

# Techniques in Molecular Genetics

Immunological Methods  
And Western Analysis

H.E. Schellhorn

# Day 4

- Immunology and Westerns
- PAGE
- Western

# The Different Branches of the Immune Response

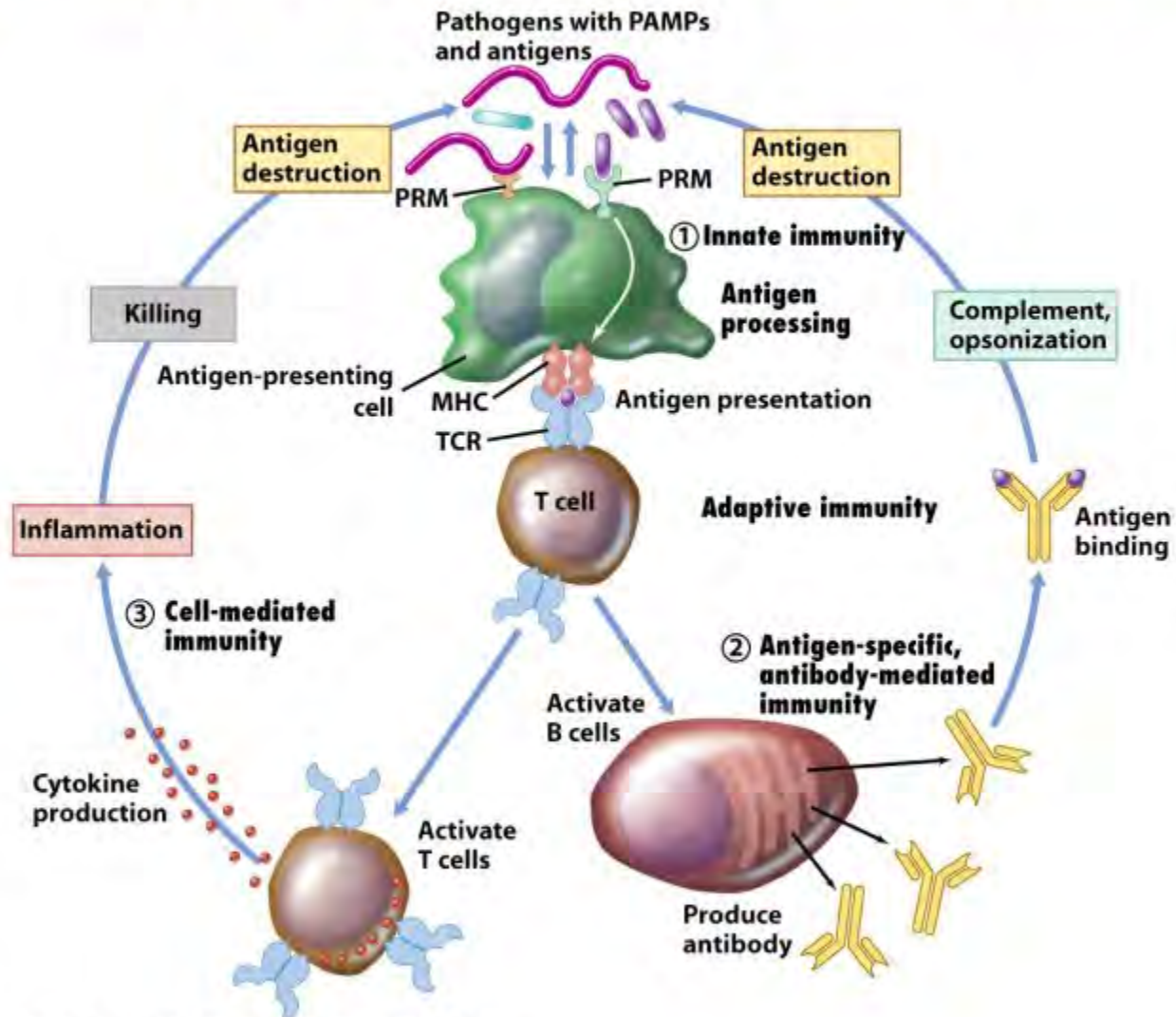


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## Two Sides of the Adaptive Immune Response

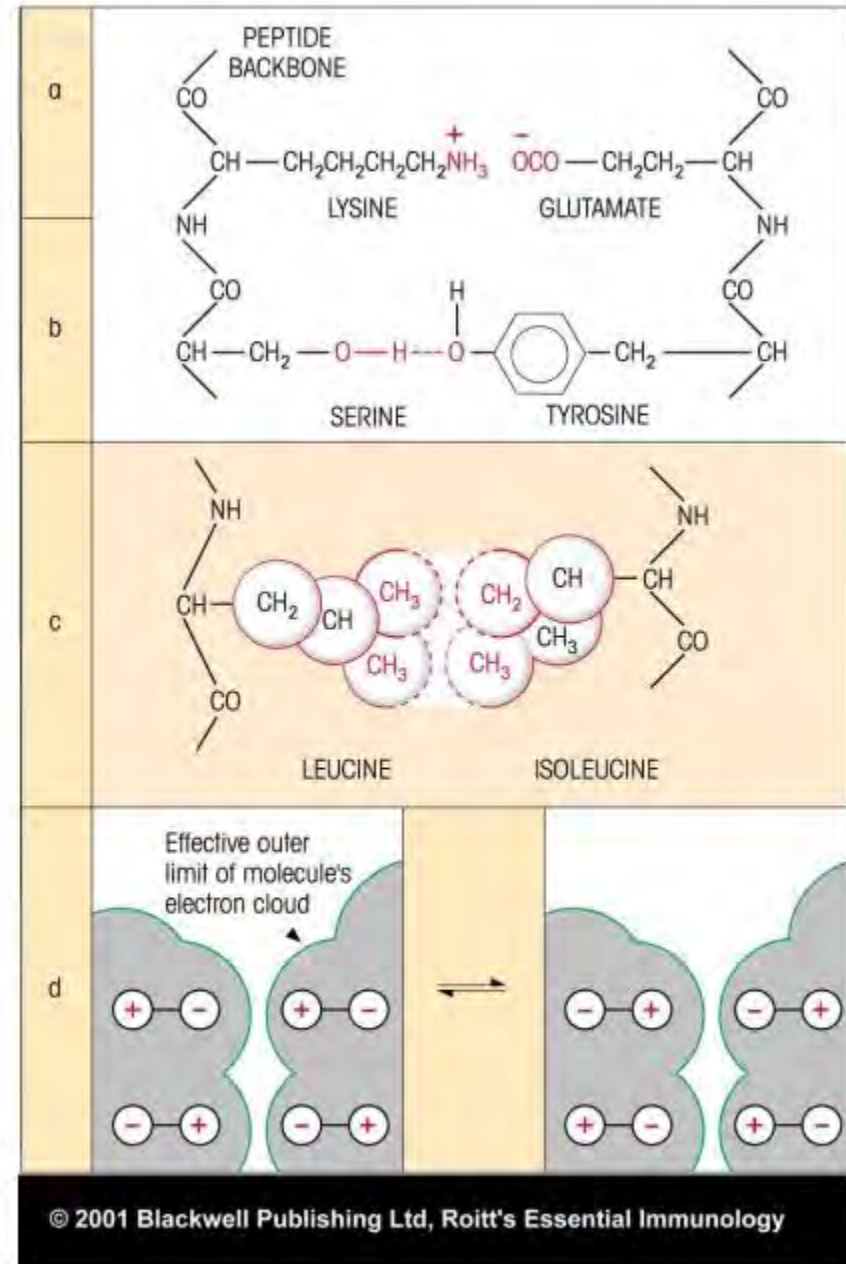
### *Humoral immune response*

- Defence against extracellular pathogens
- Soluble Effectors: Antibodies produced by B cells

