



AZERBAIJAN MEDICAL UNIVERSITY
DEPARTMENT OF MEDICAL MICROBIOLOGY and IMMUNOLOGY

Lesson 20.

**Complement fixation test (CFT). Immunofluorescence reaction (IFR).
Enzyme-linked immunosorbent assay (ELISA). Radioimmunoassay (RIM).
Immunoblotting (IB). Application of genetic methods in microbiological
diagnostics. Polymerase chain reaction (PCR). Sequencing**

FACULTY: General Medicine
SUBJECT: Medical microbiology - 1

Discussed questions:

1. Complement fixation test (CFT) and its essence
2. Mechanism of CFT and its diagnostic importance.
3. The components for using in CFT and reaction technique.
4. The essence of the reactions that take place in the presence of targeted antibodies.
5. The importance of reactions involving targeted antibodies in rapid diagnosis.
6. Mechanism of immune fluorescence reaction (Kuns reaction): direct (DIFR) and indirect (IFR) variant.
7. Immune enzyme analysis (ELISA) and its essence: direct and indirect variants.
8. Radioimmune method (competitive, reverse and indirect), application.
9. Immune blotting and mechanism.
10. Application of genetic methods in microbiological diagnosis.
11. Molecular-genetic methods, types, application in diagnosis.
12. The essence, mechanism and practical significance of the polymerase chain reaction (PCR, real-time RT PCR).
13. Molecular hybridization method, essence, mechanism and practical significance.
14. Restrictive analysis, essence, mechanism and practical significance.
15. Sequencing method, essence, mechanism and practical significance.
16. Genetic engineering, goals and objectives.

Purpose of the lesson:

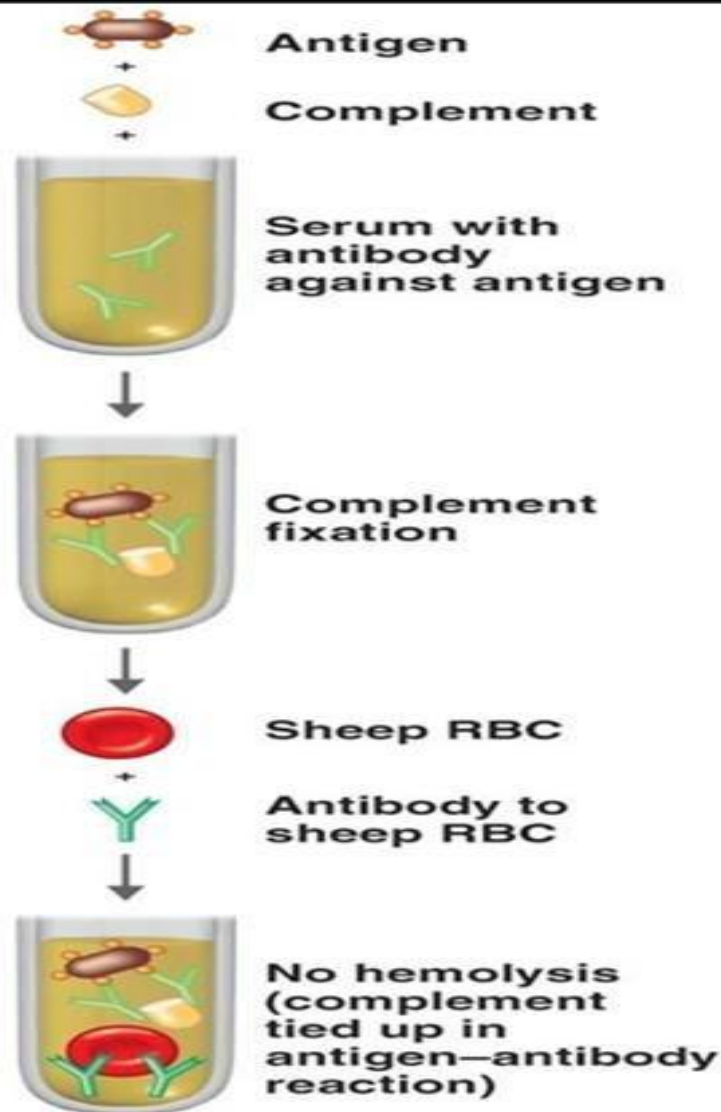
- Explain to students the reactions that occur in the presence of complement and labeled antibodies. To acquaint them with the nature, mechanism and reaction techniques of CFT, IFR, IFA, RIM, as well as immunoblotting reactions and the importance of these reactions in the diagnosis of infectious diseases. To inform students about molecular genetic methods (PCR, molecular hybridization, restriction analysis, sequencing method) and explain their application, importance and mechanisms in diagnosis.

Complement fixation test (CFT)

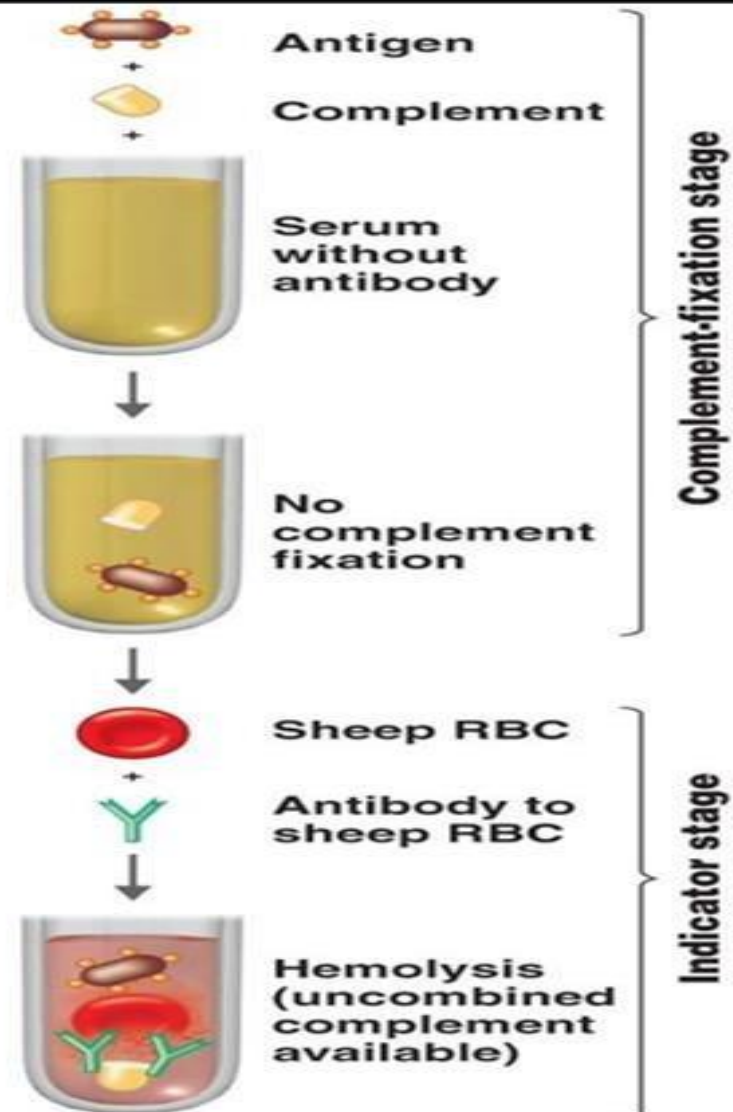
- Formation of antigen-antibody complex in this reaction is determined based on combination of complement.
- If the antigen-antibody complex is formed, i.e. when the reaction is positive, the complement binds to this complex by means of the Fc-fragment of the antibody, in other words, the fusion of the complement with the antigen-antibody complex occurs.
- If the antigen-antibody complex is not formed, then the complement remains free. Thus, it is possible to evaluate the result of the reaction based on whether the complement is combined or remains free.

