ELISA: Enzyme Linked Immunosorbent Assay

It is a highly sensitive method and has widespread application in the field of research and diagnostics.

It is quantitative as well as qualitative and can be adapted as per the need of the reaction.

It relies on antigen-antibody reaction taking place on a solid support. This solid support is usually a microwell made of the polystyrene.

Basic principle: It is built on the principle of antigen-antibody interaction and utilizes their highly specific nature.

Generally, antigen is coated on a solid support and antibody is allowed to bind it overnight. This antibody is generally monoclonal in nature i.e. will bind only one epitope.

Next day, we add enzyme labelled secondary antibody that binds to the primary antibody. After the binding of secondary antibody is complete, we add coloured substrate which is specific for enzyme.

This leads to development of a soluble coloured product that will absorb at a particular wavelength. The absorbance values so obtained are used to assess the concentration of the antigen in the sample. This is followed in case of quantitative ELISA.

In case of qualitative ELISA, mere presence or absence of the soluble coloured product is enough to inform about the presence or absence of the antigen in the reaction.

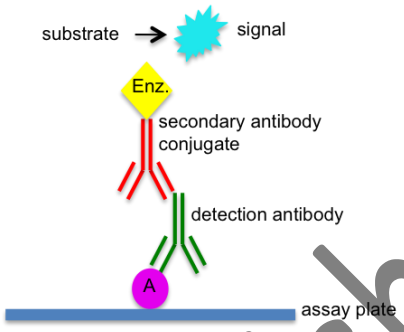
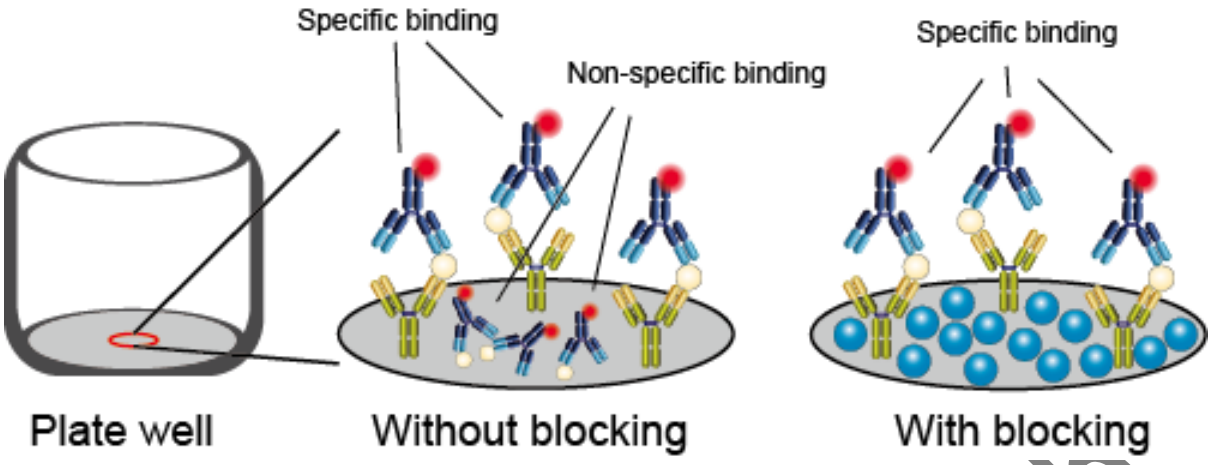


Fig: Basic Principle of ELISA

Blocking in ELISA: This is a very important process during ELISA experiments. If we fail to perform blocking, then it will lead to false positive results. **False positive results mean when our analyte is absent but test results are visible as positive, thus, indicating false presence of the analyte. In case our analyte is present, we will get higher than actual concentration for the analyte which again is erroneous.**

Blocking is performed after initial coating is over and unbound molecules have been washed away while next set of molecules have not been added. If we refer to the image shown above, blocking will be performed after the coating of A but before the addition of detection antibody.

To perform blocking, we incubate the reaction well with a 3-5% solution of non-specific proteins in buffer. E.g. 3% BSA in TPBS or 5% skimmed milk powder in TPBS. This reaction is done for about 1-2 hours and it blocks all the protein binding sites available. This ensures that detection antibody will bind only to the analyte if it is present and if analyte is absent then it will not bind to well surface in non-specific manner.



