nibrt
National Institute for Bioprocessing Research and Training

L8 Bioanalytical Techniques

Lecture 3

Protein Estimation Assays

Learning Objectives

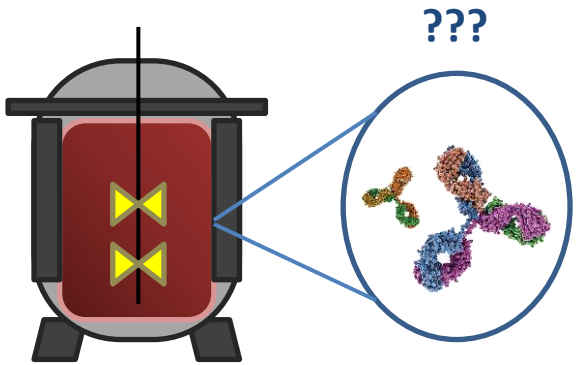
1. Discuss the principles of spectrophotometry
2. Describe the application of the Beer Lambert Law
3. Discuss the principal methods used for protein assays
4. List the advantages/disadvantages associated with assay methods
5. Discuss the importance of standard curves
6. Discuss the advantages of newer technologies such as SoloVPE

Topics

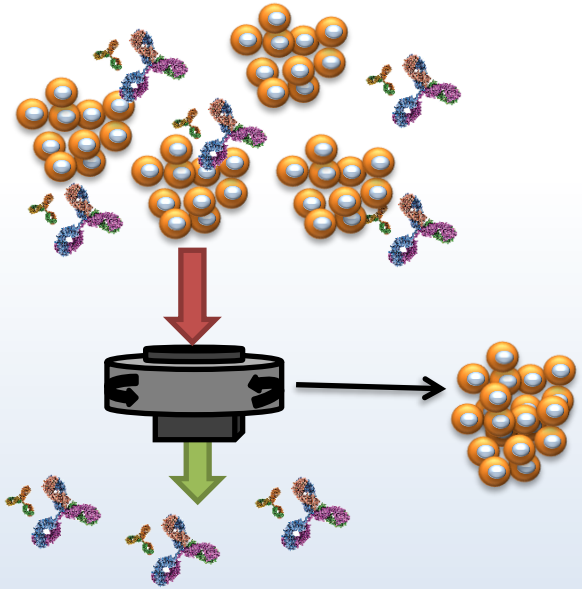
- Protein Estimation Assays
- UV Absorbance Assay
- Dye-based Colorimetric Assays
- Copper based Colorimetric Assays
- Protein Standard Curves
- Protein Estimation by SoloVPE

When is protein concentration measured in bioprocessing?

- **Upstream Processing**
  - Determine titre in bioreactor
  - Harvest can begin once sufficient biopharmaceutical produced by cells

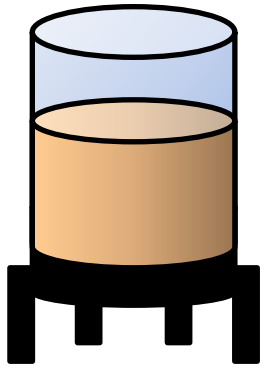


- **Harvest**
  - Determine total protein and specific biopharmaceutical titre
  - Ensure % recovery within spec

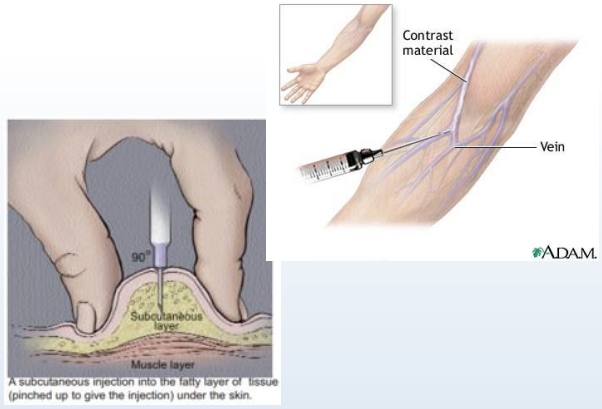


When is protein concentration measured in bioprocessing?

- **Downstream Processing**
  - Product loading: columns have limited protein binding capacity
  - % Recovery must be consistent

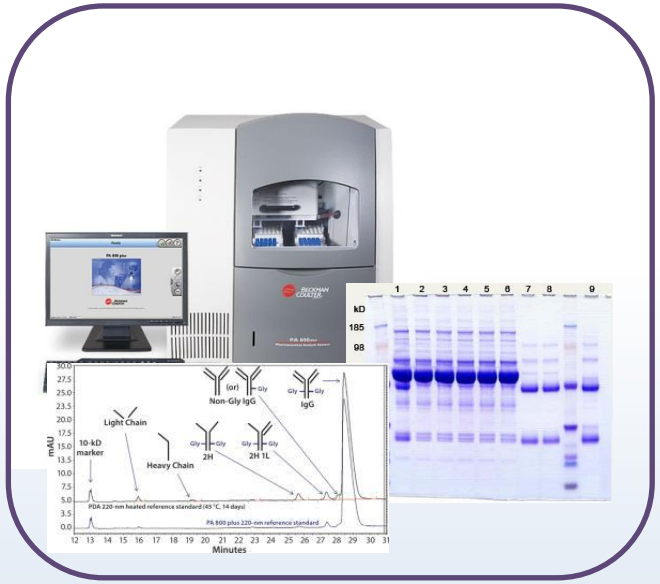


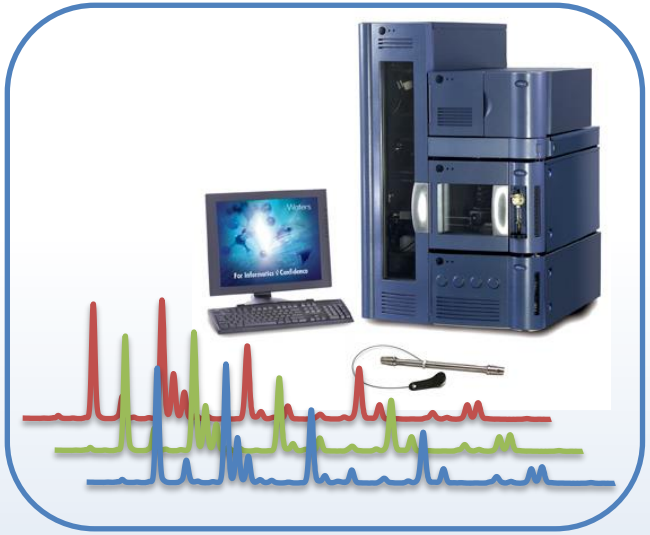
- **Formulation & lot release**
  - Therapeutic dosage is based on the concentration of protein when injected



