nibrt
National Institute for Bioprocessing Research and Training

Bioanalytical Techniques

Lecture 6

Immunoassays and Bioassays

Learning Objectives

1. Understand the differences between Immunoassays and Bioassays and why are they used

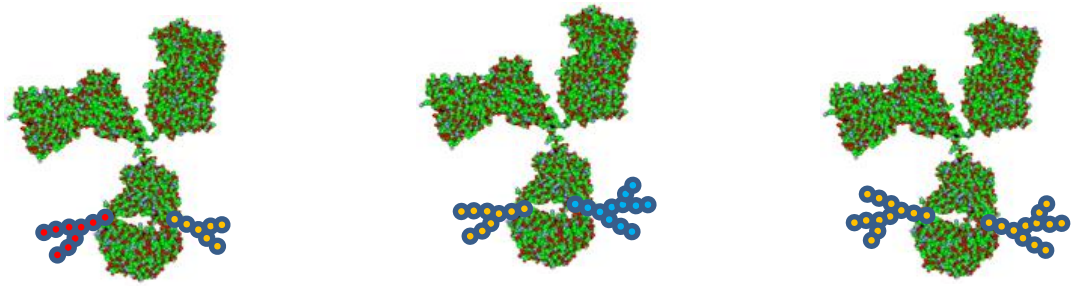
2. Understand the common types of Immunoassays used in the analysis of biologics

3. Discuss the complexity of Bioassays

Topics

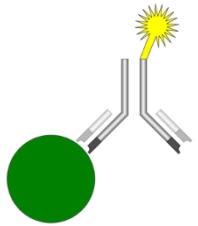
- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

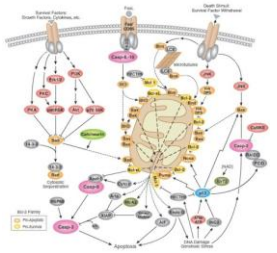
The complexity relates to structure AND function



- Biologics are **complex and heterogeneous** in composition and can exist in a number of physical and chemical conformations that can impact product safety and efficacy (e.g. glycosylation, deamidation)
- **Physicochemical analysis** can inform us about **the structure** of the biopharmaceutical
- We also need information about **the specificity and function**

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

Topics

- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

Introduction to Immunoassays

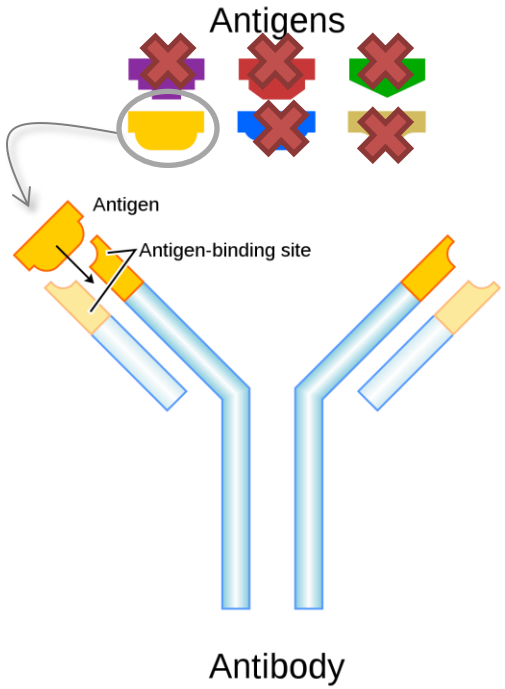
- An immunoassay is a test that uses **antibody** and **antigen** complexes as a means of generating a **measurable result.**

- An **antibody:antigen complex** is also known as an **immuno-complex.**

- Immunoassays utilize one or more **select antibodies** to **detect analytes** of interest

Antibodies

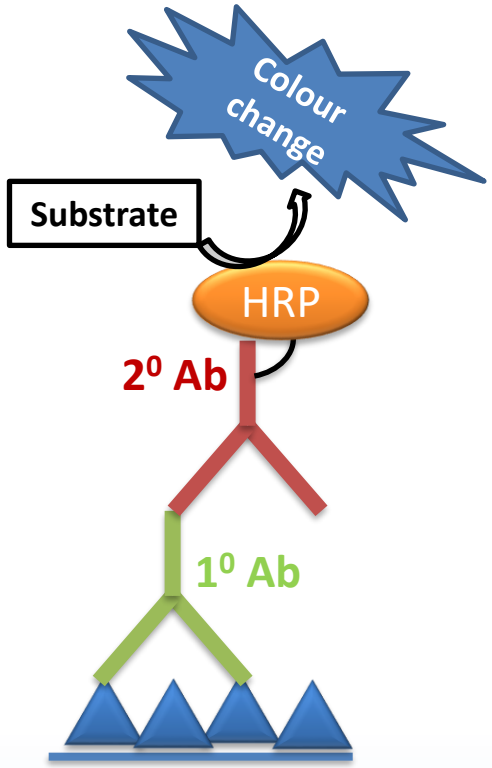
- Antibodies possess high binding
  - specificity
  - affinity



- It is the **specific binding of an antibody to an antigen** that allows the detection of analytes by a variety of immunoassay methods

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

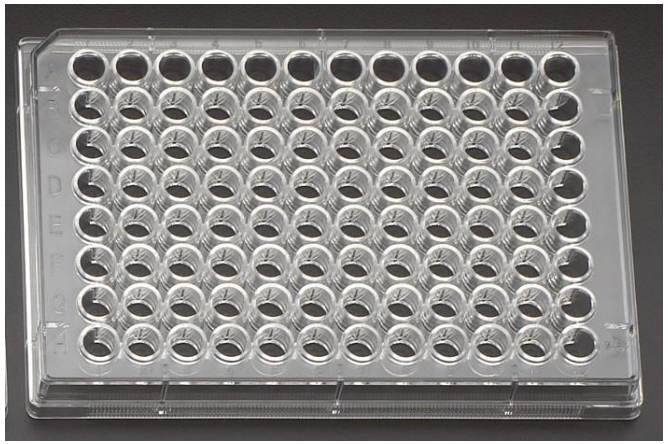
Topics

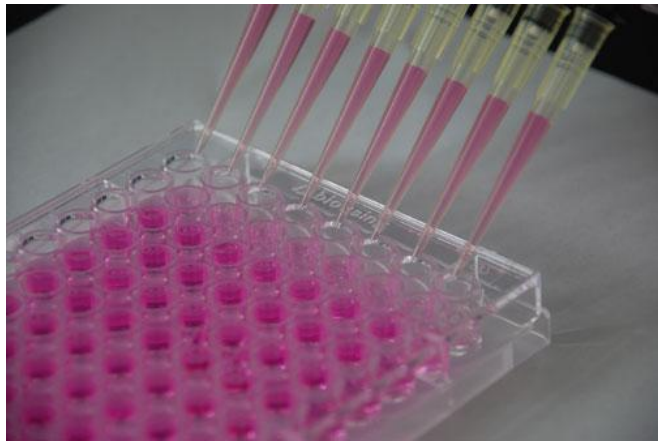
- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

