CHAPTER 6

Immunoassay in pharmacokinetic and pharmacodynamic bioanalysis

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6.1 *Summary*

This chapter outlines the general concept of immunoassay as a bioanalytical tool and describes the modern application of the technique in a bioanalytical department within the pharmaceutical industry.

The various types of immunoassay procedure are described in the context of the requirements for the development and optimisation of an immunoassay method. This includes the generation of polyclonal and monoclonal reagent antibodies and the selection of a suitable label.

Once a method has been developed it is essential that it is validated in terms of accuracy, precision, sensitivity, specificity, linearity and analyte stability, and this important requirement is described both for methods developed in-house and for commercial kits.

Although still used for the bioanalysis of small molecule drugs where appropriate, the current major application for immunoassay is in the measurement of biomarkers. The various types of marker and their importance are described and illustrated with a specific example (determination of Cox-2 selectivity in human blood).

The chapter includes a brief description of immunoassay data processing, the important points that must be considered here, and a summary of assay automation possibilities.

The other important current application of immunoassay in bioanalysis is in the measurement of biological drugs (biopharmaceuticals). The important issues that relate to the use of immunoassay in this area are described together with an actual example of an application of the technique in support of a bio-pharmaceutical product.

6.2 *The role of immunoassay in drug discovery and development*

Within the pharmaceutical industry, immunoassay has traditionally been used for the analysis of small and large drug molecules in biological fluids to support pre-clinical and clinical drug discovery and development programmes. In the 1980s radioimmunoassay (RIA) was the major form of immunoassay used but this has largely given way in the 1990s to techniques involving spectrophotometric detection such as enzyme-linked immunosorbent assay (ELISA) or time-resolved fluorescence immunoassay (TRFIA). Until the 1990s high-performance liquid chromatography (HPLC) was the main alternative technique for the bioanalysis of drugs but often it could not match the sensitivity or throughput of immunoassays. Therefore, choice of technique was made on a case by case basis depending on the structure of the drug, availability of suitable expertise and resource, and desired limit of quantification.

There are many examples of successful drug development programmes where immunoassays have been used as the bioanalytical tool of choice such as ranitidine (Zantac), acyclovir (Zovirax and Valtrex) and lamotrigine (Lamictal). Lamotrigine is still being quantified by immunoassay in post-marketing studies.

The advantages of immunoassay as a bioanalytical technique include the ability to achieve very sensitive assays with very small sample volumes. In addition, there is generally no need for a sample preparation or extraction step prior to analysis and samples can be assayed directly in an appropriate matrix. Once established, immunoassays are easily automated and are high-throughput methods that do not require expensive instrumentation. However, there are disadvantages of immunoassays, most notably the perception that immunoassays for drugs are intrinsically non-specific, particularly with respect to metabolites. This is not entirely true as specificity is dependent on appropriate assay development, particularly with respect to antiserum generation. Another major disadvantage is the comparatively long assay development time, which is usually some months, with no guarantee of success.

In recent years LC–MS–MS technology has improved to such an extent that it has become the technique of choice for small molecule drug and metabolite analysis throughout the pharmaceutical industry, largely replacing immunoassay and HPLC. These considerable improvements have led to the potential for extremely short assay development time (days), improved sensitivity (comparable to immunoassay) and extensive automation of the tedious and time-consuming extraction procedures. The question therefore must be asked is there still a role for immunoassay in future drug discovery and development programmes? The answer to this question is most definitely yes and the reasons for this are as follows.

First, there are many exciting developments ongoing in the commercial immunodiagnostic industry which will undoubtedly impact upon immunoassay use in drug discovery and development. Such efforts include the simultaneous determination of related analytes in multi-analyte immunoassays, development of ultrasensitive immunoassays (e.g. immuno-PCR) and the continuously increasing number of commercial assays and antisera for novel biomarkers. The measurement of biomarkers in drug discovery and development is becoming increasingly important and as many of these markers are macromolecules, such as proteins, immunoassay is usually the technique of choice. This developing area is discussed in detail later in the chapter.

Second, immunoassay has an important role supporting the increasing number of biological drug discovery and development programmes, e.g. therapeutic monoclonal antibodies and vaccines where it is often the only appropriate analytical technique.

Finally, immunoassay development and application may still be useful for some small molecule drugs such as in the analysis of established drugs, mainly in the later stages of clinical development. This strategic use of immunoassay can free-up valuable LC–MS–MS resource and instrumentation or can provide a cost-effective alternative to outsourcing the method. The relatively long immunoassay development time may not be an issue at this stage of drug development, and the availability of an LC–MS–MS assay provides a simple means of evaluating the specificity of the immunoassay by simply cross-validating the two methods.

To summarise, it is clear that immunoassay will have a major role to play in bioanalysis in the future and instead of diminishing in importance it is staging somewhat of a renaissance.

6.3 *Principles of immunoassay*

Immunoassay is an analytical tool that relies on the ability to generate a response as a result of an antibody–antigen interaction. An antigen is a molecule that can be bound by a specific antibody and is capable, either directly or indirectly of eliciting an immune response when injected into a living host. Part of this immune response results in the production of high-affinity antibodies which bind specifically to the

antigen. In nature this is designed to aid in the removal of foreign molecules from the host but specific and high-affinity antigen–antibody interactions can be exploited to quantify molecules by means of an immunoassay. Since the discovery of immunoassay over 40 years ago this technology has been very widely exploited for the bioanalysis of both small and large organic molecules.

In general immunoassays fall into two broad categories, competitive and non-competitive. In a competitive assay, analyte (antigen) in a sample competes with a constant amount of labelled analyte for a limiting amount of antibody. Increasing amounts of analyte in the sample will result in less-labelled antigen being bound by the antibody. Before measurement of the labelled fraction bound to the antibody, separation of antigen–antibody complex and free-labelled antigen is achieved by one of a number of methods including activated charcoal, polyethylene glycol (PEG), and a secondary antibody which binds to the primary antibody. In RIA the label is a radioactive form of the analyte (usually ^{3}H or ^{125}I) which can be determined in the antibody-bound fraction by scintillation or gamma counting (see Figure 6.1).

