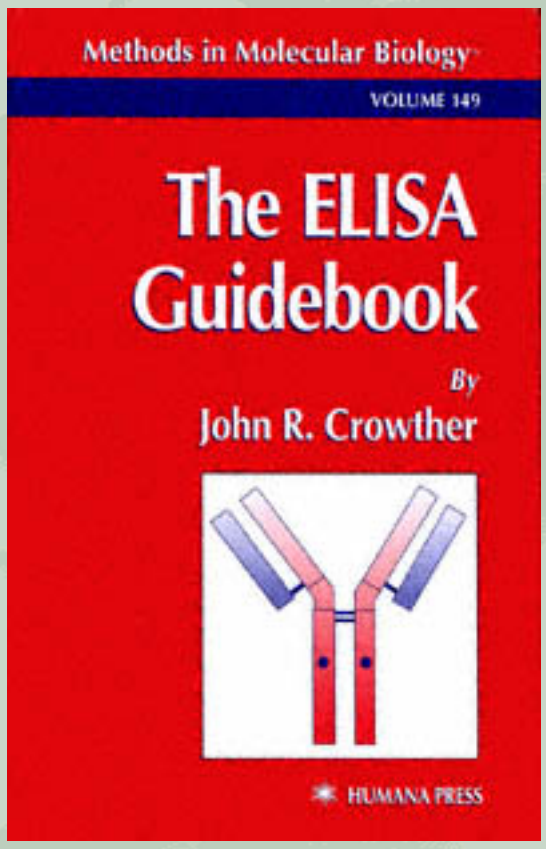


The ELISA Guidebook

By
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The International Atomic Energy Agency, Vienna, Austria

METHODS IN MOLECULAR BIOLOGY™

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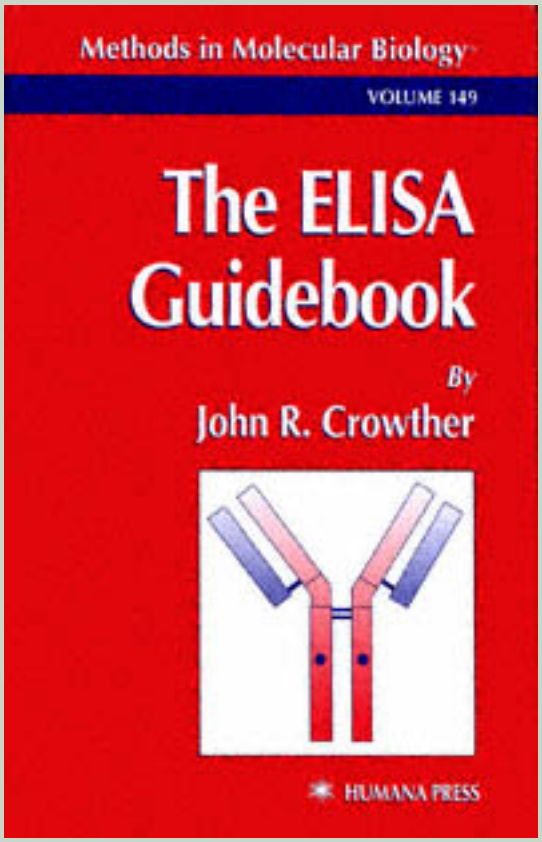
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Preface

The aim of *The ELISA Guidebook* is to expand the information concerning enzyme-linked immunosorbent assay (ELISA) published in *ELISA: Theory and Practice* by J. R. Crowther (1995), in the *Methods in Molecular Biology* series by Humana Press (vol. 42). The earlier book concentrated on the immunological background of the reagents exploited in such assays, and dealt practically with the various assays, through examples using noninfectious systems. This new volume is a major extension and updating of that book, with a reorganization of the chapters, and extra information dealing, in particular, with chessboard titration of reagents, quality control, monoclonal antibodies, validation of assays, statistics, and epidemiological considerations. Suitable for scientists with previous experience of the technique, it can, however, be used successfully by those with little experience, and as a teaching aid.

The ELISA Guidebook deals with heterogeneous enzyme-linked immunosorbent assays. The abbreviation ELISA, or in the plural ELISAs, will be used from now on to denote this kind of assay. Besides the inherent feature of all ELISAs¡ªthat there is an enzyme linked to one of the reagents¡ªheterogeneous assays involve the attachment of one reagent to a solid phase and subsequent addition of reagents that bind. The separation of bound and free components is necessary through washing steps. Such assays must be distinguished from homogeneous ELISAs, in which reagents are added simultaneously.

ELISAs remain the mainstay of testing in which the specificity inherent in antibodies is exploited. The technique is still expanding in all fields of pure and applied biology, and in particular, now constitutes a backbone diagnostic technique. Recent applications into quality assessment of foods for contaminants is testimony to the flexibility for these possible systems. There is an increasing use of automated systems in commercial applications of ELISA; however, there is still a major use for more manual techniques in the development of assays, and for routine use in laboratories with lesser facilities. A thorough understand-

ing of the principles is vital to the proper use of ELISA, even where established kits are provided.

The key to all ELISA systems is the 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 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.

Besides covering the various assay parameters, the basic reagents, and the skills needed to perform ELISA, *The ELISA Guidebook* introduces these increasingly important topics: quality control of testing; kit production; validation; statistical requirements for examination of data and for epidemiological studies; equipment choice, care, and calibration; technology transfer; and monoclonal antibodies. Wherever possible, explanations are provided in diagrammatic, as well as written, form. The text may, in places, seem repetitious. However, in the experience of the author, and through feedback from the previous publication, readers respond very differently to various approaches, so that conveying information by multiple exposures is considered pedagogically useful.

Although often reviewed, it is worth considering the beginnings of ELISA, which stemmed from investigations of the ability of enzyme-labeled antibodies ***(1¨C3)*** to identify antigens in tissue. The methods of conjugation were exploited to measure serum components in the first "true" ELISAs ***(4¨C6)***.

By far the most exploited ELISAs use plastic microtiter plates in an 8 ¡Á 12 well format as the solid phase ***(7)***. Such systems benefit from a large selection of specialized commercially available equipment including multichannel pipets for the easy simultaneous dispensing of reagents and multichannel spectrophotometers for rapid data capture. There are many books, manuals, and reviews of ELISA and associated subjects that may be examined for more practical details ***(8¨C21)***. The following table summarizes some of the features that make ELISA so sustainable a technique.

Advantages of ELISA

1. Simplicity	(a) Reagents added in small volumes
	(b) Separation of bound and free reactants is made by simple washing procedures
	(c) Passive adsorption of proteins to plastic is easy
	(d) Specialized equipment readily available
2. Reading	(a) Colored end-product can be read by eye to assess whether tests have worked (avoiding waiting for results where machine reading essential as in RIA)

	(b) Multichannel spectrophotometers quantify results that can be examined statistically
3. Rapidity	(a) Tests can be performed in a few hours (b) Spectrophotometric reading of results is rapid (96 wells read in 5 s)
4. Sensitivity	Detection levels of 0.01 to 1 µg/mL are easily and consistently achievable. These levels are ideal for most diagnostic purposes
5. Reagents	Commercially available reagents offer great flexibility in ELISA design and achievement of specific assays
6. Adaptability	Different configurations allow different methods to be examined to solve problems. This is useful in developing tests and research science
7. Cost	(a) Startup costs are low (b) Reagent costs are low
8. Acceptability	Fully standardized ELISAs in many fields are now accepted as "gold-standard" assays
9. Safety	Safe nonmutagenic reagents are available. Disposal of waste poses no problem (unlike radioactivity)
10. Availability	ELISAs can be performed anywhere, even in laboratories where facilities are less than state of the art
11. Kits	ELISA kits are widespread and successful
12. Standardization	Quantification of data allows easier standardization

All the key elements listed will be examined in detail in this book. The background needed in immunologic/serologic aspects is not dealt with extensively as a discrete chapter, rather points are included at appropriate times. Scientists involved in developing and using ELISA should be familiar with the concepts inherent in immunology. There are several excellent textbooks, including Roitt and colleagues (22), that should be read. Immunochemical methods are also important, e.g., in purifying and exploiting antigens and antibodies, and for conjugat-

ing proteins. An excellent manual covering all aspects of immunochemistry is available [Harlow and Lane (23)], which also outlines many relevant laboratory practices.

JOHN R. CROWTHER, PHD

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1¡ª
Overview of ELISA in Relation to Other Disciplines

This chapter examines what areas of science are needed to allow optimal use of ELISA and notes their relationships. This information is useful for students and those instructing students. Diagrams, with brief descriptions of key points, are used to illustrate such relationships. Inherent in this exercise are considerations of the exact requirements by the operators in using the ELISA. Attention to increasing knowledge in those areas highlighted is essential both in developmental work to produce a working ELISA and in the ultimate value of any test devised. A good deal of attention should be directed at defining, as clearly as possible, the objectives for the ELISA. The development of a diagnostic test for a specific disease requires that all other data pertaining to the biology of that disease, e.g., antigenicity and structure of the agent, antibody production in different animals following infection, qualitative assessment of antibodies by different assays, and availability of standard or control sera, are known. Some attention must be paid to the laboratory facilities available, e.g., equipment, reagents already developed, small laboratory animals, experimental large animals, cash to buy commercial products, and trained personnel. In this way, the chances of producing a sustainable test to solve the defined problem are significantly greater than when a test is developed by a dabbling technique with poor or no forward planning.

Figure 1 emphasizes that we are considering the heterogeneous ELISA involving separation steps and a solid phase. Four major advantages of ELISA are promoted, all of which add to the reasons that this form of ELISA has been, and will continue to be, successful.

Figure 2 deals with the systematics of the ELISA and shows the various stages needed and factors important in those stages.

Figure 3 emphasizes that using the equipment to perform ELISAs requires skills, and that both physical and mental processes are needed. **Figure 3** also indicates that instruments need to be maintained for optimal performance.

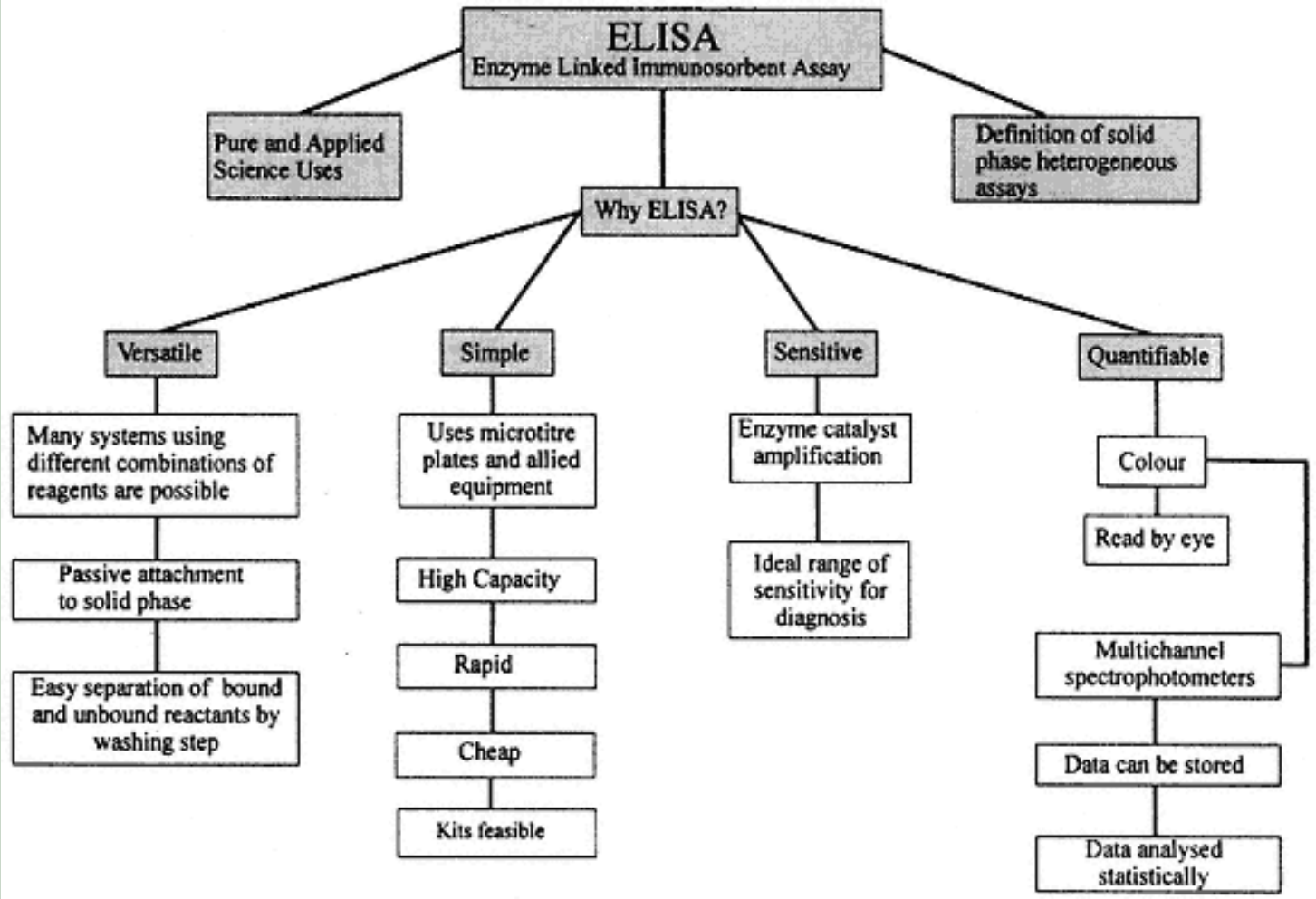


Fig. 1.
Scheme showing features of ELISA that make it advantageous for a wide range of applications.

Figure 4 deals with some of the enzymatic systems in the ELISA, and illustrates areas that need to be understood in order to allow optimal performance to be maintained. Understanding enzyme kinetics, catalysis reactions, hazards, and buffer formulation (pH control) are all essential.

Figure 5 illustrates the use of ELISAs in binding and inhibition/competition interactions to allow an understanding of a problem. It is essential that the chemical and physical nature of antibodies and antigens are understood, particularly in cases of developmental work. As full an understanding of the antigenic properties of agents being examined is needed to allow maximum exploitation of ELISA, particularly if the results are ever to be understood.

Figure 6 deals with data processing and analysis. Various essential statistical parameters must be elucidated, if data are to be interpreted. This is true in understanding how to calculate the variance in a result, and also for examining populations. Such studies actually define any ELISA's performance, allowing confidence in results to be measured, thereby allowing a meaning to be placed on results. The concepts of controlling assays with references to standards is also needed.

Figure 7 extends the use of statistical understanding into epidemiological needs. A common use of ELISA is to provide data on populations studied. The

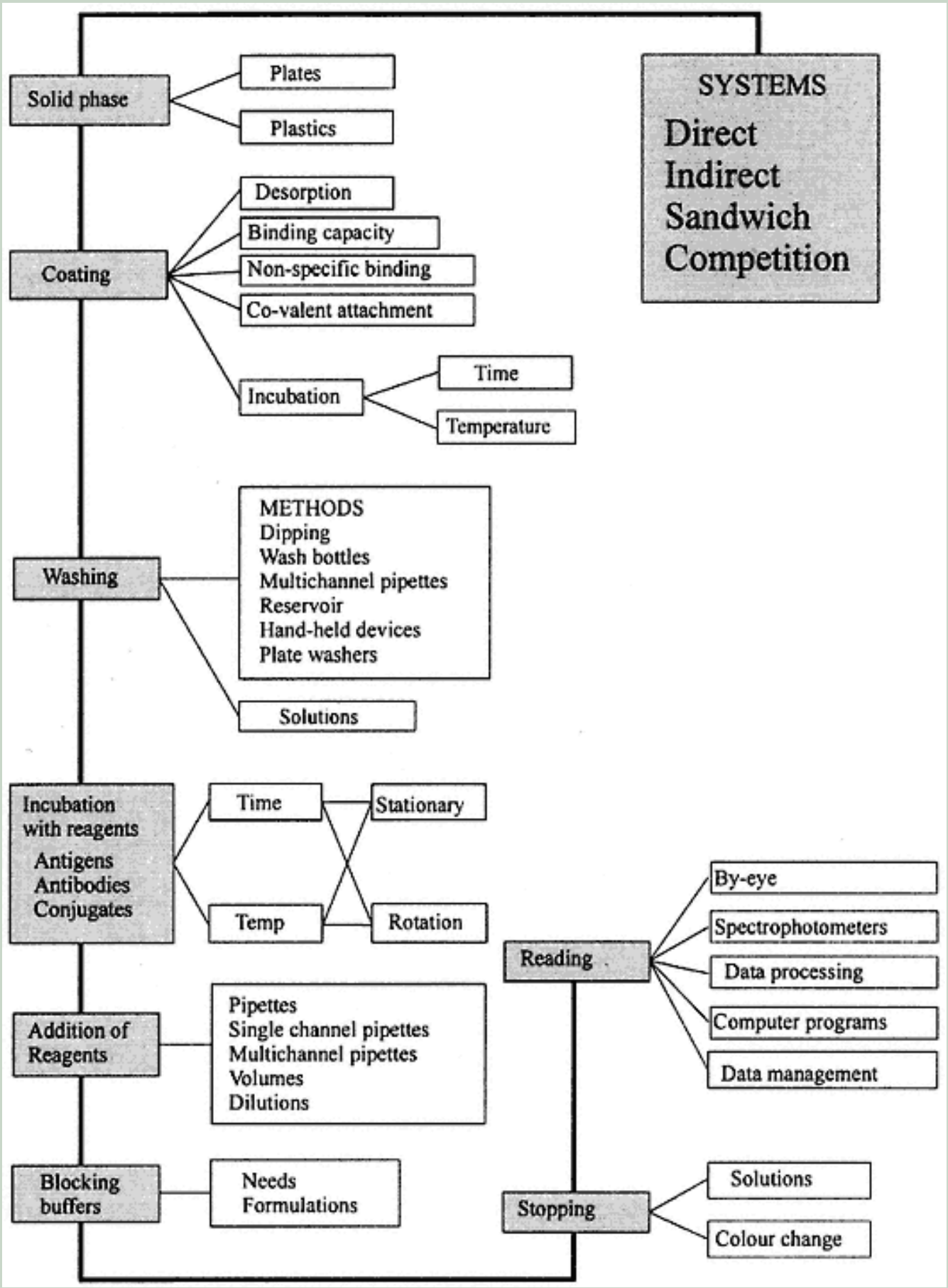


Fig. 2.
Scheme relating stages in ELISA. Specific stages vary according to the system utilized.

areas of sampling (size, number, and so forth) are vital when planning disease control strategies.

These simplified overviews should be used as reference points when considering the development and specific use of any ELISA. They should help

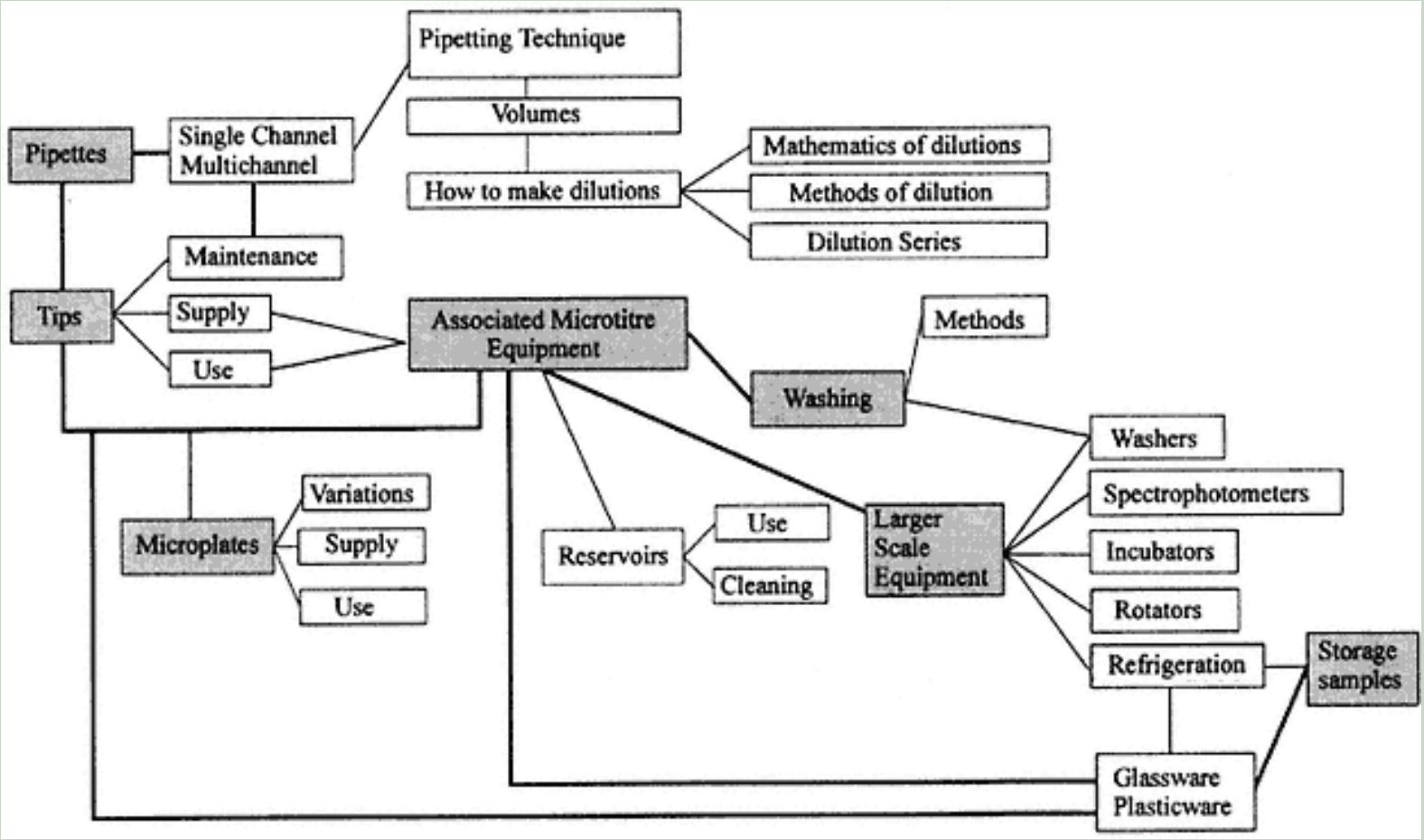


Fig. 3.
Scheme relating equipment needs and skills for ELISA.

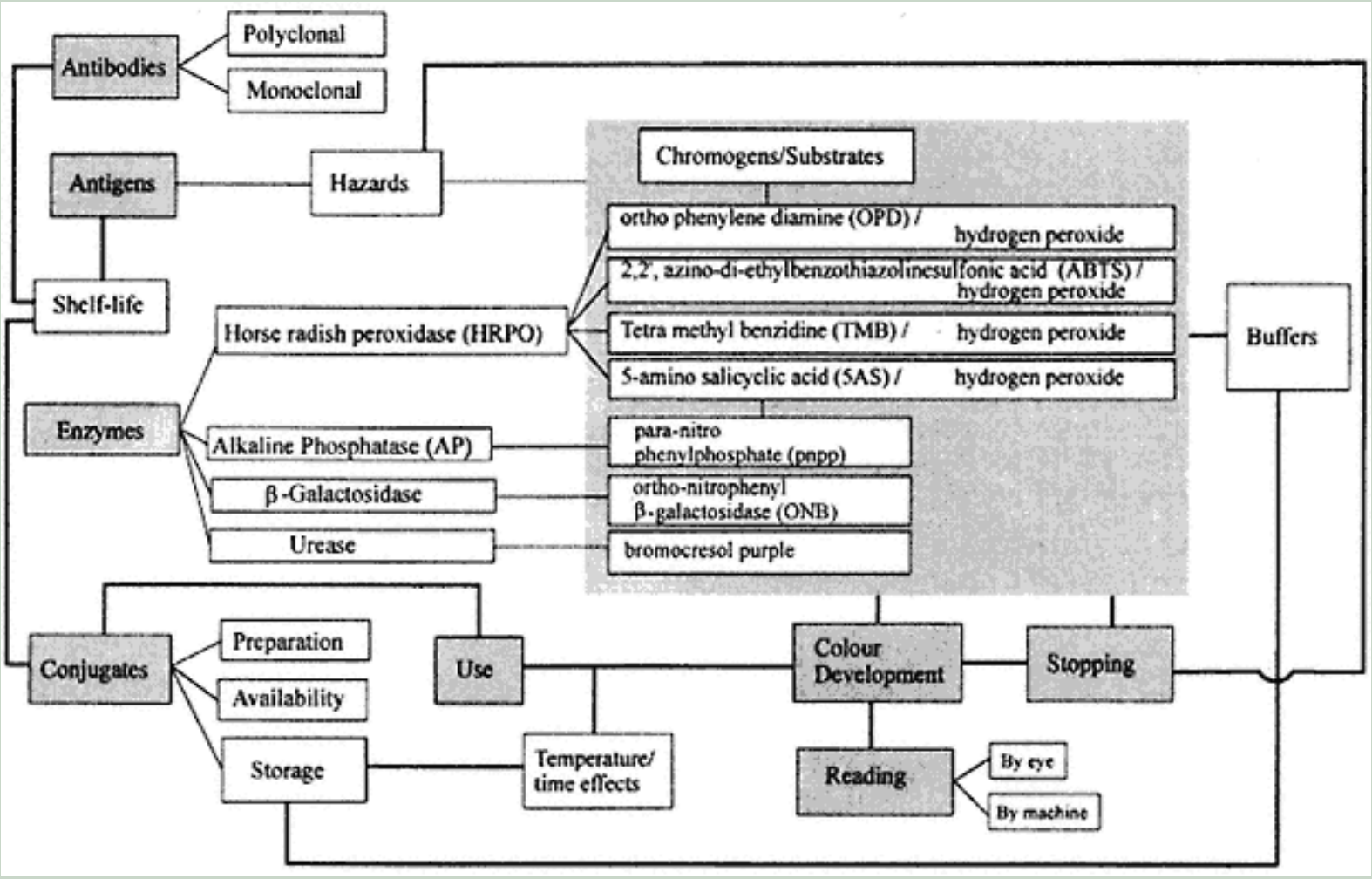


Fig. 4.
Relationships of enzyme systems to components of ELISA.

readers with limited exposure to ELISA, particularly after studying the details in later chapters. They are also useful for trainers in establishing areas of competence in students.

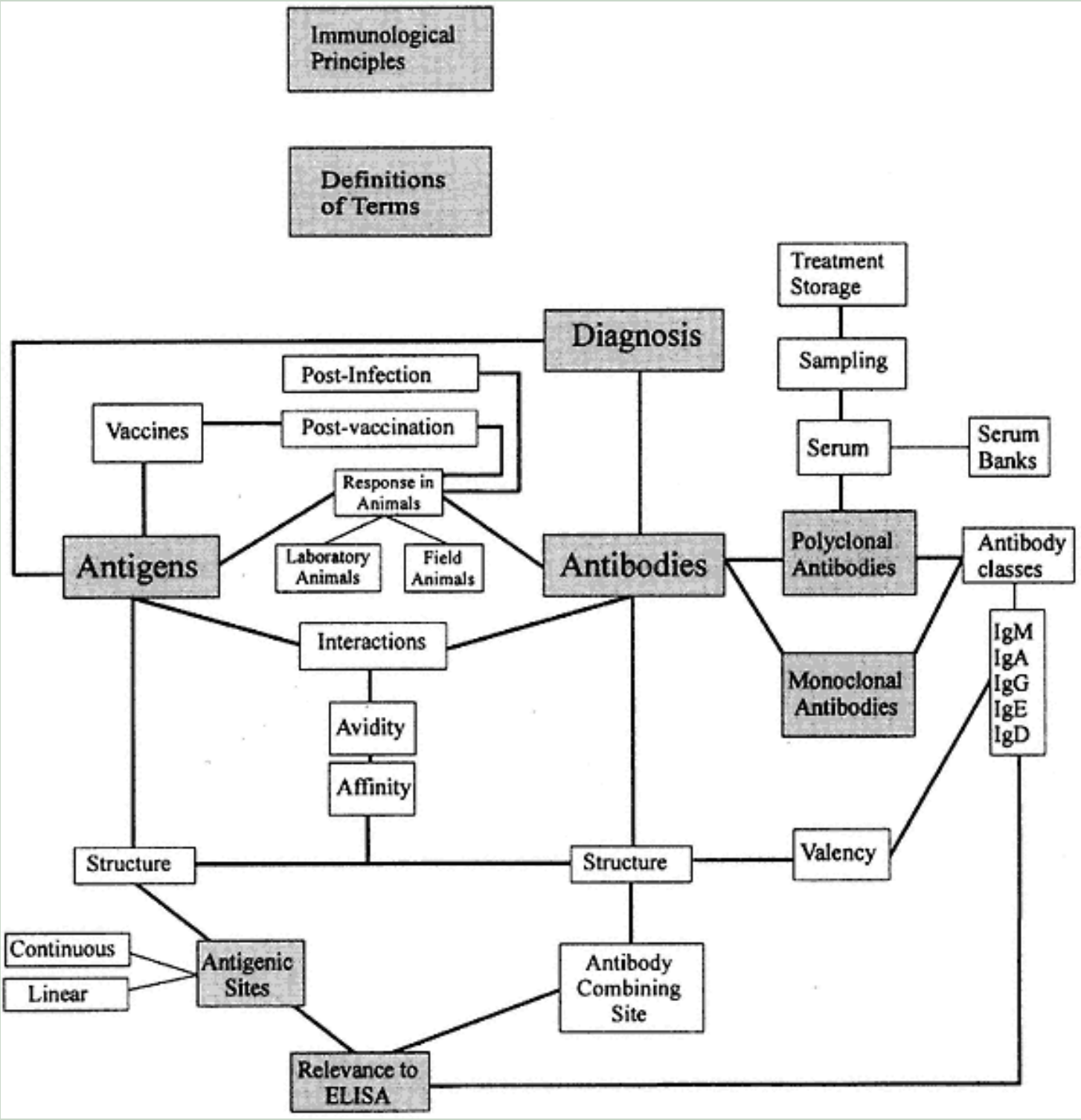


Fig. 5.
Requires features in immunological understanding in order to establish ELISA.

These are the key points to keep in mind at this early stage when considering then use of ELISA:

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

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

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

4. The capacity for testing has to be addressed. For example, when an ELISA may be used on a large scale (kit), then sufficient reagents, standard sera, conjugates (batches), and antigen preparations must be available. Research leading to suc-

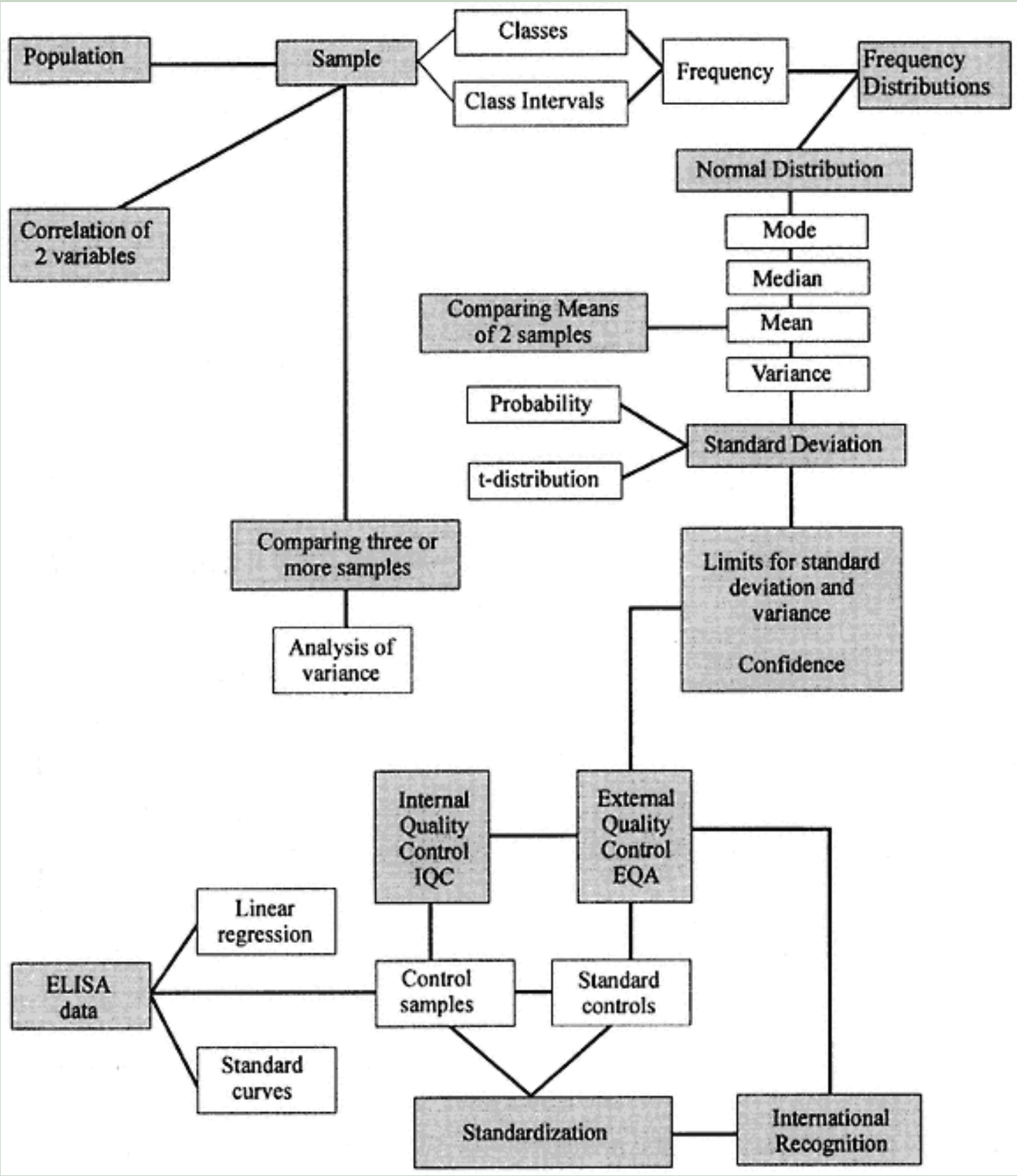
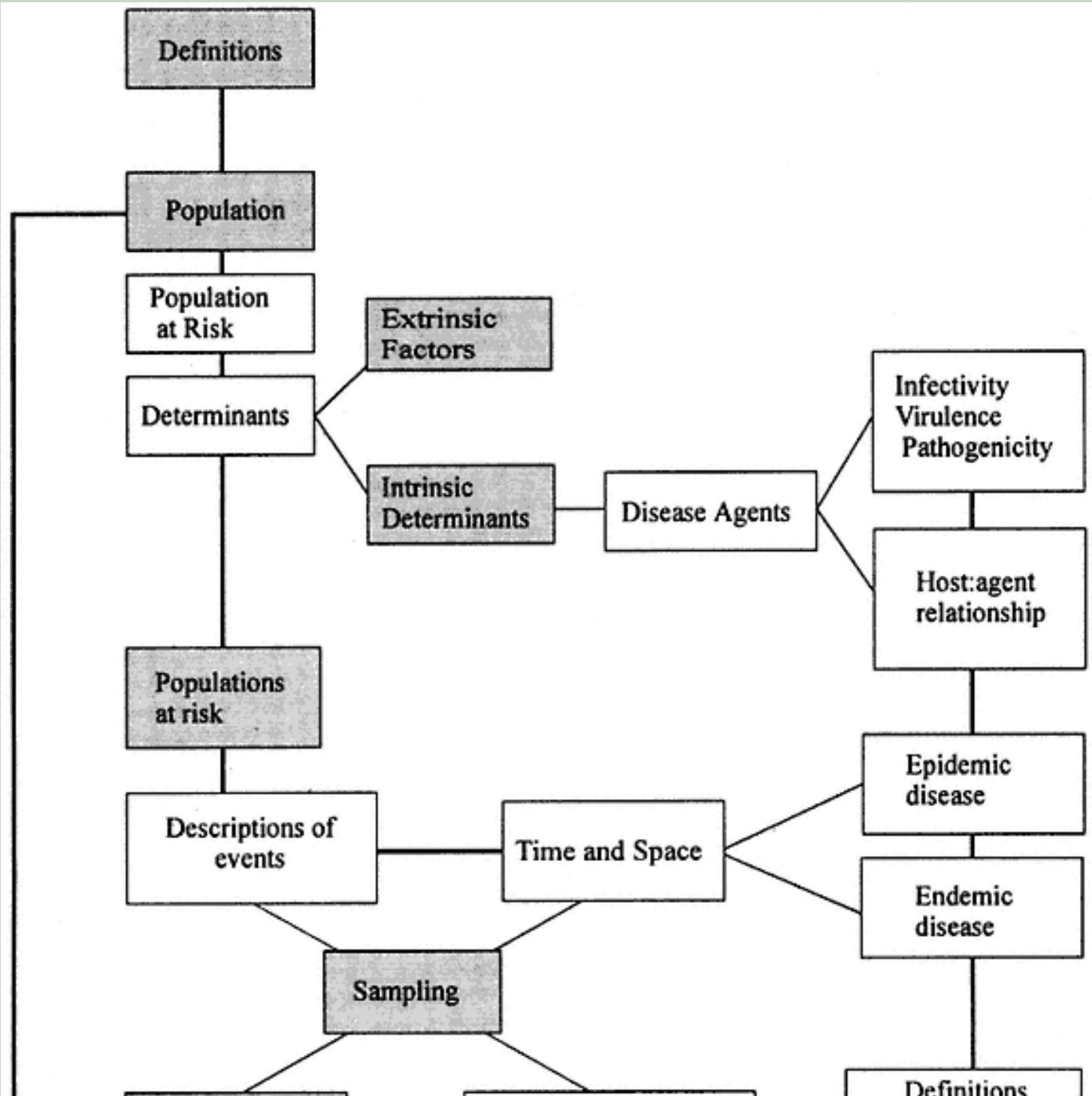


Fig. 6.
Important statistical factors needed to make use of ELISA. Note the links to quality control (internal) and the establishment of confidence in test results. Increasingly, assays need international recognition.

cessful assays in which reagents are difficult to prepare on a large scale, require extensive expertise to formulate, or are reliant on a specific limited batch of a commercial reagent are not sustainable.

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



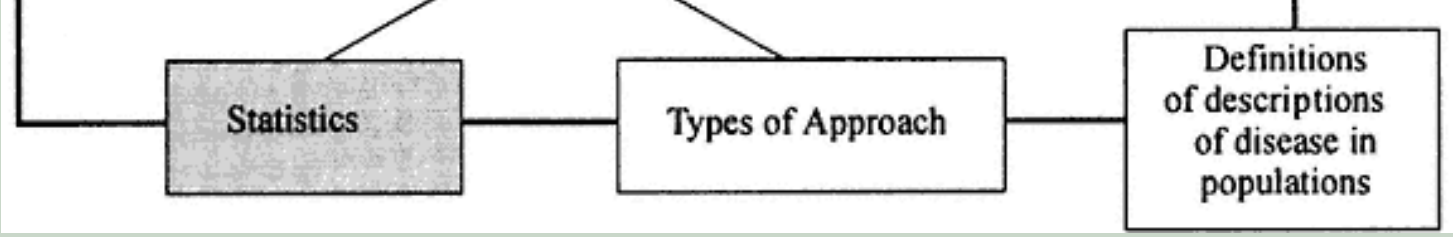


Fig. 7.
Scheme relating basic areas in epidemiology that need to be understood in the context of data obtained from ELISA. Note the strong link with statistics/sampling, which is inherent in the test design.

The knowledge and skills required to both perform ELISA and make use of the data have to be gained through a variety of sources, including textbooks. As with all other techniques, the ultimate benefit is not the technique in itself, but the meaningful gathering and analysis of the data. One factor not included in all these examples is that of common sense: the ability to really consider what one is doing, and why, and not to overlook the simplicity of what is needed by being blinded by the technology for its own sake. Most problems are relatively simple to examine after some clear thought. Thus, the good ELISA

person will consider the problem first, obtain the necessary technical skills and equipment to perform a test, and then obtain data that is from a planned perspective. As much data from all other tests and the scientific literature should also be sought. This is true for an assay developer, as well as a person using a supplied, predetermined kit. The skills required by the use of a kit are no less than those of the developer; indeed, a kit in the hands of an unskilled worker is often useless. The majority (90%) of problems observed in the practice of ELISA are operator faults caused by lack of common sense, failure to appreciate the need to stick to instructions, sloppy technique, or poorly maintained equipment. Most of the remaining percentage is caused by poor-quality water.

2¡ª
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 ¡Á 12-well format.

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

3. Results are obtained through the development of color.

1¡ª
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¡ª
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 ¡Á 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¨Cguinea 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

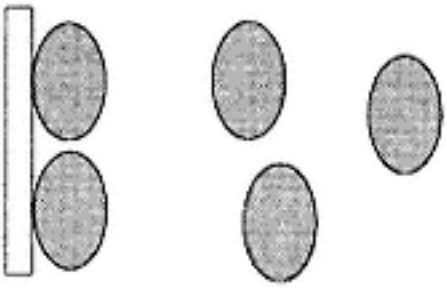
2. Indirect ELISA

3. Sandwich ELISA

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

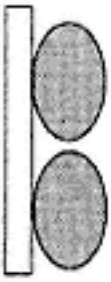
The systems (arrangement and use of reagents in the test), are illustrated herein through the use of symbols (as defined in **Table 2**), as well as in terms. In this way, it is hoped that the reader will gain a clear idea of the various systems and their relative advantages and disadvantages. A key 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¡[a]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.

(i)



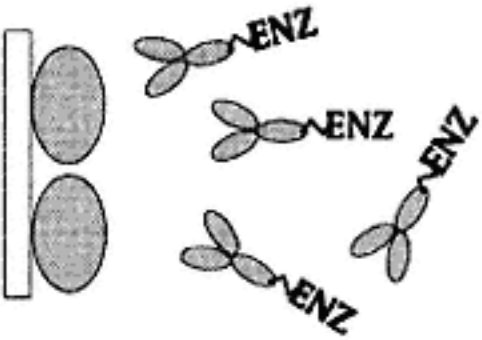
Antigen is added to the solid phase and adsorbs passively on incubation.

(ii)



After incubation, any non-bound antigen is washed away leaving the 'coated' solid phase.

(iii)



Antibodies specific for the antigen and labeled with an enzyme (conjugate) are added, and incubated.

(iv)



The conjugate binds with antigen on solid phase.

Any unbound (free) conjugate is washed away.

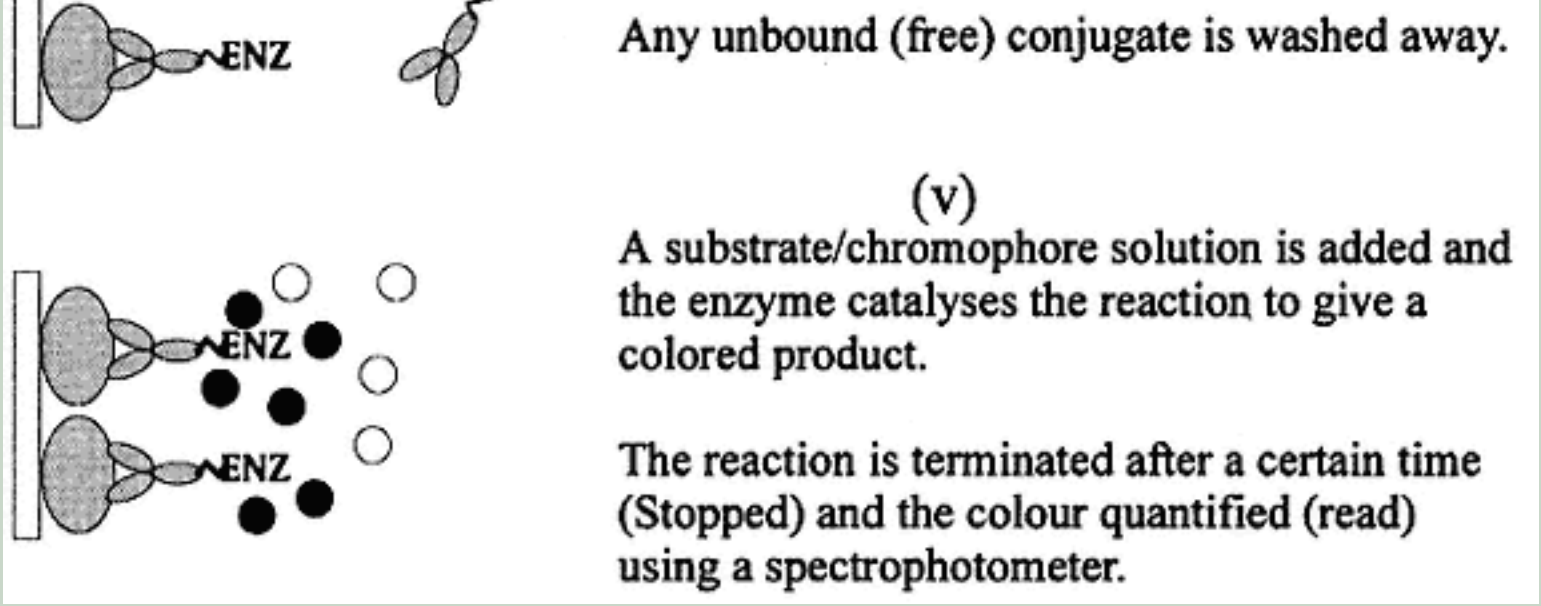


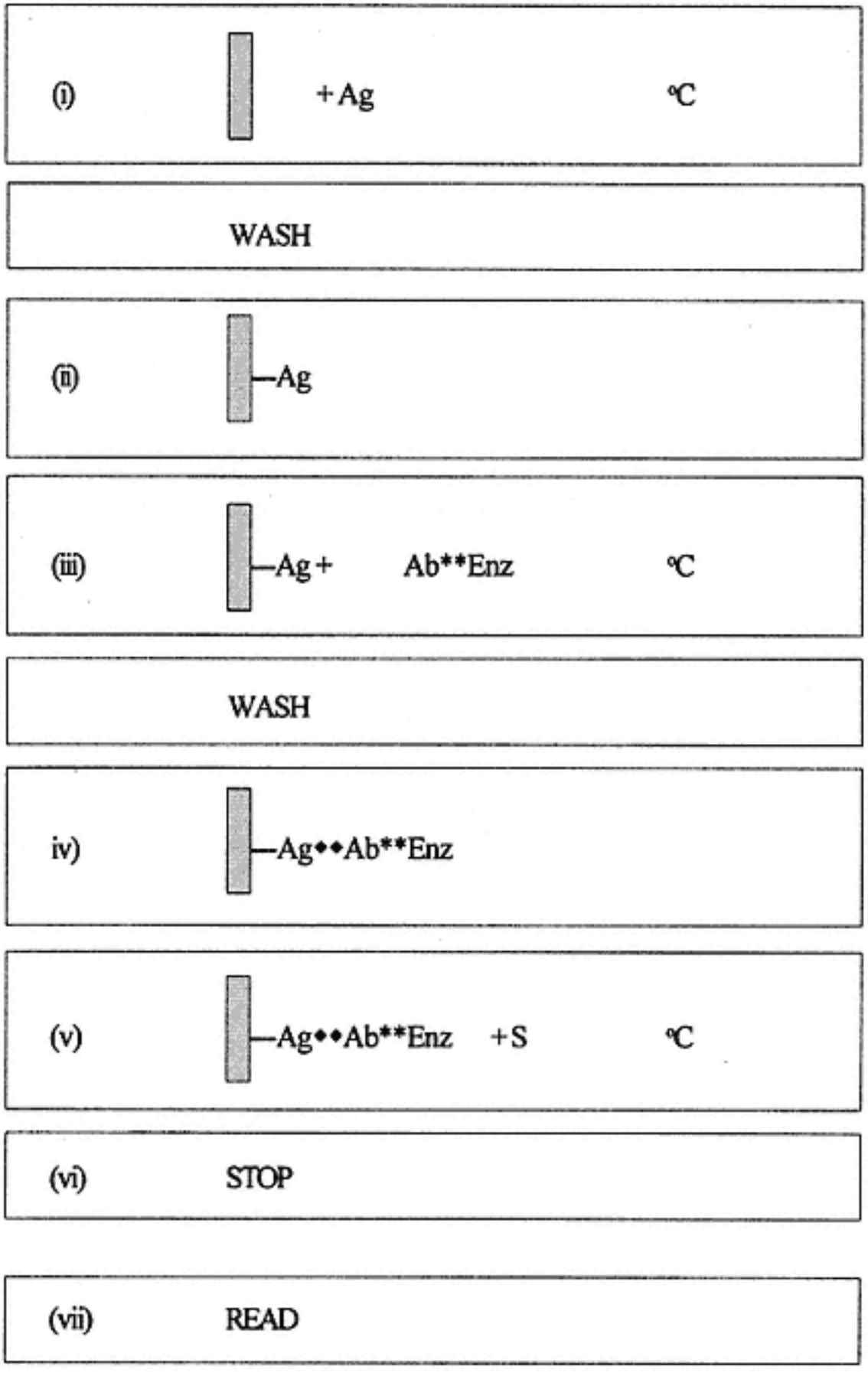
Fig. 1.
Direct ELISA. Antigen is attached to the solid phase by passive adsorption. After washing, enzyme-labeled antibodies are added. After an incubation period and washing, a substrate system is added and color is allowed to develop.

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¡ª
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¡ãC 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¨Cbound 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.*

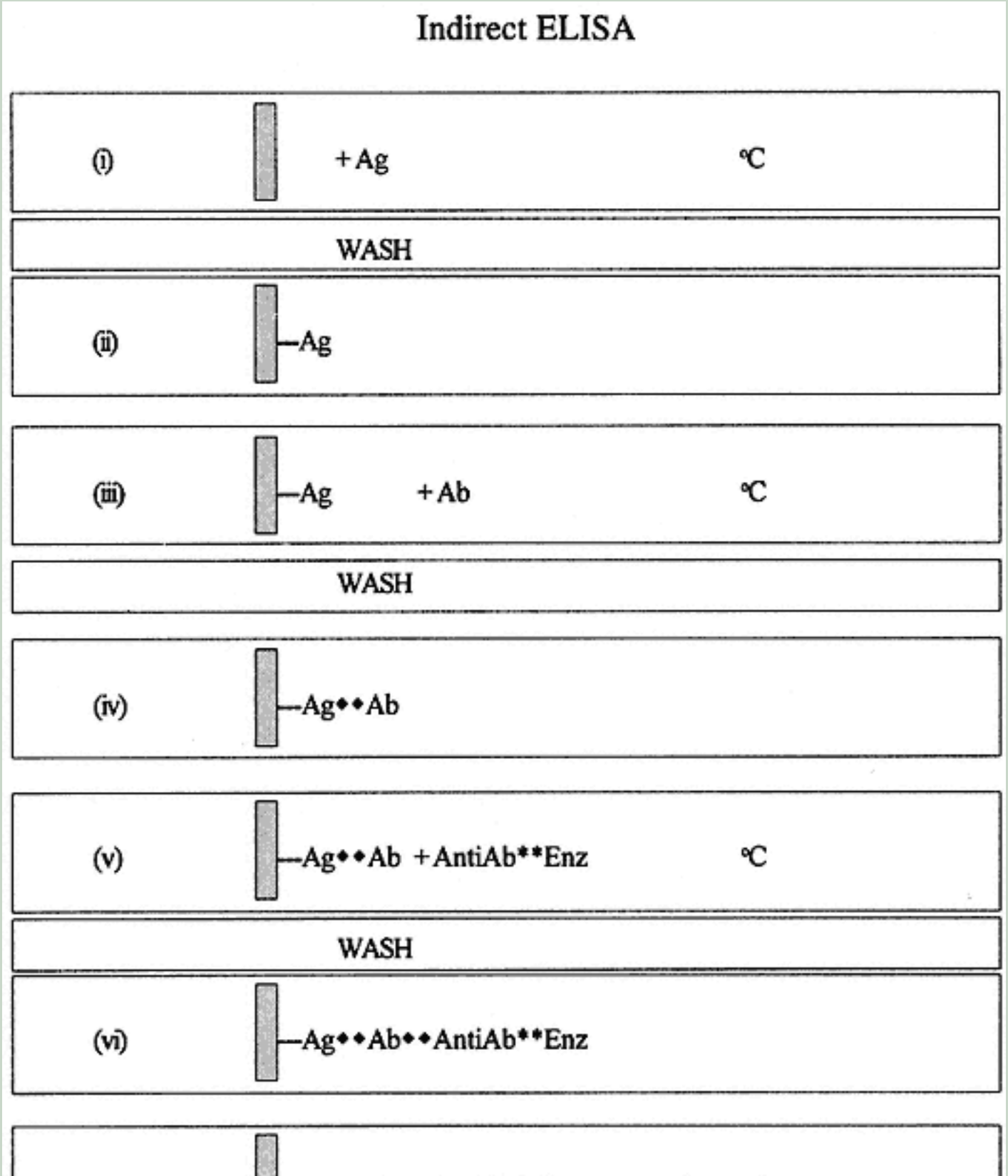
On incubation, antibodies bind to the antigen. Again, a simple washing step is then used to remove unbound antibodies (stage iv). Stage v involves the addition of a suitable substrate or substrate/chromogen combination for the particular enzyme attached to the antibodies. The objective is to allow development 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¡ª
Indirect ELISA

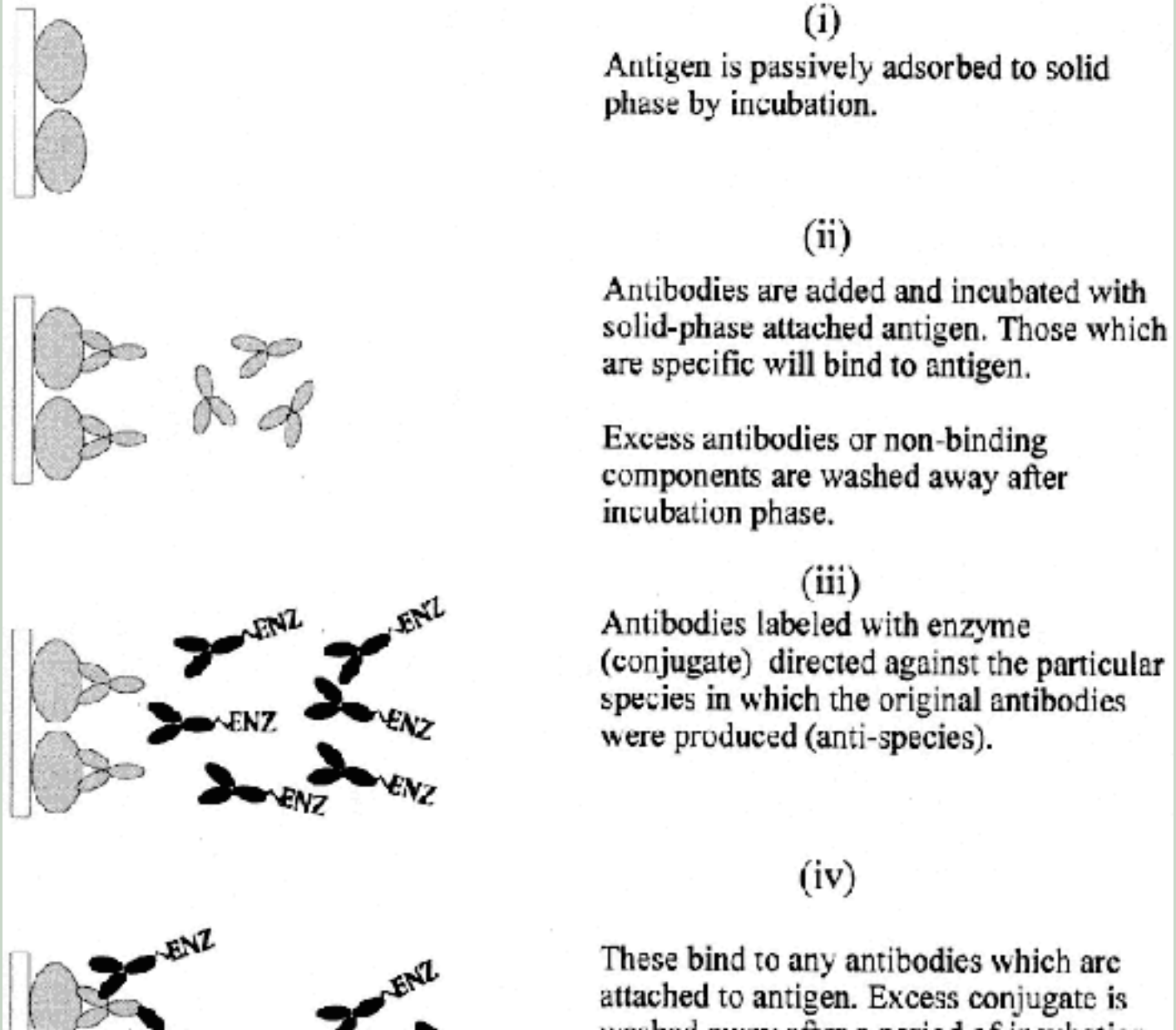
Indirect ELISA is illustrated in the following diagram and in **Fig. 2**. Stages i and ii are similar to the direct system. Stage iii involves the addition of unlabeled detecting antibodies, which are diluted in a buffer to prevent nonspecific attachment of proteins in antiserum to solid phase (blocking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



(vii)	—Ag••Ab••AntiAb**Enz	+S ℃
(viii)	STOP	
(ix)	READ	

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_1, IgG_2, and so on.



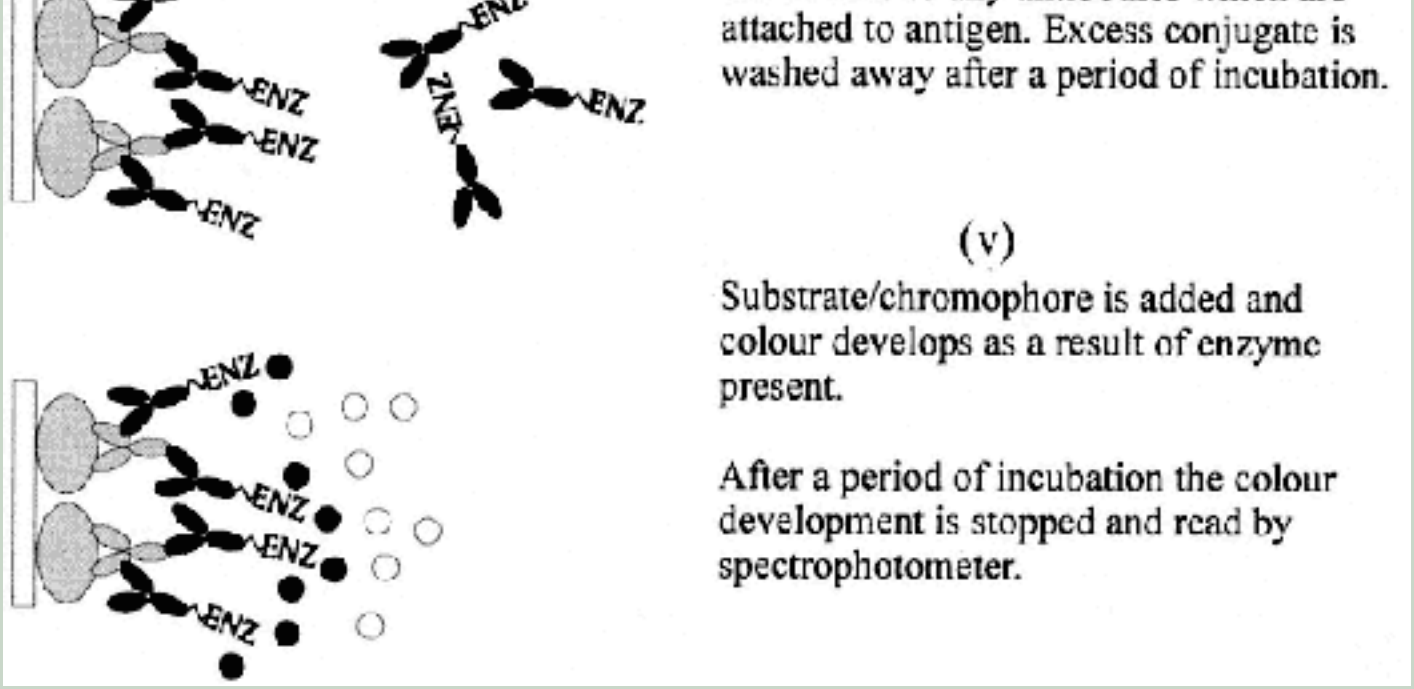


Fig. 2.
Indirect ELISA. Antibodies from a particular species react with antigen attached to the solid phase. Any bound antibodies are detected by the addition of an antispecies antiserum labeled with enzyme. This is widely used in diagnosis.

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

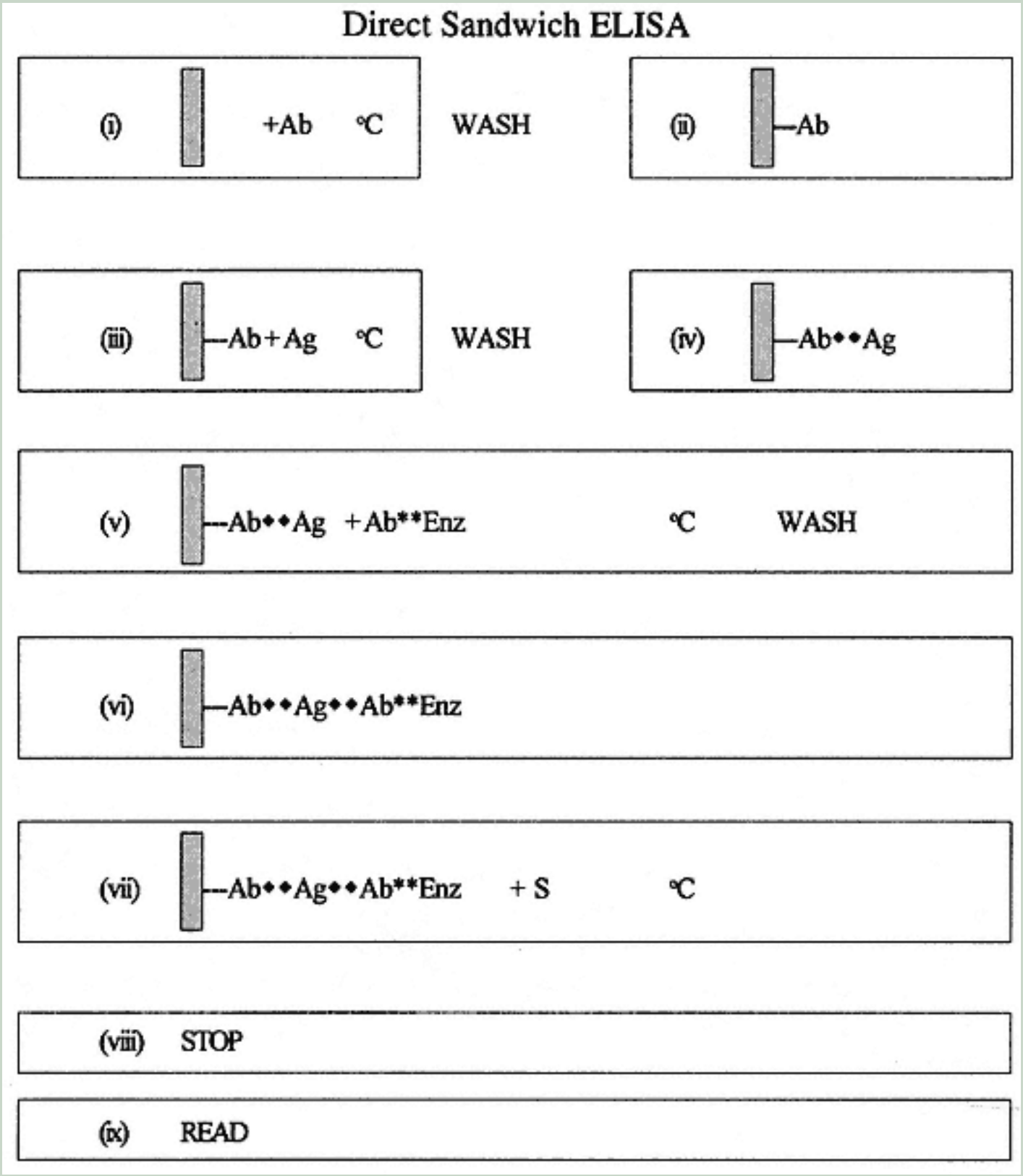
such systems have is the varying degree of nonspecific binding in individual sera. This tends to widen the dispersion (variability) in assay results and, therefore, increases the need to process many sera to assess confidence.

2.3¡ª
Sandwich ELISA

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

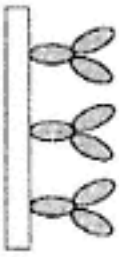
2.3.1¡ª
Direct Sandwich ELISA

The direct sandwich ELISA illustrated as follows and in **Fig. 3**.



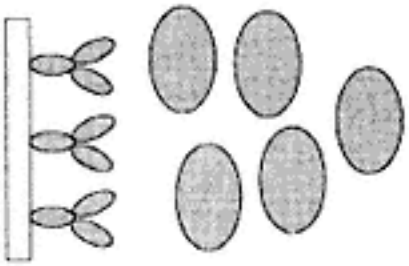
The direct sandwich ELISA, involves the passive attachment of antibodies to the solid phase (stages i and ii). These antibodies (capture antibodies) then

(i)



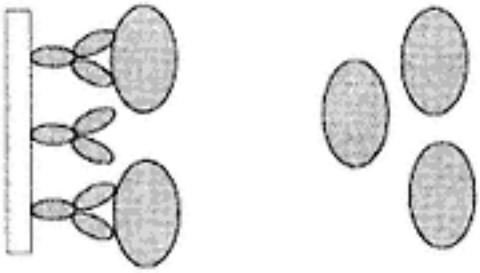
Antibodies are passively attached to solid phase by incubation in buffer.

(ii)



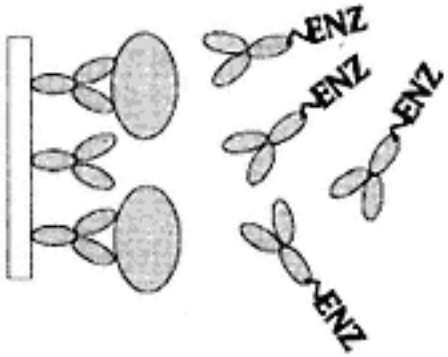
Free antibodies are washed away and then antigen added.

(iii)



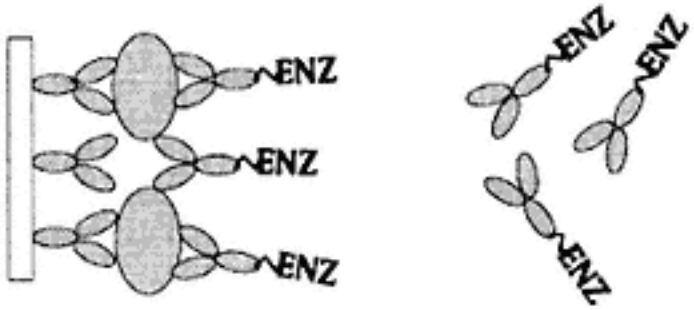
Antigen is captured by coating antibodies during incubation. Unbound antigen is then washed away.

(iv)



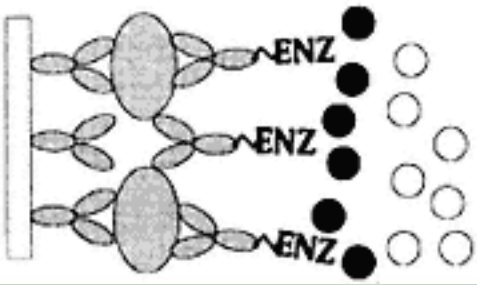
Conjugated antibodies directed against antigen are added. These antibodies can be the same preparation as used on the solid phase or from a different source (species).

(v)



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

(vi)



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

The reaction is stopped and colour quantified in a spectrophotometer.

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¨Cantigen 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¡ªe.g., each antibody preparation used must be labeled (for different antigens)¡ªin the same way as the direct ELISA was limited to single antibody preparations.

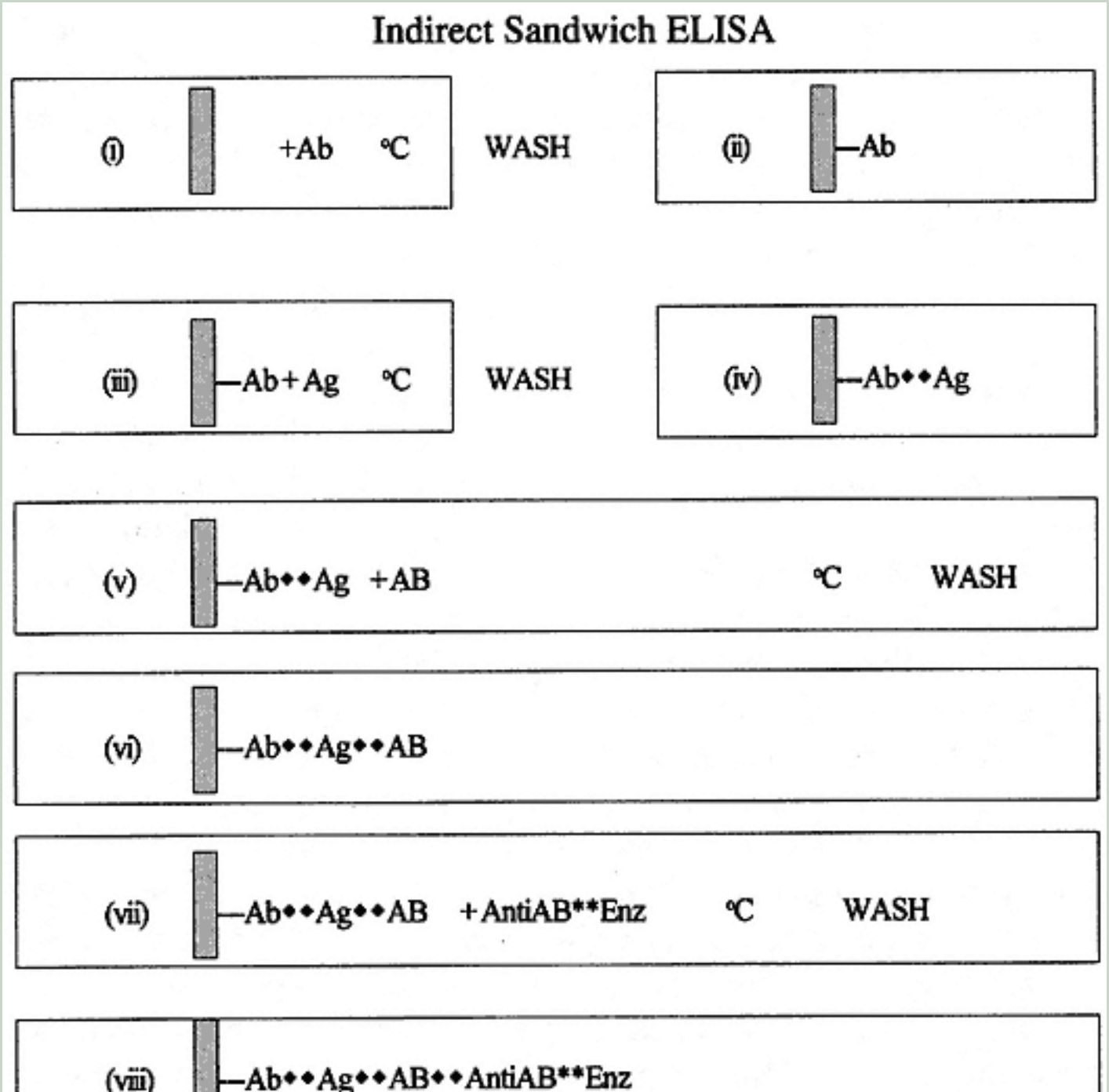
The system also is limited in that antigens must have at least two antigenic sites (epitopes), since both the capture and the detecting antibodies need to bind. This can limit the assay to relatively large antigenic complexes.

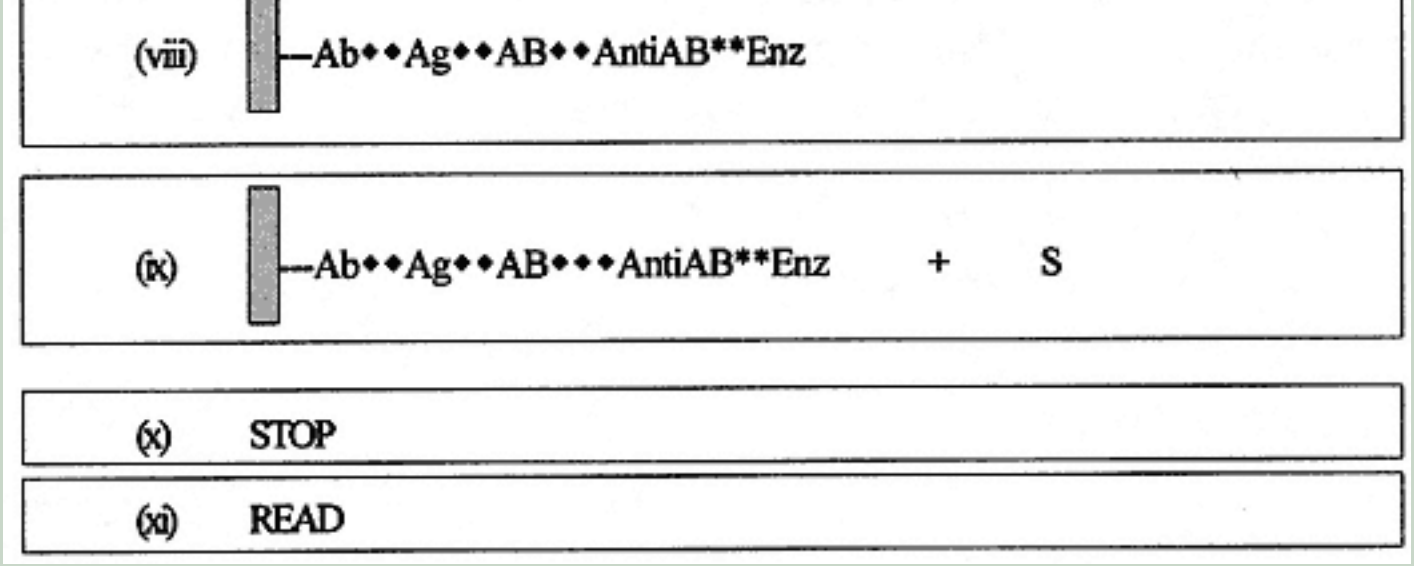
The capture antibody (on the solid phase), and the detecting antibody, can be against different epitopes on an antigen complex. This can be helpful in orienting the antigenic molecules so that there is an increased chance that the detecting antibodies will bind. It can also be an advantage when investigating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

Indirect sandwich ELISA is illustrated as follows and in **Fig. 4**. In indirect sandwich ELISA assay stages i¨Civ 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¨Cix).

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-





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 $F(ab')_2$ fraction). The detecting antibodies could be untreated. The enzyme conjugate could then be

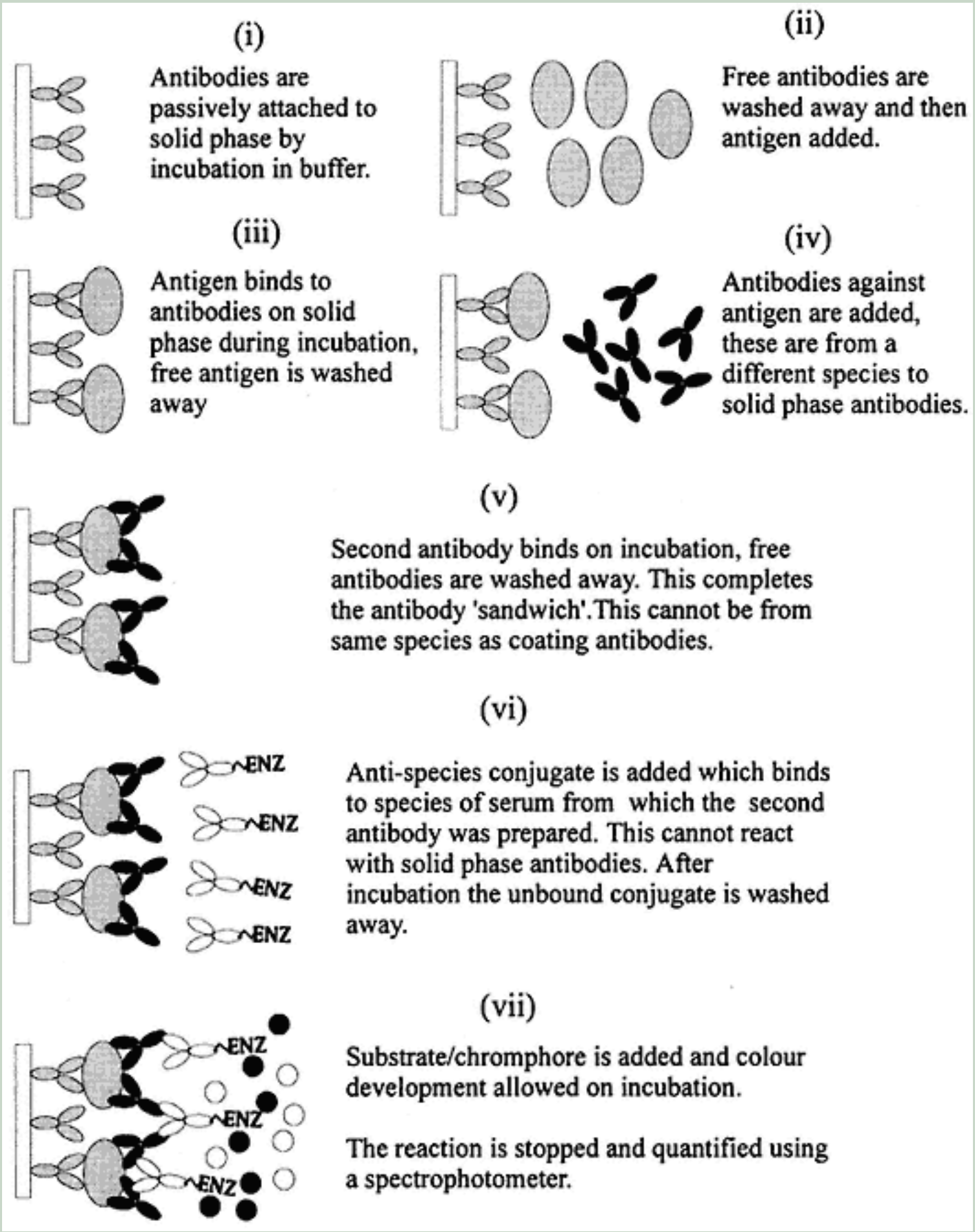


Fig. 4.
Indirect sandwich ELISA. The antigen is captured by a solid-phase antibody. Antigen is then detected using antibodies from another species. This in turn is bound by an antispecies conjugate. Thus, the species of serum for the coating and detecting antibodies must be different; the antispecies cojugate 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.

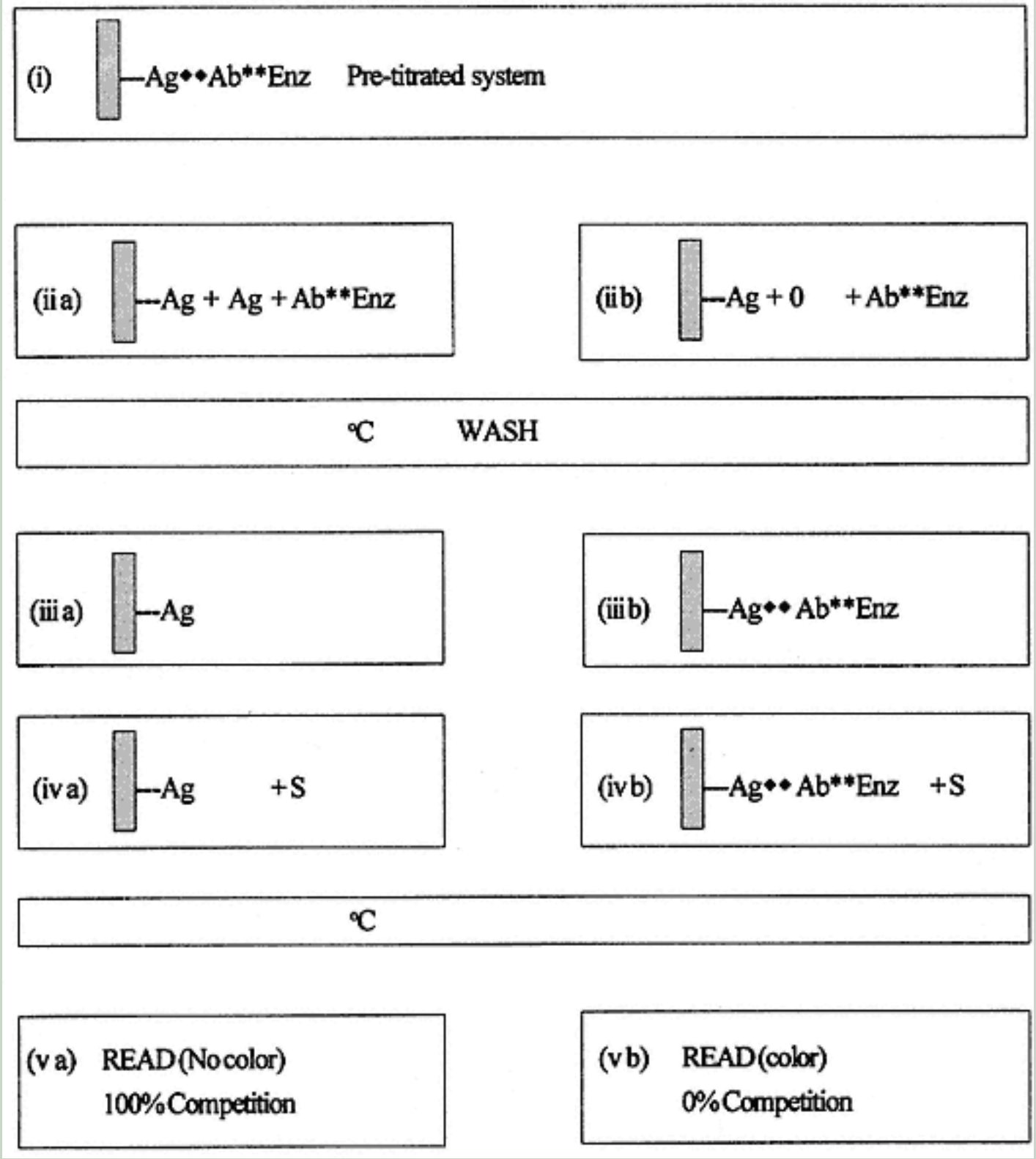
2.4¡ª
Competition/Inhibition Assays

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.¨C2.3.** 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¡ª
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

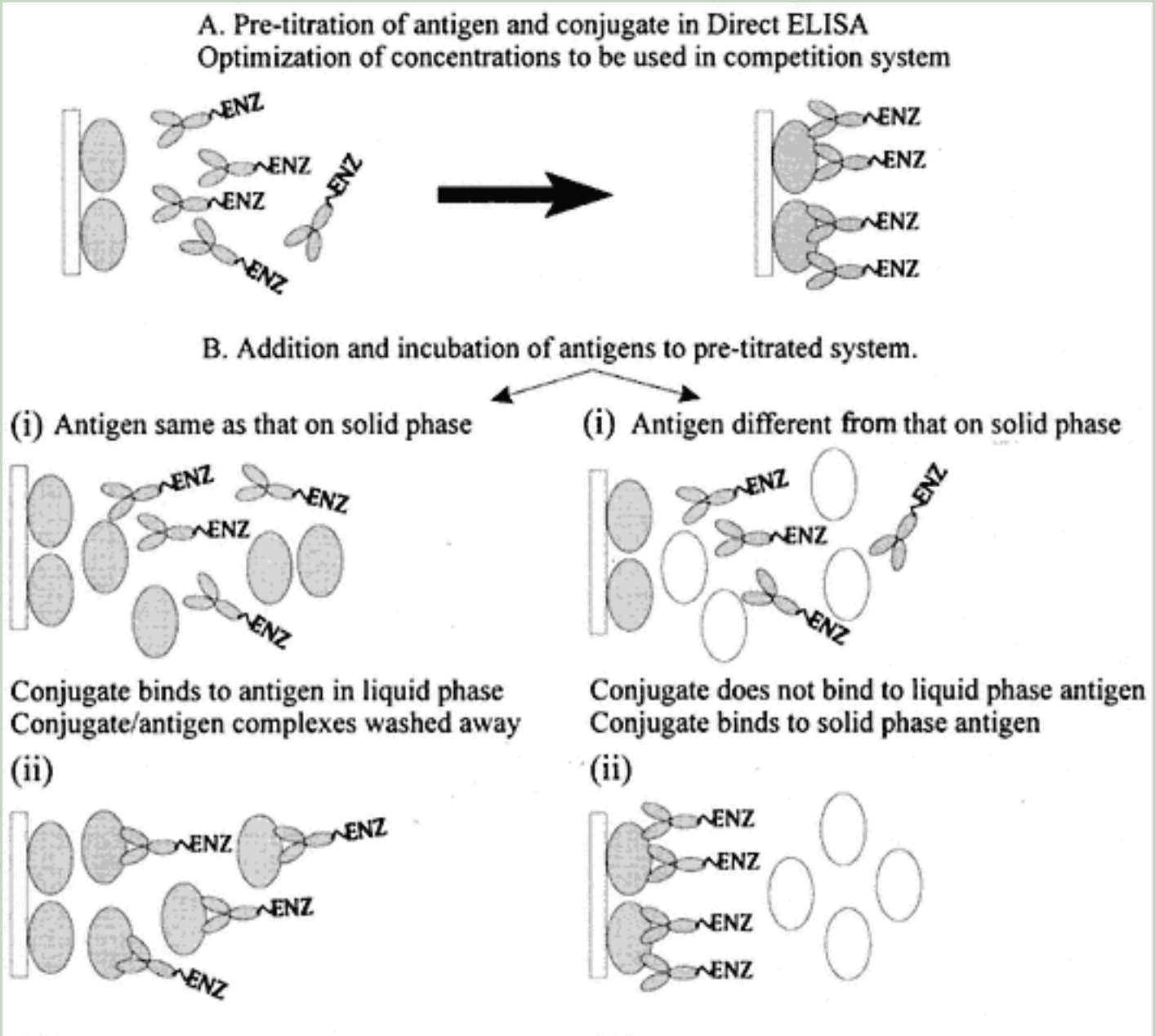
Direct C-ELISA Test for Antigen



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¡ª
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.



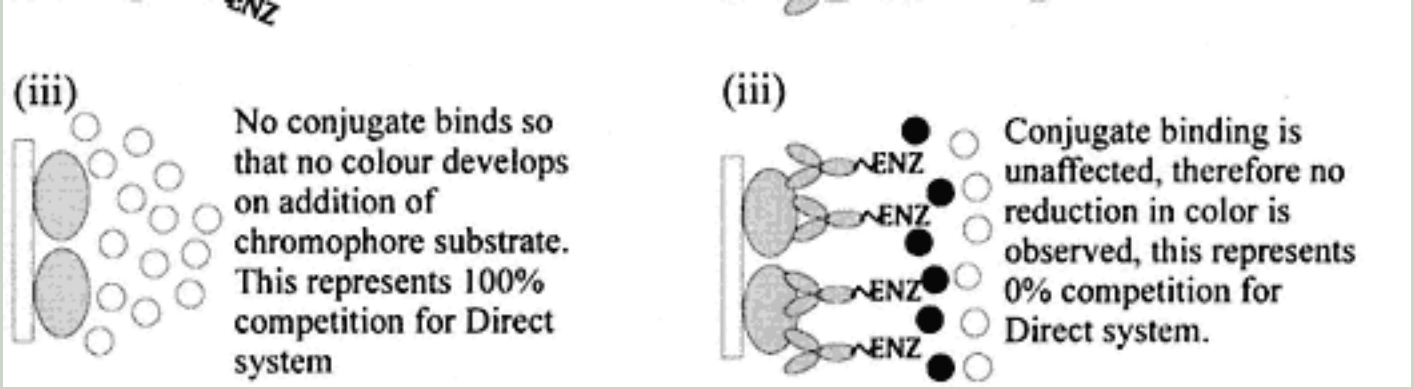
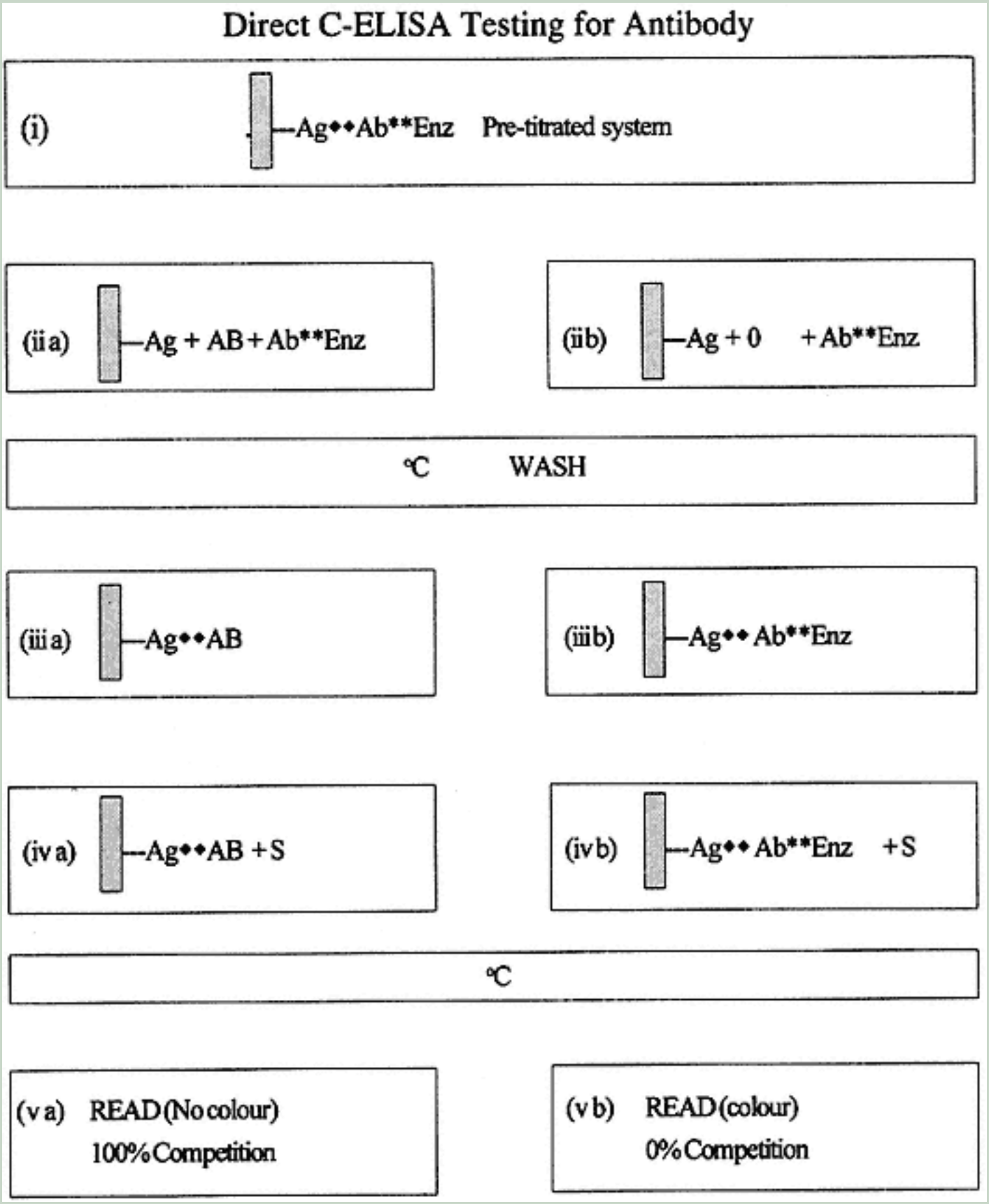


Fig. 5.
Direct C-ELISA for antigen. Reaction of antigen contained in samples with the enzyme-labeled antibody directed against the antigen on the solid phase blocks the label from binding to the solid-phase antigen. If the antigen has no cross-reactivity or is absent, then the labeled antibody binds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



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

2.4.3¡[a]
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.

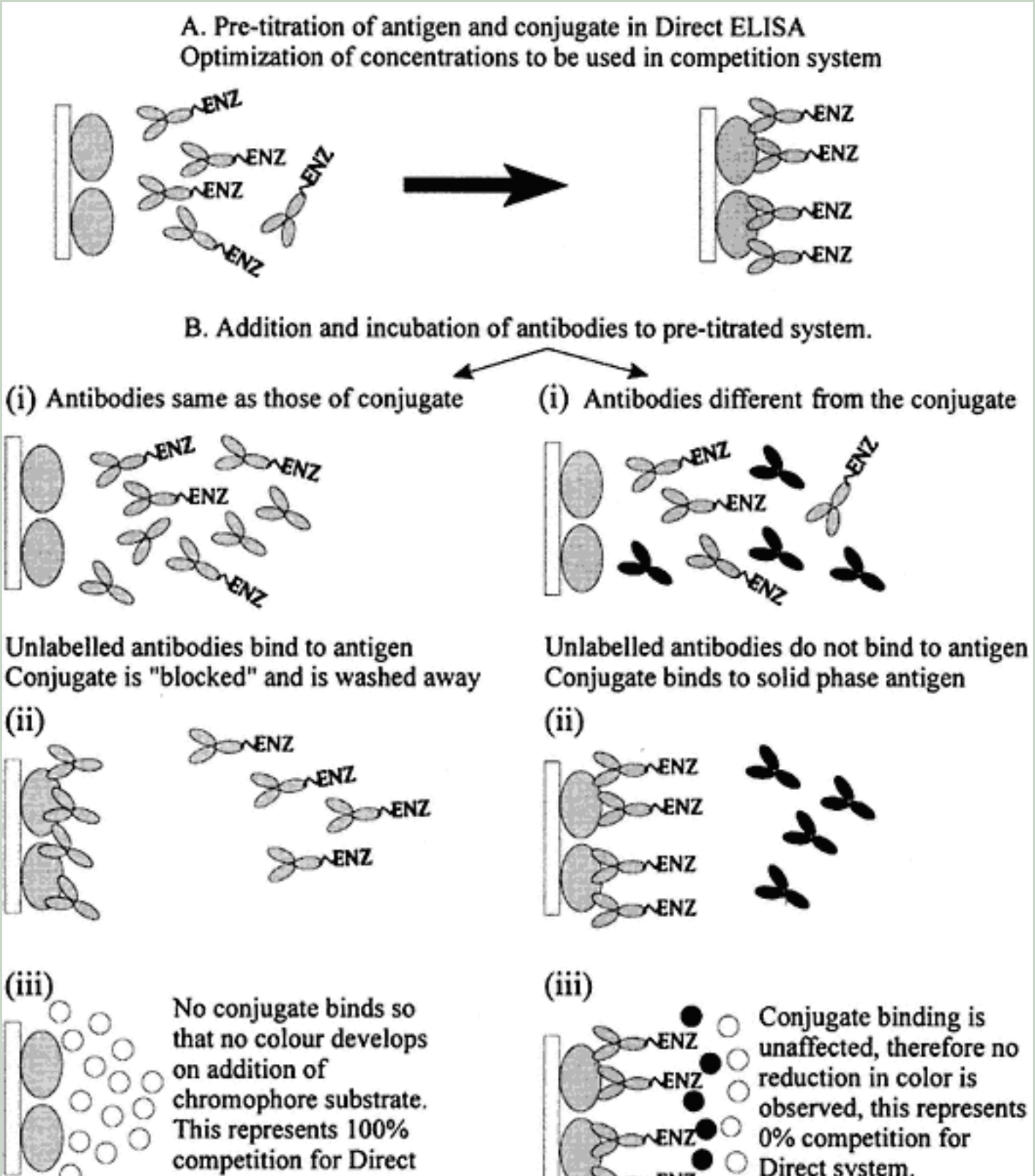




Fig. 6.
Direct C-ELISA for antibody. The degree of inhibition by the binding of antibodies in a serum for a pretitrated enzyme-labeled antiserum reaction is determined.

2.4.4¡[a]
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

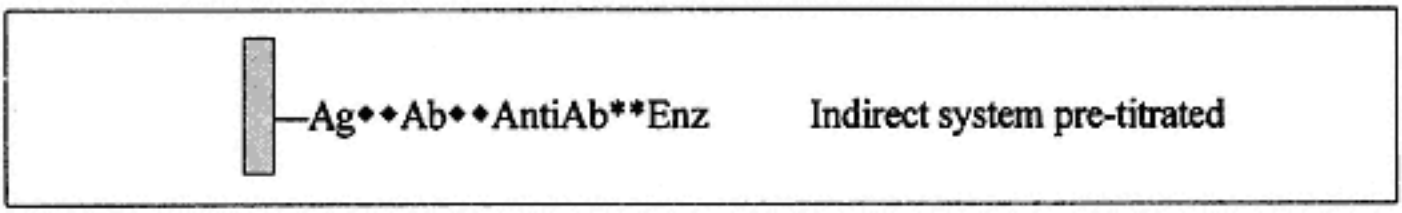
the test sample. In these ways, the advantage in terms of binding to the antigen on the wells is given to the test sample. Bound antibodies then inhibit or block the binding of the subsequently added labeled antibodies.

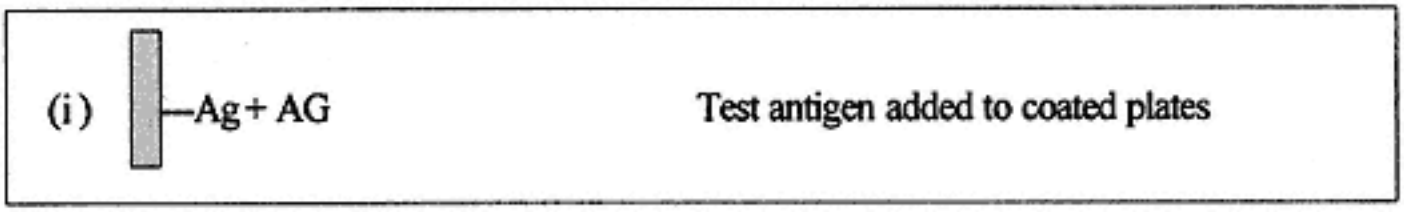
2.5¡[a]
Competitive and Inhibition Assays for Indirect ELISA

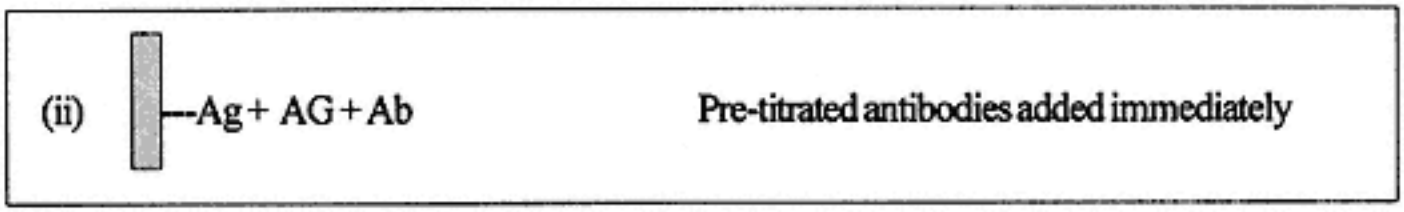
2.5.1¡[a]
Indirect C-ELISA Antigen Measurement

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

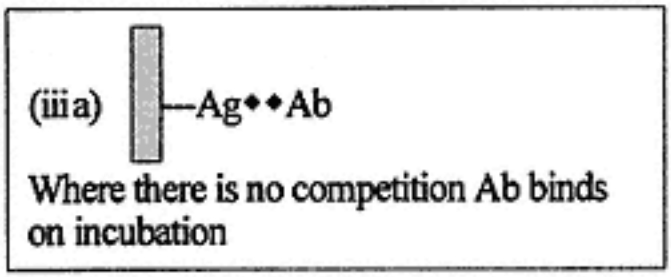
Indirect C-ELISA Antigen Measurement

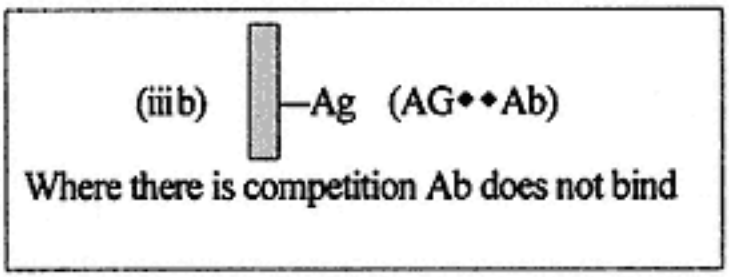


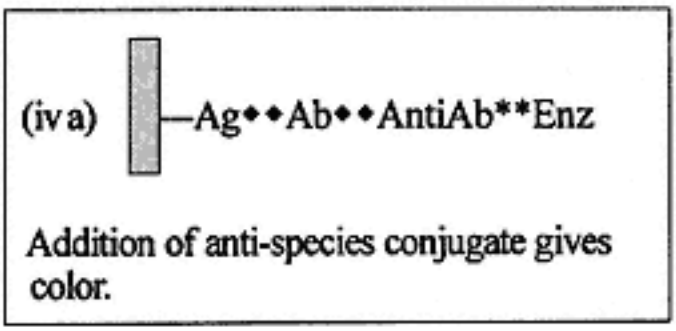


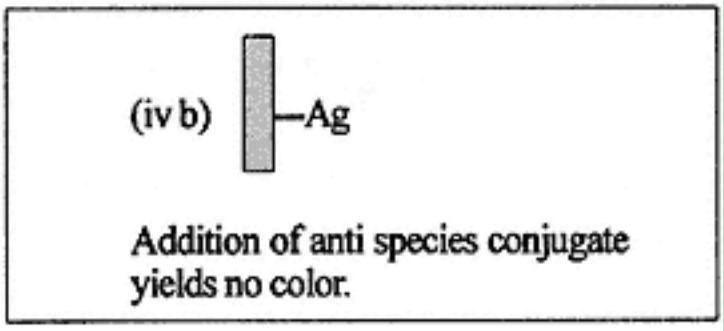


℃ WASH









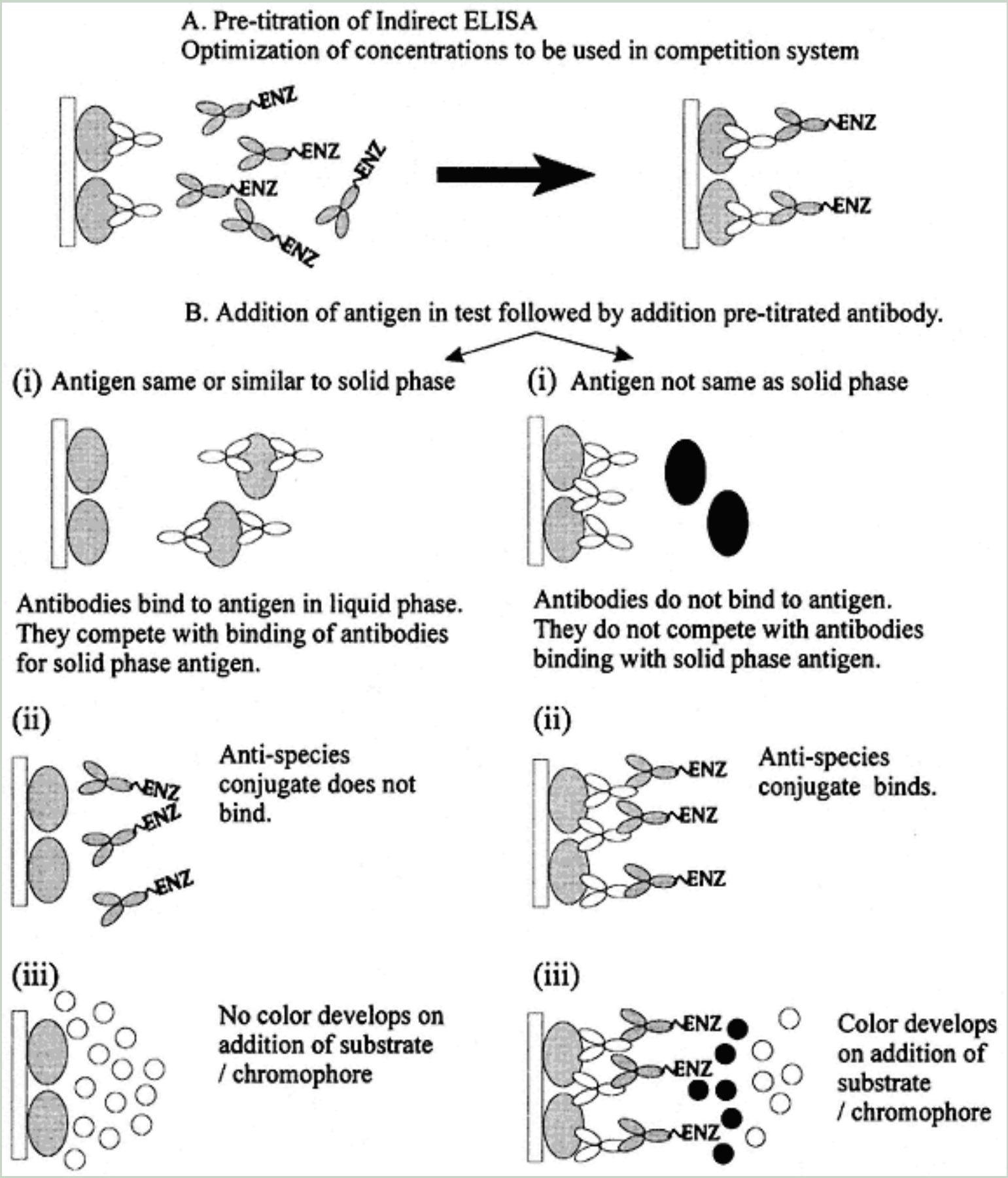


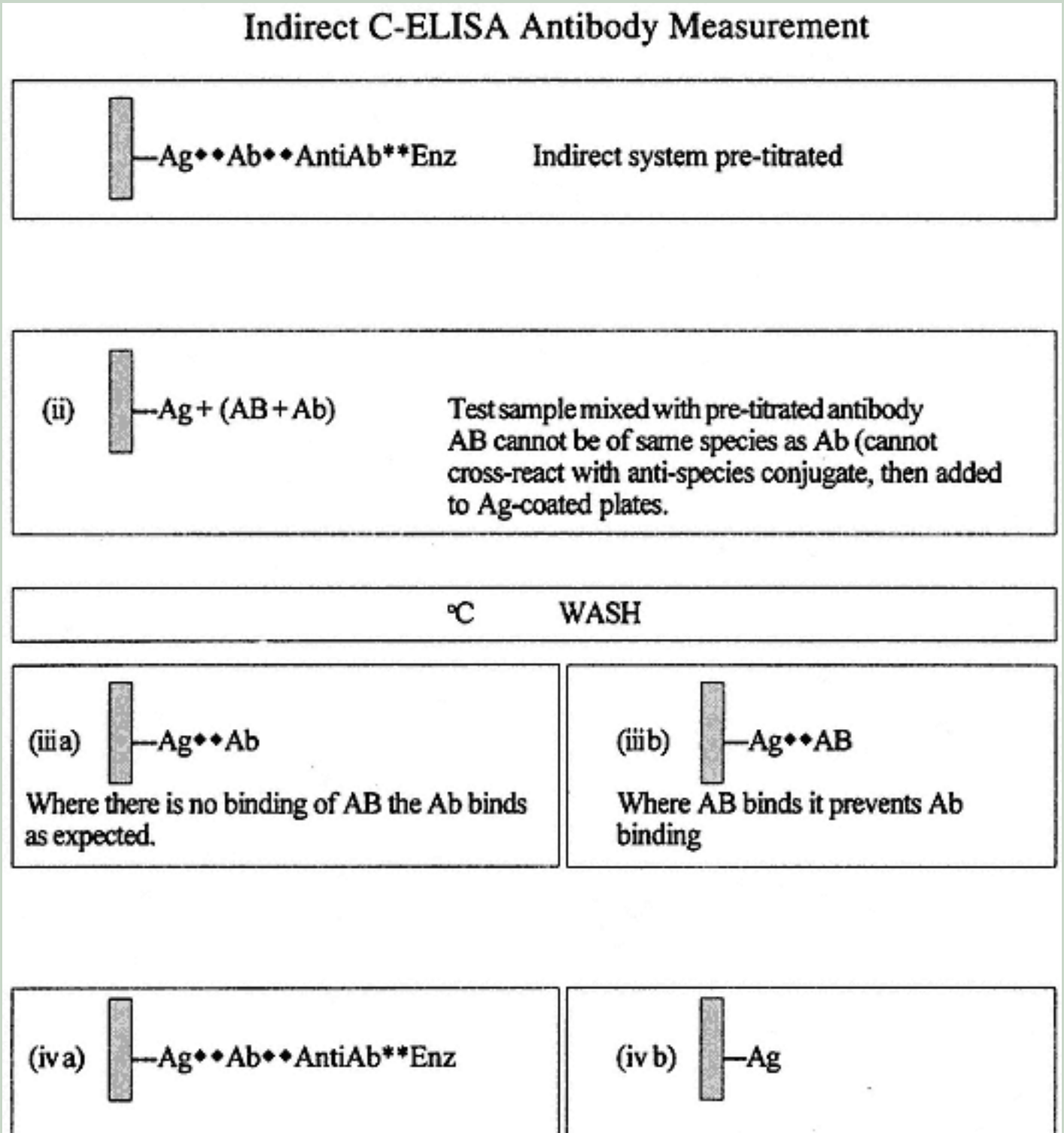
Fig. 7.
Indirect C-ELISA antigen measurement. The degree of competition by the binding of antigens in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.

2.5.2¡[a]

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

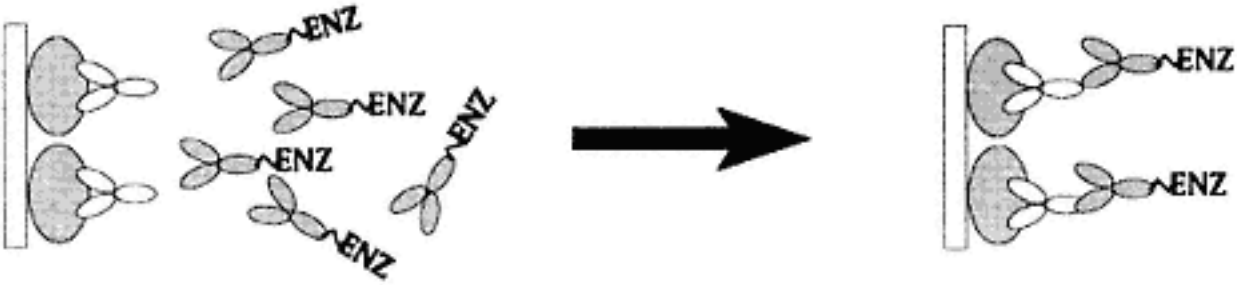


Addition of anti-species conjugate gives color.

Addition of anti species conjugate yields no color.

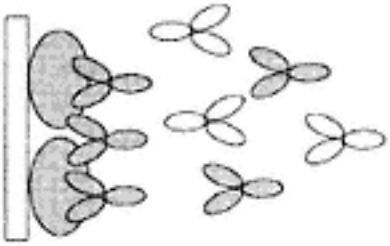
for antigen and antibody measurement can be altered with respect to the initial titration of the reagents in the pretitration phase. Thus, by using different concentrations of antibody, the effective sensitivity for competition or inhibition by antigen or antibody can be altered to favor either analytical 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).

A. Pre-titration of Indirect ELISA
Optimization of concentrations to be used in competition system



B. Mix pre-titrated and test antibodies . Add to coated plates.

(i) Antibody same or similar.



Antibodies bind to antigen on solid phase. They compete with binding of pre-titrated antibodies for solid phase antigen.

(i) Antibody not same.



Antibodies do not bind to antigen on solid phase, pre-titrated antibodies bind with solid phase antigen.

(ii)



Anti-species conjugate does not bind. The species of competing

(ii)



Anti-species conjugate binds.

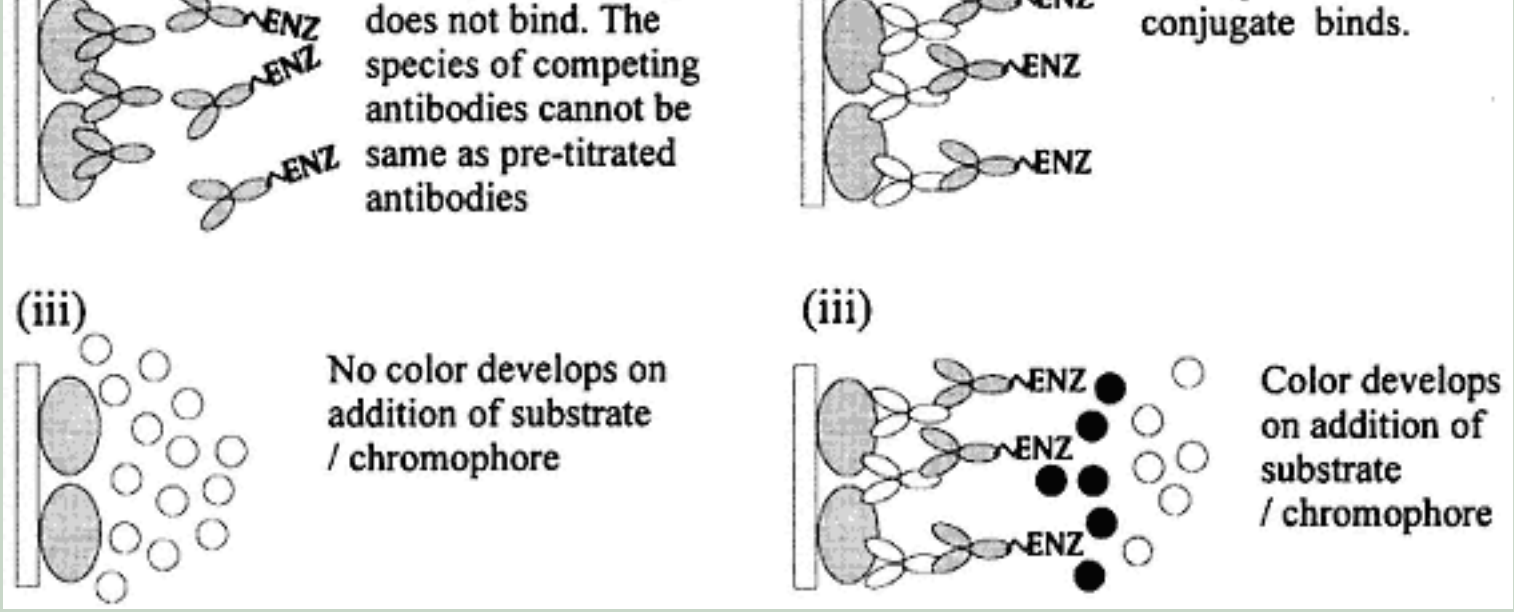
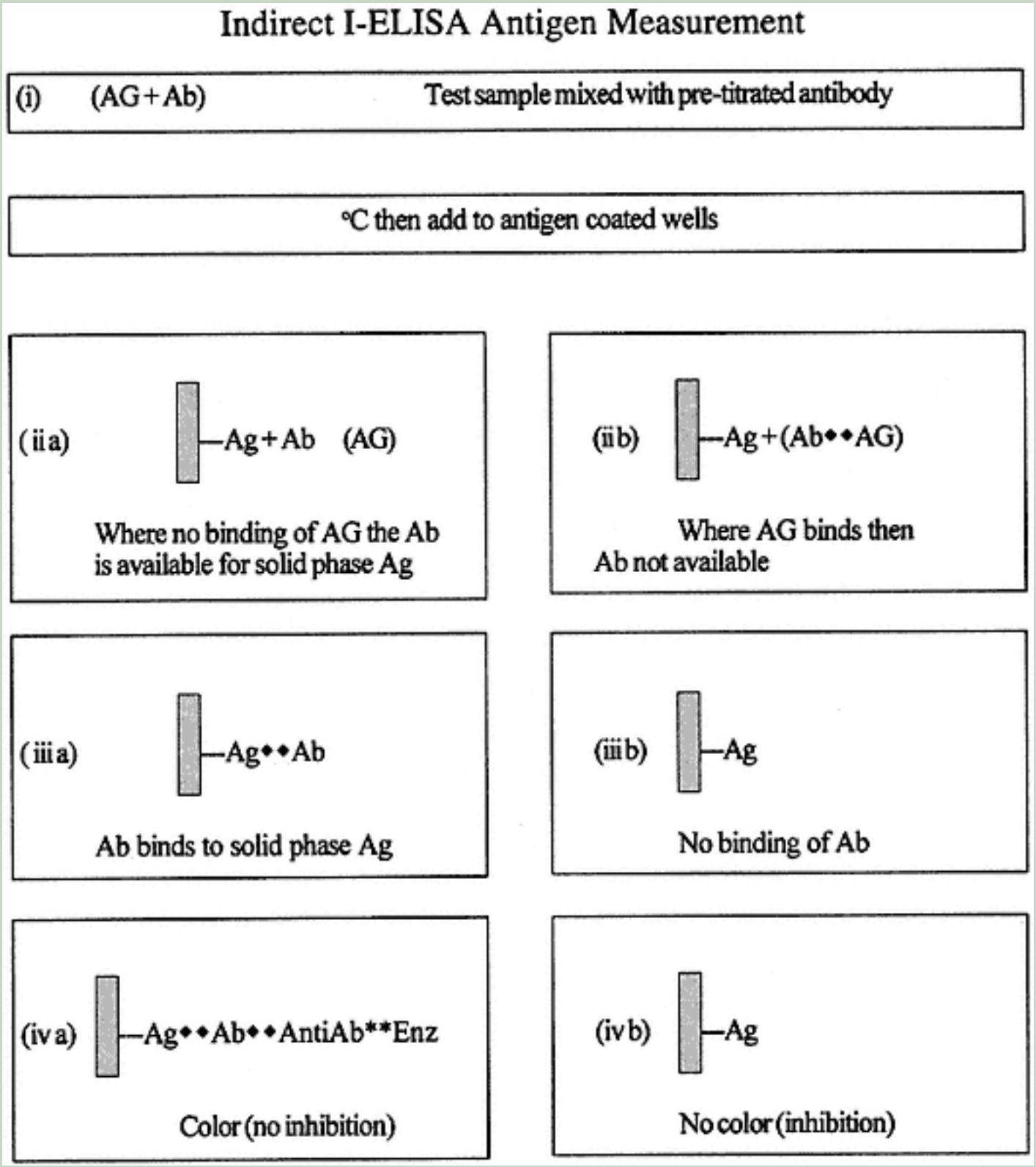


Fig. 8.
Indirect C-ELISA antibody measurement. The degree of competition by the binding of antibodies in a sample for a pretitrated enzyme-labeled antiserum reaction is determined.

2.5.3¡[a]
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.

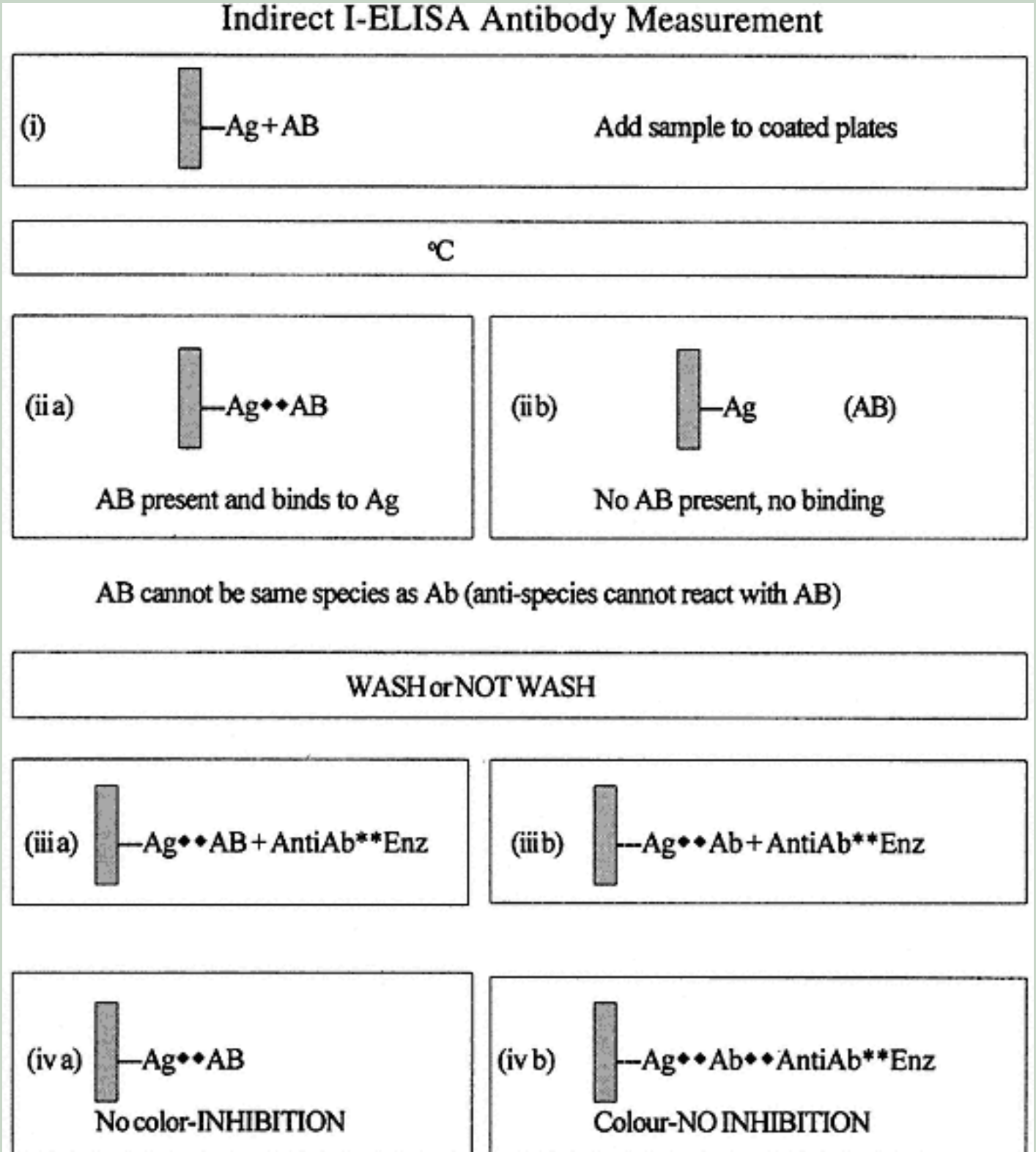


2.5.4¡[a]
Indirect I-ELISA Antibody Measurement

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¡[a]
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 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.

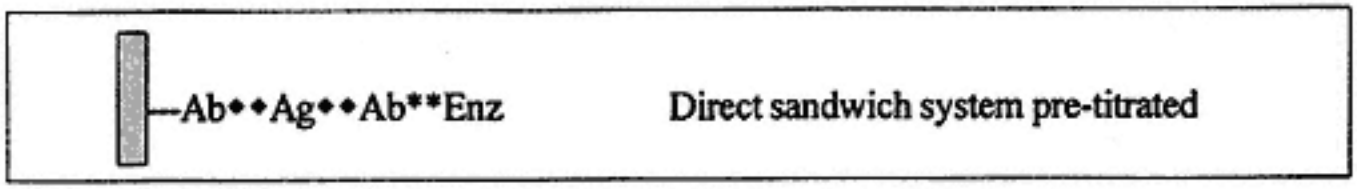
The main point about using sandwich assays is that they may be essential to presentation of antigen usually by concentrating the specific antigen from a mixture through the use of a specific capture serum. Thus, the advantages of competitive/inhibitive techniques rely on antigen capture. Whether direct 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.

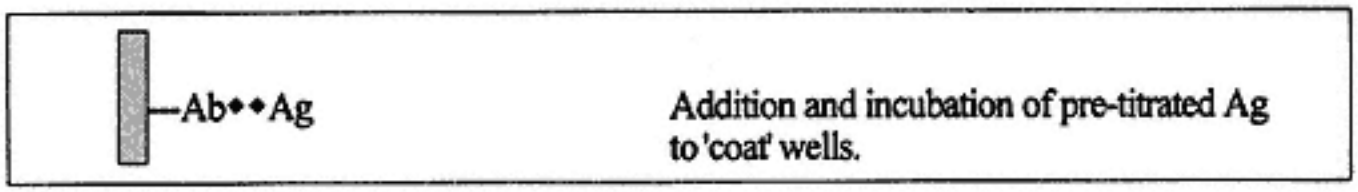
The assays are described under direct sandwich and indirect sandwich headings. Direct sandwich, involves assays utilizing a capture and a directly labeled detecting antibody (two antibody systems), and indirect sandwich involves assays utilizing three antibody systems (antispecies conjugate used to measure 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¡[a]
Direct Sandwich C-ELISA for Antibody

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

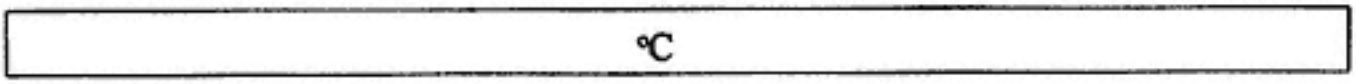
Direct Sandwich C-ELISA for Antibody

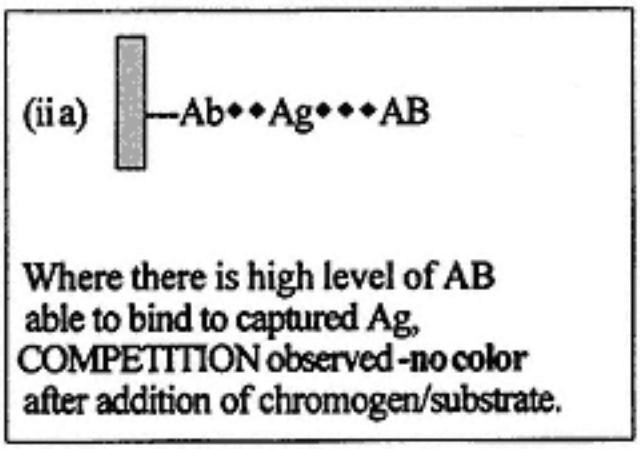


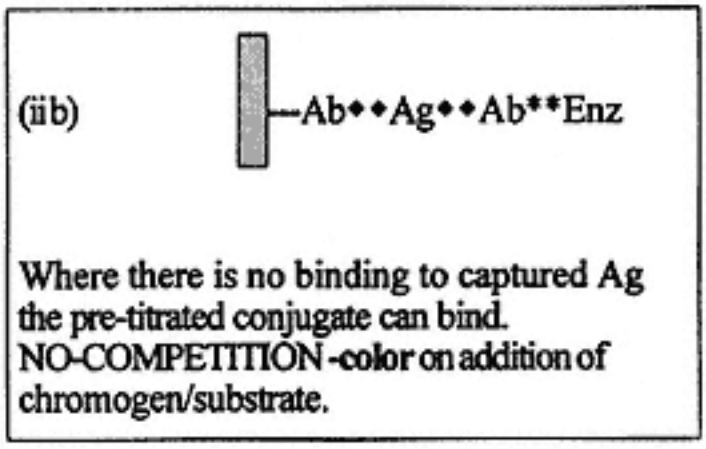


COMPETITION PHASE









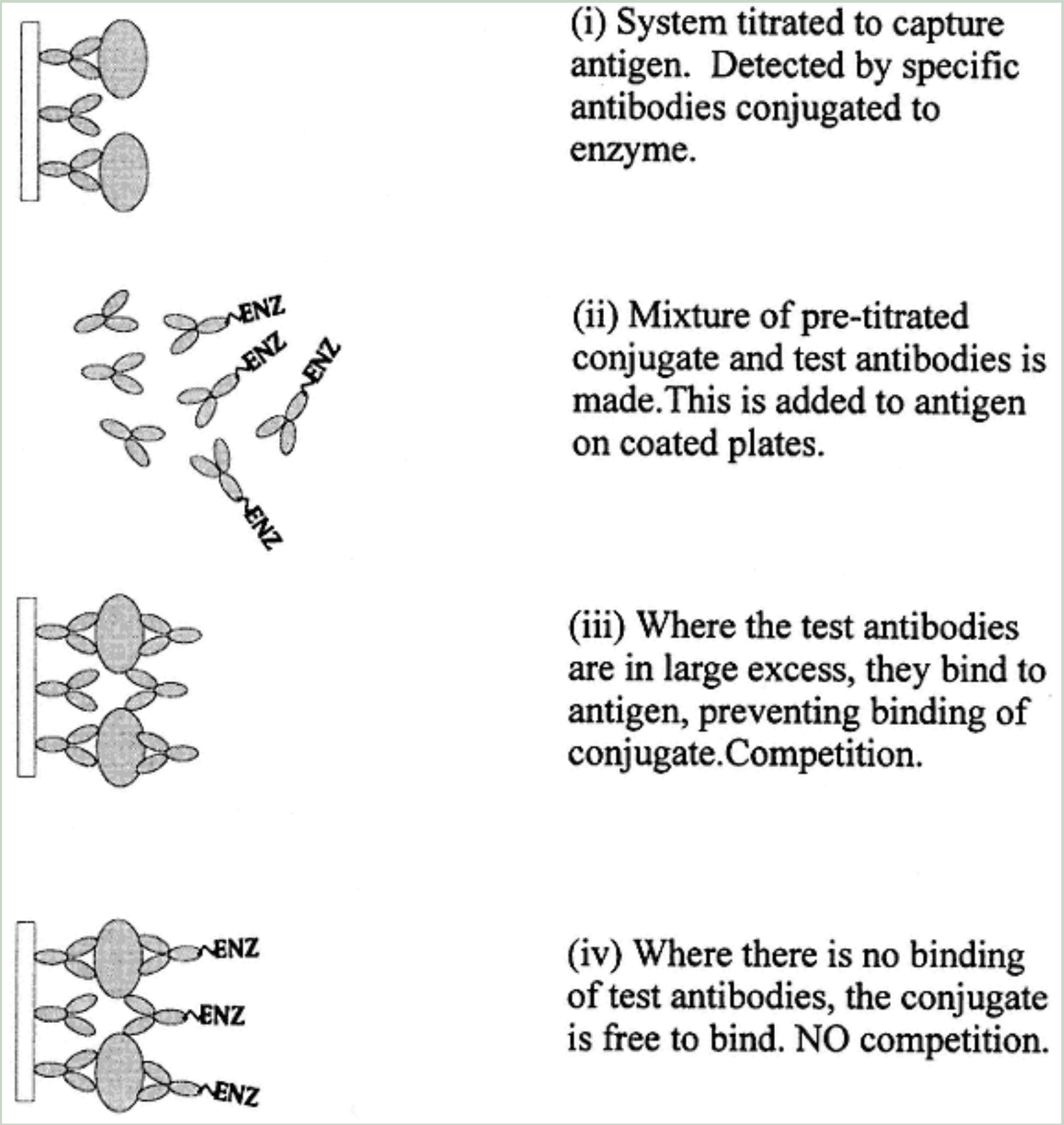


Fig. 9.
Direct sandwich competition ELISA for antibody. This system exploits the competition of antibodies in a sample for the binding of a pretitrated quantity of labeled antibody specific for the antigen captured by the coating antibodies on the wells. The extent of competition depends on the relative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

2.6.2¡ª
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¡ª
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.

