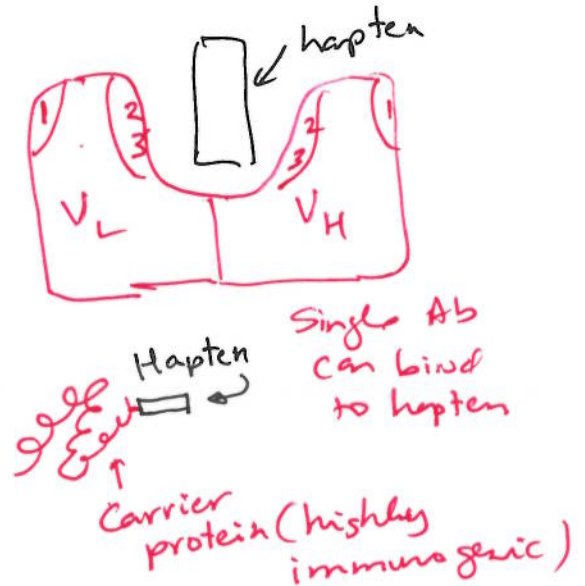


### Lecture 14. Antibodies & Immunoassays I

#### Antibody-Antigen Interactions:

##### A. Antibody against Haptens: (e.g. cocaine, PCP)

- Antibodies generated by attaching hapten to a *carrier protein* to make it immunogenic.
- Resultant antibodies can recognize hapten in the absence of the peptide with useful specificity and affinity.
- Interaction between antibody and immunoglobulin generally involves a deep binding pocket, utilizing 4-6 of the hypervariable loops, giving modest binding affinity.

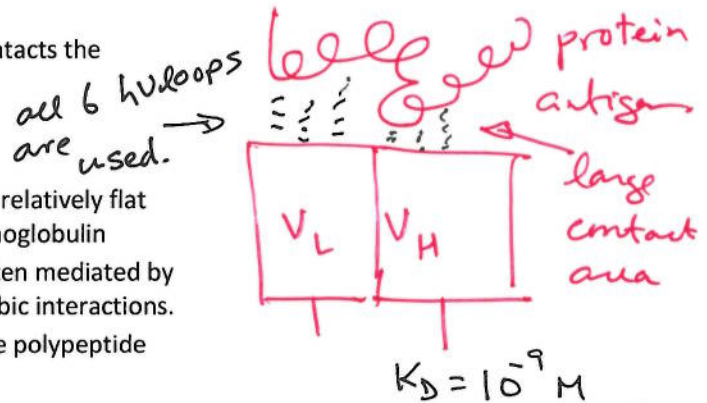


##### B. Antibody against Peptides:

- Homopolymers of amino acids are not immunogenic
- Lys, Glu, Tyr 10-20 kDa is immunogenic
- Lys, Gly, Tyr, Phe: 4 kDa is immunogenic.

##### C. Antibody against Proteins- Antibodies generally of very high affinity (nM dissociation constants).

- **B- cell epitope:** Region on the antigen that contacts the antibody.
- Interactions between antibody and antigen:
  1. Utilizes all 6 hypervariable regions.
  2. Involves extensive surface contacts between relatively flat surfaces on both the antibody and the immunoglobulin
  3. Mediated by ion-pairing, hydrogen bonds (often mediated by water), van der Waals interactions, hydrophobic interactions.
  4. Usually involve discontinuous segments of the polypeptide chain.