Protein Estimation Assays

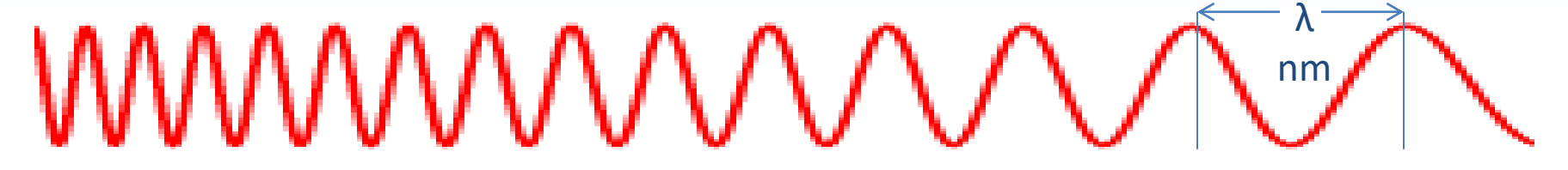
- **Testing**
  - Protein estimation is often the first step in any form of analysis (LC, Electrophoresis, MS, Immunoassays) to ensure optimum sensitivity





Measuring Protein Concentration

- Protein concentration is usually determined by measuring the **amount of light absorbed by a protein solution**
- Choose a **single wavelength (λ)** of light at which your sample has **maximum** absorbance



Measuring Protein Concentration

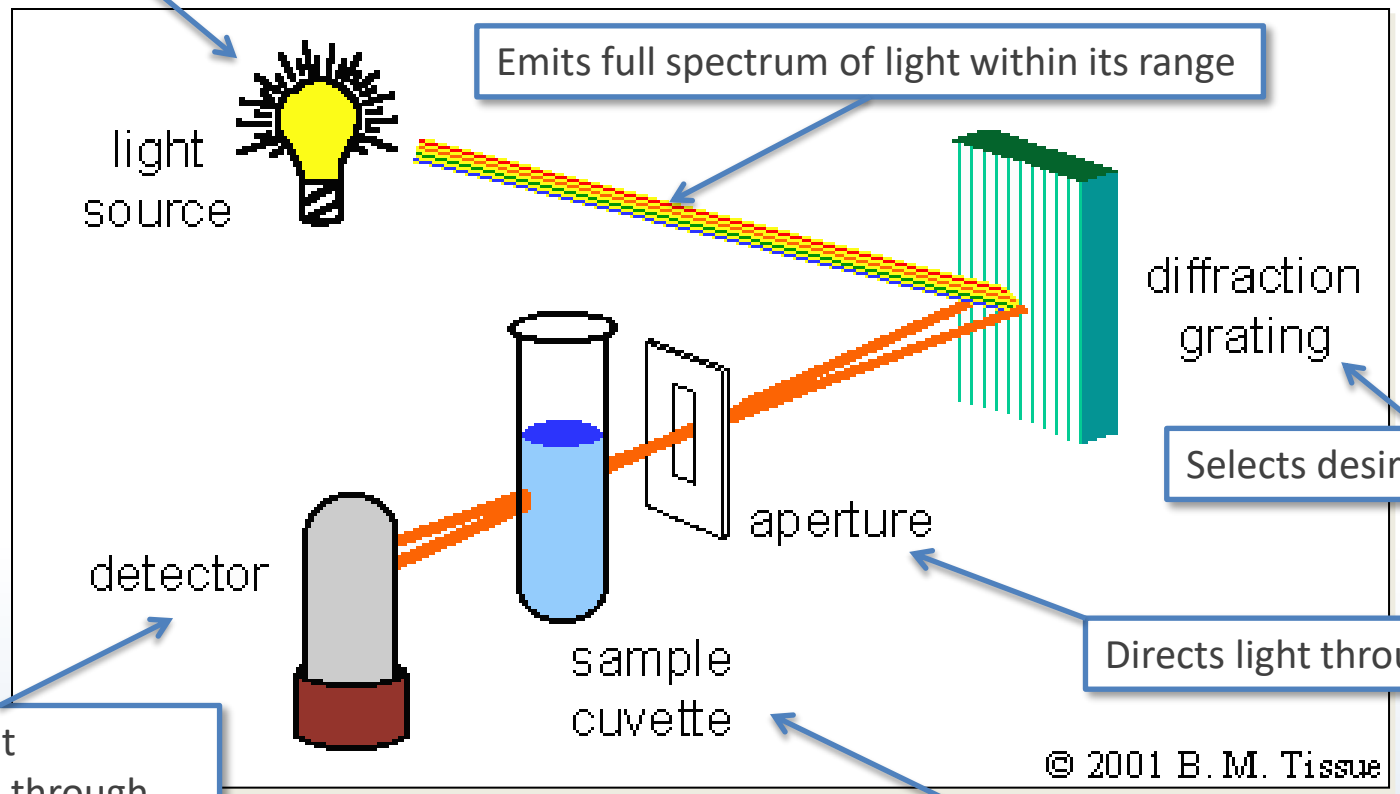
- Protein concentration is usually determined by measuring the amount of light absorbed by a protein solution
- For this, use a **spectrophotometer**

http://www.jenway.com/product.asp?dsl=289

http://www.hitachi-hitec.com/global/science/uv_vis/u2900.html

Measuring Protein Concentration

Tungsten lamp = 340 – 1100nm range (Visible)
Deuterium lamp = 168 – 360nm range (UV)



Emits full spectrum of light within its range

Selects desired wavelength

Directs light through sample

Detects light transmitted through sample

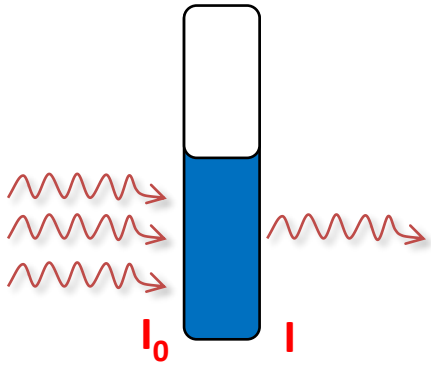
Contains sample in aqueous solution

Transmittance and Absorbance

Transmittance:

- Measure of amount of light transmitted through sample
- Ratio of light **entering** the sample and light **exiting** the sample

Transmittance (T)= I/I_0



- Low concentration of protein → High Transmittance

Absorbance:

