Quality Control Biotechemistry

NBC²

Common Process Compounds and Methods of Removal or Purification

Component	Culture Harvest Level	Final Product Level	Conventional Method
Therapeutic Antibody	0.1-1.5 g/l	1-10 g/l	UF/Cromatography
Isoforms	Various	Monomer	Chromatography
Serum and host proteins	0.1-3.0 g/l	< 0.1-10 mg/l	Chromatography
Cell debris and colloids	10^6/ml	None	MF
Bacterial pathogens	Various	$<10^{-6}$/dose	MF
Virus pathogens	Various	$<10^{-6}$/dose (12 LRV)	virus filtration
DNA	1 mg/l	10 ng/dose	Chromatography
Endotoxins	Various	<0.25 EU/ml	Chromatography
Lipids, surfactants	0-1 g/l	<0.1-10 mg/l	Chromatography
Buffer	Growth media	Stability media	UF
Extractables/leachables	Various	<0.1-10 mg/l	UF/ Chromatography
Purification reagents	Various	<0.1-10mg/l	UF

What Will Change During Scale-up?

Process Development Considerations

- Utility requirements
- Water requirement
- Cleaning/Sanitizing solution requirements
- Buffer prep
- Number of steps in cell culture scale up
- Harvest techniques
- Column packing; distribution of introduced liquid at large columns
- Equipment – bubble trap
- Automation of process
- Data collection
- Sample load

Quality Control Biochemistry

HPLC (High Pressure Liquid Chromatography)

IEF (Isoelectric Focusing)

ELISA (Enzyme-Linked Immunosorbent Assay)

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

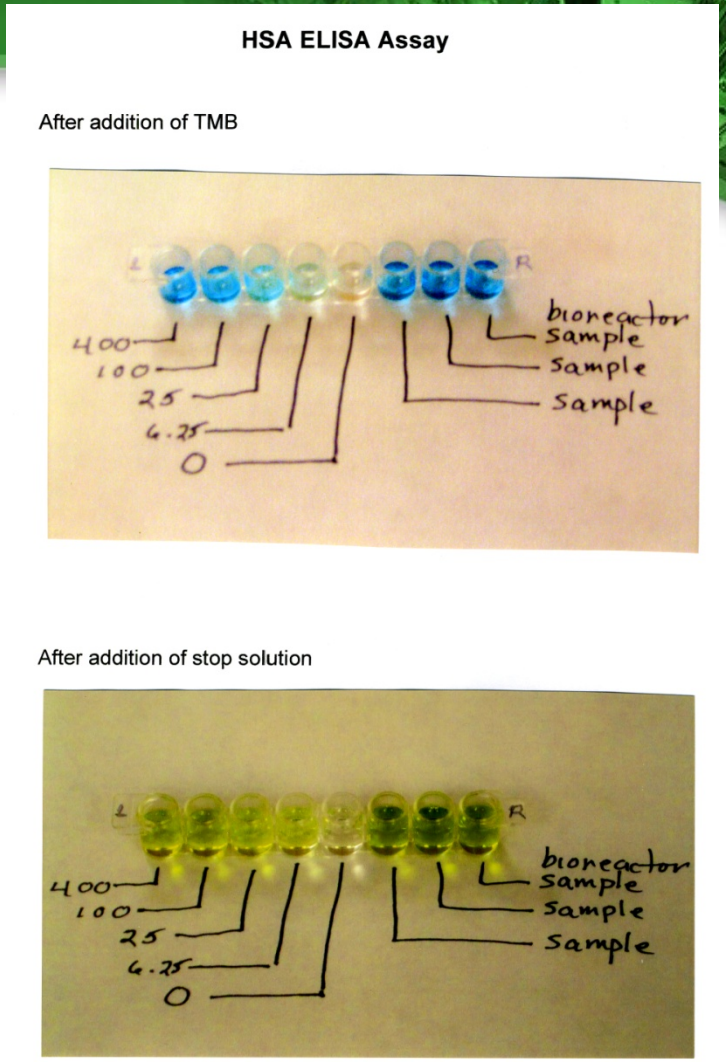
Quality Control Biochemistry: ELISAs and SDS-PAGE

Each of these methods is important in the Downstream Processing of the Protein of Interest:

- IEF (Isoelectric Focusing): Use an SDS-PAGE gel box (or CE = capillary electrophoresis) to determine the **pI** or the pH at which the protein of interest is neutral.
- ELISAs: Use antibody reagents and a microtitre plate reader to determine the **concentration** and/or the **activity** of a protein of interest.
- SDS-PAGE: Use acrylamide gel electrophoresis to separate proteins according to **molecular weight** (a single band indicates **purity – if validated to do so**).

