Antigen-antibody reactions with labeled reagents

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ANTIGEN – ANTIBODY REACTIONS WITH LABELED REAGENTS

Enzyme immunoassay
Radioimmunoassay
Immunofluorescence and flow cytometry
Immunochromatographic tests

Many immunological assays are based on the principle of antigen-antibody reactions in which, one of the reagents is labeled with certain **marker** that can be visualized or detected with suitable equipment. In most of these tests, a marker is linked to an antibody, although sometimes an antigen can also be labeled (e.g. in radioimmunoassay, or RIA, radioactive isotope is often linked to an antigen). If an antibody is labeled, a marker is chemically linked to **Fc region of an antibody**, while the Fab fragment remains free to react with an antigen (Fc region is relatively large, therefore it is possible to link several molecules of the marker to one immunoglobulin molecule, which amplifies the detected signal and increases the sensitivity of these techniques). Various markers, such as enzymes, fluorescent dyes, radioisotopes, colloidal gold, and many others, can be used for the labeling of the reagents and several different methods are distinguished by the type of the marker applied. Among them, the most commonly used are enzyme immunoassay (EIA), radio-immunoassay (RIA), immunofluorescence (IF) and rapid immunochromatographic tests.

Enzyme immunoassay

Enzyme immunoassay, **EIA** or **ELISA** (E*nzyme-linked immunosorbent assay*) represents a method based on antigen-antibody reaction in which one of the reagents (typically an antibody) is labeled with an enzyme (enzymes that are most commonly used are peroxidase and alkaline phosphatase). Upon interaction between antigen and enzyme-labeled antibody, the appropriate substrate is being added to a reaction and a colored product is formed by the catalytic action of an enzyme. Developed color can be then detected colorimetrically, whereby the intensity of the obtained signal (expressed as an **absorbance**) is proportional to the amount of tested molecule in a sample.

ELISA test is used primarily for detection of molecules (**antigens** or **antibodies**) in **liquid samples** (e.g. serum, cerebrospinal fluid, urine, etc.). It is usually performed in **microtiter plates with 96 wells**, which allows simultaneous analysis of a large number of samples (each well is used to assess one sample) (Figure 1). The test is performed through **several steps** (stages) by adding one by one of the selected reagents in the plate's well and incubating them for certain period of time. Each time the reagent is added and incubated, the well is than **washed to remove unbound molecules**. Figure 2 depicts the basic **principle of ELISA test** when an antibody (A) or an antigen (B) is detected. If the aim of a test is to **detect an antibody** specific for a particular antigen in a sample (e.g. an antibody specific for an antigen of the microorganism of interest in the serum of a patient suspected of having a disease), than this antigen must be previously bound to the bottom and the walls of a well in a plate in which the reaction is performed (In most commercially available tests, this step is done by the company that produces the test). Further, the test is carried out by incubating the sample in which the antibody of interest is sought, followed by the addition of a secondary anti-immunoglobulin

antibody labeled with an enzyme and the substrate in the end. In case when the aim of a test is to **detect an antigen** in a sample (some microbial antigen or a hormone in a serum, for example), the unlabeled antibody specific for this antigen has to be previously attached to plate (this antibody is called capture antibody). Then the sample is added, followed by the enzyme-labeled antibody (so-called detection antibody that is specific for a different epitope of tested antigen compared to capture antibody) and the substrate at the end. This type of the ELISA test is often called **"sandwich" ELISA**, since the antigen is caught between two antibodies that are specific for the same antigen (but not the same epitope in order to avoid the interference between the two antibodies). In both types of ELISA, if the sample contains tested substance (antigen or antibody), enzyme-labeled antibody will bind to it, and, after the addition of substrate, the color will develop. The presence of a color is considered as positive result, and its intensity is proportional to the amount of the substance in a sample (the more of a substance is in the sample-the more labeled antibody is bound and consequently the color is more intense), which allows ELISA to be used as a quantitative test. On the other hand, if the tested antigen or antibody in the sample is absent, neither enzyme-labeled antibody will be bound, nor the colored product will be formed after substrate addition. The absence of colored reaction is considered as a negative result of a test.

