IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

Immunoserology 1. precipitation, agglutination

Basic Immunology

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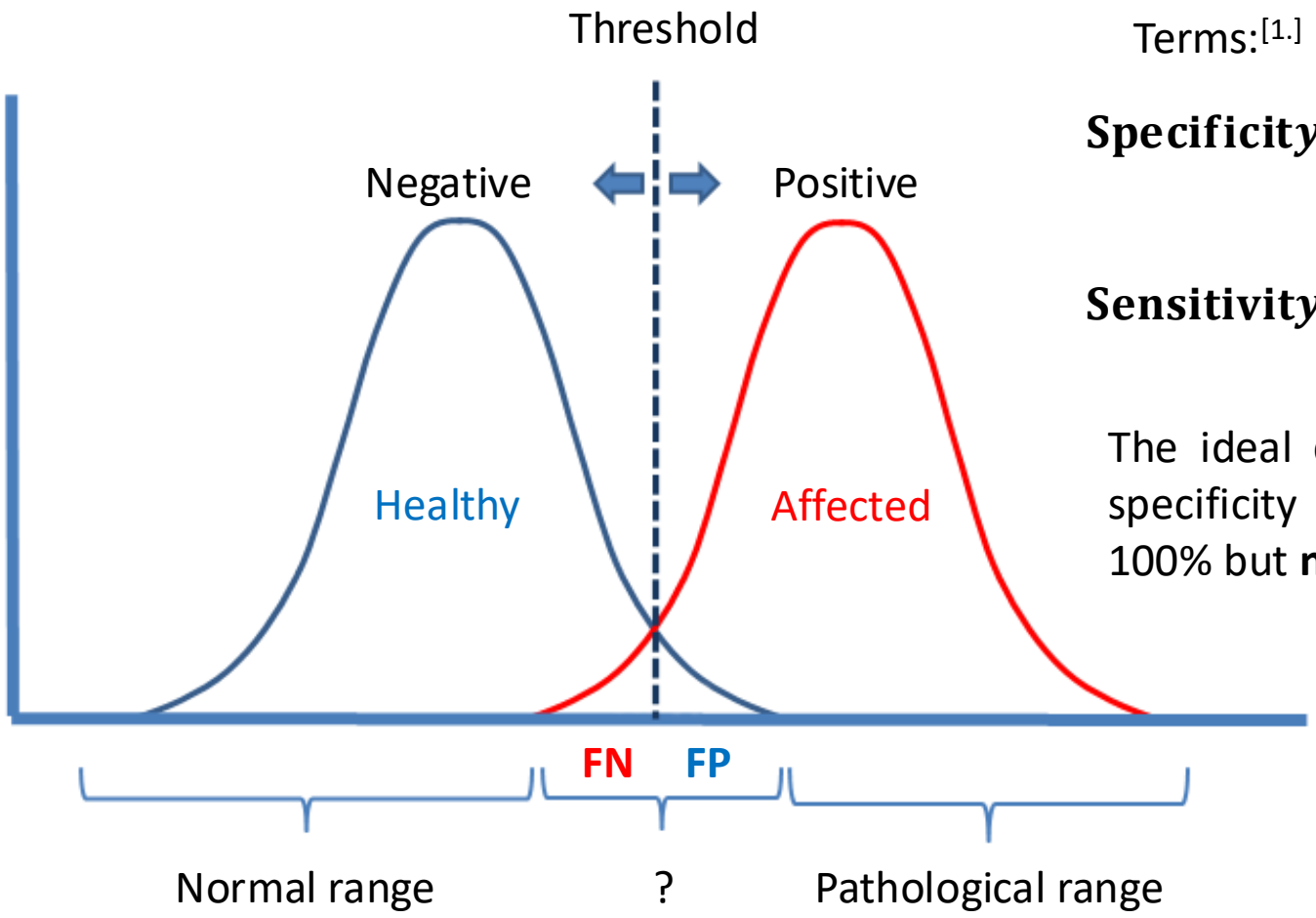
Definition of serology

- 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



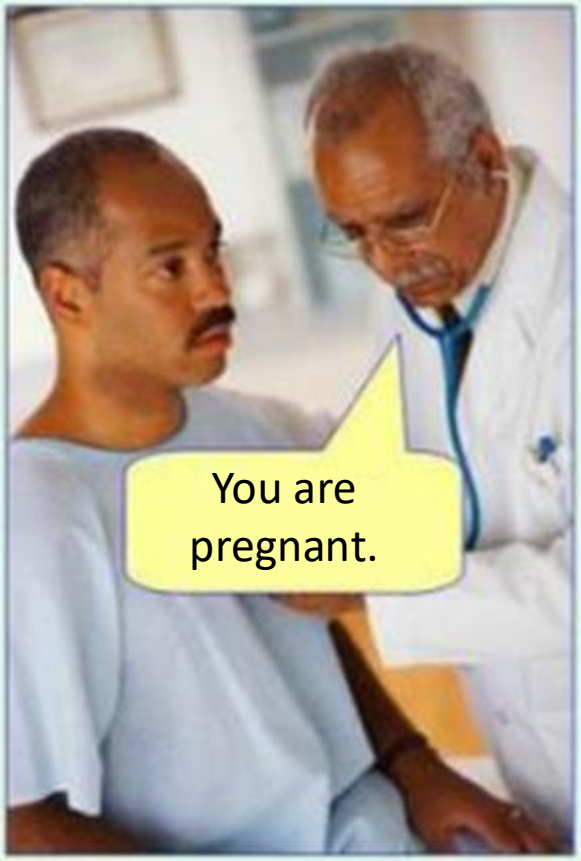
Terms:[1.]

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

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

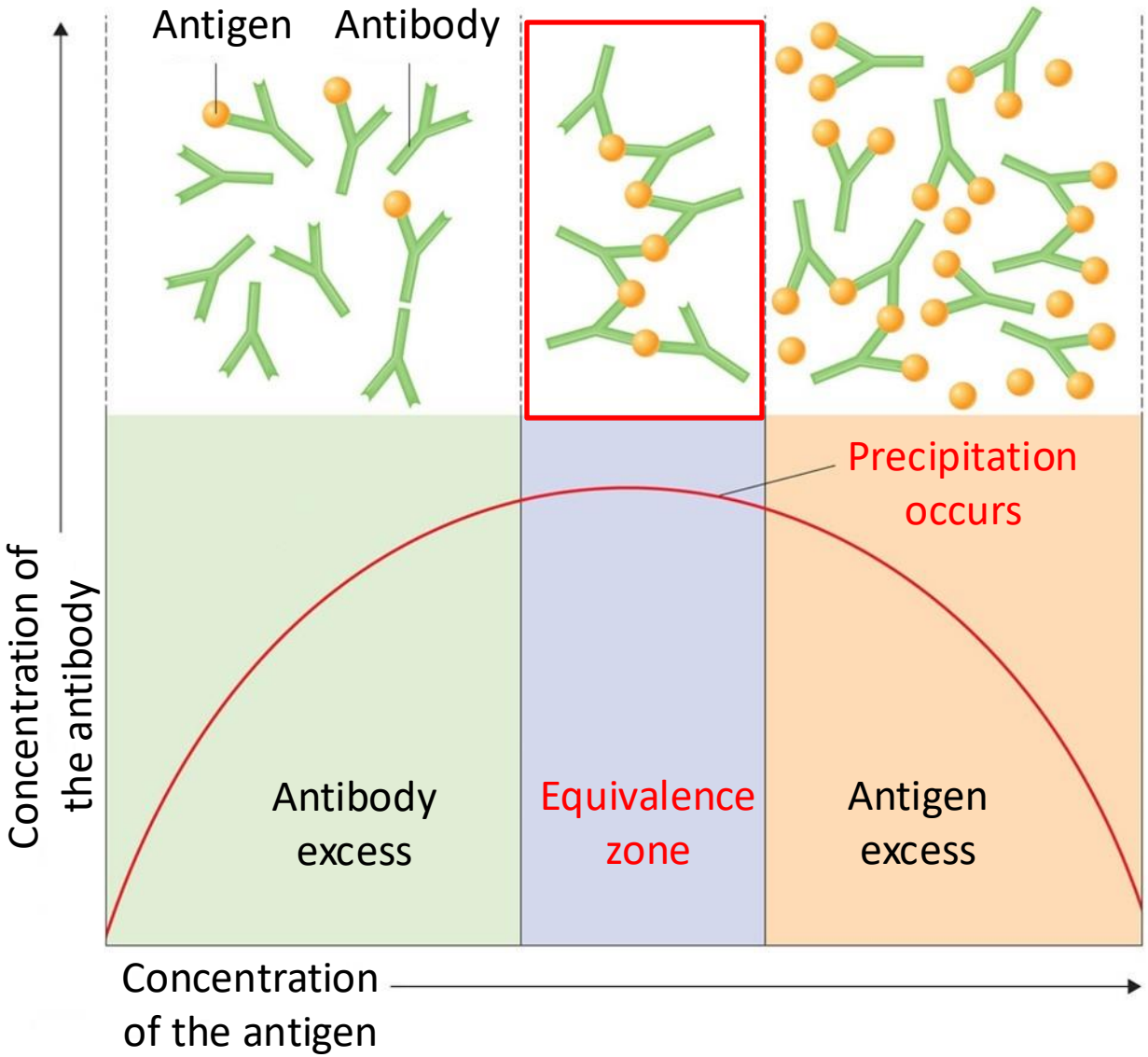
The ideal diagnostic test has a specificity and sensitivity of 100% but **no such test exists**.

