37

Enzyme Linked Immunosorbent Assay (ELISA)

John Crowther

1. Introduction

There have been very few developments that markedly affect the need to greatly revise the text from the last version of this book. This is testament to the fact that heterogeneous enzyme linked immunosorbent assays (ELISA) provide ideal systems for dealing with a wide range of studies in many biological areas. The main reason for this success is test flexibility, whereby reactants can be used in different combinations, either attached passively to a solid phase support or in the liquid phase. The exploitation of the ELISA has been increased through continued development of specifically produced reagents for example, monoclonal and polyclonal antibodies and peptide antigens coupled with the improvement and expansion of commercial products such as enzyme linked conjugates; substrates and chromogens; plastics technology and design of microwell plates; instrumentation advances and robotics. However, the principles of the ELISA remain the same.

A brief scan of the literature involving ELISA can be used to illustrate the continued success of ELISA. The number of publications with ELISA mentioned in all science areas from 1976 to 2004 is shown in **Table 37.1**. A fairly constant increase in the number of papers using ELISA methods is indicated. A breakdown of publications according to science areas in five yearly periods from 1980 in **Table 37.2** illustrates the versatility in use of the ELISA, as well as highlighting the major areas of use in Medicine and Dentistry; Immunology and Microbiology Molecular biology and Genetics and Biotechnology. It is interesting to note that the earliest exploitation of ELISA was in Immunology and Microbiology and Molecular Biology and Biotechnology, probably reflecting the greatest research areas. Medicine and Dentistry (associated by the search engine) shows the greatest rate of increase in use (probably in the Medical sphere only) from the 1990s.

The search results indicate the continued expansion of ELISA in science and there is no reason to believe that this will change even in the face of modern technologies exploiting molecular methods. The analytical and systematic characteristics of the ELISA are ideally suited to diagnosis at the screening

From: *Molecular Biomethods Handbook, 2nd Edition.*
Edited by: J. M. Walker and R. Rapley © Humana Press, Totowa, NJ

Table 37.1. Literature search in ScienceDirect database for ELISA.

Year	Number	Year	Number
1976	6	1991	743
1977	13	1992	774
1978	14	1993	820
1979	31	1994	870
1980	45	1995	1016
1981	95	1996	1093
1982	125	1997	1119
1983	216	1998	1099
1984	257	1999	1144
1985	367	2000	1118
1986	420	2001	1120
1987	547	2002	1198
1988	565	2003	1253
1989	640	2004	1591
1990	682		

Table 37.2. Breakdown of literature search in science groups.

Subject	1980–1984	1985–1989	1990–1994	1995–1999	2000–2004
Agriculture and biological sciences	87	274	615	804	827
Molecular biology, genetics and biotechnology	374	1,329	1,762	1,845	2,096
Chemistry	8	29	77	208	279
Environmental science	4	13	52	125	162
Immunology and microbiology	514	1,584	2,128	2,450	2,772
Medicine and dentistry	280	971	1,639	2,875	3,372
Neurosciences	21	124	198	380	484
Pharmacology and toxicology	24	108	247	397	497
Veterinary sciences	71	219	522	769	853

level; for surveillance where larger scale sample handling is required and for research. Many of the accepted standard assays in many scientific fields are ELISA based and have replaced other "gold standard" assays. In conjunction with the rapidly evolving use of molecular methods centering on the polymerase chain reaction (PCR) technologies there is the need to use serological confirmatory methods in a dual approach to directly identify and characterise disease agents and to assess disease prevalence through the measurement of specific antibodies or other chemical factors as a result of infection. The use

of ELISA methods in testing the environment, and animal, or plant products as safe for human and animal consumption is also a rapidly evolving area for ELISA.

ELISA therefore, has been used in all fields of pure and applied aspects of biology, in particular it forms the backbone of diagnostic techniques. The systems used to perform ELISAs make use of antibodies. These are proteins produced in animals in response to antigenic stimuli. Antibodies are specific chemicals that bind to the antigens used for their production thus they can be used to detect the particular antigens if binding can be demonstrated. Conversely, specific antibodies can be measured by the use of defined antigens, and this forms the basis of many assays in diagnostic biology.

This chapter describes methods involved in ELISAs where one of the reagents, usually an antibody, is linked to an enzyme and where one reagent is attached to a solid phase. The systems allow the examination of reactions

Table 37.3. Brief descriptions of elements common to ELISAs.

Solid phase	This is usually a plastic microtiter plate well. Specially prepared ELISA plates are commercially available. These have 8–12 well formats (even larger possibilities such as 394 well plates), the plates can be used with a wide variety of microtiter equipment, such as multichannel pipets, to allow great convenience to the rapid manipulation of reagents in small volumes.
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 simple way of immobilization of one of the reactants in ELISA is one of the keys to its success.
Washing	Simply flooding and emptying wells with a buffered solution is enough to separate bound and free reagents. Again, this is a key to the simplicity of the ELISA over methods involving complicated separation methods.
Antigen	These are proteins or carbohydrates, which, when injected into animals or as a result of the disease process, elicit the production of antibodies. Such antibodies usually react specifically with the antigen and therefore can be used to detect that antigen.
Antibody	Antibodies are produced in response to antigenic stimuli. These are mainly protein in nature. In turn, antibodies are antigenic.
Antispecies antibody	Antibodies obtained when antibodies from one animal are injected into another species. Thus, guinea pig serum injected into a rabbit would elicit a rabbit anti-guinea pig serum.
Enzyme	A substance that can act at low concentration as a catalyst to promote a specific reaction. Several specific enzymes are commonly used in ELISA.
Enzyme conjugate	An enzyme that is attached irreversibly, by chemical means, to a protein, usually an antibody. Thus, an antispecies enzyme conjugate would be guinea pig antirabbit conjugated to enzyme.
Substrate	The substrate is the chemical compound on which the enzyme reacts specifically. This reaction is used in some way to produce a signal that is read as a color reaction in ELISA.
Chromophore	This is a chemical that alters color as a result of enzyme interacting with substrate, allowing the ELISA to be quantified
Stopping	The process of stopping the action of the enzyme and substrate.
Reading	This implies measurement of the color produced in ELISA. This is quantified using special multichannel spectrophotometers reading at the specific wavelength of the color produced. Tests can be read by eye for crude assessment.

through the simple addition and incubation of reagents. Bound and free reactants are separated by a simple washing procedure. The end product in an ELISA is the development of color that can be quantified using a spectrophotometer. These kinds of ELISA are called heterogeneous assays and should be distinguished from homogeneous assays where all reagents are added simultaneously. The latter assays are most suitable for detecting small molecules such as digoxin or gentamicin.

The development of ELISA stemmed from investigations of enzyme-labeled antibodies *(1–3)*, for use in identifying antigens in tissue. The methods of conjugation were exploited to measure serum components in the first true ELISAs *(4–6)*.

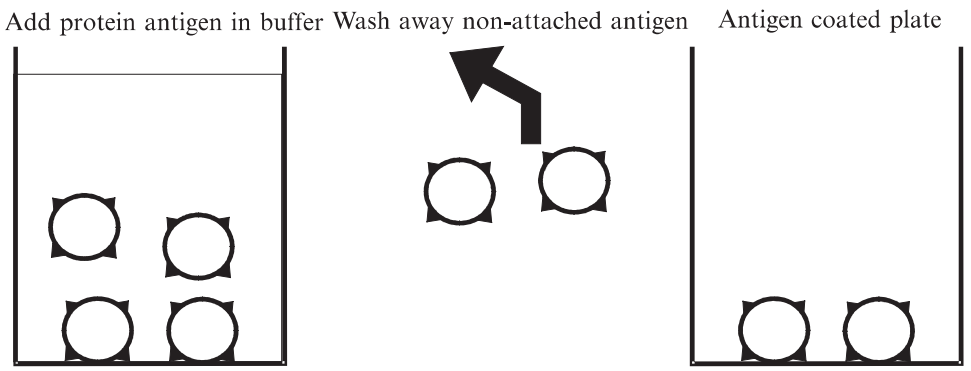
By far the most exploited ELISAs use plastic microtitre plates in an 8 × 12 well format as the solid phase *(7)*. Such systems benefit from a large selection of specialized commercially available equipment including multichannel pipets for the easy simultaneous dispensing of reagents and multichannel spectrophotometers for rapid data capture. There are many books, manuals and reviews of ELISA and associated subjects that should be examined for more detailed practical details *(8–21)*.

The key advantages of ELISA over other assays are summarized in **Table 37.3**.

