nibrt
National Institute for Bioprocessing Research and Training

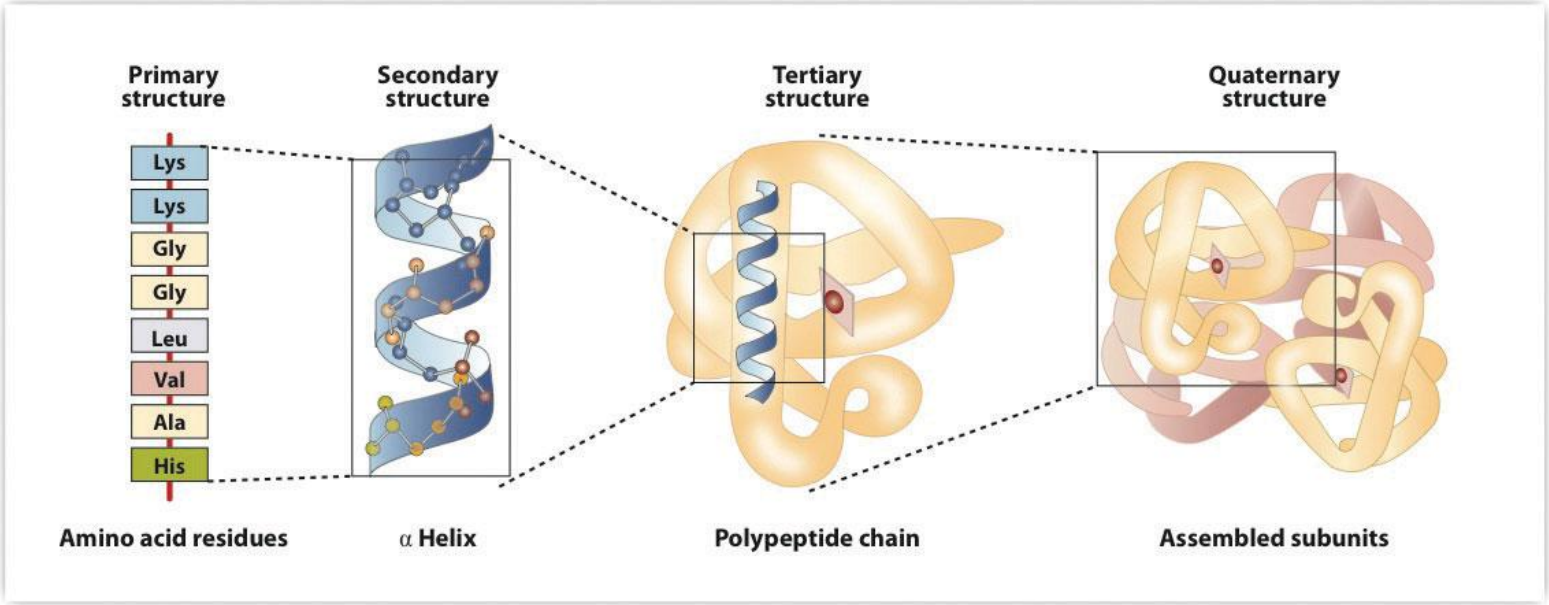
Lecture 9
RECAP

Topics

- QC Testing
- Protein Estimation Assays
- Slab Gel Electrophoresis
- Capillary Electrophoresis
- HPLC
- Immunoassays and Bioassays
- PCR

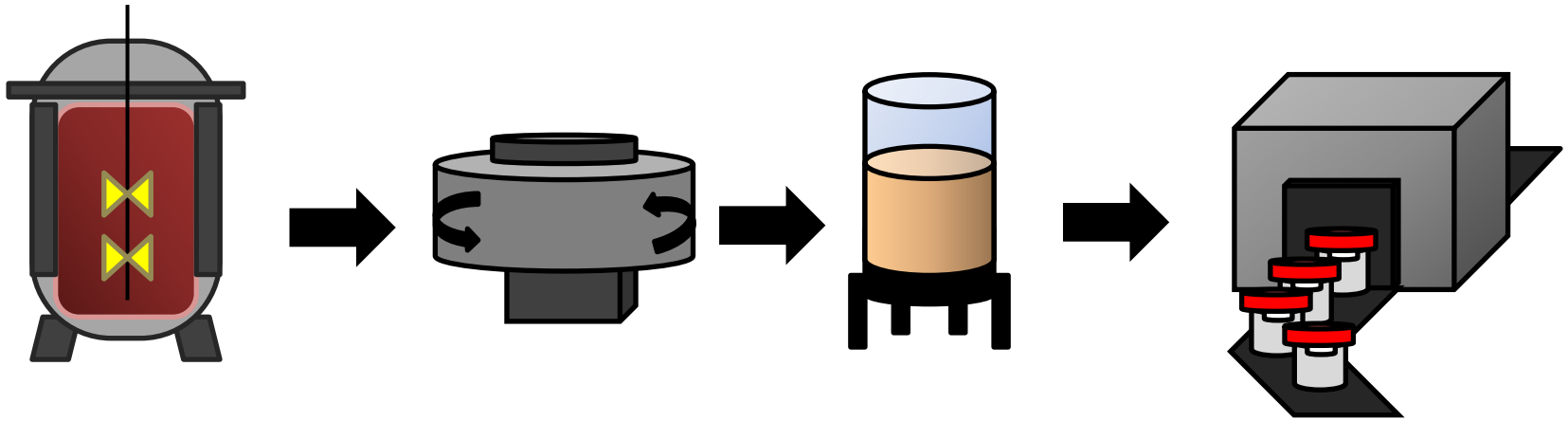
What makes a protein?

- The amino acids are arranged in a **specific sequence** which is dictated by the **gene** for that protein
- The sequence is **extremely important** for the protein to take its **biologically active shape**



Bioprocessing: Complex and Unique

Many complex **cell culture** and **purification** procedures.

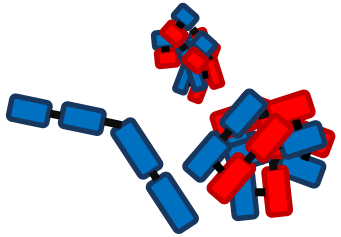


Upstream	Harvest	Downstream	Fill Finish
Cell Culture scale up and protein production	Cell removal	Protein purification	Formulation and Filling

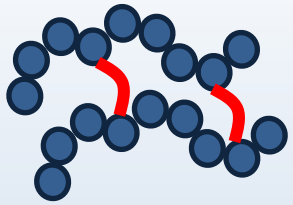
ICH Topic Q 6 B
Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

