

## 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.

### 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 definitions of terms used in ELISA. These terms are greatly amplified throughout the subsequent text.


### 2. Basic Systems of ELISA

This section describes the principles involved in the many configurations possible in ELISA. The terminology here may not always agree with that used by others, and care is needed in defining assays by name only. The specific assay parameters must always be examined carefully in the literature. The

**Table 1**  
**Brief Definition of Terms**

Term	Definition
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 antiguinea 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).
Chromophore	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.

**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

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 in terms. In this way, it is hoped that the reader will gain 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 antispecies conjugate against a subclass of immunoglobulin (Ig) is required.

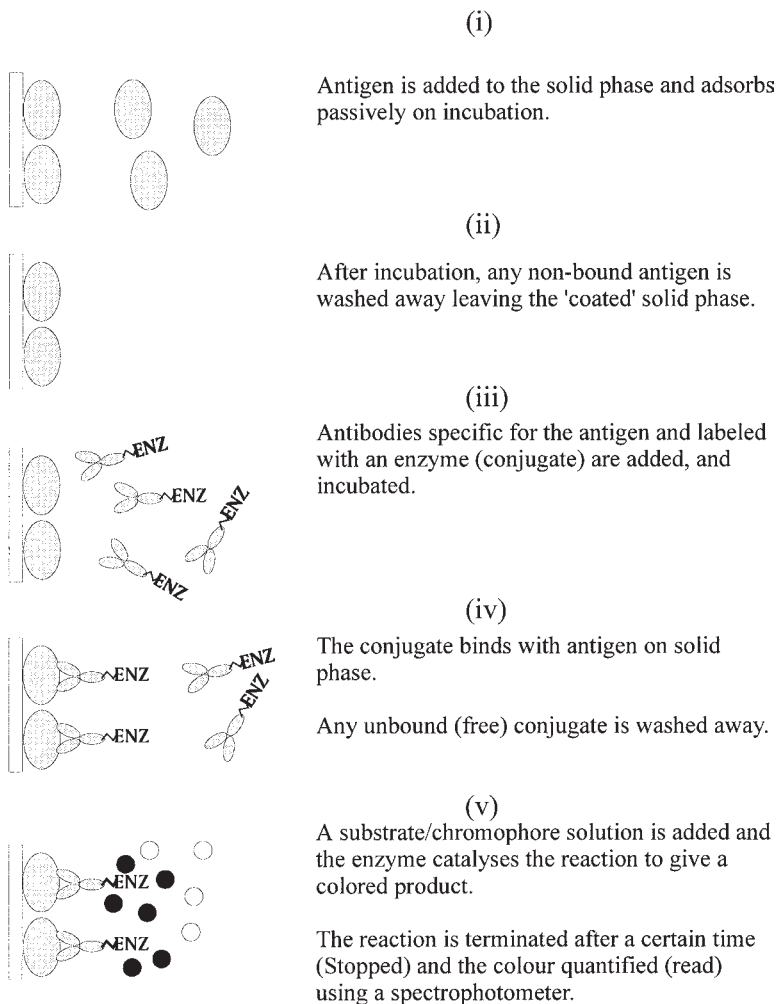


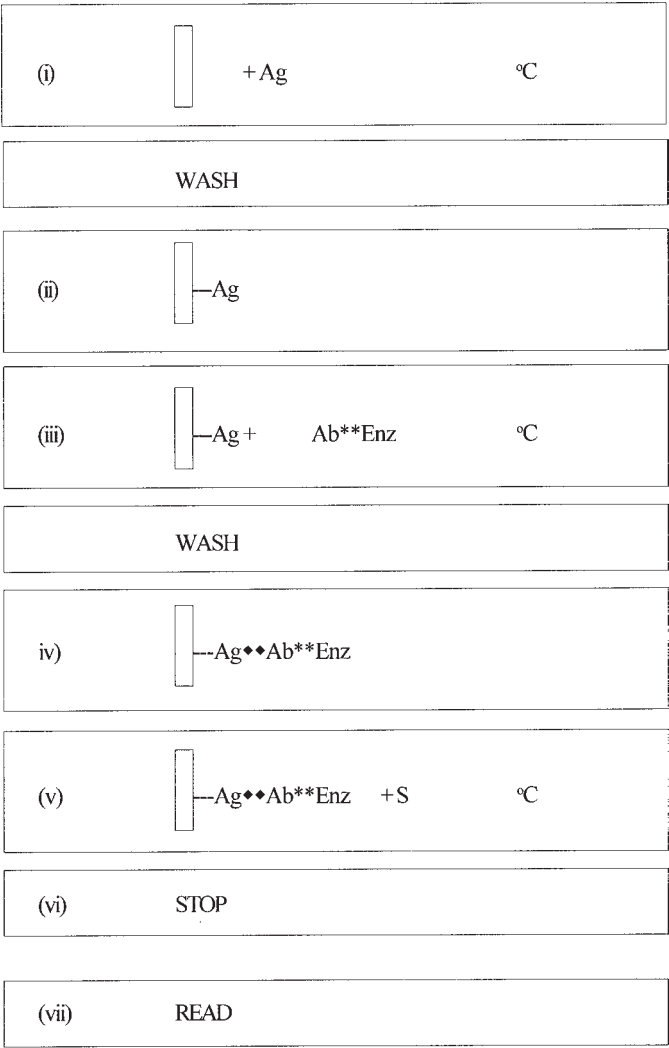
Fig. 1. Direct ELISA. Antigen is attached to the solid phase by passive adsorption. After washing, enzyme-labeled antibodies are added. After an incubation period and washing, a substrate system is added and color is allowed to develop.

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 the ELISA, and is illustrated in **Fig. 1** and in the following diagram.

Direct ELISA



Antigen is diluted in a buffer (stage i), commonly a high pH (9.6) carbonate/ 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 the incubation is 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 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.

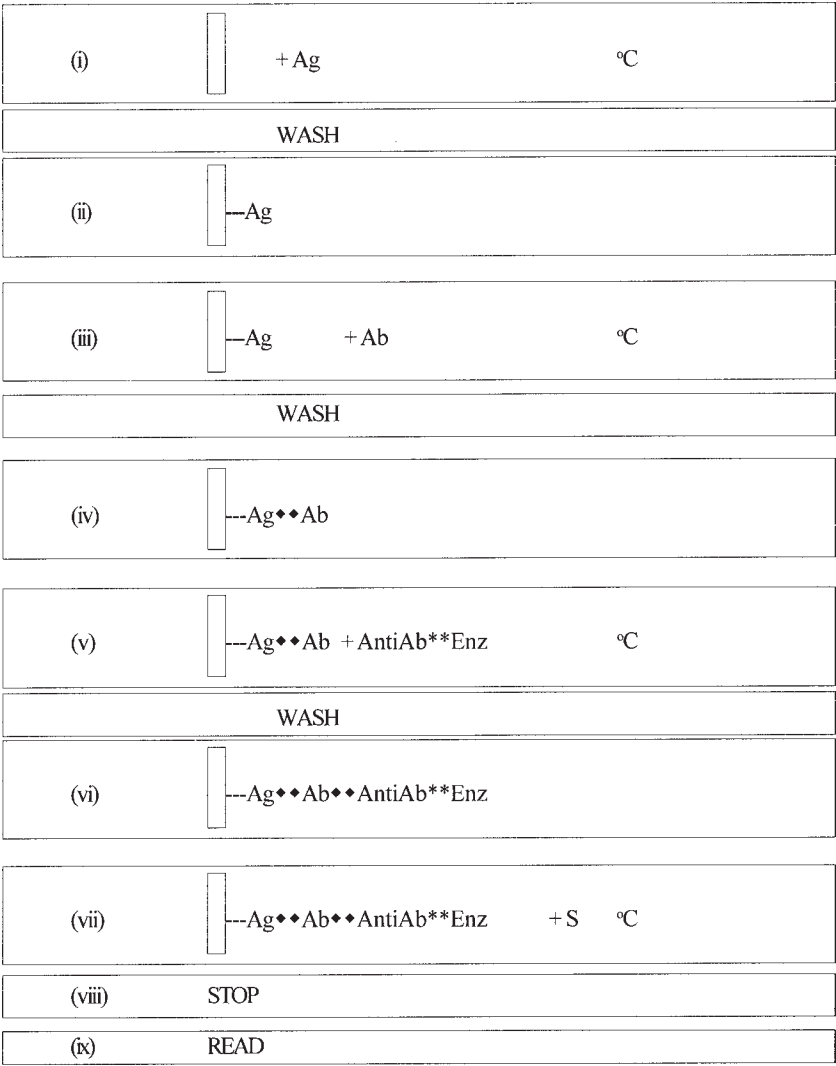
This kind of system has severe limitations when used only in this form but has assumed great importance as the “target” system in competition and inhibition assays, particularly when mAbs are conjugated and/or highly defined antigens are used.

