Serology and Immunoassays

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Introduction to Serologic Reactions

- Serology studies in vitro **reactions between antigens and antibodies**, essential for identifying infections.
- The system relies on **immune specificity**. An antibody reacts only with its inducing antigen, allowing for precise identification of pathogens.
- **Cross-reactions** can occur between related antigens limiting a test's specific utility.
- Serology diagnoses **infectious diseases** and **autoimmune disorders**. It is also critical for **blood and tissue typing.**

Major Uses of Serologic Tests

- Tests diagnose infections when organisms are hard or dangerous to culture (syphilis and hepatitis viruses, *Rickettsia)*.
- Serology helps when organisms grow too slowly. It provides answers when cultures would take weeks.
- Tests detect autoimmune antibodies against body components (Anti-DNA antibodies in lupus or rheumatoid factor).
- Known antibodies determine ABO and Rh blood types. They also identify HLA antigens before transplantation.

TABLE 64–1 Major Uses of Serologic (Antibody-Based) Tests

I. Diagnosis of infectious diseases

- When the organism cannot be cultured (e.g., syphilis and hepatitis A, B, and C).
- When the organism is too dangerous to culture (e.g., rickettsial diseases).
- When culture techniques are not readily available (e.g., HIV, EBV).
- When the organism takes too long to grow (e.g., *Mycoplasma*).

One problem with this approach is that it takes time for antibodies to form (e.g., 7–10 days in the primary response). For this reason, acute and convalescent serum samples are taken, and a fourfold or greater rise in antibody titer is required to make a diagnosis. By this time, the patient has often recovered and the diagnosis becomes a retrospective one. If a test is available that can detect IgM antibody in the patient's serum, it can be used to make a diagnosis of current infection. In certain infectious diseases, an arbitrary IgG antibody titer of sufficient magnitude is used to make a diagnosis.

II. Diagnosis of autoimmune diseases

- Antibodies against various normal body components are used (e.g., antibody against DNA in systemic lupus erythematosus, antibody against human IgG [rheumatoid factor] in rheumatoid arthritis).

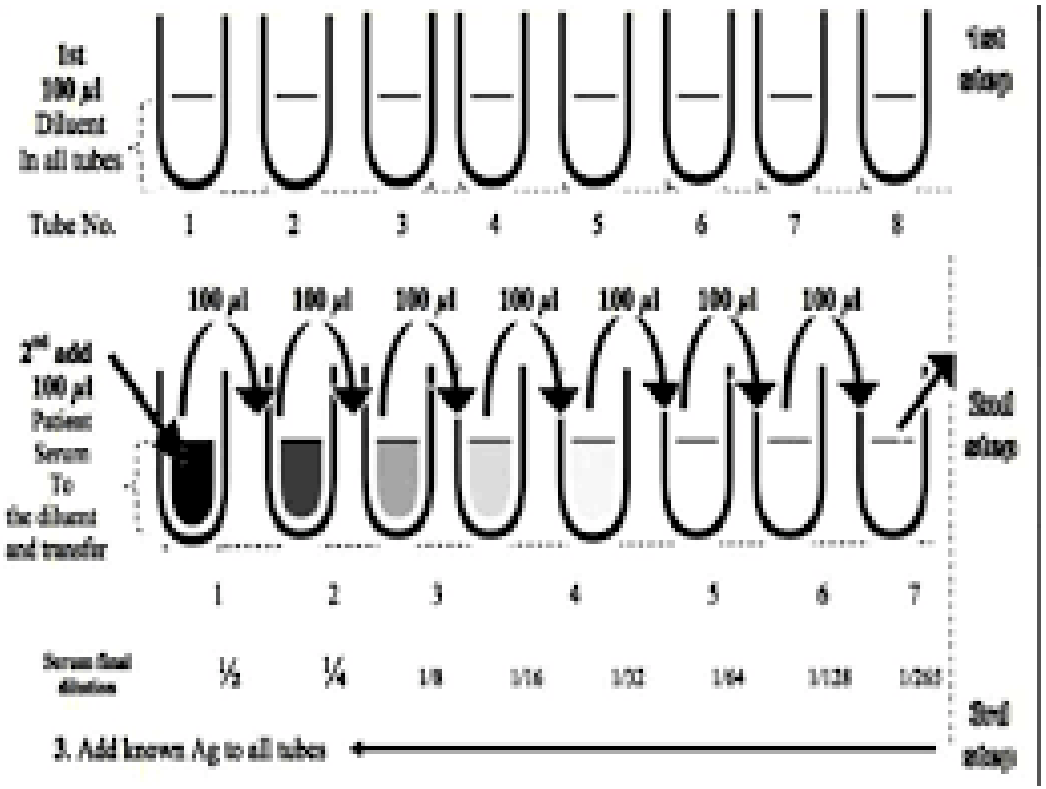
III. Determination of blood type and HLA type

- Known antibodies are used to determine ABO and Rh blood types.
- Known antibodies are used to determine class I and class II HLA proteins prior to transplantation, although DNA sequencing is also being used.

EBV = Epstein–Barr virus; HIV = human immunodeficiency virus; HLA = human leukocyte antigen.

Understanding Titers

- A titer is the highest dilution yielding a positive reaction. It quantifies the antibody concentration in serum. A higher denominator indicates more antibodies.
- A titer of 1/64 implies more antibodies than 1/4. This value estimates the strength of the immune response.
- A fourfold IgG rise between acute and convalescent samples is required.
- High (early) IgM titers suggest a current infection. IgG titers usually indicate past exposure or immunity.