In the figure above, you can see that without blocking the antibody is showing non-specific binding and this will generate false signals while in the scenario where proper blocking has been done signals are generated only by those antibodies which are binding to the antigen/analyte.

Enzymes used in ELISA: The enzymes are covalently linked or conjugated to the Fc region of the antibody. Usually this conjugation is seen in case of secondary antibody but in case of direct ELISA this can be seen on the primary antibody as well. There are two most commonly used enzymes i.e. **Alkaline phosphatase (ALP)** and **Horseradish peroxidase (HRP).**

Another enzyme that is less commonly used is **beta-galactosidase (beta-gal)**. This is not used much as it has limited substrates for use in ELISA.

Substrates used in ELISA for different enzymes are given below:

1. **Substrates for HRP:** 3,3',5,5'-tetramethylbenzidine or TMB, o-phenylenediamine dihydrochloride or OPD
2. **Substrates for ALP:** p-Nitrophenyl phosphate, Disodium salt or PNPP.
3. **Substrates for beta-galactosidase:** o-nitrophenyl-β-D-galactopyranoside or ONPG

Types of ELISA: Based on the variations in the procedure ELISA is of four basic types in increasing order of sensitivity as is given below:

1. Direct ELISA.
2. Indirect ELISA.
3. Sandwich ELISA.
4. Competitive ELISA.

These types are discussed below in required detail.

1. **Direct ELISA:** Antigen is plated on the surface of ELISA well then blocking is performed. After this enzyme labelled primary antibody is used for binding to the antigen and then after incubation and washing substrate is added for colour development.

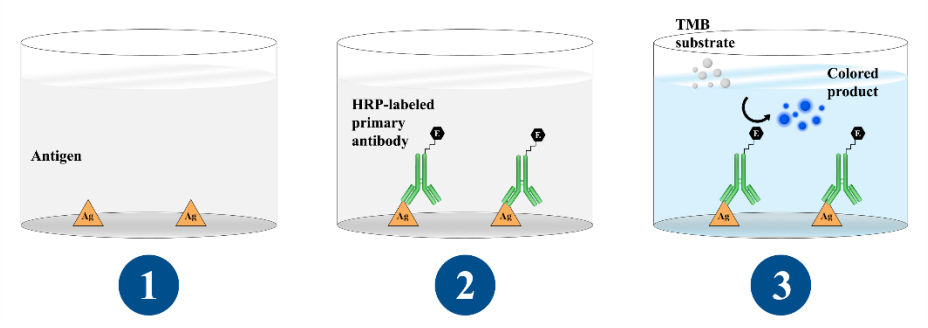


Figure: Direct ELISA

Advantages:

- Quicker than other variants of ELISA.
- Less number of steps and less reagents needed so less chances of error.

Disadvantages:

- Less flexible in adaptation as for each antigen we need enzyme labelled primary antibody which may not always be possible.
- No signal amplification due to absence of secondary antibody. This leads to reduction in sensitivity.

2. **Indirect ELISA:** In this, antigen is coated on the polystyrene well and then we perform blocking. Now, we add unlabeled primary antibody which is generally a mab and is specific for the antigen used to coat the well. It is allowed to bind the antigen overnight and then after washing the unbound primary antibody, we add secondary antibody.

   The secondary antibody is polyclonal and is labelled with enzyme. After sufficient incubation, we wash away the unbound secondary antibody and add the substrate suitable for the enzyme label. This leads to generation of a soluble coloured solution whose absorbance is recorded.

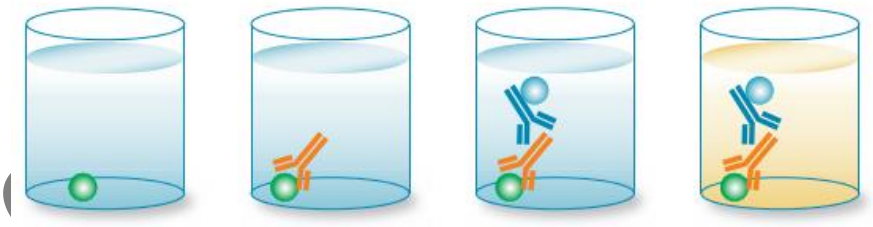


Figure: Indirect ELISA

Advantages:

- High sensitivity due to signal amplification induced by the use of labelled secondary antibody.
- Provides flexibility as single polyclonal secondary antibody can be used against many different primary antibodies as long as source animal of the primary antibody is not changed.