## 2.2. Indirect ELISA

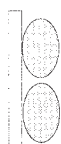
Indirect ELISA is illustrated in the following diagram and in **Fig. 2**. Stages i and ii are similar to the direct system. Stage iii involves the addition of unlabeled detecting antibodies, which are diluted in a buffer to prevent nonspecific attachment of proteins in antiserum to solid phase (blocking 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), antisppecies antibodies, diluted in blocking buffer, again followed by incubation and washing to achieve binding of 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 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 anti-

Indirect ELISA

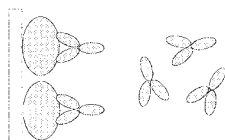


bodies are produced and are termed antispecies conjugates. Thus, if the detecting antibodies were produced in rabbits, the enzyme-labeled antibodies would have to be antirabbit Igs in nature. This allows great flexibility in use of antispecies conjugates in that different specificities of conjugate can be used to detect particular immunoglobulins binding in the assay, and there are literally thousands of commercially available conjugates available. For example, the antispecies conjugate could be anti-IgM, anti IgG<sub>1</sub>, IgG<sub>2</sub>, and so on.



(i)

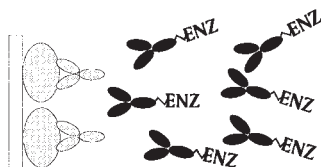
Antigen is passively adsorbed to solid phase by incubation.



(ii)

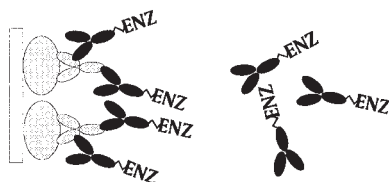
Antibodies are added and incubated with solid-phase attached antigen. Those which are specific will bind to antigen.

Excess antibodies or non-binding components are washed away after incubation phase.



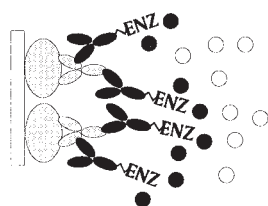
(iii)

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



(iv)

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



(v)

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

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

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 antispecies antiserum labeled with enzyme. This is widely used in diagnosis.

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



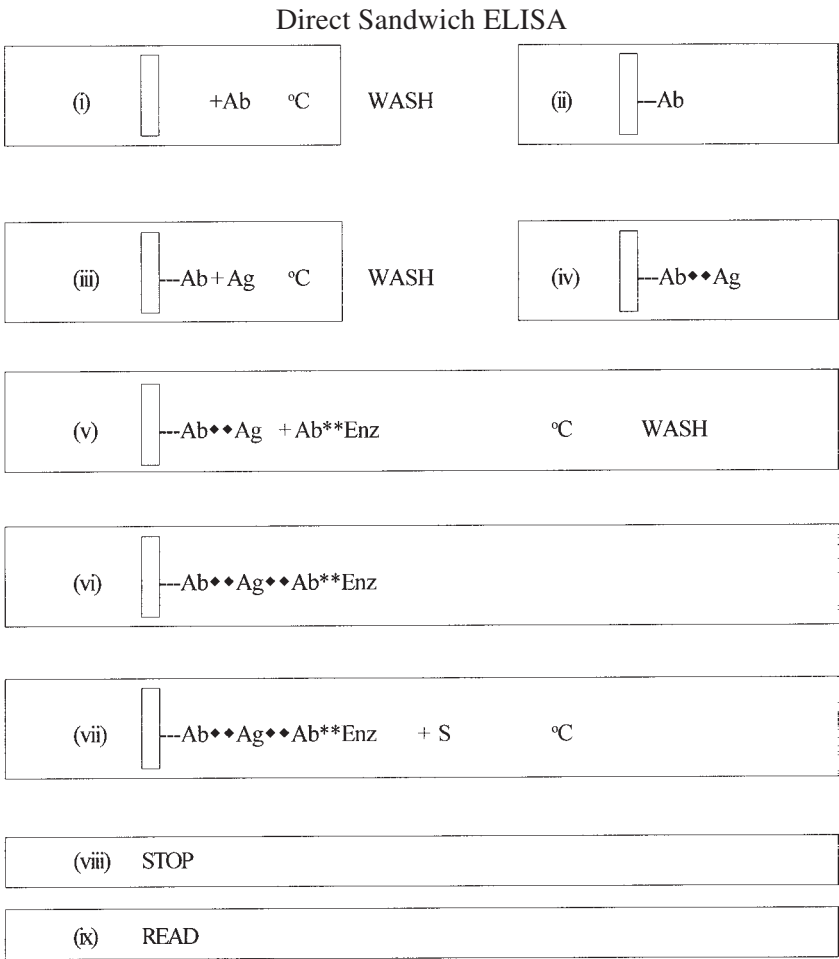
such systems have is the varying degree of nonspecific binding in individual sera. This tends to widen the dispersion (variability) in assay results and, therefore, increases the need to process many sera to assess confidence.

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 illustrated as follows 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