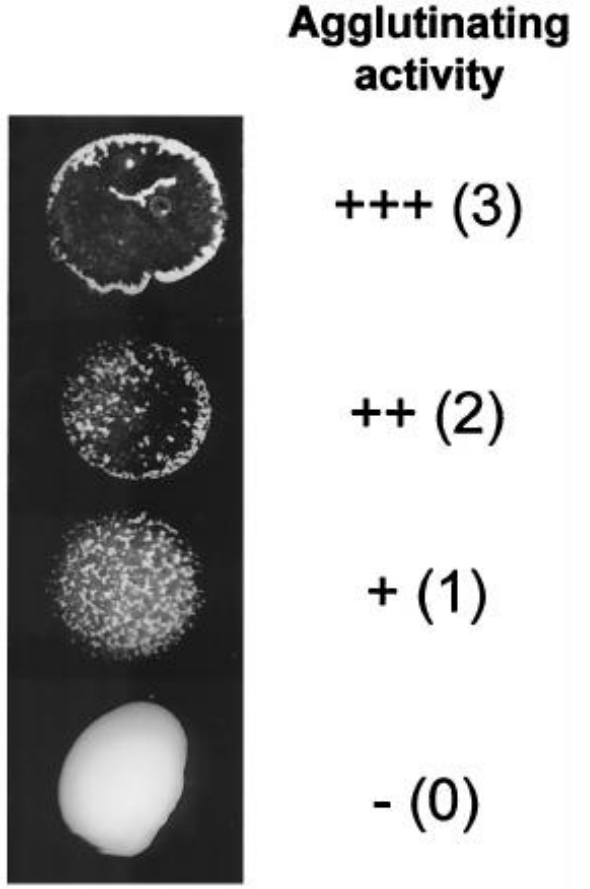


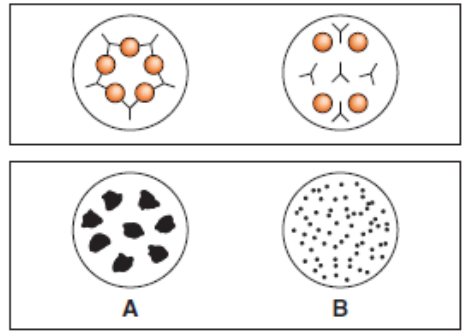
Overview of Diagnostic Test Types

- Labs use different methods based on antigen type. Agglutination tests use particulate antigens. Precipitation tests use soluble antigens.
- RIA and ELISA provide high sensitivity. They quantify minute amounts of antigens or antibodies.
- Immunofluorescence visualizes antigens directly on cells. It uses fluorescently labeled antibodies.
- Complement fixation detects antibody consumption. Western blotting confirms specific protein presence.

Agglutination Reactions

- Agglutination involves particulate antigens like bacteria. It also works with inert particles like latex beads.
- Multivalent antibodies cross-link these particles. This forms a lattice structure. The result is visible clumping on a slide.
- This reaction is simple and rapid. It requires minimal laboratory equipment.





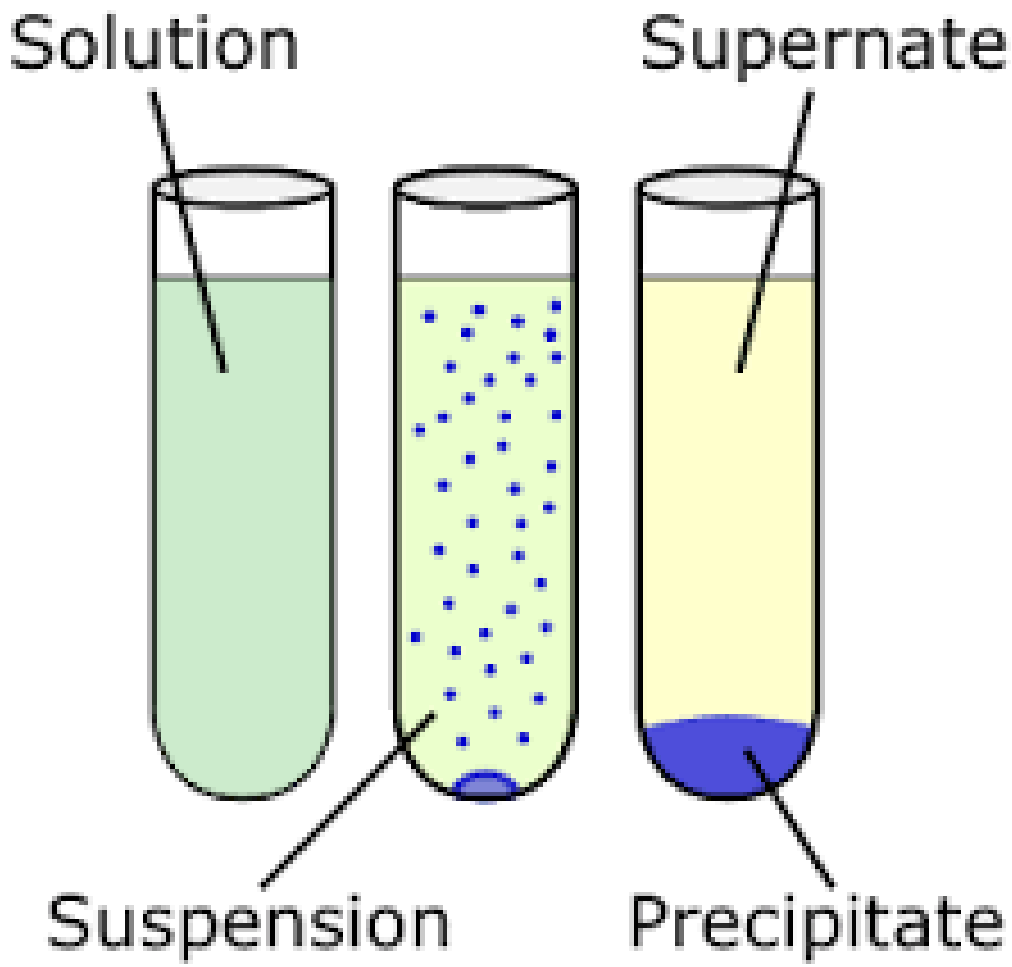
JRE 64–1 Agglutination test to determine ABO blo
On the slide at the bottom of the figure, a drop of the pa
was mixed with antiserum against either type A (**left**) o
) blood cells. Agglutination (clumping) has occurred in t
on the left containing the type A antiserum but not in th
ning the type B antiserum, indicating that the patient is
as A antigen on the red cells). The slide at the top shows
d cells (circles) are cross-linked by the antibodies ("Y" sha
op on the left but not in the drop on the right. If aggluti
curred in the right side as well, it would indicate that th
t was producing B antigen as well as A and was type AB

Clinical Applications of Agglutination

- **ABO blood** typing relies on agglutination. Patient **red blood cells clump** with specific antisera.
- **Latex agglutination** uses antigen-coated beads. This allows for quick detection of pathogens.
- **Hemagglutination** specifically refers to red cell clumping. This is distinct from blood clotting.
- These tests serve as effective screening tools. They provide immediate visual results.

Precipitation Reactions

- **Precipitation** tests use **soluble antigens**. Antibodies cross-link molecules **into large aggregates**. These aggregates fall out of solution.
- Optimal proportions of antigen and antibody are required. This state is called the **zone of equivalence**.
- **Maximal lattice formation occurs in this zone**. The reaction fails if ratios are unbalanced.
- Both **antigen and antibody must be multivalent**. This ensures the formation of a stable lattice.



The Precipitin Curve & Prozone Effect

- The precipitin curve plots reaction efficiency. The **peak represents the zone of equivalence.**
- **Antibody excess** inhibits efficient lattice formation. This is known as **the prozone effect**.
- The prozone effect can cause **false-negative** results. Labs **dilute serum** to fix this issue.
- **Antigen excess** also prevents visible precipitation **(postzone**). The resulting complexes are too small to settle.

