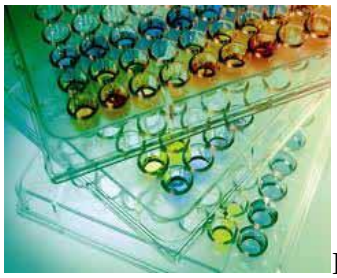
What is ELISA? Enzyme-linked immunosorbent assay (ELISA)

 Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries,such as ELISA application in food industry. In simple terms, in ELISA, an unknown amount of antigen is affixed to a surface, 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 is added that the enzyme can convert to some detectable signal, most commonly a colour change in a chemical substrate.

What is being tested using ELISA assay?

If a protein with multiple epitopes is being detected, a sandwich assay is a good choice. It usually requires two antibodies that react with different epitopes. However, if the molecule has multiple repeating epitopes, it is possible in a sandwich assay to use the same antibody for both capture and detection. Alternatively, if there is a supply of the analyte to be detected in pure form that can absorb effectively to a microwell, then one can set up a competitive assay in which the purified analyte is immobilized and analyte in the sample competes with the immobilized analyte for binding to labeled antibody. In this case it is essential to titrate the antibody so that it is limiting, or else the assay sensitivity will be lowered.

Polystyrene will bind a wide variety of proteins in an increasing amount depending on their concentration in the coating solution. The specific and optimal amount needs to be determined for each protein, but some general observations have been made for antibodies. Medium to low binding plates bind typically up to 100 - 200 ng of IgG/cm^2 while high binding plates typically can bind up to 400 - 500 ng of IgG/cm^2. In addition to proteins, polystyrene plates will absorb peptides generally of 15 - 20 amino acids in length. In order to achieve strong binding, a peptide will need both hydrophobic and hydrophilic interactions. Typically a drawback to adsorbing peptides directly is that they tend to have few epitopes, and if these are involved in interaction with the plastic, it will be difficult for an antibody to bind to them. One alternative is to attach the peptide to a larger protein through a spacer arm that provides some distance between the peptide and the protein, allowing the antibody to interact with the peptide.

An organism such as bacterial or viral assays that detect whole organisms can also use sandwich assays with the same antibody for both capture and detection. If the target molecule is small or consists of a single epitope, a modification of the formats described above is needed. Small molecules by themselves either do not adsorb well to a solid phase, or may be masked by the blocking protein added. However, small molecules can often be attached to larger proteins which provide a means to attach the desired epitope to a solid phase in a configuration that allows the epitope to be bound by an antibody.

Carbohydrates and heavily glycosylated proteins do not adsorb well to polystyrene by the forces described above because they have very little ability to participate in hydrophobic interactions. Membrane proteins released from cells and maintained in solution by detergents are also not adsorbed well in the presence of detergents. Covalent linkage or reduction of the detergent concentration are the best means for attaching these proteins. In fact, covalent linkage can be performed in the presence of detergents such as Tween-20 and Triton X-100.

What is ELISA Mechanism?

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate, see in detail in the section of ELISA device) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. The part of antibody incubation of ELISA is similar with that of western blot. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. 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 in the sample.

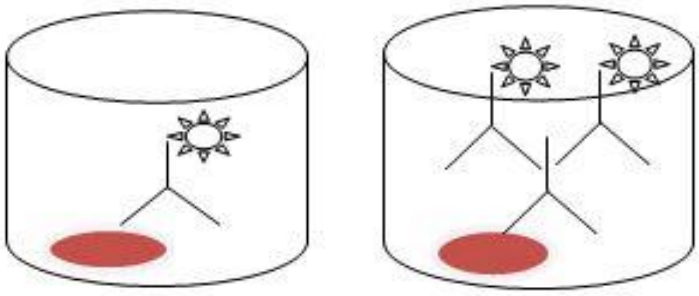
Four kinds of ELISA here are here illustrated as you may concern:

1. Direct ELISA, Simple and Time-Saving

Involve attachment of the antigen to the solid phase, followed by an enzyme-labeled antibody. This type of assay generally makes measurement of crude samples difficult, since contaminating proteins compete for plastic binding sites.