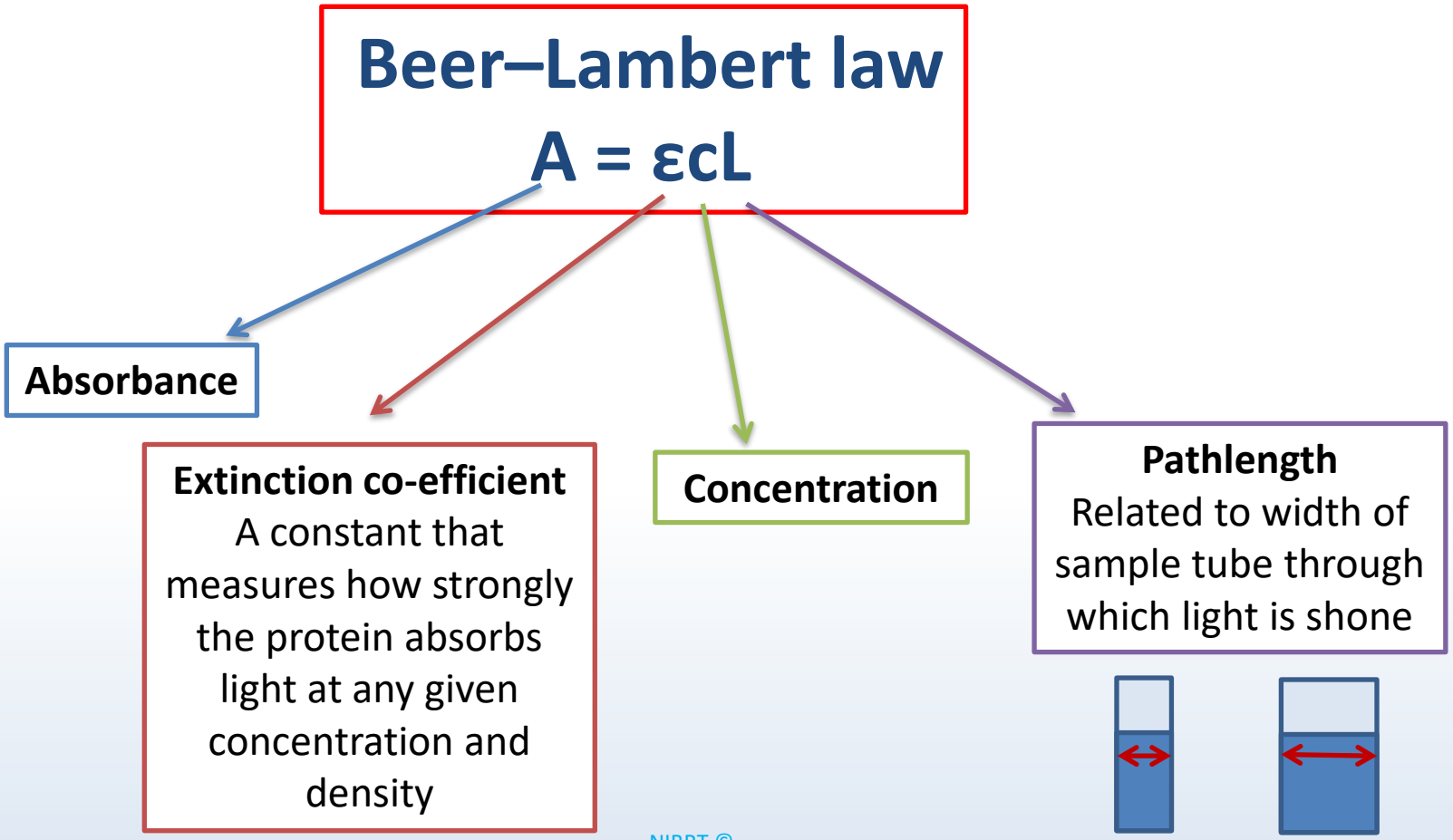
- Measure of amount of light absorbed by sample
- Calculated from transmittance

Absorbance (A) : $A = -\log_{10}T$

- Low concentration of protein → Low Absorbance

Beer-Lambert Law

- Absorbance is related to concentration by:



Protein Concentration Assays

- There is no completely satisfactory single method to determine the concentration of protein in any given sample

- The choice of the method depends on:
  - the **nature of the protein**,
  - the nature of the **other components** in the protein sample
  - desired **speed, accuracy, precision** and **sensitivity** of assay

Protein concentration assays

Methods:

- Absorbance at 280nm → No dye or chemicals
- Bradford → Dye-based Colorimetric
- Biuret
- Lowry
- BCA

→ Copper-based colorimetric

Method	Estimated working range
A280nm	0.2 – 2mg/ml
Coomassie (Bradford)	100 - 1500µg/ml (Bio-Rad website)
Modified Lowry Assay	1 - 1500µg/ml (Pierce website)
BCA	20 - 2000 µg/ml (Pierce website)

Topics

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- UV Absorbance Assay
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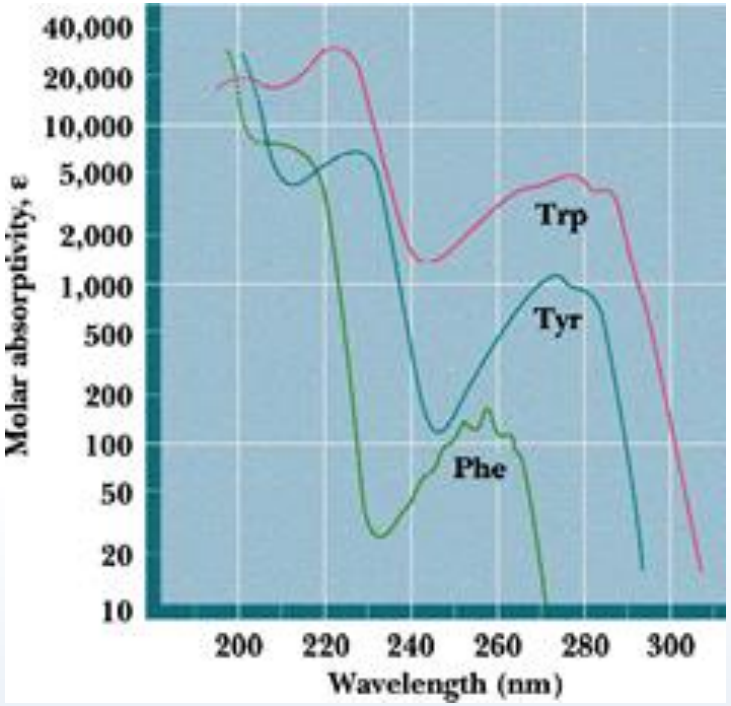
- Copper based Colorimetric Assays
- Protein Standard Curves
- Protein Estimation by SoloVPE

UV method: Abs at 280nm

- Aromatic amino acids tyrosine and tryptophan naturally absorb light at 280nm
- Can accurately measure protein concentration in 0.2-2mg/ml range
- Non-destructive method
- Used during downstream processing



Layne E. *Methods in Enzymology 3:* 447-455. 1957
Stoscheck CM. *Methods in Enzymology 182:* 50-69. 1990