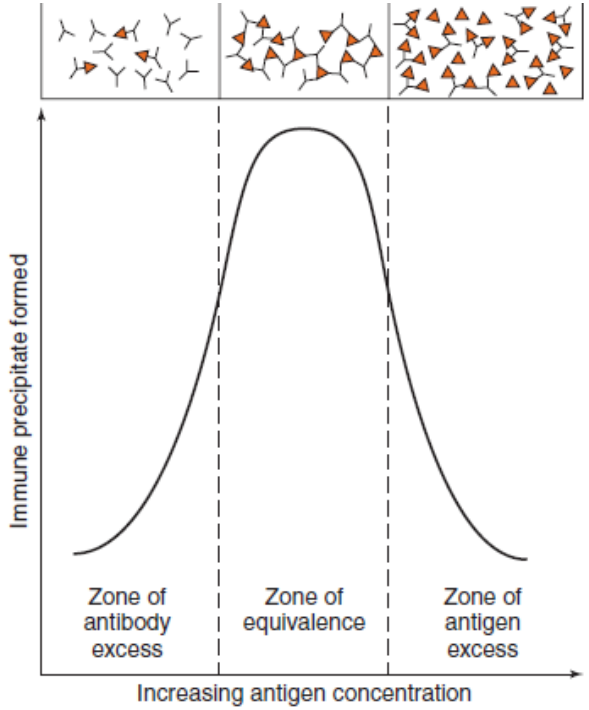


FIGURE 64–2 Precipitin curve. In the presence of a constant amount of antibody, the amount of immune precipitate formed is plotted as a function of increasing amounts of antigen. In the top part of the figure, the binding of antigen (▲) and antibody (Y) in the three zones is depicted. In the zones of antibody excess and antigen excess, a lattice is not formed and precipitation does not occur, whereas in the equivalence zone, a lattice forms and precipitation is maximal. (Reproduced with permission from Stites DP, Terr A, Parslow T, eds. *Basic & Clinical Immunology*. 9th ed. Originally published by Appleton & Lange. Copyright 1997 McGraw-Hill.)

- **Precipitation in Solution**
- Used to measure immunoglobulins in plasma.
- The lab test is called: nephelometry; amount of precipitate is measured by optical density (light refraction).
- The value is compared with a standard curve.

Immunodiffusion (Precipitation in Agar)

- **Single radial diffusion** mixes antibody into agar. Antigen diffuses from a well to form a ring.

Ring size indicates antigen concentration (e.g. IgG, IgM, complement).

- **Double diffusion** (Ouchterlony) involves two diffusing reactants. Antigen and antibody move toward each other.

- Precipitin lines form where concentrations meet. Patterns indicate antigen identity or non-identity.

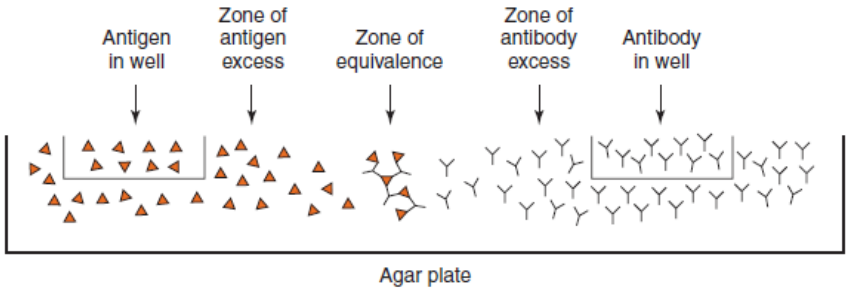


FIGURE 64–3 Double diffusion in agar. Antigen is placed in the well on the left, and antibody is placed in the well on the right. The antigen and antibody diffuse through the agar and form a precipitate in the zone of equivalence. Close to the antigen-containing well is the zone of antigen excess, and close to the antibody-containing well is the zone of antibody excess. No precipitate forms in the zones of antigen and antibody excess.

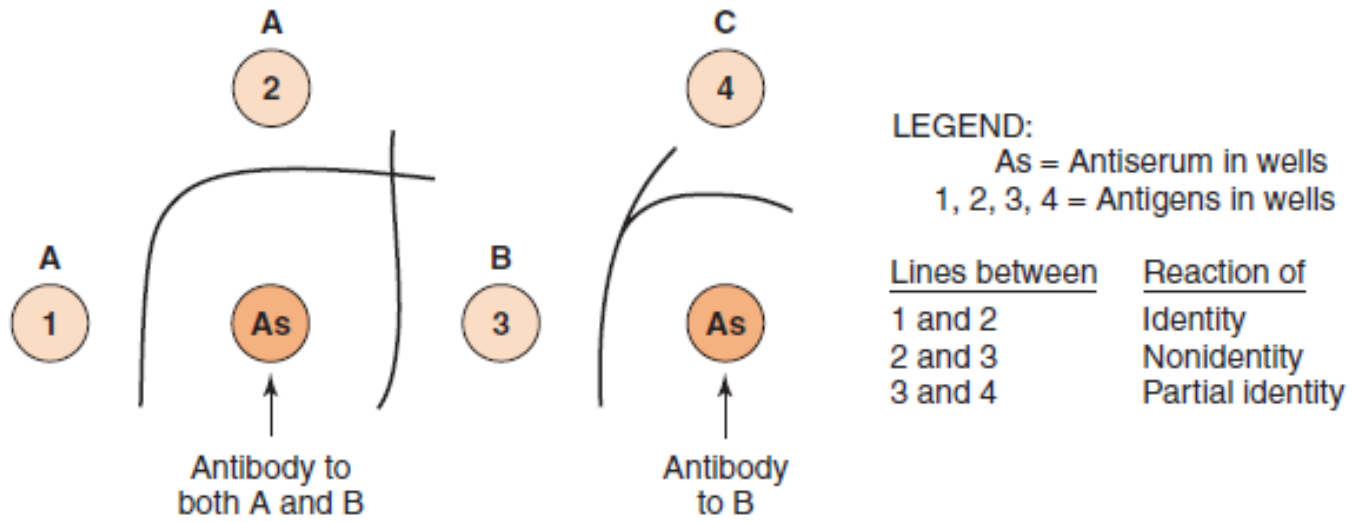


FIGURE 64–4 Double-diffusion (Ouchterlony) precipitin reactions. In these Ouchterlony reactions, wells are cut into an agar plate and various antigens and antisera are placed in the wells. The antigens and antibodies diffuse toward each other within the agar, and a line of precipitate forms in the zone of equivalence. Close to the antigen-containing well, a zone of antigen excess exists and no precipitate forms; close to the antibody-containing well, a zone of antibody excess exists and no precipitate forms. A and B are unrelated antigens (i.e., they have no epitopes in common). B and C are related antigens (i.e., they have some epitopes in common but some that are different). For example, chicken lysozyme (well B) and duck lysozyme (well C) share some epitopes because they are both lysozymes but have unique epitopes as well because they are from different species. The line of identity between B and C is caused by the reaction of the anti-B antibody with the shared epitopes on antigens B and C. The spur pointing toward well 4 is caused by the reaction of some of the anti-B antibody with the unique epitopes on antigen B in well 3. These lines of partial identity occur because antibody to B (chicken lysozyme) is polyclonal and has some immunoglobulins that react with the epitopes common to chicken and duck lysozyme and other immunoglobulins that react only with the epitopes unique to chicken lysozyme.

