Quality Control Biotechnology
<table>
<thead>
<tr>
<th>Component</th>
<th>Culture Harvest Level</th>
<th>Final Product Level</th>
<th>Conventional Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic Antibody</td>
<td>0.1-1.5 g/l</td>
<td>1-10 g/l</td>
<td>UF/Cromatography</td>
</tr>
<tr>
<td>Isoforms</td>
<td>Various</td>
<td>Monomer</td>
<td>Chromatography</td>
</tr>
<tr>
<td>Serum and host proteins</td>
<td>0.1-3.0 g/l</td>
<td>&lt;0.1-10 mg/l</td>
<td>Chromatography</td>
</tr>
<tr>
<td>Cell debris and colloids</td>
<td>10⁶/ml</td>
<td>None</td>
<td>MF</td>
</tr>
<tr>
<td>Bacterial pathogens</td>
<td>Various</td>
<td>&lt;10⁻⁶/dose</td>
<td>MF</td>
</tr>
<tr>
<td>Virus pathogens</td>
<td>Various</td>
<td>&lt;10⁻⁶/dose (12 LRV)</td>
<td>virus filtration</td>
</tr>
<tr>
<td>DNA</td>
<td>1 mg/l</td>
<td>10 ng/dose</td>
<td>Chromatography</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>Various</td>
<td>&lt;0.25 EU/ml</td>
<td>Chromatography</td>
</tr>
<tr>
<td>Lipids, surfactants</td>
<td>0-1 g/l</td>
<td>&lt;0.1-10 mg/l</td>
<td>Chromatography</td>
</tr>
<tr>
<td>Buffer</td>
<td>Growth media</td>
<td>Stability media</td>
<td>UF</td>
</tr>
<tr>
<td>Extractables/leachables</td>
<td>Various</td>
<td>&lt;0.1-10 mg/l</td>
<td>UF/ Chromatography</td>
</tr>
<tr>
<td>Purification reagents</td>
<td>Various</td>
<td>&lt;0.1-10 mg/l</td>
<td>UF</td>
</tr>
</tbody>
</table>
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).
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

<table>
<thead>
<tr>
<th>Concentration ng/ml</th>
<th>OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.071</td>
</tr>
<tr>
<td>6.25</td>
<td>0.169</td>
</tr>
<tr>
<td>25</td>
<td>0.426</td>
</tr>
<tr>
<td>100</td>
<td>0.951</td>
</tr>
<tr>
<td>400</td>
<td>1.156</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1.320</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.290</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1.290</td>
</tr>
</tbody>
</table>

HSA Standard Curve

\[ y = 0.0024x + 0.2992 \]
\[ R^2 = 0.7191 \]
ELISA Equipment

Multi-Channel Pipettor

Microtitre Plate Reader
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

Colored product

TMB
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)
- Chemiluminescent Tag
The animation may be found at:
usmlemd.wordpress.com/2007/06/12/elisa-test/
SDS-PAGE Gel Box

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
separate proteins based on molecular weight

Isoelectric Focusing
identify the pH at which a protein carries no net charge
Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis developed by Laemmli (1970)
• characterize (MW)

• quantify (densitometry)

• determine other proteins in a sample

• step in Western blot (used to identify)
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)

GenScript One-Step™ Western Blot Detection

1 hr

Pretreatment
Primary Antibody
One-Step Western Kit
Development

Classical Western Blot Detection

Blocking
Primary Antibody
Secondary Antibody
Development

4.5 hrs
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

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

Coomassie Brilliant Blue G Dye
## Protein Characterization Methods

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PURITY</td>
<td>SDS-PAGE, 2D ELECTROPHORESIS, IEF, HPLC, MS, CAPILLARY ELECTROPHORESIS</td>
</tr>
<tr>
<td>MOLECULAR WEIGHT</td>
<td>SDS-PAGE, GEL FILTRATION CHROMATOGRAPHY, MS, ANALYTICAL ULTRACENTRIFUGATION</td>
</tr>
<tr>
<td>FUNCTION</td>
<td>MUCH VARIETY</td>
</tr>
<tr>
<td>PRIMARY STRUCTURE</td>
<td>AA COMPOSITION, PEPTIDE MAPPING, N-TERMINAL SEQUENCING, COMPLETE AMINO ACID SEQUENCING</td>
</tr>
<tr>
<td>SECONDARY, TERTIARY, QUARTERNARY STRUCTURE</td>
<td>X-RAY CRYSTALLOGRAPHY, NMR, ANALYTICAL ULTRACENTRIFUGATION, FLUORESCENCE SPECTROSCOPY</td>
</tr>
<tr>
<td>POST-TRANSLATIONAL MODIFICATION</td>
<td>DEPENDS ON TYPE OF MODIFICATION</td>
</tr>
</tbody>
</table>
Quality Control Biochemistry: Some Additional Techniques

- Mass Spectrometry
- 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