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A Practical Guide

Edited by
Brian Law



Immunoassay

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Enzyme-linked immunosorbent assay (ELISA) development and optimisation

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Introduction

The term ELISA (enzyme-linked immunosorbent assay) was introduced by Engvall and Perlmann (1971) to describe a subset of the widely used immunoassay technique. ELISAs are distinguished from other immunoassays such as RIA by the use of an enzyme label linked either to the antigen or the antibody. This label in conjunction with a suitable substrate produces the assay signal. ELISAs are distinguished from other enzyme immunoassay (EIA) methods by the fact that one of the reagents is bound to a solid phase. In practice this solid phase usually takes the form of a 96-well microtitre plate. This gives the ability to handle many samples at one time and it facilitates automation of the assay. For a full discussion on all aspects of EIA the reader is referred to a number of books on the subject (Tijssen, 1985; Kemeny and Challacombe, 1988; Kemeny, 1991).

Many different enzymes have been used as tracers in ELISAs (Gosling, 1990; Porstmann and Kiessig, 1992), including urease, alkaline phosphatase, horseradish peroxidase (HRP) and β -galactosidase. Each of these can be employed with a number of substrates to generate an assay signal which usually takes the form of a coloured dye. Substrates which yield fluorescent or chemiluminescent products have also been used (Gosling, 1990).

The ELISA technique has principally been applied to the determination of proteins and other large-molecular-weight species. However, two formats have also been developed for the analysis of low-molecular-weight analytes of less than 1000 Dalton (Tijssen, 1985). These consist of one with the antigen as the immobilised component and the other with the antibody immobilised.

In the immobilised antigen ELISA, the analyte (or a suitable hapten) is linked to the solid phase indirectly. This linking is effected by preparing a hapten-protein conjugate which can be physically adsorbed onto the surface of the solid phase. The design and synthesis of this hapten-protein conjugate is crucial to the successful development of the ELISA.

There are many more steps in an antigen-immobilised ELISA than in a comparable RIA and these are shown in Figure 7.1. The first stage of the assay involves adsorbing the hapten-protein conjugate (plate conjugate) to the solid phase, which is known as sensitisation. The plate is then washed and vacant binding sites on the plate are often blocked by a suitable inert protein which is included in the buffer. The plate is then washed again and analyte-specific antiserum is then incubated in the wells together with standards or samples containing the analyte. This is the primary antiserum incubation. The antiserum used in this incubation is diluted such that the amount of antibody is limited compared with the amount of immobilised antigen bound to the plate. Added analyte then competes with the immobilised antigen for the antibody binding sites. The amount of antibody bound to the immobilised antigen at equilibrium is inversely related to the concentration of added analyte. Following a further washing stage (which is effectively the separation of bound and free) the enzyme-labelled antibody is added to detect the primary antibody bound to the immobilised antigen. This is known as the second antibody incubation. The plates are further washed and a response developed using a suitable substrate. A calibration curve is obtained similar to that of a competitive RIA with decreasing colour for increasing analyte concentrations.

The assay format with the analyte immobilised has a number of advantages over the alternative which are worthy of discussion. In the immobilised antibody approach an analyte-enzyme conjugate has to be prepared for every newly developed assay. When employing the immobilised antigen approach recommended here however, the enzyme-labelled second antibody can be obtained commercially. Furthermore, if the specific or first antibody is always raised in the same species, then the same enzyme-labelled second antibody can be used with every assay. In effect it is a 'universal tracer', lending significant advantages of cost and convenience. With the immobilised antibody approach it may be necessary to purify the antiserum before this can be linked to the solid phase, especially when only a small population of the specific antibodies are present in the sera. When the antibody is immobilised

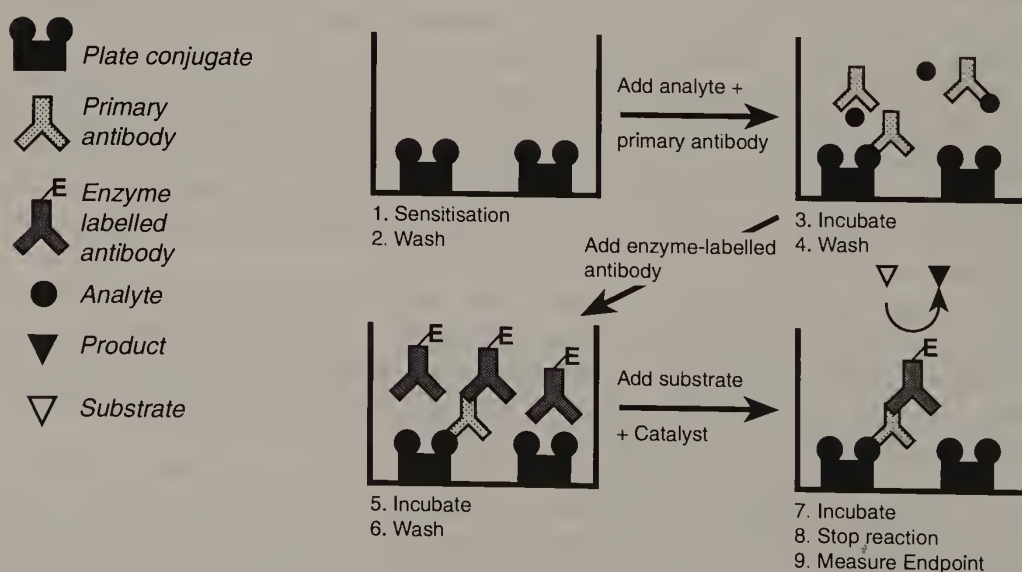


Figure 7.1 Schematic diagram for an immobilised antigen ELISA.

on the plate, there is often a significant reduction in affinity (Arends, 1971) coupled with a dramatic reduction in binding capacity (Butler *et al.*, 1992). The denaturation of the antibody which leads to the loss of binding can obviously occur with other proteins (Pesce and Michael, 1992), however with multivalent plate conjugates this should be less of a problem. Finally with the immobilised antigen method, the matrix is washed away before the enzyme-antibody complex is added. In the antibody immobilised format the enzyme-labelled antigen and the matrix are present simultaneously (Figure 7.2). Thus should the sample contain any noxious agents or irreversible enzyme inhibitors then the resultant signal could be affected and a spurious result obtained.

The immobilised antigen approach has been used extensively and successfully in the authors' laboratories and the development of ELISAs employing this approach will form the basis of this chapter. Initially the reagents and equipment particular to ELISA will be described, followed by a discussion of the major stages of assay development. Five major stages will be considered, these are:

- 1 The selection of a suitable carrier protein and production of the plate conjugate
- 2 Selection of the initial assay conditions
- 3 Assessment of the antisera
- 4 Introduction of matrix
- 5 Optimisation of assay conditions with matrix present

This chapter will concentrate in the main on stages 1, 2 and 5 in the list above, since these aspects of assay development are specific to the ELISA format. The conditions for the preparation and purification of the plate conjugate are essentially the same as those for the preparation of immunogens (Chapter 3) although some important differences will be outlined.

The assessment of the antiserum and the subsequent development and in part the optimisation (steps 3 to 5 above) are similar to those described in Chapter 6 for RIA.

