UNIVERSITY OF CAMBRIDGE

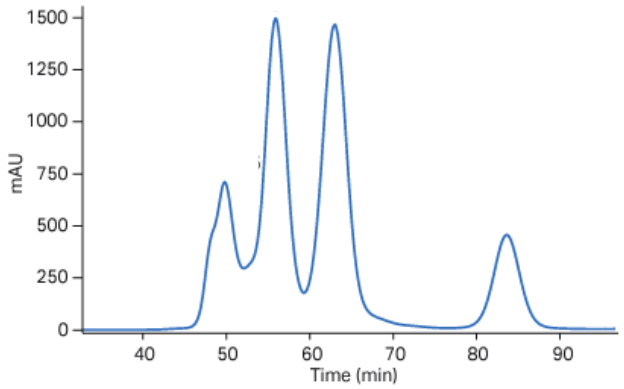
Protein Purification Part 3

Day 5: Friday 24th March

Continuing Protein Purification – Quality Control

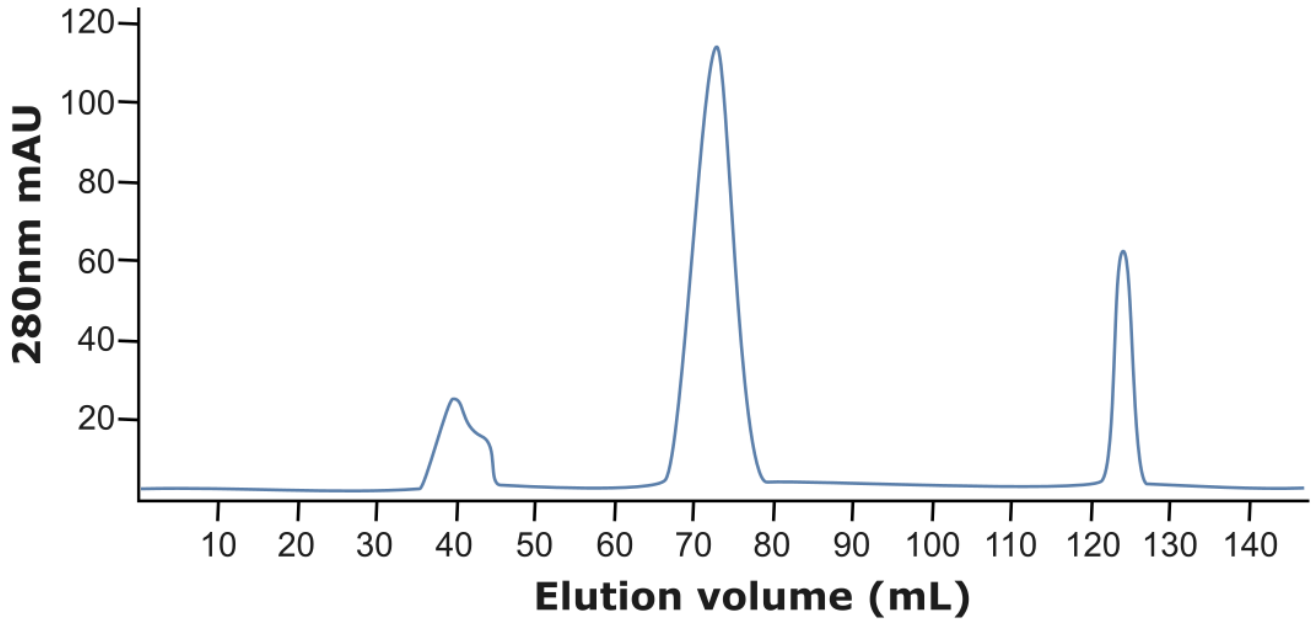
- In Parts 1 and 2 we learnt about:
  - Chromatography techniques
  - Chromatography equipment

- Today we'll learn about:
  - Interpreting your data
  - Checking your protein quality
  - Checking for correct protein folding
  - How to store your protein
  - Troubleshooting difficult samples



Interpreting Your SEC Elution Profile

- What can the elution volume tell us?



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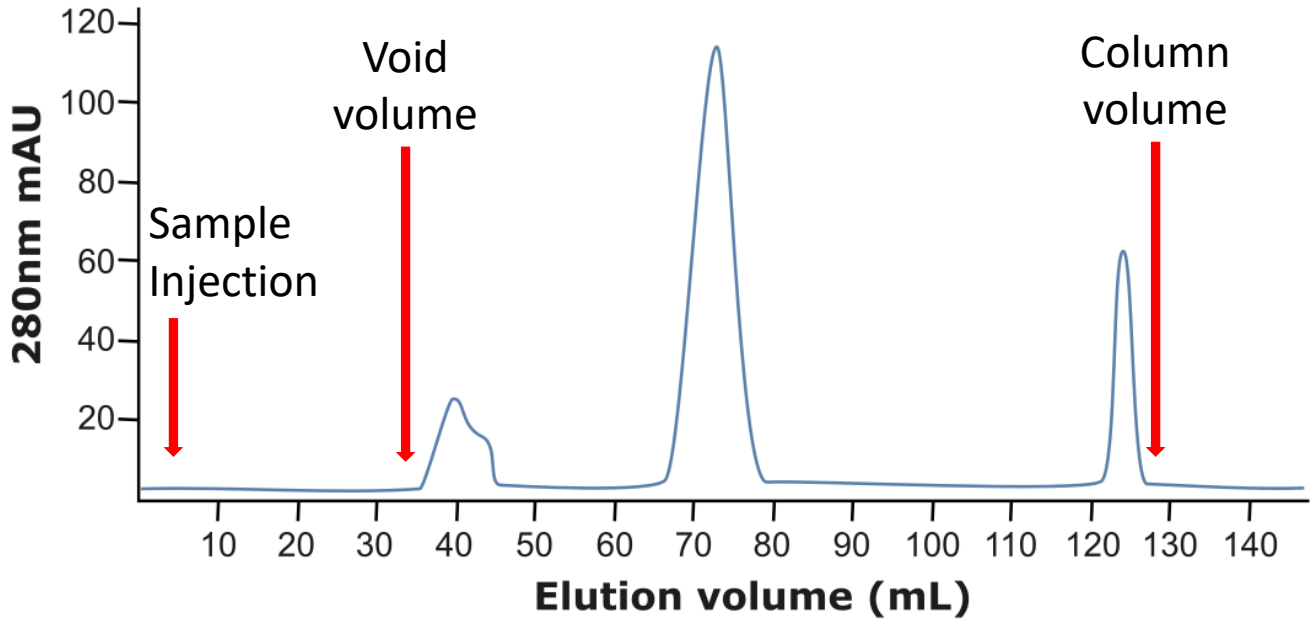

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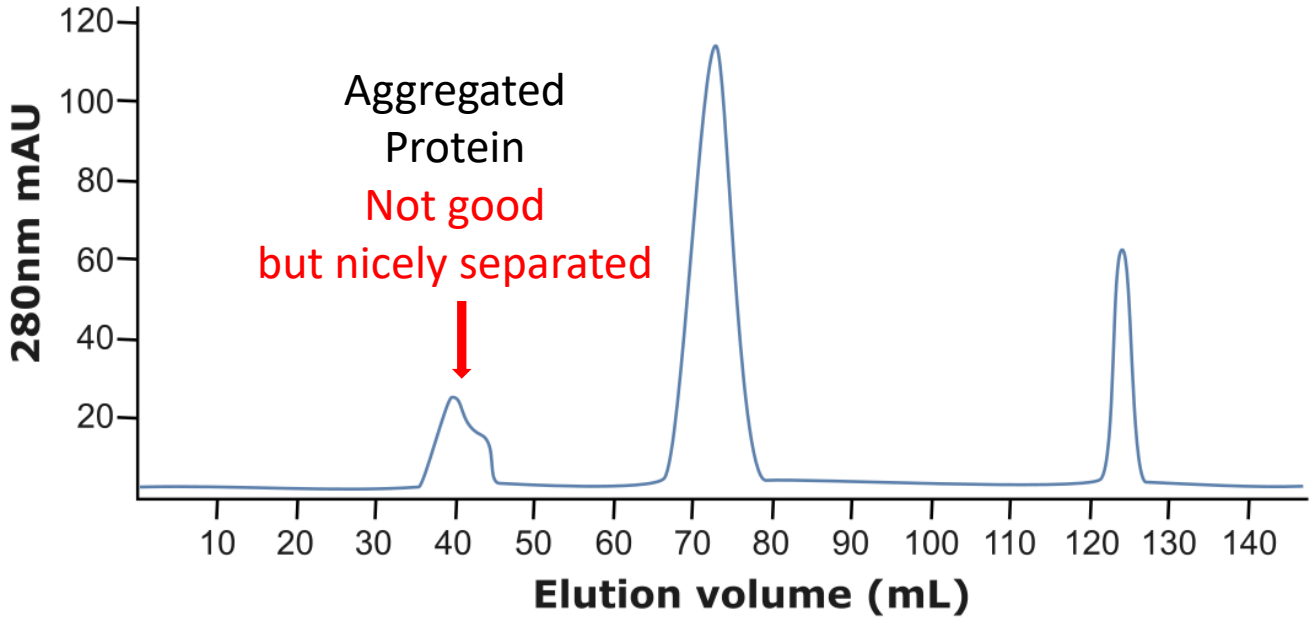

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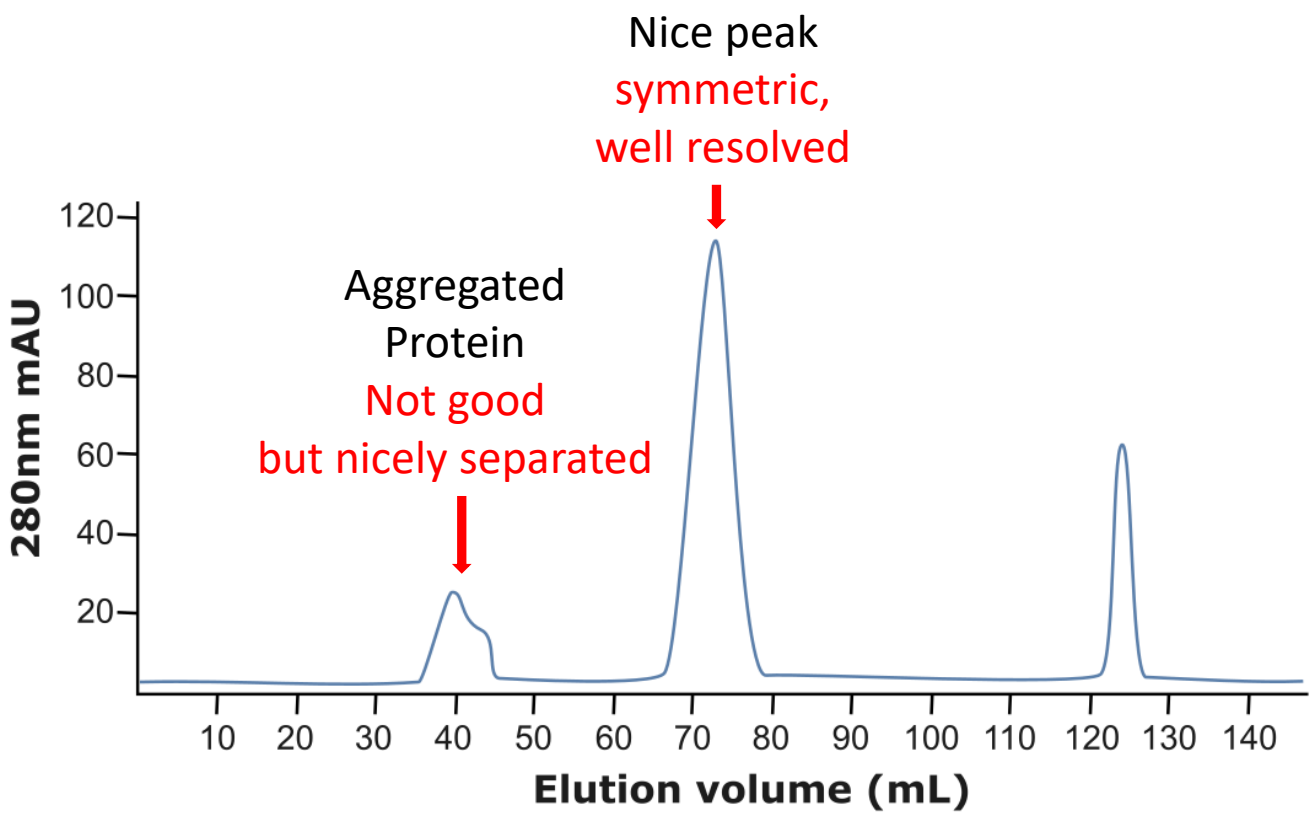

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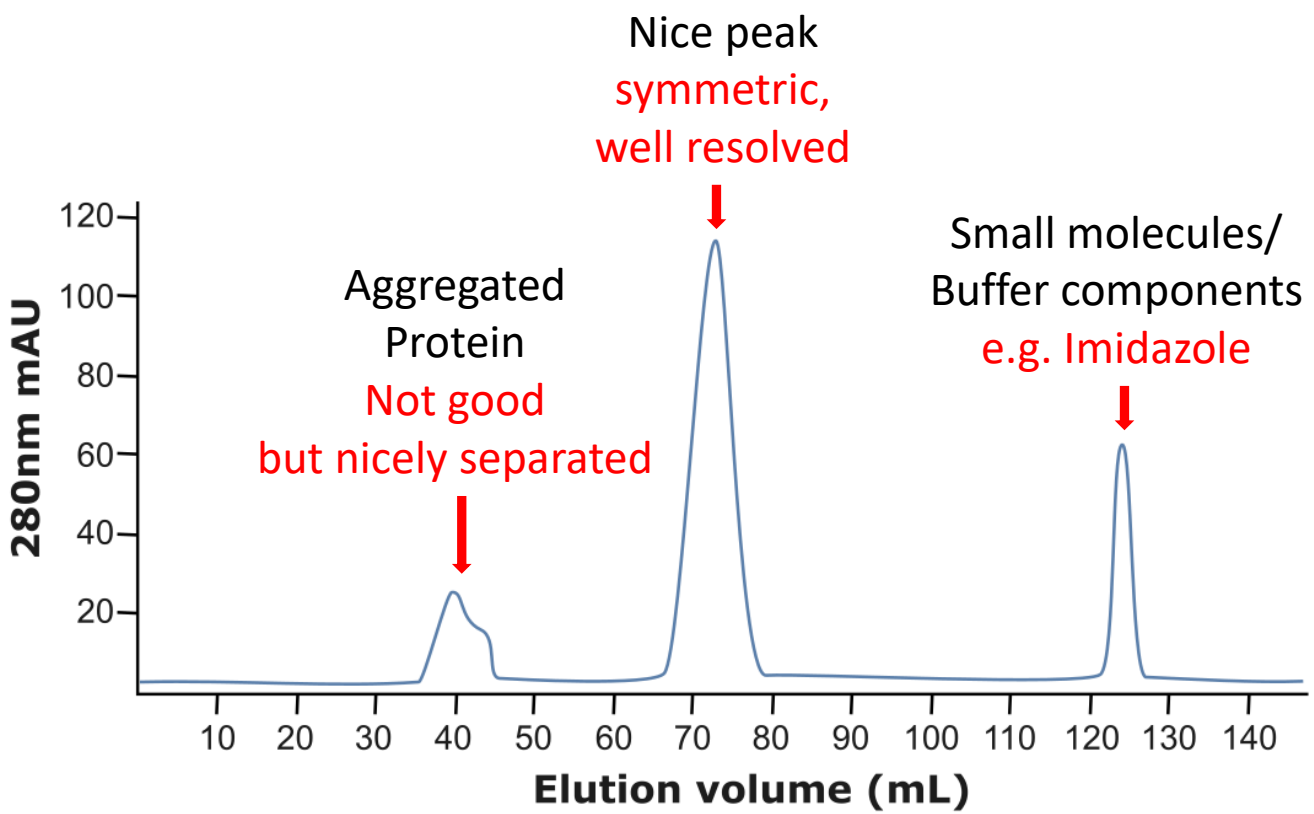
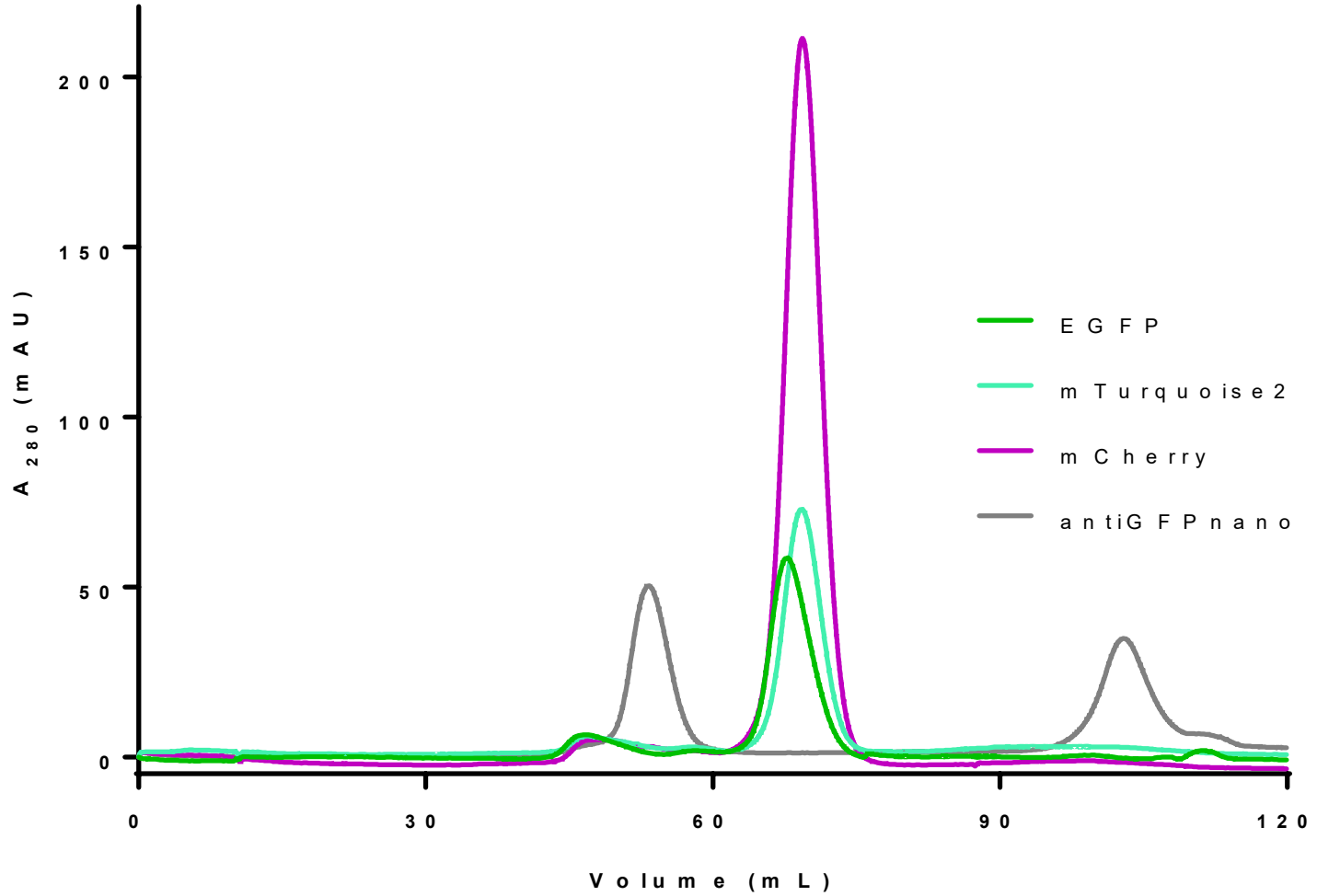
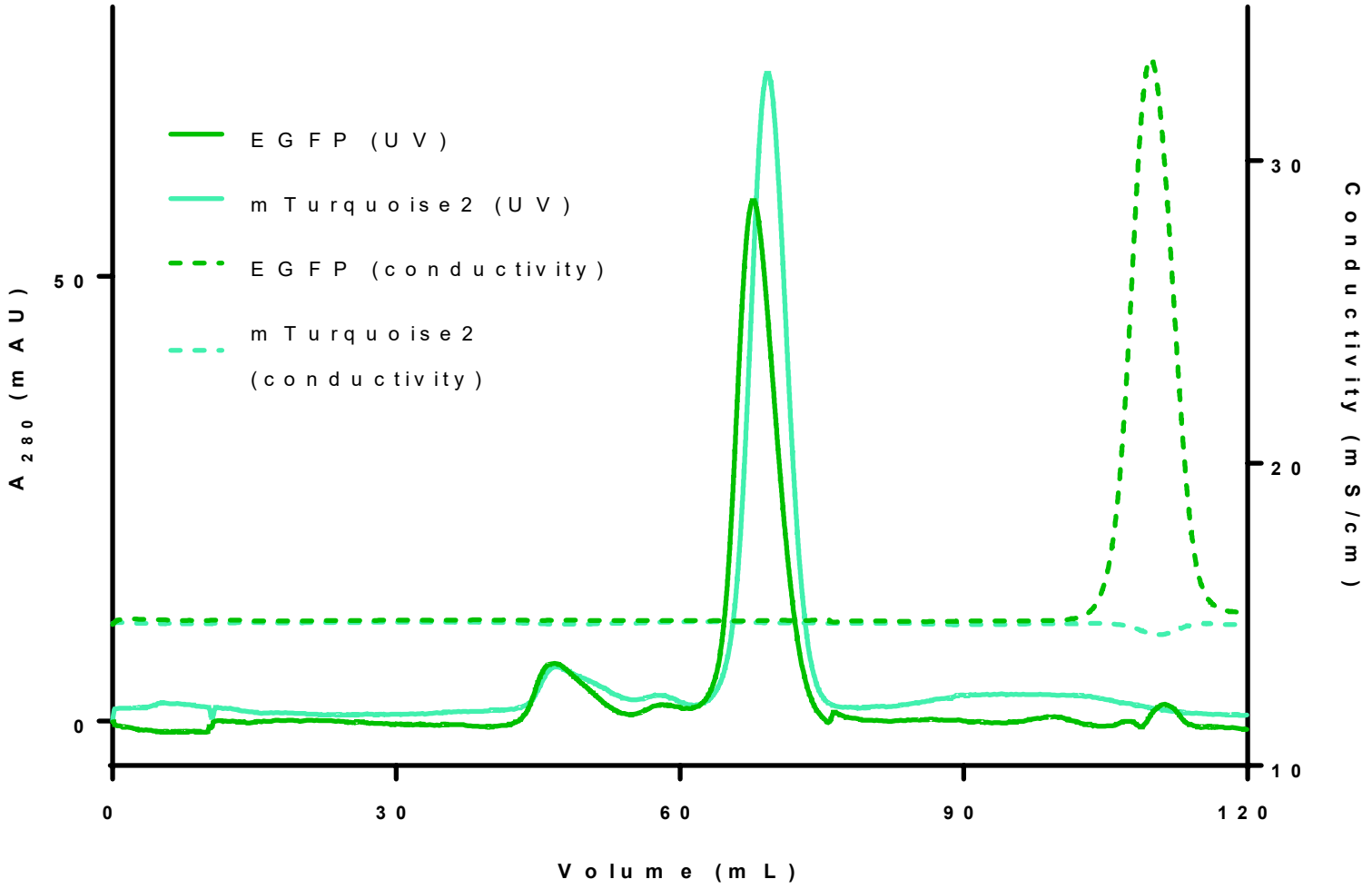