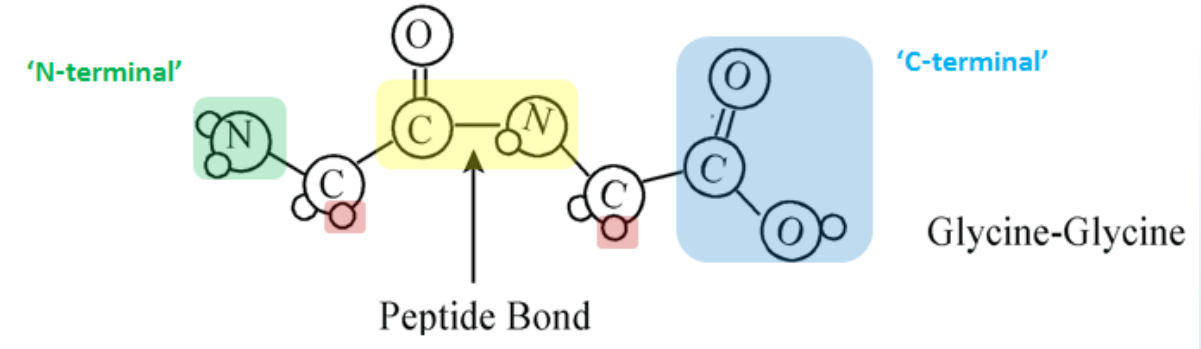
UV method: Abs at 280nm

- **Drawbacks:**
  - Protein must contain Tyr and Trp to absorb effectively
  - Absorbance depends on proportion of these amino acids – variability between proteins
  - Not very sensitive (cannot detect less than 0.2mg protein)
  - Plastic absorbs at this λ, must use quartz cuvettes
  - Need high volume of sample
  - DNA absorbs at this λ

Far UV method: Abs 190-220nm

- Peptide bonds in proteins (linkage between amino acids) naturally absorb light in the UV range (max. abs at 190-220nm)
- Because of absorption by oxygen at 190nm, usually measure at 205-220nm
- More sensitive than Abs_{280nm}
- Not dependent on amino acid content of proteins

Drawback:
Many interfering substances e.g. some buffers made to isolate proteins



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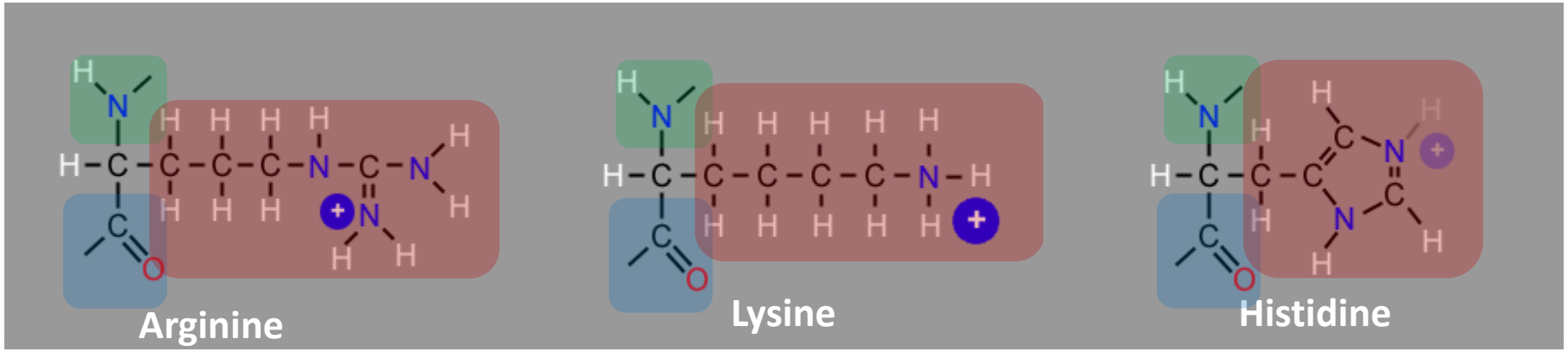
Bradford Assay

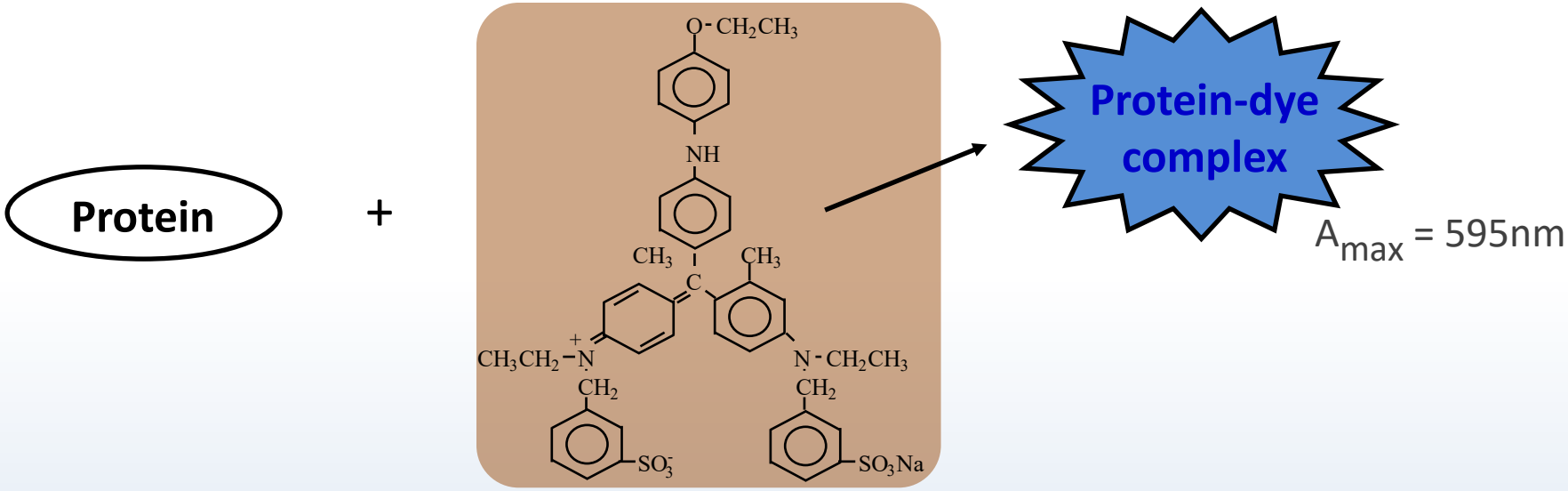
- Most popular dye-based protein assay
- Uses a blue dye: Coomassie Brilliant Blue G-250
- Dye binds to free amino groups (NH_2) in the side chains of amino acids arginine, lysine, histidine



- Protein standard AND protein of interest must contain these amino acids for this assay to work

Bradford Assay

- Dye undergoes an absorbance shift upon binding
- When unbound to protein, dye is positively charged (cationic) and has absorbance maximum at **465nm (red/brown colour)**
- When bound to protein , dye is negatively charged (anionic) and has absorbance maximum at **595nm (blue colour)**



