



IMMUNOLÓGIAI ÉS  
BIOTECHNOLÓGIAI  
INTÉZET



# 8th practice: Immunoserology 1. precipitation, agglutination

Basic Immunology

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## Humoral immune responses - Summary

1. In humoral immune responses B cells may participate in various ways, as determined by their origin (B-1/B-2) or location (follicular/FoB or marginal zone/MzB). During immune responses activated B cells undergo differentiation towards plasma cells or memory B cells.
2. B-1 cells contribute to antibody production through continuous self-renewal and immediate plasmacellular transformation. B-2 cells participate in immune responses either as antigen recognizing cells (FoB) or antigen transporting cells (MzB), during which B cells also act as antigen presenting cells for the selection and differentiation of follicular T helper cells (TFh).
3. In the induction of B-cell responses the structure/type of antigen and the lymphoid tissue microenvironment are of critical significance; in the process non-clonal receptor-ligand pairs (cytokines and their receptors, activation molecules and their partners) participate, in addition to the antigen binding by BcR.

## Humoral immune responses - Summary

4. T-independent antigens induce immediate plasmacellular differentiation outside follicles via the simultaneous cross-linking of large number of B-cell receptors; during the process short-lived plasma develop cells that produce overwhelmingly IgM.
5. The differentiation of B cells activated by T-dependent antigen is promoted by TFh-derived signals (CD40L, CD28, cytokines) and altered chemokine preference; the proliferation of activated B cells (centroblasts) is maintained via contacts with follicular dendritic cells (FDC).
6. The point mutations of the BcR Ig V-regions induced by AID upon binding to antigens preserved by FDCs (iccosome) promote the survival of high-affinity centrocytes and the formation of memory cells. The transcriptional regulation (Pax-5/BLIMP-1) of centrocytes determines the memory B or plasmacellular path of differentiation, the T-cell derived cytokines define the isotype of Ig heavy chain produced by the plasma cells.

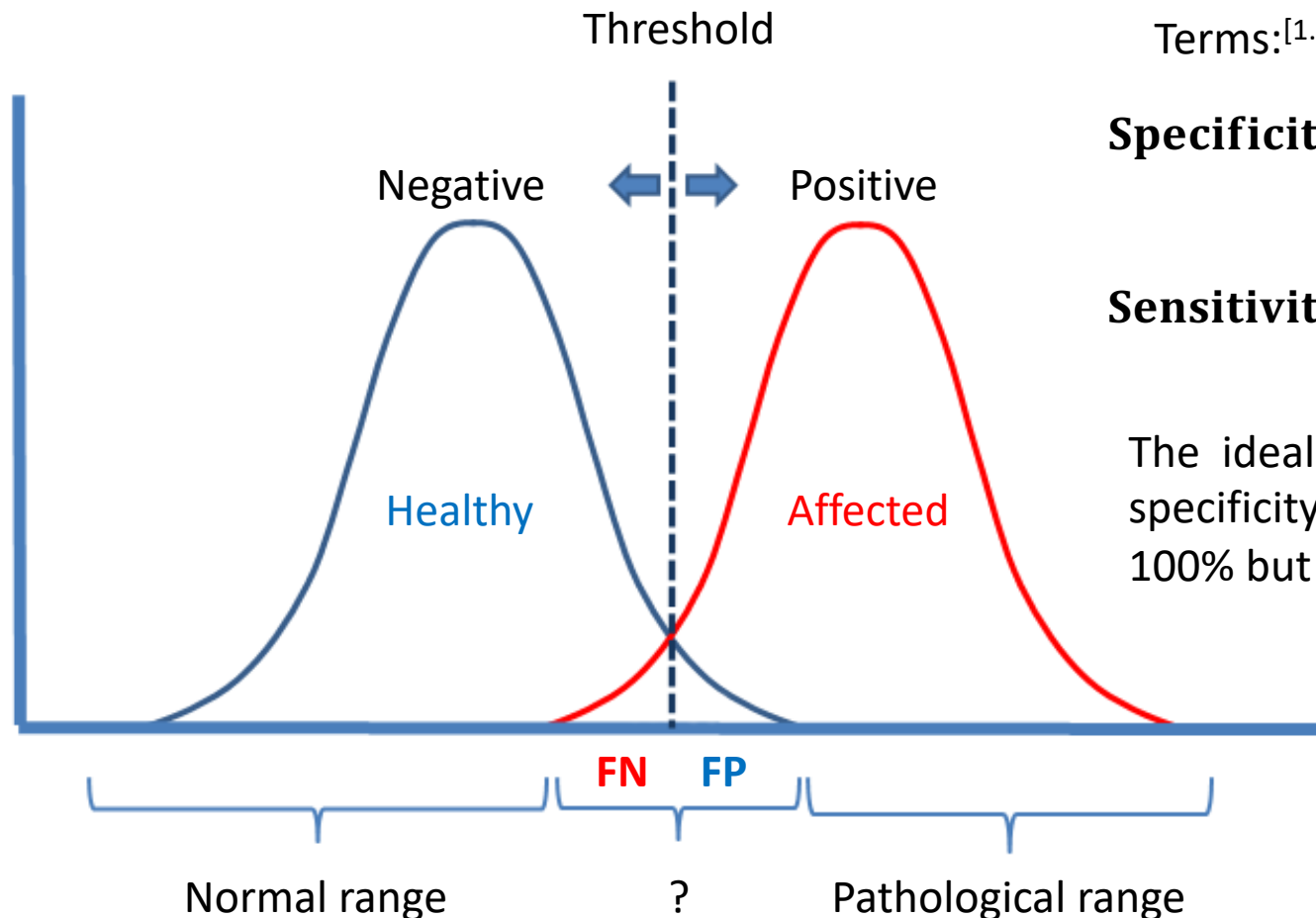
# Definition of serology

- The scientific research of **blood serum** or other body fluids; in practice it usually refers to the identification of **antibodies** in the serum.
- Do you remember?
  - **Blood plasma**: supernatant of anticoagulated blood
  - **Blood serum**: supernatant of coagulated blood
- Also based on the **antigen-antibody reaction**. (both can be detected)
- Which methods does it include?
  - Methods based on **precipitation**
  - Methods based on **agglutination**
  - **Immunoassays** (ELISA, ELISPOT, radioimmunoassay, etc., see in next practice)
  - **Immunoblot techniques** (Western blot, Dot blot, see in next practice)
  - **Indirect immunofluorescence microscopy**
- Main clinical applications:
  - Diagnostics of **infectious diseases** (e.g. detection of antibodies produced against the pathogens)
  - Diagnostics of **autoimmune disorders** (detection of autoantibodies)
  - Diagnostics of **immunodeficiencies** (measuring the levels of immunoglobulins)
  - Checking **blood types**

# Specificity, sensitivity

**FN = False negative**

**FP = False positive**



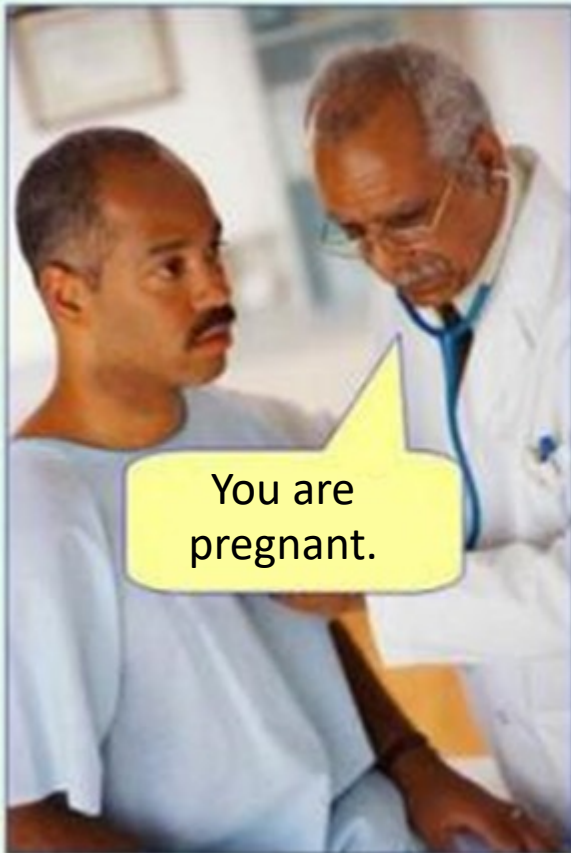
Terms:[1.]

$$\text{Specificity} = \frac{\text{Real negatives}}{\text{All negatives}}$$

$$\text{Sensitivity} = \frac{\text{Real positives}}{\text{All positives}}$$

The ideal diagnostic test has a specificity and sensitivity of 100% but **no such test exists.**

