Evaluation of commercial reproducible solid-phase enzyme immunoassays

J.M. Coll

Deparment of Sanidad Animal. Instituto Nacional de Investigaciones Agrarias. Madrid.

INTRODUCTION

Solid-phase, heterogeneous enzyme linked immunosorbent assays (ELISA) for both antigens and antibodies are being developed at a very rapid rate (Sánchez Vizcaíno and Álvarez, 1987). The acceptance of the solid-phase format in various areas of clinical or veterinary assays is due to the stability of reagents, unitized packaging, convenient and small instruments, and minimal preparation by users before testing (Burkhardt, 1987). For practical reasons, these assays are generally made in 96-well microtitre plates which allow easier handling of a large number of samples. Although enzyme immunoassays are based either on activity amplification (non-competitive) or activity modulation (competitive), the former is preferred because of easier interpretation and wider application. This chapter will focus on a review the latest advances which could be applied to the production, scaling-up and performance of commercial reproducible enzyme immunoassays.

An analysis of the different features in solid-phase ELISA, reveals four common steps (fig. 1): *1)* attachment of the immunoreactant to the solid-phase; *2)* incubation with the test sample; *3)* amplification step, and *4)* enzyme assay.

ATTACHMENT OF THE IMMUNOREACTANT TO THE SOLID-PHASE

Types of solid-phase

A variety of materials and configurations have been used as solid-phase for enzyme immunoassays (Burkhardt, 1987). Probably the most commonly used reaction vessel is the microtitration plate (Lambre and Kasturi, 1979) as it is inexpensive, convenient to use, readily available and appears to work for most assays. The standardized plate containing 96 wells (300 µl per well) also has the advantages of compact geometry, economy of rea-

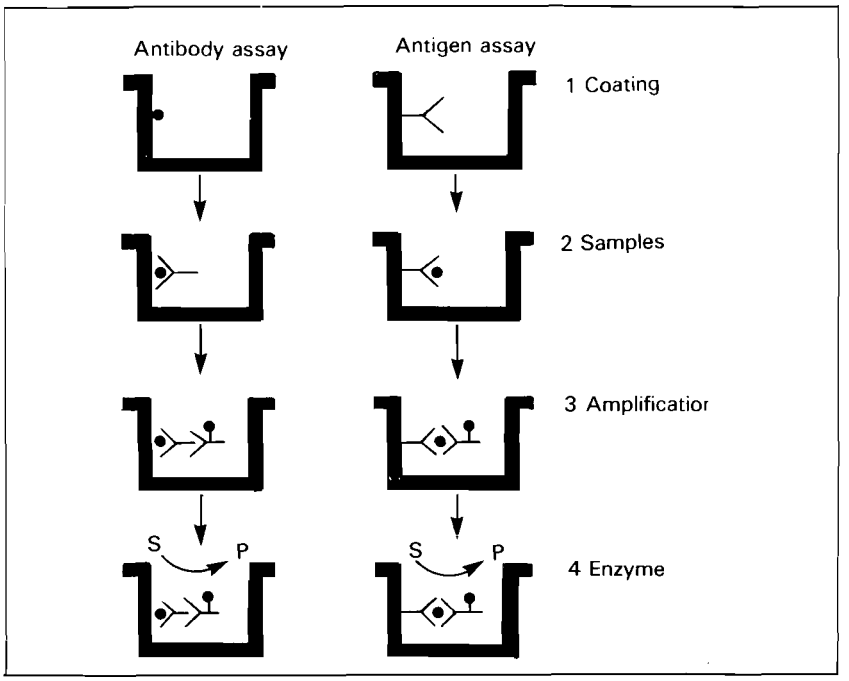

Fig. 1. Scheme of enzyme immunoassays for antibody or antigen detection in microtitre wells. All non-competitive enzyme immunoassays have four common steps: (1) coating of the solid-phase with immunoreactant (antigen, or antibody), (2) incubation with the test sample, (3) amplification step, and (4) enzyme assay. Washing is performed in between steps. Quantification is obtained by measuring the product by comparison with known calibrated standars. : antigen; : antibody; : conjugate; S: substrate; P: product.

Correspondencia y solicitud de separatas: Dr. J.M. Coll. Departamento de Sanidad Animal.
Instituto Nacional de Investigaciones Agrarias.
INIA, CRIDA 06. 28012 Madrid. España.

gents, and fixed order for each sample, thus avoiding mistakes and unnecessary labelling (Palomo et al, 1982; Ablin, 1987).

Most microtitre plates are made either of polystyrene or of polyvinyl plastic. In polyvinyil plates, slightly stronger reactions can be obtained, and the plate can be cut into pieces but the polystyrene plate is more difficult to handle and to sead. The rigid polystyrene is easier to use but produces, weaker reactions (Zouali and Stollar, 1986), costs nearly three times as much as the polyvinyl, and a complete plate has to be used for each assay. However, in recent years polystyrene plates divided in rows, with different configurations, have been available, wich easily adapt the number of assays to the daily variable number of samples, which occur in clinical laboratories.

New designs and/or materials will probably be evolved in this area. For example, Hargreaves et al (1987) have developed novel immunoassays in which the solid-phase is generated *in situ* after the specific binding reaction has occurred. In these assays, the antibody is conjugated to a monomer that is either polymerized upon reaction with the antigen or is precipitated when there is a change in the temperature. Another type of solid-phase is the microporous membrane. A higher ratio of surface area to volume within the microporous solid-phase assures shorter diffusion distances and, therefore, a rapid binding of the liquid-phase reagents. Flow of reagents through the membrane increases the speed, detectability and simplicity of the assay (Valkirs and Barton, 1985).

Attachment of immunoreactants

Physical or chemical bonds can be used for the attachment of immunoreactants (antigen or antibody) to the solid-phase (Andersen, 1986). A physical binding is made through out all parts of the molecule, whereas a specific chemical reaction, generally, orients the molecule in the solid-phase and, in addition, is stronger and more stable. However, with the best solid-phase on the market, the physical bonds can bind as strongly as the chemical bonds.

The number of immobilized molecules is a function of time, temperature, concentration of immunoreactant and buffer (Hutchens and Porath, 1987). Coating of the solid-phase with the immunoreactant takes place at either 37 °C for 4-6 h or at 4 °C overnight, in buffers at basic pH and low ionic strength (Engvall and Perlmann, 1972; Lambre and Kasturi, 1979). Alternatives for scaling-up the coating of the solid-phase with high reproducibility, in large batches are, the elimination of the liquid containing the immunoreactant by incubation at 37 °C (Iturralde and Coll, 1984) or by liophylization (Institute Pasteur Production). Both procedures are generally carried out by using distilled water for dissolving the immunoreactant. Whatever the method for coating, protein molecules attach so well to the solid-phase that subsequent exposure to 0.5 M ionic strength will not cause detectable detachment.

The quantity of antibody which can be adsorbed to the well of a microtitre plate is limited both by the fraction of specific antibodies present in the immunoglobulin preparation and by the surface of the well. Assuming a maximum adsorption of 1.5 ng/mm^2 and 150 kDA for an immunoglobulin molecule, the maximum attainable concentration for an antigen-affinity purified monoclonal antibody is about 10^{-7}M. For affinity purified polyclonal antibodies, hyperimmune antisera or post-infection sera, about 3, 10 or 100 times less specific immunoglobulin will be bound (Tijssen, 1985). The relationship between coating concentration, purity of immunoreactants, epitope density, and prozone phenomenon will have to be studied for every system to define the correct specificity and to obtain the maximum detectability of the assay (Vos et al, 1987).

For maximal sensibility, the amount of protein bound to the surface must be maximal. The optimal amount of protein to be coated, however, could be lower than the maximal attainable concentration. An excess of coated protein over the optimal concentration would increase unspecific protein-protein interactions which would increase, percentage of background, unstability, possibility of being washed, etc. Furthermore, a non-optimal amount of protein bound could cause false positives (Vizcaino and Cambra, 1987). Finding the optimal concentration depends slightly on the type of ELISA and the proteins (antigens or antibodies) used. The best method is to test different dilutions of conjugate against successive dilutions of the protein coating the plates. The best dilution produces the lowest non-specific reaction (background colour) with negative samples and the highest reaction with positive samples. As the coating dosage increases the optical density increases, reaching the maximum point generally but not always, around the maximal attainable concentration that a well is capable of adsorbing. The exact concentrations to be used for coating must, therefore, be determined in each case, by similar titrations.

