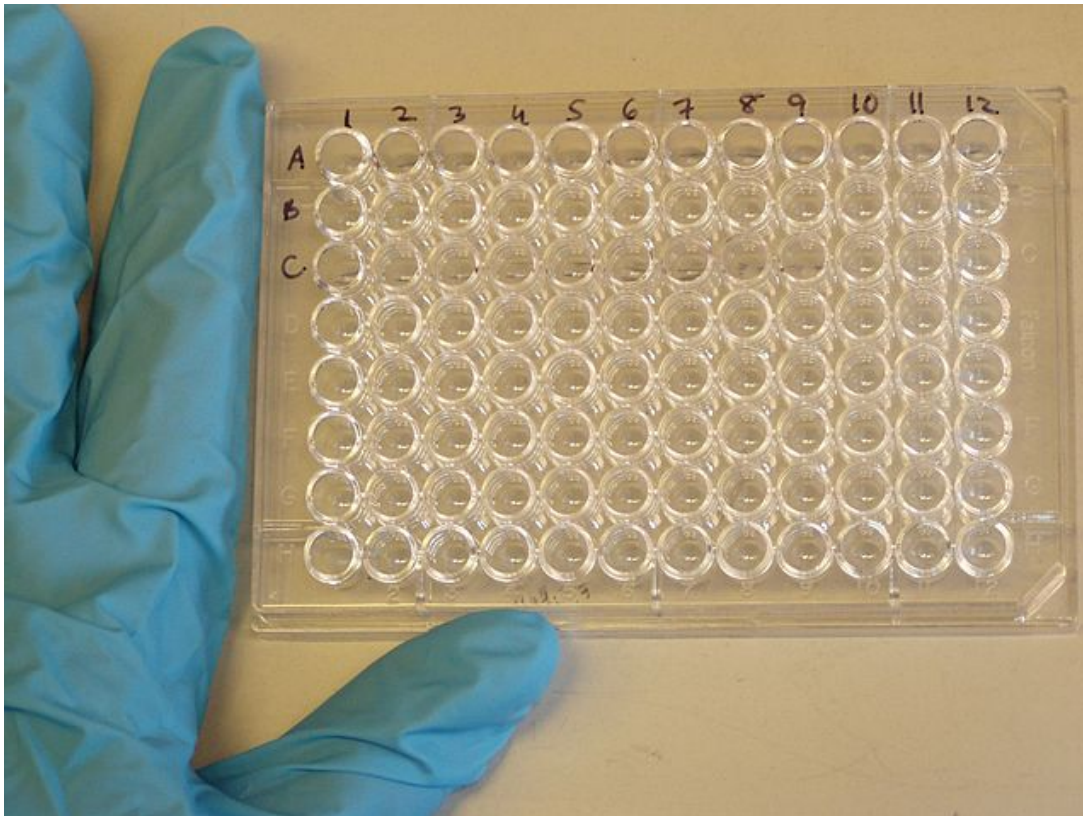


## Immunodiagnosics

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The science of Immunodiagnosics has recently progressed in leaps and bounds. As proficient clinicians, we need to be aware of some of them. This article focuses on core techniques customized for immunodiagnosis, namely Immunodiagnostic Methods (Agglutination, Nephelometry, Immunoprecipitation and Radial Immunodiffusion), Electrophoresis and Western Blot, Immunoassays (ELISA, RIA and Luminex:tm:), Immunofluorescence and Immunohistochemistry, Flow Cytometry and Fluorescence-Activated Cell Sorter (FACS), Evaluation of Cellular Activity and Preparation of Antibodies.



## Immunodiagnosics

Evidence attests to first use of immunology as a diagnosis tool when a **test for serum insulin** was performed. Since then, it has developed into a science, a discipline of using antigen-antibody reactions as primary means of detection of molecules. The can be used to detect even a small number of molecules.