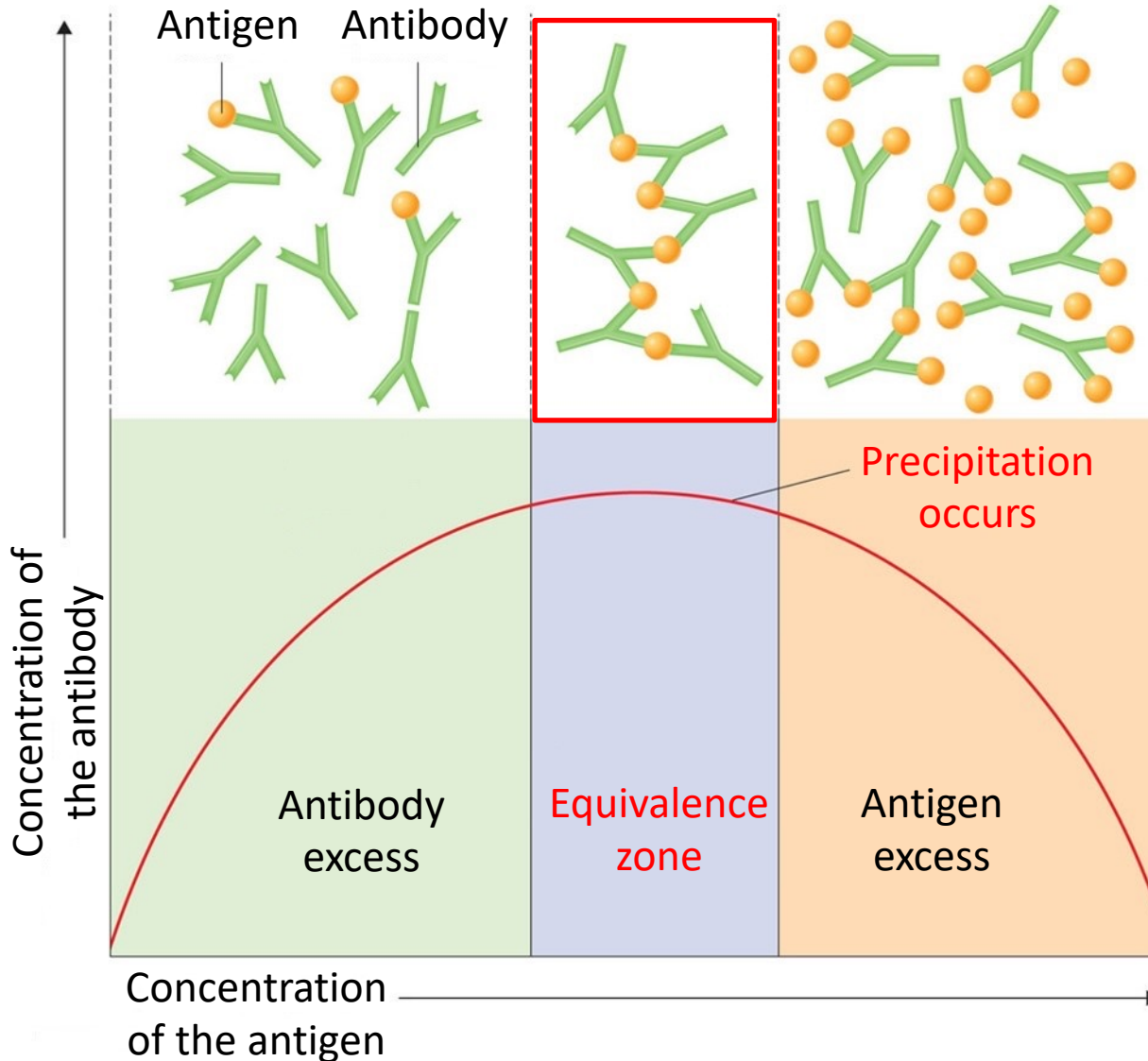
False positive  
result



False negative  
result



# Precipitation



If the antigen and the recognizing antibody are in the same solution at appropriate ratio (equivalence zone) then they will form larger immunocomplexes.



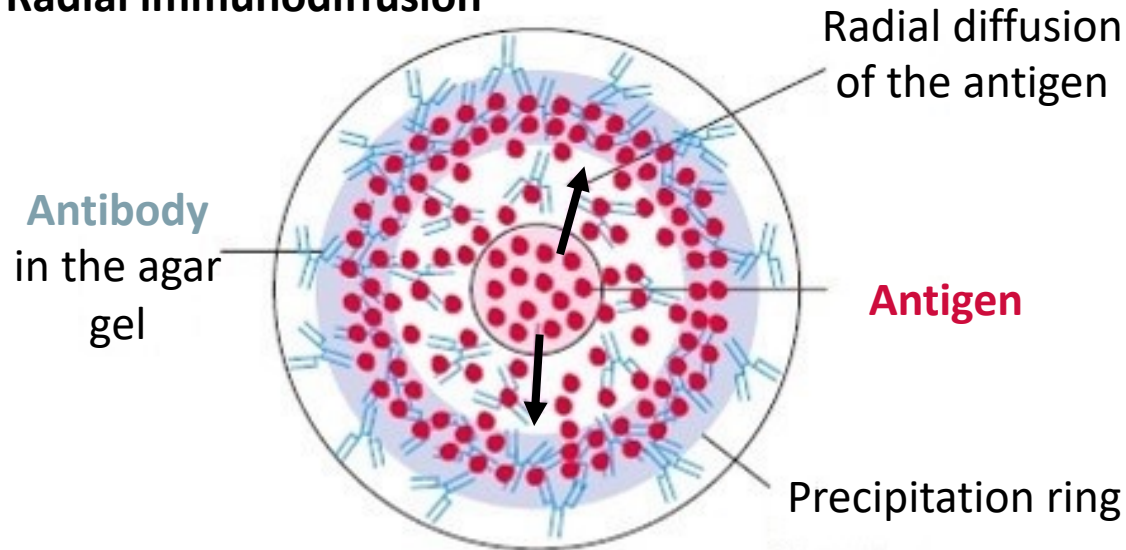
Solubility of these protein complexes decreases and they will precipitate.

Methods based on immunoprecipitation:

- **Immunodiffusion**
- **Immunoelectrophoresis**

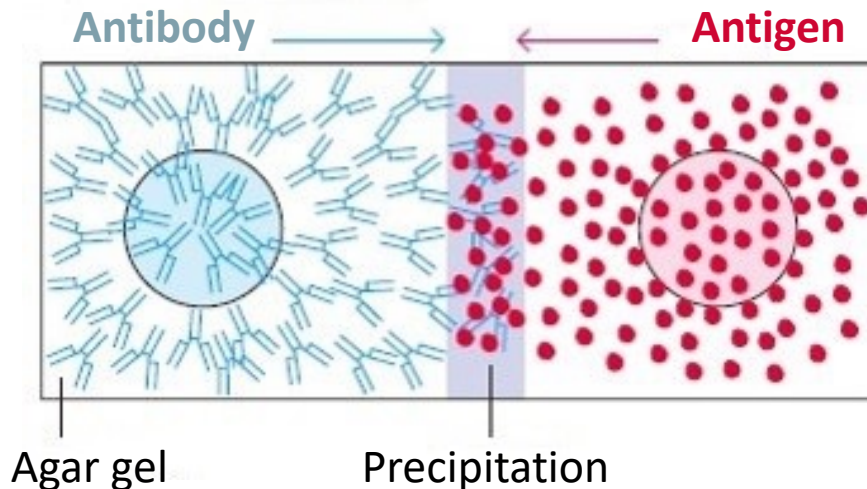
# Immunodiffusion I.

## Radial immunodiffusion



Simple but **outdated** techniques.

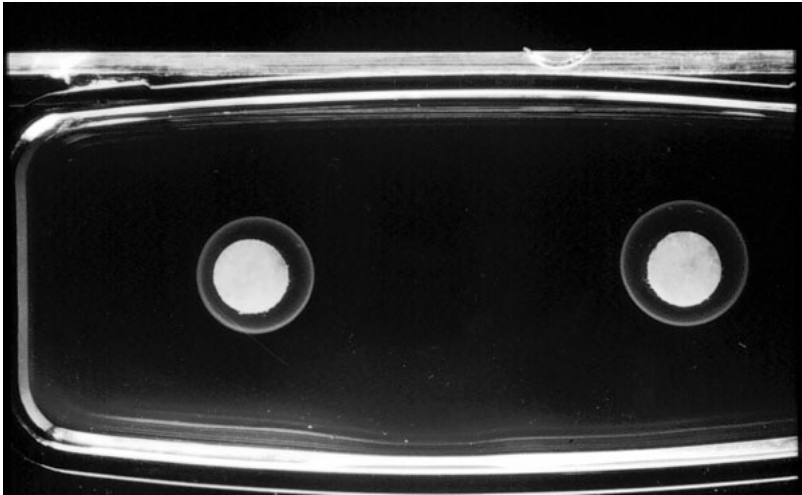
## Double immunodiffusion





# Immunodiffusion II.

**Mancini**<sup>[2.]</sup> radial immunodiffusion:



The antigen is evenly incorporated into the agar gel. Then serum sample is placed into the hole in the gel. The antibodies in the serum will diffuse radially. Once the antigen-antibody concentration reaches the equivalence zone they will form a precipitation ring.

**Semiquantitative** method.

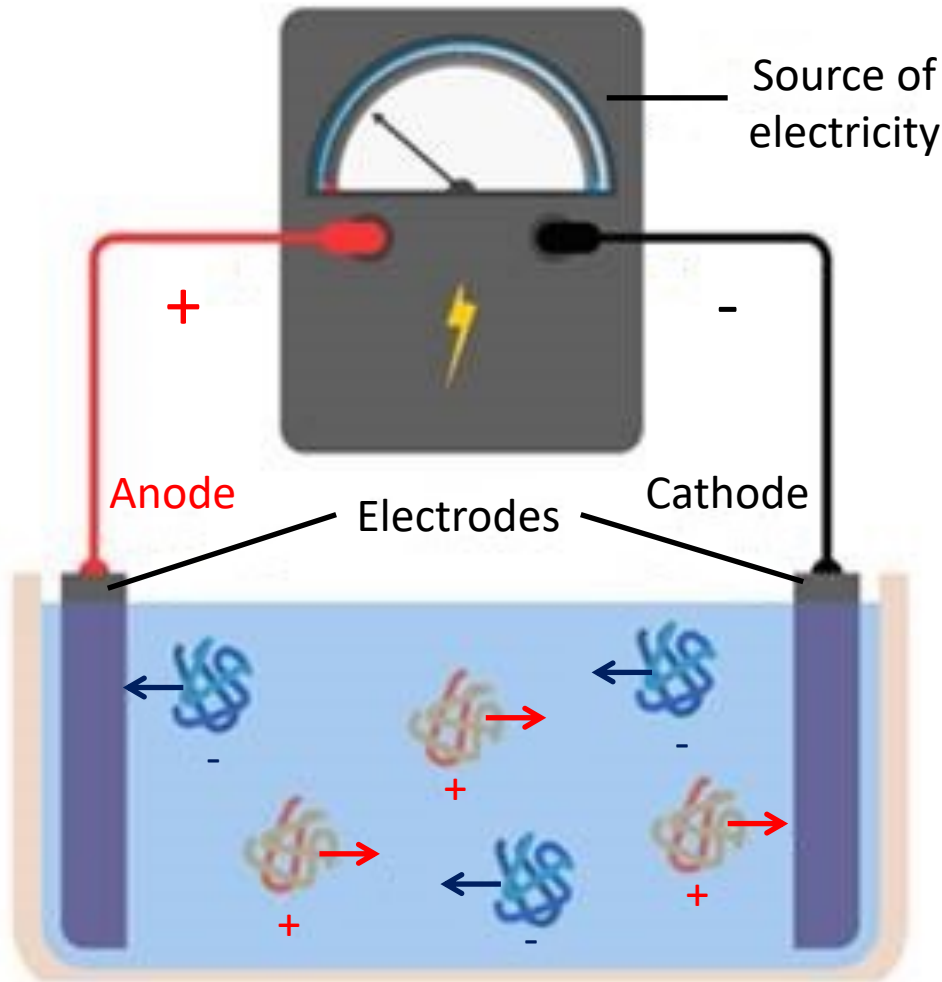
**Ouchterlony**<sup>[3.]</sup> double immunodiffusion:



The hole in the middle contains the antigen while the other surrounding holes contain the investigated sera. As the antigen and the antibodies in the sera diffuse towards each other they will precipitate once they reach the equivalence zone.