ELISA

Principles

- ELISA uses a 96-well plate in which each well is coated with a **protein** or **antibody** to which its partner will **bind**





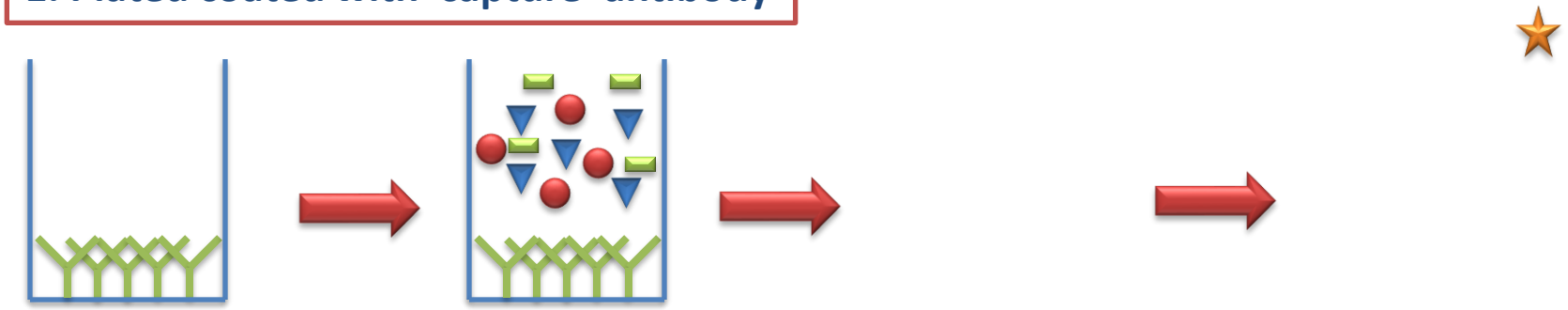
- The protein or antibody of interest can then be detected and, if desired, the amount quantified.

www.topfreebiz.com/product/2230698/96-Well-Cell-Culture-Plate.htm
www.globebio.com/96-well-plate-with-lid-individual-wrap-sterile.html