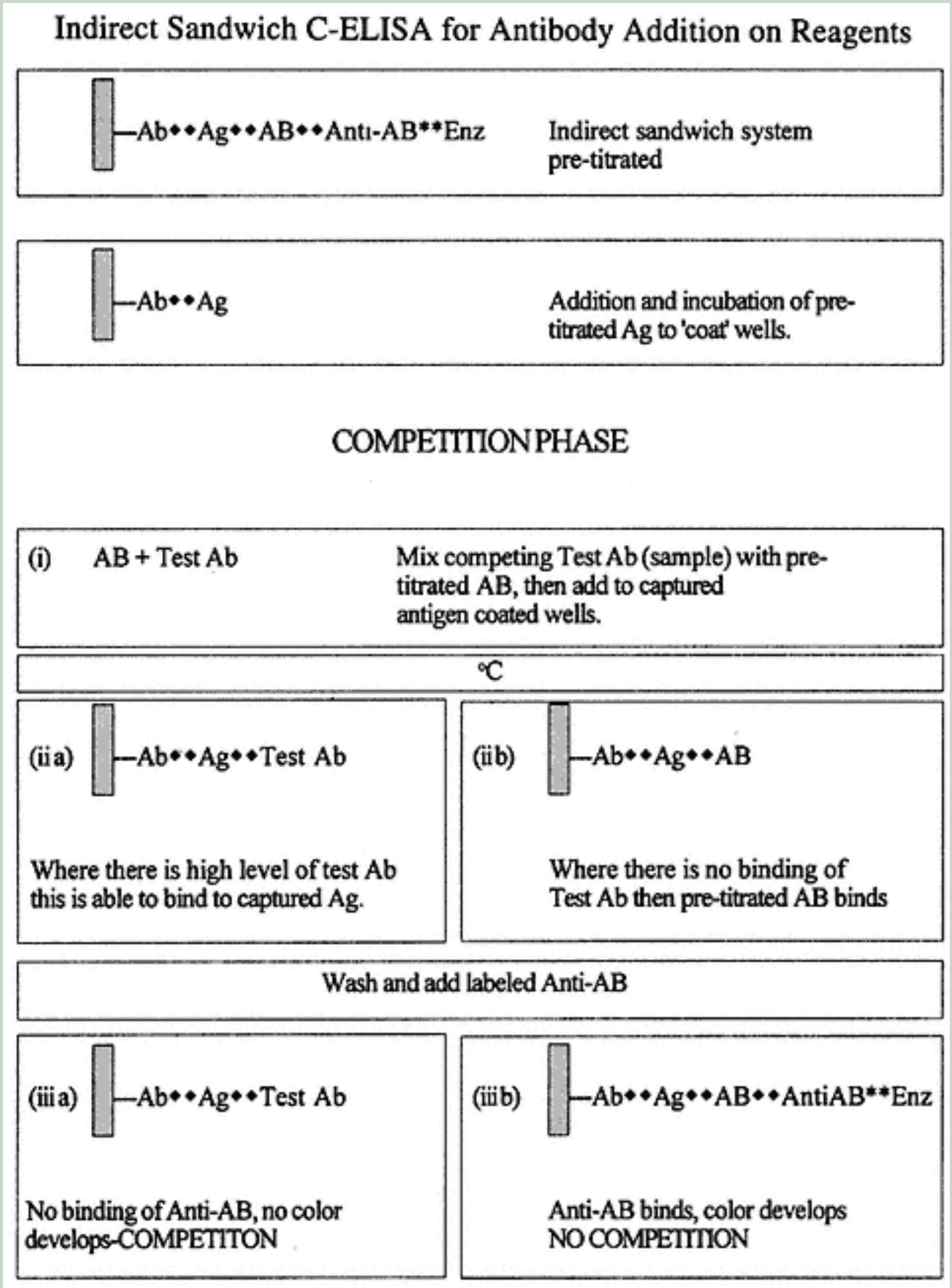
2.6.4¡ª
Indirect Sandwich C-ELISA for Antibody

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¡ª
Indirect Sandwich I-ELISA for Antibody

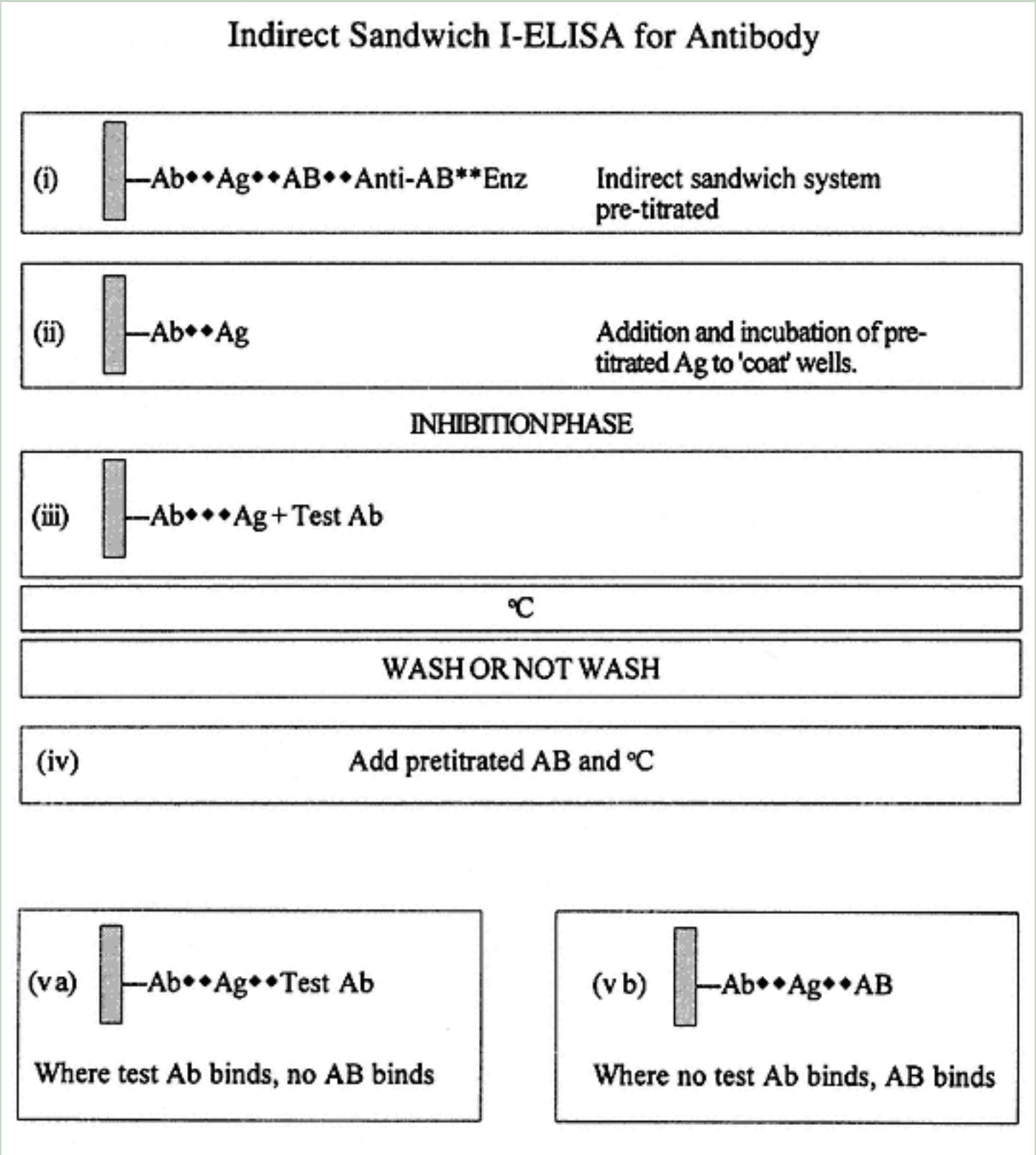
The indirect sandwich I-ELISA for antibody is similar to that for C-ELISA except that the time of addition of reagents is altered to allow a greater chance for reaction. This is illustrated in the diagram on page 37.

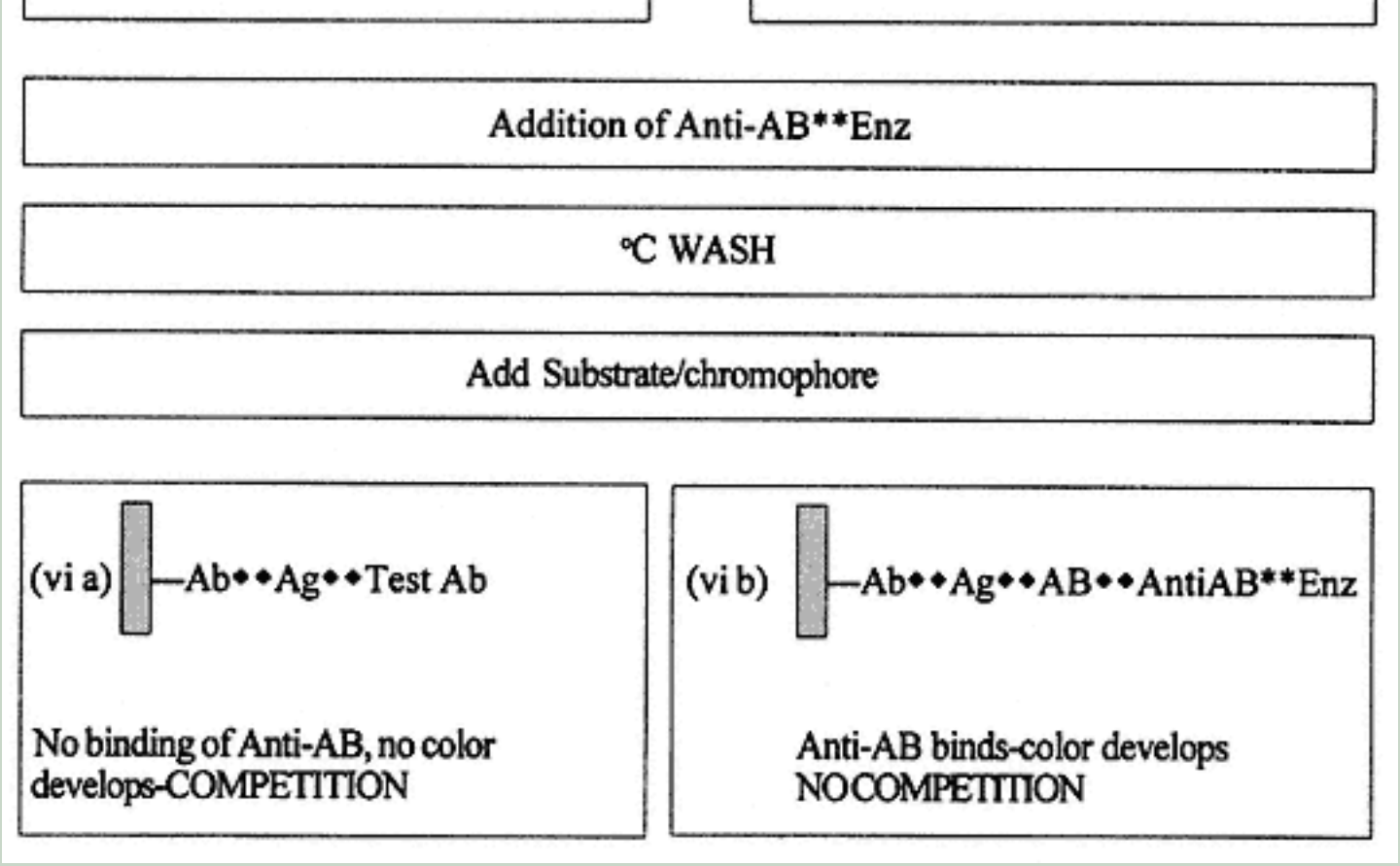


2.6.6¡[a]

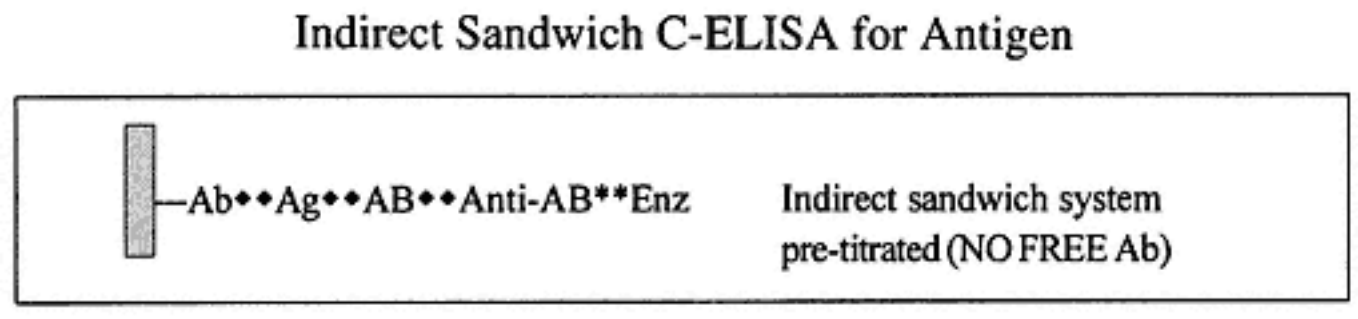
Indirect Sandwich C-ELISA for Antigen

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-



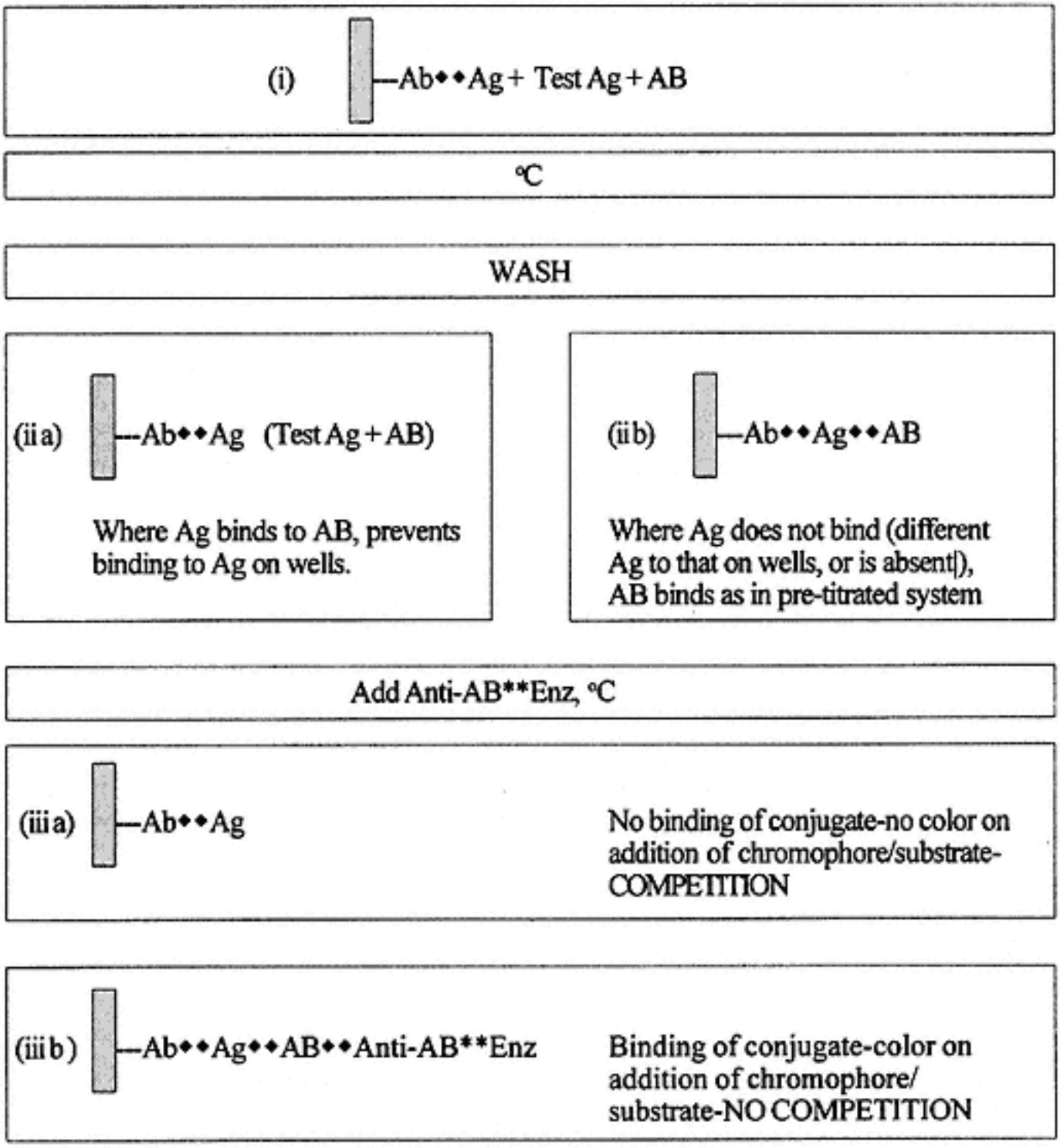


body assays examined in **Subheadings 2.6.4** and **2.6.5.** The antigen has to be in excess, as shown in the following diagram.



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¡ª 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¡ª 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¡ªproof that a test system(s) can work (phase 1).

2. Validation¡ªshowing that a test(s) is stable and that it is evaluated over time and under different conditions (phase 2).

3. Standardization¡ªquality 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¡ª 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¨Cwhole 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 µL. Therefore the maximum number of samples that can be run as single tests is 30 ¡Á 1000 ¡Á 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¡ª
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¨CC) 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 µg)
- 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. Antiguinea pig conjugate
- e. Postinfected and d 0 (uninfected) cow sera
- f. Anticow conjugate
- g. Antirabbit conjugate

2.7.2.1¡[a]
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.

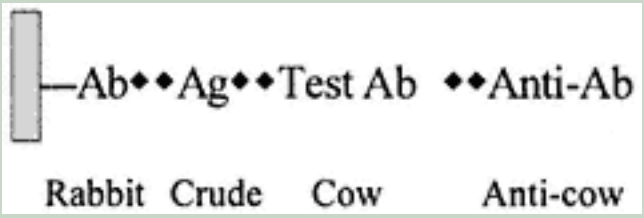
Any bound test antibody would be from cows and thus detected using an antibovine conjugate. This may cause problems since the crude antigen was used to raise the rabbit serum. Hence, antibodies against contaminating proteins may be produced in the rabbit. The cow sera being tested may react with such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

3¡ª
Stages in ELISA

This chapter gives general information on essential practical features of ELISAs. These can be summarized as follows:

1. Adsorption of antigen or antibody to the plastic solid phase

2. Addition of the test sample and subsequent reagents

3. Incubation of reactants

4. Separation of bound and free reactants by washing

5. Addition of enzyme-labeled reagent

6. Addition of enzyme detection system (color development)

7. Visual or spectrophotometric reading of the assay

1¡ª
Solid Phase

The most widely exploited solid phase is the 96-well microtiter plate manufactured from polyvinyl chloride (flexible plates), or polystyrene (inflexible rigid plates). Many manufacturers supply plates designed for ELISA and provide a standardized product. The use of a wide variety of plates from different manufacturers has been reported for a broad spectrum of biological investigations. It is impossible to recommend one product as a universally accepted plate. In cases in which specific assays have been developed, it is prudent to use the recommended plate; however, because, in practice, there is relatively little difference between plates, it is possible to perform the same test using different plates provided that suitable standardization is performed. In this respect, laboratories that deal with large numbers of ELISAs involving different antigens and antibodies can perform standardized assays using the same type of plate. Ideally, flat-bottomed wells are recommended in which spectrophotometric reading is employed to assess color development. However, round-bottomed wells can be used in which visual (by eye) assessment of the ELISA is made. Such plates can be read by a spectrophotometer but are not ideal.

The performance of plates should be examined for given assays on a routine basis, since it cannot be automatically assumed that the plates will not vary in

performance. This is particularly important when different batches of plates are received. The batch number usually can be obtained from the boxes in which the plates are provided and from documentation accompanying the plates. Some plates also have codes embossed onto the plastic to identify the particular stamps used in their manufacture. In practice, sometimes poor-quality plates are sent out even when a certificate of guarantee is provided.

1.1¡ª
Immobilization of Antigen on Solid-Phase Coating

A key feature of the solid-phase ELISA is that antigens or antibodies can be attached to surfaces easily by passive adsorption. This process is commonly called coating. Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between nonpolar protein substructures and the plastic matrix. The interactions are independent of the net charge of the protein, and thus each protein has a different binding constant. The hydrophobicity of the plastic/protein interaction can be exploited to increase binding since most of proteins' hydrophilic residues are at the outside and most of the hydrophobic residues orientated towards the inside ***(1)***.

Partial denaturation of some proteins results in exposure of hydrophobic regions and ensures firmer interaction with the plastic. This can be achieved by exposing proteins to low pH or mild detergent and then dialysis against coating buffers before coating.

The rate and extent of the coating depends on these factors:

1. Diffusion coefficient of the attaching molecule.

2. Ratio of the surface area being coated to the volume of the coating solution.

3. Concentration of the substance being adsorbed.

4. Temperature.

5. Time of adsorption.

These factors are linked. It is most important to determine the optimal antigen concentration for coating in each system by suitable titrations. A concentration range of 1¨C10 µg/mL of protein, in a volume of 50 µL, is a good guide to the level of protein needed to saturate available sites on a plastic microtiter plate. This can be reliable when relatively pure antigen (free of other proteins other than the target for immunoassay) is available. Thus, the concentration can be related to activity. However, when coating solutions contain relatively small amounts of required antigen(s), the amount attaching to a well is reduced according to its proportion in the mixture. Other contaminating proteins will take up sites on the plastic. Because the plastic has a finite saturation level the use of relatively crude antigens for coating may lead to poor assays.

Care must be taken to assess the effects of binding proteins at different concentrations, since the actual density of binding may affect results. High-den-

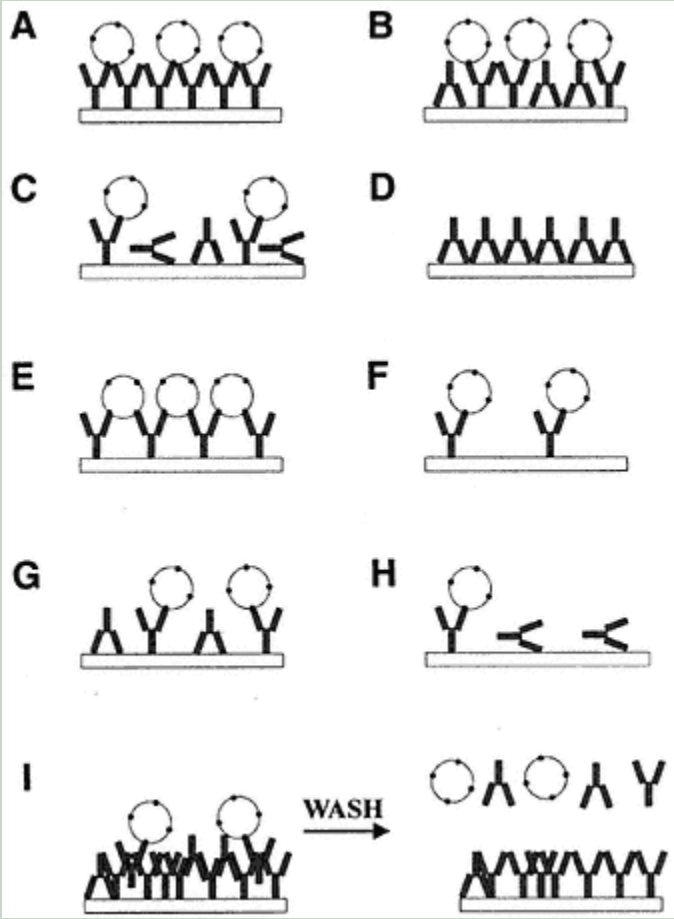


Fig. 1.
Effects on antibodies of coating. **(A)** Antibody molecules packed evenly, orientation of Fc on plate, monovalent interaction of multivalent Ag; **(B)** antibody molecules packed evenly, orientation Fc and Fab on plate, monovalent binding of multivalent Ag; **(C)** antibody binding in all orientations, monovalent binding of multivalent Ag; **(D)** antibody binding via Fab, no binding of Ag; **(E)** antibody spaced with orientation to allow bivalent interaction between adjacent antibody molecules; **(F)** antibody spaced too widely to allow adjacent molecules to bind bivalently via Fc; **(G)** as in (E) except that orientation is via Fc or Fab; **(H)** more extreme case of (C) with less antibody and more molecules inactive owing to orientation; **(I)** multilayered binding in excess leading to binding but elution on washing.

sity binding of antigen may not allow antibody to bind through steric inhibition (antigen molecules are too closely packed). High concentrations of antigen may also increase stacking or layering, which may allow a less stable interaction of subsequent reagents. Orientation and concentration of antibody molecules must also be considered because these factors affect the activity of assays. **Figures 1** and **2** examine the elements of adsorption.

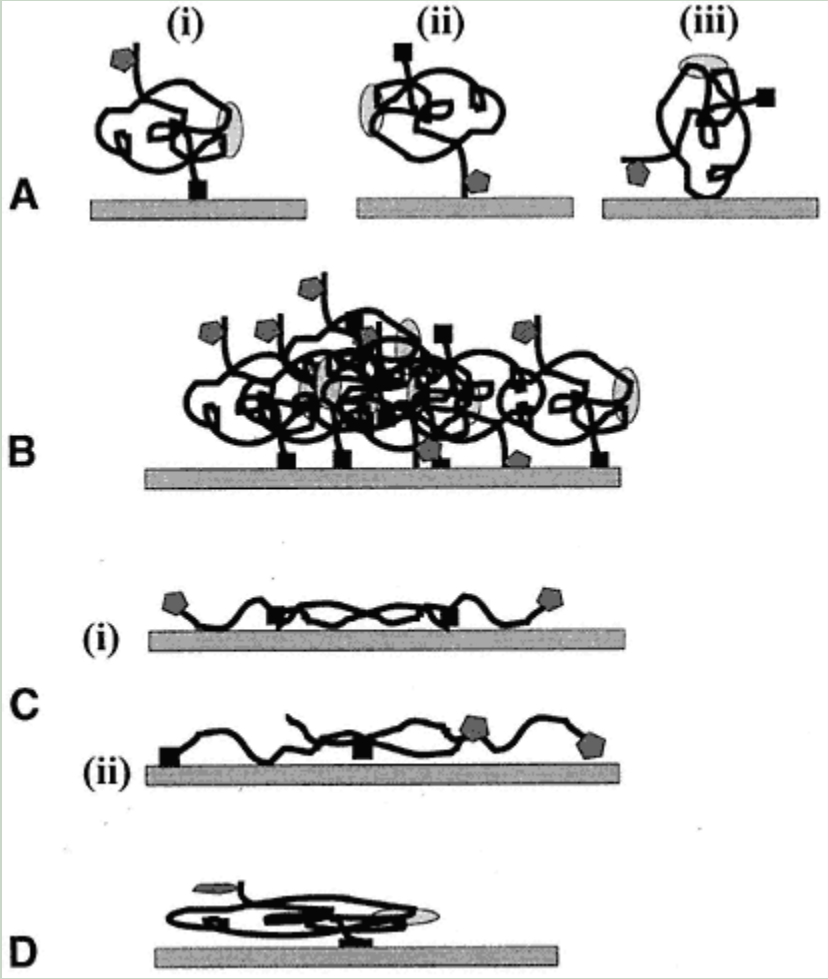


Fig. 2.
Possible effects on soluble protein of immobilization. Protein is shown as having three antigenic sites (epitopes). Two are linear (solid box and shaded pentagon), and one is conformational dependent (shaded oval). **(A)** (i) to (iii) The orientation of the molecule on the well affects the presentation of the individual epitopes. This is true of passive and covalent binding to plastic. **(B)** Aggregation of the antigen can complicate presentation and also lead to leaching following binding with detecting antibody. **(C)** The antigen may be altered through treatment before attachment. In both (i) and (ii) the conformational epitope has been destroyed. Note also that the orientation of the molecules affects the presentation and spacing between individual epitopes. **(D)** Nondenatured protein can also alter its conformation by passive adsorption to plastic.

1.2¡ª
Coating Time and Temperature

The rate of the hydrophobic interactions depends on the temperature: the higher the temperature, the greater the rate. There are many variations on incubation conditions. It must be remembered that all factors affect the coating and, thus a higher concentration of protein may allow a shorter incubation time as compared to a lower concentration of the antigen for a longer time. The

most usual regimes involve incubation at 37¡ãC for 1¨C3 h or overnight at 4¡ãC, or a combination of the two, or incubation (more vaguely) at room temperature for 1¨C3 h, *(see* **ref. *2*** for a typical study. There are many more variations, and, ultimately, each scientist must titrate a particular antigen to obtain a standardized regime. Increasing the temperature may have a deleterious effect on antigen(s) in the coating stage, and this may be selective, so that certain antigens in a mixture are affected whereas others are not. Rotation of plates can considerably reduce the time needed for coating by increasing the rate of contact between the coating molecules and the plastic.

1.3¡[a]
Coating Buffer

The coating buffers most used are 50 m*M* carbonate, pH 9.6; 20 m*M* Tris-HCl, pH 8.5; and 10 m*M* phosphate-buffered saline (PBS), pH 7.2 (2). Different coating buffers should be investigated when problems are encountered or compared at the beginning of assay development. From a theoretical point of view, it is best to use a buffer with a pH value 1 to 2 units higher than the isoelectric point (p*I*) value of the protein being attached. This is not easy to determine in practice since antigens are often complex mixtures of proteins. By direct study of the effects of different pHs and ionic strengths, greater binding of proteins may be observed. An increase in ionic strength to 0.6 *M* NaCl in combination with an optimal pH was found to give better results for the attachment of various herpes simplex viral peptides *(3).* Proteins with many acidic proteins may require a lower pH to neutralize repulsive forces between proteins and the solid phase, as shown in **ref. *3***, in which the optimal coating for peptides was pH 2.5¨C4.6. PBS, pH 7.4, is also suitable for coating many antigens. Coating by drying down plates at 37¡ãC using volatile buffers (ammonium carbonate) and in PBS is often successful, particularly when relatively crude samples are available. Some antigens pose particular problems, including some polysaccharides, lipopolysaccharides, and glycolipids. In cases in which it proves impossible to directly coat wells with reagent, initial coating of the wells with a specific antiserum may be required. Thus, sandwich (trapping) conditions must be set up. Passive adsorption has several theoretical, although not necessarily practical, drawbacks. These include desorption, binding capacity, and nonspecific binding.

1.4¡[a]
Desorption

Because of the noncovalent nature of the plastic/protein interactions, desorption (leaching) may take place during the stages of the assay. However, if conditions are standardized, leaching does not affect the viability of the majority of tests. There are some reports that the vigorousness of washing at the

various stages of assays (including that after coating) affects the assays through stripping of protein; however, I have not encountered this problem.

1.5¡[a]
Binding Capacity

It is important to realize that plastic surfaces have a finite capacity for adsorption. The capacity for proteins to attach to microplate wells is influenced by the exact nature of the protein adsorbed to the specific plate used. Saturation levels of between 50 and 500 ng per well have been found valid for a variety of proteins when added as 50-µL volumes. The effective weight of protein per well can be increased if the volume of the attaching protein is increased, effectively increasing the surface area of the plastic in contact with the coating antigen. In cases in which there is an obvious discrepancy between the actual concentration of protein added (where known) and the values just given, then the titration of the ELISA should be re-examined. Thus, if concentrations of, e.g., 1¨C10 mg/mL (or greater) of sample are needed to coat wells, this is not have an ideal situation.

1.6¡[a]
Nonspecific Binding

Unlike antigen/antibody interactions, the adsorption process is nonspecific. Thus, it is possible that any substance may adsorb to plastic at any stage during the assay. This must be considered in assay design because reagents may react with such substances. High levels of nonspecific binding can be alleviated through alteration of systems relying on direct adsorption of antigen and the use of sandwich techniques, in which specific antibodies capture and concentrate specific antigens.

1.7¡[a]
Covalent Antigen Attachment

A variety of chemicals that couple protein to plastic have been used to prevent desorption, the antigen being covalently bound. These include water-soluble carbodiimines, imido- and succinimidylesters, ethanesulfonic acid, and glutaraldehyde. Precoating of plates with high molecular weight polymers such as polyglutaraldehyde and polylysine is another alternative ***(4,5)***. These bind to plates with a high efficiency and act as nonspecific adhesive molecules. This method is particularly useful for antigens with a high carbohydrate content since these normally bind poorly to plastic.

Generally, successful assays can be obtained without the need to link antigens to plates covalently. Specially treated activatable plates are now available. The use of covalently attached proteins does offer the possibility that plates could be reused. After an assay, all reagents binding to the solid-phase attached protein could be washed away after using a relatively severe washing procedure, e.g., low pH. The covalent nature of the bonds holding the solid-

phase antigen would prevent this from being eluted. Provided this procedure did not destroy the antigenicity of the solid-phase attached reagent, the plates might be exploited after equilibration with normal washing buffers.

2¡ª
Washing

The purpose of washing is to separate bound and unbound (free) reagents. This involves the emptying of plate wells of reagents followed by the addition of liquid into wells. Such a process is performed at least three times for every well. The liquid used to wash wells is usually buffered, typically PBS (0.1 *M,* pH 7.4), in order to maintain isotonicity since most antigen¨Cantibody reactions are optimal under such conditions. Although PBS is most frequently used, lower-molarity phosphate buffers (0.01 *M)* may be used, provided that they do not influence the performance of the assay. These buffers are also more cost-efficient.

In some assays tap water has been used for washing. This is not recommended because tap water varies greatly in composition (pH, molarity, and so forth). However, assays may be possible provided the water does not drastically affect the components of the test. Generally, the mechanical action of flooding wells with a solution is enough to wash wells of unbound reagents. Some investigators leave washing solution in wells for a short time (soak time) after each addition (1¨C5 min). Sometimes detergents, notably Tween-20 (0.05%) are added to washing buffers. These can cause problems in which excessive frothing takes place producing poor washing conditions since air is trapped and prevents the washing solution from contacting the well surface. When using detergents, care must be taken that they do not affect reagents adversely (denature antigen), and greater care is needed to prevent frothing in the wells. The methods used for washing are given next.

2.1¡ª
Dipping Methods

The whole plate is immersed in a large volume of buffer. This method is rapid but is likely to result in cross-contamination from different plates. It also increases the cost of washing solution.

2.2¡ª
Wash Bottles

Fluid is added using a plastic wash bottle with a single delivery nozzle, which is easy and inexpensive. Here the wells are filled individually in rapid succession and then emptied by inverting the plate and flicking the contents into a sink or suitable container filled with disinfectant. This process is repeated at least three times. Wells filled with washing solution may also be left for about 30 s before emptying.

2.3¡ª
Wash Bottles Plus Multiple Delivery Nozzles

This is essentially as in **Subheading 2.** except that a commercially multiple delivery (usually 8) device is attached to the outlet of the bottle. This enable 8 wells to be filled at the same time.

2.4¡ª
Multichannel Pipets

The multichannel pipets used in the ELISA can be used to fill the wells carefully. The washing solution is contained in reservoirs .

2.5¡ª
Large Reservoir

The use of a large reservoir of washing solution is convenient. Here, a single or multiple nozzle can be connected to the reservoir via tubing so that the system is gravity fed. Care must be taken that large volumes of solution do not become microbially contaminated.

2.6¡ª
Special Hand-Washing Devices

Hand-washing devices are available commercially and involve the simultaneous delivery and emptying of wells by a handheld multiple-nozzle apparatus. These are convenient to use but require vacuum creating facilities. In washing plates manually, the most important factor is that each well receive the washing solution so that, e.g., no air bubbles are trapped in the well, or a finger is not placed over corner wells. After the final wash in all manual operations, the wells are emptied and then blotted free of most residual washing solution. This usually is accomplished by inverting the wells and tapping the plate on to an absorbent surface such as paper towels, cotton towels, or sponge material. Thus, the liquid is physically ejected and absorbed to the surface, which is soft and therefore avoids damage to the plate.

2.7¡ª
Specialist Plate Washers

Specialty plate washers are relatively expensive pieces of apparatus that fill and empty wells. Various washing cycles can be programmed. These are of great advantage when pathogens are being examined in ELISA because they reduce aerosol contamination. Most of the methods involving manual addition of solutions and emptying of plates by flicking into sinks or receptacles must be regarded as potentially dangerous if human pathogens are being studied, particularly at the coating stage if live antigen is used. Also, remember that live antigens can contaminate laboratories where tissue culture is practiced. The careful maintenance of such machines is essential because they are prone to machine errors such as a particular nozzle being blocked.

3¡ª
Addition of Reagents

Immunoassays involve the accurate dispensing of reagents in relatively small volumes. The usual volumes used in ELISA are in the range of 50 or 100 µL per well for general reagents, and 2¨C10 µL for samples. It is essential that the operator be fully aware of good pipetting technique and understand the relationships of grams, milligrams, micrograms, nanograms, and the equivalent for volumes, e.g., liters, milliliters and microliters. Thus, assays cannot be performed when there is no knowledge of how to make up, e.g., 0.1 *M* solutions. The ability to make accurate dilutions is also extremely important so that problems can be solved before you attempt ELISA or any other biological studies (e.g., having a 1/50 dilution of antiserum but needing to make up a 1.3500 dilution in a final volume of 11 mL).

3.1¡ª
Pipets

The microtiter plate system is ideally used in conjunction with multichannel microtiter pipets. Essentially they allow the delivery of reagents via 4, 8, or 12 channels and are of fixed or variable volumes of 25¨C250 µL.

Single-channel micropipets are also required that deliver in the range of 5¨C250 µL. Samples are usually delivered by microtiter pipets from suitably designed reservoirs (troughs) that hold about 30¨C50 mL of solution.

General laboratory glassware is needed such as 5- and 25-mL glass or plastic bottles, and 10-, 5-, and 1-mL pipets.

3.2¡[a]
Evaluating Pipet Performance

Pipetting errors are often a major cause of nonreliable test results in a diagnostic laboratory. A simple control technique is hereby proposed to circumvent this problem. At the beginning of each workday, the pipet should be checked for dust and dirt on the outside surfaces. Particular attention should be paid to the tip cone. No other solvents except 70% ethyl alcohol should be used to clean the pipet.

3.2.1¡[a]
Short-Term Performance Evaluation:
Control of Pipet Calibration Using Graduated Tips

The exercise will take only a few minutes, but it will make you absolutely confident of preventing pipetting errors related to the function of the pipet.

1. Set the volume of the pipet as indicated in the accompanying manufacturer's instructions.

2. Place a graduated tip firmly on the tip cone.

3. Aspirate the specified volume of distilled water into the tip.

4. Hold the filled tip in a vertical position for a few seconds.

Table 1
Example of Gravimetric Calibration Method Used for Three Specified Volumes Pipet (40¨C200 µL)

	Nominal volume		
	5:1	25:1	50:1
Measurement 1	5.12	25.08	50.21
Measurement 2	4.91	25.01	50.01
Measurement 3	5.07	25.27	50.19
Measurement 4	5.01	25.10	50.12
Measurement 5	4.98	24.89	50.00
Average	5.02	25.07	50.11
Accuracy (%)	0.36	0.28	0.21
Precision (%)	1.62	0.55	0.20

5. Check for leakage.

6. Check that the aspirated volume corresponds to the specified volume as indicated by tip graduation.

7. Repeat **steps 1¨C6** at least five times.

8. Practice the reversed and nonreversed pipetting techniques.

3.2.2¡[a]
Long-Term Performance Evaluation:
Control of Pipet Calibration Using Gravimetric Calibration Method

If the pipet is used daily, it should be checked every 3 mo. By means of the gravimetric calibration method (*see* **Table 1**), the pipet should be examined for leakage, accuracy, and precision.

Accuracy and precision can easily be calculated by the formulae in **Subheadings 3.2.2.1.** and **3.2.2.2.** In contrast to commercial pipet calibration computer software, the conversion factor for calculating the density of water suspended in air at the test temperature and pressure are not considered. For calibration of multichannel pipets, examine each channel separately.

3.2.2.1¡ª
Accuracy
(As Defined for This Exercise)

A pipet is accurate to the degree that the volume delivered is equal to the specified volume. Accuracy is expressed as the mean for replicate measurements:

$$\bar{E}\% = [(\bar{V} - V_0)/V_0] \times 100$$

$\bar{E}\%$ = accuracy; $\bar{V}$ = Mean volume; and V_0 = Nominal volume

3.2.2.2¡ª
Precision
(As Defined for This Exercise)

Precision refers to the repeatability of dispensed samples. It is expressed as the coefficient of variation (CV%).

$$\text{CV}\% = (S/\bar{W}) \times 100$$

in which S = standard deviation; and $\bar{W}$ = mean weighing.

3.2.2.3¡ª
Equipment Needed

The following equipment is needed:

1. Calibrated thermometer; to measure water temperature

2. Distilled water

3. Glass vessel with a volume 10 to 50 times that of the test volume

4. Analytical balance (calibrated?)

5. Pipet (labeled) and tips

Conduct the test on a vibration-free surface covered with a smooth, dark, nonglared material. Work in an area that is free of dust.

3.2.2.4¡ª
Procedure

1. Set the volume of the pipet as indicated in the accompanying manufacturer's instructions.

2. Place a pipet tip firmly on the tip cone.

3. Aspirate the specified volume of water into the tip.

4. Hold the filled tip in a vertical position for a few seconds and check for leakage.

5. Dispense the distilled water into a preweighed beaker and record the weight to the nearest tenth of a milligram. Repeat at least five times.

6. Calculate the results (accuracy, precision).

7. Record the results over time.

In theory, optimum accuracy and precision values approach zero. Note that the smaller the specified volume chosen for evaluation, the greater the effect of volume variation on accuracy and precision. Therefore, it is good laboratory practice to plot the results of accuracy and precision for a pipet's specified volume on a data chart over time.

Finally, you must decide which level of accuracy and precision can be met in your laboratory. This depends on what the pipet is used for. For the preparation of aliquots of serum bank samples, the pipetting error will not significantly matter. For ELISA, however, pipetting of a small, e.g., 10-µL volume for preparation of working conjugate dilutions requires the best accuracy and precision; bear this in mind because if you are pipetting 5 rather than 10 µL, you will obtain a double-diluted working conjugate dilution and, consequently,

Table 2
Troubleshooting of Pipetting Errors

Problem	Cause	Solution
Leakage	Tips are not compatible.	Choose compatible tips.
	Tips are incorrectly attached.	Attach firmly.
	Foreign bodies are between the piston O-ring and cone.	Clean the tip cone, and attach new tips. Clean and grease the O-ring and tip cone.
	Insufficient grease is on the tip cone and O-ring.	Apply grease.
Innaccurate	The pipet is incorrectly operated.	Follow instructions.
dispensing	Tips are incorrectly attached.	Attach tips firmly.
	High-viscosity fluids are not present.	Recalibrate the pipet using high-viscosity fluids.

an unreliable assay result. As a rule, a volume error of 1% for volumes ≥10 µL is still acceptable in a serodiagnostic laboratory.

3.2.2.5¡[a]
Recalibrating of Pipets

To recalibrate pipets, refer to the manufacturer's detailed instructions or contact the service representative.

3.2.3¡[a]
Pipet Troubleshooting

Table 2 provides the most common problems encountered with pipetting, possible causes, and solutions.

3.3¡[a]
Tips

After the microplate, the tips are the most important aspect of ELISA and also an expensive component. Many thousands of tips might be needed to dispense reagents. Many manufacturers supply tips, therefore care must be taken to find tips that fit the available microtiter pipets.

Multichannel pipet tips are best accessed by placing them in special boxes holding 96 tips in the microplate format. The tips can be purchased already boxed (expensive), and then the boxes can be refilled by hand with tips bought in bulk. Sterile tips are available in the box format. Generally, tips should not be handled directly by hand. When restocking boxes or putting the tips on pipets, plastic gloves should be worn to avoid contaminating the tips.

Tips for dispensing in single-channel pipets have to be carefully considered. In cases in which small volumes (5¨C20 µL) are pipeted, the pipet manufacturer's recommended tips should be used. It is essential that the tips fit securely on the pipets and that they can be pressed on firmly by hand (avoiding

their end). Particular care is needed when multichannel pipets are used to pick up tips from boxes since often one or two tips are not as securely positioned as the rest, which causes pipetting errors. The operator should always give a visual check of the relative volumes picked up.

When cost-efficiency is a factor, tips may be recycled after washing. However, it is not recommended that tips that have been in contact with any enzyme conjugate be recycled and these should not be placed with other tips used for other stages in ELISA. The washing of tips should be extensive, preferably in extensive acid or strong detergent solutions, followed by rinsing in distilled water. The cost/benefit of washing must be examined carefully, because the production of sufficient quantities of distilled water can be expensive. Tips should be examined regularly and damaged ones should be discarded. **Figure 3** illustrates some practical aspects of pipetting in ELISA. Note that the training in pipetting techniques is extremely vital to the successful performance of ELISA.

3.4¡ª
Other Equipment

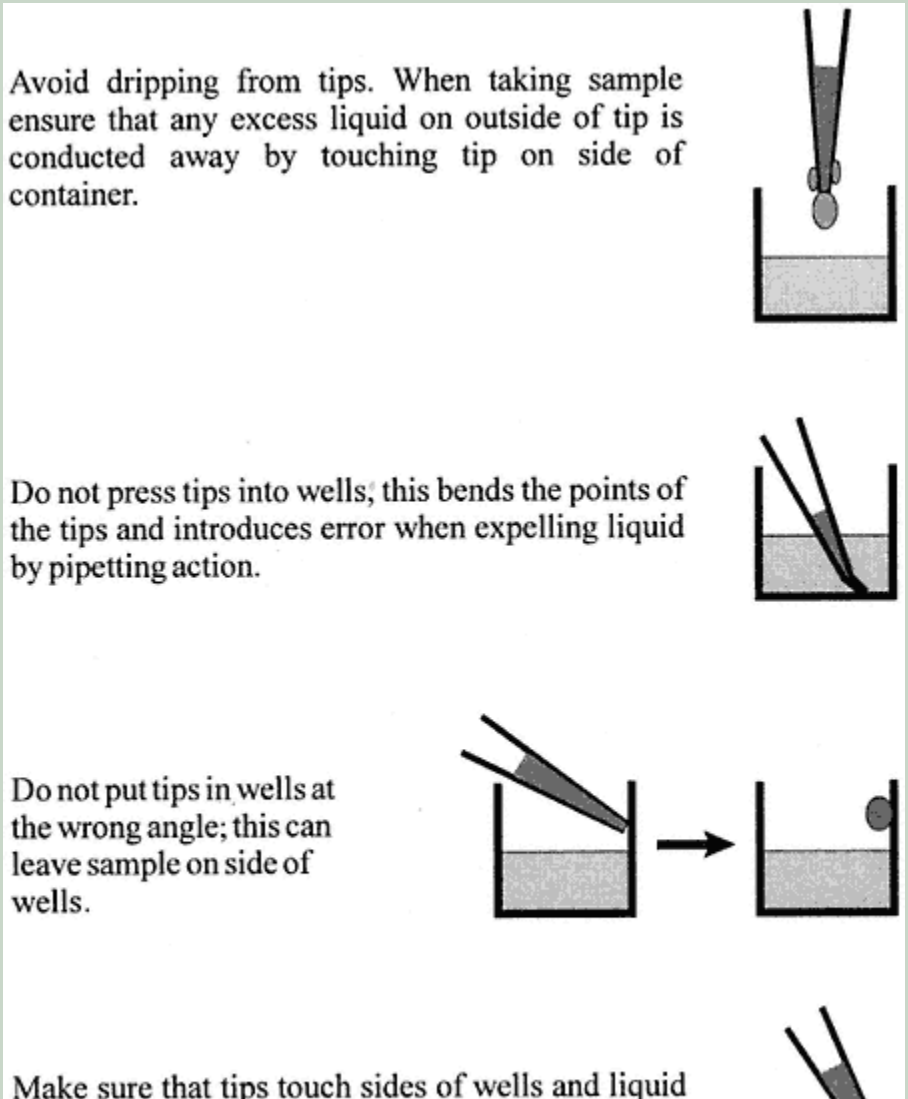
Several manufacturers supply microtiter equipment to aid multichannel pipetting, including tube and microtip holders. The former consists of a plastic box that carries 96 plastic tubes with a capacity of about I mL. The tubes are held in exactly the same format as a microtiter plate so that samples can be stored or diluted in such tubes and multichannel pipets can then be used for rapid transfer from the tubes. The tip holders involve the same principle, whereby tips for the multichannel pipets are stored in the 96 well format so that they can be placed on to multichannel pipets rapidly in groups of 8 or 12. Various reservoirs with 8 or 12 channels for separation of reagents are also available. These are useful for the simultaneous addition of separate reagents.

4¡ª
Incubation

The reaction between antigens and antibodies relies on their close proximity. During ELISA, this is affected by their respective concentrations, distribution, time and temperature of incubation, and pH (buffering conditions). In any interaction, the avidity of the antibodies for the particular antigen(s) is also important. Two types of incubation conditions are common: (1) incubation of rotating plates (with shaking) and (2) incubation of stationary plates. These conditions affect the times and temperatures required for successful ELISAs and therefore discussed separately.

4.1¡ª
Incubation of Reagents While Rotating Plates

The effect of rotating plates is to mix the reactants completely during the incubation step. Since the solid phase limits the surface area of the adsorbed



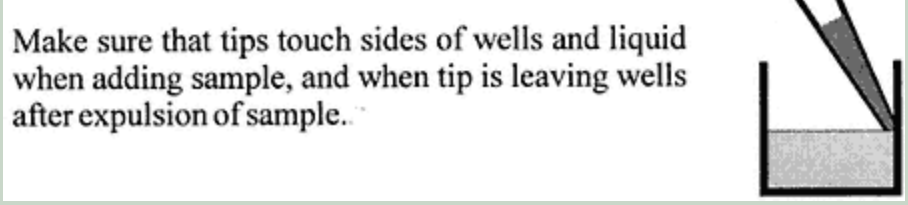


Fig. 3.
Factors influencing proper dispensing of samples into microtiter plate wells.

reactant, mixing ensures that potentially reactive molecules are continuously coming into contact with the solid phase. During stationary incubation, this is not true and mixing takes place only owing to diffusion of reagents. Thus, to allow maximum reaction from reagents in stationary conditions, greater incubation times may be required than if they are rotated. This is particularly notable when highly viscous samples, e.g., 1/20 serum, are being examined. This represents 5% serum proteins, and diffusion of all antibodies on to the solid phase may take a long time. This can be avoided if mixing is allowed throughout incubation. Similarly, when low amounts of reactant are being assayed, the contact time of the possibly few molecules that have to get close

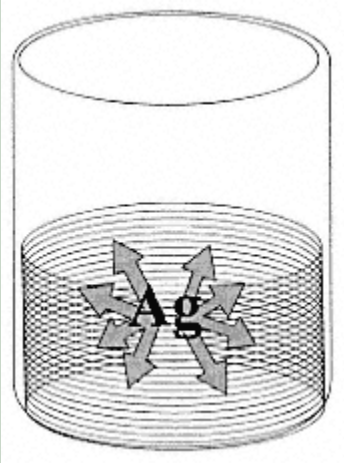


Fig. 4.
Continuous mixing enables maximum contact of molecules in liquid phase with those on solid phase. This alleviates the following problems of: temperature¡ª the close approximation of antigen and antibody is necessary to allow binding. Increasing temperature under stationary conditions increases diffusion rate of molecules and variations in temperature will affect rate and variation in the test (e.g., stacking plates); variation in handling¡ª plates may be unequally moved during incubation causing unequal mixing. Different operators use different techniques that are not controllable (e.g.., plates may be tapped or other operators may move the pates); viscosity effects¡ªwhen samples are of different viscosities, this may affect diffusion of molecules under stationary conditions; times of incubation¡ªthese can be reduced under rotationary conditions; and detection of low concentrations¡ªthis is increased by rotation.

to the solid-phase reactant is greatly enhanced by mixing throughout incubation. Simple and very reliable rotating devices are available with a large capacity for plates. Shakers with limited capacity (e.g., four plates) are also available.

Figure 4 gives the advantages of rotation. Rotation allows ELISAs to be performed independently of temperature considerations. The interaction of antibodies and antigen relies on their closeness, which is encouraged with the mixing during rotation. Stationary incubation relies on the diffusion of molecules and thus, is dependent on temperature. Therefore standardization of temperature conditions is far more critical than when rotation is used.

The effect of temperature also has implications when many plates are stacked during incubation since the plates heat up at different rates depending on their position in the stack. The wells on the inside may take longer to equilibrate than those on the outside, which has a direct effect on the diffusion conditions, which, in turn, affects the ELISA. This is negated by rotation because there is the same chance of molecular contact in all wells.

4.2¡ª
Stationary Incubation Conditions

Assays may be geared to stationary conditions, although the exact times and temperatures of incubation may vary. The temperatures for incubation are most commonly 37¡ãC, room temperature (on the bench), and 4¡ãC. Usually the time of incubation under stationary conditions reflects which incubation temperature is used. Therefore, at 4¡ãC, a longer incubation might be given (overnight). In general, most incubations for stationary assays involving the reaction of antigen and antibodies are 1¨C3 h at 37¡ãC. Sometimes these conditions are combined so that one reagent is added for, say, 2 h at 37¡ãC followed by one overnight at 4¡ãC, usually because this produces a convenient work schedule. When incubation is performed at room temperature, care must be taken to monitor possible seasonal variations in the laboratory, since temperatures can be quite different, particularly in nontemperate countries. Direct sunlight should also be avoided, as must other sources of heat, such as from machinery in the laboratory. As already stated, how the plates are placed during stationary incubation should be considered. Ideally, they should be separated and not stacked.

In assays, the plates should also be handled identically and there should be no tapping or shaking of the plates (including accidental nudging or movement by other personnel), because this will allow more mixing and interfere with the relative rate of diffusion of molecules in different plates. Regular handling can be a primary cause of operator-to-operator variation.

Under mixing conditions, most antigen/antibody reactions are optimum after 30 min at 37¡ãC, so that assays can be greatly speeded up with no loss in sensitivity. This is not true under stationary conditions. Care must be taken to consider the types of antibodies being measured under various conditions since ELISAs rarely reach classical equilibrium conditions. **Figure 5** illustrates the factors affecting ELISA under stationary conditions.

5¡ª
Blocking Conditions and Nonspecific Reactions

Measures must be taken to prevent nonspecific adsorption of proteins to wells from samples added after the coating of the solid phase before, during, or as a combination of both times. Nonspecific adsorption of protein can take place with any available plastic sites not occupied by the solid-phase reagent. Thus, if one is assessing bovine antibodies in bovine serum, and bovine proteins other than specific antibodies bind to the solid phase, antibovine conjugate will bind to these and give a high background color.

Two methods are used to eliminate such binding. One is the addition of high concentrations of immunologically inert substances to the dilution buffer of the added reagent. Substances added should not react with the solid-phase antigen nor the conjugate used. Commonly used blocking agents are given in **Table 3,** and they act by competing with nonspecific factors in the test sample

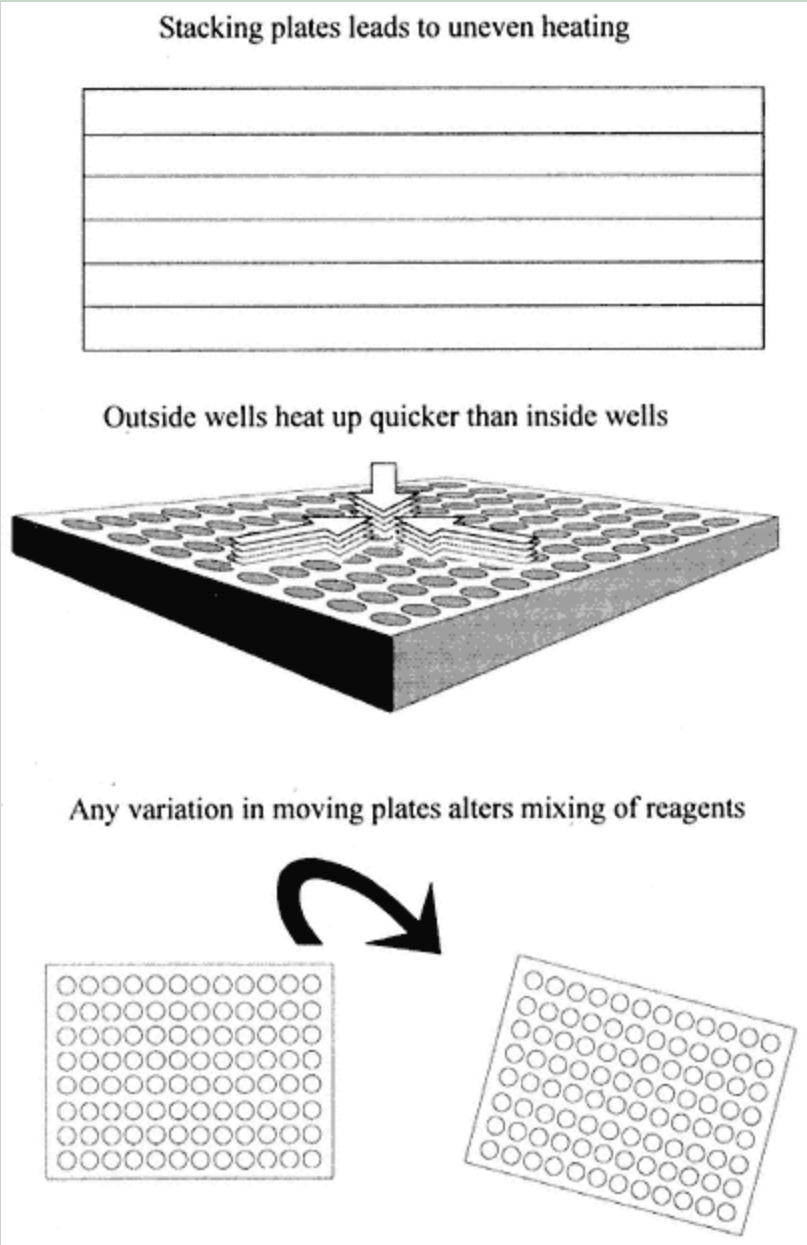


Fig. 5.
Effects when incubating stationary plates.

for available plastic sites. The concentration used often depends on the dilutions of the test samples; thus, if 1/20 serum is being tested (5% protein) then blocking agents have to be at high-concentration to compete successfully, or have an increased binding potential as compared to the nonspecific substance. Such blocking agents can also be added as a separate step before the addition of the sample; this increases the competing ability of the blocker. Nonionic detergents have also been used to prevent nonspecific adsorption. These are used at low concentration so as to allow interaction of antigen and antibody. Occasionally, both detergents and blocking substances are added together.

Table 3
Commonly Used ELISA Blocking Agents

Protein	Reference
Normal rabbit serum	***6***
Normal horse serum	***7***
Human serum albumin	***8***
Bovine serum albumin	***9***
Fetal calf serum	***10***
Casein	***11***
Casein hydrolysate	***12***
Gelatin	***13***
Detergents	
—Tween-20	***9***
—Tween-80	***14***
—Triton X-100	***15***
—Sodium dodecyl sulfate	***15***
Other	
—Dextran sulfate	***7***
—Coffee mate	***16***
—Nonfat dried milk	***17***

The best conditions for individual assays are only assessed in practice; however, the cost of such reagents should be taken into account. Skimmed milk powder (bovine source), has been used successfully in many assays and is quit inexpensive. But, that certain blocking agents may be unsuitable for different enzyme systems. For example, skimmed milk cannot be used in urease-directed ELISA, or when biotin/avidin systems are used. Contaminating substances such as bovine immunoglobulin (IgG) in bovine serum albumin (BSA), may eliminate the use of certain blocking agents from different suppliers. Most assays are validated under stated blocking conditions. However, investigators may adapt assays for use with other blocking reagents in which prescribed substances prove unobtainable or expensive.

5.1¡ª
Nonspecific Immunological Mechanisms

Reactions between solid-phase, positively charged basic proteins and added reagents owing to ionic interactions have been described ***(15)***. This was removed by the addition of heparin or dextran sulfate in the diluent. The positive charges could also be removed by the addition of a low concentration of an anionic detergent sodium dodecyl sulfate [SDS]). Such interactions have been noted for conjugates, which, although they do not bind to uncoated plates, do bind strongly to

those containing antigens. The addition of a variety of blocking buffers, e.g., containing BSA, Tween-20, or casein, does not overcome the problem. Usually a high concentration of nonimmune serum from the same species as that in which the conjugate was prepared is necessary to prevent such a reaction.

5.2¡ª
Immunological Mechanisms

There are a large number of reports in which antibody/antigen reactions have been noted where they should not have occurred. These can be termed *aspecific reactions* and are of an immunological nature. These are antibodies that are naturally present in serum which bind to antibodies from other species. They are not present in all sera and, consequently, cause problems in ELISA. For example, human heterophilic antibodies have been demonstrated against a common epitope on the $F(ab')^2$ fragment of IgG from bovine, ovine, equine, guinea pig, rat, and monkey species ***(18)***. Methods of overcoming such antibodies include the use of $F(ab')^2$ as capture antibody in which the heterophilic reaction is against the Fc portion of IgG, or the use of high levels of normal serum obtained from the same species as the ELISA antibody in the blocking buffer. A review of heterophilic antibodies, is given in **ref. *18***.

5.3¡ª
Interference by Rheumatoid Factor

Rheumatoid factor (RF) can cause a high level of false positives in the indirect ELISA. The factors are a set of the IgM class of antibodies that are present in normal individuals but are usually associated with pathological conditions. They bind to the Fc portion of IgG antibodies that either are complexed with their respective antigen or are in an aggregated form. Thus, any solid-phase/ IgG/RF will be recognized by conjugates which recognize IgM and produce a false positive. Conversely, the binding of the RF to the antigen/IgG complex has been shown to interfere with the binding of IgG-specific conjugates, producing a lower or false reaction in ELISAs.

5.4¡ª
Miscellaneous Problems

Many sera contain antibodies specific for other animal serum components; for example, antibovine antibodies are commonly present in human sera ***(19)***. Care must be taken when dealing with conjugates that may have unwanted cross-reactions of this type. Many conjugates are pretreated to adsorb out such unwanted cross-species reactions. Reagents are available for this purpose in which various species serum components are covalently linked, e.g., to agarose beads. These are added to sera, incubated for a short time, and then centrifuged into a pellet. Such beads can be reused after a treatment, which breaks the immunological bonds between the antigen and serum component with which it reacted. Such solid-phase reagents are more advantageous than meth-

ods in which normal sera are added to absorb out activities because the antibody molecules are totally eliminated from the solution.

5.5¡ª
Treatment of Samples

Many laboratories routinely heat sera to 56¡ãC. This can cause problems in ELISA and should not be pursued. Heating can cause large increases in nonspecific binding to plates. (*See* **ref. *8***).

6¡ª
Enzyme Conjugates

Intrinsic to the ELISA, is the addition of reagents conjugated to enzymes. Assays are then quantified by the buildup of colored product after the addition of substrate or a combination of substrate and dye. Usually antibodies are conjugated to enzymes; some methods are given subsequents. Other commonly used systems involve the conjugation of enzymes to pseudoimmune reactors such as proteins A and G (which bind to mammalian IgGs), and indirect labeling using biotin/avidin systems. Four commonly used enzymes will be described. **Tables 4** and **5** give the properties of enzymes, substrates, and stopping conditions.

6.1¡ª
Horseradish Peroxidase Plus Hydrogen Peroxide Substrate

Horseradish peroxidase (HRP) plus hydrogen peroxide substrate is widely used. HRP is a holoenzyme of mol wt 40,200 containing one ferritprotoprotein group per molecule. The apoenzyme is a glycoprotein of 308 amino acids and 8 neutral carbohydrate side chains attached through asparigine residues. The polypeptide chain alone has a mol wt of 33,890 and it has 4 disulfide linkages. The covalent structure consists of two compact domains sandwiching the hemin group. Seven isoenzymes of HRP have been isolated. Isoenzyme C (p*I* 8.7¨C9.0) apoprotein (6.8) is the main cationic form-constituting about 50% of the commercially available highly purified HRP. The reaction mechanism and spectral changes of peroxidase catalysis are complex, involving peroxidatic, oxidatic, catalytic, and hydroxylactic activities. For a full explanation see **ref. *20***. The substrate hydrogen peroxide is also a powerful inhibitor, so defined concentrations must be used. The reduction of peroxide by the enzyme is achieved by hydrogen donors that can be measured after oxidation as a color change. The choice of converted substrates that remain soluble is essential in ELISA so that optimal spectrophotometric reading can be made. Commonly used chemicals are given next.

6.1.1¡ª
Ortho-*Phenylenediamine*

Ortho-phenylenediamine (OPD) is prepared as a solution of 40 mg of donor/ 100 mL of 0.1 *M* sodium citrate buffer, pH 5.0. Preweighed tablets are available commercially.

Table 4
Substrates and Chromophores Commonly Used in ELISA

Enzyme label (mol wt)	Substrate	Chromophore	Buffer
HRP (40,000)	Hydrogen peroxide (0.004%)	OPD	Phosphate/citrate, pH 5.0
	Hydrogen peroxide (0.004%)	TMB	Acetate buffer, 0.1 *M*, pH 5.6
	Hydrogen peroxide (0.002%)	ABTS	Phosphate/citrate, pH 4.2
	Hydrogen peroxide (0.006%)	5-AS	Phosphate, 0.2 *M*, pH 6.8
	Hydrogen peroxide (0.02%)	Diaminobenzidine	Tris or PBS, pH 7.4
AP (100,000)	pnpp (2.5 mM)	pnpp	Diethanolamine (10 m*M*) and magnesium chloride (0.5 m*M*), ph 9.5
β-Galactosidase (540,000)	ONPG (3 m*M*)	ONPG	Magnesium chloride and 2-mercaptoethanol in PBS, pH 7.5
Urease (480,000)	Urea	Bromocresol	pH 4.8

Table 5
Enzyme Labels, Chromophores, and Stopping Conditions in ELISA

		Color		Reading (nm)		
Enzyme label	System	Not stopped	Stopped	Not stopped	Stopped	Stop solution
HRP	OPD	Green/orange	Orange/brown	450	492	1.25 *M* sulfuric acid
	TMB	Blue	Yellow	650	450	SDS (1%)
	ABTS	Green	Green	414	414	No stop
	5-As	Brown	Brown	450	450	No stop
	Diaminobenzidine	Brown	Brown	N/A	N/A	No stop
AP	pnpp	Yellow/green	Yellow/green	405	405	2 *M* sodium carbonate
β-Galactosidase	ONPG	Yellow	Yellow	420	420	2 *M* sodium carbonate
Urease	Urea bromocresol	Purple	Purple	588	588	Merthiolate (1%)

6.1.2¡ª
2,2'-Azino Diethylbenzothiazoline Sulfonic Acid

2,2'-Azino diethylbenzothiazoline sulfonic acid (ABTS) is prepared at the same concentration as OPD but in 0.1 *M* phosphate/citrate buffer, pH 4.0. Tablets are available.

6.1.3¡ª
5-Aminosalicylic Acid

Commercial 5-aminosalicylic acid (5-AS) is dissolved in 100 mL of distilled water at 70¡ãC for about 5 min with stirring. After cooling to room temperature, the pH of the solution is raised to 6.0 using a few drops of I *M* sodium hydroxide.

6.1.4¡[a]
Tetramethyl Benzidine

The optimum substrate (hydrogen peroxide) concentration of tetramethyl benzidine (TMB) depends on the hydrogen donor and the solid phase. This is usually established in preliminary tests, but concentrations between 0.010 and 0.0005% are adequate. Hydrogen peroxide is available as 30% commercially. The development of the colored product is measured at different wavelengths. The optimum wavelength may also shift if the reaction is stopped by a blocking reagent to prevent change of the optical density (OD) after a reaction period. The stopping reagents involving HRP are solutions of hydrochloric or sulfuric acid for OPD and TMB, SDS for ABTS. The optimal wavelengths for reading are 415 nm for ABTS; 492 nm for acidified OPD (420 nm for nonacidified OPD); 492 nm for 5-AS; and 655 nm for TMB (unstopped), 450 nm (acidified).

6.2¡[a]
Alkaline Phosphatase Plus p-*Nitrophenylphosphate*

Alkaline phosphatase (ALP) used in immunoassays generally comes from bovine intestinal mucosa or from *Escherichia coli*, and these sources differ considerably in their properties. ALPs are dimeric glycoproteins, and all their zinc metalloenzymes contain at least two Zn (II) per molecule. See **ref. *20*** for a detailed explanation of their structure and reaction mechanisms. ALPs are assayed in buffer depending on the source of the enzyme. For bacterial enzyme, 0.1 *M* Tris-HCl buffer, pH 8.1, containing 0.01% magnesium chloride is used. For intestinal mucosal enzyme a 10% (w/w) diethanolamine (97 mL in 1 L of a 0.01% magnesium chloride solution) buffer pH 9.8 (adjusted with HCl), is used. *p*-Nitrophenylphosphate (pnpp) is added just before use (available as preweighed pellets) to 1 mg/mL. The production of nitrophenol is measured at 405 nm. The reaction is stopped by the addition of 0.1 vol of 2 *M* sodium carbonate. Note that inorganic phosphate has a strong inhibitory effect on AP and therefore PBS or similar buffers are to be avoided.

Other chromogenic substrates can also be exploited, including phenolphthalein monophosphate, thymophthalein monophosphate, β-glycerophosphate, and uridine phosphate. Fluorigenic substrates can also be used such as β-naphthyl phosphate, 4-methylumbelliferyl phosphate, and 3-*o*-methylfluorescein monophosphate ***(21)***.

6.3¡[a]
β-*Galactosidase Plus o-Nitrophenyl*-β-D-*Galactopyranoside*

The reagent is prepared by the addition of a solution containing 70 mg of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per 100 mL of 0.1 *M* potassium phosphate buffer, pH 7.0, containing 1 mM magnesium chloride and 0.01 *M* 2-mercaptoethanol. The reaction may be stopped by the addition of 0.25 vol of 2 *M* sodium carbonate.

6.4¡[a]
Urease, pH Change, and Bromocresol Purple Indicator

The reagent is prepared by the addition of a weakly buffered solution of urea (pH 4.8) in the presence of bromocresol purple. The urea is hydrolyzed to liberate ammonia in the presence of urease, which raises the pH of the solution, resulting in a color change from yellow to purple. The reaction can be stopped by the addition of 10 µL of a 1% solution of merthiolate® (thimerosal) to each well. **Tables 4** and **5** summarize the properties of various enzyme systems.

7¡[a]
Availability of Conjugates

Conjugates may be obtained commercially or made in individual laboratories. Great care must be exercised in using the appropriate reagent in any assay. Thus, the immunological implications of various reagents must be considered and information sought. Many conjugates are directed against different species serum components. These are some of their features:

1. The species in which the antiserum is produced can be important. Thus, donkey anticow IgG denotes that a donkey has been used to prepare antiserum against cow IgG. Other examples are rabbit antipig and pig, antidog.

2. The preceding description must be refined since both the donating serum and the immunogen are probably subjected to immunochemical treatments. Thus, the donating serum can be crudely fractionated before conjugation (which is usual), or may be affinity purified, so that the conjugate is 100% reactive against the immunogen. Examples are: donkey IgG anticow IgG or donkey IgG (affinity purified) anticow IgG.

These descriptions deal with the processing of the donor serum after production and before conjugation. The description of the immunogen is also sometimes critical. Using the last example, the cow IgG may have itself been affinity purified before injection of the donkey, or specific parts of the Ig purified to raise a subclass specific antiserum, for example, heavy chain IgG may have been

injected into the animal so that the conjugate only reacts with IgG. Remember that all Ig classes share antigens, so that it is highly likely that an antiserum raised against IgGs of any species will detect IgA, IgM, as well as IgG. Thus, the specificity of the conjugate has to be considered in any assay.

3. Different sources and batches of conjugates from the same manufacturer may vary. Thus, in large-scale applications, it is good practice to obtain a successful batch sufficient for all future testing. The anti-IgG one obtains from commercial company A may give different results from that obtained from company B.

4. Conjugates must be titrated to optimum conditions and not used in excess. This is vital to obtain reliable results.

8$_i^a$
Conjugation with Enzymes

The analytical sensitivity of the ELISA depends on the ability of the antibody to bind and the specific enzyme activity of the labeled immunoreactant, the conjugate. The linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if any chemical modification of the moieties involved alters the antigenic determinants or the reactive sites on antibody molecules. Thus, chemical methods that do not affect these parameters have been chosen. Most of the techniques are straightforward and can be readily used by nonspecialists interested in developing their own enzyme immunoassays.

Not only must the immunoreactivities be maintained after conjugation, but also the catalytic activity of the enzyme. Following conjugation it is necessary to test the immunoreactivity to determine whether it has the desired specification. Before use in ELISA, it may be necessary to purify the conjugates to remove unconjugated antigen or antibody and free enzyme. Reagents used to produce conjugates are numerous and their mode of action is to modify the functional groups present on proteins. Antigens that are nonproteinaceous (e.g., steroids) can be conjugated with different means and are not dealt with here. Enzymes are covalently bound to reagents either directly by reactive groups on both the enzyme and reagent or after introduction of reactive groups (e.g., thiol or maleimid groups) indirectly via homo- or heterobifunctional reagents in two-step procedures ***(22)***. The requirements for optimal conjugation are as follows:

1. Simplicity and rapidity.

2. Reproducibility (obtaining constant molar ratio of enzyme and reagent).

3. High yield of labeled reagent and low yield of polymers of enzyme and reagent.

4. Low-grade inactivation of reagent and enzyme.

5. Simple procedures f or separation of labeled and unlabeled reagents.

6. Long-term stability without loss of immunological and enzymatic activities.

9$_i^a$
Development of Label

The substrate is usually chosen to yield a colored product. The rate of color development will be proportional, over a certain range, to the amount of

enzyme conjugate present. On a kinetic level, reactions are distinguished by their kinetic order, which specifies the dependence of reaction rate on the concentration of reactants. Under the conditions generally employed in ELISA, the reaction exhibits zero order with respect to the substrate. Too little substrate will limit the rate of production of product. Thus, sufficient substrate must be present to prevent the substrate and/or cofactors from being rate limiting. In cases in which substrate and chromogenic hydrogen donors are necessary for color development, the concentrations of both must be assessed to obtain optimum conditions.

The product must be stable within a defined time, and products that are unstable in bright light or at temperatures at which the assay is performed should be avoided. The physicochemical parameters that affect the development of color include the following:

1. Buffer composition and pH.

2. Reaction temperature.

3. Substrate and/or cofactor concentration and stability, product stability, and enzyme stability.

4. Substrate and product stability.

9.1¡ª
Horseradish Peroxidase

HRP is active over a broad pH range with respect to its substrate, hydrogen peroxide, however, the optimum pH for the development of label in the ELISA will vary depending on the chromogenic donor. Changing the pH will reduce the reaction rate, but will not affect the reaction kinetics; for example, increasing the pH to 5.0 for ABTS will slow down the rate of reaction (pH optimum 4.0), but will not affect the linearity of the kinetics. The majority of the buffers used in substrate formulation are of low-molarity citrate base. Because the reaction kinetics are dependent on pH, a stable buffering capacity is essential. The stability of HRP varies in different buffers, being more stable in 0.1 *M* citrate than 0.1 *M* phosphate buffer. High-molar phosphate buffer can be particularly damaging to HRP at low pH. Nonionic detergents exert a stabilizing effect on the enzymic activity of HRPO, and this can be enhanced by increasing reaction temperatures. Detergents have also been demonstrated as having a stabilizing effect on the enzymes.

9.2¡ª
Alkaline Phosphatase

AP is active at alkaline pH, optimum above pH 8.0. The buffer used with the substrate pnpp is diethanolamine/HCl, pH 9.6. Inorganic Mg^{2+} is essential for enzymatic activation. Nonionic detergents appear to have no effect on enzyme activation, substrate catalysis, or product development. Inactivation of the enzyme on contact with microplates does not occur.

9.3¡ª
Urease

Urease is enzymatically active over a broad pH range. The specificity of urease for its substrate (urea) is almost absolute. The urease substrate solution contains urea and a pH indicator, bromocresol purple, at pH 4.7. The urease catalyzes the urea into ammonia and bicarbonate, and the released ammonia causes an increase in pH that changes the color of the indicator from yellow to purple. The generation of color is not directly related to the amount of urea catalyzed. Because the color development is dependent on pH, it is essential to check that the pH is accurate before addition to wells in a test. It is also essential that no alkaline buffers remain after, e.g., washing (pH 7.4, PBS) because this will cause a change in color, and plates must be washed finally in water if PBS is the usual washing buffer.

9.4¡ª
Reaction Temperature

Between-well variation in an assay can cause differential rates of color development. Similarly, varying temperatures in the performance of the assay can cause variation. It is advisable, therefore, that substrates be added at a defined temperature and that plates be incubated under uniform conditions¡ªnormally room temperature. Note that this definition is rather loose and that each laboratory should be assessed since temperature can vary greatly in different countries. The best practice is to add substrate solutions at a defined temperature obtained by using solutions heated to (or cooled) to that defined temperature. This is particularly important when attempting to standardize assays among operators and laboratories in which a fixed time for stopping an assay is used.

9.5¡ª
Substrate/Cofactor Concentration and Stability

As already stated, substrate concentrations must be optimized. This is usually stated for particular systems (literature, kits, and so forth). Certain solutions can be made and stored. As an example, OPD can be made up in buffer, stored frozen in well-sealed vials, and then be thawed and used (after the addition of H_2O_2). This negates the need to weigh out small amounts of OPD for small volumes of substrate solution and aids standardization of assays. The use of preweighed chemicals in the form of tablets available commercially also greatly improves the accuracy and convenience of producing substrate solutions, although these tablets are expensive.

9.6¡[a]
Product Stability

Once the substrate has been catalyzed and a colored product achieved, it is essential that the color remain stable. In the majority of ELISAs, positive results are read by eye or by spectrophotometer as the intensity of color (OD) as com-

pared to a series of previously worked out negative values. An unstable colored product would affect the buildup of color. For spectrophotometric reading of results, it is vital that the product color remain stable without shifting the absorption spectrum, as the microplate readers assess the absorbance of the colored addition of a reagent preventing further enzymatic activity. This is dealt with below.

9.7¡[a]
Enzyme Stability

The enzymes used in the ELISA are stable with respect to their activity with defined substrates. Thus, a high degree of consistency is found using the same batch of conjugate under defined conditions.

9.8¡[a]
Substrate and Product Stability

Substrates are only soluble to a limited extent in aqueous buffers. The use of mixed aqueous/organic buffers is possible. These solvent systems can allow significantly greater amounts of substrates to be incorporated into solution and also allow their use in microplate ELISAs. Partially or totally insoluble products can be used in variants of ELISA, e.g., in the staining of sections in immunohistochemistry in which insoluble products localize the area of antigen or antibody reaction.

10¡[a]
Stopping Reactions

Reagents are added to prevent further enzymatic reaction in ELISA. This is performed at a time as determined for the specific assay. This process is usually called stopping and the reagent used is the stopping reagent. Stopping is usually done at a time when the relationship between the enzyme-substrate-product is in the linear phase. Molar concentrations of strong acids or strong bases stop enzymic activity by quickly denaturing enzymes. Other stopping reagents are enzyme specific. Sodium azide is a potent inhibitor of HRP, whereas EDTA inhibits AP by the chelation of metal ion cofactors.

Since the addition of stopping agents may alter the absorption spectrum of the product, the absorption peak must be known. For example, OPD/ELISAs stopped by sulfuric acid are read at 492 nm (450 nm before stopping). **Table 5** gives the wavelengths for reading the appropriate substrates before or after the addition of stopping agents. The addition of stopping agents can also increase the sensitivity of an ELISA. In the addition of stopping reagent, the volumes must be kept accurate, since photometric readings are affected if the total volume of reactants varies.

11¡[a]
Reading

As the product of substrate catalysis is colored, it can be read in two ways: (1) by eye inspection or, (2) using a spectrophotometer.

11.1¡ª
Reading by Eye

ELISAs can be designed for use with either reading by eye or a spectrophotometer although different conditions and controls may have to be included. The principles of ELISA must be thoroughly understood before either system is adopted. In particular testing by eye is not necessarily simpler to standardize. However, when correct standardization is used, it offers sensitive assays. When a correct plate template is used, the range of color product will be from full through partial color to no color.

Known, strong positive samples will give strong color, weak positives will give partial color, and negatives will give no color, or that of negative wells. Controls of this sort must be incorporated into the intended assays. Some difficulties arise in differentiating weak positives from negatives by eye. The interpretation of tests by eye can vary from operator to operator, and, hence, results are more subjective than by using a spectrophotometer. Some substrate/enzyme combinations favor reading by eye.

In cases in which tests have to be read by eye (when instrumentation is not available), the best assays can be produced in other laboratories that can quantify reagents using machine reading and evaluate the parameters of the reading by eye. As an example, a negative population of sera can be examined, and control negative sera, reflecting different parts of the negative OD distribution, can be adopted for controls by eye. Thus, a serum having the highest OD value may be selected as the negative control. Any sera giving discernible results by eye higher than this serum would therefore be assessed with high confidence as being positive. Assays that require comparison of closely related data, such as competition assays, are not suitable for interpretation by eye, e.g., in which the competition slope is compared.

11.2¡ª
Spectrophotometric Reading

The product of the substrate catalysis by enzyme is measured by transmitting light of a specific wavelength through the product and measuring the amount of adsorption of that light, if any, with a machine. Because different products are produced in ELISA, care must be taken to select appropriate filters for the detection of the correct wavelengths. Although microcuvets and conventional spectrophotometers can be used for this purpose there are laborious in cases in which large numbers of samples are measured. Special machines are available for the reading of colored products in microplates. These read the absorbance of each well at a preselected wavelength of light. Either one well can be read at a time (manual readers), or more suitably, a column of eight wells can be read simultaneously (semiautomatic or automatic multichannel spectrophotometers).

For semiautomatic readers, the wavelength filters are added manually, whereas for automatic readers, the wavelength filter(s) are contained as an internal filter wheel and can be selected from a control panel. In the main, the results from such machines are expressed as absorbance units and are recorded on paper rolls, or through interfacing with a computer. Various (limited but useful) ways of processing the data is usually available, such as the expression of the absorbance values as a matrix or as plus and minus against control wells or values given to the machine. Most readers can be connected to computers, and a range of software (commercial and private) is available to manipulate and store data. This is important in large-scale sample handling, or in which complicated arithmetic routines are performed on the data. An important feature of the ELISA having a colored product that can be examined by eye is that tests can be rapidly assessed before machine reading; one can see that a test has or has not worked at a glance. Thus, extensive reading time is not wasted if a careless mistake has occurred, unlike radioimmunoassay, in which it is essential to count samples before results are obtained. Such assessment by eye is also convenient when "sighting" experiments are being conducted during development of assays.

12¡ª
Practical Problems

This section discusses problems associated with the practical aspects of ELISAs that have been observed under different laboratory conditions.

12.1¡ª
Overall Observations on Running ELISA

The major cause of problems in running an ELISA is the scientist(s) involved. This has been demonstrated graphically through my involvement in training and supplying kit reagents to many laboratories worldwide. The main problem is lack of close contact training in the fundamentals of ELISA so that the scientist has the experience to identify and then solve problems in the use of reagents. There is no substitute for good training.

12.1.1¡ª
Problems Caused by Lack of General Scientific Knowledge

The biological implications of results cannot be assessed without general knowledge of several fields of science, such as, epidemiology, immunochemistry, biochemistry, and immunology, as indicated in Chapter 1.

12.1.2¡ª
Problems Are Caused by Sloppy Technique

The reproducibility of any assay relies, in part, on the accuracy of the investigators involved. This is further complicated when many people perform the assay (e.g., in a laboratory concerned with large-scale testing of sera on a routine basis). Attempts should be made to provide individual assessments to

improve reliability of techniques (e.g., accurate pipetting and timing). Quality control and quality assurance protocols should be developed, as discussed in Chapter 9.

12.1.3¡ª
Common Problems of Instrumentation and Reagents

Not all problems can be blamed directly on the operator. Although the individual steps of ELISAs are relatively simple, assays can be regarded as complex in that several steps with different reagents (all of which have to be standardized) must be made. This increases the likelihood of problems in any methodology. Reagents also have to be stored and are subject to contamination by microorganisms or from other operators introducing unwanted reagents through the use of contaminated pipet tips. A surprisingly common error is the reading of wells at the wrong wavelength. Operators must learn to read the color by eye as well as to read the OD. In cases in which operators have never seen an ELISA, then this can be excused, although when a plate does show "decent" color, the OD readings should be giving a range of values for any substrate of about 1 OD. If plates showing a strong to moderate color as assessed by eye, but only low OD readings (0.1¨C0.3 maxima), then the wavelength should be checked. Confusion about the filter can be the result of other operators placing filters in machines for other systems as well as not knowing where a particular wavelength filter is on a filter-wheel machine. Often software programs also indicate a wavelength on the readout that is not associated with the machine, but merely the wavelength at which the test should be read.

12.1.4¡ª
Water

Water can be a major problem in standardization of assays among different laboratories even when identical reagents are used. Thus, some kits supply water, at least for the initial dilutions of the stock reagents. The reasons why water affects the ELISA have not been extensively examined and no single factors have emerged as being most important. It is a good idea to use triple-distilled water, but it is not always available to less well-equipped laboratories. The type of problem encountered is that of readings higher than expected using control sera as well as plate blanks. The supply of tested water for the preparation of buffers for the initial dilutions of reagents will solve this problem. Operators should also obtain supplies from other laboratories to examine whether they solve observed problems.

12.1.5¡ª
Laboratory Glassware

Laboratory glassware should be clean and well rinsed in glass-distilled water. This avoids the introduction of contaminants or adverse pH conditions into ELISA reagents, especially when initial dilutions of conjugate is con-

cerned. The use of acids can cause problems if sufficient rinsing is not done, since enzymes are destroyed.

12.1.6¡ª
Micropipet Tips

Micropipet tips are expensive and can be in short supply in some laboratories. They can be washed, but the following steps should be taken:

1. Never wash and reuse tips that have delivered conjugate or conjugate solution.

2. Check that the ends of the tips are not damaged during use and washing.

3. Always rinse the tips very well in distilled water.

4. Dry the tips before use.

5. Get the appropriate tips to fit your micropipets. A poor fit causes problems with pipetting, which leads to inaccuracy.

12.1.7¡ª
Micropipets

Because micropipets are the instruments that deliver volumes of liquid, they are fundamentally important to the accuracy of the ELISA. They should be checked regularly for precision and accuracy of delivery volumes. Instructions on how to do this are usually included with the pipets; If not, ask the manufacturer how they are to be checked. Limited maintenance of the pipets should be made with attention to the plungers in multichannel pipets, which get contaminated. Care should be taken to make sure that liquids are not pulled into the pipet; if so, they must be cleaned. Corrosion can occur when, e.g., sulfuric acid stopping reagent is taken into the body of a pipet.

12.1.8¡ª
Plates

1. If a particular plate is recommended, then use that plate unless you retitrate given reagents in another manufacturer's plate.

2. Never use a tissue culture grade plate for ELISA. Sometimes these plates can be made to work, but they give much more variability than those specifically made for ELISA.

3. Always report which plate and which treatment of the plate has been made.

4. Reuse of plates after washing is problematic and high variability is observed. However, if economic and supply reasons deem it necessary, use 2 *M* NaOH overnight after washing the plates in tap water, and then rinse thoroughly in distilled water. You should use washed plates with many more controls than for new plates to measure variability.

12.1.9¡ª
Troughs (Reservoirs)

1. Use specific troughs only for conjugate and substrate to avoid cross contamination.

2. After use, wash the troughs in tap water, followed by distilled water and then leave them soaking in a mild detergent. For use, rinse in tap water, distilled water, and then dry with towel.

3. Never leave reagents in troughs for a long time after they have been used in an assay; rinse immediately if possible.

12.1.10¡ª
Substrate Solution

The temperature of the substrate solution is important because this affects the rate of color reaction. Therefore, try to perform the addition with the substrate always at the same temperature. This can easily be achieved if buffer tablets are used by keeping the water used at a constant temperature, or preincubating the substrate in a water bath. When substrate solution is kept frozen, you must ensure that on thawing the same temperature is achieved for every test (again by using a water bath). A range of 20¨C30¡ãC is recommended. The variation in temperature of the substrate solution will be the greatest factor in causing differences among assays performed with the same reagents.

12.1.11¡ª
Timing of Steps

Generally, individual steps should be timed accurately; thus, for a 1-h incubation step, no more than 5 min either way should be tolerated. For assays that recommend specific times, there is no reason that they cannot be met. Timing is less important when plates are rotated, although it is good practice to follow protocols accurately.

12.1.12¡ª
Incubation

We have already considered stationary vs rotated plates. The conclusion is that rotation of plates for incubation steps is highly recommended to eliminate vicosity effects; time differences; and temperature effects, including edge-well differences caused when plates are stacked and incubated stationary. However, when a rotator is not available, provided that standardization of methods is used, stationary plate assays are not a problem. The following tips are helpful when incubating plates that are not rotated:

1. Avoid stacking the plates; keep them separated.

2. Incubate at 37¡ãC.

3. Always use the same procedure for addition of reagents; that is, do not tap one plate, pick another up three times, or examine one or two plates during the incubation and not others. Using different procedures mixes the reagents over the solid phase to different degrees, thereby altering the interaction in the wells. Thus, take more care with handling the plates identically in one test and from day to day.

4. If incubation has to be done at room temperature make a note of the temperature, and its variation during the year. This may explain variation in results at different times.

Table 6
Problems and Solutions in ELISA

Problem	Solution
No/very little color even after 30-min incubation with substrate/chromophore	
—1. No hydrogen peroxide added.	Check.
—2. Hydrogen peroxide stock inactivated.	Retitrate.
—3. Added blocking buffer in adsorption step for antigen.	Check.
—4. Wrong dilution of hydrogen peroxide.	Check.
Color all over plate	
—1. Too strong conjugate.	Check dilution.
—2. Conjugate reacts with something other than target species	Check with suitable controls.
—3. Serum factors in heated sera.	Do not heat sera routinely.
Patchy color	
—1. Poor and variable coating of plates with reagents.	Check coating buffer and homogeneity of preparation.
—2. Bubbles in multichannel pipet tips.	Avoid overvigorous pipeting and detergents.
—3. Poor pipeting technique.	Practice more care.
—4. Plates faulty or non-ELISA plates.	Contact manufacturer; try alternative plates.
—5. Incubated plates in stacks.	Keep plates separated during stationary incubation
—6. Poor mixing of reagents including test sample.	Ensure mixing on sampling.
—7. Dilution series poorly done.	Practice pipeting; examine pipets for wear; recalibrate pipets.
—8. Poor washing.	Avoid detergents in wash solution; ensure no air bubbles are trapped in wells.
Color develops very quickly	

1. Conjugate too strong.	Retitrate.
2. One reagent at too high concentation.	Check dilutions.

(table continued on next page)

(table continued from previous page)

Problem	Solution
Color develops too slowly	
1. Conjugate too weak.	Check dilutions used; retitrate.
2. Contamination inhibits enzyme activity (e.g., sodium azide for peroxidase).	Avoid wrong preservatives
3. Low temperature of incubation.	Make sure temperature of substrate is correct
4. pH of substrate incorrect.	Check.
Totally unexpected results	
1. Plate format incorrect.	Check.
2. Dilution series made incorrectly.	Check.
3. Gross error in test protocol.	Check.
4. Visual estimation of color does not match ELISA reader results.	Check for contaminated/damaged filter; inappropriate filter (wrong wavelength)
High background color	
1. Nonspecific attachment of antibodies.	Check for unsuitable blocking conditions or omission of blocking buffer.
2. Antispecies conjugate reacts whether any reagent	Set up controls to assess with reagent on plate nonspecifically to any binds other in test.

12.1.13¡ª
Conjugates

Care must be taken with conjugates because since they are the signal suppliers of the whole assay.

1. Make sure you understand what the conjugate is (species made, specific antibody activity, and so forth).

2. Store at recommended temperatures.

3. Never store diluted conjugate for use at a later time.

4. Always make up the working dilution of conjugate just before you need it.

5. Always use clean tips, preferably previously unused, to dispense conjugates.

6. If the recommended dilution or titrated dilution of conjugate is very high (e.g., 1/10,000), add I µL to 10 mL to make 10 mL at working strength. You may have difficulty in making small volumes of working strength. Thus, a small dilution should be made to allow feasible pipetting of the conjugate without waste. Dilute in 50% glycerol/50% PBS to, say, 1/10 of the original. Store at ¨C20¡ãC if possible.

7. Never leave conjugates on the bench for an excessive time.

8. Preferably add sterile glycerol (equal volume) for conjugates stored at ¨C20¡ãC.

12.1.14¡[a]
Addition of Stopping Solution

Because the multichannel spectrophotometer reads through a thickness of liquid, any change in the volume in a well will result in an alteration of OD reading for the same colored solution. Thus, it is important to add stopping solution accurately to achieve the same volume in each well and limit the effect of volume changes (This, of course, is also true of addition of conjugate solution and concerns the blotting of plates to eliminate residual washing solution, all of which affect the final volume per well.)

12.1.15¡[a]
Addition of Samples

An accurate and consistent pipetting technique is a prerequisite for limiting pipetting error. Major problems are caused by the following factors:

1. Failure to put sample into the buffer in the well, leaving it on the side of the plate (particularly when plates are incubated stationary).

2. Frothing on addition of samples.

3. Lack of concentration when adding a large number of samples, causing missed wells and duplication of samples in the same well.

4. Poorly maintained pipets and tips.

5. Improperly thawing out sera (protein tends to collect at the bottom of the tubes on freezing) so that adequate mixing to ensure homogeneity is essential.

12.1.16¡[a]
Reading Plates/Data

The advantage of ELISA is that the plates can be read quickly and a large amount of data can be generated that can lead to several problems:

1. Computerization whereby the plate data are processed and the results given (e.g., ¡À56%) must be checked quickly from examination of plate data by eye. This is essential since some programs do not give warnings to check highly suspect results probably caused by a major sampling error. Thus, mean values may be calculated from the plate data by the computer and use these to ascribe positive or negative for particular samples. Unless safety features to screen for extremely different OD values in a pair are included in the program, false results are obtained (e.g., two values for a serum are 0.40 and 0.42, mean = 0.41 = positive; two values for a serum are 0.02 and 0.74, mean = 0.41 ?). Personal examination of initial plate data would easily spot this serum result as nonsense, whereas the sole reference to the computer printout of positive/negative would not. This is a facile example, but more complex analytical programs have similar hidden problems.

2. Large databases set up to store data from large-scale screening. This is related to the checking factor in which results read directly into a database are taken as reliable without examination by eye of the feasibility of those results. Researchers often wish to have results from several laboratories, and therefore programs have been supplied to facilitate this need. Such programs can easily dehumanize the diagnostic process by controlling results and denying the ability to backcheck data.

3. Cables that connect computers to spectrophotometers and printers that do not work. These are general hardware problems that must be conquered.

12.2¡[a]
Troubleshooting ELISA

Table 6 (*see* pp. 78 and 79) presents some of the problems commonly seen in ELISA development and practice and highlights areas that should be examined first when assays are proving difficult.

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4¡ª
Titration of Reagents

This chapter examines in more detail the necessary practical skills needed to facilitate the development and sustainability of ELISAs. Basic methodologies to set up all systems are discussed so that the reader can both investigate the possibilities of tests using his or her reagents and use with confidence reagents obtained from other sources. As with all other tests, it is vital that scientists realize the principles of the methods to allow good judgement based on quantifiable and controllable features. In other words, there should be a full understanding of what is being performed in the laboratory. The intention of this training is to produce new skills and understanding as well as to encourage critical assessment. Such a mentality is necessary and supported through the application of statistical criteria to results and to continuous monitoring of performance.

1¡ª
Chessboard or Checkerboard Titrations

The many ELISA systems described previously all require that the reagents used be optimized. In other words, the working concentration of each component of the test must be assessed. **Table 1** gives a simplified overview of the systems, indicating the number of reagents needed to be optimized, as a reminder.

A key feature in helping this process is through the use of chessboard or checkerboard titrations (CBTs). The use of microtiter plates is an important feature of ELISAs. This chapter describes this type of format. CBTs can be accomplished in any format in which reagents can be diluted, but the microtiter plate, with its associated equipment for ease of pipetting, is ideal. It will become clear that CBT is not the only method for optimizing reagents, and that often concentrations must be adjusted with reference to actual test conditions.

The process of CBT involves the dilution of two reagents against each other to examine the activities inherent at all the resulting combinations. The maximum number of reagents that can be titrated on a plate is two, and this is illustrated in the direct ELISA (*see* **Subheading 1.1.**). The use of CBT in some

Table 1
Basic ELISA System Components Requiring Titration

ELISA	Reagents involved	Number titrated
Direct	Antigen; antibody conjugated to enzyme	2
Indirect	Antigen; antibody; antispecies conjugate	3
Direct sandwich	Capture antibody; antigen; conjugated second antibody	3
Indirect sandwich	Capture antibody; antigen; second antibody; antispecies conjugate against second antibody	4

other systems with more than two reagents is also illustrated. The descriptions of pipetting and diluting techniques are also fundamental to the performance of ELISAs in general. As the reader becomes familiar with the methods, fewer details will be necessary to describe the tests.

1.1¡ª
Direct ELISA CBT

Figure 1 shows diagrammatically the dilution scheme. The upper part of **Fig. 1** illustrates the typical numbering and lettering associated with microtiter plates. Thus, columns are labeled 1¨C12 and rows are labeled A¨CH. This nomenclature is used henceforth to identify locations on the plates.

1.1.1¡ª
Stage (i) of Direct ELISA CBT

Stage (i) involves diluting the antigen in a coating buffer. The volumes usually used in ELISA are 50 or 100 µL. In this chapter, we use 50 µL as the standard volume added to wells. A twofold dilution range is usual at this stage, e.g., one volume taken from one well and added to the same volume in the next well, and so on. The most practical way of performing the test is as follows:

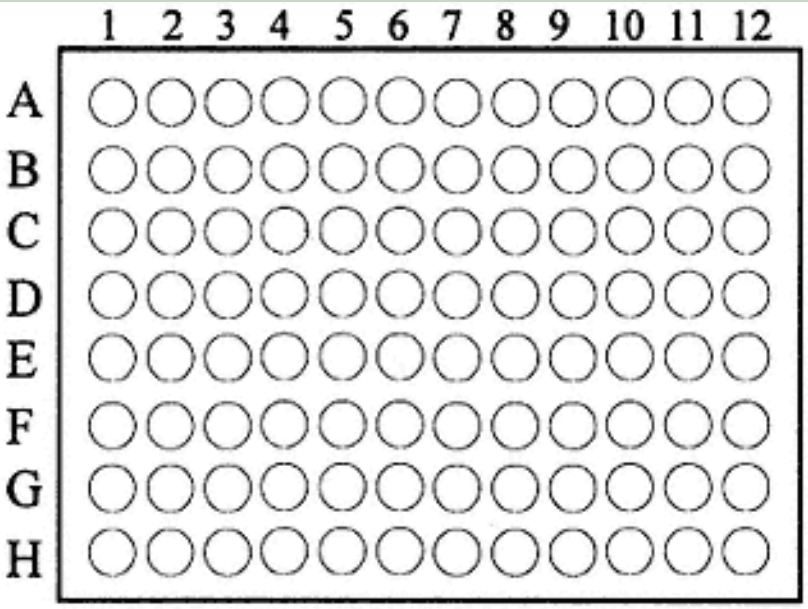
1. Add the diluent (in this stage the coating buffer) in 50-µL vol to all wells of the plate using a multichannel pipet.

2. Add 50 µL of a dilution of the antigen to all wells in column 1.

a. The initial dilution can be made in a small bottle to result in a volume necessary for addition to column 1; that is, you will need eight wells ¡Á 50 µL = 400 µL of antigen dilution. It is advisable to make slightly more of the initially diluted antigen than is theoretically needed to allow for material adhering to bottles, and so forth; in this case, 500 µL (0.5 mL) should be made.

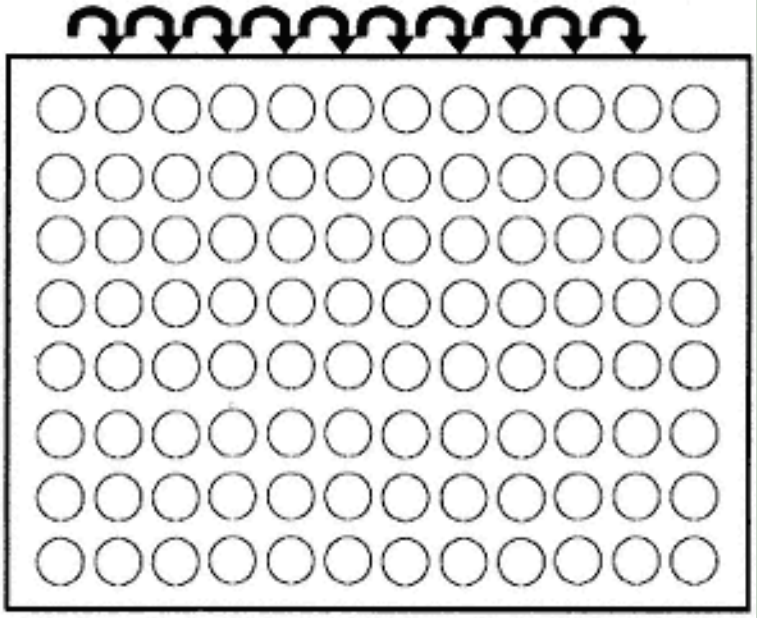
b. Assessment of the initial dilution is based on any knowledge of the likely antigen concentration (e.g., as assessed from other tests). With the CBT there is going to be a direct assessment of activity in the ELISA at a range of concentrations, and, therefore, if there is a gross under-or overestimate of antigen, another CBT can be made accounting for such problems. The likely

The numbers 1 to 12 denote columns, A-H denote rows.



Stage i). Dilute the antigen from column 1 to column 11. Column 12 receives diluent only.

Incubate. Wash wells



Stage ii) Add the labeled antibodies, dilute from row A to row G. Row H receives diluent only. Antibody is diluted in blocking buffer (containing inert protein and or detergent to prevent non-specific adsorption of protein. Incubate. Wash.

Add chromophore/substrate.

Read OD.

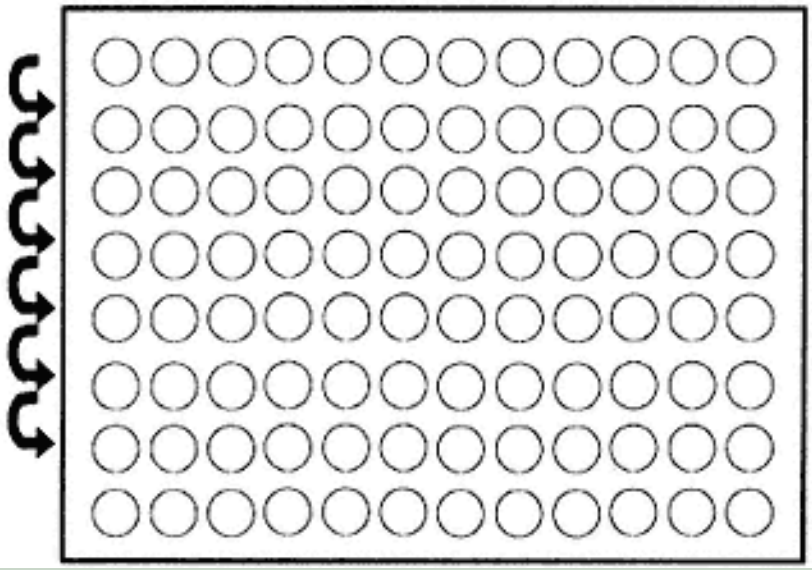


Fig. 1.
Plate layout and CBT of antigen against conjugated antibody for direct ELISA.

purity of the antigen (concentration of specific antigens as compared to contaminants) and the availability of antigen must be considered. A useful starting dilution for all antigens might be 1/10¨C20 in coating buffer. Let us assume that we add 1/20.

3. Add 50 µL of the prediluted antigen to all wells in column A. Mix with a multichannel pipet fitted with eight tips. The mixing implies that the liquid in the well is pipeted up and down in the tip at least five times. This should not be done too vigorously.

4. After the final mixing, take 50 µL of the diluted antigen from the eight wells in column A and transfer to column B. Mix as before. Repeat the procedure until column 11. Note that this means there is no antigen in column 12, and this will serve as one control (development of color with conjugate dilutions on wells containing no antigen).

5. After the final mixing action in column 11, take out 50 µL and discard. In the wells we have created a twofold dilution series of antigen in coating buffer, beginning at 1/20 in column A and ending at 1/20,480 in column 11.

6. Incubate the plate to allow time for the adsorption of antigen to the wells. The nature and time of the incubation should be the same as that used in the test proper. Most antigens will attach with incubation under stationary conditions at 37¡ãC in 2 h. However, it may be more convenient to allow overnight incubation at 4¡ãC. Whatever conditions are applied, they must be followed in the subsequent development of the test since alteration in times, temperatures, or regimes of shaking or tapping plates will alter the kinetics of adsorption.

7. Now wash the plate by flooding and emptying the wells with phosphate-buffered saline (PBS), as described in Chapter 3.

1.1.2¡[a]
Stage (ii) of Direct ELISA CBT

Stage (ii) involves making a similar dilution range of the conjugated antibody made against the antigen. In this case the dilution range is made from row A to G. The added buffer is blocking buffer (containing a relatively high concentration of inert protein to prevent nonspecific binding of proteins (*see* Chapter 3). In this case, the blocking buffer might be PBS (0.1 *M*, pH 7.6) containing skimmed milk powder (5%) and 0.05% Tween-20.

The dilution range is made using the multichannel pipet with 12 tips, directly in the wells. Again, there must be mixing between each addition. Note that there is no dilution of conjugate into row H, this acts as a control for only substrate and antigen (since the wells contain a dilution range of antigen). The initial dilution should be in the region of 1/50 for a direct conjugate.

1. Incubate the plate under rotation (best) at 37¡ãC for 1 h or stationary at 37¡ãC for 2 h. Wash the wells.

2. Add chromophore/substrate. This could be any of the ones described in Chapter 3 added with due care as to accuracy and checks on the pHs of the buffers involved. In this example, we shall assume we add H_2O_2/ortho-phenylenediamine (OPD) at 50 µL/well (in every well of the plate).

3. Leave the plate stationary for 15 min to allow color to develop. The exact timing of color development and conditions should be adhered to in subsequent assays. At this stage, it is good practice to observe the plate for the rate of color development.

Table 2
Results of CBT (OD Data)[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.1	2.2	2.1	2.1	2.0	2.1	1.9	1.7	1.5	1.3	1.0	0.6
B	2.1	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.9	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.7	1.5	1.2	0.9	0.5	0.3
D	1.8	1.8	1.7	1.8	1.5	1.2	1.0	0.7	0.5	0.3	0.2	0.1
E	1.8	1.8	1.7	1.5	1.3	1.1	0.9	0.7	0.5	0.3	0.2	0.1
F	1.5	1.5	1.4	1.3	1.1	0.9	0.7	0.5	0.3	0.2	0.1	0.1
G	0.7	0.7	0.7	0.6	0.6	0.5	0.3	0.2	0.1	0.1	0.1	0.1
H	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Note that rows D and E are highlighted and column 12 rows A to C are boxed.

4. Stop (depending on system).

5. Read the OD of color in a spectrophotometer.

1.2¡[a]
Results

Table 2 presents stylized results that might be obtained. We are attempting to assess the optimal dilutions of antigen to coat the wells and the interaction of the conjugate. **Figure 2** shows the data plotted.

1.2.1¡[a]
Analysis of Data

Each of the columns contains a constant but different dilution of antigen. Differences in color between the columns, where there is a constant addition of labeled antibody, reflects the effect of altering the concentration of antigen. The rows can be assessed for a maximum color in which there is a range of values that are similar. This can be regarded as a plateau and reflects areas where the antibody is in excess. In this area, where there is no decrease in color on dilution of the antigen, a maximum saturating level of antigen is coated to the plates. Thus, we can identify regions of excess antigen/antibody. Taking row A:

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.1	2.2	2.1	2.1	2.0	2.1	1.9	1.7	1.5	1.3	1.0	0.6

The shaded values are similar, giving a plateau maximum value of about 2.0 OD units. There is no effect on color where antigen is coated at 1/20, 1/40, 1/80, 1/160, 1/320, or 1/640 (wells 1¨C6). This indicates that in the presence of a constant dilution of antibody, there is a similar amount of antigen coating the wells to a dilution of 1/640. Following further dilution, there is a decrease in OD values on dilution of the antigen.

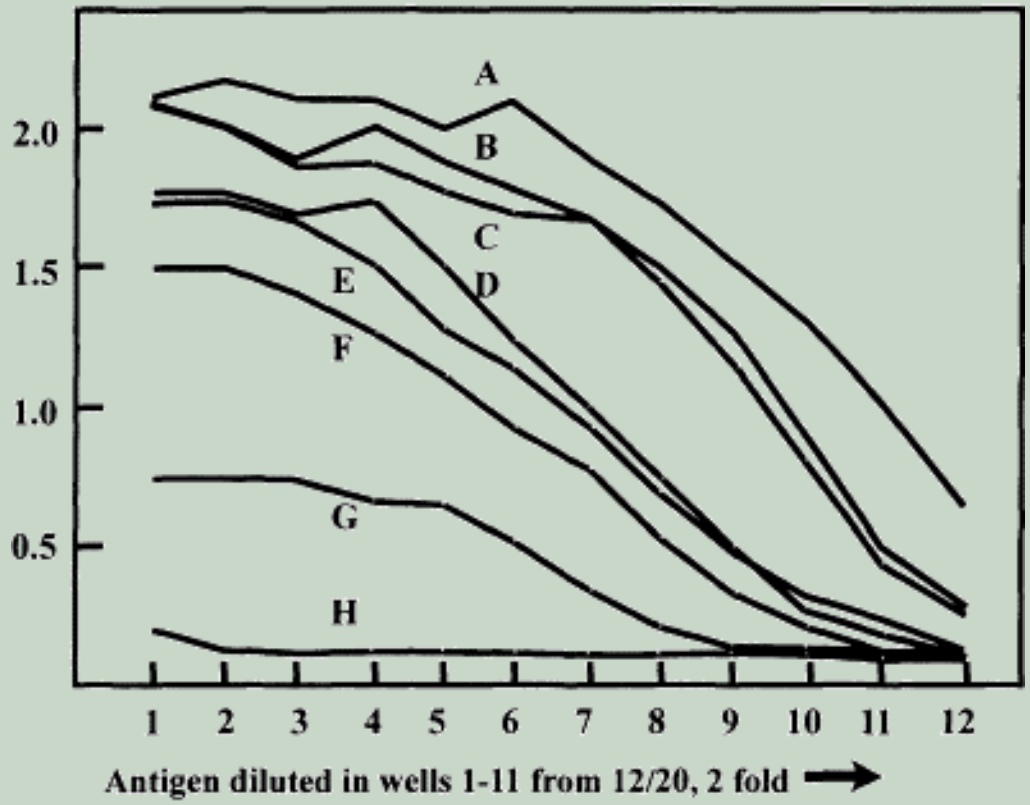


Fig. 2.
Plots of OD values obtained relating different antigen concentrations to different dilutions of labeled antibody. Row 12 received no antigen. A¨CG indicate rows containing different dilutions of labeled antibody. Row H did not receive labeled antibody.

Note that rows B and C give similar results showing a plateau from rows 1¨C6. **Figure 2** shows graphically that the curves are similar, although there are slight reductions on dilution of the conjugate. Again, this indicates that antigen is in excess, certainly at the dilution added to column 5. Thus, increasing the concentration of antigen above that contained in dilution at 1/320 only wastes antigen.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.1	2.2	2.1	2.1	2.0	2.1	1.9	1.7	1.5	1.3	1.0	0.6
B	2.1	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.9	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.7	1.5	1.2	0.9	0.5	0.3

The OD values in column 12 should be considered since this column represents the color developing where there is no antigen. The values are high for A¨CC, relative to color for D¨CG, which are low and the same. This color can be presumed to be the result of nonspecific attachment of the conjugate to the wells. The higher the amount of protein added, the greater the chance of non-specific events. Note that there is a reduction in color down each of the col-

umns on dilution of conjugate even where an excess of antigen is shown (columns 1¨C5/6). This is owing to the reduction in concentration of the conjugate (diluting out of reactive enzyme-labeled antibodies).

To obtain the most controllable results in ELISA, maximum OD values should be about 1.5.¨C1.7 OD units. Values above this are inaccurate on instrumentational grounds. Thus, optimization involves assessment of assays in which plateau maxima are near these OD values. This is based on OPD/H_2O_2 and other systems that have their own optima, but these should never approach very high ODs with respect to each substrate/chromophore system. Let us now concentrate on rows D and E, where this is a plateau height of 1.8 in the region where antigen is in excess:

D	**1.8**	**1.8**	**1.7**	**1.8**	**1.5**	**1.2**	**1.0**	**0.7**	**0.5**	**0.3**	**0.2**	**0.1**
E	**1.8**	**1.8**	**1.7**	**1.5**	**1.3**	**1.1**	**0.9**	**0.7**	**0.5**	**0.3**	**0.2**	**0.1**

For row D, there is a titration of antigen (decrease in color from the maximum on dilution of antigen) beginning at column 5, and for row E starting at column 4. The titration curves for each seen in **Fig. 2** are not as "high" as for those in A¨CC; however, the background values for both are low (0.1 OD) and identical with controls in which there is no addition of antigen or conjugate (e.g., H¨C12).

On further dilution of conjugate (rows F¨CH), there is a distinct decrease in the color obtained in which antigen has been shown to be in excess and on dilution of the antigen. Here, there is a drop in the potential analytical sensitivity of the assay (ability to detect antigen) reflected in the decrease in plateau height OD and the area under the titration curves seen in **Fig. 2**.

This has been a rather long description of a relatively simple operation. However, remember that at the beginning of the test, it was not known whether antigen bound to a plate or whether the labeled antibody would bind to antigen. We can view the results of the plate by eye very quickly and almost instantly see that the test has worked and what the optimal areas are, without reference to the actual OD results. This can be important when performing initial experiments in which a relatively large amount of work is necessary to assess a more complex situation. The ability to assess tests very rapidly by eye is a district advantage over assays in which quantification relies on instrumentation alone.

We can summarize the results as follows:

1. The antigen coats plate to give maximum reaction where antibody conjugate is in excess to 1/320¨C1/640.

2. Conjugate dilutions in rows A¨CC are too strong and give plateau maxima that are too high.

3. Rows D and E give good titration curves for antigen and have ideal plateau height maxima.

4. There is a loss in analytical sensitivity if the conjugate is diluted as in rows F and G.

Table 3
Results of CBT (OD Data)
in Which the Antigen Is Limiting Reaction

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.7	1.3	0.7	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1
B	1.7	1.3	0.5	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C	1.7	1.2	0.4	0.3	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
D	1.6	1.8	0.3	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	1.5	1.8	0.3	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
F	1.4	1.5	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
G	0.9	0.7	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

We have now completed the first sighting experiment evaluating the direct ELISA. At this stage, we can repeat the experiment with some alterations of reagent dilutions or conditions, depending on the results obtained. In the preceding example, we obtained ideal results with good activity for antigen and antibody conjugate. However, this is not always the case. Two examples of poor results necessitating alterations and reassessment are presented next.

1.2.2¡ª
Poor Results

Table 3 presents the results of an experiment identical to that described in **Subheading 1.1.** We now see that there is a good result in rows A and B and that after this dilution of antigen, very little color is produced. This indicates that there is little antigen attaching on dilutions greater than that used in row B. The conjugate appears to be usable to detect antigen until rows D and E since we obtain similar OD values where there is enough antigen coating the wells. In this case, the CBT could be repeated with a different dilution range of antigen to increase possibly the plateau maximum area in the presence of excess conjugate.

The next example (*see* **Table 4** for results) shows what may happen when the conjugate is of a low reactivity.

The results show that we have low color in the test. There is a rapid decrease in color on dilution of the labeled serum, rows A to B to C, and so on. There is, however, a plateau from columns 1 to 5 (A¨CC) indicating that there is antigen attaching at a similar level in these wells. In this case, one variation suggested from the initial CBT would be to coat plates with antigen at the dilution used in row 3 (the last dilution showing a plateau maximum value) and to titrate the conjugate, beginning at a higher concentration. In this way, a better estimate of conjugate activity would be obtained.

Table 4
Results of CT (OD Data)
in Which the Labeled Antibody Is Limiting Reaction

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.0	1.0	1.0	0.9	0.8	0.4	0.3	0.1	0.1	0.1	0.1	0.1
B	0.6	0.6	0.5	0.5	0.5	0.4	0.3	0.1	0.1	0.1	0.1	0.1
C	0.3	0.3	0.3	0.3	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1
D	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
F	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

This situation is common to direct ELISA systems because production of good conjugates depends on the specific activity of the antibodies labeled, which is a function of the weight of enzyme attached to antigen-specific antibodies. Conjugation of polyclonal sera usually results in the specific attachment of enzyme to a relatively small percentage of the total protein content of the sample that is specific antibody. The rest of the protein is labeled, leading to problems with high backgrounds.

1.2.3¡ª
Plateau OD Values

The maximum color obtained in an assay in which reagents are in excess results from the following factors:

1. The amount of antigen that can passively attach to a well.

2. The number of antigenic sites available for antibody binding.

3. The density of the antigenic components on the wells.

4. The specific activity of the conjugate in terms of how much enzyme is attached to particular antibody species in the whole serum and their respective affinities.

These factors are examined later. However, the CBT allows a rapid estimate of the feasibility of assays whose results may indicate problems associated with these factors.

2¡ª
More Complicated Systems

After the simplest case of the Direct ELISA we must consider situations in which there are three or more components to titrate. These are shown in **Table 5**. Remember that only two components can be varied by dilution in any test. The criteria for assessment of each of the stages in CBT are similar to those

Table 5
Possible Combinations for Titrations[a]

Indirect ELISA (3 components) Antigen + Antibody + Antispecies Conjugate		
1. Antigen D	Antibody D	Antispecies conjugate C
2. Antigen C	Antibody D	Antispecies conjugate D
Optimized reagents in I and 2 checked in 3		
3. Antigen C	Antibody C	Antispecies conjugate D
Sandwich ELISA Direct (3 components) Antibody + Antigen + Labeled Antibody		
1. Antibody D	Antigen D	Labeled antibody C
2. Antibody C	Antigen D	Labeled antibody D
Optimized reagents in 1 and 2 checked in 3		
3. Antibody C	Antigen C	Labeled antibody D
Sandwich ELISA Indirect (4 components) Antibody + Antigen + Antibody + Anti-antibody Conjugate		
1. Antibody D	Antigen D antibody C	Anti-antibody C
2. Antibody C	Antigen C antibody D	Anti-antibody D
Optimized reagents in 1 and 2 checked in 3 and 4		
3. Antibody C	Antigen C antibody D	Anti-antibody C
4. Antibody C	Antigen C antibody C	Anti-antibody D

[a]C, constant amount (no dilution series); D, dilution series.

described extensively for the direct ELISA. Remember also that the CBT aims to indicate optimal conditions and is quite useful in arriving rapidly at a feasible concentration range for all components in the desired assay. The objective is to examine reagents for their usefulness and lead to conditions for a fully defined test to perform a specific task.

Competition assays involve the interruption of these systems. They have to be titrated in the same way to allow competitive or inhibition techniques.

2.1¡[a]
Indirect ELISA

The indirect assay is used mainly to measure antibodies against a specific antigen either through the full titration of a sample or as a single dilution. Thus, we need a test with an optimal amount of antigen coated to wells that will successfully bind to antibodies, which, in turn, can be detected with an optimal

amount of antispecies conjugate. We can only titrate two of these variable in one assay. The most important aspects to consider are (1) that enough antigen is available for antibody binding¡ªwe do not wish to waste antigen by adding concentrations that are too high such that the wells receive a large excess of antigen compared to the amount needed to fill available plastic sites; and (2) that we have optimal amounts of conjugate to avoid high nonspecific backgrounds and to allow the detection of all bound antibody molecules to give the required analytical sensitivity to be achieved. We also need to assess the effect of diluting negative sera (from same species as test samples) on an assay to obtain an idea as to the possible backgrounds of such sera at various dilutions. Thus, we can modify the needs for setting up this assay:

1. We need a sufficient amount of antigen coating to the wells to capture antibodies.

2. We need at least one serum positive for the antigen.

3. We need at least one negative serum from the same species as the test samples.

4. We need an antispecies conjugate.

2.1.1¡ª Initial CBT

The initial test should be a CBT relating antigen dilutions to the positive and negative sera, using a commercial conjugate diluted to the recommended level. However, the individual laboratory estimation of optimal titers of antispecies conjugates is relatively easily made using the direct ELISA format. Either whole serum from the particular animal species target or a fraction (e.g., IgG) from the serum can be used to coat plates in stage (i) of the direct assay. When whole serum is used, 1/200 should be the initial dilution from rows I to 11. When IgG is prepared the weight can be measured spectrophotometrically and a starting concentration of 10 µg/mL used.

The second stage involves dilution of the conjugate in blocking buffer. Estimation of the optimal amount of conjugate is as already described. A dilution yielding a plateau maximum or about 1.8 OD units with a good titration curve should be used in indirect ELISA assessment. The dilution used can be altered later as a result of examination of results from the titration. This may be necessary since the exact nature and concentration of the specific immunoglobulins binding in the indirect ELISA to the specific antigen(s) may differ from those in the serum or serum fraction preparations.

2.1.2¡ª Stage (i) of Indirect ELISA CBT

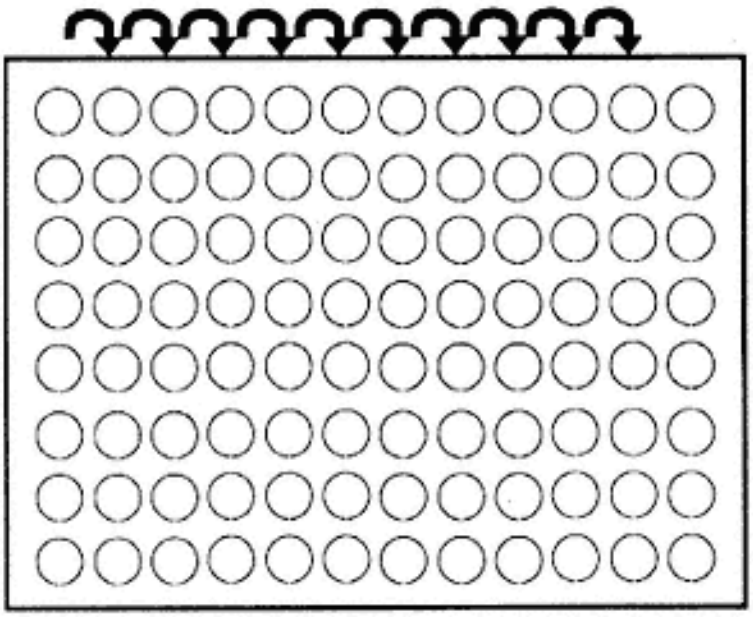
Figure 3 presents the stage (i) CBT for the indirect ELISA. This stage titrates the antigen against the positive and negative sera and can be regarded as a "sighting" exercise. Remember that conditions can be changed in a repeat CBT. Typical results might be those in **Table 6**.

Stage i)

Dilute the antigen from column 1 to column 11. Column 12 receives diluent only.

Incubate.

Wash wells



Stage ii)

Add the serum containing antibodies against antigen (one plate) or negative serum (second plate), dilute from row A to row G. Row H receives diluent only. Antibody is diluted in blocking buffer (containing inert protein and or detergent to prevent non-specific adsorption of protein.

Incubate.

Wash.

Add anti-species conjugate (given or pre-titrated) at single dilution in blocking buffer. Incubate. Wash.

Add chromophore / substrate.

Incubate. Stop reaction.

Read OD.

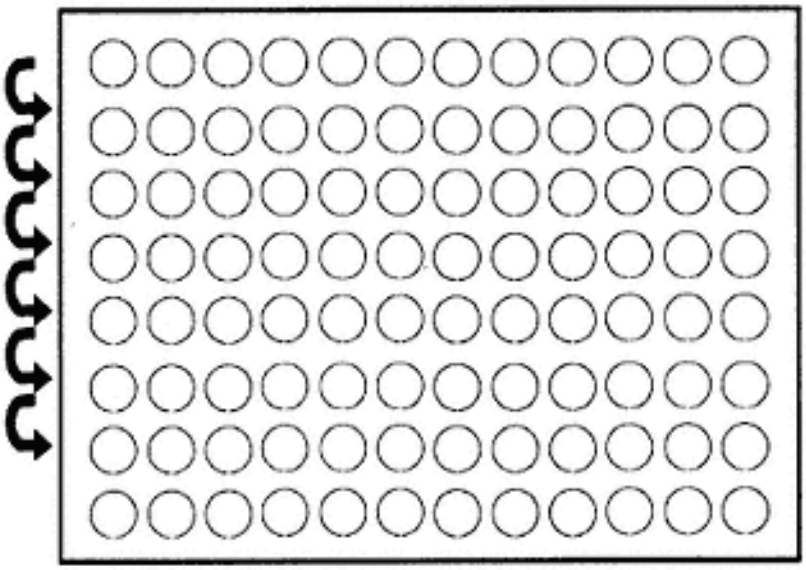


Fig. 3.
Plate layout and CBT of antigen against positive and negative sample sera for indirect ELISA. Constant dilution of antispecies conjugate was used.

In this example, we have used a titration of antigen from 1/50, twofold, and a titration of serum from 1/50, twofold. The controls for the test are shown in column 12, which contains no antigen but contains a dilution range of antibody and row H, which contains a dilution range of antigen, but no antibody. The results can be regarded as good, since we have color development at high levels and a corresponding titration as we reduce antigen or antibody. **Table 7** shows the area in gray, where there is an optimal amount of antigen coating allowing antibodies to be titrated maximally.

Table 6
Results of Stage 1 Titration of Antigen and Positive Antibody Containing Samples for Indirect ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2	2.3	2.2	2.1	2.0	2.0	1.9	1.6	1.5	1.3	0.8	0.6
A	2.2	2.3	2.2	2.1	2.0	2.0	1.9	1.6	1.5	1.3	0.8	0.6
B	2.2	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.8	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5	1.2	0.7	0.5	0.3
D	1.9	1.9	1.7	1.8	1.5	1.2	1.0	0.7	0.5	0.3	0.2	0.1
E	1.8	1.8	1.7	1.5	1.3	1.1	0.8	0.6	0.5	0.3	0.2	0.1
F	1.3	1.3	1.2	1.2	1.1	0.8	0.6	0.5	0.3	0.2	0.1	0.1
G	0.7	0.7	0.7	0.6	0.6	0.5	0.3	0.2	0.1	0.1	0.1	0.1
H	0.4	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1

Table 7
Optimal Coating Region for Titrating Antibodies

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2	2.3	2.2	2.1	2.0	2.0	1.9	1.6	1.5	1.3	0.8	0.6
B	2.2	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.8	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5	1.2	0.7	0.5	0.3
D	1.9	1.9	1.7	1.8	1.5	1.2	1.0	0.7	0.5	0.3	0.2	0.1
E	1.8	1.8	1.7	1.5	1.3	1.1	0.8	0.6	0.5	0.3	0.2	0.1
F	1.3	1.3	1.2	1.2	1.1	0.8	0.6	0.5	0.3	0.2	0.1	0.1
G	0.7	0.7	0.7	0.6	0.6	0.5	0.3	0.2	0.1	0.1	0.1	0.1
H	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Thus, antigen can be diluted to the levels in columns 5 and 6 before there is a loss in color. Note that the plateau height maxima do decrease particularly after row E, indicating that there is a reduction in antibodies binding owing to the decrease in amount of antigen coating the wells.

Examination of column 12 indicates that in the absence of antigen, the positive serum at dilutions of 1/50, 1/100, and 1/200 does bind to the plate-producing background. This is typical of serum in the indirect assay, and such nonspecific backgrounds have to be considered carefully when adapting the indirect ELISA for screening of test samples at single dilutions, since they influence the effective analytical sensitivity of assays. The same color develops in the titration of the negative serum (**Table 8**), indicating that this background stems from an interaction of the serum proteins contained in the serum.

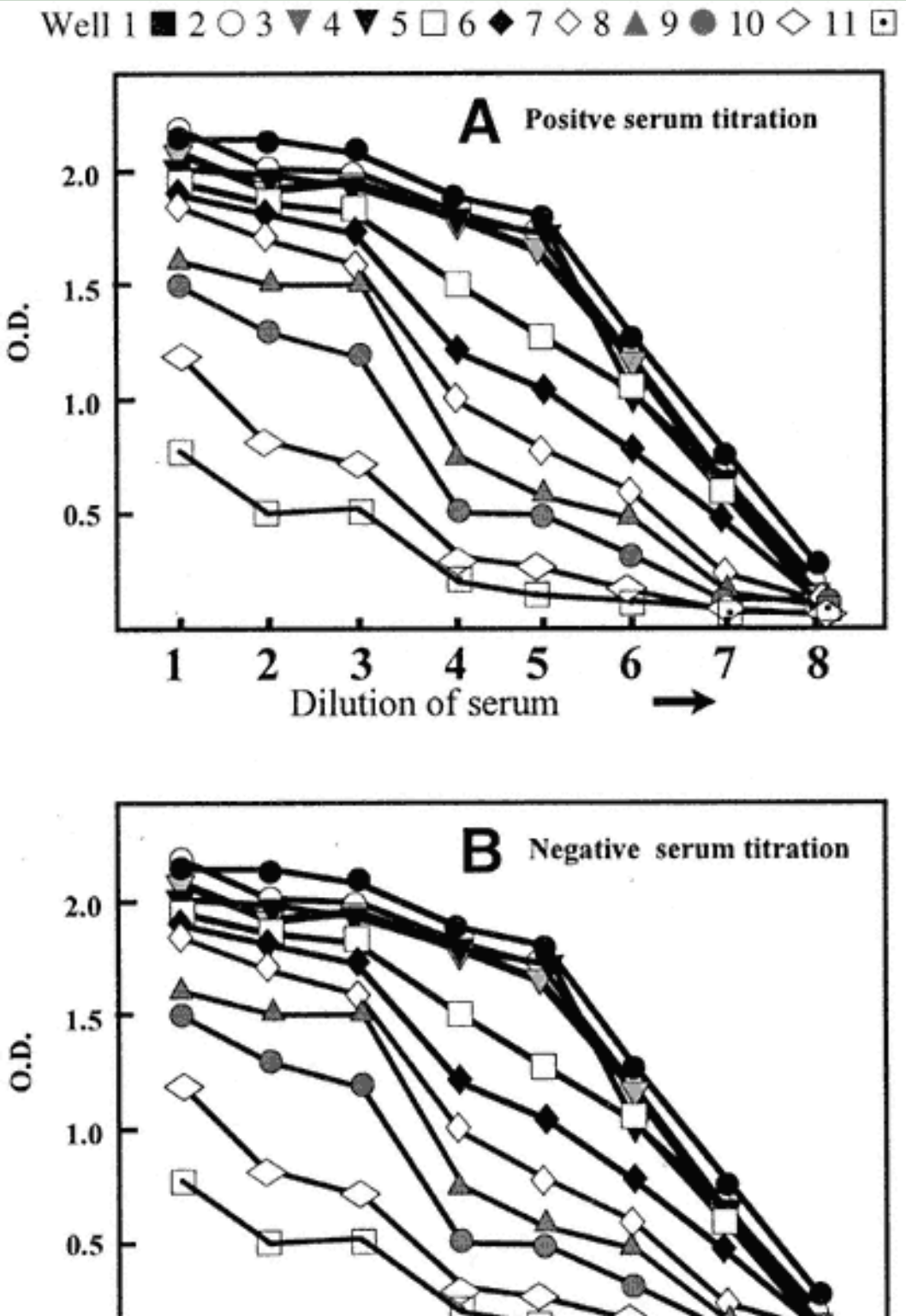
Table 8
CBT of Antigen and Negative Serum

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.7	0.7	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.6
B	0.4	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
D	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
F	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 8 given the results of titrating the negative serum. There is generally much lower color development, expected when there is no antibody binding to the antigen. There is, however, some color at various combinations, particularly when the concentrations of the negative serum are high. This color is owing to nonspecific attachment of the serum components from the species of animal being tested. Thus, the background needs to be examined and can be reassessed when retitrating the conjugate as described in stage (ii). As already indicated, there is color in A12, and it is almost as high as that seen in A1. The latter well contains antigen, indicating that there is a slight increase in binding, although it is not great. The serum dilutions of 1/50 and 1/100 do show some background, and as already indicated, this is mainly owing to interaction of the serum proteins with the plate nonspecifically. **Figure 4** plots the data for positive and negative sera.

2.1.3¡[a]
Stage (ii) of Indirect ELISA CBT:
Titration of Sera and Conjugate

From the first CBT, we can estimate an antigen dilution in which there is good color development as a result of the binding with antibodies. Because we do not wish to waste antigen by adding excess (which is washed away in the coating phase), we can select the last dilution of antigen, which gives a good titration curve for the antiserum (i.e., high plateau height maxima and high end point). **Table 7** indicates that columns 5 to 6 has enough antigen to fulfill these criteria. Thus, wells could be coated at this single dilution, and it would be expected that the positive serum would titrate with a maximum OD (in which antibodies were in excess) of approximately 2.0, and that antibodies would still be detected (on dilution) to row G.



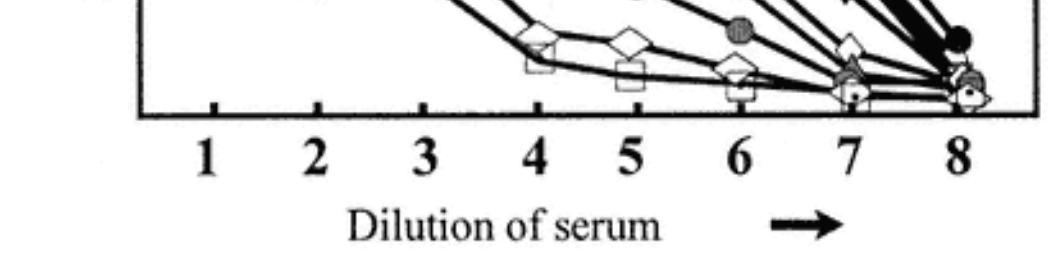


Fig. 4.
Graph relating OD values for different antigen concentrations against dilution series of antibody in CBT. Curves show titration of antiserum at different antigen dilutions from wells 1 to 11. Points from wells 4¨C11 are not shown for negative serum titrations (all 0.1).

2.1.3.1¡ª
Titration of Sera

For titration of sera, the positive serum dilution range is not extensive enough to allow titration of antibodies to an endpoint (where OD in the pres-

ence of antibodies equals background OD). This can be addressed by altering the dilution range of the positive antiserum in stage (ii). Assuming we have taken the antigen dilution as that in column 5, then the first operation in stage (ii) involves the following:

1. Coat wells of two plates with antigen at a dilution equivalent to that in column 5 (1/800). Incubate for the same times as in stage (i). Wash the wells.

2. Add a dilution range of positive or negative antisera as used in stage (i). Incubate and wash the wells. Here, as previously indicated, we can alter the range since in stage (i) we did not find the end point of the positive serum because we used a too-limited range of dilutions. We can increase the range in any of three ways:

a. Diluting the serum beginning at column 1 and diluting to column 11.

b. Starting at a higher initial dilution, e.g., 1/200.

c. Altering the dilution range to threefold (rather than twofold).

The best method has to be assessed with reference to the initial CBT. When the dilution range is far too low and high color is an obtained across the plate with indication of a titration only in the last two rows, then the use of a three-fold range is recommended.

In the present example, the dilution of positive serum began at 1/50 and was diluted twofold, in a seven-well series. Thus, we obtained dilutions of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, and 1/3200. At the last dilution, we had not obtained an end point, and by examination of the curves, we can predict that at least another four similar dilution steps would be needed before the color would be reduced to background (because the antibodies were diluted out). Thus, in stage (ii) we can use the coated plate and dilute the positive serum from 1/50 [as in stage (i)] by 11 steps to 1/51,200, using columns 1¨C11.

2.1.3.2¡[a]
Titration of Antispecies Conjugate

In stage (i) we estimated the appropriate conjugate dilution either from information given by the producers or from a preliminary CBT of the conjugate against serum coated to wells. Titration of the conjugate at this point offers an examination of its activity under the indirect assay conditions proper, allowing refinement of the dilution to maximize analytical sensitivity as a result of identifying areas where excess conjugate produces high backgrounds.

1. Add a twofold dilution range of conjugate from row A to H. Incubate and then wash. Again, we have the opportunity of adjusting the starting dilution based on stage (i) results. When the commercial company recommendation is used, it is good practice to begin the conjugate dilution approx fourfold higher than recommended and to dilute to at least fourfold lower. As an example, if the recommended dilution is 1/2000, then the conjugate should be titrated from 1/500 in twofold steps to 32,000. When there is an initial CBT against relevant serum, then the same procedure should be adopted around the initially found optimum.

Table 9
Results of CBT for Positive Serum[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.8	1.8	1.8	1.8	1.6	1.2	1.1	0.9	0.6	0.5	0.5	0.5
B	1.8	1.8	1.8	1.8	1.6	1.2	1.1	0.9	0.6	0.5	0.3	0.2
C	1.7	1.7	1.7	1.7	1.6	1.1	0.9	0.7	0.5	0.4	0.2	0.1
D	1.6	1.6	1.5	1.3	1.1	0.9	0.7	0.5	0.3	0.2	0.2	0.1
E	1.2	1.1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.1
F	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.2	0.1	0.1	0.1	0.1
G	0.5	0.5	0.5	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1
H	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Antigen was constant. Serum was diluted at 1/50, twofold, from column 1 to 11. Conjugate was diluted at 1/500, twofold, rows A¨CG.

Table 10
Results of CBT for Negative Serum[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
B	0.4	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
D	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
F	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Antigen was constant. Serum was diluted at 1/50, twofold, from column 1 to 11. Conjugate was diluted at 1/500, twofold, rows A¨CG.

2. Add relevant chromophore/substrate to the system and incubate.

3. Stop the reaction as in stage (i) and read the OD in a spectrophotometer.

2.1.4¡[a]
Results

Tables 9 and **10** shows the results for the CBTs. **Table 9** shows that there is a high background of 0.5 in row A, column 12. In this row, 1/500 conjugate was used, which indicates that it is binding nonspecifically with the antigen-coated plates. This finding is confirmed in **Table 10,** where the background value is maintained on dilution of the negative serum from A2 to A12. Although these are idealized results, they do illustrate a common phenomenon. The background is reduced in row B (dilution of conjugate 1/1000 with reference to the

value in column 12 of **Table 9**), but **Table 10** shows that there is still a higher background in B3¨CB 12, indicating some nonspecific complications.