Your size-exclusion chromatography results



You can download the data from the course website

Your size-exclusion chromatography results



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Interpreting SEC Elution Profiles

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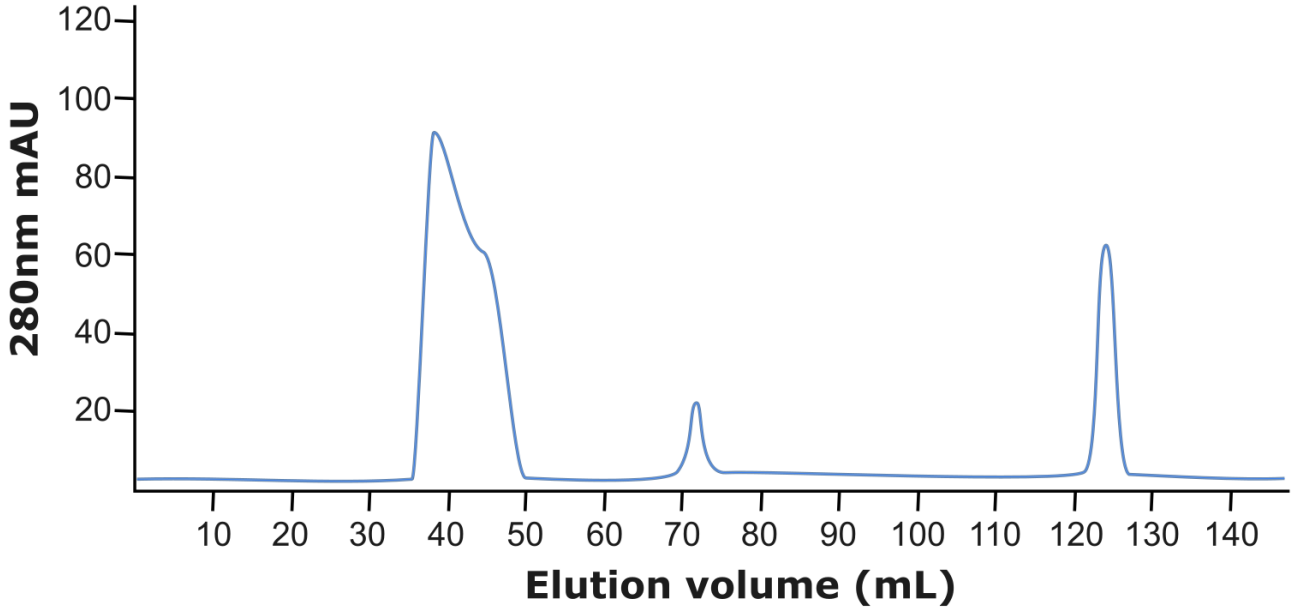

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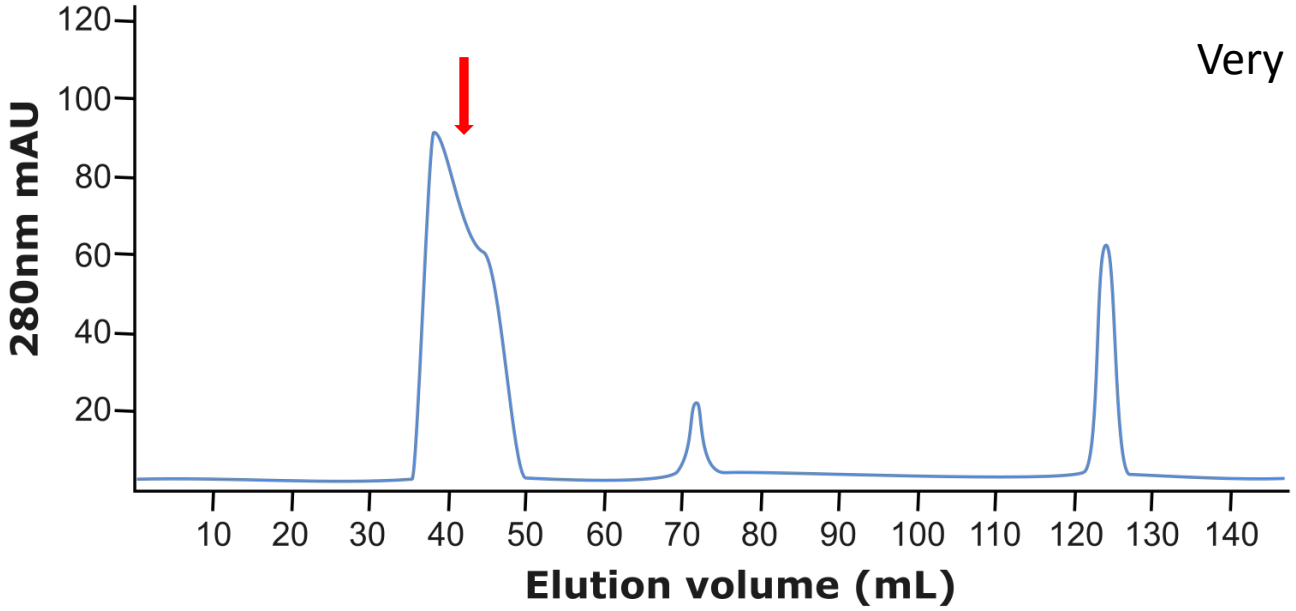

Mostly aggregate
Very little good quality protein

Interpreting SEC Elution Profiles

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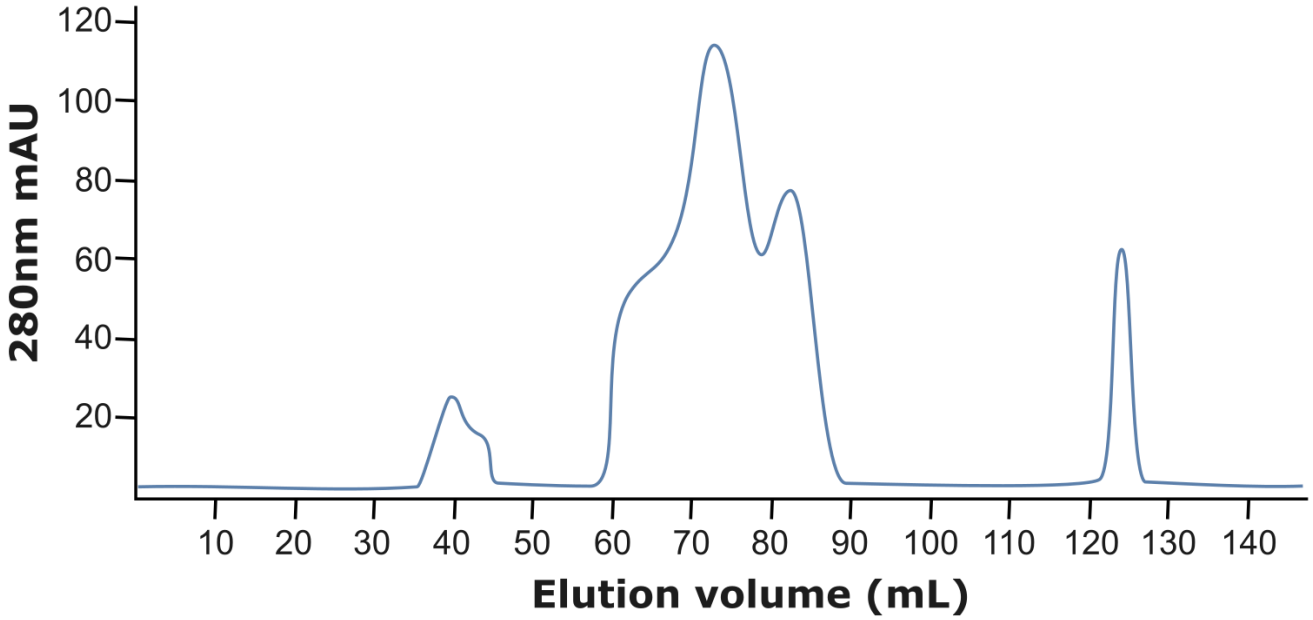

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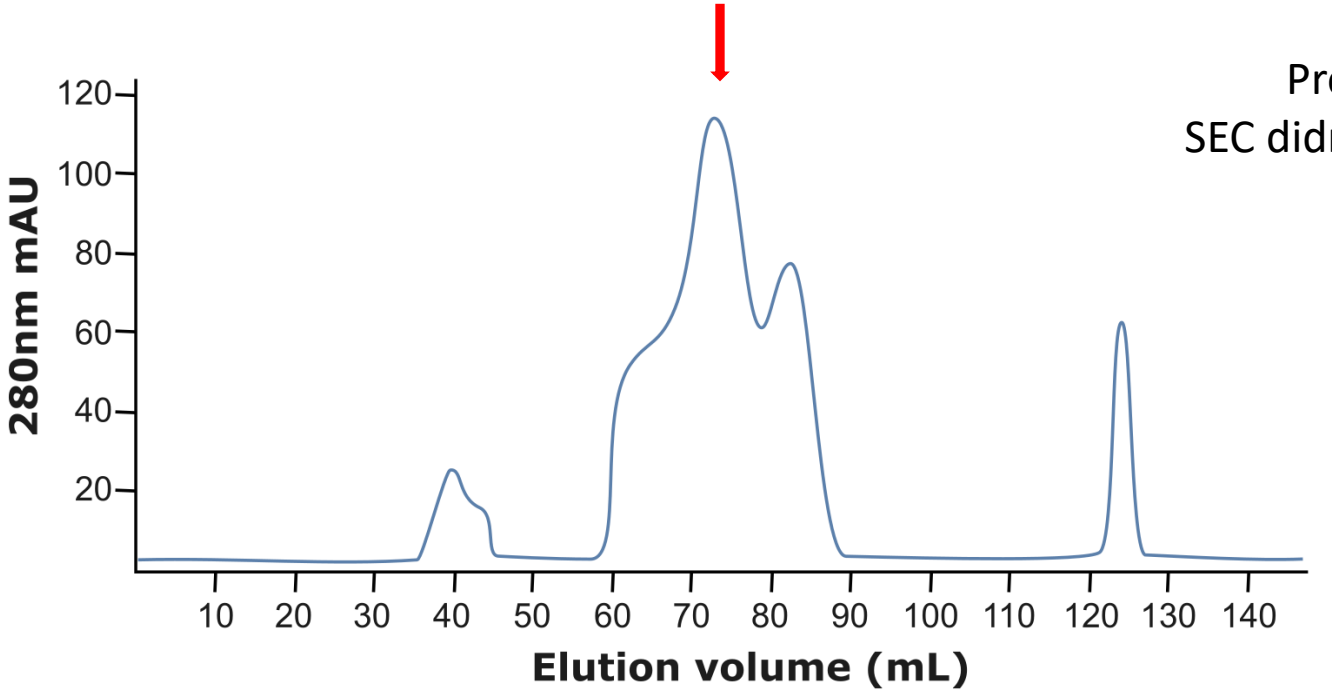

Protein not well resolved
SEC didn't really help purify protein

Interpreting SEC Elution Profiles

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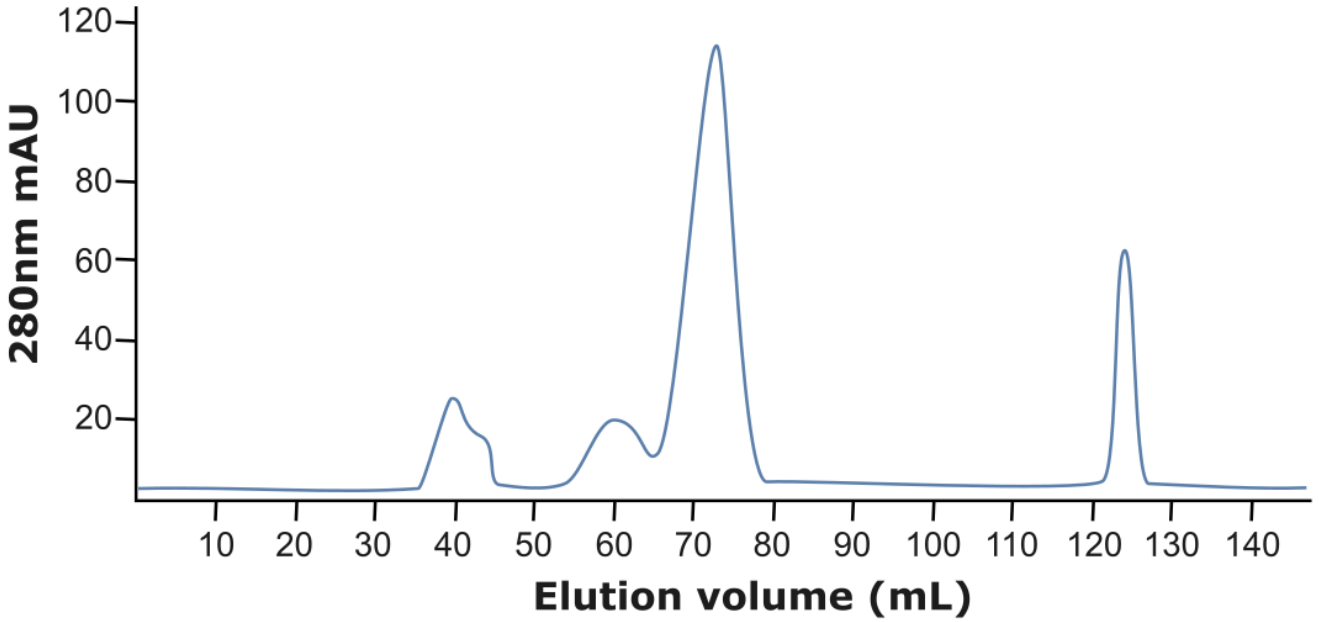