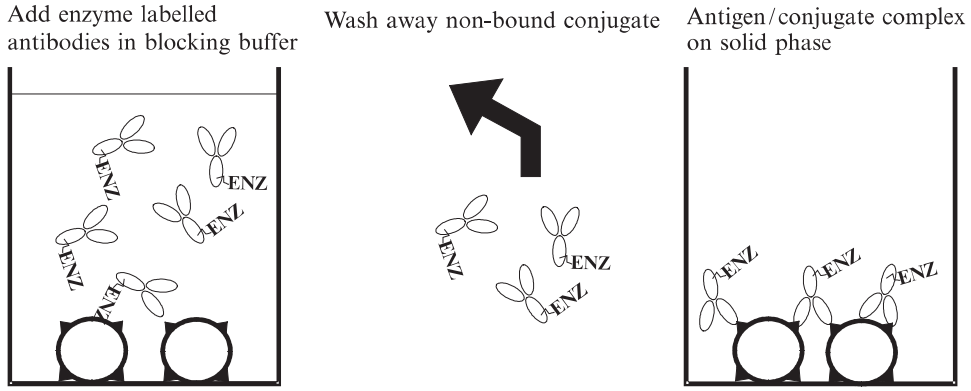
2. What is ELISA?

Figure 37.1A illustrates a protein that is adsorbed to a plastic surface. The attachment of proteins and hence the majority of all antigens in nature, to plastic, is the key to most ELISAs performed. This process is passive so that protein solutions, in easy to prepare buffer solutions, can be added to plastic surfaces and will attach after a period of incubation at room temperature. The most commonly used plastic surface is that of small wells in microtitre plates. Such plates contain 96 wells measuring approx 5 mm deep by 8 mm diameter in a 12 × 8 format. The main point here is that there has evolved a whole technology of equipment for rapidly handling materials in association with these plates. After the incubation of antigen excess unbound antigen is washed away by flooding the plastic surface, usually in a buffered solution. The plastic can then be shaken free of excess washing solution and is ready for the addition of a detecting system.

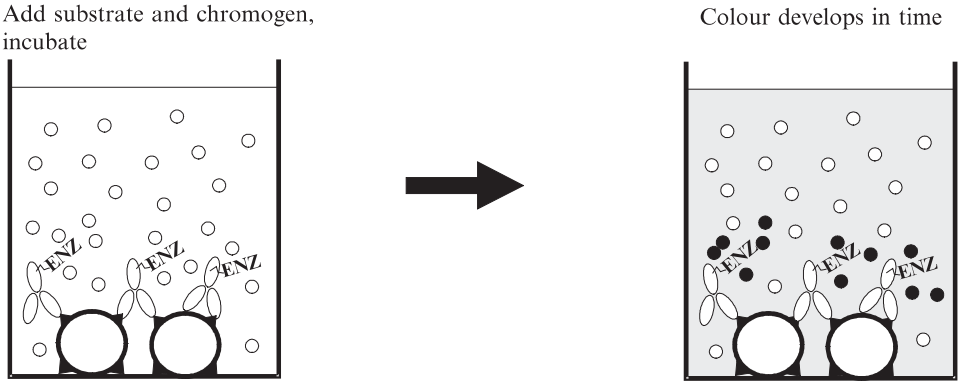
The simplest application of the ELISA is illustrated in **Fig. 37.1**. An antibody prepared against the antigen on the plastic is added (**Fig. 37.1B**). The antibody has been chemically linked to an enzyme; this is usually called a conjugate. The antibody is diluted in a buffer (e.g., phosphate buffered saline, pH around 7.2) containing an excess of protein that has no influence on the possible reaction of the antibody and antigen. A very cheap example is that of approx 5% skimmed-milk powder, Marvel, which is mainly the protein from cow milk, casein. The purpose of this excess protein is to prevent any passive attachment of the conjugate (antibody is protein) to any free sites on the plastic not occupied by the antigen. Such sites will be occupied by the excess milk powder proteins by competition with the low concentration of conjugate. These diluting reagents have been called blocking buffers.



(**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 iner 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.

Fig. 37.1. Illustration of steps in simple direct ELISA

On addition of the conjugate under these conditions the only reaction that occurs is the specific immunological binding of the antigen and antibody in the conjugate. Thus an antigen-enzyme linked antibody complex is produced and because the antigen is bound to the plastic the enzyme is bound also. Such a

process requires incubation (15–60 min) that can be at room temperature. After incubation the plastic surface is washed to remove all unreacted conjugate.

The next stage is illustrated in **Fig. 37.1C**. Here, a substrate for the enzyme is added in solution with a chromogenic chemical that is colorless in the absence of enzyme activity on the substrate. Because there is enzyme linked to the antibody, which is attached specifically to the antigen on the plastic surface, the substrate is catalyzed causing a color change. The rates of such color changes are proportional to the amount of enzyme in the complex. Thus, taking the other extreme, if no antigen were attached to the plastic then no antibody would be bound. Therefore if no enzyme is present to catalyze the substrate so no color change would be observed. The enzyme activity is usually stopped by the addition of a chemical that drastically alters the pH of the reaction or denatures the enzyme e.g., 1 *M* sulfuric acid.

Color can then be assessed by eye or quantified using a multichannel spectrophotometer that is specially designed for use with the microplates and can read a plate (96 samples) in 5 s. Such machines can be interfaced with microcomputers so that a great deal of data can be analyzed in a short time.

There are many systems in ELISA depending on what initial reagent is attached to the solid phase and what order subsequent reagents are added. Thus there is great versatility possible for adaption of ELISAs to solve applied and pure problems in science.

The basic principles of ELISA are summarized below:

1. Passive attachment of proteins to plastics
2. Washing away of unattached protein
3. Addition, at some stage, of a specific antibody linked to an enzyme
4. Use of competing inert proteins to prevent nonspecific reactions with the plastic
5. Washing steps to separate reacted (bound) from unreacted (free) reagents
6. Addition of a specific substrate that changes color on enzyme catalysis or substrate and a colorless chromophore (dye solution) that changes color owing to enzyme catalysis
7. Incubation steps to allow immunological reactions
8. Stopping of enzyme catalysis
9. Reading of the color by spectrophotometer.

Specific details of these stages can be obtained from the references in particular ***(8–16)***. The next section illustrates some of the possible variations.

3. Basic Assay Configurations

There are three basic systems used in ELISA: direct ELISA; indirect ELISA; and sandwich ELISA. All these systems can be used to perform competition of inhibition ELISAs.

These systems will be described to illustrate the principles involved with the aid of diagrams. The various stages common to ELISAs will then be described in more detail.

3.1. Direct ELISA

This is the simplest form of ELISA as shown in **Fig. 37.2**. Here an antigen is passively attached to a plastic solid phase by a period of incubation. As indicated

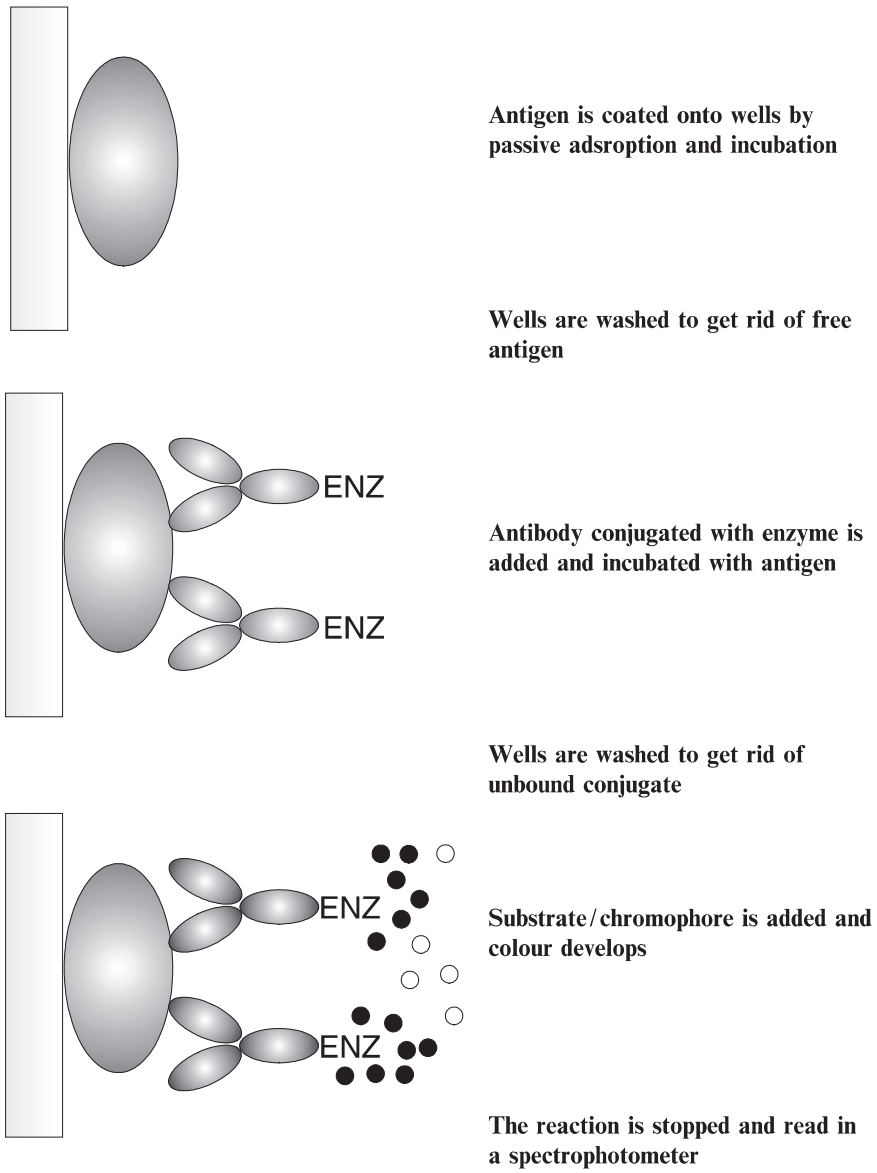


Fig. 37.2. Direct ELISA. Antigen is attached to the solid phase by passive adsorption. After washing, enzyme labeled antibodies are added. After an incubation period and washing, a substrate system is added and color allowed to develop

in Subheading 2, the most useful solid phase is a microtiter plate well. After a simple washing step, antigen is detected by the addition of an antibody that is linked covalently to an enzyme. After incubation and washing the test is developed by the addition of a chromogen/substrate whereby enzyme activity produces a color change. The greater the amount of enzyme in the system then the faster the color develops. Usually color development is read after a defined time or after enzyme activity is stopped by chemical means at a defined time. Color is read in a spectrophotometer.