In row C for both plates, the background in column 12 is low (0.1) and remains constant for the rest of the dilutions of the conjugate. This can be regarded as the minimum background for the test (plate background). **Table 9** indicates that there is a "good" titration of serum in row C, where there is a plateau (region of maximum OD), indicating a region of antibody excess. Thus, the conjugate at the dilution in row C (1/2000) can detect the bound antibodies and an optimal OD reading can be obtained. The end point (with reference to the OD obtained in the absence of serum [C12]) has not been obtained, but there is a gradual reduction in OD as the serum is diluted (titration curve). This can be contrasted to the idealized results for row C in **Table 10**. Here, no titration is observed at any dilution of the negative serum.

Reduction in the concentration of the antispecies conjugate has two effects. One is to reduce the plateau maximum color, and the other is to effectively reduce the end point of the titration. Thus, in **Table 9** we see a small reduction in plateau in row D, which becomes marked when we further dilute.

By row G, we have a low OD even where we know that there is enough antibody binding to give a strong signal in the presence of excess antibody (as seen in A¨CD, columns 1¨C4/5). The optimal dilution of the conjugate at this stage is therefore taken from assessing the plateau maximum color and the titration end point with reference to the backgrounds in the controls. In this case, a dilution of conjugate of 1/2000 to 1/4000 appears optimal with the serum dilutions used. At this dilution, there is no OD measured in the negative serum.

2.1.4.1¡[a]
Binding Ratios

Another way of examining the results is to calculate the binding ratios (BRs) relating the positive and negative titrations. This is simply the OD value at a given dilution for the positive serum divided by that of the negative serum. **Table 11** gives the BRs for the data in **Tables 9** and **10**. This process gives a clearer picture of the best conditions for setting up assays and is a feature used when tests are used for diagnostic purposes.

Table 11 illustrates the highest BRs in rows C and D, despite the OD values for the positive serum being higher in A and B. This results from the relatively low OD values for the negative serum at higher dilutions of conjugate. Note that the BRs at 1/50 and 1/100 positive serum in A1, B1, and 2 are lower than in subsequent wells. This is typical and results from the effect of high nonspecific binding at these dilutions with negative sera. In fact, it is usual for this effect to be more exaggerated in practice.

Care is needed in interpreting best conditions by this method because where there are extremely low OD values for binding with negative sera, even low

Table 11
BRs of Positive and Negative Sera from Tables 9 and 10[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.0	3.6	3.6	3.6	3.2	2.4	2.2	1.8	1.2	1.0	1.0	1.0
B	4.5	6.0	9.0	9.0	8.0	6.0	5.5	4.5	3.0	2.5	1.5	1.0
C	17.0	17.0	17.0	17.0	16.0	11.0	9.0	7.0	5.0	4.0	2.0	1.0
D	16.0	16.0	15.0	13.0	11.0	9.0	7.0	5.0	3.0	2.0	2.0	1.0
E	12.0	11.0	9.0	8.0	7.0	6.0	5.0	4.0	3.0	2.0	1.0	1.0
F	8.0	7.0	6.0	5.0	4.0	3.0	2.0	2.0	1.0	1.0	1.0	1.0
G	5.0	5.0	4.0	3.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
H	3.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 12
End Points of Serum Titrations

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.0	3.6	3.6	3.6	3.2	2.4	2.2	1.8	1.2	1.0	1.0	1.0
B	4.5	6.0	9.0	9.0	8.0	6.0	5.5	4.5	3.0	2.5	1.5	1.0
C	17.0	17.0	17.0	17.0	16.0	11.0	9.0	7.0	5.0	4.0	2.0	1.0
D	16.0	16.0	15.0	13.0	11.0	9.0	7.0	5.0	3.0	2.0	2.0	1.0
E	12.0	11.0	9.0	8.0	7.0	6.0	5.0	4.0	3.0	2.0	1.0	1.0
F	8.0	7.0	6.0	5.0	4.0	3.0	2.0	2.0	1.0	1.0	1.0	1.0
G	5.0	5.0	4.0	3.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
H	3.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

OD values for positive sera can appear to give best results. However, the lower the OD values being examined, the higher the potential variation in results, and therefore a compromise between what seems to be the highest BR values and obtaining a reasonable OD value in the positive sample is required. The assessment of end points can also be made using this method. The last dilution of serum that gives a BR of >1.0 can be judged as the end point.

In our idealized example, the end points for the various dilutions of conjugate are shown as a line in **Table 12**. Here, we can see that the effect of diluting conjugate is to reduce the end points (E¨CG). In B¨CD, the final end point has not been found, although the indication from examination of the BR data is that the conjugate dilution in C gives the highest potential analytical sensitivity since it has a BR of 4.0 at the dilution in column 10, as compared to the other results. The only controls not discussed are those in row H. These are antigen-coated wells (constant), with antibody dilutions of 1/50 twofold, but no conjugate. In this example, there is some color in the 1/50 and 1/100 positive serum wells

despite the lack of conjugate. This can affect the estimation of which serum dilution should be used in an indirect assay involving testing of samples at a single dilution. This effect disappears at 1/200 positive serum and is not observed for the negative serum.

2.1.5¡[a]
Conclusion

Although the use of CBT may seem a laborious process, the principles are easy. Initially the antigen was titrated against the antisera using an estimate of the conjugate dilution. This indicated that there was a significant difference between the two sera in the OD values obtained. The approximate antigen concentration that coated the plates was then taken and used to relate the antisera and conjugate dilutions. Thus, we can conclude the following:

1. The antigen can be used at 1/800.

2. The conjugate can be used at 1/2000 to 1/4000.

3. There is a good discrimination of positive serum from negative serum using these conditions, and a dilution of serum at 1/400 could be suggested for a test involving single dilutions of sample.

2.1.6¡ª
Developing Indirect ELISAs

The tests just described can be made in 2 days, and further refinements can be made using the reagents in additional tests in which smaller changes in dilutions can be assessed. However, as with all ELISAs, it is imperative that the ultimate purpose of the test be addressed as early as possible.

In cases in which the test is to be used to screen hundreds or thousands of sera according to positivity, based on a single dilution (in duplicate or triplicate), the titration phase must include a reasonable amount of work to recognize the factors inherent in examination of a varied population of antisera. The previous example centered on the use of a single positive and negative serum. This is patently not going to reflect differences in the population of sera to be examined. Thus, at the stage where we have a working dilution of the antigen and conjugate, we must now include more positive and negative sera (when possible) to further test the parameters for optimizing analytical sensitivity. The problem then is to assess these factors:

1. Whether the optimal antigen holds for a number of negative and positive sera.

2. The optimal antiserum dilution to use for single-sample screening. This is a balance between achieving maximum analytical sensitivity and maximal specificity.

3. The mean OD value of a negative population (and its variability). This allows the designation of positivity at different confidence levels and its variation.

The indirect assay may be used in a competitive or inhibition assay. In these cases, the problem is to screen positive sera for characteristics that best match

those for field or experimental sera in terms of antibody populations reacting with specific determinants on the antigen used. It may be necessary to screen a number of positive sera under test conditions to identify a single serum with the best properties.

Indirect assays also offer a relatively easy and rapid method of end point titration of many sera. Relatively simple titrations can give confidence in the properties of sera to be used in other tests (i.e., they can confirm positivity or negativity). This is examined next in the worked examples of the use of indirect ELISAs.

2.2¡ª
Direct Sandwich ELISA

Direct sandwich ELISA is a three-component assay. We have to titrate the following:

1. The capture antibody.

2. The antigen that is captured.

3. The detecting conjugate.

Only two components can be varied in any one test, so the same criteria as indicated in the indirect ELISA apply. Probably the most variable area in this assay is the activity of the labeled conjugate, which is usually produced in the laboratory developing the assay. Such conjugates can have quite different properties owing to the intrinsic amount of enzyme that is attached to specific antibodies within a polyclonal serum produced against the antigen. Thus, no assumptions as to the activity can be made as in the case of antispecies conjugates from commercial sources, and the initial titrations require possibly more cycles to fine-tune concentrations.

2.2.1¡ª
Aids in Developing Assays

One aid to developing such assays can be the use of indirect ELISAs to assess the relationship of antigen and antibody binding. However, the need to develop a capture ELISA (antibody on wells as reagent to capture antigen) usually stems from the need to concentrate a weak antigen (unsuitable for indirect ELISA) or to capture a specific component of an antigenic mixture. The selection of an antiserum for labeling and conjugation for use in the sandwich ELISA, could be based on estimation of the titer of a number of sera by the indirect ELISA, where the antigen can be coated in a sufficient quantity. Thus, high-titer sera can be identified. Such titrations can be made by other methods, leading to the selection of sera with the highest activities.

2.2.2¡ª
Stage (i):
Titration of Capture Antibody and Antigen

Figure 5 shows the scheme for stage (i) of direct sandwich ELISA. Whole serum is used as the capture reagent, a dilution of 1/100 should be used in

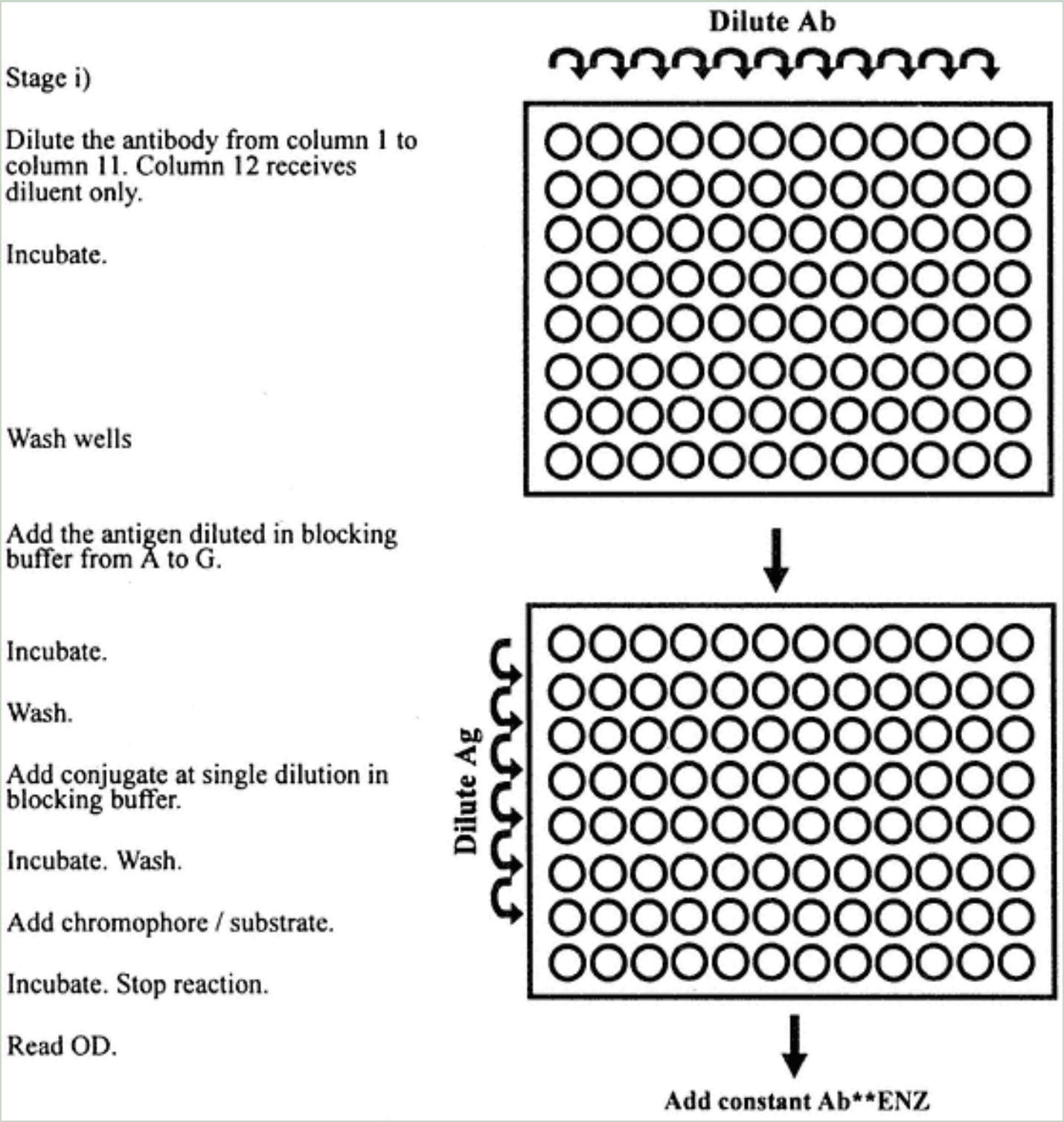


Fig. 5.
CBT of capture antibody against antigen for direct sandwich ELISA.

column 1. When IG (e.g., IgG) has been prepared from the antiserum, the concentration of protein can be easily found through reading absorbance in an ultraviolet spectrophotometer. In this case, a starting concentration of 10 µg/mL should be used (we are still using a 50 µL volume as the constant in this example). At this level of protein, the wells will be saturated so that the activity of the capture antibody relies on the relative concentration of the specific antibodies in the IgG fraction as compared to the other IgG molecules in the serum. The addition of any higher concentration is a waste and will not improve the capturing ability of the coating reagent. The dilution range should be twofold

and serum diluted to column 11. The usual diluents for antibodies are PBS, pH 7.2, or carbonate/bicarbonate buffer, pH 9.6.

After incubation (e.g., 1 to 2 h at 37¡ãC), the wells are washed. Next, the antigen is diluted from row A to row G. When there is no indication from other assays as to the likely concentration of the antigen, then the dilution range should be started at 1/50.

The assumption here is that there is a sufficient volume of antigen to allow such a dilution. When there are very small volumes of antigen of unknown concentration, then you must decide whether there is enough antigen to develop any test or whether it is of a high concentration and that 1/50 will be enough to titrate originally. The results of stage (i) will indicate whether the antigen is of a high or low concentration. For a 1/50 dilution in a 50-µL vol test, we will require 12 wells at 1/50 for row A plus 12 wells at 1/50 for dilution in row B (a total of 1200 µL [1.2 mL]) for the test. This equals 24 µL of undiluted antigen for a single stage (i) CBT. Remember to allow a little extra volume than that exactly required. Thus, 30 µL will be a more realistic volume diluted to 1500 µL. The antibody is diluted in a suitable blocking buffer to prevent nonspecific attachment of proteins to the wells. After incubation and washing, a constant dilution of the labeled detecting serum is added. Since we have no idea as to the effective activity, then add conjugate diluted to 1/200. Thus, we need a volume of 96 wells ¡Á 50 µL = 4800 µL (4.8 mL), say, 5000 µL (5.0 mL) to allow for losses. This means that we need 5000/200 µL of undiluted conjugate for a plate in stage (i) = 25 µL. The calculations are included here to remind operators to pay attention to the availability of reagents. After incubation, the relevant chromophore/substrate solution is added and the color development read with or without stopping (depending on the system).

2.2.2.1¡ª
Results of Stage (I)

Table 13 gives an example of good results. The columns contain dilutions of capture serum, and the rows contain dilutions of captured antigen. Row A contains the highest amount of antigen and examination of the OD values shows that there is a plateau of OD values from column 1 to 4. This indicates that detecting serum is in excess and that there is enough antigen to allow for a significant signal (1.9 OD units). A similar plateau is observed in row B, indicating that dilution of antigen has no significant effect on the titration of the serum. The data in A and B are identical, indicating that there is the same amount of captured antigen present in the rows. Row C shows a slightly reduced plateau OD value but the extent of the plateau (to column 4) is the same as for A and B. This reduction indicates that there is a slight reduction in the amount of captured antigen. This is also reflected by examination of the full titration

Table 13
Example of Good Test Results from Stage (i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.9	1.9	1.9	1.9	1.8	1.3	1.1	0.9	0.6	*0.5*	0.3	0.1
B	1.9	1.9	1.9	1.9	1.8	1.3	1.1	0.9	0.6	0.5	0.3	0.1
C	1.8	1.8	1.8	1.8	1.6	1.1	0.9	0.7	0.5	0.4	0.2	0.05
D	1.6	1.6	1.6	1.5	1.2	1.0	0.9	0.7	0.5	0.3	0.1	0.05
E	1.2	1.1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.05	0.05
F	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.2	0.05	0.05	0.05	0.05
G	0.5	0.5	0.5	0.4	0.3	0.2	0.1	0.05	0.05	0.05	0.05	0.05
H	0.2	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

Table 14
Binding Ratios of Serum

	1	2	3	4	5	6	7	8	9	10	11	12
A	19	19	19	19	18	13	11	9	6	5	3	1
B	19	19	19	19	18	13	11	9	6	5	3	1
C	36	36	36	36	32	22	18	14	10	8	4	1
D	32	32	32	30	24	20	18	14	10	6	2	1
E	24	22	18	16	14	12	10	8	6	4	1	1
F	16	14	12	10	8	6	4	4	1	1	1	1
G	10	10	10	8	6	4	2	1	1	1	1	1
H	4	2	1	1	1	1	1	1	1	1	1	1

range values; however, the background OD (column 12) is lower in C (0.05) than in A and B (0.1). The continued reduction in antigen concentration (D¨CG) exaggerates the loss of ability of the detecting serum to be titrated where both the plateau height maxima and the end points are reduced significantly. These data can be expressed as BRs, relating the values in the presence of serum to the control values in column 12. **Table 14** shows these values.

Examination of BR exaggerates the advantage of using the antigen at lower concentrations than those used to obtain maximum OD, since the background OD values (antigen plus only conjugate) are lower. From the data in **Tables 13** and **14**, we can estimate an optimal dilution of antigen to be that used in row C and the optimal dilution of capture antibody to be that used in column 4. This is highlighted on **Table 14**. Assuming we started the dilutions of capture serum at 1/200 and antigen at 1/50, we have optimal values for each of 1/1600 (serum) and 1/200 (antigen), respectively.

Table 15
Titration of Antigen and Detection of Labeled Serum with Constant Capture Antibody[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.1	2.1	2.1	2.1	2.0	1.9	1.8	1.7	1.2	0.6	0.4	0.4
B	1.9	1.9	1.9	1.9	1.8	1.3	1.1	0.9	0.6	0.5	0.3	0.2
C	1.9	1.9	1.9	1.9	1.8	1.3	1.1	0.9	0.6	0.5	0.3	0.1
D	1.9	1.9	1.9	1.9	1.8	1.3	1.1	0.9	0.6	0.5	0.3	0.05
E	1.8	1.8	1.8	1.7	1.5	1.3	1.1	0.8	0.5	0.3	0.2	0.05
F	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.2	0.05	0.05	0.05	0.05
G	0.5	0.5	0.5	0.4	0.3	0.2	0.1	0.05	0.05	0.05	0.05	0.05
H	0.2	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

[a]Constant capture antibody was 1/1600. Antigen was diluted from 1¨C11, beginning 1/50, two-fold. Labeled antibody was diluted from A to G, beginning 1/50, twofold.

2.2.3¡[a]
Stage (ii):
Titration of Antigen and Labeled Antibody

We can now fix the concentration of one of the reactants. Stage (i) used a constant dilution of labeled detecting serum (1/200) that gave successful results. However, we need to know the optimal titration of this reagent so as not to waste a valuable resource (by underestimation of the concentration) and to examine the effects on the ultimate analytical sensitivity of the assay. The idea is to optimize the dilution of conjugate in detecting the captured antigen.

Stage (ii) involves coating plates with a constant amount of capture serum as determined in stage (i) (equivalent to dilution in column 4 = 1/1600). After incubation and washing, the antigen is added at a twofold dilution range from a value two-to fourfold higher than found to be optimal in stage (i) from row A to G. After incubation and washing, the detecting conjugate is diluted from column 1 to 11 starting at a dilution two to fourfold higher than that used in stage (i), i.e., 1/50¨C100. After incubation and washing and addition of substrate /chromophore, the test is read. **Table 15** presents idealized data.

Reference to the rows in **Table 15** shows that at 1/50 conjugate we have a good titration range of antigen but there is a high background (0.4). On dilution, we obtain similar results in rows B¨CD indicating that the detecting conjugate is in excess until the dilution used in row D. Note also that there is a significantly lower background (column D12) than in C12 and B12. On further dilution (rows E¨CG), we lose the plateau height and end points for titrating the antigen. Again the BRs can be plotted relating the antigen concentration to conjugate concentration to clarify the impact of the observed differences in background (**Table 16**). The data indicate that the optimal conjugate dilution for use in detecting available antigen is that observed in row

Table 16
BRs for Data in Table 15[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	5	5	5	5	5	5	5	4	3	2	1	1
B	10	10	10	10	9	7	6	5	3	3	2	1
C	10	10	10	10	9	7	6	5	3	3	2	1
D	38	38	38	38	36	26	22	18	12	10	6	1
E	36	36	36	34	30	26	22	16	10	6	4	1
F	16	14	12	10	8	6	4	4	1	1	1	1
G	10	10	10	8	6	4	2	1	1	1	1	1
H	4	2	2	1	1	1	1	1	1	1	1	1

[a]Data are rounded up to one decimal place.

D (1/400). The dilution in row E (1/800) gives quite similar results and could be used to detect the antigen when the availability of the conjugate is a strong consideration.

2.2.4¡[a]
Further Refinement

Stages (i) and (ii) enable a good estimate of the concentrations of each reactant to be made. The idealized example is when the tests work well. Even here we may require further CBTs to establish more precise conditions; for example, a CBT of dilutions of capture antibody against constant antigen and dilutions of conjugate could be examined. The initial CBTs also give an opportunity to set up limited studies on field samples. Thus, if you wish to titrate antigen in samples, you could coat plates with antibody, add serial dilutions of test antigens and then detect these with the conjugate. This would investigate how proper field samples behave in a test and possibly give clues as to the need to modify conditions.

The titration of all three reactants also allows them to be used in similar assays with other reagents. Thus, we may wish to examine another antigen in the assay. The capture serum and detecting conjugate can be used at the dilutions found from CBTs, but the new antigen titrated. Similarly, other capture antibody preparations can be used in tests involving the antigen and conjugate used at the optimal dilutions, as found by CBTs.

As for the description of the direct ELISA, the purpose for which the assay is being developed should always be the strongest factor in test reagent optimization. Ultimately, the test will have to be proved to perform on particular samples and under specific conditions, and validation of ELISAs must meet such conditions.

Table 17
CBT, Low OD Data, Stage (i) Direct Sandwich ELISA[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.1
B	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.1
C	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.1	0.1	0.1
D	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.1
E	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1
F	0.4	0.4	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
G	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Capture serum diluted is in columns 1¨C11, antigen diluted is in rows A¨CH, and the constant was conjugate.

2.2.5¡[a]
Bad Results

The idealized example is typical of good results in which all reactants perform well, i.e., can be used at high dilution and give OD values that are relatively high. When one or more of the reactants is at a low concentration or has poor binding characteristics in the assay, the CBT soon indicates where the problems reside.

Although the number of examples cannot be exhaustive, demonstration of a few bad results is probably far more informative than giving the ideal situation.

2.2.5.1¡[a]
Low Color Generally over Plate

Table 17 gives the results of a similar CBT for stage (i) of the direct sandwich ELISA (results shown in **Table 13**).

Generally there is low color. There is a plateau corresponding to a maximum value of 0.4 OD units, from column 1 to 5 (highest concentrations of capture antibody), which indicates that the antigen is being captured. There are several reasons for the low OD value in this region:

1. The capture antibodies specific for the antigen are at a low concentration with respect to other serum proteins or do not bind as well as other proteins. Thus, the amount of antigen captured is limited. In this situation, there is no observed increase in OD on increasing the capture antibody concentration.

2. The amount of antigen available for capture is low. This is unlikely since dilution of antigen (from A to B to C, and so on) does not decrease the OD observed in columns 1¨C4, indicating that there is an excess of antigen to row F (after which we observe a reduction in OD).

3. The activity of the conjugate is low.

The CBT can be repeated using increased staring concentrations of the titrated components. Thus, we could begin the capture antibody concentration at 10X that used in the first CBT. It is unlikely, however, that this will increase the OD values since we did observe that there was an extensive (columns 1¨C5) plateau maximum indicating that there was maximal activity being measured that did not alter on dilution of the antigen.

In cases in which there is an observed increase in OD on increasing capture antibody, the CBT can be reassessed and a stage (ii) CBT can be performed. When there is no increase in OD values with increased concentrations of antigen or conjugate, the strongest candidate for replacement is the capture antibody. When this antibody is the same as that used for conjugation, both should be replaced.

2.2.5.2¡ª
Extremes of Color

When there is a very high color in the majority of the wells, the CBT must be repeated with lower concentrations of reactants. **Table 18** gives data from such a plate. The reagent responsible for the high readings may be directly identified from the CBT. Close examination of background values is also necessary because the results may be owing to a very high nonspecific binding of one of the reactants.

The data in **Table 18** shows a high background for the conjugate in the absence of capture serum (column 12). Row H indicates that there is no color obtained in which there is antigen and capture antibody in the absence of conjugate. Thus, there is unwanted nonspecific color through attachment of the enzyme conjugate to the wells. This could indicate that the conjugate is being used at far too high a concentration, so that the blocking buffer conditions are not preventing nonspecific adsorption of the enzyme-labeled proteins. There is a titration of antigen on diluting the capture serum, indicated in the backgrounds in column 12; for example, in row G, which has a background of 0.9, a plateau of approx 2.0 is observed with an OD above background observed in column 11 and a reduction in color gradually from columns 3 and 4 to column 11.

A further CBT can be made using the conjugate beginning with that used in row F in the first attempt. **Table 19** gives idealized results. Here, the background is eliminated by row D (results are the same in D12, E12, F12, and G12). The effect of diluting the capture antibody is to titrate the antigen after an initial plateau (region of capture antibody/antigen excess). The conjugate dilution up to row D is not suitable owing to the high backgrounds obtained. Thus, a dilution of conjugate at about that in rows D and E can be assessed in the second stage of the CBT, in which the capture antibody and antigen can be varied.

Table 18
High Color in CBT[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 * 5	2 * 6	2 * 5	2 * 7	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.6	1.6
B	2 * 8	2 * 6	2 * 5	2 * 8	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.6	1.6
C	2 * 7	2 * 5	2 * 4	2 * 6	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.6	1.6
D	2 * 6	2 * 7	2 * 4	2 * 6	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.5	1.4
E	2 * 2	2 * 3	2 * 3	2 * 6	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.5	1.3
F	2 * 1	2 * 2	2 * 2	2 * 6	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.5	1.0
G	2.0	2.0	1.9	1.9	1.8	1.7	1.4	1.3	1.2	1.6	1.3	0.9
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Capture antibody titrated is in columns 1¨C11, and constant antigen A12 to H12, conjugate diluted is in rows A¨CG. *Readings are out of accurate range for reader.

Table 19
Repeat of CBT in Which High Color Was Obtained Using More Dilute Conjugate[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 * 1	2 * 2	2 * 2	2 * 6	2 * 7	2 * 1	2.0	1.9	1.7	1.6	1.5	1.0
B	2.0	2.0	1.9	1.9	1.8	1.7	1.4	1.3	1.2	1.6	1.3	0.9
C	1.9	1.9	1.9	1.9	1.8	1.6	1.3	1.1	0.9	0.5	0.3	0.3
D	1.8	1.8	1.8	1.8	1.8	1.5	1.1	0.8	0.6	0.4	0.2	0.1
E	1.6	1.6	1.6	1.6	1.6	1.3	1.1	0.7	0.5	0.3	0.2	0.1
F	1.2	1.1	1.1	1.1	1.1	1.0	0.9	0.6	0.4	0.2	0.1	0.1
G	0.9	0.9	0.8	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Capture antibody titrated is in columns 1¨C11, constant antigen is in A12¨CH12, and conjugate diluted is in rows A¨CG (beginning with dilution used in row F in the initial CBT).

2.2.5.3¡[a]
Very Weak Reactions

In cases in which little color is observed, we run into more difficulties because there is no obvious indicator of whether one or all the reagents are not functioning.

A special case is when there is no color development even after a significant time of incubation of the substrate/chromophore. Then the most likely culprit is the operator who forgets to add substrate to the reaction mixture. This can be tested by dipping a microtip into a conjugate and putting the tip into the remaining substrate/chromophore solution. This should show a rapid color

change; if not, the operator should repeat the test making sure that the proper substrate/chromophore mixture is correct.

When there is color, the CBT can be repeated. Should very low color then be obtained, the initial CBT should be repeated beginning with much higher concentrations of the two reagents being titrated. This can be a relatively futile operation since we know the dilutions of reactants and have usually added these at a high concentration in the initial CBT. Obviously, the third reactant (conjugate) can be critical, so a higher concentration can be added. Once again, when conjugates are not reacting at dilutions from 1/100 and below, there is little practical value in pursuing their use.

Thus, low color can result from an error such as failing to add proper reagents, making a mistake in the original dilution, or having all the reactants of inadequate strength.

It is easier to assess the reason for low color when there is an indication that one of the reagents is active. This is illustrated in **Table 20**, in which there is some color development in one area of the plate associated with column 1 and rows A¨CE. Since we have a constant amount of antigen and 1.5 OD units in column 1 is observed, this indicates that both the antigen and conjugate can function although the activity of the conjugate is also rapidly diminished after row C.

The fault here lies with the capture antibody, whose ability to capture rapidly dilutes out by column 3. The CBT could be repeated with higher concentrations in column 1. As already indicated, since the capture antibodies are present as a small component of the total serum proteins, and its capture activity resides in the ability of these specific antibodies to bind to the wells, it may be impossible to achieve a better capture reagent with this serum.

2.2.6¡ª
CBT for Other Systems

The last examples are meant to indicate the first developmental steps in analyzing the suitability of available reagents. In all cases, there is always going to be the need to make adjustments to allow establishment of defined test protocols. The CBT allows only a rough estimate of activities.

More complicated systems (e.g., those relying on four reactants) rely on establishing rough parameters for two of the reagents and examining the affect of diluting the other two. Such assays can be helped greatly through the developmental work with other ELISA systems using the same reagents. Examples are given next.

2.2.6.1¡ª
Developing an Indirect Sandwich ELISA

We already have titrated capture antibodies for use in a direct sandwich ELISA. Thus, the effective concentrations of capture antibodies, antigen, and conjugate are known. Now we may wish to develop an indirect sandwich

Table 20
Results Where One or More Reagents Are Weak[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5	0.5	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
B	1.5	0.4	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C	1.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
D	0.8	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
E	0.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
F	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a]Capture antibody titrated is in columns 1¨C11, constant antigen is in A12¨CH12, and conjugate diluted is in rows A¨CG.

ELISA replacing the labeled antibodies directly prepared against the target antigen by a nonlabeled detecting serum and an antispecies conjugate. A good reason to do this would be to allow the use of many different animal sera for examination. A good starting point would be to perform a CBT using constant capture antibodies and antigen and titration of the detecting serum and conjugate. The species of the detecting serum would have to be different from that of the capture antibodies since we are adopting an antispecies conjugate (the antispecies conjugate has to be tested for non-ELISA activity against the coating antibodies). Conditions for the optimal coating and antigen concentrations can be used initially. If the test is successful, adjustments can be made by altering any one of the reactant's concentrations.

The establishment of the concentrations of reagents in tests other than those finally used is not uncommon. In fact, pretitration in other systems could be used as a deliberate tool. In the majority of cases, laboratories are working with a limited range of antigens and antibodies, and their exploitation in different systems often results from the need to improve methods defined by a specific task at hand. The reagent link extends to developments in monoclonal antibodies (mAbs) in which polyclonal antibodies may by used at some stage to help production of a more specific or sensitive test. As an example, an indirect sandwich ELISA based on polyclonal sera is available, and we are investigating the use of a detecting mAb (possibly to see whether we can increase specificity for detecting captured antigen). Here, the initial CBT should involve constant capture antibodies and antigen, and titrate the mAb and antimouse conjugate. We know for certain (based on original polyclonal-based ELISA) that we can provide enough captured antigenic target for the mAb. Similarly, the activity of the mAb as a capture reagent can be assessed using the constant components of antigen, polyclonal detecting serum, and conjugate.

5¡ª
Theoretical Considerations

This chapter examines the aspects of using ELISA to solve problems, definitions of terms met in serology, antibody structure and the production of antibodies in animals, units, dilutions, and molarities. *Antibodies¡ªA Laboratory Manual* ***(1)*** is an excellent manual of techniques relevant to ELISA and all scientists involved in experimental work involving antibodies should have this manual. The manuals given in **refs.** ***2*** and ***3*** also provide extensive relevant practical information.

1¡ª
Setting up and Use of ELISA

The main aim in the development or use of established ELISAs is to measure some reactant. The need to measure a substance is the major reason for the assay. ELISAs can be used in pure and applied fields of science, but the chief reason they are worth developing is their high sample-handling capacity, ideal analytical sensitivity, and ease of performance. Another factor is the ease of reading, so that time is not wasted when a test has gone wrong. Thus, ELISAs can be assessed by eye before machine reading; for example, time is not wasted in reading 1000 sample points before this insight is obtained (as in radioimmunoassay). Care must be taken not to discard successful tests merely because ELISAs are in fashion. The relationship between ELISA results and other test system results must be established so that a large amount of comparative work using ELISA and one or more assays might be involved in setting up the ELISA as a standard assay.

2¡ª
What Is Known Already

A body of knowledge is often available in the scientific literature on any problem faced by an investigator. Therefore, a survey is necessary. This would mainly involve the detailing of work concerning the biological agent (antigen) being examined and any work involved with its relationship to defined hosts in experimental (laboratory animals) and field studies. This

knowledge can be divided into two categories: the biochemical/molecular biological aspects of the agent, and the immunological aspects (serology and immunology *per se*).

The literature may deal with the exact agent the investigator wishes to study or a similar agent and also reveal whether ELISAs have been performed. Obviously the two aspects of biochemistry and serology are related via the host. The main task at the beginning of any study is to define the aims properly. Scientists should also examine published work with care since often assays have been poorly devised or have been validated with little data. Scientists should also seek advice from others in related fields and examine whether reagents have already been produced that might be applicable to their own problems. A particular area is the use of monoclonal antibodies (mAbs).

An excellent catalog of immunological reagents is available, Linscott's that lists and updates reagents and suppliers of thousands of polyclonal and monoclonal reagents of direct relevance to ELISA. Much information is also available in catalogs of commercial firms that often have detailed technical descriptions of the use of their products.

3¡ª Complexity of Problems

Complexity of problems is manifested throughout the interrelationship between the agent and host. The concept of the relationship between antigenicity /immunogenicity and protection should be examined in this light. Thus, the major dogmas of antibodies and antigens must be examined. This book does not investigate the theories surrounding immunology, and textbooks should be consulted for more details. This chapter highlights relevant knowledge to allow more information to be sought. The following definitions are helpful.

3.1¡ª Antigenicity

Antigenicity is the ability of proteins and carbohydrates to elicit the formation of antibodies, which, by definition, bind specifically to the antigens used for injection into animals. Antibodies may be produced as a consequence of replication of an agent or by injection of inactivated whole or parts of that agent. This can further be refined so that defined peptides or polypeptides are used. The antigens used to elicit antibodies can, in turn, be used in tests such as the ELISA.

3.2¡ª Immunogenicity

Immunogenicity is a measure of the effect of binding of antibodies elicited by any substance. More specifically, the effect is one of producing some degree of immunity against the disease agent. Generally, such measurements are made in vitro or in animal systems other than those being examined in the field.

3.3¡ª
Protection

Production of antibodies and demonstration of immunogenic responses does not necessarily mean that animals will be protected against challenge of the disease agent. The relationship of immunoassay results to protection is never straightforward because of the many other factors involved in immunological response, e.g., cellular immunity.

4¡ª
Antigenic Considerations

Inherent to the understanding of what is being measured in any ELISA is the definition of terms involving antigens and antibodies and an understanding of the implications of the size, number of possible antigenic sites (epitopes), distribution of epitopes (distance between them), variability of epitopes, effect on variation in epitopes on different assay systems, and so forth. As the size (molecular weight) of disease agents increases, complexity increases.

4.1¡ª
Size Considerations

Immunoassays must be developed with as much knowledge as possible of all previous studies. The complexity of agents generally increases with their size. On theoretical grounds, the relationship of size to possible complexity can be demonstrated by examining spherical agents of different diameters, beginning at 25 nm (e.g., the size of a foot-and-mouth disease virus [FMDV] particle).

One can calculate that the area bound by a Fab molecule (single arm combining site of antibody without Fc) is approx 20 nm^2. This can represent an antigenic site (epitope). Thus, the number of possible sites on the virus is the surface area of the virion divided by the surface area of the combining site. The surface area of a sphere is 4 ¡Á π ¡Á r^2; therefore, for FMDV 4 ¡Á 3.14 ¡Á 12.52 = 12.56 ¡Á 156.25 = 1962 nm^2. By dividing this by 20, the maximum number of Fab sites possible is 98.

If the diameter of the agent is increased by twofold to 50 nm, using the same calculation the number of sites is 392. Another twofold increase in diameter gives 1468. These are small agents. If the calculations are done for agents increasing in diameter by 10-fold steps, for Fab and whole IgG (binding bivalently effective area 60 nm^2), the data would be as given in **Table 1**.

The numbers in **Table 1** illustrate that the surface area increases as a square function of the diameter. Such a calculation is based on the facts that the whole surface is antigenic (rarely true) and that the molecules bind maximally. However, experimentally, relative figures close to the theoretical are obtained. This has implications in immunoassays since one can calculate how much antibody is needed to saturate any agent, or measure the level of antibody attachment as a function of available surface.

Table 1
Relationship of Diameter of Agents to Number of Binding Sites

Diameter	Fab sites	IgG sites
25	98	32
250	9800	3200
2500	980,000	320,000
25,000	98,000,000	32,000,000

Since we know the molecular weight of IgG (and Fab), we can calculate the weight of a number of molecules. Thus, the mol wt of IgG = 150,000. Using Avogadro's number (approx 6 ¡Á 10^{23}) we obtain the following results:

1. 1 g of IgG contains approx 6 ¡Á $10^{23}/1.5$ ¡Á 10^{5} = 4 ¡Á 10^{18} molecules.

2. 1 mg of IgG contains approx 4 ¡Á $10^{18}/10^{3}$ = 4 ¡Á 10^{15} molecules.

3. 1 µg of IgG contains approx 4 ¡Á $10^{18}/10^{6}$ = 4 ¡Á 10^{12} molecules.

4. 1 ng of IgG contains approx 4 ¡Á $10^{18}/10^{9}$ = 4 ¡Á 10^{9} molecules.

5. 1 pg of IgG contains approx 4 ¡Á $10^{18}/10^{12}$ = 4 ¡Á 10^{6} molecules.

Such a model calculation helps us to understand at the molecular level what we are dealing with when faced with different antigens.

4.2¡ª
Definitions

There may be some confusion concerning the terminology used in immunological and serological circles. This section provides some working definitions that will aid the understanding of the mechanisms involved in ELISAs.

4.2.1¡ª
Antigen

An antigen is a substance that elicits an antibody response as a result of being injected into an animal or as a result of an infectious process. Antigens can be simple (e.g., peptides of mol wt about 5000) to complex. Antibodies specific for the antigen are produced. The definition can be extended to molecules that evoke any specific immune response including cell-mediated immunity or tolerance.

4.2.2¡ª
Antigenic Site

An antigenic site is a distinct structurally defined region on an antigen as identified by a specific set of antibodies usually using a polyclonal serum.

4.2.3¡ª
Epitope

An epitope is the same as an antigenic site, but in which a greater specificity of reaction has been defined, e.g., using tests involving mAbs in which a single population of antibodies identifies a single chemical structure on an antigen.

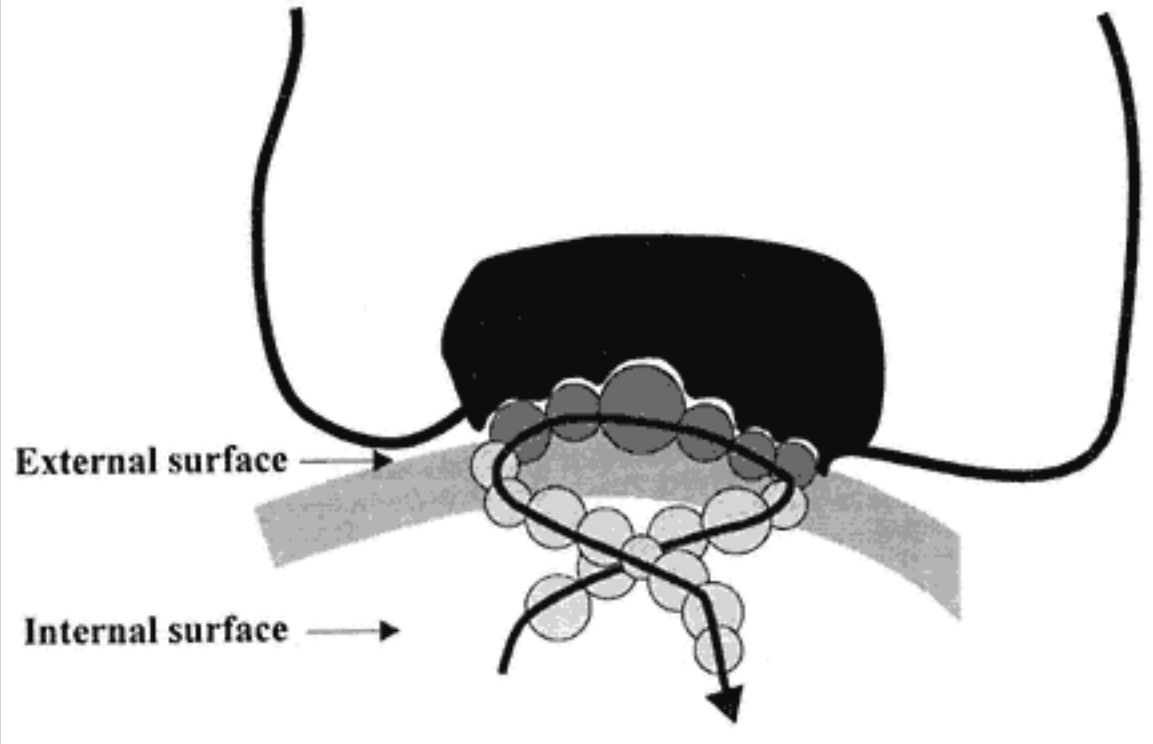


Fig. 1.
Representation of a linear of continuous site. Black area shows paratope of antibody with specificity for site.

4.2.4¡ª
Epitype

An epitype is an area on an antigen that is identified by a closely related set of antibodies identifying very similar chemical structures (e.g., mAbs, which define overlapping or interrelated epitopes). An epitype can be regarded as an area identifying slightly different specificities of antibodies reacting with the same antigenic site.

4.2.5¡ª
Continuous Epitope

A continuous epitope is produced by consecutive atoms contained within the same molecule. Such epitopes are also referred to as linear epitopes and are not usually affected by denaturation (*see* **Fig. 1**).

4.2.6¡ª
Discontinuous Epitope

A discontinuous epitope is produced from the interrelationship of atoms from nonsequential areas on the same molecule or from atoms on separate molecules. Such sites are also usually conformational in nature (*see* **Fig. 2**).

4.2.7¡ª
Linear Epitope

A linear epitope is the same as a continuous epitope, with recognition of atoms in a linear sequence (*see* **Fig. 1**).

4.2.8¡ª
Conformational Epitope

A conformational epitope is formed through the interrelationship of chemical elements combining so that the 3D structure determines the specificity and affinity. Such epitopes are usually affected by denaturation (*see* **Fig. 2**).

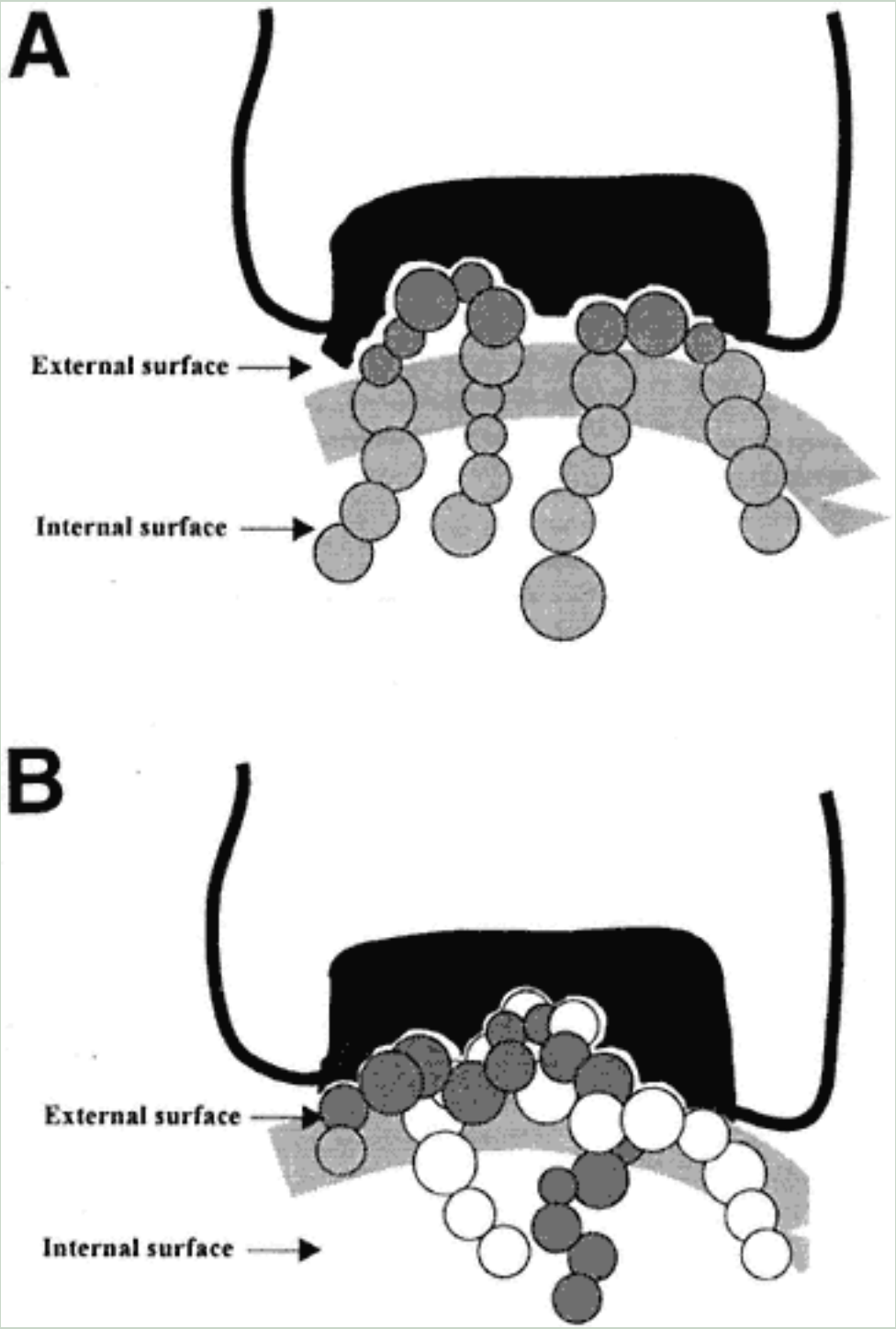


Fig. 2.
Representations of two types of conformational epitopes. Black area shows papatope of antibody molecule with specificity for the sites. **(A)** Recognition of three-dimensional (3D) relationship of atoms from nonconsecutive atoms on same protein molecule; **(B)** recognition of 3D relationship of atoms on two different protein molecules.

4.2.9¡[a]
Antibody-Combining Site

An antibody-combining site is the part of the antibody molecule that combines specifically with an antigenic site formed by the exact chemical nature of the H and L chains in the antibody molecule.

4.2.10¡ª
Paratope

A paratope is the part of the antibody molecule that binds to the epitope. It is most relevant to mAbs antibodies in which a single specificity for a single epitope can be defined.

4.2.11¡ª
Affinity and Avidity

Affinity and avidity relate to the closeness of fit of a paratope and epitope. Considered in thermodynamic terms, it is the strength of close-range non-

covalent forces. Mathematically it is expressed as an association constant (*K*, L/mol) calculated under equilibrium conditions. Affinity refers to the energy between a single epitope and paratope. Antisera usually contain populations of antibodies directed against the same antigenic site that have different affinities owing to their differences in exactness of fit. Antisera of multiple specificity (i.e., specific to many determinants on an antigen) cannot be assessed for affinity, however, they can be assessed for overall binding energy with an antigen in any chosen assay. This is termed the *avidity* of the serum. The avidity represents an average binding energy from the sum of all the individual affinities of a population of antibodies binding to different antigenic sites.

4.2.12¡ª
Polyclonal Antibodies

Polyclonal antibodies are the serum product of an immunized animal containing many different antibodies against the various mixtures of antigens injected. The antiserum is the product of many responding clones of cells and is usually heterogeneous at all levels. These levels include the specificity of the antibodies, classes and subclasses, titer, and affinity. The response to individual epitopes may be clonally diverse, and antibodies of different affinities may compete for the same epitope. This variation means that polyclonal antisera cannot be reproduced (*see* **Fig. 3**).

4.2.13¡ª
Monoclonal Antibodies

mAbs are antibodies derived from single antibody-producing cells immortalized by fusion to a B-lymphocyte tumor cell line to form hybridoma clones. The secreted antibody is monospecific in nature and thus has a single affinity for a defined epitope (*see* **Fig. 4**).

5¡ª
Antibodies

Antibodies form a group of glycoproteins present in the serum and tissue fluids of all mammals. The group is also termed *immunoglobulins* (Igs) indicating their role in adaptive immunity. All antibodies are Igs, but not all Igs are antibodies, that is, not all the Ig produced by a mammal has antibody activity. Five distinct classes of Ig molecules have been recognized in most higher mammals. These are Ig, IgG, IgA, IgM, IgD, and IgE. These classes differ from each other in size, charge, amino acid composition, and carbohydrate content. There are also significant differences (heterogeneity) within each class. **Figure 5** shows the basic polypeptide structure of the Ig molecule.

5.1¡ª
Antibody Structure

The basic structure of all Ig molecules is a unit of two identical light (L) polypeptide chains and two identical heavy (H) polypeptide chains linked

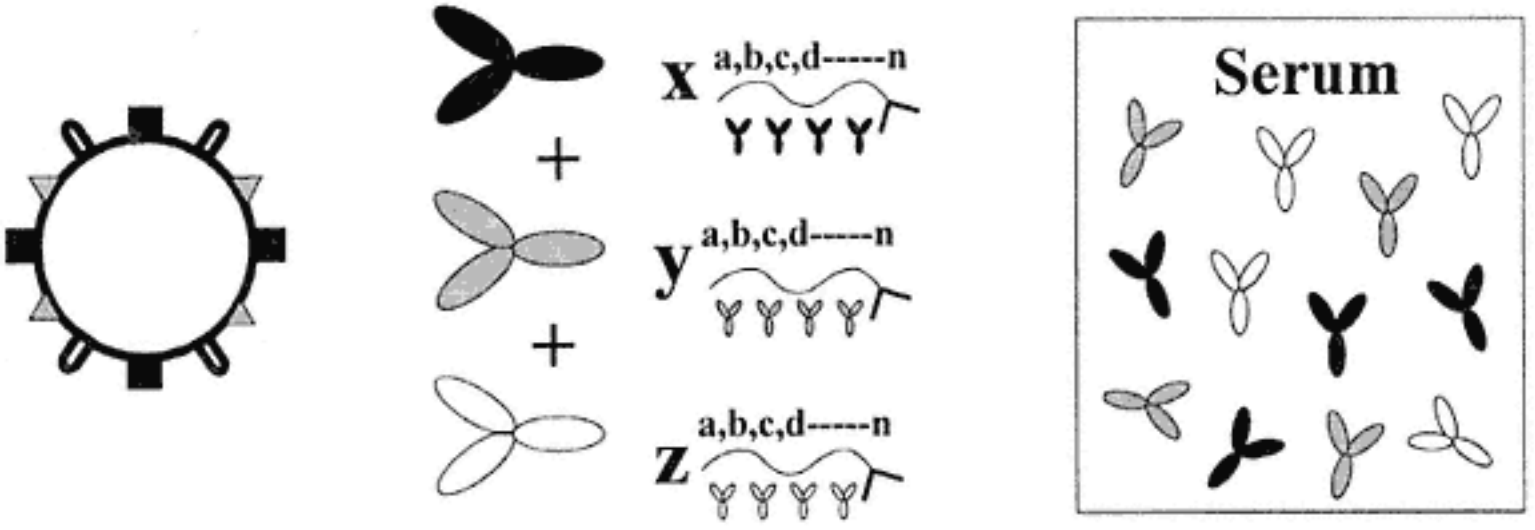


Fig. 3.
Specific antibodies are produced against different sites and can vary in affinity against the same site. This, a,b,c,d-----n represents a range of slightly different antibody populations recognizing antigenic sites x, y, and z. The antibodies have different affinities, classes, and isotypes. The resulting mixture is a polyclonal antiserum.

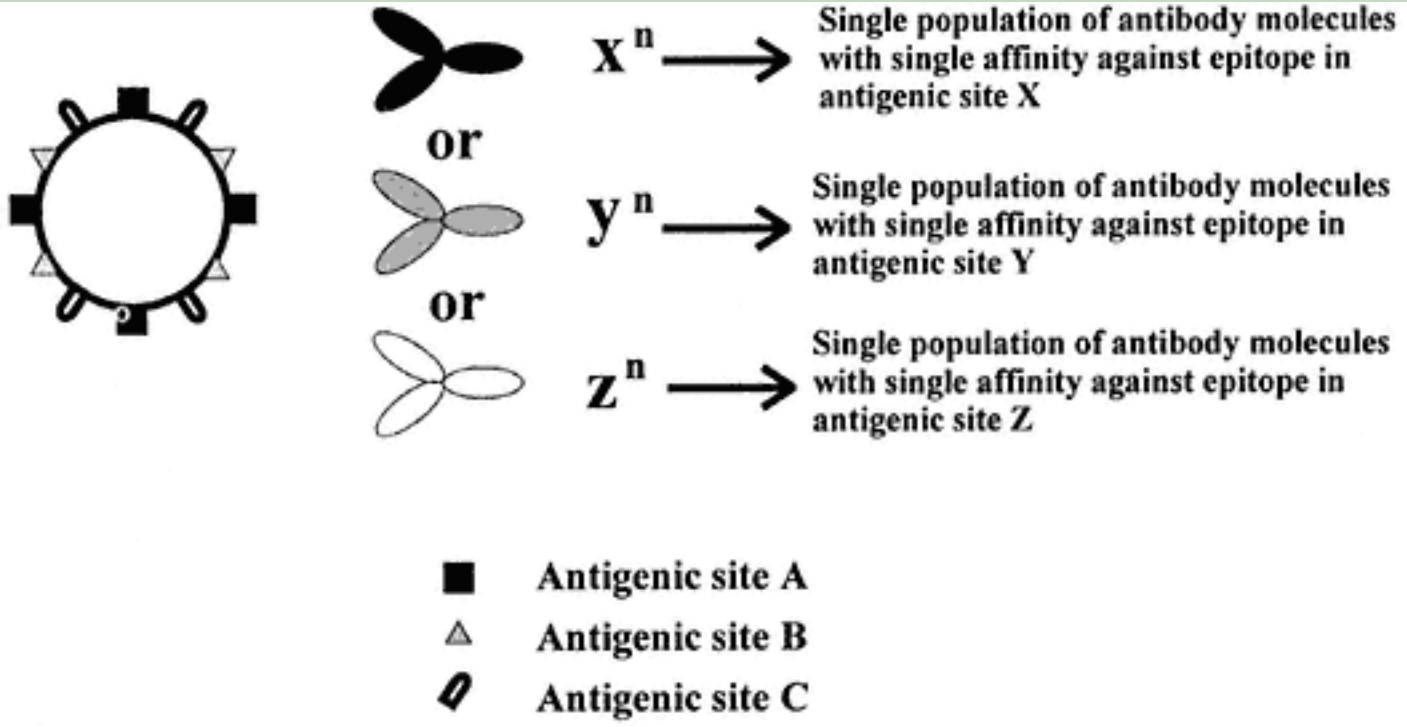


Fig. 4.
mAbs are monospecific in terms of antigenic target and affinity.

together by disulfide bonds. The class and subclass of an Ig molecule is determined by its heavy chain type. Thus, in the human, there are four IgG subclasses¡ªIgG$_1$, IgG$_2$, IgG$_3$, IgG$_4$¡ªthat have heavy chains called 1, 2, 3, and 4. The differences between the various subclasses within an individual Ig class are less than the differences between the different classes. Therefore IgG$_1$ is more closely related to IgG$_2$, and so on than to IgA, IgM, IgD, or IgE. The most common class of Ig is IgG.

IgG molecules are made up of two identical light chains of mol wt 23,000 and two identical heavy chains of mol wt 53,000. Each light chain is linked to

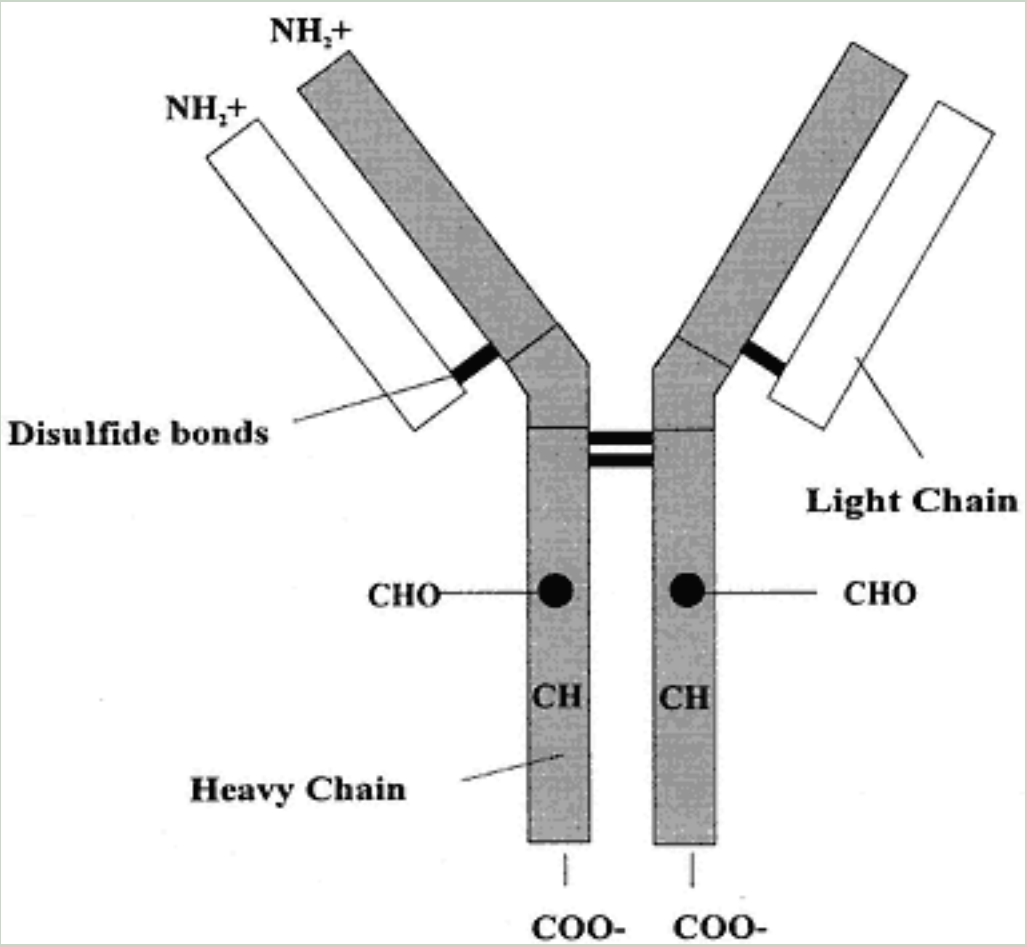


Fig. 5.
Representation of basic structure of an IgG molecule.

a heavy chain by noncovalent association, and also by one covalent disulfide bridge. For IgG, each light-heavy chain pair is linked to the other by disulfide bridges between the heavy chains. This molecule is represented schematically in the form of a Y, with the amino (N-) termini of the chains at the top of the Y and the carboxyl (C-) termini of the two heavy chains at the bottom of the Y shape. A dimer of these light-heavy chain pairs is the basic subunit of the other Ig isotypes. The structures of these other classes and subclasses differ in the positions and number of disulfide bridges between the heavy chains, and in the number of L-H chain pairs in the molecule. IgG, IgE, and IgD are composed of one L-H chain pair. IgA may have one, two, or three light-heavy chain pairs. IgM (serum) has five light-heavy chain pairs, whereas membrane-bound IgM has one. In the polymeric forms of IgA and IgM, the light-heavy chain pairs are held together by disulfide bridges through a polypeptide known as the J chain.

In both heavy and light chains, at the N-terminal portion the sequences vary greatly from polypeptide to polypeptide. By contrast, in the C-terminal portion of both heavy and light chains, the sequences are identical. Hence, these two segments of the molecule are designated variable and constant regions. For the light chain, the variable (V) region is about 110 amino acid residues in length

and the constant (C) region of the light chain is similarly about 110 amino acids in length.

The variable region of the heavy chain (V_H) is also about 110 amino acid residues in length, but the constant region of the heavy chain (C_H) is about 330 amino acid residues in length. The N-terminal portions of both heavy and light chain pairs comprise the antigen-combining (binding) sites in an Ig molecule. The heterogeneity in the amino acid sequences present within the variable regions of both heavy and light chains accounts for the great diversity of antigen specificities among antibody molecules. By contrast, the constant regions of the heavy chain make up the part of the molecule that carries out the effector functions that are common to all antibodies of a given class.

Figure 5 shows that there must be two identical antigen-binding sites (more in the case of serum IgM and secretory IgA); hence, the basic Y-shaped Ig molecule is bivalent. This bivalency permits antibodies to crosslink antigens with two or more of the same epitope. Antigenic determinants that are separated by a distance can be bound by an antibody molecule.

The antigen-combining site (active site) is a crevice between the variable regions of the light and heavy chain pair. The size and shape of this crevice can vary owing to differences in the relationship of V_L and V_H regions as well as to differences in variation in the amino acid-sequence. Thus, the specificity of antibody will result from the molecular complementarity between determinant groups (epitopes) on the antigen molecule and amino acid residues present in the active site.

An antibody molecule has a unique 3D structure. However, a single antibody molecule has the ability to combine with a range (spectrum) of different antigens. This phenomenon is known as multispecificity. Thus, the antibody can combine with the inducing antigenic determinant or a separate determinant with similar structures (crossreacting antigen). Stable antigen-antibody complexes can result when there is a sufficient number of short-range interactions between both, regardless of the total fit. This is a problem for the immunoassayist, and care must be taken to ensure that the operator is assaying for the correct or desired antigen; therefore, careful planning of negative and positive controls is essential.

5.1.1¡[a]
Antibody Digestion

Figure 6 demonstrates the digestion of IgG using papain or pepsin proteolytic enzymes. Mild proteolysis of native Ig at the hinge regions of the heavy chain by papain will cleave IgG into three fragments. Two of these fragments are identical and are called fragment antigen binding or Fab. Each Fab consists of the variable and constant regions of the light chain and the variable part of

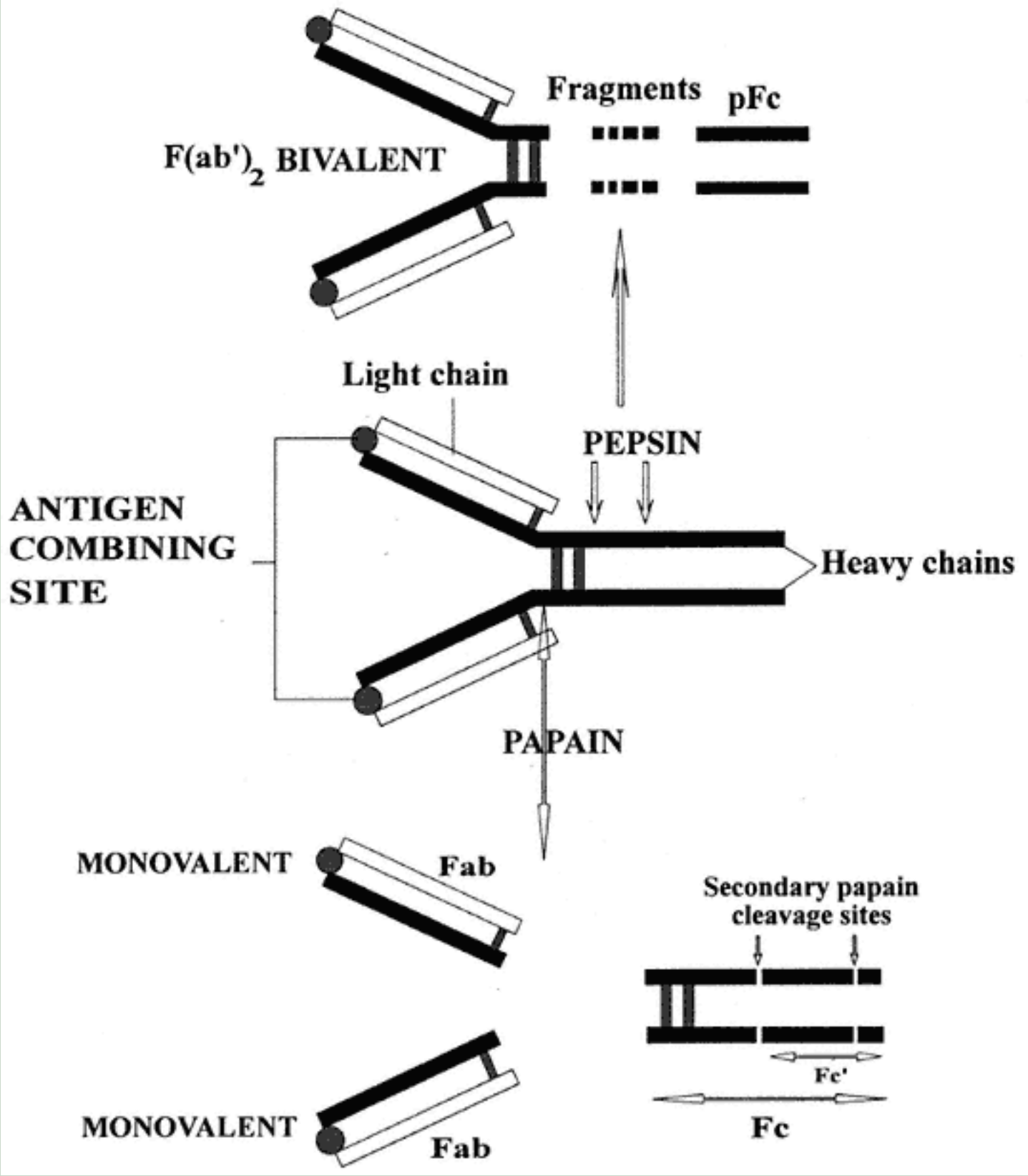


Fig. 6.
Enzymatic cleavage of human IgG. Pepsin cleaves the heavy chain to give F(Ab') and o/fc' fragments. Further action results in greater fragmentation of central protein to peptides. Papain splits the molecule in the hinge region to give two Fab_2 fragments and the Fc fragment. Further action on the Fc can produce Fc'.

the constant (ChI domain) regions of the heavy chain. Therefore, each Fab carries one antigen binding site. The third fragment, consisting of the remainder of the constant regions of the heavy chains, is readily crystallizable and is called fragment crystallizable or Fc.

Pepsin digestion cleaves the Fc from the molecule but leaves the disulfide bridge between the Fab regions. This molecule contains both antigen-combining sites and is bivalent.

5.1.2¡ª
Antibody Classes

The five immunological classes (isotypes) can be distinguished structurally by differences in their heavy chain constant regions (i.e., mainly the Fc portion). These heavy chain classes define the corresponding Ig classes IgA, IgG, IgD, IgE, and IgM. Some classes can be divided further into subclasses.

In addition, two major types of light chains exist, based on the differences in the constant region Cl and are known as kappa (κ) and lambda (λ). Igs from various mammals appear to conform to this format. However, the subclass designation and variety may not be the same in all species examined; for example, mice have IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and cows have IgG_1 and IgG_2.

5.2¡ª
Antibody Production in Response to Antigenic Stimulus

The antibodies produced in a humoral response to antigenic stimulus are heterogeneous in specificity and may include all Ig classes. This heterogeneous response is owing to the fact that most antigens have multiple antigenic determinants that trigger off the activation of different B-cells. Therefore, the serum of any mammal (vertebrate) contains a heterogeneous mixture of Ig molecules. The specificities of these Ig molecules will reflect the organism's past antigenic exposure and history.

The first antibody produced in response to a primary exposure of an immunogen is IgM. When the immunogen is persistent or the host (mammal) is reexposed to the immunogen, other classes of antibody may be produced as well as IgM. The body compartment in which the immunogen is presented can determine the predominant antibody isotype produced (e.g., IgA in the gastrointestinal [GI] tract). In general, primary exposure to an immunogen stimulates the production of IgM initially, followed by the appearance of IgG), as shown in **Fig. 7**.

If no further exposure occurs, or the immunogen is removed by the mammal, a low level of IgM and IgG can be detected. If reexposure occurs, a similar peak of IgM antibody is produced that declines in a similar kinetic manner to the primary IgM response, but the IgG response is not only more rapid (over time) but also reaches higher serum levels that persist for a longer period of time. This IgG response to reexposure is known as the anamnestic response. This is illustrated in **Fig. 7**.

In cases in which complex antigens occur, as in infectious diseases, the dosage (infection level), type of antigen (viral, bacterial, protozoan, helminthic), route of infection (oral, respiratory, cutaneous), and species of mammal infected (cow, pig, camel, human) will all affect the degree and speed by which IgG replaces IgM.

These considerations are vital for the immunoassayist who is concerned with diagnosing infectious diseases of mammals, and great care and planning should

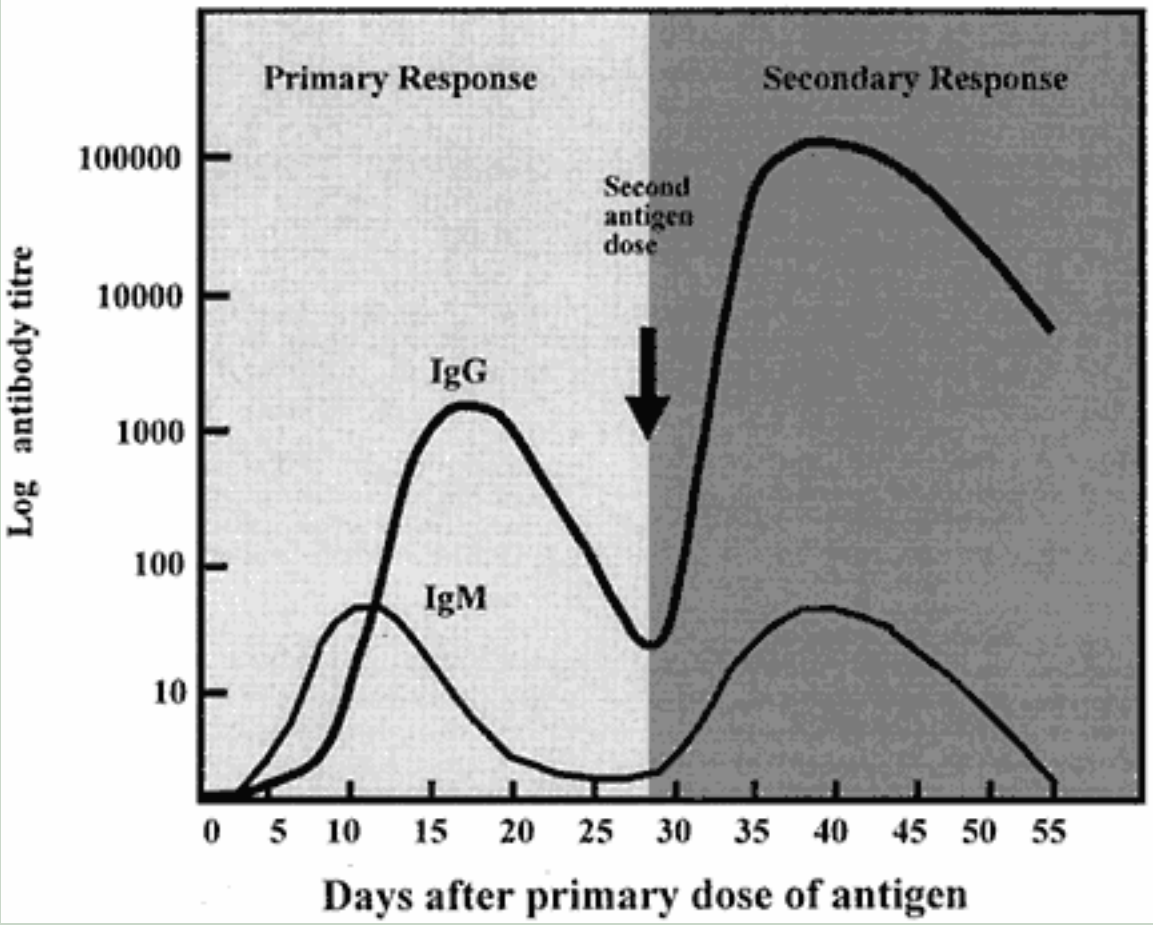


Fig. 7.
Anamnestic response following second administration of antigen. Primary response following initial antigen dose has a lag phase in which no antibody is detected (4¨C5 d). This is followed by a lag phase in which antibody is produced. A plateau phase follows in which antibody titers stabilize, after which a decline in titer is observed. On secondary stimulation, there is an almost immediate rise in titer and higher levels of antibodies are achieved that are mainly IgG.

be exercised before undertaking such immunoassays. Note also that at this stage, different infectious disease agents can stimulate different antibody isotypes. For example, certain viral pathogens stimulate predominantly IgM agglutinating responses, bacterial polysaccharides stimulate IgM (and IgG_2 in humans) antibodies, and helminthic infections stimulate the synthesis of IgE antibody.

In general, it can be stated that during the development of immunity to infectious disease agents, the antibodies produced become capable of recognizing antigens better, as demonstrated by improved antigen-antibody interaction. The multispecificity of antibody molecules (i.e., the ability to combine with a variety of epitopes containing similar molecular structures) is dependent not only on the heterogeneity of the epitope in question, but also on the molecular construction of the antigen-reactive sites (paratopes) of the antibody molecules.

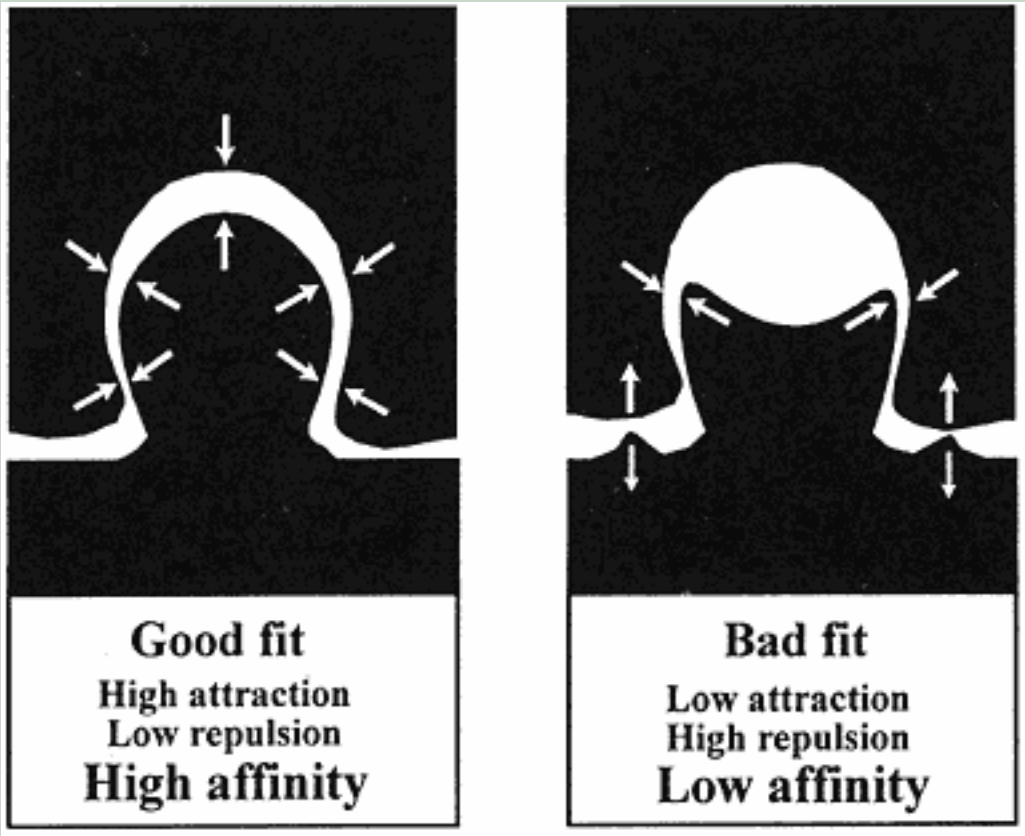


Fig. 8.
A good fit between antigenic sites and antibody-combining sites creates as environment for the intermolecular attractive forces to be created and limits the chances of repulsive forces. The strength of the single antigen/antibody bond is the affinity that reflects the summation of the attractive and repulsive forces.

5.3¡ª
Affinity and Avidity

The binding energy between an antibody molecule and an antigen determinant is termed *affinity*. Thus, antibodies with paratopes that recognize epitopes perfectly will have high affinity (good fit) for the antigen in question, whereas antibodies with paratopes that recognize epitopes imperfectly will have low affinity (poor fit). Low-affinity antibodies in which the fit to antigen is less than perfect will have fewer noncovalent bonds established between the complex, and the strength of binding will be less, as shown in **Fig. 8**.

With simple immunogens containing few epitopes, as the antibody response develops (in response) to this immunogen, its recognition by antibody will become better or closer, that is, low-affinity antibodies will be replaced by high-affinity antibodies, which will cause the interaction between antigen and antibody to be more stable. Antibodies produced later during infection are generally of higher affinity than those produced early on during infection. Hence, the IgG antibodies produced in response to reexposure will be of higher affinity than those produced in response to initial exposure.

In a serum sample in which there has been polyclonal stimulation of antibody production by antigen, a variety of affinities will be present within the

antibodies. The match (fit) between antibodies to that antigen will be variable, and the antibodies present in that serum sample will bind to antigen differentially. Thus, not only can an antigen stimulate different antibody isotypes but also antibodies with different affinities for the antigenic determinant. Avidity can be regarded as the sum of all the different affinities between the heterogeneous antibodies contained in a serum and the various antigenic sites (epitopes). It is important to realize that the avidity of a serum may change on dilution because an operator may be diluting out certain populations of antibodies.

As an example, we could have a serum containing a low quantity of antibodies showing high affinity for a particular complex antigen and a high quantity of low-affinity antibody. Under immunoassay conditions in which that serum is not diluted greatly, we would have competition for antigenic sites between the high- and low-affinity antibodies, and the high-affinity antibodies would react preferentially. On dilution, however, the concentration of the high-affinity antibodies would be reduced until we would be left only with low-affinity antibodies. Such problems are important when an operator is using immunoassays to compare antigens by their differential activity with different antisera. The dilution of any serum can affect its ability to discriminate between antigens owing to the dynamics of the heterogeneous antibody population (relative concentrations and affinities of individual antibody molecules).

Such problems of quality and quantity do not apply to mAbs, because, by definition, the Ig molecules in the population are identical. They all have the same affinity and therefore the avidity equals affinity. Thus, the population reacts identically to any individual molecule in that population. On diluting the monoclonal population, there is no alteration in the affinity/avidity of the serum, and a change noted for reaction between the mAb and antigen must be from changes on the particular antigen.

Figure 9 illustrates crossreactions between sera and different antigens. Here, specific reactions occur in which all the antibodies have "best fit." When two antigens share a similar antigen, crossreactions will be observed. The two non-identical sites may also contribute to the crossreaction. When all the antibodies show no recognition of the antigens available, no reaction will be seen. It is important to understand the concepts of variability in (1) isotype production and (2) affinity and affinity maturation when developing immunoassays for infectious disease agents that are normally more chronic than acute in duration.

As antigens are introduced into different compartments of the mammalian body they can stimulate the production of different antibody isotypes. Local antibody responses in the GI tract and the respiratory tree are predominantly IgA isotypes, whereas those in the other major compartments are predominantly IgG (IgM). Certain sites in the body (e.g., testes) are immunologically privileged and stimulate lower antibody responses to immunogens. Most

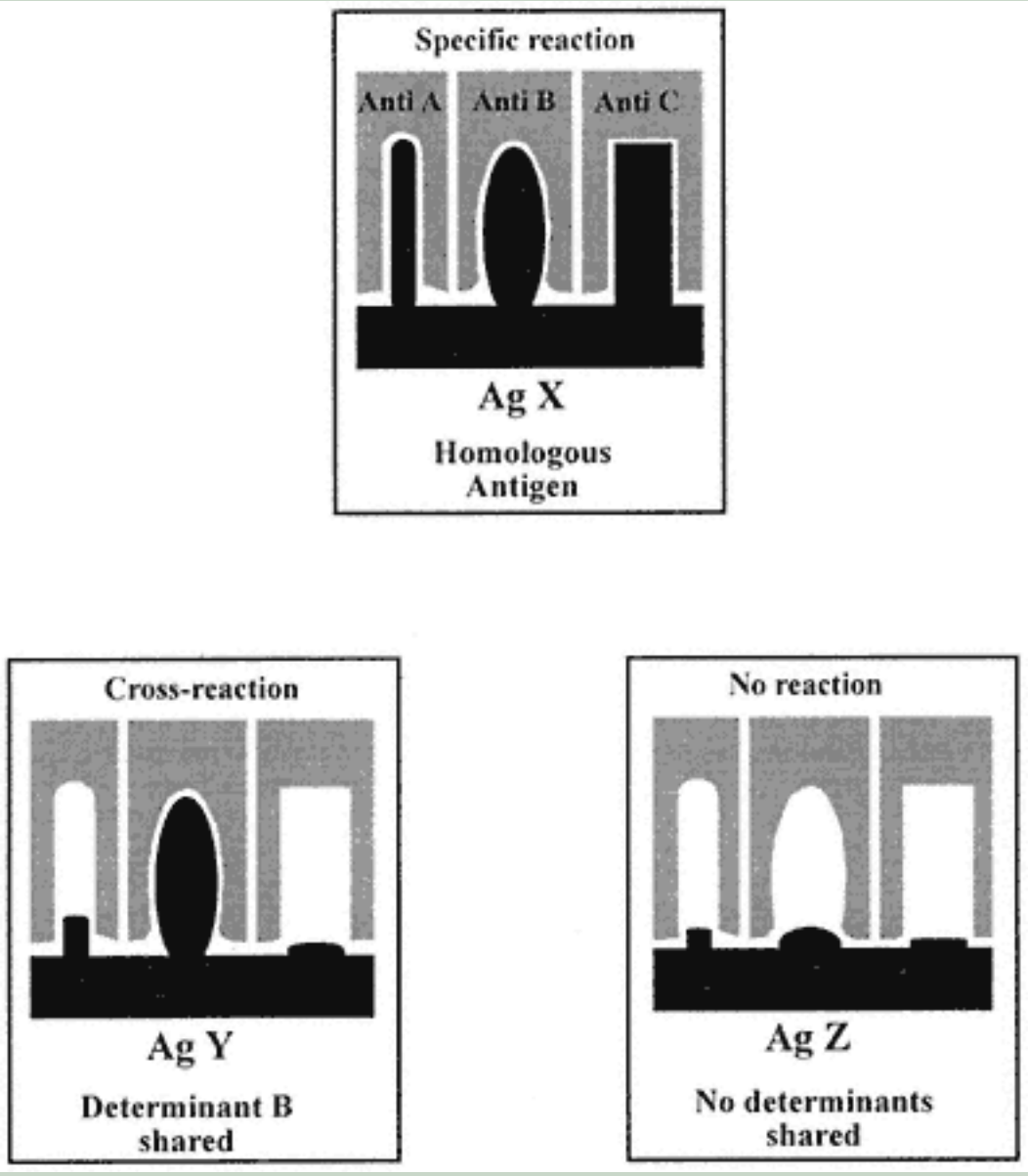


Fig. 9.
Specificity, crossreactivity, and nonreactivity. Antisera contain populations of antibodies. Each population is directed against a different determinant (A, B, and C). Antigen X and Y share a determinant (B); thus, antiserum against X will react withantigen Y (crossreact) as well as reacting specifically with antigen X. Antiserum against antigen X does not react with antigen Z since no determinants are shared.

infectious diseases are transmitted by aerosolization, close contact, or vectors; thus, their route of transmission is variable. In addition, their final location may be distant from their point of deposition. Similarly, whereas some pathogens are capable of division within the host, others are incapable of division within the mammalian host (e.g., helminths).

5.4¡[a]
Antibody Production in Response to Immunization/Vaccination

Individuals can be rendered resistant to infectious agents by either passive immunization or active immunization. In general, the beneficial effects of immunization are mediated by antibodies, and therefore the effects of immunization can be monitored by the immunoassayist.

5.4.1¡[a]
Passive Immunization.

Passive immunization is accomplished by transferring antibodies from a resistant to a susceptible host. Passively transferred antibodies confer a temporary but immediate resistance to infection, but are gradually catabolized by the susceptible host. Once passive protection wanes, the recipient becomes susceptible to infection again. Passively transferred antibodies can be acquired by the recipient either transplacentally and transcolostrally as in neonates, or by injection of purified antibodies from a resistant donor into a susceptible recipient.

5.4.2¡[a]
Active Immunization

Active immunization is accomplished by administering antigens of infectious agents to individuals so that they respond by producing antibodies that will neutralize the infectious disease agents, once contracted. Reexposure to such agents, following active immunization, will result in an anamnestic immune response, in which the antibodies or effector cells produced will be capable of neutralizing the effect of or destroying the inciting agents. Such antibodies are known as protective antibodies, and their complementary antigens as protective antigens. The protection conferred by active immunization is not immediate, as in passive immunization, because the immune system requires considerable time process such antigens and produce protective antibodies. However, the advantage of active immunization is that it is long lasting, and restimulation by the same antigens present in pathogens leads to an anamnestic response. It is important to recognize that the immunity produced to pathogens, following active immunization, is only as broad as the antigenic spectrum of the preparation used for immunization. Protection can be afforded using different approaches in the formulation of vaccines.

5.4.3¡[a]
Live Vaccines

Live vaccines may be attenuated by the passage of agents (e.g., viruses) in an unusual host so that they become nonpathogenic to vaccinated animals. Usually these are good vaccines because they supply the same antigenic stimulus as the disease agent. There can be problems of reversion to the pathogenic agent and some replication of the agent usually occurs.

5.4.4¡ª
Modified Vaccines:
Whole Disease Agent

Vaccines can be grown and then chemically modified (e.g., heat killed, nucleic acid modified [mutagens], or formaldehyde treated). These are potentially good vaccines in that full antigenic spectrum is given. The antigenic mass must be high since there is no replication to challenge the immune system. Repeat vaccinations are common to elevate antibody levels.

5.4.5¡ª
Purified Antigens

Protective antigens can be identified and used as proteins, polypeptides, and peptides as immunogens, usually with adjuvants. Usually these vaccines are not as good as those in which total antigenic spectrum is used. They have the advantages of being able to synthesize products on large scale by chemical methods (e.g., as peptides) and are noninfectious.

5.4.6¡ª
DNA Technology Products

Genes producing particular immunogens can be inserted into replicating agents so that their products are expressed. Novel approaches include use of mammalian viruses, insect viruses (baculovirus expression), yeasts, *E. coli*.

5.4.7¡ª
Generalities

Most vaccines are administered by either sc or im injections. When vaccinating large herds of animals, other techniques such as high-pressure jet injections may be employed. Obviously the risk of administering unwanted or contaminating organisms and antigens should be minimal; hence, sterile administration of vaccines is indicated. Subcutaneous or im vaccination should induce all antibody isotypes given the fact that the inciting antigens are capable of doing so. Therefore, the immunoassayist must consider whether total antibody assays, isotype-specific assays, or assays to detect antigen clearance are to be utilized to assess the effects of vaccination.

Some antigens may be administered orally (e.g., poliomyelitis vaccines in humans) by incorporation in food or drinking water (e.g., in poultry flocks) or by inhalant exposure of an aerosolized vaccine (e.g., diseases of the respiratory tract). In these instances, the production of local antibodies to prevent the ingress of pathogens through the GI or respiratory tree barriers should be sought. The immunoassayist must decide whether an assay for isotype-specific antibodies, notably IgA, may provide deeper insight into the benefits of vaccination than an assay for total antibody.

In some instances in which infectious disease agent is endemic and vaccination, especially of newborns, is indicated, it may prove difficult or impossible to differentiate the beneficial effects of vaccination because residual levels of antibody may be present in nonvaccinated stock. Such factors must be borne in mind when assays are developed to determine the immunological status of large groups of mammals.

5.5¡ª
Antibody Production in Response to Infectious Agents

It is beyond the scope of this book to catalog the humoral immune responses produced in mammals in response to the variety of infectious disease agents

such as viruses, bacteria, fungi, protozoa, helminths, and arthropods. Such information may be obtained from textbooks and specialized review articles. This section deals with the general considerations of host-parasite relationships, with specific reference to the production of antibodies to pathogens. As already mentioned, most infectious diseases are transmitted by aerosolization, close contact, or vectors, and their final location may be distant from their point of deposition. Therefore, these pathogens involve multiple organs. Similarly, many pathogens, but not all, have the capacity to divide within the mammalian body, and in such instances, the numbers and amounts of antigens produced will increase over time and be proportional to the number of pathogens at the time of sampling. When pathogens do not divide or reproduce in the mammalian host, the amount of antigens produced may be directly proportional to the infective dose. Hence, in devising assays for infectious disease agents, the immunoassayist must take into account whether high or low concentrations of antigens and antibodies are to be sought. When antibody titers of less than 1:50 are anticipated, serum dilutions of less than 1:50 or possibly less than 1:10 for the test serum must be employed. Previous knowledge of specific host-parasite systems will prove invaluable in devising more specific and sensitive enzyme immunoassays.

Although many nonimmunological mechanisms exist for the removal of pathogens from the body (e.g., lysozyme, iron-binding proteins, myeloperoxidase, lactoperoxidase, complement, basic peptides, and proteins), it is generally recognized that the immune system plays a vital role in the control and destruction of pathogens. For this reason, the measurement of antibody or antigen by sensitive assays, such as ELISA, provides a useful indicator for the assessment of immune status. When an infectious agent enters the mammalian body, the first components recognized as foreign are surface components of that pathogen. This host/pathogen interface plays a vital role in the control of infectious diseases, not only in its involvement in stimulating the early humoral immune response but also in its involvement in mediating protective immune responses. Immune responses that reduce pathogen numbers by lysis, agglutination or phagocytosis and that reduce the antigen load are normally regarded as protective responses. Such antibodies directed against specific epitopes on the pathogens can be sought by the immunoassayist in an effort to correlate protective responses with clinical betterment. However, insight into the molecular basis of such interactions is necessary before immunoassays can be developed to demonstrate protective responses (e.g., knowledge of the immunochemistry of the surface-exposed molecules and their epitopes, knowledge of specific antibody isotypes that mediate these responses). Because infectious disease agents stimulate antibody production, these antibodies can prove useful to the immunoassayist for detecting exposure to pathogens.

We have seen that when the antigens of pathogens are recognized by the host, an antibody response ensues, initially of the IgM isotype and followed by the IgG isotype, together with an increase in antibody affinity, over time. When a variety of antibody isotypes is produced in response to infection, this isotypic variation can be used to determine the chronicity of the infection since IgM antibody isotypes normally appear before IgG antibody isotypes. Similarly, increasing levels of antibodies can indicate current infections or exacerbations of infections, whereas decreasing antibody titers can indicate past infections or successful control of current infection. In the absence of detectable free circulating antibody, either free antigen or circulating immune complexes can be detected by ELISA. When antibodies specific to antigens of a pathogen are used to detect the presence of free antigen in the test sample, a direct correlation can be made between ELISA positivity and current infection. When protective mechanisms occur, destruction of the pathogen is the outcome. This is accompanied by the release of previously internal components, which, if antigenic, will stimulate the production of specific antibodies. Thus, the destruction of pathogens will lead to the production of antibodies against the antigen repertoire, both surface exposed and internal, of that pathogen. Owing to the commonness of some internal antigens (e.g., enzymes) the consensus of opinion indicates that the more specific antigens or pathogens (excluding endotoxins) are surface expressed at one time or another during development. The surface-exposed antigen mosaic is normally less complex than the internal antigen mosaic of pathogens.

5.5.1¡ª
Effect of Antibody in Viral Infection

Viruses as a group must enter a cell to proliferate, since they lack the biochemical machinery to manufacture proteins and metabolize sugars. Some viruses also lack the enzymes required for nucleic acid replication. The number of genes carried by viruses varies from 3 to about 250, and it is worth noting how small this is compared to the smallest bacterium.

The illnesses caused by viruses are varied and include acute, recurrent, latent (dormant but can recur), and subclinical. The immune response ranges from apparently nonexistent to lifelong immunity. The acute infection is probably most encountered by the immunoassayist who is interested in animal diseases, but it must be borne in mind that the total knowledge of a specific disease is needed in order to devise assays of relevant to specific problems.

Because the outer surfaces (capsids) of virus contain antigens, it is against these antigens and the envelope that the antiviral antibodies are mounted. The first line of defense (excluding interferon) is either IgM and IgG antibodies in which viruses are present in plasma and tissue fluids (vector transmitted) or secretory IgA antibodies where viruses are present on epithelial surfaces (air-

borne, close contact). Some viruses that replicate entirely on epithelial surfaces (e.g., respiratory tree, GI tract, genitourinary [GU] tract) and that do not have a viremic phase will be controlled by secretory IgA. Antibodies may destroy extracellular viruses, prevent virus infection of cells by blocking their attachment to cell receptors or destroy virus-infected cells.

5.5.2¡ª
Effect of Antibody in Bacterial Infection

The role of antibody in combating bacterial infection is diverse. Antibody to bacterial surface antigens (fimbriae, lipotechoic acid, and some capsules) prevents the attachment of the bacterium to the host cell membrane by blocking receptor sites. Antibody can neutralize bacterial exotoxins (possibly by blocking the interaction between the exotoxin and the receptor site). Normally IgG antibodies are responsible for neutralization of toxins. Antibody to capsular antigens can neutralize the antiphagocytic properties of the capsule, or in organisms lacking a capsule, antibodies to somatic antigens may serve a similar function. IgG antibodies are regarded as more effective opsonins than IgM in the absence of complement than IgG. It is the most effective antibody isotype in the presence of complement. Thus, IgM antibodies are more effective in inducing complement-mediated lysis and bacterial opsonization prior to phagocytosis. Antibody can block transport mechanisms and bacterial receptors (e.g., for iron-chelating compounds); neutralize immunorepellants (which interfere with normal phagocytosis), and neutralize spreading factors that facilitate invasion (e.g., enzymes, hyaluronidase).

5.5.3¡ª
Effect of Antibody in Protozoan Infection

In general, antibodies serve to regulate parasites that exist in the bloodstream and tissue fluids, but they are ineffective once the parasite has become intracellular. Hence, the importance of antibody varies with the infection under study. The presence of encysted morphological forms in the host also reduces the efficacy of antibodies (e.g., Toxoplasma, Entamoeba, Giardia). Many protozoan parasites undergo development in the mammalian host, which is manifested in morphologically distinct form. Such developmental forms often have associated stage-specific surface antigens.

Antibodies can damage parasites directly, induce lysis, activate complement, agglutinate extracellular forms, stimulate antibody-dependent cellular cytotoxicity, and block their entry into their host cells. IgM, IgG, and IgA antibody isotypes are involved in these reactions. The isotype specificity not only depends on in which host compartment the parasite is residing (e.g., respiratory tree, GI tract, GU tract [IgA], bloodstream, lymphoid tissue [IgM, IgG]) but also on the antigens expressed during the different developmental stages and their chronicity.

Protozoan parasites can induce chronic infections in mammals, and therefore large amounts of antibody (IgM + IgG) are produced in response to infection. The external surfaces, and the antigens contained therein, are important in the control of protozoan infections and many effective/protective antibody mechanisms are directed against them.

Protozoan parasites are capable of the polyclonal stimulation of B-cells, and, hence, in some infections (e.g., trypanosomiasis) large quantities of non-trypanosomal IgM and IgG antibodies are produced. In such instances, the immunoassayist should have the capability of distinguishing parasite-specific from nonspecific antibody responses. Protozoan parasites are also capable of depressing the immune response (immunodepression) (e.g., Babesia), and in such instances circulating antibody levels are reduced. The above phenomena must be borne in mind when devising ELISAs for protozoan parasites.

5.5.4¡[a]
Effect of Antibody in Helminth Infections

Helminths (trematodes, cestodes, nematodes, Acanthocephalans) normally have complicated life cycles, and their larger size and increased complexity cater for the increase in antigenic diversity found within them. In addition, when many developmental forms exist in the host, a stage specificity of antigens (restricted to each stage) has been demonstrated. Helminth parasites induce chronic infections, and such long-term antigen challenge exposure can produce elevated levels of circulating antibodies. Because helminth parasites are normally transmitted by close contact, vectors, water, and possibly aerosolization, they affect numerous tissues and organs of the host. Some helminths have a minimal migratory phase, but the majority are capable of migration throughout the host's soft tissues.

Antibodies of IgM, IgG, IgA, and IgE isotypes are produced in response to helminth antigens, and depending on in which physiological compartment of the host the parasite is residing, each isotype can be utilized in ELISAs for detecting antibody recognition (exposure) of parasite antigens. IgE Igs are elevated in helminth infections, and although only a proportion of the IgE Ig produced has antibody activity against parasite antigen(s), this class of Ig has been implicated in resistance to helminth. Both IgG and IgE antibodies are involved in antibody-dependent cellular cytotoxicity mechanisms against helminth parasites.

Helminth parasites are the most complex infectious disease agents infecting mammals, and hence the host's antibody responses to them are the most diverse. Unlike other infectious diseases, most helminths do not divide in the body of the final host, and therefore the antigenic load is dependent on the infective dose. Exceptions to this are *Echinococcus* spp. and *Strongyloides* spp. Antibodies are produced against antigens present within helminths (somatic

antigens), antigens on helminth outer surfaces (surface antigens) and antigens in helminth excretions or secretions (excretory-secretory [ES] antigens). The ES antigens of helminths have been shown to confer the most specificity in immunoassays. Not all stages of the helminth life cycle occur in the mammalian host, and therefore it is vital that the immunoassayist consider the pathogen of interest with reference to this. Antigens collected or extracted from stages of the parasite that do not occur in the final host are unlikely to be of use for detecting antibodies to the parasite in that host. Similarly, because helminths develop within the host, the earlier mammalian stages of development stimulate antibodies against antigens of only that stage. Later developmental stages stimulate antibodies not only to their respective stages but also to previous stages if homologous or crossreacting antigens occur in such stages. The humoral immune responses to helminths are complex and care is necessary in developing ELISAs for helminth infections in order that cross-reactions be minimized. Helminths are capable of modulating the immune response, and immunosupression of antibody responses occurs in some diseases (e.g., Haemonchus).

5.6¡[a]
Overview on Antibody Production

In the previous sections, the complexity of the humoral response to infectious disease agents was demonstrated. As the agents become structurally more complex, a greater degree of antigenic diversity arises, which can complicate immunodiagnosis. However, it is apparent that infectious disease agents stimulate the production of specific antibodies in hosts, and these antibodies can be used in ELISAs to determine the exposure of a host to a pathogen. Similarly, interactions between host and pathogen cause the release of pathogen antigens into the host's bodily fluids. These antigens can be used in ELISAs to detect current infections. In endemic areas mammals may be infected with more than one pathogen. In cases in which antigens of each pathogen are noncrossreactive with antibodies produced in response to each pathogen, diagnosis by immunoassay is straightforward. Difficulties will inevitably arise when crossreacting antigens (possibly from closely related pathogens) and antibodies occur. Care must be taken when developing ELISAs for such systems, and basic research to define the problems of crossreactions must be undertaken.

In considering antigens of potential usefulness in immunodiagnosis, it is apparent that the greater the number or variety of antigens used to detect circulating antibodies, the wider the antibody diversity detected will be, and hence the likelihood of detecting positive cases. However, owing to the complexity and cross-reactivity of antigens and antibodies, the immunoassayist must select the antigen or group of antigens that stimulate antibody production but do not produce crossreactions.

5.7¡ª
Relationship between Antigen and Antibody in Vivo

We have seen that the ingress of antigens into the host's body eventually leads to the production of circulating antibodies to them. In the case of complex antigens (e.g., infectious disease agent antigens), each antigen can stimulate the production of a variety of antibody isotypes with a variety of affinities, and these antibodies can be used in ELISA for the immunodiagnosis of infectious diseases. In certain infectious diseases (e.g., viruses, bacteria, protozoa) in which the pathogens divide in the host's body, as the pathogen burden increases, so does the antigenic load. Similarly, when pathogens are dealt with effectively (e.g., by lysis) the release of previously internalized antigens into the surrounding tissue occurs, thus increasing the free antigenic load in the host.

One function of antibodies is to mop up free antigen and cause the antigen/ antibody complex produced to be removed from the system, normally by phagocytic cells. It is important to consider the kinetics of antigen and antibody appearance and disappearance when dealing with antibody responses to infectious disease agents.

In the early stages of a primary infection, very little circulating antibody is present. When the infectious disease antigens are processed by the host, antibody synthesis occurs and these antibodies, predominantly of the IgM isotype, recognize specific antigens. In this stage of the disease process, antigen levels would be expected to be higher than antibody levels in the fluid sampled, i.e., excess antigen. Because there are relatively higher concentrations of antigen than antibody, all available antibody will be bound to antigen to form immune complexes, leaving the residual antigen free to circulate in bodily fluids. As more antibody is produced in response to continuous antigen challenge, the relative concentrations of antibody and antigen become similar, and as this antibody binds to antigen, less free antigen is present. At a point where the amount of circulating antibody equals the amount of free antigen, following antigen/antibody interaction to produce immune complexes, neither free antibody nor free antigen remains, and at this point both reactants are equivalent. When the concentration of circulating antibody exceeds the concentration of circulating antigen, no free antigen will be present, but a residual amount of antibody will be present, i.e., excess antibody.

This is a simplistic account of the interaction between antibody and antigen, but it demonstrates that whenever specific antibodies to an antigen and that antigen interact, immune complexes are produced. The antibody isotype, antibody affinity, number of epitopes on an antigen molecule, and chronicity of antigen and antibody production are all important in determining the fate of immune complexes. In general, large complexes (those found by interac-

tion of antibodies and antigen with numerous epitopes) are removed by phagocytic cells. Smaller complexes (antibodies and antigen with few epitopes) are removed slowly by phagocytes and remain in the circulation longer. The main sites for immune complex removal are the liver (Kupfer cells), spleen, and lungs. Low-affinity immune complexes are smaller than high-affinity complexes, and therefore persist longer in the circulation. Excess antigen and excess antibody immune complexes are normally smaller than antigen/antibody equivalent immune complexes and therefore remain in the circulation longer.

Some of the pathology associated with infectious diseases is owing to immune complex deposition. Thus, in certain instances the concentration of soluble (circulating) immune complexes in bodily fluids can provide insight into immune complex pathology. The immunoassayist should be capable of devising assays for soluble immune complexes. For example, in excess antigen complexes in which an antigen-specific antibody (preferably from a different species from the host) is available, the antigen can be trapped by adsorbing the specific antibody onto the ELISA plate. The reaction can be developed with an anti-host species-specific antibody enzyme conjugate.

Because infectious diseases are often chronic, immune complexes would be expected to be produced owing to persistent antigen and antibody production. In the early stages, excess antigen would be expected, and hence the immunoassayist could measure circulating antigen. Remember that the antigen chosen for measurement has to be specific for the pathogen in question. As the disease state progresses to chronicity, less free antigen will be available owing to increased antibody production and the immunoassayist should measure circulating antibodies, or circulating immune complexes. It should be borne in mind that infectious disease organisms are antigenically complex, may or may not divide within the host, may reinfect the host, or may vary antigenically, and hence the balance previously described between antigen and antibody will vary from pathogen to pathogen. Because pathogens produce numerous antigens of different immunogenicity, many antigen/antibody interactions involving both low- and high-affinity antibodies will be present.

The most useful assays will be those that take into account the preceding considerations. The pathogen/host interactions should be examined as fully as possible before ELISAs are developed. Finally, as already mentioned, many infectious disease agents have immunomodulatory effects varying from immunodepression, including reduction in humoral antibody production, to the induction of T- or B-cell tolerance. Tolerance, in which an organism becomes unresponsive to a particular antigen, may occur in a variety of ways, but the outcome is that antibodies fail to be produced. Infectious disease agents can blockade receptors on antibody-forming cells or mature antigen-specific lymphocytes and make them unresponsive to that antigen. When high levels of

antigen such as pneumococcal polysaccharide induce the event, a state of high zone tolerance exists. When low doses of monomeric antigen induce the event, a state of low zone tolerance exists. Thus, in designing ELISAs the effect of parasite antigens on the induction of tolerance must be considered.

5.8¡ª
Diagnostic Usefulness of Antigens and Antibodies in Infectious Diseases

Detection of both antigens and specific antibodies can prove useful in the laboratory diagnosis of infectious diseases. As we have seen, the production of specific antibodies in response to antigen challenge is multifactorial. Not only antigens and antibodies but also immune complexes can be detected by ELISA, and each will aid diagnosis. In general, free circulating antigen is present for shorter periods of time than free circulating antibody. Reinfection with the same pathogen or exacerbation will cause transient increases in free circulating antigen (antigenemia), followed by increased production of circulating antibodies. Both these events can be detected by ELISA, and the immunoassayist must be aware of this. Similarly, soluble immune complexes can be detected by ELISA, although the assay design is somewhat more complicated.

Because antigens of infectious disease agents stimulate the production of specific antibodies, the latter can be used in ELISAs as an indicator of infection. Normally the antibody response is long-lived and often is present in the absence of the inciting antigens. For this reason, the detection of antibodies in the host does not indicate the presence of a current infection, but does indicate exposure. Antibodies to some antigens are more persistent than antibodies to other antigens and, in some instances, may persist for the lifetime of the individual. In such instances, the question, Is the host immune or refractory to reinfection? cannot be answered by detecting antibodies to antigen mosaics of pathogens. Questions such as, Is the residual antibody effective in the prevention of reinfection? can only be answered when the biological effects of these antibodies are known.

Effects such as neutralization, agglutination, attachment onto receptors to prevent intracellular localization, and lysis are well-known biological effects of antibodies on infectious disease agents. If the mechanism of immunity is known (i.e., the effect of antibody on the target antigens), and if the target antigens can be isolated, they can be used in ELISAs to monitor the rate of production and duration of protective antibody. Few of these assays are available at present, and therefore the immunoassayist must utilize other general phenomena associated with the development of the immune response. The following situations expand this idea.

5.8.1¡ª Neonates

Maternal antibody of the IgG isotype is transmitted passively to neonates transplacentally and in the colostrum. Other isotypes are also found in colostrum and milk (IgA, IgM), but are not transmitted across the neonatal GI tract. Both IgM and IgG antibodies to antigen can be assayed for, and when IgG antibodies are present in the absence of IgM antibodies, the likelihood of them being passively transmitted is high. When humoral IgM antibodies occur, then the organism must be synthesizing them *de novo.* If antibodies are assayed for in other physiological compartments of the body (e.g., GI tract) these conclusions become invalid since both maternal IgM and IgA are secreted in milk and may still be biologically active as co-proantibodies.

5.8.2¡ª Immunologically Competent Mammals

We have seen that IgM is the first isotype of humoral antibody produced in response to antigenic challenge and that if the challenge persists it is replaced by IgG isotype. If the infection is chronic and the antigen persists, or reexposure occurs, higher levels of IgG will be produced. This information is valuable to the immunoassayist because when total antibody (all isotypes) is assayed, an increase in antibody titer over time is indicative of a current infection. This is also true when isotype-specific second antisera are used. The comparison of IgM and IgG levels is also a useful indicator.

Persistent antigen induces an isotype switch in T-cell-dependent antigens, stimulating the production of IgG rather than IgM. If IgM and IgG levels to the same antigen are compared, the following can be deduced: IgM antibody in the absence of IgG antibody means there has been recent acquisition of infection in which no isotype switch has occurred as yet. IgM and IgG antibody present means prior acquisition of infection, in which isotype switch has occurred, with IgM response present but possibly declining. Prior acquisition of infection where isotype switch has occurred, means that the host has become reexposed to the same antigen, stimulating the production of more IgM antibody.

IgG antibody in the absence of IgM antibody could mean the previous acquisition of infection, in which isotype switch has occurred, and the IgM response is below the assay detection level. In this case, the infection would be expected to be chronic.

When antigens are localized in the respiratory, the GI, tract or GU tract, stimulating local antibody responses, the detection of IgA antibody isotypes would be of value. Similarly, when it is known that pathogens stimulate isotype-specific responses (e.g., helminth), IgE-specific antibody responses can be assayed.

5.8.3¡[a]
Herd Immunity

Each individual in a group has a varying potential to respond to antigens and hence to mount a protective immune response. The range of immune responses in a group follows a normal distribution pattern whereby the majority of individuals mount an average immune response, and a small proportion mount either a very effective immune response or an ineffective immune response. When vaccination or exposure to a pathogen is concerned, <100% of the population will be adequately protected, and for those individuals concerned, this lack of protection will be serious. This lack of protection may be owing to the inability to recognize antigens or to produce antibodies (agammaglobulinemia). These individuals can act as reservoirs of pathogens for future transmission to other susceptible individuals, and the seriousness of the situation will depend on the mode of transmission, the number of susceptible individuals, and in cases in which vaccination is common practice, the efficacy of vaccination.

Less than 100% protection may be sufficient to prevent the spread of the disease within the population, since the likelihood of a susceptible individual encountering an infected individual becomes reduced. This phenomenon is known as herd immunity and is an important consideration in assessing the potential of individuals in a population to succumb to infection.

Many factors can affect the quantity and quality of an immune response, most of which have been mentioned previously. Other factors that affect the immune response are stress, pregnancy, surgery, concomitant infections, extremes of temperature, and especially malnutrition. All these factors reduce the quantity and quality of the immune response, and when the immunoassayist performs seroepidemiological surveys to assess the herd immunity among various populations, these factors must be taken into account prior to determining levels of adequate protection within a population.

For each infectious disease, antibody levels (if any) in naive, subclinically infected, clinically infected, and immune individuals in the endemic population should be sought. Only in this way can the immunoassayist relate antibody levels to infection. Once these parameters are known, the effect of vaccination in these individuals can be assayed as well as the relationship between protective antibody and immunity.

One major problem is that individuals in an endemic area have had previous exposure of an infectious disease agent, or antigens thereof, and therefore ascribing an antibody titer or threshold above which protection occurs and below which reinfection occurs is difficult, if not impossible.

The situation becomes more complex when assessing the protective effects of vaccination in such a group of individuals. Often individual antibody levels

are static owing to chronic exposure and variable owing to previously mentioned factors. In the instances in which naive individuals become immune, seroconversion occurs, but in the majority of instances where individuals have had previous exposure, increases in antibody levels are sought. These may be so small that they become statistically nonsignificant.

5.9¡ª
Antigenic Commonness

Because there is a limited number of amino acids and carbohydrates and so forth produced by living organisms, the number of combinations of these components is limited (for proteins and carbohydrates). Therefore, it is likely that different organisms will produce similar proteins, glycoproteins, and so on. When partial or complete similarities occur in the products of living organisms, a commonness in terms of antibody recognition occurs.

Structural proteins appear to be well conserved and would be expected to demonstrate immunological crossreactivity. The nature of this crossreactivity varies among similar antigenic molecules and depends on the ability of the paratope to fit the epitope.

We have already seen that the paratopes of antibodies will bind to epitopes that are recognized either completely or partially. The immunoassayist must consider the possibility that the same or similar antigens may exist in a variety of infectious agents. For example, many cell surface antigens are carbohydrate in nature and the same epitopes may be present on two cell types, one of which may be pathogenic whereas the other may be a commensal. Similarly, some carbohydrates may be present in widely differing infectious disease agents (e.g., blood group like, ascarone). When polyclonal antibodies are produced in response to infection, antibodies to noncrossreactive as well as crossreactive epitopes will be produced and hence the problem of crossreactivity can be minimized.

The problem of antigenic commonness becomes more obvious when using mAbs in diagnosis. In the developmental stage of an assay, the immunoassayist must consider which other antigens, including antigens from other infectious disease agents, might be involved in crossreactions and develop the assay accordingly, bearing in mind the type of problems associated with such crossreactions.

6¡ª Other Techniques

The performance of good immunoassays also requires practical expertise in immunochemical techniques (or at least theoretical knowledge of when such techniques are to be used). The following list provides some techniques of use in the purification and characterization of antigens and antibodies:

1. Sucrose density gradient centrifugation.

2. Polyacrylamide gel electrophoresis (PAGE).

3. PAGE followed by immunoblotting.

4. Isoelectric focusing.

5. Immunodiffusion in agar/ agarose.

6. Gel chromatography (DEAE, affinity, sephadex).

7. Salt fractionation of IgG.

8. Enzyme conjugation methods.

The nature and preparation of antibody fractions and their relevance in disease and assay should also be examined (i.e., whole molecule, Fc, Fab, $F[ab']_2$, IgM, IgA).

7¡ª Units

Successful assays depend on a good knowledge of units of volume and weight. The concepts of accuracy in dilutions and the relevance of pipetting methods also fall within the necessary practice needed for assays.

7.1¡ª Volumes

The pipets used in microtiter plate assays are graduated in microliters (µL). The relationship of volumes is given in **Table 2**.

7.2¡ª
Weights

Note the relationship of weights as given in **Table 3**.

8¡ª
Dilutions

Difficulties are often encountered in the making of dilutions. It is essential that great care be taken in making the correct dilution and that there be no wasting of expensive reagents through making up convenient dilutions into an unneeded final volume. For example, we may need to make up a 1/1200 dilution of a sample already at a 1/50 dilution in a final volume of 5.5 mL. Often operators attempt to round up volumes so that larger than necessary volumes are made, which is wastes of reagents (e.g., conjugates). Other problems, such as making a 1/20,000 dilution in a final small volume such as 3 mL arise.

All problems of dilution are eased if all volumes in the calculation are converted to microliters (µL). The following examples illustrate this.

8.1¡ª
Making a 1/100 Dilution of Neat Sample at a Final Volume of 10 mL

Neat infers a sample is undiluted. Thus, we require a final volume of 10 mL. Convert the required volume to microliters:

Table 2
Relationship of Volumes in Microtiter Assays

Volume	Symbol	Cubic centimeter	Microliters
Liter	L	1000	1,000,000
Milliliter	mL	1	1000
Microliter	µL	0.001	1

Table 3
Relationship of Weights

Unit	Relationship to gram
Gram	1
Milligram	10^{-3}
Microgram	10^{-6}
Nanogram	10^{-9}
Picogram	10^{-12}
Femtogram	10^{-15}
Attogram	10^{-18}

$$10 \times 1000\ \mu L = 10{,}000\ \mu L$$

The dilution required is 1/100. Divide this into the required final volume:

$$10{,}000/100 = 1/100$$

This is the volume of neat (undiluted sample) to be added into the required volume in microliters, which is therefore 100 µL.

In this example there would be little difficulty in using microliters. Thus, 10 mL/100 = 0.1 microliters. We would therefore add 100 µL of the neat sample to 9900 µL (9.9 mL) of diluent (final volume minus the volume of the added neat sample). The conversion of the 0.1 mL into the units of the micropipet would then have to made, i.e., 0.1 mL = 100 µL.

A slightly more complex calculation illustrates the benefit of initial conversion to microliters of all the volumes.

8.2¡ª
Making a 1/200 Dilution of a Neat Sample in a Final Volume of 4 mL

Convert the required volume to microliters = 4 mL = 4000 µL. The dilution factor is 200. Therefore, we need 4000 µL/200 µL = 20 µL of neat sample. *(A check on such calculations should always be made.)* Thus, the dilution factor ¡Á

volume of sample being diluted should equal the required volume. We therefore have 200 ¡Á 20 µL = 4000 µL (4.0 mL). Note here by that using milliliters, we would have 4/200 = 0.02 mL. This becomes a little more difficult to relate to the microliter setting of the micropipets.

8.3¡ª
Calculating a Diluted Sample

When a sample is already diluted, we have to include this in the calculation. Thus, we have an already diluted sample at 1/50. We require a final dilution of 1/1000 in a final volume of 20 mL.

This type of calculation causes most problems. Convert the required volume to microliters. The final volume required = 20 mL = 20,000 µL. The dilution factor required is 1000.

Now, assume the sample were not already diluted. Then we would add 20,000/1000 = 20 µL of neat sample. Therefore, since the sample is already diluted 1/50, we have to add more. The factor is determined by the known dilution factor (50), so we multiply the value for undiluted sample by this factor:

$$20\ \mu L \times 50 = 1000\ \mu L$$

This may seem obvious, but taking a more complex dilution.

8.4¡ª
Dilution of a Final Volume

We require a 1/18,000 dilution in a final volume of 27 mL. We already have a dilution of the sample at 1/25. By converting to microliters we have a required final volume is 27,000 µL. Assume the sample were undiluted, then we would require 27,000/18,000 = 1.5 µL. Since it is already diluted then multiply the 1.5 µL by the dilution factor:

$$1.5 \times 25 = 37.5\ \mu L$$

Check: 18,000 ¡Á 37.5/25 = 27,000 µL = 27 mL.

When high dilutions are needed in small volumes, it may be necessary to make up a limited dilution series to avoid wasting reagents.

8.5¡ª
Requiring a High Dilution

Direct addition of the undiluted sample would require 5000/100,000 = 0.05 µL. This volume is impossible to pipet. For most practical purposes, the pipetting of volumes <5 µL is not recommended. The high dilution just given can be achieved by two manipulations. There are alternatives, and these are governed by the availability of the reagent being diluted. If it is available in only small volumes, then the initial volume used to make the first dilution can be made small. Thus, a 1/100 dilution can be made into 1000 µL by adding 10 µL of neat to 990 µL of diluent. Calculating the amount of this 1/100 dilution

to be diluted as required in a final volume of 5 mL, we have 5000/100,000 ¡Á 100 (the already produced dilution factor) = 5 µL. Thus, by adding an initial dilution into 1 mL of buffer, we can produce high dilutions using acceptable pipetting volumes.

8.6¡ª
Reviewing of Method for Dilution

1. Determine the final volume required and convert this to microliters.

2. Divide this by the dilution factor. This is the sample volume to be added to the final volume of diluent in microliters.

3. When there has been predilution of the sample, follow steps 1 and 2, and then multiply the predilution factor by the volume found in step 2.

4. When high dilutions are needed, make two or three dilutions of sample in small volumes.

8.7¡ª
Compensation of Volumes

Since we require a final volume (calculated in **stage 1** of **Subheading 8.6.,** then addition of sample will obviously increase the final volume. As an extreme example, we may require a 1/10 dilution of a sample in 1 mL. This means adding 100 µL to a final volume of 1 mL. Following the procedures in **Subheading 8.1¨C8.6.**, we would end up with a volume of 1100 µL. Thus, this would not achieve an accurate 1/10 dilution¨Crather 1/11. The obvious course is to compensate for the addition of the sample volume by taking removing this from the final volume calculation. Thus, in the same example, the final volume required is 1000 µL and the sample volume calculated is 100 µL. Therefore we need to remove 100 µL of diluent to compensate for the extra 100 µL of sample added, so we need 900 µL of diluent plus 100 µL of sample to achieve a perfect 1/10.

The accuracy then of the dilution depends on removing the sample volume from the final volume. However, this depends on the actual volume to be added. As an example, we may require a final dilution of 1/100 in 1 mL. Thus, we need a final volume of 1000 µL and need to add 10 µL of sample. Here, we could compensate by removing 10 µL of diluent before adding the 10 µL of sample, but, in terms of accuracy, we can see that the difference between the dilutions with and without compensation are minimal. Hence, with compensation we have a dilution of 10/1000 = 1/100, and, without compensation we have a dilution of 10/1010 = 1/101. Effectively there is no practical difference.

8.7.1¡ª
When to Compensate

There can be no exact rule about when to compensate because there may be occasions in which the activity of a sample is affected by a very small difference in dilution. However, this is not usually true in ELISA and as a strong

guideline I suggest that when the volume of the sample being added is >2% of the required volume, compensation should always be used. Thus, to make a 1/200 dilution, 5 µL can be added to 1000 µL (0.5%); 10 µL can be added to 1000 µL (1%); 20 µL can be added to 1000 µL (2%); 40 µL should be added to 960 µL.

9¡ª
Pipets

As in all assays, accurate pipetting is vitally important to obtain consistent results. Examine the multichannel pipet. It can be of fixed volume (dispensing a fixed specified volume) or of variable volume. For variable-volume pipets, the volume delivered can be adjusted by turning the knob at the top of the pipet so that the volume read on the side of the pipet handle is altered. These are digital pipets: the volume is shown as a number on the side in microliters.

Practice setting up different volumes. Remember to note where the comma (denoting the decimal point) is. Thus, 200 = 200 µL, and 20.0 = 20 µL.

Some pipet volumes are altered using a vernier scale. Follow the instructions provided by the manufacturer. Generally these are not recommended because it is easier to make mistakes with them, and the scale tends to wear. Multichannel pipets are designed to deliver 4, 8, or 12 vol simultaneously, and therefore are ideal for microtiter plates. The pipets having 12 channels offer highest flexibility in that up to 12 channels can be utilized. Any number of the channels can be loaded with a tip (up to 12). This fact often confuses workers when they first encounter such pipets. Practice the pipetting action and putting on tips.

9.1¡ª
Pipetting Action

Remember always to use the pipet whose maximum volume is nearest to the volume you require. All pipets should be calibrated on a routine basis (every month). Techniques for performing calibrations can be obtained from commercial companies supplying the pipets. Some companies also provide a calibration service. Special calibration tips with precise volumes marked on the outside allow routine examination of the volumes being dispensed. Pipets should also be checked for damage.

9.1.1¡ª
Picking up Solutions

Press the button on top of the pipet to the first stop before you put tips in solution, and then place tips in solution. Release the button steadily. You will notice that a volume of liquid is taken up into each tip. Check that each tip has the same volume. If not, expel the liquid after noting which tip was "low" in volume. Press that tip on harder. Repeat the pipetting.

9.1.2¡[a] Dispensing Solutions

Put the points of the tips in the wells resting on the sides of the plastic, if possible. Press the knob to the first stop. Solution will be expelled, try to pull the tips out up the side of the wells. When you have finished, either pull the tips off by hand or press the tip ejector on the side of the pipet; this works with some tips but not others.

9.1.3¡[a] Single-Channel Pipets

Single-channel pipets are used to deliver single volumes of solution, particularly for small volumes. These can be the vernier or digital type, fixed or variable volumes.

9.1.4¡[a] Troughs

Reservoirs for liquid dispensing by multichannel pipets are used as reservoirs (containers) for the solutions in the ELISA for multichannel pipets. These can be homemade or commercial, and either single or multiple troughs. Some are only suitable for eight channels.

9.2¡[a] Pipetting Exercise

To get used to the pipets, perform some simple exercises in making dilution series in microtiter plates. The following materials are needed:

1. Multichannel pipet.

2. Trough.

3. Tips.

4. Solution of dye (e.g., phenol red in water, trypan blue).

5. Water.

Making a dilution series is highly important in immunoassay. In most cases, multiple dilution ranges can be made using the multichannel pipets. A dilution series is given next.

For a twofold dilution series, in 50 µL vol add 50 µL of the substance to be diluted to 50 µL of diluent, mix, transfer 50 µL of this dilution to another 50 µL of diluent, and repeat to the required range.

In microtiter plates, the required volume of diluent is dispensed in the wells with the multichannel pipet. The substance being titrated is added either at the starting dilution (in the test volume) to the first well and to the second well as an equal volume of the starting dilution plus the volume of diluent in the well, or to the first row only in the test volume with the sample at twice the required initial concentration into the test volume of diluent. The solution in the first or second row is then mixed using the multichannel pipet, and the test volume is

transferred to the next well containing the test volume of diluent, using the pipetting action of the multichannel pipet.

The process is repeated over all wells, each containing the test volume of diluent. In most cases carryover of reagent can be ignored when the same tips are used for the dilution series. Thus any number of dilution series up to 12 rows (8 dilutions) or 8 rows (12 dilutions) can be prepared simply. Thus, for the exercise, the steps are as follows:

1. Add 50 µL of diluent (water) to each well of a plate.

2. Add 50 µL of dye solution to the first row (A¨CH) of the plate using a multichannel pipet.

3. Mix by pipetting action (first stop only used to avoid frothing).

4. Transfer 50 µL of diluted dye from row 2 to 3, and mix.

5. Transfer 50 µL from row 3 to 4, and mix.

6. Repeat to row 12.

We now have 8 rows (A¨CH) of the same dilution range of the dye. This is a twofold range (equal volumes transferred) from 1/2, 1/4, 1/8, and so on, to 1/4096. You can make any fold dilution range provided the volumes you transfer are effectively large enough to be pipetted and mixed after transfer.

Examine the dilution range to ensure that the rows appear as if they have the same volume and that there is a logical dilution effect along the rows. Discard the first attempt, throw dye into sink, wash the plate under the tap, blot the plate dry, and repeat.

Try the following dilution ranges:

1. A 3-fold dilution range: 50 µL vol (25 µL carried over into 50 µL diluent).

2. A 4-fold dilution range: 50 µL vol (approx 17 µL carried over into 51 µL).

3. A 5-fold dilution range: 60 µL vol (15 µL carried over into 60 µL).

4. A 10-fold dilution range: 100 µL vol (11 µL carried over into 110 µL).

5. A 5-fold dilution range: 100 µL vol (25 µL carried over into 100 µL).

9.2.1¡ª
Effect of Different Dilution Series

The choice of which dilution range to use depends on what activities are being titrated. Thus, if there is a large quantity of antibody in a serum, then a high dilution is necessary in order to assess this.

Preliminary experiments to assess ELISAs often involve the titration of reagents of unknown strengths. In such cases, the appropriate selection of dilution ranges is important. It is simple to make two-, three-, and fourfold dilution ranges, but what are the advantages? **Table 4** gives the effective dilutions of a sample using the different ranges over eight wells (eight dilution steps) and illustrates the ranges of dilutions covered by each.

Table 4
Dilutions Obtained Using Different Dilution Series

	Well number							
	1	2	3	4	5	6	7	8
Range	Dilutions produced							
Twofold range	2	4	8	16	32	64	128	256
Threefold range	3	9	27	81	243	729	2178	6534
Fourfold range	4	16	64	256	1024	4096	16384	65536

This shows that a simple adjustment of diluting range dramatically increase the dilution of samples. The three-or fourfold range is convenient in that the range titrates samples at relatively high concentration. Thus, low-titre samples might be observed but it is also useful in case samples have high titers. In the case of the twofold range a high-titer sample would show color across all the wells and no titer would be indicated. Preliminary experiments can be followed with the most suitable ranges according to the titer established as in **Table 4**.

10¡ª
Molarities

To make up a 1 *M* solution of a compound we take the molecular weight of that compound in grams and dissolve this to I L (final volume) of liquid (usually distilled water).

Thus, the molecular weight of a particular compound must be calculated from the atomic formula or read off the reagent bottle.

Take a simple example of sodium chloride (NaCl). The molecular weight is 58.5. Therefore 58.5 g made up to 1 L in distilled water represents a 1 *M* solution. A 0.1 *M* solution would contain 58.5/10 = 5.85 g/L.

Often we do not require a large volume so that 100 mL of a 1 *M* solution of NaCl would contain 5.85 g of NaCl since 1 L of 1 *M* contains 58.5 g, and 100 mL = 1/10 L. Therefore, we require 10 times less NaCl.

Another way to calculate this is to always calculate the amount of chemical needed to give the required molarity per milliliter. Thus, 1 *M* = 58.5 g/L = 0.0585 g/mL. If we require 50 mL at 1 *M,* we therefore require 50 ¡Á 0.0585 g made up to 50 mL. This helps calculation of more difficult molarities.

For example, we require a 0.125 *M* solution of NaCl in 35 mL. Calculate the number of grams per milliliters at the required molarity: 1 *M* = 58.5 g/L and 0.125 *M* = 58.5/8 (0.125 *M*/1 *M)* = 7.35 g/L = 0.0073 g/mL. We require a final volume of 35 mL. Therefore, 35 ¡Á 0.0073 = 0.256 g = 2.57 g (rounded up).

For 2 *M* solutions, we require twice the weight of that required for a 1 *M* solution, which is 117 g/L in the case of NaCl. If we require 17 mL of a 2 *M* solution of NaCl, 1 *M* NaCl = 58.5 g/L and 2 *M* NaCl = 117.0 g/L = 117/1000 =0.117 g/mL. We need 17 mL = 0.117 ¡Á 17 = 1.99 g.

Again, a fraction of a molarity is best calculated by assessing needs per mL for example, we require a 0.15 *M* solution of NaCl in 25 mL. Therefore, 1 *M* NaCl = 58.5 g/L; 1.5 *M* = *1.5* ¡Á 58.5 g/L = 87.75 g/L; and 0.15 *M* = 87.5/10 = 8.75 g/L = .0088 g/mL. Hence, we need 25 mL = 25 ¡Á 0.0088 g = 0.22 g.

Sometimes molarities are expressed in millimolar quantities, e.g., 100 m*M*, 10 m*M*, 30 m*M*. One millimolar = 1/1000 *M*. Thus, for NaCl, 58.5/1000 = 0.0585 g/L = 1 m*M*.

Hence, a 10 m*M* solution contains 0.585 g/L, and a 100 m*M* solution contains 5.85 g/L.

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6¡ª
Practical Exercises

The aim of this chapter is to illustrate the principles of ELISA by (1) showing worked examples of each assay, including diagrams of plates and representational data from assays, (2) analyzing such data in terms of important rules that are learned at each stage, and (3) providing full working instructions for investigators to be able to perform each assay so that they obtain their own data to be analyzed.

This chapter can be used in several ways. First, researchers without access to reagents will obtain a working knowledge of ELISA through the examples. Second, it can be used in training courses in which reagents may be provided (as indicated in the text). Third, the information will be useful to investigators who have already had some experience with the technique but may have had difficulties in obtaining and analyzing data.

Remember that it is the application of the ELISA to specific problems, and not the methodology for its own sake, that is the most important reason the techniques should be mastered.

1¡ª
Test Schemes

You are already familiar with the concepts in ELISA, whereby an antigen binds to an antibody that can be labeled with an enzyme or, in turn can be detected with a species-specific antibody (enzyme labeled). All the ELISAs described are variations on this theme. Inherent in the methods of ELISA is that one of the reagents is attached to a solid phase, making the separation of bound (reacted) and unbound (nonreacted) reagents simple by a washing step. Before performing ELISA on disease agents, it is useful to train operators how to use reagents of defined reactivity, which are easily available and which provide security problems. An ideal system is to use an immunoglobulin (Ig) and, more particularly, an immunoglobulin G (IgG) as an antigen. Do not get confused since you have learned that the antibody population contains high levels of IgG acting as antibody. In the context of learning the principles, we are

using IgG as an antigenic protein, because, (1) IgG from one animal species can be injected into another animal species so that a specific antiserum to that IgG is prepared; and, (2) Such antibodies can be labeled with enzyme, or detected with a second species-specific antibody labeled with enzyme.

Such reagents are defined, easy to standardize, stable, and available commercially. The particular IgG system chosen in this chapter involves the guinea pig, but similar tests can be performed with IgG from other species using the appropriate antispecies reagents. The systems described are analogous to the ones most commonly used to examine problems associated with diagnosis.

The schemes are described as symbols and as practical exercises in full as follows:

—I	= solid-phase microtiter plate well
—Ag	= antigen
—Ag1, Ag2, etc.	= particular antigens highlighted
—I-Ag	= antigen passively attached to solid phase
—I-Ab, I-AB	= particular antibodies passively coated to wells
—Ab	= antibody
—AB	= antibody from a different species to Ab
—Ab_X, Ab_Y	= different antibodies identified by subscript letters
—Anti-Ab	= antispecies specific antibody (species in which Ab was made)
—Anti-Ab*E	= antispecies-specific antibody labeled with enzyme
—W	= washing step
—+	= addition of reagents and incubation step
—S	= substrate/chromophore addition
—R	= reading the test in spectrophotometer.

Many of the practical steps are similar. The conjugates used are all horseradish peroxidase (HRP) and the substrate/chromophore is H_2O_2/*ortho*-phenylenediamine (OPD). The following practical details are helpful:

1. Substrate/chromophore. The easiest method is to use commercial tablets that are preweighed. Also, citrate/phosphate tablets can be purchased (pH 5.0). There are commercial preparations of this substrate/chromophore that require addition of only water. I recommend 30 mg tablets, which make 75 mL of solution in buffer. Unused OPD solution (without added hydrogen peroxide) can be stored at ¨C20¡ãC but should be examined closely for discoloration on thawing. Use the completely made up solution as soon as possible. All liquids should be at optimal temperature. The hydrogen peroxide can be purchased as 3 or 6% solution and should be stored as instructed by suppliers. Tablets of urea/peroxide can also be obtained and used to make up a stock of hydrogen peroxide of defined strength rather than purchasing liquid that has certain transportation restrictions. Hydrogen peroxide should be added to the required concentration just before addition to wells; for example, add 5 µL of hydrogen peroxide (30% w/v) to every 10 mL of OPD

solution (in citrate phosphate buffer pH 5.0) or 25 µL of 6% (w/v) hydrogen peroxide. It is imperative that the strength of the hydrogen peroxide be accurate.

2. Washing solution is phosphate-buffered saline (PBS) without addition of Tween-20. Washing requires the addition and emptying of wells four times.

3. Blocking buffer is PBS containing a final concentration of 1% bovine serum albumin (BSA) and 0.05% Tween-20. This should be made in volumes necessary to complete tests as required, but can be stored at 4¡ãC if contamination is avoided, and should always be warmed and inspected for contamination before use.

4. Stopping solution is 1 *M* sulfuric acid, and care should be taken in its preparation. It can be stored at room temperature.

5. Read implies using a multichannel spectrophotometer to assess the optical density (OD) values of plates and is in all cases in this chapter read at 492 nm. Plates should also be inspected by eye before reading to determine whether there are gross errors and whether the results are feasible.

1.1¡ª
Direct ELISA:
Titration of Antigen and Antibody

1.1.1¡ª
Learning Principles

1. Measuring the optimum concentration of antigen to coat wells.

2. Measuring the optimum dilution of enzyme-linked antibody.

3. Using multichannel and single-channel micropipets.

4. Revising principles of dilution.

5. Making up and storing of buffers and solutions.

6. Learning to observe tests by eye and by using multichannel spectrophotometers.

7. Handling data.

8. Solving problems.

1.1.2¡ª
Reaction Scheme

$$\text{I-Ag}_{W} + \text{Ab*E}_{W} + \text{S–READ}$$

—I-	= microplate wells (solid phase)
—Ag	= guinea pig IgG adsorbed to wells
—Ab*E	= rabbit ant-iguinea pig conjugated with HRP enzyme
—S	= H_2O_2 + OPD (chromophore)
—READ	= observing by eye or read in spectrophotometer (before or after stopping color development with H_2SO_4)
—+	= addition of reagent and incubation at 37¡ãC or room temperature for 1 h
—W	= wash wells in PBS (four times)

1.1.3¡ª
Basis of Assay

The basis of this assay is to dilute the Ag across the plate one way in a buffer that allows passive adsorption, incubate the plate at 37¡ãC or room temperature for 2 h, wash the plate and then dilute the conjugate across the plate, the oppo-

site way to the Ag, obtaining a checkerboard titration (CBT) of Ag against Ab*. The Ab*E is diluted in a buffer to prevent nonspecific adsorption of the Ab*E to any free protein binding sites on the wells. After washing, all the wells receive a solution containing the substrate for the enzyme (H_2O_2) and a chromophore which can change color if the H_2O_2 is acted on by the enzyme. Thus, the color developing in each well depends on (1) the amount of antigen, and (2) the amount of conjugate that has bound to that antigen. The more conjugate, the more enzyme the more color.