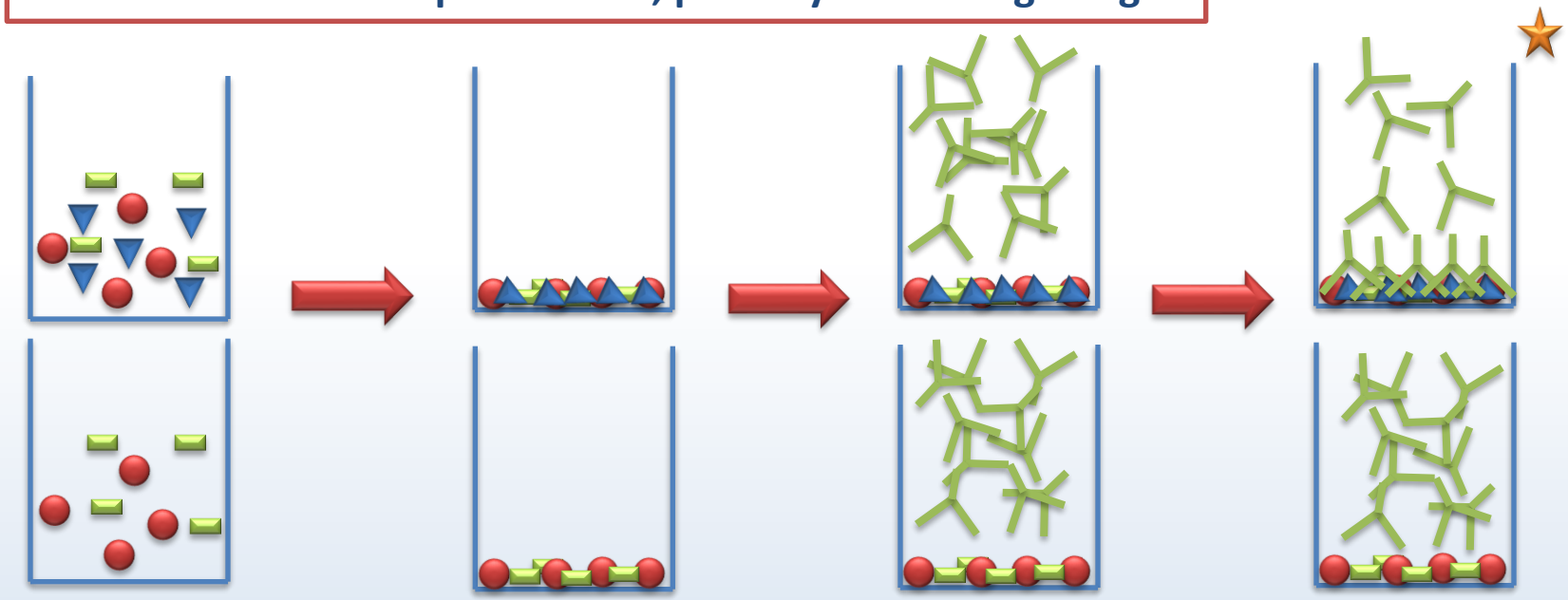
ELISA

Principles

1. Plated coated with ‘capture’ antibody

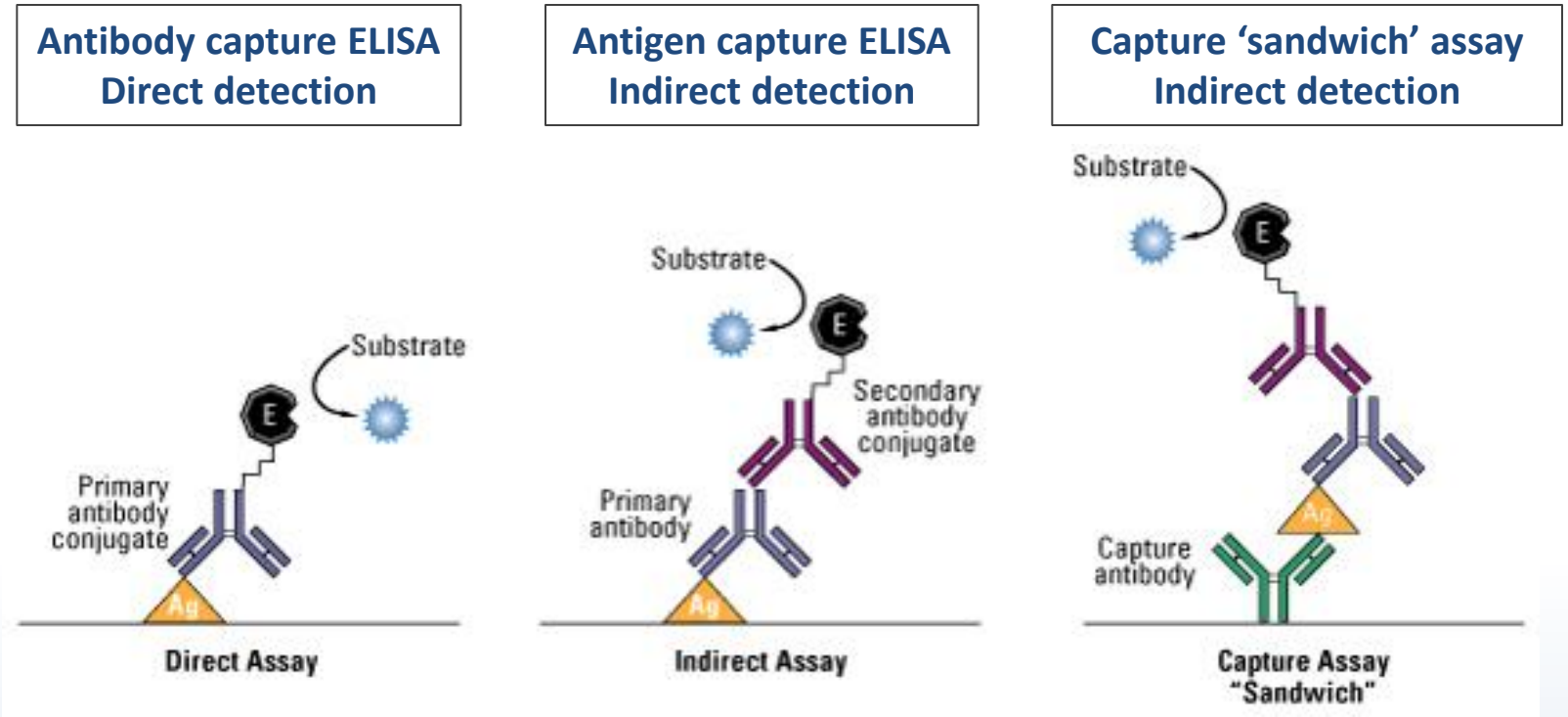


2. Plated coated with protein mix, possibly containing antigen



ELISA
Types of Detection

- Antigen can be immobilised **directly on the assay plate** or via a **capture antibody** that has been attached to the plate



- Antigen is detected either **directly** (labelled primary antibody) or **indirectly** (labelled secondary antibody)

Image from Pierce website

ELISA Controls

- **Unknown sample**
  - Under investigation; does it contain antigen ‘Protein X’?
- **Positive control**
  - Definitely contains Protein X (e.g. purified form of antigen)
  - Colour change = assay can detect antigen
  - No colour change = assay cannot detect antigen and must be repeated
- **Negative control**
  - Definitely does NOT contain Protein X
  - Colour change = antibodies might be cross-reacting and giving non-specific results , therefore results from unknown sample are unreliable
  - No colour change= results from unknown sample are likely to be reliable and not a result of non-specific binding

ELISA
Example

Results:

- Positive control (+) turned blue
- Negative control (-) did not change colour
- Sample A did not change colour, Sample B did change colour

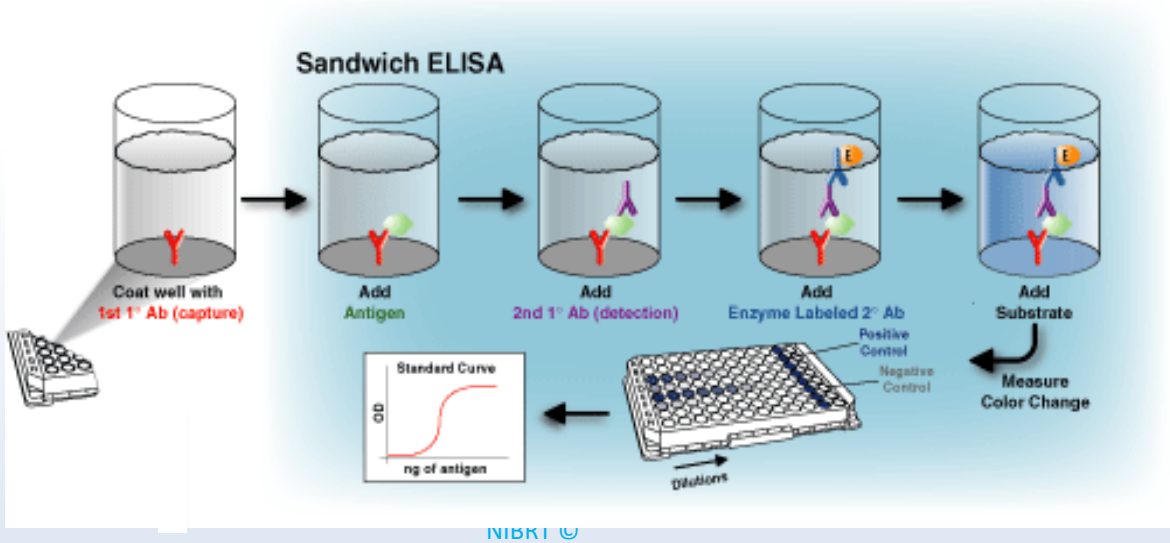
Conclusions:

1. Assay worked correctly (expected results from controls)
2. Antigen was present in Sample B but not in Sample A

Indirect Sandwich ELISA

General Protocol

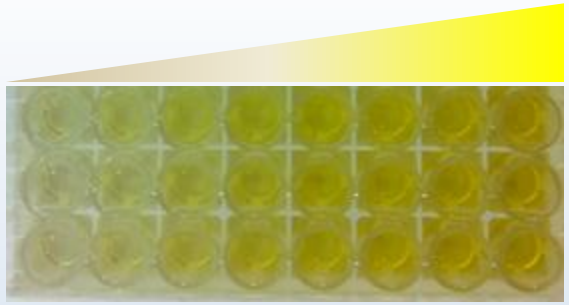
1. Apply 2-20µg/ml sample (50µl) per well, cover and incubate
2. Remove excess sample and rinse x 3 with rinsing buffer
3. Apply blocking agent and incubate
4. Remove excess blocking agent and rinse x 3 with rinsing buffer
5. Apply 1^0 Ab and incubate
6. Remove excess Ab and rinse x 3 with rinsing buffer
7. Apply 2^0 Ab and incubate
8. Remove excess Ab and rinse x 3
9. Apply substrate and observe colour change – **visual** or in **spectrophotometer**

