KITS OF ENZYME IMMUNOASSAYS IN MICROTITRE WELLS TO DETECT VIRUS AND VIRAL ANTIBODIES

J. M. COLL
Departamento de Sanidad Animal. CIT-INIA
Embajadores, 68. 28012 Madrid

SUMMARY

The solid-phase, heterogeneous enzyme linked immunosorbent assays (ELISA) for both viral antigens and antibodies (Coll, 1991a) are having a wide acceptance in clinical and veterinary laboratories. This is mainly due to the rapid assay, high detectability, high stability of reagents and no need of sophisticated equipment or disposal residues. The cell culture techniques used for amplification and the techniques most used for identification in virology such as neutralization and immunofluorescence and to a leser extent, immunoperoxidase, complement fixation, hemagglutination, electron microscopy, immunodifusion or radioimmunoassay, are being substituted by enzymeimmunoassays (immunodot and ELISA) and DNA probes after amplification by thermostable polymerases. 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. In kits these are specially important since intra-and inter-assay and inter-batch reproducibility are expected to be high. The efforts to scale up this technology would benefit not only the production of kits on a large scale but also the basic virus research into news appliations.

KEY WORDS: Kits
ELISA
Viruses
Viral antibodies
Microtitration
Plates

STEPS IN SOLID-PHASE ELISA

Solid-phase separation systems have the advantage of no additional manipulations to effect separation other than removal of unbound ligand in the liquid phase. Solid-phase can be applied to any immunoassay using labels (radioisotopic, fluorescent, chemiluminescent or enzymatic) for the amplification steps (Table 1). Microwell plates, the most standard of the commercial solid-phases, are not very well suited for radioimmunoassays but they are good for ELISA. In the future they will be used for fluorescent and luminescent immunoassays (Coll, 1991b), because of their extremely high sensitivity will allow to wide the range of

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TABLE 1

COMPARISON BETWEEN ELISA AND OTHER LABELED IMMUNOASSAYS

Comparación entre ELISA e inmunoensayos con otros marcajes

Characteristcs *Características*	Label *Marcaje*			
	Radioisotopic *Isótopos*	**Fluorescent** *Fluorescencia*	**Chemiluminescent** *Quemiluminiscencia*	**Enzymatic** *Enzimático*
Sensitivity /L *Sensibilidad*	pmol-fmol	pmol	pmol-fmol	nmol-pmol
Use in field *Uso en campo*	−	−	−	+
Stability of label *Estabilidad*	−	+	+	+
Possitibility of homogeneous assay *Ensayo homogéneo*	−	+	+	+
Iow M.W. labels *Bajo peso molecular*	+	+	+	−
Possibility of catalyzing labels *Marcaje catalizador*	−	−	+	+
Cheap instrumentation *Poca instrumentación*	−	+	+	+
Radiactive hazards *Peligro radiactivo*	+	−	−	−

All labeled immunoassays share common features irrespective of the nature of the label such as: some steps (Fig. 1), the use of high affinity antibodies, the use of a label to amplify the signal, the capacity for processing a large number of samples and the possibility of automation. The radioisotopic label is based on radioactivity disintegration. The fluorescence label is based on the light emitted by a fluorophore when excited with a light of another wavelength. The chemiluminescent label uses chemical redox reactions involving oxygen or peroxide and an oxidizable organic substrate in which energy is released as visible light. Enzyme label uses an enzyme to convert a colorless substrate into a colored product.

applications, specially of low molecular weight analytes (Table 2). These techniques however will demand special plates that have no influence in the emitted light (for instance, coloured plates).

ELISA is based on putting together the high specificity of antibodies with the high catalytic power of enzymes (Coll, 1991c). In solid-phase immunoassays, the solid-phase is first coated with one immunoreactant (viral antigen or antibody), then the sample containing the analyte is added and incubated to allow the specific antigen-antibody interaction to take place. Non-reacting sample is washed away. In the solid-phase ELISA, addition of excess of the enzyme-labeled antibody, incubation and washing of the excess non-reacted labeled reagent, follows. The solid-phase retained enzyme is then assayed by measuring the accumulation of a colored product. The amount of product in proportional to the amount of analyte in the sample. An analysis of the commercially most used designs in solid-phase ELISA, reveals four common steps (Fig. 1): attachment to the solid-phase, incubation with the sample, amplification and enzyme assay (Tijssen, 1985). A similar process could also be used with DNA probes (Coll, 1991d).

