THE SCIENCE WORLD
A Monthly e Magazine
Popular Article
ISSN:2583-2212
December, 2022; 2(12), 2024-2031

Immunochemical techniques: An Overview

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https://doi.org/10.5281/zenodo.7414349

Introduction

Immunochemical techniques are based on a reaction of antigen with antibody, or more exactly, on a reaction of an antigenic determinants with the binding site of the antibody. The antibodies used are produced by various ways. Monoclonal antibodies are products of a single clone of plasma cells derived from B-lymphocytes, prepared in the laboratory by hybridoma technology, based on cellular fusion of tumor (myeloma) cells with splenic lymphocytes of immunized mice. Monoclonal antibodies are directed against single epitope; and are all identical copies of immunoglobulin molecule with the same primary structure and specificity of antigen binding site. They typically display excellent specificity, but poor ability to precipitate antigen. Polyclonal antibodies (conventional antibodies) are prepared by immunization of animals (rabbits, goats, sheep) with the antigen. Blood serum of the immunized animal that contains antibodies against the antigen used, is called an antiserum. If one antigen (e.g. one protein) is used for immunization, monospecific antibodies (antiserum) result. However, as every epitope stimulates different clone of B cells, and complex antigens bear several epitopes, the antiserum contains mixture of monoclonal antibodies, differing in their affinity and specificity towards particular epitopes on the antigen used for immunization. Immunization of an animal with mixture of antigens results in production of poly-specific antibodies1, containing immunoglobulins against many antigens (e.g. antiserum against human serum proteins used in immunoelectrophoretic).

Precipitation methods in gel

As a support, agar or agarose gel is used most often. In single immunodiffusion only one component (i.e. antigen or antibody) diffuses from the place of sample application, while the other reaction partner is dispersed evenly in the gel. If both components of the immunochemical reaction diffuse in the gel

against each other from places of their application, the technique is called double immunodiffusion. In the area of antigen antibody reaction, a precipitation zone appears as line, crescent or circle. The immunodiffusion methods in gel are represented e.g., by the Mancini's single radial immunodiffusion and the Ouchterlony's double immunodiffusion. Other techniques combine an immunochemical reaction with electrophoretic separation, such as immunoelectrophoretic, Laurell's (rocket) immunoelectrophoretic, and immunofixation.

Single radial immunodiffusion

Single radial immunodiffusion represents a simple method without requirements for expensive instruments. The antigen is applied into wells that are cut in the agarose gel containing dispersed corresponding monospecific antibody. The agarose plate is incubated at room temperature for 48-72 hours depending on the specific protein in question. The antigen from the sample diffuses out from the wells into the agarose where the antibody concentration is constant. In a distance from the well where antigen

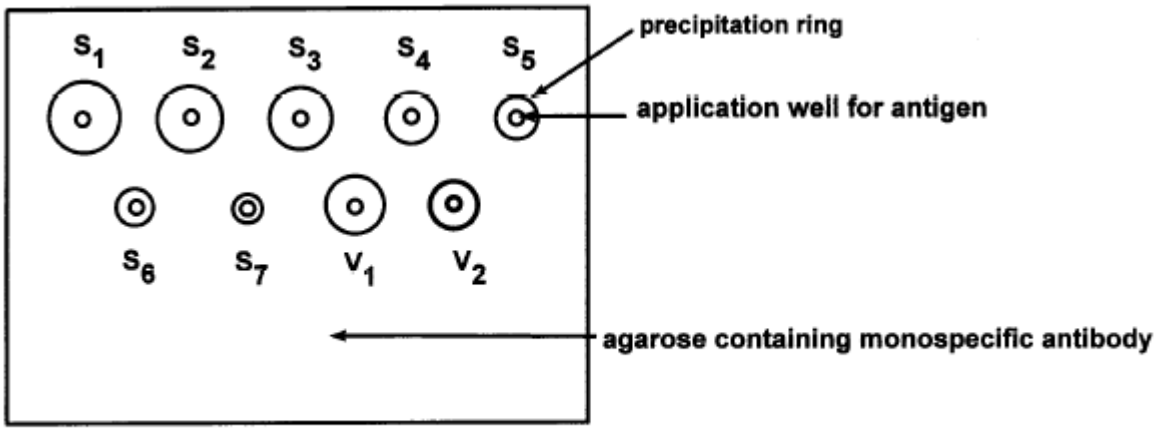

Fig. 4: Single radial imunodiffusion ($S_1 - S_7$ – standards, V_1-V_2 – samples)