ELISA may be **qualitative** or **quantitative** test. The result of the qualitative test can be positive or negative (for example, ELISA test for determining the presence of HIV infection). When using **the qualitative test** it is important to define the values of absorbance which represent the lowest limit for a positive result (i.e. the "**cut-off" value**). There are several ways to determine the "cut-off" values and it is usually determined by the manufacturer of a test and included in the assay instructions. For example, one way would be to compare values with the values of the negative and weak positive control. In that way, the "cut-off" value is determined as the mean value of absorbance of the negative and weak positive control. In **quantitative ELISA test**, result is expressed as the concentration of the antigen determined using **standard curve** (i.e. by comparing the absorbance values obtained for the sample with absorbance values obtained with the standards).

Enzyme immunoassays have **high specificity and sensitivity**. It is very **practical** test because it allows simultaneous analysis of a **large number of samples**. Furthermore, it does not require the use of expensive equipment (except for the colorimeter) and it is fairly easy to perform, which makes it suitable and affordable for most laboratories. Because of all of these advantages, this method is now one of the most widely used immunological assays and has been applied in various areas of medicine. For example, ELISA is commonly used for **the diagnosis of infectious diseases**, either for detection of soluble antigens of infectious agents or specific antibodies. ELISA is also used to determine the presence of **auto-antibodies** in patients with autoimmune diseases or **antibodies specific for various allergens** in atopic diseases. In addition to the detection of specific antibodies, ELISA test also allows the **determination of their isotype** (class) and **avidity** (a measure of the overall strength of an antibody-antigen binding). ELISA is often used for **precise measuring of the amount of different proteins** (hormones, cytokines, tumor markers and many others) in sera and other liquid samples. Due to high sensitivity of modern ELISA tests, this method has largely replaced RIA test in laboratory diagnosis and research today.

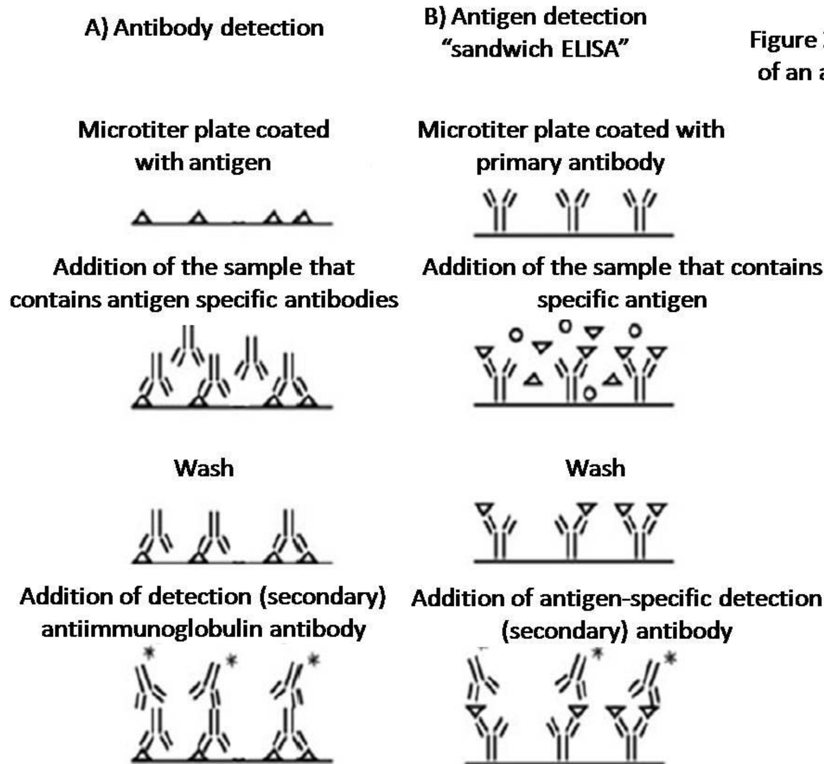


Figure 2. Comparative overview of the detection of an antibody (A) and an antigen (B), so-called "sandwich" ELISA).