1.1.4¡ª
Materials and Equipment

1. Ag: guinea-pig IgG in PBS at 1 mg/mL.

2. Anti-guinea pig IgG prepared in rabbits conjugated to HRP.

3. 96-well microplate for ELISA.

4. 12-channel (tipped) micropipet (5 to 50-µL).

5. Single-channel micropipet (5 to 50-µL) plus tips and trough.

6. 10- and 1-mL pipets.

7. Carbonate/bicarbonate buffer, pH 9.5, 0.05 *M*.

8. PBS containing 10% bovine serum albumin (BSA), 0.05% Tween-20.

9. Solution of OPD in citrate buffer.

10. Bottle of hydrogen peroxide (30% W/V, from 4¡ãC).

11. Washing solution (PBS) reservoir.

12. 1 *M* sulfuric acid in water.

13. Paper towels or thin flat sponge.

14. Small-volume bottles.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

1.1.5¡ª
Practical Details

1. Examine a plate, note the position of letters A¨CH and the numbers 1¨C12. Place the plate with A at the top left-hand corner in front of you, as in **Fig. 1**. The 8 wells labeled with letters (A¨CH) are referred to as rows. The 12 wells labeled by numbers (1¨C12) are referred to as columns.

2. Use the 12-channel pipet with 12 tips to add 50 µL of carbonate buffer to each well of the plate. Use a trough to act as a reservoir for the buffer, and add 6 mL to give extra volume needed for the whole plate.

3. Dilute the antigen (1 mg/mL) to 10 µg/mL in carbonate buffer. Make up 1 mL of the antigen at this concentration; that is, add 1 mL of buffer to a small bottle. Pipet 10 µL of antigen into this. Mix well by rotating the bottle by hand (do not be overvigorous).

4. Set a single-channel micropipet to 50 µL. Add 50 µL of diluted antigen to all the wells of column 1. You should now have 100 µL of antigen in column 1.

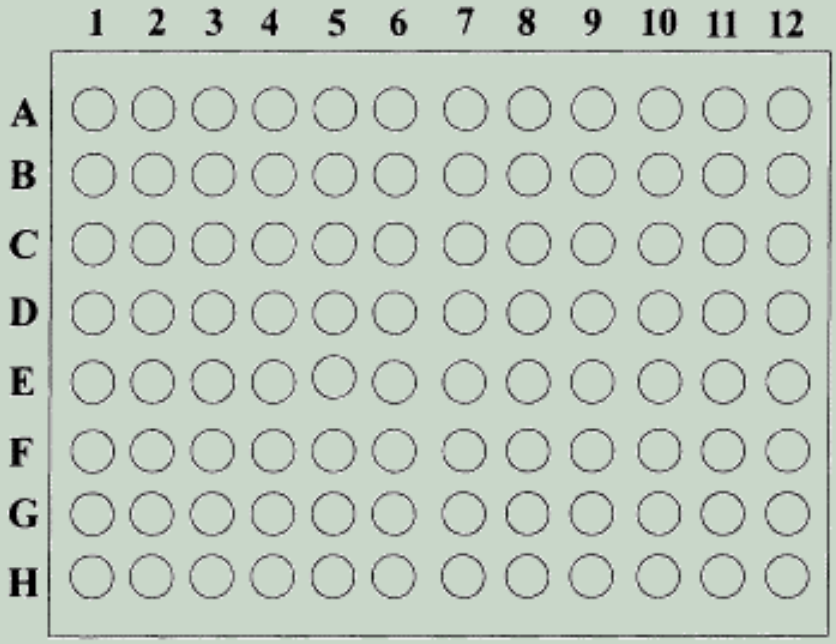


Fig. 1.
Numbering and lettering on microtiter plate.

5. Put tips into column 1, and mix the contents by pipetting up and down eight times, using the first stop of the pipet. Transfer 50 µL to column 2 (A¨CH), mix, and transfer 50 µL to column 3, and so on, to column 11. After the last mixing, discard the 50 µL left in the pipet. You should now have 50 µL of a dilution series in each row, ending with column 11. Check by eye that the volumes are similar in all the wells (**see Fig. 2**).

6. Put a lid on the plate and leave it on the bench (flat surface) for 2 h at room temperature, or at 37¡ãC for 2 h or, if more convenient, leave it at 4¡ãC overnight.

7. Wash the plate. The exact method depends on the equipment used. The principle is to discard the contents of the wells by "flicking" them into a sink (or suitable container bowl), then adding PBS and flicking this away four times. The major concern is that all the wells are filled at each stage.

8. Turn the plates onto absorbent paper (sponge), and remove the majority of the residual PBS by gently tapping the plates against the paper (picking the plate up to do this, well openings down).

9. To add the dilutions of conjugate, take the enzyme conjugate from the refrigerator. Check that it is rabbit anti-guinea pig IgG, conjugated to HRP (there may be variations in the particular species used to prepare the anti-guinea pig serum labeled: e.g., it could be sheep anti-guinea pig IgG). Make up I mL of a 1/200 dilution of the conjugate in a 5-mL bottle. Use the 5¨C50-µL single-channel pipet to add the conjugate, that is, add 5 µL of conjugate to 1 mL of blocking buffer. (**Note:** We do not wish to have any nonspecific adsorption of the conjugate to the plastic during the test.) Mix well by gentle swirling action; do not shake vigor-

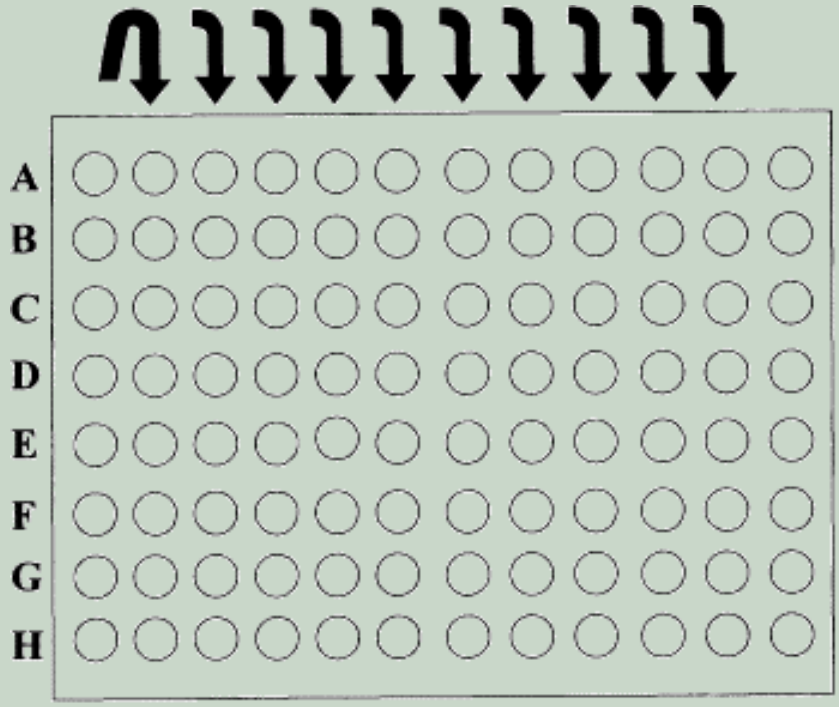


Fig. 2.
Dilution of antigen across plate from columns 1 to 11.

ously. Add 50 µL of blocking buffer to every well of the microplate using the multichannel pipet fitted with 12 tips. This is accomplished by adding about 6 mL of blocking buffer to a trough and pipetting from this. Wash the trough, after dispensing the blocking buffer, using tap water (or PBS). Dry the trough for use with conjugate with paper towel. Pour the conjugate dilution into a trough. Using the multichannel pipet with 12 tips attached, add 50 µL of the conjugate dilution into the first row (A, 1¨C12) of the plate. Thus, there is 100 µL of a 1/400 dilution of conjugate in this row. Mix using the multichannel pipet (eight times up and down). Transfer 50 µL of the conjugate from row A to B (1¨C12), and mix in row B (eight times). Transfer 50 µL to row C (1¨C12) and mix. Repeat the transfer of dilutions to the end of the plate (row H). There should now be 50 µL of conjugate dilutions in all wells, at a dilution range from 1/400 in row A to 1/51,200 in row H. This is diagrammatically shown in **Fig. 3**. Thus, a CBT has been performed relating to how the antigen and antibody have been diluted. Put a lid on the plate and leave at room temperature for 1 h, or at 37¡ãC for 1 h.

10. Wash the plate and flick free from excess washing solution (*see* **step 7**).

11. To add substrate/chromophore for color development, thaw 10 mL of citrate buffer containing OPD in a water bath or at room temperature (slower), or make up OPD solution from tablets. Ensure that the solution reaches an acceptable temperature, i.e., room temperature if this is fairly constant in your laboratory. It is a good idea to have a water bath at a temperature of 20¡ãC and to use this to equilibrate the OPD solution to achieve a standardized temperature since this

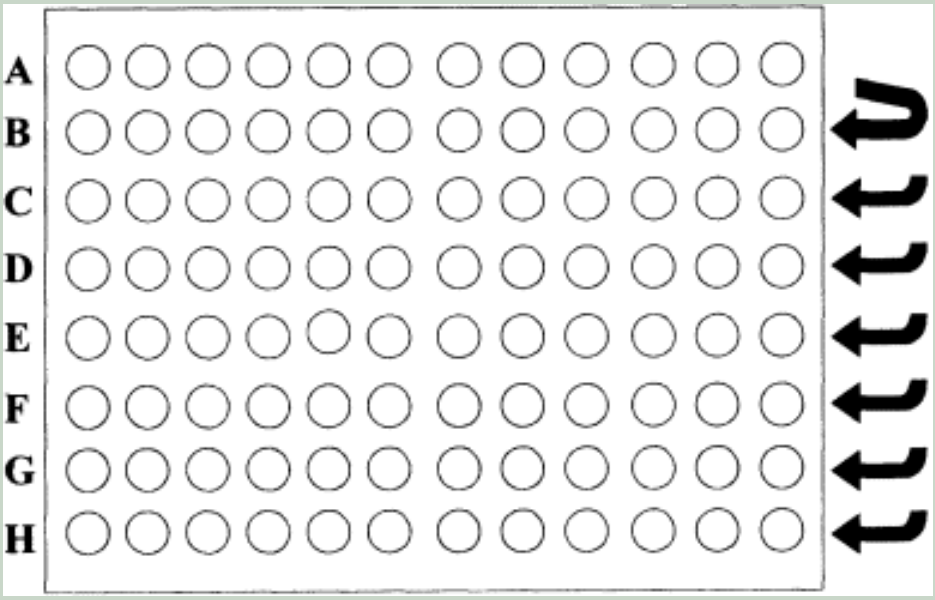


Fig. 3.
Dilution of antibody from row A to H.

affects the rate of color development of the ELISA. Add 5 µL of hydrogen peroxide (30%)/10 mL of OPD. (Immediately, put this back in the refrigerator with a top screwed on tightly.) Mix gently. Pour the solution into a trough (must be washed and free of any previous reagents). Use a multichannel pipet to add 50 µL of solution to each well (8 or 12 tips used).

12. Leave the plate on the bench and examine color changes at approx 1, 3, 5, 8, and 10 min after addition.

13. Add 50 µL of a 1 *M* solution of sulfuric acid in water (supplied) to each well after 10 min of color development (use a clean trough and multichannel pipets again) to stop color development.

14. Read the plate by eye and use a spectrophotometer.

1.1.6¡[a]
Explanation of Data

Figure 4 is a diagrammatic representation of plates set up using the same reagents as described, at different times before color development has been stopped. **Table 1** gives an assessment by eye of the development of color. **Table 2** presents the OD results for the plate stopped at 10 min. These results are analyzed graphically in **Fig. 5**, which relates the color developing in the wells with different antigen-coating concentrations for different dilutions of antispecies conjugate.

From **Fig. 5** and **Table 2**, the optimal dilution of conjugate that might be used in ELISA to detect guinea pig IgG can be determined. Also, the respective antigen concentration that might be used to detect antibodies (highly relevant in the indirect ELISA and explained fully in **Subheading 2.**) can be

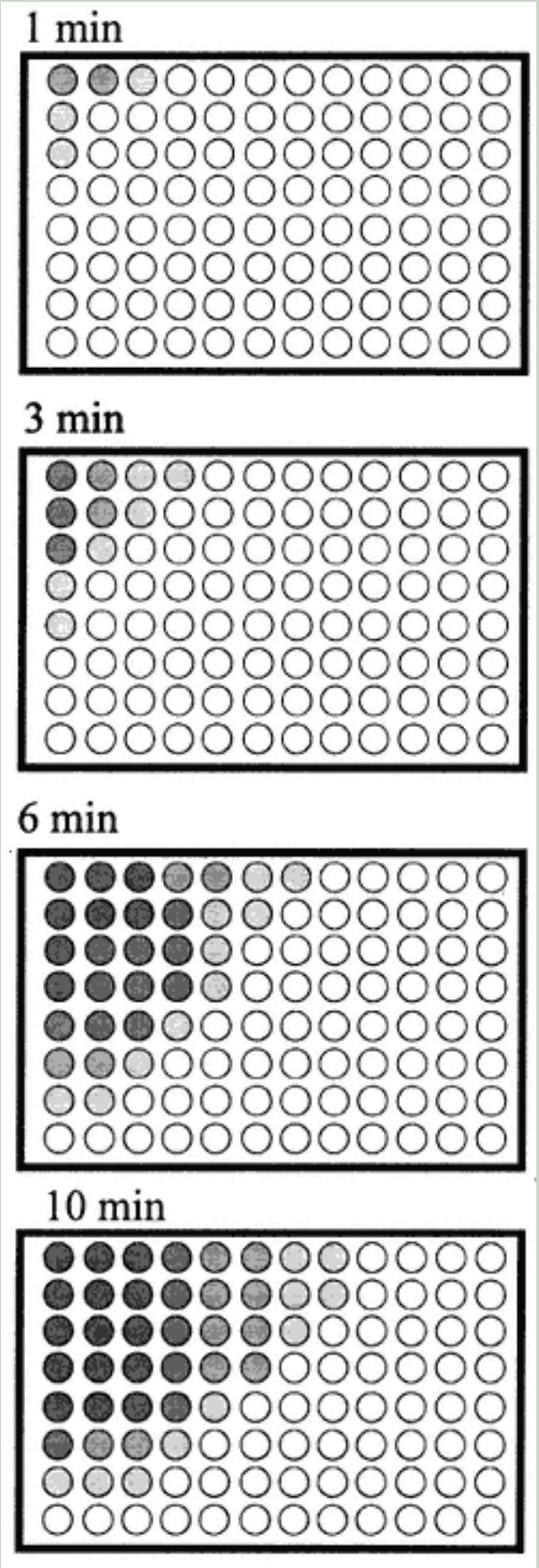


Fig. 4.
Representation of color development
at 1, 3, 6, and 10 min.

observed. These figures provide individuals who have never seen an ELISA, an idea what to expect, and can be used as a comparison with their test results. They are also useful to those who obtain the text without access to reagents, in that it allows them to work through the examples without the need for setting-up an actual assay. Individuals who have performed an assay following the

Table 1
Assessment of Plates by Eye[a]

	1	2	3	4	5	6	7	8	9	10	11	12
Observed at 1 min												
A	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
B	+	+¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
C	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
D	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
E	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
F	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
G	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
H	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
Observed at 3 min												
A	++	++	++	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C
B	++	+	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
C	++	+	+	+¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
D	+	+	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
E	+	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
F	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
G	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
H	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
Observed at 6 min												
A	++	++	++	++	++	++	+	+	+¨C	¨C	¨C	¨C
B	++	++	++	++	++	+	+¨C	¨C	¨C	¨C	¨C	¨C
C	++	++	++	++	++¨C	¨C	¨C	¨C	¨C	¨C	¨C	
D	++	++	+¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
E	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
F	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
G	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
H	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
Observed at 10 min												

A	++	++	++	++	++	++	++	++	+	+¨C	+¨C	¨C
B	++	++	++	++	++	++	+	+	¨C	¨C	¨C	¨C
C	++	++	++	++	++	+	+	¨C	¨C	¨C	¨C	¨C
D	++	++	++	++	+¨C	¨C	¨C	¨C	¨C	¨C	¨C ¨C	
E	++	++	+	+	+¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
F	+	+	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
G	+	+¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C
H	+	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C	¨C

¨C, No detectable color; +¨C, weak color; +, definite color; ++, strong color.

Table 2
OD Results for Plate Stopped at 10 Min

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.89	1.88	1.67	1.34	1.10	0.97	0.86	0.57	0.44	0.32	0.31	0.31
B	1.87	1.86	1.63	1.29	1.04	0.93	0.84	0.53	0.35	0.24	0.23	0.21
C	1.68	1.45	1.32	1.14	0.96	0.86	0.64	0.45	0.29	0.19	0.17	0.16
D	1.14	1.03	0.94	0.83	0.57	0.45	0.38	0.29	0.19	0.18	0.15	0.16
E	0.99	0.91	0.74	0.54	0.46	0.36	0.29	0.19	0.18	0.15	0.13	0.14
F	0.66	0.44	0.39	0.33	0.24	0.21	0.19	0.15	0.18	0.16	0.14	0.12
G	0.34	0.20	0.16	0.18	0.16	0.18	0.15	0.16	0.14	0.12	0.14	0.13
H	0.21	0.22	0.15	0.18	0.17	0.15	0.13	0.14	0.15	0.13	0.12	0.12

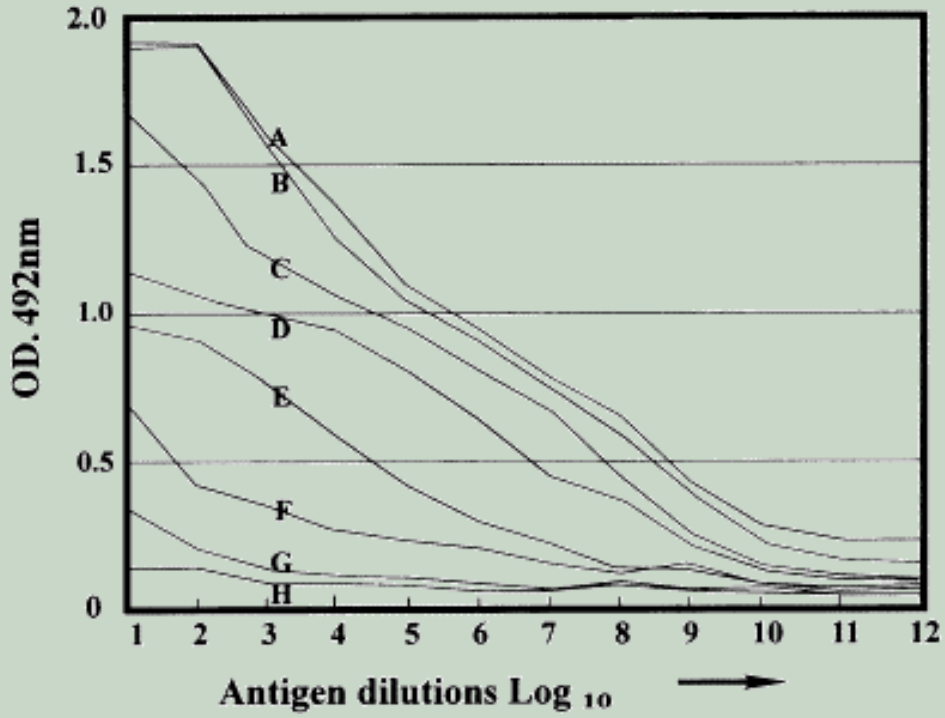


Fig. 5.
Titration curves for conjugates against different dilutions
of antigen (IgG) on columns 1¨C11 of plate.

protocols in **Subheading 1.1.**, might apply the observations on their plate to those demonstrated, and compare them critically.

1.1.7¡ª
Aspects of the Described Assay

Little happens during the first 30 s to 1 min after the addition of substrate. Color then is detected in wells 1¨C3 of rows A and B and possibly C. The strongest color is detected in the wells containing the highest concentrations of

antigen. By 3 min, the pattern should be confirmed, with detectable color in rows C¨CE. After 6 min, there is stronger color in rows A¨CD, all showing a gradual reduction in color as the antigen is diluted across the plate. The wells showing no color (no detectable antibody) for rows A, B, C, D, E, F, and G are 10, 9, 8, 6, 4, 3, and all rows, respectively.

At 10 min (the time for stopping color development), there is little change in the pattern, although the intensity of the color may have increased. Note that at the strongest concentration of the conjugate, there may be some color in the negative control well (12). Also note the color change on addition of sulfuric acid (stopping).

1.1.8¡ª
Plate Reader Data

Now let us discuss the following factors:

1. Plateau height.

2. Background, nonspecific adsorption of conjugate.

3. Plate background.

The color changes associated with each well have now been quantified, so that the exact situation can be assessed as shown in **Fig. 6**. Each line represents the titration of a different dilution of conjugate against the same dilution range of antigen. Note that rows A and B are quite similar. For wells 1 and 2, there is similar color, with no decrease in color when the antigen concentration is supposedly being decreased on the plate. This represents a plateau region (constant high color). Thus, the plate wells have a similar amount of antigen as judged by the developing color which means that at antigen levels higher than those of well 2, no more antigen can attach to the plastic of the wells. This is a factor of the binding capacity of the plastic and may vary from protein to protein.

From well 3 in rows A and B, the color decreases, corresponding to the dilution of antigen on the wells. Note that A10¨CA12 show a similar color (around 0.31). This represents the end point of the titration at the respective conjugate dilutions of 1/400.

Although row B shows a similar titration range, note that the color in rows 10¨C12 are similar to each other but lower than for row A. In particular, note well 12 for row B. This is the well that had no antigen, so the color developing in this row represents the nonspecific adsorption of conjugate.

The color diminishes in column 12 as the conjugate is diluted (e.g., C12 = 0.16), and then stays at a similar level. The conclusion here is that the 1/400 and 1/800 dilutions of conjugate cause some problems of nonspecific adsorption. Below these dilutions, there is no further problem. The residual level of color, independent of the dilution of conjugate, is the plate background and is

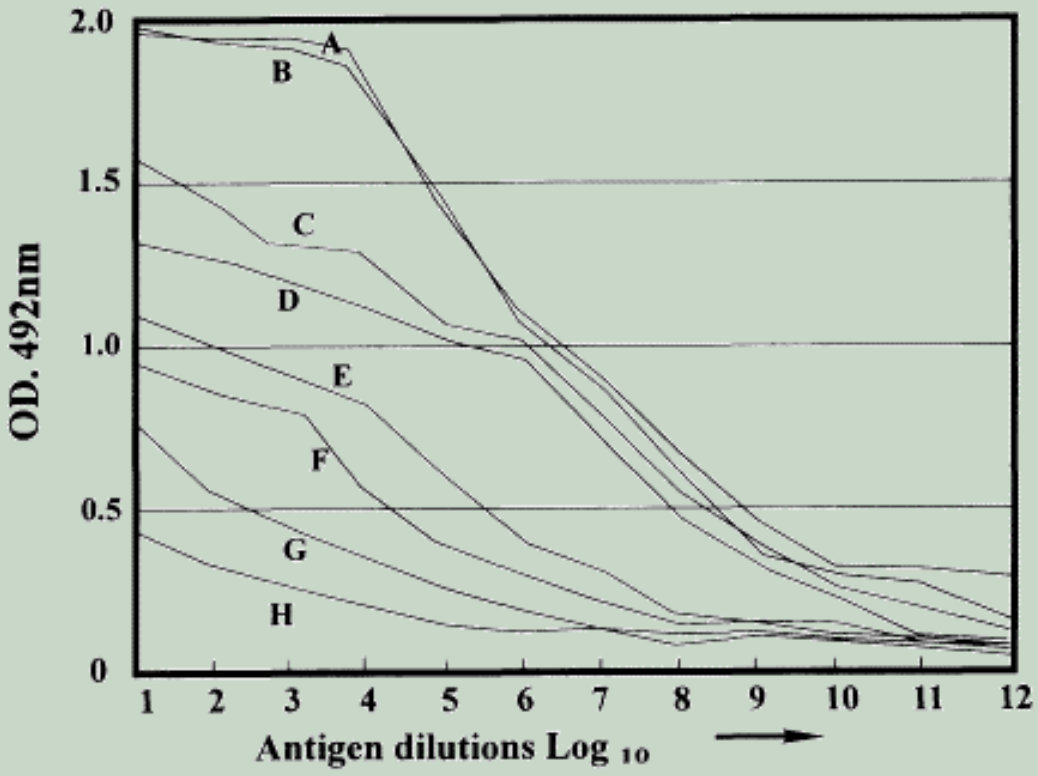


Fig. 6.
Indirect ELISA: titration curves for anti-guinea pig serum dilutions (A¨CH) against different dilutions of antigen (IgG) on columns 1¨C11 of plate.

the result of the change in color of the substrate independent of there being any enzymatic activity (oxidation owing to the air, effect of light).

Row C shows a good titration (high levels of color where there is antigen to low levels of color on antigen dilution) range of color. The end point of the titration is around well 9 (last dilution showing color above the plate background), which is similar to rows A and B (since their backgrounds are higher).

Row D also shows a good titration of antigen, although the color is weaker, and the end-point is now around well 8. This indicates that we are losing some sensitivity is being lost in the titration of the antigen at this conjugate dilution. Wells E¨CG demonstrate the loss in sensitivity on dilution of the conjugate, in particular well G, in which there is virtually no titration of the antigen.

1.1.9¡ª
Optimal Dilutions

We now may determine (1) the dilution of conjugate to be used in an ELISA to detect guinea pig IgG, and (2) what dilution of antigen (IgG) can be used on a plate in order to be used in other assays. Remember, this test is a demonstration of the principles to be used in specific antigen assays. The

same test can be used for their standardization (more clearly demonstrated in **Subheading 2.**).

1.1.9.1¡ª
Optimal Conjugate Dilution

The 1/400 and 1/800 dilutions give good titration with high backgrounds. The 1/1600 gives a similar titration curve of similar end point to the 1/400 and 1/800 with a lower background. Thus, we could use this dilution without loss of sensitivity. The 1/3200 also gives an adequate titration of antigen, although there is some loss if sensitivity (ability to react with antigen), as judged by limiting of the end point. Thus, the optimal dilution is somewhere between 1/1600 and 1/3200. In practice, a dilution of 1/2000 might be used for initial tests. This might be adjusted after later tests using particular antigens (e.g., if this assay were used to titrate antispecies conjugates that were then used in the Indirect ELISA).

1.1.9.2¡ª
Optimal Antigen Dilution

Optimal antigen dilution is relevant in other ELISAs in which specific antigens need to be titrated for use, e.g., in indirect assays. We might wish to use a constant dilution of IgG to detect antibodies against guinea pig IgG. The levels of IgG available on the wells after adsorption are reflected in the developing color. At high dilutions there is little color, and therefore little IgG is attached. In the plateau region (at plastic saturation level), there is an excess of IgG. The optimal amount to titrate antibody is when around 1¨C1.5 OD units of color are obtained using the optimal conjugate dilution. Therefore, the antigen dilutions in wells 3 and 4 are suitable for reaction with antibody. The exact value can be adjusted after actual assessments in specific assays.

1.2¡ª
Conclusion

Direct ELISA was extensively described because it introduces the investigator to the ELISA. Many of the areas covered will need less explanation, so that protocols shown for ELISAs will have less detail. The major use for the direct ELISA is to be able to titrate antispecies conjugates and thus avoid using preparations that are too strong or two weak. Some of the major principles of ELISA were introduced¨Cplateau height, end point, nonspecific reactions, backgrounds, and titration curves¡ªand they will be constantly reviewed in all the assays described.

2¡ª
Indirect ELISA

This section describes the development of the ELISA using nonpathogenic materials. The optimization of the indirect ELISA is described, followed by an

exercise in its use to titrate antibodies and then by the use of single dilutions to assess sera.

2.1¡ª
Learning Principles

1. Measuring optimal antigen concentration to coat wells.

2. Titrating antisera.

3. Using antispecies conjugates.

2.2¡ª
Reaction Scheme

$$\text{I-Ag}_{W} + \text{Ab}_{W} + \text{Anti-Ab*E}_{W} + \text{S–READ}$$

I	= microplate wells
Ag	= guinea pig IgG adsorbed to wells
Ab	= rabbit anti-guinea pig serum
Anti-Ab*E	= goat anti-rabbit serum conjugated with HRP
S	= H_2O_2 + OPD
READ	= observe by eye or read in spectrophotometer
+	= addition and incubation at 37¡ãC or room temperature for 1 h
W	= washing of wells with PBS

2.3¡ª
Basis of Assay

The basis of this assay is to titrate antibodies that have reacted with an antigen by using an antispecies conjugate. The indirect aspect therefore refers to the fact that the specific antiserum against the antigen is not labeled with an enzyme, but a second antibody specific for the particular species in which the first antibody was produced is labeled. Such assays offer flexibility and form the bases of other ELISAs. In principle, the optimization of reagents is similar to the direct ELISA. However, three factors have to be considered:

1. The optimal dilution of antigen.

2. The optimal dilutions of antisera.

3. The optimal dilution of conjugate.

The third factor was dealt with in the direct ELISA. You should now be able to titrate the conjugate (anti-rabbit in this case). The major use of indirect ELISA is to titrate antibodies against specific antigens. In this case, a constant amount of antigen is adsorbed to wells, and antisera are titrated against this as dilution ranges. Any reacting antibody is then detected by the addition of a constant amount of antispecies conjugate. Such assays can be evaluated fully from the diagnostic point of view in which numbers of field and experimental antisera (known history) are available. Therefore, they can be used to assay single dilutions of antisera, and tests can be adequately controlled using stan-

dard positive and negative antisera. Thus, the indirect ELISA has found many applications in epidemiological studies assessing disease status.

2.4¡[a]
Materials and Reagents

1. Ag: guinea pig IgG at 1 mg/mL (1 g/L).

2. Ab: rabbit anti-guinea pig serum.

3. Anti-antibody*E: Sheep anti-rabbit serum linked to HRP (rabbit IgG needed if conjugate titration not done, as for titration of anti-guinea pig conjugate).

4. Microplates.

5. Multichannel and single-channel pipets.

6. 10 mL and 1 mL pipets.

7. Carbonate/bicarbonate buffer, pH 9.6, 0.05 *M*.

8. PBS containing 10% BSA and 0.05% Tween-20.

9. Solution of OPD in citrate buffer.

10. Bottle of hydrogen peroxide (30% w/v).

11. Washing solution (PBS) in a bottle or reservoir.

12. 1 *M* sulfuric acid in water.

13. Paper towels.

14. Small-volume bottles.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

2.5¡ª
Protocol for Indirect ELISA

The first stage in this assay involves the titration of the antispecies conjugate under the conditions described in the direct ELISA. Remember that the antigen used to titrate the conjugate must be appropriate; for example, if an antibovine conjugate is to be used, then use BSA as the antigen in the original CBT. If detection of an antibovine IgG is required, then use bovine IgG as the antigen in the direct ELISA CBT.

The anti-rabbit conjugate needs to be titrated so that we know the dilution to use in the indirect assay in order to detect any reacted rabbit serum (the optimal dilution of conjugate may be given in class if this procedure has not been carried out):

1. Titrate the anti-rabbit conjugate (optimal dilution may be given).

2. Take a microtiter plate with A1 at the top left-hand corner. Add 50 µL of carbonate /bicarbonate buffer to each well using a multichannel pipet.

3. Make a dilution range of the guinea pig IgG from 5 µg/mL from column 1 (eight wells) to 11. This is made exactly as described for the direct ELISA. Add 50 µL of the guinea pig IgG at 10 µg/mL (or 1/50 if the concentration is unknown) to column 1. Mix (pipet up and down eight times with a multichannel pipet), and then transfer 50 µL of dilution to column 2. Mix and continue transferring to

column 11. Discard 50 µL remaining in tips after mixing in column 11. Thus, we have a twofold dilution range of IgG in each row A to H, excluding column 12 wells.

4. Incubate at room temperature or 37¡ãC for 2 h.

5. Wash the wells in PBS (fill and empty the wells four times).

6. Blot the plates.

7. Dilute the rabbit anti-guinea pig serum to 1/50 in blocking buffer (PBS containing 1% BSA and 0.05% Tween-20). Make up 1 mL; therefore, add 20 µL to 1 mL of buffer.

8. Add 50 µL of blocking buffer to all wells using a multichannel pipet.

9. Add 50 µL of the 1/50 anti-guinea pig serum to each well of row A. Mix and transfer 50 µL to row B, mix and transfer 50 µL to row C, and repeat this procedure to row H. We now have a twofold dilution series of antibody the opposite way to the IgG antigen.

10. Incubate the plate at room temperature or 37¡ãC for 1 h.

11. Wash and blot the plate.

12. Make up the antispecies conjugate (kept at ¨C20¡ãC) to the optimal dilution found in the direct ELISA (or as instructed) in the blocking buffer. Make up enough for all the wells of the plate plus 0.5 mL (approx 5.5 mL). This might appear wasteful but is convenient practice since it allows for minor errors in pipeting and avoids the need to make up a small volume of conjugate when one runs out on the last row (i.e., when the exact volume to fill the plate wells is made up). Add 50 µL of the dilution to each well using the multichannel pipet and a clean trough.

13. Incubate at room temperature or 37¡ãC for 1 h.

14. Wash and blot the wells.

15. Thaw out the OPD (10 mL). Add 5 µL of H_2O_2 immediately before use. Mix well. Add 50 µL of this to each well, using a multichannel pipet and clean the troughs (make sure that the trough is not contaminated with conjugate from the previous addition to the plate).

16. Incubate for 10 min (note color changes).

17. Stop any color development by adding 50 µL of 1.0 *M* sulfuric acid to each well.

18. Read the plate by eye and with a multichannel spectrophotometer after titration of antigen (guinea pig IgG) and antibody (anti-guinea pig serum) as described above.

2.6¡[a]

Results

Table 3 presents the microplate reader results. Note that these produce a similar picture to the direct ELISA results. Also, you should have observed that there was a similar development of color throughout the 10-min incubation after addition of the substrate solution. **Figure 7** shows the data graphically. Plots relating the concentration (or dilution) of the IgG (Ag) to the OD for all the different dilutions of rabbit anti-IgG are shown.

Plot the data relating the IgG concentration on the plate plotted as a $\log_{10}$ twofold series (micrograms/milliliter per well, or dilution if the actual concen-

Table 3
Plate Data from CBT of Conjugate and Antigen

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.92	1.89	1.92	1.89	1.45	1.12	0.89	0.67	0.45	0.39	0.40	0.39
B	1.94	1.89	1.91	1.86	1.47	1.09	0.87	0.59	0.39	0.38	0.31	0.29
C	1.56	1.43	1.33	1.29	1.07	0.89	0.78	0.56	0.43	0.32	0.23	0.19
D	1.34	1.23	1.14	1.09	0.97	0.75	0.68	0.49	0.29	0.21	0.17	0.15
E	1.14	1.00	0.89	0.76	0.56	0.41	0.32	0.23	0.19	0.17	0.19	0.12
F	0.92	0.83	0.73	0.54	0.43	0.32	0.21	0.17	0.19	0.16	0.16	0.14
G	0.76	0.56	0.42	0.36	0.28	0.21	0.19	0.18	0.16	0.14	0.15	0.15
H	0.45	0.32	0.29	0.21	0.17	0.14	0.15	0.18	0.16	0.15	0.16	0.10

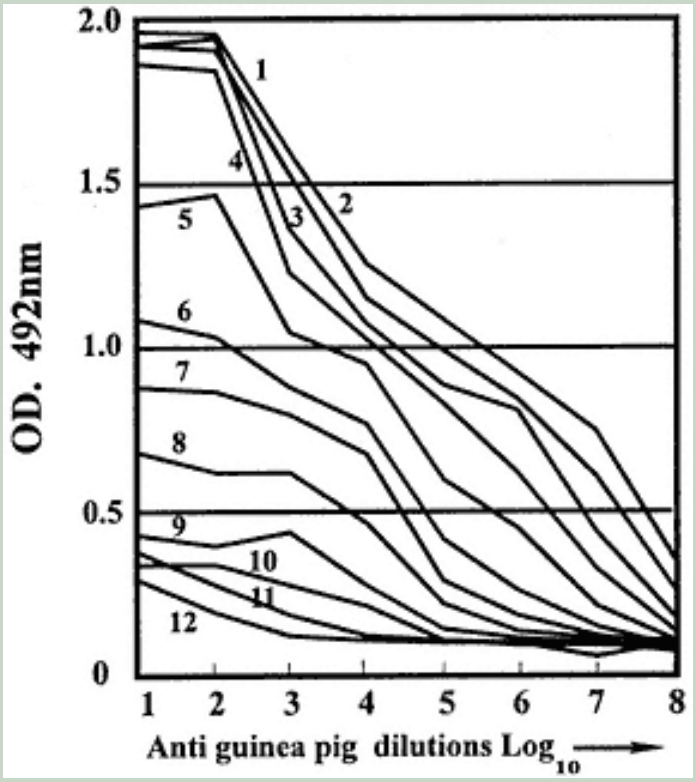


Fig. 7.
Indirect ELISA: titration curves of anti-guinea pip serum (A¨CH) against constant antigen concentrations of IgG (columns 1¨C12).

tration is unknown) against the OD for each dilution of antibody used. You should end up with eight lines on a single graph, one for each antiserum dilution. You have already observed similar results in the direct assay. Similar areas of reactivity can be identified on the indirect CBT.

1. Plateaus of similar high color are shown in rows A and B, wells 1¨C4.

2. There are higher plate background values in rows A and B (possibly C) than for more dilute serum.

3. The serum titration end points (where the OD value for a particular IgG concentration is the same as plate background) are similar for rows A¨CD. After this dilution of antiserum, there is loss in detection of IgG.

4. Loss of end point detection is matched by a loss in OD at high concentrations of IgG. For example, in rows F¨CH at 5 µg/mL of IgG, there is substantial and increasing loss in color, as compared to where maximal color (in antibody excess: row A) is observed. Note that row H barely titrates the IgG; very low color is obtained.

2.6.1$_i^a$
Optimization of Reagents

Rows A and B indicate that antibodies are in excess, and we have some problems of nonspecific attachment to the plate without antigen having been adsorbed (well 12). Note that in these rows, the plateau regions extend to well 4. Thus, no more antigen (IgG) is able to absorb to the plate above the concentration in well 4. Rows C and D give optimal titrations of the IgG in that maximum values do not exceed 1.6 OD, and high end point titers are obtained. Below these dilutions, sensitivity for the detection of IgG is lost. Thus, to detect the antigen optimally, and to use a single dilution of antiserum under the conditions of the ELISA described, use a dilution of about 1/400 to 1/800.

The optimum dilution of antigen that might be used as a single dilution to detect and maybe quantify antibodies is best assessed as the dilution (or concentration) that shows good binding across the whole range of antiserum dilutions. The best way to illustrate this is to draw a graph of the plate data, but this time, plot the dilutions of serum gainst the OD for the various antigen concentrations (or dilutions). This was done in **Fig. 7**.

At the first four concentrations (dilutions) of antigen (IgG), there is little difference in the end-point detection for the dilutions of antiserum. are reduced. After this, the OD readings and the end point detections are reduced. At the extreme, in column 10, barely any antibody is detectable, even where the serum is most concentrated. The higher values in rows A¨CC correspond to the nonspecific binding to the wells seen in row 12. Thus, the dilution of antigen found in columns 3 and 4 is optimal to detect antibodies.

3¡ª
Use of Indirect ELISA to Titrate Antibodies

The optimized reagents in **Subheading 2.4.** can be exploited to measure antibodies directed against the guinea pig IgG target.

3.1¡ª
Learning Principles

1. Titrating antibodies from positive sera using full-dilution ranges.

2. Establishing ELISA-negative antibody levels for control of nonimmune sera.

3. Duplicating samples tested.

3.2¡ª
Reaction Scheme

$$\text{I-Ag}_{W} + \text{Ab}_{W} + \text{anti-Ab*E}_{W} + \text{S–READ}$$

——I	= microplate
——Ag	= optimum concentration of antigen
——Ab	= test serum plus or minus in reaction for Ag
——AntiAb*E	= antispecies antibody linked to enzyme
——S	= substrate/color detection system
——W	= washing step
——+	= addition and incubation of reactants

In this exercise, the Ag and Anti-Ab*E are used at optimal dilution. The test or standard Abs are added as dilution ranges.

3.3¡[a]
Basis of Assay

We are now able to titrate antibodies, since we know the antigen optimum and the conjugate optimum dilutions for our given system. Thus, if sera are reacted with the antigen on the plate, and if they contain antibodies against the guinea pig IgG, they will be picked up by the subsequent addition of the conjugate. The seropositive serum titration curves may then be compared with each other and to the seronegative curves to establish antibody titers and examine the result of nonspecific reactions at the various dilutions of the negative sera, within the system.

3.4¡[a]
Materials and Reagents

1. Ag: guinea pig IgG, 1 mg/mL.

2. Ab: three rabbit serum samples after injection with guinea sera test bled at different times following inoculation with guinea pig IgG and three rabbit sera from antibody-negative animals (prebleeds).

3. Anti-Ab*E: sheep anti-rabbit serum linked to HRP.

4. Microplates.

5. Multichannel and single-channel pipets.

6. 10- and 1-mL pipets.

7. Carbonate/bicarbonate buffer, pH 9.6, 0.05 *M*.

8. PBS containing 1% BSA and 0.05% Tween-20.

9. OPD solution.

10. Hydrogen peroxide.

11. Washing solution (PBS).

12. 1 *M* sulfuric acid in water.

13. Paper towels.

14. Small-volume bottles/microdilution system.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

3.5¡ª
Titration of the Antigen Dilution or Concentration for Use in Measuring Antibodies

Titration is performed as described earlier for the indirect assay (*see* **Subheadings 2.5.** and **2.6.**), in which we also titrated the optimum dilution of conjugate. Now we are concerned with the titration of antibodies against guinea pig IgG in rabbit sera. Therefore, we make the CBT of guinea pig IgG against the positive rabbit antiserum and use a constant dilution of anti-rabbit conjugate.

Note that while is setting up an indirect ELISA, a positive serum against the particular antigen being detected is needed. Such sera are often available as determined from other serological assays, from systems whereby specific antibodies are expected (e.g., from experimentally infected or vaccinated animals or from animals during the course of an outbreak). The exact conditions of the ELISA may therefore have to be altered during the developmental stages when many sera have been examined as compared to the originally used positive serum. For now, the original "optimal" conditions are determined using a defined (experimentally derived) positive serum.

3.6¡ª
Titration of Different Sera

1. Dilute guinea pig IgG (Ag) to optimum concentration in carbonate/bicarbonate buffer, pH 9.5, 0.05 *M* (as determined in **Subheading 2.1.**).

2. Add 50 µL to each well of the plate using a multichannel pipet.

3. Incubate at 37¡ãC for 2 h.

4. Wash and blot the plate.

5. Add 50 µL of blocking buffer to all the wells using a multichannel pipet and trough.

6. Take the six sera supplied. Label the three positive sera 1, 2, and 3. Label the three negative sera 4, 5, and 6. Dilute each one to 1/20 in blocking buffer in small bottles, and make up a final volume of 0.5 mL of each (25 µL + 475 µL of blocking buffer).

7. Turn the plate with 50 µL of blocking buffer per well so that well H1 is on the left-hand top corner (*see* **Fig. 8**). Add 50 µL of serum 1 dilution to wells H1 and H2, add 50 µL of serum 2 dilution to wells H3 and H4, and add 50 µL of serum 3 dilution to wells H5 and H6. Repeat the process adding sera 4, 5, and 6 to wells H7 and H8, H9 and H10, and H11 and H12. We now have each of the sera diluted effectively to 1/40 in 100 µL of blocking buffer in the left-hand extreme row (H) of the plate (*see* **Fig. 9**).

8. Use the multichannel pipet with 12 tips attached to mix, and the dilute the sera across wells G, F, E, D, C, B, and A, transferring 50 µL of each dilution. We now

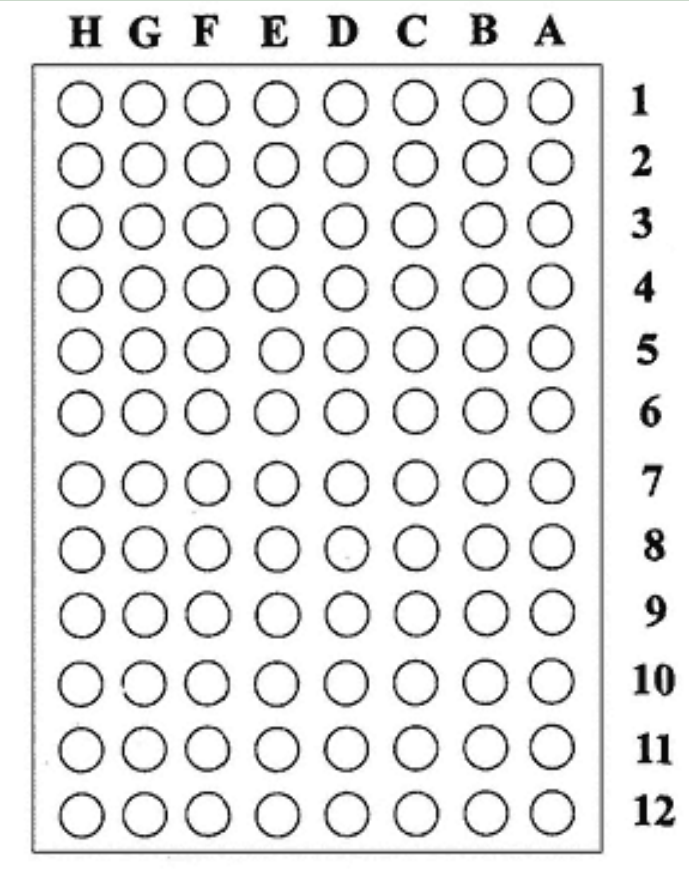


Fig. 8.
Orientation of plate for dilutions of sera.

have a twofold dilution range of the sera, in duplicate, that is, there are two dilution series of each of the sera (*see* **Fig. 9**).

9. Incubate at 37¡ãC or room temperature for 1 h (the exact conditions you used in the indirect CBT are best).

10. Wash and blot the plate.

11. Add 50 µL of anti-rabbit conjugate per well (diluted in blocking buffer).

12. Incubate at 37¡ãC (or room temperature) for 1 h (conditions as for 1-h incubation in CBT).

13. Wash and blot the plate.

14. Add substrate and chromophore (50 µL).

15. Stop color development after 10 min.

16. Read the plate in a multichannel spectrophotometer. Remember to watch the plate as the color develops and make relevant notes.

3.7¡ª
Explanation of Data

Figure 10 gives typical results from this assay, and **Table 4** presents the OD readings.

1. Serum 1: The values of the duplicate samples are quite similar. The titration shows a plateau region where the values are the same (wells H1 and H2, and

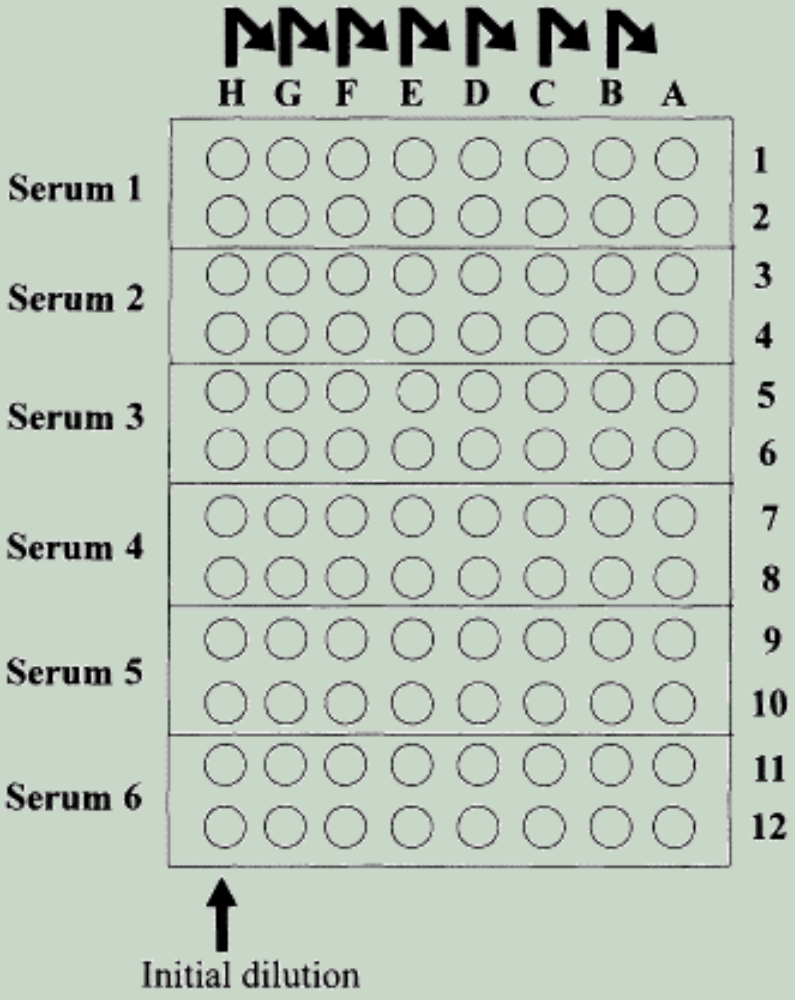


Fig. 9.
Addition and dilution steps of sera to wells.

wells G1 and G2). Thus, there is a maximum color obtained up to 1/80; increasing the concentration of antibodies has no effect on the readings. This represents the region where all the antigen is saturated with antibody. The value of the OD is dependent on the amount of antigen that has attached to the wells, which, in turn, is dependent on the adsorption characteristics of the plastic and the concentration of antigen. On further dilution, the antibodies are no longer in excess, so they are titrated, as seen by a gradual decrease in the OD.

2. Serum 2: The OD levels even at 1/40 are not equal to those where antibody was in excess in serum 1. Thus, the antibodies are not saturating the antigen on the wells, and are therefore not present in excess. The titration of the serum begins immediately on dilution. Note that the last dilutions give low OD values equivalent to the plate background, unlike serum 1.

3. Serum 3: Even at 1/40 there are low OD values as compared to the serum samples 1 and 2. There are fewer antibodies in this serum than the other two! Again, the titration begins immediately on dilution and the low OD(around 0.19) is attained

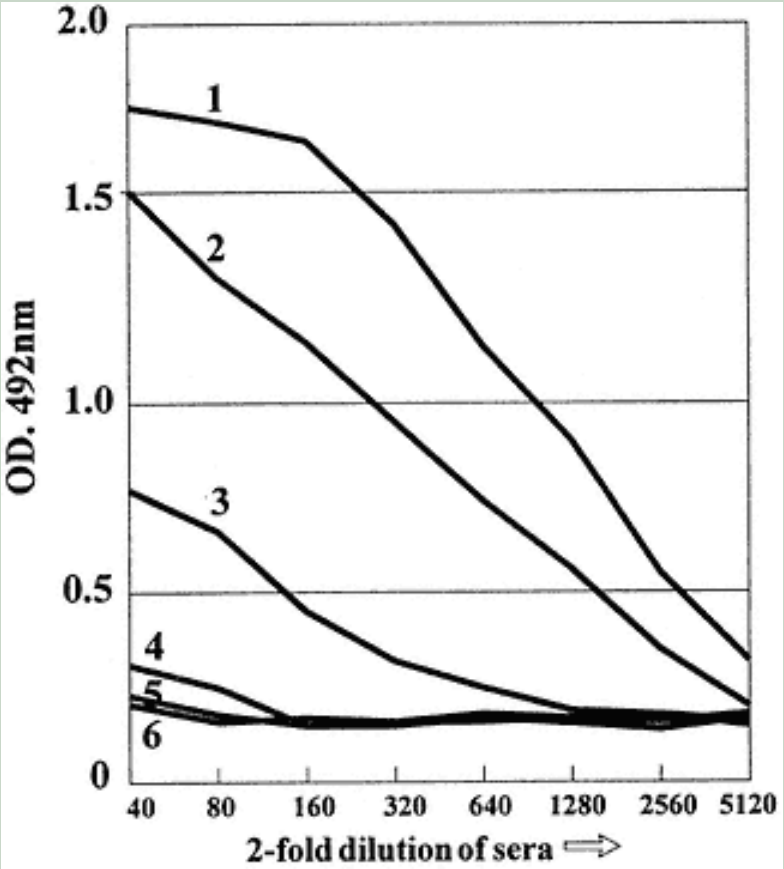


Fig. 10.
Plots of data in Table 4 showing titrations of six different serum samples diluted from 1/40 in a twofold series against constant antigen concentrations. Mean values for OD are used.

Table 4
Plate data

	Serum 1		Serum 2		Serum 3		Serum 4		Serum 5		Serum 6		
	1	2	3	4	5	6	7	8	9	10	11	12	Dilution
A	0.34	0.32	0.19	0.23	0.14	0.15	0.17	0.16	0.15	0.16	0.19	0.15	5120
B	0.54	0.56	0.34	0.36	0.17	0.19	0.14	0.14	0.16	0.18	0.16	0.14	2560
C	0.87	0.91	0.54	0.57	0.18	0.19	0.14	0.17	0.17	0.16	0.17	0.14	1280
D	1.16	1.14	0.76	0.72	0.28	0.25	0.17	0.16	0.18	0.16	0.17	0.19	640
E	1.45	1.49	0.95	0.91	0.31	0.32	0.15	0.14	0.17	0.15	0.14	0.17	320
F	1.68	1.70	1.15	1.17	0.43	0.46	0.13	0.15	0.14	0.15	0.16	0.18	160
G	1.76	1.73	1.34	1.32	0.65	0.66	0.23	0.24	0.18	0.17	0.15	0.17	80
H	1.79	1.76	1.56	1.54	0.78	0.76	0.31	0.32	0.28	0.24	0.21	0.23	40

at 1/1280. Overall, these three positive sera have different reactivities in terms of the quantity of antibody titrated. Thus, serum 1 has the highest titer, showing a plateau (is able to saturate the antigen). Serum 2 has the next highest amount of antibodies since it has an end point around 1/5120 (point where OD equals the plate background). Serum 3 has the lowest amount of antibody, with an end point of around 1/1280.

4. Serum 4: This is a negative serum (clinically). Therefore, by definition it should contain no antibodies. The color obtained reflects the nonspecific attachment of the serum to the antigen. Most nonspecific "sticking" might be expected in the least dilute sample. This is the case here, with background levels at 1/40 and 1/80 serum dilutions. Note that the levels of nonspecific color are much lower than in the positive sera, but are distinct from the assumed plate background, which can be taken as the backgrounds observed for the negative sera at their highest dilution. Note that in such wells as (E, D, C, B, A) give similar results and no titration is observed on dilution.

5. Sera 5 and 6: These give results similar to serum 4, although there is a lower amount of color in the 1/80 wells, reflecting different amounts of nonspecific adsorption of serum proteins.

3.7.1¡[a]
Curve Shapes

The plotted data, particularly for the positive sera, produce curves rather than straight lines. Generally, there is a region on the curve that contains three to five points that are more linear than the rest. The nonlinear regions occur at the top and bottom of a graph, and such sigmoidal curves are typical of serum titrations as shown in **Fig. 11**. End point determinations are difficult to assess exactly, because there is a pronounced "tail" at the low OD end of the results.

3.7.2¡[a]
Comparison of Serum Titration Curves

The amount of specific antibody in each serum has been titrated over a dilution range. The serum containing the most antibodies will have a higher dilution end point (dilution where the OD is the same as the background OD). Thus, as already indicated, the end points may be compared as representing the titer of the sera. This can be assessed by eye as well as by machine reading.

A better estimate of the end point is made by drawing a straight line through the points on the curves that are nearly in a straight line. If this was done statistically, a regression analysis of the points would be made and the best line of fit would be given mathematically (*see* **Fig. 12**). Graphically, this may be approximated to sufficient accuracy. Thus, the end points are assessed when the regression lines (or graphically produced lines) cut the measured background OD line, assuming that the curves are of similar shape (the lines are then parallel). However, this may not always be the case, since different antibody populations may be responsible for the color of the ELISA. In this case,

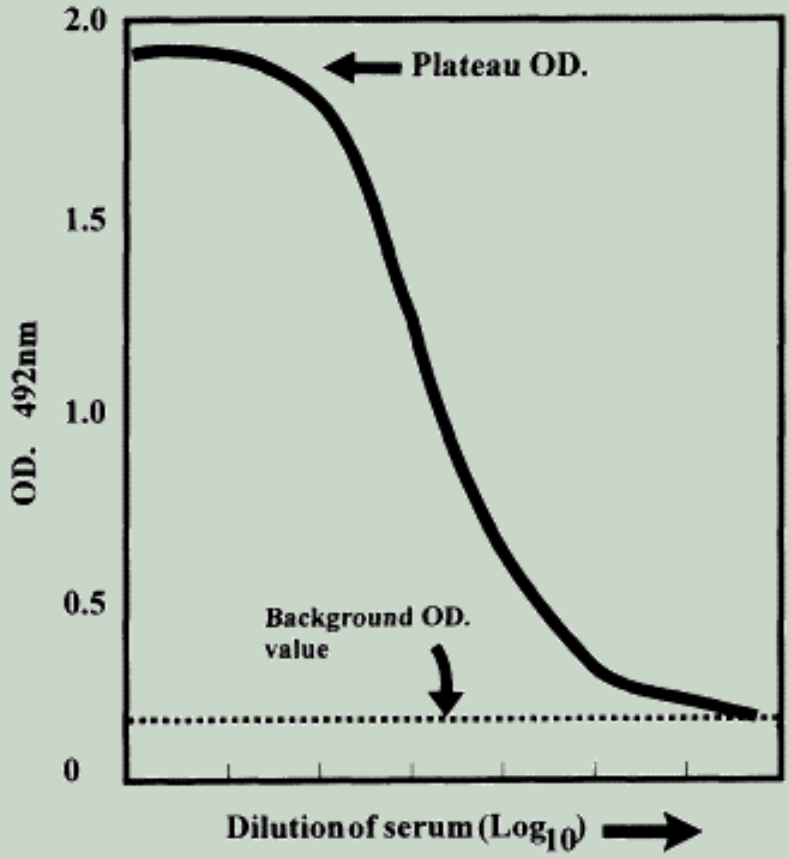


Fig. 11.
Serum titration curves showing sigmoidal nature.

differences must be noted and taken into account when the implications of the titers found are considered. Note that the curves obtained for negative sera are very flat; even so, they have an end point. Sera may also show differences in maximum plateau heights and shape (**Fig. 13**).

Figure 14 attempts to explain why there are differences in plateau heights for different sera. Here, several sera are reacting maximally with the antigen since on increasing their concentration, there is no increase in color. The plateau heights are different, however, showing that different weights of antibody have reacted with the same antigen for particular sera. This is a function of the number of reactive antigenic sites on the antigen and the quantities and specificities of the antibody populations in the sera. Although this is uncommon when using polyclonal antibodies, it is common when using monoclonal antibodies (mAbs).

Where the curves are parallel, any point can be taken on them for comparison of samples. This is illustrated in **Fig. 15**. Analysis of as many sera as possible over full-dilution series and examination of the curves should be made to establish whether there is parallelism. This is important when spot tests are

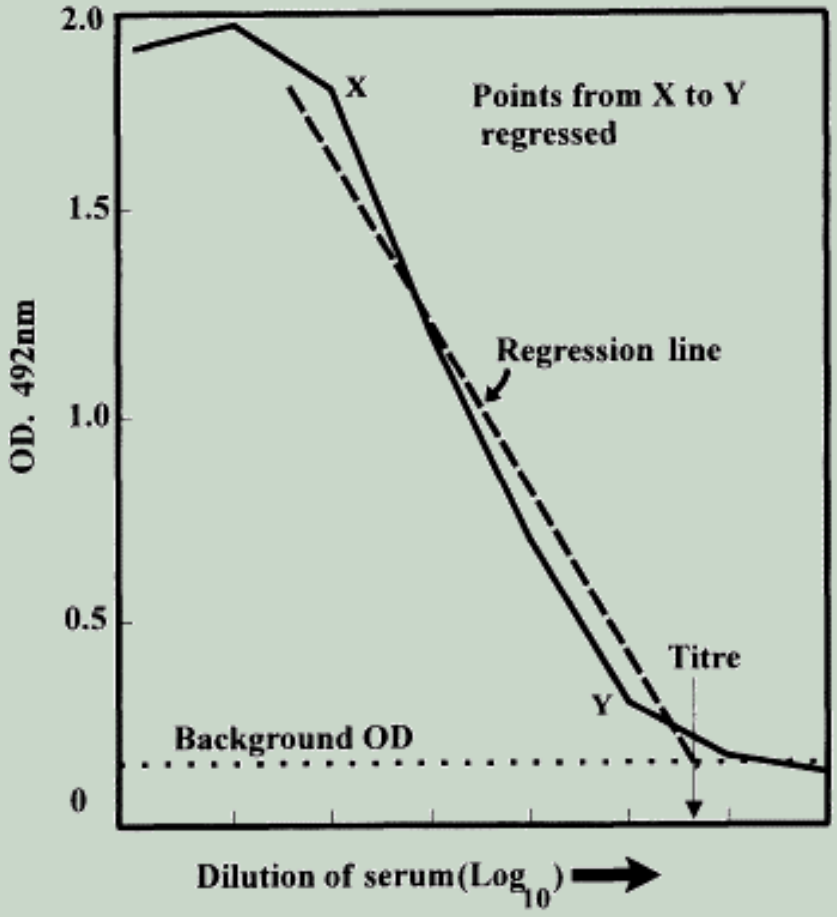


Fig. 12.
Regression of points in serum titration curve to obtain
a titer at the intersection of the background OD.

required so that a single dilution of test sample can be established. The dilution can be taken where samples give results in the parallel regions of curves.

A line is drawn at a particular OD, and the dilution of serum giving this OD for all the sera is determined, thus giving relative titers. Such relative titers may be expressed compared to an accepted standard serum, which, in turn, can be given in any units. The actual activity of the standard serum may be known (e.g., number of micrograms/milliliter of specific antibody), so that all the sera compared to this can be expressed in the same units.

3.7.3$_i$[a]
Negative Sera and Control Sera

The test made involved only one control, that of negative sera. Ideally, a plate background should be included to measure the color in the wells with only the antigen and conjugate. This should correspond to the readings beyond the titration of the antibodies, observed when a low plateau is obtained even on dilution of the samples. Such backgrounds can be subtracted from the whole

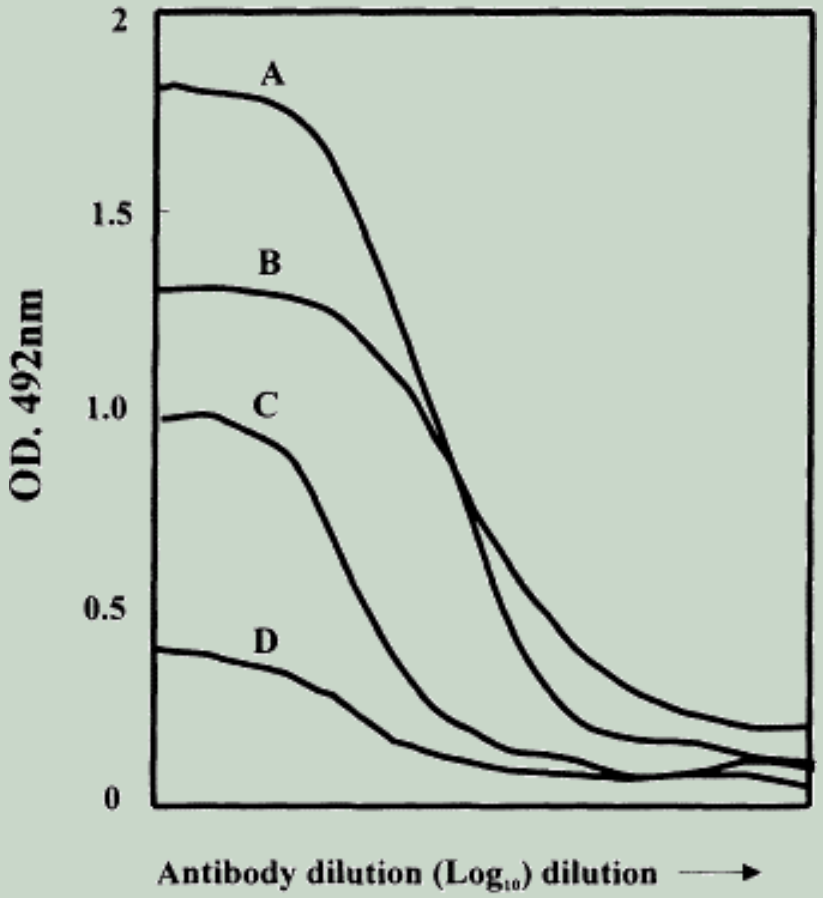


Fig. 13.
Variation in sigmoid curves for serum titrations.

plate results before any processing of the data, or used to blank the spectrophotometer before reading. The treatment of the results of the negative serum depends on what is known about the negativity in terms of other tests and clinical findings. For example, British cattle are ideal as negative sera when studying anti-foot-and-mouth disease virus (FMDV) antisera, because Britain is disease-free. This may not always be possible in countries where disease is endemic. Note also that control negative sera obtained from other countries may not reflect the same negative population of another country, since there are breed differences, complications owing to other infections, and so forth. This could affect the performance of kits in which standard negative sera are supplied to act as controls in the ELISA. Kits must be evaluated, wherever possible, in the country where they are to be used. The control value for the negative serum supplied may not reflect the mean value for negative sera.

3.7.4$_i^a$
Selection of a Single Serum Dilution to Perform a "Spot Test"

Examination of the serum titration curves for positive and negative sera can tell us which dilution might be suitable to use in the indirect ELISA so

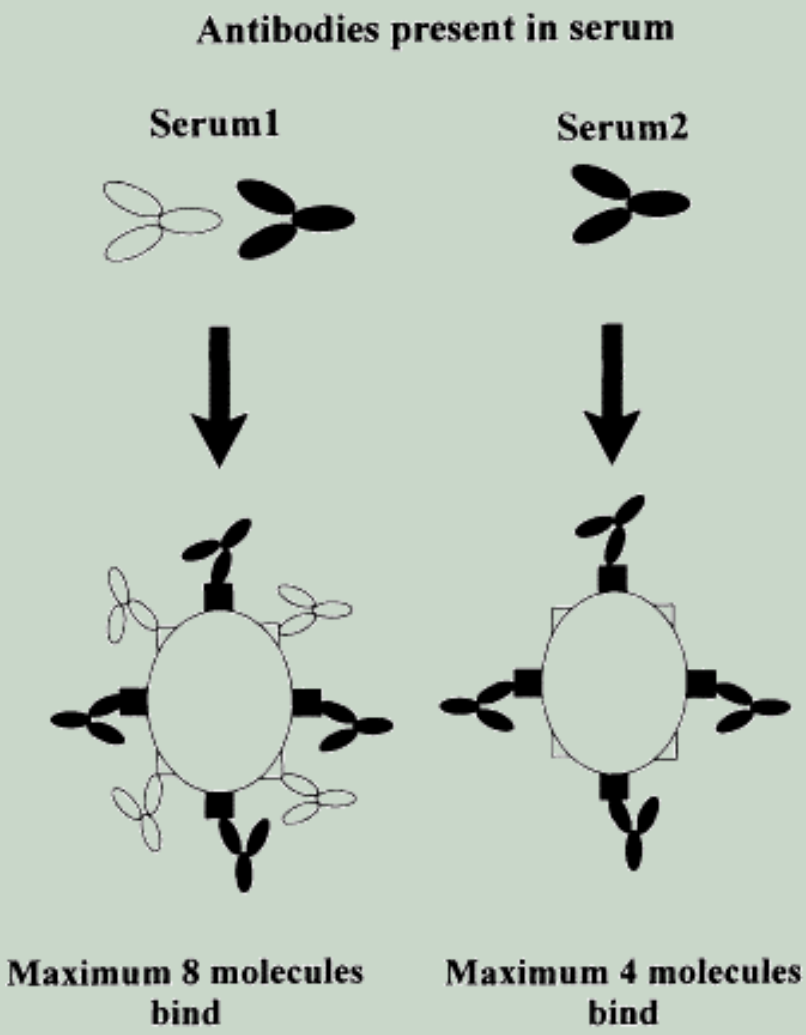


Fig. 14.
Diagram representing the maximum number of molecules of antibody that can bind to antigens. Differences in plateau height (maximum OD) can be attributed to different populations of antibodies in sera.

that antibodies may be assayed on single wells (or multiple wells using the same dilution). Thus, in **Fig. 10**, we observe that there is low nonspecific activity seen in the negative sera at 1/40 and 1/80. The positive sera still show high OD values at these dilutions so that the relative sensitivity of the assay (detection of specific antibodies) can be made at such dilutions. However, if dilutions greater than 1/80 are used, we can still measure antibody in the absence of nonspecific reactions. The sensitivity does drop, however. Remember that we are trying to balance sensitivity with low background in the presence of other serum proteins in the sample. If we had used the sera at 1/160, then we would have had values for the ELISA as shown in

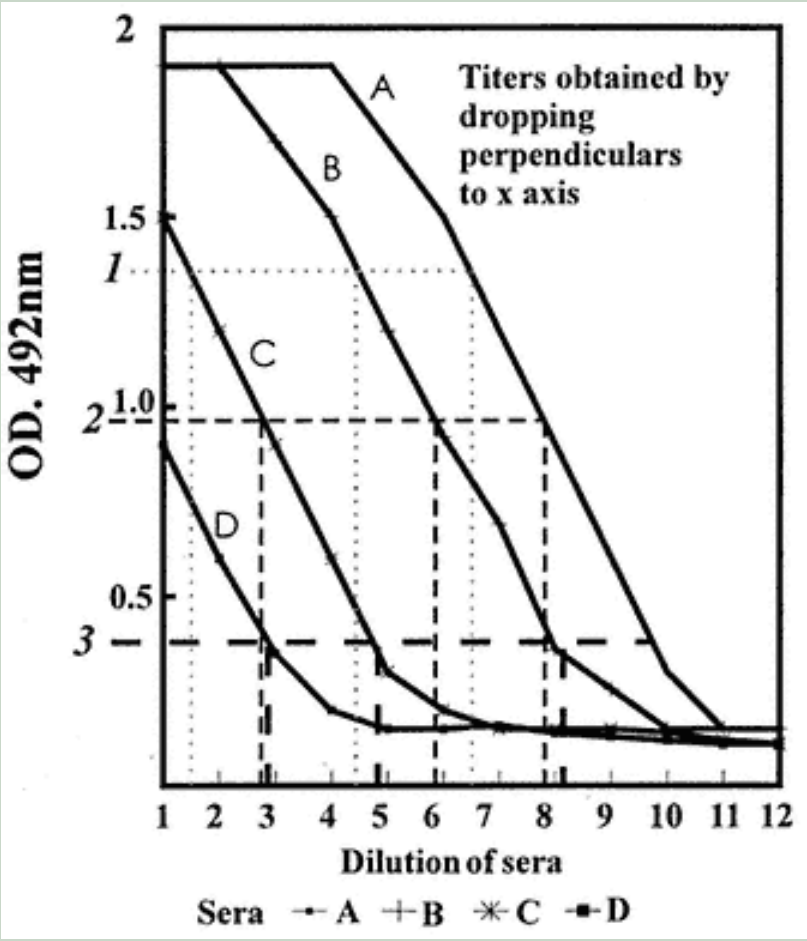


Fig. 15.
Comparison of serum titration curves to standard serum titration at three points (OD values *1*, *1*, and *3*) in parallel regions of curves. Titers can be read from the *x*-axis and related.

Table 5
Mean OD492
of Anti-Guinea Pig Sera
at 1/60 Dilution

Serum	OK
1	1.69
2	1.16
3	0.45
4	0.14
5	0.15
6	0.17

Table 5. The negative sera levels are therefore around 0.15, whereas all the positive sera are above this value. The next exercise expands on this approach.

4¡ª
Use of Indirect ELISA to Determine the Positivity of Sera at Single Dilution

4.1¡ª
Learning Principles

1. Examining negative serum populations for establishing OD limits of negativity.

2. Examining antibody-positive serum populations.

3. Examining frequency of results in a population.

4.1¡ª
Reaction Scheme

$$\underset{W}{\text{I-Ag}} + \underset{W}{\text{Ab}} + \underset{W}{\text{Anti-Ab*E}} + \text{S–READ}$$

- I = microplate
- Ag = optimum concentration of antigen
- Ab = test sera at single dilution
- AntiAb*E = anti-species antibody linked to enzyme
- S = Substrate/color detection system

In this exercise, we use Ag and Anti-Ab*E at optimal dilutions. The test sera are added at a constant dilution. Control positive antisera can be added at a constant dilution or as a dilution range to produce a standard curve relating color to dilution or concentration of antibodies added. Thus, the test sera can be related to the positive serum titration curve. The same can be done by including accepted negative control sera standards.

4.2¡ª
Materials and Reagents

1. Ag: guinea pig IgG (1 mg/mL) (or previously titrated).

2. Ab: 48 rabbit sera including high, moderate and low titer against guinea pig IgG (24) and negative sera (24).

3. AntiAb*E: sheep anti-rabbit serum linked to HRP.

4. Microplates.

5. Multichannel and single-channel pipets.

6. 10- and 1-mL pipets.

7. Carbonate/bicarbonate buffer.

8. PBS containing 1% BSA and 0.05% Tween-20.

9. OPD solution.

10. Hydrogen peroxide.

11. Washing solution.

12. Paper towels.

13. 1 *M* sulfuric acid in water.

14. Small-volume bottles/microdilution equipment.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

18. Calculator.

4.3¡ª
Protocol for Spot Test

From the previous exercises, you should have assessed the dilution of test serum that can be used to discriminate between positive and negative nonspecific results, based on the difference noted between the selected positive and negative sera titrated over full-dilution ranges. We are now going to titrate all the sera at the dilution found as duplicates (two wells per serum dilution in the indirect ELISA).

1. Add the guinea pig IgG to the wells of a microtiter plate at optimum dilution (as in earlier exercises). Incubate at 37¡ãC for 2 h (or under particular optimal conditions).

2. Wash and blot the plate.

3. Dilute the test serum samples appropriately, in blocking buffer. Sera may be diluted into small-volume bottles. However, this causes two problems: manipulation (capping and so on) is laborious, and transfer of serum dilutions must be made with single channel pipet. The latter problem is important because it takes a long time to transfer all the sera to the different wells. The initially added samples will therefore receive a longer contact time with the antigen, which may well affect the results. This can be avoided if the samples are transferred to other plates before dilution (e.g., plastic non-ELISA microtiter plates in volumes that need not be accurate). The plate can then be sampled using a multichannel pipet if the dilution factor for the sera is not too high. The initial dilution can be made directly into, say, 100 µL of blocking buffer in the non-ELISA plates. The transfer of the required volume of the diluted test sample can then be effected using a multichannel pipet. Thus, the samples are transferred at approximately the same time. Special systems have been developed for use with multichannel pipets. These are ideal for the dilution and storage of test samples. Volumes of about 1 mL can be made up, making the accurate dilution of up to 1/200 (5 µL of sample/mL) easy. The microtiter dilution system should be available for this exercise.

4. Add a volume of blocking buffer to the plastic tubes held in the tube holder. For example, if a dilution of 1/100 is required, add 0.5 mL of blocking buffer per tube, and then add 5 µL of test sample. (*See* **Fig. 16** for a pattern of samples on a plate.) Incubate for 1 h, as in the previous exercise, and follow the same steps for washing and addition of reagents to the stopping stage. Read the OD values.

4.4¡[a]
Example of Data

Table 6 gives typical results. The results obtained in your specific assay can be processed in the same way. **Figure 17** is a diagrammatic representation of a stopped plate.

Since duplicates have been made, examine the variation between the values. This should not be high; that is, there should be little difference between the

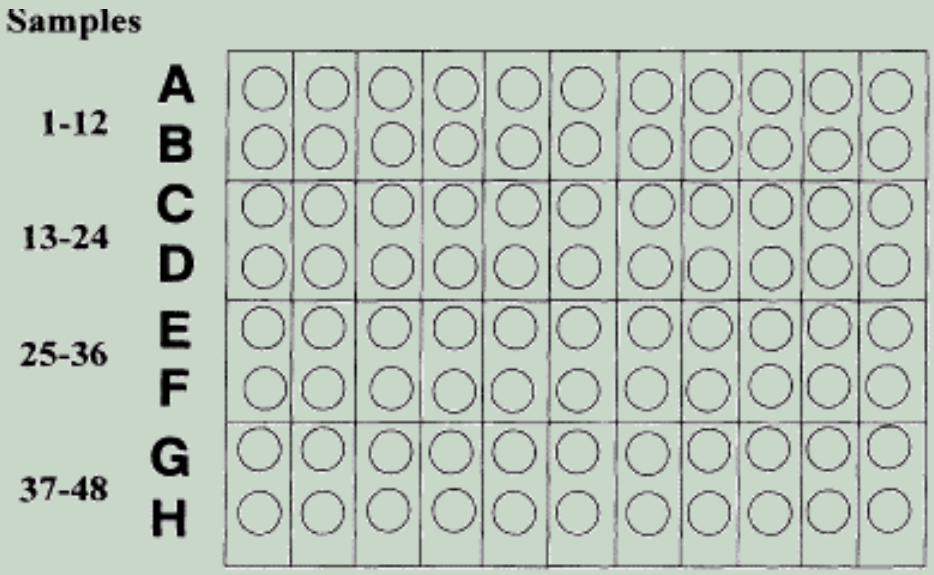


Fig. 16.
Micronics system for dilution of samples, showing the order of samples.

Table 6
Plate Data for Example Assay[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.21	1.09	0.78	0.32	0.12	0.66	0.65	0.17	0.67	0.34	1.34	1.11
B	1.19	1.03	0.69	0.31	0.16	0.64	0.62	0.16	0.64	0.37	1.28	1.17
C	1.00	0.23	0.45	0.56	0.78	0.13	0.19	0.45	0.56	0.78	1.00	0.56
D	0.97	0.27	0.49	0.54	0.72	0.16	0.20	0.44	0.53	0.75	1.01	0.55
E	0.13	0.14	0.18	0.09	0.07	0.12	0.14	0.09	0.08	0.12	0.16	0.14
F	0.12	0.13	0.16	0.09	0.08	0.11	0.13	0.10	0.09	0.11	0.13	0.15
G	0.15	0.18	0.13	0.14	0.10	0.15	0.13	0.12	0.13	0.13	0.12	0.08
H	0.13	0.16	0.13	0.15	0.12	0.13	0.12	0.14	0.15	0.12	0.11	0.09

[a]Duplicates of samples made are A1, B1; A2, B2; and so on. Suspect positive sera (24) are in rows A¨CD. Negative (prebleed sera) are in rows E¨CH.

ODs for both test wells of the same sample. Calculate the mean (average result) of the OD from both wells if the difference is not large. Variation in results are discussed later. Take the mean value to two decimal places.

4.5.1¡[a]
Mean and Standard Deviation from Mean of Negative Serum Data

Take all the means of the negative sera and calculate the mean and standard deviation (*SD*) of the negative population using the calculator. (Note: Instruction should be given on the use of a calculator.) Noncourse users of the manual

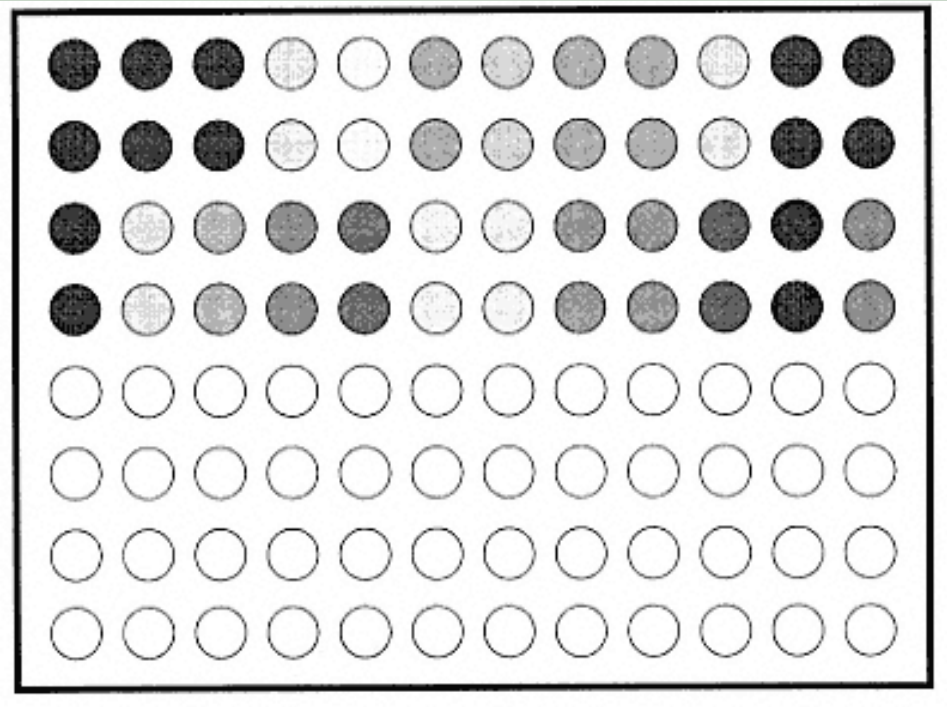


Fig. 17.
Diagrammatic representation of a stopped plate.

should obtain a calculator and follow operating instructions to calculate the same parameters.

4.5.2¡[a]
Frequency Plots of Negative Serum OD Results

Plot the results for the negative sera as shown in **Fig. 18**. These relate the number of samples giving a particular OD.

A frequency distribution is obtained so that the distribution of negative results is obtained. Create a table of OD intervals and score the numbers of sera falling into the intervals. Add up the score, and plot this against the intervals. The mean of the data for the negative sera and the *SD* of the data can be found using a calculator. Thus, the population mean of a limited (in this case) negative population is found. If the population of negative ELISA readings is distributed normally (normal distribution statistics), then the upper limits of negativity can be ascribed with defined confidence limits depending on the number of *SD*s from the mean that is used.

The mean value in this case is 0.125, and the *SD* is 0.026. Thus, if we select 3X *SD* above this mean value (= 0.084) and add this to the mean value (= 0.209), any values equal to or above this value are unlikely to be part of the measured negative population as defined by the fact that only approx 0.1% of negative sera examined tended toward this value. Limits using 2X the *SD* above the measured negative population mean reduce confidence in the results for ascribing positivity (increase possible sensitivity but reduce specificity).

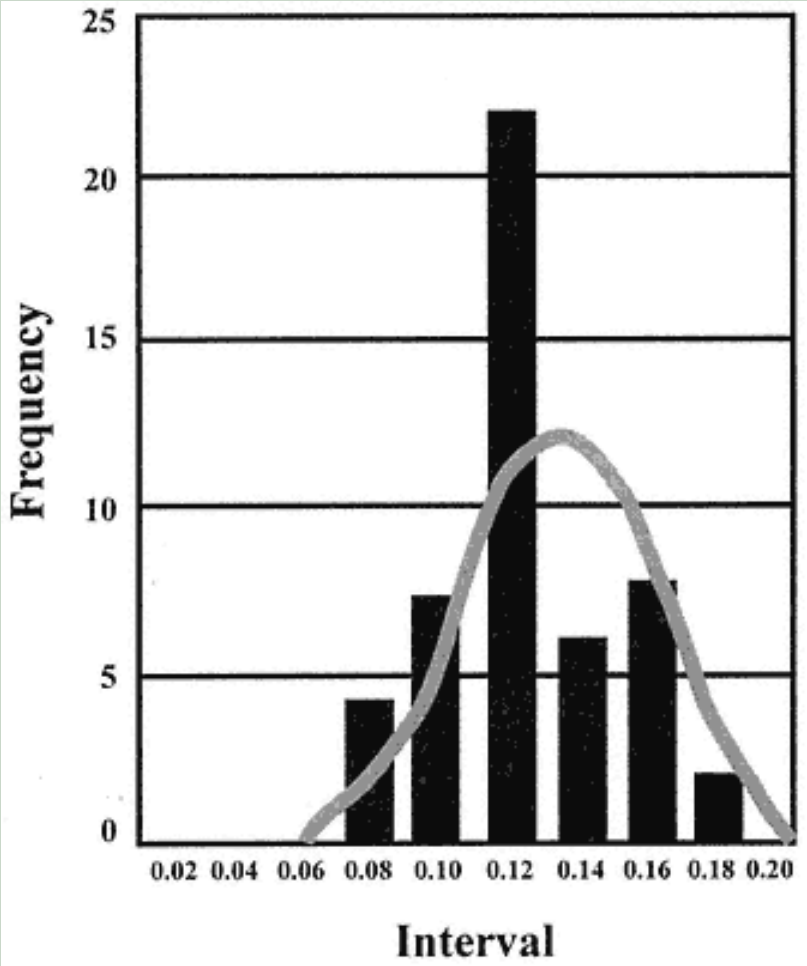


Fig. 18.
Frequency plot relating number of sera giving particular OD values. The gray curve is the normal distribution plot.

In practice, such distributions are skewed to the right-hand side, so that a tailing of results is seen at the higher ODs (*see* **Fig. 19**). To establish an OD reading that reflects the upper limit of negativity (since all negative sera have been studied), a statistical evaluation of the distribution is required. In general, since the distribution is skewed, a value of two times the mean OD for all the negative sera has been found to determine the upper limit of negativity (which corresponds to the lower limit of positivity) with a 95% confidence limit. Thus, we are 95% certain that a sample giving an OD value of equal to or greater than the value at two times the mean of the negative population OD results is positive. In practice, a confidence limit of 99% can be ascribed to results equal or greater than two times the population mean.

4.5.3$_{i}^{a}$
Problems

When examining negative populations, we are assuming such sero-negativity using one or more factors, such as other serological test results, knowledge of the clinical history of the animals, and epidemiological factors. Thus, it may

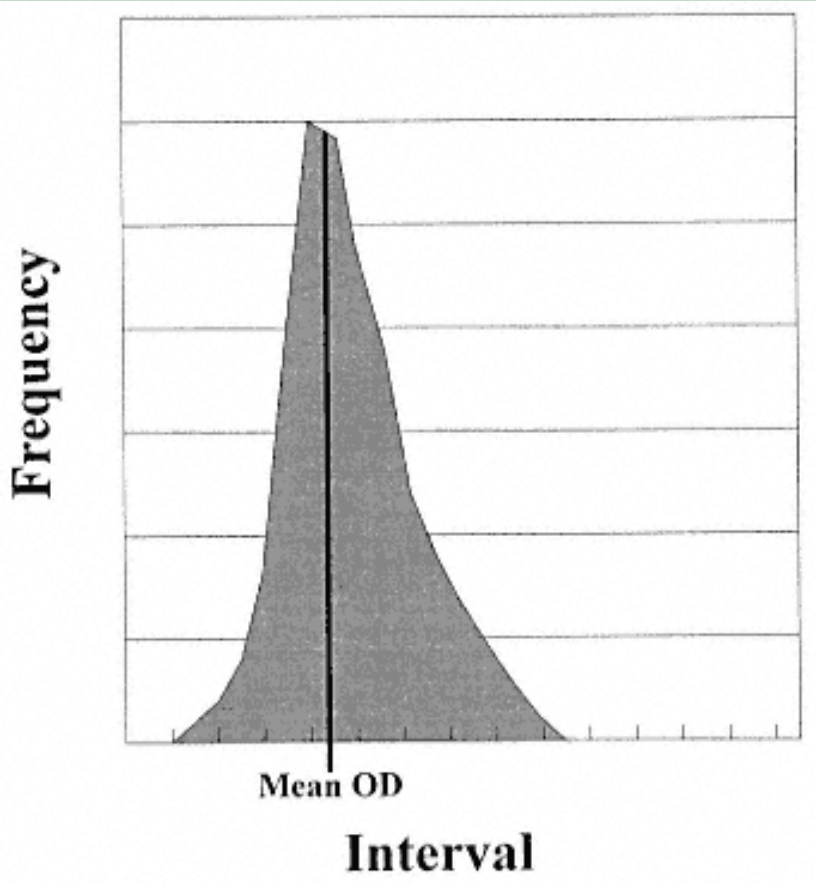


Fig. 19.
Distribution skewed to the right.

be easy to identify seronegative animals in countries where a particular disease has never been recorded. However, this may not be true for countries that have endemic disease or where vaccination campaigns have been mounted at various times (with variable amounts of antibody against specific disease agents being elicited). In such conditions, the experimenter might make the best assessment of likely negative animals. In this case, after plotting the frequency curves, one of several distributions might be obtained:

1. **Figure 19**: One peak at the low OD end of distribution. The population is probably negative with all sera showing low OD.

2. **Figure 20**: Two peaks fairly well separated at the low OD and at the higher OD end of distribution. Distinct populations of animals that are positive and negative may reflect recent infection or vaccination.

3. **Figure 21**: Twopeaks merged. There is no clear distinction between populations (the high and low ODs greatly overlap). These curves also illustrate what the picture looks like after sampling total populations containing positive and negative animals. Thus, for the example in **Fig.19**, there is no problem in ascribing an upper limit for negativity. Obviously the sera show the type of result expected of

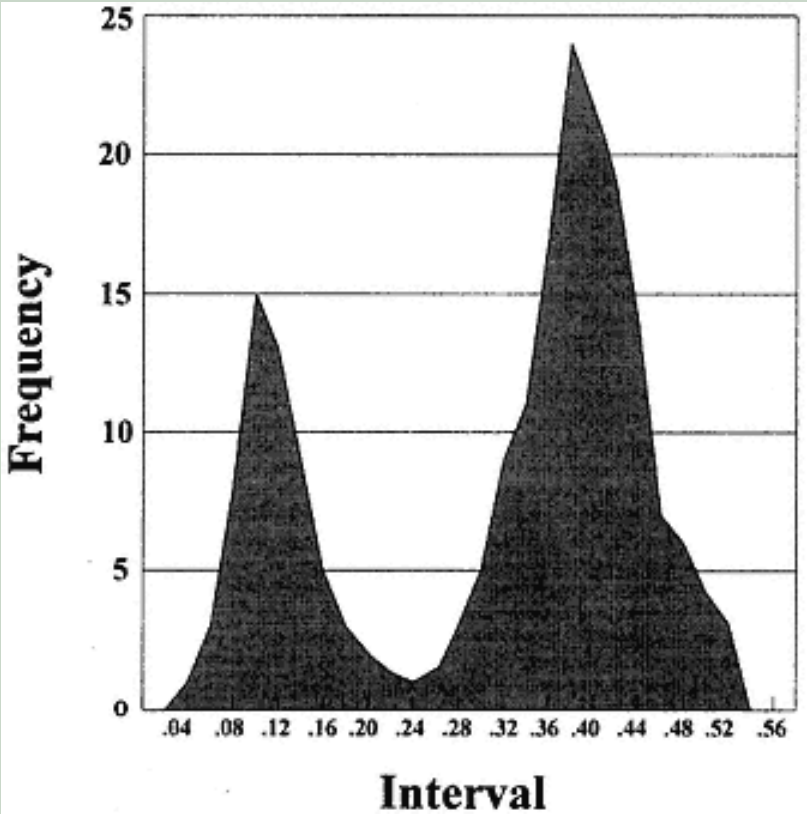


Fig. 20.
Frequency plot of OD results from analysis of sera,
with two peaks representing distinct populations.

a totally negative population. However, in **Fig. 20** we have a percentage of high OD results. These probably represent positive animals, and we can use the clear difference in the two distributions obtained to suggest strongly that the low OD results represent a negative population.

The distribution in **Fig. 21** demonstrates a situation in which there is a heterogeneous population of animals with respect to their levels of antibodies. Thus, it is probable that the low OD range (which is shown by the situations in **Figs. 19** and **20**) represents negative animals. The merging of high and low results with high numbers of animals probably indicates that antibody levels have been reduced in the population after a past infection. Such a population can be studied using a defined negative population (maybe from another source), but the negative distribution cannot be assessed from study of this type of distribution alone. Hence, the experimenter might obtain serum samples from relevant species from countries where the disease they wish to study is absent. The negative value(s) obtained from such sera may not always be the same as that of the indigenous stock, but for most exercises will suffice.

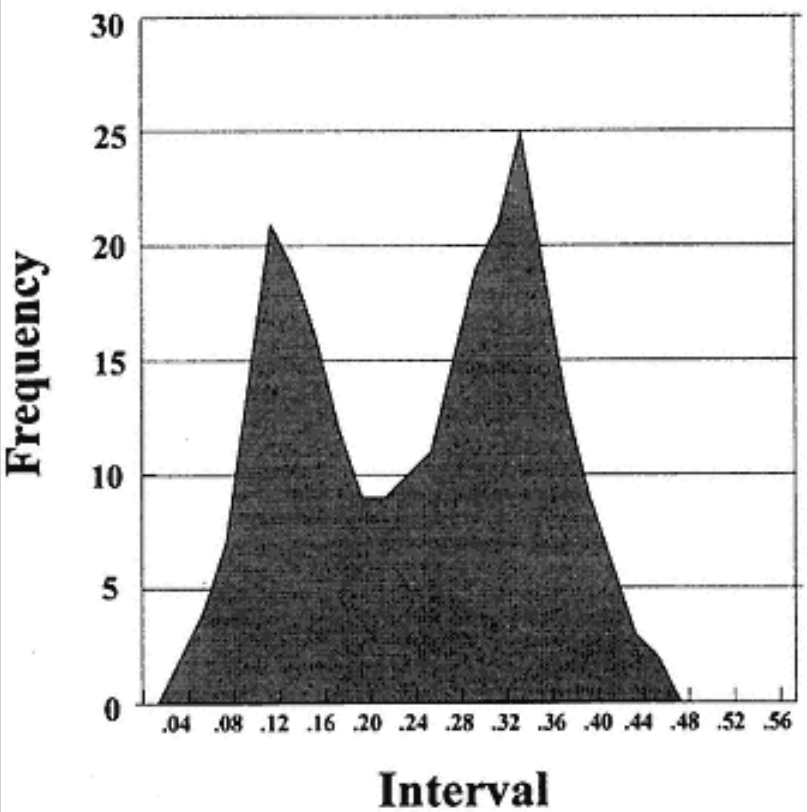


Fig. 21.
Frequency plot of OD results from analysis of sera.
No clear distinction is seen between populations.

4.6¡ª
Establishment of Control Negative Sera

It is possible to use a limited number of negative sera to act as controls in any assay of antibodies. This can only be realistically done if a distribution of many negative serum OD levels has been made (approx 100 minimum). Thus, a serum typifying the mean of the population of negative sera can be used. If this is included as a single dilution in the indirect ELISA, the OD values obtained may represent the mean value for the negative serum population. The upper limit of negativity can then be calculated by multiplying this value by 2 (since we know that this is a relevant value after studying the distribution). This approach is relevant when multichannel spectrophotometers are be used to read the color.

If assessment by eye is being used, control negative sera giving OD levels at the upper limit of negativity (around two times the mean) might be used. Color development in such assays should then be allowed until color is just detectable in the negative controls. The test should then be stopped. Therefore, any wells showing color more intense than the control wells would be positive for antibody.

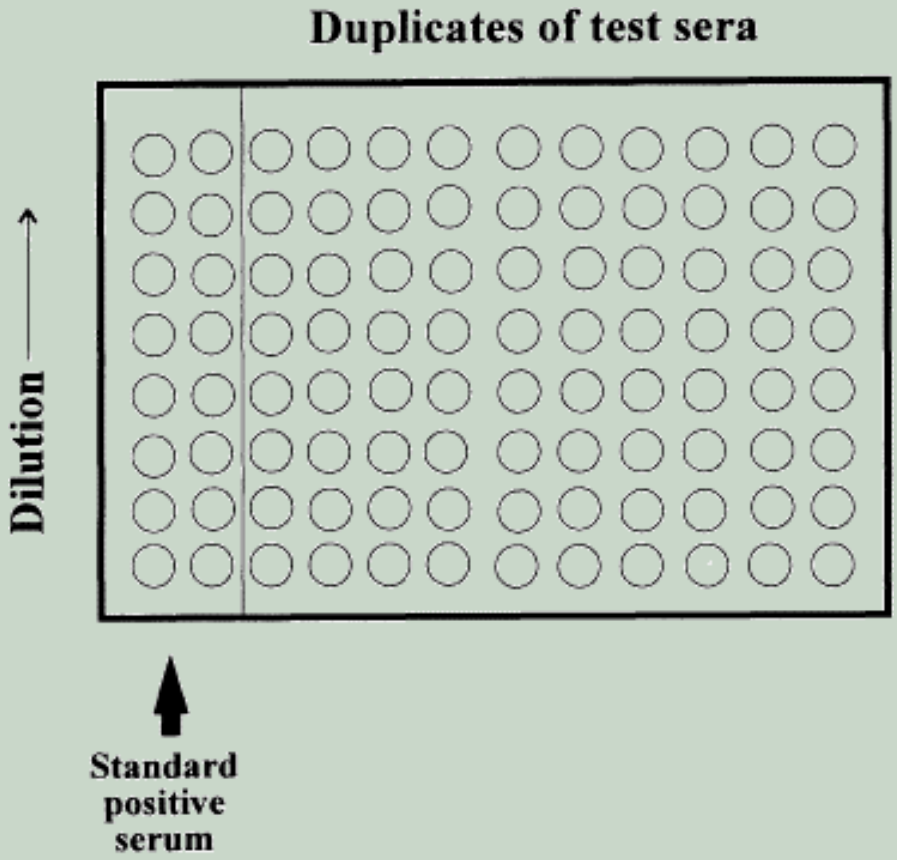


Fig. 22.
Plate layout for comparison of test sera with standard serum titration.

4.6.1¡[a]
Data Analysis

You have calculated the mean OD of the negative population as well as the *SD* from the mean of the population. Now find a serum that characterizes the mean of the population. As well as one that characterizes the upper limit (two times the mean) of the population.

4.7¡[a]
Relating Single Test Dilutions to Standard Positive Antiserum Curves

If a characterized antiserum is available, it may be used as a standard in the indirect ELISA. In this case, a full-dilution range of the serum is made and titrated under identical conditions to the single dilutions of the test sera. A typical plate format is shown in **Fig. 22**. At the end of the test, a standard curve relating the OD to the dilution of standard positive serum is constructed. The titers of the test samples can then be read from this curve so that a relative assessment of activity is obtained. This is demonstrated in **Fig. 23**. The standard serum may be given an arbitrary activity (units) so that results may be expressed in those units. Such control positive sera may be useful when standardization between laboratories is required.

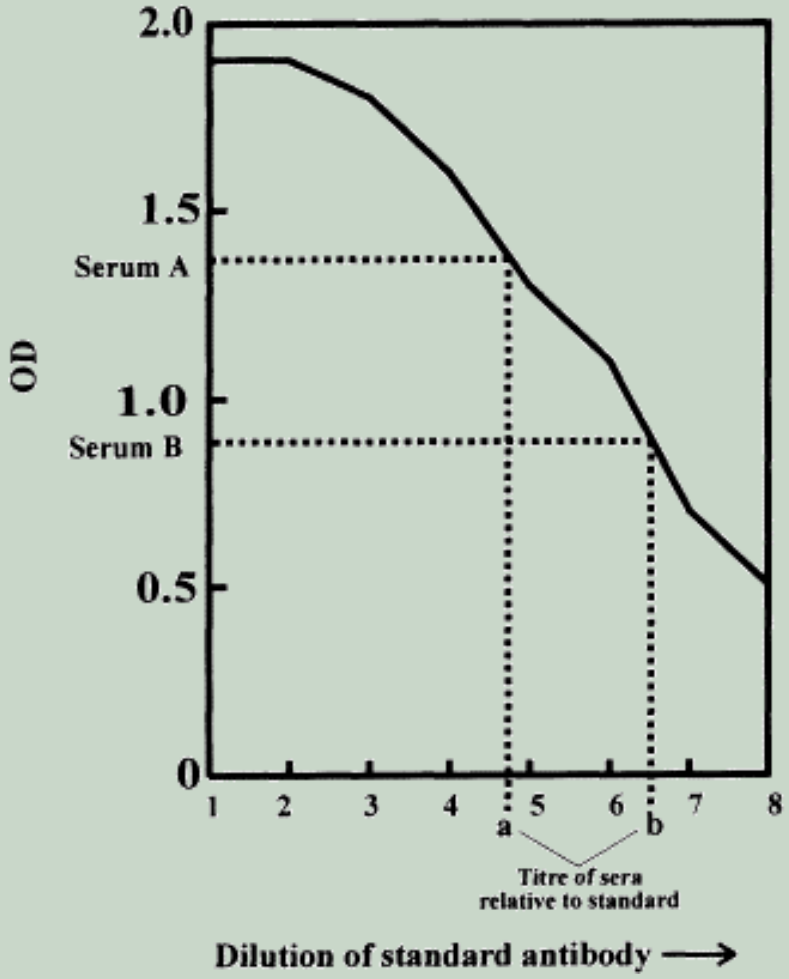


Fig. 23.
Use of standard serum titration curve to assess titers of test sera. OD values obtained from serum **A** and **B** are read from the titration curve of the standard serum (**a** and **b**).

4.8¡ª
Complications of Actual Disease

Studies on many systems have shown that false positive results are obtained in a low percentage of animals from a guaranteed noninfected population. It is difficult to determine why such reactions occur, but several reasons have been proposed, such as contamination of the serum with bacteria and fungi, dietary factors, and heating of the sera. The percentage can be of the order 1¨C2%, and they are easily read as very high ODs as compared to the majority of samples giving the typical negative distributions already discussed. This nonspecificity may be eliminated, e.g., by using different antigenic preparations. However, the number of likely false positive results should be taken into account when diagnosing disease on a herd basis. Thus, if we know that 2 animals in 100 show this response and we find that 20 animals in 100 show high responses, it

is likely that disease is diagnosed. However, if only one to three animals "positive," this finding could be owing to the identified nonspecific reactions.

5¡ª
Use of Antibodies on Solid Phase in Capture ELISA

This section examines sandwich ELISAs for measurement of antigens and antibodies.

5.1¡ª
Use of Capture ELISA to Detect and Titrate Antigen

5.1.1¡ª
Learning Principles

1. Optimizing the amount of capture antibody attached to the wells.

2. Optimizing amount of detecting antibody.

5.1.2¡ª
Reaction Scheme

$$\text{I-AB}_{X}\ \underset{W}{+}\ \text{Ag}\ \underset{W}{+}\ \text{Ab}_{Y}\ \underset{W}{+}\ \text{Anti-Ab*E}\ \underset{W}{+}\ \text{S–READ}$$

—I	= microplate wells (solid phase)
—AB_X	= capture antibody (species X) specific for Ag
—Ag	= antigen
—Ab_Y	= detecting antibody (species Y) specific for Ag
—AntiAb*E	= antispecies Y antibody linked to enzyme
—S	= Substrate/color detection system
—W	= washing step
—+	= addition and incubation of reactants

In this exercise, the capture antibody, the detecting antibody and the conjugate are used at optimal dilutions.

5.1.3¡ª
Basis of Assay

Antigens can do the following:

1. Attach poorly to plastics.

2. Be present in low quantity, e.g., in tissue culture fluids.

3. Be present as a low percentage of total protein in a "dirty" sample (e.g., in feces or epithelium samples).

4. Be unavailable for purification and concentration, since they are antigenically unstable when separated from other serum components.

In these cases, the indirect assay is unsuitable for handling the antigen, because it relies on the attachment of the antigen directly to the wells. The capture assay overcomes many of these problems, since the antigen is attached to the wells via specific antibodies. The test relies on the availability of two antisera from different species, so that the conjugate reacting with the second (detecting) antibody does not react with the capture antibody. It is also essential that the antigen have at least two antigenic sites so that antibody may bind

to allow the sandwich (the antigen being the filling). Thus, when small antigens are being used (e.g., peptides), they may not react in such assays owing to their limited antigenic targets. The test offers an advantage over the indirect assay in the quantification of antigens, since direct attachment of proteins to wells is often nonlinear (i.e., is not proportional to the amount of protein in the sample). This is exaggerated if contaminating proteins are present with the antigen (e.g., serum components), because these compete for plastic sites in a nonlinear way. Because the capture antibody is specific, it binds antigen proportionally over a large range of protein concentrations. Thus, such assays give reproducible results when quantification is required. The assay is practically identical to the indirect assay except that an extra step (the capture antibody) is added. Thus, we have three parameters to optimize:

1. The capture antibody.

2. The detecting antibody.

3. The conjugate against the detecting antibody.

5.1.4$_i$[a]
Materials

1. Capture antibody (AB_X): = sheep anti-guinea pig Ig (an IgG preparation at approx 5 mg/mL in PBS).

2. Ag: = guinea pig Ig at 1 mg/mL or as prepared by the worker.

3. Detection antibody (Ab_Y): rabbit anti-guinea pig Ig serum (Ab).

4. Anti-Ab_Y*E: sheep anti-rabbit conjugate (HRP).

5. Microplates.

6. Multichannel, single-channel, 10-mL and 1-mL pipets.

7. Carbonate/bicarbonate buffer, pH 9.6.

8. PBS 1% BSA/Tween-20 (0.05%).

9. Solution of OPD in citrate buffer.

10. Hydrogen peroxide 30% (w/v).

11. Washing solution.

12. 1 *M* sulfuric acid in water.

13. Paper towels.

14. Small-volume bottles.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

5.1.5¡ª
Methods

Since you are now familiar with the indirect assay, the steps in the optimization of the capture ELISA should be straightforward. The first essential step is to determine the amount of capture antibody to be attached to the wells. We have two situations in the laboratory depending on the availability of specific reagents. We can use capture antibody as an IgG preparation, or if sufficiently

high-titer serum is available against the antigen, as whole serum. The easiest way to avoid serum effects is to prepare the IgG. Salt fractionation is usually adequate and does not affect antibody activity. Care must be taken to assess the effect of chemical preparation of IgG from mAbs.

5.1.5.1¡ª
Use of IG Preparations

The advantage of using IG preparations is that the weight of Ig can be calculated, so that a defined quantity of reagent may be added to the plate. In general, a maximum amount of protein will attach to the wells. Because we know that a maximum possible binding of subsequently added antigen may be expected, we may add the Ig at "saturating" level. Thus, a good estimation of the activity of a capture antibody (the particular dilution/concentration to be used) can be assessed. As an example, if capture antibody is added at 5 µg/mL in 50-µL amounts, this represents the saturating amount of antibody protein that will attach to the wells. The ultimate activity will depend on the concentration of the specific Ig (against the Ag) in the capture antibody and the spacing of the capture molecules.

Some assays perform better at lower than saturating levels of capture antibody so that a titration is needed. Generally, the amount of specific antibodies in a serum as a percentage of the total protein is about 1¨C5%. The preparation of Ig eliminates a large percentage of the serum proteins not involved in the assay (e.g., serum albumins). Therefore, the activity of the Ig protein (relative increase in the IgG fraction that will attach to each well) is effectively increased. In other words, there is a greater proportion of IgG sticking to the wells to act as trapping antibody if Ig preparations are used.

5.1.5.2¡ª
Use of Whole Serum

Dilutions of untreated serum can be used. However, as already indicated above, the proportion of specific IgG is low, and other serum proteins attach in a competitive manner. One cannot assume that putting on a low dilution of serum will give a good level of capture antibody. The most usual event is that a bell-shaped curve of capture ability is obtained, with little activity at high concentrations of serum and a rise in activity as the serum is diluted. In general, serum has to be diluted to around 1/500 to 1/2000. Thus, we must have fairly high titers to be able to use whole serum. **Figure 24** demonstrates the activity of whole and IgG capture antibodies as they are diluted down, to illustrate the bell-shaped curve.

5.1.5.3¡ª
Titration of Capture Antibody Using IgG

1. Dilute the sheep anti-guinea pig IgG preparation to 5 µg/mL in carbonate buffer. Add 50 µL to each well on the plate except column 12.

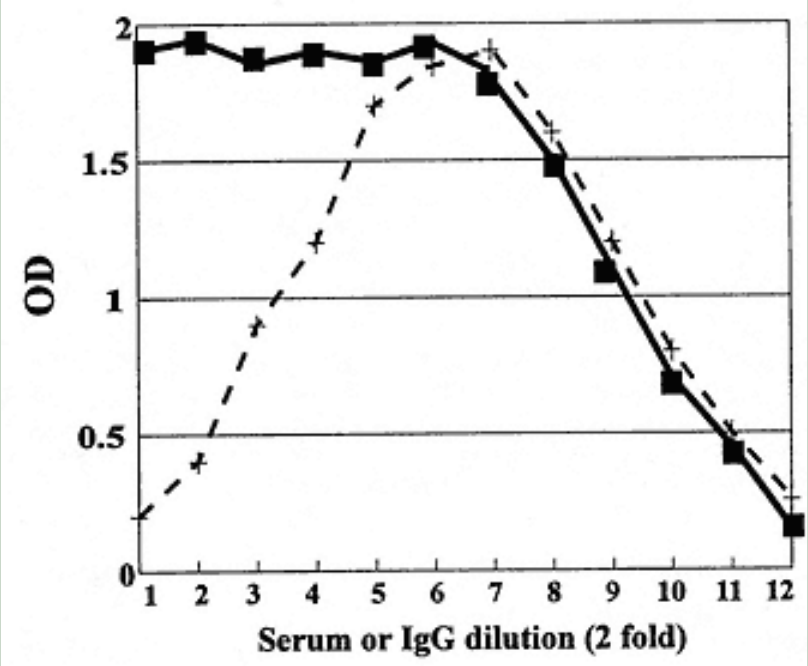


Fig. 24.
Comparison of capture of IgG using whole serum or IgG as capture antibody. □, IgG; +, serum.

2. Add adsorption buffer alone to row 12. Incubate at 37¡ãC for 2 h or overnight if more convenient (remember to put lids on the plates).

3. Wash the plates. (From now on we are performing a procedure similar to the indirect ELISA.)

4. Place the microtiter plate with well A1 at the top left-hand corner. Add 50 µL of blocking buffer to each well.

5. Make a dilution range of guinea pig IgG (the antigen of interest) from 5 µg/mL from column 1 (8 wells) to column 11 in blocking buffer. Thus, add 50 µL of guinea pig IgG at 10 µg/mL to the first row 1 using a multichannel pipet. Mix and double dilute across the plate (you should be competent at this now). Remember to discard the last 50 µL in the tips so that each well should only contain 50 µL of fluid (check!).

6. Incubate the plates at room temperature or at 37¡ãC for 1 h.

7. Wash the plates.

8. Add 50 µL of blocking buffer to each well.

9. Dilute rabbit anti-guinea pig serum to 1/100 in blocking buffer (make up 1.0 mL: add 10 µL of undiluted serum to 1.0 mL of buffer). Mix. Add 50 µL of the dilution to row A using a single-channel pipet. Dilute across rows A¨CH using a multichannel pipet. We now have a twofold dilution range from 1/200 (row A) to 1/25,600 (row H).

10. Incubate the plate at room temperature or at 37¡ãC for 1 h.

11. Wash the plate.

Table 7
CBT of Guinea Pig IgG vs Rabbit Anti-Guinea Pig IgG¡ª
Constant Capture Antibody, Constant Conjugate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.67	1.67	1.68	1.65	1.54	1.34	1.09	0.89	0.67	0.54	0.34	0.23
B	1.68	1.68	1.65	1.56	1.51	1.31	1.04	0.84	0.59	0.51	0.32	0.17
C	1.56	1.54	1.52	1.43	1.34	1.23	0.99	0.76	0.52	0.43	0.23	0.09
D	1.12	1.09	1.00	0.94	0.87	0.78	0.67	0.56	0.45	0.34	0.19	0.08
E	1.00	0.97	0.89	0.78	0.67	0.56	0.43	0.34	0.23	0.21	0.17	0.08
F	0.78	0.74	0.71	0.56	0.51	0.43	0.32	0.21	0.19	0.14	0.09	0.10
G	0.54	0.51	0.51	0.42	0.36	0.32	0.21	0.16	0.14	0.09	0.08	0.09
H	0.34	0.34	0.32	0.21	0.18	0.15	0.16	0.09	0.08	0.07	0.08	0.09

Guinea pig IgG diluted 1¨C11; anti-guinea pig IgG diluted A¨CH.

12. Make up an optimum dilution of sheep anti-rabbit conjugate. Add 50 µL to each well using multichannel pipets.

13. Incubate for the standard time as used in the optimization of conjugate (1 h at 37¡ãC or room temperature).

14. Wash the plate.

15. Add substrate and stop the color development at 10 min.

5.1.5.3.1¡ª
Data

Essentially we have made a CBT of the antigen against the detecting antibody (as in the indirect assay). Thus, we have assumed that the capture antibody, put on the plate as an IgG, is at maximal reactivity. The results are therefore similar to those obtained in the indirect assay, and can be treated similarly. Each row A¨CH had an identical dilution series of the antigen (guinea pig IgG) being captured by the same amount of antibody, thus the same amount of guinea pig IgG should be present attached via antibody to the wells 1¨C11. The rabbit antibody against the antigen has been titrated at different dilutions so that we can examine which dilution shows the best detection of IgG in rows A¨CH. The use of 5 µg/mL of capture IgG was taken as that which from experience saturates the plastic sites available on the plate wells. Once the antigen and detecting serum optima have been established using this level of capture IgG, they can be altered to examine the effect on the assay. **Table 7** gives the spectrophotometric plate readings. A representation of the plate is also shown in **Fig. 25**.

5.1.5.3.2¡ª
Plots of Data

Figure 26 shows the data plotting the OD results obtained at the different antigen dilutions for each dilution of rabbit anti-guinea pig serum.

5.1.5.3.3¡ª
Assessment of Data

Column 12 contained no antigen (guinea pig IgG), and therefore examination of the color here gives a measure of the bind-

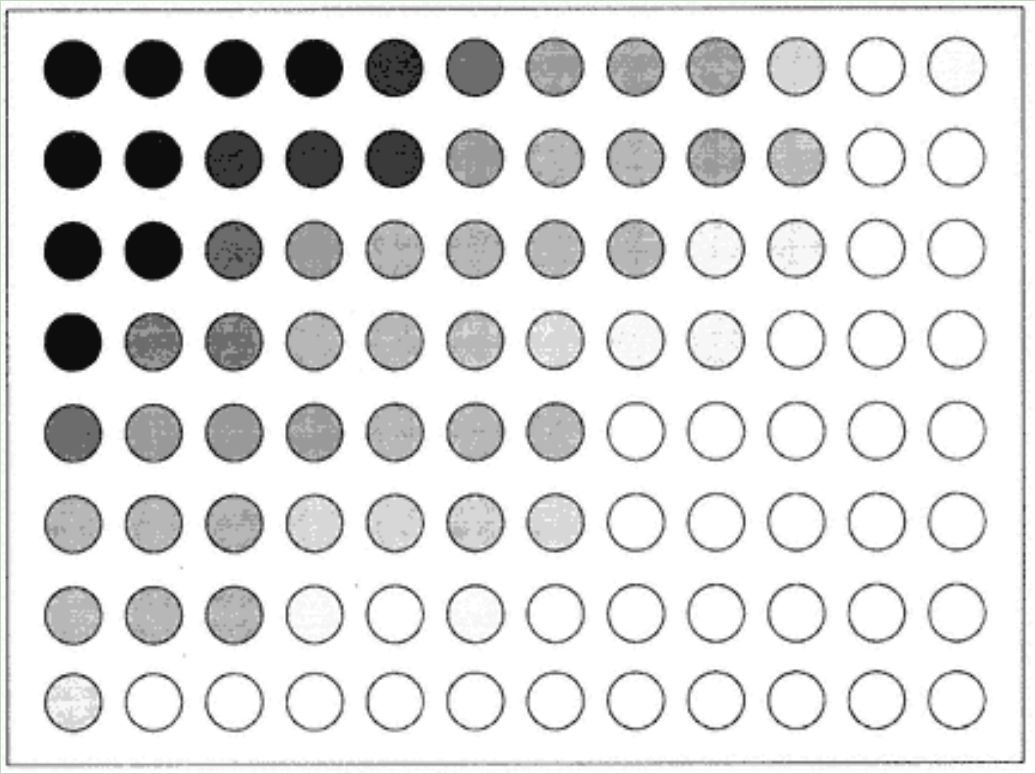


Fig. 25.
Diagrammatic representation of the plate showing results of CBT.

ing of the detection system to the plate or capture antibody. Thus, rows A and B show higher levels of color than the other rows. The value around 0.09 appears to be the plate background expected in the presence of the same dilution of conjugate. Thus, the end point detection of IgG is affected in rows A and B. Examination of the plateau heights indicates that the trapping system is saturated in columns 1¨C4, since we obtain similar OD values; as an example, we have around 1.67 for the first four wells using the 1/200 detecting antibody, although the actual plateau height value reduces on dilution of the detecting rabbit anti-guinea pig serum. Examination of **Fig. 26,** which relates the curves obtained for the detection of trapped Ig for different dilutions of the rabbit anti-guinea pig Ig, easily shows that the last dilution giving an optimal titration is in row C. After this dilution, the effect of is to reduce more markedly the OD in the plateau region (where the trapped Ig is in excess) and also to affect the sensitivity of detection of the Ig at higher dilutions, as indicated by a reduction in the end points where the test background is the same as the plate background.

5.1.5.4¡ª
Retitration of Capture IgG

The optimal dilutions chosen will depend on how the test is to be used. If an antigen is to be detected, then we might require high detection limits in the system, so that we can use a dilution of detecting antiserum to maximize this.

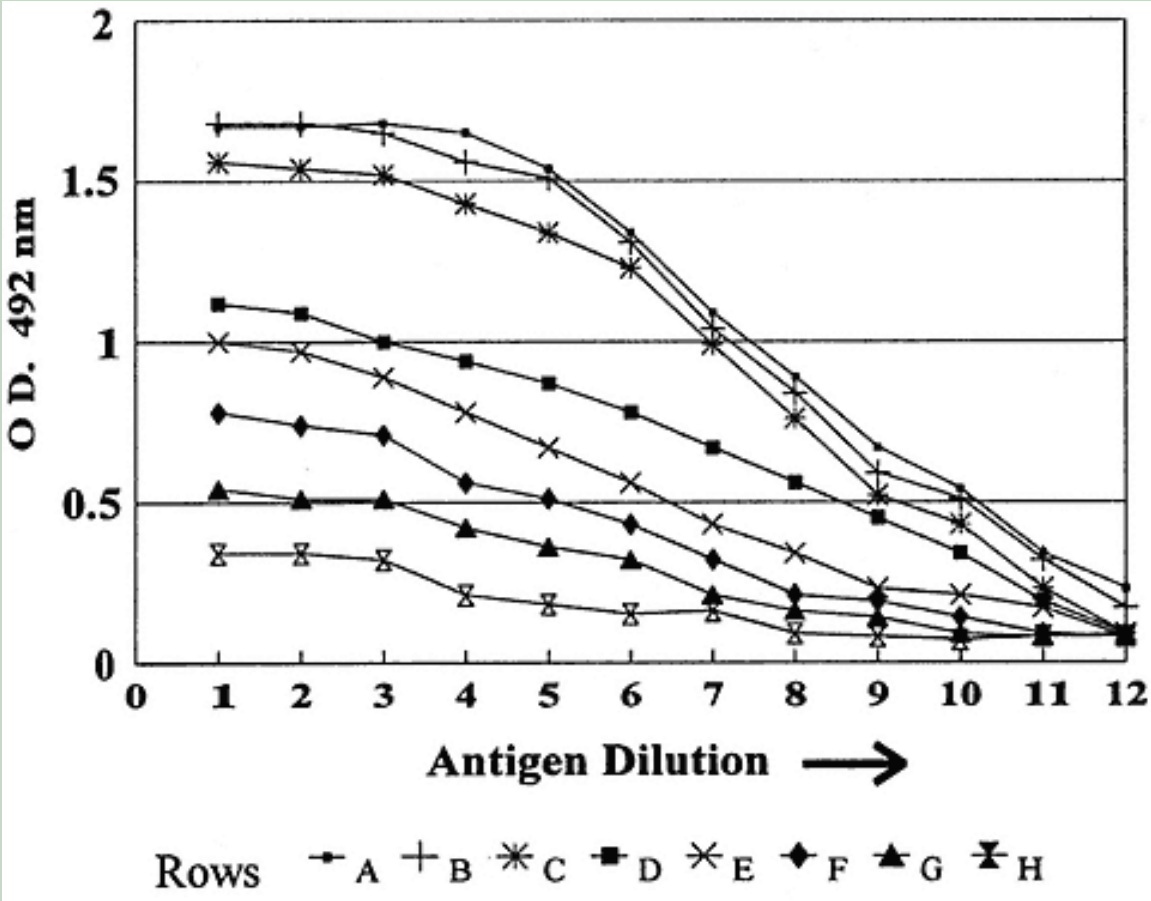


Fig. 26.
Titration of guinea pig IgG using constant capture conditions. Each line represents titration of the same dilution range of IgG using a different concentration of rabbit anti-guinea pig IgG. The conjugate dilution is constant. Data are from **Table 7**.

We will see later that capture assays are used in competitive situations in which the amount of antigen to be captured needs to be reduced so that a variation in reagent concentrations for that application may be necessary.

The established optima for the antigen and detecting serum can be reassessed using lower concentratons of capture IgG. Thus, a full CBT can be performed using 2.5, 1.25, and 0.625 µg/mL of the capture IgG. However, a simpler procedure is to coat plates with a dilution range of the capture IgG and use constant antigen, detecting antiserum and pre-titrated conjugate dilutions. **Table 8** gives the results of a typical titration of this sort. Here plates were coated with capture anti-IgG at 5 µg/mL, in a twofold range from row A to H, only columns 1 to 4; thus, quadruplicate samples were being examined. After incubation and washing, antigen (guinea pig IgG) at 0.625 µg/mL was added in blocking buffer. Following incubation and washing, the detecting antibody (rabbit anti-guinea pig IgG) was added at 1/400 diluted in blocking buffer. After incubation and washing, the anti-rabbit conjugate was added at the dilution used to optimize the reagents.

Table 8 shows that the capture IgG produces similar results at 5 and 2.5 µg/mL; thus, the latter dilution can be used in an assay to capture antigen.

Table 8
Titration of Capture IgG Against Optimal Antigen, Detecting Antibody, and Conjugate

Capture IgG concentration (µg/mL)		1	2	3	4	Mean
5.0	A	1.50	1.48	1.49	1.51	1.50
2.5	B	1.49	1.47	1.51	1.46	1.48
1.25	C	1.25	1.21	1.24	1.27	1.24
0.63	D	0.95	0.94	0.96	0.93	0.95
0.32	E	0.67	0.69	0.69	0.66	0.68
0.16	F	0.36	0.37	0.40	0.37	0.38
0.08	G	0.14	0.12	0.15	0.12	0.13
0.08	H	0.05	0.04	0.04	0.03	0.04

Lower concentrations produce lower OD values, indicating that not all the available antigen is being captured. The reduced ability to bind antigen (when in excess) is accompanied by a decreased ability to detect small amounts of antigen (the minimum detection limit is reduced).

Similar titrations of the other reagents can be made in which only one is diluted and the others are kept constant. Thus, in the previous case we know we have three conditions optimized under experimental conditions with control sera and antigen. The capture IgG can be used at 2.5 µg/mL, the antigen can be used at 0.625 µg/mL, and the rabbit detector at 1/400, with the anti-rabbit conjugate at a constant dilution as assessed originally against the relevant IgG attached to a microplate.

We may wish to reassess the conjugate dilution under standardized conditions. Thus, using the capture IgG, antigen, and detecting antiserum optima just given, replicate wells can be used to titrate different dilutions of conjugate. An example is given in **Table 9**. Here, a dilution of 2¨C400 of conjugate gives similar results. Effectively, a dilution of 1/800 gives "optimal" results (OD value around 1.45), for an assay.

5.1.5.5¡ª
Titration of Capture Antibody When Used as Whole Serum

As already stated, whole serum can be used to coat plates and act as a capture reagent. However, this is not recommended because we cannot measure the protein Ig because it is contaminated with "blocking" serum proteins that compete for plastic binding sites preferentially over the IgG. The simplest method is to perform a CBT relating dilutions of capture serum to dilutions of detecting antibody and keep the antigen constant.

Table 9
Assessment of Constant Capture System with Different Dilutions of Conjugate

Conjugate dilution		1	2	3	4	Mean
200	A	1.95	1.87	1.87	1.95	1.92
400	B	1.84	1.82	1.84	1.82	1.83
800	C	1.45	1.41	1.44	1.47	1.44
1600	D	0.95	0.94	0.96	0.93	0.95
3200	E	0.77	0.79	0.79	0.76	0.78
6400	F	0.36	0.37	0.40	0.37	0.38
12,800	G	0.15	0.14	0.15	0.14	0.15
None	H	0.05	0.04	0.04	0.03	0.04

The diagram below illustrates this:

5.1.5.5.1¡ª
Reaction Scheme

$$\text{I-Ab}_{W} + \text{Ag}_{W} + \text{AB}_{W} + \text{Anti-AB*E}_{W} + \text{S–READ}$$

- I-Ab = dilution range of trapping antibody
- Ag = constant dilution of antigen (high concentration)
- AB = dilution range of detecting antibody
- Anti-AB*E = conjugated anti species antibody
- S = substrate/chromophore
- READ = OD

This assay is not described in detail. However, a description of the test is given with relevant points highlighted. You should now have enough experience to be able to set up the exact practical details with help from the exercise titrating IgG as capture antibody.

5.1.5.6¡ª
Method

1. Dilute the serum containing capture antibody on plates in carbonate/bicarbonate buffer (begin at 1/100, twofold dilutions). Incubate and then wash the plates.

2. Add constant (excess) antigen diluted in blocking buffer. (It is difficult to specify here what excess might be for specific systems, for example, an undiluted tissue culture sample containing virus might be expected to have a high concentration of antigen.) Incubate for 1 h at standard conditions.

3. Wash and add dilutions of detecting antibodies in blocking buffer to obtain a CBT (dilute in opposite direction to the capture serum). Incubate and then wash the plates.

Table 10
Dilutions of Capture Serum 1/100 to 1/51,200

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.67	0.96	1.34	1.35	1.32	1.11	0.98	0.76	0.56	0.45	0.23	0.12
B	0.66	0.99	1.42	1.37	1.29	1.09	0.89	0.75	0.54	0.36	0.22	0.11
C	0.65	0.98	1.36	1.34	1.15	0.99	0.87	0.72	0.52	0.31	0.17	0.09
D	0.56	0.88	1.23	1.19	1.01	0.88	0.74	0.65	0.43	0.26	0.14	0.09
E	0.45	0.67	1.00	1.09	0.98	0.78	0.56	0.45	0.34	0.21	0.12	0.07
F	0.23	0.43	0.78	0.76	0.56	0.45	0.40	0.33	0.23	0.16	0.12	0.08
G	0.15	0.23	0.34	0.35	0.21	0.15	0.16	0.09	0.08	0.07	0.09	0.09
H	0.15	0.19	0.18	0.17	0.16	0.10	0.09	0.08	0.09	0.07	0.07	0.09

Detecting serum deluted A¨CH 1/200 2 fold.

4. Add optimal conjugate, incubate, and wash.

5. Add substrate and then stop at 10 min.

5.1.5.6.1¡ª
Data

Table 10 presents typical results. Data are plotted in **Fig. 27**. Rows A¨CH contain dilution ranges of the capture serum 1/100 to 1/51,200 and column 1 = 1/100, and column 12 = 1/51,200. Row A received the detecting antiserum at 1/200, row B at 1/400 and so on to row H at 1/12,800.

5.1.5.6.2¡ª
Conclusion

1. The optimal dilution of capture serum is around row 5 (last row showing maximum OD).

2. The optimal dilution of detecting second antibody is around row C¨CD (last showing maximal titration curve of antigen).

3. Bell-shaped curves are obtained where low dilutions of capture serum give low OD values (columns 1 and 2).

5.1.5.7¡ª
Notes on Use of Capture ELISAs to Detect Antigens

Once the optimal conditions have been established, the capture assays can be used in several ways.

5.1.5.7.1¡ª
Diagnosis of Specific Disease Agents

$$\text{I-Ab}_{W} + \text{Ag}_{W} + \text{AB}_{W} + \text{Anti-AB*E}_{W} + \text{S–READ}$$

Here, a sample possibly containing antigen is added to a capture system (microtiter plate wells coated with an antiserum against a specific disease). Any bound antigen is then detected by another antibody from a different species. Such assays are important in serotyping in which the second antibody

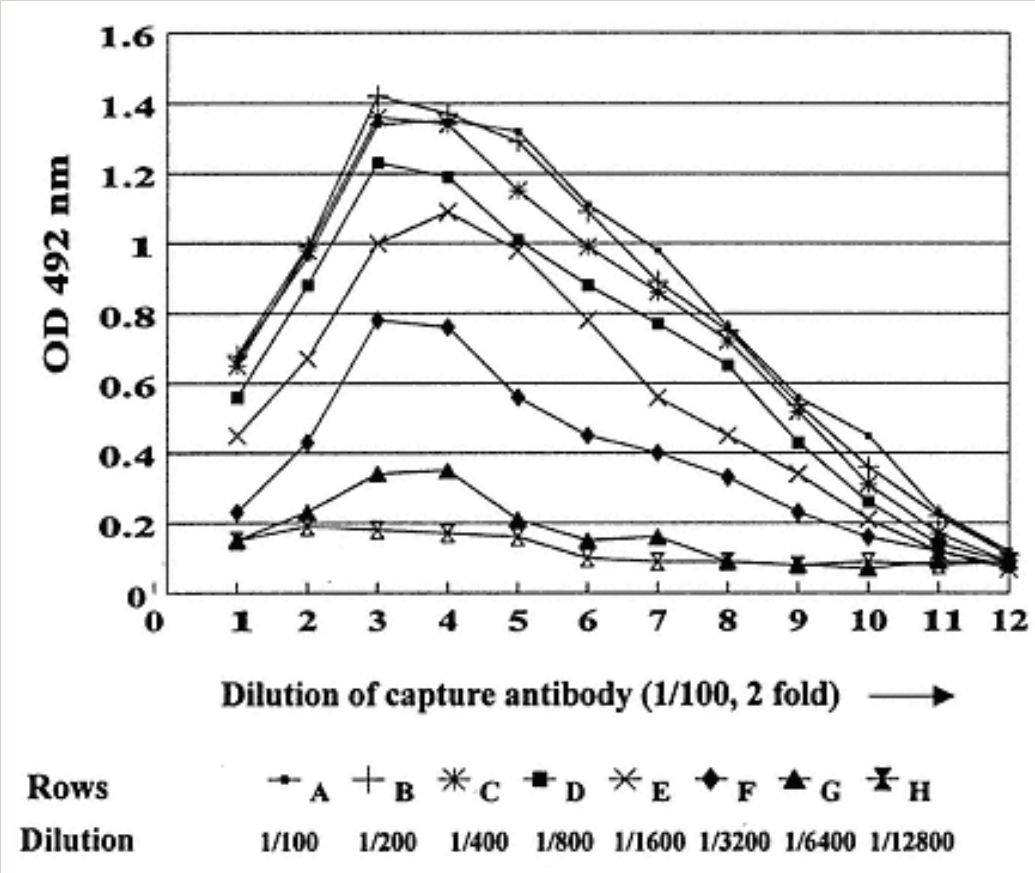


Fig. 27.
Graph of data in Table 10. Columns 1¨C12 contain dilutions of capture antibody on wells. Rows A¨CH have different dilutions of detecting antibody.

may further "divide" the disease agent into a serological grouping, such as is used routinely to serotype FMDV into one of seven distinct serotypes. The use of capture antibody means that relatively crude or contaminated samples can be used. Antigens may be quantified with reference to a standard antigen titration on the same plate.

Single dilutions of material containing the Ag can then be titrated in the same system and the developing OD read against the standard titration. Again, the use of the capture antibody ensures an efficient and proportional uptake of the antigen on to the plate, which is unaffected by contaminating proteins.

5.2¡ª
Use of Capture ELISA to Detect and Titrate Antibodies

Essentially, the same parameters have to be standardized as for capture ELISA for antigen detection. However, the test is used to measure antibodies against a fixed amount of antigen captured on the plate. Thus, we must optimize the system to have the correct amount of capture antibody and antigen necessary to bind any test or control antisera. The test offers the ability to specifically capture antigen using a solid-phase antibody. Thus, relatively crude

preparations can be used in cases in which the required antigen concentration may be low. Care must be taken to avoid reactions of the conjugate with components of the assay.

5.2.1¡[a]
Learning Principles

1. Optimizing of capture antibodies.

2. Optimizing of detecting antibody.

5.2.2¡[a]
Reaction Scheme

$$\text{I-Ab}_X \underset{W}{+ \text{Ag}} \underset{W}{+ \text{AB}_Y} \underset{W}{+ \text{Anti-Ab*E}} \underset{W}{+ \text{S–READ}}$$

—I	= microplate
—AB_X	= trapping antibody (species X)
—Ag	= antigen
—Ab_Y	= test or control sera (species Y)
—Anti-Ab*E	= antispecies Y antibody conjugated with enzyme
—S	= substrate/color detection system
—W	= washing step
—+	= addition and incubation of reagents

5.2.3¡[a]
Materials and Methods

1. Capture antibody (AB_X): sheep anti-guinea pig Ig at 5 mg/mL in PBS.

2. Ag: guinea pig Ig at 1 mg/mL.

3. Test antisera (Ab_Y: three rabbit anti-guinea pig Ig sera; also seronegative (Ab) rabbit sera.

4. Anti-Ab_Y*E: rabbit antigoat Ig conjugated to HRP.

5. Microplates.

6. Multichannel, single-channel, 10-, and 1-mL pipets.

7. Carbonate/bicarbonate buffer, pH 9.6, 0.05 *M.*

8. PBS 1% BSA, 0.05% Tween-20.

9. Solution of OPD in citrate buffer.

10. Hydrogen peroxide.

11. Washing solution.

12. Paper towels.

13. 1 *M* sulfuric acid in water.

14. Small-volume bottles.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

5.2.4¡[a]
Optimization of Test

We need to know the following:

1. What dilution of capture antibody to use.

2. What dilution of antigen to use.

3. What dilution of conjugate to use.

The aim is to have a constant system involving capture antibody (AB_X), antigen (Ag), and conjugate (Anti-Ab*E), which can then be used to titrate test sera (Ab_Y). We have already dealt with the use of capture antibody as whole serum or as IgG. For this exercise, we use sheep anti-guinea pig IgG (or the equivalent in an individual's systems). Thus, examination of the data in **Table 10** allows an estimation of the optimum capture IgG and antigen levels required to allow detection of antibodies.