TABLE 2

SOME OF THE APPLICATIONS OF ELISA

Aplicaciones del ELISA

Assay *Ensayo*	Field *Campo*	Examples of applications *Ejemplos de aplicaciones*
Antibodies *Anticuerpos*	Diagnostic	—IgM and IgG during infection. —Immune complexes.
	Vaccines	—Assesing of immunization.
	Reagents	— Antibody levels during immunization. —Screening of monoclonal antibodies.
Antigens *Antígenos*	Diagnostic	—Virus (medicine, veterinary, plants) —Bacteria and mycoplasms. —Tumor antigens. —Some low molecular weight antigens (drugs, metabolites, hormones, etc.).
	Basic research	—Follow up of protein purification —Variability of viruses. —Etc.

The non-labelled immunoassay techniques (immunodiffusion, agglutination, etc.) are limited to the assay of analytes present at relatively high concentrations, since only in such cases, the resultant antibody-antigen complexes are sufficiently large to be detectable (Coll, 1987a; Coll, 1988c). The use of enzyme-complex reagents is indicated in all cases where a high level of detectability is needed. Their use avoids the dangers and precautions involved in working with radioactive isotopes and in addition, the change in the color of a substrate can be seen by eye, thus eliminating the requirement for expensive equipment to measure radioactivity, fluorescence or chemiluminiscence. These other techniques are of use when extremely high sensitivity is needed (for instance in the case of most low molecular weight antigens).

ATTACHMENT OF THE VIRAL IMMUNOREACTANTS TO THE SOLIDPHASE

Attachment of the viral immunoreactants depends on the type of solid-phase. Most solid-phase immunoassays can be performed in beads, tubes or microtitration plates. Beads are easier to coat because the possibility of simultaneous incubation in the same solution (Coll, 1987a). However, this procedure is only simple for the kit producer but not for the user. The user has to transfer and touch the coated surface to incubate the beads individually with the samples. Tubes have the advantage of set up the exact number desired for the assay, they are easier to handle and they offer greater flexibility for volumes (up to 4 ml). In both cases either, transfer to or pipetting of, solutions has to be done one by one.

The most used reaction vessel is the microtitration plate as it is inexpensive, convenient 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 reagents, and fixed order for each sample, thus avoiding mistakes and unnecessary labelling. To pipette solutions into the wells, simultaneous automatic pipettes could be used. Microtitre plates are made either of polystyrene or of polyvinyl plastic. In polyvinyl, stronger reactions can be obtained, and the plate can be cut into pieces but it is difficult to handle and to read. The rigid polystyrene is easier to use but produces weaker reactions, costs three times more, and a complete plate has to be used for each assay. However, in recent years polystyrene plates