concentration is equivalent to the antibody concentration (i.e., zone of equivalence is reached in terms of the precipitin curve discussed above), the complex antigen-antibody precipitates and appears as a strong white ring around the well. The square of the ring diameter is directly proportional to the antigen concentration.

Double immunodiffusion

In double immunodiffusion reaction, the antigen and the antibody diffuse towards each other. The Ouchterlony's modification is the one most often used. It is based on wells punched into the agarose gel in a rosette pattern that are filled with antigen or antibody solutions, respectively. Both antigen and antibody molecules are allowed to diffuse radially into the gel surrounding the wells; and where the antigen and specific reactive antibody meet, a precipitin line forms. If antiserum to several possible antigens is placed into the central well and the outer wells are filled by different antigens, precipitin lines

of various shapes can arise. For instance, if two antigenic mixtures are applied into two adjacent wells, the following patterns of precipitin lines can be observed, independence on the relationship between the two antigenic mixtures.

- *A reaction of identity* – if two identical antigens are applied into two adjacent wells, the precipitin bands form a continuous arc.
- *A reaction of non-identity* – the precipitin bands form lines that intersect.
- *A reaction of partial identity* – it is characterized by a formation of a spur. The common hooked precipitation line arises from the reaction of the common antigenic determinants on both antigens with the antibody. The spur means that the second antigen lacks an epitope present in the first antigen that is recognized by one of the antibodies in the antiserum.

Double immunodiffusion is a qualitative technique suitable for the identification of antigens and the estimation of their mutual immunochemical relationships if the specific antisera are available. On the other hand, this method may be used for characterization of antibodies using known purified antigens.

Immunoelectrophoretic

Immunoelectrophoretic is a qualitative method that combines protein electrophoresis with immunodiffusion. It is performed in two steps. The first one involves the separation of antigens according to their charges/size in an electrical field. In the second step, a suitable antiserum (poly-specific or monospecific) is applied to grooves running parallel to the electrophoresis migration zone. The separated antigens and antibodies are allowed to diffuse into the gel towards one another. The precipitation line is formed in the area when the antigen with the reacting antibody meets.

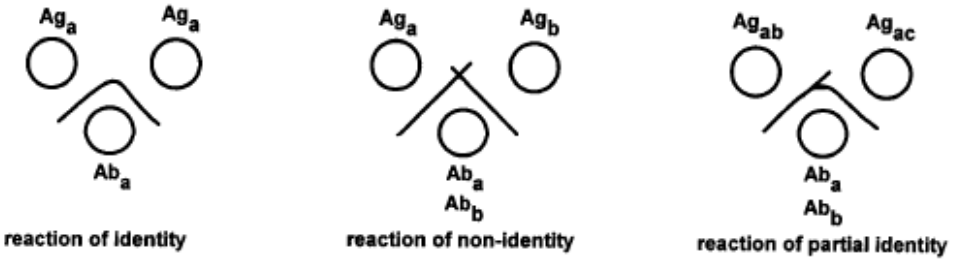

Fig. 5: Double immunodiffusion according to Ouchterlony (Ag_a, Ag_b, Ag_c – antigens with epitopes a, b, c; Ab_a, Ab_b – antibodies against epitopes a and b)