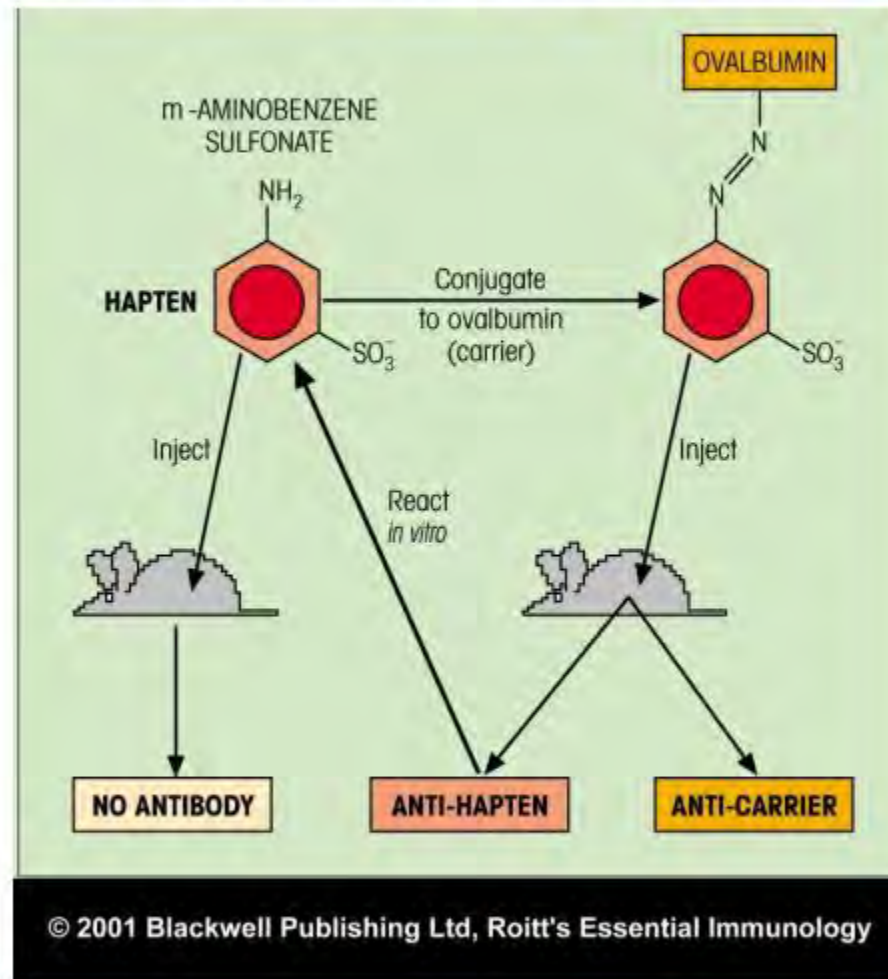
### *Cell-mediated immune response*

- Defence against intracellular pathogens (viruses and bacteria)
- Effector T cell (CD4 helper and CD8 cytotoxic)
- Dependence on antigen presentation by MHC I or II

