IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

5th practice: Immunoserology precipitation, agglutination ELISA, immunoblotting techniques

Basic Immunology

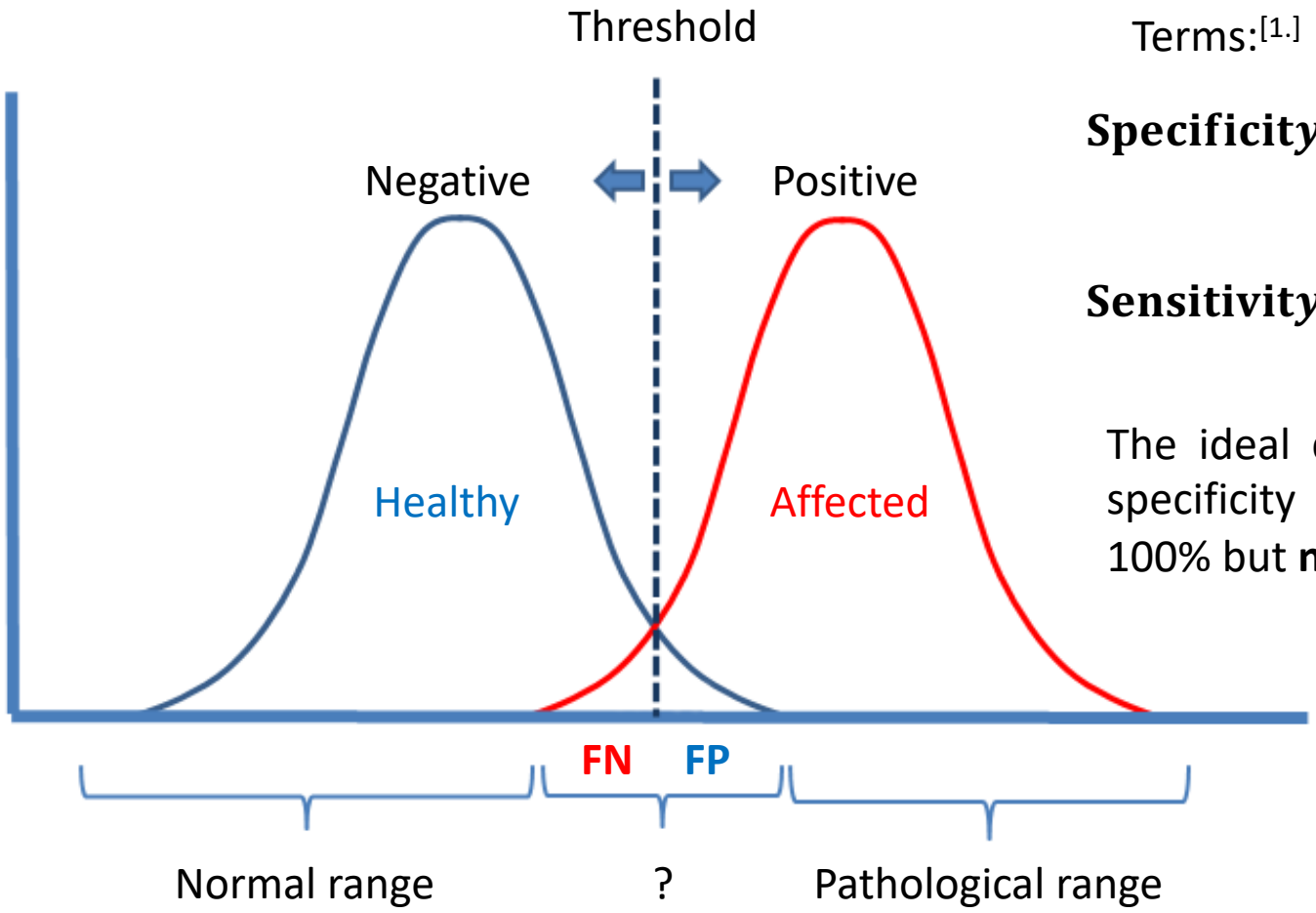
University of Pécs, Clinical Center
Department of Immunology and Biotechnology
Pécs, 2021.

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.]

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

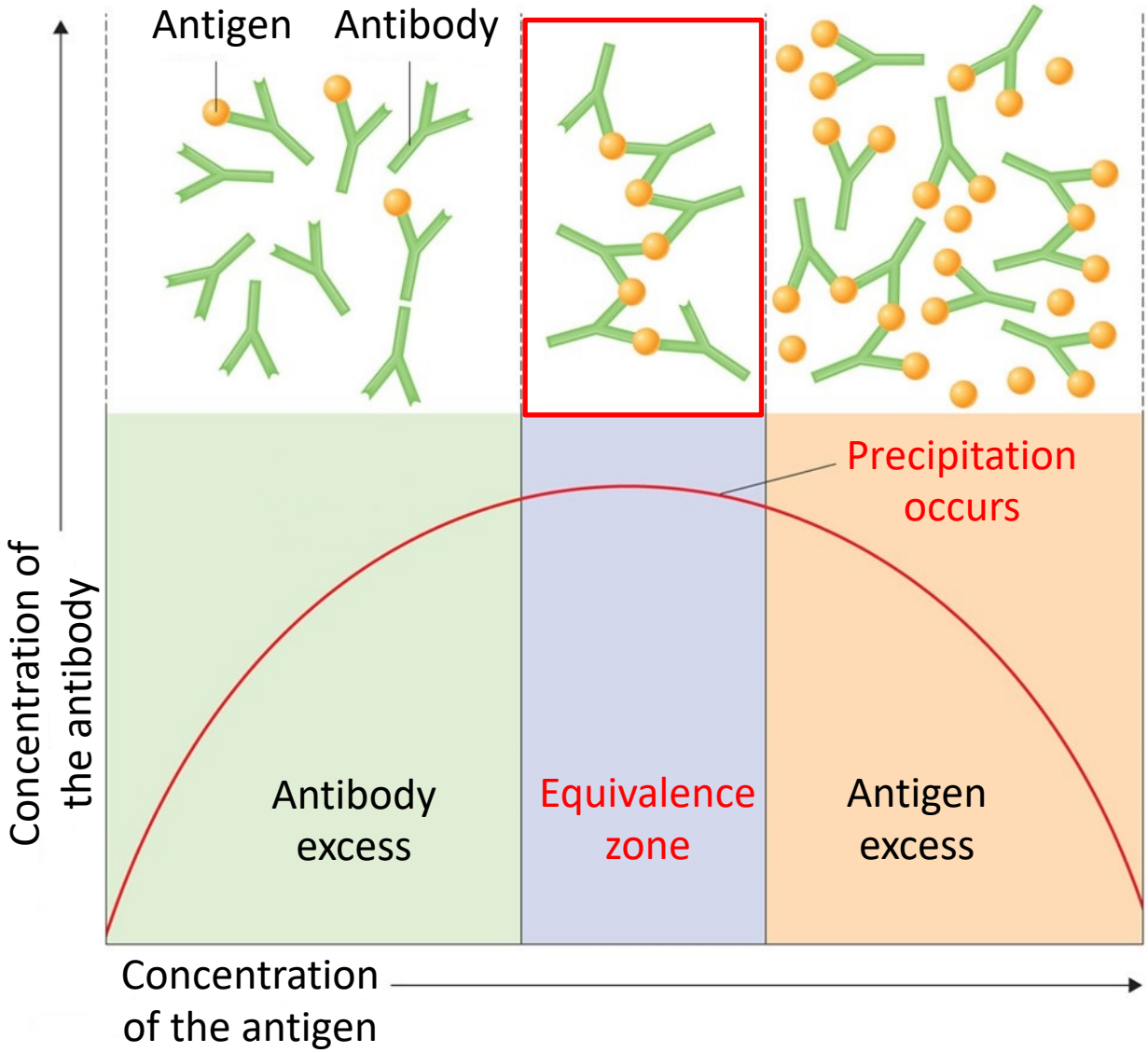
$$\textbf{Sensitivity} = \frac{\text{Real positives}}{\text{All positives}}$$

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.

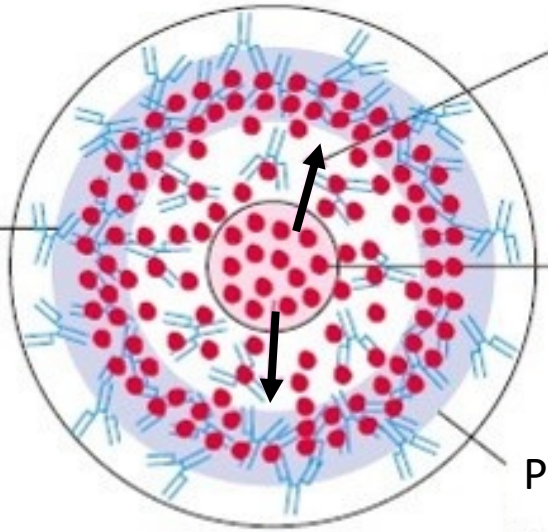
Solubility of these protein complexes decreases and they will precipitate.

Methods based on immunoprecipitation:

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

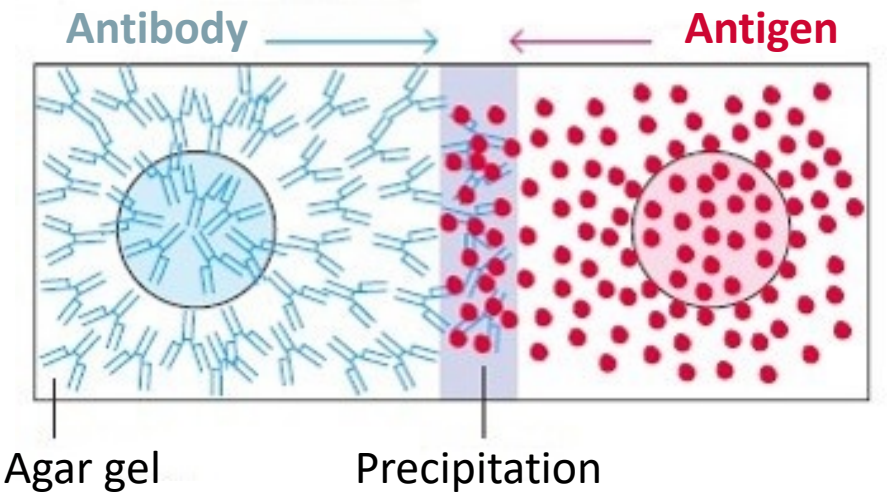
Immunodiffusion I.

Radial immunodiffusion



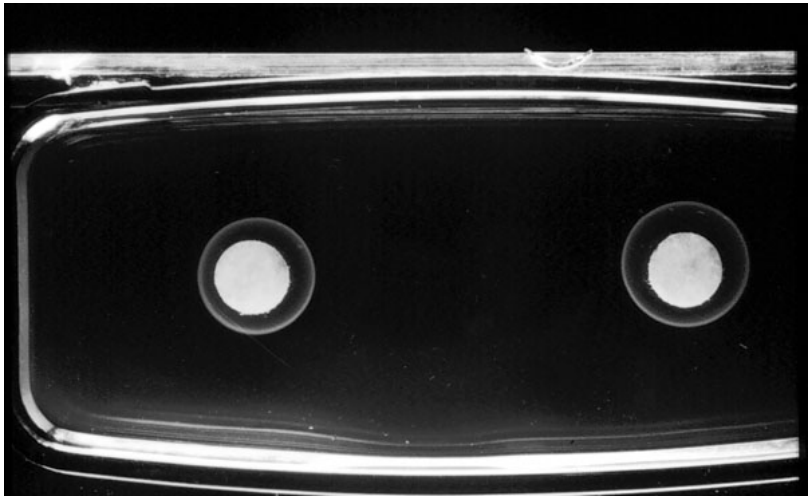
Simple but **outdated** techniques.

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.

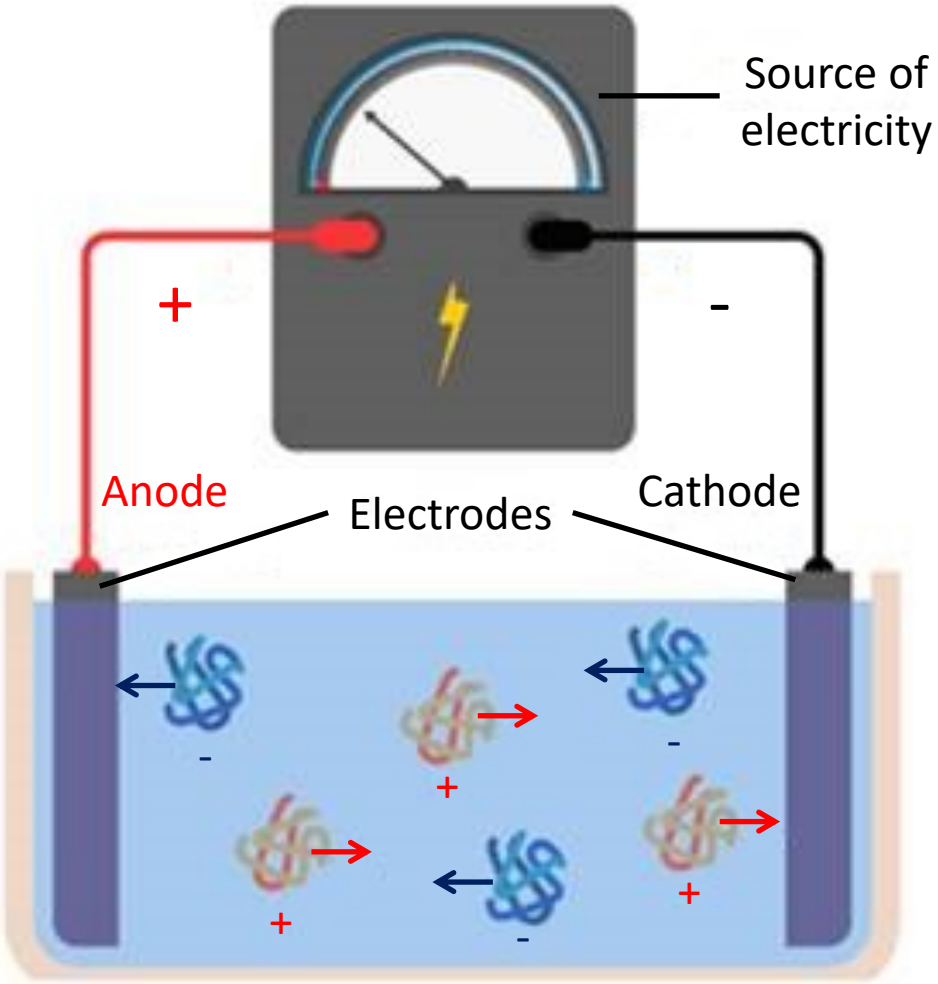
Semiquantitative method.

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.

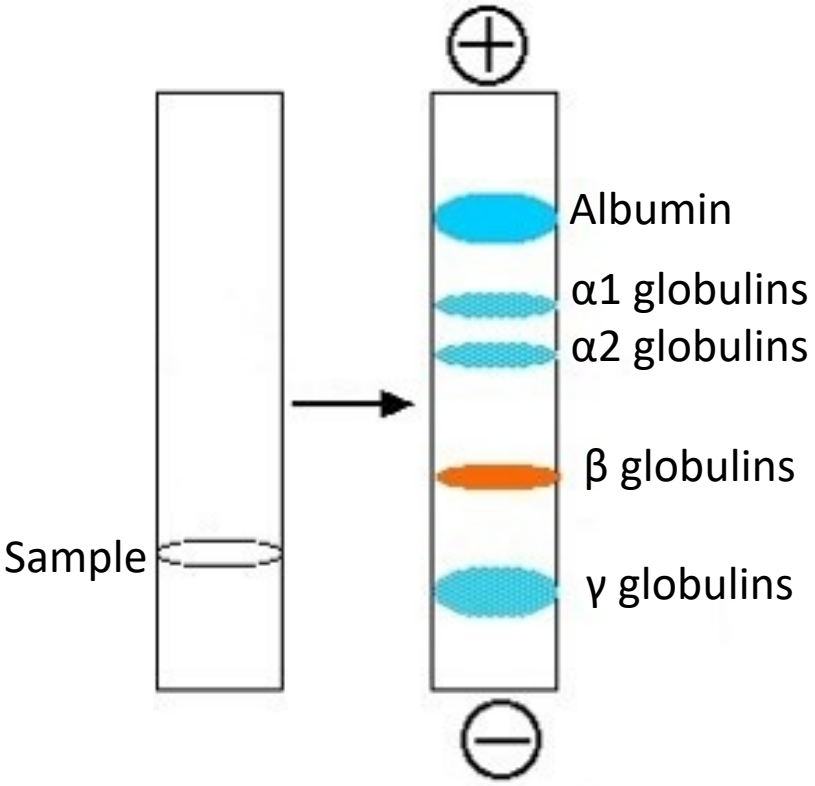
Protein electrophoresis



- 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

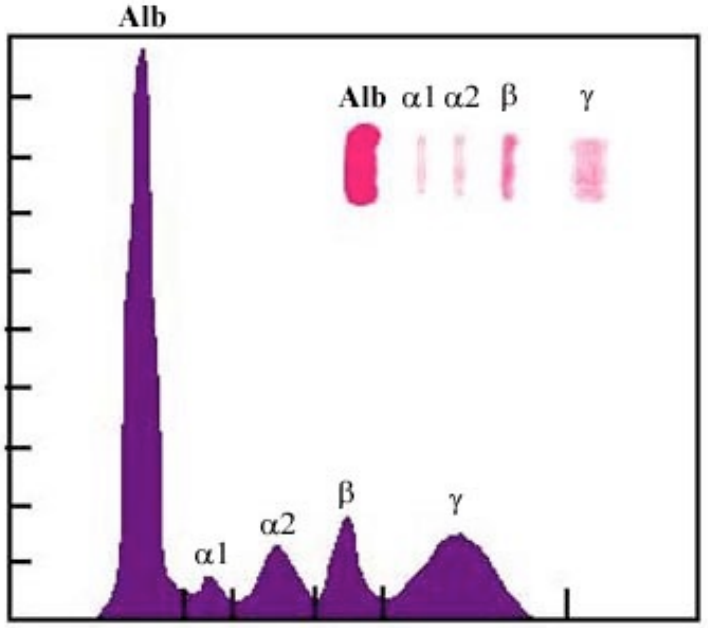
Was awarded the 1948 Nobel Prize in Chemistry:

„for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins."[5.]