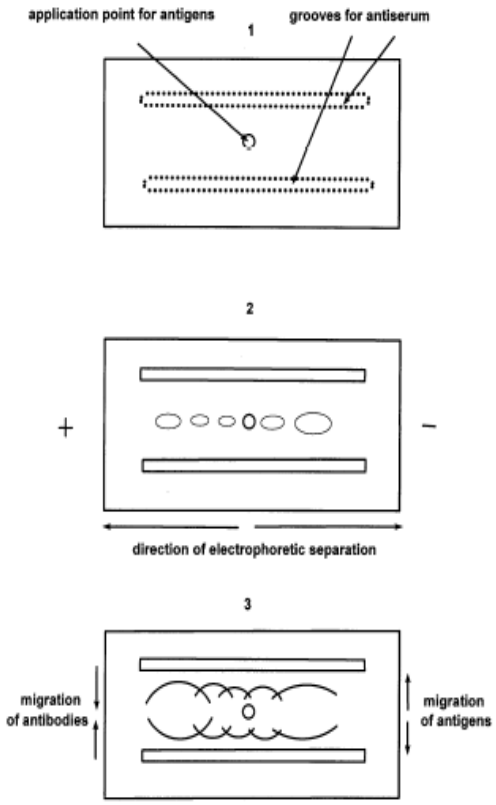

Fig.6: Immunoelectrophoresis (polyspecific antiserum used)

Immunofixation

Immunofixation is a method used for the detection and isotyping of monoclonal immunoglobulins in serum, urine and cerebrospinal fluid. Similarly, to immunoelectrophoretic, immunofixation is carried out in two stages. In the first one, serum proteins are separated by electrophoresis. In the second step, the monoclonal immunoglobulins are identified by means of immunoprecipitation with specific antibodies.

Precipitation methods in solution

Agglutination

Agglutination (from latin 'agglutino' – to glue, to attach) is an immunochemical technique in which a specific antibody reacts with the *corpuscular antigen*. Agglutination reaction is based on the formation of bridges between bivalent (IgG) or multivalent (IgM) antibodies and antigenic particles with multiple epitopes. Bivalency or multivalency of the used antibody and multiple antigenic determinants on the surface of particles are necessary for the creation of cross-linking and the formation of a high-molecular-weight lattice that is observable macroscopically. IgM antibodies with ten antigenic-combining sites permit a more effective bridging than IgG. Some antibodies react with the corpuscular antigen, but may not produce agglutination. In this case, the agglutination may be achieved if an anti-immunoglobulin

is added into the reaction mixture (the Coombs test). Hemagglutination is a variant of agglutination technique in which red blood cells are used as the antigen bearing particles. Agglutination reactions are performed on slides, in test tubes or microtiter plates. They are more sensitive in comparison with immunoprecipitation methods. The agglutination methods produce qualitative or semiquantitative results. Agglutination assays may be classified as *direct* or *indirect tests*.

Direct agglutination

In a direct agglutination test, the antigen is an integral part of the cell surface (red blood cells, bacteria). A suspension of particles is directly agglutinated by specific antibodies present in the examined sample. This assay is frequently used in the hematology for the *determination of blood group* or in the immunological diagnostics for detection of specific antibodies directed against naturally occurring antigens on the surface of some microbes (for example against *Salmonella typhi* – the *Widal test*). For the examination of antibodies, the test is usually performed with serial dilutions of the sample. The highest dilution of serum that still causes agglutination is denoted as a *titer of the antibody*.

Indirect agglutination

Indirect agglutination assay utilizes particles with the antigens that have been passively attached to their surface. Originally, red blood cells were used as carriers for antigens; lately, inert particles such as latex, colloid gold and other substances have been shown to be more versatile for the agglutination technique. Many proteins, bacterial and viral antigens are easily adsorbed onto the particle, while other substances require modification by tannic acid or chromium chloride.

Agglutination inhibition test

Agglutination inhibition test is another form of agglutination reaction that permits the determination of soluble antigens. It is based on the competition between the antigens in solution and the same antigens on the particle surface for limited number of antibodies. The specific antibodies are incubated with the test solution containing the soluble antigens. Following the addition of the particles coated with the antigens, the agglutination does not occur because most of the antibodies have been already saturated with a soluble form of the same antigen from the sample. Therefore, antibody binding sites are unavailable for bridging the coated particles. A lack of agglutination indicates a positive result. On the other hand, if the soluble antigen is not present in the tested sample, after the addition of the corpuscular antigen the agglutination develops.