CFT (principle of reaction)

- CFT, being a complex reaction, essentially consists of two serological reactions. One of these reactions is the main reaction, and the other is an indicator reaction to reveal whether the complement is combined or free.
- Appropriate antigen and complement are added to the patient's inactivated and diluted blood serum.
- After incubation, a hemolytic system is added to detect free complement in the mixture.
- Hemolytic system consists of sheep erythrocytes and antibodies against them. When complement is added, hemolysis occurs in this system.
- When the reaction is positive, the formed antigen-antibody complex binds the complement, as a result, hemolysis does not occur in the hemolytic system, since there is no complement in the mixture.
- When the reaction is negative, complement remains free, so hemolysis is observed in the hemolytic system.



(a) Positive test. All available complement is fixed by the antigen-antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

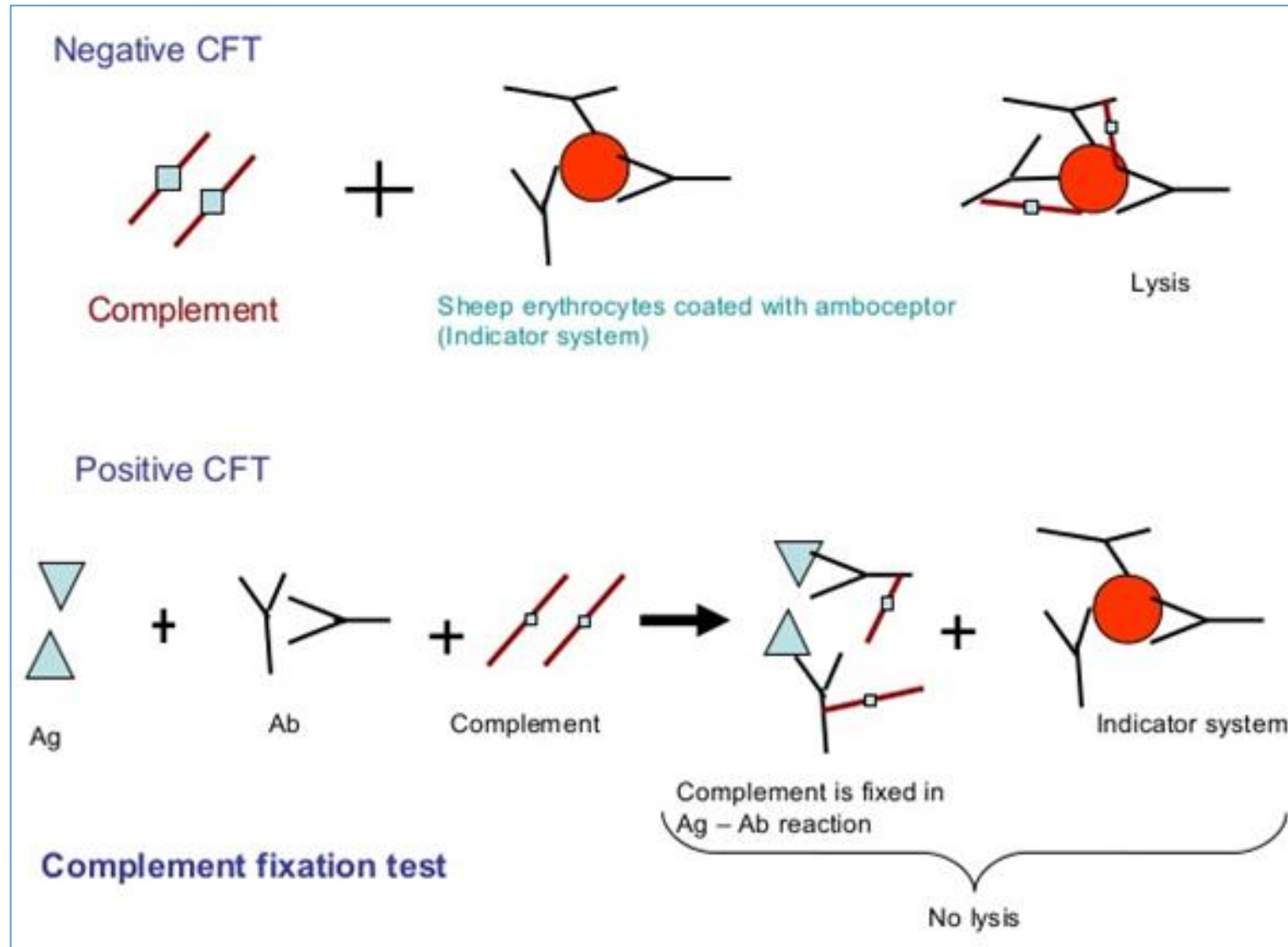


(b) Negative test. No antigen-antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

Complement-fixation stage

Indicator stage

CFT (principle of reaction)



CFT (application)

- CFT is used to detect complement-binding antibodies in blood serum during infectious diseases (toxoplasmosis, viral infections, etc.).
- CFT is distinguished by its high specificity and sensitivity. To set up the reaction, the relative amount of all the ingredients (components) - the tested serum, antigen, complement and hemolytic serum - must be determined.

CFT (ingredients)

- Sheep erythrocyte suspension is obtained from defibrinized sheep blood. For this, sheep blood erythrocytes are washed by centrifugation several times with a physiological solution, then they prepare a 3% suspension of the obtained erythrocytes in a physiological solution.
- Hemolytic serum is usually obtained commercially. In special institutions, rabbits are immunized 3-5 times intravenously (hyperimmunized) with a 50% suspension of sheep erythrocytes. Serum obtained from rabbits was heated at 56°C for 30 min. they inactivate by heating (inactivation of complement in serum). Such sera are released in vials, on which the titer of the serum is recorded, that is, its minimum dilution that completely lyses a 3% suspension of sheep erythrocytes in the presence of complement at 37°C for 1 hour. As a working dose for CFT, they take hemolytic serum in a 3-fold excess titer. For example, if the hemolytic serum titer is 1:1200, it is used at a 1:400 dilution for CFT.

CFT (ingredients)

- Hemolytic system. It consists of equal volumes of hemolytic serum and a 3% suspension of sheep erythrocytes.
- Hemolytic serum is taken in a 3-fold excess titer. For erythrocyte sensitization, this mixture is heated at 37°C for 30 min. stored in a thermostat.
- Since there is no complement in the hemolytic system, hemolysis is not observed here. The addition of complement causes hemolysis in the hemolytic system, that is, a hemolysis reaction occurs.

CFT (ingredients)

- Dried or fresh blood serum of guinea pig is used as complement.
- To determine the titer and working dose of complement, it is titrated through the hemolysis reaction. For this, guinea pig blood serum is diluted with physiological solution at a ratio of 1:10. Then, from this dilution, add 0.05 ml to 0.5 ml to several test bottles in different volumes (0.05 ml, 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml) is added. The volume of the solution in each test bottle is brought up to 1.5 ml with physiological solution. 45 min at 37°C. After incubation, 0.5 ml of hemolytic system is added to each test tube.
- Again 30 min. After incubation, the titer of complement is determined - its smallest amount that causes complete hemolysis.
- A working dose of complement is used for CFT. The working dose of complement is 20-30% higher than its titer. For example, if the complement titer is 0.3 ml, the working dose for CFT will be approximately 0.4 ml.

CFT (ingredients)

























- Antigen. Antigens for CFT are obtained from microorganisms, their lysates, tissue extracts, etc. in special institutions. they prepare. In KBR, these antigens are applied in a working dose.
- Blood serum of the patient. Immediately before the reaction, the patient's blood serum was heated at 56°C for 30 min. they inactivate by heating (inactivation of complement in serum).

CFT (result)

- The CFT result is evaluated by noting the presence or absence of hemolysis in each test tube.
- The reaction is considered positive in the absence of hemolysis, when the liquid in the test tube becomes colorless due to the sedimentation of erythrocytes.
- If the reaction is negative, the lysis of erythrocytes is observed, as a result of which the solution in the test tube acquires a red color of varying intensity.
- The degree of hemolysis is evaluated depending on the intensity of staining of the liquid and the amount of sedimentation of erythrocytes (++++, +++, ++, +).
- The dilution of the serum in the last test tube in which hemolysis was not observed is taken as the titer of the complement fixation test.



