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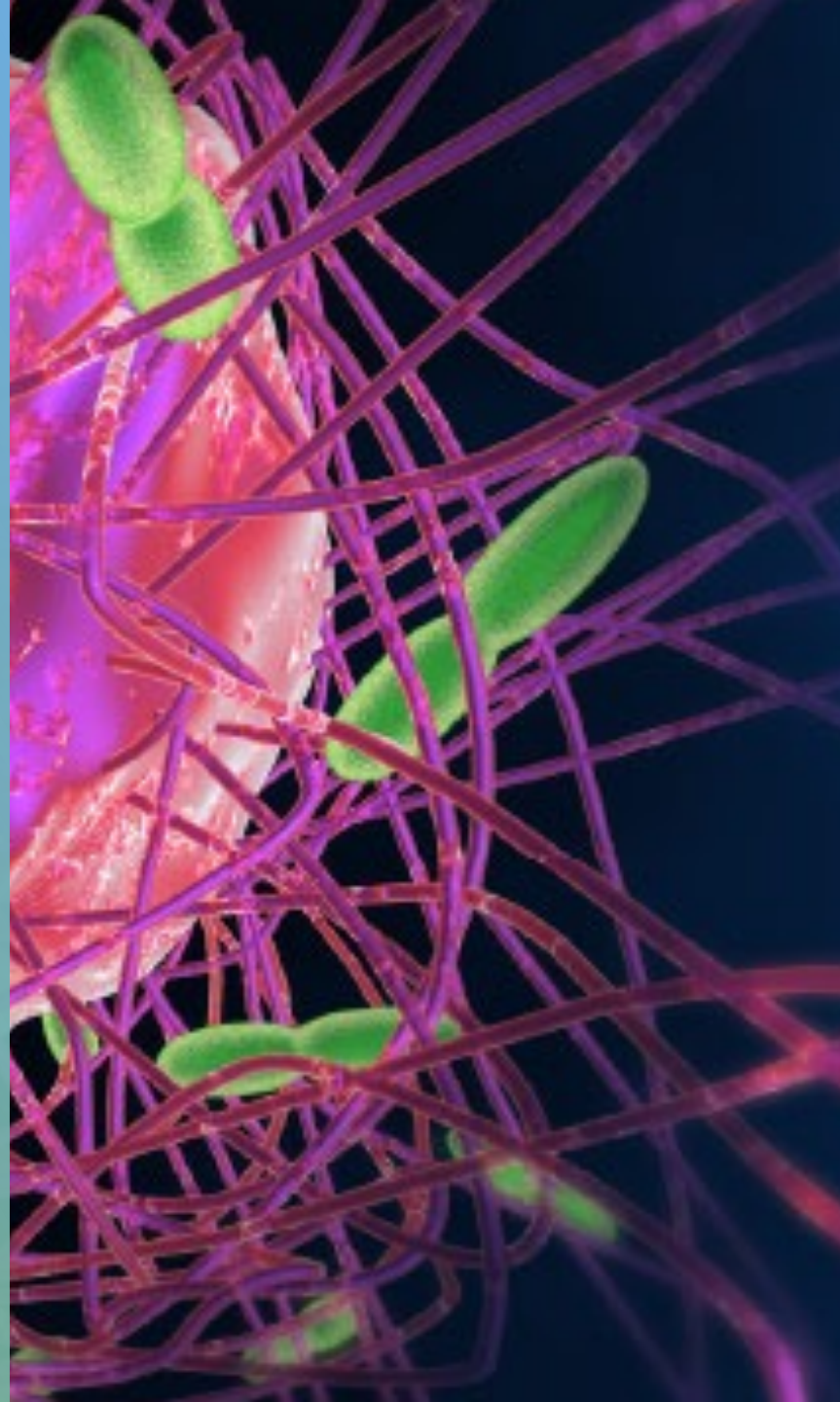
ProTech



HOW DO YOU DO IT

Quality control of recombinant proteins – how to tackle the reproducibility Crisis

Arthur Sedivy, 15.12.2023

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VBCF PROTECH BIOPHYSICS
HOW DO YOU DO IT

1. Who we are / What we do
2. The Reproducibility Crisis
3. Protein Quality Control
4. Protein Quality Optimization
5. ProTech Equipment

Vienna BioCenter
CORE FACILITIES

WHO WE ARE AND WHAT WE DO

Vienna BioCenter CORE FACILITIES

CORE FACILITIES

Vienna BioCenter

Vienna BioCenter CORE FACILITIES

Vienna Biocenter Core Facilities (VBCF)
Funded in 2011 by the Austrian Ministry of Science and Research and the City of Vienna.

The core facilities offer:

- Access to **state-of-the-art technologies**
- Joint exploration of new and emerging technologies
- **Economies of scale** through centralization
- Joint repository of **expertise**

www.vbcf.ac.at

- Advanced Microscopy
- Bioinformatics & Scientific Computing
- Electron Microscopy
- Histopathology
- Metabolomics
- Next Generation Sequencing
- Plant Sciences
- Preclinical Imaging
- Protein Technologies
- Vienna Drosophila Resource Center
- Child Care Center

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WHO WE ARE
WHAT WE DO

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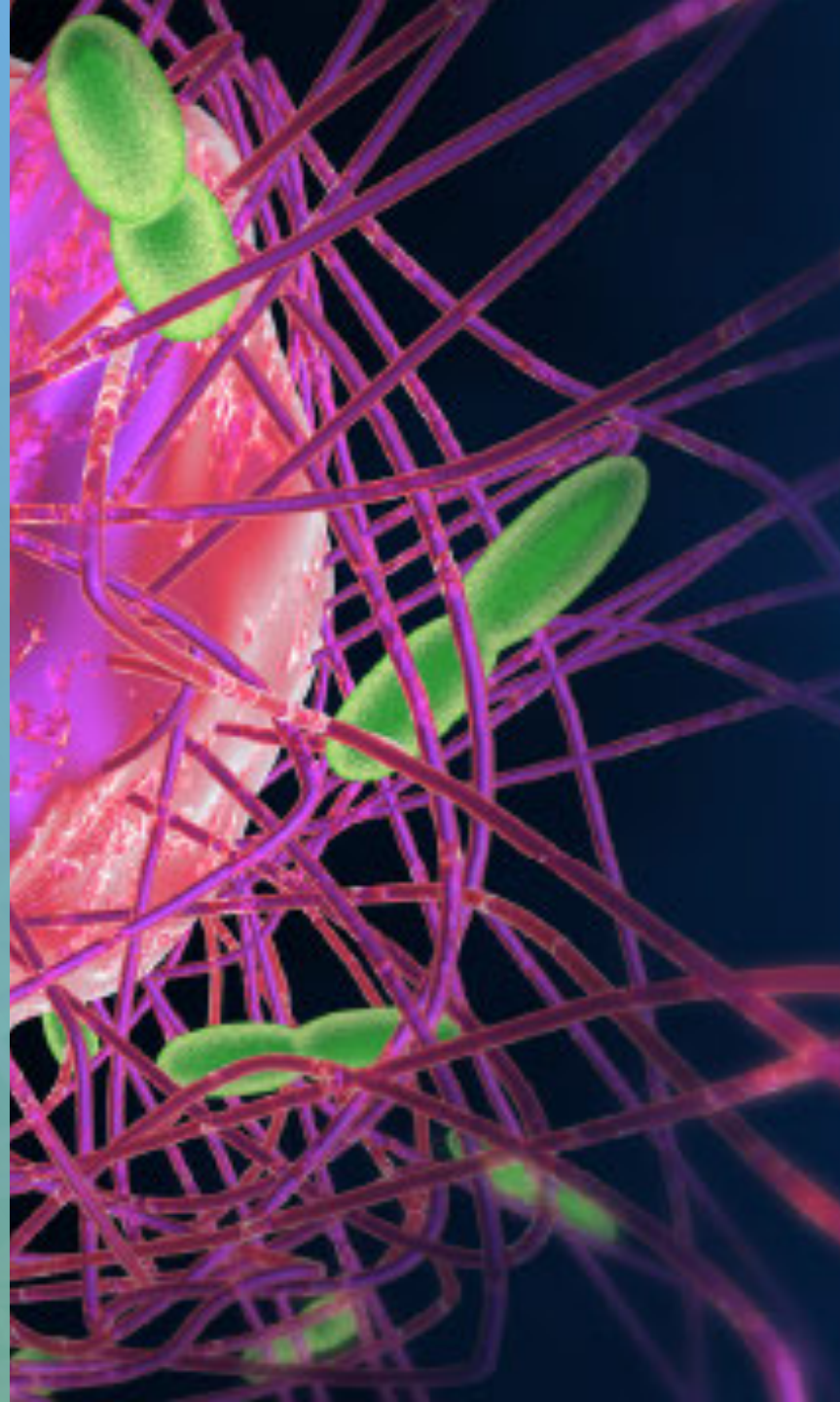
- Shareholders
  - IMP, GMI, IMBA, MPL
    - Each with their own agenda
    - Focus on services for the campus
- Funders
  - City of Vienna
    - Focus on services for companies within Vienna
  - Austrian Government
    - Focus on services for all scientists within Austria
- Scientific Community
  - Good laboratory practice, benchmarking
    - Focus on promoting world-wide science
- ProTech Services
  - Cloning
    - Different strategies, multi-gene expression, …
  - Expression
    - E.coli, insect cells, HEK cells
    - Small scale (construct/expression strategy screens)
    - Large scale (liters)
  - Purification
    - Small scale (to screen for optimal expression/purification strategies)
    - Large scale (for subsequent use)
  - Biophysical characterization
    - Details later on

THE REPRODUCIBILITY CRISIS

What is the current situation



ANALYSIS OF THE SITUATION AT CORE FACILITIES IN DIFFERENT EUROPEAN INSTITUTIONS

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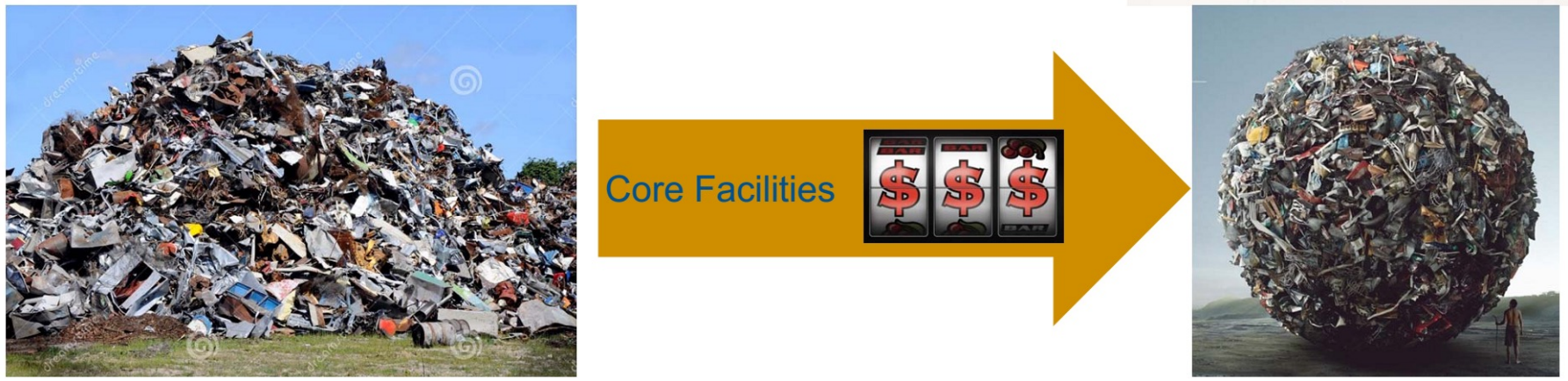
- **Lot of time is spent on poor quality samples**



- **The best experiments in the world will turn garbage in expensive garbage**

- **Improving the quality of the samples is essential to improve the quality of the results we produce**

THE INTERNATIONAL POSITION

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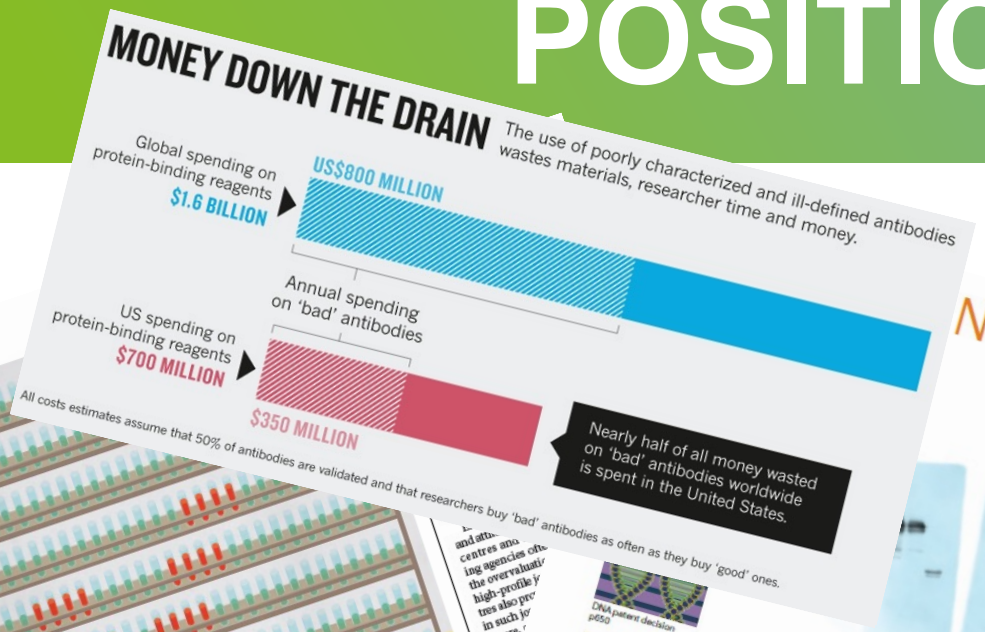

NEWS & ANALYSIS

THIS WEEK

unite for reproducibility

Reliability of 'new drug target' claims called into question

Bayer halts nearly two-thirds of its target-validation projects because in-house experimental findings match up with published literature claims, finds a first-of-a-kind analysis on data irreproducibility