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.

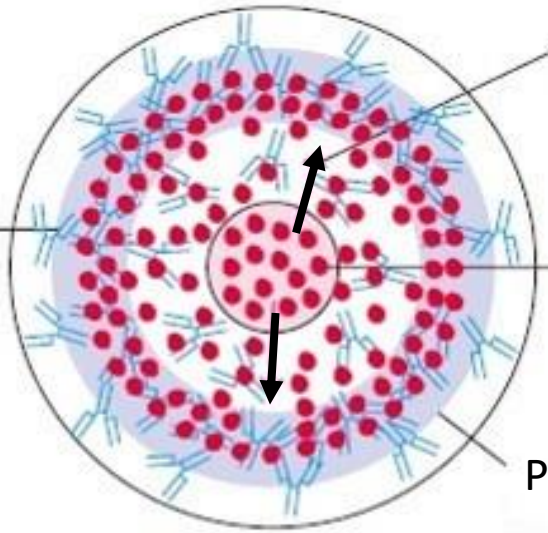
Solubility of these protein complexes decreases and they will precipitate.

Methods based on immunoprecipitation:

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

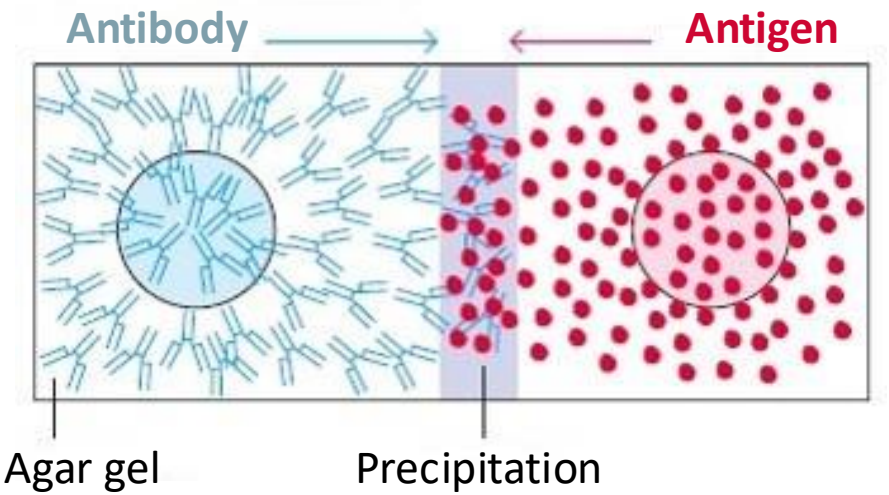
Immunodiffusion I.

Radial immunodiffusion



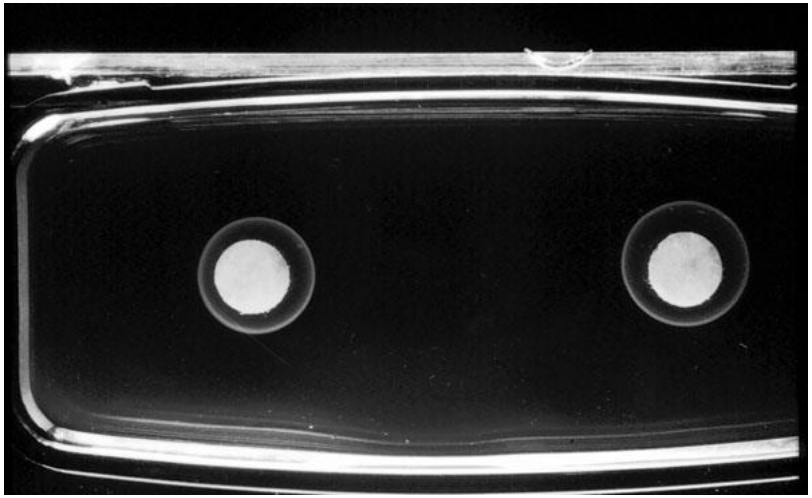
Simple but **outdated** techniques.

Double immunodiffusion



Immunodiffusion II.

Mancini[2.] 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.

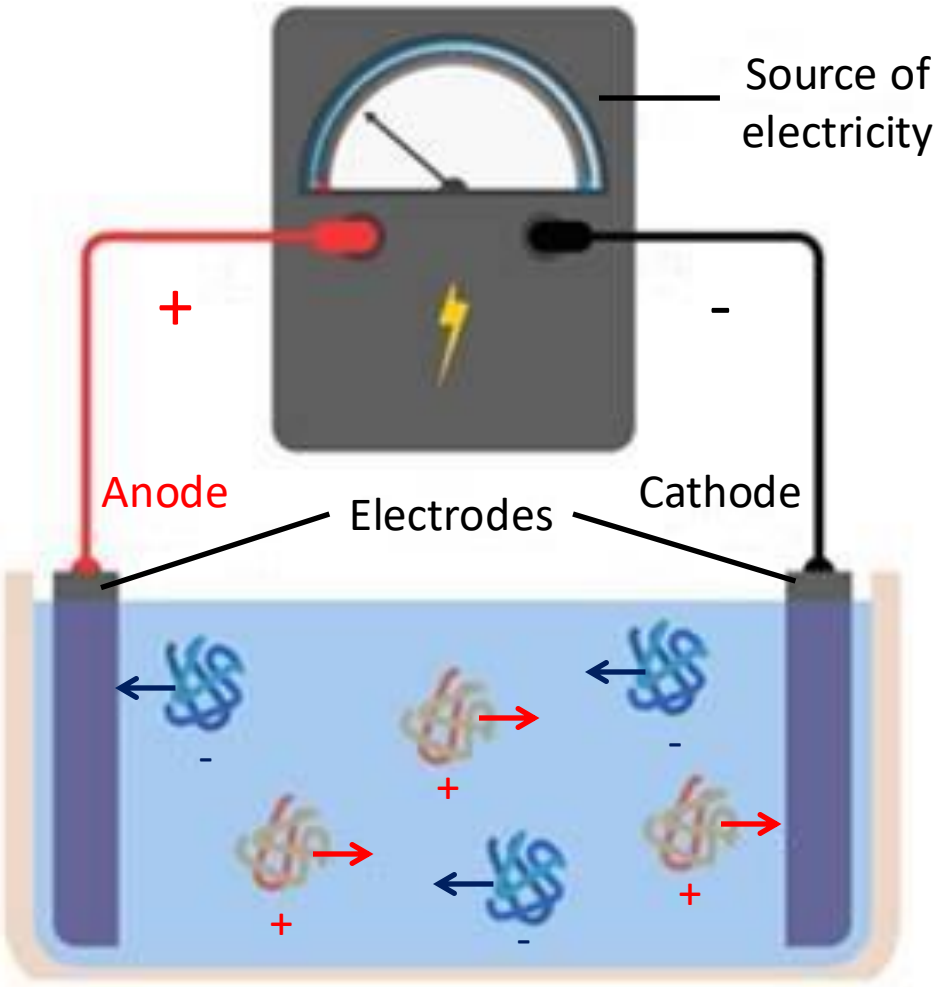
Semiquantitative method.

Ouchterlony[3.] 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.