CFT in microplate (result)

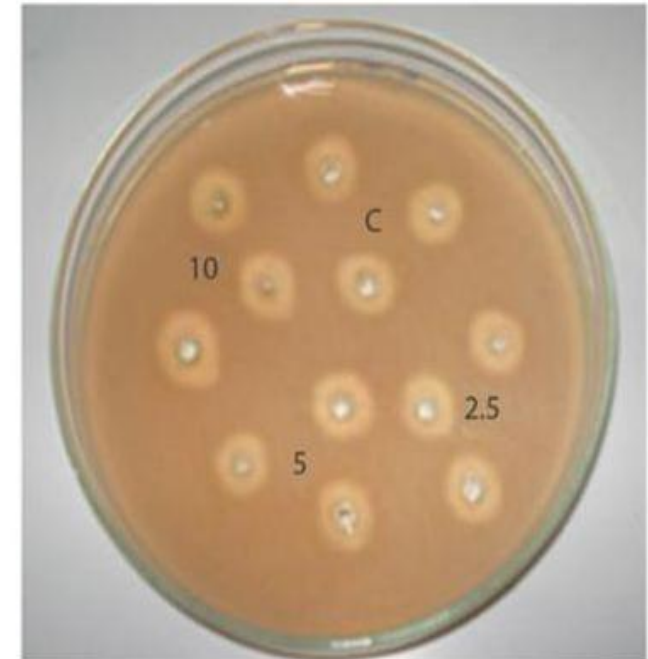
	1/16	1/32	1/64	1/128	1/256	Control serum	Titer
Active Acute							Titer <16
Recovering							Titer ≥256
Active Acute							Titer <16
Recovering							Titer =128

CFT in test tubes (result)



Radial hemolysis reaction (RHR)

- Combination of antigens adsorbed to erythrocytes with corresponding antibodies causes activation of complement and lysis of erythrocytes.
- Adsorbed erythrocytes with appropriate antigens and complement are mixed with melted agar gel cooled to 40°C. Agar is poured onto a glass plate, after solidification, the patient's blood serum is added to the wells opened here.
- When the reaction is positive, a radial hemolysis zone is formed around the cells as a result of agar diffusion of antibodies. Through this reaction, many viral diseases - flu, measles, tick-borne encephalitis, etc. it is possible to determine specific antibodies in the blood serum of patients during

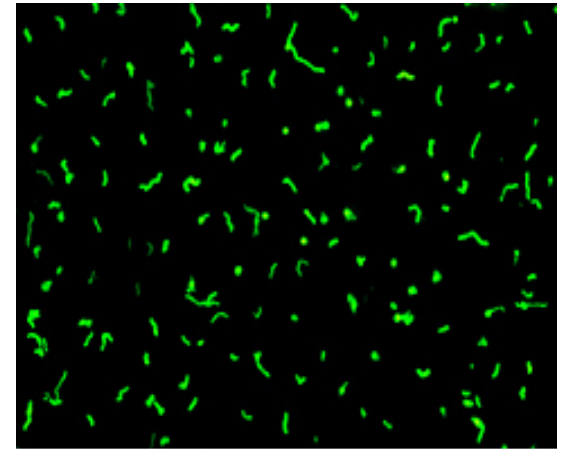


Reactions involving labeled antibodies and antigens

- To determine the antigen-antibody complex formed in serological reactions, in some cases, antibodies or antigens involved in these reactions are pre-marked.
- Fluorochromes, enzymes and radioactive isotopes are used for targeting. Targeting is based on the chemical combination (conjugation) of antibodies or antigens with the indicated agents.
- Different reactions are distinguished depending on the type of agent used for labeling.

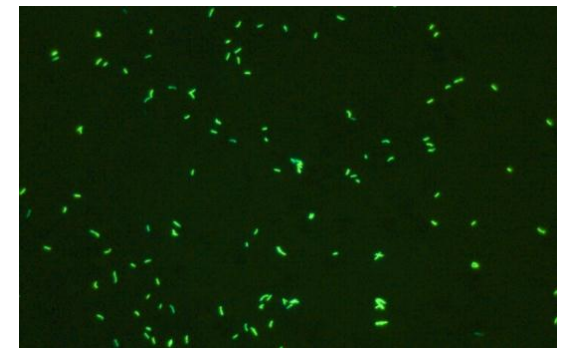
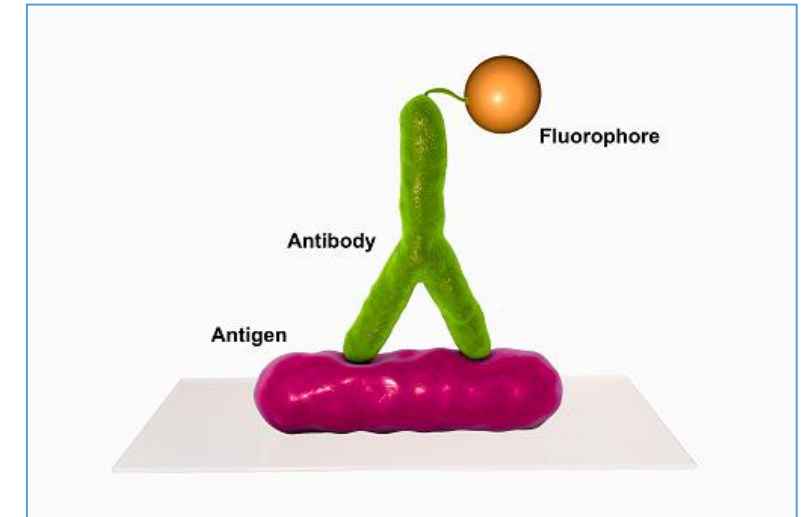
Immunofluorescence reaction (IFR)

- In this reaction called Kuhn's method, known antibodies are labeled with substances called fluorochromes (for example, isothiocyanate).
- These substances emit yellow-green light under the influence of ultraviolet rays. The immunological specificity of antibodies is preserved even after labeling and they can react with corresponding antigens. The resulting antigen-antibody complex can be easily determined under a luminescence microscope.
- Kuhn's reaction is an expression-diagnostic method used to detect microbial antigens or antibodies. There are several variants of the method.



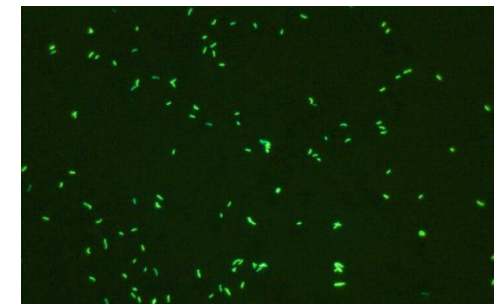
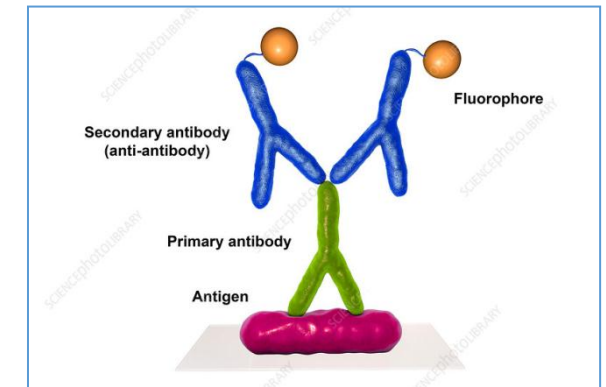
IFR (direct)

- The direct variant is used in smears treated with fluorochrome-labeled antibodies, histological preparations, etc. based on the detection of microorganisms.
- After chemically fixing the prepared smear, fluorescent serum is added to it, and after a certain period of incubation, they are carefully washed.
- When the reaction is positive, the formed antigen-antibody complex is observed under a luminescence microscope as a green light.
- When the reaction is negative, no fluorescence is observed because the labeled antibodies are removed by washing.