**Semiquantitative** method.

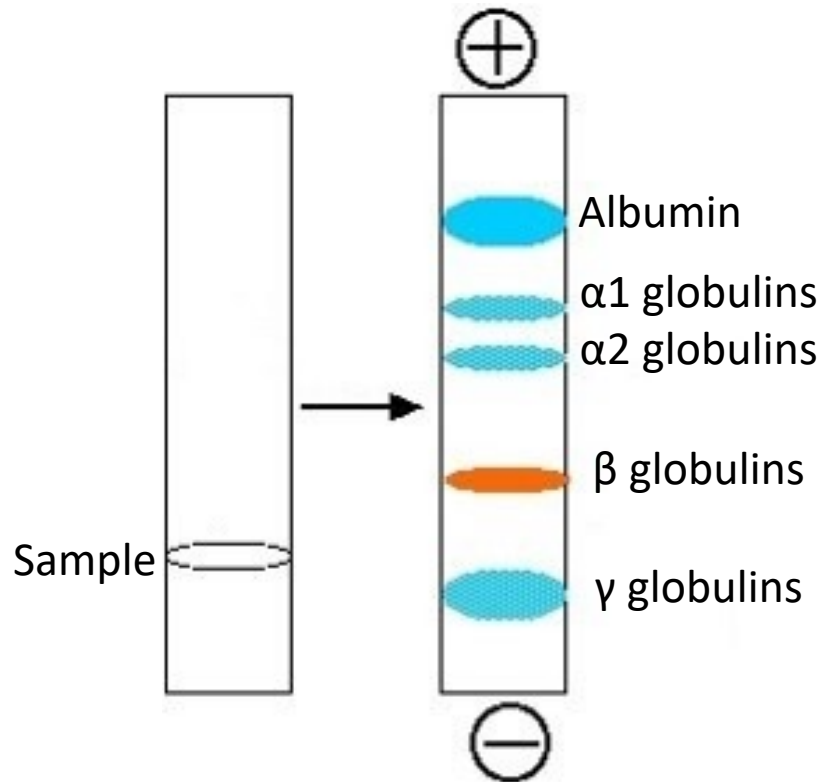
# Protein electrophoresis



- Molecules with electric charges (including proteins) will migrate towards the opposite charge if put into an electric field.
- The speed of their migration depends on:
  - The resistance of the matrix (can be standardized)
  - The voltage applied (can be standardized)
  - The **size** and the **charge** of the proteins (the latter **depends on pH**)
- Proteins that migrate with different speeds can be physically **separated**.
- The matrix can be:
  - Solid (e.g. paper, nitrocellulose)
  - Semi-fluid (e.g. agarose or polyacrylamide gel)
  - Fluid

# Serum protein electrophoresis

- The electrophoresis of the serum is performed under alkaline pH. The majority of the proteins in such conditions will migrate towards the positive electrode. The proteins can be detected by adding non-specific dyes.<sup>[4.]</sup>

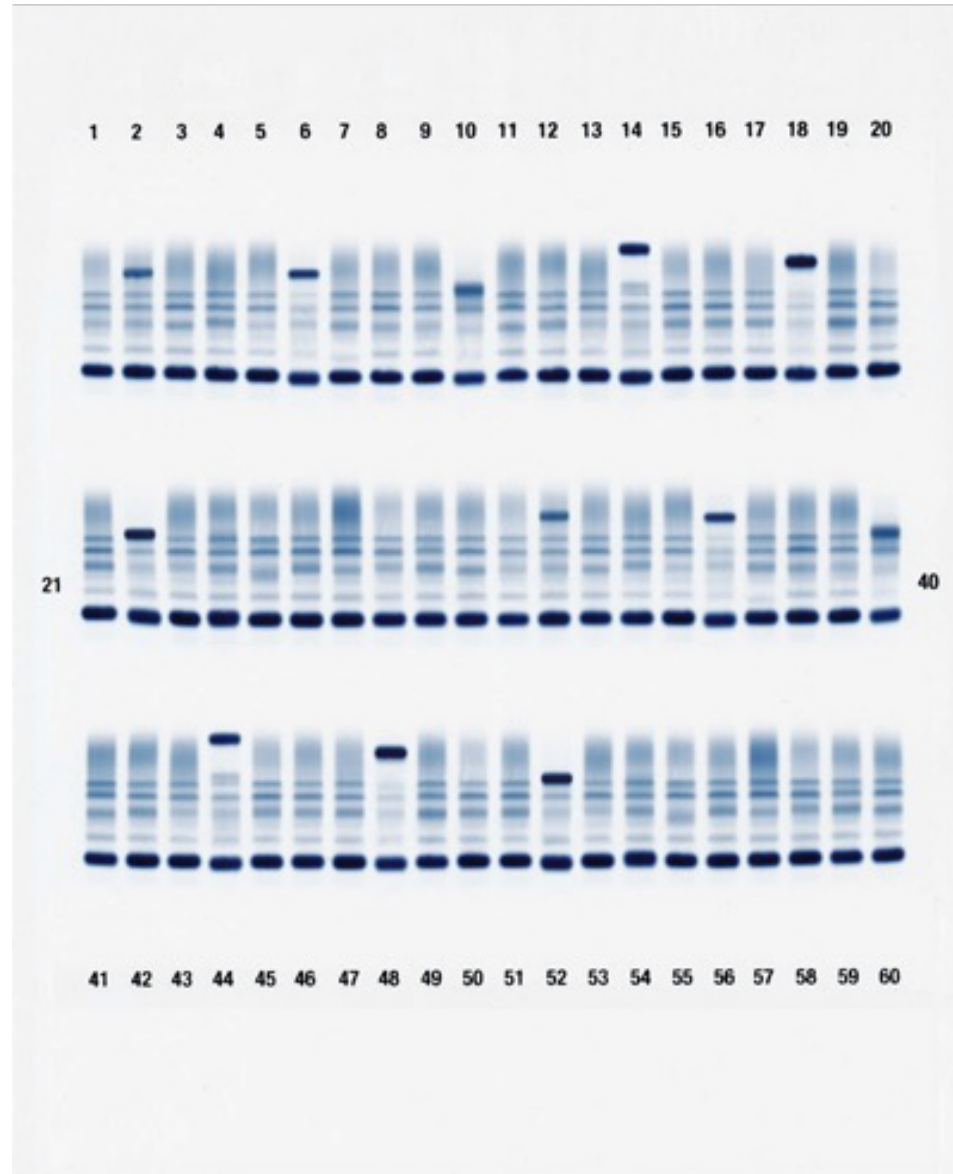


Arne Tiselius

Was awarded the 1948 Nobel Prize in Chemistry:

„for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins.”<sup>[5.]</sup>

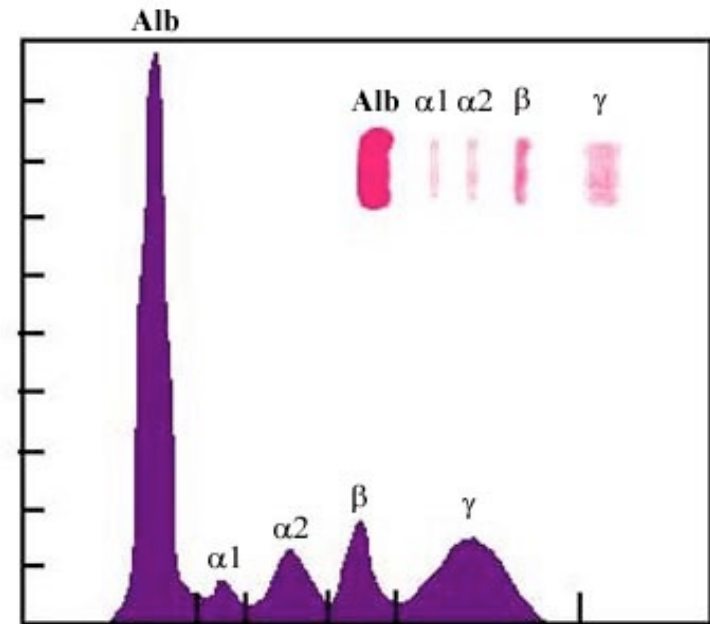
# Agarose gel electrophoresis



# Analysis of serum electrophoresis

Some examples of the proteins found in the different fractions:<sup>[6.]</sup>

- The biggest fraction is the **albumin**. ↓
- $\alpha 1$  globulins:
  - **$\alpha 1$ -antitrypsin** ↑
  - **Serum amyloid A** ↑
  - **Retinol-binding protein** ↓
  - **Transcortin** ↓
- $\alpha 2$  globulins:
  - **Ceruloplasmin** ↑
  - **Angiotensinogen**
  - **Haptoglobin** ↑
- $\beta$  globulins:
  - **$\beta 2$ -microglobulin** ↑
  - **Transferrin** ↓
  - **Plasminogen**
- $\gamma$  globulins:
  - **Immunoglobulins**



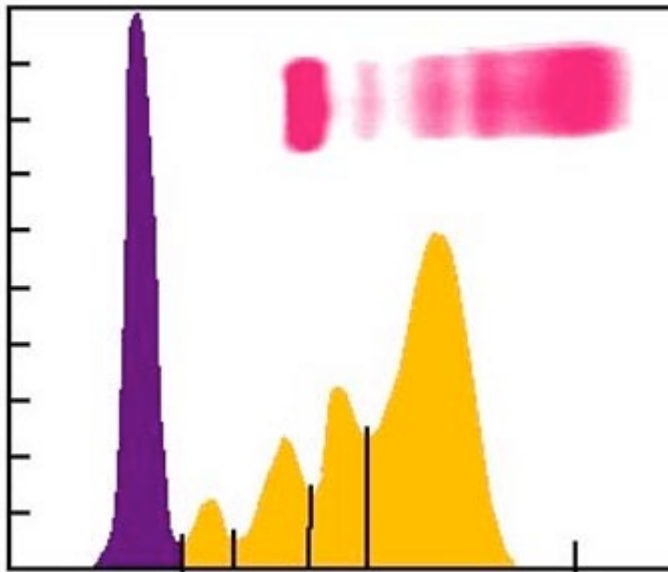
The normal pattern of serum electrophoresis and corresponding **densitometric diagram**.

