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## Preface

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. There has been some rearrangement of chapters plus addition of three new ones dealing with charting methods for assessing the indirect ELISA, ruggedness and robustness of tests-aspects of kit use and validation, and internal quality control and external quality management of data, respectively. These reflect the need to control what you are doing with ELISA and to exploit the method to its full extent. I do not apologize for dealing with the same areas in different ways a number of times, as it is imperative that principles are understood to allow planning, operation, and control of ELISA.

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 1. A fairly constant increase in the number of research works using ELISA methods is indicated. A breakdown of publications according to the areas of science in 5 yearly periods from 1980 given in Table 2 illustrates the versatility in the use of ELISA, as well as highlights 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 ELISA are ideally suited to diagnosis at the screening 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 a need to use serological confirmatory methods in a dual approach to directly identify and characterize 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.

**Table 1**  
**Literature search in ScienceDirect database for ELISA**

Year	Number
1976	6
1977	13
1978	14
1979	31
1980	45
1981	95
1982	125
1983	216
1984	257
1985	367
1986	420
1987	547
1988	565
1989	640
1990	682
1991	743
1992	774
1993	820
1994	870
1995	1,016
1996	1,093
1997	1,119
1998	1,099
1999	1,144
2000	1,118
2001	1,120
2002	1,198
2003	1,253
2004	1,591

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.

**Table 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

The book describes the 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 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, which 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, which should be examined for more detailed practical details (8–21).

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. Their 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, which 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, which 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, since it is a binding assay requiring defined antibodies and antigens, all of which can be provided. Table 3 illustrates some applications of ELISA with relevant references.

**Table 3**  
**Applications of ELISA**

<b>General</b>	<b>Specific</b>	<b>References</b>
Confirmation of clinical disease	Titration of specific antibodies	(21–35)
	Single dilution assays	(27, 30–34, 36)
	Relationship of titer to protection against disease	(29, 37)
	Kits	(28, 32, 33)
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	(25, 26, 32, 34, 36, 38–40)
	Antibody class measurement (IgM, IgG, IgA, IgD, IgE)	(41–44)
	Antibody subclass measurement (IgG1, IgG2b, IgG3)	(42)
	Antibody IG2a, affinity	(28, 45, 46)
	Relative binding antibodies	(25, 26, 34, 40, 47)
Antigenic comparison	Affinity differences in binding of antibodies	(40, 45, 48–50)
	Measurement of weight of antigens	(28, 34, 46, 48, 51–56)
	Examination of treatments to antigen (inactivation for vaccine manufacture, heating, enzyme treatments)	(46)

(continued)

**Table 3**  
**(continued)**

<b>General</b>	<b>Specific</b>	<b>References</b>
	Identification of continuous and discontinuous epitopes by examination of binding of polyclonal and MAbs to denatured and nondenatured proteins	(28, 55, 57, 58)
	Antigenic profiling by MAbs	(28, 57, 59–61)
	Comparison of expressed and native problems	(5, 55, 62, 63)
	Use of MAbs to identify paratopes in polyclonal sera	(58, 62, 64)
Monoclonal antibodies	Screening during production	(57, 59)
	Competitive assay-antibody assessment	(62)
	Comparison of antigens	(28, 32, 57, 58, 60, 62)
	Use of MAbs to orientate antigens	(55)
Novel systems	High-sensitivity assays (Amplified-ELISA)	(65)
	Fluorogenic substrates	(66)
	Biotin-avidin systems	(67)
More recent references	Food analysis	(68–70)
	Fish	(71–75)
	AIDS	(75–77)
	SARS	(78)
	Bird flu	(79, 80)
	Allergens	(81, 82)
	Emerging diseases	(83)
	Psychiatry	(84)
	Review	(85, 86)
	Snakes	(87)
	Environment	(88)
	Chemoluminescence	(89)

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 (90). References (91, 92) are excellent text books on immunology. An invaluable source of commercial immunological reagents is available in (69). 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 nowadays, since 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.

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# Chapter 2

## Systems in ELISA

This chapter defines the terms and examines the configurations used for most applications of ELISA. Such a chapter is important because the possibilities inherent in the systems of ELISA must be understood in order to maximize their versatility in assay design. All heterogeneous systems have three basic parameters:

1. One reactant is attached to a solid phase, usually a plastic microtiter plate with an  $8 \times 12$ -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.

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### 1. Definition of Terms

Immunoassays involve tests using antibodies as reagents. Enzyme immunoassays make use of enzymes attached to one of the reactants in an immunoassay to allow quantification through the development of color after the addition of a suitable substrate/chromogen.

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

An enzymatic reaction is utilized to yield color and to quantify the reaction, through the use of an enzyme-labeled reactant. **Table 1** gives the definitions of terms used in ELISA. These terms are greatly amplified throughout the subsequent text.

**Table 1**  
**Brief definition of terms**

<b>Term</b>	<b>Definition</b>
Solid phase	Usually a microtiter plate well. Specially prepared ELISA plates are commercially available. These have an 8×12- well format and can be used with a wide variety of specialized equipment designed for rapid manipulation of samples, including multichannel pipets.
Adsorption	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	The simple flooding and emptying of the wells with a buffered solution to separate bound (reacted) from unbound (unreacted) reagents in the ELISA. Again, this is a key element to the successful exploitation of the ELISA.
Antigens	A protein or carbohydrate that when injected into animals elicits the production of antibodies. Such antibodies can react specifically with the antigen used and therefore can be used to detect that antigen.
Antibodies	Produced in response to antigenic stimuli. These are mainly protein in nature. In turn, antibodies are antigenic.
Antispecies antibodies	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 that 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 that is attached irreversibly to a protein, usually an antibody. Thus, an example of antispecies enzyme conjugate is rabbit anti-guinea linked to horseradish peroxidase.
Substrate	A chemical compound with which an enzyme reacts specifically. This reaction is used, in some way, to produce a signal that is read as a color reaction (directly as a color change of the substrate or indirectly by its effect on another chemical).
Substrate	A chemical that alters color as a result of an enzyme interaction with substrate.
Stopping	The process of stopping the action of an enzyme on a substrate. It has the effect of stopping any further change in color in the ELISA.
Reading	Measurement of color produced in the ELISA. This is quantified using special spectrophotometers reading at specific wavelengths for the specific colors obtained with particular enzyme-chromophore systems. Tests can be assessed by eye.

**2. Basic Systems of ELISA**

This section describes the principles involved in the many configurations possible in ELISA. The terminology used here may not always agree with that used by others, and care is needed in defining the assays by name. The specific assay parameters must always be examined carefully in the literature. The following set of definitions attempts to clear up the myriad of published approaches to describing the systems used in a few words such as “double-sandwich competitive ELISA” and “indirect sandwich inhibition ELISA.” The aim is to have a clear approach. Three main methods form the basis to all ELISAs:

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

All three systems can be used to form the basis of a group of assays called competition or inhibition ELISAs.

The systems (arrangement and use of reagents in the test) are illustrated herein through the use of symbols (as defined in **Table 2**) as well as terms. In this way, it is hoped that the reader will gain

**Table 2**  
**Definition of Symbols or terms used to describe assays**

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

a clear idea of the various systems and their relative advantages and disadvantages. A key feature of the flexibility of ELISA is that more than one system can be used to measure the same thing. This allows some scope to adapt assays to suit available reagents as well as to note areas of improvement through the identification of the need to prepare additional reagents – e.g., that monoclonal antibodies (mAbs) may be needed to give an assay the required specificity, or that a particular anti-species conjugate against a subclass of immunoglobulin (Ig) is required.

Practical details of the various stages, e.g., solid phase, buffers, incubation, and conjugates, are dealt with in detail in Chapters 3 and 4.

**2.1. Direct ELISA**

Direct ELISA can be regarded as the simplest form of ELISA, and is illustrated in **Fig. 1** and in **Diagram 1**.

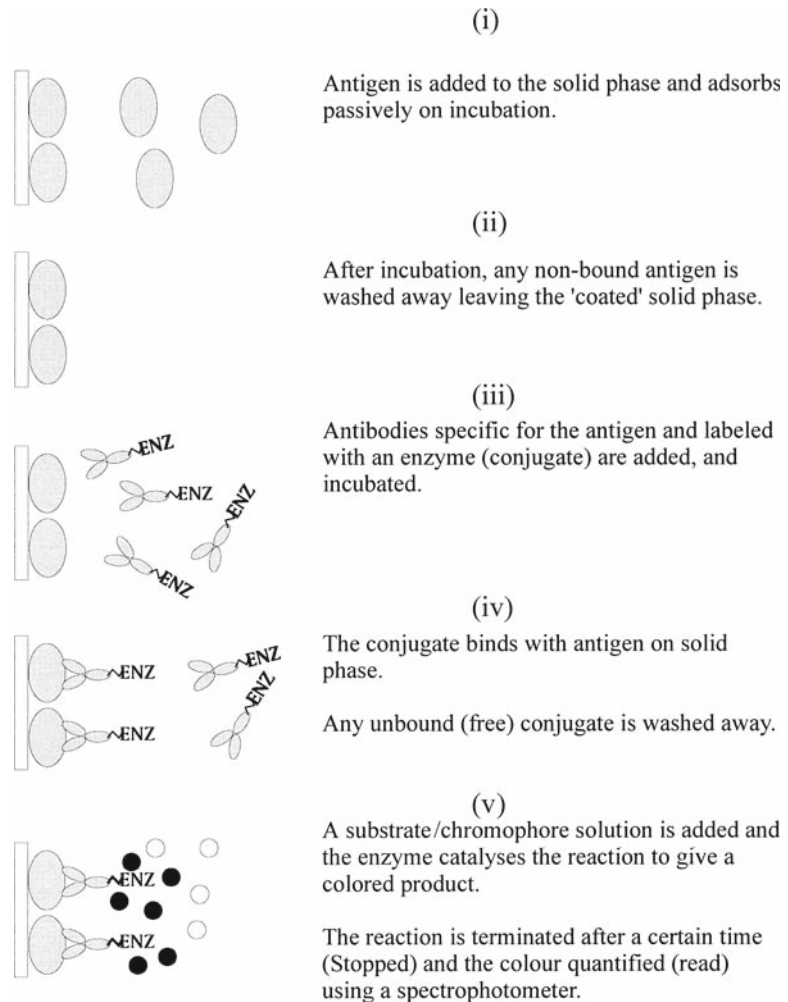


Fig. 1. Direct ELISA. Antigen is attached to the solid phase by passive adsorption. After washing, enzyme-labeled antibodies are added. After an incubation period and washing, a substrate system is added and color is allowed to develop.

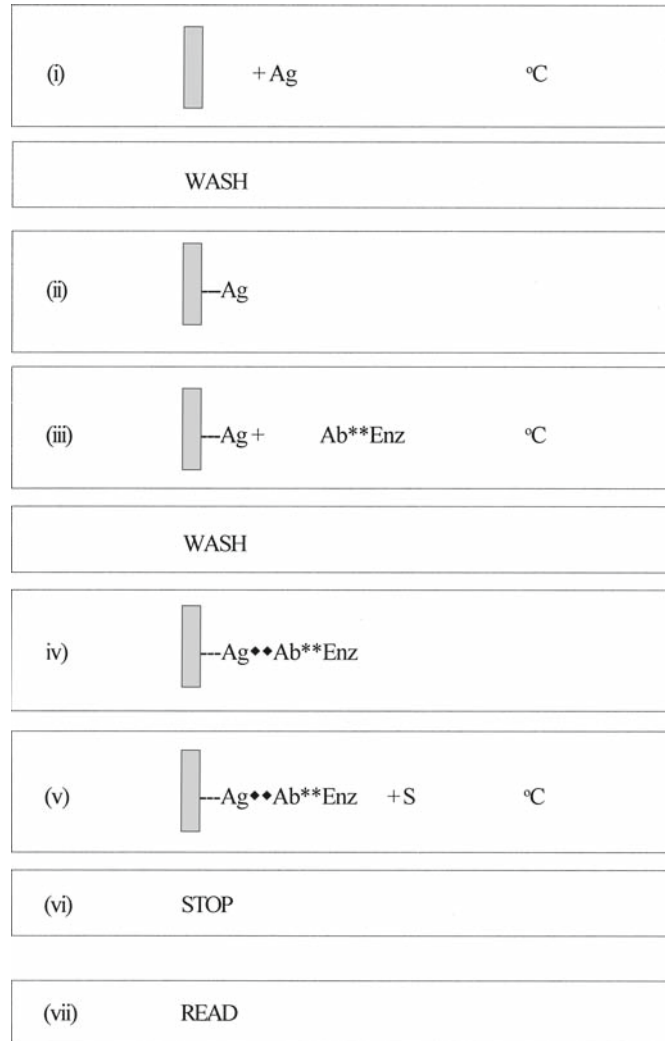


Diagram 1. Direct ELISA.