Direct immobilization of antibodies or antigens on surfaces limits the usefulness of solid-phase assays for at least three reasons: *a)* variation in the physical structure of the surfaces are extended to the analytical system; *b)* lateral surface interactions of the adsorbed protein could distort the molecules, and *c)* interaction of lipophilic domains of the proteins with the hydrophobic solid-phase might either include the binding site or provoke steric hindrance for the binding. These are some of the reasons why indirect immobilization methods are being used in some systems. These include, for example, plates coated with protein G (Nilson et al, 1986), protein A (Schramm et al, 1987a), anti-immunoglobulin antibodies (Sankolli et al, 1987), specific binding proteins (Delpech et al, 1987), or specific ligands (Martínez and Coll, 1987). Schramm et al (1987b), demonstrated a higher reproducibility for enzyme immunoassays based on

monoclonal antibodies bound to protein A-coated polystyrene plates than for those immobilized directly on the plates.

Solid-phase enzyme immunoassays were of limited use for assaying hydrophobic immunoreactants such as biological membrane proteins, because of difficulty in disrupting the structures of the analyte while preserving the binding capacity of the solid-phase immunoreactant. Very recently, however, the coating of polystyrene wells with membrane proteins in the presence of high-critical micelle concentration detergents (zwittergent 3-08 or octyl-β-glucoside) has been reported (Gardas and Lewarthowska, 1988).

Covalent attachment of the immunoreactant to the solid-phase has the theoretical advantages of increased capacity, added stability and higher reproducibility. A 50 to 100-fold enhanced total capacity has been demonstrated by using gamma irradiation and carboxylation of both polystyrene and polyvinyl surfaces (Larsson et al, 1987). Glutaraldehyde, either alone or with poly-lysine, has also been used to increase stability by covalent attachment of immunoreactants (Zouali and Stollar, 1986; Martínez and Coll, 1987).

To perform the coating on a large scale, automatic dispensing devices are needed so that variations from well to weli and from plate to plate are low enough to allow commercialization. Some of these apparatus have recently become available in the market. To perform the incubations needed for the coating, air forced incubators with homogenous temperatures are preferred, to decrease individual plate variations. With the use of both of the above mentioned methods, variability of the enzyme immunoassay due to coating could be in the range of 2 %-4 % CV from well to well and less than 6 % CV from plate to plate (CV, coefficient of variation, SD as percentage of the mean).

Blocking

Nonspecific binding of immunoglobulin, conjugate or interfering proteins to the solid-phase can reduce both specificity and detectability (Ekins, 1981). During incubation periods, all proteins will bind to a certain extent to available sites remaining in the solid-phase after attachment of the immunoreactants. Methods to reduce nonspecific binding (blocking) are designed both to occupy remaining bindings sites after adsorption of either antigens or antibodies and to prevent adsorption of undesired molecules by adding agents to the appropiate buffers (Hutchens and Porath, 1987). The optimal blocking agent(s) for any particular ELISA system, must be determined by empirical testing. By testing nine different proteins, Vogt et al (1987) found milk and casein to be the best blockers for nonspecific binding in a peroxidase-o-phenylenediamine ELISA. For commercial purposes, however, pretested batches of bovine serum albumin are preferred because of their much higher stability in ready-to-use buffers. In many cases, the blocking can be reduced to a washing step with an appropiated buffer, and then drying (unpublished at the moment of writing the article).

Kit presentation

The aspect for storage of the plates deals with stability problems when it have to be kept in a kit at 4 °C.

Water content of the atmosphere where they are to be kept is critical for the stability of most coated plates. Plates kept in a humid atmosphere for a few weeks not only lost their binding capacity, but also increased their intra-assay coefficient of variation (unpublished observations). Alternatives for storing coated plates in the research laboratory include freezing them at −20 °C, sealing them with silica gel at −4 °C, keeping them in boxes under vacuum, or keeping them vacuum sealed in individual containers. For commercial purposes, the plates are best kept in individual plastic or aluminium containers vacuum sealed at 4 °C. Under these conditions most coated plates are stable for up to two years and they can be stored at room temperature for months.

INCUBATION AND WASHING PROTOCOLS

Incubation

In solid-phase techniques, molecules must make contact with a surface. To increase the probability of theim doing so, diffusion distances should be low (as in microtitre wells), while time, temperature and concentrations should be high (Andersen, 1986).

Incubations at 37 °C give a physiologycal temperature that the molecules should be able to tolerate as well as a high mobility, so that equilibrium is soon reached. A common mistake, however, is to use a buffer or a sample coming from a cold room and then incubate it at 37 °C. In a microtitre plate of 96 wells, these changes mean a cooler inner portion and a warmer outer portion, which causes different diffusion velocities and high intra assay variation (Ekins, 1981).

Buffers, solid-phase and samples should, therefore, be used at the incubation temperature. A good alternative is to use room temperature for incubation. Although this slows down the assay, higher reproducibility can thus be obtained (unpublished observations). Using short-time incubations, small (less than 10 percent) but detectable differences are recorded between the first and last pipetted wells. Also, the unequal evaporation with longer incubation times (more than 1 h), between the center and the outside wells poses some problems which could be easily solved by performing incubations in home-made humid chambers (Bookbinder and Panosian, 1986). Incubation times should be optimized.

Solid-phase enzyme immunoassays have been plagued both by non specific binding to the solid-phase, necessitating multiple washing steps, and by slow reaction kinetics relative to reactants that are free to diffuse in solution. Some of the non-specific binding can be minimi-

zed by the dilution buffer used. (Hutchens and Porath, 1987). The dilution buffer contains the same bovine serum albumin batch use for blocking the plates, in high (0.5 %-1 %) concentration in relation to potentially interfering proteins, thus significantly reducing the binding of molecules which can add background to the system. Foetal calf serum (used because of the absence of immunoglobulins) or milk are not suitable for commercial dilution buffers due to their instability (unpublished observations). A non ionic detergent (Tween 20 or 80) is usually added to the buffers to prevent weak, non specific, hydrophobic interactions with the plastic surface (Engvall and Perlmann, 1972; Gardas and Lewartowska, 1988). The pH is not critical when close to physiological values and when polyclonal antibodies are used, but it has to be optimized if monoclonal antibodies, lectin, or other specific ligand interactions are used in the assay. Merthiolate is a good antimicrobial agent because of its stability and non-interference with the assay. Phenol red or any other colorant can be added to allow easy visualization of wells, pipetted onto the 96 wells plates, and it also serves for continuous monitoring of pH (Martínez and Coll, 1988). Quality control of the dilution buffer during production includes measuring the pH and the conductivity. The complete dilution buffer should be filtered to sterility and kept in appropiate tinghtly closed containers for kit presentation.

Because of the high detectability of many enzyme-immunoassays, in many assays the samples have to be diluted 1000-10000 fold in dilution buffer (Hamilton, 1987; Highton and Hessian, 1984; Kramer et al, 1987). Such high dilutions are time-consuming and very susceptible to error. To avoid these massive dilutions, the same immunoreactant that is bound to the solid-phase could be added to the dilution buffer. The added immunoreactant would compete with the analyte in the sample shifting the calibration curve to higher analyte concentrations, as it has been reported for particle-counting immunoassays (Collet-Cassart et al, 1983).

When using human serum for standards of the calibration curve, pooled serum negative for both hepatitis B surface antigen and HIV antibodies should be used. Standards are best prepared, when possible, by removing the analyte from the pooled serum, and then adding the purified analyte to the dilution buffer and to the diluted analyte-free pooled serum, used at the same dilution as that for the individual test samples (Martínez and Coll, 1987; Meyer and Keller, 1988).

Washing

Washing represents one of the most important problems of ELISA, incomplete washing causing interference in background and low reproducibility. Washing after conjugate incubation is the most critical. Homogeneous ELISA, where no washing is needed in order to measure the specific reaction, are not yet reproducible enough to be commercialized (Ekins, 1981).

Flooding and shaking with washing buffer is inadequate unless carefully done. Each well might allow droplets of buffer or air bubbles to be trapped and so prevent some wells from being filled, causing unequal washing of individual wells. By delivering buffer to each well, air bubbles may still form (Ashorn and Krohn, 1986). Removing the buffer from well to well is even more difficult. Inverting and shaking the plate might not be homogeneous unless done by vigorously rapping the plate on an absorbent towel placed on a flat surface. When working with infectious agents, however, a vacuum suction tube attached to a side-arm flask half-filled with a disinfectant solution should be used. More recently, automation has been introduced for washing, using several commercially available appliances which sequentially aspirate and fill all twelve wells in a row simultaneously. At each of the twelve positions there is an aspiration and a delivery tip. A vacuum pump aspirates the solution into a side-arm flask that may contain disinfectant. Some models can deliver measured amounts of washing buffer (25-300 µl per well) or can aspirate and fill the 96 wells simultaneously.

