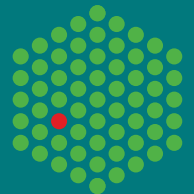


Sample and buffer preparation

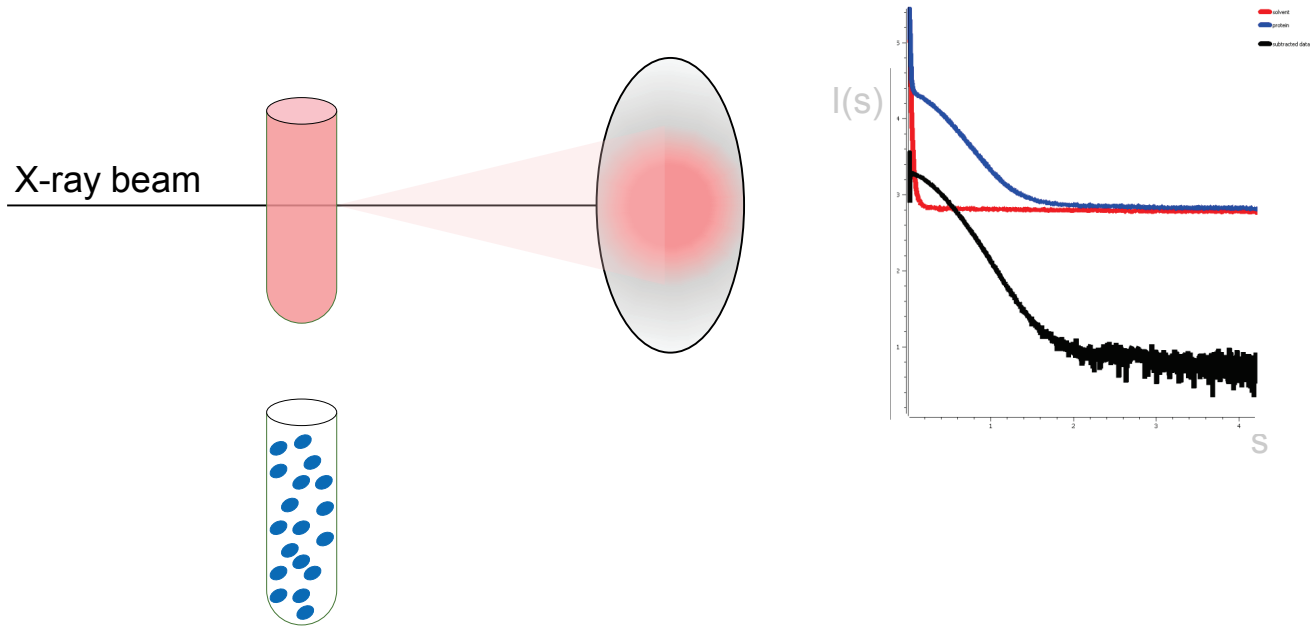
Melissa Graewert
Tuesday, June 21st

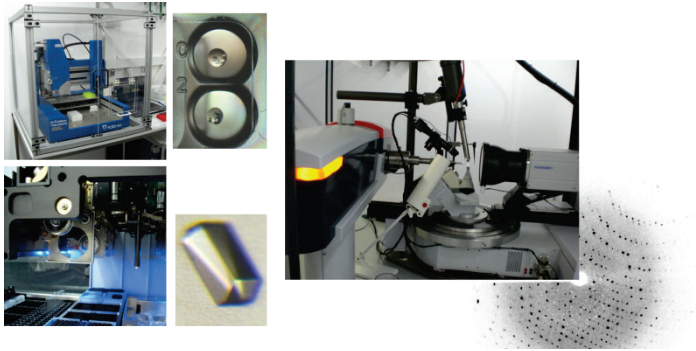
EMBL



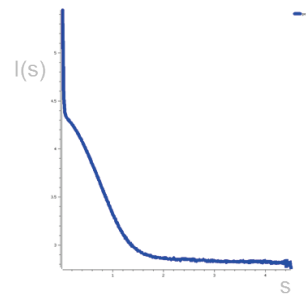
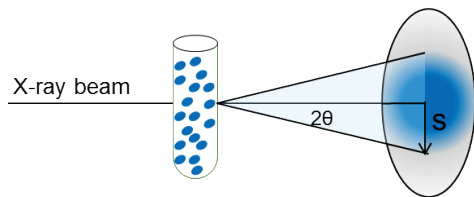
- Planning an Experiment
 - Assessing Sample suitability
- Preparing an Experiment
 - Best sample
 - Best buffer
- Performing an Experiment
 - Trouble shooting at the beamline: 5 test cases

- The SAXS (small angle X-ray scattering) Experiment





Signal is amplified
High resolution $d \approx \lambda$

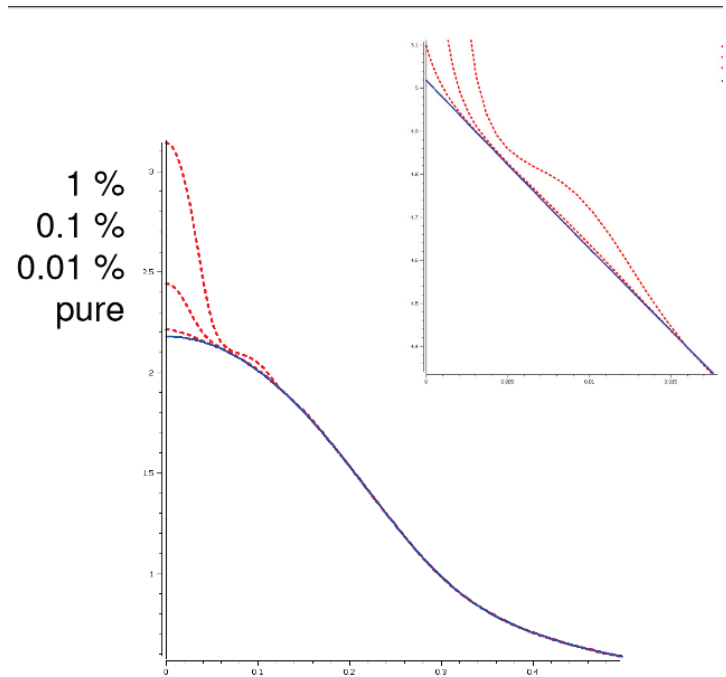
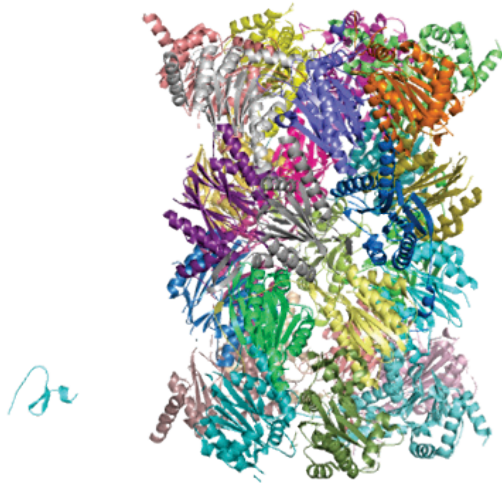


$I(s)$ is isotropic
Concentrates around the primary beam
Low resolution $d \gg \lambda$

No need to grow crystals
No crystallographic packaging forces
Not limited by MW
Physiological conditions
Observe response to changes

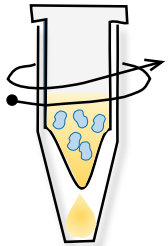
- Sample requirements
 - Amount: 7 μ l; 30-50 μ l per sample
 - Concentration: dependent on MW
 - Buffer: as less additives as possible
 - Sample quality

Foldon vs Proteasome

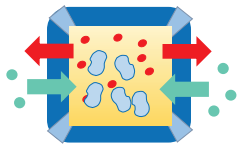


■ Sample preparation strategies

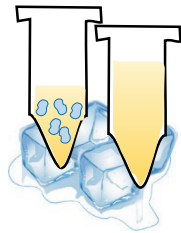
■ Sample stable at high concentrations



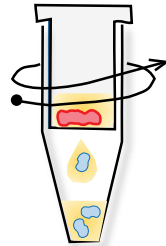
Concentrate



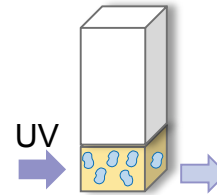
Dialyze



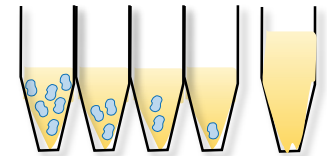
(Store/Ship)



Filter

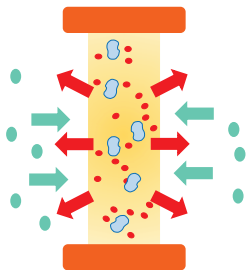


Conc. determination

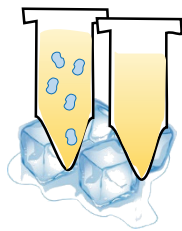


SAXS from dilutions & buffer

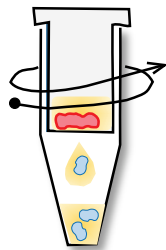
■ Sample not stable at high concentrations



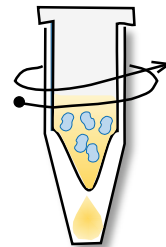
Dialyze



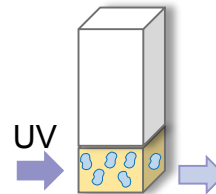
(Store/Ship)