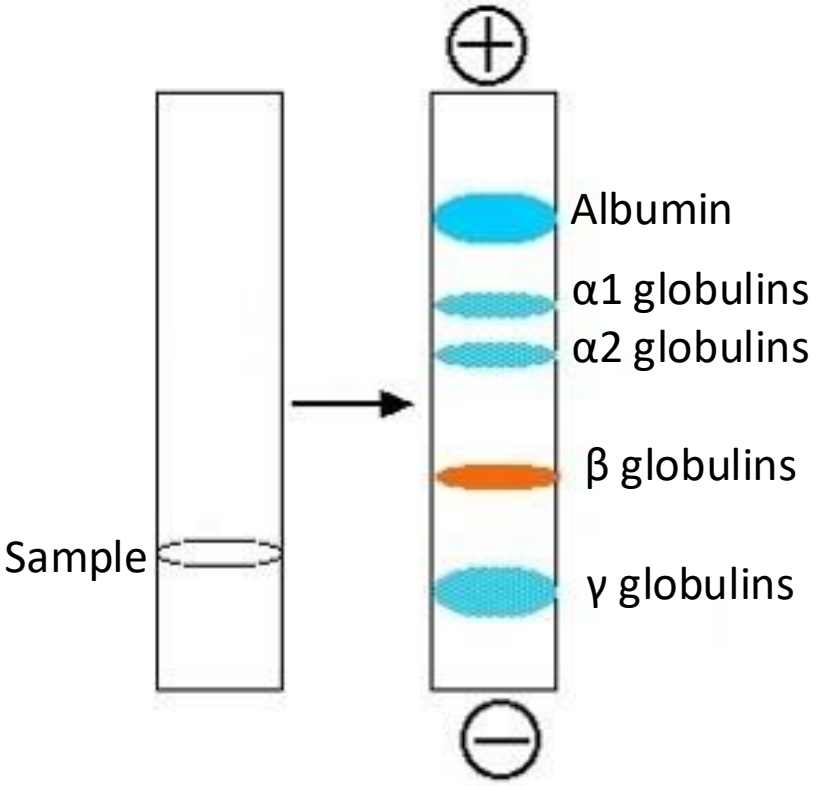
Protein electrophoresis



- 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

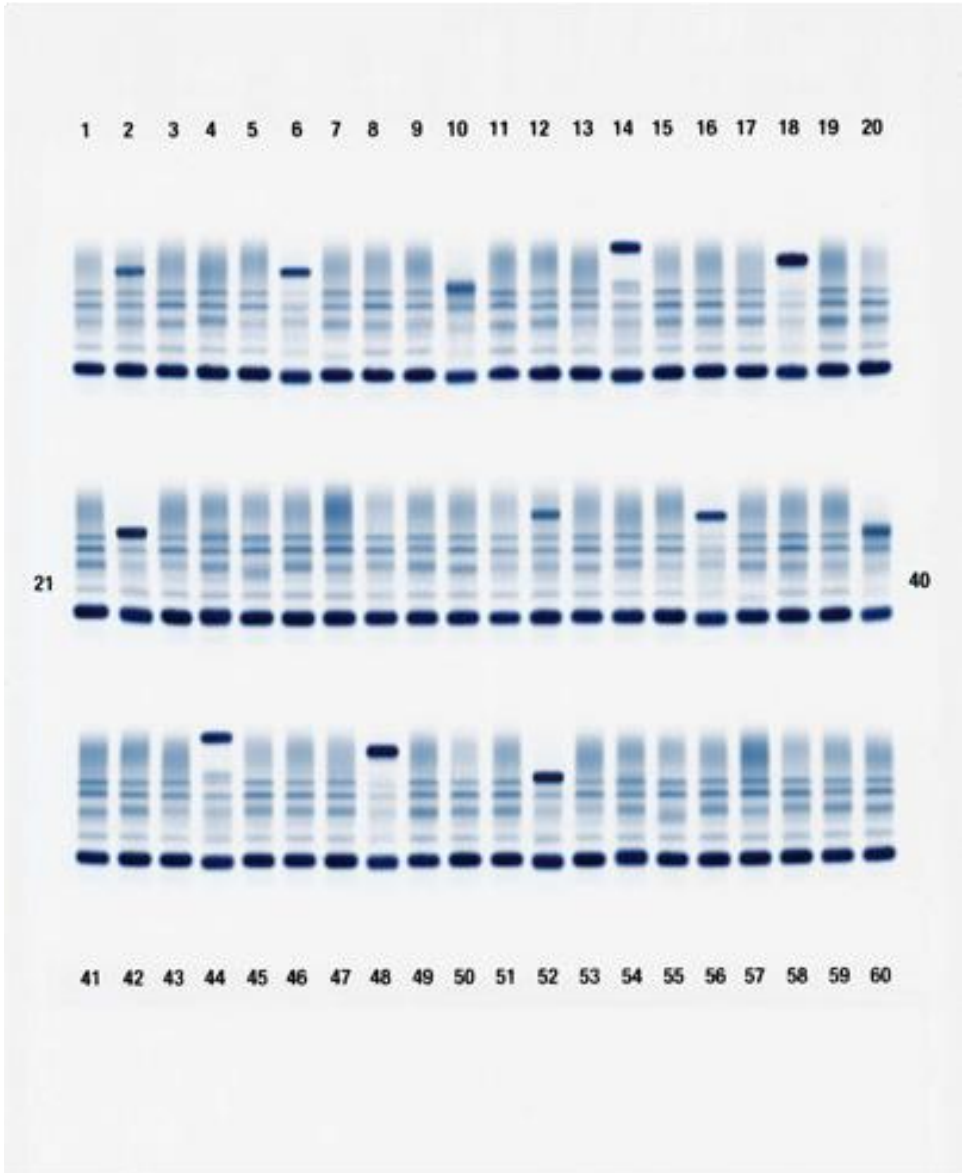
- The electrophoresis of the serum is performed under alkaline pH. The majority of the proteins in such conditions will migrate towards the positive electrode. The proteins can be detected by adding non-specific dyes.[4.]



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."[5.]

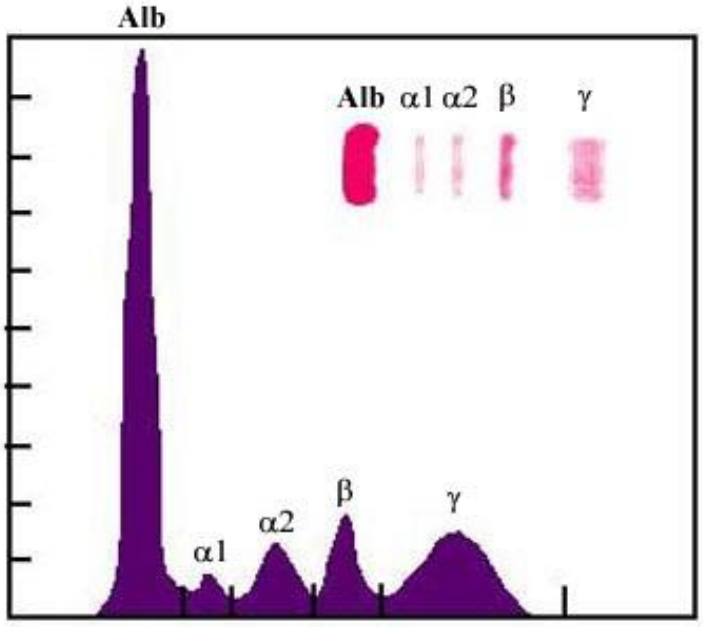
Agarose gel electrophoresis



Analysis of serum electrophoresis

Some examples of the proteins found in the different fractions:[6.]

- The biggest fraction is the albumin. ↓
- α1 globulins:
  - **α1-antitrypsin ↑**
  - Serum amyloid A ↑
  - Retinol-binding protein ↓
  - Transcortin ↓
- α2 globulins:
  - Ceruloplasmin ↑
  - Angiotensinogen
  - Haptoglobin ↑
- β globulins:
  - **β2-microglobulin ↑**
  - Transferrin ↓
  - Plasminogen
- γ globulins:
  - **Immunoglobulins**



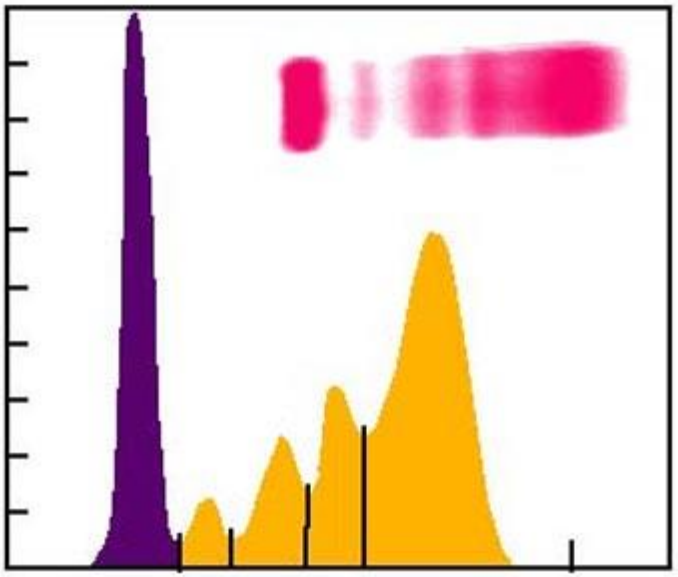
The normal pattern of serum electrophoresis and corresponding **densitometric diagram**.

Their levels in the blood change during the **acute phase reaction** due to inflammatory cytokines (e.g. TNFα, IL1, IL-6):

- **Increase** (also called positive **acute phase proteins**, their most prominent member being **CRP** which can be found between the β and the γ fractions[7.])
- **Decrease**

Examples of abnormal electrophoretic patterns I.

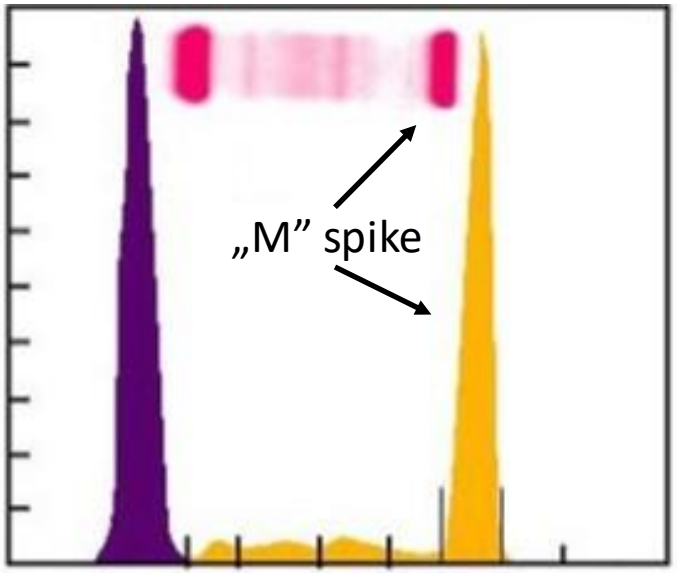
Polyclonal gammopathy



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

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

Monoclonal gammopathy

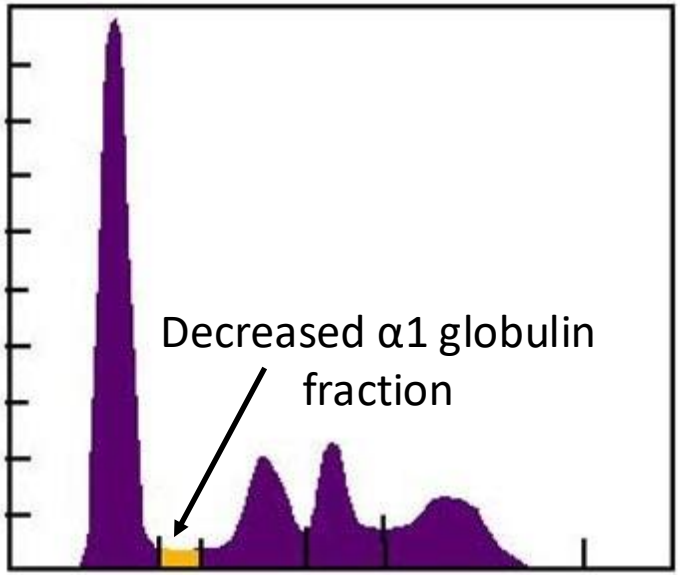


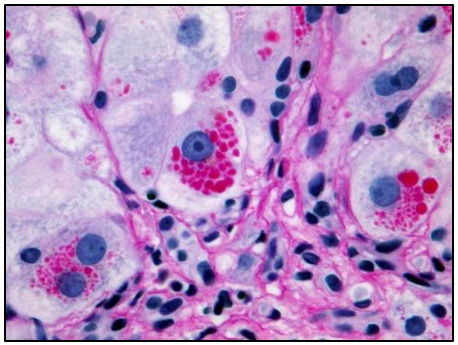
An excess of immunoglobulins produced by a **single B cell clone**. Found in **plasma cell neoplasms**:[7.]

