



An Introduction to Immunoassay

Immunoassay is an important laboratory technique that can be used to measure concentrations of many different substances found in saliva, blood, or other body fluids. It does this by making use of antibodies, which are special proteins made in the body in response to invading micro-organisms or other foreign substances.

Most immunoassays share two basic steps:

- Antibodies prepared beforehand against a specific antigen are used to capture molecules of the antigen present in samples. Alternatively, antigens may be used to capture antibodies present in samples. The substance of interest that is captured is known as the analyte.
- A means of measuring the concentration of the captured analyte must be included. This is most often done by introducing some type of measurable label that directly or indirectly indicates the presence of the analyte.

Most modern assays employ a labeling design known as enzyme immunoassay, which uses enzymes that have been coupled to antibodies or antigens (the enzyme conjugate). The conjugated enzymes act on compounds known as substrates to modify them chemically. Salimetrics uses TMB (3,3',5,5'-tetramethylbenzidine) as the substrate in its immunoassay kits. TMB is often the preferred colorimetric substrate because it gives the highest color intensity and low background values.ⁱ Salimetrics uses horseradish peroxidase (HRP) as the conjugated enzyme in most of its immunoassay kits. It is one of the most favored enzymes used in immunoassay because it acts on the substrate at a high rate, which increases the amount of color produced.ⁱⁱ

In addition to colorimetric assays, there are also chemiluminescent immunoassays (CLIA or ChLIA), which use substrates that yield products that emit chemically-produced light.ⁱⁱⁱ Chemiluminescent substrates are expensive, however, and they require a specialized instrument for measuring the light emitted. For most purposes, and particularly with competitive enzyme immunoassays (EIA, see below), colorimetric measurement can give sufficiently low detection limits,^{iv} and accurate results.

Immunometric Assays

Numerous schemes have been developed that use antibodies to capture and measure analytes. The details of some can be complex, but most are designed around two basic strategies. Perhaps the easiest to understand is the immunometric assay (Figure 3):

- Antibodies immobilized onto a plastic surface (most often a 96-well microtiter plate) are used to capture the target antigen present in the sample.
- A second antibody linked to an enzyme (the conjugate) is then added. It binds to a different location on the target antigen.
- Plate wells are washed to remove unbound components.
- Substrate is added. Bound enzyme present reacts with the substrate, yielding color.
- The enzymatic reaction is stopped in order to establish a consistent time period for all wells. After stopping, the color is measured.

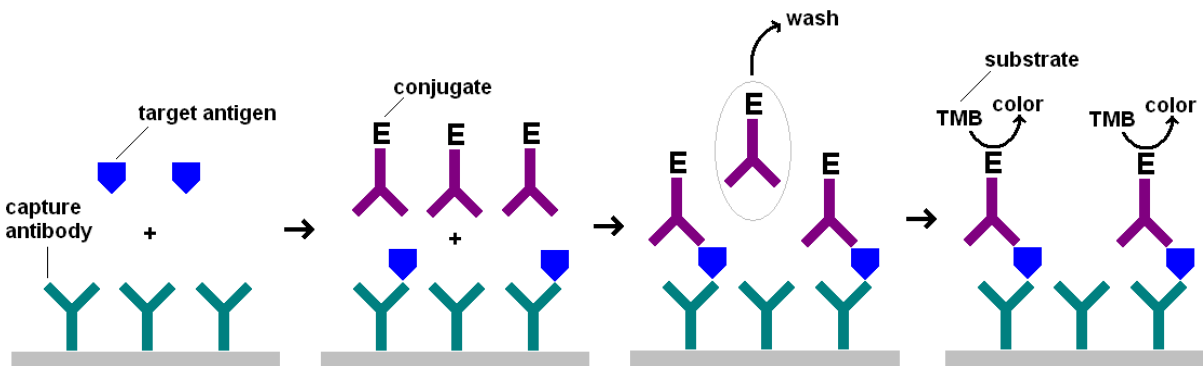


Figure 3. Immunometric assay to measure antigen.

In an immunometric assay the color generated is directly proportional to the amount of analyte present (Figure 4).

