About this learning guide...

**Intended Audience**
This Learning Guide is intended to serve the basic educational needs of healthcare professionals dealing with laboratory medicine. Anyone associated with the specialty of immunoassay testing—sometimes referred to as “special chemistry”—will find this monograph of even greater interest.

Labradorians, those who use the laboratory’s services, and those who service the laboratory will find this guide useful. This includes but is certainly not limited to laboratory technicians and technologists, supervisors and managers, nurses, suppliers, and other physician office and laboratory support personnel.

**How to Use This Learning Guide**
Each section begins with a Section Overview that summarizes the goals and content for each section. Next, Learning Objectives will help you focus on the key concepts presented in each section. At the end of each section, there is a short Section Review and Section Review Quiz to help you recall the main concepts. If you answer a quiz question incorrectly, review the appropriate portions of the text before moving to the next section.

For quick reference, a Glossary is included at the end of this Learning Guide, as well as a Bibliography and Suggested Reading section, listing additional reading sources for further study.

This Learning Guide ends with a Learning Guide Satisfaction Survey. If you complete the survey and return it to Abbott Laboratories, Diagnostics Division, we can use your feedback to ensure that future editions will be as beneficial as possible.

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Learning Objectives
After completion of this chapter, you will be able to:

- define immunoassay
- describe the structure and preparation of antibodies
- define four categories of immunoassay methodology (competitive and noncompetitive, and homogeneous and heterogeneous)

Introduction
An immunoassay is a test that uses antibody and antigen complexes as a means of generating a measurable result. An antibody:antigen complex is also known as an immuno-complex. "Immuno" refers to an immune response that causes the body to generate antibodies, and "assay" refers to a test. Thus, an immunoassay is a test that utilizes immunocomplexing when antibodies and antigens are brought together.

Immunoassays are different from other types of laboratory tests, such as colorimetric tests, because they use antibody:antigen complexes to generate a signal that can be measured. In contrast, most routine clinical chemistry tests utilize chemical reactions between the reagent (a solution of chemicals or other agents) and patient sample to generate a test result.
Immunoassay: Antibodies, Antigens and Analytes Defined

Antibodies and Antigens
An antibody is a protein that is produced by the body in response to an “invading” (foreign) substance¹. Antibodies are produced as part of the body’s immune response to protect itself. For instance, some immunoassays test for the presence of antibodies to cancer molecules. Thus, if the antibodies are present, it means invading cancer cells are too.

An antigen is the substance that the body is trying to “fight off” (eliminate or reduce) by mounting an immune response. Some immunoassays test for antigens directly, rather than looking for the antibodies. In a test to measure the concentration of a therapeutic drug, for example, the drug is the antigen that binds to the antibody.

An analyte is anything measured by a laboratory test. In immunoassay testing, the analyte may be either an antibody, or an antigen.

Summary of Immunoassay Techniques
Immunoassays utilize one or more select antibodies to detect analytes of interest. The analytes being measured may be those that are naturally present in the body (such as a thyroid hormone), those that the body produces but are not typically present (such as a cancer antigen), or those that do not naturally occur in the body (such as a medication or abused drug).

Antibodies possess high specificity and affinity for a specific antigen. It is the specific binding of an antibody to an antigen that allows the detection of analytes by a variety of immunoassay methods.

Structure of Antibodies
Antibodies (Ab) are a type of protein called immunoglobulins. The most common one is immunoglobulin G (IgG). IgG is a protein comprised of two main structural and functional regions:

FIGURE 1-1 Antibody Structure and Functional Sites

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¹ An exception is the case of autoimmune diseases, where the body produces antibodies to naturally occurring proteins rather than foreign substances.
Preparation of Polyclonal and Monoclonal Antibodies

Antibody reagents are developed from either polyclonal or monoclonal antibodies. Polyclonal antiserum (serum from blood containing the desired antibodies) is generated in animals, most commonly sheep, rabbits, or goats. The animals produce the antiserum—just as a human would—as a defense mechanism when exposed to an antigen. Antiserum contains a mixture of antibodies, each of which may bind to different antigen binding sites, or epitopes.

The process of making an antiserum begins by injecting a solution that contains the antigen of interest into an animal. This antigen of interest is sometimes called an immunogen, because it can stimulate an immune response. Over time, and in some cases with multiple injections, the immune system of the animal produces antibodies to the antigen that was injected. Blood is collected from the animal, and serum is isolated from the blood. This serum is usually rich in antibodies that recognize the antigen, and is called the antiserum.

FIGURE 1-2 Multiple antigen specificities of polyclonal antibodies

Antiserum usually contains a mixture of antibodies that recognize and bind to the same antigen, but they may attach to different epitopes (see Figure 1-2). An antigen that has multiple sites for antibodies to bind is called a multivalent antigen. These types of antibodies, present as a diverse mixture, are called polyclonal antibodies.

Monoclonal antibodies differ from polyclonal antibodies in that they are highly specific for a single epitope on a multivalent antigen (see Figure 1-3). They are produced from a single cell line using hybridoma technology and mouse myeloma cell lines. Hybridomas are antibody-producing tumor cells that produce many copies of the same antibody and grow easily in laboratory cell culture.

An advantage of monoclonal antibodies is that the hybridoma cell line that produces them is potentially “immortal” and can produce the same antibodies consistently and indefinitely. A polyclonal antiserum produced by immunization of animals can vary from animal to animal, and a useful antiserum may no longer be available if the single animal that produces it dies.

FIGURE 1-3 Uniform specificities of monoclonal antibodies
Hybridomas are produced in a multi-step procedure (see Figure 1-4):

- injecting a specific antigen into a host animal (typically a mouse);
- isolating antibody-producing cells (B cells) from the spleen of the mouse;
- fusing these B cells with a specific type of tumor cell that grows easily in culture and produces antibodies;
- isolating successful hybridomas (fused cells) that produce antibodies specific for the antigen of interest.

In immunoassays, both monoclonal and polyclonal antibodies are used for detecting antigens, each with specific strengths for particular applications. Immunoassays that detect antibodies in patient sera are likely to involve detection of polyclonal antibodies generated by the patient’s immune system.

**FIGURE 1-4 Procedure for generating monoclonal antibodies**
Categories of Immunoassay Methodologies

In this section, four categories of immunoassay methodology will be discussed. They are noncompetitive and competitive immunoassays, and homogeneous and heterogeneous immunoassays.

All immunoassays require the use of labeled material in order to measure the amount of antigen or antibody present. A label is a molecule that will react as part of the assay, so a change in signal can be measured in the blood:reagent solution. Examples of a label include a radioactive compound, an enzyme that causes a change of color in a solution, or a substance that produces light. The label can be applied during the manufacture of the reagent to either the antibody (Ab*, see Figure 1-5) or antigen (Ag*, see Figure 1-6). Immunoassay technologies utilize different formats to distinguish the bound antigen-antibody complex from the free unbound label.

**FIGURE 1-5** Labeled antibodies allow detection of antigen/antibody complexes in immunoassays

**FIGURE 1-6** Labeled antigen also allows detection of antigen/antibody complexes in immunoassays
Competitive and Noncompetitive Immunoassays

The measurement of analyte in an immunoassay is achieved by using either a competitive or a noncompetitive format.

Competitive Format

In competitive formats, unlabeled analyte (usually antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay. The unlabeled antigen blocks the ability of the labeled antigen to bind because that binding site on the antibody is already occupied. Thus, in a competitive immunoassay, less label measured in the assay means more of the unlabeled (test sample) antigen is present. The amount of antigen in the test sample is inversely related to the amount of label measured in the competitive format (Figure 1-7).

In the one step competitive format (see Figure 1-8), both the labeled antigen reagent (Ag*) and the unlabeled specimen (or test sample analyte) compete for a limited amount of antibody.