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**



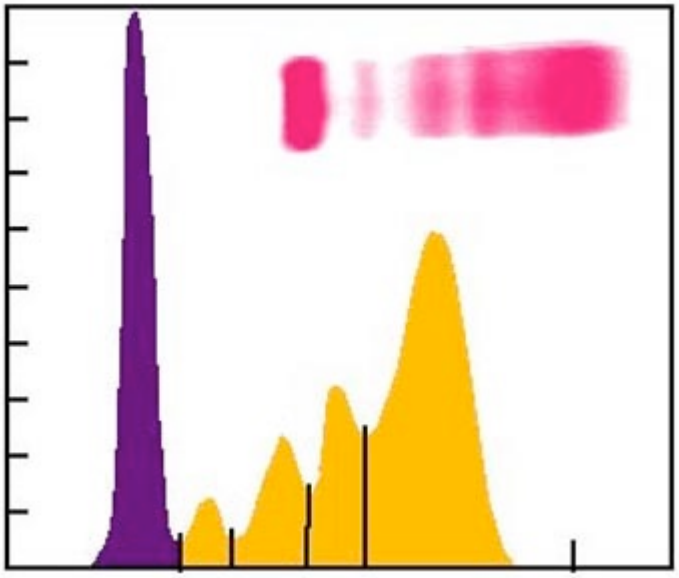
The normal pattern of serum electrophoresis and corresponding **densitometric diagram**.

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.

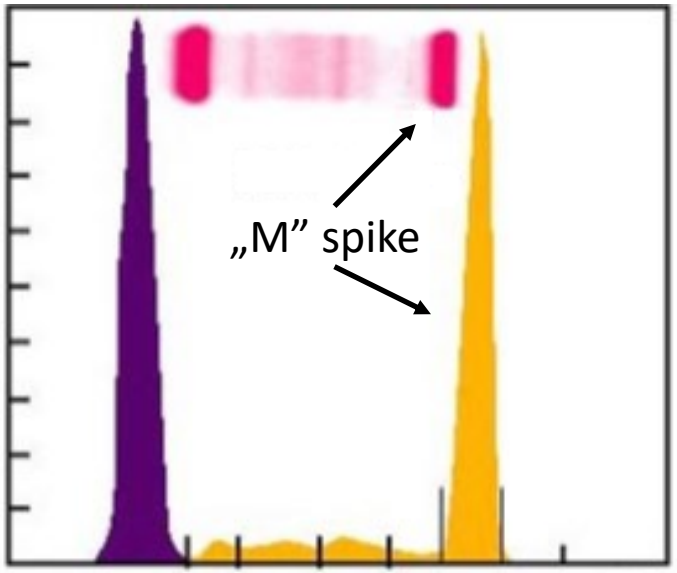
Polyclonal gammopathy



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

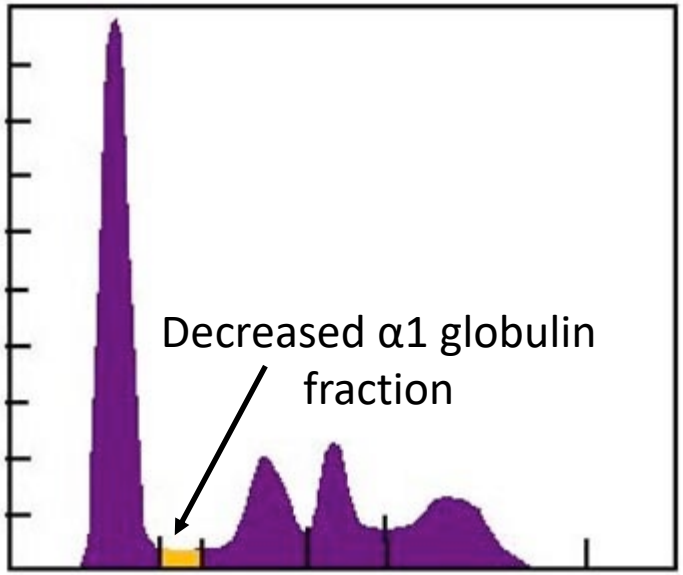


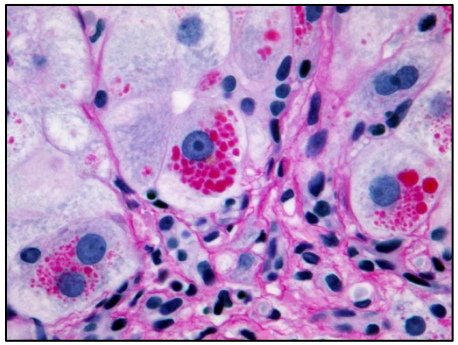
An excess of immunoglobulins produced by a **single B cell clone**. Found in **plasma cell neoplasms**:[7.]

- 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:

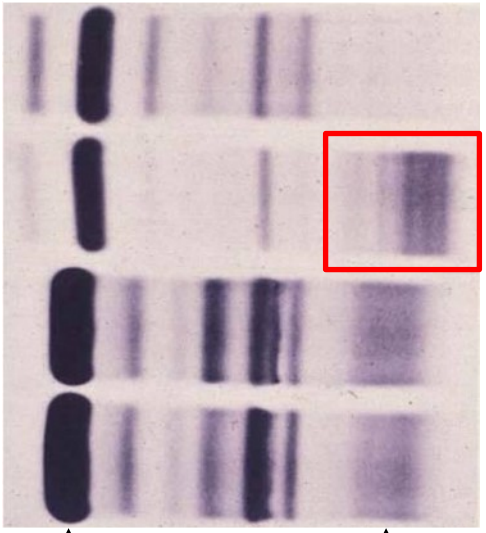
- **It is a genetic disorder.**
- Liver cells are unable to secrete α1-antitrypsin which accumulates in their cytoplasm.
- The level of **α1-antitrypsin** greatly decreases in the blood which will lead to complications:

↓

- **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)

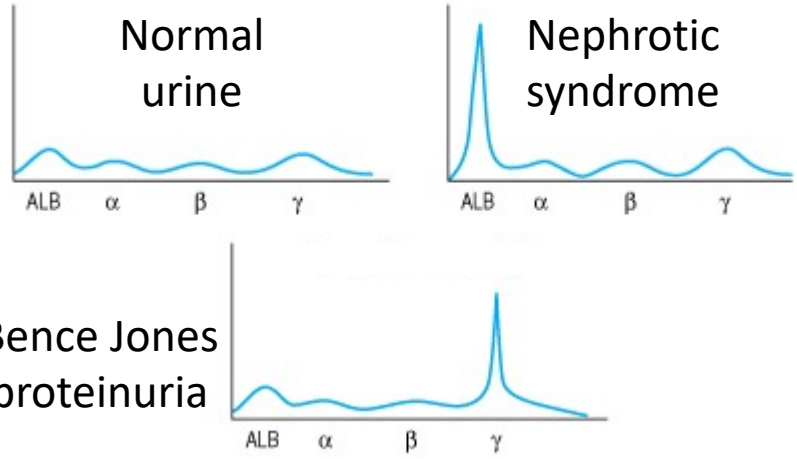


In the CSF of the patient individual bands can be seen in the gamma globulin fraction. The detected pattern differs from that seen in the patient's serum.

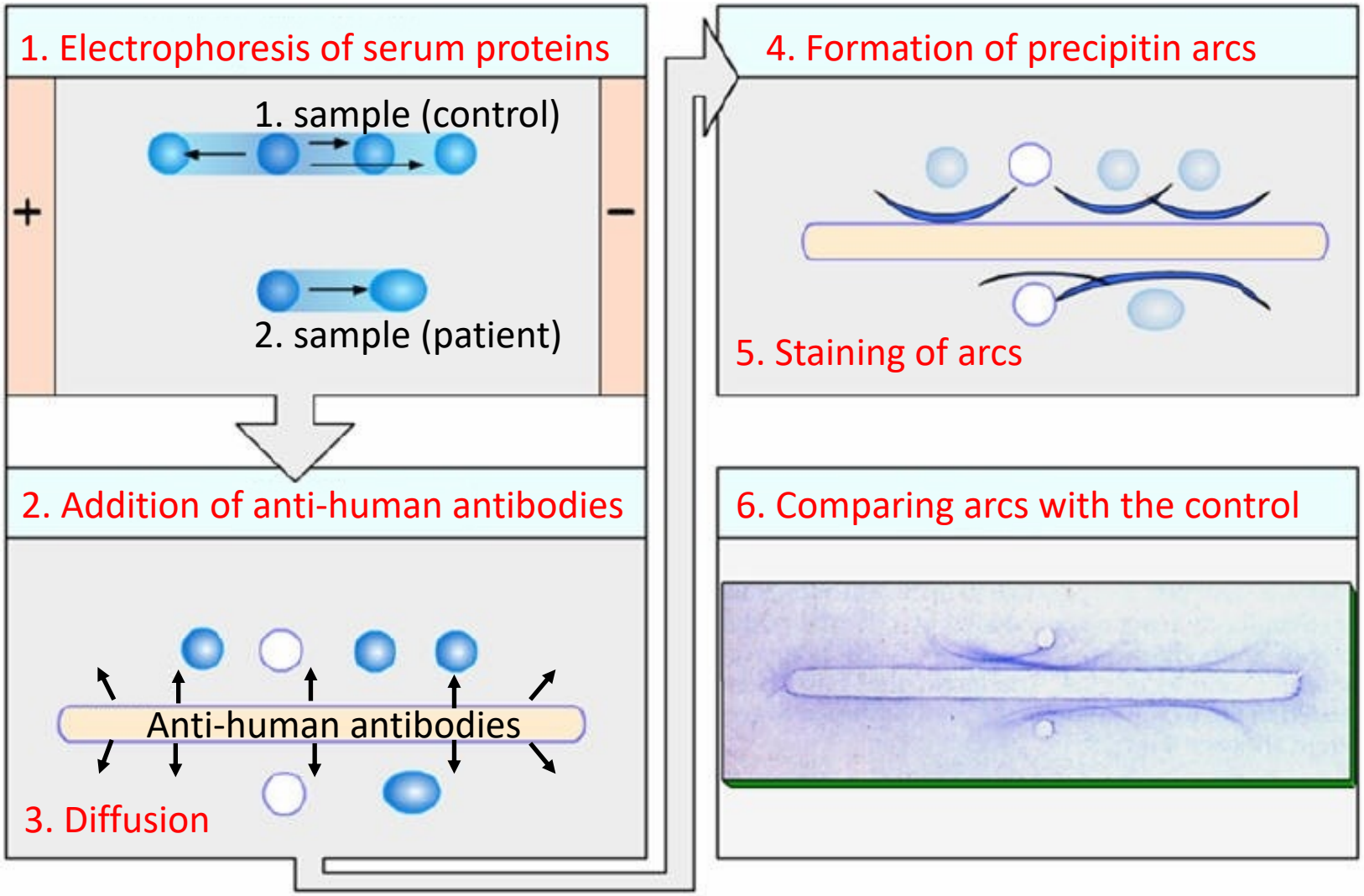
Immunoglobulins are produced locally in the central nervous system of the patient. (**oligoclonal gammopathy**, e.g. in **multiple sclerosis**[9.])

Urine electrophoresis:

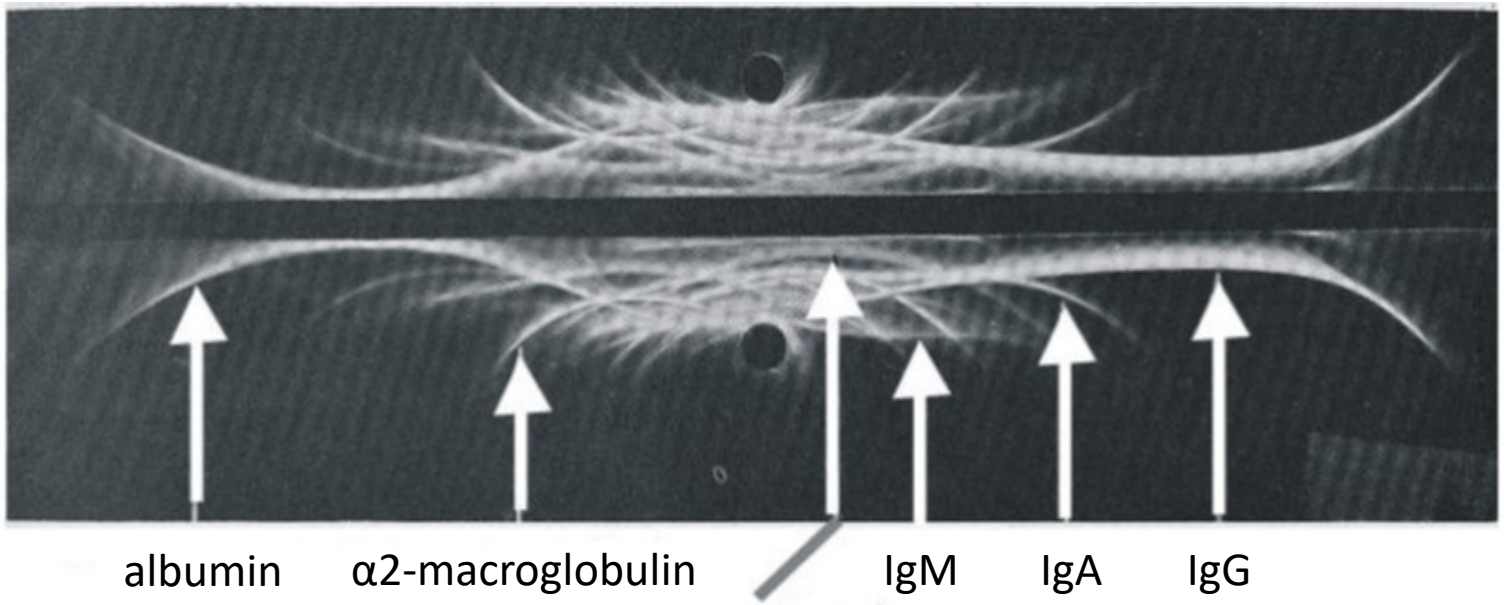
Performed simultaneously with serum electrophoresis when **multiple myeloma** is suspected. They try to detect the immunoglobulin light chain (Bence Jones protein[10.]) in the urine.



Immunoelectrophoresis I.

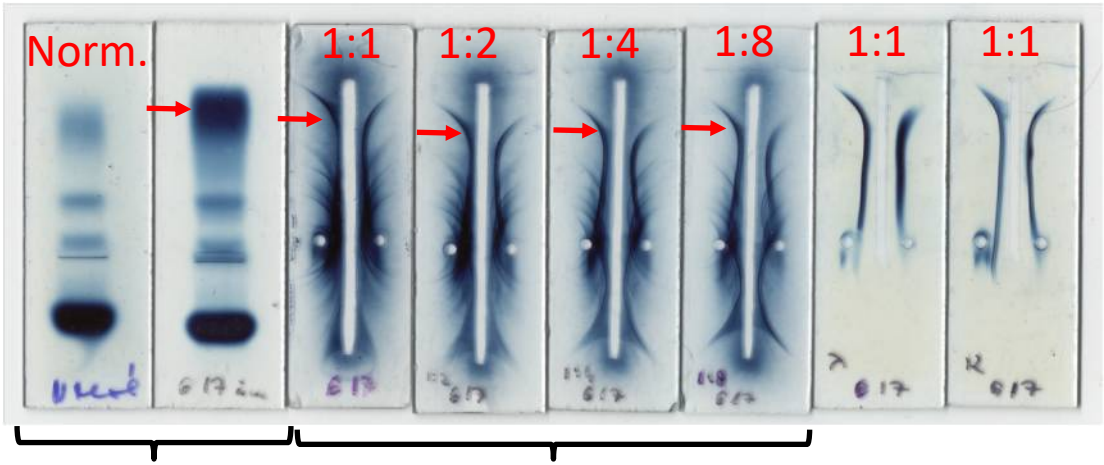


Immunoelectrophoresis II.