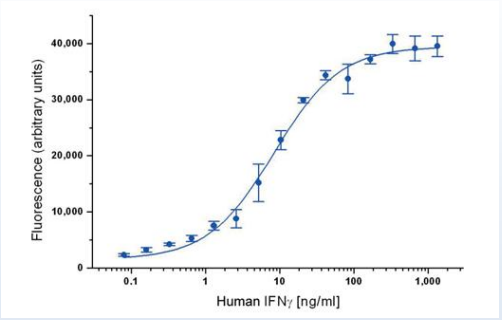


Types of ELISA
Qualitative v Quantitative

- **Qualitative ELISA:**
  - Gives yes or no result
  - Results can be estimated visually without use of spectrophotometer

- **Quantitative ELISA:**
  - Gives concentration of antigen present
  - Standard curve of known concentrations of antigen is required
  - Results can be estimated using spectrophotometer



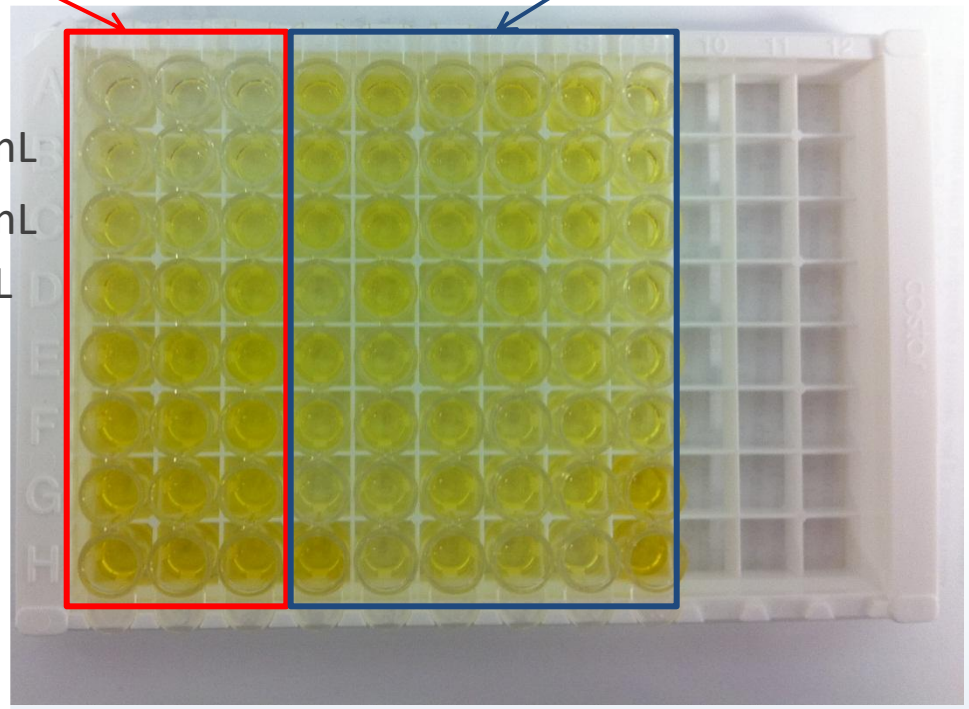


Quantitative ELISA
Example

Known concentrations of α1 acid glycoprotein in triplicate

Samples of unknown concentration of α1 acid glycoprotein

0
0.063µg/mL
0.125µg/mL
0.25µg/mL
0.5µg/mL
1µg/mL
2µg/mL
4µg/mL



ELISA
Advantages

- Relatively fast
- Sensitive (as little as 10 pg/mL detected)
- Specific
- Many samples can be processed at once (96-well plate)
- Small sample size required
- Colorimetric results: easily observed and measured (spectrophotometer)
- Test for presence of antigen or antibody
- Easy to learn, simple procedure
- Easily automated for HTS (high throughput screening)

ELISA
Disadvantages

- Many sources of variability
- Setting up new ELISA may require significant optimisation

Table 1. Factors that affect ELISA signal generation.

Factor	Variable Characteristic
Assay Plate	material, well shape, pre-activation
Coupling Buffer	composition, pH
Capture Antibody	specificity, titer, affinity, incubation time and temperature
Blocking Buffer	composition, concentration, cross-reactivity
Target Antigen	conformation, stability, available epitope(s), matrix effects
Detection Antibody	specificity, titer, affinity, incubation time & temperature, cross-reactivity
Enzyme Conjugate	type of enzyme, type of conjugate, activity, concentration, cross-reactivity
Washes	buffer composition, volume, duration, frequency
Substrate	sensitivity, manufacturer lot, age
Signal Detection	filters, imaging instrument, exposure time

ELISA Applications

- **Product characterisation:**
  - Can help determine binding affinities for product variants
  - Detection of anti-drug antibodies (immunogenicity) during trials
- **In-process testing:**
  - Quantify concentration of biopharmaceutical in a sample from bioreactor (which contains all other host cell proteins (HCPs) and media components)
- **Release testing:**
  - HCPs
  - Residual Protein A (for MAbs only)