Alternatively, an enzyme conjugate, where a suitable enzyme such as alkaline phosphatase has been covalently attached to the analyte whilst retaining its catalytic activity, is used as the label (enzyme immunoassay, EIA). The amount of enzyme in the bound

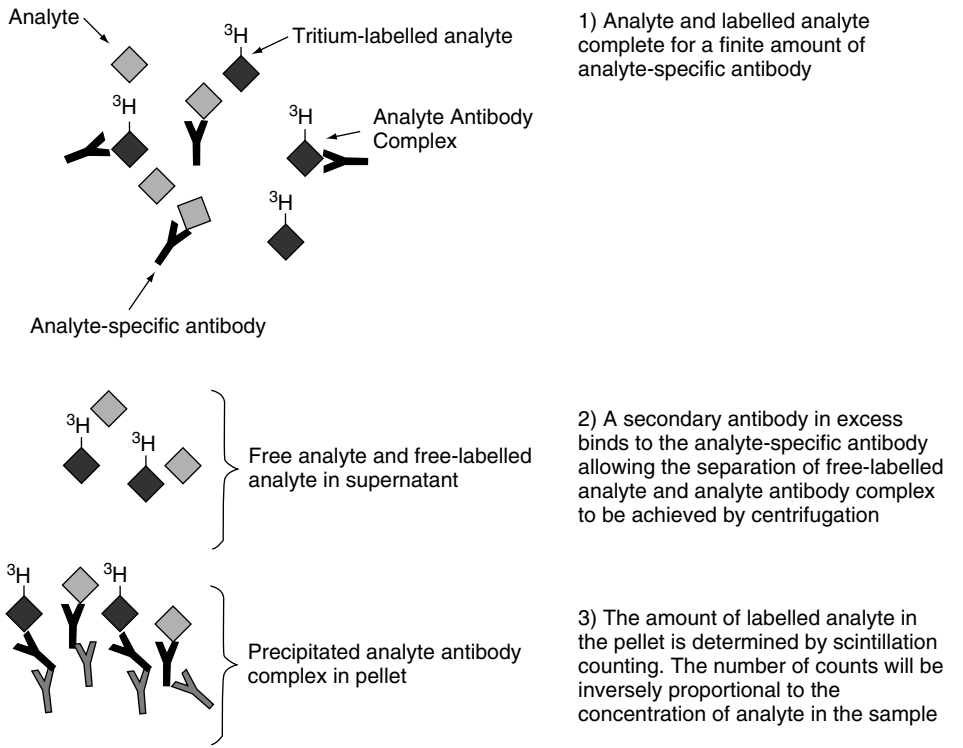

FIGURE 6.1 *An RIA utilising a second antibody to enable separation of bound and free fractions.*

fraction (using a separation system that retains the bound fraction in the supernatant) is determined by the addition of a substrate resulting in the formation of a coloured product. The intensity of the colour is determined spectrophotometrically.

A non-competitive immunoassay, in effect, involves the capture of all the analyte in the sample by excess antibody. The capture antibody is usually immobilised on a solid phase such as a polystyrene bead, a coated tube or more commonly the surface of a microtitre plate resulting in the most popular immunoassay format currently used, ELISA. Following washing of the solid phase a secondary antibody, which is also specific for the analyte but at a different site (epitope) and is typically conjugated to an enzyme, is added. The secondary antibody binds to the captured analyte forming a 'sandwich'. Further washing to remove unbound secondary antibody and addition of enzyme substrate result in the development of colour, the intensity of which is directly proportional to the concentration of analyte in the sample (Figure 6.2). Because this 'sandwich' assay format is dependent on the analyte being large enough to accommodate two different antibody molecules it is usually only applicable to macromolecule analytes.

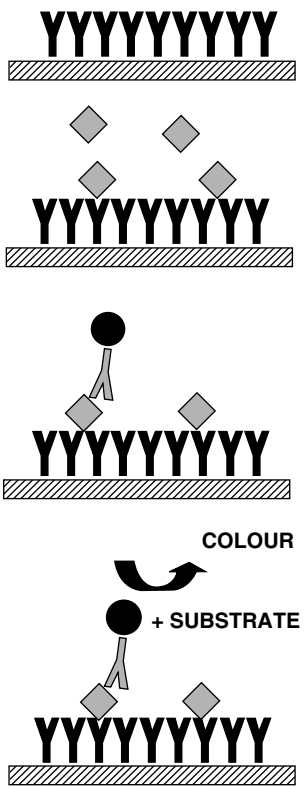

FIGURE 6.2 *A non-competitive ELISA format.*

6.4 *Assay development*

The first consideration in assay development and optimisation should be the intended application of the assay as this will influence the development goals such as desired accuracy, precision and sensitivity.

Immunoassay, compared to other bioanalytical techniques, requires a relatively long time for assay development. The step that takes by far the most time is generation of the reagent antibody, be it monoclonal or polyclonal, which can take a number of months with no guarantee that a suitable reagent will be produced. There are often commercially available immunoassay kits and/or reagent antibodies that can be used directly, or adapted for use in the analysis of biomarkers. These kits and reagent antibodies therefore save a lot of time and effort and if applicable would be used in preference to developing assays in-house. For novel biomarkers, academia may be the best source of antibody reagents.

There are a variety of immunoassay types (e.g. RIA, ELISA) and a number of formats for each, and it is therefore not possible to describe the development of all of these here. There are many excellent books and references which describe the assay development process for immunoassays in detail. The following section will briefly describe production of antisera, introduce the different types of label (often called tracer) available and list the steps necessary for assay development and optimisation.

6.5 *Production of reagent antibodies*

The antiserum is the key reagent in any immunoassay as it governs the selectivity, sensitivity, precision and accuracy of the method. Macromolecules, such as high molecular weight foreign proteins and polypeptides, are naturally immunogenic whereas lower molecular weight haptens (<2,000 Daltons) will require coupling to a protein to make them immunogenic.

A decision is required regarding production of monoclonal or polyclonal antiserum. As a rule of thumb, monoclonals are best suited for large molecules for use in two-site sandwich assays (one monoclonal to capture the molecule of interest and a second against a different non-overlapping epitope) and polyclonals for small molecules. For competitive immunoassay formats such as RIA, polyclonals are often superior because of their generally greater affinity.

For monoclonal antibody production the standard method involves a series of immunisations of mice with antigen over the course of several weeks to enhance the activation and proliferation of mature B cells producing antigen-specific antibodies localised within the splenic capsule. Several mice are usually immunised, and serum is periodically tested to determine antigen-specific antibody titre. When a suitable titre is achieved, spleen cells are removed and somatically fused with immortal

hybridoma or myeloma cells which are diluted and cultured in microtitre plates. Once the desired antibody-secreting wells are identified, the cells are expanded and antibody harvested. The time frame for generating monoclonal antibodies is generally 3–9 months but an alternative more rapid method is now available using a novel repetitive, multiple-site immunisation strategy called RIMMS. RIMMS can produce reagent antibodies in a month.

