IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

Immunoserology 2.
ELISA, immunoblotting techniques

Basic Immunology

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

Indirect ELISA practice

Required materials and equipment

- 96-well microtiter plate pre-coated with antigen, filled with gelatine blocking solution (300 µl/well)
- Wash buffer
- Samples (dilution factors indicated in parentheses) and control solutions in labelled tubes (100 µl/tube):

1. (1x)	**2.** (5x)	**3.** (25x)	**4.** (50x)	**5.** (100x)	**6.** (200x)	neg. crtl. (wash buffer)	poz. ctrl. (IgG 1:1000)

- Conjugate: anti-mouse IgG peroxidase (PO), 1:10,000
- Substrate: TMB (3,3',5,5'-tetramethylbenzidine)
- STOP solution

Procedure

1. Remove the gelatine blocking solution from the plate. Pour it off with a firm motion. Turn the plate upside down and tap it onto a clean paper towel.

2. Washing (3x)

- Add **300 µl** of wash buffer to each well. Pour it off with a firm motion. Turn the plate upside down and tap it onto a clean paper towel. Repeat this step two times. Follow the same procedure for all subsequent washing steps.

3. First incubation (antibody binding)

- After the third wash, add **100 µl** of each sample and the positive and negative controls into the wells.
- Add the samples in the following order:

1.	1x
2.	5x
3.	25x
4.	50x
5.	100x
6.	200x
7.	neg. crtl. (wash buffer)
8.	poz. ctrl. (IgG 1:1000)

- **Incubate** at room temperature for **35 minutes**.

4. Washing 3x

- Repeat the washing step 3 times as described above (step 2).

5. Incubation with the secondary antibody

- Add **100 µl** of anti-mouse IgG PO conjugate to each well.
- IIncubate at room temperature for **35 minutes**.

6. Washing 3x

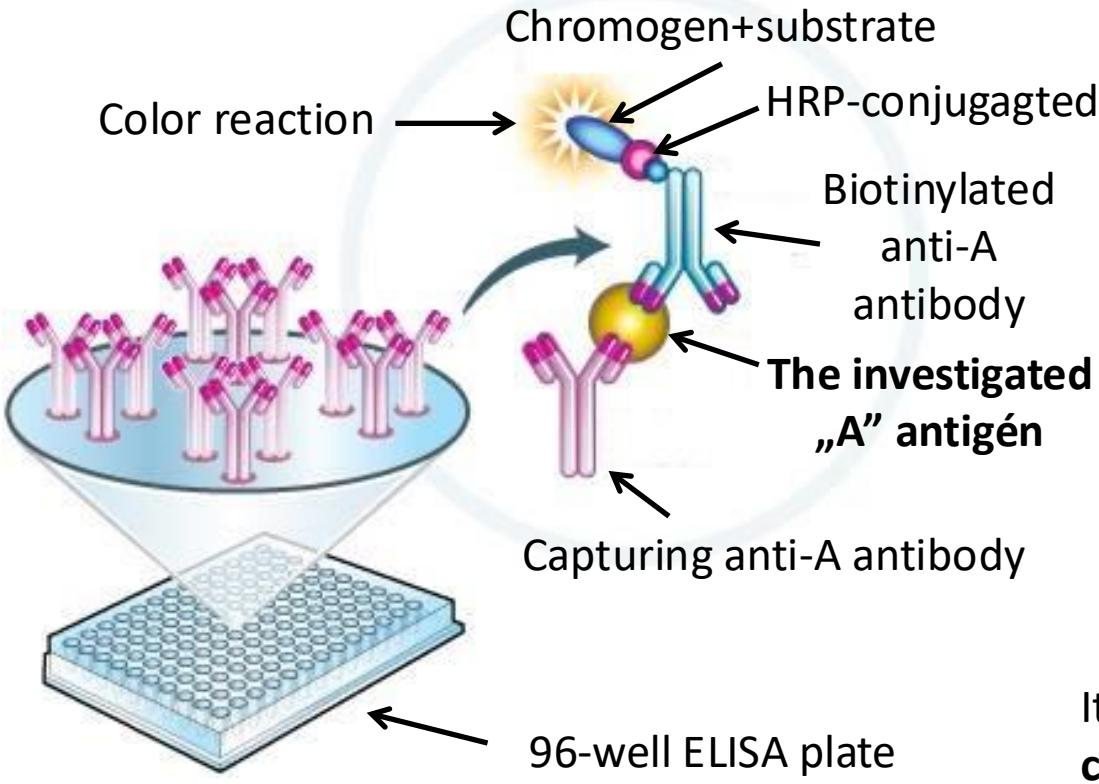
- Repeat the washing step 3 times as described above (step 2).

7. Colour development and stopping the reaction

- **Substrate addition**: Add **100 µl of TMB** solution to each well. Wait until a blue colour develops.
- **Stopping**: Add **50 µl of STOP** solution. The blue colour will change to yellow.

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):

