**Lec1 Immunological technique**

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**2- Chemiluminescent Immunoassays**

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**References:**

* **Miller,L.M.and Stevens,C.D.(2021) Clinical immunology & Serology, Alaboratory perspective,5th ed FA Davis, Philadelphia.**

Labeled Immunoassays

Immunoassay Labels

Labeled immunoassays differ from unlabeled techniques by including a detection molecule (label) in the test system. Most current techniques utilize non-isotopic labels to generate a light signal. Depending on manufacturer design, labels may include:

**1- a colorimetric substrate,2- fluorescent compound (fluorophore), or3-luminescent molecule**. Earlier generation immunoassays used radioactive isotope elements as the label, but these techniques are less commonly used in clinical laboratories today. Generation of the detection signal also varies across manufacturers. Labels are sometimes referred to as “tracer” molecules because they allow for tracing of the detection signal.

One technique common to many labeled immunoassay methods is enzyme mediated catalysis of a reagent substrate to generate a light signal.

A wide variety of test designs have been developed by different manufacturers, and the selection of the label is aimed at the testing needs for measuring the analyte of interest.

**General Immunoassay Formats**

**Heterogeneous Versus Homogeneous Immunoassays**

There are two major formats for all labeled immunoassays, *heterogeneous*

and *homogeneous.* Separation of bound and free tracer label before signal

measurement is a requirement in all immunoassay designs. The approaches

to achieve this can be broadly categorized as heterogeneous or

homogeneous.

In **heterogeneous immunoassays,** physical separation of bound and free components is required. Separation of components is achieved by a variety of methods, including 1-**centrifugation,2- binding to solid phase material, or 3-magnetic separation**. Solid-phase materials are the most common and include polystyrene reaction wells, micro particle beads, latex beads, and plastic tubes.

**homogeneous immunoassays.** Immunoassay methods that do not require a physical separation step.

Although homogeneous immunoassay designs have this property in common, the methods by which they measure the detection signal and patient analyte concentration can vary extensively across manufacturers.

Additional sub classifications are made to describe whether immunoassay

method designs are ***competitive* or *noncompetitive****,* based on the principle of

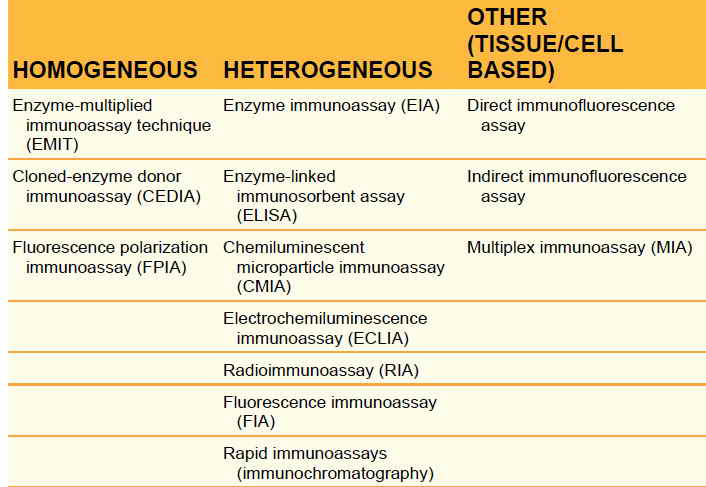
the test reaction. **Table 1**.

**CompetitiveVersusNoncompetitive Immunoassays Competitive Immunoassays**

In **competitive immunoassays,** the test system reagents consist of limited antibody (Ab), tracer or labeled antigen (Ag\*), and reagent substrate. The only variable in the reaction is the concentration of patient antigen (Ag),which is the analyte of interest. All the reactants are mixed together simultaneously; labeled tracer antigen (Ag\*) competes with unlabeled patient antigen (Ag) for a limited number of binding sites for antibody (Ab).

The concentration of the labeled analyte (Ag\*) is in excess to ensure all binding sites on the antibody will be occupied whether or not sample antigen is present. After washing to remove unbound label, the amount of antibody-bound label (Ag\*Ab) is measured and used to determine the amount of patient antigen present. If patient antigen is present, some binding sites will be occupied with unlabeled analyte, thus decreasing the amount of bound label detected **(Fig1).** Therefore, the amount of bound label is inversely proportional to the concentration of the labeled antigen, which means that the more labeled antigen detected, the less antigen is present in the patient sample.