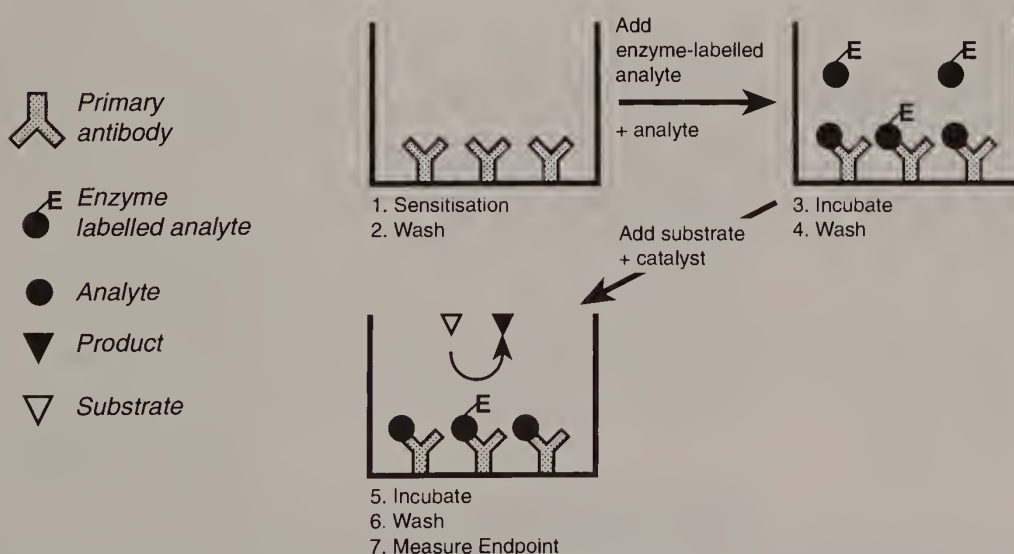


Figure 7.2 Schematic diagram for an immobilised antibody ELISA.

Reagents and buffers for ELISA

Enzyme-labelled antibodies

Although it can be relatively simple to prepare antibody–enzyme conjugates (see Tijssen, 1985; Kemeny, 1991 for methods), for those delving into ELISA for the first time the use of pre-prepared reagents is recommended. A wide range of enzyme-labelled anti-IgG reagents are commercially available for use as indicator reagents in immobilised antigen ELISAs. The most common of these contain HRP, alkaline phosphatase or β -galactosidase. These are available linked to IgG (often from donkey) and directed against IgG from the major species used for reagent antibody production, i.e. sheep, rabbit and goat.

Each of these enzymes can be utilised with a variety of substrates. Most of the commonly used substrates give rise to soluble coloured products which can be readily detected by spectrophotometric means. Table 7.1 lists the three most commonly used enzymes along with their possible substrates. HRP is probably the most popular, and its use is recommended here and by other workers (Porstmann and Kiessig, 1992) principally because of its low cost and the fact that it gives a stable coloured end product.

Some workers have used substrates which give rise to fluorescent or chemiluminescent products (e.g. Shalev *et al.*, 1980). For example, 4-methylumbelliferyl derivatives can give reaction products with alkaline phosphatase that can be detected at 100-fold lower concentrations than the conventionally used nitrophenyl phosphate. The increased sensitivity of detection for these materials however is rarely converted into a comparable increase in assay sensitivity (Porstmann and Kiessig, 1992) and hence their use is not recommended at this stage.

Table 7.1 Commonly used enzymes in ELISA and their substrates

Enzyme	Substrate	Comment
Alkaline phosphatase (EC 3.1.3.1)	<i>p</i> -Nitrophenyl phosphate	Enzyme present at low levels in serum
Horseradish peroxidase (HRP) (EC 1.11.1.7)	<i>m</i> -Phenylenediamine/H ₂ O ₂	Carcinogen
	<i>o</i> -Phenylenediamine (OPD)/H ₂ O ₂	Mutagen
	2,2'-Azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) disodium salt (ABTS)/H ₂ O ₂	Mutagen
	3,3',5,5'-Tetramethylbenzidine (TMB)/H ₂ O ₂	Non-carcinogenic*
	<i>o</i> -Toluidine/H ₂ O ₂	Non-mutagenic*
	<i>o</i> -Dianisidine/H ₂ O ₂	Carcinogen?
β -Galactosidase (EC 3.2.1.28)	<i>p</i> -Nitrophenyl galactose	Carcinogen
	4-Methylumbelliferyl- β -D-galactose (MUG)	Chemiluminescent Enzyme not normally present in mammalian fluids Fluorescent

* Bos *et al.* (1981).

Most manufacturers of indicator antisera suggest dilutions to be used with their reagents. However, whilst the recommended dilution may give an acceptable rate of colour development, this may be at the expense of a high degree of non-specific colour. It is recommended that the most appropriate dilution for a particular assay/reagent combination is investigated and optimised.

In our experience manufacturers often make 'improvements' to their products, such as changing the enzyme:antibody ratio or providing the reagent in a purer form. These changes may alter the non-specific binding characteristics of the product and also the rate of colour development. In such circumstances we have usually found it necessary to re-optimize the incubation conditions for the enzyme-labelled reagent. It is strongly recommended therefore that all new preparations are checked, and if necessary the appropriate assay conditions re-optimised to regain the required assay characteristics.

Assay buffers

A wide variety of buffers are used in ELISAs, including those for sensitisation, primary and secondary incubations as well as for blocking, washing and developing the colour. Although there appears to be a great degree of latitude in the type of buffers that can be used, on occasions even for the sensitisation buffer (Geerligs *et al.*, 1988; Porstmann and Kiessig, 1992), the use of the appropriate buffer at certain stages can be important for successful assay development. For example, the colour development buffers often have their own specific characteristics, especially with respect to the pH optima (Porstmann and Kiessig, 1992). Because sodium azide can be an inhibitor of HRP it is not generally used as a preservative in the substrate development buffer, and for convenience many workers prefer to avoid this preservative completely when working with this enzyme. Where a preservative is required, thiomersal at a concentration of around 0.1 per cent can be used. In practice however we do not use a preservative but prepare buffers on a weekly or 2 weekly basis.

Detergents are included in all buffers (with the exception of the sensitisation buffer) where they act to reduce non-specific binding of proteins to the plastic surface (Engvall and Perlmann, 1972). Their action is thought to be the result of a reduction of the surface tension of the incubation media, thus inhibiting hydrophobic interactions between proteins from the reagents, sample or buffers with the uncoated plastic surface. If a detergent is used effectively then it may be possible to omit the use of a blocking buffer (Bullock and Walls, 1977). However the detergent concentration should be kept as low as possible since too high a concentration may interfere with the antibody-antigen binding.

A variety of detergents have been used in ELISAs including the Tweens: 20, 40 and 80 as well as Brij 35 and Triton X-100. By far the most widely used detergent is Tween 20 which is effective at concentrations of less than 0.05 per cent.