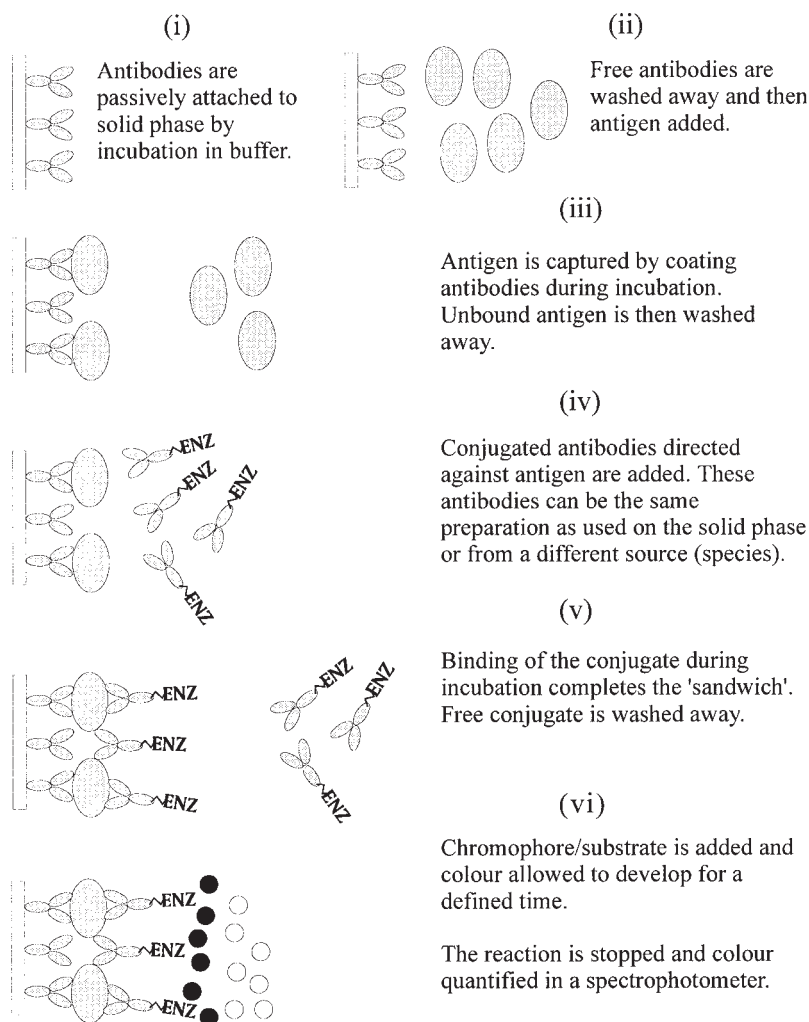


Fig. 3. Direct sandwich ELISA. This system exploits antibodies attached to a solid phase to capture antigen. The antigen is then detected using serum specific for the antigen. The detecting antibody is labeled with enzyme. The capture antibody and the detecting antibody can be the same serum or from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

bind antigen(s) that are added in stage 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).

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.

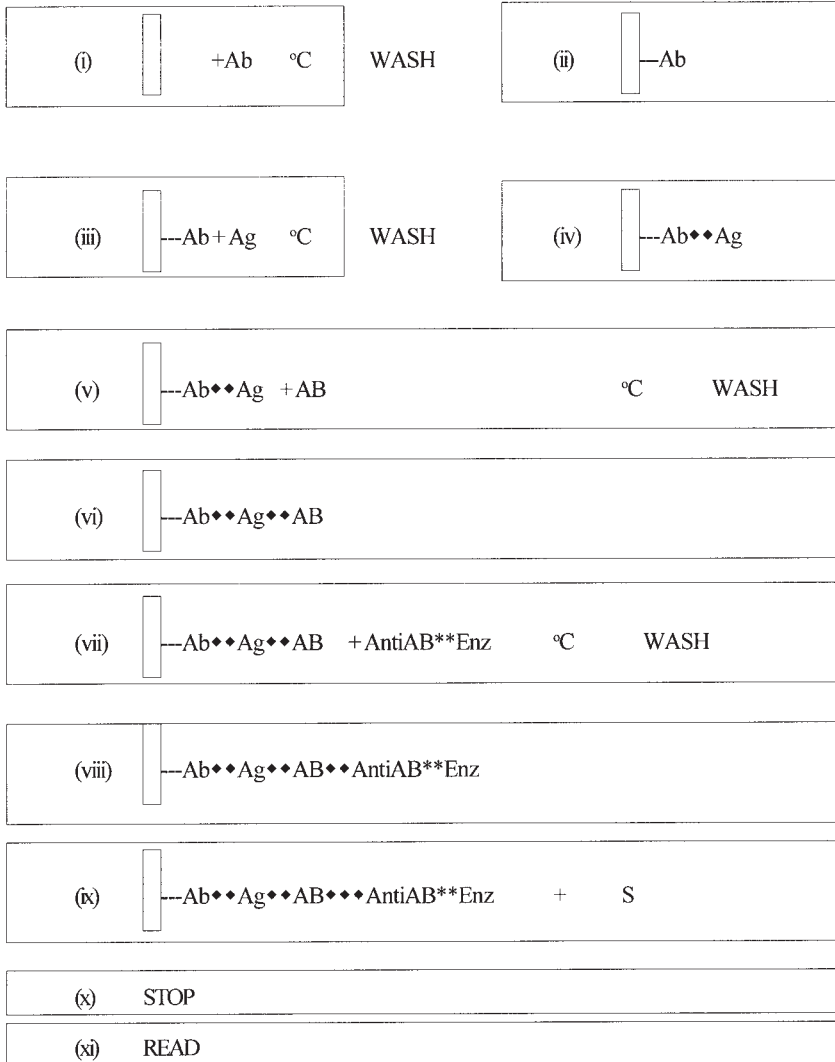
The capture antibody (on the solid phase), and the detecting antibody, can be against different epitopes on an antigen complex. This can be helpful in orienting the antigenic molecules so that there is an increased chance that the detecting antibodies will bind. It can also be an advantage when investigating 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 is also critical and can greatly affect the assay.

### **2.3.2. Indirect Sandwich ELISA**

Indirect sandwich ELISA is illustrated as follows and in **Fig. 4**. In indirect sandwich ELISA assay stages i–iv are quite similar to 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 antispecies enzyme conjugate (stage vii). The bound conjugate is then processed as described in the other systems (stages xiii–ix).

The advantage to this assay is that any number of different sources of antibodies (samples) can be added to the captured antigen, provided that the spe-

### Indirect Sandwich ELISA



cies in which it was produced is not the same as the capture antibody. More specifically, the enzyme conjugated antispecies 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

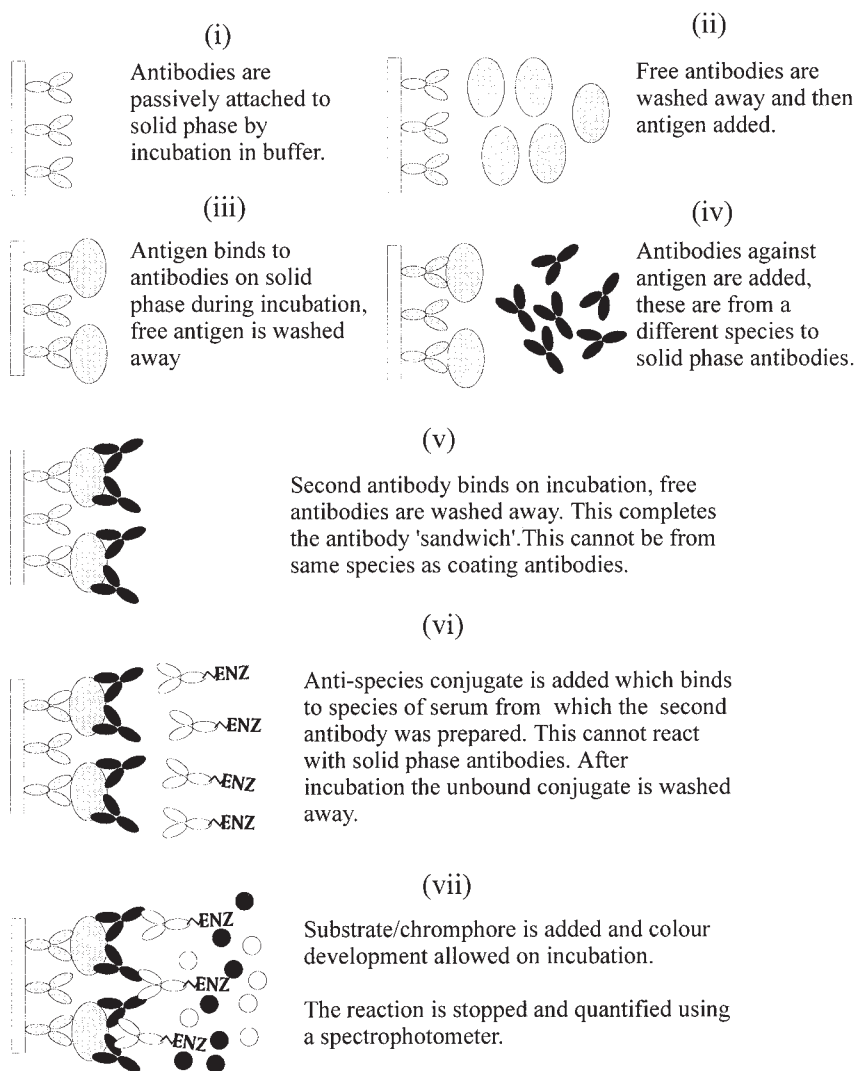


Fig. 4. Indirect sandwich ELISA. The antigen is captured by a solid-phase antibody. Antigen is then detected using antibodies from another species. This in turn is bound by an antispecies conjugate. Thus, the species of serum for the coating and detecting antibodies must be different; the antispecies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

an antispecies 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 to 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 the case use of  $F(ab')_2$  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 antimouse Fc completes the requirements.