Asher Mullard

NIH plans to enhance reproducibility

Francis S. Collins and Lawrence A. Tabak

ANNOUNCEMENT

Reducing our irreproducibility

RESEARCHER OPINION

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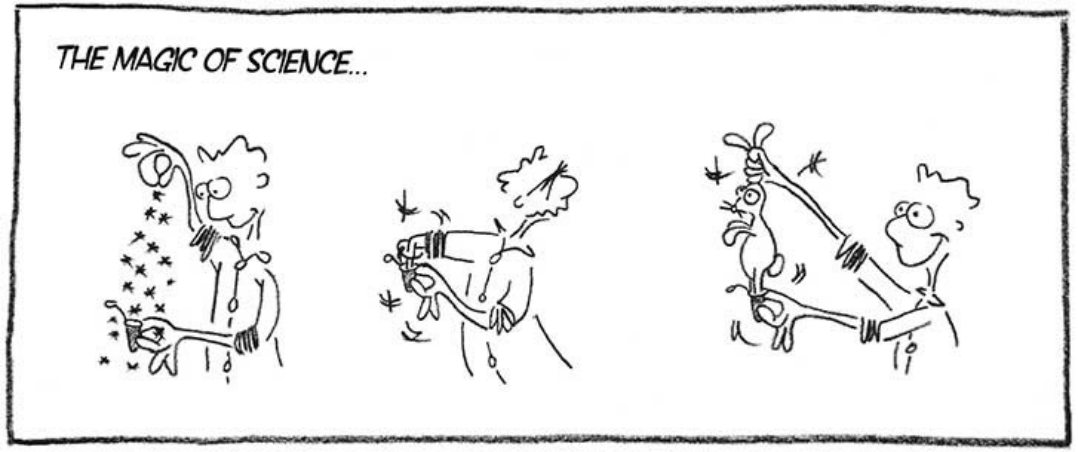
- **“I do not have time…”**
- **“My boss thinks it is a waste of time…”**
- **“It is the way we have prepared samples in the lab for the last ten years….”**
- **“But some experiments have worked with this sample…”**
- **“I do not know how to do it…”**
- **“I will do the experiment anyway it may work…”**



THE REALITY:

QUOTING PEOPLE'S EXPERIENCE WITH SAMPLE NOT FULLY VALIDATED

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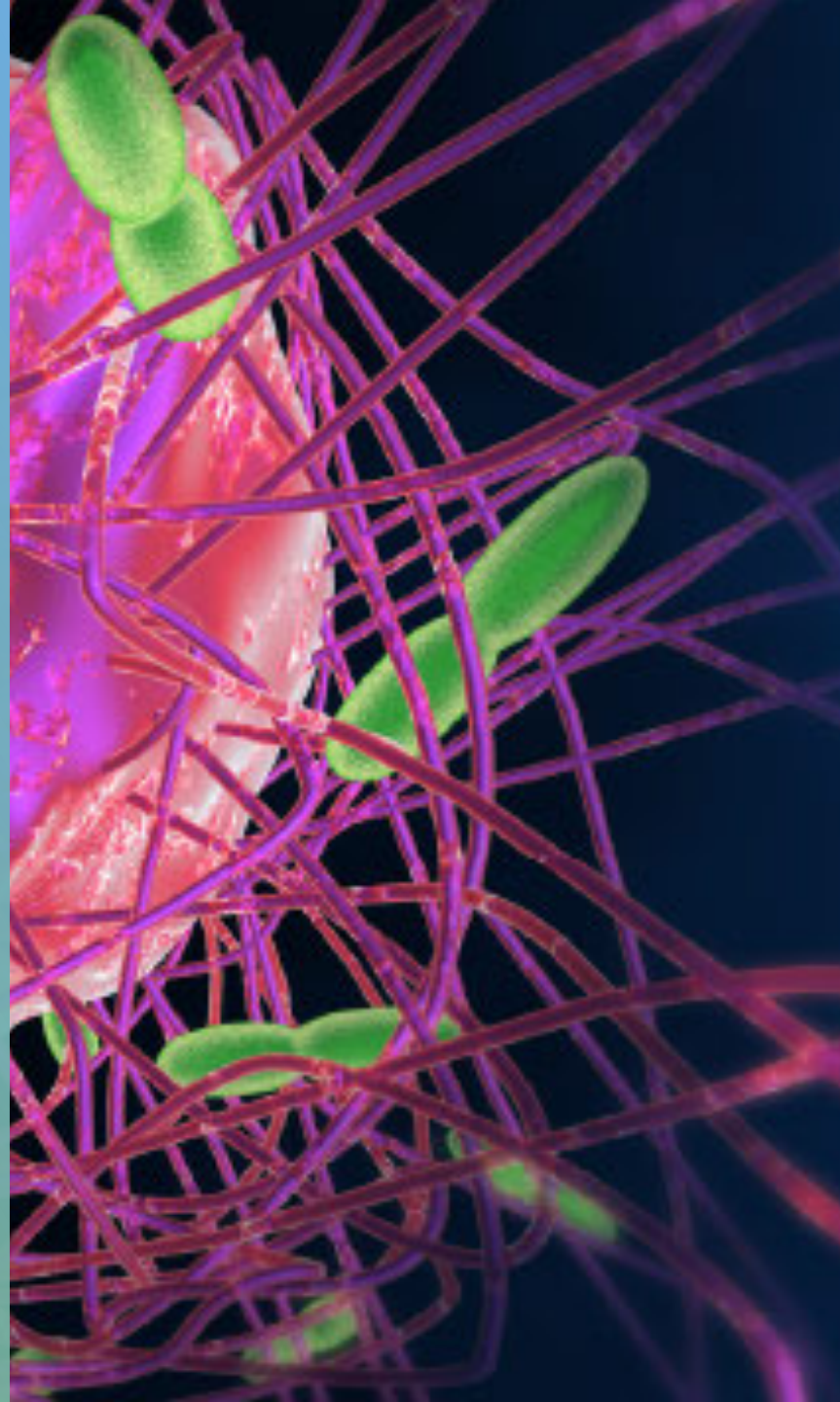
- **" We expressed a human protein in insect cells. We visualize by Coomasie stain in PAGE-SDS a protein band with a "correct" MW. We succeed to crystallize, and only then we "discover" that we did not crystallize our target. It was an ubiquitous insect cell protein "**
- **" We expressed a yeast protein. We visualize by Coomasie staining in SDS-PAGE a protein band with a "correct" MW. We did interaction studies and could not reproduce our previous data. After a test by mass spectrometry we have discovered that we were missing 4 amino acids at one end that were essential for the interaction."**
- **" I have done many unsuccessful tests of crystallization with my protein. I finally decide to do a simple quality test. I noticed that the protein was not homogenous in the buffer I used for purification. After buffer optimization, I have obtained a homogenous protein that crystallized."**

PROTEIN QUALITY CONTROL

Why checking protein quality matters



PROTEIN QUALITY CONTROL

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Association of **R**esources for **B**iophysical **R**esearch in **E**urope

https://www.arbre-biophysics.eu/

Protein **P**roduction and **P**urification **P**artnership in **Eu**rope

http://structuralbiology.eu/networks/p4eu

- List of minimum information you should know about your protein
- Survey on protein quality control for statistical analysis

WHAT? - MINIMUM INFORMATION ABOUT YOUR PROTEIN

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- **Protein name** and **full primary structure**, by providing a NCBI or UniProt accession number and cloning strategy

- Protein **concentration**
  - Specifying the method used for quantification and the molar extinction coefficient at 280nm, if applicable

- Storage conditions
  - i.e. final **buffer composition** (pH, buffers, salts and additives), storage temperature or lyophilization conditions

WHAT? – ADDITIONAL INFORMATION ABOUT YOUR PROTEIN

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- Gain additional information from primary structure using SW tools
  - Molecular weight
  - Amino acid / atomic composition
  - Number of Residues
  - Potential foldstate
  - …
- ExPASy ProtParam (http://web.expasy.org/protparam/)
  - pI
  - In vivo / in vitro stability
- Marius Clore Group NIH Protein Calculator (https://www.gmclore.org/clore/)
  - Molecular weight (including isotopes)
  - Molar Absorptivity @ 280nm AND @205nm
- Alphafold
  - Possible structures

PROTEIN QUALITY CONTROL

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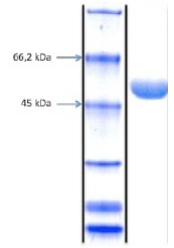
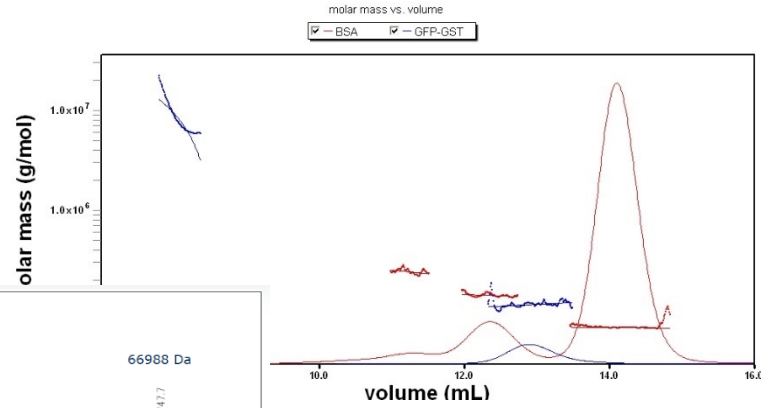
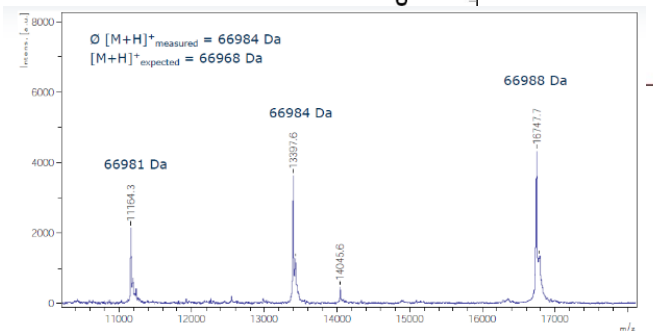
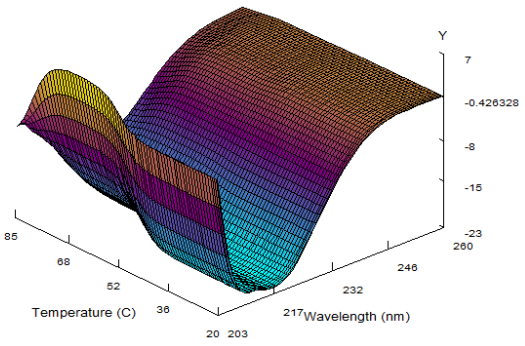
minimum

- Purity
- Homogeneity
- Identity
- Fold State
- Batch to Batch consistency





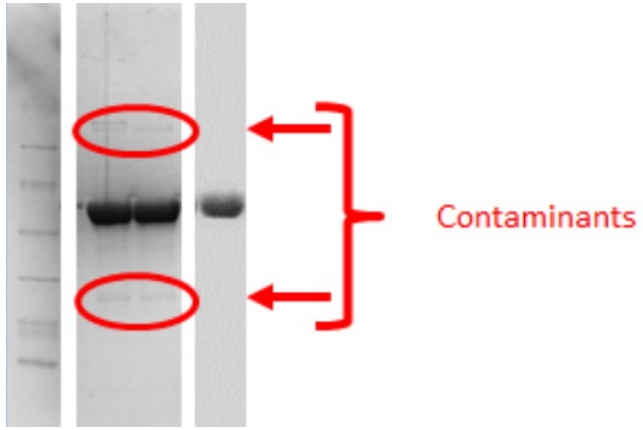




PURITY

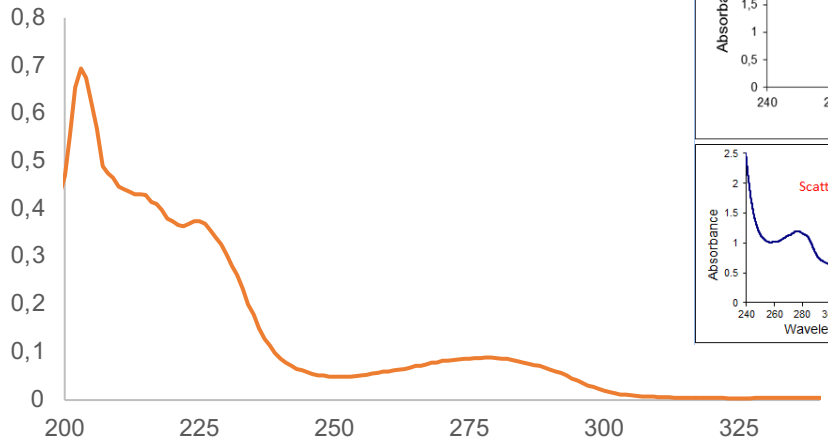
- SDS-PAGE or similar (Protein contamination)
  - Fluorescent / silver staining recommended!

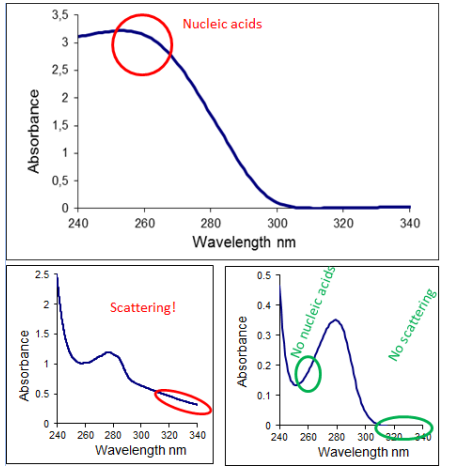
length of primary sequence



- UV-vis spectrum from 200 nm to 340 nm or higher
  - DNA / small molecule contamination + protein concentration as bonus!
  - Don't use the Nano drop (<10mg/ml)
    1cm cuvettes are your friend!

absorbance of whole sample





HOMOGENEITY

- Size Exclusion Chromatography +

  Static Light Scattering (SEC-MALLS)