A large excess of either antigen or antibody is required to obtain a reasonable saturation of the antibody or antigen bound to the solid-phase (Engvall and Perlmann, 1972). Removing that excess during the washing steps dissociates the antibody-antigen complex to reestablish their equilibrium as dictated by their K value. Low affinities (10^{-6} M) result in rapid disocciation even with one washing, whereas, with high affinities (10^{-8}-10^{-9} M), the antibody-antigen complex is highly stable and the activities obtained are truly representative for initial concentrations. It is therefore recommended that high affinity antibodies be used in solid-phase ELISA (Tijssen, 1985). Cross reacting antigens (or antibodies) with lower affinities than the main ligand, elute more rapidly through repeated washing than the specific antigen (or antibody). Successive washings can, therefore, be beneficial for the elimination of cross reacting immunoreactants (Boscato and Stuart, 1988).

Choosing and adequate washing buffer can be of great help in reducing nonspecific reactions. Alternatives for the washing buffers go from tap water (Ashorn and Krohn, 1986) or deionized water (Delpech et al, 1987) to dilution buffer (Coll, 1987 c). An intermediate composition is often preferred for washing buffer either as diluted dilution buffer or incomplete (no albumin, for example) dilution buffer, because of its comparably higher reproducibility with tap water (tap water composition varies greatly from laboratory to laboratory and in the same laboratory even from day to day).

Kit presentation of the washing buffer poses problems because of the large volumes that are needed relative to the other components to be included into the kit. Some manufacturers choose to include the washing buffer in powder form (without Tween) whereas others prefer to use 10-20 fold concentrated solutions.

The optimal plate incubation and wash protocol will therefore include, short diffusion distances; an assay facilitated by not too low concentrations; a buffer with low viscosity; incubation at room temperature; prewarmed components; washing with a controlled buffer for an adequate number of times, and a not too short incubation time. Inter- and intra-assay reproducibilities should be less than 10 % CV, provided that standards are used with every run and the incubation times are long enough (at least 1 h) to minimize differences between opposite ends of the plates.

AMPLIFICATION STEP

Design

The amplification step is generally obtained by the reaction of the bound analyte with a specific antibody-enzyme complex, the conjugate. The conjugate is generally obtained by the covalent coupling of antibodies and enzymes (Avrameas, 1969). Good performance of conjugates largely depends on high specific activity or purity of both antibodies and enzymes (Ekins, 1981). Although some more complicated desings are theoretically possible (for example, use of universal anti-immunoglobulin antibodies species specific coupled to enzymes, use of avidin or streptavidin complexes, etc.), the use of specific antibody conjugates in commercial kits is most favoured because of added simplicity in handling (only one incubation-wash for this step) and interpretation (lowest number of controls). Alternatives include the use of protein A (Marril et al, 1986) or protein G (Nilson et al, 1986) coupled to enzymes.

The source and degree of purification of the antigen to be used to obtain the specific antibody; source and degree of purification of the specific poly-or monoclonal antibody (Fraeyman et al, 1987; Kelsten et al, 1988); source and degree of purification of the enzyme; coupling method; and degree of purification of the resulting antibody-enzyme complex (Boorsma and Streefkerk, 1976 a), all influence the final performance of the conjugates in the amplification step. Stability, specific activity, maximum ELISA signal and background (non-specific binding) are some of the measurable characteristics that have to be optimized in order to scale-up conjugate production with reproducibility (Engvall and Perlmann, 1972; Boscato and Stuart, 1988; Avrameas and Ternynck, 1969; Andersen, 1986; Clark and Price, 1987; Palomo et al, 1982).

Antibodies

Although many variation of solid- or liquid-phase, non-competitive or competitive binding immunoassay formats have been sucessfully used, the non-competitive two-site immunometric assay using two monoclonal antibodies was the most favoured in a recent review of human immunoglobulin quantitation (Hamilton, 1987). Ideally, antibodies used for the preparation of conjugates should be of the highest specific activity available, that is the highest ratio of specific antibody to total antibody. These are probably the monoclonal antibodies purified by affinity chromatography over solid-phase coupled antigen (Martínez and Coll, 1988). This method gave the highest yield (table I) with good performances and stability. Purification by affinity chromatography over protein A is probably the best alternative actually available (Marril et al, 1986).

The use of and new developments in monoclonal reagents (Campbell, 1984; Samoilovich et al, 1987; Rueda and Coll, 1988) have enabled the performance of assays in solid-phase (Schramm et al, 1987a); which are more specific than the corresponding polyclonal-based assays (Felder et al, 1987); they are very rapid (5 min) (Chandler et al, 1987); in only one step (Houwers and Schaake, 1987); have a high precision (Shahangian et al, 1987); with very low volumes down to 5 µl (Macario et al, 1986); are polipeptide chain specific (Iijima et al, 1988); stereospecific (Bjerke et al, 1986); using dry, ready-to-use reagents (Macario et al, 1987); and have the lowest background (Martínez and Coll, 1988).

Methods for the *in vivo* production of monoclonal antibodies in mice are used to obtain small volumes of highly concentrated antibody even when there is a demand for larger quantities (Iturralde, 1983; Iturralde and Coll, 1984). *In vitro* methods still yield concentrations that are several orders of magnitude lower than those produced *in vivo* (Reuveny et al, 1986a, 1986b; Vélez et al, 1986). Some methods designed to increase ascitic fluid production in mice have recently been reported. For example, large amounts of polyclonal antibody were obtained by inducing ascitic fluid with a combination of pristane, Freund's complete adjuvant, immunogen and non-producer myeloma cells (Lacy and Voss, 1986). Moreover, priming with incomplete Freund's adjuvant instead of pristane permitted production of larger amounts of monoclonal antibody in a short time, using low numbers of hibrydomas (Mueller et

TABLE I. **Yield of antibody-peroxidase conjugates by different methods of antibody purification**

Source	Purification	Yield
Goat antiserum	Total serum	2.5
Goat antiserum	Affinity chromatography	2.0
Rabbit antiserum	Total immunoglobulin	1.5
Mouse ascites	Total ascites	7.2
Mouse ascites	Affinity chromatography	66.6

The yield is expressed as ml of conjugate of the same titre per ml of antiserum or ascites (1/1000). Titre of conjugate was defined as the dilution at which an absorbance of 2 (A492-620 nm) was reached in the binding of human C-reactive protein to solid-phase phosphorylethanolamine at 160 mg/l of C-reactive protein as described in Martínez and Coll, 1987.
Antiserum from hyperimmunized goats and rabbits had about the same amount of antibodies against C-reactive protein (unpublished results).

al, 1986; Gillete, 1987). In another recent study, crosses between two different strains yielded mice producing four times more ascitic fluid than the BALB/c parent (Brodeur and Tsang, 1986). There are, however, some production problems when large amounts of antibody are required. For example, the maintenance of a large colony of animals for the purpose of collecting and processing milliliter quantities of ascitic fluid from each animal lacks industrial efficiency because this is a time-consuming effort. However, injection of sterile physiological saline, followed by extraction of the diluted ascitic fluid facilitates and speeds up further processing of ascitic fluid such as centrifugation. The procedure is especially useful for the recovery of large amounts of ascitic fluid for production purposes (Coll, 1987 b).

Before monoclonal antibodies became generally available, most enzyme immunoassays (Engvall and Perlmann, 1972) used the immunoglobulin fraction of a hyperimmune serum to prepare the conjugates. The best hyperimmune sera have only about ten percent of specific antibody (Palomo et al, 1982); which means that about a 10-fold decrease in specific activity is to be expected and that some background and/or croos reactivity problems probably cannot be avoided (Highton and Hessian, 1984; Ng et al, 1987; Plebani et al, 1986). The affinity purified polyclonal antibodies are also a good reagent with which to prepare conjugates though yield is not as high as with polyclonals (table I). The advantage of using pure, isolated antibodies for conjugates, is debatable since they exhibit potential instability and a tendency to aggregation (Snoijink, 1987). In addition, immunoglobulin fractions of antisera have been used by many laboratories with adequate results in respect to specificity, performance, and reproducibility.

Another way of obtaining conjugates with high specific activity is to use protein A (Marrill et al, 1986) or protein G (Nilson et al, 1986), both from *Staphylococcus aureus*, and coupled to the enzymes. Proteins of this kind specifically react with the Fc portion of some immunoglobulin classes and, therefore, they can be used to detect immunoglobulins (Sánchez Vizcaíno and Álvarez, 1987). The use of Lectin-antibody conjugates have also been described (Guesdon and Avrameas, 1983).

Enzymes

The relative detectability and nonspecific binding of the conjugates obtained with peroxidase, alkaline phophatase and β-galactosidase (the three enzymes most used in ELISA) varied with different assays (Ishikawa, 1983; Scharpe et al, 1987; Kato et al, 1975), substratechromogens (Al-Kaissi and Mostratos, 1983), and coupling reagents (Ishikawa, 1983; Tijssen, 1985; Boorsma and Streefkerk, 1976b). Optimization of enzyme, coupling and substrate must be performed for every type of assay (King and Kochoumian, 1979; Ford et al, 1978).