Process-related impurities

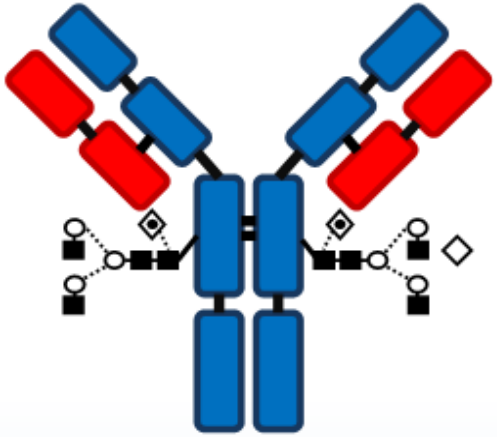
Product-related impurities



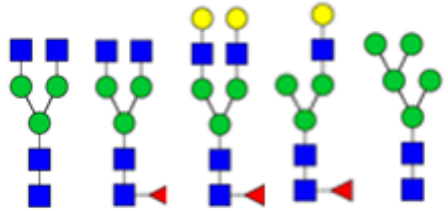
Disulfide bridges



Intact mass
Higher Order Structure
Potency
Binding properties

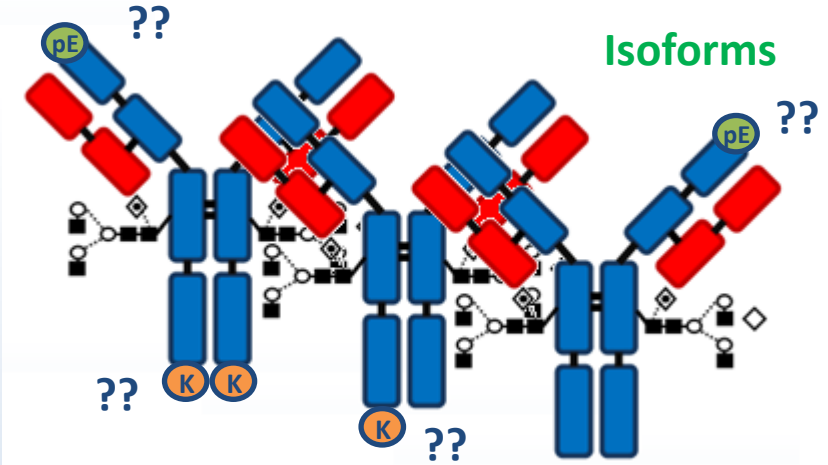


Glycan profile



Amino acid sequence
Mutations
PTM

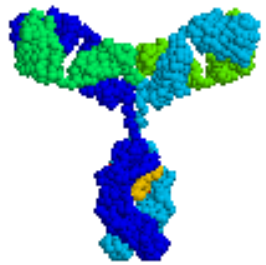
Isoforms



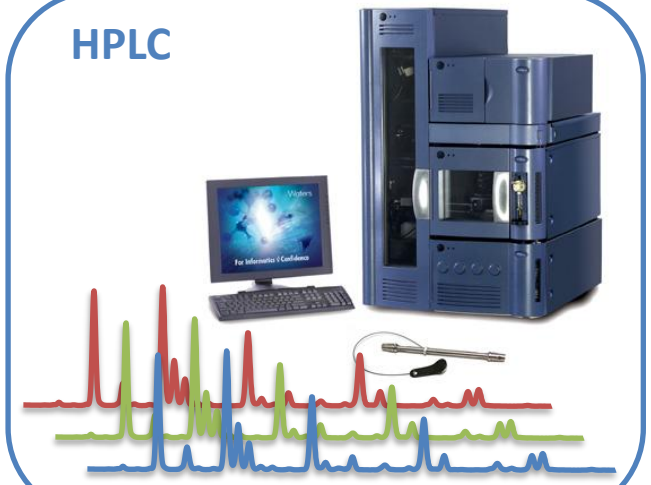
Vial inspection

pH, conductivity, osmolality

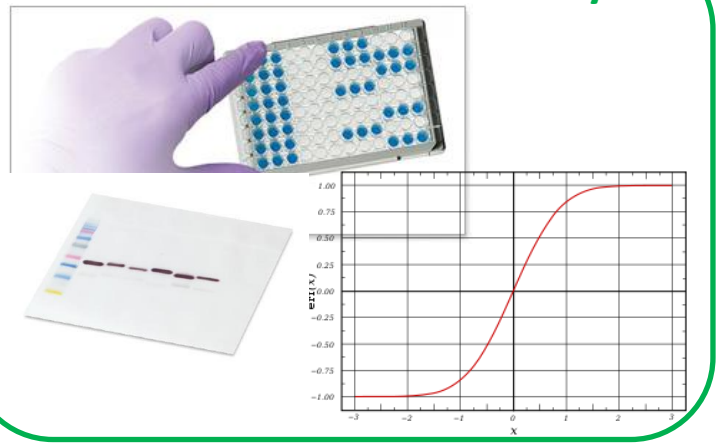
Electrophoresis



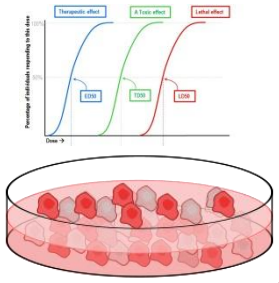
HPLC



Immunoassays



Bioassays



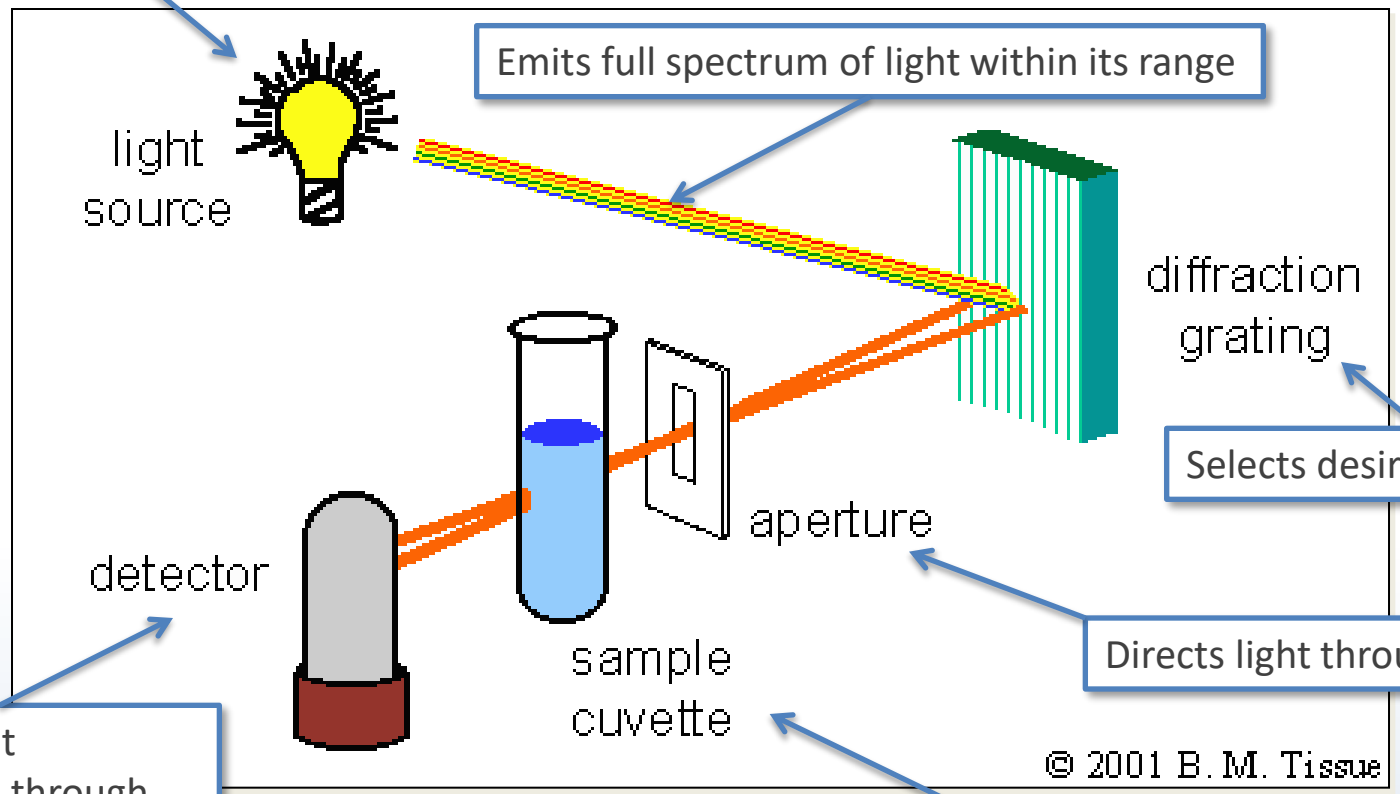
Microbial testing

Topics

- QC Testing
- Protein Estimation Assays
- Slab Gel Electrophoresis
- Capillary Electrophoresis
- HPLC
- Immunoassays and Bioassays
- PCR