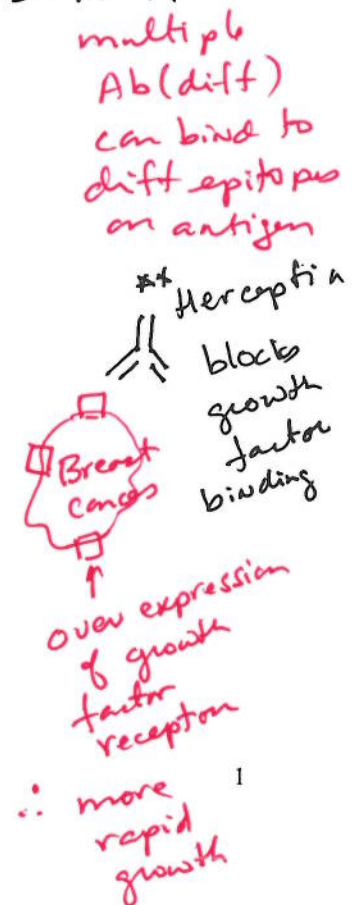
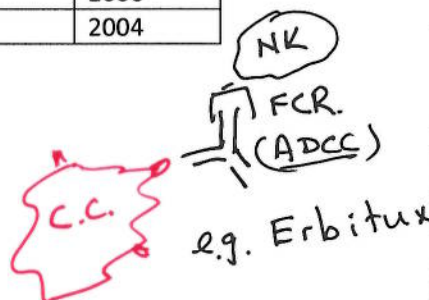
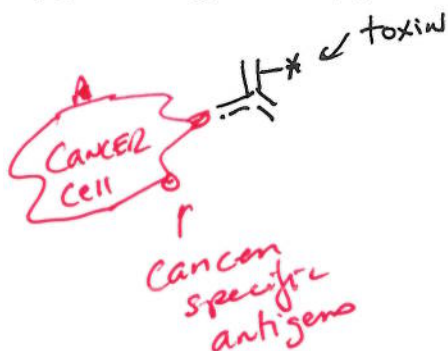
#### General Uses of Antibodies:

- Protein purification
- Tumor detection and imaging
- Diagnostic reagents.
- Catalytic antibodies
- Drug Detoxification
- Tumor killing

#### Antibodies in Clinical Use:

Trade Name	Used to Treat:	Approved in:
Rituxan	Non-Hodgkin lymphoma	1997
Herceptin*	Breast cancer	1998
Mylotarg*	Acute myelogenous leukemia (AML)	2000
Zevalin*	Non-Hodgkin lymphoma	2002
Bexxar*	Non-Hodgkin lymphoma	2003
Erbitux	Colorectal cancer Head & neck cancers	2004 2006
Avastin	Colorectal cancer	2004

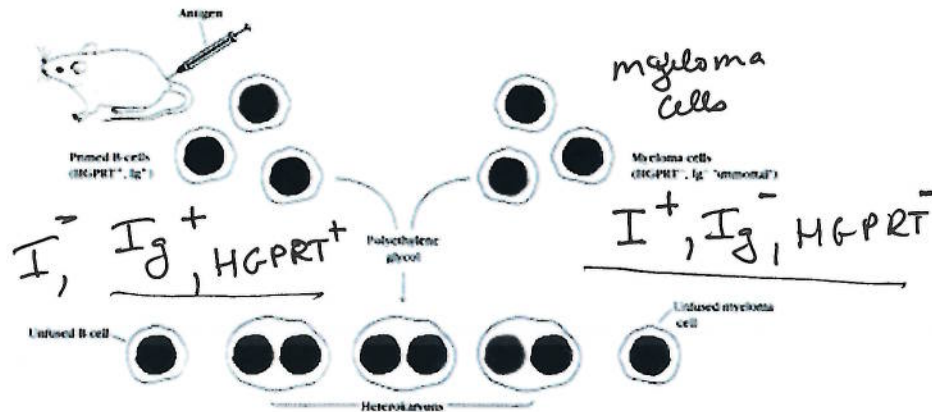
\*Conjugated to drug, radioisotope, or toxin.



**Generation of Monoclonal Antibodies**

Animals (e.g. rabbits) are not the best source of antibodies for several reasons; here are some of the scientific ones:

- It is difficult to obtain large quantities of antibodies.
- The antibodies will be **polyclonal**, they are a heterogeneous pool of antibodies that will recognize many different epitopes on the same antigen or many different antigens on the same pathogen.