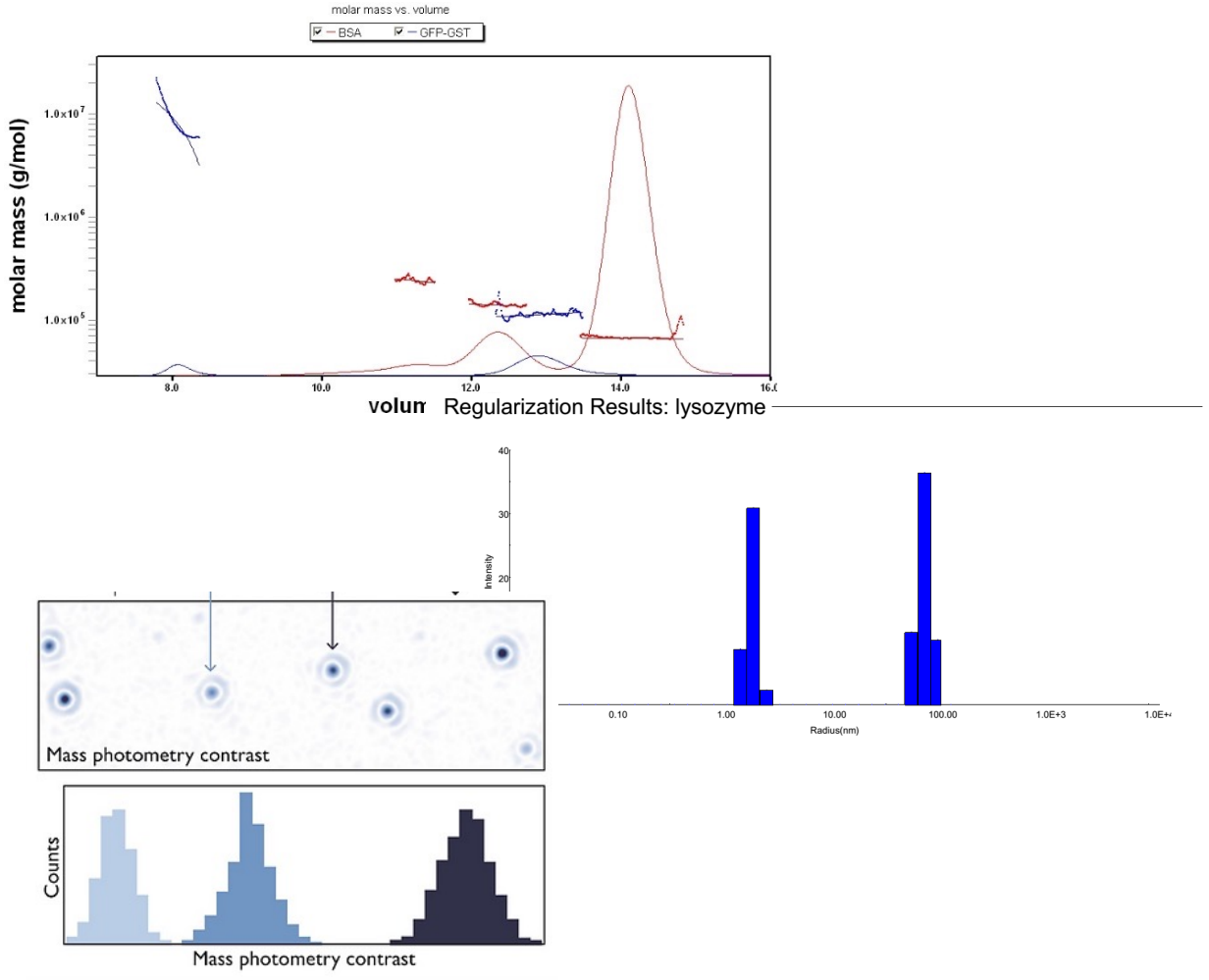
  size / shape / column interaction + mass

- Dynamic Light Scattering (DLS)

  diffusion / size

- Mass photometry (Refeyn One)

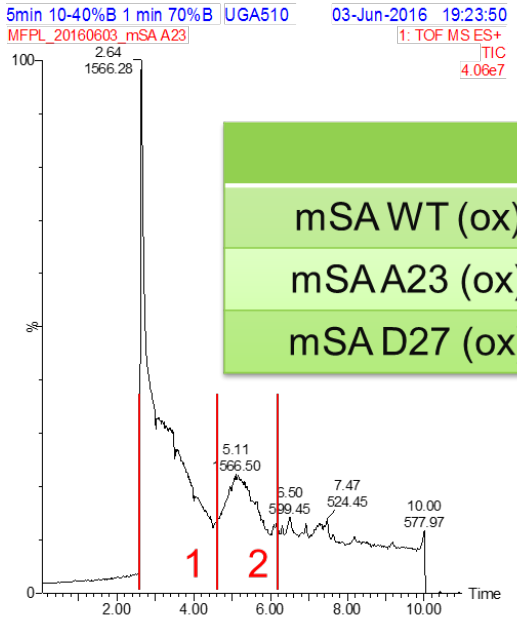
  Single particle mass distribution



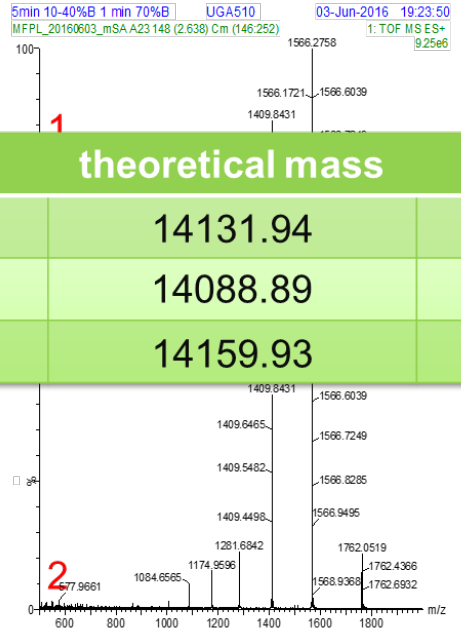
IDENTITY

- Intact mass spec — molecular mass
- Peptide mass fingerprint mass spec — peptide sequences (from mass)
  - mSA A23 MS

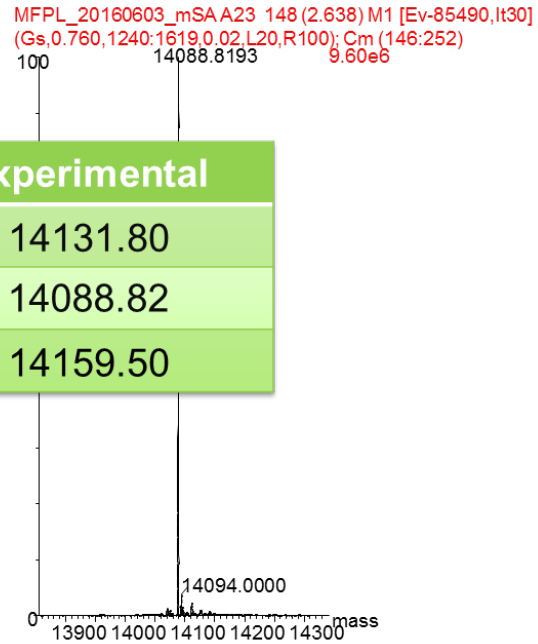
MS chromatogram



Raw Spectrum



Deconvoluted Spectrum



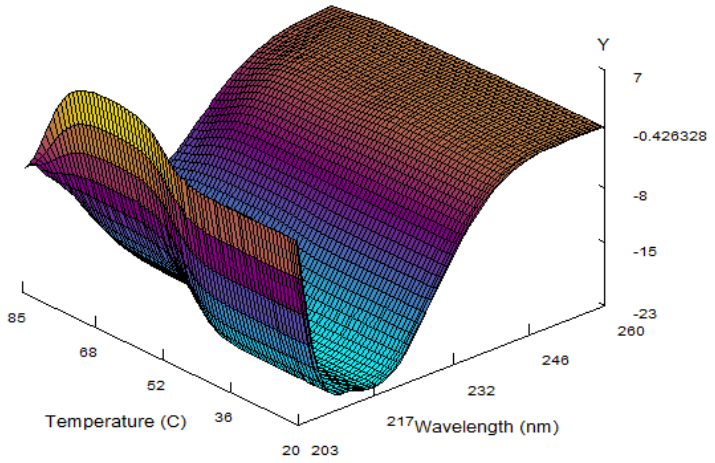
	theoretical mass	experimental
mSA WT (ox)	14131.94	14131.80
mSA A23 (ox)	14088.89	14088.82
mSA D27 (ox)	14159.93	14159.50

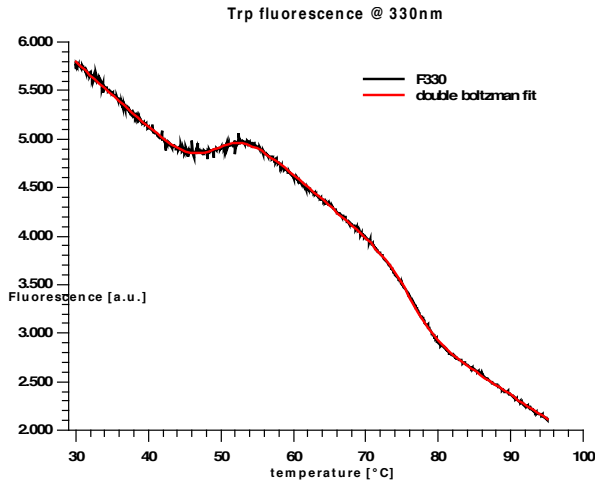
FOLD STATE

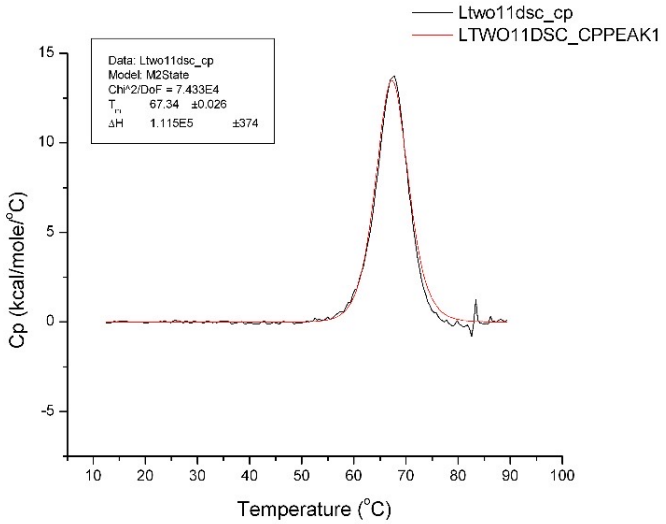
- “Melting” using CD / Thermofluor / nanoDSF (Prometheus) / DSC etc.

secondary / tertiary structure

temperature dependent “steps”







- **Batch to Batch consistency**
  - **CD** / DLS or any method mentioned before

SURVEY ABOUT QC

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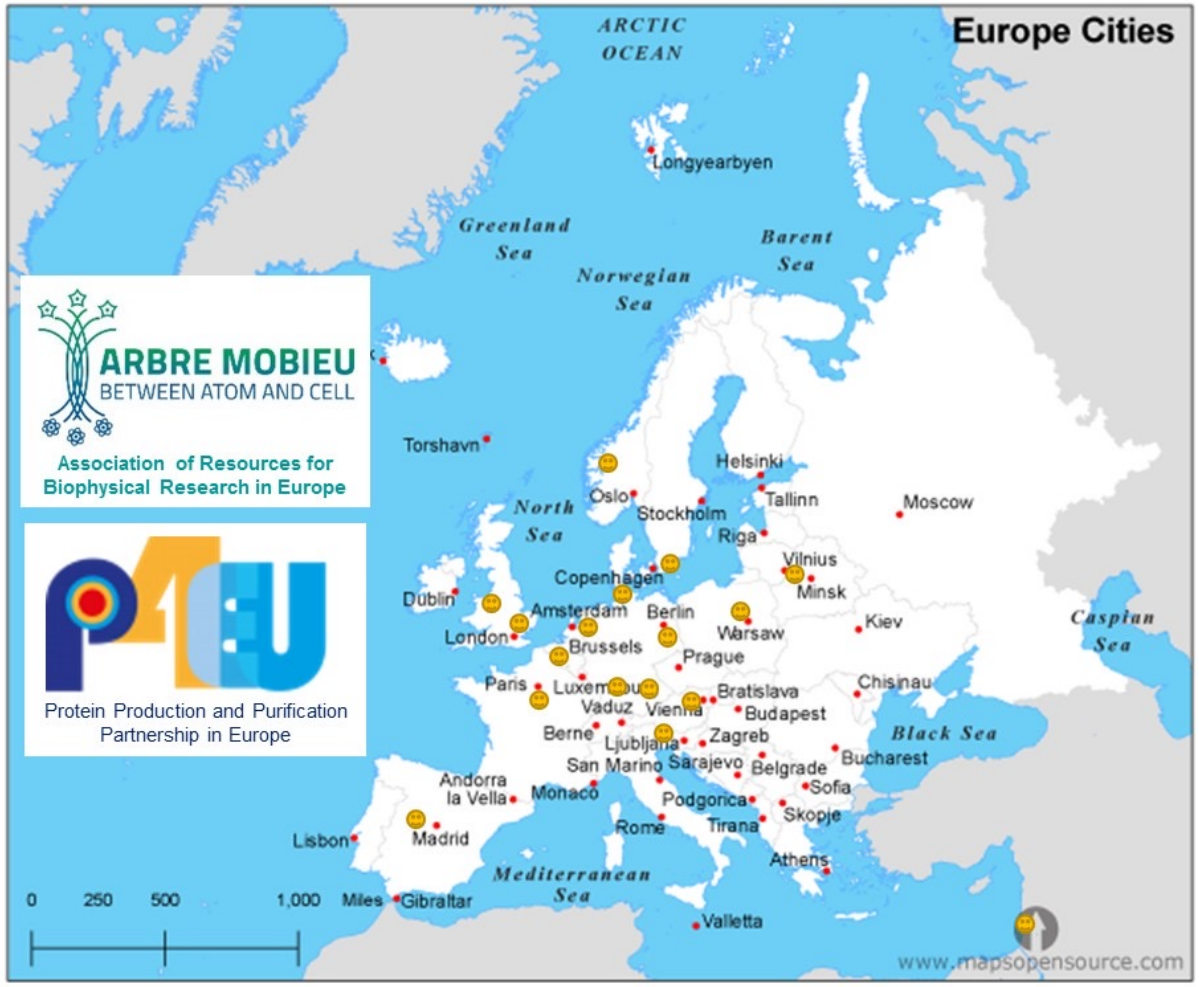
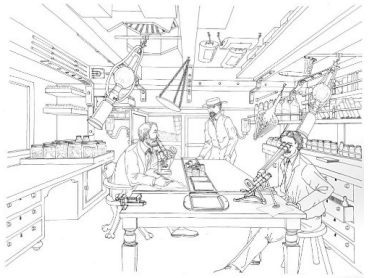


