Enzyme Linked Immunosorbent Assay

ELISA
I
Principles

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SECTION ONE

What do we need to know to run a good ELISA?

Various disciplines in science are needed to allow optimal use of ELISA and these are outlined here. Attention to increasing knowledge in the areas highlighted is essential both in developmental work and in the ultimate value of any test devised.

Defining, as early as possible, the **OBJECTIVES FOR THE USE OF THE ELISA** is essential.

The operator has to have an understanding all other published work concerning the "problem". Development of a diagnostic test for a specific disease requires that all other data pertaining to the biology of that disease, e.g., antigenicity and structure of the agent; antibody production in different animals following infection; qualitative assessment of antibodies by different assays and availability of standard or control sera, etc are examined.

Attention to laboratory facilities is essential, e.g., equipment; reagents already developed; laboratory small animals; experimental large animals; cash to buy commercial products; trained personnel, etc. In this way, the chances of producing a sustainable test to solve the defined problem are significantly greater than where a test is developed by a "dabbling" technique with poor or no forward planning.

Diagram 1

The heterogeneous ELISA is considered in this course, involving separation steps and a solid phase.

Four major advantages of ELISA are promoted in Diagram 1. All of which add to the reasons why this form of ELISA has been, and will continue to be, successful.

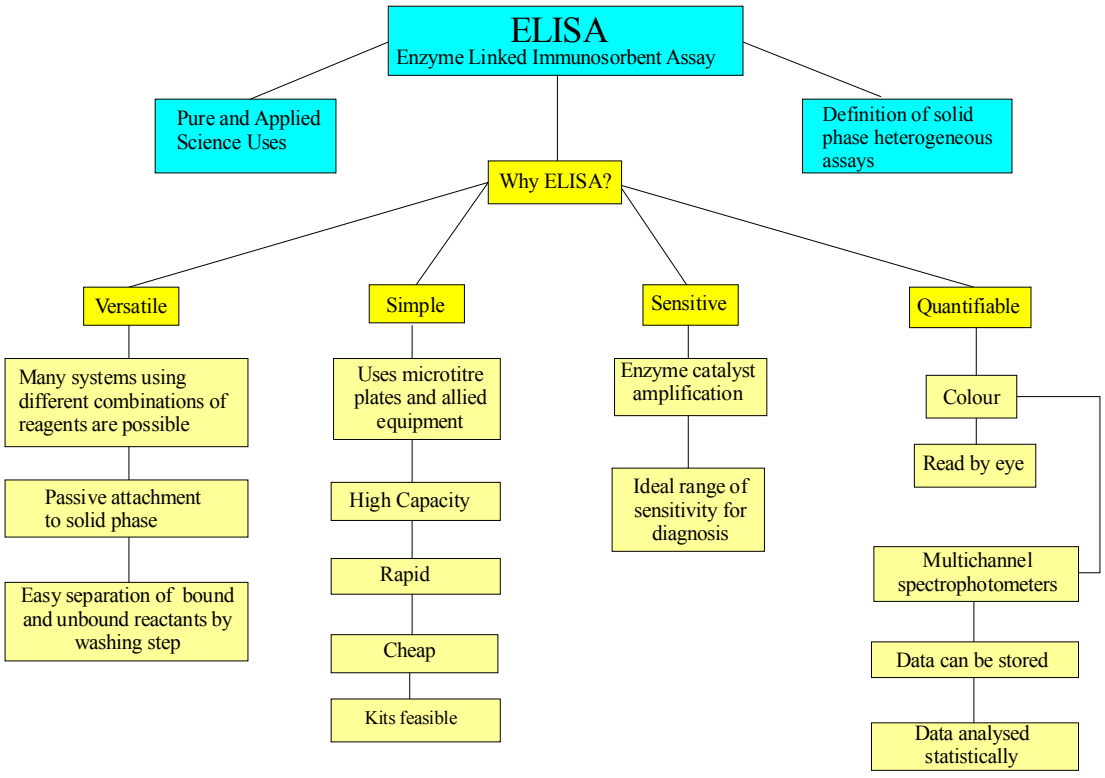


Diagram 2

The systematics of the ELISA and shows the various stages needed and factors important in those stages.

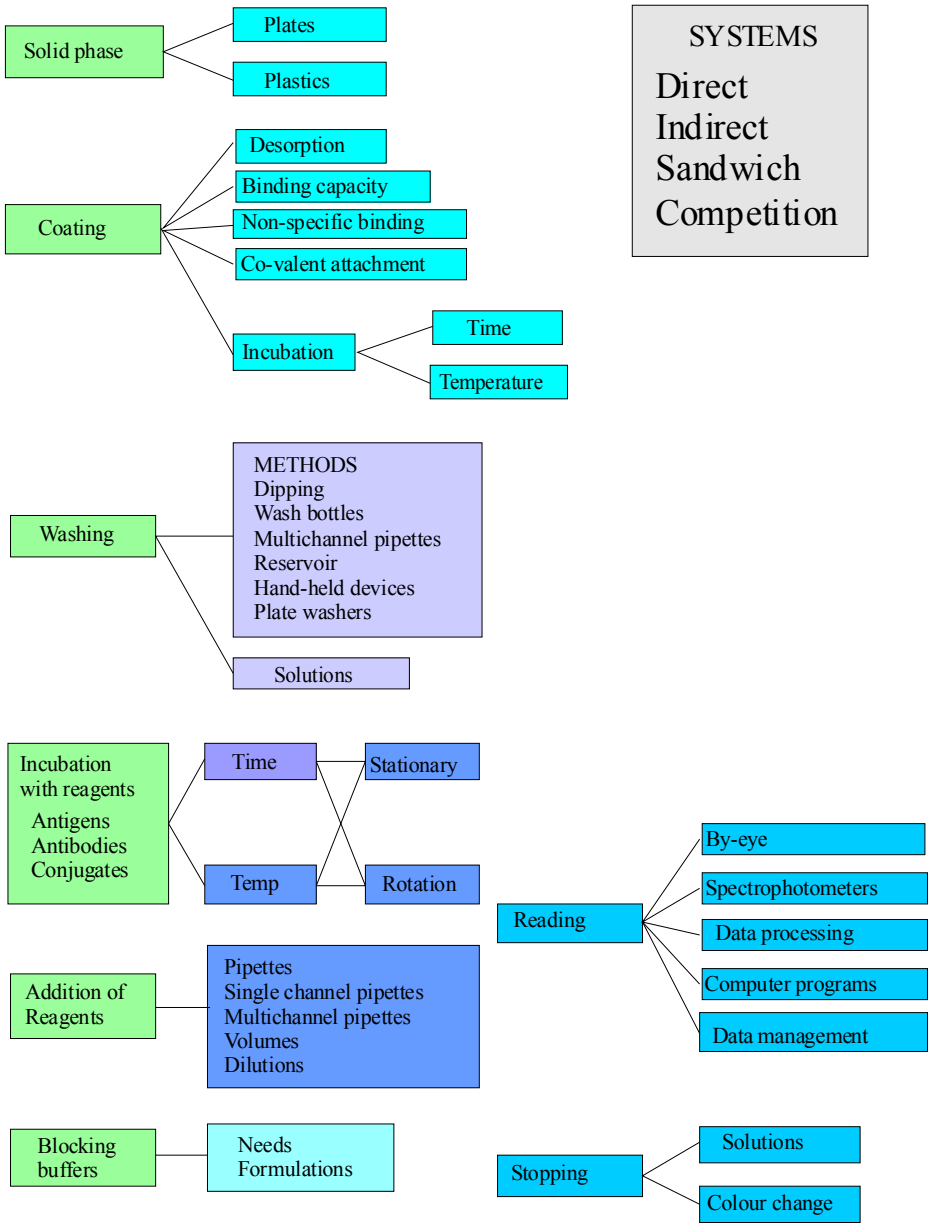


Diagram 3

Emphasizes that using **the equipment** to perform ELISAs requires **skills**, and that both **physical** and **mental** processes are needed. The figure also can be used to indicate that instruments need to be maintained for optimal performance.