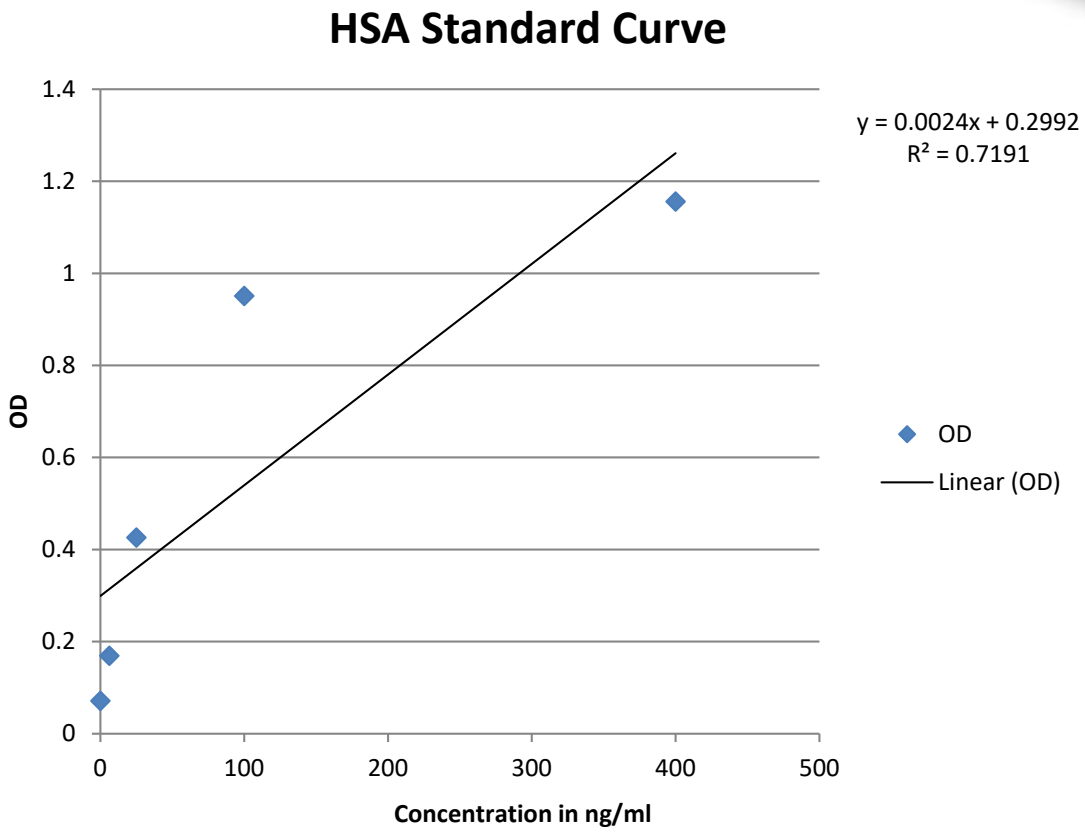
ELISAs

There are several types of ELISAs including direct (sandwich), indirect, competitive and activity ELISAs. ELISAs are read on a microtitre plate reader which is a mini-spectrophotometer that determines the absorption or transmission of a beam of light of a particular wave length passing through a solution of the protein of interest. Using standards to generate a standard curve, one can determine the concentration of the protein of interest in a sample.



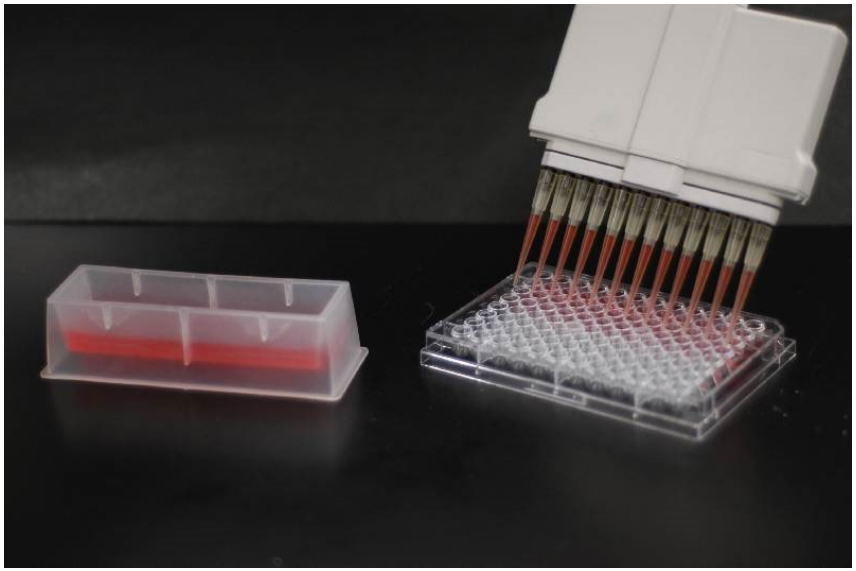
HSA ELISA Results
Spring 2009 Data

Concentration ng/ml	OD
0	0.071
6.25	0.169
25	0.426
100	0.951
400	1.156
Sample 1	1.320
Sample 2	1.290
Sample 3	1.290