Polyclonal antibodies can be generated in a number of species with rabbits and sheep being the most commonly used. Generation of polyclonal antisera is less labour intensive than generation of monoclonal antibodies. In short, animals are immunised (prime) with the immunogen in a suitable adjuvant (material to enhance the immune response, e.g. Freund's) and then after a delay are boosted several times over a number of months and serum harvested when a suitable titre has been achieved. Immunisation and boosting are usually carried out intradermally, intramuscularly or subcutaneously.

Reagent antibodies can also be produced using molecular biology techniques without the use of animals but currently these are not widely used in the pharmaceutical industry.

6.6 *Selection and production of label*

Labelling of reactants is one of the most critical factors of immunoassay development and can be relatively labour intensive and technically difficult for those new to immunoassay. For these reasons commercial sources of label are generally sought and only if these are not available would labels be developed in-house.

A number of different labels have been used in immunoassays but there are essentially four types of label commonly used in the pharmaceutical industry:

- Radiolabels
- Enzyme labels
- Fluorescent labels
- Chemiluminescent labels.

Choice of label is dependent on the assay requirements (e.g. high sensitivity), ease of availability (e.g. commercial source), detection capabilities, site radioactivity regulations and restrictions (may preclude use of radiolabels) and experience of the bioanalyst.

6.6.1 Radiolabels

Radiolabels were used in the very first immunoassays and despite a decline in their usage there are still many assays using a radiolabel as a tracer. It is possible to radiolabel the antigen as used in traditional RIAs or the antibody as used in immunoradiometric assays (IRMAs). There are several radioisotopes that could

theoretically be used but in practice it is only ^{125}I, and to a lesser extent tritium (^{3}H), that are commonly used. The latter, however, has found favour in the pharmaceutical industry for small molecular weight drugs where bioanalytical sensitivity requirements are often relatively modest (>1 ng/mL).

All molecules of interest contain hydrogen atoms, and it is usually possible to synthesise a version of the molecule in which one or more of these atoms have been replaced with tritium to create a tracer. However, one major limitation is the preparation of the label which requires specialist input from a radiochemistry perspective. In addition, separation of the antibody-bound and free fractions of the analyte and the counting times required to achieve acceptable precision can also be bettered by alternative labels. There is however, the possibility of developing homogeneous RIAs suitable for tritium tracers by utilising Amersham's scintillation proximity assay (SPA) technology. SPA also provides the opportunity for using radiolabels in microtitre plate immunoassays.

In contrast to tritium tracers, it is far easier to prepare iodinated tracers, and specialist input may not be necessary provided that adequate facilities are available. The vast majority of commercial RIAs or IRMAs use ^{125}I as a tracer as it is generally the only suitable radiolabel for proteins. An additional advantage of iodinated tracers is the higher specific activity which gives the potential for more sensitive assays and simpler, more rapid radioactivity counting. Iodination procedures can either be direct, where the analyte (or an analogue) is labelled by replacing an atom of hydrogen with ^{125}I, or indirect by linking a suitable pre-iodinated molecule (radio-tag) to the analyte. The main disadvantages of iodinated tracers are safety issues such as monitoring exposure, monitoring contamination and stringent disposal procedures. In addition, the labels have a relatively short shelf-life due to the short half-life of ^{125}I with labels only really being viable for a few months at best before re-synthesis is required.

6.6.2 Enzyme labels

Enzyme labels were introduced into immunoassays in the early 1970s and have now become established as the most versatile and popular class of label. Enzymes are covalently coupled to a protein (e.g. an antibody to the analyte of interest) and enable amplification of a signal by creation of a coloured product from a substrate. The most commonly used enzyme labels are horseradish peroxidase and alkaline phosphatase. A wide range of antibody–enzyme conjugates are available commercially that can be used as detection reagents in ELISAs and therefore there is usually no need for label production during assay development. The main advantages of enzymes as labels are their availability, suitability for microtitre plate-based assays and the fact that they are measurable by many methods with very high sensitivities. The main disadvantage is their size; large enzyme-containing complexes diffuse slowly leading to longer incubation times and may bind non-specifically to reaction vessels.

6.6.3 Fluorescent labels

The use of time-resolved fluorescence has provided a viable alternative to radiolabels and enzyme labels for immunoassays, with the potential for lower background values and greater sensitivity. Perkin Elmer Life Sciences (formerly Wallac Ltd) provides readily available commercial kits and reagents for this assay technology as dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®). The technology uses chelates of the lanthanide metal ions which have long-lived flourescence under some circumstances. In DELFIA these lanthanide metal ions, particularly Europium, are used to label the molecule of interest. Preparation of fluorescent labels, other than those available commercially, can be accomplished in-house with limited training, and the assays are ideally carried out in microtitre plate format.

6.6.4 Chemiluminescent labels

Chemiluminescent labels are extremely popular in the immunodiagnostic industry with a wide range of kits and analysers available, mainly to support clinical chemistry applications. At present, the labels are not widely used in conventional bioanalysis departments other than in EIAs to quantitate enzyme labels (e.g. horseradish peroxidase, alkaline phosphatase).

6.6.5 The streptavidin–biotin system

The streptavidin–biotin (or avidin–biotin) system is widely used in immunoassay. Streptavidin is a binding protein isolated from *Streptomyces* that has an extremely high affinity and specificity for the water-soluble vitamin B6, biotin. Avidin is a protein found in egg white that has similar properties to streptavidin but is more prone to non-specific binding. Biotin is relatively polar and thus can be easily coupled to antibodies, and streptavidin (or avidin) can be coupled to solid phases, fluorochromes and enzymes. Streptavidin is a tetramer and has four biotin-binding sites per molecule. Therefore, use of the streptavidin–biotin system in immunoassay can greatly improve sensitivity of the assay by dramatically amplifying the signal.

6.7 *Assay development and optimisation*

After generation or purchase of reagent antibodies and a suitable label there are a number of further steps required to develop and optimise an immunoassay. Assay

development work will be affected by a number of variables some of which are listed here:

- Quality of antiserum and label
- Type of buffer
- Protein additives
- Incubation volume
- Concentration of reactants
- Plate coating conditions (if appropriate)
- Time and temperature
- Separation step selected
- Sample matrix.