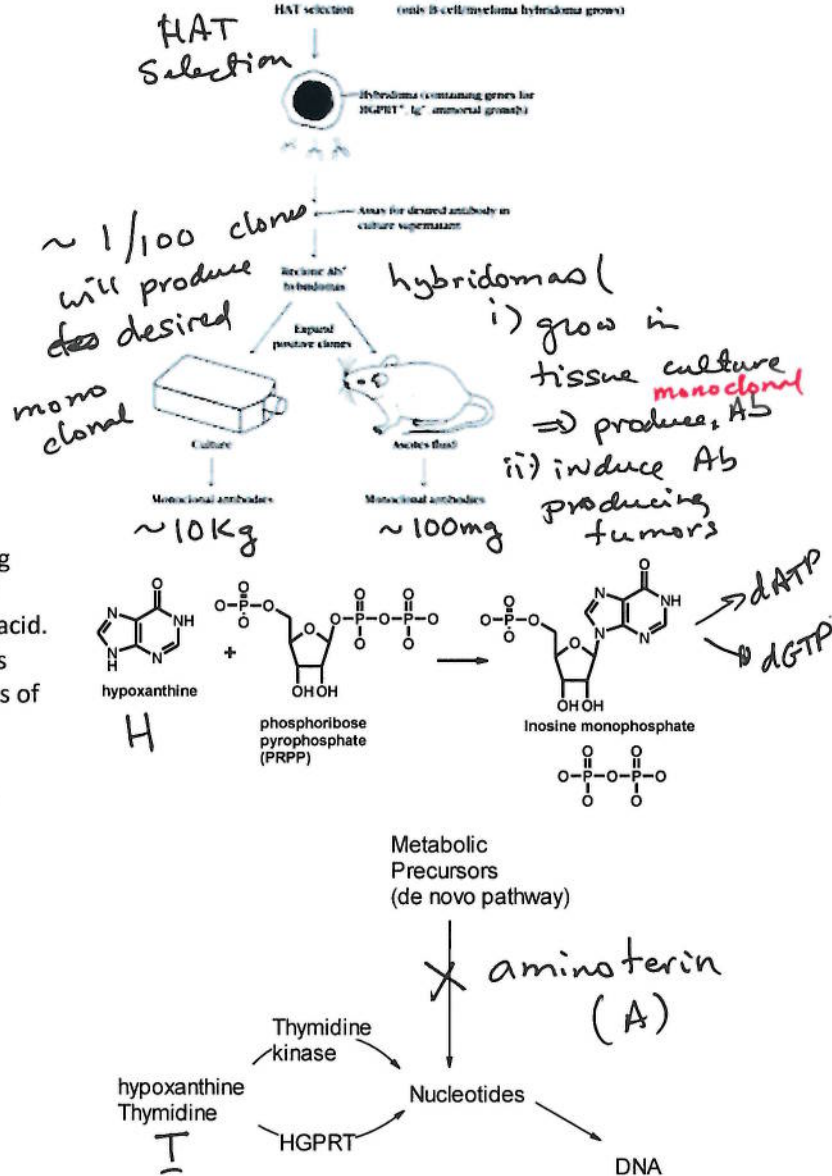
**Steps for generation of Hybridomas:**

1. Immunization of mouse.
2. Isolation of primed B-cells (spleen)
3. Fusion with myeloma cells.
- 4a. HAT selection
- 4b. Resolution of heterokaryons.
5. Assay for production of antibody
- 6a. Growth in tissue culture (kg)
- 6b. Growth in mice (gm)

Selection for hybridomas (HAT Media):

- Nucleotides are produced by two pathways in mammalian cells:
  - i) a de novo synthesis pathway
  - ii) a salvage pathway.
- The de novo pathway can be inhibited using **aminopterin**, which inhibits the transfer of methyl groups from activated dihydrofolic acid.
- If the de novo pathway is blocked then cells need Hypoxanthine and Thymine as sources of purines and pyrimidines for the salvage pathway.
- Incorporation of hypoxanthine require the presence of hypoxanthine-guanine phosphoribosyl transferase (HGPRT).
- Myeloma cells are HGPRT<sup>-</sup> and cannot utilize hypoxanthine in the salvage pathway.
- Plasma cells are HGPRT<sup>+</sup> and can utilize hypoxanthine in the salvage pathway.

Therefore, growth of the cells on HAT media (hypoxanthine, aminopterin, thymidine) will select for immortal cells that have acquired the HGPRT gene via fusion with a plasma cell.