ELISA Equipment

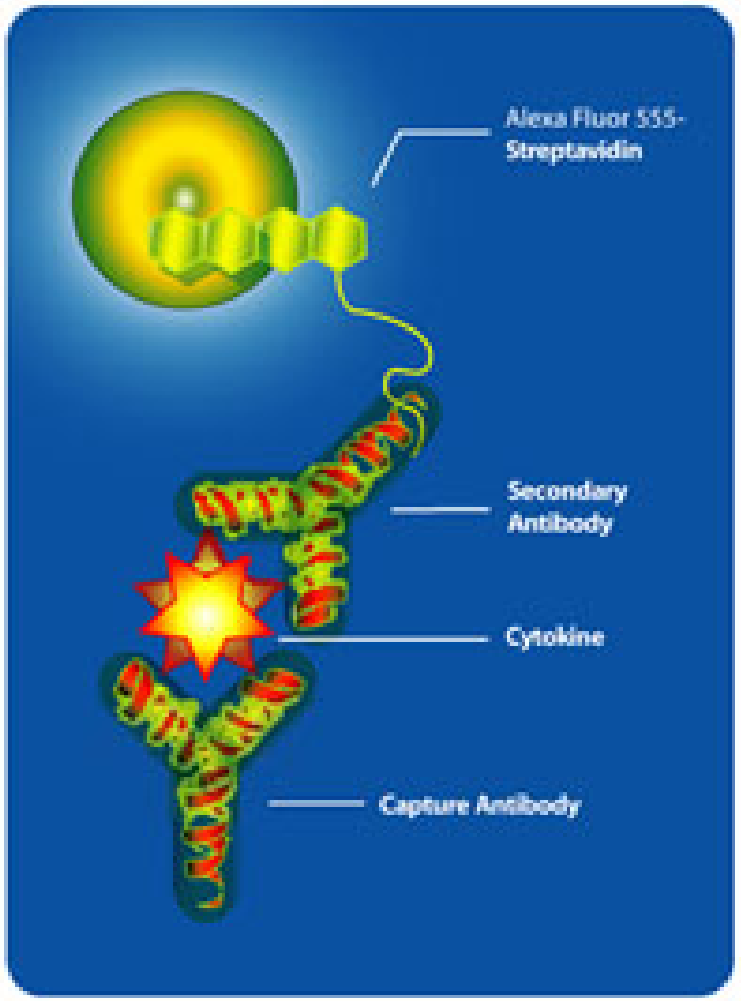
Multi-Channel Pipettor



Microtitre Plate Reader

ELISA Process

To make an ELISA, one must utilize antibodies to the protein of interest. The first antibody recognizes the protein of interest. The second antibody recognizes another epitope on the protein of interest and carries an enzyme that will be used to quantify the protein of interest.