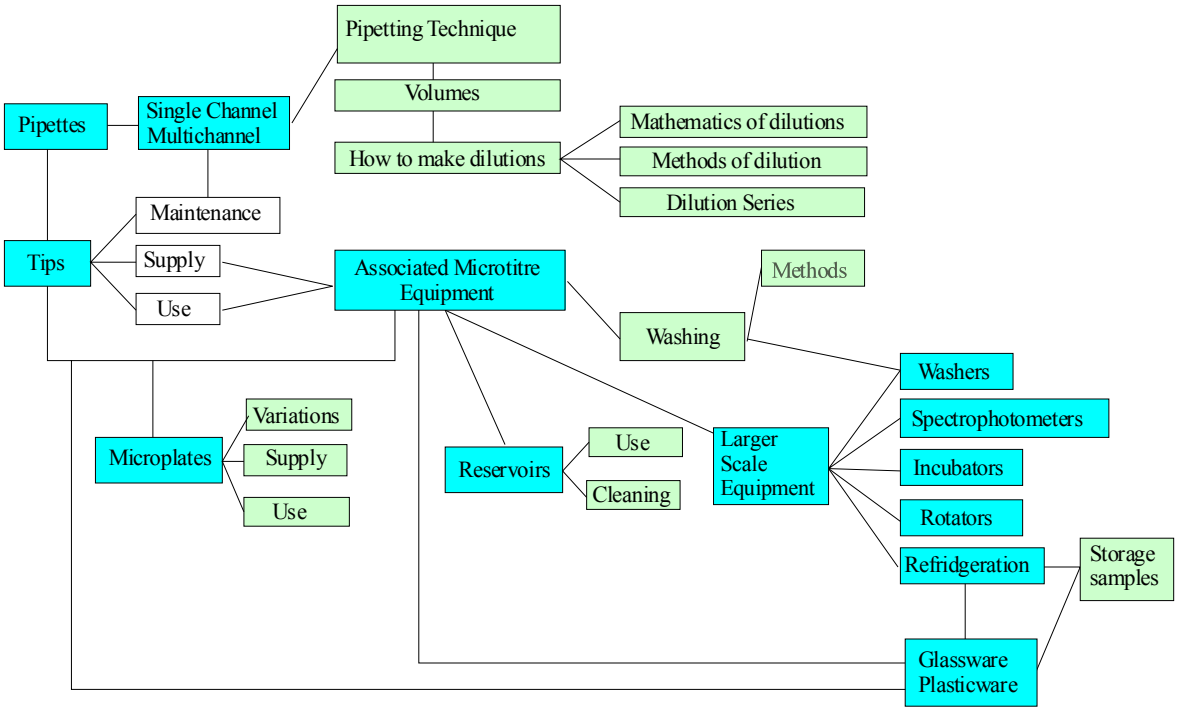


Diagram 4

Deals with the some **enzymatic systems in the ELISA**, and illustrates areas which need some understanding to allow optimal performance to be maintained. Understanding of enzyme kinetics, catalysis reactions, hazards and buffer formulation (pH control), are all essential.

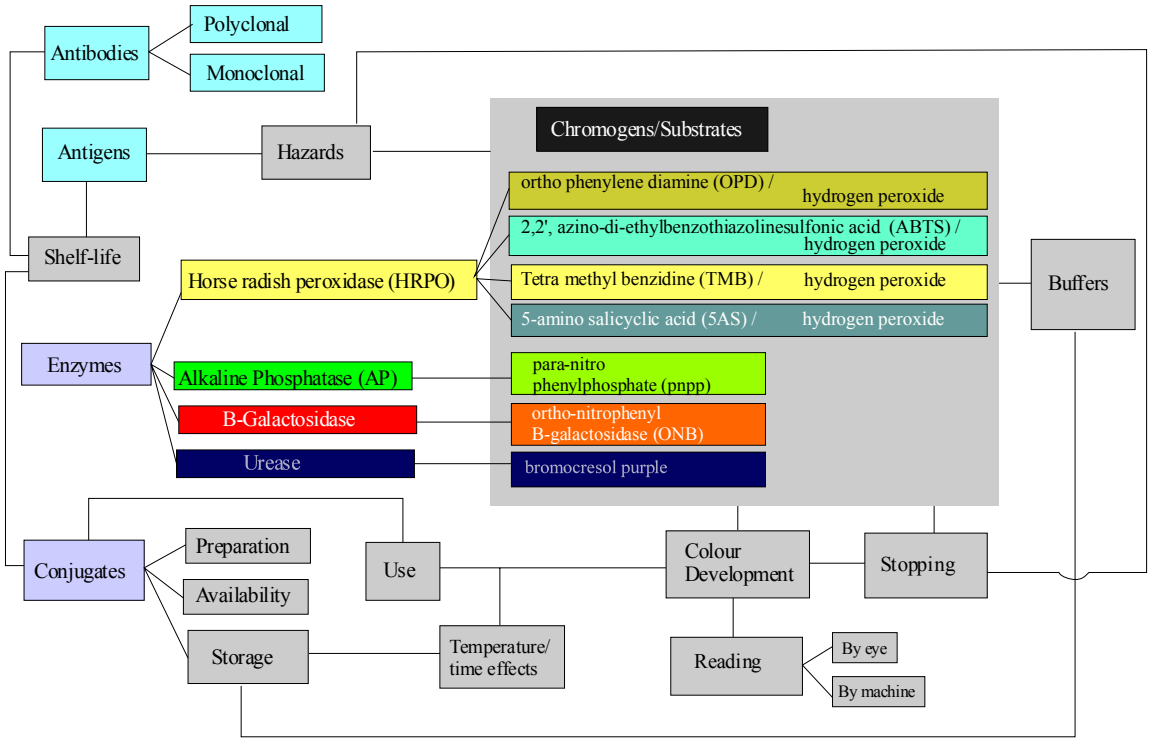


Diagram 5

Leads more into the use of **ELISAs in binding and inhibition/competition interactions** to allow an understanding of a problem. It is essential that the chemical and physical nature of antibodies and antigens are understood, particularly where developmental work is being made. As full an understanding of the antigenic properties of agents being examined is needed to allow maximum exploitation of ELISA, particularly if the results are ever to be understood.

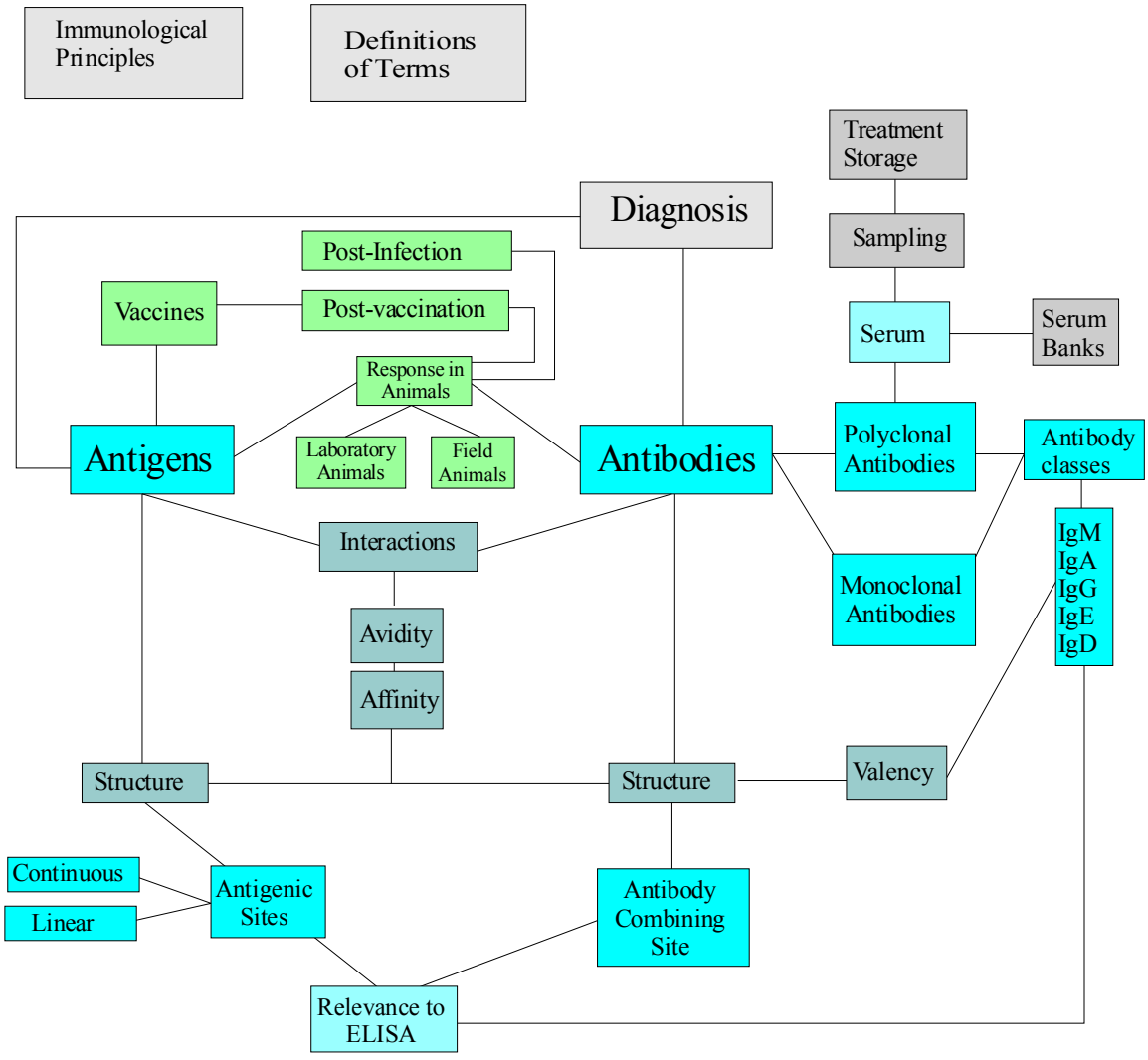


Diagram 6

Deals with **data processing and analysis**. Various essential statistical parameters have to be elucidated, if data is to be interpreted. Such studies actually define any ELISA's performance, allowing confidence in results to be measured, so allowing a meaning to be placed on results. The concepts of controlling assays with references to standards is also needed.