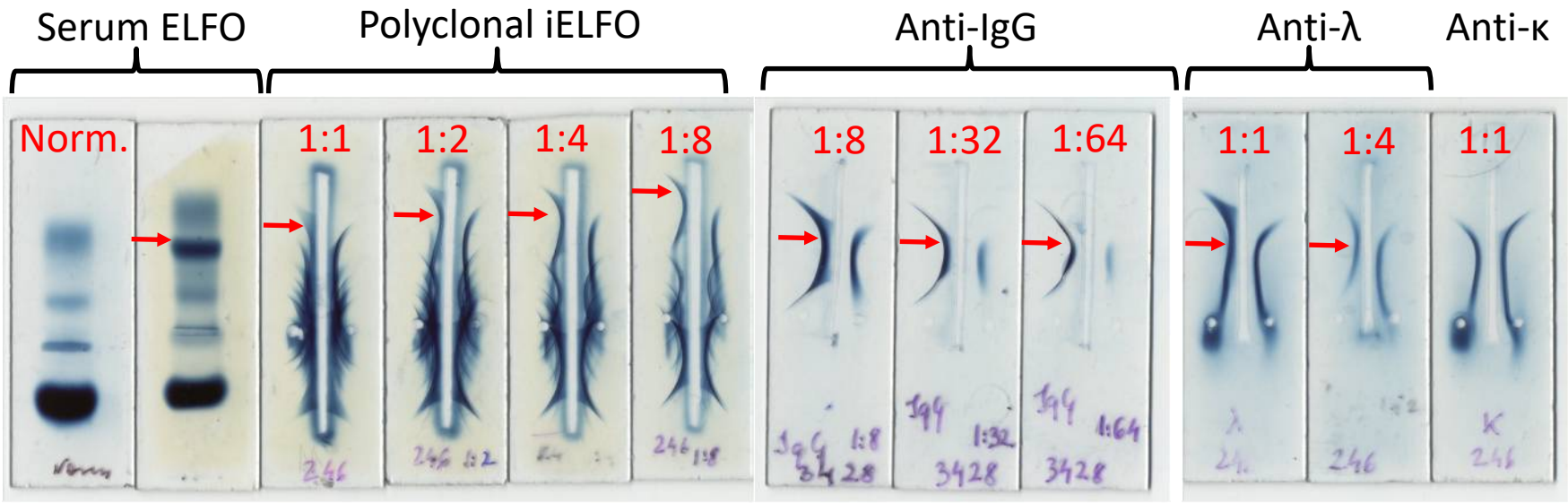


The precipitin arcs of the sample are always compared with a normal control.[11.]
When adding polyclonal antibodies the number of arcs depends on what sort of anti-human serum was used. (horse, rabbit, goat, etc.).
Serum proteins can be specifically identified with the use of monoclonal antibodies.
It is a **semiquantitative test** but is only rarely used in the clinical practice.

1. Polyclonal hypergammaglobulinemia (Rheumatoid arthritis)

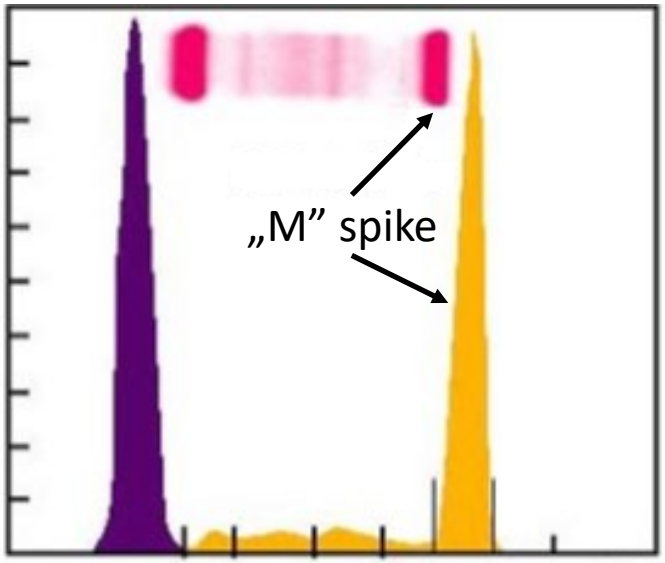


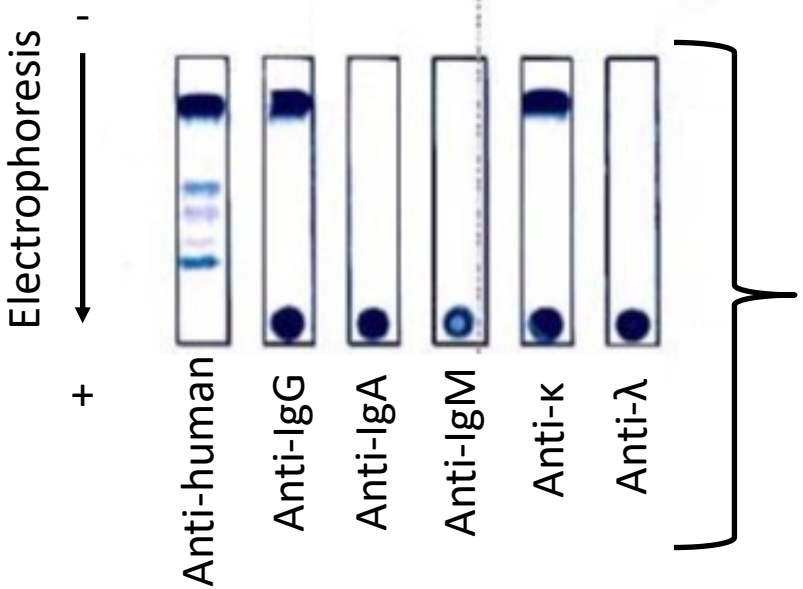
(Explanation:
The 1st patient had a polyclonal B cell activation due to an autoimmune reaction which led to polyclonal antibody production. In the 2nd case clones of a single cancerous plasma cell produce monoclonal immunoglobulins of the very same isotype.)



2. Monoclonal gammopathy (multiple myeloma producing **IgG λ** immunoglobulin)

Immunofixation I.





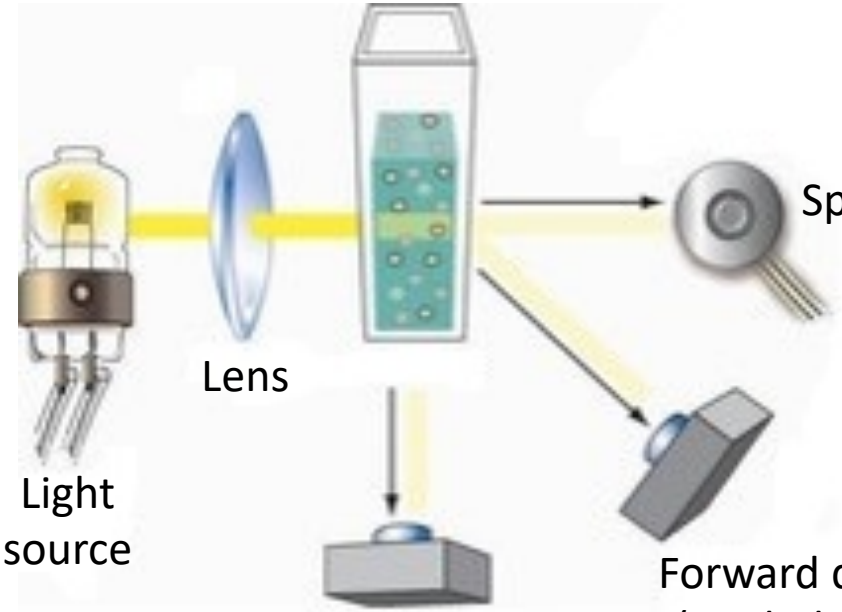
1. The electorphoresis is performed simultaneously by dividing the serum into several parallel samples.[12.]
2. The specific proteins are detected in different gels using different antibodies. (The added antibodies precipitate with the antigen which is usually detected with dyes. The antigens are the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

Nephelometry, turbidimetry



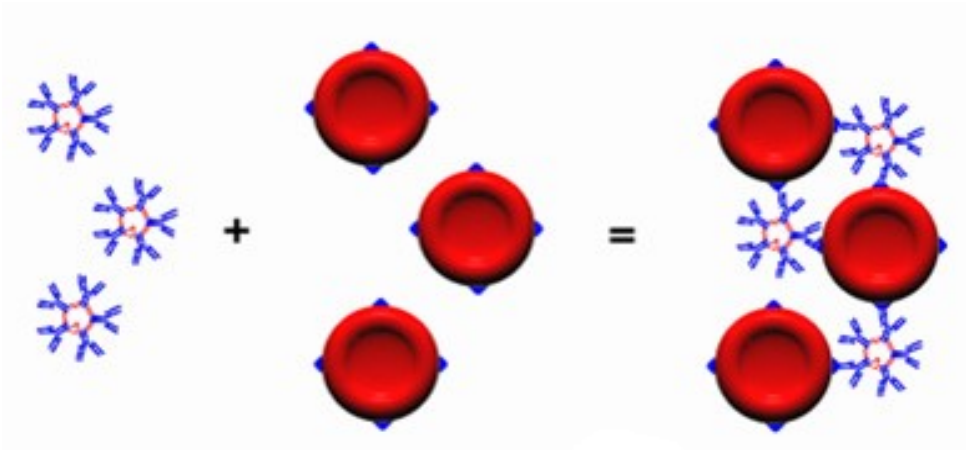
Macromolecules (such as immunocomplexes) in **solutions scatter light**. The scattering is proportional to size of the particles.

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.]

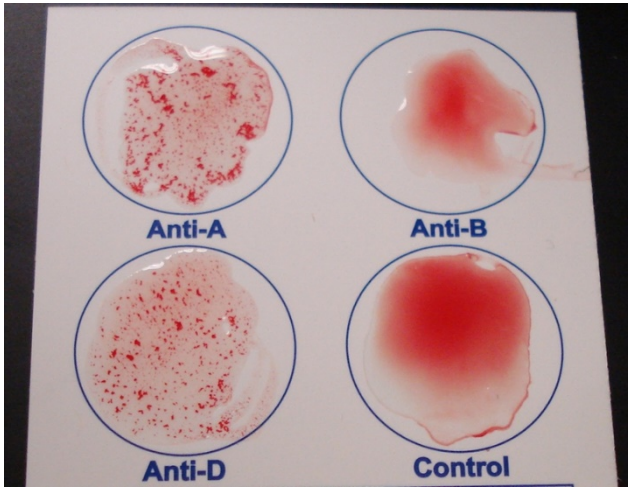
Application: Measuring the concentrations of immunocomplexes, e.g. IgA, IgM, IgG levels or the levels of light chains (e.g. in multiple myeloma), complement levels

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.

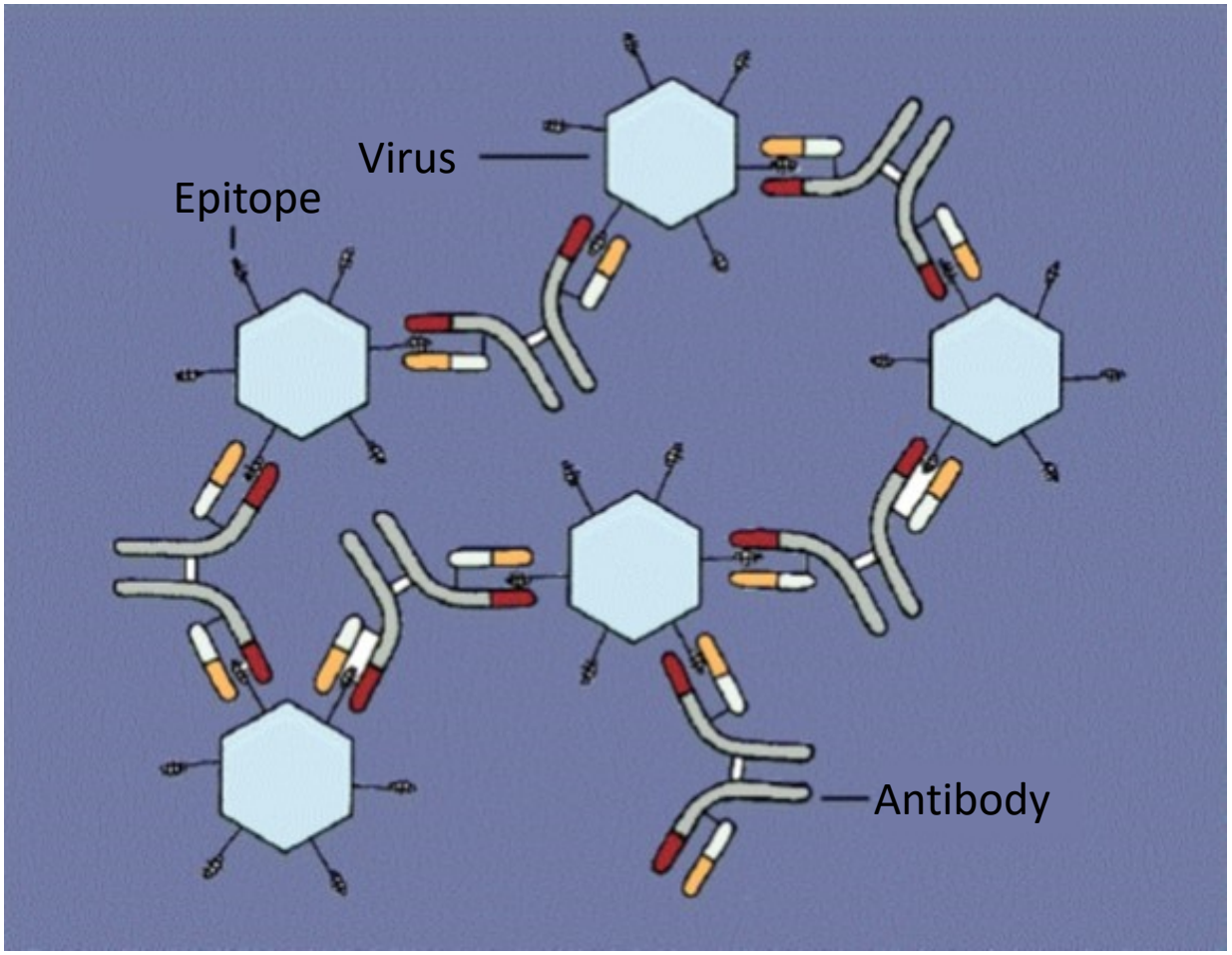


Anti-„A" IgM RBC with „A" antigen Hemagglutination



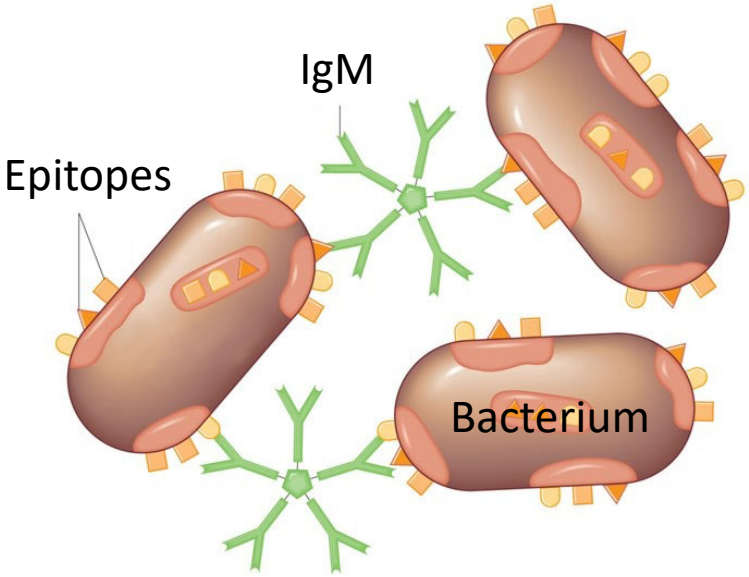
Blood type test: A, Rh(D) positive

Physiological role of agglutination



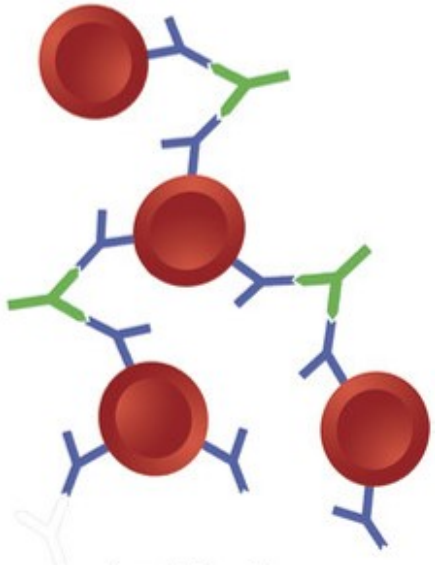
Direct or indirect

Direct agglutination:



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

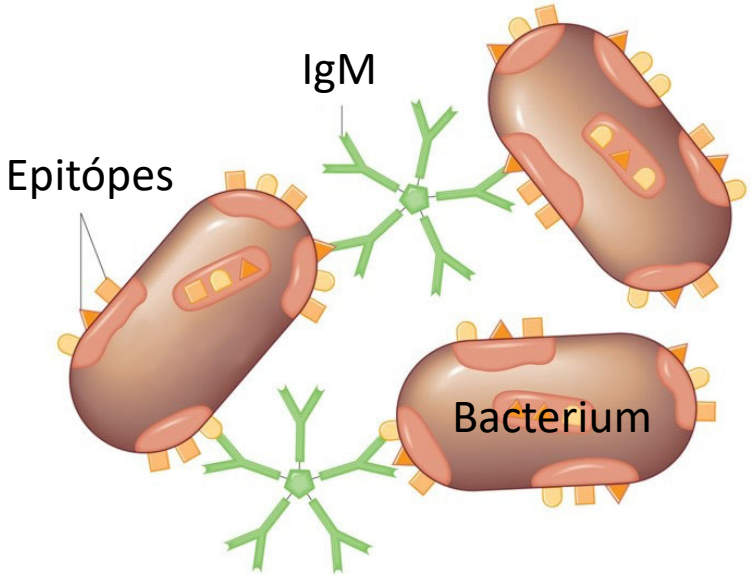
Indirect agglutination:



- 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.

