Research methods in Immunology

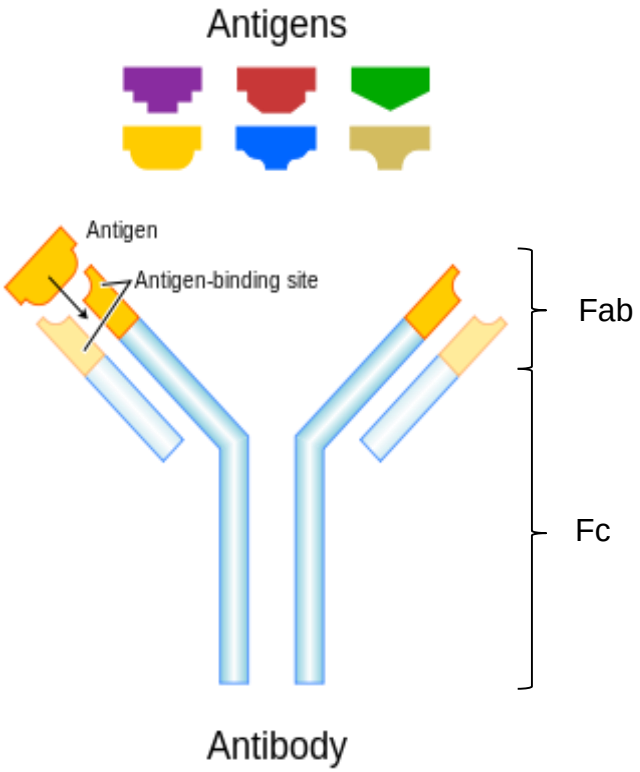
Medical studies in English

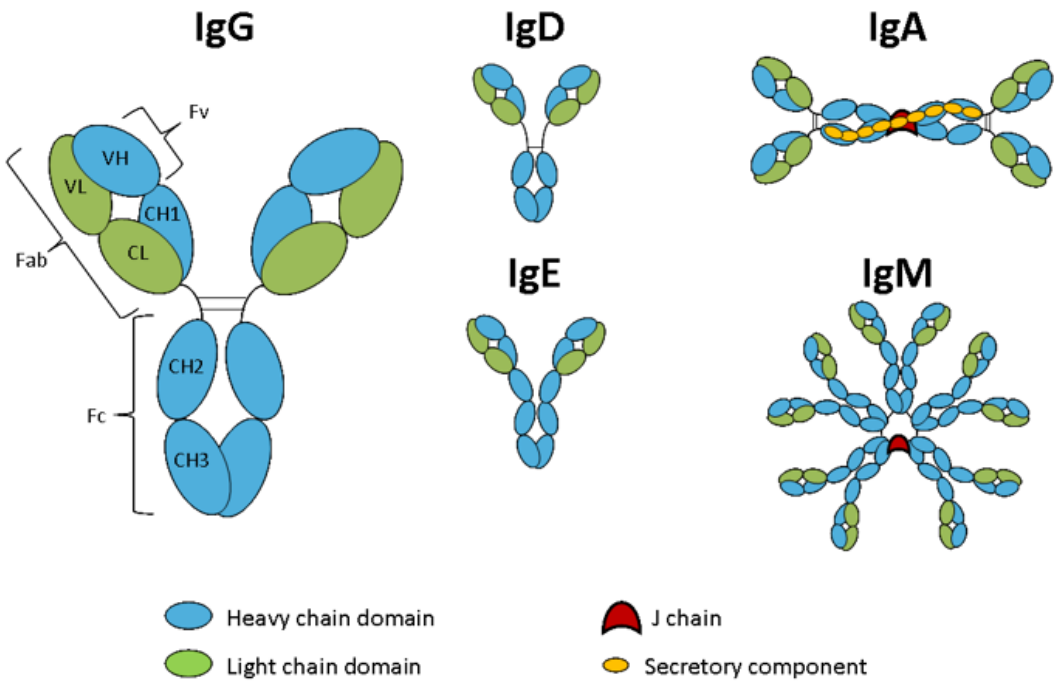
MEFST, 2020.

Techniques in Immunology

- Monoclonal and polyclonal antibodies production
- Flow cytometry
- ELISA - *Enzime linked immunosorbent assay*
- RIA – *Radio immunosorbent assay*
- IP – immunoprecipitation
- Western blot, immunofluorescence
- Blood group typing
- Coombs test (direct and indirect)
- Affinity chromatography
- Other techniques: Haematopoietic cells concentration, complement fixation test, contact precipitation, gel immunodiffusion, protein electrophoresis…

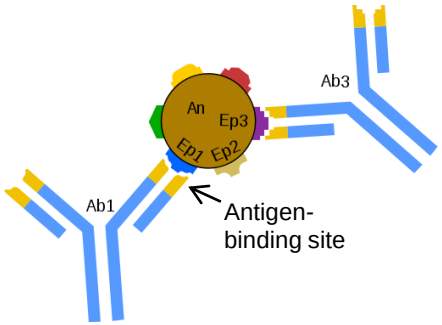
Antibodies





Specificity, Affinity, Avidity

Polyclonal antibodies



Aminal selection: Base on the need of the amount of antibodies, select animal based on their size.

Antigent injected into the aminal body

antigen

B cell

Antigen activated B cell.

Clone of memory B cells

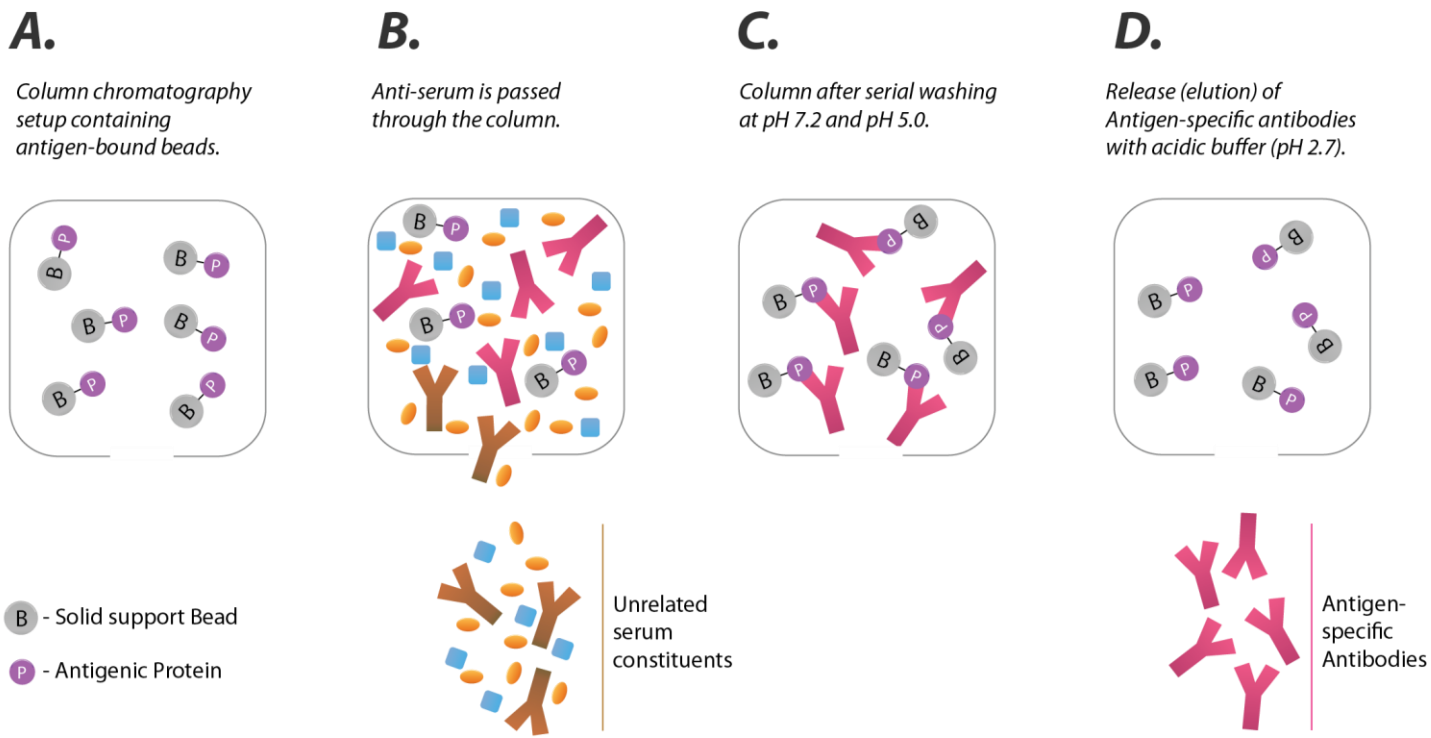
Clone of plasma cells

Secreted antibodies

Polyclonal antibodies from different B cells are produced.

polyclonal antibodies containing serum is taken out from the animal's bodies

(Immuno)affinity chromatography



(Immuno)affinity chromatography

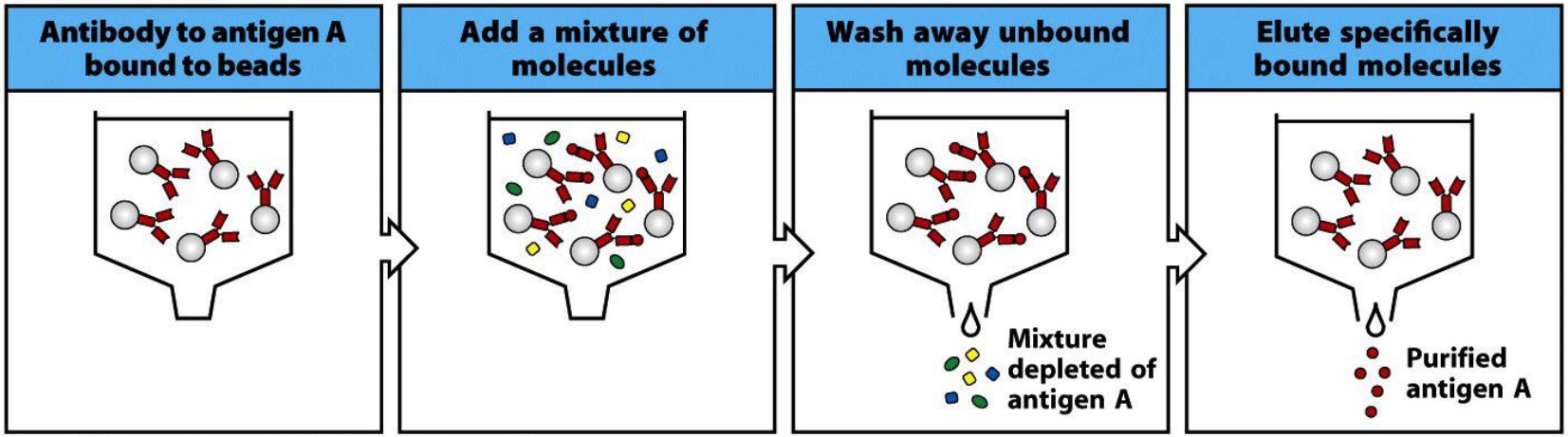


Figure A-5 Immunobiology, 7ed. (© Garland Science 2008)

- Monoclonal antibodies are bound to gel matrix
- Mixture of proteins is passed through the column
- Column is washed and unbound proteins are discarded
- By changing the pH of the elution buffer, ag/ab bound is broken, antigens are washed away from the antibodies and collected in a tube

