**Lec2 Immunological technique**

**Prof. Dr. Ekhlass N. Ali**

**Antibody Interference**

**Autoantibodies**

Antibodies produced in vivo may interfere with immunoassays whenever

the antibody produced is similar to that of the test kit reagent. Production of

autoantibodies (antibodies directed against self-antigens) by individuals

with an autoimmune disease is one possible source of interference. A clinical example is in patients with rheumatoid arthritis who test positive for rheumatoid factor (RF). If present, RF can cause false-positive results in clinical tests that use immunoglobulin G (IgG) antibodies. If this is suspected, serum can be pretreated with a blocking reagent to avoid this problem. As an example, animal IgG antibodies may be added to the patient sample to bind RF before testing. Importantly, these “blocking antibodies" must be of a different species than the capture and detection antibodies used in the immunoassay test

**Heterophile Antibodies**

Similar interference mechanisms may occur in individuals who have **heterophile antibodies** that react with animal proteins because the detection antibodies used in test reagents are generated in nonhuman species (mouse, rabbit, goat). Heterophile antibodies may be produced by patients with certain infections, patients who have received therapeutic animal globulins, or patients who have frequent contact with animals.

Heterophile interference occurs when the individual produces antibodies similar to those used in the test reagent kit, such as anti-mouse, -rabbit, or - goat antibodies. Heterophile antibodies will often cross-link reagent capture and detection antibodies in the absence of antigen (test analyte) to produce a falsely increased test signal **(Fig. 1).** In rarer cases, heterophile antibodies can cause a falsely decreased test result by binding to the antigen of interest or by binding to the labeled detector antibody, preventing the formation of an immune complex

****

Fig 1:Interference by heterophile or anti-animal ntibodies. Heterophile or antianimal antibodies (red) can cause both false decreases and false increases, depending on

their reactivity against the antibody species used in an immunoassay. However, false increases caused by linking the capture antibodies (blue) and the detection antibodies (black) together are most likely, as shown

**Biotin Interference**

When test reagent kits also use a biotin-SAv complex, even more unpredictable interferences can occur. The most pressing concern facing clinical laboratorians using this immunoassay format is when the individual being tested has recently taken high-dose biotin as a nutritional supplement or medication (usually at a dose equal to or greater than 1 milligram/1,000 micrograms). The ingested biotin causes interference in the immunoassay,and depending on the immunoassay design, test results may be falsely increased (competitive design) or decreased (noncompetitive

immunoassay). Such interference is usually discovered when reported test

results are found to be inconsistent with the clinical picture. For example, a markedly abnormal thyroid-stimulating hormone (TSH) test result may be observed in a person with no other evidence of pituitary or thyroid disease.Fortunately, the solution to correct or prevent this problem is often quite simple, by having the patient refrain from taking biotin for at least 8 to 48 hours before blood sample collection. Alternatively, measuring the same analyte on a different platform that does not use the biotin-SAv reaction could be performed on the sample to obtain reliable results. Biotin interference in the original test result would be suspected if the result on the re-tested sample normalizes when the alternative method is used.

**Other Technical Concerns**

*Cross-reactivity* describes detection of a substance other than the analyte of interest. This often occurs because of similar structure to the analyte(s) used to calibrate the test method immunoassay. When cross-reactivity occurs, it is typically observed as a false-positive result. Multiple case reports of cross-reactivity in drug immunoassays have been reported in the literature.

One case example was a false-positive opiate immunoassay screen result in which naloxone (an opiate antagonist) was detected as an opiate by a homogeneous EIA method (CEDIA, described in a later section). In such instances, an alternative method must be used to confirm the initial result obtained by the immunoassay.

Finally, the clinical laboratorian must be cautious whenever interpreting immunoassay test results across different manufacturers, as direct comparisons may not be possible. Because test manufacturers often develop capture and detection antibodies to slightly different epitopes, the reference interval (normal range) established for an immunoassay can vary widely across manufacturers, even for the same clinical test. This is especially critical when serial measurements of tumor marker proteins are being performed to monitor medical treatment of cancer patients.

**Homogeneous Enzyme Immunoassays**

The chief use of homogeneous immunoassays has been in the determination of low-molecular-weight analytes (haptens), such as hormones, therapeutic drugs, and drugs of abuse, in both serum and urine. Homogeneous immunoassays **(Fig. 2)** may provide quicker test results because wash steps are not required, and as mentioned, these immunoassays tend to be less prone to analytical interferences. Despite the advantages of homogeneous immunoassays, their analytical sensitivity is not as robust as heterogeneous immunoassays.