5.2.5¡[a]
Data

Using the titrations established in **Subheading 5.1.**, we can obtain the optimal amount of antigen (guinea pig Ig in this case) that gives a high plateau OD where the detecting antiserum is in excess. Turn to the data shown in **Table 7**. We can see that the plateau height is maintained to around column 4, showing that there is a maximum level of antigen to react with the antibodies in the positive serum. This concentration (or dilution) can be used in the capture assay under the same conditions to titrate antibodies from any sera. We can therefore use the following quantities:

1. The capture antibody at 2.5 µg/mL if used as an Ig preparation or at the titrated level as found in **Subheading 5.1.5.4.** (**Table 8**).

2. The antigen at the concentration or dilution used in columns 4 to 5 (**Table 7**).

3. The conjugate as titrated initially as in **Table 9**.

5.3¡[a]
Methods for Titration of Antibodies

We can examine sera for antibodies by using full dilution ranges or at a single dilution (spot test). This approach has already been described for the indirect ELISA (*see* **Subheadings 3.6.** and **3.7.4.**). The methodology for capture assays is similar except that the antigen is presented to the test sera after being captured by an antibody coating the microtiter wells. The optimum amounts of capture antibody and antigen are determined as in **Subheadings 5.1.5.1.¨C5.2.5.** After plates have been prepared with an optimal amount of antigen, the stages following this are very similar to the indirect ELISA.

Set up the capture assay to present optimal amounts of guinea pig IgG. After washing the plates, use the same already examined in **Subheading 3.6.** (rabbit anti-guinea pig sera) in a similar way as described from stages 6¨C15 in **Subheading 3.6.** This entails making dilution ranges of sera, incubation, washing plates, addition, and incubation with anti-rabbit conjugate, washing, and addition if substrate/chromophore. Compare the data obtained with that in **Subheading 3.6., Table 4.**

Repeat the same procedure but this time making spot dilutions of the various rabbit sera as in **Subheading 4.3.** Compare the data with that in **Subheading 4.4., Table 6.**

5.4¡ª
Problems Using Capture Assays

1. Care must be taken to examine whether any of the reagents interact. Unexpected crossreactions can be found with immunological reagents. For example, the conjugated antibodies might react with other species other than which they were prepared. There are crossreactions between certain species so that conjugates against cow proteins will react with sheep and goat proteins. Thus, in such a system the use of sheep or goat Ig as a capture antibody precludes the use of antibovine conjugates to detect the reaction of bovine antibodies with a particular antigen.

2. When relatively crude antigens are captured, contaminating proteins may also be trapped, which interfere with the assay. As an example, when purified FMDV is injected into an animal there is a specific response against the virus, but also a response against contaminating bovine serum proteins that are present in extremely low amounts, which comes from the tissue culture medium. Such sera used as capture reagent will capture not only virus but also bovine proteins. Thus, in typing exercises using tissue culture or bovine epithelial samples, a high quantity of bovine protein is captured. The use of antibovine conjugates to detect bound bovine serum in a trapping assay therefore also binds to the trapped bovine protein giving high backgrounds. In the typing assay proper, guinea pig sera are prepared as the second typing detecting sera. These also bind bovine proteins and therefore detect bound bovine protein to the capture antiserum. Again specific typing is affected. However, the second antibody can be treated to remove the crossreactivity either by adding a high concentration of the crossreactive protein to the reagent (in this case 1 mL of normal nonimmune bovine serum is added to 1 mL of typing guinea pig serum), or by using affinity reagents in which bovine serum is attached to a solid phase (e.g., agarose beads), which can be incubated with the serum so that the crossreactive antibodies are removed after incubation and separation of the beads by centrifugation. Or, as is most common, the test may be made using blocking buffers containing high levels (around 5%) of the crossreactive protein.

6¡ª
Competitive ELISA

The direct, indirect, and capture ELISAs have now been examined. You should be able to optimize the conditions of the tests and be able to use them to measure antigen or antibody in a variety of formats. Competitive ELISAs involve the principles of all these types of assay.

Basically, they involve methods that measure the inhibition of a reactant for a pretitrated system. The degree of inhibition reflects the activity of the unknown. We can therefore measure antibody or antigen, and even compare

small differences in the binding of antigens or antibodies so that antigenic subtyping may be performed by comparing the relative avidity of one antiserum for two antigens in the same system. As a reminder, let us consider the competitive assays based on the indirect test and the trapping test for the detection of antigens or antibodies in a diagrammatic way.

6.1¡[a]
Indirect Assay:
Antigen Detection by Competition

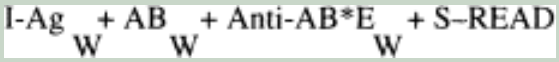
6.1.1¡[a]
Reaction Scheme



A pretitrated indirect assay with optimal Ag1, AB, and conjugate, is competed for by Ag2, as a dilution range in the liquid phase. If Ag2 can bind AB, it will prevent AB binding that would normally react with Ag on the plate. The maximum expected OD for the pretitrated system without competitor (Ag2) is therefore reduced in the presence of the competitor Ag2. The degree of inhibition of the pre-titrated reaction is proportional to the relative amount of the competitor.

6.2¡[a]
Indirect Assay:
Antibody Detection by Competition

6.2.1¡[a]
Reaction Scheme

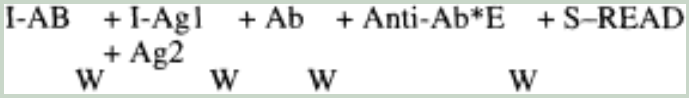


A pretitrated system is challenged by a dilution range of Ab. The competing antibody is from a species that is not the same as that of the AB in the optimized system, and obviously the Anti-AB*E should not react with the Ab. The degree of inhibition of the pretitrated system depends on the concentration and interaction of the Ab competitor with the Ag, this time on the solid phase.

The direct assay could also be used for both these assays. Note that in the direct assay, any species of competing antibody can be used since the AB is labeled with conjugate. Such assays are becoming increasingly relevant when mAbs are used.

6.3¡[a]
Capture Assay:
Antigen Detection by Competition

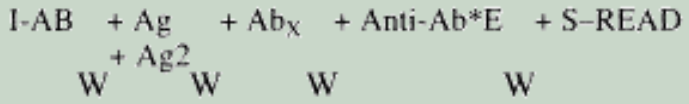
6.3.1¡[a]
Reaction Scheme



The capture assay is optimized to detect the Ag I trapped on the plates using Ab. The competition is achieved in which Ag2 is mixed with the Ab in the liquid phase. If this reacts, the amount of Ab available for reaction with the trapped Ag1 is reduced.

6.4¡ª
Capture Assay:
Antibody Detection by Competition

6.4.1¡ª
Reaction Scheme



The capture antibody is optimized to bind Ag, which is detected by a constant amount of Ab_X (from animal species X). The competition involves the reaction of the Ag with antisera from species Y (which should not interact with the conjugate anti-Ab_X), in the liquid phase. The remaining Ag is then trapped and titrated with the Ab_X and the conjugate. A reduction in the expected OD for the system without any Ab_Y represents competition.

Next, we discuss the following assays:

1. Direct competition assay¡ªantigen detection and quantification.

2. Indirect assay¡ªantigen competition.

3. Indirect competition assay¡ªantibody detection.

a. Full titration curves.

b. Spot test assessment of sera.

7¡ª
Direct Competitive ELISA for Antigen Detection and Quantification

The direct assay for antigen detection and quantification has assumed an increased importance with the development of mAbs. A single mAb can be the one reagent that dominates a diagnostic assay, and therefore mAbs are worth labeling for use in an assay. The specificity of the assay is ensured and relatively crude antigenic preparations can be coated for use in a direct test format (provided enough antigen attaches). This is also relevant to polyclonal antibodies. The demonstrated assays involve IgG/anti-IgG systems.

7.1¡ª
Learning Principles

1. Optimizing of homologous system.

2. Understanding competition curves.

7.2¡[a]
Reaction Scheme

$$\text{I-Ag1} \underset{W}{} + \text{Ab*E} \underset{+\text{Ag2}}{} + \text{S–READ} \underset{W}{}$$

- I-Ag1 = microplate with optimum concentration of antigen attached
- Ag2 = competing antigen as a dilution range
- Ab*E = conjugate specific for the Ag1
- S = substrate/color detection system
- READ = spectrophotometric reading
- + = addition and incubation steps
- W = washing step

Table 11
Data from CBT of Guinea Pig IgG and
Anti-Guinea Pig Enzyme Conjugate in Subheading 1.1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.89	1.88	1.67	1.33	1.10	0.97	0.86	0.57	0.44	0.32	0.31	0.31
B	1.87	1.86	1.63	1.29	1.04	0.93	0.84	0.53	0.34	0.24	0.23	0.21
C	1.68	1.45	1.32	1.14	0.96	0.86	0.64	0.45	0.29	0.19	0.17	0.16
D	1.14	1.03	0.94	0.83	0.57	0.45	0.38	0.29	0.19	0.18	0.15	0.16
E	0.99	0.91	0.74	0.54	0.46	0.36	0.29	0.19	0.18	0.15	0.13	0.14
F	0.66	0.44	0.39	0.33	0.24	0.21	0.19	0.15	0.18	0.16	0.14	0.12
G	0.34	0.20	0.16	0.18	0.16	0.18	0.15	0.16	0.14	0.12	0.14	0.13
H	0.30	0.19	0.15	0.16	0.15	0.17	0.13	0.12	0.13	0.13	0.15	0.16

This exercise will most simply demonstrate the principles involved with competitive assays.

7.3¡[a]
Materials and Reagents

1. Ag1: guinea pig IgG at 1 mg/mL for attachment to solid phase.

2. Ag2: Two samples: (a) guinea pig IgG (known concentration) and (b) rabbit IgG at 1 mg/mL.

3. Ab*E: rabbit anti-guinea pig IgG conjugated to HRP.

4. Microplates.

5. Multichannel, single-channel 10- and 1-mL pipets.

6. Carbonate/bicarbonate, pH 9.6, 0.05 *M*.

7. PBS 1% BSA, 0.05% Tween-20.

8. Solution of OPD in citrate buffer.

9. Hydrogen peroxide.

10. Washing solution.

11. Paper towels.

12. Small-volume bottles.

13. 1 *M* sulfuric acid in water.

14. Multichannel spectrophotometer.

15. Clock.

16. Graph paper.

17. Calculator.

7.4¡ª
Practical Details

Repeat the exercise in **Subheading 1.1.**, involving the CBT of antigen and enzyme-linked antibody. You should obtain a similar picture. Compare your results to those in **Table 11**.

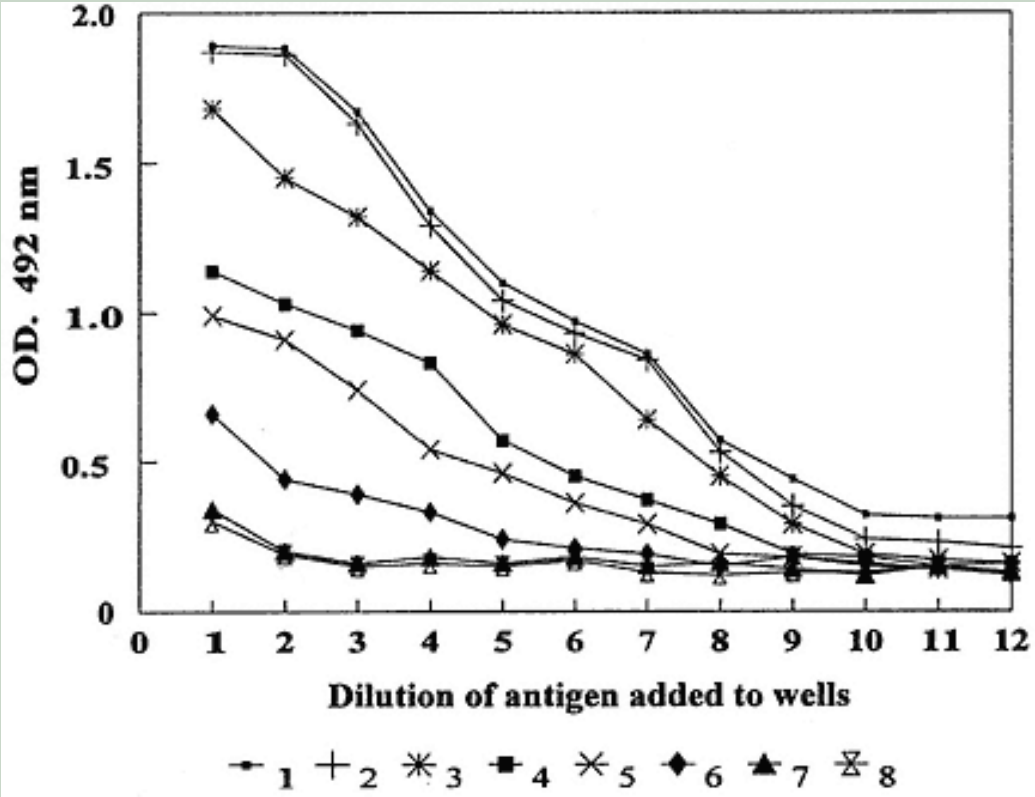


Fig. 28.
Data from **Table 11** relating antigen titrations
at different concentrations of conjugate.

The labeled conjugate dilutions are made from A to H, IgG is diluted 1¨C11, 12 has no antigen. **Figure 28** presents a plot of a CBT of guinea pig IgG against rabbit anti-guinea pig conjugates.

7.4.1¡[a]
Assessment of Data and Choice of Conditions for Competition

We are trying to compete the same antigen (guinea pig IgG) and a different antigen (IgG from the rabbit), for a pretitrated homologous solid-phase reaction. The ultimate sensitivity of the assay depends on the exact relationship of the antibody and antigen attached to the solid phase. If we use too much antibody, so that it is in excess of that required to saturate the antigen, we will have a quantity of free antibody that may bind to the competitor, and there will still be an amount left to react with the solid-phase IgG. Thus, competition will only occur when extremely high concentrations of competitor are used.

This can be illustrated by examination of the titration curves in **Fig. 29**. Note that the plateau region represents excess antibody for any given antigen concentration. The OD and extent of these plateau regions vary according to the exact amount of antigen attached to the solid phase. As we reduce the antigen the plateau height values decrease. At the highest concentrations of antigen, the titration curves are similar for different antibody concentrations,

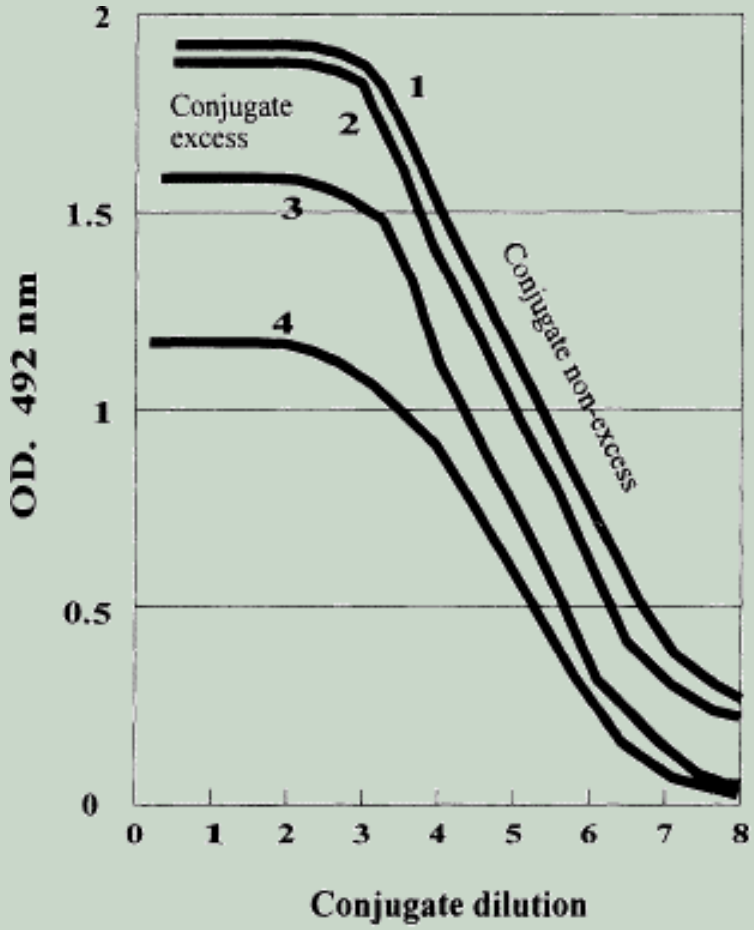


Fig. 29.
Illustration of regions of conjugate excess and nonexcess when titrating conjugate against constant concentration of antigen.

indicating that the antigen and antibody are behaving at maximum saturating levels. On dilution of the antigen, we see that the plateau height is reduced, even where we know that the antibody is available for higher OD values (curves 3 and 4). Here, the antigen is the limiting factor in color development. In the competition assay, a maximum plateau height dependent on the amount of antigen attached of around 1.0¨C1.5 OD should be selected. In other words, find out which dilution of antigen produces serum titration curves giving a maximum plateau of these values, e.g., curves 3 and 4. From this titration curve, we need to estimate the dilution of antibody yields about 70% of the maximum plateau OD. Using curve 1, we can illustrate this, as shown in **Fig. 30**.

7.4.2¡ª
Estimation of Antibody Dilution to Be Used in Competition Assay

The conditions are now set for competition. We have: (1) the antigen dilution as for curve, and (2) the antibody dilution estimated as shown in **Fig. 30**.

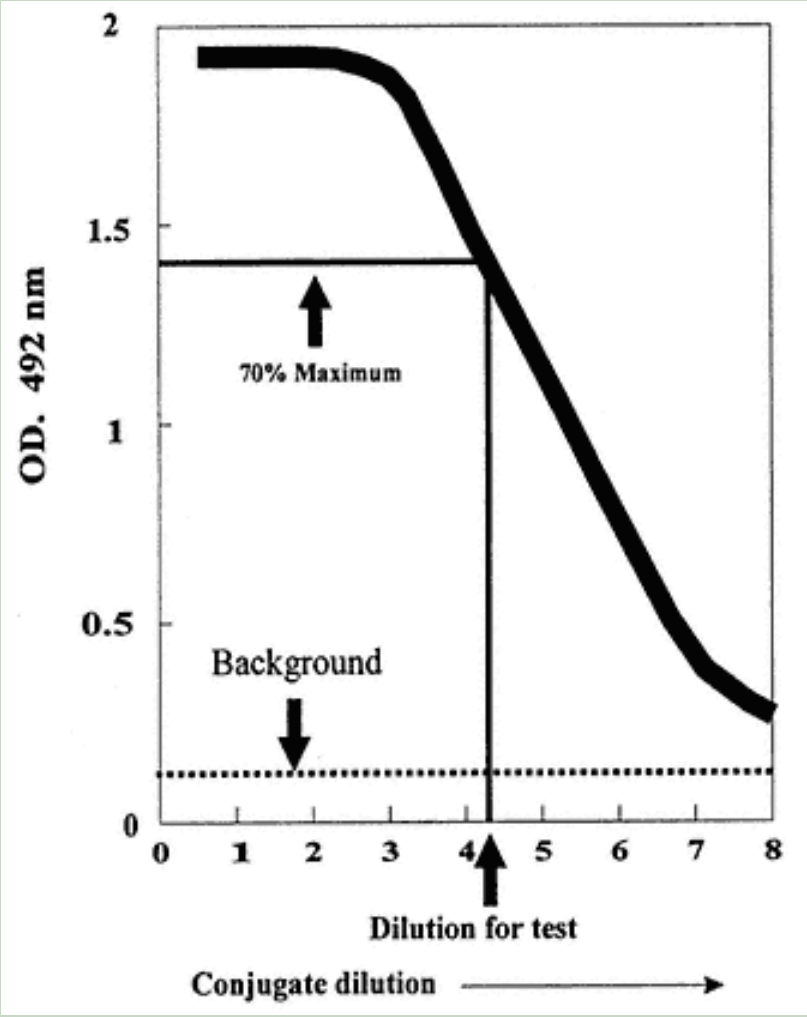


Fig. 30.
Estimation of conjugate dilution for use in competition stage.

7.5¡ª
Competition Assay Proper

1. Prepare the optimum antigen coated with guinea pig IgG.

2. Wash the plates.

3. Dilute the guinea pig Ig (homologous competitor) and the rabbit Ig (heterologous competitor) to 40 µg/mL in blocking buffer.

4. Add 50 µL of blocking buffer to each well of the Ig-coated plate.

5. See the plate design in **Fig. 31**. Make a twofold dilution range of the guinea pig and rabbit Ig by adding 50 µL of the Igs to the first row 1. Do this in triplicate (three rows for the guinea pig Ig¡ª1A, B, and C; and three rows for the rabbit Ig¡ª1D, E, and F).

6. Double dilute the IgGs across the plate (1¨C11).

7. Dilute the anti-guinea pig conjugate (pretitrated) in blocking buffer. Make up 6 mL.

8. Add 50 µL of the diluted conjugate to rows A¨CG. Do not add to row H. Mix the contents of the plates by gentle tapping. Add 50 µL of blocking buffer to row H.

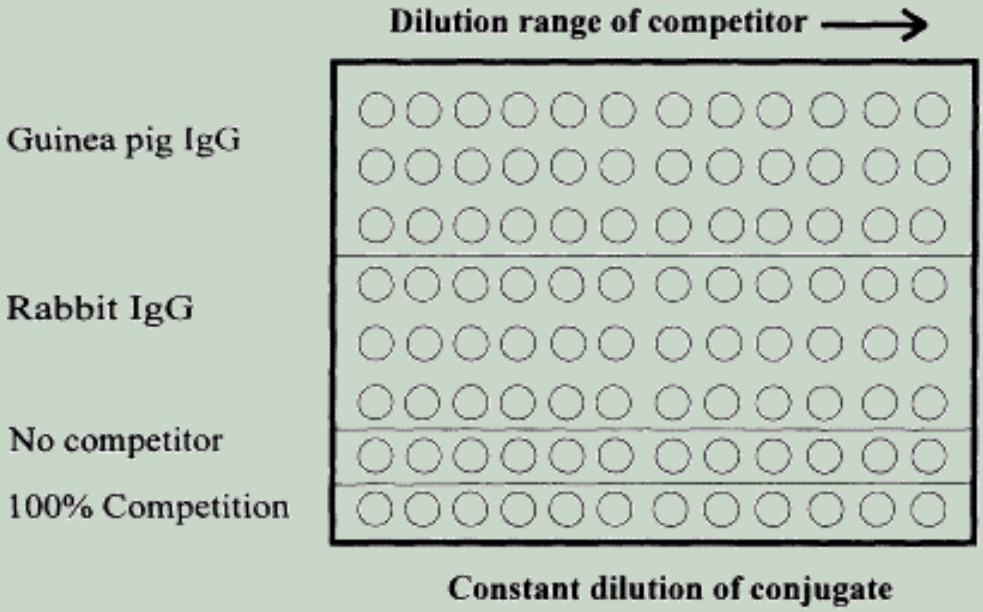


Fig. 31.
Plate design for performance of competition assay.

9. Incubate for 1 h at room temperature (or under conditions you used to titrate the conjugate). Rotate the plate to mix reagents every 10 min.

10. Wash the plates.

11. Add OPD/H_2O_2 solution.

12. Stop the reaction after 10 min by addition of 50 µL of 1 *M* H_2SO_4.

13. Read the OD in spectrophotometer at 492 nm.

7.5.1¡[a]
Data:
Typical Results

Table 12 presents the results from the spectrophotometer readings of the plates. **Figure 32** relates the OD values to the various concentrations of the competitors added. The results are processed intially as the mean OD for the triplicate estimations.

7.5.2¡[a]
Further Processing of Data

1. Calculate the mean OD reading of row G. This represents the OD resulting from the reaction of the conjugate with only the solid-phase Ig and the conjugate. This value should be similar to that obtained when you titrated the conjugate. It represents the 0% competition OD, where most color is obtained.

2. Calculate the mean of the OD from row H. This represents the 100% competition level, i.e., where there is a total inhibition of the binding of antibody (not a strictly true 100% control since the conjugate was excluded from the test, but it approximates very well). Thus we have the 100% competition (degree of inhibition) and the 0% competition OD values.

3. Convert the OD values obtained for the wells that contained the two competitors into percentage of competition using the two values just calculated.

Table 12
Plate Data from the Competition of Samples of Guinea Pig and Rabbit IgG for Direct ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.05	0.07	0.10	0.23	0.35	0.56	0.78	0.98	1.12	1.34	1.34
B	0.06	0.06	0.08	0.12	0.25	0.41	0.61	0.79	1.01	1.14	1.35	1.38
C	0.07	0.05	0.09	0.13	0.21	0.43	0.58	0.81	1.05	1.17	1.36	1.34
D	1.12	1.23	1.34	1.35	1.34	1.36	1.29	1.37	1.36	1.41	1.32	1.34
E	1.14	1.24	1.35	1.35	1.36	1.39	1.34	1.36	1.33	1.34	1.32	1.38
F	1.13	1.25	1.34	1.38	1.38	1.41	1.42	1.35	1.33	1.38	1.34	1.32
G	1.35	1.34	1.41	1.35	1.36	1.32	1.29	1.34	1.37	1.39	1.32	1.45
H	0.05	0.06	0.05	0.07	0.08	0.04	0.07	0.05	0.04	0.07	0.08	0.04

A¨CC: guinea pig IgG, D¨CF: rabbit IgG.

7.5.3¡[a]
Example from the Data in Table 12

The mean of row G = 1.35, which is equivalent to 0% competition (a lot of color). The mean of row H = 0.07, which is equivalent to 100% competition (little color). Subtract the mean of row H from all the values obtained. If the value is minus then call it 0. This determines the 100¨C0% OD competition values (i.e., the range is from 0 to 1.29 OD). Using a simple formula, the percentage of competition of the samples can be calculated. **Table 13** presents the processed data with respect to subtraction of background for all data, using the following equation

$$\% \text{ competition} = 100 - \left[\frac{(\text{Test OD-background})}{\text{Range}} \times 100\right]$$

As examples for further processing and estimation of competition, we have for the guinea pig Ig competition experiment (backgrounds already taken off test results and range calculated):

Range = 1.29

Taking row 5 we have:

$$100 - [(0.16/1.29) \times 100] = 87.7\%$$

Taking row 6 we have:

$$100 - [(0.33/1.29) \times 100] = 75.0\%$$

Taking row 7 we have:

$100 - [(0.51/1.29) \times 100] = 60.0\%$

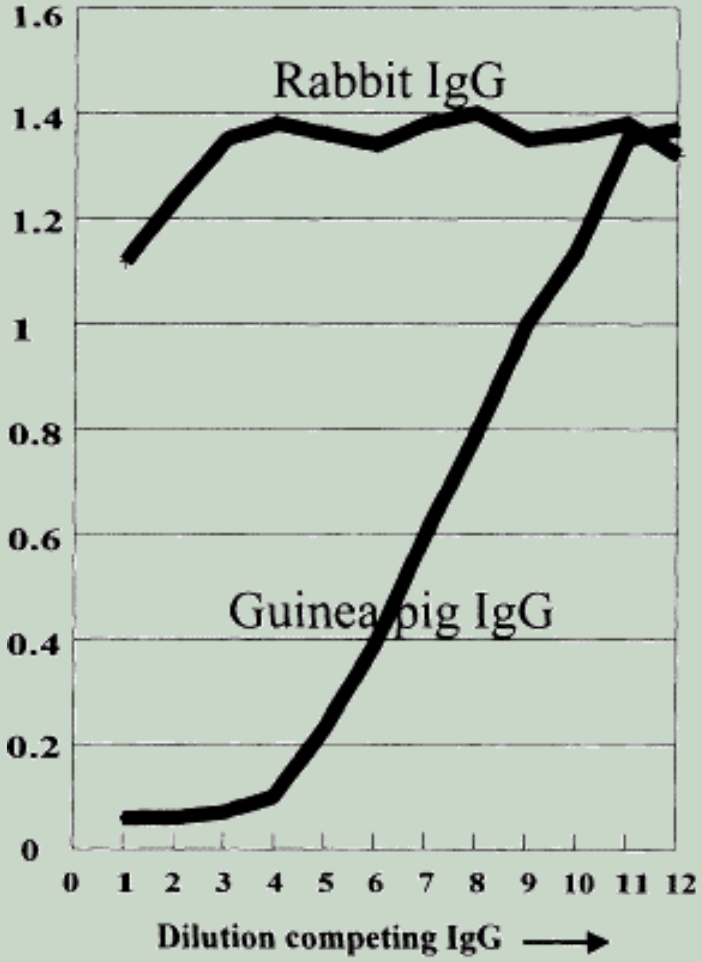


Fig. 32.
Competition of guinea pig IgG and rabbit IgG for guinea pig system.

Repeat this exercise for your data. Plot the data relating the percentage of competition against the concentration or dilution of the IgGs as in **Fig. 33**.

7.5.4¡ª
Analysis of Data

1. Note that as you dilute out the homologous competitor (the guinea pig Ig), the competition reduces.

2. Note that the plateau of 100% competition is where the competing Ig is in large excess over that on the plate.

3. Suggest what is happening at the 50% competition point.

4. Note that the competition curve is sigmoidal.

5. Note that the rabbit Ig hardly competes. Why?

6. Suggest how the sensitivity of the assay might be altered? A clue here is to examine what happens if we reduce (1) the amount of antigen on the solid phase and (2) the amount of conjugate in the test.

Table 13
Mean Values in Table 12
for Various Dilutions of Competing Antigens[a]

	Mean ABC minus row H guinea pig IgG	Mean DEF minus row H rabbit IgG
1	0	1.17
2	0	1.18
3	0	1.29
4	0.01	1.35
5	0.16	1.28
6	0.33	1.27
7	0.51	1.27
8	0.73	1.29
9	0.93	1.28
10	1.10	1.29
11	1.28	1.26
12	1.29	1.27

[a]Mean row G ¨C mean row H = 1.29 = the range.

7.6¡[a]
Indirect Assay:
Antigen Competition

This exercise is similar to the direct competition assay for antigen except the antigen is detected by an unlabeled antiserum (rabbit anti-guinea pig IgG), which in turn is detected using an antispecies conjugate. Here, the heterologous antigen is bovine IgG. Thus, the indirect assay is optimized as in **Subheading 2.** You can use the results of the CBT in this chapter (**Table 3**) to assess the following:

1. The best guinea pig concentration/dilution to adsorb to wells.

2. The optimum amount of antibody to give about 70% binding to the optimum amount of antigen found as shown in **Fig. 30**.

7.6.1¡[a]
Materials

1. Ag1: guinea pig IgG at 1 mg/mL for attachment to solid phase.

2. Ag2: samples of (a) guinea pig IgG standard solution (known concentration of 1 mg/mL, (b) three solutions of guinea pig IgG at unkown concentration, and (c) bovine IgG solution at 1 mg/mL.

3. Ab: rabbit anti-guinea pig IgG.

4. Ab*E: pig anti-rabbit pig IgG conjugated to HRP.

5. Microplates.

6. Multichannel and single-channel 10 mL, 1 mL pipets.

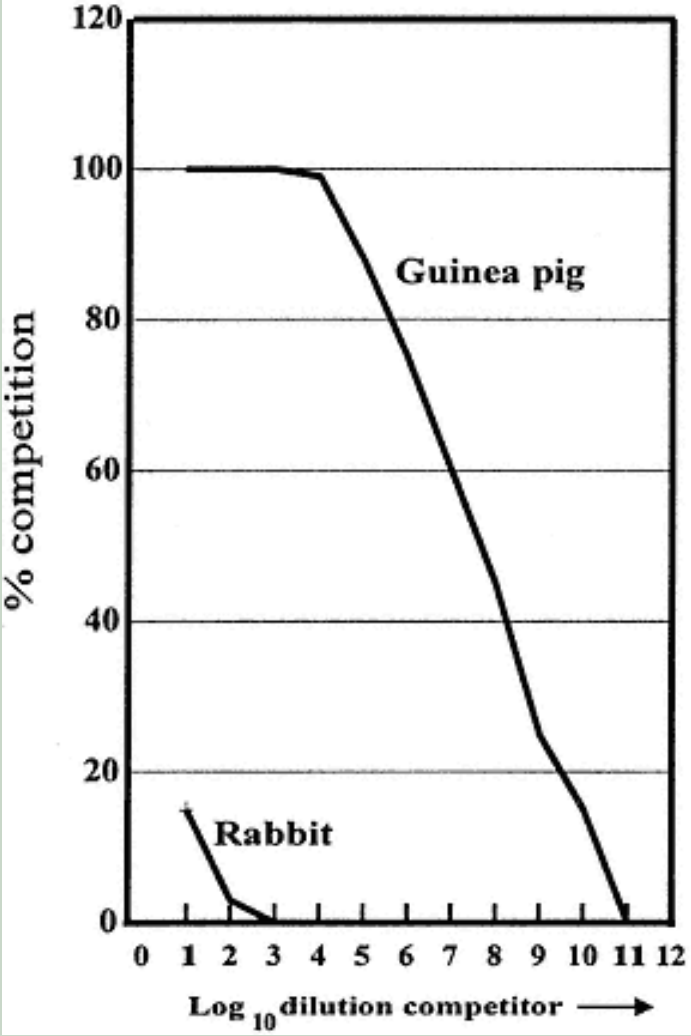


Fig. 33.
Percentage of competition plots of guinea pig and rabbit IgG competing for the guinea pig system.

7. Carbonate/bicarbonate, pH 9.6, 0.05 *M*.

8. PBS 1% BSA, 0.05% Tween-20.

9. Solution of OPD in citrate buffer.

10. Hydrogen peroxide.

11. Washing solution.

12. Paper towels.

13. Small volume bottles.

14. 1*M* sulfuric acid in water.

15. Multichannel spectrophotometer.

16. Clock.

17. Graph paper.

18. Calculator.

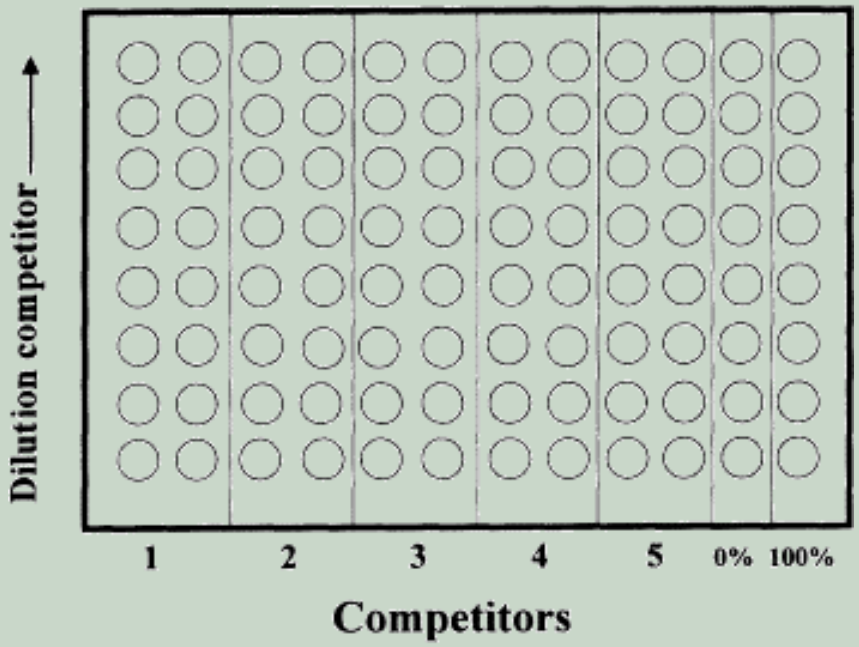


Fig. 34.
Plate design for addition of competitors. 1, Guinea pig control; 2, bovine IgG; 3, sample A; 4, sample B; 5, sample C.

These competition steps are identical to the direct assay and the data are processed in the same way. The extra step is to detect any reacted antibody with the antispecies conjugate.

7.6.2$_i$[a]
Method

1. Coat the wells of a microplate with 50 µL, of guinea pig Ig at optimum concentration found from CBT.

2. Wash the wells.

3. Dilute the homologous guinea pig IgG competitor of known concentration and the bovine Ig sample to 40 µg/mL in blocking buffer.

4. Take the 3 samples of unknown levels of guinea pig Ig and dilute 1/10 in blocking buffer.

5. Add 50 µL of blocking buffer to all the wells of the microplate that is coated with the optimum guinea pig Ig.

6. Make a twofold dilution range of all the diluted samples. Thus, add 50 µL of the initial dilution as shown in the plate plan in **Fig. 34**. Prepare duplicate columns of each for eight wells.

7. Add 50 µL of the pre-titrated antibody against the guinea pig Ig to columns 1¨C11. Add 50 µL of blocking buffer to column 12.

8. Incubate under conditions in which initial CBT were performed. Mix contents every 10 min (unless overnight incubation is being used).

9. Wash the wells and blot.

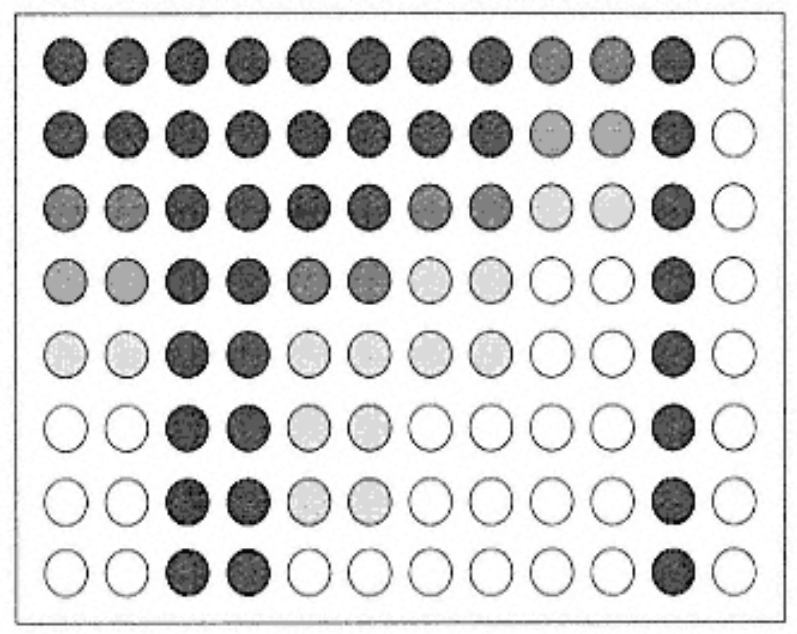


Fig. 35.
Representation of place of competition assay.
See the data in **Table 12**.

10. Add 50 µL of the the optimal diluton of the pig anti-guinea pig conjugate, per well and incubate at 37¡ãC for 1 h. Wash the plates.

11. Add the OPD substrate/chromophore solution (50 µL per well), and then stop the reaction by addition of 50 µL of 1 *M* H_2SO_4 after 10 min.

12. Read the OD using a spectrophotometer at 492 nm.

7.6.3¡ª
Data: Typical Results

Figure 35 represents the appearance of the stopped plate. Plate data as shown in **Table 14**.

7.6.4¡ª
Treatment of Data

1. Take the mean value of the OD from row 12 (0.08 in data from **Table 14**).

2. Subtract this from all the ODs of the rest of plate.

3. For each of the duplicate wells, find the mean OD for each competitor dilution. Thus, we have the values given in **Table 15**.

4. Take the mean result of column 11 in **Table 15** = 1.26. This is the 0% competition value. Use the following formula to calculate the percentage of competition of each IgG dilution:

$$\% \text{ competition} = [(100 - \text{OD in presence of competitor})/1.26 \text{ (range)} \times 100]$$

Thus, for data in **Table 15**, we have the processed data in **Table 16**. Plot the competition curves relating competition to $\log_{10}$ dilution or concentration as shown in **Fig. 36**.

Table 14
Plate Data for Indirect Competition ELISA to Measure Antigen

	Guinea pig test control		Bovine		Guinea pig 1		Guinea pig 2		Guinea pig 3		0%	100%
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.31	1.30	1.31	1.33	1.32	1.32	1.26	1.34	1.12	1.10	1.34	0.06
B	1.12	1.14	1.32	1.34	1.28	1.29	1.21	1.18	0.88	0.83	1.32	0.08
C	0.79	0.77	1.29	1.28	1.09	1.10	1.00	0.98	0.68	0.66	1.34	0.09
D	0.57	0.54	1.27	1.19	0.85	0.79	0.76	0.74	0.44	0.43	1.32	0.06
E	0.33	0.36	1.25	1.24	0.67	0.69	0.47	0.48	0.22	0.23	1.29	0.08
F	0.18	0.15	1.25	1.26	0.41	0.45	0.26	0.27	0.09	0.09	1.34	0.09
G	0.09	0.10	1.21	1.22	0.30	0.29	0.16	0.17	0.08	0.09	1.33	0.08
H	0.08	0.07	1.15	1.18	0.13	0.15	0.09	0.07	0.07	0.06	1.35	0.06

7.6.5¡[a]
Examination of Data

1. The bovine IgG competition is very low, and the slope of the curve is very different from those of homologous control guinea pig IgG.

2. The curves for all guinea pig competitors are of similar shape.

3. The curves for test guinea pig IgG A, B, and C are displaced as compared to control IgG curve.

A standard curve relating the concentration of guinea pig IgG competitor in the liquid phase to the competition achieved is shown by the control IgG. The concentration of IgG in the other samples can be determined with reference to this standard curve. Since the general slope of the curves is similar, we can compare the relative concentration at any point on the standard curve. Ideally, the best comparison point is at 50% competition. Thus, draw a line across the 50% competition point on your graphs, as indicated in **Fig. 37**.

Read the dilution of the test IgGs that gives 50% competition, and then relate this to the known IgG concentration necessary to give 50% competition as determined from the standard curve at this point.

Thus, assuming a starting concentration of guinea pig IgG at 2 µg/mL, we have a standard IgG 50% competition = 1/64; a dilution for IgG A = 1/20; a dilution for IgG B = 1/40; and a dilution for IgG C = 1/140. Multiply the dilution factor by the 2 µg to get concentration/milliliters in the test IgG:

1. IgG C = 140/64 ¡Á 2 µg = 4.4 µg/mL.

2. IgG B = 40/64 ¡Á 2 µg = 1.25 µg/mL.

3. IgG A = 20/64 ¡Á 2 µg = 0.63 µg/mL.

Table 15
Mean Values of Plate Data Shown in Table 14

	1 2	3 4	5 6	7 8	9 10	11
A	1.22	1.24	1.24	1.22	1.05	1.26
B	1.05	1.25	1.21	1.12	0.81	1.24
C	0.70	1.21	1.01	0.91	0.59	1.26
D	0.47	1.15	0.74	0.65	0.36	1.24
E	0.27	1.15	0.59	0.38	0.14	1.21
F	0.08	1.14	0.34	0.19	0.00	1.26
G	0.00	1.13	0.21	0.09	0.00	1.25
H	0.00	1.07	0.06	0.00	0.00	1.27

Table 16
Competition Percentage Values from Data Shown in Table 15

	% Competition (%)				
	Guinea pig control 1¨C2	Bovine IgG 3¨C4	Guinea pig 1 IgG 5¨C6	Guinea pig 2 IgG 7¨C8	Guinea pig 3 IgG 9¨C10
A	6	5	5	7	20
B	20	5	7	12	36
C	35	9	20	28	53
D	67	10	42	49	71
E	79	10	53	70	100
F	93	11	75	87	100
G	100	15	87	100	100
H	100	20	95	100	100

Remember that the dilution range is in $\log_{10}$ steps so that the antilog of the value has to be taken to obtain the dilution factor at 50%.

7.6.6¡[a]
Conclusions

1. We have used a standard curve relating a known concentration of homologous competitor to its competing ability, to measure unknown concentrations of the same IgG in samples. This has analogies to the radioimmunoassay approaches used in the quantification of hormones.

2. Note that if it is known that the substance for detection and quantification is the same immunologically (homologous) as the standard substance used to compute the standard curve, then single dilutions of test could be used and their competing ability read from a standard curve.

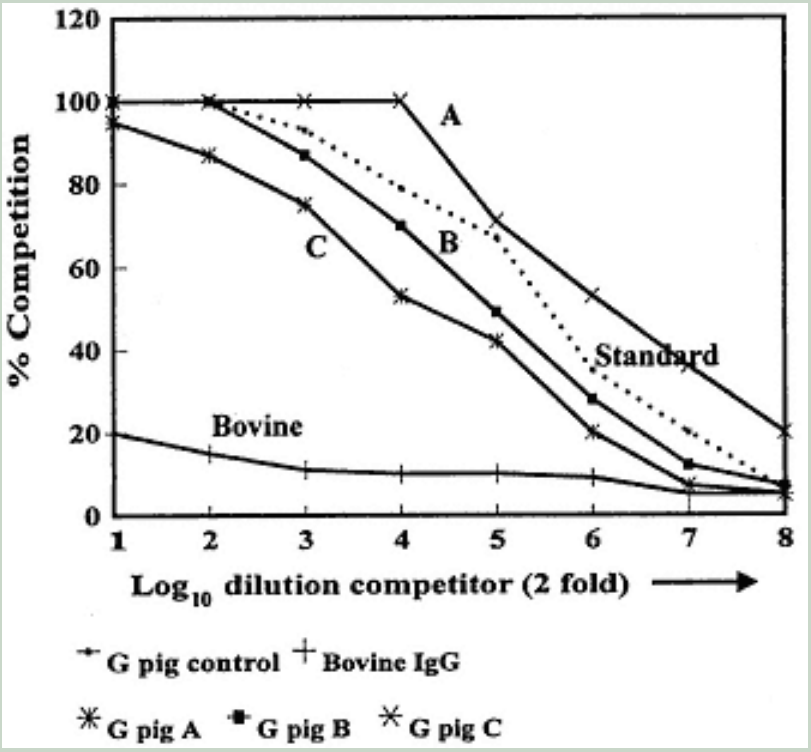


Fig. 36.
Competition curves for various competitors.
Data is shown in Table 12.

3. Such competition assays can be used to determine the similarity of antigens in the same system competing for a single antiserum. The slopes of the competition lines can be compared to obtain a measure of antigenic relatedness.

7.7¡[a]
Indirect Competition Assay for Antibody Detection

7.7.1¡[a]
Reaction Scheme

I-Ag + Ab + anti-Ab*E + S–READ
W + AB W W

- I = microplate
- Ag = antigen
- Ab = pretitrated antibodies to Ag (species X)
- AB = competing antibody (from species different from Ab)
- Anti-Ab*E = conjugated antispecies in which Ab was produced
- S = substrate and chromophore
- W = washing step
- + = addition and incubation of reagents
- READ = reading in a spectrophotometer

In this exercise, the indirect assay is used to pretitrate the homologous antibody. The optimized system is then competed with a dilution range of antibod-

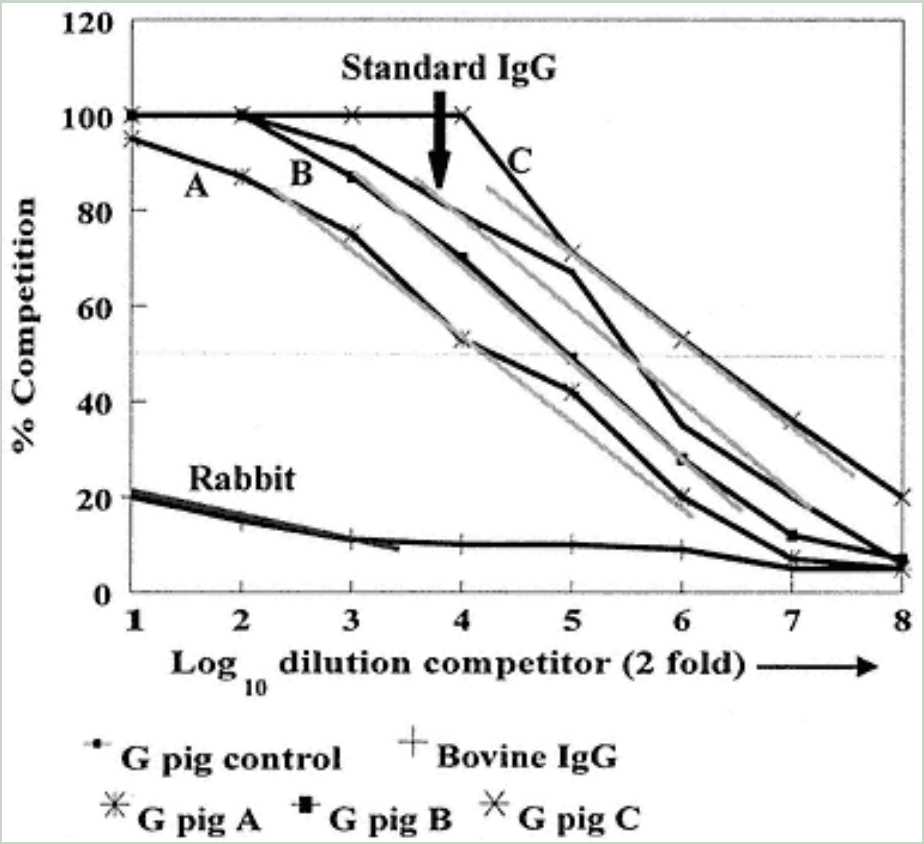


Fig. 37.
Regression of competition curves for various competitors.
Data are from Table 14. Regression lines are gray.

ies from another species (the conjugate must not react with the competing antibodies). In this assay, the pretitration of the homologous serum is slightly different from the antigen competition indirect ELISA in that we need to add the amount of homologous antibodies that just saturate the antigen coated on the plate, since we do not wish to leave excess free antigenic sites that could react with the competing antibody and have little influence on the binding of the homologous antiserum. Note that this kind of assay can be made using the direct ELISA using a conjugated homologous serum, as for the direct antigen competition ELISA. Such assays are becoming more common with the advent of the use of mAb reagents.

7.7.2¡ª
Data

Figure 38 is a graph relating the antibody titration curves to the IgG concentrations on the wells. From these data we can do the following:

1. Assess the best antigen concentration for use in the competition assay, and select the IgG concentration that gives a plateau maximum (in antibody excess) of around 1¨C1.5 OD. (curves 4 and 5).

2. Select the dilution of serum that just saturates this level of IgG (approx 1/100).

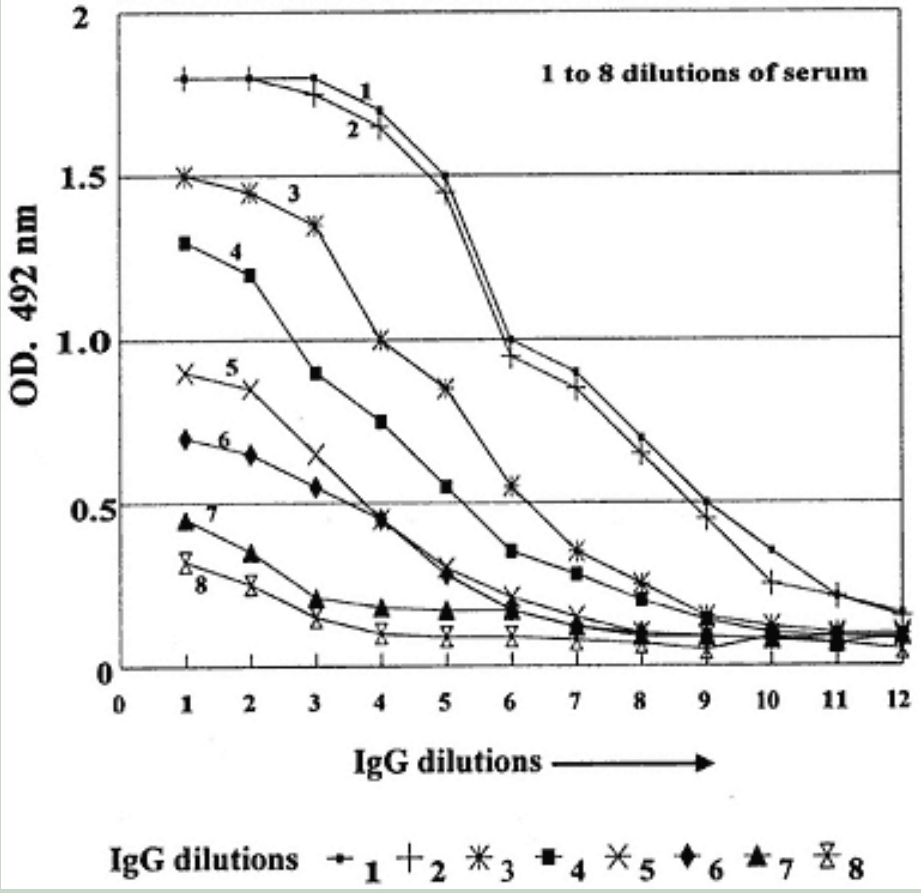


Fig. 38.
Titration curves relating IgG dilutions on wells against different serum dilutions.

7.7.3¡ª
Increasing the Confidence of the Titration Curve Results

Since in the CBT we are using only a single-dilution range of antibody against the antigen, it is essential to titrate the antiserum in multiple rows against the antigen level found to be optimal. That is, we adsorb IgG at a level equivalent to the fourth or fifth dilution used in the preceding test, and then titrate in quadruplicate a dilution series of serum against it. In this way, we can observe the variation in results and assess the confidence in the titer of antibody that just saturates the antigen used in the competition assay proper. This may be necessary when, e.g., one obtains poor competition in the test proper with low sensitivity, indicating that too high or much too low a concentration of antiserum was used.

7.7.4¡ª
Competition Assay Proper

1. Add 50 µL of guinea pig IgG to plates at optimal concentration found in stage 1, incubate, and wash the plates.

2. For the rabbit anti-guinea pig sera, label the standard antiserum 1, label the two unknown titer sera 2 and 3. Label the two sero-negative rabbit sera 4 and 5.

3. Dilute the rabbit sera to 1/50 in blocking buffer (make up 0.5 mL of each; i.e., add 10 µL of serum to 0.5 mL of buffer).

4. Add 50 µL of blocking buffer to all the antigen-coated plate wells.

5. Add 50 µL of rabbit serum I to wells HI and H2. Add duplicate rows of other sera in row H (serum 2 in H3 and 4; serum 3 in H5 and 6; serum 4 in H7 and 8; serum 5 H in 9 and 10). Dilute the sera using a multichannel pipet, transferring and mixing 50 µL for each step. We thus have a dilution range from 1/100 (row H) to 1/12,800 (row A) for each of the sera.

6. Incubate for 30 min at 37¡ãC. Do not wash the plate.

7. Add 50 µL of the swine anti-guinea pig serum at the optimal dilution, found in stage 1, to each well from columns 1 to 11. Do not touch liqid in the wells when adding reagent. Add 50 µL of blocking buffer to column 12.

8. Incubate for 1 h at 37¡ãC.

9. Wash the wells.

10. Add 50 µL of antiswine conjugate to each well diluted in blocking buffer.

11. Incubate at 37¡ãC for 1 h.

12. Add 50 µL well of substrate and OPD per well, and incubate for 10 min.

13. Stop the reaction by addition of 50 µL of 1M H2SO4 to each well.

7.7.4.1¡ª
Typical Data

Figure 39 is a representation of the ELISA plate after stopping. **Table 17** presents the data.

7.7.5¡ª
Processing Data

Processing of the data is similar to the other competition assays performed:

1. Column 12 = 100% competition value, take the mean OD = 0.08

2. Subtract this from OD values of all wells.

3. Take the mean OD of the duplicates for the competitors. This is shown in **Table 18**.

4. Plot the data, and relate the log10 dilution of each antiserum to the percentage of competition as illustrated in **Fig. 40**.

7.7.6¡[a]
Analysis of Data

1. Note that the curves for the rabbit antisera are similar. All the samples compete. Sample 3 is a strong competitor since it gives high competition at higher dilutions than the standard (1). Sample 2 is a weaker competitor than the standard.

2. The negative sera give little or no competition even at low dilutions.

3. The activity of each of the two test sera can be compared to the standard competing antiserum (1). Arbitary units can be ascribed to the standard serum so that serum titires could be expressed against this. As an example, let the titer of the standard serum at 50% competition be 1000. The relative titers of the other two

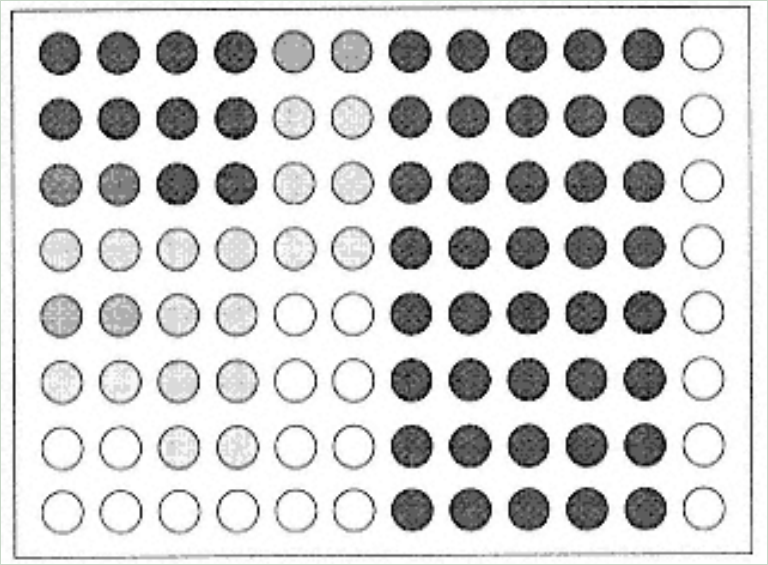


Fig. 39.
Representation of plate showing competition assay. Data are given in Table 17.

test sera can then be related to this. Since the same dilution range was used for the samples we have at 50% competition for serum 2, it is two times stronger than the standard. Therefore, we need twice as much antiserum to compete to the same level as the standard, so the relative titer of the serum is 1/500. For serum 3 at 50%, we require five times less antiserum to give the same result as the standard so that the titer is 5000. The difference in the dilution factors necessary to give 50% competition is easily assessed from the graphs in **Fig. 40**.

4. Note that this processing holds true only if the competition curves show similar characteristics (shape). Considerable variation in slopes indicates that there is a different population of antibodies in the competing serum. As in all assays, the general picture of titration curves is best examined by the assay of as many sera as possible.

7.8¡[a]
Indirect Assay Competition for Antibody Detection Using a Single Dilution of Test Serum

Here we use the standard competing rabbit serum as a full titration range in three rows of the plate. The rest of the plate contains a number of rabbit sera of high, medium and low titer against guinea pig IgG as used in **Subheading 7.6.** Not all the sera can be used in this exercise owing to the limited space on the plate. The assay is identical to that in **Subheading 7.6.** except that duplicate samples of sera are assessed at a single dilution for their competing ability. The titer of the serum is then read from the standard curve obtained on full dilution of the standard serum. The test therefore has two stages: (1) the titration of the homologous antiserum and solid-phase antigen in a CBT indirect ELISA, and (2) the competition proper.

Table 17
Plate Data from Exercise 7.7. in Subheading 7.7.4.
Showing Competition of Indirect Assay by Antibodies

Standard serum	1	2	3	4	100%	0%

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.12	1.16	1.21	1.20	0.78	0.84	1.14	1.13	1.14	1.15	1.11	0.07
B	1.07	1.09	1.21	1.19	0.56	0.58	1.15	1.12	1.16	1.14	1.15	0.09
C	0.89	0.91	1.10	1.09	0.34	0.32	1.13	1.09	1.15	1.12	1.17	0.07
D	0.63	0.61	0.87	0.89	0.21	0.19	1.10	1.09	1.13	1.15	1.16	0.06
E	0.42	0.41	0.63	0.65	0.09	0.08	1.16	1.09	1.14	1.13	1.15	0.08
F	0.23	0.26	0.43	0.45	0.08	0.07	1.13	1.14	1.14	1.16	1.15	0.07
G	0.13	0.12	0.23	0.25	0.07	0.08	1.15	1.12	1.16	1.15	1.17	0.06
H	0.08	0.09	0.12	0.10	0.08	0.07	1.14	1.16	1.14	1.15	1.15	0.07

Table 18
Mean Values of Data in Table 17

	1¨C2	3¨C4	5¨C6	7¨C8	9¨C10	11
A	1.05	1.12	0.71	1.06	1.07	1.08
B	1.00	1.12	0.49	1.06	1.07	1.07
C	0.90	1.01	0.25	1.03	1.06	1.09
D	0.52	0.80	0.12	1.01	1.06	1.08
E	0.34	0.56	0.00	1.04	1.06	1.07
F	0.16	0.36	0.00	1.06	1.07	1.07
G	0.05	0.16	0.00	1.06	1.07	1.09

H	0.00	0.03	0.00	1.07	1.06	1.07

7.8.1¡[a] Materials and Reagents

As for **Subheading 7.7.** except that the rabbit sera are replaced by 32 rabbit sera including seronegative, low, medium, and high titers against guinea pig IgG.

7.8.2¡[a] Pretitration Stage

Repeat the first stage as in **Subheading 7.7.**, or use these data to establish conditions. From the data the best antigen concentration, and the dilution of swine antibody that just saturates the IgG, is determined.

7.8.3¡[a] Competition Assay Proper

1. Add 50 µL of guinea pig IgG to plates at optimal dilution Incubate and wash the plates.

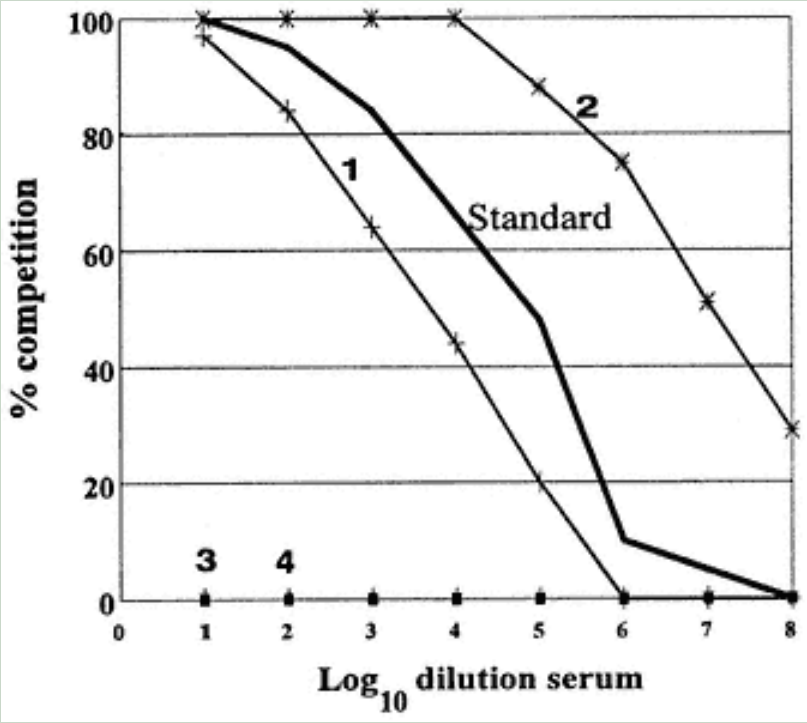


Fig. 40.
Competition curves for various competing sera.
Data are from Table 18.

2. Dilute the rabbit test sera to 1/50 in blocking buffer. Use the micronics racks for dilutions so that the samples can be added using a multichannel pipet. Dilute the standard rabbit antiserum to 1/50.

3. Add 50 µL of blocking buffer to columns 1, 2, 11, and 12.

4. Add 50 µL of the diluted standard rabbit serum to H1 and H2. Make a twofold dilution range of the serum to A1 and A2.

5. Add the test samples to the wells as duplicates, as indicated in **Fig. 41**.

6. Incubate the plates for 30 min at 37¡ãC.

7. Add 50 µL of the optimum dilution of swine anti-guinea pig serum in blocking buffer, incubate for 1 h at 37¡ãC, and mix the contents of the plates every 10 min. Do not add this serum to column 12. Instead, add 50 L of blocking buffer.

8. Wash the plates.

9. Add the anti-swine conjugate diluted in blocking buffer (optimum dilution). Incubate for 1 h at 37¡ãC.

10. Wash the plate.

11. Add substrate/OPD, and incubate for 10 min.

12. Stop the reaction by addition of 50µL of 1 *M* H2SO4 per well.

13. Read plate using spectrophotometer. (*See* **Fig. 42** for a representation of a stopped plate.)

7.8.4¡[a]
Typical Data

Table 19 presents typical data for a spot test.

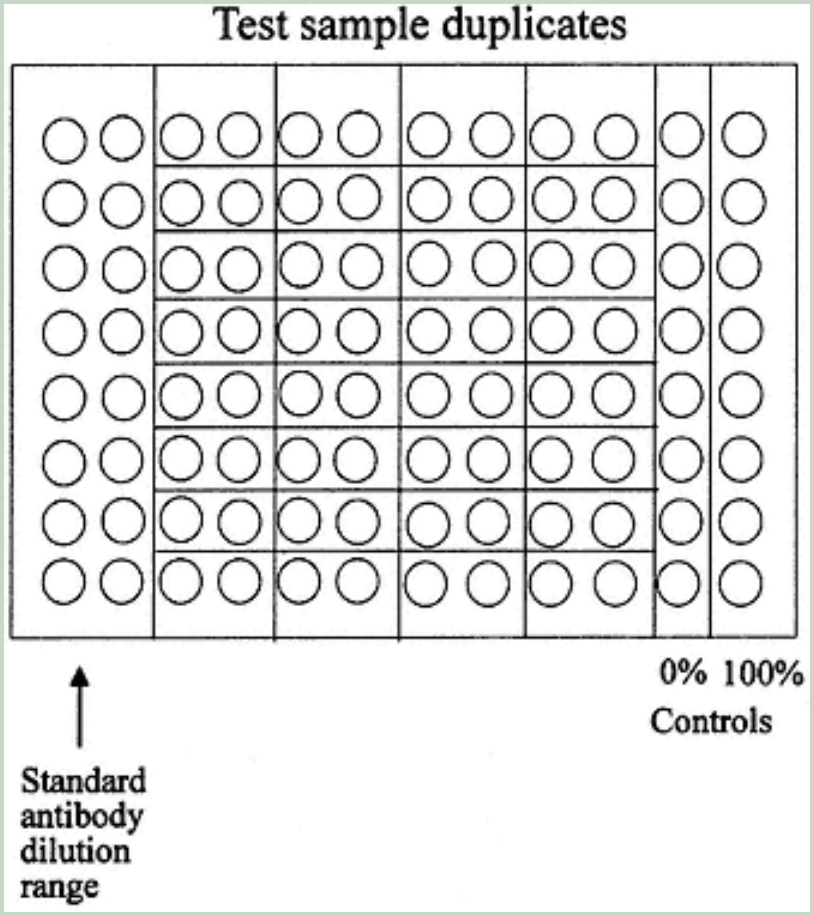


Fig. 41.
Plate layout for spot testing in competition assay.

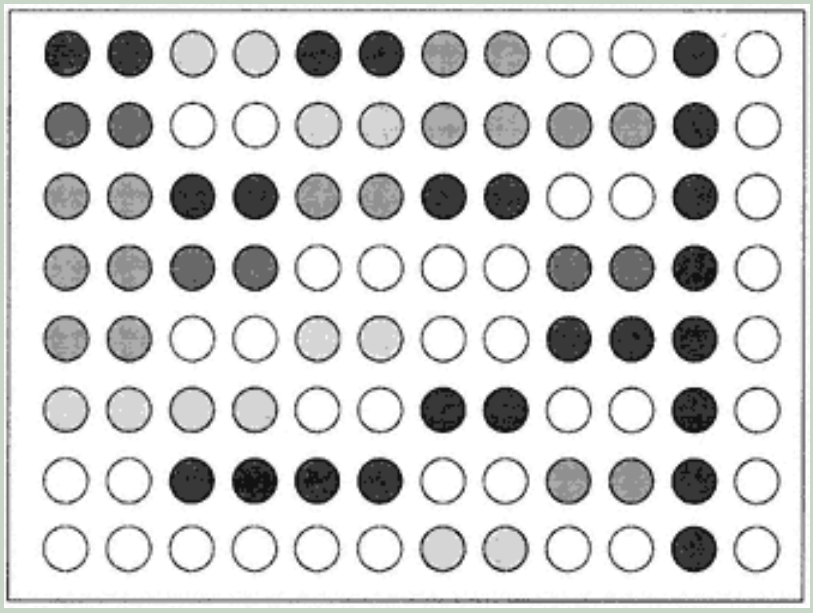


Fig. 42.
Representation of a spot test competition assay.
Data are given in Table 19.

Table 19
Plate Data from Spot Test

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.31	1.30	1.31	1.33	1.32	1.32	1.26	1.34	1.12	1.10	1.34	0.06
A	1.21	1.24	0.34	0.32	1.23	1.21	1.12	1.12	0.09	0.09	1.23	0.07
B	1.03	1.05	0.19	0.18	0.43	0.45	0.56	0.58	0.78	0.78	1.21	0.09
C	0.91	0.90	1.13	1.15	0.67	0.69	1.11	1.13	0.12	0.14	1.24	0.08
D	0.76	0.73	0.98	0.96	0.13	0.12	0.16	0.13	0.78	0.80	1.24	0.09
E	0.53	0.54	0.06	0.04	0.34	0.36	0.16	0.18	1.23	1.21	1.23	0.08
F	0.31	0.34	0.34	0.36	0.14	0.16	1.17	1.19	0.08	0.10	1.21	0.07
G	0.12	0.13	1.21	1.23	1.14	1.11	0.09	0.07	0.67	0.69	1.26	0.05
H	0.06	0.08	0.06	0.09	0.15	0.12	0.23	0.27	0.10	0.12	1.23	0.06

7.8.5¡[a]
Treatment of Data

For the other competition results, make the following calculations:

1. Calculate the mean of column 12, and subtract this from all results from the other wells.

2. Calculate the mean from column 11 (after subtraction in **step 1**).

3. Express the other ODs as a percentage of the range 0 to the mean column 11, i.e., from 0 to 1.16 in the example above.

4. Take the mean OD of the duplicate wells.

5. Use the following formula to calculate the percentage of competition in each well:

$$100 - [(OD/1.16) \times 100]$$

Plot the standard serum competition activity relating competition to $\log_{10}$ of the dilution.

6. Read the relative titers of the other competition results from the curve.

7. Another approach to evaluation of spot testing is that in which accepted negative sera are assessed as controls. Several sera can be included in a test so that their mean competition value and its variation can be assessed. Thus, sera giving higher values of competition under the same conditions (with prescribed confidence limits) can be assessed for positivity. Studies on a large number of negative sera give better population data as described for the other assays, so that chosen negative controls may be added from the defined population (*see* **Table 20**). In **Table 20**, the sera with asterisks could be the negative controls in order to test whether the system was ideal. The percentage value of their mean plus a defined interval as a percentage of this mean (as directed from large population studies) could be given. Here, we have mean = 3%. Assume that two times this mean is an acceptable upper limit for negative competition values. Therefore, sera could be ascribed as positive with competition values ≥6%.

Table 20
Mean Values of Test Sera from Table 19
Processed as Competition Values

	Competition results (%)				
	1 2	3 4	5 6	7 8	9 10
A	0	73	2[a]	16	99
B	16	90	68	57	39
C	28	7[a]	48	9	95
D	41	23	96	93	38
E	59	100	76	91	0
F	78	76	93	4[a]	98
G	100	100	95	84	97

[a]Sera could be used as negative controls.

7.8.6¡[a]
Notes

We have used a dilution of 1/100 for the test sera in the example. This is based on preliminary studies establishing the dilution as being optimal for distinguishing positive and negative values. This must be attempted in your laboratories for specific disease studies. The approach to examination of negative populations has already been discussed. In the case of competition assays, a lower dilution of test serum might be used (effectively increasing the sensitivity of the assay) since nonspecific factors detected in the indirect assay do not seem to affect competition assay results. Construction of full-scale serum titration competition curves of many negative and positive sera will nominate the best dilution (with definable confidence limits) of serum to be used. The sources of such sera have already been discussed.

Thus, for any particular dilution used in the competition assay, an upper limit of negativity should be definable (as a competition value) above which positivity of antibody will be detected. Once competition assays have been characterized in central laboratories, it is usually simple to read the assays by eye, with good levels of precision and sensitivity. In these cases, the selection of appropriate negative controls that define upper limits of negativity as determined by eye is important.

7.8.7¡[a]
Conclusions about Competition Assays

1. Competition assays provide a relatively simple method once the homologous systems have been titrated.

2. These assays can be read by eye, with some loss of sensitivity and reduction in confidence limits.

3. In all the examples given, we have used 50 µL of competitor and 50 µL of homologous serum as a mixture to compete for only 50 µL of antigen on the solid phase. You can alter the volumes to suit. For example:

a. 100 µL of antigen solid phase vs 50 µL of homologous serum and 50 µL of competing antigen (or antibody). In this case, the pretitration would be with 100 µL of solid-phase antigen vs 50 µL of serum dilutions + of 50 µL blocking buffer.

b. 50 µL of solid-phase antigen vs 25 µL of homologous serum + 25 µL competing antigen (or antibody). In this case the pretitration would be between 50 µL of solid-phase antigen and 25 µL of antibody dilutions + 25 µL of buffer.

c. The competitor and homologous serum can be mixed together in another plate before addition to the solid-phase antigen plate. These types of assay can be termed inhibition assays since the reagents are not directly competing in the same system.

4. Differences in results can be observed by alteration of the sequence of reagents, i.e., when true competition and inhibition methods are used. In practice, the mixing of reagents in a true competition assay gives the most sensitive assays and best reflects avidity differences among reagents.

7¡ª
Monoclonal Antibodies

The ability to produce and exploit monoclonal antibodies mAbs has revolutionized many areas of biological sciences. The unique property of an mAb is that it is a single species of immunoglobulin (IG) molecule. This means that the specificity of the interaction of the paratopes on the IG, with the epitopes on an antigenic target is the same on every molecule. This property can be used to great benefit in immunoassays to provide tests of defined specificity and sensitivity, which improve the possibilities of standardization. The performance of assays can often be determined relating the actual weight of antibody (hence the number of molecules) to activity. Often the production of an mAb against a specific epitope is the only way that biological entities can be differentiated. This chapter outlines the areas involving development of assays based on mAbs. The problems involved address the physical aspects of mAbs and how they may affect assay design and also the implications of results based on monospecific reagents. Often these are not fully understood, leading to assays that are less than satisfactory, which does not justify the relatively high cost of preparing and screening of mAbs.

There are many textbooks and reviews dealing with the preparation of mAbs, the principles involved, and various purification and manipulative methods for the preparation of fragments and conjugation. There has been little general information attempting to summarize the best approaches to assay design using mAbs. Much time can be wasted through bad planning, and this is particularly relevant to mAbs. A proper understanding of some basic principles is essential. It is beyond the scope of this chapter to discuss all aspects, but major areas are highlighted.

1¡ª
Purpose of the Assay Involving the mAb

The fundamental question, "What is the purpose of the assay involving the mAb?" may never be properly considered in sufficient detail. The first consid-

erations presented in the following list address the "state of knowledge" about a problem (disease organism, serology, antigenic structure, other tests) and available reagent base. These are in common with other test developments but are particularly relevant to mAbs:

1. How much knowledge do we have about other current assays?

2. How much information have do we about the target antigen (organism)?

3. What information is there in the literature?

4. What time frame have we got for development of an assay?

5. What advantages and disadvantages will an mAb-based assay have over conventional techniques or polyclonal systems?

6. What polyclonal reagents do we have available from their use in other tests?

Analysis of this information should indicate whether mAbs are needed to provide the necessary properties of a test.

1.1¡[a]
Availability of mAbs

mAbs are available from two sources. The first is from other laboratories. Such mAbs will have been subjected to various degrees of characterization, and it is vital that all data pertaining to the mAbs be obtained. This will allow a better selection process for candidate mAbs for use in designing assays. The second source is to produce them either in-house where there are facilities, or through a contract with a specialist laboratory acting in a service capacity.

The cost of setting up and maintaining an mAb production facility is great, and usually resources are made available only where there is a need to produce a variety of mAbs against a number of agents. A typical laboratory making mAbs must consider carefully the logistical problems not only of tissue culture maintenance to a high standard but cloning, screening, amplification of mAb (increasing the amount of mAb produced), and storage of cell lines. In other words, careful planning with a full knowledge of the implications of mAb production is essential. Production methods have become easier as technologies surrounding the methods have improved, so it is feasible to set up production in a relatively small laboratory. The basics of production must include at least one dedicated worker responsible for maintaining the mAb production, animal facilities (with appropriate legal approvals), and enough financial support to have a sustainable work program.

A salutary truth is that there is no certainty of success when embarking on production. However, the first essential factor in planning is to decide exactly what the purpose of an mAb will be, so that the correct mAbs can be identified as quickly as possible and the work effort concentrated on these. It is far too tempting in practice to try to handle all the mAbs produced in a fusion.

Table 1
Scheme for Preparation and Characterization of mAbs Using ELISA

1. Growth of cells in microplates; secretion of immunoglobulin into maintenance medium	Spot test for levels; spot test for specific activity of mAbs
2. Growth of selected hybridomas; stabilization; secretion of IG into medium	Spot test for levels of IG; spot test for specific activity
3. Larger-scale production in tissue culture, or ascites; secreted mAb or as ascites fluid.	Specific activity for each mAb determined using one or more antigen (epitope); isotyping
4. Purification of mAbs; preparation of fragments?; conjugation; assay design	Specific activity of each mAb determined using different epitopes; quantification of IG; performance of mAbs as capture reagent; examination of conjugates

5. Use of mAbs	Standardized reagents; sifferentiating reagents; specific diagnostic reagents; panels used to profile epitopes in epidemio logical studies; epitope characterization

1.2¡ª
Planning

Clear planning involves consideration of the immunization regime that might best give a range of mAbs most relevant to their intended task. Again, reference to developments in this field will give clues as to the best methods. Basically, mice (usually) have to be exposed to a specific antigen(s) and must stimulate antibody-producing cells. The approach can be "shotgun" in which a relatively complex and "crude" mixture of antigens is used as the immunizing agent, or it can involve administration of a pure, defined antigen. The first approach has implications when the antibody secreted from hybridomas (fused cells secreting antibodies) need to be screened for activity. Screening and selection of appropriate clones is time-consuming, requires assay development, and should always have the aim of finding as quickly as possible the antibodies of direct relevance to the preconceived objectives of the required function. **Table 1** presents a typical scenario for the screening and eventual isolation and use of mAbs.

1.3¡ª
Screening mAbs after Fusion

Screening should be selective as early as possible. Thus, the appropriate antigen has to be used in screening to test for antibody binding. The ELISA systems are ideal for this and, often at this stage, rely on data from other

polyclonal assays. As an example, the concentration of an antigen that gives a good level of coating and signal in a polyclonal serum-based assay might be known. This concentration can be used to screen for mAbs using an indirect ELISA.

The essential work in the early stages is to have actively growing hybridomas secreting IG, and to ensure that these stem from single cells. Usually at the early stages of producing stable hybridomas, only a small amount of tissue culture fluid is available for testing, so that assays are made using a single dilution. This limits the possibilities of screening against a variety of antigens, which would allow some differential analysis of binding to be made at this stage. Therefore, it may be more useful to select an antigenic mixture at this stage. The specificity of the mAbs could then be determined when they are available in higher amounts.

The antimouse conjugate used must detect all isotypes of mouse IG equally well (these are commercially available). Mouse IGs have a number of specific isotypes and in order not to miss any possible binding, the conjugate must detect IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 isotypes.

There is a similarity in stages 2 and 3 in **Table 1**. This indicates that screening can be made quite early when it is reasonably certain that colonies of cells have been derived from single cells, depending on the methods used.

When single cells have been picked, there may be fewer clones to examine, but confidence in their clonality is high. Thus, screening here may be more relevant than for limit dilution methods. From a practical point of view, it might be useful to give a few figures for good and bad fusion results. An initial target of 200¨C500 clones (representing the handling of two-to four-microtiter plates) is reasonable. These reflect my experience and depend greatly on exactly which methods are used and the experience of the operators involved as well as the biological system being examined. They also depend on exactly how much work is put into the production. There must always be a realistic balance between achieving a target mAb and the maintenance of thousands of clones. Of these clones, many will turn out to be unproductive for various reasons, and hence useless. The maintenance and testing of 200¨C500 clones in the early stages is manageable by a single person. Increasing this number runs the risk of poor management. The essential target of this part of the operation is to produce colonies of cells that are secreting mAb. The testing of the mAb as early as possible will highlight candidate mAbs for further testing, stabilization, and use.

This scenario is further complicated if mAbs against several antigens are being produced by different operators. It is extremely difficult to control operations in which several runs are being made, each requiring operative procedures at different times.

The examination of whether colonies are secreting mouse IG might sometimes be useful, especially when there is little activity against target antigens in screening. Nonsecretors can also be discarded. This can be accomplished easily through developing a competitive assay. The competitive assay for determining mouse IgG is described next, for reference and to introduce techniques and terms that will be generally used.

2¡ª Competitive Assay for Determination of Mouse IgG

2.1¡ª Requirements

1. Mouse Igs isolated from whole mouse serum.

2. Antimouse enzyme-labeled conjugate (commercially available reacting with all isotypes of mouse Igs).

3. Microtiter plates (96-well ELISA plates).

4. Micropipets (5- to 50-µL variable volume), single and multichannel pipets (12 channel), and tips.

5. Reservoirs for reagents.

6. 5- to 20-mL bottles.

7. Relevant substrate/chromogen solution for enzyme conjugate.

8. Phosphate-buffered saline (PBS) (0.1 *M*, pH 7.4).

9. Blocking buffer: 5% skimmed milk powder in PBS.

10. Relevant agent/conditions for stopping reaction after addition of chromogen/substrate.

11. Multichannel spectrophotometer.

12. Samples for testing.

13. Washing solution (PBS, pH 7.4, 0.02¨C0.1 *M*).

2.2¡ª
Preparation of Mouse Ig

Preparation of mouse Ig requires a minimum of 2 mL of mouse serum. A relatively simple technique for the preparation of total mouse Ig from serum, and one that gives adequately pure Ig for this test, is to use ammonium sulfate precipitation. Once the Ig is obtained and dialyzed against, e.g., PBS as used in ELISA, the concentration can be assessed either by protein estimation using chemical kits or, more easily, by reading the absorbance at 280 nm in an ultraviolet (UV) spectrophotometer. The concentration of Ig can be related to the absorbance using the following formula:

$$\text{Absorbance (280 nm)} \times 0.7 = \text{mg/mL of Ig}$$

However, the formula is only true for relatively pure IgG. Once this has been accomplished, a solution containing a known concentration of mouse Ig is available. It should be stored in small aliquots at ¨C20¡ãC. For the sake of this example, we can make the assumption that a sample with 1 mg/mL (i.e., 1000 µg/mL) has been obtained directly or has been diluted to this concentration.

This process should take a few h to precipitate Ig followed by overnight dialysis allowing for changes in dialyzing solution.

2.3¡ª
Stage 1:
Optimization of the Mouse/Antimouse System

The objective of this stage is to titrate the mouse Ig, when attached to the solid phase, against the antimouse conjugate. Optimal conditions specifying the dilution of mouse Ig (antigen) and conjugate will be determined. The system will then be challenged by the addition of known Ig concentrations to the conjugate on addition to the mouse Ig¨Ccoated wells. A standard curve relating the concentration of mouse Ig added and the degree of inhibition of pretitrated reaction between the mouse Ig antigen on the plate and the conjugate can be established. Readings of inhibition by samples possibly containing mouse Ig can then be used to estimate the concentration of mouse Ig. Although this may seem complicated, the test can be set up in 2 d. Such a system is also relevant for determination of concentrations of Ig for any antiserum species. The prepared Ig will act as the standard solution for all competitive tests, so it needs to be protected from contamination by storing at low temperature.

2.3.1¡ª
Practical Details of Stage 1:
Chessboard Titration

1. Dilute the mouse Ig to 5 µg/mL in PBS. Prepare a volume of 1 mL of this solution. For our example, we have 1000 µg/mL so we add 5 µL of this to 1 mL (1000 µL) of PBS. Mix well without causing frothing.

2. Add 50 µL of PBS to columns 2¨C12 of a microtiter ELISA plate.

3. Add 50 µL of Ig solution to columns A and B, using a 50-µL pipet.

4. Place eight tips on a multichannel pipet, and set the volume to 50 µL. Place the tips in column 2. Mix the contents (50 µL of PBS and 50 µL of Ig solution) by gentle pipeting action four times. Take up 50 µL of mixed solution in column 2 and transfer to column 3. Repeat the mixing exercise in column 3. Transfer to column 4, repeat, and so on until column 11. Discard the last 50 µL after final pipeting action in column 11. We now have the same dilution range of Ig in 50-µL vol, added to rows A¨CH, beginning at 5 µg/mL and diluted twofold to column 11.

5. Incubate the plates for 2 h at 37¡ãC.

6. Wash the plates by flooding and emptying wells 5 times in PBS. Then blot almost dry by tapping plates onto a sponge of paper towel.

7. Obtain conjugate and read the titer recommended by the commercial company. Make a dilution of approximately eight times that recommended; for example, if the recommended dilution is 1/400, make up 1/500. A final volume of 1 mL (1000 µL) is needed in this stage. Thus, add 2 µL of conjugate to 1000 µL of PBS. An examination of the appropriate pipet to use with small volumes is required. Where there is no pipet to deliver 2 µL accurately, then a 5-µL vol can be used into the appropriate volume; however, this does waste conjugate. Mix the conjugate solution without undue force.

Table 2
Data from CBT of Ig and Conjugate[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2	2.3	2.2	2.1	2.0	2.0	1.9	1.6	1.5	1.3	0.8	0.6
B	2.2	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.8	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5	1.2	0.7	0.5	0.3
D	1.9	1.9	1.7	1.8	1.5	1.2	1.0	0.7	0.5	0.3	0.2	0.1
E	1.8	1.8	1.7	1.5	1.3	1.1	0.8	0.6	0.5	0.3	0.2	0.1
F	1.3	1.3	1.2	1.2	1.1	0.8	0.6	0.5	0.3	0.2	0.1	0.1
G	0.7	0.7	0.7	0.6	0.6	0.5	0.3	0.2	0.1	0.1	0.1	0.1
H	0.4	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1

[a]Conjugate diluted from A to H (e.g., 1/500 in above example). Mouse Ig is diluted from 1 to 11 (5 µg/mL starting concentration in this example).

8. Add 50 µL of blocking buffer to all the wells of the Ig-coated microplates using a multichannel pipet.

9. Add 50 µL of the diluted conjugate to each well of row A.

10. Using a multichannel pipet with 12 tips, mix the contents of row A. Transfer 50 µL to row B, mix, transfer to row C, and so on until row H. Discard the last 50 µL in the tips. We now have a dilution of conjugate in blocking buffer (50 µL per well) from 1/1000 (since we added 50 µL at 1/500 to 50 µL of buffer) to 128,000.

11. Incubate the plates for 2 h at 37¡ãC.

12. Wash the plates, and blot.

13. Add the appropriate substrate/chromogen solution, leave for the prescribed time, and stop the reaction.

14. Read the OD values on a spectrophotometer.

2.3.2¡ª Results

Table 2 gives typical results obtained with a horseradish peroxidase (HRP) conjugate/H_2O_2-*ortho*-phenylenediamine (OPD) system.

Chessboard titrations (CBTs) have already been considered in some detail *(see* Chapter 4). In **Table 2**, row E in this plate indicates that there is a good level of color development with the conjugate at 1/8000. The plateau maximum value in the presence of excess antigen (mouse Ig) is acceptable, and the Ig is detected to column 11 (with respect to a plate background value of 0.1).

For the final competitive assay we require a single dilution of coating antigen. Ultimately, the sensitivity of the assay depends on the concentrations of conjugate and Ig, so that we are seeking a level of coating antigen Ig that does not saturate the wells, and a conjugate dilution that just reacts with all the coating Ig. The box in **Table 2** reflects the area where this is relevant. Stage 2

involves closer examination of the concentrations under competitive conditions to assess the best system in practice. This procedure takes only 1 d. The box in **Table 2** covers three antigen dilutions and two conjugate dilutions, which can be examined in stage 2.

2.4¡ª Practical Details Stage 2: Competition of Standard IgG with Titrated Conjugate and Coating Ig

2.4.1¡ª Materials

The materials needed are the same as those used in stage 10. Two microtiter plates are needed: plate 1 for the competition conditions, and plate 2 to set up controls for 100% competition values.

2.4.2¡ª Method

The diagram in **Fig. 1** shows a template indicating the addition of reagents to plate 1.

1. Coat the wells of the plate (50 µL per well in PBS) with IgG at the three dilutions identified in stage 1. The microplate can be rotated so that the long edge is vertical and to the left of the operator (row H). Add the first dilution of IgG to wells 1¨C4, A¨CH. Add the second dilution to wells 5¨C8, A¨CH. Add the third dilution to wells 9¨C12, A¨CH. We have 1.5, 0.75, and 0.375 µg/mL for the three dilutions of Ig to coat the wells. The same conditions for coating concerning incubation and washing are as already described.

2. Wash the wells with PBS.

3. Add competing Ig as a dilution range from 5 µg/mL across seven wells. The Ig is diluted in blocking buffer to prevent it from coating the wells.

4. Add the conjugate (50 µL per well) as shown in **Fig. 1**. The dilutions x and y represent the dilutions identified in stage 1.

5. Incubate the plates for 2 h at 37¡ãC as in stage 1.

6. Wash the wells.

7. Add the appropriate substrate/chromogen, and stop the reaction.

8. Read the results on a spectrophotometer.

Plate 2

1. In place of the coating step with Ig, add 50 µL of coating buffer to wells H¨CA, 1¨C4. Incubate as for coating.

2. After washing, add PBS diluent (50 µL) to each well as a substitute for the competing Ig.

3. Add the two dilutions of conjugate used in plate I in 50-µL vol to the wells: 1/*x* dilution in blocking buffer from H¨CA, 1 and 2; 1/*y* dilution to wells H to A, 3 and 4. Incubate as for the competition stage in plate 1.

4. Wash and add substrate/chromogen, incubate, and stop the reaction as for plate 1. Read the results.

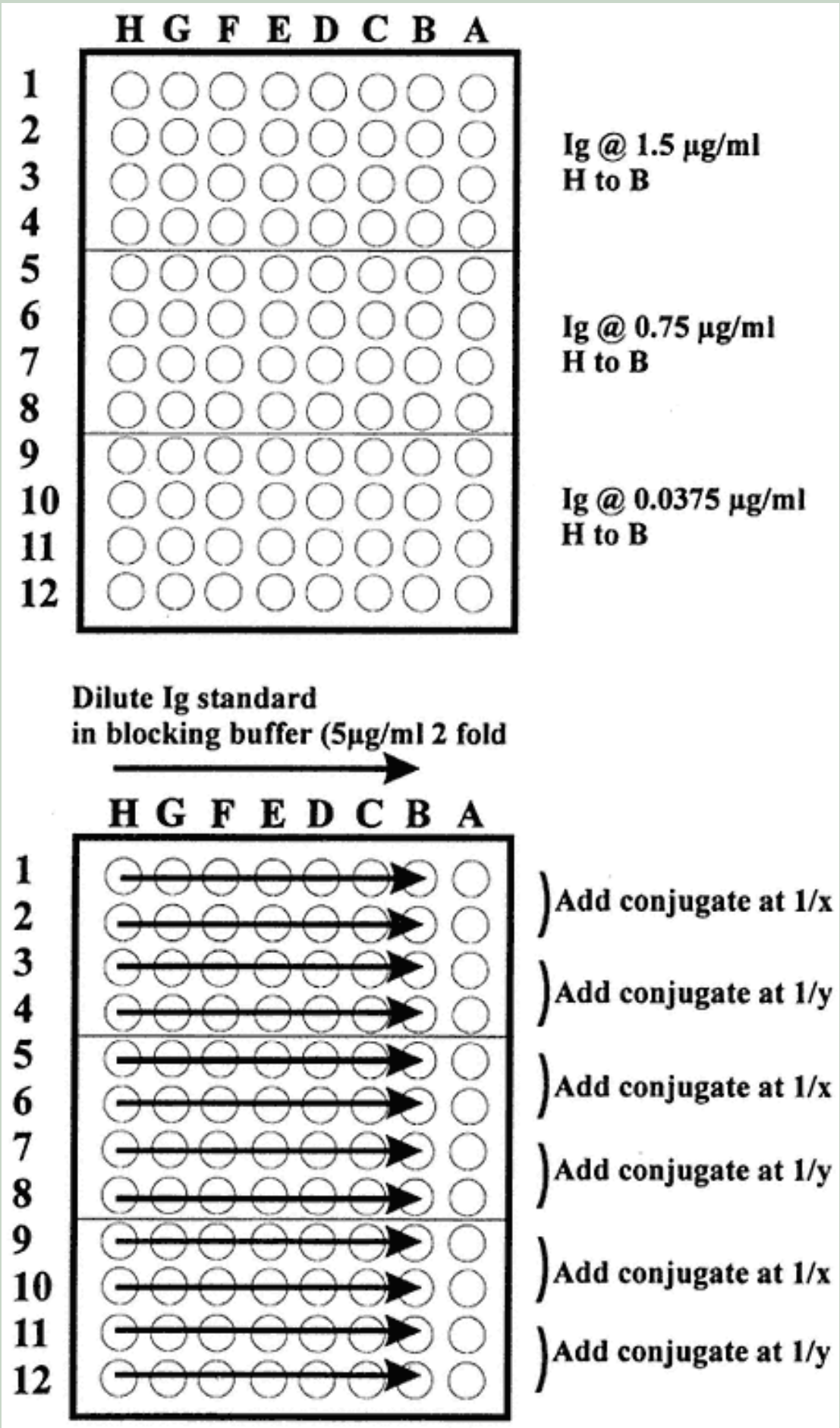


Fig. 1.
Scheme for addition of reagents to assess optimal concentrations for competition assay.

2.4.3¡[a]
Results

Typical results are given in **Table 3**. The mean values for the replicates are shown in bold. In plate 1, the data show that the addition of Ig as a competitor for the labeled antimouse conjugate had the effect of reducing color when it was added at high concentrations, as indicated in the wells H, G, and F. As the

Table 3
Plate 1 and 2 Data

	H	G	F	E	D	C	B	A	Ig mg/mL
					Plate 1 data				
1	0.30	0.35	0.51	1.25	1.45	1.81	1.80	1.81	1.5
2	0.32	0.37	0.53	1.25	1.47	1.77	1.82	1.83	1.5
Mean	**0.31**	**0.36**	**0.52**	**1.25**	**1.46**	**1.79**	**1.81**	**1.82**	**1/*x* conjugate**
3	0.24	0.33	0.64	1.05	1.21	1.39	1.45	1.49	1.5
4	0.26	0.35	0.68	1.05	1.21	1.37	1.45	1.51	1.5
Mean	**0.25**	**0.34**	**0.66**	**1.05**	**1.21**	**1.38**	**1.45**	**1.50**	**1/*y* conjugate**
5	0.17	0.21	0.33	0.44	0.67	0.89	1.14	1.16	0.75
6	0.19	0.23	0.29	0.42	0.69	0.87	1.12	1.18	0.75
Mean	**0.18**	**0.22**	**0.31**	**0.43**	**0.68**	**0.88**	**1.13**	**1.17**	**1/*x* conjugate**
7	0.09	0.14	0.23	0.35	0.67	0.89	0.93	0.98	0.75
8	0.07	0.16	0.25	0.37	0.71	0.85	0.97	1.00	0.75
Mean	**0.08**	**0.15**	**0.24**	**0.36**	**0.69**	**0.87**	**0.95**	**0.99**	**1/*y* conjugate**
9	0.06	0.09	0.18	0.27	0.45	0.67	0.76	0.76	0.375
10	0.04	0.11	0.16	0.29	0.47	0.63	0.78	0.80	0.375
Mean	**0.05**	**0.10**	**0.17**	**0.28**	**0.46**	**0.65**	**0.77**	**0.79**	**1/*x* conjugate**
11	0.04	0.06	0.10	0.17	0.29	0.46	0.51	0.58	0.375
12	0.04	0.08	0.12	0.21	0.29	0.44	0.55	0.58	0.375
Mean	**0.04**	**0.06**	**0.11**	**0.19**	**0.29**	**0.45**	**0.53**	**0.58**	**1/*y* conjugate**
					Plate 2 data				
1	0.07	0.09	0.08	0.10	0.07	0.07	0.05	0.07	
2	0.06	0.04	0.08	0.05	0.05	0.06	0.05	0.08	
1/*x* mean of 16 wells = 0.07									
3	0.04	0.05	0.06	0.05	0.06	0.04	0.08	0.05	
4	0.06	0.08	0.05	0.4	0.05	0.03	0.04	0.05	
***1/y* mean of 16 wells = 0.05**									

competing Ig was reduced the effect was also reduced. Plate 2 results show that there is a low color representing the background.

To calculate the degree of competition, a calculation of the range of values with 100% and 0% competition is needed. The 100% competition values for both dilutions of conjugate used can be taken from mean values on plate 2. We have $1/x = 0.07$; and $1/y = 0/05$.

The 0% values for each of the conjugate dilutions and each of the IgG coating conditions are read for the respective concentrations in column A of plate 1. These are contained within the line drawn on the results of plate 1.

Table 4
Mean Values in Competition Assay Minus the Background Values for Relevant Conjugate Dilution Used

	0.31	0.36	0.52	1.25	1.46	1.79	1.81	1.82	
=	0.24	0.29	0.45	1.18	1.39	1.72	1.74	1.75	¨C1/*x* conjugate (0.07)
	0.25	0.34	0.66	1.05	1.21	1.38	1.45	1.50	
=	0.20	0.29	0.61	1.00	1.16	1.33	1.40	1.45	¨C1/*y* conjugate (0.05)
	0.18	0.22	0.31	0.43	0.68	0.88	1.13	1.17	
=	0.11	0.15	0.24	0.36	0.61	0.81	1.06	1.10	¨C1/*x* conjugate (0.07)
	0.08	0.15	0.24	0.36	0.69	0.87	0.95	0.99	
=	0.03	0.10	0.19	0.31	0.64	0.82	0.90	0.94	¨C1/*y* conjugate (0.05)
	0.05	0.10	0.17	0.28	0.46	0.65	0.77	0.79	
=	¨C0.02	0.03	0.10	0.21	0.39	0.58	0.70	0.72	¨C1/*x* conjugate (0.07)
	0.04	0.06	0.11	0.19	0.29	0.45	0.53	0.58	
=	¨C0.01	0.01	0.06	0.14	0.24	0.40	0.48	0.53	¨C1/*y* conjugate (0.05)

These results show the color obtained for a particular coating concentration of Ig with the conjugates at different dilutions.

2.4.4¡[a] Calculations

The mean value of the conjugate control for both the dilutions of conjugate obtained in plate 2 is subtracted from the values obtained in which that dilution of conjugate was used. This is shown in **Table 4**.

The values for the reaction between the conjugate and various concentrations of coating Ig without competition (0% competition) are shown in the gray boxes. This value is now used in the following formula to calculate the effect of adding the competing Ig:

$$\text{Competition} = 100 - [(\text{OD test}/\text{OD conjugate}) \times 100]$$

As an example: taking the first line of data we have the following values:

Value

0.24 100 ¨C [(0.24/1.75) ¡Á 100] = 100 ¨C (0.14 ¡Á 100) = 100 ¨C 14 = 86%

0.29 100 ¨C [(0.29/1.75) ¡Á 100] = 100 ¨C (0.17 ¡Á 100) = 100 ¨C 17 = 83%

0.45 100 ¨C [(0.45/1.75) ¡Á 100] = 100 ¨C (0.26 ¡Á 100) = 100 ¨C 26 = 74%

0.92 100 ¨C [(1.18/1.75) ¡Á 100] = 100 ¨C (0.67 ¡Á 100) = 100 ¨C 67 = 33%

1.27 100 ¨C [(1.39/1.75) ¡Á 100] = 100 ¨C (0.79 ¡Á 100) = 100 ¨C 79 = 21%

1.47 100 ¨C [(1.72/1.75) ¡Á 100] = 100 ¨C (0.98 ¡Á 100) = 100 ¨C 98 = 2%

1.74 100 ¨C [(1.74/1.75) ¡Á 100] = 100 ¨C (0.99 ¡Á 100) = 100 ¨C 99 = 1%

For the second row or results we have a different value for the % competition OD: 1.5 as follows:

Value

0.20 100 ¨C [(0.20/1.45) ¡Á 100] = 100 ¨C (0.13 ¡Á 100) = 100 ¨C 13 = 87%

0.29 100 ¨C [(0.29/1.45) ¡Á 100] = 100 ¨C (0.19 ¡Á 100) = 100 ¨C 19 = 81%

0.44 100 ¨C [(0.66/1.45) ¡Á 100] = 100 ¨C (0.46 ¡Á 100) = 100 ¨C 46 = 54%

0.84 100 ¨C [(1.05/1.45) ¡Á 100] = 100 ¨C (0.72 ¡Á 100) = 100 ¨C 72 = 28%

1.08 100 ¨C [(1.21/1.45) ¡Á 100] = 100 ¨C (0.83 ¡Á 100) = 100 ¨C 83 = 17%

1.21 100 ¨C [(1.38/1.45) ¡Á 100] = 100 ¨C (0.95 ¡Á 100) = 100 ¨C 95 = 5%

1.40 100 ¨C [(1.40/1.45) ¡Á 100] = 100 ¨C (0.97 ¡Á 100) = 100 ¨C 97 = 3%

Similar calculations can be made on all the other results. These are shown in **Table 5**, which lists all the data.

From these data we can determine optimal coating and conjugate dilutions to be used to test Ig in samples. Although it looks complicated, the whole procedure is accomplished in 2 d and is usually straightforward. Again, the calculations, seem problematic, but careful consideration will indicate that they are relatively simple.

In practice, examination of the data in the tabular form should be enough to set up optimal conditions. The optimal conditions can be assessed best if data are plotted. **Figure 2** illustrates features of the competition curves. All the curves illustrate that there is competition for the expected binding of the conjugates. The best combination of reagents to analyze samples is obtained by considering the effective analytical sensitivity of each combination. The estimate of sensitivity is made by examining the weight of competing Ig at the 50% competition point. Thus, a line drawn across the 50% point is shown in **Fig. 2**. The weight of Ig standard needed to give 50% inhibition of the conjugate binding for each combination can be measured as shown in **Fig. 3**, by plotting the perpendicular to the *x*-axis where the curve crosses the 50% competition line. Ideally, the weight of competing Ig at the 50% competitive point should be the same as that added to coat the wells, assuming that all the Ig added was bound to the wells.

Note that it is much easier to plot the competition data on semilog graph paper. The competition is plotted on an arithmetic scale and related to $\log_{10}$ of the competing Ig concentration. In this way, the values for the various concentrations can be directly read from the scale. This approach also simplifies all other data plotting in which the *x*-axis is a scale of activity. **Figure 4** presents the same data plotted in this way. The values for the combination are shown in **Table 6**.

2.4.5¡[a]
Conclusion

All conditions show that the conjugate was competed for on the addition of the known standard concentrations of Ig. A standard curve relating added Ig concentration to competition is obtained. The choice of best conditions is a

Table 5
Percentage Competition Values

Conjugate	Competing antigen (µg/mL)							Coating Ig
dilution	5	2.5	1.25	0.63	0.32	0.16	0.08	(µg/mL in 50 µL)
1/*x*	86	83	74	33	16	2	1	1.5
1/*y*	87	81	54	28	17	5	3	
1/*x*	90	86	78	67	45	26	4	0.75
1/*y*	97	89	80	67	32	13	4	
1/*x*	101	96	86	71	46	19	3	0.375
1/*y*	101	98	89	74	63	25	15	

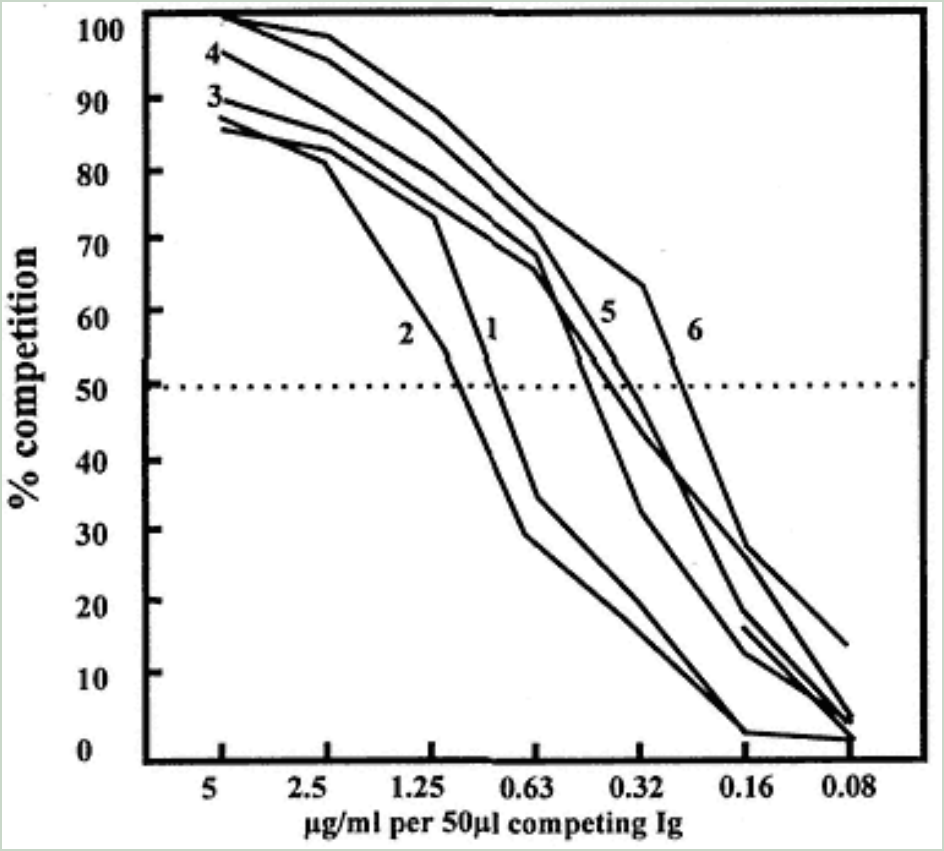


Fig. 2.
The six lines show the competition data for the various combinations of coating concentrations and conjugate dilution. Lines 1 and 2 show data for 1.5 µg/mL, 3 and 4 for 0.75 µg/mL, and 5 and 6 for 0.375 µg/mL coating. The conjugate dilution for 1, 3, and 5 was 1/*x;* for 2, 4, and 6, the conjugate dilution was 2/*y*. The 50% competition point is indicated by the dashed line.

balance between required sensitivity and likely accuracy of the test. Screening for secretion of mouse Ig does not require high precision, the results are relative within the standard system used with reference to the conjugate. The sen-

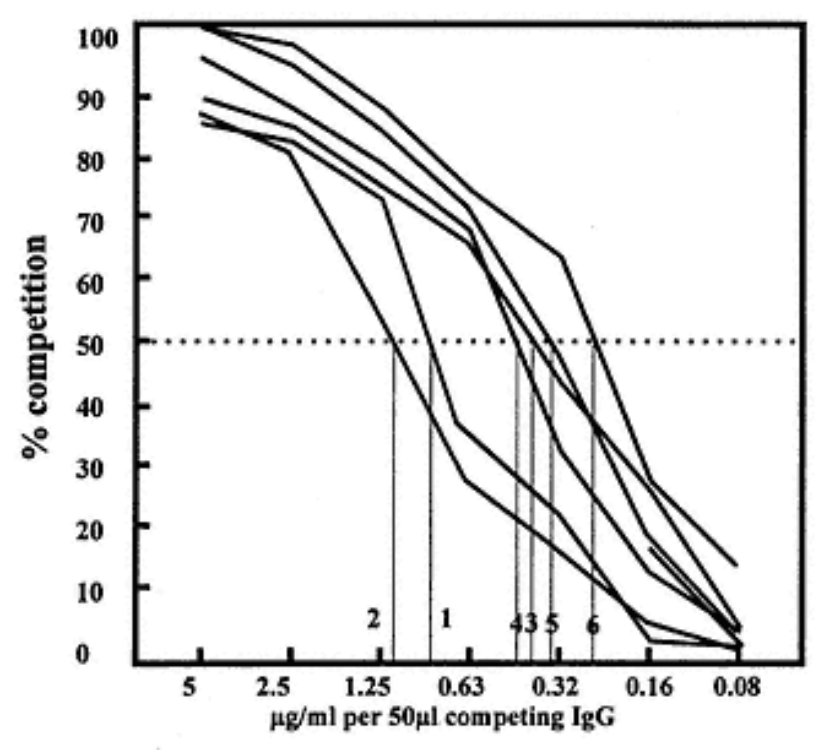


Fig. 3.
The six lines show the competition data for the various combinations of coating concentrations and conjugate dilution. The competing Ig at 50% is indicated for each of the combinations 1¨C6.

sitivity is lowest when there are high levels of antigen Ig coating and higher levels of conjugate. In this case, there is more conjugate and antigenic target to be competed for. Reference to the OD values obtained for the 0% control indicates that this is rather high (OPD substrate system). The intermediate values of coating plates result in good titrations over the range of Ig added with OD values for 0% competition in a good area for this substrate. The sensitivity is increased here from about 1 µg/mL in 50 µL to about 0.4 µg/mL. The curves are also extended more into the area below which 50% competition is achieved. The third coating concentration gives a slightly higher sensitivity, but the OD values for control 0% competition can be regarded as too low. The variation in results increases with reaction in the colored product. An optimal dilution of coating Ig at about 0.75 µg/mL with a conjugate dilution equivalent to 1/*y* appears suitable for screening.

2.5¡ª
Use of Competition Assay to Assess Samples

The conditions established can now be used routinely for screening unknown samples for the presence of mouse Igs. The conditions for coating, washing,

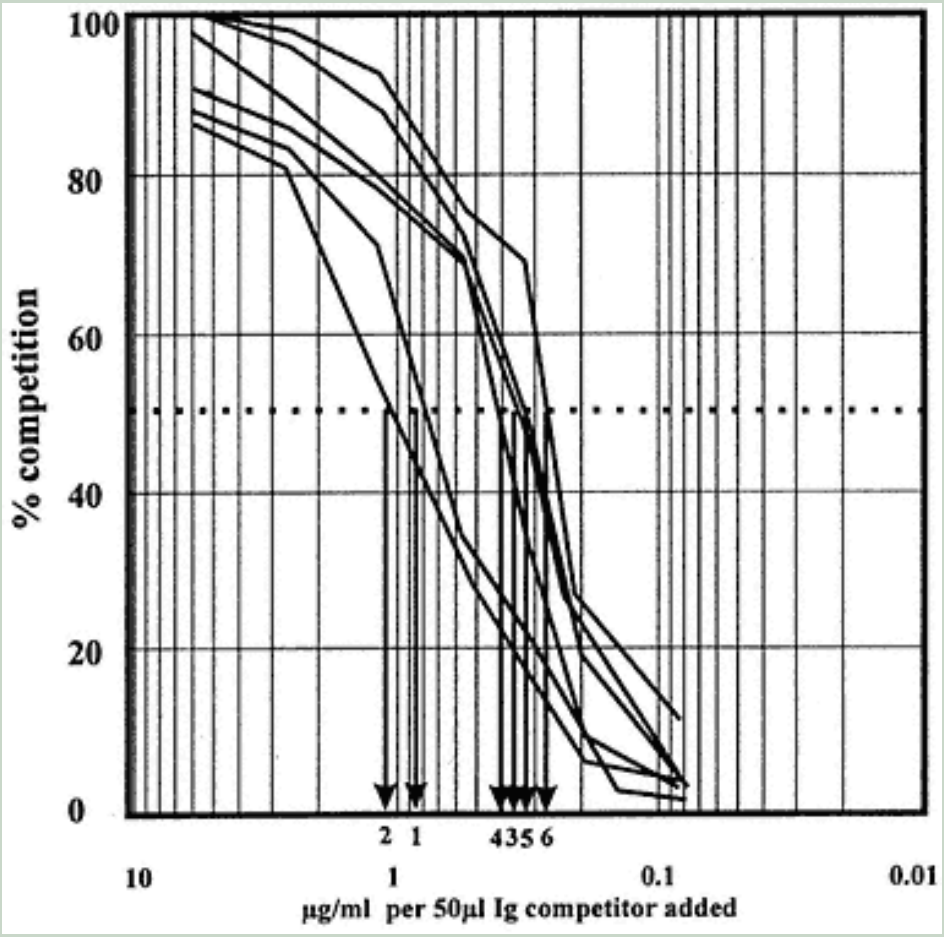


Fig. 4.
Competition data plotted on long-semilog scale.
The weight of competing Ig can be read directly from
the *x*-axis. Combinations 1¨C6 are shown.

buffers, incubation, and so forth, are those described previsouly. A suitable template for addition of samples and controls (known Ig standards) should be developed. An example is shown in **Fig. 5**.

1. Wells should be coated with Ig at 0.75 µg/mL (50 µL per well). After incubation and washing, samples from mAb hybridoma studies can be added. A template as shown in **Fig. 5** is suitable.

2. Add 25 µL of blocking buffer to each well except the control wells (*see* below). Add 25 µL of each mAb sample. Test mAb in duplicate if possible.

3. Add a dilution range of standard Ig preparation from 5 µg/mL by seven wells as in stage 2 (duplicate row).

4. Set up 0% controls (wells with blocking buffer added to Ig-coated wells).

5. Set up 100% competition controls.

6. Add conjugate to relevant wells (50 µL at 1/*y)* found in first stages.

7. Incubate, wash, add substrate, develop color, and stop.

8. Read the plates.

Table 6
Values for 50% Competition

Combinations of conjugate and coating IgG	Competing Ig giving 50% competition (μg/mL per 50 μL)	Plate concentration of Ig (μg/mL per 50 μL)
1	0.90	1.5
2	1.10	1.5
3	0.40	0.75
4	0.45	0.75
5	0.35	0.375
6	0.28	0.375

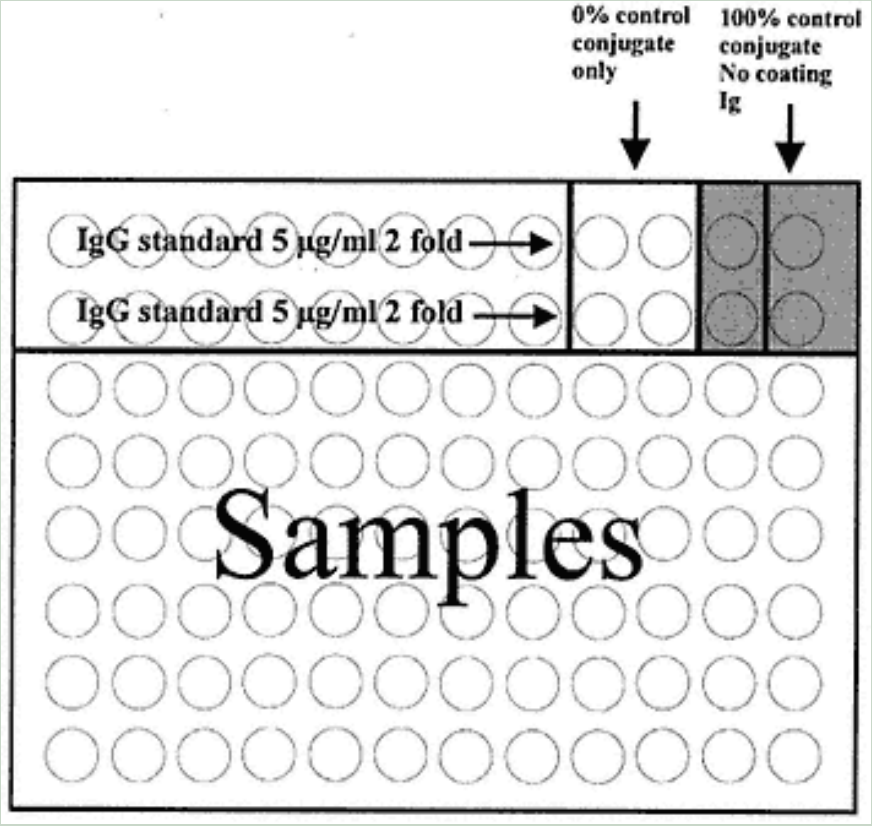


Fig. 5.
Plate layout for measuring mouse Ig from samples. The gray box indicates no coating with IgG made. The standard dilution of Ig was make as a duplicate two-fold dilution range. Zero percent control competition measures the reaction between coated wells and conjugate, and 100% competition is the reaction between conugate and uncoated wells.

2.5.1¡ª
Results

Typical results are shown in **Table 7**. **Figure 5** shows the template used. Duplicate samples of mAb are titrated vertically.

For accurate treatment of data, the means of the results are calculated. The mean of the 100% competition data (A11 and 12 and B1 and 12) is subtracted

Table 7
Examination of Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.05	0.13	0.22	0.37	0.68	0.89	0.99	1.07	1.09	1.08	0.05	0.05
B	0.06	0.15	0.20	0.35	0.66	0.91	0.97	1.09	1.08	1.07	0.04	0.06
C	0.04	1.08	0.56	0.43	0.04	0.91	0.89	0.67	0.56	0.05	1.07	1.04
D	0.06	1.06	0.58	0.47	0.06	0.89	0.91	0.65	0.60	0.07	1.09	1.02
E	1.08	1.10	1.12	1.00	1.12	0.98	0.67	0.32	0.43	1.09	1.07	1.06
F	1.10	1.06	1.08	0.98	1.10	0.98	0.65	0.28	0.41	1.07	1.05	1.06
G	0.56	0.45	0.32	1.07	1.06	0.76	0.45	1.04	1.09	1.21	0.12	0.13
H	0.58	0.51	0.30	1.11	1.10	0.78	0.43	1.10	1.03	1.09	0.10	0.11

from all means. The percentage competition is then calculated for the data using the same formula as in **Subheading 2.4.4.**

The standard curve can be plotted on semilog paper. The percentage values for the samples can be used to read off the relative activity as compared to the standard curve. This is illustrated in **Fig. 6**, in which only three samples are shown being read from the standard curve. When the sample values are equal to 100%, the effective concentration in the samples cannot be calculated. When they are lower then effectively the concentration of Ig can be assessed. Examination of the stopped plate by eye is useful and may be enough to indicate which wells or samples contain Ig. Thus, assessment as to wells which have no or very little color is enough to indicate Ig secretion. Similarly where wells have color equivalent to that in the 0% control wells then they can be deemed "negative" with respect to Ig secretion to the limits of sensitivity as defined by the standard curve. The standard curve should also be examined in a by eye assessment and should indicate a gradual increase in color as expected by a decreasing level of competition.

Table 8 shows the data in **Table 7** and represents mean of replicates minus the mean value for the 100% competition controls, and the % competition values. The mean of replicates of 100% competition data is 0.05

3¡[a]
Screening of mAbs for Specific Activity

A reminder is needed that the purpose of producing mAbs is to solve a particular problem. The screening of mAbs must reflect this purpose as early as possible. Reference to **Table 1** indicates when specific screening is best attempted. ELISAs measuring binding of fusion products to antigens are ideal for testing a high number of clones in a 96-well format. The screening does require some pretesting of reagents to allow confidence that a system will

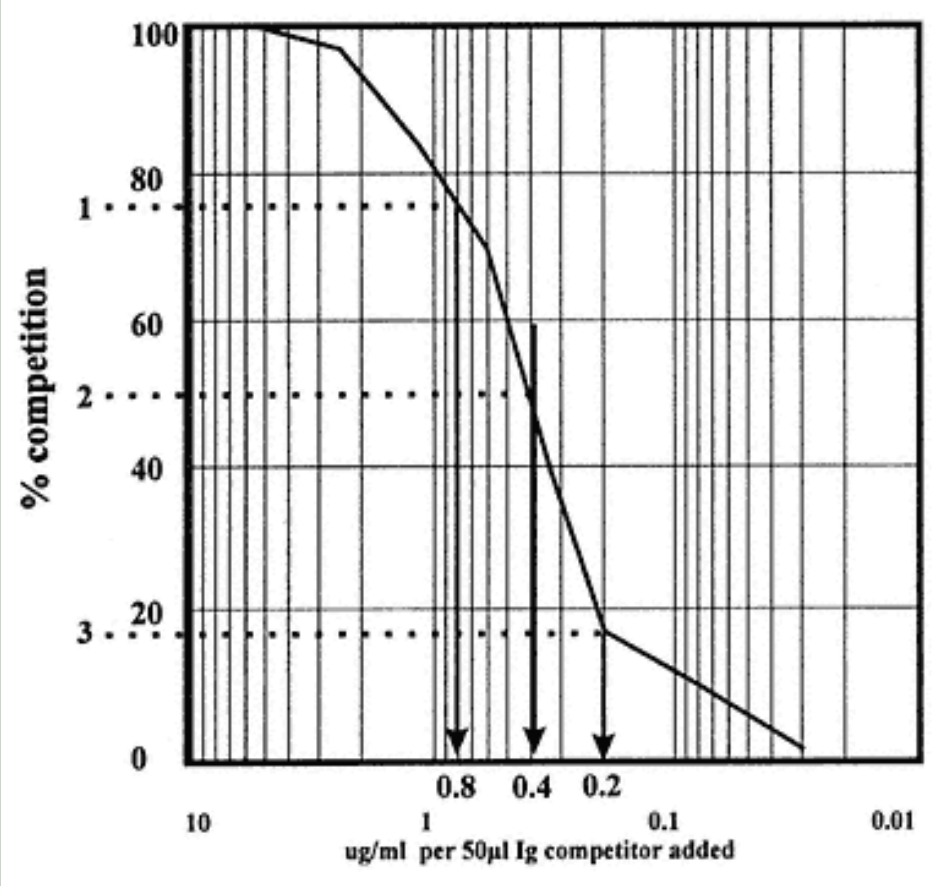


Fig. 6.
Competition data for standards plotted on log-semilog scale. The weight of competing IgG in samples can be read directly from the *x*-axis. Three examples of test samples are shown, as indicated in the text. The estimated micrograms/milliliter values are shown for each sample below the *x*-axis.

detect mAb if produced. The usual source of developmental reagents comes from collecting serum from mice primed and vaccinated in the same way (or the actual mice used) as was used to prepare the mAbs. The polyclonal serum should contain antibodies that bind to a selected target antigen(s). This and preimmune serum can be used for developing an ELISA system for screening purposes.

A review of what is available in terms of likelihood, specificity, physical parameters, and availability is needed in order to assess the most suitable test. This also infers that there may be other reagents already exploited in ELISAs (e.g., polyclonal capture antibodies) that can be used. **Table 9** shows some considerations. The development of assays to detect bound mouse antibodies is no different from that for other assays. We need to be certain that a test will measure this binding and be reasonably assured that the test has good analytical sensitivity (can detect low levels of antibody).

Table 8
Competition Data

	1	2	3	4	5	6	7	8	9	10	11	12
	0	0.04	0.16	0.31	0.62	0.85	0.93	1.03	1.03		100	
%	**100***	**96**	**84**	**70**	**40**	**17**	**10**	**0**	**0**			
	0	1.02	② 0.52	0.40	0	③ 0.85	0.85	0.61	0.53	0.01	1.03	0.98
%	**100**	**1**	**50**	**61**	**100**	**17**	**17**	**41**	**50**	**100**	**0**	**5**
	1.04	1.03	1.05	0.94	1.06	0.93	0.61	① 0.25	0.97	1.03	1.01	1.01
%	**0**	**0**	**0**	**10**	**0**	**11**	**41**	**76**	**5**	**0**	**1**	**1**
	0.52	0.43	0.26	1.04	1.03	0.72	0.39	1.02	1.01	1.11	0.06	0.07
%	**50**	**71**	**77**	**0**	**0**	**31**	**63**	**0**	**1**	**–7**	**94**	**93**

*Figures in bold represent % competition data.
◯ = Samples used in **Fig. 6.**

Table 9
Considerations of Components of mAb Screening

Question	Considerations
Is there an antigenic target?	Large antigenic complex, polypeptide, peptide, denaturable, single epitope expressed, multiple epitopes expressed
Is capture needed?	Is polyclonal available, species, concentration known?
Can it coat directly?	Concentration known?
What amount is available?	Examples: I mL used at 1/10; 1 mL can be diluted 1/2000, 5 mg used at 2 µg/mL
What is the degree of purity?	Amorphous mixture containing different antigens, purified product
Can it be used for vaccination?	With any of the above, assesses likelihood of spectrum of mAbs produced and complications using reagents specified

The most obvious ELISA for general screening is the indirect system involving detection of bound mouse Ig using an antimouse conjugate. A commercial preparation reacting with all isotypes should be purchased. The antigenic target may well have been characterized in some other ELISA using polyclonal reagents so that the effective concentration can be assessed for use in detecting mAbs. Several scenarios will be described to indicate strategies.

These are probably available in laboratories equipped to produce mAbs because they have a research base. When reagents are unavailable (e.g., polyclonal antiserum raised against antigen[s]), then the mouse antibody detection system has to be worked up from basic principles. This is also illustrated. A key point in screening is that results are relative in the initial phases. One attempts to identify mAb-producing cells as early as possible and to avoid maintenance of large numbers of cells in a blind fashion.

The factors considered in **Table 9** are relevant to the development of all ELISAs. Certain factors are more important when mAbs are to be exploited owing to the unique single specificity inherent in that reagent. This specificity can be both a great benefit and a problem, and care is needed to avoid unsuitable systems. The systematics of ELISA will be discussed after a few examples of how previous experience with the antigen helps in screening. These are meant only to illustrate principles and are not explored in great detail. The particular methods (e.g., CRTs, dilution ranges) are all examined in other sections.

3.1¡ª
Previous Experience in Helping to Develop an Indirect Assay

Our previous experience in developing an indirect assay involved antigen (Ag) coated directly to wells and a detecting serum in turn detected by an antispecies conjugate. In that case, the Ag concentration was known, and at this concentration, we know that a substantial amount of the antibodies bind, and that a good signal is seen on addition of conjugate and substrate/chromogen. This concentration of antigen can be tested in the indirect ELISA; the mouse serum can be tested before and after the vaccination regime for producing the mAbs.

Assuming that the antigen in the indirect ELISA is the same as that used to prime and vaccinate mice, the polyclonal ELISA reagents can be used to assess antigen coating. The pre- and postvaccinal mice sera can be titrated in a CBT against the antimouse conjugate. Some adjustment can then be made to the concentration of antigen and conjugate to allow optimization of all three components. The antigen is therefore controlled. There may be problems inherent in the presentation of the antigen to monospecific mAbs in screening, as compared to what is observed with polyclonal antibodies. This is explained in the **Subheading 9.**, dealing with the implications of mAbs in different assays.

3.1.1¡ª
Assay

1. A dilution (concentration) of antigen should be used on plates that gives a high OD in the presence of excess mouse positive serum. This will allow maximum binding of mAb from tissue culture preparations.

Table 10
Typical Results from Indirect ELISA screening of mAbs

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.45	0.04	0.05	1.31	0.08	0.09	0.23	0.26	0.34	0.78	0.03	0.09
B	1.41	0.05	0.04	1.38	0.09	0.07	0.19	0.30	0.38	0.69	0.07	0.12
C	0.80	0.23	0.45	0.08	0.09	1.31	0.08	0.09	0.09	0.56	0.12	0.13
D	0.07	0.27	0.49	0.06	0.12	1.26	0.05	0.09	0.06	0.58	0.16	0.19
E	0.19	0.89	0.97	0.67	0.45	0.09	0.04	0.06	0.04	1.21	0.09	0.45
F	0.23	0.90	0.89	0.76	0.56	0.08	0.07	0.05	0.07	1.32	0.07	0.39
G	0.07	0.05	1.10	1.23	1.34	1.56	0.08	1.21	0.58	0.57	0.63	0.08
H	0.07	0.08	1.21	1.25	1.23	1.59	0.09	1.19	0.59	0.57	0.65	0.09

[a]AB 1 is positive serum control. CD 1 is negative serum control. Samples are as vertical duplicates.

2. A volume of 25 µL of each mAb should be added in blocking buffer (as used in CBT of mouse serum). The mAbs can be added to wells already containing 25 µL of a solution of blocking buffer in which the blocking agent is at twice the normal concentration. This will compensate for the dilution of the mAb. A single dilution or, better, a duplicate sample should be used.

3. The mouse positive and negative sera should be added to some wells as controls to be indicators that the test is working. A dilution of positive serum that gives just gives the maximum OD (plateau height maximum in the presence of excess mouse serum) can be added. A dilution of negative serum should be used to give an OD value that reflects background.

4. The incubation step to allow binding of mAb to antigen should be that used in the CBT. One hour at 37¡ãC while plates are being rotated is usually adequate.

5. After washing, the addition of antimouse conjugate (optimal concentration) and incubation are as in CBT.

6. Washing, substrate/chromogen addition, and stopping areas are as for the CBT.

3.1.2¡[a]
Results

A typical plate might give results as shown in **Table 10**.

3.1.3¡[a]
Conclusion

1. The control serum values indicate the test worked in that the wells were coated with antigen (gray box in **Table 11**).

2. High OD values are seen for certain mAb samples almost achieving plateau height seen with polyclonal serum (shown in boxes in **Table 11**). In G/H 5, a value greater than the polyclonal value is seen.

3. There are values with lower OD values but still high relative to others showing values around that of control negative wells **(Table 12)**.

Table 11
Highlighting Strong Positive Samples in mAb Screening

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.45	0.04	0.05	1.31	0.08	0.09	0.23	0.26	0.34	0.78	0.03	0.09
B	1.41	0.05	0.04	1.38	0.09	0.07	0.19	0.30	0.38	0.69	0.07	0.12
C	0.80	0.23	0.45	0.08	0.09	1.31	0.08	0.09	0.09	0.56	0.12	0.13
D	0.07	0.27	0.49	0.06	0.12	1.26	0.05	0.09	0.06	0.58	0.16	0.19
E	0.19	0.89	0.97	0.67	0.45	0.09	0.04	0.06	0.04	1.21	0.09	0.45
F	0.23	0.90	0.89	0.76	0.56	0.08	0.07	0.05	0.07	1.32	0.07	0.39
G	0.07	0.05	1.10	1.23	1.34	1.56	0.08	1.21	0.58	0.57	0.63	0.08
H	0.07	0.08	1.21	1.25	1.23	159	0.09	1.19	0.59	0.57	0.65	0.09

[a]Gray box indicates positive control values. Open boxes show high OD readings.

Table 12
Highlighting Other Clearly Positive Samples in mAb Screening

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.45	0.04	0.05	1.31	0.08	0.09	0.23	0.26	0.34	0.78	0.03	0.09
B	1.41	0.05	0.04	1.38	0.09	0.07	0.19	0.30	0.38	0.69	0.07	0.12
C	0.80	0.23	0.45	0.08	0.09	1.31	0.08	0.09	0.09	0.56	0.12	0.13
D	0.07	0.27	0.49	0.06	0.12	1.26	0.05	0.09	0.06	0.58	0.16	0.19
E	0.19	0.89	0.97	0.67	0.45	0.09	0.04	0.06	0.04	1.21	0.09	0.45
F	0.23	0.90	0.89	0.76	0.56	0.08	0.07	0.05	0.07	1.32	0.07	0.39
G	0.07	0.05	1.10	1.23	1.34	1.56	0.08	1.21	0.58	0.57	0.63	0.08
H	0.07	0.08	1.21	1.25	1.23	1.59	0.09	1.19	0.59	0.57	0.65	0.09

[a]Open boxes show values indicating positive samples. Gray boxes indicate low but probably still positive values.

At this stage the concentration of the mAb is likely to be unknown, although the mAbs can be titrated in terms of mouse antibody, as explained earlier. Thus, the activities of the individual samples cannot be directly compared. A low OD may only mean that there is a small quantity of specific mAb, as compared to those that gave high ODs. The low OD values may have come from colonies with few cells. Therefore, all mAbs showing positivity at this stage have to be "grown on" to allow for more cells to be produced that secrete antibody.

The maximum OD of any mAb depends on the epitope density on the sample attached to the wells. The maximum OD for the polyclonal serum may well be greater than that for any mAb screened (unlike the example shown in **Table 10**.). This depends, to a large extent, on the number of different epitopes ex-

pressed on any solid phase "antigen" and the number of different polyclonal antibodies produced against it. When the single epitopes that mAbs identify are present in a low concentration, then the maximum binding of mAb as well as the maximum plateau will be low. This cannot be established in a single-dilution screening, but will be explained further below, when dealing with characterization of mAbs. Knowledge about the relative complexity of an antigen used in screening is very helpful in predicting the likely difference between polyclonal and mAb binding plateau maxima. As an example, at one extreme, in which a peptide is used with a single epitope, polyclonal sera will bind, giving maximum plateau similar to those of mAbs, and there will be a single saturating event in the presence of excess antibody from either source. Indeed, the mAbs may react better owing to their uniform structure.

Screening against more than one antigen may be relevant. It is possible that more than one antigen can be used at the first screening stage in order to establish some level of specificity of the mAbs at a very early stage. Thus, two specific antigens may be used and the relative binding of any secreting mAb determined. This can also serve to increase the likelihood of picking up mAbs that may not be detected because they have a low affinity against a particular antigenic preparation. If an operator is unsure as to the best antigen to use in a final assay, more than one can be tried. It must be emphasized that the objective in screening is to limit the number of clones to manageable proportions while successfully finding products for a defined purpose.

When the concentration of the mAbs is known, the activities can be compared with respect to the concentrations of Ig.

3.2¡ª
The Next Stage

Once you have identified mAbs that are secreting antibody, these can be selected for further growth, amplification of product, and complete characterization. This requires that supernatant fluids be retested to determine whether they are still secreting Ig. The same test already described in **Subheading 3.1.1.** However, there is now a large increase in the volume of supernatant, which allows for additional testing. This stage may be the safest to begin accurate characterization. In this case, the more stabilized clones can be stored in liquid nitrogen to allow their restoration once characterization has identified the required mAbs.

A word of caution on screening using indirect ELISA is necessary. mAbs do show exquisite specificity, by definition. Solid-phase coating of antigens can affect certain epitopes in that they change their conformation. This prevents some mAbs from reacting (being dependent on conformation). Thus, in some cases, mAbs will be missed in the indirect ELISA.

3.3¡ª
Other ELISA Screening Systems

The exploitation of other ELISAs depends on the number of assays already developed and hence reagents available. The antigen can be captured via polyclonal antibodies, allowing indirect sandwich ELISAs. Here again, the system can be worked out for a completely polyclonal set of conditions and the effective capture antibody concentration used to present antigen to the mAbs. There are some problems that depend on the nature of antigen. As an example, when a small peptide is captured, the particular epitope targeted by an mAb may be already bound by the trapping serum. Thus, mAbs specific for that epitope would not be detectable. The presentation of more complex antigens with repeating identical epitopes will not affect screening. The capture of antigens also may protect the conformation of certain epitopes as compared to those directly coating wells.

Competitive assays may also be exploited. Thus, a fully validated polyclonally based assay can be used and challenged by samples containing mAbs. This is only successful with smaller, more limited antigenic targets, since mAbs, by definition, will only compete for a single epitope with a single affinity. Polyclonal sera contain many antibodies directed at a variety of antigenic determinants. The antibodies themselves form complex interactions depending on the variable affinities of the individual molecules in the mixture (avidity). Thus, with more complex antigens, an mAb may completely compete off polyclonal antibodies from a single epitope, but, of course it fails to block reactions with all the other sites. The effect is that only a low maximum inhibition of binding is seen by mAbs. Owing to consideration as to the nature of the antigen (e.g., its physical state, the number of likely antigenic sites), its denaturability and size, will give a clue as to the best bet for screening.