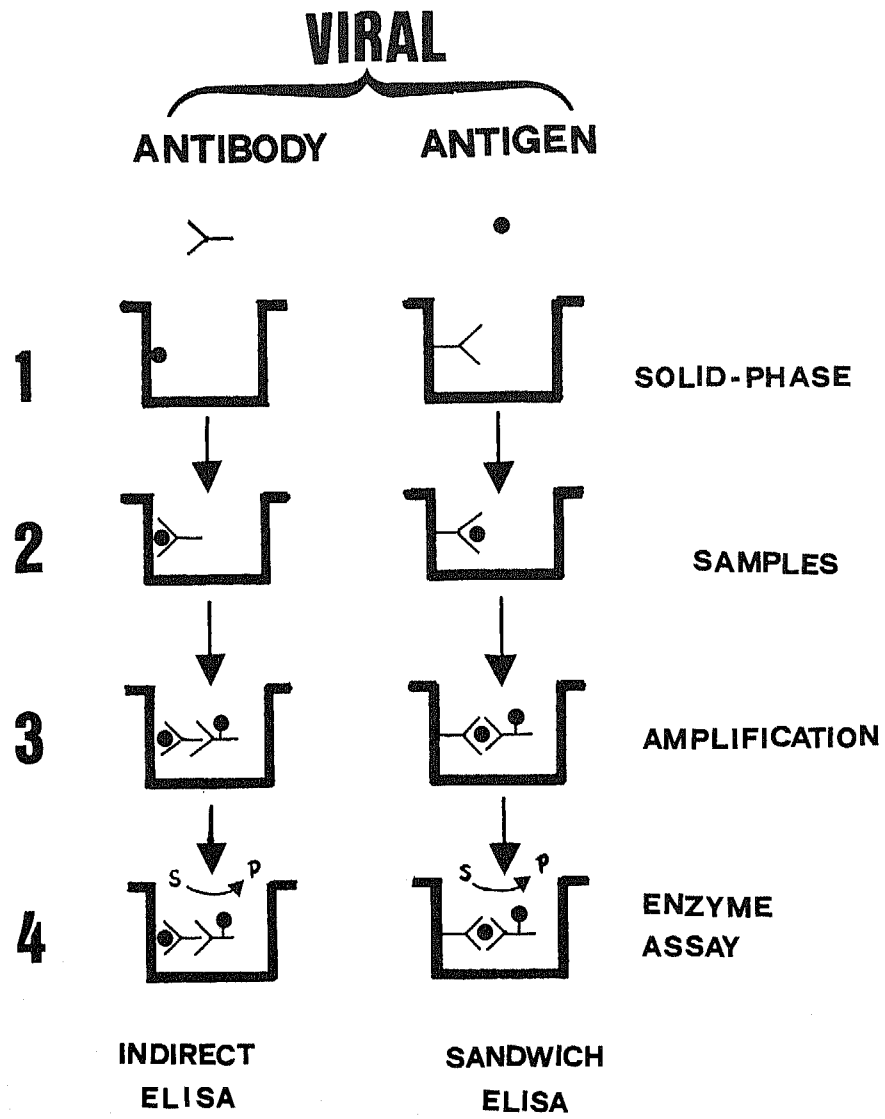

Fig. 1.—Scheme of enzyme immunoassays for antibody or antigen detection in microtitre wells.

Esquema de los inmunoensayos enzimáticos para detección de anticuerpos o antígenos en placas de microtitulación.

The ELISA is based on the addition of the samples containing the analyte (substance to be measured) to a solid-phase coated by an immunoreactant. After incubation, the excess of non-reacting analyte is eliminated by washing and a conjugate of antibody and enzyme is added to the solid-phase (amplification step). After incubation, the excess of conjugate is eliminated by washing and the bound enzyme is assayed. All non-competitive enzyme immunoassays have four common steps: (1) coating of the solid-phase with immunoreactant (antigen for the antibody assay, or antibody for the antigen, assay); (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 colored product by comparison with known calibrated standards. •, antigen; γ, antibody; γ•, conjugate; S, substrate; P, product. All labeled immunossays (radiosotopic, fluorescent, chemiluminescent and enzyme) share the first three steps.

divided in rows, which adapt the number of assays to the daily variable number of samples have appeared. New designs and/or materials will probably be evolved in this area as shown by the solid-phase generated in situ (Hargreaves *et al.*, 1987) or the microporous membranes (Valkirs, Barton, 1985).

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/or low ionic strength (Andersen, 1986; Harlow, Lane, 1988). Alternatives for scaling-up with high reproducibility, are the elimination of the distilled water used to dissolve the immunoreactant by incubation at 37 °C (Coll, 1989a) or by lyophylization. Assuming a maximum adsorption of 1,5 ng/mm^2 (in 200 µ per well of 7 mm of diameter) and 150 KDa for an immunoglobulin molecule, the maximum concentration for an antigen-affinity purified monoclonal antibody is about 10^{-8} M (Andersen, 1986). For affinity purified polyclonal antibodies, hyperimmune antisera or post infection sera, 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 maximun detectability when needed, depending on each particular case (Vos *et al.*, 1987). To increase detectability, plates coated with protein G (Nilson *et al.*, 1986), protein A (Schramm *et al.*, 1987), anti-immunoglobulin antibodies (Sankolli *et al.*, 1987), small peptides from viral relevant epitopes, specific binding proteins (Delpech *et al.*, 1987), or specific ligands (Coll, 1988b) are being used.