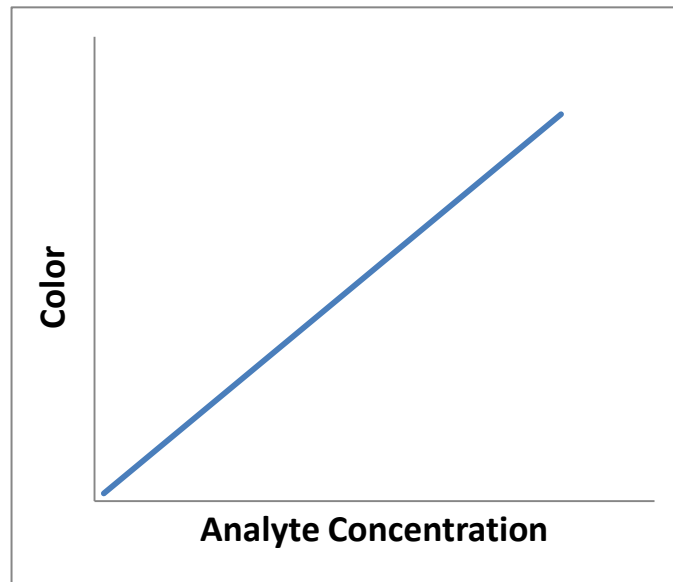


Figure 4. Immunometric assay signal response.

Immunometric assays are also commonly used to measure antibodies as the analyte (Figure 5). In this case a capture antigen is fixed to the plastic surface, and the target antibody binds to it. An antibody-enzyme conjugate that binds to the target antibody is then added, the plate is washed, and the conjugate reacts with the substrate to produce the color.

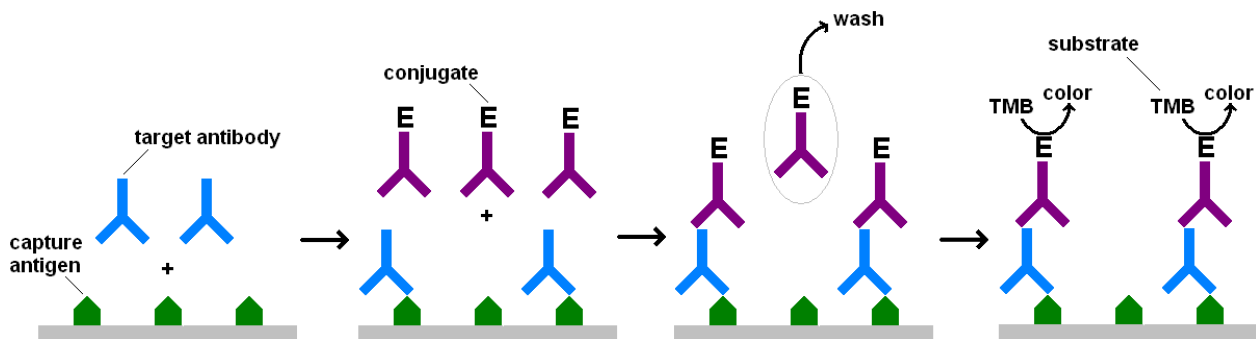


Figure 5. Immunometric assay to measure antibodies.

Because the analyte in an immunometric assay is surrounded on two sides, the procedure is often referred to as a sandwich assay. The acronym ELISA (Enzyme Linked Immuno Sorbent Assay) is also often associated with sandwich assays, but some authorities prefer to use the term in a more general sense for all sorts of microtiter plate immunoassays that involve enzymatic labels.^v The acronym IEMA is used more formally to refer to the Immunoenzymometric assay.

Competitive Assays

Immunometric assays work well when the analyte is a molecule large enough to bind two separate antibodies at one time. Drugs and many hormones are small molecules, however, and they require a different assay design, known as a competitive assay (Figures 6,7).^{vi} As in the immunometric assay, a capture antibody is used, and it may be attached beforehand to the surfaces of the plastic test wells. It is available only in limited amounts, however, and no secondary signal antibody used. The steps are as follows:

- Antigen (analyte) and antigen molecules conjugated to an enzyme compete for the available antibody sites.
- Plate wells are washed to remove all unbound analyte and conjugate.
- Substrate is added. Bound enzyme present reacts with substrate, yielding color.
- After stopping the reaction, color is measured.