Sensitisation buffers

For sensitisation of the plate most workers employ a carbonate buffer (pH 9.8, 0.1 M). Some workers claim adsorption to be relatively independent of pH, ionic strength or buffer type (Geerligs *et al.*, 1988; Porstmann and Kiessig, 1992) whilst

others hold the opposite view (Tsang *et al.*, 1980). We have found phosphate buffer (pH 7, 0.1 M) with or without the addition of sodium chloride to be useful in a number of assays and this will be used here.

Incubation buffers

The nature of the incubation buffer used can have a marked effect on both specific and non-specific binding. Generally, neutral or near-neutral buffers are used, the most popular being phosphate, phosphate/citrate and tris(hydroxymethyl)amino-methane (Tris). We recommend a standard phosphate buffered saline (PBS), and to help minimise matrix interference the overall concentration is kept high with the phosphate at least 0.05 M, and the sodium chloride up to 0.3 M.

Blocking buffers

A variety of agents have been successfully employed as blocker in ELISA including gelatin, casein, BSA and even powdered milk, usually in the concentration range 0.1 to 1.0 per cent. The buffer is normally phosphate or Tris based. When required we have found phosphate buffered saline containing sodium caseinate (0.1 per cent) to be very effective.

Although blocking buffers can be used in a separate blocking step, when required it is generally convenient to include the blocking protein in the incubation buffers.

Washing buffers

For plate washing, the same phosphate buffer as used for incubation is recommended without the sodium chloride but with the addition of the detergent Tween 20 (0.05 per cent).

Substrate development buffers

Most enzyme/substrate combinations have their own special buffer requirements particularly with respect to pH and the need for cofactors. It is important that these are determined before trying an enzyme for the first time. For the development of the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) disodium salt (ABTS) employed in the examples quoted here, an acetate buffer (pH 4.2, 0.1 M) is used along with hydrogen peroxide as the cofactor. When working with the enzyme HRP, a non-ionic detergent (e.g. Tween 20 or Triton X-100) is usually included in the substrate development buffer as this allows development at a temperature above the normal optimum of 15°C (Tijssen, 1985).

Stop solution

In the assays described here using HRP, sodium azide at a concentration of 10 mM is used as the stop solution to end the enzyme reaction. A number of alternatives have been employed such as sulphuric acid (4 M), although the use of sodium cyanide is not recommended.

Commercially available reagents

A number of companies, in particular Sigma (Poole, Dorset, UK) and Pierce Warriner (Chester, Cheshire, UK) supply a wide range of reagents, many specifically designed for ELISA work. These include not only enzymes, antibody–enzyme conjugates, activated proteins and purified detergents but even pre-prepared blocking buffers. Although the cost of some of these materials may be higher than those prepared in-house, their use does help minimise the risk of failure for anyone intending setting up an ELISA for the first time.

Equipment for ELISA

Microtitre plates

ELISAs can be carried out in a variety of ways with the solid phase in the form of a tube, dip-stick, bead or membrane. However the convenience and low cost associated with the use of the microtitre plate is responsible in part for the rapid development and growth of ELISA. Microtitre plates are self-contained units usually consisting of 96 wells, arranged in 8 rows (horizontally) by 12 columns (vertically). Each well has a maximum capacity of approximately 300 µl of which up to 200 to 250 µl is effectively usable.

Microtitre plates are widely available in one of two plastics, namely polystyrene and polyvinylchloride (PVC). Flexible PVC microtitre plates have been reported to have the greatest capacity to bind protein (Kemeny, 1991). They also have the advantage that they can be cut up with scissors if the radioactive content of wells is required to be counted, for example when checking protein binding capacity. However, for most work we have successfully used rigid polystyrene plates and our choice is also reflected in a survey of the recent literature which shows polystyrene to be preferred over PVC. A number of manufacturers now produce individual wells which can be inserted into a suitable holder. These can be useful where there is a need to set up small numbers of tests and a full plate is not required.

Batch-to-batch differences between plates is much less common than it used to be. Nevertheless, it is a good idea to buy plates in bulk and test new batches in parallel with old ones. This should be carried out using a robust established, but discriminating, assay. Some manufacturers supply certificated microtitre plates where the adsorption of IgG across the 96 wells has a coefficient of variation of less than 5 per cent and all results within 10 per cent of the mean. The use of these certificated plates is recommended.

Another common problem with microtitre plates is the occurrence of edge effects which are manifested in increased signal in the outer wells. These effects are thought to be caused by temperature differentials across the plates and are evident when chilled reagents are used. Because of the insulating properties of the polystyrene, the outer wells reach ambient temperature faster than those in the middle of the plate. This problem can usually be eliminated by ensuring all reagents are at room temperature prior to adding to the wells. However, if the problem persists then the use of the outer wells of the plate should be avoided such that the plate is effectively reduced to 60 wells, i.e. 6 × 10. It is also recommended that plates are not stacked as this can effectively insulate the inner wells, further increasing the temperature

differential. The use of long incubation times or continuous agitation should help ensure that thermal equilibrium is attained across the plate.

In our laboratories we use MaxiSorp F96 certificated polystyrene plates from Nunc (obtained in the UK through Life Technologies Ltd, Paisley, UK).

To prevent evaporation and possible contamination during the long incubations the plates should be covered. Many workers use purpose-made adhesive films such as Seal Plate Films (Anachem Ltd, Beds, UK). However where these are highly adhesive there may be problems actually removing them and for this reason a number of workers simply use domestic 'cling film'.

Pipettes

For most work it is suitable to use air displacement pipettes capable of delivering from 10 µl up to 100 µl. To minimise pipetting operations the use of multi-channel pipettes is recommended. These are similar to the standard single pipettes but with either 12 or 8 channels to allow multiple dispensing or pipetting, either across or down a microtitre plate. It needs to be borne in mind however that calibration of the multipipettes can be a tedious task.

Washing apparatus

ELISAs involve numerous wash steps which can be conveniently and simply carried out by flooding the plate using a squeeze bottle. After a defined period the waste solution is merely flicked out of the wells into a waste container.

Where more precise control or a less messy procedure is desired then one of the proprietary wash systems can be used. Such a device is the Immunowash-12 (Nunc), which has 24 nozzles arranged in pairs which fit across the 12 rows of the plate. One set of nozzles are connected to a reservoir and are used to dispense buffer into the wells using a simple siphon system. The other set of nozzles are connected to waste via a water-jet-driven vacuum pump and are used to aspirate the wash solution to waste.

Detectors

The quantification of the assay end point merely involves reading the absorbance of the coloured solution in the plate well. A range of plate readers are available which incorporate a suitable spectrophotometer to allow precise measurement of this coloured solution. These readers are fully automatic such that the whole plate can be read and 96 optical density measurements returned within 2 to 3 min. To allow detection of the full range of coloured reaction products most plate readers incorporate a series of filters with different wavelength cut-offs. A plate reader with filters set at 405, 450 and 490 nm will be suitable for the vast majority of the enzyme/substrate combinations which are used. Some automated systems such as the Biomek 1000 (see below) also have in-built detection systems.

As an alternative to stopping the reaction prior to reading, a kinetic plate reader can be used. With this type of system the rate of the enzyme reaction is determined

rather than the colour at the end point. This approach should give greater precision since the tight control of the time course of the enzyme reaction is no longer required. Where the stopped reaction approach is to be employed, access to a kinetic plate reader during assay development can also be useful since this can allow the substrate incubation process to be studied and the stop time to be optimised.