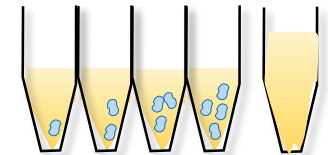
Filter



Step-wise
concentration



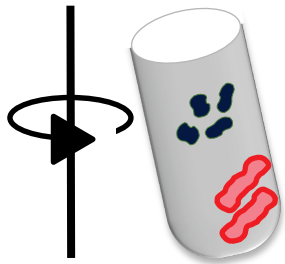
Conc. determination



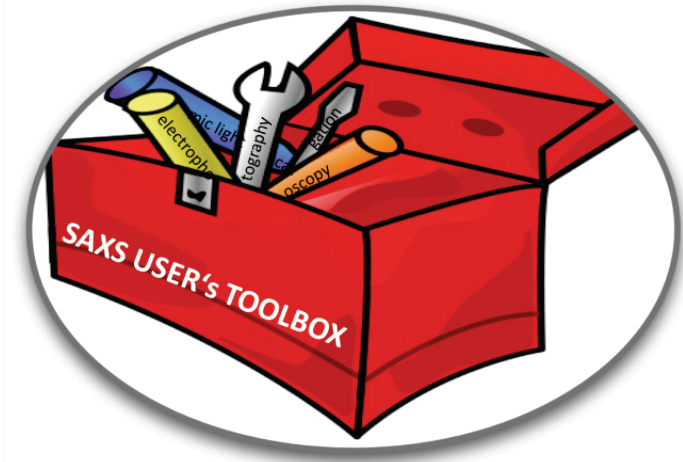
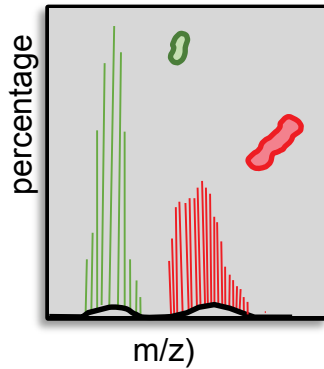
SAXS from different conc.
centration steps & buffer

■ Sample Characterization

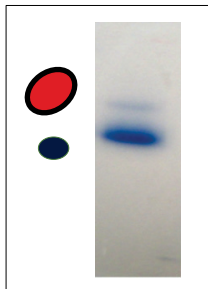
Analytical ultracentrifugation



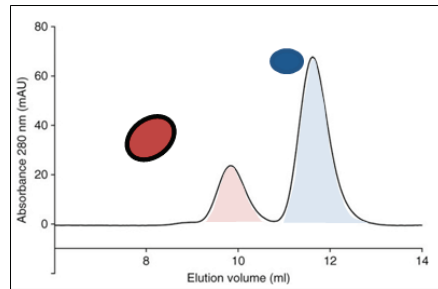
nativeMS



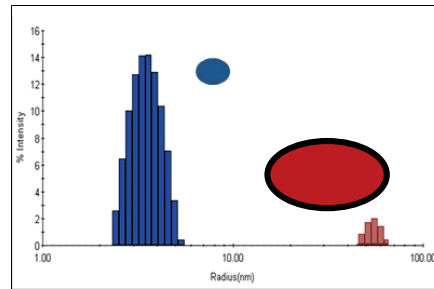
Native Gel Electrophoresis



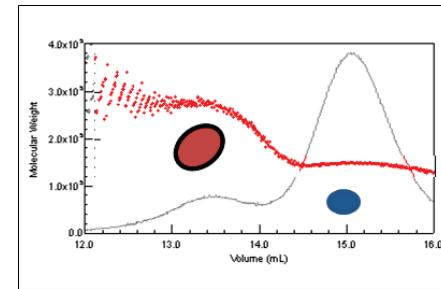
Size Exclusion Chromatography



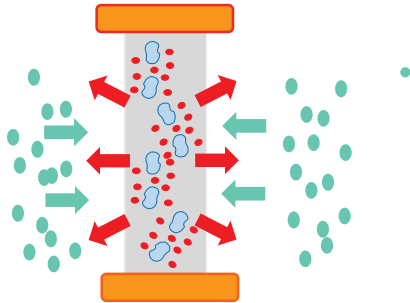
Dynamic Light Scattering



Static Light Scattering



- Buffer Preparation
 - Method of choice: Dialysis



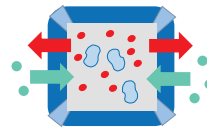
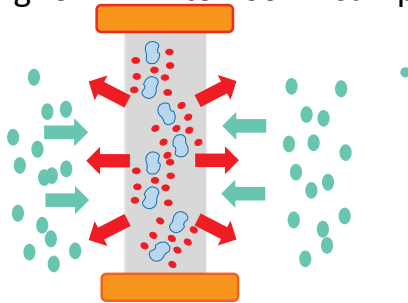
- diffusion coefficients
- temperature
- time
- concentration of species
- sample volume
- dialysate (buffer) volume (100:1)
- number of dialysate changes (2-3)
- membrane surface area
- membrane thickness
- molecular charges
- dialysate agitation (stirring)

→ standard protocol: 16 to 24 hours

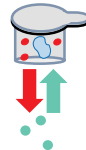
- Buffer Preparation

- Method of choice: Dialysis

Tubing for ~2mL to 100mL samples



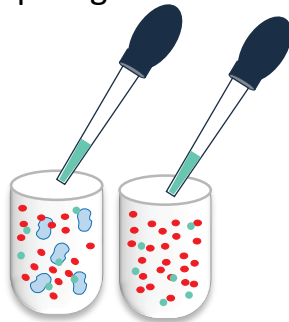
Cassettes for
~0.5mL to
70mL



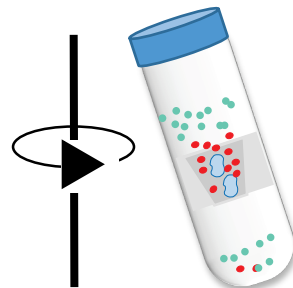
Cup Devices for
~10µL to 2mL

- Alternative methods

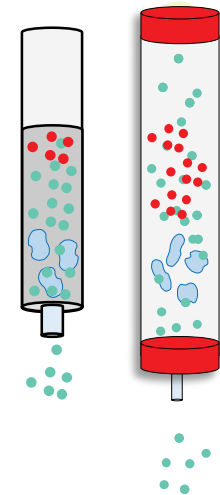
Spiking



Diafiltration

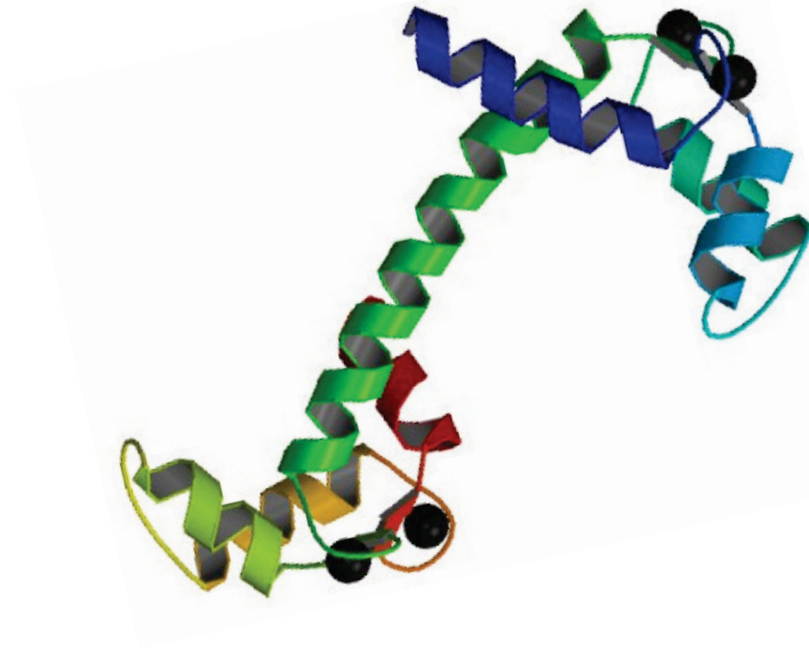


Desalting/SEC column



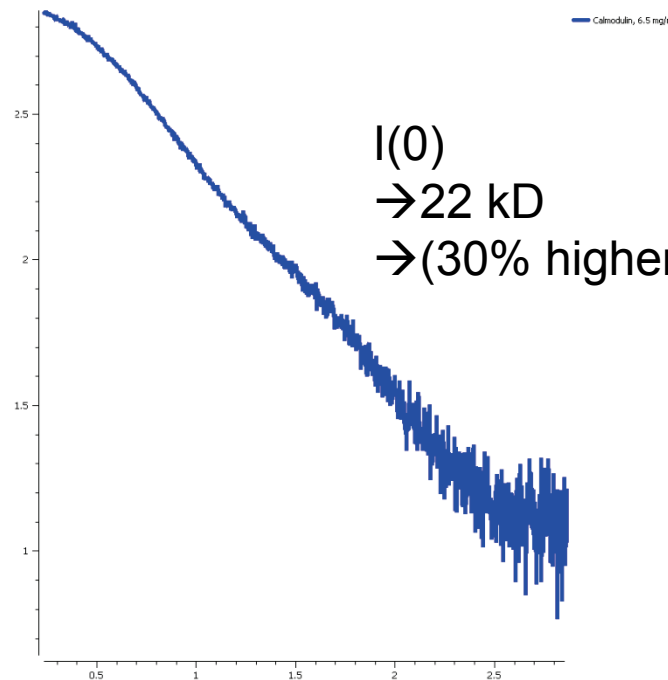
- Case 1: the Relaxed Scientist

- Sample: Calmodulin; 100 ul 6.5 mg/ml (UV-Vis)
- Question: confirm monomeric state of protein

