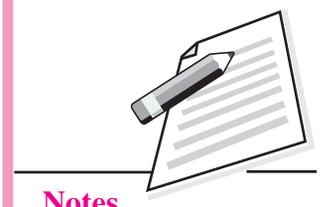


63

EIA AND RIA

63.1 INTRODUCTION

The ELISA, Enzyme linked Immunosorbent assay, also sometimes known as EIA i.e. Enzyme Immuno Assay is a rapid test used for detecting and quantifying antibodies or antigens in specimen against viruses, bacteria and other materials. This method is used to detect/diagnose infectious, autoimmune and other diseases.

ELISA is carried out on solid matrix, in 96 well microtitre plates or strips (12 wells or 8 wells each) made of polystyrene/commercially available coombs/ cartridges (Rapid ELISA). The protein antigen is affixed to any of above mentioned surfaces, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance/substrate specific for the enzyme is added that the enzyme can convert to some detectable signal, most commonly a colour change. Performing ELISA, like other antigen antibody reactions, involves at least one antibody with specificity for a particular antigen. Depending upon whether we want to detect antibody or antigen the type of ELISA will vary. For example the antigen in a sample can be detected either by direct ELISA , sandwich ELISA or competitive ELISA and antibody is usually detected by indirect ELISA. I know it seems very confusing to you now but as we go along describing the various types of ELISA technologies, each principle will become clear to you. Another thing to note is that between each step of assay, whatever the ELISA format is, the plate is washed with a mild detergent solution to remove any un reacted proteins or antibodies. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen/antibody in the sample and the reaction is read either with naked eye or with an ELISA Reader. The result is expressed as OD (Optic Density) value (Reader) or titre.

Notes

Newer ELISA-like techniques have been developed which utilize fluorogenic, electro chemi luminescent, and real-time PCR reporters to create quantifiable signals. The new reporters are more sensitive. These newer techniques do not use enzymes as in ELISA but use other reporter molecules as given above. We will discuss ELISA in this chapter. Recently an ultrasensitive, enzyme-based ELISA test using nanoparticles as a chromogenic reporter has been developed which can detect attograms of analyte in the specimen and result can be read as colour signal with naked-eye.

OBJECTIVES

After reading this lesson, you will be able to:

- define ELISA
- describe the history of ELISA
- discuss the process of ELISA
- describe the various types of ELISA
- describe the methods of various types of ELISA with examples
- practice "Internal quality control" for ELISA
- read the result of ELISA with naked eye and with ELISA Reader
- describe the various applications of ELISA

63.2 DEFINITION

Collins English Dictionary describes enzyme-linked immunosorbent assay as " An immunological technique for accurately measuring the amount of a substance, for example in a blood sample".

American Heritage English Language Dictionary defines ELISA as "A sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein, especially an antigen or antibody". It is often used as a diagnostic test to determine exposure to a particular infectious agent, such as the AIDS virus, by identifying antibodies present in a blood sample.

Random House Kernerman Webster's College Dictionary defines ELISA as "A diagnostic test for detecting exposure to an infectious agent, as the AIDS virus, by combining a blood sample with antigen of the agent and probing with an enzyme that causes a color change when antibody to the infection is present in the sample".

You see in all the above definitions the common thing is that ELISA is an antigen-antibody reaction that uses enzyme/s and specific substrate wherein the

presence of unknown substance which may be an antigen or antibody or a protein in the specimen is detected by colour change at the end of the reaction/test. The test is performed on solid phase –the polystyrene microtitre plates/strips and the final result is mostly read on the ELISA Reader as OD value.

63.3 THE HISTORY OF ELISA

Before the development of the EIA/ELISA, the only option for conducting an immunoassay was Radioimmunoassay, a technique which used radioactively-labeled antigens or antibodies for diagnostics before the advent of ELISA. Rosalyn Sussman Yalow and Solomon Berson published a paper in 1960 on Radioimmunoassay. Radioactivity was the reporter providing the signal which indicated the presence of analyte being sought in the sample. However, radioactivity posed a potential health threat, so safer reporter alternatives were sought.