Their levels in the blood change during the **acute phase reaction** due to inflammatory cytokines (e.g.  $\text{TNF}\alpha$ , IL1, IL-6):

- **Increase** (also called positive **acute phase proteins**, their most prominent member being **CRP** which can be found between the  $\beta$  and the  $\gamma$  fractions<sup>[7.]</sup>)
- **Decrease**

# Examples of abnormal electrophoretic patterns I.

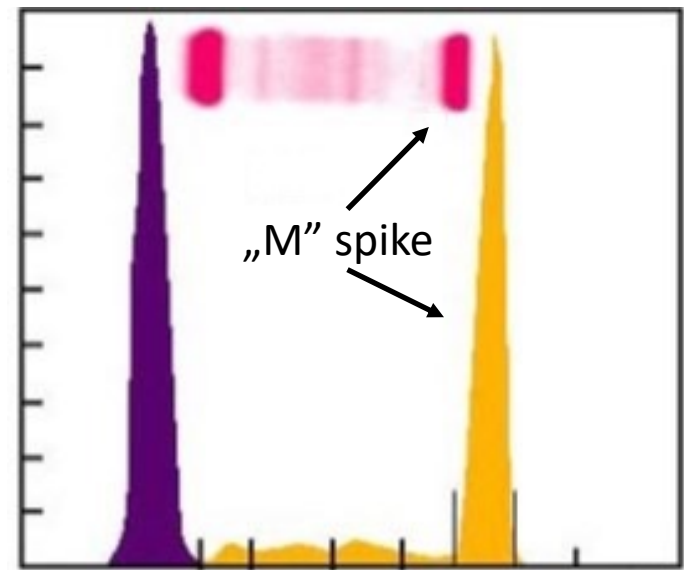
## Polyclonal gammopathy



An excess of immunoglobulins produced by **various B cell clones** in **inflammatory conditions**:<sup>[7.]</sup>

- Infections
- Autoimmune disorders
- Cancers
- Liver diseases (e.g. hepatitis, cirrhosis)

## Monoclonal gammopathy

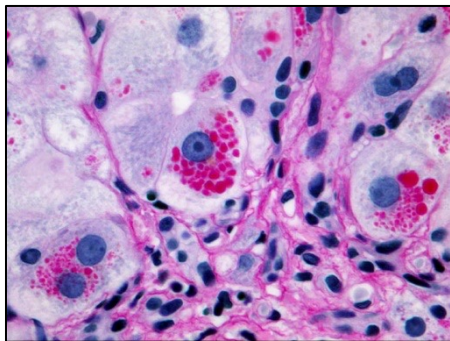
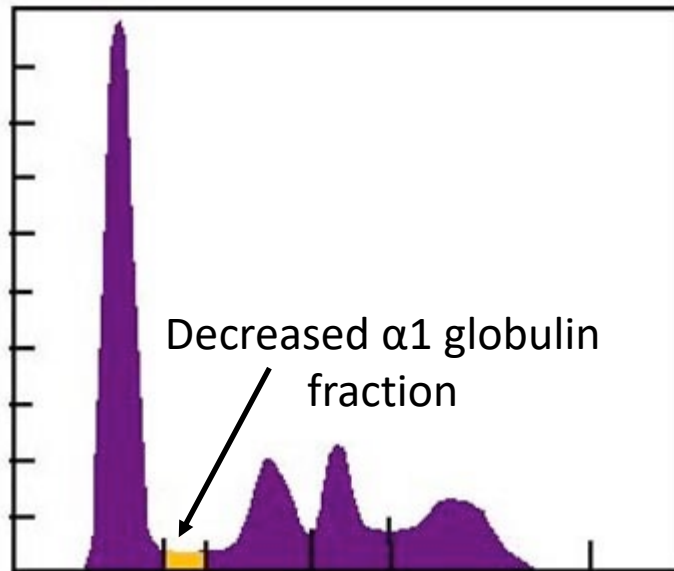


An excess of immunoglobulins produced by a **single B cell clone**. Found in **plasma cell neoplasms**:<sup>[7.]</sup>

- Multiple myeloma
- Waldenström macroglobulinemia
- MGUS (Monoclonal gammopathy of undetermined significance)

# Examples of abnormal electrophoretic patterns II.

## $\alpha$ 1-antitrypsin deficiency<sup>[8.]</sup>



Accumulated A1AT can be seen as PAS-positive granules inside hepatocytes.

## $\alpha$ 1-antitrypsin (A1AT):

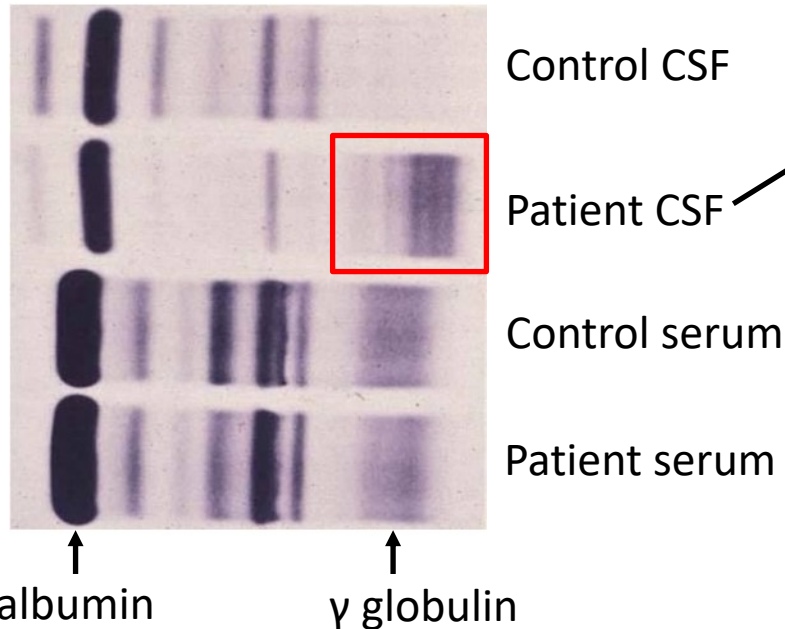
- It is produced by the liver.
- It neutralizes the elastase enzyme produced by neutrophils during inflammation.

## $\alpha$ 1-antitrypsin deficiency:

- It is a genetic disorder.
- Liver cells are unable to secrete  $\alpha$ 1-antitrypsin which accumulates in their cytoplasm.
- The level of  **$\alpha$ 1-antitrypsin** greatly decreases in the blood which will lead to complications:
  - **Liver damage** (because of A1AT deposition)
  - **Damage of the lungs** (inflammatory reactions will cause serious tissue damage without the inhibitory effects of A1AT)
  - **Chronic pancreatitis** (because of the absence of A1AT)

# Electrophoresis of other body fluids

## Cerebrospinal fluid (CSF)



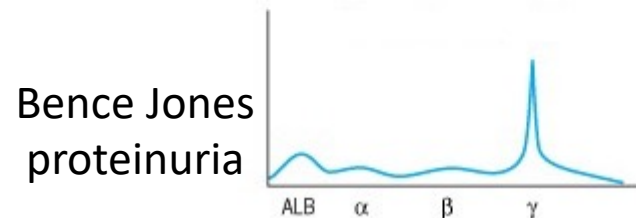
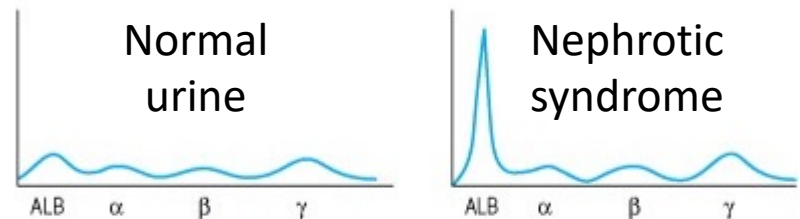
In the CSF of the patient individual bands can be seen in the gamma globulin fraction. The detected pattern differs from that seen in the patient's serum.



Immunoglobulins are produced locally in the central nervous system of the patient. (**oligoclonal gammopathy**, e.g. in **multiple sclerosis**<sup>[9.]</sup>)

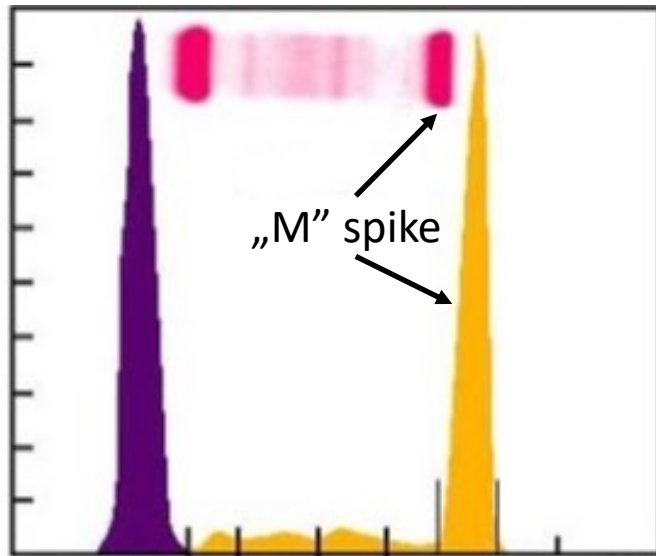
## Urine electrophoresis:

Performed simultaneously with serum electrophoresis when **multiple myeloma** is suspected. They try to detect the immunoglobulin light chain (Bence Jones protein<sup>[10.]</sup>) in the urine.