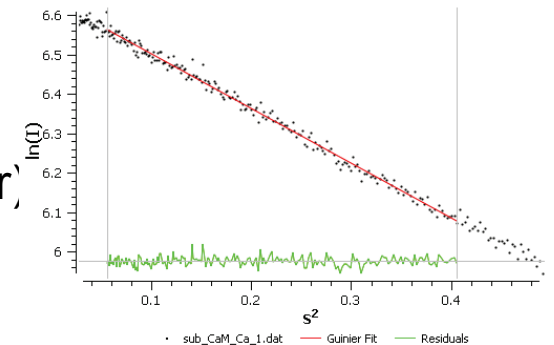


■ Case 1: the Relaxed Scientist

- Sample: Calmodulin; 100 ul 6.5 mg/ml (UV-Vis)
- Question: confirm monomeric state (16.8 kD)
- Result:



$I(0)$
→ 22 kD
→ (30% higher)



$$MM_p = I(0)_p / c_p \frac{MM_{st}}{I(0)_{st} / c_{st}}$$

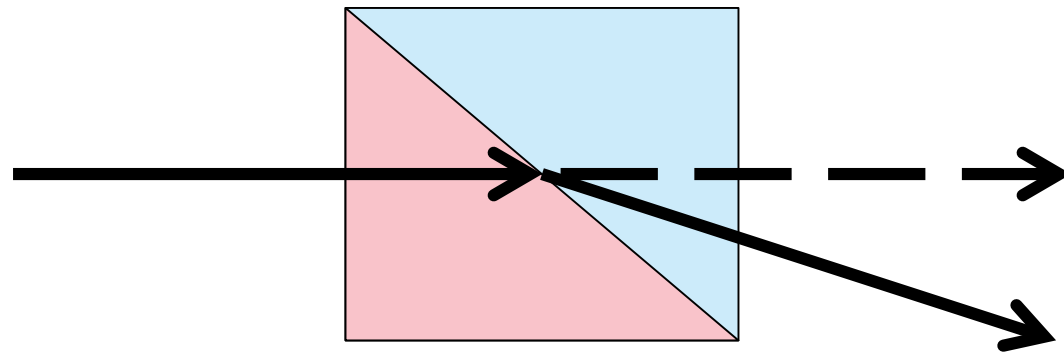
■ Case 1: the Relaxed Scientist

- Sample: Calmodulin; 100 ul 6.5 mg/ml (UV-Vis)
- Question: confirm monomeric state of protein
- Result: ambiguous; $MW_{\text{expected}} = 16.8 \text{ kD}$
 $MW_{l(0)} = 22 \text{ kD}$ (30% higher)
- Explanation: unsuitable method for determining c

ADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTEAELQDMINEV
DADGNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVFDKDGNGYISAAELRHVM
TNLGEKLTDEEVDEMIREANIDGDGQVNYEEFVQMMTAK → $\epsilon = 0.17$

protparam: Experience shows that this (no Trp) could result in more than 10% error in the computed extinction coefficient

Method	Principle	Advantages	Limitations
Lowry Assay	<ul style="list-style-type: none"> - Biuret chromophore (copper ion complex with amide bonds) - Cu^{+1}, Tyr, Trp reduce Folin-Ciocalteu reagent (660 nm) 	<ul style="list-style-type: none"> - Relative sensitive: - 1 to 100 ug 	<ul style="list-style-type: none"> -interfering compounds such as detergents, carbohydrates, glycerol, Tris, EDTA... -- content of Tyr, Trp - time consuming
BCA Assay bicinchoninic acid	<ul style="list-style-type: none"> - protein backbone chelates Cu^{2+} ions and reduces them to Cu^{1+} which shifts color of dye (562 nm) 	<ul style="list-style-type: none"> -less sensitive to the types of amino acids in the protein - suitable for most detergents & denaturants 	<ul style="list-style-type: none"> - Cysteine rich samples (temp) - reducing agents (DTT, 2-ME) -time consuming
Bradford Assay	<ul style="list-style-type: none"> - Color shift of Coomassie brilliant blue G-250 dye upon binding arg and aromatic residues 	<ul style="list-style-type: none"> -simple, rapid, cheap, sensitive - micro: 1-20 ug - macro: 20-100 ug - copes with reducing agents 	<ul style="list-style-type: none"> -- content of Arg (eg. histones) -- non linear curve (absorbance of free dye) - Choice of standard, pH - "sticky proteins" precipitate
UV(280 nm)	<ul style="list-style-type: none"> Ultraviolet absorbance according to Beer's law, $A \sim c \cdot \epsilon$ 	<ul style="list-style-type: none"> - quick - sample recovery 	<ul style="list-style-type: none"> - sequence dependent - protein complexes, mixtures -- sensitive to pH and ions
Differential Refractometry	<ul style="list-style-type: none"> index of refraction according to Snell's law 	<ul style="list-style-type: none"> - total/ pure protein - quick - sample recovery 	<ul style="list-style-type: none"> -Magic number -Temperature sensitive



dual cell, deflection design

Rudolph Research Analytical J357 refractometer

■ Assay choice

- Goal: accuracy (compared to precision)
- Consider sample composition (sequence)
- Consider buffer composition (pH, additives)
- Consider a precipitation step to remove buffer
- Sample volume (High-through put)

- Protocol for choosing suitable method,
eg. Olson, Markwell; Curr Protoc Protein Sci. 2007(3)

■ Case 2: the Lazy Scientist

- Sample: 11 mg/ml Lysozyme in 30 and 90 mM NaCl
- Question: effect of addition of salt

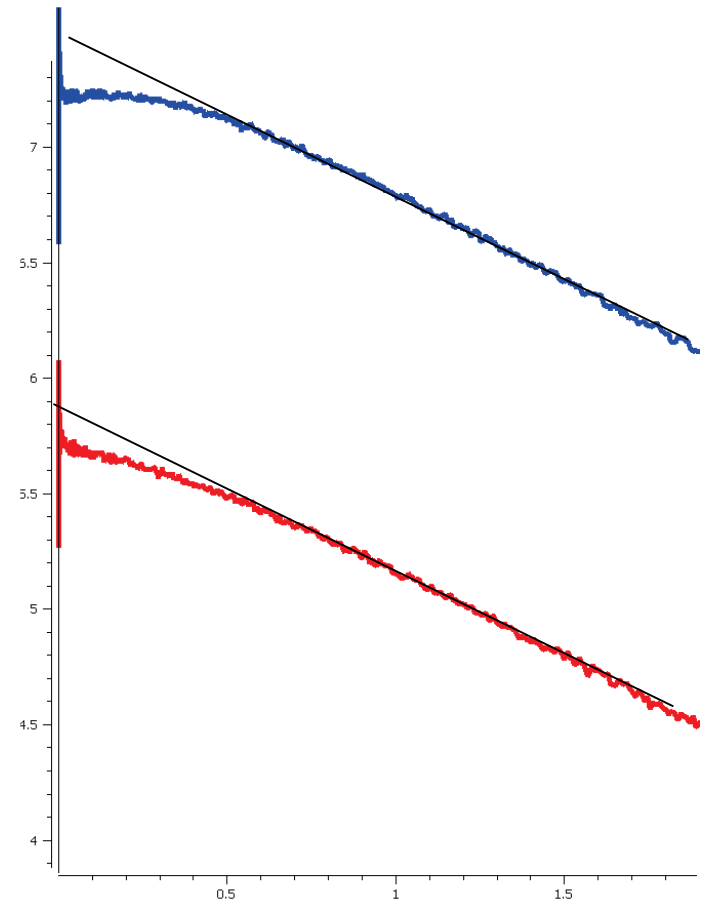
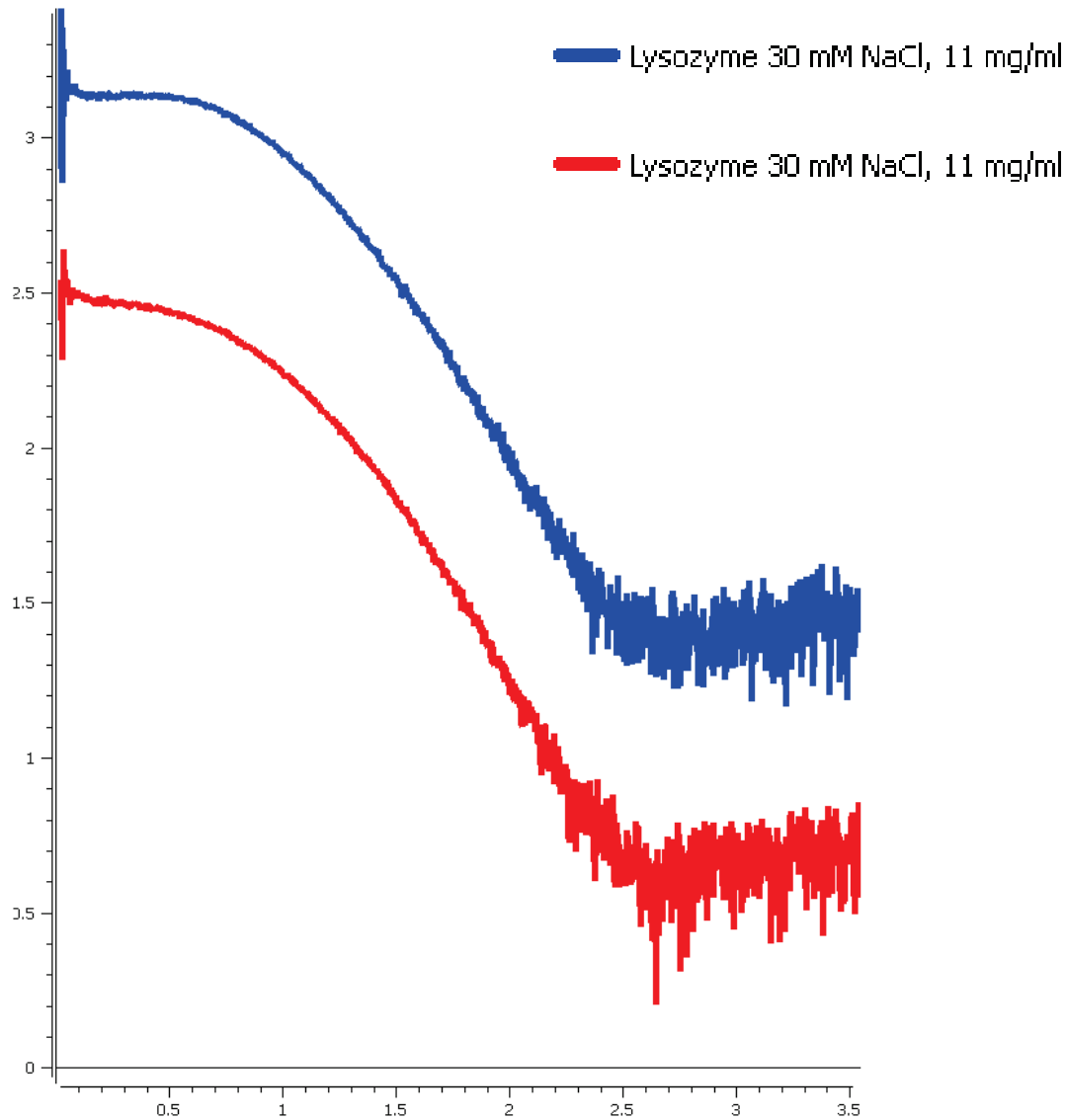
Code	Conc. mg/ml	Log plot	Kratky plot	Guinier points	Quality	R_g nm	D_{max} nm	V_{Porod} nm ³	V_{DAM} nm ³	$MW_{(0)}$ kDa	MW_{Porod} kDa
b_30	11.0			153 266	68%	1.0 ± 0.5	3	19	21	12	12
d_90	11.0			118 282	77%	1.2 ± 0.2	4	19	22	12	12