Indirect variant (indirect immunofluorescence reaction - IIFR)

- The direct version of IFR requires the presence of antibodies labeled against each microorganism. Therefore, the indirect version of the reaction is used more often.
- This variant is based on the detection of the antigen-antibody complex with the help of antiglobulin antibodies labeled with fluorochrome.
- They add diagnostic rabbit serum to the fixed smear and wash it carefully after incubation for a certain period of time. Then, antiglobulin (against rabbit globulin) serum labeled with fluorochrome is added to the smear and washed again after incubation for a certain period of time.
- When the reaction is positive, the formed complex (microorganism-antibody-fluorochrome-labeled antiglobulin complex) is detected under a luminescent microscope.



Enzyme linked immunosorbent assay (ELISA)

- ELISA is based on the detection of the corresponding antigen with the help of antibodies labeled with enzymes. More peroxidase, alkaline phosphatase, etc. enzymes are used.
- The antigen-antibody complex formed after the labeled antibodies combine with the corresponding antigens is determined by the enzyme.
- For this, a substrate (hydrogen peroxide) that can be decomposed by an enzyme (for example, peroxidase) and an indicator (chromogen, for example, 5-aminosalicylic acid, orthophenyldiamine, etc.) are added to determine the substance formed as a result of decomposition (atomic oxygen).
- If the substrate is broken down by an enzyme, the color of the indicator changes, and the intensity of the color change is directly proportional to the amount of the antigen-antibody molecule. The color change formed as a result of the reaction is evaluated by the colorimetric method.
- Solid-phase ELISA, performed by sorption of one of the components of the reaction - antigen or antibody, to a solid carrier, for example, to the well of a polystyrene plate, is more commonly used.

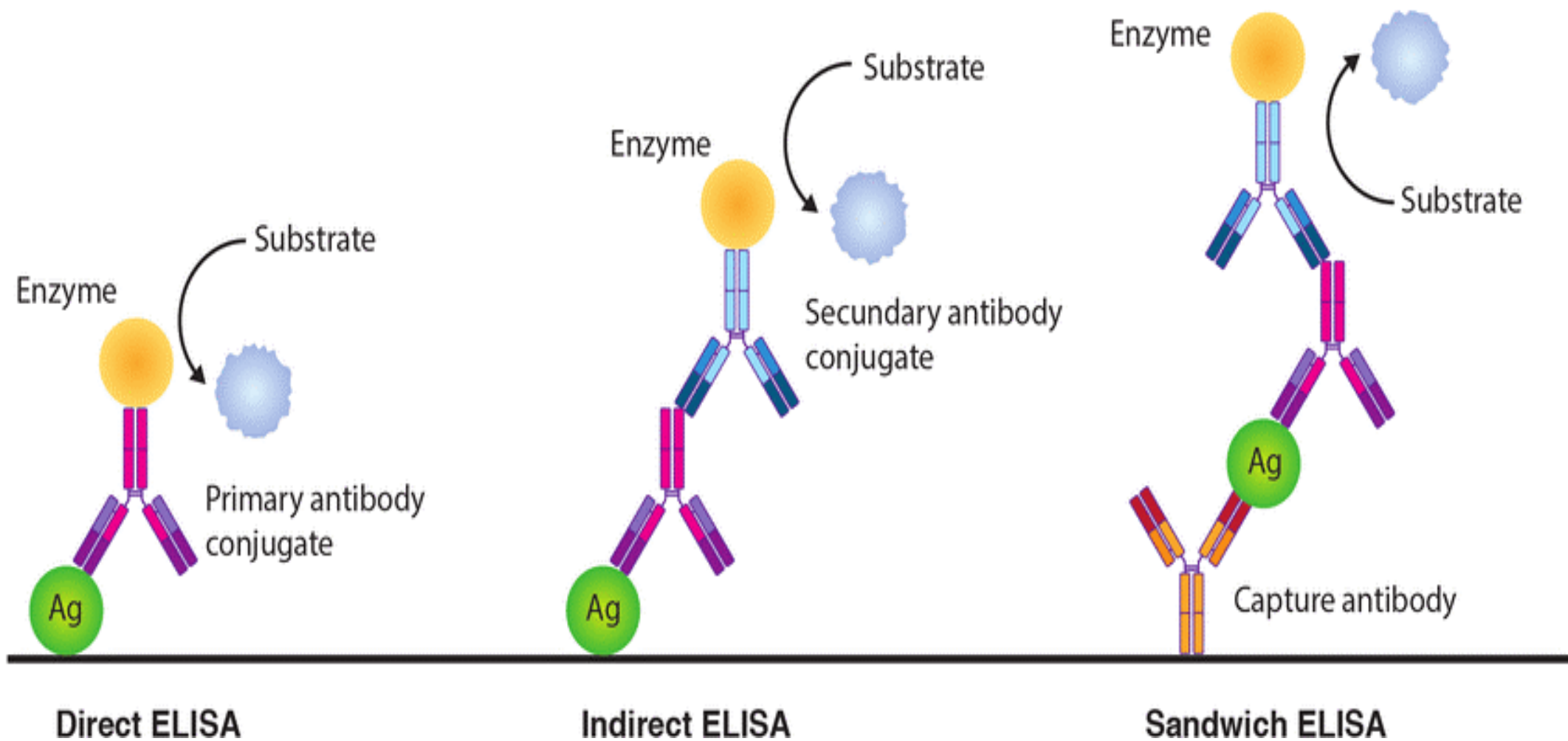
Solid phase ELISA (indirect variant)

- During the solid-phase ELISA, which is used to determine the antibody, a known antigen is adsorbed to the well of the plate.
- The indirect version of the reaction is more applicable. For this purpose, the patient's blood serum, enzyme-labeled antiglobulin serum, substrate to be broken down by the enzyme, and chromogen are sequentially added to the well of the tablet on which the known specific antigen has been sorbed. After each addition of the next component, unbound reagents are removed from the well by washing.
- When the reaction is positive, the antibodies in the patient's blood serum combine with the antigen and stay on the wall of the cell, and the enzyme-labeled antiglobulin also combines the antibodies with itself and stores them here. Finally, the added substrate is broken down by the action of the enzyme, the formed substance changes the color of the indicator (chromogen).

Solid-phase ELISA (Sandwich method)

- During the solid-phase ELISA, which is used to determine the antigen, a known antibody is adsorbed to the well of the tablet.
- The sandwich method is more commonly used. For this purpose, unlabeled specific immune serum against the sought antigen, enzyme-labeled antiglobulin serum, and enzyme-degraded substrate and chromogen are sequentially added to the well of the tablet.
- The further course of the reaction is as shown above.

Scheme of solid-phase ELISA



The technique of ELISA

At present, kits of equipment and reagents used in the diagnosis of each disease are commercially available



The technique of ELISA

- Certain dilutions of the examined material are prepared in a special buffer solution. The material from each dilution is added to two wells of the tablet (known antigen or known antibody has been sorbed into the well of this tablet) and incubated in a thermostat at 37°C for 1-3 hours.
- After incubation, the wells of the tablet are carefully washed with a special buffer solution to remove unbound antigen or antibodies. Then, enzyme-labeled antibody against the desired antigen or enzyme-labeled antiglobulin in a buffer solution of 0.1 ml of the working dilution is added to the wells, incubated in a thermostat at 37°C for 2 hours, washed 3 times with buffer solution to remove uncombined conjugates.
- After that, a mixture of substrate (H_2O_2) and chromogen (for example, orthophenyldiamine) in volume of 0.1 ml is added to the wells, kept in the dark at room temperature for 30 minutes.
- During the incubation process, an enzyme (peroxidase) bound to the wall of the wells decomposes the substrate (H_2O_2) into atomic oxygen, which oxidizes the chromogen and changes its color (a yellow color is formed). Stop reagent (0.1ml 1N H_2SO_4 or 1N NaOH) is added to the wells to stop the substrate decomposition reaction.