Antigen is diluted in a buffer (stage i), commonly a high pH (9.6) carbonate or bicarbonate buffer or neutral phosphate-buffered saline (PBS). 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 will attach passively to the plastic during a period of incubation. The temperature and time of incubation are not so critical, but standardization of conditions is vital, and the use of incubators at 37°C is favored (since they are widely available in laboratories). After incubation, any excess antigen is removed by a simple washing step (stage ii), by flooding and emptying the wells, using a neutral buffered solution (e.g., PBS). Antibodies conjugated with an enzyme can now be added (stage iii), and

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

On incubation, antibodies bind to the antigen. Again, a simple washing step is then used to remove unbound antibodies (stage iv). Stage v involves the 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 enzymatic catalysis. The reaction is allowed to progress for a defined period, after which the reaction is stopped (stage vi) by altering the pH of the system, or by adding an inhibiting reactant. Finally, the color is quantified by the use of a spectrophotometer reading (stage vii) at the appropriate wavelength for the color produced.

This kind of system has severe limitations when used only in this form but has assumed great importance as the “target” system in competition and inhibition assays, particularly when mAbs are conjugated and/or highly defined antigens are used.

## 2.2. Indirect ELISA

Indirect ELISA is illustrated in **Diagram 2** and in **Fig. 2**. Stages i and ii are similar to the direct system. Stage iii involves the addition of unlabeled detecting antibodies, which are diluted in a buffer to prevent nonspecific attachment of proteins in antiserum to solid phase (blocking buffer). This is followed by incubation and washing away of excess (unbound) antibodies, to achieve specific binding (stage iv). Stage v 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 the conjugate (stage vi). Substrate/chromophore is then added to the bound conjugate (stage vii) and color develops, which is then stopped (stage viii) and read (stage ix) in a spectrophotometer.

The indirect system is similar to the direct system in that the 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 are termed anti-species conjugates. Thus, if the detecting antibodies were produced in rabbits, the enzyme-labeled antibodies would have to be anti-rabbit Igs in nature. This allows great flexibility in the use of anti-species conjugates in that different specificities of conjugate

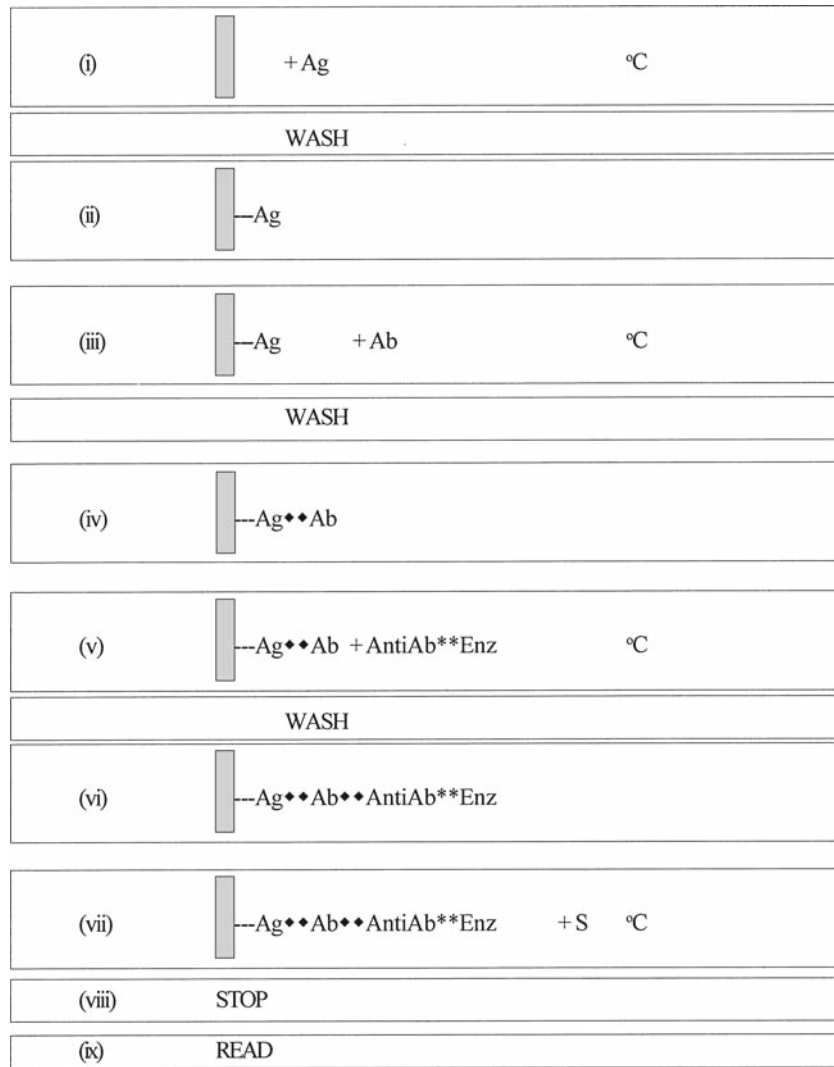


Diagram 2. Indirect ELISA.

can be used to detect particular immunoglobulin's binding in the assay, and there are literally thousands of conjugates available commercially. For example, the anti-species conjugate could be anti-IgM, anti-IgG<sub>1</sub>, anti-IgG<sub>2</sub>, and so on.

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. One problem that such systems have is the varying degree of nonspecific binding in individual sera. This tends to widen the dispersion (variability) in assay results and, therefore, increases the need to process many sera to assess confidence.



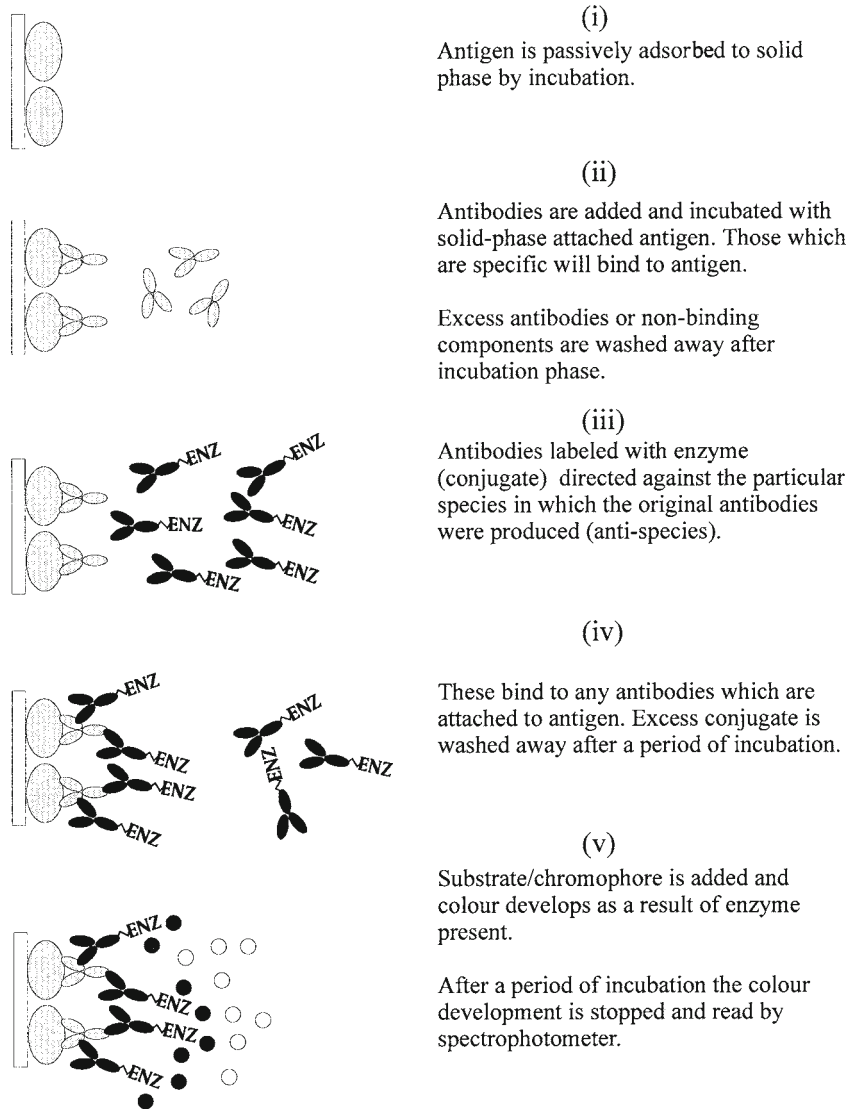


Fig. 2. Indirect ELISA. Antibodies from a particular species react with antigen attached to the solid phase. Any bound antibodies are detected by the addition of an anti-species antiserum labeled with enzyme. This is widely used in diagnosis.

**2.3. Sandwich ELISA**

Sandwich ELISA can be divided into two systems, which have been named the direct sandwich ELISA and the indirect sandwich ELISA.

**2.3.1. Direct Sandwich ELISA**

The direct sandwich ELISA is illustrated in **Diagram 3** and in **Fig. 3**.

The direct sandwich ELISA involves the passive attachment of antibodies to the solid phase (stages i and ii). These antibodies (capture antibodies) then bind antigen(s) that are added in stage

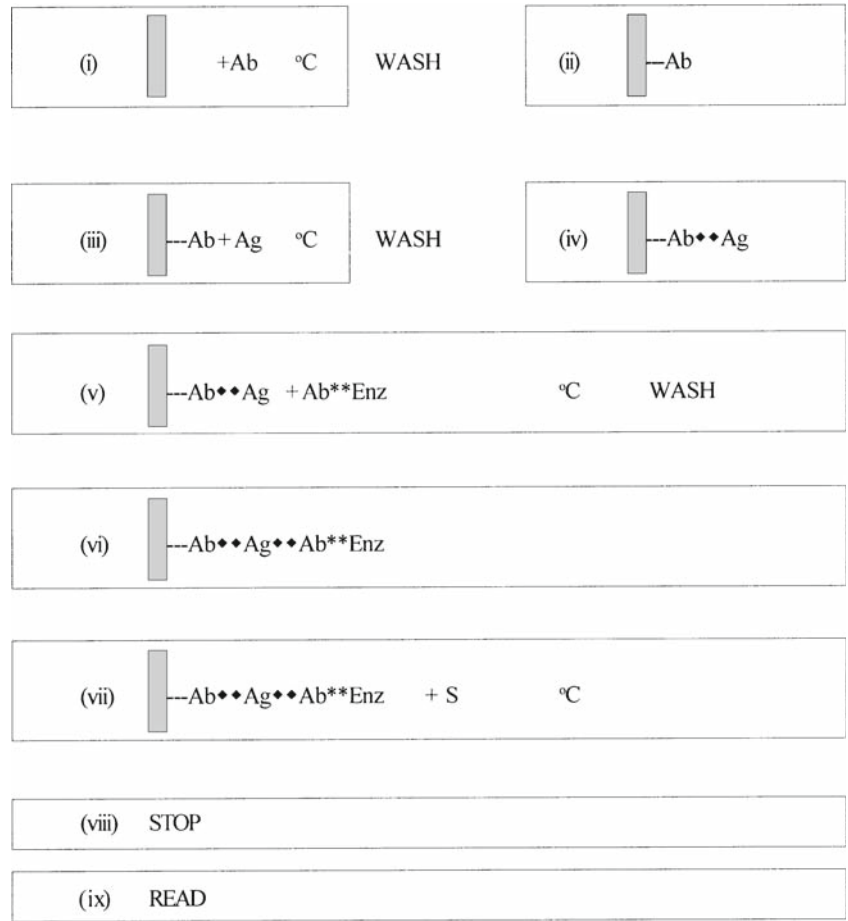


Diagram 3. Direct sandwich ELISA.

iii. The antigen(s) are diluted in a blocking buffer to avoid nonspecific attachment to the solid phase. Here, the components of the blocking buffer should not contain any antigens that might bind to the capture antibodies. After incubation and washing, an antibody-antigen complex is attached to the solid phase (stage iv).