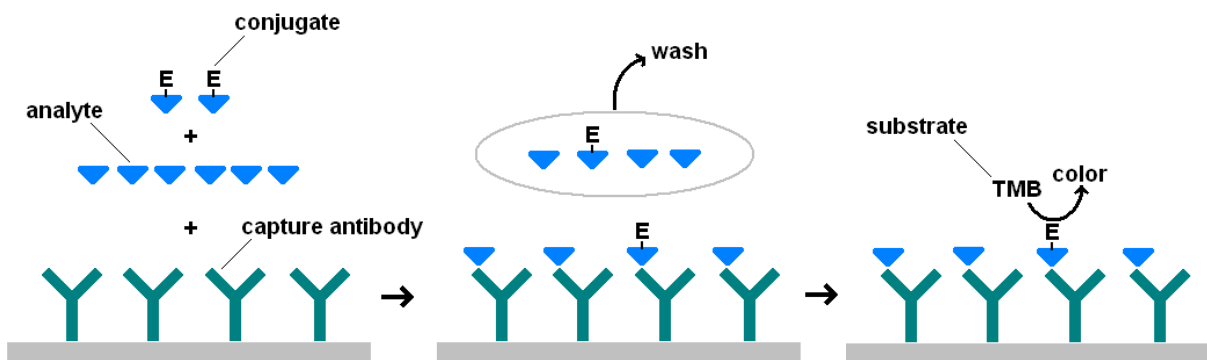


Figure 6. Competitive assay, high concentration of analyte.

If the concentration of the analyte in the sample is high (Figure 6), relatively less conjugate will bind to the antibodies in the plate well, and the color generated will be low. If the concentration of the analyte in the sample is low (Figure 7), relatively more conjugate will bind, and the color generated will be high. The color in a competitive assay is therefore inversely proportional to the amount of analyte present in the sample (Figure 8).

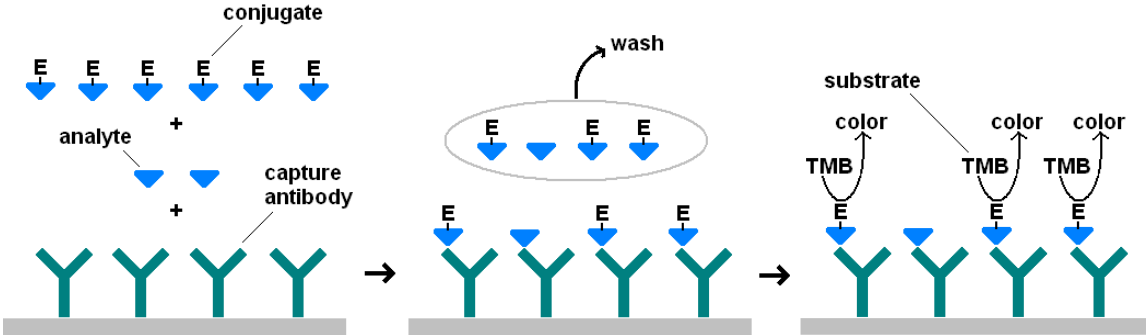


Figure 7. Competitive assay, low concentration of analyte.

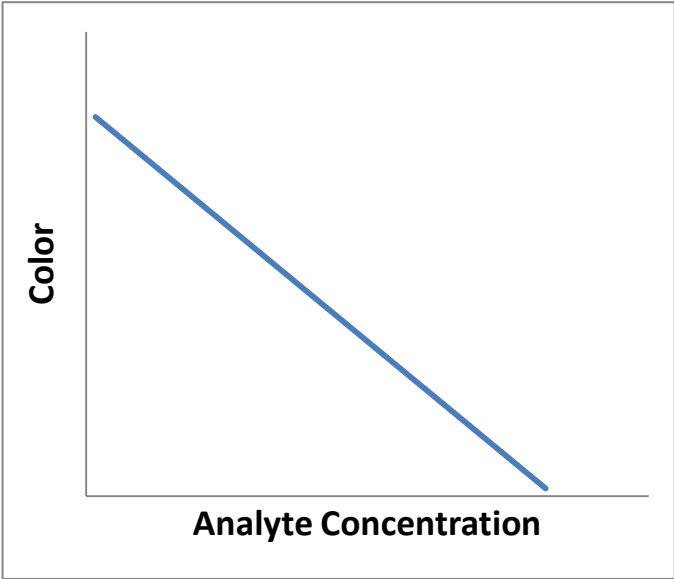


Figure 8. Competitive assay signal response.