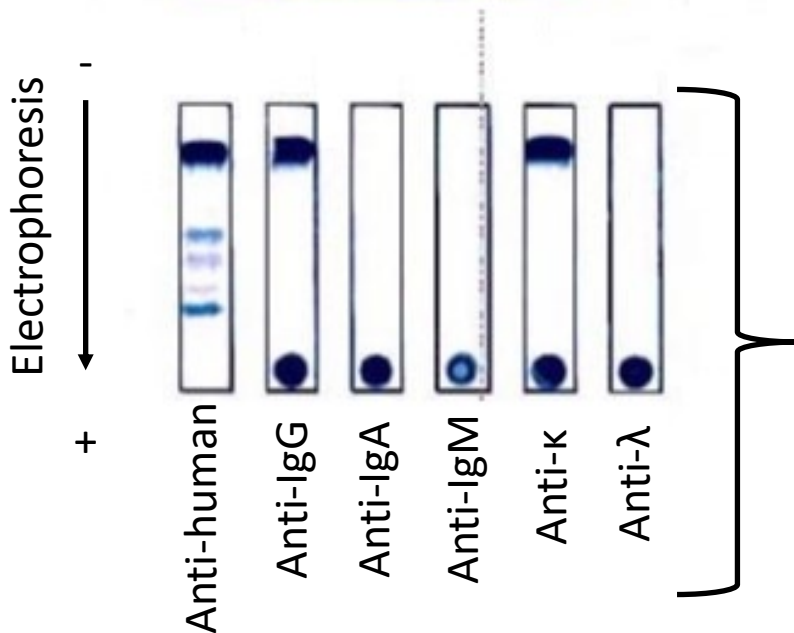
# Immunofixation



1. The electrophoresis is performed simultaneously by dividing the serum into several parallel samples.<sup>[12.]</sup>
2. The specific proteins are detected in different gels using different antibodies. (The added antibodies precipitate with the antigen which is usually detected with dyes. The antigens are the human immunoglobulins themselves in most cases.)

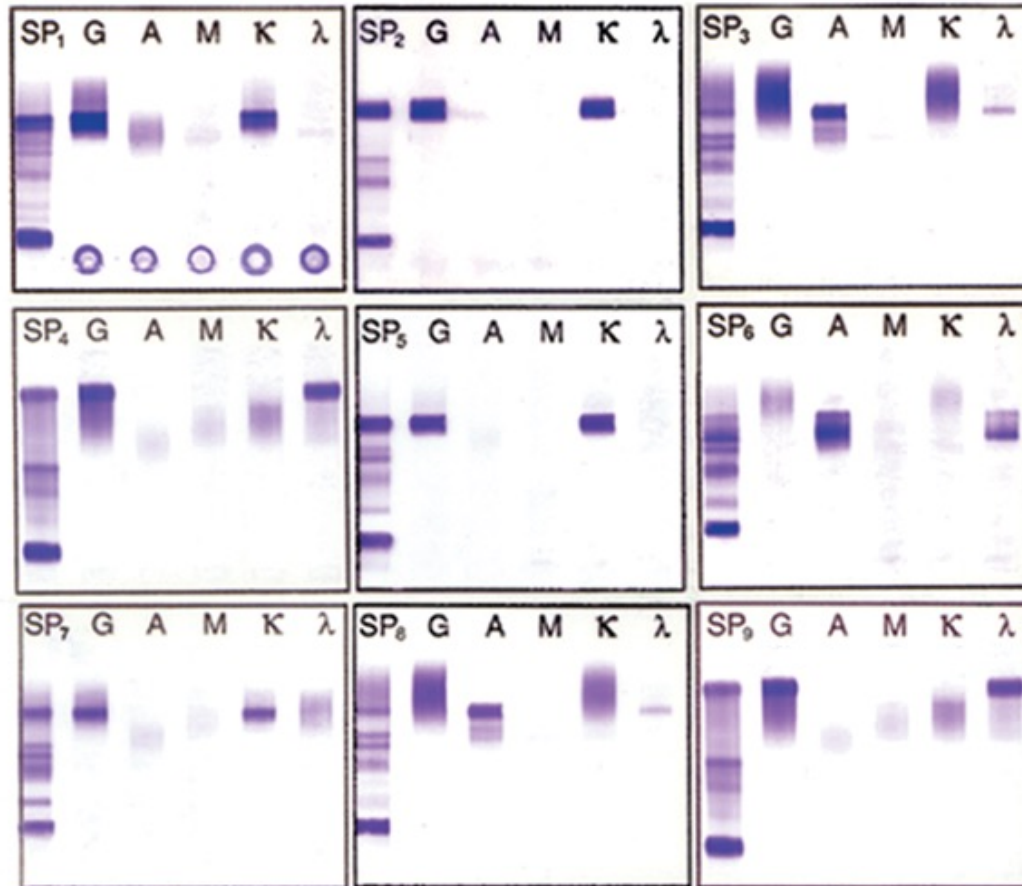
Application:

- Diagnostics of plasma cell neoplasms by detecting the abnormal monoclonal antibodies („**paraproteins**”) in the serum they produce.<sup>[13.]</sup>



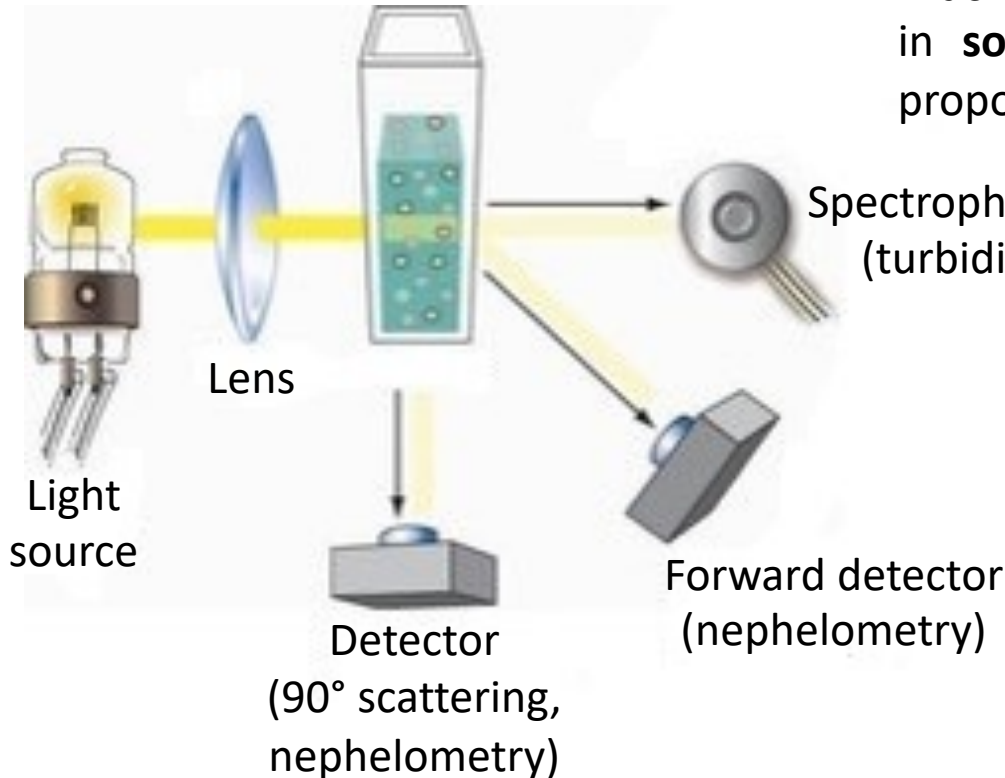
Multiple myeloma producing monoclonal antibodies of IgG κ isotype.

# Immunofixation



1. ELFO
2. + Anti-IgG anti-IgA, anti-IgM anti-kappa (K) anti-lambda (λ)

# Nephelometry, turbidimetry



**Macromolecules** (such as immunocomplexes) in **solutions scatter light**. The scattering is proportional to size of the particles.

The analyte can be identified based on the **light scattering** with nephelometry. As light passes the cuvette the **light intensity will decrease** which is detected by turbidimetry.<sup>[14.]</sup>

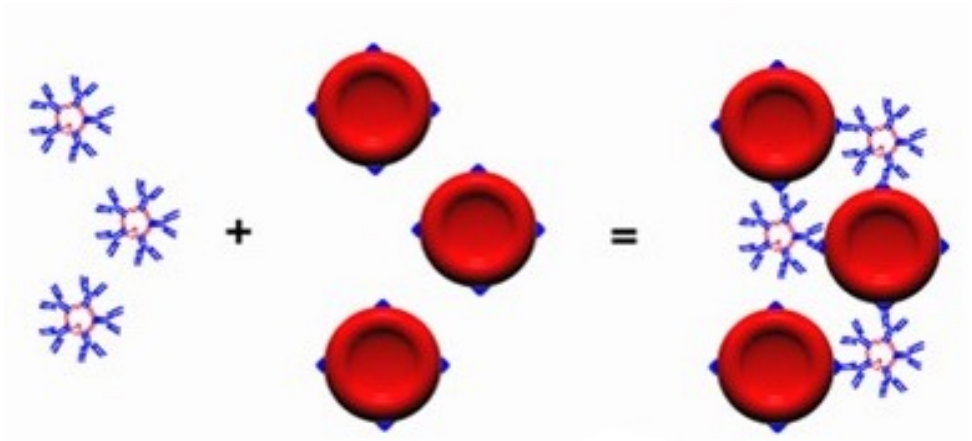
**Application:** Measuring the concentrations of immunocomplexes, e.g. IgA, IgM, IgG levels or the levels of light chains (e.g. in multiple myeloma), complement levels

# Nephelometry

- Nephelometry, a method to detect the concentration of serum proteins including immunoglobulin, is based on the concept that particles in solution will scatter light passing through the solution rather than absorbing the light. Nephelometers record the degree of scatter, and scientists correlate this with the quantity of protein in the solution.
- In 1971 L.M. Killingsworth and John Savory working at the University of Florida in Gainesville described a method for the detection and quantitation of immunoglobulin isotypes in human serum. They diluted human serum with saline and mixed these dilutions with antibody specific for IgG, IgA, or IgM. The turbidity of these mixtures was measured in a nephelometer, an instrument that measures the scatter of light from a laser passing through the solution. Known quantities of the immunoglobulin being measured are mixed with the antibody to develop a standard curve from which the concentration of the protein in the unknown sample can be deduced.
- Nephelometry is the method of choice in the clinical laboratory to measure the concentration of **immunoglobulin isotypes (IgG, IgA, IgM, and IgE) as well as other serum proteins including hemoglobin, C-reactive protein, albumin, haptoglobin, and others.**
- Polyclonal increases in **immunoglobulin concentrations** are associated with infections, autoimmune diseases, and chronic inflammation, while monoclonal increases suggest multiple myeloma or Waldenström macroglobulinemia. Decreased concentrations may signal immunosuppression, kidney failure, or protein losing enteropathies.

