Development and Validation of Flow-Injection (Continuous-Flow) ELISA Techniques.

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Development and validation of flow-injection (continuous-flow) ELISA techniques

Declan Gerard Spillane
Medical Sciences Section

"Development and validation of flow-injection (continuous-flow) ELISA techniques"

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Submitted to the National Council for Education Awards for the Degree of Doctor of Philosophy

September 1998
Abstract

A flow-through enzyme-linked immunosorbent assay was developed based on affinity chromatography using the determination of ferritin in serum as a model system. In this method, samples and standards are introduced to separate columns containing immobilised anti-ferritin antibody, and antigen bound by solid-phase antibody is subsequently detected using an anti-ferritin-alkaline phosphatase conjugate. To detect immobilised label, p-nitrophenyl phosphate is added and product is developed in the column at room temperature. Following elution of product from the column, the absorbance is measured and the columns are regenerated using a low pH elution.

The final developed system requires approximately 1.5 h for the simultaneous assay of standards and up to forty samples. The lower limit of detection using a 200 μl assay volume is $1.82 \times 10^{-15}$ mol or $9.11 \times 10^{-12}$ mol/l (4.1 μg/l). However, there is potential to increase the assay sensitivity further through the use of amplification systems for alkaline phosphatase label. In addition, the present assay gives accurate results, good precision, and is easy to perform. The immunoaffinity columns have been shown to be stable for at least ten assays and presumably could be used for an even greater number. By using different immobilised and labelled antibodies, this method could easily be adapted for use with other analytes.
Acknowledgements

I thank Dr. John O’Mullane for giving me the opportunity to carry out this research, for his interest shown in my work, and for his excellent help and advice throughout.

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Lastly, for the comforts of home, I owe a debt of gratitude to my parents.
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1 Introduction
1 INTRODUCTION

Quantitative and qualitative methods using antibodies or antigens as primary reagents are now integral to many clinical, pharmaceutical, and basic scientific investigations. Such popularity arises either because they are the most effective and suitable of the methods available or because no other type of assay system is feasible. A diverse array of formulations exists, many of which are the subject of discussion in this review.

1.1 Naming Immunoassays

The nomenclature of immunoassays varies greatly, reflecting the immense diversity of assay formulations. Most assay names contain the combining word *immuno*, indicating the assay is antibody-antigen based, another combining word indicating the type of label used (*enzyme* or *enzymo, radio, fluoro*, etc.), along with the word *assay* (Gosling, 1994). Examples include radioimmunoassay (RIA), enzyme immunoassay (EIA), and fluoroimmunoassay (FIA).

These names usually refer to limited-reagent competitive immunoassays, whereas reagent-excess assays are commonly distinguished by reversing the order of the combining forms, e.g., immunoradiometric assay (IRMA), immunofluorometric assay (IFMA), and immunoenzymometric assay (IEMA) (Gosling, 1994). Enzyme-linked immunosorbent assay (ELISA) is a term used to describe solid-phase immunoassays incorporating enzyme as label, irrespective of whether the assay is competitive or non-competitive.
1.2 Classification of Immunoassay

Immunoassays may be classified according to the type of analysis, test sample, assay system, and assay conditions (Miyai, 1991). Table 1.2.1 outlines the criteria that may be used in immunoassay classification.

Table 1.2.1 Classification of immunoassay

<table>
<thead>
<tr>
<th>Type of Analysis</th>
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<tbody>
<tr>
<td>Quantitation</td>
<td>v</td>
</tr>
<tr>
<td>(semi-quantitative, qualitative)</td>
<td></td>
</tr>
<tr>
<td>Characterisation</td>
<td></td>
</tr>
<tr>
<td>(epitope mapping)</td>
<td></td>
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<table>
<thead>
<tr>
<th>Test Sample</th>
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<tbody>
<tr>
<td>Antigen</td>
<td>v</td>
</tr>
<tr>
<td>(macromolecule, peptide, hapten)</td>
<td></td>
</tr>
<tr>
<td>Antibody</td>
<td></td>
</tr>
<tr>
<td>(antibody to exogenous antigen, autoantibody)</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Assay System</th>
<th></th>
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<tbody>
<tr>
<td>Labelled</td>
<td>v</td>
</tr>
<tr>
<td>Unlabelled</td>
<td></td>
</tr>
<tr>
<td>Competitive (limited-reagent)</td>
<td>v</td>
</tr>
<tr>
<td>Non-competitive (reagent-excess)</td>
<td></td>
</tr>
<tr>
<td>Separation (heterogeneous)</td>
<td>v</td>
</tr>
<tr>
<td>Non-separation (homogeneous)</td>
<td></td>
</tr>
<tr>
<td>Visual assessment</td>
<td>v</td>
</tr>
<tr>
<td>Instrumentation</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay Conditions</th>
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<tbody>
<tr>
<td>Liquid phase</td>
<td>v</td>
</tr>
<tr>
<td>Solid phase</td>
<td></td>
</tr>
<tr>
<td>Equilibrium</td>
<td>v</td>
</tr>
<tr>
<td>Non-equilibrium</td>
<td></td>
</tr>
<tr>
<td>Manual</td>
<td>v</td>
</tr>
<tr>
<td>Automated</td>
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(Modified from Miyai, 1991)

While all these factors could potentially be used to classify immunoassays, authors tend only to exploit the differences in assay systems when categorising methods. For example, reviews dealing specifically with enzyme immunoassay (Blake and Gould, 1984, Oellerich, 1984) divided assays on the basis of whether they required a
separation step (heterogeneous) or not (homogeneous). They further divided the two groups into competitive and non-competitive assays, where applicable. Porstmann and Kiessig (1992) discussed enzyme immunoassays in three groups, the two-site enzyme immunoassay for macromolecular antigen detection, the homogeneous enzyme immunoassay, which was divided further into competitive and non-competitive methods, and immunoassays for specific antibodies. Surprisingly, the competitive ELISA for antigen was not accommodated by this classification. Gosling (1990) devised a six group classification from the publications appearing in Clinical Chemistry between 1980 and 1990 using the type of assay system and test sample as his criteria. For example, groups 1 to 4 and 6 are mainly assays of macromolecular antigens or haptens, while group 5 assays are for specific antibodies. Assays in group 4 and most assays in groups 3 and 5 involve the use of excess reagents, whereas those in groups 1 and 2 involve limited reagent concentrations, particularly limited antibody. Group 6 assays can make use of either limited or excess reagents in homogeneous format.

*Group 1* includes immunoassays of macromolecular antigens and haptens, in which labelled analyte is used. They involve the use of limited concentrations of antibody and are universally termed competitive immunoassays.

*Group 2* assays make use of limited concentrations of labelled antibody and immobilised analyte in the measurement of macromolecular antigens and haptens (competitive).

*Group 3* assays include precipitation, nephelometric, and turbidimetric immunoassays, as well as particle agglutination and particle-counting immunoassays. In general, the endpoints involve a direct detection of immune complexes and some are characterised by the lack of any labelled reagent.
Group 4 includes assays involving labels, in which all the principle reagents are in excess (non-competitive).

Group 5 incorporates assays for quantifying specific antibodies, while group 6 includes methods not requiring separation steps (homogeneous). They have features in common that result in a modulation of the signal from label by the binding reaction.

For simplicity, labelled immunoassays can be divided into two large groups, homogeneous or heterogeneous, with each group capable of further division into non-competitive and competitive methods. The term competitive indicates that the analyte (hapten, macromolecular antigen or antibody) is in direct competition with another molecule for binding to an antigen or antibody. Immunoassays relying on the direct detection of immune complexes (e.g., turbidimetry and nephelometry) can be treated separately.
1.3 Heterogeneous Immunoassays

Immunoassays requiring bound from free label separation.

1.3.1 Competitive (limited-reagent)

The category includes the classical competitive immunoassay for macromolecular antigens and haptens (Figure 1.3.1), in which the analyte competes with a labelled analyte for a limited number of antibody binding sites (Österman et al., 1979; Rappuoli et al., 1981; Kaibe et al., 1990; Luppa et al., 1995; Munro and Stabenfeldt, 1984; Veneziale et al., 1981). Following an incubation step, antibody-bound label and free label are separated to allow one of them to be quantified, usually the bound. Plotting the concentration of analyte against the signal obtained gives an inverse plot if bound label is measured or a direct relationship if one measures the free fraction. The labelled analyte is not necessarily identical to the analyte being analysed, but it must be able to compete with the analyte for sites on the antibody (Luppa et al., 1995). Generally, limited concentrations of labelled analyte are used, however, to lower the detection limit, one may add the label in excess some time after the mixing of analyte and antibody (Gosling, 1990). This has the effect of promoting disequilibrium, giving an instantaneous titration of binding sites unoccupied by analyte (Gosling, 1990).

Antibodies of a particular isotype can also be measured in a competitive format using a labelled antibody in competition for a limited number of sites on solid-phase anti-isotype immunoglobulin (Engvall and Perlmann, 1971). Also, heterogeneous competitive assays can detect specific antibodies independently of their isotype using enzyme-labelled specific antibodies and antigen immobilised on solid phase (Porstmann...
and Kiessig, 1992). However, the sensitivity of these assays is limited greatly by the affinity of the antibody to be detected (Porstmann and Kiessig, 1992).

![Diagram](image)

**Either free or bound label measured**

*Figure 1.3.1* Classical competitive immunoassay for macromolecular antigen and hapten. All components are mixed with a limited amount of antibody specific for analyte. Separation of bound from free label is necessary before the measurement of either.

Other competitive immunoassays exist which use a limited concentration of labelled antibody and immobilised analyte in the measurement of macromolecular antigen (Ogbonna et al., 1995) and hapten (Sturgess et al., 1986). These assays have the advantage that labelled antigens with, for example, low solubility in aqueous media can be avoided. Immobilised analyte competes with free analyte in the sample for a limited number of binding sites on the labelled antibody in solution (Figure 1.3.2). To ensure that immobilised analyte is present in a constant limited amount in each assay vessel, the analyte is coupled to a protein and this is used to coat the solid phase. Ogbonna et
al. (1995), in an assay for apolipoprotein B100 (apoB), used an apoB-rabbit immunoglobulin conjugate in competition with apoB in sample for binding to acridinium N-hydroxysuccinimide-labelled anti-apoB antibody. Goat anti-rabbit IgG, immobilised to magnetic particles, was used to separate the apoB-rabbit IgG conjugate bound to labelled antibody from labelled antibody bound to apoB from the sample. Bound chemiluminescence was measured, giving an inverse calibration curve.

Figure 1.3.2 Competitive immunoassay for macromolecular antigen and hapten using a limited amount of immobilised analyte and labelled antibody. After washing, the label associated with solid phase is measured.

Thompson et al. (1985) developed an interesting competitive immunoassay for adenosine deaminase binding protein (ABP), which differs slightly from those mentioned above. The method uses a biotin-labelled anti-idiotype antibody in competition with ABP for a solid-phase monoclonal antibody (URO-4), which is capable of recognising ABP and being recognised by the biotin-labelled antibody. When no ABP is present in sample, binding of the biotinylated anti-idiotype antibody is
maximal, which is then detected using an avidin-horseradish peroxidase conjugate. Increasing ABP decreases the binding of the anti-idiotype antibody to URO-4, thus decreasing the amount of bound enzyme.

1.3.2 Non-competitive (reagent-excess)

The category includes the one-site immunoassay for haptens, which employs a moderate excess of labelled antibody incubated with sample, followed by an excess of immobilised hapten to remove unreacted labelled antibody (Miles and Hales, 1968; cited in Piran et al., 1995). The signal associated with the supernate is measured after transfer to a second reaction cuvette, giving a direct relationship (Figure 1.3.3). Piran et al. (1995) applied this principle to the measurement of triiodothyronine ($T_3$) using a chemiluminescent label. Acridinium ester (AE)-labelled anti-$T_3$ is incubated with sample, producing AE-anti-$T_3$/$T_3$ complexes. Unreacted AE-anti-$T_3$ is removed using an excess of controlled-pore glass particles (CPG) with immobilised diiodothyronine ($T_2$). Only AE-anti-$T_3$ possessing two unoccupied sites is bound by the $T_2$-coated CPG. Paramagnetic particles (PMP) with immobilised anti-AE are then added to the same cuvette to capture AE-anti-$T_3$/$T_3$ complexes. AE-anti-$T_3$ immobilised to the CPG cannot bind due to steric hindrance. The PMP are magnetically separated from the CPG and the chemiluminescence associated with the PMP is measured, giving a direct relationship. The authors stressed that this system has advantages over previous one-site assays, since there is no need to transfer material to a new cuvette and the label is measured in the absence of possible sample interferences.
**Step 1**

Excess of labelled antibody bound to analyte

- Analyte from sample
- Excess of labelled antibody

**Step 2**

Excess amount of solid-phase analyte

- Free labelled antibody removed by immobilised analyte

---

**Figure 1.3.3** One-site immunoassay for haptens. Analyte from sample is reacted with an excess of labelled antibody (step 1). To remove unoccupied labelled antibody, an excess of solid-phase analyte is added (step 2). The signal associated with the supernate is measured following transfer to a new reaction vessel.

The category also includes the two-site sandwich immunoassay for macromolecular antigens, in which an excess of solid-phase antibody captures analyte in solution, with subsequent detection using an excess of antibody-label (Figure 1.3.4) (Ishikawa et al., 1980; Papoian et al., 1991; Noé et al., 1992; Masayuki et al., 1993). Therefore, signal obtained is directly proportional to the amount of analyte present in the sample. While this type of assay format is generally used for the measurement of macromolecular antigens, it has also been used to measure human IgE using goat antibodies as capture and label (Ruan et al., 1987). Generally, two-site assays involve the sequential incubation of sample and labelled antibody, but one-step assays involving simultaneous incubations have been developed (Nomura et al., 1983; Burgi et al., 1988; Tanebe et al., 1992; Brailly et al., 1994). However, the high-dose hook effect becomes problematic in these systems, since at high analyte concentrations there is competition between free analyte and conjugate-bound analyte for reaction with the solid-phase antibody (Nomura et al., 1983; Porstmann and Kiessig, 1992). Theoretically, but not practically, the problem may be avoided through the use of a large amount of...
immobilised and labelled antibody (Nomura et al., 1983). Otherwise, one may use the sample to be tested in two dilutions differing from each other by at least a factor of 100 (Nomura et al., 1983; Porstmann and Kiessig, 1992). If speed of assay is not a priority and sensitivity is, one may use an unlabelled secondary antibody to detect the immobilised analyte, followed by an anti-species conjugate. Ko et al. (1992), in an assay for human interleukin (IL)-8, used a mouse monoclonal antibody as primary capture, rabbit anti-IL-8 as the secondary antibody, and an alkaline phosphatase-labelled goat anti-rabbit antibody as conjugate. To prevent cross-reaction, it is important that the primary capture antibody and the secondary antibody are derived from different species.

Figure 1.3.4 The principle of the two-site sandwich assay for macromolecular antigen. Antigen in the sample is mixed with an excess of solid-phase antibody. After washing the solid phase, labelled antibody is added and label remaining bound after washing is measured.

Heterogeneous non-competitive immunoassay is also used for the measurement of specific antibodies. In the “antibody capture” assay (Figure 1.3.5), serum is incubated with an excess of solid-phase antigen and the amount of bound antibody is detected using an excess of labelled class-specific antibody (Chlang et al., 1989).
"Antibody capture" immunoassay for specific antibody of particular isotype. Antibody in sample is mixed with an excess of solid-phase antigen. After washing, an excess of labelled anti-class antibody is added. Signal from bound antibody is measured after washing.

"Antigen capture" assays (Figure 1.3.6) use immobilised anti-class antibodies directed against relevant antibodies from the sample (Gosling, 1990). Subsequently, labelled antigen is captured only by the immunoglobulin of interest or one may use unlabelled antigen, which can be detected through the use of a labelled antibody directed against the bound antigen (Gosling, 1990).
1.4 Homogeneous Immunoassays

Homogeneous immunoassays do not require bound from free label separation. Assays in this category generally rely on a modulation of the signal from the label by the antigen-antibody reaction. However, separation-free immunoassays are generally less sensitive than their heterogeneous counterparts (Gosling, 1990), with detection limits ranging from $10^{-5}$ to $10^{-11}$ mol/l (Jenkins, 1992; Price and Newman, 1991). This is largely due to signal measurements in the presence of potential sample interferents (Jenkins, 1992). In any case, the assays tend to be used to monitor concentrations of analytes such as drugs when low detection limits are not required.

1.4.1 Competitive

1.4.1.1 Enzyme multiplied immunoassay technique (EMIT)

Rubenstein et al. (1972) developed the first homogeneous enzyme immunoassay, which relied on the modulation (inhibition) of enzyme activity of a morphine-lysozyme conjugate when bound by anti-morphine antibodies. Addition of free morphine to this mixture reduced the inhibition of enzyme activity in direct proportion to the amount of morphine added (Figure 1.4.1). The small size of some haptens permits intimate interaction of the antibody and enzyme within the complex, thus providing a mechanism for modulation of enzyme activity through steric exclusion (Rubenstein et al., 1972). By contrast, this is more difficult to achieve using enzyme conjugates of large molecules, unless the substrate is sufficiently large. Gibbons et al. (1980) employed the same principle to the assay of IgG antibodies using an IgG-β-galactosidase conjugate in competition with IgG for sites on rabbit anti-human γ-chain.
antibodies. The commonly used substrate, o-nitrophenyl-β-galactoside (oNPG), was coupled via the o-nitrophenyl group to high molecular weight polymers (dextrans), producing macromolecular substrates required (10-2,000 K). When a conjugate with 4.3 IgG's per enzyme was incubated with excess rabbit anti-human IgG, the activity with 0.4 mmol/l 10 K, 40 K, 70 K, and 2,000 K substrates was inhibited by 42, 83, 87, and 95%, respectively.

![Chemical diagram]

**Figure 1.4.1 Principle of the EMIT system of homogeneous enzyme immunoassay.** Conjugation of hapten or macromolecular antigen does not destroy enzyme activity, but combination with specific antibody causes a marked inhibition. The measured enzyme activity is dependent on the relative amounts of free analyte and enzyme-labelled analyte.

It is also possible to observe an increase in enzymatic activity upon antibody binding. Thompson (1989; cited in Jenkins, 1992), in an assay for thyroxine (T₄), used a conjugated ligand which was the inhibited form of malate dehydrogenase. Binding of antibody resulted in the reversal of enzyme inhibition.
In addition to the use of enzymes as labels, prosthetic groups (Morris et al., 1981), substrates (Burd et al., 1977; Wong et al., 1979), inhibitors (Bacquet and Twumasi, 1984; Finley et al., 1980), and enzyme fragments (Khanna et al., 1989; Engel and Khanna, 1992) have been conjugated to ligands in homogeneous enzyme immunoassay.

1.4.1.2 Prosthetic group-labelled immunoassay (PGLIA)

Morris et al. (1981) designed a homogeneous competitive immunoassay for theophylline using FAD as label. In this system, sample ligand and FAD-labelled ligand compete for sites on antibody of limited concentration. In the free form, the ligand-FAD conjugate has the ability to regenerate the active holoenzyme, glucose oxidase, but when bound by antibody it is sterically restricted from associating with apoenzyme. Therefore, glucose oxidase activity is at its lowest when no ligand is present in the sample.

1.4.1.3 Reactant-labelled fluorescent immunoassay (RLFIA)

Homogeneous methods using substrate or reactant as label have also been described (Burd et al., 1977; Wong et al., 1979). The hapten is labelled with a fluorogenic substrate, β-galactosyl umbelliferone, which is not fluorescent under assay conditions. The hapten/dye conjugate is used as substrate for the enzyme β-galactosidase, generating the fluorescent product, umbelliferone. In the absence of hapten in sample, the conjugate is bound by specific antibody, sterically restricting access of the enzyme to substrate and little fluorescence results. Fluorescence increases in direct proportion to the added hapten. Worah et al. (1981) developed an assay for human IgM using the same principle with N-(6-aminohexyl)-7-β-galactosyl-coumerin-3-carboxamide as fluorogenic substrate. This type of scheme is limited in that it lacks the amplification
feature of other enzyme-based immunoassays, since the system produces only one product molecule per label. Therefore, its detection limit is dependent upon the detection limit of the fluorescent product.

1.4.1.4 Inhibitor-labelled immunoassays

Irreversible inhibitors have also been used as labels in the development of homogeneous immunoassays (Bacquet and Twumasi, 1984; Finley et al., 1980). Bacquet and Twumasi (1984) used an avidin-5,5-diphenylhydantoin (DPH) conjugate in competition with free DPH for a limited number of antibody binding sites. The interaction of anti-DPH with the avidin-DPH conjugate sterically inhibits avidin inactivation of pyruvate carboxylase. Therefore, when the DPH hapten is absent from sample, the avidin-DPH conjugate is bound by antibody and enzyme activity is maximal. The activity decreases with increasing DPH, i.e., an inverse relationship exists.

The same rationale was applied to an assay for T₄ using a cholinesterase inhibitor as label (Finley et al., 1980).

1.4.1.5 Cloned enzyme donor immunoassay (CEDIA)

This elegant homogeneous immunoassay system uses enzyme fragments prepared by recombinant DNA technology (Khanna et al., 1989; Engel and Khanna, 1992). Two separate genes are engineered to produce two separate polypeptide fragments, enzyme donor (ED) and enzyme acceptor (EA), which can come together to produce active β-galactosidase enzyme. The ED can be coupled to ligands without affecting the activity after complementation. If the hapten-ED component is bound by antibody, the recombination process cannot occur and so enzyme activity is lost. The system is typically competitive, with hapten-ED and hapten in sample competing for limited
antibody. When hapten is absent, binding of conjugate is maximal and the hapten-ED is unavailable to bind with EA. With increasing free hapten, the hapten-ED is available to combine with the EA fragment, resulting in an active enzyme and an increase in signal. CEDIA assays have been successfully developed for theophylline, phenobarbitol, B12, and folate, as well as large molecular weight analytes like ferritin (Engel and Khanna, 1992).

1.4.1.6 Fluorescence-based homogeneous immunoassay

Fluorescent labels may also be used in homogeneous competitive immunoassay formats for haptens, macromolecular antigens, and antibodies. As with enzyme-based assays, signal is modulated as a result of the binding reaction. Methods include fluorescence polarisation immunoassay (Young et al., 1984; Sidki et al., 1988), fluorescence quenched immunoassay (Kobayashi, 1980; Nargessi et al., 1979), and the fluorescence excitation transfer immunoassay (Van der Werf and Chang, 1980; Calvin et al., 1986). The fluorescence quenched assays in the "direct" format are used for haptens, whereas the "indirect" method can be used for both macromolecular antigen and antibody. Fluorescence excitation transfer is useful for all analytes.

1.4.2 Non-competitive

The category includes the enzyme channelling immunoassay (Litman et al., 1980), enzyme enhancement assay (Gibbons et al., 1981), and liposomal immunoassay (Frost et al., 1996).

1.4.2.1 Enzyme channelling immunoassay

The assay allows for the quantitation of large antigens and antibodies using a specific hexokinase-labelled antibody and agarose beads, the latter is coated with specific
antibody and glucose-6-phosphate dehydrogenase. The subsequent binding reactions result in an accelerated formation of NADH from glucose, ATP, and NAD due to the close proximity of the two enzymes. By contrast, there is a pronounced lag phase in the reaction when the enzymes are separated. The assay measures the rate of NADH formation fluorometrically and gives a direct relationship.

1.4.2.2 Enzyme enhancement immunoassay

Gibbons et al. (1981) designed the enzyme enhancement assay for polyvalent ligands and antibodies. A limited amount of β-galactosidase-labelled antibody combines with a polyvalent analyte and then an excess of negatively charged antibody is added. The binding of the second succinylated antibody in excess increases the local charge around the enzyme, directly affecting the rate of formation of product from dextran-linked oNPG. Product manifests itself by forming a second light scattering phase, in the form of small droplets, which can be determined by turbidimetry. The relationship is direct and has been used for the measurement of human IgG and C-reactive protein (CRP).