3.2. Indirect ELISA

Antigen is passively attached to wells by incubation. After washing, antibodies specific for the antigen are incubated with the antigen. Wells are washed and any bound antibodies are detected by the addition of antispecies antibodies

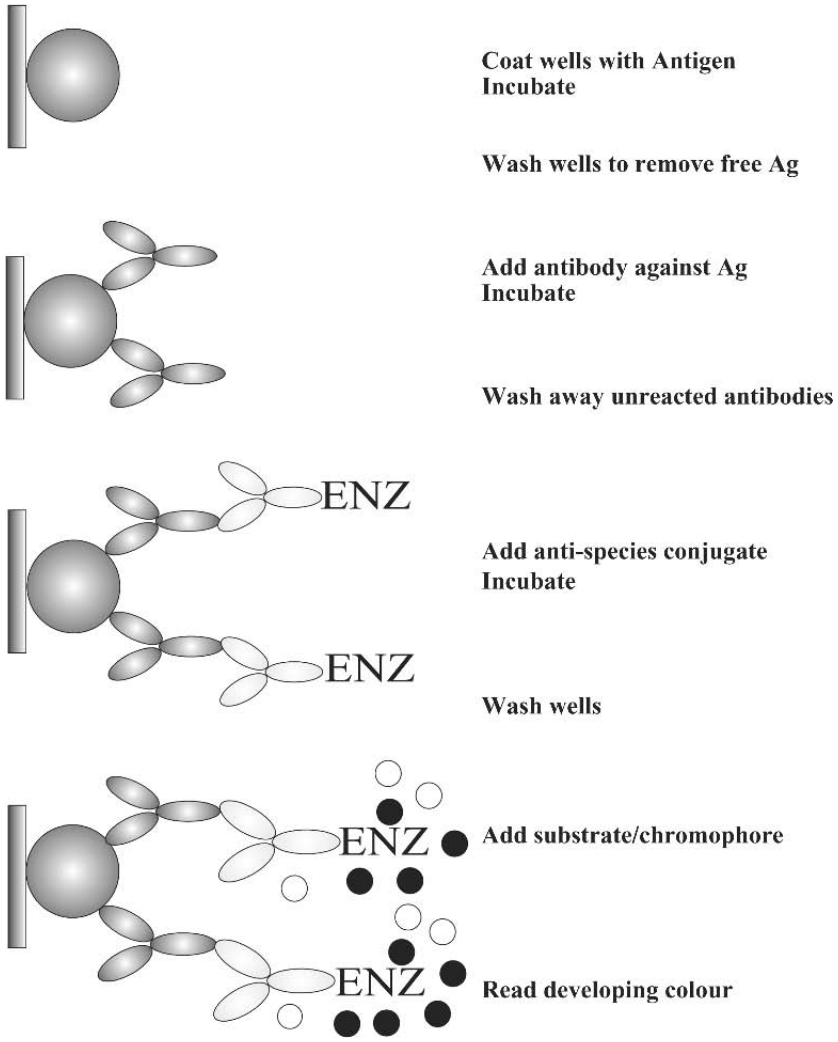


Fig. 37.3. Indirect ELISA. Antibodies from a particular species react with antigen attached to the solid phase. Any bound antibodies are detected by the addition of an antispecies antiserum labeled with enzyme, this is widely used in diagnosis

covalently linked to an enzyme. Such antibodies are specific for the species in which the first antibody added were produced. After incubation and washing, the test is developed and read as described in Subheading 3.1. The scheme is shown in **Fig. 37.3**.

3.3. Sandwich ELISA

There are two forms of this ELISA depending on the number of antibodies used. The principle is the same for both whereby instead of adding antigen directly to a solid phase, antibody is added to the solid phase, and then acts as to capture antigen. These systems are useful where antigens are in a crude form (contaminated with other proteins) or at low concentration. In these cases the antigen cannot be directly attached to the solid phase at a high enough concentration to allow successful assay based on direct or indirect ELISAs. The sandwich ELISAs depend on antigens having at least two antigenic sites so that at least two antibody populations can bind.

3.3.1. Direct Sandwich ELISA

This is shown in **Fig. 37.4**. Antibodies are protein in nature and can be passively attached to the solid phase. After washing away excess unbound antibody, antigen is added and is specifically captured. The antigen is then detected by a second enzyme labeled antibody directed against the antigen. This antibody can be identical to the capture antibody reacting with a repeating antigenic site or an antibody from a different species directed against the same or a different site. Thus a "sandwich" is created. This type of assay is useful where a single species antiserum is available and where antigen does not attach well to plates.

3.3.2. Indirect Sandwich ELISA

This is similar in principle to the last system but involves three antibodies. **Fig. 37.5** illustrates the scheme. Coating of the solid phase with antibody and capture of antigen are as in Subheading 3.1–3.3, however, here the antigen is detected with a second unlabeled antibody. This antibody is in turn detected using an antispecies enzyme labeled conjugate. It is essential that

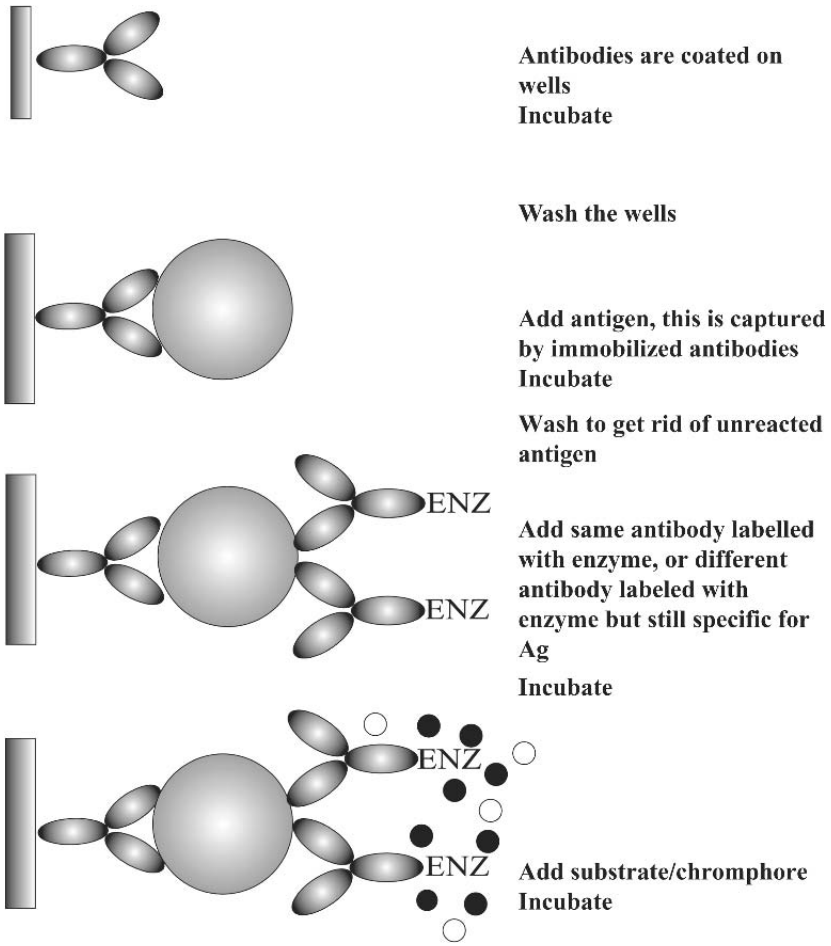


Fig. 37.4. Sandwich ELISA-direct. This system exploits antibodies attached to a solid phase to capture antigen. The antigen is then detected using serum specific for the antigen. The detecting antibody is labeled with enzyme. The capture antibody and the detecting antibody can be the same serum or from different animals of the same species of from different species. The antigen must have at least two different antigenic sites