Fig 2:Homogeneous immunoassay. Reagent antibody is in solution. Patient antigen and enzyme-labeled antigen are added to the test tube. Patient antigen and enzyme labeled antigen compete for a limited number of binding sites on the antibodies. When patient antigen is present, the enzyme label on the reagent antigen is not blocked, so color,development is observed. Sample A has a low concentration of patient antigen, whereas Sample B contains more patient antigen and has stronger color development.

The original homogeneous immunoassay design, which is still in use today on ltiple platforms, is the **enzyme-multiplied immunoassay technique (EMIT)** first developed by the Syva Corporation (now part of Siemens). The EMIT immunoassay is based on the principle of change in enzyme activity as specific antigen–antibody interaction occurs in solution.Reagent antigen is bound to an enzyme tag, commonly glucose-6-phosphate dehydrogenase (G6PDH). When reagent antibody binds to specific determinant sites on the enzyme–antigen pair, the active site on the enzyme is blocked, resulting in a measurable loss of activity. Free analyte (antigen in the patient sample) competes with enzyme-labeled analyte for a limited number of antibody-binding sites. G6PDH catalyzes the reduction of NAD+ to NADH, leading to increased absorbance in the UV-wavelength region

(340 nm). The resulting enzyme activity and signal generated are directly

proportional to the concentration of patient antigen or hapten present in the

test solution **(Fig. 3).**The **cloned-enzyme donor immunoassay (CEDIA)** is another example of a homogeneous immunoassay. CEDIA was developed using genetic engineering techniques to produce the β-galactosidase enzyme in two parts:

acceptor and donor. When separated, the enzyme is not fully formed, and there is no enzymatic activity; when the acceptor–donor pair combine,enzymatic activity is restored, and reagent substrate is catalyzed to generate a product signal that is measured by photometry. To achieve this reaction, the hapten antigen (Ag) is attached to the enzyme donor piece. The enzymedonor Ag competes with patient Ag (analyte) for limited antibody binding spots. As patient Ag binds to reagent Ab, it leaves the enzyme donor free to bind with the enzymeacceptor molecule. Therefore, enzymatic activity and signal are directly proportional to patient Ag concentration. This reaction occurs in solution without need of a solid-phase material,which makes it a homogeneous immunoassay. Its most common clinical use is to detect or quantitate drug levels (both therapeutic and abused).

**Chemiluminescent Immunoassays**

The majority of immunometric assays on the market today are based on

chemiluminescent label detection. These are collectively referred to as **chemiluminescent immunoassays. Chemiluminescence** is the emission of

light caused by a chemical reaction, typically an oxidation reaction,producing an excited molecule that releases light during return to its original ground state. Large numbers of molecules are capable of chemiluminescence, and some of the most common substances used in test systems are luminol, acridinium esters, ruthenium derivatives, and nitrophenyl oxalates. When these substances are oxidized, intermediates are produced that are of a higher energy state. These intermediates

spontaneously return to their original ground state, emitting photon energy

(light) in the process. Light emissions range from a rapid flash of light to a

more continuous glow that can last for hours. For example, when acridinium esters are oxidized by hydrogen peroxide under alkaline conditions, they emit a quick flash of light. When luminol is used in the reaction, the light signal remains for a longer time period.Chemiluminescent technology is the basis for several types of automated immunoassays, which are used to detect a wide variety of clinical analytes,including antigens and antibodies. The design can be applied to

heterogeneous or homogeneous immunoassay formats. Haptens such as therapeutic drugs and steroid hormones are measured using competitive immunoassays, whereas the sandwich or capture format is used for larger analytes such as protein hormones. These immunoassays also demonstrate excellent analytical properties for measurement of serum antibodies produced in several types of infectious diseases and autoimmune disorders.



Gig3:EMIT immunoassay design. *(Figure courtesy of Dr. Paul Johnson*

Chemiluminescent immunoassays have superior analytical sensitivity and a wide dynamic range, the latter implying that the immunoassay performs well at both very low and very high analyte concentrations. This provides excellent sensitivity to detect very low levels while also reducing the need for sample dilution because extremely elevated results can be accurately measured. Detecting high antigen concentration without need for sample dilution can be especially useful when measuring tumor markers or autoantibodies in diseased patients, where concentration levels can be 10-fold or greater than the upper limit of the normal cutoff value.

