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Students Collaborative Report

Expression, purification and basic characterization of target protein samples for drug binding studies

Monday 8 September – Wednesday 10 September 2025

Latvian Institute of Organic Synthesis, Riga, Latvia

Training School of the COST Action CA21111
One Health drugs against parasitic vector borne diseases in Europe and beyond
OneHealthdrugs

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Students Collaborative Report

Day 1 – 08/09/2025

Lecture 1: Introduction to protein expression in bacteria

Dr. Teodors Pantelejevs (Latvian Institute of Organic Synthesis)

This lecture covered protein expression in E. coli, focusing on why researchers produce proteins in-house and the key steps involved. It explained considerations such as expression host selection, plasmid design, promoters, affinity tags, and factors affecting protein yield and solubility. Finally, it highlighted the importance of optimizing conditions like temperature, induction timing, and media to achieve successful expression before scaling up production.

Laboratory activities: Protein Production

E. coli cell transformation

In day one, we aimed at growing enough recombinant cells expressing the protein of interest (ID: FPOX_X02C).

The construct containing the gene inserted into the vector pET-17b and the ampicillin resistance selection marker was mixed with *E. coli* cells and heat shocked. After incubation at 37 ºC and centrifugation, cells were resuspended and plated on agar. Overnight incubation led to colony formation (**Figure 1.1**).

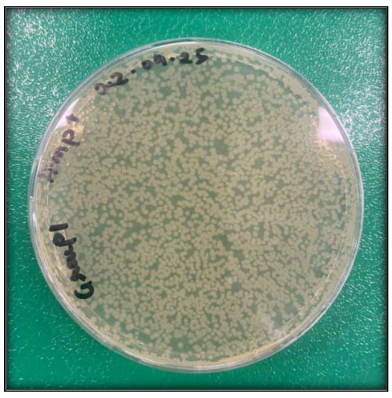


Figure 1.1 Colonies on agar plate after overnight incubation.

We proceeded to pick a single colony from the agar plate and inoculated in liquid medium for small-scale culture (50 mL). This was incubated overnight at 37 ºC.

For large-scale culture, we first measured the OD_{600} value of the small-scale culture, which was 8.9 (**Figure 1.2**).

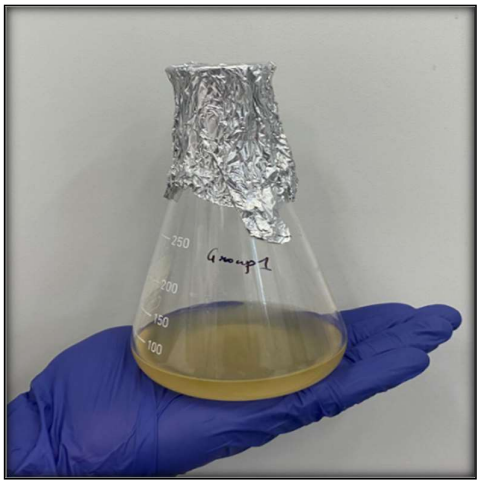


Figure 1.2. Small-scale culture after incubation

We diluted it to a final OD_{600} of 0.1 into a final volume of 500 mL and incubated it at 37 ºC until OD_{600} reached 0.4 (around 1.5 h). Then, we induced protein expression by adding IPTG into the culture and incubated at 18 ºC overnight.

Cells were harvested by centrifugation (**Figure 1.3**) and a pellet of 1.68 g was collected and stored at -20 ºC.

Figure 1.3. Pellet after large-scale culture centrifugation

Day 2 – 09/09/2025

Lecture 2: Introduction to protein expression in bacteria

Prof. Emilio Parisini (Latvian Institute of Organic Synthesis, University of Bologna)

This lecture focused on protein purification techniques, explaining why purification is necessary and outlining the general workflow from expression to assessment of purity. It discussed key methods such as affinity chromatography (including IMAC and various tags like His, GST, FLAG, MBP), size-exclusion chromatography, and ion-exchange chromatography. The lecture emphasized the importance of understanding protein properties and selecting appropriate purification strategies to achieve high yield and purity.

Laboratory activities: Protein Purification

During the second day, we first focused on breaking open the bacterial cells to release the expressed protein, and then on purifying it using different liquid chromatography methods.
To obtain the cell lysate and release the protein into solution, we used a sonicator with the following settings: 15 sec on / 20 sec off, 70% power, for a total of 10 minutes. The lysate was then centrifuged at 46,000 g for 45 minutes, and the supernatant was collected.

The purification workflow included:

- IMAC (Nickel Affinity Chromatography);
- SEC (Size Exclusion Chromatography);
- IEX (Ion-Exchange Chromatography);
- SDS-PAGE analysis.

IMAC

The first purification step was performed using Ni-NTA slurry as the solid phase (**Figure. 2.1**). The resin was washed with 5 CV of Milli-Q water and equilibrated with 5 CV of Buffer B (50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol, 20 mM imidazole). We then loaded the clarified lysate obtained in the previous step and washed the resin with 5 CV of Buffer B. The protein of interest was eluted using 5 mL of Buffer C (50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol, 400 mM imidazole).
Samples collected for SDS-PAGE included pellet, lysate, flow-through, and IMAC elution. The concentration of the final fraction was measured with a Nanodrop.

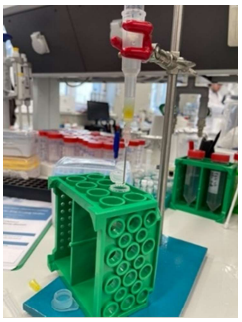


Figure 2.1. IMAC purification performed with Ni-NTA beads in a gravity column

SEC

To further purify the protein, we separated it based on size and shape using a Sephacryl S-100 HR column (**Figure 2.2**). At the end of the first day, the column had been equilibrated with Buffer A (50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol). On the following day, the separation was performed using the ÄKTA system. Fractions were collected starting at 0.38 CV and stored at 4 °C. The concentration of the fractions was then measured.

Figure 2.2. Size-exclusion chromatography (SEC) performed with a Sephacryl S-100 HR column using the Äkta

IEX

To separate proteins based on surface charge, we used ion-exchange chromatography. The column was equilibrated with 5 CV of Buffer D, after which the protein sample was loaded. Elution was performed with Buffer E, and the eluted fractions were collected for SDS-PAGE analysis.

SDS-PAGE analysis

To check the purity, size, and presence of the protein at different purification steps, we performed SDS-PAGE with the following samples: lysate, pellet, flow-through, IMAC elution, SEC elution, and IEX elution (**Figure 2.3**).