In the two step competitive format, the antibody concentration of the reaction solution is present in excess in comparison to the concentration of antigen. Antibody reagent is first incubated with specimen containing antigens of interest; then in the second step, labeled antigen is added. Remember that in the competitive format, less bound labeled antigen indicates more antigen present in the test sample. Two step competitive assay formats provide several fold improved assay sensitivity compared to one step assay formats.
Noncompetitive (Sandwich) Method

Noncompetitive assay formats generally provide the highest level of assay sensitivity and specificity (see Glossary for definition of these terms) and are applied to the measurement of critical analytes such as cardiac and hepatitis markers (see Chapter 3). This format is referred to as a “sandwich” assay because analyte is bound (sandwiched) between two highly specific antibody reagents (Figure 1-10).

Noncompetitive assay formats can also utilize either one step or two step methods, as with the competitive assay. The two step assay format employs wash steps in which the sandwich binding complex is isolated and washed to remove excess unbound labeled reagent and any other interfering substances.

The two step noncompetitive format usually offers the highest specificity and sensitivity of all the assay formats discussed here.
In noncompetitive assays, the measurement of labeled analyte, usually antibody, is directly proportional to the amount of antigen present in the sample. This can be represented by a dose response curve (Figure 1-11). The X-axis plots concentration of an antigen. The Y-axis plots response, which in this case is signal. Thus, the more antigen that is present, the more labeled antibody that will bind. This direct proportionality is in contrast with the indirect proportionality of competitive immunoassays discussed earlier.

**Homogeneous and Heterogeneous Immunoassay Methods**

Immunoassay methods that require separation of bound Ab-Ag* complex are referred to as heterogeneous immunoassays. Those that do not require separation are referred to as homogeneous immunoassays.

Homogeneous methods have been generally applied to the measurement of small analytes such as abused and therapeutic drugs. Since homogeneous methods do not require the separation of the bound Ab-Ag* from the free Ag*, they are generally much easier and faster to perform.

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**FIGURE 1-11** Amount of antigen is directly related to the amount of label (signal) in competitive formats.

**FIGURE 1-12** Homogeneous and heterogeneous immunoassays.
Chapter 1 Summary

Immunoassays are tests that use antibody and antigen complexes (also called immunocomplexes) to measure the presence of a specific analyte in a sample.

Antibodies are proteins that are normally produced by the immune system in response to a foreign substance.

Antigens are the molecules that antibodies bind to, which in the body could be an invading pathogen, or the foreign molecules injected into an animal to trigger the immune response.

Antibodies are comprised of two major regions, the Fab region (antigen specific) and the Fc region.

Antibody preparations are either polyclonal antisera, which recognize multiple sites on antigens, or monoclonal antibodies, which recognize single sites on antigens.

In immunoassays, the antibody or antigen is labeled in order to have a measurable signal that corresponds to the concentration of the analyte.

Immunoassays can be either competitive or noncompetitive. In competitive immunoassays, the amount of antigen is inversely proportional to the amount of signal. In noncompetitive immunoassays, the amount of antigen is directly proportional to the amount of signal.

Homogeneous immunoassays do not require separation of unbound complexes from the bound complexes, and thus are faster and easier to perform than heterogeneous immunoassays.

Heterogeneous immunoassays require the separation of unbound complexes, often utilizing a solid phase reagent such as a magnetic particle or plastic bead.
Quiz questions for Chapter 1
Circle the correct answer or fill in the blank.

1. Monoclonal antibodies and polyclonal antibodies:
   a. Can provide specificity and sensitivity for measuring analytes
   b. Are immunoglobins
   c. Bind antigen through the Fab binding region
   d. All of the above

2. An individual monoclonal antibody will bind several different sites (epitopes) on an analyte.
   a. True
   b. False

3. In noncompetitive immunoassay formats, the measured signal is typically ________________to the analyte concentration in specimen.
   a. Directly proportional
   b. Inversely proportional

4. Which immunoassay methodology is preferred to provide the highest sensitivity and highest specificity?
   a. One step competitive
   b. Two step noncompetitive
   c. Two step competitive
   d. One step noncompetitive
Learning Objectives
Upon completion of this section, you will be able to:

- Describe the methodologies of RIA, EIA, FPIA, MEIA and CMIA
- Identify assays that utilize these methods

Early Methods of Immunoassay: RIA and EIA
The development of practical immunoassays began in the 1960s with the application of radioimmunoassays (RIAs). RIAs utilize radioactive isotopes as a label (Figure 2-1), and the amount of radioactivity measured is indicative of the amount of analyte present. Remember that in the noncompetitive sandwich format on the left in Figure 2-1, the amount of label directly correlates with the amount of antigen present; while in the competitive format on the right in Figure 2-1, the amount of antigen is inversely correlated with the amount of label.

RIAs are still used today, particularly for detection of very low quantities of analytes. However, due to the inherent complications of handling and disposing of radioactive materials, RIAs in the clinical laboratory are used less often than a different type of immunoassay, called enzyme immunoassay (EIA).

In EIAs, enzyme labels are used in place of radioactive labels. Typical enzyme labels are alkaline phosphatase, horseradish peroxidase, and β-galactosidase. Whereas RIAs use radioactivity to measure the concentration of analyte, EIAs typically use a change in color, emission of light, or other signal. Specific equipment is required to quantitate the amount of enzyme present by measuring the specific change that occurred.
ELISA, or Enzyme Linked Immunosorbent Assay, represents a popular application of solid phase heterogeneous sandwich immunoassay that combines enzyme-antibody label reagent with a solid phase bound antibody. An ELISA is a type of EIA. Initially, either microtiter plates or 1/4 inch beads were utilized as the solid phase material. A microtiter plate is simply a square plastic dish with shallow wells that are coated with reagent.

Starting in the late 1970s and throughout the 1980s–90s, major advances in automation and sensitivity of immunoassays, and EIA in particular, were achieved. Fluorescence Polarization Immunoassay (FPIA) and Microparticle Enzyme Immunoassay (MEIA) technologies represented the predominant technologies for some years. More recently, Chemiluminescent Magnetic Immunoassay (CMIA) technology has been routinely applied due to inherent increased sensitivity. These methods will each be discussed in more detail.

**Fluorescence Polarization Immunoassay - FPIA**

*Fluorescence Polarization Immunoassay (FPIA)* is a type of homogeneous competitive fluorescence immunoassay. With competitive binding, antigen from the specimen and antigen-fluorescein (AgF) labeled reagent compete for binding sites on the antibody. As a homogeneous immunoassay, the reaction is carried out in a single reaction solution, and the bound Ab-AgF complex does not require a wash step to separate it from “free” labeled AgF.

**FIGURE 2-2** Competitive fluorescence polarization immunoassay (FPIA)

FPIA is utilized to provide accurate and sensitive measurement of small toxicology analytes such as therapeutic drugs, and drugs of abuse, toxicology and some hormones. The application of FPIA to the Abbott TDx® system greatly automated formerly labor-intensive manual immunoassays.

The FPIA reagent includes:

- **S:** Antibody Reagent: Antiserum to analyte;
- **T:** Tracer: Fluorescein-labeled analyte;
- **P:** Pretreatment detergent: facilitate release of drug from serum proteins.
FPIA utilizes three key concepts to measure specific analytes in a homogeneous format: **fluorescence, rotation of molecules in solution, and polarized light**.

**Fluorescence**: Fluorescein is a fluorescent label. It absorbs light energy at 490nm and releases this energy at a higher wavelength (520nm) as fluorescent light (Figure 2-3).

![Figure 2-3 Detection of fluorescence in fluorescein-conjugated complexes](image)

**Rotation of Molecules in Solution**: Larger molecules rotate more slowly in solution than do smaller molecules. This principle can be used to distinguish between the smaller antigen-fluorescein molecule, AgF, which rotates rapidly, and the larger Ab-AgF complexes, which rotate slowly in solution.