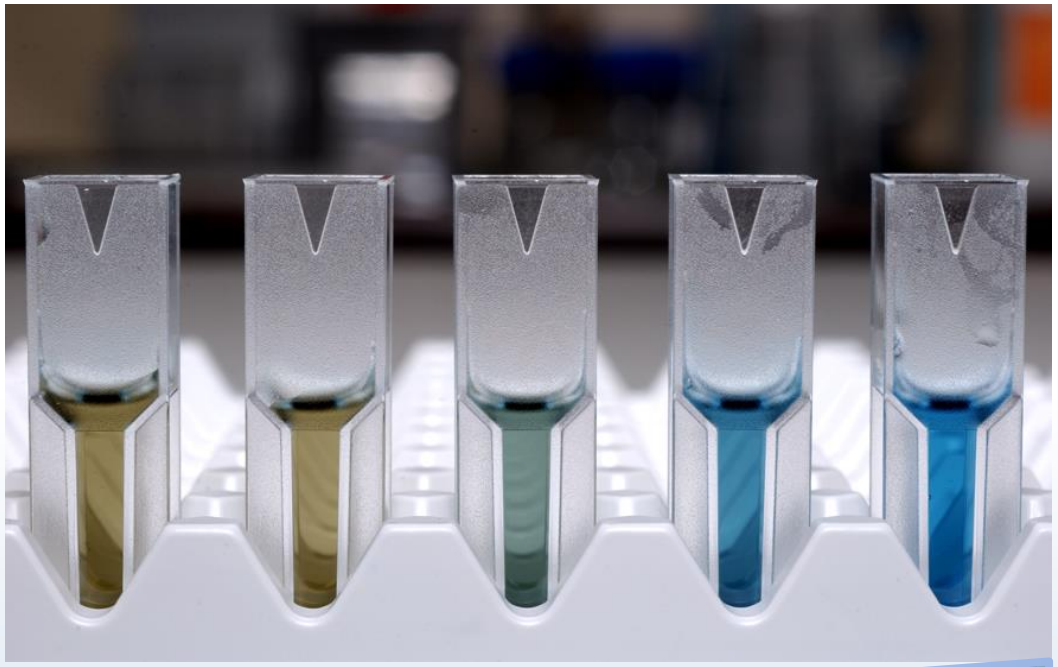
Coomassie G-250

Bradford, M. (1976). *Anal. Biochem.* 72, 248-254

Bradford Assay

- Dye undergoes an absorbance shift upon binding
- When unbound to protein, dye is positively charged (cationic) and has absorbance maximum at **465nm (red/brown colour)**
- When bound to protein , dye is negatively charged (anionic) and has absorbance maximum at **595nm (blue colour)**

Working range
0.1 – 1.5mg/ml



http://www.eiroforum.org/media/photo_galleries/embl/index.html

Bradford Assay

Advantages

+ Simple, easy-to-use, one-reagent system
+ Fast and inexpensive
+ Almost instantaneous colour development at room temperature
+ Highly specific for protein
+ Very sensitive
+ Free amino acids, peptides and low MW proteins (<3000Da) do not interfere
+ Can be adapted to work in µg range (1 – 20ug)

Disadvantages

- Colour response at high concentrations may be non-linear
- High protein-to-protein variation, choice of standard very important
- Unstable working reagent
- Incompatible with many detergents

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Biuret Assay

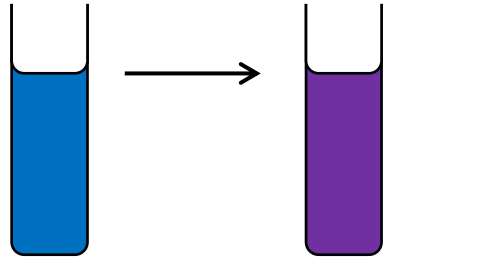
- A colorimetric assay at A_{max} = 540nm
- Direct complex between peptide bonds in proteins and Cu^{2+} ions, forming a purple-coloured complex

Advantages

+ Most linear method
+ Reproducible
+ Few interfering agents

Disadvantages

- Not highly sensitive, requires large amount of protein
- Limited shelf-life of reagents
- Precipitation can occur



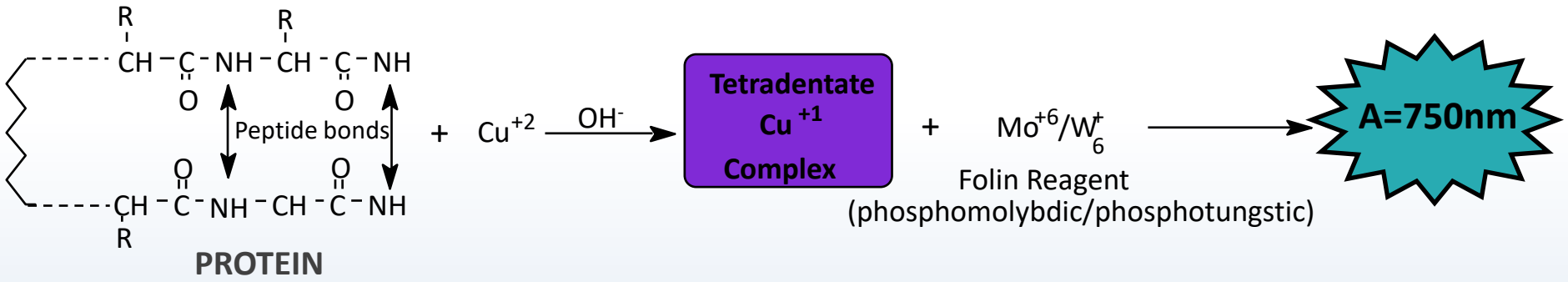
- Biuret assay was modified by researchers and new assays were developed

Lowry Assay

- Modification of Biuret Assay
- A colorimetric assay at A_{max} = 750nm
- Combines 2 reactions: copper ions with peptide bonds (Biuret assay) & oxidation of aromatic amino acids (Folin reagent, for enhanced colour development)

Reaction 1: Cu^{2+} + peptide bonds → Cu^{1+}-peptide bond complex **purple**

Reaction 2: Folin reagent + Cu^{1+}-complex → reduced Folin reagent **blue-green**



Lowry OH, *et al.*, (1951) *J. Biol. Chem.* 193, 265-275

Lowry Assay

Advantages

+ Sensitive over a wide range
+ Can be performed at room temperature
+ Reproducible

Disadvantages

- Many substances interfere with the assay (strong acids, ammonium sulfate, calcium, magnesium)
- Time-consuming to perform
- Inconvenient, lots of fresh solutions required
- Photosensitive, so must avoid illumination during the assay
- Amount of colour varies with different proteins
- Requires precisely timed addition of Folin reagent