# Immunological Methods: Stabilization of Antigen-Antibody Interactions

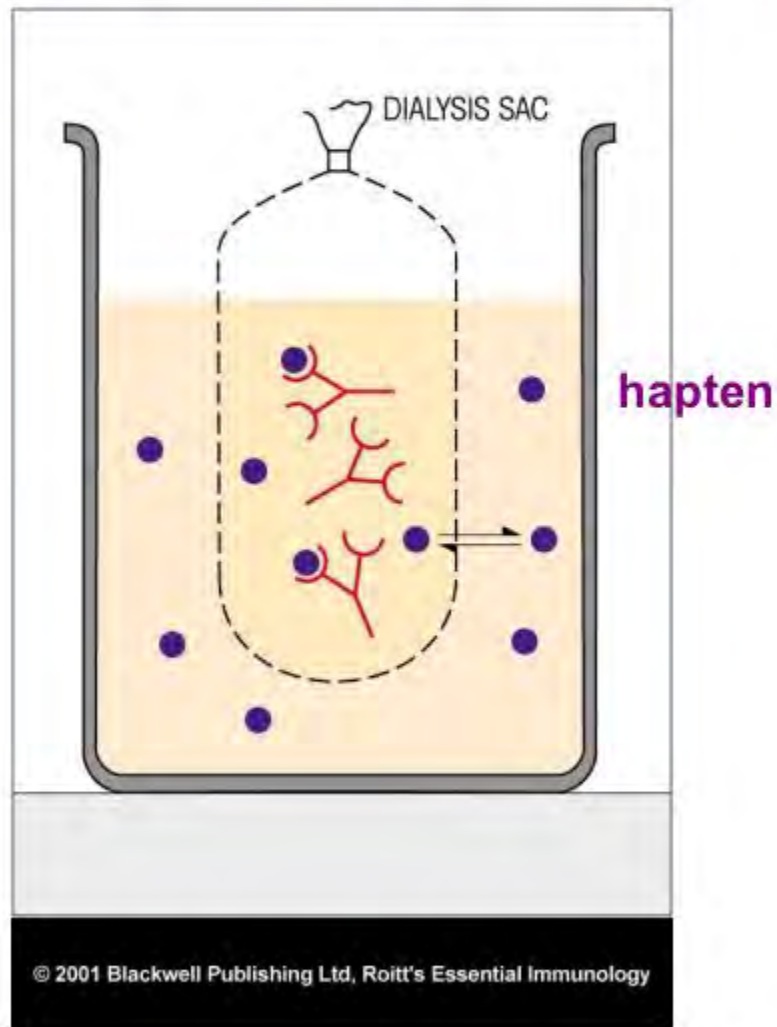


# Immunological Methods: Immunization with Haptens

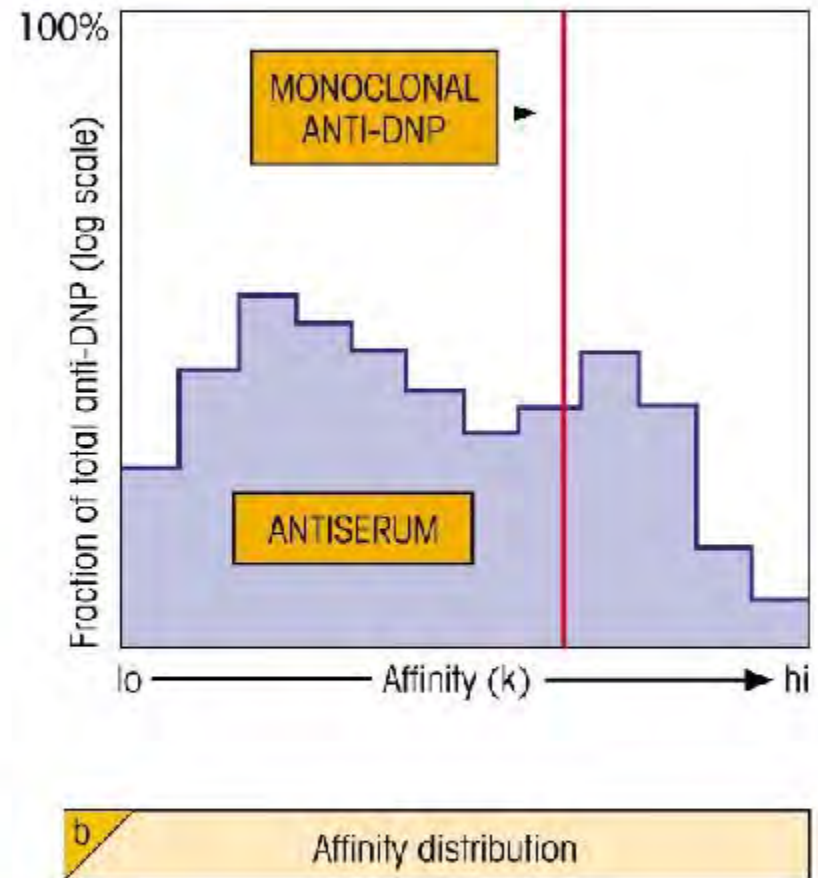
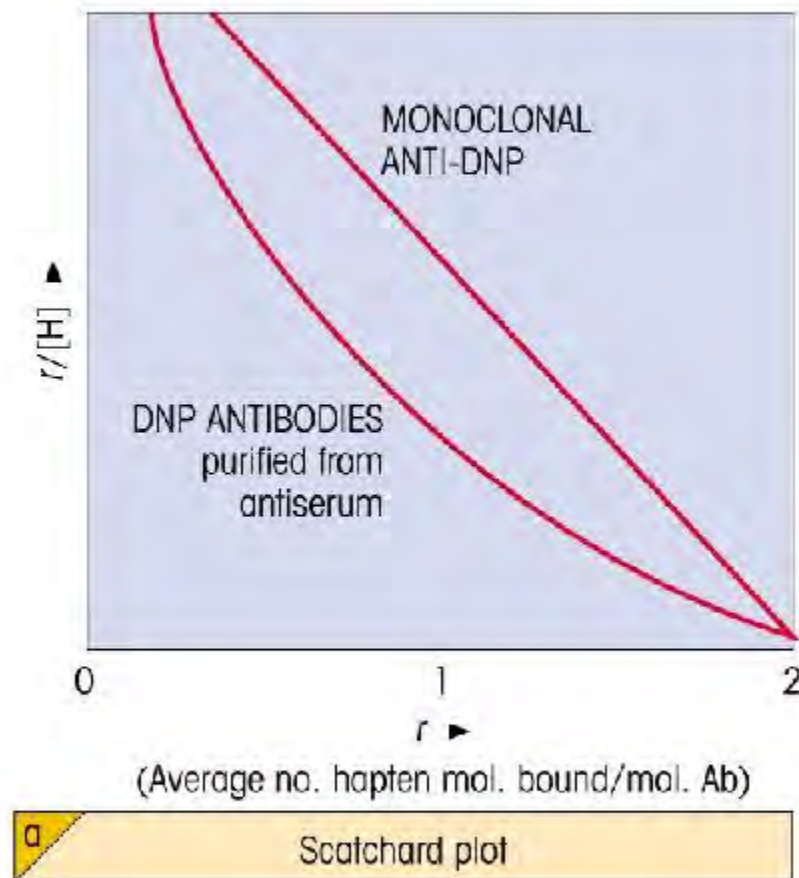


**Application: Creation of peptide antisera**

# Immunological Methods: Characterization of Antigen-Antibody Interactions



# Immunological Methods: Antisera Contain Antibodies with Different Affinities



**Monoclonal antibodies vs polyclonal antiserum**



# Immunological Methods: Principle of Immunoprecipitation - IP

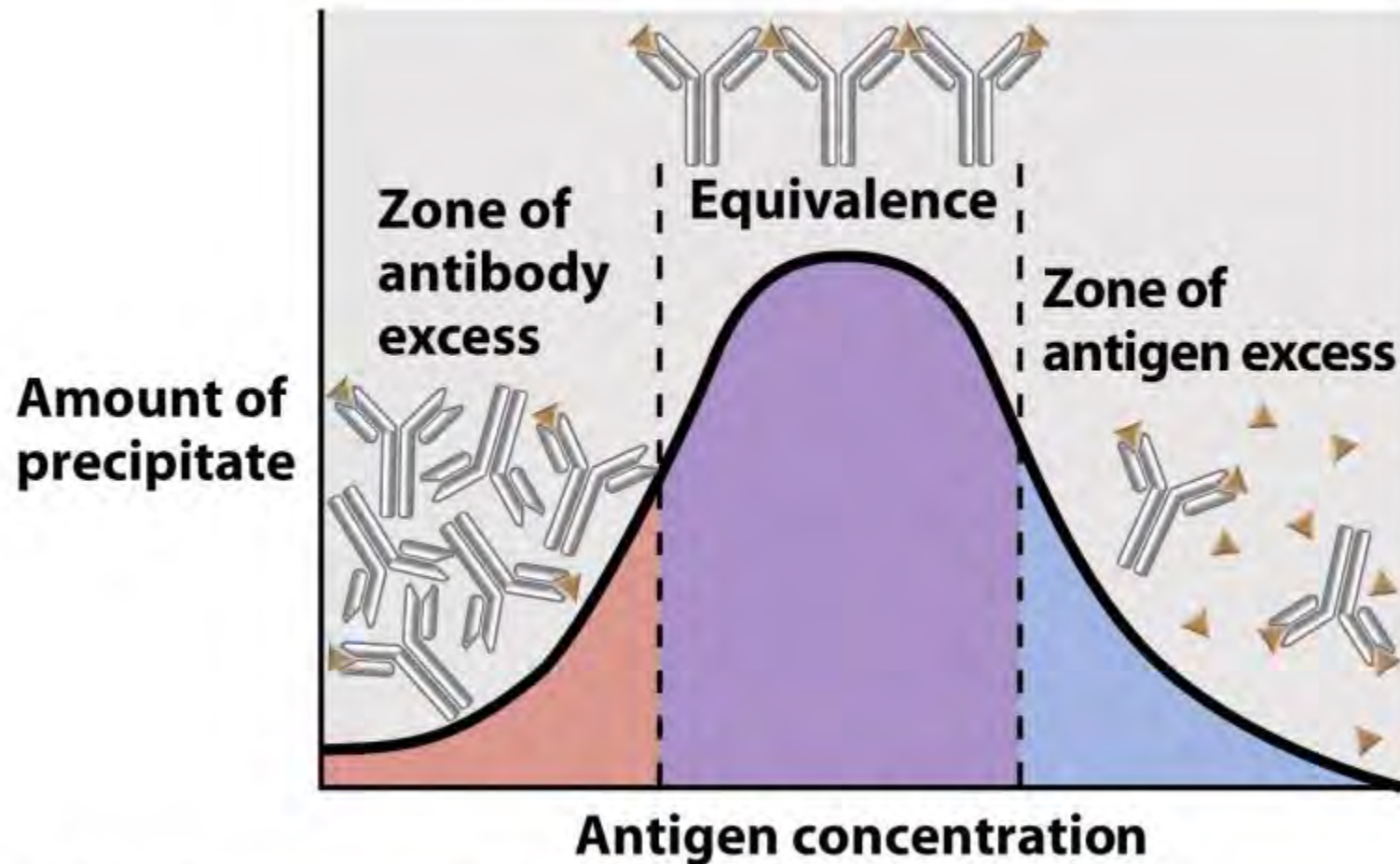


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## Immunological Methods: IP Application - Immunodiffusion

