Week 36 - Overview

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Weekly summary:

Week 36 was dedicated to evaluating efficiency of solubility tags for AKR1D1 constructs cultivated at 37ºC with 3h induction. Expression cultures of AKR1D1-His, AKR1D1-SUMO and AKR1D1-TrxA from previous week were sonicated and analysed by SDS-PAGE. Additionally, ci2350-MBP and its double mutants (V263I and R215A) were purified, together with a control sample containing empty pET-11a, for use as a negative control in testosterone reduction assays.

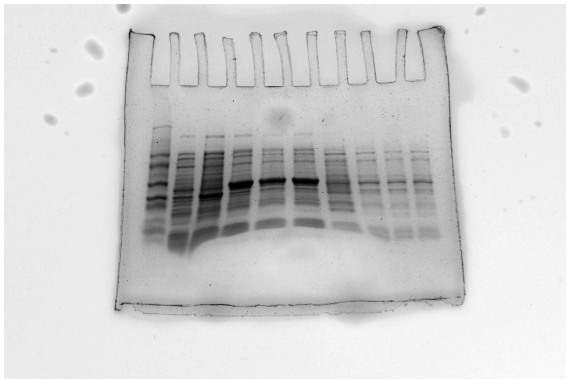
Main results of the week:

On the SDS-PAGE gels, the bands across the gel formed a ‘frowning’ pattern, the middle lanes migrated less than outer lanes. The current during the run was also very low (~7mA). After troubleshooting with our PI Johan, we linked this to an incorrectly prepared SDS running buffer. The stock was assumed to be 50X and was diluted 1:50 prior to running the gel. It was in fact a 10X stock, so the working buffer was too dilute. The resulting low ion concentration gave the buffer low conductivity, explaining the low current and the poor run. When the buffer was instead diluted 1:5, the gel ran better and the current was higher (~50 mA). Aside from this, the bands of our overexpressed proteins were clearly visible, mostly in the whole-cell lysate fractions, and less in the soluble fractions.

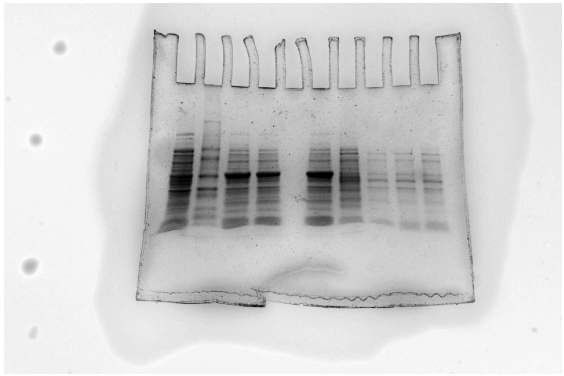
Insert the table of the well in the figure description.

Well	Gel 1 (AKR-Trx)	Gel 2 (AKR-SUMO)	Gel 3 (AKR-His)
1	PageRuler Broad Range Unstained	Total uninduced	Total uninduced
2	pET11 - total fraction	PageRuler Broad Range Unstained	Total induced 1

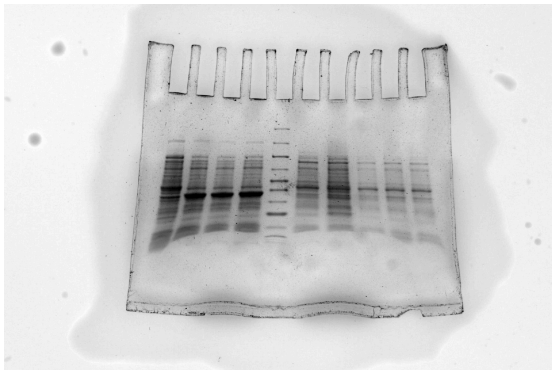
3	Total uninduced	Total induced 1	Total induced 2
4	Total induced 1	Total induced 2	Total induced 3
5	Total induced 2	Empty	PageRuler Broad Range Unstained
6	Total induced 3	Total induced 3	pET11 - soluble fraction
7	Supernatant uninduced	Supernatant uninduced	Supernatant uninduced
8	Supernatant induced 1	Supernatant induced 1	Supernatant induced 1
9	Supernatant induced 2	Supernatant induced 2	Supernatant induced 2
10	Supernatant induced 3	Supernatant induced 3	Supernatant induced 3



Gel 1 (AKR-Trx)



Gel 2 (AKR-SUMO)



Gel 3 (AKR-His)

Protein concentrations of each sample were measured by Bradford assay and multiplied by the target band's fraction of the total lane intensity on SDS-PAGE, allowing us to estimate the mass concentration of the expressed recombinant protein in the whole-cell and soluble fraction for each AK1D1 construct.