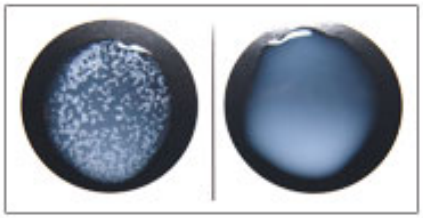
Example:

- 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

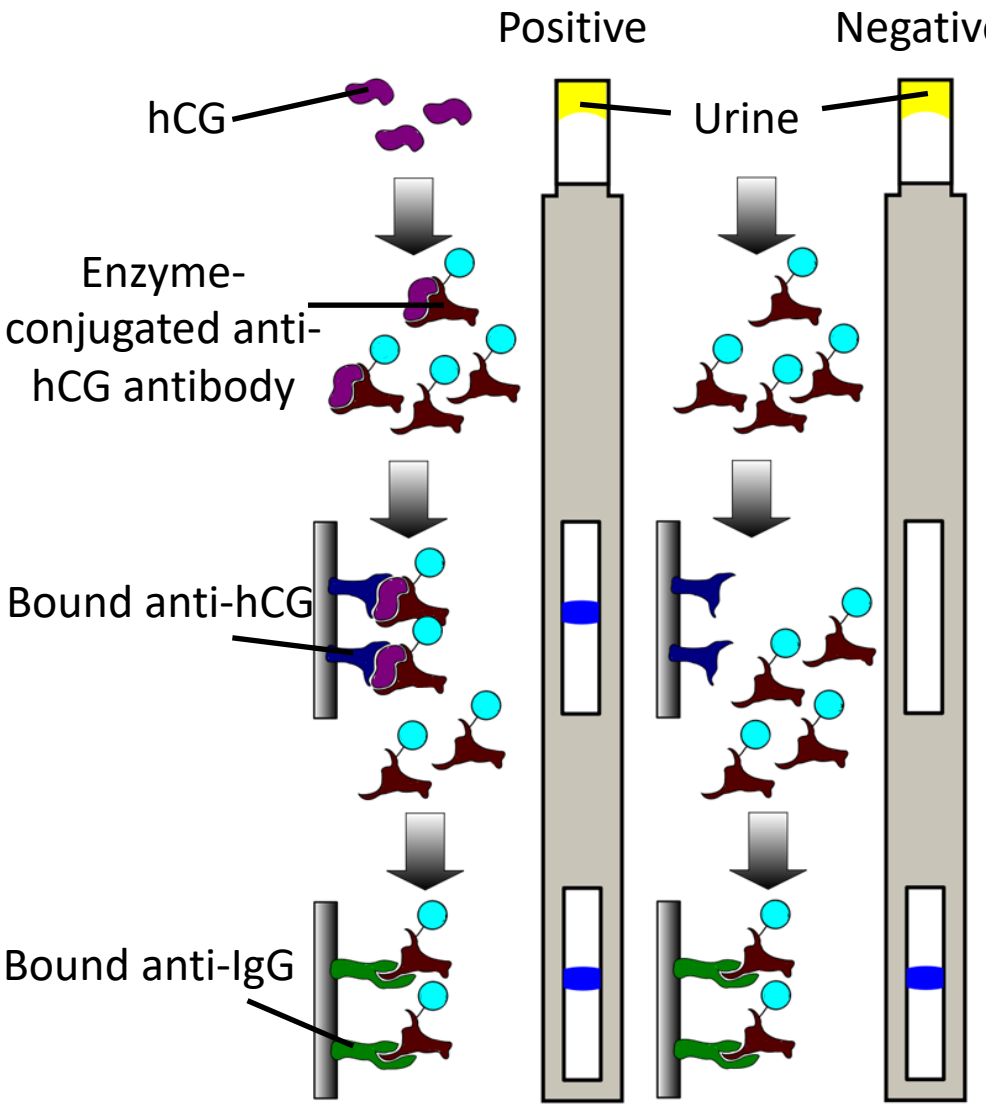
The antigen/antibody that participates in the reaction is bound to the surface of **latex beads.**

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

Applications:

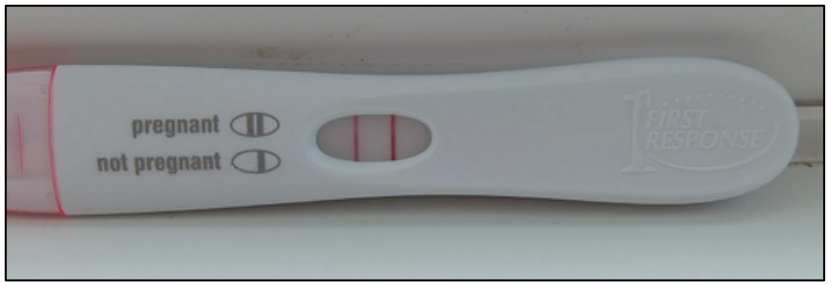
- **Diagnostics of autoimmune disorders**, e.g.:
  - Rheumatoid arthritis (rheumatoid factor, RF[16.]) , SLE (various autoantibodies)
- **Diagnostics of infectious diseases**, e.g.:
  - Detection of antibodies against microbial antigens (e.g. anti-streptolysin O antibody, ASO/AST[17.])
  - Detection 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.]

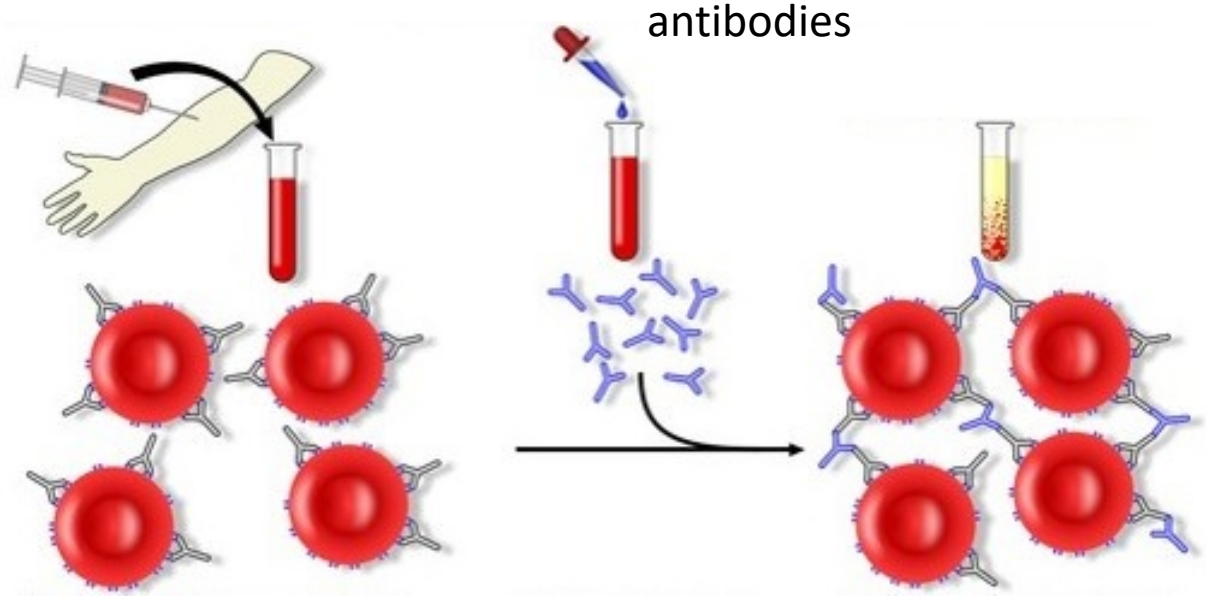


Band forms only if the enzyme-conjugated antibody is bound. If hCG is not present in the urine then only the anti-IgG will bind the labeled antibodies and only one band will appear.

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

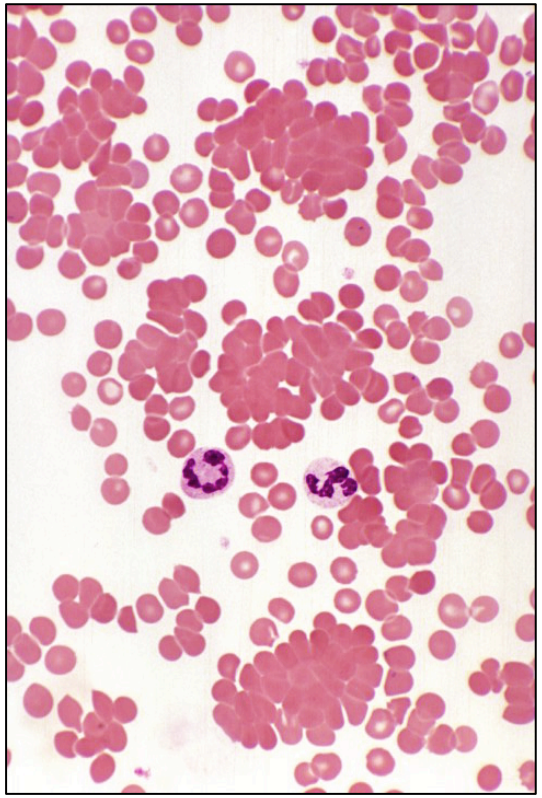
Venopuncture

+ Anti-human immunoglobulin antibodies



Autoreactive antibodies on the patient's RBCs

Hemagglutination

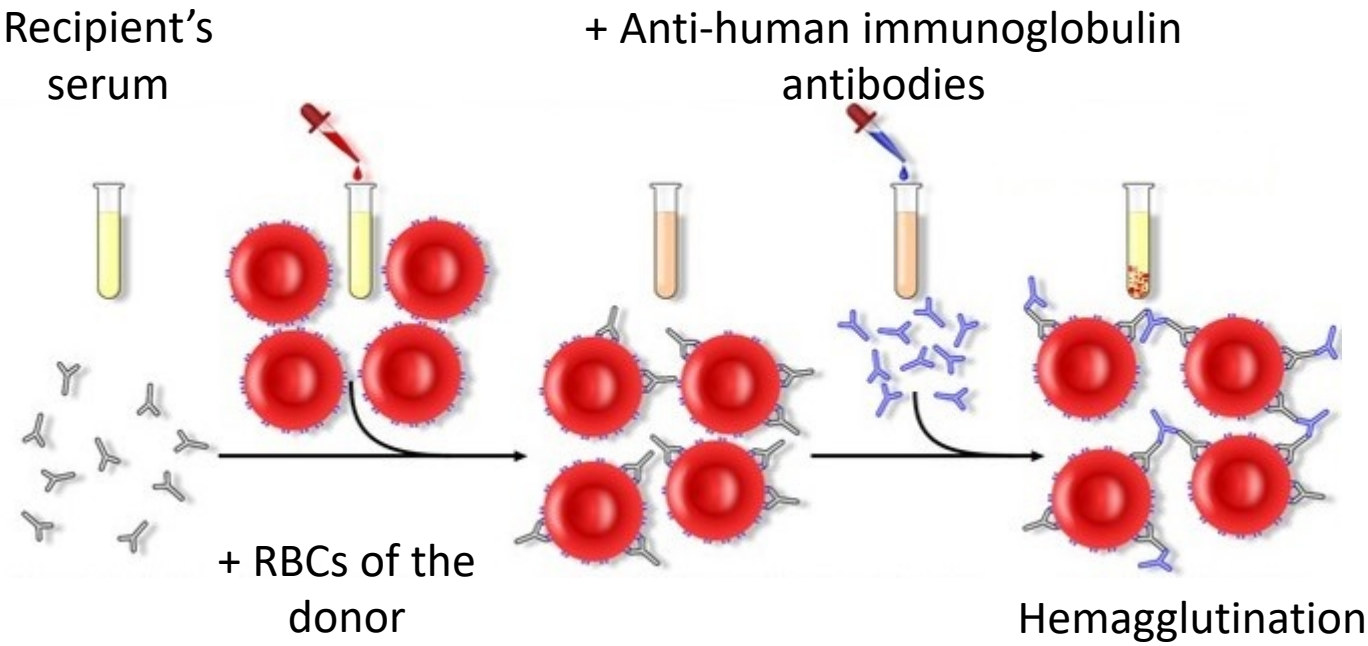


In vivo hemagglutination in a patient with AIHA.

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

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

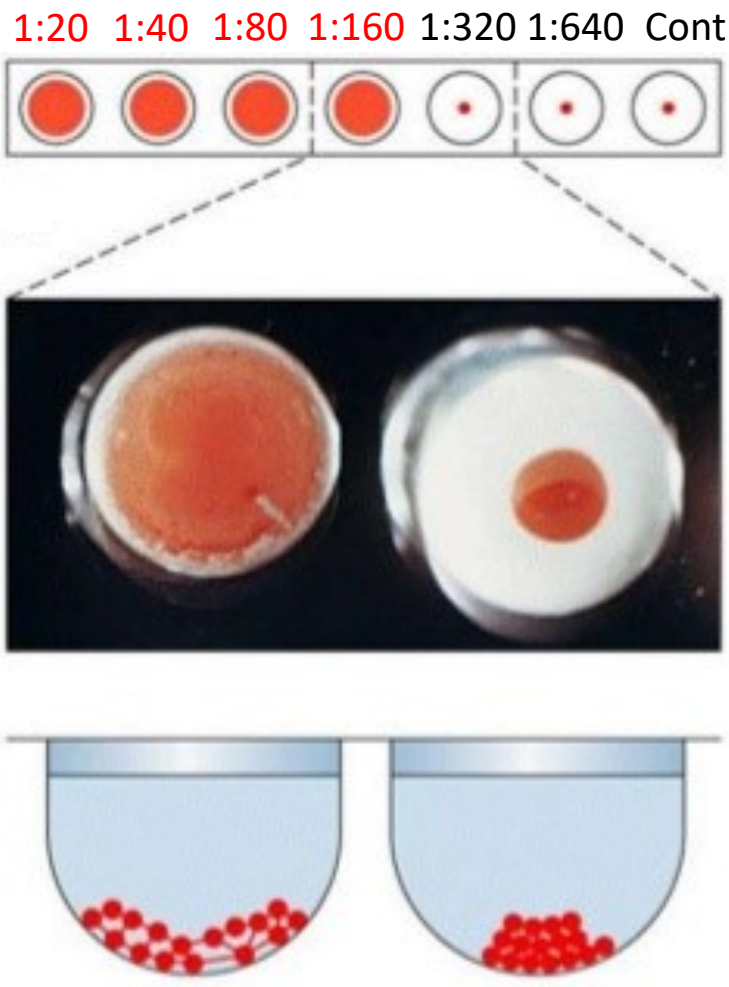
Indirect Coombs test (Indirect antiglobulin test)



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

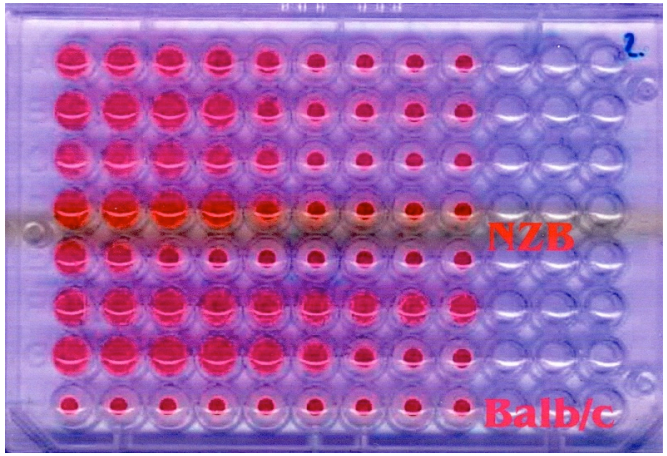


Hemagglutination Negative

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)



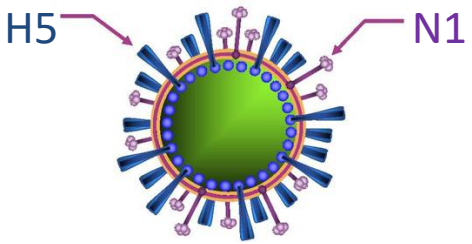
- NZB: New Zealand Black mouse strain[25.] (murine model of AIHA)
- BALB/c: albino strain of house mouse (control)

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.:

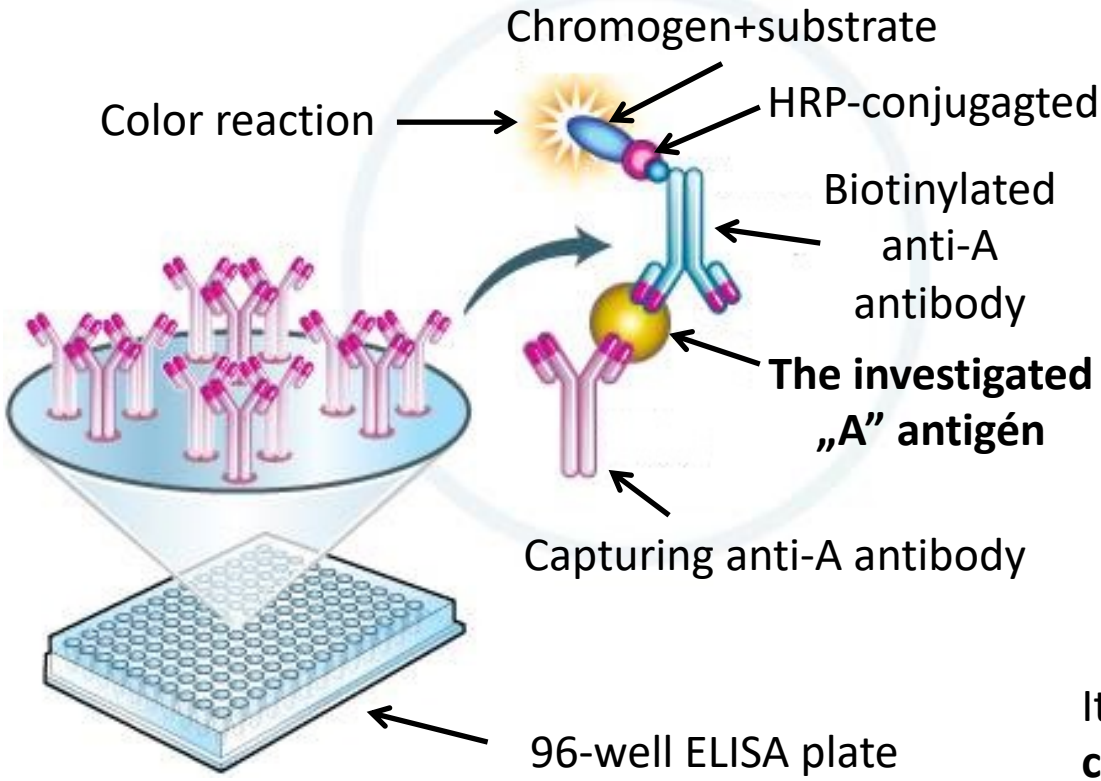
- **Influenza hemagglutinin**
- Measles hemagglutinin
- Mumps hemagglutinin



- 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.]