70% frames

→ Conclusion increase in NaCl,
unfavorable for the protein

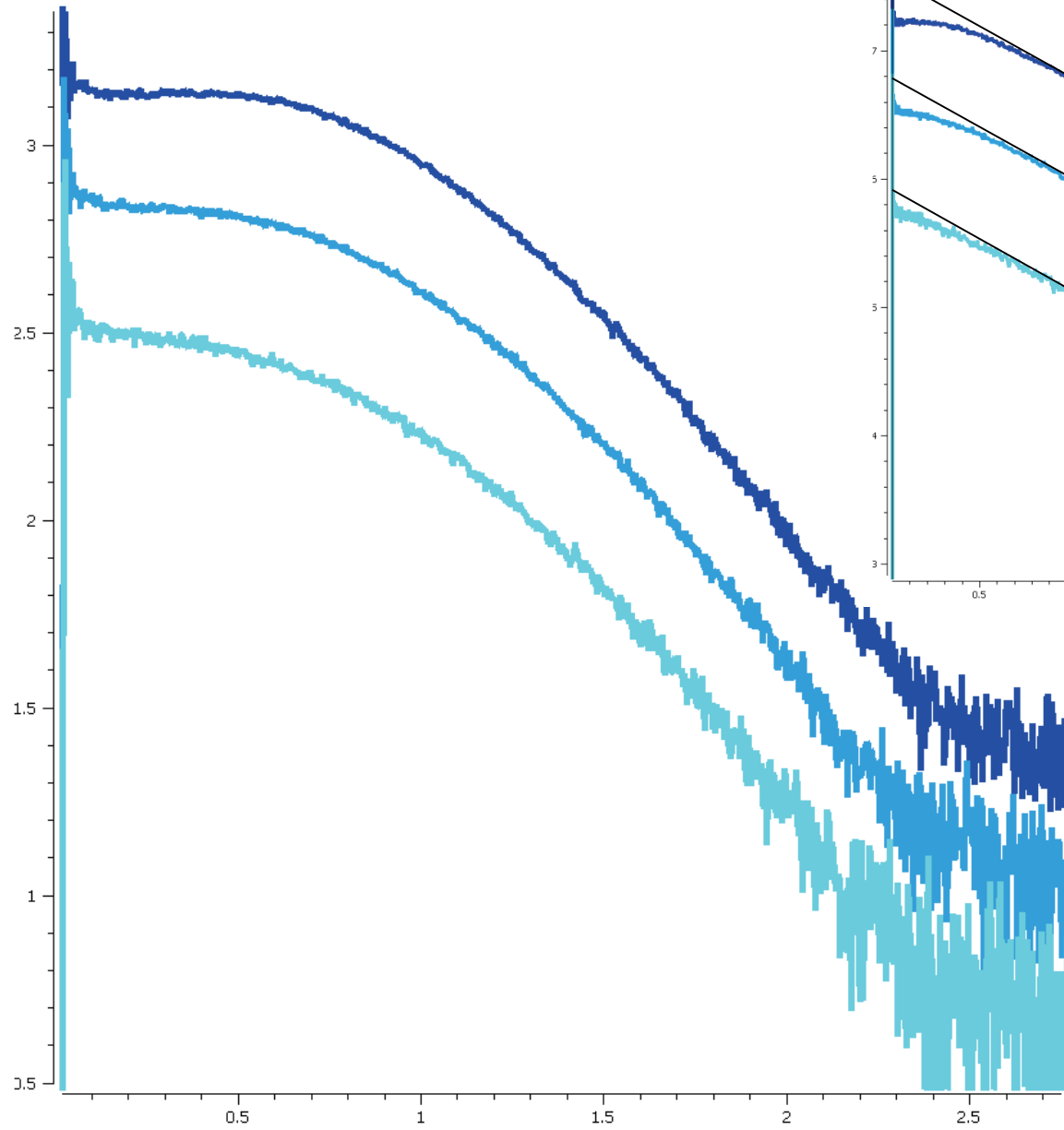
■ Case 2: the Lazy Scientist

- Sample: 11 mg/ml Lysozyme in 30 and 90 mM NaCl
- Question: effect of adding salt
- Result: ambiguous; $Rg_{30\text{mM}} = 1.0 \text{ nm}$
 $Rg_{90\text{mM}} = 1.2 \text{ nm}$
→ $Rg_{\text{expected}} = 1.4 \text{ nm}$



PERFORMING THE EXPERIMENT

11 mg
5.5 mg
2.75 mg



PERFORMING THE EXPERIMENT

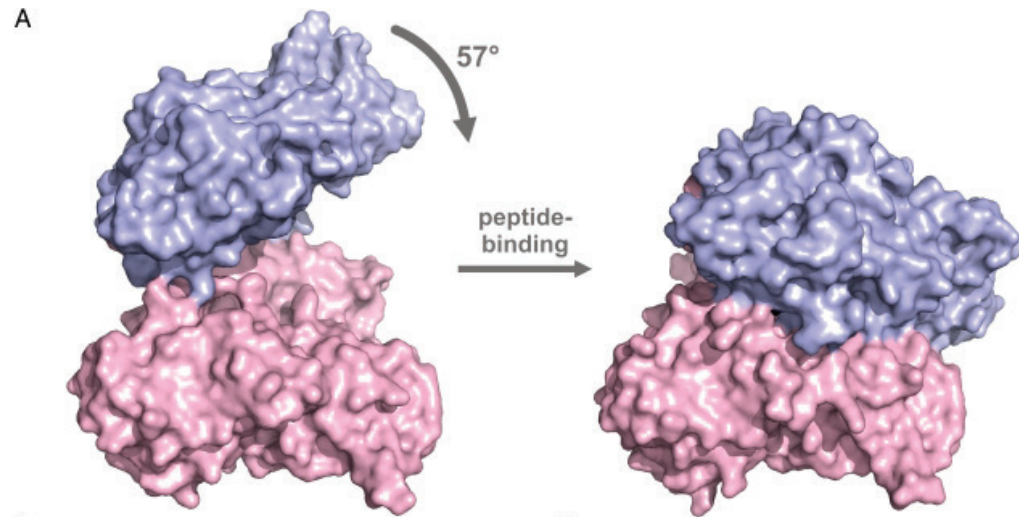


■ Case 2: the Lazy Scientist

- Sample: 11 mg/ml Lysozyme in 30 and 90 mM NaCl
- Question: effect of addition of salt
- Result: ambiguous;
 $RG_{\text{expected}} = 1.4 \text{ nm}$
 $RG_{30\text{mM}} = 1.0 \rightarrow 1.4 \text{ nm}$
 $RG_{90\text{mM}} = 1.2 \rightarrow 1.4 \text{ nm}$
- Explanation: concentration effects
- Solution: measure different concentrations

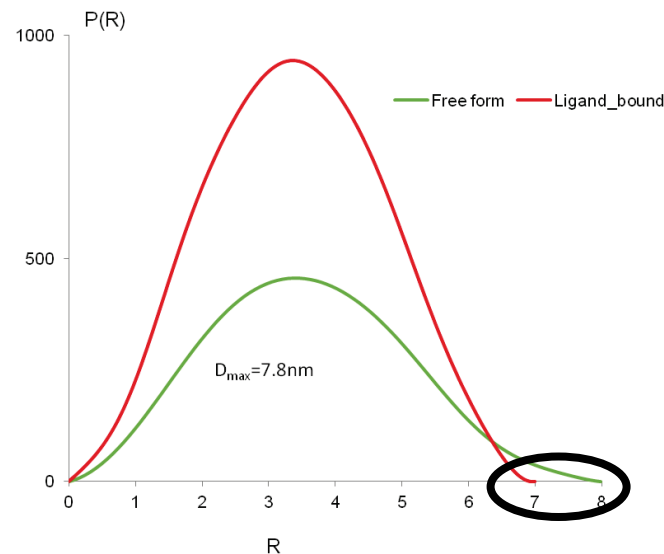
■ Case 3: the Ambitious (Hasty) Scientist