A comparison of the relative detectability for several enzymes, when considering the best substrates, methods of chemical conjugation and their relative costs, clearly favours peroxidase obtained from horseradish roots (Tijssen, 1985). Highly purified horseradish peroxidase is much cheaper than any other enzyme used to-date and, furthermore, it is easily conjugated by several methods; it is very stable either as a free enzyme or after coupling to the antibody and its activity is easily detected (Kramer et al, 1987). The uselfulness of peroxidase in ELISA is attested by its wide use in research laboratories. Because of simplicity, purity, price and commercial availability, the preparation of peroxidase conjugates is generally preferred in commercial kits (table I).

Coupling reagents

Two coupling reagents are currently being used quite frecuently, either glutaraldehyde (Avrameas, 1969) or periodate (Nakane and Kawaoi, 1974). Glutaraldehyde can be used in the one-step or two-step method (Boorsma and Kalsbeeck, 1975).

Although neither glutaraldehyde nor periodate are the only coupling reagents (Wold 1972; Weston et al, 1980; Endo et al, 1987; King et al, 1978; Tijssen, 1985; Deedler and de Water, 1981), because glutaraldehyde is very simple to use and very gentle, it continues to be the most popular for commercial purposes. Some disadvantages are: the general claim that antibody function is seriously affected during conjugation (Boorsma and Streefkerk, 1976 b); the heterogenicity of the molecular weight of the resulting conjugates (Tijssen, 1985); and the abundant free peroxidase remaining after the reaction (un published observations). Despite these reported problems, we found (Coll, 1987 c) that the use of old glutaraldehyde (polyglutaraldehyde) (Rembaum et al, 1978) gave results similar to those obtained when using periodate to obtain conjugates and, furthermore, the use of old glutaraldehyde was much more simple and reproducible for either poly-or monoclonal antibodies. The disadvantages of glutaraldehyde as a coupling reagent are few and are shared with other cross-linking agents. It is to be expected thant any chemical modification related to cross linking of amino acid side chains will have some effect in altering the activity of immunoglobulins, especially with monoclonal antibodies (Endo et al, 1987). Since the carbohydrate part of the immunoglobulins is not involved in their antigen-binding properties, its modification (as with the method for periodate) should not affect antigen binding. These theoretical considerations are being developed in the search for new methods of coupling (O'Shannessy and Quarles, 1987). The heterogenicity of the high molecular weight of peroxidase conjugates obtained by the glutaraldehyde method, can be viewed either as a disadvantage (for example, because of decreasing penetrability in immunohistology) or as an advantage (for example, to increase detectability in ELISA). Assays require optimization to reach a decision (Nakane, 1979; Montoya and Castell, 1987).

When confronted with reproducible scaling-up, further advantages of the one-step old-glutaraldehyde method were noted. The greatest volumes per batch could be obtained only by this method, which saved time and minimized the characterization steps to standarize the reagent for the largest possible number of kits. Stability was very good. The two-step old-glutaraldehyde procedures makes scaling-up to production more difficult and irreproducible.

Kit presentation

An optimum concentration of labelled antibody yielding maximum assay detectability exists, the exact value of which depends both upon the equilibrium constant of the antibody, and the extent of its non specific binding to the solid-phase (Engvall and Perlmann, 1972). An increase in specific activity will, in general, increase assay detectability if non-specific binding is low (Ekins, 1981). Most conjugate preparations are optimally used in 1000-10000-fold dilution of the concentration used for storage.

Storage of concentrated conjugates has generally been carried out at −20 °C in the presence of glycerol and protein in research laboratories. Several procedures for keeping highly concentrated peroxidase conjugates in research laboratories were found to be unsuitable for long-term storage. The best conditions were obtained by using ammonium sulphate precipitates at 4 °C for up to 2 years (Montoya and Castell, 1987). However, the clinical use of ELISA kits requires highly stable conjugates which can be stored at 4 °C but at ready-to-use highly diluted concentrations. Some of the practical problems associated with the storing of a few microlitres of conjugate include: buffer evaporation, the necessity of using very small containers, the difficulty in processing on a large scale, the need for reconstitution steps, and low reproducibility. The problem arises because the activity of diluted conjugates decays more rapidly than that of 100-1000 fold concentrated preparations. The concentration-dependent loss of activity of antibody-peroxidase conjugates seems to relate to the decrease in peroxidase activity (Shannon et al, 1966; Kay et al, 1967), since this loss could be prevented by the addition of heme in buffers containing 1 percent of protein. Inclusion of heme in the storage buffer preserved the peroxidase activity more efficiently than for similar conjugates kept without heme (Coll, 1987c). By using this method, the peroxidase conjugates could probably be included in the ELISA kit at the required dilution.

In order to increase volume and dilution, one could allow the free peroxidase to remain in the storage buffer; however, the elimination of free peroxidase permitted a reduction in background level in some systems. Lyophilization also presents problems because of the low volumes, reconstitution steps, standardization from batch to batch and non-reproducible loss of activity (unpublished results).

ENZYME ASSAY

Enzyme-substrate-chromogen combinations

Choice of an anzyme-substrate-chromogen alternative is important if optimal results are desired. The relative merits of some combinations have been studied (Ford et al, 1978; Deedler and de Water, 1981; Ishikawa, 1983) and substancial differences in both dose-response kinetics and assay reproducibility have been demonstrated. Given a workable assay, the sensitivity, the detectability and the difficulties for scale-up to production may also influence the choice. Two combinations are now most widely accepted for use in commercial ELISA kits: alkaline phophatase/p-nitrophenylphosphate (Zouali and Stollar, 1986) and horseradish peroxidase/o-phenylenediamine (Gallati and Brodbeck, 1982; Lambre and Kasturi, 1979; Voogdet et al, 1980). For the reasons put forward before, we favoured the peroxidase-o-phenylenediamine ELISA system; this will be discussed further.

Peroxidase/o-phenylenediamine

Peroxidase catalyses the reduction of H_2O_2 with the concurrent oxidation of a chromogen producing an optically measurable colour. Among the many chromogens tested, o-phenylenediamine seems to give the highest absorbance readings but also one of the highest backgrounds (Al-Kaissi and Mostratos, 1983). O-phenylenediamine is a very suitable hydrogen donor; the oxidized form (orange) can be measured at low concentrations at 450 nm (pH, 5) or at 492 nm (pH, 1-2) (Gallati and Brodbeck, 1982). For minimal background, o-phenylenediamine should be white, and stored in the absence of metal ions, in the cold and in the dark, since it is highly photosensitive. The source of o-phenylenediamine is also important. The HCl form considerably decreases the pH of citrate/phosphate buffers and thus lowers the activity. The use of the recently available commercial preweighted pills of o-phenylenediamine has increased reproducibility and simplicity.

The substrate generally used (H_2O_2) is not only a substrate but also an inhibitor for peroxidase (Tijssen, 1985). Optimal and reproducible results are obtained only in a limited concentration range. The real concentration of H_2O_2 can be obtained by absorbance at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$).

When the substrate buffer containing H_2O_2 and o-phenylenediamine are combined, any traces of iron and, above all any light, initiate the reaction and, in a few hours, the o-phenylenediamine is spontaneously decomposed. Most probably this is one of the reasons why the ELISA based on peroxidase/o-phenylenediamine system has high backgrounds and relatively high inter-assay variations (Gallati and Brodbeck, 1982). The low stability of the final mixture makes it necessary to keep the o-phenylenediamine separated from the rest of the substrate buffer components in commercial kits. Substrate buffers containing H_2O_2 are commonly stored by using tightly closed and dark containers.

TABLE II. **Comparison of a low-background substrate buffer with commercial buffers**

	Absorbances at 492 nm			
	Abbot	Pasteur	Behring	Substrate buffer
Spontaneous decomposition	0.67	0.58	0 .44	0.062
ELISA + CRP	0.90 ± 0.01	0.67 ± 0.01	0.66 ± 0.04	0.69 ± 0.04
ELISA - CRP	0.20 ± 0.03	0.10 ± 0.01	0.12 ± 0.03	0.07 ± 0.01
ELISA (%) background	(22)	(14.9)	(18)	(10)

Substrate buffer from Abbot (carcynoembryonic antigen kit) contained citrate/phosphate buffer pH 5.6 (pH 5 after addition of 1 mg/ml OPD). Substrate buffer from Pasteur (Kit for hepatitis B) contained 50 mM citric/citrate buffer pH 5.5 (pH 5 after addition of 1 mg/ml OPD). Substrate buffer from Behring (Kit for T 4) contained 100 mM citrate/phosphate buffer pH 5 (pH 4.5 after addition of 1 mg/ml OPD) it also contained sodium perborate, not H_2O_2. Low-background substrate buffer was 50 mM citrate 150 mM phosphate pH 5.5 (pH 4.5 after addition of 1 mg/ml OPD) 0.01 percent merthiolate, 1.5 mM acetanilide, 5 mM NTA, 0.14 β-mercaptoethanol and 3 mM H_2O_2. OPD used came from Sigma. All substrates were assayed by the ELISA as described (Coll, 1987c). Averages from duplicates and ranges are given. Spontaneous decomposition was measured after adding OPD to the substrate buffer, and exposing the mixture to 1 h of incident light (unpublished results).

