | 38.7 ng/uL |
| D.M. D | 95 ng/uL | 2x | 47.5 ng/uL |
| D.M. F | 116 ng/uL | 3x | 58 ng/uL |
| D.M. G | 137 ng/uL | 3x | 45.7 ng/uL |

# Day 45: [08/27, 2026], Thursday

**Weekly responsible:** Giulia
**Lab participants:** Shun, Hema, Ruiqi

## Daily overview of experiments

Cultivated AKR1D1-SUMO and empty pET11a controls under different induction conditions for subsequent solubility analysis. CIi2350-MBP double mutant D was selected (after sequencing analysis) and inoculated for protein expression. Additional testosterone assays were performed, and purification buffers were prepared.

| ***Activity*** | ***Protocol File*** | ***Comments*** |
|---|---|---|
| Inoculation of good mutants for fridays protein expression | | *double mutations |
| Production of all AKR1D1 constructs (+ controls) | | *at 37C, to assess the efficiency of different tags |
| Buffer preparation for protein purification | | |
| Testosterone assay | | for ciMBP, AKR1D1-TRX and negative control |

## Results and interpretation

### Akr1d1-SUMO and pet11a cultivation

Cultivation was carried out for AKR1D1-SUMO and empty pet11a in E.coli Bl21. Cultures of 50 mL were carried out in triplicate for akr1d1 sumo at 37°C degrees for three hours after induction with 1 mM IPTG. An uninduced control was also included with the same conditions besides IPTG. samples were taken for SDS and Bradford assay and pellet stored at -80°C. The goal of

this culture is the comparison of the three tags for solubility evaluation. Empty Pet11a was also cultured in duplicate at 17°C overnight + uninduced sample. The scope is purification + assay testing as a negative control. One culture was also carried out at 37°C for 3 hours to have a baseline for the solubility evaluation of akr1d1 with different tags.

See excel file for details on culture.

## Buffer Preparation for Protein Production

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
    - For 250 mL: 41.7 mL of 3M NaCl + 5 mL of 1M sodium phosphate
- Wash buffer 2 (washing the columns): same as binding buffer
- Binding buffer: 500mM NaCl, 20mM sodium phosphate, 30mM imidazole
    - For 500 mL: 83 mL of 3M NaCl + 10mL of 1M sodium phosphate + 15mL of 1M Imidazole
- Elution Buffer: 500mM NaCl, 20mM sodium phosphate, 500mM imidazole
    - For 250 mL: 42 mL of 3M NaCl + 5mL of 1M sodium phosphate + 125mL of 1M Imidazole

500 mL of binding buffer and 250 mL of wash buffer was prepared.

## Inoculation of double mutants for production

After checking the sequencing results (see dedicated tab), ci-MBP double mutant (D) was deemed the best candidate and inoculated for production on Friday and glycerol stock preparation.

## Testosterone assay for ciMBP, AKR1D1-TRX and negative control

The testosterone assay was carried out for ciMBP with the same conditions as the day before. An increase in absorbance is visible compared to the previous day with the same conditions. A negative control with everything besides enzyme was also carried out (Wavescan only, no kinetics), to have a baseline of the absorbance at lower NADPH concentration (20 uL instead of 50 uL).

## Prep for Bradford and SDS page of cultivations, retrieval of cultivations after 3 hours.

1 mL samples were taken from both cultures at time of induction and at the end of culture (3 hours). The OD of the cultures are listed in the table below. For SDS analysis, a smaller volume was aliquoted from the 1 mL sample, centrifuged and supernatant was removed. The pellet was then resuspended with MQ water to an OD of 20. When SDS is performed, an equal amount of SDS loading dye (with added betamercaptoethanol as 5% of the total) should be added, so that the sample reaches an OD of 10 and subsequently boiled and used for the assay. All the samples for the assay are in the pink rack in the freezer and the volume is also written on the side. For the overnight samples, see next day table.