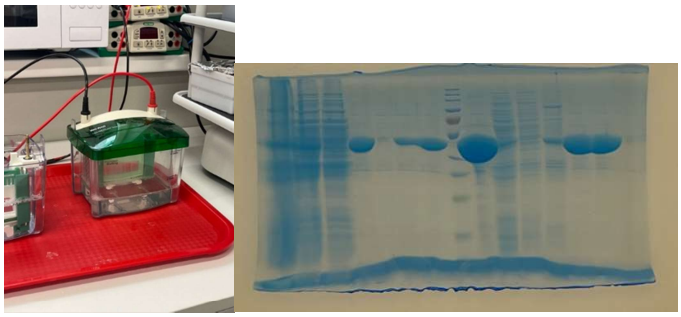


Figure 2.3. SDS-PAGE apparatus (left) and acrylamide gel showing the main fractions from the different purification steps (right)

Day 3 – 09/09/2025

Lecture 3: Biophysical assessment of protein quality

Prof. Cecilia Pozzi (University of Siena)

This lecture introduced biophysical techniques used to assess protein quality, including methods for analyzing structure, stability, size, and interactions. It focused on Circular Dichroism (CD) spectroscopy, Differential Scanning Fluorimetry (DSF), and MALDI-TOF mass spectrometry, explaining their working principles and applications. Together, these methods enable researchers to evaluate protein folding, detect conformational changes, measure stability, and identify proteins with high sensitivity.

Laboratory activities: Protein Characterization

Circular Dichroism (CD)

The secondary structure of the protein FPOX_X02C was assessed using CD (**Figure 3.1**). 80.64 µL of the fraction N14 previously purified via Size Exclusion Chromatography (SEC) were diluted in 419 µL of Tris buffer containing 50 mM NaCl, in order to reach a protein concentration of 5 µM. 400 µL of the diluted protein was inserted in a quartz cuvette and the sample was measured at 200-250 nm, 3 times at 20 °C.

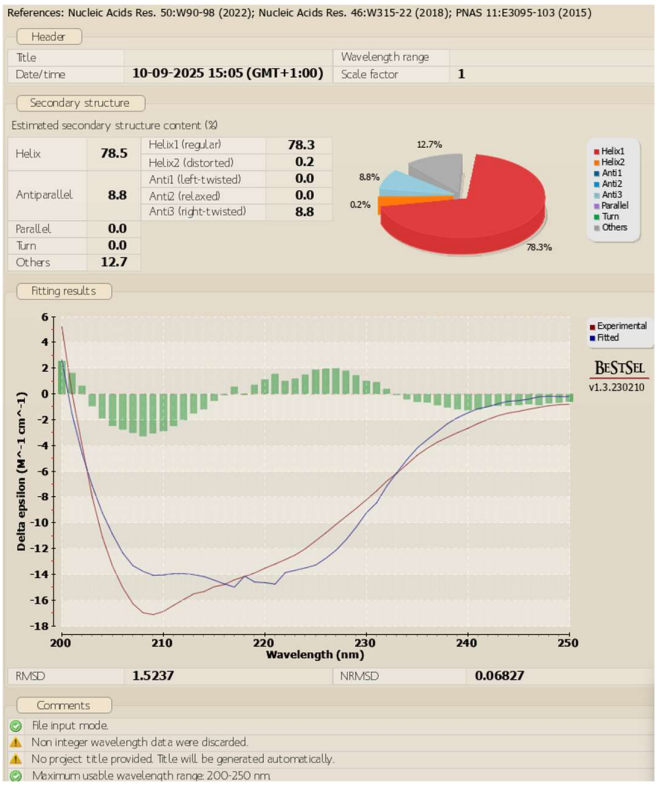


Figure 3.1. CD spectrum of the protein at 20°C. The secondary structure content determined by CD spectroscopy is in agreement with the crystal structure of the protein (PDB code: 5T1E)

Differential scanning fluorimetry (DSF)

The protein stability was assessed by preparing a sample consisting of 81 µL of a 31 µM fraction solution (N14), purified by Size-Exclusion Chromatography, and 419 µL of Tris buffer containing 50 mM NaCl, to achieve a final concentration of 10 µM. A 50 µL aliquot of the sample solution was mixed with 50 µL of SYPRO Orange dye solution (diluted by 10) to prepare the DSF sample solution. Additionally, 50 µL of Tris buffer were mixed with the dye solution to prepare the blank for the DSF analysis. From sample solution, 20 µL were loaded into PCR plate wells in triplicate, while two blanks were prepared with the blank solution. The plate containing all samples was sealed with optical film and centrifuged for one minute. Finally, the analysis was performed by running a temperature gradient of 1 °C/min using the JTSA software (**Figure 3.2**).

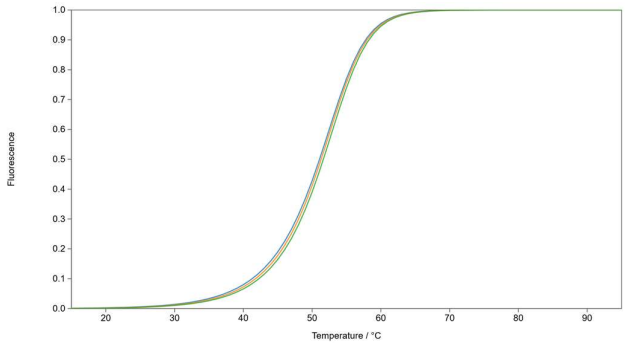


Figure 3.2. DSF curve of the protein, showing a Tm = 53°C

Mass-Spectrometry (Matrix-Assisted Laser Desorption Ionization – Time-Of-flight MALDI-TOF).

For the endpoint investigation of the protein identity, we determined the purified protein (FPOX_XO2C) by applying MALDI-TOF to analyse the molecular weight as well as confirming the purity of the content by mixing 5 µL of protein approximately (5mg/mL) into 5 µL 0.1% trifluoroacetic acid (**TFA**) in acetonitrile (**ACN**). This solution was further mixed into a matrix composed of sinapinic acid (**SA**) in a 1:1 ratio of 1 µL each. Thereafter, the 2 µL solution was transferred onto a MALDI plate and was air dried at room temperature for 1.5 hours. Subsequently, the MALDI analysis were performed on a Bruker Autoflex maX system, which was used to acquire mass-to-charge ratio (m/z) (**Figure 3.3**).