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

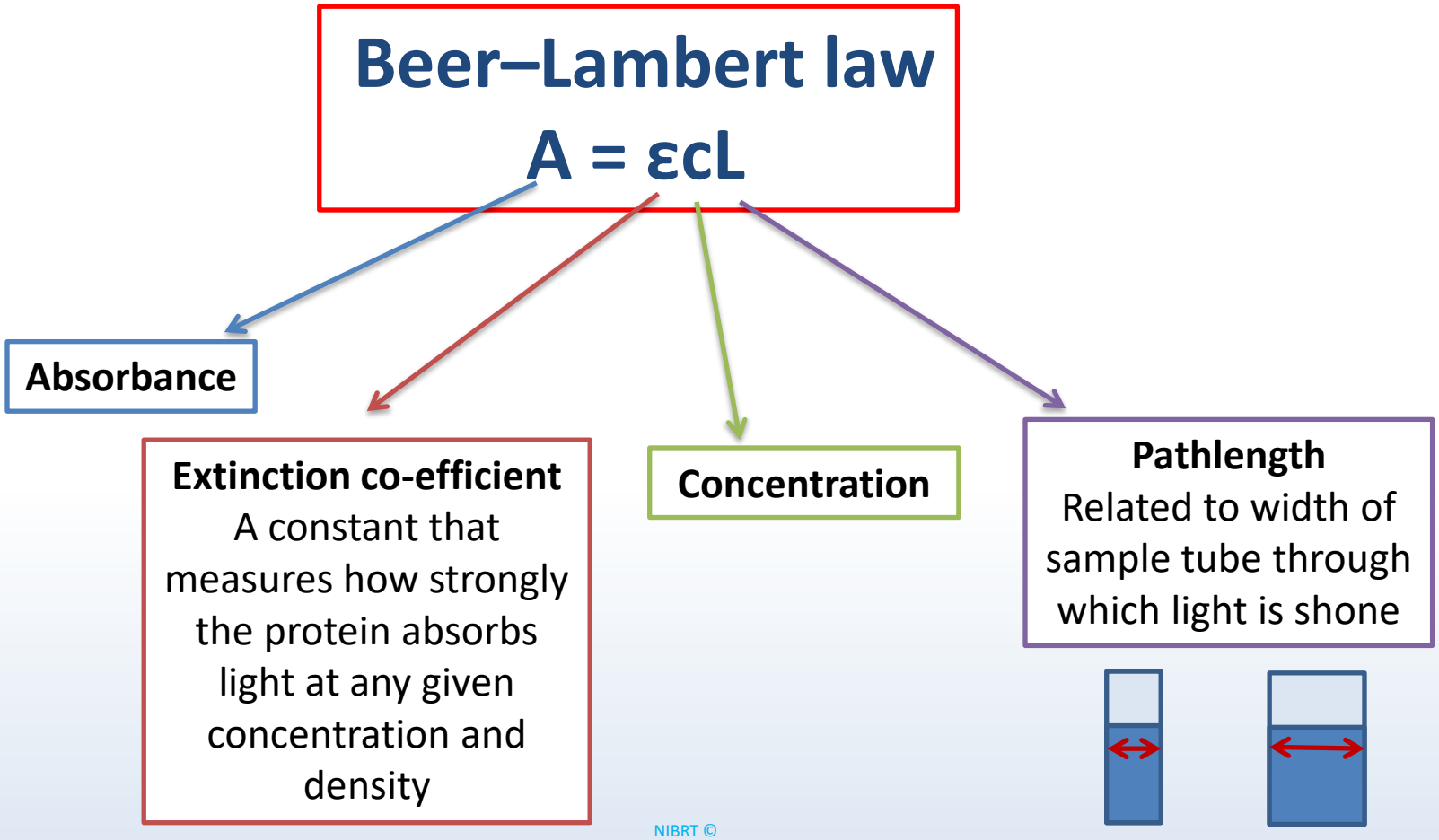
Directs light through sample

Detects light transmitted through sample

Contains sample in aqueous solution

Beer-Lambert Law

- Absorbance is related to concentration by:



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)

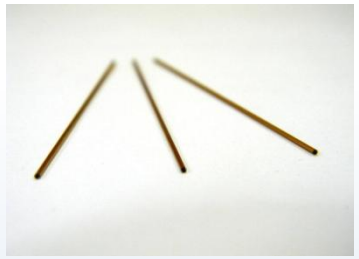
SoloVPE

- 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

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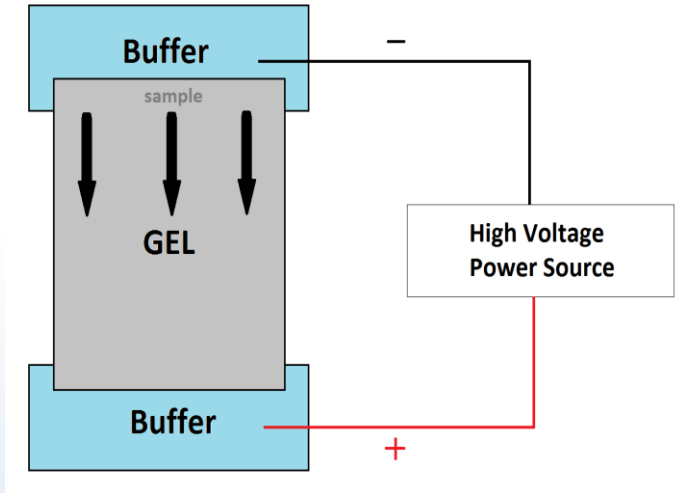
Electrophoresis

- Usually performed by placing sample in a non-charged **gel** through which an **electrical current** is applied

- The **electrical charge** and **mass** of the biomolecule affect its **mobility through the gel** during electrophoresis

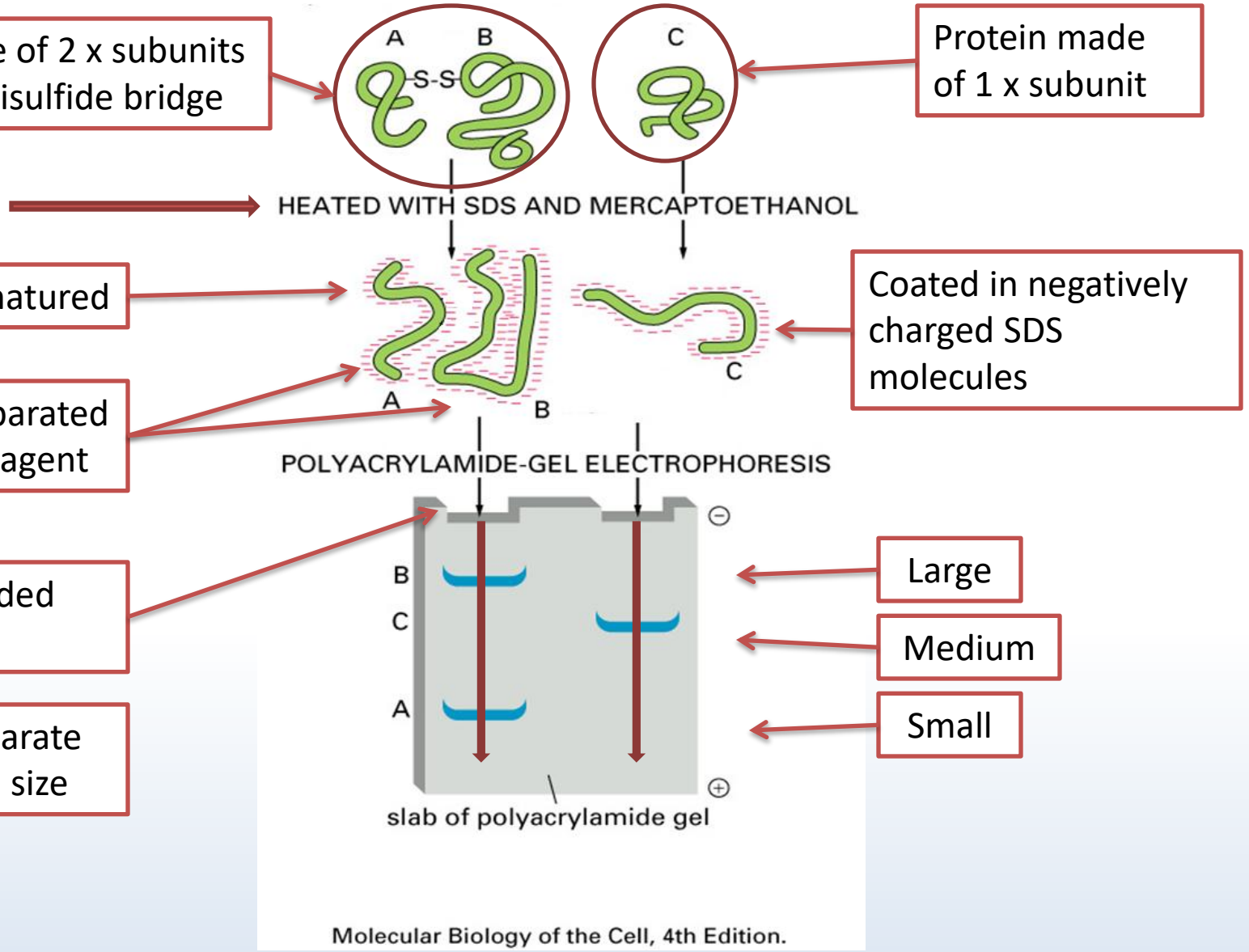
- Can separate based on:
  - Size
  - Isoelectric point
  - Size:charge ratio