ELISA (result)

- It is possible to evaluate the reaction with the naked eye. For this, it is enough to compare the color change of the main experiment with the control. The greatest dilution of the material is noted in which the intensity of the color formed in this dilution is more intense than in the corresponding dilution of the control.
- To evaluate the reaction more accurately, the photoelectrocolorimetric method is used.
- As a positive result, the largest dilution of the examined material is taken so that its extinction level is at least 2 times higher than the extinction level of the corresponding dilution of the control.



ELISA (result)

Currently, microplate photometers (readers) that allow automatic evaluation of the result are widely used



Microplate photometer (STAT FAX®) for automatic evaluation of enzyme immunoassay results

Radioimmune method (RIM)

- High-sensitivity RIM detects microbial antigens, as well as hormones, enzymes, medicinal substances, immunoglobulins, etc. in very small concentrations in examination materials. allows to set.
- However, due to the possibility of radioactive radiation, this method is not widely used.

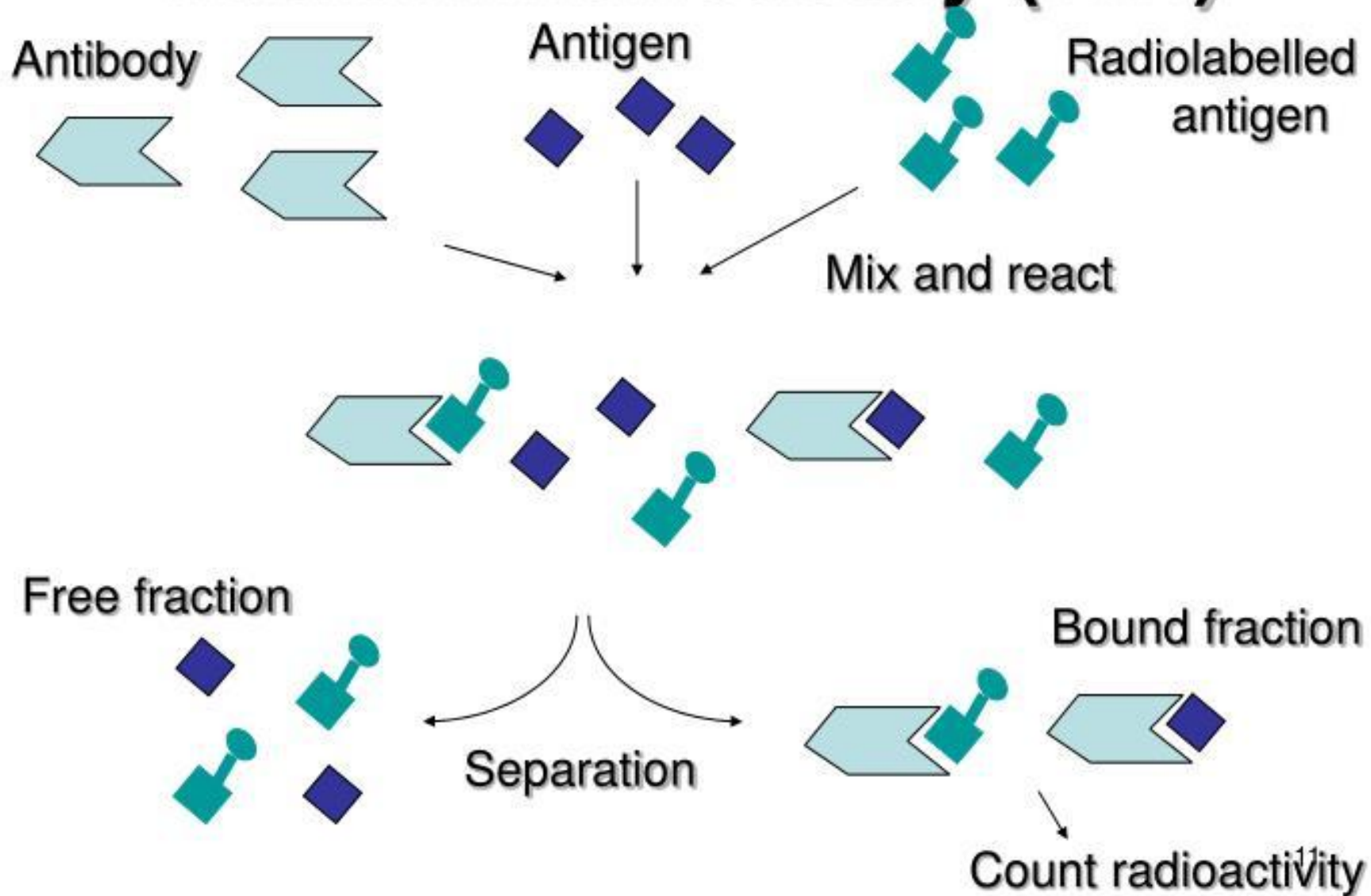
Radioimmune method (RIM)

- RIM is based on the use of antigens or antibodies labeled with radioisotopes, mainly ^{125}I .
- The antigen-antibody complex formed after the isotope-labeled antibodies combine with the corresponding antigens is determined by measuring the radioactivity. The intensity of radioactivity is directly proportional to the amount of the formed antigen-antibody complex.
- Solid-phase RIM performed by adsorbing one of the reaction components - antigen or antibody on a solid carrier, for example, in the well of a polystyrene microplate, is basically similar to IFA. However, the result of the reaction is evaluated not by determining the activity of the enzyme, but by measuring the radioactivity.

Radioimmune method (RIM)

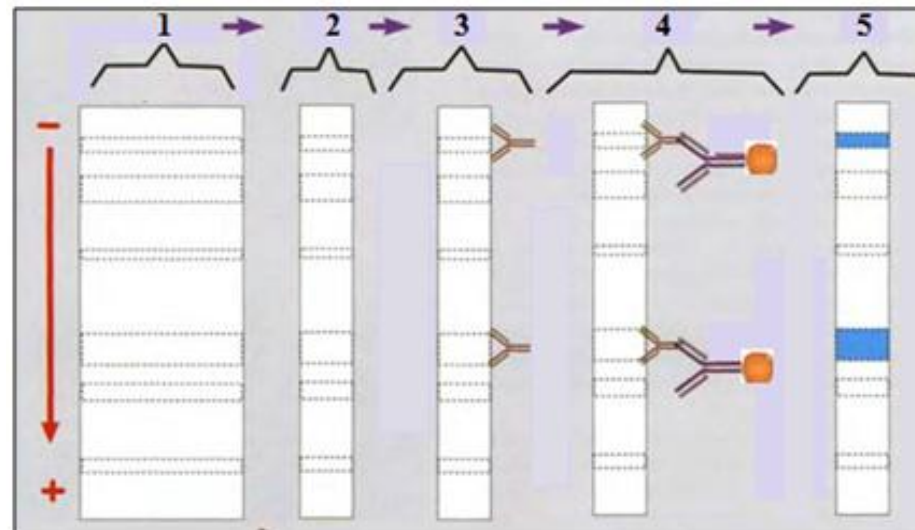
- The competitive version of the method is used to determine the amount of antigen in the examination material.
- For this, a known antibody is adsorbed into the well of a polystyrene microplate. The examined material is added to this well, and after a certain period of incubation, an antigen labeled with a radioactive isotope, having the same specificity as the antigen to be determined, is added.
- If there is a relevant antigen in the test material, it blocks it by combining with antibodies adsorbed on the cell wall. As a result, there is no significant change in the amount of added labeled antigen after the reaction.
- If there is no relevant antigen in the test material, the antibodies adsorbed on the cell wall remain free. Therefore, the amount of labeled antigen that is subsequently added is significantly reduced after the reaction.