**Polarized Light**: Fluorescence polarization technology distinguishes antigen-fluorescein (AgF) label from antibody bound-antigen-fluorescein (Ab-AgF) by their different fluorescence polarization properties when exposed to polarized light (Figure 2-4). Polarized light describes light waves that are only present in a single plane of space. When polarized light is absorbed by the smaller AgF molecule the AgF has the ability to rotate its position in solution rapidly before the light is emitted as fluorescence. The emitted light will be released in a different plane of space from that in which it was absorbed and is therefore called unpolarized light. With the larger sized Ab-AgF complex, the same absorbed polarized light is released as polarized fluorescence because the much larger Ab-AgF complex does not rotate as rapidly in solution. The light is released in the same plane of space as the absorbed light energy, and the detector can measure it.

![Figure 2-4 Measurement of large complexes using fluorescence, rotation, and polarized light in FPIA](image)
FPIA results in an inverse dose response curve such that lower levels of patient analyte result in a higher signal (in this case, the signal is polarized light). High signal at low patient analyte levels results in a highly sensitive assay (Figure 2-6).

**FIGURE 2-5** Smaller complexes in FPIA result in lower fluorescence signal

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**Microparticle Enzyme Immunoassay - MEIA**

*Microparticle Enzyme Immunoassay (MEIA)* is an immunoassay method that utilizes the isolation of antibody/antigen complexes on a solid phase surface of small beads called microparticles. MEIA has been widely adapted to automate the measurement of large molecules such as markers associated with cardiac, fertility, cancer, metabolic, hepatitis, and thyroid testing.

The components of MEIA include the following, all suspended in a specific buffer optimized for the assay (Figure 2-7):

- **Microparticle-Antibody Solid Phase**: Latex microparticles that are coated with antibody to bind the specific analyte being measured;
- **Antibody-Enzyme Conjugate**: Alkaline Phosphatase enzyme conjugated to antibody;
- **Enzyme Substrate**: Fluorescent 4-Methylumbelliferone Phosphate (MUP) in solution that is available for a reaction with the enzyme on the antibody.

**FIGURE 2-7** Components of the MEIA
A summary of how the components work in combination with the sample to produce a signal and corresponding test result is shown in Figure 2-8.

Notice how the glass fiber matrix serves to anchor the complexes. Also note that this method uses a noncompetitive format, so that the amount of analyte is directly proportional to the amount of signal.

Chemiluminescent Magnetic Immunoassay—CMIA
Chemiluminescent compounds can also be used to label analytes. Chemiluminescent compounds are distinct from radioactive, fluorescent, and enzymatic labels. A chemiluminescent label produces light when combined with a trigger reagent. Although many instruments in the clinical laboratory are based on chemiluminescent technology, the specific type of label varies and is often patented, and thus performance can vary. In the case of the Abbott ARCHITECT® (from Abbott Laboratories, Chicago, Illinois, USA), for example, the label is a patented acridinium derivative. This label produces high light emission, and thus high sensitivity (it is easier to measure a large amount of light). The chemical principal is illustrated in Figure 2-9.
The reaction steps for ChemiFlex® (the trademarked name for Abbott’s version of CMIA) are very similar to the MEIA described above, and are compared in Figure 2-10. They both utilize noncompetitive sandwich assay technology to measure analytes. Remember that, in both of these noncompetitive immunoassays, the amount of signal measured is directly proportional to the amount of analyte present in the sample.

**FIGURE 2-9** Abbott ARCHITECT® chemiluminescence label for CMIA

**FIGURE 2-10** Comparison of MEIA and Chemiflex methods
The methods of MEIA and CMIA both use microparticles to anchor antibodies, but there are other similarities and differences as well (Table 2-11). The label, separation step and measurement technology differ between these two methods.

<table>
<thead>
<tr>
<th>Technology</th>
<th>Solid Phase</th>
<th>Separation Step</th>
<th>Label</th>
<th>Detection Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEIA</td>
<td>Latex Microparticle</td>
<td>Glass Fiber Matrix</td>
<td>Alkaline Phosphatase Enzyme</td>
<td>Fluorescence Detector</td>
</tr>
<tr>
<td>CMIA</td>
<td>Magnetic Microparticle</td>
<td>Magnet</td>
<td>Chemiluminescent Compound</td>
<td>Chemiluminescence Photomultiplier Tube</td>
</tr>
</tbody>
</table>

**Nephelometry**

Nephelometry is based on the phenomenon of light scattering (Rayleigh scattering) produced when an incident light beam hits a particle, or an antigen-antibody complex, in solution. The resulting amount of scattered light is used to quantitate the analyte of interest. It is a common immunoassay technique as it is easy to produce immune complexes in solution. Light scattering is affected by several factors, including particle size, molecular weight, analyte concentration, the wavelength of the incident light, the distance from the particle to the detector, and the detector observation angle. The Rayleigh equation accounts for these factors and guides the design of nephelometers. Light scatter is often measured at an angle of 90° (right angle) to the incident beam, although other angles, such as 30°, may also be used. Nephelometry is typically used to measure specific proteins, which form immune complexes with antibodies that bind to them, resulting in crosslinked particles in solution.

The antigen excess (prozone or prezone phenomenon) is a concern as the intensity of scattered light will decrease after an antigen-antibody complex exceeds a certain size. It is important that nephelometric methods be able to detect antigen excess. This can be accomplished by measuring dilutions of the patient sample and measuring changes in the kinetics of immune complex formation using sophisticated algorithms. Matrix effects are also a concern. Lipemia in particular can be a problem as lipoproteins and chylomicrons scatter light and can result in a high background signal. Rate nephelometry is a means of minimizing matrix background interference.

Due to the nature of this methodology, nephelometers tend to be specialized, stand alone analyzers that perform a limited menu of more esoteric tests. It is difficult to integrate a nephelometric optical system with a general purpose clinical chemistry analyzer, which primarily uses spectrophotometry. Rayleigh scattering is useful for small particles that are less than 40 nm in size, and that range includes many of the specific proteins, thus limiting the number of analytes that can be measured.

**Turbidimetry**

Turbidimetry is a methodology related to nephelometry and is based on the interaction between an incident light beam and particles such as immune complexes. The amount of light that passes through a solution of particles decreases as the turbidity of the solution increases. The basic concept is the same as for absorption spectrophotometry, that is, the amount of transmitted light decreases with the concentration of particles (i.e., turbidity of the solution). Turbidity is measured at a 0° angle from the incident light beam, as with spectrophotometry. Thus, a general purpose clinical chemistry analyzer can perform both traditional spectrophotometric assays and turbidimetric assays. Turbidimetric tests, as with nephelometric procedures, require the formation of immune complexes between an antigen and antibodies to the antigen. Thus, both turbidimetry and nephelometry are useful for essentially the same analytes, typically specific proteins. Turbidimetry may use both end point and rate methods. An advantage of turbidimetry is that allows for consolidation of more esoteric tests on a standard, high volume clinical chemistry analyzer as it does not require a specialized, separate instrument. Specific protein testing, can be performed along with routine spectrophotometric assays using a sample from the same primary specimen collection tube, avoiding the need to collect a separate tube from the patient or to prepare an aliquot from the primary tube.
Chapter 2 Summary
There are seven methods of immunoassay that were summarized in this chapter.

Radioimmunoassay (RIA): Antibody or antigen is labeled with radioactivity, and used in a noncompetitive or competitive format.

Enzyme Immunoassay (EIA): Antibody or antigen is labeled with an enzyme that converts a substrate to a product with a resulting signal that is measured, such as a change in color.

Fluorescence Polarization Immunoassay (FPIA): Typically antigen is labeled with fluorescent label and competes with unlabeled antigen from the specimen. The relatively slow rotation of large molecules as well as the ability of slow-moving particles to polarize light are utilized to obtain a measure of the number of large antibody-antigen-fluorescein particles in solution. In this competitive format, the concentration of analyte present is indirectly proportional to the amount of signal measured.