The captured antigen (sometimes referred to as trapped) is then detected by the addition and incubation of enzyme-labeled specific antibodies in blocking buffer (stage v). 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 (stage vi), the bound enzyme is developed by the addition of substrate/chromogen (stage vii), then stopped (stage viii), and finally read using a spectrophotometer (stage ix).

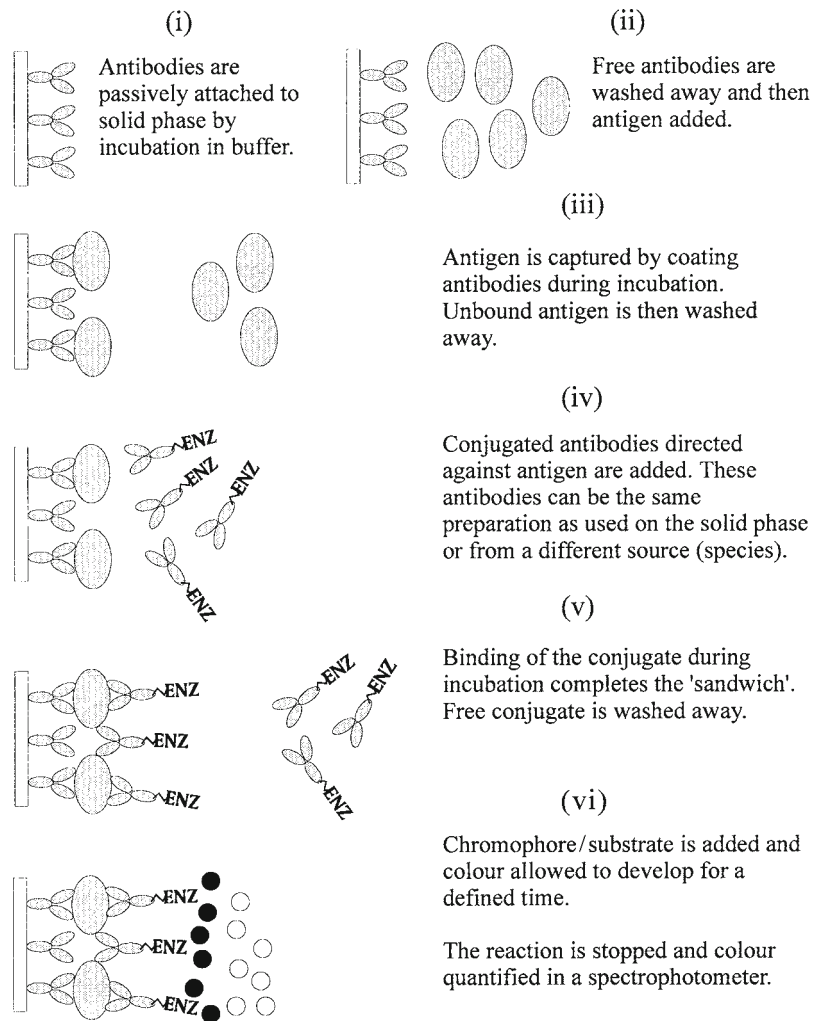


Fig. 3. Direct sandwich ELISA. This system exploits antibodies attached to a solid phase to capture antigen. The antigen is then detected using serum specific for the antigen. The detecting antibody is labeled with enzyme. The capture antibody and the detecting antibody can be the same serum or from different animals of the same species or from different species. The antigen must have at least two different antigenic sites.

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 labeled (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 must have at least two antigenic sites (epitopes), 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 the use of different detecting antibodies and a common capture antibody, and more versatile and hence appropriate systems are dealt with in **Subheading 2.3.2** The use of exactly the same antibodies for capture and detection (e.g., mAbs) can lead to problems, whereby there is a severe limitation of available binding sites for the detector. The size and the spatial relationship (topography) of the epitopes on the antigenic target are also critical and can greatly affect the assay.

2.3.2. Indirect Sandwich ELISA

Indirect sandwich ELISA is illustrated in **Diagram 4** and in **Fig. 4**. In indirect sandwich ELISA assay, stages i-iv are quite similar to

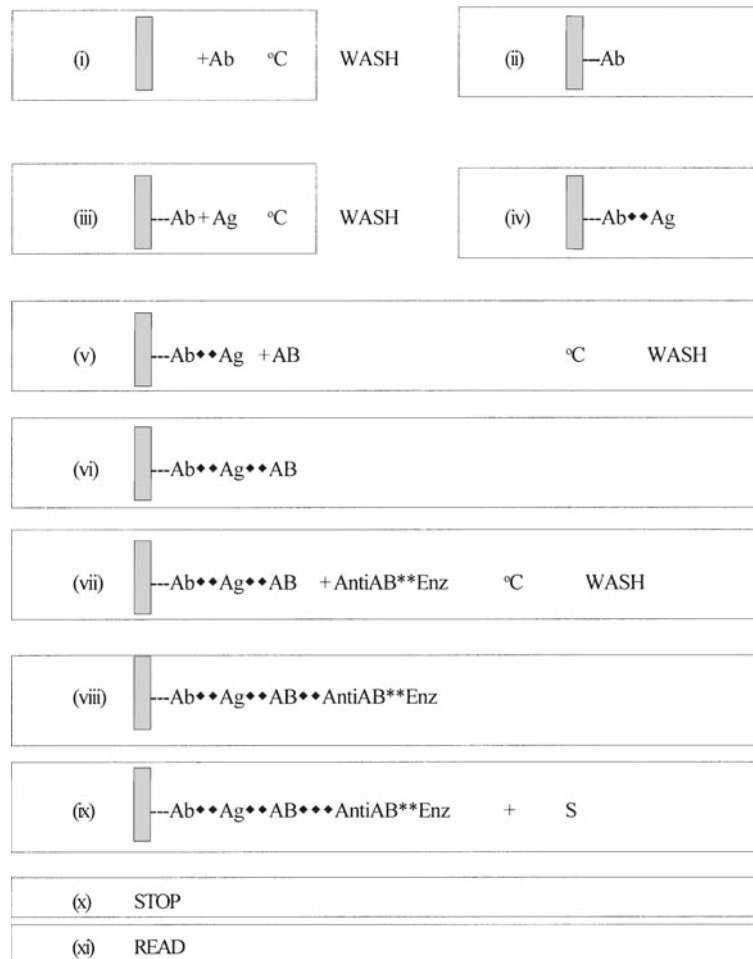


Diagram 4. Indirect sandwich ELISA.

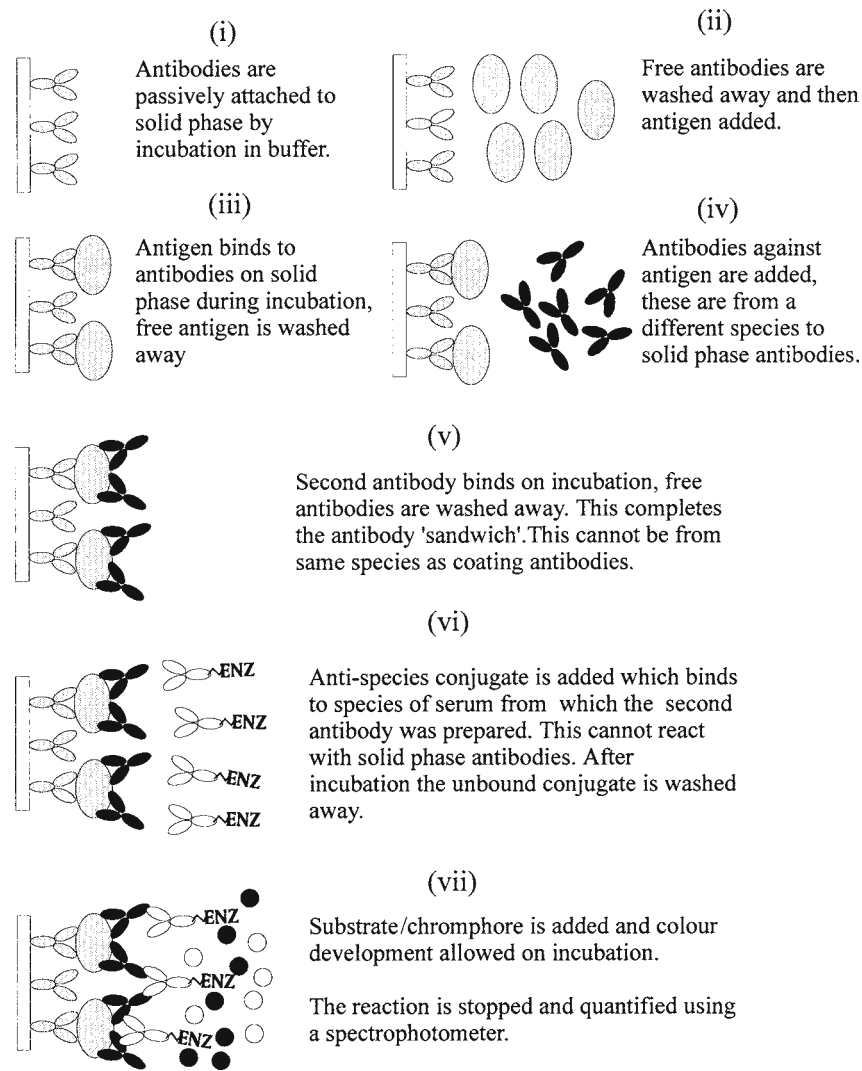


Fig. 4. Indirect sandwich ELISA. The antigen is captured by a solid-phase antibody. Antigen is then detected using antibodies from another species. This in turn is bound by an anti-species conjugate. Thus, the species of serum for the coating and detecting antibodies must be different; the anti-species conjugate cannot react with the coating antibodies.

those of the direct sandwich ELISA. Thus, antibodies are passively attached to the solid phase and antigen(s) are captured. However, stage v involves the addition of detecting antibodies. In this case, the antibodies are not labeled with enzyme. After incubation and washing (stage vi), the detecting antibodies are themselves detected by addition and incubation with an anti-species enzyme conjugate (stage vii). The bound conjugate is then processed as described in the other systems (stages xiii–ix).

The advantage of this assay is that any number of different sources of antibodies (samples) can be added to the captured antigen, provided that the species in which it was produced is not

the same as the capture antibody. More specifically, 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 (also called F(ab')<sub>2</sub> fraction). The detecting antibodies could be untreated. The enzyme conjugate could then be an anti-species anti-Fc portion of the Ig molecule. Thus, the conjugate would react only with antibodies containing Fc (and therefore not the capture molecules). The need to devise such assays depends on the reagents available.

It may be that a mAb is available that confers a desired specificity as compared with polyclonal sera or that one wishes to screen a large number of mAbs against an antigen that must be captured (it may be at a low concentration or in a mixture of other antigens). In this case, the use of F(ab')<sub>2</sub> polyclonal sera is unsuccessful; therefore, the preparation of fragments for the capture antibody is worthwhile, and in fact, relatively easy-to-use kits are available for this purpose. The use of a commercially available anti-mouse Fc completes the requirements.

#### **2.4. 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 pretitrated system. The systems involve all the other ELISA configurations already described. The assays can also 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. This section describes the possible applications of such methodologies, indicating the advantages and disadvantages. C-ELISA (competition ELISA) and I-ELISA (inhibition ELISA) are used to describe generally the assays involving the elements described in **Subheading 2.1–2.3** 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.