**Chemiluminescent Microparticle Immunoassay**

In the **chemiluminescent microparticle immunoassay (CMIA)** design,antibody-coated microparticles are used in the reagent. In this method,patient antigen competes with a hapten (Ag) labeled with an acridinium ester. Magnets are used to attract the microparticles for physical separation and allow for unbound substances to be washed off. As such, CMIA is a heterogeneous immunoassay because the particles are physically separated by magnets. As antigen concentration increases in the patient sample, it prevents labeled Ag molecules from binding to the Ab-coated

microparticles. Conversely, low patient antigen causes more labeled Ag tobind to the microparticles. A final test signal is generated by adding hydrogen peroxide to the alkaline solution, which destabilizes the acridinium ester, resulting in a flash of light (chemiluminescence). The resulting signal is indirectly proportional to the antigen concentration in the patient sample. CMIA technology has been largely applied for use in drug measurements, for example, phenytoin, a common anti-epileptic drug measured in therapeutic drug monitoring (TDM), to ensure the patient’s drug levels are within the effective dose range, and are not at toxic or

subtherapeutic levels.

**Electrochemiluminescence Immunoassay**

A newer modification of the traditional chemiluminescent immunoassay is

the **electrochemiluminescence immunoassay (ECLIA).** Ruthenium, a chemical substance used as an indicator, can be conjugated to antibody used in capture immunoassays. It undergoes an electrochemiluminescent reaction with another chemical substance, tripropylamine (TPA), at the surface of an electrode. When the ruthenium is oxidized and then returned to its reduced state through interaction with TPA, it gives off light that can be measured by a photomultiplier tube. Magnetic beads or micro particles are often used as the solid phase to capture the labeled antibody.

**Lec3 Immunological technique**

**Prof. Dr. Ekhlass N. Ali**

**Fluorescent Immunoassays**

In 1941, Albert Coons demonstrated that antibodies could be labeled with

molecules that fluoresce. These fluorescent compounds, called **fluorophores** or **fluorochromes,** can absorb energy from an incident light source and convert that energy into light of a longer wavelength and lower energy as the excited electrons return to the ground state.

Fluorophores are typically organic molecules with a ring structure; each has a characteristic optimal absorption range. The time interval between absorption of energy and emission of fluorescence is very short and can be measured in nanoseconds.

Ideally, a fluorescent probe should exhibit high intensity, remain stable in

solution, and be distinguished from background sample fluorescence. The

two compounds most often used are fluorescein and rhodamine, usually in

the form of isothiocyanates, because these can be readily coupled to antigen

or antibody. Fluorescein absorbs maximally at 490 to 495 nm and emits a

green color at 520 nm. It has high intensity, good photostability, and a high

quantum yield. Tetramethylrhodamine absorbs at 550 nm and emits red light at 585 nm. Because their absorbance and emission patterns differ,fluorescein and rhodamine can be used together.Fluorescent-labeled antibodies or antigens have been used in a variety of immunoassays, including direct and indirect immunofluorescence assays,

multiplex immunoassays (MIAs), and fluorescence polarization

immunoassays (FPIAs). In contrast to the “reagent-based” systems

discussed in preceding sections, some fluorescent immunoassays take place

on a microscope slide or on the surface of live cells. These techniques are called **immunofluorescence assays (IFAs).** The principles of laboratory methods that use fluorescent labels and examples of their clinical applications are presented in the text that follows.

**Direct Immunofluorescence Assays**

Fluorescent staining methods can be categorized as either direct or indirect,

depending on whether the antibody specific for the antigen has a fluorescent

tag attached to it. **Figure 1** depicts the difference between the two

techniques.

**Direct immunofluorescence assays** may be used to detect antigens on tissue sections fixed onto a microscopic slide or in live cell suspensions. In a direct IFA performed on a microscope slide, antibody that is conjugated with a fluorescent tag is added directly to sample antigen fixed onto the slide. After incubation and a wash step to remove unbound antibody, the slide is read using a fluorescence microscope. The bound fluorescent probe is detected under UV light (see **Fig. 2A**). In this manner, multiple antigens can be detected with a high degree of sensitivity and specificity.