Interpreting EIA Results

In order to determine the concentrations of analyte in samples, several known concentrations of analyte must be analyzed as part of each test plate as standards (or calibrators). Results from these wells are used to establish a calibration curve, from which the results for the unknown samples can be found. The amount of color, or optical density (OD), measured for each sample and standard (B) is divided by the OD of a sample with no analyte present (B_0), and the ratios (B/B_0) plotted against the concentration of analyte (on a logarithmic scale). The result is an inverse S-shaped curve (Figure 9). The process of fitting a curve to the data points from the standards is most accurately accomplished by using specialized computer software in conjunction with the plate reader. Salimetrics advises using a 4-parameter sigmoid minus curve fit.

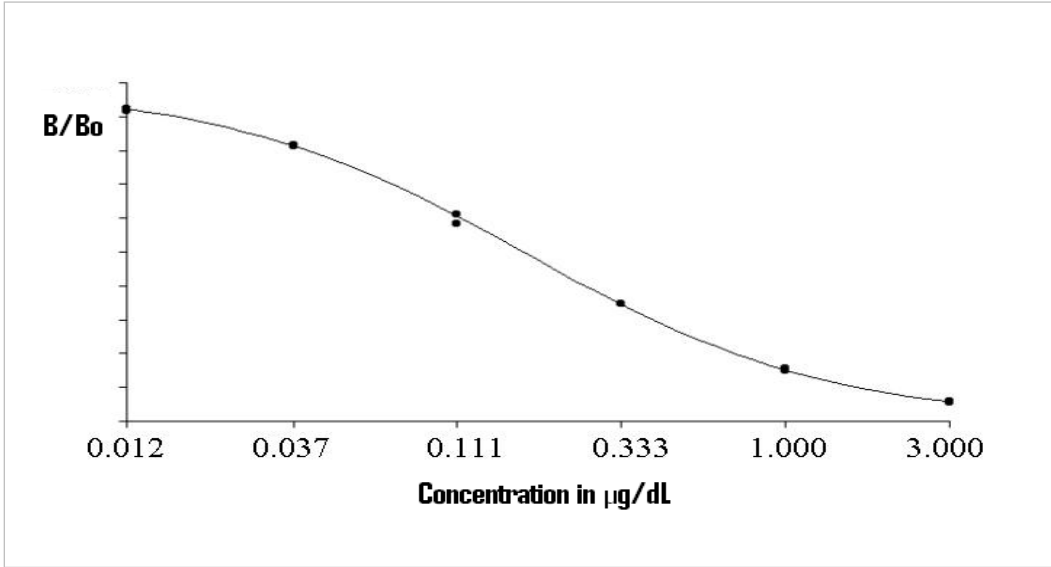


Figure 9. Typical Cortisol standard curve, 4-parameter sigmoid minus curve fit.



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Variants of Assay Design

Some competitive assays are done with plates not pre-coated with capture antibody. In these assays the capture antibody is added in solution to each test well at the same time as the other components, rather than being immobilized to the plate ahead of time. During incubation, the antibodies bind to the analyte or conjugate, and also attach to the surface of the test well. Afterwards, the plates are washed as usual, and substrate is added for the color generation phase.

Another assay variation is the indirect competitive assay, used by Salimetrics in the kit for SIgA (Figure 10):

- Samples and standards are initially incubated in separate tubes with a constant amount of antibody-enzyme conjugate. The conjugate binds to the analyte (SIgA).
- The amount of conjugate left unbound is inversely proportional to the concentration of SIgA that is present in the samples or standards.
- The amount of unbound conjugate remaining is determined by taking a sample from the first tube and transferring it to a microtiter plate with SIgA immobilized in the wells. Unbound conjugate from step 1 adheres to the SIgA in the well.
- Conjugate previously bound to SIgA in step 1 is not free to attach to the well, and is washed away, leaving the unbound conjugate from step 1 adhering to the wells.
- Substrate is added. Bound enzyme present reacts with the substrate, yielding color.
- After stopping the reaction, color is measured.

The color generated corresponds to the amount of unbound conjugate from step 1, and it is therefore inversely proportional to the concentration of the analyte.