Initially in a **direct ELISA** test which is considered to be the simplest type of ELISA the antigen is adsorbed to a plastic plate, then an excess of another protein (normally bovine serum albumin) is added to block all the other binding sites. While an enzyme is linked to an antibody in a separate reaction, the enzyme-antibody complex is applied to adsorb to the antigen. After excess enzyme-antibody complex is washed off, enzyme-antibody bound to antigen is left. By adding in the enzyme's substrate, the enzyme is detected illustrating the signal of the antigen.

However, in terms of **direct ELISA versus indirect ELISA**, in an indirect ELISA, the steps are similar, but with important differences and an additional step. After the antigen is adsorbed to the plate (and after the BSA step), the next antibody to be added is the antibody that recognizes the antigen (this antibody does not have the enzyme attached to it). Then, an enzyme-antibody conjugate is prepared, which is added to the plate and detects the antibody that is adsorbed to the antigen (in a direct ELISA, the enzyme-antibody conjugate directly adsorbs to the antigen), then the substrate is added which detects the presence of the enzyme and thus the antigen. So, in the indirect ELISA, the enzyme-antibody conjugate uses an antibody against the type of antibody that is used to detect the antigen, kind of like a sandwich. For instance, if the antigen is HIV-1 gp120, then an anti-HIV antibody (HIV-1 gp120 Antibody) is prepared (let's say from a mouse). Then, in a separate reaction, an enzyme is attached to an anti-mouse antibody. So, in order to detect the HIV in the assay, an anti-mouse antibody is used to detect the antibody attached to the antigen.



Direct ELISA Schema Indirect ELISA Schema

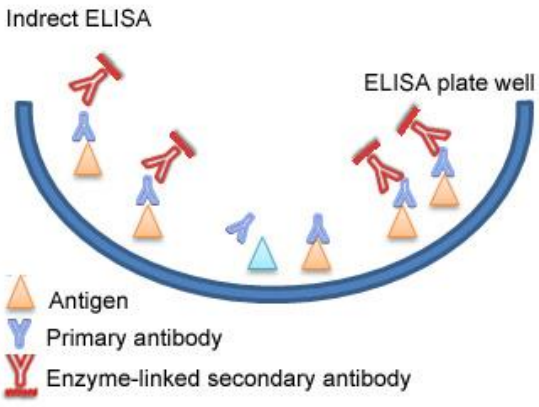
Direct ELISA, when compared to other forms of ELISA testing, is performed faster because only one antibody is being used and fewer steps are required. This can be used to test specific antibody-to-antigen reactions, and helps to eliminate cross-reactivity between other antibodies.

Disadvantages of direct ELISA

The primary antibody must be labeled individually, which can be time-consuming and inflexible when performing multiple experiments. Also, the signal is less amplified in direct ELISA, which means a lower sensitivity and could be viewed as a disadvantage to some.

2. Indirect ELISA, conventional but efficient

Indirect ELISAs also involve attachment of the antigen to a solid phase, but in this case, the primary antibody is not labeled. An enzyme-conjugated secondary antibody, directed at the first antibody, is then added. This format is used most often to detect specific antibodies in sera.



Indirect ELISA is a two-step ELISA which involves two binding process of primary antibody and labeled secondary antibody. The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody. However, this may lead to nonspecific signals because of cross-reaction that the secondary antibody may bring about.

1. Micro-well plates are incubated with antigens, washed up and blocked with BSA.

2. Samples with antibodies are added and washed.

3. Enzyme linked secondary antibody are added and washed.

4. A substrate is added, and enzymes on the antibody elicit a chromogenic or fluorescent signal.

Indirect ELISA advantages:

- High sensitivity: More than one labeled antibody is bound per antigen molecule;
- Flexible: Different primary detection antibodies can be used with a single labeled secondary antibody;
- Cost-saving: Fewer labeled antibodies are required.