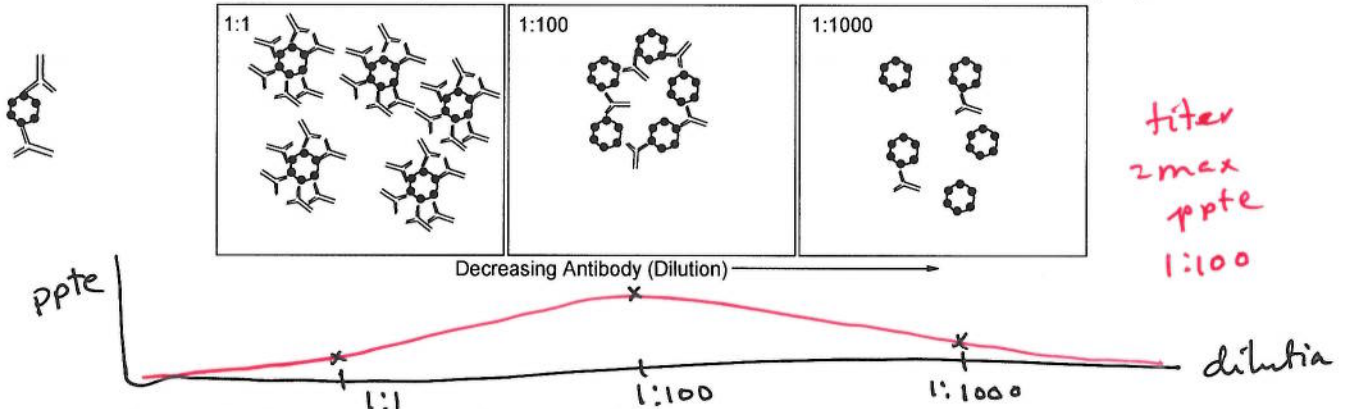


**Immuno-Assays – Detection of antibody or antigen.**

**Precipitation Reactions:** These methods rely on the ability of the antibody to form large crosslinked antibody-antigen complexes that precipitate out of solution. These methods are becoming less frequently used as solid-phase assays become more prevalent. Since crosslinking is required the following two criteria must be met:

- The antibody must have at least two binding sites.
- The antigen must have at least two binding sites.

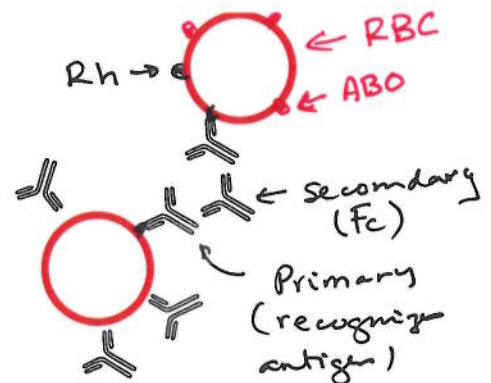
A fixed amount of antigen is mixed with a set of serial dilutions of serum (containing Ab).



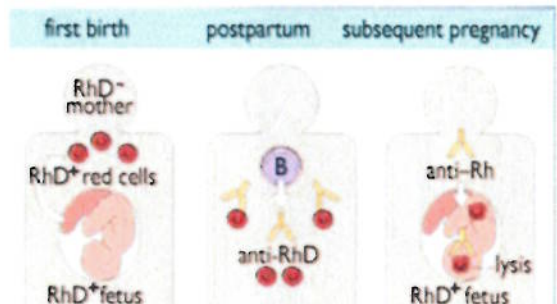
- When antibody is in excess each antigen becomes coated with antibody, preventing the efficient formation of cross-linkages between their antigen particles.
- When antibody and antigen are about 1:1 (equivalence zone), efficient crosslinking and precipitation occurs. The dilution of serum that still gives precipitation is referred to as the **titer** of the serum.
- When antigen is in excess, all antibodies are complexed to individual antigen molecules, so no aggregation or precipitation occurs.

**Agglutination Reactions:** These are similar to precipitation reactions, except that they involve the precipitation of cells.