Bicinchoninic Acid (BCA) Assay

- Modification of Lowry assay
- A colorimetric assay at A_{max} = 562nm
- Combines the reaction of copper ions with peptide bonds (Biuret reaction) with the reaction with BCA reagent
- Requires peptide bonds and presence of cysteine (SH), cystine (S-S), tryptophan and/or tyrosine

Originally described by Smith PK, *et al.* (1985) *Anal. Biochem.* 150, 76-85
http://www.somatco.com/medkit.htm

Bicinchoninic Acid (BCA) Assay

Advantages

+ Very sensitive
+ Compatible with most detergents (up to 5% v/v)
+ Working reagent is stable
+ Very little variation in response between different proteins
+ Broad working range in response curve
+ Large sample numbers can be analysed

Disadvantages

- 30min incubation required
- Requires incubation at high temperature
- Substances that affect copper can interfere with the assay (thiols, reducing sugars, ascorbic acid, copper chelating agents)
- Requires preparation of solutions before use

Topics

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Relating Absorbance to Concentration

Absorbance = ??? mg/ml

- Use a **standard curve**
- A standard curve is constructed by measuring the absorbance of a range of protein samples of ***known*** concentration
- The protein sample of ***unknown*** concentration can be compared to this curve to determine how much protein is present

Relating Absorbance to Concentration

Unknown Sample: Sample from bioreactor

Protein used to prepared samples of known concentration: Bovine serum albumin (BSA)

Protein concentration	Abs at 595nm
0 mg/ml	0
0.3mg/ml	0.18
0.6mg/ml	0.35
0.9mg/ml	0.49
1.2mg/ml	0.62
1.5mg/ml	0.72

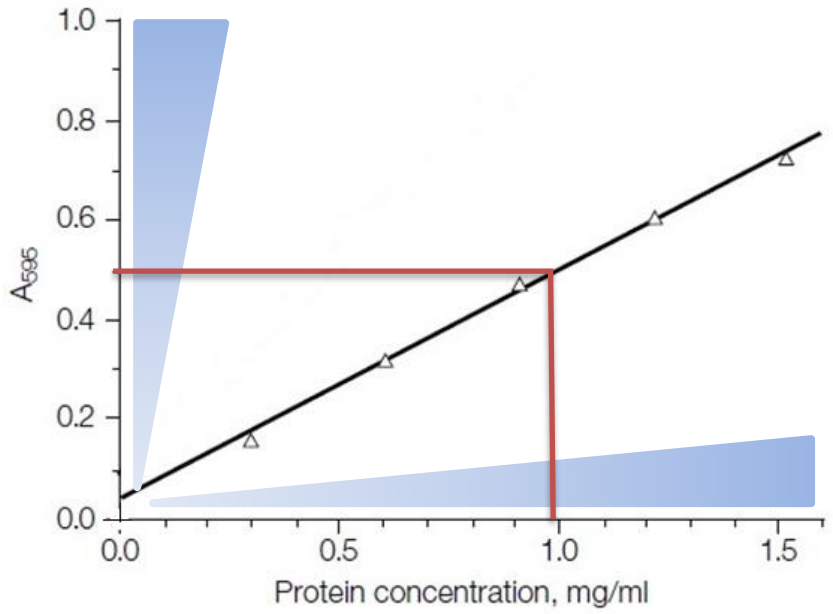


Fig. 2. Typical standard curves for the Bio-Rad protein assay: bovine serum albumin (□) and γ-globulin (△).

Absorbance of bioreactor sample = 0.50

∴ concentration of protein in cell lysate sample = 0.99 mg/ml

Linear Regression using Standard Curves

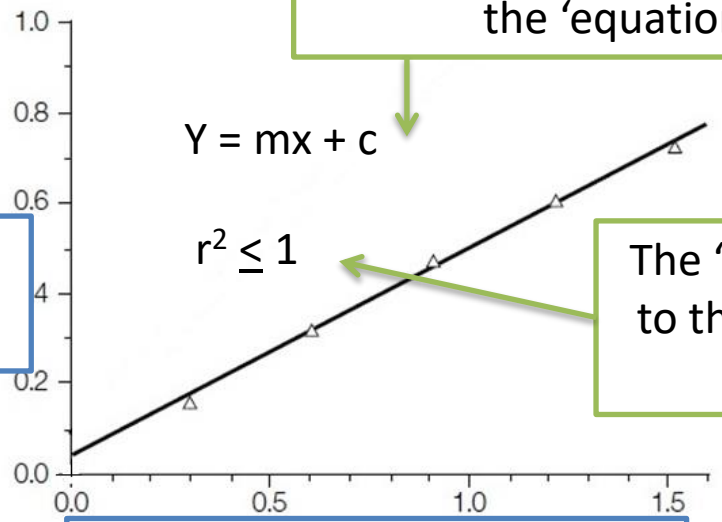
- Linear regression: predicting one variable as a function of another

This relationship of Y to X is described by the 'equation of the line'

$Y = mx + c$

$r^2 \leq 1$

The 'goodness of fit' of the data to the trendline is described by the r^2 value



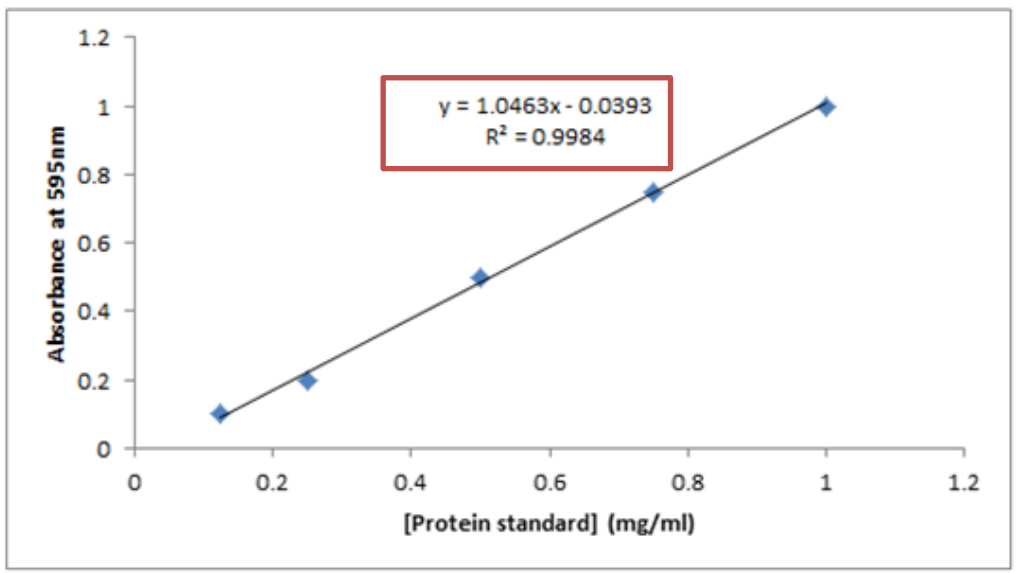
Y
Criterion variable = the one we are measuring