##### **2.4.1. Direct C-ELISA: Test for Antigen**

Direct C-ELISA testing for antigen is described and shown in **Diagram 5** and in **Fig. 5**. A pretitrated, direct system is challenged by the addition of antigen. The effect of the addition is measured by a decrease in expected color of the pretitrated system (used as a control). Thus, the competition stages proper start at stage iii, in which a sample is added to a solid phase that has the system antigen already passively attached. This sample is diluted in blocking buffer to prevent antigen binding to the solid phase

nonspecifically. At this stage, nothing should happen in terms of binding. The pretitrated dilution of labeled antibody (specific for the solid-phase antigen) is then added. The competitive phase now begins where, if the test antigen introduced is the same or similar to the solid-phase antigen, it will bind with the introduced labeled antibodies (stage ii a). 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 the addition of substrate, and so forth. When there is no antigen in the test sample, or when the antigenic similarities are limited, there is no binding with the labeled antibodies (stage ii b); thus, there is nothing to prevent (compete with) the binding of the labeled antibodies (stage iii). The net result is that, for samples containing antigen, there is

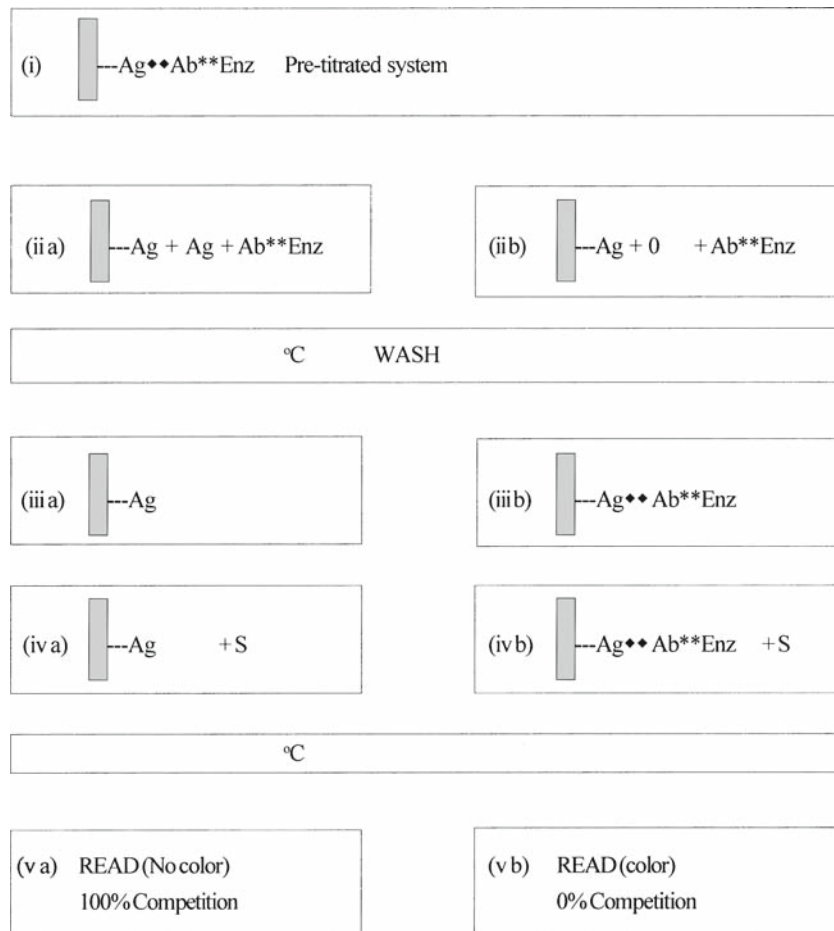


Diagram 5. Direct C-ELISA test for antigen.

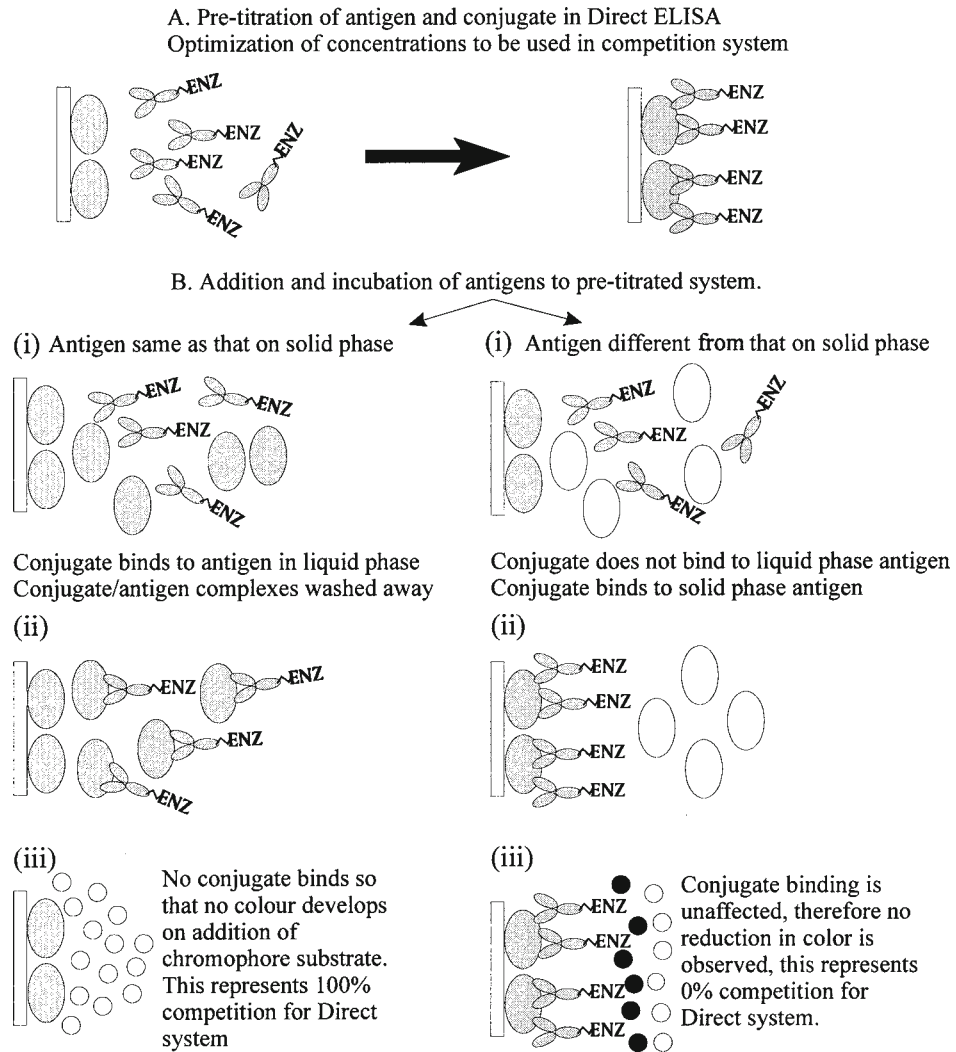


Fig. 5. Direct C-ELISA for antigen. Reaction of antigen contained in samples with the enzyme-labeled antibody directed against the antigen on the solid phase blocks the label from binding to the solid-phase antigen. If the antigen has no cross-reactivity or is absent, then the labeled antibody binds to the solid-phase antigen and a color reaction is observed on developing the test.

competition affecting the pretitrated expected color, whereas in negative samples there is no effect on the pretitrated color.

2.4.2. Direct C-ELISA:  
 Test for Antibody

Direct C-ELISA testing for antibody is illustrated in **Diagram 6** and in **Fig. 6**. The system here is the same as that for the test of 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.



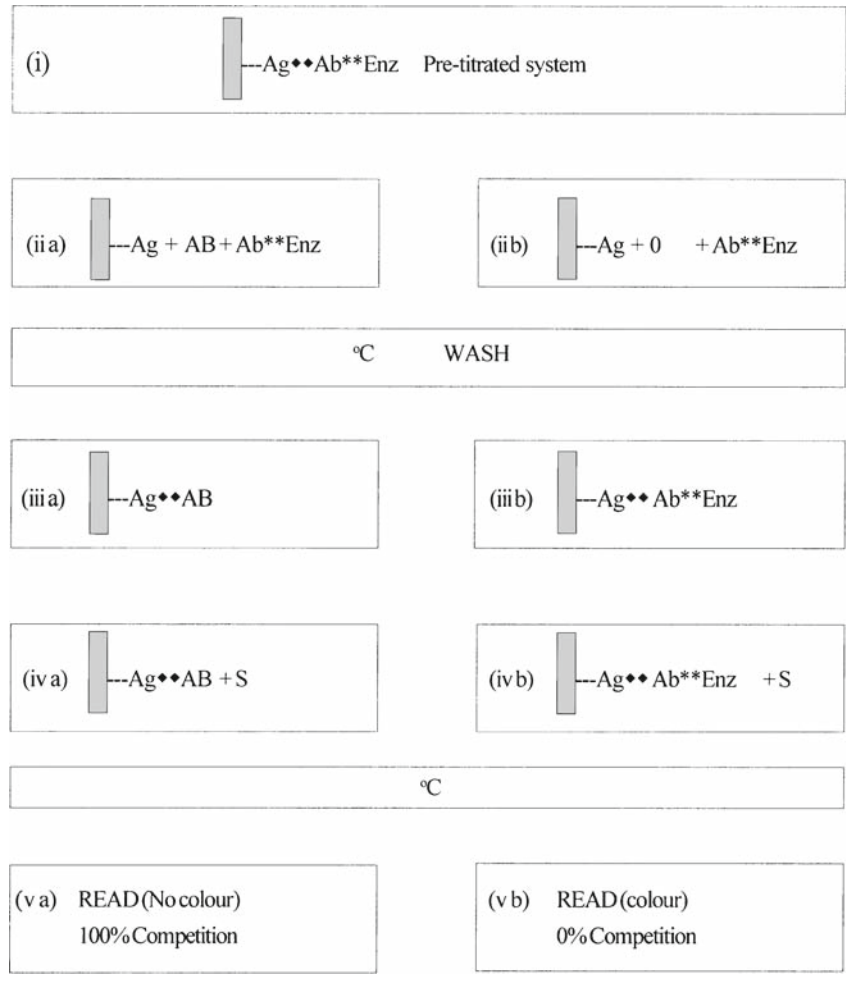


Diagram 6. Direct C-ELISA testing for antibody.

The competitive aspect here is between any antibodies in the test sample and the labeled specific antibodies for antigenic sites on the solid-phase bound antigen. The test sample and pretitrated labeled antibodies are mixed before adding to the antigen-coated plates.

2.4.3. Direct I-ELISA:  
Test for Antigen

Direct I-ELISA for antigen testing is not an available alternative, since test antigen has to be mixed with pretitrated labeled antibody. Thus, competitive conditions apply. One variation is that test antigen can be premixed with the labeled antibody and incubated for a period before the mixture is applied to the antigen-coated plates. In practice, this makes no difference to the assays in which antigen is added to the coated plates initially.

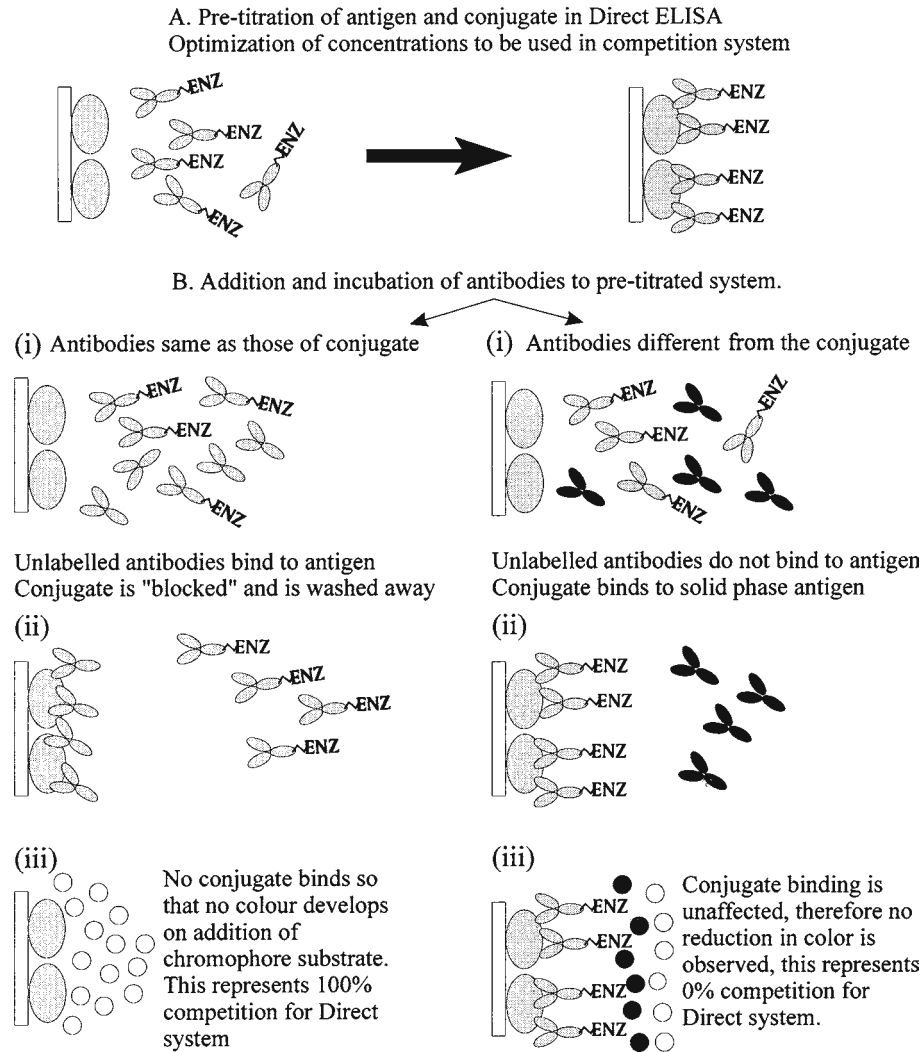


Fig. 6. Direct C-ELISA for antibody. The degree of inhibition by the binding of antibodies in a serum for a pretitrated enzyme-labeled antiserum reaction is determined.