- **Immunoelectrophoresis**
- Combines electrophoresis with immunodiffusion. **Serum proteins are first separated by charge** (electrical current).
- Antibody then diffuses to form precipitin arcs. The shape of arcs reveals specific proteins.
- It detects abnormal globulin production (e.g.myeloma proteins).
- **Counter-immunoelectrophoresis** uses electricity. It **drives antigen and antibody** together rapidly.

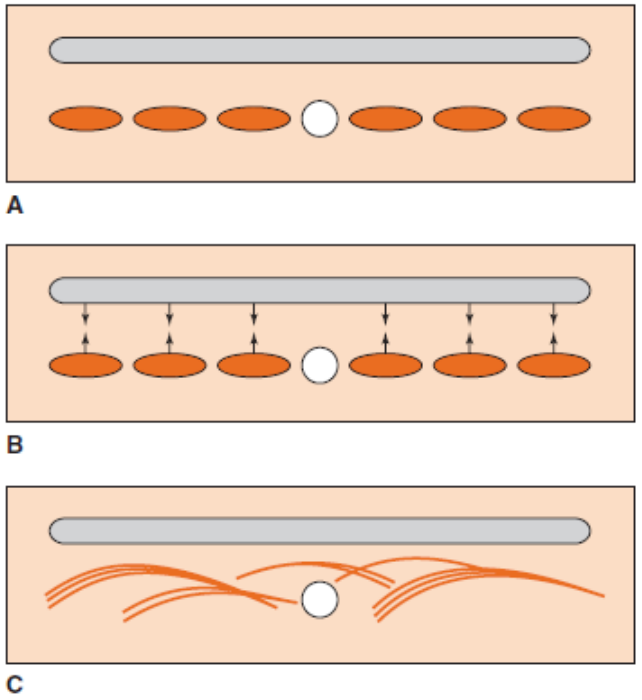
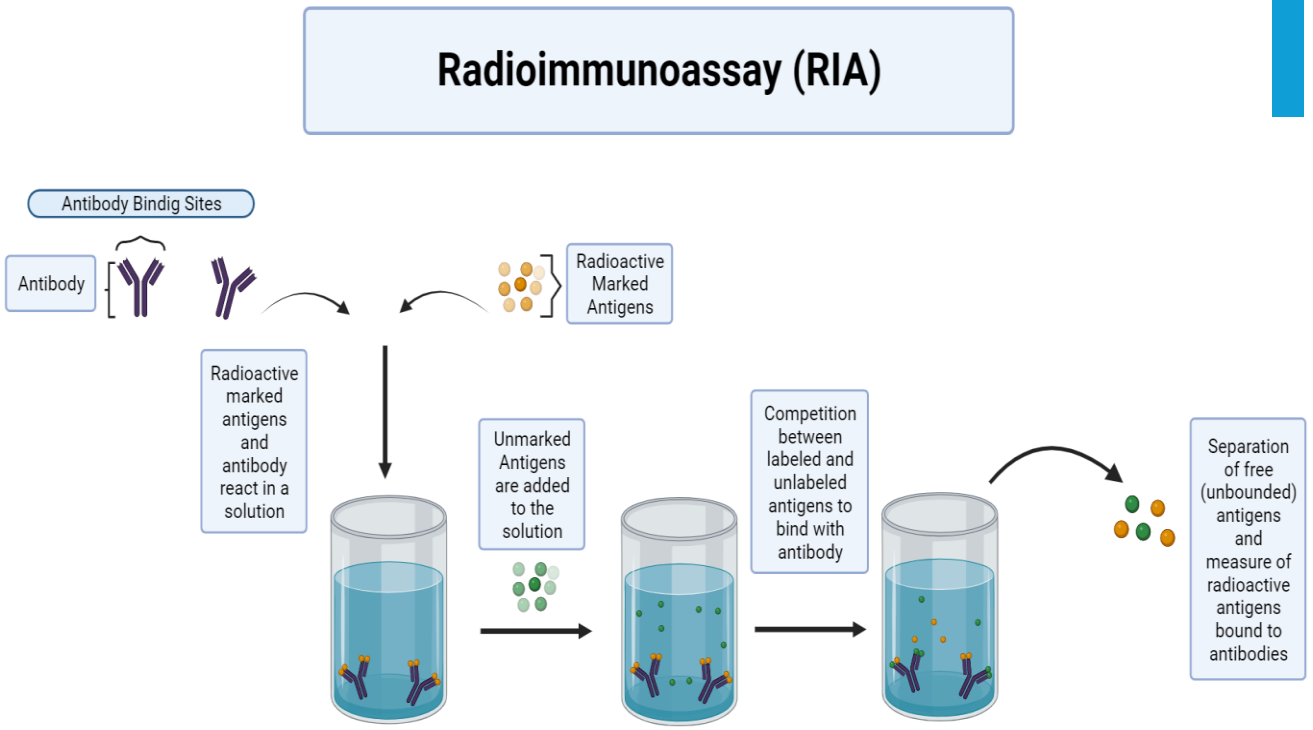


FIGURE 64–5 Immunoelectrophoresis. **A:** Human serum placed in the central well is electrophoresed, and the proteins migrate to different regions (orange ellipses). Antiserum to human serum is then placed in the elongated trough (gray areas). **B:** Human serum proteins and antibodies diffuse into agar. **C:** Precipitate arcs (orange lines) form in the agar. (Reproduced with permission from Stites DP, Terr A, Parslow T, eds. *Basic & Clinical Immunology*. 9th ed. Originally published by Appleton & Lange. Copyright 1997 McGraw-Hill.)

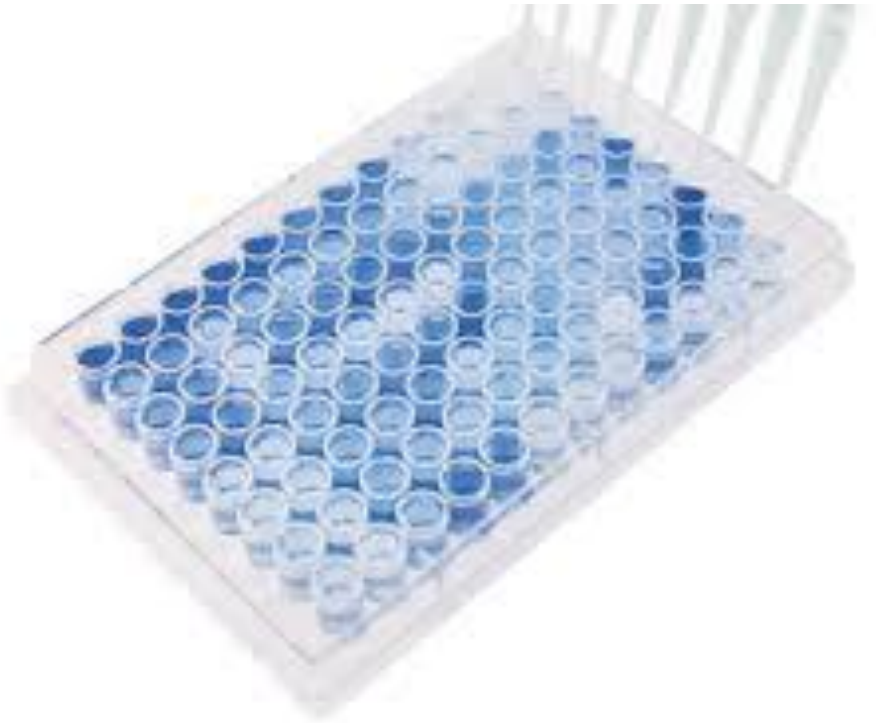
Radioimmunoassay (RIA)

- RIA is a **highly sensitive** quantitative method. It measures antigens in low concentrations.
- Radio-labeled antigens compete with patient antigens for antibody binding sites.
- **Less radioactivity** in the complex means **more patient** antigen. The relationship **is inversely** proportional.
- RIA measures hormones and drugs. The radioallergosorbent test **RAST** is a specialized RIA for IgE antibodies.