X
Predictor Variable = the one we know

Using the Equation of the Line

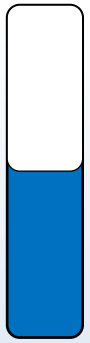
Standard Curve

- R^2 value should be 0.97 – 1.0



Test sample

- Abs reading = 0.506



= ??? mg/ml

x=(y+0.0393)/1.0463

= 0.52 mg/ml

Equation of line: y = mx + c y = absorbance of test sample m = slope of line **x = concentration of test sample** c = intercept of line on y axis *Solve equation for x*
Equation of line from graph: y = 1.0463x - 0.0393 x = (0.506 + 0.0393)/1.0463 = 0.52
Concentration of test sample = 0.52mg/ml

The Equation of the Line

- Non-linear regression is used when the standard curve is non-linear
- Typically performed by software!
- Many methods use only the linear portion of the curve to simplify regression

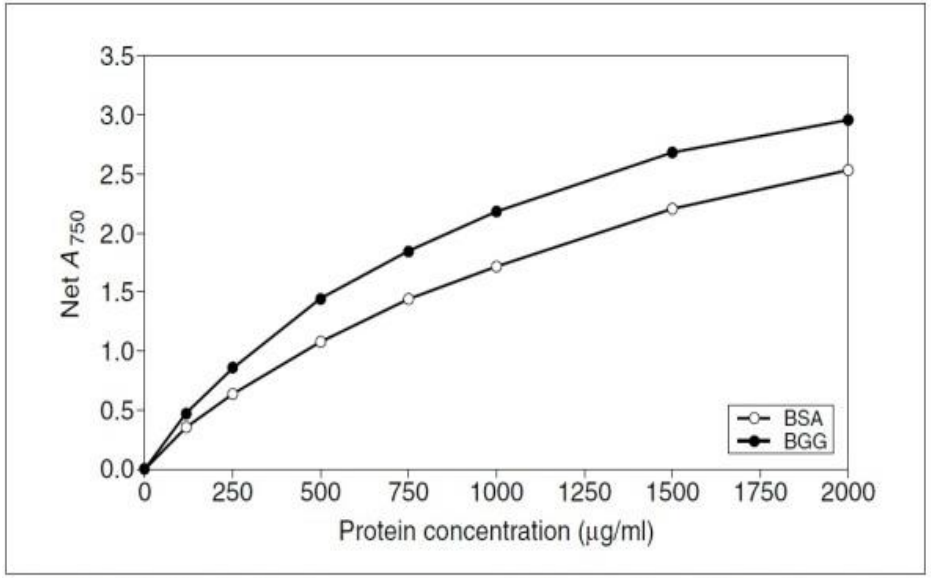


Figure A.3H.2 Graph of the color response curves obtained with Pierce's Modified Lowry Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 750 nm in a Hitachi U-2000 spectrophotometer. Krohn, R. (2011) Curr Prot Cell Biol . 52, A3H1-A3H28

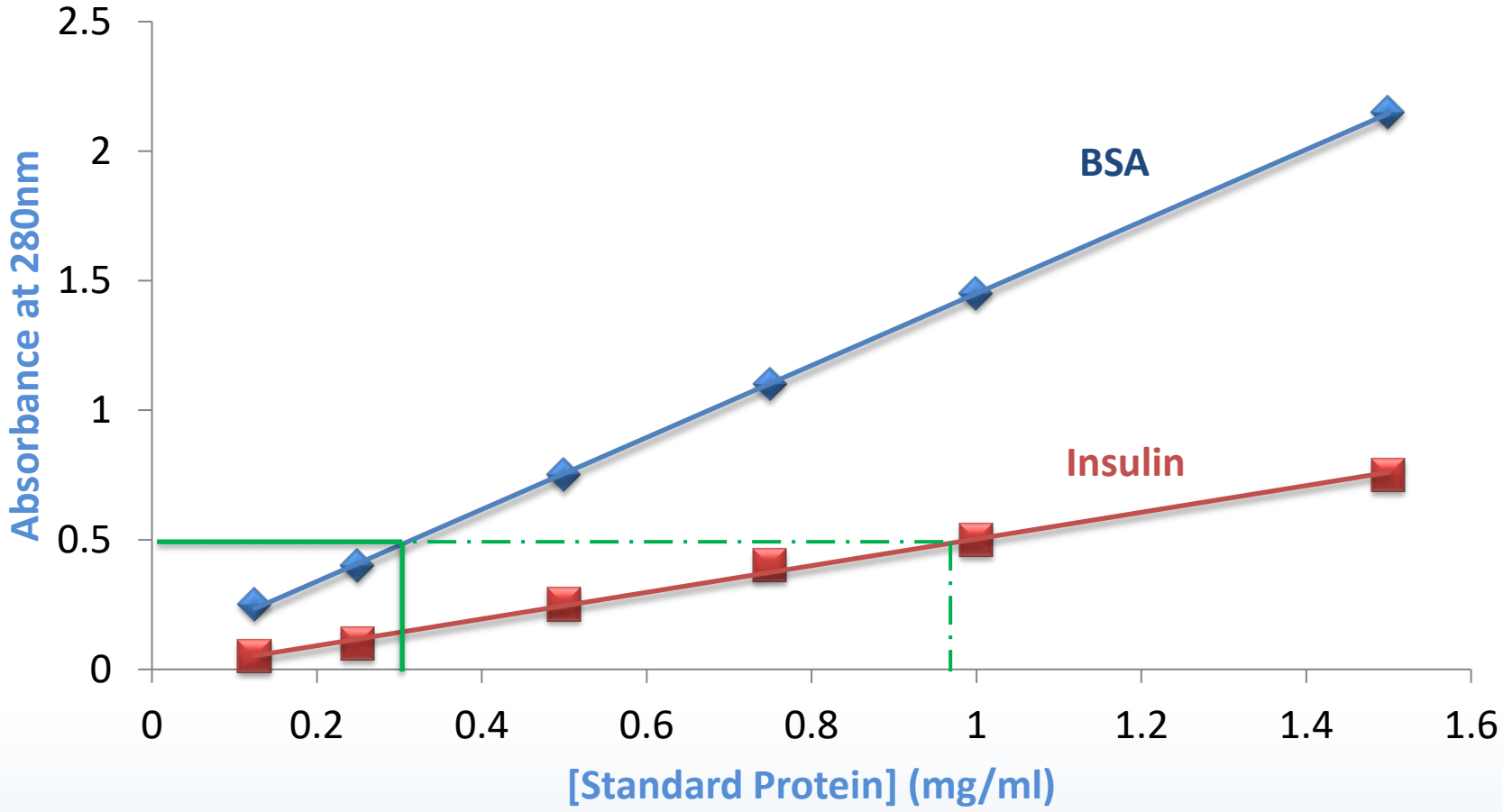
Choice of Protein Standard

- **Bovine serum albumin** (BSA) is a good protein standard for an unknown sample of a mixture of several proteins
  - distribution amino acids is comparable to many other general proteins

- As biopharmaceutical becomes increasing purer during manufacturing, need to consider changing protein used for standard curve.