Microparticle Enzyme Immunoassay (MEIA): A solid phase microparticle is coated with antibodies against an antigen of interest, and is used to capture the analyte. The antibody for detection is labeled with an enzyme as in the EIA. The concentration of analyte is proportional to the amount of signal measured. A noncompetitive sandwich format yields results that are directly proportional to the amount of analyte present.

Chemiluminescent Magnetic Immunoassay (CMIA): A chemiluminescent label conjugated to the antibody or antigen, and it produces light when combined with its substrate. This method is very similar to MEIA, though the chemiluminescent reaction offers high sensitivity and ease of measurement. A noncompetitive sandwich format yields results that are directly proportional to the amount of analyte present.

Nephelometry: Antigen and antibody combine in a solution to form an antigen-antibody complex, or lattice. Incident light is scattered at various angles by the complex. The amount of scattered light is proportional to the antigen concentration.

Turbidimetry: The methodology is similar as nephelometry except the decrease in light transmission caused by the antigen-antibody complex is measured as the incident light passes through a cuvette.
Quiz questions for Chapter 2
Circle the correct answer or fill in the blank.

1. List four immunoassay detection technologies
   a 
   b 
   c 
   d 

2. In the FPIA technology...
   a Measured signal is directly proportional to analyte concentration
   b The Ag-Fluorescein molecule rotates rapidly and emits polarized light
   c Both labeled analyte and unlabeled specimen analyte compete for antibody binding sites
   d All of the above

3. FPIA technology is applied using which of the following:
   a Homogeneous assay
   b Noncompetitive format
   c MEIA
   d Heterogeneous assay

4. MEIA utilizes what type of solid phase:
   a Magnetic microparticles
   b Microtiter plates
   c Latex microparticles
   d 1/4 inch beads

5. Which of the following typically represent the most sensitive label?
   a Chemiluminescence
   b Enzyme
   c Fluorescence
   d Microparticle

6. List three classes of labels used in immunoassay methodologies:
   a 
   b 
   c 

Learning Objectives
After completing this section, you will be able to:

• Describe accuracy and precision as it relates to immunoassays
• Discuss the use of calibrators and controls in these assays
• Understand potential pitfalls to immunoassay testing, particularly interferences from the high dose hook effect and human anti-mouse antibodies (HAMA)

Introduction
Manufacturers of immunoassays spend significant time determining which immunoassay method is most appropriate for a specific analyte to ensure the highest quality results. In many instances, a particular method is modified, perhaps with the addition of a special reagent component, to combat some common roadblocks to obtain optimal results. Sometimes these challenges are due to interfering substances present in the blood, problems inherent in the measuring technology, and/or to laboratory technique. This section will discuss how immunoassays are optimized to ensure high quality results that result in optimal patient care. This section includes discussion of the impact of calibration, control material, interferences, and prozone (high dose hook) effects.

Immonoassays Must Be Accurate and Precise
Accuracy and precision in immunoassays is crucial to their usefulness (Figure 3-1). Accuracy means that the assay is giving the laboratorian and clinician the right answer, and describing the concentration of analyte that is actually present. It is often pictured in terms of an arrow hitting a target’s bull’s eye.

Precision in immunoassays means that the combination of reagents, analyzer, and other influencing factors can yield reproducible results. It can be compared to hitting the same spot on a target, though not necessarily the bull’s eye, over and over.

Accuracy is the ability to measure the correct concentration of analyte in a sample. Immunoassays may be accurate but imprecise, precise but inaccurate, or both accurate and precise.
Clinical sensitivity and specificity are typically thought of as subsets of accuracy and precision. If an assay has the ability to accurately and reproducibly generate results that don’t produce false positives, then it is considered specific. A false positive means that a result may incorrectly indicate a certain patient condition exists (the “positive” result does not really identify a patient who is positive). In hepatitis testing, for example, a false positive means the assay indicated a patient is positive for hepatitis, when he/she is not. This can occur for many reasons, but in most instances it is because the assay methodology cannot distinguish between the desired analyte being measured and one that behaves like it (they may share similar molecular structures, but physiologically have different effects on the body). An assay is considered to exhibit clinical sensitivity when it can accurately and reproducibly ensure that false negatives do not occur. Using hepatitis testing again as an example, the laboratorian does not want to give the clinician a result that incorrectly indicates that a patient does not have hepatitis when he/she does. If the patient is truly positive for hepatitis, an assay with poor clinical sensitivity that can’t detect very low levels of hepatitis (is not sensitive enough) may falsely indicate the patient is hepatitis negative (does not have hepatitis).

Calibration and Controls in Immunoassays

**Calibrator Impact on Immunoassays**

Calibrators are solutions with known values that establish the relationship between the amount of signal produced in the assay and analyte concentration. By running a set of calibrators, a “calibration curve” (Figure 3-2) (basically a dose:response curve as discussed earlier) is set up in the instrument’s software and correlates certain values of signal to known analyte concentrations. By comparing levels of signal produced by patient samples to this calibration curve, a patient analyte concentration value, or result, can be determined. It is very important that the calibration material is treated properly per the manufacturer’s package insert, and that all acceptance criteria are appropriate so a correct calibration curve is set. If the calibration is not correct, then the corresponding patient results will not be correct. Most aspects of calibration are automated on today’s immunoassays analyzers, although liquid, refrigerated, and ready-to-use calibrators ensure less potential operator error compared to those that need to be manually reconstituted or thawed.
It is also important that the manufacturer choose the right matrix for the calibrators. The matrix is the solvent containing the analyte in the calibrator (or control). This is to ensure that the signal response of the calibration curve mimics the signal from patient samples. Matrices may be animal serum-based, aqueous, or derived from other materials.

**Calibrator Traceability**

It is not unusual to obtain different results if the same patient specimen is tested for the same analyte using two different immunoassays. The difference may be small or large, but discordance can be a source of confusion to healthcare providers when trying to diagnose or monitor patients, and may even cause physicians to question which test result is “correct” and whether the other is erroneous. Confusion is compounded by the use of different medical decision levels for immunoassays, as in “cutoffs” for cardiac marker assays. For example, an emergency room patient may present with a Troponin-I value that is consistent with a myocardial infarction if tested using one assay, but the troponin value may be below the cutoff for a cardiac event by a different assay. If serial specimens from a patient are tested by two laboratories using different methods and produce discordant values, there’s definitely a chance for clinical misinterpretation unless the proper method specific cutoffs are applied.

There are various reasons for non-equivalent immunoassay results, but a prominent cause is differences in antibody specificity. Antibodies may recognize and bind to different antigens (epitopes), producing different analytical responses, and manufacturers typically do not use identical antibodies. Another common cause is a lack of metrological traceability and standardization for kit calibrators. Calibrators should be prepared from well-recognized reference materials, or from secondary reference materials linked to primary materials, or calibrator values should be assigned by analysis using an accepted reference method. If manufacturers use different reference materials and/or reference methods for the production or value assignment of kit calibrators, immunoassay test results can naturally be expected to vary.