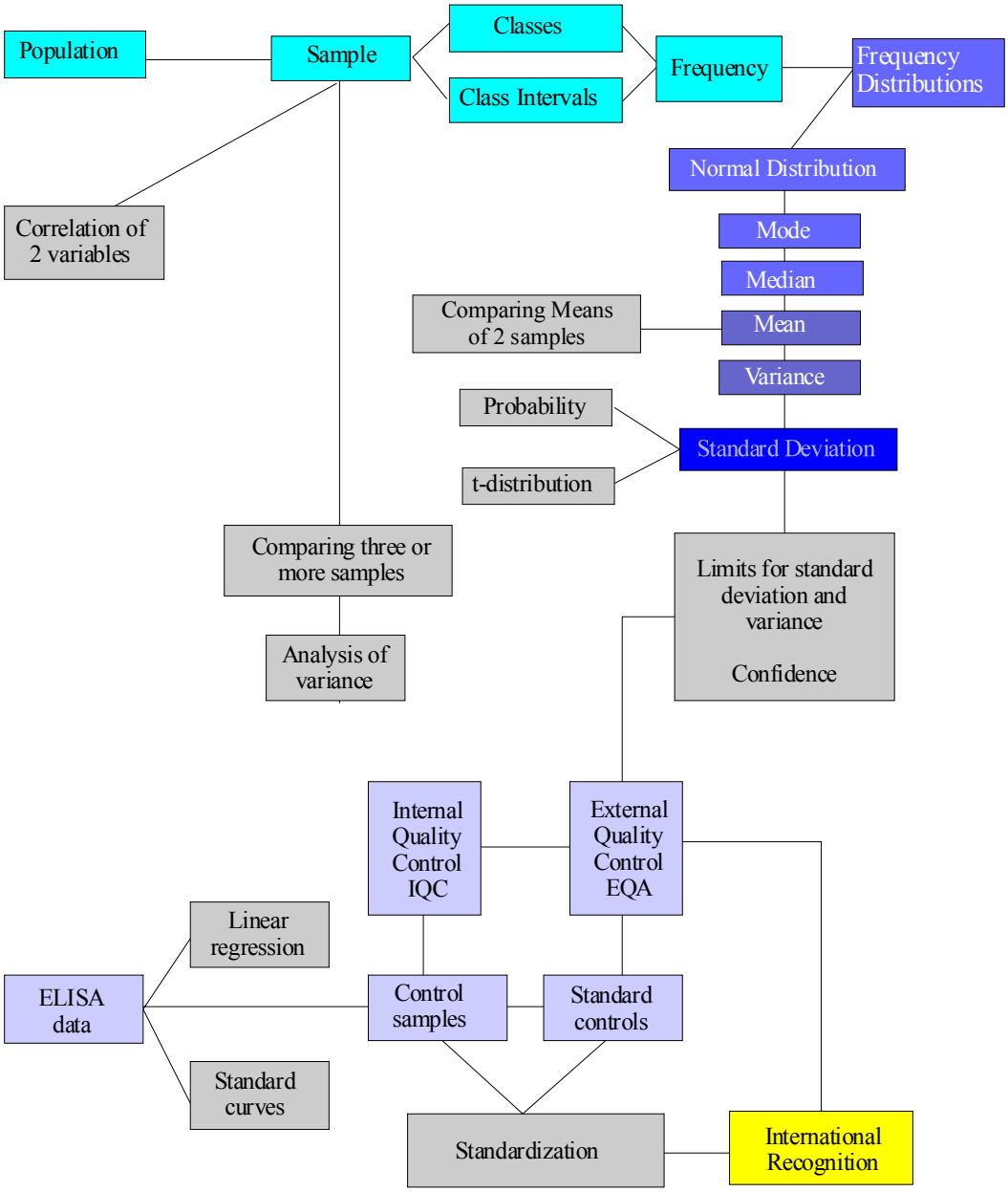
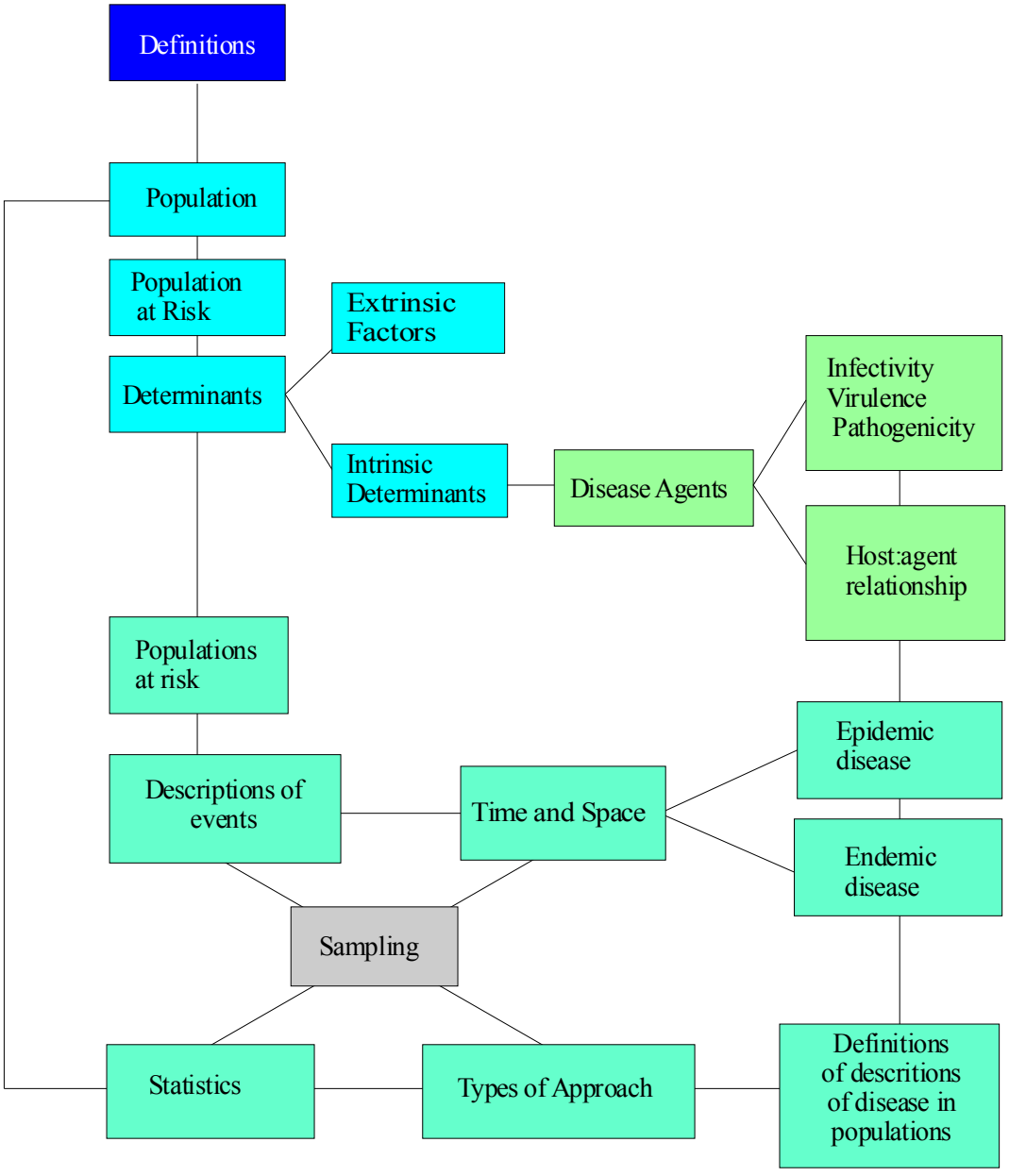


Diagram 7

Extends the use of **statistical understanding** into epidemiological needs. A common use of ELISA is to provide data on populations studied. The areas of sampling (size, number etc.), are vital where planning is made.