Radioimmunoassay (RIA)



Immunoblotting

Immunoblotting (eng. blot - spot) - is based on the detection of antigens previously separated into components by electrophoresis by means of ELISA or RIM. For this, the antigen is separated into its constituent parts by electrophoresis in polyacrylamide gel (1), then it is transferred from the gel to nitrocellulose membrane strips (2). The patient's blood serum is added to such strips (3), after a period of incubation, they are carefully washed to remove unbound antibodies, and an antiglobulin serum labeled with an enzyme against human immunoglobulin is added (4). Since the antibodies in the examined blood serum combine with the antigens on the nitrocellulose membrane strips, an antigen-antibody-labeled antiglobulin complex is formed here. Addition of substrate and chromogen to such bands leads to the formation of colored spots in the corresponding areas (5).



Genetic engineering

- The basis of genetic engineering is the transfer of DNA to prokaryotic and eukaryotic cells after splitting it into nucleotide sequences.
- As a result, such cells - hybrids, having foreign gene fragments, ensure the expression of the transferred gene.
- One of the ultimate goals of genetic engineering is to provide the product or signal encoded by a particular gene in the recipient organism.



Genetic engineering (obtaining a gene)

- For this, first, the gene (DNA molecule) encoding that product or symbol is obtained or synthesized. After that, they break the DNA molecule into fragments using enzymes called restrictase. This enzyme belongs to endonucleases and has the ability to cleave the DNA molecule only in certain places.
- Fragments of the DNA molecule obtained by the action of restriction enzymes are called restrictions. If necessary, it is also possible to connect the ends of the restrictions by means of DNA-ligases.

Genetic engineering (gene transfer)

- DNA fragments are connected to the vector. A vector is an agent that transfers a foreign DNA fragment to a recipient cell.
- Plasmids, phages, or their combination - cosmids and phazmids - are mostly used as vectors.
- Transformation, transfection and microinjection methods are used to transfer recombinant DNA (rDNA) to the recipient cell by means of a vector.

Genetic engineering (gene transfer)

- Through natural transformation, rDNA can be transferred to some strains of *Bacillus subtilis*, *Streptococcus pneumoniae*, and *E.coli*.
- Transfer of rDNA to prokaryotic and eukaryotic cells by phage is called transfection. In some cases, they infect eukaryotic cells with a vector virus. In this case, polyoma and SV-40 viruses are mostly used as vectors.
- Through the microinjection method, they transfer the DNA molecule, as well as the rDNA, to the cultured cells of animals and plants using glass microscopes.
- rDNA can also be transferred to recipient cells by means of liposomes. Liposomes are prepared on the basis of an equal mixture of phosphatidylserine and cholesterol. They sonicate the rDNA and liposome mixture and then incubate it with the recipient cell.

Genetic engineering (obtaining the final product)

- They transfer rDNA to recipient cells called permissive cells. These cells are such cells that the transferred rDNA remains in the composition of that cell without splitting and replication of the vector is possible, in other words, the expression of rDNA is observed.
- E.coli and B.subtilis are used more than prokaryotes and Saccharomyces cerevisiae yeast than eukaryotes in genetic engineering.
- Currently, insulin, somatotropin hormone, interferons, interleukins, etc., based on the expression of the corresponding genes in rDNA superproducers of producing bacteria and yeast strains have been obtained and used in biotechnology.

Modern possibilities of genetic engineering

- It was possible to obtain transgenic animals by microinjection of rDNA into animal germ cells (or egg cells). Due to the presence of certain genes of the donor in the genome of such organisms, they acquire new characteristics.
- Phytopathogenic microorganisms, cold, etc. with the same rule. transgenic plants with resistant properties to the factors were also obtained. Fruit and carrot varieties containing certain "vaccines" were obtained by transferring the genes of immunodominant antigens of some microorganisms to plants.
- One of the recent successes of genetics is the creation of genetic clones, that is, genetic copies. Genetic cloning was first created at the end of the last century by Scottish scientists Jan Wellmuth and Ken Campbell.

Application of genetic methods in diagnostics

- Polymerase chain reaction
- Molecular hybridization method
- Restriction analysis
- Sequencing method

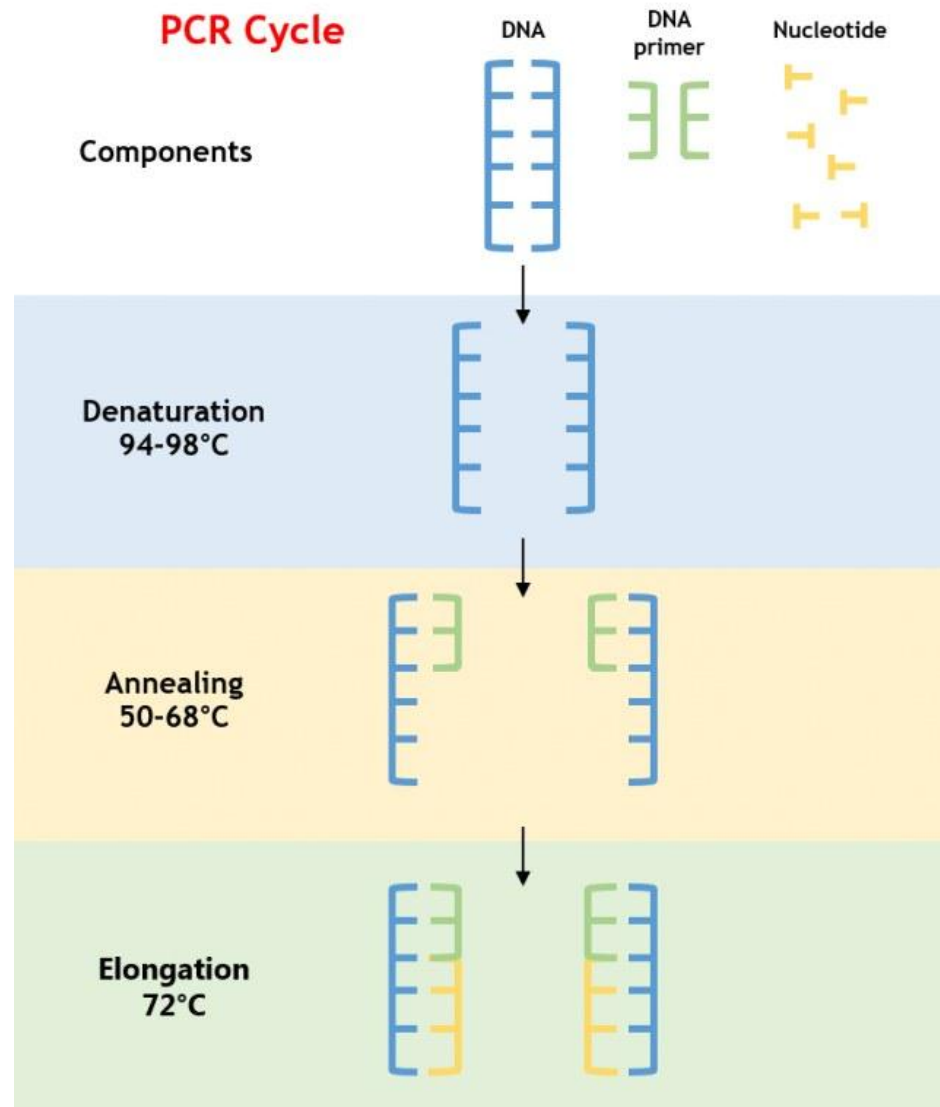
Polymerase chain reaction (PCR)

- it is a method based on the detection of DNA or RNA fragments of the causative agent by multiplying them in the examined materials. Having high sensitivity and specificity, PCR allows to detect even very small parts of DNA or RNA fragments of the causative agent in the examined materials. A special set of equipment is used to conduct ZPR.
- First, the double-stranded DNA-matrix is heated to 92-96°C (or up to 98°C if a thermally stable polymerase is used) for 0.5-2 minutes to separate the DNA strands. This stage is called denaturation, the hydrogen bonds between the DNA strands are broken. Usually, before the first cycle, the reaction mixture is heated for 2-5 min to completely denature the matrix and primers.
- The desired DNA primer and polymerase enzyme are then added, and the primer binds to the DNA strand when it is complementary to it. The indicated cycle is usually repeated 70-80 times. If the test material contains the desired DNA fragments, its amount increases many times due to the formation of new DNA chains (amplification).
- After that, electrophoretic identification of DNA is carried out.