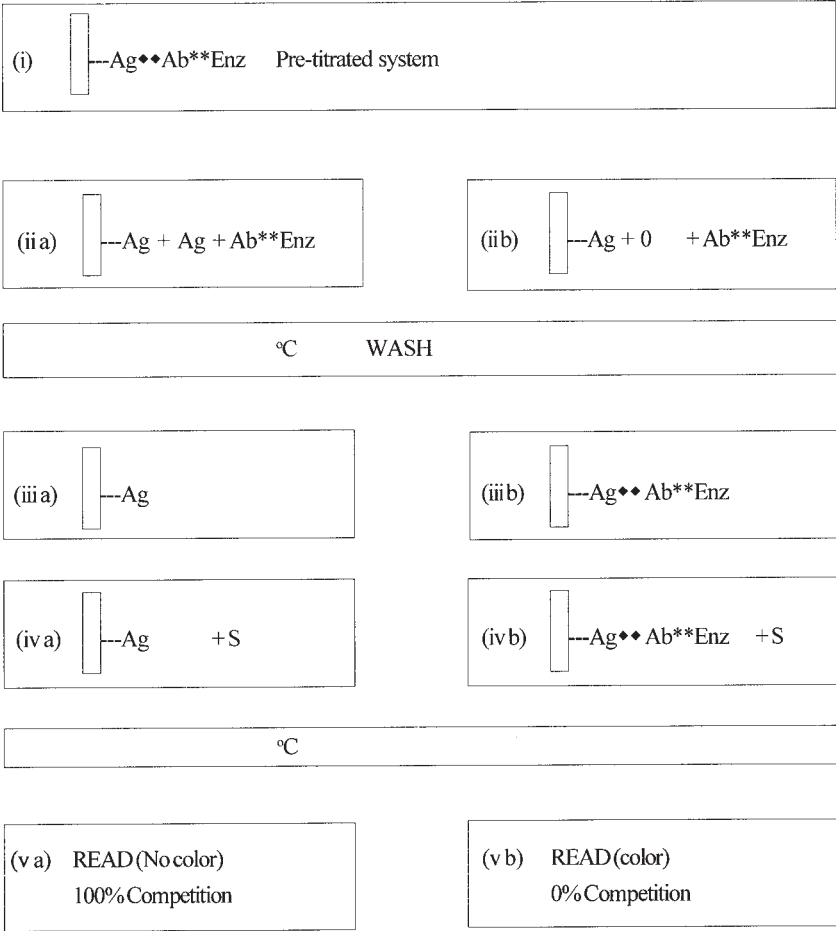
## 2.4. Competition/Inhibition Assays

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 also can be used for the measurement of either antibody or antigen. The terminology used in the literature can lead to confusion the term blocking-ELISA is also frequently used to describe such assays. 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 the following diagram 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 addition of substrate, and so forth. When

Direct C-ELISA Test for Antigen

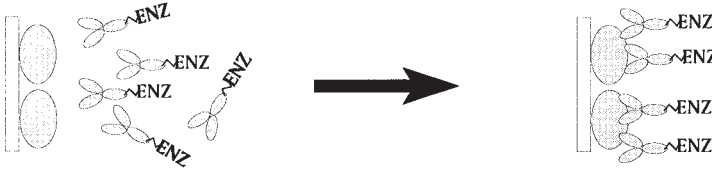


there is no antigen in the test sample, or when the antigenic similarities are limited, there is no binding with the labeled antibodies (stage ii b); thus, there is nothing to prevent (compete with) the binding of the labeled antibodies (stage iii). The net result is that for samples containing antigen, there is 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

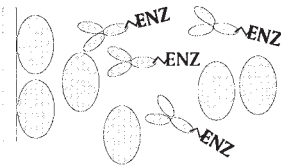
Direct C-ELISA testing for antibody is illustrated in the diagram at the top of page 25 and in **Fig. 6**. The system here is the same as that for the test of antigen; however, the measurement or comparison of antibodies is being made.

A. Pre-titration of antigen and conjugate in Direct ELISA  
Optimization of concentrations to be used in competition system



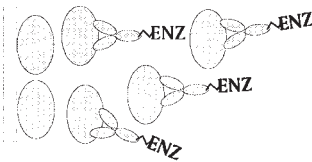
B. Addition and incubation of antigens to pre-titrated system.

(i) Antigen same as that on solid phase



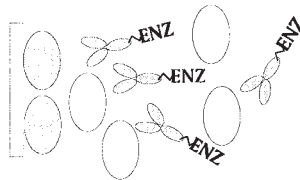
Conjugate binds to antigen in liquid phase  
Conjugate/antigen complexes washed away

(ii)



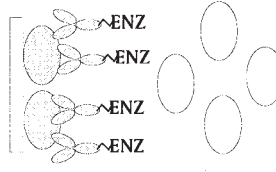
(iii) No conjugate binds so that no colour develops on addition of chromophore substrate. This represents 100% competition for Direct system

(i) Antigen different from that on solid phase



Conjugate does not bind to liquid phase antigen  
Conjugate binds to solid phase antigen

(ii)



(iii)

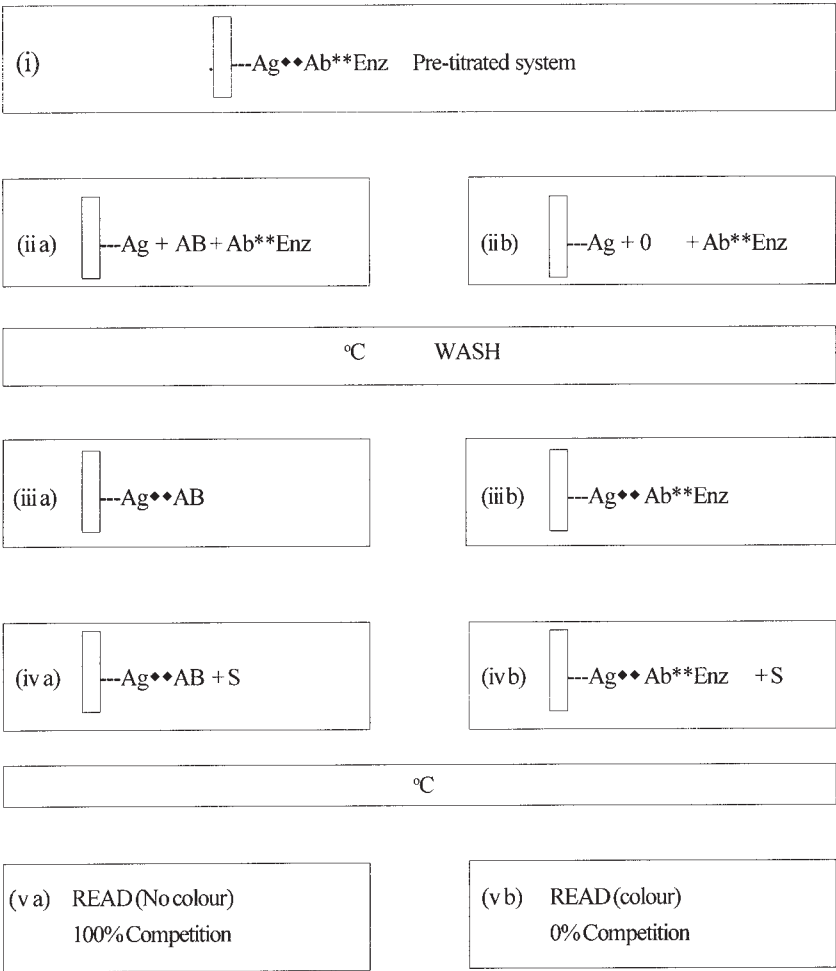
Conjugate binding is unaffected, therefore no reduction in color is observed, this represents 0% competition for Direct system.