Disadvantages:

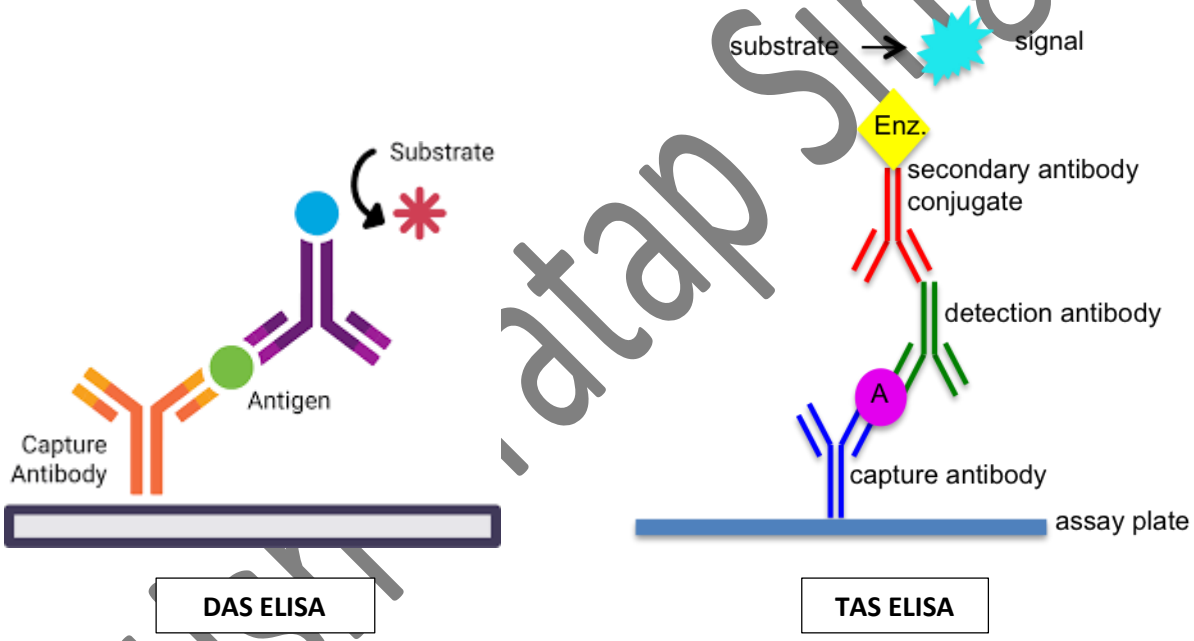
- Background noise in signal due to possible cross reactivity by the polyclonal secondary antibody.

3. **Sandwich ELISA:** It involves at least two different antibodies and these antibodies must be pair matched i.e. they should bind to different epitopes on the antigen.

   The reaction surface is coated with the capture antibody and then blocking is performed. Now, the sample containing antigen is added and antigen binds to the capture antibody. After this, we add detection antibody which binds to the antigen but on a different epitope than the one bound by the capture antibody.
   Thus, antigen becomes sandwiched between capture and detection antibody and ELISA method is called as sandwich ELISA.

   The detection antibody can be labelled with enzyme for generating the signal and in this case the method is called as double antibody sandwich ELISA or DAS-ELISA.

   Alternatively, we can add another enzyme labelled antibody that binds to the detection antibody. In this case, method is called as triple antibody sandwich ELISA or TAS-ELISA.



Advantages:

- 2-5 times more sensitive than indirect ELISA.
- High specificity as two different antibodies are used for the capture and detection.
- No need of purified antigen and gives results for analysis of complex samples such as serum or plasma.

Disadvantages:

- Needs testing of antibody pair for purpose of pair matching.
- Costly as two different monoclonal antibodies are used during capture and detection.