1.4.2.3 Liposomal immunoassay

Liposomes entrapping the dye sulphorhodamine were used to develop an assay for anti-cardiolipin antibodies (ACAs) (Frost et al., 1996a). IgG ACAs induce liposomal lysis in the presence of magnesium ions and the resulting absorbance changes are directly proportional to the amount of ACAs present. The dye, when trapped in the liposomes, forms dimers at high concentrations which absorb at 530 nm. When released from the liposomes into aqueous media, the absorption spectrum reverts to that of the free dye (565 nm). Therefore, one can monitor the decrease in absorbance at 530 nm (inverse) or increase at 565 nm (direct).
1.4.2.4 *Substrate inhibition immunoassay*

Suzuki et al. (1989) developed a homogeneous enzyme immunoassay for protein C, in which aggregation of the enzyme label overcame substrate inhibition by H\textsubscript{2}O\textsubscript{2}. The assay uses horseradish peroxidase-labelled antibody to produce aggregates with protein present in sample. These aggregates have peroxidase activity in the presence of excess H\textsubscript{2}O\textsubscript{2}, whereas free labelled antibody does not. Therefore, the relationship between protein C concentration and enzyme activity is direct.
1.5 Immunoassays Relying on Direct Detection of Immune Complexes

The category includes immunodiffusion, nephelometric, and turbidimetric immunoassays, as well as particle-aided immunoassays. These systems make use of labelled and unlabelled reagents, and involve the direct detection of immune complexes.

Single radial immunodiffusion (SRID) involves pipetting a volume of sample into holes cut into a buffered agar gel containing specific antibody and, after incubation, measuring the diameter of precipitin rings visible around each well, the diameter being directly proportional to the amount of analyte present (Salabè et al., 1996). “Rocket” electrophoresis is based on the same principle, however, the time of reaction is reduced significantly through the use of electric charge (Nunez et al., 1997). Peaks or rockets form in the gel, the height of which are directly proportional to the concentration of analyte present.

More refined approaches used to monitor immune complex formation are turbidimetry (Weets et al., 1996) and nephelometry (Pernet et al., 1996). The principles of light scattering techniques such as turbidimetry and nephelometry are discussed comprehensively by Price et al. (1983). Nephelometry measures the scattering species (immune complexes) in solution by a means of the increase in light intensity at some angle θ when the incident beam is passed through the sample (Figure 1.5.1a). The angle is usually 90°, but some nephelometers are designed to measure scattered light at an angle other than 90°, in order to take advantage of the increased forward scatter intensity caused by high scattering from larger particles (immune complexes). When light scatter units are measured and plotted against known concentrations of analyte, the relationship is non-linear. Turbidimetry, on the other hand, measures the scattering
species in solution by means of a decrease in intensity of the incident beam as it passes through the sample (Figure 1.5.1b). Light has been lost due to reflection, absorption, and scatter. Linear standard curves are used in turbidimetry to convert transmittance to concentration, giving inverse plots.

Figure 1.5.1 Principles of nephelometry (A) and turbidimetry (B).

To increase the sensitivity of immune complex detection, turbidimetry and nephelometry can also be carried out using labelled antibody or antigen in particle-enhanced immunoassays. The particle-enhanced turbidimetric immunoassay (PETIA) and the particle-enhanced nephelometric immunoassay methods have both been used to measure protein antigens using antibody-coated latex particles (Medcalf et al., 1990a; Medcalf et al., 1990b; Borque et al., 1993; Borque et al., 1995). Antibodies have also been measured using either antibody coated particles (Thakkar et al., 1991) or particles coated with specific antigen (Harchali et al., 1994).

Particle-aided immunoassays of this type (direct agglutination) are suitable only for the measurement of antibody and multivalent antigen, since haptens may not allow immune complex formation with bivalent antibody. However, particle-enhanced inhibition assays (turbidimetric and nephelometric) have been developed using analyte-coated
particles in competition with analyte from sample for antibody binding sites. This format is particularly useful for haptens, since numerous hapten molecules can coat the solid-phase particle, thus each particle is essentially acting as multivalent antigen. Resultantly, immune complex formation with bivalent antibody is made possible. Such assays have been used in the measurement of small molecules such as digoxin (Davey et al., 1997) and theophylline (Litchfield et al., 1984), as well as albumin (Thakkar et al., 1997), alpha lactalbumin (Cuillière et al., 1997), and immunoglobulins G, A, and M (Cuillière et al., 1991).

Particle-counting immunoassay (PACIA) differs slightly in that it involves measuring the decrease in number of unagglutinated particles during the course of an immunoreaction (Masson and Holy, 1986; Mathieu et al., 1989; Collet-Cassart et al., 1981). This type of light scattering immunoassay appears to be the most sensitive, giving detection limits in the picomolar range or lower, e.g., 1.5 pmol/l (Mathieu et al., 1989), 0.2 pmol/l (Wilkins et al., 1988; cited in Gosling, 1990).

Generally, immunoassays relying on direct detection of immune complex formation have to use polyclonal antisera, since monoclonal antibodies alone cannot precipitate antigen unless it has multiple repeating epitopes. However, mixtures of monoclonal antibodies can be used to assay macromolecular antigens (Mathieu et al., 1989). Moreover, single monoclonal antibodies can be used in inhibition formats for hapten and macromolecular antigen, since numerous molecules of analyte are coupled per particle (Davey et al., 1997; Thakkar et al., 1997).

In addition, to maximise and accelerate precipitin formation in light-scattering immunoassays, polyethylene glycol 6,000 and 8,000 may often be used (Litchfield et al., 1984; Collet-Cassart et al., 1981; Thakkar et al., 1997).
Red blood cells have also been used as labels in direct detection immunoassays, for example, chicken or sheep red cells coated with *Treponema pallidum* antigen are used in the haemagglutination test for detection of specific antibody (Kasahara, 1992). Presence of antibody in patient serum gives visible agglutination of the red cells (haemagglutination). Dilution of patient serum allows one to titre the antibody. Haemagglutination tests are now available in kit form for detecting rheumatoid factor and antibody to hepatitis B virus (HBV) surface, e, and c antigens.

Latex particles have also been used in simple agglutination assays for, for example, human chorionic gonadotropin (hCG), fibrin degradation products (FDP's), streptolysin O, and human immunodeficiency virus (HIV) antibodies. The assays are rapid (minutes) and visual, and can be used either qualitatively or semi-qualitatively. They are used widely in hospital laboratories in kit form.
1.6 Reagents

1.6.1 Antibodies

1.6.1.1 Polyclonal antibody

Polyclonal antisera represents the total population of antibodies present in animal serum, with each antibody representing the secretory product from a single lymphocyte clone. When an immunogen containing many epitopes is injected into an immunocompetent host, each epitope is recognised by a single lymphocyte clone via specific antibody displayed on its surface. Each clone is thus stimulated to differentiate and proliferate (often with the help of T-cells) into antibody-producing plasma cells. Therefore, many different antibodies are produced in response to the immunogen (i.e., polyclonal), each one having specificity for a single epitope. Polyclonal antisera to a specific immunogen is not only heterogeneous with respect to specificity, but is also heterogeneous with respect to affinity. Therefore, the affinity is an average of the combined affinities of the individual antibodies for their specific epitopes.

There is no formal reliable guidelines for polyclonal antibody production, but there are a number of factors which must be taken into account, for example, the nature of the antigen, immunological adjuvants, choice of animal, routes of immunisation, dosage of immunogen, and immunisation schedule (Burrin and Newman, 1991; Dunbar and Schwoebel, 1990; Abdul-Ahad and Gosling, 1994; Schimpl, 1993).

Not all antigens can induce a good immune response (haptens) and therefore need to be coupled to larger molecules such as bovine serum albumin (BSA) to render them immunogenic. The immunogen preparation must be of very high purity, since in a
protein preparation that contains as little as 1% contamination, the majority of antibodies may recognise that contaminant if it is highly immunogenic.

Adjuvants, substances which non-specifically enhance the immune response to an immunogen, are also of importance in the production of polyclonal antisera. The most frequently used adjuvants are the water-in-oil emulsions with the immunogen in the aqueous phase (e.g., Freund’s incomplete adjuvant), or one may use a microbial antigen in the mixture to further enhance the adjuvant properties, e.g., heat-killed *Mycobacterium tuberculosis* in Freund’s complete adjuvant. Other adjuvants include alum hydroxide (alum), saponins complexed to membrane protein antigens, bacterial products such as lipopolysaccharide (LPS) and muranyl dipeptide (MDP), liposomes, and latex particles.

The major species used for the production of polyclonal antisera are rabbits, sheep, goat, guinea pig, and donkey, with the larger animals being more popular for commercial-scale production. The age of the animal is important, since newborn animals are unable to synthesise antibody and old animals show a reduced response when compared to that seen in their prime. Some particular strains or breeds of animal may offer advantages over others with respect to the response against a particular immunogen, e.g., guinea pigs for insulin antisera.

Routes of immunisation and doses used vary greatly, but generally primary doses involve low concentrations of immunogen injected intradermally. Secondary treatments may involve subcutaneous routes at two to four sites. A suitable primary dose for a rabbit or guinea pig is about 100 μg (200-500 μg for sheep and goats), with 10-50% of the primary dose used for booster doses. Low doses produce higher affinity antibody, since at high concentrations of immunogen, B-cells with low affinity immunoglobulin receptors may be recruited. After the primary injection, the antiserum titre rises
gradually and reaches a plateau after 4-6 weeks, after which a slow decline occurs. Following the booster injection, the titre reaches a maximum after 10-14 days, at which time the antibody should be harvested. Subsequent boosters may or may not produce improvements in titre.

Once the animal has been bled, partial purification of immunoglobulin from the antiserum may be obtained using ammonium sulphate fractionation plus DEAE (dimethylaminoethyl) ion-exchange chromatography, or using protein A/G affinity chromatography. However, for the isolation of a pure population of antibodies, affinity extraction with immobilised antigen or antibody is desirable.

Figure 1.6.1 Possible immunisation schedule for polyclonal antibody production.

1.6.1.2 Monoclonal antibody
Monoclonal antibodies are defined as antibodies derived from one lymphocyte clone, and are thus homogeneous. They are not inherently different from antibodies making up polyclonal antisera, since polyclonal antisera is only a mixture of monoclonal antibodies. Monoclonal antibodies offer many advantages over polyclonal antibodies, including an improved continuity of supply and better defined specificity. As a result,
they are widely used in immunoassay (Brailly, 1994; Piran et al., 1995; Thompson et al., 1984). However, they are not without their limitations (Table 1.6.1). The increasing popularity of monoclonal antibodies amongst workers has been illustrated by Gosling (1990). He has shown that the percentage of new immunoassays (published in Clinical Chemistry) using monoclonal antibodies has risen from 0% in 1980 to >50% in 1990. One would assume that the percentage is even higher today.

Table 1.6.1 Comparison of monoclonal and polyclonal antibodies

<table>
<thead>
<tr>
<th>Advantages of monoclonal over polyclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Indefinite supply of antibody with constant characteristics.</td>
</tr>
<tr>
<td>2. Obtained from impure immunogen. Require highly purified immunogen for polyclonal antibody production.</td>
</tr>
<tr>
<td>3. Monoclonal antibody is more specific.</td>
</tr>
<tr>
<td>4. Affinity and fine specificity are better defined.</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Advantages of polyclonal over monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Higher affinities predominate.</td>
</tr>
<tr>
<td>2. Easier and cheaper to produce.</td>
</tr>
<tr>
<td>3. Form precipitin lattices with most antigens. However, monoclonal antibodies are often used in mixtures to overcome this problem.</td>
</tr>
<tr>
<td>4. Monoclonal antibodies rely on detection of only a single epitope.</td>
</tr>
</tbody>
</table>

(Compiled using data from Siddle, 1985; Dunbar and Skinner, 1990)

The technique of monoclonal antibody production (Abdul-Ahad and Gosling, 1994; Burrin and Newman, 1991; Dunbar and Skinner, 1990) relies on the fusion of normal B-cells (plasma cells) with myeloma cells (cancerous cells of the B-cell lineage) to produce hybridomas (Figure 1.6.2). The hybridoma has properties of both cells, possessing the antibody producing ability of plasma cells and the immortality of
myeloma cells. The majority of monoclonal antibodies are generated by means of mice spleen (BALBc) and murine myeloma cells, but rat/rat, rat/mouse, sheep/mouse, and human/human hybridomas have also been developed.

Immunisation of the mouse is usually with 10-100 µg of immunogen in 250 µl of emulsion with Freund's complete adjuvant injected intradermally or subcutaneously, followed by boosters of similar amount after 4-6 weeks using Freund's incomplete adjuvant. Basically, isolated spleen cells from the mouse are fused with myeloma cells using polyethylene glycol (PEG). All cells are grown in hypoxanthine, aminopterin, and thymidine medium (HAT), which is selective only for spleen-myeloma hybrids. Since successful fusions are rare, only a few wells contain small clusters of viable cells, however, not all produce antibody, and even if they do, many will not secrete antibody specific for the antigen of interest. After subculture, wells are then screened for antibody of interest, usually using solid-phase antibody capture assays, followed by subculture of antibody positive clones to ensure purity. Production of antibody can be carried out in homogeneous suspension in flat tissue culture flasks, or hybridomas can be established in the peritoneal cavity of BALB/c mice (with 10% contamination from non-specific antibody).

With the advent of monoclonal antibodies has come the production of anti-idiotype antibodies, antibodies with specificity for the paratope of a specific immunoglobulin. If a homogeneous (monoclonal) antibody is injected into an immunocompetent animal, antibodies may be produced with specificity for the paratope (Dunbar and Skinner, 1990). These antibodies have been used in competitive assay format, as described earlier (Thompson et al., 1985).

In addition, Bugari et al. (1990) have reported on the use of bispecific antibodies, obtained through the fusion of two hybridomas producing antibodies to β-
galactosidase and human lutropin. The resultant antibody, used as tracer antibody in a sandwich ELISA format, had more reactivity of both antibody and enzyme when compared to traditional antibody-enzyme conjugates.

![Diagram of monoclonal antibody production]

**Figure 1.6.2** Monoclonal antibody production.

1.6.1.3 Antibody fragments

Antibodies are essentially bifunctional molecules, containing specific sites for interaction with antigen (located in the Fab region) and interaction with the other components of the immune system (Fc region) (Figure 1.6.3). Antibodies also interact with specific proteins such as proteins A and G, via the Fc region, a property which has been exploited successfully in immobilising antibodies to solid supports (Lu et al.,
1996) and in the production of labelled antibody using a protein A-enzyme conjugate (Kobatake et al., 1990).

However, despite these developments, the Fc portion of the IgG molecule is often unnecessary and even undesirable in an antibody to be used as an immunoassay reagent. The Fc in a labelled antibody may bind to either complement or rheumatoid factor present in the sample, thus increasing the amount of label non-specifically bound (Gosling, 1990). Moreover, the presence of Fc in an immobilised antibody may correspondingly act as a binding site for the same proteins and result in blocking of the analyte-specific binding sites (Gosling, 1990). Also, the Fc portion of the IgG molecule is hydrophobic, causing high non-specific binding of IgG when used as label (Ishikawa et al., 1989).

The Fc portion can be removed, without impairment of antigen binding, by digestion with either papain or pepsin to yield monovalent Fab or divalent F(ab)'₂, respectively (Figure 1.6.3) (Ishikawa, 1987). Reduction of F(ab)'₂ yields monovalent Fab' (Ishikawa, 1987).

Figure 1.6.3 The various regions of IgG, produced by papain and pepsin cleavage.
Many workers have turned to univalent Fab' fragments as labels to reduce non-specific binding (Ishikawa et al., 1982; Ruan et al., 1987; Hashida and Ishikawa, 1990; Aubin et al., 1997). In these cases, the Fab' fragment is conjugated to enzyme by selective use of thiol groups in the hinge of Fab', distal to the antibody binding site. Fab' fragments labelled by the non-hinge method have higher non-specific binding and lower antigen binding (reduced by 50-65%) than those prepared by the hinge method (Ishikawa et al., 1989).

Fragments of antibodies have also been utilised in non-enzymatic immunoassays, in which F(ab)₂ fragments are coupled to latex particles for use in particle-aided immunoassays (Borque et al., 1993; Collet-Cassart et al., 1981; Borque et al., 1995). In addition, Fab'-coated liposomes containing sulphorhodamine B have been used in a sandwich-type format for urinary microalbumin (Frost et al., 1996b).

### 1.6.2 Labels

The principal factors determining the suitability of a labelling substance include specific activity, ease of labelling, ease of endpoint determination, associated hazards, and possibilities for convenient assay formulation or homogeneous operation (Gosling, 1990). The specific activity is of primary importance, since high specific activity is essential for developing immunoassays with low detection limits.

#### 1.6.2.1 Radioisotopes

Radioisotopes were introduced into immunoassay in 1960 (Yalow and Berson, 1960) and very much represent the traditional label. However, their use has declined significantly over recent years due primarily to associated radiation hazards. Gosling (1990) has shown that between 1980 and 1990 the use of radioisotopes as labels
decreased from 50% to an apparently stable 25% of new assays. Despite this fact, about 70% of commercial assays in 1994 still used isotopic labels (Gosling, 1994).

The criteria for the selection of a suitable radioactive tracer are half-life, specific radioactivity (disintegration/second or Becquerels), and practicability of labelling (Simonnet and Guilloteau, 1993). Since the half-life of $^{131}\text{I}$ is only 8 days and the specific radioactivity of $^{14}\text{C}$ is $2.33 \times 10^3$ TBq/mmol (Table 1.6.2), neither is employed as label in immunoassay (Simonnet and Guilloteau, 1993). $^{125}\text{I}$, with a half-life of 60 days, is a good compromise and is therefore the most popular radioisotope used (Simonnet and Guilloteau, 1993). However, because the iodine atom is relatively large, it proved unsuitable for small molecules such as steroids, as it interfered with antibody binding (Edward, 1992). Although $^3\text{H}$ can be substituted directly for a hydrogen present in the molecule to be labelled, making it suitable for low molecular weight molecules, its relatively expensive liquid scintillation detection and low specific radioactivity means that $^{125}\text{I}$ is often used for labelling steroids despite the steric hindrance (Edwards, 1992).

**Table 1.6.2** Half-lives and specific radioactivity of radioisotopes

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Half-life in days</th>
<th>Specific radioactivity (TBq/mmol*)</th>
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<tbody>
<tr>
<td>$^{131}\text{I}$</td>
<td>8</td>
<td>600</td>
</tr>
<tr>
<td>$^{32}\text{P}$</td>
<td>14.3</td>
<td>336</td>
</tr>
<tr>
<td>$^{125}\text{I}$</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>$^{57}\text{Co}$</td>
<td>271.4</td>
<td>18</td>
</tr>
<tr>
<td>$^3\text{H}$</td>
<td>4475</td>
<td>1.07</td>
</tr>
<tr>
<td>$^{14}\text{C}$</td>
<td>$2.06 \times 10^6$</td>
<td>$2.33 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

*TBq = terabecquerel = $10^{12}$ becquerel

(From Simonnet and Guilloteau, 1993)
The specific activity of a tracer is a direct consequence of the ratio of radioisotope to carrier molecule in the tracer and the activity of the radioisotope. Theoretically, the specific activity could be increased by using several molecules of isotope per molecule of substance. However, since this often leads to a loss in immunoreactivity, the ratio rarely exceeds 1:1 (Edwards, 1992; Simonnet and Guilloteau, 1993).

1.6.2.2 Enzymes

Over recent years, the decline in the popularity of radioisotopes has been associated with an increase in the use of enzymes as labels (Gosling, 1990), since enzymes offer many advantages over radioisotopes, including the lack of radiation hazards and longer shelf-life (Table 1.6.3). In addition, a single enzyme molecule can act upon its substrate (present in large excess) to generate many product molecules in a short time. Conversion rates of 100-1,000 molecules per enzyme molecule per second gives a large degree of signal amplification (Gudgin Dickson et al., 1995). Product molecules are commonly detected by colorimetry, but if even greater sensitivity is required, fluorogenic substrates may be used. Alternatively, one may use luminescent (bio- and chemi-) assays, in which light is emitted through the direct action of enzyme on substrate or indirectly through the channelling of the product into further reactions.

The ideal enzyme has a high turnover number, low Km, is stable upon storage (both in free and conjugated form), is pure or easy to prepare, is easy to conjugate and easily detectable, and is preferably absent from organisms from which samples are taken (particularly for homogeneous assay) (Porstmann and Kiessig, 1992; Tijssen, 1993). In addition, its chromogenic substrates should be water soluble, stable, odourless, colourless, non-mutagenic, non-toxic, and form product with a high molar extinction
coefficient and a broad absorbance maximum between 400 and 600 nm (Porstmann and Kiessig, 1992). Unfortunately, no such enzyme exists.

Table 1.6.3 Comparison of enzyme with radiolabels in immunoassay

a) Advantages of enzyme labels

(i) No radiation hazards occur during labelling or disposal of waste.
(ii) Enzyme-labelled products can have a long shelf-life, e.g., 1 year or more.
(iii) Equipment for enzyme assay can be inexpensive and is generally available.
(iv) Homogeneous assays can be completed in a few minutes.
(v) Heterogeneous assays are ideal for visual qualitative tests.
(vi) Multiple simultaneous assays are possible.

b) Disadvantages of enzyme labels

(i) Plasma constituents may affect enzyme activity.
(ii) Assay of enzyme activity can be more complex.
(iii) Less control of enzyme labelling reactions.
(iv) At present homogeneous enzyme immunoassays have limited sensitivity.

(Modified from Blake and Gould, 1984)

A large number of enzymes are currently available, but in approximately 65% of all assays horseradish peroxidase is used, whereas alkaline phosphatase is used in about 25% of assays (Tijssen, 1993). This is in partial agreement with Gosling's (1990) observation that, of all new enzyme immunoassays published in 1990, 50% used horseradish peroxidase and 25% used alkaline phosphatase. Other enzymes used include β-galactosidase, urease, acid phosphatase, glucose oxidase, glucoamylase, carbonic anhydrase, acetylcholinesterase, xanthine oxidase, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase, ribonuclease (Tijssen, 1993),
metapyrocatechase (Kobatake et al., 1990), thrombin (Merenbloom and Oberhardt, 1995), and hexokinase (Litman et al., 1980).

### 1.6.2.2.1 Horseradish Peroxidase

Horseradish peroxidase (HRP) is by far the most common label used in enzyme immunoassay, its popularity at least partly stemming from its high turnover number, the variety of sensitive assay systems available, its suitability for diverse conjugation procedures, and its small molecular size (40,000 Da) (Gosling, 1990). Peroxidase reacts with its substrate, H$_2$O$_2$, decomposing it to H$_2$O by a hydrogen donor (DH$_2$), the resulting oxidised hydrogen donor representing the final product, measured by its absorbance in the visible spectrum (Figure 1.6.4) (Tijssen, 1993; Johannsson, 1991). Reactions are carried out at neutral or slightly acidic pH (Johannsson, 1991).

\[
\text{H}_2\text{O}_2 + 2\text{DH}_2 \xrightarrow{\text{HRP}} 2\text{H}_2\text{O} + \text{D}_2\text{H}_2
\]

**Figure 1.6.4**

Several chromogenic substrates have been used in the assay of horseradish peroxidase, including $o$-phenylenediamine ($o$PD), 3,3',5,5'-tetramethylbenzidine (TMB), and 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (Johannsson, 1991). The specific activity with ABTS is about 1,000 U/mg at 25°C, and that of other substrates is a few fold higher (Johannsson, 1991). To increase the sensitivity of horseradish peroxidase detection, the fluorogenic substrates, $p$-hydroxyphenylacetic acid (HPAA) and 3-($p$-hydroxyphenyl) propionic acid (HPPA), may be used (Porstmann and Kiessig, 1992). In addition, horseradish peroxidase detection may be enhanced via the
luminescent oxidation of cyclic diacylhydrazides such as luminol (Figure 1.6.5) (Kricka, 1991; Truchaud and Garcera, 1993).

![Luminol + H₂O₂ → HRP → Light](image)

**Figure 1.6.5**

However, high background signals, low light intensity, and a rapid decay of light emission limit the reaction (Kricka, 1991; Truchaud and Garcera, 1993). With the addition of small amounts of phenols, naphthols, and amines as "enhancers", the light intensity of the emission is increased by several orders of magnitude (2,500 fold after 30 seconds), there is a reduction in the background emission from the luminol-peroxide blank, and the light emission is a long-lived glow (>30 min) (Kricka, 1991; Truchaud and Garcera, 1993). Li et al. (1993) used a chemiluminescence detection system that included the luminol derivative, L-012, and 4-(4-hydroxyphenyl)thiazole as an enhancer. The detection limit of the assay for human basic fibroblast growth factor (hbFGF) was ten times more sensitive than a previous immunoassay employing oPD as substrate.