- Sample: well characterized mutants, different ligands
- Question: understanding the binding mechanism



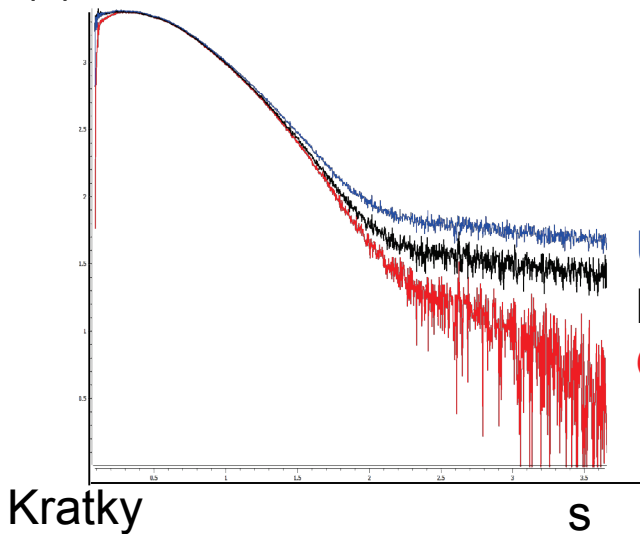
■ Case 3: the Ambitious (Hasty) Scientist

- Sample: well characterized mutants, different ligands
- Question: understanding the binding mechanism
- Result: look at automated pipeline



Run #	File	Conc., mg/ml	Description	R _g , nm	I(0)	Guinier points	D _{max} , nm	MM, kDa	Volume, nm ³	Quality, %	Comments
7	bsa_007.dat	4.4	bsa	3.25±3%	21.66	33 - 138 (106)	11.4	66	157	86	12:46
10	ivypw_010.dat	10.0	hdpp3_e451a+ivy	2.62±1%	20.31	36 - 178 (143)	7.8	62	130	88	14:10
12	ivypw_012.dat	10.0	hdpp3_2xmut+ivy	2.73±1%	21.27	38 - 169 (132)	8.1	65	133	87	14:13
14	ivypw_014.dat	10.0	k670a+ivyp	2.76±1%	16.67	35 - 164 (130)	8.6	51	138	88	14:15
16	ivypw_016.dat	10.0	r669a+ivyp	2.68±1%	20.04	49 - 171 (123)	8.0	61	136	84	14:17
19	vypw_019.dat	10.0	hdpp3_e451a+vvy	2.55±1%	19.98	35 - 153 (119)	7.6	61	124	89	14:21
21	vypw_021.dat	10.0	r669a+vvy	2.65±1%	19.87	29 - 171 (143)	7.5	61	138	90	14:23
24	vypw_024.dat	10.0	hdpp3_e451a+vyp	2.63±1%	20.15	30 - 177 (148)	7.8	61	128	90	14:27
26	vypw_026.dat	10.0	hdpp3_2xmut+vyp	2.76±1%	21.45	43 - 164 (122)	8.4	65	142	86	14:29
28	vypw_028.dat	10.0	k670a+vypw	2.80±1%	16.73	31 - 164 (134)	9.2	51	147	89	14:32
30	vypw_030.dat	10.0	r669a+vypw	2.69±2%	20.03	39 - 128 (90)	8.0	61	138	86	14:34
33	valo_033.dat	10.0	hdpp3_e451a+val	2.59±2%	20.44	33 - 163 (131)	7.5	62	127	89	14:38
35	valo_035.dat	10.0	hdpp3_2xmut+val	2.74±1%	21.67	42 - 169 (128)	8.8	66	142	86	14:40
37	valo_037.dat	10.0	k670a+valo	2.75±1%	16.79	57 - 161 (105)	8.0	51	134	82	14:43
39	valo_039.dat	10.0	r669a+valo	2.69±1%	20.20	43 - 168 (126)	7.8	62	149	86	14:45
42	lw_042.dat	10.0	hdpp3_e451a+lw	2.57±1%	20.26	42 - 151 (110)	7.5	62	129	87	14:49
44	lw_044.dat	10.0	hdpp3_2xmut+lw	2.74±1%	21.44	36 - 167 (132)	8.6	65	140	88	14:51
46	lw_046.dat	10.0	k670a+lw	2.75±1%	16.63	48 - 168 (121)	8.4	51	136	84	14:53
48	lw_048.dat	10.0	r669a+lw	2.69±1%	20.79	45 - 171 (127)	7.8	63	148	86	14:56
51	p0_051.dat	10.0	hdpp3_e451a_a0	2.63±1%	20.60	46 - 176 (131)	7.6	63	137	85	14:59
53	p0_053.dat	10.0	hdpp3_2xmut_b0	2.75±1%	21.73	45 - 166 (122)	8.8	66	131	85	15:02
55	p0_055.dat	10.0	k670a_c0	2.79±1%	16.89	44 - 163 (120)	9.1	51	132	85	15:04
57	p0_057.dat	10.0	r669a_d0	2.74±1%	20.35	35 - 169 (135)	8.4	62	149	88	15:06
Run #	File	Conc., mg/ml	Description	R _g , nm	I(0)	Guinier points	D _{max} , nm	MM, kDa	Volume, nm ³	Quality, %	Comments

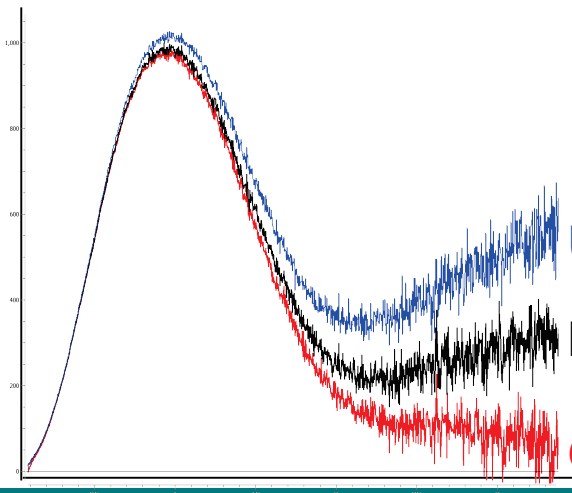
Wrong Buffer



Under subtraction (Tris)
Matching buffer (Tris + glycine)
Over subtraction (Tris + glycerol)

Kratky

s



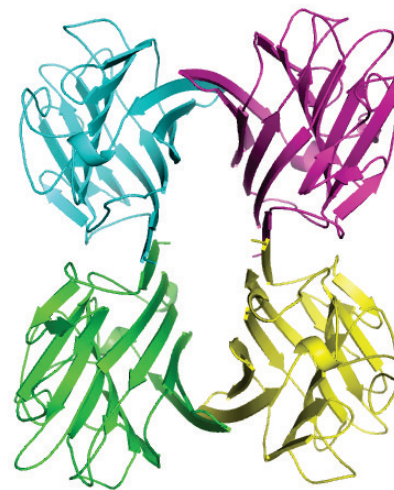
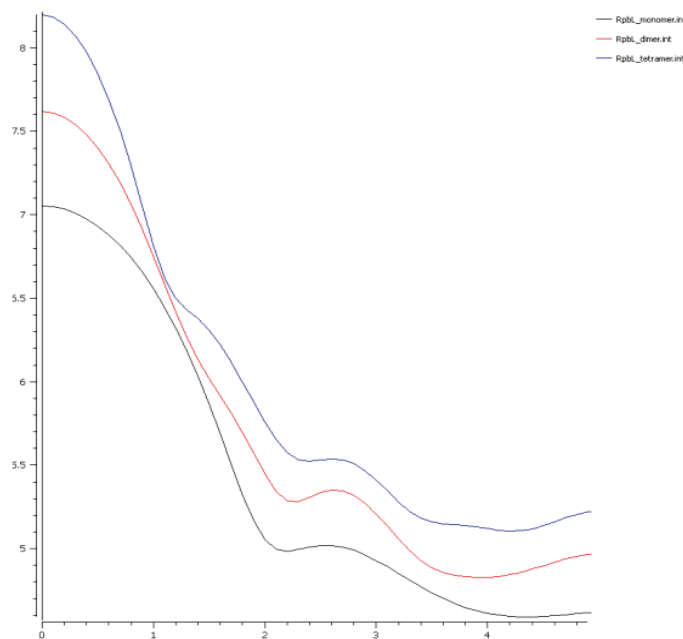
Under subtraction (Tris)
Matching buffer (Tris + glycine)
Over subtraction (Tris + glycerol)

■ Case 3: the Ambitious (Hasty) Scientist

- Sample: well characterized mutants, different ligands
- Question: understanding the binding mechanism
- Result: ambiguous; $D_{\text{max}_{\text{unbond}}} = 8 \text{ nm}$
 $D_{\text{max}_{\text{bond}}} = 7 \text{ nm}$
 $D_{\text{max}} = 7.5\text{-}9.2 \text{ nm}$
 - Explanation: incorrect buffer subtraction
 - Solution: dialysis, SEC, (centricon)