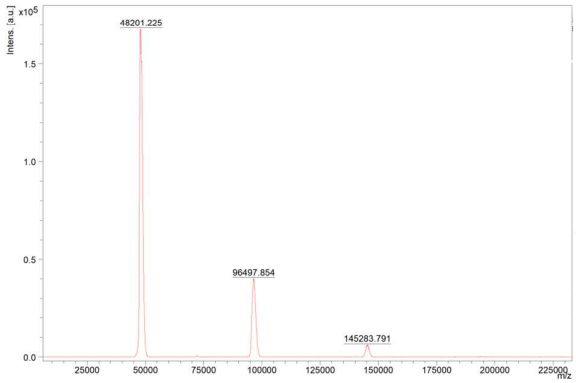


Figure 3.3. MALDI-TOF spectrum showing that the protein has the expected molecular weight (Mw = 48201.225 D) Peacks of the dimer and trimer of the protein are also visible in the spectrum

Conclusion

During the training school, participants were divided into small groups to improve the effectiveness of the learning experience, and this division was maintained throughout the course. At the conclusion of the program, each group prepared and delivered a flash presentation summarizing the key concepts, techniques, and activities covered during the training. The slides from these presentations are included as an annex to this document for completeness and future reference.

Annex I

Slides of group presentations done at the end of the school are attached.

- Presentation from Group 1
- Presentation from Group 2
- Presentation from Group 3
- Presentation from Group 4

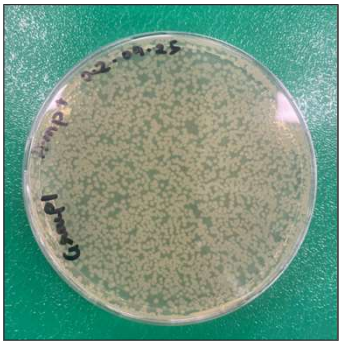
Expression, Purification and Basic Characterization of Target Protein Samples for Drug Binding Studies

Group 1 Laboratory Activities
Francesco Calzaferri
Walliyulahi Ajibola
Şener Çintesun

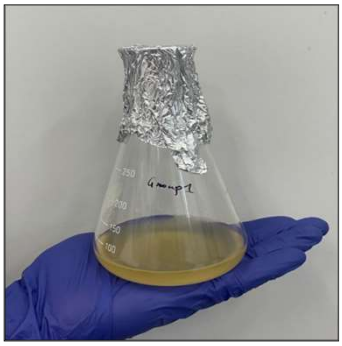
Latvian Institute of Organic Synthesis, Riga, Latvia

Day 1

1) Transformation



2) Small-scale culture



After 8 h, OD_{600} = 2.5

Group 1

3) Large-scale culture, induction and cell harvesting

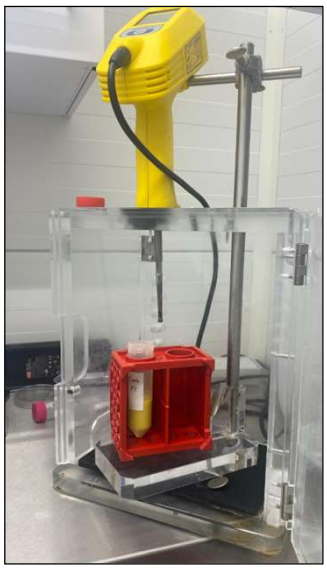
- Small scale (from Shapla ☺) → OD_{600} = 8.9 → 5.6 mL in 500 mL of LB
- After 40 min, OD_{600} = 0.01 → we added 10 mL ☺
- After 1.5 h, OD_{600} = 0.43
- Induction IPTG
- 20 min → centrifugation → pellet of 0.78 g

Group 1

Day 2

1) Sonication

6.28 g of pellet (from Shapla ☺)



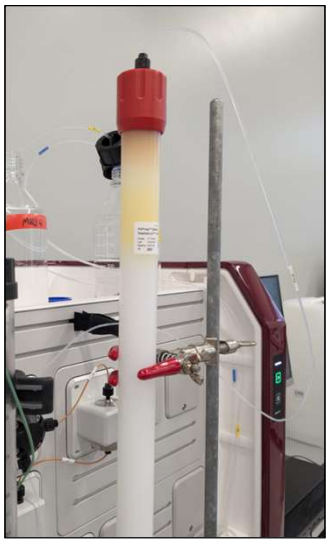
2) IMAC

Flow-through
Wash
E1
E2

OD_{600} = **4.067** mg/mL ($A_{260/280}$ = **0.79**)

Group 1

3) SEC



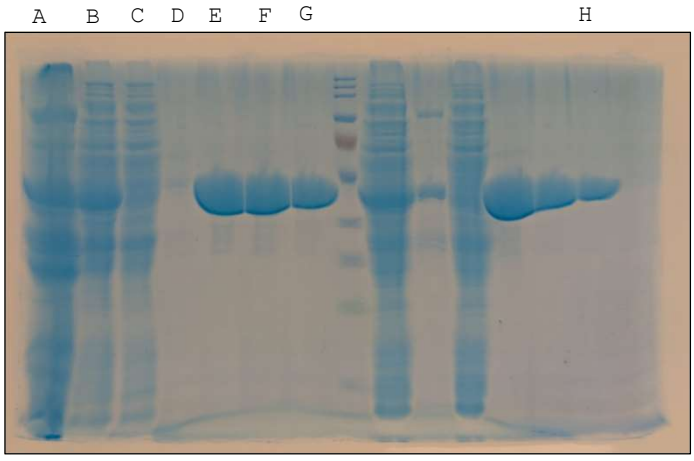
Fractions	mg/mL	$A_{260/280}$
F3	0.957	0.67
F4	2.341	0.66
F5	2.426	0.67
F6	1.075	0.68
F7	0.257	0.80

4) IEX

Group 1

5) SDS-PAGE

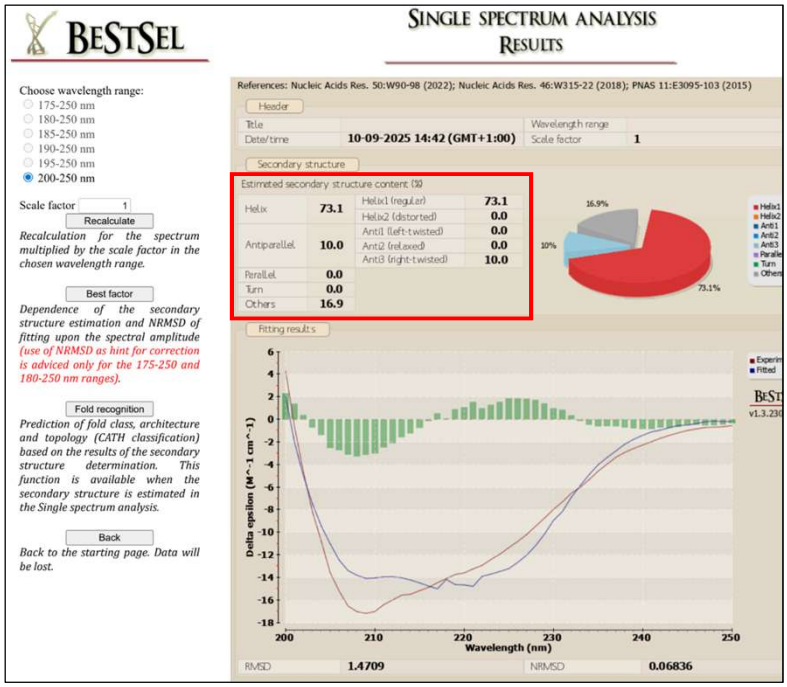
A Insoluble
B Soluble
C Flow-through
D Wash
E IMAC E1
F IMAC E2
G SEC F4
H IEX E