The practical detection limit of horseradish peroxidase using chromogenic substrates is in the region of 10^{-14} to 10^{-17} mol, which is comparable to the commonly employed radioisotope \(^{125}\)I (Johannsson, 1991). This appears to be in partial agreement with Ishikawa (1987), who suggests that 25 and 50 amol of peroxidase can be detected in 10 min using oPD and TMB, respectively. With longer incubation (100 min), 5 amol of horseradish peroxidase can be detected using TMB (Ishikawa, 1987). Using a fluorogenic substrate (HPPA) in a 100-min incubation, the sensitivity may be increased by 10-50 fold (Ishikawa, 1987).
1.6.2.2.2 Alkaline phosphatase

Alkaline phosphatase (AP) is a dimeric glycoprotein of approximately 140 K molecular mass and has a large number of free amino groups, which can be used for conjugation without loss of enzyme activity (Johannsson, 1991). It hydrolyses numerous phosphate esters of primary alcohols, phenols, and amines, with an optimum activity in the pH range 9.5-10.5 (Johannsson, 1991). Using 1 mmol/l diethanolamine buffer, pH 9.8, containing 0.5 mmol/l MgCl₂ and 15 mmol/l p-nitrophenyl phosphate (pNPP), the specific activity is 2,000 U/mg at 37°C (Johannsson, 1991; Tijssen, 1993). At 25°C, activity is reduced to about half, but pNPP suffers from spontaneous hydrolysis at temperatures greater than 30°C (Tijssen, 1993). As alkaline phosphatase is larger than peroxidase and the millimolar extinction coefficient of the nitrophenol product (18.3) is lower than that of TMB product, the detectability of alkaline phosphatase with pNPP is not as good as that of peroxidase (Johannsson, 1991). According to Ishikawa (1987), the detection limit of alkaline phosphatase using pNPP in a 10-min incubation is 10,000 amol, while Johannsson (1991) reports that as little as 30 amol of alkaline phosphatase can be detected after 30 min with 150 µl of pNPP substrate.

To increase the detectability of alkaline phosphatase using colorimetry, authors have turned to amplification systems involving nicotinamide dinucleotide phosphate (NADP) as substrate (Self, 1985; Moss et al., 1985; Stanley et al., 1985; Johannsson et al., 1986; Dhahir et al., 1992). Alkaline phosphatase dephosphorylates NADP to NAD, the catalytic activator for a NAD/NADH redox cycle (Figure 1.6.6). NAD is reduced to NADH by the action of alcohol dehydrogenase. NADH is then subsequently oxidised by the action of diaphorase, which simultaneously reduces p-iodonitrotetrazolium violet (INT) to formazan. Reformed NAD is free to enter the cycle repeatedly,
resulting in the production of several formazan molecules per molecule of NAD. Stanley et al. (1985) used this amplification system in an assay for human thyroid stimulating hormone (hTSH), obtaining a 70-fold increase in sensitivity over the conventional assay using pNPP as substrate. Moss et al. (1985), in an assay for prostatic acid phosphatase, reported an increase in sensitivity of the order of 175 times that of conventional methods, while Dhahir et al. (1992) achieved a 10-fold improvement on the sensitivity of proinsulin determination over previous assays. Johannsson et al. (1986) claim that the system can detect 0.01 amol of alkaline phosphatase after a 3-h incubation.

![Diagram of the amplification of alkaline phosphatase label](image)

**Figure 1.6.6** Amplification of alkaline phosphatase label. NAD, produced by the catalytic action of alkaline phosphatase on NADP, is reduced to NADH through the action of alcohol dehydrogenase. NADH is subsequently oxidised by the action of diaphorase, which simultaneously reduces a tetrazolium salt to a coloured product.

In addition, alkaline phosphatase may be detected through the use of bioluminescent and chemiluminescent assays (Kricka, 1991), or using the fluorogenic substrate, 4-methyllumbelliferyl phosphate (Ishikawa, 1987). The latter increases the sensitivity of alkaline phosphatase detection by up to 1,000 fold (10 amol v 10,000 amol) when compared to pNPP using the same incubation times (Ishikawa, 1987).
bioluminescent assay, alkaline phosphatase cleaves the phosphate group from firefly D-
luciferin-\(O\)-phosphate to liberate D-luciferin, which is a substrate for the
bioluminescent firefly luciferase reaction (Figure 1.6.7) (Kricka, 1991).

\[
\begin{align*}
\text{Firefly luciferin-\(O\)-phosphate} & \xrightarrow{AP} \text{Firefly luciferin} \\
\text{Firefly luciferin} & \xrightarrow{\text{Firefly luciferase}} \text{Light}
\end{align*}
\]

**Figure 1.6.7** Detection of alkaline phosphatase label using bioluminescence.

The most sensitive and widely investigated chemiluminescent detection system for
alkaline phosphatase involves an adamantyl 1,2-dioxetane aryl phosphate, which is
deprophosphorylated to a phenoxide intermediate that decomposes further to form
adamantanone and an aryl ester (the emitter) (Kricka, 1991). The light emission is in
the form of a glow and lasts for \(>1\) h, and can be enhanced through the use of a variety
of substances (Kricka, 1991). Kricka (1991) suggests that as little as 0.01 and 0.001
amol of alkaline phosphatase can be detected using firefly D-luciferin-\(O\)-phosphate
bioluminescence and adamantyl 1,2-dioxetane enhanced chemiluminescence,
respectively.

The sensitivity of alkaline phosphatase detection has also been enhanced using
amperometric detection of reaction products. Ciana et al. (1996), in an assay for \(\alpha\)-
fetoprotein in human serum, used \(p\)-hydroxyphenyl phosphate as substrate. The
hydrolysis product, hydroquinone, was detected by oxidative amperometry after a 10-
min incubation, giving a detection limit for alkaline phosphatase of 60 zmol (60 \times 10^{-21}
mol) or 36,000 molecules. The detection limit for \(\alpha\)-fetoprotein using amperometry,
0.07 ng/ml, was fourteen times lower than by photometry. Bauer et al. (1996) used a
phenol-indicating biosensor, consisting of a Clark-type electrode covered by a membrane with co-entrapped tyrosinase and quinoprotein glucose dehydrogenase. Alkaline phosphatase cleaves phenyl phosphate to phenol, which is oxidised at the sensor membrane by the oxygen-consuming tyrosinase via catechol to \( o \)-quinone (Figure 1.6.8). The quinone is then reconverted to catechol by glucose dehydrogenase, resulting in a 350-fold amplified sensor response to phenol. The oxygen consumption of the enzyme couple in the presence of phenol is monitored as a decrease in current. Using this recycling system, 320 zmol of alkaline phosphatase can be detected after a 57.5-min incubation with phenyl phosphate.

![Substrate reaction and biosensor](image)

**Figure 1.6.8** Schematic representation of the principle of alkaline phosphatase measurement using amperometry. \( \text{PQQ} = \text{pyrroloquinolinequinone} \).

1.6.2.2.3 \( \beta \)-galactosidase

\( \beta \)-galactosidase (\( \beta \)-GAL) from *E. coli* is a tetramer of 465 K molecular mass (Tijssen, 1993). The most commonly employed substrate is \( \text{oNPG} \) and is hydrolysed at a rate of
about 500 U/mg at 25°C using 50 mmol/l phosphate buffer, pH 6.8, containing 1 mmol/l MgCl₂ and 2.6 mmol/l substrate (Tijssen, 1993).

Johannsson (1991) suggests that β-galactosidase does not appear to offer advantages over alkaline phosphatase, however, Ishikawa (1987) reports that 1,000 amol of β-galactosidase can be detected after a 10-min incubation using oNPG as substrate, as compared to 10,000 amol of alkaline phosphatase under comparable conditions. Best sensitivity is obtained using a bioluminescent assay involving oNPG as substrate (0.0002 amol), but this requires numerous reagents and lengthy incubation steps (Figure 1.6.9) (Kricka, 1991).

\[
\begin{align*}
\text{o-Nitrophenyl-β-D-galactoside} & \xrightarrow{β-GAL} \text{β-D-Galactose + o-Nitrophenol} \quad (1) \\
\text{β-D-Galactose + NAD} & \xrightarrow{Galactose dehydrogenase} \text{Galactonate + NADH} \quad (2) \\
\text{NADH + FMN + H⁺} & \xrightarrow{NADH dehydrogenase} \text{NAD + FMNH₂} \quad (3) \\
\text{FMNH₂ + O₂} & \xrightarrow{Luciferase + Long-chain aldehyde} \text{Light + FMN} \quad (4)
\end{align*}
\]

**Figure 1.6.9** Detection of β-galactosidase label using bioluminescence.

Alternatively, β-galactosidase label can be detected using an adamantyl 1,2-dioxetane phenyl galactoside in an analogous manner to adamantyl 1,2-dioxetane aryl phosphate used for alkaline phosphatase detection (Kricka, 1991). Using the fluorogenic substrate, 4-methylumbelliferyl-β-D-galactopyranoside, as little as 0.2 and 0.002 amol of β-galactosidase can be detected after 10-min and 1,000-min incubations, respectively (Ishikawa, 1987).
1.6.2.2.4 Amplification of signal

Modest signal amplification can be achieved through the direct coupling of a number of enzyme molecules to the antibody or antigen (Johannsson, 1991). Alternatively, one can use indirect labelling, in which the label determined at the end of the assay is not the primary label (Avrameas, 1992). For example, in a two-site sandwich assay a biotinylated secondary antibody may be detected using avidin as bridge between the antibody and labelled biotin, or using labelled avidin (Avrameas, 1992). Since each antibody may carry several biotin molecules and since avidin can bind biotin at ratio of 1:4, there is a large degree of signal amplification, resulting in between a 2 to 100-fold increase in sensitivity over conventional procedures (Avrameas, 1992). As described earlier, recycling systems may be used to enhance the detectability of enzyme label, the most common being the NAD/NADH cycling system for the measurement of alkaline phosphatase.

There are several naturally occurring examples of biological amplifiers, the well-known cascade mechanism of blood clotting being a prime example. Coagulation cascades offer enormous amplification potential, because each enzyme molecule, in turn, activates multitudes of other enzymes, leading to the formation of a fibrin clot (Merenbloom and Oberhardt, 1995). Merenbloom and Oberhardt (1995) exploited this natural amplification system in a homogeneous immunoassay of whole-blood samples using thrombin as the label. However, this serine protease is the last cascade step, representing the lowest level of amplification, but according to Merenbloom and Oberhardt (1995), the technology is potentially applicable to immunoassays using multiple cascade levels. With biotin as model analyte, a competitive homogeneous immunoassay was developed using a biotin-thrombin conjugate in competition with
biotin in control plasma for a limited number of sites on a monoclonal antibody. For each test, a sample, inhibited biotin-thrombin conjugate, anti-biotin monoclonal antibody, and paramagnetic iron oxide particles were mixed together and introduced to a test-card reaction chamber. Because the conjugate is inhibited by $p$-amidinophenyl ester of cinnamate, no clotting takes place during the initial immunoreaction. After a brief incubation period, the card is illuminated with ultraviolet light, reversing thrombin inhibition. However, only thrombin not bound by antibody can convert fibrinogen to a fibrin clot. The clotting time is established photometrically through the change in motion of the paramagnetic particles, which become trapped in the forming clot. Because a high concentration of analyte results in a greater amount of free biotin-thrombin conjugate and a faster clotting time than a low concentration, the clotting time is inversely proportional to the concentration of analyte in the sample. The detection limit of the assay was 200 to 1,000 nmol/l, with potential to decrease to 26 pmol/l using factor Xa as label.

1.6.2.3 Enzyme-related labels

As discussed earlier, in addition to the use of enzymes as labels, prosthetic groups (Morris et al., 1981), substrates (Burd et al., 1977; Wong et al., 1979), and inhibitors (Bacquet and Twumasi, 1984; Finley et al., 1980) have been used as labels in immunoassay.

In 1976, Carrico et al. described a cycling system for the sensitive detection of nicotinamide 6-(2-aminoethylamino) purine dinucleotide (AENAD) used as label (Figure 1.6.10). AENAD, serving as cofactor for lactate dehydrogenase, is reduced to AENADH, with the simultaneous conversion of lactate to pyruvate. Through the action of diaphorase, thiazolyl blue is reduced, the label serving as reducing agent.
Resultantly, the label is restored to its original state, thus allowing it to participate in further reactions.

![Reaction Diagram]

1. Lactate dehydrogenase
2. Diaphorase

**Figure 1.6.10** Amplification system for the detection of AENAD cofactor used as label. The progress of the reaction is monitored spectrophotometrically at 570 nm.

Similarly, Schroeder et al. (1976) developed a system for the detection of ligand-AENAD conjugates involving a bioluminescent reaction. Ligand-AENAD is reduced through the action of alcohol dehydrogenase using ethanol as reducing agent (Figure 1.6.11, Reaction 1). AENADH is then channelled into reactions involving the use of the NAD(P)/FMN-dependent luciferase from *Photobacterium fisheri* (Figure 1.6.11, Reactions 2 and 3).

**Alcohol dehydrogenase**

\[
\text{Ligand-AENAD} + \text{Ethanol} \rightarrow \text{Ligand-AENADH} + \text{Acetaldehyde} \quad (1)
\]

\[
\text{NADH} + \text{FMN} + \text{H}^+ \xrightarrow{\text{NADH dehydrogenase}} \text{NAD} + \text{FMNH}_2 \quad (2)
\]

\[
\text{FMNH}_2 + \text{O}_2 \xrightarrow{\text{Luciferase}} \text{Light} + \text{FMN} \quad (3)
\]

**Figure 1.6.11** Use of the bacterial luciferase reaction to detect cofactor conjugates.
Carrico et al. (1976) and Schroeder et al. (1976) report that the ligand-AENAD conjugates are not available for participation in their respective reactions when bound by antibody, thus making them ideal for use in competitive homogeneous formats.

1.6.2.4 Luminescence

1.6.2.4.1 Fluorescence

Luminescence is the most general term for light emission from a chemical species, fluorescence representing a specific type of luminescence, namely light absorption followed by rapid light emission. In fluorescence, a single photon of light is absorbed by a molecule (excitation) and re-emitted at a slightly longer wavelength (Stoke’s shift) (Gudgin Dickson et al., 1995). Usually, the energy is emitted over a broad band in the ultraviolet to visible range of the electromagnetic spectrum (Gudgin Dickson et al., 1995). Theoretically, this should be the most sensitive detection method, since a single label can be measured many times during a relatively short measurement interval (Gudgin Dickson et al., 1995). In comparison, the signal provided by $^{125}$I represents only one detectable event per second per $7.5 \times 10^6$ molecules, so that only 0.000013% of the label is detected every second (Wood, 1991). In addition, fluorescent assays do not require expensive and unstable reagents, complex instrumentation or licensing, and containment or disposal procedures, associated with the use of radioisotopes (Quinn, 1993).

A wide range of fluorescent molecules have been employed as labels in immunoassay, the most widely used being the isothiocyanate derivatives of fluorescein and rhodamine (Quinn, 1993). Fluorescein is regarded as one of the most useful low molecular weight fluorophores available because of its high quantum yield in aqueous solutions (Quinn, 1993). Fluorescein and rhodamine have Stoke’s shifts of only 28 and 35 nm,
respectively, and a time scale of emission in the order of $10^{-12}$ to $10^6$ seconds (Wood, 1991; Gudgin Dickson et al., 1995). As a consequence, a major limitation of these labels is that background fluorescence tends to be severe due to light scattering and background fluorescence from sample over a wide wavelength range of approximately 300-600 nm (Wood, 1991; Gudgin Dickson et al., 1995). In addition, the presence of two fluorophores in close proximity can cause self-quenching if there is overlap between emission and excitation energies (Wood, 1991; Gudgin Dickson et al., 1995). These limitations can be overcome through the use of lanthanide chelates, consisting of an organic molecule bound to a metal ion, e.g., europium ($\text{Eu}^{3+}$), samarium ($\text{Sm}^{3+}$), terbium ($\text{Tb}^{3+}$), and ruthenium ($\text{Ru}^{2+}$) (Gudgin Dickson et al., 1995). These labels have several advantages over organic labels (Table 1.6.4), including a long-lived fluorescence (microseconds to milliseconds), which allows selective detection by delaying measurement until a set time after the excitation pulse, when all short-lived species have decayed (Wood, 1991; Gudgin Dickson et al., 1995). In addition, favourable emission characteristics such as a large Stoke's shift (>200 nm for $\text{Eu}^{3+}$) and narrow emission band permits additional discrimination against other luminescent species and scattered excitation light, through the use of wavelength filtering (Quinn, 1993; Gudgin Dickson et al., 1995).

The first time-resolved immunoassay system to be developed commercially was the DELFIA system (dissociation-enhanced ligand fluorescence immunoassay), in which antibody or antigen is labelled with a stable europium chelate (Wood, 1991). The europium ion has weak fluorescence in this form, but on completion of the immunoassay, the europium is released from the hydrophilic chelator into a highly lipophilic environment, which enhances the fluorescence (Wood, 1991). Reagents used include fluorinated aromatic $\beta$-diketones to promote absorption and energy transfer to
the lanthanide ion, and synergistic reagents to increase the luminescence yield (Gudgin Dickson et al., 1995).

Table 1.6.4
Advantages and disadvantages of organic and lanthanide chelate fluorescent labels

<table>
<thead>
<tr>
<th>Fluorescent species</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical organic</td>
<td>Chemically stable, high fluorescence yield.</td>
<td>Small Stoke's shift and short fluorescence</td>
</tr>
<tr>
<td>labels such as</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fluorescein</td>
<td>Can be used with conventional fluorescence</td>
<td>interference from background signals.</td>
</tr>
<tr>
<td></td>
<td>instrumentation.</td>
<td></td>
</tr>
<tr>
<td>Lanthanide chelates</td>
<td>Large Stoke's shift, narrow band emission and</td>
<td>Metal-complex stability may be low; requires</td>
</tr>
<tr>
<td></td>
<td>long luminescence lifetime.</td>
<td>special handling to ensure chelate binding.</td>
</tr>
<tr>
<td></td>
<td>These properties permit selective discrimination against usual background signals by wavelength and temporal filtering.</td>
<td>Relatively low fluorescence yield compared with the best organic fluorophores.</td>
</tr>
<tr>
<td></td>
<td>Luminescence relatively insensitive to environment, temperature.</td>
<td>Specifically tailored time-gated instrumentation required for selective detection.</td>
</tr>
</tbody>
</table>

(From Gudgin Dickson et al., 1995)

Interestingly, Madersbacher et al. (1993) compared chelates of europium with horseradish peroxidase and $^{125}$I as detector labels used in solid-phase two-site immunometric determination of follicle-stimulating hormone (FSH). Using the same
monoclonal antibodies in all assays, IFMA achieved the best detection limit (2 ng/l v's 100 ng/l with IRMA and 8 ng/l with IEMA), the widest measuring range (2-160,000 ng/l), and the greatest signal-to-noise ratio (13,000:1 at 160,000 ng/l). The authors accredit this superiority to the characteristics of the europium-conjugated monoclonal antibodies and to the fluorescence detection system, i.e., the dissociative enhancement principle for europium in combination with a fluorometer for time-resolved detection. With standard labelling procedures, about 10-15 molecules of europium chelate, but only 2-3 atoms of $^{125}$I and only 2-3 molecules of horseradish peroxidase, can be covalently linked to monoclonal antibody (Madersbacher et al., 1993).

1.6.2.4.2 Chemiluminescence and bioluminescence

Chemiluminescence is light emission that arises during the course of a chemical reaction, whereas bioluminescence is a special type of chemiluminescence found in nature, in which light emission is facilitated by a catalytic protein (Kricka, 1991). These detection systems are attractive, since they are sensitive, fast (signal generated in a few seconds), simple, and use non-hazardous reagents (Kricka, 1991). The sensitivity largely stems from the fact that no external light source is required, as would be the case for fluorescence or colorimetric measurements, meaning that all light reaching the detector is as a result of the reaction (Kricka et al., 1991). The majority of labels used in luminescence immunoassays are chemiluminescent in nature (usually derivatives of luminol, isoluminol, and acridine), since they are stable, and relatively cheap and easy to couple to proteins without loss of activity (Wood, 1993). The efficiency of the chemiluminescent reactions (number of photons emitted/number of molecules reacting) is generally very low (<5%), however, specially synthesised oxamides have an efficiency of greater than 30% (Wood, 1984). In any event, the detection limits are still
equal to or better than that of radioisotopic labels ($^{125}$I), with acridinium esters achieving a detection limit of 0.8 amol (Wood, 1984; Kricka et al., 1991). Added disadvantages include signal quench effects by serum/plasma components when labels are used in liquid-phase immunoassays, an inability to repeat measurements once the chemical reaction has taken place, and short-lived luminescence (e.g., ~25 seconds for luminol and isoluminol) (Wood, 1984; Kricka et al., 1991).

Generally, chemiluminescent reactions proceed without biological intervention, however, peroxidase has been used successfully to provide "active oxygen" to oxidise luminol label (Wood, 1993). The light is produced continuously in the form of glow as the peroxidase generates nascent oxygen from $\text{H}_2\text{O}_2$ (Wood, 1993). As discussed previously, chemiluminescent reactions may be used to detect enzyme labels, e.g., horseradish peroxidase, alkaline phosphatase, and $\beta$-galactosidase.

Bioluminescence is light emission associated with biological systems. The components involved include a luciferin in reduced form as substrate, together with a luciferase (label) as specific enzyme (Wood, 1993). There are two main bioluminescent systems, one of which is ATP-dependent (Figure 1.6.7, Reaction 2) and the other of which is NAD(P)/FMN-dependent (Figure 1.6.11, Reactions 2 & 3) (Wood, 1991; Wood, 1993). The former is derived from the American firefly, the most commonly used luciferin-luciferase system, and the latter is present in marine bacteria such as *Vibrio* species (Wood, 1991; Wood, 1993). Using the firefly system, the detection efficiency is much higher than for radioisotopes, since light efficiency can approach 100% (Kricka et al., 1991). Limitations of these systems include expense of reagents, unstability of enzymes, and complications in preparation of labels (Wood, 1984).
1.6.2.5 *Alternative labels*

Synthetic lipid bilayer vesicles (liposomes) containing carboxyfluorescein (CF) have been used as label in a homogeneous sandwich-type immunoassay to determine ferritin in human serum (Ishimori and Rokugawa, 1993). The liposomes, containing immobilised monoclonal anti-ferritin antibody and encapsulated CF, were incubated in sample containing ferritin. After incubation, a second antibody and complement were added, resulting in the formation of a sandwich of antibody, ferritin, and complement on the liposome surface. The fluorescence from the liposomes lysed by the action of complement was monitored, whilst CF trapped in liposomes was not fluorescent because of self-quenching. Using this system, ferritin in the range of 10-1,000 µg/l could be determined in human sera. Similarly, liposomes entrapping the dye sulforhodamine B were used to develop an assay for anti-cardiolipin antibodies (Frost et al., 1996a) and urinary microalbumin (Frost et al., 1996b). The use of liposomes as primary labelling substances leads to amplification, because the lysis of each vesicle releases many molecules of the trapped indicator (Gosling, 1990).

DNA has also been used successfully as label in a solid-phase two-site sandwich immunoassay for the measurement of hTSH and hCG (Joerger et al., 1995). The DNA label is detected using specific primers in the polymerase chain reaction (PCR), producing amplification products with length and sequence identical to those of the original label. Amplification products are then analysed by agarose gel electrophoresis. Detection limits for hTSH (10^{-19} mol) and hCG (5 \times 10^{-18} mol) exceeded those of conventional enzyme immunoassays by two to three orders of magnitude (Joerger et al., 1995). These principles have been extended to the simultaneous detection of hTSH, hCG, and β-galactosidase in a solid-phase two-site sandwich immunoassay format, using three specific monoclonal antibodies as capture and three specific
oligonucleotide-labelled monoclonals (Hendrickson et al., 1995). The labels, which are of differing lengths, possess the same primer binding sequences, thus permitting the use of the one primer for the detection of all three labels. Once the PCR is complete, amplification products are analysed using agarose gel electrophoresis. Since the oligonucleotide labels are of differing lengths, the amplification products differ in length and mobility on the agarose gel. The latter characteristic allows perfect discrimination between labels. Similarly, authors have used enzyme-coding DNA fragments as labels (Christopoulis and Chiu, 1995). The DNA label is detected with high sensitivity by measuring the enzyme activity of firefly luciferase after expression. Sensitivity is derived mainly from the combined transcription/translation process, since transcription produces several mRNA molecules per DNA template, and translation, in turn, produces more than one protein molecule from each transcript. Therefore, for each DNA label there is a large yield of protein. In addition, since the DNA label encodes an enzyme, the amplification is further enhanced due to substrate turnover. Using this system, the authors detected as little as 3,000 molecules of DNA label and 50,000 molecules of antigen.
1.7 Separation Methods

Several methods can be used to separate bound from free label in heterogeneous immunoassay. In competitive immunoassays, liquid-phase separation can be achieved using either polyethylene glycol (PEG) (Bailey, 1994; Österman et al., 1979), second antibody (Al-Bassam et al., 1978; Blake et al., 1982; Bailey, 1994), or both (Carstens et al., 1997). Using low concentrations of PEG, one can bring about the precipitation of antibody molecules, whilst leaving antigen in solution (Bailey, 1994). Once the precipitate is formed, the contents are centrifuged, the supernatant poured off, and the signal associated with either is measured (Bailey, 1994; Österman et al., 1979). The alternative method relies on the use of antiserum directed against the first antibody, for example, Al-Bassam et al. (1978) used donkey anti-sheep antibody to precipitate a sheep antibody employed in a competitive assay for nortriptyline. In addition, charcoal has also been used to absorb free label, with separation again being achieved using centrifugation (Gosling, 1990).