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.

Again there is a requirement to titrate the direct ELISA system, which is then challenged by the addition of test antibodies. The competitive aspect here is between any antibodies in the test sample and the labeled specific antibodies for antigenic sites on the solid-phase bound antigen. The test sample and



Direct C-ELISA Testing for Antibody



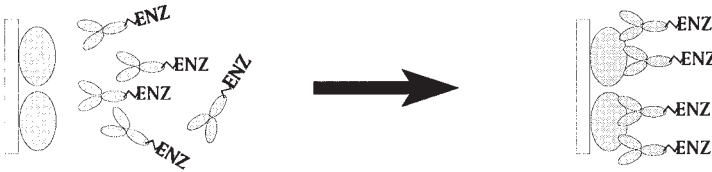
pretitrated labeled antibodies are mixed before adding to the antigen-coated plates.

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.

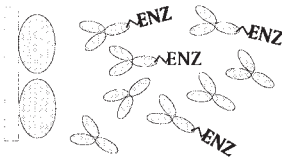
## A. Pre-titration of antigen and conjugate in Direct ELISA

Optimization of concentrations to be used in competition system



## B. Addition and incubation of antibodies to pre-titrated system.

## (i) Antibodies same as those of conjugate

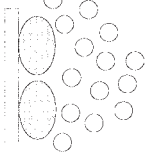


Unlabelled antibodies bind to antigen  
Conjugate is "blocked" and is washed away

## (ii)



(iii) No conjugate binds so that no colour develops on addition of chromophore substrate. This represents 100% competition for Direct system

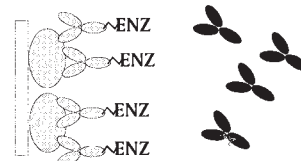


## (i) Antibodies different from the conjugate



Unlabelled antibodies do not bind to antigen  
Conjugate binds to solid phase antigen

## (ii)



## (iii)

Conjugate binding is unaffected, therefore no reduction in color is observed, this represents 0% competition for Direct system.

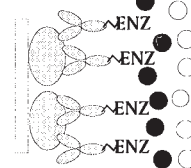


Fig. 6. Direct C-ELISA for antibody. The degree of inhibition by the binding of antibodies in a serum for a pretitrated enzyme-labeled antiserum reaction is determined.

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

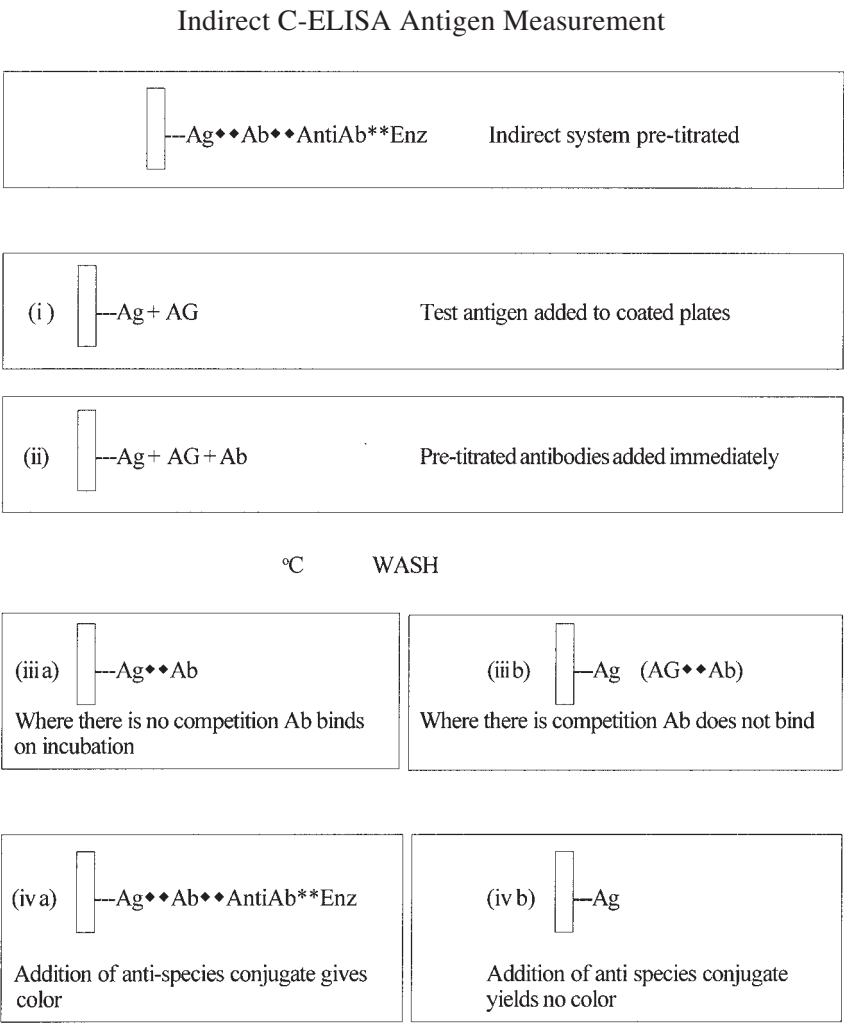
The test sample possibly containing antibodies specific for the antigen on the plates is added and incubated for a period. There are then two alternatives: (1) the wells can be washed and then the pretitrated labeled antibody can be added, or (2) pre-titrated labeled antibody can be added to the wells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

2.5.1. Indirect C-ELISA Antigen Measurement

Indirect C-ELISA antigen measurement is illustrated in the following diagram and in **Fig. 7**.