Although depending on each particular case, covalent attachment of the immunoreactant to the solid-phase has the reported advantages of increased capacity, added stability and higher reproducibility (Coll, 1991d). A 100-fold enhanced capacity has been demonstrated by using irradiation of both polystyrene and polyvinyl surfaces (Larsson *et al.*, 1987; Zoval, Stollar, 1986). Glutaraldehyde, either alone or with poly-lysine, has also been used to increase stability by covalent attachment (Martínez, Coll, 1987).

There is some variability as a result in the manufacture of the polystyrene plates which results in variability in their adsorptive properties. To test for the resulting reproducibility, a solution of conjugate can be adsorbed, the enzyme activity measured and the optical density and standard deviations of all wells of the same plate or of different plates can be compared. The values differ for different manufacture companies. Only those with < 5 p. 100 CV from plate to plate should be used (Tijssen, 1985).

To perform the coating on a large scale, automatic dispensing devices are needed so that variations from well to well and from plate to plate are low enough. For the coating, air forced incubators with homogeneous temperatures are preferred. By using both methods, variability due to coating could be 4 p. 100 from well to well and 6 p. 100 CV from plate to plate (Coll, 1991c; Martínez, Coll, 1988).

During incubation periods, all proteins will bind to a certain extent to the solid-phase after attachment of the immunoreactants. Methods to block nonspecific binding are designed both to occupy remaining binding sites after adsorption and to prevent adsorption of undesired molecules. In many cases, the blocking can be reduced to a washing step with an optimal buffer. The optimal blocking agent(s) for any particular ELISA system, must be determined by testing.

Plates kept in humid atmosphere for a few weeks, lose their binding capacity and increased intra-assay CV (Coll, 1989a). Alternatives for storing coated plates, include: freezing at − 20 °C, sealing with silica gel at 4 °C, or sealing under vacuum. The plates are best kept in individual plastic or vacuum-sealed aluminun containers. Under these condition most coa-

ted plates are stable for up to 2 years at 4 °C and they can be stored at room temperature for months (Harlow, Lane, 1988).

Incubation and washing protocols

To increase the probability of molecules contacting to the solid-phase, diffusion distances should be low (as in microtitre wells), while time, temperature and concentrations should be high.

Buffers, solid-phase and samples should be used at the incubation temperature. A good alternative is to use room temperature for incubation. Using short-time incubations, small but detectable differences are recorded between the first and last pipetted wells. Also, the unequal evaporation with more than 1h incubation times, between the center and the outside wells posses some problems which could be easily solved by performing incubations in humid chambers and with at least 30 min incubation times.

Some non-specific binding can be minimized by using the same bovine serum albumin batch used for blocking the plates in the dilution buffer in high (0.5-1 p. 100) concentration in relation to potentially interfering proteins (Harlow, Lane, 1988). Foetal calf serum (no immunolobulins) or milk are not suitable for dilution buffers due to their instability. A non-ionic detergent (Tween 20) is usually added to the buffers to prevent hydrophobic interactions with the plastic surfaces (Gardas, 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. Merthiolate is a good antimicrobial agent because of its stability and non interference with the assay. Phenol red can be added to allow visualization of wells pipetted and it also serves for continous monitoring of pH (Martínez, Coll, 1988; Sanz, Coll, 1992). Quality control of the dilution buffer during production includes measuring the pH and the conductivity. In addition, the complete dilution buffer should be filtered to sterility and kept in closed containers for kit presentation. Detectability of viral antigens can be dramatically increased by including dissociating agents in the sample buffer. For instance, the inclusion of high ionic strength buffers allowed more than 100-fold increase in detectability to assay for rhabdoviruses (Sanz, Coll, 1990, 1991a b).