However, by far the most convenient and efficient methods involve the use of solid phases, with solid-phase methods representing 70% of all new immunoassays in 1990 (Gosling, 1990). Kaibe et al. (1990) used antibody complexed to bacterial cell walls in a competitive immunoassay format for the epileptic drug, Zonisamide. Once the immunocompetition had taken place, the insolubilised antibody was removed via centrifugation and the signal associated with precipitate was measured. Similarly, Ko et al. (1992) used a second antibody coupled to iron to precipitate the first antibody. However, since these types of assay require the use of a centrifuge, their usage has declined over the years to <10% of new solid-phase assays in 1990 (Gosling, 1990).
More commonly, antigens and antibodies are immobilised to solid phases which allow separation to be achieved via several washing steps. Table 1.7.1 highlights some of these solid matrices employed in heterogeneous immunoassay. By far the most popular solid-phase system employed is the 96-well plastic microtitre plate, representing 70% of all new solid-phase assays in 1990 (Gosling, 1990). While a range of plastics may be used, polystyrene appears to be the most popular. The popularity of microtitre plates largely stems from the large batches of samples that can be assayed on one plate conveniently and quickly using purposefully designed multi-channel pipettes, automated plate washers, and readers.

**Table 1.7.1** Examples of some solid matrices used in heterogeneous immunoassay

<table>
<thead>
<tr>
<th>Particulate</th>
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</thead>
<tbody>
<tr>
<td>Polystyrene balls</td>
</tr>
<tr>
<td>Glass balls</td>
</tr>
<tr>
<td>Controlled-pore glass beads</td>
</tr>
<tr>
<td>Magnetic beads</td>
</tr>
<tr>
<td>Sepharose</td>
</tr>
<tr>
<td>Sephadex</td>
</tr>
<tr>
<td>Poros beads</td>
</tr>
<tr>
<td>(Ishikawa et al., 1990; Hashida and Ishikawa, 1990; Ishikawa et al., 1982)</td>
</tr>
<tr>
<td>(Ruan et al., 1987)</td>
</tr>
<tr>
<td>(Sportsman et al., 1983; Ogbonna et al., 1995)</td>
</tr>
<tr>
<td>(Ogbonna et al., 1995; Pollema and Ruzicka, 1994)</td>
</tr>
<tr>
<td>(Nilsson et al., 1993)</td>
</tr>
<tr>
<td>(Freytag et al., 1984)</td>
</tr>
<tr>
<td>(Kronkvist et al., 1997)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Solid surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose membranes</td>
</tr>
<tr>
<td>Plastic tubes</td>
</tr>
<tr>
<td>Plastic microtitre plates</td>
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<tr>
<td>(Newman and Price, 1991)</td>
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<td>(Thijssen et al., 1991; Bargi et al., 1988)</td>
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<td>(Brailly et al., 1994; Li et al., 1993; Aubin et al., 1997; Luppa et al., 1995)</td>
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In recent years, separation has been achieved with a high degree of reproducibility using capillary electrophoresis (CE) (Schmalzing et al., 1995; Novotny, 1996). Schmalzing et al. (1995), in a competitive immunoassay for cortisol, used CE to
separate free cortisol-fluorescein conjugate from cortisol-fluorescein conjugate bound to specific antibody. The signal associated with either could then be measured in the capillary using laser-induced fluorescence. Alternatively, direct CE may be used in one-site immunoassay to separate labelled antibody-antigen complexes from an excess of unreacted labelled antibody (Schmalzing et al., 1995). However, the molecule being measured needs to be large enough to effect a change in the mobility of the antibody when bound and is therefore of little use for small molecules such as cortisol (Schmalzing et al., 1995).
1.8 Immobilisation of Biomolecules on Surfaces

Biomolecules (antibodies and antigens) may be attached to surfaces using either passive adsorption, consisting of primarily hydrophobic interactions or hydrophobic/ionic interactions between the biomolecules and the surface (Gibbs, 1995a), or covalent attachment (Gibbs, 1995a; Butler, 1992; Cabral and Kennedy, 1991). Using the latter method, molecules may be attached to surfaces via amino, thiol, carboxyl, phenolic, guanido, imidazole, disulphide, indole, thioether, and hydroxyl functional groups (Cabral and Kennedy, 1991). Support materials generally do not possess reactive groups, but rather hydroxyl, amino, amide, and carboxyl groups, which have to be activated using functional cross-linking reagents in order to immobilise the biomolecules (Cabral and Kennedy, 1991). For example, amino surfaces can be activated through gluteraldehyde pre-treatment and a carboxyl surface may be activated using a carbodiimide (Douglas and Monteith, 1994). However, for polystyrene, carboxyl and amine groups have to grafted to the surface before using the functional cross-linker (Gibbs, 1995a). In addition, functional and covalently reactive groups such as N-oxysuccinimide (to couple amine groups), maleimide (to couple free sulphhydryl groups), and hydrazide (to couple periodate-activated carbohydrate moieties) can be grafted onto a polystyrene surface (Gibbs, 1995a).

For the activation of polysaccharides such as Sepharose, numerous methods may be used to activate the surface, one of which involves the use of cyanogen bromide (CNBr). The popularity of CNBr-activated Sepharose is due to its high capacity for immobilised reactants, for example, 25-60 mg of protein can be immobilised on 1 ml of gel (CNBr-activated Sepharose 4B Instructions, Pharmacia-Biotech, Uppsala, Sweden). At high pH, CNBr introduces very reactive cyanate esters (-O-C≡N) and less
reactive imidocarbonates (>C=NH) into the matrix by reacting with endogenous hydroxyl groups (Cabral and Kennedy, 1991). Therefore, the reactive matrix is then suitable for the coupling of amine-containing ligands (Cabral and Kennedy, 1991). Sepharose is now supplied commercially in the reactive form, thus eliminating the need to use CNBr, which is extremely toxic. Nilsson et al. (1993) used tresyl chloride activation for Sepharose CL-4B, a process which converts the hydroxyl groups of the matrix into active sulphonates, thus making it suitable for coupling amine-containing ligands (Cabral and Kennedy, 1991).

While covalent coupling may be obligatory for the coating of most solid phases, polystyrene and other plastics may be coated using passive adsorption (Gibbs, 1995a). The interactions that take place are greatly affected by the concentration of biomolecule being adsorbed, the nature of the buffer used (ionic strength and pH), and the time and temperature of incubation (Gibbs, 1995b). In addition, the binding of molecules is prevented by non-ionic detergents (Gardas and Lewartowka, 1988).

For immobilisation to plastics, authors generally use a 0.05 mol/l carbonate buffer, pH 9.6, with incubations carried out overnight at 4°C (Munro and Stabenfeldt, 1984; Ko et al., 1992; Chlang et al., 1992; Papoian et al., 1991), however, the conditions used may vary between workers. For example, coating has also been achieved using carbonate buffers incubated for 4 h at 4°C (Ii et al., 1993) and for 6 h at 25°C (Thompson et al., 1985), or using phosphate buffers (pH 7.2-7.4) incubated overnight at room temperature (Aubin et al., 1997), for 3 h at 37°C (Rappuoli et al., 1981), and for 2 h at room temperature (Noé et al., 1992). In contrast, Krachmalnicoff et al. (1990) used a Tris buffer, pH 9.0, incubated at 4°C overnight. Generally, incubation times may be shortened using increased temperatures (Gibbs, 1995b), however, studies using
polystyrene have shown that 90% of maximum binding occurs within 2 to 6 h, even at 4°C (Pesce et al., 1977).

The optimum concentration for coating plastic microtitre plates and tubes typically lies between 0.4-10 μg/ml (Krachmalnicoff et al., 1990; Papoian et al., 1992; Noé et al., 1992; Aubin et al., 1997; Dhahir et al., 1992; Munro and Stabenfeldt, 1984). Higher concentrations may lead to an inefficient immunoassay, for example, Kobatake et al. (1990) noticed a reduced signal in a two-site sandwich assay when using a primary antibody concentration of 1 mg/ml. This is because at high concentrations the molecules may bind each other loosely, forming multiple layers, which are unstable and can peel off during the assay (Kemeny, 1992). To coat polystyrene and glass balls with IgG, higher concentrations (100 μg/ml) have been used (Ishikawa et al., 1980; Ishikawa et al., 1982; Ruan et al., 1987), since several balls provide a larger surface area for binding.

When using passive adsorption, the binding capacity of polystyrene is approximately 100-200 ng IgG/cm² (Gibbs, 1995a), which is equivalent to 4 x 10⁹ to 8 x 10⁹ molecules/cm². Conradie et al. (1983) reported that partial denaturation of antibody using low pH (2.5), 3 mol/l urea, and temperatures as high as 82°C enhanced the ELISA reaction colour in two-site sandwich assays for ferritin and hepatitis B surface antigen. They attributed this to an increased exposure of new hydrophobic domains, leading to binding to regions on the polystyrene surface normally not coated by non-perturbed molecules. Moreover, irradiation of the polystyrene surface incorporates carboxyl groups capable of ionic interactions with positively charged groups on the biomolecules (Gibbs, 1995a). Resultantly, the binding capacity is greatly increased to approximately 400-500 ng IgG/cm², due to the fact that ionic interactions require that smaller portions of the molecule be in contact with the surface to achieve stable
immobilisation (Gibbs, 1995a). Also, when polystyrene plates were coated with adhesive polyphenolic protein purified from the mussel *Aulacomya ater*, the capacity of the plates to bind antigens such as hCG was enhanced significantly (Burzio et al., 1996).

However, there is a significant loss of biological recognition following immobilisation of antibodies and antigens to surfaces (Lu et al., 1996; Butler, 1992). Covalent immobilisation of antigen may lead to loss of one or more epitopes through formation of the covalent linkage or steric hindrance, whilst passive adsorption may lead to epitopes being sterically buried (Butler, 1992). When antibodies are covalently attached to supports, their specific binding capacity is usually less than that of soluble antibodies (Lu et al., 1996). The reason for this reduction is attributed to the random orientation of the antibody, resulting in the exclusion of the antibody binding site in the Fab region (Lu et al., 1996). After passive adsorption, it has been reported that <3.0% of the binding sites of monoclonal antibodies and approximately 5-10% of those of polyclonal antibodies were capable of capturing antigen (Butler et al., 1992). Lu et al. (1996) have reported on a number of methods to alleviate the problem of random orientation of IgG antibodies. Firstly, proteins A/G can be used to bind the antibody via the Fc region, thus leaving the antigen binding sites free, or alternatively, antibody can be coupled to the solid support via an oxidised carbohydrate moiety associated mainly with the Fc region. Finally, monovalent Fab’ fragment may be bound to the insoluble support via a sulphydryl group in the C-terminal region of the fragment. Oriented immobilisation can also be achieved using antiglobulin or avidin-coated surfaces to bind biotinylated antibody, the former method giving a higher percentage of active sites per well (in some cases >70%) (Butler et al., 1992). However, the actual number of sites is low, resulting in similar or fewer numbers of active sites per well
when compared to the avidin-biotin method or passive adsorption (Butler et al., 1992). Antibodies immobilised by the avidin-biotin method generally gave the highest number of binding sites per well when compared to antibodies immobilised by the other two methods (Butler et al., 1992). When compared to covalent immobilisation, antibodies immobilised via the avidin-biotin bridge also showed increased activity (Peterman et al., 1988; cited in Butler et al., 1992).
1.9 Alternative Immunoassays

1.9.1 Immunosensors

Immunosensors are defined as “analytical devices that detect the binding of an antigen to its specific antibody by coupling the immunochemical reaction to the surface of a device known as a transducer” (Gizeli and Lowe, 1996). An immunosensor is a form of biosensor, in which the antigen or antibody represents the sensor molecule, with the signal transducer providing an indication that the ligand has bound to the sensor (Morgan et al., 1996). Four main types of transducer have been used, exploiting changes in electrochemical, mass, heat or optical properties (Morgan et al., 1996). Direct immunosensors involve the direct detection of the antibody-antigen reaction in real time, whereas indirect immunosensors involve the use of a label to detect the antibody-antigen reaction (Gizeli and Lowe, 1996; Morgan et al., 1996). However, Morgan et al. (1996) suggest that the latter is not a true immunosensor. True immunosensors are attractive alternatives to conventional immunoassay, allowing rapid, real-time monitoring of antibody-antigen reactions in homogeneous formats, the most sensitive immunosensor being in the order of $2 \times 10^{-13}$ mol/l for hepatitis B surface antigen measurement in serum (Morgan et al., 1996). The latest addition to the immunosensor family involves the use of optical tweezers to detect the antibody-antigen reaction, however, it is not a true immunosensor, since the system does not allow real-time monitoring. Optical tweezers are focussed laser beams used to trap and remotely manipulate dielectric particles, and have recently been used to measure the force required to separate antigen-antibody bonds in a competitive immunoassay for BSA (Helmerson et al., 1997). In the assay, the optical tweezers is used to trap polystyrene microspheres coated with antigen and then pull the microspheres away
from a silanized glass coverslip coated with specific antibody. Throughout, the average force applied by the optical tweezers to break the antigen-antibody bonds and pull the microspheres away from the surface is measured. When free antigen is applied to the system, the force required is decreased in inverse proportion to the amount of antigen added, thus allowing a standard curve to be prepared. With this competitive or displacement-type system, $1.45 \times 10^{-15}$ mol/l of BSA could be measured after a 2-h pre-incubation. However, the authors would need to verify this detection limit using real samples.

Similarly, single antigen-antibody recognition events can be detected using atomic force microscopy (AFM) (Davies et al., 1994). In this technique, a surface of interest is moved past a very small tip attached to a flexible cantilever; the bending of the cantilever as the tip rises and falls gives an indication of the surface roughness or topography (Kricka, 1997). Davies et al. (1994) used AFM to compare microtitre wells coated with passively absorbed anti-ferritin antibody with those coated with biotinylated anti-ferritin antibody linked via streptavidin. Both types of surface were analysed before and after incubation with ferritin antigen, with the extent of ferritin binding being represented by an increase in surface roughness. Their studies, while showing the advantages of using the latter method for coating antibodies to polystyrene, also demonstrated the potential application of AFM in quantitative analysis. Recently, Perrin et al. (1997) realised this potential, adapting the AFM to the quantitative analysis of ferritin. Silicon wafers were covalently coated with anti-ferritin antibodies to a surface density of 1.4 ng/mm$^2$. To demonstrate ferritin binding, an ELISA was performed on the coated wafers using an alkaline phosphatase-labelled anti-ferritin antibody as detector and $p$NPP as substrate. Subsequently, the suitability of AFM to quantitative measurements was demonstrated using coated wafers.
incubated with a range of ferritin concentrations, followed by scanning with the AFM. As expected, the authors noticed that the surface roughness of the wafers increased in direct proportion to the concentration of ferritin added. However, at this stage of development, the sensitivity of the AFM immunoassay does not compare well with conventional ELISA, i.e., 60 ng/ml v 1.2 ng/ml.

1.9.2 Novel homogeneous immunoassay

Recently, Meyerhoff et al. (1995) described a non-separation electrochemical enzyme immunoassay (NEEIA) for detecting marker proteins in undiluted blood using a gold-coated microporous membrane. The gold coating served simultaneously as a solid phase for immobilising the capture antibody and an amperometric detector for monitoring, via an oxidation or reduction reaction, the amount of enzyme product generated immediately adjacent to the gold surface. In the assay, analyte is incubated simultaneously with horseradish peroxidase-labelled antibody on the gold side of the membrane, resulting in the formation of a sandwich on the surface during a fixed period of incubation. The substrate is then introduced through the back side of the porous membrane, encountering the immobilised enzyme-conjugate first. During the short electrochemical measurement period after substrate addition (1 min), very little substrate diffuses into the bulk solution, hence, no separation step is required. As discussed earlier, Merenbloom and Oberhardt (1995) developed a novel homogeneous immunoassay for whole-blood samples using thrombin as the label.
1.9.3 Dual and multianalyte immunoassays

Immunoassay systems generally measure only one analyte type in a sample, however, the potential advantages of shortened overall assay times, use of less sample, and reduction in reagent costs has spurred authors to develop immunoassays that simultaneously measure two or more analytes.

Blake et al. (1982) developed a classical competitive enzyme immunoassay that simultaneously measured the thyroid hormones, T₃ and T₄, in one tube. This simultaneous format was facilitated by the use of two different enzyme systems that generated products distinguishable by absorption spectrophotometry. Alkaline phosphatase and β-galactosidase were used as the labels and were detected using phenolphthalein monophosphate and oNPG, respectively. When the substrates were hydrolysed, they generated products with different spectral characteristics. In fact, the absorbance of phenolphthalein at 420 nm was only 1.7% of its absorbance at 540 nm, while o-nitrophenol had <0.7% of its maximum absorbance (~420 nm) when studied at 540 nm. Although this model demonstrated the principle of simultaneous immunoassay very well, it did have one minor flaw. Due to the differing pH optima of the two enzymes, colour development had to be carried out using separate incubations. However, this is a flaw with the model system and not with the principle itself, and could potentially be rectified using enzymes with similar pH optima. The authors also suggest that the model could be expanded so that more than two analytes could be measured simultaneously.

Macri et al. (1992) developed a sandwich enzyme immunoassay for the simultaneous measurement of α-fetoprotein and free-β hCG using alkaline phosphatase and peroxidase as labels. However, while all the steps of the sandwich assay were carried
out simultaneously, colour development again required separate incubations. In any case, the assay did reduce reagents costs and sample volume requirements.

In 1992, Hoshino and Miyai developed a simultaneous homogeneous enzyme immunoassay for the measurement of ferritin and α-fetoprotein using a single incubation, in which sample was mixed with two specific horseradish peroxidase-labelled antibodies and with substrate. No separation was required, since only antibody aggregates showed catalytic activity. From separate dose-response curves for both analytes, the authors were able to establish absorbance cut-off points for the simultaneous assay of samples, below which both analytes were within normal limits (<20 ng/ml for α-fetoprotein and <200 ng/ml for ferritin) and above which one or both analytes were high. Since the individual absorbances of 20 ng/ml of α-fetoprotein and 200 ng/ml of ferritin were 0.015, the first cut-off point was 0.015. Therefore, any samples giving absorbances below 0.015 in the simultaneous assay were considered negative. If a sample gave an absorbance >0.03, it was considered positive, because one or both analytes must be high. Samples giving absorbances between 0.015 and 0.03 were treated as suspicious, since both analytes may be normal or one may be above the cut-off point of 0.015. For positive and suspicious samples, both analytes were measured separately to determine which parameter was increased. Therefore, while the assay only allowed semi-quantitative analysis, it did prove a useful screening method for the two tumour markers.

As discussed earlier, Hendrickson et al. (1995) developed a solid-phase two-site sandwich immunoassay for the simultaneous measurement of hTSH, hCG, and β-galactosidase using three specific monoclonal antibodies as capture and three specific oligonucleotide-labelled monoclonals (Hendrickson et al., 1995).
1.10 Optimisation of Immunoassay

1.10.1 Development of a two-site sandwich immunoassay

1.10.1.1 Optimisation of antibody concentrations

Firstly, one needs to determine the optimum concentration for coating the capture antibody to the solid phase. To do this, a range of antibody concentrations are used for immobilisation and standard curves are prepared at each concentration using a constant excess of labelled antibody (Kemeny, 1992; Gibbs, 1995b). When the standard curves are plotted (concentration v's specifically bound signal), one should notice an increase in steepness of the curves with increasing antibody concentration, until an antibody concentration is reached above which no improvement in signal is achieved (Figure 1.10.1). This may be due to the fact that excess reagent concentrations prevail, in which case further increases serve only to increase the background signal (non-specific binding) (Micallef and Ahsan, 1994).

Alternatively, the binding capacity of the solid phase may have been reached. For instance, the optimal concentration for coating antibody to plastic by passive adsorption typically lies between 0.4 to 10 μg/ml. Further increases leads to unstable multilayer formation from protein-protein interactions, thus leading to a reduction in assay sensitivity and precision (Kemeny, 1992). Once the coating concentration has been established, the same procedure can be used in the optimisation of conjugate concentrations.
Micallef and Ahsan (1994) used a slightly different approach to optimising the concentration of antibody used to coat magnetic particles. Various concentrations of immobilised antibody were reacted with excess labelled antibody in the presence of zero analyte and at the highest concentration of analyte specified to be measured (Figure 1.10.2). As before, the specific signal for the highest standard reaches a maximum value with increasing antibody concentration (in this case excess reaction concentrations prevail). Further increases in antibody serve only to increase non-specific binding, leading to a reduced signal-to-noise ratio and decreased sensitivity. Again, the labelled antibody may be similarly tested at serial dilutions using the optimised immunoextractant concentration. This approach has been used by a number of workers to optimise concentrations of antibody for use in two-site immunoassays (Noé et al., 1992; Dhahir et al., 1992).

**Figure 1.10.1** Determination of the optimum coating concentration for primary antibody.
1.10.1.2 Method of immobilisation

As discussed earlier, the immobilisation method is also of importance when optimising immunoassays. Passive adsorption and most covalent methods render a high percentage of bound antibodies inactive. Alternative methods, in which there is oriented immobilisation of the antibody molecules, may generate a higher number of functional sites, e.g., biotin-avidin systems, protein A/G, etc.

1.10.1.3 Incubation conditions

To help ensure accuracy in the measurement of samples, it is advisable to use a calibrant matrix which is identical to (Papoian et al., 1991), or closely resembling (Dhahir et al., 1992), the sample matrix with respect to composition and pH. If buffer is to be used as calibrant matrix, one should at least ensure that the pH approximates
that of the samples to be measured. However, it is likely that use of such a buffer in conventional two-site sandwich immunoassay will lead to poor comparison between sample and standard, since there are differences in composition. For the incubation of conjugate antibody, a buffer with pH between 6 and 9 should be used, since antigen-antibody reactions normally take place within this range (Kemeny, 1992).

To achieve maximum signal, it is usual to incubate for sufficient time to allow all reactions to achieve or approach equilibrium. Generally, the higher the incubation temperature used, the shorter the times required. For example, Papoian et al. (1991), in a sandwich ELISA for IL-3, performed the incubations for both analyte and conjugate at 37°C for 1 h, while Noé et al. (1992) incubated analyte at room temperature for 4 h and conjugate overnight at 4°C in a sandwich ELISA for erythropoietin.

As described earlier, analyte and labelled antibody can be reacted simultaneously with immunoextractant in a single step or, alternatively, analyte and labelled antibody may be incubated sequentially using two separate reactions. The former saves on time, but it does have problems with the hook effect (Nomura et al., 1983).

1.10.2 Development of a limited-reagent assay

Selection of the appropriate antibody and labelled analyte concentrations is performed by making serial dilutions of both, and testing the combinations of each antibody dilution with every labelled analyte dilution in the presence of zero standard and at anticipated lowest and highest analyte concentrations (Micallef and Ahsan, 1994). The initial optimum concentrations are those giving the greatest, and most reproducible, changes in bound signal with increasing analyte concentrations (Figure 1.10.3), and the
final concentrations selected are those giving the best precision in the clinical diagnostic range (Micallef and Ahsan, 1994).

![Graph showing antibody dilution and displacement curves at a fixed concentration of labelled analyte.](attachment:graph.png)

**Figure 1.10.3** Antibody dilution and displacement curves at a fixed concentration of labelled analyte. More curves are prepared for other labelled analyte concentrations.