- Traditionally used to detect antigens on the surface of red blood cells, such as Rh factor (see below).
- Usually, indirect crosslinking is used to form the aggregation. A **secondary antibody** is added that binds to the **primary antibodies** that have bound to their epitope on the surface of the cell. The increased distance between cells prevents electrostatic repulsion between cells that may occur with using just the primary antibody.
- Antigen-coated beads can substitute for RBCs, allow the detection of a wide range of haptens.

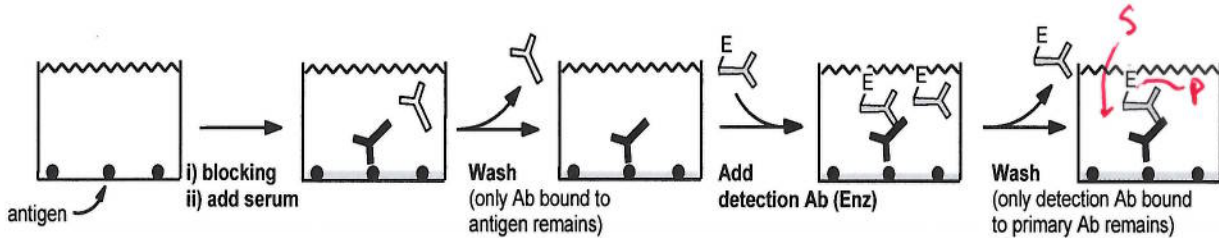


**Rh factor** is an antigen found on the surface of RBCs. If a Rh<sup>-</sup> mother has a child with an Rh<sup>+</sup> father there is a chance that the child will be Rh<sup>+</sup>. During childbirth there is mixing of the fetal and maternal blood, and some of the Rh<sup>+</sup> fetal blood can enter the mother – this antigen will be seen as foreign by the mother and she will produce anti-Rh antibodies. Any subsequent children who are Rh<sup>+</sup> will develop anemia *in utero* due to IgG from the mother crossing the placenta.

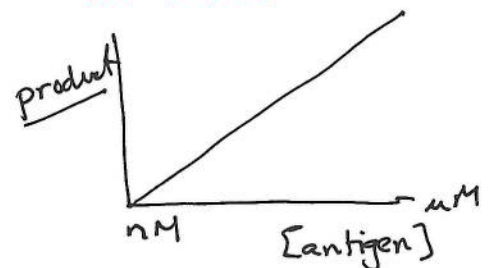
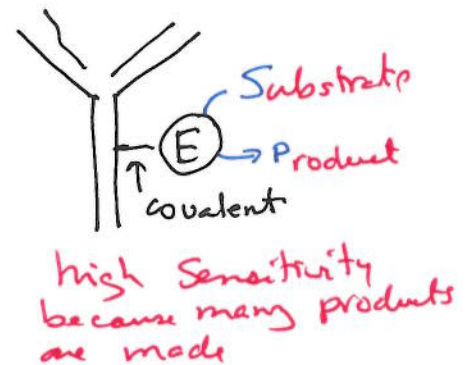


**ELISA (Enzyme-Linked Immunosorbent Assay):**

**Indirect ELISA (Detection of *Antibody*):** Example application - Used to detect HIV antibodies in infected individuals.