ELISA basics I.

- **ELISA** = **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay[1.]
- An example of how ELISA works: (so-called sandwich ELISA, see on the next slides):

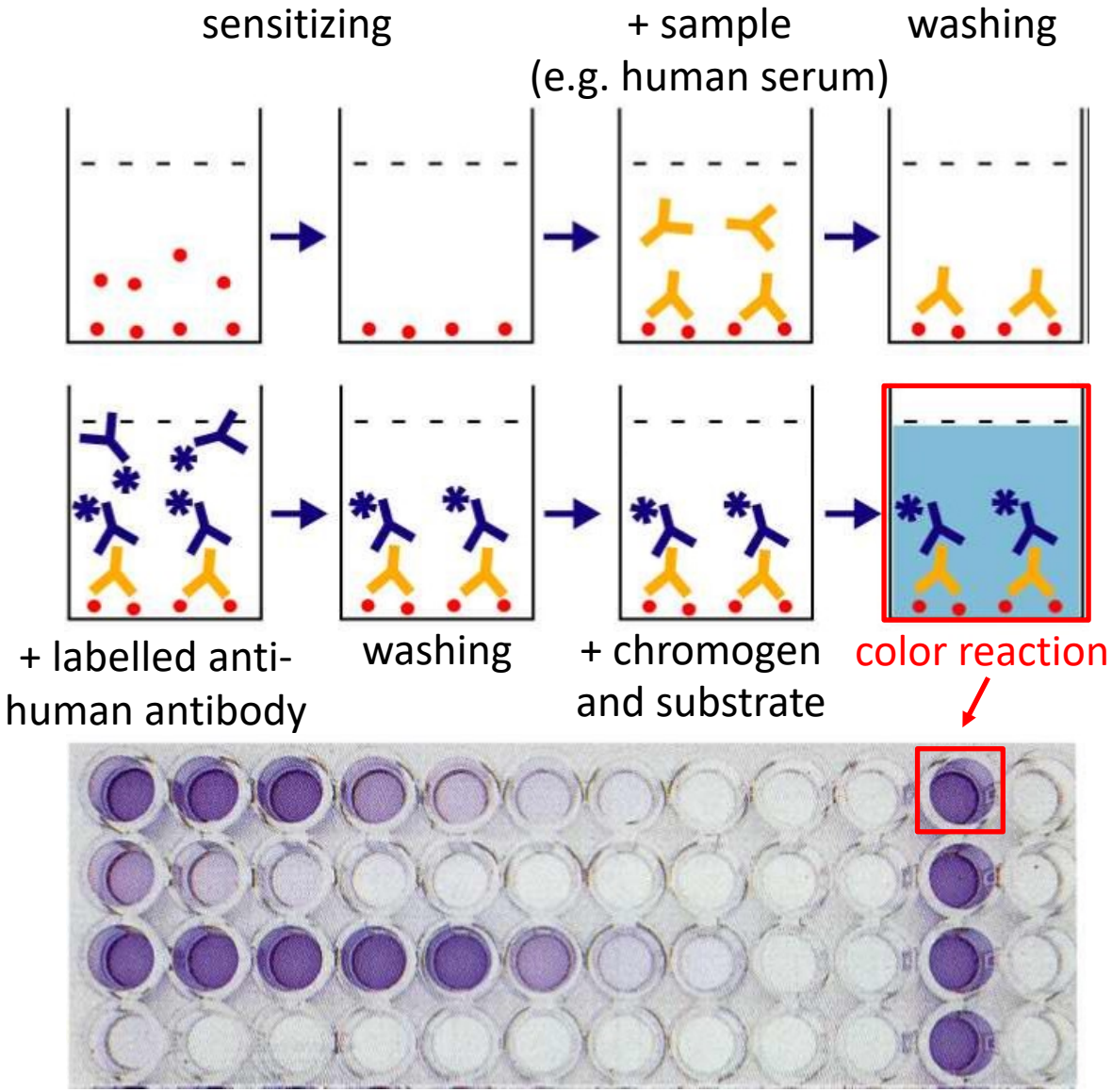


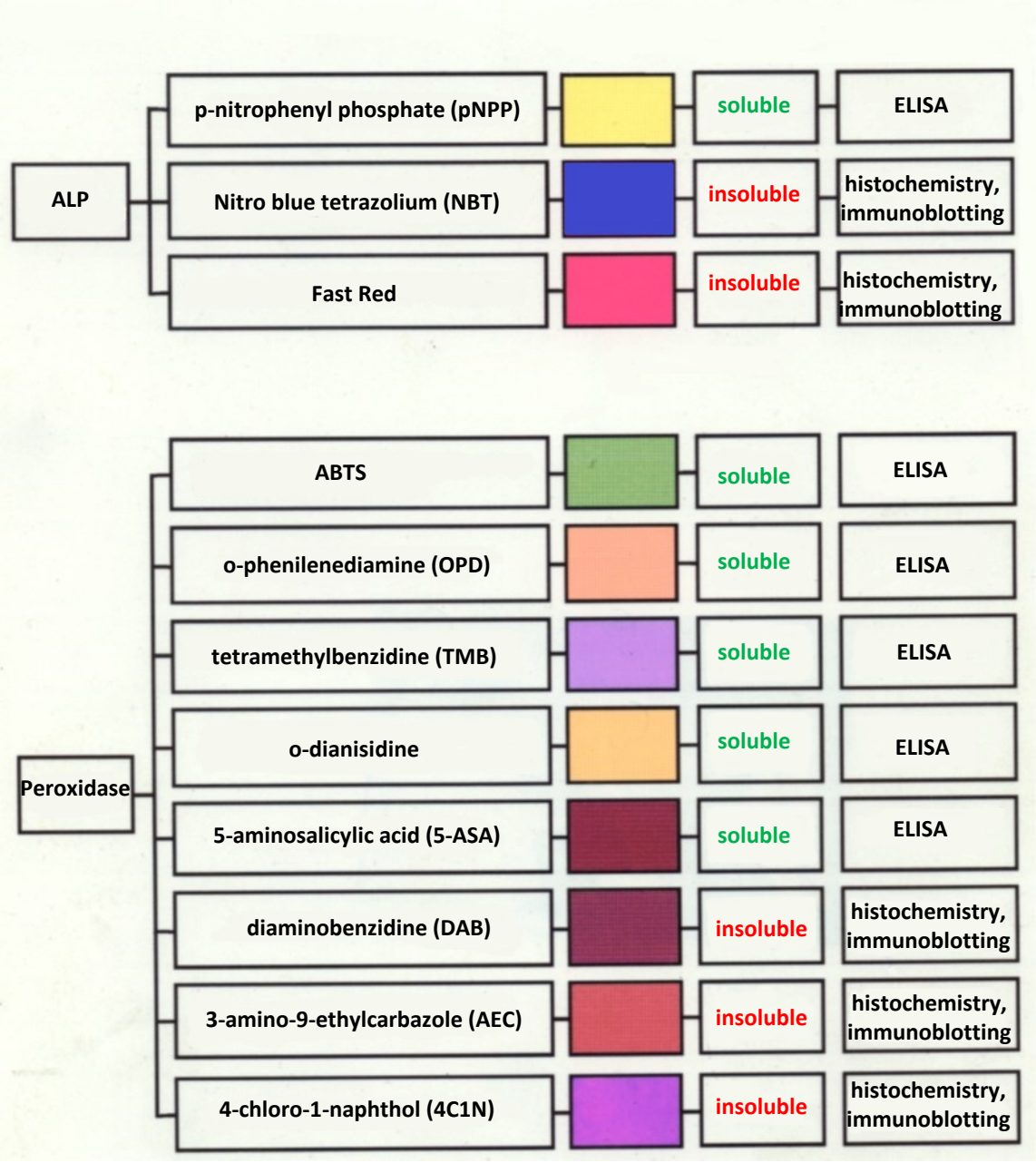
It is a **quantitative** method, the exact **concentartion of the antigen** can be determined based on the intensity of the color reaction.

ELISA basics II.

- It is based on the **antibody-antigen reaction**, **both** of them **can be detected**.[2.]
- **Sensitization**: One of the participants is bound to solid surface.
- **Blocking**: Blocking of non-specific binding sites.
- The participant of interest (either the antigen or the antibody) is in a **soluble form**. (e.g. blood serum)
- The capturing antigen/antibody will bind its soluble ligand and **bound immunocomplexes will form**.
- Components not bound to the surface are removed by washing.
- The bound immunocomplexes can be detected with enzymatic color reactions either directly or indirectly.
- The colored end-product of the chromogen is soluble and diffuses in the solution.
- The **concentration** of the investigated participant **can be calculated** by measuring the **light absoprtion** of the solution and using **standard samples** with known concentrations. → **It is a quantitative method**!

Principle of ELISA (indirect ELISA)

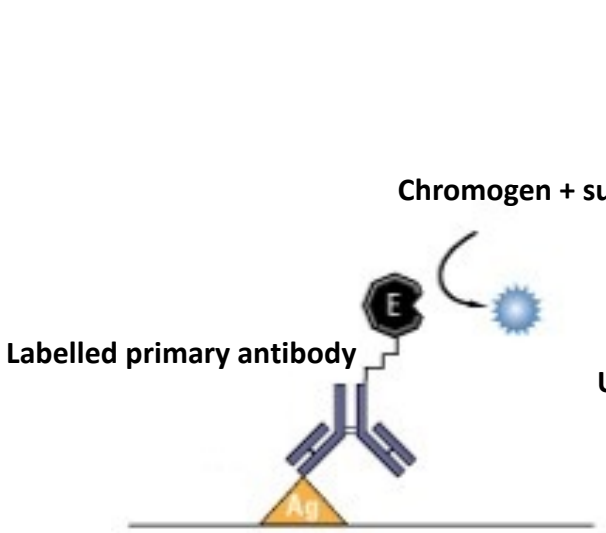




In the case of **ELISA** the **colored end-product** of the chromogen must be **soluble**. The end-product will randomly **diffuse** in the solution changing the **light absorption** properties of the solution. Light absorption is then measured well by well by the ELISA reader.[2.]

In case of **enzyme IHC** and **immunoblotting techniques** (e.g. Western blot) the end-product **must be insoluble**, otherwise it will diffuse away. An insoluble end-product will stay at the site of the reaction allowing the visualization of antigen-antibody reaction.

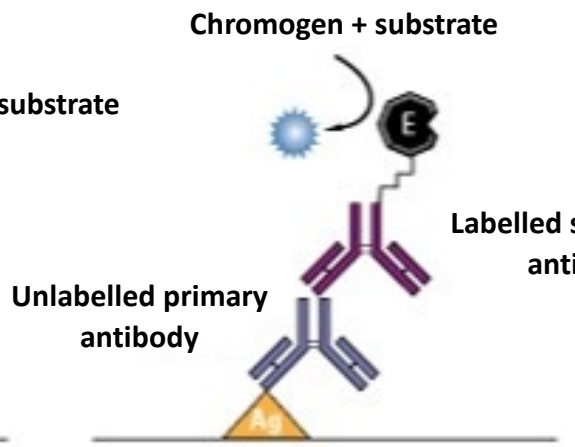
Main types of ELISA



Direct ELISA

↓

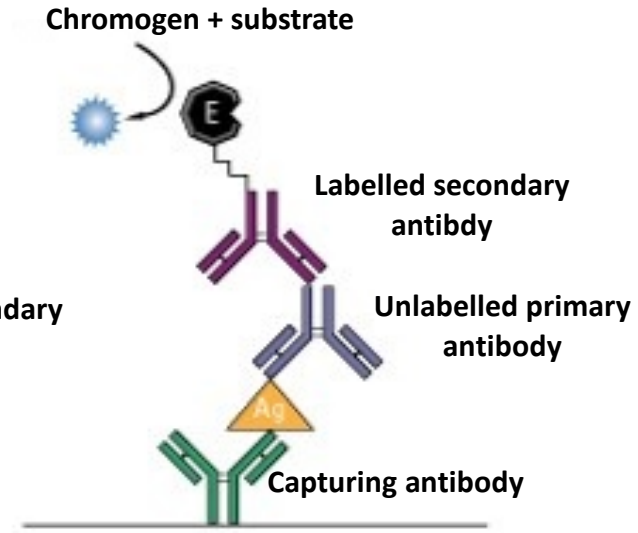
Expensive, weak signal.



Indirect ELISA

↓

Cheaper, signal is stronger.



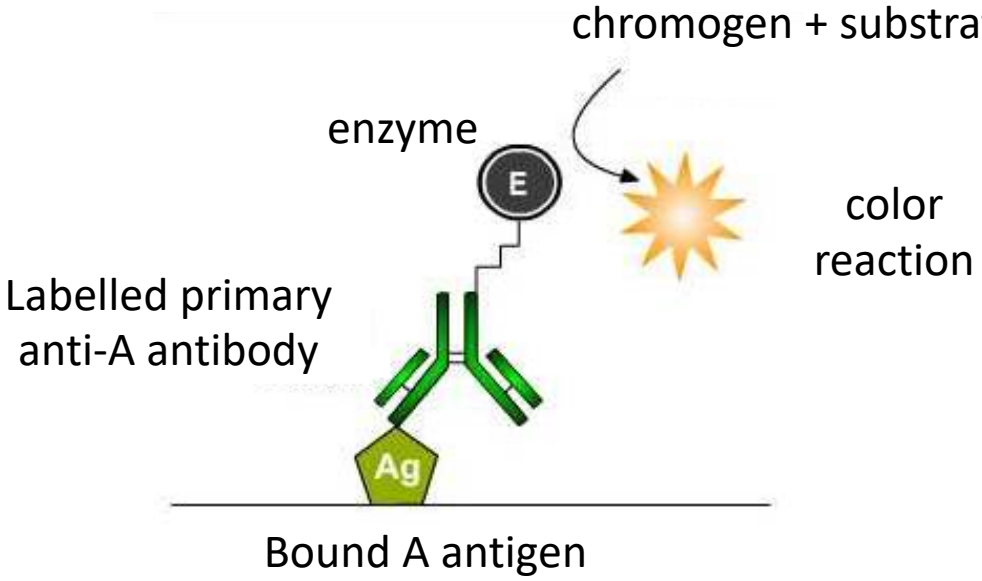
Sandwich ELISA

↓

The capturing and the primary antibodies recognize **different epitopes** of the very same antigen!