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

Examples of abnormal electrophoretic patterns II.

α1-antitrypsin deficiency[8.]





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

α1-antitrypsin (A1AT):

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

α1-antitrypsin deficiency:

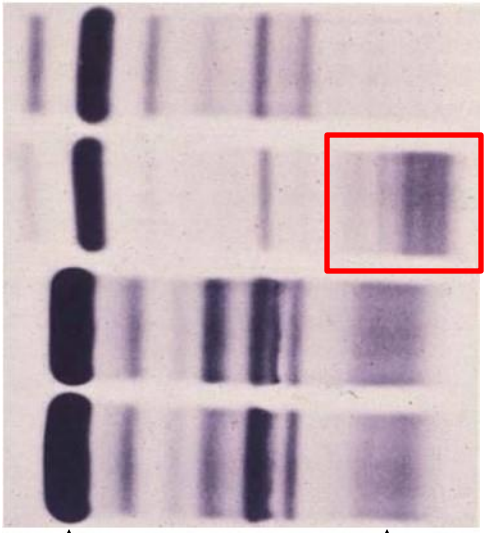
- **It is a genetic disorder.**
- Liver cells are unable to secrete α1-antitrypsin which accumulates in their cytoplasm.
- The level of **α1-antitrypsin** greatly decreases in the blood which will lead to complications:

↓

- **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)

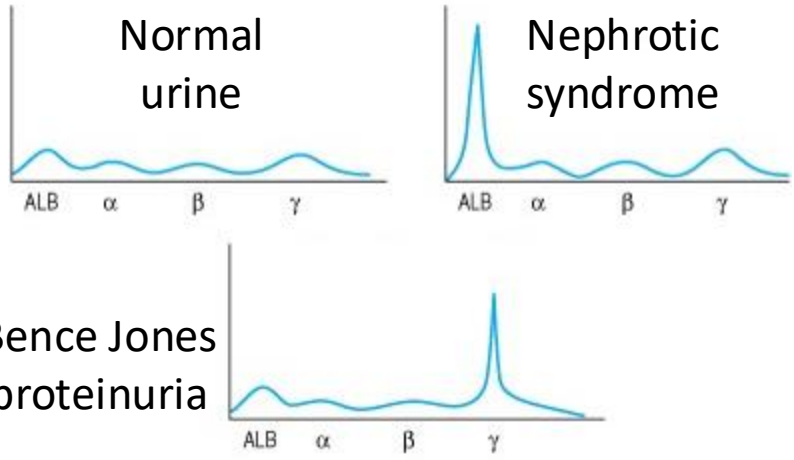


In the CSF of the patient individual bands can be seen in the gamma globulin fraction. The detected pattern differs from that seen in the patient's serum.

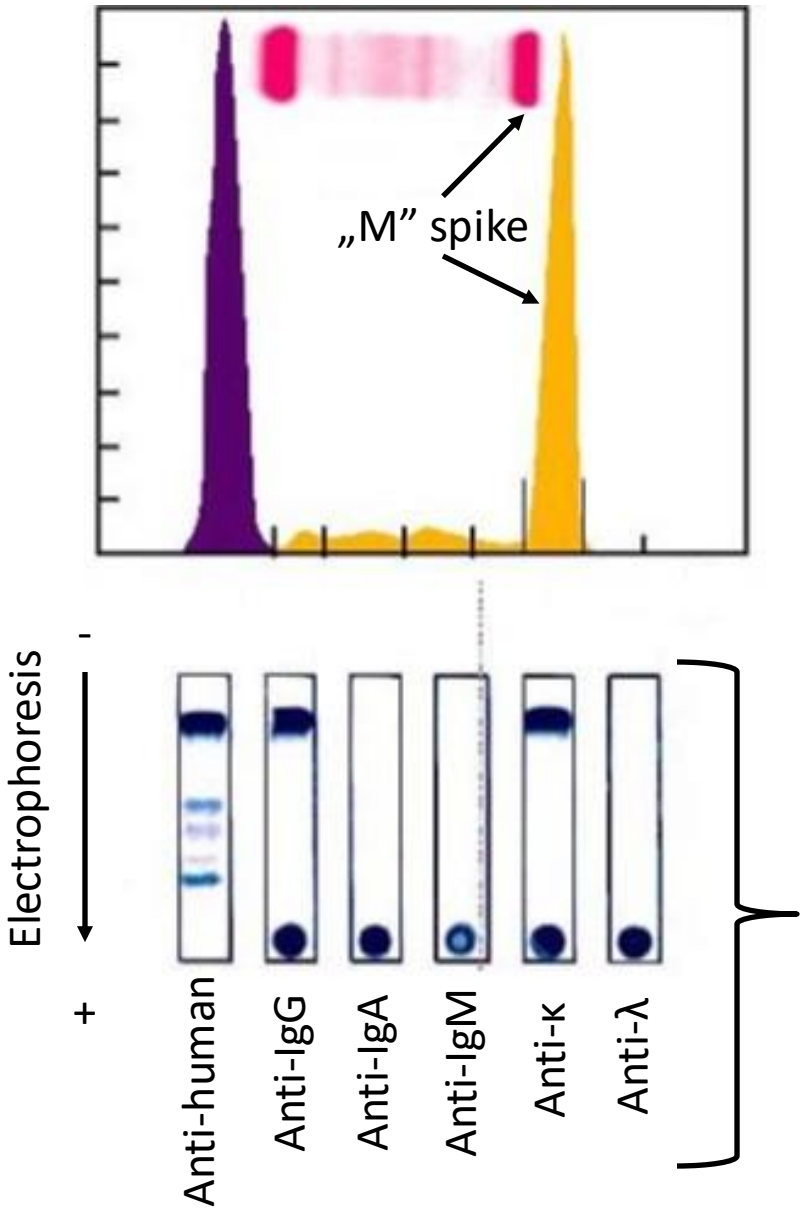
Immunoglobulins are produced locally in the central nervous system of the patient. (**oligoclonal gammopathy**, e.g. in **multiple sclerosis**[9.])

Urine electrophoresis:

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



Immunofixation



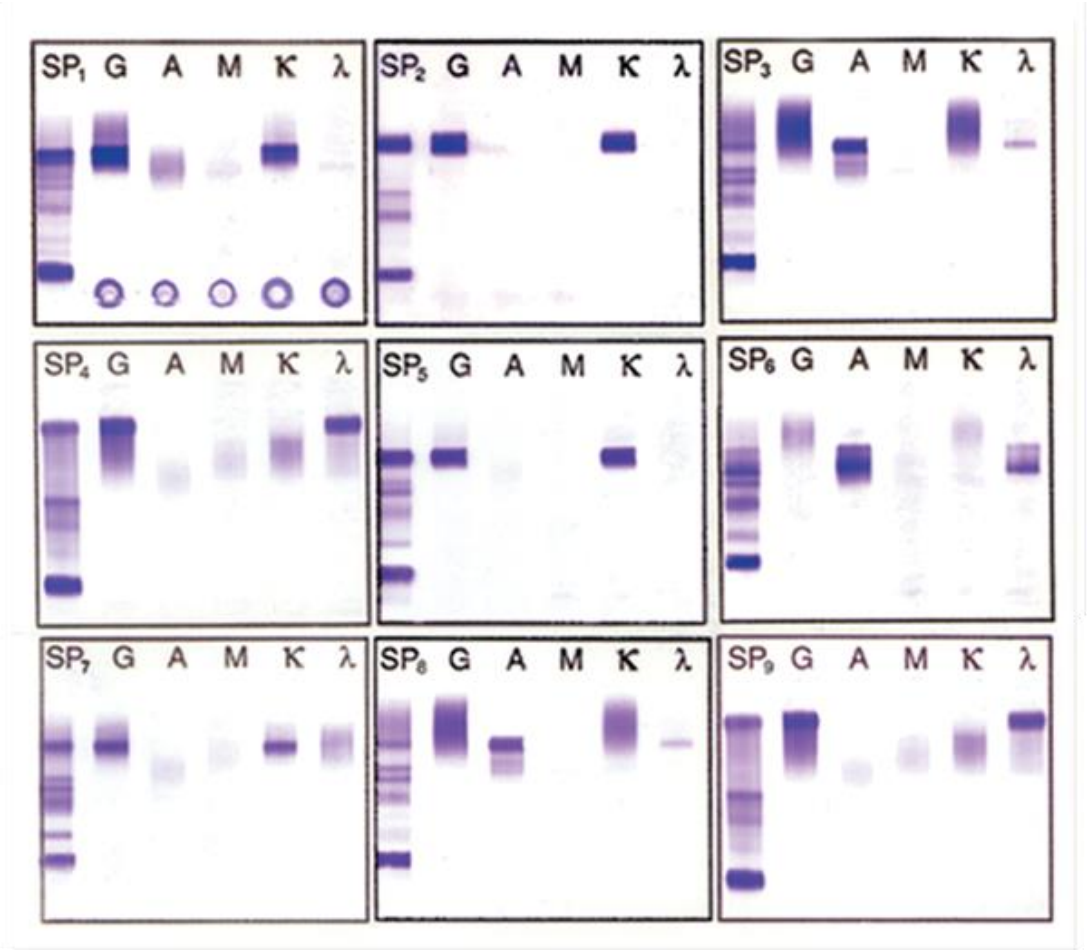
1. The electorphoresis is performed simultaneously by dividing the serum into several parallel samples.[12.]
2. The specific proteins are detected in different gels using different antibodies. (The added antibodies precipitate with the antigen which is usually detected with dyes. The antigens are the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