Generally, workers prepare antibody dilution curves without added unlabelled analyte and pick the antiserum dilution giving approximately 30-50% (Bailey, 1994) or 40-50% (Parker, 1990) binding of labelled analyte, since displacement of the label by a small increment of unlabelled analyte is usually at its greatest here (maximal slope at zero-dose response). For competitive immunoassays using radioactive label, the radioactive antigen giving about 8,000 to 10,000 cpm is generally used (Parker, 1990; Bailey, 1994), whereas in immunoassays with antigen-enzyme conjugates, the goal is to obtain about 40-50% binding in uninhibited samples with enough enzyme activity to detect decreases in binding down to about 5% or less (Parker, 1990).

Ekins (1991) suggests that assays constructed in such a manner are likely to give adequate sensitivity and an acceptable working range, but are formulated without regard for the effect antibody concentration has on assay precision and sensitivity. He suggests preparing antibody dilution curves (similar to above) using 1) zero standard

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and 2) a small increment of unlabelled analyte (ΔA), sufficient in magnitude to cause significant displacement of the resulting antibody dilution curve. Each point of the antibody curve is determined in replicates of five to ten. The labelled analyte concentration is fixed and represents the lowest usable concentration needed to register a measurable signal down to, for example, 1% binding of label. The curve obtained is similar to above (Figure 1.10.3), but without high standard. Ekins indicates that the antiserum dilution giving the greatest displacement should not be used without regard to the (im)precision, since reproducibility of the zero-dose response may be poor here. He suggests plotting $\delta_b/\Delta b$ (standard deviation in measuring bound signal at zero dose divided by the change in bound signal due to the displacement by unlabelled analyte) as a function of $bo$ (the signal bound at zero dose) (Figure 1.10.4). From this curve, an antiserum concentration can be chosen which gives a low standard deviation in the measurement of zero dose (but with good displacement).

![Figure 1.10.4](image)

**Figure 1.10.4** Curve representing the quotient $\delta_b/\Delta b$ plotted as a function of $bo$. The value of $bo$ at which $\delta_b/\Delta b$ is minimal ("x" on graph) indicates the antibody dilution giving maximal sensitivity (Ekins, 1991).
1.11 Validation of Immunoassay

1.11.1 Specificity

To test the specificity of an assay system, dose-response curves are constructed in the presence of the analyte and potential cross-reactants tested at concentrations greater than likely pathophysiologic concentrations (Micallef and Ahsan, 1994). For example, Ko et al. (1992), in a non-competitive ELISA for IL-8, tested serial dilutions ranging from 1 µg/ml to 0.1 ng/ml of recombinant IL-8 (rIL-8) and potential cross-reactants such as monocyte chemotactic and activating factor (MCAF), rIL-1α, and recombinant tissue necrosis factor (rTNF). Brailly et al. (1994) used the same procedure in a two-site ELISA for IL-6, but they also tested potential cross-reactants added at 100 µg/l to rIL-6 solutions. The latter procedure is particularly important for two-site immunoassays, since a cross-reactant that binds only one of the two antibodies will give little or no assay response when presented alone, but may reduce the response of analyte if simultaneously present in sufficiently high concentrations (Micallef and Ahsan, 1994).

For competitive immunoassays, serial dilutions of analyte and cross-reactant may also be tested as above, with cross-reactivity expressed as the percentage ratio of concentrations of analyte and cross-reactant that produces 50% displacement of tracer (Carstens et al., 1997; Luppa et al., 1995).

1.11.2 Sensitivity (detection limit)

The determination of the detection limit (sensitivity) also incorporates an assessment of the reproducibility (within-run precision) of an assay. For example, Brailly et al. (1994)
assayed a range of IL-6 standard concentrations in replicates of twelve, thus allowing a calculation of the signal coefficient of variation (%C.V.) for each concentration. The resultant information was then used to prepare a precision profile by plotting the %C.V. (y-axis) against the analyte concentration (x-axis), thus allowing determination of the working range of the assay. To calculate the minimum detectable dose, the authors used the dose-response curve, extrapolating from the y-axis at the signal equal to non-specific signal (blank) plus two standard deviations (S.D.). However, Micallef and Ahsan (1994) recommend a factor of three be universally adopted.

The assay detection limit, which is defined as the (im)precision in measurement of the zero-dose response, is now widely accepted as the only valid indicator of immunoassay sensitivity (Ekins and Chu, 1991). The authors claim that the slope of the dose-response curve as an indicator of sensitivity is misleading, since a curve of lower slope could possibly have a lower imprecision of zero-dose response, and so a lower detection limit, than a curve of greater slope.

Ekins and Chu (1991) indicate that the sensitivity of competitive immunoassays are limited primarily by the affinity of the antibody, with the specific activity of the label of secondary importance. Assuming that 1) the error of signal measurement to be zero (label of infinite specific activity), 2) the use of $^{125}\text{I}$ as label with the radioactivity being counted for 1 min, 3) the radioactivity of the antibody-bound labelled analyte is measured, and 4) the experimental error in the measurement of the bound fraction ($\delta_{Ro}/Ro$) is 1%, the potential sensitivity attainable in such an assay is $\delta_{b}/KRo$, where $\delta_{b}/Ro$ represents the error in the measurement of zero-dose response (Ro). For example, if $K$ is $10^{12}$ l/mol, maximal assay sensitivity is $10^{-14}$ mol/l or ~6 x $10^9$ molecules/l. According to Ekins and Chu (1991), it is near impossible in practice to achieve sensitivities greater than about $10^{10}$ molecules/l using a competitive approach,
assuming an upper limit of antibody affinity at $10^{12}$ l/mol, irrespective of the nature of label used.

From the literature, the practical limit of competitive immunoassays appears to be in the picomolar range. Luppa et al. (1995) achieved a detection limit of 55 pmol/l (2.5 fmol/well) in an immunoassay for total estrone, while Ogbonna et al. (1995), in their competitive immunoassay for apolipoprotein B100, attained a detection limit of 4.0 pmol/l or 400 amol/tube. However, since these authors failed to record the affinities of the antibodies used, it is difficult to assess how close these assays came to Ekins’ and Chu’s (1991) theoretical limits. Carstens et al. (1997), in a radioimmunoassay for urodilatin in human urine, measured 500 amol/tube. This corresponds to a urine concentration of 7.5 pmol/l, if 400 µl of urine is extracted and resuspended in 600 µl of assay buffer, using 100 µl for assay. Applying the affinity of the polyclonal antibody used ($1.05 \times 10^{11}$ l/mol) to the model as proposed by Ekins and Chu (1991), the theoretical limit is $-10^{13}$ mol/l or 0.1 pmol/l, which is seventy-five times lower than the limit achieved. Interestingly, Helmerson et al. (1997) measured as low as 1 fmol/l (1 zmol/assay) using an optical tweezers-based competitive immunoassay for BSA. While this appears to be lower than theoretical limits, the antibody affinity was not stated.

According to the models proposed by Ekins and Chu (1991), the potential sensitivity of a non-competitive immunoassay is given by $(R_o/[Ab]K)(\delta_{R_o}/R_o)$, with $R_o$ in this case representing the labelled antibody misclassified as bound, commonly referred to as “non-specifically bound” antibody. The authors stress the importance of minimising non-specific binding and using high affinity antibodies with non-isotopic labels of high specific activity in order to achieve the maximal potential sensitivity.

In accordance with the theoretical model as proposed by Ekins and Chu (1991), the assays with the lowest detection limits are indeed the non-competitive reagent-excess
assays. In 1982, Ishikawa et al. measured as little as 0.2 amol of ferritin (1.33 x 10^{-15} mol/l), 2.4 amol of IgE (1.6 x 10^{-14} mol/l), and 5.7 amol of TSH (3.8 x 10^{-14} mol/l), using antibody-coated polystyrene balls and affinity-purified Fab'-β-galactosidase conjugate in a sandwich format. Using antibody-coated glass balls in a similar format, Ruan et al. (1987) attained even better sensitivities, measuring 1 zmol of ferritin (2 x 10^{-16} mol/l) and 0.03 amol of IgE (6 x 10^{-15} mol/l). Hashida and Ishikawa (1990) also measured 1 zmol of ferritin (3.33 x 10^{-17} mol/l), but using a more complicated format called immune complex transfer two-site immunoassay. Ferritin was reacted simultaneously with affinity-purified dinitrophenyl biotinyl anti-ferritin IgG and affinity-purified anti-ferritin Fab'-β-galactosidase conjugate. The complex formed was trapped onto anti-dinitrophenyl group IgG-coated polystyrene balls. After washing the balls to remove excess conjugate, the complex was eluted from the balls using an excess of α-N-dinitrophenyl-L-lysine and transferred to streptavidin-coated balls. In this way, the non-specific binding was reduced with a lesser decrease in specific binding, giving improved sensitivity when compared to the previous assay using IgG-coated polystyrene balls (Ishikawa et al., 1982). In addition, the assay showed little ill-effects when 5 µl and 10 µl of serum was added to the reaction mixture. The authors suggest that even better sensitivity could be achieved through the use of a label with higher specific activity and another transfer of the complex. However, it would be of interest to see how these non-competitive immunoassays would fare with real samples.
1.11.3 Precision

Within-run and between-run precision are simply assessed using samples analysed in several replicates, usually at low, medium, and high concentrations of analyte (Luppa et al., 1995; Ogbonna et al., 1995; Tanebe et al., 1992; Dhahir et al., 1992).

1.11.4 Accuracy

Generally, samples, especially biological samples (serum, plasma, cerebrospinal fluid, urine), constitute complex mixtures, with certain components of the matrix possibly interfering non-specifically with the immune binding reaction of the assay (Baker et al., 1985). These interferences are particularly problematic when standards are prepared in buffer diluents (Noé et al., 1992; Luppa et al., 1995; Tanebe et al., 1992), since there is no guarantee that such a matrix will behave identically to the sample matrix. Preferably, the calibrant matrix should be identical to the fluid in the samples to be tested (Papoian et al., 1991), as the assay response may vary in different media. As an alternative, one could use a closely resembling matrix, for example, Dhahir et al. (1992) used bovine serum as calibrant matrix in a two-site sandwich assay for the measurement of proinsulin in human serum.

To determine whether the system can accurately measure analyte in samples when compared to standard, one can perform recovery, linearity, and comparison studies.

1.11.4.1 Recovery

For study of analytical recovery, workers usually add graded doses of calibrant spanning the dose range of the assay to samples (Bugari et al., 1990; Dhahir et al., 1992; Noé et al., 1992). When the samples are analysed, the observed results should equal the endogenous concentrations plus the amounts of calibrant added. Recovery is
then calculated by dividing the observed value by the expected value and multiplying by 100. A mean recovery of \(-100\%\) indicates that the matrices of the samples and standards are equivalent (absence of matrix effects) (Baker et al., 1985).

This method is likely to give false recovery results, since the endogenous concentration is measured using the assay under investigation, with recovery assumed to be 100\%. For example, Bugari et al. (1990) used their system to measure the endogenous concentration of lutropin in a serum pool (4.4 I.U./l) and added 3.125 I.U. of lutropin per litre to give an expected value of 7.525 I.U./l. Subsequently, the measured value was 7.2 I.U./l, giving a recovery of 90\%. However, if for example, the recovery associated with the endogenous concentration was initially 80\% (actual 5.5 I.U./l), then the actual recovery would only be 83.5\%.

A more correct approach would be to use sample matrix devoid of analyte under investigation (Aubin et al., 1997).

1.11.4.2 Linearity

Simply, samples may be progressively diluted in calibrant matrix and analysed to calculate the amount of material present. Any substances present in the samples that affect assay response, but are not identical to the calibrant, may give sample and standard dose-response curves that are non-parallel (Micallef and Ahsan, 1994). There are several ways to assess parallelism. Firstly, from the measured concentration in the undiluted sample, one can calculate the expected values of the diluted samples and plot the expected versus measured values (Bugari et al., 1990). The resultant plot should give a straight line of negligible intercept and a correlation coefficient \((r)\) of 1.00. Alternatively, one may plot the effective sample volume (x-axis) against the measured concentration on the y-axis (Luppa et al., 1995; Tanebe et al., 1992), with the points
obtained falling on a straight line of negligible intercept. Any deviation from linearity at higher volumes (lower dilutions) would clearly indicate that the sample volumes or dilutions routinely determined should be limited to those safely within the linear region (Micallef and Ahsan, 1994).

1.11.4.3 Comparison with reference methods

A number of samples are analysed and compared to an established reference method using regression analysis. While Luppa et al. (1995) and Burgi et al. (1988) used sample numbers greater than thirty, others have failed to use statistically significant numbers of samples. Ogbonna et al. (1995) used only ten samples for comparison purposes with an immunoturbidimetric assay, while Noé et al. (1992) used only seventeen for correlation with RIA.
1.12 Some Interferences in Immunoassay

Pesce and Michael (1992) outlined the common interferences associated with immunoassay and divided them into exogenous and endogenous causes. The exogenous interferences are due to effects before the test reaction occurs, whereas endogenous interferences are due to materials inherent in the biological test solution.

Exogenous interferences include sample collection reagents, for example, anticoagulants such as heparin and EDTA. The effects of these reagents can be observed by performing recovery studies with added interferent. In addition, the authors identify solid-phase binding as a possible cause of exogenous interference, in that binding of antigen or antibody to a surface may change the properties of that surface, thus leading to non-specific binding. Graves (1988) showed that the non-specific binding of rabbit IgG to a protein-coated surface was highly charge dependent, with binding being high to positively charged surfaces. Also, incomplete saturation of solid-phase binding sites for antigen and antibody may lead to non-specific binding, thus reagents may need to be added to block these vacant sites. A study by Pratt and Roser (1989) compared casein, BSA, and newborn calf serum for their ability to block unoccupied sites on polystyrene solid phase. Results showed casein to be the most effective blocking agent, a result which was later confirmed by Esser (1991).

Heterophilic antibodies, which represent one of the endogenous interferences, are antibodies present in serum/plasma samples capable of reacting with various species of immunoglobulins (Kohse and Wisser, 1990; Levinson, 1992). Kohse and Wisser (1990) and Levinson (1992) outlined the possible mechanisms of interference by these antibodies in immunoassays. In a double antibody method, the heterophilic antibody can promote binding between the first and second antibodies, even in the absence of
analyte, leading to an erroneously high result. On the other hand, the heterophile antibody may bind only one antibody, thus sterically hindering binding of the analyte to this antibody. An erroneously low value results. In addition, heterophilic antibodies may also cause problems in competitive immunoassay when double-antibody solid-phase separation systems are used (Boscato and Stuart, 1988; Kohse and Wisser, 1990). Presumably, the presence of the heterophilic antibody in the sample could lead to inhibition of antibody binding to the second antibody immobilised to solid phase, thus leading to reduced bound tracer and an erroneously high analyte value. The interference caused by heterophilic antibodies may be eliminated using a non-specific immunoglobulin from the animal species used to raise the antibodies. However, this does not always work. Polyclonal mouse IgG and polymerised monoclonal IgG failed to eliminate interferences from heterophilic antibodies in a two-site assay for creatine kinase MB (CKMB) using two monoclonal antibodies (Vaidya and Beatty, 1992). When the authors analysed eighty-one samples using an intact capture anti-B antibody and intact anti-CKMB-β-galactosidase conjugate, eighteen out of eighty-one samples gave falsely high CKMB values due to heterophilic antibodies. Addition of polymerised monoclonal IgG and polyclonal mouse IgG failed to eliminate the false positive results. However, when the intact IgG-enzyme conjugate was replaced by an F(ab')2-conjugate, fourteen of the eighteen samples gave values within the normal range. Use of the polymerised or polyclonal IgG along with the F(ab')2-enzyme conjugate gave normal results for all samples. Therefore, the results of the study indicate that the heterophilic antibodies largely had specificity for the Fc region of the monoclonal antibodies. However, the use of fragmented antibodies would not always eliminate heterophilic antibody interferences, since some have been shown to be directed against epitopes residing on the Fab region (Levinson, 1992).
Some other endogenous interferences include those due to rheumatoid factors and autoantibodies present in serum or plasma. Rheumatoid factors are IgM-type antibodies that bind to multiple antigenic determinants on the Fc portion of IgG (Levinson, 1992; Kohse and Wisser, 1990). The consequences of this binding in relation to immunoassay have been discussed earlier ("Antibody fragments"). In addition, some individuals can produce autoantibodies to the test analyte, the most frequently occurring antibodies being those to thyroglobulin, with antibodies against T₃ and T₄ occurring less commonly (Kohse and Wisser, 1990). Such antibodies can lead to erroneously high or low hormone concentrations when measured using competitive immunoassay depending upon the separation method used (Kohse and Wisser, 1990).
**1.13 Column-Based Flow-Through Immunoassay**

Although immunoassay techniques offer the advantages of selectivity and sensitivity, they have the disadvantage in that they usually require prolonged incubation times, since the rate of antigen-antibody interaction is diffusion dependent. To circumvent this limitation, many have turned to affinity chromatography, thus reducing the contact time between antigen and antibody (de Alwis and Wilson, 1985; Lejeune et al., 1990; Hage and Kao, 1991; Freytag et al., 1984; Janis and Regnier, 1989; Cassidy et al., 1992; Nilsson et al., 1994; Kronkvist et al., 1997). In addition, many column-based immunoassays are automated, thus reducing operator input and eliminating the need for experienced personnel.

De Alwis and Wilson (1985) designed a high-performance immunoaffinity chromatography (HPIAC) sandwich ELISA for the measurement of mouse anti-bovine IgG using a flow-through immunoreactor packed with bovine IgG immobilised on Reactigel-6X. To carry out the assay, a standard was injected into a flowing stream of assay buffer and carried through the immunoreactor to waste. This injection was followed by two injections of a goat anti-mouse IgG-glucose oxidase conjugate spaced 2 min apart. Again, excess material was carried to waste by the flowing stream. Subsequently, three aliquots of 1% glucose were injected at 3-min intervals and the resulting product ($H_2O_2$) was diverted to an amperometric detector. The output from the detector was processed and the peak area was used as the basis of analysis. Such analysis took 15 min. Minutes after the last addition of substrate, the column was exposed to disruption buffer to remove analyte-second antibody conjugate, followed by assay buffer to equilibrate the column. This disruption and equilibration process took approximately 15 min. The lower limit of detection of the assay was in the
femtomolar range when the standards were prepared in buffer or in 6 g/dl bovine serum albumin in buffer, however, when control serum was used the limit increased to 1 pmol or $3.33 \times 10^{-8}$ mol/l.

In the column-based sandwich immunoassay designed by de Alwis and Wilson (1985), the advantages of the chromatographic process appears only in the first step, i.e., ligand binding by a large excess of antibody solid-phase, whereas the second step (addition of labelled antibody) is run with a lack of stationary phase excess. This results in the loss of the chromatography advantages at the second step. Consequently, several additions of the labelled antibody are required for reproducible binding. This lead Lejeune et al. (1990) to design an automated reversible antibodies capture immunoassay (RACIA) for human growth hormone. Their system used an affinity column packed with two superimposable gels, the first labelled with a phosphonic group (a ligand of phosphatase) and the second one with aminophenyl thiogalactoside (a $\beta$-galactosidase ligand). Two anti-human growth hormone antibodies, conjugated to $\beta$-galactosidase and alkaline phosphatase and directed at different epitopes on the antigen, were then passed through the column. A standard prepared in buffer containing 0.5% or 6% BSA was passed through the column, resulting in the binding of antigen to the antibody-alkaline phosphatase conjugate in the first part of the column. Addition of an excess of mineral phosphates displaced the enzymatically labelled antibody-antigen complex, which was then captured by the excess antibody in the second part of the column. After elution using an alkaline buffer, the complexes were collected and assayed for phosphatase activity using amplified detection. The revelation time was 3.25 h. During the elution process, the column goes back to its native state and is ready to receive antibody-enzyme conjugates directed against the same or different analytes. The optimised system allowed the measurement of human
growth hormone in the range of 0.1 amol to 100 fmol in a 4-h time period overall. However, it remains to be seen whether such sensitivity could be attained using real samples.

To improve on the sensitivity obtained by de Alwis and Wilson (1985), Hage and Kao (1991) used HPIAC to develop a sandwich assay for parathyroid hormone (PTH) using chemiluminescent detection. In this method, a standard prepared in plasma and acridinium ester-labelled anti-(1-34 PTH) antibodies were injected onto a column containing anti-(44-68 PTH) antibodies. The column was then washed, with excess reagents going to waste. After desorption with elution buffer, the labelled antibody was combined with alkaline peroxide reagent in a post-column reactor and the resulting light production was measured. The column was then switched back to assay buffer and allowed to equilibrate before the next standard was added. The assay took 1 h 6 min per sampling, allowing 1 h for pre-incubation of the standard and labelled antibody and 6 min per plasma injection. The limit of detection of the assay was 16 amol, which equals $2.4 \times 10^{-13}$ mol/l using a 66 μl assay volume.

Freytag et al. (1984) designed a single-antibody non-competitive affinity-column-mediated immunoassay (ACMIA), which could be performed on the DuPont aca® III discrete analyser. To determine digoxin, standards prepared in drug free serum were mixed with an anti-digoxin-β-galactosidase conjugate in the sample cup. After a 10-min pre-incubation at room temperature, an aliquot of each mixture was aspirated and passed through a disposable affinity column containing immobilised analyte. The enzyme activity that passed through the column was quantified using oNPG as substrate, with the enzyme activity being measured as the rate of change in absorbance at 405 nm. Therefore, the concentration of digoxin was directly proportional to enzyme activity. The optimised assay using oubain resin, F(ab′)2-β-galactosidase
conjugate, and a flow rate of 34 μl/s had a total analysis time of 18 min, giving a limit of detection for digoxin of 0.2 mg/l (2.56 x 10⁻⁷ mol/l) using a 200 μl volume.

In 1989, Janis and Regnier made use of two columns, an affinity and a reverse-phase chromatography (RPC) column, in an automated non-competitive system called dual-column immunoassay (DCIA). The immunoaffinity column was prepared by reversible adsorption of antibodies on a column with covalently immobilised protein G. The use of protein G means that the columns could be used for a wide range of immunoassays (universal support). For transferrin quantitation, rabbit anti-human transferrin was injected first onto the protein G column, followed by an injection of serum sample or standard containing transferrin. The antigen was then desorbed using elution buffer and quantitated by RPC. Loading buffer was used to equilibrate the protein G column. The complete cycle from the addition of antiserum to quantitation of antigen by RPC took 30 min. Since specific antiserum is added prior to antigen injection, one can measure a different analyte once a cycle is complete. The immunoassay for transferrin had a detection limit of 0.7 pmol, which equals 3.5 x 10⁻⁸ mol/l using a 20 μl volume. The authors stated that DCIA was more sensitive than conventional immunoaffinity chromatography, since the antigen eluted from the first column as a broad peak, was concentrated on the second more efficient column. When compared to ELISA, the precision was better, but ELISA was more sensitive. However, the method does allow one to better discriminate between specific analyte and cross-reactants. As with ELISA, the procedure also has the potential for multianalyte testing.

Similarly, Cassidy et al. (1992) used a “universal support” to reversibly immobilise any antibody in an automated competitive assay that also does not require tagged or labelled molecules. In the kinetic immunochromatography sequential addition (KICQA) immunoassay for albumin or transferrin, specific antibody and
standard/serum sample were sequentially added onto a protein A column, followed by injection of label. The amount of label passing through the column was detected by absorption in the UV region, giving a direct relationship for calibrants when absorbance (peak area) was plotted against the concentration of antigen. The label in KICQA is a known amount of pure antigen and does not possess a tag, e.g., fluorescent, since the antigen in sample and label contact the antibody at different times. Once the assay is complete, the antibody-antigen complexes are desorbed using acidic elution buffer. The authors indicate that the detection limit for human albumin was 40 ng (1.5 x 10^{-14} mol) using this system, but since they failed to give the sample volume used, one cannot calculate the molar concentration. However, the authors do state that this should not be taken as the lower limit of detection for KICQA, since the limit is subject to the limitations of the detector used and the absence of a tag for the label. The total assay time is extremely fast, with results available in less than 1 min (0.9 min).