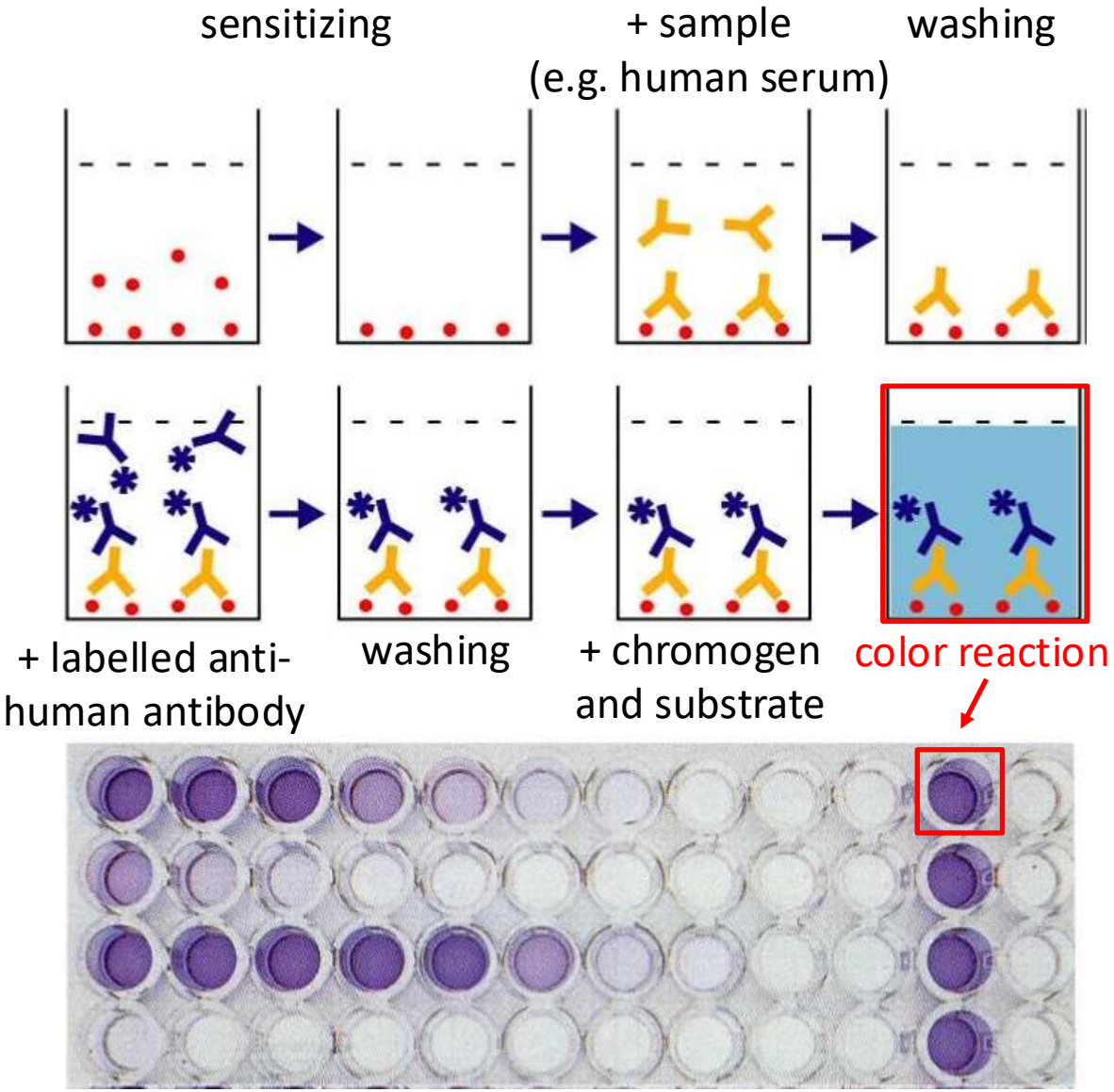


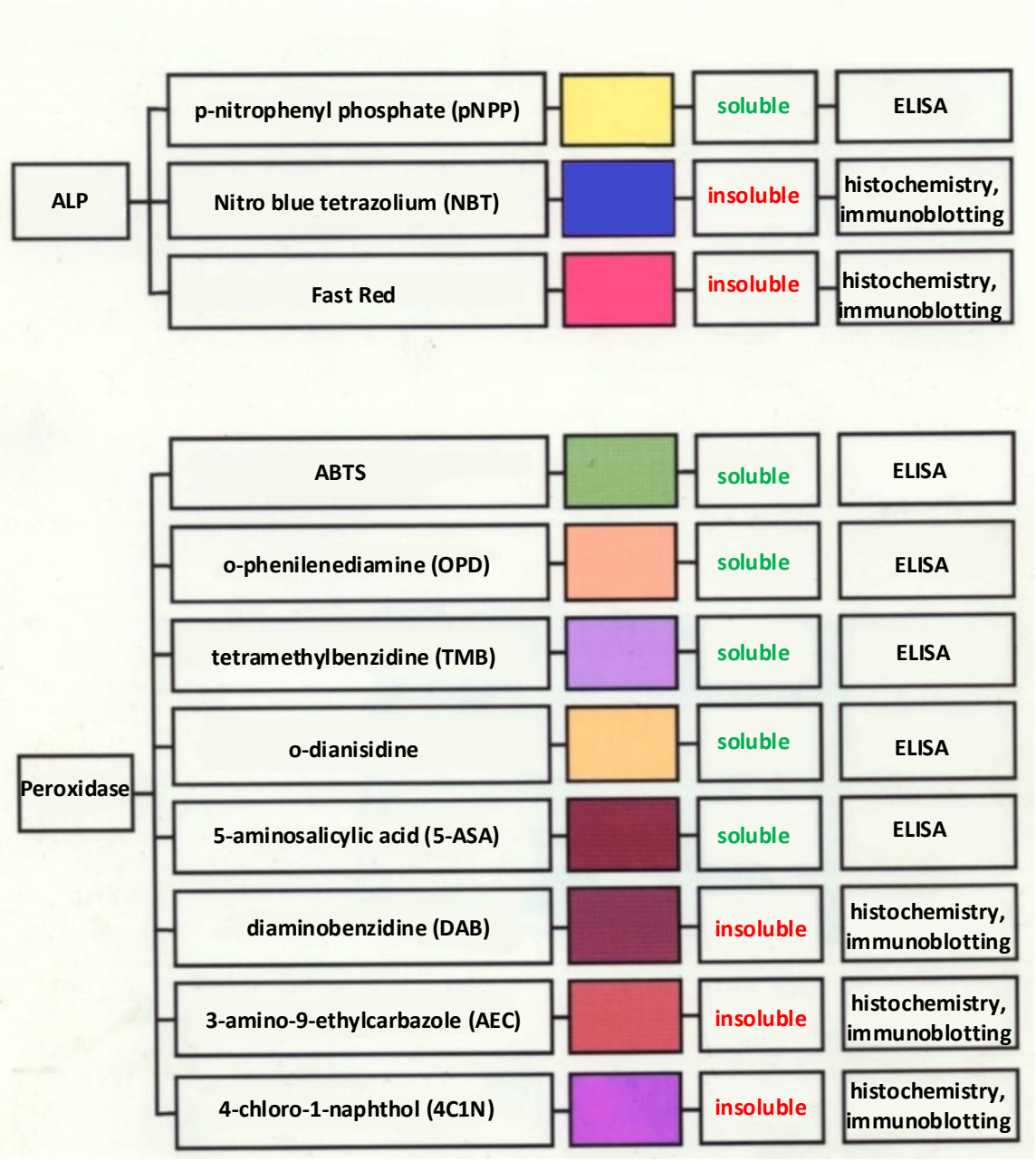
It is a **quantitative** method, the exact **concentartion of the antigen** can be determined based on the intensity of the color reaction.

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)

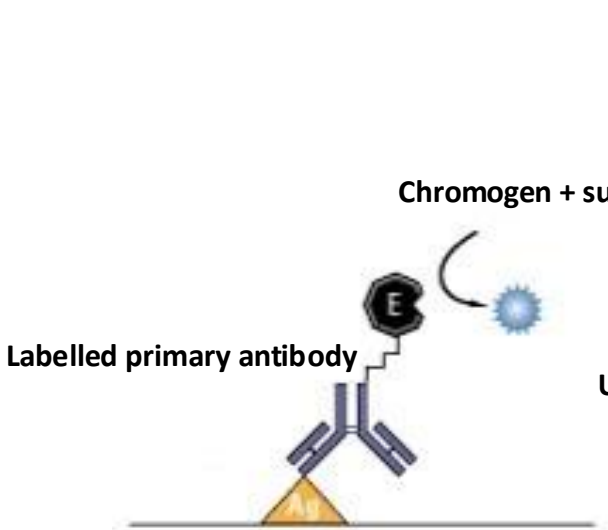




In the case of **ELISA** the **colored end-product** of the chromogen must be **soluble**. The end-product will randomly **diffuse** in the solution changing the **light absorption** properties of the solution. Light absorption is then measured well by well by the ELISA reader.[2.]

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.