In the indirect ELISA test, the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labeled, anti-species globulin conjugate. The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is directly proportional to the amount of bound sample antibody. The more antibody present in the sample, the stronger the color development in the test wells. This format of indirect ELISA is suitable for determining total antibody level in samples

3.Competitive ELISA:

The third type of ELISA is the Competition Assay, which involves the simultaneous addition of 'competing' antibodies or proteins. The decrease in signal of samples where the second antibody or protein is added gives a highly specific result.

Basic Principles

The central event of competitive ELISA is a competitive binding process executed by original antigen (sample antigen) and add-in antigen. The procedures of competitive ELISA are different in some respects compared with Indirect ELISA, Sandwich ELISA and Direct ELISA. A simplized procedure list is as follow:

1. Primary antibody (unlabeled) is incubated with sample antigen.
2. Antibody-antigen complexes are then added to 96-well plates which are pre-coated with the same antigen.
3. Unbound antibody is removed by washing the plate. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
4. The secondary antibody that is specific to the primary antibody and conjugated with an enzyme is added.
5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.

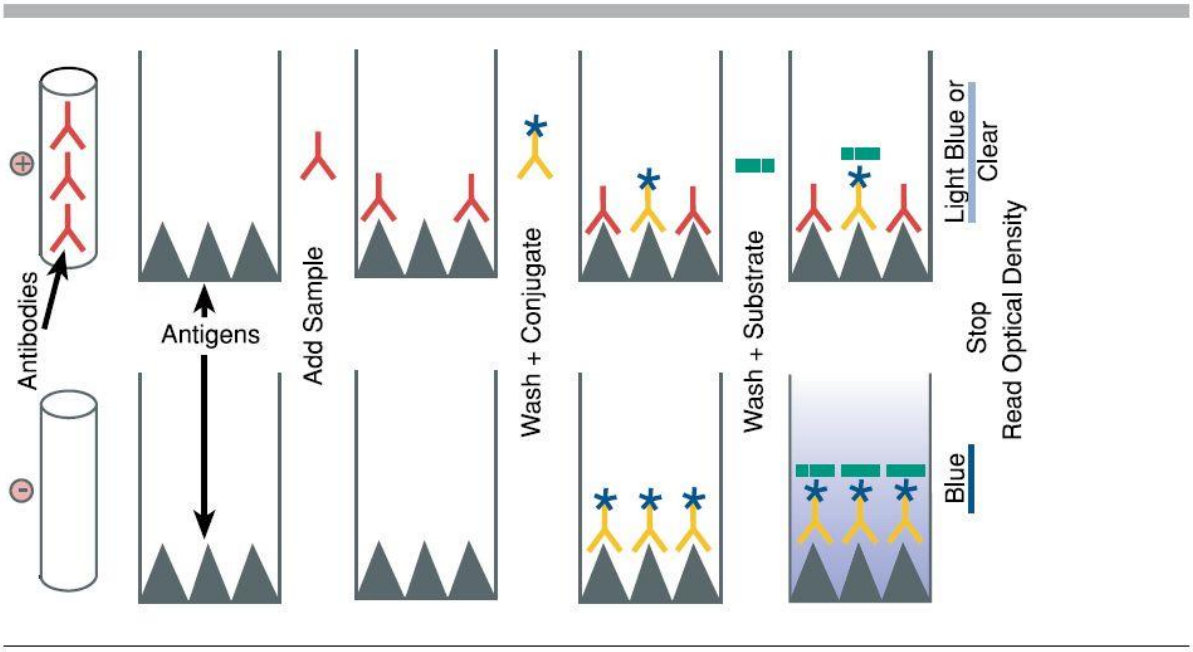
For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present.

(Note that some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with your sample antigen (unlabeled). The more antigen in the sample the less labeled antigen is retained in the well and the weaker the signal).

It is common that the antigen is not first positioned in the well.