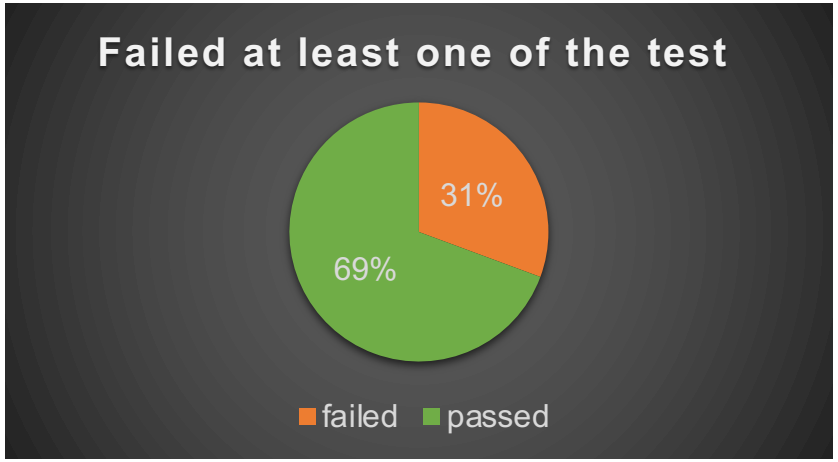
31 institutions



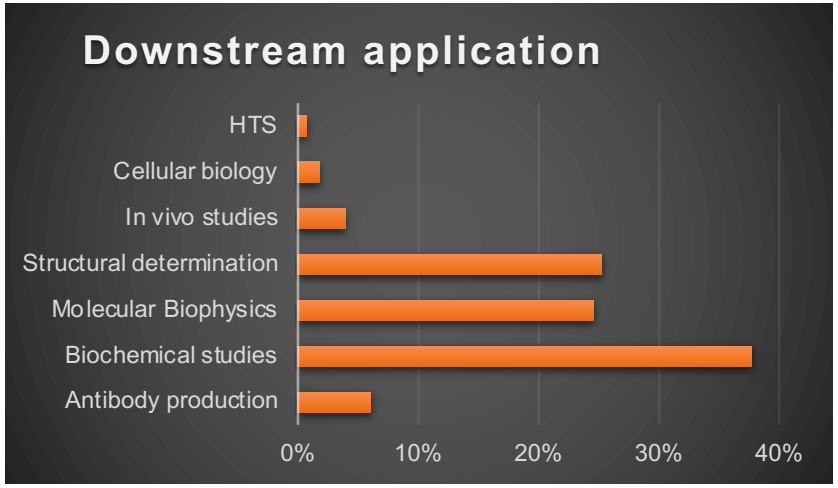
47 laboratories

186 samples

RESULTS

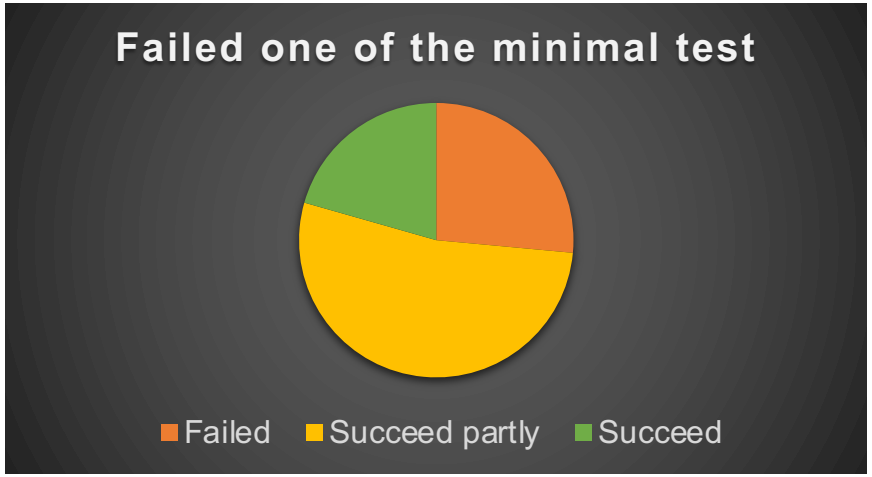


Out of the one that have been tested for purity, homogeneity, and identity, 1/3 of the samples did not pass all the tests



The downstream application was known for 130 samples out of 186.

EFFECT ON DOWNSTREAM APPLICATION



26% failed in the downstream application
53% succeeded only partly in the downstream application
21% succeeded in the downstream application

In conclusion only a 1/5 did succeed fully

6% failed in the downstream application
20% succeeded only partly in the downstream application
74% succeeded in the downstream application

In conclusion 3/4 did succeed fully

PROTEIN QUALITY CONTROL

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- **Purity**
- **Homogeneity**
- **Identity**
- Fold State
- Batch to Batch consistency

All the methods within ONE day!

ropean Biophysics Journal (**2021**)

REFERENCE ON PROTEIN QUALITY CONTROL

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Lebendiker *et al. BMC Research Notes* 2014, **7**:585
http://www.biomedcentral.com/1756-0500/7/585

BMC Research No

CORRESPONDENCE Open Acce

The Trip Adviser guide to the protein science world: a proposal to improve the awareness concerning the quality of recombinant proteins

Mario Lebendiker[1†], Tsafi Danieli[1†] and Ario de Marco[2*]

Protein production and purification

Structural Genomics Consortium[1–3], Architecture et Fonction des Macromolécules Biologiques[4], Berkeley Structural Genomics Center[5], China Structural Genomics Consortium[6,7], Integrated Center for Structure and Function Innovation[8], Israel Structural Proteomics Center[9], Joint Center for Structural Genomics[10,11], Midwest Center for Structural Genomics[12], New York Structural GenomiX Research Center for Structural Genomics[13–17], Northeast Structural Genomics Consortium[18,19], Oxford Protein Production Facility[20], Protein Sample Production Facility, Max Delbrück Center for Molecular Medicine[21], RIKEN Structural Genomics/ Proteomics Initiative[22] & SPINE2-Complexes[23,25]

NATURE METHODS | VOL.5 NO.2 | FEBRUARY 2008 | 135

Commentary Open A

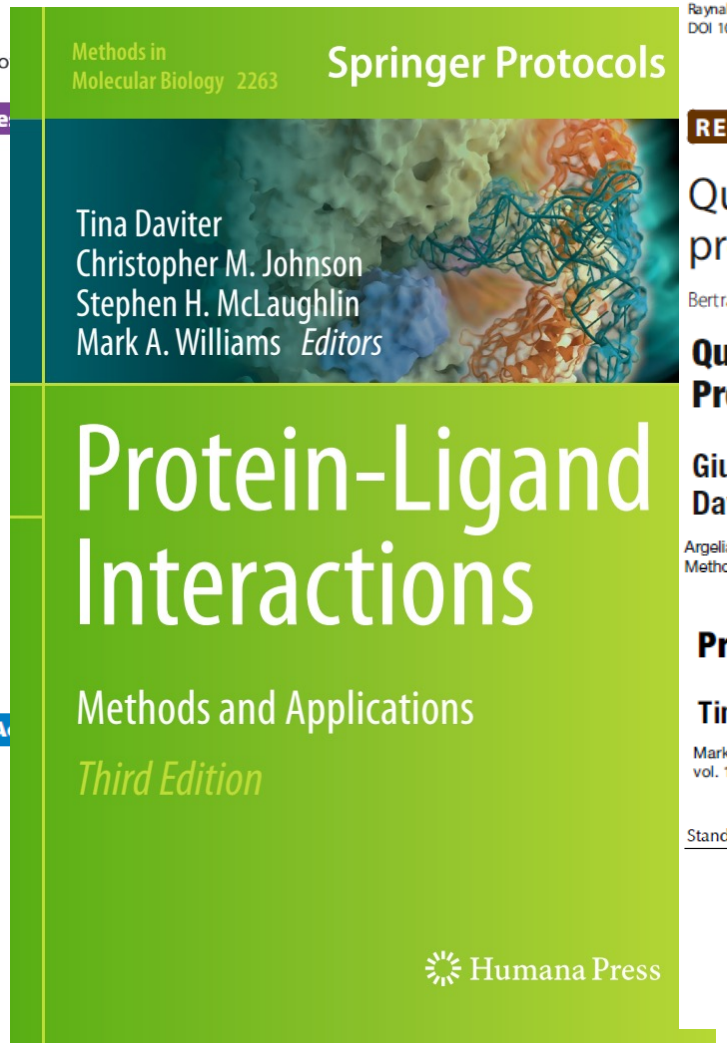
Minimal information: an urgent need to assess the functional reliability of recombinant proteins used in biological experiments

Ario de Marco

Address: COGENTECH, via Adamello 16, 20139, Milano, Italy
Email: Ario de Marco - ario.demarco@ifom-ieo-campus.it

Published: 23 July 2008
Microbial Cell Factories 2008, **7**:20 doi:10.1186/1475-2859-7-20

Received: 24 June 2008
Accepted: 23 July 2008



Raynal *et al. Microbial Cell Factories* (2014) 13:180
DOI 10.1186/s12934-014-0180-6

MICROBIAL CELL FACTORIES

REVIEW Open Access

Quality assessment and optimization of purified protein samples: why and how?

Bertrand Raynal[1,2*], Pascal Lenormand[1,2], Bruno Baron[1,2], Sylviane Hoos[1,2] and Patrick England[1,2*]

Quality Assessment of Recombinant Proteins Produced in Plants

Giuliana Medrano, Maureen C. Dolan, Jose Condori, David N. Radin, and Carole L. Cramer

Argelia Lorence (ed.), *Recombinant Gene Expression: Reviews and Protocols, Third Edition*, Methods in Molecular Biology, vol. 824, DOI 10.1007/978-1-61779-433-9_29, © Springer Science+Business Media, LLC 2012

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Protein Sample Characterization

Tina Daviter and Rémi Fronzes

Mark A. Williams and Tina Daviter (eds.), *Protein-Ligand Interactions: Methods and Applications*, Methods in Molecular Biology, vol. 1008, DOI 10.1007/978-1-62703-398-5_2, © Springer Science+Business Media New York 2013

35

Standards in Genomic Sciences (2011) 5:195-197 DOI:10.4056/sigs.1834511

Recombinant protein quality evaluation: proposal for a minimal information standard

Ashley M. Buckle[1,15], Mark A. Bate[1], Steve Androulakis[2], Mario Cinquanta[3], Jerome Basquin[4], Fabien Bonneau[4], Deb K. Chatterjee[5], Davide Cittaro[3], Susanne Gräslund[6], Alicja Gruszka[7], Rebecca Page[8], Sabine Suppmann[9], Jun X. Wheeler[10], Deborah Agostini[3], Mike Taussig[11], Chris F. Taylor[12], Stephen P. Bottomley[1], Antonio Villaverde[13], Ario de Marco[14,*]

PROTEIN QUALITY CONTROL DONE

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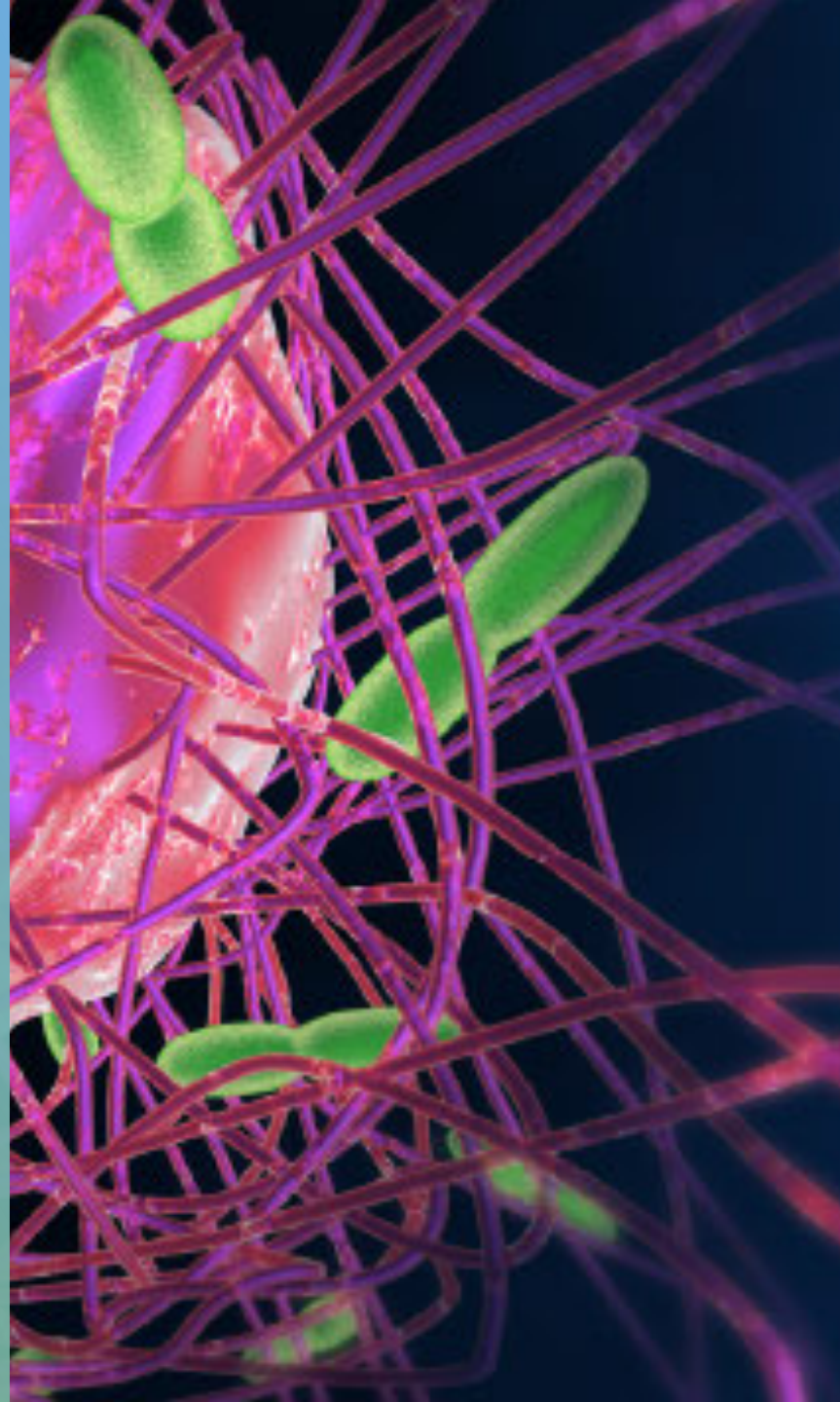
- Sample in good shape -> great!

- Sample in bad shape -> ☹
- What to do? -> Optimization!

PROTEIN QUALITY OPTIMIZATION

How to make your proteins happy



HOW TO FURTHER OPTIMIZE?

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- Optimization of experimental / storage conditions

Buffers
pH
Salt / ions
detergents
Cofactors / ligands
Protein concentration
…

Protein is out of cellular environment!

Screening of different conditions

WHAT TO OPTIMIZE FOR?