| AT TIME OF INDUCTION | OD | Volume of the culture used | Water added after centrifugation |
|---|---|---|---|
| Pet11a sample 1 | 1.26 | 200 uL | 12.6 uL |
| Pet11a sample 2 | 1.49 | 200 uL | 14.9 uL |
| Pet11sample 3 | 1.61 | 200 uL | 16.1 uL |
| Pet11a no induction | 1.42 | 200 uL | 14.2 uL |
| AKR1D1-sumo 1 | 1.21 | 200 uL | 12.1 uL |
| AKR1D1-sumo 2 | 1.17 | 200 uL | 11.7 uL |
| AKR1D1-sumo 3 | 1.13 | 200 uL | 11.3 uL |
| AKR1D1-sumo no induction | 1.52 | 200 uL | 15.2 uL |
| END OF CULTURE | | | |
| Pet11a induced 3h culture | 3.44 | 100 uL | 17.2 uL |
| AKR1D1-sumo 1 | 5.88 | 100 uL | 29.4 uL |
| AKR1D1-sumo 2 | 4.74 | 100 uL | 23.7 uL |
| AKR1D1-sumo 3 | 5.26 | 100 uL | 26.3 uL |
| AKR1D1-sumo no induction | 8.42 | 50 uL | 13.15 uL |

The cultures were stopped after 3 hours, centrifuged at room temperature for 5 minutes and stored at -80°C after weighing the pellet.

| Sample | Pellet Weight (g) |
|---|---|
| pET-11a | 0.542 |
| AKR1D1-sumo 1 | 0.444 |
| AKR1D1-sumo 2 | 0.442 |
| AKR1D1-sumo 3 | 0.479 |
| AKR1D1-sumo no induction | 0.858 |

# Day 46: [08/28, 2026], Friday

**Weekly responsible:** Giulia
**Lab participants:** Hamsi, Hema, Patrik, Ruiqi

## Daily overview of experiments

Protein expression of AKR1D1-His and AKR1D1-Trx at 37C to determine solubility tag efficiency. Expression of ci2350-MBP for purification and assay purposes. Sonication and purification of empty pET-11a, atcABC, and AKR1D1-His.

| ***Activity*** | ***Protocol File*** | ***Comments*** |
|---|---|---|
| Protein expression of all good mutants + AKR1D1-his and AKR1D1-trx | | |
| Prep for Bradford and SDS page of cultivations, retrieval of cultivations after 3 hours. | | |
| Cell sonication of pET-11a empty vector cultures | | From previous day culturing |
| Sonication and purification of atcABC and AKR1D1-his | | Some data needs to be filled in. |

## Results and interpretation

### BL21 DE3 cultivation of AKR1D1-trx and AKR1D1-His + double mutant of ci2350-MBP (D)

Cultivation was carried out for AKR1D1-trx and AKR1D1-His. Cultures of 50 mL were carried out in triplicate at 37°C degrees for three hours after induction with 1 mM IPTG. An uninduced control was also included with the same conditions besides IPTG. samples were taken for SDS and Bradford assay and pellet stored at -80°C. The goal of this culture is the comparison of the three tags for solubility evaluation, together with AKR1D1-sumo from Thursday cultivation.

ci-MBP was also cultivated overnight at 17°C, with 0.8 mM IPTG induction, for further testing of the mutants, in 75 mL cultures.

For details on the cultures, see excel file in protocols folder.

## Prep for Bradford and SDS page of cultivations, retrieval of cultivations after 3 hours.

1 mL samples were taken from both cultures at time of induction and at the end of culture (3 hours). The OD of the cultures are listed in the table below. For SDS analysis, a smaller volume was aliquoted from the 1 mL sample, centrifuged and supernatant was removed. The pellet was then resuspended with MQ water to an OD of 20. When SDS is performed, an equal amount of SDS loading dye (with added betamercaptoethanol as 5% of the total) should be added, so that the sample reaches an OD of 10 and subsequently boiled and used for the assay. All the samples for the assay are in the pink rack in the freezer and the volume is also written on the side.

| AT TIME OF INDUCTION | OD | Volume of the culture used | Water added after centrifugation |
|---|---|---|---|
| AKR1D1-His 1 | 1.47 | 200 uL | 14.8 uL |
| AKR1D1-His 2 | 1.48 | 200 uL | 14.8 uL |
| AKR1D1-His 3 | 1.41 | 200 uL | 14.2 uL |
| AKR1D1-His no induction | 0.96 | 200 uL | 9.6 uL |
| AKR1D1-TRX 1 | 2.27 | 200 uL | 22.8 uL |
| AKR1D1-trx 2 | 1.57 | 200 uL | 15.8 uL |
| AKR1D1-trx 3 | 1.28 | 200 uL | 12.8 uL |
| AKR1D1-trx no induction | 1.69 | 200 uL | 16.9 uL |
| ci-MBP (double mutant) S1 | 1.47 | 200 uL | 14.8 uL |
| ci-MBP (double mutant) S2 | 1.97 | 200 uL | 19.8 uL |