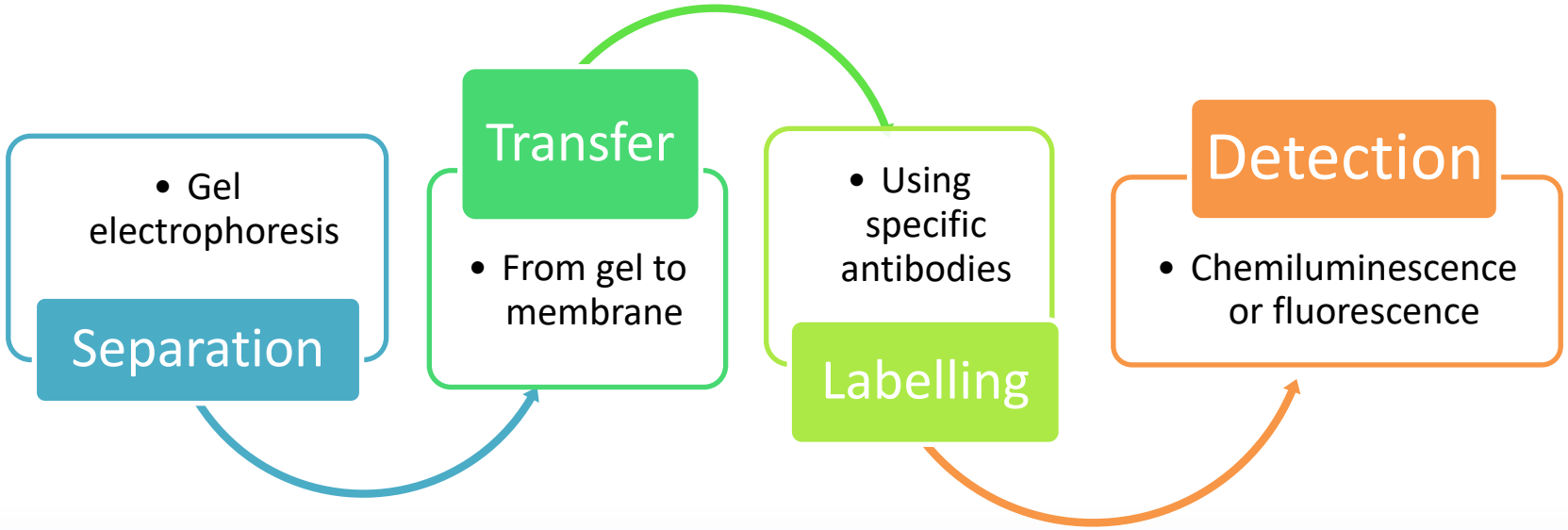
Topics

- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

Western Blotting Introduction

- Transfer of proteins from **gel** to **membrane** and detection of **specific proteins using antibodies**
- Introduced by Towbin *et al.* (1979) and is now a routine technique for protein analysis
- Analytical tool to provide information on **identity** and **sample integrity** at **lot release**, **stability testing** and product **characterisation** stages

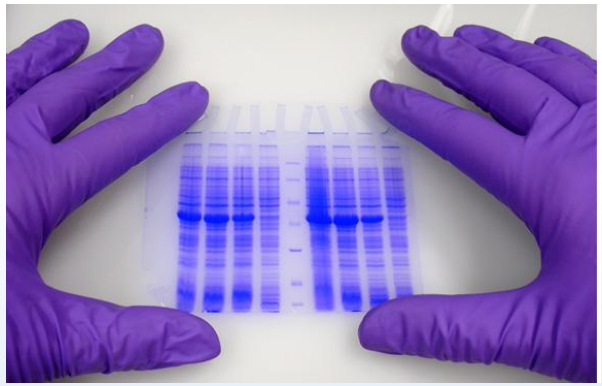
Western Blotting workflow

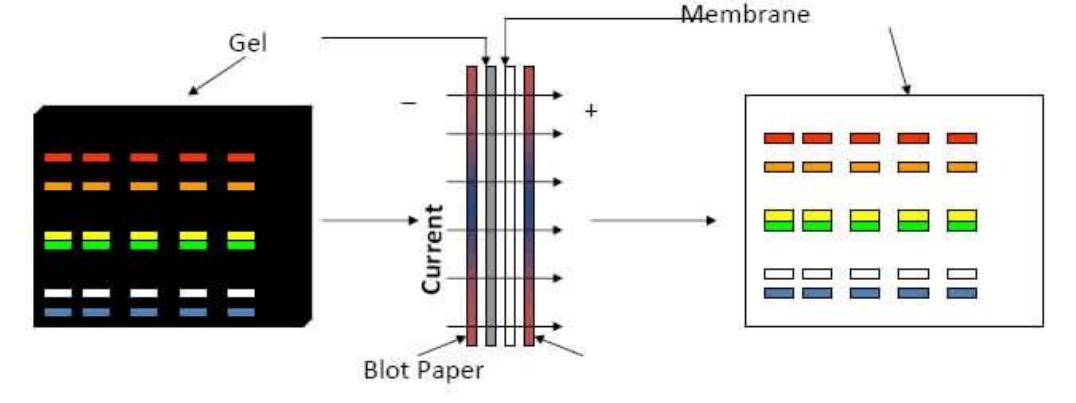


Separation and Transfer

- After the gel electrophoresis (e.g. SDS-PAGE) proteins are **transferred from the gel onto a solid membrane support** (electroblotting)

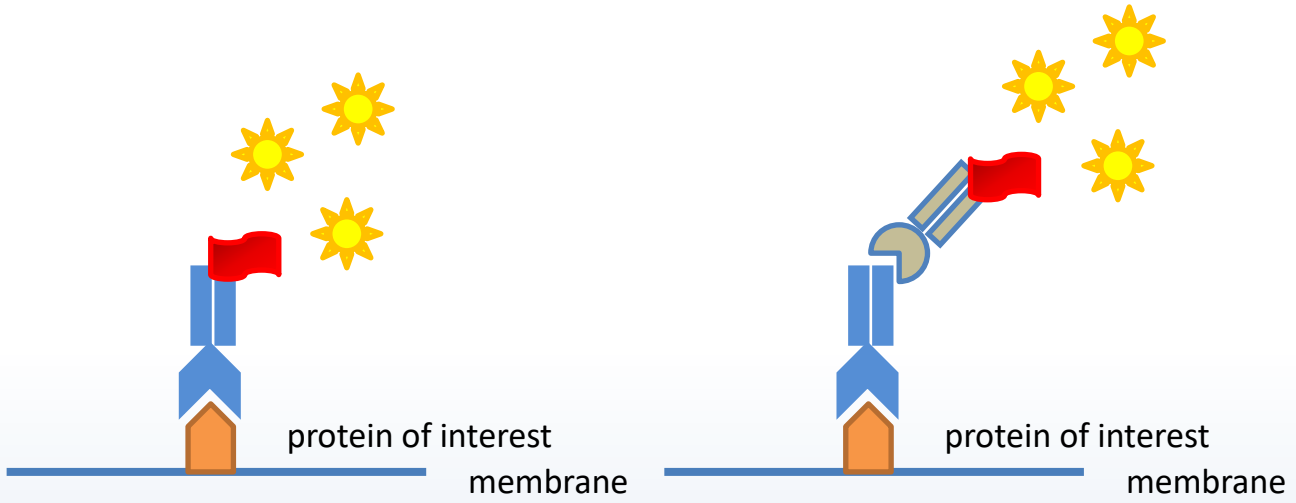
- The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel





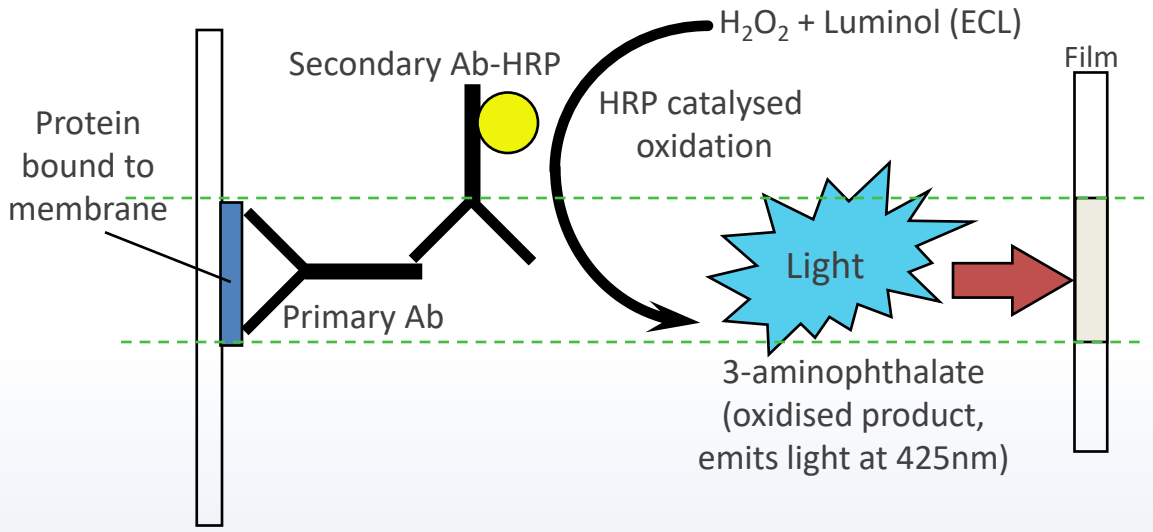
Labelling

- **Direct** – uses a labelled primary antibody to identify the target protein
- **Indirect** – uses an unlabelled primary followed by labelled secondary antibodies



Enhanced Chemiluminescent Detection

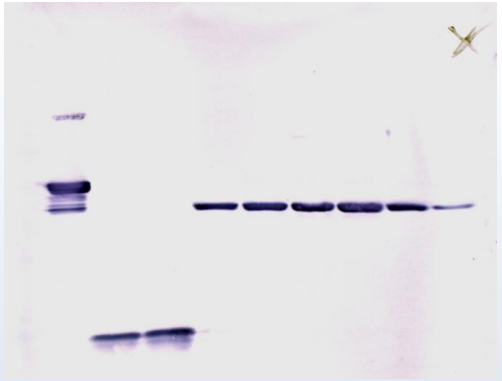
- Most sensitive detection method
- Light is produced when substrate meets enzyme
- Transient light signal that can be captured using film or a special camera
- The intensity of the signal correlates with abundance of the antigen on membrane



Applications of Western Blot

- **Release testing:**
  - Provides information on identity and sample integrity/purity at lot release

- **Characterisation and Stability testing:**
  - Used to detect different isoforms (glycosylated, phosphorylated) and breakdown products



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

Topics

- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

Bioassays

- A *bioassay* is defined as an analytical procedure measuring a biological activity of a test substance based on a **specific, functional, biological response** of a test system.

  (WHO/NIBSC, J. Immunol. Methods (1998), 216, 103-116. International consensus, Dev. Biol. Standard. (1999) vol 97)

- A quantitative measurement of the biological response is referred to as **potency**

- Biological activity is a critical quality attribute, therefore potency is an essential component of quality control

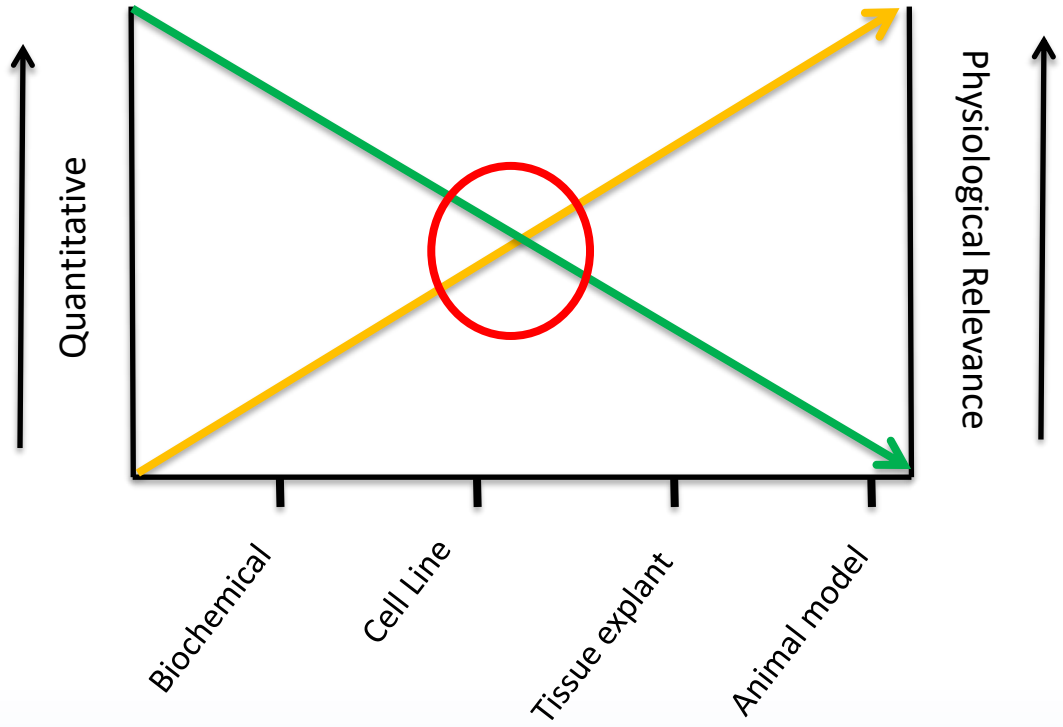
http://www.mbswonline.com/upload/presentation_5-24-2012-9-17-24.ppt

Types of Potency Assays

- **Cell-based bioassays**, which measure biochemical or physiological response at the cellular level;
- **Biochemical assays;** measure enzyme reaction rates or biological responses induced by immunological interactions.
- **Ligand and receptor binding assays**
- **Animal-based bioassays,** which measure an organism‘s biological response to the product

http://www.ich.org/fileadmin/Public_Web_Site/Training/ASEAN_Q5C_workshop_May_2011/SESSION_III_ICHQ6B_Specifications.pdf

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

Bioassay development
Mechanism of Action (MOA)

- Design of a relevant bioassay begins with understanding **how a drug affects the target**
  - Single MOA or complex MOA?
  - What are the **critical biological pathways** and can they be exploited to develop a bioassay?
- Choice of cell type/tissue/animal relevant to disease
- Cellular response, or measured parameter (reporter gene, proliferation, cell viability, etc.) relevant to mechanism of action of drug and disease

Bioassay development
Cell Selection

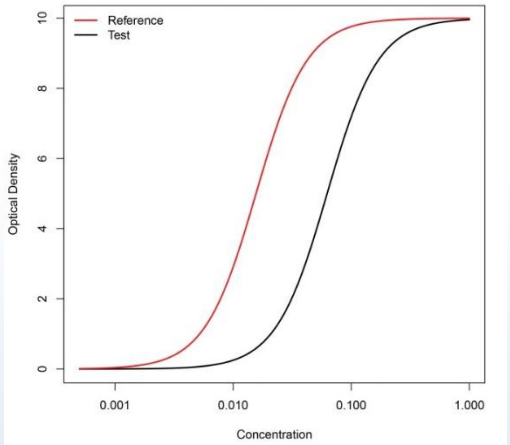
The biggest issue with bioassays is that they require the use of cells, either cultured or primary cells derived from blood.