The application of metrological concepts to immunoassays is promoting harmonization of test results. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) is an international consortium that identifies recognized reference materials and reference methods of the highest metrological order. These methods and materials are suitable for calibrator traceability purposes. If manufacturers use them to construct traceability chains for their assay kit calibrators, greater harmonization of test results from different methods can be expected. However, in some cases, more than one reference material or reference method may be available and, if manufacturers trace to different materials or methods, discordant test results may be generated. Even if manufacturers use identical calibrator traceability chains, non-equivalent test values may still exist due to the other method differences (i.e., antibody specificity, analytical signal type, etc.). Use of identical, or at least similar, reference materials and reference methods has been shown to be effective for assay harmonization, decreasing the variability in results when the same specimens are tested by two or more methods.
An example of how metrological traceability can promote harmonization of immunoassays is use of NIST (National Institute for Standards and Technology) SRM 2921, Troponin I (cTnI). The NIST developed this standard reference material to meet the need for a secondary reference material that can be used to evaluate the accuracy of Troponin I tests. SRM 2921 was purified from human heart tissue (as opposed heart tissue from a different species) which contains three forms of cardiac troponin: cTnT, cTnI, and cTnC. The concentration of cTnI in the SRM was determined using amino acid analysis and liquid chromatography. The use of physicochemical methods instead of immunoassays is significant as the resulting quantitative cTnI value can be reproduced within a single laboratory or in different laboratories using the same methods. Thus, the analytical results don’t depend on antibody-antigen binding and are unaffected by variability in the specificity of the antibodies used by different assays, or by lot-to-lot variability for any given assay. The cTnI concentration of SRM 2921 is 31.2 mg/L (+/- 1.4 mg/L) and manufacturers can verify the accuracy of their kit calibrators if linked to the SRM by a traceability chain. If SRM 2921 is proven to be commutable (i.e., responds equivalently to different analytical systems and mimics the response of fresh, human specimens), it allows different assays to be harmonized and to produce comparable results independent of the method, the analyzer, and the laboratory performing the testing. This in turn allows laboratories to use standard medical decision limits (“cutoffs”) for the diagnosis and treatment of myocardial infarction instead of method specific cutoffs.

Control Impact on Immunoassays

Controls are samples that contain known concentrations of analyte. They are used to monitor the accuracy and precision performance of an assay and analyzer. If the control is “in range,” it is assumed the reagents and analyzer are performing correctly and patient testing can begin. Controls are typically assayed every run, shift, or day depending upon the analyte and/or the analyzer, and the manufacturer’s package insert. Analysis is typically performed using a Levey Jenning Chart (Figure 3-3). This sort of chart plots control values and indicates if any potential concerning trends are evolving. If controls are trending up or down (or otherwise behaving in a manner that is not acceptable), it likely indicates a reagent, calibration, or analyzer issue that may be affecting patient results in a similar manner.

FIGURE 3-3 The Levey Jenning chart for analysis and tracking of controls
**Assay Interferences**

One step immunoassays can be prone to interferences that affect both sensitivity and specificity. Interferences most often are due to agents that interfere with the binding of antibodies to antigens for a variety of reasons. In this section, interferences due to high dose hook effects and human anti-mouse antibodies will be discussed. In general, sequential assays (two step) are more likely to yield accurate results by eliminating the adverse contribution of binding proteins, endogenous interfering substances and general matrix effects due to the extra wash step.

**High Dose Hook Effect: Antigen Excess (Prozone Phenomenon)**

Potential for a high dose hook effect is a phenomenon that is inherent with one step “sandwich” assay designs. Very high concentrations of antigen in the patient sample bind to all available sites—saturating them—on both the antibody-solid phase and the antibody-labeled conjugate and thereby prevent the “sandwich” formation. Under these conditions, the measured level of analyte may be significantly lower than the actual level present in the sample. The high dose hook effect refers to the hook that is observed in the dose response curve when data are plotted as a signal versus analyte concentration (Figure 3-4). This high dose hook effect is also called the prozone or prezone effect.

**Human Anti-Mouse Antibodies (HAMA)**

Interference in immunoassays is also caused by the presence of human anti-mouse antibodies (HAMA). These antibodies can be present in human blood as a consequence of the body's immune response to exposure to mouse antigens. There are various reasons that a human's immune system would produce antibodies to “fight off” invading antigens that are derived from exposure to mice. For example, mouse monoclonal antibodies are used in some cancer treatments. A human immune system may also produce a response to mice if the patient handles mice. Environmental exposure to mice is also common.

Patient specimens containing HAMA may produce falsely elevated (false positive) or suppressed (false negative) results in immunoassays that utilize mouse monoclonal antibodies. Sandwich assays are usually the most susceptible to HAMA interference.
**False Positives Due to HAMA:** In the case of false positives, an assay sandwich is formed and thus signal produced, even though no patient analyte is present (Figure 3-5). The HAMA binds to both the solid phase capture antibody as well as the labeled antibody reagent, so it appears as if the analyte in the patient sample is present and causing the complex.

**False Negatives Due to HAMA:** HAMA may cause false negative assay results by two mechanisms: binding and blocking the solid-phase capture antibody, or binding and blocking the labeled antibody reagent.

Manufacturers utilize various techniques to minimize the impact from HAMA including:

- True two step design to wash away interference
- Blockers to reduce if not eliminate interference by occupying the binding sites on HAMAs.
Interferences

**Heterophile Antibodies**

Heterophile antibodies are produced against antigens from other species, such as those from animal red blood cells or animal serum. Patients may be exposed to animal antigens in a variety of ways, including inoculation with a vaccine based on animal serum or by handling animals or even just by being exposed to them (e.g., hair or dander). Heterophile antibodies are commonly found in various diseases such as mononucleosis (non-Epstein-Barr antibodies), and, more rarely, in leukemia, cytomegalovirus infection, Burkitt's lymphoma, rheumatoid arthritis, and viral hepatitis. Heterophile antibodies can interfere with immunoassays in the same manner as HAMA, binding to antibodies used in assays and potentially causing either falsely elevated or falsely decreased results. There is not a practical means of predicting the presence of these antibodies in patients as it's unknown if an individual has been exposed to the kinds of foreign materials producing a heterophilic immune response. In addition, individual immune responses to these materials varies greatly. As with HAMA, immunoassays may avoid, or at least minimize, interference by heterophile antibodies by using a two-step design to wash away these antibodies before they can bind to reagent antibodies, or by adding blocking substances to which the heterophile antibodies will bind. Other means of dealing with heterophile antibodies are to test dilutions of a patient's specimen (antibody is diluted to a concentration so low that it does not interfere) or by testing the patient's specimen using one or more assays from other manufacturers (other tests may use different reagent antibodies that are unaffected by the heterophile antibody). When faced with unexpected immunoassay test results that appear to be inconsistent with other laboratory findings or the clinical presentation, the possibility of heterophile antibody interference causing a false positive or false negative result should be considered.
Chapter 3 Summary

**Accuracy** means that the assay is giving the right answer, measuring the amount of analyte that is actually present in the sample.

**Precision** means that the assay measures the amount of analyte in a reproducible manner every time.

**Clinical sensitivity** and **clinical specificity** refer to the ability of the assay to accurately and reproducibly generate results that minimize the numbers of false positives and false negatives that occur.

**Calibrators** are samples of known concentration used to set analyzer or instrumentation parameters.

**Controls** are samples of known concentration that are used to monitor the accuracy and precision of an analyzer and assay.

One type of interference that can reduce the accuracy and precision of immunoassays is the **high dose hook effect**, also called the prozone effect. Under conditions of antigen excess, the assay reads out less antigen concentration than is actually present.

Another type of interference is due to the presence of **human anti-mouse antibodies** (HAMA) that may be present in the serum of patients who have been treated with mouse antibodies for therapy, or exposed to mice in other ways.

**Two-step immunoassays** can reduce interference from a high dose hook effect.
Quiz questions for Chapter 3
Circle the correct answer or fill in the blank.

1. The ability of an assay to reproducibly hit a target concentration is referred to as:
   a) Specificity
   b) Sensitivity
   c) Precision
   d) Accuracy

2. Antigen excess "hook effect" interference may be eliminated with which of the following:
   a) Two step immunoassay methodology
   b) One step immunoassay methodology
   c) Decreased antibody reagent
   d) Decreased solid support reagent

3. Interference from HAMA can be eliminated by utilizing two step immunoassay methodologies.
   a) True
   b) False

4. HAMA antibodies occur as a result of:
   a) Patient exposure to mice or mouse proteins
   b) Contamination of the sample with mouse proteins
   c) Immunization
   d) Food allergies

5. Calibrators are samples from individuals known to be in the normal range that are used to check instrumentation set-up.
   a) True
   b) False
Learning Objectives
After completing this section, you will be able to:

- Name several classes of tests that utilize immunoassay technology.
- Describe use of specialized tests for endocrine hormones, cardiovascular damage, hepatitis antigens, and tumor antigens.²

Introduction
By completing sections one through three of this Learning Guide, you have learned various technologies and techniques used in the application of immunoassays. This section will discuss application of these various immunoassay techniques to specific laboratory tests. There are four major classes of specialized immunoassays that will be covered: endocrine hormones, cardiac markers, hepatitis antigens, and tumor antigens.