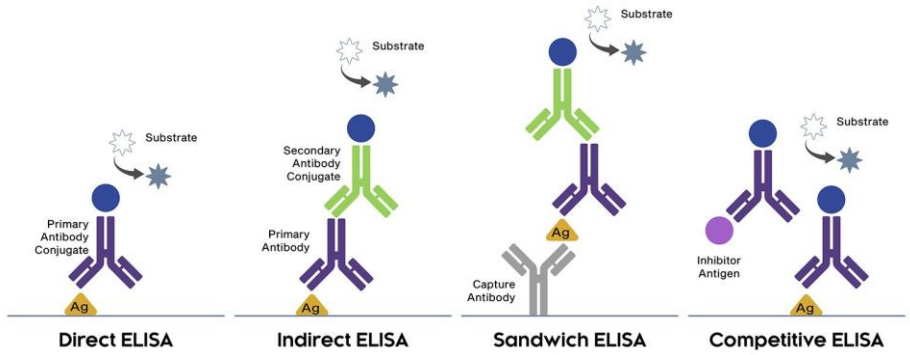
Enzyme-Linked Immunosorbent Assay (ELISA)

- ELISA quantifies antibodies without radiation. It uses **enzyme-linked conjugates** instead.
- The method is as sensitive as RIA. It requires no radioactive waste handling.
- Enzymes convert **a substrate into a colored product**, measured by a spectrophotometer (optical density-absorbance).
- A standard tool for screening, safe and widely available.



ELISA Methodology

- A known antigen is attached to a surface. Patient serum is added to the well.
- Patient antibodies bind to the fixed antigen. An enzyme-linked anti-human IgG is then added.
- This secondary antibody binds the patient's antibody. A substrate is added to react with the enzyme.
- The resulting color indicates the antibody level. Intensity correlates with the amount of patient antibody.



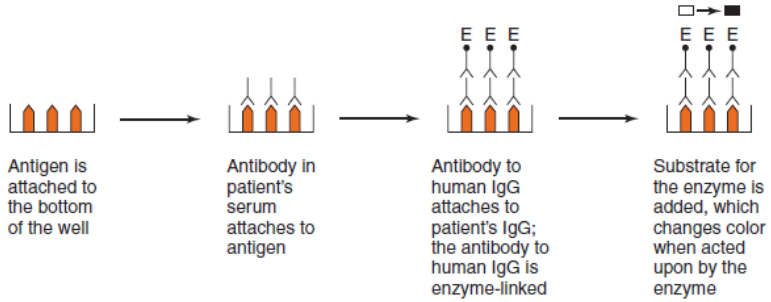


FIGURE 64–6 Enzyme-linked immunosorbent assay (ELISA). The term enzyme-linked refers to the covalent binding (linking) of an enzyme to antibody to human IgG. If the patient has antibodies to the microbial or viral antigen, those antibodies will bind to the microbial or viral antigens. The antibody to human IgG linked to the enzyme will then bind to the patient's antibodies. Then when the substrate of the enzyme is added, the substrate changes color, indicating that the patient's serum contained antibodies.

Western Blot (Immunoblot)

- Western Blot **confirms** positive screening results. .
- Viral proteins **are separated by electrophoresis**. They are **transferred onto filter paper**.
- Patient **serum is applied to the paper**. Antibodies bind to specific protein bands.
- **Enzyme-labeled anti-IgG** visualizes the bands. This confirms specific antibodies are present.

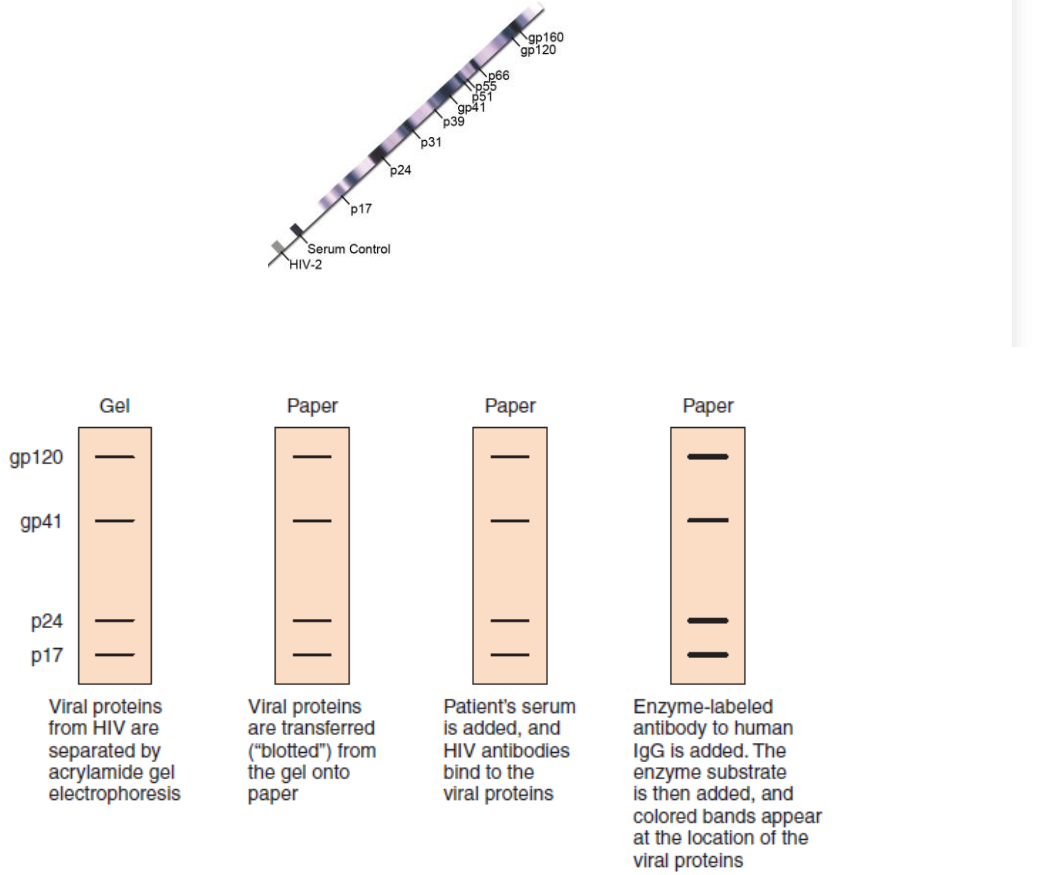
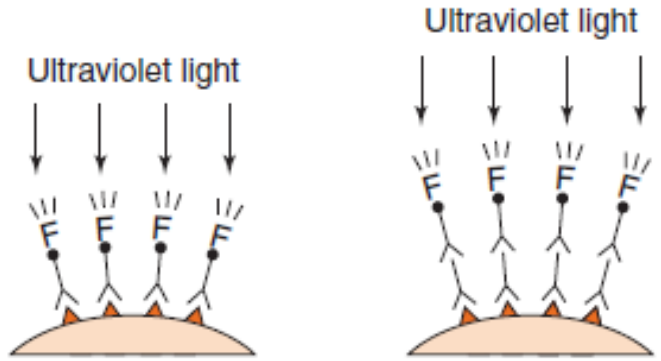