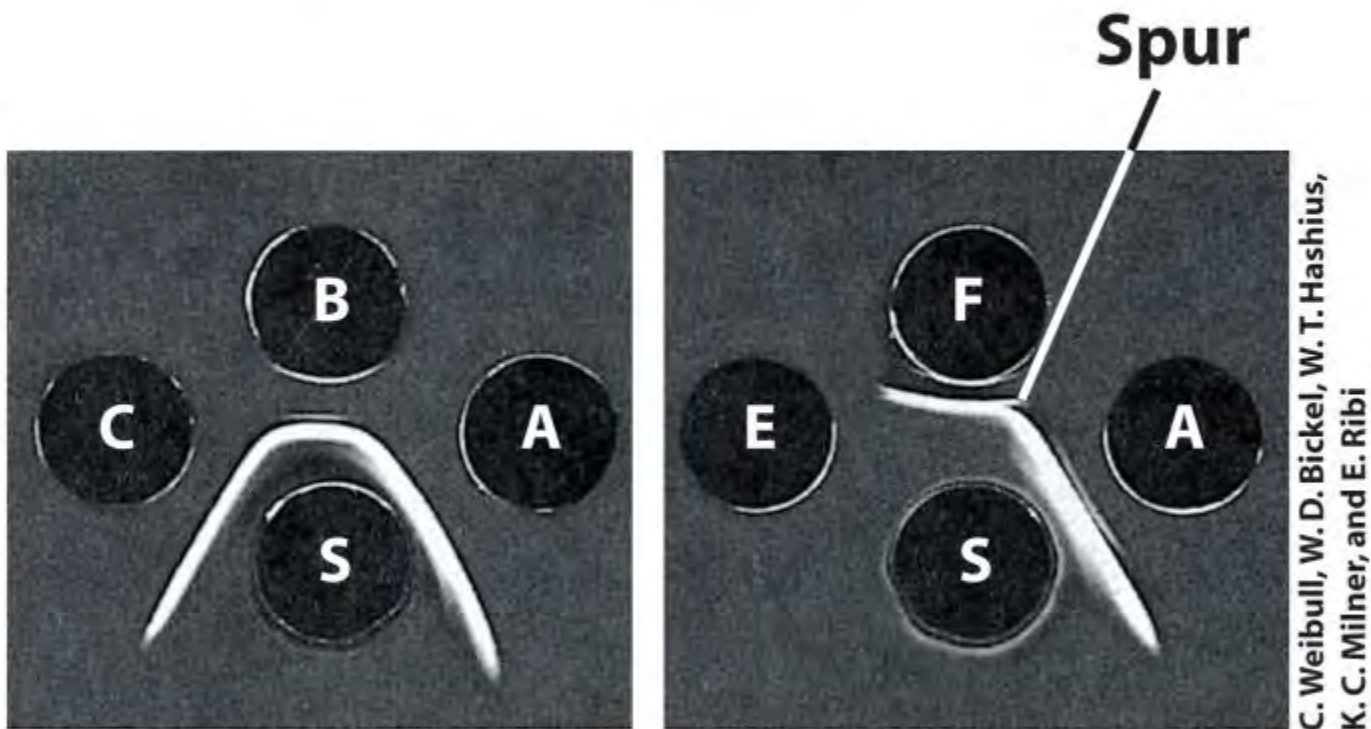
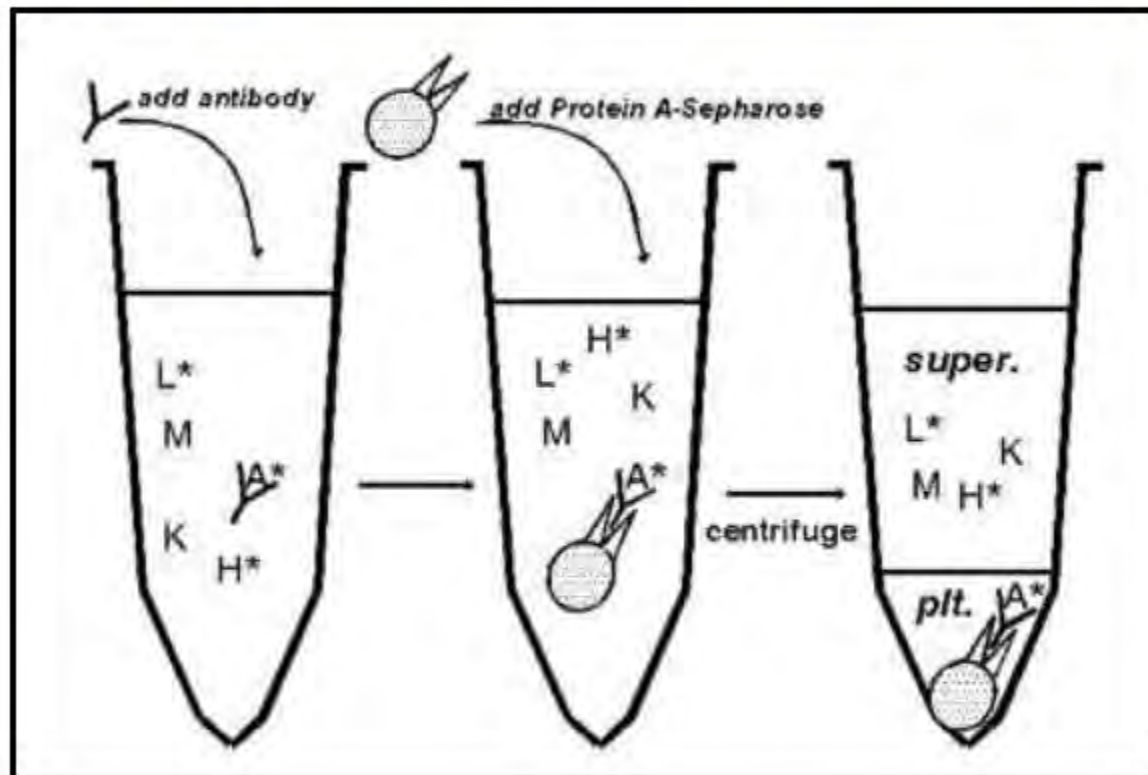


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C. Weibull, W. D. Bickel, W. T. Hashius,  
K. C. Milner, and E. Ribi