Application:

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

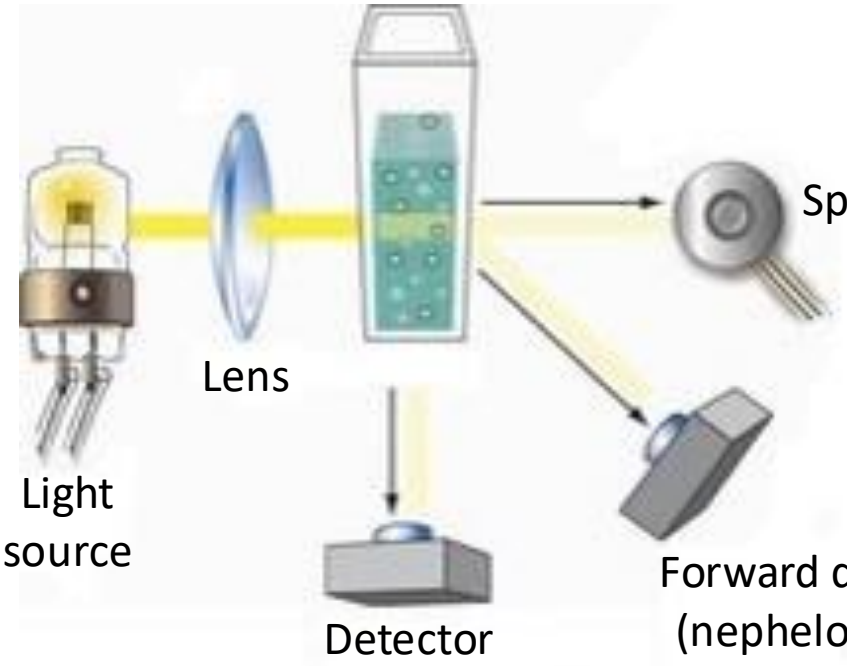
Multiple myeloma producing monoclonal antibodies of IgG κ isotype.

Immunfixation



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.[14.]

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

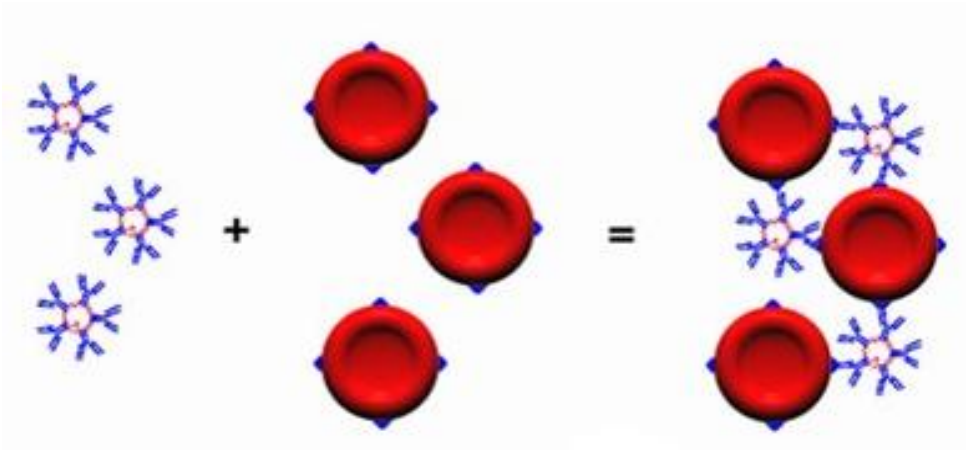
- Nephelometry, a method to detect the concentration of serum proteins including immunoglobulin, is based on the concept that particles in solution will scatter light passing through the solution rather than absorbing the light. Nephelometers record the degree of scatter, and scientists correlate this 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

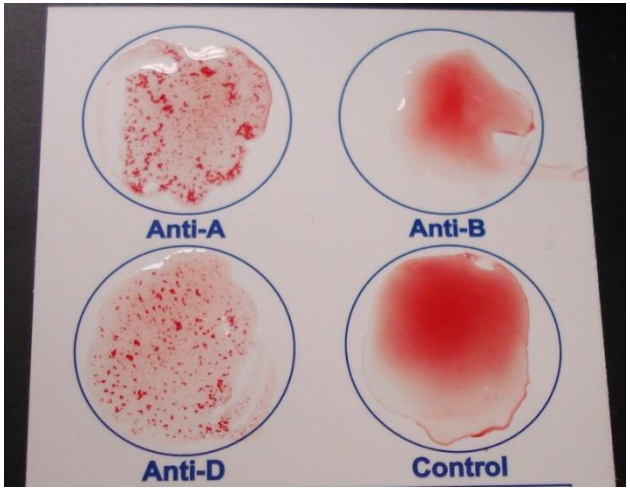
- If antibodies cross-bind larger particles and it leads to the aggregation of these particles = **agglutination** (if these particles are red blood cells it is called **hemagglutination**)
- Agglutination is one of the **physiological functions** of antibodies, agglutination of pathogens prevents the spread of infections.[15.]
- 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