**2.4.4. Direct I-ELISA:**  
*Test for Antibody*

The test sample possibly containing antibodies specific for the antigen on the plates is added and incubated for a period. There are then two alternatives: (1) the wells can be washed and then the pretitrated labeled antibody can be added, or (2) pretitrated labeled antibody can be added to the wells containing the test sample. 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.

**2.5. Competitive and Inhibition Assays for Indirect ELISA**

Indirect C-ELISA antigen measurement is illustrated in **Diagram 7** and in **Fig. 7**.

*2.5.1. Indirect C-ELISA Antigen Measurement*

*2.5.2. Indirect C-ELISA Antibody Measurement*

Indirect C-ELISA antibody measurement is illustrated in **Diagram 8** and in **Fig. 8**.

Note that the same pretitrated 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 pretitration phase. Thus, by using different concentrations of antibody, the effective sensitivity for competition or inhibition by antigen or antibody can be altered to favor

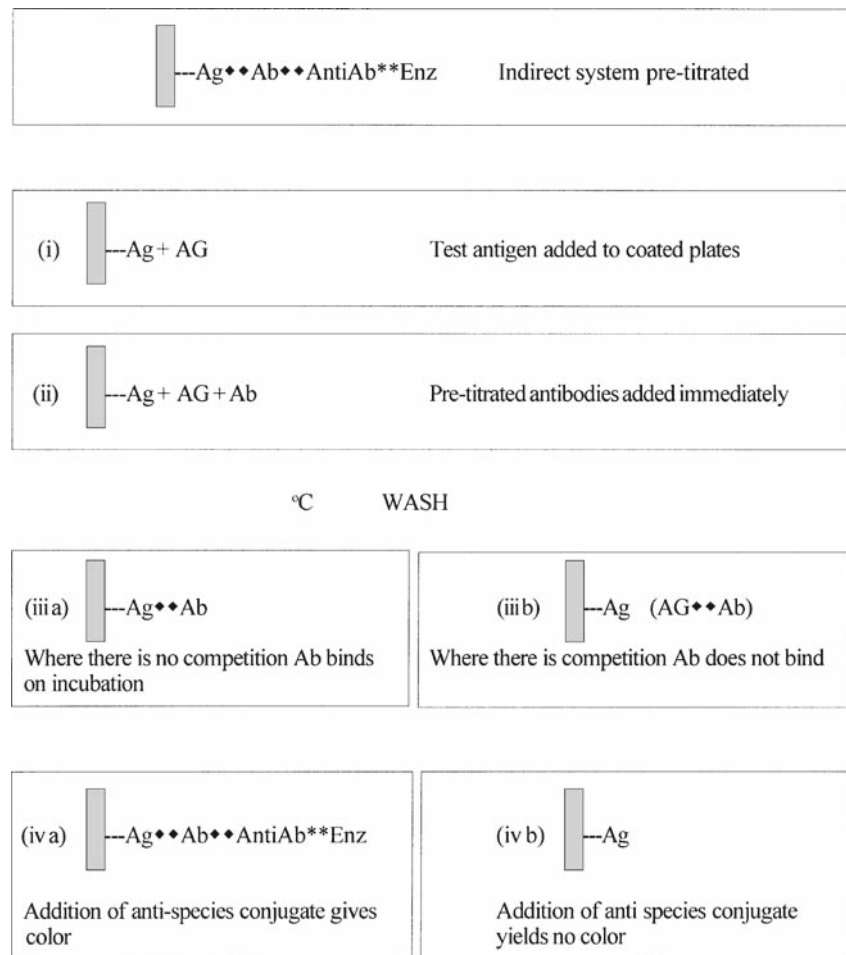


Diagram 7. Indirect C-ELISA antigen measurement.

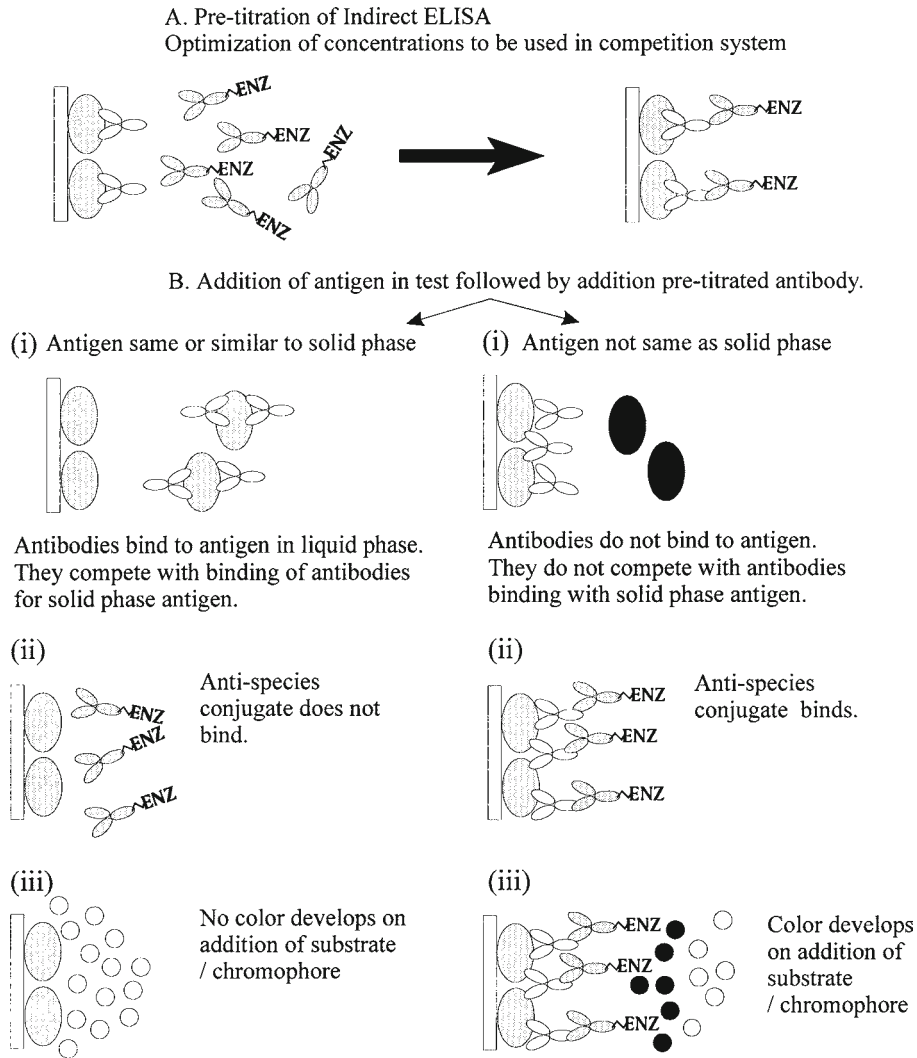


Fig. 7. Indirect C-ELISA antigen measurement. The degree of competition by the binding of antigens in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.

either analytical sensitivity or specificity. It is important to realize 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 idealized tests to suit the analytical parameters needed (degrees of required specificity and sensitivity). This is particularly important when devising assays based on polyclonal antibodies, which are markedly affected through the use of different dilutions of sera (alterations in quality of serum depending on relative concentrations of antibodies against specific antigenic determinants).

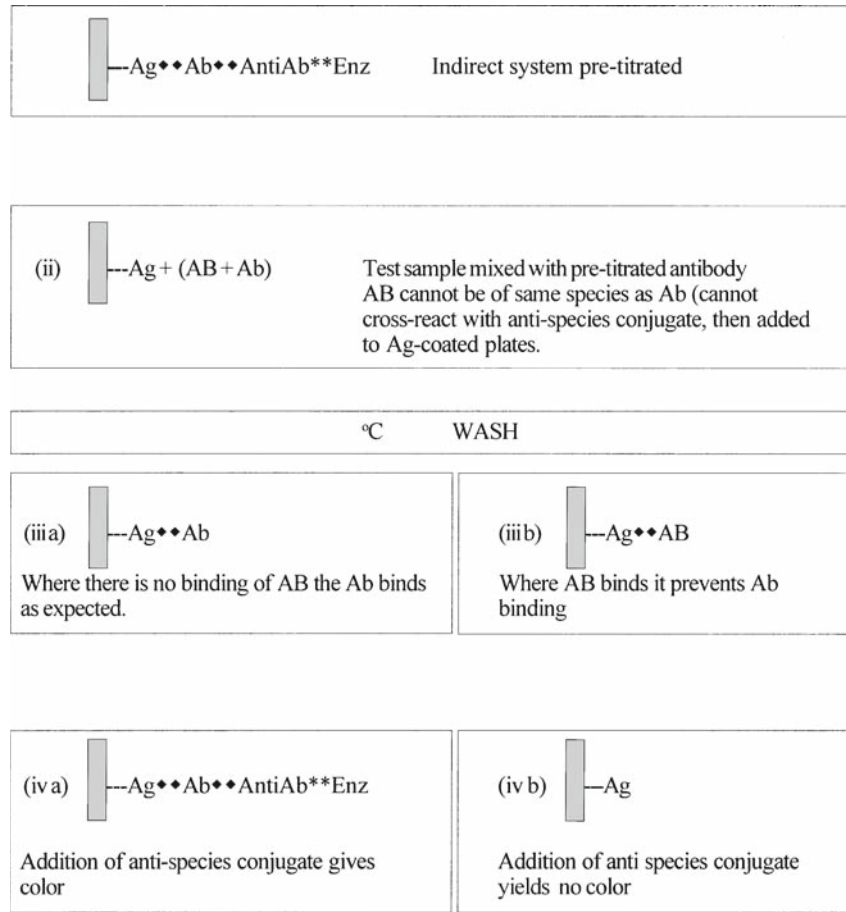


Diagram 8. Indirect C-ELISA antibody measurement.

2.5.3. Indirect I-ELISA  
Antigen Measurement

The test sample containing antigen can be premixed with the pretitrated antibody and incubated. The mixture can then be added to antigen-coated plates. The advantage of binding with the antibody is then in favor of the test sample. This is illustrated in **Diagram 9**.

2.5.4. Indirect I-ELISA  
Antibody Measurement

Principles of indirect I-ELISA antibody measurement are shown diagrammatically as follows. The sample containing AB is added to the antigen-coated plates and incubated. There are then two alternatives: (1) a washing step followed by the addition of pretitrated antibody, or (2) no washing step and the addition of pretitrated antibody to the mixture. This is illustrated in **Diagram 10**. Once again the advantage of binding is afforded to the sample.