Issues associated with the use of cells as analytical reagents:

1. Sterility requirement for culture, risk of microbial contamination, suitability for QC testing??
2. Training, staffing and technical expertise requirements.
3. Analytical performance, may be influenced by passage number, confluent state, cell age, position in cell cycle.
4. Availability and reproducibility of primary cells if required, time limitation following isolation.
5. Handling, especially of concern for primary cells.
6. Cell line integrity – issues with cell line contamination; mycoplasma and HeLa contamination.

Bioassays
Data analysis

- By measuring the potency, we can infer the ‘structural integrity’ of the biopharmaceutical
  - A product that has lost structural integrity will lose potency
- The results of biological assays are expressed in units of activity calibrated against an international or national reference standard or a characterised in-house reference material



For biologics we are estimating the potency of our test sample relative to that of standard (reference) = Relative Potency

Cell Based Bioassays (mAB examples)

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

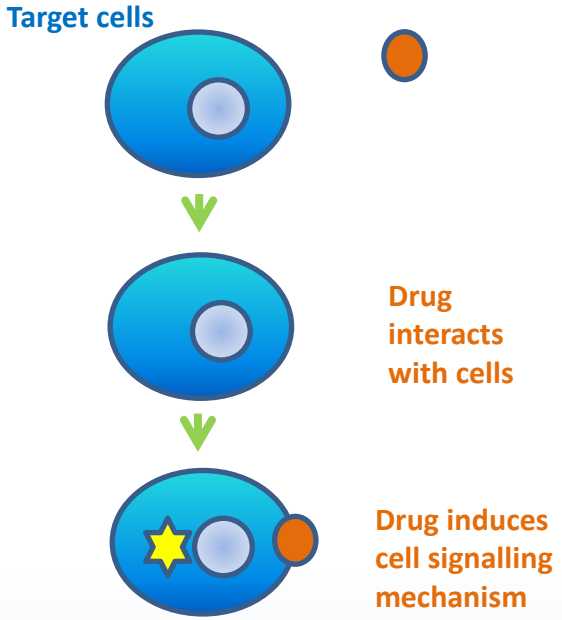
Each Bioassay is unique as it depends on the drug and its MOA

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

Cell Signalling Assay

- Biopharmaceuticals can **activate a signalling cascade** within a cell which results in a specific therapeutic effect.

- The **proteins** in the signalling cascade can then be **measured**, as active or inactive, via a number of techniques



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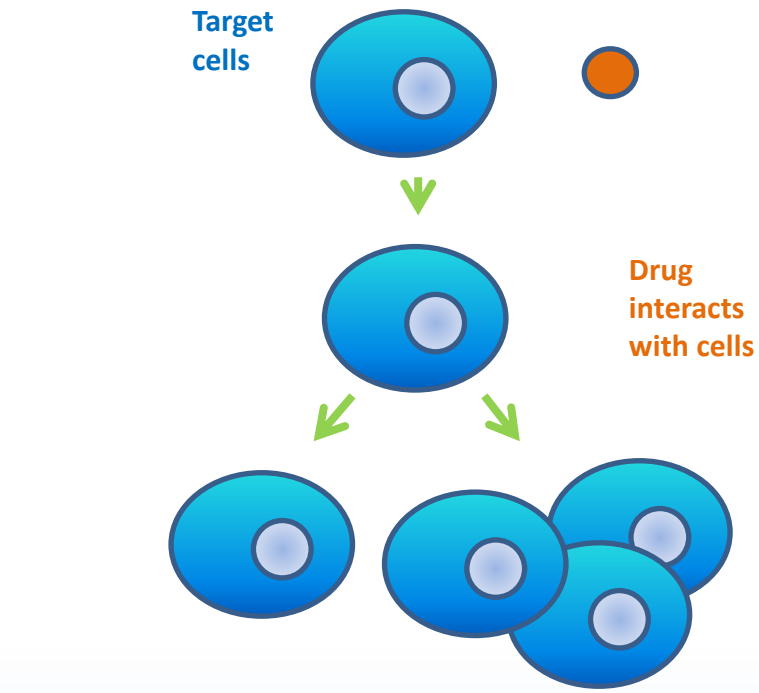
Proliferation Assay

- Some drugs are designed to **inhibit or promote the target cell's ability to proliferate**.

- The final readout for this assay is a cell count which can be measured using spectroscopic techniques (e.g. fluorescence, luminescence or colorimetric assays)



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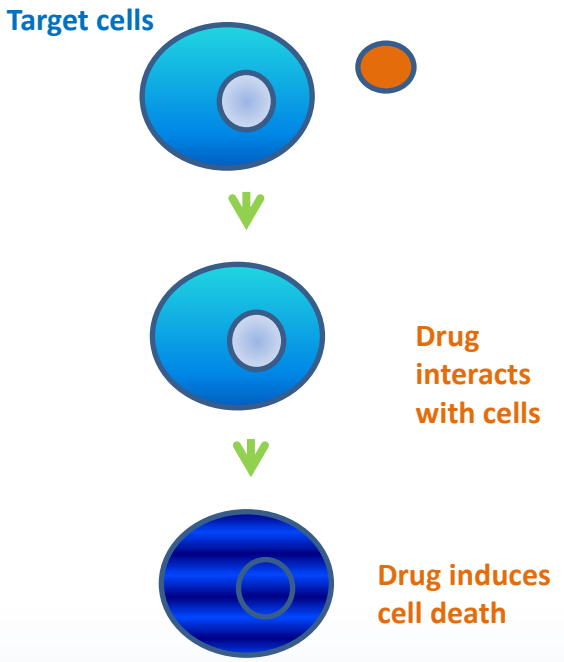
Cytotoxicity Assay