Spontaneous decomposition of the o-phenylenediamine is lowe at low pH. On the other hand, peroxidase activity has an optimal pH which depends on the isoenzyme composition (Shannon et al, 1966; Kay et al, 1967). Therefore, it should be possible, to further decrease the background by using peroxidase isoenzymes of lower optimal pH (Porstmann et al, 1987).

To reduce the background of a peroxidase/o-phenylenediamine ELISA (Martínez and Coll, 1988) a systematic study of the effect of concentrations of o-phenylenediamine, H_2O_2, citrate and H^+ in the substrate buffer was undertaken. The addition of acetanilide (a stabilizer used for H_2O_2 preservation) and/or β-mercaptoethanol to the substrate buffers further delayed spontaneous decomposition. Nitrilotriacetate (an iron quelant) was also included to neutralize possible traces of iron. The resulting low-background substrate buffer reduced 5-10 fold the spontaneous decomposition (decreasing the inter-assay variation about 3-fold) and about 2-fold the percentage of background in ELISA as compared with other commercially available substrate buffers (table II).

Measurement of enzyme activity

Most tests are designed so that, after a short incubation period (for example, 30 min), the build-up of coloured product is enough for measurements. These procedures requiere the stopping of the reaction for all samples at the same time. For reproducible results, it appears important to ensure an equal distribution of enzyme products throughout the wells before the absorbance is measured. Within-assay variation can be greatly decreased simply by shaking the plates before measurement. The degree of variation also depends on the time of incubation with the enzymatic substrate, because the enzyme products become distributed by diffusion. Absorbance values could be 2-fold higher when the plate has been shaken because the enzyme products concentrated near the surface of wells are dispersed into the center, were the light path of the spectrophotometers is situated (Kemp et al, 1985). Reported alternatives include ultrasound to accelerate and distribute the enzyme products (Chen et al, 1984).

The enzymes most commonly used in ELISA are very effective catalysts; a single molecule of enzyme converts 10^3-10^4 even 10^6, molecules of substrate into product per minute. Hence, analytes can be detected down to femtomol levels (Puget et al, 1977). If analytes to be measured are in still lower concentrations, the methods used to increase detectability include: luciferin derivatives (quantified by a luminometric detection system) which allow determinations of about sixty-fold lower concentrations (Miska and Geiger, 1987); cascade systems in which the first enzyme produces a substrate for a second enzyme/substrate system with detectabilities about 30 to 50-fold higher (Carr et al, 1987); optimization of assay conditions such as increasing sample size, incubation time, reaction time, temperature, and decreasing final reaction volume with about a 5-fold increase in detectability (Shahangian et al, 1987); and photodensitometry which consists in photographing the plates and then densitometizing the photographs with a 5 to 10-fold increase in detectability (Labrousse and Avrameas, 1987). Other methods which in theory should be far superior, such as fluoroimmunoassay, have not demonstrated any improvement in detectability (Schmidt and Steinmetz, 1987). A new approach which would probably facilitate automation is the use of electrodes to detect the enzyme products. An electrode that detects phenol as the product of alkaline phosphatase has been described (Wehemeyer et al, 1985).

There has been no report so far on the theoretical potential of using double wavelenght measurements to detect two analytes in the same ELISA. However, dual staining has been used in histochemistry and in blotting. This was achieved either by sequentially applying two different enzymes, e.g., peroxidase and alkaline phosphatase (Falini, 1986) or different substrates for the same enzymes, e.g., peroxidase with 3,3' diaminobendizine, brown, 4-chloro-1naphtol, purple, and 5-bromo-4-chloro-3-indoxil phosphate, blue (Tabibzadeh and Gerber, 1986; Lee et al, 1988).

Automation and reporting results

ELISA is gradually proceeding towards automation. In the mean time progress is being made in several directions (La Belle, 1987; Quee and Johnson, 1984; Karpinski et al, 1987).

ELISA has been measured mainly by using end-point analysis.

This method is the simplest since the basic requirements to measure initial linear rates of reaction are difficult to obtain practicably. These include, uniform distribution of the chromogen, uniform temperature within the reading chamber, short reading intervals and highly automated data-processing capability. Although new photometric instruments with the above mentioned characteristics are beginning to be marketed, they are not yet in general use (Kung and Humphries, 1987).

The use of simplified calibration with standards is another indication of progress (Fraser, 1987). Meyer and Keller (1988) described a procedure to recalibrate preceding curves by using a single point. The validity of the procedure was demonstrated by comparing it to full calibration with several standards.

Computer programs are also now available which speed-up the processing of data. For example, Beatty et al (1987) described a computer program utilizing logit-log linear regression analysis of sigmoid serial dilution curves plus a weighted least-squares best curve fit analysis and an iterative manipulation to eliminate errant data points. On the other hand, three laboratories recently evaluated a highly automated commercial ELISA processor with good overall results. Parameters evaluated include, precision, interference, stability of conjugate, carry-over of reagents in the dispenser system, carry-over by the aspiration comb, efficiency in washing, linearity, spectrometer, dispenser and statistical procedures used by the processor (Steinmann et al, 1988).

Results in quantitative ELISA may be given in two forms, expressing the activity of the antiserum in international units or expressing the concentration of antigen (mg/ml). Standard unit-response curves must be constructed with aliquots of a highly positive serum or purified antigen diluted with a negative sample. The aliquots are then assayed, calibrated with international standards and expressed in international units. Calibration aliquots of a positive sample diluted with a negative sample should be then included as internal markers for between-run, between-laboratory and between-method normalizations to provide analytical consistency to the measurements.

The selection of a suitable cut-off value is important to minimize false responses in qualitative assays. This level can be moved upwards or downwards to incur more in false positive or in false negative errors, depending on which adjusment has less grave consequences. Results in the intermediate area classified as doubtful can be re-assayed in more replicates to increase the confidence of the result. The general use of standard deviation to define cut-off values is strictly speaking not valid because of the longer tail observed towards higher values in negative standards. Due to this effect false positives occur with significant higher frequency than expected from a normal distribution. Various systems have been proposed to determine cut-off levels taking account of the daily variations in a particular system. The cut-off level is sometimes set at 2 or 3 times the mean of the results with sera from the negative group, at the mean plus 2-3 standard deviations of the mean if a large number of normal reference sera could be tested or at 0.2-0.3 absorbance units. The percent of positives method expresses the absorbance of the test sample as a percentage of that of a positive reference sample measured simultaneously. Background absorbance mean value is sometimes added to that percentage. In each case it remains, nevertheless, necessary to decide at which level the response is to be considered positive by testing a large number of samples classified by other methods (Martínez and Coll, 1987).

STANDARDIZATION OF ASSAYS

ELISA is highly dependent on the quality of reagents for its sensitivity and specificity (Fraser, 1987). Where possible, purification procedures should be applied to antigens, antibodies, and conjugates so that reproducibility is assured (Bookbinder and Panosian, 1986). In commercial assays this is especially important since reproducibility is expected to be high not only between intra-and inter-assay but also from batch to batch (Brubaker et al, 1987). To-date, it has been necessary for users to select reagents on the basis of specifications provided by the manufacturer and then to undertake extensive "in house" checks of performance (Snoijink et al, 1987). Quality control and standardization are not yet functional realities in ELISA.

With the rapid acceptance of ELISA (Legros et al, 1988), more emphasis must be placed on preparing standard reagents and on formulating a unified standard procedure. No ELISA method can be succesfully applied until reagents are commercially available, which means that standardization and, therefore, quality control are required (Fraser, 1987; Fraser and Singer, 1985). International collaborative studies on standarization of reference material should be carried out more frequently (Smith et al, 1987; Brubaker et al, 1987; Zugaza et al, 1987; Legros et al, 1988; Steinmann et al, 1988). The use of reference material in enzyme immunoassays has the potential of greatly improving the world-wide comparison of measurements by adjusting to the requirements suggested by the World Health Organization Expert Committee on Biological Standards (Schramm et al, 1987a).