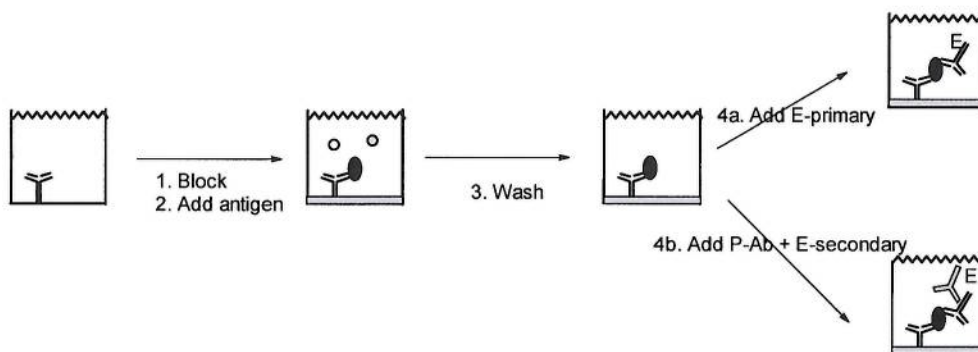


1. Antigen is bound to the bottom of a microtiter plate, plate is coated with blocking protein to prevent non-specific binding.
2. Specific antibody (primary antibody) is added that binds to the antigen. The usual source of this antibody is serum of a patient.
3. Detection:
  - i) An enzyme-linked secondary antibody is added. The secondary antibody is covalently linked to an enzyme, such as alkaline phosphatase, horseradish peroxidase, or p-nitrophenyl phosphatase. These enzymes give colored or light producing (chemiluminescence) products, amplifying the signal due to enzymatic turnover.
  - ii) The original primary antibody can also be enzyme linked in certain cases. The usual application of this is to detect bound antigen. The antibody must be available in pure form.
4. Amount of product produced is proportional to the amount of bound primary antibody.



**Sandwich ELISA (Detection of *Antigen*)**

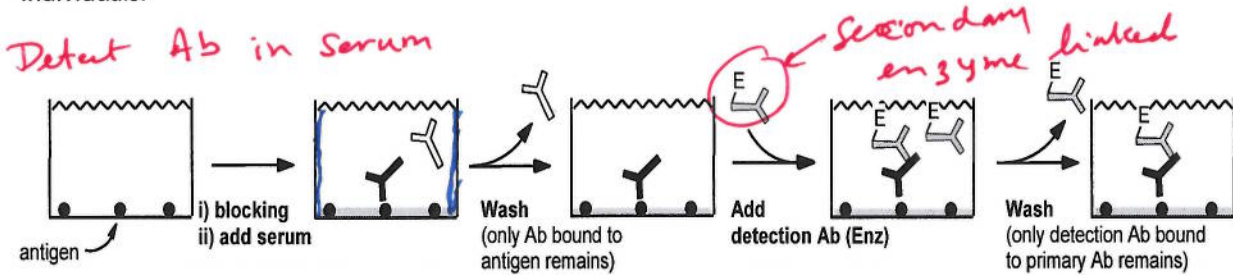
1. Antigen specific primary antibody is bound to the bottom of the well, non-specific blocking protein is added.
2. Antigen in solution is added.
3. Detection – two approaches.
  - i) Enzyme-coupled primary antibody that is specific for the antigen is added.
  - ii) A second, non-enzyme coupled, primary antibody added, followed by detection antibody with enzyme attached. The detection antibody **must** only recognize the Fc region of the 2<sup>nd</sup> primary antibody.
4. Product produced is proportional to the amount of bound antigen.