Monoclonal antibodies

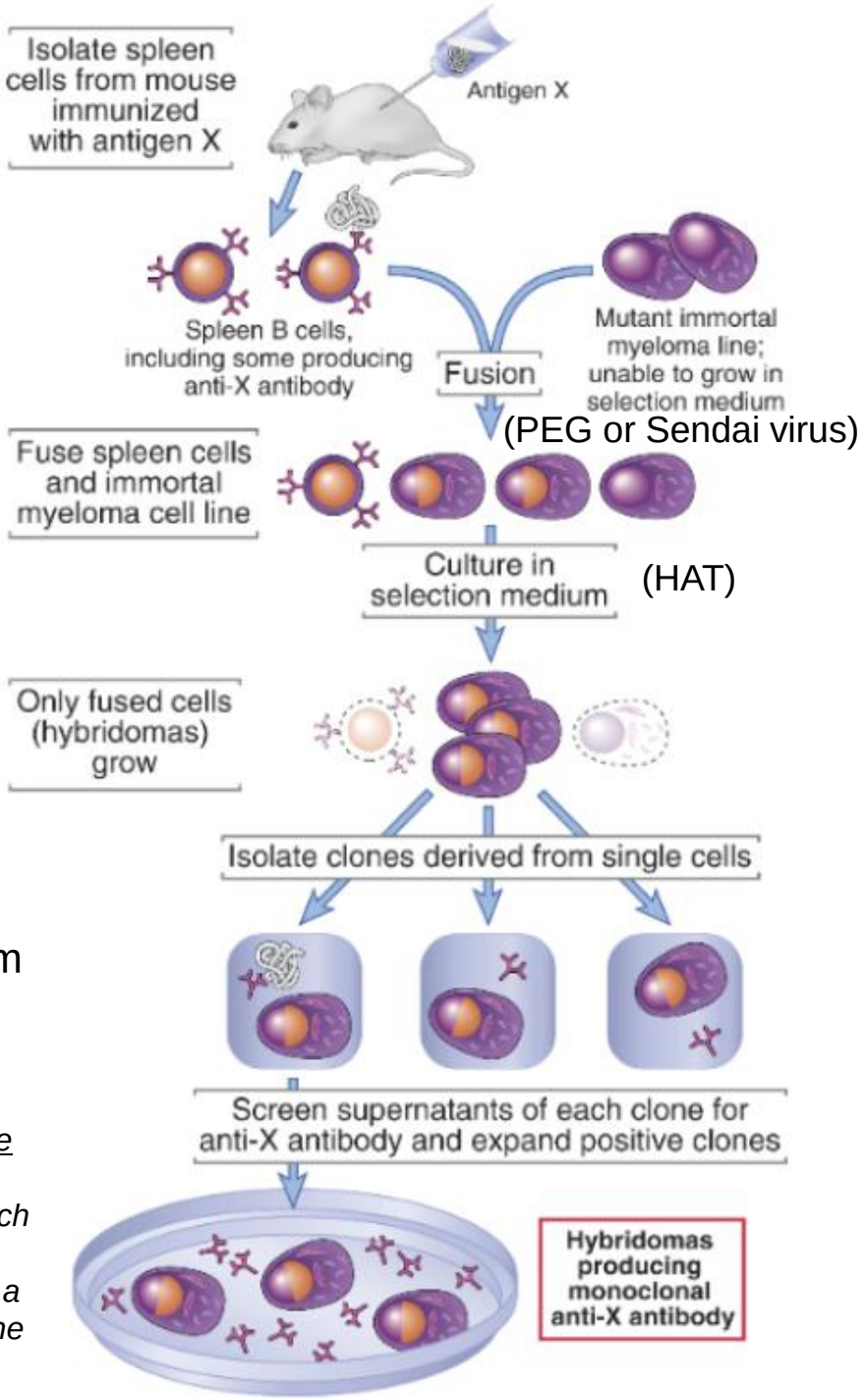
Antigens:

- have many epitopes
- Inoculated to experimental animal induce production of polyclonal antibodies
- By isolating **plasma cells** from spleen and fusing them with **B myeloma cells** (which do not produce antibodies), clones of plasma cells are immortalized → **hybridomas**.
- Upon selection in specific media, single cell clones are propagated, and checked for antibody production and quality

Monoclonal antibodies are used for:

- Radio labeled or fluorescent labeled application (ELISA, RIA)
- Immunofluorescence (IF, IHC), IP, WB, experimental use
- Antigen specific and immunotoxic tumor therapy (Herceptin)
- Many other applications

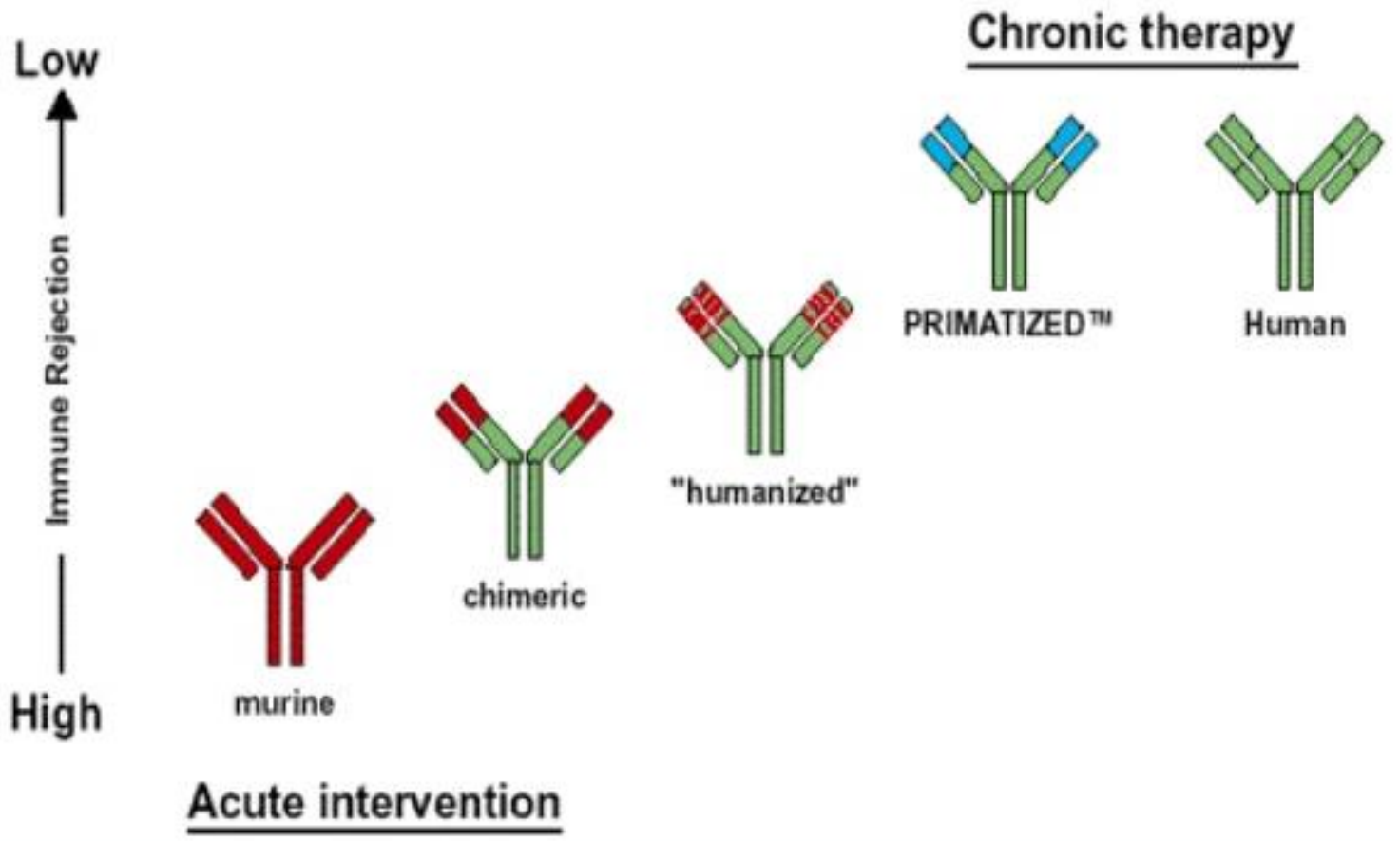
Monoclonal antibody production



PEG: polyethilen glycol
HAT : hypoxanthine-aminopterin-thymidine medium

Aminopterin blocks DNA de novo synthesis, which is absolutely required for cell division to proceed, but hypoxanthine and thymidine provide cells with the raw material to evade the blockage (the "salvage pathway"), provided that they have the right enzymes, which means having functioning copies of the genes that encode them. HGPRT - Hypoxanthine-guanine phosphoribosyltransferase - plays a central role in the generation of purine nucleotides through the purine salvage pathway.

Monoclonal antibodies used in therapy



https://en.wikipedia.org/wiki/List_of_therapeutic_monoclonal_antibodies

Sensitivity of immunoassays

Sensitive methods
- **Precise**
- **Expensive**

Immunoelectron microscopy
Western blotting
Flow cytometry
Immunofluorescence
ELISA
RIA

Less sensitive methods
- **Semi-quantitative results**
- **Cheaper**

Agglutination inhibition
Passive agglutination
Hemagglutionation
Immunoelectrophoresis
Radial immunodiffusion
Precipitation reaction in fluids

Sensitivity of various immunoassays

Assay	Sensitivity* (μg antibody/ml)
Precipitation reaction in fluids	20–200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10–50
Ouchterlony double immunodiffusion	20–200
Immunoelectrophoresis	20–200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006–0.06
Agglutination inhibition	0.006–0.06
Radioimmunoassay	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	<0.0001–0.01
ELISA using chemiluminescence	<0.0001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.06–0.006

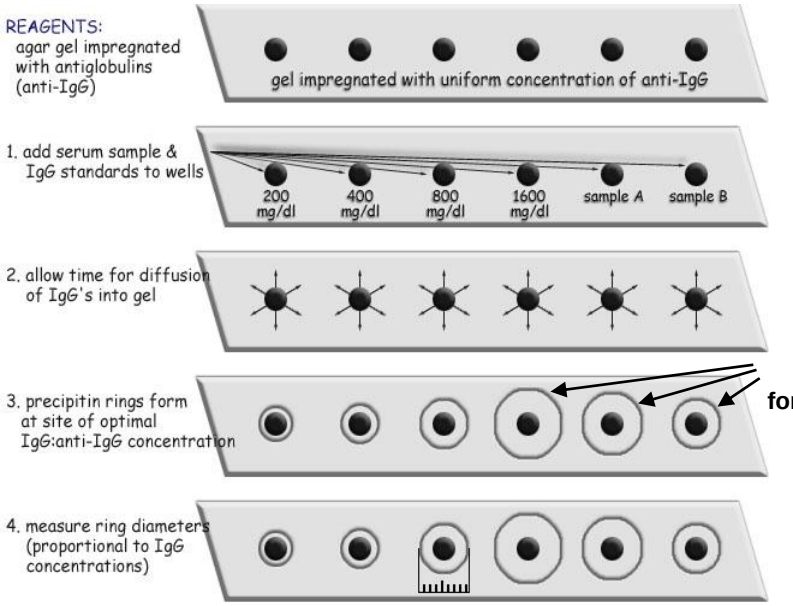
*The sensitivity depends upon the affinity of the antibody as well as the epitope density and distribution.

†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology*, 5th ed., American Society for Microbiology, Washington, D.C.