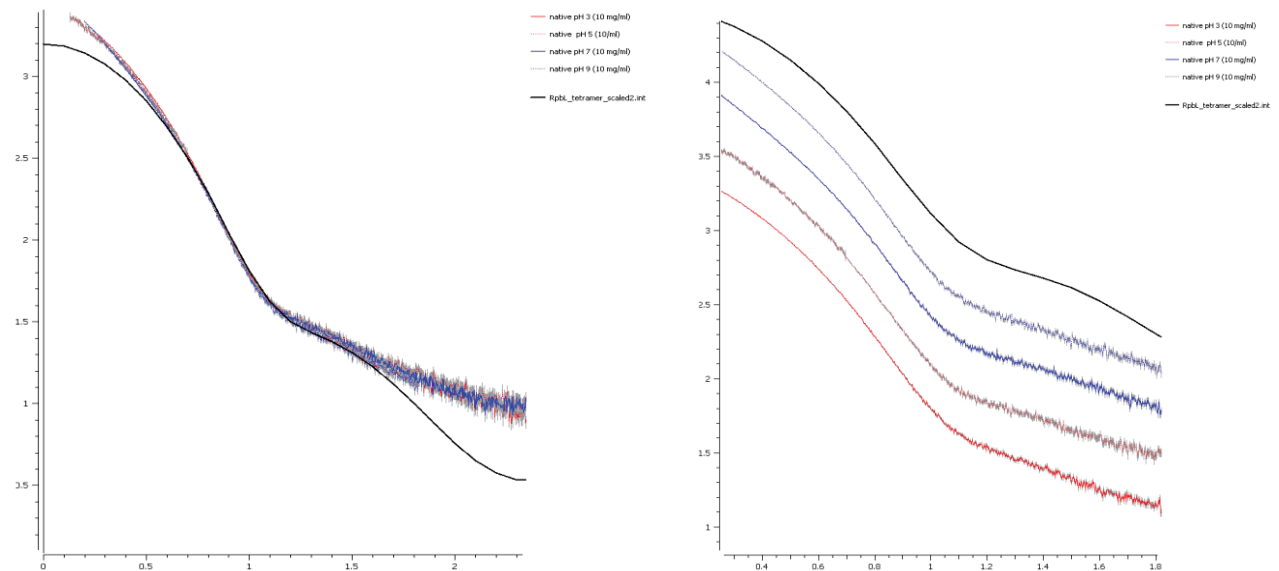
■ Case 4: the Super Scientist

- Sample: excellently prepared, fully analyzed
- Question: pH dependent oligomerization

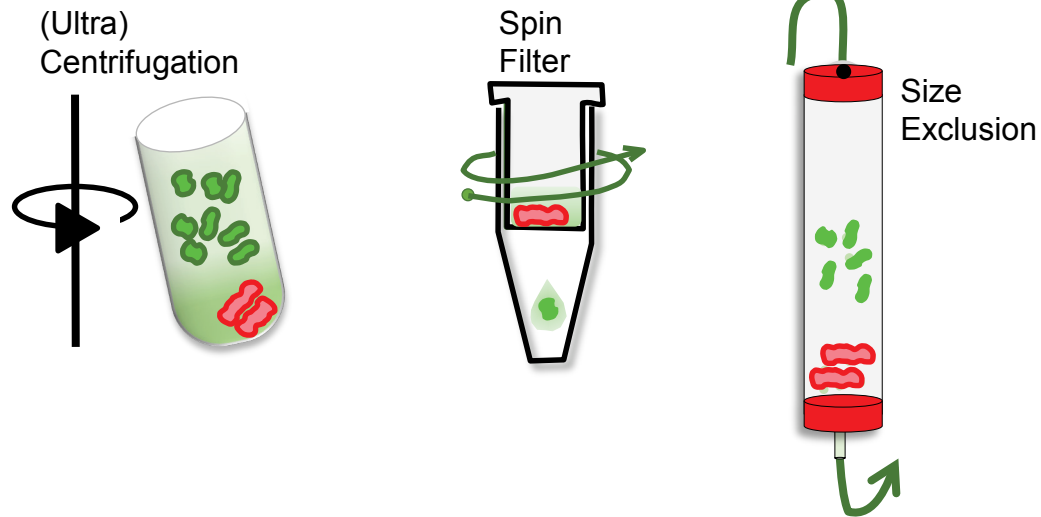


■ Case 4: the Super Scientist

- Sample: excellently prepared, well analyzed sample
- Question: pH dependent oligomerization
- Result:



- Removal of aggregates



- Time/ storage/ transport
 - Stability
 - pH
 - Concentration
 - Additives
 - Proteases
 - Freezing/ Thawing
 - Tube size, concentration

Only use additives when it is really necessary!

Class of additive	example	concentration	purpose
Salts	NaCl, KCl, (NH ₄) ₂ SO ₄	50-150 mM	maintain ionic strength of medium
Detergents	Deoxycholate, Triton X-100	0.1-1% <i>As close as possible to cmc!</i>	solubilization of poorly soluble proteins
Glycerol		5- 10 %	stabilization
Glucose or sucrose		25 mM <i>contrast!</i>	Stabilize lysosomal membranes, reduce protease release
Metal chelators	EDTA, EGTA	1 mM	reduce oxidation damage, chelate metal ions
Reducing agents	DTT, DTE 2-Mercaptoethanol	1-10 mM 0.05%	reduce oxidation damage <i>low shelf-life!</i>
Ligands, metal ions	Mg ²⁺ , ATP, GTP	1-10 mM	stabilization <i>absorption</i>

https://www.embl.de/pepcore/pepcore_services/protein_purification/extraction_clarification/lysis_buffer_additives

Table 1. Comparison of Protein Storage Conditions

Characteristic	Storage Condition			
	Solution at 4°C	Solution in 25-50% glycerol or ethylene glycol at -20°C	Frozen at -20° to -80°C or in liquid nitrogen	Lyophilized (usually also frozen)
Typical shelf life	1 month	1 year	Years	Years
Requires sterile conditions or addition of antibacterial agent	Yes	Usually	No	No
Number of times a sample may be removed for use	Many	Many	Once; repeated freeze-thaw cycles generally degrade proteins	Once; it is impractical to lyophilize a sample multiple times

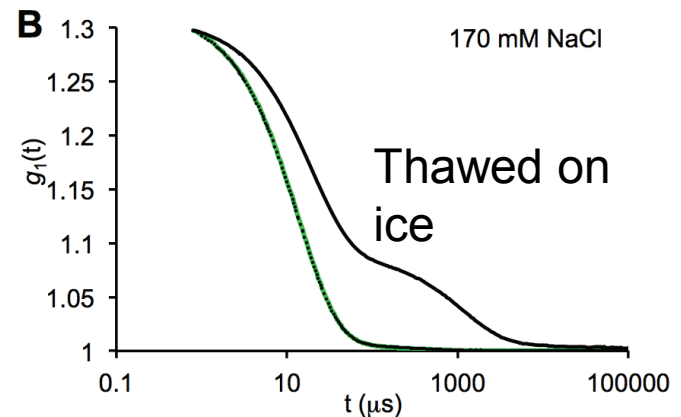
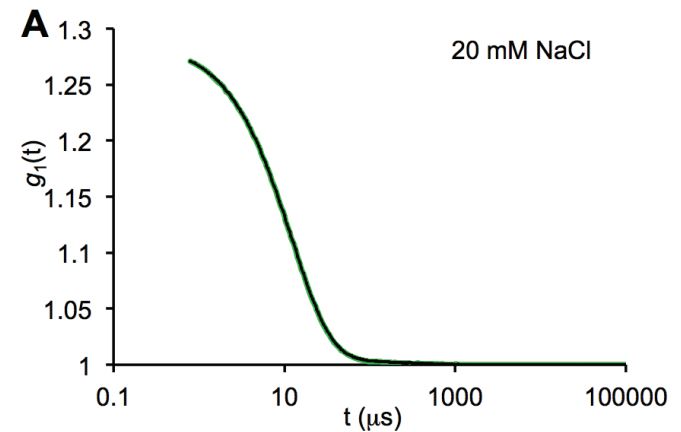
Protein Concentration:

Dilute protein solutions (< 1 mg/ml) are more prone to inactivation and loss as a result of low-level binding to the storage vessel. Therefore, it is common practice to add “carrier” or “filler” protein, such as purified bovine serum albumin (BSA) to 1-5 mg/ml (0.1-0.5%), to dilute protein solutions to protect against such degradation and loss.

@ Pierce

- Time/ storage/ transport

- Stability
- Freezing/ Thawing
 - Speed
 - Additives
 - Dried ice
- Tube size, concentration





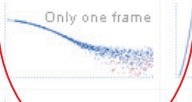
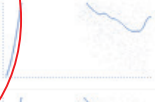


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■ Case 4: the Super Scientist