FIGURE 64–9 Western blot (immunoblot test). In this test, microbial or viral proteins are separated on an acrylamide gel and then transferred (blotted) onto paper. The patient's serum then interacts with the separated proteins. If antibodies are present in the patient's serum, they bind to the proteins. The patient's antibodies are then detected by using labeled antibody to human IgG.

Immunofluorescence (Direct)

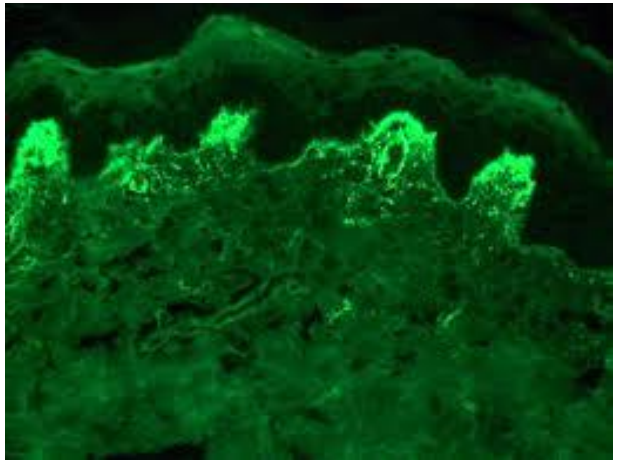
- This method uses **fluorescent dyes on antibodies**. Dyes like fluorescein glow under **UV light**.
- Labeled antibodies are applied directly to tissue. They bind to specific antigens if present.
- The reaction is viewed with a **fluorescence** microscope.
- This is a "one-step" rapid diagnosis. It detects antigens in biopsy specimens.



A. Direct fluorescent-antibody test

B. Indirect fluorescent-antibody test

FIGURE 64–7 Fluorescent antibody test. **A:** In the direct fluorescent antibody test, the fluorescent dye is attached directly to the antibody that is interacting with the antigen (dark triangles) on the surface of the cell. **B:** In the indirect fluorescent antibody test, the fluorescent dye is attached to antibody made against human IgG.



Immunofluorescence (Indirect)

- Indirect immunofluorescence is a two-stage test. **Patient serum reacts with a fixed (known) antigen first**.
- A **labeled anti-human IgG is added** second. This binds to the patient's antibody.
- This method is more sensitive than the direct test.

Complement Fixation Test

- Ag-Ab complexes fix (consume) complement (test principle).
- Patient serum is mixed with known antigen and complement. If antibodies are present, complement is fixed.
- If antibodies are absent, complement remains free. The second stage tests for this free complement.
- Endogenous serum complement must be inactivated first. This is done by heating the serum.

Interpreting Complement Fixation

- **Sensitized red blood cells are the indicator**. They lyse in the presence of free complement.
- **No hemolysis** indicates a **positive result**. It means complement was consumed by patient antibodies.
- **Hemolysis indicates** a **negative result**. It means complement was left free to attack the RBCs.

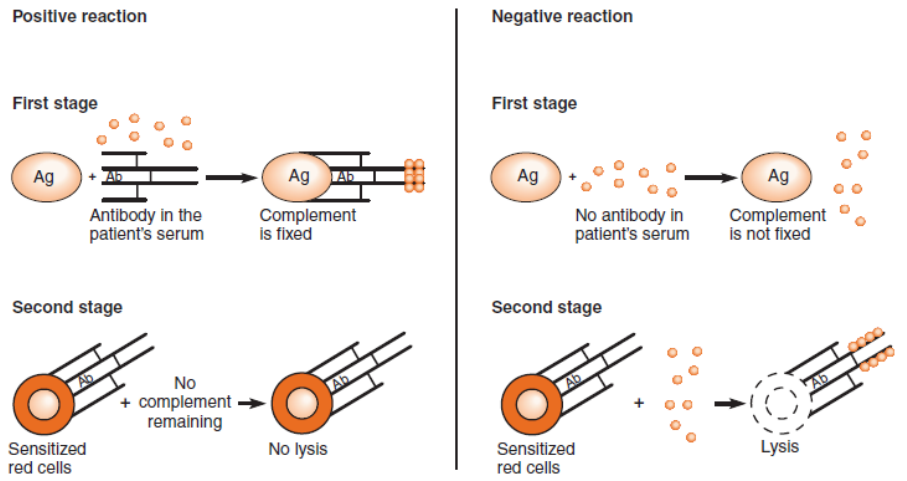
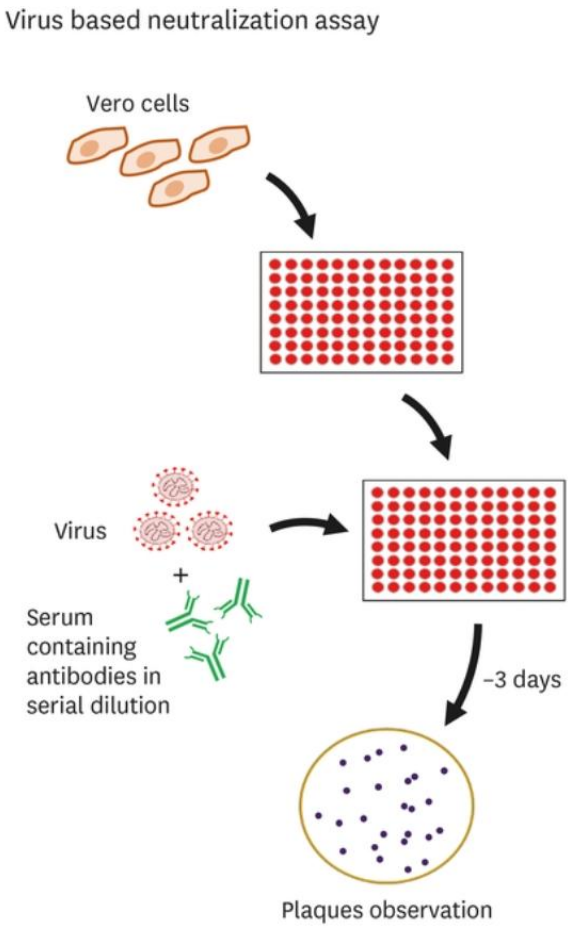


FIGURE 64–8 Complement fixation. **Left:** Positive reaction (i.e., the patient's serum contains antibody). If a known antigen is mixed with the patient's serum containing antibody against that antigen, then complement (solid circles) will be fixed. Because no complement is left over, the sensitized red cells are *not* lysed. **Right:** Negative reaction. If a known antigen is mixed with the patient's serum that does *not* contain antibody against that antigen, complement (solid circles) is *not* fixed. Complement is left over and the sensitized red cells are lysed. Ab, antibody; Ag, antigen.