Day 3

Group 1

1) CDsf F4

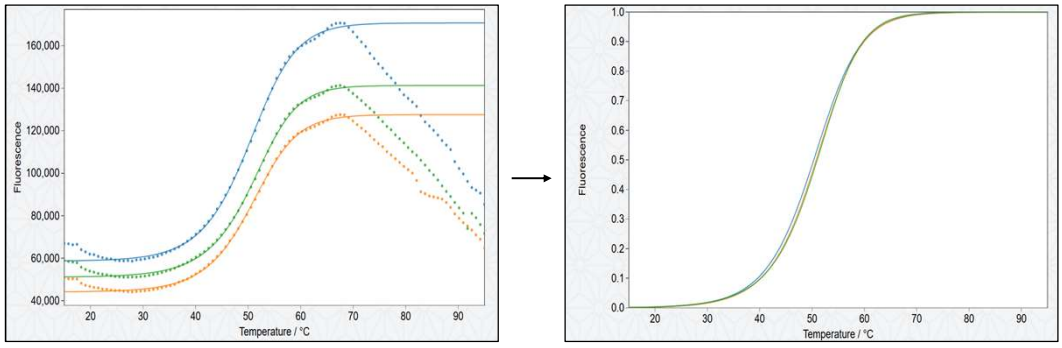


Day 3

Group 1

Group 1

2) DSF F4

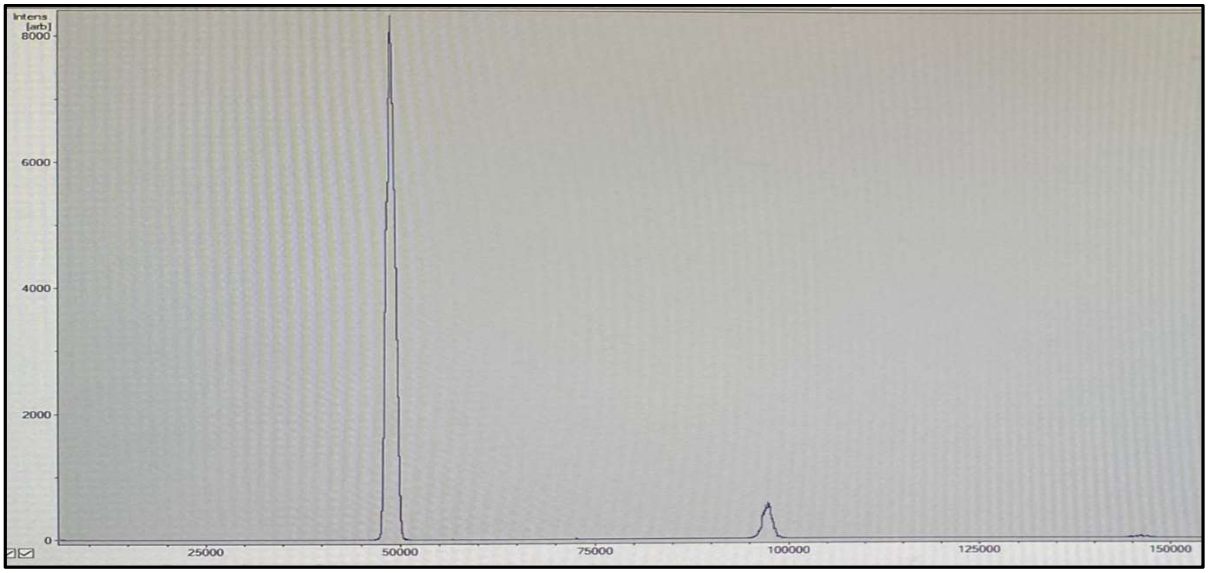


Name	Type	Tm	ΔTm	ΔTm / σ	R^2	Min T	Max T	Min F	Max F
A01	Assay	50.3			1.000	27.9	67.5	58,620	170,703
A02	Assay	50.8			1.000	27.9	67.5	44,122	127,550
A03	Assay	51.0			1.000	27.1	67.5	51,068	141,276

Group 1

3) MALDI-TOF F4

MW: 48.2 kDa



THANK YOU!

6th TRAINING SCHOOL - Expression, purification and basic characterization of target protein samples for drug binding studies.

GROUP 2

Cagla Burcin Bicakci
David Cisneros
Hatçe Asli BEDEL

1st Day
Transformation and Scale culture

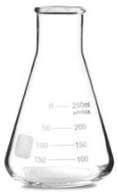
Transformation

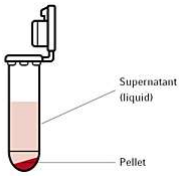
1st Day
Transformation and Scale culture