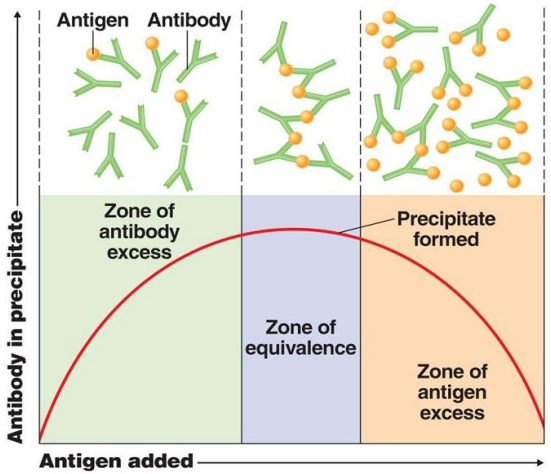
Precipitation based immunoassays

Immunodiffusion

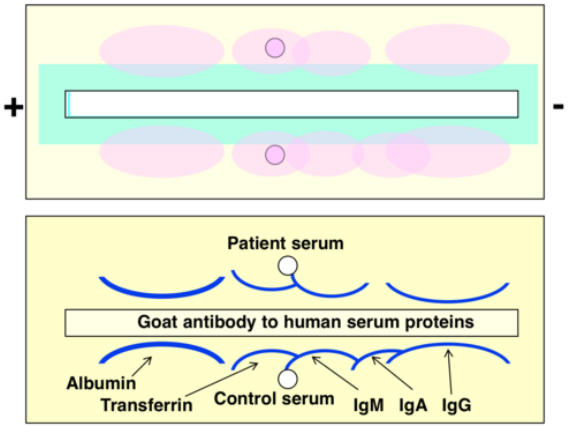


Zone of equivalence: formation of large complexes

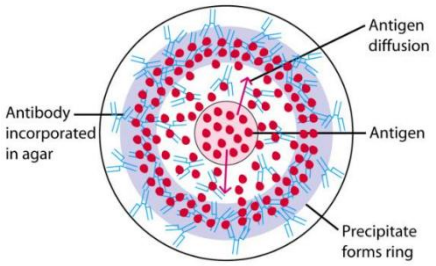
Precipitation curve



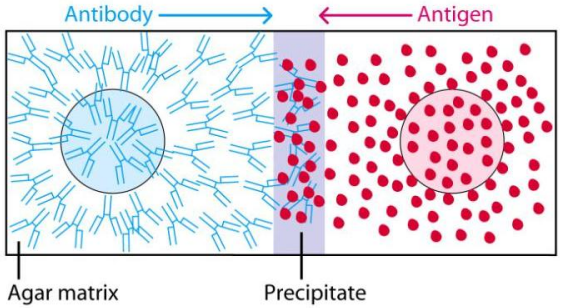
Immunoelectrophoresis



Radial immunodiffusion



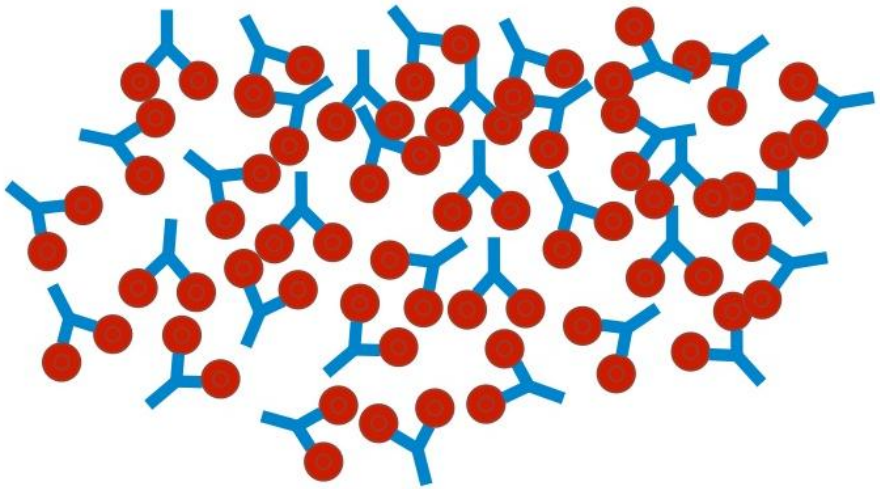
Double immunodiffusion



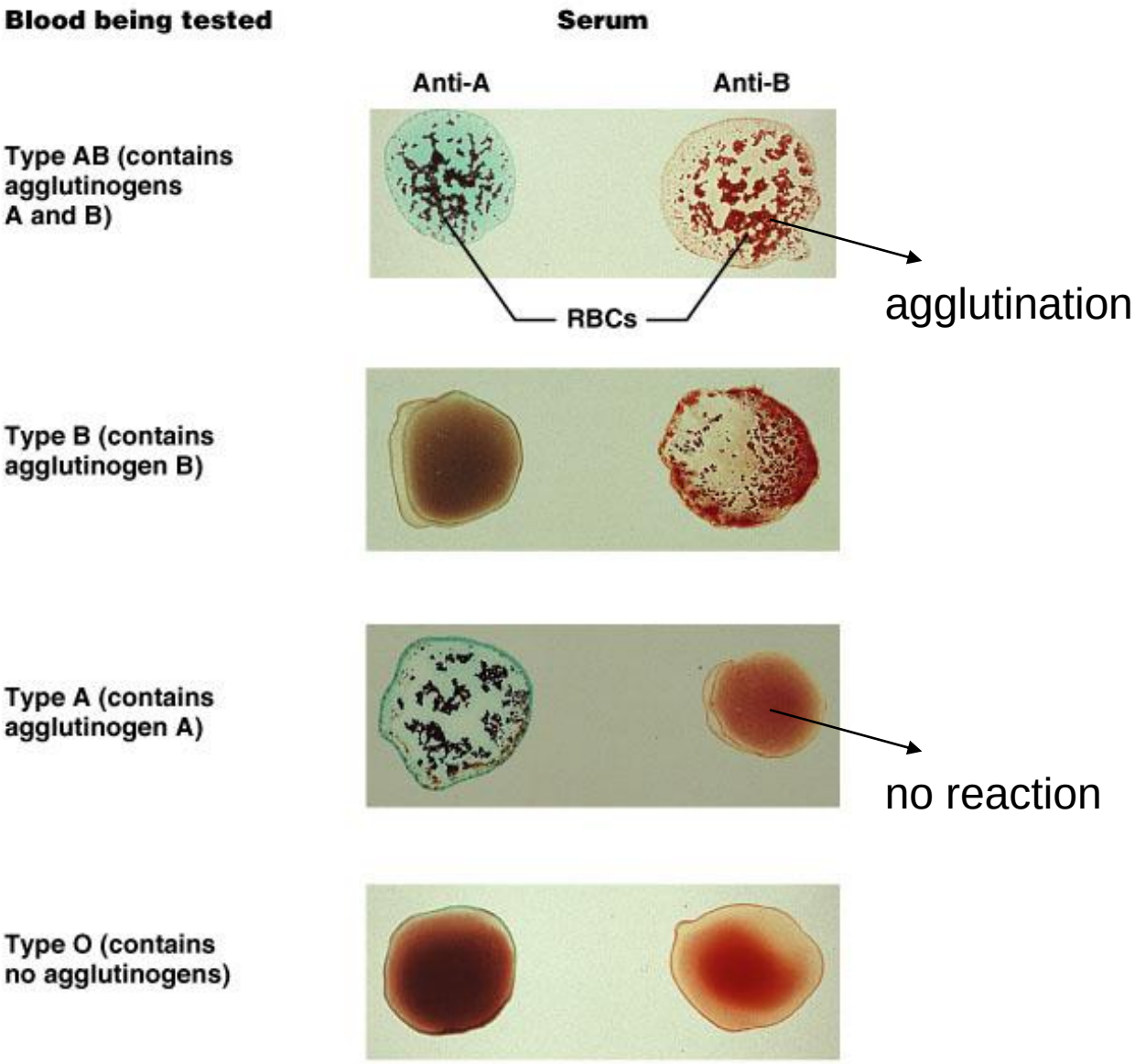
Agglutionation based immunoassays

- Agglutination is defined as the formation of clumps of cells or inert particles by specific antibodies

- Hemagglutination
- Direct Coombs test
- Indirect Coombs test



Hemagglutination - blood group typing



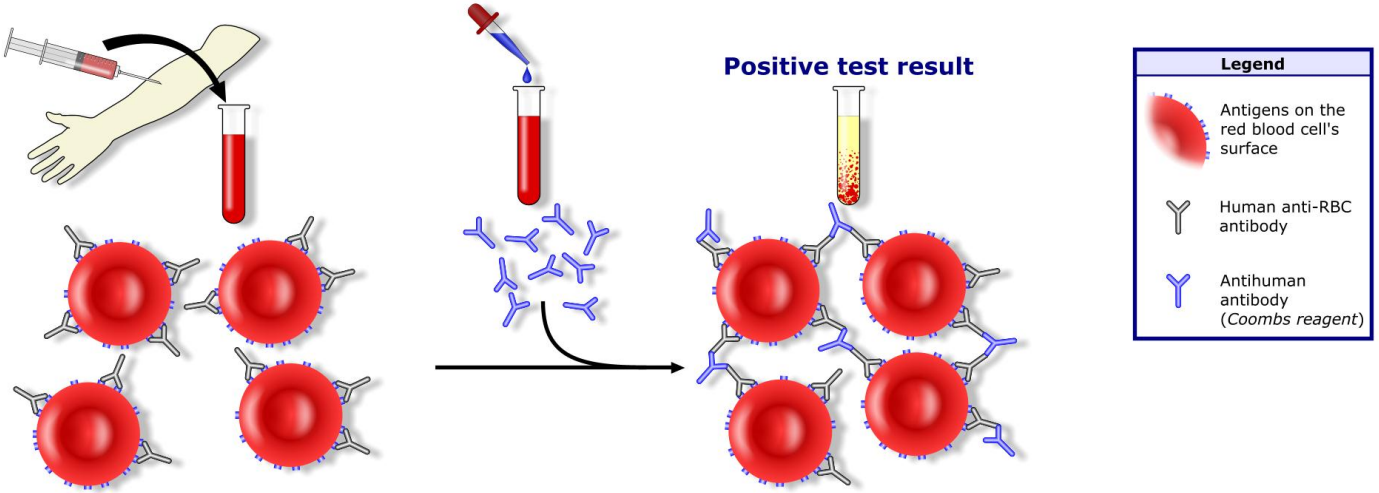
Coombs test

= Testing the presence of antibodies against red blood cells. (Coombs reagent = anti human IgG)

- **Direct Coombs test**
  - Testing if IgG and/or complement are bound on erythrocyte membrane (hemolytic anemia)
- **Indirect Coombs test**
  - Testing if there are unbound antibodies in the serum (anti-Rh IgG). Example:
    - Testing for **pregnant woman (Rh sensibilization)**
    - blood testing prior to transfusion.

  (Visualization = hemagglutination)

Direct Coombs test / Direct antiglobulin test

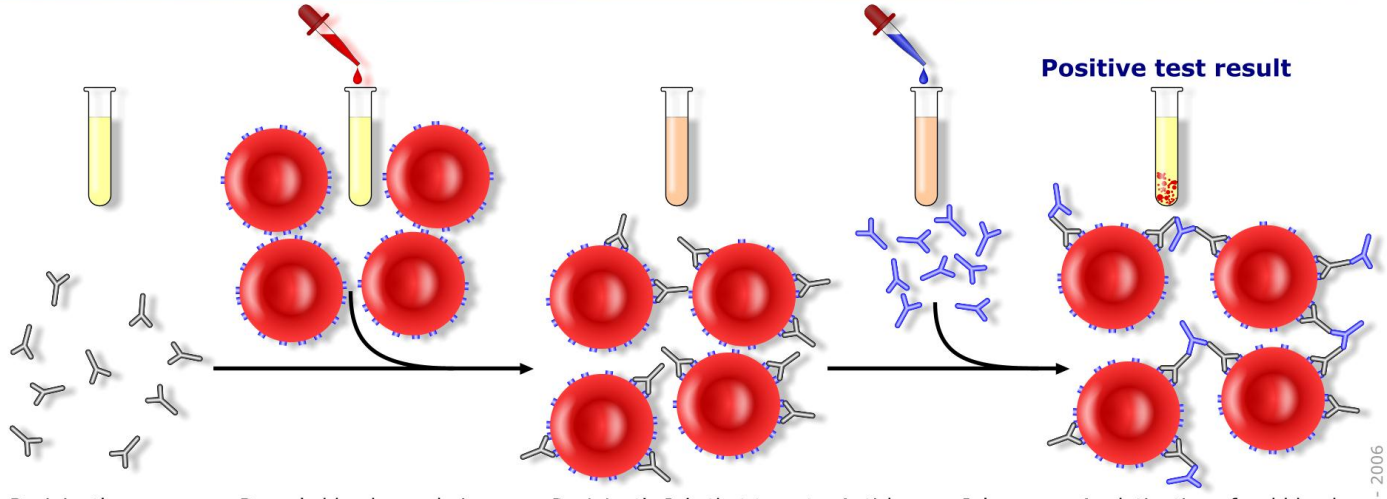


Blood sample from a patient with immune mediated haemolytic anaemia: antibodies are shown attached to antigens on the RBC surface.

The patient's washed RBCs are incubated with antihuman antibodies (*Coombs reagent*).

RBCs agglutinate: antihuman antibodies form links between RBCs by binding to the human antibodies on the RBCs.

Indirect Coombs test / Indirect antiglobulin test



Recipient's serum is obtained, containing antibodies (Ig's).

Donor's blood sample is added to the tube with serum.

Recipient's Ig's that target the donor's red blood cells form antibody-antigen complexes.

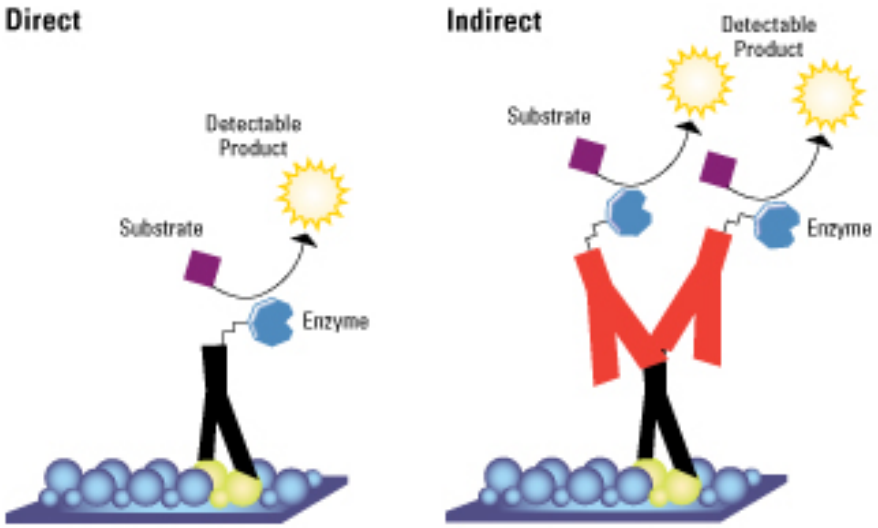
Anti-human Ig's (*Coombs antibodies*) are added to the solution.

Agglutination of red blood cells occurs, because human Ig's are attached to red blood cells.