In 1993, Nilsson et al. designed an automated immunochemical flow-ELISA for α-amylase using a sequential competitive format. Calibrant or sample containing α-amylase was injected through the injection valve and passed through a column containing rabbit anti-α-amylase Sepharose CL-4B. This was followed by an injection of an α-amylase-horseradish peroxidase conjugate onto the column. The conjugate bound to antibodies not occupied by sample α-amylase. After washing, substrate solution was passed through the column and product eluting from the column was measured spectrophotometrically at 540 nm. Bound α-amylase was then eluted from the column using acidic buffer. A complete cycle took 330 s. The column binding capacity decreased slightly every time the column was used due to denaturation caused
by the low pH buffer and substrate solution. Therefore, a standard curve measured on a fresh column was much higher than a curve measured on the same column after it had been used for a while. However, the authors state that the shape of the curve does not change, so that one calibration peak with a known amount of α-amylase was enough to fit a previously measured curve to the current status of the column. The nature of calibrants and samples analysed, plus the detection limit of the assay, was not stated by the authors.

Most of the systems described require regeneration of columns as part of the sampling cycle, therefore limiting the lifetime of the affinity column and reducing the sample throughput of the system. Kronkvist et al. (1997) designed an ingenious system which eliminates the need for time-consuming regeneration. Two different types of assay were evaluated in their study. The first was based on competitive enzyme immunoassay, where the analyte (cortisol or budesonide) and a known amount of alkaline phosphatase-conjugated analyte bind competitively to a limited amount of antibody. After a 1-h incubation, the standard was injected into the flow where the antibodies bind to protein G immobilised on the column. The free enzyme conjugate passing through the column was mixed with substrate from a merging flow and allowed to react in the post-column reactor. Product was detected downstream by amperometry or fluorometry. The second enzyme immunoassay was a displacement assay requiring no pre-incubation step. Alkaline phosphatase-labelled cortisol was injected into the flow system to overload the capacity of the affinity column, packed with immobilised anti-cortisol antibodies. After the addition of standard, the displaced fraction of labelled cortisol was allowed to react with substrate from the merging flow and detected downstream.
In both systems, another sample or standard may be added after product has been detected without the requirement for column regeneration, therefore extending the lifespan of the column and increasing sample throughput. Resultantly, the displacement assay for cortisol took about 8 min per analysis. The competitive flow-injection assay for cortisol and budesonide required at least a 1-h incubation, thus limiting overall analysis time. However, excluding the pre-incubation, sample throughput was twenty per hour. Moreover, the conditions for the enzyme reaction for both systems can be optimised without having to consider the stability of the affinity column, since the substrate flow does not pass through the column.

Best sensitivity was achieved using the competitive flow-injection immunoassay, which allowed measurement of 0.02 pmol of budesonide \((8 \times 10^{-8} \text{ mol/l})\) using amperometric detection. A similar assay for cortisol using fluorescence detection allowed measurement of 2.5 pmol of cortisol \((10^{-7} \text{ mol/l})\). The displacement assay was not as sensitive, allowing measurement of only 12.5 pmol of cortisol. However, assessment with real samples (serum or plasma) was neglected. This system does have one major drawback in that the enzyme reactions are carried out in the presence of sample matrix components, which may interfere with enzyme activity or detection of product. For instance, pure plasma interfered greatly with amperometric detection and to a lesser extent with fluorescence measurements. This limitation can only be overcome using sample pre-treatment, thus extending the overall analysis time of samples.
1.14 Project Objectives

- Primarily, to develop an affinity-column-mediated flow-through two-site sandwich ELISA using ferritin as model analyte. Such a system would be designed with simplicity and speed in mind, while retaining the performance characteristics associated with conventional immunoassay systems.
- To ensure the latter through validation studies, e.g., recovery, linearity, etc.
- To partially optimise a conventional two-site sandwich ELISA in order to provide evidence of antigen recognition by the chosen antibody pair. In addition, such study should demonstrate the potential sensitivities attainable using these antibodies in a column-based immunoassay.
2 Materials
2 MATERIALS

2.1 Equipment

2.1.1 pH meter
Orion model 420A pH/mV/temperature meter (Orion Research (ATI Orion), Boston, MA, U.S.A.). The meter is microprocessor-controlled and features pH autocalibration, sealed keypads and simultaneous temperature display.

2.1.2 Microtitre plate reader
Dynatech MR7000 (Dynatech Laboratories, West Sussex, U.K.). The reader measures the light absorbance of solutions in each of the 96 wells of a microtitre plate. Reading is very rapid, with absorbance values available in <1.7 seconds for a single wavelength and <2.2 seconds for dual readings. Results can be presented graphically or as optical density matrices via an Epson FX80 compatible dot-matrix printer (Epson America Inc., Torrance, CA, U.S.A.). The memory allows storage of fifty user programs and data from twenty microplates.

2.1.3 Balance
A&D HR200 (A&D Co. Ltd., Tokyo, Japan). The HR200 is a high performance analytical balance with a resolution of 0.1 mg and a capacity of 210 g.
2.1.4 Microtitre plates

Nunc Maxisorp 96-well microtitre plates (Nunc, Roskilde, Denmark). All wells are within \(\pm 0.005\) absorbance units from the mean and show good homogeneity in the absorption of IgG - C.V. less than 5% and all results within \(\pm 10\%\) from mean.

2.1.5 Water purification system

Millipore-Milli-U10 (Millipore Corporation, Bedford, MA, U.S.A.). The system produces grade 2 (according to ISO3696/B53978) water directly from deionisation using a mixed bed ion-exchange resin. Product is low in inorganic ions, dissolved organic compounds, bacteria, and colloidal matter.

2.1.6 Mixer

Denley Spiramix 5 (Denley, Bolney, Sussex, England).

2.1.7 Columns

Disposable polystyrene columns (0.5-2.0 ml) (Pierce, Rockford, IL, U.S.A.). Supplied in packages of 100, with 200 polyethylene discs (45 μ pore size) and 100 each of top and end caps.

2.1.8 Centrifuge

The Jouan C4.12 benchtop centrifuge (Jouan Inc., Winchester, VA, U.S.A.).
2.2 Chemicals

Merck KGaA, Darmstadt, Germany

- Sodium dihydrogen phosphate (NaH₂PO₄).
- Disodium hydrogen phosphate (Na₂HPO₄).
- Sodium hydrogen carbonate (NaHCO₃).
- Sodium carbonate (Na₂CO₃).
- Glycine (C₂H₅NO₂).
- Tris-(hydroxymethyl)-aminomethan (Tris) (C₄H₁₁NO₃).

BDH Chemicals Ltd., Poole, England

- Hydrochloric acid (HCl) - [density = 1.15 g/ml].
- Sodium hydroxide (NaOH).
- Magnesium chloride (MgCl₂).
- Ethanol (C₂H₅OH).
- Ethanolamine (C₂H₇NO) - [density = 1.015 g/ml].
- Acetic acid (C₂H₄O₂).
- Sulphuric acid (H₂SO₄).

Sigma Chemical Co., St. Louis, MO, U.S.A.

- Diethanolamine (C₄H₁₁NO₂) - [density = 1.0881 g/ml].
- Polyoxyethylenesorbitan monolaurate (Tween 20).
- Sodium acetate (NaC₂H₃O₂).
- Sodium chloride (NaCl).
2.3 Reagents

Sigma Chemical Co., St. Louis, MO, U.S.A.

- Alcohol dehydrogenase (E.C. 1.1.1.1) from equine liver - [1.7 units/mg].
- Diaphorase (E.C. 1.8.1.4) from *Clostridium kluyveri* - [5.4 units/mg].
- β-Nicotinamide dinucleotide phosphate (βNADP).
- *p*-Iodonitrotetrazolium violet (INT).
- Bovine serum albumin (BSA), Fraction V.

Pharmacia-Biotech, Uppsala, Sweden

- Cyanogen bromide-activated Sepharose 4B, supplied freeze-dried in the presence of additives. The product is a pre-activated gel for immobilisation of ligands containing primary amines, thus it is applicable to the immobilisation of antibodies. The coupling reaction is spontaneous, rapid, and convenient.

Fluka Chemie AG, Buchs, Switzerland

2.4 Immunoglobulins and Antigen

Biomerieux, Lyon, France

- Mouse monoclonal IgG to human ferritin (clone B8).
- Mouse monoclonal anti-human ferritin-alkaline phosphatase conjugate (clone B8). The alkaline phosphatase used as label was isolated from calf intestine.

Calbiochem-Novabiochem, La Jolla, CA, U.S.A.

- Ferritin from human liver.

Cork University Hospital, Cork, Republic of Ireland

- Human serum samples
3 Methods
3 METHODS

3.1 Conventional Two-Site Sandwich ELISA for Ferritin

3.1.1 Preparation of buffers

3.1.1.1 pH-adjusting solutions

- 6 mol/l HCl. Add 190 ml of concentrated HCl to 810 ml of distilled water (dH2O).
- 6 mol/l NaOH. Dissolve 240 g of NaOH in 1 litre of dH2O.

3.1.1.2 Coating buffer

- 0.05 mol/l bicarbonate, pH 9.6. Dissolve 3.44 g of NaHCO3 and 0.95 g of Na2CO3 in 800 ml of distilled water. Adjust pH with 6 mol/l HCl/NaOH. Make up to 1 litre with dH2O.

3.1.1.3 Wash buffer

- 0.05 mol/l phosphate buffered saline, pH 7.2, containing 0.1% (v/v) Tween 20. Dissolve 1.69 g of NaH2PO4 and 5.11 g of Na2HPO4 in 800 ml of dH2O. Add 1 ml of Tween 20 and adjust pH with 6 mol/l HCl/NaOH. Make up to 1 litre with dH2O.

3.1.1.4 Assay buffer

- 0.05 mol/l phosphate buffered saline, pH 7.2. Dissolve 1.69 g of NaH2PO4 and 5.11 g of Na2HPO4 in 800 ml of dH2O. Adjust pH with 6 mol/l HCl/NaOH and make up to 1 litre with dH2O.
3.1.1.5 Substrate buffer

- 0.1 mol/l Tris-HCl, pH 9.8, containing 1.5 mol/l NaCl. Dissolve 12.11 g of Tris and 87.66 g of NaCl in 800 ml of dH₂O. Adjust the pH with 6 mol/l HCl and make up to 1 litre with dH₂O.

3.1.2 General procedure for the measurement of ferritin

1. Sensitise wells of a microtitre plate with 100 µl of anti-ferritin antibody diluted in coating buffer. Incubate @ 4°C.
2. Empty the contents of each well. To wash the wells, add 400 µl of wash buffer and decant. Repeat the procedure three more times. Finally, blot dry.
3. Add 200 µl of 1% BSA (1 g/dl dH₂O) to each well and incubate @ 37°C for 1 h.
4. Wash the wells as in step 2.
5. To prepare a standard curve, dissolve ferritin antigen in assay buffer and double dilute this working standard in assay buffer on the microtitre plate, to give a final volume of 100 µl in each well. To detect non-specific binding, use 100 µl of assay buffer as blank. Incubate for 1 h @ 37°C.
6. Wash as in step 2.
7. Add 100 µl of anti-ferritin-alkaline phosphatase conjugate diluted in assay buffer. Incubate for 1 h @ 37°C.
8. Wash as in step 2.
9. Pipette 100 µl of a freshly prepared substrate solution (2 mg of pNPP per ml of substrate buffer) and incubate at room temperature for 20 min.
10. Read the absorbances of the wells @ 405 nm.
3.2 Affinity-Column-Mediated Flow-Through ELISA for Ferritin

3.2.1 Preparation of buffers

3.2.1.1 Buffers used in the preparation of the Sepharose gel

- 0.1 mol/l acetate, pH 4.0, containing 0.5 mol/l NaCl. Dissolve 1.23 g of NaC$_2$H$_3$O$_2$, 97.24 ml of C$_2$H$_4$O$_2$, and 29.22 g of NaCl in 800 ml of dH$_2$O. Adjust pH with 6 mol/l HCl/NaOH and make up volume to 1 litre with dH$_2$O.

- 0.1 mol/l Tris-HCl, pH 8.0, containing 0.5 mol/l NaCl. Dissolve 12.11 g of Tris and 29.22 g of NaCl in 800 ml of dH$_2$O. Adjust the pH using 6 mol/l HCl and make up to 1 litre with dH$_2$O.

- 1 mmol/l HCl. Pipette 32 µl of concentrated HCl into 1 litre of distilled water.

3.2.1.2 Coupling buffer

- 0.1 mol/l bicarbonate, pH 8.5, containing 0.5 mol/l NaCl. Dissolve 8.23 g of NaHCO$_3$, 0.21 g of Na$_2$CO$_3$, and 29.22 g of NaCl in 800 ml of dH$_2$O. Adjust pH with 6 mol/l HCl/NaOH and make up volume to 1 litre with dH$_2$O.

3.2.1.3 Blocking buffer

- 1 mol/l ethanolamine, pH 8.0. Add 60.78 ml of ethanolamine to 800 ml of dH$_2$O. Adjust pH using 6 mol/l HCl and make up to 1 litre.
3.2.1.4 **Carrier buffer**

- 50 mmol/l Tris-HCl, pH 7.4, containing 0.5 mol/l NaCl, 0.1% (w/v) BSA and 0.035% (v/v) Tween 20. Dissolve 6.06 g of Tris and 29.22 g of NaCl in 800 ml of dH₂O and adjust the pH to 7.4 using 6 mol/l HCl. Dissolve 1 g of BSA and 350 µl of Tween 20, and make up the volume to 1 litre using dH₂O.

3.2.1.5 **Substrate buffer**

- 0.1 mol/l Tris-HCl, pH 9.8, containing 1.5 mol/l NaCl. Dissolve 12.11 g of Tris and 87.66 g of NaCl in 800 ml of dH₂O. Adjust the pH with 6 mol/l HCl and make up to 1 litre with dH₂O.

3.2.1.6 **Regeneration buffer**

- 0.1 mol/l glycine, pH 2.1. Dissolve 7.5 g of glycine in 800 ml of dH₂O. Adjust the pH with 6 mol/l HCl and make up to 1 litre with dH₂O.

3.2.1.7 **Amplifier substrate buffer**

- 50 mmol/l diethanolamine/HCl, pH 9.5, containing 1 mmol/l MgCl₂. Pipette 4.93 ml of diethanolamine into and dissolve 0.095 g of MgCl₂ in 800 ml of dH₂O. Adjust pH to 9.5 using 6 mol/l HCl and make up to 1 litre using dH₂O.

3.2.1.8 **Amplifier buffer**

- 20 mmol/l phosphate buffer, pH 7.2, containing 4% ethanol and 5 mg/ml BSA. Dissolve 0.68 g of NaH₂PO₄ and 2.03 g of Na₂HPO₄ in 800 ml of dH₂O. Adjust pH to 7.2 with 6 mol/l HCl/NaOH. Add 40 ml of ethanol and 5 g of BSA. Make up to 1 litre with dH₂O.
3.2.2 Coupling anti-ferritin antibody to CNBr-activated Sepharose 4B

3.2.2.1 Preparation of Sepharose gel

1. Weigh out the required amount of freeze-dried powder (1 g of powder = 3.5 ml of gel) and suspend it in 1 mmol/l HCl.

2. The swollen gel should be washed for 15 min with 1 mmol/l HCl on a sintered glass funnel. The manufacturer recommends the use of approximately 200 ml of 1 mmol/l HCl per gram of freeze-dried powder, added in several aliquots.

3.2.2.2 Coupling the ligand

1. Dissolve the ligand to be coupled (anti-ferritin antibody) in coupling buffer. Use 5 ml of coupling solution per gram of freeze-dried powder.

2. Mix the coupling solution containing the antibody with the washed gel in a stoppered vessel. Rotate the mixture end-over-end for 1 h at room temperature or overnight at 4°C. A magnetic stirrer should not be used.

3. Wash away excess ligand with at least five gel volumes of coupling buffer.

4. To block any remaining reactive groups, transfer the gel to 1 mol/l ethanolamine buffer, pH 8.0. Let it stand for 2 h.

5. Wash the product first with 0.1 mol/l acetate buffer, pH 4.0, containing 0.5 mol/l NaCl, and then with 0.1 mol/l Tris-HCl, pH 8.0, containing 0.5 mol/l NaCl. Repeat the cycle two more times. Use at least five gel volumes for each buffer.

1 Manufacturer’s instructions.
3.2.3 Packing the gel into a polystyrene column

1. Place the bottom cap on the end of the column.
2. Place the column in a test tube (16 x 150 mm) and add 3.0 ml of dH₂O.
3. Float one of the porous polyethylene discs on top of the liquid within the column.
4. Depress the disc evenly to the bottom of the column using the insertion rod supplied with the columns.
5. Empty the column of water and add a volume of gel slurry required to give a final volume of 0.5 ml of settled gel.
6. Allow 30 min for the gel to settle.
7. Place a second polyethylene disc on top of the liquid within the column and depress it to just above the settled gel. Leave approximately 1-2 mm of space between the top of the gel bed and the bottom of the top disc.
8. Wash the inside top part of the column to clean out any gel that may have remained along the sides during packing.
9. For storage, keep approximately 2 ml of dH₂O over the top disc to prevent drying out. Place the top cap on the column and store upright at 4°C.

3.2.4 Using the packed column

1. To use the column, remove the top cap first. This prevents bubbles being drawn into the gel. Next, remove the bottom cap and place the column in a suitable holder, e.g., 16 x 150 mm test tube. Allow the water to drain completely from the column.

---

2 Modification of the manufacturer's instructions.
3 Manufacturer's instructions.
2. If air bubbles are entrapped in the gel, spin the column first at 1,000 g for 10 min. This procedure is important, since air bubbles significantly reduce flow rates.

3.2.5 General procedure for the measurement of ferritin

1. Equilibrate all columns with at least 3 ml of carrier buffer.

2. Pipette 200 µl of standards (ferritin dissolved in carrier buffer) or samples into separate columns and allow them to pass through under gravity. Add 500 µl of carrier buffer to each column to wash antigen further into the gel.

3. Pass 500 µl of the anti-ferritin-alkaline phosphatase conjugate diluted in carrier buffer through each column.

4. Wash the excess through using carrier buffer.

5. Add 500 µl of substrate solution (2 mg of pNPP per ml of substrate buffer) to each column and incubate at room temperature. The timing of substrate incubation should be started upon addition of substrate to the first column.

6. Transfer the columns to clean test tubes (10 x 100 mm) and elute pNP product using 1 ml of carrier buffer. To ensure that the timing of incubation of substrate within each column is precise, elution buffer should be added to the batch of columns in the same sequence as the addition of substrate. Therefore, if one assumes that the speed of pipetting substrate approximates the speed of pipetting elution buffer, the time of incubation of substrate should be the same within each column.

7. Pipette 100 µl of the eluate in each tube to a microtitre well and read their absorbances at 405 nm using the Dynatech MR7000.
4 Results
4 Results

4.1 Conventional Two-Site Sandwich ELISA for Ferritin

4.1.1 Comparison of antibody concentrations for immobilisation to solid phase

a) Standard curves were prepared at various anti-ferritin antibody concentrations:
   1.63, 3.25, 6.5, 13, 26 µg/ml. Immobilisation was carried out overnight.

b) Ferritin antigen concentrations: 0, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 µg/l.

c) Anti-ferritin-alkaline phosphatase conjugate concentration: 0.098 µg/ml.

d) Each standard curve was prepared in triplicate.

Table 4.1.1 Comparison of coating antibody concentrations: results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean absorbance at 405 nm (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Primary antibody concentration (µg/ml)</strong></td>
</tr>
<tr>
<td></td>
<td>1.63</td>
</tr>
<tr>
<td>1,000</td>
<td>0.541</td>
</tr>
<tr>
<td>500</td>
<td>0.495</td>
</tr>
<tr>
<td>250</td>
<td>0.535</td>
</tr>
<tr>
<td>125</td>
<td>0.552</td>
</tr>
<tr>
<td>62.5</td>
<td>0.484</td>
</tr>
<tr>
<td>31.25</td>
<td>0.396</td>
</tr>
<tr>
<td>15.6</td>
<td>0.269</td>
</tr>
<tr>
<td>7.8</td>
<td>0.182</td>
</tr>
<tr>
<td>3.9</td>
<td>0.131</td>
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<tr>
<td>1.95</td>
<td>0.094</td>
</tr>
<tr>
<td>Blank</td>
<td>0.061</td>
</tr>
</tbody>
</table>
Figure 4.1.1 Comparison of antibody concentrations for immobilisation to solid phase.
4.1.2 Comparison of conjugate antibody concentrations

a) Standard curves were prepared using various anti-ferritin-alkaline phosphatase conjugate concentrations: 0.049, 0.065, 0.098, 0.196, 0.49 µg/ml.

b) Anti-ferritin antibody concentration used for coating solid phase: 6.5 µg/ml. Antibody immobilisation was carried out using an overnight incubation.

c) Ferritin antigen concentrations: 0, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 µg/l.

d) Each standard curve was prepared in triplicate.

Table 4.1.2 Comparison of conjugate antibody concentrations: results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean absorbance at 405 nm (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conjugate antibody concentration (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>0.049</td>
</tr>
<tr>
<td>1,000</td>
<td>0.345</td>
</tr>
<tr>
<td>500</td>
<td>0.366</td>
</tr>
<tr>
<td>250</td>
<td>0.396</td>
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<tr>
<td>125</td>
<td>0.378</td>
</tr>
<tr>
<td>62.5</td>
<td>0.330</td>
</tr>
<tr>
<td>31.25</td>
<td>0.273</td>
</tr>
<tr>
<td>15.6</td>
<td>0.233</td>
</tr>
<tr>
<td>7.8</td>
<td>0.201</td>
</tr>
<tr>
<td>3.9</td>
<td>0.170</td>
</tr>
<tr>
<td>1.95</td>
<td>0.152</td>
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<tr>
<td>Blank</td>
<td>0.141</td>
</tr>
</tbody>
</table>
Figure 4.1.2 Comparison of conjugate antibody concentrations.
4.1.3 Reduction of the incubation time for the immobilisation of anti-ferritin antibody to solid phase

a) Two standard curves were prepared using primary antibody coated at 4°C overnight (O/N) and at 4°C for 2 h.

b) Anti-ferritin antibody concentration: 6.5 μg/ml.

c) Ferritin antigen concentrations: 0, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 μg/l.

d) Concentration of conjugate antibody: 0.196 μg/ml.

e) Each standard curve was prepared in triplicate.

Table 4.1.3 Reduction of incubation time for coating antibody to solid phase: results

<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Mean absorbance at 405 nm (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation conditions</td>
</tr>
<tr>
<td></td>
<td>4°C O/N</td>
</tr>
<tr>
<td>1,000</td>
<td>1.195</td>
</tr>
<tr>
<td>500</td>
<td>1.207</td>
</tr>
<tr>
<td>250</td>
<td>1.212</td>
</tr>
<tr>
<td>125</td>
<td>1.041</td>
</tr>
<tr>
<td>62.5</td>
<td>0.774</td>
</tr>
<tr>
<td>31.25</td>
<td>0.527</td>
</tr>
<tr>
<td>15.6</td>
<td>0.339</td>
</tr>
<tr>
<td>7.8</td>
<td>0.200</td>
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<tr>
<td>3.9</td>
<td>0.129</td>
</tr>
<tr>
<td>1.95</td>
<td>0.095</td>
</tr>
<tr>
<td>Blank</td>
<td>0.057</td>
</tr>
</tbody>
</table>
Figure 4.1.3 Reduction of the incubation time for the immobilisation of anti-ferritin antibody to solid phase.
4.1.4 Precision and limit of detection (L.O.D.)

a) Each ferritin standard and blank (0.975-500 μg/l) was analysed in replicates of twelve.

b) Anti-ferritin antibody was coated for 2 h at 4°C using a concentration of 6.5 μg/ml.

c) Anti-ferritin-alkaline phosphatase conjugate concentration: 0.196 μg/ml.

d) The limit of detection was estimated from the standard curve as the concentration of ferritin giving an absorbance corresponding to the absorbance of the blank plus three standard deviations.

