



TITLE: Methodology: Immunoassays

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Slide 1: Introduction

This portion of the Pearls of Laboratory Medicine series will serve as an introduction to clinical immunoassays. Topics for discussion will include basic antibody structure, immunoassay designs, common types of immunoassays found in the clinical laboratory, and mechanisms of interferences seen with clinical immunoassays.

Slide 2: Antibody Introduction

Antibodies are glycoproteins commonly referred to as immunoglobulins and are produced by the immune system when a foreign molecule is detected in the body. Antibodies have constant regions and variable regions, with the antigen binding site located in the variable region. Monoclonal antibodies are produced by a single clone of plasma cells and all bind to the same epitope of an antigen. Polyclonal antibodies are produced by many plasma cell clones and bind to a wide variety of epitopes on an antigen. How tightly an antibody binds to an antigen is termed the affinity while the number of potential binding sites is the avidity.

Analytical methods that use antibodies for detection are referred to as immunoassays. The reasonably specific binding of antibodies to their targets makes immunoassays effective methods for detection of analytes in complex biological matrices. Several different types of immunoassays are used clinically and differ in design, detection mechanism and how the assay reagents are combined with the sample.

Slide 3: Heterogeneous vs. Homogeneous Assays

Heterogeneous immunoassays require separation of the analyte-antibody complex from the remaining sample prior to final analysis. This can be accomplished using precipitating chemicals, cross-linking with other antibodies or use of an antibody bound to a solid phase. Once the remaining matrix components are washed away, the remaining assay components are added for final detection.

Homogenous assays can distinguish between free and antibody-bound analyte and do not require a physical separation of analyte-antibody complex from the remaining sample.

Slide 4: Competitive vs. Noncompetitive immunoassays

Immunoassays also differ on the type of reaction method used. Competitive immunoassays restrict the number of antigen binding sites, resulting in a competition for antibody binding between the endogenous analyte and a detectable, labeled analogue. As a result, the amount of labeled analogue bound is inversely proportional to the amount of analyte in the sample. As the amount of analyte in the sample increases, the detectable signal decreases. Competitive immunoassays can be classified as simultaneous addition where all components are added at once or sequential addition where the sample is incubated with the antibody before the labeled analogue is added.

Noncompetitive immunoassays are designed to have excess antibody binding sites and produce a signal directly proportional to the amount of analyte in the sample. In sandwich immunoassays, two separate antibodies are used. A capture antibody is bound to a solid support and is used to extract the analyte of interest from the sample. A second antibody is labeled to allow detection and binds to a separate site on the analyte, resulting in an antibody sandwich, with the analyte positioned in the middle of the two antibodies. As the amount of analyte in the sample increases, the detectable signal increases.

Slide 5: Immunoassay Detection Methods: Fluorescence

For final detection, a wide variety of immunoassay detection methods are currently used in the clinical laboratory, including fluorescence, chemiluminescence, radioactivity, and enzyme-based detection.

Fluorescence detection uses a dye-labeled antibody directed against an analyte of interest, with the amount of detected fluorescence proportional to the amount of analyte in the sample. Another common type of fluorescent immunoassay is a fluorescence polarization immunoassay that uses fluorescently labeled analogues rather than a labeled antibody. When labeled analogue is unbound by antibody it is free to rotate and there is low polarization of light. When the labeled analogue becomes bound by antibody, its rotation is slowed by the mass of the antibody and there is an increase in polarized light. The change in polarization is directly proportional to the amount of analyte in the sample.

Slide 6: Immunoassay Detection Methods: Enzymatic

The most common enzyme-based antibody labels include alkaline phosphatase, peroxidases, glucose-6-phosphate dehydrogenase and beta-galactosidase. Enzymatic immunoassays are advantageous due to the amplification inherent to the enzymatic process. The substrate to product reaction can produce a colored product, fluorescent product, a photon or even an intermediate substrate for a second enzymatic reaction.

Enzyme-linked immunosorbent assays are common in the clinical laboratory and use a capture antibody and an enzyme-labeled detection antibody. Many automated instruments use an enzyme-labeled detection antibody and a chemiluminescent substrate producing a photon for measurement.

Slide 7: Immunoassay Detection Methods: EMIT and CEDIA

Two other types of enzyme detection include EMIT and CEDIA. An enzyme-multiplied immunoassay or EMIT uses an enzyme-labeled analogue that competes with endogenous analyte binding to a test antibody. When the antibody binds the enzyme-labeled analogue it is unable to convert substrate to product. The change in enzymatic activity is proportional to the concentration of the analyte in the sample, with high concentrations having high enzyme activity.

A cloned enzyme donor immunoassay or CEDIA uses genetically engineered inactive fragments of beta-galactosidase. One fragment is bound to an analogue, and if the test antibody binds the labeled antigen, reformation of the enzyme fragments is inhibited. Conversely, if the labeled antigen does not interact with the antibody, as is the case with high concentration of the analyte in the sample, the enzyme fragments recombine and produce product from provided substrate.

Slide 8: Turbidimetry vs. Nephelometry

Aggregation of antibody and antigen into large complexes is another common method used in clinical immunoassays. Both turbidimetry and nephelometry are based on the concept of light scattering. Turbidimetry measures a reduction in light transmission as a result of antigen-antibody complex formation. As antigen-antibody complexes form, light scattering increases while light transmission decreases.

Conversely, nephelometry detects a portion of the light that is scattered at a chosen measurement angle by the antigen-antibody complex. As antigen-antibody complexes form, light scattering at the measured angle increases. Both methods make use of a spectrophotometer for the final measurement.

Slide 9: POC Immunoassay Designs

An important application of immunoassays is seen with various point-of-care assays. Point-of-care immunoassays are popular in emergency situations and for in-home testing. Variations in design exist amongst vendors, with many including a porous membrane to draw in liquid sample and a labeled antibody. The antibody-analyte complex can be detected with a second capture antibody and a color development step or the use of colored microparticles. Alternatively, optical immunoassays use a change in light reflection when the thickness of a thin antibody film increases with antigen binding. An important feature of many point-of-care immunoassays is the inclusion of a built-in quality monitor to verify proper storage and appropriate device operation.

Slide 10: Assay Cross-reactivity and Interference

With immunoassays comprising a substantial component to clinical laboratory testing, their advantages and disadvantages are well documented. One advantage with the use of antibody-based detection methods is the ability to screen for structurally similar compounds in a single assay. This cross-reactivity is utilized in drug screening methods that provide rapid turn-around-time but less overall certainty to the specific identity of the measured analyte. This same cross-reactivity can also be a significant problem with immunoassays if a highly specific measure of only a single analyte is needed.

Anti-reagent antibodies, often referred to as heterophile antibodies, present a major challenge to the current use of immunoassays in diagnostic medicine. Anti-reagent antibodies are problematic for sandwich immunoassays where antibodies present in the patient sample bridge the detection and the capture antibodies even in the absence of analyte. Importantly, both false negative and false positive results can occur due to anti-reagent antibody.

The hook effect is another well-known interference that occurs when high concentrations of analyte are present in the sample. The saturation of both capture and detection antibodies reduces sandwich formation leading to falsely decreased levels that are only detected by reanalysis of a diluted sample.

A last major obstacle is the variation in antibodies used in different vendor assays for the same analyte. Each manufacturer's antibody will likely bind to different epitopes of the same antigen, resulting in different values of analyte for the same sample. This problem is circumventable to some degree by the continued use of the same vendor's assay, but provides ongoing difficulty in universal reference interval establishment.

Slide 11: References