One such alternative reporter tested was enzyme peroxidase which reacted with appropriate substrates (such as ABTS or 3, 3', 5, 5'-Tetramethylbenzidine) to produce a change in colour, which could be used as a signal. The next step in history was the linking of an antibody /antigen to the enzyme. This linking process was independently developed by two different scientists Stratis Avrameas and G.B. Pierce. The next development was the idea of fixing the antigen/ antibody to prepare the immunosorbent surface as it is necessary to remove any unbound antibody or antigen by washing. Wide and Porath developed the technique and published the same in 1966. Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in The Netherlands used the knowledge published by Wide and Porah to develop ELISA and EIA tests independently and published the technologies in 1971. The clinical impact of EIA/ELISA as immunoassays has been overwhelming. Perlmann, Schuurs, Engvall, and van Weemen were honored with the German scientific award of the "Biochemische Analytik" for their inventions in 1976. EIA/ELISA used the principles of conventional radioimmunoassay, with the key difference that the antibodies are labelled with an enzyme, rather than radioisotopes. Now other reporter molecules like fluorogenic, electro chemi luminescent, and real-time PCR and appropriate signal systems have been developed and available in Medicine for diagnostics.

A well-established in vitro diagnostic industry has developed based on these technologies and are marketing a huge number of EIAs/ELISAs.

63.4 PRINCIPLE AND PROCESS

As you know already ELISA is a biochemical technique used mainly in infectious diseases and immunology to detect the presence of an antibody or an

Notes

antigen in a sample. ELISAs also can provide a useful measurement of antigen or antibody concentration in a sample.

63.4.1 Principle

ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number.

Simply put for ELISA an antigen is adsorbed/fixed to the surface of a solid matrix which may be a well on a micro titration plate/ well strip, beads or may be a special type of paper made of Nitrocellulose. Then, a specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the specific substrate for the enzyme is added. The subsequent reaction produces a detectable signal, a color change in the substrate.

ELISA technology has advanced a lot. To make this immunoassay more and more sensitive as well as specific various advances have been made and now we have different types of ELISAs. There are slight differences in the technology though basically ELISA remains an antigen antibody reaction performed as wet test using different reporter and signal systems.

63.4.1.1 Types of ELISAs

ELISAs can be divided into the following categories based on the principle used:

- Indirect
- Sandwich
- Competitive
- Antigen and antibody capture ELISA

63.4.1.2 Indirect ELISA

HIV antigens are attached covalently to the solid phase support allowing corresponding/specific antibodies present in the specimen to bind, and these bound antibodies are subsequently detected by enzyme labelled anti-human immunoglobulin and specific substrate system. If the test specimen contained the antibodies specific to the antigen fixed on the solid phase colour reaction will take place. The colour reaction can be read with naked eye or with ELISA Reader at the specified UV light using the special filters. The result is expressed as OD (optical Density) value.

63.4.1.3 Sandwich ELISA

One of the most useful of the immunoassays is the two antibody “sandwich” ELISA. This assay is used to determine the antigen concentration in unknown

samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample.

To utilize this assay, one antibody (the "**capture**" antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Specimen containing antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the "**detection**" antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate.

63.4.1.4 Competitive ELISA

In this assay the antibodies present in the specimen compete with the enzyme conjugated antibodies in the reagent for binding to the antigen on the solid phase. If the test specimen contains corresponding/specific antibodies, these will compete with the labelled antibodies in the reagent for binding to antigen. So that less or not labelled antibodies can attach to the solid phase. Hence, faint or no colour is produced on addition of substrate if specimen contains antibodies against the antigen on the solid phase.

63.4.1.5 Antigen and antibody capture ELISA

Antigen capture ELISA can be based on principle of indirect or competitive ELISA, only difference being in the initial step of attaching antigen to the solid phase. A monoclonal antibody directed against an antigen is bound to the solid support. Next step is addition of antigen supplied as reagent in the test kit. This antigen is captured by the monoclonal antibody bound to the solid phase. Test specimen appropriately diluted is added next. Antibodies if present in the specimen bind to the antigen on solid support. Remaining principle is same as indirect ELISA Only advantage of antigen capture ELISA is that it is more specific than indirect assay.

INTEXT QUESTIONS 63.1

Match the following

1.	Indirect ELISA	(a)	Determines antigen concentration
2.	Sandwich ELISA	(b)	Antibodies compete to bind with antigen
3.	Competitive ELISA	(c)	Captures monoclonal antibody
4.	Antigen & Antibody capture ELISA	(d)	Detects bound antibodies

63.5 PROCESS OF ELISA

Although many variants of ELISA have been developed and used in different situations, they all depend on the same basic elements:

- **Coating/Capture:** direct or indirect immobilization of antigens to the surface of polystyrene microplate wells.
- **Plate Blocking:** addition of irrelevant protein or other molecule to cover all unsaturated surface-binding sites of the microplate wells.
- **Probing/Detection**: incubation with antigen-specific antibodies that affinity-bind to the antigens.
- **Signal Measurement:** detection of the signal generated via the direct or secondary tag on the specific antibody.