Performing SDS PAGE

•Protein made of 2 x subunits
•Joined by a disulfide bridge

Protein made of 1 x subunit



Proteins denatured

Coated in negatively charged SDS molecules

Subunits separated by reducing agent

Samples loaded onto gel

Proteins separate according to size

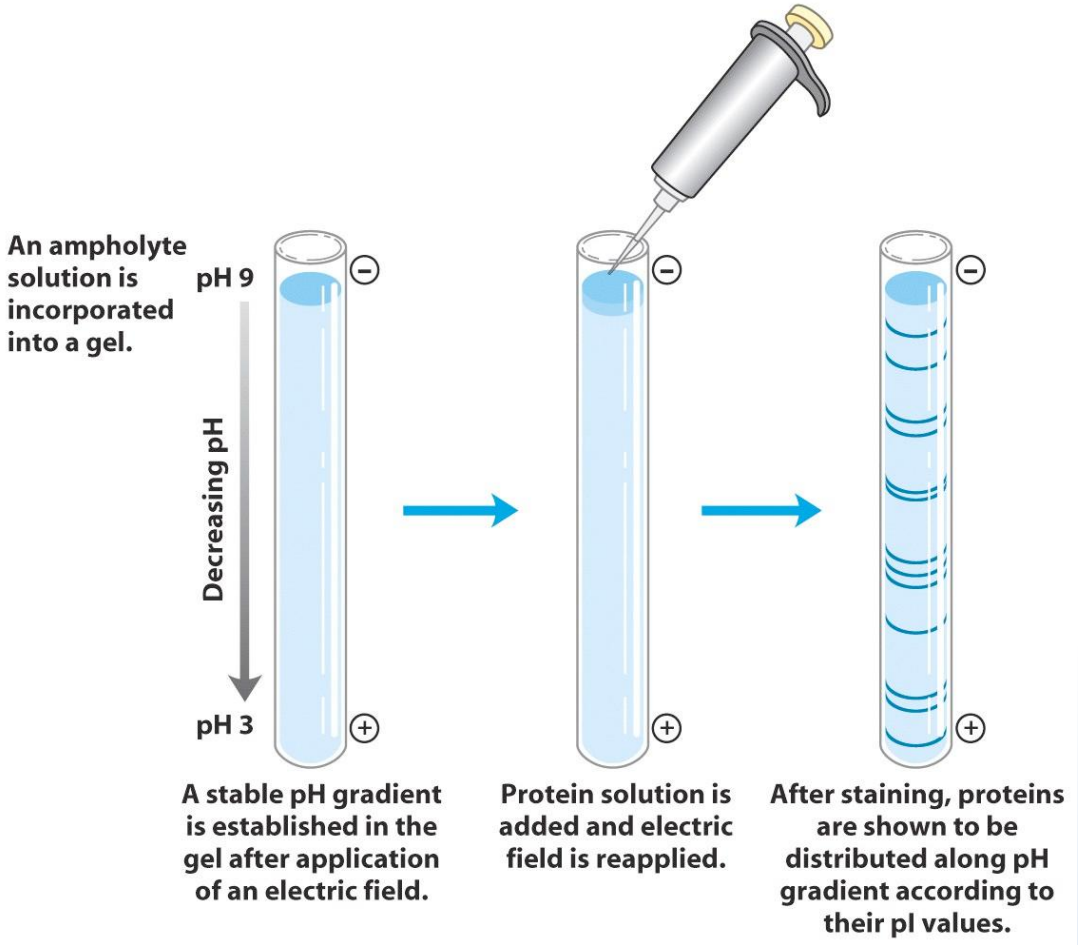
Large

Medium

Small

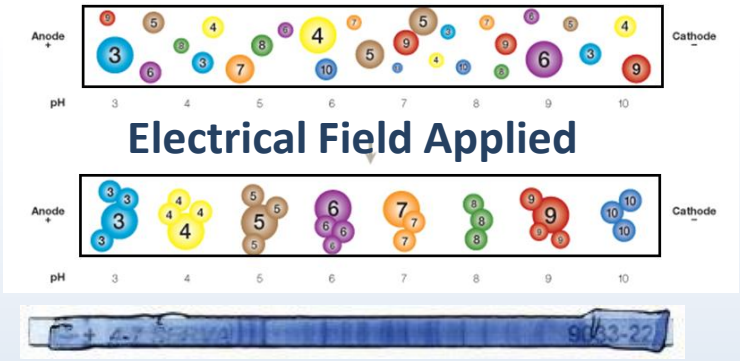
Isoelectric Focusing (IEF)

- Protein mixture is loaded and electrophoresed until the proteins in the sample reach the pH that is equal to their pI.



- When the protein in the pH gradient reaches a zone in which the net surface charge is zero, it will no longer move.

- At this point the protein becomes “focused” and a band is formed in the gel.



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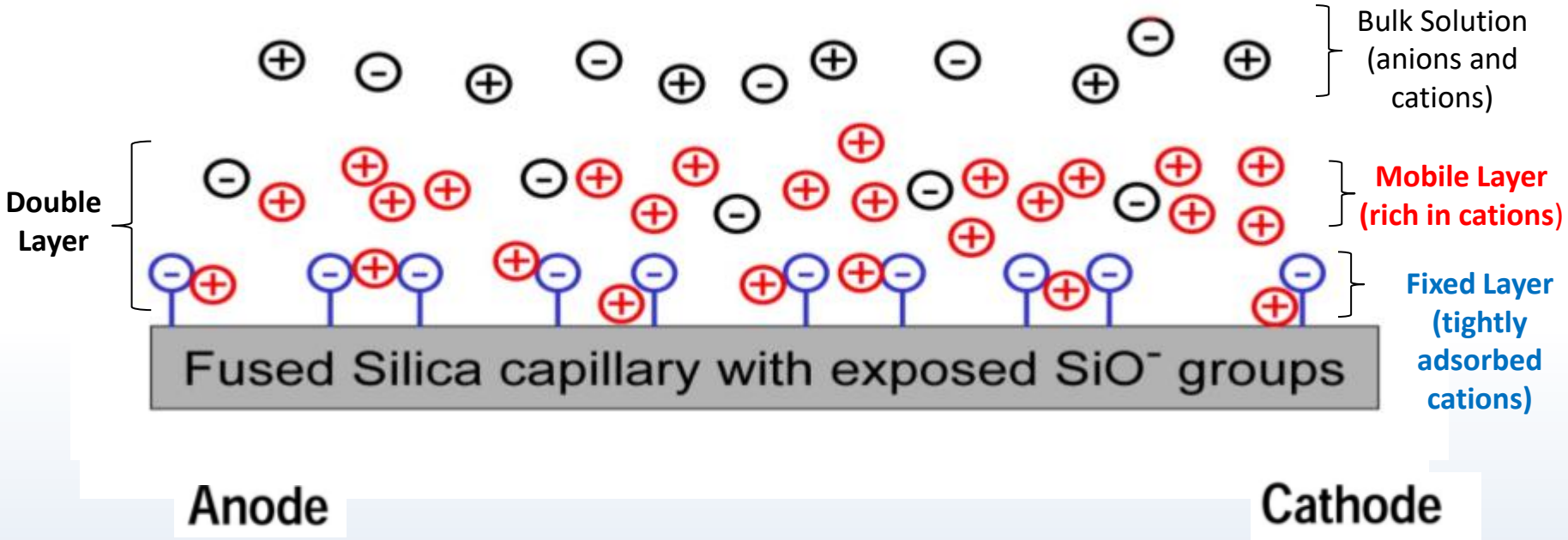
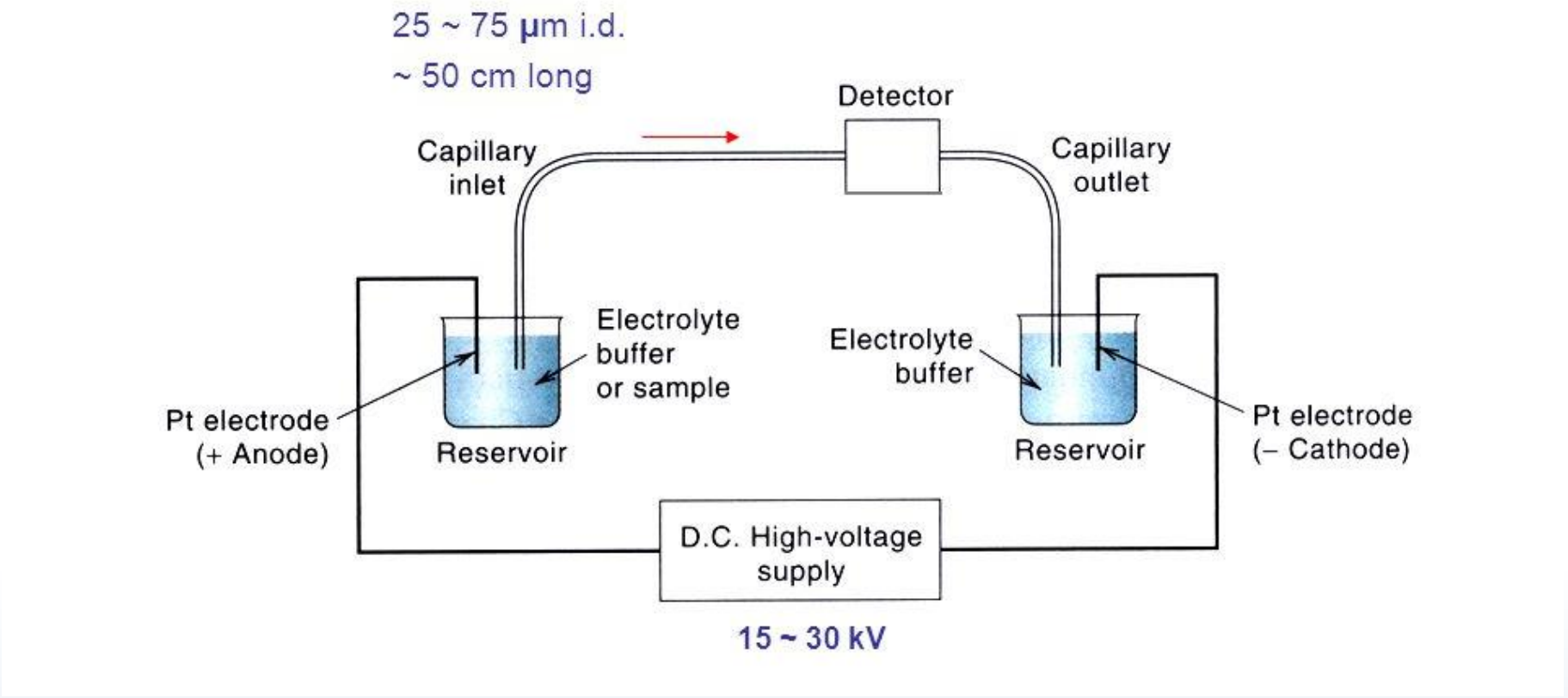
EOF