When using human serum for standards, pooled serum negative for both hepatitis B surface antigen and human immunodeficiency viral antibodies should be used (Martínez, Coll, 1987, 1988).

Washing, specially after conjugate incubation, is one of the most important problems of ELISA, incomplete washing causing background and low reproducibility. Homogeneous ELISA'S were no washing is needed are not yet reproducible enough to be standarized. Flooding and shaking generates droplets of buffer or air bubbles which cause unequal washing. By delivering buffer to each well, air bubbles may still form. 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. Automation has been introduced for washing, using several commercially available appliances with sequentially aspirate and fill twelve wells in a row simultaneously.

To avoid any loss of sensitivity, an excess of conjugate concentration is required to obtain a saturation of the analyte (viral antibody or antigen) bound by the immunoreactant of the solid-phase. While removing that excess of conjugate during the washing steps, the complex could be dissociated. Under the same buffer and physical conditions, antibodies

with low affinities (10^{-6} M) tend to dissociate more rapidly than antibodies with high affinites (10^{-8}–10^{-9} M). Provided that near equilibrium had been reached after each wash, by using high affinity antibodies the measured activity is more representative of the initial analyte concentration. Since cross-reactivity of antibodies with non-specific molecules is very often of lower affinity, cross reacting antigens (or antibodies) with lower affinities than the main ligand, elute rapidly through repeated washing (Boscato, Stuart, 1988). This phenomenon is of value to increase specificity when polyclonal rather than monoclonal antibodies are used for the assays. Alternatives for the washing buffers go from tap water or deionized water to dilution buffer (Coll, 1989a).

Because of the large volumes of washing buffer that are needed relative to the other components to be included in 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.

AMPLIFICATION STEP

The amplification step is obtained by the reaction of the bound analyte with a specific antibody-enzyme complex, the conjugate. The conjugate is obtained by the covalent coupling of antibodies and enzymes. The use of specific antibody conjugates in 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 G (Nilson *et al.*, 1986) or protein A (Schramm *et al.*, 1987) coupled to enzymes. The non-competitive two-site immunometric assay using two monoclonal antibodies was the most favoured design in a review of ELISA quantitation (Hamilton, 1987). Ideally, antibodies used for the preparation of conjugates should be of the highest ratio of specific antibody to total antibody. These are the monoclonal antibodies purified by affinity chromatography over antigen. This method gave the highest yield (Table 3) with good performances and stability. Purification by affinity chromatography over protein A or G is probably the best alternative actually available (Campbell, 1984).

Some of the new developments in monoclonal antibodies (Coll, 1987b; Estepa *et al.*, 1990; Hurtado, Coll, 1986; Iturralde, Coll, 1984; Rueda, Coll, 1988; Sanz *et al.*, 1991) have enabled the performance of assays in solid-phase which are more specific than the corresponding polyclonal-base assays, only one step, have a high precision, with very low volumes are polypeptide chain specific (Jijima *et al.*, 1988), stereospecific (Bjerke *et al.*, 1986), use dry ready-to-use reagents and have the lowest background (Martínez, Coll, 1988).

Before monoclonal antibodies became available (Palomo *et al.*, 1982), enzyme immunoassays used the immunoglobulin fraction of a hyperimmune serum. The best hyperimmune sera have only about 10 p. 100 of specific antibody which means about a 10-fold decrease in specific activity and some background and/or cross reactivity problems. The affinity purified polyclonal antibodies are also a good reagent with which to prepare conjugates, though yield is not as high as with monoclonals (Table 3).

The relative detectatibily and nonspecific binding of the conjugates obtained with peroxidase, alkaline phosphatase and β-galactosidase (the three enzymes most used in ELISA) varied with different assays, substrate chromogens, and coupling reagents. Optimization of enzyme, coupling and substrate must be performed for every type of assay (Coll, 1988a).