The Bradford assay results showed that protein concentration in the soluble fraction was almost equal to, or slightly higher than, that of the whole-cell fraction. This is not physically possible, as since the soluble fraction is a portion of the whole-cell fraction, an error must have occurred somewhere in the experiment. The most likely cause is incomplete resuspension of the insoluble material in the whole-cell samples before the Bradford assay, which would have led to underestimating the concentration of whole-cell protein. If the experiment were to be repeated, we would ensure complete solubilization of the insoluble fraction prior to quantification, for example boiling the sample in a buffer compatible with the Bradford assay, to obtain consistent and reproducible results.

TOTAL FRACTION

Sample	1	2	3	Mean	Standard Dev	CV	Prot. Conc (mg/mL)	Volume Ratio (%)	Concentration of recombinant protein (µg/mL)
AKR-His N	0.053	0.054	0.067	0.058	0.008	13.466	0.274	7.876	21.59
AKR-His 1	0.035	0.065	0.034	0.045	0.018	39.439	0.233	26.618	61.92
AKR-His 2	0.042	0.054	0.057	0.051	0.008	15.563	0.252	34.399	86.80
AKR-His 3	0.056	0.051	0.051	0.053	0.003	5.481	0.258	29.722	76.54
AKR-SUMO N	0.128	0.071	0.095	0.098	0.029	29.202	0.399	9.732	38.81
AKR-SUMO 1	0.079	0.051	0.041	0.057	0.020	34.557	0.271	30.427	82.47
AKR-SUMO 2	0.079	0.032	0.048	0.053	0.024	45.086	0.259	28.585	73.91
AKR-SUMO 3	0.083	0.048	0.034	0.055	0.025	45.889	0.265	31.486	83.37
AKR-Trx N	0.112	0.12	0.121	0.118	0.005	4.192	0.460	4.231	19.46
AKR-Trx 1	0.056	0.057	0.053	0.055	0.002	3.762	0.266	32.19	85.57
AKR-Trx 2	0.057	0.053	0.061	0.057	0.004	7.018	0.271	39.933	108.23
AKR-Trx 3	0.059	0.047	0.056	0.054	0.006	11.565	0.262	39.785	104.11
pet11a	0.097	0.105	0.109	0.104	0.006	5.894	0.416	0	0.00

Soluble FRACTION

Sample	1	2	3	Mean	Standard Dev	CV	Prot. Conc (mg/mL)	Volume Ratio (%)	Concentration of recombinant protein (µg/mL)
AKR-His N	0.082	0.081	0.088	0.084	0.004	4.525	0.354	12.423	43.99
AKR-His 1	0.02	0.017	0.021	0.019	0.002	10.767	0.154	14.304	21.98
AKR-His 2	0.036	0.022	0.033	0.030	0.007	24.300	0.188	13.236	24.88
AKR-His 3	0.025	0.021	0.021	0.022	0.002	10.341	0.163	9.485	15.46
AKR-SUMO N	0.123	0.106	0.115	0.115	0.009	7.417	0.451	6.534	29.45
AKR-SUMO 1	0.031	0.042	0.043	0.039	0.007	17.220	0.214	6.781	14.51
AKR-SUMO 2	0.035	0.041	0.039	0.038	0.003	7.970	0.213	11.568	24.63
AKR-SUMO 3	0.039	0.042	0.048	0.043	0.005	10.657	0.227	8.509	19.35
AKR-Trx N	0.089	0.105	0.075	0.090	0.015	16.741	0.373	13.988	52.15
AKR-Trx 1	0.043	0.044	0.035	0.041	0.005	12.130	0.220	22.241	48.96
AKR-Trx 2	0.031	0.049	0.026	0.035	0.012	34.236	0.204	21.609	43.98
AKR-Trx 3	0.058	0.031	0.033	0.041	0.015	36.994	0.220	24.77	54.53
pet11a	0.061	0.073	0.07	0.068	0.006	9.184	0.305	0	0.00

Comparing the proportion of target protein in the soluble fraction (based on SDS-PAGE band intensities), AKR1D1-SUMO had the lowest soluble fraction ratio and AKR1D1-TrxA the highest. Surprisingly, since SUMO tag is commonly used to enhance solubility, AKR1D1-SUMO appears to have lower solubility compared with AKR1D1-HIs. The uninduced culture showed little to no target protein, as expected. The last column of the table (insert which table), ‘Concentration of recombinant protein’ is unreliable because of the issues with the Bradford assay described above.