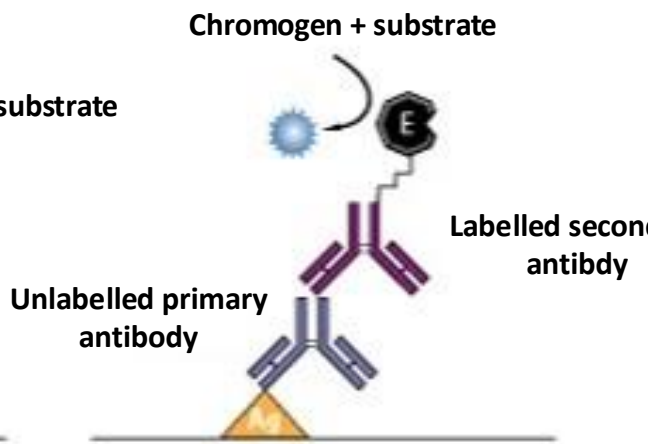
Main types of ELISA



Direct ELISA

↓

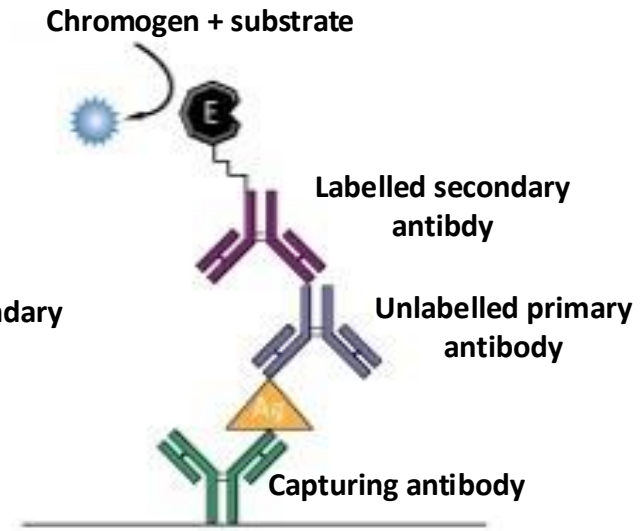
Expensive, weak signal.



Indirect ELISA

↓

Cheaper, signal is stronger.



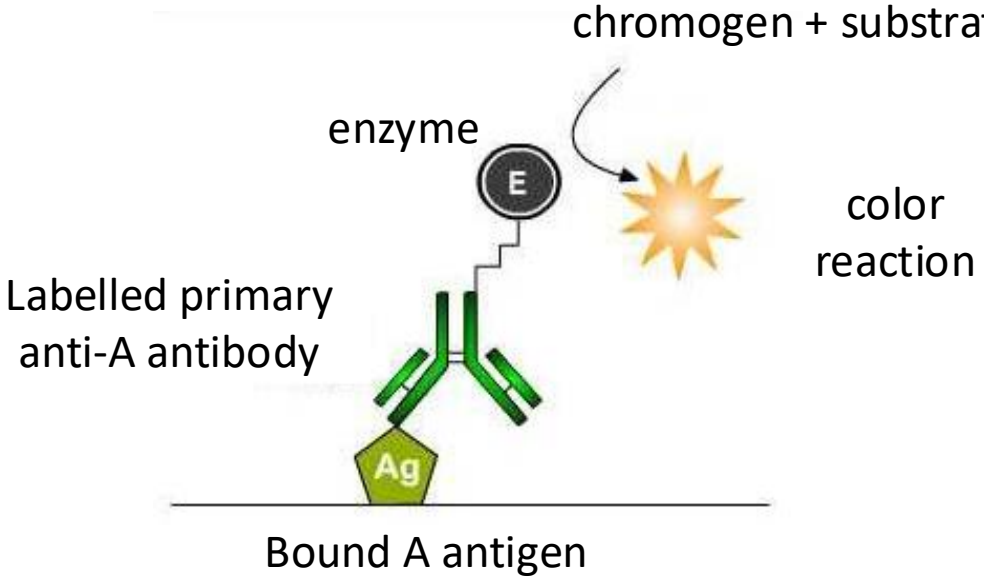
Sandwich ELISA

↓

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.]



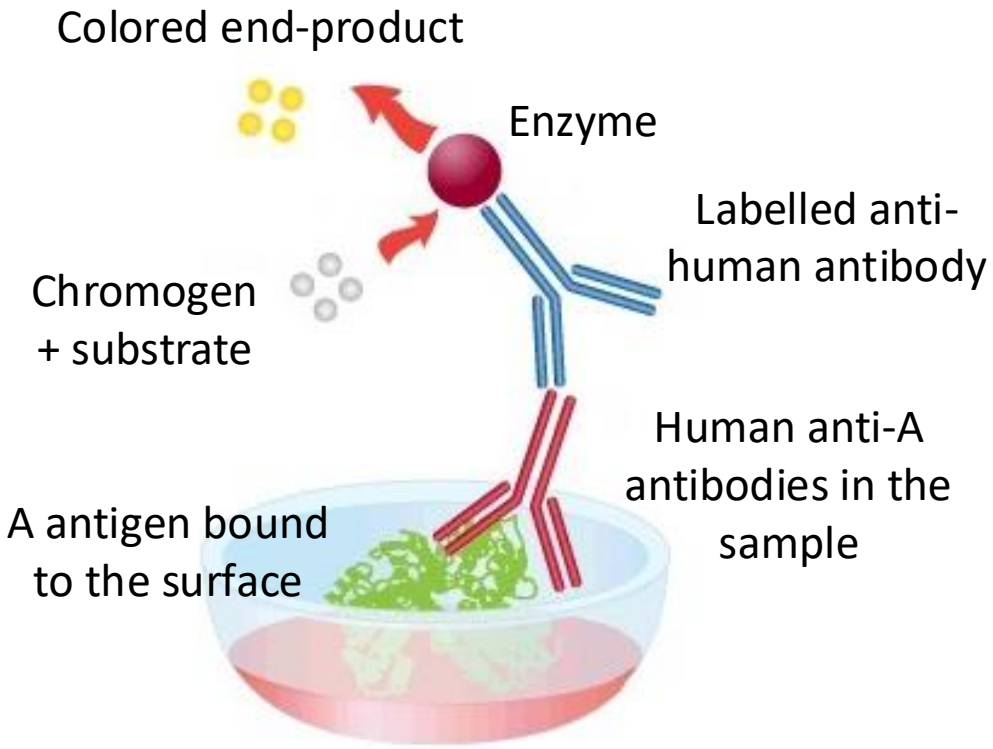
Advantages:

- **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)