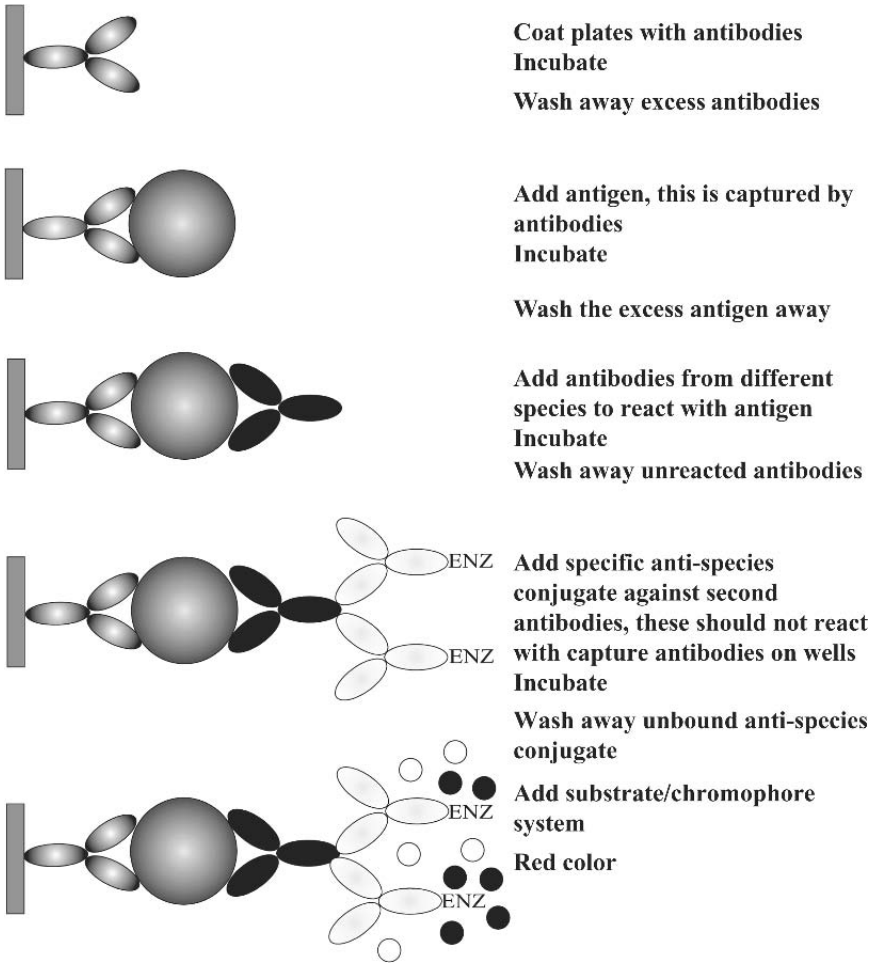


Fig. 37.5. Sandwich ELISA-Indirect. The detecting antibody is from a different species to the capture antibody. The anti-species enzyme-labeled antibody binds to the detecting antibody specifically and not to the capture antibody

the antispecies conjugate does not bind to the capture antibody, therefore the species in which the capture antibody is produced must be different. The same considerations about the need for at least two antigenic sites to allow the "sandwich" are relevant. The advantage of this system is that a single antispecies conjugate can be used to evaluate the binding of antibodies from any number of samples. This is not true of the Direct Sandwich where each serum tested would have to be labeled with enzyme.

4. Competition/Inhibition ELISAs

The systems described in Subheadings 3.1–3.3 are the basic configurations of ELISA. All of these can be adapted to measure antigens or antibodies using competitive or inhibition conditions. Thus each the assays described above require pretitration of reagents to obtain optimal conditions. These optimal conditions are then challenged either by the addition of antigen or antibody. These will be described.

4.1. Direct ELISA Antigen Competition

This is shown in **Fig. 37.6**. The Direct ELISA is optimized whereby a defined amount of antigen coating the plate is bound by an optimal amount of enzyme labeled antibody. Wells coated with the optimal amount of antigen are then set up. This "balanced" situation can then be "challenged" by the addition of samples that could contain the same (or similar) antigen as that attached to the plate. On addition of the enzyme-labeled conjugate the test antigen reacts and prevents that antibody binding to the antigen on the solid phase. Thus the added antigen in the liquid phase and the solid phase antigen, compete for the labeled antibody. The higher the concentration of identical antigen in the test, the greater is the degree of competition. Where the antigen added in the test sample is not the same as the solid phase antigen, then it does not bind to the

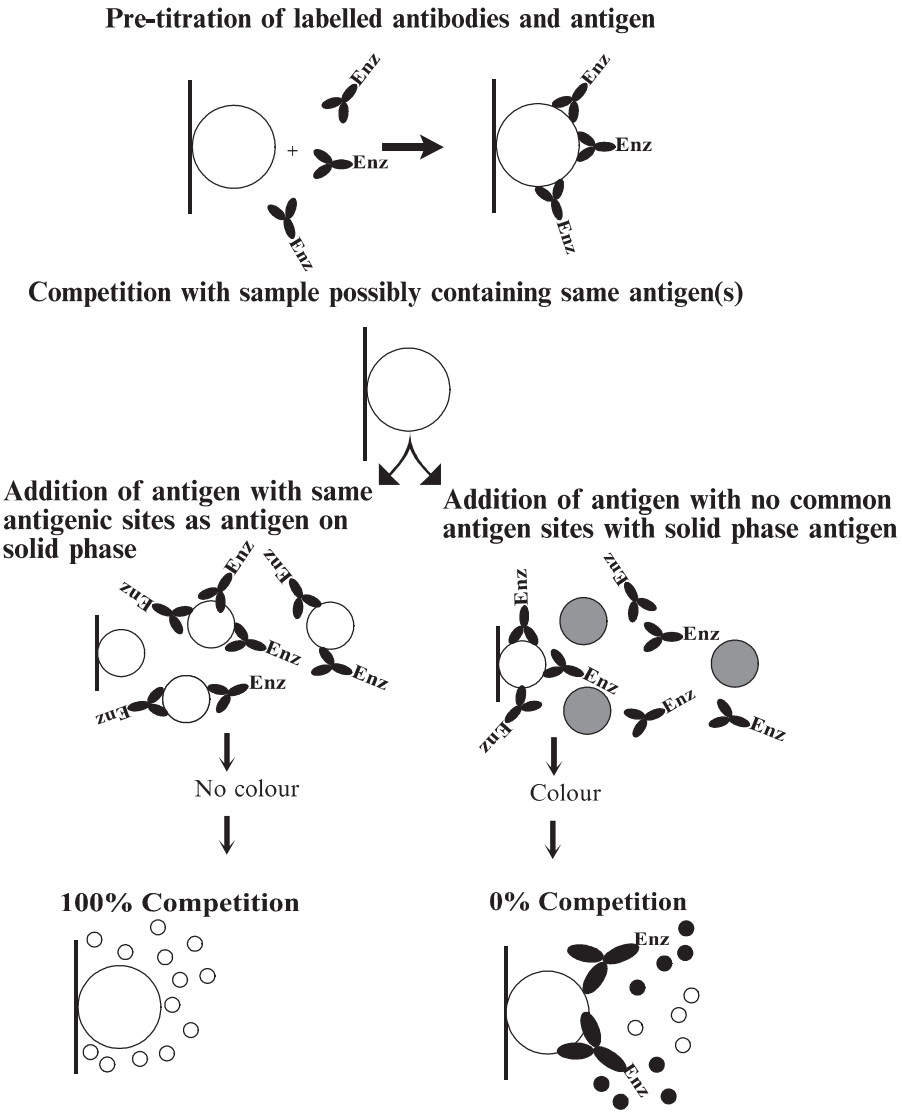


Fig. 37.6. Competition ELISA-direct antigen. Reaction of antigen contained in samples with the enzyme-labeled antibody directed against the antigen on the solid phase blocks the label from binding to the solid phase antigen. If the antigen has no cross-reactivity or is absent, then the labeled antibody binds to the solid phase antigen and a color reaction is observed on developing the test

added conjugate and this can consequently bind without competition to the solid phase antigen.

4.2. Direct ELISA Antibody Competition

This is very similar to the assay in Subheading 4.1, except that test samples are added containing antibodies possibly directed towards the antigen coated on the solid phase. This is shown in **Fig. 37.7**. Thus high concentrations of identical antibody mean that the conjugated pretitrated antibody is inhibited and thus no color reaction is observed (as expected from the pretitration exercise). Such assays are increasing in usefulness with the development of monoclonal antibody (MAb) based tests.