Neutralization Tests

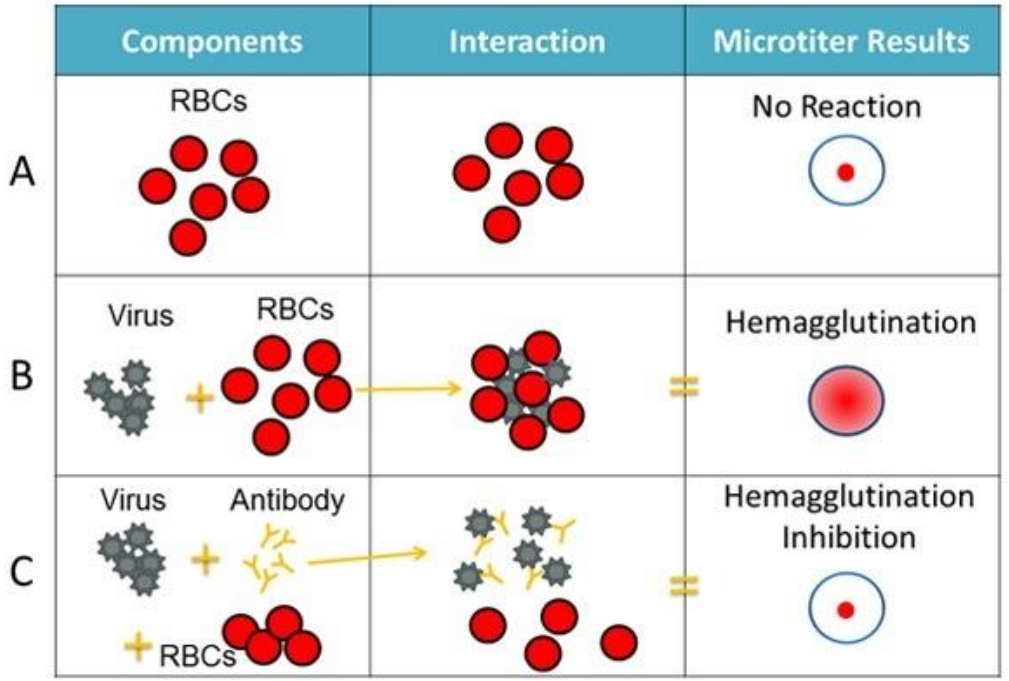
- Antibodies can block toxin or viral effects. This biological activity is measured in neutralization tests.
- Antibodies prevent viral damage in cell culture (inhibition of cytopathic effects).
- Tests can also use host animals. Antibodies protect mice from lethal toxins by neutralizing them.
- These tests are highly specific.

e.g Plaque Reduction Neutralization Test (PRNT).



Hemagglutination Tests

- Some viruses naturally clump red blood cells (Influenza viruses). This is called active hemagglutination.
- Patient antibodies can inhibit this clumping (basis of **Hemagglutination Inhibition tests)**
- **Passive hemagglutination** uses antigen-coated RBCs. The RBCs act as carriers for soluble antigens.
- Antibodies cross-link these cells to cause clumping. This makes precipitation reactions visible.



Antiglobulin (Coombs) Test

- Some patients with certain diseases (e.g., hemolytic disease of the newborn [Rh incompatibility] and drug-related hemolytic anemias) become sensitized but do not exhibit symptoms of disease.
- Antibodies against Red Blood cells are formed and bind to the red cell surface but do not cause hemolysis.
- The Coombs reagent is an anti-human immunoglobulin. It binds to these human antibodies on RBCs causing agglutination.
- The test diagnoses hemolytic anemias. It detects antibodies on RBCs (direct Coombs test) or in serum (indirect Coombs).

Direct vs. Indirect Coombs Test

- **Direct Coombs detects bound antibodies** in vivo. It tests washed patient red blood cells (autoimmune hemolytic anemia, hemolytic disease of the newborn).
- **Indirect Coombs test**, the patient's serum is mixed with normal red cells, and antiserum to human immunoglobulins is added. **If antibodies are present in the patient's serum, agglutination occurs**.
- This is crucial for blood transfusion cross-matching. It prevents transfusion reactions.

Flow Cytometry

- Flow cytometry **counts cells in a fluid stream**. It uses laser analysis of single cells.
- Cells **are labeled with fluorescent monoclonal antibodies**. These target surface markers like CD4.
- The machine **analyzes cell size and fluorescence**. It **quantifies** specific immune cell populations.
- fluorescence-activated cell sorter (FACS) is a specialized form of this technology. It physically sorts cells based on markers.

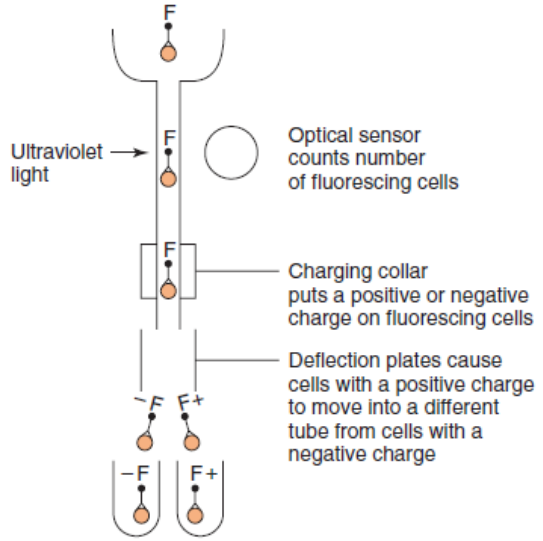


FIGURE 64–10 Flow cytometry. At the top of the figure, a cell has interacted with monoclonal antibody labeled with a fluorescent dye. As the cell passes down the tube, ultraviolet light causes the dye to fluoresce and a sensor counts the cell. Farther down the tube, an electrical charge can be put on the cell, which allows it to be deflected into a test tube and subjected to additional analysis.