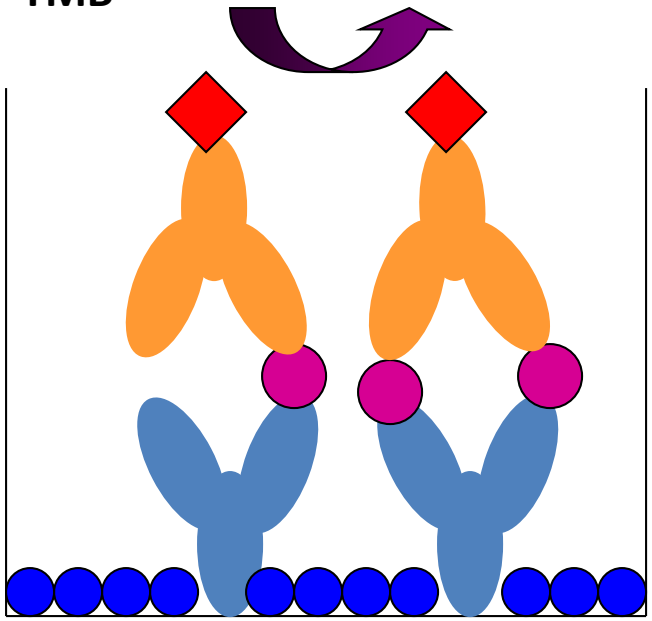


ELISA Process – Colorimetric Reaction

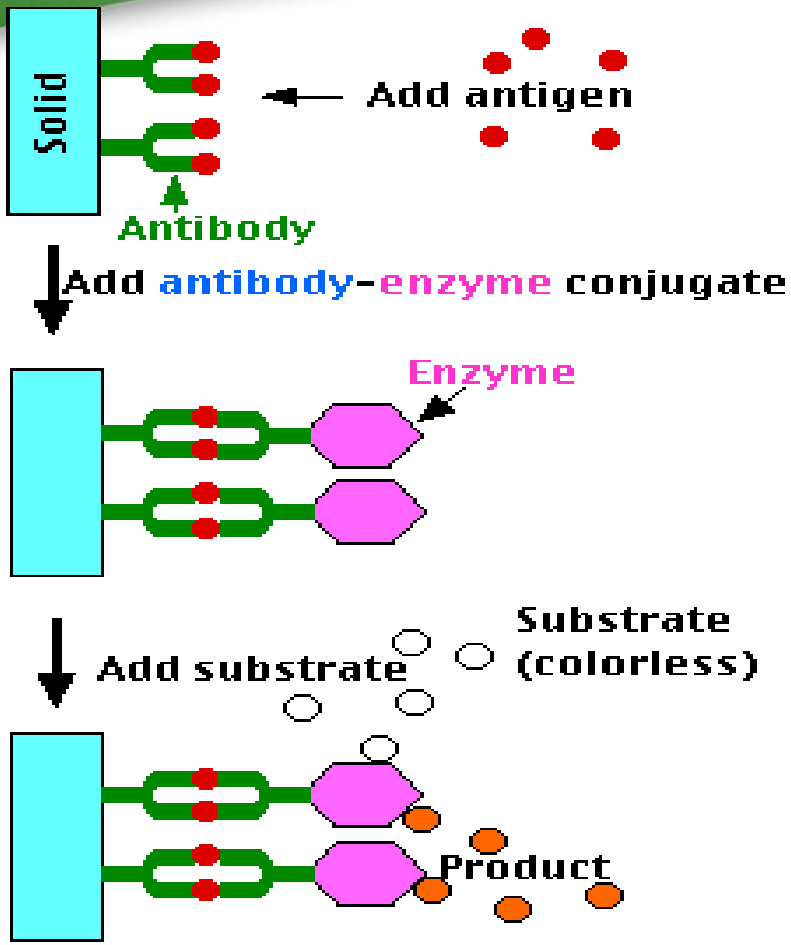
Colorless substrate

TMB

Colored product



ELISA Continued



ELISA = Antibody Sandwich

Antibodies as Reagents

ELISAS are Immunoassays which use an antibody (Ab) to detect and quantify substances

Ab are extremely specific – ADVANTAGE

Ab can not be detected, need a marker:

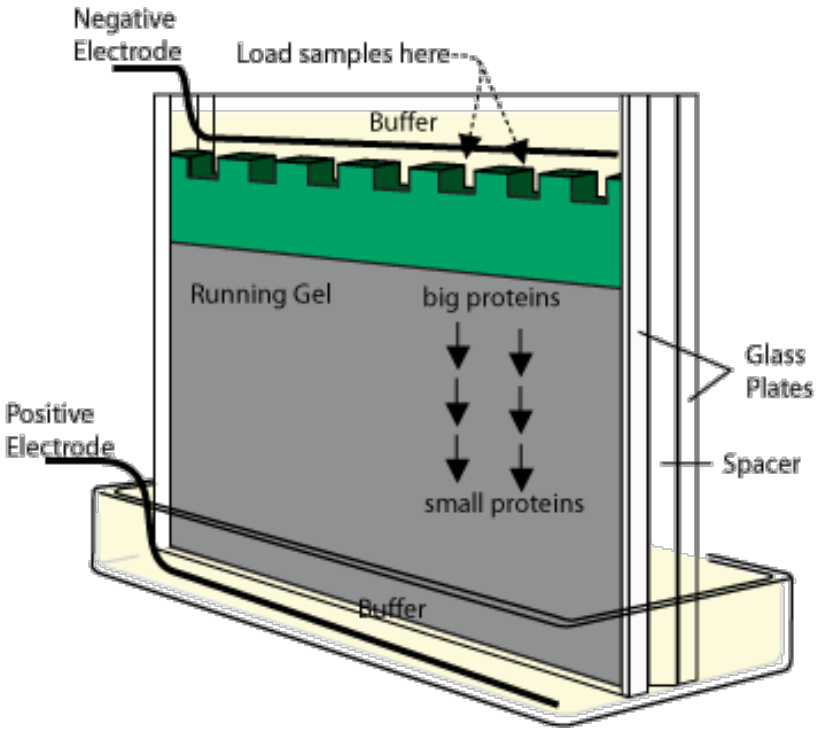
- Radioactive labels (RIA)
- Enzymes (EIA) – Horseradish Peroxidase; Alkaline Phosphatase
- Fluorescent Tag (FIA)
- Chemiluminescencent Tag

ELISA Animation

- The animation may be found at: usmlemd.wordpress.com/2007/06/12/elisa-test/

SDS-PAGE

SDS-PAGE Gel Box



SDS-PAGE Overview

SDS Polyacrylamide gels (SDS-PAGE) are called "denaturing gels" because they contain sodium dodecyl sulfate (SDS), an ionic detergent that binds to the amino acid residues in the proteins. Due to its ionic properties, SDS confers a net negative charge on all the proteins, overcoming any intrinsic charge; in this way the proteins uniformly migrate toward the positive electrode. SDS also disrupts the secondary and tertiary structure of the proteins, essentially destroying their globular configuration and making them into linear molecules that then migrate in the electric field on the basis of their size. PAGE is a very powerful technique because even small differences in molecular weights produce distinguishable bands on a gel.