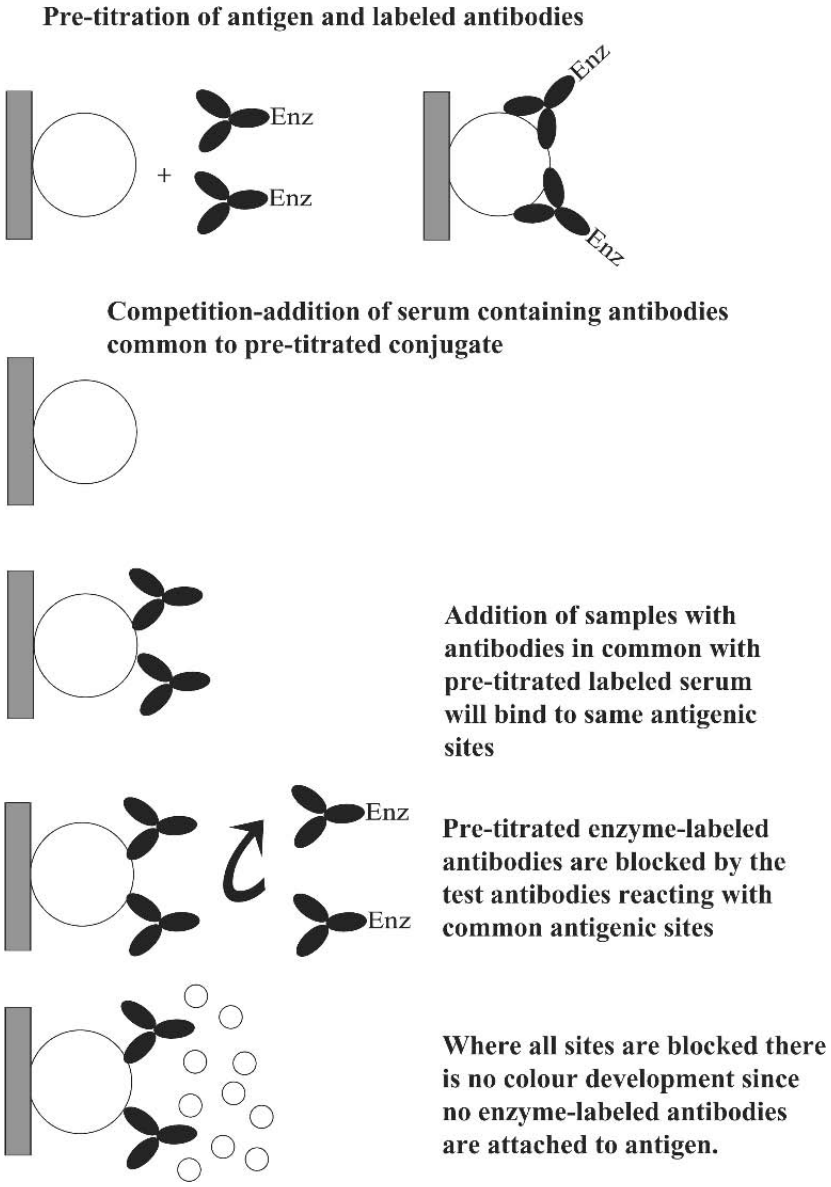


Fig. 37.7. Competition ELISA-direct antibody. The degree of inhibition by the binding of antibodies in a serum for a pretitrated enzyme-labeled antiserum reaction is determined

4.3. Indirect ELISA Antigen Competition

Figure 37.8 illustrates the principles of this assay. The system relating the antigen, primary antibody and labeled antispecies conjugate is pretitrated. There is inhibition of the binding of primary antibody on addition of test samples containing the same antigen as that coated on the wells. Conversely, where the antigen added does not bind to the primary antibody, then no inhibition occurs and on subsequent addition of the conjugate the expected pretitrated level of color is observed.

4.4. Indirect ELISA Antibody Competition

This is very similar to the assays described in Subheading 2.3. Here test samples containing antibodies that can bind to the solid phase antigen inhibit the pretitrated primary antibody, as shown in Fig. 37.9. The key problem with

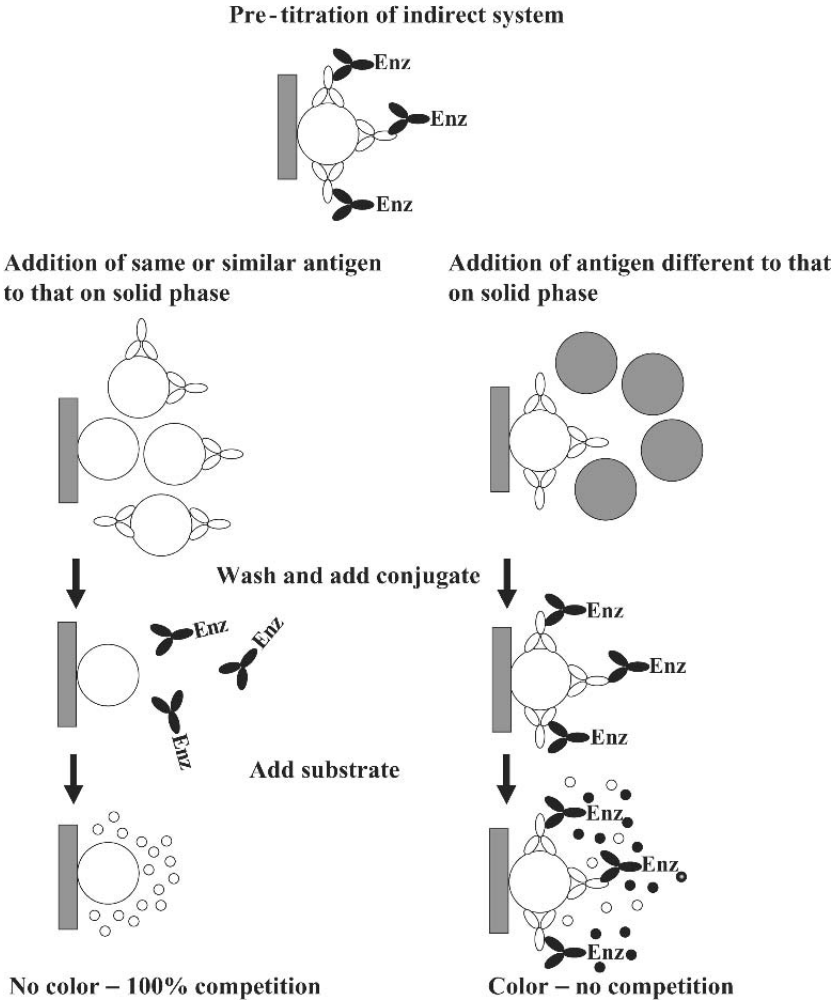


Fig. 37.8. Competition ELISA-indirect antigen. The pretitrated indirect ELISA is competed for by antigen. If the antigen shares antigenic determinants with that of the solid phase antigen, then it binds to the pretitrated antibodies and prevents them binding to the solid phase antigen. If there is no similarity then the antibodies are not bound and can react with the solid phase antigen. Addition of the antispecies enzyme conjugate quantifies the bound antibody

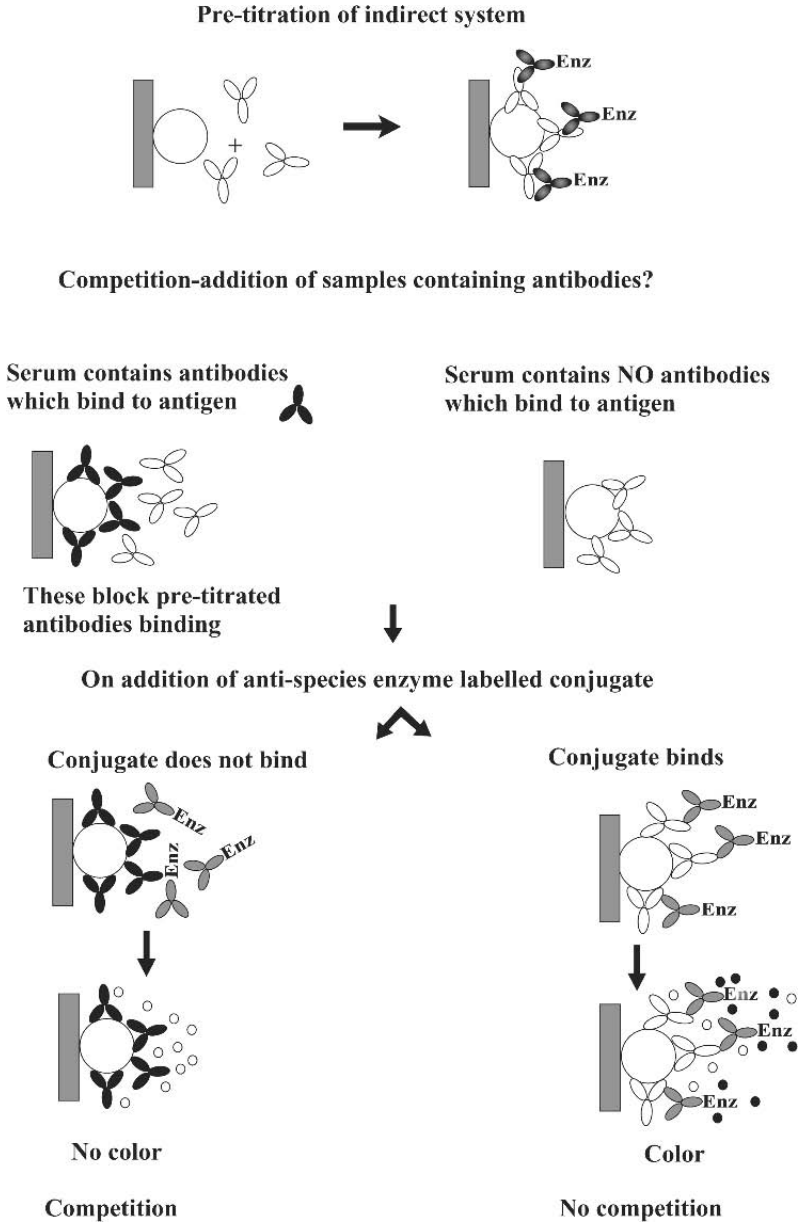


Fig. 37.9. Indirect ELISA-antibody inhibition involves the pretitration of an antigen and antiserum in an indirect ELISA. The addition of a serum containing cross-reactive antibodies will upset the "balance" of the pretitrated system. Because an antispecies conjugate is used, the species from which the sample of serum is taken cannot be the same as that used for the pretitration (the homologous system)