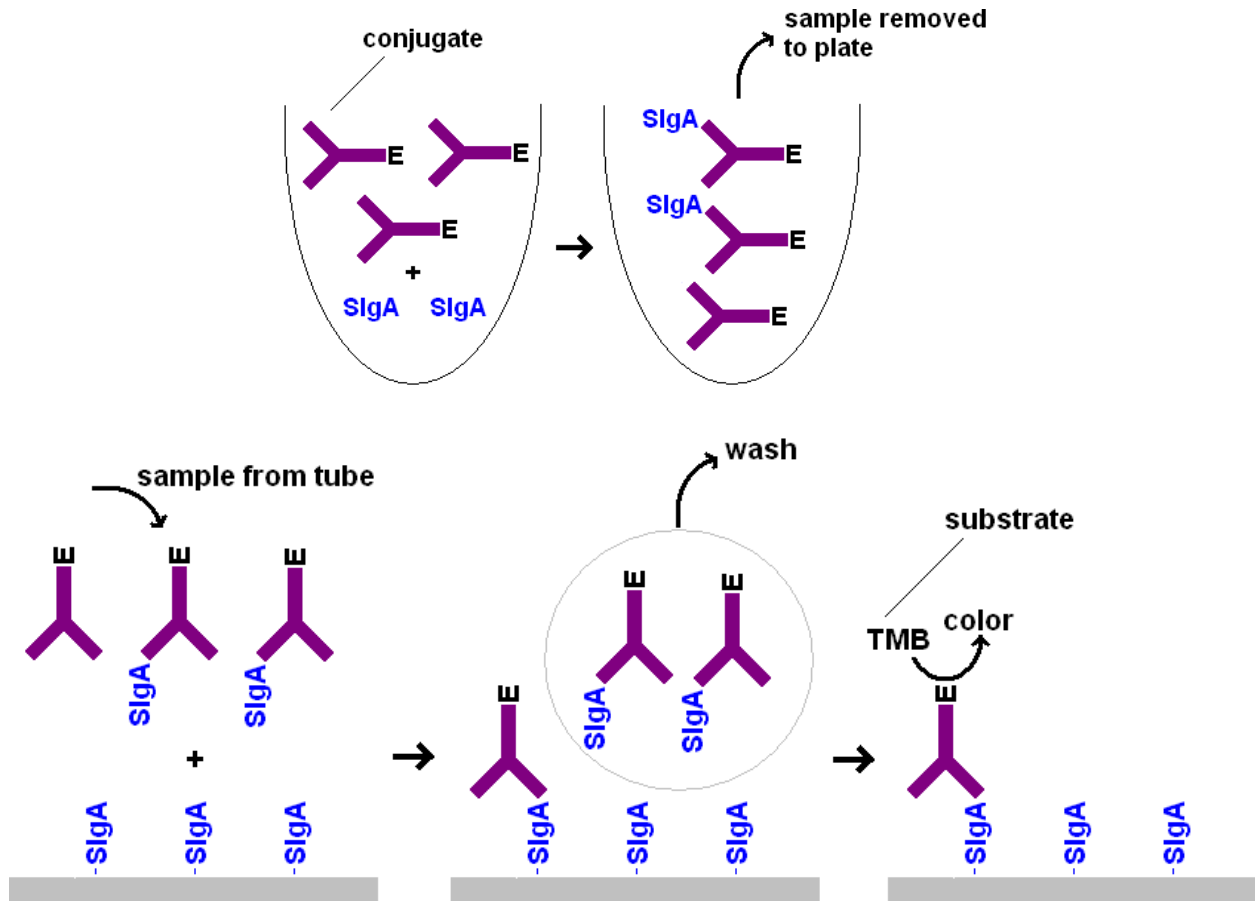


Figure 10. Indirect Competitive Assay for SlgA.

ⁱ David Wild, ed., *The Immunoassay Handbook*, 2nd ed. (New York, 2001), p. 162.

ⁱⁱ Wild, p. 162.

ⁱⁱⁱ Wild, p. 165.

^{iv} James P. Gosling, *Immunoassays: A Practical Approach* (Oxford, 2000), p. 93.

^v Gosling, p. 14.

^{vi} Gosling, p. 13.