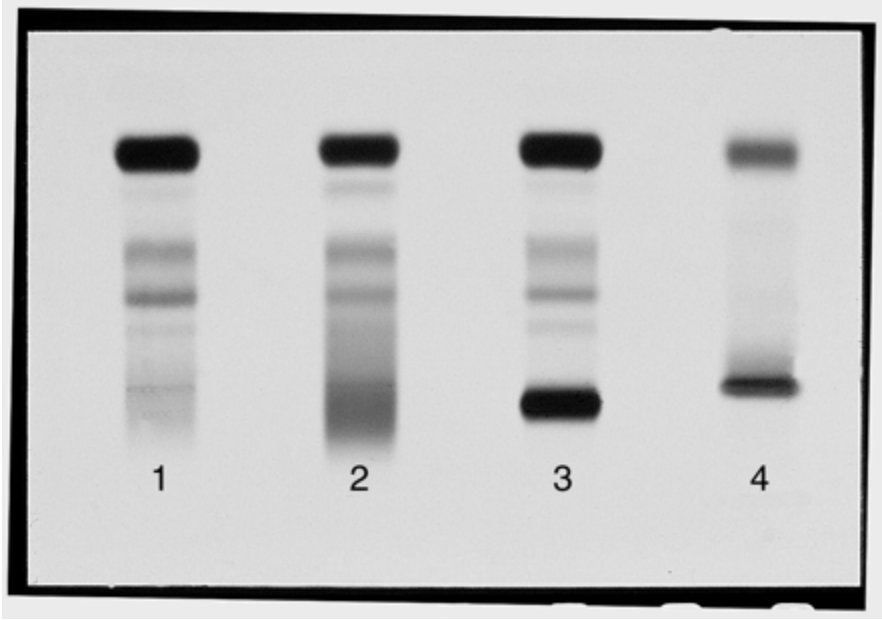
SDS-PAGE Continued

SDS-PAGE
separate proteins based on molecular weight

Isoelectric Focusing
identify the pH at which a protein carries no net charge

SDS-PAGE Continued

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis developed by Laemmli (1970)



SDS-PAGE Continued

- characterize (MW)
- quantify (densitometry)
- determine other proteins in a sample
- step in Western blot (used to identify)

SDS-PAGE Continued

Coomassie Blue Stain (0.1 ug)
Silver Stain (2 ng)

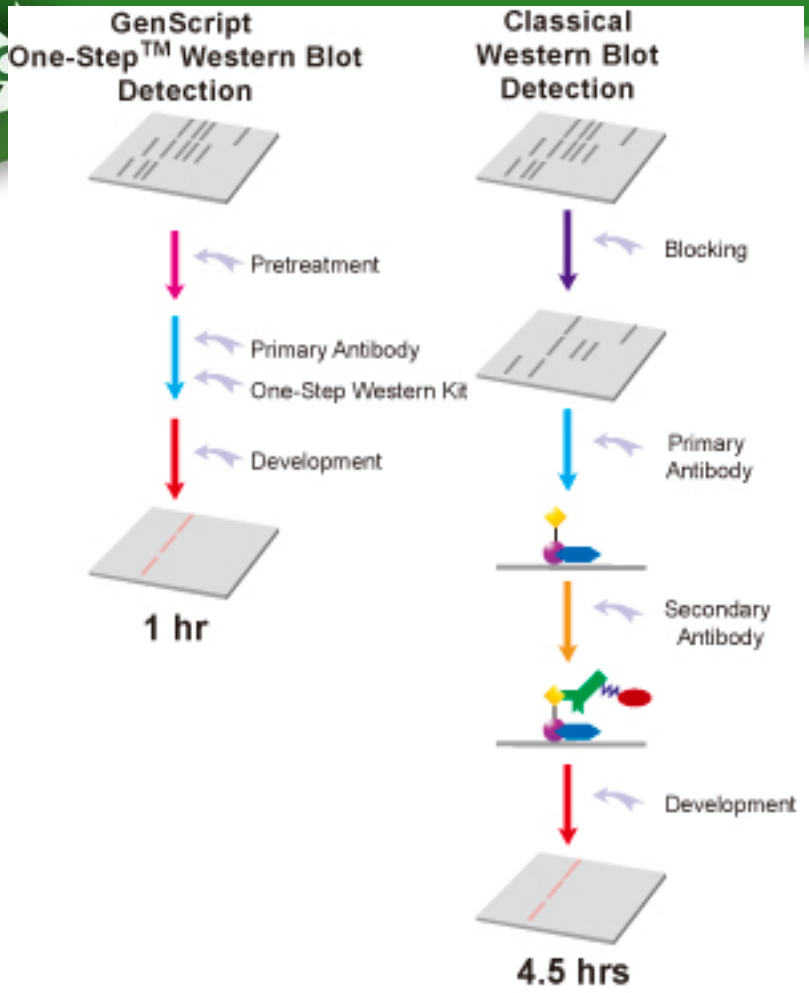
How to Quantify Proteins?

Densitometry

Molecular Weight Determination

- Run SDS PAGE with known standards (MW markers)
- Graph
- Measure distance unknown protein traveled
- Compare on standard curve

Immunoblots (Westerns)



A280

Tryphophan
Phenylalanine
Tyrosine

ALL ABSORB LIGHT AT 280 nm

Crude, not necessarily quantitative

Same amount of protein will show different A280 depending on amount of above amino acids