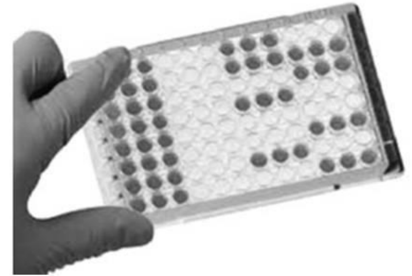


Figure 1. Microtiter plate.

Radioimmunoassay

Radioimmunoassay or **RIA** is an immunological technique used for detection of tested molecules using specific antibodies labeled with **radioactive isotope**, based on a similar principle as enzyme immunoassay. There are also variations of this method where antigen is labeled with radioisotope. In RIA, signal is detected by measuring radioactivity (i.e. radioactive decay rate of an isotope) with specific radioactivity **counter** (which counts a number of times a radioactive decay occurs in the sample) and the amount of tested antigen is determined by comparison with the standards.

RIA is a **very sensitive quantitative test** used for measuring **very low concentrations of antigen** in **liquid samples**, such as certain hormones in the blood. This is the reason why RIA, when it was developed in the late 50's, revolutionized medicine (especially endocrinology). Until then, there was no technique that would measure small quantities of substances in body fluids, such as IgE antibodies, that are bound to mast cell receptors and are present in the blood in very low concentrations. Indeed, **RAST method** (RadioAllergoSorbentTest), a variant of RIA test, was widely used in the past for detection of IgE antibodies specific for certain allergens in people with allergies.

RIA is **very precise** and **reliable** test, and beside high sensitivity its advantages are **high specificity** and **affordable price**. Nevertheless, this technique also has some **serious drawbacks**, such as problems with providing of radio-labeled reagents, half life of radioisotopes, **exposure to radiation** and **disposal of radioactive waste**. Due to these limitations, and the fact that the sensitivity of the new generation of enzyme immunoassays has markedly increased lately, RIA (including RAST method) has been mostly abandoned today and replaced with enzyme immunoassays or some other sensitive immunological methods.

Immunofluorescenece

Immunoflourescence (IF) is a technique based on the antigen-antibody reaction for detection of particular molecule that uses antibodies labeled with **fluorescent dye (fluorochrome)**. **Fluorescence** is the emission of light by a substance that has absorbed light or other electromagnetic radiation (It is a form of luminescence). In most cases, the emitted light has a longer wavelength, and therefore lower energy, than has absorbed radiation. This technique is primarily used for detection of **antigens on the cell surface** (or sometimes inside the cell), but can also be used for **antibody detection** (both in serum and bound to cells of different tissue as occurs in some autoimmune diseases). It can be performed on **microscope slides** using **fluorescent microscope** which differs from the conventional optical microscope by the light source (fluorescent microscope usually uses intense near-monochromatic source of illumination, such as xenon and mercury lamps) and by the presence of particular filters. Each of these filters emits specific wave length thus enabling more precise image. The other possibility is performing immunoflourescence using cells suspended in a liquid samples (i.e. peripheral blood cells) and analyzing them by **flow cytometry** (see below).

Immunoflourescence is **qualitative** technique wherein the presence of color is considered as a positive result and its absence as a negative result. Under certain circumstances, immunoflourescence can also be **semi-quantitative** (e.g. grading of a positive sample as weak or strong positive or when determining the titer of antibody in the serum). Sometimes, immunofluorescenece can even be used as **quantitative** method, especially when average fluorescence intensity of analyzed particles is determined by the flow cytometry.