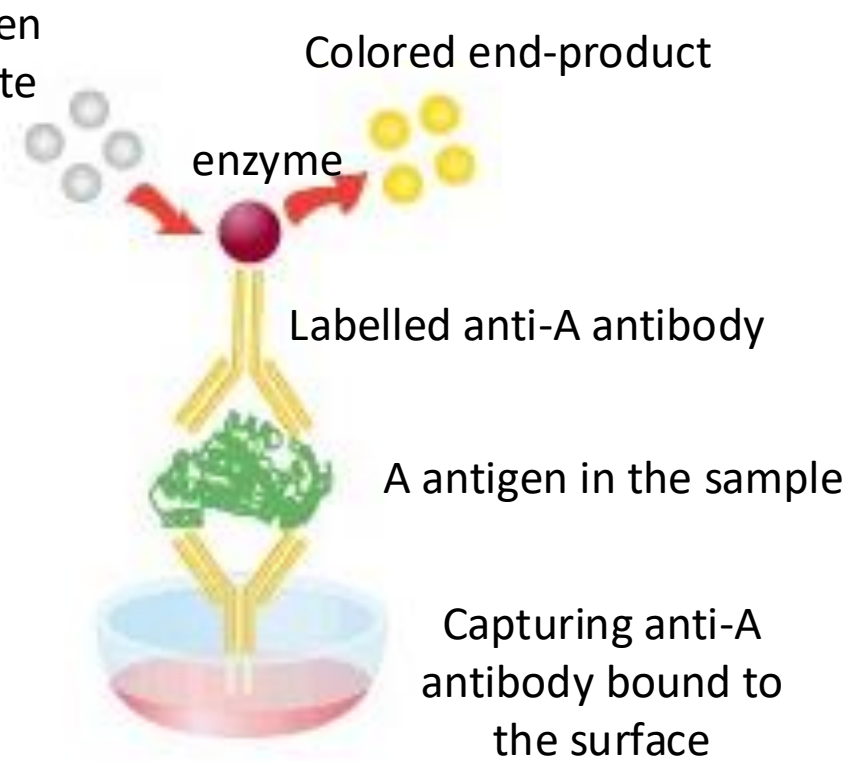
Indirect ELISA



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



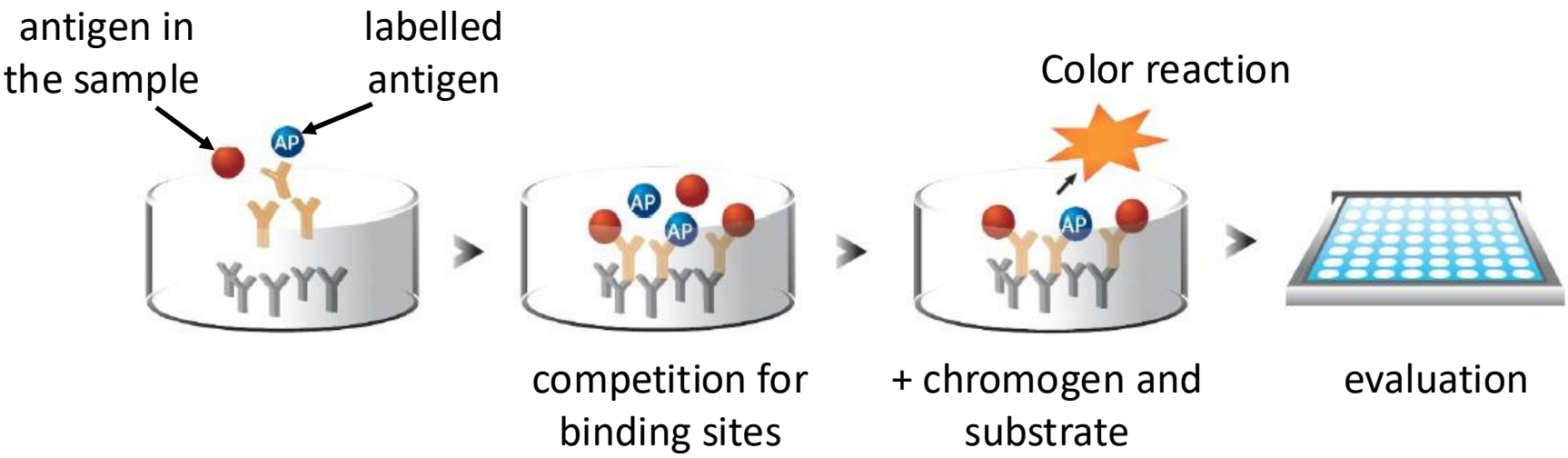
Applications: Detection of a specific antigen in the sample.

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



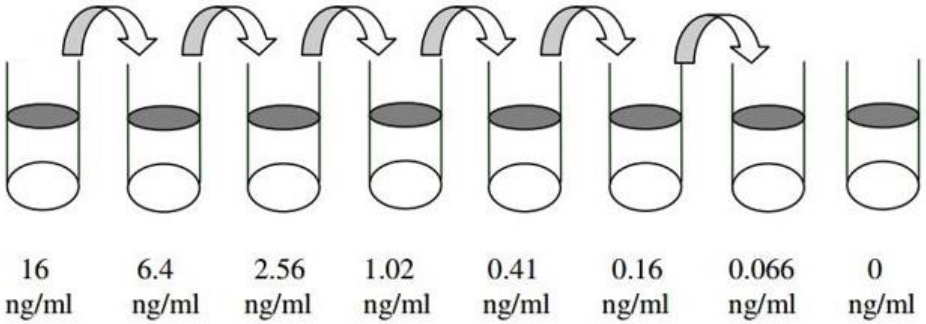
Application: Detection of a **specific antigen** in the sample.

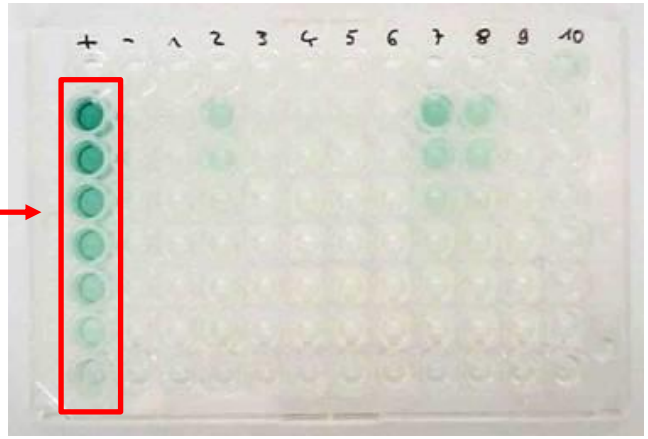
Principle:

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.

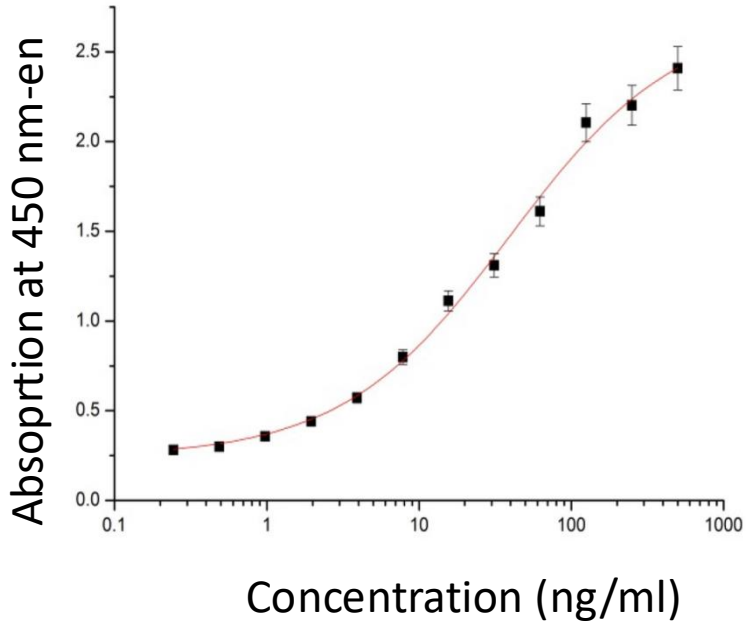
Creating standards with known concentrations:





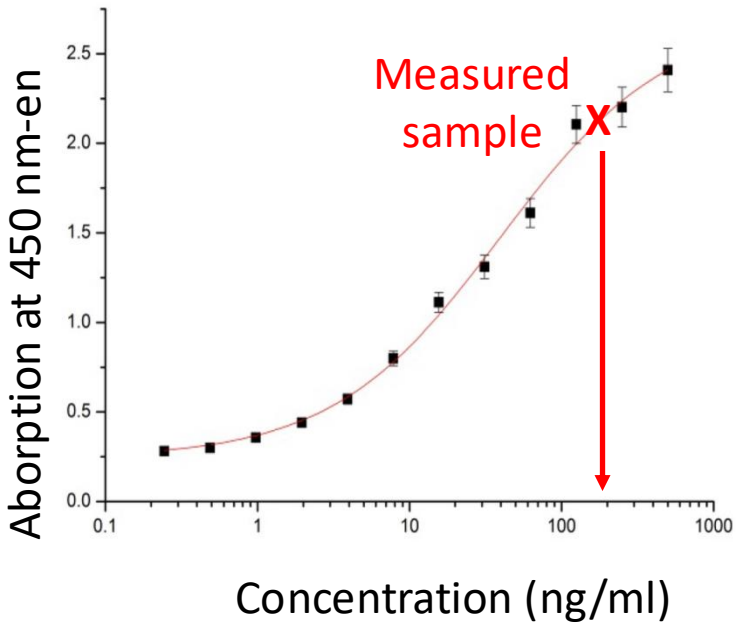
An ELISA reader which measures the **light absorption** in well of the ELISA plate.