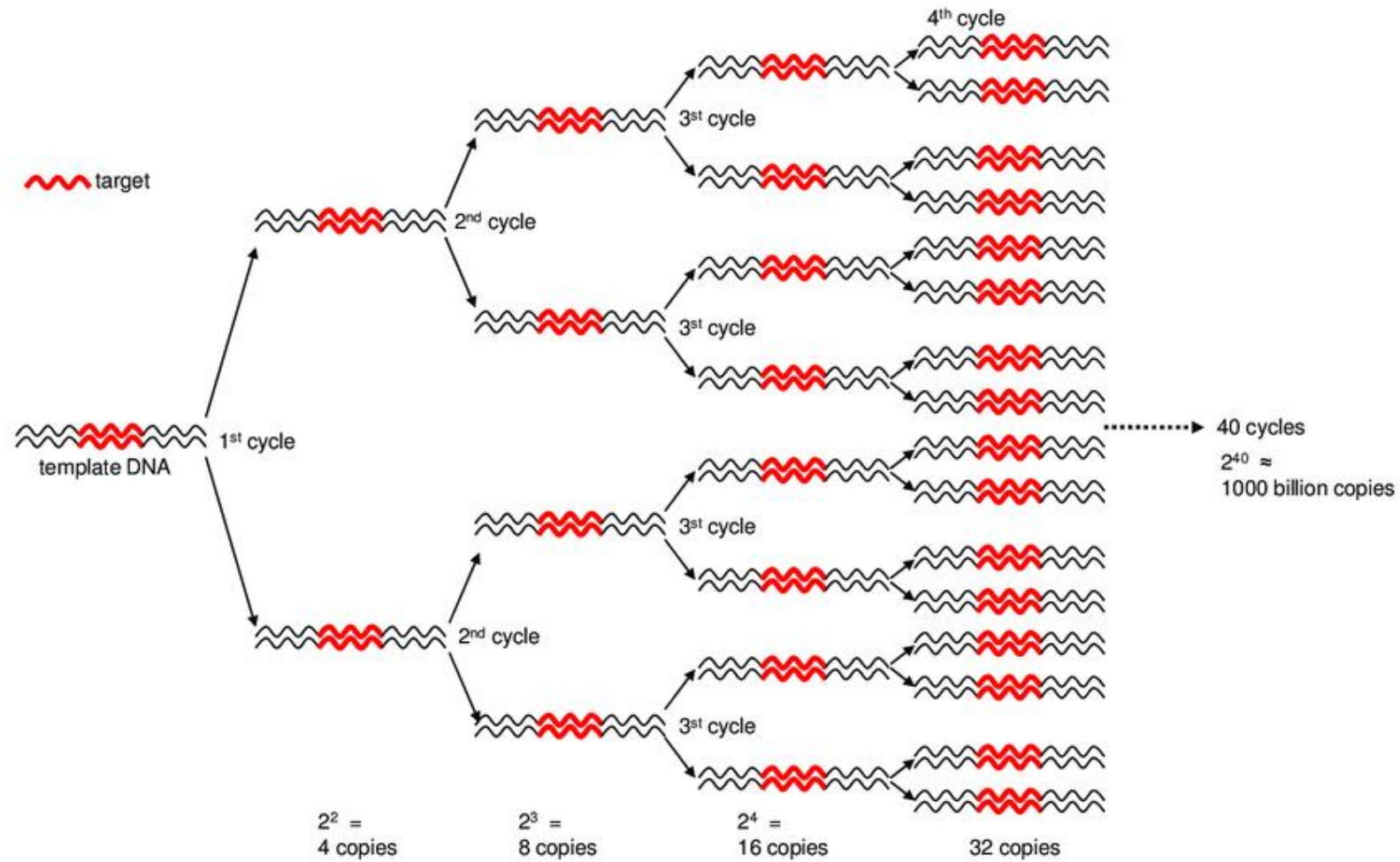
Real-time PCR

- Real-time PCR (real-time PCR) allows detection of accumulation of amplification products directly during amplification.
- Since the kinetics of amplicon collection directly depends on the number of matrix copies under investigation, it allows quantitative measurements of DNA and RNA of the causative agent.
- The obtained information can be used to control the effectiveness of the treatment