	TOTAL PROTEIN		SOLUBLE PROTEIN		
Sample	Volume Ratio (%)	Raw volume	Volume Ratio (%)	Raw volume	Solubility (%)
AKR-His N	7.876	502	16.499	687	136.85
AKR-His 1	26.618	1633	15.05	242	14.82
AKR-His 2	34.399	1679	14.419	370	22.04
AKR-His 3	29.722	1764	10.255	253	14.34
AKR-SUMO N	9.732	589	6.534	246	41.77
AKR-SUMO 1	30.427	1933	6.781	87	4.50
AKR-SUMO 2	28.585	1774	11.568	253	14.26
AKR-SUMO 3	31.486	2091	8.509	230	11.00
AKR-Trx N	4.231	225	13.988	551	244.89
AKR-Trx 1	32.19	2364	22.241	647	27.37
AKR-Trx 2	39.933	2400	21.609	591	24.63
AKR-Trx 3	39.785	2551	24.77	672	26.34

The uninduced cultured showed unexpectedly high protein concentrations measured by Bradford assay, even though all samples had been normalized to OD 20 before the assay. Because of the high total protein content in these samples, SDS-PAGE lanes may also have been overloaded, increasing background and affecting baseline determination and band

quantification (volume ratios and raw volumes). In future runs, we would ensure complete solubilization before quantification and loading an equal amount of total protein per lane.

The solubility percentages of the uninduced cultures were implausible, due to the quantification problems described above, and were therefore excluded from the analysis. The induced cultures gave more plausible values, although solubility was low overall. AKR1D1-SUMO had the lowest solubility (~10%), and AKR1D1-TrxA the highest (~26%). Given the issues with the Bradford assay and band quantification, these values should be considered estimations.

AKR1D1-TrxA is the most promising candidate for continued work amongst AKR1D1 constructs. However, solubility alone does not establish the best construct, since soluble yield and enzymatic activity also matter, and the quantification problems this week make the values approximate. Further experiments are needed to confirm these results, to compare soluble yield and activity across different constructs along with testing expression and activity under hypoxic conditions, as this experiment was performed aerobically.

The testosterone reduction assays were inconclusive, as the empty pET-11a control samples have also shown a decrease in NADPH concentration. Possible explanations include native E. coli reductases which might have been purified by accident that use NADPH as a cofactor, or cross-sample contamination. The assays need to be repeated, with additional controls, such as heat-inactivated proteins, and paying extra care not to contaminate samples during purification.

Day - : [August 31, 2026], Monday

Participants: Giulia, Patrik

Daily overview of experiments

This day has been evaluating on how to proceed with the analysis of AKR1D1 solubility tag efficiency. No actual lab work has been done.

Activity	***Protocol File***	***Comments***
Making a protocol	Lysis by B-PER + Br…	

Results and interpretation

Creating a Lysis by B-PER Protocol

Use the new protocol pipeline Lysis by B-PER + Bradford for combined lysis and SDS - Bradford sampling. Hope it works and that its good

Day 48: [September 1, 2026], Tuesday

Participants: Giulia, Patrik

Daily overview of experiments

Preparation of samples for Bradford assay and SDS page. Chemical cell lysis using B-PER.

Activity	***Protocol File***	***Comments***
Preparing AKR1D1 cultures for Solubility tag analysis	Lysis by B-PER + Br… Cultures for tag asse…	Cultures from 27th and 28th August. Samples taken for Bradford analysis of total cell protein and Bradford and SDS for soluble protein determination. *SDS for the total cell protein sample has already been taken during culturing.
Start of Bradford assay, calibration curve, AKR1D1-sumo first replicate run	Cultures for tag asse… Protein Quantificatio…	

Results and interpretation

Preparing AKR1D1 cultures for solubility tag analysis

In total 39 samples had to be made starting from 13 cultures (empty pET-11, AKR1D1-SUMO (no induction + 3 induced), AKR1D1-Trx (no induction + 3 induced), AKR1D1-His (no induction + 3 induced)). Each culture had 3 samples prepped and aliquoted (Bradford analysis of total cell protein, Bradford analysis of soluble fraction and SDS analysis of soluble fraction).

Workflow was followed according to the Lysis by B-PER + Bradford protocol, with exact volumes calculated in Cultures for tag assessment.xlsx.

Bradford assay

Bio-rad indications were followed for the Bradford assay. A calibration curve was prepared with BSA. 0.0014 g of BSA were weighed in an eppendorf tube and dissolved in a 1.4 mL cell wash buffer for a final concentration of 1 mg/ml (cell wash buffer, compatible with Bradford reagent). This stock was then used for serial dilution to the selected concentrations: 1, 0.8, 0.6, 0.4, 0.2, 0.1 mg/mL.