## Immunological Methods: IP Application - Immunoprecipitation



A\* - antigen

L\*, H\*, M, K - other proteins

Source: <http://www.animal.ufl.edu/hansen/protocols/imp98.prt.htm>

# Immunological Methods: Immunofluorescence Analysis - IF

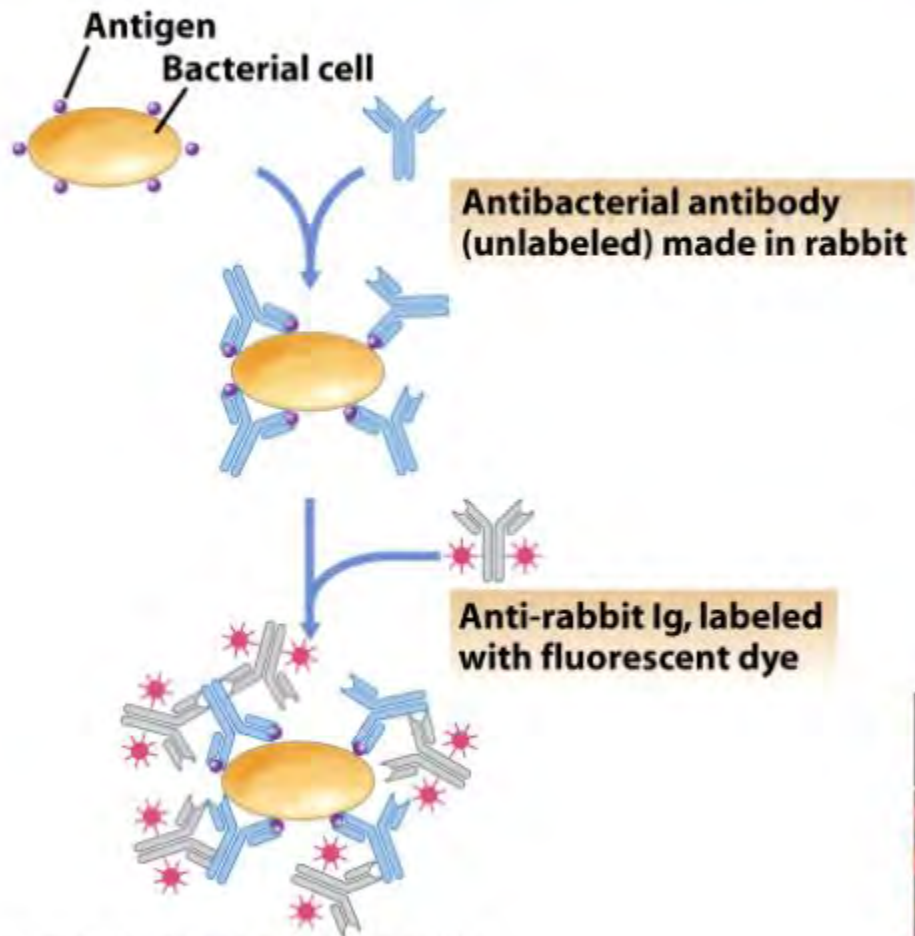


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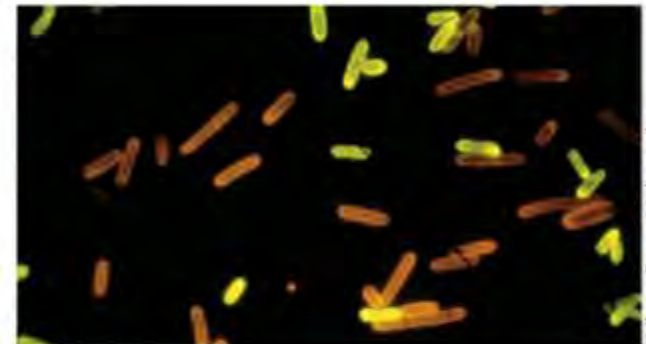
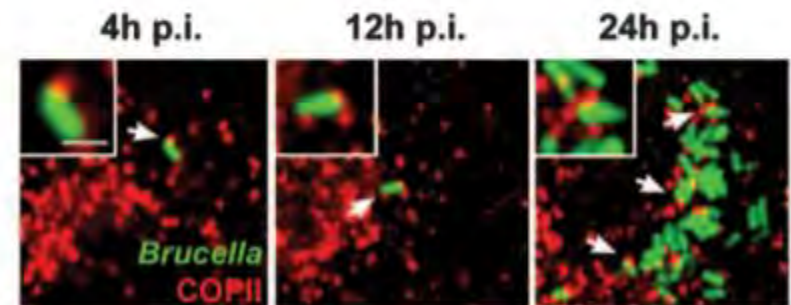


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Wellcome Research Laboratories



# Immunological Methods: Fluorescence-Activated Cell Sorter - FACS

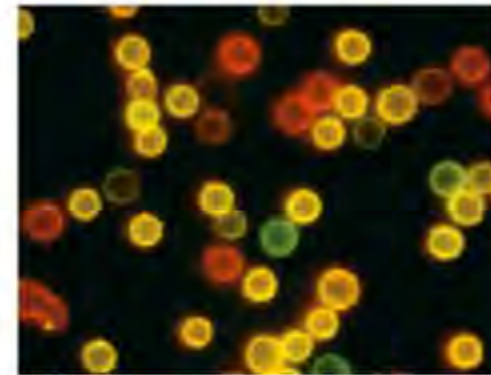
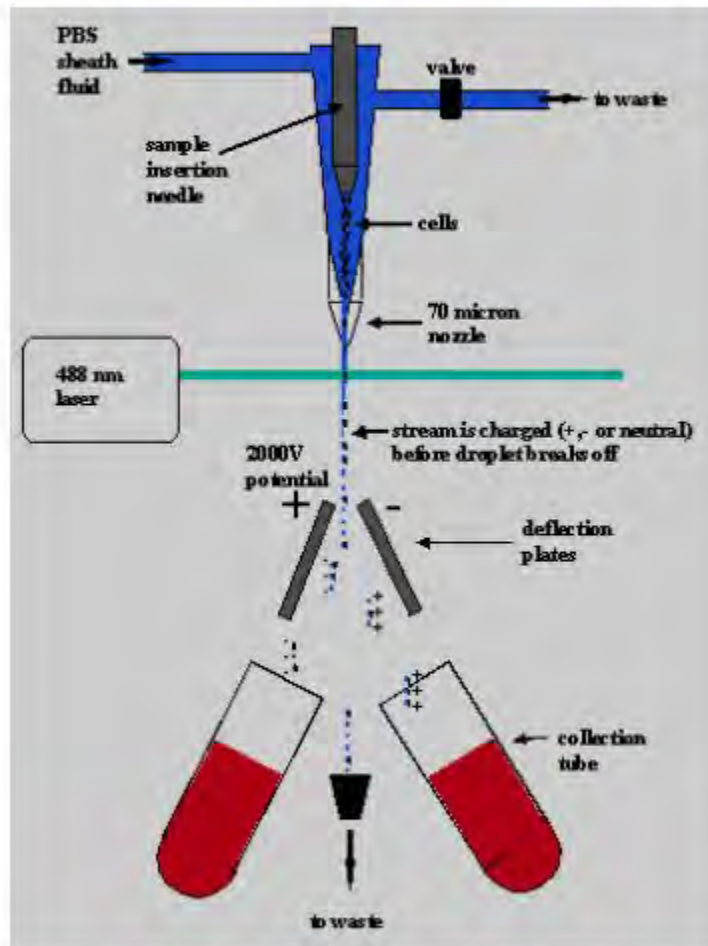


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Richard Lewis

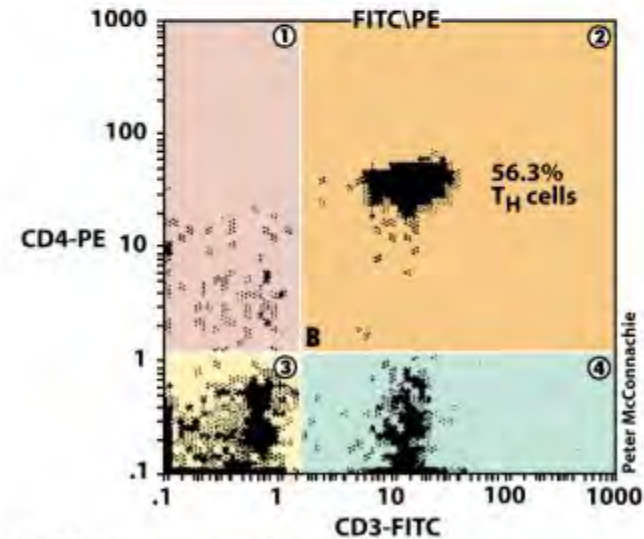


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Peter McConnachie

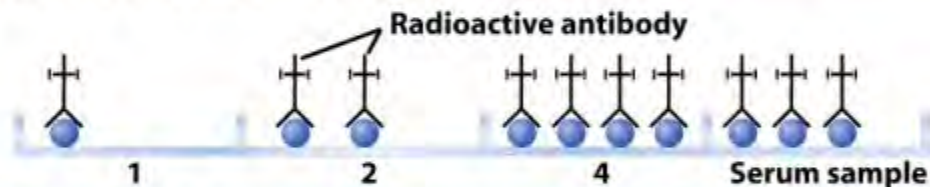
Courtesy Vanderbilt Medical Center  
[www.mae.wmich.edu/faculty/liou/wp\\_MEmicro04p2\\_ManikK.ppt](http://www.mae.wmich.edu/faculty/liou/wp_MEmicro04p2_ManikK.ppt)

# Immunological Methods: Radioimmunoassay - RIA

1. Bind insulin to wells of microtiter plate



2. Add excess anti-insulin antibodies that are labeled with  $^{125}\text{I}$ ; wash to remove unbound antibody



3. Count radioactivity in gamma radiation counter. Wells labeled 1, 2, and 4 establish a standard curve with known amounts of antigen (insulin). The radioactivity in the last well indicates, by comparison to the standard curve, how much insulin is present in a known amount of serum.