Classes of Specialized Tests
Endocrine Hormones
Hormones are generally produced in specialized endocrine glands and are transported to cells via the blood circulatory system. Examples of endocrine glands include the hypothalamus, anterior pituitary lobe, thyroid gland, ovary, testes, adrenal cortex, placenta, and pancreas. Hormones play a variety of roles in maintaining normal functioning of the body, and measuring levels of these hormones can be crucial for diagnosing an illness or for monitoring treatment. Several key hormones are typically measured by immunoassay technology (see Table 4-1).

² The sponsor of this educational Learner Guide, Abbott Laboratories, does not make any representations of its or other products in the U.S. or outside the U.S.
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Testing Category</th>
<th>Primary Site of Action</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid Stimulating Hormone (TSH)</td>
<td>Thyroid</td>
<td>Thyroid gland</td>
<td>Stimulation of thyroid hormone secretion</td>
</tr>
<tr>
<td>Thyroxine (T4) and Triiodothyronine (T3)</td>
<td>Thyroid</td>
<td>General body tissue</td>
<td>Stimulation of oxygen consumption and metabolic rate of tissue</td>
</tr>
<tr>
<td>Free Thyroxine (T4) and Free Triiodothyronine (T3)</td>
<td>Thyroid</td>
<td>General body tissue</td>
<td>Free forms of thyroid hormones found in serum that are not bound to other proteins, hence “free”; thought to be more accurate measure of thyroid status</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Metabolic</td>
<td>General body tissue</td>
<td>Metabolism of carbohydrates, proteins and fats</td>
</tr>
<tr>
<td>Follicle-Stimulating Hormone (FSH)</td>
<td>Fertility</td>
<td>Ovary, testes</td>
<td>Growth of follicles and secretion of estrogens and ovulation</td>
</tr>
<tr>
<td>Luteinizing Hormone (LH)</td>
<td>Fertility</td>
<td>Ovary, testes</td>
<td>Ovulation, secretion of progesterone</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Fertility</td>
<td>Mammary gland</td>
<td>Growth of mammary gland, initiation of milk production</td>
</tr>
<tr>
<td>Estrogen</td>
<td>Fertility</td>
<td>Female accessory sex organs</td>
<td>Development of secondary sex characteristics</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Fertility</td>
<td>Female accessory reproductive structure</td>
<td>Preparation of the uterus for ovum implantation, maintenance of pregnancy</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Fertility</td>
<td>Male accessory sex organ</td>
<td>Development of secondary sex characteristics</td>
</tr>
<tr>
<td>Human chorionic gonadotropin (hCG)</td>
<td>Pregnancy/Fertility</td>
<td>Ovary, testes</td>
<td>Maintenance of corpus luteum function, ovulation, secretion of progesterone</td>
</tr>
</tbody>
</table>
**Indicators of Cardiovascular Damage**

Assessment of cardiac damage during or following a myocardial infarction (or heart attack) is strongly aided by immunoassay testing. Immunoassay testing for specific proteins released during and after a heart attack is often performed for diagnosis and monitoring of the heart condition. Such markers include troponin, myoglobin, CK-MB, and, most recently B-type, Natriuretic Peptide (BNP) and high sensitivity C-Reactive Protein (hsCRP). The markers are listed in Table 4-2.

**TABLE 4 Markers of Cardiovascular Damage**

<table>
<thead>
<tr>
<th>Marker of Cardiac Damage</th>
<th>Description</th>
<th>How levels correlate with cardiovascular damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin I</td>
<td>A protein that is released from dead and injured heart muscle cells</td>
<td>Levels increase as early as 4 hours following myocardial infarction; alone or better when combined with tests for CK-MB (see below) this test is a very reliable diagnostic test; remains elevated for up to 8 days following damage</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>A protein found in heart and other muscles, important for oxygen transport to muscle cells</td>
<td>Released upon damage of any muscles; levels increase as early as 2 hours following myocardial infarction; levels return to reference ranges by about 24 hours; considered a very sensitive early diagnostic test, especially in conjunction with other markers of myocardial infarction</td>
</tr>
<tr>
<td>Creatinine Kinase-MB (CK-MB)</td>
<td>A form of the enzyme found mostly in heart muscles and released upon damage</td>
<td>Levels increase as early as 4 to 6 hours following myocardial infarction; rapid immunoassays are useful for emergency room diagnosis; considered a standard test in diagnosis of myocardial infarction</td>
</tr>
<tr>
<td>B-type Natriuretic Peptide (BNP)</td>
<td>A protein hormone released from the left ventricle of the heart when damaged</td>
<td>Increased levels are indicative of injury or stress to the left ventricle of the heart, which includes myocardial infarction, hypotension (low blood pressure), or stress on the ventricular muscle from accumulated blood and fluid as in congestive heart failure</td>
</tr>
<tr>
<td>High Sensitivity CRP (hsCRP)</td>
<td>A protein released into serum in response to injury, infection, and inflammation</td>
<td>May be useful for predicting risk of another myocardial infarction if one has already occurred; however, multiple triggers for CRP production must be considered when interpreting results</td>
</tr>
</tbody>
</table>
**Hepatitis**

Hepatitis is inflammation of the liver, and can be caused by viruses, bacteria, drugs, toxins or excess alcohol intake. In part, due to the development of immunoassays, five distinct viruses have currently been identified that cause hepatitis: Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, and Hepatitis E. Each virus can be identified individually using immunoassay methods (see Table 4-3).

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Routes of Transmission</th>
<th>Onset</th>
<th>Incubation</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis A virus (HAV):</td>
<td>Fecal Oral Route</td>
<td>Usually abrupt</td>
<td>15 – 50 days</td>
<td>≤ 39 years: ≤ 0.3%</td>
</tr>
<tr>
<td>Picornaviridae; Small, single-stranded RNA virus</td>
<td></td>
<td></td>
<td></td>
<td>≥ 40 years: 2.1%</td>
</tr>
<tr>
<td>Hepatitis B virus (HBV):</td>
<td>Percutaneous</td>
<td>Usually insidious</td>
<td>Average: 60 – 90 days</td>
<td>0.5 – 1.0%</td>
</tr>
<tr>
<td>Hepadnaviridae; Partially double-stranded DNA virus</td>
<td></td>
<td></td>
<td>Range: 45 – 180 days</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (HCV):</td>
<td>Percutaneous, Permucosal</td>
<td>Insidious</td>
<td>14 – 182 days</td>
<td>About 60 – 85% infected individuals became chronic; In the U.S. 10,000 to 12,000 die each year from HCV associated chronic liver disease</td>
</tr>
<tr>
<td>Flaviviridae; single-stranded RNA virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis D virus (HDV):</td>
<td>Percutaneous, Permucosal</td>
<td>Usually abrupt</td>
<td>21 – 49 days</td>
<td>2 – 20%</td>
</tr>
<tr>
<td>single-stranded RNA virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis E virus (HEV):</td>
<td>Fecal Oral Route (especially contaminated water)</td>
<td>Usually abrupt</td>
<td>Average: 40 days</td>
<td>About 1 – 3%, 15 – 25% in pregnant women</td>
</tr>
<tr>
<td>Single-stranded RNA virus</td>
<td></td>
<td></td>
<td>Range: 15 – 60 days</td>
<td></td>
</tr>
</tbody>
</table>

All types of viral hepatitis affect liver cells. Individuals infected with a hepatitis virus tend to have generalized symptoms, which in the early stages are similar to the flu. Symptoms include fatigue, joint/muscle pain, loss of appetite, nausea, diarrhea, fever, and jaundice. Because symptoms are not specific, immunoassays are required to distinguish between specific viruses. Hepatitis tests are utilized for both screening and monitoring purposes. Please refer to the Abbott Diagnostics Hepatitis Learning Guide for a more detailed primer on hepatitis.