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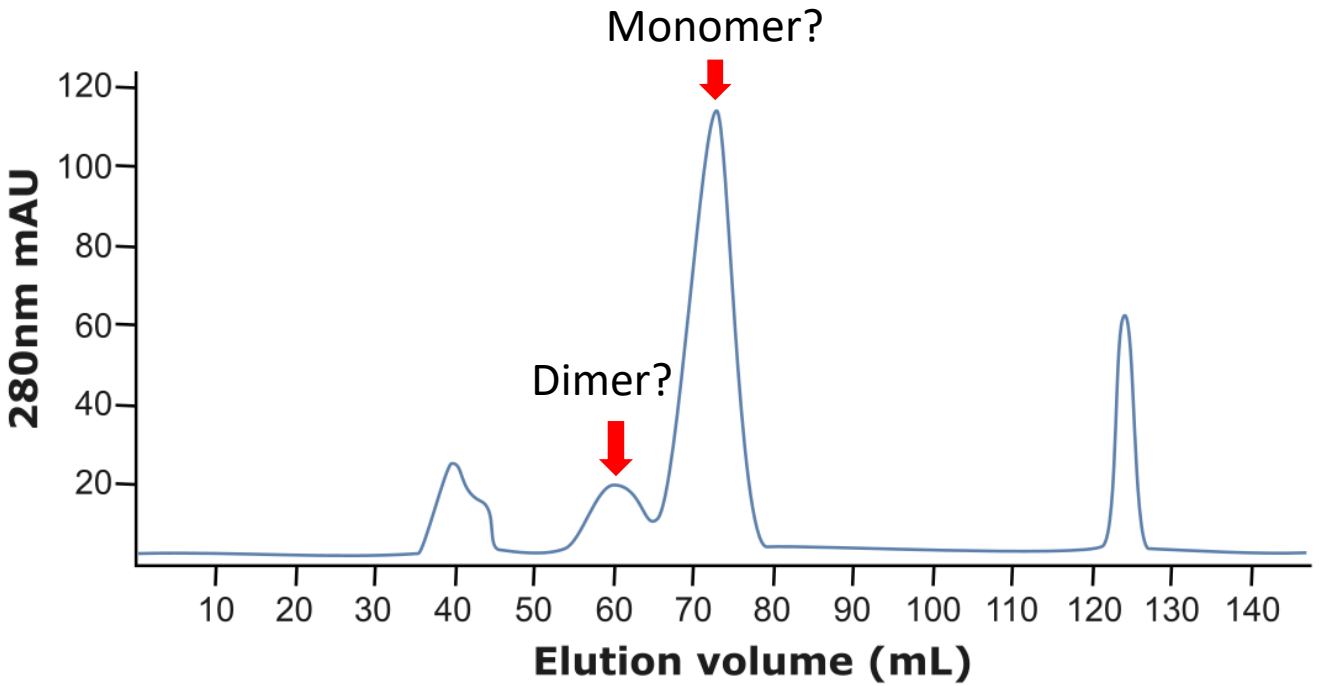
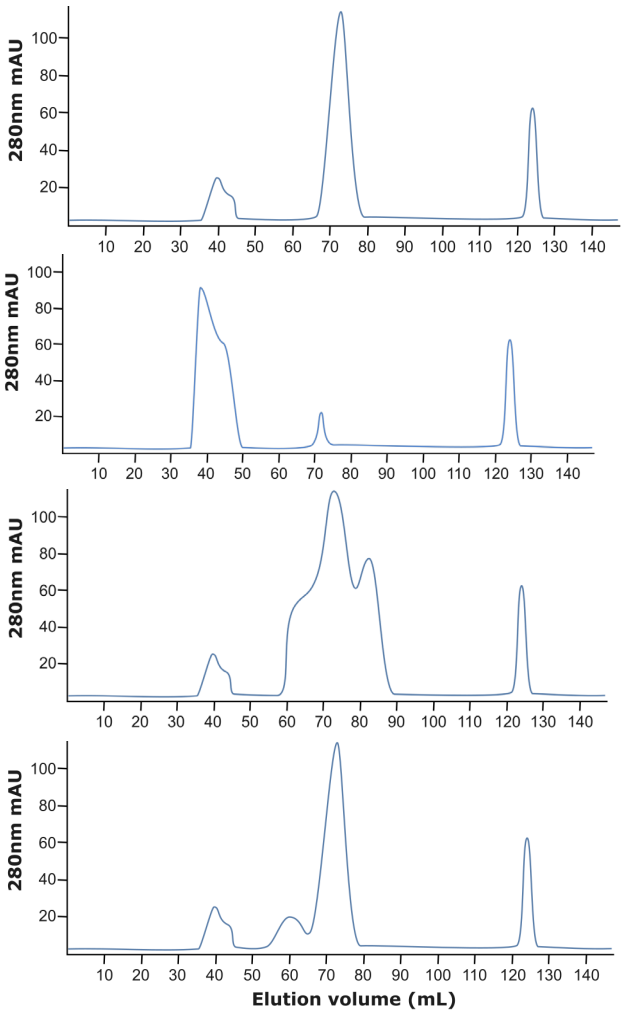


Could be forming dimer and monomer

How might we check these hypotheses?



Nice, pure protein?

Aggregated protein?

Impure protein?

Dimer?

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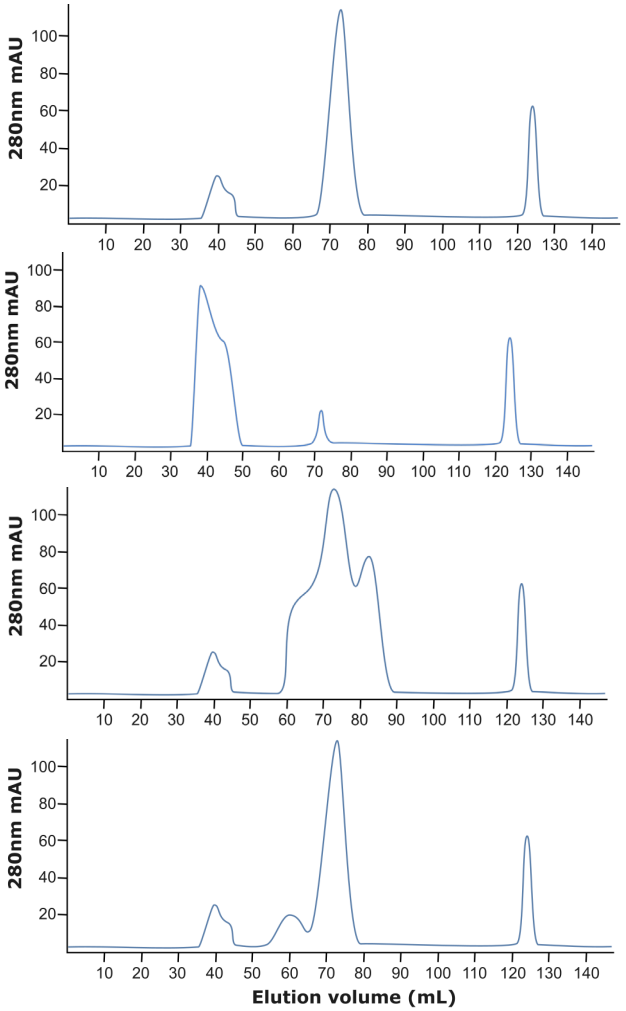

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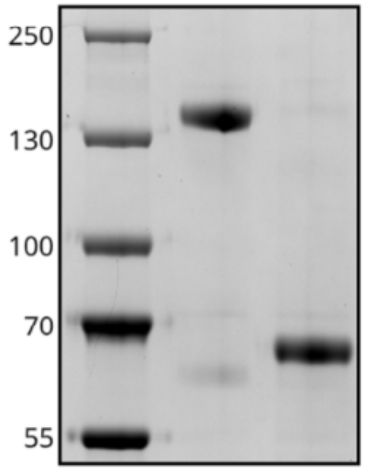
SDS-PAGE

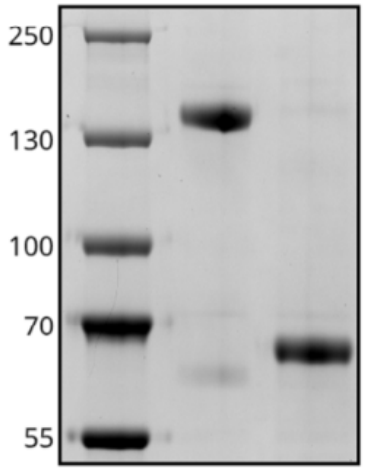


SDS-PAGE

- Allows separation of proteins based on MW

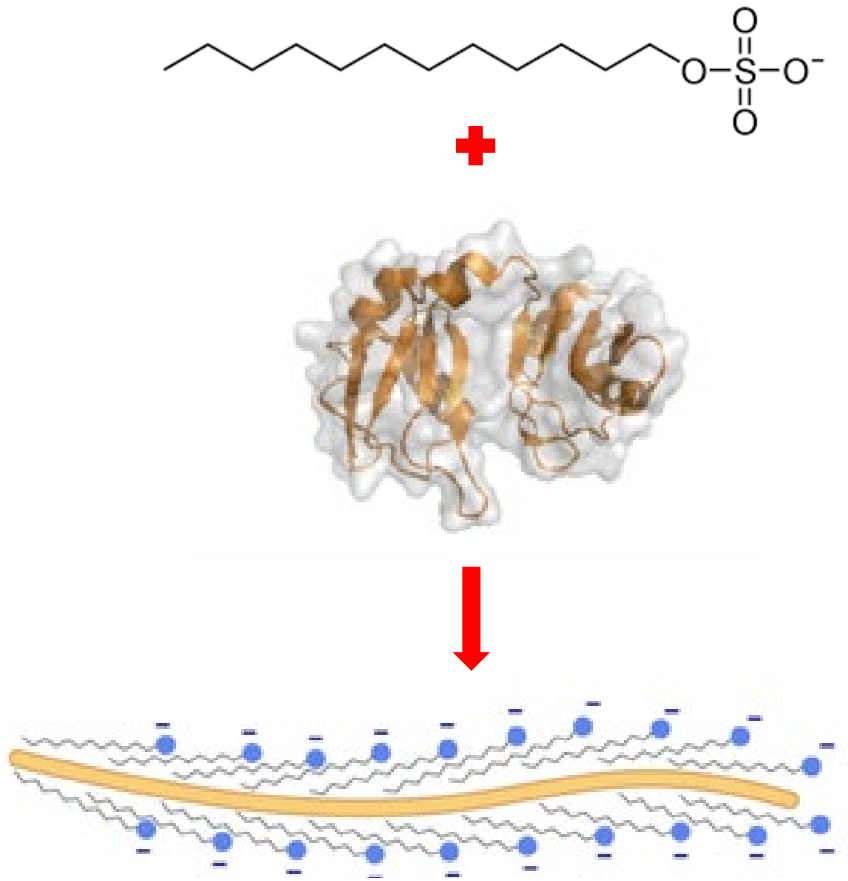
- Sample preparation
  - Why do we use SDS?
  - Why do we add DTT?
  - Why do we boil?

- Sample separation
  - Why do we use acrylamide?
  - How do we select the %acrylamide?
  - Why do we use a resolving gel?
  - Why use gradient or Tricine gels?



SDS – sodium dodecyl sulfate

- SDS possesses –ve charge
- About 1 SDS molecule per 2 amino acids
- Masks the protein's intrinsic charge
- Protein is now charged based on mass



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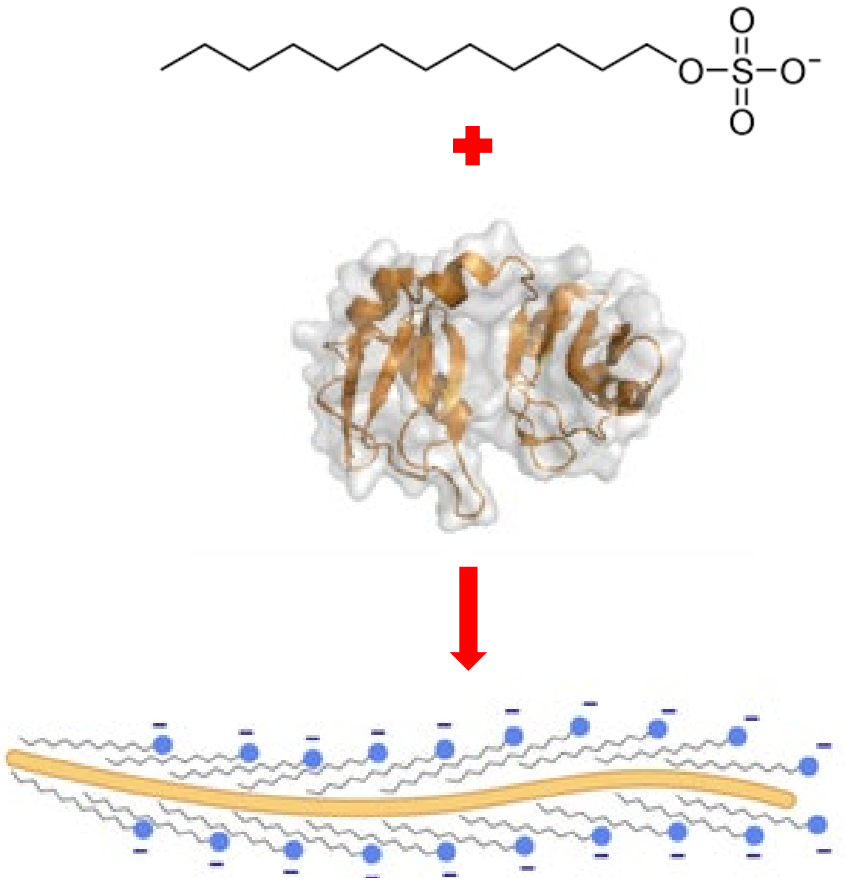
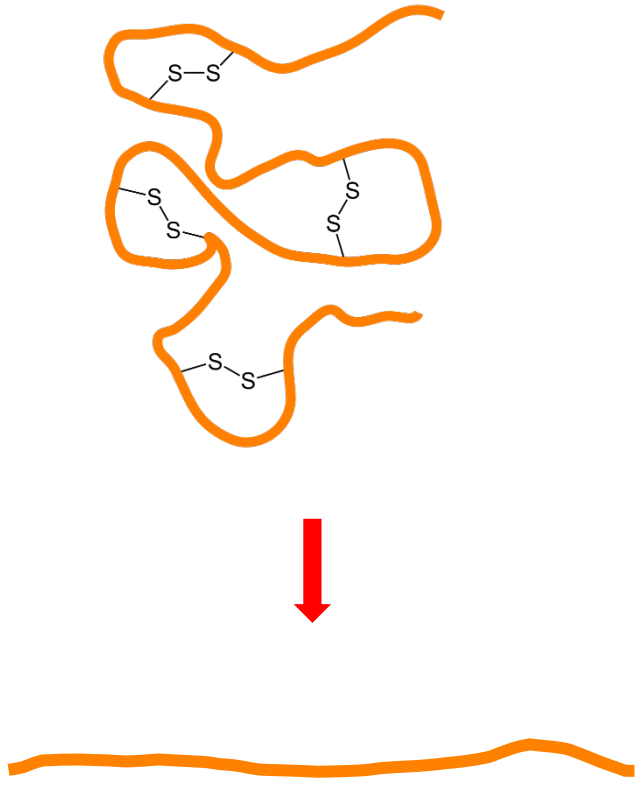
- Amphipathic nature of SDS allows it to unfold polar and non-polar structures
- Highly denaturing, disrupt complexes as well as protein fold



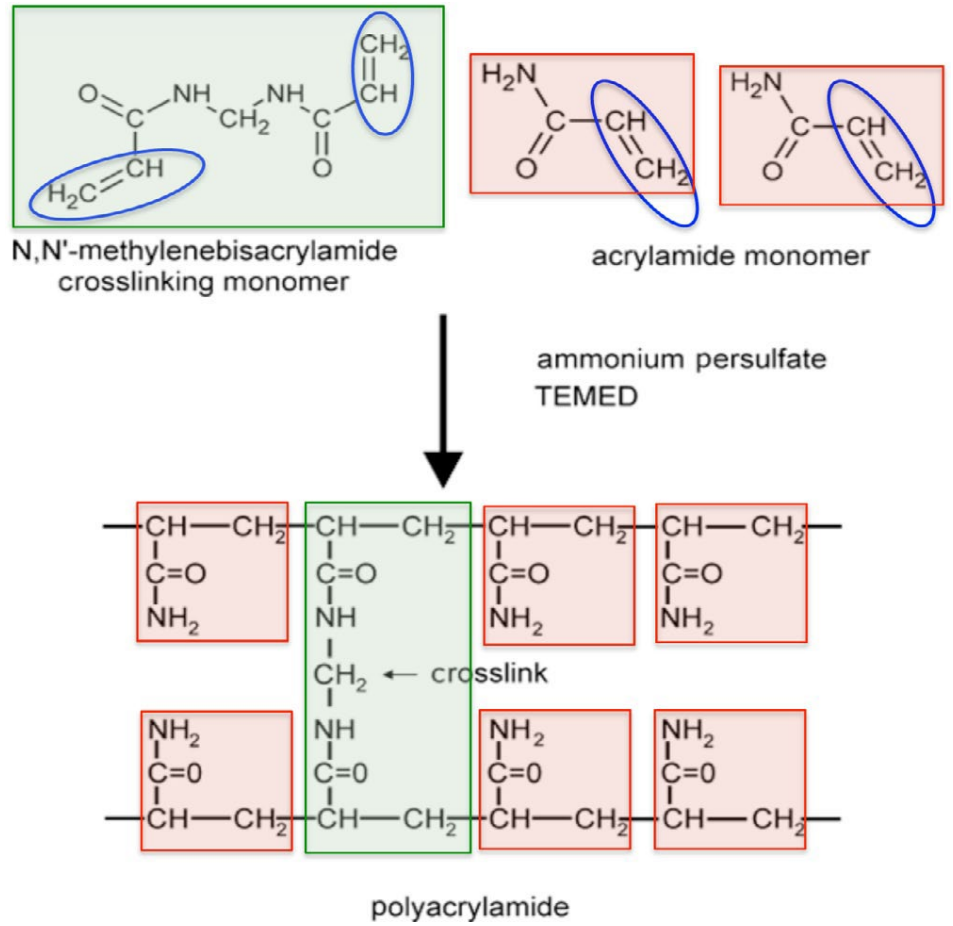
Why add DTT and why boil?