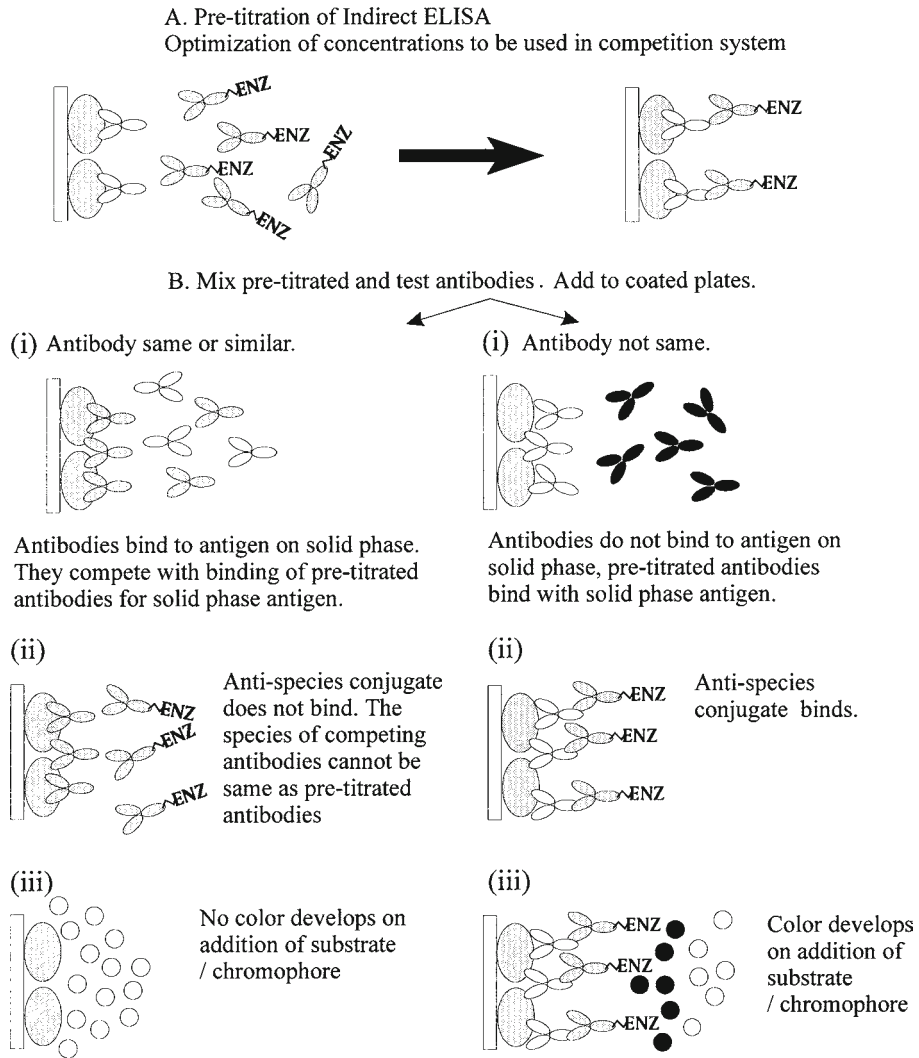


Fig. 8. Indirect C-ELISA antibody measurement. The degree of competition by the binding of antibodies in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.

**2.6. Competition and Inhibition Assays for Sandwich ELISAs**

Reference to previous sections reminds us that sandwich ELISAs are performed with both direct and indirect systems; that is, both involve the use of an immobilized antibody on the solid phase to capture antigen. For the direct sandwich ELISA, the detecting antibody is labeled with enzyme, whereas in the indirect system the detecting antibody is not labeled, which is in turn detected using an anti-species conjugate.

Both systems are more complicated than those described previously in that there are more stages involved. Consequently,

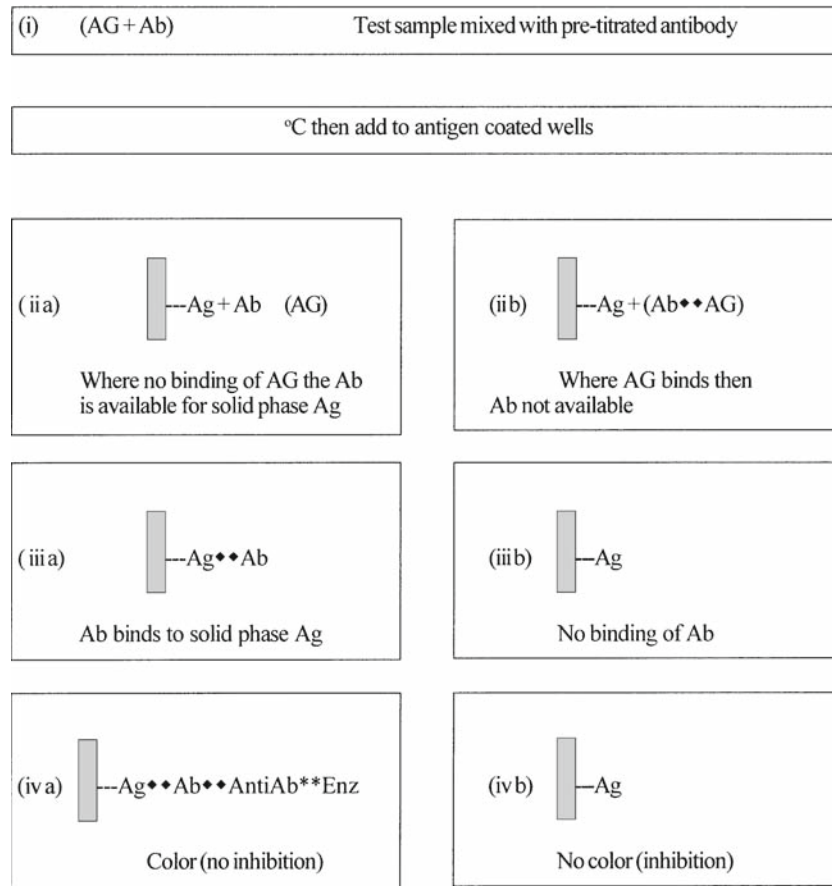


Diagram 9. Indirect I-ELISA antigen measurement.

the possibilities for variation in competing or inhibiting steps are increased. Attention must be focused on why a certain system is used as compared with others.

The main point about using sandwich assays is that they may be essential for presentation of antigen, usually by concentrating the specific antigen from a mixture through the use of a specific capture serum. Thus, the advantages of competitive/inhibitive techniques rely on antigen capture. Whether direct or indirect measurement of detecting antibody is used depends on exactly what kind of assay is being used. This section covers the principles, which in turn highlight the problems that must be addressed. Unsuitable systems are also illustrated.

The assays are described under direct sandwich and indirect sandwich headings. Direct sandwich involves assays utilizing a capture and a directly labeled detecting antibody (two antibody systems), and indirect sandwich involves assays utilizing

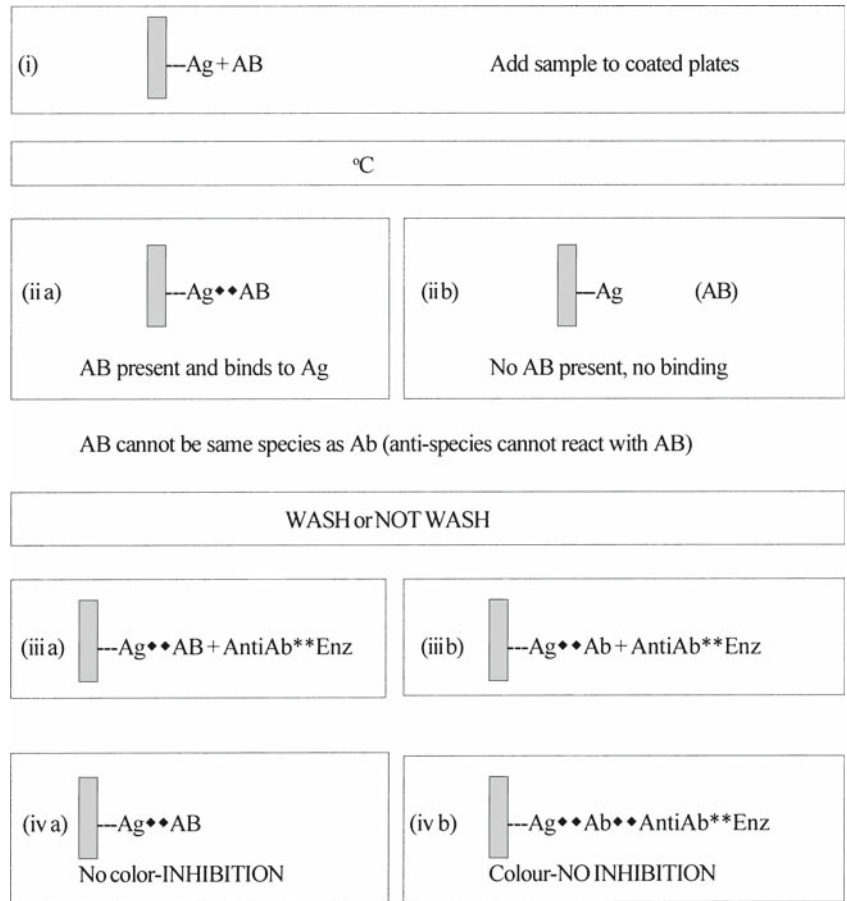


Diagram 10. Indirect I-ELISA antibody measurement.

three antibody systems (anti-species conjugate used to measure detecting serum). They are described for detecting antigen or antibody, as in the previous sections. The use of competition (C) and inhibition (I) assays is also described. Care should be taken to revise the basic sandwich systems since each must be titrated to optimize conditions before being applied in the competition/inhibition assay.

2.6.1. Direct Sandwich C-ELISA for Antibody

Direct sandwich C-ELISA for antibody is illustrated in **Diagram 11** and in **Fig. 9**.

2.6.2. Direct Sandwich I-ELISA for Antibody

The direct sandwich I-ELISA for antibody is as described for the previous competitive system except that the sample under test is added to the captured antigen for a time preceding the addition of the labeled antibodies. Following this incubation step, there are two alternatives. The first is to add the pretitrated labeled



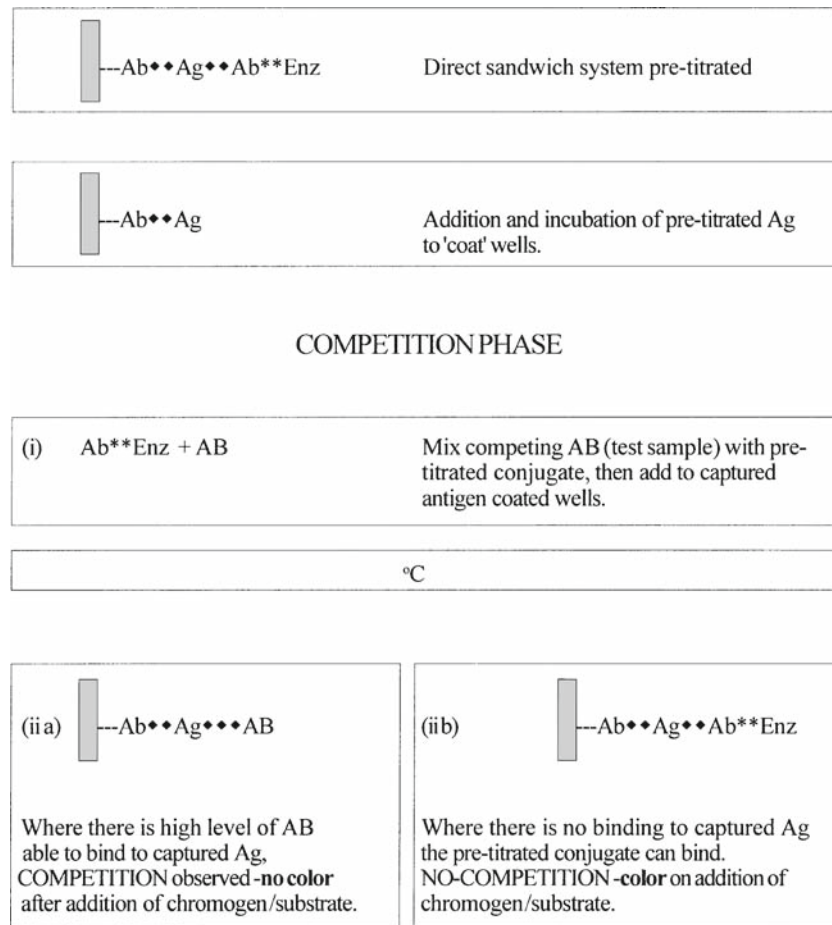
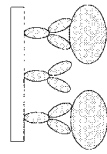


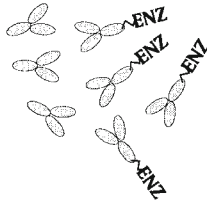
Diagram 11. Direct sandwich C-ELISA for antibody.

antibodies directly to the reaction mixture followed by incubation. The second 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 labeled antibodies followed by washing and then addition of substrate/ chromophore solution. The results are read according to the reduction in color as seen in controls in which no test sample was added. The greater the concentration of test antibodies that bind, the greater the degree of inhibition of the labeled antibodies.