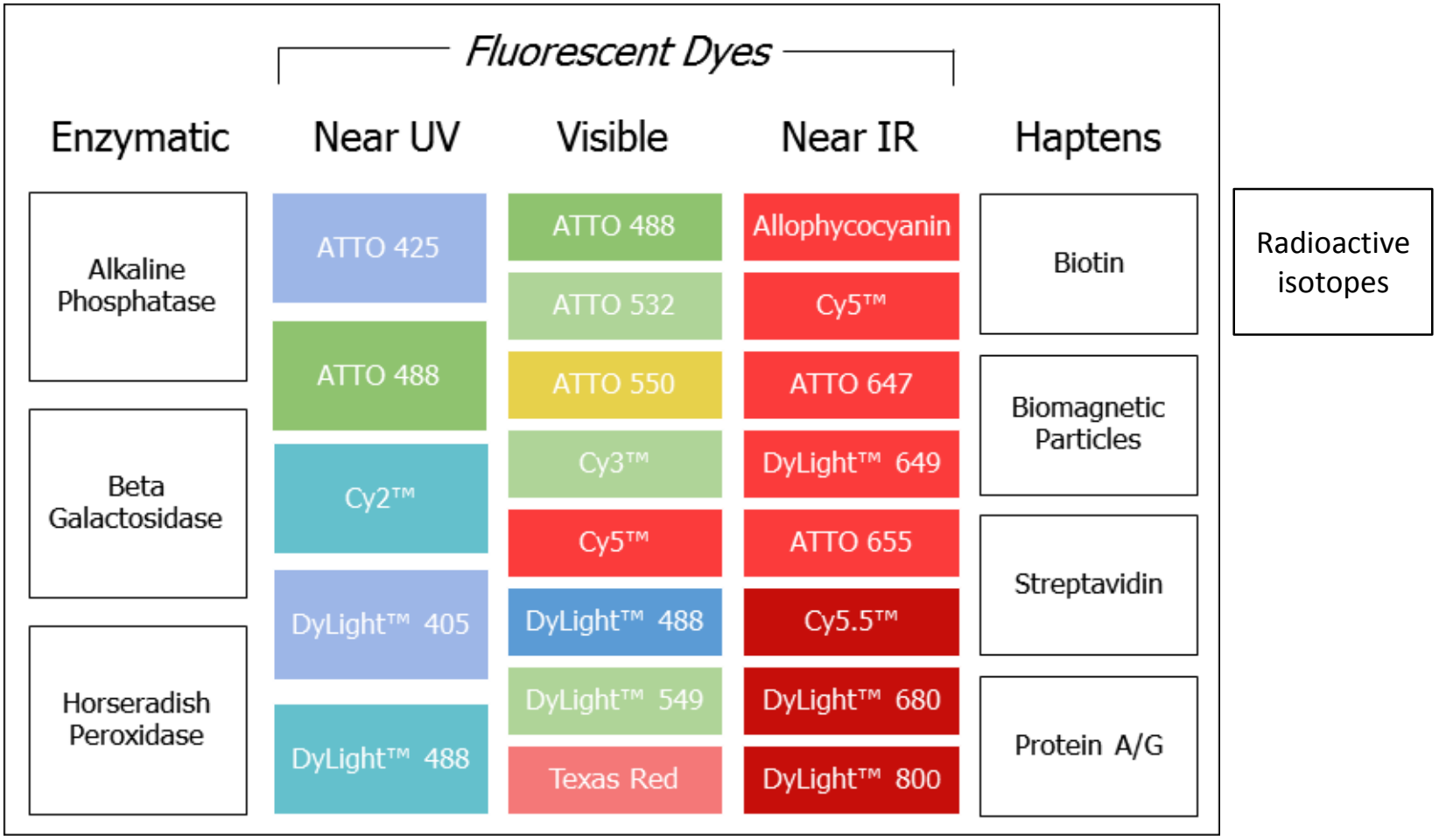
Titrations - By diluting a serum containing antibodies the quantity of the antibody in the serum can be gauged. This is done by using doubling dilutions of the serum and finding the maximum dilution of test serum that is able to produce agglutination of relevant RBCs

Immunoassays using labeled antibodies

- ELISA
- RIA
- Western blotting
- Immunoprecipitation
- Immunohistochemistry
- Immunofluorescence
- Flow cytometry
- Immunoelectron microscopy

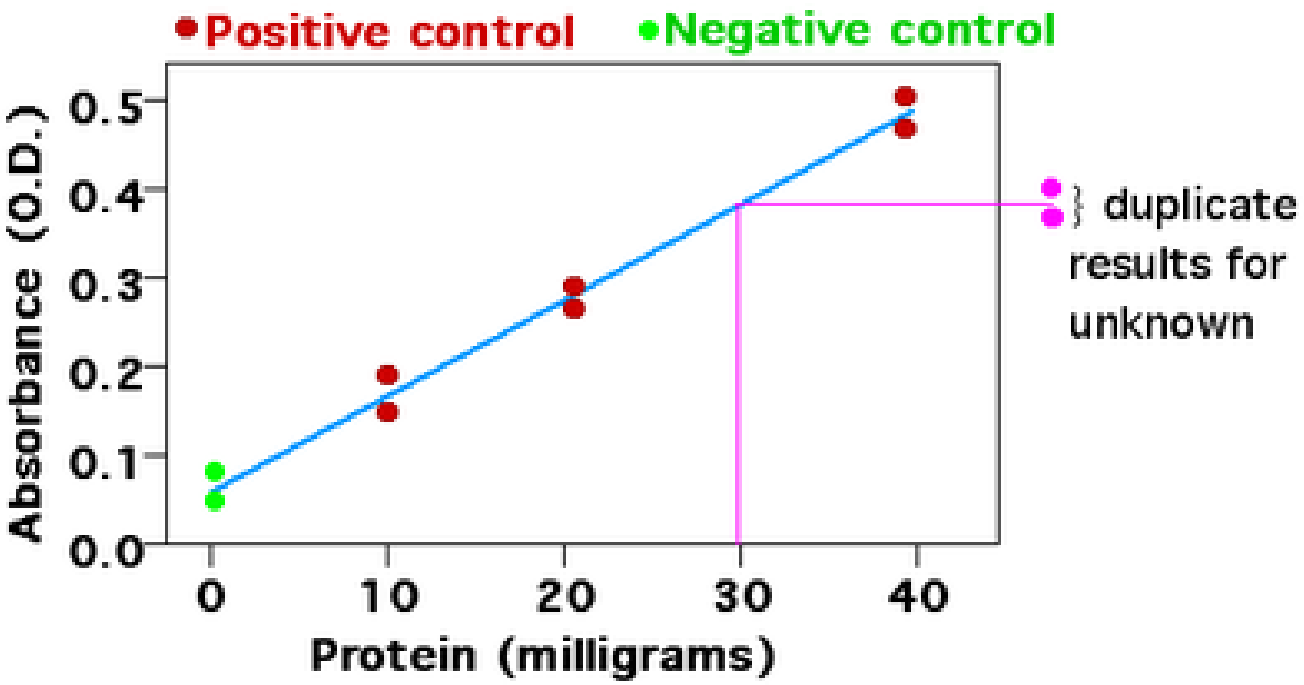


Antibody labeling



- Detection – fluorescence, colorimetric, luminescence

Standard curve

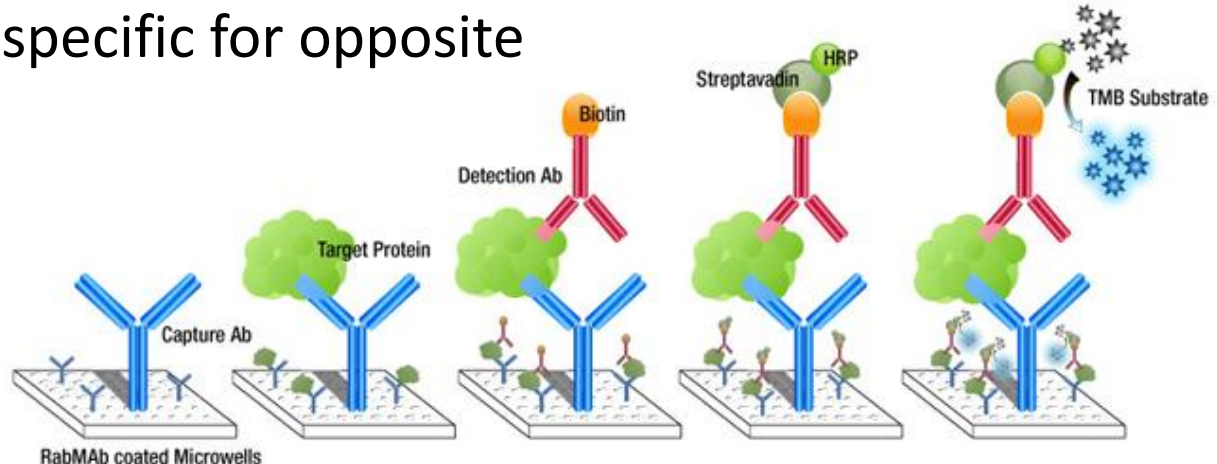


ELISA

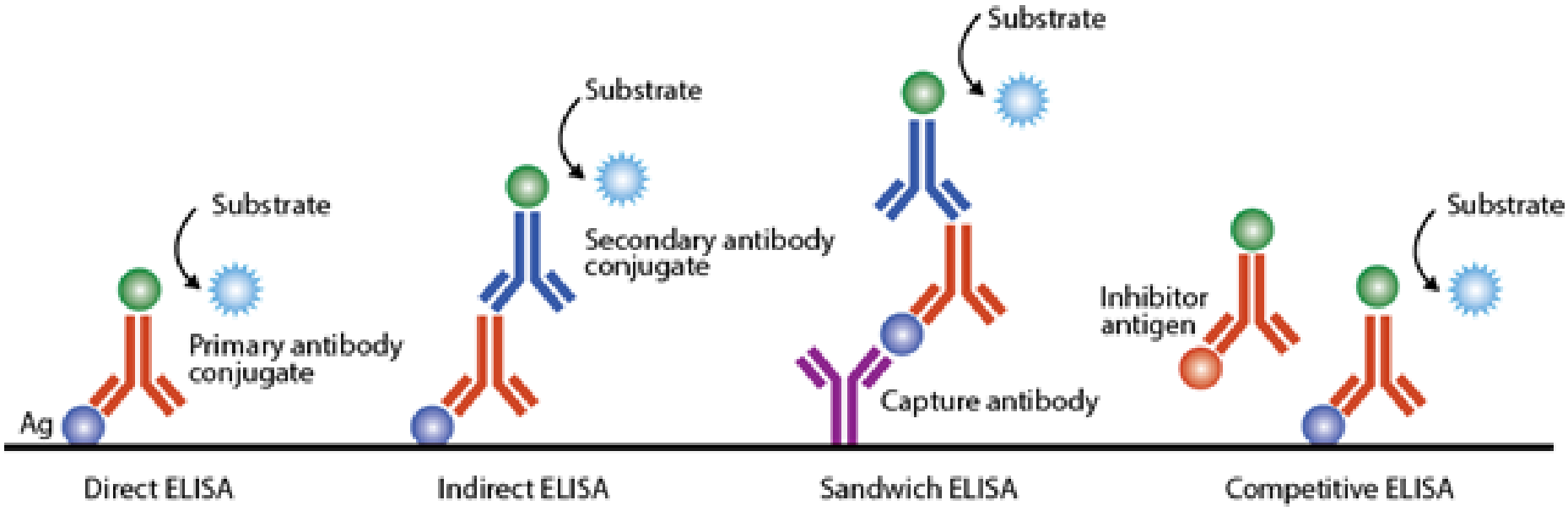
- **Enzyme-Linked ImmunoSorbent Assay**
- Each sample is pored into a well with immobilised antibodies specific for tested molecule (hormone, enzyme)
- A second specific antibody is added to each well in excess. This ab is bound to a color producing enzyme
- Well is washed out from unbound components
- A substrate specific for an enzyme is than added, and **color development** is measured by spectrophotometry

Sandwich ELISA- for very low concentration antigens (ex. Cytokines)