**ELISA (Enzyme-Linked Immunosorbent Assay):**

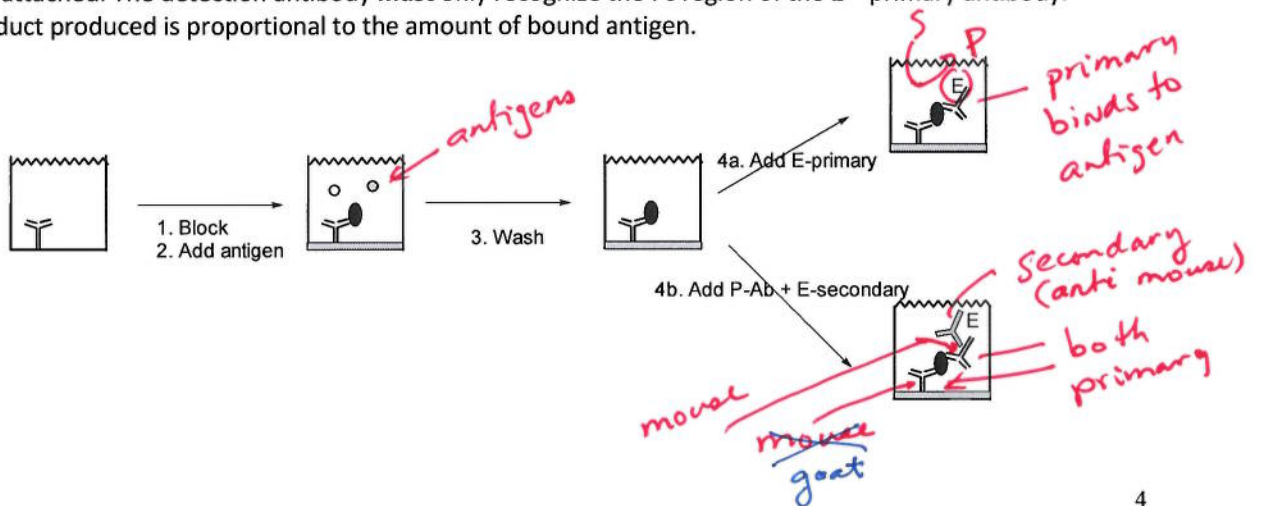
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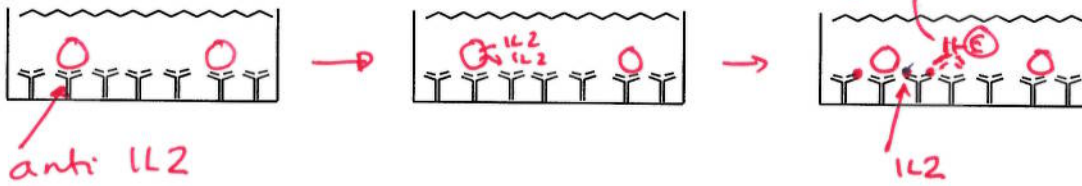
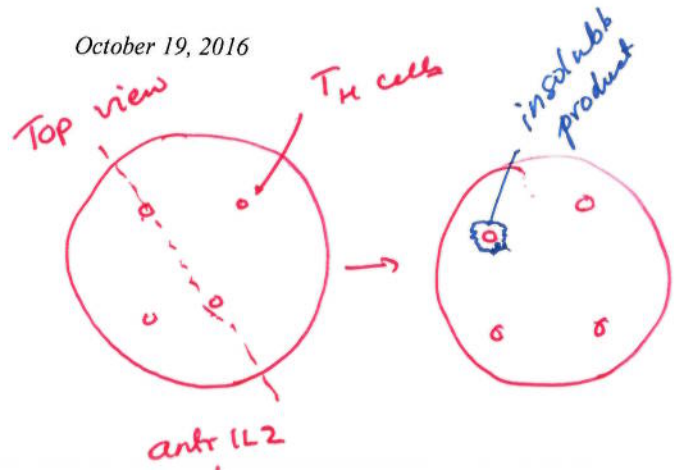
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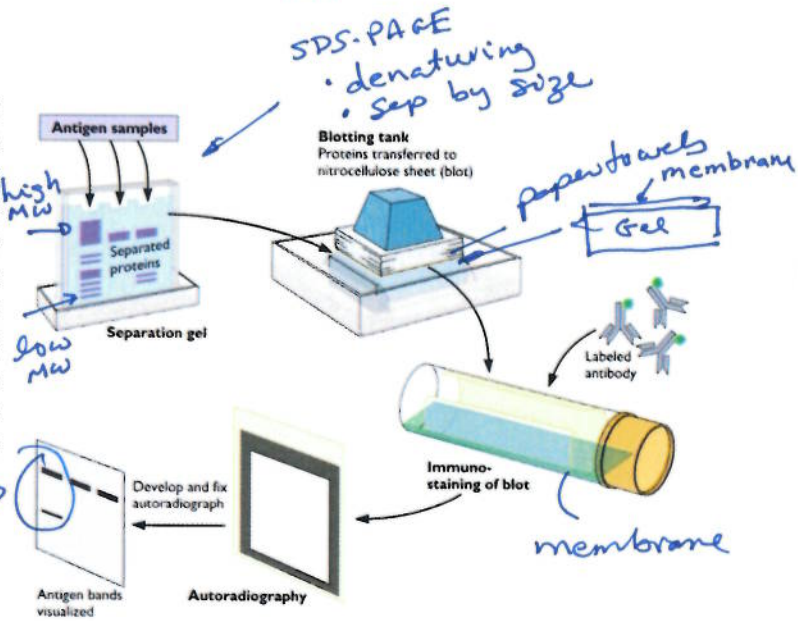


**ELISPOT Assay – Sensitive Detection of Cytokine release.**

1. Coat well with primary anti-cytokine antibodies.
2. Seed well with cytokine secreting cells.
3. Secreted cytokines bind to antibody near cell location.
4. Detect bound cytokines with secondary, enzyme linked, anti-cytokine antibody.