Due to estimated concentration of the proteins in each sample of 5 mg/mL (rule of thumb, see protocol), the AKR1D1-sumo samples were then diluted 10 times in the cell wash buffer (10 uL in 90 uL), in order to fall in the linear range of the calibration curve. Bradford reagent was diluted 5 times in the cell wash buffer. Following the Bio-rad protocol and Abchem indications, 1 mL of Bradford reagent was added to each cuvette + 20 uL of the previously diluted sample (or standard in case of the calibration curve). The measurements were run in triplicates to ensure better precision. Blank was prepared with 20 uL of cell wash buffer into 1 mL of 1x Bradford reagent.

CALIBRATION	RUN 1	RUN 2	RUN 3
1	0.380	0.337	0.378
0.8	0.304	0.257	0.302
0.6	0.246	0.231	0.238
0.4	0.165	0.143	0.165
0.2	0.095	0.083	0.08
0.1	0.039	0.041	0.049

Total Fraction	1	2	3
AKR-His N	0.053	0.054	0.067
AKR-His 1	0.035	0.065	0.034
AKR-His 2	0.042	0.054	0.057
AKR-His 3	0.056	0.051	0.051

AKR-SUMO N	0.128	0.071	0.095
AKR-SUMO 1	0.079	0.051	0.041
AKR-SUMO 2	0.079	0.032	0.048
AKR-SUMO 3	0.083	0.048	0.034
AKR-Trx N	0.112	0.12	0.121
AKR-Trx 1	0.056	0.057	0.053
AKR-Trx 2	0.057	0.053	0.061
AKR-Trx 3	0.059	0.047	0.056
pet11a	0.097	0.105	0.109

Total Fraction	1	2	3
AKR-His N	0.082	0.081	0.088
AKR-His 1	0.02	0.017	0.021
AKR-His 2	0.036	0.022	0.033
AKR-His 3	0.025	0.021	0.021
AKR-SUMO N	0.123	0.106	0.115
AKR-SUMO 1	0.031	0.042	0.043
AKR-SUMO 2	0.035	0.041	0.039
AKR-SUMO 3	0.039	0.042	0.048
AKR-Trx N	0.089	0.105	0.075
AKR-Trx 1	0.043	0.044	0.035
AKR-Trx 2	0.031	0.049	0.026
AKR-Trx 3	0.058	0.031	0.033
pet11a	0.061	0.073	0.07

Day 49: [September 2, 2026], Wednesday

Participants: Patrik, Rytis, Giulia

Daily overview of experiments

Finishing Bradford Assay for AKR1D1 cultures, and pET-11 control culture.

Activity	***Protocol File***	***Comments***
Bradford Assay	Protein Quantificatio…	Finishing samples from yesterday
Solubility Calculations	File	

Results and interpretation

Bradford Assay

All procedures have been done the same way as the previous day. Results of the readings have been put into the results from yesterday.

Solubility calculations

Using Bradford Assay results from multiple AKR1D1 cultures, both soluble and total protein fractions, and SDS-PAGE results, we managed to calculate the mass of our recombinant proteins for different tags.

When looking at Bradford results, it seems that the protein amount in the soluble fraction is higher than the protein amount in the total protein fraction. This cannot be true, and our experiment has gone wrong somewhere. There were probably issues with resuspension of the insoluble part in whole-cell lysate samples, so the protein amount seemed lower than it actually was. If we were to redo this experiment, boiling the samples to solubilize the insoluble fraction would be required.

TOTAL FRACTION

Sample	1	2	3	Mean	Standard Dev	CV	Prot. Conc (mg/mL)	Volume Ratio (%)	Concentration of recombinant protein (µg/mL)
AKR-His N	0.053	0.054	0.067	0.058	0.008	13.466	0.274	7.876	21.59
AKR-His 1	0.035	0.065	0.034	0.045	0.018	39.439	0.233	26.618	61.92
AKR-His 2	0.042	0.054	0.057	0.051	0.008	15.563	0.252	34.399	86.80
AKR-His 3	0.056	0.051	0.051	0.053	0.003	5.481	0.258	29.722	76.54
AKR-SUMO N	0.128	0.071	0.095	0.098	0.029	29.202	0.399	9.732	38.81
AKR-SUMO 1	0.079	0.051	0.041	0.057	0.020	34.557	0.271	30.427	82.47
AKR-SUMO 2	0.079	0.032	0.048	0.053	0.024	45.086	0.259	28.585	73.91
AKR-SUMO 3	0.083	0.048	0.034	0.055	0.025	45.889	0.265	31.486	83.37
AKR-Trx N	0.112	0.12	0.121	0.118	0.005	4.192	0.460	4.231	19.46
AKR-Trx 1	0.056	0.057	0.053	0.055	0.002	3.762	0.266	32.19	85.57
AKR-Trx 2	0.057	0.053	0.061	0.057	0.004	7.018	0.271	39.933	108.23
AKR-Trx 3	0.059	0.047	0.056	0.054	0.006	11.565	0.262	39.785	104.11
pet11a	0.097	0.105	0.109	0.104	0.006	5.894	0.416	0	0.00