The methods are admired for their speed, accuracy, and simplicity in arriving at the diagnosis of the disease. The various tools in the armamentarium of immunodiagnosics

are described in the sections below.

## Immunodiagnostic Methods

### Agglutination

Originating from the Latin word “**agglutinare**,” which stands for “gluing to,” agglutination is said to occur if an antigen is mixed in a solution containing its corresponding antibody; also known as “**Isoagglutinin**.” Agglutination of antigen coated particles is frequently used for detection of specific antibodies. Common examples of the use of agglutination are blood grouping techniques and identification of Rheumatoid factor as well as the autoantibody to Fc component of gamma globulin in Rheumatoid arthritis assessment.

#### Agglutination and Precipitation

| Method                                    | Use  |
|---|--|
| Agglutination of antigen-coated particles | Detection of specific antibodies                                   |
| Nephelometry                              | Quantification of proteins in serum, plasma or cerebrospinal fluid |
| Immunoprecipitation                       | Isolation of specific antigen from a mixture of antigens           |
| Radial Immunodiffusion                    | Quantification of antigen  |

### Nephelometry

Nephelometry is used for quantification of proteins in the serum, plasma or cerebrospinal fluid. The principle is to measure forwarded scattered light when a laser beam passes through a sample and the light is deflected by the particles. proportional to the turbidity of the solution. The amount of scatter is measured, compared to known mixtures, and then the amount of unknown is determined using standard curves.

The specific polyclonal antibody and the analyte in the test sample interact and immune complexes are formed. Light from a laser light source results in scattering of the molecules. The light scattered onto the receiving photodiode is proportional to the number of complexes (that is, concentration of the analyte).

**There are 2 types of nephelometry, summarized as below:**

| Type                               | Explanation  |
|------------------------------------|--|
| <b>End point nephelometry</b>      | Tests are run by allowing the antibody/antigen reaction to run through to completion                 |
| <b>Kinetic nephelometry (rate)</b> | The rate of scatter is measured right after the reagent is added. Kinetic nephelometry is preferred. |

### Immunoprecipitation

Used for isolation of a specific antigen from a mixture of antigens, this technique entails adding an excess of immobilized antibody specific for the antigen of interest in the solution, collecting the immobilized antibody by centrifugation, washing with fresh solution to remove unbound antigens and then subsequently denaturing the antibody to elute the antigen.

A prerequisite for immunoprecipitation is potential coupling of the antibody to a solid substrate at some point of time during the procedure.

**The types of immunoprecipitation can be summarized as follows:**

| Type   | Explanation   |
|--|---|
| <b>Chromatin immunoprecipitation</b>               | It is used to determine the specifications of DNA binding sites on the genome coding the protein of interest. |
| <b>Individual protein immunoprecipitation (IP)</b> | Uses antibody specific for a known protein  |
| <b>Protein complex immunoprecipitation (Co-IP)</b> | Intact protein complexes of antigens with other proteins bound to it are isolated.                            |
| <b>Tagged proteins</b>                             | Different proteins are tagged at either end to enable immunoprecipitation by using these molecules.           |
| <b>RNP immunoprecipitation (RIP)</b>               | Ribonucleoproteins are tagged .   |

## Immunodiffusion

It is based on the principle of differential segregation via diffusion through a substance such as agar.

## Ouchterlony Double Immunodiffusion

It is also known as agar gel immunodiffusion or passive immunodiffusion. It is an immunological technique used to identify and quantify immunoglobins and extractable nuclear antigens.

It is done by making a series of wells on the gel agar and antigen is placed on one and the test sera placed on the other to check for the presence of antigens.

The plate is left for 48 hours as the contents diffuse across and meet and if the antibody and antigen reaction takes place a white precipitate is formed in form of a straight line known as precipitate line.

## Radial immunodiffusion

Radial immunodiffusion is used for quantification of antigens. Agarose gel containing a specific polyclonal antibody is allowed to stand for 24 to 48 hrs. As the analyte diffuses into gel, the specific antibody binds to the antigen of interest and a precipitate forms. The size of the precipitation ring depends on the quantity of loaded analyte.