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- Stability
  - unfolding / degradation / aggregation
  - during the time of your experiment (>10 h)
  - Temperature dependence (Tm)
  - Freeze – thaw cycles
- Crystalizability
- Homogeneity

SCREENING METHODS

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- Thermofluor (DSF) syprOrange/ANS — fluorescent dye + RT-PCR
- nanoDSF — Turbidity / trp fluorescence
- DLS — diffusion / size NO LABEL
- Mass photometry — Mass distributions NO LABEL

THERMOFLUOR

Differential Scanning Fluorimetry (DSF) Thermal Shift Assay (TSA)

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THERMOFLUOR

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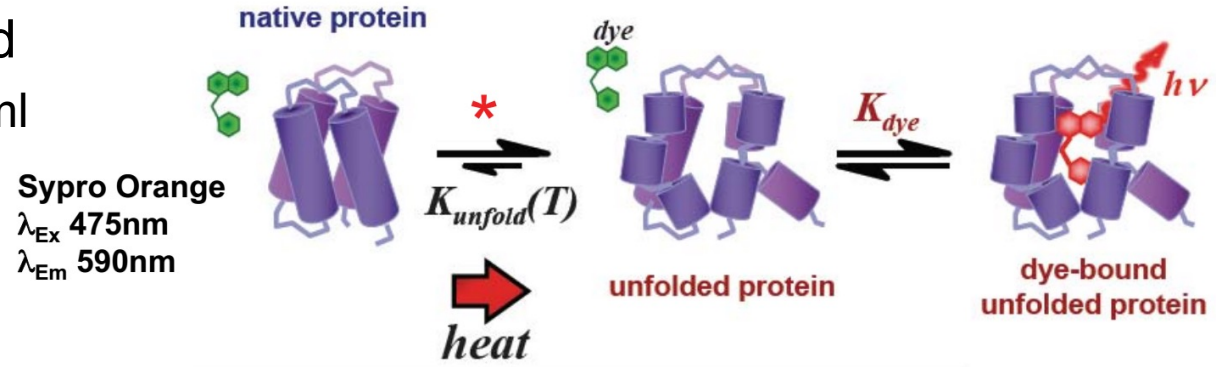
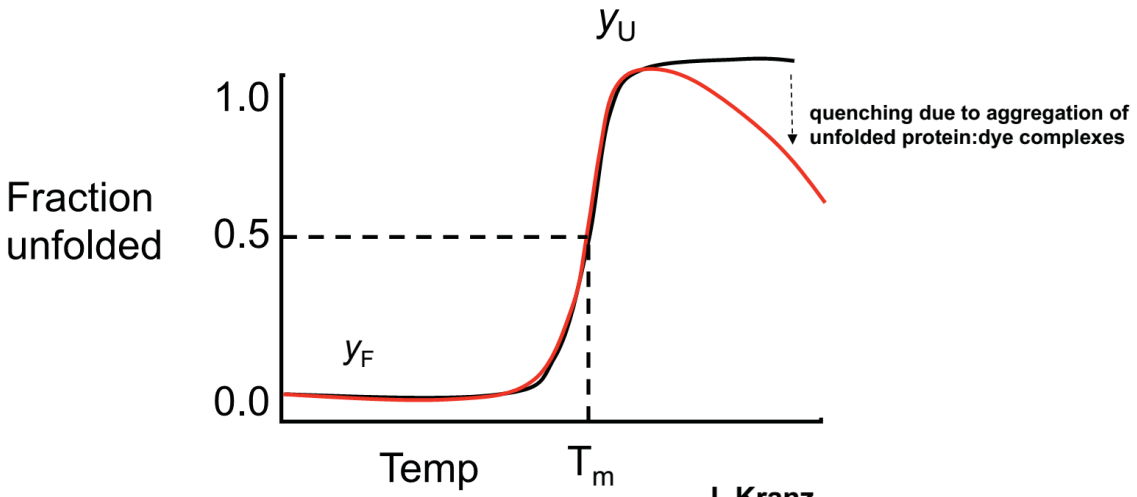
- 96-well plates
- RT-PCR machine
- ~1hour
- Disadvantages
  - Dye
  - Just Tm value
  - (ΔH possible)
  - No membrane proteins

- Material needed
  - 4µl 0.3-2mg/ml





J. Kranz
Johnson & Johnson Pharmaceutical Research & Development

NANODSF

Prometheus

http://www.nanotemper-technologies

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NANODSF – PROMETHEUS

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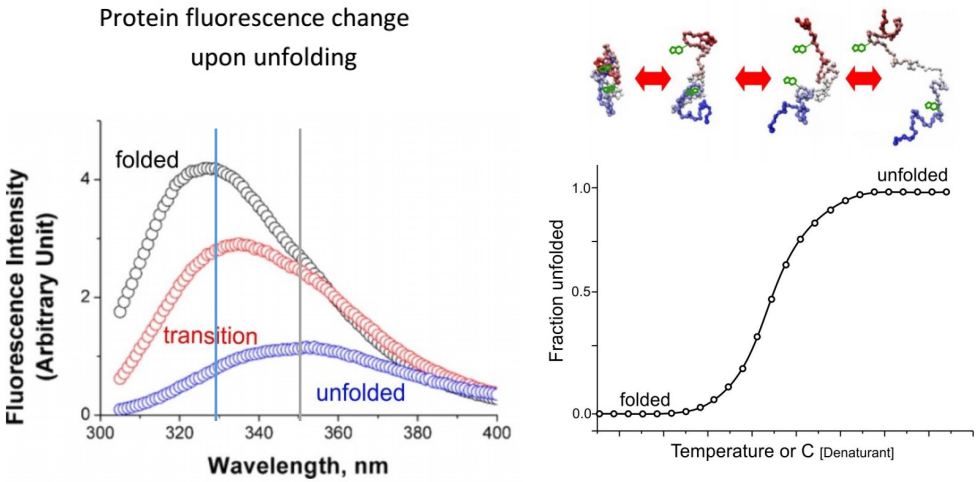
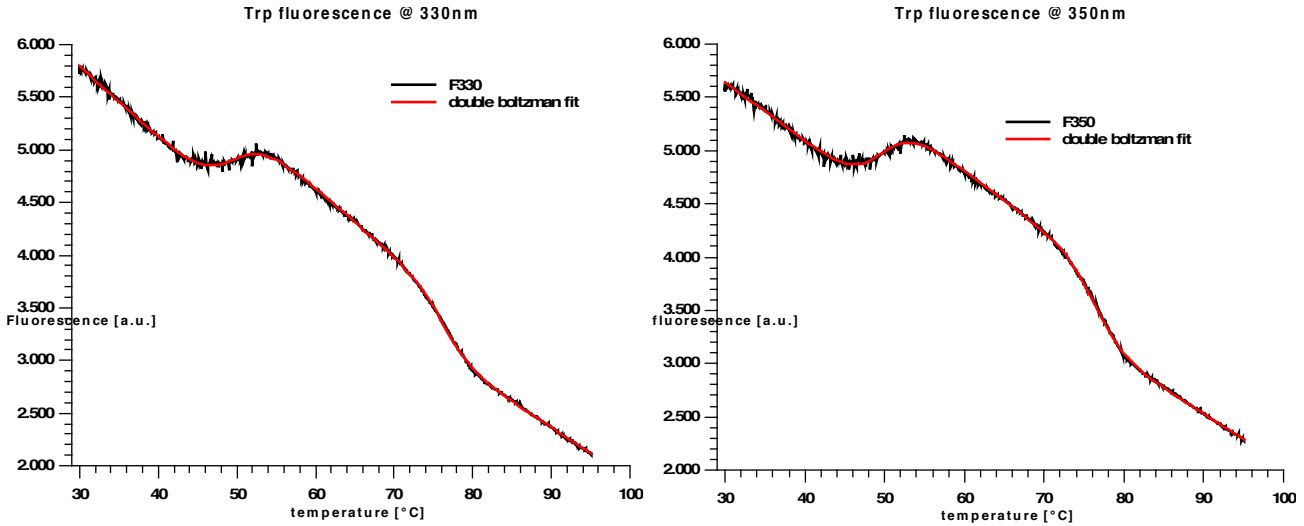
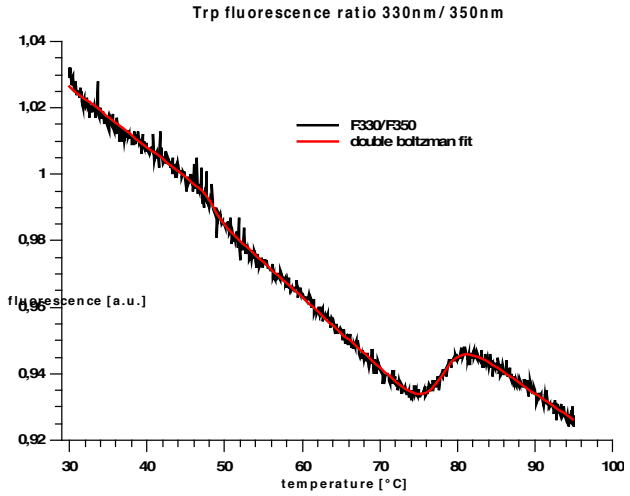
- TRP fluorescence @330nm and 350nm
- Protein folding



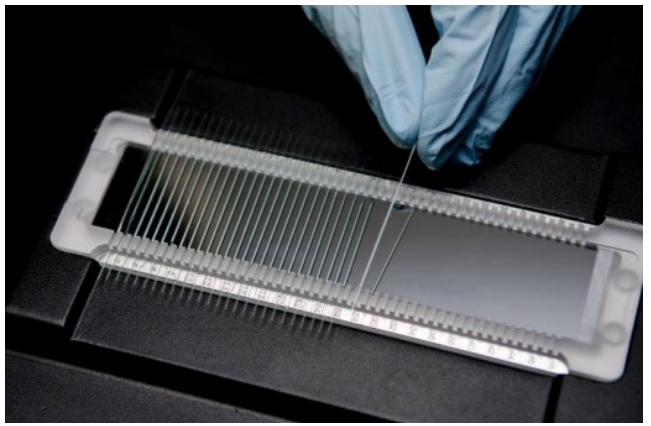




NANODSF – PROMETHEUS

Vienna BioCenter CORE FACILITIES

- ~100nM @ 10µl x 48 samples
- Thermal unfolding (Tm)
- Chaotrop denaturation (GndCl, Urea, ...)
- Time dependence
- Similar to thermofluor (DSF), but label free



DLS PLATE READER

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DLS PLATEREADER

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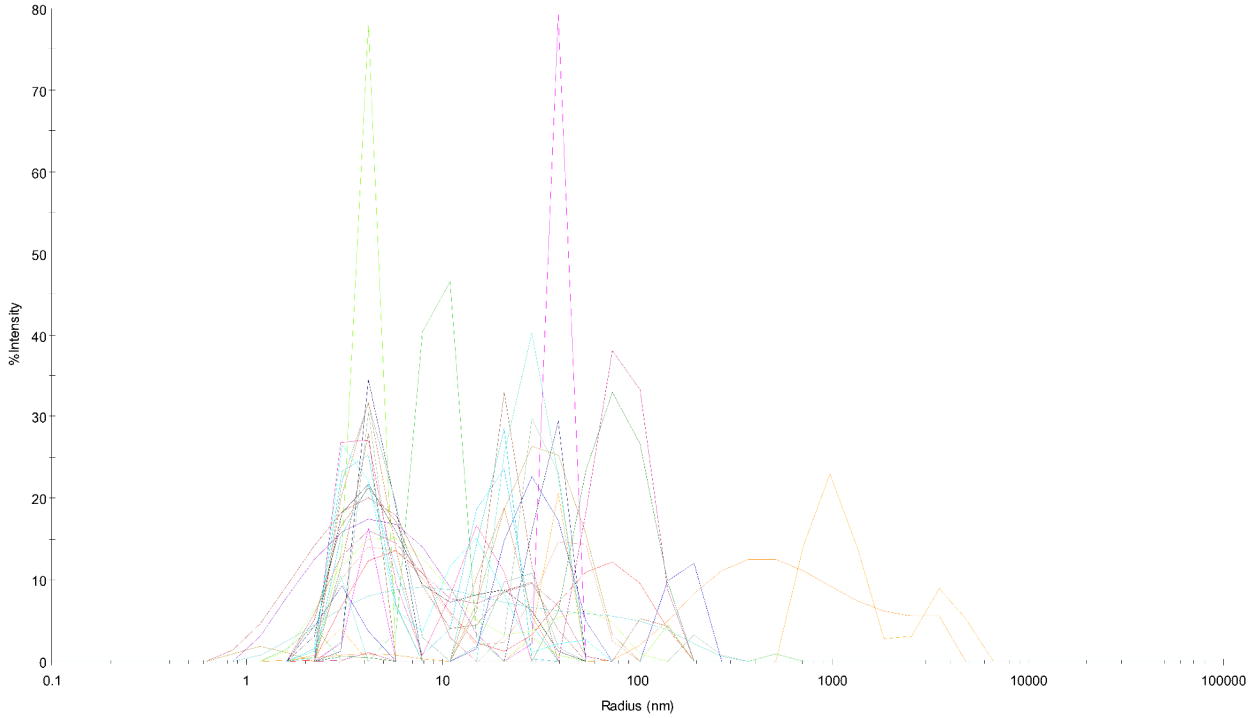
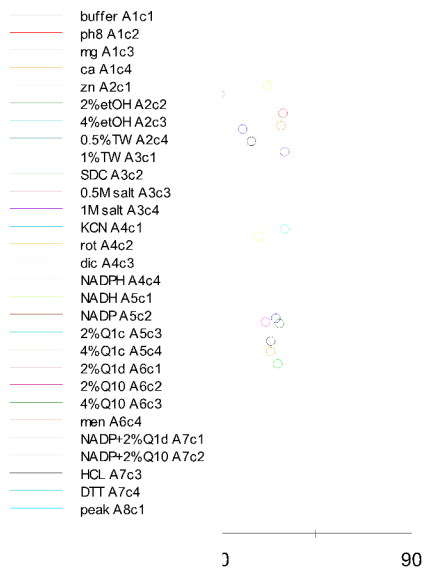
- Light scattering (label free)
- 6µl sample >0.2mg/ml in 1536 well plate
- Time dependent / temperature depend aggregation