4¡ª
Characterization of mAbs

Characterization of mAbs depends exactly on the purpose of the mAb. The indirect ELISA with the detection of binding to a solid-phase antigen usually screens most mAbs of ultimate use, even when the mAbs are used in different systems. At this stage, there is the advantage that a larger volume of supernatant is available, so that more tests can be performed. We also now have fewer concerns about sterility in sampling since cells can be grown to allow supernatant production for testing alone.

5¡ª
Consideration of Use of mAbs

Table 13 lists some of the uses of mAbs and highlights their potential advantages and disadvantages. These stem from the single specificity of mAbs and the ability to standardize a product. The ultimate use must be considered to

Table 13
Uses of mAbs in ELISA

1. Differentiating closely related strains.	Single mAb or mAb panels 2 or more 100's. All ELISAs.
2. Ability to capture defined antigen from mixture.	Sandwich ELISA as capture antibody.
3. To specifically detect polyclonally captured antigen.	Detecting mAb directly labeled or in indirect system (antimouse conjugate).
4. To use a reference standard.	Activity defined by weight. Any ELISAs.
5. To link sequence and structural data.	Defining epitopes; mAb escape mutant studies. All ELISAs.
6. To define conformational and linear sites.	Binding studies; All ELISAs.
7. To use a specific systems for quantification.	mAbs used as capture and detecting reagents.
8. To assess affinities against specific epitopes; compare epitopes and specific paratopes.	Competitive ELISAs.
9. To use as isotype-specific assays.	Use of anti-isotype conjugates to examine binding mixtures or single mAbs. Indirect and indirect sandwich ELISAs.
10. To use as multiple assays detecting different antigens.	Mixture of mAbs; different labeled conjugates.

evaluate the need for sufficient quantities and the purity. Examples of these applications will be shown later.

6¡[a] Purification of mAbs

Purification of mAbs may be necessary to increase the concentration of Ig or to free the Ig from other proteins. The source of the mAb is important (how it is grown). **Table 14** presents some sources and typical concentrations of Igs in mAbs and polyclonal sera.

Contaminants in mAbs depend largely on the method used to amplify the amount of Ig and the purification methods. These include non-antibody substances such as serum proteins, proteases, lipids, endotoxins, nucleic acids, and viruses. The actual purity of mAb depends on the ultimate use, and 95% is suitable for most assays. The purification procedure should be made as soon as possible. When this is not possible, mAb preparations can be stored. **Table 15** gives some conditions for storage.

Table 14
Sources of Antibodies

Material	Serum	mAb tissue culture 10% calf serum	mAb serum free	mAb ascites in vivo
Antibody produced	Polyclonal	Monoclonal	Monoclonal	Monoclonal
Total antibody	5¨C10 mg/mL	0.5¨C1.0 mg/mL	0.05 mg/mL	1¨C15 mg/mL
Specific antibodies	1¨C5%	5%	100%	80¨C90%
Contamination	Serum proteins/ antibodies	Calf serum/proteins antibodies	None	Mouse antibodies/ proteins
Purity specific antibody possible	10%	>95%	>95%	90%

Table 15
Storage Conditions for mAbs

	Action	Storage	Stability				
Cell culture supernatants	Concentrated (X10), filter sterilized	2¨C5¡ãC ¨C20¡ãC ¨C70¡ãC	Few days 1 yr 1¨C5 yr	Ascites	Centrifuged, fat-free filter sterilized	2¨C5¡ãC ¨C20¡ãC ¨C70¡ãC	Few days 1 yr 1¨C5 yr

Note again that mAbs are proteins and an excellent substrate for microbiological growth. Since mAbs are a single molecule, any physical or biological effect on one molecule will affect the entire antibody population. Care must be taken to store mAbs in buffered solutions and at suitable temperatures. Excessive freeze thawing should not be done. Ascites fluids are well buffered at the source, but purification procedures remove this buffer. Filter sterilization is recommended but possible losses must be taken into account.

Stabilization of mAbs is important if their reactivity is to be maintained. External factors such as excess of heat, pH, shaking, detergents, high salt, and chelating agents should obviously be avoided. Other chemical effects over longer times can also cause problems, such as, hydrolysis, crosslinking, and oxidation. However, these are less likely to induce problems in assays in which efficient use of controls and well-stored mAbs are maintained. Problems of aggregation and adsorption also reduce mAb activity. Approaches to stabiliz-

ing mAbs include freeze drying, addition of stabilizers in which proteins, sugars, amino acids, and fatty acids ***(1)***. Storage at a reduced temperature is still the most recommended procedure. mAbs are best stored in a neutral pH buffer solution containing approx 0.1 *M* salt concentration. Solutions should be relatively concentrated (approx 1 mg/mL), and higher if possible.

The addition of glycerol is recommended generally (1 vol glycerol to 1 vol mAb) with storage at ¨C20 to ¨C70¡ãC.

The addition of preservatives is also recommended, e.g., merthiolate at a concentration of 1/10,000, or sodium azide (0.02¨C0.1%). Care is necessary in using both preservatives. Sodium azide also inhibits some ELISAs, and attention to its final concentration in any assay is necessary.

Lyophilization can provide a stable mAb preparation particularly when supplied in kits, in which transportation and collection of reagents causes storage problems. Successful procedures for lyophilization are necessary to achieve a stable product.

6.1¡ª
Methods for Purification of mAbs

A wide variety of methods for purification are available. For most ELISA applications a purity approaching 95% may be sufficient to remove unwanted proteins. The method depends on the particular source material and its volume, and ultimately how much is needed. Techniques depend on fractional precipitation, electrophoretic, ion-exchange, ultracentrifugation, and affinity methods.

Generally, for immunoassays relatively small amounts (order of milligrams) are needed for developmental and applied work. Ultimately, the activity of any mAb can be associated with a defined weight of relatively pure product. Therefore, the available amount of mAb can be calculated with respect to any defined assay. This activity can be assumed on any subsequent preparation of mAb and the necessary steps taken to produce the required amount. As an example, a mAb is titrated for use in an ELISA at a dilution of 1/10,000 in a 50-µL vol. There is 1 mL of the preparation at 1 mg/L. This would allow 10,000 mL of reagent ¡Á20 = 200,000 assay points. It can be determined whether this is enough to serve the needs in a given time. Note that the activity here can be related to the weight, i.e., 1/10,000 = 1 mg/mL. Preparation of the same mAb in the same way means that each milligram of mAb produced (and purified in the same way) should have a titer of 10,000. Such calculations based on mAbs are feasible as compared to polyclonal antibody considerations. Thus, when an mAb-based test is envisaged for use in 20 laboratories all examining 10,000 samples per year for 5 yr, the effective amount of mAb required can be calculated and production tailored to meet this need. This is an important consideration in all aspects of serology and avoids the embarrassment of validating particular sera for use (e.g., as standards in assays) only to find that, although a perfect serum

is identified, only 1 mL usable at 1/800 is available. The distinct advantage of mAbs in this area is that an identical preparation can be produced on demand from the hybridomas.

6.1.1¡ª
Fractional Precipitation

Fractional precipitation methods can have damaging effects on certain mAbs. Pilot studies can examine this possibility. Methods classically involve the use of ammonium sulfate, sodium sulfate, caprylic acid, Rivanol, and polyethylene glycol. These separate mAb from the majority of other proteins in ascites. Following dialysis, this procedure may well be sufficient for use in ELISA.

6.1.2¡ª
Electrophoretic Separation

Electrophoretic separation techniques involve a good deal of instrumentation and also involve free-boundary electrophoresis, zone electrophoresis on celluloses acrylamide, and so forth, as well as isoelectric focusing techniques.

6.1.3¡ª
Ion-Exchange and Gel Filtration Chromatography

Anion and cation exchangers as well as gel filtration can be used to selectively purify mAbs¡ªe.g., sephadex G series; sepharoses 2B, 4B, 6B, CVL; Sephacryl; as well as a variety of agarose or polyacrylamide beads.

6.1.4¡ª
Affinity Chromatography

Affinity chromatography offers efficient large and small-scale applications¡ªe.g., the use of protein A, protein G, and protein A/G on a variety of matrices such as glass beads, avidin-biotin systems, antigen affinity columns to selectively purify mAb, and hydroxylapatite. There are many configurations exploiting affinity purification on the commercial market. Kits for small-scale purification and reagents to produce larger scale capacities can be purchased. Such techniques tend to favor the purification of specific Ig, and care must be taken to establish that particular mAbs are bound with sufficient affinity to the ligands. Recently kits have become available from Pharmacia (E-Z-SEP) that selectively precipitate Igs from heterogeneous mixtures by a volume exclusion technology through the use of nonionic linear polymers and special buffers. The kits are optimized for precipitation of globulins from ascites fluid, cell culture supernatants, bioreactor fluids, and animal sera. The isolation is independent of the species or subclass.

7¡ª
Characterizing mAbs by Isotype

The determination of the isotype of a particular mAb can be important in assessing its potential as a reagent, as well as allowing different possibilities of

assay design. Early characterization of the isotype of an mAb may be important for a number of reasons. First, an isotype can determine the simplest method of purification. For example, IgG_{2a} and IgG_{2b} bind protein A, whereas IgG_1 mAbs do not bind protein A very well under standard conditions. Second, some isotypes are better suited to certain immunological techniques than others. For example, IgG_{2b} antibodies are the best isotype for complement-mediated killing of in vitro cells carrying the epitope. Third, isotype characterization also reveals the structure of the antibody, which may make it undesirable in some applications. For example, IgM exists as a pentamer composed of five 180-kDa subunits; therefore, IgM monoclonals are often too large for applications that require monomers. Fourth, an isotype determines the best method for preparing Fab fragments by proteolysis.

Several commercial kits are available to measure isotypes as well as specific anti-isotype reagents for mouse, rat, and human mAbs. The kits are expensive and care should be made to justify the need for isotype determination at any particular stage. Kits are based on various formats:

1. Agar gel immunodiffusion.

2. Coated latex particle strip assays, e.g., Boehringer Mannheim IsoStripTM, which is made simple by the kit's two components. Each development tube supplied with the kit contains colored latex beads bearing antimouse κ and antimouse λ antibodies, which will react with any mouse mAb regardless of its isotype. The isotyping strip bears immobilized bands of goat antimouse antibodies corresponding to each of the common mouse antibody isotypes (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA) and to the [kappa] and [lambda] light chains. Both sides of the strip also bear a positive control band. The diluted sample is added to the development tube, where the mouse mAb resuspends and forms a complex with the antibody-coated latex beads. When the isotyping strip is placed in the development tube, this complex flows up the strip (via capillary action) until it is bound by the immobilized goat antimouse antibody specific for the mAb's isotype and light chain. This takes approx 5 min to perform.

3. Use of specific anti-isotype antibody measurement of mAbs in the ELISA format of choice, either directly with mAb after binding to a target antigen, e.g., Biomeda-Mouse Hybridoma IsoType Kit. The kit is designed to be used for determination of the class and subclasses of mouse mAbs in mouse hybridoma supernatants or mouse ascites using enzyme immunoassay. The reagents in this package detect primary antibodies of mouse isotypes IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM origin, as well as to identify the κ or λ light chain antibodies in one simple, antigen-mediated immunoperoxidase assay. Purified antigen is absorbed to the microtiter plate wells. The remaining sites for protein binding on the microtiter plate are saturated with blocking protein solution. This complex is incubated with the antibody secreting hybridoma supernatants or diluted ascites. The mouse Igs bind to the antigen absorbed to the wells. Goat antimouse Ig

isotype-specific antibodies are applied for isotype determination in the hybridoma supernatants. Signal is detected by incubation with HRP labeled rabbit antigoat IgG. This step is followed by incubation with ready-to-use 3,3',5,5' tetramethylbenzidine chromogen.

Pierce offers ELISA-based mAb isotyping kits using HRP/2,2'-azino diethylbenzothiazoline sulfonic acid and Alkaline Phosphatase/*p*-nitro-phenylphosphate with two basic types of screening procedures. Antigen-dependent screening is as previously described above and involves coating the antigen on a microtiter plate. The hybridoma supernatant is then added, and the mAb is detected using an enzyme-conjugated antimouse antibody.

An antigen-independent screening method is also supplied when soluble antigens are difficult to obtain. ELISA plates are coated with an antibody to mouse immunoglobulin. This antibody then serves to capture the mAbs from the hybridoma supernatant. The presence of positive clones is proven with enzyme-conjugated antimouse immunoglobulin.

8¡ª
Processing of mAbs

Applications involving the use of Ig fractions may offer advantages. The proteolytic cleavage and purification of products from mAbs (particularly of mouse origin) can be accomplished through the use of kits designed to give good yields. **Figure 7** gives the details of proteolytic cleavage of human IgG molecules. This illustrates the three major components of use and relevance in ELISA; the Fab_2 and Fab and Fc fractions. Care must be taken concerning to the digestion of mouse IgG molecules since each isotype has a different degree of resistance to proteolytic cleavage. A good kit can be obtained from Pierce. These kits selectively cleave Ig molecules into Fab and $F(ab')_2$ fragments using papain, pepsin, ficin, and trypsin immobilized on a crosslinked agarose support. Attaching the enzymes to a solid phase eliminates the problems often encountered with soluble enzymes to allow easy separation of enzyme from antibody fragments, no contamination with autodigestion products from the enzyme, and reproducible results.

9¡ª
Assay Formats Using mAbs

mAbs may be used in any assay format and they offer certain advantages over polyclonal reagents when used similarly. Care must be taken to assess the use in terms of the exact preparation (purity, isotype, fraction) of mAbs with due attention to the specificity of the interaction of the mAb and the epitope to which it binds and the nature of the antigen(s) that are involved in any assay. Certain formats used with mAbs can be used uniquely as compared to polyclonal sera. The valency of the mAb or mAb preparation can affect assays; **Table 16** outlines parameters.

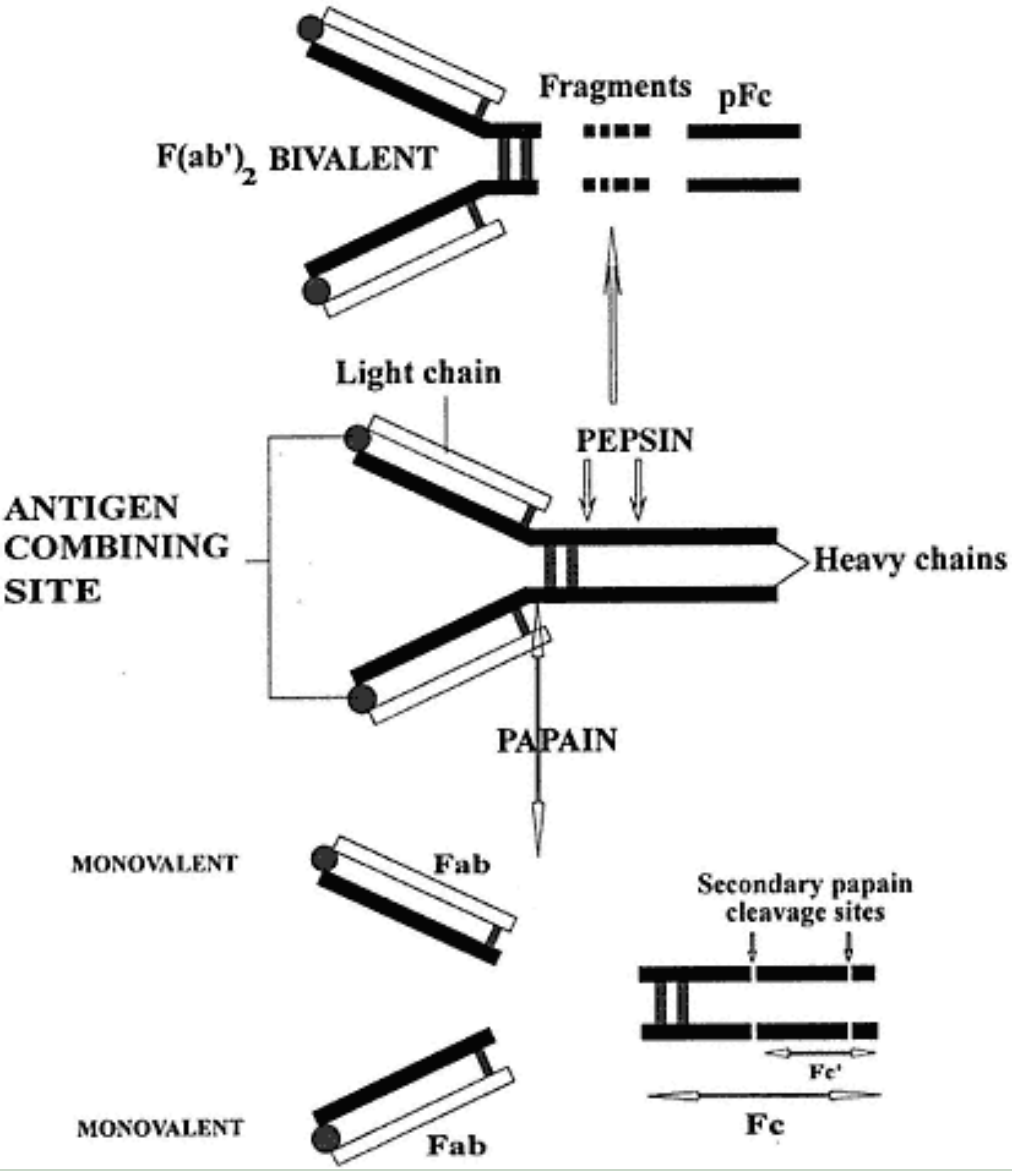


Fig. 7.
Enzymatic cleavage of IgG. Pepsin cleaves the heavy chain to give F(Ab') and pFc' fragments. Further action results in greater fragmentation of the central protein to peptides. Papain splits the molecule in the hinge region to give two Fab fragments and the Fc fragment. Further action on the Fc can produce Fc'.

Table 17 shows the direct, indirect, and sandwich systems using symbols for the reactants. Each system can be challenged with antigen or antibody and thus, used to perform competitive or inhibition assays. Certain possible advantages and disadvantages of assays using mAb-based reagents as compared to polyclonal antibody reagents are shown.

The key features that can make mAbs potentially absolute reagents stem from their specificity. Therefore mAbs can be used effectively to measure only the epitopes against which they are directed and indirectly assess any other antibodies from other sources that bind with that epitope. The nature of the epitope (e.g., whether or not it is conformation dependent) and its overall ex-

Table 16
Factors Involved
in Assay Design Using mAbs

Valency	Example
Monovalent	Fab fragments
Bivalent	Whole $IgG_{1\text{-}C3,}$ Fab_2
Multivalent	IgM/IgA
Mixture	Not successfully cloned

Table 17
Use of mAb Reagents in ELISA Systems[a]

Direct ELISA

—I-Ag + mAb*Enz

—I-Ag + Fab_2*Enz

—I-Ag + Fab*Enz

Indirect ELISA

—I-Ag + mAb + Antimouse*Enz

—I-Ag + Fab_2 + Antimouse*Enz or Anti-Fab*Enz

—I-Ag + Fab + Antimouse*Enz or Anti-Fab*Enz

Direct sandwich ELISA

—I-mAb1 + Ag + mAbl*Enz (same mAb detecting)

—I-Fab_2 + Ag + mAb*Enz (same mAb detecting)

—I-mAb1 + Ag + mAb_2*Enz (different mAb detecting)

—I-mAb + Ag + PC*Enz (polyclonal antibody detector)

—I-PC + Ag + mAb*Enz (polyclonal capture antibody)

Indirect sandwich ELISA

—I-$mAb_{isotype1}$ + Ag + mAb $_{isotype2}$ + Antiiso2*Enz

—I-Fab_2 + Ag + mAb + AntiFc*Enz

—I-mAb + Ag + PC + AntiPC*Enz

—I-Fab_2 + Ag + PC + AntiPC*Enz

—I-PC + Ag + mAb (+Fab_2 or Fab) + Anti mAb*Enz (or Anti-Fab*Enz)

[a]I- = solid phase with attached reagent; mAb = whole mAb molecules; Fab_2 = bivalent mAb minus Fc; Fab = monovalent fraction; + = addition of reagent in sequence; Ag = antigen; PC = polyclonal antibodies; *Enz = reagent conjugated to enzyme; Fc = Fc fraction of mAb Ig.

pression are also important, and the antigen used in any assay has to be considered carefully. Factors affecting affinity are changes in antigen (alterations of epitopes by physical factors or differences among samples and their density of expression) and the valency of the mAb preparation. The epitope density also affects the binding according to the exact systems used. Such factors

can be examined with reference to some of the assay systems described in **Table 17**.

9.1¡ª
Direct ELISA

The following list is a reminder of the systems as given in **Table 17**:

1. I = Ag + mAb*Enz.

2. I = Ag + Fab_2*Enz.

3. = Ag + Fab*Enz.

Direct ELISA depends on being able to successfully bind antigen to a solid phase while maintaining a reaction with mAb. Binding of antigen can affect the availability and orientation of epitopes (presentation to antibody) and also alter the antigenicity, particularly in which mAbs are directed against conformation-dependent epitopes. The mAb or fractionated mAb also has to be conjugated to enzyme. Conjugation may affect the binding of mAbs. The specific activity of the mAb detector is related to the amount of enzyme. This type of assay is best suited for a competitive/inhibition format in which an mAb has been identified that provides useful information about a single property. Since the initial screening of mAbs probably involves the indirect ELISA, it is likely that mAbs identified will bind to the coated-well antigen (if the same antigen preparation is used). The key is that several mAbs may have to be labeled until the best is identified. This is laborious and the use of the indirect ELISA, utilizing an antispecies conjugate, is recommended when many mAbs can be assessed in competition format.

9.2¡ª
Indirect ELISA

The following list is a reminder of the systems as given in **Table 17**.

1. I-Ag + mAb + Anti-mouse*Enz.

2. I-Ag + Fab_2 + Anti-mouse*Enz or anti-Fab*Enz.

3. I-Ag + Fab + Anti-mouse*Enz or anti-Fab*Enz.

mAbs screened by indirect ELISA already should be suitable for this test. The main application is in competitive tests in which both antigen and antibodies can be assessed in samples. This involves pretitration of the system (Ag and mAb). Samples can then be added and mixed with the pretitrated concentration of mAb and incubated, or added simultaneously. The binding of Fab fractions may well show different characteristics to whole mAb since bivalency of antibody molecules is lost. This affects the relative affinity of binding to the antigen. It may allow a greater density of binding of Fab molecules as compared to whole molecules. The use of Fab molecules in competition assays may reduce or enhance sensitivities depending on the exact distribution and effective relative affinities of binding to antigen.

9.3¡ª
Direct Sandwich ELISA

The following list is a reminder of the systems as shown in **Table 17**:

1. I-mAb1 + Ag + mAbl*Enz (same mAb detecting).

2. I-Fab_2 + Ag + mAb*Enz (same mAb detecting).

3. I-mAb1 + Ag + mAb2*Enz (different mAb detecting).

4. I-mAb + Ag + PC*Enz (polyclonal antibody detector).

5. I-PC + Ag + mAb*Enz (polyclonal capture antibody).

The advantage of this system is linked with the specificity of the mAb in possibly capturing only the targeted antigen. Bivalent mAbs usually capture well, but have to be tested individually for required performance.

The use of the same Mab for capture and detection (as in examples 1 and 2) can lead to problems in which there is a limited number or single (e.g., peptide) epitope being expressed since the capture antibody may effectively preclude any further reaction. However, the use of the same Ab can be exploited to achieve highly specific assays for the detection of particular complexes bearing an antigen, as is illustrated for specific detection of whole particles of foot-and-mouth disease virus (FMDV) from subunits. In this example, the key is that the initial capture by the mAb orientates the antigen complexes. The consideration of the effects of orientation is necessary in all tests involving mAb capture.

When more than one mAb is available, sandwich assays can be made by labeling mAbs and using them as detectors and can alleviate problems of orientation and limited epitopes. This can also lead to very specific assays.

Examples 4 and 5 show the use of a polyclonal serum to either capture or detect. As a capture serum, mAbs can be used to detect specific epitopes and increase the specificity of assays. Again, with antigens showing limited epitopes, the polyclonal capture may result in prevention of any more binding saturation of epitopes. When polyclonal antibodies are used as a general detector, they allow a number of mAbs to be screened for effective capture of antigens. The specificity of the initial capture depends on the mAb. Here, orientation effects are more limited (as shown in data, e.g., with foot-and-mouth disease virus (FMDV) in **Subheading 10.1**).

The assays developed (pretitrated) can all be used with competitive/inhibition systems for the detection of antibodies. Examination of antigens is more difficult since it must be ensured that the capture antibody is saturated with antigen because addition of competing antigen increases the effective concentration and free capture antibodies will bind to this. The same is applicable in indirect sandwich systems.

9.4¡ª
Sandwich ELISA¡ªIndirect

The following list is a reminder of the systems given in **Table 17**:

1. I-$mAb_{isotype1}$ + Ag + $mAb_{isotype2}$ + Anti-iso2*Enz.

2. I-Fab_2 + Ag + mAb + Anti-Fc*Enz.

3. I-mAb + Ag + PC + Anti-PC*Enz.

4. I-Fab_2 + Ag + PC + Anti-PC*Enz.

5. I-PC + Ag + mAb(+Fab_2 or Fab) + Anti mAb*Enz (or Anti-Fab*Enz).

In cases in which mAbs are available in pairs and their isotype is known, mAbs with different isotypes can be used to capture and detect antigens, as shown in example 1. This is made possible by the use of an antimouse isotype-specific conjugate, which allows a higher level of screening of mAbs provided that the enzyme conjugates are affordable. When the bivalent Fab_2 is prepared, the whole molecule mAb or different mAbs can be used. These are detected using an anti-Fc specific conjugate (example 2). Thus, a large number of mAbs can be screened using a single successful capture reagent.

The use of polyclonal antibodies and mAbs is shown in examples 1¨C3. Examples in 3 and 4 show the benefit of screening mAbs for capture activity using a general detecting reagent and antispecies conjugate, and example 3 is probably the most widely used application.

When polyclonal antibodies are used to capture antigens, the screening of mAbs is relatively easy, and whole mAb or fractions can be used with appropriate antispecies conjugates (example 5).

The preparation of polyclonal reagents in experimental animals or characterization from field sera is important in the development of assays. Such sera can be used directly as components of assays and also as reagents defining mAb reactivity. This is particularly important in research areas. Often the best assays involve the use of polyclonal and mAb reagents, one allowing generalized reactivity and the other high specificity.

The systems can all be used in competition/inhibition ELISA for examination of antibodies. The target antigen can be captured first and then competition performed for the detecting antibody.

In summary, the use of mAbs and mAb-polyclonal systems offers a large number of possibilities. The particular advantage of any one has to be determined in the feasibility stages of the development of assays. The production of polyclonal reagents is recommended to allow greater flexibility and possibly avoid too great a specificity of reaction at the various phases of the ELISAs; for example, polyclonal reagents may serve as a general capture reagent for a polyvalent antigen and the specificity of the mAb detector for a particular epitope exploited. Assays can be evolved with limited reagents, e.g., the use of

mAb combinations using isotype-specific conjugates. Care must be taken to examine the use of mAbs in combination with respect to the orientation of antigens and subsequent elimination of binding of the detector. Some knowledge of the antigen(s) should be sought (molecular weight, density) to aid the designing of the assays.

10¡[a]
Examples of the Use of mAbs

Examples of the use of mAbs are now given in detail and involve studies on FMDV:

1. The quantification of whole virus particles in the presence of subunits bearing the same epitope using the same mAb as capture and detector (direct sandwich ELISA).

2. The use of panels of mAbs at a single dilution to differentiate antigenic differences among many virus isolates, involving polyclonal capture sera, mAb detectors and antimouse conjugate (indirect sandwich).

3. The use of mAb-based competition assay for detection of antibodies against rinderpest virus and the development of kits.

The methods illustrate the interface of different technologies and disciplines needed to produce a successful ELISA for a specific purpose. This typifies the interaction of research facilities necessary to develop assays and a thorough understanding of the biological entities being examined.

10.1¡[a]
Quantification of Whole Virus Particles of FMDV in the Presence of Virus Subunits, Using mAbs in a Sandwich ELISA

10.1.1¡[a]
Background

The immunogenicity (ability to elicit protective antibodies in animals) of FMDV vaccines depends, to a large extent, on the production of whole virions (146S particles, so named because of their sedimentation characteristics in sucrose density gradients) in tissue culture and the stability of these particles after virus inactivation procedures and formulation into vaccines. The immunogenicity of subunits (12S particles) is very poor, weight for weight, compared to the 146S particles. Both whole and subunit particles are produced in the infectious process during the manufacture of vaccines. The specific quantification of 146S particles is made using physical methods using either $CsCl_2$ or linear sucrose density centrifugation methods. Both these methods are laborious, take a relatively long time, are subject to standardization problems, require expensive equipment, and do not assess whether virus has been affected by proteolytic enzymes.

Serological methods for estimating the specific weight of 146S are complicated since whole particles and subunits share most of the same epitopes. Thus,

polyclonal sera produced against purified 146S particles cross react with subunits and, therefore, cannot be used directly to assess the weight of whole particles specifically.

Virus-neutralizing mAbs offer serological systems that can overcome the problems of crossreactivity. Neutralizing mAbs against most serotypes of FMDVs have been prepared and characterized in many laboratories worldwide. Such reagents have been used to compare virus isolates antigenically, to prepare and characterize mAb escape mutants allowing the identification of epitopes at the amino acid level, and as reagents in assays to measure antibodies. From such studies, the antigenic makeup of the surface of FMDVs is becoming more clear, particularly when studied in conjunction with X-ray crystallographic data.

One strategy for the specific detection of 146S would be to select an mAb that bound only to the whole virion and not to the subunit particle. This has been proved possible, but such mAbs are not commonly isolated. Another strategy is to use the same mAb as capture and detector. This strategy has another advantage when commonly produced mAbs of a particular specificity have been defined. The use of centrifugation methods involves the sedimentation of virus and its assessment by reading the absorbance of fractions at 259 nm in a spectrophotometer. This measures the RNA content of the fraction, which is then used to calculate the protein weight using a formula. The association of protein to RNA of the correct sedimentation value indicates that virions are being quantified, however, it does not indicate whether the proteins in the virion are cleaved. Cleavage of protein VP1 in the virion capsid can dramatically alter the immunogenicity of the vaccine. If cleavage is to be estimated, then the peak fractions measured from the gradient have to be analyzed by polyacrylamide gel electrophoresis (PAGE), which is a laborious and limiting procedure. The complete procedure of centrifugation and analysis on PAGE, which gives full confidence in the vaccine, does not allow the prospect of on-line testing for virus as it is being produced during the vaccine run. The use of mAbs similar to those identified in this chapter will not only quantify 146S specifically but will identify whether the VP1 protein has been cleaved. The system could also be adapted to the on-line continuous testing for 146S virus, which would allow a greater control of the manufacturing process so that virus could be harvested at the time of maximum production.

10.1.2¡ª Materials and Methods

10.1.2.1¡ª Viruses

Type O1 Kaufbeuren FMDV was grown in BHK-21 cells in the absence of bovine serum. Infected tissue culture fluid was clarified by low-speed centrifu-

gation and the protein precipitated by the addition of an equal volume of saturated ammonium sulfate (pH 7.4, controlled by the addition of NaOH). After 1 h at room temperature, the precipitated protein was collected by centrifugation (approx 6000*g*; Mistral 6L centrifuge). The sediment was resuspended in a minimum volume of PBS and clarified by centrifugation at 10,000g. The supernatant was then centrifuged at 100,000*g* for 2.5 h to pellet the virus. One milliliter of PBS was added to cover the pellet, which was then left overnight at 4¡ãC to allow the pellet to rehydrate. The pellets were then resuspended by agitation with a pipet and brief sonication in a water bath sonicator. Purification was made on linear 15¨C45% sucrose density gradients after the addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.1%. The concentration of purified virus (146S) was established by examination of the RNA adsorption at 259 nm. Peak samples were stored at ¨C70¡ãC without further additions.

10.1.2.2¡ª
Preparation of 12S Subunit Particles

One milliliter of purified virus containing 200 µg of virus was acidified by the addition of 2 mL of 0.1 *M* NaH_2PO_4. Phenol red indicator solution was added (0.05 mL), and the mixture was left at room temperature for 10 min, after which the pH was adjusted to 7.4 by the addition of 0.1 *M* NaOH.

10.1.2.3¡ª
Preparation of Trypsin-Treated Virus

To 200 µg of purified virus in 1.0 mL of sucrose was added to 50 µL of trypsin solution (2 mg/mL in 0.1 *M* phosphate buffer, pH 7.4). The mixture was incubated at 37¡ãC for 15 min. The virus was then diluted to the assay concentrations in the relevant buffer with no further treatment.

10.1.2.4¡ª
Preparation of Denatured Virus

To 1 mL of purified virus containing 200 µg was added 10 mg of SDS (giving a final concentration of 1%) and 20 µL of mercaptoethanol (to a final concentration of 2%). The mixture was heated for 3 min in a boiling water bath. The virus preparation was dialyzed against PBS, and the volume after dialysis was noted to allow an accurate determination of concentration of the protein relative to the starting material.

10.1.2.5¡ª
Antisera

Guinea pig and rabbit polyclonal antisera against type O and SAT 2 FMDVs were prepared after multi-vaccination of animals with purified inactivated virus containing antibodies with a wide spectrum of activity against all FMDVs components.

10.1.2.6¡ª
Monoclonal Antibodies

mAbs were prepared as described in **ref. 2**. Acites fluids were prepared by ip inoculation of the hybridoma cells into mice previously sensitized with Freunds' Complete Adjuvant (FCA). Ascites fluids were clarified by centrifugation and stored at ¨C20¡ãC. The anti¨Ctype O mAbs B2, C8, C9, and D9 have been extensively characterized using serological tests and mAb escape mutant studies ***(3¨C5)***.

10.1.2.7¡ª
Enzyme Conjugation of mAbs

Ascites fluids were labeled with HRP using the method described in **ref. 6**.

10.2¡ª
ELISAs

10.2.1¡ª
Titration of mAbs as Capture Antibodies

The ascites and the polyclonal rabbit serum were diluted in 0.05 *M* carbonate /bicarbonate buffer, pH 9.5, in 50-µL vol into wells of a microtiter ELISA plate (Nunc Maxisorb). Twofold dilution series from 1/20 were made across 11 wells, in quadruplicate. The plates were incubated at 4¡ãC overnight or at 37¡ãC for 2 h. Plates were then washed by flooding and emptying the wells four times with PBS. Plates were blotted almost dry and 50 µL of the respective purified FMDV, 12S, trypsin-treated virus (TTV), or denatured virus (DNV) were added to each well at 2 µg/mL, diluted in PBS containing 5% bovine skimmed milk powder (Marvel) and 0.1 % Tween-20 (blocking buffer to prevent nonspecific attachment of protein). The plates were incubated at 37¡ãC for 1 h while being rotated. Plates were then washed and 50 µL of the relevant type of specific polyclonal guinea pig anti-FMDV serum was added at optimal dilution to each well, diluted in the blocking buffer just described. Plates were then incubated at 37¡ãC for 1 h while being rotated. Anti-guinea pig whole IgG HRP conjugate was then added, 50 µL per well diluted in blocking buffer, and the plates were incubated for 1 h at 37¡ãC while being rotated. Plates were then washed and 50 µL per well of OPD/H_2O_2 chromogen/substrate was added. Color was allowed to develop for 10 min ,and then the reaction was stopped by the addition of 50 µL of 1 *M* H_2SO_4. The results were quantified by reading the plates on a multichannel spectrophotometer. Data relating the activity of each mAb dilution to capture virus as detected by the polyclonal system were plotted. Optimal dilutions of each mAb were measured to allow a single dilution to be assessed in virus quantification studies. The effect of using different concentrations of mAbs as capture reagents was also examined when assessing the various antigen preparations.

10.2.2¡ª
Titration of Enzyme-Labeled mAbs

Plates were coated with 50 µL per well of an optimal dilution of the relevant rabbit anti-FMDV serum in carbonate/bicarbonate buffer (1/5000). After incubation overnight at 4¡ãC or at 37¡ãC for 2 h, the plates were washed. Purified 146S and 12S (50 µL) were then added at 2 µg/mL in blocking buffer and incubated for 1 h at 37¡ãC while being rotated. Each of the conjugated mAbs was tested on the relevant virus capture plates by titration in twofold dilution series from 1 to 10 across 11 wells. The conjugates were diluted in 50 µL of blocking buffer as just described and incubated for 1 h at 37¡ãC while being rotated. Plates were then washed and the chromogen/substrate steps followed as in **Subheading 10.2.1.** Data were obtained relating the activity of the conjugates on dilution to estimate the optimal dilution to be used in the virus quantification studies.

10.2.3¡ª
Titration of Virus Using Dilutions of mAb as Capture and Detecting Reagents

Optimal dilutions of each of the mAbs, as assessed **Subheadings 10.2.1.** and **10.2.2.**, were used to coat wells under the same conditions. After washing, known concentrations of 146S, 12S, TTV or DNV were added as twofold dilution series in 50 µL of blocking buffer (beginning at 5 µg/mL). Plates were incubated at 37¡ãC for 1 h. Labeled mAbs were then added at optimal dilutions in 50 µL of blocking buffer. After 1 h of incubation at 37¡ãC, the plates were washed and substrate/chromogen was added, followed by stopping and reading in a spectrophotometer. Different combinations of mAbs were used as capture and detecting reagents. Virus and virus preparations were also captured using the rabbit serum and then detected with the mAb reagents. Guinea pig serum was also used to quantify the antigens when mAbs and rabbit serum had been used as capture reagents.

10.2.4¡ª
Titration of 146S in the Presence of 12S

The effect of adding high concentrations of 12S to a constant amount of 146S was examined using different concentrations of mAb capture reagents. The same systems were also examined using guinea pig polyclonal detecting serum.

10.2.5¡ª
Standardization of 146S Titrations

A standard preparation of known concentration was established using purified 146S virus quantified by UV spectrophotometry. This standard was stored in small volumes at ¨C70¡ãC, and samples were thawed and used once in the assays. The standard was diluted in quadruplicate in 50 µL of blocking buffer

to produce a standard curve relating the weight of 146S to OD, and the variation at each point was calculated. For the assay of virus contained in tissue culture samples, four different infected tissue culture supernatants were diluted 1/2, 1/4, 1/8 and 1/16 in blocking buffer and then assayed in quadruplicate in 50-µL vol. The weight of the virus in the samples was then calculated for the different dilutions by referring to the curve obtained for the standard 146S titration. The standard 146S acted as a control for each test plate, and examination of the cumulative data from all the test plates allowed the variation of the assay to be determined.

Determinations of the concentrations of the virus from three of the tissue culture samples were made over ten tests on ten separate days.

10.3¡ª
Results

Figure 8 shows the titration curves obtained using different dilutions of mAbs and the polyclonal rabbit serum as capture reagents for constant amounts of the various antigens. Captured antigens were detected using polyclonal guinea pig serum/anti-guinea pig conjugate. All the mAbs captured both 146S and 12S, although the plateau maximum OD for 12S was significantly lower than that for 146S, particularly for mAbs B2 and D9. This was also true for the rabbit capture/guinea pig detector system. Neither TTV nor DNV was detected when the B2 and D9 mAbs were used as capture reagents, whereas mAbs C8 and C9 were capable of presenting TTV to the detecting antibody. The polyclonal system detected all antigens, although dilution of the serum was needed to achieve optimal capture. The optimal dilution for each of the mAbs and the polyclonal sera for the capture of 146S are arrowed.

Figure 9 shows the titration curves for each of the mAb-enzyme conjugates where constant amounts of 146S and 12S were captured by the optimal dilution of polyclonal rabbit serum indicated in **Fig. 1**. All the mAb conjugates were capable of detecting both antigens, the plateau height differences between 12S and 146S resulted from the limiting factors of the polyclonal rabbit serum to capture 12S as explained diagrammatically in **Fig. 10**. The reduction in plateau height for the titration of the same weight of 12S was also shown in the polyclonal capture/detection system.

Figure 11 shows the titrations of various antigens using optimal dilutions of the same mAbs as both capture and detecting reagents. The rabbit capture/ guinea pig detection system is also included for the same materials assessed. This shows that B2 and D9 detected 146S but not 12S, TTV or DNV; whereas C8 and C9 detected 12S and TTV. The polyclonal system demonstrated that all the antigens were present in the samples and that there were marked effects on the maximum OD obtainable for 12S and DNV, with a small effect on TTV.

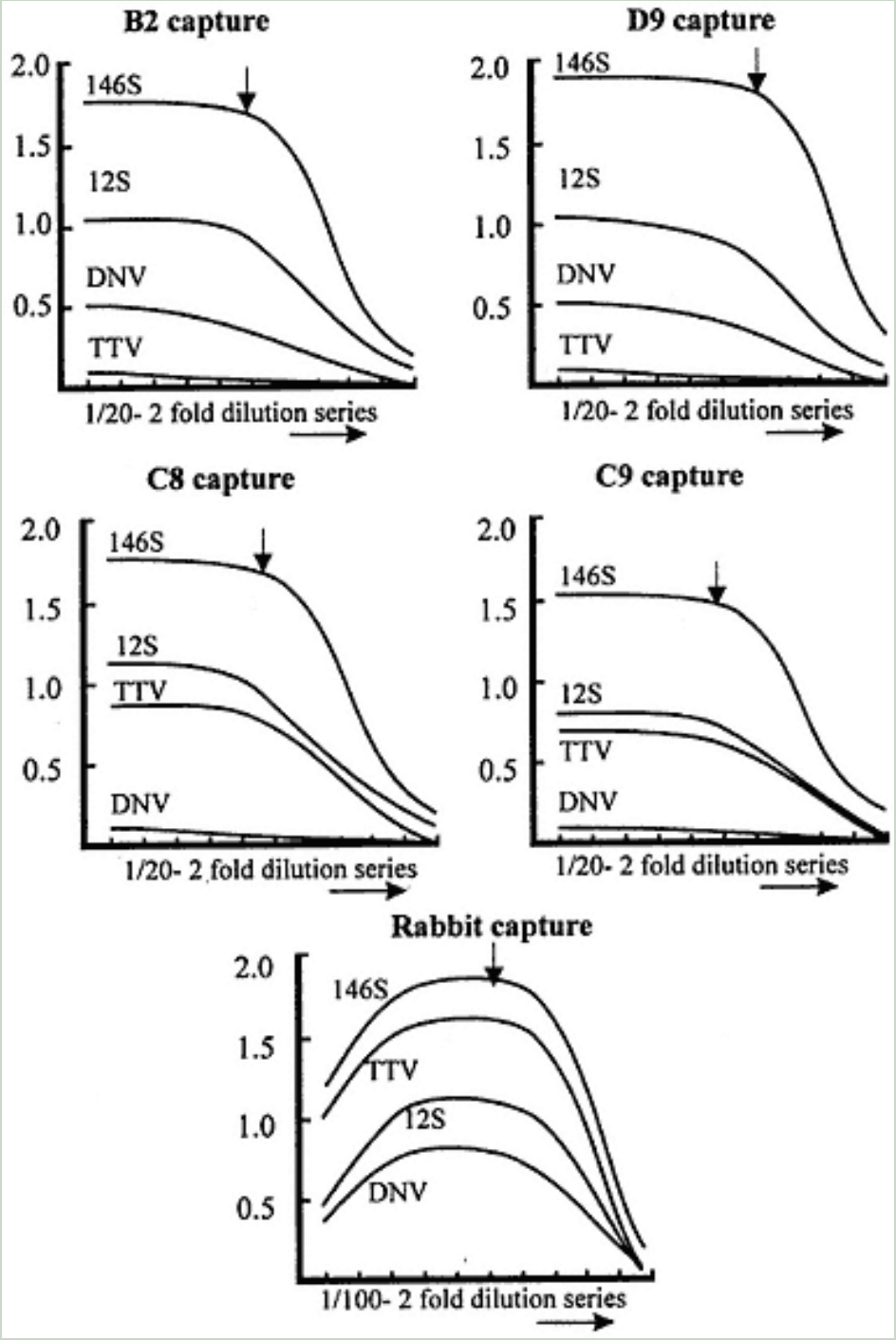


Fig. 8.
Use of mAbs and rabit sera on solid phase to capture various antigenic preparations of FMDV. ↓ = optimal dilution of capture reagents.

Figure 12 shows the effect of measuring two different concentrations of 146S in the presence of a dilution series of 12S. The rabbit/guinea pig system demonstrated that the 12S was titratable. There was no significant effect on the detection of the constant amount of each of the 146S samples in any of the mAb capture/detector systems. Error bars representing 2 ¡Á *SD* from the mean OD of each sample are shown.

Figure 13 shows the results of titrating 146S in the presence of a relatively high concentration of 12S. The rabbit/guinea pig system indicates the level of reaction owing to the 12S observed after the 146S is diluted to a level below that of the 12S. No such plateau is observed in the mAb systems.

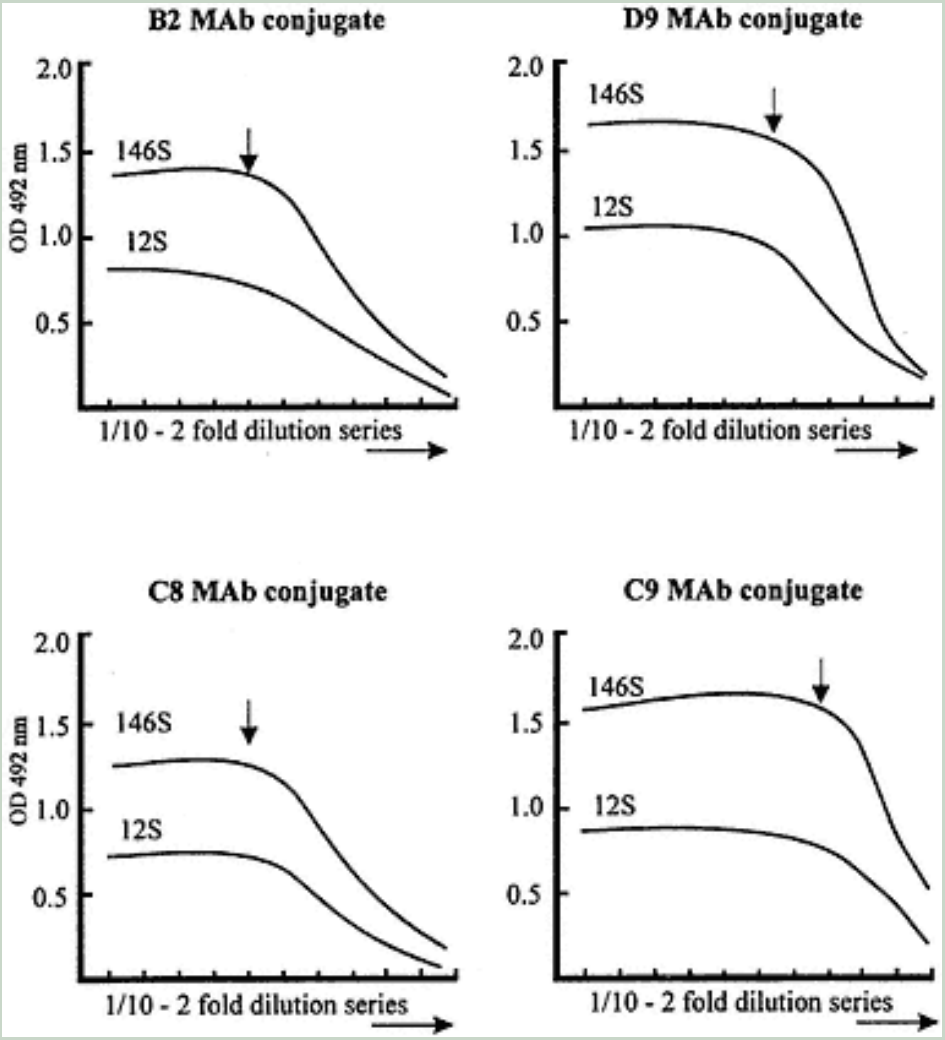


Fig. 9.
Titration of mAb enzyme conugates against 146S and 12S captured by rabbit serum. ↓, Optimal dilution to detect maximum amount of 146S virus.

Figure 14 shows a curve of cumulative data obtained on 10 estimations of the standard 146S performed on different plates over 2 wk using the B2 and D9 mAb systems. The variation in results is shown as bars (1 ¡Á *SD* mean) for each of the titrations (quadruplicate estimates). A line has been drawn through 1.0 OD to highlight the mean and upper and lower limits of the data for each mAb. This represents the most precise area on the standard curve.

The concentration of virus in unknown samples was best estimated when OD values of 0.6¨C1.3 were obtained, corresponding to a range of approx 0.03¨C0.5 µg/mL of virus on the standard curve. **Table 18** shows the results for the determinations of the 146S in four infected tissue culture samples. **Table 19** shows data for three of the samples assessed at a single dilution over 10 tests.

Figure 15 is a diagrammatic representation illustrating the relationship B) to 12S subunits derived from those particles (**Fig. 15C**). The relative sizes of the particles are drawn to scale along with an IgG molecule. A side view of the

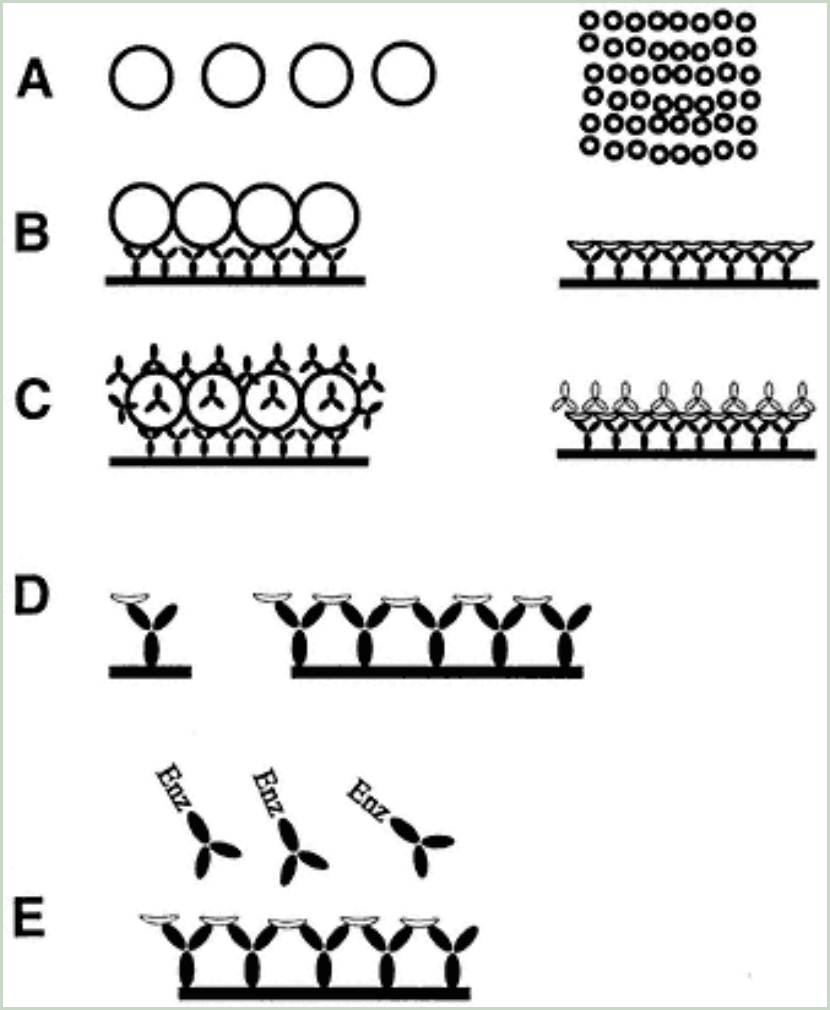


Fig. 10.
Consequences of binding virus and subunits via mAbs or polyclonal sera. **(A)** Relative subunit number to four virus particles. **(B,C)** Capture of 146S virus particles and subsequent detection presents more antigenic sites that with optimal capture of subunits generated from the same number of particles, thus affecting plateau height maxima. **(D)** mAbs orientate subunits to present internal epitopes, unlike presentation of captured 146S particles. **(E)** Detection of mAb captured subunits with same mAb as used to capture does not work, as relevant epitopes are already bound, unlike detection of epitopes on whole particles in the same system.

12S pentamer (**Fig. 15D**) illustrates that epitopes are contained internally as well as externally (in common with 146S external sites). The consequences of mAb or polyclonal antibody capture/detection is examined in **Fig. 15E** and **F**.

Note in **Fig. 10**. **Figure 10A¨CC** that for the same antigenic mass (illustrated as four virus particles), a higher number of subunits are available for capture. However, the ability of the capture system to bind all the units released on

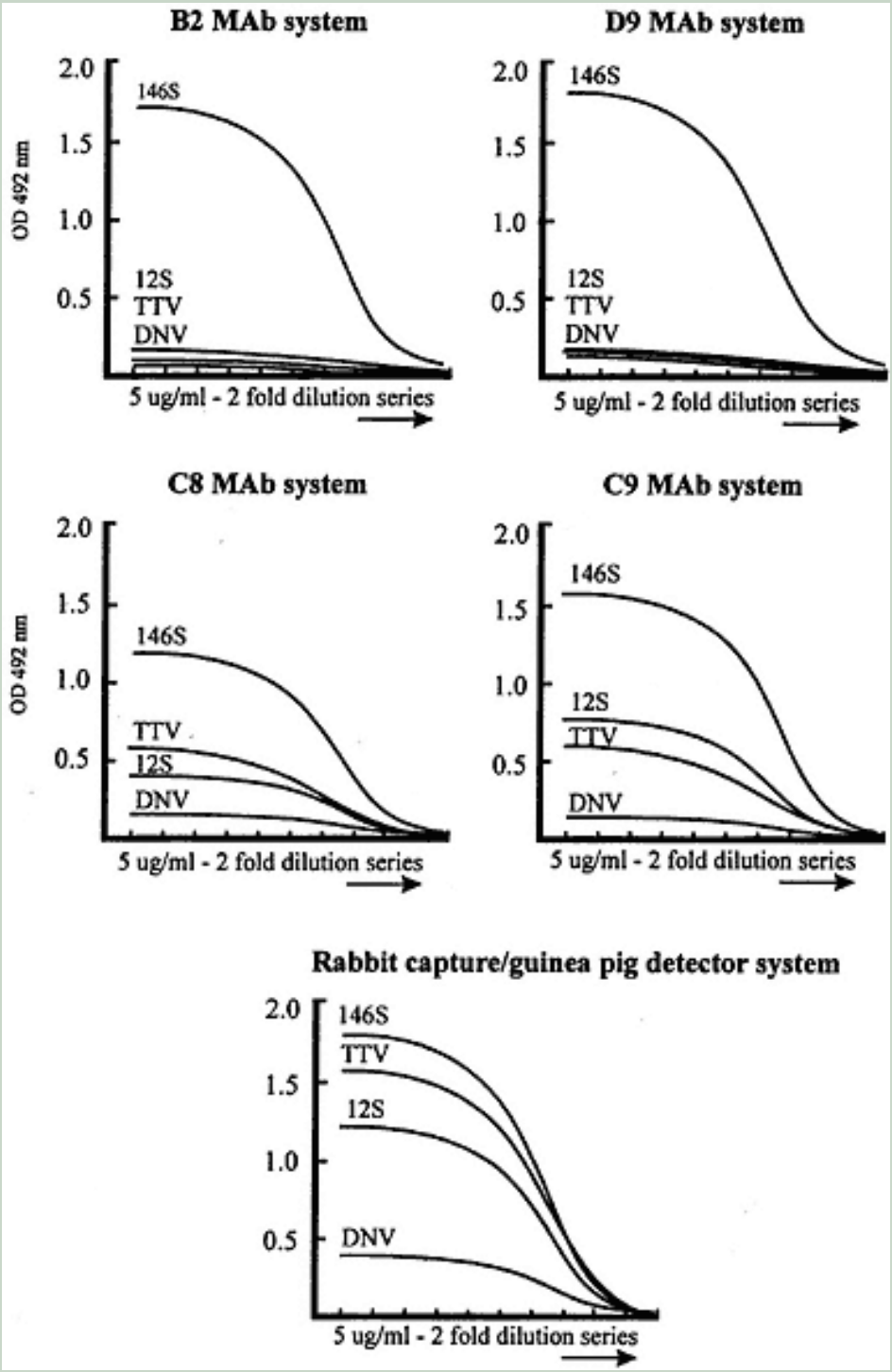


Fig. 11.
Titration of mAbs as capture and detection systems
against dilutions of 146S, 12S, TTV, and DNV.

producing 12S is more limited. This affects the number of antibodies binding as compared to the antigens exposed on virions. **Figure 10D** illustrates that the binding of capture mAbs to external 12S epitopes in common with 146S has

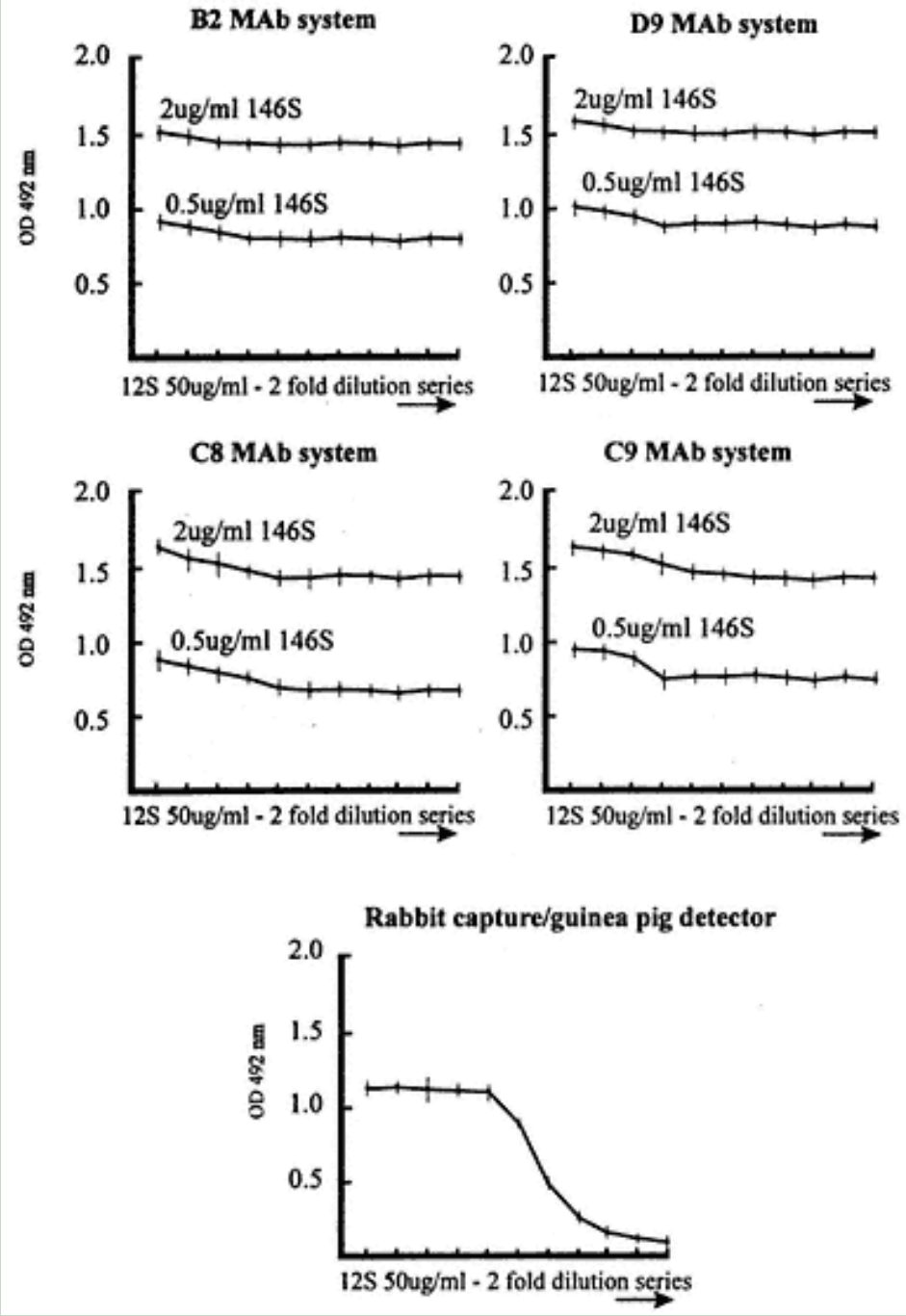


Fig. 12.
Measurement of 146S in the presence of a dilution series of 12S using mAb capture and detection system.

the effect of preventing the same mAb binding, thus allowing differentiation of 12S and 146S particles, even though they share the same epitope.

10.4¡ª
Discussion

A novel method is described for the detection and quantification of whole 146S particles of FMDV using mAbs as capture and detecting reagents. The

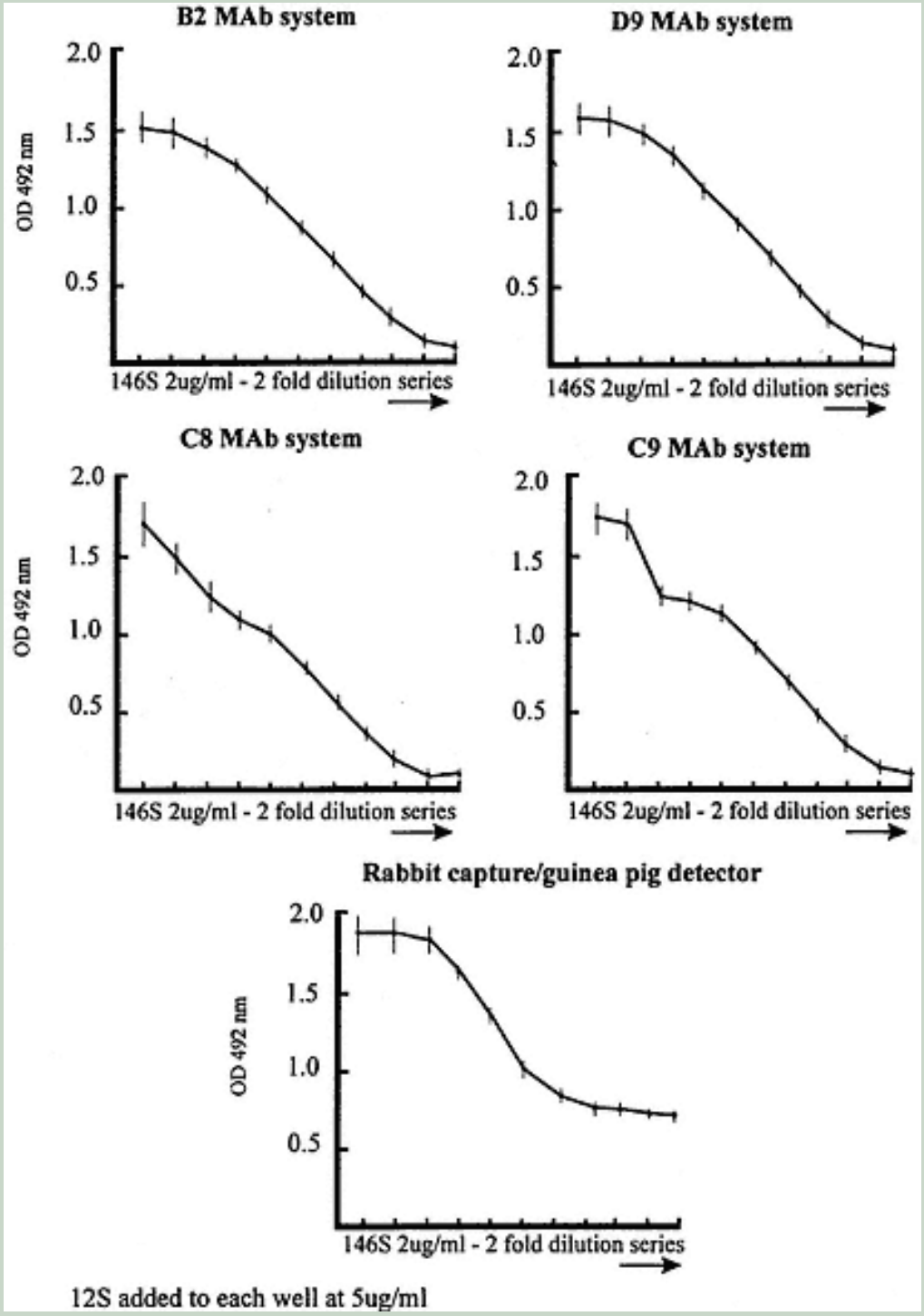


Fig. 13.
Titration of 146S in the presence of a constant amount
of 12S using mAb capture and detection systems.

key to the success of the system relies on the fact that, although the epitopes identified by the mAbs are common to 12S and 146S, they are on the outer capsid surface. The subsequent presentation of the mAb-bound 146S and 12S to the same mAb allows detection of only 146S since the crossreactive epitopes

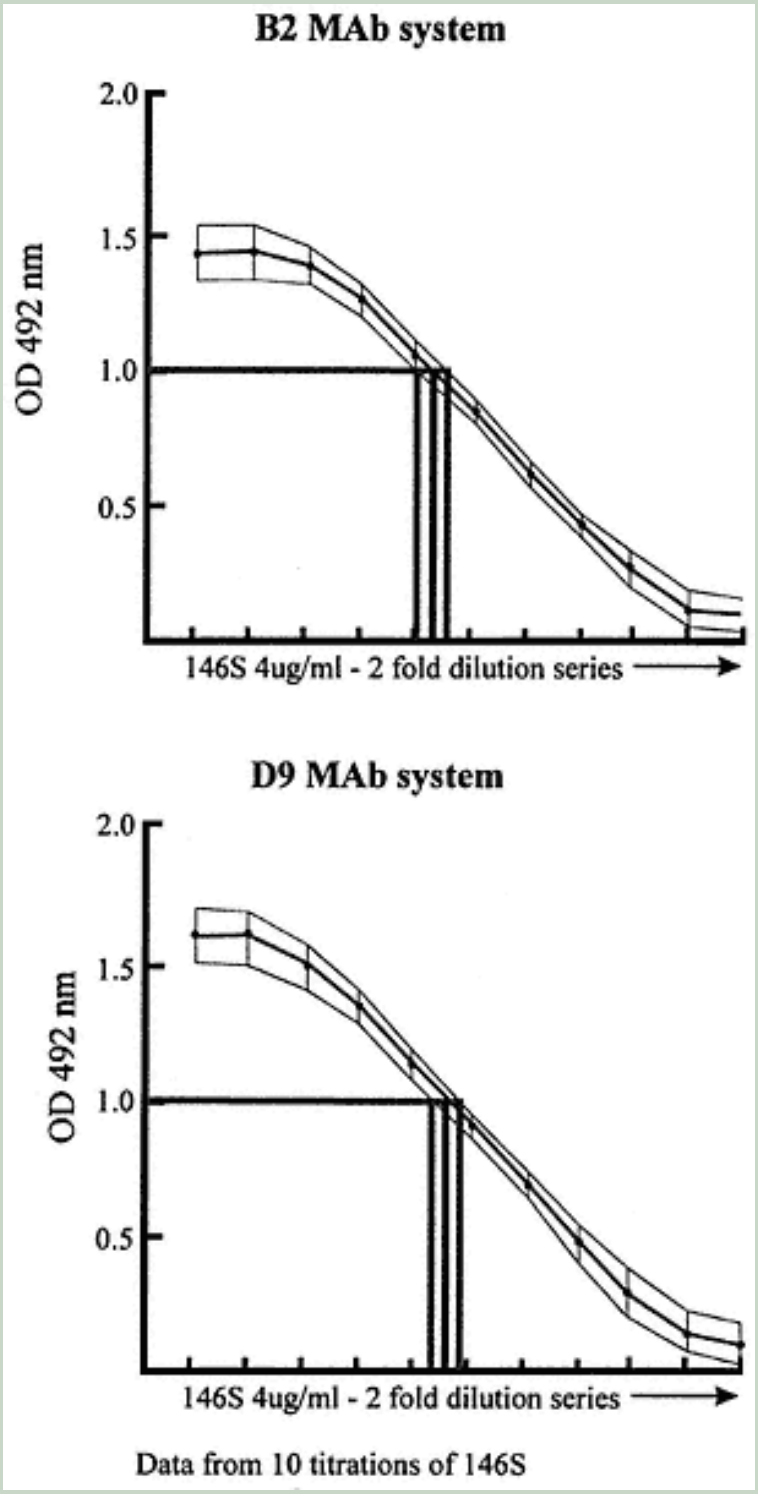


Fig. 14.
Cumulative data for titration of 146S
using mAb capture and detection systems.

on the 12S particles are orientated toward the plate by interaction with the capture mAb.

The capture of 12S by the mAbs and polyclonal serum was shown through its detection using the polyclonal guinea pig antiserum detector in **Fig. 9**. Note the different reaction for the same weights of 146S and 12S by all the systems.

Table 18
Quantification of 146S Virus
from Four Tissue Culture Samples at Different Dilutions

Sample		OD	*SD*	Weight from mean value read from standard curve (µg/mL)[a] Mean	*SD*	Concentration in original (µg/mL)[b] Mean	¡À*SD*
A	1/2	1.51	0.09	>4	¡[a]	>4	¡[a]
	1/4	1.45	0.09	1.71	0.10	6.84	6.44¨C7.24
	1/8	1.30	0.06	0.83	0.07	6.64	6.08¨C7.20
B	1/2	1.35	0.07	1.01	0.12	2.02	1.78¨C2.26
	1/4	1.29	0.05	0.81	0.05	3.24	3.04¨C3.44
	1/8	1.10	0.04	0.37	0.03	2.96	2.72¨C3.20
C	1/2	1.12	0.04	0.39	0.02	0.78	0.74¨C0.82
	1/4	0.89	0.03	0.20	0.01	0.80	0.76¨C0.84
	1/8	0.76	0.02	0.11	0.01	0.88	0.80¨C0.96
D	1/2	0.11	0.01	0.008	0.004	0.016	0.008¨C0.024
	1/4	0.10	0.01	0.007	0.004	0.028	0.012¨C0.044

[a]Weights measured using mean value from sample. The variation of standard curve at this OD was used to establish the standard deviation (*SD*) for the test sample.

[b]Obtained by multiplying the weights of virus obtained for the mean and *¡ÀSD* by dilution factor.

Table 19
Assessment of 146S Virus from Three Tissue Culture Samples
Using 10 Separate Tests

Sample	Dilution	Weight (µg/mL)	SD (µg/mL)	CV(%)[a]
A	1/16	7.1	0.51	7.1
B	1/8	3.1	0.19	6.1
C	1/8	0.8	0.05	6.1

[a]Coefficient of variation (%).

In polyclonal capture/detection systems in the sandwich ELISA, there is always a reduction in the plateau height (a constant maximum OD observed for a range of concentrations in which the detecting serum is in excess), since there is both a reduction in the number of 12S particles (antigenic mass) that can be captured from the equivalent weight of 146S owing to physical reasons, and an orientation factor for 12S (similar to that described for the mAb) depending on the exact nature of the polyclonal antibodies and the extent that the capture and detecting antibodies react with internal and external epitopes. The sandwich

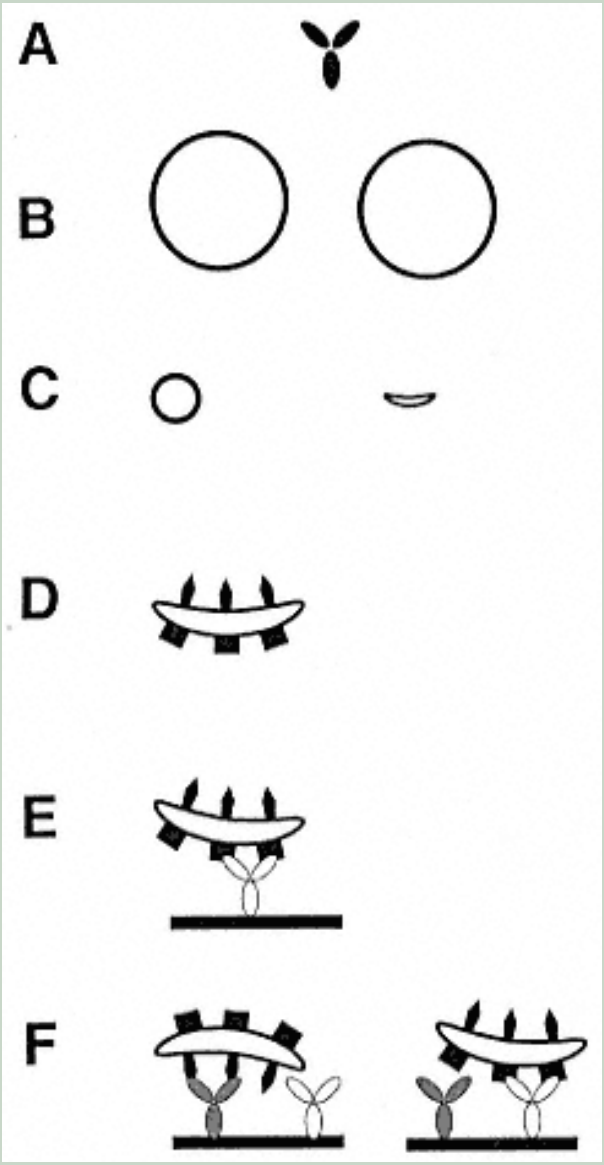


Fig. 15.
Outline of antigen(s) detected in ELISA.
(A) Relative sizes of whole antibody molecules, virus (146S), and subunits (12S); **(B)** profile of virus and subunits; **(C)** 12S subunit with internal and external (as exposed on 146S) epitopes; **(D)** capture of 12S by mAbs directed against external epitopes with orientation of internal epitopes to detecting antibodies; **(E)** polyclonal serum containing antibodies against both internal and external epitopes orientate 12S in both ways.

ELISA has been used successfully as an analytical method for the estimation of total degradation of the 146S using a defined polyclonal system in the analysis of the pH stability of 146S and 75S particles.

All the mAbs examined were capable of detecting 146S and 12S when used as detecting reagents, as shown in **Fig. 9**. The reduction in plateau height was again observed. However, mAbs used in combination did not detect 12S

even at high concentration, but were capable of specifically measuring 146S. **Figure 12** illustrates this and also shows that mAbs B2 and D9 did not detect TTV or DNV, although both these mAbs have been shown to react with continuous epitopes (linear determinants) on the VP1 loop of type O FMDV in the indirect ELISA. However, the sandwich conditions presumably inhibit any second antibody binding since denaturation and disruption of the virus produces small polypeptides and peptides containing the linear epitopes that bind exclusively to the capture antibodies.

The use of such virus-neutralizing mAbs reacting with linear epitopes on VP1 on the outside of the capsid that are sensitive to proteolytic cleavage allows not only the quantification of virus but also a qualitative assessment of the antigen. This is vital in preparing vaccines that show poor immunogenicity when cleavage of VP1 has occurred. mAbs that react with similar epitopes have been characterized, so it is envisaged that there will be little difficulty in identifying reagents suitable for this assay for the assessment of vaccines. The prerequisite is that the mAb can bind to the vaccine strain of interest.

Titration of 146S in the presence of large excesses of 12S could interfere with the specific quantification of 146S. This could occur since the capture mAbs bind 12S, which may affect the capture potential for 146S. The results in **Figs. 12** and **13** confirm that this was not a problem. There was a slight increase in the expected OD values for the 146S , when 12S was added at 100 and 50 times the weight of 146S, particularly using the C8 and C9 systems. Such ratios are not expected to be present in infectious tissue culture samples prepared during the manufacture of vaccine.

The use of standard curves for calculation of virus weight should be successful if precautions are taken to avoid thermal and chemical effects on standard preparations. Thus, once a purified virus has been assessed spectrophotometrically and stored in small aliquots at ¨C70¡ãC or in liquid nitrogen, and when used as single batches, it should be possible to standardize assays precisely. This was shown by titration of the same virus on 10 different days where the best range for the estimation for virus was when OD values were from approx 1.2 to 0.4, corresponding to 0.5 and 0.03 µg/mL of virus/mL. The data in **Tables 18** and **19** indicate that reproducibility of the assay is acceptable for the purpose of assessing of 146S in vaccines.

11¡ª Use of mAbs to Examine Antigenic Variation in Type A FMDV

11.1¡ª Background

Any mAbs produced against members of serotype A FMDVs can be been used to examine antigenic differences. The mAbs used in this study were obtained from various laboratories in Europe and South America. A microtiter plate sandwich ELISA was used to measure the binding of the mAbs with virus

field isolates, vaccine strains, and mAb escape mutants relative to binding with homologous virus. Different amounts of serological and biochemical data were available as to the characterization of the mAbs, particularly for the identification of the critical amino acid sequences bound by individual mAbs. The use of a relatively high number of viruses allowed mAbs of similar reactivities to be compared and grouped using multivariate statistics. The antigenic relationships between the viruses were also evaluated in the same way, and the relevance of results to the epidemiology of strains was examined. The study has allowed the frequency of different epitopes on type A viruses to be examined and the consideration of the epidemiological significance of the findings. Recommendations are made for the use of a limited number of mAbs to act as a standard panel to allow rapid antigenic analysis of isolates.

mAbs against most serotypes of FMDVs have been prepared and characterized in many laboratories worldwide. Such reagents offer the potential for rapid antigenic characterization of virus isolates in simple binding assays such as ELISA. Antigenic variation of FMD viruses is important in many areas involving the control of diseases, such as assessing field strains for their potential threat to animals vaccinated with vaccine strains, comparing vaccine strains among producers, monitoring the vaccine strain throughout production, examination of challenge strains used to evaluate vaccine efficacy which are produced by passage in animals, and examining of persistent viruses (carrier state).

This study shows how mAbs produced against serotypes A5, A10, A22, and A24 viruses, from different laboratories, can be used to group viruses according to their similar properties; examine the distribution and variation of the epitopes; recommend an assay for the rapid comparison of epitopes on type A viruses; and to define a limited panel of type A mAbs that might be useful in comparing type A field, vaccine, and challenge strains.

11.2¡ª
Materials and Methods

11.2.1¡ª
Viruses

The viruses were obtained from the World Reference Laboratory (WRL), at Pirbright, UK. Certain isolates were selected as representatives of vaccine strains. The isolates were amplified by growth in tissue culture usually through bovine thyroid (BTY) primary cells, then passaged in continuous monolayer cultures of baby hamster kidney (BHK-21). Some of the viruses also were passaged in continuous renal swine cells (RS). The passage history of most the isolates is indicated by the number following the cell line. Most of the samples for use in the ELISA were obtained by a further passage of seed stock virus in BHK cells, but the last manipulation of the virus is shown by the last cell line indicated. When monolayers were totally disrupted, the mixture was cen-

trifuged (2000g for 10 min) to remove debris, and the supernatant was stored at ¨C70 or ¨C20¡ãC after the addition of an equal volume of sterile glycerol.

11.2.2¡ª
Antisera

Rabbit and guinea pig polyclonal antisera against type A5, A22, and A24 viruses were prepared as described in **ref. *2***. These sera, used as capture antibodies and detecting antibodies, respectively, in ELISAs were produced after multivaccination of animals with purified inactivated virus, containing antibodies with a wide spectrum of activity against all FMDVs components.

11.2.3¡ª
Monoclonal Antibodies

The mAbs used in this study came from the IAH, Pirbright, UK, and from various laboratories in Europe and South America. These were obtained as ascites fluids or tissue culture preparations.

11.2.4¡ª
Data Obtained for mAbs

Different methods were used to characterize the various mAbs, including use of various ELISAs with various antigenic preparations of the viruses. The data were also used to evaluate the findings of the sandwich ELISA.

11.3¡ª
Sandwich ELISA to Compare Binding of mAbs to FMDVs: Antigenic Profiling

The sandwich ELISA was conducted as described in **ref. *7***. Briefly, microtiter plate wells were coated with a pooled mixture of rabbit antibodies produced against type-purified isolates characterizing A24, A22, and A5 FMD virus subtypes. Such a mixture has been shown to be effective in capturing most of the type A FMDV isolates examined in the WRL at Pirbright. The rabbit antiserum was added in 50-µL vol to the wells and diluted in 0.05 *M* carbonate/bicarbonate buffer, pH 9.6. Plates were incubated overnight at 4¡ãC or 1 h at 37¡ãC. Plates were then washed with PBS, and the various virus preparations were added in duplicate in 50-µL vol, (as shown in **Fig. 16**) diluted in 50 µL of blocking buffer (PBS containing 3% bovine serum albumin, 0.1% Tween-20, and 5% nonimmune [normal] bovine serum). The homologous virus was always included on row A of each plate to demonstrate the maximal interaction of the mAbs so that relative assessments of binding could be examined, as discussed next.

Plates were washed and mAbs were added as shown in **Fig. 17**. The mAbs were diluted in blocking buffer as described for the virus dilution. The dilution of mAb used was determined from the studies on mAb binding to homologous virus using indirect and sandwich ELISAs; an excess of mAb was always used. The last two columns of the plates received guinea pig serotype-specific serum

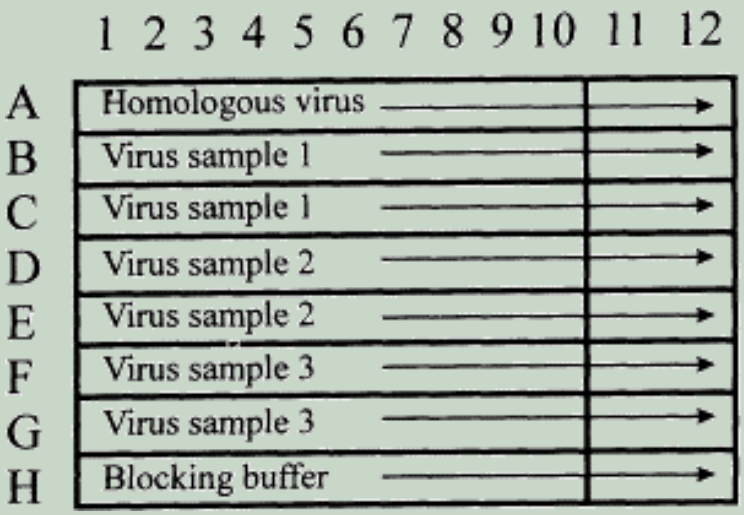


Fig. 16.
Sandwich ELISA for mAb profiling. All wells of the plates are coated with polyclonal anti-FMDV-type specific serum. A mixture of rabbit antibodies against A5, A22, and A24 serotypes is used. After incubation and washing, a single dilution of different virus suspensions is added (in blocking buffer) as shown, in duplicate rows for test samples (B, C; D, E; and F, G). Row A contains virus responsible for eliciting mAbs used, and row H receives only blocking buffer.

at a pretitrated dilution in blocking buffer. This serum was broadly reactive (produced in a way similar to the rabbit capture antibodies), and has been tested to react with all viruses within a serotype. The values in these columns served to estimate the amount of each virus captured. The last row received no virus but the respective column mAb. This acted as the background control for any mAb interaction. Plates were incubated at 37¡ãC for 1 h while being rotated. Plates were washed, and then each well that had mAb added received antimouse IgG enzyme conjugate at a pretitrated optimal dilution in blocking buffer. The last two columns received anti¨Cguinea pig conjugate as described for the virus titrations. The plates were incubated at 37¡ãC for 1 h with rotation and then the OPD substrate solution was added. The color development was stopped after 10 min and the color quantified by a multichannel spectrophotometer.

The dilution of each virus was usually determined by previous titration in a sandwich ELISA in which plates were coated and viruses diluted in triplicate as twofold series, in blocking buffer. After incubation with rotation, the plates were washed and a pretitrated antiserotype-specific guinea pig serum was added diluted in blocking buffer. The plate was then incubated as for the virus stage and washed, and then each well received a dilution (in blocking buffer) of anti-guinea pig HRP conjugate. After incubation as before, H_2O_2/OPD substrate was added. Color development was stopped at 10 min. The plates were read in a multichannel spectrophotometer (492 nm). The developed color

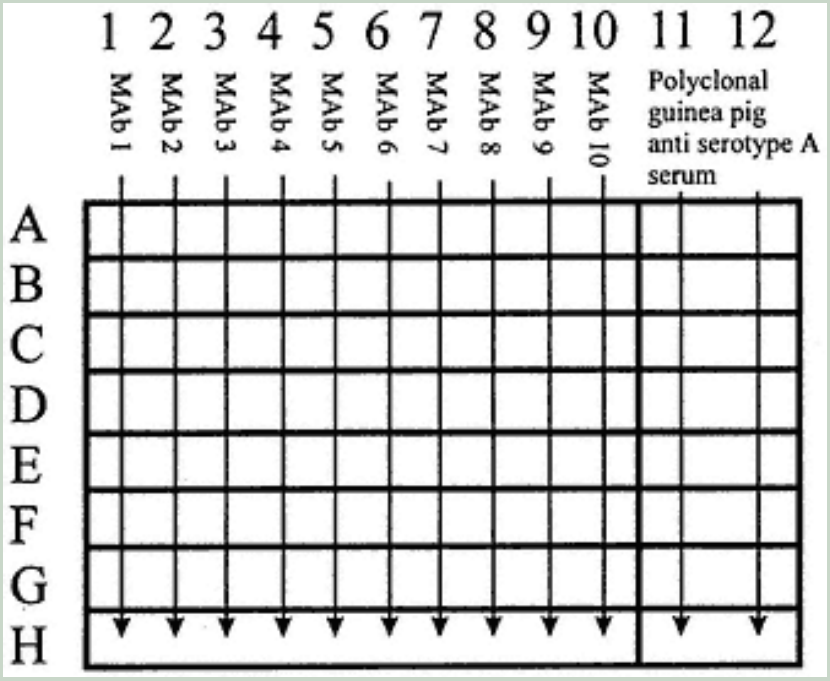


Fig. 17.
Addition of mAbs to profiling plates. mAbs 1¨C10 are added in columns 1¨C10 at a single dilution in blocking buffer. Polyclonal anti¨Ctype A serum is added in columns 11 and 12. Plates are incubated and washed, after which antimouse enzyme conjugate is added to columns 1¨C10 and anti¨Cguinea pig enzyme conjugate to columns 11 and 12. After incubation and washing, substrate chromophore is added and color development stopped. The OD values are read and the relative binding of mAbs to viruses is determined with reference to the reactions of the mAbs with the homologous virus. The OD values in row H are subtracted from each OD value in the respective column. The OD values for each duplicate mAb reaction are then averaged. This mean OD value is then expressed as a percentage of the mean value obtained for the virus/ guinea pig binding for each respective virus. Finally, this percentage value is expressed as a percentage of the value obtained for the homologous virus. This, the relative weights of each virus are taken into consideration by reference to the examination of the guinea pig polyclonal readings, and results are therefore comparing the relative binding of the same mAbs to different viruses as compared to the homologous virus.

was related to the dilution, and the dilution giving 1.2 to 1.5 OD was used in the antigenic profiling ELISA described in **Subheading 11.3.1.** The plates were incubated for 1 h at 37¡ãC while being rotated at approximately three revolutions per second. In practice, it was found that most tissue culture preparations contained high levels of virus and that dilutions of 1/3 to 1/16 could be used to provide excess virus for the trapping rabbit serum. Therefore, the amount of rabbit serum was limiting in this step.

11.3.1¡ª Processing of Data

Processing of the data is described in **ref. 7**. Briefly, the OD values in the last row (mAb negative antigen control) were subtracted from each column.

The OD values for each duplicate were then averaged. This mean value was then expressed as a percentage of the mean value obtained for the virus/guinea pig binding for each respective virus. Finally, this percentage value was expressed as a percentage of the value obtained for the homologous virus. Thus, the relative weights of each virus were taken into consideration by reference to the examination of the guinea pig polyclonal readings. Results are therefore comparing the relative binding of the same mAbs to different viruses as compared to the homologous virus. This is illustrated in **Tables 20** with simplified results.

The criteria for assessing the results and statistical considerations were examined in **ref. *7***. The data were analyzed with a computer-based package using multivariate statistics to perform hierarchical analysis (block method, complete linkage). The mAbs were then assessed according to their reactions with the viruses, and the viruses were related according to their reactions with the mAbs. Cluster analyses were made grouping the reactions of the mAbs and viruses, respectively. The reactions were related to obtain dendrograms of the mAb and the virus groups.

11.3.2¡[a]
Results

All the mAbs were examined initially for their binding characteristics against 20 selected field strains, using the antigenic profiling sandwich ELISA. From all data, 30 of the mAbs were selected for use in larger-scale antigenic profiling studies, in which a higher number of virus isolates was examined. The conclusive final relationships for the mAbs and the antigenic relationships of the viruses were based on the use of these 30 mAbs and 60 viruses. The data in the dendrograms in **Fig. 18** show the cluster analysis for the mAbs. Ten clusters were selected as being distinct; these are indicated in **Fig. 19** (in which the origin of the mAbs is also indicated). That the mAbs in these clusters reacted with different epitopes was further confirmed through the examination of other available data. In **Fig. 19,** mAb L13 is boxed in because it reacts with a conformational epitope whereas the other mAbs bind to a linear epitope.

The relationship of the viruses as elucidated from the binding pattern with the mAbs is shown as a dendrogram in **Fig. 20**. The distance at which the viruses within a cluster are assessed as being very similar but different to another cluster has been put at 5.0. This is demonstrated by the line drawn across the dendrogram in **Fig. 20**. This value is based on assessing the relevance of the observed clusters to existing epidemiological knowledge of virus isolates. Sixteen clusters are produced, as shown in **Fig. 21**. The virus profiling data showed two major clusters separating A22-like viruses from the A24-like and A5/A10-like viruses, the latter two clusters were more closely related. The first cluster group indicates closely related South American A24 Cruzerio iso-

Table 20
Stylized Data and Methods for Calculation

(i) Untreated sylized OD data (duplicates made the same)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.1	1.5	1.3	1.6	1.0	0.8	0.9	1.1	1.2	1.3	1.6	1.6
B	0.1	1.4	1.3	1.1	0.1	0.1	0.7	1.1	0.1	0.1	1.5	1.5
C	0.1	1.4	1.3	1.1	0.1	0.1	0.7	1.1	0.1	0.1	1.5	1.5
D	1.1	1.4	1.2	0.1	0.1	0.1	0.1	1.2	1.0	1.0	1.5	1.5
E	1.1	1.4	1.2	0.1	0.1	0.1	0.1	1.2	1.0	1.0	1.5	1.5
F	0.5	1.1	1.3	0.1	0.5	0.8	0.9	1.1	0.1	0.1	1.2	1.2
G	0.5	1.1	1.3	0.1	0.5	0.8	0.9	1.1	0.1	0.1	1.2	1.2
H	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2

(ii) OD minus row H value in each column

	1	2	3	4	5	6	7	8	9	10	11/12
A	1.0	1.3	1.0	1.5	0.9	0.7	0.8	1.0	1.1	1.2	1.4
B	0	1.2	1.0	1.0	0	0.1	0.6	1.0	0	0	1.3
C	0	1.2	1.0	1.0	0	0.1	0.6	1.0	0	0	1.3
D	1.0	1.2	0.9	0	0	0.1	0	1.1	0.9	0.9	1.3
E	1.0	1.2	0.9	0	0	0.1	0	1.1	0.9	0.9	1.3
F	0.4	0.9	1.0	0	0.4	0.7	0.8	1.0	0	0	1.0
G	0.4	0.9	1.0	0	0.4	0.7	0.8	1.0	0	0	1.0

(iii) Means of OD values after subtraction

	1	2	3	4	5	6	7	8	9	10	11/12
A	1.0	1.3	1.0	1.5	0.9	0.7	0.8	1.0	1.1	1.2	1.4
B/C	0	1.2	1.0	1.0	0	0.1	0.6	1.0	0	0	1.3
D/E	1.0	1.2	0.9	0	0	0.1	0	1.1	0.9	0.9	1.3
F/G	0.4	0.9	1.0	0	0.4	0.7	0.8	1.0	0	0	1.0

(iv) Percentage value of OD mAb/OD value polyclonal, for that virus in 11/12

	1	2	3	4	5	6	7	8	9	10	11/12
A	71	93	71	107	64	50	57	71	79	86	100
B/C	0	92	77	77	0	8	46	77	0	0	100
D/E	77	92	70	0	0	8	0	84	70	70	100
F/G	40	90	100	0	40	70	80	100	0	0	100

(v) mAb percentage in each column as percentage of homologous virus value

	1	2	3	4	5	6	7	8	9	10
A	100	100	100	100	100	100	100	100	100	100
B/C	0	99	108	72	0	16	80	108	0	0
D/E	108	99	99	0	0	16	0	118	89	81
F/G	56	97	140	0	61	140	158	140	0	0

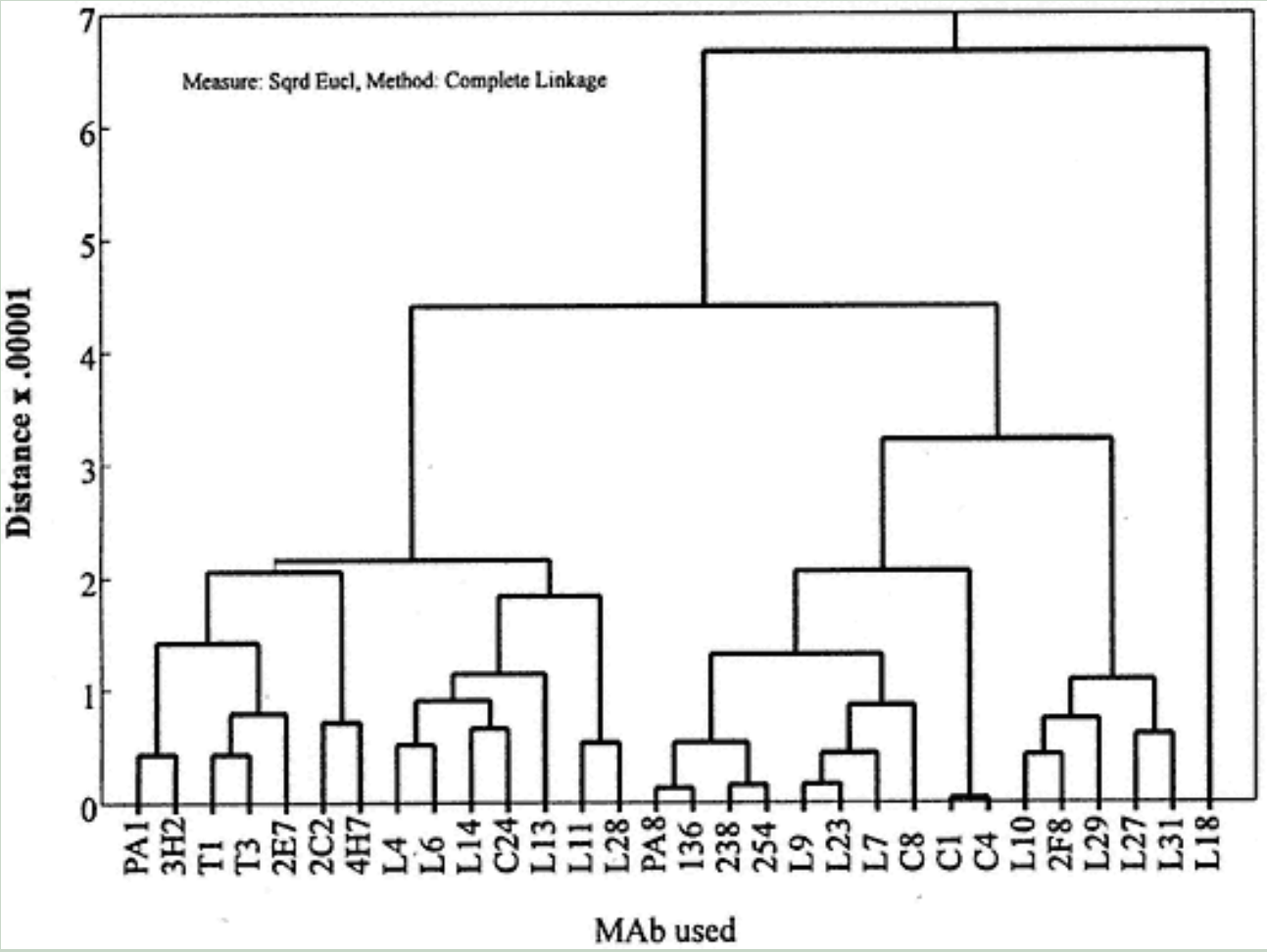


Fig. 18.
Grouping of mAbs according to reaction patterns against viruses. Analysis was by multivariate statistics.

lates. The profiling confirms the identity of two vaccine and challenge strains produced after passage in cattle and indicates their similarity to the A24 Cruz reference strain. Viruses from Peru in 1971 and 1972 appear to be similar to each other and to the reference strain. The second cluster within the A24-like viruses comprises two virus isolates from Brazil in 1979 and A24 Venceslau in 1970. Both these clusters have a similar relationship to clusters 3 and 4. These clusters contain South American viruses expected to be A24-like (A Col/Sab/85 and A Col/Boy/89); however, viruses of African origin (A Egypt 1/77, A Cam 5/75, and A Sau 1/76) are also included. Cluster 5 has more similarity to the A5/A10 viruses and again contains both South American (A Bra/68, A27 Col/67) and African (A Libya 3/79, A Alg 5/75) viruses. This cluster is similar to clusters 6 and 7, which contain early European (A5 West/51, A Greece 1/76), Middle Eastern (A Sau 23/68) and African (A Ken 1/76) viruses. Cluster 8 is more distinct than cluster 1¨C7 and comprises the A10 Holl/42, A10 Arg/61 (known to be related), and A5 Spa/73 virus. In turn, this group is strongly related to A5 viruses from Italy and France.

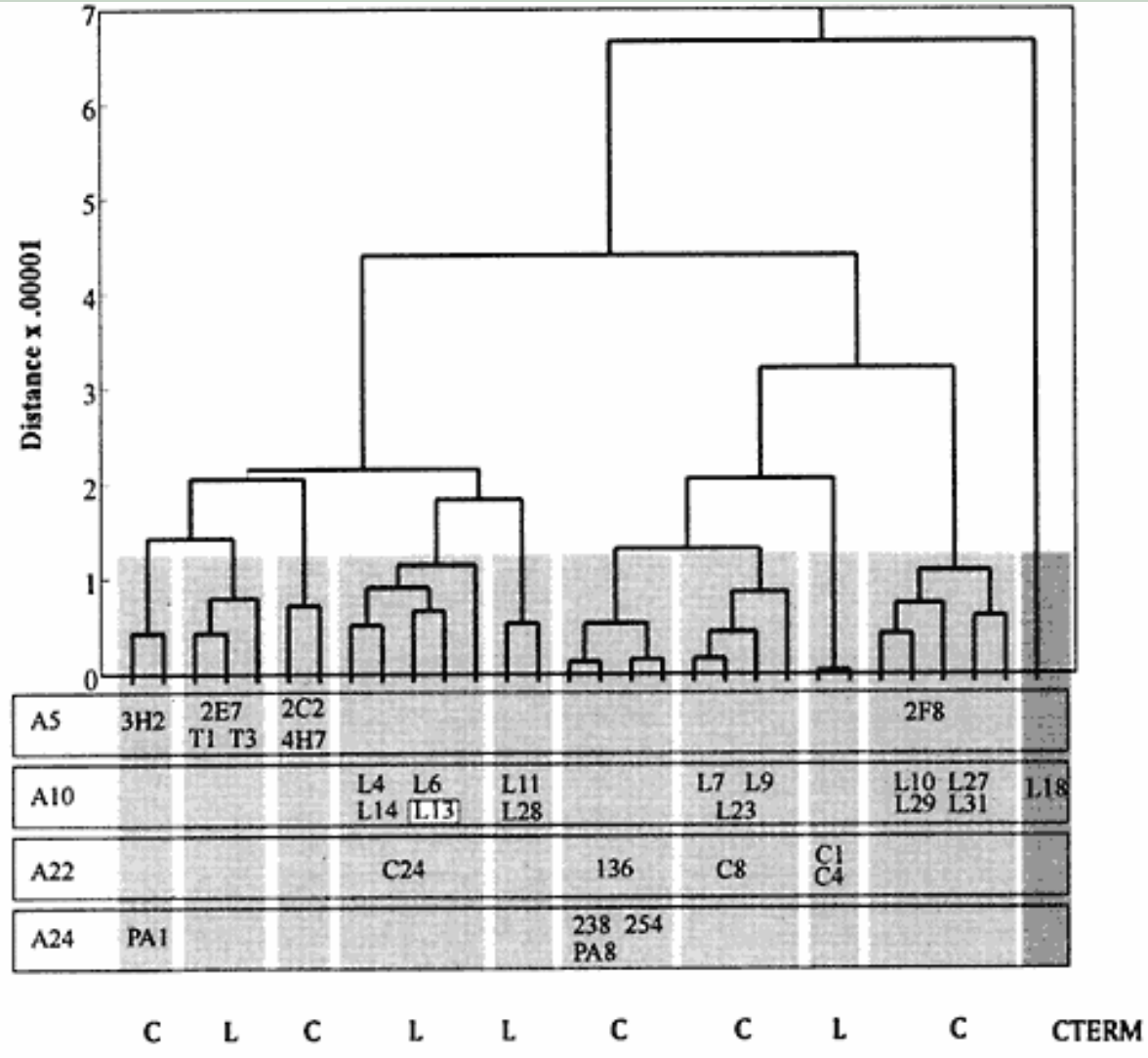


Fig. 19.
Grouping of mAbs according to reaction patterns against viruses. Analysis was by multivariate statistics. There was clustering of mAbs with similar reactivities. Data were examined in light of the origin of mAb and known reactions from other tests. Serotypes used to generate mAbs are shown as A5, A22, A24, and A10. C, mAbs binding to conformation-dependent epitopes; L, mAbs binding to conformation-independent epitopes (linear); CTERM, mAbs demonstrated to react with the C-terminus of structural protein VP1.

A representative mAb profile from some of the clusters is shown in **Fig. 22A¨CD**. Such profiles relating binding of mAbs are typically produced. Their evaluation is more difficult. Comparison by eye estimation is not valid; this section has attempted to introduce statistical methods for the easy comparison of data.

11.3.3¡ª
Discussion

The method used for the rapid analysis of virus isolates relied on the specificity of mAbs. In this study, a relatively large panel was used, the mAbs of which were prepared against several classical type A viruses representing epidemiologically important groups. Thus, one aspect of the study allows an

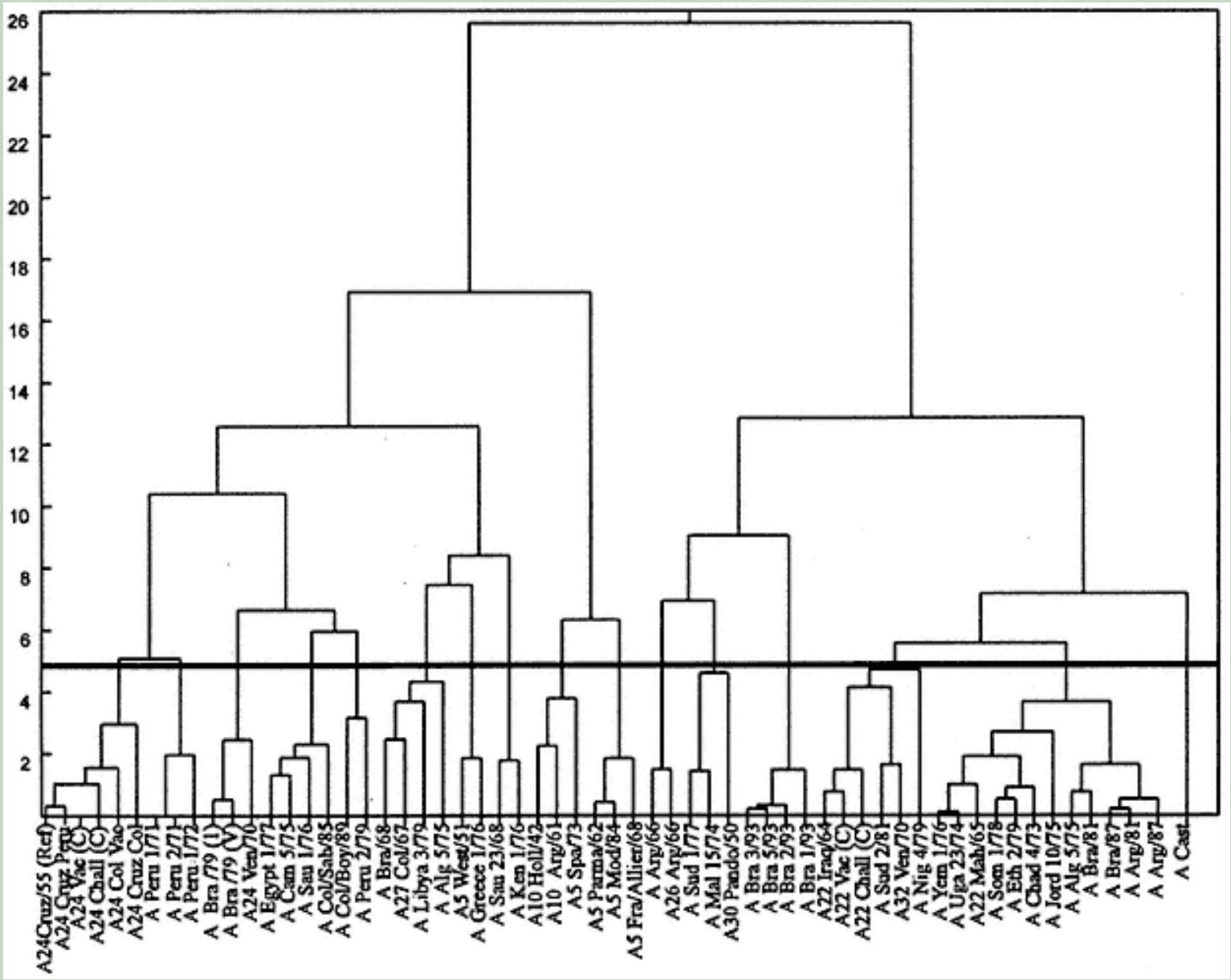


Fig. 20.
Grouping of viruses according to their similarity of binding profiles with mAbs.

examination of the distribution of epitopes identified by the mAbs. The characterization of the specific mAbs in terms of, e.g., reaction with conformational and linear sites, amino acid sequences important to binding, and trypsin sensitivity, allows various antigenic properties to be ascribed to the viruses where binding of antibody is observed. Such properties can be used to directly compare possible biological properties of isolates and thus predict problems in vaccine formulation, identify specific changes to viruses during the manufacture of vaccine and examine viruses and virus proteins produced and expressed using molecular biological techniques. The data collected for the mAbs is included to allow a comprehensive list of properties to be available to other researchers.

The use of a large number of virus isolates also allowed the rapid comparison of all the mAbs. Thus, the variation in antigenic properties of the isolates allowed identification of patterns of reaction of the mAbs, thereby producing groups (clusters) of identically or similarly reactive mAbs and distinguishing them from other clusters. This approach is only possible where a large number

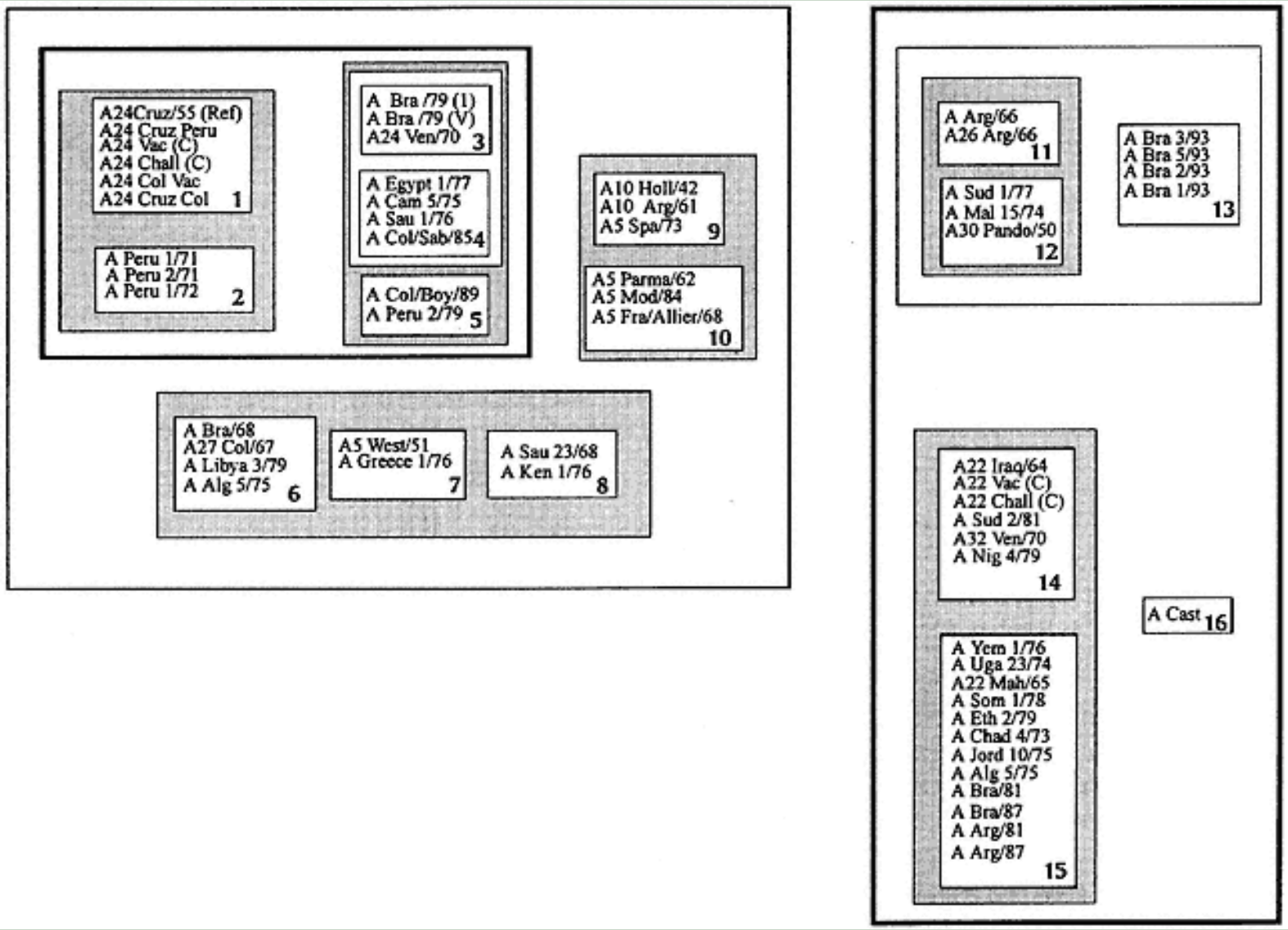
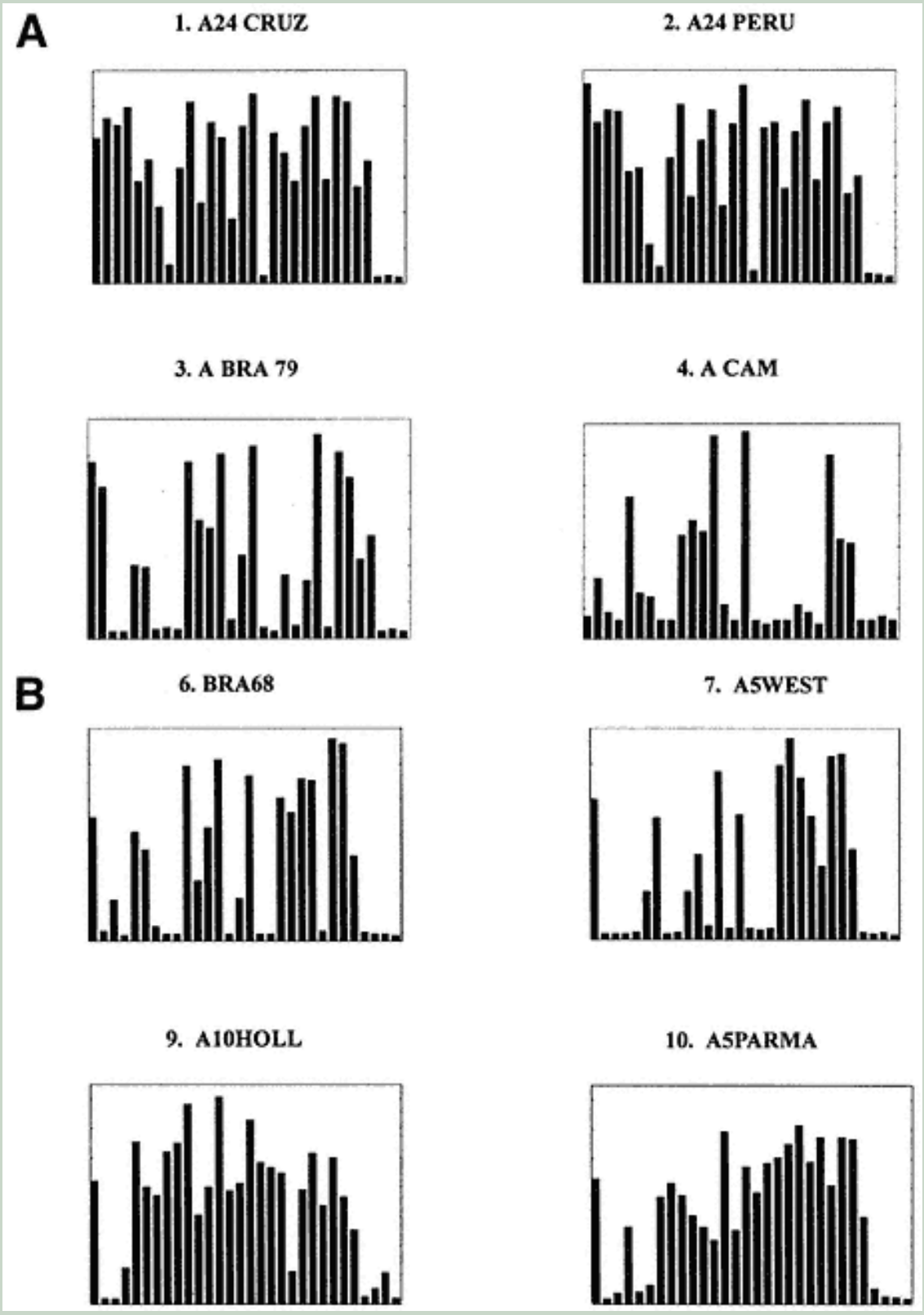


Fig. 21.
Virus clusters (1¨C16) taken from data in Fig. 21. The closeness of relationships is indicated by boxes.


A
1. A24 CRUZ
2. A24 PERU
3. A BRA 79
4. A CAM
B
6. BRA68
7. A5WEST
9. A10HOLL
10. A5PARMA

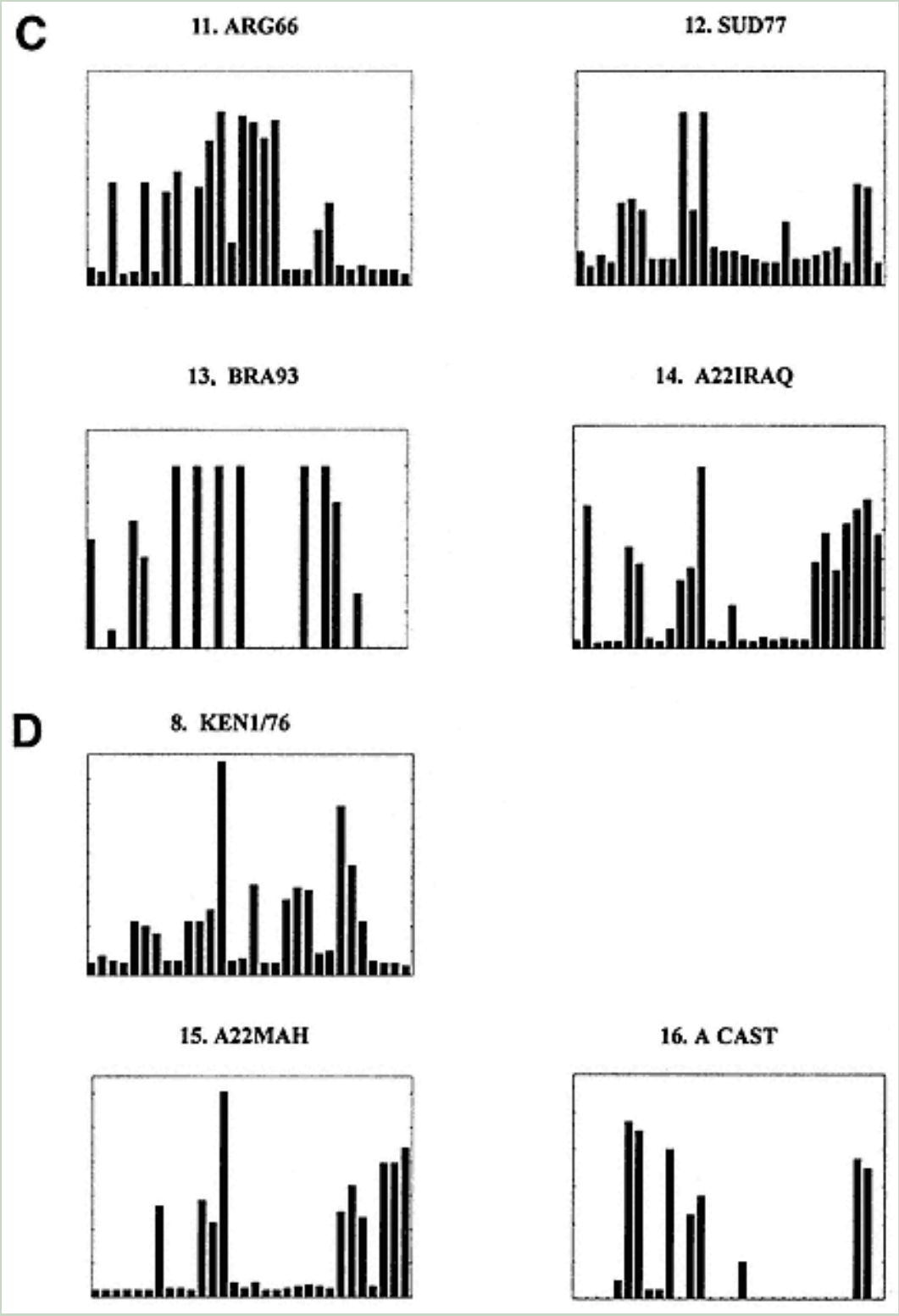


Fig. 22.
(A) Profiles showing individual mAb reactions with particular viruses. 1¨C4, members of those cluster groups specified in **Fig. 22**. **(B)** Profiles showing individual mAb reactions with particular viruses. 6, 7, 9, and 10: members of those cluster groups specified in **Fig. 22**. **(C)** Profiles showing individual mAb reactions with particular viruses. 11¨C14: Members of those cluster groups specified in **Fig. 22**. **(D)** Profiles showing individual mAb reactions with particular viruses. 8, 15, and 16: Members of those cluster groups specified in **Fig. 22**. Scale (*y*-axis) is from 0¨C120% binding with respect to homologous binding of mAb to parental strain.

of epidemiologically distinct isolates is available (as is true of the WRL), and a limitation of the variation in antigenic makeup of the isolates would have an effect on the patterns of reaction of the mAbs. Thus, if only two strains were used in this study, it would be likely that only two to three groups of mAbs would be observed. The clusters observed for the mAbs through examination of many isolates are verified with reference to data from this and previous studies on mAb escape mutants as well as reference to the binding characteristics of the mAbs.

This exercise also allows a limited panel to be designated whereby the relationships between isolates can be made with a low number of mAbs, which greatly simplifies assays. Confidence that limited panels reflect true antigenic differences comes only through a thorough examination of mAbs against a large number of viruses. Such an approach is also quite useful to workers in laboratories without access to a large number of isolates because the mAbs they produced can be compared to others by standardization laboratories. The mAbs can be identified as fitting into particular clusters (already defined), and properties common to those clusters can be ascribed.

The method used to examine the virus/mAb binding used mAb in excess (i.e., at a high concentration). This can have a distinct effect on the binding profiles observed. The relative affinity of each of the mAbs depends on the exact differences between the epitopes presented on the heterologous viruses as compared to the homologous virus. The assay used is essentially a binding assay, and small differences in affinity constants among isolates are not reflected by differences in binding in which there is a large excess of antibody molecules. Thus, any differences noted in this study reflect relatively large differences in affinity (a significant difference in epitope). Such differences also fit in with the examination of the virus-neutralizing capacity of the mAb for homologous and heterologous viruses. Not all mAbs that bind 100% to heterologous isolates will neutralize that virus (results not shown). This is a result of the differences in the conditions in the virus neutralization test (VNT). In the VNT, the amount of virus used represents about 100 TCID50 (approx 10^5 virus particles assuming 1 TCID50 is equivalent to 10^3 noninfectious particles). In the ELISA, approx 0.05 µg is present to bind with mAb (approximately 8 ¡Á 10^9 virus particles). The same concentration of mAb is used in both assays; thus, by the Law of Mass Action, the ELISA tends to effect reaction owing to the high concentration of virus (approx ¡Á 80,000 times that in the VNT). Therefore, mAbs with reduced affinity tend to have the reaction driven in the ELISA but not in the VNT.

Differences in affinity for strongly binding mAbs can be assessed easily using competitive assays in which a homologous system involving pretitrated

virus and homologous mAb (submaximal binding concentration) is challenged by the addition of heterologous virus. Here, the relationship of competition slopes comparing homologous and heterologous viruses reflects the relative affinity of the mAb for the two isolates (data not shown).

12¡ª
Conclusion

Figure 23 shows the features of mAbs relevant to the ELISA. Although the mAb is, by definition, a specific reagent with respect to the binding to epitopes, the physical state of the mAb, its density, and the distribution of epitopes all affect assay performance.

Let us review the diagrams in **Fig. 23**:

1. Diagram 1: This indicates that the orientation of the mAb on the plastic surface can affect subsequent binding to antigens (e.g., in capture ELISA). Since the mAb is a single population of molecules with identical chemical structure, any tendency for such orientations (depending on the exact nature of plastics used and the solutions used to bind the mAb), will be translated to all molecules. This means that a variety of plates and solutions of different ionic strengths and pHs can be used in cases in which an mAb apparently does not perform well as a capture reagent.

2. Diagram 2: This indicates that the density of mAb can affect binding, even when the orientation is correct and Fab molecules are present. Full-dilution ranges of mAbs should be performed to allow an assessment of binding properties, since it is possible that lower concentrations of mAb are better spaced to allow capture. This is also dependent on the nature of the antigen.

3. Diagram 3: This reminds us that the isotype of an mAb can be important. Generally, IgM molecules are poor capture reagents. The use of mAbs of different isotypes can be exploited through the use of specific antimouse isotype reagents.

4. Diagram 4a: When bivalent molecules of mAb are reacting with epitopes on a complex, the spacing of the epitopes has a profound effect on the actual affinity of the mAb. When epitopes are spaced too far to allow bivalent binding, effectively a single Fab interaction takes place. The orientation of the epitopes is also important.

5. Diagram 4b: When small molecules (e.g., polypeptides) are coated, mAb may also have optimal bivalent binding in which the spacing (and presentation/orientation) is optimal.

6. Diagram 4c: Here, the spacing is too large to allow bivalent binding and hence the effective affinity is reduced as compared to diagram 4a.

7. Diagram 4d: The deliberate processing of mAbs to Fab fragments affects the affinity. Here, the spacing of the molecules is not as important as in diagrams 4a and 4b, since the Fab fragments are free to interact in solution.

8. Diagram 4f: This probably reflects the most common situation in which mAbs are used as a relatively impurified mixture of bivalent and monovalent molecules.

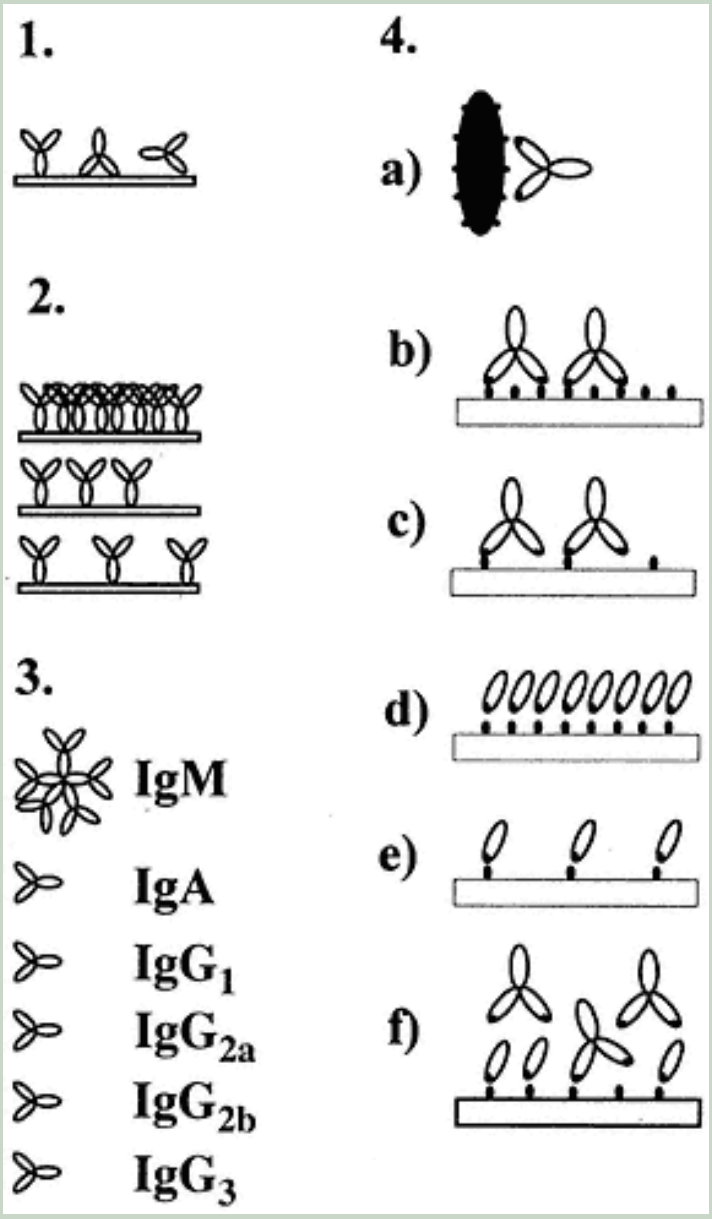


Fig. 23.
Properties of mAbs relevant to performance of ELISA. **1,** The orientation of molecules affects capture properties; **2,** density of molecules affects performance through interference. The correct spacing of mAbs also is important with reference to epitope spacing and density; **3,** the isotype of mAbs can be important; **4a,** optimal binding of bivalent mAb needs spacing of epitopes on multivalent antigen target; **4b,** spacing of small molecules is important to maximal bivalent binding; **4c,** where the distance between FAB fragments is too large, only monovalent binding takes place; **4d¨C4e,** Fab fragments are free to bind and the reaction is limited only by concentration of epitopes; **4f,** the most common mixture of bivalent and Fab molecules used in assays.

Here, the binding of Fab fragments and bivalent molecules can be regarded as competitive. Assays developed with such reagents may suffer since the distribution of Fab to bivalent molecules is different from batch to batch and owing to physical changes on storage. When a purified product is used, the results may be

different. This is also relevant when considering mAbs as capture reagents (as in diagram 1).

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8¡ª
Validation of Diagnostic Tests for Infectious Diseases

1¡ª
Validation

Validation involves all processes that determine the performance of an assay to achieve a defined set of objectives. Only when actual data have been obtained can test parameters be assessed and confidence in results be assigned in a statistical sense. Validation is a continuous process, in which increasing knowledge about an assay is gained each time it is run. The continuous process also involves data obtained when the test is performed in hitherto untried scenarios. Since most assays begin in the research arena, the use of validated assays in the form of kits by a wider range of scientists in laboratories varying widely in expertise, equipment, and climatic conditions can cause problems. The objective in validation is to be able to define an assay in terms of statistically quantifiable parameters with measured confidence. The designation of "validated assay" is only merited when it has been defined in terms of its capacity to classify samples with regard to the presence or absence of a particular analyte. Validation relies on examination of as many factors as possible. At any stage, quantifiable parameters must be defined describing the test and mechanisms to reevaluate be put into place.

A validated assay, therefore, depends on the characteristics of assay design that ensure the results. This leads to a robust assay (not easily affected by physical factors, operators, or geographical location where used or where samples came from). Such assays generate data that can be compared directly irrespective of which laboratory uses it, and to what population of animals it is applied.

In the context of ELISA, the development and validation of an assay is usually made using a limited number of tests, on samples from a selected group(s) of animals or patients, and made over a short time frame. The data define the performance of the assay, and these performance characteristics are published, and possibly certified by governing authorities. In this case, what constitutes a validated assay obviously depends directly on the experiences limited to the

samples analyzed. The conditions established during this validation phase can also be modified based on experience with the assay's capacity to correctly classify the infection status of animals from various populations over a longer time period. Such a situation is unavoidable since a single laboratory cannot have access to all samples at all times. However, the validation methods made must be clearly described so that at least variations from the accepted criteria can be determined and possibly accounted for.

1.1¡ª
Definition of a Validated Assay

The concept of a validated assay has many shades of meaning among laboratory diagnosticians and veterinary clinicians. For this chapter, a validated assay is described in terms of its use as an assay that provides results that consistently identify animals as being positive or negative for the presence of a specific analyte (antibody or antigen), and by inference accurately predict the infection status of animals with a known (measurable) degree of statistical certainty. The principles underlying the development and maintenance of such a validated assays are examined herein.

1.2¡ª
Components of Assay Validation

The development and validation of an assay is a multicomponent operation consisting of at least three general areas:

1. Feasibility of the method including choice and optimization of reagents and protocols.

2. Determination of the assay's performance characteristics.

3. Continuous monitoring of assay performance during routine use.

The third component may not be immediately considered as part of assay validation, but it is included because a test can be considered valid only when the data generated and their interpretation are, respectively, accurate and meaningful and updated. The development of an indirect ELISA for antibody detection can be used to illustrate points 1¨C3. This is a test format that can be difficult to validate since there is signal amplification owing to both specific and nonspecific components.

1.3¡ª
Feasibility Studies

In our ELISA example, feasibility studies are first made to examine whether the selected reagents have the capacity to distinguish between a range of antibody concentrations and the infectious agent in question, while providing minimal background activity. This can be a rapid process (a few weeks) and uses a minimum of samples. It establishes whether the test is feasible for further examination.

1.3.1¡ª
Samples for Feasibility Studies:
Serum Controls (Standards)

Developments in ELISA rely on the availability of some reagents of relevance to the problem at hand. Thus, other tests can provide information, e.g., about antibodies measured in a serum, which allows them to be used in ELISA development. In the case of the indirect assay, we are trying to estimate antibodies against a specific agent through their binding to that agent (on a plate), and subsequent detection with an antispecies conjugate. We are also trying to produce an assay that can differentiate between samples containing (positive) and not containing (negative) antibodies.

The availability and selection of four or five samples (positive sera in our example) that range from high to low levels of antibodies against the infection/ infectious agent in question is quick useful. The availability of such samples relies on a continuity of work at a given institution or their preparation in animals with specific disease agents. In addition, a sample(s) containing no antibody is required. Such control positive and negative samples should be taken, wherever possible, from known infected or uninfected samples from a representative population of animals for which the eventually validated assay will be applied (target population). Preferably, the samples should have given expected results in one or more serological techniques other than the one being validated. These same samples are used to optimize reagents throughout the feasibility studies. The samples are preferably taken from individual animals but they may represent pools of samples from several animals. A good practice is to prepare or obtain a significant volume (e.g., 10 mL) of each sample and divide it into 0.1-mL vol, to be stored at ¨C20¡ãC. One volume of each is thawed, used for experiments, and stored at 4¡ãC between experiments until depleted. Then, another aliquot may be thawed for further experimentation.

This procedure aims to provide the same sample source of sera, in which the same number of freeze/thaw cycles is maintained for all experiments. This precaution is a strong element in reducing any variation that may be introduced, since freeze thawing can denature protein and hence antibodies. Excess shaking of samples is also to be avoided since the shearing action in overvigorous mixing also denatures protein. Shaking also causes frothing (excess bubbles), which produces partitions of proteins so that antibody may be enriched in the bubbles (hence, depleted in the main volume of liquid).

Care is necessary to ensure that samples taken from the freezer are mixed thoroughly, because freezing causes the protein content of serum to separate to the bottom of tubes. Basically, samples (including test samples) should be treated gently.

Note that the qualitative nature of antibodies making up a serum may be altered greatly even though the quantity of antibody measured appears, by some

tests, to stay the same. As an example, shaking may destroy a high-affinity IgM population of a serum, allowing a more stable but lower-affinity IgG population to react with a target antigen. The net result (titer) may not alter in a specific assay, but the overall avidity of the serum has.

Conversely, other test systems may detect the drop in IgM, or increase in IgG, and hence show great alterations in respective titers. Note also that the problems with physical handling are more acutely important when levels of antibodies are low since there is a low level of positive antibody (protein), and small amounts of denaturation can turn a weak positive control into a negative one.

The approach of using the same sera has the added advantage of generating a data trail for the repeatedly-run samples. After the assay is validated, one or more of the samples can become the serum control(s) that may be the basis for data expression and repeatability assessments both within and between runs. They may also serve as serum standards if their activity has been predetermined by other accepted methods; such standards provide assurance that runs of the assay are producing accurate data.

1.3.2¡ª
Expression of Data

The method use to normalize and express data should be decided preferably no later than at the end of the feasibility studies. Comparisons of results from day to day and among laboratories are most accurate when done using normalized data. For example, in ELISA systems, optical density (OD) values are absolute measurements that are influenced by ambient temperatures, test parameters, and photometric instrumentation. Therefore, results need to be calculated and expressed as a function of the reactivity of one or more serum control samples that are included in each run of the assay.

Classically, normalization of data is accomplished in indirect ELISA by expressing OD values in one of several ways¡ªe.g., by expressing the OD values as a percentage of a single high-positive serum control that is included on each plate. This method is adequate for most applications.

More rigor can be brought to the normalization procedure by calculating results from a standard curve generated by several serum standards. This requires a more sophisticated algorithm such as linear regression or log-logit analysis to calculate the normalized value for each test sample. These approaches are more satisfactory because they do not rely on only one high-positive control sample for data normalization, but, rather, utilize several serum controls to plot a standard curve from which the sample value is extrapolated. This allows for some experimental error correction; for example, if one of the control samples was omitted or gave a high variation from the expected value, then the test may be accepted provided the other controls were acceptable.

Whatever the type of assay, it is essential to include additional controls for any reagent that may introduce variability and thus undermine attempts to achieve a validated assay.

1.3.3¡[a]
Repeatability:
Preliminary Estimates

Evidence that an assay is repeatable is necessary for further development. This is accomplished by calculating the intra-and interplate variation using the same samples run in different plates and on different days (and with different operators). Ideally, such tests should be run on at least 10 plates on 10 separate occasions. Coefficients (CVs) of variation (standard deviation [*SD*] of replicates of mean of replicates of equal to or less than 15% for the raw OD values indicate adequate repeatability at this stage of assay development. Such data obtained on a number of different plates and days also allows confidence limits to be ascribed to the variation observed (comparison of different means with their respective variations). However, if there is evidence of excessive variation (>20%) within and/or between runs of the assay, more preliminary studies should be made. This either will confirm that stabilization of the assay is possible or will determine ultimately whether the test format should be abandoned. This is extremely important because an assay that is inherently variable has a high probability of not withstanding the rigors of day-to-day testing on samples from the targeted population of animals.