- DTT
  - Reduce disulfide bonds
  - If these are retained, the sample will not run according to its mass
- Boiling
  - Remove any SDS-resistant structure
  - Ensure sample is fully unfolded
- The proteins will now run according to MW



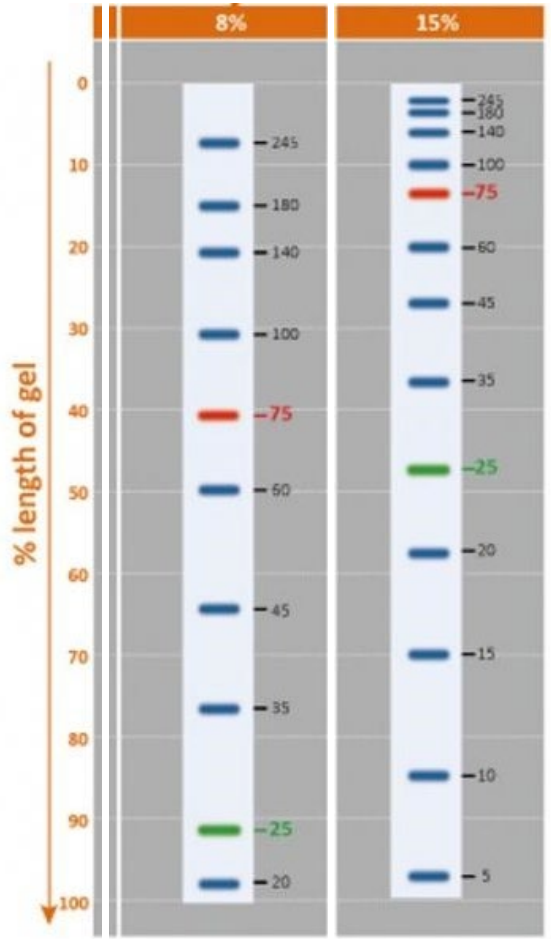
Acrylamide to polyacrylamide

- Acrylamide/Bis solution
- Free radical polymerisation
- Polymerisation of bis/acrylamide is carried out by adding:
  - *catalyst* TEMED and
  - *radical initiator* ammonium persulfate (APS)



Protein separation

- Negatively charged molecules migrate toward the +ve anode
- The polymerised acrylamide acts like a sieve
- The % acrylamide determines what sizes are best resolved

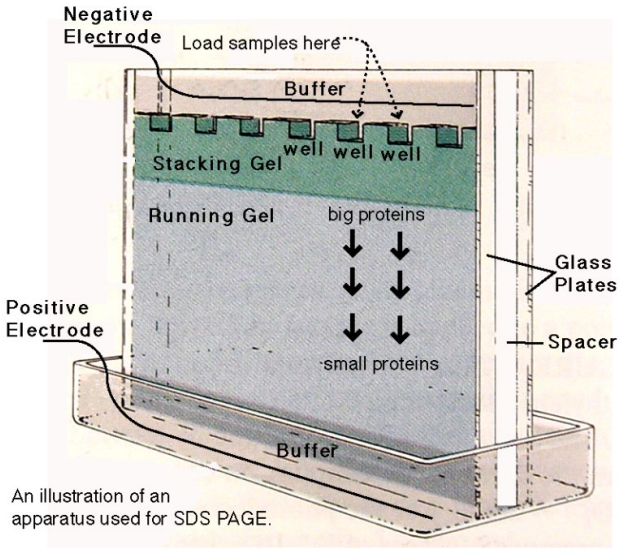


Protein separation

- Negatively charged molecules migrate toward the +ve anode
- The polymerised acrylamide acts like a sieve
- The % acrylamide determines what sizes are best resolved
- Higher % better for small proteins

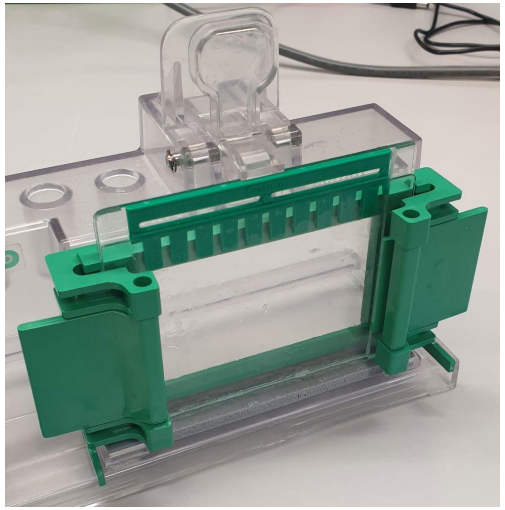
Acrylamide (%)	Mw Range (KDa)
7	50–500
10	20–300
12	10–200
15	3–100

Assembling and pouring the gel



An illustration of an apparatus used for SDS PAGE.

Take two glass plates
Use a holder to clamp them
Use a stand to seal the bottom
Pour in the resolving gel mix
Let it set
Pour in the stacking gel mix with a well comb



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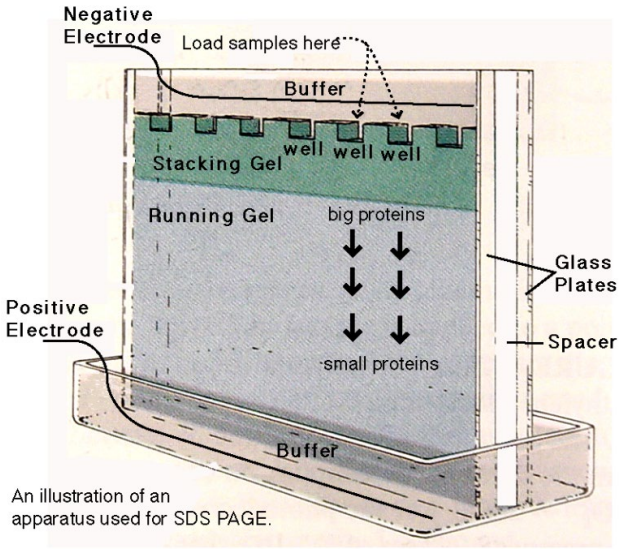

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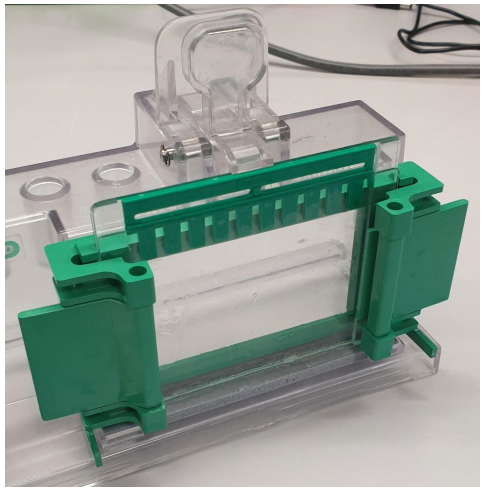
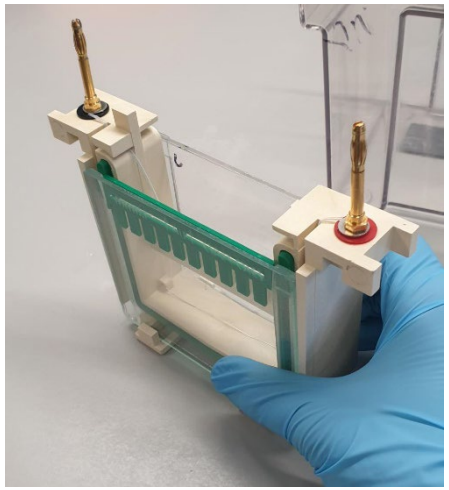
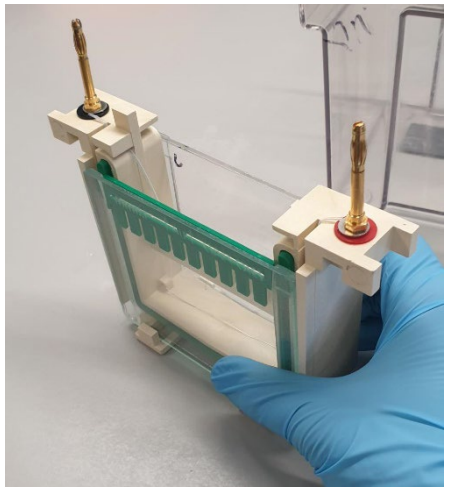


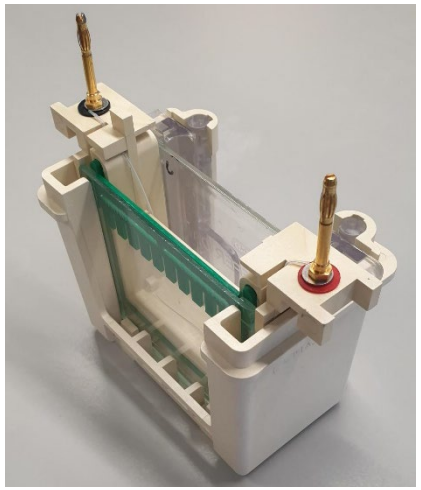


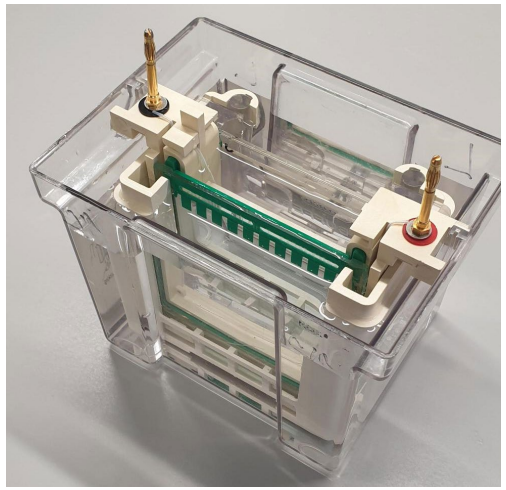
Once it's set, remove it from the stand and holder
Insert it into the central tank apparatus
Add buffer dam plate to seal the chamber

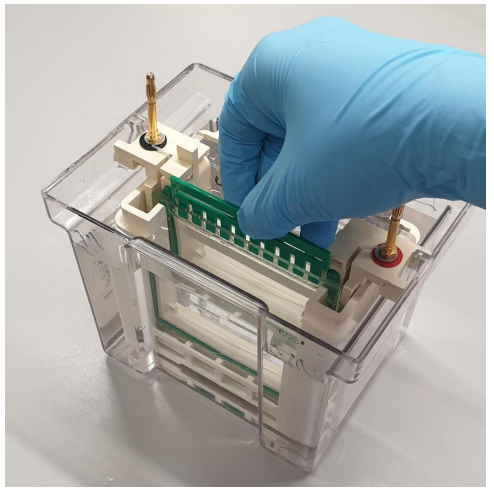
Assembling and pouring the gel

Assemble the remainder of the gel tank
Add running buffer to the tank
Remove the well comb



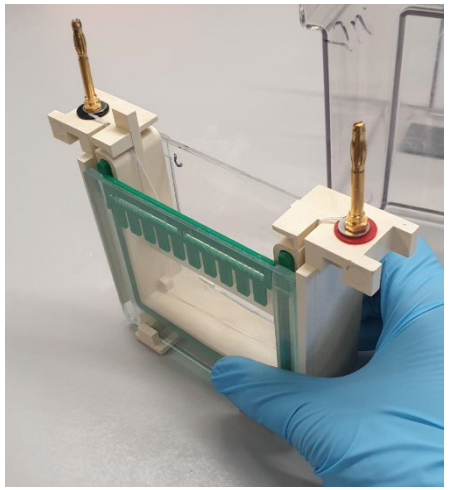


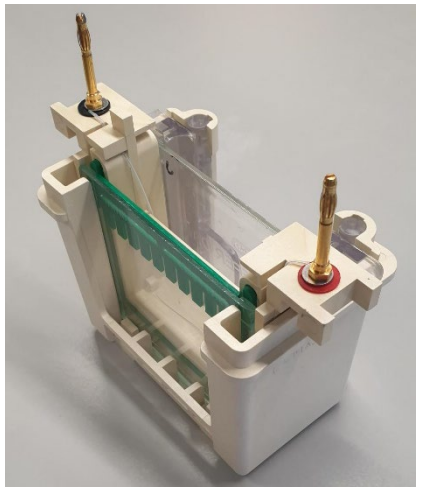


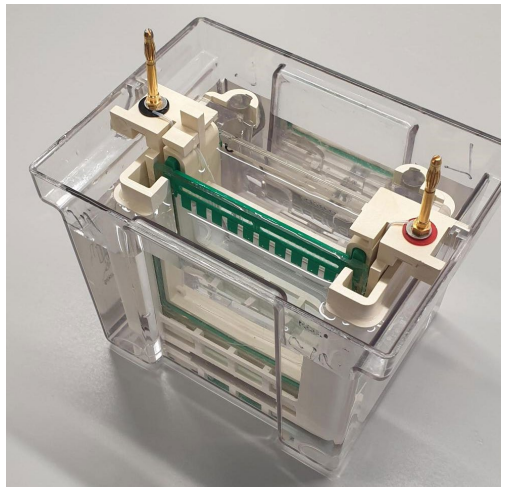


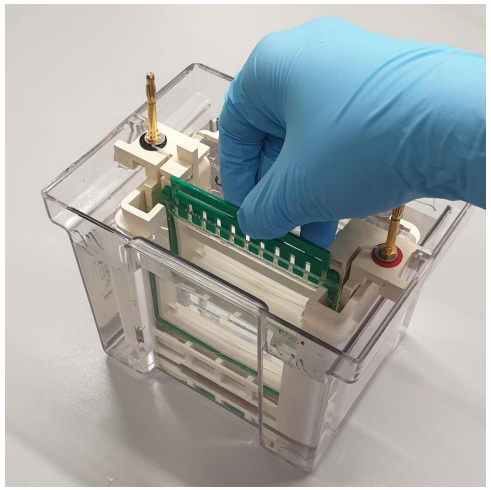
Assembling and pouring the gel

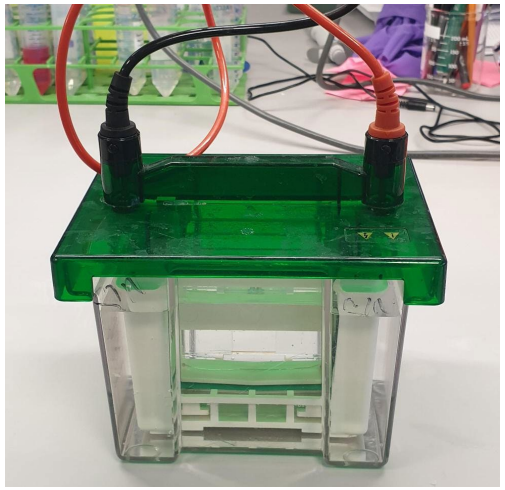
Assemble the remainder of the gel tank
Add running buffer to the tank
Remove the well comb
Add samples
Run the gel







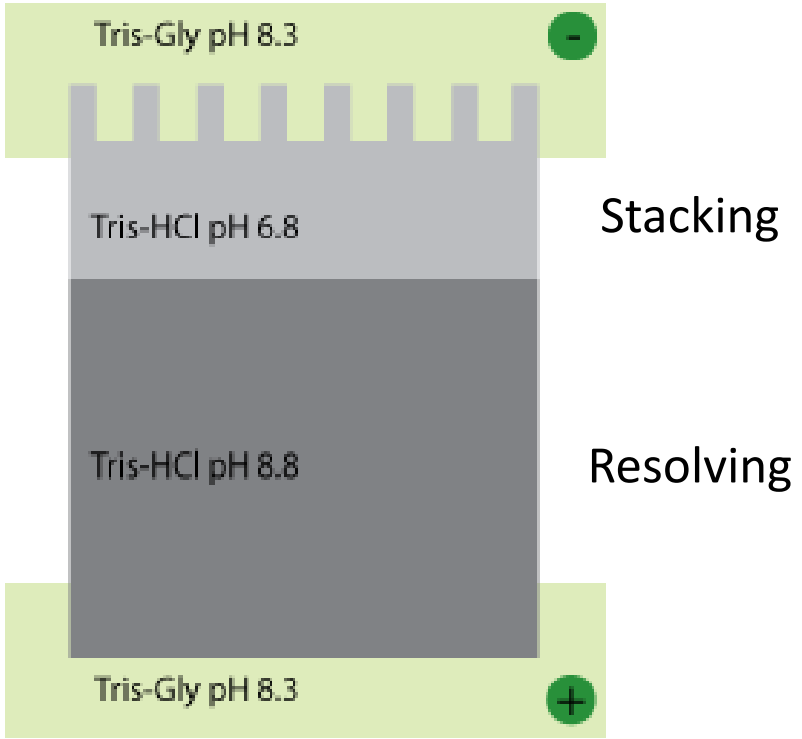




Protein separation – stacking and resolving gels

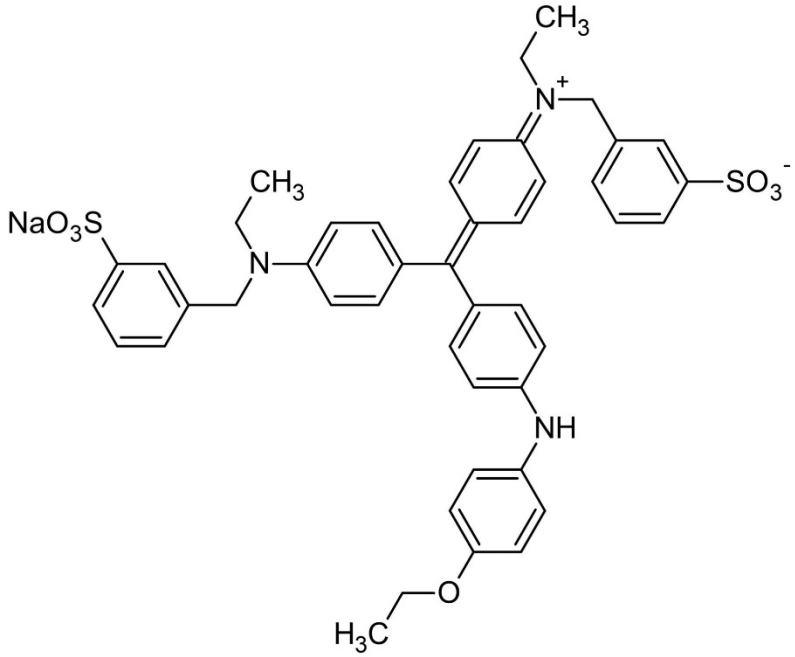
- "Discontinuous Buffer System"
  - Buffer in the tank and gel differ in terms of composition and pH

- Stacking gel: pH 6.8 and low % acrylamide
  - Don't want proteins to separate (low %)
  - Glycine –ve at pH 8.3, enter low pH stacking gel, switch to neutral so moves very slowly
  - The Cl^- ions from Tris-HCl highly mobile
  - Proteins intermediate mobility, concentrated into narrow band between these two fronts

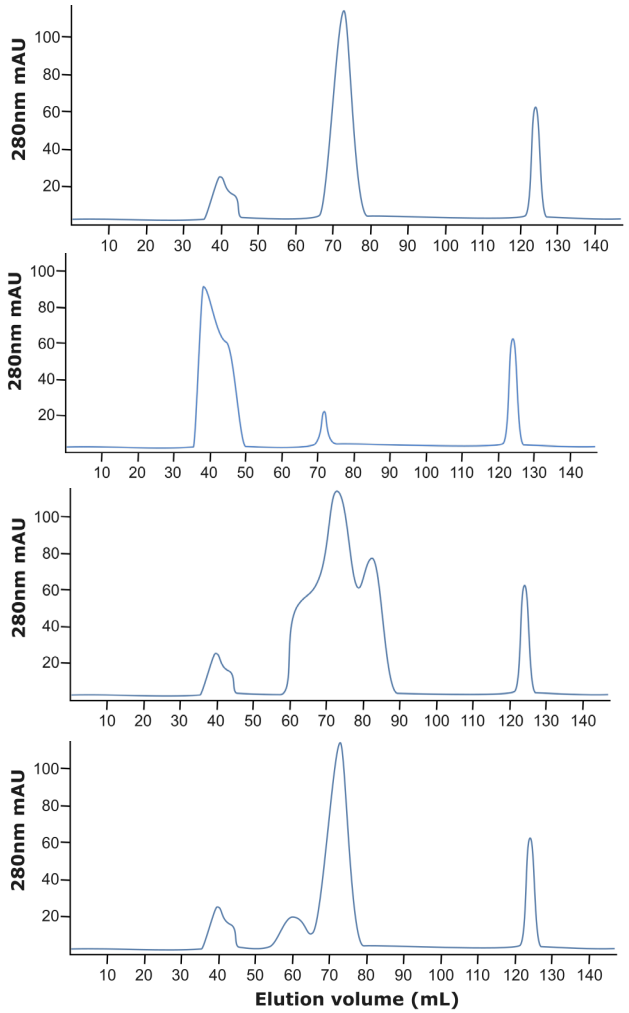


Gel staining

- Coomassie
  - Most common
  - Cheap
  - Ionic interaction between sulfonic acid groups and positive protein amine groups
- Silver stain or Sypro Orange stain
  - More sensitive
- Unlike Western blots, you see everything



How can we use SDS-PAGE to check our SEC samples?