- If concentration of one particular protein in a mixture is above **60%** then should consider using **this protein** to generate standard curve
  - E.g. a sample is 40% host cell proteins and 60% Mab, use purified Mab as your protein for your standard curve

Choice of Protein Standard



Absorbance of test sample = 0.5

Concentration using BSA = 0.3mg/ml

Concentration using Insulin= 0.98mg/ml

=> ~3-fold difference!

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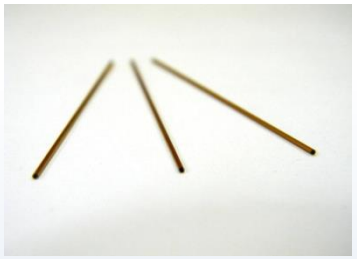
SoloVPE

- SoloVPE is a **slope spectrophotometer** developed by C-Technologies
- It uses **UV–Vis measurement** platform and **variable pathlength technology**
- Composed of **Agilent Cary 60 spectrophotometer** and **fibre optic coupler**
- **Fibre optic cable** carries light from spec to the fibre platform to a piece of optical fibre called a "**Fibrette**"
- Light passes **through fibrette into the sample** and various pathlength measurements taken.

To process a sample you need :

Vessel

+



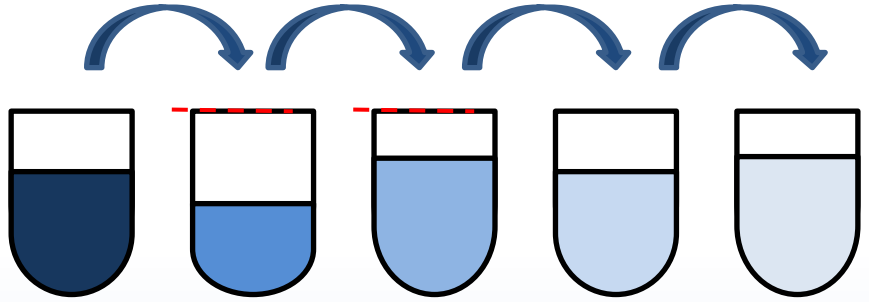
Fibrette

+ **Protein Sample** +

SoloVPE

Why SoloVPE?

- Traditional spectroscopy lacks the ability to analyse **concentrated samples.**
- Performing sensitive dilutions of concentrated samples is a **primary source for error**.



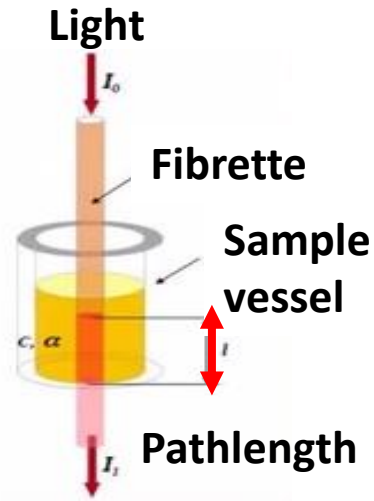
- There is **no sample dilution requirement** with the SoloVPE.

Why SoloVPE?

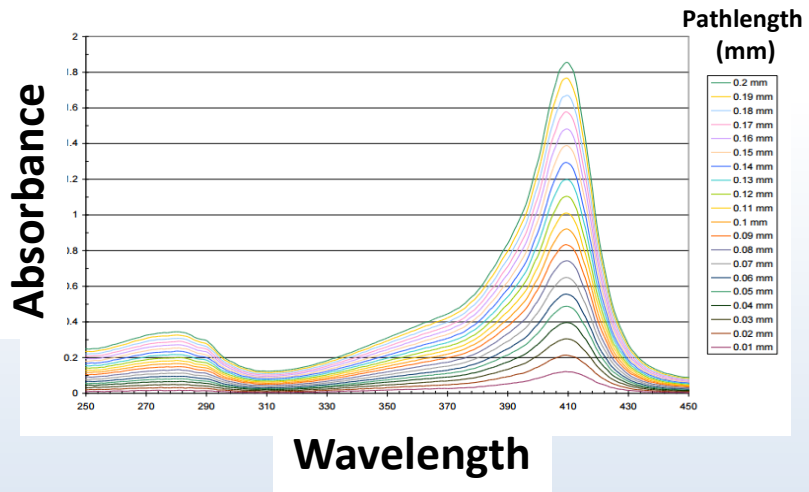
- Takes measurements at **multiple pathlengths** (traditional methods use a fixed pathlength).
- Determines the **most appropriate pathlength** for each sample and ensures linearity (absorbance vs. pathlength) at higher protein concentrations.
- As with traditional methods the SoloVPE uses the principles of the **Beer–Lambert law.**
- Using the protein's molar extinction coefficient and the slope of the line generated in the graphs by the SoloVPE the concentration of a protein can be determined.

Variable Pathlength technology

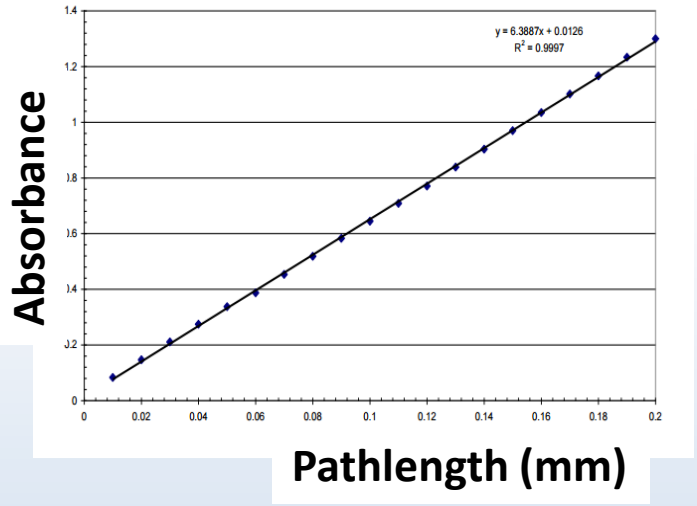
- **Pathlength:** the distance between the tip fibrette and the inside bottom of the sample vessel.
- The SoloVPE takes absorbance measurements at multiple pathlengths by moving the fibrette up and down through the sample.
- These absorbance values are plotted against the pathlength and the slope of the line is determined.
- Beer–Lambert's law and the molar co-efficient are used to calculate the protein concentration.



Absorbance varies at different pathlength



Absorbance vs. pathlength
Note: linear range



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Thank You