These overviews should be used as reference points when considering the development and specific use of any ELISA. They are also useful for trainers in establishing areas of competence in students.

Key points

1. The ELISA is a tool to solve a problem.

2. Any problem should be defined, as clearly as possible, with reference to all previous work defining the specific agent involved and related agents.

3. Other methods for analysing the problem should be reviewed, particularly where tests are already established. This has implications if the ELISA is to "replace" existing tests.

4. The capacity for testing has to be addressed. For example, where an ELISA may be used on a large scale (kit), then sufficient reagents, standard sera, conjugates (batches), and antigen preparations, have to be available. Research leading to successful assays, where reagents are difficult to prepare on a large scale, or require extensive expertise to formulate, or are reliant on a specific limited batch of a commercial reagent, are not sustainable.

5. Where a test may be of use to a wider group of scientists, the possible conditions (laboratory facilities, expertise), should be considered when developing assays. Such technology transfer factors are relevant particularly in developing country laboratories.

The knowledge and skills required to perform ELISA and make use of data have to be gained through a variety of sources, including text books. The ultimate benefit is not the technique in itself, but the meaningful gathering and analysis of the data.

One factor not included in all these examples is that of "common sense".

The ability to really consider what one is doing, and why, and not to overlook the simplicity of what is needed by being blinded by the technology for its own sake.

Most problems are relatively simple to examine after some clear thought. Thus, the good "ELISA-person", will consider the problem first, obtain the necessary technical skills and equipment to perform a test and then obtain data which is from a planned perspective.

As much data from all other tests and the scientific literature is also to be sought. This is true for an assay developer, as well as a person using a supplied, predetermined kit. The skills required by the kit user are no less than the developer, indeed a kit in the hands of an unskilled worker is often useless.

NOTE!

The majority, (90%), of problems observed in practice of ELISA are operator faults caused by lack of common sense, failure to appreciate the need to stick to instructions, sloppy technique or poorly maintained equipment. Most of the remaining percentage is caused by poor quality water!

Systems in ELISA

All heterogeneous systems have three basic parameters.

1. One reactant is attached to a solid phase, usually a plastic microtiter plate with a 8 x 12 well format.

2. Separation of bound and free reagents, which are added subsequently to the solid phase attached substance, is by a simple washing step.

3. Results are obtained through the development of color.

Terms

Immunoassays involve tests using antibodies as reagents.

Enzyme immunoassays (EIA) make use of enzymes attached to one of the reactants in an immunoassay to allow quantification through the development of color after addition of a suitable substrate/chromogen.

As indicated above ELISAs involve the step-wise addition and reaction of reagents to a solid phase bound substance, through incubation, and separation of bound and free reagents using washing steps.

An enzymic reaction is utilized to yield color and is used to quantify the reaction, through use of an enzyme labeled reactant.

A very brief definition of terms used in ELISA is needed.

TERM	Definition
Solid phase	This is usually a microtiter plate well. Specially prepared ELISA plates are commercially available. These have an 8 x 12 well format and can be used with a wide variety of specialized equipment designed for rapid manipulation of samples including multichannel pipettes
Adsorption	This is the process of adding an antigen or antibody, diluted in buffer, so that it attaches passively to the solid phase on incubation. This is a simple way for immobilization of one of the reactants in the ELISA and one of the main reasons for its success
Washing	Simply flooding and emptying the wells with a buffered solution is enough to separate bound (reacted) from unbound (unreacted) reagents in the ELISA. Again this is a key element to the successful exploitation of the ELISA.
Antigen	These are proteins or carbohydrates which when injected into animals elicit the production of antibodies. Such antibodies can react specifically with the antigen used and therefore can be used to detect that antigen
Antibodies	Antibodies are produced in response to antigenic stimuli. These are mainly protein in nature. In turn antibodies are antigenic
Anti-species antibody	These are produced when proteins (including antibodies) from one species are injected into another species. Thus, guinea pig serum injected into a rabbit elicits the production of rabbit anti-guinea pig antibodies
Enzyme	A substance which can react at low concentration as a catalyst to promote a specific reaction. Several specific enzymes are commonly used in ELISA with their specific substrates
Enzyme conjugate	An enzyme which is attached irreversibly to a protein, usually an antibody. Thus, an example of anti-species enzyme conjugate would be rabbit anti-guinea linked to horse radish peroxidase
Substrate	The substrate is a chemical compound with which an enzyme reacts specifically. This reaction is used in some way to produce a signal which is read as a color reaction (directly as a color change of the substrate or indirectly by its affect on another chemical)
Chromophore	This is a chemical which alters color as a result of enzyme inter--action with substrate
Stopping	The process of stopping the action of enzyme on the substrate, has the effect of stopping any further change in color in the ELISA
Reading	This implies measurement of color produced in the ELISA. This is quantified using special spectrophotometers reading at specific wavelengths for the particular colors obtained with particular enzyme/chromophore systems. Tests can be assessed by-eye

Basic Systems of ELISA

Many configurations are possible in ELISA. The terminology here may not always agree with that used by others so care is needed in defining assays by "name-only".

The specific assay parameters must always be examined carefully in the literature.

This set of definitions attempts to clear up the myriads of published approaches to describing the systems used in a few words such as "double-sandwich competitive ELISA" and "indirect sandwich inhibition ELISA.

There are three main methods that form the basis to all ELISAs.

1. DIRECT ELISA

2. INDIRECT ELISA

3. SANDWICH ELISA

All these systems (1-3) can be used in assays called:

4. COMPETITION OR INHIBITION ELISA

1. Direct ELISA

Symbol	Description
I	Solid phase microtiter well
-	Attachment to solid phase by passive adsorption
Ag	Antigen
Ab	Antibody
AB	Antibody (different species donor than Ab)
AntiAb	Anti-species antiserum against species from donor Ab
AntiAB	Anti-species antiserum against species from donor AB
**ENZ	Enzyme linked to reactant
S/C	Substrate/chromophore system
WASH	Washing step
oC	Incubation
READ	Read color in spectrophotometer
+	Addition of reagents
	Binding of reagents
STOP	Stopping of color development

Direct ELISA

This is the simplest form of the ELISA .

Step	Action	Phase
1	I	Solid phase e.g. Plastic well
2	I- + Ag	Coating. Add antigen.
3	I-Ag oC	Adsorption. Incubate
4	Wash	Washing Separate bound and free Ag
5	I-Ag	Coated solid phase
6	I—Ag + Ab**Enz	Conjugate addition Add specific antibody labeled with enzyme. Diluent contains blocking agent. Incubate
7	Wash	Separate bound and free conjugate
8	I-Ag-Ab**Enz	Conjugate bound toAg
9	I-Ag-Ab**Enz + S/C	Addition of substrate /chromphore and incubation
10	Colour	Colour development
11	STOP	Addition of stopping reagent
12	READ	Read Absorbance or by eye

Coating (Steps 2 and 3)

Antigen is diluted in a buffer and added to the solid phase, commonly high pH (9.6) carbonate/bicarbonate buffer or neutral phosphate buffered saline. The key is that the buffer contains no other proteins that might compete with the target antigen for attachment to the plastic solid phase. Antigens are mainly protein in nature, and attach passively to the plastic during a period of incubation. The temperature and time of the incubation is not so critical, but standardization of conditions is vital and the use of incubators at 37^0C is favoured, (since they are widely available in laboratories).

Washing (Steps 4 and 7)

Washing is necessary to separate free from bound reagents. In Step 4, any unadsorbed antigen is removed, in Step 7 it is necessary to remove free antibody enzyme from that bound specifically to the antigen. Washing can be simply flooding and emptying wells, using a neutral buffered solution (e.g., PBS).

Conjugate addition (Step 6)

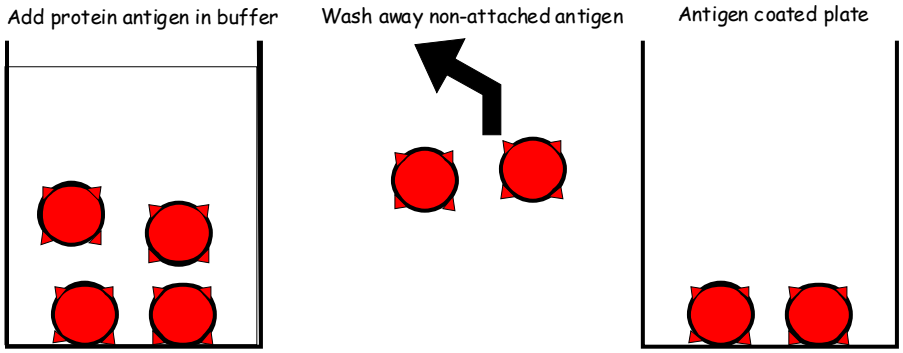
Antibodies conjugated with an enzyme are added directed specifically against antigenic sites on the solid-phase bound reagent. The conjugated antibodies are diluted in a buffer containing some substances which inhibits passive adsorption of protein, but which still allows immunological binding. Such substances are either other proteins, which are added at high concentration to 'compete' for the solid phase sites with the antibody protein; or detergents at low concentration and are termed "blocking " agents, and the buffers they help formulate are called **"blocking buffers"**. On incubation, antibodies bind to the antigen.