- Antibodies used are specific for opposite sides of molecule



ELISA

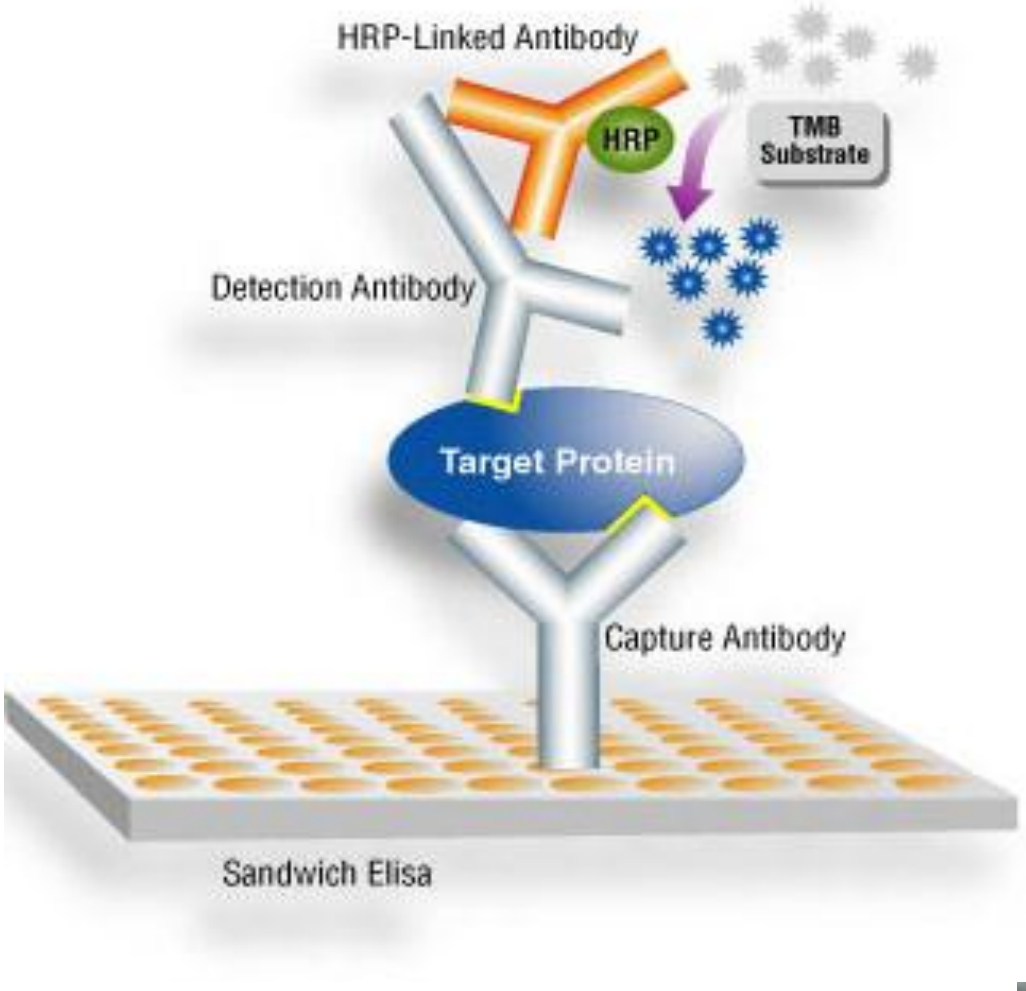


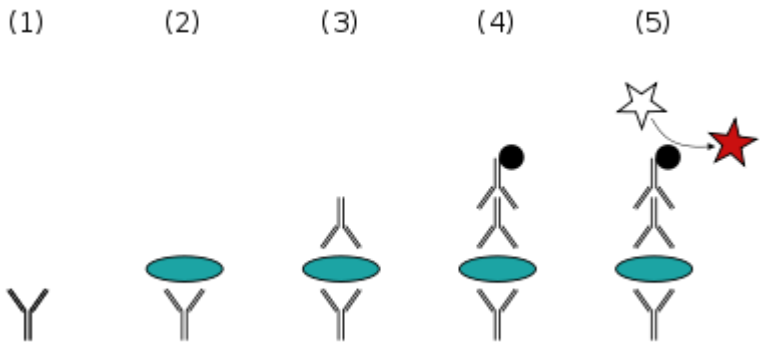
Antibodies
(HIV,
Mycobacterium)

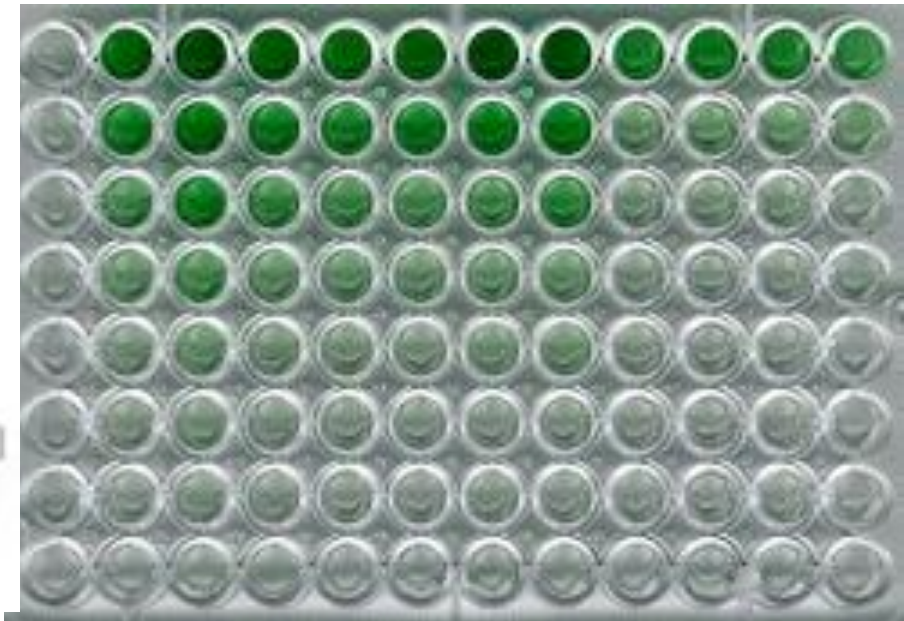
Hormones
Tumor markers
Serum proteins
Cytokines
Drugs

T3, T4
Progesterone
Free testosterone

Sandwich - ELISA



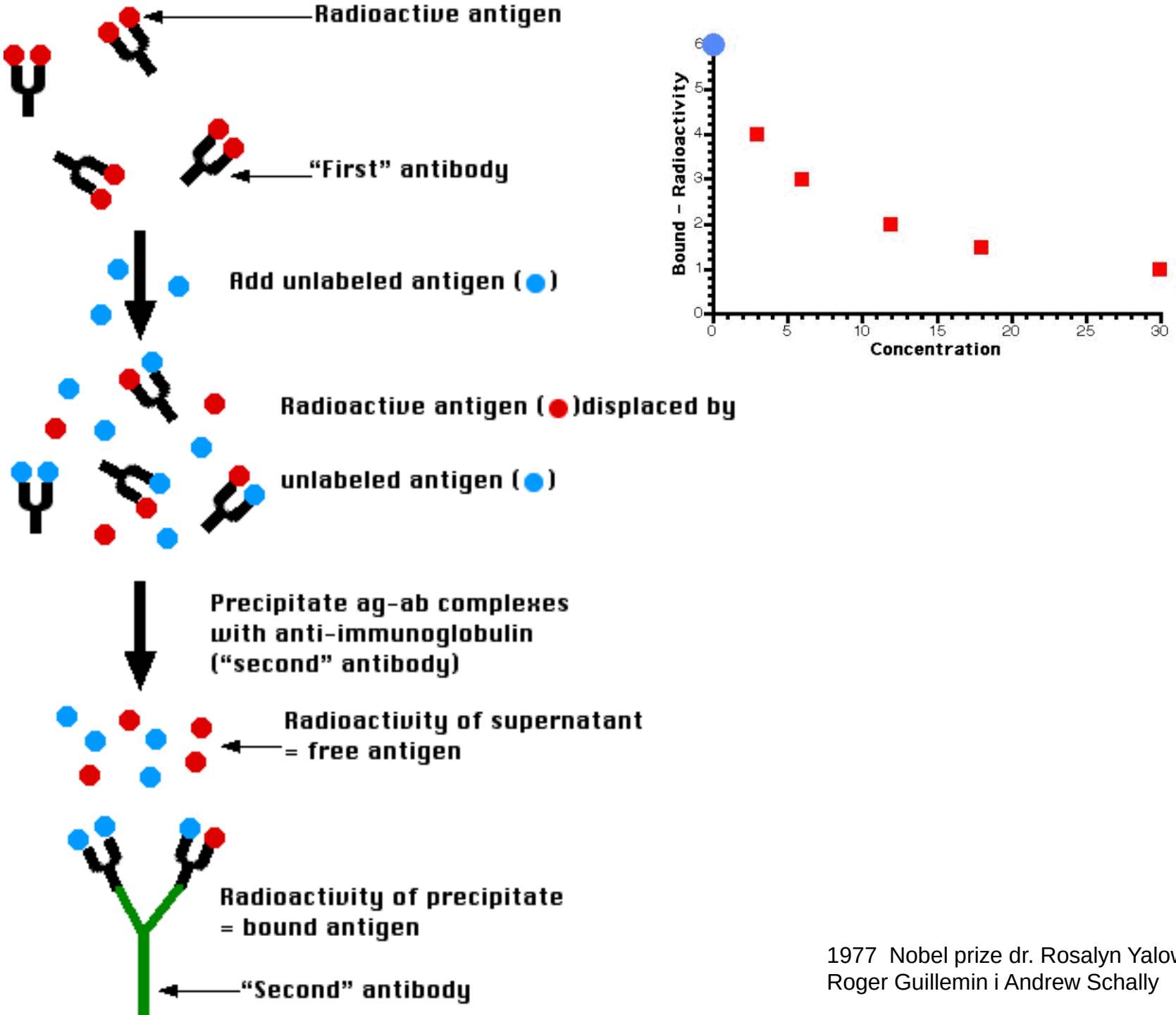




RIA

Radioimmunoassay

- More sensitive than ELISA test
- Used for measuring blood hormone concentration
- Procedure: Standard curve preparation
  - Standard radioisotope bound (marked) antigen (egz. hormone) binds to antibody
  - After adding unmarked antigen in a known concentration, some of the marked antigen is displaced from antibody, due to competition.
  - A binding curve of radioactivity is plotted for several known concentrations.
- Now, an unknown hormone concentration can be measured in tested sample (serum, urine, saliva, etc.) by comparing results to plotted curve.