this assay is that the test antibody cannot be from the same species as the primary antibody because this is detected by an antispecies conjugate.

4.5. Sandwich ELISA Direct Antibody Competition

This is shown in **Fig. 37.10**. The situation begins to look a little more complex because more reagents are involved. The figure illustrates two methods where a pretitrated direct sandwich system is competed for by antibody in test samples. The first involves mixing and incubation of the pretitrated antigen with test serum before addition to wells containing the solid phase antibody.

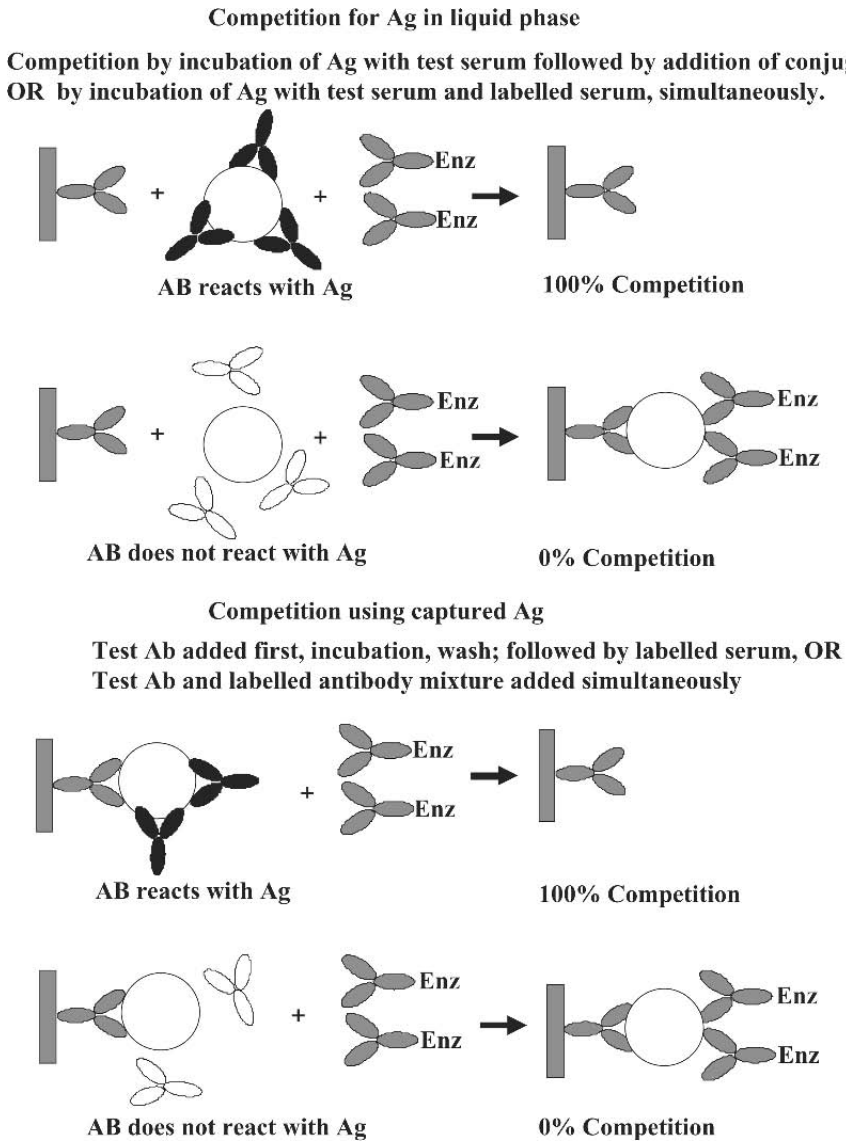


Fig. 37.10. Competition Sandwich ELISA-direct for antibody

Here, if the antibodies in the test sample bind to the antigen, then they stop the antigen binding to the solid phase antibody. On addition of the pretitrated conjugate there is no color. The second situation involves the use of antigen attached to wells via the capture antibody. After washing the test antibody is added. If this reacts with the captured antigen then it blocks the binding of subsequently added conjugate. In fact both these forms of assay whereby test antibodies are allowed to bind before the addition of detecting second antibody should be termed inhibition or blocking assays because strictly competition refers to the simultaneous addition of two reagents. This can be illustrated in the second situation where the test antibody and second conjugated antibody could be mixed together before addition to the antigen captured on the wells; this is competitive. Note that because we have an enzyme labeled detecting serum then any species serum can be used in the competitive system.

4.6. Sandwich ELISA Indirect Antibody Competition

Figures 37.11 and **37.12** illustrate methods for performing this ELISA. Again we have a more complex situation because five reagents are involved. Basically the Indirect sandwich ELISA is pretitrated. Test antibodies are then added either to the antigen in the liquid phase (**Fig. 37.11**) or to antigen already captured (**Fig. 37.12**). If test antibodies bind to the antigen in either system then the subsequent addition of the second antibody and the antispecies conjugate will be negated. Note here that, as with the Indirect ELISA competition, the species from which the test sera came cannot be the same as that used to optimize the assay i.e., the antispecies conjugate cannot react with the test antibodies.

The assays described are inhibition or blocking assays and can all be further "complicated" with reference to when addition of reagents are made. Thus in **Fig. 37.11**(i) the antigen, test antibodies and detecting antibody could be added together (competition). In **Fig. 37.11**(ii), the test and detecting antibodies could be added together (competition). Similarly for **Fig. 37.12**, the test and detecting antibodies could be premixed to offer competitive conditions.

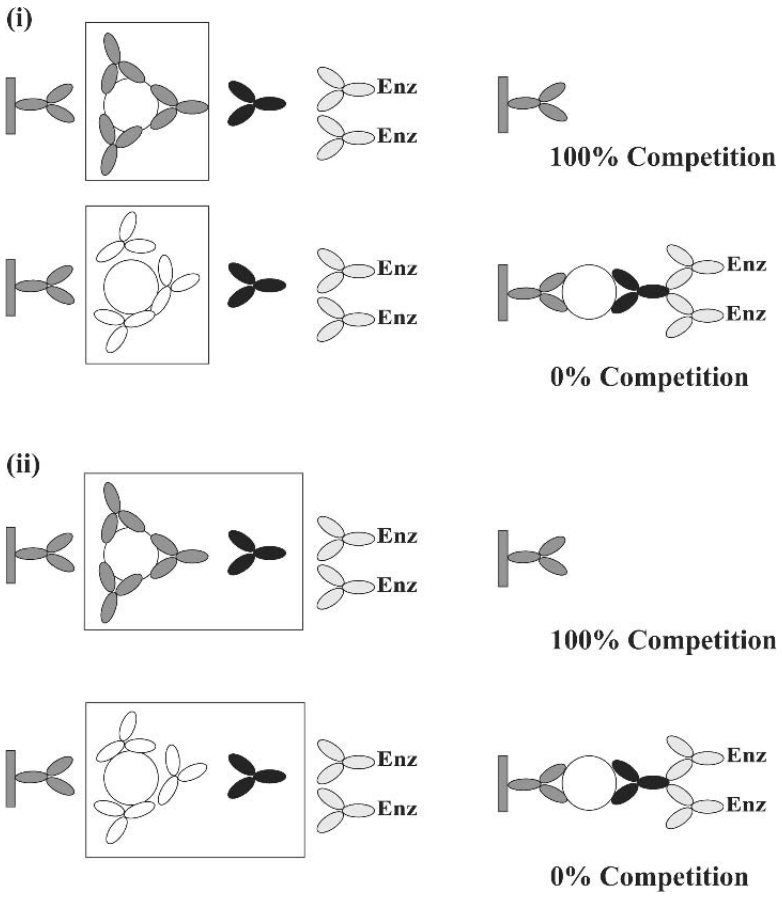


Fig. 37.11. Competition for sandwich ELISA-Indirect (liquid phase antigen) Ag is reacted with competing antibodies followed by addition of second antibody (i) or Ag, competing serum and second antibodies are mixed (ii). Bound second antibody is detected by antispecies against second antibody. This cannot react with species in which test antibody was raised