A simple washing step is then used to get rid of unbound antibodies **(Step 7).** The complex then on plate is antigen combined with specific enzyme labeled antibody.

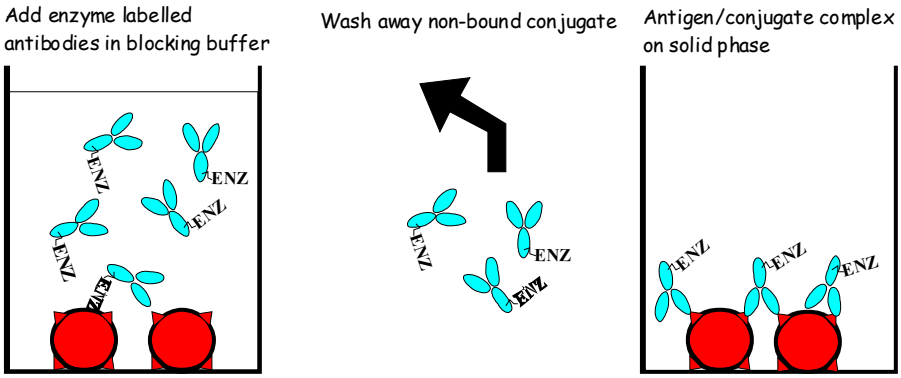
Substrate/Chromphore Addition (Step 9)

This involves addition of a suitable substrate or substrate/chromogen combination for the particular enzyme attached to the antibodies. The objective is to allow development of a color reaction through enzymic catalysis. The reaction is allowed to progress for a defined period after which the reaction is stopped by the alteration in pH of the system, or addition of an inhibiting reactant. Finally, the color is quantified by the use of a spectrophotometer reading at the appropriate wavelength for the color produced.

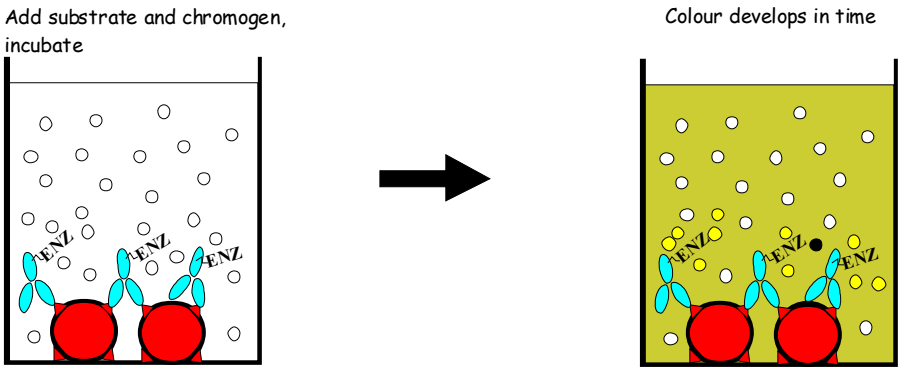
Another WAY to show Direct ELISA



a) Antigen is added in buffer. The protein attaches passively to plastic surface of microtitre plates well. After a period of incubation the non-adsorped protein is washed away.

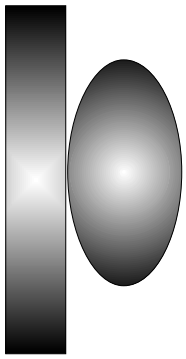


b) Antibodies with enzyme co-valently linked (conjugate) is added in a solution containing inert protein and detergent (to prevent non-specific attachment of the antibodies to plastic wells. The antibody binds to the antigen on well surface. After incubation, non-bound antibodies are washed away.



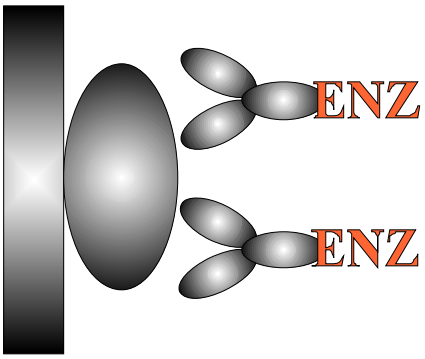
c) Add substrate and chromogenic dye solution. Substrate interacts with enzyme to affect dye solution to give a colour reaction.

Another way



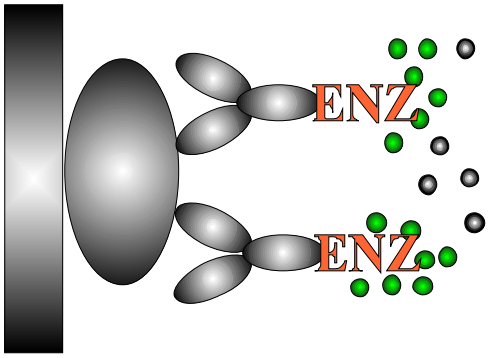
Antigen is coated onto wells by passive adsrption and incubation.

Wells are washed to get rid of free antigen



Antibody conjugated with enzyme is added and incubated with antigen

Wells are washed to get rid of unbound conjugate



Substrate/chromophoreis added and colour develops

The reaction is stopped and read in a spectrophotometer

2. Indirect ELISA

Step	Action	Phase
1	I	Solid phase e.g. Plastic well
2	I + Ag	Coating. Add antigen.
3	I-Ag ℃	Adsorption. Incubate
4	Wash	Washing Separate bound and free Ag
5	I-Ag	Coated solid phase
6	I-Ag + Ab	Antibody addition. Add specific antibody from particular species e.g. rabbit/cow/sheep etc. Diluent contains blocking agent. Incubate
7	Wash	Separate bound and free conjugate
8	I-Ag-Ab	Antibody is bound to antigen
9	I-Ag-Ab + AntiAb**Enz	Addition of enzyme labeled antibodies against particular species in which Ab was produced, incubate
10.	Wash	Separate bound and free conjugate
11.	I-Ag-Ab-AntiAb**Enz	Bound conjugate only
12.	I-Ag-Ab-AntiAb**Enz + S/C	Addition of substrate /chromphore and incubation
13	Colour	Colour development
14	STOP	Addition of stopping reagent
15	READ	Read Absorbance or by eye

Steps 1-5 are similar to the Direct system where wells are coated with antigen.

Step 6 involves the addition of unlabeled antibodies, which are diluted in a buffer to prevent non-specific attachment of proteins in antiserum to solid phase, (blocking buffer).

This is followed by incubation and washing away excess (unbound) antibodies, to achieve specific binding

Step 7 involves washing away unbound antibodies leaving the complex in **Step 8**, consisting of antigen and antibody, attached to solid phase.

Step 9 is the addition of the conjugate (enzyme-labeled), anti-species antibodies, diluted in blocking buffer, again followed by incubation and washing to achieve binding of conjugate. After washing (**Step 10**) the complex on the solid phase has increased with the enzyme conjugate detecting the specific species of antibody detecting the antigen (**Step 11**)

Step 12 involves the addition of the substrate/chromophore and allowing the colour to develop (**Step 13**). Stopping the color is **Step 14** and reading is **Step 15**.

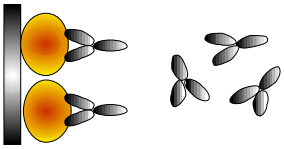
The Indirect system is similar to the Direct system, in that antigen is directly attached to the solid phase and 'targeted' by added antibodies, (detecting antibodies). However, these added antibodies are not labeled with enzyme but are themselves 'targeted' by antibodies linked to enzyme. Such antibodies are produced against the immunoglobulins of the species in which the detecting antibodies are produced and termed "anti-species" conjugates.

Thus, if the detecting antibodies are produced in rabbits, the enzyme labelled antibodies would have to be anti-rabbit immunoglobulin in nature. This allows great flexibility in use of anti-species conjugates in that different specificities of conjugate can be used to detect particular immunoglobulins binding in the assay and there are literally thousands of commercially available conjugates available. For example, the anti-species conjugate could be anti-IgM, anti IgG_1, IgG_2 , etc.