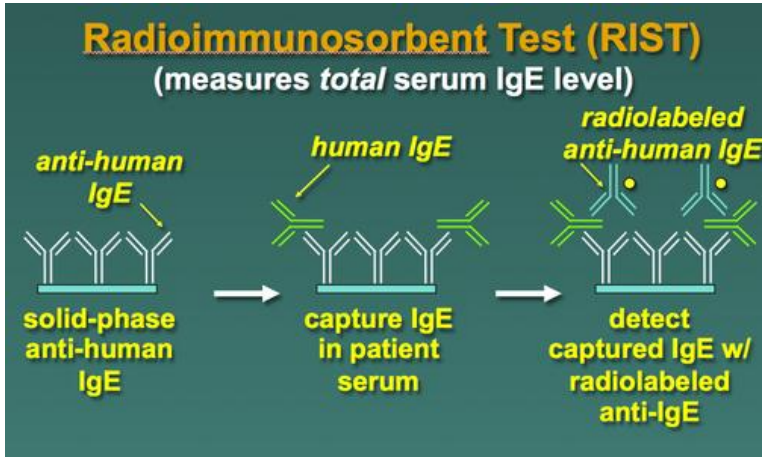


1977 Nobel prize dr. Rosalyn Yalow, Roger Guillemin i Andrew Schally

Allergic response testing

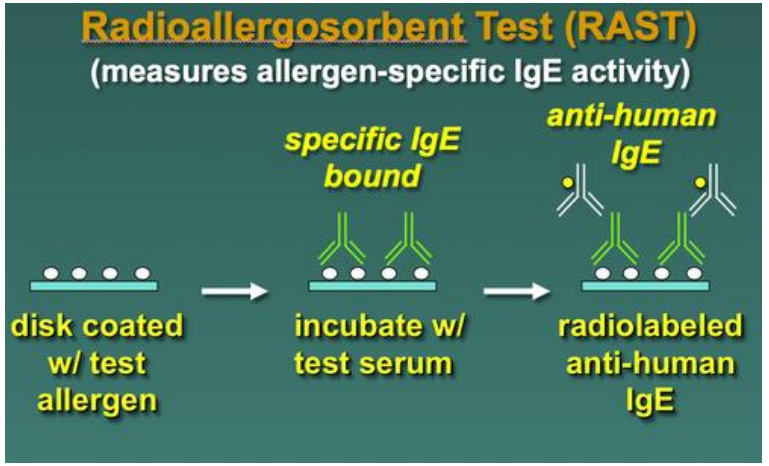
RIST

- Measures the total serum IgE

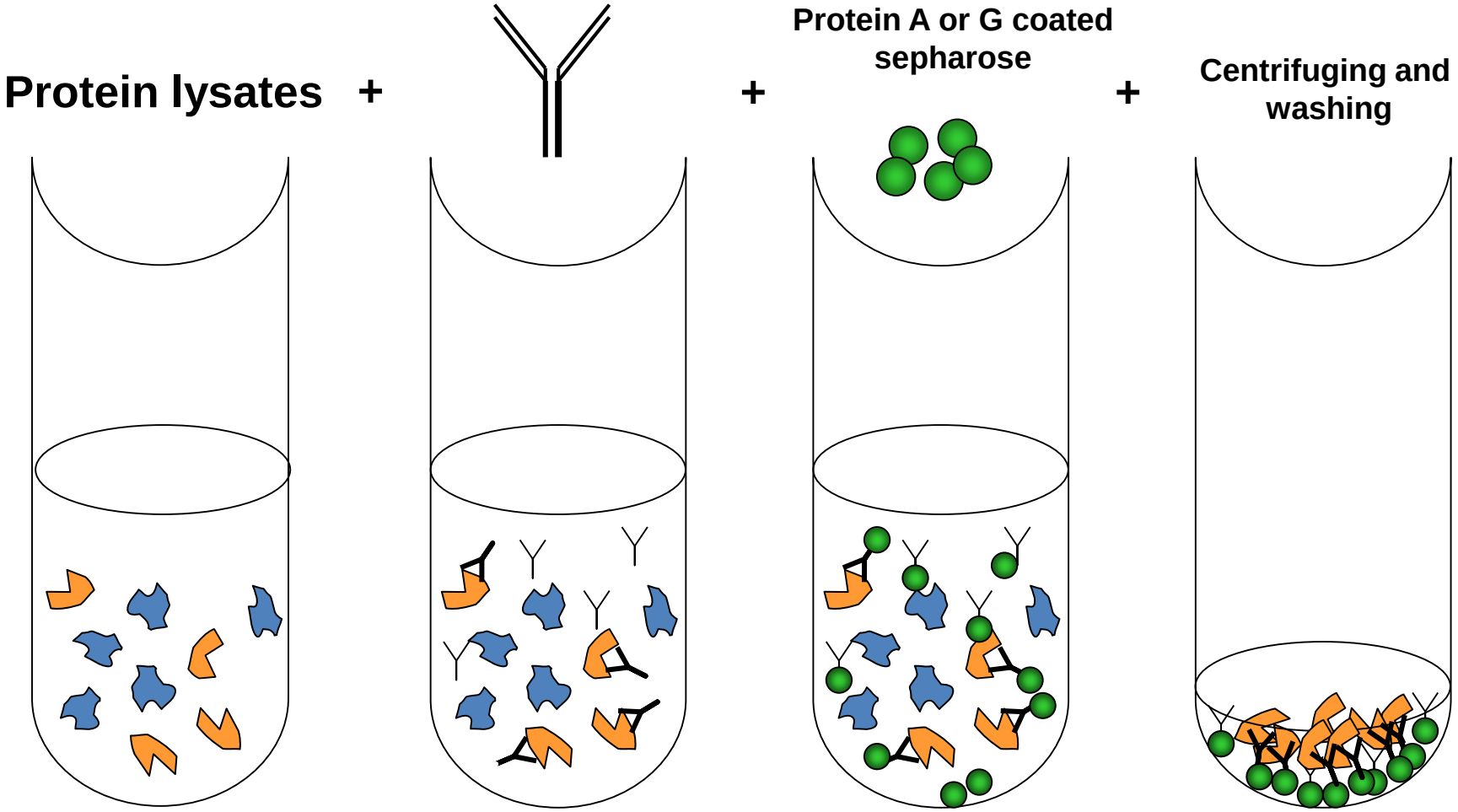


RAST

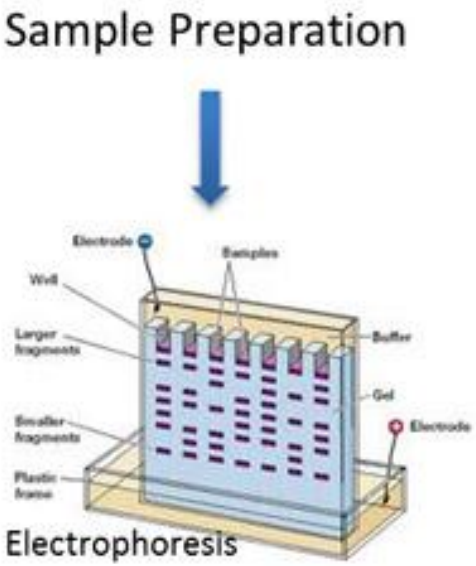
- Measures the antigen specific IgE

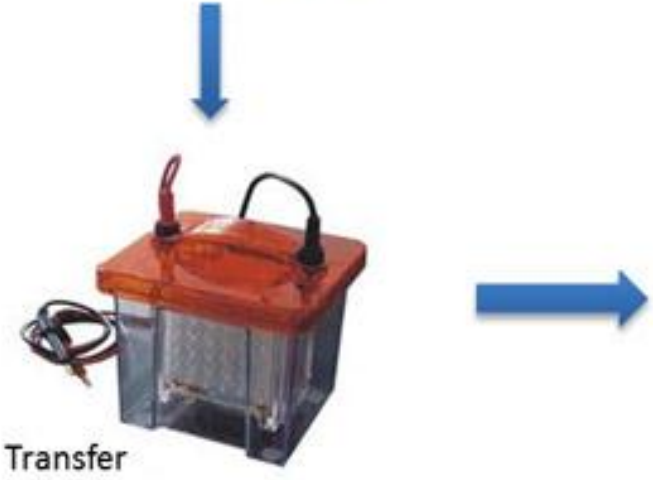


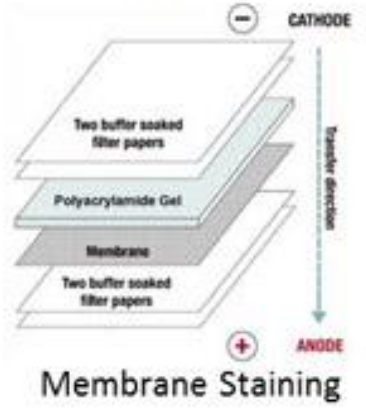
Immunoprecipitation

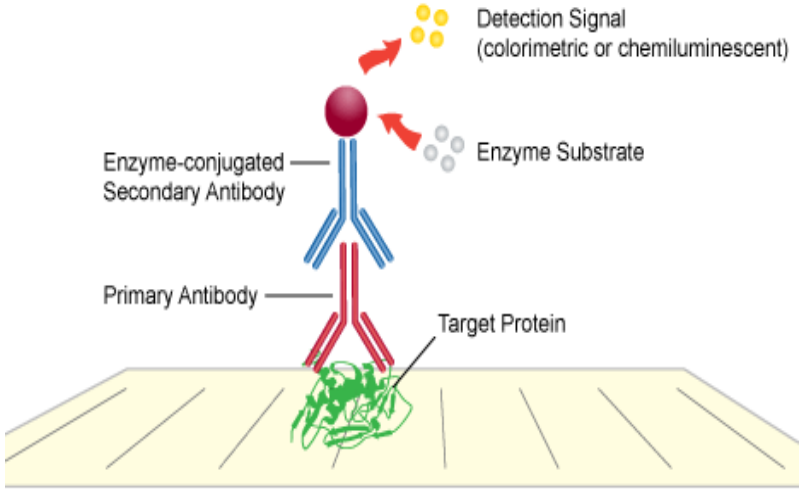


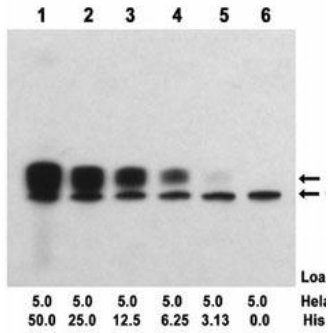
Western blotting

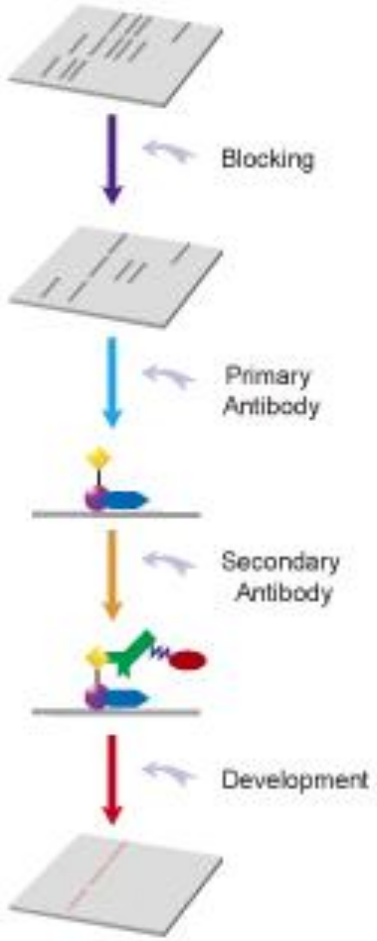




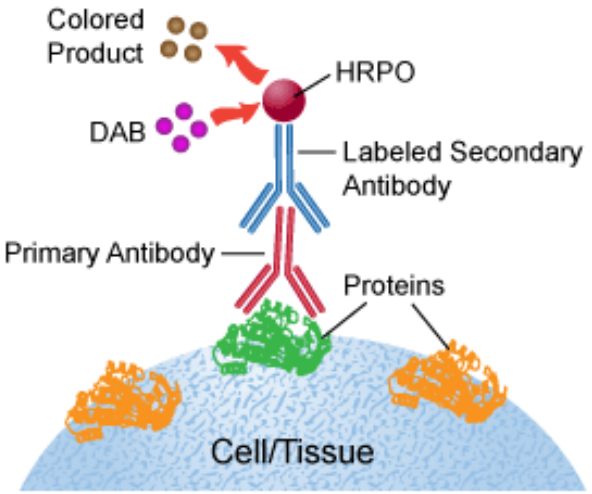


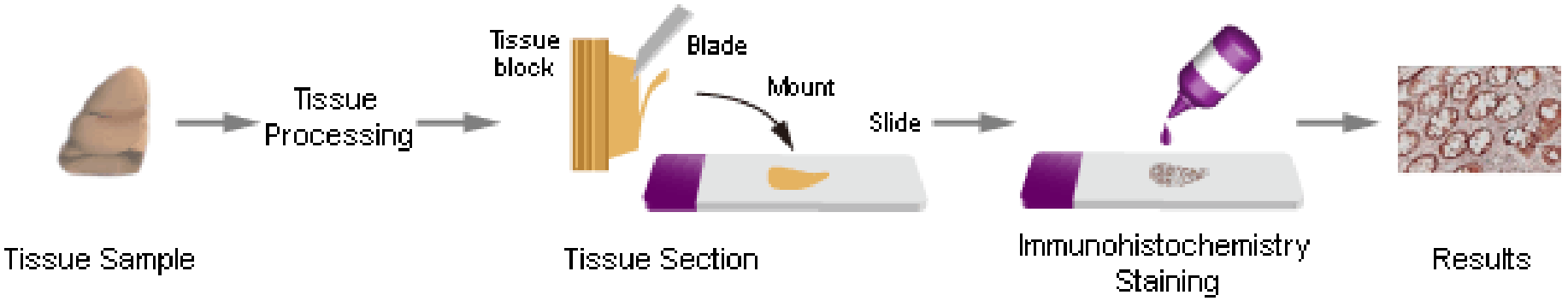


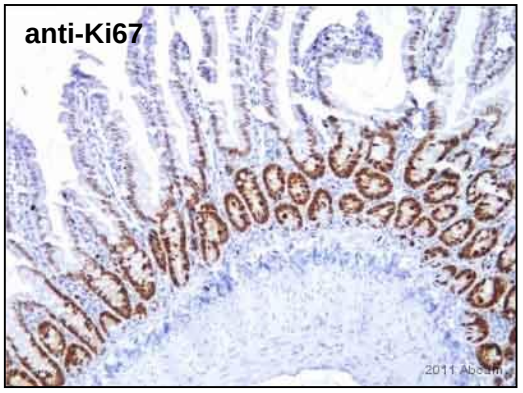


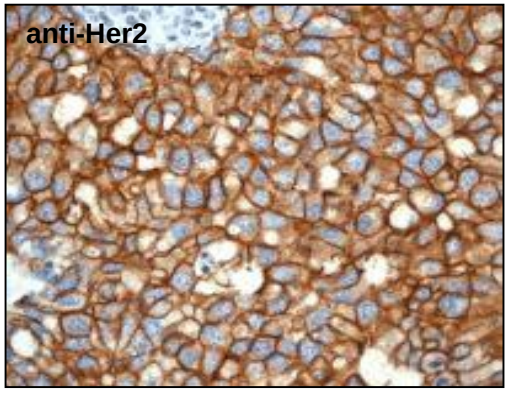


Immunohistrochemistry