Small scale culture

OD600 (6h incubation) = 2.3
OD600 (O/N incubation) =5.4

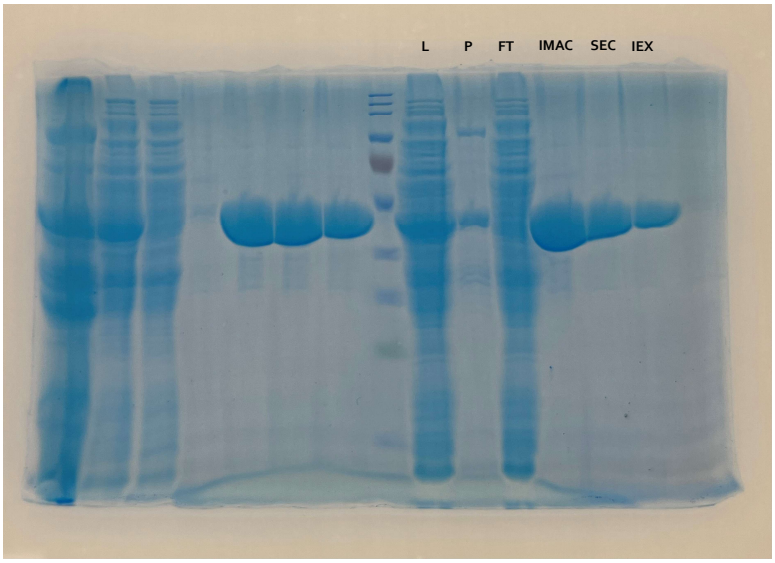
Large scale culture



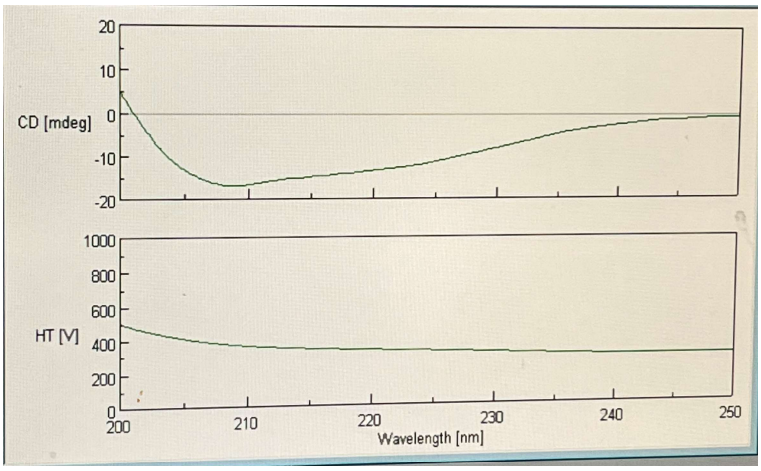


Pellet mass = 5.84g
Yield = 18.5 mg/L

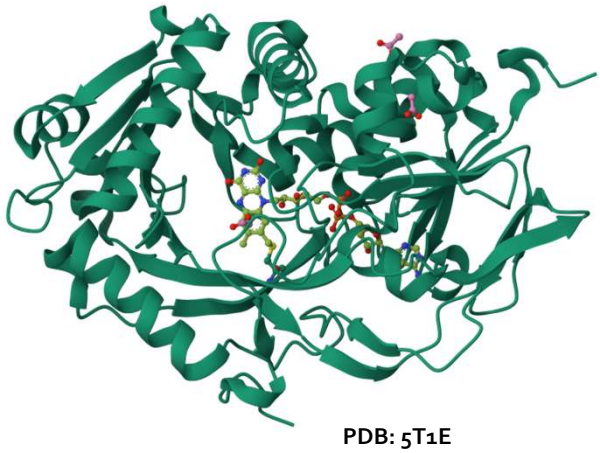
2nd Day
Cell lysis and protein purification
-IMAC
-SEC
-IEX



3rd Day
Biophysical measurements
-Circular Dichroism (CD)

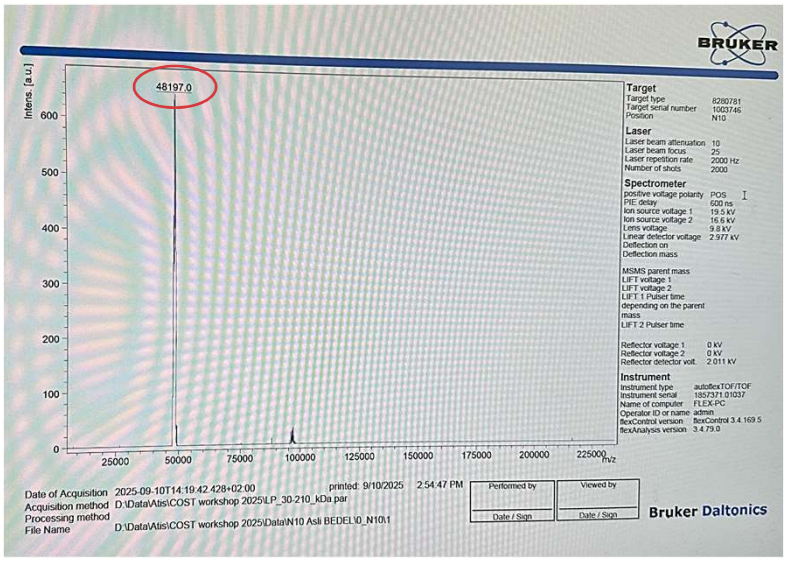


3rd Day
Biophysical measurements

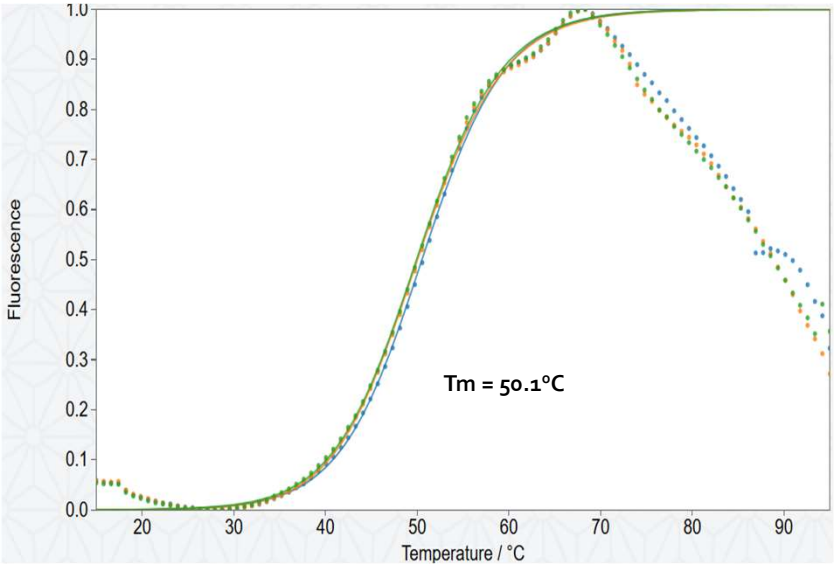


PDB: 5T1E
Phaeospaeria nodrum fructosyl peptide oxidase

3rd Day
Biophysical measurements
-MALDI-TOF



3rd Day
Biophysical measurements
-DSF



6th TRAINING SCHOOL - Expression, purification and basic characterization of target protein samples for drug binding studies.