There are obviously specific challenges and issues for each assay format and label type but the following five phases are applicable to most formats:

1 Selection of operating conditions and reagents, e.g. assay format, buffer to be used, commercial enzyme-label, production of plate conjugate for ELISA, etc.
2 Selection of initial assay conditions (e.g. incubation temperature) and separation system (if appropriate).
3 Assessment and selection of antisera with respect to specificity, titre and potential sensitivity.
4 Introduction of matrix to determine matrix effects on the assay.
5 Optimisation of assay conditions to obtain the desired sensitivity, specificity, precision and accuracy and to limit non-specific interference.

It is important to note that matrix is not introduced until a working assay has been established in buffer to avoid complicating the assay development process.

6.8 *Assay validation*

The published proceedings from the Crystal City Conference on the validation of bioanalytical methods have been generally accepted as guidelines in the pharmaceutical industry. However, it is clear that these proceedings do not adequately address the special issues pertaining to the validation of immunoassays (e.g. non-linear calibration curves) and therefore there is a clear need for specific guidelines. To this effect there has been an excellent review recently published in the *Journal of Pharmaceutical and Biomedical Analysis* (Findlay *et al.*) and also a specific conference was held in March 2000 on the validation of assays for macromolecules (sponsored by the American Association of Pharmaceutical Scientists, AAPS). There is a

workshop report arising from the meeting which will form the basis of guidelines specific to the validation of methods for the bioanalysis of macromolecules.

The validation requirements and assay acceptance criteria for immunoassays will vary depending on the analyte and the intended application of the method. For example, an immunoassay method intended for use for the analysis of a drug in pre-clinical safety or clinical evaluation would require a full validation package as well as cross-validation to a reference method. In contrast, an immunoassay for use in exploratory discovery, when rapid turnaround of results is required, does not need to be fully validated provided that the users are satisfied that the method is suitable for the intended purpose. If an immunoassay has been purchased as a kit then the objective is to verify performance of the kit rather than to validate it from scratch.

Immunoassays are generally less precise than chromatographic assays and therefore the criteria for accuracy and precision for assay acceptance may need to be more lenient than for chromatographic assays.

6.9 *Immunoassays developed in-house*

The purpose of assay validation is to establish confidence that the result obtained in each assay will always reflect the 'true' value. To do this a series of assays are carried out to determine a number of criteria as described below.

Accuracy is a measure of how close the observed result is to the 'true' value. It can be determined by 'spiking' reference analyte material into control biological matrix to create a series of validation controls (VCs) each with a known concentration of analyte. The reference analyte stock solution should be different to that used to prepare the assay standards. In addition the VCs should reflect the anticipated concentration range of the unknown samples and should span the assay standard curve. The VCs are assayed in replicates of six in a three to six separate assays. From the observed results for each VC within and between assays and calculating the percentage difference from the known concentration of analyte, a percentage bias can be obtained which indicates the inter- and intra-assay accuracy of the method.

Precision is a measure of the ability of an assay to reproduce an observed result. VCs should be prepared and assayed in the same way as that described in the determination of accuracy. The mean and standard deviation within and between assays should be determined and the percentage coefficient of variation of the mean (CV) calculated. The CV provides an indication of the variability and therefore the inter- and intra-assay precision of the method. Precision is independent of accuracy and a method can be inaccurate but reproducible and vice versa.

Specificity is defined as the ability of the assay to distinguish the analyte from other substances in the sample. This is a particularly important factor in the validation of an immunoassay as these assays are perceived as being less specific

than physicochemical techniques. This latter perception is because it is possible for an antibody to bind to a number of different molecules if the latter all share some of the same structural features. The best way to determine whether an assay is specific for the analyte is to cross-validate it where possible with a gold standard technique such as MS. However, the use of MS will not be possible if the analyte is a macromolecule. The alternative is to carry out accuracy measurements in the presence of potential cross reactants; these could include drug metabolites, co-administered drugs or molecules which are structurally related to the analyte.

The limits of quantification are defined as the lowest and highest concentrations of the analyte that can be determined with both acceptable accuracy and precision. These values are determined by using VCs at concentrations near the expected limits of the assay.

The stability of the target analyte in biological matrix should be determined for various storage conditions over the period of time that the samples are likely to be stored prior to analysis. The effect of multiple freeze-thaw cycles on the analyte should also be investigated as in most cases samples need to be stored frozen prior to assay.

If a sample extraction step is required prior to assay the analyte recovery should be determined. This is important because a low extraction recovery could reduce assay accuracy, sensitivity and precision. Recovery is checked by passing a known concentration of the analyte in the appropriate biological matrix through the extraction procedure and determining the amount of analyte recovered. This is usually expressed as a percentage of the concentration of the analyte originally spiked into the biological matrix.

Linearity of dilution has to be assessed to make sure that if a sample containing a high concentration of the analyte is diluted into the range of the assay the correct result is obtained. Effects of biological matrices are diminished with increasing sample dilution in assay buffer, and this may cause the apparent concentration of the sample to increase or decrease. To determine whether matrix interference occurs, a VC with a high concentration of the analyte should be assayed undiluted and then at increasing dilutions in assay diluent until the lower limit of quantification of the assay is reached. The results when corrected for dilution should be the same as the result obtained for the undiluted VC. If this is not the case then samples must be diluted in blank sample matrix and an equal volume of blank matrix incorporated into the calibration curve.

The above list of considerations is not exhaustive and constitutes the minimum investigations that should be carried out when attempting to validate an immunoassay. When carrying out these investigations it is important to have as good a knowledge of the assay system and analyte as possible and to have already established that the assay is fully optimised and performing reliably. Potential problems with the assay can be predicted and validation experiments carried out to determine the limits of the assays' use.

6.10 *Commercial kit immunoassay*

The objective of the validation is to verify that the performance of the method in-house is satisfactory for the intended purpose and in agreement with the claims of the manufacturer.

Commercial immunoassay kits will contain an instruction booklet or kit insert that will detail the use of the kit. This booklet usually contains information on assay performance, the detail of which varies from kit to kit and manufacturer to manufacturer. Some kits are well established and accepted by regulatory authorities as 'gold standard' analytical methods whereas others are less well characterised and are intended for 'research purposes only'. It is the suitability and performance of the latter category that usually requires the closest scrutiny. The validation is also particularly important if the kit is modified in some way, e.g. it is to be used for a different matrix to the one stipulated by the manufacturer.