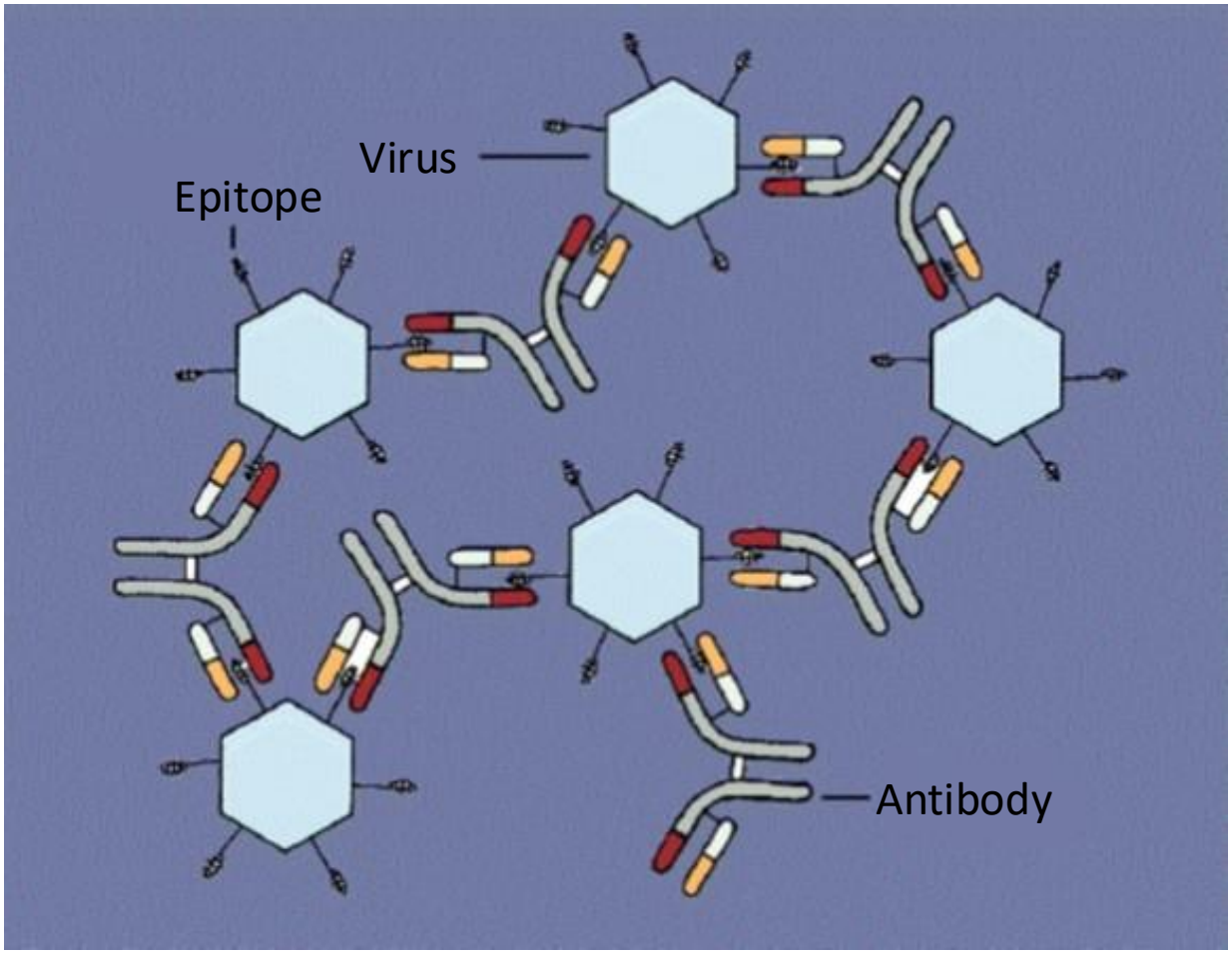
RBC with „A" antigen

Hemagglutination



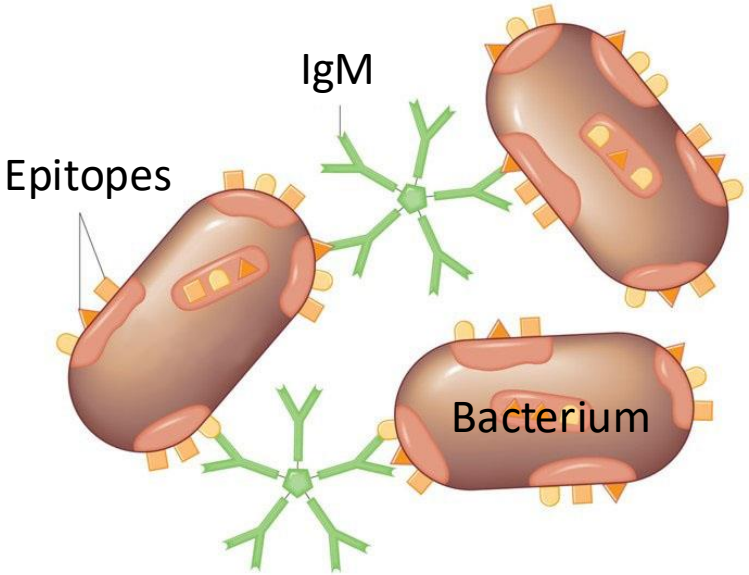
Blood type test: A, Rh(D) positive

Physiological role of agglutination



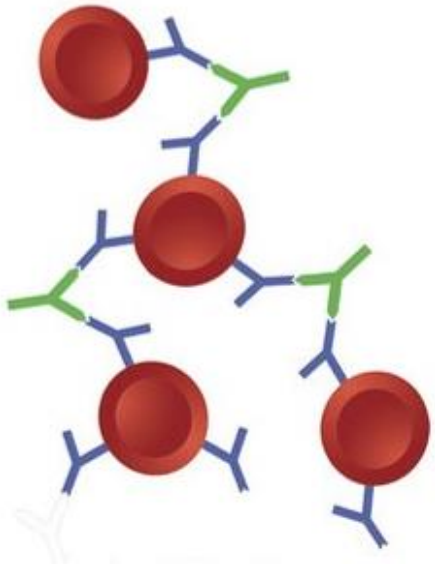
Direct or indirect

Direct agglutination:



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

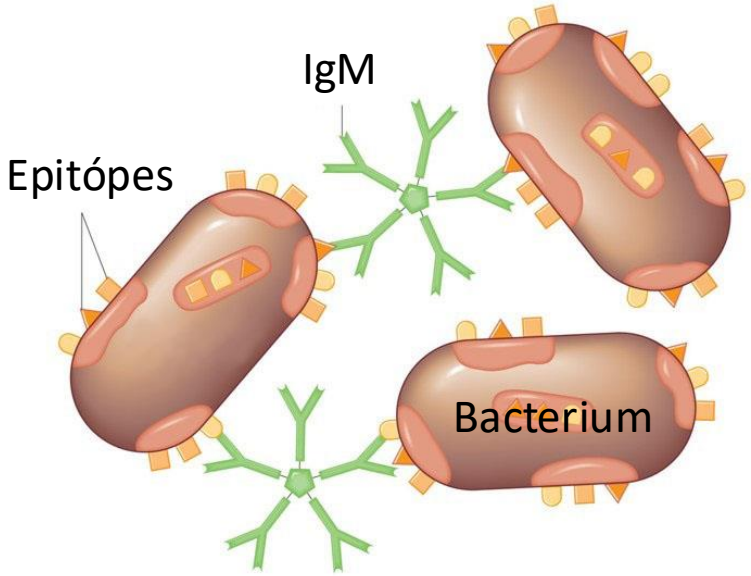
Indirect agglutination:



- 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

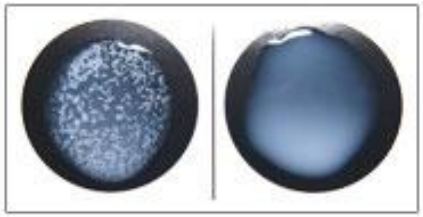
Passive agglutination:

- 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

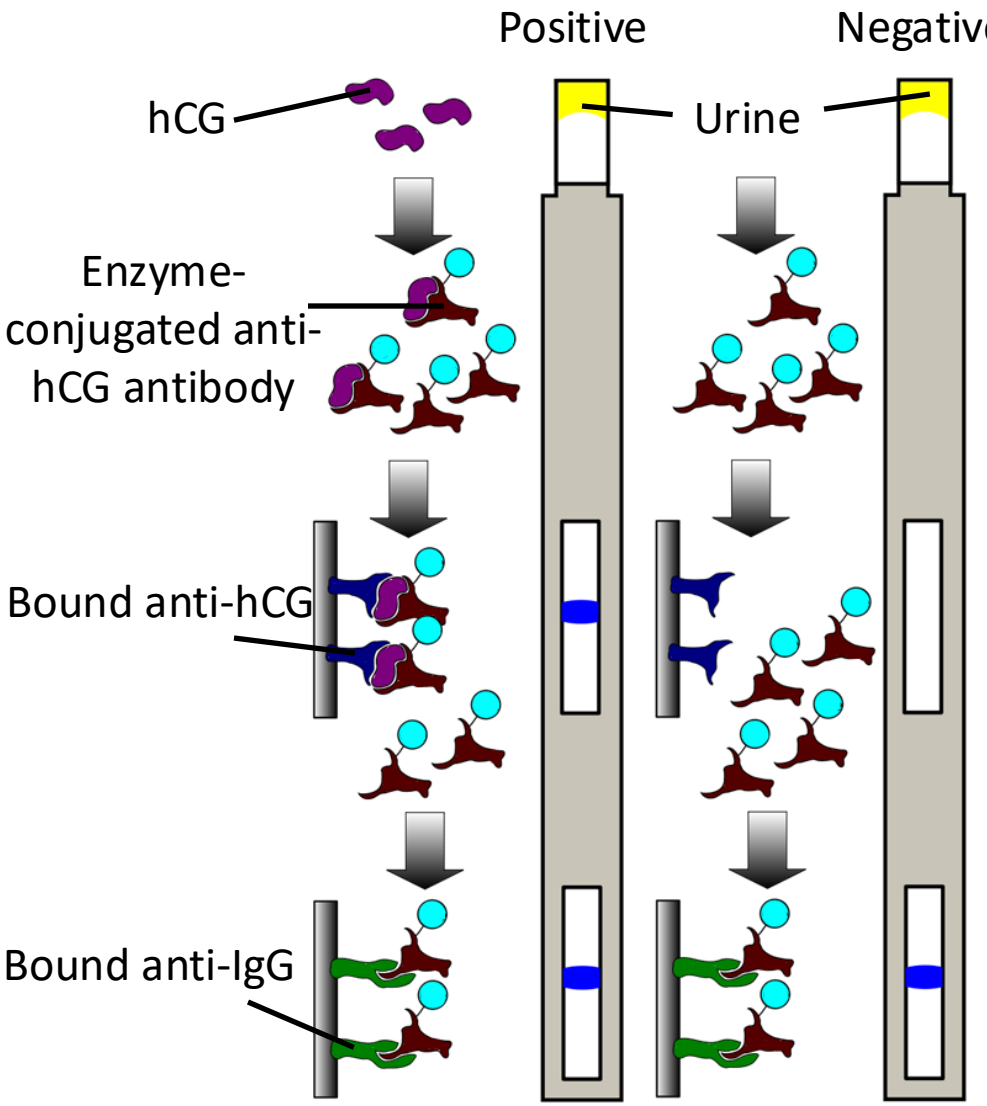
The antigen/antibody that participates in the reaction is bound to the surface of **latex beads.**

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

Applications:

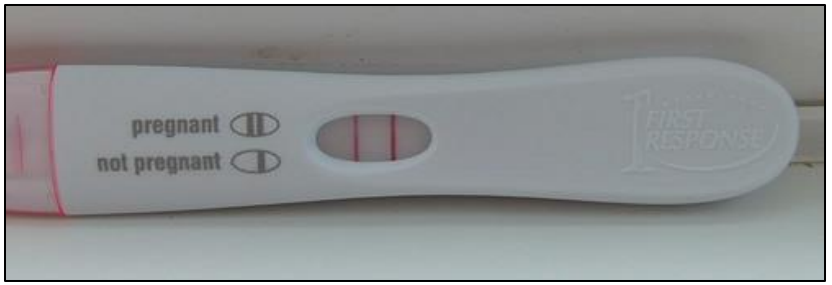
- **Diagnostics of autoimmune disorders**, e.g.:
  - Rheumatoid arthritis (rheumatoid factor, RF[16.]) , SLE (various autoantibodies)
- **Diagnostics of infectious diseases**, e.g.:
  - Detection of antibodies against microbial antigens (e.g. anti-streptolysin O antibody, ASO/AST[17.])
  - Detection of bacterial antigens
- Detection of other proteins, e.g.:
  - **C-reactive protein** (CRP, acute phase protein[18.]), D-dimer[19.] (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.**[20.]