This first layer of cations (**fixed layer**) is not of sufficient density to totally neutralise the negative charges of the silanoate, so a second outer layer of cations forms (mobile layer)



CE Instrumentation

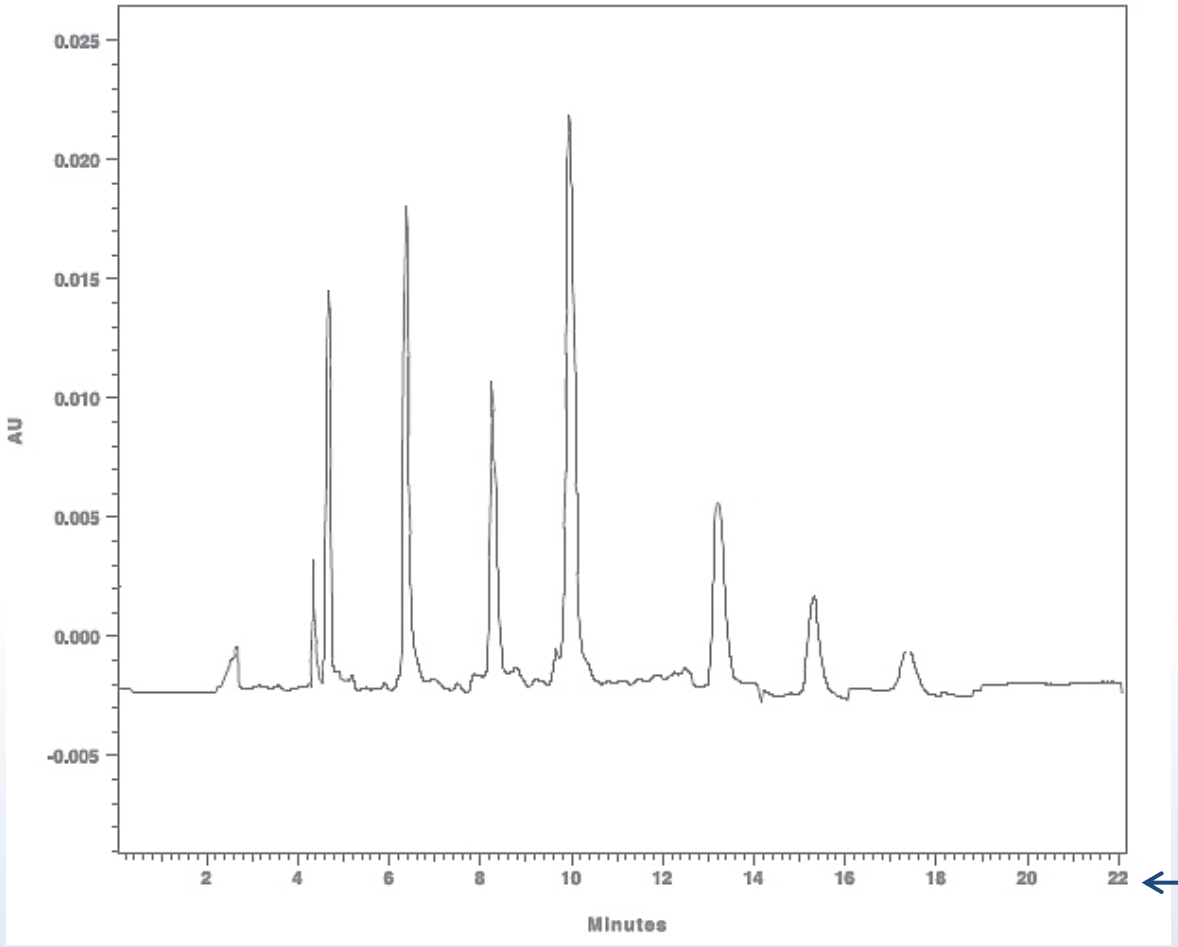


G. D. Christian, Analytical Chemistry, 6th ed., John Wiley, 2004, p. 632

CE Results

- Results are shown as an **electropherogram** (similar to a chromatogram in HPLC): time v absorbance/fluorescence units

Detector response



Migration Time

CE separation techniques

- Several separation techniques can be used depending on the capillary and the electrolytes used

  - **Capillary Zone Electrophoresis (CZE):** simplest form of CE, separation is by charge-to-mass ratio
  - **Capillary Gel Electrophoresis (CGE):** separation occurs in a gel in the capillary e.g. polyacrylamide gel. SDS can be used to coat protein in negative charge so separation is by size
  - **Capillary iso-electric focusing (CIEF):** separation is by charge & occurs along a pH gradient so proteins migrate depending on their isoelectric point

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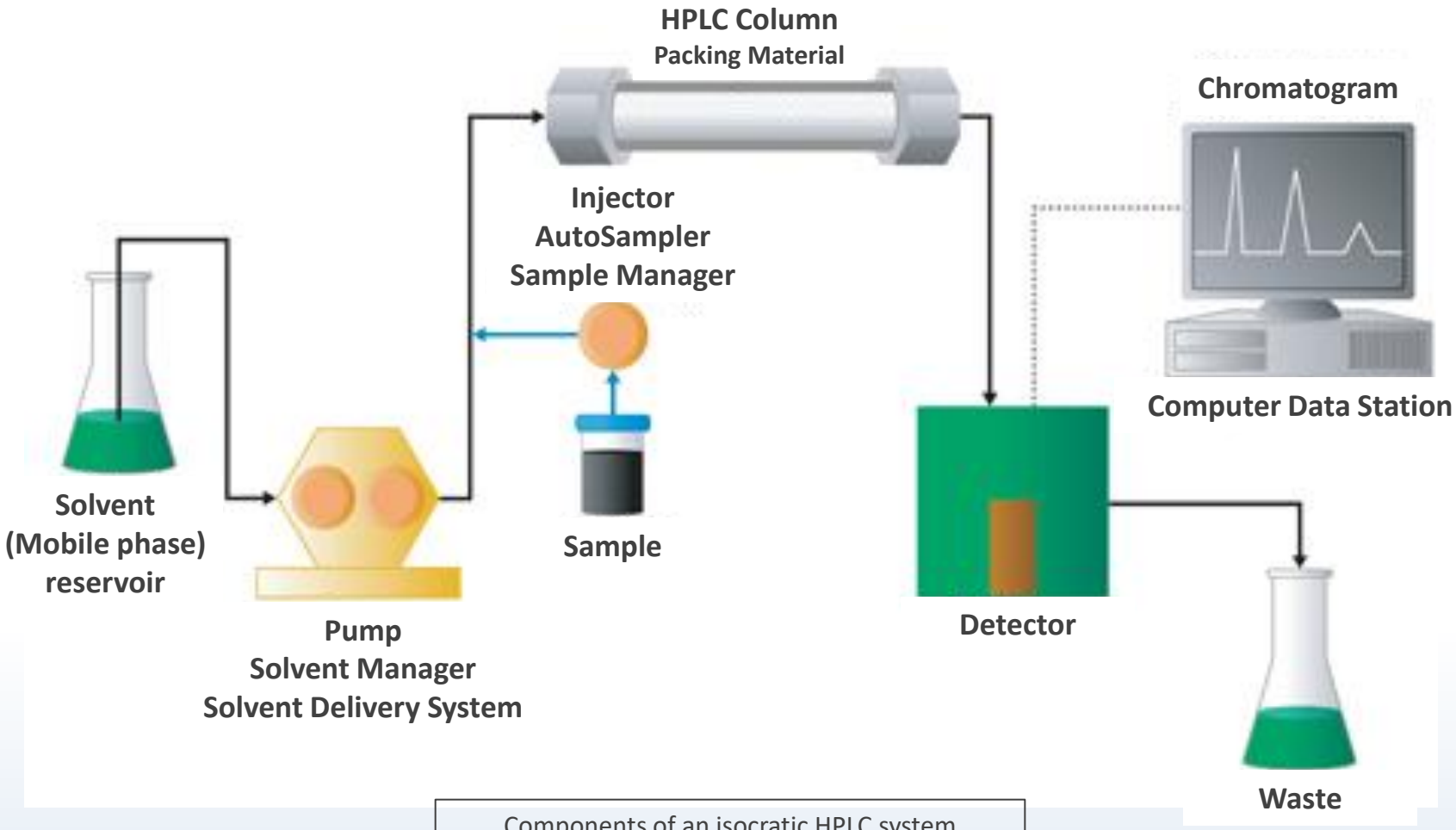
HPLC
Equipment