A comparison of the relative detectability for those enzymes when considering the best

TABLE 3

YIELD OF ANTIBODY-PEROXIDASE CONJUGATES AFTER DIFFERENT METHODS OF ANTIBODY PURIFICATION

Rendimiento de conjugados anticuerpo-peroxidasa después de varios métodos de purificación de los anticuerpos

Source *Origen*	Purification *Método de purificación*	Yield *Rendimiento*
Goat mantiserum	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. 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 mono-clonal antibody, source and degree of purification of the enzyme; coupling method; and degree of purification of the resulting antibody-enzyme complex, all influence the final performance of the conjugates in the amplification step. Yield, stability, specific activity, maximun ELISA signal, and lowest background are some of the measurable characteristcs that have to be optimized in order to scale up conjugate production with reproductibility.

substrates, methods of chemical conjugation and their relative cost, clearly favours peroxidase obtained from horseradish roots (Tijssen, 1985). Any increase in specific activity will increase assay detectability if non specific binding is low and highly purified peroxidase can be easily obtained. Highly purified peroxidase is much cheaper, 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. The usefulness of peroxidase in ELISA is attested by its wide use in laboratories. Because of simplicity, purity, price and commercial availability, the preparation of peroxidase conjugates is generally preferred in the kits (Table 1).

Three coupling reagents are being used quite fraquently, either glutaraldehyde, biotin or periodate. Because glutaraldehyde is very simple to use and very gentle, it is the most popular. Some disadvantages are: the claim that antibody function is affected during conjugation, the heterogenicity of the molecular weight of the resulting conjugates, and the abundant free peroxidase remaining after the reaction. Despite these reported problems, we found that the use of one-step aged-glutaraldehyde (polyglutaraldehyde) (Coll, 1987) gave results similar to those obtained when using biotin or periodate. On the other hand, the disadvantages of glutaraldehyde as a coupling reagent are shared with other cross-linking agents. It is to be expected that 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. Since the carbohydrate part of the immunoglobulins is not involved in their antigen-binding properties, its modification should not affect antigen binding. These theoretical considerations are being developed in the search for new methods of coupling (O'Shannessy, Quarles, 1987). Assays will require some optimization to reach a decision in each case (Campbell, 1984).

When confronted with reproducible scaling up, further advantages of the one-step aged-glutaraldehyde method were noted. The greated volumes per batch could be obtained only by this method, which saved time and minimized the characterization steps to standarize the reagents for the largest possible number of kits. Stability was also very good (Martínez, Coll, 1987).

Most conjugate preparations are optimally used in 1,000-fold dilutions of the concentration used for storage. Storage of concentrated conjugates in research laboratories has generally been carried out at – 20° C in the presence of glycerol and protein. Several procedures for keeping kighly concentrated peroxidase conjugates in research laboratories were found to be unsuitable for long-term storage in the kit form. The best conditions were obtained by using ammonium sulphate precipitates at 4 °C for up to 2 years (Montoya, 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 dificulty in processing on a large scale, the need for reconstitution steps, and their low reproducibility. The problem arises because the activity of diluted conjugates decays more rapidly than that of 1,000-fold concentrated preparations. The concentration-dependent loss of activity seems to relate to the decrease in peroxidase activity, since this loss could be prevented by the addition of 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 the reduction of backgrounds. Lyophilization, because of the low volumes, reconstitution steps, standarization from batch to batch and non-reproducible loss of activity is not used (Coll, 1987c).

ENZYME ASSAY

The relative merits of some enzyme/substrate combinations have been studied and substantial differences in both dose-response kinetics and assay reproducibility have been demonstrated (Andersen, 1986). Given a workable assay, the sensitivity, the background, 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 ELISA kits: alkaline phosphatase/p-nitrophenylphosphate and horseradish peroxidase/o-phenylenediamine. For the reasons put forward before, the peroxidase/o-phenylenediamine ELISA system is favoured and this will be discussed further.