Basic steps are as shown below:

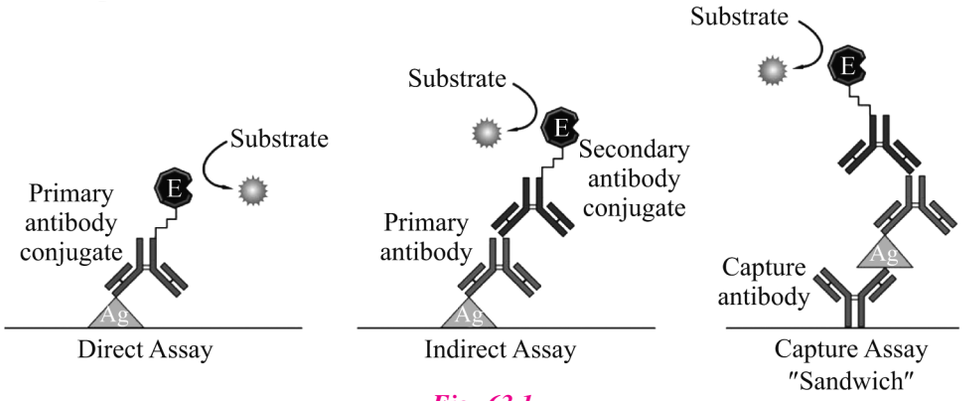


Fig. 63.1

In a typical assay designed to detect an antigen in a sample, either the antigen is immobilized by direct adsorption or first antibody is adsorbed and then antigen is adsorbed on the well surface of the ELISA plate. The plate is blocked with albumin. The antigen is probed with a specific detection antibody. The detection antibody may be directly labeled with a signal-generating enzyme or fluorophore or it may be secondarily probed with an enzyme- or fluor-labeled secondary antibody. For enzymatic detection, the appropriate enzyme substrate is added. The signal observed is proportional to the amount of antigen in the sample. Every step of test is followed by washing to remove the un-reacted reactants, only specific (high-affinity) binding remains that causes the detection signal at the final step.

63.6 METHODS OF ELISA

We will see now how the different types of ELISA are performed. We will take example of HIV ELISAs.

63.6.1 Indirect ELISA

Always follow the instructions of the manufacturer of the kit which are given in the kit literature and develop a protocol/SOP. One example is given below.

Principle

HIV antigens are attached covalently to the solid phase support allowing HIV antibodies present in the specimen to bind, and these bound antibodies are subsequently detected by enzyme labelled anti-human immunoglobulin and specific substrate system. If the test specimen contained antibodies colour reaction will take place.

Materials required:

- Single / multichannel pipettes with disposable tips: 5-50ul , 50-200ul
- Incubator (37+20C)
- ELISA reader with or without washer
- Powderless disposable gloves
- Absorbent paper
- Deionised water
- Discard jar with hypochlorite solution
- Wash bottles

Protocol:

Add appropriate amount of diluted sample in various wells
↓
Incubate for required time at room temperature
↓
Empty plate and tap out residual liquid. Wash 3-5 times
↓
Appropriately diluted enzyme conjugate is added and incubated as specified
↓
Wash the plate (3-5 times) and tap out residual liquid
↓
Add appropriate amount of substrate solution to each well
↓
Incubate as specified
↓
Add required amount of stop solution Sodium hypochlorite solution (5.2%)
↓
Read plate with plate reader/read colour change with naked eye.

63.6.2 Sandwich ELISA

One of the most useful of the immunoassays is the two antibody "sandwich" ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample.

Notes

Principle

To utilize this assay, one antibody (the "**capture**" antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the "**detection**" antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate.

Materials

As for Indirect ELISA test.

Protocol

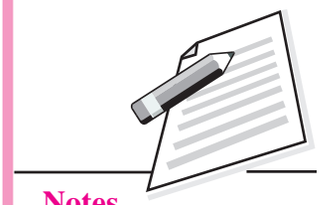
The following is the general protocol for a sandwich ELISA. Follow manufacturers instructions for precise steps.