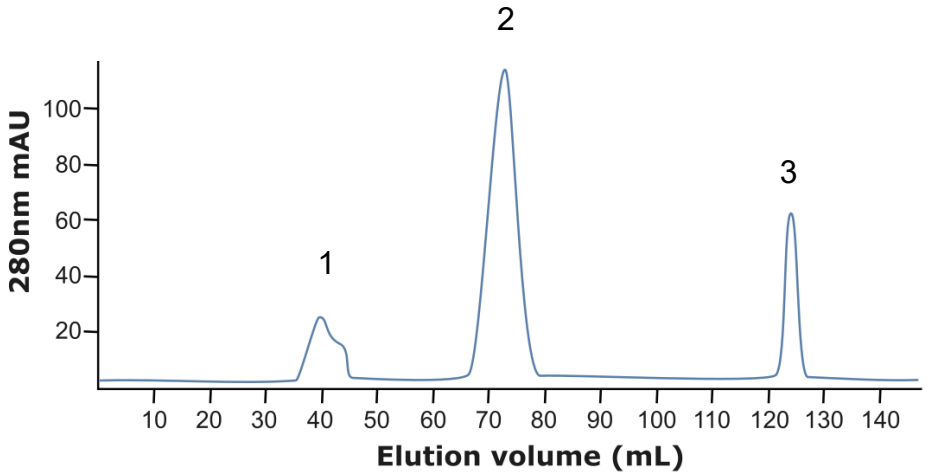
Nice, pure protein?

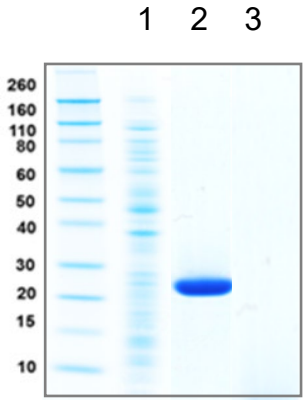
Aggregated protein?

Impure protein?

Dimer?

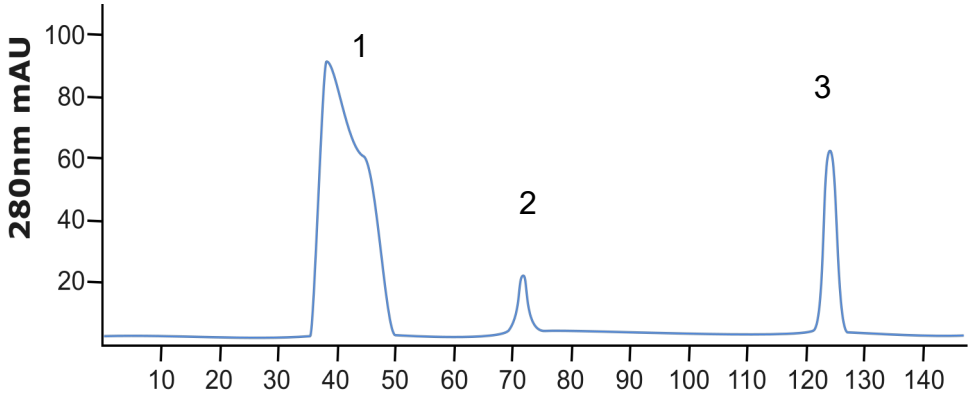
SDS-PAGE Analysis

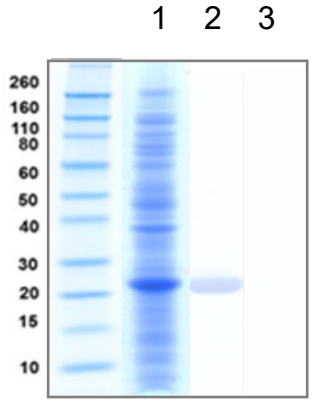




Expected MW of protein

SDS-PAGE Analysis

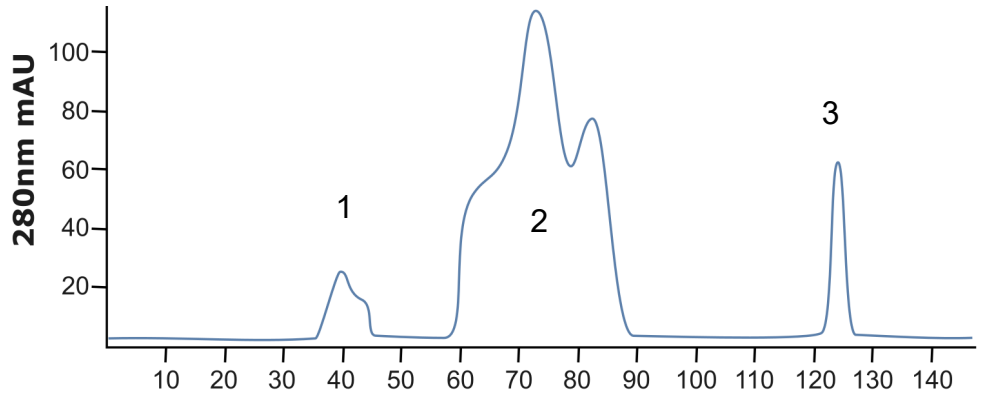




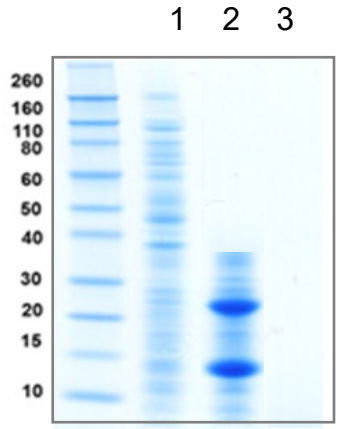
Most of protein in aggregate peak

Aggregated protein?

SDS-PAGE Analysis

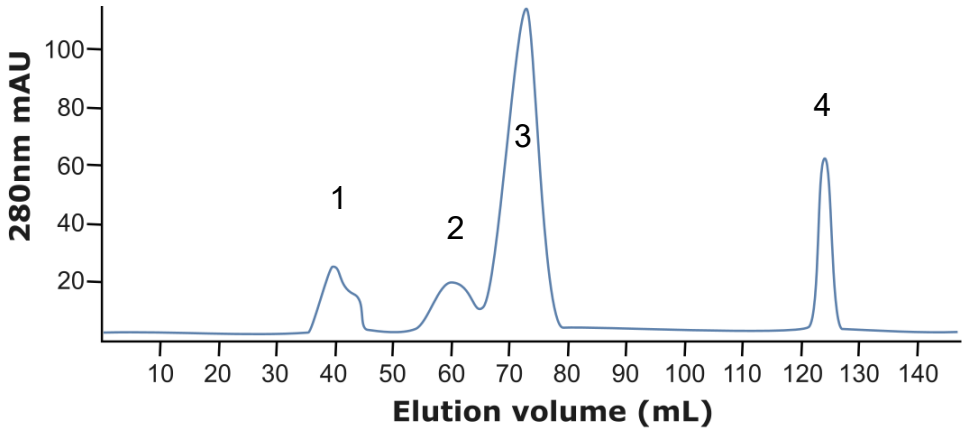


Impure protein?

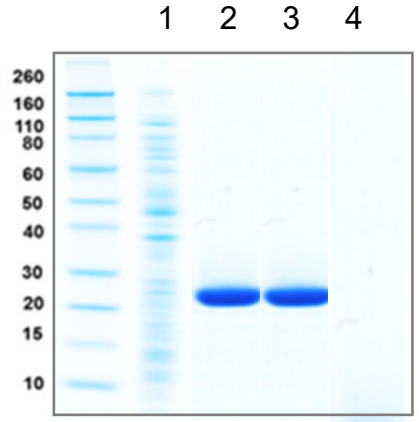


Protein not pure

SDS-PAGE Analysis

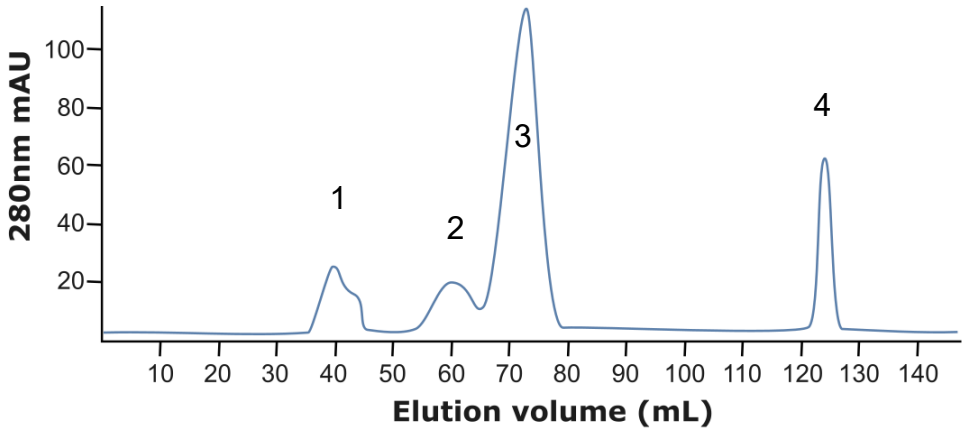


Dimer?



Same pure protein in both peaks

SDS-PAGE Analysis



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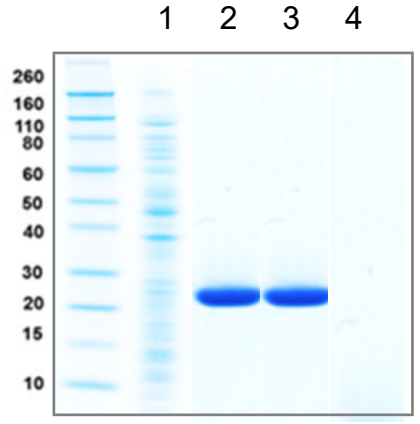
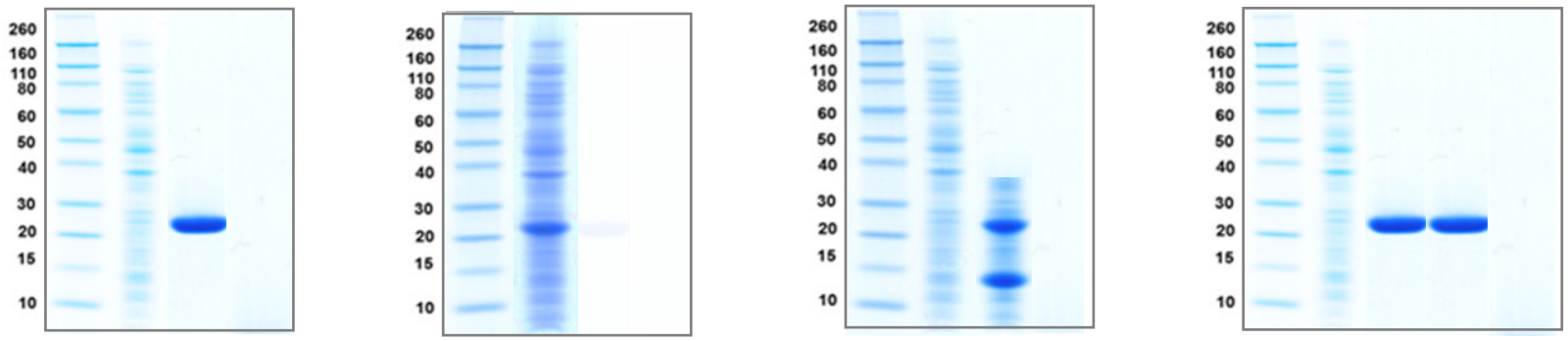


Same pure protein in both peaks

Remember SDS-PAGE denatures proteins so dimeric protein will run as a monomer

SDS-PAGE Analysis

- Combined with SEC, normal SDS-PAGE can tell us a lot



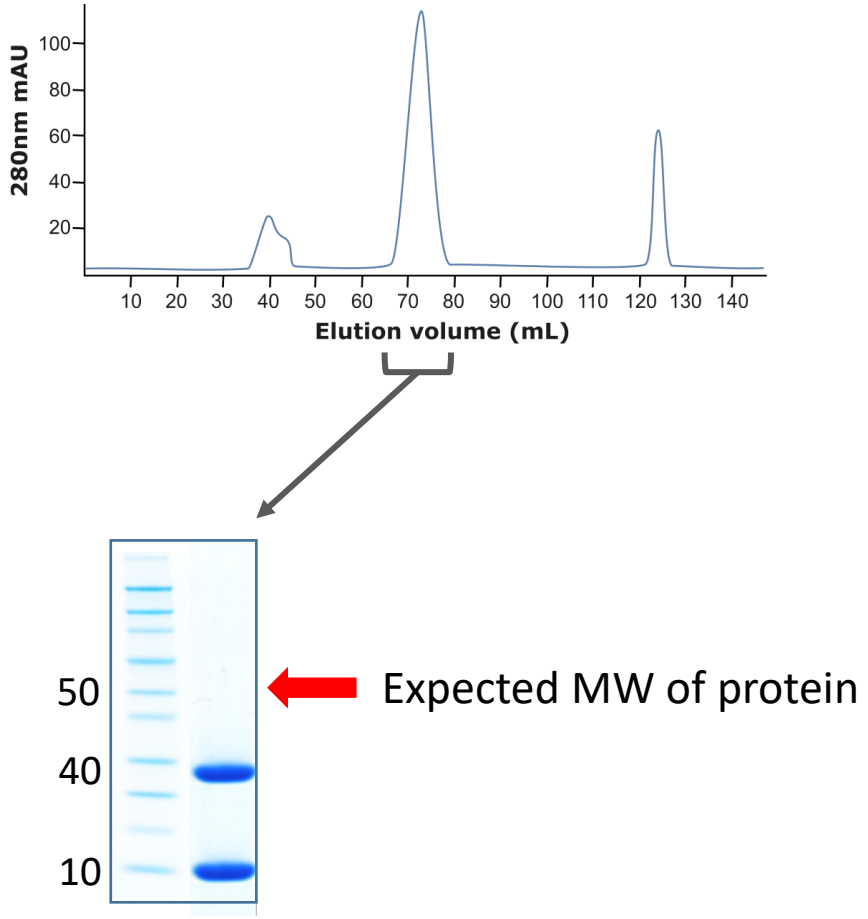
- There are some other useful things we can learn using SDS-PAGE

SDS-PAGE Analysis

- Based on our knowledge of how SDS-PAGE works we can answer additional questions:
  - Has my protein been clipped/processed?
  - Does my protein have disulfide bonds?
  - Does my protein have any post-translational modifications such as glycosylation or phosphorylation?