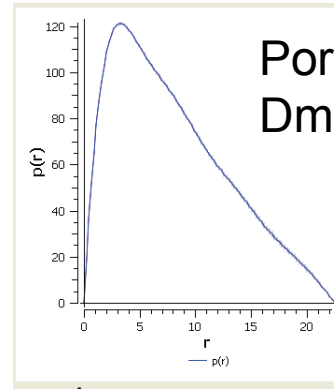
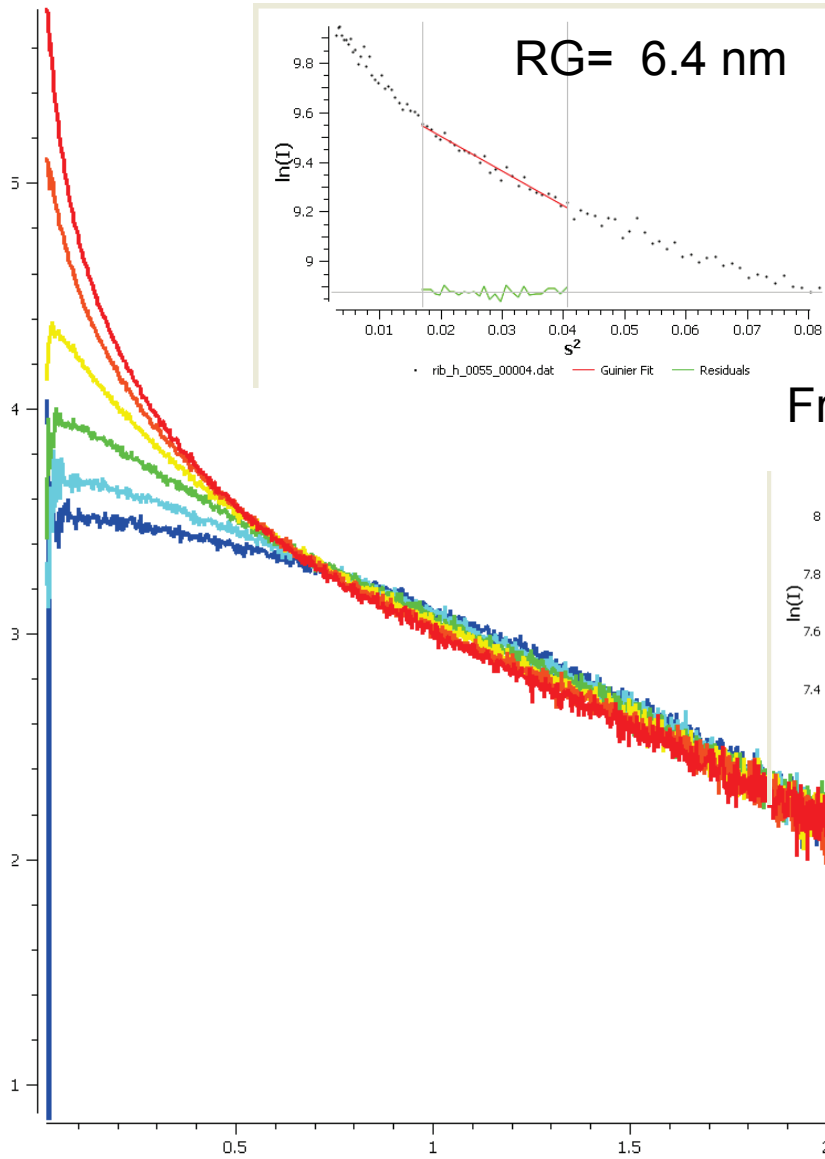
- Sample: excellently prepared, well analyzed sample
- Question: pH dependent oligomerization
- Result: polydisperse sample
 - Explanation: time/storage/transport dependent alterations
 - Solution: re-characterize the sample, 'first aid' at the beamline

■ Case 5: the Brilliant Scientist

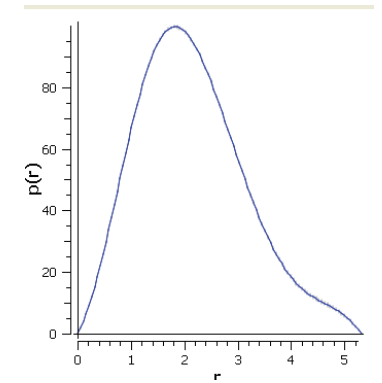
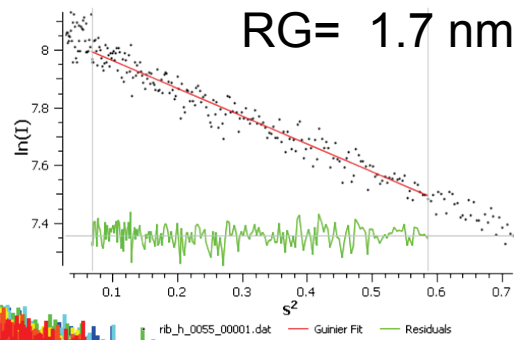
- Sample: dilution series of Ribonuclease
- Question: *ab initio* Model
- Result: Radiation Damage

Run#	Description	Code	Conc. mg/ml	Log plot	Kratky plot	Guinier points	Quality	R _g nm	D _{max} nm	V _{Porod} nm ³	V _{DAM} nm ³	MW _{I(0)} kDa	MW _{Porod} kDa
238	ribo_10	r_h_2	10.0			66 271	76%	1.7 ± 0.3	▲	6	17	16	11
240	ribo_10	r_h_2	10.0			87 288	70%	1.7 ± 0.3	▲	6	17	16	10
242	ribo_10	r_h_2	10.0			43 288	80%	1.7 ± 0.3	▲	6	18	16	11

Frame 4



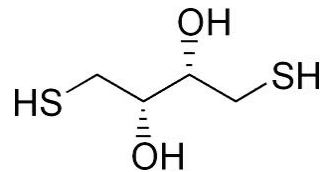
Frame 1



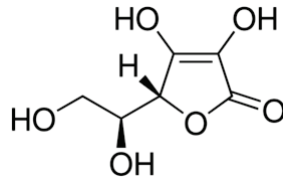
Porod V = 17.39 nm³
Dmax = 5.32 nm

■ Scavengers

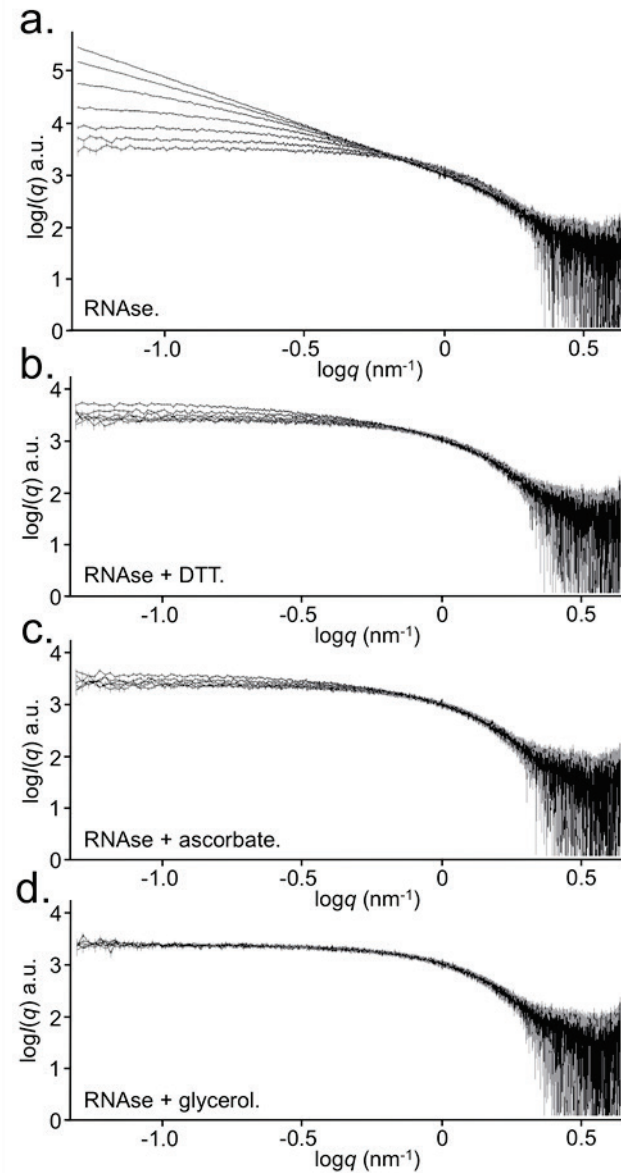
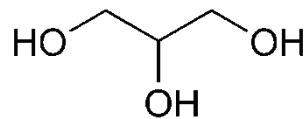
- DTT (b)



- Ascorbic Acid (c)