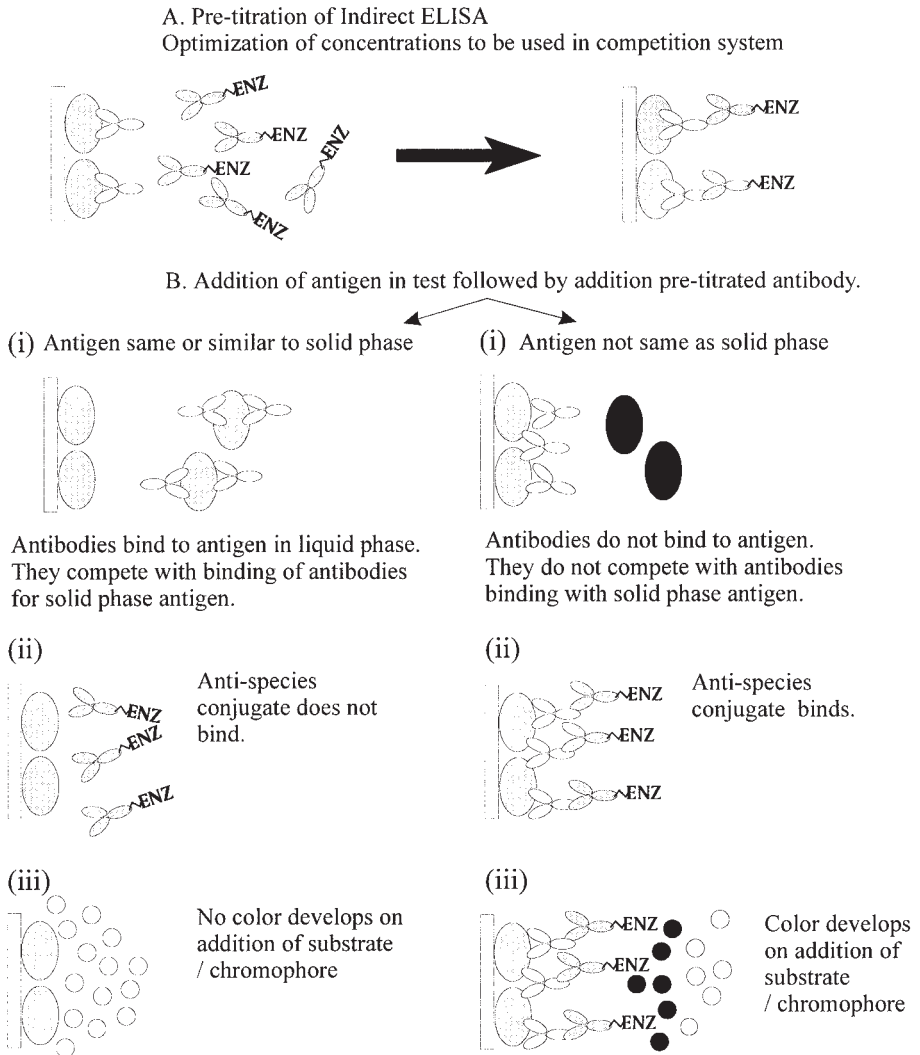


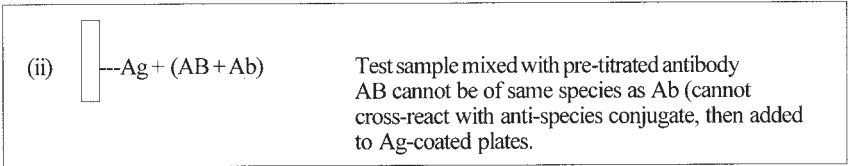
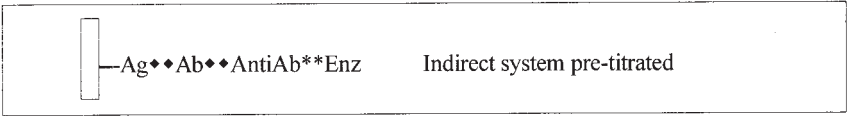
Fig. 7. Indirect C-ELISA antigen measurement. The degree of competition by the binding of antigens in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.

### 2.5.2. Indirect C-ELISA Antibody Measurement

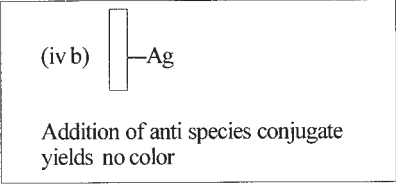
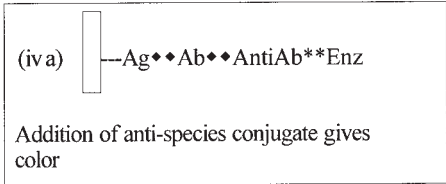
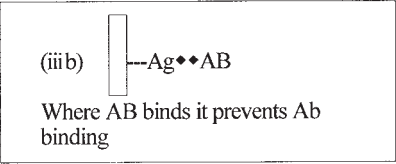
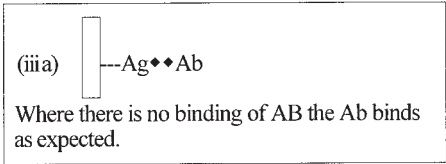
Indirect C-ELISA antibody measurement is illustrated in the following diagram and in **Fig. 8**.

Note that the same pretitrated system can be used for both antigen and antibody titration. The respective analytical sensitivities of the systems as adapted

Indirect C-ELISA Antibody Measurement



°C	WASH
----	------



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 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 dramatically affected through the use of different dilutions of sera (alterations in quality of serum depending on relative concentrations of antibodies against specific antigenic determinants).

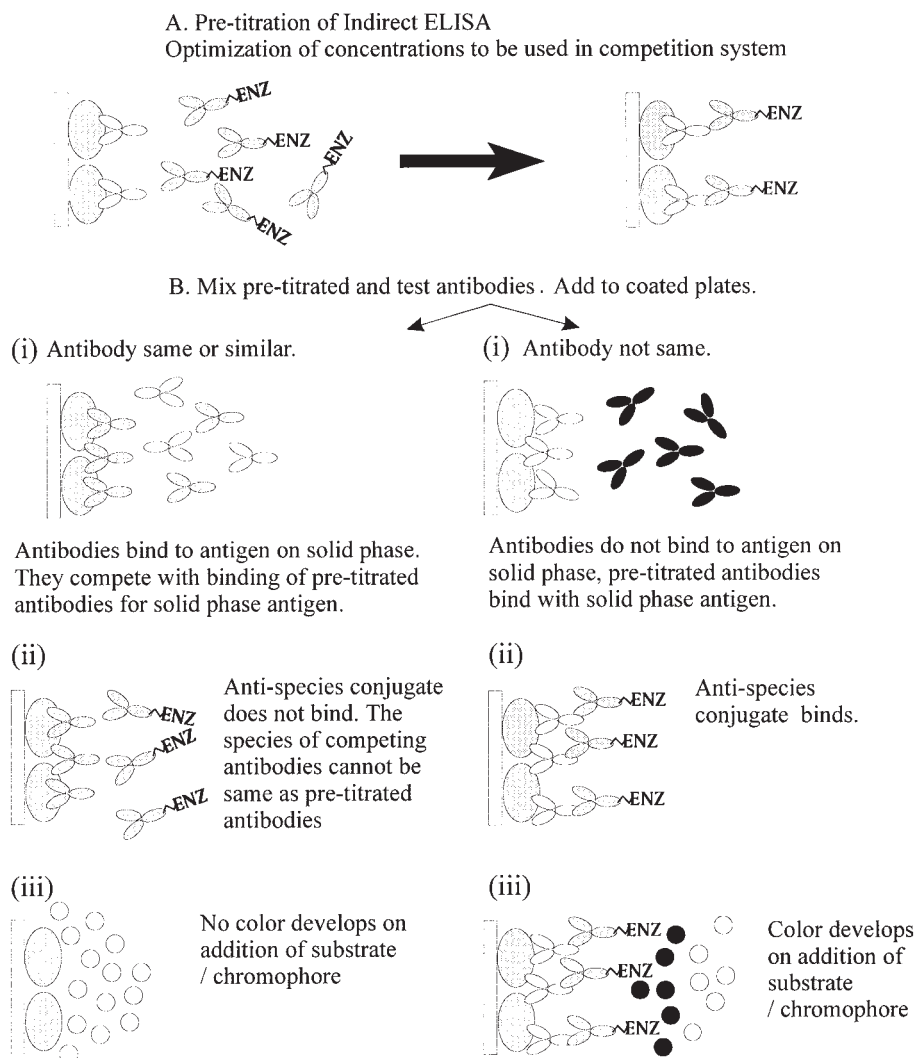


Fig. 8. Indirect C-ELISA antibody measurement. The degree of competition by the binding of antibodies in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.