Components of an isocratic HPLC system
Adapted from Waters website

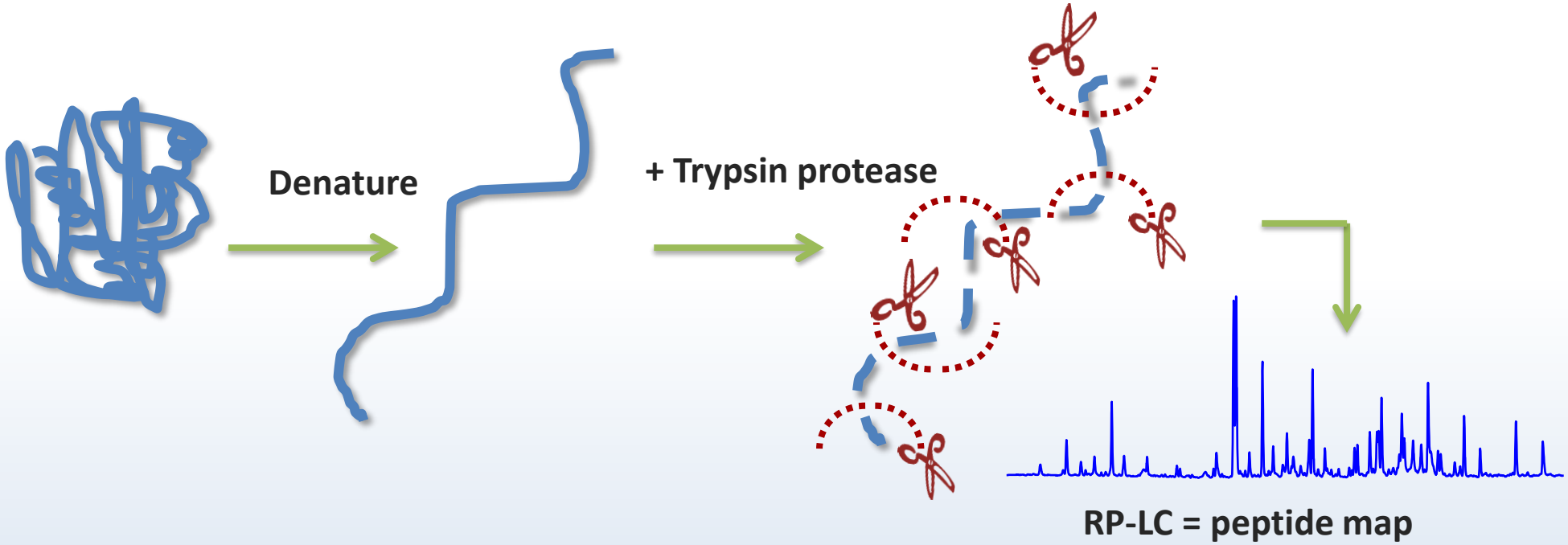
Key Analytical methods
Chromatography

Isolation Method	Feature
• Normal phase chromatography (NP-HPLC)	Hydrophilicity
• Reversed phase chromatography (RP-HPLC)	Hydrophobicity
• Affinity chromatography	Biorecognition
• Size exclusion chromatography (SEC)	Size
• Ion exchange chromatography (IEX)	Charge
• Hydrophilic interaction chromatography (HILIC)	Hydrophilicity

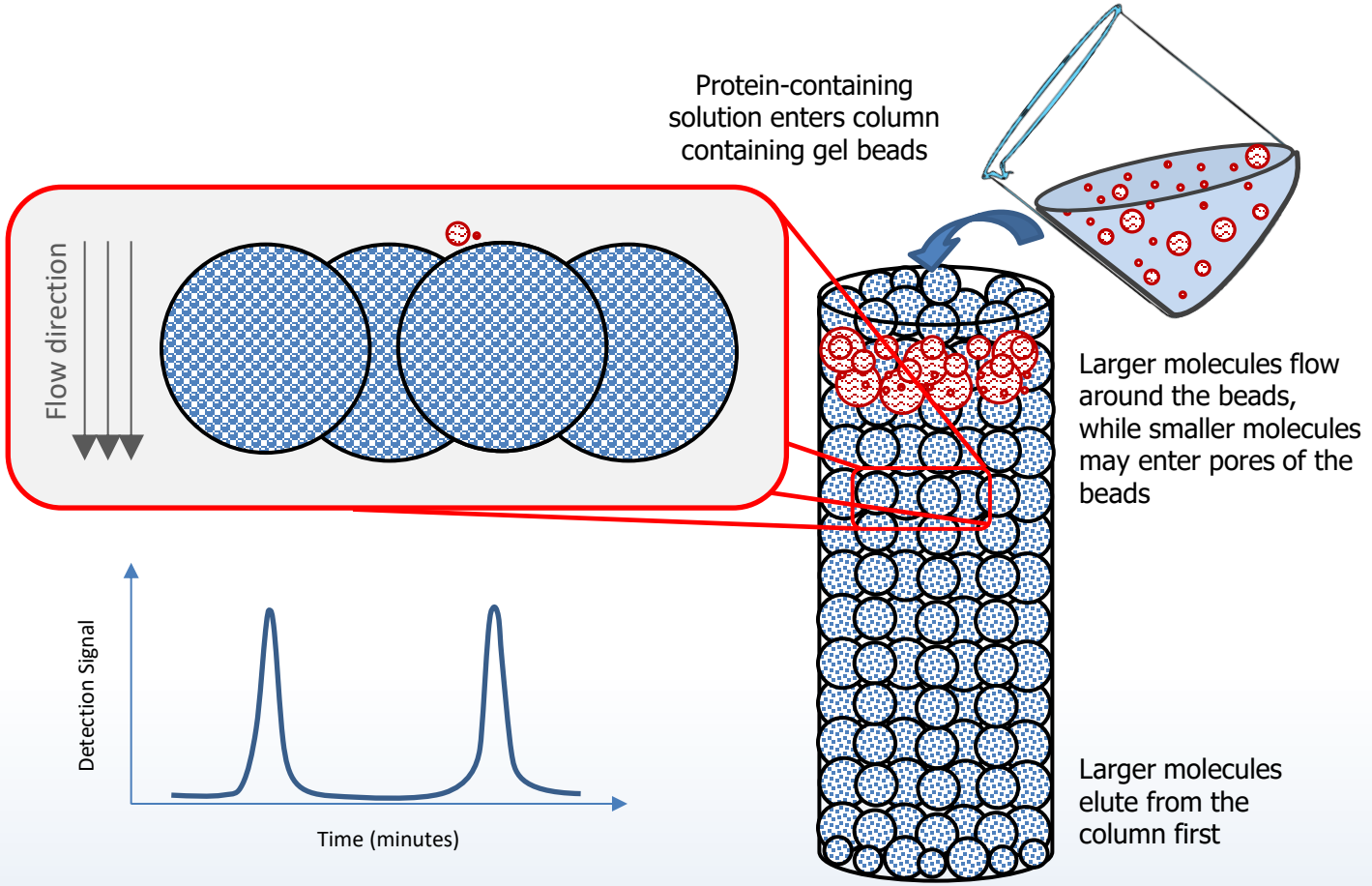
Analytical: Peptide mapping

- Peptide mapping involves **digesting** a protein with a **protease** such as **trypsin** to create specific **peptides**

- Used to produce a **unique 'fingerprint'** of an individual protein when analysed by RP-LC



Size Exclusion Chromatography



Topics

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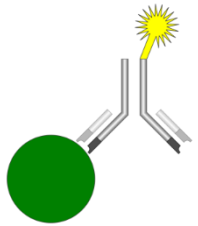
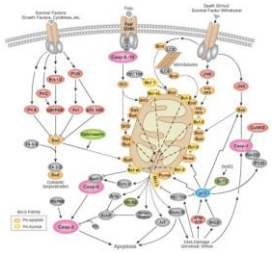
Biological Characterisation