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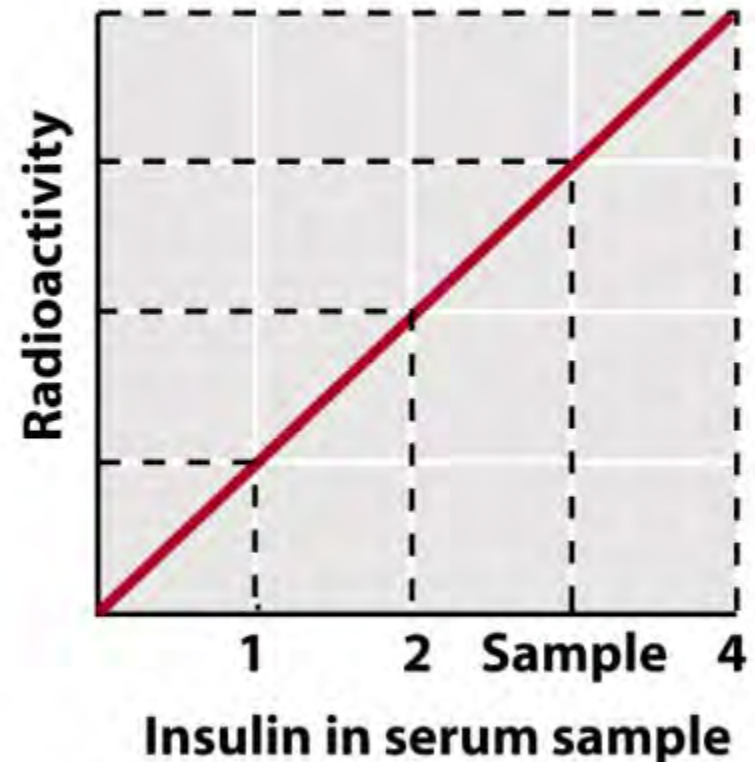


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# Immunological Methods: Enzyme-Linked Immunosorbant Assay ELISA

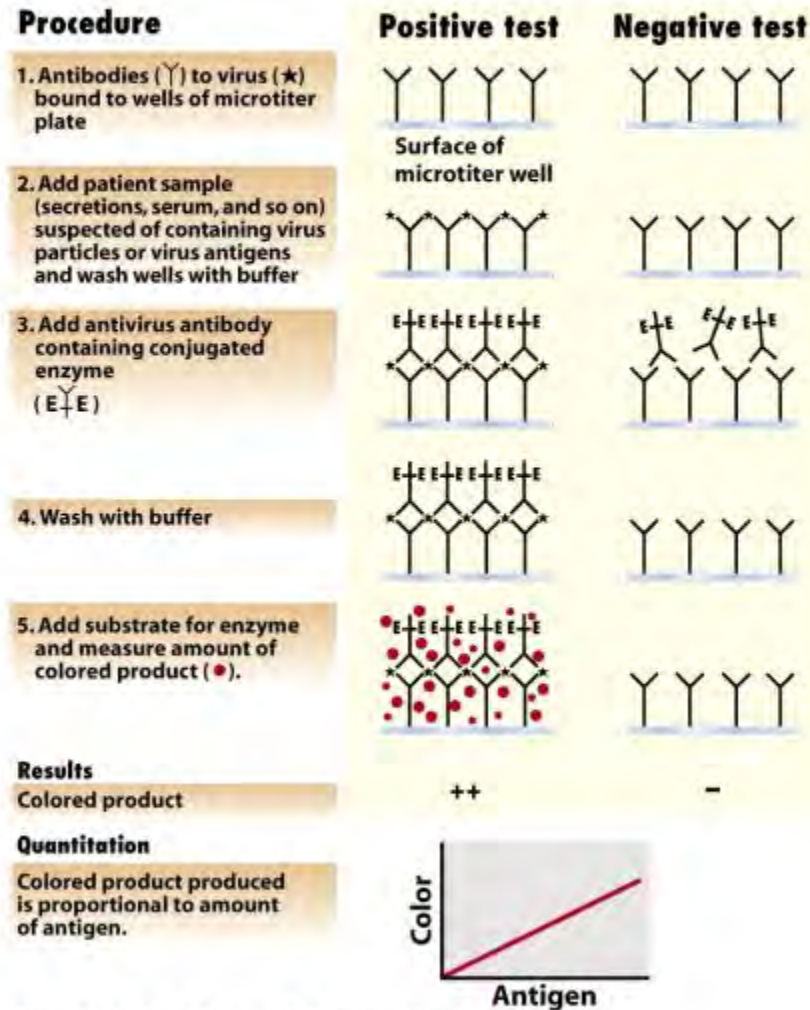
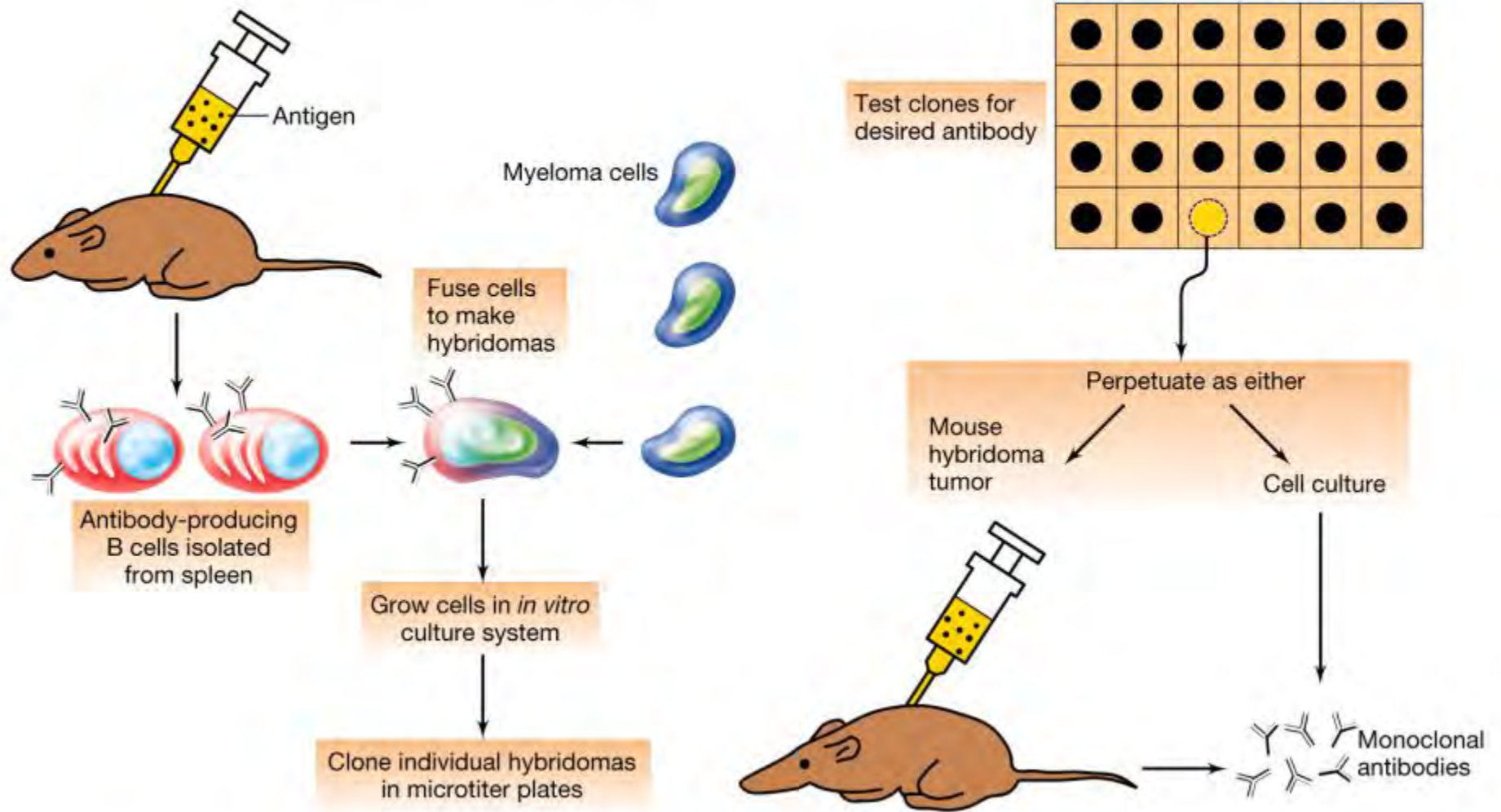