Protein processing – clipped loops

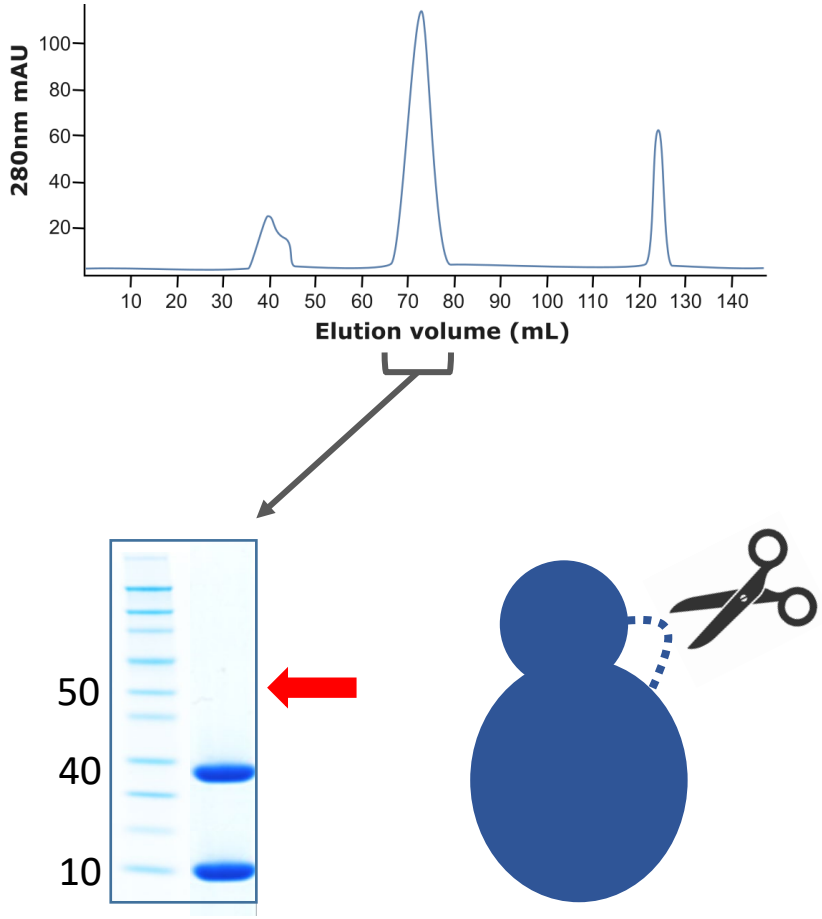
- What if I had a lovely peak off SEC but it's two bands of the wrong size



Protein processing – clipped loops

- What if I had a lovely peak off SEC but it's two bands of the wrong size

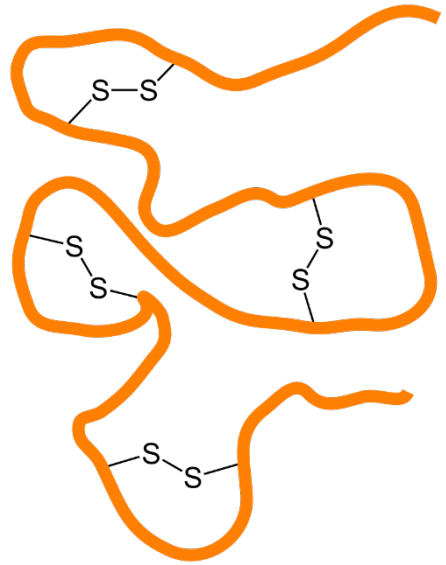
- SDS-PAGE unfolds and separates protein so if there is a clipped surface loop you're protein can be correctly folded but look like this



Disulfide bond formation

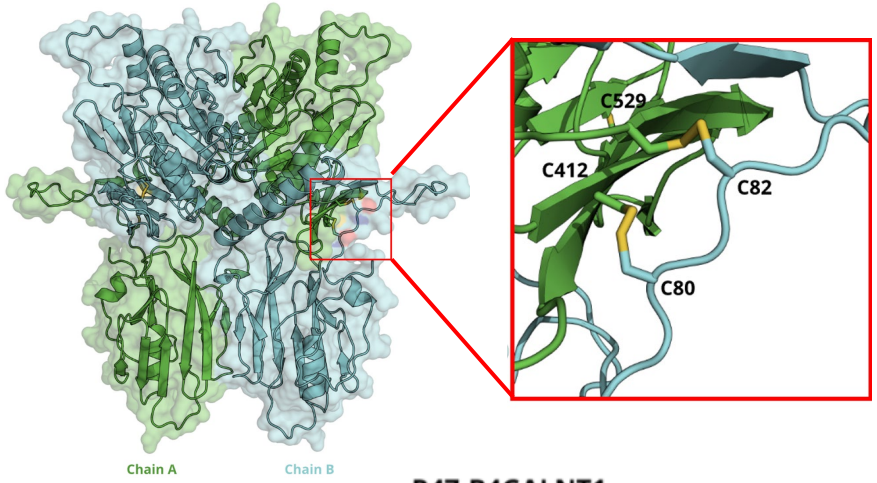
- When preparing SDS-PAGE samples we not only boil in SDS, we also add reducing agent (DTT) to break disulfide bonds

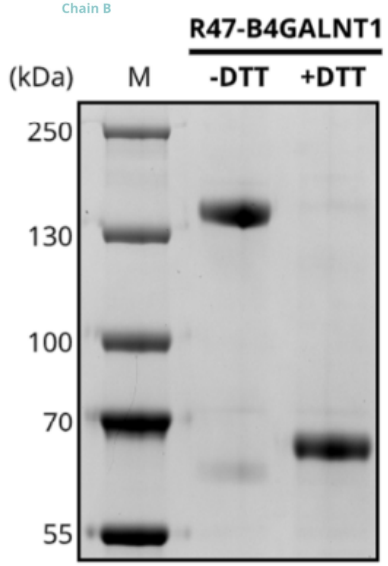
- This makes sure they run at their correct molecular weight



Disulfide bond formation

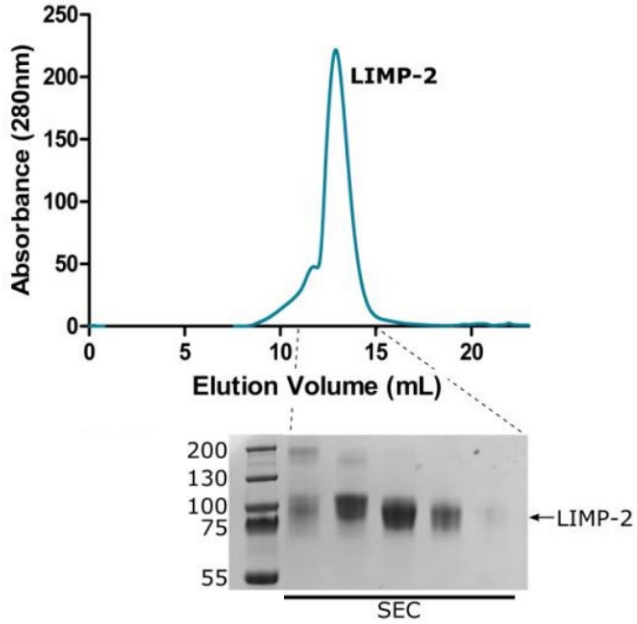
- Non-reducing SDS-PAGE (without DTT) can reveal if your protein forms:
  - Intramolecular disulfide bonds
  - Intermolecular disulfide bonds





Post-translational Modifications

- If your protein is glycosylated on SDS-PAGE it might:
  - run much larger than expected
  - appear smeary



Expected MW 45 kDa

Post-translational Modifications

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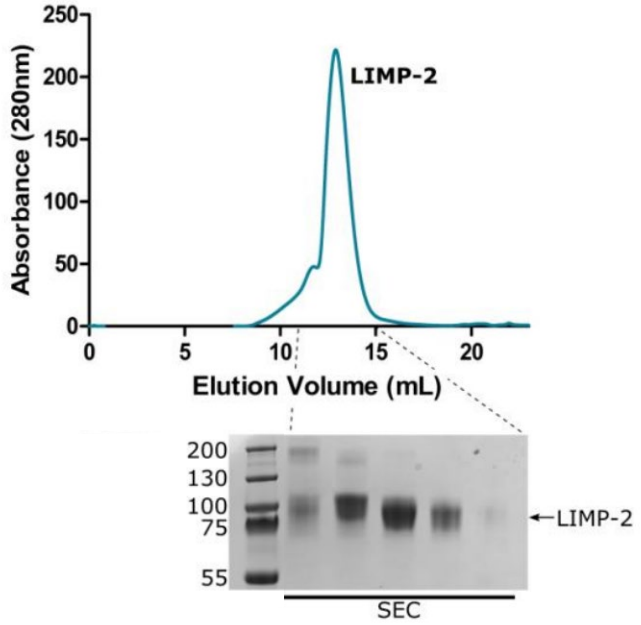
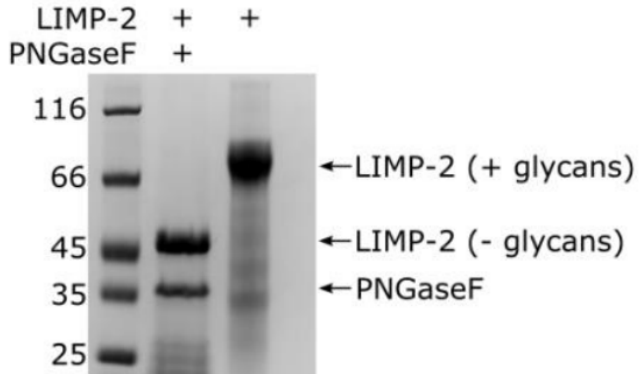
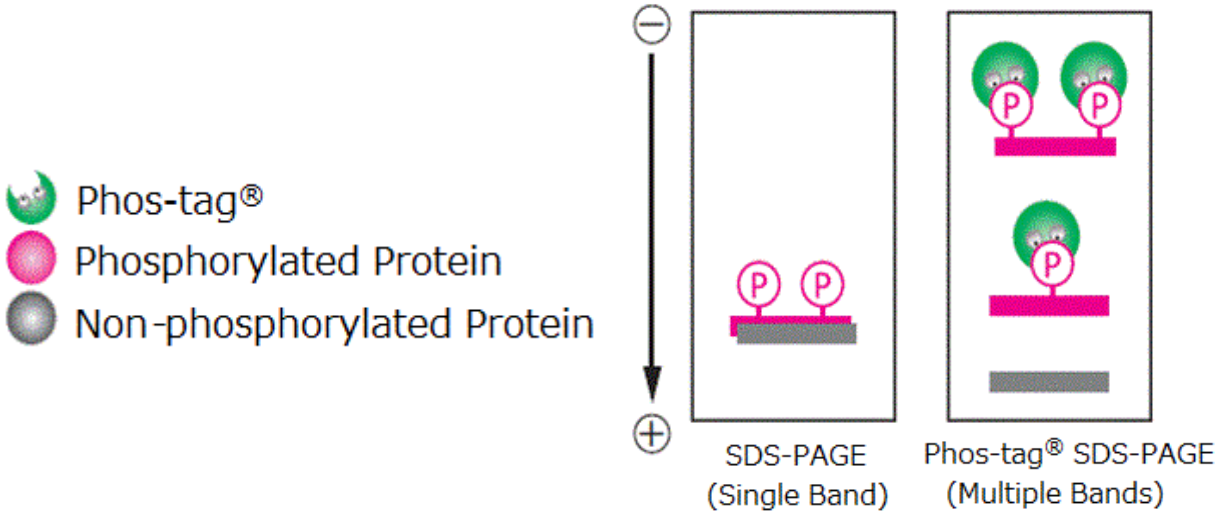


- Deglycosylation using PNGaseF can confirm this, reducing your protein to its predicted MW



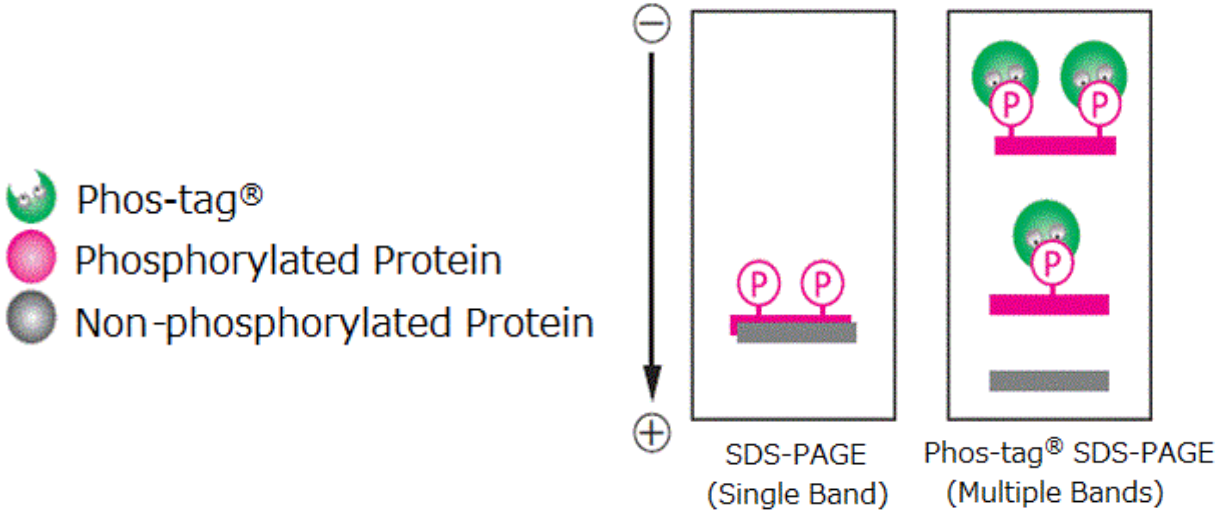
Post-translational Modifications - phosphorylation

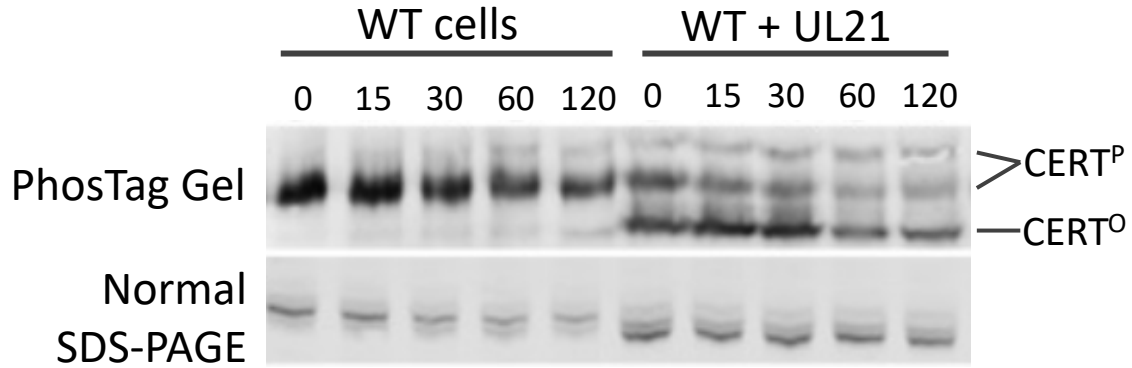
- Add a Phostag reagent into SDS-PAGE gel mix
- Retards phosphorylated proteins so run higher



Phosphotag Gels

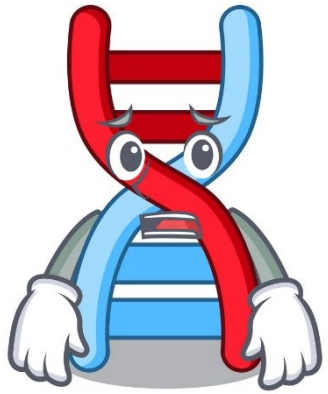
- Add a Phostag reagent into SDS-PAGE gel mix
- Retards phosphorylated proteins so run higher
- The more phospho sites the higher they run

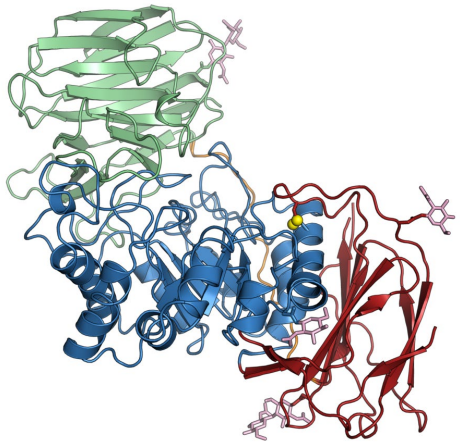




What can’t SDS-PAGE tell us about our sample?

- Using SDS-PAGE we can’t:
  - Identify DNA contamination
  - Reveal if your protein is actually folded
- How do we check this?





Estimating Purity Using UV Spectrophotometry

- UV spectrophotometry and the A260:280 ratio

Estimating Purity Using UV Spectrophotometry

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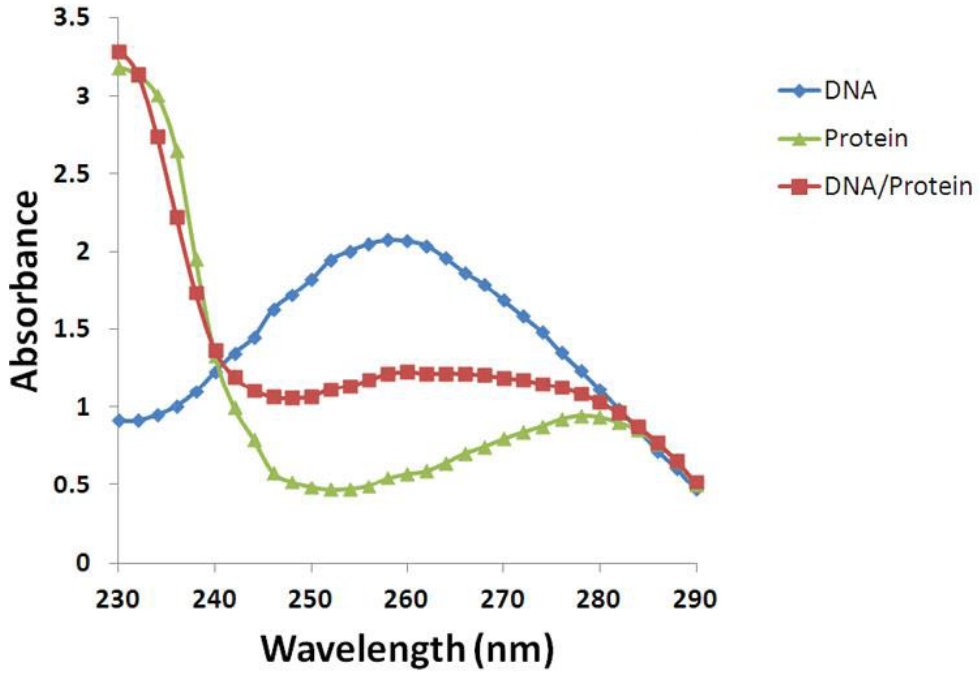
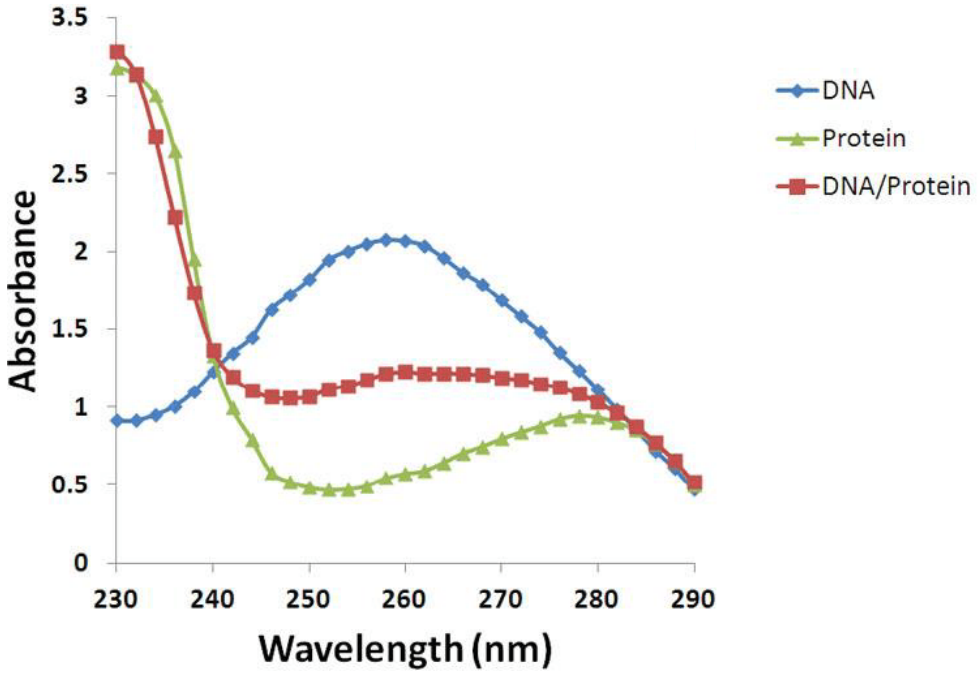