Some of the variables which must be reported, evaluated and standardized for each analyte (Delfert et al, 1987; Sung and Neeley, 1985; Frase and Singer, 1985) are:

1. The assay conditions, such as the solid-phase upon which the reaction is performed; the type and purity of the immunoreactant attached to the surface; the method of washing; the incubation conditions; the type of conjugate to be prepared with what enzyme; the effects and advantages of the various substrates or

chromogens; the method of reading and reporting results and the costs per unit of sample.

2. The characteristics of the calibration or standard curve including both the change of response per unit, amount of reactant or sensitivity which corresponds to the slope, and the lowest concentration of reactant which can be measured or detectability, which mainly depends on background (Karpinski et al, 1987; La Belle, 1987; Linnet, 1987; Meyer and Keller, 1988; Vos et al, 1987; Rodbard, 1978; Thibaut et al, 1987).

3. The variabilities or interference involved in the sera being tested, include serum dilution (Boscato and Stuart, 1988), immunoglobulins (Plebani et al, 1986; Clark and Price, 1987), excess of lipids (Martínez and Coll, 1987), inhibitors of coagulation, reconstitution experiments, and excess of specific, potentially interfering components such as rheumatoid factors (Highton and Hessian, 1984).

4. The precision, including intra-assay, inter-assay and batch to batch are expressed relative scatter of values around the mean which is normally expressed as the variation coefficient CV, in percentage (Bookbinder and Panosian, 1986; Fraser, 1987).

5. Accuracy, which includes studies with reference to a previous method or to a specific disease, to estimate the conformity of the new results with a standard or true value, respectively (Fraser, 1987; Linnet, 1987; Brubaker et al, 1987). These quantitative studies can be expressed as correlation coefficients and regression lines (Bookbinder and Panosian, 1987). The distribution of analyte values obtained by ELISA in samples from healthy individuals must also be compared to those obtained with previosly used assays (Coll, 1987a) and to those from individuals with diagnosed diseases. Clinical significance of the data can thus be evaluated (Zugaza et al, 1987; Thibaut et al, 1987) and cut-off values between negative and positive samples defined (Delfert et al, 1987).

6. Identification of the types of errors. Errors should be minimized by the design of the assay and its presentation form in a kit. Errors can be systematic, experimental or mistakes. A systematic error or bias, can be corrected by regular evaluation and quality control of reagents. These are for example, storage effects, improper calibration, non specific binding, inhibitors of enzymes, etc. Experimental or random errors are for example, temperature, evaporation, light conditions, composition of samples, etc. They can be corrected by including internal standards run with each assay. Mistakes are experimental errors that can be prevented by a simple design of the assay, clear labelling of each reagent or a good instruction manual. These are, for example, pippetting twice in the same well, bad reconstitution of reagents, poor washes, etc.

TABLE III. **Evaluation criteria for commercial ELISA**

Step	Concepts	Best solution
Solid-phase	Geometry	96-well polystyrene plates
	Flexibility	Plates divided in rows
	Stability	Vacuum closed containers
Incubation-washings	Sample preparation	10-100-fold optimal dilutions
	Controls	+/− included
	Stability	Merthiolate included
	Pippetting/pH	Colour indicator included
	Temperature	Room temperature
	Time	Incubations $\geq$ 30 min
	Simplicity	$\leq$ 3 washing
	Washing buffer	10-fold concentrated included
Amplification	Stability	500-fold concentrated
	Sensibility	Peroxidase/OPD
	Reproducibility	Monoclonal reagents
Enzyme-assay	Simplicity	Preweighted pills
	Interassay reproducibility	CV $\leq$ 20 %
	Intraassay reproducibility	CV $\leq$ 10 %
	Sensibility	Low background ($\leq$ 0.1)
	Interferences	Absent
	Linearity	At least 2 logs

CONCLUSIONS

Quantitation of antigens or antibodies by ELISA requires high inter-and intra-assay reproducibility and low background. To obtain these results all reagents must be tested, not only for performance but also for stability. To perform an ELISA in the research laboratory one must obtain all the reagents, freeze aliquots of the reagents that are not stable enough or prepare them fresh just before use. In addition, the analysis is performed by highly trained technicians in a well equiped laboratory with results interpreted by highly skilled personnel. In a clinical or veterinary laboratory resources are limited. At best, there is probably limited space at 4 °C, average level technicians and some pieces of normal equipment. Furthermore, samples to be assayed come daily in unpredictable numbers, and there is not much time to process them.

These are some of the reasons why the assay and the reagents for the practical every-day use of quantitative ELISA, should (table III):

Be stable at 4 °C and able to stand room temperature for limited periods of time.

Be presented in liquid solutions to avoid reconstitution steps that are unnecessary sources of errors and lead to additional working-time.

Be presented in a small and well identified number of bottles, preferably in the form of a kit containing all that is needed to perform the assay.

Be able to process a variable number of samples and to be automated.

Have a minimum number of steps and a short processing time.

Not require sophisticated equipment.

Be easy of interpretation.

Because of the high detectability, high stability of reagents and no required sophisticated equipment or disposal of residues, the ELISA technique has been the target of recent experimentation to produce kits that are more reproducible, more specific and more simple to employ, so that it will be used increasingly, with a higher degree of reliability and accuracy.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. José Ángel Martínez Escribano and Dr. Javier Domínguez Juncal, both at the Sanidad Animal of Department of the Instituto Nacional de Investigaciones Agrarias of Madrid, for reviewing the manuscript, Michael Austin Sevener for reviewing the English and Julio Coll Pérez for the typing and the drawing.

References

Ablin J. Improved enzyme immunoassay for factor VIII-related antigen (von Willebrand factor antigen). Clin Chem 1987; 33:1.935-1.936.

Andersen J. Guide to solid phase Immuno Assays. Kamstrup, Dinamarca, Nunc GMBH publications, 1986; 35.

Al-Kaissi E, Mostratos A. Assessment of substrates for horseradich peroxidase in enzyme immunoassay. J Immunol Methods 1983; 58:127-132.

Ashorn P, Krohn K. Washing of ELISA plates with running tap water. J Immunol Methods 1986; 88:141-142.

Avrameas S. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 1969; 6:43-52.

Avrameas S, Ternynck T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 1969; 6:53-66.

Beatty JD, Beatty BG, Vlahos WG, Hill LR. Method of analysis of non-competitive enzyme immunoassays for antibody quantification. J Immunol Methods 1987; 100:161-172.

Bjerke RJ, Cook G, Rychlik N, Gjika HB, Van Vunakis H, Langome JJ. Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. J Immunol Methods 1986; 90:203-213.

Boscato LM, Stuart MC. Heterophilic antibodies: A problem for all immunoassays. Clin Chem 1988; 33:27-33.

Bookbinder MJ, Panosian KJ. Correct and incorrent estimation of within-day and between-day variation. Clin Chem 1986; 32:1.734-1.737.

Bookbinder MJ, Panosian KJ. Using the coefficient of correlation in Method-Comparison studies. Clin Chem 1987; 33:1.170-1.176.

Boorsma DM, Kalsbeek GL. A comparative study of horseradish peroxidase conjugates prepared with a one-step and a two-step method. J Histochem Cytochem 1975; 23:200-206.

Boorsma DM, Streefkerk JG. Peroxidase-conjugate chromatography isolation of conjugates prepared with glutaraldehyde or periodate using polyacrylamide-agarose gel. J Histochem Cytochem 1976; 24:481-486 (a).

Boorsma DM, Streefkerk JG. Periodate or glutaraldehyde for preparing peroxidase conjugate. J Immunol Methods 1976; 30:245-255 (b).

Brodeur BR, Tsang PS. High yield monoclonal antibody production in ascites. J Immunol Methods 1986; 86:239-241.

Brubaker DB, Blick KE, Romine M. Four immunoassay methods and standards compareed for measuring fibronectin. Clin Chem 1987; 33:126-129.

Brukhardt AE. Chemical assays. New opportunities. Clin Chem 1987; 33:1.574-1.578.

Campbell AM. En: Burdon RH, Van Knippenberg PH, eds. Laboratory Techniques in Biochemistry and Molecular Biology. Monoclonal Antibody Technology. Amsterdam, Elsevier, 1984; 33-64.

Carr RI, Mansour M, Sadi D, James H, Jones JV. A substrate amplification system for enzyme-linked immunoassays. Demonstration of its general aplicability to ELISA systems for detecting antibodies and immune complexes. J Immunol Methods 1987; 98:201-208.

Chandler HM, Fuller SA, Nakagawa GH, Nagainis PA, Hurrell JGR. An accelerated enzyme immunoassay for human choriogonadotropin in urine, involving reflow of specimen through capillary tubes. Clin Chem 1987; 33:498-501.

Chen R, Weng L, Sizto NC, Osorio B, Hsu C, Rodgers R, Litman DJ. Ultrasound-accelerated immunoassay, as exemplified by enzyme immunoassay of choriogonadotropin. Clin Chem 1984; 30:1.446-1.451.