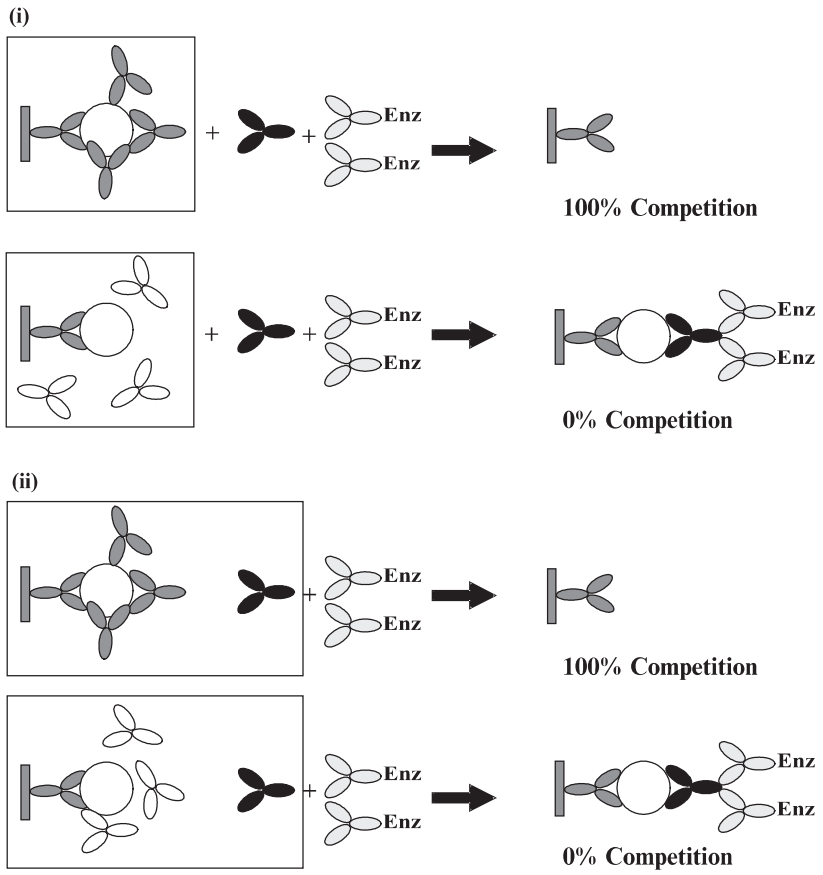


Fig. 37.12. Competition for Sandwich ELISA-Indirect (solid phase antigen)Ag is captured first and then either (i) competing antibodies are added, incubated and then second antibody added, with or without a washing step to remove unbound test antibody and complexes of this antibody and antigen (i) or competing serum and second antibodies are mixed to compete directly (ii). Bound second antibody is detected by antispecies against second antibody. This cannot react with species in which test antibody was raised

4.7. Sandwich ELISAs for Antigen Competition

These have not been illustrated. There is an intrinsic difficulty in that wells are coated with capture antibody. Addition of competing antigens thus serves to increase the concentration of antigen that can be captured resulting in no competition.

5. Summary of Uses of Various Methods Used in ELISA

This section will consider the reasons how and why different systems need to be applied and summarizes the interrelationships of the methods. An overview of the most commonly used systems is shown in **Fig. 37.13**. Although not all applications can be covered here, this will serve to illustrate the versatility of ELISA. Boxes A, B, C and D in the figure cover uses of the systems used in noncompetitive ways.

5.1. A-Direct ELISA

The drawback here is that antibodies from each serum have to be labeled. Antispecies conjugates can be titrated in this method using specific serum proteins from the target species. The Direct ELISA can be used to standardize

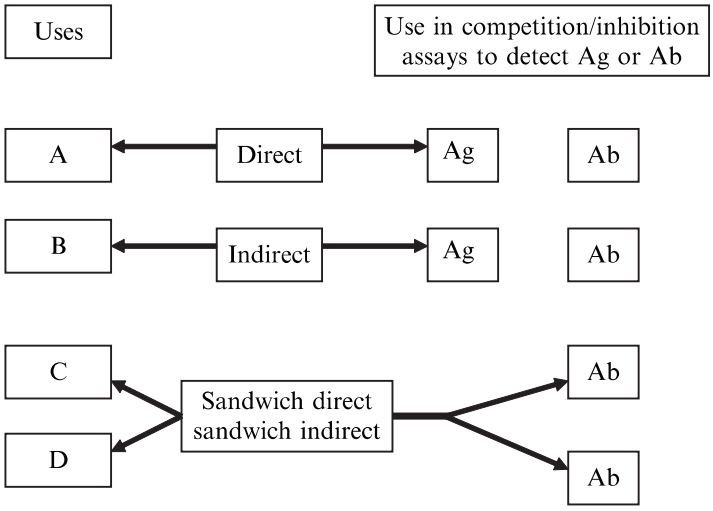


Fig. 37.13. Relationship of methods used in ELISA. Some uses of assays in non competitive ways (A, B and C) are discussed in text. Ag = antigen; Ab = antibody

antispecies conjugates from batch to batch and as a way of estimating the working dilution of conjugate to be used in other ELISA systems.

5.2. B-Indirect ELISA

This is a far more flexible test in that a single antispecies conjugate will detect antibodies. Thus various sera with different specificities can be detected using the same reagent. The Indirect ELISA has been used widely in diagnosis of diseases through the detection of antibodies. The method depends on the availability of enough specific antigen(s) at a suitable concentration for coating plates. Where antigens are at low concentration or in the presence of other contaminating proteins, the test might be impossible to perform. Thus antigens have to be relatively pure and be unaffected by adsorption on to the solid phase.

5.3. C-Sandwich ELISA-Direct

The use of antibodies coated to plates as capture reagents is essential where antigens are at low concentration or are contaminated with other proteins e.g., stool samples. The system also favors presentation of some antigens in a better way than when they are directly coated to plates and also limits changes to conformational epitopes owing to direct coating. The test can be used to detect antibodies via specifically captured antigens, or antigens through the specificity of the capture antibodies. The direct sandwich relies on the conjugation of specific antibodies against the target antigen that causes some problems with variability where new batches are prepared. The antibodies can be identical to the capture antibodies. The test does rely on there being at least two combining sites (epitopes) on the antigen.

5.4. D-Sandwich ELISA-Indirect

This system offers similar advantages as in Subheading 3.3, in that low concentrations of antigen can be specifically captured. This system offers the

advantage over the Direct Sandwich ELISA in that a single antispecies conjugate can be used to bind to detecting sera, thus a variety of different species detecting antibodies can be examined. The antispecies conjugate cannot be allowed to react with the initial coating antibodies so that care must be taken to avoid cross-reactions and sera must be prepared in at least two species. If only a single species is available then Fab2 fractions can be prepared from the capture antibodies and a specific anti-Fc conjugate used to develop the test.

5.5. Competition/Inhibition ELISA for all Systems

The advantages examined in the basic ELISAs- in Subheadings 5.1–5.3, all are relevant to the adaptations of the assays in competition/inhibition ELISAs. Thus the affects of coating on antigen, low concentrations of antigen, contaminating proteins, flexibility of using a single conjugate to detect many sera and orientation of antigens are all pertinent to finding the best system for solving problems. Generally competitive methods offer advantages over systems where antibodies or antigens are detected directly. The greatest increase in methods has come through the exploitation of monoclonal antibodies (MAbs) in competition/inhibition systems. **Table 37.4** briefly defines some elements of ELISA.

Table 37.4. shows the most commonly used enzymes and substrate/chromophore systems used in ELISA and the color changes with relevant stopping agents.

A. Commonly used conjugate/substrate systems

Enzyme label	Substrate	Dye	Buffer
Horse radish peroxidase	H_2O_2 (0.004%)	OPD (ortho-phenylene diamine)	Phosphate/ citrate pH 5.0
	H_2O_2 (0.004%)	TMB (tetra methyl benzidine)	Acetate buffer (0.1 *M*) pH 5.6
	H_2O_2 (0.002%)	ABTS (2,2′-azino di-ethyl	Phosphate/citrate, pH 4.2
	H_2O_2 (0.006%)	5AS (5-aminosalicylic acid) thiazolinesulfonic acid	Phosphate (0.2M), pH 6.8
	H_2O_2 (0.02%)	DAB (diamino benzidine)	Tris or PBS, pH 7.4
Alkaline phosphatase	pnpp	pnpp (paranitrophenyl phosphate)	Diethanolamine (10 m*M*) plus $MgCl_2$(0.5 m*M*), pH 9.5.