The validation assays should be designed to assess the following:

- Accuracy
- Precision
- Specificity and matrix effects
- Elements of stability
- Linearity of dilution (as necessary)
- Equivalence of clinical material to that in the kit (as necessary)
- Recovery (as necessary).

Most kits will contain all reagents necessary for the assay. In some circumstances it may be necessary to prepare standards using clinical trial material instead of using the calibration standards provided in the kit. This is important when the analyte being measured and the standard in the kit are not equivalent (e.g. different binding affinities).

In common with in-house developed immunoassays, the preparation of VCs is required and these VC samples should be prepared in the biological matrix for which the method will be validated. A vast majority of commercial kit immunoassays will be for the determination of biomarkers. Unlike conventional drugs, biomarkers are usually present endogenously in the matrix of interest which causes additional complications during assay validation. Essentially, the analyst has two choices: attempt to remove the endogenous analyte prior to VC preparation, or not to remove it before adding a known amount of analyte and correct the data accordingly. For example, some analytes can be removed by stripping the matrix (e.g. charcoal for low molecular weight analytes) or affinity purification using antibodies (for immunogenic large molecular weight analytes).

6.11 *Data handling*

At the endpoint of an immunoassay an appropriate instrument will capture label signals such as optical densities or counts derived from radioisotopic decay. It is then necessary to process these raw data and produce a standard curve allowing the interpolation of unknown sample concentration. This can be simply carried out by plotting the standard curve manually using graph paper. However there are certain characteristics of an immunoassay standard curve that make this difficult and it is also not a practical approach in terms of reproducibility or high throughput in the bioanalytical laboratory.

There is a non-linear relationship between the measured response in the assay and the analyte concentration with the result that immunoassay standard curves tend to be sigmoidal. There is no universal mathematical function that will uniquely fit the best curve through the standard points, so care must be taken to avoid the introduction of bias. An additional problem is that there is greater error associated with the standard points at the extremes of the calibration curve where it becomes asymptotic, than in the central linear region of the curve. For these reasons computer software is used which offers a choice of curve fitting procedures based on either the 'best fit' through the actual standard points produced (e.g. spline fit) or on a mathematical model which reflects the physical principles underlying immunoassays (e.g. four-parameter logistic fit). The latter are generally preferred as they are less subject to bias resulting from any inaccurate standard points. MultiCalc, produced by Perkin & Elmer Life Sciences, is an industry standard immunoassay data processing software package which has a range of curve fitting options that can be applied to the raw data. Examples of standard curves produced using Multicalc are shown in Figures 6.3 and 6.4.

6.12 *Automation*

Automation allows higher sample throughput, potentially increases assay precision, negates the need to manually assay high-risk samples from infected patients and also frees scientists from having to spend large amounts of time carrying out routine analysis. Hospital Clinical Chemistry Laboratories, where very large numbers of samples are analysed on a routine basis, have been taking advantage of automated immunoassay systems for some time. These have tended to centre on large dedicated autoanalysers such as the Abbott Laboratories AxSym, which can carry out many of the standard clinical biochemistry tests. Samples are collected, labelled with a barcode and loaded onto the analyser. The barcode carries all the information required for the analyser to carry out the requested analysis using a common immunoassay format and report the results to a database of patient results. The analyser will also periodically run quality control samples and carry out appropriate calibration. In this way thousands of samples can be analysed per day. However, the modern bioanalytical facility within a drug

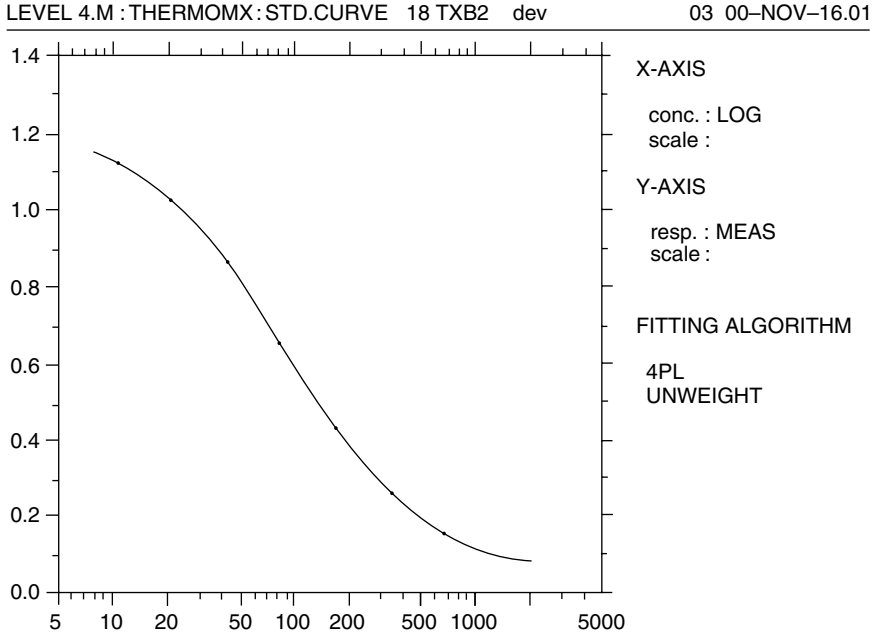

FIGURE 6.3 *Competitive EIA using four-parameter logistic curve fitting procedure (unweighted).*