Estimating Purity Using UV Spectrophotometry

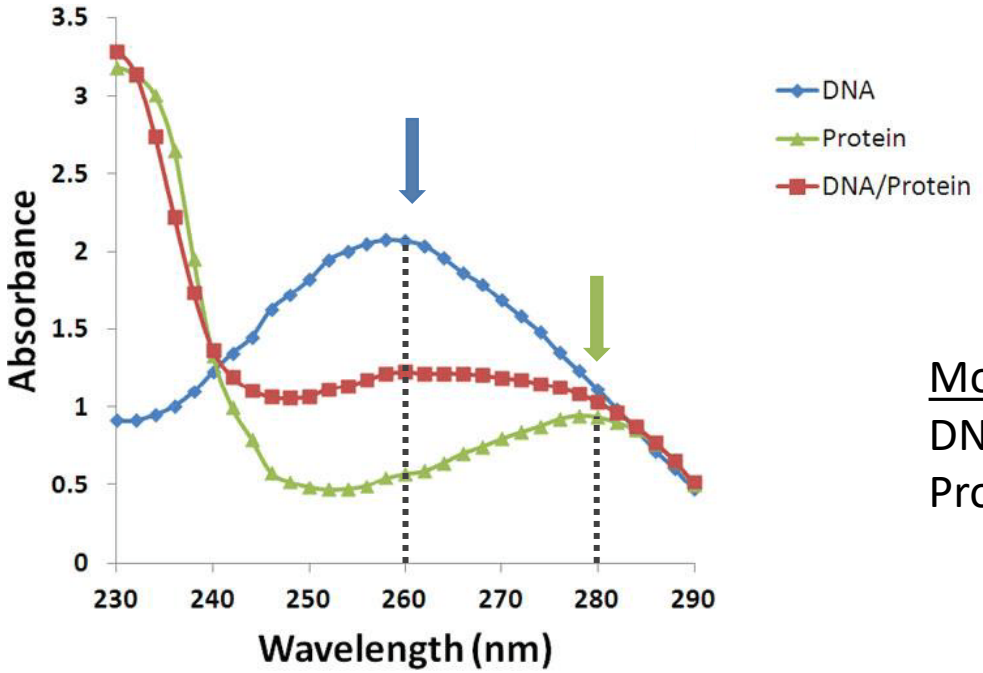
- UV spectrophotometry and the A260:280 ratio



Analyse the UV spectrum of your sample

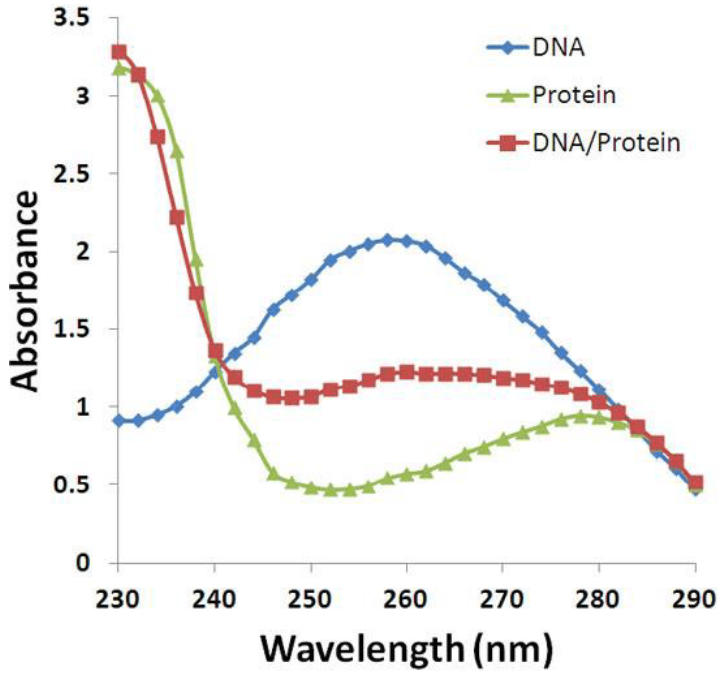
Estimating Purity Using UV Spectrophotometry

- UV spectrophotometry and the A260:280 ratio



Molecule	260/280
DNA	1.8
Protein	≤ 0.6

Estimating Purity Using UV Spectrophotometry



Molecule	260/280
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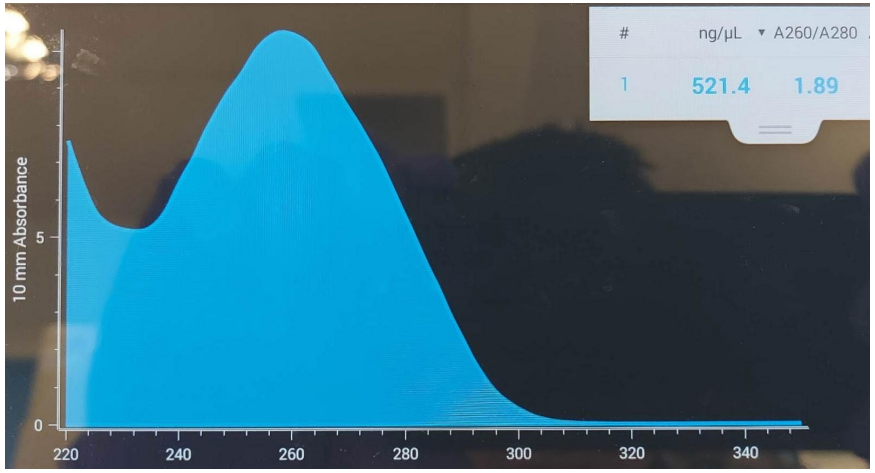
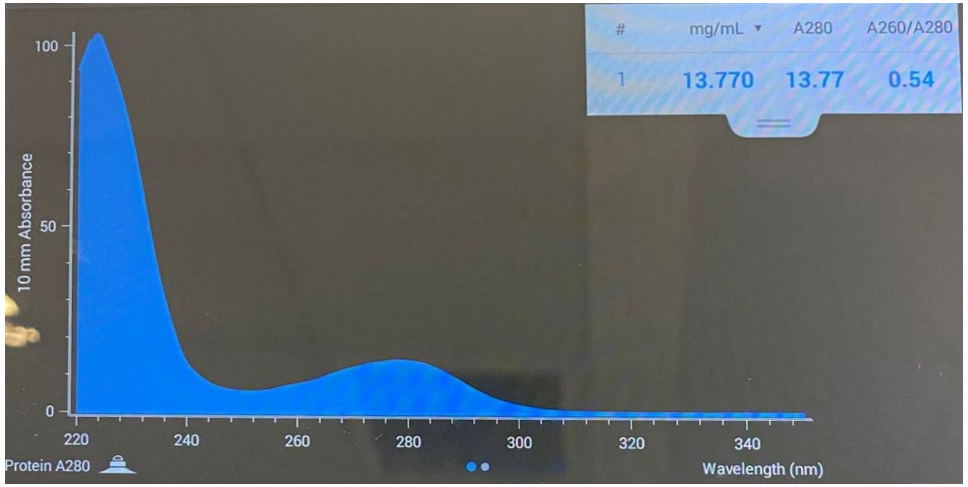
Pure DNA sample on Nanodrop

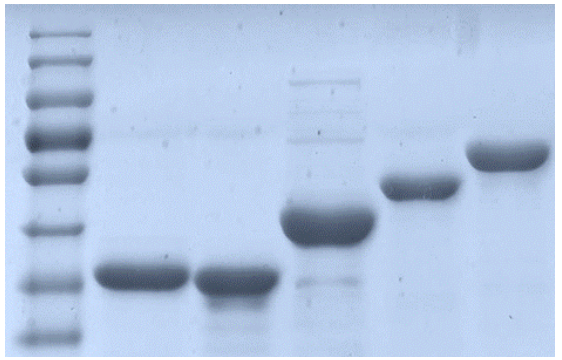


Pure protein sample on Nanodrop

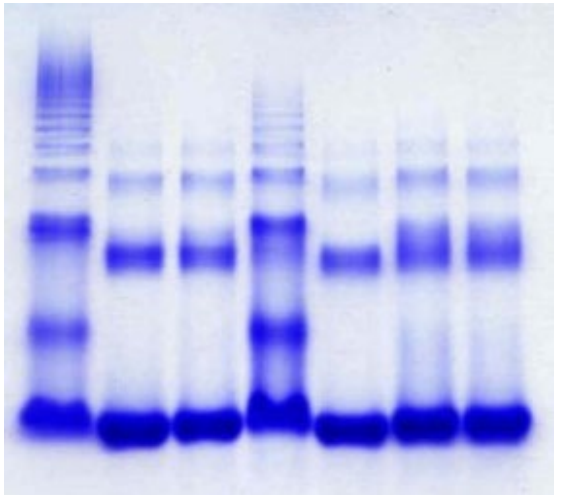


Is my protein folded?

- For normal SDS-PAGE the sample is boiled in SDS so is unfolded to allow for good size separation

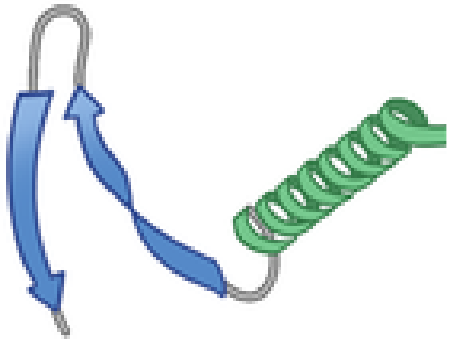


- Native-PAGE doesn't unfold the protein but is mostly useful for monitoring interactions or oligomerisation



Is my protein folded?

- Secondary structure
  - Check this using circular dichroism



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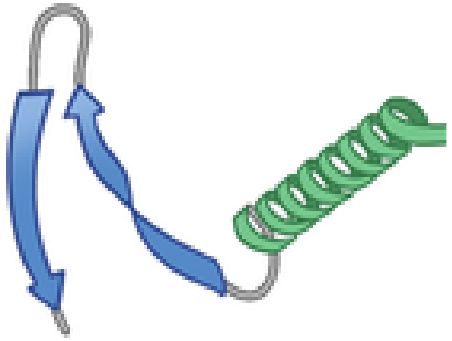
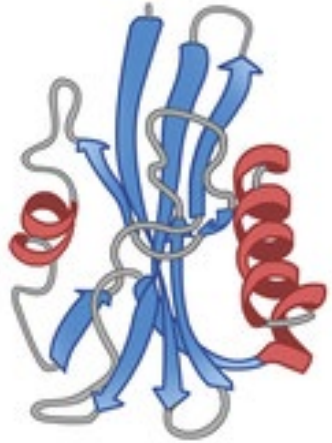
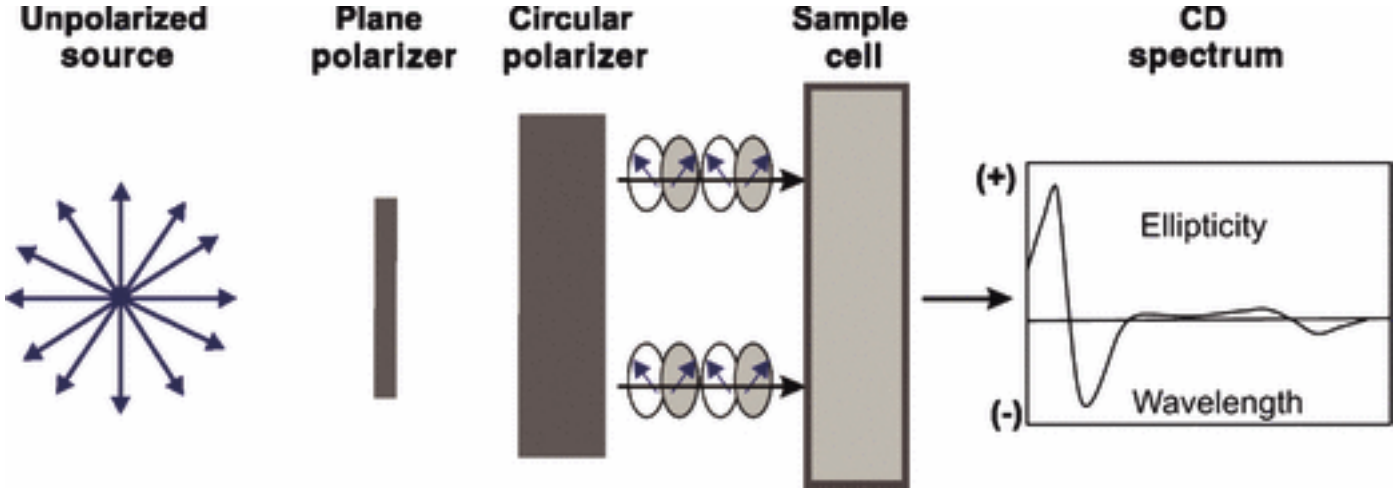


- Tertiary structure
  - Check this using differential scanning fluorimetry



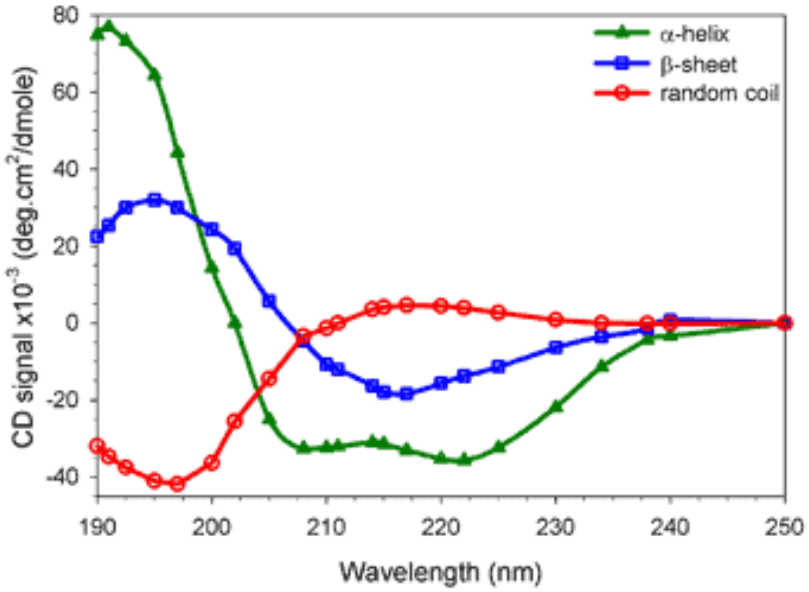
Circular dichroism (CD) spectroscopy

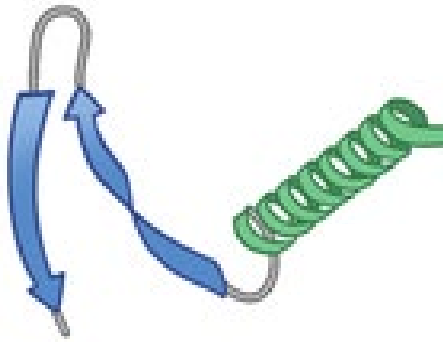
- Light can be circularly polarised (left- or right-handed)
- CD spectroscopy measures difference in absorption of left- and right-handed circularly polarised light



CD of proteins

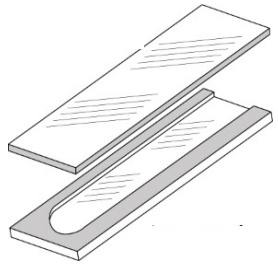
- Protein secondary structural elements have characteristic CD spectra
- CD can tell you the secondary structure of your protein





Measuring CD spectra

- Put sample into thin quartz cuvette
  - Need buffer with low optical activity and low salt



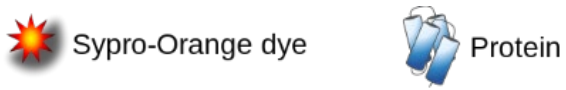
- Measure spectra

- Tells you secondary structure, **not** folding
  - Although you can do ‘melt curves’

Measuring folding: Thermal Shift Assay

- Differential scanning fluorimetry

- Heat protein in presence of hydrophobic dye



Measuring folding: Thermal Shift Assay

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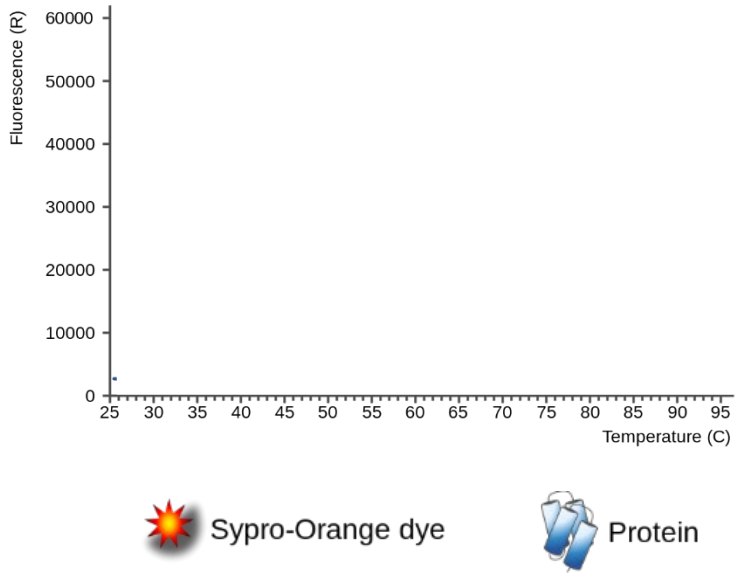
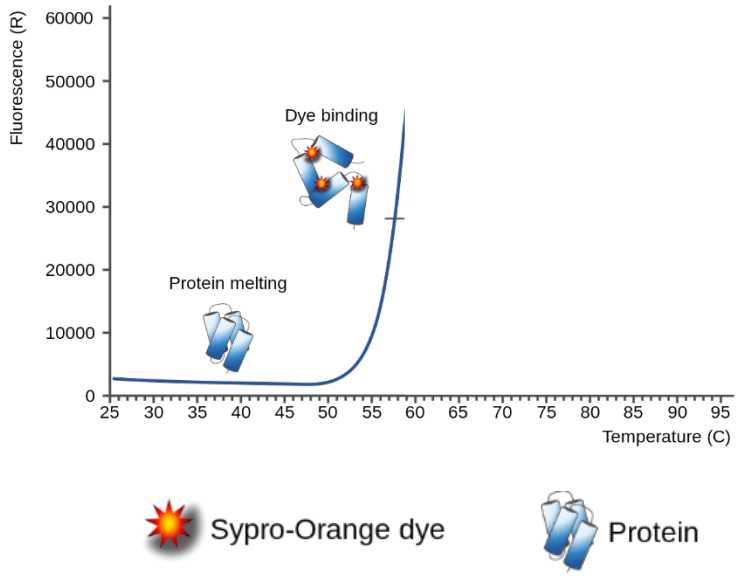