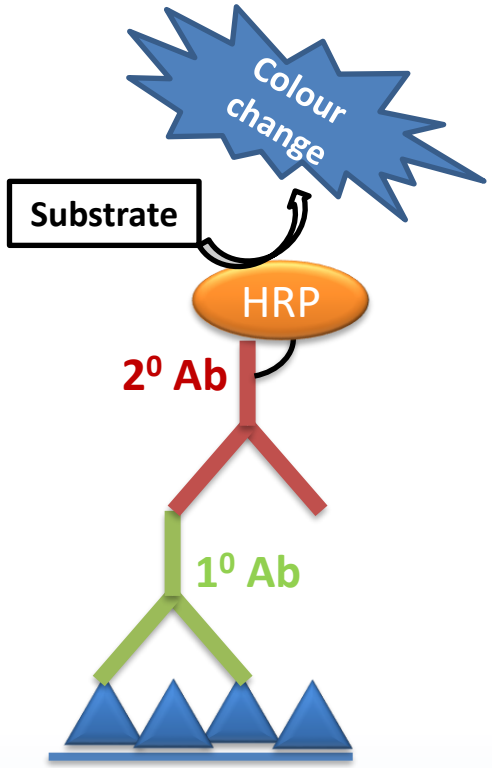
Immunoassay	Bioassay
An immunoassay is a quantitative or qualitative analytical method that relies on the **binding of an antibody to an antigen**	A bioassay is a quantitative analytical method that measures a **defined biological effect in a living system**, such as an intact cell.

VS

Antibodies used in immunoassays

- The antibody that detects the antigen is called the **primary antibody (1^{o})**
- For detection of this interaction, a second antibody is required **(the secondary antibody, 2^{o})**
- The **2^{0} Ab** is designed such that its **binding partner** is the **1^{0} Ab**
- The 2^{0} Ab is labelled (conjugated) with an **enzyme**
  - E.g. horseradish peroxidase (HRP)

- Adding the substrate of the enzyme leads to a **measurable response** (e.g. colour change)
- Can also use a **fluorophore** (non enzymatic detection)

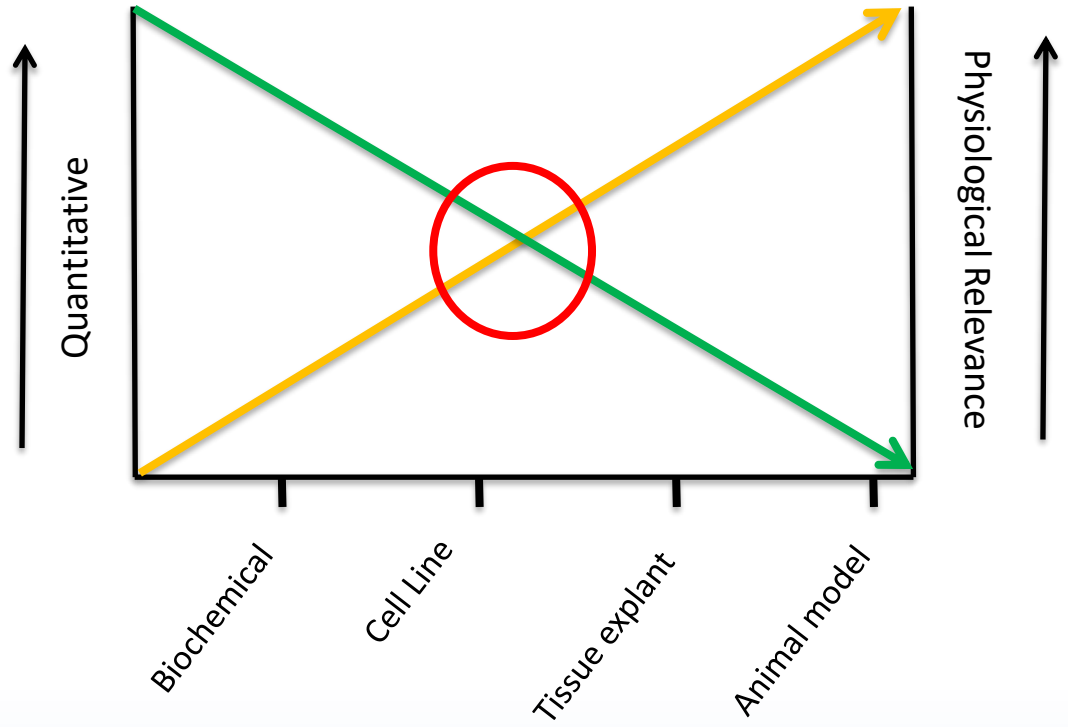


www.mandel.ca/products/protein_analysis/Enzymes_Substrates/SureBlue_Reserve_TMB_Microwell_Substrate/index.html

Western Blotting –v- ELISA

Western Blotting	ELISA
Immobilised antigen on membrane	Immobilised antigen on plastic plate
Qualitative and Semi-quantitative	Qualitative and Quantitative
Detection of protein of interest	Detection of protein of interest
Provides information on purity and MW of protein	Does not provide information on purity or MW of protein

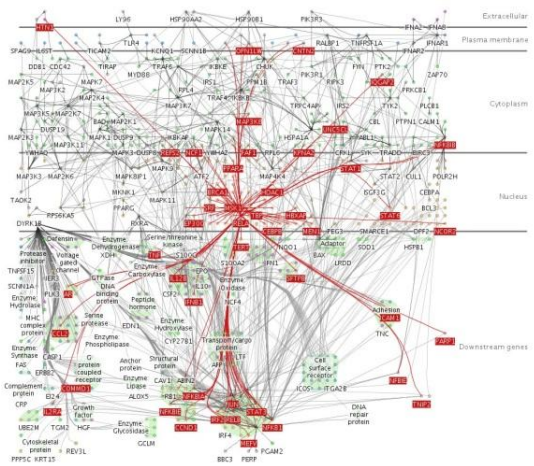
Types of Potency Assays



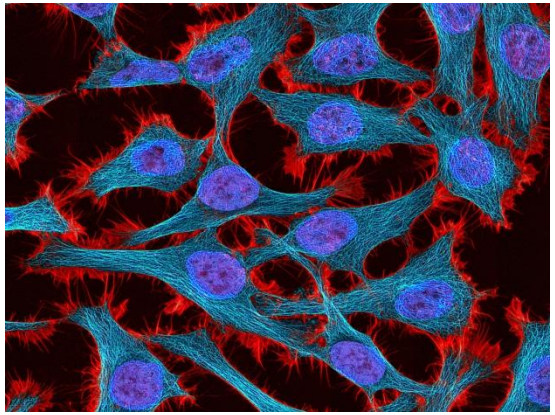
Cell based bioassays can be both quantitative and physiologically relevant and represent a good balance between the two extremes

http://www.mbswonline.com/upload/presentation_5-24-2012-9-17-24.ppt

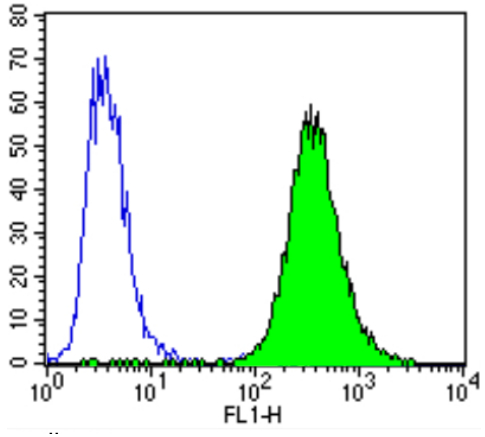
The Problem with Bioassays



1. Functional bioassays rely on the translation of complicated cellular biochemistry into an analytical read out.



2. Functional bioassays require the use of cultured or primary cells. How does *in vitro* reflect *in vivo*??



3. Analytical component dependent upon the performance of cells and biochemistry, does it answer the question posed?

Informative cell based functional bioassays require careful experimental design, optimization, validation and operation to generate consistent and reliable data.