Bradford Assay

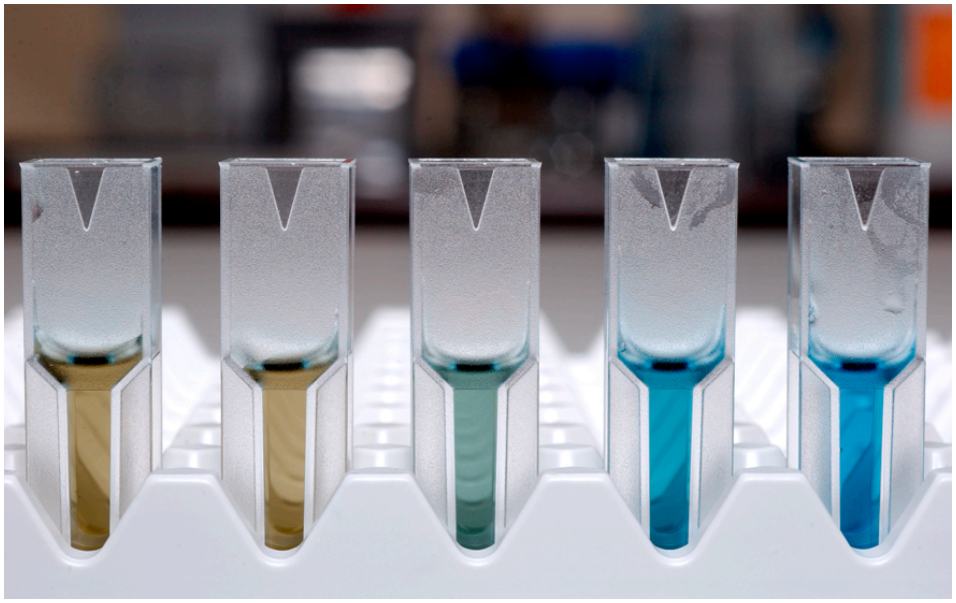
SECOND MOST CITED PAPER IN SCIENCE JOURNALS

Bradford, M. M. (1976)

A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding.

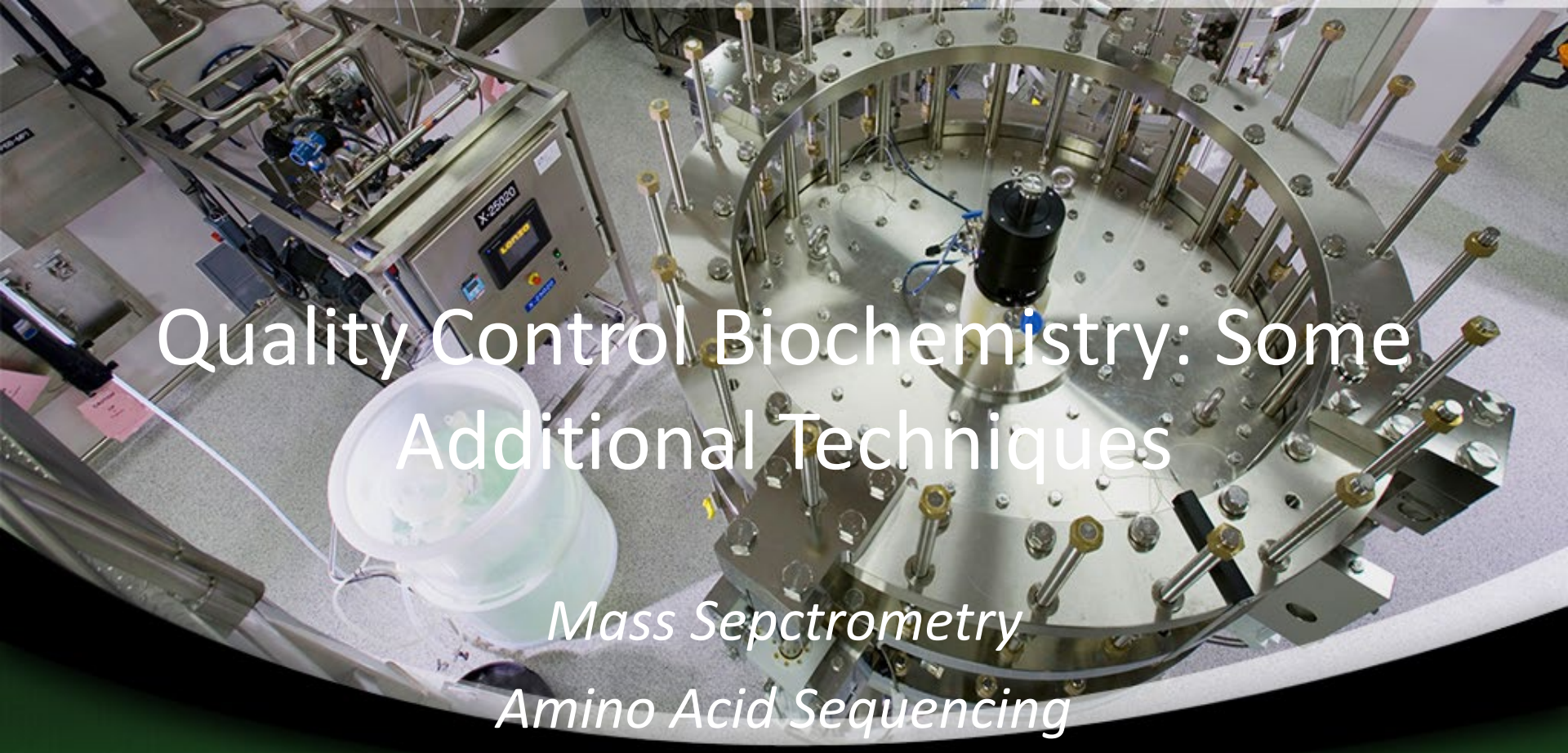
Anal. Biochem. 72:248-254.