## Electrophoresis and Western Blot

Electrophoresis is commonly used to achieve separation of serum proteins.

First observed by **Ferdinand Frederik Reuss from Moscow**, this electrokinetic experience is based on the motion of particles relative to the suspending medium under the influence of an electric field. Electrophoresis of cations is termed as **Cataphoresis**, while that of negatively charged particles is known as **Anaphoresis**.

Polyacrylamide gel provides improved clarity and segregation and, hence, is used in quantitative analysis of DNA and RNA.

Western Blotting separates protein antigens by SDS-polyacrylamide gel electrophoresis. It also goes by the name of "**protein immunoblot.**" "Western" blot is based on the eponymously named "**Southern Blot**"—an RNA separation technique originally

contributed by **Edwin Southern**.

Electrophoretic transfer of proteins to the membrane is followed by labeling of the proteins in the membrane using a primary antibody specific for the antigen of interest as well as a secondary one specific for the primary antibody. The latter is tagged with an enzyme. A substrate is added, which emits light when cleaved by the enzyme. The use of an antibody-labeled membrane, with light emitting substrate added to expose film, completes the process.

## Immunoassays

Biochemical tests using antibodies or, occasionally, antigens to detect macromolecules in a solution constitute "immunoassays." The entity thus detected is known as "**analyte**."

Immunoassays typically involve the use of specific molecules which have the potential to conjugate to the molecule of interest. These molecules are termed as "labels." Different types of labels used define the type of immunoassay performed. The significant ones can be summarized as follows:

| Label used   | Immunoassay performed   |
|--|---|
| Enzymes as labels  | <b>Enzyme-Linked ImmunoSorbent Assays(ELISAs), also called Enzyme Immuoassays(EIAs)</b> |
| Molecules along with enzymes which lead to light emitting mechanisms | <b>Chemiluminescence</b>  |
| Radioactive isotopes as labels                                       | <b>Radioimmunoassay(RIA)</b>  |

Few more terms related to immunoassays earn a mention.

## Sandwich immunoassay

In these tests, the antibody is first bound to the well of microtiter plate, then varying amounts of antigen is added. Copious washing about brings the removal of the unbound antigen. A labeled second antibody, specific for non-overlapping epitopes of the antigen, is then introduced. The unbound, labeled second antibody is removed by liberal washing. The amount of bound second antibody is measured. The bound second antibody, as a function of the concentration of antigen added by construction of a standard curve, helps in determination of the concentration of the antigen of interest.

## Fluorescent microsphere (Luminex) assay

These high-yield assays involve the use of pre-coated, color-coded beads, which are covered with analyte-specific antibody for segregation of the molecule of interest. They find use in the diagnosis of Hepatitis A, for instance, where the amount of signal emitted at the end is proportional to anti-Hep A antibody in the patient's serum.

## Immunofluorescence and Immunohistochemistry

These methods are primarily used on body tissues to determine specific biomolecule targets conjugated to antibodies with tagged fluorescent dyes. Immunofluorescence is a very important diagnostic tool and can be considered a subset of immunohistochemistry where fluorophores are used to determine the location of specific antigens and antibodies.

**There are 2 types of immunofluorescence as summarized below:**

## Primary (Direct)

Direct immunofluorescence uses a single, primary antibody, chemically linked to a fluorophore. In this test, the biopsy sample is placed on a slide, and fluorescein-labeled sheep antibodies against the antigen of interest are added. Under ultraviolet light, the fluorescein label emits visible light over the antigen of interest. The tissue used can be of animal or human origin, as long as it contains the antigen. Then, the patient's serum is added. Next, autoantibodies bind to the antigen. Fluorescein-labeled sheep anti-human IgG is added, which binds to the IgG. Bound IgG is detected under ultraviolet light.