Add required volume of sample containing antigen to the wells
↓
Incubate for required amount of time
↓
Wash the plate three to four times
↓
Add the labeled second antibody
↓
Incubate at room temperature for required amount of time
↓
Wash the plate 3-4 times
↓
Add substrate as indicated by manufacturer
↓
Read on the ELISA reader after suitable incubation time.

63.6.3 Competitive ELISA

Principle

In this assay the HIV-antibodies present in the specimen compete with the enzyme conjugated antibodies in the reagent for binding to the antigen on the solid phase. If the test specimen contains HIV antibodies, these will compete with the labelled antibodies in the reagent for binding to antigen. So that less or not labelled antibodies can attach to the solid phase. Hence, faint or no colour is produced on addition of substrate if specimen contains HIV antibodies.



Materials

These are same as used in case of indirect / sandwich ELISA techniques

Protocol:

Add appropriate amount of standard/ sample solution to the wells

↓

Allow to incubate for required amount of time (as in kit insert)

↓

Add appropriate amount of conjugate solution to the wells

↓

Wash the plate with wash solution 3-4 times

↓

Add substrate as indicated by manufacturer.

↓

After suggested incubation time has elapsed,

↓

Optical densities at target wavelengths can be measured on an ELISA reader

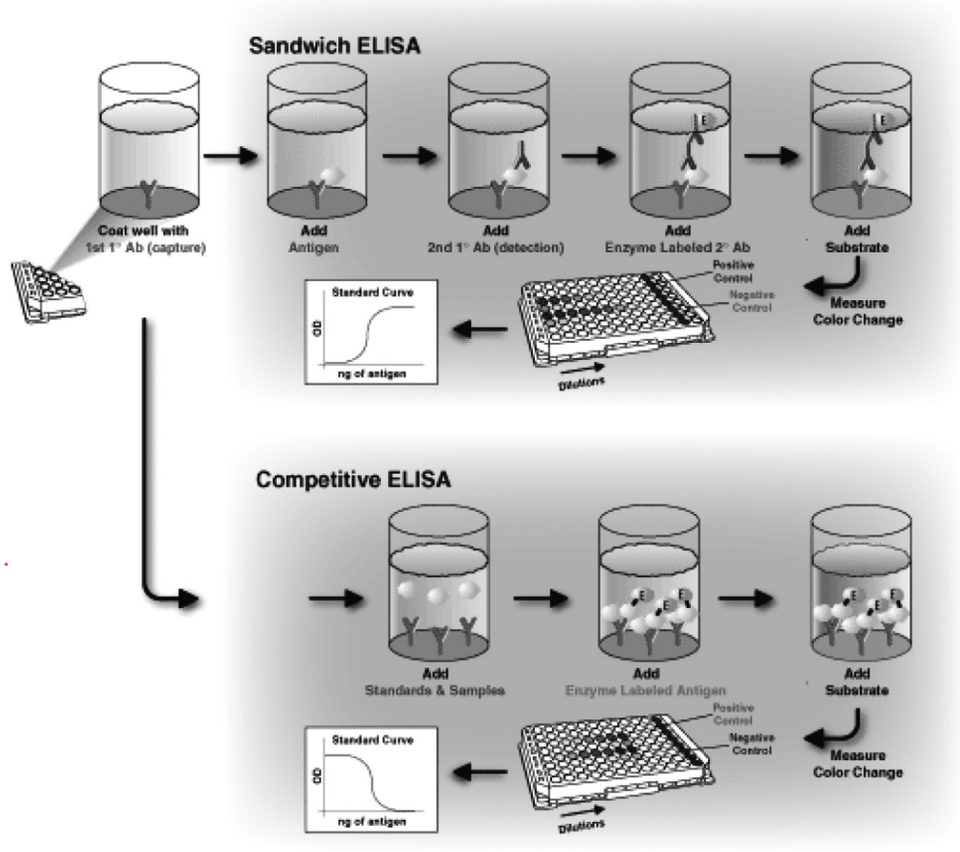


Fig. 62.2 Sandwich and Competitive ELISA techniques

Notes

63.6.4 Antigen and antibody capture ELISA

Antigen capture ELISA can be based on principle of indirect or competitive ELISA, only difference being in the initial step of attaching antigen to the solid phase. A monoclonal antibody directed against an antigen is bound to the solid support. Next step is addition of the standard antigen supplied as reagent in the kit. This antigen is captured by the monoclonal antibody bound to the solid phase. Test specimen appropriately diluted is added next. Antigen specific antibodies if present in the specimen bind to antigen on solid support. Remaining principle is same as indirect ELISA. Only advantage of antigen capture ELISA is that it is more specific than indirect assays.