1.3.4¡[a]
Choice of Optimal Assay Parameters

Optimal concentrations/dilutions of the antigen adsorbed to the plate, serum, enzyme-antibody conjugate, and substrate solution are determined through chessboard titrations (CBTs) of each reagent against all other reagents after confirming the best choice of reaction vessels (usually evaluation of two or three types of microtiter plates, each of which has different binding characteristics).

Additional experiments determine the optimal temporal, chemical, and physical variables in the protocol, including incubation temperatures and durations; the type, pH, and molarity of diluent, washing, and blocking buffers; and equipment used in each step of the assay (e.g., pipettors and washers that give the best reproducibility). There are numerous publications detailing the reagents and protocols available for assay development. Often these publications give examples of assays dealing with similar antigens and species of sera being examined.

1.3.5¡[a]
Analytical Sensitivity and Specificity

Experiments to establish the analytical sensitivity of the assay (the smallest detectable amount of the analyte in question) and the analytical specificity (the degree to which the test does not crossreact with analytes associated with

other infections) are needed. Note that sensitivity and specificity here are not strictly the same as when being considered in a purely immunological way, but are an attempt to quantify the "detection" level of any assay (sensitivity) that is affected by unwanted crossreactivity (specificity considerations).

Analytical sensitivity can be assessed by end point dilution analysis, which measures the dilution of serum at which antibodies are no longer detectable. Analytical specificity is best assessed by examining test performance using a panel of sera derived from animals that have experienced related infections that may stimulate crossreactive antibody. If, e.g., the assay does not detect antibody in limiting dilutions of serum with the same efficiency as other assays, or crossreactivity is common when sera from animals with closely related infections are tested, the reagents need to be recalibrated or replaced, or the assay abandoned.

1.4¡[a]
Determining Assay Performance Characteristics

When feasibility studies indicate that an assay has potential for field application, the next step is to characterize the assay's performance characteristics. Estimates are needed of diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP).

D-SN is the proportion of known infected reference animals that test positive in the assay; infected animals that test negative are deemed false negative results. D-SP is the proportion of uninfected reference animals that test negative in the assay; uninfected animals that test positive are deemed false positive results. The number and source of reference samples used to derive D-SN and D-SP are thus of paramount importance if the assay is ever to be properly validated for use in the general population of animals targeted by that assay.

These are primary parameters obtained during validation of an assay. They are the basis for calculation of other parameters from which inferences are made about test results. It is important that estimates of D-SN and D-SP be as accurate as possible. Ideally, they are derived from testing a series of reference samples from reference animals of known infection status relative to the disease or infection in question.

1.4.1¡[a]
Intended Use of the Assay

Determination must be made of how many reference samples must be tested in order to achieve statistically significant estimates of D-SN and D-SP with an acceptable error. This depends on the intended use of the assay. When a screening test is needed for application to a highly pathogenic disease, the threshold that separates seropositive from seronegative animals can be set at a low level, so that it is unlikely that any infected animals will be misclassified as uninfected. However, a consequence of the low threshold is that uninfected

animals showing nonspecific activity will be misclassified as infected. This will directly contribute to a lowering of assay specificity.

Alternatively, if the test is for a highly endemic but less pathogenic disease, generally the threshold can be set relatively high because it is important that the test not classify an animal as infected when in fact it is uninfected. Because of its high specificity, such a test is often used as a confirmatory test. Having determined whether high sensitivity or high specificity is the primary requirement for the assay, it is theoretically possible to calculate the number of samples required to establish valid estimates of D-SN and D-SP.

1.4.2¡ª
Size of Reference Serum Panel Required for Calculations of D-SN and D-SP

The optimal way to determine D-SN and D-SP of any assay is to test a large panel of reference sera that represents two groups of animals. One group should be proven to be infected with the agent in question. The second group should be known to be free of infection. In theory, the number of infected animals tested to achieve the desired diagnostic sensitivity of the test (¡À allowable error) can be approximated by the following formula:

$$n = \{[4 \times ds \times (1 - ds)]/e2\}$$

in which n = the number of animals that need to be tested in the new assay; ds = the diagnostic sensitivity that is sought (i.e., the expected proportion of infected animals that will test positive); and e = the amount of error allowed in the estimate of diagnostic sensitivity.

For instance, if a 95% diagnostic sensitivity is desired with ¡À5% error allowed in that estimate, the theoretical number of animals that is needed in the test validation = { [4 ¡Á 0.95 ¡Á (1 ¨C 0.95)]/.052}, which is 76 infected animals. If one wishes to increase the diagnostic sensitivity to 99% ¡À 2%, then the theoretical number of animals required is only 99.

These estimates of sample size may be misleading because they assume that the reference animals represent the same and a normal frequency distribution in the total population. This is unlikely since the latter population is influenced by many unquantifiable biological and environmental variables. Factors such as breed, age, sex, stage of infection, differing responses of individuals to infectious agents, differing host responses in chronic versus peracute infections, and the effect of diet and environment are but a few examples. All may have an impact on antibody production. Additionally, antibody to closely related infectious agents may cause crossreactions in the assay, and if this combination of agents is found only in one portion of the total population targeted by the assay, but is not represented in the panel of reference sera, then obviously the estimates of D-SN and D-SP derived from the reference panel will be

wrong. It is therefore impossible to represent fully all variables found in a target population of, say, 25 million animals using a sample of 100 animals.

The way to reduce the error in any statistical estimate is to increase the sample size (the larger the sample size, the more confident one can be in the estimate of the population). The experience of people validating assays indicates that is necessary to evaluate sera from several hundred known infected animals to account for many of the variables in a large population. Since the number of variables is indeterminate, we would recommend that at least 500¨C1000 samples be selected randomly from throughout the target population in which the assay will eventually be used. Such an exercise serves to define a population and may be further refined if distinct environmental regions can be regarded as having similarly influenced animals. In this way defined populations can be compared as to their distribution statistics. The extension of assays through active use in the evaluation of different populations and comparative testing against other methods also serves to allow a reestimation of the sensitivity and specificity of ELISAs.

The calculated number of uninfected reference animals required to establish diagnostic specificity of the assay is even greater. To validate an antibody detection test that will be 99% specific (only one false positive per 100 uninfected animals), an extremely large population of uninfected animals must be tested, representing as many biological and environmental variables as possible. This will allow an estimate of confidence in the test specificity. Again, the assumptions in the statistical calculations are a major concern; therefore, one should think in terms of at least 1000, and preferably upward of 5000 samples from animals that are known to be uninfected and not vaccinated with the agent in question, to establish a reasonable estimate of specificity.

1.4.3¡ª
The Gold Standard for Classifying Animals as Uninfected

The term *gold standard*, refers to the method or composite of methods giving results that are regarded as unequivocally classifying animals as infected or not infected. The results obtained from the new method are compared to those obtained using the gold standard during the validation process. In statistical terms, the gold standard results are regarded as the independent variable whereas the result from the new assay is the dependent variable. The results of the new assay are deemed correct or incorrect relative to the gold standard.

Classifying a population of animals as unequivocally uninfected with the agent in question using culture or isolation techniques or serology is not possible. One cannot rule out the possibility of false negative results, but it is possible to combine several sources of information to determine the probability that reference animals have never experienced an infection with the agent.

Accordingly, reference animals selected to represent the uninfected group in the assessment of assay specificity need to be selected as follows:

1. From geographical areas where the disease has not been endemic for the at least 3 yr.

2. From herds from those areas that have not had clinical signs of the disease during the past 3 yr, nor herds that have been vaccinated against the agent in question.

3. From herds that are closed to importation of animals from endemic areas and do not have infected neighboring herds.

4. From areas where there is no evidence of antibody to the agent in question based on repeated testing over the past 2¨C3 yr.

If all of these criteria are met, one can be reasonably certain that these animals have not experienced the agent in question. Sera from such animals could then be used as the reference sera for the uninfected reference animal group.

1.4.4¡[a]
Gold Standard for Classifying Animals as Infected

Several standards have been described that can be used with varying success to characterize the animals that serve as a source of reference sera:

1. Verification of infection: an absolute gold standard. The only true gold standard for classifying an animal as infected is the isolation of infectious agents or unequivocal histopathological criteria. Sera from such animals then are used to establish analytical and diagnostic sensitivity of a new assay designed to detect antibody to that agent.

2. Comparative serology: a relative standard of comparison. It may be impractical, technically difficult, or impossible to obtain definitive proof of infection via culture or isolation techniques. In the absence of such a gold standard, less exacting methods must serve as the standard of comparison with the new assay. If the other tests have already established assay performance characteristics (e.g., the Rose Bengal screening test followed by the complement fixation confirmatory test for detection of antibody to *Brucella bovis)*, their results taken together provide a useful composite-based standard by which the new assay may be compared.

When the new test is evaluated by comparison with another serological test or combination of tests, the estimates of D-SN and D-SP for the new test are called *relative diagnostic sensitivity* and *relative diagnostic specificity.* These standards of comparison, however, have their own established levels of false positivity and false negativity that are sources of error carried over into the new assay. Therefore, the relative D-SN and D-SP for the new test will be underestimated. It follows that the greater the amount of false positivity and false negativity in the test that is used as the standard of comparison, the more the new assay's performance characteristics will be undermined. In other

words, care must be taken when the "new" test in fact shows a better diagnostic capability than those previously accepted.

1.4.5¡[a]
Experimental Infection or Vaccination:
An Adjunct Standard of Comparison

Another standard for assessment of antibody response is sera taken sequentially, over several months, from experimentally infected or vaccinated animals. The strength of this standard is that it measures the ability of the assay to detect early antibody production and to follow the kinetics of antibody production to the agent in question. This also can be relative to preintervention treatment through the taking of samples before treatment. If it is evident that animals become infected, shed organisms in low numbers, but have no detectable antibody during the first 2 to 3 mo using the new assay, the analytical sensitivity of the assay may be inadequate, and estimates of diagnostic sensitivity will be low. Alternatively, if antibody appears quickly after inoculation of the infectious agent, and earlier than in the conventional assays used as standards of comparison, the new assay may have greater analytical sensitivity (and associated increased diagnostic sensitivity) than the conventional assay.

The interpretation of experimentally derived infected/vaccinated antibody response must be done carefully. The particular strain of organism, route of exposure, and dose are just a few variables that may stimulate an antibody response be that is quantitatively and qualitatively atypical of natural infection in the target population. The same is true of vaccination. Therefore, it is essential that experimentally induced antibody responses are relevant to those occurring in natural outbreaks of disease caused by the same infectious agent, otherwise the estimates of relative D-SN and D-SP may be in error. Because of the difficulty of equivalence in responses of naturally infected and experimentally infected/vaccinated animals, the relative D-SN and D-SP data derived from such animals should be considered as an adjunct criterion and should not be used alone to determine a new assay's relative D-SN and D-SP.

1.5¡[a]
Random Testing of Samples from a Population Endemic for Disease:
No Standard of Comparison

Validation of assays can be made in the absence of a standard. The validation then relies on statistical tools such as cluster or mixture analysis. Assuming that a few sera of known status are available to establish the feasibility of the assay system, it is possible to obtain a rough estimate of the assay's performance characteristics. Then, several thousands of animals in the target population can be tested in the absence of known infection status data other than possibly scattered clinical observations. If a clear bimodal frequency distribution becomes evident with a large peak consisting of many animals at the low

end of the antibody scale, and a second peak extended over a wide range of higher antibody responses, it may be possible to estimate a cutoff in antibody response that separates presumed uninfected from presumed infected animals. Since in this scheme there is no proof of the infection status of the animals, this approach should be done as a last resort with later confirmation after definitive standard(s) of comparison become available. This process also is inherent in the cumulative analysis of data from the field on continuous use of a kit.

1.6¡ª
Repeatability and Reproducibility:
Calculations

During feasibility studies, preliminary estimates of repeatability should be obtained. Selected sera from a bank of reference sera used to determine the assay's D-SN and D-SP can be tested using a series of runs of the assay within the same laboratory. It is useful to have several operators of the assay system do this exercise independently. This will provide an indication of assay repeatability that addresses the robustness of the assay.

Similarly, reproducibility of the assay (agreement among results of samples tested in different laboratories) needs to be established by testing the same sera in several other laboratories. The evaluation of both repeatability and reproducibility should be made on normalized data. For repeatability data, CVs for replicates should not exceed 10%, and regression analysis of normalized reproducibility data among laboratories generally should not give significant differences at the 95% confidence level.

1.7¡ª
Selection of the Positive/Negative Threshold (Cutoff)

After all the reference sera are tested, frequency distributions of results from infected and uninfected populations can be established. Both distributions are plotted on the same graph with the vertical axis representing the number of animals having test results that fall within each of 20 or so intervals of result values plotted on the horizontal axis.

For instance, when the data are expressed as a percentage of the value for the high-positive control sample (PP), 20 intervals of five units each (0¨C4%, 5¨C9%, 10¨C14%, and so on) could represent the horizontal axis. There is usually an overlap in these frequency distributions. The selection of a cutoff value for the new test will fall somewhere within this overlapping region.

The extent of the overlap may vary considerably from one assay to another. If only a small percentage (e.g., 2%) of the results from infected and uninfected animals are overlapping, and the cutoff selected is at the midpoint of the overlapping region, then the D-SN and the D-SP will both be 99%.

Alternatively, if the overlap involves a greater percentage of animals (e.g., 10%), then the cutoff chosen may be shifted to the left to minimize the false negative results (favoring greater D-SN), or to the right to minimize the false

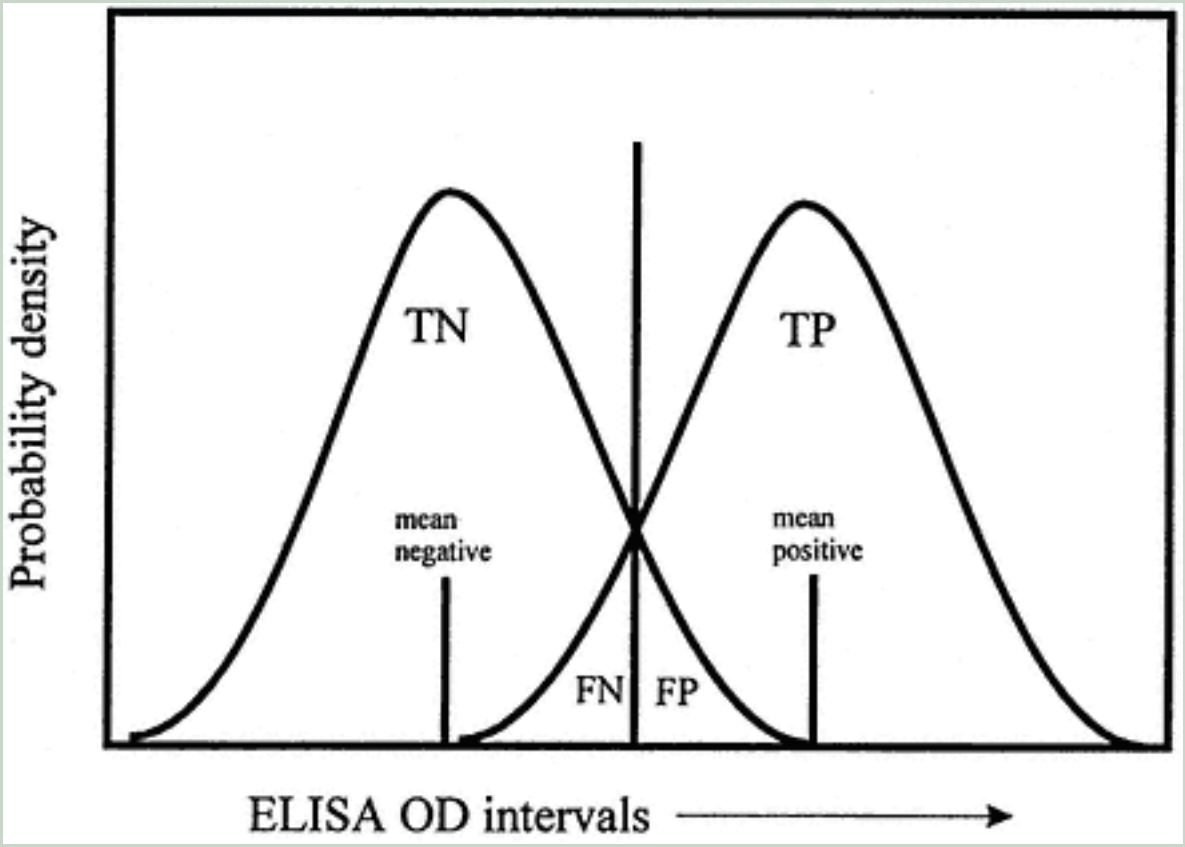


Fig. 1.
Hypothetical distribution density of ELISA results of populations of noninfected (left-hand curve) and infected (right-hand curve) individuals. The FN and FP area is referred to as "gray area," and results falling here must be regarded as suspect. The importance of retesting depends on how important the result is to classification of the test unit. The setting up of cutoff values depends on knowledge of such overlaps and the variability of the test used. FN, False negative; FP, false positive; TN, true negative; TP, true positive.

positive results (favoring greater D-SP), depending on the intended application of the assay. Once selected, the cutoff will determine the D-SN and D-SP, which, in turn, are the bases for calculating predictive values for positive and negative test results.

1.7.1¡[a]
Details

When giving further consideration to cutoff values one needs to recognize that there is invariability an overlap (as already stated) between populations containing negative and positive test results. Thus, the estimation of a perfect discriminatory cutoff is not possible. **Figure 1** presents a hypothetical (but typical) overlap of positive and negative distributions of ELISA. The cutoff value is the point set on the test scale that determines whether the response is positive or negative. The observed overlap reduces confidence in such statements for certain samples. The importance of false negative or false positive results depends on the required levels of diagnostic sensitivity against specificity. As already indicated, setting a cutoff of two or three times the *SD* of the negative control group is accepted practice. This assumes that there is a normal

distribution statistic in both types of population and that a representative sampling procedure for the whole population is being measured. The rationale here is that approx 95% of normally distributed observations are expected to fall within a range of mean 2 ¡Á *SD*, and would test negative. However, as already indicated, the normal distribution is not always realized, and the distribution of positives (particularly) is neglected. This may be, and often is, biased. The major flaw in this method is that it deals with the specificity of the assay results but not the sensitivity. The value can be regarded as a good reference value. The continuous validation of assays, with reference to evaluating a wider number of epidemiological niches using the same reagents, often modifies the cutoff value using this criterion. The relationship between sensitivity and specificity cannot be forgotten in any consideration. Raising the cutoff value increases specificity, and reducing it increases diagnostic sensitivity (reduces specificity). Whether this matters depends on the problem being tackled, and what types of results can be accepted. For example, false positive results may be unacceptable. This is true in, say, in the diagnosis of AIDS. However, it is important that someone not be missed either. False negative results may be acceptable in cases in which another test can be used in parallel. The first screening of samples could be with an ELISA set to have low sensitivity, but would be specific and reduce the number of clear positives, to relieve the burden on another more sensitive assay (e.g., polymerase chain reaction [PCR]).

Borderline or intermediate results require cautious in interpretation. Statistically this gray zone defines a range of cutoff values that would result in a sensitivity or specificity less than a predefined level. Although there are techniques to attempt to compensate for unknown factors, the best approach is to assimilate as much data from all sources. This allows explanation and then manipulation (selection) of data to help eliminate bias. The flexibility in altering the cutoff value is data dependent and a continuous process in test validation.

1.8¡[a]
Calculation of D-SN and D-SP:
Predictive Value of Diagnostic Tests

The inferences from test results rather than observed test values are of interest to the diagnostician. Thus, the use of the assay rather than the technology itself is key. Measurement of disease state, level of protective antibody, and transition of disease status are all objectives. Rarely is there a perfect correlation between disease status and any test result. Thus, there is always some degree of false positivity or negativity. The establishment of the correlation is regarded as diagnostic evaluation. The standard for presenting such results is in **Table 1**, which relates true positive (TP), true negative (TN), false positive (FP), and false negative (FN) results represented as frequencies of the four possible decisions. These can be shown in a 2 ¡Á 2 *(see* **Table 1**).

Table 1
Relationships of Disease Status and Test Results[a]

	True disease state	
Test	D+	D¨C
T+	TP	FP
T¨C	FN	TN

[a]Diagnostic (clinical) sensitivity = [TP/(TP + FN)] ¡Á 100. Diagnostic (clinical) specificity is [TN/ (TN + FP)] ¡Á 100.

D-SN and D-SP can be estimated from test results on a panel of reference sera chosen early in the validation process. Calculations of D-SN and D-SP, therefore, only apply to the reference sera and can be extrapolated to the general population of animals only insofar as the reference sera fully represent all variables in that targeted population. The paramount importance of the proper selection of reference sera is thus self-evident.

Other terms based on these values can be defined as follows.

1.8.1¡[a] Prevalence of Disease (P)

Prevalence of disease denotes the probability of a subject as having a disease:

$$\text{General probability (P)} = \Pr(D+)$$

$$P = (TP + FN)/N$$

P is an unbiased estimator of the prevalence in a target population if animals can be randomly selected and subjected to both the reference test (to establish D) and the new test (T).

1.8.2¡[a] Apparent Prevalence

Apparent prevalence (AP) denotes the probability of a subject to have a positive test result:

$$AP = \Pr(T+)$$

$$AP = (TP + FP)/N$$

1.8.3¡[a] Predictive Value

The predictive value of a positive test gives the percentage of subjects suffering from disease correctly classified as positive by the test, and defined as follows:

$$[TP/(TP + FP)] \times 100$$

The predictive value of a negative test gives the percentage of healthy subjects correctly classified as negative by the test, and defined as follows:

$$[TN/(TN + FN)] \times 100$$

1.8.4¡ª
Components of Diagnostic Accuracy

Accuracy is the ability of a test to give correct results (diagnostic sensitivity). As already indicated, this can be estimated by the observed agreement of the new and reference test. There are two components.

1.8.4.1¡ª
Sensitivity

Sensitivity (Se) is the probability of a positive result (T+) given that the disease is present (D+):

$$Se = TP/(TP + FN)$$

1.8.4.2¡ª
Specificity

Specificity (Sp) is the probability of a negative results (T¨C) given that the disease is not present (D¨C):

$$Sp = TN/(TN + FP)$$

1.9¡ª
Combined Measures of Diagnostic Accuracy

1.9.1¡ª
Efficiency

The overall efficiency (Ef) of a diagnostic test, defined as the percentage of subjects correctly classified as diseased or healthy, with a given prevalence of P, is estimated using the following relationship:

$$Ef = [(TP + TN)/(TP + FP + TN + FN)] \times 100$$

1.9.2¡ª
Youden's Index

Youden's Index (J) is the measure of the probability of correct classifications that is invariant to prevalence. In terms of Se and Sp we have:

$$J = Se + Sp - 1$$

1.9.3¡ª
Likelihood Ratios

Likelihood ratios (LRs) are important measures of accuracy that link estimations of pre- and posttest accuracy. They express the change in likelihood of disease based on information gathered before and after making tests. The degree of change given a positive result (LR+) and given a negative result (LR¨C) depends on the *SD*e and Sp of the test.

1.9.3.1¡ª
Likelihood Ratio of a Positive Test Result

The likelihood ratio (LR) of a positive test result is the ratio of the probability of disease to the probability of nondisease given a positive test result, divided by the odds of the underlying prevalence [odds (P) = (P/1 ¨C P)]:

$$LR+ = Se/(1 - Sp)$$

1.9.3.2¡ª
Likelihood Ratio of a Negative Test Result

The likelihood ratio of a negative test result is the ratio of the probability of disease to the probability of nondisease given a negative test result, divided by the odds of the underlying prevalence:

$$LR- = (1 - Se)/Sp$$

1.9.3.3¡ª
Odds Ratios

Odds ratios (ORs) combine results from studies:

$$OR = (TP \times TN)/(FP \times FN)$$

1.10¡ª
Examples Relating Analytical and Diagnostic Evaluation

In a new ELISA, 250 sera have been examined from nonexposed animals and 92 infected animals. **Table 2** presents the results.

The following data reflect some of the features already discussed:

1. Sensitivity (Se)	=	76/92	=	0.826
2. Specificity (Sp)	=	250/250	=	1.000
3. Efficiency (Ef)	=	326/342	=	0.953
4. Youden Index (J)	=	0.826 + 1 ¨C 1	=	0.826
5. Likelihood ratio (LR+)	=	0.826/0	=	cannot be defined
6. Likelihood ratio (LR¨C)	=	0.174/1. 000	=	0.174

As already stated, the perfect test would exhibit 100% sensitivity, specificity, and efficiency. This is not possible in practice. Thus, the values of each depend on the decision level or point chosen (cutoff values, reference values, and so forth). The setting of the criteria for decisions is not solely based on statistics since there are ethical, medical, and financial implications, particularly in the human testing fields. The relationship of sensitivity to specificity has to be considered. A high cutoff favoring specificity reduces diagnostic sensitivity. Increasing sensitivity leads to false positive results. Different tests can be performed on samples in which there is to be increased confidence such that results are false positives, for example, the use of PCR may provide a higher degree of specificity. This pairing of sensitivity and specificity is inherent in all assays. The two parameters can be evaluated in terms of receiver operating characteristics (ROC) curves.

Table 2
Relationship of Disease Status and Test Results

	Infection status	
Test	D+	D¨C
T+	76	0
T¨C	16	250

1.11¡ª
ROC Analysis

ROC analysis was developed in the early 1950s and extended into medical sciences in the 1960s. It is now a standard tool for the evaluation of clinical tests. The underlying assumption in ROC analysis is that the diagnostic variable is to be used as the discriminator of two defined groups of responses (e.g., test values from diseased/nondiseased animals, or infected/noninfected animals). ROC analysis assesses the performance of the system in terms of Se and 1 ¨C Sp for each observed value of the discriminator variable assumed as a decision threshold, e.g., cutoff value to differentiate between two groups of responses. For ELISA, which produces continuous results, the cutoff value can be shifted over a range of observed values, and Se and 1 ¨C Sp are established for each of these. Setting this as *k* pairs, the resulting *k* pairs ([1 ¨C Sp] Se) are displayed as an ROC plot. The connection of the points leads to a trace that originates in the upper right corner and ends in the left lower corner of the unit square. The plot characterizes the given test by the trace in the unit square, irrespective of the original unit and range of the measurement. Therefore, ROC plots can be used to compare all tests, even when the tests have quite different cutoff values and units of measurement. ROC plots for diagnostic assays with perfect discrimination between negative and positive reference samples pass through coordinates (0; 1), which is equivalent to Se = Sp = 100%. Thus, the area under such ROC plots would be 1. 0, and the study of the area under the curve (AUC), which evaluates the probabilities of (1 ¨C Sp) and Se, is the most important statistical feature of such curves.

1.11.1¡ª
Area under the ROC Curve

The theoretical exponential function underlying the ROC plot is estimated on the assumption that data from two groups are distributed normally (bimodally). The ROC function is then characterized by parameter *A* (standardized mean difference of the responses of two groups) and the parameter *B* (ratio of *SD*s). Thus, for a set of data for a positive and negative reference group with means of x_0 and x_1, in which $x_0 < x_1$ and the *SD*s are s_0 and s_1, respectively,

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$$A = (x_1 - x0)/S_1$$

$$B = S_0/S_1$$

The AUC can be estimated making assumptions about the distribution of test results. Therefore, a nonparametric test is based on the fact that the AUC is related to the test statistic *U* of the Mann-Whitney rank sum test:

$$\mathrm{AUC}_1 = U/n_1 n_0$$

With $U = n_1 n_0 + n_0 (n_0 + 1) / 2$ ¨C *R*, *R* = rank sum of squares.

A parametric approach can be taken. Here,

$$\mathrm{AUC}_2 = \theta\,[A/(1 + B^2)^{(0.5}]$$

Here we can see that for AUC = 0.5, then $A = 0$. Equal values for negative and positive reference populations indicate a noninformative diagnostic test. Theoretically, AUC < 0.5 if A is negative. In practice, such situations are not encountered, or the decision rule is converted to obtain positive values for *A.*

The bimodal distribution may not be justified for a given set of data, and other methods have been developed based on maximum likelihood estimates of the ROC function and the AUC.

1.11.2¡ª
Optimization of Cutoff Value Using ROC Curves

The optimal pair for sensitivity and specificity is the point with the greatest distance in a Northwest direction, from the diagonal line Se = (1 ¨C Sp) (*see* **Figs. 2** and **3**).

1.11.3¡ª
Example of ROC Analysis

The principle of ROC analysis is to generate plots using a spreadsheet (e.g., EXCEL) in which:

1. A grid of possible cutoff values is generated.

2. For each cutoff value, the resulting sensitivity (Se) and 1 ¨C Sp are calculated.

3. The values of Se are plotted against 1 ¨C Sp for all grid points.

Many statistical packages can be used to construct ROC plots and shareware is available in the public domain. In this example (taken from **ref. *1***), the AUC is estimated for an ELISA for T3 using a test reference (T1), given the mean values of 1.04 and 0.11; the *SD*s 0.47 and 0.13; and the sample sizes 25 (positive subpopulation) and 75 (negative subpopulation). The rank sum and the *U* statistic are as follows:

$A = (1.04 - 0.11)/0.47 = 1.97$
$B = 0.13/0.47 = 0.276$
$R = 2864$

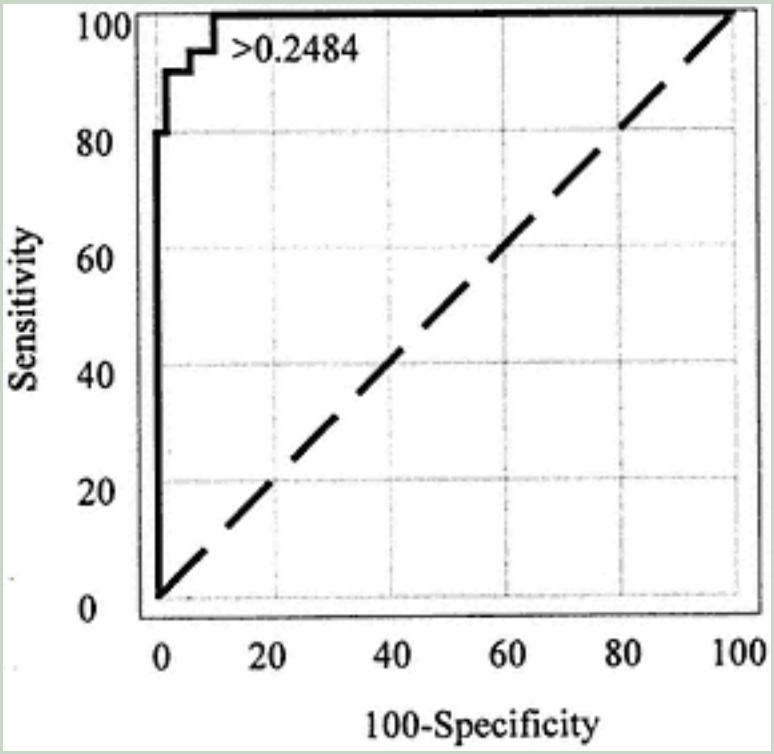


Fig. 2.
ROC plot for T3 data (T1 assumed as reference test, *n* = 100). Area under plot = 0.993; standard error (SE) = 0.012; 95% confidence interval (CI) = 0.950¨C0.998.

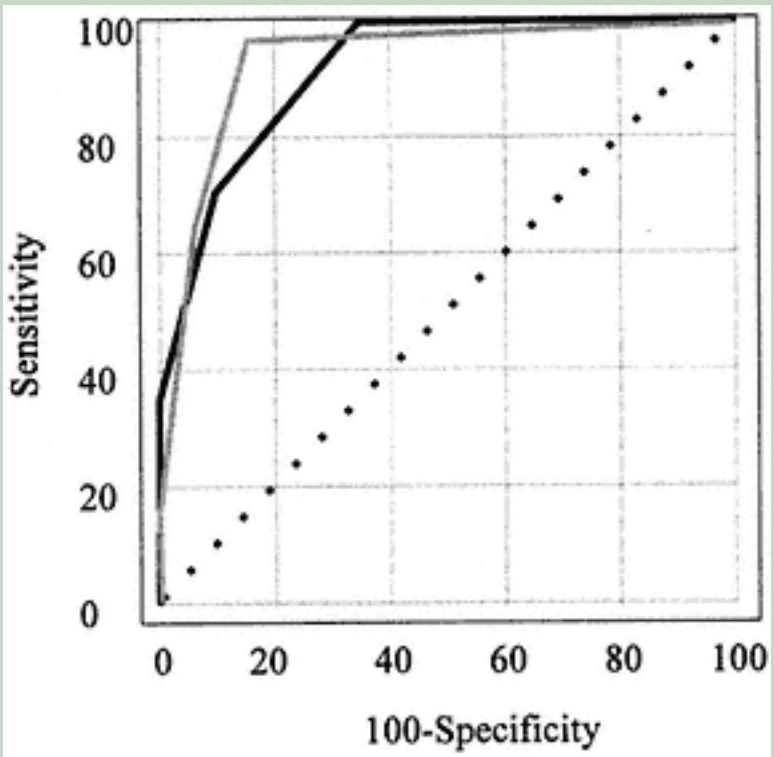


Fig. 3.
ROC plot for data T7 and T10 (T1 assumed as reference test, n = 100). T7 area under plot = 0.920; SE = 0.039; 95% CI = 0.848¨C0.965. T10 area under plot = 0.957; SE = 0.029; 95% CI = 0.896¨C0.987. Difference between areas = 0.037; SE = 0.029; 95% CI = ¨C0.046 to 0.130. Significance level = 0.437. The difference between the areas is not significant.

$$U = 2575 + 75\,(75 + 1)/2 - R = 1861$$
$$AUC_1 = 1861/1875 = 0.9935$$
$$AUC_2 = \theta\,[1.97/(1 + 0.13^2)^{0.5}] = \theta\,(1.953) = 0.9745$$

Thus, the nonparametric test and parametric estimates of the AUC for T3 data are 0.9935 and 0.9745, respectively. **Figures 2** and **3** show examples of ROC plots.

1.12¡ª
Monitoring of Assay Performance during Routine Use

If we accept the proposition that a validated assay implies provision of valid test results during routine diagnostic testing, it follows that assay validation must be an ongoing process consistent with the principles of internal quality control (IQC). Continuous evaluation of repeatability and reproducibility are thus essential for helping to ensure that the assay is generating valid test results.

Repeatability between runs of an assay within a laboratory should be monitored by plotting results of the control samples to determine whether the assay is operating within acceptable limits. It is useful to maintain a running plot of the serum control values from the last 40 runs of the assay and to assess them after each run of the assay to determine whether they remain within 95% CI. If results fall outside of the CIs or are showing signs of an upward or downward trend, there may be a problem with repeatability and/or precision that needs attention. Similarly, when several laboratories assess samples for interlaboratory variation, reproducibility of the assay is monitored. The assay should also be subjected to tests for accuracy. This may be done by including samples of known activity in each run of the assay or by periodic testing of such samples. In addition, it would be desirable to enroll in an external quality assurance program in which a panel of samples, supplied by a third party, are tested blind to determine proficiency of the assay. Inclusion of such QC schemes in routine use of an assay that was validated at one point in time, ensures that the assay maintains that level of validity when put into routine use.

1.13¡ª
Updating Validation Criteria

We have stressed that an assay is valid only if the results of selected sera from reference animals represent the entire targeted population. Because of the extraordinary set of variables that may affect the performance of an assay, it is highly desirable to expand the bank of gold standard reference sera whenever possible. This follows the principle that variability is reduced with increasing sample size; therefore, increases in the size of the reference serum pool should lead to better estimates of D-SN and D-SP for the population targeted by the assay. Furthermore, when the assay is to be transferred to a completely differ-

ent geographic region (e.g., from the Northern to Southern Hemisphere), it is essential to revalidate the assay by subjecting it to populations of animals that reside under local conditions. This is the only way to ensure that the assay is valid for that situation.

1.14¡ª
Assay Validation and Interpretation of Test Results

It is wrong to assume that a test result from a validated assay correctly classifies animals as infected or uninfected based solely on the assay's D-SN and D-SP. The calculations of D-SN and D-SP are based on testing the panel of reference sera and are calculated at one point in time. This is unlike the ability of a positive or negative test result to predict the infection status of an animal because this is a function of the prevalence of infection in the population being tested. Prevalence of disease in the target population, coupled with the previously calculated estimates of D-SN and D-SP, is used to calculate the predictive values of positive and negative test results.

For instance, assume a prevalence of disease in the population targeted by the assay of only 1 infected animal per 1000 animals, and a false positive rate of the test of 1 per 100 animals (99% D-SP). Of 1000 tests run on that population, 10 will be false positive and only 1 will be true positive. Therefore, when the prevalence of disease is this low, positive test results will be an accurate predictor of the infection status of the animal in only about 9% of the cases. Thus, if assay validation is concerned with accuracy in test results *and their inferences*, then proper interpretation of test results is an integral part of providing valid test results. It is beyond the scope of this chapter to discuss the many ramifications of proper interpretation of test results generated by a validated assay.

1.15¡ª
Validation of Assays Other Than ELISA

Although the example we have used was an indirect ELISA test, the same principles apply in the validation of any other types of diagnostic assay. Feasibility studies, assay performance characteristics, the size and composition of the animal populations that provide reference sera, and the continuing assessment and updating of the performance characteristics of the validated assay are all essential if the assay is to be considered thoroughly and continuously validated.

2¡ª
More Principles of Validation

This section reexamines the process of validation in a slightly different way. Other terms are introduced and explained. The initial steps in assessing the performance of an assay is really a technical evaluation. Various experimental procedures can be used to assess aspects affecting the performance of assays. These examine the following areas:

1. Precision (reproducibility).

2. Sensitivity.

3. Accuracy.

4. Specificity.

These factors help cover potential sources of error in assays that may be:

1. Systematic: errors that consistently affect repeated measurements of the same sample.

2. Random: errors affecting individual measurements randomly causing a scatter.

2.1¡ª
Precision

Precision can be regarded also as reproducibility and is a statistical measure of the variation in samples on repeat determinations of the same sample either within the same run or from day to day or operator to operator in time. This is always examined first in any assay development because an assay with great imprecision in the early stages is not likely to be of any routine use, despite later attempts to improve this factor. Precision testing involves testing samples many times to accumulate data for analysis within and between runs. Different samples should be examined reflecting the target population in which the test is to find practical use.

The statistics involve (1) the mean $(\bar{X})$, (2) standard deviation (*SD*), and (3) coefficient of variation (CV), which is expressed as follows:

$$CV = \%CV = (SD/\bar{X}) \times 100$$

The performance of an assay can be examined through profiling the precision measured for different sample (analyte) concentrations and conditions. Such assessment at any stage can be regarded as a precision profile.

2.1.1¡ª
Precision Profile

Precision profiles are obtained by plotting the values of %*CV* against the concentration of measured analyte. To construct such curves, between 10 and 20 replicates should be run of each standard concentration (dilution). At least three dilutions should be examined representing high medium and low signal in the ELISA. These should include samples representing approx 80, 50, and 20% of maximal activity measured. This can give useful information about the reproducibility for different concentrations. The minimum acceptable precision can be defined, and estimates of about a maximum of 10% should be accepted. Differences in reproducibility are evident in assays at different concentrations of reagents used in development as well as for different concentrations of analyte being detected. **Figure 4** shows an example of %*CV* plots against concentration of analyte detected. Usually nonuniform error is seen across the concentration range used as illustrated. The acceptable precision is

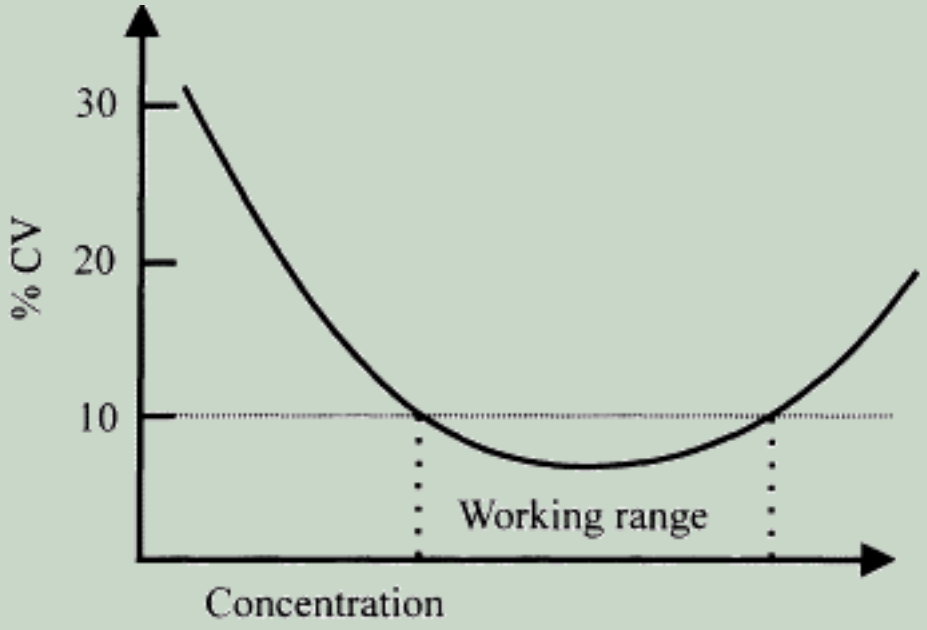


Fig. 4.
The precision profile of an assay showing nonuniform error. The *CV* is plotted against the analyte concentration. The working range of the assay can be defined as the range where imprecision is below a preset level such as 10%, as shown.

drawn on the graph, and this would define a "working" range within which there were acceptable limits for the variation in the assay. On further validation extending development into a kit format, this degree of variation can be measured and limits acceptable to the kit imposed.

Precision should be estimated not only within runs but also between runs from day to day. Usually there is more variation day to day (and operator to operator), but the continuous exercise of precision analysis allows limits to be determined statistically that define the test. The variation measured during development is the sum of all the errors that affect the test.

2.2¡ª
Sensitivity

Sensitivity is the assay's capacity to measure the smallest amount of target analyte under the standard conditions defined. For a full treatment of the approaches to determining the theoretical sensitivity of assays, *see* **ref.** ***1***. This explains the Yalow and Berson and Ekin models defining antigen and antibody interactions and points to the features inherent in assays that affect the sensitivity. The required sensitivity is a consideration here and practically depends on the balance between obtaining maximal sensitivity and the precision of the results compared to suboptimal conditions of sensitivity conditions. It may be advantageous to reduce the sensitivity for certain assays to improve both accuracy and specificity. Thus, conditions can be assessed that reflect the likely concentration of analyte being determined. The factors involve the examina-

tion of the concentration of reagents, the times for incubation, the effects of temperature, the mixing of reagents, the sequence that reagents are added, and, in order to improve precision, the number of replicates run.

2.3¡ª
Accuracy

Accuracy is the concept of being able to measure a true value of the analyte. The use of control standards, which, by definition indicate the true value, can give a measure of accuracy and evaluate any bias in the functional aspects of the use of an ELISA.

The bias may be proportional when the results indicate a constant percentage higher (positive bias) or lower (negative bias). The assay may involve both types of bias depending on the range of standards assessed. **Figure 5** shows the relationship of precision, accuracy and bias.

Accuracy can be affected by all components of an assay. Generally, accuracy has to be determined by comparing results to a reference method. However, in most cases, only an indirect assessment is possible, and several methods are used. Including calibration standards, recovery studies and parallelism.

2.3.1¡ª
Calibration Standards

The provision of standards for ELISA is not as simple as for other assays involving more physical methods (e.g., in which a defined substance can be measured by weight or wavelength, and in which it is known that test samples contain the same substance known to be identical in structure). Even in these cases, methods used to extract the sample or other physical treatments may alter the measurements in a test to increase imprecision. In the ELISA we rely on measuring activity through many steps and assessing the activity of reagents such as antisera, which show variability in their own right. The inability to supply standards in biological fields, which can reflect all activities of similar reactants, is a drawback to using calibration studies. Some areas have better chances of assessing accuracy with respect to standards, e.g., monoclonal antibodies (mAbs), which can be defined exactly in terms of epitope recognition and physical structure. In this case, a standard preparation can be classified through considerations of weight to activity measured.

2.3.2¡[a]
Recovery Studies

A recovery study determines the ability of a test to measure a known incremental amount of standard analyte from a sample matrix. Thus, in practice, a known amount of analyte (*A*) is added to base (*B*) and the recovery (*C*) is calculated as a concentration after performing the assay. The percentage of recovery is as follows:

$$(C - B)/A \times 100)$$

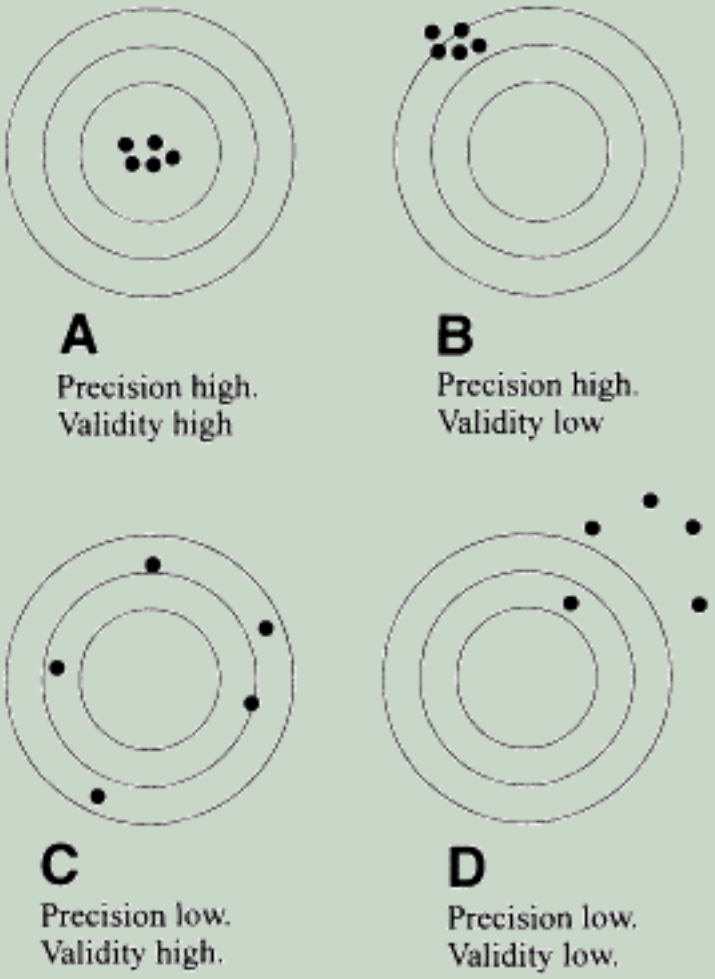


Fig. 5.
Representations of precision (reproducibility) and validity (accuracy). The target for accuracy of a test result is represented by the central disc. For example, this is the correct mean for a sample analyzed by ELISA. Results from five tests are shown as black dots. **(A)** Data are grouped tightly (reproducible) and all are in the correct result disc (accurate). **(B)** Although the data are precise (all showing similar results), they all are inaccurate and biased toward the upper left. **(C)** This shows a wide dispersion of data (not reproducible), but the average result of the data predicts the accurate result; confidence in these data is low. **(D)** This shows both irreproducible results in which the control mean is not reflected by the average of all the results; the test is inaccurate and variable.

This system is applicable to standards which can be highly defined and whose purity can be guarantee, e.g., drugs, steroids, peptides. Again this method suffers in typical uses of ELISA for diagnosis where purity and heterogeneity of samples are the norm.

2.3.3¡[a] Parallelism

Parallelism relies on dilutions of standards and testing these in an assay. Correction of the measurements for samples, with respect to the dilution fac-

tors, should show that there is equivalent "activity." The easiest way to treat results is to simply multiply the found concentration by the dilution factor. The results can be plotted against dilution, and a parallel response is inferred from an observed horizontal line. Statistics can be used to examine the significance of the correlation of activity to dilution.

In assays of antibody, both the concentration and avidity of the serum have to be considered. Parallelism will only be demonstrated when the avidity of the test antibodies corresponds exactly to those in the assay calibrators. Thus, samples with high-affinity antibodies will show overrecovery on dilution whereas those with low-affinity antibodies will show underrecovery. Hence, the serial monitoring of antisera and the consequential nonlinearity of the antibody titers can give rise to clinical interpretation. One method of minimizing this is to use reference preparations that best reflect the average avidity (sum of all affinities of antibodies in a sample). In this way samples dilute correctly on "average" in which approximately half will show apparent underrecovery and half overrecovery. In simple terms, standards are "sought" from a population that best reflect the average avidity of sera in a population. Diluents affect such considerations, and the dilution matrix should be maintained throughout the range chosen.

2.4¡ª
Specificity

The accuracy of an assay ultimately relies on its specificity. Thus, the ability for accuracy relies on the assay determining only the required parameter, probably when mixed with other components. With polyclonal antibodies, the specificity is complicated owing to the heterogeneity of the antibodies, which have varying affinities even against the same epitope. mAbs offer better reagents because they are, by definition, reactive as a single population of antibody with a single affinity. However, even in this case, minor variations in the same epitope affect the binding of an mAb and hence the specificity. The existence of crossreactivities and the variation in a specific response against a required single antigenic site of choice complicate the measurement of a specific activity. Such factors include the existence of endogenous molecules that are structurally similar to the principle analyte, the in vivo production of metabolites of the principle analyte with common crossreactive epitopes, and the possible administration of similar analytes as vaccines or medications (*see* **ref. 2**).

2.5¡ª
Testing of Ruggedness (Robustness of Test)

The entire process of developing assays and their validation involves determining the effect of all operational parameters and the tolerances for their control. This includes all the systematic factors, assay temperatures, times for incubation, volumes, separation procedures for samples, order of addition of

reagent, and signal detection. In short, all the factors that can be examined and the effect of changes in conditions should be understood. The examination of the tolerance of the assay to changes is a measure of the ability of the test to resist changes and maintain its test results within tolerable limits. The tolerances can be measured against different conditions depending on the intended environment of the assay. A robust assay might be viewed as one whose reagents are stable at high temperatures, which might be more suitable for use in countries where the environment is hot and where laboratory temperature control is not available. The suitability of an ELISA may in fact be dominated by its intended end user, and the relevant factors should be examined to allow the best chance of a successful assay. The development of kits to be used worldwide, should consider the robustness or ruggedness carefully. Some factors for consideration are examined in the next section. Often, validation requires that a set of reagents and a defined protocol be field tested to allow an estimation of robustness. Indeed, it may not be possible to test all factors in a central laboratory to account for the types of challenge to the assay experienced through its dispersion. Often changes have to be made to assay components, systems, methods of sending materials, and so forth, according to the feedback from extended validation.

3¡[a] Kits

The definition of what comprises a kit rests on considerations on test validation, the perceived objective of the kit, the market or end users who are to exploit the kit, and, the factors involved in sustainability. The last area is perhaps the most important and needs examination in the light of commercial interests and international bodies supplying help through technology transfer to developing countries. Thus, the equation for a kit is complex, involving technical performance, supply and profit motives, and continuity. Kits, at best, also have to be accepted by international bodies to fulfill their ultimate role of standardization of a given approach and allow harmonization with other tests measuring the same or similar factors. It would be useful here to examine some generalized ideas about kits in all fields of human and veterinary applications. The separation of developments in humans and animals is also relevant and is examined subsequently.

Where do kits stem from? It could be assumed that kits always supply a need identified by careful assessment of existing problems and the current solutions, and thus that there is a direct route from need to development to end product. This is not generally true. Rarely is there such a clean scenario. Rather, some developments in research are harnessed to prove feasibility of an approach, which then leads to a relatively moderate amount of validation followed by exploitation. Who exploits the reagents and in what form usually determines

the success of the kit and its long-term prospects and, more important, its actual benefit. This area has to take into account the profit motive as well as technical aspects. The possibilities for profit are great in the human medical sphere and concentrated on relatively few diseases, whereas the veterinary market is fragmented, centered more on application in developing countries, and hence lacks appeal to the commercial sphere.

Having indicated that kits can be relatively poorly thought-out entities, it is probably incumbent that I define the ultimate kit. Such a definition or statements may then be examined against kits being used by readers or used to help design better kits. Having said this, there is no perfect kit that deals with biological systems. The section on validation of assays strongly indicates that the process is continuous and that data derived from the use of kits constantly redefines the particular test. The gathering of information from kits and the modification of reagents/conditions/protocols is necessary to account for the many variables that cannot be assessed at a single time point or in a single laboratory. The validation also involves changes in the biological systems examined, such as alteration in the antigenicity of agents examined, which necessitates action.

3.1¡[a]
A Definition of a Perfect Kit

1. A kit should contain everything needed to allow testing including software packages for storage, processing, demonstration, and reporting of data.

2. The reagents should be absolutely stable under a wide range of conditions of temperature (rugged, robust).

3. The manual describing the use of the kit should be foolproof.

4. The kit should be validated in the field as well as in research laboratories.

5. All containers for reagents should be leakproof.

6. IQC samples should be included.

7. External quality assessment should be included in the kit package.

8. Data on the relationship of kit results to those from other assays should be included.

9. Attention should be made to ensuring that all equipment used in the kit is calibrated (spectrophotometers, pipets).

10. Training courses should be organized in use of kits.

11. Information exchange should be set up to allow rapid on-line help and evaluation of results when there are perceived problems.

12. The internationally sanctioned supply and control of standards used in kits should be maintained.

3.2¡ª
Other Considerations for Kits

Allied to validation and ruggedness testing, the implication for a kit is that the reagents can be supplied over an extended time. This can mean that differ-

ent batches or lots of materials are produced at different times. It is essential that there be monitoring of reagents to ensure consistency. The degree of inaccuracy owing to lot variation is ultimately determined by the manufacturer (the tolerance limits). Typical tolerance limit could be of 2¨C10% or 1 to 2 *SD*s of the difference among lots. Examples of lot changes include all materials in an ELISA such as changing the antispecies conjugate. This can have a profound effect on an assay and care should be taken to retitrate conjugates to equivalent activities. In fact, all aspects are relevant from the solid phase, control antisera, buffers, substrates, and so on. The control of this element of kits was originally the supplier's responsibility. QC is essential and measurement of the new errors with respect to those of the previously established lots. Changes in lots should also be reported to users, who may encounter problems owing to local conditions. If these are reported to the supplier, the supplier may indicate that changes are made. Microtiter plates can be a problem, and it should not be assumed that different batches supplied from manufacturers are the same for any specific assay, since the tests used to establish batch-to-batch variation by plastics manufacturers are not the same as those for any particular assay. Thus, a statistically valid number of plates should be tested when they are from a different batch number.

3.3¡ª
Quality Assurance/Quality Control

Some consideration of Quality Assurance (QA) and QC is relevant. It is also considered with reference to controlling a single assay. IQC should be designed to ensure that results are within acceptable (given) limits of accuracy and precision. Thus, all aspects that might influence assay performance should be monitored, such as routine assessment of equipment performance, reagent stability, technique, assay conditions, and sample handling. QC samples should be included in every assay at regular intervals (if not every time, and such samples should reflect the concentration range where "clinical" decisions can be made with regard to samples from a "population." The QC samples therefore control retrospectively the within assay, between assay, bias, drift, and shift in results.

The most common method of presenting of data and the statistical analyzes is through Shewhart or Levy-Jennings control charts ***(3)***. These charts require the estimation of the mean and *SD* for each control used.

3.4¡ª
External Quality Assurance Schemes

External Quality Assurance (EQA) schemes attempt to provide an independent assessment of a laboratory's performance usually with respect to a defined assay. Such schemes complement (and use) ICQ. The basis of the schemes is

that an organizer sends the same control samples to all participating laboratories for testing at regular intervals. The samples are tested in the routine cycle of the laboratory and the results are transmitted back to the organizer.

Successful schemes need not require many samples (e.g., five). The transmission of IQC data and a questionnaire also add a great deal to the EQA (*see* **refs.** ***4¨C7*** for reviews on IQC and EQA).

3.5¡ª
Standards

An ELISA can be developed to measure a substance that has not been previously examined. Thus, a reference preparation may not exist. Generally, the substance may not be characterized by a single chemical structure. A well-defined compound of high quality, purity, and stability, can be adequate as a standard. However, in ELISA, many biologically active substances are only available in crude forms. Three types of reference preparations are commonly used for standardization of immunoassay kits.

3.5.1¡ª
International Standards

An international standard (IS) or international reference standard (IRS) must be used to calibrate a new method for biological analytes. As examples, materials for such standards are collected, tested, and stored by the World Health Organization International Laboratory for Biological Standards. An international unit for activity is assigned to these preparations, and collaborative efforts among several laboratories maintain these as reliable reference preparations. The IRS status is reserved to designate preparations that do not meet the very demanding criteria for an IS. The ISs are available in limited quantity for a small charge for calibration purposes of national or reference preparations. They are not available in sufficient quantities to serve as routine standards.

3.5.2¡ª
Reference Materials

Reference materials are not as extensively tested as ISs, but they do have certain potency and purity data. Such preparations are useful in cases in which substances cannot be completely characterized by physical means alone.

3.5.3¡ª
In-House Reference Preparations

In-house reference preparations are produced by the laboratory that developed the assay or are those that have been acquired without reliable potency estimates. Frequently, these materials are calibrated against an IS.

3.5.4¡ª
Working Standards

Working standards are very important forms of standards and are prepared in the laboratory in relatively large volumes. The extensive testing and validation

required for the introduction of reference standard is not normally necessary, but the donor laboratory must assume responsibility for maintaining appropriate quality. This form of standard is common in animal disease diagnosis.

4¡ª
Shelf Life and Reagent Considerations

QC relies on the source and accurate preparation of reagents. The quality of reagents for certain stages can be more critical than others. With in-house assays, new reagents must be thoroughly characterized, and once this is done the controls can be restricted to those used for monitoring assay performance. Regular checks are needed to examine deterioration in reagents. Assays based on kit material also require attention even though reagents are provided, because often producers do not provide complete test formats of individual reagent batches. A major problem is that test kit reagents have to be transported, and this can affect reagents (factors of robustness have already been mentioned). Each kit delivered must be checked. Kit overlapping is important, in which the new kit is evaluated against the old by testing the same samples. Reagents from the old kit can be substituted in order that the activity of new reagents can be determined. Instructions for the kits must be followed exactly, e.g., the reconstitution of freeze-dried reagents. These must be opened very carefully. because powdered material can easily be lost owing to the difference in pressure (lower) in the vial. There are manufacturing guidelines concerning reagents ***(8)***. These can be summarized as follows:

1. Liquid and freeze-dried reagents should be stored at 2¨C8¡ãC.

2. Reagents transported in dry ice should be stored at ¨C20¡ãC.

3. After reconstitution, freeze-dried reagents should be stored at 2¨C8¡ãC for short times, or as samples at ¨C20¡ãC.

4. Thawed reagents should not be refrozen.

4.1¡ª
Liquids

Water quality plays a critical role for some reagents. Untreated water can contain inorganics, organics, dissolved gases, suspended solids, colloids, microorganisms, and pyrogens. Deionization and reverse osmosis are used to prepare water for laboratory use. Cation and anion-exchange resins are used to remove all dissolved ionizable substances and provide a primary water source. When coupled to specific ion-exchange resins or activated carbon resins, all organic or colloidal matter can be removed. Reverse osmosis uses a semipermeable membrane to separate substances from the water.

Most immunoassays can be performed with a water quality of 5 Ω/cm at 25¡ãC with an organic content of <2 ppm. Thus, use of deionization controlled by a conductivity meter is adequate to obtain water for assay and washing use in ELISA.

Water quality can be a major problem in some countries. Attention has been focused on devising special units suitable for developing countries.

4.1.1¡ª Aqueous Protein Solutions

Proteins can be relatively fragile in aqueous solution so that enzymes and antibodies need to be handled with care. High temperatures, and acid or alkaline solutions should be avoided. Temperatures above 40¡ãC cause denaturation of proteins. Solutions of proteins that are stirred vigorously are denatured by shearing action. The shelf life of proteins is prolonged by cold storage but attention should be focused on the state of the protein. For most enzymes in a dry phase, storage at 2¨C8¡ãC is good. Other enzymes may be unstable even dry and should be stored at ¨C20¡ãC. Repeated thawing is disastrous. Organic solvents should be avoided with enzymes except at concentrations of <3%. Enzyme labels can be supplied as liquids but need addition of cryoprotectants such as glycerol and polyethylene glycols, (approx 40% final concentration).

4.1.2¡ª Preservatives

Common preservatives in diagnostic reagents are as follows:

1. Thimerosal (0.01%): This is expensive, difficult to dispose of (mercuric compound), and can affect assays.

2. Sodium azide (0.02¨C0.1%): This is biostatic, difficult to dispose of, and can inhibit enzyme reactions.

Although both are used for various reagents in ELISA, they do pose problems of safety and disposal. A commercial product, ProClin™, from Rohm and Haas, Spring House, PA, is recommended in **ref. *8***. It is reported to be a broad-spectrum biocide, having good compatibility and stability and low toxicity at in-use levels. It eradicates bacteria, fungi, and yeast cells at very low concentration, does not interfere with enzyme reactions, and can be disposed of without restrictions.

4.2¡ª
Shelf Life Evaluation

Evaluation of shelf life is related to the ruggedness of a test. The shelf life is a measure of the time within which the performance characteristics of a test are maintained under specified handling conditions. The change in quality is a function of factors such as storage temperature, humidity, package protection, and formulation. These are key factors in kits, which have to be dispatched and which may not be collected or used under the predetermined optimal conditions. This is particularly important in some developing countries, so that kit formulations must be tested for performance under a wider set of variables. There is an attempt to measure product expiration times with commercial kits

through practical determination of stability. This is also tied up with governmental regulations. For example, the Food and Drug Administration requires written testing programs designed to examine the stability of products based on these factors:

1. The sample size and the test intervals for each attribute measured.

2. Reliable, specific, and meaningful tests to assess quality.

3. The conditions under which held-back samples are stored.

4. Testing to be conducted under the same conditions as in the intended market.

5. Tests to be conducted at the time of dispensing, as well as after reconstitution of reagents.

4.2.1¡ª
Types of Stability

Physical, bacteriostatic, and functional stabilities are important. Physical appearance such as discoloration and precipitation is undesirable. Most products contain bacteriocidal or bacteriostatic compounds to prevent deterioration through microbial growth. Thus, on storage, the active component must remain at a sufficiently high level to work. Functional changes are damaging to the assay's performance (can reduce analytical and diagnostic sensitivity and specificity). These can involve functional changes owing to antibody degradation, as well as chemical changes affecting function, e.g., in chromophore quality owing to oxidation or reduction.

4.2.2¡ª
Criteria for Shelf Life

A typical quality criterion for shelf life is that the product must retain at least 90% of its original value throughout its life. This performance is often applied to assessing the results of stability testing. Reagents used in ELISA, and diagnostic kits in general, must have a long shelf life. Most reagents are stable when unopened. It is desirable to have single lots of reagents, which can be used over a long period in many laboratories. Stability involves factors of degradation. The rates of degradation for given reagents are defined by the laws of chemistry and physics. The dominating factor for a given pH, ionic strength, composition, and so on, is temperature. Thus, degradation of any product can be monitored at high temperatures and the information extrapolated to the anticipated storage temperature, to determine the usable shelf life.

4.3¡[a]
Real-Time Stability Testing

The testing of products in real time has to be regarded as the gold standard for determination of expiry dates. However, this is not practical in most cases. Products must be left to allow a degradation to be observed. Methods to measure at least a 1% degradation, as distinct from interassay variation, must be available. The reliability can be increased if a single lot reference is included

with each test point. Sample recovery among samples can be normalized to this reference to minimize the impact of systematic drift and imprecision. The reference materials themselves should be sufficiently stable so that a single lot provides unchanging performance throughout stability testing. In brief, the real-time data collection is complicated by drift or changes in the testing method used over a period of time.

4.4¡[a]
Accelerated Stability Testing

Accelerated stability testing is often used when developing clinical reagents to provide an early indication of shelf life. The method involves subjecting products to several high temperatures. The amount of heat input needed to cause product failure is determined. An efficient system requires at least four "stress" temperatures ***(10)***. Temperatures that cause denaturation should not be used. This is particularly true for labile proteinaceous reagents, such as antibodies and enzymes. One advantage is that samples can be subjected to elevated temperatures, stored at low temperature, and then assayed at the same time as unstressed controls. There are several approaches to analysis, involving different mathematical methods.

4.4.1¡[a]
Protocol for Accelerated Stability Testing

Four parameters for accelerated stability testing must be considered ***(9)***:

1. Preparation of samples: The samples must be as close to, if not identical to those to be used in assays. This includes containers.

2. Storage conditions: Four temperatures for storage should be examined. The highest temperature is determined by the type of substance being examined (e.g., to avoid denaturation of proteins, and by the length of time available to devote to the assays). Lower temperatures may be needed when the containers themselves are affected.

3. Analytical procedures: The analytical method used must be meaningful for the active reagent of the product. Chemical assays are reliable up to 2¡ãC but biological assays only to 5¡ãC. It is vital that the initial assay be titrated extremely accurately in multiple replicates to allow the determination of degradation.

4. Analysis and interpretation of data: Methods are reviewed in **ref. *8*, Chapter 10**. These exploit the Arrhenius relationship, which states that the functional relationship between time and stability of a product stored under constant conditions is dependent on the order of reaction and the rate constant that determines the speed of reaction. A general guideline is shown in **ref. *8*, Chapter 10** which delineates the steps as follows:

a. Select four temperatures. Define degradation, e.g., loss of total function or clinical parameter (enzyme concentration, percentage of binding of a reference material to an antibody at a predetermined concentration, and so on). Place the reagents in incubators at the predefined temperatures. If degrada-

tion is not observed within a reasonable time (e.g., 1¨C2 mo), then select higher temperatures. There must be degradation for determination of shelf life.

b. Place a sufficient amount of reagent in the final containers, at the selected temperatures, for specified time periods. This will depend on the estimated rate of degradation. The volume/amount will depend on test method requirements, but there should be enough to allow testing to run duplicate assays for each point of a minimum three-point assay curve. Higher temperatures should be sampled more frequently than lower. The following protocols are suggested: 37¡ãC once every 5 d; 45¡ãC once every 4 d; 50¡ãC once every 3 d; 60¡ãC once every 2 d; and, 80¡ãC once every day.

c. Samples should be cooled on removal from elevated temperatures and analyzed promptly.

d. Measurements can be made on any critical components.

e. Data plotted against day number should give an approximate straight line using semilog graph paper. Conventionally, the slope is measured. However, the measure of the length of time for activity (potency) to drop by 90% of the original value, or any other appropriate criterion, can be selected and determined from the graph. The real situation requires an estimation of the maximum allowable drop in activity that would not affect the functioning of the product and thus the assay performance.

f. From such a set of data, one can predict the time required to reach 90% potency at the desired temperature. The logarithms of the t_{90} values are related linearly to the reciprocal of the absolute temperature.

g. A semilog plot of t_{90} values (ordinate) and $1/T$ in Kelvins (abscissa) should give a straight line. From this, the predicted shelf life at the desired storage temperature can be calculated.

Based on the experiences of the author in **ref. *8***, here are some guidelines to approximate the shelf life of a product at a desired temperature.

1. One month at 50¡ãC is equivalent to 1 yr at room temperature.

2, Two months at 50¡ãC is equivalent to 2 yr at room temperature.

3. Three months at 37¡ãC is equivalent to 1 yr at room temperature.

4. Six months at 37¡ãC is equivalent to 2 yr at 4¡ãC.

5. Seven days at 37¡ãC is equivalent to 1 yr at 4¡ãC

6. Three days at 37¡ãC is equivalent to 3-6 months at 4¡ãC.

5¡[a]
Literature

Further information on the various aspects of validation can be obtained in **refs. *10*** and ***11***. In addition, an excellent review of advanced methods for test validation and interpretation in veterinary medicine is available in **ref. *12***.

6¡[a]
Statistics

This section reviews some statistical terms and principles. The purpose of ELISA is to measure quantities or compare antigens or antibodies through

measuring the level of binding or inhibition of binding of some labeled material. Statistical analyzes are applicable to both the assay itself, through examination of the variation inherent under different conditions and to the data generated and its relevance to the problem investigated. Thus, statistical analysis is inherent in ELISA development, for continuous use (monitoring performance), and to analyze the results for specific samples (e.g., whether a sample falls into a positive or negative population). Statistics also assess results in terms of confidence in the values obtained.

It is vital to understand that statistical examination should never be regarded as the last thing to do when faced with data. Consideration of what statistical methods are to be used must be made during the planning stages, so that data can be immediately assessed, particularly since computer software is often used. A key is to ask, How can anyone prove anything associated with an assay? Proof statistically is, by definition, a measure of the probability of an event. The stronger the measure of the statistical probability, the more true the measure is.

6.1¡ª
Populations

A major use of ELISA is to obtain data from sample analysis and assess whether a particular sample is positive (contains a particular analyte) or negative (does not contain analyte). This requires both the examination of the specific assay parameters (assessing sensitivity and specificity) and the defining of a specific population in terms of data obtained and with reference to other data from other tests. The establishment of a population statistic requires that the data be obtained and analyzed. The analysis determines the type of distribution of data, and hence any sample value can be assessed with reference to all data in that population. The amount of data obtained to establish a population affects the certainty of any result, as does where the samples were taken from to establish the population (sampling/survey statistics).

6.2¡ª
Basic Terms

These are a few basic terms:

1. The *mean* is the arithmetic mean of a set of data.

2. The *median* defines a value or interval range of values in the middle of a set of values in a population.

3. The *mode* defines the value or interval range that is most frequent in any population.

These are illustrated in **Fig. 6** as a plot of the data shown in **Table 3**. These are OD results from the analysis of a supposedly negative population of samples. The interval has been selected as 0.02 OD units represented on the x-axis, and the numbers of samples in any defined interval are plotted on the y-axis.

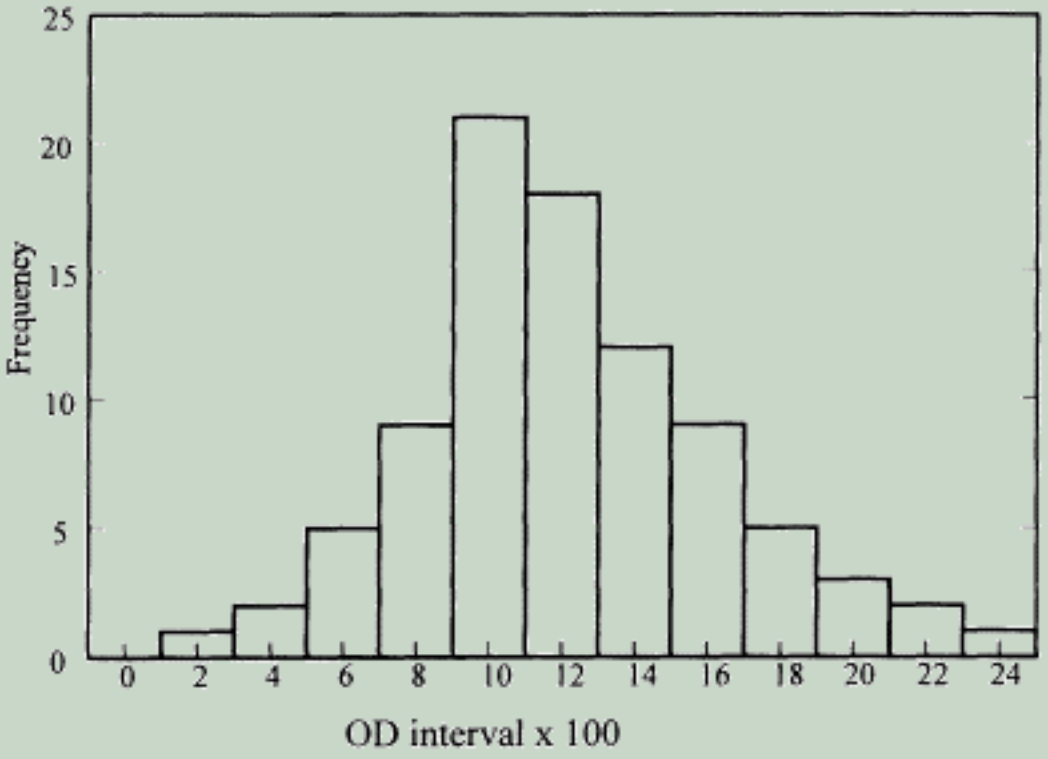


Fig. 6.
Frequency histogram of data in Table 3.

Table 3
Data from Analysis of Samples

OD interval	Number (frequency)
0.00¨C0.02	1
0.02¨C0.04	2
0.04¨C0.06	5
0.06¨C0.08	9
0.08¨C0.10	21
0.10¨C0.12	18
0.12¨C0.14	12
0.14¨C0.16	9
0.16¨C0.18	7
0.18¨C0.20	4
0.20¨C0.22	2
0.22¨C0.24	2
0.24¨C0.26	0

Figure 6 represents actual data for 77 results taken from a population of samples. We can see that there is a peak where most of the data are placed from intervals about 0.08¨C0.16. Lower numbers are observed on either side of this.

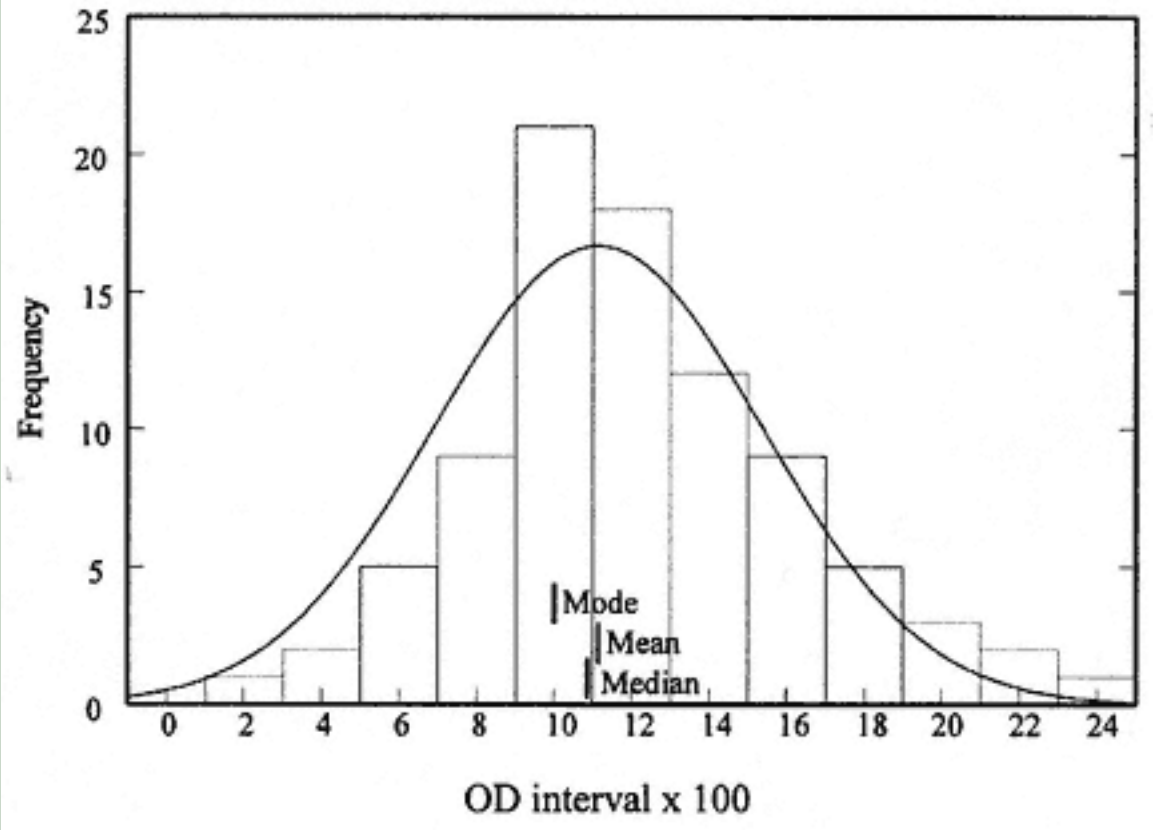


Fig. 7.
Frequency histogram of data in Table 3 with normal distribution plotted and the mean, median, and mode highlighted.

The mean, median and mode can be described for this data as shown in **Fig. 7**. The data is shown as a distribution curve and these can be defined mathematically according to which distribution they fit. Some distribution curves are shown in **Fig. 8**. The statistics involving normal distribution curves are illustrated in **Fig. 9**.

6.2.1¡ª
Normal Distribution

When the distribution is symmetrical, it is referred to statistically as a Gaussian or normal distribution. In a perfect case, the mean, mode, and median are identical. Although this perfect situation is never achieved in practice, most situations for ELISA regard distributions as normal. The normal distribution can be described in mathematical terms with regard to the mean (X) and the *SD* of the observed values. The use of sampling techniques and the examination of distribution in samples attempts to determine the true mean (u) and variance (S^2) of the entire population from which the samples are taken. The breakdown in the statistical considerations of a normal distribution are shown in **Fig. 9.** The key here is that whatever the measured values, a certain percentage of the sample is always contained within the *SD* values. One *SD* on either side of the mean contains 68% of all values under the curve of that distribution. In the

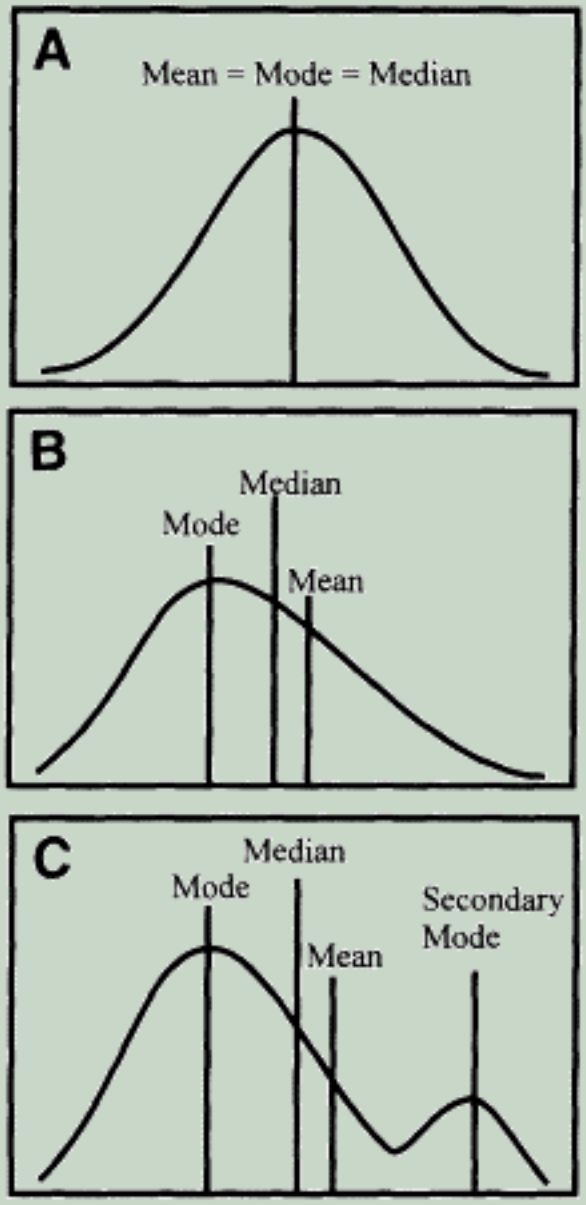


Fig. 8.
Population distributions. **(A)** Gaussian or "normal" distribution. This is symmetrical about the mean and the values of the mean, median, and mode are the same. **(B)** A long "tail" of higher values, this represents a nonsymmetrical distribution in which there are different values for the mean, median, and mode **(C)**.

same way, 1.96 ¡Á *SD* on either side of the mean contains 95% of the entire population. The 99% limits fall 2.58 *SD*s to either side of the mean.

The methods involved in calculating the mean, median, mode, and *SD* are usually made through the use of a statistical package, and the theory of their calculation can be examined in any fundamental book on statistics.

The confidence in results is important in the examination of distributions. This is considered in the calculation of the distribution statistics *per se*, but generally the greater the number of samples analyzed to calculate a distribu-

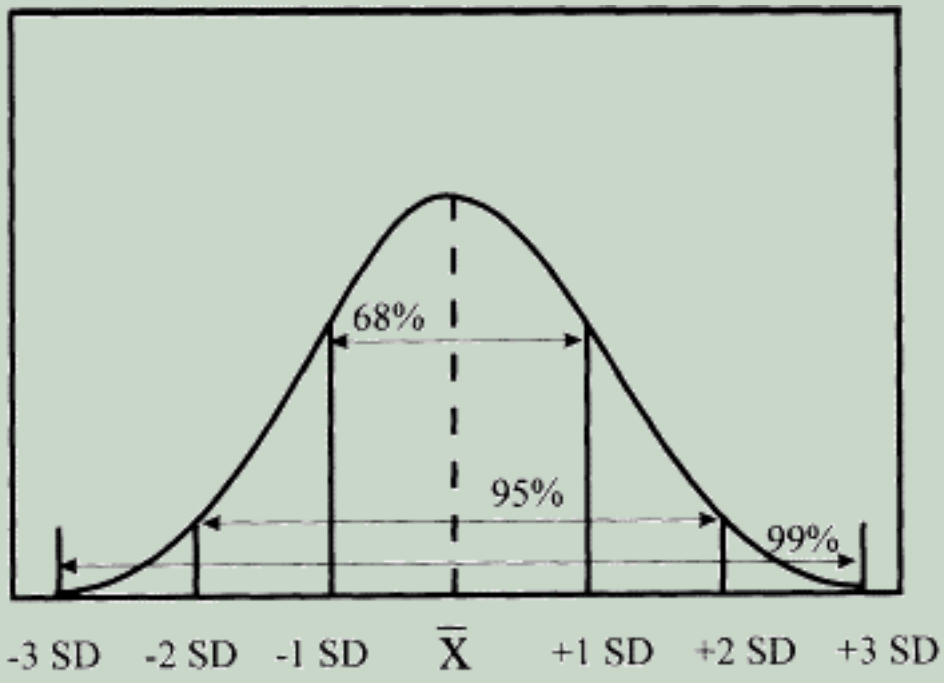


Fig. 9.
Probability distribution for a population defined by Guassian curve.

Mean $(\bar{X})$, and plus or minus 1, 2, 3 ¡Á *SD* encompases the percentages indicated. Thus, e.g., 95% of all results in a population would be distributed about the mean between +2 and ¨C2 ¡Á *SD*.

tion, the greater the confidence one can have in evaluating any result with respect to that distribution. The confidence value for any sample can be measured and used to ascribe confidence in a result with regard to the distribution statistic, although this is not common.

6.2.2¡[a]
Nonnormal Distributions

The analysis of nonnormal distributions requires more complex mathematical considerations. Differences among mean, median, and mode alert the operator to nonsymetrical distributions.

6.3¡[a]
Variation in Practice

The mean $(\bar{X})$, *SD*, and %*CV* are used as indicators of the variation on data. The formula for $\bar{X}$ is as follows:

$$\bar{X} = X_i/N$$

in which *X* is an individual component and *N* is the sample size.

The formula for *SD* and %*CV* are as follows:

$$SD = (X_i - X)^2/(N - 1)$$

$$\%CV = (SD/X) \times 100$$

Worked examples of the use of statistics is most worthwhile.

6.3.1¡[a]
Example Data

A number of supposedly negative samples (*n* = 421) have been analyzed by an indirect ELISA for the detection of antibodies. A single dilution was measured for each sample and the data were collected as OD values and based on a validated assay. Note that one concept in validation is the examination of populations to establish what we think of as a negative result. The distribution of data from a selected "negative" population will help describe any other test result as falling into or out of that population. Whether the population is actually negative with respect, e.g., of detecting a specific antibody is important and not always easy to ascertain. Examination of a large data set will indicate possible problems in determining whether there is wider dispersion (range) of results than might be expected. What is expected in a negative population is that all results will be low (OD) with a low variation. This can be examined, and any results with very high ODs can be reexamined. The object here is to plot the data as a frequency distribution and then calculate the mean and *SD* of that population. The selection of a negative population is easier from countries where there has never been a particular disease, but this does not rule out nonspecific results nor that the parameters measured, say, in cattle from England are

the same as those in the Sudan.

6.3.1.1¡ª
Data

Figure 10 shows all the data plotted as a frequency distribution. Since we have taken a relatively large population, this might be reasonably expected to represent the distribution inherent in the whole population. However, the sample must be regarded with respect to the estimated total number of animals in a population and the possible geographical variations.

Note that the calculated mean, median, and mode are similar, indicating a normal distribution. We can therefore use statistics to set various limits as to values with respect to the mean and *SD*. Thus, from the data shown in **Fig. 10**, we see that the mean is 13.18 and that the *SD* is 5.76. Hence, 95% of all results lay between plus and minus 2 ¡Á *SD* of the mean. Therefore, the mean plus 2 ¡Á *SD* = 24.7, and the mean minus 2 ¡Á *SD* = 1.68. Any values outside the upper value of 24.7 would be more unusual in the population of negatives, and above 3 ¡Á *SD* very unusual. The population examined appeared to give results that were normally distributed. The mean value of this population and the *SD* can be used to determine the positivity of samples. The confidence we can have in any datum point (sample) can be determined with respect to the calculations based on the negative population examined. This requires the consideration of some statistical terms.

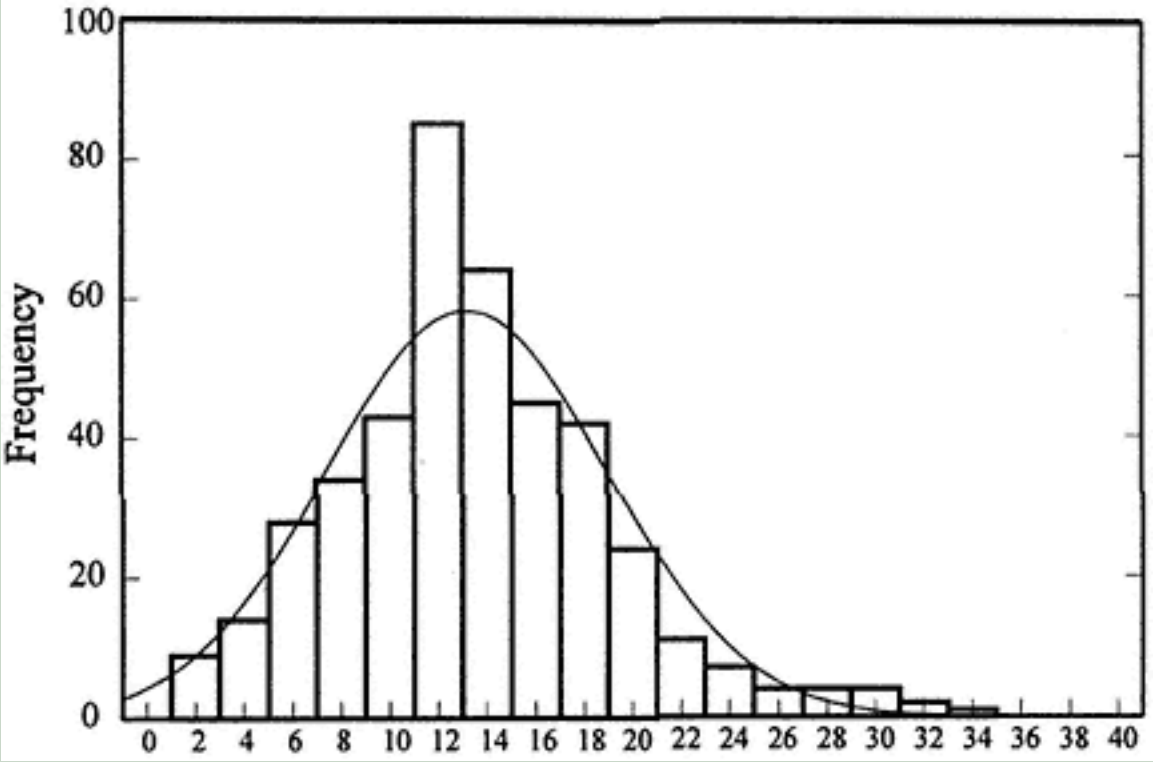


Fig. 10.
Distribution plot of 421 samples. Data were analyzed in a statistical program and showed the following: mean = 13.18; mode = 1 1.68; median = 12.00; lower quartile = 10; upper quartile = 16; interquartile range = 6; standard error of mean (SEM) - 0.28; variance = 33.20; *SD* = 5.76; *CV* = 0.44.

6.4¡[a]
Confidence Interval

The CI for a mean essentially describes where the population mean *(u)* lies with respect to the sample mean $\bar{X}$ with a given probability. If several means are available from different groups of measurements of the same sample, the individual means will also be distributed normally around the grand mean. The random variation in a population of means is described by the SEM:

$$S_{\bar{X}} = SD/\sqrt{N}$$

By using the SE of the experimentally determined mean one can give a CI that has a known probability of including the true population mean. This depends on the number of measurements (*N*), the *SD* and the level of confidence desired:

$$\mu = \bar{X} \pm (tSD/\sqrt{N})$$

or

$$\mu = \bar{X} \pm (tS_{\bar{X}})$$

The value of *t* is taken from a table and depends on the level of confidence required or the level of probability (*p*) that *u* is outside the CI because of chance

alone and the number of degrees of freedom (v, which is one less than the sample size) associated with the estimation of *SD.* Thus, values such as *p* = 0.05 indicate a 95% confidence limit [100 (1 ¨C *p*)%], that the interval includes the true mean. Basically, this means that we examine the variation in the result for a single sample (its mean and variation as error), and see where this fits in the distribution (with its internal variation).

The t values also fit a normal distribution. Here, one is making an assumption that the true population mean and *SD* are known, and because the CI is being made to include the true mean, the 0.5 probability that µ is beyond the calculated limits a is spread over both ends of the distribution (both tails). Here a two-sided interval, *p* = 0.5 *t* value, is used. The uses in which a single tail (above or below the mean) is considered, a one-sided *t* value (*p* = 0.025 is used (equivalent to the two-sided *p* = 0.05). The object then is (1) to have a defined total population statistic and (2) to examine where and with what confidence a sample value fits into this population. Some points need to be made concerning confidence limits.

1. No error can be calculated in which a single sample alone is measured. One element during the development of an assay is that the random error can be measured for many samples. This generally tends to be a compound of the internal variations of all aspects of sample analysis, and thus an overall generalized error can be calculated and "imposed" on all samples.

2. The *SD* of the population mean (S_x) is smaller than the sample *SD*. The *SD* decreases as the square root of the number of samples is averaged; thus, obviously, the mean of the population is a better approximation of the true mean *(u)* than any single sample. As the number of samples increases, the CI for the mean increasingly decreases, because of the decreasing *SD* of the mean and of the decreasing value of *t*.

3. The defining of confidence limits requires the analyst to make a subjective choice of a level of confidence. The higher the level of confidence, the wider the limits.

As indicated already, the calculation of confidence limits does depend on the random errors following a Gaussian distribution, and this can be tested by χ^2 squared or Kolmogorov-Smirnov testing, but in the main immunoassay, data follow the Gaussian statistic, which can often be proved throughout assay validation through repeat testing of samples.

7¡[a]
Optimization of ELISA Procedures: New Approach

Recently, a method for optimizing ELISA procedures has been demonstrated ***(13)***. This method attempts to compare the net effects of different conditions using an experimental design called the Taguchi method. The method attempts to reduce the effects of the interactions of optimized variables, making it possible to access the optimal conditions even in cases in which there are large

interactions among variables. The proposed scheme is said to allow calculation of the biochemical parameters of the ELISA. Thus, the calibration curve and the intra-and the interassay variability can be calculated in one step. This compares with the step-by-step approach of CBTs. The method is exemplified using an ELISA required to be optimal for the detection of the single-chain fragment of variable phages. The calculations are made through a spreadsheet program. This procedure is worth examining when considering the optimization of ELISAs and is one of the few designs intended to allow optimization based on a simple statistical evaluation. The design is based on that used to optimize the PCR ***(14,15)***.

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9¡ª
Charting Methods for Internal Quality Control

This chapter deals with relatively simple ways to use control charts to monitor the performance of ELISAs. A rinderpest competition ELISA, for the estimation of antibodies in serum samples, is used to demonstrate the methods. This assay is available in kit form. Constant evaluation of the use of the kit is part of what is called internal quality control (IQC). **Figure 1** shows an overview of the ELISA scheme described in this chapter. The details of the procedure, which involves plotting the data graphically (charting methods), are explained herein. The objectives of charting data are as follows:

1. To keep a constant record of all data.

2. To monitor the assay from plate to plate in any one day's testing.

3. To monitor the tests made from day to day, week to week, year to year.

4. To allow rapid identification of unacceptable results.

5. To allow recognition of reagent problems.

6. To identify trends in results (increasingly poor performance).

7. To identify when a new set of kit reagents is necessary.

8. To allow identification of differences in operators of the assay.

9. To fulfill various criteria for good laboratory practice.

10. To fulfill necessary requirements for external recognition that tests are being performed at an acceptable level (increasingly important when results are used for international trading purposes).

1¡ª
Good Practice of IQC

IQC methods allow test operators as individuals to monitor the performance of their test. When there is more than one operator, the method produces a unification of approach, to allow control over results, and allows discrepancies among performance to be identified. It also promotes the idea of "open" results that can be viewed by anyone, including outside scientists, interested in evaluating the status of a laboratory involved in providing results on which management decisions concerning disease control are made.

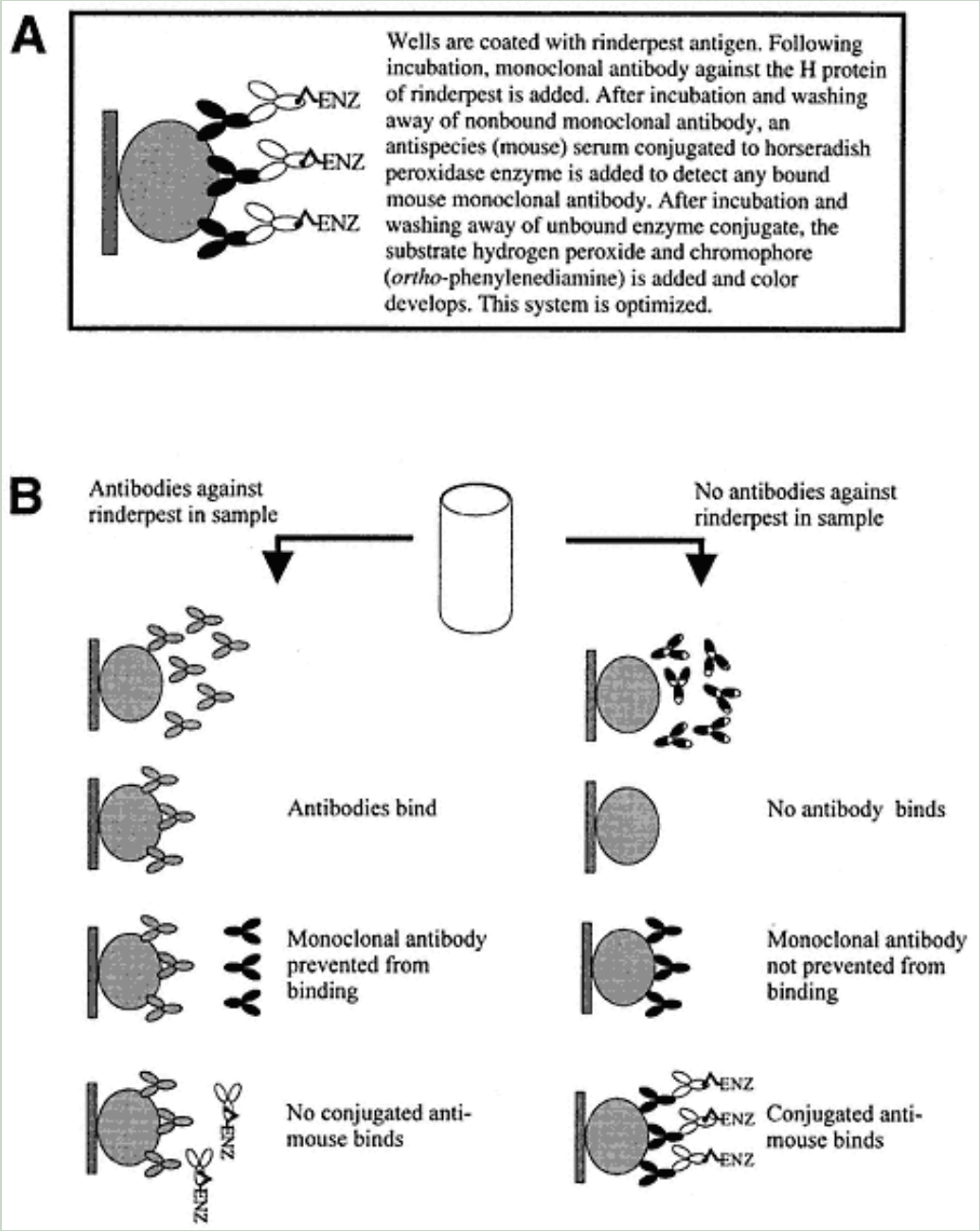


Fig. 1.
Principles of competitive ELISA for measuring antibodies against rinderpest virus: **(A)** pretitration of indirect ELISA; **(B)** blocking of ELISA to detect antirinderpest antibodies in serum samples.

The method described here is not a deep statistical analysis of data. Rather, it attempts to visually assess results, so as to increase awareness of operators about what they are doing on a daily, monthly, and yearly cycle of work. The

most important feature of testing in a laboratory is that operators have a very good understanding of the principles of the test they are using, and that they fully understand the nature of their test results and the need to process data. There is no substitute for this understanding, but the charting method recommended is an aid to simplifying the process of monitoring test performance. At first glance, this approach may seem to be overcomplicated. However, it is to be assumed that people involved in testing have had some trained and are running the assays on a fairly routine basis.

1.1¡ª
Principles of ELISA for Detection of Antibodies against Rinderpest

Figure 1 shows the principles of the ELISA used. The basis of the test is the prevention of pretitrated monoclonal antibody (mAb) from binding to rinderpest antigen on the wells of a microtiter plate by polyclonal antibodies in samples (serum). The degree of prevention is measured with reference to controls with no sample added (Cm) in which maximum optical density (OD) values are observed, and to wells in which is only conjugate is added (Cc) where low color is observed. Percentage inhibition (PI%) values are calculated with reference to these values.

Control serum samples are provided in the kits, which give strong (C++) and moderate (C+) PI% values. These controls can be used for assessment of the test from plate to plate and day to day. The plate design for the kits is shown in **Fig. 2**. For each plate, 40 samples are examined in duplicate, at a single dilution, and controls are placed as shown. Each PI% value is calculated with reference to the controls of individual plates. Studies on large numbers of negative sera and experimentally infected animals have determined the PI% values that ascribe positivity to samples.

On receipt of kits, operators should have everything to be able to perform the assay. The control sera enable operators to monitor the assay routinely, and the use of these data in control charts is the basis of this chapter. The necessary data for plotting on various charts is obtained through the calculation of the mean and standard deviation (*SD*), from the mean of control samples as raw OD data or PI% values.

2¡ª
Charts

Two kinds of charts are recommended: (1) Daily Detailed Data (DDD) charts, used to plot data from single plates; and (2) Summary Data Charts (SDC), used to plot data from all plates performed on any day.

The use of such charts at various points of the rinderpest ELISA is illustrated in **Fig. 3**. The processing of data for plotting on the charts is important, and illustrative tables suitable for this purpose are presented in **Figs. 4¨C7**. Raw

Cc	Cc	1	5	9	13	17	20	25	29	33	37
C++	C++	1	5	9	13	17	20	25	29	33	37
C++	C++	2	6	10	14	18	22	26	30	34	38
C+	C+	2	6	10	14	18	22	26	30	34	38
C+	C+	3	7	11	15	19	23	27	31	35	39
Cm	Cm	3	7	11	15	19	23	27	31	35	39
Cm	Cm	4	8	12	16	20	24	28	32	36	40
C-	C-	4	8	12	16	20	24	28	32	36	40

Fig. 2.
Plate layout for rinderpest competition ELISA. Controls are C++ (strong antibody positive); C+ (weaker antibody positive); Cm (mAb control, no sample); and C¨C (conjugate control, no sample or mAb).

(nonprocessed) OD values, for each plate, are plotted for the Cm controls only on DDD charts and SDC charts. The PI% values are monitored for all controls on DDD and SDC charts. In terms of practical use of charts, a few rules can be given. These encourage the best use of the charts to allow easy monitoring and transparency of results to permit identification of problems on a continuous basis.

2.1¡ª
General Recommendations for Charting Methods

1. Charts should be displayed openly (on walls) and copies also kept on file.

2. A specific individual should be appointed to oversee the charts. This individual should ensure that all people performing the assays fill in the tables and chart the results. This individual should ensure that only relevant data are added to the charts, for example, plates used for developmental work or research should not be included, only those involved in running a routine assay.

3. The results on the charts should be discussed regularly with all involved in laboratory testing, and any trends identified and appropriate action taken.

2.2¡ª
Initial Examination of Kits

A great deal of care has been taken to ensure that the reagents and materials will work in laboratories worldwide with different local conditions, and that

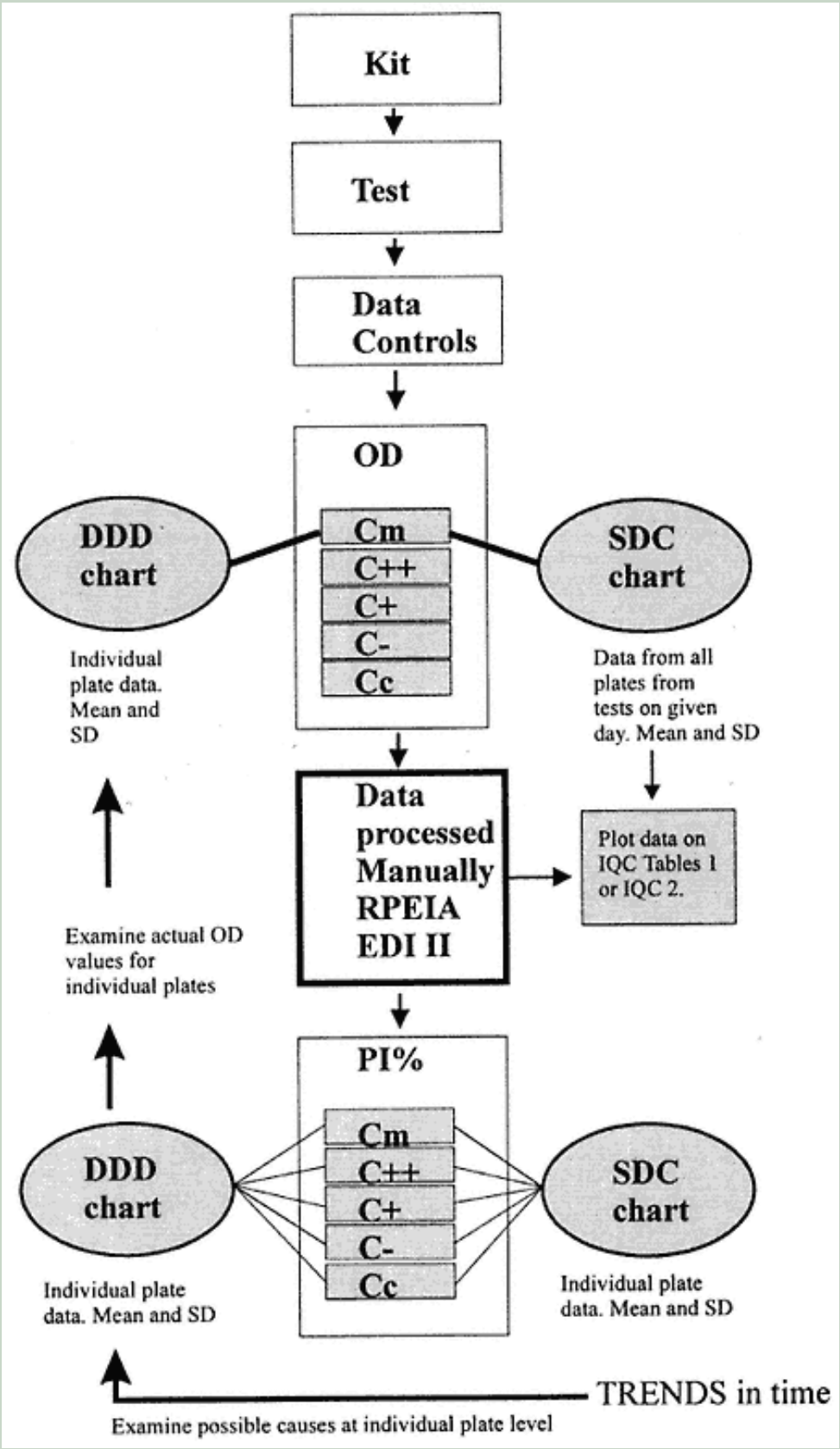
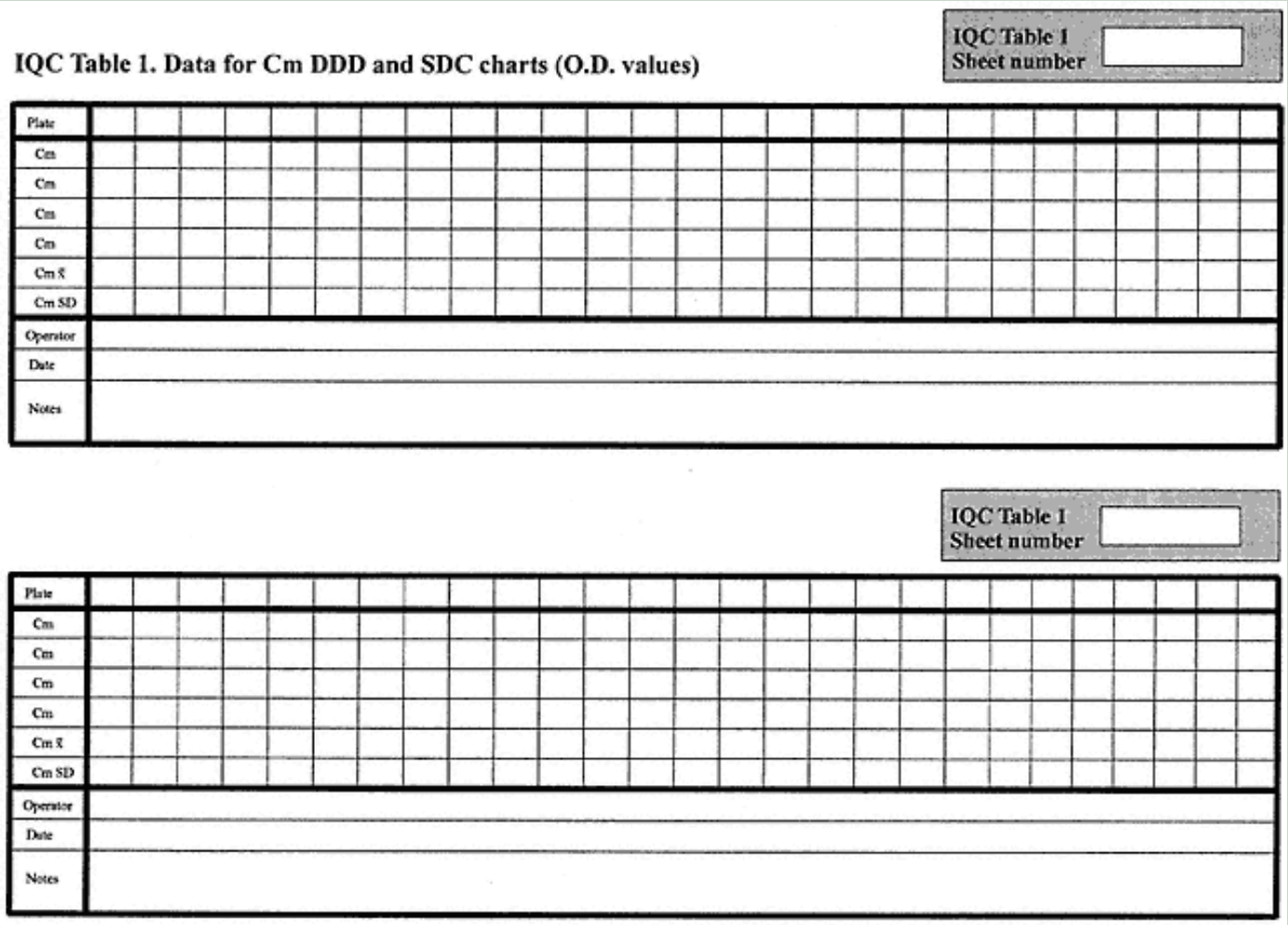


Fig. 3.
Scheme for plotting data on charts.

the kit will travel without deterioration of performance. On receipt of a kit, there are two initial questions: (1) How do we know that the kit reagents are



IQC Table 1. Data for Cm DDD and SDC charts (O.D. values)

IQC Table 1
Sheet number

Plate																											
Cm																											
Cm																											
Cm																											
Cm																											
Cm $\bar{x}$																											
Cm SD																											
Operator																											
Date																											
Notes																											

IQC Table 1
Sheet number

Plate																											
Cm																											
Cm																											
Cm																											
Cm																											
Cm $\bar{x}$																											
Cm SD																											
Operator																											
Date																											
Notes																											

Fig. 4.
Tables of Cm controls as OD values for DDD and SDC analysis.

IQC Table 2 Data for Cm, C++, C+, C-, Cc means and SD (PI% values)

IQC Table 2
Sheet number

Plate																														
Cm																														
Cm																														
Cm																														
Cm																														
Cm $\bar{x}$																														
Cm SD																														
C++																														
C++																														
C++																														
C++																														
C++ $\bar{x}$																														
C++ SD																														
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Cc																														
Cc																														
Cc $\bar{x}$																														
SD Cc																														
Date																														
Operator																														
Notes																														

Fig. 5.
Table for data from Cm, C++, C+, C¨C, and Cc controls as PI%.

Plate	1	SDC Data	1	2	3	4	SDC Data
Cm	0.77		0.82	0.81	0.84	0.78	
Cm	0.75		0.80	0.83	0.87	0.78	
Cm	0.79		0.76	0.76	0.82	0.89	
Cm	0.81		0.72	0.86	0.81	0.81	
Cm x̄	0.78	0.78	0.78	0.82	0.84	0.82	0.82
Cm SD	0.03	0.03	0.04	0.04	0.03	0.05	0.04
	19.2.97		23.2.97				
	Norman		Anna				

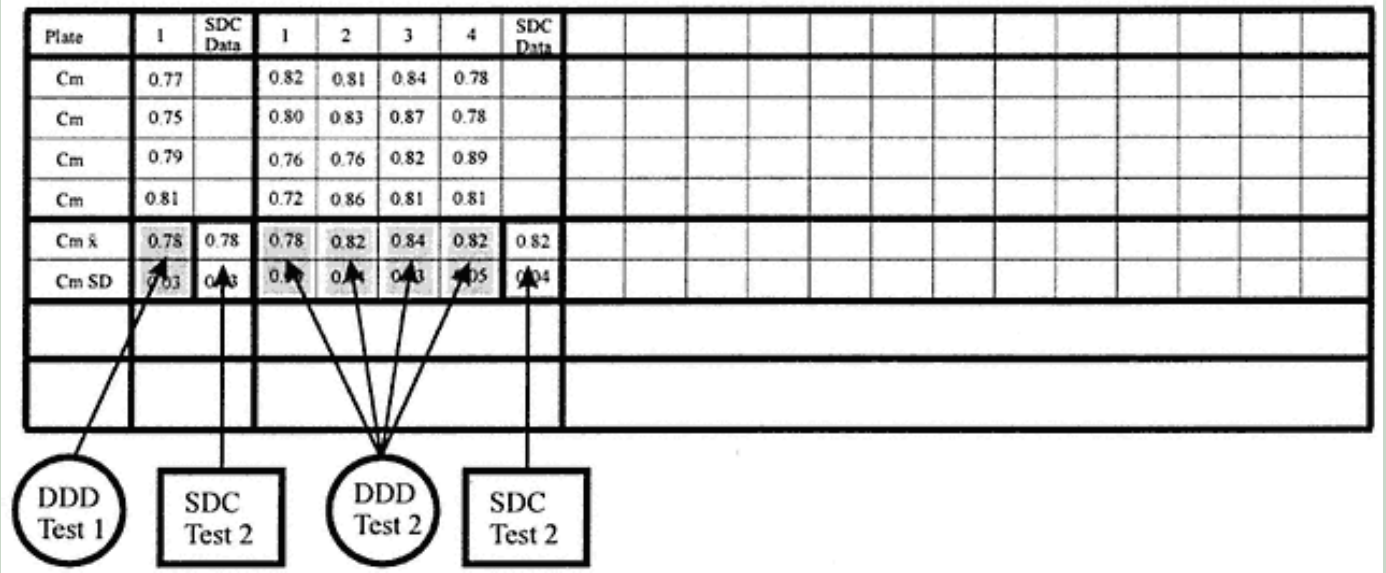


Fig. 6.
Illustration of use of IQC Table 1 for obtaining DDD and SDC data.

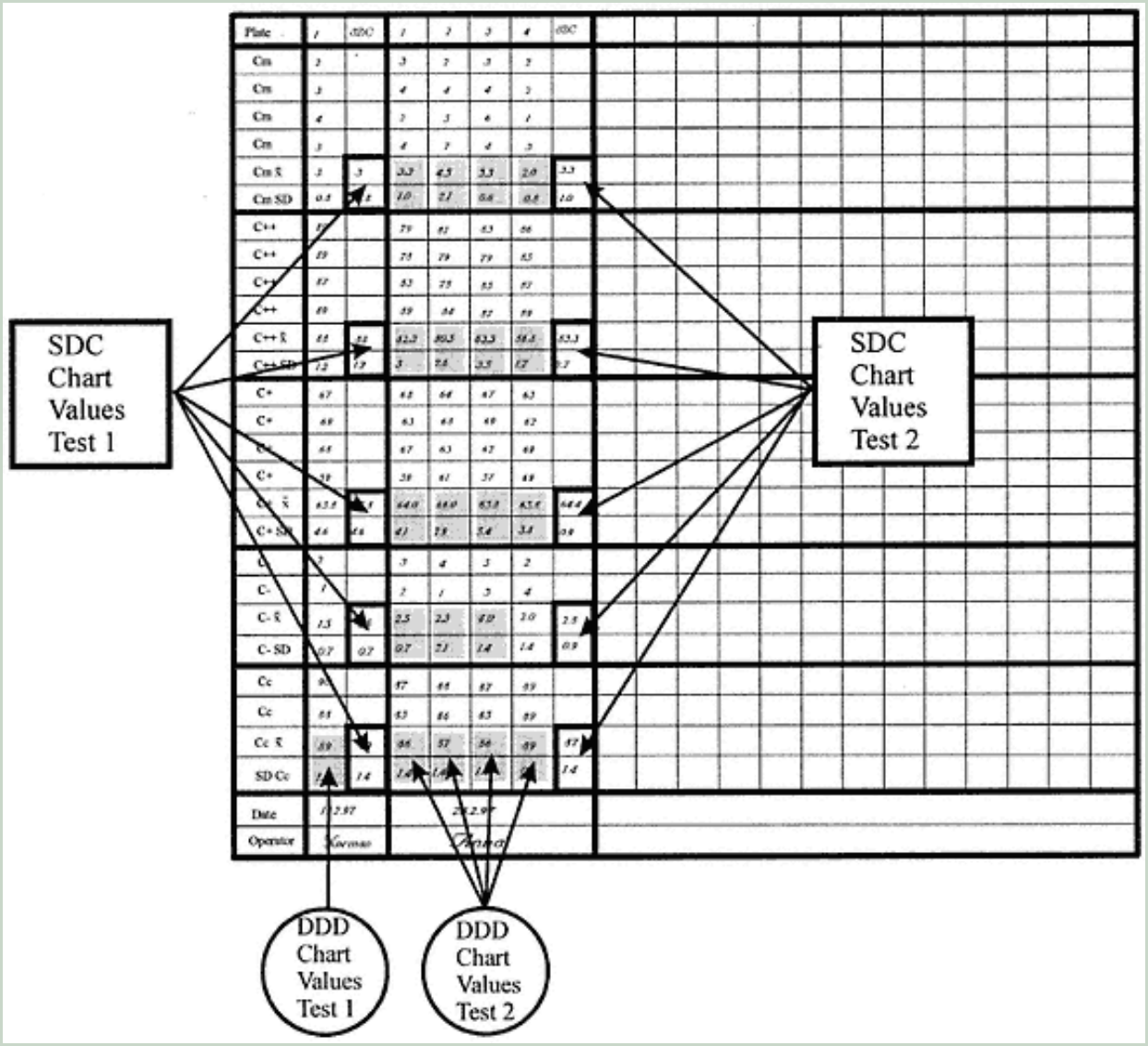


Fig. 7.
Illustration of use of IQC Table 2 for processing data.

working as expected? and, (2) How can we ensure that the kit keeps on working, i.e., the established diagnostic criteria are maintained?

The first task then is to run an assay with the kit using available control reagents and to examine the results in the context of parameters given in the manual. This tells us whether the kit is performing as expected.

2.3¡ª
Running the Assay for the First Time

The following controls have been examined in the test. Test operators should familiarize themselves with the controls and their purpose. This information should be contained in the manual accompanying the kit.

Controls on ELISA plates are as follows:

1. Cm, mAb control.

2. C++, strong positive control.

3. C+, moderate positive control.

4. C¨C, negative serum control.

5. Cc, conjugate control (no serum/no monoclonal).

Note the following conditions of the test:

1. The results expected (and the limits allowed) have been worked out by the suppliers of the kits.

2. These controls have been assessed many times and the results examined statistically.

3. The results using the control reagents have fixed limits.

4. If the results obtained are the same or within allowable limits, then your test is good.

5. If the results are outside the limits, then something is wrong.

2.4¡ª
Data Processing Fundamentals

Let us deal with the fundamentals of what the data processing does. Initial checks should determine that the controls have been placed in particular positions on the plate as shown in **Fig. 2**. A typical set of OD data for the controls is shown in **Table 1**. The data is processed to calculate PI% values and are shown in **Table 2**. The processing can be by hand (calculator) or through the use of dedicated software (as in the case of rinderpest ELISA). The formula for calculating the PI% value is as follows:

$$PI\% = 100 - [(\text{mean of replicate OD controls/median OD Cm}) \times 100]$$

2.4.1¡ª
Calculation of Mean and Standard Deviation

The mean for each control sample is obtained by adding up all the individual values and dividing by the number of values used (the number of values is usually ascribed the letter *n*). The sign for the average (mean) is $\bar{x}$. Thus, for the previously cited example, the mean OD for the Cm is 0.455 + 0.612 + 0.533 + 0.655 = 2.255 ¡Â 4 = 0.564.

Table 1
Data for Control Sera on One Plate

Control	Data(OD)
Cc	0.010(0.009)
C++	0.077(0.063)
C++	0.072(0.071)
C+	0.278(0.296)
C+	0.313(0.280)
Cm	0.685(0.673)
Cm	0.636(0.641)
C¨C	0.668(0.667)

Table 2
Control Samples Data After Processing

	OD_1	OD_2	OD_3	OD_4	$PI\%_1$	$PI\%_2$	$PI\%_3$	$PI\%_4$>
C++	0.077	0.063	0.072	0.071	88	90	89	89
C+	0.278	0.296	0.313	0.280	58	55	52	57
C¨C	0.668	0.667	¡ª	¡ª	¨C2	¨C2	¡ª	¡ª
Cc	0.011	0.09	¡ª	¡ª	98	99	¡ª	¡ª
Cm	0.685	0.673	0.636	0.641	¨C4	-2	¨C9	¨C8

The standard deviation (SD) in mathematical terms is the positive square root of the variance of the data. The variance is measured by subtracting the mean of the test wells data ($\bar{x}1$, $\bar{x}2$, $\bar{x}3$, and so on) from the overall mean value (X) and squaring that value. Each of these squared values is then added. The resulting value is divided by the total number of datum points used minus one. We therefore have:

$$\text{Variance} = (X - \bar{x}1)^2 + (X - \bar{x}2)^2 + (X - \bar{x}3)^2 + (X - \bar{x}4)^2 / n - 1$$

The *SD* is the square root of this value. As a demonstration, if we have OD data for Cm controls of 0.453, 0.612, 0.533, and 0.625, then we have for calculation of the mean and *SD* of OD values for Cm:

$$0.565 - 0.453 = (0.109)^2 = 0.012$$
$$0.565 - 0.613 = (-0.048)^2 = 0.002$$
$$0.565 - 0.534 = (0.031)^2 = 0.001$$
$$0.565 - 0.656 = (-0.091)^2 = 0.008$$

By adding up the values, we have 0.012 + 0.002 + 0.001 + 0.008 = 0.023. When we divide by *n ¨C* 1 = 3, we have 0.023/3 = 0.0076. This is the variance.

The *SD* is the square root of this, i.e., 0.0076 = 0.087. The Cm OD mean and *SD* are thus expressed as 0.564 ¡À 0.087.

This illustrates the principles of the calculation. In practice, this calculation is easily made using a statistical pocket calculator or using dedicated computer software associated with kits for direct analysis of data.

2.4.2¡ª
Recording Data in Tabular Form:
Before Plotting

The key to successful monitoring is attention to detail, the accurate manipulation of data, and the constant ability to check data. The next examples show how the data can be controlled by using tables, before plots are made. In this way, the operator can check the data as it is copied into the tables as well as be able to record the results of the calculations needed. The data can be stored in a file for instant reference in association with the charts.

We have to obtain the data for DDD and SDC charts. Two kinds of tables are shown to illustrate this:

1. IQC Table 1 (shown in **Fig. 4**). This records the actual OD data for the Cm values as well as the mean and *SD* for each plate and the overall mean and *SD* for any given test.

2. IQC Table 2 (shown in **Fig. 5**). This records the PI values for all the controls, the mean PI values and *SD* for each plate, and the overall mean and *SD* of all controls on any test.

Figure 6 illustrates filling in the data in the IQC Table 1 and **Figure 7** illustrates filling in the data in IQC Table 2.

The relationship of the data according to DDD and SDC charts is shown in Fig. 3. This shows the necessary data needed to be plotted in terms of the controls. From Fig. 5, it can be seen that the data obtained in IQC Tables I and 2 is need for plotting at various times. Once in tabular form, the data can be easily plotted. Illustrations of plots are shown in **Figs. 8¨C20.** Tables can be filed for future reference.

The data in **Fig. 6** are described according to how it is to be plotted. Values and processed data from two tests with different operators are indicated. Column 1 shows the four Cm controls, the mean value calculated from these four controls in any single plate, and the *SD* of the data. The second column shows the data from a test involving a single plate. The mean value (0.78) and the *SD* (0.03) are put into relevant boxes after their calculation. These are plotted on the DDD charts (gray shaded boxes). The date and operator are added. The SDC value is calculated for the sum of all the mean values on all plates in a given test (in this case 1 plate so that SDC data are identical to the DDD data).

A second test is shown (four plates). Again the OD values are recorded and the mean and *SD*s calculated and placed in boxes along with the *SD* of data (shaded boxes). These are plotted on DDD charts.

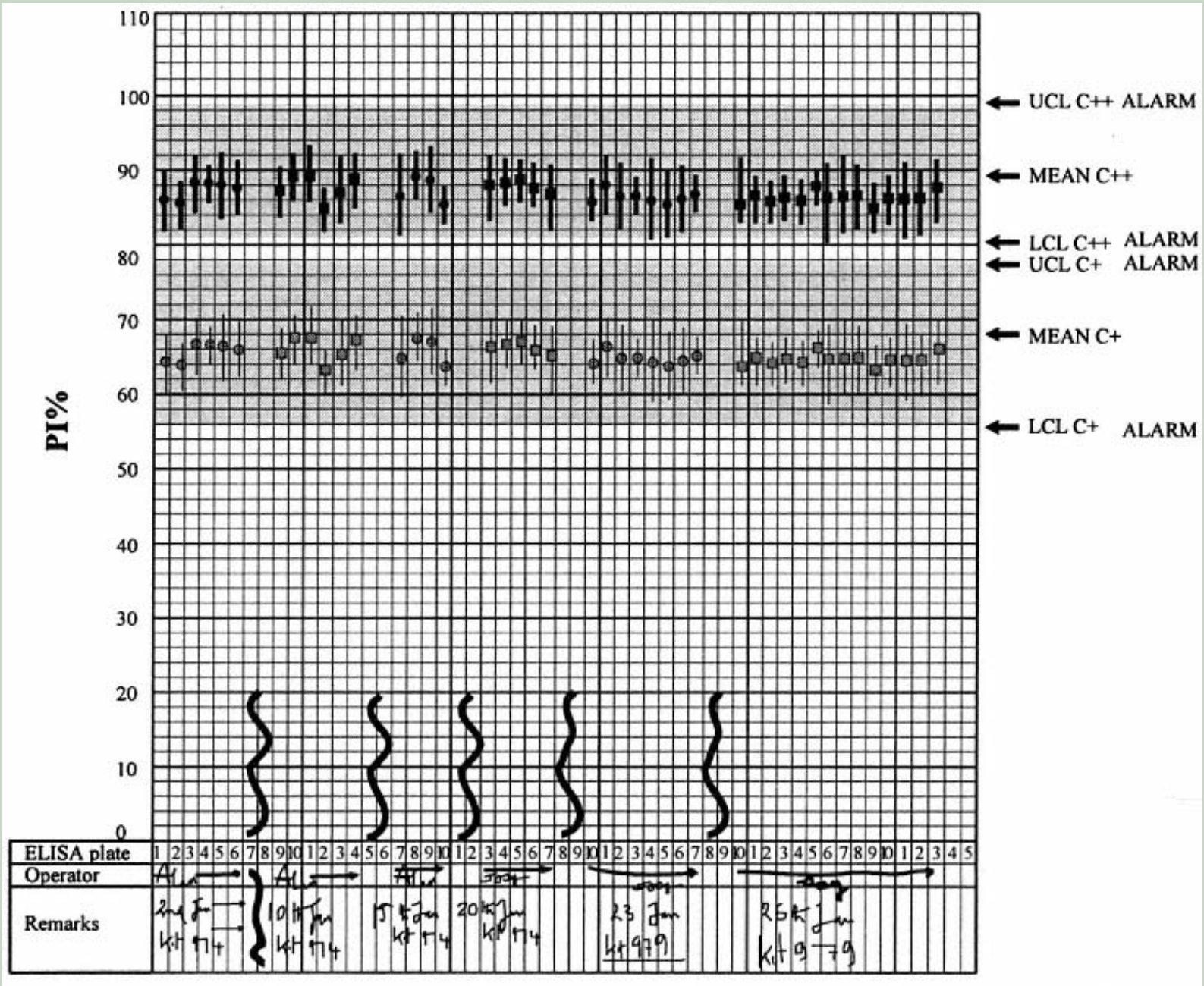


Fig. 8.
Illustrative chart for DDD plots of Cm data.

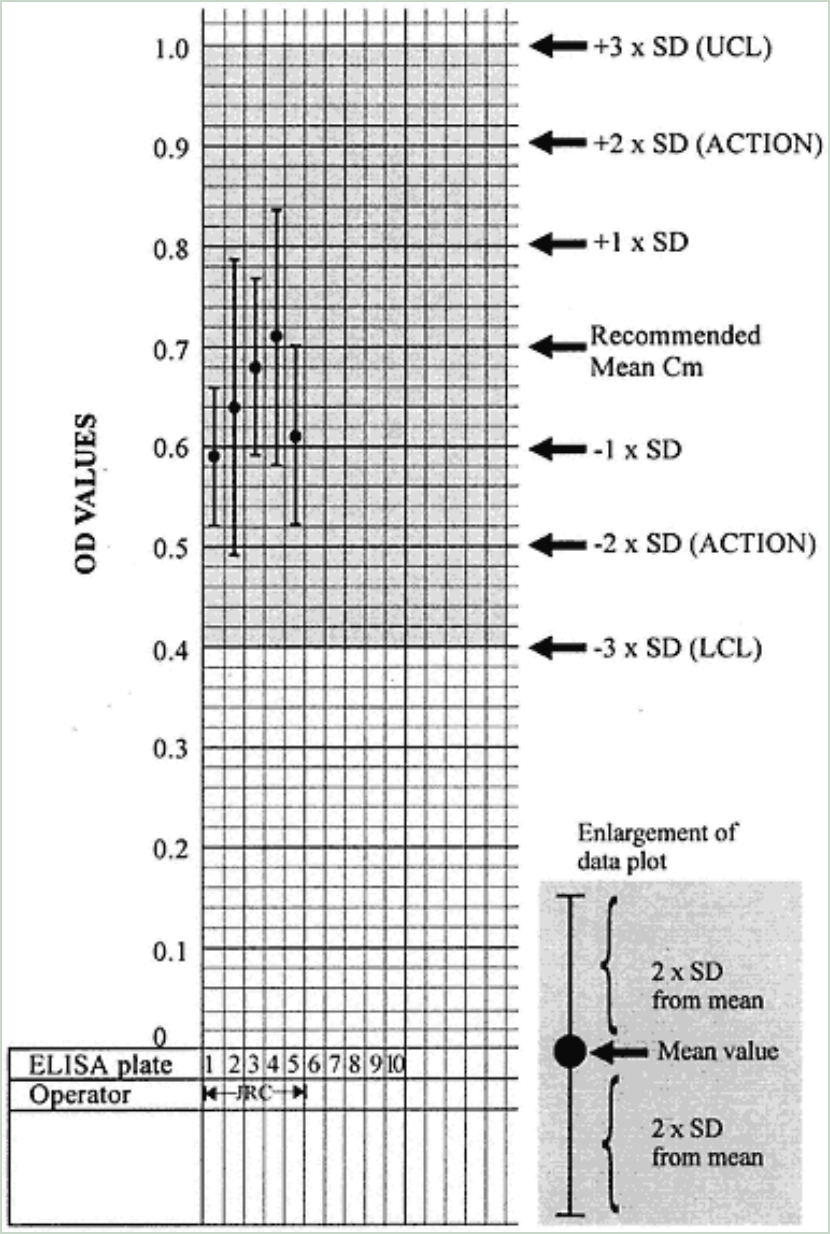


Fig. 9.
Ilustrative close-up of plots of OD values on DDD chart for Cm.

The SDC data in this case is the value obtained after calculating the mean of all the individual plate PI means (DDD data means). Note that the *SD* of the SDC data is based only on analysis of the mean PI of individual plates; it is not the mean of the *SD* shown for each plate in the gray boxes.

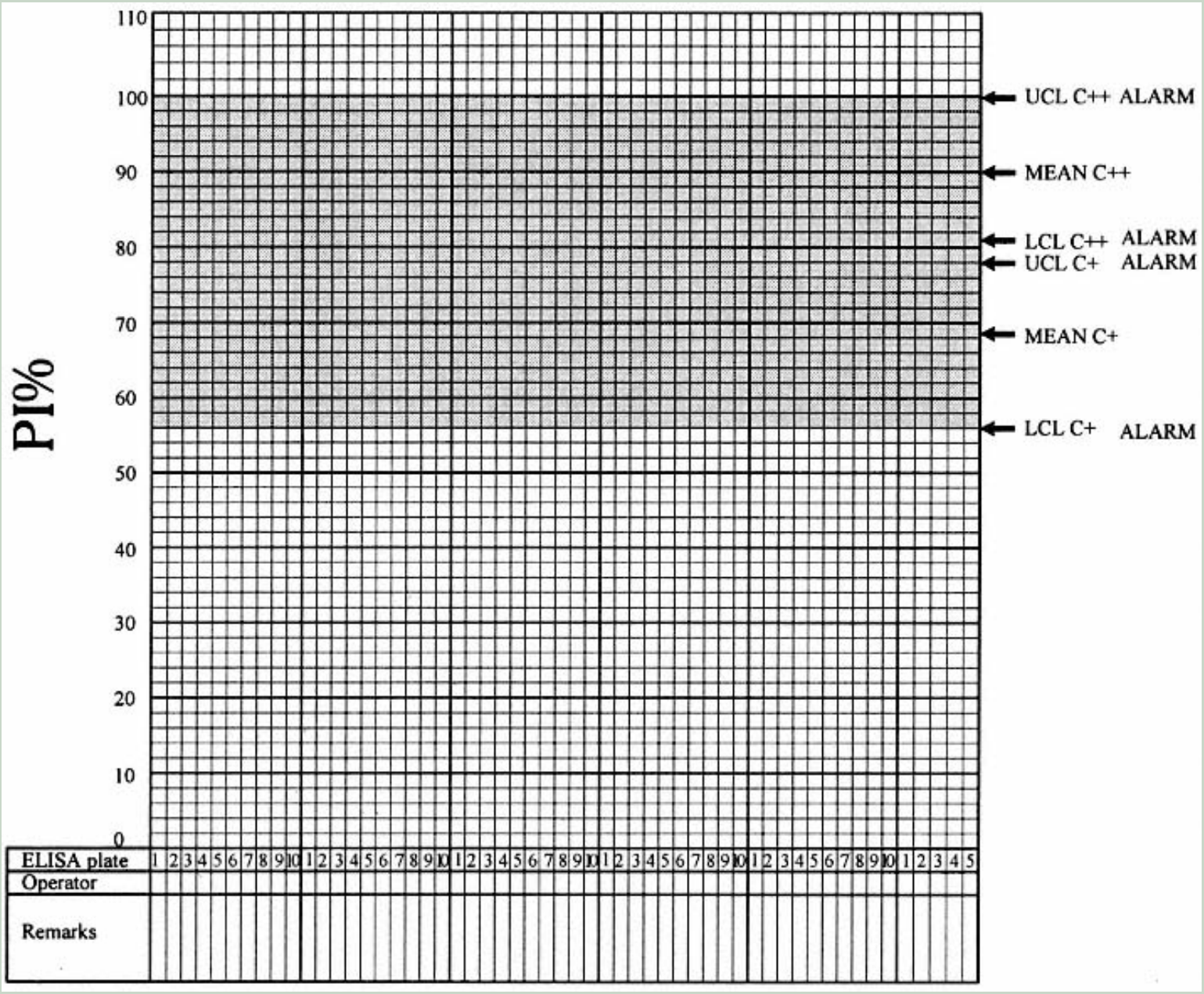


Fig. 10.
Illusrative chart for DDD plots of PI% C++ and C+ data.

Figure 7 shows data in the IQC Table 2. PI% results for two assay runs are shown. The two test days involved a single plate and four plates, respectively. The data required for SDC plots are shown in the thick lined boxes (overall mean and *SD* of results for all the plates run on that day). The data for the DDD charts (individual plate data) are also shown.

3_i^a
Controlling the Assay Using Graphs:
Plotting the Data

We make use of the fact that the same control sera are set up on each plate. We can examine differences in results among plates to keep a check on the test performance. Two kinds of data are available, as already indicated (1) actual OD readings, and (2) PI% values calculated by the software and used to assess samples.

Remember that a convenient method of viewing data is to plot values on a chart. This can be examined easily and can give a view of the data over a time period. Two kinds of charts are recommended for quality control.