Plate shakers

One limitation of solid-phase assays is the slow attainment of equilibrium compared with a solution-based system: this often leads to long incubation times (Franz and Stegemann, 1991). To a degree this can be overcome by gently agitating the plates during the incubation phases. The use of some sort of shaker or mixer is considered essential at the colour development stage to ensure that the colour is distributed evenly in the well and precise absorbance measurements are made. A quick mix is also recommended prior to the start of each incubation. A number of suitable mixers/shakers are available commercially and some of the automated systems (see below) and modern plate readers have a variable shaking facility built in.

Automated systems

Where high sample throughput is required then the use of some form of automation is strongly recommended. Because the separation of bound and free in an ELISA merely involves washing the plate, total automation of the process is possible in contrast to conventional RIA methods.

Most automated ELISA instruments are essentially liquid handling devices which are capable of carrying out all the pipetting operations of an ELISA. One widely used instrument which offers full automation is the Biomek 1000 Automated Laboratory Workstation (Beckman Instruments (UK) Ltd, Berks, UK). This instrument can dispense volumes in the range 20 to 200 µl with excellent accuracy and precision with the facilities to vary the rate of aspiration and dispensing which can be useful when handling viscous samples. In addition, this particular system will wash the plates and measure the optical density response at the end of the assay. The use of such equipment not only gives better assay precision compared with manual analysis but the monotony associated with carrying out the assay is virtually eliminated.

Plate conjugates

Small-molecular-weight species, particularly polar molecules, do not readily bind to plastics. Where they do, their small size precludes the ready access of an antibody, such that direct attachment of an antigen to a solid phase to give an antigen-bound ELISA is not possible. Most proteins however show considerable hydrophobic character which results in strong non-covalent binding to plastic surfaces. Thus, providing the analyte can be chemically linked to a protein, the resultant conjugate can be bound to the surface of a plastic microtitre plate. A variety of proteins have been used as carriers for the antigen and the methods of conjugation are similar to those employed in immunogen formation.

Table 7.2 Molecular masses and iso-electric points (pI) of various proteins used in the preparation of plate conjugates

Protein	Molecular mass (Dalton)	Iso-electric point
Bovine thyroglobulin	670 000	4.6
Immuno gamma globulin	150 000	4–5
Casein	375 000	4–5
Human serum albumin	68 000	4.7
Ovalbumin	43 000	4.7
Poly-D-lysine	1 000–300 000	NA
Haemocyanin	1 000 000	5–6.4
Bovine serum albumin	68 000	4.6–4.9

NA = not applicable.

Carrier proteins

Table 7.2 shows the variety of proteins which have been used as carriers in immobilised antigen ELISAs, together with their molecular masses and iso-electric points (pI). Of the proteins tabulated, the most commonly used are bovine serum albumin (BSA), bovine thyroglobulin, keyhole limpet haemocyanin (KLH) and poly-D-lysine. Assays based on these different proteins vary considerably in their characteristics, especially the non-specific binding and detection limits.

Since any antisera raised against a hapten–protein conjugate will contain antibodies to the protein as well as the hapten, it is essential that the protein used in the production of the immunogen is completely different to that used in the plate conjugate. Failure to do so will lead to increased non-specific binding and seriously compromised sensitivity, since antibodies will also bind to the carrier protein (Briand *et al.*, 1985).

Incorporation ratio

The conjugation or incorporation ratio of the plate conjugate has been reported to be an important factor controlling the assay sensitivity. The degree of incorporation determines the physical distance between the adjacent hapten or analyte molecules which are chemically linked to the surface of the protein. If the distance between adjacent hapten molecules is similar to the distance between the two binding sites of the antibody then bivalent binding of the antibody to the plate conjugate can occur. Bivalent binding interactions have been reported to have affinity constants which are 100 to 1000 times greater than the corresponding monovalent binding (Karush and Hornick, 1973). Thus if the incorporation ratio is very high, a poor sensitivity will result since the analyte will be unable to compete effectively with the hapten–protein conjugate for the antibody. It is recommended therefore that incorporation ratios are kept low, certainly less than those used in the production of immunogens.

In our laboratory, plate conjugates involving poly-D-lysine (molecular mass 50 000 Dalton) having an incorporation ratio of 6 : 1 and bovine thyroglobulin (molecular mass 670 000 Dalton) with a ratio of 22 : 1 have produced assays with similar sensitivity to that of RIA. Thus incorporation ratios of this order are recommended.

Preparation of plate conjugates

Methods for producing plate conjugates are essentially the same as those for immunogen preparation described in Chapter 3 and any of the methods described there can be used. In order to keep the incorporation ratio to a minimum it is recommended that the hapten to protein reactant ratio is reduced by at least a factor of two compared with that used to produce immunogens. Alternatively, the reaction time can be reduced.

It is strongly recommended that the incorporation ratio is measured using one of the available techniques (Chapter 3) so that the influence of the incorporation ratio on sensitivity can be determined.

Bridge recognition

To minimise bridge recognition (see Chapter 6) it is important that the chemical bridge linking the hapten to the protein in the immunogen and the plate conjugate are different. This is even more important than in RIA since in ELISA both the immunogen and the plate conjugate have the same chemical nature. It is particularly important in ELISA therefore to have a range of linking procedures available and if possible a range of haptens bearing different reactive groups. However, it appears to be accepted that bridge recognition can be reduced or minimised when the length of the bridge in either conjugate is less than four atoms.

Other literature indicates that the degree of bridge recognition can vary across a series of polyclonal antisera from different animals immunised with the same immunogen and that the effect is generally less of a problem for antisera taken later in an immunisation schedule. Thus careful screening and selection of the 'right' antiserum is an extremely important factor in determining the assay quality.

Irrespective of which conjugate is nominally the immunogen and which the plate conjugate, it is recommended that both are employed as immunogens and injected into animals. Any sera obtained against one conjugate can be evaluated using the other as the plate conjugate and *vice versa*. It is important to bear in mind in evaluating sera produced in this way that meaningful results will only be obtained if both the chemical linking group, i.e. the bridge, and the protein are different in the immunogen and the plate conjugate (Briand *et al.*, 1985).

Assay development

Defining the starting conditions

Like the development of an RIA, the first experiment in setting up an ELISA can also be critical, with inappropriate selection of these starting conditions possibly leading to perfectly good reagents being rejected. In contrast to RIA, ELISA has many more variables which need to be fixed before beginning the assay development. Foremost amongst these is the plate conjugate concentration, which along with the incorporation ratio effectively controls the mass of hapten in the assay system. Varying the plate conjugate concentration is therefore equivalent to varying the mass of tracer in a RIA.

The optimum conjugate concentration for a particular assay is dependant on a number of factors and certain assumptions have to be made at this stage. In terms of assay sensitivity the optimum concentration should be as low as can be precisely detected using an enzyme-labelled antibody. However, a very low concentration of conjugate will leave the plate surface uncoated, which may lead to high non-specific binding if appropriate measures are not adopted. Conversely, a high concentration of conjugate not only results in reduced assay sensitivity but it can also lead to poor precision since some of the conjugate will only be loosely held by protein-protein interactions once the plate surface is saturated. These protein-protein interactions are weaker than those between the plastic and the protein and desorption of the plate conjugate can occur. It should be noted that the optimum concentration of protein to saturate the surface is dependent on the nature of the protein (Cantarero *et al.*, 1980).