Coomassie Brilliant Blue G Dye



Protein Characterization Methods

CHARACTERISTIC	METHOD
PURITY	SDS-PAGE, 2D ELECTROPHORESIS, IEF, HPLC, MS, CAPILLARY ELECTROPHORESIS
MOLECULAR WEIGHT	SDS-PAGE, GEL FILTRATION CHROMATOGRAPHY, MS, ANALYTICAL ULTRACENTRIFUGATION
FUNCTION	MUCH VARIETY
PRIMARY STRUCTURE	AA COMPOSITION, PEPTIDE MAPPING, N-TERMINAL SEQUENCING, COMPLETE AMINO ACID SEQUENCING
SECONDARY, TERTIARY, QUARTENARY STRUCTURE	X-RAY CRYSTALLOGRAPHY, NMR, ANALYTICAL ULTRACENTRIFUGATION, FLUORESCENCE SPECTROSCOPY
POST-TRANSLATIONAL MODIFICATION	DEPENDS ON TYPE OF MODIFICATION



Quality Control Biochemistry: Some Additional Techniques

Mass Sepctrometry

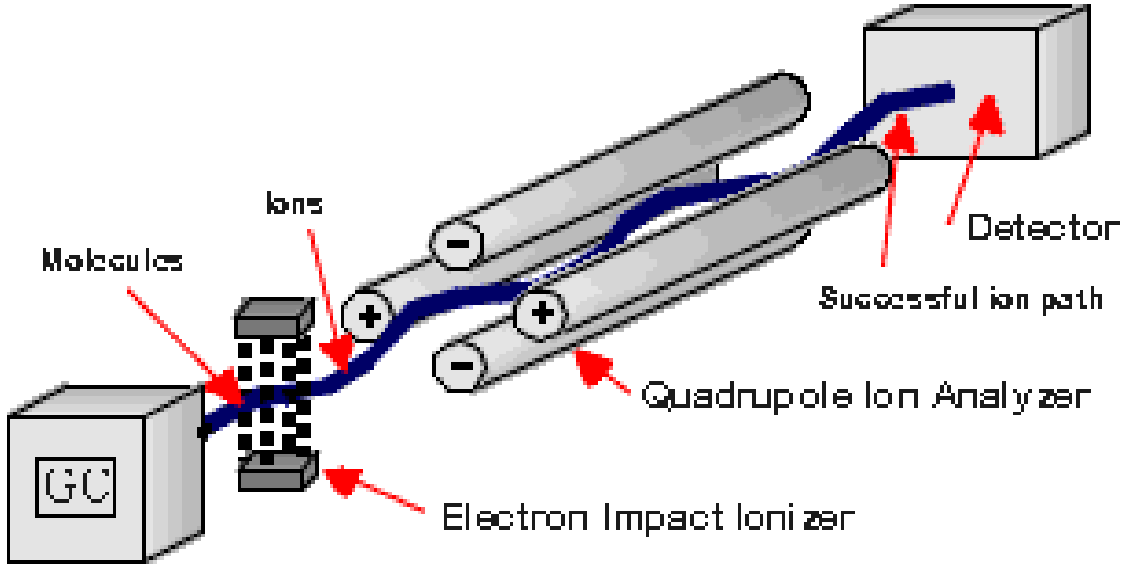
Amino Acid Sequencing

X-ray Crystallography

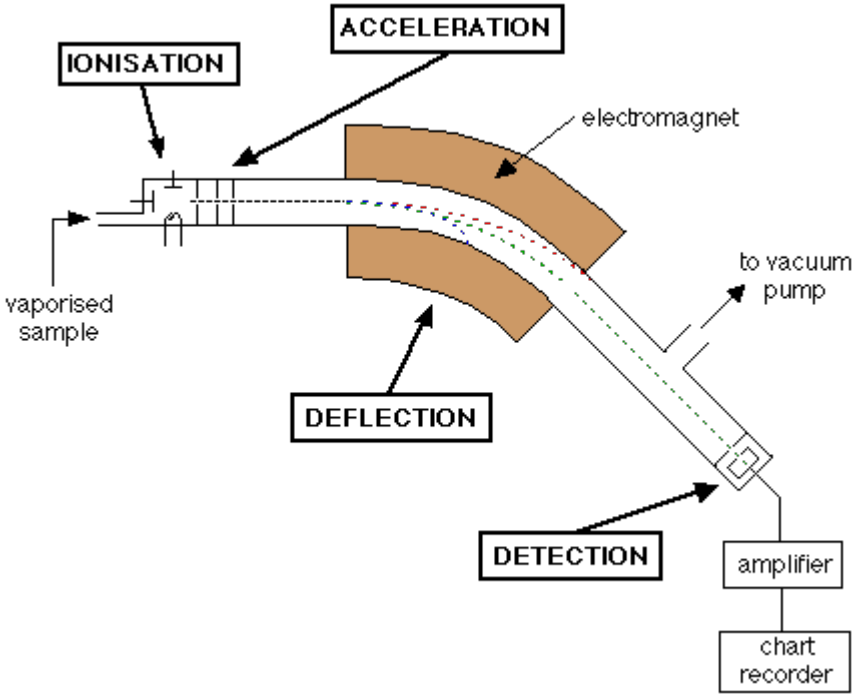
Nuclear Magnetic Resonance

Mass Spectrometry

- Molecular Weight determination
- More accurate than SDS PAGE
- Less protein needed for analysis than SDS PAGE