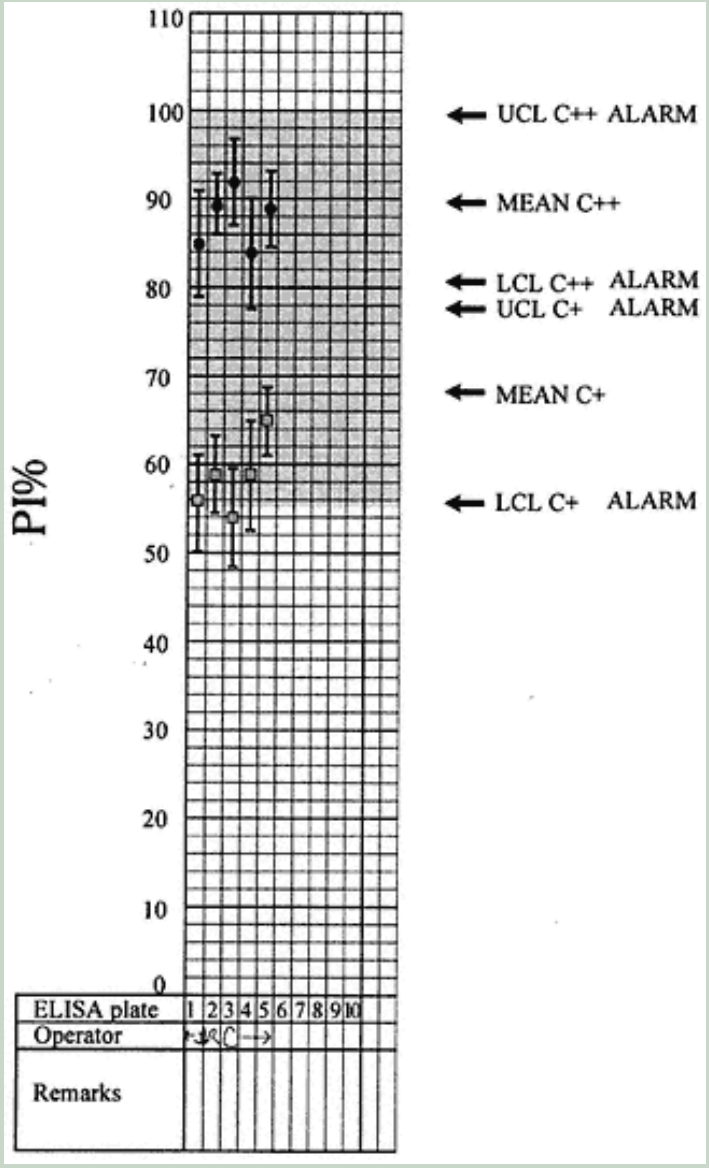


Fig. 11.
Illustrative enlargement of chart for
DDD plots of C++ and C+ PI% data.

1. A chart that keeps the actual data for various test parameters for each plate used. This is called a DDD.

2. A chart that summarizes data for any particular tests done on a given day. This is called an SDC.

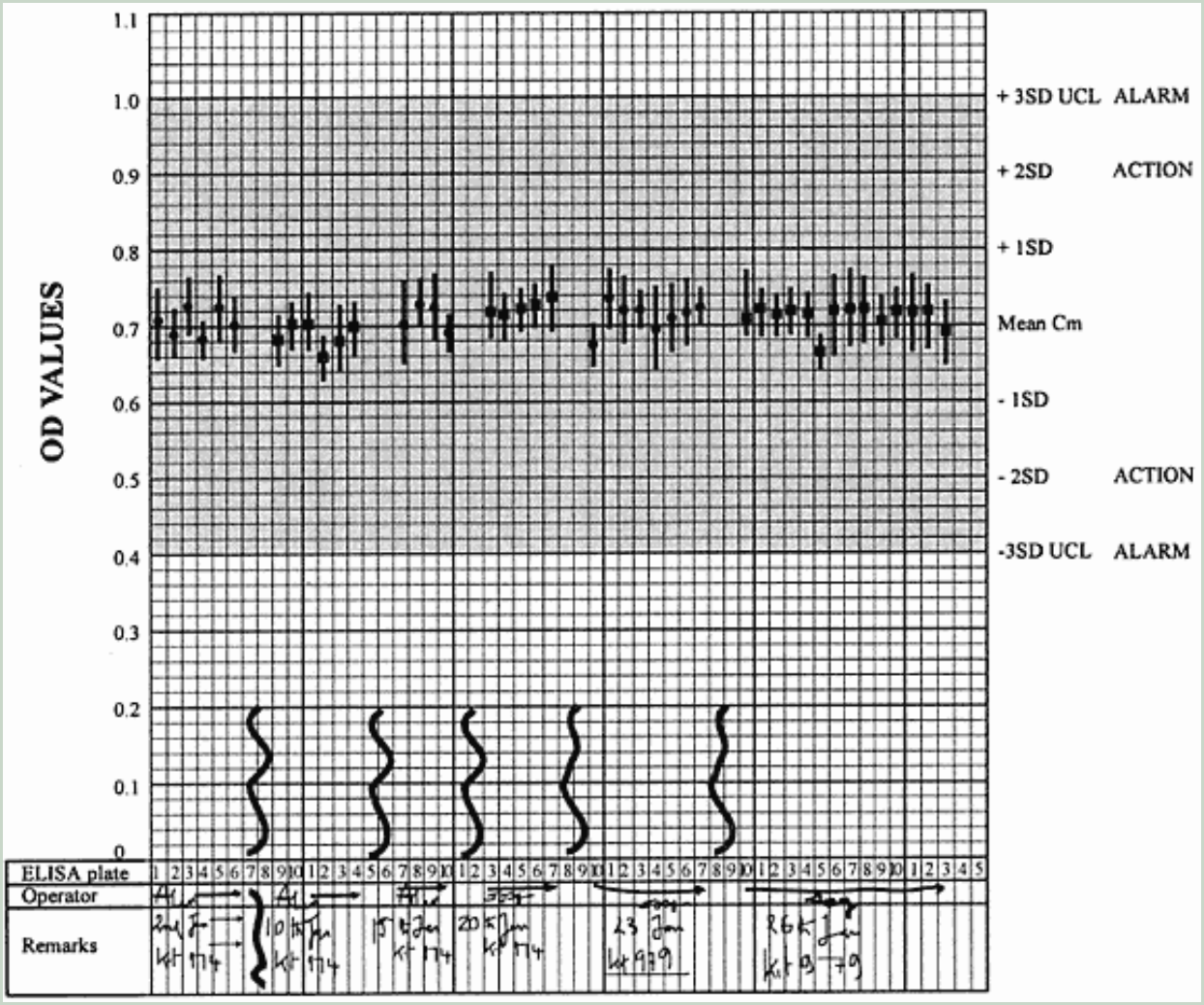


Fig. 12.
Illustrative DDD chart for longer-term plots of several tests of Cm OD data.

3.1¡ª
Daily Detailed Data Charts

DDDs plot the OD data on the control Cm samples for each plate and the %PI values for other controls. Three charts are needed to cover all controls:

1. Cm: OD mean and *SD*.

2. C++ and C+: PI% mean and *SD*.

3. C¨C and Cc: PI% mean and *SD*.

3.1.1¡ª
Examples

Table 3 shows data from a test. Let us take the data of a plate read out as shown in **Table 3**:

Cm data	0.685	0.673	0.636	0.641

Calculate the Cm mean and *SD* of this data: mean = 0.659; *SD* = 0.024. Calculate the mean and *SD* for PI values given for C++, C+, C¨C, and Cc (*see* **Table 4**).

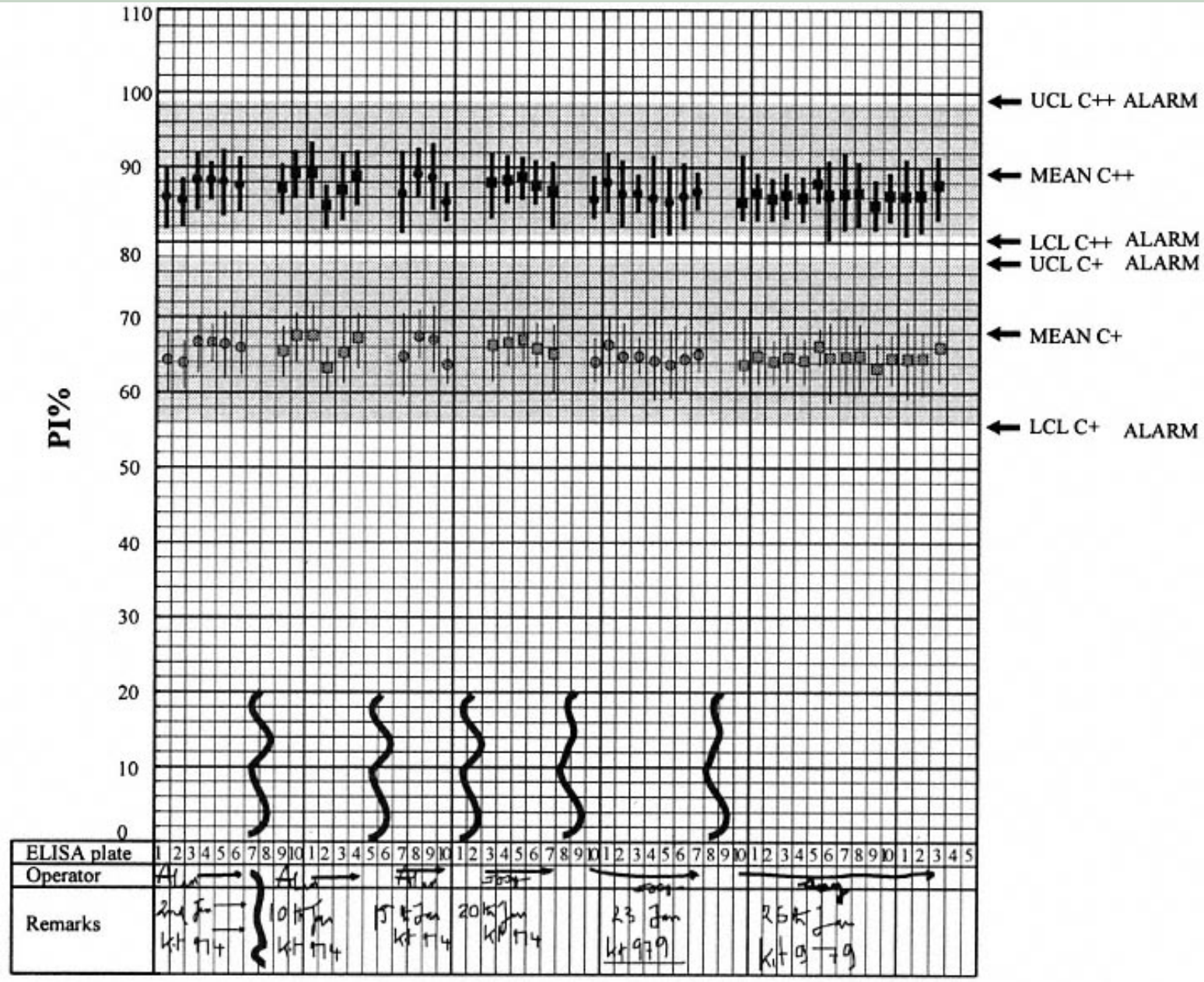


Fig. 13.
Illustrative DDD chart for plots of C++ and C+ PI% data.

Multiply the *SD* by 2 and make a complete table as shown in IQC Tables 1 and 2 (*see* **Table 5**). The calculation of *SD* gives information about the variation observed in the data and is important in assessing confidence about the calculated mean.

3.2¡ª
Plotting on DDD Charts

Note that we may have run more than one plate on any given day. The DDD charts examine data from all plates run so that reference to each plate can be made. Let us assume that we have run five plates on one day. The data in **Table 6** are an example of what might be expected from the five plates. The parentheses show the value of 2 ¡Á the *SD* for each point. Thus, for each plate the actual Cm values are noted and the PI values for each of the controls along with the SDs, are calculated. Now the data can be plotted.

3.3¡ª
DDD Chart Design

Figure 8 shows designs for plotting Cm OD. **Figure 9** shows an enlargement of an area with data plotted. **Figure 10** shows a design for a DDD control

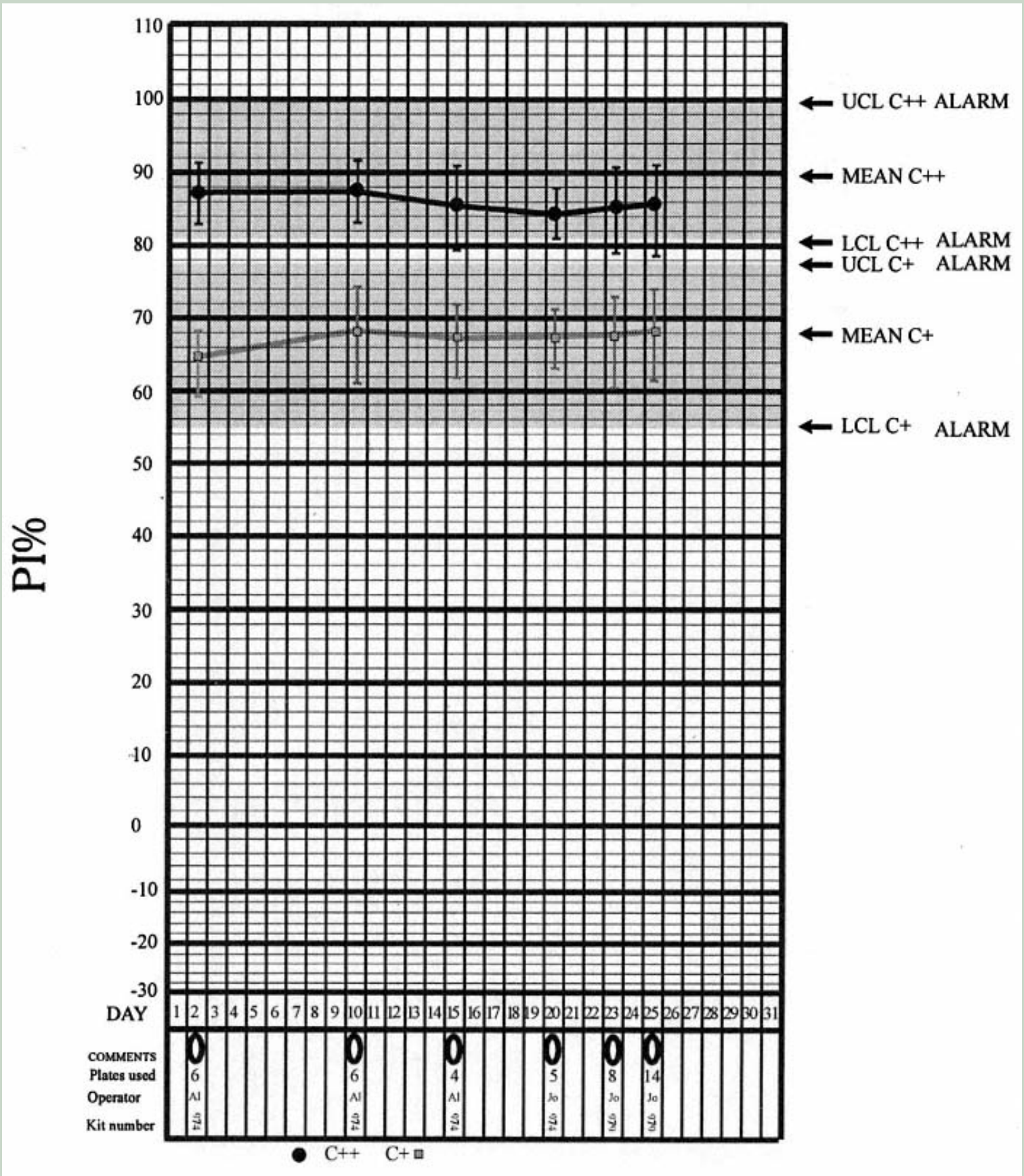
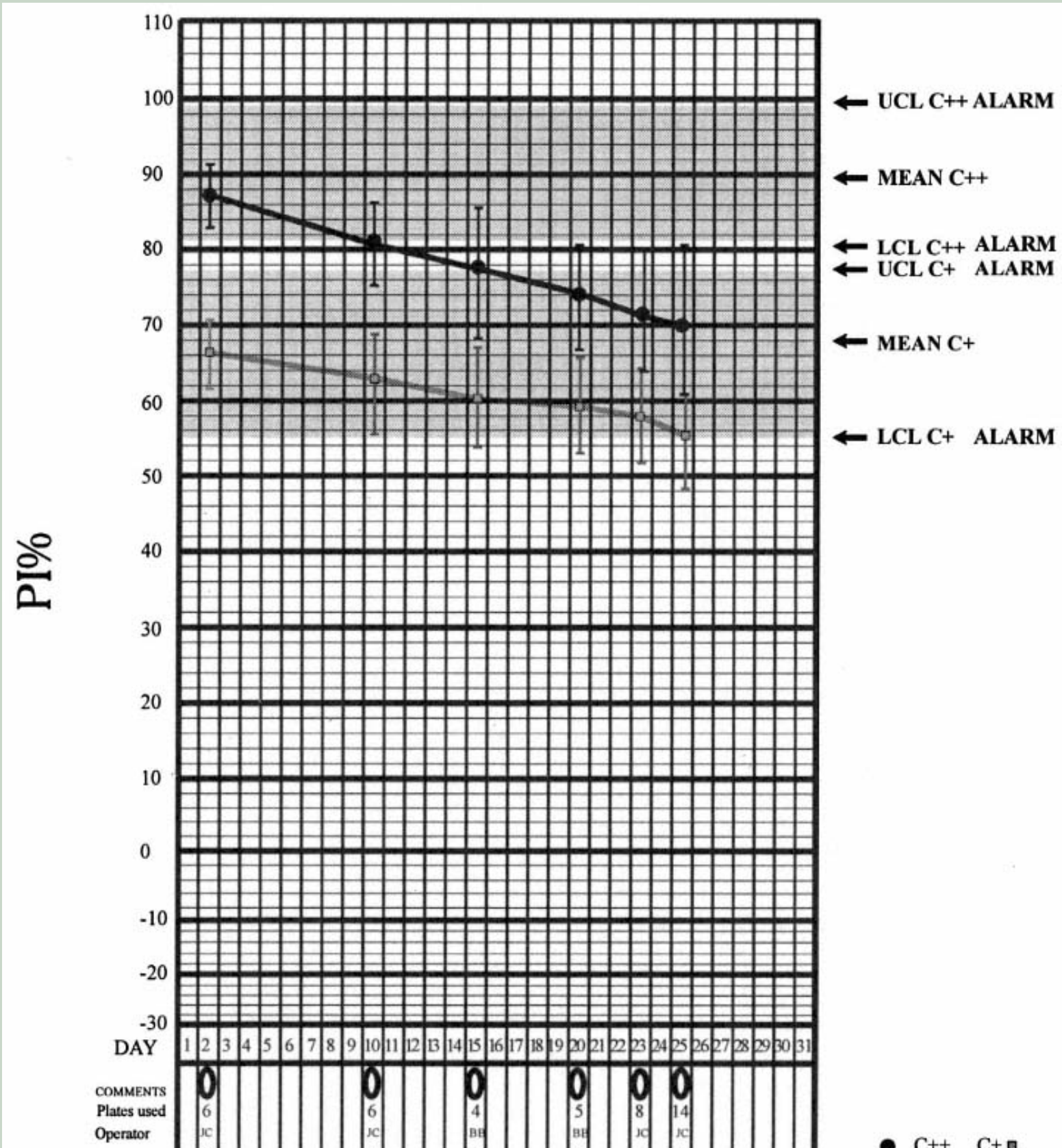


Fig. 14.
Illustrative SDC chart for plots of C++ and C+ PI% data.

chart for PI% values. **Figure 11** illustrates an enlarged plot of data. Note that (1) the charts should be updated every time a test is made by each operator, and (2) the charts should be displayed preferably near where tests are made.

In **Fig. 8**, the *y*-axis shows OD units. The *x*-axis contains columns, each one representing the data from a single plate. This chart is for plotting OD data from the Cm control. The expected mean of the Cm (given in the kit manual) and the allowed variation from the mean values are shown in the gray areas. This represents 1, 2, and 3 *SD* from the mean. The test values obtained on a



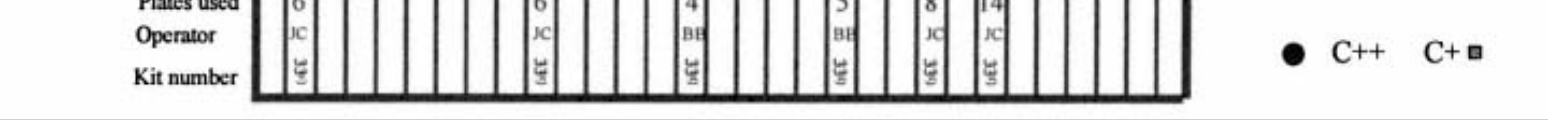


Fig. 15.
Illustrative SDC chart of C++ and C+ PI% data showing downward trend.

plate should be within the ¡À2 ¡Á *SD*, preferably as close to the indicated mean as possible. The ACTION notice at ¡À3 ¡Á *SD* from expected mean indicates that results are at too great a variance from the expected mean and that the test parameters should be examined. UCL and LCL are ALARM points representing the mean ¡À3 ¡Á *SD* values.

Figure 9 shows an enlarged graph of the DDD chart with Cm data plotted. The data from the five plates examined above are plotted as mean and ¡À2 ¡Á *SD* from the mean Cm OD values. Note that the operator is indicated in the lower

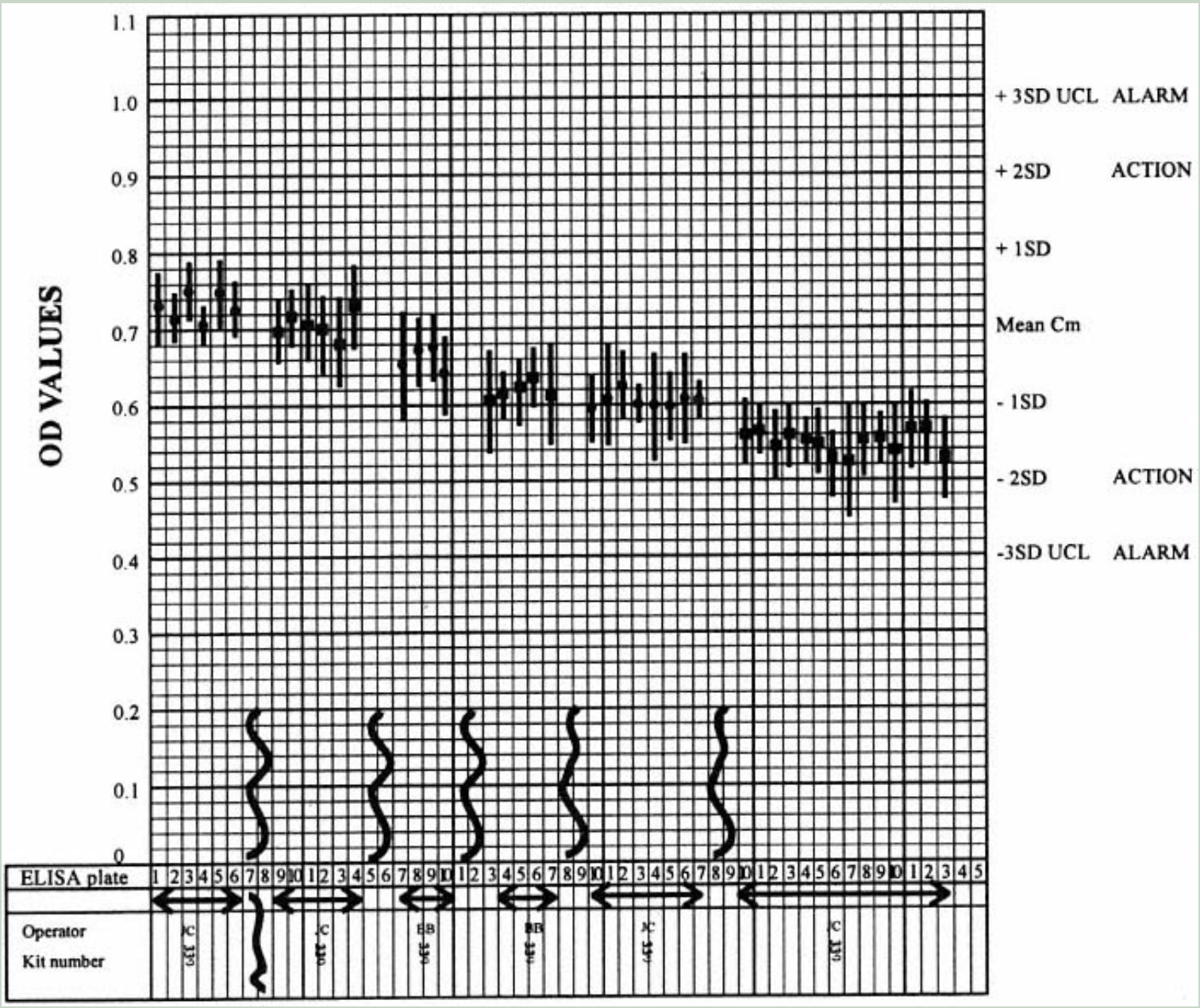


Fig. 16.
Illustrative DDD chart for Cm OD values showing downward trend.

left-hand corner. This is important when tracing any problems (identifying specific problems to particular operators). Note also that this space should be used to indicate the date (to identify when the test was made). Similar considerations are shown in **Figs. 10** and **11**, in which the PI% values are shown. The limit values are also shown.

4¡ª
Summary Data Charts (SDC)

The DDD charts are plotted as an accurate reference of the test performance on each plate. The assessment of the whole test performed on a day-to-day, week-to-week, month-to-month, and year-to-year basis is examined by plotting data on SDC charts.

The SDC charts differ fundamentally from the DDD charts in that the data plotted represent the mean and variation from all plates used in an assay treated as a group. Thus, if only 1 plate is used on a particular day, then the data are used to plot the SDC chart points for that day. If 5 plates are used, then these will be used to obtain the SDC data for that day. If 35 plates are used, they will

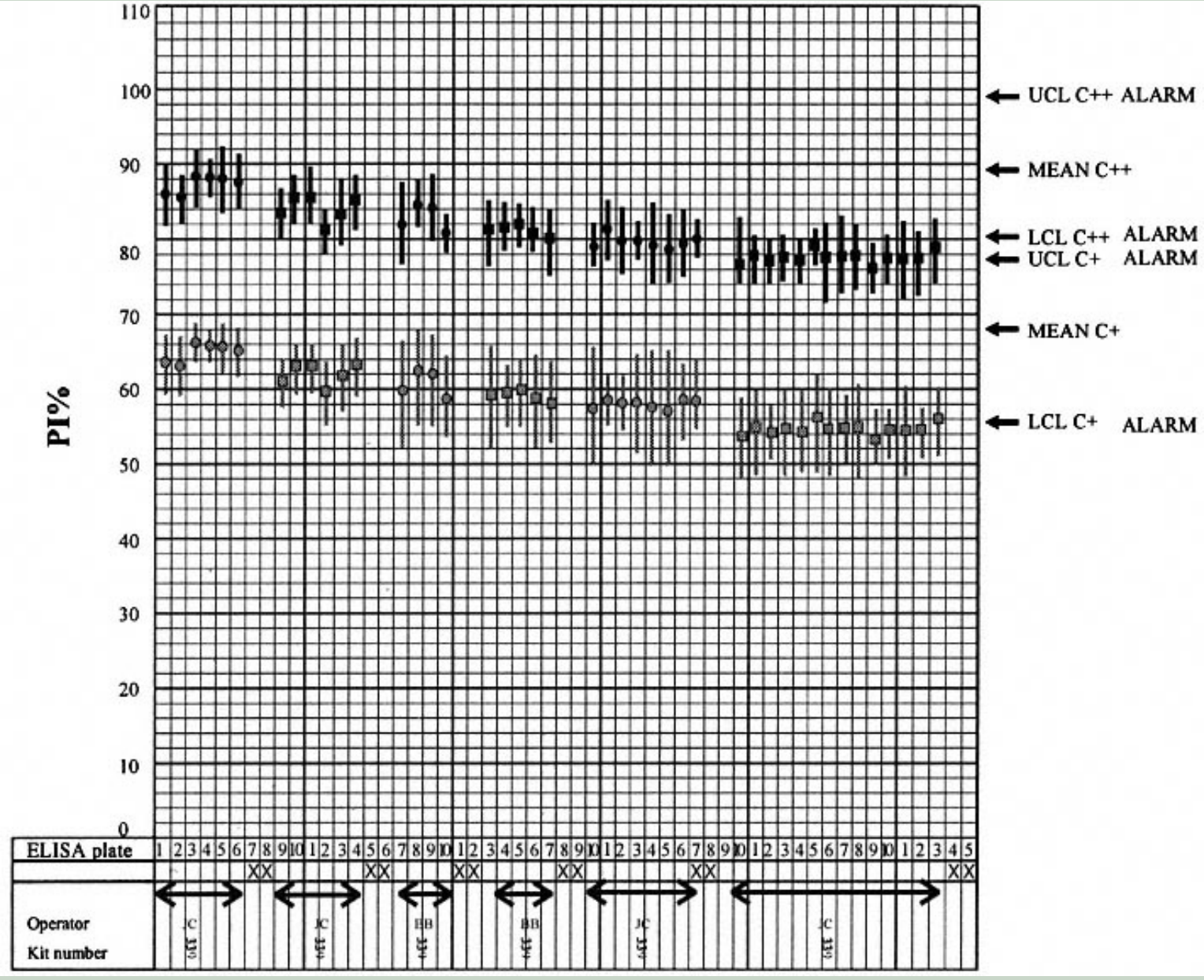


Fig. 17.
Illustrative DDD chart for C++ and C+ PI% data showing downward trend.

collectively give the SDC chart data for that day. If two series of tests are made on the same day, representing 5 and 6 plates, respectively, then the data from all 11 plates are processed to give the SDC data.

4.1¡ª
Data Processing for SDC Charts

Printouts from software packages or by-hand calculations give the PI% values for the various control sera used for each plate. Thus, we have PI% values for Cm, C++, C+, C¨C, and Cc. The SDC data are obtained by taking all the given mean values for any of the control sera and calculating the mean and *SD* of that data. The Cm OD data are also plotted on an SDC. The mean of all the Cm values for a set of plates is calculated and the *SD* from this mean is plotted.

4.1.1¡[a]
Example

We may have made a test on five plates. The mean PI% values on each plate are as given in **Table 7**. Calculate the mean and *SD* for each column (*see* **Table 8**).

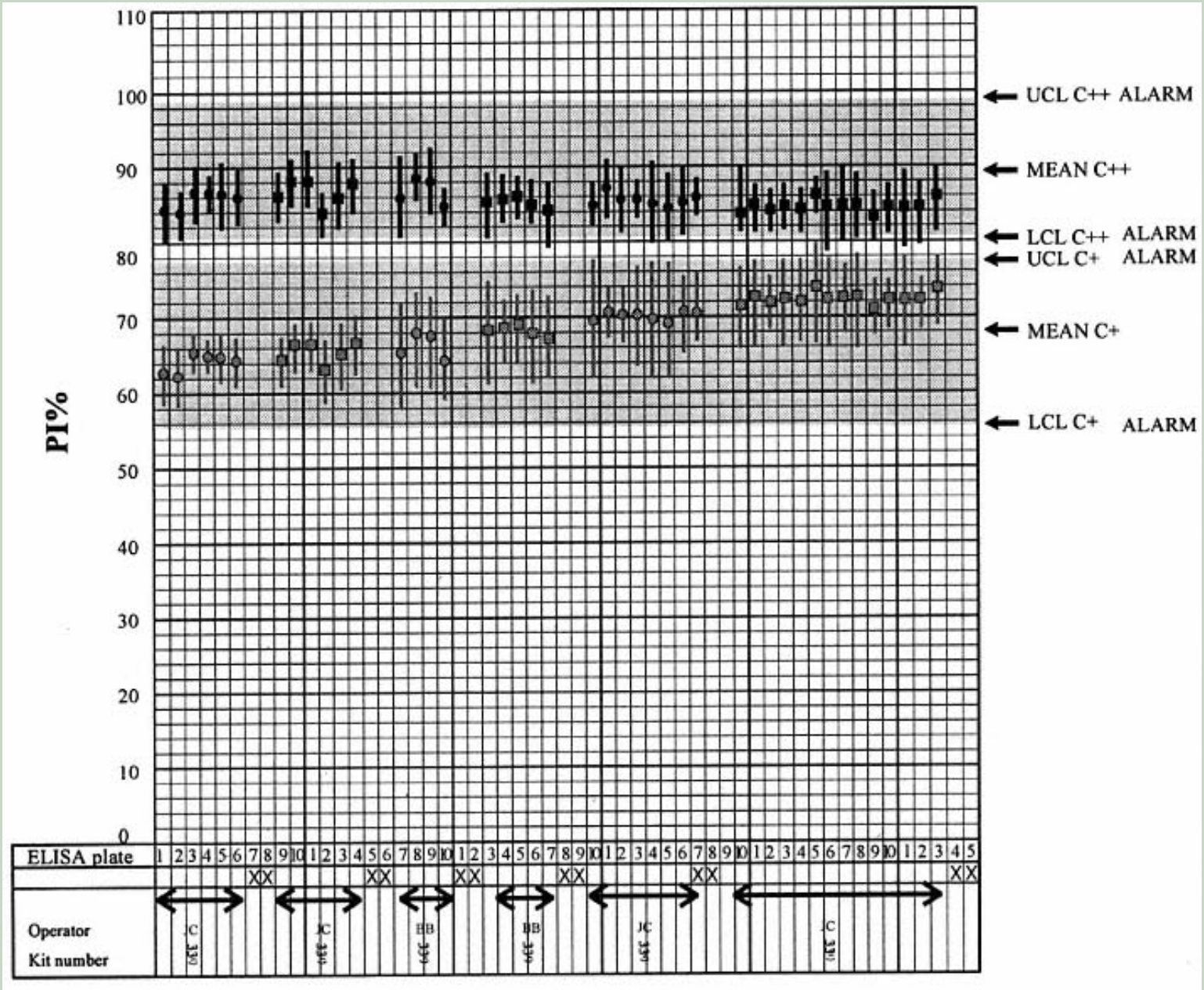


Fig. 18.
Illustrative DDD chart for C++ and C+ PI% data showing upward trand for C+ and constant acceptable limits for C++.

4.2¡[a]
Plotting Data

The values shown in **Table 8** are plotted on the SDC charts. As with the DDD, the plotting is split into different charts to cover the controls. The main idea here is to plot the summary data in real time.

The charts each have 31 d, so that the actual day in the month when the test is performed can be plotted:

1. Plot Cm data on one chart as summary OD values.

2. Plot C++ and C+ data on one chart as summary PI% values.

3. Plot Cc and C¨C data on one chart as summary PI% values.

The data are plotted on a calendar chart that measures the exact date when tests are performed. These data are first plotted on the example charts as shown. Each chart can plot data from d 1 of any particular month.

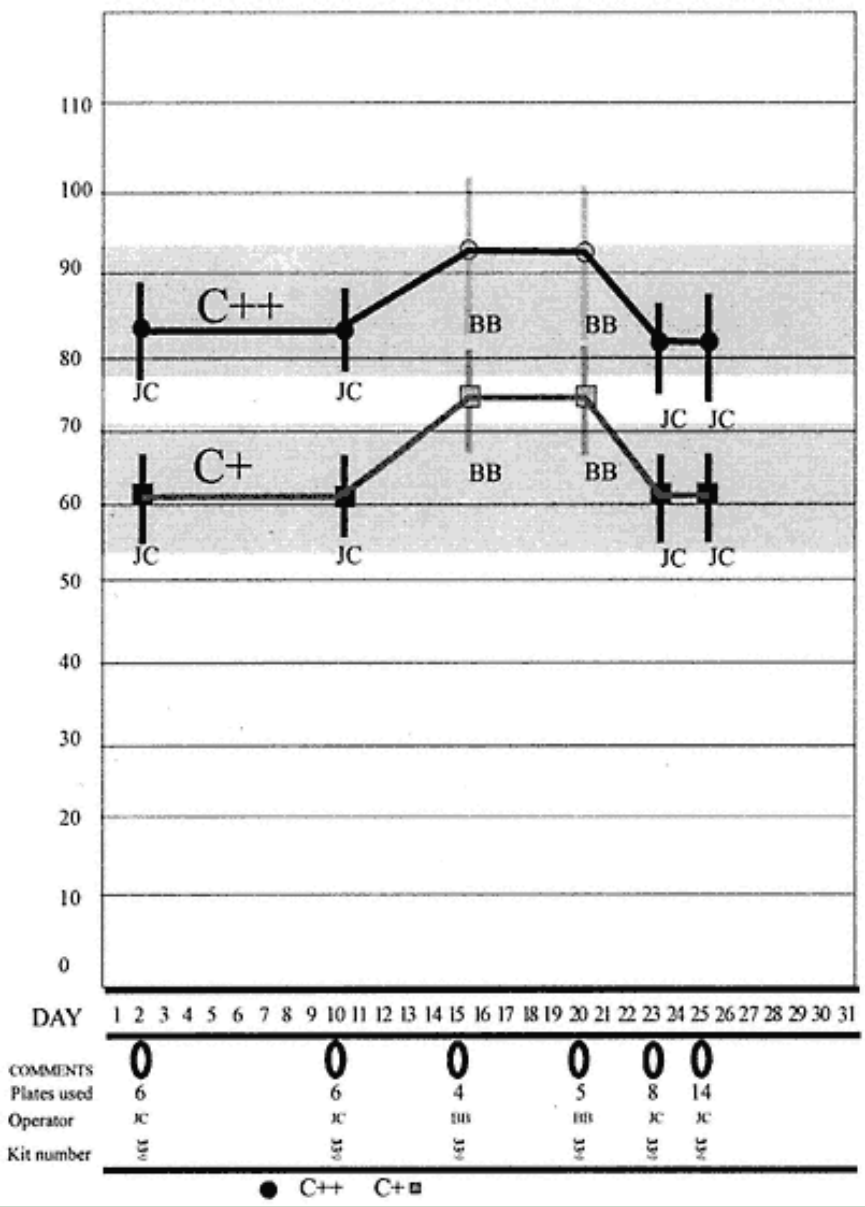


Fig. 19.
Illustrative SDC chart for C++ and C+ PI% data showing effect of different operators.

5_i^a
Interpretation of Charts

The purpose of charting the data is to help monitor the performance of the kit in its use to measure antibodies against rinderpest. The points of assessment are always with reference to the performance of the control sera and reagents supplied.

The data on these reagents have been obtained after multiple testing so that expected values and variation from these values have been recorded and calcu-

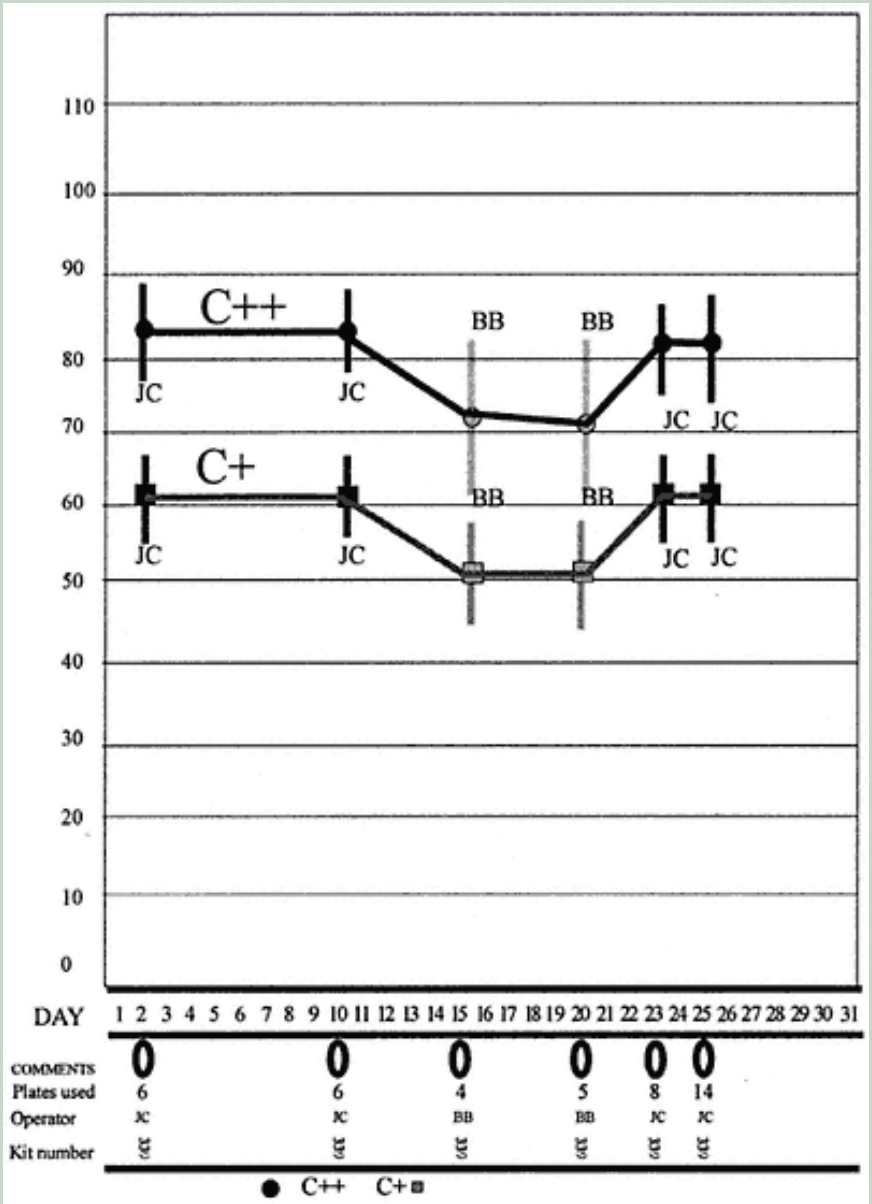


Fig. 20.
Illustrative SDC chart for C++ and C+ PI% data showing effect of different operators.

lated. The key assumption is that these are constants in the assay, i.e., that all the reagents and controls will remain the same physically throughout the time that they are used in the tests. Note that because of this assumption we have to be very careful not to handle the control samples poorly since they set the control limits of the assay.

The control serum samples represent sera containing an excess of antirinderpest antibodies (C++) and a relatively weak serum (C+) that should not give maximal inhibition but always be above the given cutoff value. We also have other "constants" that should be the same on each plate, i.e., the mAb (Cm), the C¨C, and the Cc.

Table 3
Control Samples Data After Processing

	OD_1	OD_2	OD_3	OD_4	$PI\%_1$	$PI\%_2$	$PI\%_3$	$PI\%_4$
C++	0.077	0.063	0.072	0.071	88	90	89	89
C+	0.278	0.296	0.313	0.280	58	55	52	57
C¨C	0.668	0.667	¡ª	¡ª	¨C2	¨C2	¡ª	¡ª
Cc	0.011	0.009	¡ª	¡ª	98	99	¡ª	¡ª
Cm	0.685	0.673	0.636	0.641	¨C4	¨C2	¨C9	¨C8

Table 4
Mean and *SD* for PI Values of Control Data

	Mean	*SD*
C++	89.0	2.7
C+	55.5	2.7
C¨C	98.5	0.7
Cc	¨C1	0

Table 5
Completed Calculation of Mean and 2 ¡Á *SD* of Control Data

	Mean	*SD*	2 ¡Á *SD*
C++	89.0	2.7	5.4
C+	55.5	2.7	5.4
C-	98.5	0.7	1.4
Cc	¨C1	0	0

5.1¡ª Variation

There will be variation in the results obtained in the tests. The variation will be from plate to plate on the same day, from day to day, from operator to operator. The measurement of the variation is what the charts help the operator investigate. We can (and have to) accept a degree of variation. The acceptance limits are those given in the manual. Thus, for the Cm:

1. A target mean OD reading is given.

2. Upper and lower limits for OD are given.

3. A target PI% value is given.

4. Upper and lower control limits for PI% are given.

Table 6
Example Data from Five Plates with Required Parameters Calculated for Inclusion in DDD Charts

Plate	Cm (OD)	C++	C+	C¨C	Cc
1	0.59 (.07)	85 (6.0)	56 (5.0)	97 (1.8)	1 (0.2)
2	0.64 (.15)	89 (4.0)	59 (4.5)	94 (2.2)	2 (0.2)
3	0.68 (.09)	92 (5.2)	54 (5.5)	91 (3.2)	4 (0.1)
4	0.71 (.13)	84 (6.3)	59 (6.3)	89 (4.5)	5 (0.3)
5	0.61 (.09)	89 (4.5)	65 (3.9)	93 (6.8)	3 (0.4)

Table 7
Control Data from Five Plates

	Cm	C++	C+	C¨C	Cc
Plate 1	¨C2	88	64	6	99
Plate 2	4	84	62	¨C3	91
Plate 3	3	87	59	¨C2	94
Plate 4	¨C1	89	63	4	93
Plate 5	¨C4	84	57	4	87

Table 8
Calculations of Mean and *SD* from Data in Table 7

	Mean	*SD*	2 ¡Á *SD*
Cm	0	3.4	6.8
C++	86.4	2.3	4.6
C+	61.0	2.9	5.8
C¨C	1.3	4.4	8.8
Cc	92.8	4.4	8.8

For the C++, C+, C¨C, and Cc the mean and limits are expressed only in terms of the PI% figures.

Note that the first task when receiving a kit is to run the controls under the conditions described in the manual and to see what results are obtained. These should give mean values within the described limits in the manual.

Once this is established, the routine monitoring of the test on a daily, weekly, monthly, and yearly basis is vital to answer the question, Is the test the same each time? The charts merely plot the data that are generated each time a test is made. They are a visual representation of the data collected and concisely pre-

sented. The charts should be updated immediately. The charts should be in full view so that all individuals involved in the test can see them.

5.2¡ª
What can we see from the charts?

The main constant monitoring device is the SDC chart data. This gives a time-bound view of the test. Points can be drawn connecting data from one test day to another. This can give early warning of a trend in the test indicating that something is wrong. This is illustrated under **Subheading 6.**, where worked examples of different scenarios are examined.

The use of the DDD charts is important in that every single plate data is logged. A particular day's testing can be isolated for close examination from the SDC charts. The DDD charts can then be closely examined and reasons for excessive variation in the results examined. This is also examined under **Subheading 6**.

6¡ª
Worked Examples on the Uses of DDD and SDC Charts

Several examples of what might happen in laboratories in time are given:

1. Good tests: no real problems.

2. Some worries: us test drifting?

3. Operator problems: ups and downs.

6.1¡ª
Good Tests:
No Real Problems

Figure 12 shows a DDD chart with plots of the OD means and ¡À2 *SD* of the Cm for plates run by two different workers (Alan and Josy), on different days, with different kits (974 and 979).

6.1.1¡ª
Observations

1. The *SD* bars are a similar length for all the plates and that the different operators have obtained similar mean values.

2. There are no large differences in *SD* or means for the kits.

3. The operators have identified themselves.

4. The operators have left a gap of 2 between different test days.

5. The operators have written in test dates.

6. The operators have denoted which kit was used. No further comments were worth noting.

The conclusion here is that the means of the OD values were near those specified in the manual and that there were no real differences between the operators' results. The same test's data was plotted as PI values on DDD charts. There were no large differences in *SD* or means for the kit controls. This would

indicate that samples analyzed on the individual plates would give good results within the expected limits.

Figure 13 shows an example of a DDD with data plots showing PI% means and ¡À2 *SDs* of the mean PI% of plates run by the two different operators, on different days, with different kits. The PI% values are plotted for each plate used. The operators are identified as well as the test kits. Gaps have been left between plates used in testing on different days. The key is to be able to identify individual plate data. Note that the variation is similar from plate to plate irrespective of the kit used or the operator.

The workers have plotted SDC charts for the different days of testing (**Fig. 14**). Remember that they took the respective mean values required from all the plate data from tests performed on the same day. The results show that there is a clear difference between the C++ and C+ data. The means are in the expected range. There are no differences between operators or kits used (they had no effect on performance of tests). Data points have been connected to show any trend in data (going up or down).

6.2¡ª
Some Worries:
Test Drifting

Figure 15 shows an SDC chart for a laboratory for the C++ and C+ controls. This is an example of an SDC chart with data plots showing PI% means and ¡À2 *SDs* of the mean PI% of C++ and C+ on six different days. The same kit is used throughout. Note that there seems to be a difference from the start of the month to the end. There was a gradual drift in the expected values from the first test in which the results were as expected to levels that are signaled as ALARM. The operators in this case should investigate the reasons that there is drift with reference to DDD chart data and examine operator differences and kit batch elements.

6.2.1¡ª
Examination of DDD Charts to Help Identify Problems

Reference to the DDD charts may highlight some reasons for the increased variation or drifting of an assay. Thus, the specific tests performed on the various days and plotted as overall SDC data can be examined in the light of individual plate data for any specific day's test.

Factors such as changes in conditions, operators, and kit batches also have to be examined, so that notes made on the charts highlighting these are important in this exercise. Note that the same kit has been used in the example, but with different operators.

The DDD chart in **Fig. 16** shows the actual OD data for the Cm for the plates shown in **Fig. 15**. The chart illustrates a situation in which there was an observed reduction in the mean of the PI values for the C++ and C+ in consecutive tests in SDC charts (**Fig. 15**). Thus, the reductions in OD observed in

the raw data for Cm (**Fig. 15**) are matched with alterations in the control PI values. This indicates that the performance of the test is changing over time; that is, the values are reducing with each test performed. Something has to be done to restore the recommended parameters with reference to the controls supplied. Again, the same kit was used for all tests so that differences cannot be ascribed to the change in reagents. The two operators had little effect on the reduction in expected PI values.

Figure 17 shows a DDD chart of C++ and C+ values of plates illustrating a situation in which there is a gradual reduction in values over time (using the same kit). This shows actual individual plate data for C++ and C+ PI values. There is no great difference in the variation seen for any particular plate for either value (C++ or C+), but there is a gradual alteration in the mean from test to test. The observed reductions in actual OD for Cm are matched with reductions in expected PI values. The conclusion here would be that the test results obtained for the particular kit being used are not good. This is irrespective of operator factors; both obtain similar variation. There seems to be a gradual reduction in expected OD and PI values over time. Both the C++ and C+ controls are now at ALARM levels even though the Cm OD values are just within allowable limits.

Reference to the DDD charts may also give a different picture in the context of having OD values observed for the Cm that are reducing.

The chart in **Fig. 18** illustrates a situation in which the reduction in actual Cm OD is matched by an increase in PI values, particularly for the C+. The variation in the tests is similar; that is, each test gives a mean with a similar variation. However, there is a continuous increase with time in the mean for the C+. This clearly indicates that as the test OD value goes down, the apparent sensitivity (ability to detect antibodies) goes up.

6.3¡ª
Operator Problems:
Ups and Downs

Examination of the DDD and SDC charts can highlight operator problems. We can make the assumption that the kits generally remain stable. The most dominant factor in causing variation in test results is the operator. Whether this variation is acceptable or not can be decided after results are examined.

Figure 19 is an illustration of an SDC chart (shown diagrammatically, since we should now be quite familiar with the concepts from the previous more detailed illustrations). The data show PI values for C++ and C+ and indicate that there are different results according to the operator, because the same kit is being used. The reasons can be determined by examination of all the control values plotted as DDD charts. Note also that operator BB has a higher variation in results as judged by the larger *SD* bars. In fact, operator BB has C++ and C+ values that are quite similar to each other, whereas there is a clear

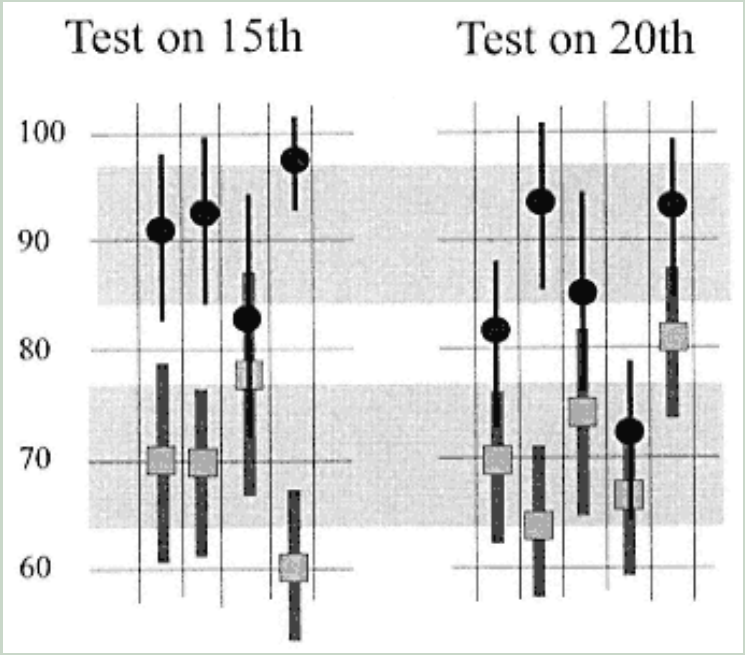


Fig. 21.
Enlarged portion of DDD plots of PI% C++ and C+.

difference in the operator JC's figures. Operator BB has higher mean and variation values using the same kit than operator JC. Operator BB's values are unacceptable. Attention to the plate data on DDD charts will determine whether variation is general for any test or whether there is high variation in one or two plates tested. This is shown in **Fig. 20**.

Care should be taken in assessing SDC charts without reference to DDD charts. Remember, the SDC charts record all the results from a given test. They record only the sum of all the mean data and the variation inherent in that data. They do not consider the variation in each plate, so we could have a situation in which we have the correct mean (in limits) for SDC charts, but this had been arrived at through tests having very high and compensating very low control data means. The variation bars in the SDC charts will indicate whether this is inherent, so attention to observed variation of the means is required, followed by specific examination of DDD charts.

Figure 21 shows DDD data for operator BB on tests performed on the 15th and 20th. Actual plate data of the mean and variation for each plate are shown. The gray areas denote limits for C++ and C+ controls. On the 15th, there were two plates out of limits for C+, one above and one below, although the actual mean of all the means for C+ (as plotted on the SDC chart) was about 70%. Thus, this operator had two plates that should be rejected. Similarly, on the

same date two of the C++ controls were outside limits. On the 20th, we see a similar situation with two plates giving control C+ outside limits and three giving C++ outside limits. The overall conclusion should be that there is unacceptable variation in the tests of operator BB and that several of the plates used in the tests should have been rejected (not included in the testing). The plotting of all data in DDD charts immediately highlights this situation.

The chart in **Fig. 21** shows that the reverse trend for an operator can be seen, in which there is a reduction in expected PI values in comparison with other workers. The tests performed by one operator are not acceptable and since the test was performed as expected by another operator, it must be a factor of the failing operator that causes the problems with the assay.

7¡ª Further Aid to Interpreting Charts

This section is intended to help the laboratory worker continuously interpret the charts as they are being plotted, i.e., as the "story" of the data unfolds. The rest of the kit's manual should be read first and the principles thoroughly understood. The intention here is to educate people in the ability to analyze charts to enable decisions to be made concerning day-to-day performance. The analysis infers that testing is being made over a significant time so that comparative results are obtained. This section uses "thumbnail" approaches at representing the different data that might be observed in practice. Analysis should lead to indications as to whether there is a need to take action and what actions are needed.

The advantages of charting data are that it can be viewed as a single entity and that trends and fluctuations can be rapidly observed through examination of SDC data and details at any time point can be expanded through examination of DDD data. This approach has already been explained. This section attempts to simplify likely scenarios in charts and indicate solutions where necessary based on observations.

7.1¡ª Examination of SDC Charts: Individual Points as Plotted

Individual plots summarize all data on a given day and test and relate the results in actual time so that trends or irregularities can be noted on a continuous basis.

1. The plots reflect the mean value of any control and its variation in all the plates examined in a given day's testing.

2. The bar plotted shows the variation.

3. The mean value and the error bars should fall within the given limits for the assay for the various control samples both for actual OD values and for the processed PI% data.

Thus, the SDC plots can alert operators to unacceptable means and errors, causing them to examine closely the individual plate data for that test to exam-

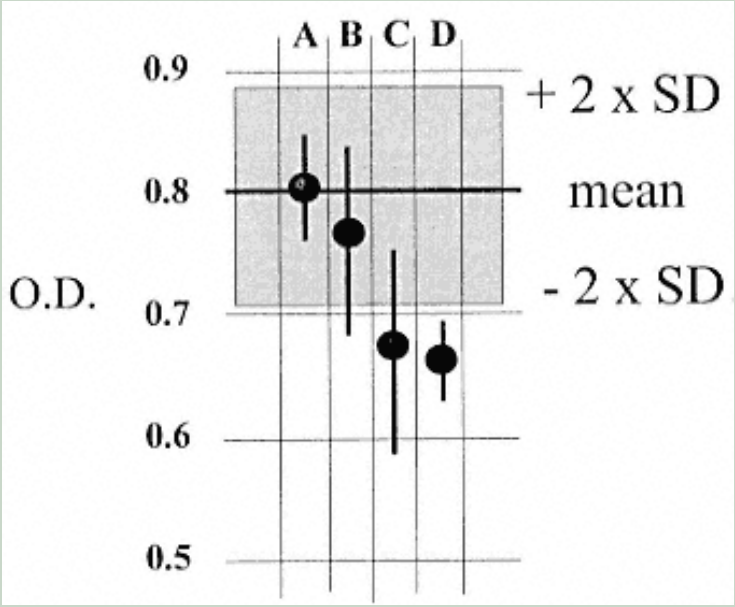


Fig. 22.
Illustration of mean values and error bars in SDC OD plots. Gray box shows the upper and lower limits for values (plus and minus 2 ¡Á *SD)* for the particular control sample examined.

ine what factors produced the variation (which affects the mean value and size of error bars).

Figure 22 illustrates the various situations that could be encountered:

1. Plot A shows the mean to be in limits and the error bar to be short and also within limits. This is ideal with reference to the sample tested.

2. Plot B shows the mean to be in limits but one error tail of the bar to be out of limits. The error is probably higher than acceptable. Reference to individual data for that day may reveal the reason; for example, a single plate control could have been missed or given an out-of-limit result, which both reduces the overall SDC mean plot and increases error.

3. Plot C shows the mean to be out of limits and most of the error bar too. Reference to individual plate data will probably reveal that most if the plates gave mean values that were too low and that readings differ greatly. This is unacceptable.

4. Plot D shows the mean value to be too low and out of limits but that the error is small, indicating little variation in results for all the plates used. This is unacceptable. The data must also be examined with reference to other controls to determine whether the low value was reflected in other data. The reduction, say, of both mean values for SDC of strong and weak positive might indicate a systemic (general error), e.g., too short an incubation time with substrate or a major general dilution error.

7.2¡ª
Examination of SDC Cm, C++, and C+ over Time

The SDC plots cover both the actual OD and the processed PI% values. It is important that both types of plot be examined together. Thus, the charts should be placed in close proximity. This should immediately alert operators to unusual fluctuations from the expected values in both charts as well as deviations in one chart but not in the other. When the Cm OD values are within limits, it is expected that the control serum values for OD and hence PI% will be within limits. When the Cm OD values do start to increase or decrease significantly (approach limits), it is possible that "alterations" in OD value are not mirrored by a significant change in the control PI% plots. In other words, when Cm OD values are showing trends, as shown in **Fig. 23**, the control C++ and C+ PI% charts should be consulted to measure the effect of the OD changes on the PI% values.

As examples, the effect of a reducing OD value for 0% competition (maximum expected color from antigen binding to mAb [mAb control]) may (1) have no effect on the PI% results for the two control antisera, (2) affect both of these only when the OD falls below a certain level, or (3) only affect one control and not the other.

Figure 23 shows trends in time for SDC OD values for, say, the Cm, but would be pertinent to all plots of data for all controls. Basic large-scale trends are shown:

1. Situation A: This can be regarded as an ideal situation in which all means and error bars are within limits. The test values are constant, and it would not be expected that the test is altering in sensitivity.

2. Situation B: The curve is irregular with large swings in mean OD values throughout time. This reflects a good deal of variation probably owing to differences associated with particular operators (differences in variability).

3. Situation C: The curve is irregular but contains areas of similarity of means. This can often be ascribed to changes in operators performing assays and/or notable changes in reagents. This type of curve should be analyzed in terms of identifying whether there are such factors that can be associated with the data.

4. Situation D: There is a fairly constant downward trend (or could be upward) irrespective of operators and probably signifies that the reagents are altering in time. This is particularly indicated when several operators perform assays. The retitration of certain reagents may be necessary to ascertain whether the accepted limits can be achieved. This identification also includes the necessity to obtain fresh reagents.

7.3¡ª
Control C++ and C+ PI% Data

C++ and C+ controls examine the competitive ability of the control sera with respect to the maximum reactivity of the mAb and antigen. They control

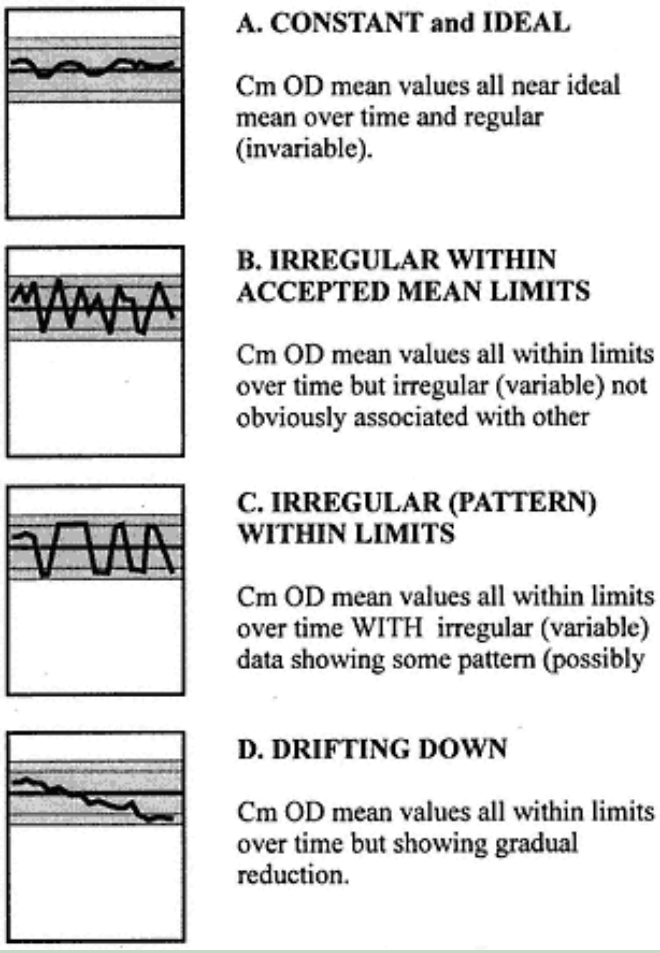


Fig. 23.
(**A¨CD**) SDC plots in time showing different trends. The trends are observed in real time. Action points represent points where the expected means fall outside limits set and where the error bars begin to fall outside the limits at the upper or lower values.

the test and can be used to assess the test's performance in terms of analytical sensitivity. The actual OD data are related to the PI% data through processing with respect to the Cm control data.

Attention to examination of both OD SDC Cm and PI% SDC for the C++ and C+ is essential as already indicated. **Figure 24** summarizes what might be observed with respect to SDC OD data and PI% data in the charts. The arrows reflect the overall trend observed as shown in **Fig. 23**:

1. The horizontal arrow indicates within mean acceptable values (as in situation A in **Fig. 23**).

2. The downward-angled arrow indicates a trend downward in values (situation B in **Fig. 23**).

3. The upward-angled arrow indicates an upward trend in results (not shown).

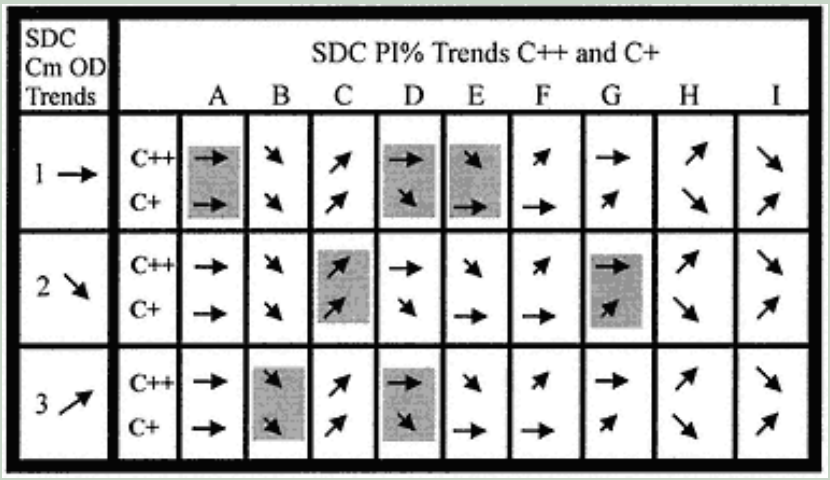


Fig. 24.
Possible relationship of trends in OD SDC Cm and PI% SDC C++ and C+. Boxes 2¨C3 show trends observed in SDC OD data in time for Cm. Boxes A¨CI indicate possibilities in trends of PE% date for each of the controls C++ and C+. Examination of OD and PE% SDC trends together gives a clue as to likely reasons for relationships. The gray boxes show most likely effects on C++ and C+ for respective Cm OD trends.

Irregular data are not shown. In **Fig. 24** the possibilities for SDC OD Cm data are represented by arrows (boxes 1¨C3), as well as all the possible, combinations of trends for the C++ and C+ PI% data. **Figure 24** is meant to illustrate all combinations. The gray boxes represent the most likely combinations for SDC OD and PI% SDC data:

1. Box 1: The Cm OD values are within limits and constant. It is not expected that the C++ and C+ PI% values will be affected, and the expected trends in PI% are those indicated in box 1A. This situation is the one expected and mostly obtained. Box 1D shows a situation in which the C++ PI value is maintained but the C+ loses competing ability. This (increase in expected OD for C+) is a result of the control deteriorating and losing antibodies. The effect is not noted in the C++, probably because there is an excess of antibodies contained in the sample and the loss can be sustained without affecting the competing number of antibody molecules. Box 1E is also observed, but is more unusual, indicating that the C++ has lost antibody activity. This is most likely owing to poor storage of that sample. The other combinations are unlikely.

2. Box 2: The Cm OD values are decreasing, possibly indicating that the antigen or mAb reactivity, or both, is being lost. The reduction in OD will be reflected by a relative increase in competing ability shown by the increase in PI% values. The results of PI% SDC will be most likely reflected as in box 2C, in which both

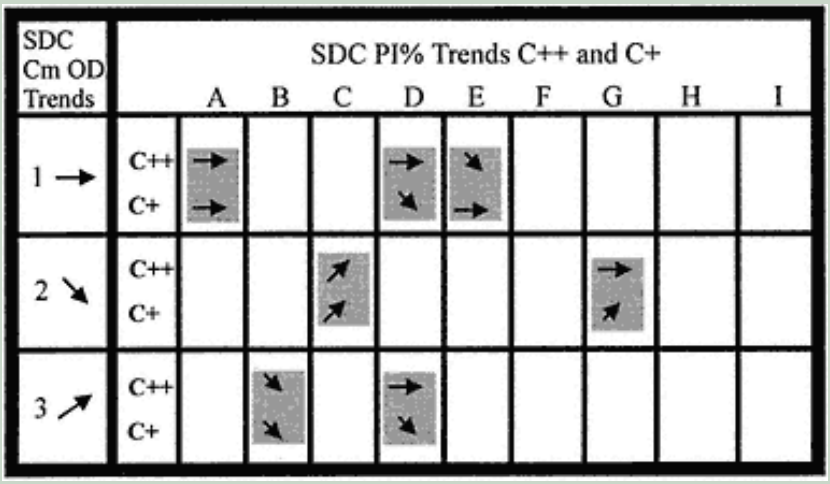


Fig. 25.
Summary of most likely relationship of trends
in OD SDC Cm and PI% SDC C++ and C+.

controls increase in PI%. Although, since the C++ contains an excess of antibodies, a situation as in box 2G is also possible.

3. Box 3: The Cm controls increase in OD value in time. This would be unusual. However, here the increase in reaction could reduce both the C++ and C+ control sera's ability to compete as in box 3B. Again, the excess antibodies in the C++ might maintain the C++ PI% against that of the C+ as in box 2G.

An overall summary of the most likely scenario for the SDC OD and PI% relationship is shown in **Fig. 25**.

7.3.1¡[a]
Irregular Plots

The same criteria for evaluating and comparing the Cm OD data and the C++ and C+ PI% data can be used to evaluate any time points plotted. This will establish any effects of the increase or decrease in Cm OD at any time on the expected PI% values.

When irregular results are obtained with high interest variation, one can note which of the results (at a given time) are affected most. Examination of the operator, methods, actual plate data, and day-to-day variables can be associated with good or bad results. Reference can be made to the individual plate data. Measures to solve the problems could then be used and results obtained after corrections compared.

7.4¡[a]
Sources of Variation

As already stated, the kit is a complex association of reagents and equipment in the hands of different operators. The sources of variation thus come

from these sources. Experience with this kit in many countries indicates that the chief source of errors (manifestation of variation) comes from the operators and not from the reagents. The continuous assessment of the kit is a good way to identify whether it is the reagents and/or equipment that are causing high variation as opposed to the operators. Some of the factors have already been examined, but still need to be emphasized.

7.4.1¡[a] ELISA Readers

7.4.1.1¡[a] Cleaning

ELISA readers are seldom examined on a routine basis. The optical devices for reading the wells can become contaminated with chemicals and should be cleaned regularly. This is a simple process. They should not be cleaned with abrasive materials or those containing solvents affecting plastics.

7.4.1.2¡[a] Filters

Filters can deteriorate rapidly in humid conditions. They should be stored with desiccant in a bag between uses. Machines with internal filter wheels have fewer problems. In cases in which there is a constant machine error indicating a failure to read, then another filter should be obtained. A spare filter should always be available (of appropriate wavelength). A careful check on the appropriate wavelength of filter should be made initially. Some individuals use the wrong filter which, although allowing some OD to be measured (e.g., a 450-nm filter will give a readout for a 492 nm color, the readings will be very low as compared to those using the correct filter.

7.4.1.3¡[a] OD Readings

Individuals should know how the color they see relates to an OD readout. Some people have observed "good" color, which is in the expected range for the kit, but have then received very low OD readings from the machine.

7.4.2¡[a] Pipets

A chief source of error leading to variation is the use of pipets, which can be the chief cause of variation among operators. Even for an individual there is an "internal" bias toward pipetting in a certain way, either always having an amount slightly over or under. This assumes that pipets are calibrated properly (i.e., deliver the volume they are set for), however, this is seldom the case. Greater care to standardize the pipeting technique will eliminate some variation in laboratories, particularly when several people are responsible for testing. This is particularly important in taking the samples from the field container to the wells in which a small volume is transferred. Care must be taken to allow an adequate time to pipet with an identical technique each time.

Table 9
Possible Problems with Reagents

Reagent	Adverse parameters	Error	Effect
Ag for coating plates	High-temperature storage	Low Ag; low color	Change in sensitivity; test failure
H_2O_2	High-temperature storage; stopper left off; used at too high concentration	Reduced or no color	Test failure
mAb	High-temperature; denaturation; dilutions made up and used next day	Reduced color	Changes in sensitivity

Conjugate	High-temperature storage; changes; wrong dilution	Reduced color Increased or decreased color	Change in sensitivity
Blocking buffer	Wrong formulation	Color too high in controls	Test failure
Washing buffer	Wrong pH	Alteration in controls	High variation in controls

7.4.3¡ª
Reagents

The protocols given in the kit's manual should be strictly adhered to. Failure to maintain required minimal temperatures or to alter dilutions can severely harm the assay parameters. Such harm can be assessed with reference to the test performance before and after an identified abuse of reagent. However, such abuses are either not identified or not reported for other reasons. Note should be taken of likely susceptibilities of reagents to various physical conditions (*see* **Table 9**).

7.4.4¡ª
Water Quality

Water quality is consistently the chief source of problems in cases in which results (OD values) are generally much lower than expected. Attempts to obtain water from other sources should be made. The reasons for the alteration in color are not clear, and it is better to obtain water from other sources rather than waste time in curing an internal problem.

7.4.5¡ª
Poor Washing

Poor washing applies to washing tips and glassware and plasticware. It is better not to reuse anything that has had contact with enzyme. When tips are reused, they must be acid washed and then very thoroughly rinsed in distilled water. The pH of the water should be checked because it can often be very

acidic (poor plant-producing water). Poorly rinsed vessels in which original dilutions of reagents are made also adds to variation because activities can be reduced or totally destroyed in a highly alkaline or acidic pH range.

7.4.6¡ª
Mathematics

Errors in diluting reagents are a major problem. Calculations should always be checked and all bottles and so forth clearly labeled with the name of the reagent and dilution. The fundamental principles involved in good performance of ELISA should be learned through in-house training, external training, and reading of the literature. There is no substitute for training, which leads to improvements in practical skills and a thorough understanding of what one is doing.

7.5¡ª
More Problem Solving

Most problems are not really taken care of until it is too late. Operators must examine results constantly. The object of this section is to detail what should be done and when actions should be taken. The very reason for ICQ charts is to examine all results at all times. Actions are dictated by the results. **Figure 26** highlights measures to be taken when certain observations are made.

7.5.1¡ª
Stage 1:
Running the Kit for the First Time

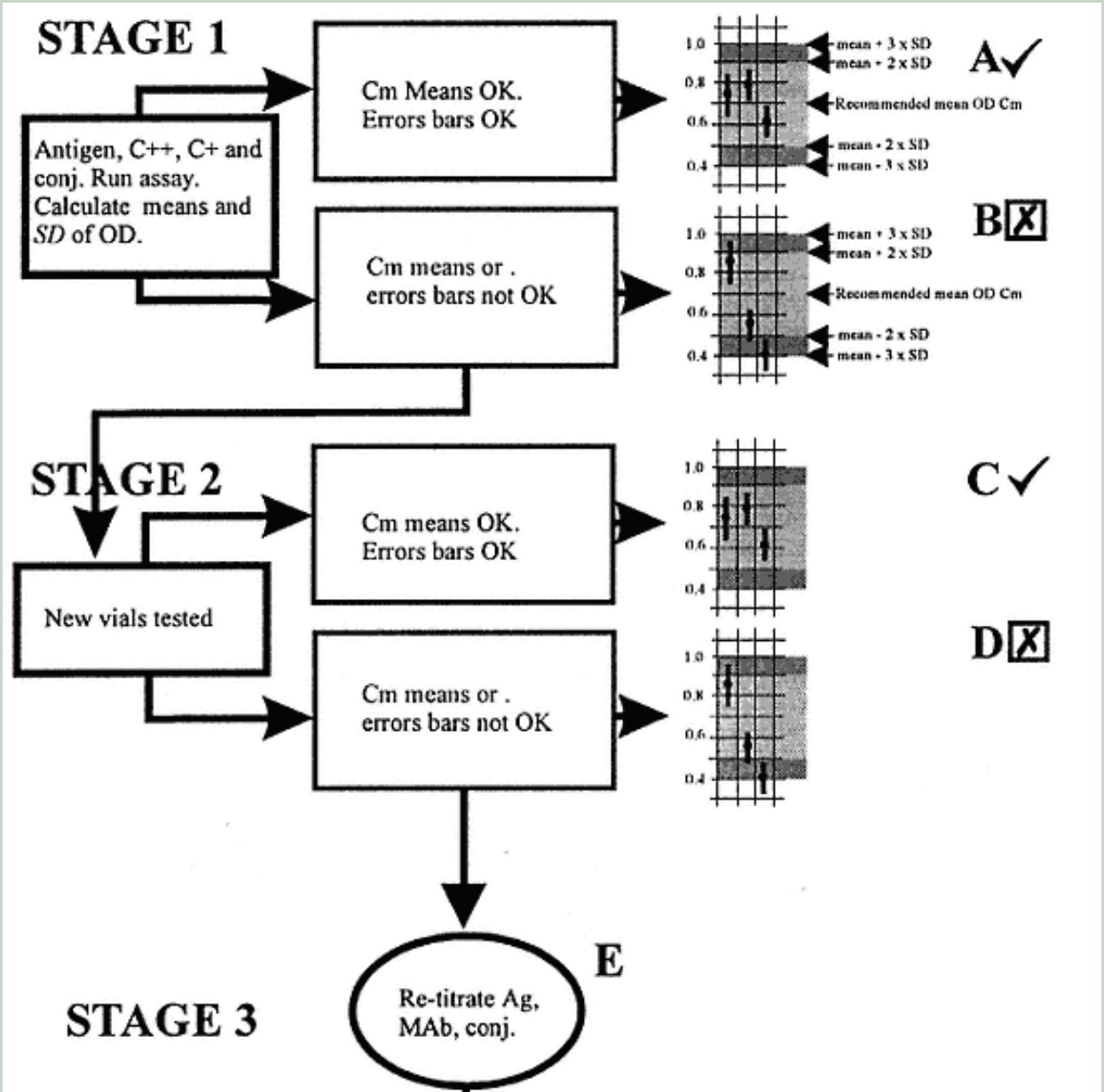
In stage 1, a single vial for conjugate, Ag, and control C++ and C+ as well as mAb is selected. The test should be made exactly as described in the kit's manual. Results should be read and processed. The correct value for Cm OD, and allowable limits, are shown in the manual. The expected mean OD is 0.7, with the variation given at 1, 2, and 3 *SD*s from this mean expected value. The initial running of the reagents should give this mean, or a mean within the 2 ¡Á *SD* range recommended. Be concerned in cases in which the mean is outside the 2 ¡Á *SD* limits set, or when the mean is within the limits but when one end of the error bars plotted is inside the upper or lower mean or ¨C3 ¡Á *SD* limits. Usually the OD readings are low when there is a problem.

7.5.1.1¡ª
Situation A

In situation A, the results are good. The mean is between the 2 ¡Á *SD* limits. The extremes of the error bars are also within the 2 ¡Á *SD* limits. The reagents are performing well and testing can begin. The PI% values for C++ and C+ should be examined to check that they are within prescribed limits.

7.5.1.2¡ª
Situation B

In situation B, the means are wither low or high. The usual situation is that they are too low. Even when the means are within the 2 ¡Á *SD* limits, note that



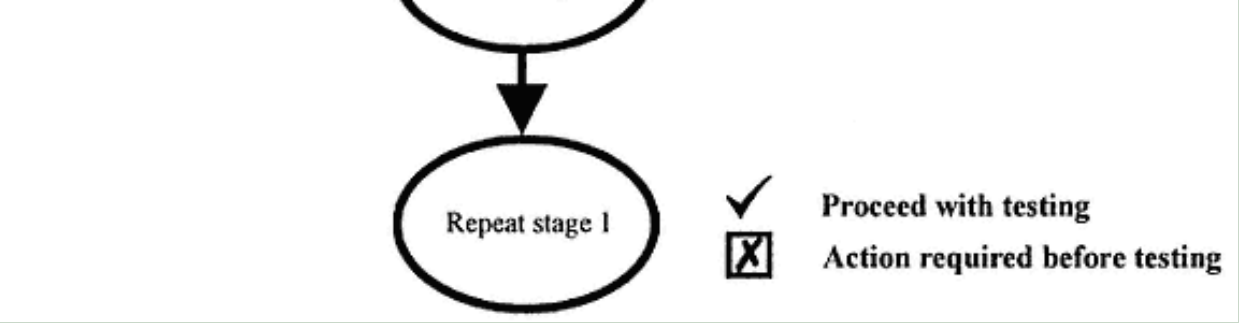


Fig. 26.
Scheme for evaluating kits.

we should be cautious when the error bars are in the 2 to 3 ¡Á *SD* range. Reasons for low color may be that the reagent was not made up properly, either by incorrect dilution or losing material on opening the vial (freeze-dried material can be lost); that the reagents were damaged transport; or that the laboratory has poor water. A poor result means that stage 2 must be run.

7.5.2¡ª
Stage 2

In case there was a general poor dilution or problem of loss, it is good practice to select new vials of all reagents, and retest. The initial vials can be stored. Be extra cautious in diluting the new vial reagents accurately. Also be careful

all material is solubilized and that none is lost when the vials are opened, or left on caps on dilution.

7.5.2.1¡ª
Situation C

If the results are good, then this points to the errors in stage 1 being dilution and making up reagents. Proceed with the testing.

7.5.2.2¡ª
Situation D

In cases in which there is no improvement, then some retitration and examination of the water in the laboratory is necessary in stage 3.

7.5.3¡ª
Stage 3

7.5.3.1¡ª
Simple First Checks

1. Are you using the correct filter? This should be 492 nm. If you have relatively strong color but the machine is reading only 0.1¨C0.4, then check the filter.

2. Make sure the substrate (H_2O_2) and chromophore *ortho*-phenylenediamine are made up properly. Check the viability by dipping the pipet tip into antimouse conjugate and putting tip into freshly made up substrate/chromophore. This should turn dark brown very quickly.

3. Double-check all dilutions you are making from vials.

4. Check the pH of the substrate buffer after the addition of chromophore!

7.5.3.2¡ª
Obtaining Another Source(S) of Distilled Water and Repeating Stage 1

Water can have a limiting effect on color development. If replacement of water allows results as in **Subheading 7.5.3.1.** then proceed with a secured source of water.

7.5.3.3¡ª
Retitrate the Antigen

Often there can be some lowering of antigen activity owing to denaturation (also on continued testing) and through aggregation. A simple examination of antigen can be made by keeping all other reactants constant.

1. Dilute antigen 1/25, 1/50, 1/100, and 1/200, as indicated in table 10.

2. Coat the plates under standard conditions.

3. Wash.

4. Add mAb at the recommended dilution. Add C++ and C+ for reference. Cc is included in column 5.

5. Incubate.

6. Wash

7. Add conjugate.

8. Incubate.

Table 10
Possible Results from Titrations (a)

	Diluted antigen[a]	1/25	1/50	1/100	1/200	0
						No Ag
	Wells	1	2	3	4	5
A	mAb (Cm)	0.92	0.72	0.45	0.32	0.03
B	mAb (Cm)	0.91	0.69	0.43	0.34	0.04
C	C++	0.14	0.11	0.08	0.05	0.03
D	C++	0.15	0.12	0.06	0.04	0.04
E	C+	0.31	0.23	0.18	0.05	0.06
F	C+	0.32	0.24	0.15	0.06	0.07
G	0 mAb	0.03	0.04	0.02	0.05	0.03

[a]mAb at recommended dilution.

9. Add substrate/chromophore.

10. Stop and read at 492 nm.

Some possible results can be examined.

7.5.3.3.1¡ª
Result (a)

Table 10 presents possible results from titrations (a). If there is an increase of OD at 1/25 and/or 1/50, this indicates that there is a loss in activity of the antigen so that recommended dilution is not suitable.

Action:

1. Use the antigen at a retitrated dilution that gives the mean OD for Cm within recommended limits. Check that the PI% values for C++ and C+ are within limits. In the example, a dilution of 1/50 is better.

2. Obtain a new batch of antigen.

Different results may be found as shown in result (b).

7.5.3.3.2¡ª
Result (b)

Table 11 presents possible results from titrations. Here, increasing the antigen concentration has no effect on increasing the OD.

Action: Retitrate the antigen, mAb, and conjugate.

7.5.4¡ª
Retitration of Antigen, Conjugate and Mab

The antigen (Ag), mAb, and conjugate can be titrated on one plate (*see* **Fig. 27**). The antigen is diluted beginning at 10 times the recommended concentration into wells in columns 1 and 7. The antigen is then diluted in a twofold range over four wells (columns 1¨C4 and 7¨C10. Columns 5, 6, 11, and 12 receive only buffer. After incubation and washing, the mAb is added and then diluted beginning at 10 times the recommended dilution in rows A and E. The mAb is diluted in blocking buffer in a twofold range from A1¨C5, A7¨C11 to D1¨C5,

Table 11
Possible Results from Titrations (b)

	Diluted antigen[a]	1/25	1/50	1/100	1/200	0
						No Ag
	Wells	1	2	3	4	5
A	mAb	0.40	0.41	0.43	0.32	0.03
B	mAb	0.43	0.40	0.41	0.34	0.04
C	C++	0.09	0.11	0.08	0.05	0.03
D	C++	0.08	0.12	0.06	0.04	0.04
E	C+	0.15	0.16	0.18	0.05	0.06
F	C+	0.16	0.17'	0.15	0.06	0.07
G	O mAb	0.03	0.04	0.06	0.04	0.05

[a]mAb at recommended dilution.

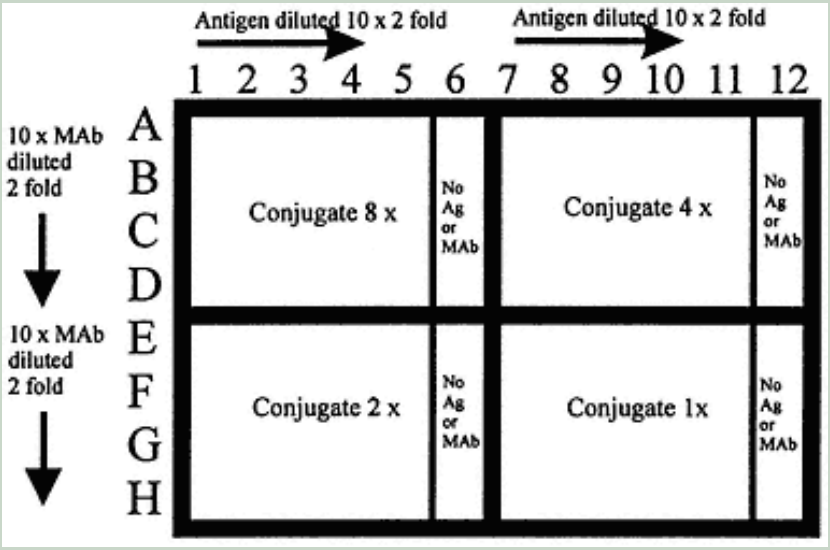


Fig. 27.
Scheme for titration of all reagents.

D7¨C11; and E1¨C5, E7¨C11 to H1¨C5, H7¨C11, respectively. Columns 6 and 12 receive only blocking buffer. After incubation and washing, the conjugate is added in all wells of the four quarters of the plate indicated at four different dilutions representing 8, 4, 2, and 1 times the recommended dilutions. After incubation and washing, substrate/chromophore is added and the late stopped at the recommended time. The color developed is then read at 492 nm. **Table 12** presents combinations of probable results. We have already assessed that under the standardized conditions, the antigen appears weak, so we are investigating mainly the probable situations in columns 5¨C8 in **Table 12**. However,

Table 12
Possible Combinations of Results[a]

1	2	3	4	5	6	7	8
Ag OK	Ag OK	Ag OK	Ag OK	Ag bad	Ag bad	Ag bad	Ag bad
M OK	M OK	M bad	M bad	M OK	M OK	M bad	M bad
C OK	C bad	C OK	C bad	C OK	C bad	C OK	C bad

[a]M, mAb; Ag, antigen, C, conjugate.

the titration recommended will investigate all the scenarios. Sketch graphs of the plates as they might appear in the various scenarios are given below in **Figs. 28** (1¨C3) and **29** (4¨C6). The situations 7 and 8 are not illustrated since there is no color obtained and in this situation (which would be very unusual), new kit regents should be tested and the materials returned to source. From the pattern obtained it should be apparent where the problem is in the reagents.

7.6¡[a]
Some Points to Help Testing

The following tips will help in testing:

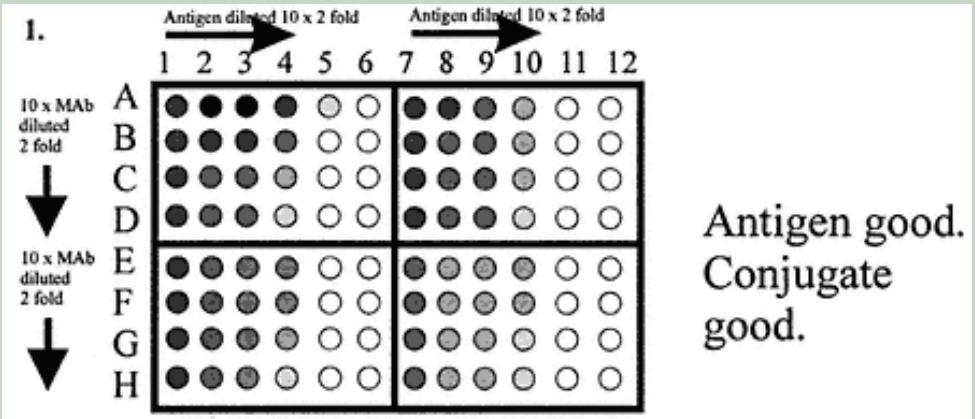
1. *Check the antigen since it is the factor that most often causes problems.* Make sure that none is lost on opening a vial (also true for all other reagents), and that all is resuspended. Check that no reagent escapes or is stuck to lids.

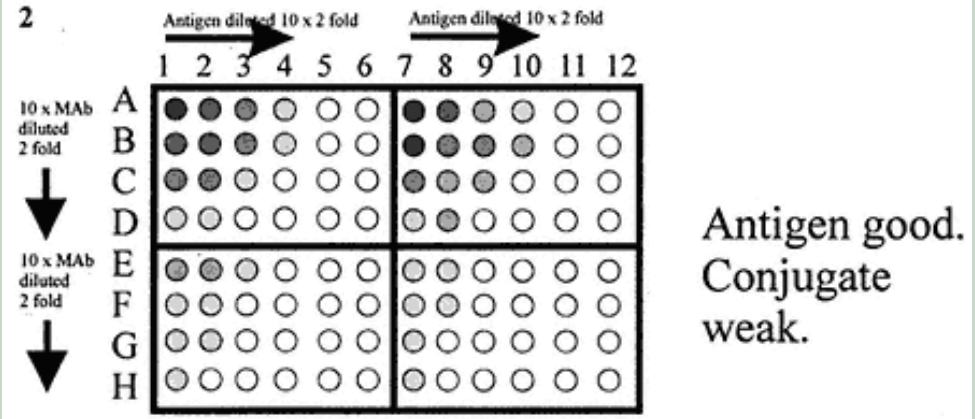
2. *Check the pH of all buffers and if possible make up reagents.* The wrong pH for substrate/chromophore will affect color development.

3. *Practice good pipeting technique at all times.* There can be large errors when pipeting small volumes. This is particularly true when tests are first run by inexperienced operators. The variation in testing should go down on practice. This will be seen also with reference to data plotted on charts. The target maximum error between replicates is 10¨C15%. If there are many duplicates for controls and test sera with error >10%, then tighten up your technique for reagent transfer. Slow down the testing (transfer).

4. *Examine data obtained with respect to actual OD values generated (unprocessed).* Ignore the computer-generated processed data while examining OD values. Assess each plate by eye with respect to controls. Reject those in which Cm values are obviously too small. Reassess the processed plate data with respect to all results for all plates in a test. This can also be done when data are plotted on charts. Do you agree with the action recorded by software-processed data?

5. *What are the results from sera tested?* Are you getting clear positive sera (high PI% competition of 80¨C100%), even when the Cm OD is low or out of limits? In this case, results may be acceptable, e.g., when in sero monitoring. However, attempt to increase Cm OD. Are you getting clear negatives (competition PI% values of¨C20 to 20%) even when the OD for Cm is low? Again, results may be acceptable. How many sera are in the problem area (e.g., 40¨C55% PI) from





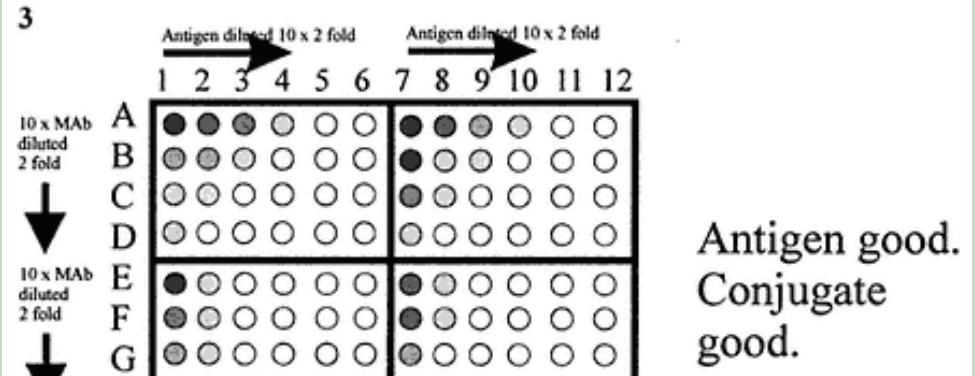
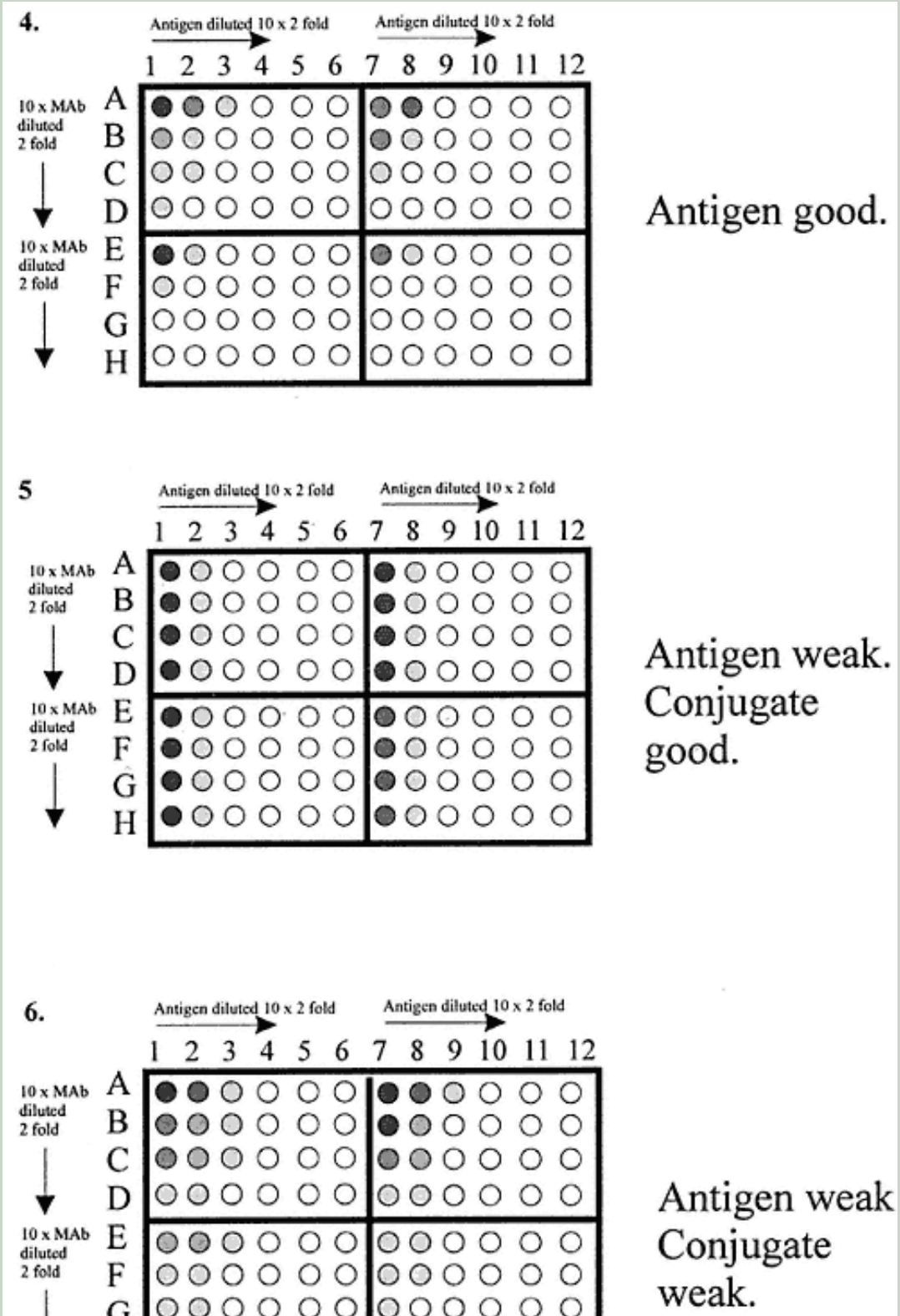




Fig. 28.
Situations 1¨C3 on titration of reagents.

the samples tested. All? A few? If there are many, then the whole system should be retitrated.

6. *Plot all processed data as they are processed on to the IQC charts.* The continuous examination and transparent recording of data is essential.



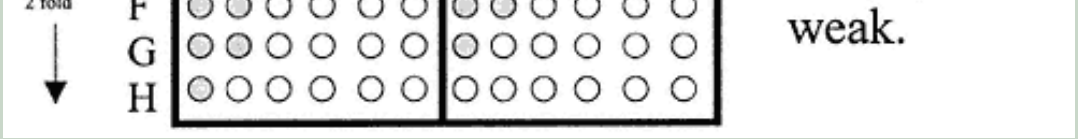


Fig. 29.
Situations 4¨C6 on titration of reagents.

7. The methods described here should be used to routinely examine reagents throughout their lifetime of use as a kit for testing. When there is an identified drift to lower OD values for Cm, then the reasons can be investigated using the

methods. The danger signals are when the mean OD of the Cm approaches the allowable mean ¡À2 ¡Á *SD* limit. Remember to observe the lower limit of error bars (2 ¡Á *SD* error bar of Cm mean per plate). If this falls inside the is within the mean Cm value ¨C3 *SD* limits, then the system should be examined.

10¡ª
Immunochemical Techniques

The scope of this book does not allow a complete description of the many techniques available for purification and treatment of reagents for facilitating immunoassays in general. There is a large amount of literature covering techniques, and these can be consulted for specific problems. The examination of many of the catalogs produced by commercial companies is useful since they often include good technical sections describing methods using their products. This chapter contains the practical basics of conjugation (a large field in itself), and details other immediately useful techniques that might be first desired in starting an ELISA. The book *Antibodies: A Laboratory Manual* ***(1)*** should be regarded as definitive in the laboratory because it is extremely "digestible" and covers a large field of methods, all of which are relevant to ELISA.

1¡ª
Labeling Antibodies with Enzymes

Antibodies can be readily labeled by covalent coupling to enzymes ***(2¨C7)***. The ideal product for any coupling reaction should have a 1:1 ratio of antibody to enzyme with no loss of specific activity of either reactant, but this is technically unachievable. However, owing to the amplification of the signal by the enzyme action, even relatively poor conjugates have required sensitivities. A large number of enzymes have been used to label antibodies. The most commonly used are horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-galactosidase. The ideal enzyme considerations are cost, stability, size, and ease of conjugation. The enzyme should have a high catalytic activity and a range of substrates that yield both soluble and insoluble products (for immunoblotting and immunocytochemical techniques). The purchasing of enzyme-linked reagents from commercial sources is recommended but for laboratory-produced specific reagents, such as monoclonal antibodies (mAbs) or affinity-purified antibodies, conjugates will need to be prepared in the laboratory.

1.1¡ª
Coupling Antibodies to HRP

Two general methods are used for the preparation of antibody peroxidase conjugates: the two-step glutaraldehyde method and the periodate method. Good batches of HRP can be determined by measuring the ratio of the HRP absorbance at 403 and 280 nm (RZ = OD 403 nm/OD 280 nm). This ratio should be at least 3.0. Good reagents designed for coupling are available commercially.

1.1.1¡ª
Glutaraldehyde Coupling

In the two-step glutaraldehyde method, glutaraldehyde is first coupled to pure HRP via the relatively few reactive amino groups available on the enzyme. By performing this step in high gluaraldehyde concentrations, very few HRP-HRP conjugates are formed. The HRP-glutaraldehyde mixture is then purified and added to antibody in solution. This method has a low coupling efficiency, so the HRP-antibody conjugates need to be separated from unconjugated material for optimum sensitivity. The HRP must be pure to minimize crosslinking of enzyme molecules to contaminating proteins during the first step of the procedure.

1. Dissolve 10 mg of HRP in 0.2 mL of 1.25% glutaraldehyde (electron microscopic grade) in 100 m*M* sodium phosphate (pH 6.8). **Caution:** glutaraldehyde is hazardous. Work in a fume hood.

2. After overnight incubation at room temperature, remove excess free glutaraldehyde by gel filtration. To do this, use a gel matrix with an exclusion limit of 20,000¨C50,000 for globular proteins. Use medium-sized beads (approx 100 µm in diameter). Prepare a column with 5 mL of bead volume according to the manufacturer's instructions. To make the column easier to load and run, first add 20 µL of glycerol and 20 µL of 1% xylene cylanol. The column should be prerun with a minimum of 10 column vol of 0.15 *M* NaCl. Allow the column to run until the buffer level drops just below the top of the bed resin. Stop the flow of the column. Carefully load the column with the glutaraldehyde-treated HRP. Release the flow and allow the HRP to run into the column. Just as the level of the HRP solution drops below the top of the column, carefully add 0.15 *M* NaCl. Run the column with 0.15 *M* NaCl. Pool the fractions that look brown. These contain the active enzyme.

3. Concentrate the enzyme solution to 10 mg/mL (1 mL final volume) by ultrafiltration or by dialysis against 100 m*M* sodium carbonate/sodium bicarbonate buffer (pH 9.5) containing 30% sucrose. Change the buffer to 100 m*M* sodium carbonate-bicarbonate (pH 9.5) either by dialysis or by washing on the ultrafiltration membrane.

4. Add 0.1 mL of antibody (5 mg/mL in 0.15 *M* NaCl) to the enzyme solution and check that the pH is >9.0.

5. Incubate at 4¡ãC for 24 h.

6. Add 0.1 mL of 0.2 *M* ethanolamine (pH 7.0). Incubate at 4¡ãC for 2 h. At this stage there will be present in the solution, the uncoupled HRP, the uncoupled antibody, and the HRP-antibody conjugate. For some assays, no further purification is necessary. In these cases, the uncoupled HRP will not bind to any antigen and will be lost during any washes prior to enzyme detection. Further purification will require separation based on the differences among the three species. The easiest separation will be between the uncoupled HRP and the two antibody-containing fractions. If the antibody binds to protein A, the antibodies can be removed simply, by low pH treatment. Separation between the two antibody fractions can be achieved by gel filtration (a 50-mL S300, or equivalent) or affinity chromatography on a concanavalin A column (eluted with 0.2 *M* glucose or methyl-mannoside). Alternatively, the whole separation can be achieved on the basis of size by gel filtration. Column eluates can be assayed by enzyme activity, absorbance at 403 nm, or absorbance at 280 nm.

1.1.2¡[a]
Periodate Coupling

Periodate treatment of carbohydrates opens the ring structure and allows them to bind to free amino groups. Coupling antibodies and HRP with periodate linkage is an efficient method. This method is based on **ref. *4*** and ***5***.

1. Resuspend 5 mg of HRP in 1.2 mL of water. Add 0.3 mL of freshly prepared 0.1 *M* sodium periodate in 10 m*M* sodium phosphate (pH 7.0).

2. Incubate at room temperature for 20 min.

3. Dialyze the HRP solution against i m*M* sodium acetate (pH 4.0) at 4¡ãC with several changes overnight.

4. Prepare an antibody solution of 10 mg/mL in 20 m*M* carbonate.

5. Remove the HRP from the dialysis tubing and add to 0.5 mL of the antibody solution.

6. Incubate at room temperature for 2 h.

7. The Schiff's bases that have formed must be reduced by adding 100 µL of sodium borohydride (4 mg/mL in water). Incubate at 4¡ãC for 2 h.

1.1.2.1¡[a]
Nakani and Kawaoi (4) Method of Enzyme Activation

1. Dissolve the HRP (HRPO, Sigma Type VI, RZ = 3) in 1.0 mL of freshly prepared 0.3 *M* sodium bicarbonate, pH 8.1 (should be this pH on making up). Note the milligrams/milliliter on the bottle of HRPO.

2. Add 0.1 mL of a 1% solution (v/v) of fluorodinitrobenzidine in absolute ethanol. Mix for 1 h (leave on bench and gently swirl every 10 min).

3. Add 1.0 mL of 0.08 *M* sodium periodate ($NaIO_4$) in distilled water. Mix gently for 30 min at room temperature (swirl every 5 min).

4. Add 1.0 mL of 0.16 M ethylene glycol (ethanediol) in distilled water. Mix gently (as in **step 3**) for 1 h.

5. Dialyze against 0.01 *M* sodium carbonate/bicarbonate buffer, pH 9.5, at 4¡ãC (three changes using 500¨C1000 mL each change).

1.1.2.2¡ª
Conjugation

1. Add the IgG (or other protein) dialyzed against 0.01 *M* carbonate/bicarbonate buffer, pH 9.5) at a ratio of 5 mg of IgG (protein) to 1.33 mg of activated enzyme. (Note: You know the volume of your activated enzyme and know the original milligrams/milliliter, and, therefore you know the effective concentration of the activated enzyme and can add so many milligrams in a certain volume. Mix and stand at room temperature for not less than 3 h (overnight is suitable).

2. Add 1 mg of sodium borohydride ($NaBH_4$)/mg of enzyme used. Make the $NaBH_4$ up fresh to about 200 mg/mL and add a relevant volume containing the correct number of milligrams.

3. Dialyze against phosphate-buffered saline (PBS).

4. You may wish to separate the free enzyme by methods already described, but in most ELISAs this is not necessary.

1.2¡ª
Coupling Antibodies to AP

Conjugation of antibodies to AP can be made using a one-step procedure with glutaraldehyde. The conjugates retain good immunological and enzymatic activity but can be large and heterogeneous in nature. The major drawbacks are the high cost of the enzyme and the need to use very concentrated solutions of enzyme and antibody.

1. Mix 10 mg of antibody with 5 mg of AP in a final volume of 1 mL. AP is usually supplied as a suspension in 65% saturated ammonium sulfate, which should be centrifuged at 4000*g* for 30 min (5 min in a microfuge). The antibody solution can then be added to resuspend the enzyme pellet.

2. Dialyze the mixture against four changes of 0.1 *M* sodium phosphate buffer (pH 6.8) overnight. This is essential to remove free amino groups present in the ammonium sulfate precipitate.

3. Transfer the enzyme-antibody mixture to a container suitable for stirring small volumes. In a fume hood add a small stir bar and place on a magnetic stirrer. Slowly, with gentle stirring add 0.05 mL of a 1% solution of electron microscopy, grade glutaraldehyde. **Caution:** Glutaraldehyde is hazardous.

4. After 5 min, switch off the stirrer and leave for 3 h at room temperature. Add 0.1 mL of 1 *M* ethanolamine (pH 7.0).

5. After an additional 2 h of incubation at room temperature, dialyze overnight at 4¡ãC against three changes of PBS.

6. Centrifuge at 40,000g for 20 min.

7. Store the supernatant at 4¡ãC in the presence of 50% glycerol, 1 m*M* $ZnCl_2$, 1 m*M* $MgCl_2$, and 0.02% sodium azide.

The procedure may be scaled down to the 1-mg antibody level if the antibody and enzyme concentration is reduced by a factor of 10. Here, the time allowed for coupling should be increased to at least 24 h. The yield of conjugate may be reduced.

1.3¡[a]
Avidin/Biotin Systems in ELISA

The specific binding between avidin (an egg white protein) and biotin (a water-soluble vitamin) has been exploited in ELISA. Avidin is a tetramer containing four identical subunits, each of which contains a very high-affinity binding site for biotin. The binding is not disturbed by extremes of salt, pH, or chaotropic agents such as guanidine hydrochloride (up to 3 *M*). The avidin/ biotin system is well suited for use as a bridging or sandwich system in association with antigen/antibody reactions. The biotin molecule can be easily coupled to either antigens or antibodies, and avidin can be conjugated to enzymes (and other immunological markers such as fluorochromes, colloidal markers, and ferritin). This section deals briefly with applications of the biotin/ avidin system to ELISA. An excellent outline of reagents and biotin/ protein-labeling methods (biotinylation) can be found in (**ref. *8***). Three basic systems are outlined.

1.3.1¡[a]
LAB System

An antigen immobilized on a microtiter well is detected by incubation with a primary antibody. After washing, this is detected by incubation with an antispecies antibody that is biotinylated (linked to biotin molecule[s]). Again, after washing, the complex is detected by the addition of avidin that is linked to enzyme followed by the addition of the relevant substrate.

1.3.2¡[a]
BRAB System

The BRAB system is essentially the same as the LAB system except that the avidin is not conjugated to an enzyme. Here, the avidin acts as a bridge to connect the biotinylated secondary antibody and biotinylated enzyme. Since the avidin has multiple biotin binding sites, this system allows more biotinylated enzyme to be complexed with a resulting amplification of signal, thus making the system potentially more sensitive than the LAB system.

1.3.3¡[a] ABC System

The ABC system is almost identical to the BRAB system except that it requires preincubation of biotinylated enzyme with avidin to form large complexes that are then incubated with the secondary antibody. In this way, there is a large increase in signal owing to the increase in enzyme molecules.

1.4¡[a] Methods for Labeling with Biotin

There are many biotinylated commercial reagents designed for use in ELISA. Avrameas and Uriel ***(7)*** will illustrate the various methods for the introduction of biotin onto reagents for use in ELISA using a variety of chemi-

Table 1
Biotinylating Reagents

Biotinylation reagent	What the reagent is reactive against
NHS-LC-biotin	Primary amines
NHS-biotin	Primary amines
SULFO-NHS-biotin	Primary amines
NHS-LC-biotin	Primary amines
NHS-SS-biotin	Primary amines
Photoactivatable	Nucleic acids
Biotin-HPDP	Thiols
Iodoacetyl-LC-biotin	Thiols
Biotin hydrazide	Carbohydrates
Biotinylated	Antimammalian IgG Protein A

cals. **Table 1** illustrates the versatility of labeling methods for proteins, carbohydrates, and nucleic acids.

2¡ª Preparation of Immunoglobulins

About 10% of serum proteins are immunoglobulins (Igs). After immunization, the specific antibodies produced are about 1¨C5% of this fraction, so the required Ig (in ELISA) may be from 0.1 to 2.5% of the total protein in a serum. Some assays are favored by the relatively crude fractionation of serum to obtain Igs, e.g., for use in binding to plates in trapping (sandwich assays) to avoid competition for plastic binding sites by other serum proteins. Several methods for separation of Igs are available for use in ELISA. These procedures are suitable for polyclonal antibodies but not necessarily for mAbs. The isolation of total Igs as compared to the purification of specific Igs, is relatively simple.

2.1¡ª Salt Fractionation

Two salts are used for selective Ig precipitation: ammonium sulfate and sodium sulfate. The concentration of ammonium sulfate is expressed as a percentage of saturation whereas the concentration of sodium sulfate is expressed as percentage (w/v). The concentration of salt at saturation depends on temperature, particularly for sodium sulfate (five times less at +4¡ãC). The isolation of mammalian IgG and IgA by ammonium sulfate precipitation depends on the volume of the serum being processed. For large volumes, the salt is added directly, whereas for small volumes, the salt is added as a con-

centrated solution. As already indicated, proteins are precipitated by different amounts of ammonium sulfate. This is a method that can be used to obtain samples of sufficient purity for most ELISAs. The initial volume of serum given here is 10 mL. Adjust the volumes accordingly to suit the starting volume of your serum.

1. To 10 mL of serum add 2.7 g of $(NH_4)_2SO_4$. Add a small quantity in steps. Stir constantly at room temperature.

2. Incubate at room temperature for 1 h while stirring.

3. Centrifuge at approx 5000g at 4¡ãC for 10¨C15 min.

4. Discard the supernatant fluid.

5. Dissolve the pellet in 2¨C3 mL of distilled water.

6. Add 0.5 g of $(NH_4)_2SO_4$, and stir constantly at room temperature.

7. Centrifuge as in **step 3**.

8. Dissolve the pellet in 10 mL of distilled water or PBS.

9. Dialyze against the appropriate buffer for use in ELISA or dialyze against distilled water and then freeze-dry.

2.2¡ª
Ion-Exchange Chromatography

After salt fractionation, IgG can be purified further on DEAE-cellulose, DEAE-Sephadex A-50, or DEAE-Sephacel. Such methods are not described in this book, but much literature is available.

2.3¡ª
Protein A

Protein A (SpA) is isolated from the cell walls of Cowan 1 or other strains of *Staphylococcus aureus.* It consists of a single polypeptide chain (mol wt of approx 42,000). Protein A has a high affinity *(K* = 10^8 L/mol) for the Fc of most mammalian IgGs and can be used for their isolation. A genetically engineered recombinant form of protein A (mol wt 32,000) is marketed, in which most of the nonessential regions have been, removed leaving four IgG binding domains intact.

Although protein A as used in immunoassays has little practical use in detecting sheep, bovine, and goat IgGs, they can be purified when the protein A concentrations are high, as in the commercially available protein A-Sepharose or protein A conjugated to Affi-gels (Bio-Rad) or glass beads. Such reagents are quite useful in rapid separation of most mammalian IgGs. Briefly, 5 mL Protein A columns are equilibrated with PBS. Serum or crude IgG is then added and elution with PBS is maintained. The IgG attaches to the column (via reaction to the protein A bound to the inert matrix), and the other serum proteins pass through the column. The bound IgG is then eluted by using a 0.9% sodium chloride solution containing 0.6% acetic acid or by adding a solution of sodium

thiocyanate (2¨C5 *M*). Such methods are particularly useful in the purification of mouse IgGs from mAb ascites preparations.

2.4¡ª
Protein G

Protein G is isolated form group G *Streptococcus* sp. The protein is similar to protein A in that it binds to a variety of mammalian Igs through their Fc region, but generally with a higher affinity. Unlike protein A, protein G binds strongly with bovine, ovine, and caprine IgGs.

2.5¡ª
Protein A/G

Protein A/G is a genetically engineered protein produced by a gene fusion product from a nonpathogenic *Bacillus* strain. The protein is engineered to have four Fc binding domains of protein A and two of protein G per molecule. The product binds to all classes of mouse IgG, but not with IgA or IgM.

3¡[a]
Immunosorbents

A breakthrough in the ease of use of immunosorbents was made with the availability of reagents such as *n*-Hydroxysuccinimide-derivatized agarose (Bio-Rad). This gel can be washed three times in cold distilled water and then used to covalently attach any protein, merely by incubation of that protein(s) in a wide variety of buffers (such as 0.1 m sodium carbonate buffer). Blocking of unreacted active sites on the gel is achieved by the addition of ethanolamine or by merely leaving the gel overnight. Such gels are thus quite easy to prepare. Antisera can then be added in neutral buffers, and the addition of some detergents (e.g. 0.5% Tween-80) minimizes nonspecific adsorption of serum proteins. Desorption of bound antibodies can then be achieved by the addition of chaotropic ions (sodium thiocyanate), organic acids with low surface tension or pH extremes. Thus, such affinity techniques can be used to remove unwanted crossreactions from sera. For example, if a serum has antibovine IgG activity, this can be adsorbed out by passing that serum over an affinity column with bound bovine serum or IgG. In this case, the antibodies passing through the column will be free from antibovine activity.

Other immunosorbents are available commercially based on beaded agarose or glass. A wide variety of proteins such as whole serum or IgG can be purchased attached covalently to beads and are extremely convenient (but expensive) for, e.g., the removal of unwanted cross reactive antibodies from small volumes of antisera. The beads are simply added to an antiserum and after a short incubation are separated by centrifugation (in a microfuge at 10,000*g* for 10¨C30 s). The agarose beads thus capture any unwanted antibodies on the solid phase, leaving the antiserum free of that contaminant. This method has the advantage over blocking by addition of high levels of specific protein (against

which the unwanted antibodies react) because there is complete separation of immune complexes, which may interfere with ELISAs. Such reagents can be reused by eluting the immunologically bound protein using similar methods (e.g., low pH), followed by extensive washing. The section on immunoaffinity purification in **ref. *1*** should be consulted for extensive practical details of methods.

4¡[a]
Production of Antisera

The raising of antisera in laboratory animals could fill a manual in itself. The variability in immune response within and between species and the various antigens used mean that no brief rules can be given, and reading of the relevant scientific literature is essential. Generally, the administration of a nonreplicating agent requires the addition of an adjuvant, whose effect is to stimulate the immune system so that efficient presentation of the antigen takes place.

4.1¡[a]
Immunization

The purpose of immunization is to obtain high-titer antisera that bind strongly to antigen (high avidity). The properties of antisera are determined by the genetic composition of the animal injected (particularly the Ir genes). This means that there can be great variation in the quantitative and qualitative aspects of antisera from among species and even among individuals of the same species. This should be borne in mind when considering the use for which the serum is being made. In preparing sera one should (1) always obtain a preimmunization serum, and (2) never automatically pool sera. Point (2) is particularly important if a defined property of an antiserum is required (e.g., in discrimination of antigens).

Up to a certain degree, an increase in the dose (weight) of antigen will increase antibody titer; however, this may also increase crossreactivity. Adjuvants also increase the immunogenicity of proteins. Haptens should be labeled with carrier proteins to elicit an immune response. The carrier protein should be foreign to the host to be recognized by the T-cells. For most immunogens, the interaction of T- and B-cells is essential for antibody production.

The animal species chosen can be important. The animal species most often used in laboratories are rabbits, goats, guinea pigs, pigs, sheep, and rats. Commercial companies may favor horses and donkeys for large-scale preparations. Many animals contain crossreactive antibodies in their serum before immunization; this could complicate their use in ELISA, and some simple absorption technique may be required (or may have been performed in commercial preparations) to block such reactions. Another point to remember is that many smaller animals can be immunized as compared to only a few larger animals,

owing mainly to cost considerations. Because of the variations in sera from the previously mentioned animals, smaller animals offer advantages in which relatively small volumes of serum are required.

For most immunization regimes, the immunogen (at about 2 mg/mL) in an isotonic salt solution is mixed vigorously with an equal volume of Freund's complete or incomplete adjuvant, to obtain an emulsion that is stable in water. It is essential that the antigen be added in small aliquots in a stepwise fashion to the adjuvant, with vigorous mixing between each addition (e.g., using a vortex mixer). On complete addition of the antigen, the emulsion must be tested for stability. This is easily done by placing a drop of the emulsion on to the surface of some distilled water in a beaker. This should spread out over the surface. However, a second drop added (or sometimes a third) should not spread and remain as a distinct drop, and the edges of the drop should show no signs of dissolution.

4.2¡[a]
Immunogen Dose

The amount of immunogen needed to induce an immune response depends on the exact nature of the antigen and the host species used. A typical dose/ response is sygmoidal in nature, whereby very large and very small doses of material elicit a weak or no response. Generally, the lowest effective dose of an antigen is preferred when raising antisera for immunoassays since this tends to elicit antibodies of highest affinity and produce polyclonal sera of high avidity. General values can be given; however, a range of concentrations of antigen is often needed to allow an estimation of its potency. Rabbits and guinea pigs usually require approx 100 µg of protein with an adjuvant, whereas doses of between 500 and 1000 µg are required for larger animals. Generally, subsequent booster doses are lower and given without adjuvant.

4.3¡ª
Improving Antigenicity of Antigens

Adjuvants are substances that enhance the immune response. A range of adjuvant methodologies is available, including the following:

1. Water-in-oil emulsions such as Freund's complete adjuvant, Freund's incomplete adjuvant. The complete adjuvant contains heat-killed *Mycobacterium tuberculosis* bacteria whereas the incomplete does not. Both have the basis of an immune modulator, e.g., branched glucose polymers or methylated bovine serum albumin (Pierce's AdjuPrime™).

2. Minerals in which the antigen can be absorbed on aluminum hydroxide, bentonite, or quaternary ammonium salts.

3. Bacterial species such as Bacille Calmette-Gu¨¦rin, *Bordetella pertussis,* or *Corynebacterium parvum.*

4. Bacterial products such as endotoxins, lipopolysaccharides, and liposomes.

5. Polynucleotides such as poly I-poly C, and poly (A-U).

The adjuvant actions generally stimulate immune responses nonspecifically by increasing antigen presentation and the number of collaborating cells involved. This effectively reduces antigen doses required and enhances immunogenicity of proteins. Adjuvant may alter the spectrum of antibodies produced in terms of both isotypes and specificities. Adjuvants also act to produce depots of antigen that are released slowly, thereby promoting continuous stimulation of the immune system. In addition, adjuvants may protect the immunogen from rapid removal and cleavage from host enzymes (*see* **ref. *9*** for review of adjuvants).

In cases in which there is no response in animals after multiple injections, alternative animal species should be tried and the dose of antigen(s) increased. If this does not succeed, then attempts to enhance the antigenicity by direct modification methods can be tried. An excellent practical description of these techniques is found in **ref. *7***. Common methods include the following:

1. The addition of small modifying groups such as dinitrophenol or arsenate.

2. Denaturation of antigen by heat treatment and/or sodium dodecyl sulfate treatment.

3. Coupling of antigen to small synthetic peptides that are sites for T-cell receptor class II protein binding.

4. Coupling of antigens to large particles such as sheep red blood cells or agarose beads.

5. Purification of antigens with other antibodies and injection of immune complexes.

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11¡ª
Test Questions

This chapter consists of a test concerning ELISA and the sciences needed to perform assays. The test can be used to gage knowledge on ELISA. Suggested points are given for the benefit of educators. Of course, the test can be adapted. All the answers can be obtained by reading this book. It may be useful to test oneself before and after referring to the book. Answers follow the test as a guide to marking.

Questions

1. What does ELISA stand for?
2. Name two substrates used in ELISA.
3. What is H_2O_2?
4. What is a blocking buffer?
5. Name three enzymes commonly used in ELISA.
6. What is IgG?
7. Complete the following list, filling in gaps in words, according to units in decreasing weight series:

   gram, milligram, _____, nanogram, _____, _____
8. Draw a diagram representing an indirect ELISA.
9. Draw a diagram representing a sandwich ELISA involving the same antibody.
10. Name a use for sulfuric acid in ELISA.
11. Name four immunoglobulin classes.
12. Name the method of titrating two reagents against each other.
13. What does OPD stand for?
14. What does the washing step do in ELISA?

15. How many microliters are in a liter of liquid?
16. What is meant by a competition ELISA?
17. What is the process of internal monitoring of a test called?
18. Name three agents added to solutions to make blocking buffers.
19. What is the most commonly used solid phase in ELISA?
20. Why do you need to tap plates when you add coating reagents?
21. Give three advantages of rotation during the incubation phase of ELISA as cmpared to stationary conditions.
22. Draw a diagram representing a sandwich ELISA involving an indirect method of detecting the second antibody.
23. Give three reasons why there might be *no* or hardly any color development after the addition of substrate/chromogen in an ELISA.
24. Calculate the following (give the answers in microliters):
    a. You need a 1/500 dilution of serum in 12 mL of buffer. How much undiluted serum must I add?
    b. You need a 1/500 dilution of serum in 12 mL. The serum given is already diluted 1/10. How do you do this?
    c. You need 110 mL of a reagent at 1/20,000. The reagent is already at 1/50. How do you do this?
25. What is an antigen?
26. Give two differences between monoclonal and polyclonal antibodies.
27. Give the substrate and name two chromogens for horseradish peroxidase enzyme.
28. What term describes the region of the antibody molecule that binds to an antigen?
29. What is a conformational epitope?
30. What is meant by the following terms?
    a. EQA
    b. SD
    c. Mean
31. What is meant by the term *stopping*?
32. The plate OD values given below show a checkerboard titration of antigen against a specific conjugate. Antigen is diluted from 1/50 from column 1 to 11 in a twofold dilution range. Conjugate is diluted from row A to G from 1/200 in a twofold range.

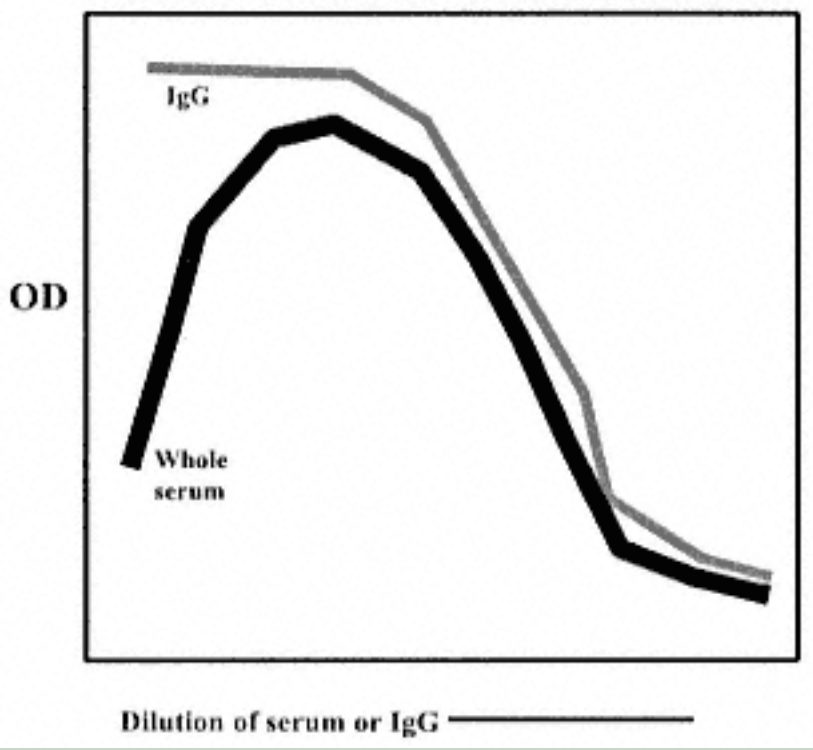
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2	2.3	2.2	2.1	2.0	2.0	1.9	1.6	1.5	1.3	0.8	0.6
B	2.2	2.0	1.9	2.0	1.9	1.8	1.7	1.5	1.3	0.8	0.5	0.3
C	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5	1.2	0.7	0.5	0.3
D	1.9	1.9	1.7	1.8	1.5	1.2	1.0	0.7	0.5	0.3	0.2	0.1
E	1.8	1.8	1.7	1.5	1.3	1.1	0.8	0.6	0.5	0.3	0.2	0.1
F	1.3	1.3	1.2	1.2	1.1	0.8	0.6	0.5	0.3	0.2	0.1	0.1
G	0.7	0.7	0.7	0.6	0.6	0.5	0.3	0.2	0.1	0.1	0.1	0.1
H	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Answer the following questions based on the data.

a. Why is color (OD) similar in A1–A5?
b. Explain possibly why the OD in A12 is high?
c. Where would you assess the optimal antigen concentration for coating wells expressed as a dilution?
d. What is the optimal dilution of conjugate? Give an approximate range here.

33. An antiserum is already diluted in blocking buffer to 1/50. You need to make a volume of 90 mL of this antiserum at 1/12,500. Show calculation to perform this dilution.

34. Explain the following titrations where whole serum is used as a capture antibody compared to the IgG fraction of the serum shown in the following diagram:

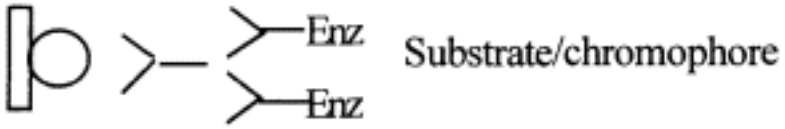


35. Define the following terms:
    a. Epitope
    b. Fab fragment
    c. Affinity
    d. Avidity
    e. Monoclonal antibody
36. How could you remove antibodies against bovine serum proteins from an antiserum, in which these are causing problems of crossreactivity?
37. You require 1 mL of a dilution of reagent at 1/1,000,000. How could you make this using only 3 mL of diluent?
38. What is the main difference between a heterogeneous and homogeneous ELISA?
39. What is an anamnestic response?
40. What does 492 nm refer to?

Answers

1. Enzyme-linked immunosorbent assay. *(2 points only for completely correct answer)*
2. Hydrogen peroxide or H_2O_2; *p*-nitrophenylphosphate or pnpp; or ortho nitrophenyl β-galactosidase. *(2 points for each correct answer, maximum 4 points)*
3. Hydrogen peroxide. Also allow substrate for horseradish peroxidase conjugates. *(2 points)*
4. Buffered solution to which substances are added to preventing nonspecific adsorption of reagents in ELISA. *(2 points)*
5. Horseradish peroxidase, alkaline phosphatase, β-galactosidase, ABTS, urease. *(2 points for each correct answer, maximum of 6 points)*
6. A class of immunoglobulin from serum, immunoglobulin G. *(2 points)*
7. Gram, milligram, microgram, nanogram, femtogram, attogram. *(2 points for each correct answer)*
8. Must contain a solid phase, attached antigen (coating), antibody-detecting antigen and anti species conjugate. Allow symbols or drawings such as the following:

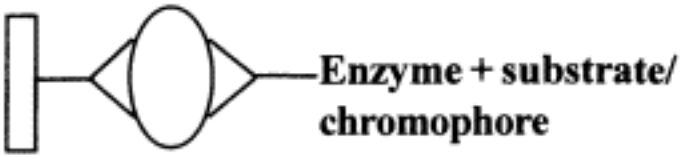
I-Ag + Ab + Anti-Ab + Anti-Abconjugate



(6 points for complete answer)

9. Must contain a solid phase, capture antibody, antigen, detecting antibody labeled with conjugate, and chromophore/substrate. Allow symbols or drawings such as the following:

I-Ab + Ag + Ab-enzyme + substrate/chromophore



(2 points for complete answer)

10. As a stopping reagent—to stop enzymatic reaction. *(2 points)*

11. IgG, IgM, IgA, IgD, or IgE. *(1 point, up to 4 maximum)*

12. Allow chessboard or checkerboard titrations. *(2 points)*

13. *Ortho*-phenylenediamine. *(3 points)*

14. Separates bound and free reagents. *(3 points)*

15. One million (1000,000). *(3 points)*

16. When a pretitrated system is challenged by the addition of a substance that competes for binding. *(3 points)*

17. Internal quality control or IQC. *(3 points)*

18. Proteins generally, BSA, gelatin, milk powder, detergents, sodium dodecyl sulfate. *(2 points for each correct answer, maximum 6 points)*

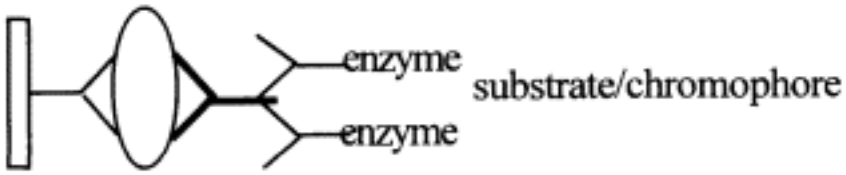
19. 96-well microtiter plates. *(2 points, allow 1 point for plastics)*

20. To ensure mixing and adequate entry of reagents. *(2 points)*

21. Ensures mixing; reduces viscosity effects; reduces temperature effects on reaction rates owing only to diffusion mixing; avoids irregular heating effects of stationary conditions owing to insulation by plastic; allows stacking of plates; or reduces time needed for incubation under stationary conditions. *(2 points for each correct answer, maximum of 6 points)*

22. Should contain a solid phase, capture antibody, antigen, detecting antibody and antispecies conjugate with the addition of substrate/chromphore. Allow symbols or drawings such as the following:

I-Ab + Ag + AB + AntiAB-Enzyme + substrate/chromphore



(6 points)

23. No conjugate added; no substrate added; one other reagent omitted; conjugate activity destroyed; or wrong plates used. *(2 points for each correct answer, maximum of 6 points)*

24. (a) 12 mL = 12,000 µL, 1/500 of 12,000 is 24 µL The answer is 24 µL. *(2 points)*; (b) 12 mL = 12,000 µL. If serum was undiluted you would need 12,000/500 = 24 µL, but since it is diluted 1/10, you need 10 times more: 10 × 24 µL = 240 µL. The answer 240 µL. *(3 points)*; (c) 110 mL = 110,000 µL. If the reagent were not prediluted, you would require 1/20,000 of this: 110,000/20,000 µL = 5.5 µL. Since the reagent is diluted 1/50, you need 50 times more: 50 × 5.5µL = 275 µL. The answer is 275 µL. *(3 points)*

25. A substance which when injected into an animal elicits antibody production. *(3 points)*

26. mAbs are single affinity reagents; mAbs are homologous population of antibodies; or mAbs react with a single epitope. *(2 points each for reasonable answers, maximum of 4 points)*

27. Hydrogen peroxide is the the substrate (allow H_2O_2). Chromogens are HRPO or horseradish peroxidase, ABTS, TMB, AS, or 5-AS. *(2 points for substrate, 2 points each per correct chromogen, maximum of 4 points)*

28. Allow antibody-combining site, paratope, or Fab. *(4 points)*

29. An epitope that relies on a distinct three-dimensional relationship of the same or different proteins to allow interaction with antibodies, or an antigenic site which is denaturable. *(4 points for a reasonable answer)*

30. (a) external quality assurance *(2 points)*; (b) standard deviation *(2 points)* (c) average of a set of data *(2 points)*

31. The process by which enzymatic development of color is stopped before reading data in a spectrophotometer. *(3 points)*

32. (a) The antigen although diluted gives the same color. Thus, there is excess antigen added here as detected with constant 1/200 dilution of conjugate. *(2 points)*; (b) Here the conjugate is at 1/200 and is sticking nonspecifically where there is no antigen as compared to later dilutions C12 D12, and so on). Thus, three is a high conjugate background. *(2 points)*; (c) 1/3200 to 1/6400. *(2 points)*; (d) 1/200 to 1/400. *(2 points)*

33. If antiserum were not diluted, I would need 90 mL, i.e., 90,000 µL/12,500 = 7 µL. However, it is already diluted 1/50 so we need 50 times more: 50 × 7 = 450 µL. Answer is 450 µL. *(5 points)*

34. The whole serum contains other proteins that block the immunoglobulins involved in capture. The IgG is separated from such proteins and can attach to plastic. The whole serum thus has a prozone of poor capture activity. When the

serum is diluted, the effect of the other proteins is negated and where high concentrations of IgG are contained, maximal capture can occur. The IgG shows a plateau of activity where IgG is in excess. *(6 points)*

35. (a) Another name for an antigenic site though better defined as an entity through monoclonal antibodies. *(3 points)*; (b) A fragment of immunoglobulin molecule resulting from digestion. This is the single antibody-combining site of the molecule. *(3 points)*; (c) Defines force between antibody and antigen. Affinity refers strictly to interaction between one species molecule and single site. *(3 points)*; (d) Represents sum of all affinities of antibody molecules. *(3 points)*; (e) A single population of antibody molecules against a single epitope. *(3 points)*

36. Use affinity reagents consisting of bovine serum or bovine IgG attached to the solid phase, columns, use of addition of excess bovine serum. *(4 points)*

37. Dilute 10 µL into 1 mL, mix, take 10 µL of this and add to 1 mL of diluent, and mix. Take 10 µL of this and add to 1 mL of diluent. Each diution step is 10 µL in 1000µL = 1/100. Three dilution steps of 1/100 = 1/1,000,000. *(3 points)*

38. Heterologous ELISAs have a washing step to separate bound and free reactants, whereas homogeneous assays do not. *(2 points)*

39. The response to a second injection of an antigen. The primary response involves a delay in antibody production whereas the anamnestic response is immediate, with increasing titer. *(3 points)*

40. This is a wavelength produced by using a filter. It is used to read OPD chromophores with horseradish peroxidase enzyme and hydrogen peroxide substrate. *(3 points)*

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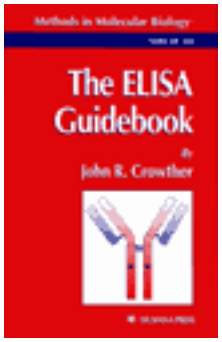
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of science are needed to allow optimal use of ELISA and notes their relationships. A good deal of attention should be directed at defining, as clearly as possible, the objectives for the ELISA. Four major advantages of ELISA are promoted, all of which add to the reasons that this form of ELISA has been, and will continue to be, successful. 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. Antigens A protein or carbohydrate that when injected into animals elicits the production of antibodies.



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