diffusion / size





BUFFER SCREEN AGGREGATES/STABILITY

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96 conditions

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sodium Citrate pH 5 10% Glycerol 200 mM NaCl	Sodium Citrate pH 5.5 10% Glycerol 400 mM NaCl	Sodium Citrate pH 5 10% Glycerol 200 mM NaCl 1 mM TCEP	Sodium Citrate pH 5 10% Glycerol 400 mM NaCl 1 mM TCEP	Sodium Citrate pH 5 10% Glycerol 400 mM KCl 1 mM TCEP	Sodium Citrate pH 5. 10% Glycerol 400 mM NaCl 1 mM DTT	Sodium Citrate pH 5. 10% Glycerol 400 mM NaCl 5 mM DTT	Sodium Citrate pH 5 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	Sodium Citrate pH 5 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	Sodium Citrate pH 5 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	Sodium Citrate pH 5 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	Sodium Citrate pH 5.5 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu
B	MES pH 6.2 10% Glycerol 200 mM NaCl	MES pH 6.2 10% Glycerol 400 mM NaCl	MES pH 6.2 10% Glycerol 200 mM NaCl 1 mM TCEP	MES pH 6.2 10% Glycerol 400 mM NaCl 1 mM TCEP	MES pH 6.2 10% Glycerol 400 mM KCl 1 mM TCEP	MES pH 6.2 10% Glycerol 400 mM NaCl 1 mM DTT	MES pH 6.2 10% Glycerol 400 mM NaCl 5 mM DTT	MES pH 6.2 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	MES pH 6.2 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	MES pH 6.2 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	MES pH 6.2 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	MES pH 6.2 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu
C	Phosphate pH 6.5 10% Glycerol 200 mM NaCl	Phosphate pH 6.5 10% Glycerol 400 mM NaCl	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 1 mM DTT	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 5 mM DTT	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 1 mM EDTA	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 5 mM EDTA	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 1 mM DTT 5 mM EDTA	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 1 mM DTT 0.05% CHAPS	Phosphate pH 6.5 10% Glycerol 400 mM NaCl 1 mM DTT PEG400	Phosphate pH 6.5	Phosphate pH 6.5 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	Phosphate pH 6.5 10% Glycerol 200 mM NaCl 1 mM DTT 0.15 mM Arg/Glu
D	PBS from Alex 10% Glycerol 200 mM NaCl	PBS from Alex 10% Glycerol 400 mM NaCl	PBS from Alex 10% Glycerol 200 mM NaCl 1 mM DTT	PBS from Alex 10% Glycerol 400 mM NaCl 5 mM DTT	PBS from Alex 10% Glycerol 400 mM KCl 1 mM EDTA	PBS from Alex 10% Glycerol 400 mM NaCl 5 mM EDTA	PBS from Alex 10% Glycerol 400 mM NaCl 1 mM DTT 5 mM EDTA	PBS from Alex 10% Glycerol 400 mM NaCl 1 mM DTT 0.05% CHAPS	PBS from Alex 10% Glycerol 400 mM NaCl 1 mM DTT PEG400	PBS from Alex	PBS from Alex 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	PBS from Alex 10% Glycerol 200 mM NaCl 1 mM DTT 0.15 mM Arg/Glu
E	HEPES pH 7.5 10% Glycerol 200 mM NaCl	HEPES pH 7.5 10% Glycerol 400 mM NaCl	HEPES pH 7.5 10% Glycerol 200 mM NaCl 1 mM TCEP	HEPES pH 7.5 10% Glycerol 400 mM NaCl 1 mM TCEP	HEPES pH 7.5 10% Glycerol 400 mM KCl 1 mM TCEP	HEPES pH 7.5 10% Glycerol 400 mM NaCl 1 mM DTT	HEPES pH 7.5 10% Glycerol 400 mM NaCl 5 mM DTT	HEPES pH 7.5 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	HEPES pH 7.5 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	HEPES pH 7.5 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	HEPES pH 7.5 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	HEPES pH 7.5 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu
F	Tris pH 8 10% Glycerol 200 mM NaCl	Tris pH 8 10% Glycerol 400 mM NaCl	Tris pH 8 10% Glycerol 200 mM NaCl 1 mM TCEP	Tris pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP	Tris pH 8 10% Glycerol 400 mM KCl 1 mM TCEP	Tris pH 8 10% Glycerol 400 mM NaCl 1 mM DTT	Tris pH 8 10% Glycerol 400 mM NaCl 5 mM DTT	Tris pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	Tris pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	Tris pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	Tris pH 8 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	Tris pH 8 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu
G	EPPS pH 8 10% Glycerol 200 mM NaCl	EPPS pH 8 10% Glycerol 400 mM NaCl	EPPS pH 8 10% Glycerol 200 mM NaCl 1 mM TCEP	EPPS pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP	EPPS pH 8 10% Glycerol 400 mM KCl 1 mM TCEP	EPPS pH 8 10% Glycerol 400 mM NaCl 1 mM DTT	EPPS pH 8 10% Glycerol 400 mM NaCl 5 mM DTT	EPPS pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	EPPS pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	EPPS pH 8 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	EPPS pH 8 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	EPPS pH 8 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu
H	CHES pH 9.0 10% Glycerol 200 mM NaCl	CHES pH 9.0 10% Glycerol 400 mM NaCl	CHES pH 9.0 10% Glycerol 200 mM NaCl 1 mM TCEP	CHES pH 9.0 10% Glycerol 400 mM NaCl 1 mM TCEP	CHES pH 9.0 10% Glycerol 400 mM KCl 1 mM TCEP	CHES pH 9.0 10% Glycerol 400 mM NaCl 1 mM DTT	CHES pH 9.0 10% Glycerol 400 mM NaCl 5 mM DTT	CHES pH 9.0 10% Glycerol 400 mM NaCl 1 mM TCEP 0.05% CHAPS	CHES pH 9.0 10% Glycerol 400 mM NaCl 1 mM TCEP PEG400	CHES pH 9.0 10% Glycerol 400 mM NaCl 1 mM TCEP 5 mM CaCl2	CHES pH 9.0 10% Glycerol 200 mM NaCl 0.15 mM Arg/Glu	CHES pH 9.0 10% Glycerol 200 mM NaCl 1 mM TCEP 0.15 mM Arg/Glu

BUFFER SCREEN AGGREGATES/STABILITY

Vienna BioCenter CORE FACILITIES

Radius measured at the beginning

96-Well	1	2	3	4	5	6	7	8	9	10	11	12
A	51,33	46,20	62,97	49,90	44,77	52,70	45,60	7,30	53,40	53,35	74,27	67,03
B	103,10	76,67	198,33	59,90	#DIV/0!	48,97	52,50	5,37	59,27	69,03	70,97	68,70
C	50,43	53,25	91,50	#DIV/0!	50,15	56,10	40,10	5,10	46,05	45,80	88,70	42,20
D	49,90	51,45	52,65	61,05	44,35	48,45	47,90	3,70	40,85	57,10	53,95	43,85
E	57,65	46,40	51,20	42,95	45,60	55,55	42,90	6,45	60,50	99,05	57,65	40,30
F	54,10	42,30	60,80	56,35	48,10	44,65	53,90	5,60	57,85	242,25	50,65	50,25
G	63,40	44,80	50,40	75,05	51,85	293,90	49,45	5,30	51,20	127,10	49,90	45,80
H	63,00	63,30	47,85	53,50	51,50	48,70	50,85	5,20	110,60	855,90	39,75	607,30

Radius measured after 2 days

96-Well	1	2	3	4	5	6	7	8	9	10	11	12
A	122,68	145,82	#DIV/0!	#DIV/0!	#DIV/0!	55,90	134,50	22,30	#DIV/0!	#DIV/0!	75,16	113,80
B	9,90	45,26	264,80	103,17	164,84	72,22	99,38	3,15	133,96	121,48	148,12	247,07
C	23,62	18,72	#DIV/0!	9,83	29,38	65,52	11,88	158,73	52,68	22,93	131,28	37,18
D	20,92	15,10	10,32	13,00	12,06	9,66	9,86	2,73	16,86	13,08	13,84	6,66
E	#DIV/0!	12,94	26,33	24,16	22,92	43,36	18,08	2,28	28,70	207,45	12,64	24,42
F	41,76	10,30	25,52	7,62	11,04	8,58	11,42	2,35	9,12	162,94	4,56	6,96
G	35,30	2,98	9309,93	8,08	11,38	2575,72	6,60	2,38	9,38	205,52	3,96	6,62
H	#DIV/0!	3,20	14,28	4,00	4,76	6,36	7,92	0,64	116,36	185,58	5,14	0,40

SAMPLE STABILITY OVER TIME

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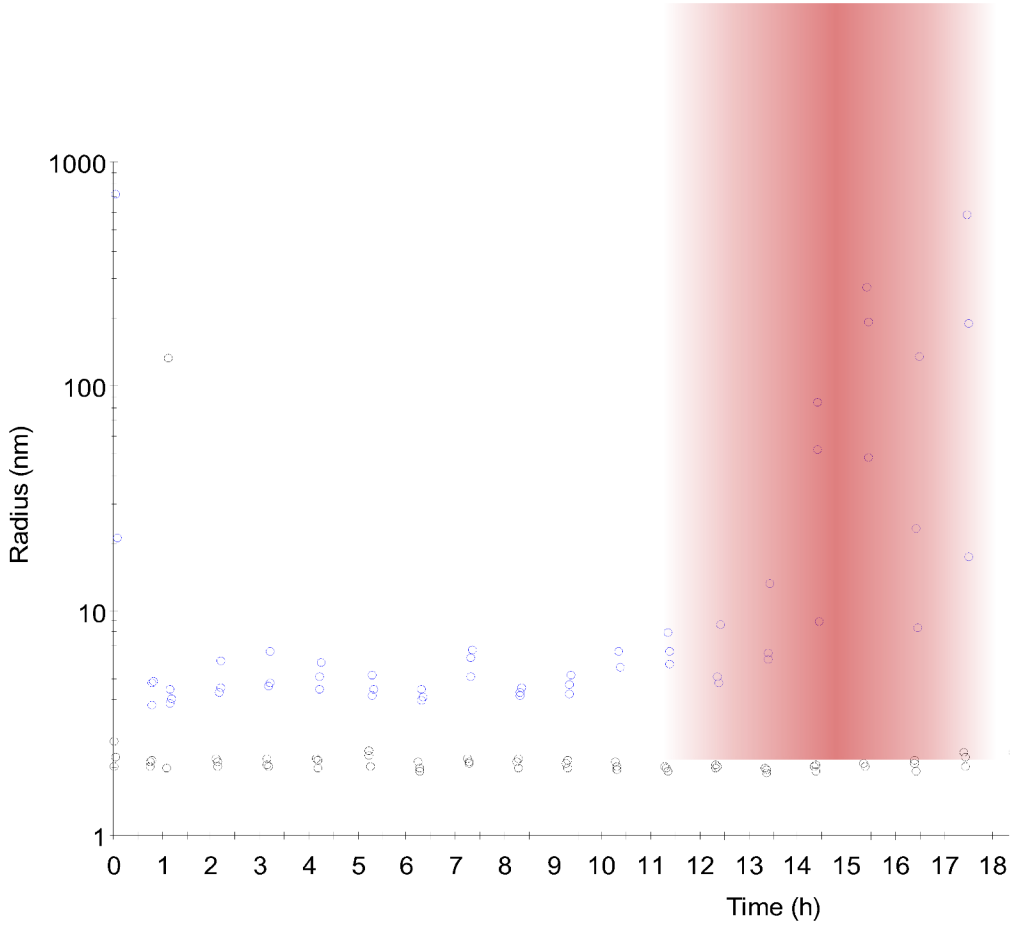
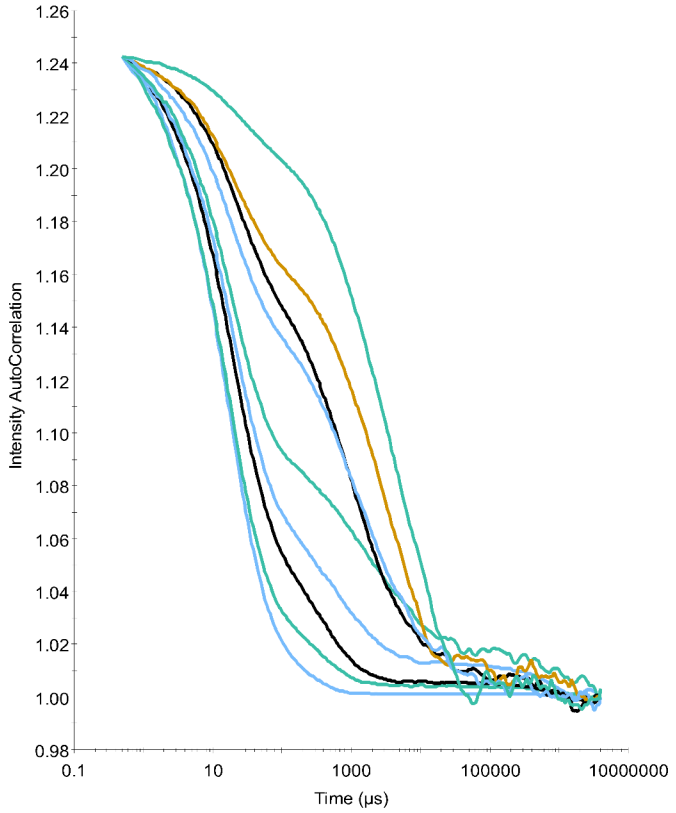


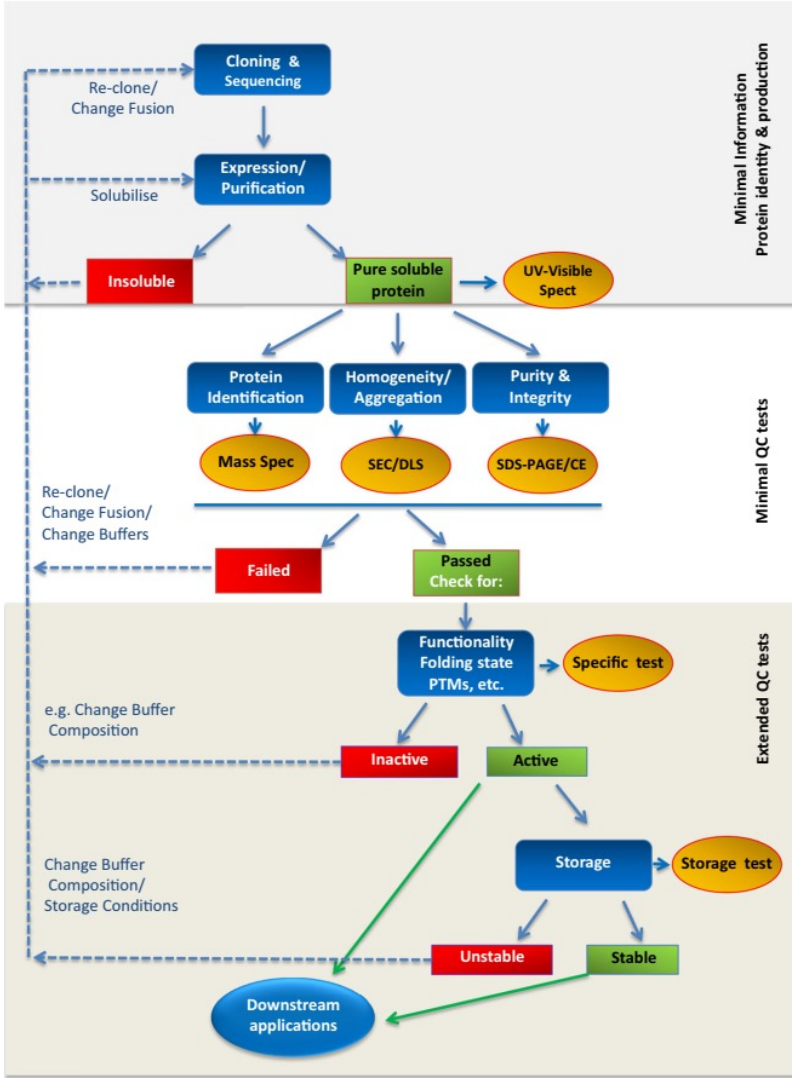


SUMMARY

- Protein Quality Control
  - Purity
  - Homogeneity
  - Identity
  - Fold State
  - Batch to batch consistency

- Advanced Quality Control – optimization
  - Buffer optimization for
    - Refolding
    - Stability
    - Crystalizability
    - Homogeneity