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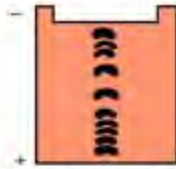
# Immunological Methods: Generation of Monoclonal Antibodies



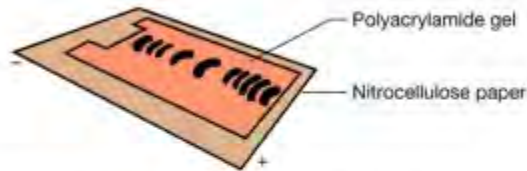


# Immunological Methods: Western Blot as Diagnostic Tool

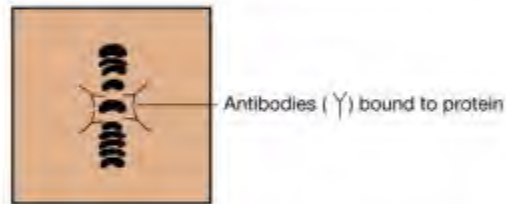
1. Denature proteins by boiling in detergent



2. Subject to electrophoresis; proteins separate by molecular weight

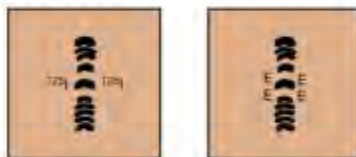


3. Blot the separated proteins from the gel to nitrocellulose paper



(a)

4. Treat nitrocellulose paper containing blotted proteins with antibodies; each antibody recognizes and binds to a specific protein, labeling the protein for detection



5. Add marker to bind to antigen-antibody complexes, either (left) radioactive *Staphylococcus* protein A-<sup>125</sup>I, or (right) antibody containing conjugated enzyme



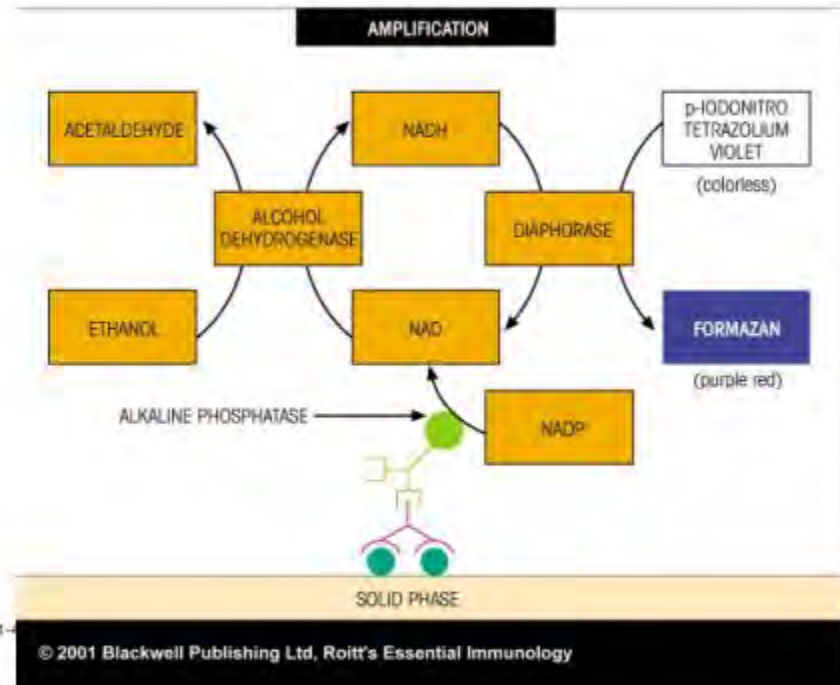
X-ray film

Nitrocellulose with enzyme-produced colored spot

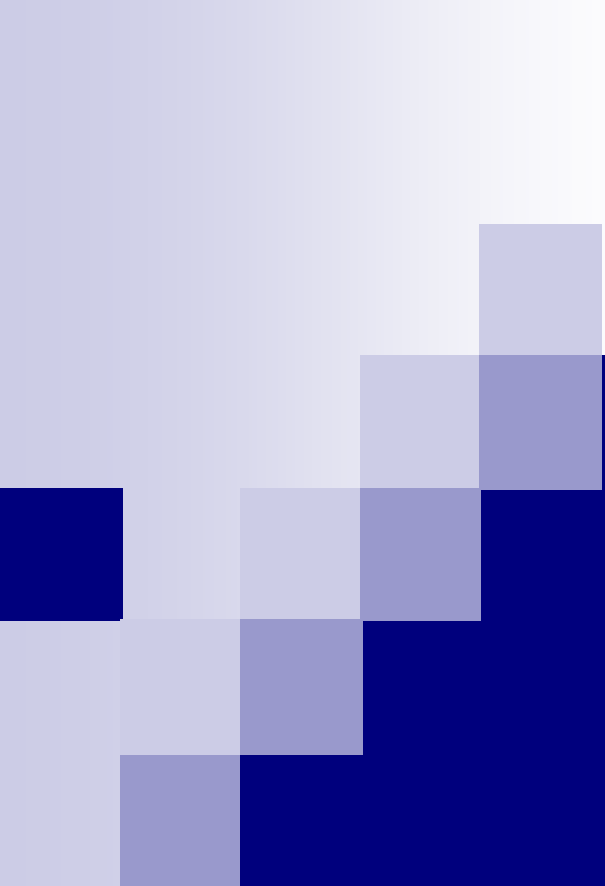
(a)



(b)