**TT-Table 1: Homogeneous and Heterogeneous Immunoassay Formats**

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**Fig 1: Principle of a competitive immunoassay**

This relationship can be illustrated by considering the ratio of tracer bound

antigen to sample antigen (analyte) in the example that follows.

Suppose labeled antigen (Ag\*) and unlabeled antigen (Ag) occur in three

different ratios: 2:1, 1:1, and 1:2. Binding to a limited number of antibody

sites will take place in the same ratios. The reaction between the antigens and their corresponding antibody can be depicted in an equation where the reactants are listed on the left side of the arrow and the products on the right. Labeled antigen (Ag\*) and reagent antibody (Ab) are held constant, at 100 units and 50 units, respectively. By altering the patient antigen (Ag) concentration, the amount of labeled antigen bound to antibody will change according to the patient antigen concentration. Thus, on the right side of the equation, one-third (33%) of binding sites are occupied by labeled antigen (Ag\*Ab) at a 2:1 Ag\*:Ag ratio, 25% of binding sites are occupied at a 1:1 ratio, and 17% at a 1:2 ratio. Additional ratio values of labeled antigen (Ag\*) to patient antigen (Ag) help to further illustrate this relationship, as depicted in **Figure 2.** It can be observed that the detection signal is

inversely proportional to the patient antigen concentration on a nonlinear scale. The relationship can be mathematically linearized to generate a standard curve, using calibrators with known amounts of antigen, in order to extrapolate the signal to the concentration of antigen in the patient sample.

**Noncompetitive Immunoassays**

In a standard **noncompetitive immunoassay,** reagent antibody (often called

*capture antibody*) is first passively absorbed onto a solid-phase material ,such as microtiter plates, nitrocellulose membranes, or plastic beads. Excess capture antibody is present to ensure that any patient antigen present will be bound. Unknown patient antigen reacts with (i.e., is captured by) the solid phase antibody. After washing to physically remove unbound antigen, a labeled antibody, directed against a different epitope of the antigen, is added to the reaction **(Fig3).** The amount of label measured is directly proportional to the amount of patient antigen present and is represented by a mathematical linear relationship.







Fig2: Example of the effect of the ratio of labeled antigen to unlabeled antigen on

the percentage of labeled antigen bound to antibody in a competitive immunoassay

**Radioimmunoassay (RIA)**

The original immunoassay developed was based on a competitive principle,

using a radioisotope label. This technique, called **radioimmunoassay (RIA),** was pioneered by Yalow and Berson in the late 1950s, who developed the immunoassay to measure insulin hormone levels in humans.Yalow was honored with the 1977 Nobel Prize in Physiology or Medicine for her groundbreaking work. The success of this original immunoassay design ultimately led researchers to develop additional RIA methods to measure other clinical analytes, including several hormones (e.g.,aldosterone, cortisol), serum proteins, drugs, viral antigens, and

immunoglobulins.Radioactive elements have nuclei that decay spontaneously into other elements and, in the process, release energy, which is measured by detectors

such as a gamma counter. Depending on the immunoassay design,radiolabeled antigens can be tagged with various radioactive elements,including tritium (3H), carbon (14C), or iodine (125I or 131I). Detectors are able to measure very low quantities of radioactivity, which gives these immunoassays extremely high analytical sensitivity.

However, the chief disadvantages of RIA methods include health hazards involved in working with radioactive substances, radioactive waste disposal problems, and the limited shelf life of some elements (for example, 125I has a half-life of about 60 days). For these reasons, RIA testing in clinical laboratories has become limited.

**Enzyme Immunoassays (EIAs)**

**Enzyme immunoassays (EIAs),** using enzymes as labels, were developed as alternatives to RIA. **Enzymes** are naturally occurring molecules that catalyze biochemical reactions. When used in a test system, they convert reagent substrates to produce chemically modified products that can be detected. Substances produced and detected in this method include colored or visible light, ultraviolet (UV) light, fluorescent light, and luminescence.

These methods have sufficient analytical sensitivity for many clinical tests

and eliminate the concerns of disposal problems or health hazards associated with radioactive isotopes. Because one molecule of enzyme can generate many molecules of product, the addition of an enzyme label further improves analytical sensitivity.