Direct ELISA

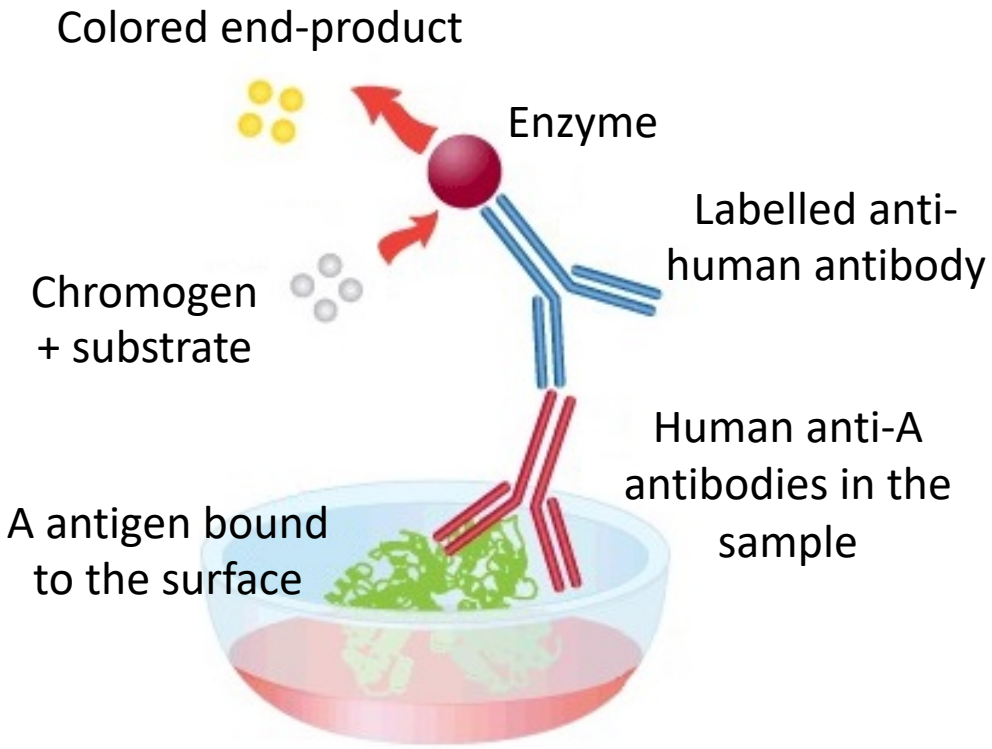
1. They bound A antigen from the sample to the plate.
2. They detect the antigen with enzyme-labelled anti-A antibody.[3.]



Advantages:
- **Fast**

Disadvantages:
- **Expensive** (requires a labelled primary antibody)
- **The signal is weak** because proteins in the sample compete with each other during the senzitization step. (Solution: Sandwich ELISA)

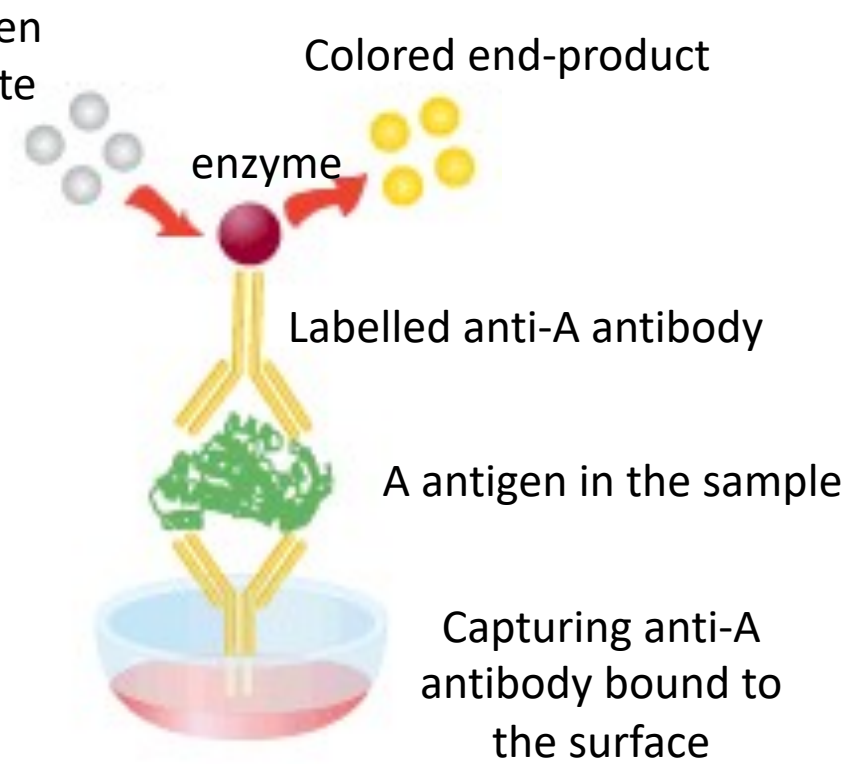
Indirect ELISA



Application: Detection of **antibodies** in the sample, e.g.:

- Testing **hybridoma supernatants**[4.]
- Detection of antigen-specific antibodies in body fluids (e.g. detecting autoantibodies in the serum in **autoimmune disorders**, see later)

Sandwich ELISA



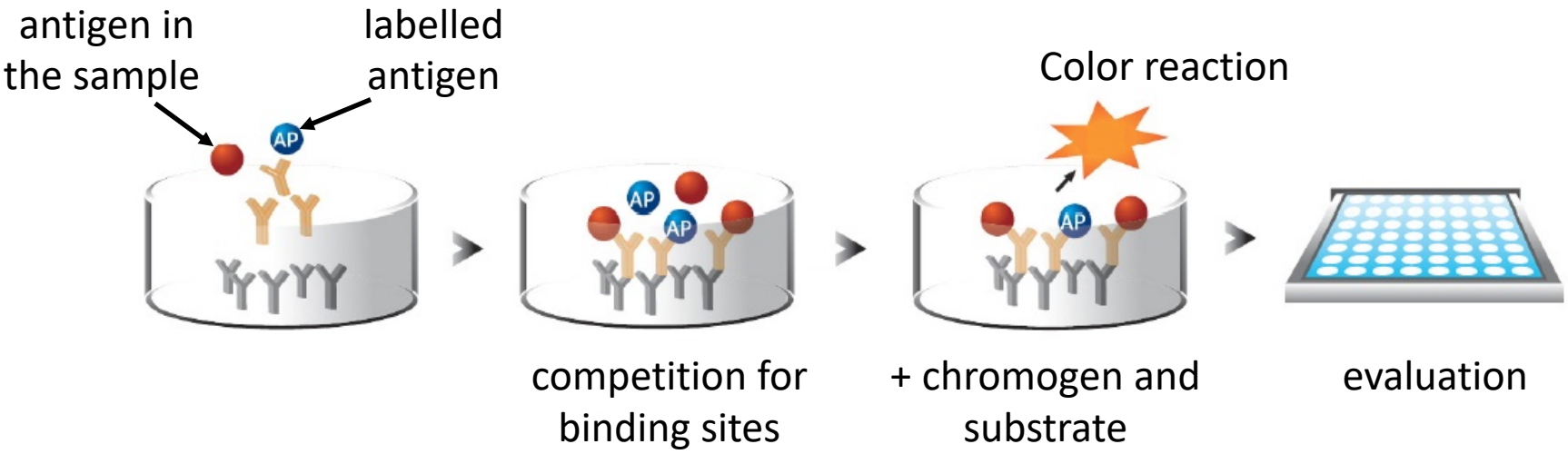
Applications: Detection of a specific antigen in the sample.

E.g.:

- Cytokines
- Tumor markers
- Hormones
- Etc.

Requirement: The capturing and the primary antibodies must recognize **different epitopes** of the very same antigen.

Competitive ELISA

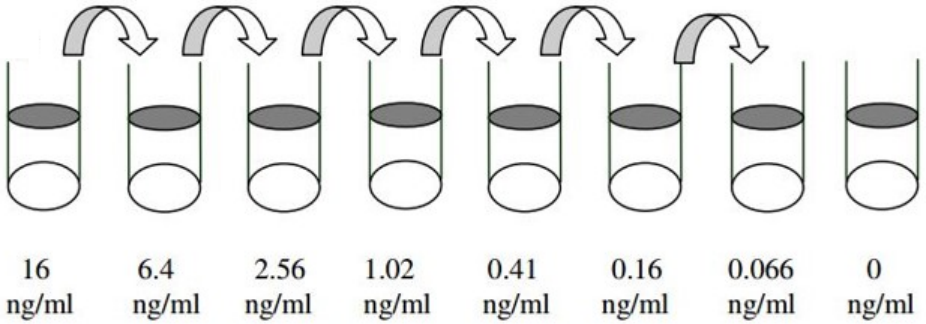


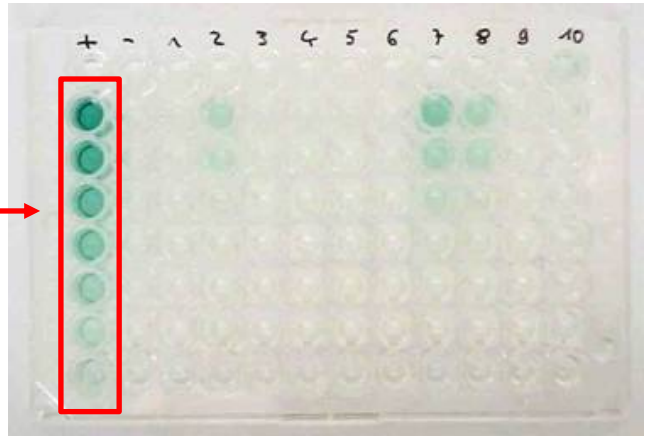
Application: Detection of a **specific antigen** in the sample.
Principle:

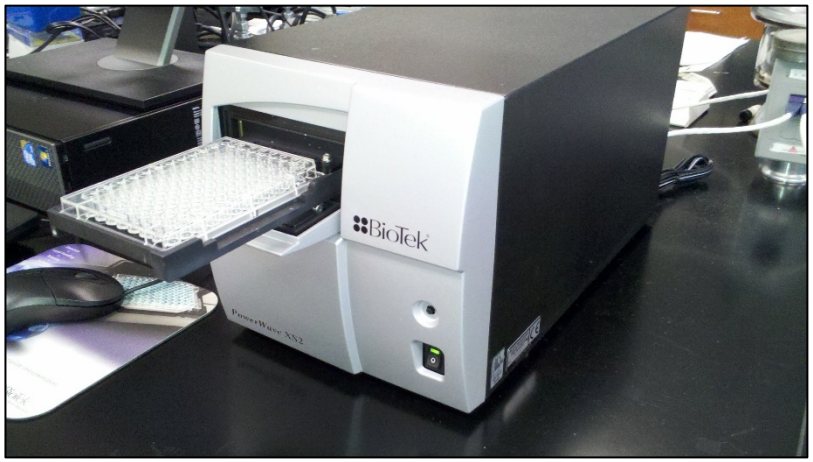
1. Binding of anti-A antibody to the plate.
2. They add a known amount of labelled antigen to the sample.
3. The unlabelled antigen in the sample will **compete with the labelled** ones for the binding sites.
4. The unbound componenets are removed by washing.
5. The intensity of the color reaction is inversely proportional with the concentration of the antigen in the sample. (The less antigen there was in the sample, the more enzyme-labelled antigen could bind to the coated antibodies.)

ELISA evaluation I.

Creating standards with known concentrations:

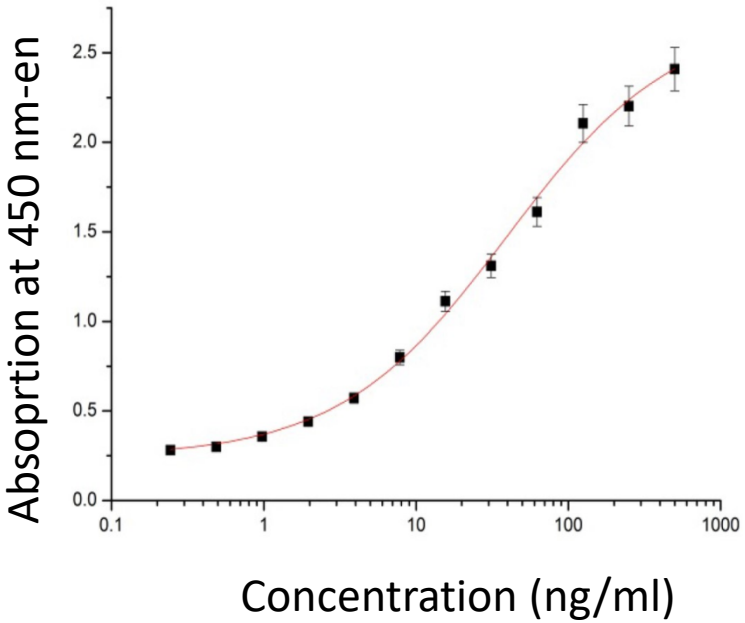






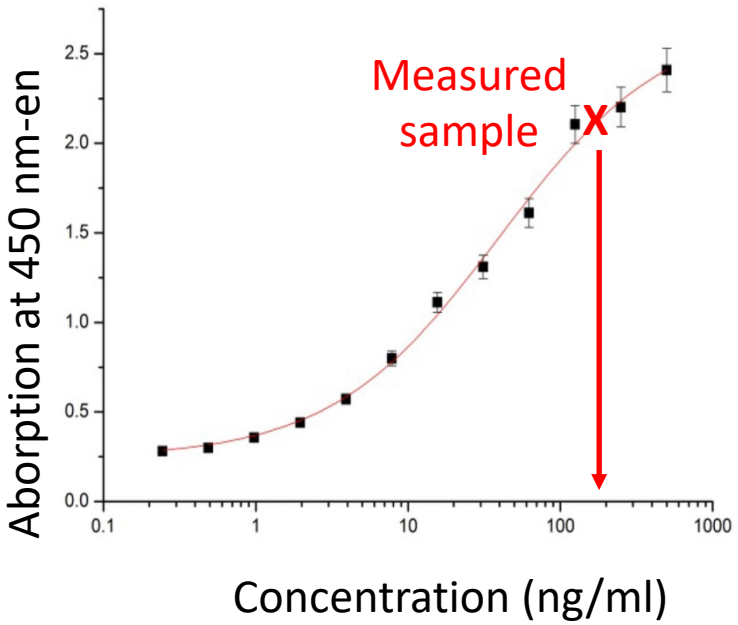
An ELISA reader which measures the **light absorption** in well of the ELISA plate.

ELISA standard curve:

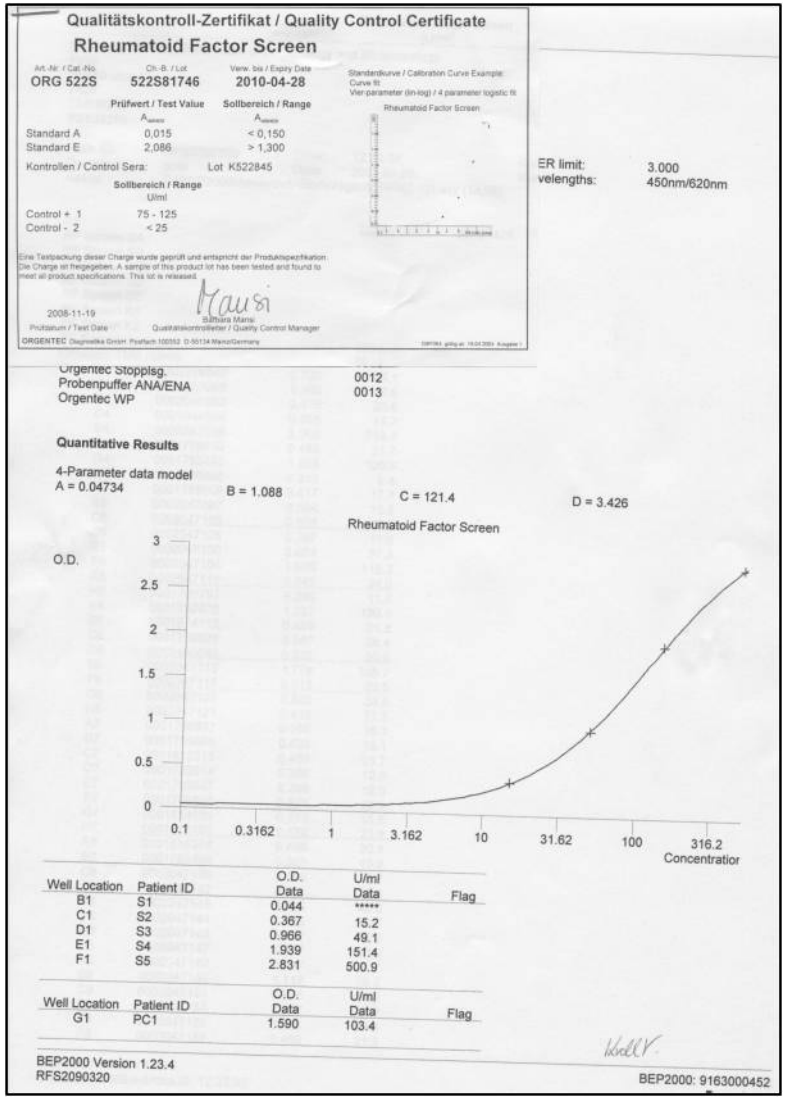


ELISA evaluation II.

Standard curve:



The concentration of the antigen is calculated based in the light absorption of the sample with help of the standard curve.