Competitive ELISA advantages:

- High specificity, since two antibodies are used the antigen/analyte is specifically captured and detected
- Suitable for complex samples, since the antigen does not require purification prior to measurement
- Flexibility and sensitivity, since both direct and indirect detection methods can be used



4.Sandwich ELISA, Highly Sensitive

The last type of assay is the sandwich ELISA. Sandwich ELISAs involve attachment of a capture antibody to a solid phase support. Samples containing known or unknown antigen are then added in a matrix or buffer that will minimize attachment to the solid phase. An enzyme-labeled antibody is then added for detection.

Sandwich ELISA is a less common variant of ELISA, but is highly efficient in sample antigen detection. Moreover, many commercial ELISA pair sets are built on this sanwich ELISA.

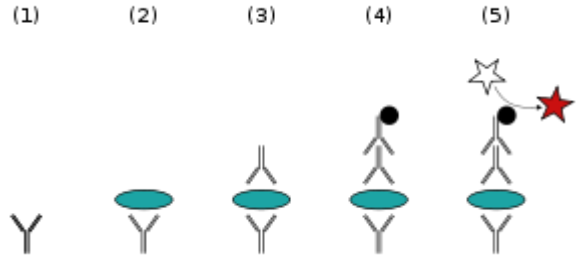
The sandwich ELISA quantify antigens between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic epitope capable of binding to antibody, since at least two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect ELISA), but lower than ELISpot.

Sandwich ELISA procedures can be difficult to optimize and tested match pair antibodies should be used. This ensures the antibodies are detecting different epitopes on the target protein so they do not interfere with the other antibody binding.

The steps are as follows:

1. Prepare a surface to which a known quantity of capture antibody is bound.
2. Block any nonspecific binding sites on the surface.
3. Apply the antigen-containing sample to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. A specific antibody is added, and binds to antigen (hence the 'sandwich': the Ag is stuck between two antibodies);
6. Apply enzyme-linked secondary antibodies as detection antibodies that also bind specifically to the antibody's Fc region (non-specific).
7. Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
8. Apply a chemical that is converted by the enzyme into a color or fluorescent or electrochemical signal.
9. Measure the absorbency or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

The image at the bottom includes the use of a secondary antibody conjugated to an enzyme, though, in the technical sense, this is not necessary if the primary antibody is conjugated to an enzyme. However, use of a secondary-antibody conjugate avoids the expensive process of creating enzyme-linked antibodies for every antigen one might want to detect. By using an enzyme-linked antibody that binds the Fc region of other antibodies, this same enzyme-linked antibody can be used in a variety of situations. Without the first layer of "capture" antibody, any proteins in the sample (including serum proteins) may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized. Use of the purified specific antibody to attach the antigen to the plastic eliminates a need to purify the antigen from complicated mixtures before the measurement, simplifying the assay, and increasing the specificity and the sensitivity of the assay.



Sandwich ELISA Schematic Procedure:

(1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

Sandwich ELISA advantages:

- High specificity, since two antibodies are used the antigen/analyte is specifically captured and detected

- Suitable for complex samples, since the antigen does not require purification prior to measurement
- Flexibility and sensitivity, since both direct and indirect detection methods can be used

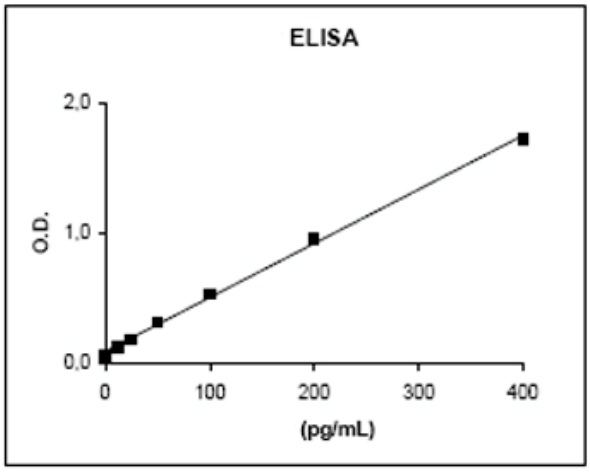
The ELISA method is a benchmark for quantitation of pathological antigens and there are indeed many variations to this method. ELISAs are adaptable to high-throughput screening because results are rapid, consistent and relatively easy to analyze. The best results have been obtained with the sandwich format, utilizing highly purified, prematched capture and detector antibodies. The resulting signal provides data which is very sensitive and highly specific.