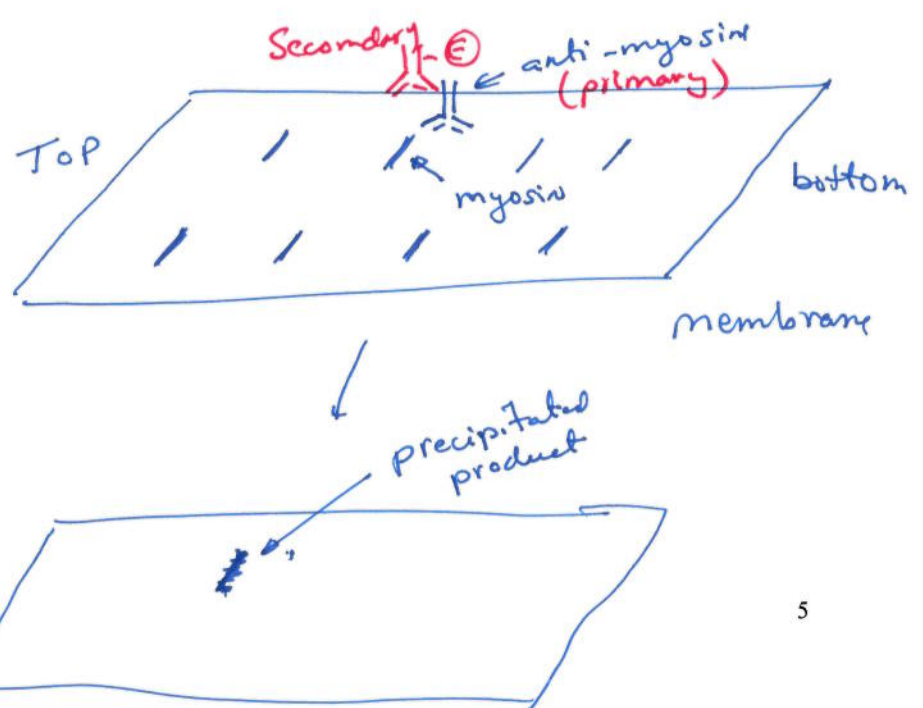
**Western Blot** is useful for detecting antigen in the presence of a complex mixture of proteins. A standard SDS gel is run to separate proteins according to size. These proteins are transferred to a membrane of nitrocellulose, forming an image of the gel. After blocking with a non-specific protein, the membrane is then washed with the primary antibodies that will recognize the desired antigens. These antibodies bind to the immobilized and denatured protein. The membrane is treated with a secondary antibody, with an attached enzyme, that recognizes the constant region of the primary antibody.



Why multiple same antigen bands.

- cleavage (both have epitope)
- different glycosylation
- diff phosphorylation states.

• cross reactivity  
- diff proteins recognized by same Ab





- No enzyme
- Radioactive form of antigen.

**Radioimmunoassay** is usually used to detect antigen. Antigen specific antibodies are immobilized in plastic wells in microtiter plates, or on beads. The assay required three steps, with only the last step required to measure the level of antigen in unknown samples.

i) A **binding curve** is generated by allowing a known amount of radioactive antigen to bind to the antibody, after washing the unbound antigen away, the bound radioactivity is then measured. A non-saturating concentration of antigen is usually selected to maximize sensitivity.

ii) In the **calibration** reaction **known** amounts of unlabeled antigen are mixed with the concentration of radioactive antigen determined from step i. After washing, the measured radioactivity is used to generate a calibration curve. The amount of radioactivity bound to the antibody will decrease because the unlabeled material can compete for binding.

iii) In measurement of **samples with unknown** amount of antigen, serum (or other fluid) that containing an unknown amount of the antigen is mixed with the same amount of radioactive antigen that was used for calibration. This mixture is bound to the antibodies. The amount of bound radioactivity is measured after washing off the excess antigen, the concentration of antigen in the fluid is obtained by comparing the radioactivity to the calibration curve.