### 2.5.3. 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 the following diagram.


Indirect I-ELISA Antigen Measurement


(i)	(AG + Ab)	Test sample mixed with pre-titrated antibody
-----	-----------	--


°C then add to antigen coated wells
-------------------------------------


(ii a)		Where no binding of AG the Ab is available for solid phase Ag
--------	---	---

(ii b)		Where AG binds then Ab not available
--------	---	--------------------------------------

(iii a)		Ab binds to solid phase Ag
---------	---	----------------------------

(iii b)		No binding of Ab
---------	---	------------------

(iv a)		Color (no inhibition)
--------	--	-----------------------

(iv b)		No color (inhibition)
--------	--	-----------------------

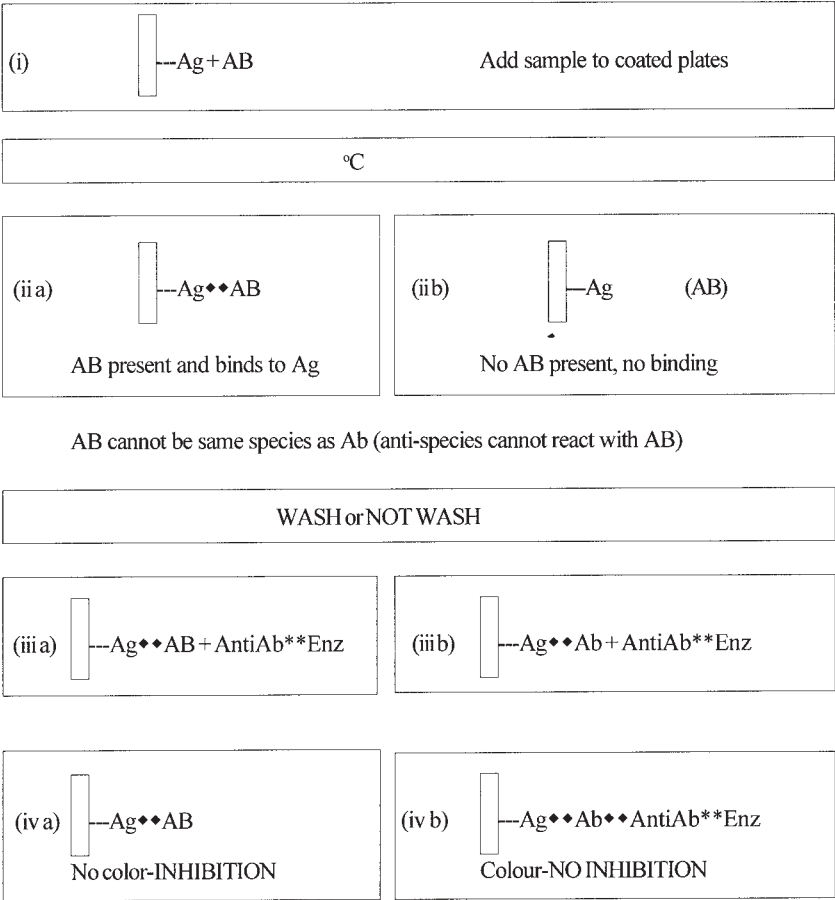
2.5.4. Indirect I-ELISA Antibody Measurement

Principles of indirect I-ELISA antibody measurement are shown dramatically 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 the following diagram. Once again the advantage of binding is afforded to the sample.

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 I-ELISA Antibody Measurement



indirect system the detecting antibody is not labeled, which is in turn detected using an antispecies conjugate.

Both systems are more complicated than those described previously in that there are more stages involved. Consequently, the possibilities for variation in competing or inhibiting steps are increased. Attention must be focused on why a certain system is used as compared to others.

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

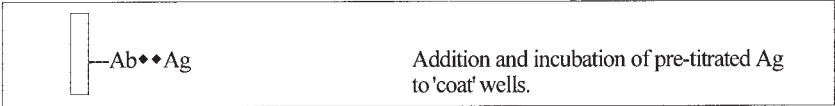


The assays are described under direct sandwich and indirect sandwich headings. Direct sandwich, involves assays utilizing a capture and a directly labeled detecting antibody (two antibody systems), and indirect sandwich involves assays utilizing three antibody systems (antispecies conjugate used to measure detecting serum). They are described for detecting antigen or antibody, as in 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.

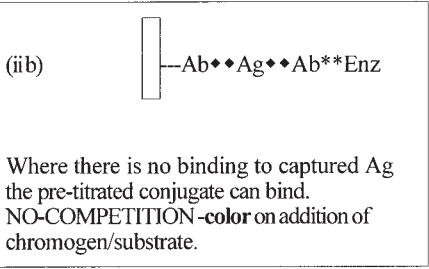
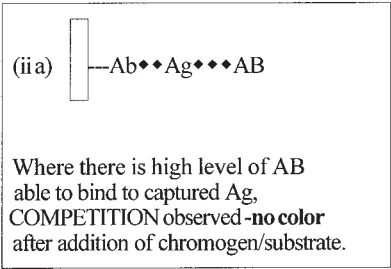
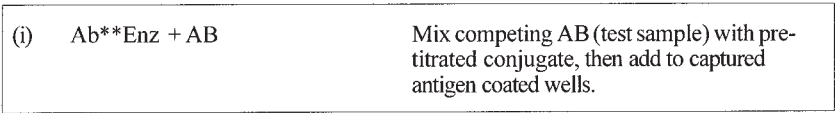
2.6.1. Direct Sandwich C-ELISA for Antibody

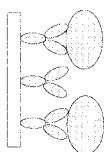
Direct sandwich C-ELISA for antibody is illustrated in the following diagram and in **Fig. 9**.

Direct Sandwich C-ELISA for Antibody

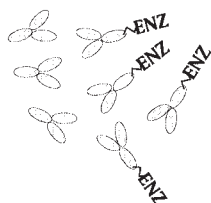


COMPETITION PHASE

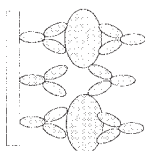




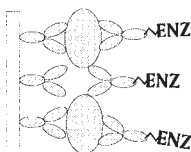
(i) System titrated to capture antigen. Detected by specific antibodies conjugated to enzyme.



(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.

### 2.6.2. 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 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.

### *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*

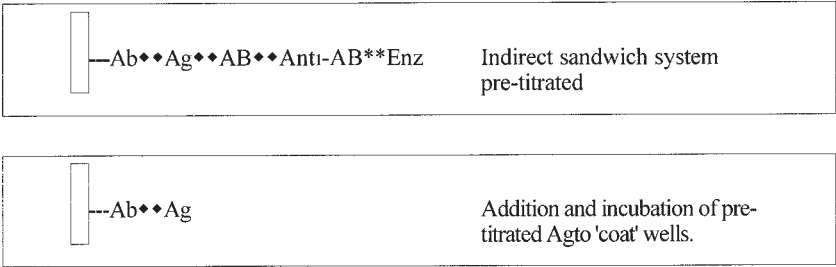
The reader should reexamine the components of the indirect sandwich ELISA. Here, as in the direct sandwich system, antigen is captured by antibodies bound to the wells. The difference is that the antigen is detected first with an unlabeled antibody, which, in turn, is detected and quantified using an antispecies conjugate. The exact time at which reagents/samples are added determines whether the system is truly examining competition or inhibition. The diagram on page 36 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 contained in the test sample. The degree of competition is proportional to the amount of antibodies contained in the test sample. The system offers greater flexibility in the use of different detecting antibodies (AB) for the captured antigen as compared to the direct sandwich assay. The system avoids having to produce 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.