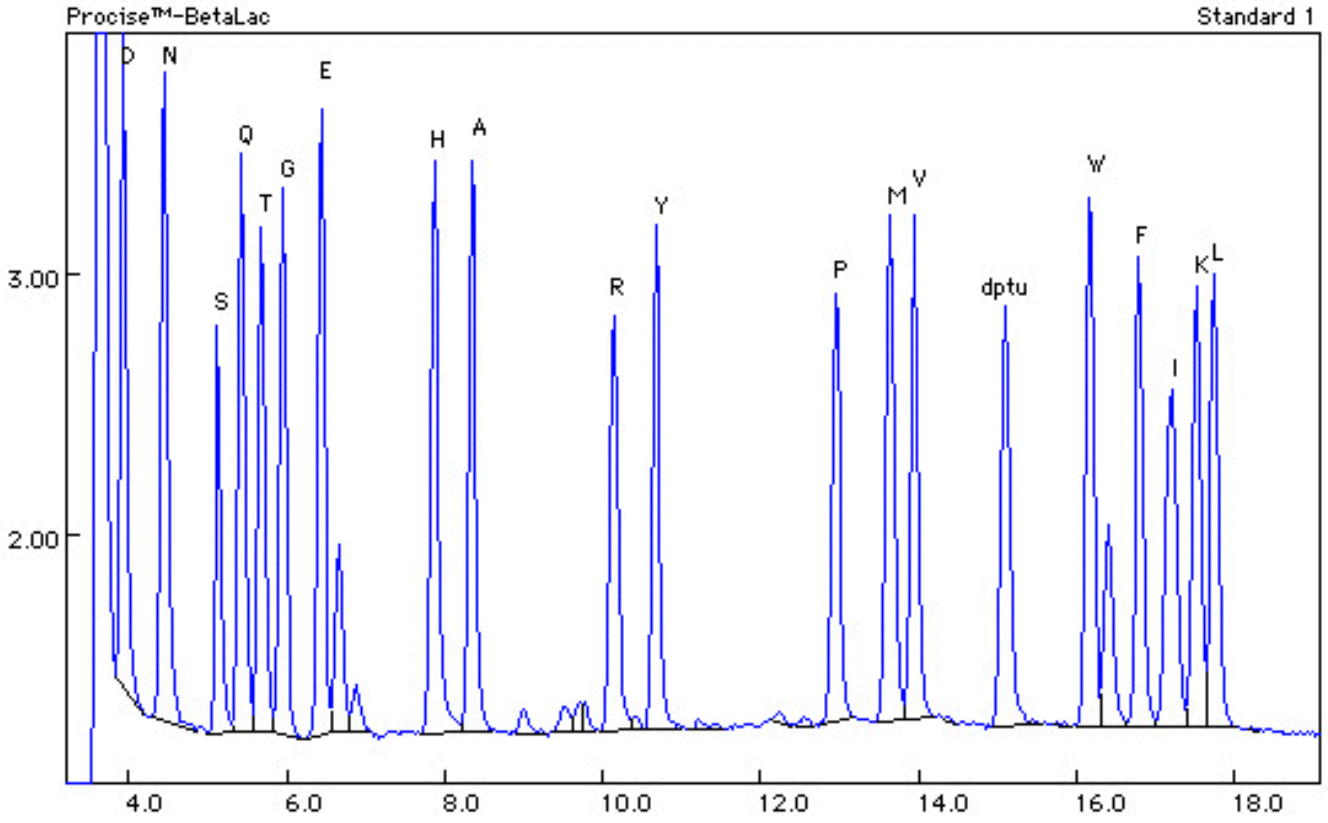


Mass Spectrometry



Amino Acid Sequencing

- Edman Degradation



Amino Acid Composition

- Treat with HCl (hydrolysis)
- Separate individual aa by ion exchange chromatography
- Analyse with HPLC

3D Structure Determination

- X Ray Crystallography
  - need to crystallize (Difficult)
- NMR
  - small proteins 25kD

X-ray Crystallography

X ray diffraction

- beam of x rays directed at protein (incident)
- beam is diffracted by electrons of atoms in protein (scattered)
- these beams hit a film detector
- computer analysis to create electron density map

Nuclear Magnetic Resonance

- uses radio frequency pulses
- energy is absorbed such that electrons move from ground to excited states
- atomic nuclei spin - create their own magnetic field
- emit radiation - differs based on atoms or chemical groups