Measuring folding: Thermal Shift Assay

- Differential scanning fluorimetry

- Heat protein in presence of hydrophobic dye
  - Fluorescence goes up when dye binds unfolded protein



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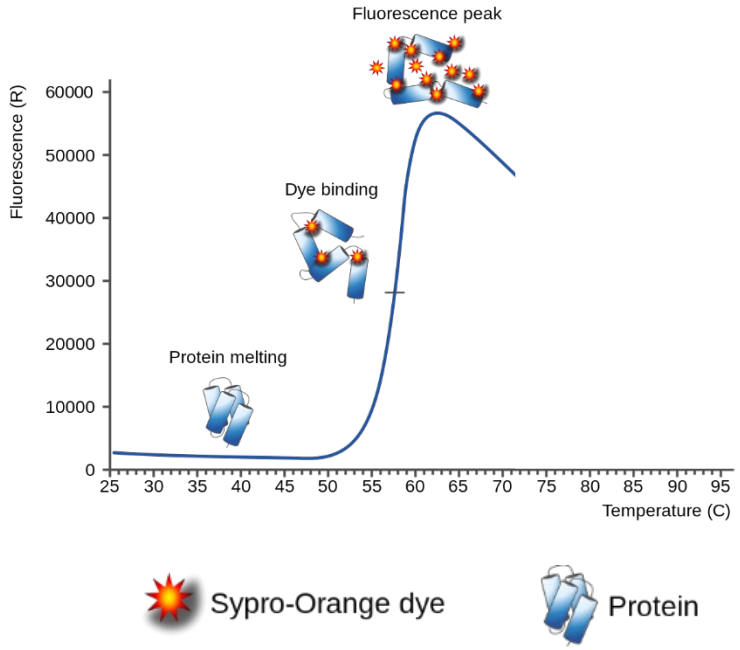

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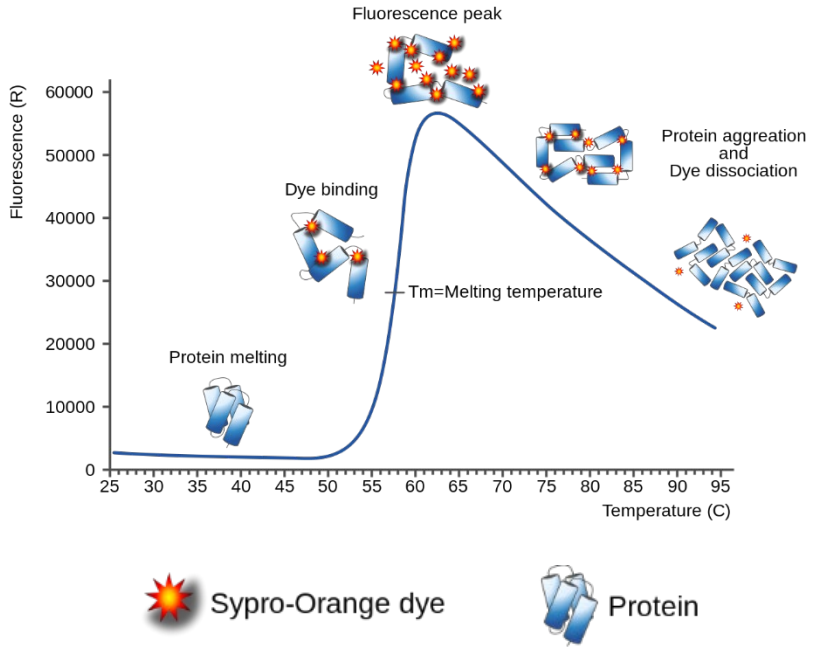
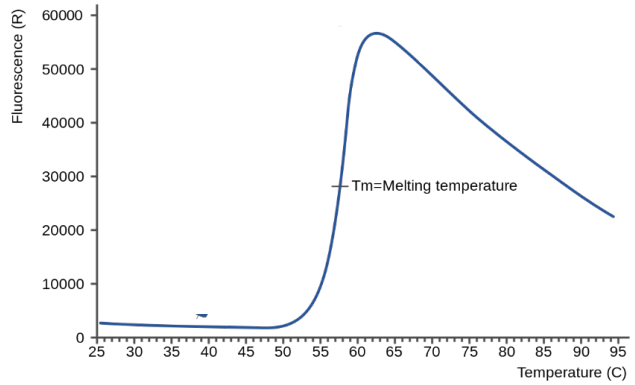


Measuring folding: Thermal Shift Assay

- Differential scanning fluorimetry
- Heat protein in presence of hydrophobic dye
  - Fluorescence goes up when dye binds unfolded protein
- Cheap and easy
  - qPCR machine

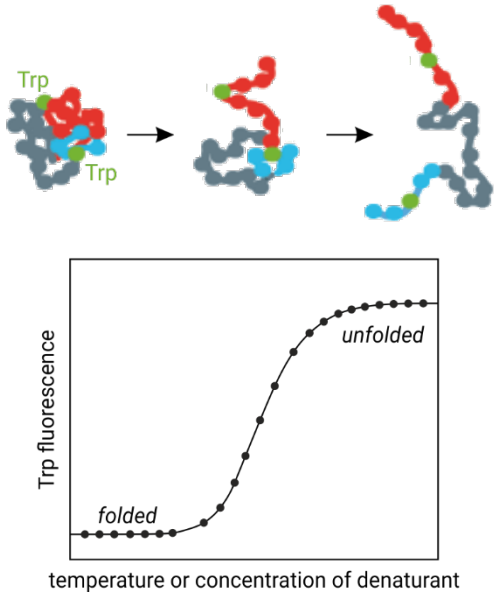


Measuring folding: Thermal Shift Assay

- Differential scanning fluorimetry

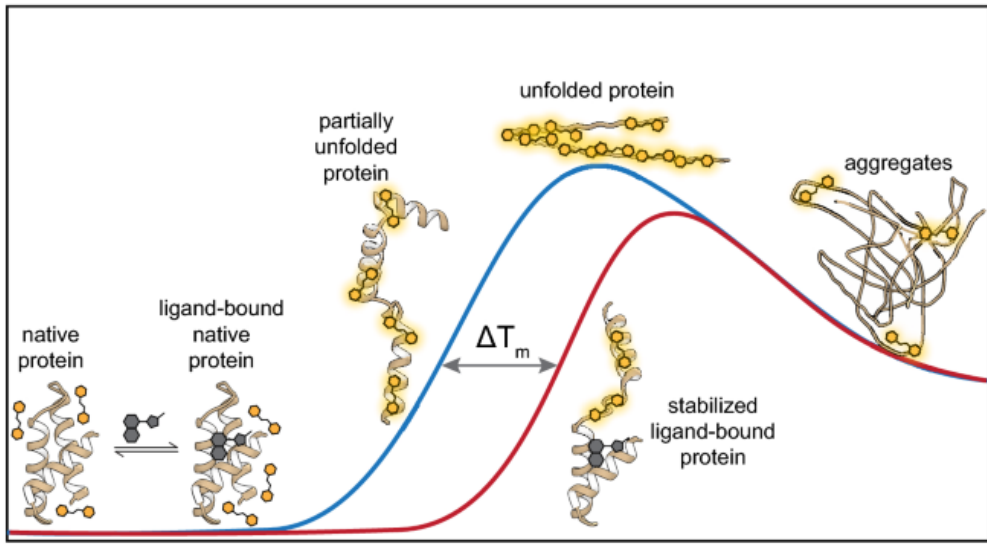
- Heat protein in presence of hydrophobic dye
  - Fluorescence goes up when dye binds unfolded protein

- Cheap and easy
  - qPCR machine
  - Can do label-free, Trp fluorescence



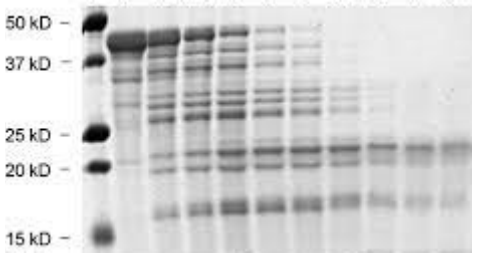
Why measure T_m?

- Does your mutation disrupt the fold?
- What is the best buffer for my protein?
- Does your protein bind a ligand?
  - Ligand binding can stabilise proteins

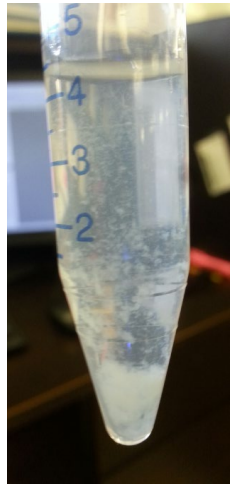


Protein Storage

- Purified proteins are not always stable for very long at 4°C



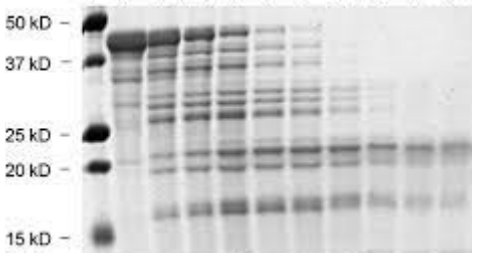
- Over time they can degrade or precipitate

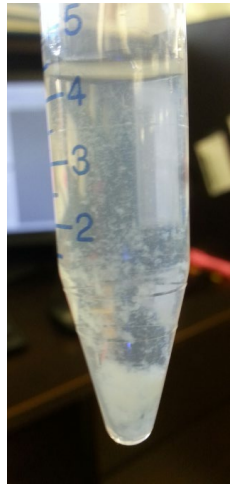


- Proteins can also precipitate if they are at too high a concentration

Protein Storage

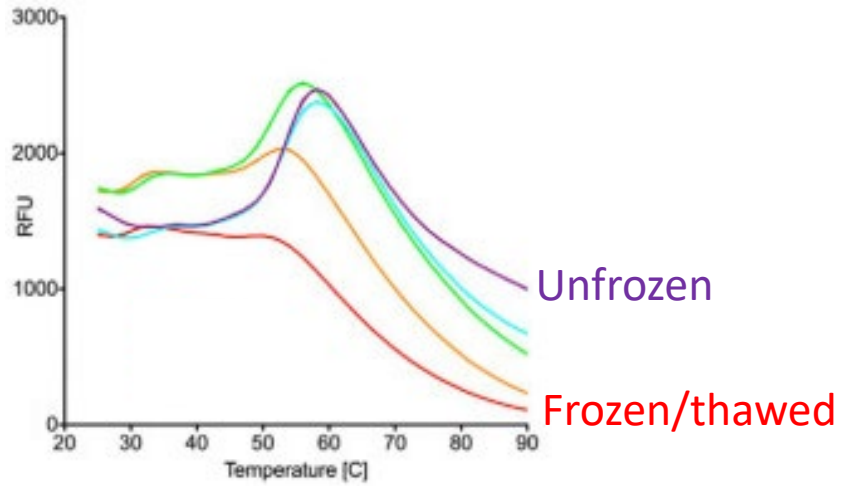
- Ideally use your protein when it is fresh
- If you need to store it you can snap-freeze it in liquid nitrogen in small aliquots (50 uL)
- Either freeze it at high conc > 2 mg/mL or add some glycerol (5-20%)
- However, some proteins don't freeze well…





Checking quality of frozen protein

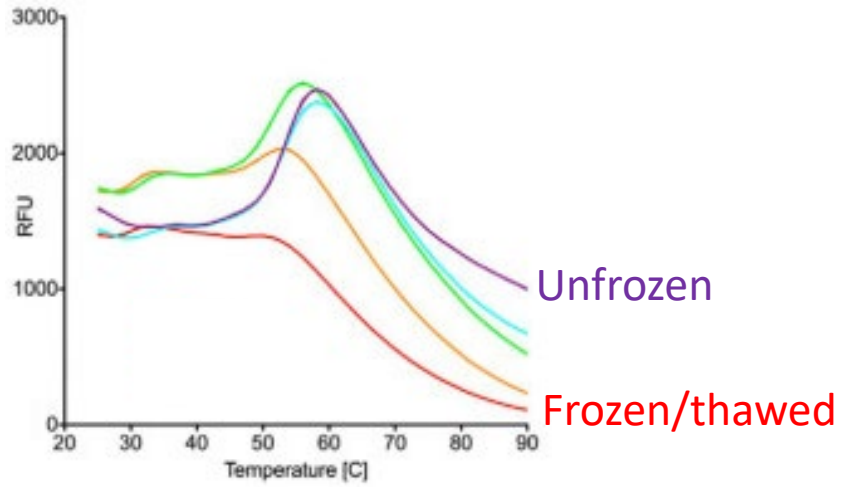
- Redo melt curve, DSF

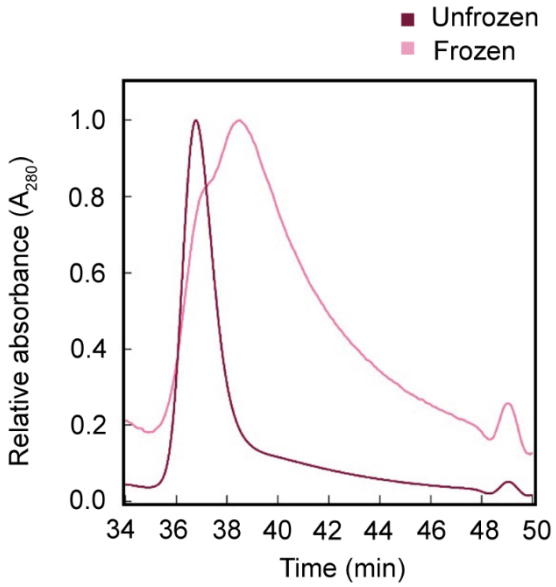


Checking quality of frozen protein

- Redo melt curve, DSF

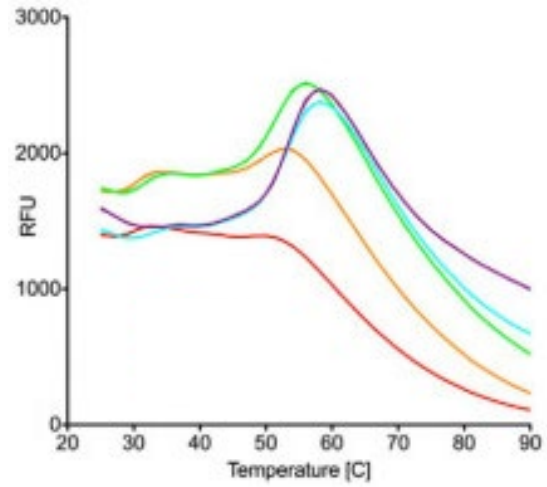
- Run SEC again to see if there is an aggregate peak

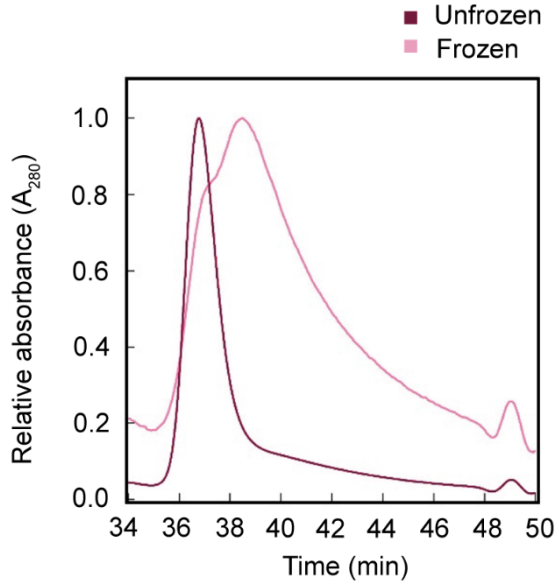




Checking quality of frozen protein

- Redo melt curve, DSF
- Run SEC again to see if there is an aggregate peak
- Run an activity assay
- Do a pulldown experiment with a binding partner



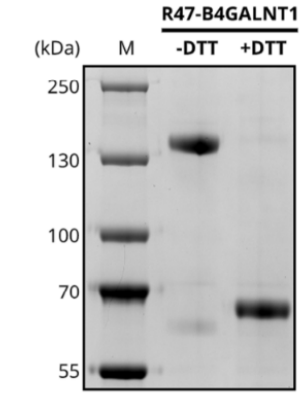


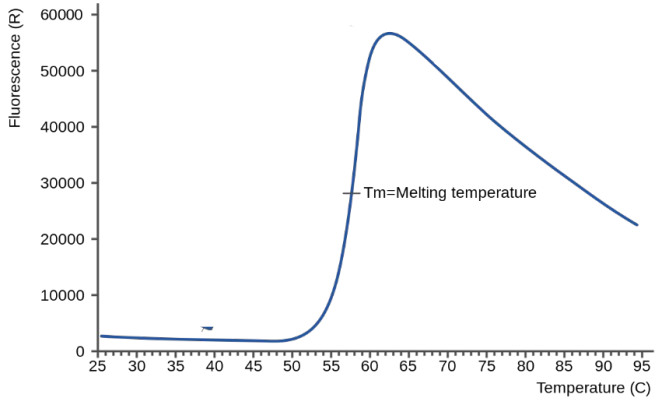
Troubleshooting and Optimisation

- Different buffers during purification
- Ensure your buffer is ≥1 pH unit from the pI of your protein
- Some proteins are more stable with addition of:
  - 5-10% glycerol
  - Increased [NaCl], up to 1M
  - Divalent cations: Ca, Mg
- Design a new construct!

Protein Purification Part 3 – Quality Control

- Today we learnt about:
  - Interpreting your data
  - Checking your protein quality
  - Checking for correct protein folding
  - How to store your protein
  - Troubleshooting difficult samples

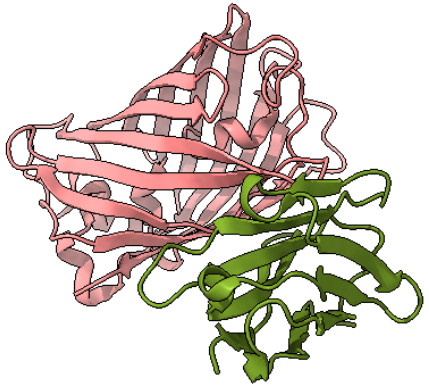




End of Week 1 Lectures

- We've learnt about:
  - Protein expression
  - Protein purification
  - Chromatography
  - Checking protein quality

- Next week we learn about:
  - Biophysics
  - Protein-protein interactions
  - Looking at protein structures
  - Solving and predicting protein structure



Questions?

Then let’s do some SDS-PAGE