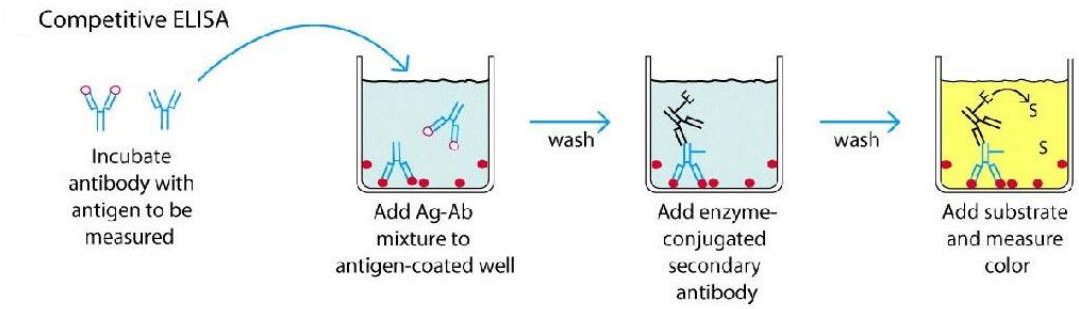
4. **Competitive ELISA:** This is also known as inhibition ELISA. In this, we mix the sample containing antigen of unknown concentration with a fixed concentration of the antibody. When allowed to incubate, we get Ag-Ab complex and some amount of free, unbound antibody.

   The amount of free, unbound antibody is inversely proportional to the concentration of the Ag in the sample. Thus, if sample has high [Ag] then amount of free, unbound antibody will be less and vice versa.

   After incubation, the whole reaction mixture containing the Ag-Ab complex and free, unbound Ab are added to the reaction well which has been coated with the same Ag.

   We incubate to allow the free, unbound Ab to bind to the coated Ag and after that well is washed to remove any unbound Ab and the Ag-Ab complex. Post the wash we add enzyme labelled secondary Ab that binds to the primary Ab and is used to develop the signal after the addition of substrate.

   The amount of signal is inversely related to the [Ag] in the original sample. If the [Ag] was high in the original sample, then the signal will be low. This happens because high [Ag] in the original sample will leave very less amount of free, unbound Ab to react with the coated Ag in the next step. This in turn will lead to less number of secondary Ab being bound to the primary Ab and hence low signal strength being generated on the addition of the substrate.



Advantages:

- No sample processing is needed so crude or impure samples can be directly used.
- Less prone to sample dilution and sample matrix effects than other ELISA variants which makes it more sensitive than other methods.
- Results show very less variation between duplicate samples due to which it is preferred method of choice to measure even minor variations in the antigen concentration.

Disadvantages:

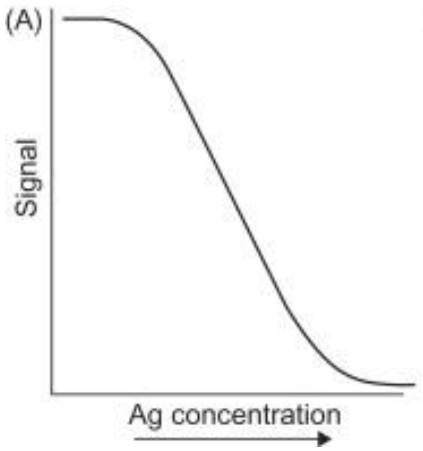
- Number of steps are more and it introduces greater chances of errors.

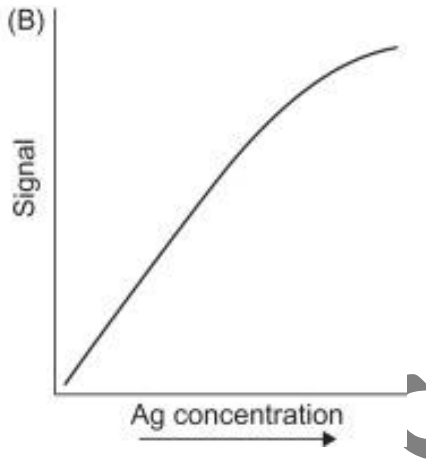
Note:

- All these variants of ELISA rely on the generation of a soluble coloured product whose absorbance is recorded. Thus, they are all called as chromogenic ELISA.
- Sensitivity of chromogenic ELISA is in the range of 10^{-10}M – 10^{-15}M.
- To increase the sensitivity, we can use luxogenic substrates such as luminol for signal generation. This substrate generates photons or light on reaction with enzyme HRP. If such adaptation is being

carried out, then ELISA is called as chemiluminescent ELISA with sensitivity range being 10^{-15}M - 10^{-18}M

Graph patterns in ELISA: When we take the absorbance readings and plot it in the form of a graph so as to find out the concentration of the unknown, we get two basic patterns in the ELISA reactions.





Graph A is showing the pattern in case of the competitive ELISA.

Graph B is showing the graph pattern in case of direct, indirect and sandwich ELISA.