## Secondary (Indirect)

Indirect immunofluorescence uses two antibodies; the unlabeled first (primary) antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it. Measurement of ANA levels in pt with SLE commonly employs indirect immunofluorescence.

Fluorescein-labeled anti-human immunoglobulin antibody and the patient's serum are used with substrate tissue from rat liver, kidney, and stomach.

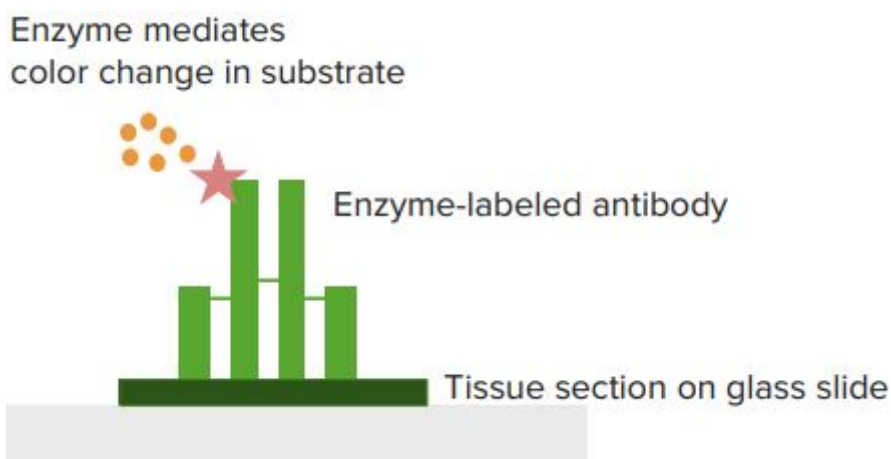
## Immunohistochemistry (IHC)

First envisaged by **Albert Coons** in 1941, IHC involves detecting antigens in tissue sections by utilizing antigen-antibody specific interactions.

**The 2 kinds of IHC assays can be tabulated as follows:**

### Direct

Direct IHC implies one-step staining methodology with a labeled antibody. The substrate for enzyme, enzyme-labeled antibody and the tissue section are all placed on a glass slide. If present, the enzyme mediates color change as a result of formation of the substrate—enzyme-labeled antibody—tissue section conjugated complex on a glass slide.

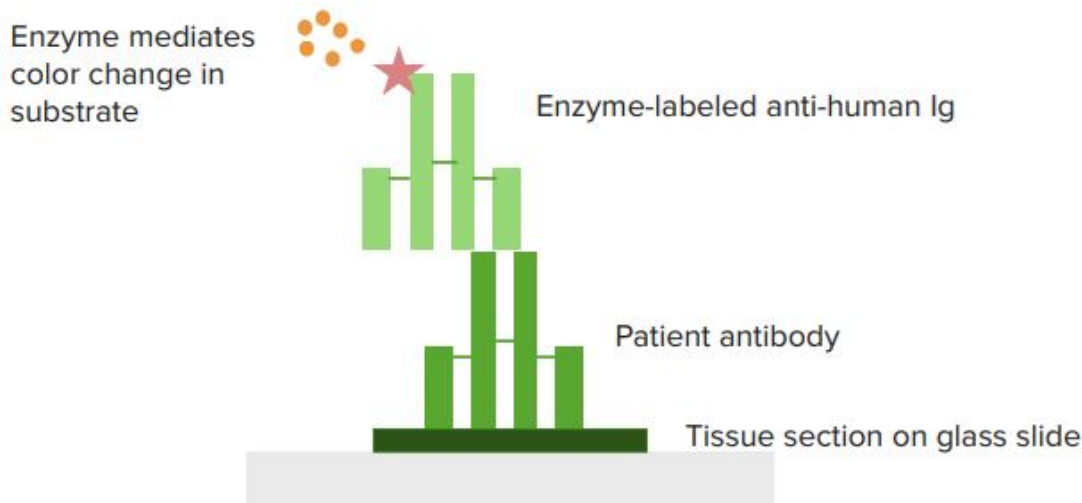


“Direct Immunohistochemistry” Image created by Lecturio

### Indirect

Indirect IHC uses a primary antibody specific to the antigen of interest and a secondary labeled antibody, which chemically binds to the primary antibody. Indirect IHC is more sensitive than direct IHC.