There are two types of this technique, **direct immunofluoresence (DIF)** and **indirect immunofluorescence (IIF)**. DIF technique uses primary antibodies (those that are specific for an antigen) that are labeled with fluorescent dye, while IIF uses unlabeled specific antibodies that bind the antigen and anti-immunoglobulin antibodies (secondary antibodies, specific for primary antibodies), which are labeled with fluorescent dye. **Direct IF** is used to detect antigens on or within the cells of different tissues (or antibodies deposited in tissues), by using labeled antibodies specific for those antigens (Figure 3a). For example, the DIF test is used for determining the presence of antigens of *Chlamydia trachomatis* in urethral swab in the case of chlamydial infection, or the presence of deposits generated by activation of the complement and immunoglobulins in the skin or kidney samples in various diseases. **Indirect IF** may also be used for determining the presence of the antigens in the sample, and is usually performed when specific labeled antibodies are not available, so the combination of specific unlabeled and secondary labeled anti-immunoglobulin antibody is used. In this case, indirect IIF can be more sensitive than DIF, since several molecules of a secondary antibody can bind to one molecule of a primary antibody generating in this way the stronger signal (Figure 3b), but IIF is generally less specific than DIF. Unlike DIF, IIF can also be used for **detection of specific antibodies in serum** and other body liquids. To this end, it is necessary to provide microscope slides with the tissue or cells as a substrate for binding the antibody from the tested sample. Such an approach is used for detection of various **autoantibodies** in different **autoimmune diseases**. For example, antinuclear antibodies (ANA) are detected in this way as a part of laboratory evaluation of systemic diseases of connective tissues (in this case, nucleated cells represent a substrate), or antineutrophil cytoplasmic antibodies (ANCA) detection in vasculitides (neutrophils are a substrate). Similarly, the antiviral antibodies may be detected in the blood sample (virus-infected cells are fixed on microscope slides and used as a substrate).

Immunofluorescence is **reliable** qualitative technique, of **relatively high specificity and sensitivity**, which is why it is often used in research and diagnostic laboratories, especially when

tissue samples are analyzed. However, this technique has certain disadvantages. Considering that the results are obtained by observation of samples under microscope, it is hard to avoid **subjectivity in evaluation**, and the **price** of the fluorescent microscope itself is rather high. Also, the necessity to examine every sample in detail limits the number of the samples that can be analyzed with this technique and makes it **inconvenient** for analyses of a **large number** of samples.

Figure 3a. Direct IF

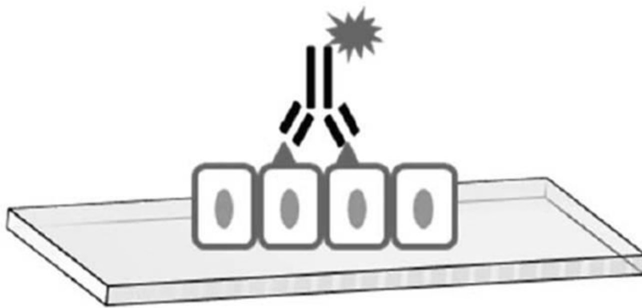
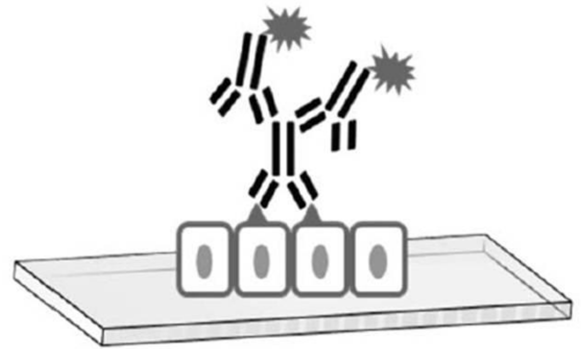


Figure 3b. Indirect IF



Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of **single particles**, usually **cells**, **labeled with fluorescent dye**, as they **flow** in a **fluid stream** through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined based on how the cell or **particle scatters laser light** and **emits fluorescence**. The colors that are commonly used for labeling antibodies are fluorescent isothiocyanate (FITC, green fluorescence) and phycoerythrin (PE, orange fluorescence), but many other fluorescent colors have been developed today. Using fluorescently labeled antibodies specific for different surface molecules, it is possible to determine population of cells in the suspension using a specific cell marker (e.g. the number of $CD4^+$ helper T cells in the blood using antibodies for the CD4 molecule). Characterization of cells based on surface markers is called **immunophenotyping**.