Clar PMS, Price CP. Removal of interference by immunoglobulins in an enzyme-amplified immunoassay for thyrotropin in serum. Clin Chem 1987; 33:414.

Coll JM. Microtitre latex C-reactive protein test for screening large numbers of sample. Clin Chem 1987; 33:1.258 (a).

Coll JM. Injection of physiological saline facilitates recovery of ascitic fluids for monoclonal antibody production. J Immunol Methods 1987; 104:219-222 (b).

Coll JM. Heme increases peroxidase-antibody activity in aged conjugates. J Immunol Methods 1987; 104:259-263 (c). Collet-Cassart D, Marschal JC, Sindic CJM, Tomasi JP, Masson PL. Automated particle-counting immunoassay of C-reactive protein and its application to serum, cord serum, and cerebrospinal fluid samples. Clin Chem 1983; 29:1.127-1.131.

Deedler AM, De Water RA. A comparative study on the preparation of immunoglobulin-galactosidase conjugates. J Histochem Cytochem 1981; 29:1.273-1.280.

Delfert DM, Rea MR, Kessler G, Siegfried BA, Valdés R. Criteria for evaluating nonquantitative assays: Application to serum choriogonadotropin. Clin Chem 1987; 33:150-153.

Delpech B, Bertrand P, Chanzy C. An indirect enzymoinmmunological assay for hyaluronidase. J Immunol Methods 1987; 104:223-229.

Ekins R. Towards immunoassays of greater sensitivity, specificity and speed. An overview. En: Albertini A, Ekins R, eds. Monoclonal antibodies and developments in Immunoassay. Holanda, Elsevier, 1981; 3-21.

Endo N, Umemoto N, Kato Y, Takeda Y, Hara T. A novel covalent modification of antibodies at their amino groups with retention of antigen-binding activity. J Immunol Methods 1987; 104:253-258.

Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-Immunoglobulin in antigen-coated tubes. J Immunol 1972; 109:129-135.

Falini B. Description of a sequential staining procedure for double immu-

noenzymatic staining of pairs of antigens using Monoclonal antibodies. J Immunol Methods 1986; 93:265-273.
Felder RA, MacMillan RH, Bruns DE. Two monoclonal-based assays for carcinoembryonic antigen compared. Clin Chem 1987; 33:700-704.
Ford DJ, Radin R, Pesce AJ. Characterization of glutaraldehyde coupled alkaline phosphatase-antibody and lactoperoxidase-antibody conjugates. Immunohistochemistry 1978; 15:237-243.
Fraeyman NH, Van de Velde EJ, De Smet FH. A monoclonal antibody-baed enzyme-linked immunosorbent assay of α_1-acid glycoprotein. Clin Chem 1987; 33:2.275-2.277.
Fraser CG. Desirable standards of performance for therapeutic drug monitoring. Clin Chem 1987; 33:387-389.
Fraser CG, Singer R. Better Laboratory evaluations of instruments and kits are required. Clin Chem 1985; 31:667-670.
Gallati VH, Brodbeck H. Peroxidase aus Meerrettich: Kinetisch studien uns optimierung der aktivitätsbestimmung mit den substraten H_2O_2 und o-phenylenediamine. J Clin Chem Biochem 1982; 20:221-225.
Gardas A, Lewartowska A. Coating of proteins to polystyrene ELISA plates in the presence of detergents. J Immunol Methods 1988; 106:251-255.
Gillete RW. Alternatives to pristane priming for ascitic fluids and monoclonal antibody production. J Immunol Methods 1987; 99:21-23.
Guesdon JL, Avrameas S. En: Langone JJ, Van Vunakis H, eds. Methods in Enzymology. Nueva York, Academic Press, 1983; 489-498.
Hamilton RG. Human IgG subclass measurements in the Clinical Laboratory. Clin Chem 1987; 33:1.707-1.725.
Hargreaves KA, Houghton RL, Monji N, Priest JH, Hoffman AS, Nowinski RC. Phase-separation immunoassays. Clin Chem 1987; 33:1.509-1.516.
Highton J, Hessian P. A solid-phase enzyme immunoassay for C-reactive protein: Clinical value and the effect of rheumathoid factor. J Immunol Methods 1984; 68:185-192.
Houwers DJ, Schaake J. An improved ELISA for the detection of antibodies to ovine and caprine lentiviruses, employing monoclonal antibodies in a one-step assay. J Immunol Methods 1987; 98:151-154.
Hutchens TW, Porath JO. Protein recognition of immobilized ligands: Promotion of selective adsorption. Clin Chem 1987; 33:1.502-1.508.
Iijima Y, Endo Y, Hata N, Fujita H, Unoki M, Miyai K. Highly specific enzyme immunoassay for the β-subunit of human thyrotropin with use of monoclonal antibodies. Clin Chem 1988; 34:98-102.
Ishikawa E. Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. J Immunoassay 1983; 4:209-327.
Iturralde MN. Obtención de anticuerpos monoclonales contra la proteína C-reactiva. Master Thesis. Universidad de Navarra, España 1983; 111.
Iturralde M, Coll JM. High stability hybrids producing monoclonal antibodies against human C-reactive protein. Rev Esp Fisiol 1984; 40:279-288.
Karpinski KF, Hayward S, Tryphonas H. Statistical considerations in the quantitation of serum immunoglobulin levels using the enzyme-linked immunosorbent assay (ELISA). J Immunol Methods 1987; 103:189-194.
Kato K, Hamaguschi Y, Fukui H, Ishikawa E. Enzyme-linked Immunoassay II. A simple method for synthesis of the rabbit antibody-β-D-galactosidase complex and its general applicability. J Biochem 1975; 78:423-425.
Kay E, Shannon LM, Lew JY. Peroxidase isozymes from horseradish roots. Catalytic properties. J Biol Chem 1967; 242:2.470-2.473.
Kemp M, Husby S, Jensenius JC. Enzyme immunoassay plates should be shaken before absorbance is measured. Clin Chem 1985; 31:1.090-1.091.
Kelsten ML, Chan DW, Bruzek DJ, Rock RC. Monitoring hepato cellular carcinoma by using a monoclonal immunoenzymometric assay for alpha-fetoprotein. Clin Chem 1988; 34:76-81.
King TP, Kochoumian L. A comparison of different enzyme-antibody conjugates for enzyme-linked immunosorbent assay. J Immunol Methods 1979; 28:201-210.
King TP, Li Y, Kochoumian L. Preparation of protein conjugates via intermolecular disulfide bond. Biochemistry 1978; 17:1.499-1.506.
Kramer BK, Jesse U, Ress KM, Schmulling RM, Risler T. Enzyme-linked immunosorbent assay for urinary albumin at low concentrations. Clin Chem 1987; 33:609-611.
Kroll MH, Ruddel M, Blank DW, Elin RJ. A model for assessing interference. Clin Chem 1987; 33:1.121-1.123.
Kung VT, Humphries GMK. Kinetic ELISA in microtiter plates. Clin Chem 1987; 33:1.573.
La Belle M. Computer assisted collection and analysis of enzyme-linked immunosorbent assay data. J Immunol Methods 1987; 103:9-14.
Labrousse H, Avrameas S. A method for the quantification of a colored or fluorescent signal in enzyme immusoassays by photodensitometry. J Immunol Methods 1987; 103:9-14.
Lacy MJ, Voss EW. A modified method to induce immune polyclonal ascites fluid in BALB/c mice using Sp2/0-Ag 14 cells. J Immunol Methods 1986; 87:169-177.
Lambre C, Kasturi KN. A microplate immunoenzyme assay for anti-influenza antibodies. J Immunol Methods 1979; 26:61-67.
Larsson PH, Johansson SGO, Hult A, Gothe S. Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays. I. Binding capacity and characteristics of grafted polymers. J Immunol Methods 1987; 98:129-135.
Lee N, Zhang S, Testa D. A rapid multicolor western blot. J Immunol Methods 1988; 106:27-30.
Legros N, Grillo F, Leclercq G. Assessment of Abbott's enzyme immunoassay for progesterone receptor in breast-cancer samples. Clin Chem 1988; 34:175-176.
Linnet K. Two-stage transformation systems for normalization of reference distributions evaluated. Clin Chem 1987; 33:381-386.
Macario EC, Jovell RJ, Macario AJL. Multiple solid-phase system for storage of dry ready-to-use reagents and efficient performance of immunoenzymatic and other assays. J Immunol Methods 1987; 99:107-112.
Macario EC, Macario AJL, Jovell RJ. Slide immunoenzymatic assay for human IgE (SIA-IgE). J Immunol Methods 1986; 90:137-141.
Marrill L, Motté P, Pernas P, Troalen F, Bohuon C, Bellet D. Evaluation of protocols for purification of mouse monoclonal antibodies. Yield and purity in two-dimensional gel electrophoresis. J Immunol Methods 1986; 90:25-37.
Martínez J, Coll J. Preliminary clinical studies on C-reactive protein quantification by ELISA. Clin Chem 1987; 33:2.185-2.190.
Martínez J, Coll J. Selection and clinical performance of monoclonal anti-C-reactive protein in ELISA quantitative assay. Clin Chim Acta 1988; 176:123-132.
Meyer HD, Keller H. One-point recalibration of heterogeneous enzyme immunoassays with non-linear calibration curves. Clin Chem 1988; 34:113-117.
Miska W, Geiger R. Synthesis and characterization of luciferin derivates for use in bioluminescence enhanced enzyme immunoassays. New ultrasensitive detection system for enzyme immunoassays. J Clin Chem Clin Biochem 1987; 25:23-30.
Montoya A, Castell J. Long-term stora-