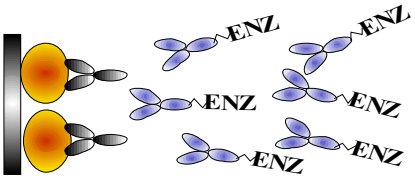
The Indirect system offers the advantage that any number of antisera can be examined for binding to a given antigen using a single anti-species conjugate. Such systems have been heavily exploited in diagnostic applications particularly when examining (screening) large numbers of samples.

Another view of Indirect ELISA

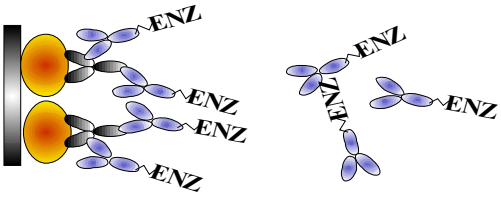
Antigen is passively adsorped to solid phase by incubation.



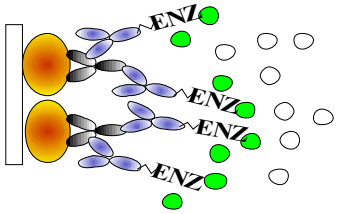
Antibodies are added and incubated with solid-phase attached antigen. Those which are specific will bind to antigen.Excess antibodies or non-binding components are washed away after incubation phase.



Antibodies labeled with enzyme (conjugate) directed against the particular species in which the original antibodies were produced (anti-species).



These bind to any antibodies which are attached to antigen. Excess conjugate is washed away after a period of incubation.



Substrate/chromophore is added and colour develops as a result of enzyme present.

After a period of incubation the colour development is stopped and read by spectrophotometer.

3. Sandwich ELISA

The term sandwich comes from the fact that antigens are sandwiched between detecting antibody both on the solid phase and acting as an enzyme labeled conjugate. The antibody attaching to solid phase is sometimes called-Capture antibody, the detecting antibody-Detector.

The sandwich systems vary depending on the source of the antibodies used. To simplify terminology, the following definitions will be used.

Sandwich Direct
Sandwich Indirect

Sandwich Direct

A. **The detecting antibody is labelled (conjugate)**
B. **The capture and detecting antibodies can be from the same sample**
C. **The detecting antibody can be from different species**

Sandwich Indirect

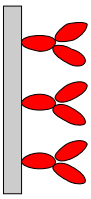
A. **The detecting antibody is NOT labelled**
B. **The detecting antibody is not prepared in same species as capture antibody**
C. **The detecting antibody is detected using an anti-species conjugate**

There are complications using the same specificities outlined after the overviews.

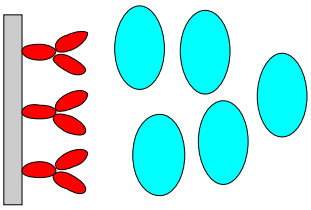
Sandwich Direct

Step	Action	Phase
1	I	Solid phase e.g. Plastic well
2	I + Ab	Coating. Add antibody in coating buffer
3	I-Ab °C	Adsorption. Incubate
4	Wash	Washing Separate bound and free Ag
5	I-Ab	Coated solid phase
6	I-Ab + Ag °C	Antigen addition. Diluent contains blocking agent. Incubate
7	Wash	Separate bound and free conjugate
8	I-Ab-Ag	Antigen is bound to antibody
9	I-Ab-Ag + Ab**Enz °C	Addition of enzyme labeled antibodies against antigen diluted in blocking buffer. Incubate
10.	Wash	Separate bound and free conjugate
11.	I-Ab-Ag-Ab**Enz	Bound conjugate only
12.	I-Ab-Ag-Ab**Enz + S/C	Addition of substrate /chromphore and incubation
13	Colour	Colour development
14	STOP	Addition of stopping reagent
15	READ	Read Absorbance or by eye

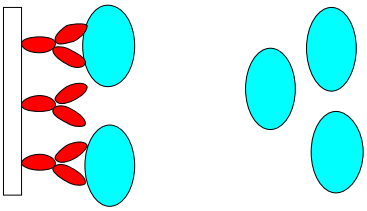
Sandwich Direct



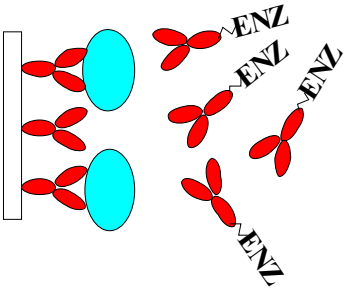
1. Antibodies are passively attached to solid phase by incubation in buffer.



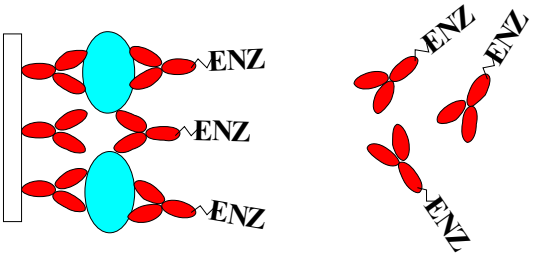
2. Free antibodies are washed away and then antigen added.



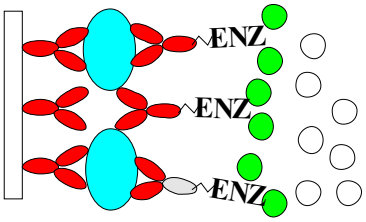
3. Antigen is captured by coating antibodies during incubation. Unbound antigen is then washed away.



4. Conjugated antibodies directed against antigen are added. These antibodies can be the same preparation as used on the solid phase or from a different source (species).



5. Binding of the conjugate during incubation completes the 'sandwich'. Free conjugate is washed away.



6. Chromophore/substrate is added and colour allowed to develop for a defined time.

7. The reaction is stopped and colour quantified in a spectrophotometer.

The Sandwich ELISA-Direct

This involves the passive attachment of antibodies to the solid phase. These antibodies then bind antigen(s). The antigen(s) are diluted in a blocking buffer to avoid non-specific attachment to the solid phase. Here the components of the blocking buffer should not contain any antigens which might bind to the capture antibodies.
After incubation and washing an antibody-antigen complex is attached to the solid phase.
The captured antigen (sometimes referred to as "trapped"), is then detected by addition and incubation of enzyme labelled specific antibodies in blocking buffer
Thus, this is a direct conjugate binding with the antigenic targets on the captured antigen.
This second antibody can be the same as that used for capture, or be different in terms of specific animal source or species in which it was produced.
After incubation and washing,the bound enzyme is developed by the addition of substrate/chromogen and then stopped and read using a spectrophotometer.

- Since a single enzyme-conjugated antibody is used, the system is limited to the specificities and properties inherent in that particular antibody 'set'. This limits the versatility of the test, e.g., each antibody preparation used must be labelled (for different antigens), in the same way as the Direct ELISA was limited to single antibody preparations.

- The system also is limited in that antigens have to have at least two antigenic sites, since both the capture and the detecting antibodies need to bind. This can limit the assay to relatively large antigenic complexes.

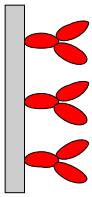
- The capture antibody (on the solid phase) and the detecting antibody, can be against different epitopes on an antigen complex. This can be helpful in orienting the antigenic molecules so that there is an increased chance that the detecting antibodies will bind. It can also be an advantage when investigating small differences between antigenic preparations by use of different detecting antibodies and a common capture antibody.

- More versatile and hence appropriate systems are dealt with below (Sandwich ELISA-Indirect), for this purpose. The use of exactly the same antibodies for capture and detection (e.g., use of monoclonal antibodies) can lead to problems whereby there is a severe limitation of available binding sites for the detector.

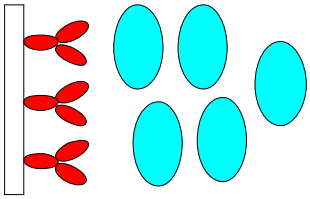
Sandwich Indirect

Step	Action	Phase
1	I	Solid phase e.g. Plastic well
2	I + Ab	Coating. Add antibody in coating buffer
3	I-Ab ºC	Adsorption. Incubate
4	Wash	Washing Separate bound and free Ag
5	I-Ab	Coated solid phase
6	I-Ab + Ag ºC	Antigen addition. Diluent contains blocking agent. Incubate
7	Wash	Separate bound and free conjugate
8	I-Ab-Ag	Antigen is bound to antibody
9	I-Ab-Ag + AB ºC	Addition of different species antibody diluted in blocking buffer. Incubate
10.	Wash	Separate bound and free conjugate
11.	I-Ab-Ag-AB	Bound antibody (AB) only
12.	I-Ab-Ag-AB + Anti AB**Enz ºC	Addition of anti species (AB) conjugate and incubation
13	I-Ab-Ag-AB-Anti AB**Enz	Bound conjugate
14	I-Ab-Ag-AB-Anti AB**Enz + S/C	Addition substrate / chromophore
15	Colour	Colour development
16	STOP	Addition of stopping reagent
17	READ	Read Absorbance or by eye

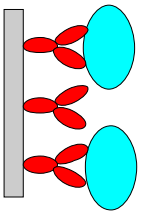
Sandwich Indirect



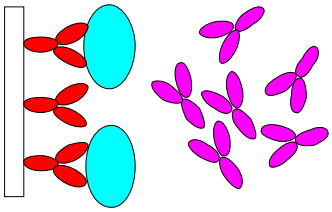
1. Antibodies are passively attached to solid phase by incubation in buffer.