GROUP 2

Cagla Burcin Bicakci

David Cisneros

Hatçe Asli BEDEL

Latvian Institute of Organic Synthesis

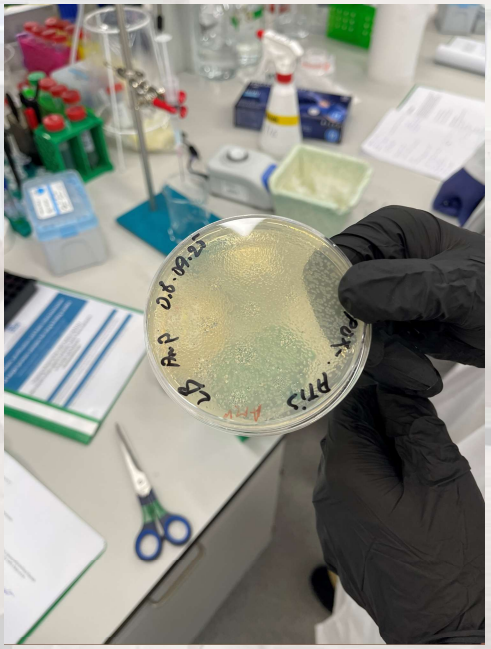
COST-Action School

Group 3
Filippo Piazza
Mindaugas Lesanavicius
Hanifi Atif Arslan

Supervisor: Atis ☺

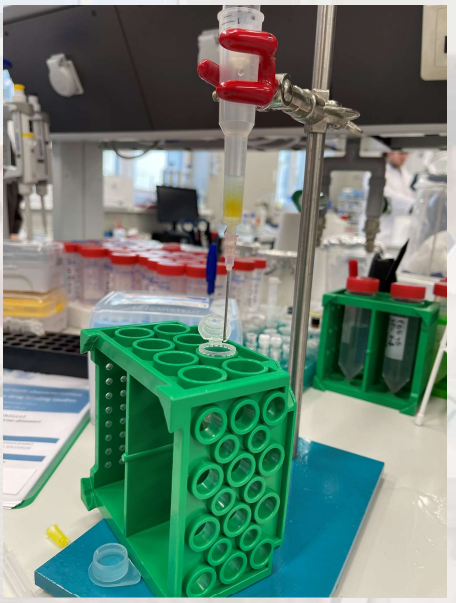
Expression, purification and basic characterization of target protein samples for drug binding studies

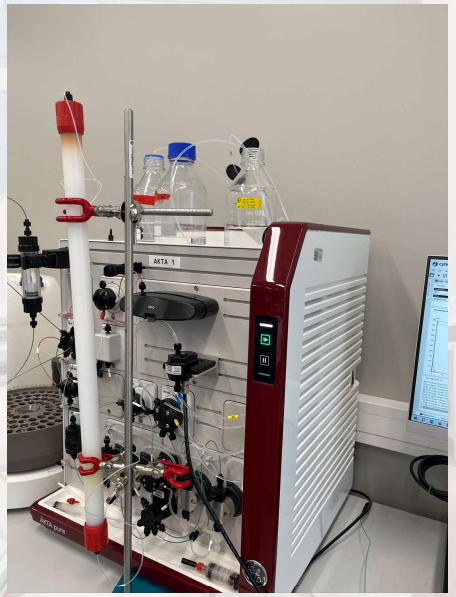
LV-1006 Riga - Latvia



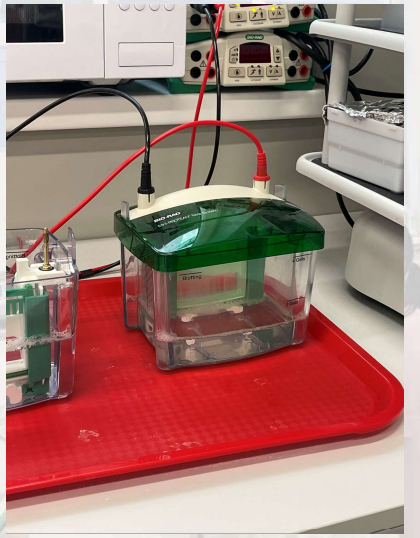
Large scale pellet weight 0.93 g

5.84 g in 1 L (after harvesting)