In practice we would use an initial plate conjugate concentration of 5 µg/ml. This is within the concentration range of 1 to 10 µg/ml recommended by Voller *et al.* (1979) and is similar to that recommended by other workers (Tsang, *et al.*, 1980; Herrmann, 1981). This approach has worked well with a range of plate conjugates derived from small synthetic polymers such as poly-D-lysine (60 000 Dalton) to large natural proteins such as bovine thyroglobulin (670 000 Dalton).

In order to minimise non-specific binding to the plate a detergent is incorporated into all buffers subsequent to the sensitisation stage. No blocking protein is used at this initial stage although its use may be indicated later (see below). The concentration of the indicator reagent (second antibody-enzyme conjugate) is fixed using the manufacturer's recommended dilution. Incubation times are kept constant, and to facilitate rapid evaluation during this early stage in the development process, these are kept as short as is practicable.

Antiserum assessment

The first stage in the assay development is to assess the antisera for titre, specificity and sensitivity, very much as described for RIA in the previous chapter. This involves carrying out a series of antibody dilution curves, along with displacement curves for the analyte of interest and any compounds for which cross-reactivity is considered critical. A suggested method for antiserum assessment is given in PROCEDURE 1 below. The method describes the evaluation of two different sera using a single microtitre plate. The antibody dilution curves give the titre, and by running displacement curves with solutions of the analyte at the desired limit of detection an indication of the likely sensitivity is obtained. The process is carried out in a similar manner to that for RIA with all tests being performed in duplicate.

PROCEDURE 1 Initial assessment of the primary antiserum for titre and sensitivity

Reagents

- Sensitisation buffer is phosphate (0.05 M, pH 7.0) prepared from Na₂HPO₄ (4.17 g), KH₂PO₄ (2.81 g) and water (1000 ml)
- Incubation buffers for both primary and secondary incubations are phosphate (pH 7.0, 0.1 M) containing sodium chloride (0.15 M) and Tween 20 (0.05 per cent). These are pre-

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pared from Na_2HPO_4 (8.34 g), KH_2PO_4 (5.62 g), sodium chloride (8.77 g), Tween 20 (0.5 ml) and water (1000 ml)

- Wash buffer is the same as that used for the incubation
- Substrate development buffer is acetate (pH 4.2, 0.1 M) containing Tween 20 (0.1 per cent v/v) prepared from sodium acetate trihydrate (0.325 g), acetic acid (0.436 ml), Tween 20 (0.1 ml) and water (100 ml)
- Plate conjugate dissolved in the sensitisation buffer at a concentration of 5 $\mu\text{g/ml}$
- Specific antiserum serially diluted in the incubation buffer 1/10 to 1/10⁸
- A control, non-immune serum diluted in a similar manner to the antiserum
- Analyte dissolved in the incubation buffer at a concentration near the preferred assay limit of detection
- Horseradish peroxidase–second antibody conjugate (Sigma, Dorset, UK) diluted in PBS containing Tween-20 (0.05 per cent) according to the manufacturer's instructions
- Hydrogen peroxide solution (30 per cent)
- Substrate solution: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) disodium salt (ABTS) (Sigma, Dorset, UK) dissolved in substrate development buffer (1.1 mg/ml)
- Stop solution prepared by dissolving sodium azide (0.65 g) in water (100 ml)

Equipment

- Polystyrene 96-well microtitre plates (certificated)
- Cellophane microtitre plate sealers
- Multi-head pipettes (8 or 12 channel) with capability to dispense up to 250 μl
- Washing apparatus
- Plate shaker
- Microtitre plate reader with 405 nm filter

Method

Add the plate conjugate solution (220 μl , 5 $\mu\text{g/ml}$) to all the wells of the plate, cover and incubate overnight at 4°C. The following day, aspirate the sensitisation solution to waste and wash the plate three times with wash buffer (300 μl). It is recommended that the wash solution is left in the well for a fixed time, typically 15 to 30 s, prior to aspirating to waste. Down the first two columns of the plate add the non-immune serum (200 μl), starting with the 1/10 dilution in the first row, the 1/100 dilution in the next row and so on with the 1/10⁸ in the last, the eighth row. Repeat the procedure for each of the antisera to be evaluated, filling four columns of the plate (two for the dilution curve and two for the displacement curve) with each serum. In this example the last two columns of the plate are not used. To the first two columns of the plate and all antibody dilution curve wells, that is the first two columns of each antiserum, add the simple buffer solution (20 μl) and to the displacement curve wells (the second two columns of each antiserum) add the analyte solution (20 μl). Cover the plate, mix gently and incubate at room temperature overnight. Aspirate to waste and wash three times with wash buffer.

Add to each well the second antibody–enzyme conjugate solution (220 μl) and incubate at room temperature for 3 h. Aspirate to waste and wash the plate three times with wash buffer. Mix a volume of hydrogen peroxide with the ABTS solution (18 μl to 10 ml) and then add this prepared substrate solution (220 μl) to each well and incubate at room temperature

for a predefined interval (approximately 15 min) until a suitable colour intensity develops. Stop the reaction by the addition of the stop solution (20 μ l) to each well and shake gently to ensure the colour is uniformly distributed in the wells and read the colour intensity at 405 nm.

Plot the optical density in each well against the log of the antiserum dilution to get an antibody dilution curve or a displacement curve, as shown previously in the assessment of antisera by RIA.

The layout of the plate can be readily altered from that described above. For example, to accommodate a narrower range of antisera dilutions ($1/10$ to $1/10^6$) these can be set up across the rows (rather than down the columns) with duplicates in adjacent wells in the same row. In this way it would be possible to test three sera per plate.

Unlike the RIA dilution curves shown in Chapter 6, there is often reduced binding at low antibody dilution in ELISA which appears as a 'hook' in the antibody dilution curve. The hook effect has been the subject of much study although the exact reason for this phenomenon is still unclear. In practice it is of little consequence however since most assays work with antiserum dilutions of greater than $1/1000$. In contrast to RIA there is no 'total' signal which can be used to reference the sample and standard binding against. In ELISA the response for a given dilution of serum is compared with the maximum binding that is observed. Although the non-specific binding may be high at low serum dilution it rapidly falls off as the serum is diluted.

The data generated from these experiments is provisional and highly dependent on the experimental conditions used. However, a good indication should be obtained of which antisera contain useful antibodies. Furthermore, from the difference in optical density between the dilution curve and the displacement curve the antisera capable of giving the most sensitive assay can be selected. If displacement curves using potential cross-reactants are also carried out, then data on antiserum specificity will also be obtained.

When the initial experiment has gone well and one or two antisera are indicated as offering the requisite sensitivity and specificity, then calibration curves should be generated using these chosen sera at the indicated dilution. The assay conditions should be the same as those used in PROCEDURE 1. The first column of the plate can be set up with zero standards and non-specific binding wells (each in quadruplicate). Eight standards can then be set up down the next and subsequent columns depending on how many replicates are required. At this stage a more complete evaluation of sera specificity should also be carried out by evaluating the cross-reactivity of related materials and/or metabolites.