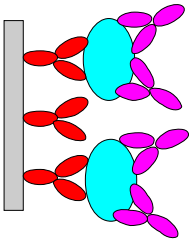
2. Free antibodies are washed away and then antigen added.



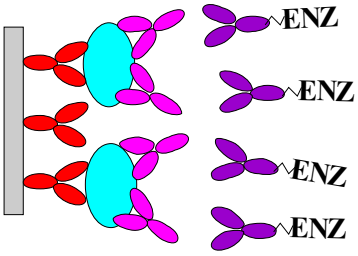
3. Antigen binds to antibodies on solid phase during incubation, free antigen is washed away



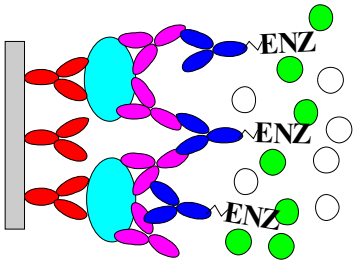
4. Antibodies against antigen are added, these are from a different species to solid phase antibodies.



5. Second antibody binds on incubation, free antibodies are washed away. This completes the antibody 'sandwich'.This cannot be from same species as coating antibodies.!



6. Anti-species conjugate is added which binds to species of serum from which the second antibody was prepared. This cannot react with solid phase antibodies. After incubation the unbound conjugate is washed away.



7. Substrate/chromphore is added and colour development allowed on incubation.

8. The reaction is stopped and quantified using a spectrophotometer.

The Sandwich ELISA-Indirect

This has similar steps to the Sandwich Direct for coating with capture antibody and addition of antigens. Thus, antibodies are passively attached to the solid phase and antigen(s) are captured.

However, the detection of the antigen involves the addition of unlabelled antibodies.

After incubation and washing, the detecting antibodies are themselves 'detected' by addition and incubation with an anti-species enzyme conjugate.

The bound conjugate is then processed as described in the other systems.

The advantage of this assay is that any number of samplescontaining antibodies can be added to the captured Ag, provided that the species in which it was produced is not the same as the capture Ab. More specifically, that the enzyme conjugated anti-species antibody, does not react with the antibodies used to capture the antigen.

It is possible to use the same species of antibody, if immunochemical techniques are used to select and produce particular forms of antibodies and with attention to the specificity of the enzyme conjugate used.

Thus, as an example, the capture antibody could be processed to a bivalent molecule without the Fc portion (a so called FAb2 fraction). The detecting antibodies could be untreated. The enzyme conjugate could then be an anti-species anti-Fc portion of the immunoglobulin molecule. Thus, the conjugate would only react with antibodies containing Fc (and not therefore the capture molecules).

The need to devise such assays depends on the reagents available. It may be that a monoclonal antibody is available that confers a desired specificity as compared to polyclonal sera or that one wishes to screen a large number of monoclonal antibodies against an antigen which must be captured (it may be at low concentration or in a mixture of other antigens). In this case use of polyclonal sera is unsuccessful, therefore the preparation of FAb2 fragments for the capture antibody is worthwhile, and in facts relatively easy to use kits are available for this purpose. The use of a commercially available anti-mouse Fc completes the requirements.

Competition/Inhibition Assays

The terms **"competition"** and **"inhibition",** describe assays in which measurement involves the quantification of a substance by its ability to interfere with an established pre-titrated system.

The systems involve all the other ELISA configurations already described.

The assays also can be used for the measurement of either antibody or antigen.

The terminology used in the literature can lead to confusion, the term "blocking-ELISA is also frequently used to describe such assays.

The abbreviation C-ELISA (for competition ELISA) and I-ELISA (for Inhibition ELISA) will be used to generally describe assays involving the elements described above and the particular application of competitive or inhibition assay dealt with specifically for each different system examined.

Reference should be made to the preceding descriptions of the basic systems for Direct, Indirect and Sandwich ELISAs which are the basis of the C-I assays.

C-Direct ELISA, Test for Antigen

Step	WITH competing antigen	WITHOUT competing antigen
	I-Ag-Ab**Enz Pre-titrated system	I-Ag-Ab**Enz Pre-titrated system
1	I-Ag + Agc + Ab**Enz Add competing Agc in blocking buffer and incubate with Ab**Enz on addition to I-Ag	I-Ag + 0 + Ab**Enz Add control negative 0 Ag
2	∝	∝
3	Wash	Wash
4	I-Ag Agc-Ab**Enz washed away	I-Ag-Ab**Enz Not prevented from binding to I-Ag
5	I-Ag NO COLOUR	I-Ag-Ab**Enz COLOUR
6	STOP	STOP
7	READ(100% competiton	0% competition

The **Direct system,** already titrated to optimise the amount of antigen on the solid phase and amount of conjugate, is challenged by the addition of antigen by addition to the conjugate simultaneously as it is added to the plate.

If the antigen is the same or similar to that on the solid phase, the conjugate binds to this. Where the concentration of the competing antigen is high, it prevents any conjugate binding to the solid phase antigen (100% competition). As the concentration of the competing antigen is reduced e.g., on dilution, a decrease in competition is observed. Thus the effect of the addition of any sample, is measured by the decrease in expected color of the pre-titrated system (used as a control).

The competitor can be added directly to the solid phase if diluted in blocking buffer before the addition of the conjugate.

The degree of competition in time depends on the relative concentration of molecules of the test and solid phase antigen (and to the degree of antigenic similarity).

After incubation and washing, the amount of labeled antibodies in the test is quantified after addition of substrate etc. Where there is no antigen in the test sample, or where the antigenic similarities are limited, there is no binding with the labelled antibodies thus, there is nothing to prevent (compete with) the binding of the labelled antibodies. The net result is that for samples containing antigen there is competition affecting the pre-titrated expected color, whereas in negative samples there is no effect on the pre-titrated color.

C-Direct ELISA, Test for Antibody

Step	WITH competing antibody	WITHOUT competing antibody
	I-Ag-Ab**Enz	Pre-titrated system
1	I-Ag + AB + Ab**Enz Add competing AB in blocking buffer and incubate with Ab**Enz on addition to I-Ag	I-Ag + 0 + Ab**Enz Add control negative 0 AB
2	∝	∝
3	Wash	Wash
4	I-Ag-AB Ab**Enz washed away	I-Ag-Ab**Enz Not prevented from binding to I-Ag
5	I-Ag-AB NO COLOUR	I-Ag-Ab**Enz COLOUR
6	STOP	STOP
7	READ(100% competiton	0% competition

The system here is the same as for C-Direct antigen however, the measurement (or comparison) of antibodies is being made. Again there is a requirement to titrate the Direct ELISA system which is then 'challenged" by the addition of test antibodies. The competitive aspect here is between any antibodies in the test sample and the labelled specific antibodies for antigenic sites on the solid phase bound antigen. The test sample and pre-titrated labeled antibodies are mixed before additon to the antigen coated plates.

Competitive and Inhibition Assays for Indirect ELISA

C-Indirect Antigen

Step	WITH competing antigen	WITHOUT competing antigen
	I-Ag-Ab-AntiAb**Enz Pre titrated Indirect ELISA	I-Ag-Ab-AntiAb**Enz Pre titrated Indirect ELISA
1	I-Ag + Abc + Ab Add competing ABc and pre titrated Ab to wells at same time Competing ABc NOT same species as Ab	I-Ag + 0 + Ab Add negative sample and pre titrated Ab
2	°C then wash	°C then wash
3	I-Ag-ABc	I-Ag-Ab
4	I-Ag-ABc + AntiAb**Enz	I-Ag-Ab + AntiAb**Enz
5	°C then wash	°C then wash
6	I-Ag-ABc	I-Ag-Ab-AntiAb**Enz
7	I-Ag-Abc + S/C	I-Ag-Ab-AntiAb**Enz + S/C
8	STOP	STOP
9	READ	READ
10	NO COLOUR	COLOUR
11	COMPETITION	NO COMPETITION

Samples possibly containing antibodies specific for the antigen on the plates are added simultaneously with the pre-titrated Ab in a competitive system.