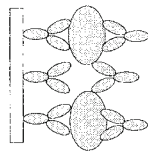
The number of components for the indirect sandwich ELISAs is increased and consequently the number of reagent combinations. The reader should by now be familiar with the descriptions in diagrammatic form so that the next series of assays exploiting the indirect sandwich ELISAs can be examined more briefly,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



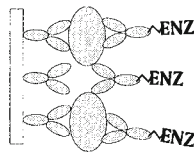
(i) System titrated to capture antigen. Detected by specific antibodies conjugated to enzyme.



(ii) Mixture of pre-titrated conjugate and test antibodies is made. This is added to antigen on coated plates.



(iii) Where the test antibodies are in large excess, they bind to antigen, preventing binding of conjugate. Competition.



(iv) Where there is no binding of test antibodies, the conjugate is free to bind. NO competition.

Fig. 9. Direct sandwich competition ELISA for antibody. This system exploits the competition of antibodies in a sample for the binding of a pretitrated quantity of labeled antibody specific for the antigen captured by the coating antibodies on the wells. The extent of competition depends on the relative concentrations of the test and labeled antibodies.

2.6.3. Direct Sandwich C- and I-ELISA for Antigen

The direct sandwich C- and I-ELISA for antigen is not suitable for the examination of antigen contained in test samples.

2.6.4. Indirect Sandwich C-ELISA for Antibody

The reader should reexamine the components of the indirect sandwich ELISA. Here, as in the direct sandwich system, antigen is captured by antibodies bound to the wells. The difference is that the antigen is detected first with an unlabeled antibody, which in turn, is detected and quantified using an anti-species conjugate. The exact time at which reagents/samples are added determines whether the system is truly examining competition or inhibition. **Diagram 12** illustrates where sample can be added to compete with the pretitrated indirect sandwich system.

It is critical that the antibody (AB) enzyme conjugate does not bind with the antibodies present in the test sample. The degree of competition is proportional to the amount of antibodies present

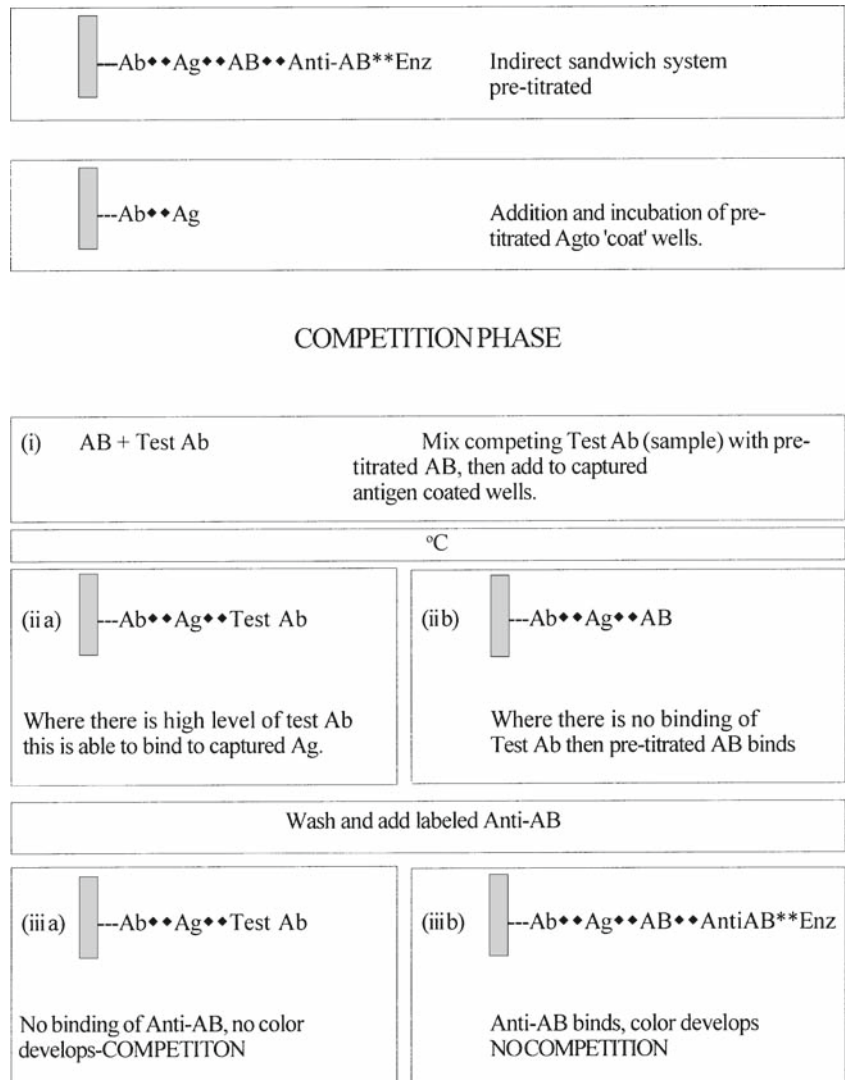


Diagram 12. Indirect sandwich C-ELISA for antibody addition on reagents.

in the test sample. The system offers greater flexibility in the use of different detecting antibodies (AB) for the captured antigen as compared with the direct sandwich assay. The system avoids producing specific conjugates for each of the sera used as detecting antibody (AB). Intrinsically, this also favors a more native reaction, since the introduction of enzyme molecules directly onto antibodies can affect their affinities (hence overall avidity of detecting AB). Thus, such a system is ideal in which the antigen must be captured and in which a number of detecting sera must be analyzed without chemical or physical modification. This also applies to the ELISA system described next.

2.6.5. Indirect Sandwich I-ELISA for Antibody

The indirect sandwich I-ELISA for antibody is similar to that of C-ELISA except that the time of addition of reagents is altered to allow a greater chance for reaction. This is illustrated in **Diagram 13**.

2.6.6. Indirect Sandwich C-ELISA for Antigen

The main problem with this form of antigen assay (indirect sandwich I-ELISAs) is that the wells are coated with antibodies that capture

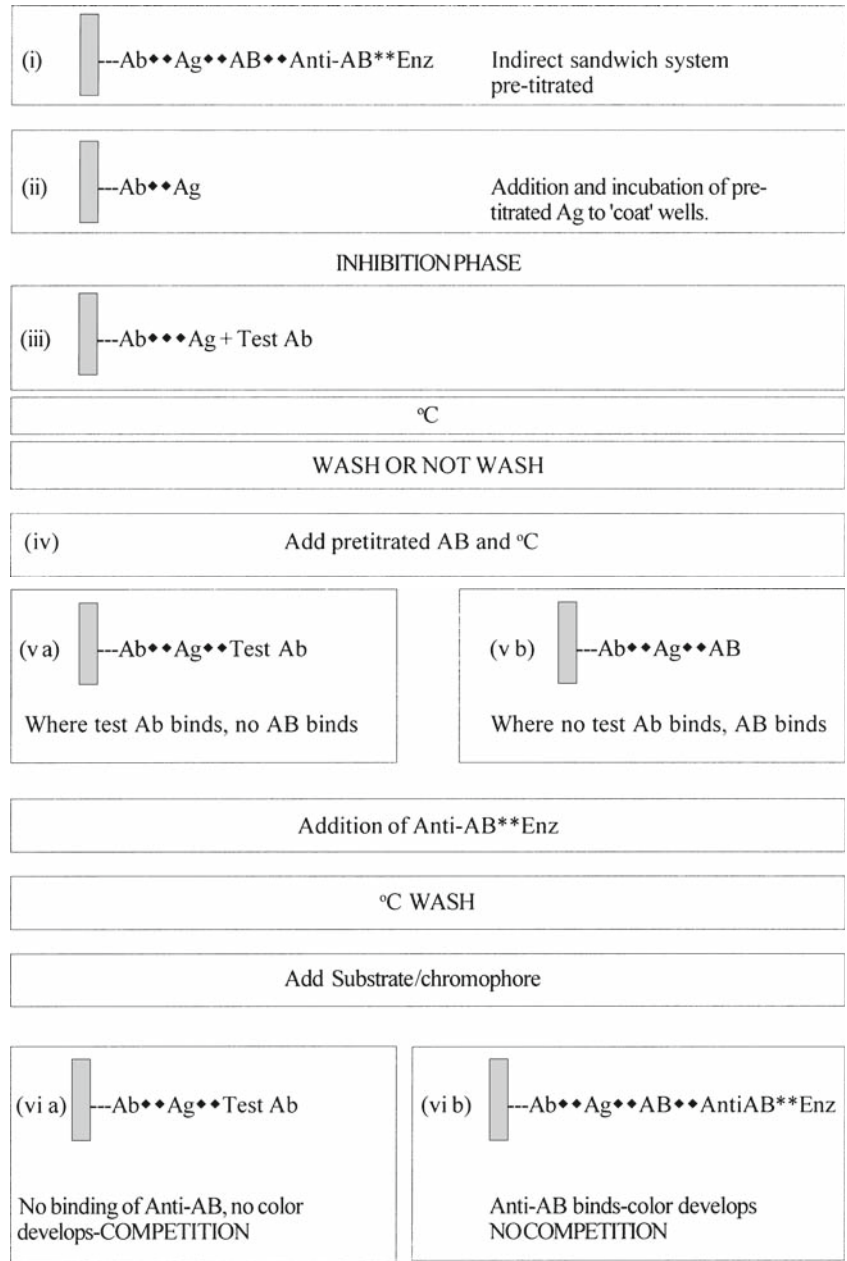


Diagram 13. Indirect sandwich I-ELISA for antibody.

antigen. Thus, any subsequent addition of antigen in a test sample will be bound to the wells if it is not fully saturated with the initially added coating antigen. The pretitration of the system then requires that there be no free antibodies coating the wells. Hence, the exact conditions for pretitration may differ from that for the antibody assays examined in **Subheadings 2.6.4** and **2.6.5**. The antigen has to be in excess, as shown in **Diagram 14**

The competitive phase occurs between the added test sample possibly containing antigen and the detecting second antibodies (AB), as shown in **Diagram 15**

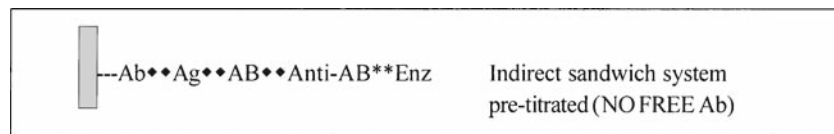


Diagram 14. Indirect sandwich C-ELISA for antigen.

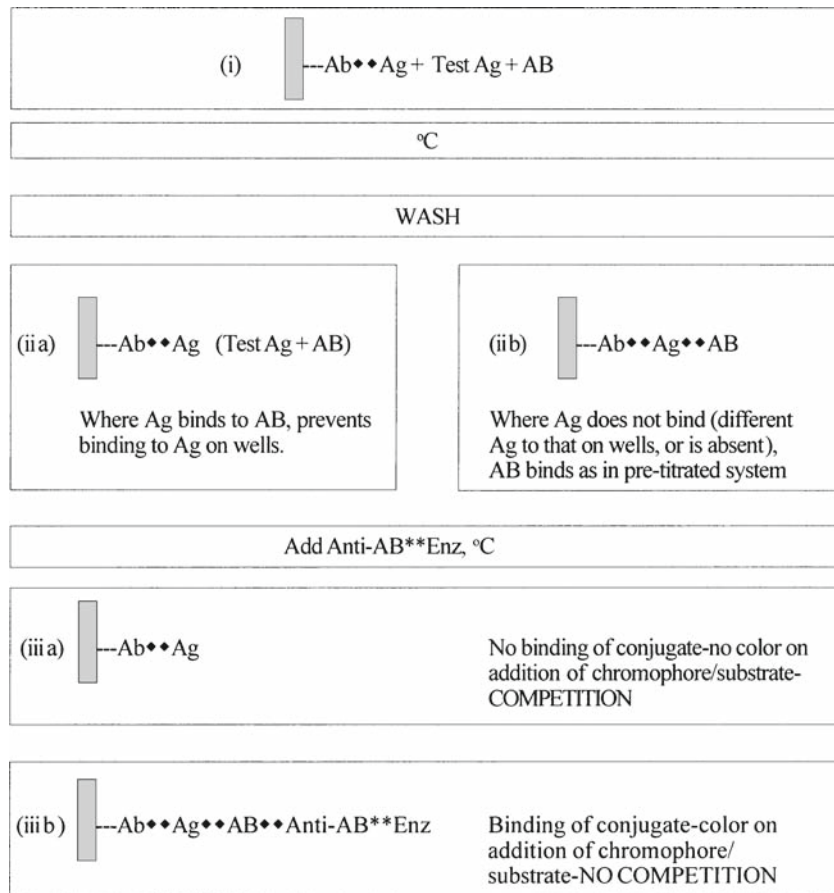


Diagram 15. Competitive phase between sample and antibodies.