ELISA Test

As to better illustrate how ELISA test works, this section took a deeper insight into: a definition of the 'sensitivity', standard curve calculation, and control samples in ELISA test, followed by a list of diseases in which ELISA test could be applied.

ELISA Test-Sensitivity

The factors that determine the ultimate sensitivity of a competitive ELISA test are the antibody affinity constant and the experimental errors. The detection limit of the substrate is not typically limiting. It has been calculated theoretically that with a $K = 10^{12}$ M^{-1} (an extraordinarily high equilibrium constant for an antigen-antibody interaction) and a 1% co-efficient of variation (CV) for the response at zero dose, the lowest detection limit possible would be 10^{-14} M. A more easily achievable limit would be 10^{-9} - 10^{-10} M. The factors limiting the sensitivity of a sandwich ELISA test are the affinity of the antibody, the experimental errors and the nonspecific binding of the labeled antibody, expressed as a percentage of the total antibody. It has been estimated that with a $K = 10^{12}$ M^{-1}, 1% CV of the response at zero dose, and a 1% nonspecific binding of the labeled antibody, the detection limit can be as low as 10^{-16} M. In addition, this can be enhanced further by using more sensitive detection substrates.



ELISA Test-Calculation from the standard curve

Create a standard curve by plotting the mean absorbance for each standard concentration (x axis) against the target protein concentration (Y axis). Draw a best fit curve through the points in the graph (A suitable computer program is suggested for this). We recommend including a standard on

each ELISA test plate to provide a standard curve for each plate used. To determine the concentration of target protein concentration in each sample:

First, find the mean absorbance value of the sample. From the X axis of the standard curve graph, extend a horizontal line from this absorbance value to the standard curve. E.g if the absorbance reading is 1, extend the line from this absorbance point on the X axis. If the samples that have an absorbance value falling out of the range of the standard curve, these samples should be diluted before proceeding with the ELISA test staining. For these samples, the concentration obtained from the standard curve when analyzing the results must be multiplied by the dilution factor.

The coefficient of variation (CV) is the ratio of the standard deviation σ to the mean μ:

$$c_v = \frac{\sigma}{\mu}$$

This is expressed as a percentage of variance to the mean and therefore indicates any inconsistencies and inaccuracies in the results. This sets a standard for the quality of the validated results. Computer programs can be used to calculate the CV values from ELISA test results.

ELISA Test-Control samples

- Positive control

As for the positive control in an ELISA test, use either an endogenous soluble sample known to contain the protein you are detecting, or a purified protein or peptide known to contain the immunogen sequence for the antibody you are using. A positive result from the positive control, even if the samples are negative, will indicate the procedure is optimized and working. It will verify that any negative results are valid. Any tissues, cells or lysates that have been used successfully can be considered a suitable positive control. Try looking at the Swiss-Prot or Omnigene database links on the datasheet. These databases will often have a list of tissues that the protein is expressed in. These can also be considered suitable positive controls. Check the GeneCards entry for the protein. This will usually provide you with relative levels of expression in various tissues. If you still have difficulty finding a suitable control, we recommend doing a quick literature search on PubMed to see which tissues and cells express the protein of interest.

- Negative control

The negative control in an ELISA test means a sample known not to express the protein you are detecting. This is to check for non-specific binding and false positive results. Each plate you use should contain a negative control sample in order to validate the results.

- Standard

A sample containing a known concentration of the target protein from which the standard curve can be obtained.