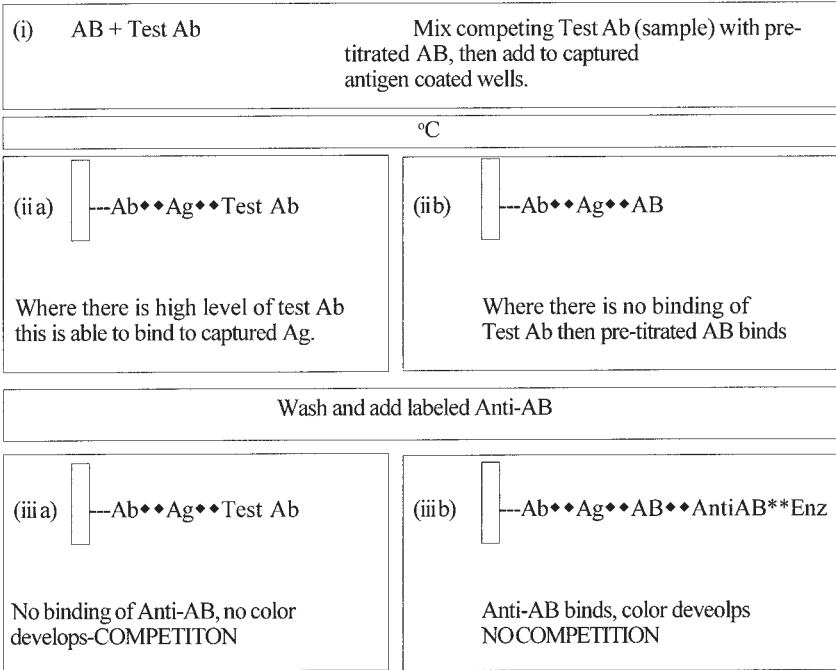
### *2.6.5. Indirect Sandwich I-ELISA for Antibody*

The indirect sandwich I-ELISA for antibody is similar to that for C-ELISA except that the time of addition of reagents is altered to allow a greater chance for reaction. This is illustrated in the diagram on page 37.

Indirect Sandwich C-ELISA for Antibody Addition on Reagents



COMPETITION PHASE




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 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 anti-

Indirect Sandwich I-ELISA for Antibody

(i)



Indirect sandwich system  
pre-titrated

(ii)



Addition and incubation of pre-  
titrated Ag to 'coat' wells.

INHIBITION PHASE

(iii)




°C

WASH OR NOT WASH

(iv)


Add pretitrated AB and °C

(v a)



Where test Ab binds, no AB binds

(v b)




Where no test Ab binds, AB binds

Addition of Anti-AB\*\*Enz

°C WASH


Add Substrate/chromophore

(vi a)



No binding of Anti-AB, no color  
develops-COMPETITION

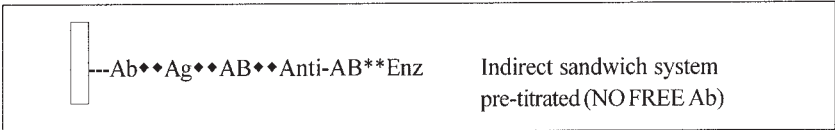
(vi b)



Anti-AB binds-color develops  
NO COMPETITION

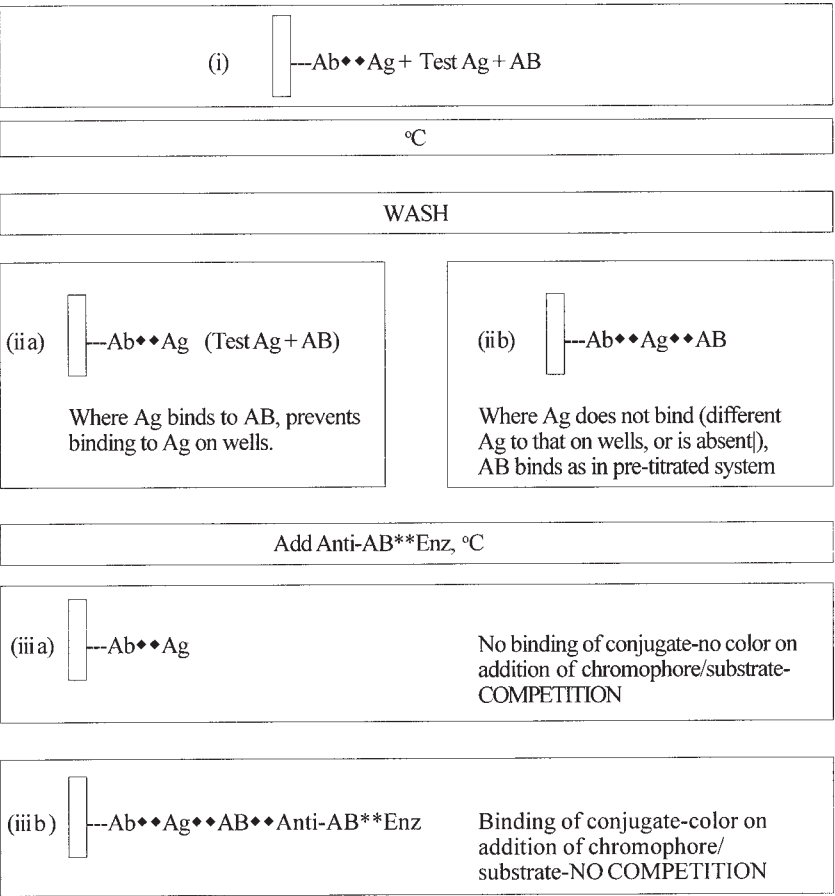
body assays examined in **Subheadings 2.6.4** and **2.6.5**. The antigen has to be in excess, as shown in the following diagram.

Indirect Sandwich C-ELISA for Antigen



The competitive phase occurs between the added test sample possibly containing antigen and the detecting second antibodies (AB), as shown in the following diagram.

Competitive Phase between Sample and Antibodies



### 2.6.7. Indirect Sandwich I-ELISA for Antigen

The indirect sandwich I-ELISA for antigen is essentially the same as for that for C-ELISA except that the AB and test antigen are mixed and incubated separately before addition 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, 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 about or very little.
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 an 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 antispecies 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 which 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 to 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 1/1000. The test volume used is 50  $\mu$ L. Therefore the maximum number of samples that can be run as single tests is  $30 \times 1000 \times 20 = 600,000$ .

This may be enough for universal testing for 10 laboratories (60,000 samples per year) for one year, or if it runs tests on 6000 samples a year, the reagent is satisfactory for 10 yr. 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 also should 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 in order 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.

1. Scenario A
  - a. Crude antigen (multiple antigenic sites)
  - b. Antibody raised against crude antigen in rabbits
  - c. Anticow conjugate
  - d. Postinfected and d 0 (uninfected) cow sera
2. Scenario B
  - a. Purified antigen (small amount, e.g., 100  $\mu$ g)
  - b. Crude antigen (large amount)
  - c. Antibody raised in rabbits against pure antigen
  - d. Antirabbit conjugate
  - e. Anticow conjugate
  - f. Postinfected and d 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. Antiginea pig conjugate
  - e. Postinfected and d 0 (uninfected) cow sera
  - f. Anticow conjugate
  - g. Antirabbit conjugate

### 2.7.2.1. 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.

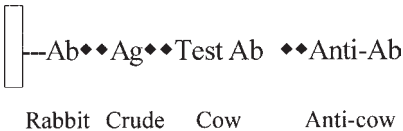
Any bound test antibody would be from cows and thus detected using an antbovine conjugate. This may cause problems since the crude antigen was used to raise the rabbit serum. Hence, antibodies against contaminating proteins may be produced in the rabbit. The cow sera being tested may react with such 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 antbovine antibodies is produced as well as antiviral antibodies. 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 nega-

tive sera allows assessment of the difference between negative and positive sera at different dilutions of serum. The following diagram 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.

2.7.2.2. 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 above.

The availability of the antirabbit conjugate may allow 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/antirabbit 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.

2.7.2.3. 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 antispecies 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 to cattle sera.

#### 2.7.2.4. 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.



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