B. Common enzyme systems, color changes and stopping reactions

	Color change			Reading wavelength		
Enzyme	System	Unstopped	Stopped	Unstopped	Stopped	Stopping solution
Horseradish peroxidase	OPD	Light orange	Orange	450 nm	492 nm	1.25 *M* H_2SO_4
	TMB	Blue	Yellow	650 nm	450 nm	1% SDS
	ABTS	Green	Green	414 nm	414 nm	No stop
	5AS	Black/brown	Black/brown	450 nm	450 nm	No stop
	DAB	Brown	Brown	N/A	N/A	No stop
Alkaline phosphatase	pnpp	Yellow/green	Yellow/green	405 nm	405 nm	2 *M* sodium carbonate

6. Uses of ELISA

The purpose of developing ELISAs is to solve problems. These can be divided into pure and applied applications, although the two are interdependent. Thus, a laboratory with a strong research base is essential in providing scientific insight and valuable reagents to allow more routine applications. The methods outlined show the flexibility of the systems. There effective use is up to the ingenuity of scientists. Recent advances in science have given the immunoassayist greater potential for improving the sensitivity and specificity of assays, including ELISA. In particular the development of MAb technology has given us single chemical reagents (antibodies) of defined specificity that can be standardized in terms of activity as a function of their weight. The development of gene expression systems has also given the possibility of expressing single genes as proteins for use in raising antibodies or acting as pure antigens. This technology goes hand-in-hand with developments in the Polymerase Chain Reaction (PCR) technologies, that enables the very rapid identification of genes and their manipulation. In turn improvements in the fields of rapid sequencing and x-ray crystallographic methods has led to a far more intimate understanding of the structure/function relationship of organisms in relation to the immunology of disease. The ELISA fits in rather well in these developments because it is a binding assay requiring defined antibodies and antigens, all of which can be provided. **Table 37.5** illustrates some applications of ELISA with relevant references.

Table 37.5. Applications of ELISA.

General	Specific	References
Confirmation of clinical disease	Titration of specific antibodies.	***21–24,26–28,30,31,41,44,50,53, 54,62–64,69***
	Single dilution assays.	***35,41,53,54,62–64***
	Relationship of titer to protection against disease.	***50,55***
	Kits.	***44,62,63***
Analysis of immune response to whole organisms, purified antigens extracted from whole organisms, expressed proteins (e.g., vaccinia, baculo, yeast, bacteria), measurement. polypeptides, peptides	Antibody quantification.	***30,31,35,38,57,60,62,64***
	Antibody class measurement (IgM, IgG, IgA, IgD, IgE).	***25,33,34,(new 94–old 95)***
	Antibody subclass measurement (IgG1, IgG2b, IgG3).	***33***
	Antibody IG2a, affinity.	***39,49,44***
Antigenic comparison	Relative binding antibodies.	***30,31,60,64,73***
	Affinity differences in binding of antibodies.	***39,43,51,52,60***
	Measurement of weight of antigens.	***29,32,36,37,43,44,48,49,56,64***
	Examination of treatments to antigen (inactivation for vaccine manufacture, heating, enzyme treatments).	***49***

(continued)

Table 37.5. (continued)

General	Specific	References
	Identification of continuous and discontinuous epitopes by examination of binding of polyclonal and MAbs to denatured and non denatured proteins.	***44,46,48,59***
	Antigenic profiling by MAbs.	***40,42,44,46,61***
	Comparison of expressed and native problems.	***47,48,5,92***
	Use of MAbs to identify paratopes in polyclonal sera.	***47,59,79***
Monoclonal antibodies	Screening during production.	***40,46***
	Competitive assay-antibody assessment.	***47***
	Comparison of antigens.	***42,44,46,47,59,62***
	Use of MAbs to orientate antigens.	***48***
Novel systems	High-sensitivity assays (Amplified-ELISA).	***58***
	Fluorogenic substrates.	***45***
	Biotin/avidin systems.	***68***
More recent references	Food analysis	***70,71,89***
	Fish	***84–87,90***
	AIDS	***78, 82, 90***
	SARS	***93***
	Bird flu	***76,77***
	Allergens	***74,80***
	Emerging diseases	***72***
	Psychiatry	***75***
	Review	***81,(new 95),96***
	Snakes	***91***
	Environment	***83***
	Chemoluminescence	***88***

The ability to develop ELISAs depends on as closer understanding of the immunological/serological/biochemical knowledge of specific biological systems as possible. Such information is already available with reference to literature surveys. Basic skills in immunochemical methods are also a requirement and an excellent manual for this is available ***(65)***. References (***66,67***) provide excellent text books on immunology. An invaluable source of commercial immunological reagents is available in ***(68)***. The references from 70 onwards are more recent and reflect newer fields into which ELISA has expanded and also the new problems arising as for example, Avian influenza and SARS. It is difficult to see that there will be a significant reduction in the rate of use of ELISA directly or as part of other molecular systems, but this can only be assessed when the next edition of this book is written. The main danger is methods involving ELISA are now regarded easy to develop. This, as for all tests, is not true and good training in ELISA is even more important today, because there is an incredible spectrum of reagents available for the development of tests. The linking of molecular methods to ELISA and other detection systems based on solid phase assays is exciting and full of potential, but there is a great need to attend to the basic understanding and principles of ELISA.

References

1. Avrameas S, Uriel J (1966) Methode de marquage d'antigenes et d'anticorps avec des enzymes et son application en immunodiffusion. Comptes Rendus Hendomadairesdes Seances de l'Acadamie des Sciences: D: Sciences naturelles (Paris) 262:2543–2545
2. Nakane PK, Pierce GB (1966) Enzyme-labelled antibodies: preparation and application for the localization of antigens. J Histochem Cytochem 14:929–931
3. Avrameas S (1969) Coupling of enzymes to proteins with gluteraldehyde. Use of the conjugates for the detection of antgens and antibodies. Immunochemistry 6:43–52
4. Avrameas S, Guilbert B (1971) Dosage enzymo-immunologique de proteines a l'aide d'immunosadorbants et d'antigenes marques aux enzymes. Comptes Rendus Hendomadaires des Seances de l'Acadamie des Sciences: D: Sciences naturelles (Paris) 273:2705–2707
5. Engvall E, Perlman P (1971) P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871–874
6. Van Weeman BK, Schuurs AHWM (1971) Immunoassay using antigen enzyme conjugates. FEBS Lett 15:232–236
7. Voller A, Bidwell DE, Huldt G, Engvall E (1974) A microplate method of enzyme linked immunosorbent assay and its application to malaria. Bull Wld Hlth Org 51:209–213
8. Burgess GW (ed) (1988) ELISA technology in diagnosis and research. Graduate School of Tropical Veterinary Science, James Cook University of North Queensland, Townsville, Australia
9. Collins WP (1985) Alternative immunoassays. Wiley, Chichester, UK
10. Collins WP (1985) Complimentary immunoassays. Wiley, Chichester, UK
11. Crowther JR (1995) *ELISA*: theory and practice. Humana, Totowa, NJ
12. Ishikawa E, Kawia T, Miyai K (1981) Enzyme immunoassay. Igaku-Shoin, Tokyo, Japan
13. Kemeny DM, Challacombe SJ (1988) ELISA and other solid-phase immunoassays. Theoretical and practical aspects. Wiley, Chichester, UK
14. Maggio T (1979) *The enzyme immunoassay*. CRC, New York
15. Ngo TT, Leshoff HM (1985) Enzyme-mediated immunoassay. Plenum, New York
16. Voller A, Bidwell DE, Bartlett A (1979) The enzyme-linked immunosorbent assay *(ELISA)*. Dynatech Europe, London, UK
17. Avrameas S, Ternynck T, Guesdon JL (1978) Coupling of enzymes to antibodies and antigens. Scand J Immunol 8 Suppl. 7:7–23
18. Blake C, Gould BJ (1984) Use of enzymes in immunoassay techniques. A review. Analyst 109:533–542
19. Guilbault GG (1968) Use of enzymes in analytical chemistry. Anal Chem 40:459
20. Kemeny DM, Challacombe SJ (1986) Advances in ELISA and other solid-phase immunoassays. Immunol Today 7:67
21. Voller A, Bartlett A, Bidwell DE (1981) Immunoassays for the 80s. MTP, Lancaster, UK
22. Kemeny DM (1987) Immunoglobulin and antibody assays. In: Lessoff MH, Lee TH, Kemeny DM, (eds) Allergy: an international textbook. Wiley, Chichester, UK, p 319
23. Kemeny DM, Chantler S (1988) An introduction to ELISA. In: Kemeny DM, Challacombe SJ (eds) ELISA and other solid phase immunoassays. Theoretical and practical aspects. Wiley, Chichester, UK, p 367
24. Kemeny DM, Challacombe SJ (1988) Micrototitre plates and other solid-phase supports. In: Kemeny DM, Challacombe SJ (eds) ELISA and other solid-phase immunoassays. Theoretical and practical aspects Wiley, Chichester, UK p 367