# Agglutination

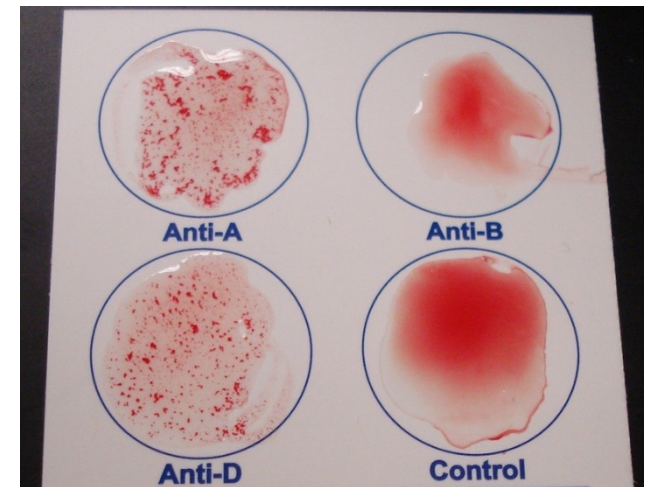
- If antibodies cross-bind larger particles and it leads to the aggregation of these particles = **agglutination** (if these particles are red blood cells it is called **hemagglutination**)
- Agglutination is one of the **physiological functions** of antibodies, agglutination of pathogens prevents the spread of infections.<sup>[15.]</sup>
- Can be **direct** or **indirect** and **active** or **passive**.
- Several diagnostic tests are based on agglutination reactions in which the clumping of the particles is directly visible.



Anti-„A” IgM

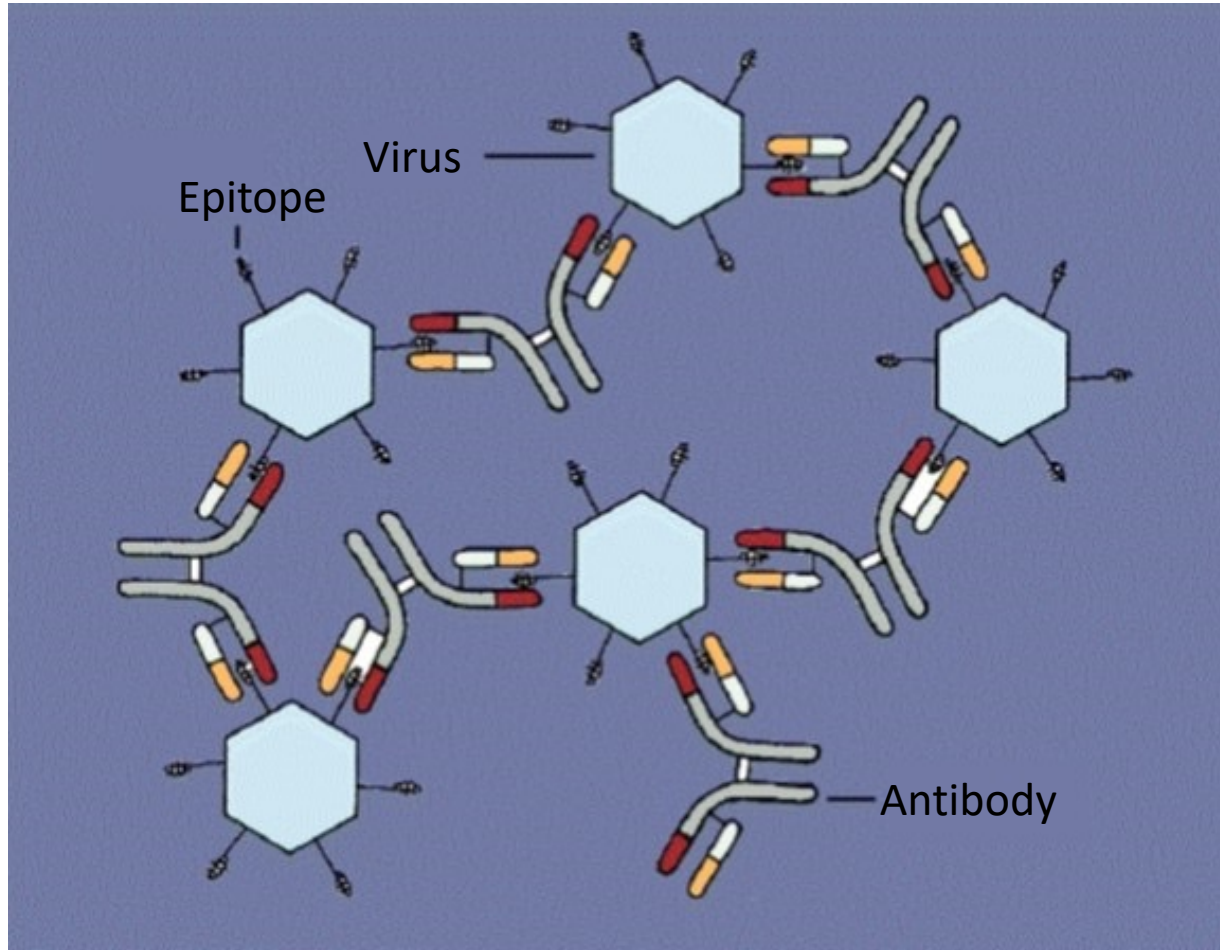
RBC with „A”  
antigen

Hemagglutination



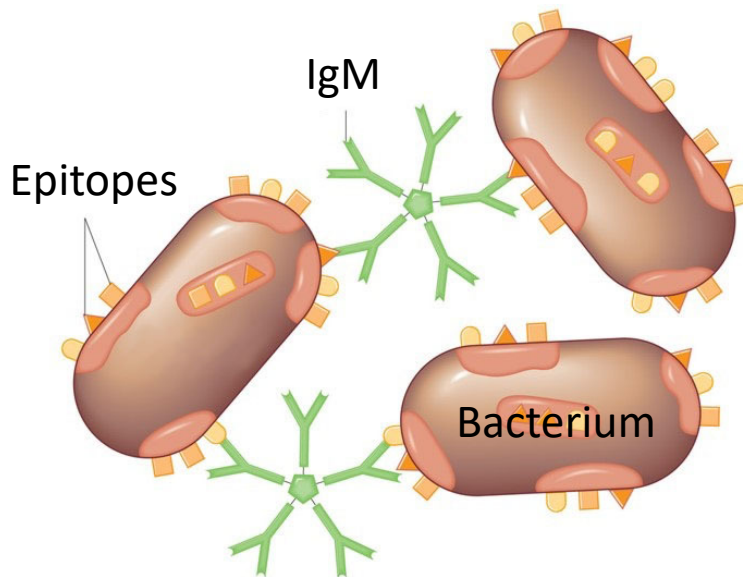
Blood type test: A, Rh(D)  
positive

# Physiological role of agglutination



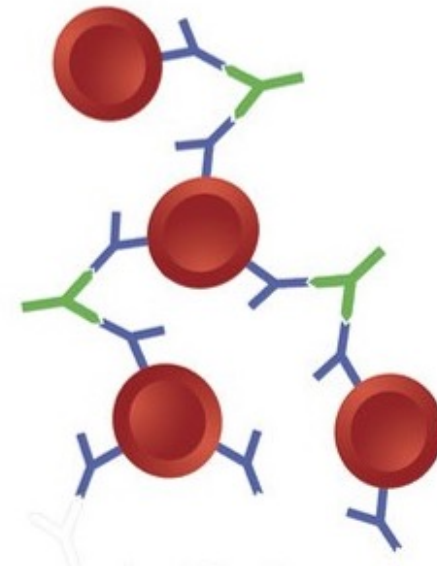
# Direct or indirect

## Direct agglutination:



- The particles are directly cross-linked by the primary antibodies.
- Antibodies of the **IgM** isotype can effectively agglutinate particles.

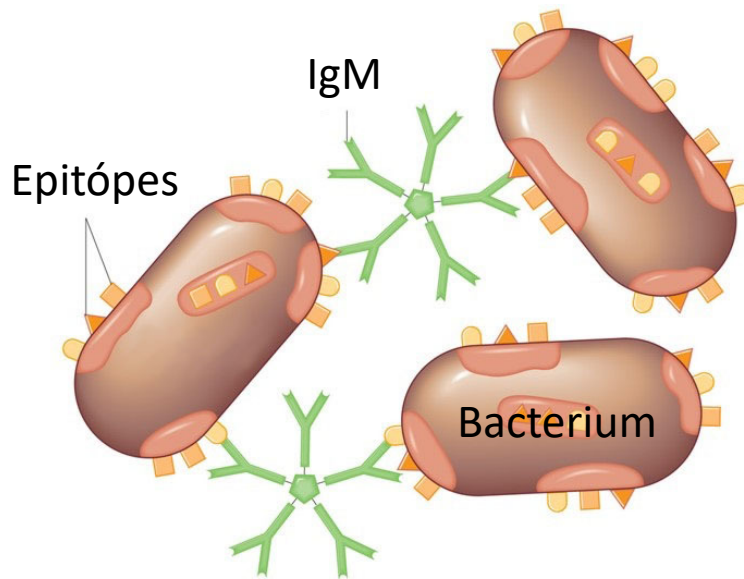
## Indirect agglutination:



- Secondary antibodies cross-link the particles.

# Active or passive

## Active agglutination:



- The cell/particle participates in the reaction with its **own** surface **antigen**.
- Example:
  - Blood group testing
  - Detection of bacterial cell surface antigens

## Passive agglutination:



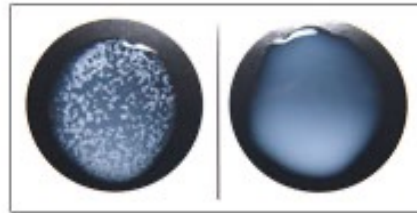
- The antigen is **artificially bound** to the particles that participate in the reaction.
- Example:
  - Latex agglutination reactions (see on the next slides)



# The clinical significance of agglutination

- One of the physiological functions of antibodies in the defense against pathogens.
- In vivo hemagglutination may occur in certain diseases. (e.g. autoimmune hemolytic anemia, AIHA)
- Diagnostic tests:
  - **Latex agglutination tests:**
    - **Autoimmune disorders** (detection of autoantibodies)
    - **Infections** (detection of microbial antigens or the antibodies that recognize them)
    - Detection of other proteins (e.g. CRP, hCG, D-dimer)
  - **Tests based hemagglutination :**
    - **Testing blood groups**
    - **Coombs test (antiglobulin test)**
    - Hemagglutination assay
  - Hemagglutination inhibition assay:
    - Identification of viral hemagglutinins
    - Testing antibodies that can inhibit viral hemagglutinins

# Latex agglutination test



Positive Negative

The antigen/antibody that participates in the reaction is bound to the surface of **latex beads**.

