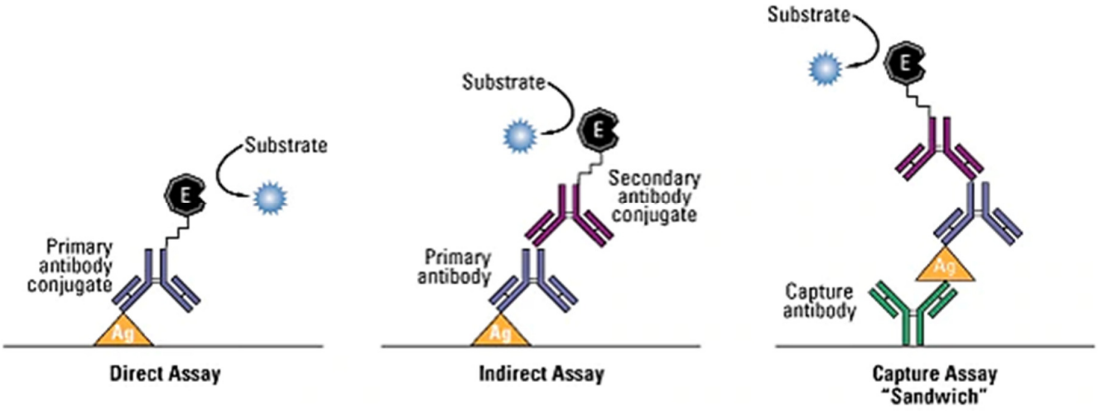
Enzyme-Linked Immunosorbent Assay

Aim

Determination of concentration of antibody by ELISA.

Principle

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying specific soluble substances such as peptides, proteins, antibodies, and hormones in complex mixture. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA, the antigen or target macromolecule is immobilized on a solid surface or microplate and then complexed with an antibody that is linked to a reporter enzyme. Detection is accomplished by measuring the activity of the reporter enzyme via incubation with the appropriate substrate to produce a measurable product. There are several formats used for ELISAs. These fall into either direct, indirect, or sandwich capture and detection methods. The key step is immobilization of the antigen of interest, accomplished by either direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (labelled primary antibody) or indirectly (such as labelled secondary antibody). The most widely used ELISA assay format is the sandwich ELISA assay, which indirectly immobilizes and indirectly detects the presence of the target antigen. This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between two primary antibodies, each detecting a different epitope of the antigen–the capture antibody and the detection antibody. The sandwich ELISA format is highly used because of its sensitivity and specificity.



Materials

1.Microtiter Coated Plate

2.Recombinant Human TNF-α

3.Human TNF-α Biotin Conjugated Detection Antibody

4.Concentrated Avidin Horseradish Peroxidase

5.WashBuffer

6.Assay DiluentA

7.Assay DiluentB

8.Avidin-HRP Diluent

9.TMB Substrate

10.Stop Solution

11.Microplate Reader

12.Adjustable pipettes

13.Deionized (DI) water

14.Wash bottle

15.Tubes to prepare standard/sample dilutions.

16.Absorbent paper

Specimen Collection and Handling

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

- *Cell Culture Supernatant:* If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at temperature < -20°C. Avoid repeated freeze/thaw cycles.
- *Serum:* Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum layer and assay immediately or store serum samples at temperature < -20°C. Avoid repeated freeze/thaw cycles.
- *Plasma:* Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store plasma samples at temperature < -20°C. Avoid repeated freeze/thaw cycles.

Procedure

1.Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2.Add 100 µl/well of standards and samples to the plate. Perform two-fold serial dilutions of the 500pg/ml top standard, either within the plate or in separate tubes. Thus, the Human TNF-αstandard concentrations are 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.63pg/ml, 7.81pg/ml, 0pg/ml. Assay Diluent A serves as the zero standard (0pg/ml). Seal plate and incubate for 2 hours at37℃.

3. Aspirate and wash plate 4 times with Wash Buffer(1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.

4.Add 100µl of diluted Detection Antibody solution to each well, Seal plate and incubate for 1 hour at 37℃.5.Wash plate 4 times with Wash Buffer(1X) as in step 3.

6.Add 50 µl of Assay Diluent B followed by addition of 100 µl of diluted Avidin-HRP solution to each well, seal plate and incubate for1 hour at 37℃.

7.Wash plate 4 times with Wash Buffer(1X) as in step 3. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help to minimize background.

8.Add 100 µl of TMB substrate solution and incubate in the dark for 15-30 minutes at 37°C. Positive wells should turn bluish in color. It is not necessary to seal the plate during this step.

9.Stop reaction by adding 100 µl of Stop Solution to each well. Positive wells should turn from blue to yellow.

10.Read absorbance at 450 nm within 30 minutes of stopping reaction.

Calculation of Results

Determine the mean absorbance for each set of duplicate or triplicate standards and samples. Subtract the mean absorbance of the zero standards (background) from each well. Using Semi-log graph paper or computer programs, plot the optical densities of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit straight line through the standard points. To determine the unknown protein concentrations, find the unknowns mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the protein

concentration. If samples were diluted, multiply by the appropriate dilution factor. Computer based curve-fitting software may be preferred.

Safety Precautions

•Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.

•Source materials maybe derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.

•Since the kit contains potentially hazardous materials, the following precautions should be observed

-Do not eat or drink while handling kit material

-Always use protective gloves

-Never pipette material by mouth

-Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.