*2.6.7. Indirect Sandwich  
I-ELISA for Antigen*

The indirect sandwich I-ELISA for antigen is essentially similar to that of C-ELISA except that the AB and the test antigen are mixed and incubated separately before being added to the wells containing captured antigen.

**2.7. Choice of Assays**

The most difficult question to answer when initiating the use of ELISAs is which system is most appropriate? This section attempts to investigate the relationships among the various systems to aid in assessing their suitability. The following questions must be addressed:

1. What is the purpose of the assay?
2. What reagents do I have?
3. What do I know about the reagents?
4. Is the test to be developed for a research purpose to be used by me alone, or for applied use by other workers?
5. Is the test to be used in other laboratories?
6. Is a kit required?

These questions have a direct effect on the phases that might be put forward as a general rule for the development of any assay. For example:

1. Feasibility – proof that a test system(s) can work (phase 1).
2. Validation – showing that a test(s) is stable and that it is evaluated over time and under different conditions (phase 2).
3. Standardization – quality control, establishment that a test is precise and can be used by different workers in different laboratories. At this stage a generalized examination of the availability of reagents and the effect this has on setting up a variety of systems will be made (phase 3).

*2.7.1. Assessing Needs*

It is assumed that there is some interest in the field in which an ELISA has to be developed. This infers that there is an understanding of the problem being addressed in terms of the biology involved and an appreciation of the literature concerning the target antigens and possible interactions of any agent with animals. If such knowledge is lacking, it should be sought through contact with other workers and by reading literature relevant to the field and associated areas, which includes the critical assessment of previously developed assays (including any ELISAs). Although this may seem obvious, unfortunately, information that is readily available to allow more rapid development of “new” assays and also comparative data assessment is often neglected.

For example:

1. We may have an antigen and may know a great deal or very little about it.
2. We may have a high concentration of a defined protein/polypeptide/peptide of known amino acid sequence or have

a thick soup of mixed proteins containing the antigen(s) at a low concentration contaminated with host cell proteins.

3. We may have an antiserum against antigen. This could be against purified antigen or against the crude soup. The antibody may have been raised in a given species, e.g., rabbit. We may have an IgG fraction of the antiserum (or could easily make one).
4. We may have field sera against the antigen (bovine sera). We may have a mAb. We may have antisera from different species, e.g., rabbit and guinea pig sera. ELISAs for similar systems may have been developed and can be found in the literature.
5. We may require an enzymatic reaction in the assay, and therefore will need an anti-species conjugate (commercial most probably) or will have to label an antigen-specific serum with enzyme (are there facilities to do this?). We must decide which commercial conjugate to buy. This will depend on the desired specificity of the conjugate (anti-whole molecule IgG, anti-H-chain IgG, anti-H chain IgM, and so on). The choice is somewhat determined by the aims of the assay and its design. Thus, we may wish to determine the IgM response of cattle to our antigen, which will require an anti-IgM (specific) somewhere in the ELISA protocol.

Obviously the basic needs for performing the ELISA must be addressed in terms of plates, pipets, buffers, reader, and so forth. In addition, if there is a need to develop a set of reagents that might be used as a universal assay, an assessment as to the scale of requirements is needed as early as possible. Thus, an estimate as to the likely usage of an assay should be made in terms of test units required in a defined time. This is translated into needed volumes of antigen, antisera, and conjugate (plates, pipet tips, and so forth). This need can be compared with what has been developed (or what needs to be produced).

For example, a test may be developed that is dependent on a single rabbit antiserum. The final volume may be 30 mL. The titer used in an assay may be 1/1,000. The test volume used is 50  $\mu$ L. Therefore the maximum number of samples that can be run as single tests is  $30 \times 1,000 \times 20 = 600,000$ .

This may be enough for universal testing for ten laboratories (60,000 samples per year) for one year, or if it runs tests on 6,000 samples a year, the reagent is satisfactory for 10 years. However, if the rabbit serum titer was 1/100, this effectively gives only enough reagent for testing 60,000 samples, which may be too little for a universal test.

Although this is a simplistic approach, early recognition as to why an ELISA is being developed is essential, which is often forgotten until the universal demands are examined. This approach should also be taken with considerations of antigen production, particularly when this may be difficult. Such considerations can also modify the selection of specific systems used. Thus, although

a successful indirect ELISA using purified antigen may be obtained, the yield of the antigen may be low and the processing laborious and expensive, such that any larger-scale use of the test is prohibitive. This problem may be alleviated through the use of capture antibodies and crude (more easily obtained) antigen preparations in the development of sandwich assays.

This approach extends to conjugates in which there may be certain commercial products or locally produced reagents that define the success of ELISAs. This is to ensure continuity of supply and standardization of reagents; sufficient quantities must be available to meet long-term needs.

*2.7.2. Examination of Possible Assays with Available Materials*

Obviously the reagents available must be examined first, as previously stated. This section examines some extremes so as to illustrate the relationship of the assays available and their particular advantages. Scenarios are described (A–C) in which different reagents are available, and these will probably cover most of those that are met in practice. Let us assume that there are sera to test from infected and noninfected animals. Further subtleties can be examined by defining the specificities of the conjugates (anti-IgG, IgM, or whether they are H-chain specific). The increase in choice of reagents and the possibilities for performing different ELISA configurations are given below.

1. Scenario A

- (a) Crude antigen (multiple antigenic sites)
- (b) Antibody raised against crude antigen in rabbits
- (c) Anti-cow conjugate
- (d) Postinfected and day 0 (uninfected) cow sera

2. Scenario B

- (a) Purified antigen (small amount, e.g., 100 µg)
- (b) Crude antigen (large amount)
- (c) Antibody raised in rabbits against pure antigen
- (d) Anti-rabbit conjugate
- (e) Anti-cow conjugate
- (f) Postinfected and day 0 (uninfected) cow sera

3. Scenario C

- (a) Crude antigen (as in A)
- (b) Antibody against pure antigen (rabbit)
- (c) Antibody against pure antigen (guinea pig)
- (d) Anti-guinea pig conjugate
- (e) Postinfected and day 0 (uninfected) cow sera
- (f) Anti-cow conjugate
- (g) Anti-rabbit conjugate



#### Scenario A

The use of crude antigen directly in an ELISA might be unsuccessful since it may be at a low concentration relative to other proteins and thus attach only at a low concentration. This would make unavailable the ELISA approaches as shown in **Subheadings 2.1 and 2.2** and thus competitive methods based on these as in **Subheadings 2.4 and 2.5**

Since a rabbit serum against the antigen is available, this may be used as a capture serum (or as capture IgG preparation), coated on the wells to capture the crude antigen to give a higher concentration to allow the bind. Thus, systems in **Subheadings 2.3 and 2.6** become available.

Any bound test antibody would be from cows and thus detected using an anti-bovine conjugate. This may cause problems since the crude antigen was used to raise the rabbit serum. Hence, antibodies against contaminating proteins may be produced in the rabbit. The cow sera being tested may react with such captured contaminants. However, when the antigen is an infectious agent, antibodies against the contaminating proteins may not be produced, thus eliminating the problem.

When the antigen is used as a vaccine, whereby relatively crude preparations similar to the crude antigen are used to formulate the vaccine, then this problem will be present. Attempts can be made to make the rabbit serum specific for the desired antigenic target.

Solid-phase immunosorbents involving the contaminating crude elements (minus the desired antigen) can be used to remove the anticrude antibodies from the rabbit serum, which could then be titrated as a capture serum. An example can be taken from the titration of foot-and-mouth disease virus antibodies. The virus is grown in tissue culture containing bovine serum. Even when virus is purified from such a preparation, minute amounts of bovine serum contaminate the virus. When this purified virus is injected into laboratory animals as an inactivated preparation, a large amount of anti-bovine antibodies as well as anti-virus antibodies are produced. This serum cannot be used in a capture system for specifically detecting virus grown as a tissue culture sample (containing bovine serum) because it also captures bovine serum. The capture serum is also unsuitable for capturing relatively pure virus for the titration of bovine antibodies from bovine serum samples because the capture antibodies react strongly with the detecting cow serum. Thus, the capture serum has to be adsorbed with solid-phase immunosorbents produced through the attachment of bovine serum to agarose beads.

Once the specificity of the capture serum is established, the optimization of the crude antigen concentration can be made using a known or several known positive cow sera in full dilution ranges. Inclusion of dilution ranges of negative sera allows

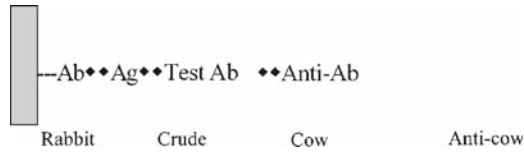


Diagram 16 Use of reagents to set up a sandwich ELISA

assessment of the difference between negative and positive sera at different dilutions of serum. Diagram 2.16<COMP: Insert Diagram 2.16 near here> illustrates the use of the reagents to set up a sandwich ELISA. The assay is made possible through the specific capture of enough antigen by the solid-phase rabbit serum.

Scenario B

This scenario is not so different from scenario A; however, there are more reagents. The antigen is available purified for use in raising antibodies in rabbits. Thus, with due reference to the reservations already described for scenario A, there is a basis for setting up a capture ELISA since the rabbit antibodies may capture the antigen at a high concentration from the crude antigen preparation, which is present in a large amount. The developmental system of the capture ELISA is as shown earlier.

The availability of the anti-rabbit conjugate may allow the development of competitive assays if enough specific antigen binds to plates, although this is unlikely, as already indicated. The antigen and rabbit serum could be titrated in an indirect ELISA (*see Subheading 2.2*) in a checkerboard fashion enabling the optimization of the antigen and serum. These optimal dilutions could be used to set up competitive ELISAs (*see Subheading 2.5.2*) in which cow sera would compete for the pretitrated antigen/rabbit/anti-rabbit conjugate system. Again, it must be emphasized that this is unlikely since the antigen is crude and some form of capture system will be needed to allow enough antigen to be presented on the wells.

Because scenario B has some purified antigen, it could be used in the development of a similar competitive assay. This will depend on the availability of this antigen, which can be determined after the initial checkerboard titrations in which the optimal dilution of antigen is calculated. The chief benefit of obtaining purified antigen is to obtain a more specific serum in rabbits allowing specific capture of antigen from the crude sample. In many cases, there is enough antigen of sufficient purity to be used in assays.

Scenario C

Here, all the possibilities of the first two situations plus the production of a second species (guinea pig) of serum against the purified antigen are present.

This allows the development of sandwich competitive assays (*see Subheading 2.6*) using either the rabbit or guinea pig as capture serum or detector with the relevant anti-species conjugate.

Different species may have better properties for acting as capture reagents and also show varying specificities. This can be assessed in chessboard titrations and is relevant because we require results on the detection and titration of cattle sera so that the competitive phase relies on the interruption of a pretitrated antibody as close to the reaction of cattle serum with antigen as possible. Rabbit or guinea pig serum may differ in their specificities as compared with cattle sera.

#### Further Comments

The assays shown in **Subheading 2.4.2** (competition for direct ELISA) are probably inappropriate owing to the possession of crude antigen (for reasons described earlier). However, if it can be shown that enough antigen can attach and that cattle sera react specifically (and not through excess antibodies directed against contaminants in the crude antigen), then we can set up assays based on this system. This requires identification of a positive cow serum and labeling of this serum with an enzyme.

Of more practical value could be the use of a positive cow serum labeled with enzyme. The serum can then be used both as capture, particularly as an IgG fraction) and for detection. In this way the competitive assay shown in **Subheading 2.6.1** is feasible and may have an advantage in that the reaction being competed against is homologous (cow antibody against antigen). This avoids complications through the use of second-species antisera produced by vaccination. The system is suitable for measuring the competition by other cow sera because the detecting antibody is labeled. Thus, a worker with relatively few reagents and the ability to label antibodies with an enzyme may have enough materials to develop assays. This brief description of system possibilities has concentrated on antibody detection. Note that most of these comments are relevant to antigen detection.