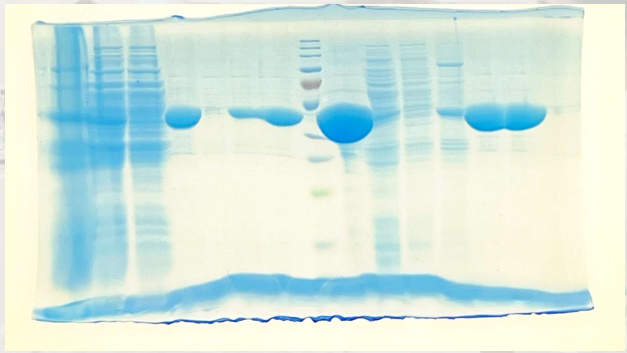





Hanifi Atif Arslan



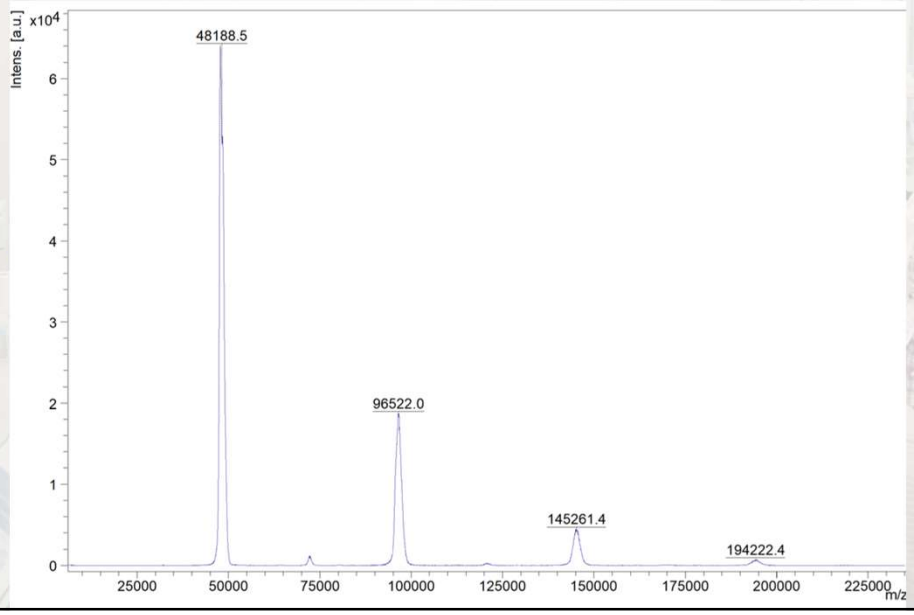
SDS-PAGE



Day 3: protein characterization

- MALDI-TOF MS
- DSF
- CD

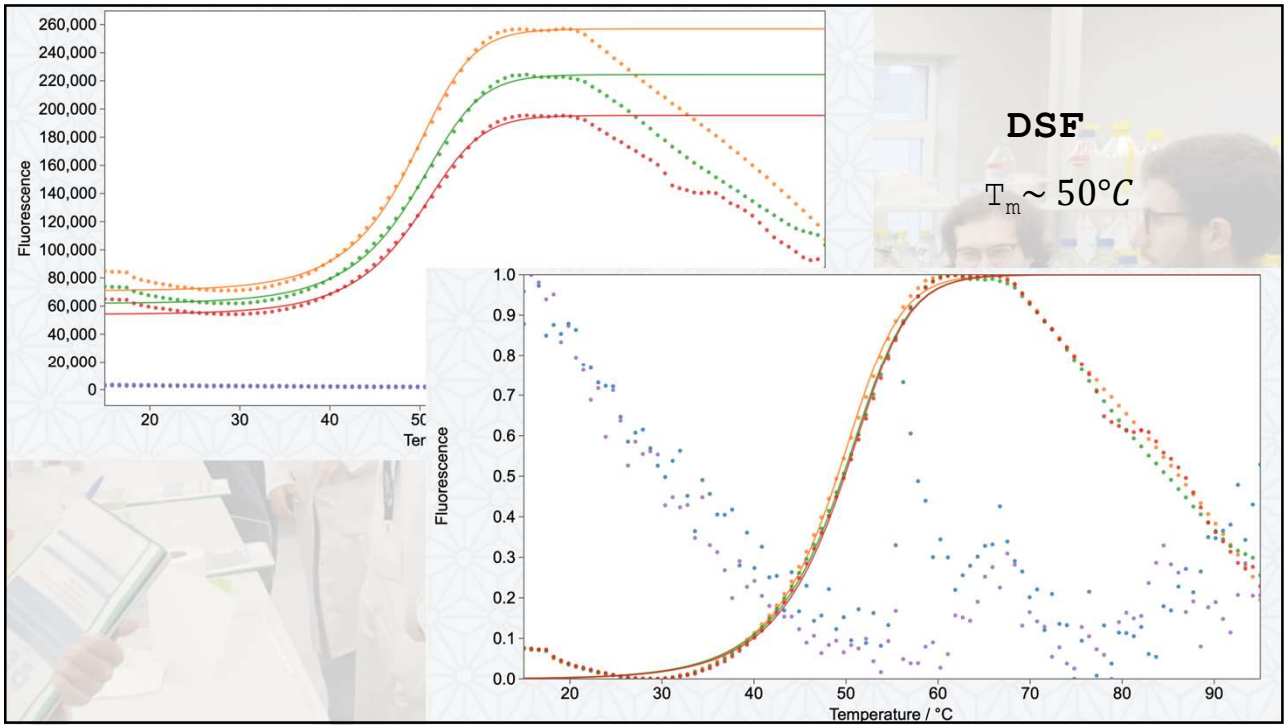
MALDI-TOF MS



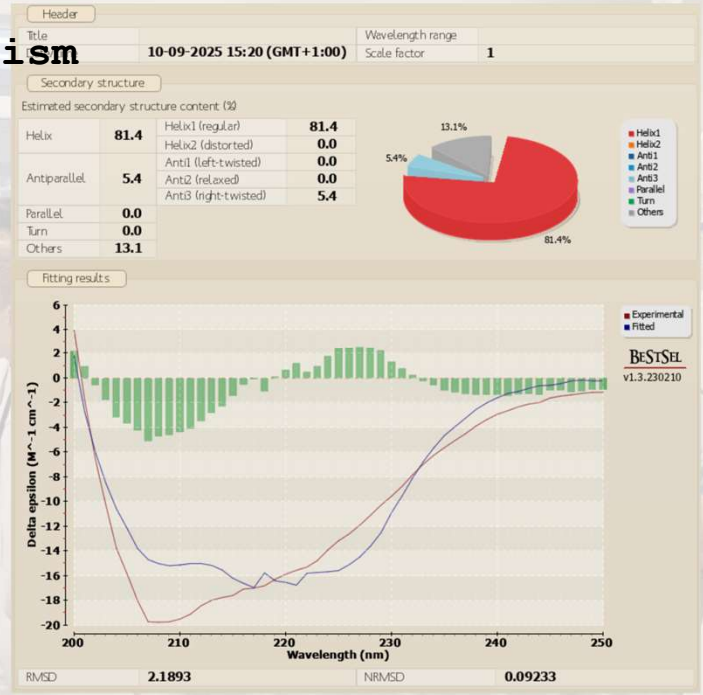
M.W. 48.2 kDa

DSF

$T_m \sim 50°C$



Circular Dicroism



Thank you for your attention!!!

Mindaugas

6th Training School

Riga, Latvia

OHD ONE HEALTH DRUGS

Latvian Institute of Organic Synthesis

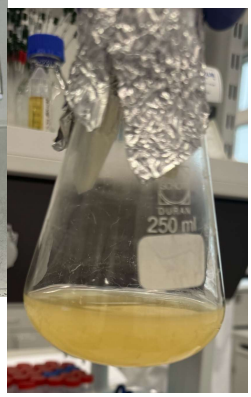
GROUP 4
Johander Inacio Dos Ramos Azuaje
Michael Lyngbæk Christensen
Foteini Batsolaki

TRANSFORMATION

Protein ID: FPOX_X02C

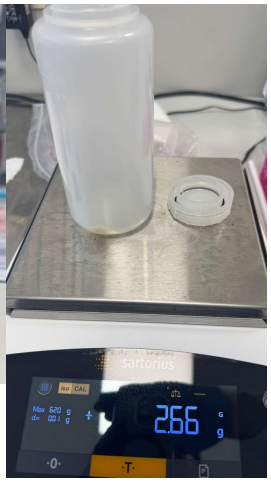
PDB code: 5T1E

SMALL & LARGE SCALE CULTURES



t = 3.5 h
OD = 2.42

t = 24 h
OD = 6.2

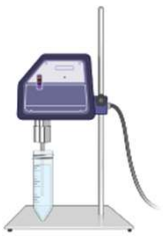


T = 1.5h
OD = 0.43

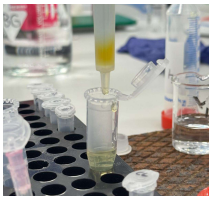
m = 2.66 g

SONICATION & NICKEL AFFINITY CHROMATOGRAPHY

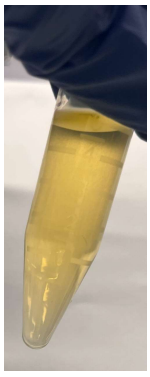
1



2



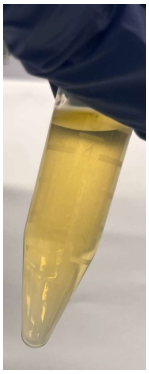
3



Fraction containing the target protein

SIZE EXCLUSION CHROMATOGRAPHY

1



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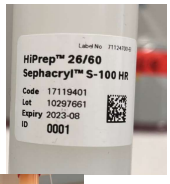
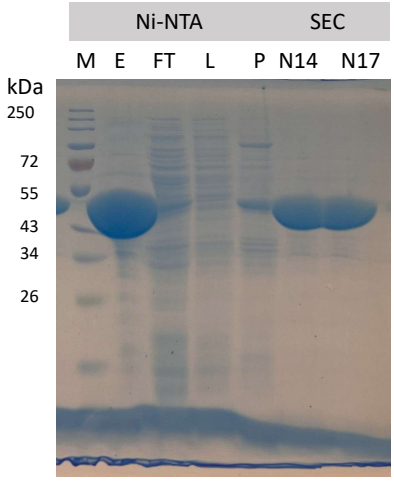