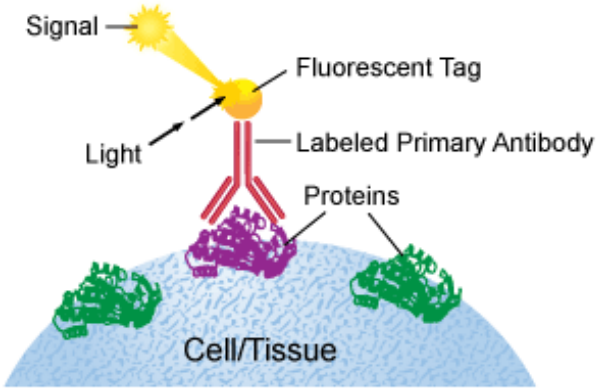


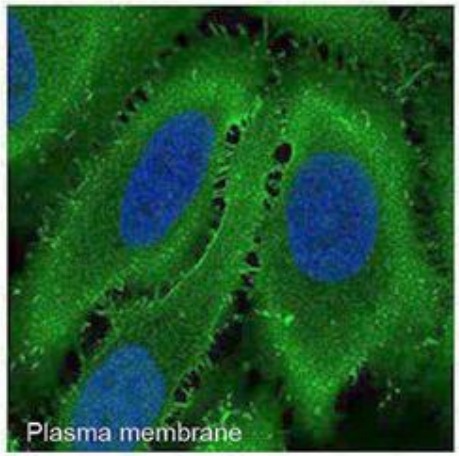


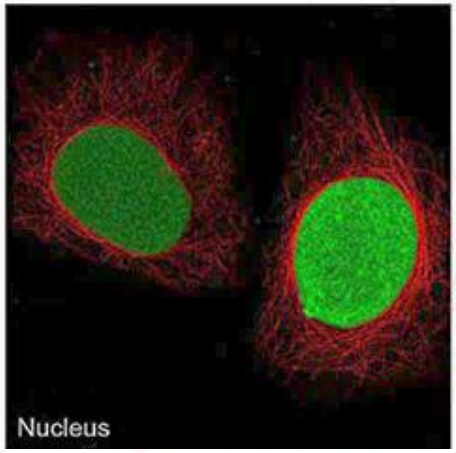


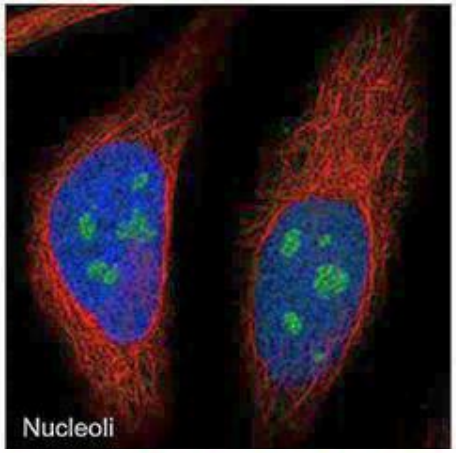


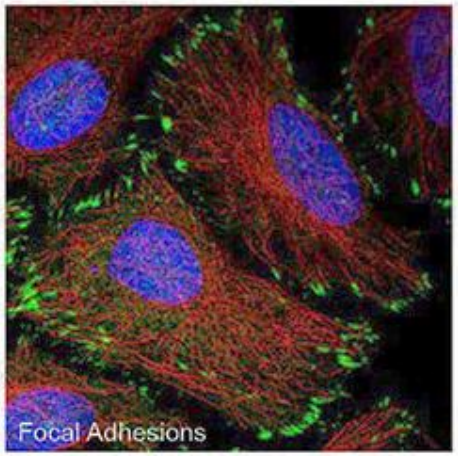
Immunofluorescence

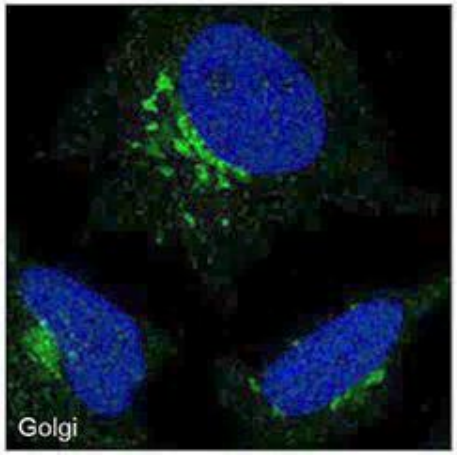


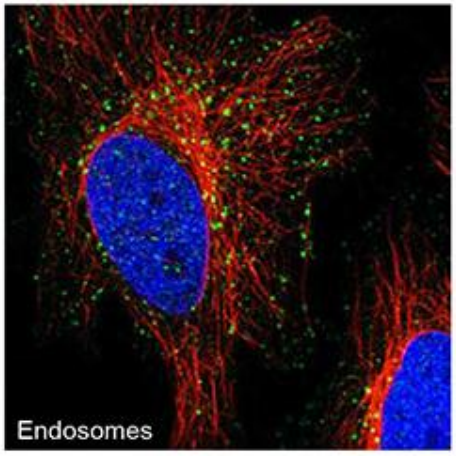


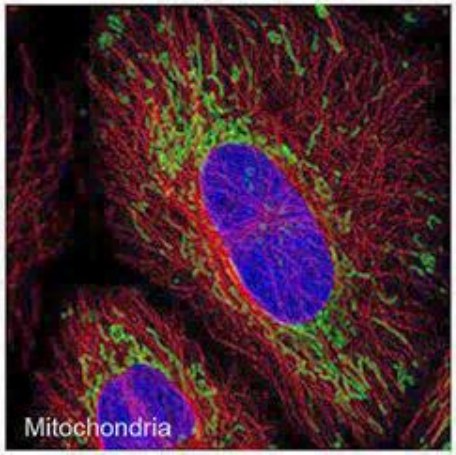


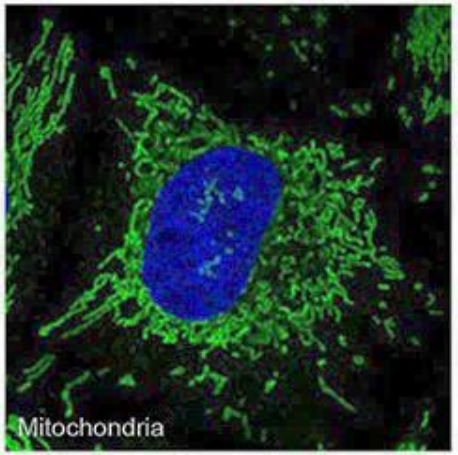






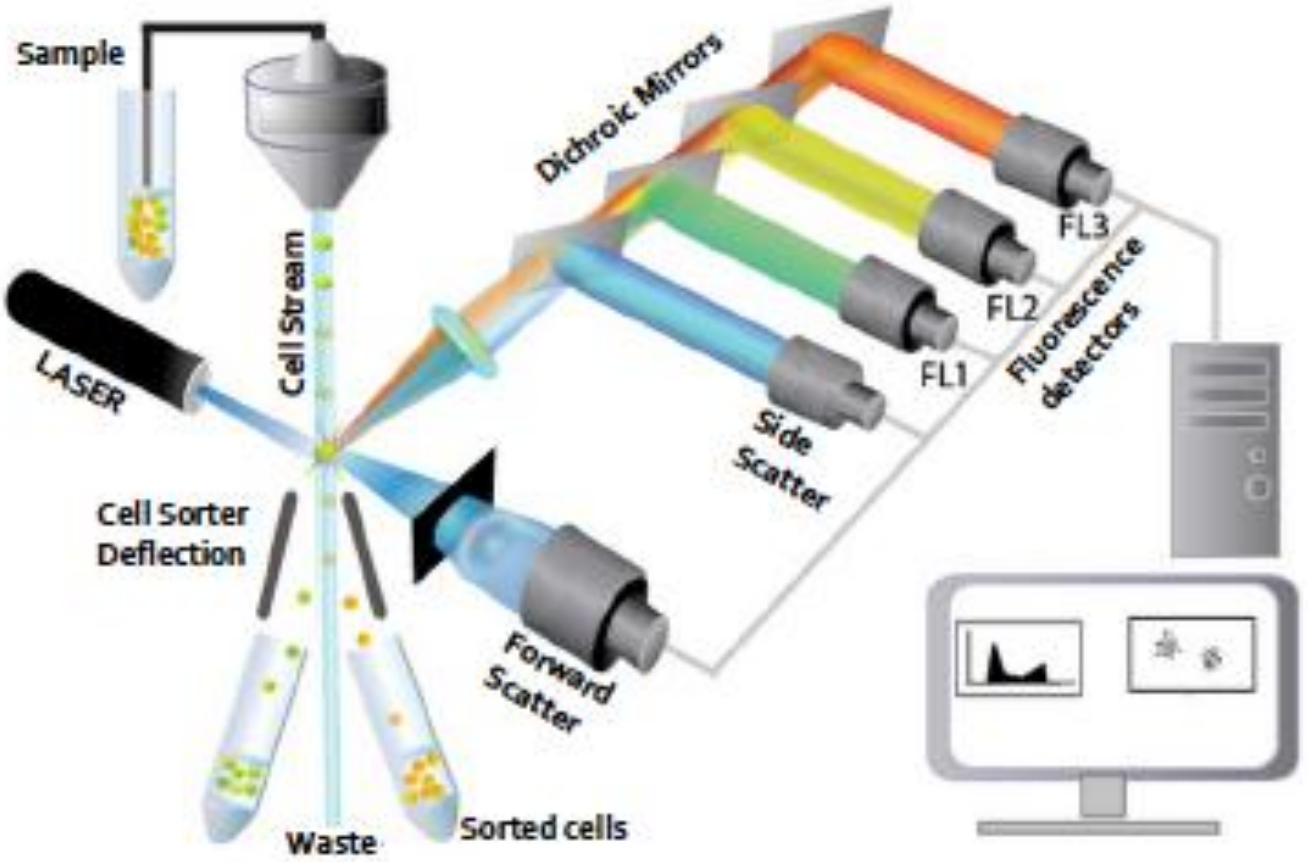


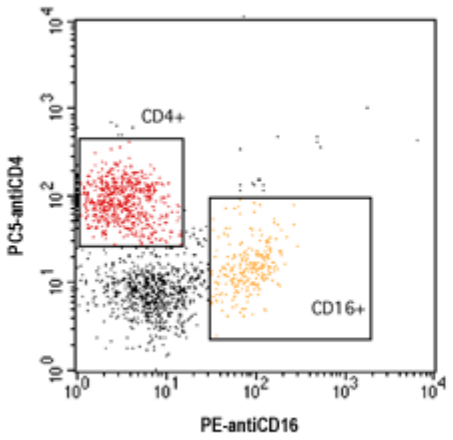


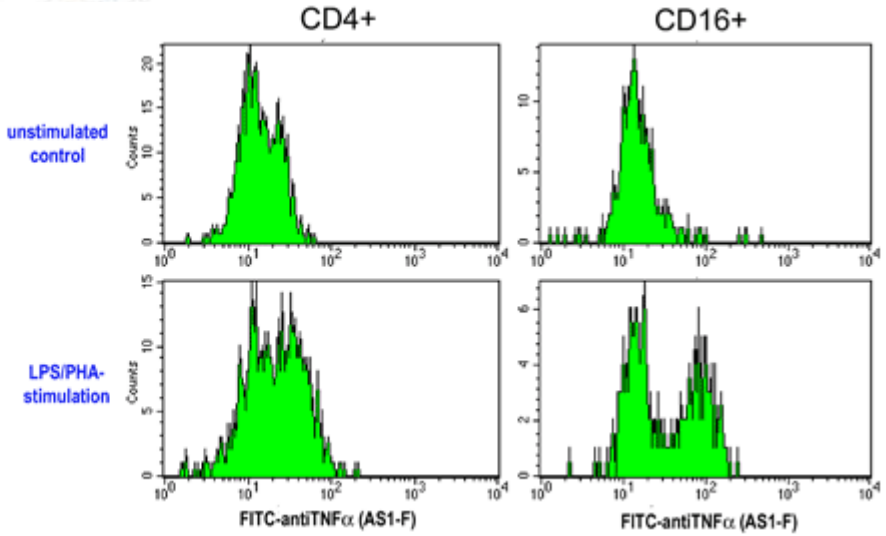


http://www.sigmaaldrich.com/

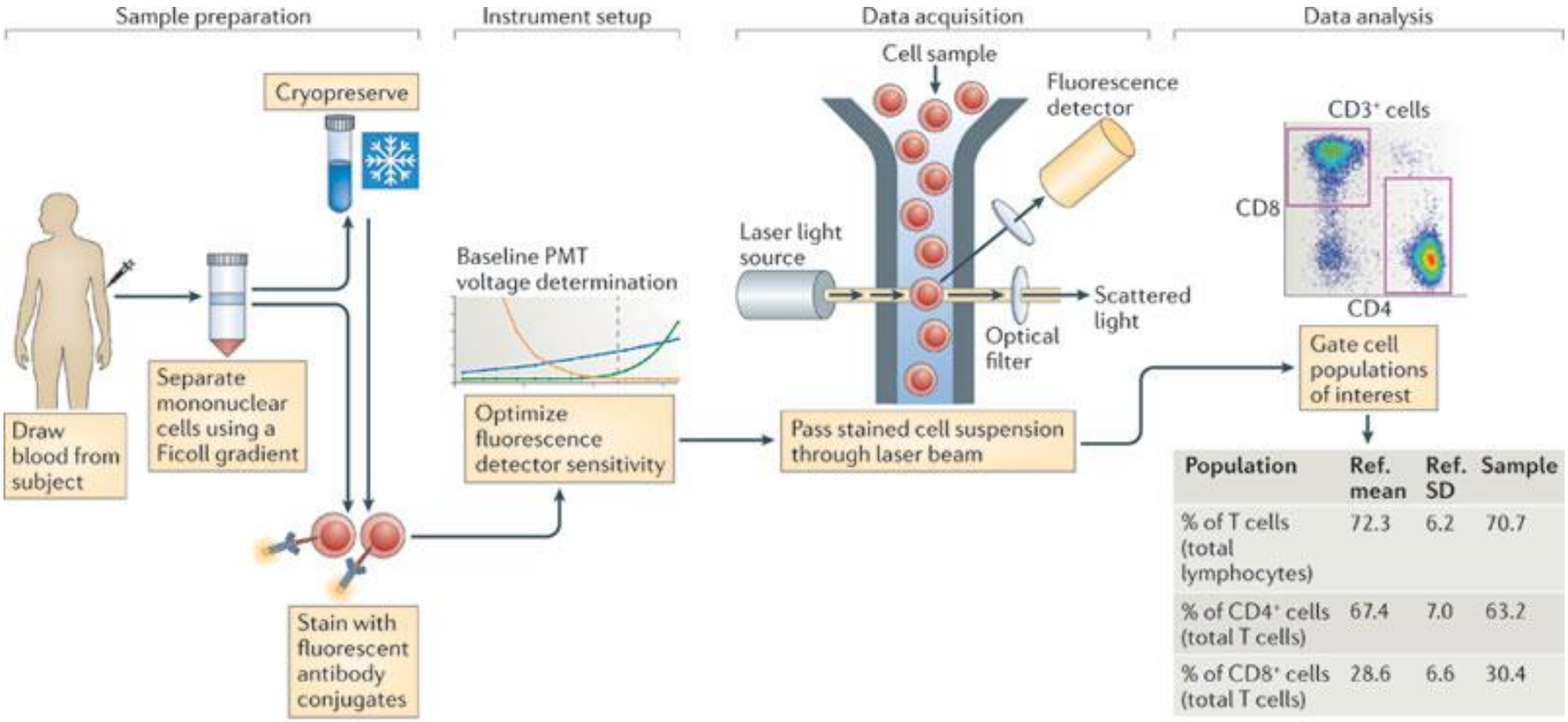
Flow cytometry







Immunophenotyping



Population	Ref. mean	Ref. SD	Sample
% of T cells (total lymphocytes)	72.3	6.2	70.7
% of CD4⁺ cells (total T cells)	67.4	7.0	63.2
% of CD8⁺ cells (total T cells)	28.6	6.6	30.4