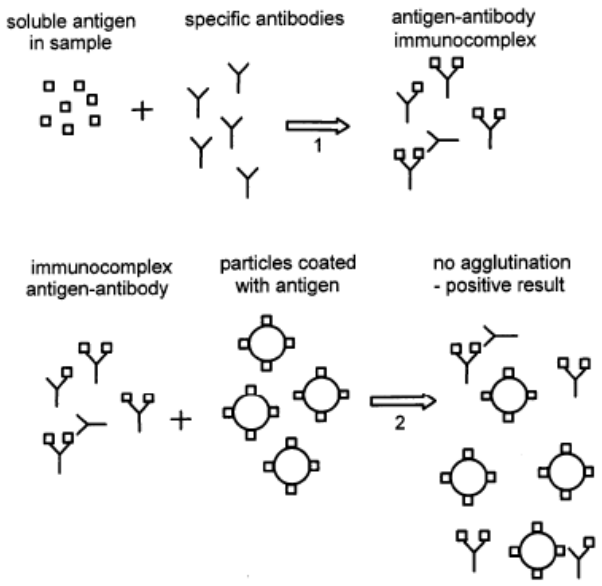

Fig. 11: Agglutination inhibition
1st reaction – a sample containing the soluble antigens is added to the specific antibodies, the antigen-antibody complexes arise
2nd reaction – after the addition of particles coated with antigens, the agglutination does not occur

Enzyme immunoassay

State-of-the-art immunoanalytical techniques achieve high sensitivity by labelling of one reacting component– antibody or antigen – with a substance which detection is more sensitive than detection of immunoprecipitated. The label can be a radioisotope (radioimmunoassay, RIA), an enzyme (enzyme immunoassay, EIA), a fluorescent or chemiluminescent substance. Detection limits of these techniques can be as low as 10−15 – 10−20 mol/L. Enzyme immunoassays utilize enzymes, usually peroxidase or alkaline phosphatase, to detect and quantify immunochemical reactions. Both antibodies or antigens can be labelled with an enzyme in order to aid detection. A heterogeneous enzyme immunoassay method is also called enzyme-linked immunosorbent assay (ELISA). In this type of assay, one of the immunochemical reaction components (antigen or antibody) is first non-specifically adsorbed to the surface of a solid phase. Tubes, wells of microtiter plates, and magnetic particles may be used as the solid phases. The solid phase facilitates separation of bound- and free-labelled reactants. A homogeneous enzyme immunoassay is a sort of enzyme multiplied immunoassay technique (EMIT) that does not require a separation of bound and free labelled antibodies or antigens. It is simple to perform and has been used for estimation of drugs, hormones and metabolites. Sample containing the estimated antigen is mixed with a known quantity of the same antigen labelled with enzyme (conjugate); and limited amount of specific antibody is added. The unlabeled antigen from the sample competes with the conjugate for the antibody. Binding of antibody on the conjugate results in loss of enzyme activity due to blocking the enzyme active site or change of its conformation. The more unlabeled antigen is present in the solution, the less conjugate

will bind to the antibody, and more enzyme activity will be preserved in the solution. Therefore, the enzyme activity is proportional to the antigen concentration in the sample. The reaction scheme in these immunochemical assays can follow either competitive, or non-competitive approach.

Competitive enzyme immunoassay

This assay is always performed under condition of antigen excess. The enzyme-labelled antigen (conjugate) is mixed with serum sample containing the unknown amount of antigen. The serum antigen and enzyme-labelled antigen compete for binding sites of a limited quantity of specific antibodies bound to the solid phase. Labelled and non-labelled antigen bind to the antibody in the same proportion as is their proportion in the reaction mixture. In other words, the more non-labelled antigen is contained in the mixture the less labelled antigen is bound. Under these conditions the probability of the antibody binding the labelled antigen is inversely proportional to the concentration of unlabeled antigen. The higher the amount of unlabeled antigen in the sample, the more labelled antigen remains free (unbound). After an incubation, all the unbound both enzyme-labelled and unlabeled antigens are removed by washing along with all other serum constituents. In the subsequent indicator reaction for detection of enzyme activity a chromogenic substrate for the enzyme label is added. The intensity of color is inversely proportional to the concentration of the antigen in serum sample. The results are obtained from a calibration curve constructed with the standards of known concentration of antigen.