Table 4.1.4 Determination of precision and detection limit: results

<table>
<thead>
<tr>
<th>Conc. (μg/l)</th>
<th>Mean abs_{405 nm}</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1.203</td>
<td>0.036</td>
<td>2.99</td>
</tr>
<tr>
<td>250</td>
<td>1.239</td>
<td>0.037</td>
<td>2.99</td>
</tr>
<tr>
<td>125</td>
<td>0.974</td>
<td>0.042</td>
<td>4.31</td>
</tr>
<tr>
<td>62.5</td>
<td>0.707</td>
<td>0.029</td>
<td>4.10</td>
</tr>
<tr>
<td>31.25</td>
<td>0.487</td>
<td>0.036</td>
<td>7.39</td>
</tr>
<tr>
<td>15.6</td>
<td>0.272</td>
<td>0.015</td>
<td>5.51</td>
</tr>
<tr>
<td>7.8</td>
<td>0.168</td>
<td>0.009</td>
<td>5.36</td>
</tr>
<tr>
<td>3.9</td>
<td>0.125</td>
<td>0.011</td>
<td>8.80</td>
</tr>
<tr>
<td>1.95</td>
<td>0.104</td>
<td>0.006</td>
<td>5.77</td>
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<tr>
<td>0.975</td>
<td>0.091</td>
<td>0.007</td>
<td>7.69</td>
</tr>
<tr>
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<td>0.081</td>
<td>0.007</td>
<td>8.64</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = ≤1.95 μg/l or $4.33 \times 10^{-12}$ mol/l (4.33 x $10^{-16}$ mol)
Table 4.1.5 Determination of precision and detection limit: raw data

<table>
<thead>
<tr>
<th>Conc. (µg/l)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>500</td>
<td>1.231</td>
<td>1.149</td>
<td>1.154</td>
<td>1.238</td>
<td>1.219</td>
<td>1.196</td>
<td>1.223</td>
<td>1.152</td>
<td>1.176</td>
<td>1.226</td>
<td>1.238</td>
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<td>1.286</td>
<td>1.191</td>
<td>1.200</td>
<td>1.243</td>
<td>1.270</td>
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<td>125</td>
<td>1.032</td>
<td>0.953</td>
<td>0.913</td>
<td>0.931</td>
<td>0.962</td>
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<td>1.029</td>
<td>0.947</td>
<td>0.962</td>
<td>0.950</td>
<td>1.039</td>
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<td>62.5</td>
<td>0.741</td>
<td>0.668</td>
<td>0.743</td>
<td>0.757</td>
<td>0.691</td>
<td>0.689</td>
<td>0.714</td>
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<td>0.721</td>
<td>0.673</td>
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<td>0.699</td>
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<td>31.25</td>
<td>0.571</td>
<td>0.472</td>
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<td>0.519</td>
<td>0.478</td>
<td>0.481</td>
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<td>0.503</td>
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<td>0.272</td>
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<td>0.269</td>
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<td>0.167</td>
<td>0.160</td>
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<td>0.164</td>
<td>0.171</td>
<td>0.173</td>
<td>0.181</td>
<td>0.175</td>
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<td>0.150</td>
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<td>3.9</td>
<td>0.136</td>
<td>0.140</td>
<td>0.126</td>
<td>0.117</td>
<td>0.133</td>
<td>0.122</td>
<td>0.123</td>
<td>0.119</td>
<td>0.137</td>
<td>0.129</td>
<td>0.114</td>
<td>0.104</td>
</tr>
<tr>
<td>1.95</td>
<td>0.111</td>
<td>0.109</td>
<td>0.103</td>
<td>0.095</td>
<td>0.109</td>
<td>0.103</td>
<td>0.105</td>
<td>0.099</td>
<td>0.111</td>
<td>0.108</td>
<td>0.098</td>
<td>0.094</td>
</tr>
<tr>
<td>0.975</td>
<td>0.107</td>
<td>0.088</td>
<td>0.096</td>
<td>0.090</td>
<td>0.097</td>
<td>0.087</td>
<td>0.087</td>
<td>0.083</td>
<td>0.094</td>
<td>0.096</td>
<td>0.087</td>
<td>0.080</td>
</tr>
<tr>
<td>Blank</td>
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<td>0.084</td>
<td>0.076</td>
<td>0.090</td>
<td>0.087</td>
<td>0.091</td>
<td>0.082</td>
<td>0.070</td>
<td>0.084</td>
<td>0.081</td>
<td>0.078</td>
<td>0.070</td>
</tr>
</tbody>
</table>
Figure 4.1.4 Standard curve.
Figure 4.1.5 Precision profile.
4.2 Development of the Affinity-Column-Mediated ELISA

4.2.1 Preparation of a standard curve

a) Freshly prepared columns were used. Coupling antibody concentration: 500 µg per gram of gel.

b) Ferritin antigen concentrations: 0, 31.25, 62.5, 125, 250, and 500 µg/l.

c) Conjugate concentration: 0.98 µg/ml.

d) Volume of carrier buffer used to remove residual label: 5 ml.

e) Substrate incubation time: 30 min.

Table 4.2.1 Standard curve: results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1.550</td>
</tr>
<tr>
<td>250</td>
<td>0.961</td>
</tr>
<tr>
<td>125</td>
<td>0.580</td>
</tr>
<tr>
<td>62.5</td>
<td>0.348</td>
</tr>
<tr>
<td>31.25</td>
<td>0.220</td>
</tr>
<tr>
<td>0</td>
<td>0.106</td>
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</table>
Figure 4.2.1 Column ELISA: standard curve.
4.2.2 Comparison of coupling antibody concentrations

a) Standard curves were prepared using three different coupling antibody concentrations: 1,000 µg/g, 500 µg/g, and 250 µg/g.

b) Freshly prepared columns were used.

c) Ferritin antigen concentrations: 0, 31.25, 62.5, 125, 250, and 500 µg/l.

d) Conjugate concentration: 0.98 µg/ml.

e) Volume of carrier buffer used to remove residual label: 5 ml.

f) Substrate incubation time: 30 min.

Table 4.2.2 Comparison of coupling antibody concentrations: results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coupling antibody concentration (µg/g)</td>
</tr>
<tr>
<td></td>
<td>250</td>
</tr>
<tr>
<td>500</td>
<td>1.202</td>
</tr>
<tr>
<td>250</td>
<td>0.706</td>
</tr>
<tr>
<td>125</td>
<td>0.417</td>
</tr>
<tr>
<td>62.5</td>
<td>0.273</td>
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<tr>
<td>31.25</td>
<td>0.167</td>
</tr>
<tr>
<td>0</td>
<td>0.082</td>
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</table>
Figure 4.2.2 Comparison of coupling antibody concentrations.
4.2.3 Comparison of conjugate antibody concentrations

a) Standard curves were prepared using three different conjugate antibody concentrations: 1.96, 0.98, and 0.49 μg/ml.

b) Freshly prepared columns were used. Coupling antibody concentration: 500 μg/g.

c) Ferritin antigen concentrations: 0, 31.25, 62.5, 125, 250, and 500 μg/l.

d) Volume of carrier buffer used to remove residual label: 5 ml.

e) Substrate incubation time: 15 min.

Table 4.2.3 Comparison of conjugate antibody concentrations: results

<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conjugate antibody concentration (μg/ml)</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>500</td>
<td>0.408</td>
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<td>250</td>
<td>0.269</td>
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<td>125</td>
<td>0.192</td>
</tr>
<tr>
<td>62.5</td>
<td>0.149</td>
</tr>
<tr>
<td>31.25</td>
<td>0.110</td>
</tr>
<tr>
<td>0</td>
<td>0.082</td>
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</tbody>
</table>
Figure 4.2.3 Comparison of conjugate antibody concentrations.
4.2.4 Reduction of buffer volume used for removal of excess conjugate antibody

a) Various volumes of carrier buffer were compared for their ability to remove excess conjugate antibody from the column.

b) To assess the efficacy of each buffer volume, conjugate antibody was added to a column, the column was washed, and pNPP substrate was added. Therefore, any absorbance reading would represent conjugate antibody non-specifically bound.

c) Freshly prepared columns were used. Coupling antibody concentration: 500 µg/g.

d) Conjugate concentration: 1.96 µg/ml.

e) Substrate incubation time: 30 min.

Table 4.2.4 Reduction of washing buffer volume: results

<table>
<thead>
<tr>
<th>Volume of buffer (ml)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
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<tr>
<td>0.5</td>
<td>0.229</td>
</tr>
<tr>
<td>1</td>
<td>0.133</td>
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<tr>
<td>2</td>
<td>0.107</td>
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<tr>
<td>3</td>
<td>0.098</td>
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<td>0.087</td>
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<tr>
<td>1 + 1</td>
<td>0.078</td>
</tr>
<tr>
<td>1 + 2</td>
<td>0.080</td>
</tr>
<tr>
<td>1 + 2 + 2</td>
<td>0.089</td>
</tr>
</tbody>
</table>
4.2.5 Efficacy of regeneration in removing bound antigen and label

a) Three standard concentrations, 1,050, 2,100, and 4,200 µg/l, plus a blank were assayed in triplicate.

b) Freshly prepared columns were used. Coupling antibody concentration: 500 µg/g.

c) Conjugate concentration: 1.96 µg/ml.

d) Washing volume for removal of excess conjugate: 1 + 1 ml.

e) Substrate incubation for 10 min.

f) To regenerate the columns, 1 ml of regeneration buffer was introduced into each column, followed by 2 ml of carrier buffer. This cycle was repeated two more times.

g) After regeneration, all columns were used to assay blanks using the conjugate concentration of 1.96 µg/ml, a washing volume of 1 + 1 ml, and a substrate incubation time of 30 min.

Table 4.2.5 Efficacy of regeneration: results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Absorbance at 405 nm</th>
<th>Absorbance at 405 nm post regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>2</td>
</tr>
<tr>
<td>4,200</td>
<td>&gt;3.00</td>
<td>&gt;3.00</td>
</tr>
<tr>
<td>2,100</td>
<td>2.690</td>
<td>2.690</td>
</tr>
<tr>
<td>1,050</td>
<td>1.521</td>
<td>1.510</td>
</tr>
<tr>
<td>0</td>
<td>0.081</td>
<td>0.082</td>
</tr>
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</table>
4.3 Validation of the Affinity-Column-Mediated ELISA

4.3.1 Final assay conditions - Supplementary to general protocol (Section 3.2.5)

a) Coupling antibody concentration: 500 μg/g.

b) Conjugate concentration: 1.96 μg/ml.

c) Washing volume for removal of excess conjugate: 1 + 1 ml.

d) Substrate incubation: 30 min.

e) Regeneration using three cycles of 1 ml of regeneration buffer, followed by 2 ml of carrier buffer.

4.3.2 Determination of the limit of detection and working range

a) Ferritin standards ranging from 4.1-525 μg/l and a blank were assayed in replicates of five.

b) The limit of detection was estimated from the standard curve as the concentration of ferritin giving an absorbance corresponding to the absorbance of the blank plus three standard deviations.

c) The working range was derived from the precision profile.

d) To assess the effect of the regeneration process on the detection limit and on the working range, the assay was performed a further nine times using the same columns.
### Table 4.3.1 Determination of the limit of detection and working range: results run 1

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean abs$\scriptstyle_{405\text{ nm}}$ (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.320</td>
<td>0.016</td>
<td>0.069</td>
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<tr>
<td>262.5</td>
<td>1.450</td>
<td>0.020</td>
<td>1.379</td>
</tr>
<tr>
<td>131.2</td>
<td>0.926</td>
<td>0.031</td>
<td>3.348</td>
</tr>
<tr>
<td>65.6</td>
<td>0.543</td>
<td>0.004</td>
<td>0.737</td>
</tr>
<tr>
<td>32.8</td>
<td>0.349</td>
<td>0.010</td>
<td>2.875</td>
</tr>
<tr>
<td>16.4</td>
<td>0.221</td>
<td>0.004</td>
<td>1.810</td>
</tr>
<tr>
<td>8.2</td>
<td>0.166</td>
<td>0.002</td>
<td>1.205</td>
</tr>
<tr>
<td>4.1</td>
<td>0.130</td>
<td>0.003</td>
<td>2.308</td>
</tr>
<tr>
<td>0</td>
<td>0.095</td>
<td>0.002</td>
<td>2.105</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 0.7 µg/l or $1.55 \times 10^{-12}$ mol/l ($3.11 \times 10^{-16}$ mol)

**Figure 4.3.1 Standard curve: run 1.**
Figure 4.3.2 Precision profile: run 1.
Table 4.3.2 Determination of the limit of detection and working range: results run 2

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean abso.405nm (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.964</td>
<td>0.033</td>
<td>1.113</td>
</tr>
<tr>
<td>262.5</td>
<td>2.038</td>
<td>0.051</td>
<td>2.502</td>
</tr>
<tr>
<td>131.2</td>
<td>1.244</td>
<td>0.029</td>
<td>2.331</td>
</tr>
<tr>
<td>65.6</td>
<td>0.766</td>
<td>0.015</td>
<td>1.958</td>
</tr>
<tr>
<td>32.8</td>
<td>0.474</td>
<td>0.012</td>
<td>2.532</td>
</tr>
<tr>
<td>16.4</td>
<td>0.296</td>
<td>0.010</td>
<td>3.378</td>
</tr>
<tr>
<td>8.2</td>
<td>0.200</td>
<td>0.002</td>
<td>1.000</td>
</tr>
<tr>
<td>4.1</td>
<td>0.152</td>
<td>0.002</td>
<td>1.316</td>
</tr>
<tr>
<td>0</td>
<td>0.099</td>
<td>0.004</td>
<td>4.040</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.0 µg/l or 2.22 x 10^-12 mol/l (4.44 x 10^-16 mol)

Figure 4.3.3 Standard curve: run 2.
Figure 4.3.4 Precision profile: run 2.
Table 4.3.3 Determination of the limit of detection and working range: results run 3

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean $\text{abs}_{405\text{nm}}$ (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.101</td>
<td>0.038</td>
<td>1.809</td>
</tr>
<tr>
<td>262.5</td>
<td>1.324</td>
<td>0.038</td>
<td>2.870</td>
</tr>
<tr>
<td>131.2</td>
<td>0.752</td>
<td>0.023</td>
<td>3.059</td>
</tr>
<tr>
<td>65.6</td>
<td>0.466</td>
<td>0.003</td>
<td>0.644</td>
</tr>
<tr>
<td>32.8</td>
<td>0.283</td>
<td>0.002</td>
<td>0.707</td>
</tr>
<tr>
<td>16.4</td>
<td>0.190</td>
<td>0.005</td>
<td>2.632</td>
</tr>
<tr>
<td>8.2</td>
<td>0.140</td>
<td>0.001</td>
<td>0.714</td>
</tr>
<tr>
<td>4.1</td>
<td>0.119</td>
<td>0.001</td>
<td>0.840</td>
</tr>
<tr>
<td>0</td>
<td>0.096</td>
<td>0.003</td>
<td>3.125</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.6 µg/l or $3.56 \times 10^{-12}$ mol/l ($7.11 \times 10^{-16}$ mol)

*Figure 4.3.5 Standard curve: run 3.*
Figure 4.3.6 Precision profile: run 3.
Table 4.3.4 Determination of the limit of detection and working range: results run 4

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean absorbance (nm=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.076</td>
<td>0.030</td>
<td>1.445</td>
</tr>
<tr>
<td>262.5</td>
<td>1.236</td>
<td>0.045</td>
<td>3.641</td>
</tr>
<tr>
<td>131.2</td>
<td>0.729</td>
<td>0.025</td>
<td>3.429</td>
</tr>
<tr>
<td>65.6</td>
<td>0.438</td>
<td>0.008</td>
<td>1.826</td>
</tr>
<tr>
<td>32.8</td>
<td>0.275</td>
<td>0.005</td>
<td>1.818</td>
</tr>
<tr>
<td>16.4</td>
<td>0.183</td>
<td>0.003</td>
<td>1.639</td>
</tr>
<tr>
<td>8.2</td>
<td>0.143</td>
<td>0.001</td>
<td>0.699</td>
</tr>
<tr>
<td>4.1</td>
<td>0.119</td>
<td>0.002</td>
<td>1.681</td>
</tr>
<tr>
<td>0</td>
<td>0.095</td>
<td>0.001</td>
<td>1.053</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 0.6 µg/l or $1.33 \times 10^{-12}$ mol/l ($2.67 \times 10^{-16}$ mol)

**Figure 4.3.7 Standard curve: run 4.**
Figure 4.3.8 Precision profile: run 4.
### Table 4.3.5 Determination of the limit of detection and working range: results run 5

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean absorbance at 405 nm (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.307</td>
<td>0.065</td>
<td>2.818</td>
</tr>
<tr>
<td>262.5</td>
<td>1.417</td>
<td>0.075</td>
<td>5.293</td>
</tr>
<tr>
<td>131.2</td>
<td>0.761</td>
<td>0.015</td>
<td>1.971</td>
</tr>
<tr>
<td>65.6</td>
<td>0.464</td>
<td>0.015</td>
<td>3.233</td>
</tr>
<tr>
<td>32.8</td>
<td>0.232</td>
<td>0.006</td>
<td>2.586</td>
</tr>
<tr>
<td>16.4</td>
<td>0.169</td>
<td>0.004</td>
<td>2.367</td>
</tr>
<tr>
<td>8.2</td>
<td>0.133</td>
<td>0.002</td>
<td>1.504</td>
</tr>
<tr>
<td>4.1</td>
<td>0.115</td>
<td>0.001</td>
<td>0.870</td>
</tr>
<tr>
<td>0</td>
<td>0.096</td>
<td>0.003</td>
<td>3.125</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.9 µg/l or $4.22 \times 10^{-12}$ mol/l ($8.44 \times 10^{-16}$ mol)

![Standard curve: run 5](image)

**Figure 4.3.9 Standard curve: run 5.**
**Table 4.3.7** Determination of the limit of detection and working range: results run 6

<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Mean abs\textsubscript{405 nm} (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.489</td>
<td>0.058</td>
<td>2.330</td>
</tr>
<tr>
<td>262.5</td>
<td>1.547</td>
<td>0.042</td>
<td>2.715</td>
</tr>
<tr>
<td>131.2</td>
<td>0.919</td>
<td>0.048</td>
<td>5.223</td>
</tr>
<tr>
<td>65.6</td>
<td>0.536</td>
<td>0.009</td>
<td>1.679</td>
</tr>
<tr>
<td>32.8</td>
<td>0.329</td>
<td>0.009</td>
<td>2.736</td>
</tr>
<tr>
<td>16.4</td>
<td>0.212</td>
<td>0.003</td>
<td>1.415</td>
</tr>
<tr>
<td>8.2</td>
<td>0.155</td>
<td>0.003</td>
<td>1.935</td>
</tr>
<tr>
<td>4.1</td>
<td>0.130</td>
<td>0.005</td>
<td>3.846</td>
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<tr>
<td>0</td>
<td>0.102</td>
<td>0.004</td>
<td>3.922</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.8 μg/l or $4 \times 10^{-12}$ mol/l ($8 \times 10^{-16}$ mol)

**Figure 4.3.11** Standard curve: run 6.
Figure 4.3.12 Precision profile: run 6.
Table 4.3.8 Determination of the limit of detection and working range: results run 7

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean abs$_{405\text{nm}}$ (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.894</td>
<td>0.039</td>
<td>1.348</td>
</tr>
<tr>
<td>262.5</td>
<td>1.967</td>
<td>0.029</td>
<td>1.474</td>
</tr>
<tr>
<td>131.2</td>
<td>1.183</td>
<td>0.038</td>
<td>3.212</td>
</tr>
<tr>
<td>65.6</td>
<td>0.685</td>
<td>0.011</td>
<td>1.606</td>
</tr>
<tr>
<td>32.8</td>
<td>0.416</td>
<td>0.007</td>
<td>1.683</td>
</tr>
<tr>
<td>16.4</td>
<td>0.256</td>
<td>0.007</td>
<td>2.734</td>
</tr>
<tr>
<td>8.2</td>
<td>0.179</td>
<td>0.004</td>
<td>2.235</td>
</tr>
<tr>
<td>4.1</td>
<td>0.142</td>
<td>0.004</td>
<td>2.817</td>
</tr>
<tr>
<td>0</td>
<td>0.102</td>
<td>0.002</td>
<td>1.961</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 0.6 µg/l or $1.33 \times 10^{-12}$ mol/l ($2.67 \times 10^{-16}$ mol)

Figure 4.3.13 Standard curve: run 7.
Figure 4.3.14 Precision profile: run 7.
Table 4.3.8 Determination of the limit of detection and working range: results run 8

<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Mean abs_{405 nm (n=5)}</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.489</td>
<td>0.111</td>
<td>4.460</td>
</tr>
<tr>
<td>262.5</td>
<td>1.552</td>
<td>0.080</td>
<td>5.155</td>
</tr>
<tr>
<td>131.2</td>
<td>0.833</td>
<td>0.011</td>
<td>1.321</td>
</tr>
<tr>
<td>65.6</td>
<td>0.483</td>
<td>0.007</td>
<td>1.449</td>
</tr>
<tr>
<td>32.8</td>
<td>0.301</td>
<td>0.003</td>
<td>0.997</td>
</tr>
<tr>
<td>16.4</td>
<td>0.198</td>
<td>0.003</td>
<td>1.515</td>
</tr>
<tr>
<td>8.2</td>
<td>0.147</td>
<td>0.002</td>
<td>1.361</td>
</tr>
<tr>
<td>4.1</td>
<td>0.119</td>
<td>0.002</td>
<td>1.681</td>
</tr>
<tr>
<td>0</td>
<td>0.092</td>
<td>0.002</td>
<td>2.174</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.0 μg/l or 2.22 \times 10^{-12} \text{mol/l} (4.44 \times 10^{-16} \text{mol})

Figure 4.3.15 Standard curve: run 8.
Figure 4.3.16 Precision profile: run 8.
Table 4.3.9 Determination of the limit of detection and working range: results run 9

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean abs&lt;sub&gt;405 nm&lt;/sub&gt; (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.523</td>
<td>0.043</td>
<td>1.704</td>
</tr>
<tr>
<td>262.5</td>
<td>1.496</td>
<td>0.031</td>
<td>2.072</td>
</tr>
<tr>
<td>131.2</td>
<td>0.885</td>
<td>0.005</td>
<td>0.565</td>
</tr>
<tr>
<td>65.6</td>
<td>0.519</td>
<td>0.014</td>
<td>2.697</td>
</tr>
<tr>
<td>32.8</td>
<td>0.291</td>
<td>0.001</td>
<td>0.344</td>
</tr>
<tr>
<td>16.4</td>
<td>0.200</td>
<td>0.003</td>
<td>1.500</td>
</tr>
<tr>
<td>8.2</td>
<td>0.157</td>
<td>0.002</td>
<td>1.274</td>
</tr>
<tr>
<td>4.1</td>
<td>0.126</td>
<td>0.003</td>
<td>2.381</td>
</tr>
<tr>
<td>0</td>
<td>0.104</td>
<td>0.002</td>
<td>1.923</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.2 µg/l or 2.67 x 10<sup>-12</sup> mol/l (5.33 x 10<sup>-16</sup> mol)

**Figure 4.3.17 Standard curve: run 9.**
Figure 4.3.18 Precision profile: run 9.
Table 4.3.10 Determination of the limit of detection and working range: results run 10

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Mean absorbance at 405 nm (n=5)</th>
<th>S.D. (A.U.)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.233</td>
<td>0.095</td>
<td>4.254</td>
</tr>
<tr>
<td>262.5</td>
<td>1.321</td>
<td>0.031</td>
<td>2.347</td>
</tr>
<tr>
<td>131.2</td>
<td>0.804</td>
<td>0.032</td>
<td>3.980</td>
</tr>
<tr>
<td>65.6</td>
<td>0.469</td>
<td>0.020</td>
<td>4.264</td>
</tr>
<tr>
<td>32.8</td>
<td>0.272</td>
<td>0.004</td>
<td>1.471</td>
</tr>
<tr>
<td>16.4</td>
<td>0.182</td>
<td>0.003</td>
<td>1.648</td>
</tr>
<tr>
<td>8.2</td>
<td>0.144</td>
<td>0.004</td>
<td>2.778</td>
</tr>
<tr>
<td>4.1</td>
<td>0.123</td>
<td>0.004</td>
<td>3.252</td>
</tr>
<tr>
<td>0</td>
<td>0.094</td>
<td>0.003</td>
<td>3.191</td>
</tr>
</tbody>
</table>

From the graph, the L.O.D. = 1.3 µg/l or $2.89 \times 10^{-12}$ mol/l ($5.78 \times 10^{-16}$ mol)

Figure 4.3.19 Standard curve: run 10.
Figure 4.3.20 Precision profile: run 10.
4.3.3 Assessment of within-run precision

a) Three serum pools (A, B, & C), representing different levels of the working range, were assayed in replicates of ten using freshly prepared columns.

b) Standard ferritin concentrations: 0, 8.2, 16.4, 32.8, 65.6, 131.25, 262.5, and 525 μg/l.