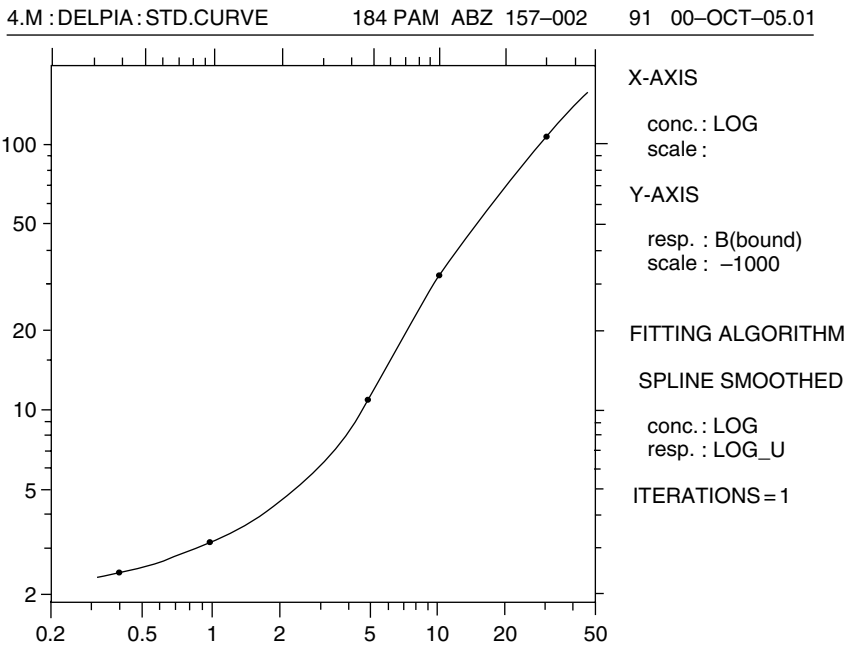

FIGURE 6.4 *Non-competitive DELFIA using a smoothed spline curve fitting procedure.*

discovery and development department requires a much more flexible approach to immunoassay automation. This is because the types of assay carried out change regularly as new candidate drugs are evaluated and new analytes are identified. Unlike the situation in a clinical chemistry facility there is no standard set of long-term routine tests, constituting the majority of the workload. Also, within a bioanalytical immunoassay lab an array of assay formats (EIA, RIA ELISA) would be carried out in different sized tubes and in a variety of microtitre plates. For this reason robotic sample processors have been chosen for automating the majority of immunoassays in the bioanalytical facility as these instruments provide the most flexibility.

Robotic sample processors such as the TECAN genesis (see Figure 6.5) or the Packard Multiprobe enable immunoassays developed in a variety of formats to be automated relatively quickly. In the past only homogeneous assays which did not require a separation phase were amenable to full automation. However plate washers, incubators and plate readers can now be added to a system allowing entire heterogeneous assay procedures to be carried out by the robot. The development of scheduling software has been a further bonus as it is now possible to carry out a number of different assays at the same time on the same robot.

6.13 *Biomarkers*

Biomarkers are becoming increasingly important in the pharmaceutical industry to aid the efficient development of new therapeutics. Biomarkers provide information

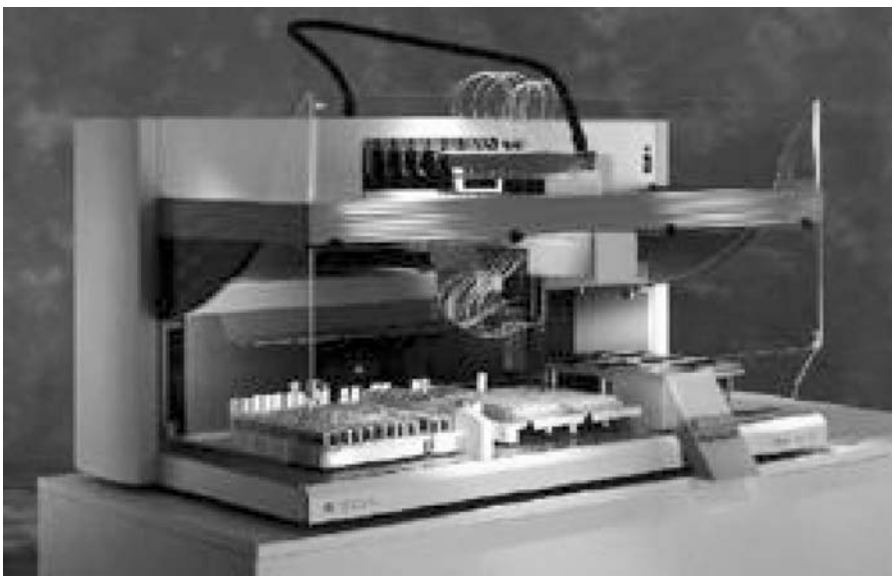

FIGURE 6.5 *TECAN genesis robotic sample processor.*

on drug mechanisms and potential efficacy and can aid in study design and appropriate dose selection. With many complex chronic disorders such as stroke, chronic obstructive pulmonary disease (COPD) and osteoarthritis, it would be necessary to treat thousands of patients over a number of years to prove the efficacy of a candidate drug. Measurement of appropriate biomarkers of drug efficacy or safety can substantially shorten this clinical drug development time or the time taken to reach a critical decision point in drug discovery. Indeed, the information provided by a good biomarker, or a panel of biomarkers, in any clinical development programme contributes to informed decisions on progressing the best drug candidates into full development quickly. The converse is also true; the poorer molecules can be de-selected more rapidly freeing up valuable resource and saving money.

The terms biomarker and surrogate endpoint are now widely accepted and the term surrogate marker, which is often used generically for all types of marker, is discouraged. Some definitions have been developed by a working group of the National Institutes of Health Director's Initiative on Biomarkers and Surrogate Endpoints and are provided below.

- A *Biomarker* is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention.
- A *Surrogate* endpoint is a biomarker that is intended to substitute for a clinical endpoint.
- A *Prognostic marker* is a test or set of tests which indicates the likely progression of a specific disease.
- A *Diagnostic marker* is a test or set of tests that determines the presence or absence of a specific disease.

There is a 'hierarchy' of validity in association with clinical disease that grows from a biomarker through a surrogate to a prognostic. A given test or set of tests thus may 'mature' through this progression as the supporting clinical validation becomes progressively stronger.

Physiologic functions and imaging have been used as surrogate endpoints for some time (e.g. electrocardiograms, blood pressure, X-ray) but it is for the determination of macromolecular biomarkers in biological fluids, for example cytokines or eicosanoids, that immunoassay is important.

In the drug discovery and development arena, biomarkers are currently commonly measured by immunoassay. This is because macromolecules in biofluids are generally not amenable to bioanalysis at high sensitivity using chromatographic approaches but elicit a good immune response for the generation of reagent antibodies for immunoassay development. This has been exploited by commercial suppliers and a vast array of commercial immunoassay kits or antisera are available which can be utilised when appropriate.

A case study is summarised below which illustrates the usefulness of measuring biomarkers (thromboxane B2 and prostaglandin E2) by immunoassay in support of a drug discovery project.