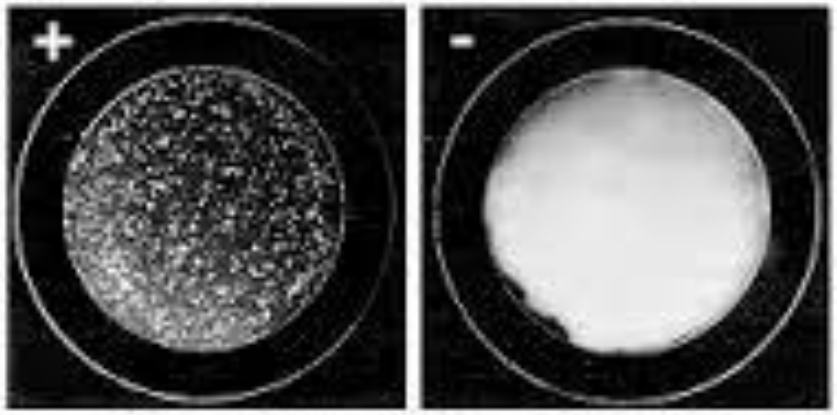


Band forms only if the enzyme-conjugated antibody is bound. If hCG is not present in the urine then only the anti-IgG will bind the labeled antibodies and only one band will appear.

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.

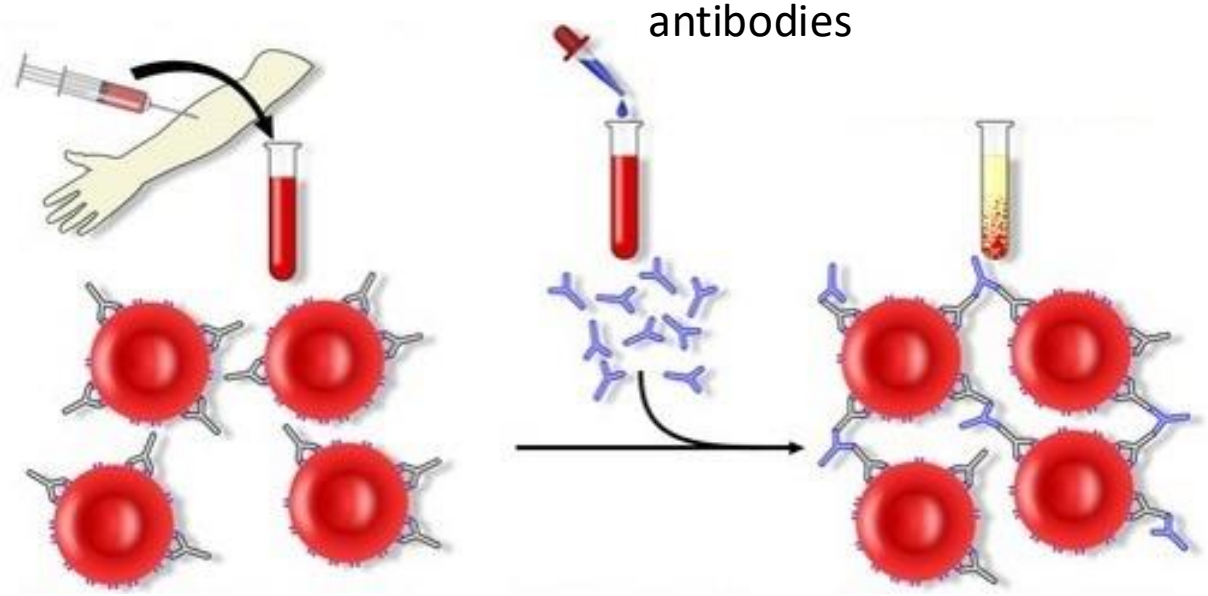


WEAR GLOVES!

Direct Coombs test (Direct antiglobulin test[21.])

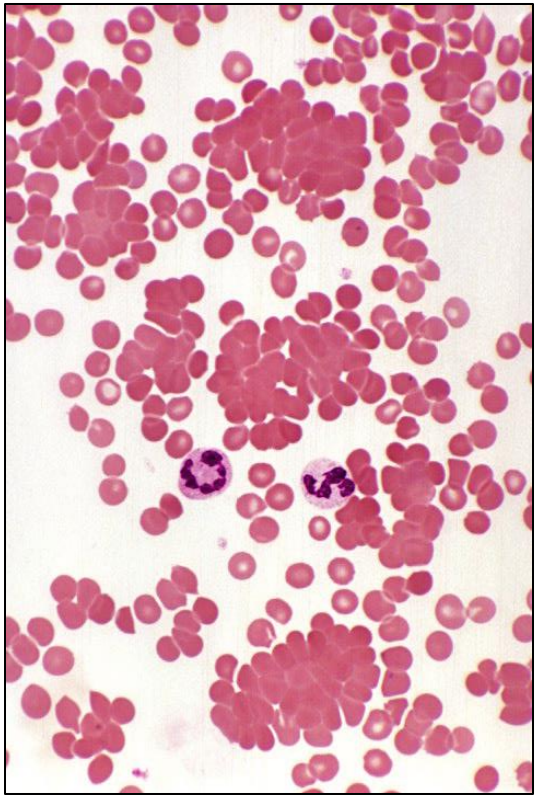
Venopuncture

+ Anti-human immunoglobulin antibodies



Autoreactive antibodies on the patient's RBCs

Hemagglutination

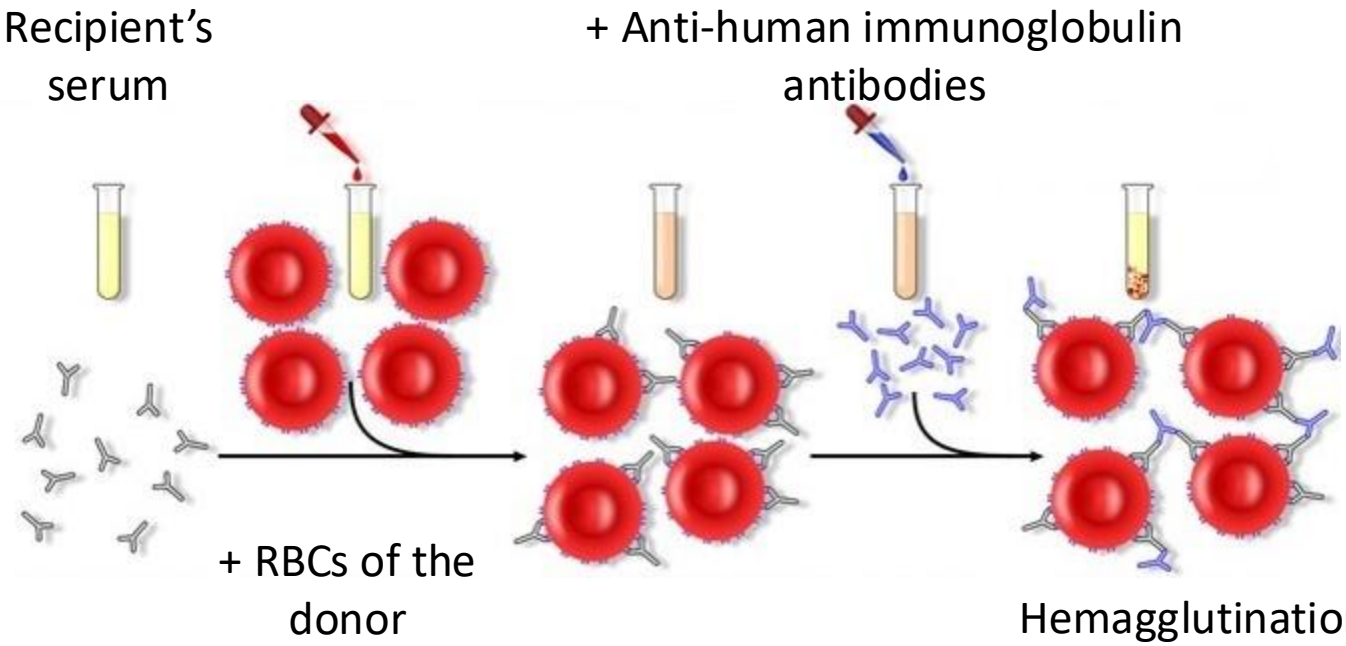


In vivo hemagglutination in a patient with AIHA.