**HIV**

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2). Within one to two months after infection with HIV, individuals may experience acute symptoms that are similar to other viral infections (fever, headache, tiredness, enlarged lymph nodes) and do not persist. More severe and persistent symptoms of infection do not generally appear until years after infection. HIV kills cells of the immune system leading to a progressive deterioration of the body’s ability to fight infections and certain cancers. There is no cure for HIV, however, highly active antiretroviral therapy (HAART) can slow disease progression and greatly prolong a person’s life expectancy.

HIV infection is most commonly diagnosed using immunoassays to detect antibodies to HIV.
TABLE 4-4 HIV Viruses

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Routes of Transmission</th>
<th>Onset</th>
<th>Incubation</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1: Retroviridae; single strand RNA lentivirus</td>
<td>Most commonly transmitted through sexual intercourse. Also transmitted by contaminated needles, unscreened blood products, and mother-to-child via perinatal transmission or breast feeding.</td>
<td>Insidious, the symptom-free phase varies in length from several months to more than 10 years.</td>
<td>20 – 60 days</td>
<td>100%, delayed by HAART</td>
</tr>
<tr>
<td>HIV-2: Retroviridae; single strand RNA lentivirus</td>
<td>Transmitted by the same routes as HIV-1; however, HIV-2 is less infectious and transmission frequency is lower. Insidious, progression to disease is slower than for HIV-1 infections and symptoms are generally milder.</td>
<td></td>
<td>20 – 60 days</td>
<td>100%; asymptomatic phase longer and life expectancy higher than for HIV-1</td>
</tr>
</tbody>
</table>

Cancer Tumor Markers

Tumor cells can produce proteins that are detected in serum by immunoassay, and these proteins are called tumor markers. Tumor markers are usually specific for a certain type of cancer. Measuring tumor markers can be useful to diagnose and monitor different types of cancer, and these tests can be used to detect cancer and monitor progression of cancer treatment (see Table 4-5).

TABLE 4-5 Tumor Markers Detected by Immunoassay

NOTE: not all immunoassays on the market are approved by government regulators for all indications listed below.

<table>
<thead>
<tr>
<th>Classes</th>
<th>Example</th>
<th>Site of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormones</td>
<td>Human Chorionic Gonadotropin (hCG)</td>
<td>Testicular, Breast, GI Tract, Lung, Ovary</td>
</tr>
<tr>
<td>Oncofetal Antigens</td>
<td>Alpha Fetoprotein (AFP)</td>
<td>Testes</td>
</tr>
<tr>
<td></td>
<td>Carinoembryonic Antigen (CEA)</td>
<td>Colorectal, GI Tract, Lung, Breast</td>
</tr>
<tr>
<td></td>
<td>Prostate Specific Antigen (PSA)</td>
<td>Prostate</td>
</tr>
<tr>
<td>Carbohydrate Markers</td>
<td>CA 15-3, CA 549, CA 27-29</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td></td>
<td>CA 125</td>
<td>Ovarian and Endometrial Carcinoma</td>
</tr>
<tr>
<td>Blood Group Antigens</td>
<td>CA 19-9</td>
<td>Pancreas, GI tract</td>
</tr>
<tr>
<td></td>
<td>CA 50</td>
<td>Pancreas, GI tract</td>
</tr>
<tr>
<td></td>
<td>CA 72-4</td>
<td>Ovary, GI tract</td>
</tr>
<tr>
<td></td>
<td>CA 242</td>
<td>Pancreas, GI tract</td>
</tr>
<tr>
<td>Proteins</td>
<td>Monoclonal IgA, Monoclonal IgG</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td></td>
<td>Monoclonal IgM, B2-Microglobulin</td>
<td>Multiple Myeloma, Waldenstrom Macroglobulinemia</td>
</tr>
</tbody>
</table>
Ideally, tumor markers would be both 1) specific for the target organ, and 2) produced in a sufficiently large concentration to be accurately detected in patients with cancer but not in a normal population. Unfortunately, with the possible exception of PSA (a common marker used to indicate presence of prostate cancer), both characteristics are rarely achieved. Therefore, in clinical practice, tumor markers are most useful to aid in diagnosis in combination with other diagnostic tests (e.g. MRI), and for monitoring progression of disease after detection and treatment.

The rate and magnitude of tumor marker reduction is used to evaluate the effectiveness of treatment. After treatment, the tumor marker should reflect whether the treatment has been successful. A decrease in the level of the tumor marker should reflect a positive response to treatment while a minimal change in the level of the tumor marker would reflect a poor response to treatment. If treatment is successful, then once the tumor marker level has stabilized to a new lower level, regularly scheduled follow-up measurements of the marker are helpful to assess stability of the disease.

**Congenital Tests**

The acquisition of certain infectious agents during pregnancy can result in the intrauterine transmission of disease from the pregnant woman to the developing fetus. Transmission of a primary CMV infection, an acute toxoplasmosis, or a primary Rubella infection, especially during the first trimester, can cause significant fetal morbidity and mortality. Patients with impaired immune systems due to AIDS, cancer therapy, and those undergoing immunosuppressive therapy following transplantation are also at risk of morbidity due to these infectious agents.

Tests are available to detect specific antibodies in response to these infectious agents as shown in Table 4-5. A positive test result for IgG antibodies indicates previous exposure to the virus or parasite. A positive test result for IgM antibodies may indicate a primary or acute infection is present but further confirmatory testing by an IgG avidity assay is required in order to assess the stage of infection and potential risk to the fetus. The IgG avidity assay measures the functional binding affinity of the IgG class of antibody in response to infection and helps distinguish between acute or primary infection versus non-primary infection.

**TABLE 4-6 Tests for Congenital Factors**

<table>
<thead>
<tr>
<th>Test</th>
<th>Factor that is measured</th>
<th>Medical conditions which can be caused by infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rub IgG and Rub IgM</td>
<td>IgG or IgM antibodies to Rubella</td>
<td>Causes measles that is usually mild in children and adults; infection during pregnancy can be detrimental to the fetus and result in miscarriage, death, and birth defects</td>
</tr>
<tr>
<td>Toxo IgG, Toxo IgM</td>
<td>IgG or IgM antibodies to <em>Toxoplasma gondii</em></td>
<td>Lymphadenopathy, chorioretinitis, congenital birth defects including hydrocephalus, chorioretinitis, mental retardation</td>
</tr>
<tr>
<td>Toxo IgG Avidity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV IgG, CMV IgM</td>
<td>IgG or IgM antibodies to Cytomegalovirus</td>
<td>Interstitial pneumonia, mononucleosis, abortion, congenital birth defects including mental retardation, blindness and deafness</td>
</tr>
<tr>
<td>CMV IgG Avidity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Metabolic Tests

Immunoassays are also available for a wide variety of indicators for metabolic functions and nutritional adequacy. Specific deficiencies can arise as a result of diet inadequacy, or as an indication of physiological disease, such as anemia. There are multiple forms of anemia that require careful diagnosis and treatment. Some examples of these kinds of tests and their indications are listed in Table 4-7.