6.14 *Case study: determination of COX-2 selectivity in human blood*

Non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively as analgesics; however, their use is associated with side effects. The mechanism of action of the NSAIDs is through their inhibition of the enzyme cyclooxygenase (COX) which is involved in the metabolism of arachidonic acid with subsequent prostanoid formation. COX has two isoforms, COX-1 which is constitutive and COX-2 which is induced during inflammation. A currently accepted hypothesis is that COX-2 inhibition provides the anti-inflammatory activity of NSAIDs, whereas COX-1 inhibition is responsible for most of their adverse effects such as disruption of the cytoprotection of the stomach, kidney function and platelet aggregation. COX-1 and COX-2 are structurally distinct, therefore the development of drugs that selectively inhibit COX-2 might lead to a new generation of anti-inflammatory drugs with increased tolerability.

The aim of this work was to determine the COX-2 selectivity of the NSAID Naproxen and a selective COX-2 inhibitor Celecoxib (Searle) in human whole blood *in vitro* according to a published method (Brideau *et al*., 1996). This could enable the establishment of a model system for the evaluation of candidate COX-2 inhibitors in drug discovery.

To determine COX-1 activity, control human blood was collected from a number of healthy volunteers and test compound (Naproxen or Celecoxib) added at a range of concentrations to separate sub-samples. After incubation, serum was harvested and samples were assayed for thromboxane B2 (TxB_2) using a commercial enzyme-immunoassay.

To determine COX-2 activity heparinised whole blood samples (from the same volunteers and occasions as for COX-1 activity) were sub-divided and a range of test compound (Naproxen or Celecoxib) concentrations added. Lipopolysaccharide (LPS) was added to all samples to stimulate an inflammatory response, following which plasma was harvested for prostaglandin E2 (PgE_2) analysis using a commercial enzymeimmunoassay.

The inhibitory potency of the test compounds was expressed as an IC_{50} value. This is defined as the concentration of the compound required to inhibit either the LPS-induced PgE_2 release (measure of COX-2 inhibition) or clotting-induced TxB_2 release (measure of COX-1 inhibition) by 50 per cent. The selectivity ratio of inhibition of COX-1 versus COX-2 was calculated by comparing respective IC_{50} values.

The results shown in Table 6.1 demonstrate that the COX-2 selectivity of the NSAID Naproxen was poor compared to Celecoxib as indicated by the relatively

TABLE 6.1 *COX-2 selectivity*

		IC_{50} (nM)		
Compound	**Volunteer**	**COX-2 (PgE_2)**	**COX-1 (TxB_2)**	**Selectivity ratio**
Naproxen	1	12000	72000	6
	2	10900	10100	1
	3	55000	100000	2
	Mean	–	–	*3*
Celecoxib	1	120	10600	88
	2	400	50000	125
	3	140	65000	464
	4	300	8000	27
	Mean	–	–	*176*

low selectivity ratios of the former compared to the latter. Early clinical data has indicated that the selective COX-2 inhibitor Celecoxib has fewer adverse effects than non-selective NSAIDs.

This whole blood assay can be used *in vitro*, as described in this example, to determine the COX-2 selectivity of compounds in the drug discovery phase as well as *ex vivo* during clinical development.

6.15 *Biological drugs*

Although most drugs in the discovery and development portfolios of the major pharmaceutical companies are small molecules there is a significant and growing interest in biological drugs. These products, which are also often referred to as biotechnology products or biopharmaceuticals, embrace gene therapy products, peptides, monoclonal antibodies, vaccines, enzymes and other biologically active proteins. Although some biological drugs have been on the market for many years (e.g. interferon, insulin) there has been a massive growth in development of these products in recent years with many new products now on, or about to enter, the market.

With the exception of gene therapy products, immunoassay techniques are the method of choice, and often the only viable methods available, for the bioanalysis of these complex macromolecules. In some cases, immunoassay is used to measure concentration (more correctly immunoreactive concentration), but in other cases it is used indirectly as an endpoint in a bioassay to measure the biologically active concentration, or potency, of the drug. In addition to the measurement of the parent drug in biofluids there is often a requirement to measure other analytes either expressed or induced by the treatment. This is because many of these biological products, since they are recognised as a foreign protein by the treated

individual, induce an immune response leading to the production of antibodies against the parent drug. In some cases this is a desirable response (e.g. vaccines, development of anti-idiotype networks) but in other cases it is undesirable (e.g. production of neutralising antibodies) as it abrogates the drug's activity. It is often very important to measure and characterise these immune responses to biological drugs and immunoassay; usually ELISA is the primary technique used.

Although many of the validation requirements of immunoassays in general are relevant to the bioanalysis of biological drugs, there are some additional requirements that must be considered in the bioanalysis of these molecules. For instance, in pharmacokinetic studies it must be considered whether a bioassay, which would measure the biologically active molecule in the bio-fluid of interest, might be more appropriate than measuring immunoreactive concentration by immunoassay. In the latter case, the immunoassay may be measuring degraded and inactive forms of the drug in addition to the parent molecule and in general there is no way of knowing if this is the case.

Another example where additional or different validation criteria are required is in the measurement of the antibodies produced against biological drugs. The methods used need to be able to clearly distinguish between neutralising and non-neutralising antibodies and in the case of the former, since they are clinically very important, the validation study must also demonstrate that the assay is able to detect all classes of antibodies and all antibody affinities likely to be present in an antibody positive sample.

Wellferon, an interferon alpha preparation, provides an example of where immunoassay has been used for the bioanalysis of a biological drug. This example also illustrates some of the typical challenges that often have to be overcome in developing and validating bioanalytical techniques for measuring complex biological molecules.

Wellferon^{TM} (interferon α-N1) is a highly purified mixture of at least nine subtypes of human interferon alpha (h)IFNα produced from a human lymphblastoid cell line. This preparation had been on the market for several years for the treatment of hairy cell leukaemia and for certain patients with chronic active hepatitis B. However, when a new master cell bank was laid down to replace the original it became necessary to conduct a clinical study to demonstrate bioequivalence of the products from the two master cell banks. The ideal way of establishing clinical bioequivalence for this type of product is to use a relevant bioassay to compare the biological activity of Wellferon in blood following administration of the two preparations to healthy volunteers on a cross-over basis. However, in this case, this was not a viable proposition because there was no suitable bioassay with adequate precision and accuracy available. The best alternative approach was to use a pseudo-pharmacokinetic endpoint by using a commercial immunoassay kit to compare immunoreactive concentrations of IFNα, derived from the two Wellferon preparations, in serum.