THE DONOR SPECIES OF COMPETING ANTIBODIES CANNOT BE THE SAME AS THE DETECTING ANTIBODY SINCE THE ANTI SPECIES CONJUGATE WOULD BIND.

Inhibition

The AB can be added first to the coated wells and incubated. The wells may then be washed or unwashed and the detecting Ab added.

In these ways, the 'advantage' in terms of binding to the antigen on the wells is given to the test sample. Bound antibodies then inhibit or 'block' the binding of the subsequently added labeled antibodies.

Note

- The same pre-titrated system can be used for both antigen and antibody titration.
- The respective analytical sensitivities of the systems as adapted for antigen and antibody measurement can be altered with respect to the initial titration of the reagents in the pre-titration phase.
- Thus, by using different concentrations of Ab the effective sensitivity for competition or inhibition by antigen or antibody can be altered to favour either analytical sensitivity or specificity.
- It is important to realise this when devising assays based on competition or inhibition, whereby they can be adapted to be used to measure either antigen or antibody.
- Alterations in the concentrations of reactants can offer more idealised tests to suit the analytical parameters needed (degrees of requires specificity and sensitivity).
- This is particularly important when devising assays based on polyclonal antibodies which are dramatically affected through use of different dilutions of sera (alterations in quality of serum depending on relative concentrations of antibodies against specific antigenic determinants).

Competitive and Inhibition Assays for Sandwich ELISAs

Sandwich ELISAs are performed with both direct and indirect systems, i.e. both involve the use of an immobilized antibody on the solid phase to capture antigen.

For the direct sandwich the ELISA the detecting antibody is labelled with enzyme whereas in the indirect system the detecting antibody is not labeled and this is in turn detected using an anti-species conjugate.

Both systems are more complicated consequently the possibilities for variation in competing or inhibiting steps are increased. Attention has to be paid to why a certain system is used as compared to others.

The main point about using sandwich assays is that they may be essential to presentation of antigen usually by concentrating the specific antigen from a mixture through the use of specific capture serum.

Thus, the advantages of competitive/inhibitive techniques have to rely on antigen capture. Whether direct or indirect measurement of detecting antibody is used depends on exactly what kind of assay is being used.

The assays will be described under sandwich-direct and sandwich-indirect headings.

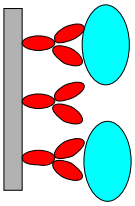
Sandwich-direct, involve assays utilizing a capture and a directly labelled detecting antibody (2 antibody systems)

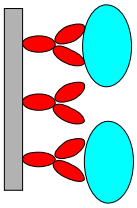
Sandwich-indirect involve assays utilizing three antibody systems (anti-species conjugate used to measure detecting serum).

They will be described for detecting antigen or antibody as in previous sections. The use of competitive (C) and inhibition (I) assays will also be described.

Direct Sandwich C-ELISA for Antibody

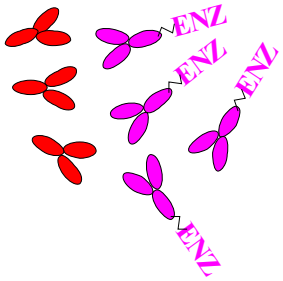
Pre titrated capture system with antigen combined with antibody.





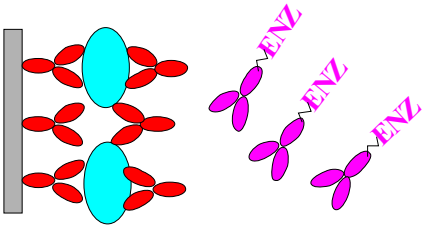
Sample contains competing antibodies

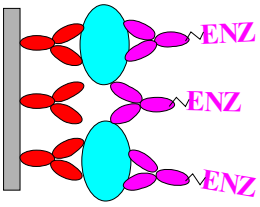
Sample contains NO competing antibodies



Competing antibodies bind to antigen

NO competing antibodies bind to antigen





Conjugate does not bind

NO COLOUR = COMPETITION

Conjugate BINDS

COLOUR MEANS NO COMPETITION

The sample under test is added to the captured antigen mixed with the detecting antibody conjugate. **(COMPETITION REACTION).** After incubation and washing, the colour develops according to the amount of conjugate binding. Where there is no competitor, the colour is high.

If the sample is added and a period of incubation allowed before addition of the conjugate this would be an **INHIBITION ASSAY**. Following this incubation step there are two alternatives.

- The first is to add the pre-titrated labelled antibodies directly to the reaction mixture followed by incubation.
- The second alternative is to wash the wells thereby washing away any excess test antibodies before the addition of labeled antibodies.

For each alternative there is an incubation step for the labelled antibodies followed by washing and then addition of substrate/chromophore solution.

The results for both systems are read according to the reduction in color as seen in controls where no test sample was added.

The greater the concentration of test antibodies that bind, the greater the degree of inhibition of the labeled antibodies.

The number of components for the Indirect Sandwich ELISAs is increased and consequently the number of reagent combinations. The reader should by now be used to the descriptions in diagrammatic form so that the next series of assays exploiting the Indirect Sandwich ELISAs will be examined more briefly, with the principles involved being highlighted.

Indirect Sandwich C-ELISA for Antibody

Step	COMPETITION	NO COMPETITION
1	I-Ab-Ag-AB-Anti AB**Enz Pre-titrated system	I-Ab-Ag-AB-Anti AB**Enz Pre-titrated system
2	I-Ab-Ag Add competing Abc mixed with pre-titrated AB	I-Ab-Ag Add negative sample mixed with pre-titrated AB
3	°C	°C
4	Wash	Wash
5	I-Ab-Ag-Abc ABc blocks AB binding	I-Ab-Ag-AB No blocking of AB
6	I-Ab-Ag-Abc + AntiAB**Enz Add anti AB conjugate	I-Ab-Ag-AB + AntiAB**Enz Add anti AB conjugate
7	°C	°C
8	Wash	Wash
9	I-Ab-Ag-Abc No binding Anti AB*Enz	I-Ab-Ag-AB-AntiAB**Enz Binding AntiAB**Enz
10	+ S/C	+ S/C
11	I-Ab-Ag-Abc	I-Ab-Ag-AB-AntiAB**Enz
12	NO COLOUR	COLOUR
13	STOP	STOP
14	READ	READ
	COMPETITION	NO COMPETITION

Review of Systems

System	Representation		Competition/Inhibition for System
Direct	I-Ag-Ab**Enz		
		AG	I-Ag-(*AG*_AntiAb**Enz)
		AB	I-(Ag_*AB*)-Ab**Enz
Indirect	I-Ag-Ab-Anti Ab**Enz		
		AG	I-Ag-(*AG*_Ab)-Anti Ab**Enz
		AB	I-(Ag_*AB*)-Ab-Anti Ab**Enz
Sandwich			
Sandwich Direct (i)			
	I-Ab-Ag-Ab**Enz		
		AG	I-(Ab_*AG*)-Ag-Ab**Enz
		AB	I-Ab-(*AB*_Ag)-Ab**Enz
Sandwich Direct (ii)	I-Ab-Ag-AB**Enz		
		AG	I-(Ab_*AG*)-Ag-AB**Enz
		AB	I-Ab-(*AB*_Ag)-AB**Enz
Sandwich Indirect	I-Ab-Ag-AB-AntiAB**Enz		
		AG	AntiAB**Enz
			Not easy since I-Ab captures Ag and *AG*
		AB	I-Ab-Ag-(*AB*.AB)-AntiAB**Enz
			AB, *AB* and Ab have to be three different species and Anti AB** Enz cannot react with Ab or *AB*

Choice of Assays

The most difficult question to answer when initiating the use of ELISAs is which system is most appropriate.

Questions which must be addressed are:

- **What is the purpose of the assay?**
- **What reagents do I have?**
- **What do I know about the reagents?**
- **Is the test to be developed for a research purpose to be used bya single user, or for applied use by other workers?**
- **Is the test to be used in other laboratories?**
- **Is a kit required?**

These questions have a direct affect on the phases that might be put forward as a general rule for the development of any assay i.e.

- **Phase 1**
  Feasibility-proof that a test system(s) can work.

- **Phase 2**
  Validation-showing that test(s) is 'stable' and that it is evaluated over time and under different conditions.

- **Phase 3**
  Standardisation, quality control, establishment that test is precise and can be used by different workers in different laboratories. At this stage a generalised examination of the availability of reagents and the effect this has on setting up a variety of systems will be made.