<table>
<thead>
<tr>
<th>Test</th>
<th>Role of factor in maintaining health</th>
<th>Indications of inappropriate levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td>Protein that stores iron for later use by multiple tissues in the body</td>
<td>Levels of serum ferritin correlate with amount of iron stored; low levels indicative of anemia or diet deficiency</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>A vitamin, cobalamin, that helps form red blood cells which carry oxygen to the tissues in the body</td>
<td>Deficiencies can cause macrocytic anemia, neurologic disease, and be an indication of autoimmune and other disorders of the digestive tract</td>
</tr>
<tr>
<td>Folate</td>
<td>A vitamin that is a cofactor in several metabolic pathways</td>
<td>Deficiencies during pregnancy may cause spinal birth defects; deficiency of both B12 and folate can lead to megaloblastic anemia</td>
</tr>
</tbody>
</table>

Specific Proteins

There are a large number of specific protein analytes that are commonly measured by immunoassays, including Apo A1, Apo B, Beta 2 Microglobulin, C3, C4, CRP, ferritin, haptoglobin, immunoglobulins (IgA, IgG, IgM), RF (rheumatoid factor), prealbumin, and transferrin. New specific protein analytes, such as anti-CCP for rheumatoid arthritis, can be expected to become routine tests as proteomic research links unique proteins with specific diseases.

Many specific proteins are non-specific markers of a general inflammatory response, such as C3, C4, CRP, and prealbumin. Others are associated with specific pathological conditions or well-defined diseases and are very useful for diagnosis when coupled with a patient’s medical history and clinical presentation. A common example is increases in an immunoglobulin fraction due to multiple myeloma. Table 4-8 lists some of the more common specific proteins and their diagnostic value.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Pathological Condition/Disease</th>
<th>Analyte Response</th>
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<tbody>
<tr>
<td>Anti-CCP (anti-cyclic citrullinated peptide antibody)</td>
<td>Rheumatoid arthritis</td>
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<tr>
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<td>Cardiovascular disease</td>
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<tr>
<td>Apolipoprotein B (Apo B)</td>
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<td>Complement C3 &amp; C4 (C3, C4)</td>
<td>Inflammation</td>
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<tr>
<td>C-Reative Protein (CRP)</td>
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<td>Ferritin</td>
<td>Iron deficiency anemia</td>
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<td>Immunoglobulin G (IgG)</td>
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<tr>
<td>Prealbumin</td>
<td>Malnutrition</td>
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<tr>
<td>Rheumatoid Factor (RF)</td>
<td>Rheumatoid arthritis</td>
<td>Increased</td>
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Other Tests and Immunoassay Technologies
Many other analytes are tested using immunoassays. Immunoassays are also used to test levels of therapeutic drugs in serum, which can be helpful for ensuring proper dosing of medication for an individual patient. Drug of abuse testing is another application of immunoassays.

Chapter 4 Summary
This chapter focused on examples of immunoassays commonly used in the clinical setting. There were six categories of tests reviewed here.

**Endocrine Hormones:** Assays for a range of thyroid, fertility, and pregnancy hormones include tests for thyroid stimulating hormone, follicle stimulating hormone, testosterone, and more.

**Indicators of Cardiovascular Damage:** Assays for proteins released by damaged heart muscle following a heart attack are useful for diagnosis and treatment of this condition. Tests include those for measuring troponin, myoglobin, creatinine kinase-MB, B-type Natriuretic Peptide, and other cardiac markers.

**Hepatitis Antigens:** Assays for each of five different strains of Hepatitis viruses are utilized for diagnosis and monitoring purposes.

**Tumor Antigens:** Assays for specific antigens produced by cancer cells can be used for diagnostic and monitoring purposes, and include prostate specific antigen (PSA) for prostate cancer and alpha fetoprotein (AFP) for testicular cancer, among other examples.

**Congenital Infectious Agents:** Serum assays for IgM and IgG antibodies in people exposed to infectious agents such as rubella, toxoplasmosis, and cytomegalovirus (CMV) are useful for diagnosis and monitoring of possible infection, particularly in pregnant women in order to assess the risk of birth defects in their children.

**Metabolics:** Assays for proteins and vitamins involved in metabolism can be measured, including ferritin as a measure of iron storage levels, and the vitamins B12 and folate.
Quiz questions for Chapter 4
Circle the correct answer or fill in the blank.

1. Tests for which of the following are not routinely quantified using immunoassays:
   a. TSH
   b. AFP
   c. Glucose
   d. Folate

2. A test for hCG would be classified as a:
   a. Thyroid assay
   b. Hepatitis marker
   c. Pregnancy marker
   d. Cancer marker

3. Which of the following are the five major viruses known to cause viral hepatitis:
   a. HBV1, HBV2, HBV3, HBV4, and Epstein-Barr Virus
   b. HAV, HBV, HCV, HDV, HEV
   c. Jaundicemia and its four sub-types
   d. None of the above

4. Viral hepatitis panels are used to:
   a. Diagnose
   b. Monitor
   c. Screen
   d. All of the above

5. A patient who is positive for IgG antibodies to Rubella was recently exposed to the virus and is mounting an early immune response.
   a. True
   b. False
### Appendix A: Answers to Quiz Questions

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### Appendix B: Bibliography and Suggestions for Further Reading


Appendix C: Glossary of Terms

Affinity – a measure of the attraction between a single antigenic site and a single antibody to that site.

Analyte – the substance, set of substances, or "factor" to be assayed.

Antibody – a glycoprotein produced by B lymphocyte cells in response to exposure to an antigen and binds specifically to that antigen.

Antigen – a substance that is capable, under appropriate conditions, of inducing a specific immune response and of reacting with the product of that response (antibody or specifically sensitized T-lymphocyte).

Antigen excess – the presence of excess antigen in relationship to antibody concentration, resulting in underestimation of antigen concentration (sometimes call "prozone effect").

Antiserum – a serum containing antibodies.

Avidity – the combined intensity of reactivities of an antibody and antigen, representing net affinity of all binding sites in the antiserum.

Calibrator – a material of known characteristics (concentration, activity, reactivity) used to calibrate or adjust an assay procedure. The material should have the same performance characteristics as the test samples in that procedure.

Chemiluminescence – emission of light through a chemical reaction involving the oxidation of an organic compound by an oxidant (such as hydrogen peroxide).

Dose response curve – describes the relationship between assay signal and analyte concentration.

Epitope – a unique region on an antigen that binds to a specific antibody.

ELISA (Enzyme-linked immunosorbent assay) – an immunoassay utilizing an antibody labeled with an enzyme marker. The change in enzyme activity as a result of the enzyme-antibody-antigen reaction is proportional to the concentration of antigen and can be measured.

False positive assay result – a positive test result obtained from a truly negative sample.

False negative assay result – a negative test result obtained from a sample truly containing analyte.

Fluorescence polarization – occurs when a molecule absorbs light at one wavelength and re-emits light at a longer wavelength.

HAMA (Human Anti Mouse Antibody) – produced in humans as an immune response following exposure to mouse antibodies.

Immune complexes – the complexes formed by the binding of antigen and antibody molecules, with or without complement fixation.

Immunoglobin – a protein comprised of heavy and light chains and functioning as an antibody.

Monoclonal antibodies – antibodies produced in vitro by a cell line arising from a single cell. All molecules are of a single class and subclass and have a single antigenic specificity.

Noncompetitive immunoassay – an immunoassay in which specimen analyte and labeled analyte are presented sequentially to the reaction antibody.

Precision – the extent to which replicate analyses of a sample agree with each other. In statistics, it is the inverse of the variance of a measurement or estimate.

Radioimmunoassay (RIA) – a quantitative assay for the detection of antigen-antibody reactions using radioactively labeled substance to measure the binding of unlabeled substance to a specific antibody or receptor.

Sandwich assay – used to describe an immunoassay, which binds analyte between two specific antibodies.

Clinical Sensitivity – the ability of a test to correctly classify patients who have a disease by producing a true positive test result.

Clinical Specificity – the ability of a test to correctly classify patients who do not have a disease by producing a true negative test result.