The most suitable analytical method was the Amersham Biotrak^{TM} kit for the determination of human IFNα in serum. The method is a solid phase ELISA, which utilises an antibody for human recombinant interferon alpha (h)IFNα bound to

the wells of a microtitre plate for capture of interferon alpha in the sample. The captured interferon alpha is then detected by means of a labelled second antibody, also specific for the analyte and, under normal circumstances, the concentration of IFNα in the sample is read off a standard curve, prepared with standards of recombinant IFNα supplied in the kit.

Although the immunoassay utilises antibodies raised against recombinant human IFNα it has been shown to be suitable for the quantitation of both recombinant and natural (e.g. Wellferon) preparations of IFNα. However, there is a complication in that it was demonstrated that different subtypes of Wellferon had markedly different affinities for the anti-IFNα antibodies in the kit. As the relative subtype composition and specific activity of Wellferon can vary significantly from batch to batch, within the specification of the product, different batches of Wellferon would have different analytical responses when measured using the method. There was therefore a high probability that if the method had been used to compare the concentration of IFNα in serum in a bioequivalence study of two different Wellferon preparations then the preparations would not have been demonstrated to be bioequivalent.

In an attempt to overcome the potential difficulties in the measurement of IFNα derived from Wellferon it was decided that the two batches of material to be compared in the clinical bioequivalence study would have been used to prepare the respective standards for the analyses of the blood samples resulting from the administration of each preparation. This enabled the concentration data to be normalised with respect to variations in sub-type and specific activity between the two batches of Wellferon being compared. This strategy was successful as it enabled bioequivalence of Wellferon derived from the new and original master cell banks to be established.

Vaccines constitute another class of bio-pharmaceutical product where immunoassay plays a major role in their pre-clinical and clinical development. Most vaccines are used prophylactically for the prevention of infectious disease and their efficacy depends on the production of an adequate humoral immune response (seroconversion) to the bacterial or viral antigen used for immunisation. This is characterised by the production of specific antibodies to the antigen and the development of immunological memory so that when the individual is later exposed to the appropriate disease-causing organism expressing the antigen a protective immune response can be rapidly mounted to combat the disease. The concentration (titre) of specific antibodies is usually determined by ELISA and for most vaccines there is a range of commercial diagnostic kits and dedicated instrumentation available.

In addition to prophylactic vaccines there is increasing interest in the development of immunotherapeutic vaccines. These will essentially be used for treating already infected patients by enhancing the patient's existing immune response. In addition to the humoral response generated these vaccines enhance the cellular (T-lymphocyte) immune response which is essential in clearing the existing infecting organism. Immunoassay also plays an important part in measuring this cellular immune response and one of the methods that is currently used is ELISpot.

ELISpot is essentially a variant of ELISA in which the proportion of T-lymphocytes that have been induced to respond to the viral or bacterial antigen used for immunisation can be determined. This is done by counting the number of cells capable of secreting a cytokine on stimulation by the antigen *in vitro*. The method involves the addition of a lymphocyte suspension prepared from the blood of a vaccinated individual to a microtitre plate coated with antibodies to an appropriate cytokine such as gamma-interferon (IFN-γ). The antigen is also added and the plate is incubated. Cells that are specific for the antigen respond by producing cytokine on their surface and this binds to the antibody on the plate in close proximity to the secreting cell. After washing, a second antibody detection system is used as in an ELISA assay. However, in this case a number of coloured spots are detected on the bottom of the plate wells, each spot corresponding to a single positive (antigen specific) cell. By counting the number of spots an assessment can be made of the extent of the specific cellular immune response in that individual. The principles of ELISpot are illustrated in Figure 6.6.

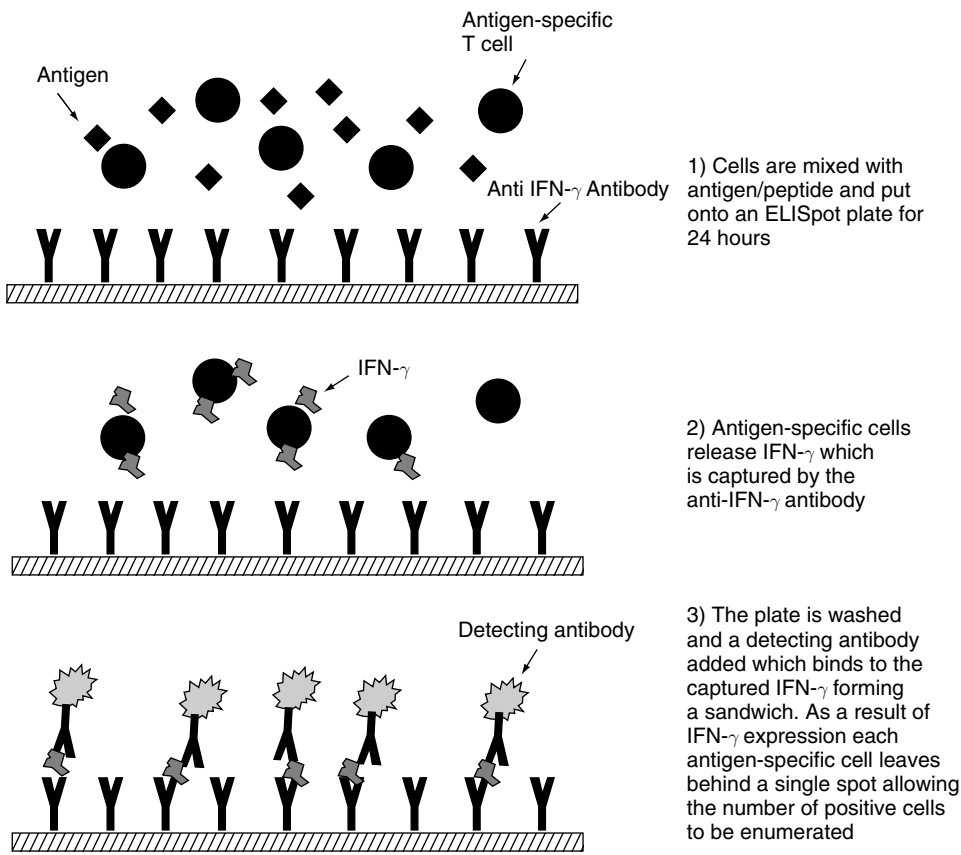

FIGURE 6.6 *Measurement of the cellular immune response by ELISpot.*

6.16 *References*

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