Soluble FRACTION

Sample	1	2	3	Mean	Standard Dev	CV	Prot. Conc (mg/mL)	Volume Ratio (%)	Concentration of recombinant protein (µg/mL)
AKR-His N	0.082	0.081	0.088	0.084	0.004	4.525	0.354	12.423	43.99
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AKR-His 2	0.036	0.022	0.033	0.030	0.007	24.300	0.188	13.236	24.88
AKR-His 3	0.025	0.021	0.021	0.022	0.002	10.341	0.163	9.485	15.46
AKR-SUMO N	0.123	0.106	0.115	0.115	0.009	7.417	0.451	6.534	29.45
AKR-SUMO 1	0.031	0.042	0.043	0.039	0.007	17.220	0.214	6.781	14.51
AKR-SUMO 2	0.035	0.041	0.039	0.038	0.003	7.970	0.213	11.568	24.63
AKR-SUMO 3	0.039	0.042	0.048	0.043	0.005	10.657	0.227	8.509	19.35
AKR-Trx N	0.089	0.105	0.075	0.090	0.015	16.741	0.373	13.988	52.15
AKR-Trx 1	0.043	0.044	0.035	0.041	0.005	12.130	0.220	22.241	48.96
AKR-Trx 2	0.031	0.049	0.026	0.035	0.012	34.236	0.204	21.609	43.98
AKR-Trx 3	0.058	0.031	0.033	0.041	0.015	36.994	0.220	24.77	54.53
pet11a	0.061	0.073	0.07	0.068	0.006	9.184	0.305	0	0.00

	TOTAL PROTEIN		SOLUBLE PROTEIN		
Sample	Volume Ratio (%)	Raw volume	Volume Ratio (%)	Raw volume	Solubility (%)
AKR-His N	7.876	502	16.499	687	136.85
AKR-His 1	26.618	1633	15.05	242	14.82
AKR-His 2	34.399	1679	14.419	370	22.04
AKR-His 3	29.722	1764	10.255	253	14.34
AKR-SUMO N	9.732	589	6.534	246	41.77
AKR-SUMO 1	30.427	1933	6.781	87	4.50
AKR-SUMO 2	28.585	1774	11.568	253	14.26
AKR-SUMO 3	31.486	2091	8.509	230	11.00
AKR-Trx N	4.231	225	13.988	551	244.89
AKR-Trx 1	32.19	2364	22.241	647	27.37
AKR-Trx 2	39.933	2400	21.609	591	24.63
AKR-Trx 3	39.785	2551	24.77	672	26.34

Day 50: [September 4, 2026], Friday

Participants: Cecilia, Patrik, Giulia, Rytis, Eleonora

Daily overview of experiments

SDS-PAGE gel electrophoresis has been done on AKR1D1 construct with different tags, assessing the solubility efficiency of different tags (SUMO, Trx and no tag). Furthermore, sonication and purification of ci_MBP double mutant and empty pET11-a was done, and testosterone assays with those samples.

NOTE: when doing purification, **if negative controls are present, purify them first**, so positive ones don't contaminate the controls in case of column clogging

Activity	***Protocol File***	***Comments***
SDS of AKR1D1 with different tags	SDS-PAGE	Assessing solubility tag efficiency combined with Bradford assay from Wednesday
Sonication of ci_MBP, ci double mutants, two negative controls		
Purification of ci_MBP, ci double mutants, two negative controls		
Assays on pet11, CI2350 double mutants		