3

Fraction ID	Concentration (mg/mol)
N12	0.234
N13	0.699
N14	1.503
N17	1.636
N18	1.017

SDS-PAGE



CIRCULAR DICHROISM (CD)

References: Nucleic Acids Res. 50:W90-98 (2022); Nucleic Acids Res. 46:W315-22 (2018); PNAS 11:E3095-103 (2015)

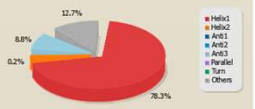
Header

Title		Wavelength range	
Date/time	10-09-2025 15:05 (GMT+1:00)	Scale factor	1

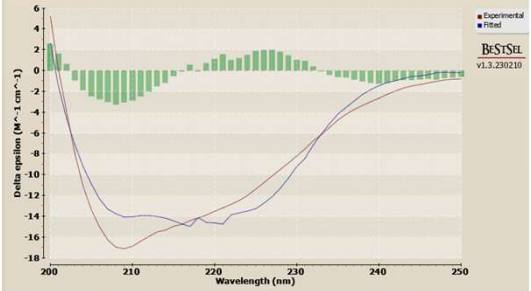
Secondary structure

Estimated secondary structure content (%)

Helix	78.5	Helix1 (regular)	78.3
		Helix2 (distorted)	0.2
Antiparallel	8.8	Anti1 (left-twisted)	0.0
		Anti2 (relaxed)	0.0
		Anti3 (right-twisted)	8.8
Parallel	0.0		
Turn	0.0		
Others	12.7		



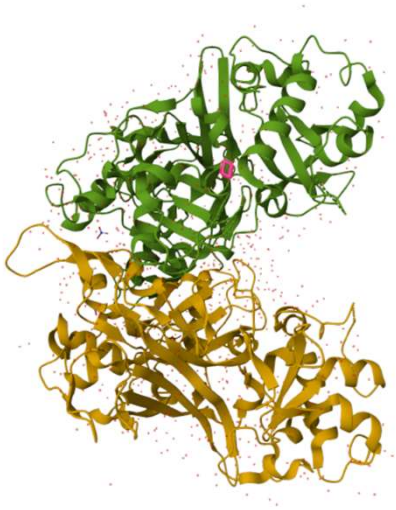
Fitting results



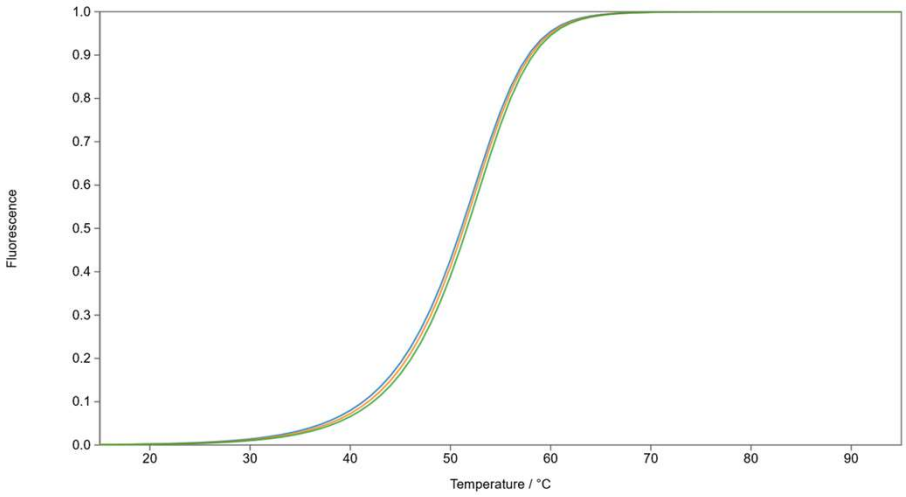
RMSD	1.5237	NRMSD	0.06827

Comments

- File input mode.
- Non integer wavelength data were discarded.
- No project title provided. Title will be generated automatically.
- Maximum usable wavelength range: 200-250 nm.



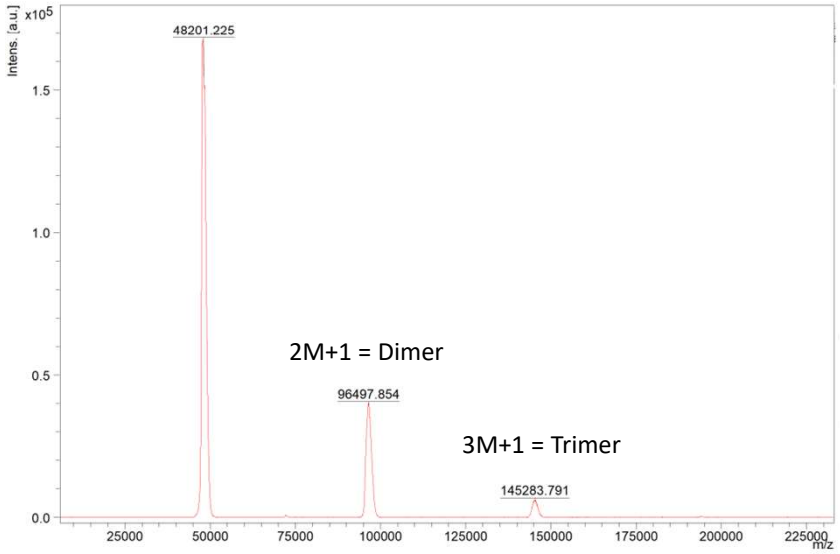
DIFFERENTIAL SCANNING FLUORIMETRY (DSF)



Well	T_m
D01	51.1
D02	51.3
D03	51.6

MALDI-TOF

M+1 = Monomer



THANK YOU FOR YOUR TIME!