63.6.5 Enzyme Linked Fluorescent Assay (ELFA)

This is the new technology utilizing the fluorescence as the detection system instead of the enzyme substrate in ELISA. The test requires a special equipment which is an automated system (VIDAS and MINI VIDAS, Biomerieux). Here also the specific instructions of the manufacturer are followed to perform the test.

63.7 QUALITY CONTROL AND CONSIDERATIONS WHILE PERFORMING ELISA

Flow chart for performing ELISA using quality control aliquot

Selection of ELISA kit	Licensed Quality checked
Performance of ELISA test	As per manufacturer's guidelines Internal controls are checked Inclusion of external control indaily run
E-ratio of external contro	Recorded O.D. value of external control /cut off value (kit)
Daily run QC sample	The OD value of the QC sample should be within the specified OD range of the QC sample.

63.8 APPLICATIONS OF ELISA

You now understand that ELISA is a highly sensitive and specific test. Since the discovery of ELISA many advances have been made to make this assay more and more sensitive and specific. ELISA is employed in Medicine to detect and diagnose microbial infectious diseases, autoimmune diseases, detection of antigen in a given sample.

- ELISA can be applied to detect and measure antibody in serum against viruses, bacteria, parasites
- ELISA has also been used in home pregnancy test (rapid ELISA)
- ELISA is used in food industry to detect potential food allergens such as milk, peanuts, walnuts, almonds, and eggs.
- ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.
- The ELISA was widely used in various areas such as immunology, Biological Pharmacy, Diagnostic industry, and so on.

INTEXT QUESTIONS 63.2

Match the following

1. Coating	(a) Addition of irrelevant protein
2. Plate blocking	(b) Incubation with antigen-specific antibodies
3. Signal measurement	(c) Immobilization of antigens
4. Probing	(d) Detection of signal generated by antibody

WHAT YOU HAVE LEARNT

- The ELISA, Enzyme linked Immunosorbent assay, also sometimes known as EIA i. e. Enzyme Immuno Assay is a rapid test used for detecting and quantifying antibodies or antigens in specimen against viruses, bacteria and other materials. This method is used to detect/diagnose infectious, autoimmune and other diseases.
- ELISA is defined variously by different authors . The common definition that emerges from all the definitions is that ELISA is an antigen-antibody reaction that uses enzyme/s and specific substrate wherein the presence of unknown substance which may be an antigen or antibody or protein in the specimen is detected by colour change at the end of the reaction/test. The test is performed on solid phase –the polystyrene microtitre plates/strips and the final result is mostly read on the ELISA Reader as OD value.
- Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in The Netherlands used the knowledge published by Wide and Porah to develop ELISA and EIA tests independently and published the technologies in 1971.

- ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number. Simply put for ELISA an antigen is adsorbed/fixed to the surface of a solid matrix which may be a well on a micro titration plate/ well strip, beads or may be a special type of paper made of Nitrocellulose. Then, a specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the specific substrate for the enzyme is added. The subsequent reaction produces a detectable signal, a color change in the substrate. ELISA/EIA can be divided into various types based on the principle used: Indirect; Sandwich; Competitive; Antigen and antibody capture ELISA.
- Applications of ELISAs include: Detection and measurement of antibody/ antigen in serum against viruses, bacteria, parasites; Home pregnancy test (rapid ELISA); Detection of potential food allergens such as milk, peanuts, walnuts, almonds, and eggs in food industry; As a rapid presumptive screen in toxicology for certain classes of drugs; Immunology, Biological Pharmacy, Diagnostic industry, and so on.

TERMINAL QUESTIONS

1. What is ELISA/EIA? How do you define ELISA?
2. Why and how was ELISA discovered?
3. Name different types of ELISAs based on technologies employed. Describe in brief the principle of each type of ELISA
4. Briefly describe principle and method of indirect ELISA.
5. Briefly describe principle and method of sandwich ELISA.
6. Briefly describe principle and method of competitive ELISA.
7. Briefly describe the quality control process for ELISA.
8. Enumerate the different applications of ELISA.

ANSWERS TO INTEXT QUESTIONS

63.1

1. (d) 2. (a) 3. (b) 4. (c)

63.2

1. (c) 2. (a) 3. (d) 4. (b)