Flow cytometry is widely used in diagnostics. It can help in diagnosis, choice of treatment and/or follow-up and prognosis of some diseases like lymphoproliferative diseases and immunodeficiencies (e.g., leukemia, primary immunodeficiencies and the HIV-infection). For example, a B-cell lymphomas, which expresses CD20, can be treated with rituximab (anti-CD20 monoclonal antibody), and it is necessary to perform immunophenotyping of tumor cells before the treatment starts. Also, flow cytometry is important for monitoring therapeutic response in different diseases (e.g. decline in number of $CD4^+$ T lymphocytes suggests progression of HIV infection or increase of marker-bearing tumor cells indicates an inadequate therapeutic response and recurrence of a tumor).

In addition to diagnostics, flow cytometry has a very important role in research laboratories. Besides immunophenotyping and determining the cell type, the maturation stage of the cells, as well as its activation status, can be determined based on surface markers (some molecules are lost, and the other begin to express at certain stage, as for the alpha chain of the receptor for IL-2 that appears on the T cells upon activation). Apart from surface markers, many

intracellular molecules can also be detected by flow cytometry. Intracellular staining requires previous permeabilization of the cell membrane, which allows labeled antibody to enter the cell. In this way, it can be determined whether the population of $CD4^+$ helper T cells under certain experimental treatment synthesizes IFN-γ or other cytokines. In addition to fluorescent labeled antibodies, other chemical compounds that exhibit fluorescence can be used as reagents. This has been applied for the analysis of the cell cycle, detection of apoptosis, oxidative stress, and changes in cell concentration of Ca^{2+}, and for many other purposes.

There are devices (the so-called **sorters)** that are able, not only to identify cells based on markers, but also to separate (sort) the various cell populations from a heterogeneous mixture of cells (e.g. those that posses certain marker, from those without it).

Rapid immunochromatographic test

Immunochromatographic technique involves the use of antibodies that are labeled with a certain non-fluorescent dye. Different molecules can be used as **markers of antibodies**, such as **colloidal gold** (i.e. colloidal gold nanoparticles conjugated with antibodies) whose use is widespread due to its high stability. The principle of this technique is based on the **binding of a soluble antigen** in the sample to colored antibody conjugate, and the subsequent **migration** of these **antigen-antibody complexes** in solution under the influence of capillary forces through the porous membrane or polymeric three-dimensional structure (so-called **matrix**), which contain other antibodies of different specificities (Those antibodies are unlabeled and are fixed at certain spots in the matrix). When these complexes encounter another antibody (specific for the same antigen, but different epitope) that is unlabeled and fixed to the membrane/matrix, antigen-antibody complexes accumulate at this spot in high amounts, which manifests as a clear, **visible band** of specific color (presence of the bands is interpreted as a positive result, while its absence is interpreted as a negative result). In the similar way, **antibodies** in the sample of various specificities (e.g. IgE antibodies specific for different allergens) can be detected by adding a secondary anti-immunoglobulin color labeled antibody, which binds to already formed complexes on the membrane/matrix.

Immunochromatographic tests are **qualitative** and used for detection of different antigens or specific antibodies in liquid samples (i.e. serum or urine). They can also be used for non-liquid samples, such as stool, but such samples must be previously suspended in a suitable buffer, which makes migration through the membrane or matrix possible. Considering the fact that monoclonal antibodies are used, these tests are **reliable** and have **high specificity** and **sensitivit**y. They are also **easy to perform**, results can be obtained very **fast** (usually in a few minutes), and they have **affordable price**. Therefore, many commercially available tests, especially those made for screening purposes, are based on the principle of immunochromatography: for example, tests for the detection of various infectious agents and their products, such as influenza virus, viruses that cause gastroenteritis (rotavirus and adenovirus), toxins of *Clostridium difficile*, group A streptococcus, etc. Rapid pregnancy tests are also based on a similar principle (detection of βhCG in urine), as are the tests for detection of blood traces in forensic medicine (detection of hemoglobin). ImmunoCAP test belongs to this group of tests and is used for detection of IgE antibodies specific for various allergens. This test has largely replaced RAST method (see RIA test) used until recently.