ELISA standard curve:

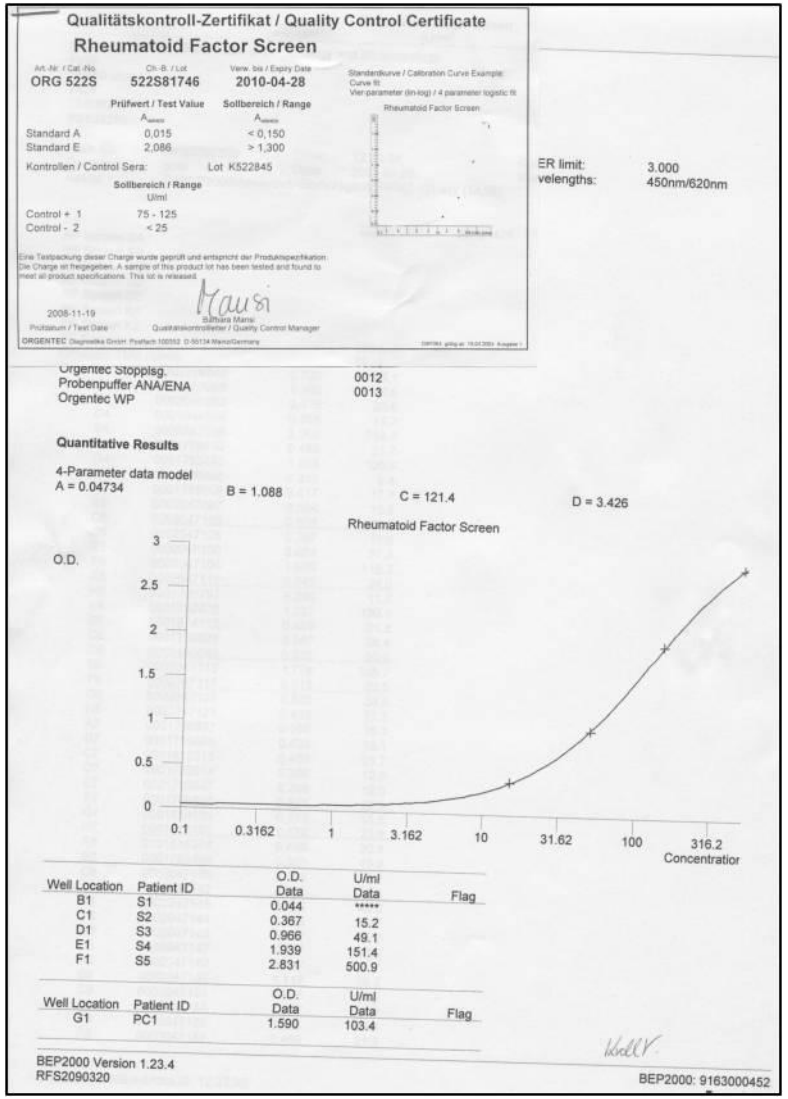


ELISA evaluation II.

Standard curve:



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

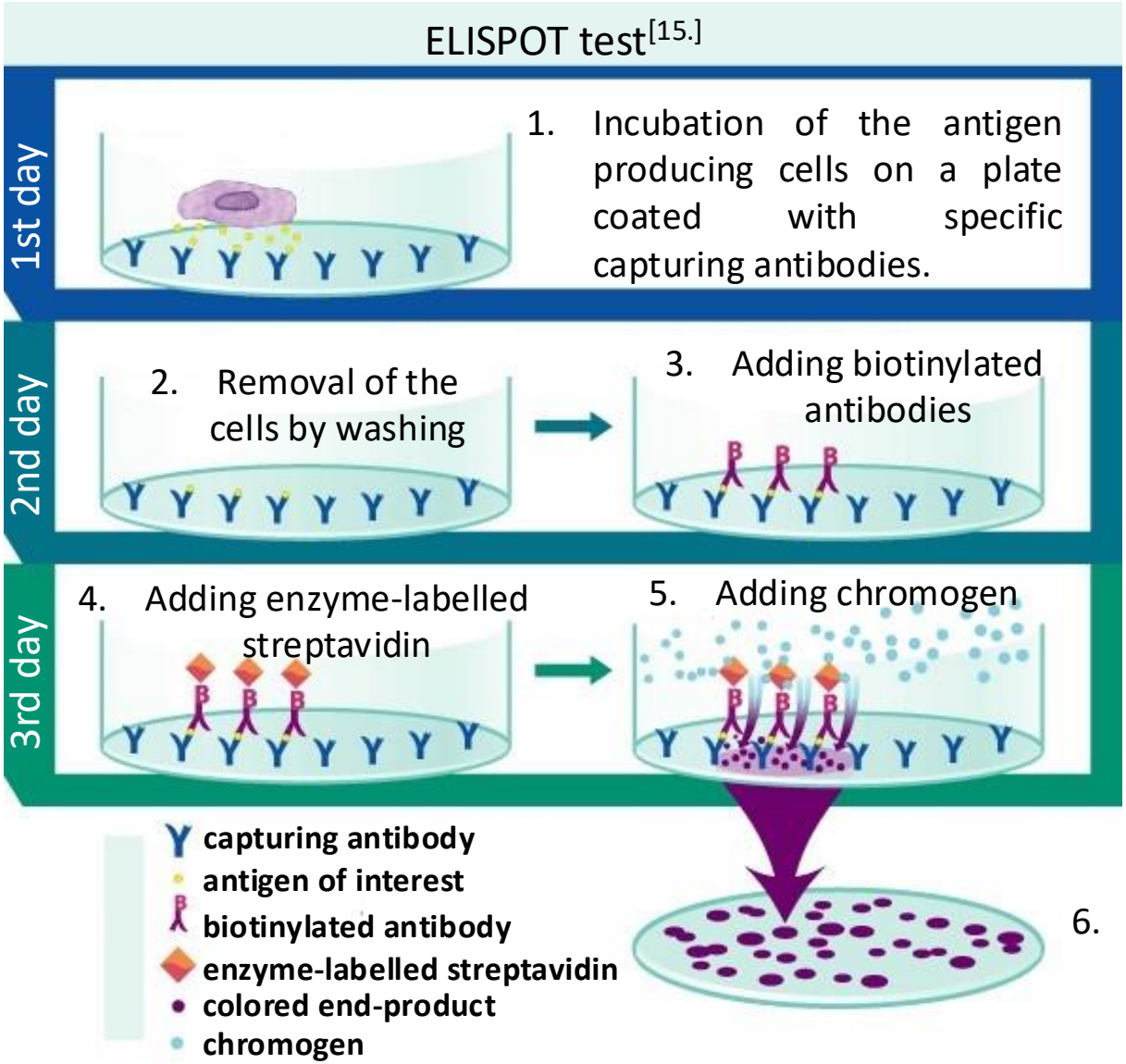


Result of a routine diagnostic ELISA (measured rheumatoid factor)