Results and interpretation

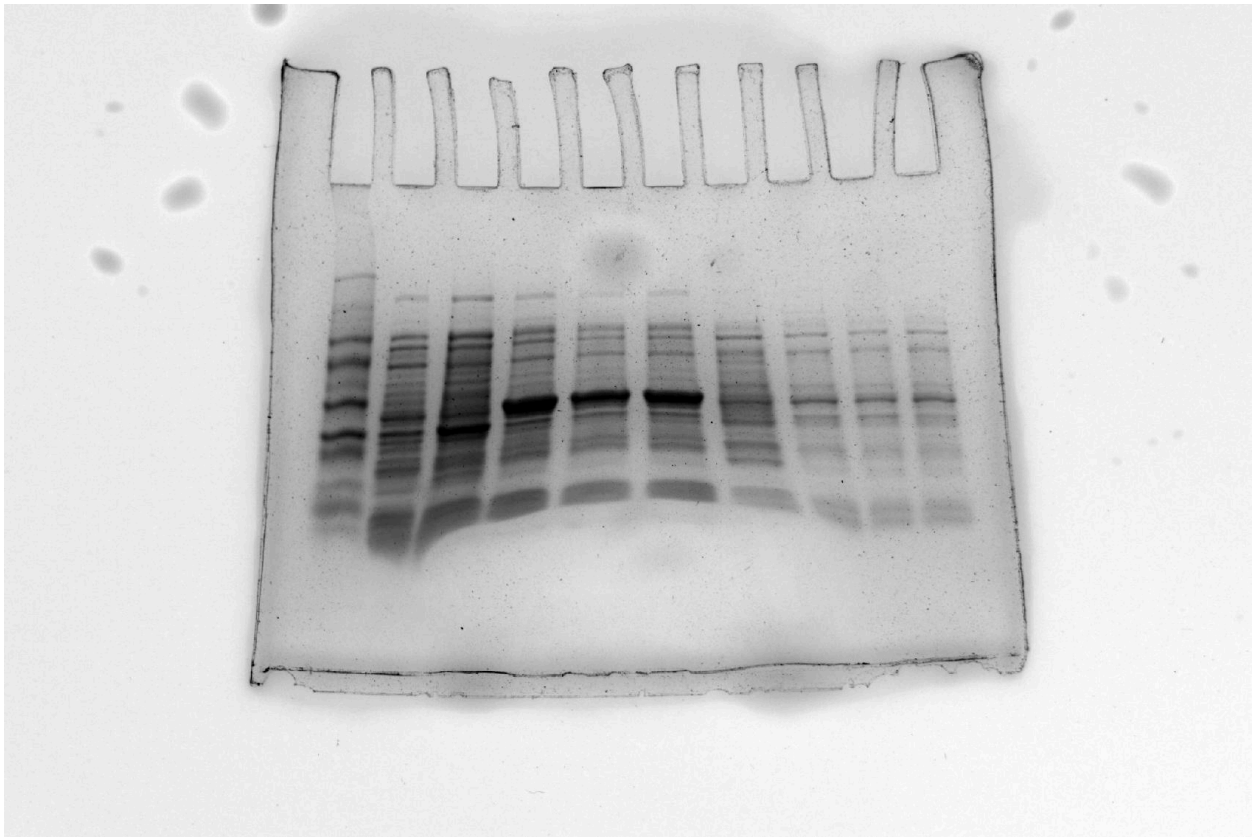
SDS of AKR1D1 with different tags

1 mL of SDS Loading Dye (2x) was made by combining 100 uL of beta-mercaptoethanol and 900 uL of the SDS Loading Dye. Premade samples were mixed with the loading dye in 1:1 ratio,