The enzyme mediates a color change when the substrate, enzyme-labeled anti-human antibody, the patient antibody and the tissue section form a conjugated complex on the glass slide.



"Indirect Immunohistochemistry" Image created by Lecturio

## Flow Cytometry and Fluorescence-Activated Cell Sorter (FACS)

### Flow cytometry

Flow cytometry is a laser or impedance based technology employed in cell counting, cell sorting and applied in protein engineering. The slow stream passage of fluid containing various molecules of cells of interest through an electronic detection apparatus allows simultaneous appreciation of the multitude of physical and chemical characteristics of about a thousand particles per second.

Flow cytometry finds application in diagnosis of cancer markers, biomarker detection, cytology, and proteomics. Additional linking of the analytical capability of the flow cytometry to a cell sorter is also possible for purification and separation of proteins of interest.

#### **Some of the common applications of flow cytometry are mentioned below:**

- Detection of cell surface markers (e.g., CD4 and CD8 T-cells) using Anti Cd-8 fluorescein isothiocyanate
- Detection of double positive thymocytes
- Detection of specific T-cells using pMHC tetramers, a fluorescently labeled tetramer of 4 identical HLA molecules loaded with a peptide from Epstein-Barr virus

### Fluorescence-activated Cell Sorter (FACS)

Being a scientific instrument of crucial significance, FACS employs flow cytometry to sort a heterogeneous mixture of different kinds of biological cells based on light scattering and fluorescent properties of the cells in question.

A mixed population of cells labeled with fluorescent antibodies is put forward the scatter detector. Parallel plates are used to detect flowing cells. An analyzer computer makes final analysis, with the results showing on the computer display.

## Evaluation of Cellular Activity

There are various tests of direct clinical relevance for evaluation of cellular activity based on the modern concepts of immunohistochemistry, immunofluorescence. Therefore, immunodiagnosics can be summarized as follows:

| Test   | Explanation   |
|--|---|
| <b>Nitroblue Tetrazolium Test (NBT)</b>              | The pale yellow color of NBT changes to dark blue in neutrophils stimulated with phorbol myristate acetate(PMA) to induce reactive oxygen species. Production of reactive oxygen species can also be measured using dihydrorhodamine 123. NBT is instrumental in the diagnosis of <b>Chronic Granulomatous Disease and diseases of phagocyte malfunction.</b> |
| <b>T-cell Receptor Excision Circle (TREC) Assay:</b> | This assay is used to quantify recent thymic emigrants as a measure of T-cell output from the thymus.   |
| <b>Lymphocyte Proliferation Assay:</b>               | Phytohaemagglutinin(PHA) mitogen, polyclonally activated T-cells and <sup>3</sup> H labeled thymidine are mixed. Subsequently, <sup>3</sup> H-thymidine titers incorporated during cell division are evaluated to determine the proliferation of the lymphocytes in the first place.  |
| <b>Measuring Cytotoxic T-Cell Activity</b>           | Target cell and effector cell are incubated together for 4 to 5 hours. Subsequent radioactivity in the culture fluid is evaluated. The same is proportional to target cell lysis.   |
| <b>ELISPOT Assay to measure cytokine release</b>     | Peripheral blood cells, lymphocytes, monocytes and soluble antigen are co-cultured for 2 days. TH2 secrete IL-4 in response to soluble test antigen. IL-4 is captured by the antibody. Un-captured cells are removed. Cytokine is revealed by the second antibody whose chemical reaction typically gives rise to spots.                                      |

## Preparation of Antibodies

Commercial production of antibodies falls into 2 categories: **polyclonal and monoclonal**. While polyclonal antibodies represent antibodies secreted by different B-cells in the body, monoclonal antibodies epitomize antibodies from a single cell lineage.