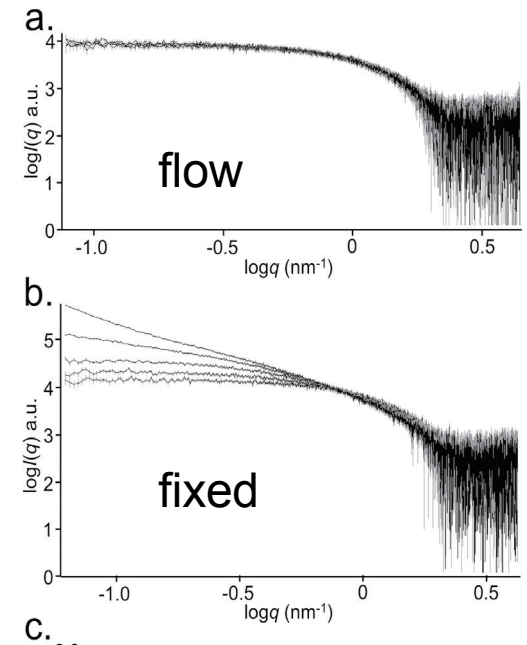


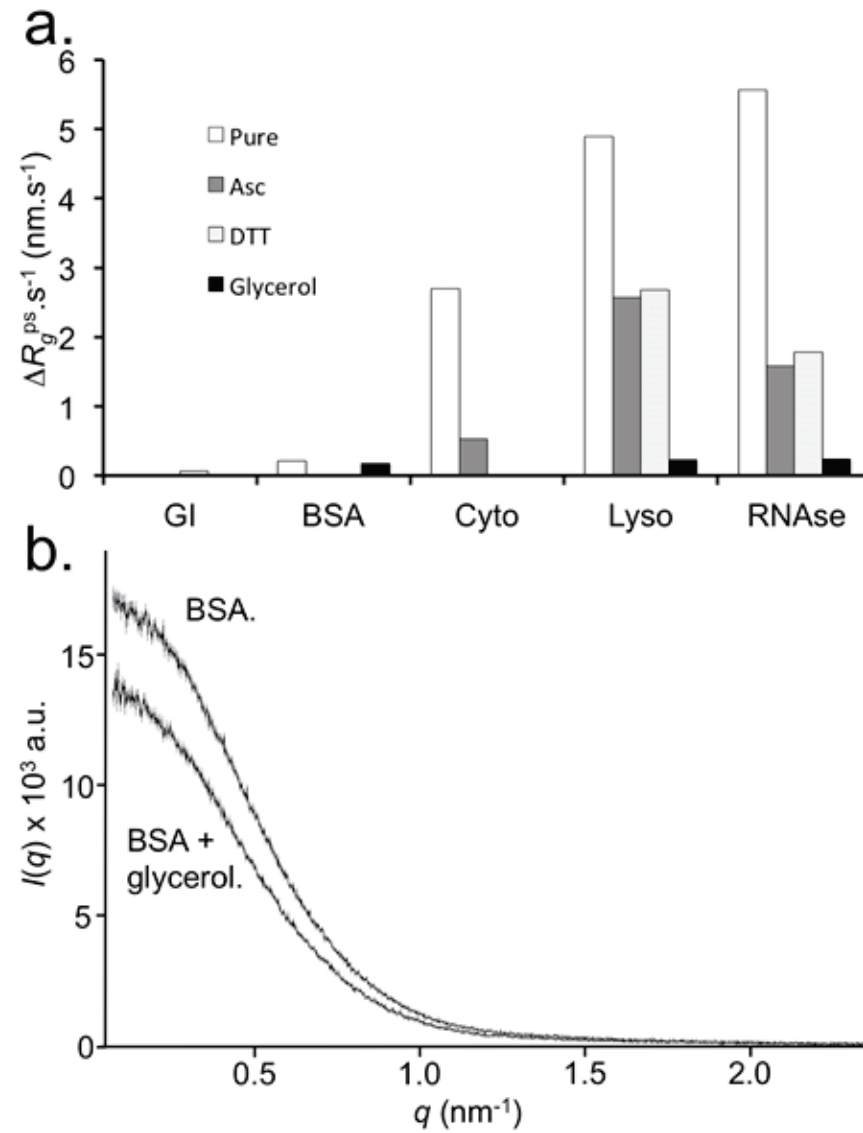
- Glycerol (d)



- Data collection strategy

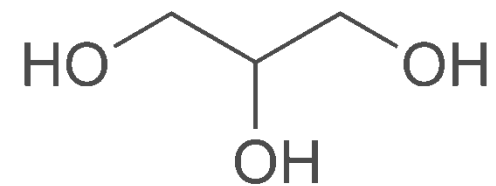
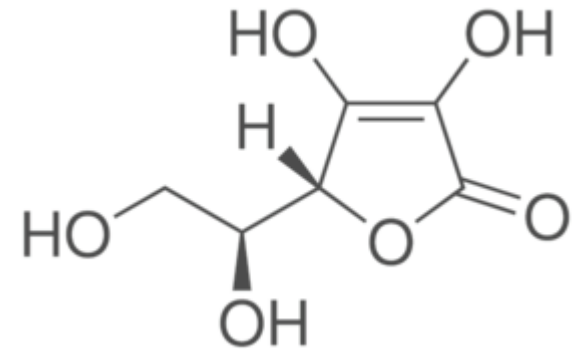
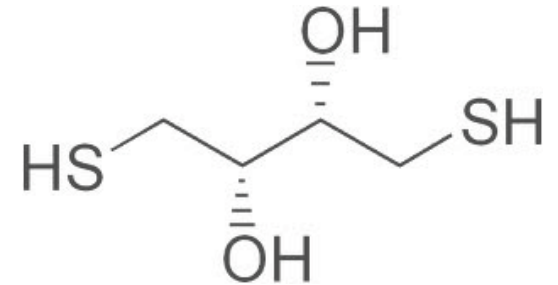
- Increase sample flow
 - Requires more material
- Attenuate the beam
 - Influences signal to noise



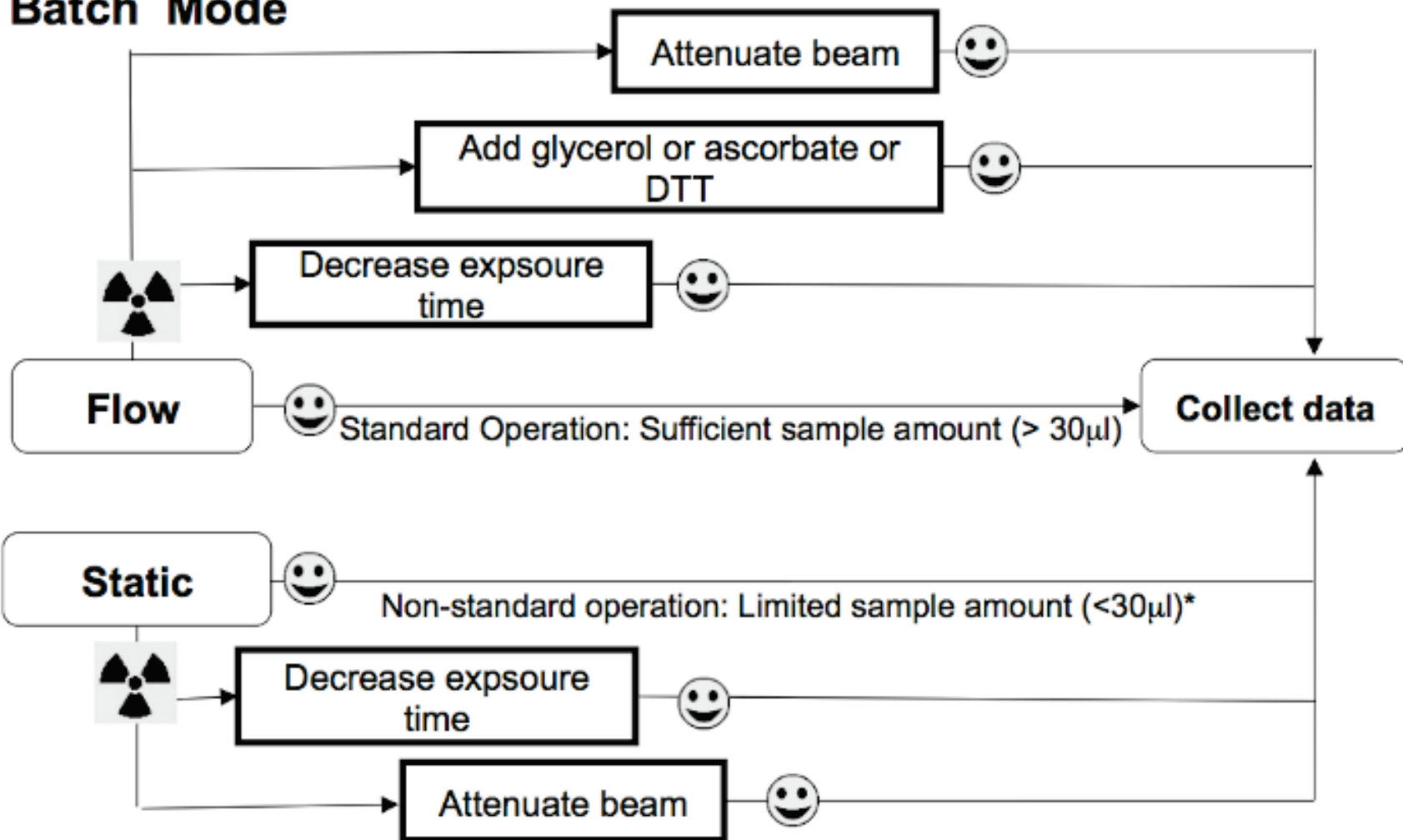


■ Scavengers

- DTT
 - Short shelf-life
 - Reduces disulfide bridges
- Ascorbic Acid
 - Only works in some cases
- Glycerol
 - Decrease in contrast
 - Difficult to pipette



Batch Mode



*It is very difficult to accurately add additives to sample volumes $< 30\mu\text{l}$

- Case 5: the Brilliant Scientist
 - Sample: dilution series of Ribonuclease
 - Question: *ab initio* Model
 - Result: Radiation Damage
 - Solution: modifications of buffer and/or data collection strategy

■ Not so- ideal samples

□ Case 1: wrong concentration

- determine best suitable method depending on proteins

□ Case 2: concentration effect (repulsion, interparticle interference)

- measure concentration series

□ Case 3: incorrect buffer subtraction

- dialysis, SEC

□ Case 4: Polydisperse sample

- Biophysical, biochemical characterization

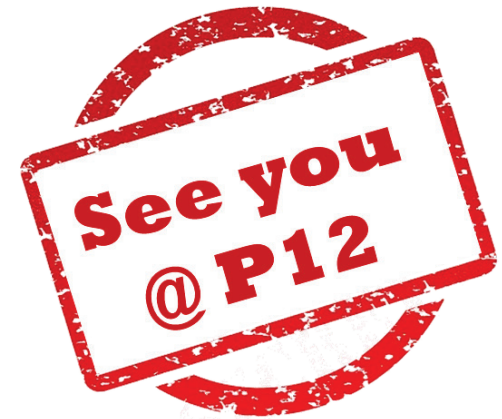
□ Case 5: Radiation damage

- Alter data collection strategy and buffer composition

■ Take home message

- Don't be too RELAXED
- Don't be too LAZY
- Don't work too HASTY (plan your experiments well)
- Be prepared for the unforeseen
- Remember (just like you) the Beam is Brilliant, so be prepared

- Know your sample!
- Know your question!



■ What do We do at P12 to improve data collection?

- Data quality (tricky samples) (*Melissa: Friday*)
- Reduced sample volume
- Higher spatial resolution
- Higher temporal resolution (*Clement: Saturday*)