Peroxidase catalyses the reduction of H_2O_2 with the oxidation of a chromogen producing a measurable colour. Among the many chromogens tested, o-phenylenediamine seems to perform best. O-phenylenediamine is a hydrogen donor; the oxidized form (orange) can be measured at low concentrations at 450 nm (pH 5) or at 492 nm (pH 1-2). For minimal background, o-phenylenediamine should be white, stored in the absence of metal ions, in the cold and in the dark, since it is photosensitive. The HCl form should be avoided since decreases the pH of citrate/phosphate buffers and thus lowers the activity. The use of commercial preweighted pills has increased reproducibility and simplicity (Hamilton, 1987).

The substrate generally used is H_2O_2 which is unstable in stock solutions and is also photosensitive. Since H_2O_2 is not only a substrate but also an inhibitor for peroxidase, 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 ($E = 43.6\ M^{-1}/cm^{-1}$). When the substrate buffer containing H_2O_2 and o-phenylenediamine are combined, any traces of iron and 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 has high backgrounds and relatively high inter-assay variations. The low stability of the final mixture makes it necessary to keep the o-phenylenediamine separated from the rest of the components in the kits. Substrate buffers containing H_2O_2 are commonly stored by using tightly closed and dark containers (Coll, 1989b).

Since spontaneous decomposition of the o-phenylenediamine is lower at low pH and peroxidase activity has an optimal pH which depends on the isoenzyme composition it should be possible, to decrease the background by using peroxidase isoenzymes of lower optimal pH (Portsmann, 1987).

Most tests are designed so that, after a 30 min incubation period, the build-up of coloured product is enough for measurements. These procedures require the stopping of the reaction for all samples at the same time. The degree of variation also depends on the time of incubation, because the enzyme products become distributed by diffusion. For instance, within-assay variation can be decreased simply by shaking the plates before measurement. Absorbance values could be 2-fold higher when the plate has been shaken because the enzyme products concentrated near the surface of the walls of the wells are dispersed into the center, were the light path of the spectrophotometers is situated. Reported alternatives include ultrasound to accelerate and distribute the enzyme products.

In a solid-phase immunoassay there is a proportionality between the number of bound molecules and the number of molecules detected. This proportionality is responsible for the sensitivity of the analysis where background also plays an important part. When background increases, it is difficult to distinguish samples containing low concentrations of analyte. Background is favoured by a multitude of factors, such as, endogenous enzymatic activity in the samples, non-specific binding of labeled reagents, spontaneous decomposition of substrate, cross-reactivity of reagents, etc. A major factor affecting the sensitivity of ELISA in the most used designs shown in Fig. 1, is the enzyme activity. A single molecule of enzyme converts 10^5 molecules of substrate into product per minute. Hence, analytes can be detected down to femtomol levels. In the case of viruses, Table 4 shows the ranges of maximal sensitivity obtained. If the analytes to be measured are in still lower concentrations, some of the methods used to increase detectability include: luciferin derivatives, cascades in which the first enzyme produces a substrate for a second enzyme, optimization of the assay such as increasing sample size, reaction time, temperature, or decreasing volume and photodensitometry which consists in photographing the plates and then densitometizing the amplified photographs. A new approach which would probably facilitate automation is the use of electrodes to detect the enzyme products (Wehmeyer *et al.*, 1985).

There has been no report so far on the potential of using double wavelength measurements to detect two viruses or anti-viruses 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 (Falini, 1986), or different substrates for the same enzyme (Lee *et al.*, 1988).

ELISA is gradually proceeding towards automation (Catty, Raykundalia, 1989), ELISA has been measured mainly by using end-point analysis 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, Humphries, 1987). The use of simplified calibra-

TABLE 4

COMPARISON OF SENSITIVITY OF SANDWICH ELISA FOR THREE VIRUSES

Comparación de la sensibilidad de sandwich ELISA para 3 virus

VIRUS	Ab+ µg/well	PV ng/ml	CCS TCID/ml	TE* TCID/g
IPN	0.5 P	10	10^5	10^4
	100 P	50	10^4	10^4
	1.0 P	–	10^3	–
	0.2 M	10	10^3	–
VHS	100.0 P	–	10^5	10^5
	1.0 M	1	10^5	10^5
	1.0 M	0.2	10^3	10^3
IHN	100.0 P	–	10^5	10^6
	100.0 P	–	10^6	10^6