Alternative used in the course:  
Chemoluminescent reaction



# Lecture Topic: Western Blot Analysis

Date: Thursday May 9<sup>th</sup> 2013



# Introduction

- Immuno (Western) Blotting is a commonly used technique to detect specific proteins from a complex mixture.
- It provides information on:
  - Protein expression (relative to a control sample)
  - Protein size (based on a marker protein run along with your sample)



# Steps in Western Blotting

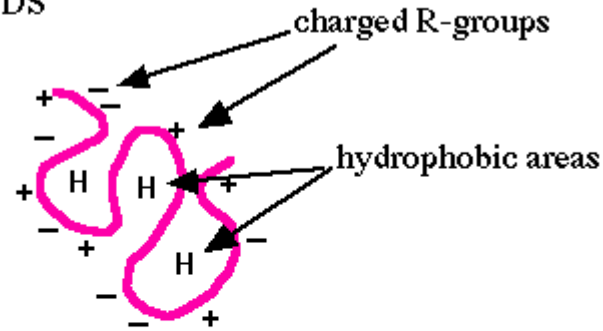
1. Sample Preparation
2. Polyacrylamide Gel Electrophoresis (PAGE)
3. Transfer from gel to membrane
4. Incubation with antibody
5. Detection



# Sample Preparation

- Cells are grown to desired density (OD)
- Samples are centrifuged to collect cells and separate media (discard supernatant)
- Wash samples in buffer to remove salts
- Coat samples in SDS-loading buffer
- Boil samples for 5 minutes to denature proteins

BEFORE SDS



AFTER SDS



SDS dissociates hydrophobic areas and renders proteins highly electronegative so that their migration through the gel is independent of their isoelectric point.

# SDS-PAGE

- Discontinuous Gel
  - Top: Stacking Gel
  - Bottom: Resolving Gel
- Proteins run from negative (anode) end to positive (cathode) end
- The percentage of gel used determines the pore size, the larger the percentage the more cross linking and the smaller the pore size

# Western Transfer

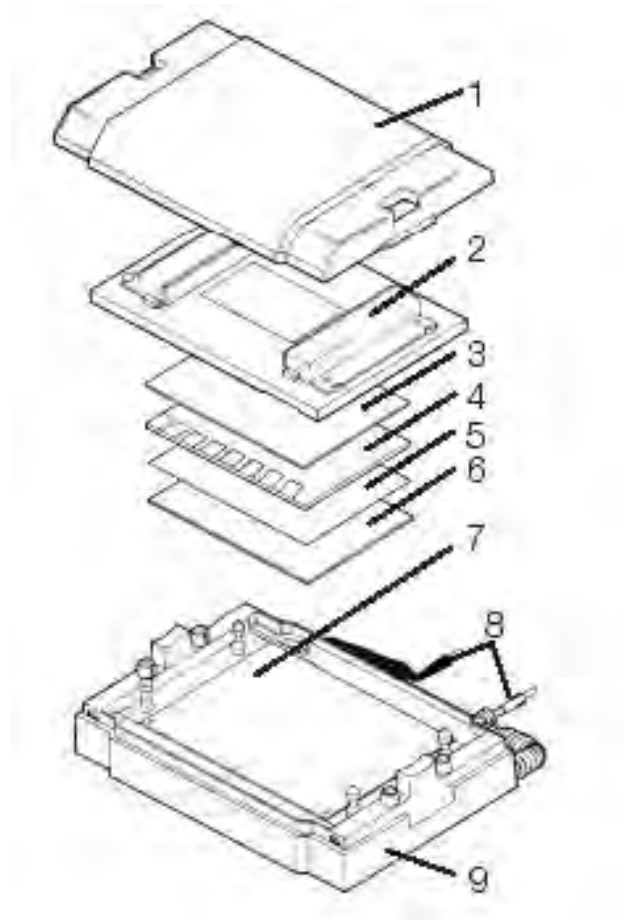
- Transfer from gel to PVDF (polyvinylidene fluoride membrane)
  - PVDF has good protein binding capacity (170-200ug/cm<sup>2</sup>), physical strength and enhanced binding in the presence of SDS
- Two Types of Transfer Units:
  1. Semi-dry Unit
  2. Mini Trans-Blot



# Semi-Dry Electrophoretic Transfer Cell (BioRad)

- How to Set Up Transfer:

1. Safety Cover
2. Steel Cathode Assembly
3. Thick Blot Paper
4. Gel
5. Membrane (PVDF)
6. Thick Blot Paper
7. Platinum Anode
8. Power Cables
9. Base

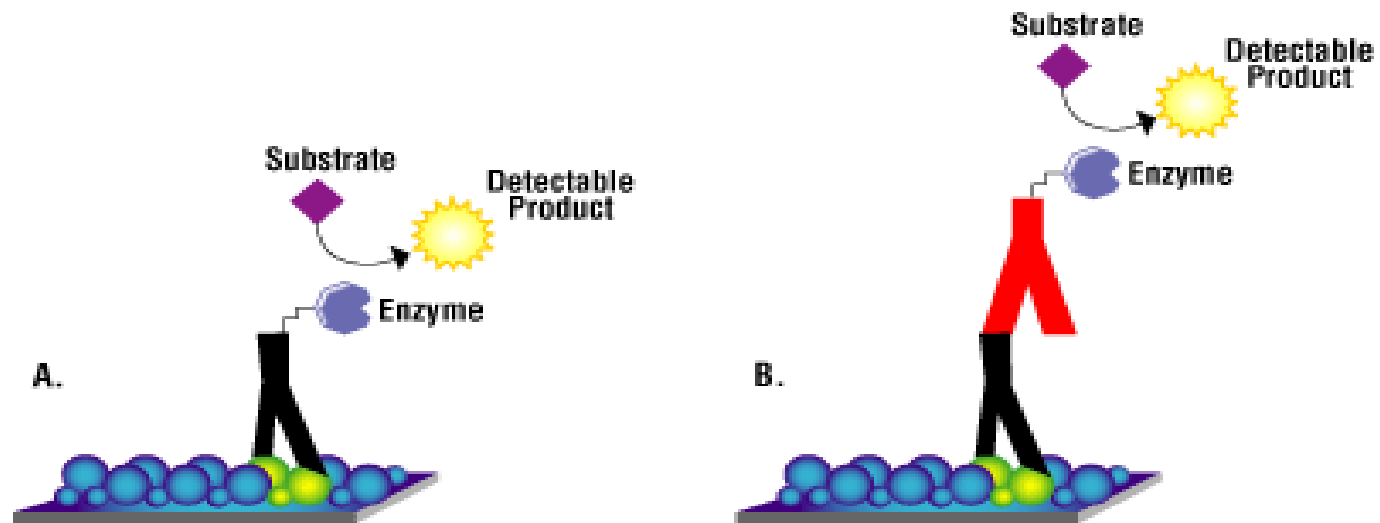




# Antibody Incubation

- After proteins are transferred from gel to membrane, the membrane is blocked using 5% milk.
- Blocking prevents non-specific interactions
- After blocking, the membrane is incubated in **primary** antibody

# Direct and Indirect Detection



Draw this outshamr

# Detection

- The **secondary** antibody is attached to HRP (horse radish peroxidase) enzyme
- HRP catalyzes the oxidation of luminol (substrate)
- Oxidation of luminol will put it in an excited state followed by decay to ground state accompanied by the emission of LIGHT
- The light is captured on a special film
- The intensity of the light is correlated with the abundance of protein present
- Enhanced Chemiluminescence occurs in the presence of chemical enhancers such as phenol.
- Signal is increased by 1000 fold