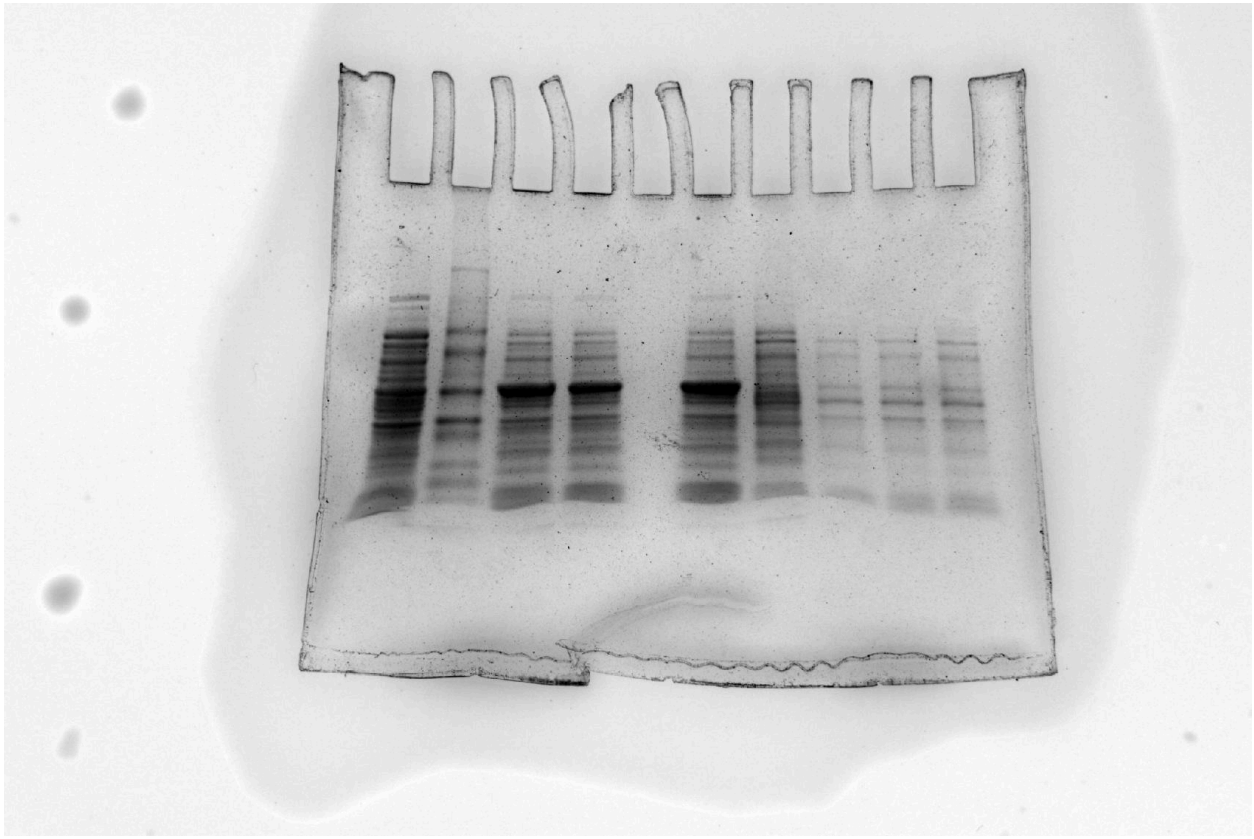
followed by incubation at 95 ºC for 5 minutes. Samples were loaded onto the gel (10 uL samples with 5 uL of ladder), and ran at 80V for 20 minutes, followed by 160V for 70 minutes . For gel Trx and SUMO, Cytiva rainbow full range ladder was used, and for His gel, PageRuler Broad Range Unstained was used, due to spending the other ladder.

AKR1D1-Sumo - 50.2 kDa
AKR1D1-TrxA - 50.7 kDa
AKR1D1-His 38.7 kDa

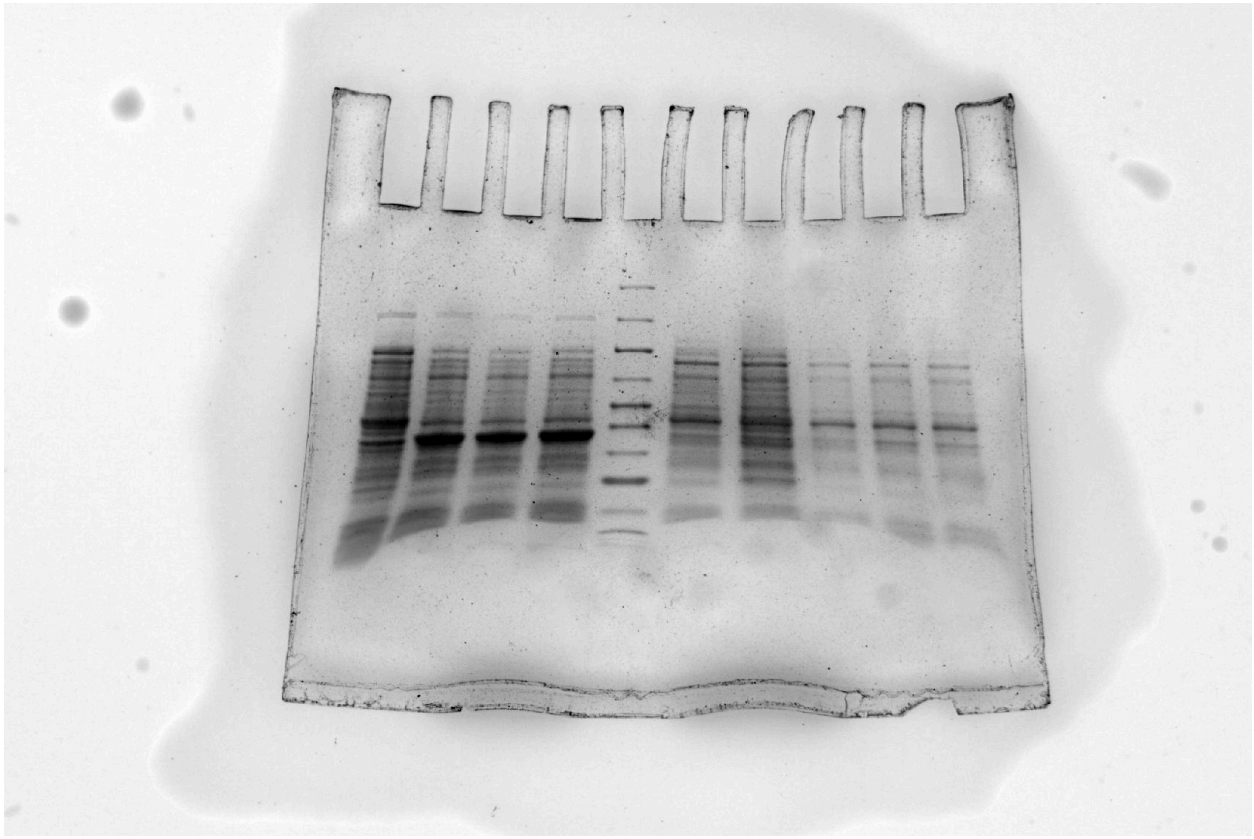
Well	Gel 1 (AKR-Trx)	Gel 2 (AKR-SUMO)	Gel 3 (AKR-His)
1	Ladder	Total uninduced	Total uninduced
2	pET11 - total fraction	Ladder	Total induced 1
3	Total uninduced	Total induced 1	Total induced 2
4	Total induced 1	Total induced 2	Total induced 3
5	Total induced 2	Broken Well	Ladder
6	Total induced 3	Total induced 3	pET11 - soluble fraction
7	Supernatant uninduced	Supernatant uninduced	Supernatant uninduced
8	Supernatant induced 1	Supernatant induced 1	Supernatant induced 1
9	Supernatant induced 2	Supernatant induced 2	Supernatant induced 2
10	Supernatant induced 3	Supernatant induced 3	Supernatant induced 3