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:

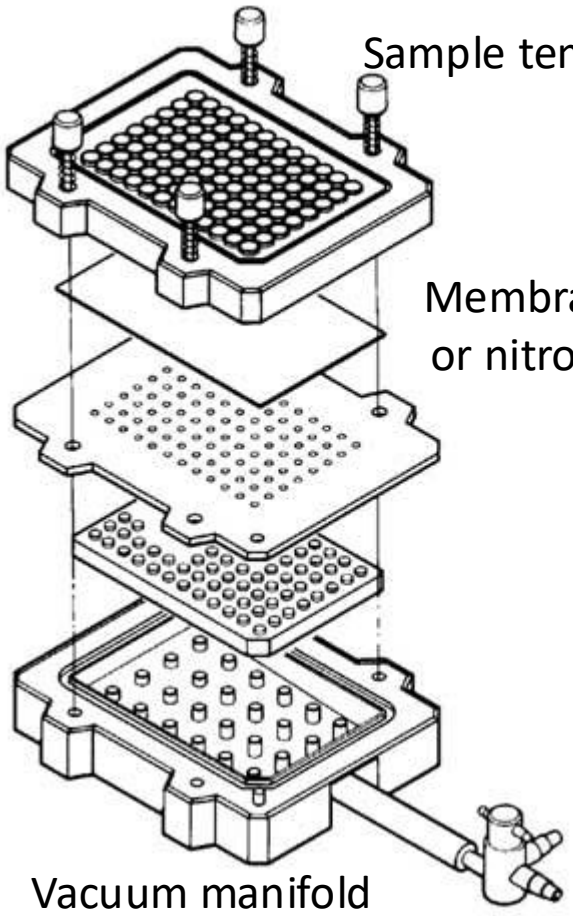
0 spot

T cells stimulated with an anti-CD3 antibody:

760 spots

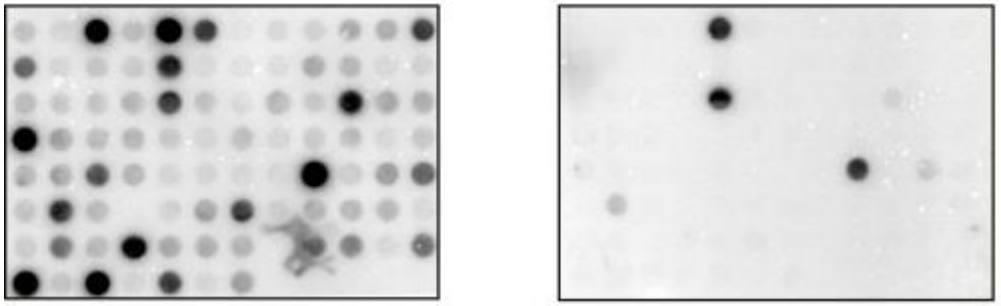
Detection of interferon gamma (IFNγ) with **ELISPOT**. The cells were put to a plate. The IFNγ they produced was instantly bound by the capturing antibody. The bound IFNγ was detected with enzymatic reaction. The stimulated T cells became activated and produced large amounts of IFNγ.

Dot blot



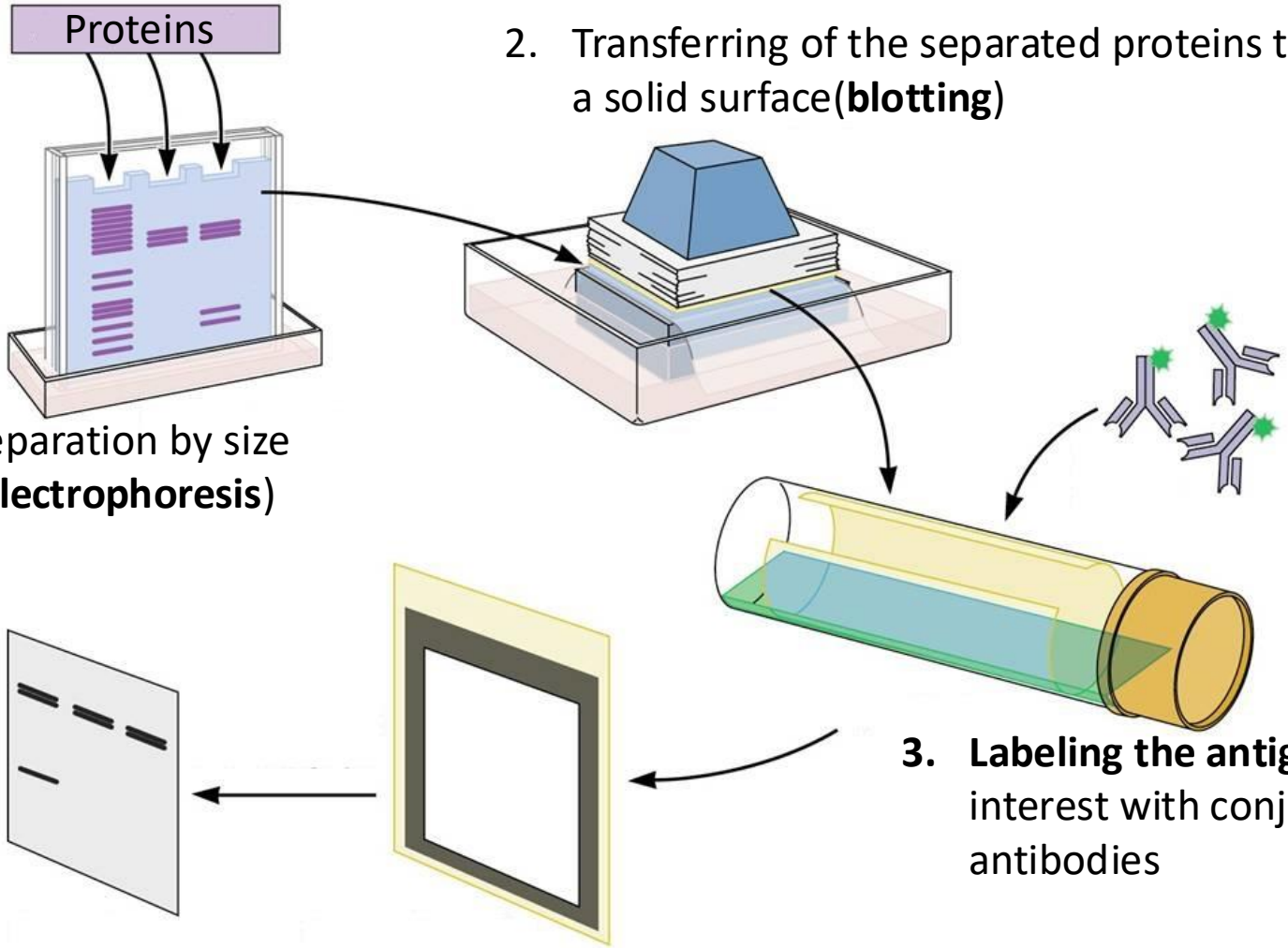
1. They put one drop of the sample containing the antigen to a solid surface (membrane).
2. The antigen attached to the surface is detected with the use of a labelled antibody either with a chromogen or with chemiluminescent reaction (see later).

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.]