Although the assay may still be in prototype, once a calibration range has been defined the precision over this range should be determined by assaying multiple calibration series and generating a precision profile (see Chapters 6 and 9).

In a number of instances the experiment described in PROCEDURE 1 may not deliver the required result: binding may be low, sensitivity or precision may be poor, or non-specific binding so high as to mask the specific binding. In certain instances the cause may be readily apparent, for example a high level of absorbance in every well may indicate the use of too high a concentration of second antibody-enzyme

conjugate. Where the reason is not immediately apparent then it will be necessary to carry out some investigation or optimisation of the experimental conditions. The most problematical stage in any ELISA is usually the sensitisation step (Kemeny, 1991). This should generally be investigated first since if non-optimal, it can affect all the above-mentioned parameters.

Optimisation of the sensitisation stage

Where the sensitisation stage is considered suspect then the experiment outlined in PROCEDURE 1 should be repeated using a range of plate conjugate concentrations with or without modifications to the sensitisation buffer (see below).

Reducing the concentration of the plate conjugate should improve sensitivity, whilst increasing the concentration will increase the limit of detection. There is an optimum with regard to the concentration of plate conjugate however. The stability of the adsorbed protein is greatest when adsorption takes the form of a monomolecular layer. When the protein concentration exceeds that required for monolayer formation a multilayered structure is produced. The resulting protein-protein interactions are weaker and can lead to poor precision as the protein desorbs during the assay procedure.

If the duplicates showed poor precision then this may be related to general handling procedures and pipetting etc. However it may also indicate the presence of protein-protein interactions (i.e. a too high a concentration of plate conjugate has been used) or that the interaction between the protein and plastic is poor.

Proteins are thought to adsorb to the plastic surfaces mainly by hydrophobic interactions, although charge is also thought to play a role (Shirahama and Suzawa, 1985; Kemeny and Challacombe, 1988). Therefore any change in the conditions which increases the hydrophobicity of the protein can be employed at the sensitisation stage. For example components which cause partial denaturation of proteins, e.g. 6 M guanidine hydrochloride (Lewis *et al.*, 1992), leading to exposure of hydrophobic regions may be suitable for inclusion in sensitisation buffers. Alternatively the ionic strength of the sensitisation medium can be increased. It is commonly believed that ionic components enhance the stability of the protein-plastic interactions by shielding repulsive charge-based interactions between neighbouring protein molecules, and several authors use high ionic concentrations as a consequence. However, the opposite has also been observed (Tsang *et al.*, 1980; Shirahama and Suzawa, 1985). If necessary the ionic strength of the sensitisation medium can be varied using sodium chloride or some other simple salt.

In the example in PROCEDURE 1 a sensitisation buffer of pH 7 phosphate was used, whereas most reports in the literature make use of carbonate (pH 9.8, 0.1 M). The reason for this is unclear but it probably relates to the fact that the first reported immunoassay using IgG-coated plastic was carried out using a carbonate buffer (Catt and Tregar, 1967). A range of proteins have been successfully immobilised using sensitisation buffers based on phosphate (Geerligs *et al.*, 1988), citrate (Geerligs *et al.*, 1988) and tris(hydroxymethyl)aminomethane (Tris) (Tsang *et al.*, 1980) under pH conditions often close to neutrality. If the sensitisation stage is considered to be problematic then it may be worth carrying out some basic optimisation experiments using a range of buffer types with and without added salts. A suitable carbonate buffer can be conveniently prepared by mixing a solution of

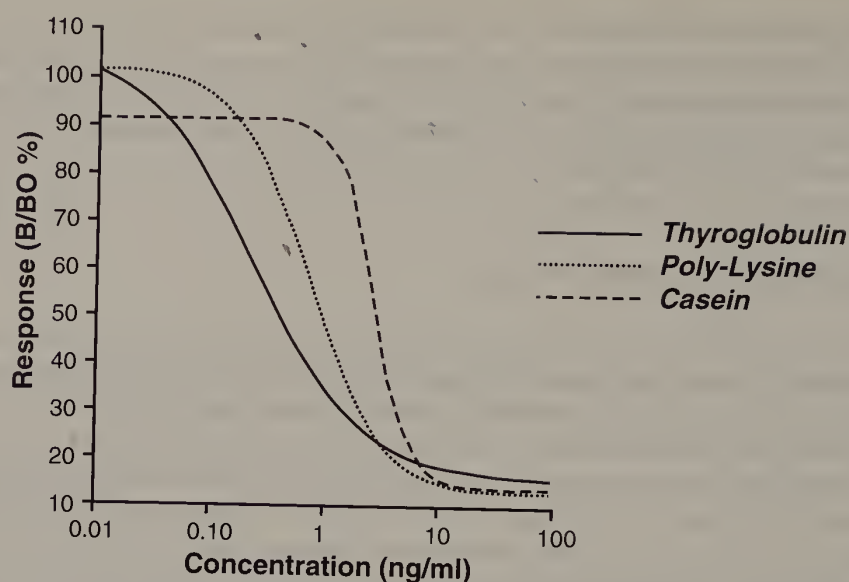


Figure 7.3 Calibration curves for an immobilised antigen ELISA using different proteins for the plate conjugate.

sodium carbonate (40 ml, 1.06 g/100 ml) with a solution of sodium bicarbonate (60 ml, 0.84 g/100 ml).

It is important to remember also that the nature of the protein itself can have a marked albeit unpredictable effect on the assay parameters, including the sensitivity. This is clearly shown by the data in Figure 7.3 which presents three ELISA calibration curves generated using identical conditions except for the nature of the conjugate protein. Although these results may change if different incubation buffers and pHs are used, they do demonstrate how the carrier protein can have a marked effect on assay sensitivity and dynamic range.

Optimisation of non-specific binding

If the non-specific binding is high, i.e. greater than 10 per cent of the maximum specific binding signal, then it is likely that the detergent is not being totally effective and some optimisation of the assay conditions is required. Although the concentration of detergent can be increased above that recommended (0.05 per cent) doing so may affect the antibody binding adversely at either the first or second stage.

In the PROCEDURE above we recommended three washes with a relatively short wash contact time. It may be possible to reduce non-specific binding by increasing the number of washes to five for example. Employing a greater number than this is unlikely to lead to any further reduction in non-specific binding and it may actually reduce specific binding and adversely affect precision and sensitivity. Increasing the length of the contact time, bearing in mind that some workers recommend contact times of up to 3 min (Bullock and Walls, 1977) or 5 min (Tijssen, 1985), and agitating the plate during washing can have a useful effect. However, some workers (Beumer *et al.*, 1992) have shown that if the washing conditions are carefully optimised it may be possible to reduce both the contact time and the number of washes.

When the simple approaches fail then the use of a blocking protein is probably required. Blocking proteins act by binding to uncoated plastic and thus preventing non-specific adsorption of the indicator antiserum. A separate blocking stage can be carried out after the sensitisation, but alternatively and more commonly, the blocking protein can be included in the first and second incubation buffers.