Sensitivity was defined as the minimal amount of virus that produced double absorbance than the background. PV, purified virus; CCS, cell culture supernatant; TE, tissue extract. +, Each well was coated with 100 µl by humid absortion at 37°C with the immunoglobulin fraction. *, For IHNV and VHSV calculated from 5 x 10^6 TCID/g of tissue and 5-fold diluted extracts. P, polyclonal; M, monoclonal, Ab, antibody; –, not determined. From Sanz and Coll, 1991b. IPN, infectious pancreatic necrosis; VHS, viral haemorrhagic septicaemia; IHN, infectious haematopoietic necrosis. $TCID_{50}$, tissue culture infective dosage at 50 p. 100.

tion with only one standard is another indication of progress (Meller, Keller, 1988). Computer programs are also available which speed-up the processing of data. Three laboratories evaluated an automated commercial ELISA processor with good overall results. Parameters evaluated include, precision, interference, stability of conjugate, carry-over of reagents in the dispenser system or in the aspiration comb, efficiency in washing, linearity, spectrometer, and statistical procedures (Steinmann *et al.*, 1988).

CONCLUSIONS

The most practical viral diagnosis is still obtained through isolation of virus in monolayers of cells, followed by neutralization of the virus with PAbs. Unfortunately, this is also be most time consuming technique. Immunofluorescence has provided a rapid yet specific alternative, however it is much less sensitive. Detection of virus by ELISA with MAbs depends on the stage of infection (acute) and on the onset of host Ab formation. Virus titre (either alive or non-infectious) may be depresed below the sensitivity margin in pooled samples containing organs of Ab-producing animals, therefore being advisable to examine individual rather than pooled organ samples in such cases.

Quantitation of viral antigens or antibodies by ELISA requires high inter-and intra-assay reproducibility and low background (Sanz, Coll, 1991a y b). 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. Kits must be able to solve all these problems. The study and use of procedures to produce kits is of mutual benefit to both research and development laboratories. New ideas first appear in research laboratories and then are scale up in development laboratories. The adaptation of the new assays to the kit phylosophy involves, mainly, increasing the stability of reagents, the reproducibility and the simplicity of manipulations. All these, are very valuable to the research laboratory which in turn will work in new ideas faster and easier than before.

Provided that all the above considerations are met most of the sandwhich ELISA based on monoclonal antibodies would be able to detect virus in acute-cases (high virus concentration), however to detect virus in carrier-cases, other techniques such as the ones based on viral genome amplification by polymerase chain reactions (Sobrino *et al.*, 1989) could only be used.

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RESUMEN

Los ensayos inmunoenzimáticos en fase sólida (ELISA) para antígenos y anticuerpos virales están teniendo amplia aceptación en laboratorios tanto clínicos como veterinarios. Ello es debido a la rapidez, sensibilidad, estabilidad de reactivos, simplicidad de aparataje y no existencia de residuos. Las técnicas de cultivo celular usadas para la amplificación y las técnicas más usadas para la identificación virológica, tales como la neutralización y la inmunofluorescencia, además de otras menos usadas, como la inmunoperoxidasa, fijación de complemento, hemaglutinación, microscopía electrónica, inmunodifusión o radioinmunoensayo, están siendo sustituidas por enzimoinmunoensayos (inmunodot y ELISA) y por sondas de DNA. La técnica ELISA ha sido el objetivo de la reciente experimentación para producir kits más reproducibles, específicos y sencillos de utilizar. Ello redundará en un incremento de su uso con una mayor fiabilidad y exactitud. En el desarrollo de kits, todo ello es importante debido a que la reproducibilidad intra e inter ensayo, además de la inter lote, deben ser altas. Los esfuerzos para escalar esta tecnología beneficiarán no sólo la producción de kits a gran escala, sino también la investigación básica de virus y las nuevas aplicaciones.

PALABRAS CLAVE: Kits
ELISA
Virus
Anticuerpos antivirales
Placas

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