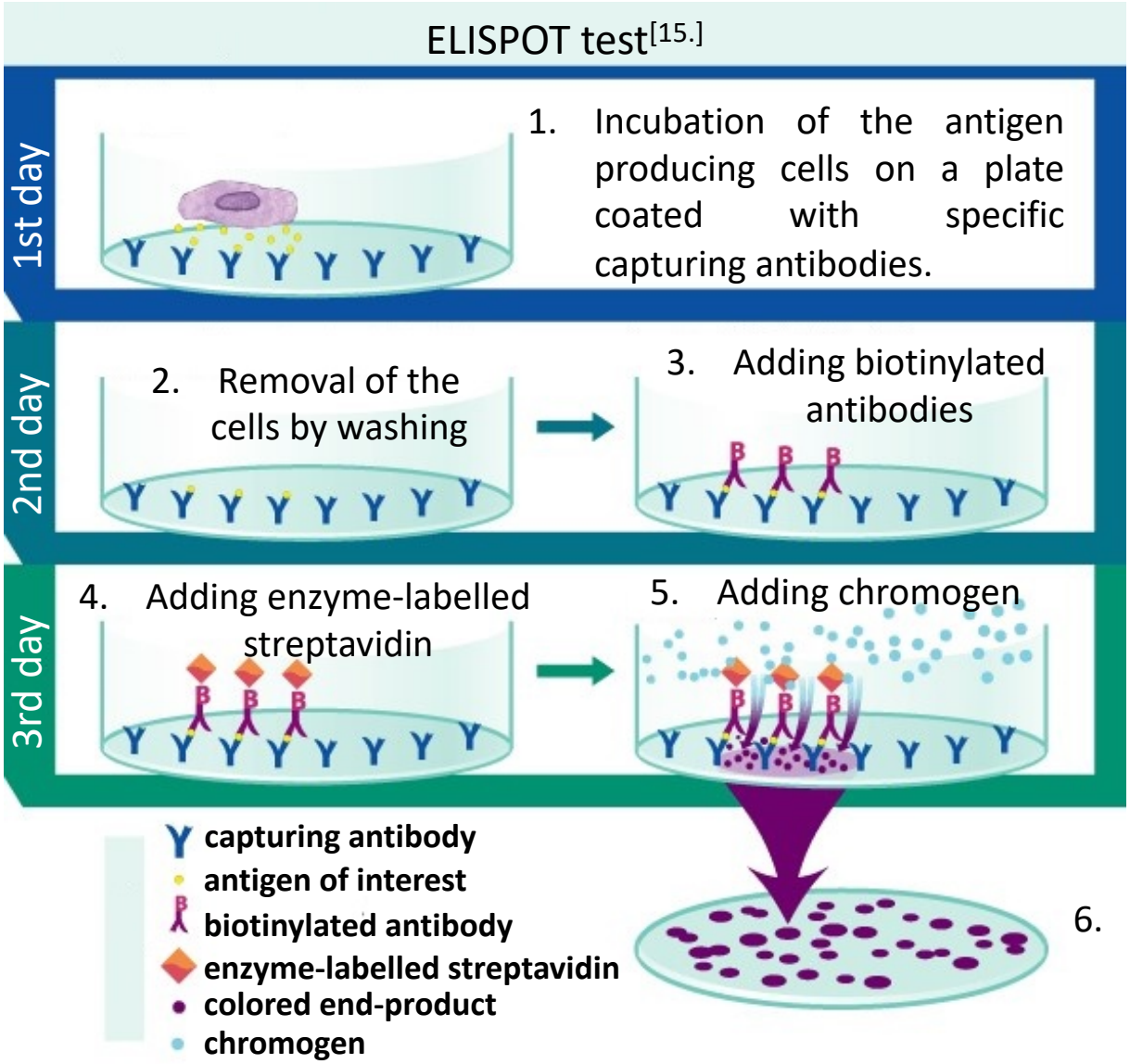


Result of a routine diagnostic ELISA (measured rheumatoid factor)

The significance of ELISA

- Medical diagnostics:
  - Diagnostics of **autoimmune disorders**[5.] (detection of autoantibodies, see later)
  - Diagnostics of **infectious diseases**[6, 7.] (detection of either microbial antigens or the antibodies produced against them, e.g. detection of anti-HIV antibodies in **HIV screening**)
  - Measuring the concentrations of specific **serum proteins** e.g. CRP, hormones[8.] (β-hCG, TSH, etc.) cytokines, tumor markers[9, 10.] (e.g. AFP, PSA, CEA, etc.)
- Industrial uses:
  - Detection of **food allergens**[11, 12.] (e.g. gluten, peanut, milk proteins, etc.)
  - Detection of **toxins** in foods[13.]
  - Testing antibody production of **hybridomas**[4.]
  - Detection of certain industrial pollutants in environmental and industrial wastes[14.]
- Research

ELISPOT



It is used to measure the **antigen secretion** of cells.

E.g.:

Cytokine production

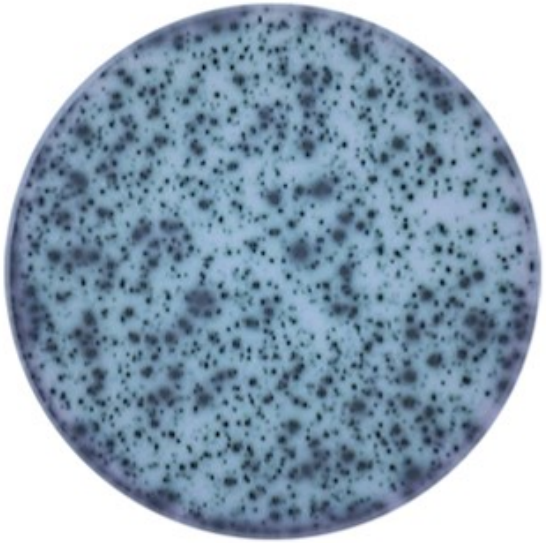
6. Formation of an **insoluble end-product** at the site of antigen production.

IFNγ production in T cells

untreated T cells:

0 spot

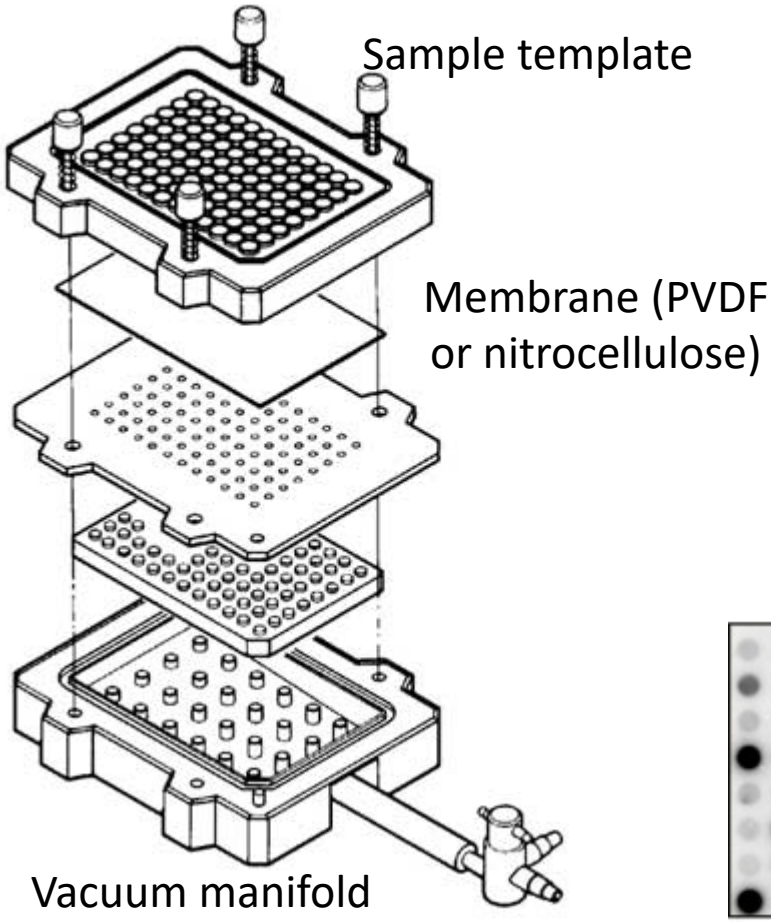
T cells stimulated with an anti-CD3 antibody:



760 spots

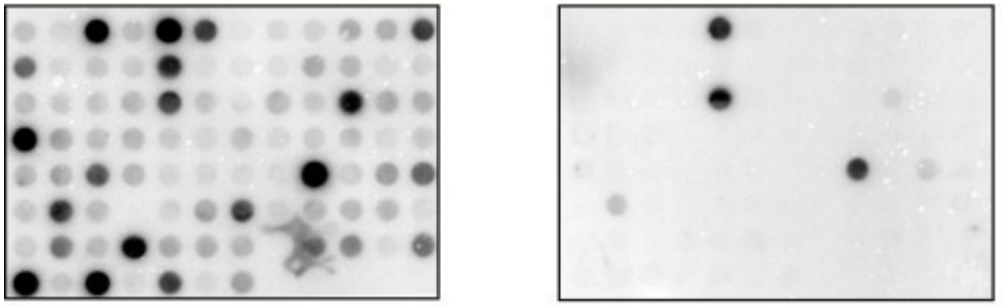
Detection of interferon gamma (IFNγ) with **ELISPOT**. The cells were put to a plate. The IFNγ they produced was instantly bound by the capturing antibody. The bound IFNγ was detected with enzymatic reaction. The stimulated T cells became activated and produced large amounts of IFNγ.

Dot blot



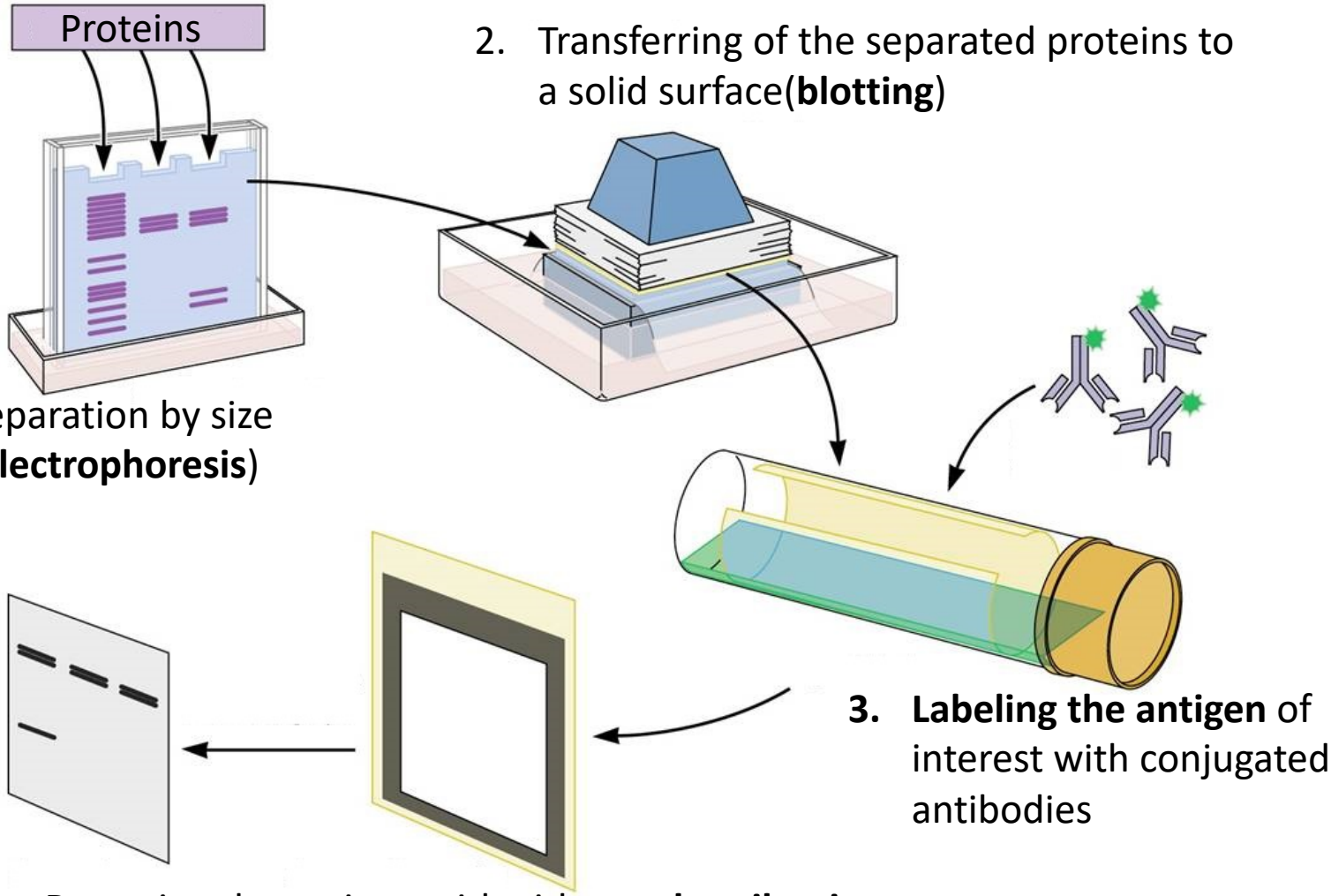
1. They put one drop of the sample containing the antigen to a solid surface (membrane).
2. The antigen attached to the surface is detected with the use of a labelled antibody either with a chromogen or with chemiluminescent reaction (see later).

Application: Detection of specific proteins in a sample of mixed proteins.



Comparison of two different samples for the same proteins with Dot blot.

Western blot[16.]

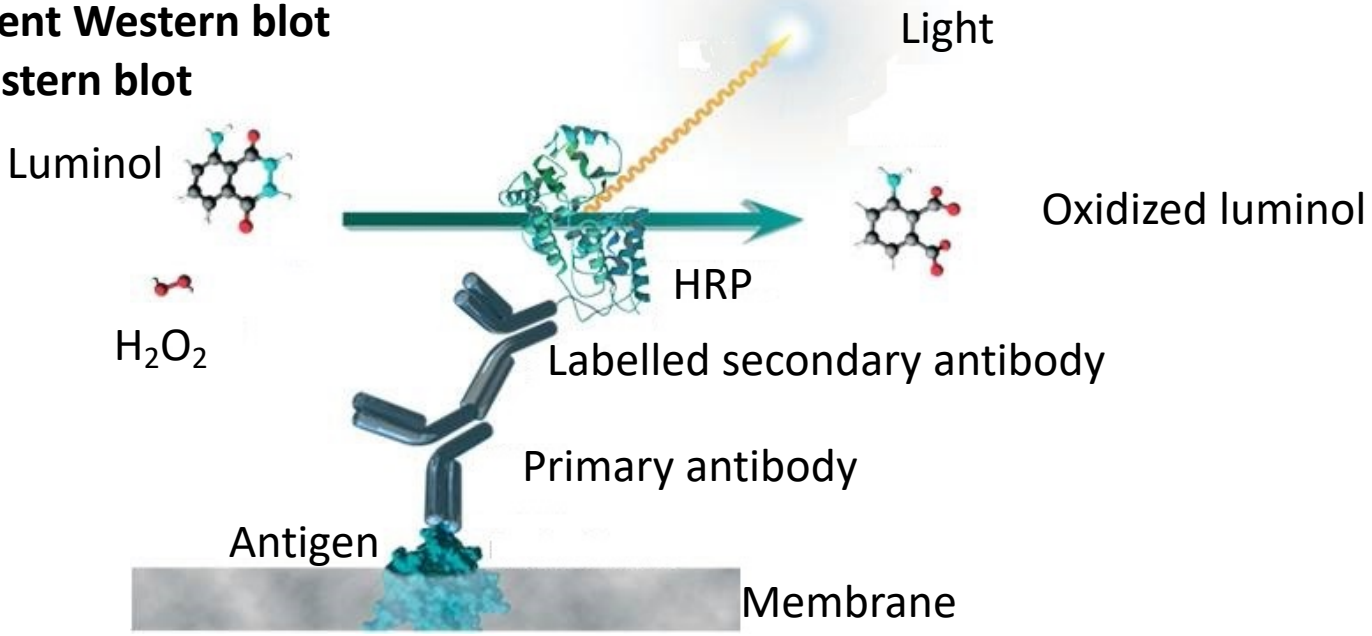


1. Separation by size (**electrophoresis**)
2. Transferring of the separated proteins to a solid surface(**blotting**)
3. **Labeling the antigen** of interest with conjugated antibodies
4. Detecting the antigen with either a **chemiluminescent reaction** or **immunofluorescence**

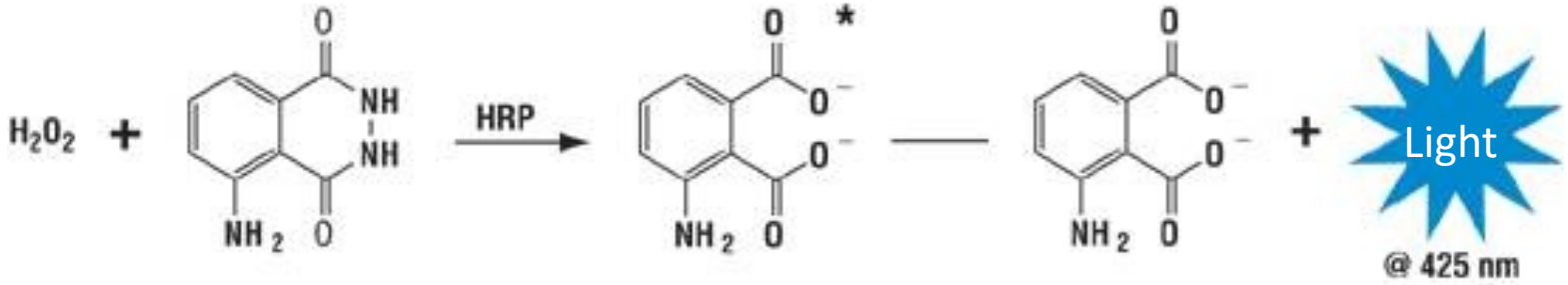
Detection of the antigen

There are several methods to visualize the bound antigens, the most frequently used are[17.]:

- **Chemiluminescent Western blot**
- **Fluorescent Western blot**

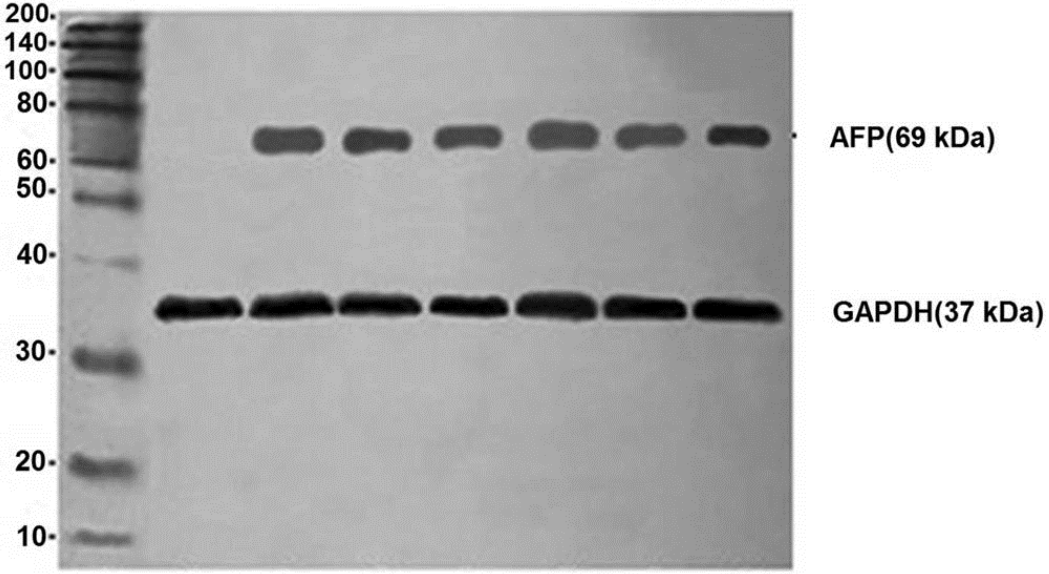


The chemiluminescent reaction of luminol:

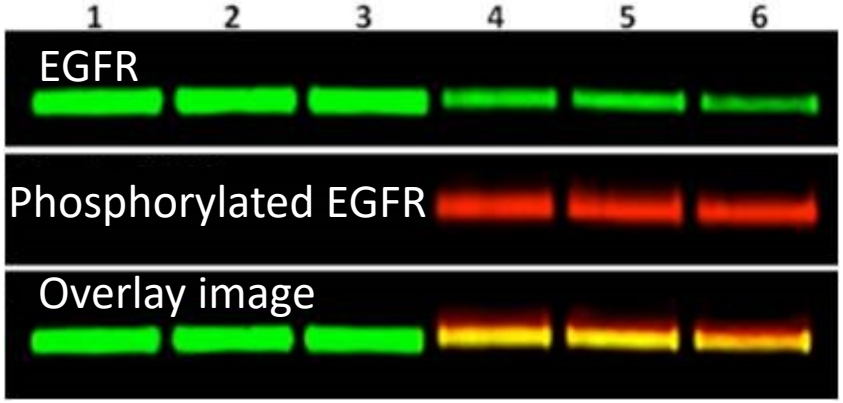


Examples

Simultaneous detection of AFP and GADPH (quantity control) with **chemiluminescent technique**:



Investigation of EGFR phosphorylation with **fluorescent Western blot**:

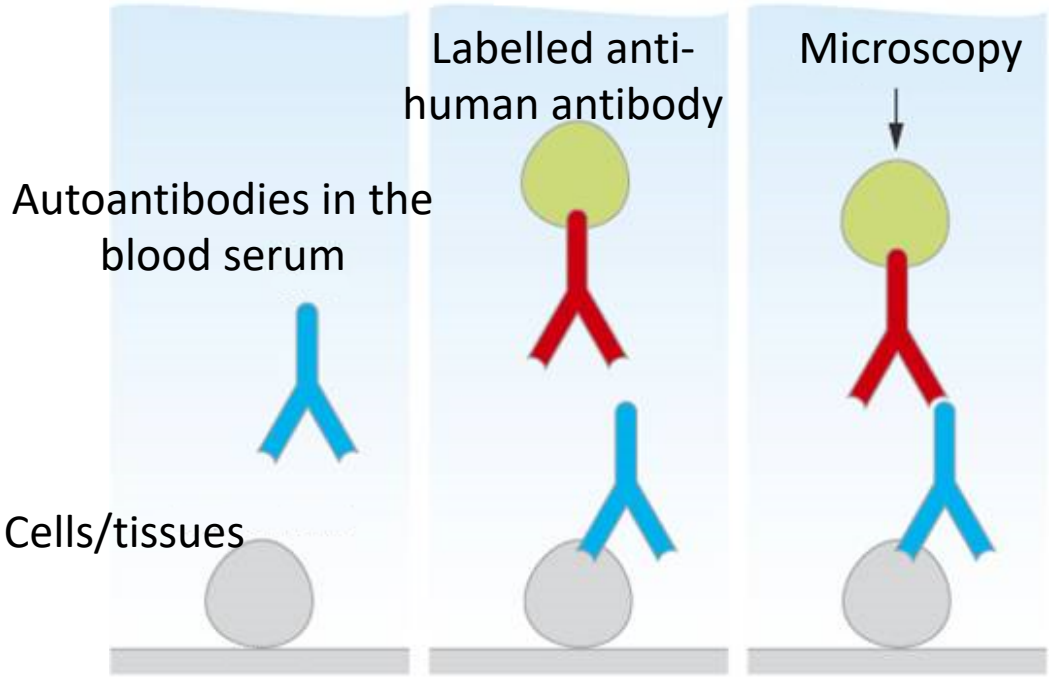


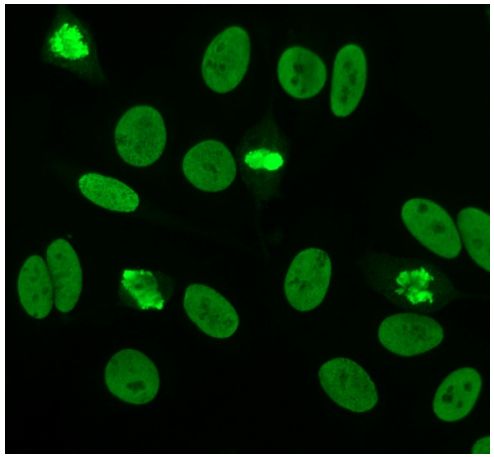
Significance of Western blot:

- What is it capable of?
  - It **can specifically detect proteins** in a **mixed protein sample** and also provides information of both the **size** and the quantity of the protein of interest. (**semiquantitative** method)
  - **Protein-protein interactions** can be detected with immunoprecipitation.
  - Can be used for **functional tests**, such as investigating protein phosphorylations in cells.
- It is extensively used in research.
- Its use for diagnostic purposes is limited because it is **hard to standardize**.[18.]
- Some examples of diagnostic uses:
  - Confirmation of certain **infectious diseases**, e.g.:
    - Lyme disease[19.]
    - BSE (Bovine spongiform encephalopathy, „mad cow disease”)[20.]
    - Confirmation of HIV infection in case of a positive ELISA screening test.[21.]

Indirect immunofluorescence microscopy as a serological test

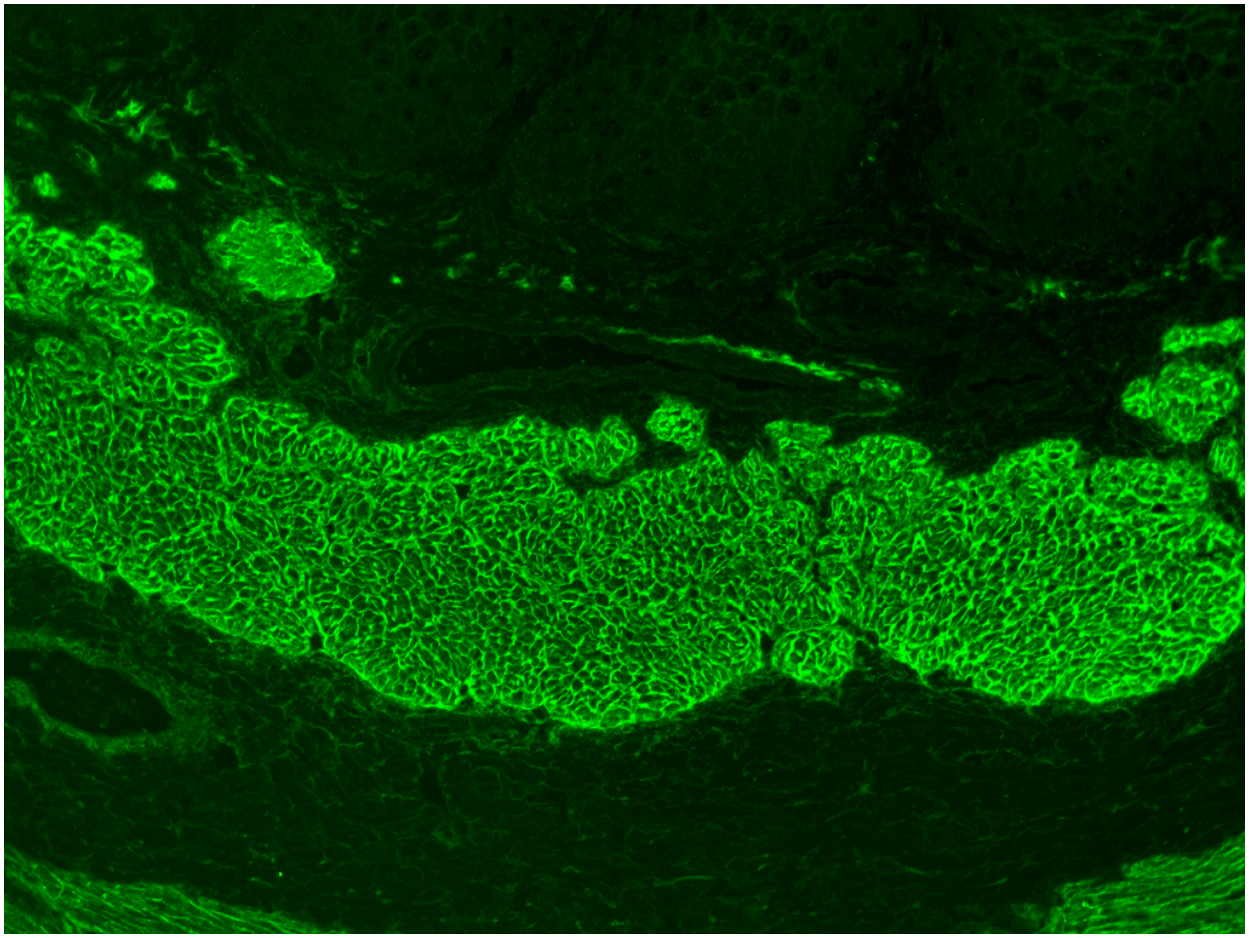
- Immunofluorescence microscopy → see 4th practice
- Application: **Diagnostics of autoimmune disorders** (see later in more detail)
- The serum of the patient is added to a cell culture or tissue. Autoantibodies in the serum will cross-react with the tissue or cultured cells which can be detected with flurochrome-conjugated anti-human antibodies.





Detection of anti-double stranded DNA (dsDNA) antibodies in a cell culture.[22.]

Indirect immunofluorescence example



Detection of anti-endomysium autoantibodies (EMA) from the serum of a patient with celiac disease on a monkey esophagus. The esophagus section was first incubated with the serum of the patient. Then fluorochrome-conjugated (**FITC**) anti-human antibody was added.[23.]

Comparing the treshold of different serological methods

Method	Estimated sensitivity (µg protein/ml sample)
Precipitation in fluids	20-200
Ouchterlony double immunodiffusion	20-200
Immunoelectrophoresis	20-200
Mancini radial immunodiffusion	10-50
Rocket immunelectrophoresis	2
Immunofluorescence	1
Direct agglutination	0,3
Passive agglutination	0,006-0,06
ELISA	0,0001-0,01

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.

References 4.

1. Lequin RM[1]: **Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA).** *Clin Chem.* 2005 Dec;51(12):2415-8. Epub 2005 Sep 22.
2. John R. Crowther: **The ELISA Guidebook** © 2001 Humana Press Inc.
3. Lin AV[1]: **Direct ELISA.** *Methods Mol Biol.* 2015;1318:61-7. doi: 10.1007/978-1-4939-2742-5_6.
4. Delaunay T[1], Louahed J, Bazin H: **Rat (and mouse) monoclonal antibodies. VIII. ELISA measurement of Ig production in mouse hybridoma culture supernatants.** *J Immunol Methods.* 1990 Jul 20;131(1):33-9.
5. Aggarwal A[1]: **Role of autoantibody testing.** *Best Pract Res Clin Rheumatol.* 2014 Dec;28(6):907-20. doi: 10.1016/j.berh.2015.04.010. Epub 2015 May 23.
6. Ghosh M[1], et al.: **Detection of hepatitis B virus infection: A systematic review.** *World J Hepatol.* 2015 Oct 18;7(23):2482-91. doi: 10.4254/wjh.v7.i23.2482.
7. Sun GG[1], et al.: **Early serodiagnosis of trichinellosis by ELISA using excretory-secretory antigens of Trichinella spiralis adult worms.** *Parasit Vectors.* 2015 Sep 23;8(1):484. doi: 10.1186/s13071-015-1094-9.
8. Islam KN[1], et al.: **Micro open-sandwich ELISA to rapidly evaluate thyroid hormone concentration from serum samples.** *Bioanalysis.* 2010 Oct;2(10):1683-7. doi: 10.4155/bio.10.125.
9. Schneider J[1], et al.: **Comparison of the tumor markers tumor M2-PK, CEA, CYFRA 21-1, NSE and SCC in the diagnosis of lung cancer.** *Anticancer Res.* 2000 Nov-Dec;20(6D):5053-8.
10. Barak V[1], et al.: **The Diagnostic and Prognostic Value of Tumor Markers (CEA, SCC, CYFRA 21-1, TPS) in Head and Neck Cancer Patients.** *Anticancer Res.* 2015 Oct;35(10):5519-24.
11. Valdés I[1], García E, Llorente M, Méndez E: **Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol.** *Eur J Gastroenterol Hepatol.* 2003 May;15(5):465-74.
12. Jayasena S[1], et al.: **Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens.** *J Agric Food Chem.* 2015 Feb 18;63(6):1849-55. doi: 10.1021/jf504741t. Epub 2015 Feb 4.

References 5.

13. Liang M[1], et al.: **Development of an indirect competitive enzyme-linked immunosorbent assay based on the multiepitope peptide for the synchronous detection of staphylococcal enterotoxin A and G proteins in milk.** *J Food Prot.* 2015 Feb;78(2):362-9. doi: 10.4315/0362-028X.JFP-14-323.
14. Hirobe M[1], et al.: **The use of enzyme-linked immunosorbent assays (ELISA) for the determination of pollutants in environmental and industrial wastes.** *Water Sci Technol.* 2006;54(11-12):1-9.
15. Kalyuzhny AE[1]: **Chemistry and biology of the ELISPOT assay.** *Methods Mol Biol.* 2005;302:15-31.
16. Hnasko TS[1], Hnasko RM: **The Western Blot.** *Methods Mol Biol.* 2015;1318:87-96. doi: 10.1007/978-1-4939-2742-5_9.
17. Mathews ST[1], Plaisance EP, Kim T: **Imaging systems for westerns: chemiluminescence vs. infrared detection.** *Methods Mol Biol.* 2009;536:499-513. doi: 10.1007/978-1-59745-542-8_51.
18. Gassmann M[1], Grenacher B, Rohde B, Vogel J: **Quantifying Western blots: pitfalls of densitometry.** *Electrophoresis.* 2009 Jun;30(11):1845-55. doi: 10.1002/elps.200800720.
19. Gerritzen A[1], Brandt S: **Serodiagnosis of Lyme borreliosis with bead based immunoassays using multiplex technology.** *Methods.* 2012 Apr;56(4):477-83. doi: 10.1016/j.ymeth.2012.02.007. Epub 2012 Mar 3.
20. Porcario C[1]: **Evaluation of two sets of immunohistochemical and Western blot confirmatory methods in the detection of typical and atypical BSE cases.** *BMC Res Notes.* 2011 Sep 29;4:376. doi: 10.1186/1756-0500-4-376.
21. Torian LV[1], et al.: **Comparison of Multispot EIA with Western blot for confirmatory serodiagnosis of HIV.** *J Clin Virol.* 2011 Dec;52 Suppl 1:S41-4. doi: 10.1016/j.jcv.2011.09.017. Epub 2011 Oct 12.
22. Buchner C[1], et al: **Anti-nuclear antibody screening using HEp-2 cells.** *J Vis Exp.* 2014 Jun 23;(88):e51211. doi: 10.3791/51211.
23. Amara W[1], Husebekk A: **Improved method for serological testing in celiac disease--IgA anti-endomysium antibody test: a comparison between monkey oesophagus and human umbilical cord as substrate in indirect immunofluorescence test.** *Scand J Clin Lab Invest.* 1998 Nov;58(7):547-54.