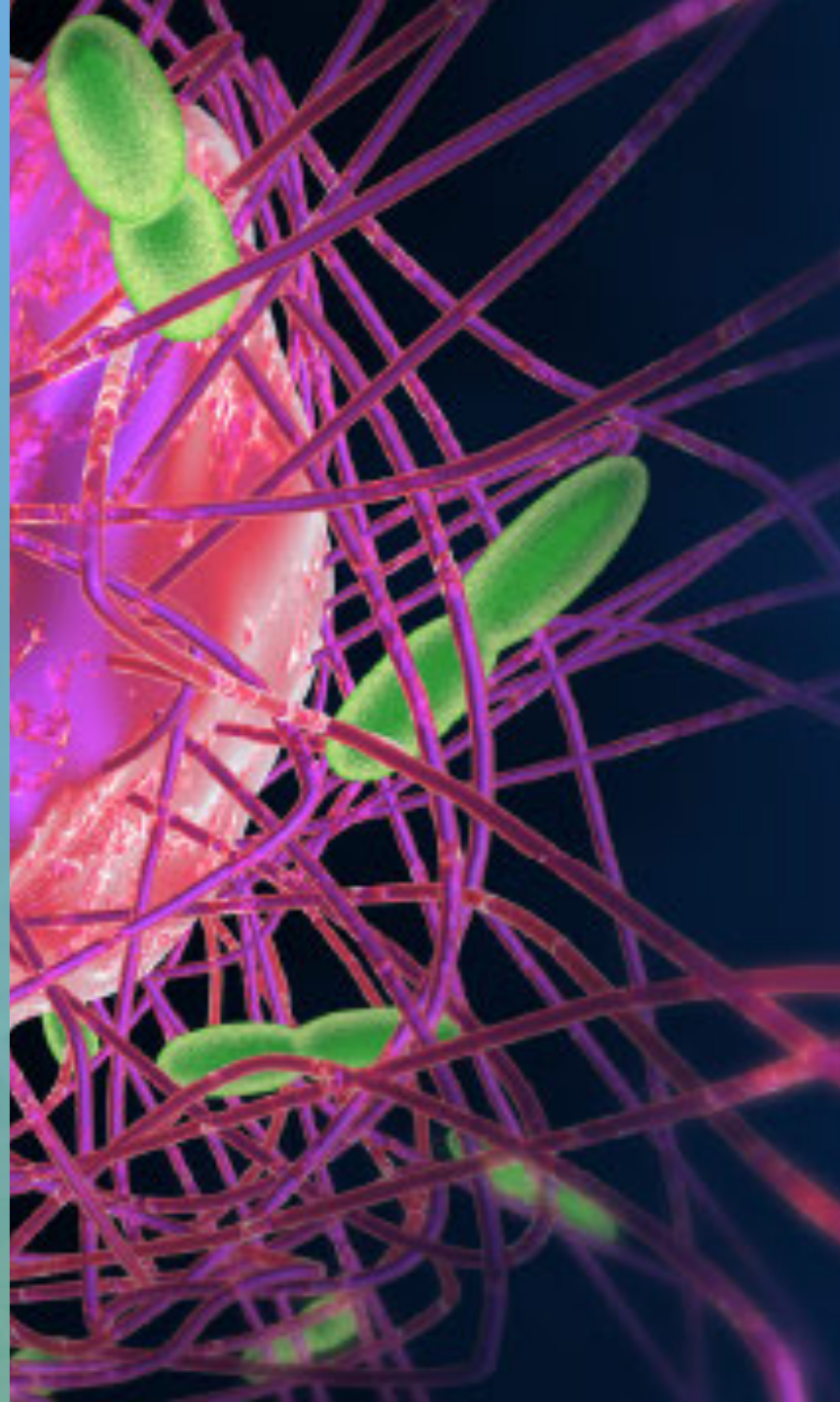
De Marco et al., nature communications (**2021**)



PROTECH EQUIPMENT

Protein Characterization / screening techniques

Molecular Interaction techniques



PROTEIN CHARACTERIZATION / SCREENING T

- UV-vis spectrometer
- DLS (Plate reader) (screening
- SEC-MALLS + viscometer (OM
- CD spectrometer
- nanoDSF (Prometheus) (screeni

PROTEIN QUALITY ASSESSMENT

Circular Dichroism Spectroscopy (CD)

- CD uses circularly polarized light to study the structure of chiral molecules, such as proteins.
- The CD spectrum of a protein can be used to determine protein secondary structure or some aspects of tertiary structure.
- CD is used to study protein stability e.g. thermal melt analysis to determine protein transition temperatures (T_m).

Typical Sample amount:
30µg
Available instrument:
Chirascan Plus (Applied Photophysics)

Dynamic Light Scattering (DLS)

- DLS measures the hydrodynamic radii of particles in a bulk solution.
- No labeling is required.
- DLS is very sensitive to aggregation.
- Changes in DLS over time can be used to measure protein stability in the presence of different buffers or additives.

Typical Sample amount:
0.5µg per condition
Available instrument:
DynaPro PlateReader II (WYATT)

PROTEIN QUALITY ASSESSMENT

Size Exclusion Chromatography coupled to Static Light Scattering (SEC-SLS)

- SEC-SLS can be used to determine the oligomeric state and precise molecular weights of different species present in a protein sample.
- No labeling is required.
- The sample is first separated on a size exclusion column. Superdex 75, Superdex 200, and a Superose 6 column are available.

Typical Sample amount:
20µg
Available instrument:
OMNISEC (Malvern)

Differential Scanning Fluorimetry (DSF or Thermofluor)

- DSF can be used to study protein stability in different buffers.
- The protein is mixed with the dye Sypro Orange to detect temperature indeced unfolding.

Typical Sample amount:
1µg per condition

PROTEIN QUALITY ASSESSMENT

PROTEOPLEX (protein complex screen)

- PROTEOPLEX is a modified DSF experiment to find buffers with highest protein complex integrity for further experiments (e.g. cryo-EM).

Typical Sample amount:
50µg per screen

Intact Mass Determination

- The exact molecular weight of an intact purified protein can be determined by mass spectrometry.
- Identity can be confirmed, and truncations detected.
- No labeling is required.

Typical Sample amount:
<1µg

Full Quality Check Service (from summer 2019)

- Purity, Identity, Homogeneity and Thermal Stability of a protein sample are checked by UV-vis, intact mass-spec, SEC-SLS, DLS and DSF.
- A full report is derived elaborating on the status of the sample.

Typical Sample amount:
25 µg

MOLECULAR INTERACTION TECHNIQU

- Isothermal Titration Calori
- Microscale Thermophoresis
- Grating Coupled Interferome

BIOMOLECULAR INTERACTION

MicroScale Thermophoresis (MST)

- MST measures the surface change of molecules upon interaction with a ligand in a microscale temperature gradient.
- Molecular interaction can be monitored by fluorescent labeling or via tryptophan fluorescence.
- binding constants in the range of nM to mM can be measured.
- Nanomolar amounts of sample are required.

Typical sample amounts:
Target (labeled): 1-100µg
Ligand: ~125µg
Available instruments:
NT.115 (blue/red), NT.LabelFree (NanoTemper Technologies)

Isothermal Titration Calorimetry (ITC)

- ITC measures the change in enthalpy upon ligand binding
- No labeling is required
- Interactions in the high nanomolar to high micromolar range can be determined using the PEAQ-ITC instrument.
- Binding constants in the nanomolar to high micromolar range can be measured.
- Stoichiometry and ΔH are also determined

Typical sample amounts:
Target (Cell): ~150µg
Ligand (Syringe): ~600µg
Available instrument:
PEAQ-ITC (Malvern)

BIOMOLECULAR INTERACTION

Surface Plasmon Resonance (SPR)

- SPR measures the mass change of a surface captured ligand upon analyte binding.
- No labeling is required.
- ligand must be coupled to the SPR chip.
- Kinetic on and off rates can be determined

Typical sample amounts:
Ligand (surface): <1µg
Analyte: ~100µg
Available instrument:
MASS-1 (Sierra Sensors/Bruker)

Instruments available for booking after training

Measurements available as full service, just bring your samples

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ProTech

Characterize your protein

Determine your biomolecular interaction

at the

VBCF Protein Technologies Facility

LOCATION

Campus Vienna Biocenter 2
Viehmarktgasse 2a
1st Floor, Rm. 1 OG 10
1030 Vienna

CONTACT

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david.drechsel@vbcf.ac.at

www.viennabiocenter.org/facilities/protein-technologies/

MORE

- Establishing standard operating procedures and making them available
- Instrument trainings
- Technology scouting – instruments for facilities/for research groups
- Organizing instrument demos
- Working as a technology hub (where is which technology available)
- Biophysical Journal April 2021 (ARBRE-MOBIEU special issue)

Volume 50, Issue 3–4

May 2021

Special Issue: MOlecular BIophysics in EUrope – Integrating Molecular Biophysics Approaches in Biology, Chemistry and Healthcare; COST Action 15126

Issue Editors: Margarida Bastos, Ramón Campos-Olivas, Thomas A. Jowitt, Stefan H. Knauer, Adriana E. Miele

https://link.springer.com/journal/249/volumes-and-issues/50-3

GET IN CONTACT

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- VBC6 (old IMP)

  Offices in rooms 171, 172, 175

- Contact us via the ticketing system
  - https://vbc.atlassian.net/servicedesk/customer/portal/10

- Contact us via email
  - ProteinTechnologies@vbcf.ac.at

THANK YOU !

Vienna BioCenter
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ACCURATE PROTEIN CONCENTRATION DETERMINATION

of purified / recombinant proteins

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IMPORTANCE OF CONCENTRATION?

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- Non important
  - Gels, DLS, NMR, X-ray crystallography, Tm, ΔH, ΔG, mass spec, …
- <10% accuracy important
  - Calorimetry (DSC, ITC)
  - Compare normalized spectra (CD, UV-vis, …)
  - Interaction measurements
    - K_D accuracy ≥ titrant concentration accuracy
- SEC-MALLS MW determination
  - Utmost importance, usually conc. is measured from refractive index!

SEVERAL METHODS TO DETERMINE PROTEIN CONCENTRATION

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Table 2
Methods for determination of protein concentration

Method	Amount of protein required (µg)	Complexity of method[a]	Response of identical masses of different proteins	Reference protein used	Major sources of interference
Biuret	500–5000	2	Very similar	Yes	Tris, NH_4^+, glycerol
UV (280 nm)	100–1000	1	Variable	No	Nucleic acids and other chromophores
UV (205 nm)	5–50	1	Similar	No	Many buffer components and other solutes
Lowry	5–100	3	Variable	Yes	Amino acids, NH_4^+, thiol compounds, certain buffers and detergents
Bicinchoninic acid (BCA)	5–100 (1–10 in microprotocol)	3	Variable	Yes	Glucose, NH_4^+, EDTA
Coomassie blue binding	5–50 (1–10 in microprotocol)	2	Variable	Yes	Triton, SDS
Amino acid analysis	10–200	4	Variable	No	Other contaminating proteins

Data adapted from Price [63], which should be consulted for further details and references for each method.
[a] Graded on a 1–4 scale; 1 involves pipetting of the sample only; 2 involves mixing the sample with one reagent solution; 3 involves mixing the sample with >1 reagent solution; 4 involves lengthy manipulation of the sample and specialised equipment.

Kelly et al. How to study proteins by circular dichroism, BBA (2005)

SEVERAL METHODS TO DETERMINE PROTEIN CONCENTRATION

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- Bradford (or similar method using reference proteins)
  - completely random, order of magnitude ok
- IR-Spectroscopy (again using reference protein)
  - No tryptophane needed
- UV-Spectroscopy (280nm) (fast, easy)
  - <20% if ε calculated, <10% if fold corrected, **<5% if ε measured**
  - >50% if Nanodrop stand
  - >100% if no tryptophanes present or if co-enzymes or metals bound
- UV-Spectroscopy (205nm)(fast)
  - <10% if ε calculated (even without tryptophanes)
- Quantitative Amino Acid Analysis
  - **<5%, expensive, time consuming**
- Refractive index measured (very accurate)
  - <5% for fixed dn/dc, **<2% for calculated dn/dc**

UV 280NM

Lambert-Beer law: OD = c ε d

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ε_{280} = 5500·#Trp + 1490·#Tyr + 125·#Cystines [$M^{-1}cm^{-1}$]

Pace et al.
Protein Science (1995)

calculated for “average protein fold” in water
(Usually within 20%)

e.g. online using ExPASy ProtParam (http://web.expasy.org/protparam/)

ε_{280} = 5690·#Trp + 1280·#Tyr (+ 120·#Cystines) [$M^{-1}cm^{-1}$]

Edelhoch
Biochemistry (1967)

calculated for 20mM phosphate buffer,
pH6.5, 6M GndCl

Compare native to 6M GndCl to correct for folded protein!
(Usually within 10%)

UV 205NM

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$\varepsilon_{205} \approx 32$ [$mg^{-1}ml$] (usually within 25%)

$\varepsilon_{205} = \boldsymbol{f}$ (AA content, #peptide bonds) [$M^{-1}cm^{-1}$]

- not only trp dependent
- very similar for all proteins (usually within 10%)
- good spectrometer needed
- not suitable for all buffers

Table III. *Molar Absorptivity Values at 205 nm (ε_{205}) Used in This Study for Protein Side Chains and the Backbone Peptide Bond*

Side chain/feature	ε_{205} ($M^{-1}\cdot cm^{-1}$)
Tryptophan	20,400
Phenylalanine	8600
Tyrosine	6080
Histidine	5200
Methionine	1830
Arginine	1350
Cysteine	690
Asparagine[a]	400
Glutamine[a]	400
Cystine[b]	2200
Backbone peptide bond[c]	2780 ± 168

[a] Values for asparagine and glutamine come from Saidel *et al.*[12] All other values are from Goldfarb *et al.*[7]
[b] If the protein has a disulfide bond, add 820 $M^{-1}\cdot cm^{-1}$ (2200 $M^{-1}\cdot cm^{-1} - 2 \times 690\ M^{-1}\cdot cm^{-1}$) to its ε_{205}.
[c] Best-fit value determined as described in the text and given as the average ±1 standard deviation.