- Standard in sample matrix (spike control)

When testing serum samples in ELISA tests, include a standard in normal diluent buffer as usual. But we recommend to also include a standard diluted in serum from the species you are testing. The two can then be compared to ensure there is no effect on the standard curve from other proteins in the serum. This is known as a spike control.

- Endogenous positive control

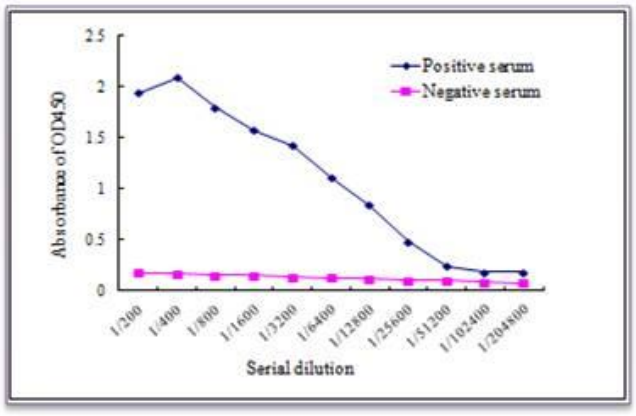
We recommend including an endogenous positive control if you are testing a recombinant protein sample. This should be an essential component of your experiment.

There are inherent difficulties with antibody detection of recombinant proteins that need to be considered. Folding of the recombinant protein may be different from the endogenous native form, and may prevent antibody access to the epitope. This is particularly the case with tagged proteins. Always ensure tags are placed on the N or C terminal end of the recombinant protein.

Most importantly, always ensure the recombinant protein includes the immunogen sequence of the antibody you are using. An endogenous positive control is important to validate the results, as well as to indicate how well the reagents (eg antibodies) and procedure are working.

ELISA Application example

Take one of the applications of ELISA for example, indirect ELISA can be applied in the **determination of antibody titer**:



Many types of immunoassays can be used to detect and quantitate both antigens and antibodies, but there are differences in the avidity requirements for the antibodies, the signal strengths of the labels, and the amount of background for each of these types of assays. Indirect ELISA is the most appropriate technique for measuring the titer of the antisera you have generated. The only difference from general indirect ELISA is that what need to be doubling diluted are antisera (antibodies) other than antigens. In this type of ELISA (Enzyme-Linked Immunosorbant Assay), the antigen (peptide or protein) is bound to the polystyrene microtiter plate first. The antiserum is then doubling diluted by for example, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600, 1/51200, and so on. The antiserum containing the anti-peptide antibody is then added to the well and allowed to bind. Note that, negative serum should also be diluted and added to the well in the same manner with antiserum at the same time. Finally, a secondary antibody, specific for the primary antibody and labeled for detection, is added to the well and allowed to bind. The secondary antibody is usually conjugated with an enzyme. This enzyme catalyzes the formation of colored substance from a colorless substrate, such as TMB. This colored substance is then quantified. The

value of OD450nm of each well is measured by a microplate reader, and then, all the values of A450 for positive serum and nagative serum are recorded in the form of line chart, as is shown above. Calculate the ratio of the absorbance of the positive serum and nagative serum (P/N). If the ratio of P/N is less than 1.5, the result is taken as negative; if $1.5 \leq P/N < 2.1$, the result is doubtful; and only when the value of P/N is more than 2.1, the result can be taken as positive. That is, as the dilutability of serum is getting larger, the dilution point when the ratio of P/N begins to get less than 2.1, is just the titer of antibody for the antiserum.

The technique of ELISA was created by Doctor Dennis E Bidwell and Alister Voller, and the first purpose was to detect various kind of diseases, such as Malaria, Chagas' disease, and Johne disease. For further detailed information about ELISA applications in **disease diagnosis**, please read about the section of ELISA related diseases. If you would like to know about some disease therapeutic targets, suggested here are some useful tools for research on diseases, including Cancer, Alzheimer's, Autoimmune disease, Diabetes, Heart disease, Vascular disease, Parkinson's, etc. Other applications of ELISA will be described respectively as following links.