| END OF CULTURE | | | |
|---|---|---|---|
| AKR1D1-his 1 | 4.45 | 100 uL | 22.26 uL |
| AKR1D1-his 2 | 4.78 | 50 uL | 11.96 uL |
| AKR1D1-his 3 | 3.85 | 100 uL | 19.26 uL |
| AKR1D1-his no induction | 6.11 | 50 uL | 15.28 |
| AKR1D1-trx 1 | 7.5 | 50 uL | 18.76 uL |
| AKR1D1-trx 2 | 5.19 | 50 uL | 12.98 uL |
| AKR1D1-trx 3 | 5.19 | 50 uL | 12.98 uL |
| AKR1D1-trx no induction | 8.22 | 50 uL | 20.56 uL |
| ci-MBP (double mutant) overnight 17°C, S1 | 9.86 | 30 uL | 14.8 uL |
| ci-MBP (double mutant) overnight 17°C, S2 | 9.64 | 30 uL | 14.46 uL |

## Retrieval of overnight cultures of pet11a (2 cultures + 1 uninduced control)

After finishing the overnight incubation at 17C, flasks were transferred to 50mL Falcon tubes, then centrifuged at 5000g at 4ºC for 10 minutes. Additional Falcon tubes filled with water were made as counterbalances to the pET-11a falcon tubes. After centrifugation, supernatant was discarded, the pellets weighed and tubes were put into the -80 freezer.

| Sample | Pellet Weight (g) |
|---|---|
| pET-11a - 1 | 0.934 |
| pET-11a - 2 | 1.019 |
| pET-11a - uninduced | 1.507 |

1 mL samples were taken for further SDS and Bradford analysis. The OD of overnight culture are listed in the table below. For SDS analysis, a smaller volume was aliquoted from the 1 mL sample, centrifuged and supernatant was removed. The pellet was then resuspended with MQ water to an OD of 20. When SDS is performed, an equal amount of SDS loading dye (with added betamercaptoethanol as 5% of the total) should be added, so that the sample reaches an OD of 10 and subsequently boiled and used for the assay. All the samples for the assay are in the pink rack in the freezer and the volume is also written on the side.

| | OD | Volume of the culture used | Water added after centrifugation |
|---|---|---|---|
| Pet11a sample 1 | 6.14 | 50 uL | 15.35 uL |
| Pet11a sample 2 | 7.72 | 50 uL | 19.3 uL |
| Pet11a no induction | 17.12 | 25 uL | 21.4 uL |

## Sonication and purification of atcABC and AKR1D1-his

4 tubes of the samples from (when) cultivation of atcABC and 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 5000 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 | | |
| S2 | | |

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 atcABC and 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. The tubes used for vivaspin were pre-washed with approximately 5 ml of cell wash buffer. All 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.

# Day 47: [August 29th, 2026], Saturday

**Lab participants:** Giulia, Patrik

## Daily overview of experiments

Finishing overnight cultivation of ci2350-MBP double mutant. Samples were stored in the -80 freezer.

| ***Activity*** | ***Protocol File*** | ***Comments*** |
|---|---|---|
| Finishing overnight cultivation of ciMBP double mutant | | |

## Results and interpretation

### Finishing ciMBP double mutant cultivation

After taking the flasks out of the incubator, medium was transferred to Falcon tubes, then centrifuged at 5000g at 4ºC for 10 minutes. Each flask was divided into 2 Falcon tubes, due to the medium being more than 50 mL. Supernatant was discarded, cell pellet weight was measured. Cells were washed with a 10 mL cell washing buffer, then centrifuged again under the same conditions. Supernatant was discarded, and the pellets stored in the -80 freezer.

| Sample | Pellet Weight |
|---|---|
| 1a | 1.288 |
| 1b | 1.094 |
| 2a | 0.801 |
| 2b | 1.567 |

After finishing the incubation, aliquot was taken for SDS and OD measurements (which were diluted 20 times).

| Sample | OD (diluted) | OD (final) |
|---|---|---|
| 1 | 0.493 | 9.86 |
| 2 | 0.482 | 9.64 |