Buffers containing BSA at concentrations of 0.05 to 0.5 per cent are commonly used as blocking agents. However, it should be realised that many adults have IgG antibodies to BSA in their blood (Dise and Brunnell, 1987) which can result in high background readings when human samples are assayed. Another factor limiting the usefulness of BSA as a blocking protein is that many antisera are produced using BSA-analyte conjugates. Thus any resulting sera will also contain anti-BSA antibodies, once again leading to high background readings.

Other proteins suitable for blocking are gelatin and casein, at concentrations of around 0.1 to 1 per cent. In our experience gelatin often results in high background colours, possibly because of the presence of natural antibodies in sera which recognise collagen fragments. However, it should be noted that gelatins do vary markedly in their effectiveness depending on their source. The use of fish-skin gelatin has been recommended by Vogt *et al.* (1987). The use of casein for blocking is also popular and in our experience gives assays with the lowest background colour. Casein is thought to owe its effectiveness to its heterogeneous composition involving proteins of differing size, amino acid sequence and ionic charge.

The data in Table 7.3 (unpublished results) shows the effectiveness of various proteins in reducing non-specific binding to poly-D-lysine coated polystyrene. Whilst several of the proteins tested clearly reduce non-specific binding, others make the situation worse, namely bovine gamma globulin (BGG). This effect however is probably concentration dependent and this factor should be investigated for any protein used. In our experience, assays with poly-D-lysine as carrier give the lowest background colours using phosphate buffer containing sodium caseinate (0.1 per cent), and this is a combination we recommend.

Table 7.3 The effect of various buffer proteins on the binding of enzyme-labelled anti-sheep IgG to poly-D-lysine adsorbed onto polystyrene

Buffer protein	Binding*	
	Protein absent	Protein present†
Haemocyanin	0.44	0.22
Gelatin	0.41	0.23
Bovine gamma globulin	0.43	1.37
Polyglutamic acid	0.48	0.22
Sodium caseinate	0.46	0.19

* Binding is measured by the optical density response at 405 nm using the substrate *p*-nitrophenyl phosphate.

† The concentration of protein in the buffer was 2 per cent with the exception of sodium caseinate which was used as a saturated solution (approx. 0.1 per cent).

Optimisation of the primary antibody incubation

The time taken for a solid-phase immunoreaction to reach equilibrium is usually longer than that for the corresponding solution-phase reaction, since it is limited by the rate of diffusion of the antibody to and from the solid phase (Stenberg and Nygren, 1988; Franz and Stegemann, 1991). Consequently, long incubation periods, often overnight, are employed for the primary antiserum incubation stage of an ELISA. Indeed, it has been reported in the literature that in the absence of vigorous agitation an incubation time of 18 to 30 h is required compared with 20 to 30 min for an equivalent solution-phase assay (Franz and Stegemann, 1991). However, with agitation of the microtitre plate, reaction rates are increased and equilibration times can be significantly reduced (Boraker *et al.*, 1992; Franz and Stegemann, 1991; Pesce and Michael, 1992).

The reaction kinetics should be investigated and optimised for each assay. For this experiment a series of wells should be coated to allow for non-specific binding, zero standards and a concentration around the mid-point on the calibration curve to be measured at each time point in duplicate. At pre-defined times the contents of six wells would be emptied, washed and the necessary reagents added to give a non-specific binding, zero standard and mid-point standard test. The times would typically be 0, 3, 6, 9 and 23 h, i.e. the experiment would be spread across two days. One hour after the last batch of wells has been set up the contents of all wells are aspirated to waste and the assay completed as normal. Carried out in this way, the experiment will give primary incubation times of 1, 15, 18, 21 and 24 h. If the assay is found to have come to equilibrium by 15 h then the experiment should be repeated using a shorter overall time, i.e. 8 to 12 h. A similar approach would be adopted if elevated temperature or agitation is employed.

ELISA procedures are often carried out at 4°C although there appears to be no good reason for this. Incubations may be carried out at higher temperatures (e.g. 20°C to 40°C) for shorter periods of time without loss in assay sensitivity, and in the authors' laboratories, room temperature incubation is commonly employed.

Introduction of matrix

As discussed in Chapter 6 on RIA development the early introduction of matrix to the assay can lead to confounding effects which makes the development of the assay more difficult. The most straightforward approach in the authors' opinion is first to establish the basic assay conditions using standards prepared in buffer. It should then be apparent whether the assay target in terms of sensitivity, specificity and precision are at least achievable. The matrix can then be introduced and, providing there are no gross changes in the major assay parameters, limited further optimisation can then be carried out. Once the best sera have been selected a comparison should be made between standards prepared in buffer and matrix.

It is often found that matrix interference in ELISA is more pronounced than in RIA. This is because the total incubation volume in ELISA is generally smaller and there are many more factors that can be affected. As well as interfering with the antibody-antigen interaction the matrix components may alter the activity of the enzyme or potentiate non-specific binding effects. It is important therefore to prepare calibration standards in the same matrix as the samples. Furthermore, since

matrix effects may vary from sample-to-sample, a pooled matrix should be used where possible. This will average or minimise the assay bias although sample to sample bias may still be evident.

Where the samples are highly proteinaceous, have high ionic strength or are generally 'dirty' then it is recommended that the proportion of matrix is kept below about 10 per cent of the total assay volume to minimise undesirable effects. Because of the limited usable volume of the microtitre plate wells (200 to 250 μ l), only relatively small sample volumes, typically up to 20 or 25 μ l, can therefore be assayed. This is in contrast to RIA, which is generally carried out in tubes where the total incubation volume can be increased to over 1 ml, and the volume of sample increased accordingly. Where the samples are relatively clean however, e.g. river water, the volume of sample added to the ELISA can be increased and the volume of first antibody decreased accordingly.

One report (Gissendorf, 1990) suggests that the incorporation of certain ions such as magnesium and calcium into ELISA buffers may help minimise matrix effects.

In the immobilised antigen ELISA the enzyme never comes into direct contact with the matrix so the potential for a matrix-induced change in the end point is reduced. HRP, the most commonly used enzyme marker, is relatively insensitive to small changes in pH, with activity varying very little over a pH range 2 units either side of the optimum. The peroxidase enzymes are, however, particularly sensitive to contaminating bacteria as well as sodium azide which is widely used as a preservative in buffers and sera.

Where the samples contain large-molecular-weight species such as proteins, then these may bind to exposed sites on the plastic. These same species can also bind to the plate conjugate itself, possibly occluding the hapten. Should they occur, these interactions can lead to changes in non-specific binding, sensitivity and precision. Following the introduction of matrix therefore, it is important to check for any variation in assay parameters such as those mentioned above. If variation is seen then re-optimisation may be necessary. As a minimum it is recommended that sensitivity and precision are re-checked following inclusion of the matrix.

Further assay optimisation

Once the basic assay has been established fine tuning of the assay parameters may be necessary to give the desired performance characteristics. Much of this work is comparable to that for RIA as discussed in the previous chapter.