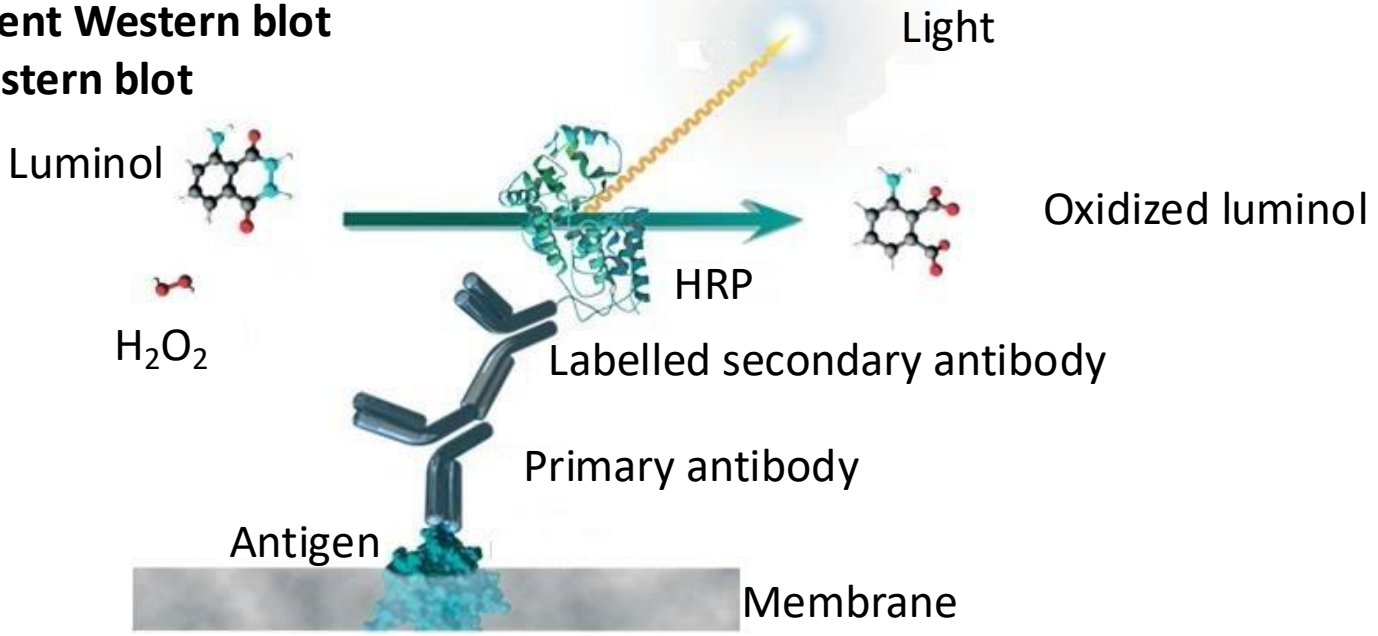


1. Separation by size (**electrophoresis**)
2. Transferring of the separated proteins to a solid surface(**blotting**)
3. **Labeling the antigen** of interest with conjugated antibodies
4. Detecting the antigen with either a **chemiluminescent reaction** or **immunofluorescence**

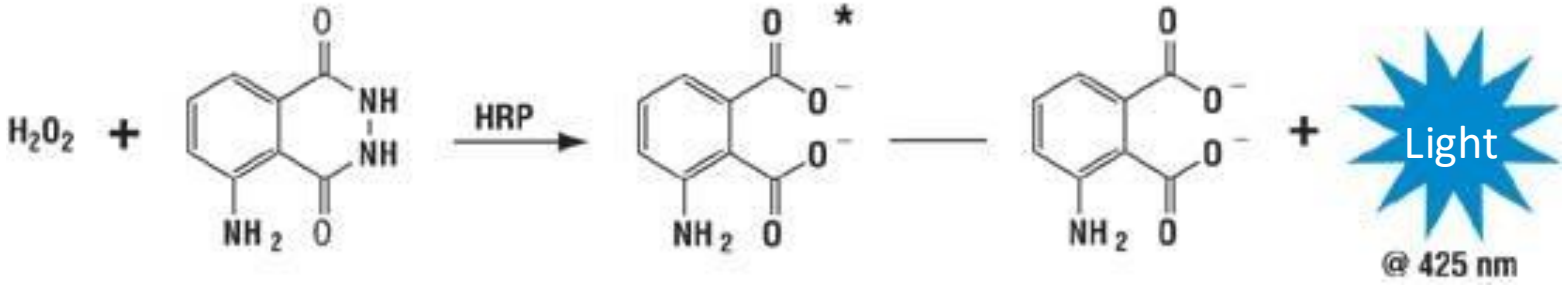
Detection of the antigen

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

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

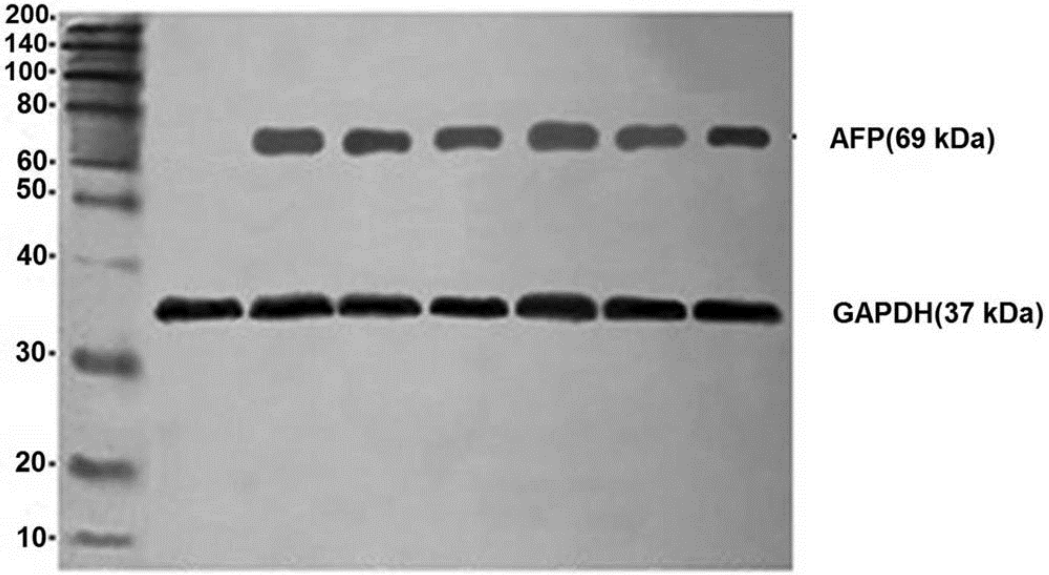


The chemiluminescent reaction of luminol:

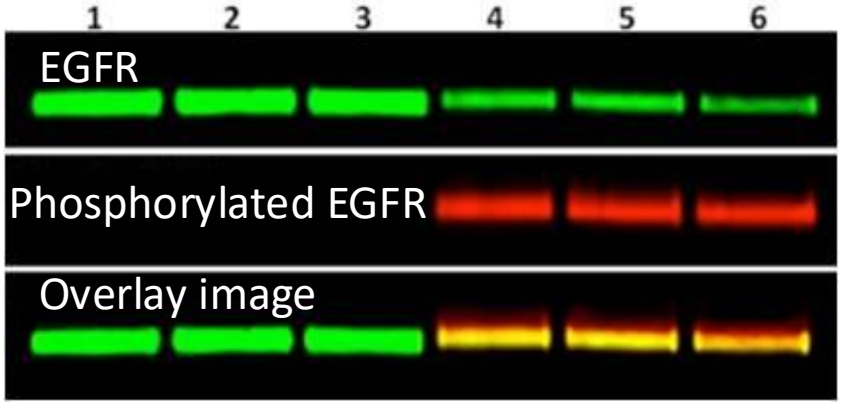


Examples

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



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

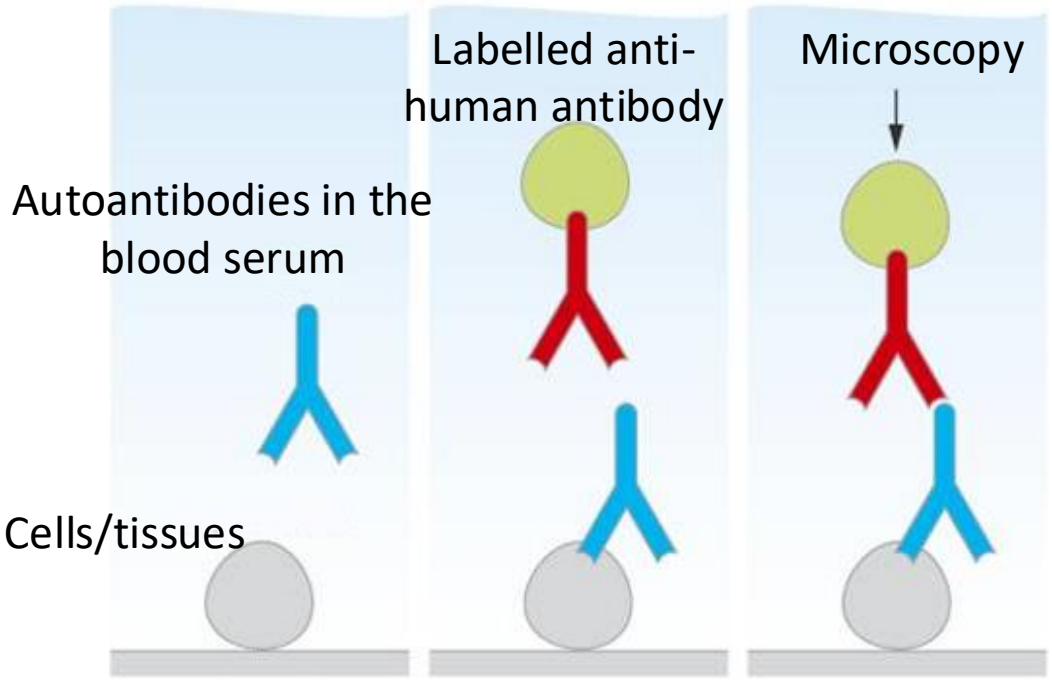


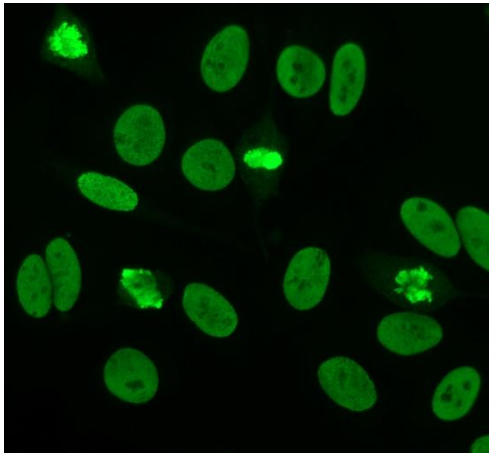
Significance of Western blot:

- 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

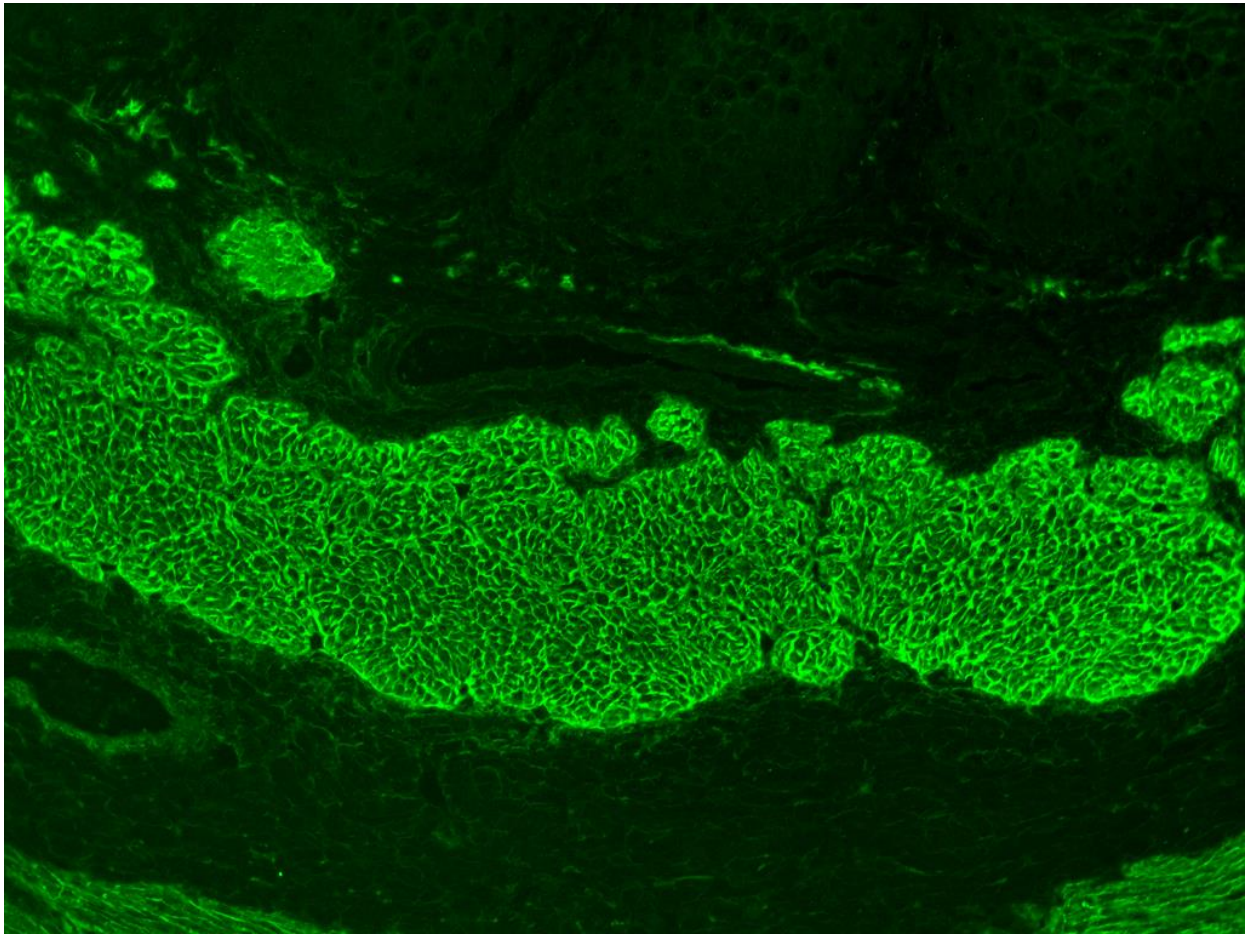
- Immunofluorescence microscopy → see 4th practice
- Application: **Diagnostics of autoimmune disorders** (see later in more detail)
- The serum of the patient is added to a cell culture or tissue. Autoantibodies in the serum will cross-react with the tissue or cultured cells which can be detected with 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. 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 2.

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.