Antigens are typically visualized as bright-apple-green or orange-yellow

objects against a dark background. Examples of antigens detected by direct

IFA methods include bacterial pathogens such as *Legionella pneumophila*

and *Chlamydia trachomatis.*

Cell-based IFA methods combine the principles of hematology cell counters with fluorescent-labeled antibodies to better classify cells. An important application of direct IFA is to differentiate cell populations of the immune system, such as T and B lymphocytes, based on their cluster of differentiation (CD) antigens by incubation of the cells with fluorescentlabeled antibodies specific for those markers. For example, this assay is very useful in the detection and quantitation of abnormal cell populations observed in leukemias. The results are analyzed by automated **flow cytometry**

Fig 2:Direct versus indirect immunofluorescent assays. (A) In a direct fluorescent

immunoassay, the patient antigen is fixed to a microscope slide and incubated directly with a fluorescent-labeled antibody. The slide is washed to remove unbound antibody. If specific antigen is present in the patient sample, fluorescence will be observed. (B) In indirect

immunofluorescence, well-characterized tissues or cells are fixed to slides. Specific

antibody in patient serum (red) binds to the antigens on the slides. A wash step is performed, and a labeled anti-human immunoglobulin is added. After a second wash step to remove any uncombined anti-immunoglobulin, the fluorescence of the sample is determined. The amount of fluorescence is directly in proportion to the amount of patient antibody present.

****

Alternatively, more recent advances in flow cytometry include *mass*

*cytometry* profiling of cells based on CD marker identification. In this

method, fluorescent labels are replaced with metal isotope labels for

detection. Metal isotopes provide for simultaneous detection of a greater

number of cellular markers as compared with traditional fluorescent labels.

**Indirect Immunofluorescence Assays**

In general, a direct immunofluorescence assay is only used for antigen

detection in cells or tissues, whereas **indirect immunofluorescence (IIF)**

**assays** can be used for either antigen or antibody identification, depending

on the intended clinical application. IIF assays involve two steps, in a

manner similar to reagent-based capture immunoassays. In the first step of

IIF assays used to detect antibody, patient serum is incubated on a

microscope slide to which a known antigen has been attached. Tissue

antigen is usually affixed to the slide; for example, human epithelial cells

are used for anti-nuclear antibody (ANA) testing. The slide is then washed,

and an anti-human immunoglobulin containing a fluorescent tag is added.

This labeled immunoglobulin combines with the first antibody to form a

sandwich, which localizes the fluorescence (**Fig. 2B).** In this

manner, one antibody conjugate can be used for many different types of

reactions, eliminating the need for numerous purified, labeled reagent

antibodies.

IIF assays generate increased staining because multiple molecules can

bind to each primary molecule, making this a more sensitive technique.

Such immunoassays are especially useful in antibody identification and

have been used in syphilis testing to detect treponemal antibodies, viral

antibodies, and autoantibodies such as ANAs and anti-neutrophil cytoplasmic antibodies (ANCAs). Reading immunofluorescent slides is partly a subjective interpretation because the technologist reports results based on the visual presence or absence of signal as well as signal intensity in positive samples. Technical experience is essential for accurate and reliable reporting of slide test results. For example, there are several nuclear patterns observed in ANA testing that the technologist must be able to distinguish when interpreting slide results .

**Multiplex Immunoassay (MIA)**

A more recently developed platform, based on fluorescent labeling and

detection, is the **multiplex immunoassay (MIA).** This high-throughput,

automated method has improved ease of clinical testing, especially in testing for autoimmune diseases. Polystyrene beads are used as the solid phase. When the technique is used to detect antibodies in patient serum, beads conjugated to different antigens are used. These beads can be distinguished by their unique shade of red, created by a specific combination of infrared and fluorescent dyes. Patient sample is added to the bead mixture, and antibodies in the sample are detected with a fluorescent tagged anti-human immunoglobulin. The beads containing the immune complexes are identified by flow cytometry. A benefit of MIA technology is that it allows for multiple antibodies to be detected simultaneously. Clinical applications of MIA include ANA testing and detection of antibodies in transplant patients to donor antigens . MIA can also be used to simultaneously detect multiple antigens in a test sample when the polystyrene beads are coated with the corresponding antibodies. One application is the detection of cytokines produced by cells cultured under different conditions.

**Fluorescence Polarization Immunoassays**

Application of fluorescent labels for homogeneous immunoassay designs

led to the development of the **fluorescence polarization immunoassay**

**(FPIA).** This technique is of more historical interest today because in 2008

the manufacturer (Abbott) announced it was discontinuing its FPIA-based

instruments. The company has since changed many of its previous platforms to chemiluminescent technology (discussed in an earlier section).Students should remain aware of FPIA technology; however, owing to reduced availability and manufacturer support for FPIA methods, their use has rapidly declined.