Optimisation of the second antibody incubation

The enzyme-labelled second antibody incubation should ideally be optimised such that it is close to equilibrium and a maximal response obtained from the specific binding wells, together with low non-specific binding. Under these conditions the lowest limits of detection and quantification will be obtained. In PROCEDURE 1 an incubation time of 3 h along with the reagent dilution as suggested by the manufacturer (typically 1/1000 to 1/5000) was recommended. Although appearing to give a satisfactory and a workable assay these parameters should be fully investigated and optimised.

Typically second antibody incubation times from 30 min to 6 h would be investigated using a series of antiserum dilutions spanning those suggested by the manufacturer. A convenient and simple experiment would involve setting up a series of zero standard and non-specific binding wells for each time point/reagent dilution combination. These would then be given a long first incubation to ensure the system was at equilibrium. At a number of set intervals over a 5 h period (e.g. 0, 2, 4, 5 and 5.5 h) the appropriate wells would be emptied, washed and enzyme-labelled second antibody solutions added. Thirty minutes after the final addition (i.e. 6 h after the start) the contents of all wells would be aspirated to waste and the entire assay completed. Each enzyme-labelled antibody solution will then have been given an incubation time of 30 min, 1, 2, 4, and 6 h.

Specificity

In certain instances, buffer type and pH, as well as the characteristics of the assay proteins can have a possible though unpredictable effect on specificity. Where the required specificity is not apparent then these parameters should be investigated initially. If the requisite specificity cannot be achieved through assay modification then the use of an extraction procedure or an HPLC clean-up can be investigated. These approaches also have the advantage that they can help overcome matrix interference problems. Ultimately however, it may be necessary to try different antisera, re-immunise or even prepare new immunogens.

Sensitivity

Of the factors which control assay sensitivity the roles of the following have already been discussed:

- the plate conjugate protein
- the hapten-protein ratio in the plate conjugate
- the concentration of plate conjugate used for sensitisation

Like all immunoassays the sensitivity is ultimately linked to the affinity of the antibody. However, where this is high (i.e. greater than 10^{12} l/mole) and not considered limiting, then sensitivity can be affected by the quality of the second antibody-enzyme reagent. In such circumstances evaluation of reagents from other sources should be carried out or reagents can be synthesised in-house.

The obvious method of improving assay sensitivity through the use of a larger sample volume, will be limited by potential non-specific interference by the matrix. However if this secondary problem of matrix interference can be eliminated or reduced through changes in the assay conditions (e.g. buffer pH, plate conjugate protein) then significant improvements in sensitivity may be possible.

Precision

Poor pipetting precision or a sloppy or rough handling technique at any of the large number of pipetting or wash steps can have a serious negative impact on assay

precision. A careful and methodical approach is therefore essential if good precision and low limits of quantification are to be achieved. The use of automated equipment can be particularly useful in ensuring that pipetting operations are reproducible and critical time periods are adhered to.

Microtitre plates which allow the covalent attachment of proteins may offer improvements in assay precision, since desorption of the plate conjugate during washing steps is eliminated. One such plate, Covalink (Nunc Ltd) has the polystyrene surface modified with secondary amine groups, which is reported to be ideal for binding molecules having carboxylic acid moieties. Conjugates can be linked to these derivatised plates using a suitable coupling procedure such as carbodiimide reaction (Sondergard-Andersen *et al.*, 1990).

Convenience

Although the number of stages and washes etc. may make ELISA seem unattractive compared with the relative simplicity of RIA, further work can often simplify the assay significantly. For example, where the assay shows low non-specific binding, it may be possible to reduce the number of washes used or the wash contact time. Either of these factors is relatively easy to study and optimise.

Through the use of a detector which is capable of kinetic measurements, the need for a stop solution can be dispensed with thus simplifying the assay procedure and possibly leading to improved precision. Where the end product is unstable or the stop reagent is not fully effective then the use of kinetic measurements could also lead to improved precision.

If assay speed is a major consideration then the assay can be run at a higher temperature; around 37°C. As well as resulting in faster attainment of equilibrium at the first and second antibody incubation stages, working at 37°C can result in sensitisation times being reduced significantly (Mushens and Scott, 1990) and even down to 1 h (Tsang *et al.*, 1980). Similarly agitation of the plate during the incubations will also result in faster attainment of equilibrium and a shorter overall assay time (Mushens and Scott, 1990). Reducing the incubation volume will also bring the assay to equilibrium faster with the possible disadvantage that matrix effects could be increased. In practice total assay volumes should not be less than 50 µl because of potential problems of evaporation which can be significant with such low volumes and uneven coating as a result of surface tension effects.

Before implementing any assay modification it is important to test its effect fully on the main assay parameters, such as precision and sensitivity. The precision profile as discussed in Chapters 6 and 9 is a powerful tool for the assessment of the effect on assay precision and limit of quantification.

Practical aspects of ELISA

Storage of coated microtitre plates

There is limited data on the storage of microtitre plates containing bound proteins. In the author's (BB) laboratory coated plates have been successfully stored at 4°C in a suitable buffer for up to 1 month. It has also been pointed out by Voller *et al.*

(1979) that dried protein-coated plates can be stored for a year or more with apparently no degradation if kept in air-tight waterproof packs. In practice we normally coat plates fresh the day before use, at least in the early stages of development. For convenience however, once the assay has been established evaluation of plate storage conditions should be carried out. One important factor controlling stability is the storage buffer which should contain some protein, typically as used in a blocking buffer.

Conclusions

Although ELISAs involve more stages than a comparable RIA they can be just as easy to perform and unlike most conventional RIAs they can be fully automated. The fact that the immunochemical reaction takes place at a solid phase often means that incubation times are longer, certainly longer than would be found with a liquid-phase assay. However, if plates are coated and stored then a well-optimised assay should be able to be completed in a typical 8 h working day.

The use of a solid-phase reagent means that the separation of bound and free can be effected by simply washing the plate. This allows simple and full automation of the whole assay procedure, from adding the samples to the wells to the generation of the calibration plot and assay results. Providing the primary sera for all assays are raised in a single species then one commercially available enzyme-antibody reagent can be successfully used with any analyte.

The ease of full automation plus the universal nature of the indicator reagent combine with the elimination of radioactivity to make ELISA a most valuable and versatile analytical tool.

It should be stressed that in our laboratories we have developed ELISAs which have similar sensitivity, specificity and precision to that of conventional liquid-phase RIAs. This has been achieved by employing the principles laid out in this chapter, the major features of which are summarised below.

A different protein must be used for the immunogen and the plate conjugate and to minimise bridge recognition different linking chemistries should also be employed. The plate sensitisation stage is the most critical and the one which generally causes the most problems. Whilst the conditions described here (pH 7 phosphate buffer, 0.1 M) have worked well in our laboratories, full optimisation of these factors may occasionally be necessary.

A plate conjugate involving a large-molecular-weight carrier, e.g. bovine thyroglobulin or poly-D-lysine (670 000 Dalton) is recommended. To attain maximal sensitivity the conjugation ratio should be low to avoid bivalent binding of antibody. A ratio of less than 20:1 is recommended for bovine thyroglobulin. The number and concentration of buffer additives should initially be kept to a minimum. Where a blocking protein is required, the type of protein and its concentration should always be determined. In this respect we have found sodium caseinate to be particularly effective in a number of assays.

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