Nature Reviews | Immunology

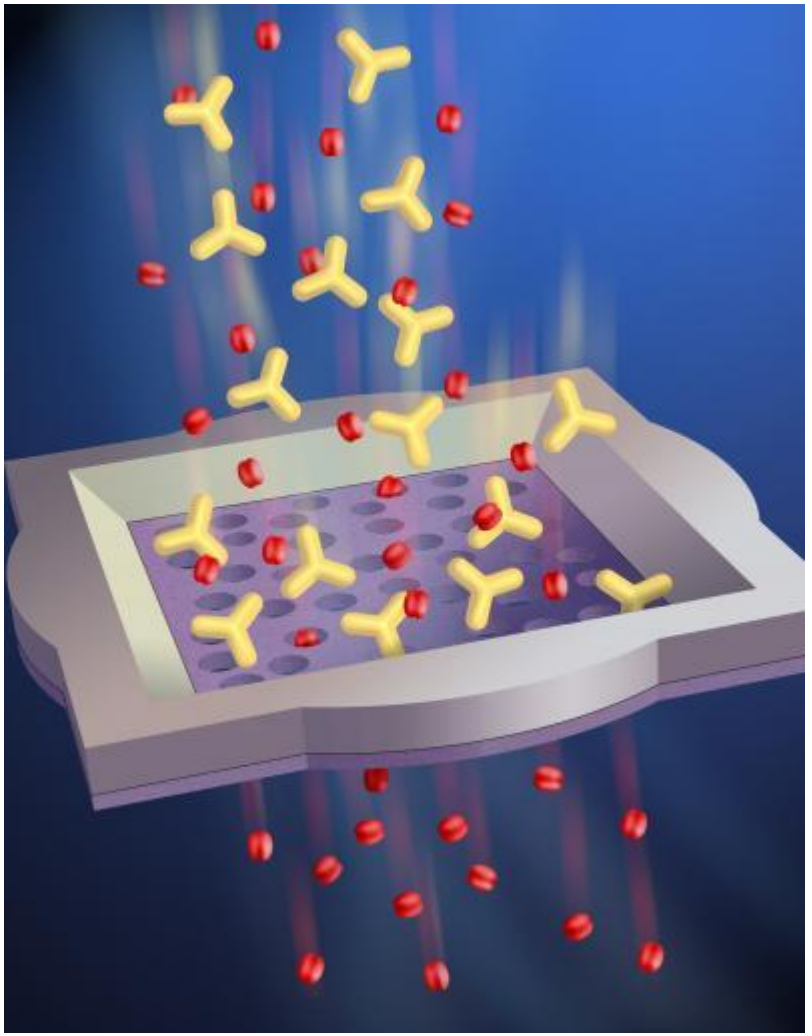
Haematopoietic stem cells

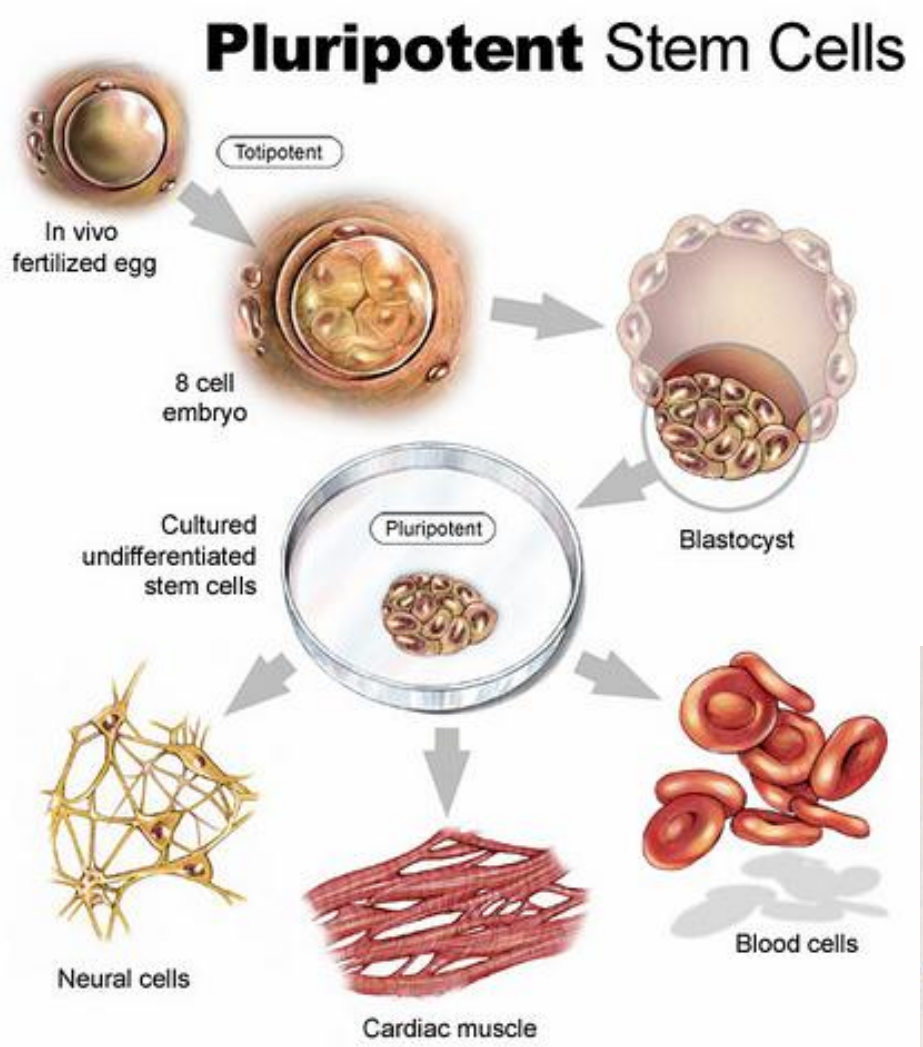
- Hematopoietic stem cell therapy – for patients with dysfunctional hematopoiesis (leukemia, irradiation)
- Injected cells find their way to bone marrow through blood stream
- Approximately 10% of donor bone marrow is transplanted
- Umbilical cord blood stem cells

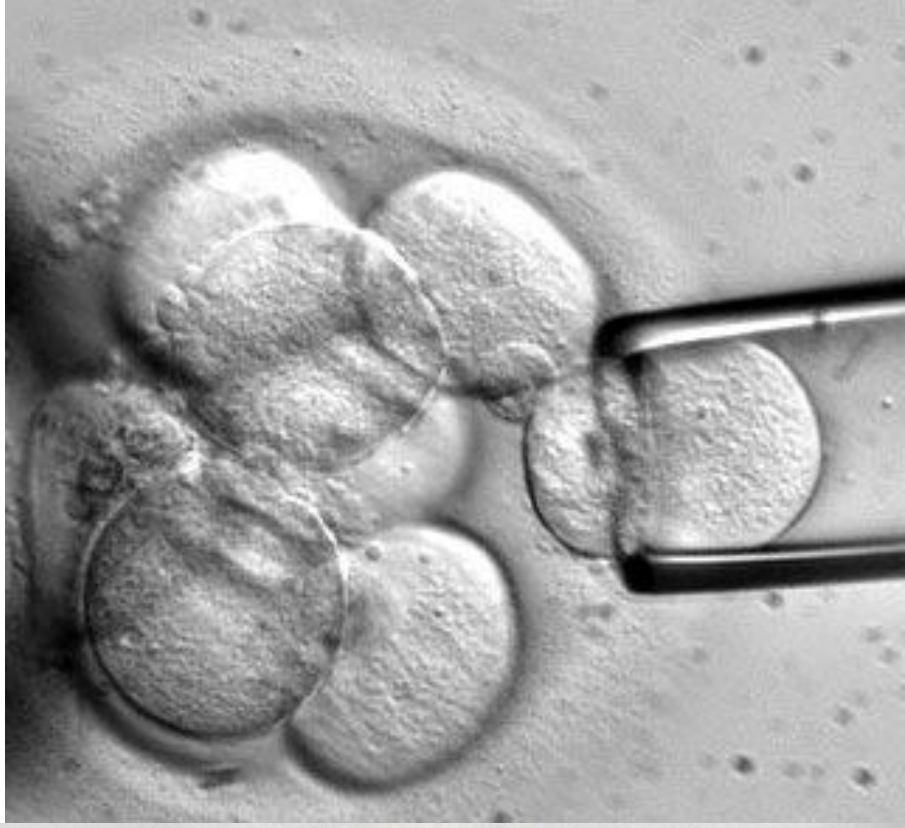
- Transplant types:
  - **Autologous** (frozen, or genetically manipulated)
  - **Syngeneic** (identical twins)
  - **Allogeneic** (common, possibility of GVHD)

Haematopoietic stem cells concentration

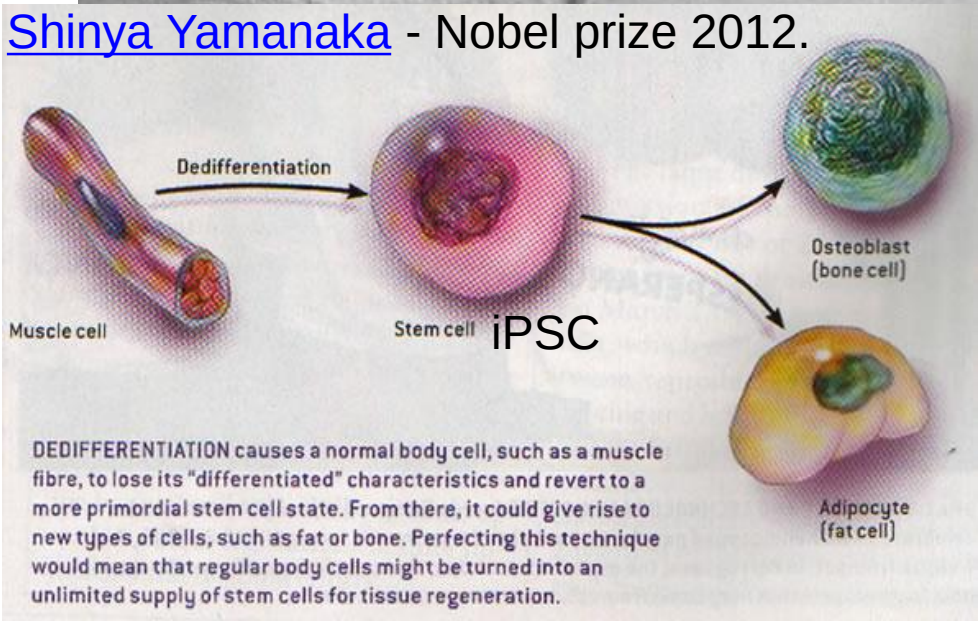
- Bone marrow is mixed with antibodies against mature red and white blood cells
- Using flow cytometry cell sorter (FACS) ab marked cells are selected and discarded
- Unmarked nondifferentiated hematopoietic stem cells are concentrated







[Shinya Yamanaka](#) - Nobel prize 2012.



Stem Cells

Stem cell type	Description	Examples
Totipotent	Each cell can develop into a new individual	Cells from early (1-3 days) embryos
Pluripotent	Cells can form any (over 200) cell types	Some cells of blastocyst (5 to 14 days)
Multipotent	Cells differentiated, but can form a number of other tissues	Fetal tissue, cord blood, and adult stem cells