Enzymes used as labels for immunoassays are chosen according to their physical properties, including the number of substrate molecules converted per molecule of enzyme, ease and speed of detection, and stability. The availability and cost of the enzyme and substrate may factor into the design of a particular enzyme reagent. Common enzymes used as labels in EIA include horseradish peroxidase (HRP), alkaline phosphatase (ALP), β-Dgalactosidase, and glucose-6-phosphate dehydrogenase (G6PDH). EIAs can be further classified as either heterogeneous or homogeneous on the basis of whether a separation step is necessary, as previously mentioned.

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**Fig 3:** Capture, sandwich, or immunometric assay.

**Heterogeneous Enzyme Immunoassays**

**Competitive Enzyme Immunoassays**

The first EIAs, developed by Eva Engvall and Peter Perlmann in 1971,

were based on the competitive principles of RIA. Capture antibodies were

adsorbed onto plastic tubes, which led these investigators to name this new

method **enzyme-linked immunosorbent assay (ELISA).** Alkaline phosphatase (enzyme) was conjugated to the antigen of interest. In competitive ELISAs, sample antigen competes with enzyme-conjugated antigen for a limited number of binding sites on antibody molecules attached to a solid phase, such as plastic tubes or microtiter plate wells.After careful washing to remove unbound antigen, enzyme activity present

in the final reaction catalyzes the conversion of the substrate to a detectable

product. The product signal is inversely proportional to the concentration of

the test substance. In the original ELISA method, sample antigen was accurately detected at a concentration as low as 1 ng/mL, achieving comparable analytical sensitivity to RIA. Since the time that ELISA methods were introduced, they have dramatically increased in popularity and have become a mainstay of testing in research and clinical laboratories.

In practice, an ELISA is an EIA, so these terms may be used interchangeably.

**Noncompetitive Enzyme Immunoassays**

Alternately, ELISA/EIA methodology may be based on a noncompetitive

design. Noncompetitive immunoassay formats can be used to detect the

presence of either an antigen or an antibody in the test sample.

Immunoassays that detect antibody are most commonly **indirect ELISAs,**

whereas those that detect antigen are termed “capture immunoassays.”

ELISA test kit procedures often use 96-well microtiter plates as the solid

phase. In addition to microtiter plates, a variety of solid-phase supports

have been developed, including plastic reaction well tubes, plastic beads,

magnetic particles, and latex particles. Most automated immunoassays in

the laboratory today are designed on similar noncompetitive principles.

This type of immunoassay is one of the most frequently used immunoassays

in the clinical laboratory because of its sensitivity, specificity, and simplicity of use.

**Indirect ELISAs**

Indirect ELISAs are most commonly used to detect a patient antibody of

interest. They are termed “indirect” because the enzyme-labeled reagent

does not participate in the initial antigen–antibody reaction. In these

immunoassays, the associated antigen is bound to the solid phase, and

patient serum with an unknown antibody concentration is added. Incubation

time allows for specific interaction between solid-phase antigen and patient

antibody. After a wash step, an enzyme-labeled anti-globulin, or secondary

antibody, is added. This second antibody reacts with any patient antibody

that is bound to the solid phase. If no patient antibody is bound to the solid

phase, the second labeled antibody will not be bound and will be removed

during a second wash step. This is followed by incubation with the enzyme

substrate, and finally, a stop solution to cease enzyme activity after a specified period of time. The amount of color, fluorescence, or luminescence generated by action of the enzyme label on the substrate is measured using a detection device and is compared with known amounts of antigen according to a standard curve. In the noncompetitive format, signal detection is directly proportional to the amount of antibody in the specimen **(Figs. 4** and **5).** Clinical applications include measurement of antibody production to infectious agents that are difficult to isolate in the laboratory; for example, this technique is useful as a screening tool for detecting patient antibodies to viruses such as HIV, hepatitis B, and hepatitis C. Another clinical application is detection of autoantibody production as the cause of a disease; for example, detection of autoantibodies to insulin and glutamate decarboxylase helps to support a diagnosis of type 1 diabetes mellitus.

**Capture (Sandwich) Immunoassays**

When antigen is the analyte of interest, antibody is bound to the solid phase.