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

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

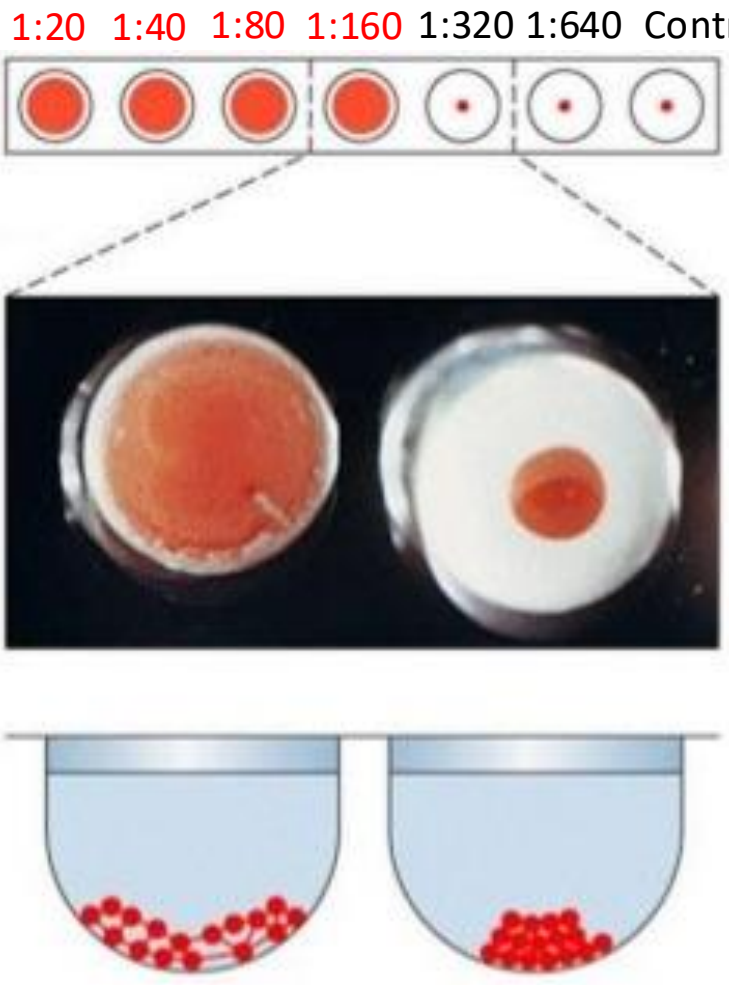
Indirect Coombs test (Indirect antiglobulin test)



Applications:

- Screening for antibodies before **blood transfusions**[23.] (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.[24.]

Hemagglutination assay

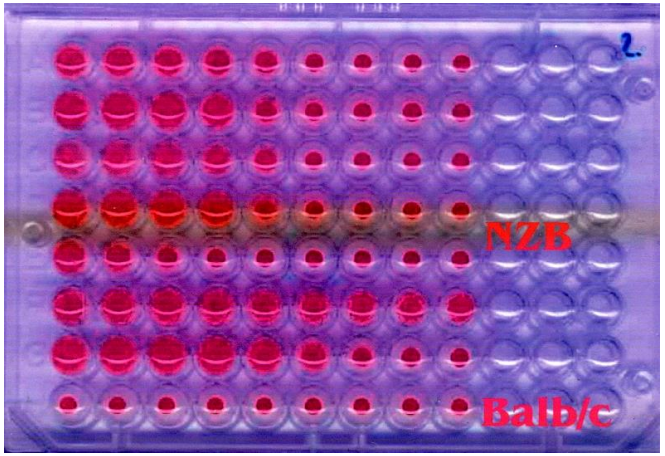


Hemagglutination Negative

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)



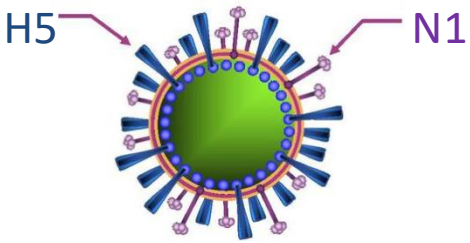
- NZB: New Zealand Black mouse strain[25.] (murine model of AIHA)
- BALB/c: albino strain of house mouse (control)

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**,[26.] 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.[26.]

References 1.

1. Akobeng AK[1]: **Understanding diagnostic tests 1: sensitivity, specificity and predictive values.** *Acta Paediatr.* 2007 Mar;96(3):338-41.
2. Mancini G, Carbonara AO, Heremans JF: **Immunochemical quantitation of antigens by single radial immunodiffusion.** *Immunochemistry.* 1965 Sep;2(3):235-54.
3. Ouchterlony O: **In vitro method for testing the toxin-producing capacity of diphtheria bacteria.** *Acta Pathol Microbiol Scand.* 1948;25(1-2):186-91.
4. Tiselius A[1]: **Electrophoresis of serum globulin: Electrophoretic analysis of normal and immune sera.** *Biochem J.* 1937 Sep;31(9):1464-77.
5. Nobelprize.org: **The Nobel Prize in Chemistry 1948** (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1948/)
6. Jain S[1], Gautam V, Naseem S: **Acute-phase proteins: As diagnostic tool.** *J Pharm Bioallied Sci.* 2011 Jan;3(1):118-27. doi: 10.4103/0975-7406.76489.
7. O'Connell TX[1], Horita TJ, Kasravi B: **Understanding and interpreting serum protein electrophoresis.** *Am Fam Physician.* 2005 Jan 1;71(1):105-12.
8. Stoller JK[1], Aboussouan LS: **Alpha1-antitrypsin deficiency.** *Lancet.* 2005 Jun 25-Jul 1;365(9478):2225-36.
9. Link H[1], Huang YM: **Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness.** *J Neuroimmunol.* 2006 Nov;180(1-2):17-28. Epub 2006 Sep 1.
10. Marshall T[1], Williams KM: **Electrophoretic analysis of Bence Jones proteinuria.** *Electrophoresis.* 1999 Jun;20(7):1307-24.

References 2.

11. Csako G[1]: **Immunoelectrophoresis: a method with many faces.** *Methods Mol Biol.* 2012;869:339-59. doi: 10.1007/978-1-61779-821-4_28.
12. Csako G[1]: **Immunofixation electrophoresis for identification of proteins and specific antibodies.** *Methods Mol Biol.* 2012;869:147-71. doi: 10.1007/978-1-61779-821-4_13.
13. Rajkumar SV[1], Kyle RA[1]: **Protein electrophoresis and immunofixation for the diagnosis of monoclonal gammopathies.** *JAMA.* 2014 Nov 26;312(20):2160-1. doi: 10.1001/jama.2014.8237.
14. Mali B[1], Armbruster D, Serediak E, Ottenbreit T: **Comparison of immunoturbidimetric and immunonephelometric assays for specific proteins.** *Clin Biochem.* 2009 Oct;42(15):1568-71. doi: 10.1016/j.clinbiochem.2009.06.016. Epub 2009 Jun 26.
15. Cooper NR, Nemerow GR: **The role of antibody and complement in the control of viral infections.** *J Invest Dermatol*. 1984 Jul;83(1 Suppl):121s-127s.
16. Anuradha V[1], Chopra A: **In the era of nephelometry, latex agglutination is still good enough to detect rheumatoid factor.** *J Rheumatol.* 2005 Dec;32(12):2343-4.
17. Kodama T[1], Ichiyama S, Morishita Y, Fukatsu T, Shimokata K, Nakashima N: **Determination of anti-streptolysin O antibody titer by a new passive agglutination method using sensitized toraysphere particles.** *J Clin Microbiol.* 1997 Apr;35(4):839-42.
18. Komoriya T[1], Terashima Y, Ogawa M, Moriyama M, Kohno H: **Development of a high-sensitivity latex reagent for the detection of C-reactive protein.** *J Immunol Methods.* 2011 Oct 28;373(1-2):63-6. doi: 10.1016/j.jim.2011.08.001. Epub 2011 Aug 26.
19. Froehling DA[1], Elkin PL, Swensen SJ, Heit JA, Pankratz VS, Ryu JH: **Sensitivity and specificity of the semiquantitative latex agglutination D-dimer assay for the diagnosis of acute pulmonary embolism as definedby computed tomographic angiography.** *Mayo Clin Proc.* 2004 Feb;79(2):164-8.
20. Braunstein GD[1]: **The long gestation of the modern home pregnancy test.** *Clin Chem.* 2014 Jan;60(1):18-21. doi: 10.1373/clinchem.2013.202655. Epub 2013 Sep 11.

References 3.

21. Zantek ND[1], Koepsell SA, Tharp DR Jr, Cohn CS: **The direct antiglobulin test: a critical step in the evaluation of hemolysis.** *Am J Hematol.* 2012 Jul;87(7):707-9. doi: 10.1002/ajh.23218. Epub 2012 May 6.
22. Barcellini W[1]: **Immune Hemolysis: Diagnosis and Treatment Recommendations.** *Semin Hematol.* 2015 Oct;52(4):304-12. doi: 10.1053/j.seminhematol.2015.05.001. Epub 2015 May 19.
23. British Committee for Standards in Haematology[1], Milkins C, Berryman J, Cantwell C, Elliott C, Haggas R, Jones J, Rowley M, Williams M, Win N: **Guidelines for pre-transfusion compatibility procedures in blood transfusion laboratories. British Committee for Standards in Haematology.** *Transfus Med.* 2013 Feb;23(1):3-35. doi: 10.1111/j.1365-3148.2012.01199.x. Epub 2012 Dec 6.
24. Abbey R[1], Dunsmoor-Su R: **Cost-benefit analysis of indirect antiglobulin screening in Rh(D)-negative women at 28 weeks of gestation.** *Obstet Gynecol.* 2014 May;123(5):938-45. doi: 10.1097/AOG.0000000000000224.
25. Yoshida S[1], Castles JJ, Gershwin ME: **The pathogenesis of autoimmunity in New Zealand mice.** *Semin Arthritis Rheum.* 1990 Feb;19(4):224-42.
26. Pedersen JC[1]: **Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus.** *Methods Mol Biol.* 2014;1161:11-25. doi: 10.1007/978-1-4939-0758-8_2.