Cell Based Bioassays (mAB examples)

Each Bioassay is unique as it depends on the drug and its MOA

- Cell signalling assay
- Proliferation assay
- Cytotoxicity assay
- Antibody dependant cell-mediated cytotoxicity (ADCC)
- Complement dependent cytotoxicity (CDC)

If the biopharmaceutical product has more than one biological activity, it will be necessary to measure all functions

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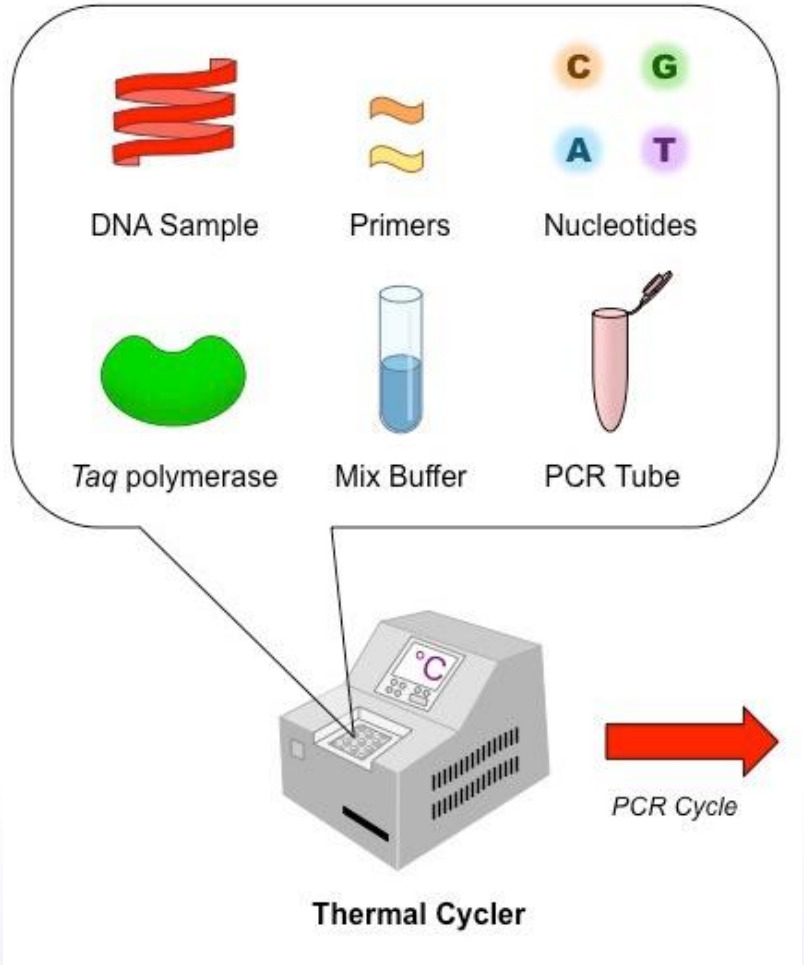
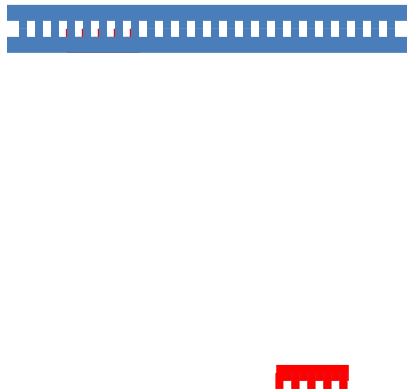
PCR Components



http://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html

Thermal Cycling



Taq polymerase extends the primers, synthesizing new strands of DNA.

Template Denaturation (96°C):

Causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Primer Annealing (55 - 65°C)

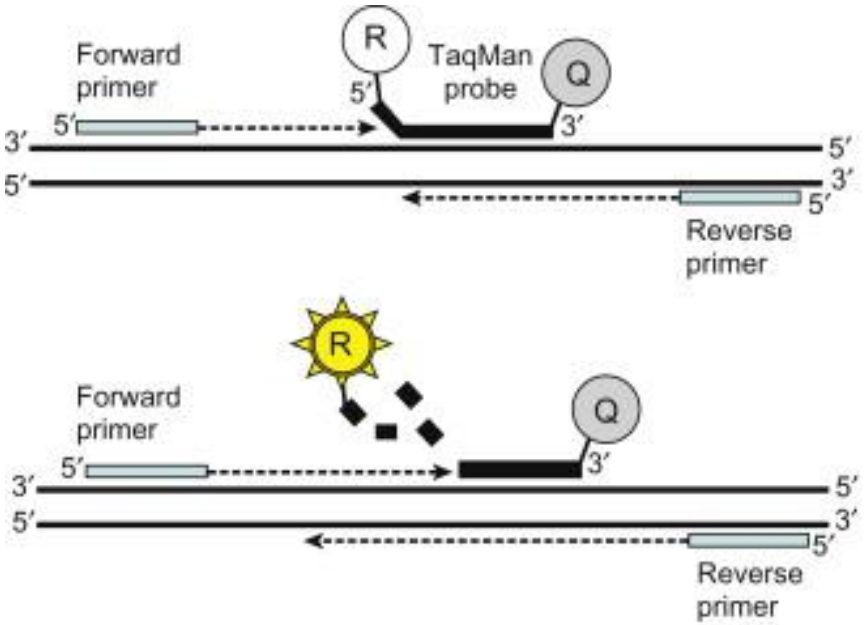
Primers bind to their complementary sequences on the single-stranded template DNA.

Primer Extension (72°C)

30-40x

TaqMan® PCR technology

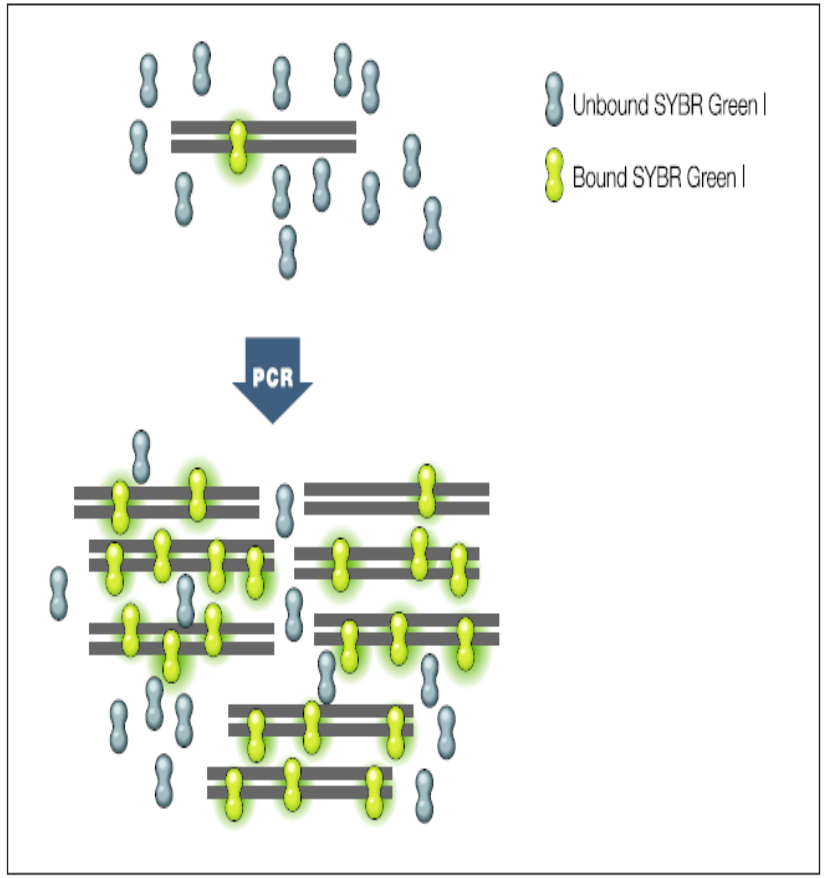
- Sequence-specific probe that **anneals between the forward and reverse primers**.
- Labelled with a **reporter (R)** and a **quencher (Q) dye.**
  - R is usually a green dye (e.g. fluorescein)
  - Q is typically a red dye (e.g. TAMRA)
- When the probe is intact **Q interferes with R - little to no fluorescence**.
- As the PCR primer extends, the probe is cleaved from the DNA strand
- **Cleavage separates R and Q – increase in fluorescence.**



https://doi.org/10.1016/B978-0-12-374513-2.00003-8

SYBR Green I

- SYBR Green I is a DNA dye that binds non-discriminately to double-stranded DNA (dsDNA).
- SYBR Green I exhibits **minimal fluorescence when it is free in solution**, but its **fluorescence increases** dramatically (up to 1000-fold) upon **binding to dsDNA** (new DNA)
- As more double stranded amplicons are produced, SYBR Green dye signal will increase respectively.



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LAQ Exam Format

- Open from 12pm on **Wednesday 16th April** (closes on Friday 18th April at 12pm)
- Time allowed = 1 hour 30min
- You will be given a choice of **4 questions and you only need to answer 3**
- DO NOT attempt all 4 questions as I will only correct 3 per student

Example long answer questions for Final Exam

- An analyst wishes to use an ELISA method of detect for the presence of HCPs in a drug substance test sample. Discuss in your own words how this assay should be carried out. Include the following information in your answer:
  1. A description of the reagents and instrumentation required
  2. Describe how the assay is working

LAQ

- All questions will focus on a specific technique but will draw on your knowledge of protein chemistry and general quality testing

- Answers should be written in your own language. Do not copy from other sources. Answers that are copied will receive zero marks

Thank You