Goldfarb et al.
J. Biol. Chem. (1951)

Anthis and Clore
Protein Science (2013)

e.g. online using Marius Clore Group NIH Protein Calculator
(http://spin.niddk.nih.gov/clore/)

REFRACTIVE INDEX

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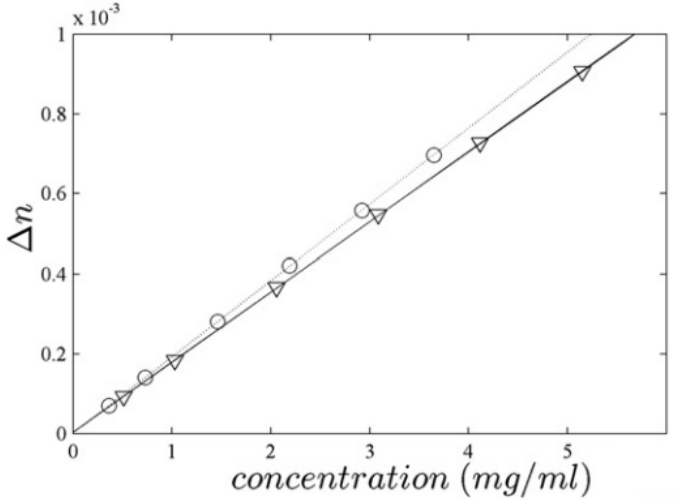
The index of refraction n is a measure of the velocity of light in a material.

speed of light: $$v_{liquid} = \frac{v_{vacuum}}{n}$$

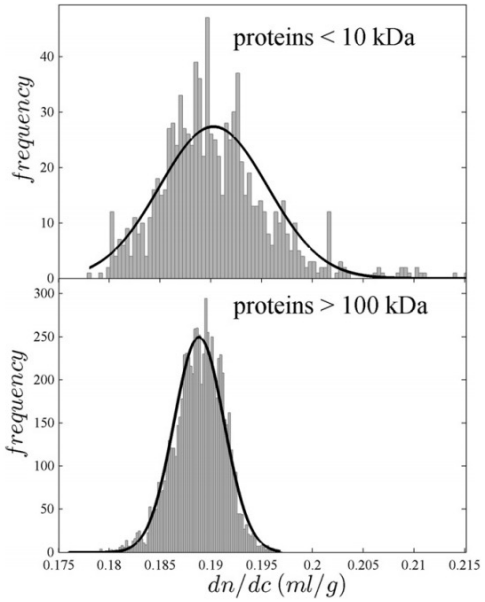
For solutes, the polarizability is expressed as the specific refractive index increment *dn/dc* (how much does the refractive index change for a given concentration of sample in solution).

$\frac{dn}{dc}$ variation for proteins <5%



ε_{280}, ε_{205}, $\frac{dn}{dc}$ calculated from AA sequence in SEDFIT (by Peter Schuck)
(http://www.analyticalultracentrifugation.com/download.htm)

On the Distribution of Protein Refractive Index Increments
Zhao et al Biophysical Journal 2011



$\frac{dn}{dc}$ FOR BIOLOGICAL SOLUTES

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Table of select dn/dc values		
Sample/Solid Phase	**Solvent/Liquid Phase**	**dn/dc [mL/g]**
Biomolecules	Aqueous Buffer	Average: 0.185
Proteins	Aqueous Buffer	0.16-0.20, average: 0.185
DNA	Aqueous Buffer	0.17
RNA	Aqueous Buffer	0.17-0.19
Alanine	Aqueous Buffer	0.19
Polysaccharides	Aqueous Buffer	Average: 0.15
Chitosan	Aqueous Buffer	0.16-0.18
Dextrane	Aqueous Buffer	0.14-0.15
Hyaluronic Acid	Aqueous Buffer	0.16-0.18
Pullulan	Aqueous Buffer	0.14-0.16
Starch	Aqueous Buffer	0.15
Glucose, Maltose, Lactose, Sucrose	Aqueous Buffer	0.14-0.15

http://www.materials-talks.com/blog/2013/06/18/refractive-index-increment-dndc-values/

TABLE 1 Refractive index properties of amino acids

Amino acid	Molar residue refractivity* (cm^3)	$\bar{v}$ (ml/g)†	*dn/dc*(ml/g)‡
Arg	39.47	0.70	0.206
His	34.62	0.67	0.219
Lys	34.10	0.82	0.181
Asp	26.06	0.60	0.197
Glu	30.07	0.66	0.183
Ser	19.16	0.63	0.170
Thr	23.82	0.70	0.172
Asn	26.09	0.62	0.192
Gln	30.37	0.67	0.186
Cys	48.58	0.63	0.206
Gly	12.81	0.64	0.175
Pro	23.74	0.76	0.165
Ala	17.15	0.74	0.167
Ile	31.87	0.90	0.179
Leu	31.59	0.90	0.173
Met	34.45	0.75	0.204
Phe	42.21	0.77	0.244
Trp	55.24	0.74	0.277
Tyr	44.34	0.71	0.240
Val	26.73	0.86	0.172

On the Distribution of Protein Refractive Index Increments
Zhao et al Biophysical Journal 2011

SEC-MALLS SETUP TO MEASURE E_{280}, E_{205}

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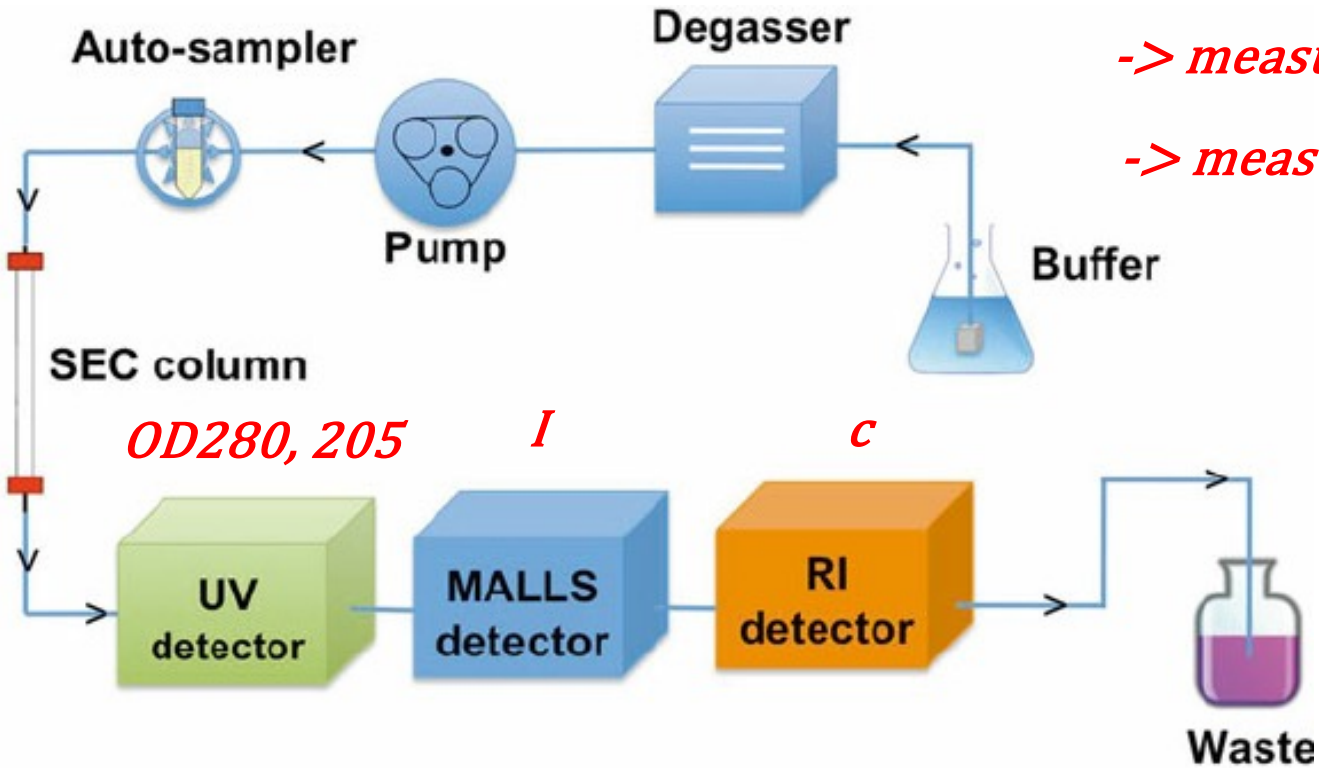
$$I \sim M_W \cdot c \cdot \left(\frac{dn}{dc}\right)^2 \cdot \frac{1}{\lambda^4}$$



-> measured M_W

-> measured ε

https://www.researchgate.net/publication/269716983_Methods_for_the_Successful_Crystallization_of_Membrane_Proteins

CONCENTRATION NORMALIZED SPECTRA

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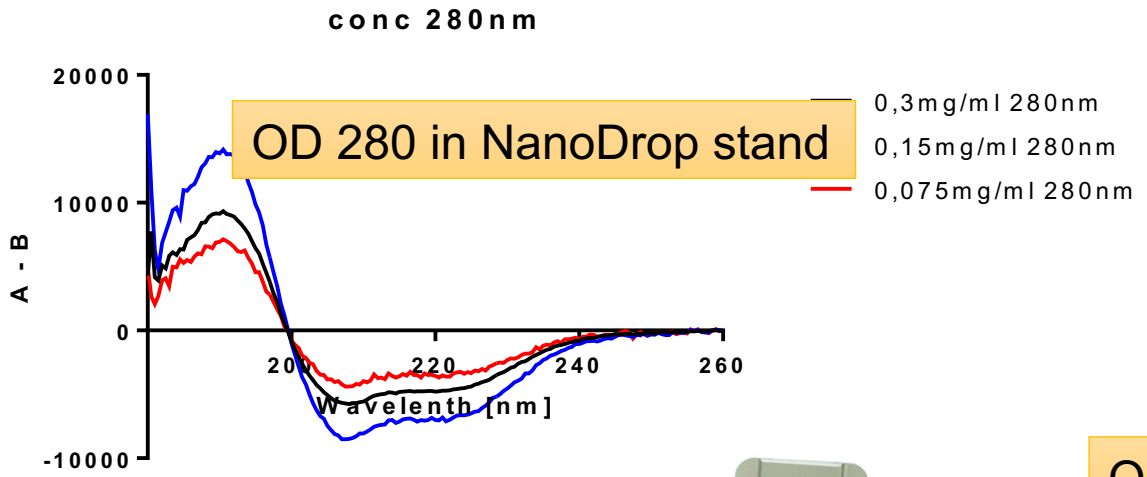
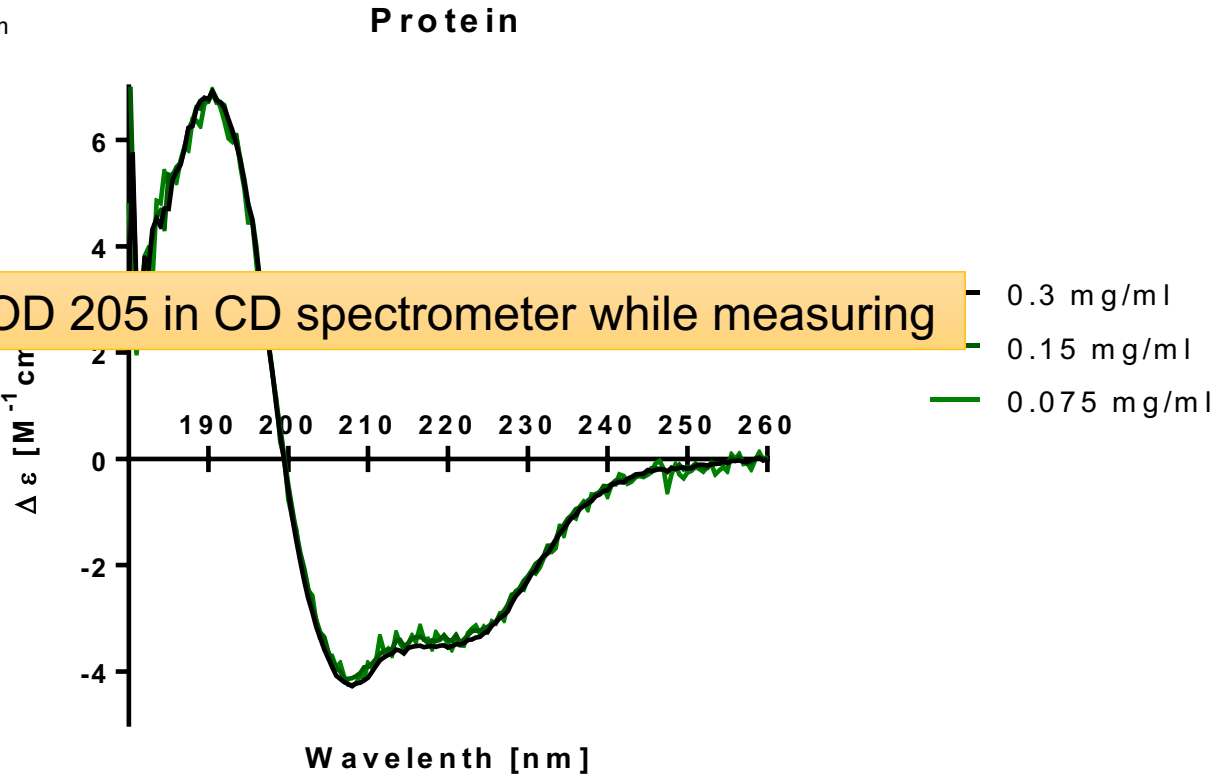


OD 280 in NanoDrop stand



OD 205 in CD spectrometer while measuring

WHY MEASURING YOUR PROTEIN CONCENTRATION WITH NANODROP STAND IS A BAD IDEA

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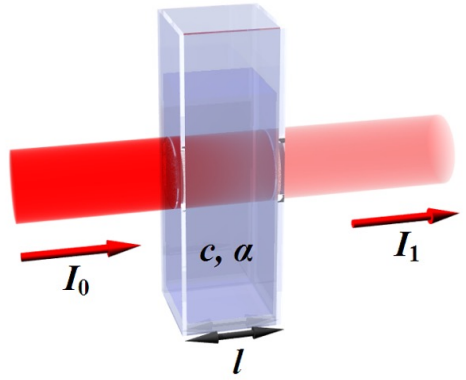
- $OD = c\,\varepsilon\,l = log[I_0/I_1]$

Movable parts:
$l \leq 0.1cm \pm ?$



- $I_0/I_1 = 10^{OD}$

≈1 mg/ml | 10% concentration accuracy | 20% detection accuracy

- OD=1 $I_0/I_1 = 10$ *Buffer-Sample difference=900%*

≈ 0.1 mg/ml | 10% concentration accuracy | 2% detection accuracy

- OD=0.1 $I_0/I_1 = 1.25$ *Buffer-Sample difference=25%*

≈ 0.01 mg/ml | 10% concentration accuracy | 0.24% detection accuracy

- OD=0.01 $I_0/I_1 = 1.0233$ *Buffer-Sample difference=2.33%*

WHAT NANODROP STAND DOES MEASURE

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- Path length reproducibility
- Sample inhomogeneity
  - Volume nanodrop stand ≤ 1µl (l ≤ 0.1cm)
  - Volume cuvette = 100µl (l = 1cm)
- Sample loading reproducibility
  - microbubbles

> 10 mg/ml up to 700mg/ml

- What to use the stand for than?
- OD=10 … 100 (NMR/crystal solution)
- Fast approximate measurements for lot of samples (DNA)

HOW TO PROPERLY MEASURE CONCENTRATION WITH UV-VIS SPECTROSCOPY

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- Spin your sample (even better: filter 0.2µm or smaller)
- Use cuvettes (1cm, 1mm, …)(most accurate d!)
- Check full spectrum (at least up to 400nm) $OD_{280nm,corr} = OD_{280nm} - 2 \cdot OD_{333nm}$
  - light scattering or other absorbing species (DNA, imidazole, …)
- Correct for buffer (blank with water, especially for 205nm!)
- Correct for light scattering