Non-competitive enzyme immunoassay (sandwich methods)

This kind of enzyme immunoassay can be adopted for measurement of either antigens or antibodies. It is a heterogeneous immunoassay using a solid phase coated with antibody or antigen, which must always be in excess over the analyte being measured.

Non-competitive enzyme immunoassay for determination of antigen

This non-competitive enzyme immunoassay is suitable for the measurement of large antigens with several antibody-binding sites. Two different molecules of antibodies directed against various epitopes are necessary for performing the assay. The first antibody is in excess adsorbed to a solid phase. The serum sample or calibrators containing the desired antigen are added to the well with immobilised antibody. The first immunochemical reaction occurs. Since the antibody is in excess, all antigen molecules should bind. After an incubation, all the non-reacting material in the sample is washed away. Then a second enzyme-labelled antibody (different from the first antibody) is added in excess. In the second immunochemical reaction, another antigen epitope binds to the second labelled antibody. “Sandwich complex” consisting of solid-phase antibody – antigen – enzyme labelled antibody is formed. After washing of all the unreacted enzyme-labelled antibody, the substrate is added. The intensity of the finally measured coloured product of the enzyme reaction is directly proportional to the amount of antigen.

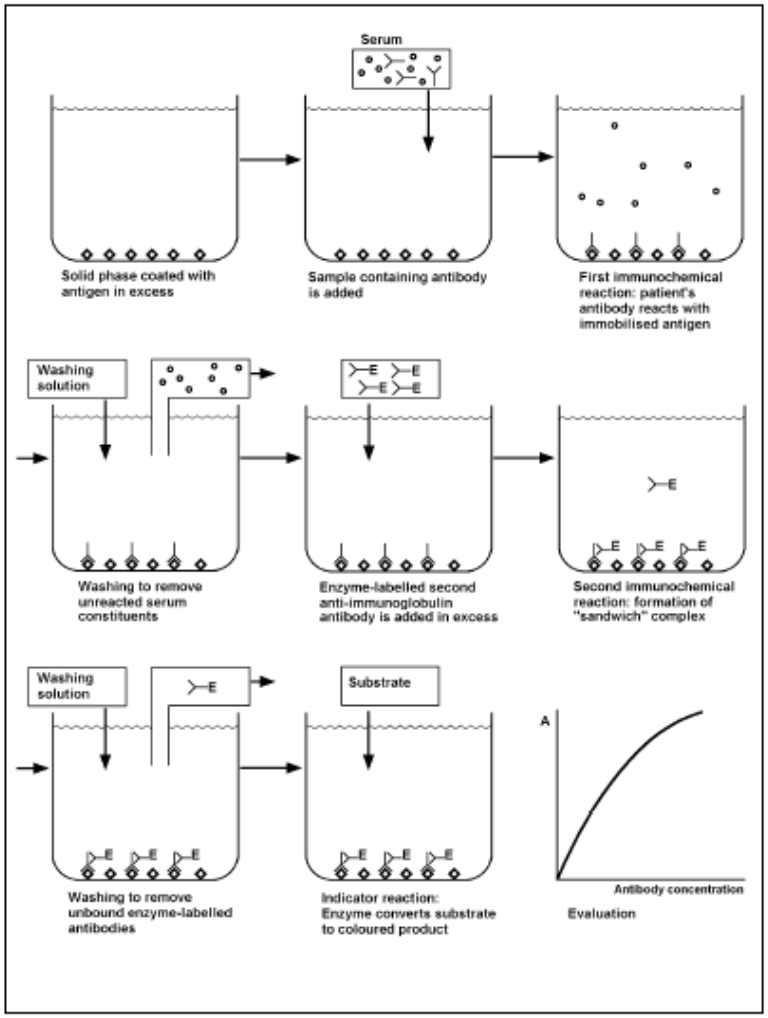

Fig 15: Non-competitive enzyme immunoassay for determination of antibody

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