**The production of various antibodies can be summarized as follows:**

### Production of Polyclonal Antibodies

Animals are inoculated with the antigen of interest. After multiple injections, the animals are supposed to have developed antibodies against the antigen. Blood is extracted from the animals and required polyclonal antibody is isolated, purified and refined to serve in diagnostic and therapeutic ways. Animals used are mammals: mouse, rabbit, sheep and goat.

### Hybridoma technology (monoclonal Antibodies)

The cells are cultured in a selective medium such as the HAT medium. After several days, only fused cells will survive. Cells are diluted, so that one cell is plated per well. Cells are grown in individual culture plate wells, and culture supernatants from wells containing growing hybrid cells are screened for presence of desired antibody by ELISA. This clone

hybridoma is an immortal producer of the desired monoclonal antibody.

### **Phage display production of monoclonal antibody**

Recent advances in molecular biology techniques such as **Phage display, Single B-cell culture, Single cell amplification and Single plasma cell interrogation techniques** use amplification of antibody genes by PCR to develop antibodies with recombinant technology.

In “phage display,” the gene contributing to production of the antigen of interest is introduced into a phage coat protein gene. This causes the gene to “display” the protein externally, while the gene is present internally. These displaying phages are systematically screened and then isolated to form the source of production of the required antigen.

## Summary

Immunodiagnosics as a science has progressed rapidly and extensively. Various techniques, based on the unique antigen-antibody reaction, are developed to isolate, quantify or produce the specific molecule of interest.

Immunodiagnosis involves the use of tests like Agglutination, Nephelometry, Immunoprecipitation and Radial Immunodiffusion to determine the desired antigen. Electrophoresis and Western Blot use protein separation techniques based on differential migration of molecules across an electric field to separate different genres of proteins present in a given sample.

Immunoassays such as ELISA, RIA and Luminex:™ use labeled antibodies to isolate and identify antigens of interest. The labels commonly used are enzymes, radioactive isotopes or chemiluminescent substances.

Immunofluorescence and Immunohistochemistry use appropriately labeled antibodies to detect the antigen of interest, especially in biological tissues. Flow Cytometry involves simultaneous evaluation of a multitude of properties of a myriad number of molecules as the solution suspending them is passed slowly across an electronic detection apparatus.

Fluorescence-Activated Cell Sorter (FACS) is a scientific instrument based on flow cytometry, with extensive applications in cytology like cell counting, cell sorting and study of cellular property. Cellular activity can be measured with the help of immunodiagnosics.

Various antibodies such as polyclonal and monoclonal can be commercially produced using advanced molecular biological techniques like production of hybridomas, phage display and cell culture.

## Review Questions

The correct answers can be found below the references.

### **1. The technique of cell counting based on flow cytometry is:**

- A. Flow cytometer
- B. Flow sorter
- C. Fluorescence-Activated Cell Sorter (FACS)
- D. Flow cytometry dependent counter



## 2. Mark the true statement:

- A. Agglutination involves the formation of antigen clumps on suspension of a fluid.
- B. PCR-based antibody production is used in immunofluorescence.
- C. Scattering of light principle is used in electrophoresis.
- D. ELISA is an immunoassay using enzymes as labels.

## 3. Mark the false statement:

- A. Nephelometry uses segregation of molecules by differential migration across an applied electric field.
- B. IHC uses antigen-antibody reactions to detect desired antigens in tissue systems.
- C. Hybridoma is formed by fusion of mouse spleen cells and myeloma cells.
- D. Phage display technique is used for production of monoclonal antibodies.

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**Correct answers:** 1C, 2D and 3A

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