Gel 1 (AKR-Trx)



Gel 2 (AKR-SUMO)



Gel 3 (AKR-His)

Sonication

Four samples were used for sonication: ci_MBP (0.8 mM IPTG, 17°C, cultivated on 22.07), ci_double mutants (0.8 mM IPTG, DATE), pET11a control (0.8 mM IPTG, 17°C overnight, 27.08), pET11-a control (no IPTG, 17°C overnight, 27.08). Cell pellets were thawed on ice, washed with 10 mL of cell wash buffer (500 mM NaCl, 20 mM sodium phosphate) per tube, and resuspended. The samples were centrifuged at 4700 x g for 10 minutes at 4°C. The resulting pellets were weighed, and lysis buffer (500 mM NaCl, 20 mM sodium phosphate, 30 mM imidazole; same as binding buffer) was added at a ratio of 5 mL of buffer per 0.4 g of pellet weight.

Sample	Pellet Weight (g)	Lysis Buffer Added (mL)
ci_MBP	1.208	15.10
ci_mutant	1.42	17.75
pET11-a control with IPTG	1.20	15.0
pET11-a control no IPTG	1.44	18.0

Samples were resuspended in lysis buffer and sonicated. The sonicator was operated at 30% amplitude for 3 cycles per sample, with each cycle consisting of 2 minutes of active time using a 3-second on/off pulse interval.
The lysate was subsequently clarified via centrifugation at 20,000 x g for 1.5 hours at 4°C. After centrifugation, each sample was filtered a total of four times: 1.2µm -> 0.8µm -> 0.45µm -> 0.2µm.

Purification of ci_mbp, double mutants and controls via Column Binding

Immobilized metal affinity chromatography (IMAC) column was stripped and cleaned (CIP) by Camille. Since the column was stored in 20% ethanol, it was washed with a 10 mL binding buffer, followed by 10mL distilled water. The re-charging of the column was done with 0.5 mL of 0.1M NiCl2. After, the column was washed with 5 mL of distilled water and 5 mL binding buffer. The flowthrough containing NiCl2 needs to be collected and put into the chemical waste canister in the fume hood for nickel waste.

The column was equilibrated with a 10 mL binding buffer. The sample flow rate was set to 1 mL/min throughout the run. All clarified lysate was processed via column (flowthrough). Then, the column was washed with 10 mL of binding buffer (wash), and the target protein was eluted using 5 mL of elution buffer (500 mM NaCl, 20 mM sodium phosphate, 500 mM imidazole). This procedure was done for all four samples.

Note: the flow rate was set to 1 mL/min, but the volume was collected far slower. The reason might be a clogged column.

To exchange the buffer and concentrate the protein, 4 mL of the eluate was loaded onto a Vivaspin centrifugal concentrator along with 4 mL of potassium phosphate buffer (pH 6.5). The mixture was centrifuged at 4000 x g for 30 minutes. This step was repeated a second time.