- Some **drugs can induce cell death**
- Live and dead cells can be measured using **differential uptake of dyes**
- Dead or dying cells have reduced membrane integrity and can take up dyes such as trypan blue or propidium iodide
- Also, the rate of cell death can be determined by measuring the **release of lactate dehydrogenase** (LDH) from the dead cells



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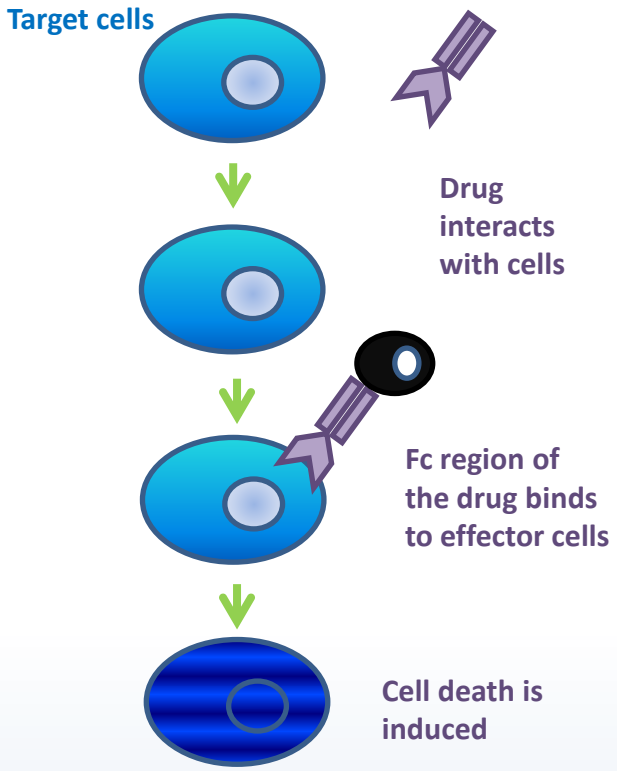
Antibody-dependent Cell-mediated Cytotoxicity (ADCC)

- **ADCC is an immune defense mechanism** whereby a particular set of immune cells (effector cells) actively engage and lyse a target cell that has been “marked for destruction” by an antibody

- Target cells are incubated with the therapeutic antibody and then co-incubated with the effector cell.

- Target cell lysis can be subsequently measured by counting or spectrophotometry.



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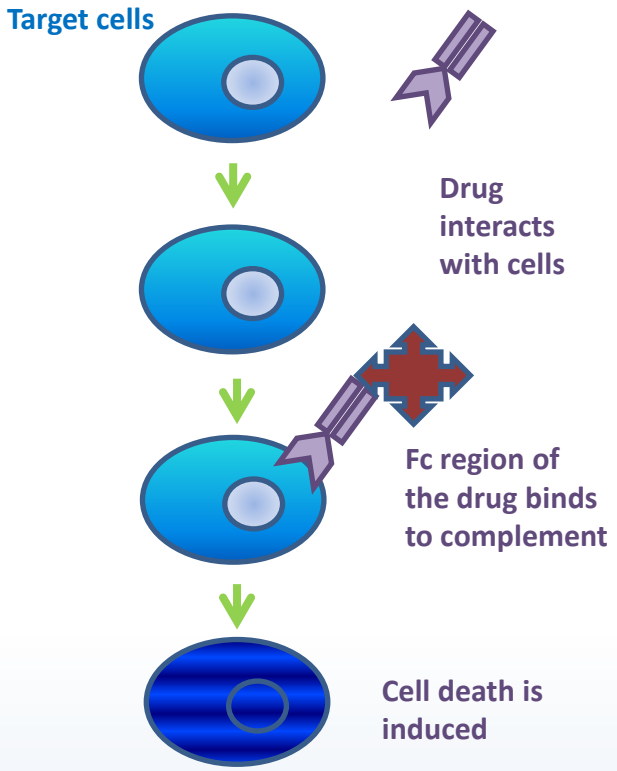
Complement Dependent Cytotoxicity (CDC)

- **The complement system** is part of the innate immune system, whereby a series of inactive **small circulatory blood proteins** become activated by antigen/antibody binding.
- The ultimate result of this system is the activation of the **membrane attack complex** (MAC) on the surface of the target cell which **induces the cell death**
- Similar to ADCC assay - the dead cells are usually measured as an endpoint.



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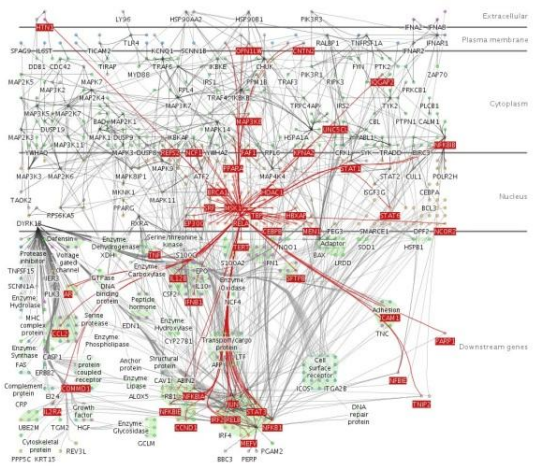
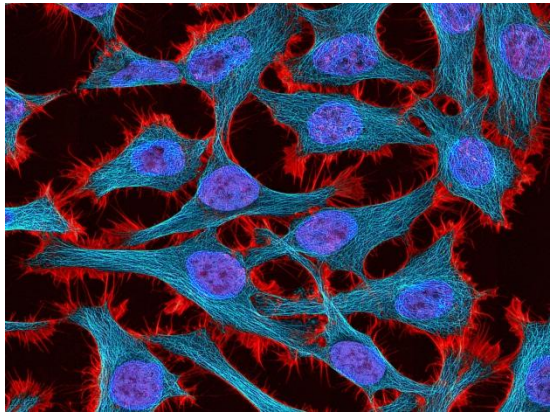
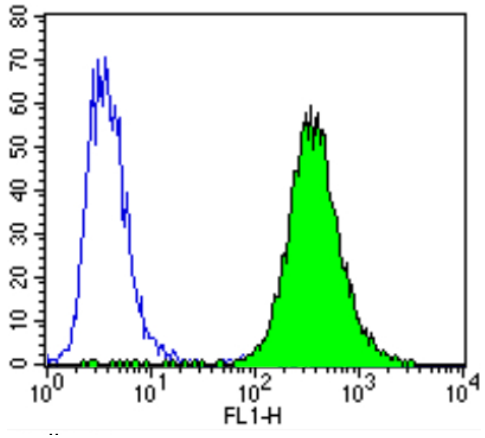
The Problem with Bioassays



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.

Topics

- Introduction to Immunoassays and Bioassays
- Immunoassays
- ELISA
- Western Blotting
- Bioassays

Thank You