These immunoassays are also termed **sandwich immunoassays, capture**

**immunoassays,** or **immunometric assays.** Antigens captured in these

immunoassays must have multiple epitopes. Excess antibody attached to the

solid phase is allowed to combine with the test sample to capture any

antigen present. After an appropriate incubation period, enzyme-labeled

antibody is added. This secondary antibody recognizes a different epitope

or binding site than the solid-phase antibody. The final complex formed

with sample antigen in between the two reagent antibodies creates the

“sandwich.” Depending upon the particular enzyme used, either a colored,

fluorescent, or chemiluminescent reaction product is detected. Product

formation is directly proportional to the amount of antigen present in the

test sample (see Fig. 3). The use of monoclonal antibodies has made this

a very sensitive test system.

Capture immunoassays are best suited to antigens that have multiple determinants, such as cytokines, proteins, tumor markers, and microorganism antigens. When used to detect microorganisms, the epitope must be unique to the organism being tested and ideally, must be present in all strains of that organism. Another use of capture immunoassays is in the measurement of immunoglobulin's, especially those of certain classes. For instance, the presence of immunoglobulin M (IgM) can help indicate an acute infection. When capture immunoassays are used to measure

immunoglobulins, the specific immunoglobulin class being detected is technically acting as the antigen, and the reagent antibody is anti-human immunoglobulin.

**Biotin-Avidin Labeling**

One way to achieve increased analytical sensitivity of labeled immunoassays is by complexing biotin to the capture antibody and streptavidin to the solid-phase material. **Biotin,** also known as vitamin B7 or vitamin H, is a vitamin of the B-complex family, whereas avidin is a protein enriched in egg yolks. The discovery of this interaction led researchers to isolate a bacterial version of avidin, called **streptavidin (SAv),** which was demonstrated to have stronger binding affinity for biotin. The formation of biotin-SAv complexes is the rationale for using these molecules in immunoassay design, especially as each molecule of SAv has multiple highaffinity binding sites for biotin. The end result is signal amplification with

enhanced detection when coupled to one of the previously discussed nonisotopic

labels. Biotin-SAv labeling can be used in both indirect ELISAs

and capture immunoassays. When a biotin-SAv design is used in a capture

immunoassay, it is sometimes referred to as a *delayed capture immunoassay*

because the final incubation step includes binding of the sandwich complex

to SAv on the solid phase.

Although all these immunoassay formats are useful in measuring antigen

or antibody levels in patients, there are certain limitations for which the

clinical laboratorian must be aware. Commonly encountered immunoassay

test interferences.

**Interferences in Immunoassays**

False-positive or false-negative results in immunoassays may occur for a

variety of reasons. They can be produced simply because of certain physical

properties of the specimen itself, as some biological materials such as urine

or plasma may cause quenching of light emission or exhibit background

fluorescence. However, additional test interferences may occur for several

different reasons related to immunoassay design. Most of the interferences

described are predominantly observed when heterogeneous methods are

used to measure the test analyte, rather than homogeneous methods.

**Antigen Interference**

The improved turnaround time, speed, and sensitivity of capture immunoassays have made them excellent tools for measuring several biological substances. However, they are subject to certain limitations. The most commonly observed of these is the **high-dose hook effect,** or *postzone* effect, where excess patient antigen causes falsely decreased detection,leading to an analyte concentration that appears to be low or normal. This effect is illustrated in **Figure 6.** Note that the curve that depicts the relationship between the analyte and the intensity of the reaction signal

takes on the shape of a “hook.” The high-dose hook effect occurs

predominantly when there are not enough capture antibody sites for antigen

binding because the majority of binding sites are filled, so the remainder of

patient antigen has no place to bind and gets removed during the wash step.

When a hook effect is suspected, the technologist should dilute the sample

to the point where the concentration is within the analytical measuring

range (calibration range). Increased antigen concentration after dilution

provides evidence that a hook effect was present in the original undiluted sample.

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**Fig 4: Noncompetitive, indirect immunoassay**

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Fig5: **Sample wells from indirect ELISA to detect patient antibody (in this example,antibody to the rubella virus). Well A1 is the reagent blank; well B1 is the positive control;well C1 is the negative control; and wells D1, E1, and F1 are calibrators run in triplicate. The remaining wells are individual patient samples. Note that the negative wells remain colorless, whereas wells ontaining samples that are positive for the antibody produce ayellow color following addition of the stop solution. Actual absorbance values are read on a**

**spectrophotometer. *(Figure courtesy of Dr. Linda Miller.)***

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Fig6: The high-dose hook effect. Antigen excess can saturate antibodies, and the

intended “sandwich” configurations cannot form, leading to a false decrease in signal.