ABO Blood Groups (Antigens)

- ABO antigens are specific surface sugars (genetically encoded).
- The H antigen is the base structure. Group A adds *N*-acetylgalactosamine.
- Group B adds galactose to the H antigen. Group O lacks these modifying enzymes.
- Group O cells display only the H antigen. This is the precursor for A and B antigens.

TABLE 64–2 ABO Blood Groups

Group	Antigen on Red Cell	Antibody in Plasma
A	A	Anti-B
B	B	Anti-A
AB	A and B	No anti-A or anti-B
O	No A or B	Anti-A and anti-B

ABO Blood Groups (Antibodies)

- People have antibodies against missing antigens. Type A blood has Anti-B antibodies.
- Type O blood has both Anti-A and Anti-B. This follows Landsteiner's Law.
- These are "**natural" IgM antibodies**, detectable **in the first 3-6 months of age**, develop against **cross-reacting bacterial antigens.**
- They **activate complement** and cause hemolysis. They are detectable early in life.

TABLE 64–3 Compatibility of Blood Transfusions Between ABO Blood Groups[1]

	Recipient			
Donor	**O**	**A**	**B**	**AB**
O	Yes	Yes	Yes	Yes
A (AA or AO)	No	Yes	No	Yes
B (BB or BO)	No	No	Yes	Yes
AB	No	No	No	Yes

[1]Yes indicates that a blood transfusion from a donor with that blood group to a recipient with that blood group is compatible (i.e., that no hemolysis will occur). No indicates that the transfusion is incompatible and that hemolysis of the donor's cells will occur.

Transfusion Compatibility

- Transfusions must match donor and recipient. Incompatibility causes immediate cell lysis.
- **Group O** is **the universal red cell donor**. Their cells lack A and B antigens.
- **Group AB** is the **universal recipient**. Their plasma lacks A and B antibodies.
- Mismatches trigger shock and hemolysis. This is due to complement activation.

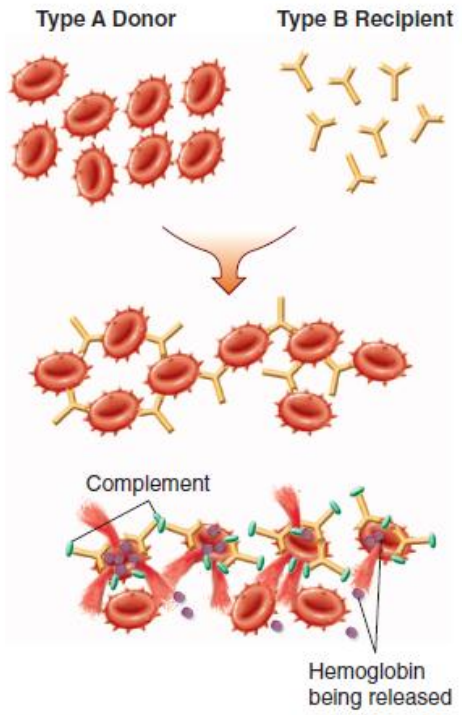


FIGURE 64–12 Transfusion reaction. **Top panel:** Red blood cells bearing A antigen are transfused into a person who is type B and therefore has antibodies to A antigen. **Middle panel:** Anti-A antibodies bind to A antigen on the red cells causing agglutination of red cells that can block movement of blood through capillaries causing anoxia to tissue. **Bottom panel:** Complement is activated by the antigen–antibody complexes and the membrane attack complex lyses the red cells, causing hemolysis and anemia. (Reproduced with permission from Cowan MK, Talaro KP, eds. *Microbiology: A Systems Approach*. New York: McGraw-Hill; 2009.)

Rh Blood Group System

- The Rh system is defined by **the D antigen**. Presence of D means Rh-positive (**85% of humans** are Rh positive).
- Rh antibodies are not naturally occurring. They are IgG antibodies.
- They form after **exposure to Rh-positive RBCs**. This occurs via transfusion or pregnancy.
- Rh incompatibility is a major clinical concern. It differs from ABO in antibody type.

Hemolytic Disease of the Newborn –erythroblastosis fetalis (HDN)

- HDN involves Rh incompatibility. An Rh-negative mother carries an Rh-positive fetus.
- Maternal IgG antibodies cross the placenta. They attack Rh positive fetal red blood cells.
- This causes hemolysis in the newborn (the Direct Coombs test is positive).
- Prevention involves high titers Anti-Rh immunoglobulins RhoGAM injection at 28 weeks of gestation and upon delivery . This prevents maternal sensitization.

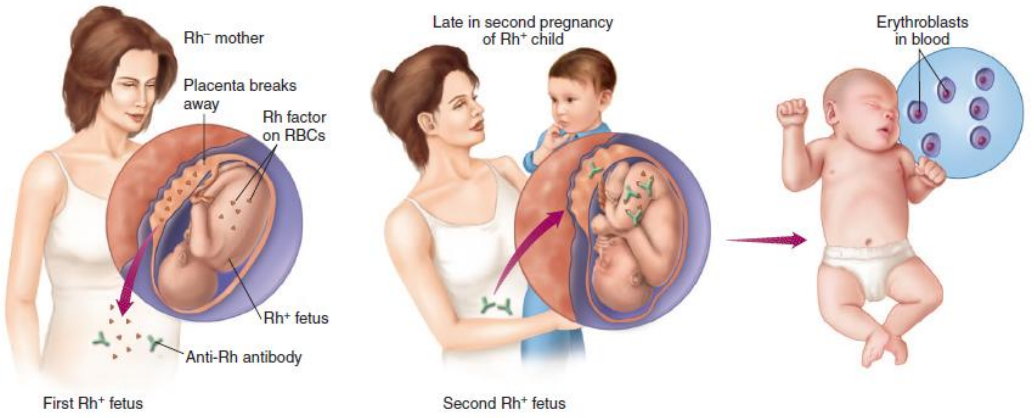


FIGURE 64–13 Hemolytic disease of the newborn (erythroblastosis fetalis). **Left panel:** Fetal red cells (RBCs) bearing the Rh antigen enter the mother's blood when the placenta separates during the birth of the first Rh-positive child. IgG antibodies to Rh antigen are then produced by the mother. **Center panel:** During a second pregnancy with an Rh-positive fetus, IgG antibodies pass from the mother into the fetus via the placenta. The antibodies bind to the fetal red cells, complement is activated, and the membrane attack complex lyses the fetal red cells. **Right panel:** Anemia and jaundice occur in the fetus/newborn. As a result of the anemia, large numbers of erythroblasts are produced by the bone marrow and are seen in the blood of the newborn. (Reproduced with permission from Cowan MK, Talaro KP, eds. *Microbiology: A Systems Approach*. New York: McGraw-Hill; 2009.)

Immune Complexes in Diagnosis

- Immune complexes are antigen-antibody lattices. They can deposit in tissues and be detected by Fluorescent labeled complement.
- Serum complexes bind to complement as C1q or specific cells in culture.
- Detection aids in diagnosing inflammatory diseases. It identifies pathology like glomerulonephritis.