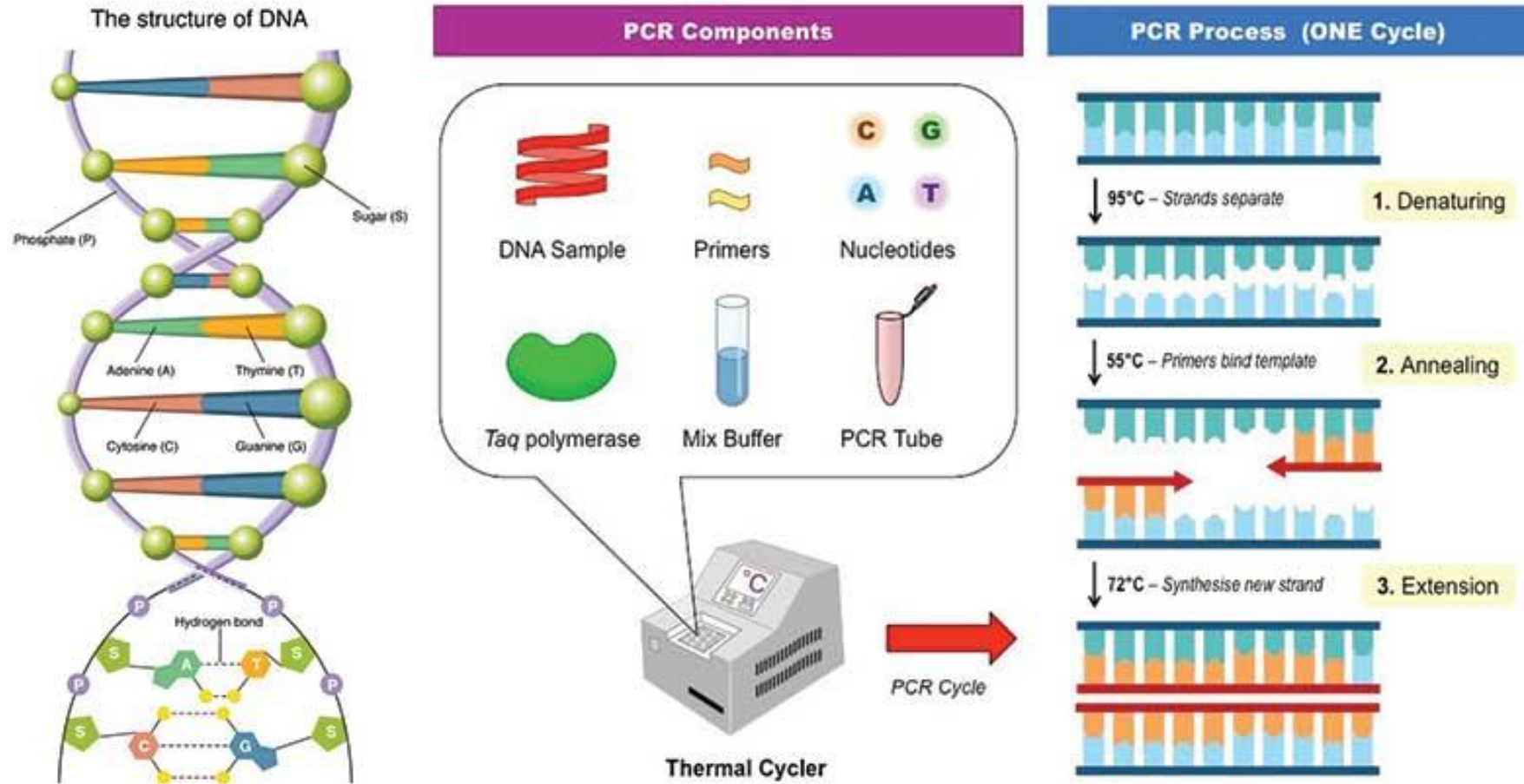
The principle of amplification



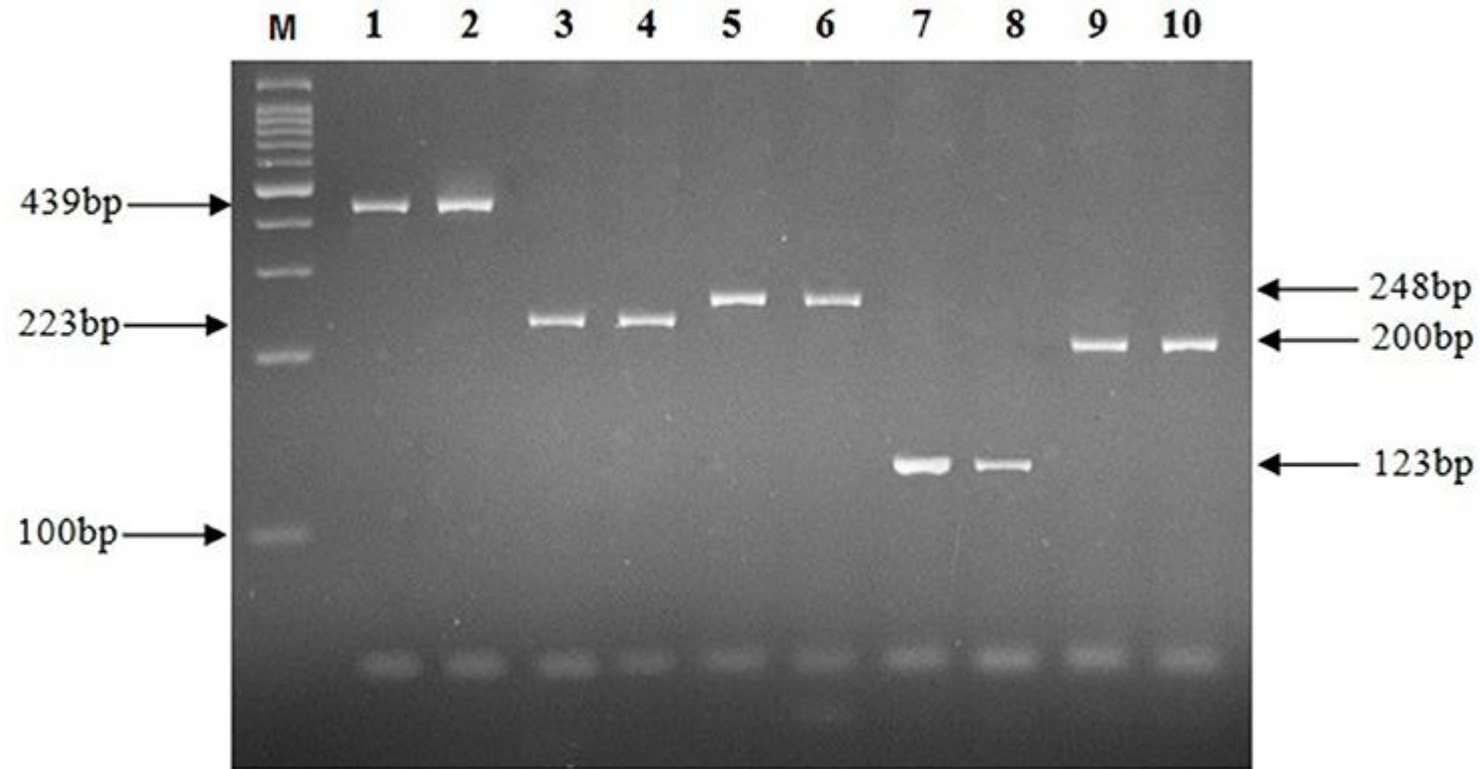
Amplification scheme



Principle of polymerase chain reaction



Electrophoretic identification of DNA (2% agarose gel electrophoresis)



M, Molecular size marker; Lanes 1, 2, 439 bp fragment of hsp65 gene; Lanes 3, 4, 223 bp fragment of mtp40 gene; Lanes 5, 6, 248 bp fragment of IS1081 gene; Lanes 7, 8, 123 bp fragment of IS6110 gene; Lanes 9, 10, 200 bp fragment of mpb64 gene

PCR amplifier device

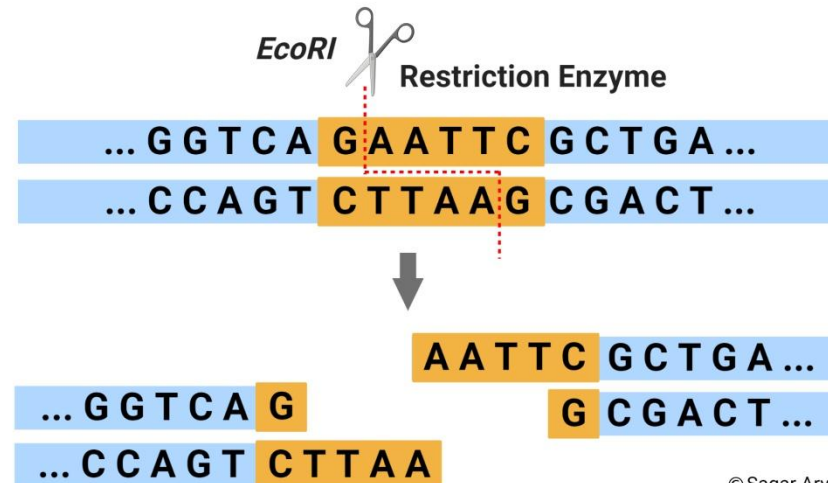


Molecular hybridization method

- In the material examined by this method, nucleic acids are detected by means of probes marked with a radioactive isotope or enzyme.
- A single-stranded DNA or RNA molecule complementary to the nucleic acid molecule being searched is used as a probe.
- If there is a corresponding (complementary) nucleic acid in the test material, they combine (hybridize) and form a double helix.
- Hybridized molecules are detected with the help of radioimmunoassay or immunoenzymatic analysis.

Restriction analysis

- Restriction analysis is mainly used in the identification of microorganisms.
- The principle of the analysis consists of electrophoretic identification of DNA fragments cleaved by restriction enzymes.
- Enzymes called restrictionase belong to endonucleases and have the ability to cleave the DNA molecule only at certain places.



Sequencing method

It allows determining the sequence of nucleotides in the DNA molecule (eng., sequence).

Sanger sequencing is more commonly used. For this purpose, the examined DNA molecule is first broken into fragments of different lengths by means of alkaline hydrolysis.

Different types of labeled dideoxynucleotides (adenine, thymine, guanine and cytosine) are added to the obtained mixture. Targeted dideoxynucleotides combine with complementary nucleotides at the 3'-end of each DNA fragment. Thus, helical DNA fragments of different lengths are labeled differently depending on which nucleotide is present at the 3'-end.

Then the labeled DNA fragments are subjected to electrophoresis in a vertical polyacrylamide gel. At this time, the fragments travel different distances depending on their molecular mass, and then the labeled dideoxynucleotides are arranged according to the sequence of nucleotides in the examined fragment. The electrophoresis process is carried out in automatic special devices with computer analysis - sequencers.