The FPIA method was based on the change in polarization of fluorescent

light emitted from a labeled molecule when it is bound to antibody. Incident

light directed at the specimen is polarized with a lens or prism so that the

waves are aligned in one plane. If a molecule is small, it will rotate quickly,

causing the emitted light to become unpolarized. If, however, the labeled

molecule is bound to antibody, the molecule is unable to tumble rapidly, and

light will remain polarized. Thus, the degree of polarized light detected corresponds to the amount of antibody-labeled antigen complex formed in solution. As the method is based on a competitive design, where labeled antigen competes with patient sample antigen for a limited number of antibody-binding sites, the fluorescence polarization detected is inversely proportional to the sample analyte concentration.

**Rapid Immunoassays**

**Rapid immunoassays** are membrane-based tests that are easy to perform and give reproducible results. Although designed primarily for point-of-care testing, many of these have found use within clinical laboratories because of their faster turnaround time of results, ease of use, and little space needed for setup. These tests are used on urine or serum samples and are designed as single-use, disposable immunoassays in a plastic cartridge.

The original rapid immunoassays are based on a flow-through design.The membrane and its large surface area enhance immunofiltration of thesample to provide speed and a high level of sensitivity. This method is a two-step immunoassay in which antigen or antibody in the patient sample is first absorbed onto the membrane containing the corresponding antibody or antigen. Following a wash step, a detection reagent is added. The reaction is then read by looking for the presence of a colored reaction product.

Newer **immunochromatography** methods combine all the previously mentioned steps into one step. The analyte is applied at one end of the strip and migrates toward the distal end, where there is an absorbent pad to maintain a constant capillary flow rate. The labeling and detection zones are set between the two ends. Sample is added to an application point; the application point also contains a labeled antigen or antibody conjugated to colored latex or colloidal gold particles. The sample reconstitutes the conjugate, where the two form a complex that migrates across the membrane. An antigen or antibody immobilized in the detection zone captures the immune complex and forms a colored line for a positive test

when the immunoassay is of a noncompetitive format **(Fig. 3).** This type of test device has been used to detect diverse analytes of interest.

Prototypical examples of rapid immunoassay uses include detection of the

human chorionic gonadotropin (hCG) hormone as an indicator of pregnancy

**(Fig. 4);** identification of microorganisms such as *Streptococcuspyogenes,* the cause of streptococcal pharyngitis and cardiac troponin to diagnose a heart attack.



Fig3:Immunochromatographic assay (noncompetitive). (A) Patient sample is

added to a cassette containing antibody labeled with colloidal gold. (B) Sample combines

with antibody and is moved along by capillary flow. (C) Monoclonal antibody to the analyte captures the patient antigen attached to gold-labeled antibody. (D) Control line has antibody

that captures the colloidal gold-labeled antibody. *(Courtesy of University of Nevada School of Medicine.)*



Fig4:Rapid immunoassay for human chorionic gonadotropin (hCG). The negative

control (left) has a line in the control region only. The positive control (right) has lines in both

the control (C) region and the test (T) region. The control line must be present for the results

to be valid, regardless of the test result. *(Photo courtesy of Dr. Paul Johnson.)*

The immunochromatographic design illustrated in Figure 3 is based on a capture or noncompetitive design. Some newer test systems, which still use similar materials, are based on a competitive format. These results must be carefully interpreted because the observed line indicates a negative result, and absence of the line is a positive result. Rapid screening tests to detect drugs of abuse provide one example of this format. A **single-step,competitive, immunochromatographic method** contains detection antibody adsorbed onto colloidal gold particles, which is then dried onto the entire membrane surface. Drug conjugates (drug bound to bovine serum albumin) are made for each drug of detection, all of which are immobilized

to a unique test-line position on the membrane. When the sample (urine) is

added, it wicks along the entire surface of the white membrane. As it passes along each test lane, drug-free urine sample dissolves the antibody–gold particle complex at each lane, resulting in observation of purple-red colored lines. Any drug present in the sample will inhibit that reaction, such that no line will be observed on the white membrane background. As this method is based on a competitive design, it may lead to initial confusion when interpreting test results. This is because the color line indicator is observed only when there is no antigen or drug present in the sample

(negative result), while the absence of the line indicator is a positive result. The test system may also include use of a strip-reading device, which aids the laboratorian in interpreting the final results. In addition, all single-use test kit immunoassays include a control line to ensure the test kit cartridge is functioning properly before reporting out results (Fig. 4).