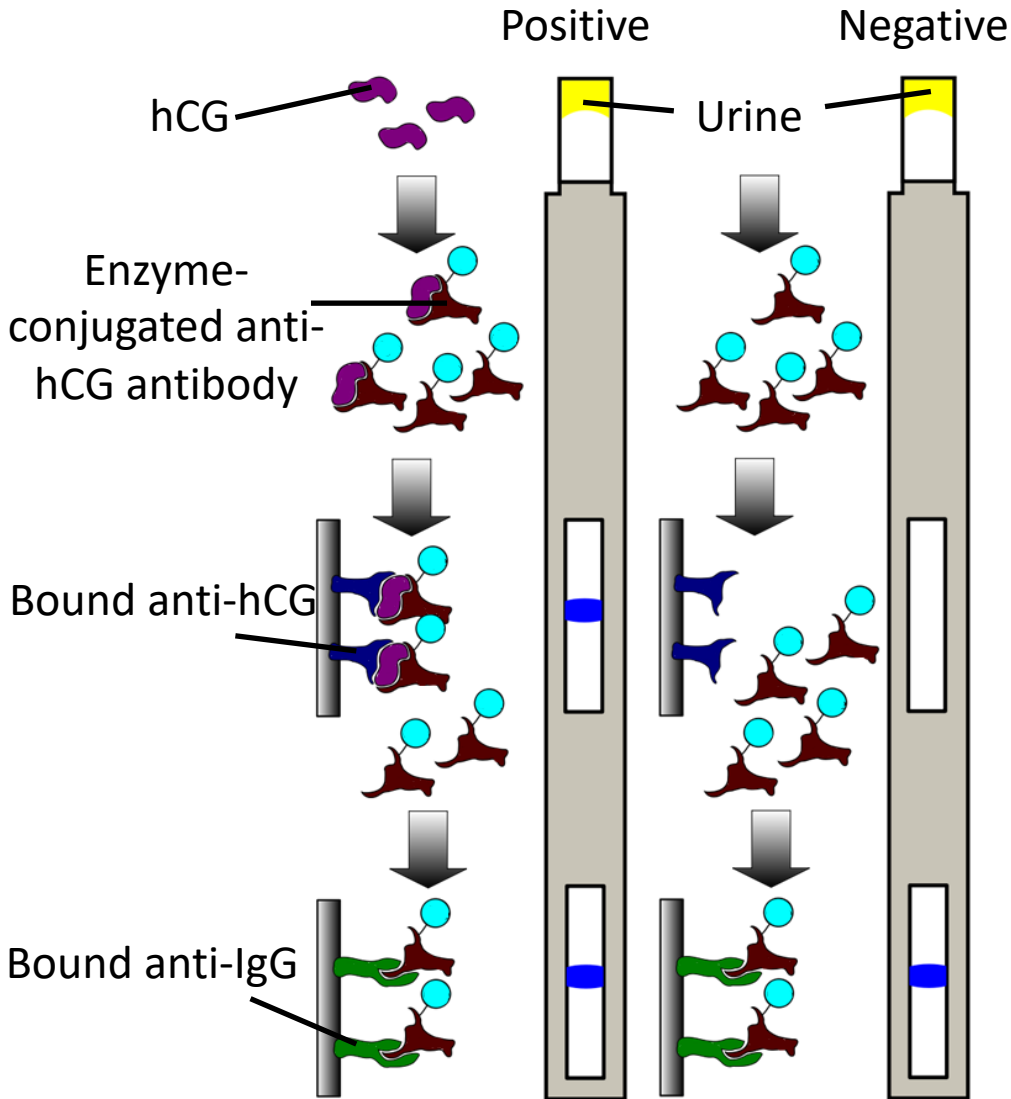


If the investigated antibody/antigen is present in the sample then it will cause the aggregation of the beads.

## Applications:

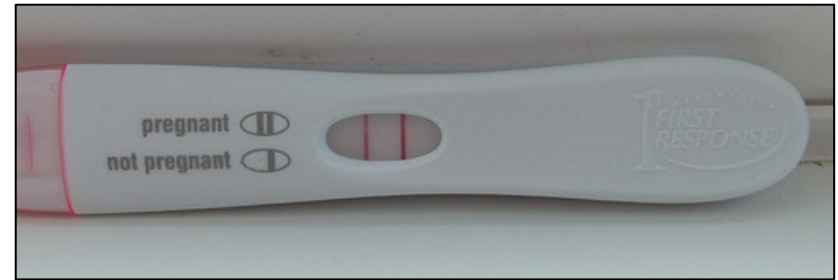
- **Diagnostics of autoimmune disorders, e.g.:**
  - Rheumatoid arthritis (rheumatoid factor, RF<sup>[16.]</sup>), SLE (various autoantibodies)
- **Diagnostics of infectious diseases, e.g.:**
  - Detection of antibodies against microbial antigens (e.g. anti-streptolysin O antibody, ASO/AST<sup>[17.]</sup>)
  - Detection of bacterial antigens
- Detection of other proteins, e.g.:
  - **C-reactive protein** (CRP, acute phase protein<sup>[18.]</sup>), D-dimer<sup>[19.]</sup> (indicates blood clot formation), **human chorionic gonadotropin** (hCG, in pregnancy)

# Home pregnancy test



After fertilization hCG produced by the trophoblasts appears in the urine of the mother.

hCG can be detected by several immunological methods (such as ELISA or agglutination) but home tests are based on **chromatography**.<sup>[20.]</sup>

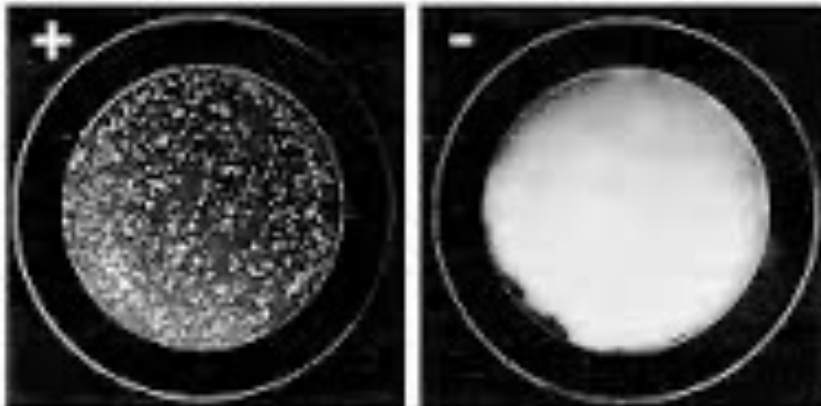


Band forms only if the enzyme-conjugated antibody is bound. If hCG is not present in the urine then only the anti-IgG will bind the labeled antibodies and only one band will appear.

# Agglutination practice

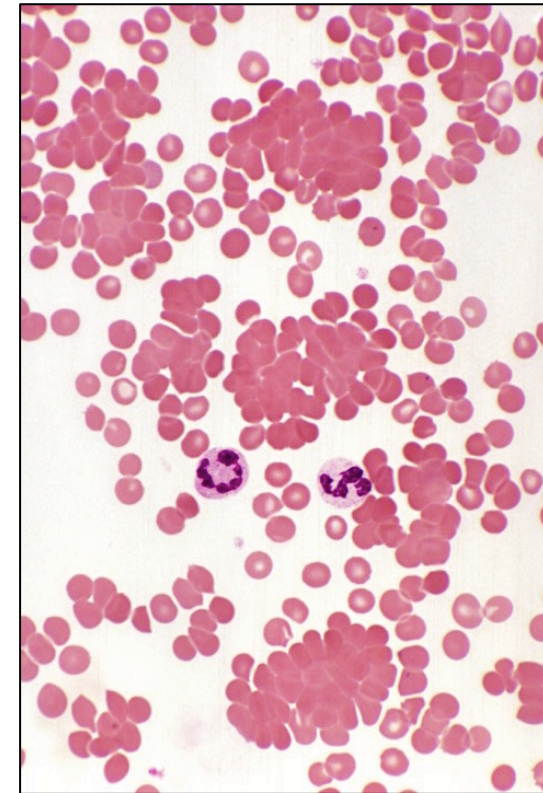
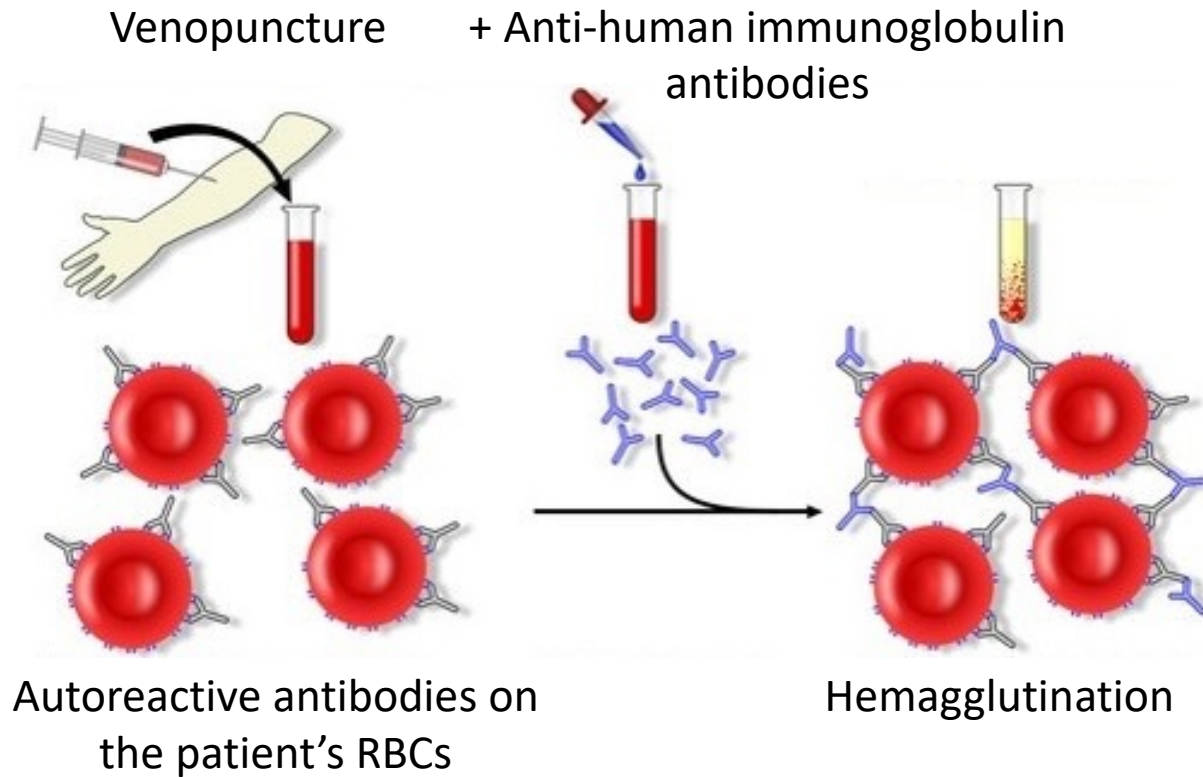
## Steps of the practice:

1. You can find different agglutination kits on your desks.
2. No actual samples were prepared, so you will only test the positive and the negative controls,
3. Squeeze the vial of the beads to deliver a drop into 2 reaction circles. Add 1 drop of the positive control into one of the circles and 1 drop of the negative control into the another. (according to the user manual)
4. Mix and blend the beads and the controls by rubbing the surface of the cards with the sticks.
5. Visible clumping of the beads in the positive control should appear.