ge of peroxidase-labelled immunoglobulins for use in enzyme immunoassay. J Immunol Methods 1987; 99:13-20.
Mueller VW, Hawes CS, Jones WR. Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. J Immunol Methods 1986; 87:193-196.
Nakane P. En: Nakamura RM, Dito WR, Tucker ES, eds. Immunoassays in the Clinical laboratory. Nueva York, Alan R. Liss, Inc., 1979; 81-87.
Nakane PK, Kawaoi A. Peroxidase-labeled antibody. A new method of conjugation. J Histochemistry Cytochemistry 1974; 22:1.084-1.091.
Ng ML, Rajna A, Khalid BAK. Enzyme Immunoassay for simultaneous measurement of autoantibodies against thyroglobulin and thyroid microsome in serum. Clin Chem 1987; 33:2.286-2.288.
Nilson B, Bjorck L, Akerstrom BO. Detection and purification of rat and goat immunoglobulin G antibodies using protein G-based solid-phase radioimmunoassays. J Immunol Methods 1986; 91:275-281.
O'Shannessy DJ, Quarles RH. Labeling of the oligosaccharide moieties of immunoglobulins. J Immunol Methods 1987; 99:153-161.
Palomo C, Martin SH, Coll JM. Obtención de anticuerpos monoclonales y posible uso en diagnóstico clínico. Immunologika 1982; 3:322-347.
Plebani A, Ugazio AG, Avanzini AM, Monafo V, Burgio CR. An enzyme-linked immunosorbent assay for cow's milk protein-specific IgE using biotinylated antigen. Avoidance of interference by specific IgG. J Immunol Methods 1986; 90:241-246.
Porstmann T, Porstman B, Evers U, Wietschke R, Nugel E, Schmechta H. Coupling of different isoenzymes of horseradish peroxidase influences the sensitivity of enzyme immunoassay. Biomed Biochim Acta 1987; 46:867-877.
Puget K, Michelson AM, Avrameas S. Light emission techniques for the microestimation of femtogram levels of peroxidase. Anal Biochem 1977; 79:447-456.
Quee CK, Johnson GF. Automation and analysis of the orthophenylenediamine (OPD)-horseradish peroxidase (HRP) indicator reaction for enzyme immunoassay (EIA). Clin Chem 1984; 30:1.055-1.056.
Rembaum A, Margel S, Levy JB. Polyglutaraldehyde a new reagent for coupling proteins to microspheres and for labeling cell-surface receptors. J Immunol Methods 1978; 24:239-250.
Reuveny S, Vélez D, Macmillan JD, Miller L. Factors affecting cell growth and monoclonal antibody production in stirred reactors. J Immunol Methods 1986; 86:53-59 (a).
Reuveny S, Vélez D, Miller L, Macmillan JD. Comparison of cell propagation methods for their effect on monoclonal antibody yield in fermentors. J Immunol Methods 1986; 86:61-69 (b).
Rodbard D. Statistical stimation of the minimal detectable concentration (sensitivity) for radioligand assays. Anal Biochem 1978; 90:1-2.
Rueda A, Coll J. Cloning myelomas and hybridomas in fibrin-clots. J Immunol Methods 1988; 114:213-217.
Samoilovich SR, Dugan ChB, Macario AJL. Hybridoma technology: New developments of practical interest. J Immunol Methods 1987; 101:153-170.
Sánchez-Vizcaíno JM, Álvarez MC. Enzyme Immunoassay Techniques, ELISA, in Animal and Plant diseases. 2.ª ed. París, Office International Epizooties and Instituto Nacional de Investigaciones Agrarias, 1987; 54.
Sankolli GM, Stott RAW, Kricka LJ. Improvement in the antibody binding characteristics of microtitre wells by pretreatment with anti-IgG Fc immunoglobulin. J Immunol Methods 1987; 104:191-194.
Scharpé S, Verkerk R, Sasmito E, Theeuws M. Enzyme Immunoassay of angiotensin and renin. Clin Chem 1987; 33:1.774-1.777.
Schmidt B, Steinmetz G. Time-resolved fluoroimmunoassay *vs* ELISA for determination of α_2-interferon. Clin Chem 1987; 33:1.070.
Schramm W, Yang T, Midgley R. Monoclonal antibodies used in solid-phase assays, as exemplified by progesterone assay. Clin Chem 1987; 33:1.331-1.337 (a).
Schramm W, Yang T, Midgley R. Surface modification with protein A for uniform binding of monoclonal antibodies. Clin Chem 1987; 33:1.338-1.342 (b).
Shahangian S, Fritsche HA, Hughes JI. A double-monoclonal immunoenzymometric assay for alpha-fetoprotein modified for increased analytical precision. Clin Chem 1987; 33:583-586.
Shannon LL, Kay E, Lew JY. Peroxidase isozymes from horseradish roots. Isolation and physical properties. J Biol Chem 1966; 241:2.166-2.172.
Smith SJ et al. An international collaborative study on standardization of apolipoproteins A-1 and B. I. Evaluation of a lyophilized candidate reference and calibration material. Clin Chem 1987; 33:2.240-2.249.
Snoijink JJ, Shields IG, Turner MW. Selection of ELISA reagents. J Immunol Methods 1987; 96:283-285.
Steinmann J, Milbrandt H, Steinkopf A et al. Multi-Centre Evaluation of the Behring ELISA processor II. J Clin Chem Clin Biochem 1988; 26:43-56.
Sung E, Neeley WE. A cost-effective system for performing therapeutic drug assays I. Optimization of the theophylline assay. Clin Chem 1985; 31:1.210-1.215.
Tabibzadeh SS, Gerber MA. Immunohistologic analysis of lymphoid cells by a rapid double staining method. J Immunol Methods 1986; 91:169-174.
Thibaut E, Amigorena S, Moncuit J, Fridman WH, Teillaud JL. Software for the quantitative evaluation of *in vitro* monoclonal antibody production from ELISA data. J Immunol Methods 1987; 104:15-24.
Tijssen P. Laboratory Techniques in Biochemistry and Molecular Biology. En: Burdon RH, Knippenberg PH, eds. Practice and Theory of Enzyme Immunoassays. Vol. 15. Amsterdam, Elsevier, 1985; 549.
Valkirs GE, Barton R. Immunoconcentration: A new format for solid-phase immunoassays. Clin Chem 1985; 31:1.427-1.431.
Vélez D, Reuveny D, Miller L, Macmillan JD. Kinetics of monoclonal antibody production in low serum growth medium. J Immunol Methods 1986; 86:45-52.
Vogt RF, Phillips DL, Henderson LO, Whitfield W, Spierto FW. Quantitative differences among various proteins as blocking agents for ELISA microtiter plates. J Immunol Methods 1987; 101:43-50.
Voogd CE, Van der Stel JJ, Jacobs IA. On the Mutagenic Action of some enzyme immunoassay substrates. J Immunol Methods 1980; 36:55-61.
Vos O, Klasen EA, Haaijman JJ. The effect of divalent and univalent binding on antibody titration curves in solid-phase ELISA. J Immunol Methods 1987; 103:47-54.
Wehmeyer KR, Halsall HB, Heineman WR. Hetereogeneous enzyme immunoassay with electrochemical detection competitive and sadwich-type immunoassays. Clin Chem 1985; 31:1.546-1.549.
Weston PD, Devries JA, Wrigglesworth R. Conjugation of enzymes to immunoglobulins using dimaleimides. Biochem Biophys Acta 1980; 612:40-49.
Wold F. Bifunctional Reagents. Methods Enzymol 1972; 25:623-651.
Zouali M, Stollar BD. A rapid ELISA for measurement of antibodies to nucleic acid antigens using uv-treated polystyrene microplates. J Immunol Methods 1986; 90:105-110.
Zugaza C, Rodríguez C, Mirabel JL, Ruiz R, Martínez C. An enzyme immunoassay method for HCG in serum evaluated. Clin Chem 1987; 33:1.256.