**WEAR GLOVES!**

# Direct Coombs test (Direct antiglobulin test<sup>[21.]</sup>)

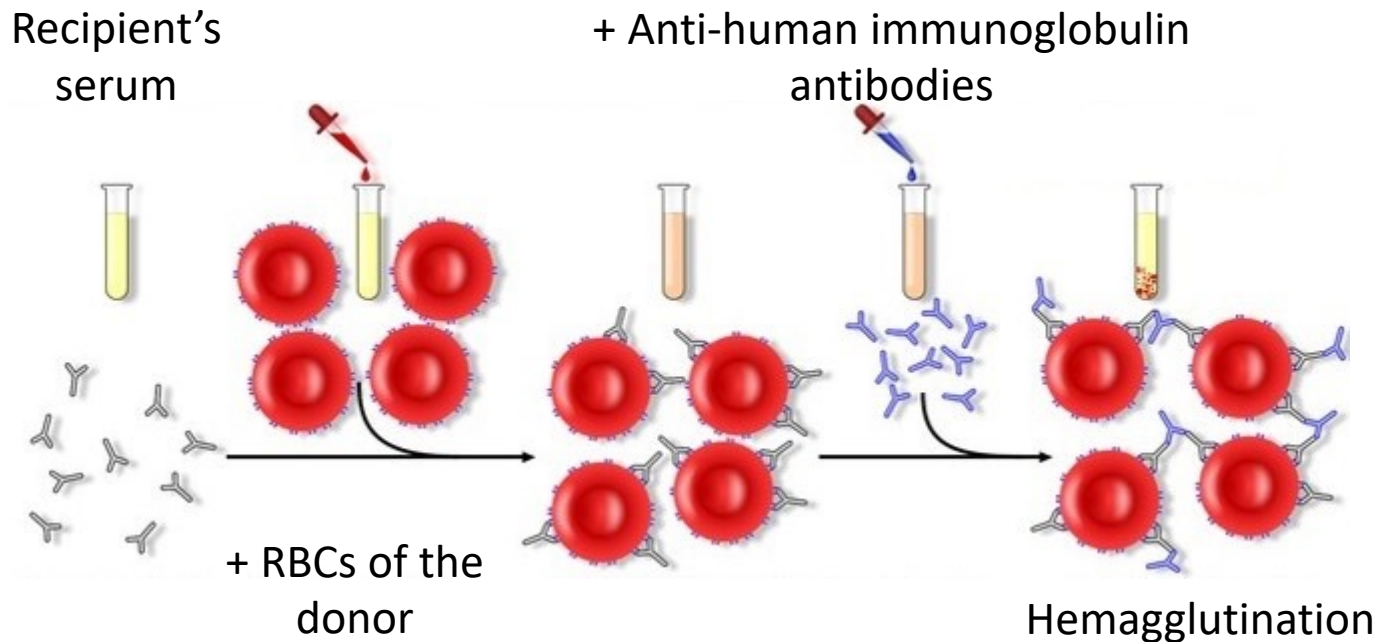


Application: Diagnostics of **immune-mediated hemolysis**,<sup>[22.]</sup> e.g.:

- AIHA (autoimmune hemolytic anemia, anemia= RBC numbers ↓)
- Erythroblastosis fetalis (Hemolytic disease of the newborn, HDN)

In vivo hemagglutination in a patient with AIHA.

# Indirect Coombs test (Indirect antiglobulin test)

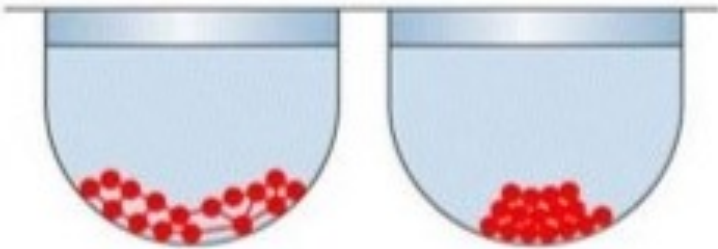
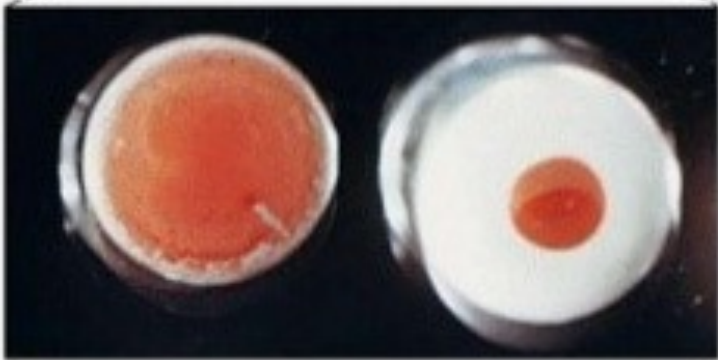


## Applications:

- Screening for antibodies before **blood transfusions**<sup>[23.]</sup> (to detect antibodies that recognize several rare blood groups other than the ABO or Rh groups systems)
- To **screen pregnant women** for anti-Rh(D) antibodies that can cross the placenta and cause erythroblastosis.<sup>[24.]</sup>

# Hemagglutination assay

1:20 1:40 1:80 1:160 1:320 1:640 Control



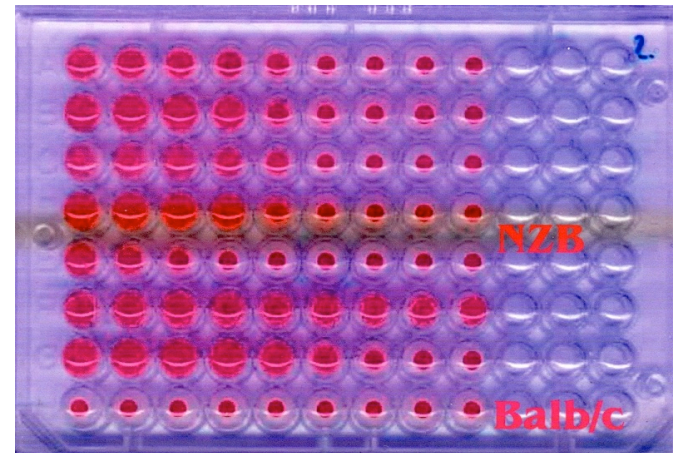
Hemagglutination

Negative

Equal amount of RBCs are put into each well. 2-fold dilutions of the sample are then created and added to the wells.

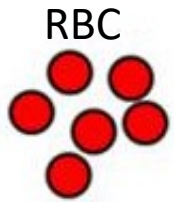

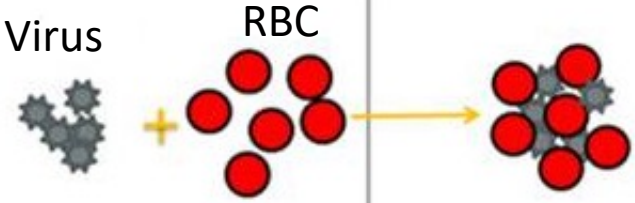

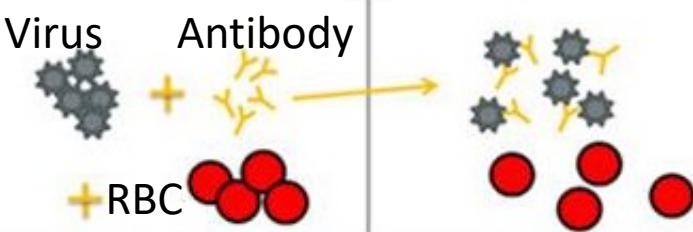



In case of a **positive reaction** the RBCs aggregate and therefore cannot settle to the bottom of the well. (HA titer: the smallest concentration of the sample which still causes agglutination)



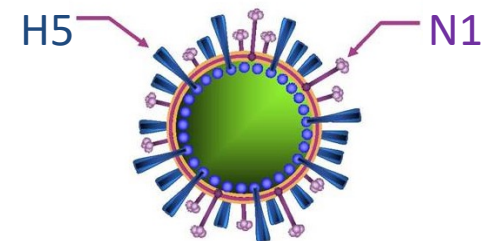
- NZB: New Zealand Black mouse strain<sup>[25.]</sup> (murine model of AIHA)
- BALB/c: albino strain of house mouse (control)

# Hemagglutination inhibition assay

Components	Reaction	Result
RBC		No reaction 
Virus + RBC		Hemagglutination 
Virus + Antibody + RBC		No reaction 

Some viruses possess proteins that can cause hemagglutination in vitro. („hemagglutinins”) E.g.:

- Influenza hemagglutinin
- Measles hemagglutinin
- Mumps hemagglutinin



- The method can be used to **classify viruses** based on their **viral hemagglutinin antigens**,<sup>[26.]</sup> e.g.: H5N1 = Influenza virus with type 5 hemagglutinin (and type 1 neuraminidase).
- Can also be used to test the levels of anti-hemagglutinin antibodies in people who received vaccinations against such viruses.<sup>[26.]</sup>



# References 1.

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