Table 4.3.11 Within-run precision: standard curve results

<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Absorbance at 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>2.761</td>
</tr>
<tr>
<td>262.5</td>
<td>1.606</td>
</tr>
<tr>
<td>131.25</td>
<td>1.002</td>
</tr>
<tr>
<td>65.6</td>
<td>0.601</td>
</tr>
<tr>
<td>32.8</td>
<td>0.362</td>
</tr>
<tr>
<td>16.4</td>
<td>0.236</td>
</tr>
<tr>
<td>8.2</td>
<td>0.148</td>
</tr>
<tr>
<td>0</td>
<td>0.095</td>
</tr>
</tbody>
</table>
Figure 4.3.21 Within-run precision: standard curve.
Table 4.3.12 Within-run precision: results for serum pools A, B, and C

<table>
<thead>
<tr>
<th>Pool A (7-18 μg/l)</th>
<th>Pool B (57-167 μg/l)</th>
<th>Pool C (257-596 μg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Absorbance</em>&lt;sub&gt;405 nm&lt;/sub&gt;</td>
<td>Conc. (μg/l)</td>
<td><em>Absorbance</em>&lt;sub&gt;405 nm&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.153</td>
<td>8.7</td>
<td>0.766</td>
</tr>
<tr>
<td>0.156</td>
<td>8.9</td>
<td>0.803</td>
</tr>
<tr>
<td>0.158</td>
<td>9.1</td>
<td>0.727</td>
</tr>
<tr>
<td>0.167</td>
<td>9.9</td>
<td>0.767</td>
</tr>
<tr>
<td>0.152</td>
<td>8.6</td>
<td>0.718</td>
</tr>
<tr>
<td>0.148</td>
<td>8.2</td>
<td>0.775</td>
</tr>
<tr>
<td>0.156</td>
<td>8.9</td>
<td>0.771</td>
</tr>
<tr>
<td>0.167</td>
<td>9.9</td>
<td>0.769</td>
</tr>
<tr>
<td>0.153</td>
<td>8.7</td>
<td>0.770</td>
</tr>
<tr>
<td>0.156</td>
<td>8.9</td>
<td>0.799</td>
</tr>
</tbody>
</table>

| Mean | 8.98 | 90.20 | 350.60 |
| S.D. (μg/l) | 0.54 | 4.10 | 16.98 |
| C.V. (%) | 6.01 | 4.55 | 4.84 |
4.3.4 Assessment of between-run precision

a) The three serum pools (A, B, & C) were assayed singly for a further nine assays using the same columns used in the assessment of within-run precision.

b) Standard ferritin concentrations: 0, 5.5, 11, 22, 43.75, 87.5, 175, 350, and 525 μg/l.

c) The mean concentrations from the assessment of within-run precision were also used in the calculations of between-run precision.

Table 4.3.13 Between-run precision: results for serum pools A, B, and C

<table>
<thead>
<tr>
<th>Assay</th>
<th>Measured concentration (μg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool A</td>
</tr>
<tr>
<td>Within-run</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.98</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>9.5</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
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<td>10.5</td>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>10.5</td>
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<tr>
<td>Mean (μg/l)</td>
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<td>S.D. (μg/l)</td>
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<tr>
<td>C.V. (%)</td>
<td>10.76</td>
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</table>
4.3.5 Analytical recovery

a) Human serum devoid of ferritin was spiked with known amounts of ferritin standard and analysed by the present assay on columns regenerated once.

b) Standard ferritin concentrations: 0, 4.1, 8.2, 16.4, 32.8, 65.6, 131.25, 262.5, and 525 µg/l.

Table 4.3.14 Analytical recovery: standard curve results

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/l)</th>
<th>Absorbance at 405 nm</th>
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<td>16.4</td>
<td>0.217</td>
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<td>8.2</td>
<td>0.142</td>
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<td>4.1</td>
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Table 4.3.15 Analytical recovery: results

<table>
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<th>Absorbance at 405 nm</th>
<th>Ferritin concentration (µg/l)</th>
<th>Recovery (%)</th>
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<tr>
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<td>Expected</td>
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<td>1.913</td>
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<td>1.402</td>
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<td>185</td>
</tr>
<tr>
<td>0.748</td>
<td>87</td>
<td>93</td>
</tr>
<tr>
<td>0.652</td>
<td>73</td>
<td>67</td>
</tr>
<tr>
<td>0.435</td>
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<td>47</td>
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<td>0.261</td>
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</tr>
<tr>
<td>0.119</td>
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Figure 4.3.22 Recovery study: standard curve.
4.3.6 Linearity

a) Three human serum samples (A, B, & C) with high ferritin concentrations were diluted in the standard matrix, i.e., carrier buffer, and were analysed using the present assay.

b) Standard ferritin concentrations: 0, 4.1, 8.2, 16.4, 32.8, 65.6, 131.25, 262.5, and 525 µg/l.

c) Freshly prepared columns were used.

Table 4.3.16 Linearity study: standard curve results

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<th>Ferritin concentration (µg/l)</th>
<th>Absorbance at 405 nm</th>
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<td>1.664</td>
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<td>131.25</td>
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<td>65.6</td>
<td>0.533</td>
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<tr>
<td>32.8</td>
<td>0.314</td>
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<tr>
<td>16.4</td>
<td>0.204</td>
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<td>8.2</td>
<td>0.139</td>
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<td>4.1</td>
<td>0.111</td>
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Figure 4.3.23 Linearity study: standard curve.
Table 4.3.17 Linearity study: results for samples A, B, and C

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<th>Dilution</th>
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<th>Sample B</th>
<th></th>
<th>Sample C</th>
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<td>Absorbance_{405}</td>
<td>Measured</td>
<td>Absorbance_{405}</td>
<td>Measured</td>
<td>Absorbance_{405}</td>
<td>Measured</td>
</tr>
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<td>440</td>
<td>2.809</td>
<td>498</td>
<td>2.705</td>
<td>476</td>
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<tr>
<td>1/2</td>
<td>1.461</td>
<td>224</td>
<td>1.650</td>
<td>260</td>
<td>1.574</td>
<td>246</td>
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<tr>
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<td>1.008</td>
<td>142</td>
<td>1.185</td>
<td>172</td>
<td>1.129</td>
<td>162</td>
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<td>0.703</td>
<td>91</td>
<td>0.751</td>
<td>99</td>
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<td>97</td>
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<td>63</td>
<td>0.509</td>
<td>62</td>
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<td>1/15</td>
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<td>33</td>
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<td>0.127</td>
<td>6.4</td>
<td>0.132</td>
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Figure 4.3.24 Linearity of column ELISA.
Figure 4.3.25 Parallelism of column ELISA.
4.3.7 Correlation study

a) Thirty serum samples were measured using the present assay and the results were compared to those obtained using the chemiluminescence immunoassay on the ACS:180 (Ciba Corning Diagnostics, Medfield, MA, USA).

b) Samples with high ferritin values were diluted 1/2.

c) The columns were previously used seven times.

d) Standard ferritin concentrations: 0, 8.2, 16.4, 32.8, 65.6, 131.25, 262.5, and 525 µg/l.

Table 4.3.18 Correlation study: standard curve results

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<th>Ferritin concentration (µg/l)</th>
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**Figure 4.3.26** Correlation study: standard curve.
<table>
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<th>Sample no.</th>
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<th>Conc. (μg/l)</th>
<th>Observed</th>
<th>Expected</th>
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Figure 4.3.27 Correlation curve.
4.3.8 Amplification of signal

a) In an attempt to increase the sensitivity of the assay, a standard curve was prepared using a modification of the amplification system for alkaline phosphatase detection (Self, 1985; Moss et al., 1985; Stanley et al., 1985; Johannsson et al., 1986; Dhahir et al., 1992).

b) Once excess conjugate antibody was removed, 500 µl of substrate (0.2 mmol βNADP per litre of amplifier substrate buffer) was added to each column and the columns were incubated at room temperature for 20 min.

c) The columns were transferred to clean test tubes (10 x 100 mm) and the NAD product was eluted using 1 ml of carrier buffer.

d) Eluate (50 µl) from each column was pipetted into a well on a microtitre plate.

e) Subsequently, 50 µl of amplifier (2.5 mmol INT, 675 units of alcohol dehydrogenase, and 1,620 units of diaphorase per litre of amplifier buffer) was added to each well and the plate was incubated for 5 min at room temperature.

f) The reaction was stopped using 50 µl of 2 mol/l H₂SO₄ and the absorbance of each well was read at 490 nm.

g) Standard ferritin concentrations: 0, 0.5, 1, 2, 4.1, 8.2, 16.4, 32.8, 65.6, 131.25, 262.5, and 525 µg/l.

h) The assay volume used was 50 µl.

i) The columns had been previously used thirteen times.
<table>
<thead>
<tr>
<th>Ferritin concentration (μg/l)</th>
<th>Absorbance at 490 nm</th>
</tr>
</thead>
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<td>4.1</td>
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Figure 4.3.28 Amplification of enzyme label: standard curve.
5 Discussion
Discussion

Immunoassays are renowned for their specificity, sensitivity, and reliability (Ekins, 1985). However, probably their greatest limiting factor is the requirement for prolonged incubations, resulting in assays taking several hours to complete. With this limitation in mind, the objective of this work was to develop and validate a flow-through two-site sandwich ELISA, which eliminates the requirement for lengthy incubations of antigen and antibody, while still maintaining the sensitivity and reliability associated with conventional immunoassay. System simplicity and reusability were also of consideration.

To ensure that the antibodies used could recognise their specific antigen, a conventional two-site sandwich ELISA was developed using 96-well microtitre plates as solid phase. Anti-ferritin antibody immobilised onto the walls of the plastic wells was used to capture ferritin antigen, which was then detected using the anti-ferritin-alkaline phosphatase conjugate. Subsequently, bound conjugate was detected using \( p \)-nitrophenyl phosphate as substrate. Therefore, the assay was equivalent to assays belonging to Group 4 of Gosling’s classification (Gosling, 1990). This development was not intended to constitute a full or comprehensive optimisation, but it was merely intended to provide proof of antigen recognition by both antibodies and, thus, their suitability for use in the column-based immunoassay.

The starting point with any solid-phase immunoassay is the determination of the optimum conditions for biomolecule immobilisation. Probably, the most convenient and indeed the most popular medium is the plastic microtitre plate. For example, in 1990 plastic microtitre plate technology could be found in 70% of all new solid-phase
immunoassays. Their popularity is most probably due to the large batches of assays that can be carried out using automated instruments purposefully designed for microtitre plates, e.g., pipettors, washers, and readers. Therefore, for the purposes of this study, it was decided to use polystyrene microtitre plates supplied by Nunc as solid phase.

As discussed earlier (Section 1.8), biomolecules may be bound to surfaces either through covalent attachment or via passive adsorption. By far the easiest method to coat the microtitre plate is to do so directly by passive adsorption to the surface of the plastic (Kemeny, 1992). Since a large surface area is needed to immobilise biomolecules in this manner, the binding capacity of polystyrene is approximately 100-200 ng IgG/cm² (Gibbs 1995a). However, it has been shown that irradiation of the polystyrene surface can increase the binding capacity to between 400 and 500 ng IgG/cm² (Gibbs 1995a). According to Gibbs (1995a), the mechanism of immobilisation in this case is by passive adsorption through hydrophobic and ionic interactions. Gibbs (1995a) also states that the latter require a smaller portion of the molecule be in contact with the surface, thus increasing the binding capacity. A negative aspect of passive adsorption is that <3.0% of the binding sites of monoclonal antibodies and approximately 5-10% of those of polyclonal antibodies are capable of binding antigen (Butler et al., 1992). However, in the interests of simplicity, passive adsorption was used in this study to coat the anti-ferritin antibody to the solid phase.

According to Kemeny (1992), the optimum concentration for coating antibodies to plastic microtitre plates by passive adsorption typically lies between 1 and 10 µg/ml. In the literature, concentrations between 0.4 and 10 µg/ml have been quoted for adsorbing antibody to plastic microtitre plates and tubes (Krachmalnicoff et al., 1990; Papoian et al., 1992; Noé et al., 1992; Aubin et al., 1997; Dhahir et al., 1992; Munro
and Stabenfeldt, 1984). At higher concentrations, molecules tend to bind loosely, forming multiple layers, which are unstable and peel off during the assay. A comparison of anti-ferritin antibody concentrations in this study (Section 4.1.1) revealed that there was little difference between antibody concentrations of 1.63 to 26 μg/ml in binding ferritin antigen up to approximately 30 μg/l. It was therefore evident from the results that an increase in antibody concentration beyond 1.6 μg/ml would not improve on the lower limit of detection, but it would permit the assay of higher concentrations of ferritin with greater sensitivity, and without an associated increase in background noise. In fact, the results showed that antibody binding was at its greatest when a concentration of 26 μg/ml was used, a finding which may be considered somewhat contradictory to the findings of previous studies (e.g. Aubin et al., 1997). Such contradiction may be due to a larger binding capacity of the Nunc Maxisorp plates used as compared to conventional systems. Therefore, higher antibody concentrations are required to obtain maximum binding and a confluent monolayer. Alternatively, the nature of the coating buffer used (0.05 mol/l carbonate, pH 9.6) may have influenced the affinity of the antibodies for the plastic surface. Further study may have shown different pH coating buffers to be more effective, e.g., phosphate, pH 7.4 or acetate, pH 5.0. In any case, for further work it was decided to immobilise using 6.5 μg of antibody dissolved in 1 ml of 0.05 mol/l carbonate buffer, pH 9.6, simply because it gave a satisfactory curve and was more economical to use than a concentration of 26 μg/ml.

Generally, signals obtained in two-site sandwich immunoassay increase in proportion to increasing labelled antibody concentration, until a point is reached at which further increases in label serve only to increase non-specific binding. Antibody in this situation
is in excess. To determine the optimum anti-ferritin-alkaline phosphatase conjugate concentration for use in this system, a series of standard curves were prepared using various concentrations of conjugate antibody (Section 4.1.2). As expected, the steepness of the curves increased with increasing label concentration and without an undesirable increase in the background signal. Best results were obtained using a concentration of 0.49 µg/ml, however, further increases in concentration may have proven advantageous. In any case, it was decided to use the sub-optimal concentration of 0.196 µg/ml for further study.

For the previous studies, antibody immobilisation was carried out using an overnight incubation at 4°C. Since such a procedure had an obvious disadvantage, it was attempted to reduce the time of incubation from overnight to 2 h. The time taken to coat a microtitre plate not only depends on the concentration of coating solution, but also on the temperature at which it is carried out (Kemeny, 1992). Generally, with increasing temperature the time of incubation can be reduced, however, it has been shown that 90% of maximum binding occurs with 2-6 h, even at 4°C (Pesce et al., 1977). Results presented here (Section 4.1.3) appear to be in agreement with this finding, since a reduction of the incubation time from overnight to 2 h produced standard curves which differed only slightly.

Of importance also are the incubation conditions used. To achieve maximum signal sizes and to minimise drifts in results, it is usual to incubate for sufficient time to allow reactions to achieve or approach equilibrium (Micallef and Ahsan, 1994). Generally, the higher the incubation temperature, the shorter the times required. In addition, antigen-antibody reactions normally take place at between pH 6.0 and 9.0 (Kemeny, 1992), so it obviously important to use an incubation/assay buffer within this pH range. Therefore, for this study, incubation of antigen and conjugate antibody were carried
separately in a 0.05 mol/l phosphate buffered saline, pH 7.2, for 1 h at 37°C. No attempt was made to determine the times required to achieve maximal binding, since it was assumed that all reactions were close to equilibrium under such incubation conditions. Moreover, for most antigens and antibodies near maximal binding occurs within 2 h (Kemeny, 1992).

The final assay showed acceptable within-run precision for standards with concentrations ranging from 0.975 to 500 µg/l and gave a detection limit of 1.95 µg/l, equivalent to 4.33 x 10^{-12} mol/l or 8.67 x 10^{-16} mol per 100 µl assay volume. However, this sensitivity was achieved using standards prepared in buffer and would likely be higher if real (serum) samples were used. Although the system was far from fully optimised and lacked a complete validation study, it demonstrated the suitability of the antibody pair for use in the column-mediated ELISA, together with the potential sensitivity attainable.

Column-based flow-through immunoassay has the distinct advantage over conventional immunoassay systems in that it does not necessitate prolonged incubations of antibody and antigen in order to achieve binding (de Alwis and Wilson, 1985; Lejeune et al., 1990; Hage and Kao, 1991; Freytag et al., 1984; Janis and Regnier, 1989; Cassidy et al., 1992; Nilsson et al., 1994; Kronkvist et al., 1997). Since the nature of the chromatographic process ensures a rapid contact between agonist (in mobile phase) and the solid-phase ligand in excess, with minimal reliance on diffusion, the reaction rate between macromolecules is actually improved (Lejeune et al., 1990). In addition, mathematical models have shown that agonist binding by a sufficient excess of solid-phase ligand can reach 100% irrespective of the agonist concentration or ligand affinity (Lejeune et al., 1990).
With these benefits in mind, it was attempted to apply the principles of the conventional two-site sandwich immunoassay to a flow-through format using columns loaded with a sufficient excess of anti-ferritin antibody. Theoretically, such an immunoreactor would be used to immobilise ferritin antigen passing through the column in the mobile phase. Any bound ferritin would subsequently be detected using a mobile-phase conjugate antibody, followed by pNPP substrate to quantitate the bound label. However, the final addition would have to be accompanied by an incubation period so that pNP product may be allowed to form within the column. Once the product has formed, it would have to be eluted from the column before absorbance readings could be taken.

To obtain a sufficient excess of ligand (antibody) within the column, one needs to select a coupling medium with a high binding capacity. For this purpose, it was decided to use CNBr-activated Sepharose 4B. Indeed Sepharose has several characteristics which made it attractive for use in this setting. Firstly, it has a high binding capacity, enabling coupling of up to 60 mg of α-chymotrypsin/ml of drained gel (CNBr-activated Sepharose 4B Instructions, Pharmacia-Biotech, Uppsala, Sweden). In addition, the coupling procedure is very simple and safe, requires no specialised equipment, and can be completed in a matter of hours. The gel is stable within a pH range of 2-11 (CNBr-activated Sepharose 4B Instructions, Pharmacia-Biotech, Uppsala, Sweden), thus permitting regeneration of the solid phase using a low pH elution. In addition, Sepharose has a low non-specific binding (Nilsson et al., 1994).

Of consideration also was the type of column used. To keep the assay on a small scale, disposable polystyrene columns capable of holding gel volumes of between 0.5 and 2 ml were used. When packed as directed (Figure 5.1), the columns have a unique stop-flow action. Aqueous solution applied to a column will automatically stop at the disc
positioned above the gel when the liquid level reaches it, so the column gel will not dry out if left unattended. Also, the nature of the system ensures that the quantity of solution applied to the column will always be equal to the amount of eluate. The volume of gel decided to pack the columns was the minimum permitted, i.e., 0.5 ml. Following the design of the column, the general assay protocol was formulated (Section 3.2.5). For the preparation of the standards and conjugate antibody, it was decided to use a 50 mmol/l Tris-HCl buffer, pH 7.4, containing 0.5 mol/l NaCl (Nilsson et al., 1994). To make the standards resemble serum samples more closely and to prevent potential binding of labelled antibody to the support, BSA and Tween 20 were also added to the buffer at concentrations of 0.1% (w/v) and 0.035% (v/v), respectively. This buffer was also chosen for all washing steps. As was the case for the conventional ferritin immunoassay, a 0.1 mol/l Tris-HCl buffer, pH 9.8, containing 1.5 mol/l NaCl (Ternynck and Avrameas, 1990), was chosen for the incubation of the pNPP substrate within the column, since its pKa of 8.1 enables Tris to act as an excellent buffer for alkaline phosphatase (Tijssen, 1993). In addition, it was decided to carry out the incubation step at room temperature using a concentration of 2 mg/ml, since pNPP is more prone to spontaneous hydrolysis at higher temperatures (Tijssen, 1993) and at higher concentrations.

From the outset, the volumes to be used for sample, conjugate antibody, and substrate were set at 200 µl, 500 µl, and 500 µl, respectively. Ideally, to permit the measurement of low antigen concentrations one should use large volumes of sample, however, when dealing with serum or plasma there are obvious restrictions. A volume of 200 µl was deemed an acceptable volume to use. For conjugate antibody, volume seemed less important, since one can adjust the amount of antibody to suit. Finally, a volume of 500 µl was chosen for the addition of substrate so that it could penetrate the entire
volume of the gel (0.5 ml). It appeared that little was to be gained by using larger volumes.

**Figure 5.1 Polystyrene column packed with gel.**

To test the general assay protocol, a standard curve was prepared (Section 4.2.1) using a coupling concentration of 500 μg/g, together with a conjugate concentration of 0.98 μg/ml and a substrate incubation time of 30 min. The standard curve obtained was satisfactory and non-specific binding was negligible when using a buffer volume of 5 ml to remove residual label.

Pharmacia-Biotech recommends that approximately 5-10 mg of protein be used for coupling to 1 ml of gel, equivalent to 17.5-35 mg per gram of freeze-dried powder (since 1 g yields about 3.5 ml of gel). However, this seemed excessive for the purposes of this assay. To investigate if a coupling concentration of 500 μg/g was achieving maximal binding of antigen or if lower amounts of antibody could be used without affecting the signals obtained, standard curves were prepared on columns containing approximately 140 μg (1,000 μg/g), 70 μg (500 μg/g), and 35 μg (250 μg/g) of antibody (Section 4.2.2). The results demonstrated that a coupling concentration of 500 μg/g was indeed sub-optimal in that higher concentrations gave better signals.
without an associated increase in background. However, it would be hard to justify choosing a coupling concentration of 1,000 μg/g over a concentration of 500 μg/g on economic grounds, since only a slight increase in signals was obtained for a 100% increase in antibody.

While the chromatographic process gives very efficient binding of antigen rapidly by an excess of solid-phase antibody, the second step (addition of conjugate antibody) is run with a lack of stationary phase excess, since the stationary phase level is controlled by bound antigen (Lejeune et al., 1990). According to Lejeune et al. (1990), antigen binding by labelled antibody in column formats is not as efficient as binding by label in conventional systems, even when a significant excess of antibody is added in several additions. In fact, this procedure only served to increase non-specific binding in their assay, leading to a decreased signal-to-noise ratio and lower sensitivity. For this assay, conjugate antibody concentrations of 0.49 and 1.96 μg/ml were compared to the concentration of 0.98 μg/ml used initially (Section 4.2.3). As expected, increasing the concentration beyond 0.98 μg/ml produced higher signals, fortunately, without an associated increase in non-specific binding. Presumably, higher concentrations of label would produce even better signals, but economy and the risk of increased background noise eliminated their use. However, the latter aspect would be of a lesser concern, since the increased amount of residual label could be potentially be removed using larger volumes of carrier buffer during the washing step. For further assays a concentration of 1.96 μg/ml was used.

In addition, as part of assay development, an attempt was made to lower the volume of buffer used previously to remove excess antibody label in earlier assays (Section 4.2.4). This aspect of the procedure was of particular interest, since 5 ml of buffer passed
through the columns quite slowly, thus limiting the overall speed of the assay. The results showed that a 2 ml volume of buffer, added in two 1 ml washes was sufficient to remove any excess label.

Up to this point in assay development, all studies were performed using freshly prepared columns. However, the use of fresh columns for each assay proved expensive, since approximately 70 μg of antibody was used per column. The columns, however, were reusable. In order to permit the reuse of packed columns after assays, it was proposed to treat the columns with three cycles of 1 ml of 0.1 mol/l glycine buffer, pH 2.1, followed by 2 ml of carrier buffer. Having tested the efficiency of such a treatment (Section 4.2.5), it was evident that the process failed to remove antigen fully from the columns when concentrations greater than 1,050 μg/l were assayed. Therefore, another treatment would be required in this case.

Before one can measure samples using a developed assay, it is important that the assay be validated, most importantly for accuracy and precision. Firstly, it is useful to test for inconsistency in the analysis of standards, since any problems here will point to potential deficiencies in the precision and, therefore, accuracy of sample analysis. The resultant information may be represented graphically as a plot of coefficient of variation (y-axis) against the standard analyte concentration (x-axis). This precision profile allows one to determine the range of analyte concentrations that give acceptable levels of imprecision, for example, C.V. ≤10%. Incorporated into this analysis is the determination of the assay sensitivity, the operational definition being “the smallest detectable dose at which the assay response is significantly different from zero” (Micallef and Ahsan, 1994). This limit may be measured from the dose-response curve by extrapolation from the y-axis at the signal equal to the background noise plus three standard deviations (Brailly et al., 1994; Micallef and Ahsan, 1994).
To determine the working range and the limit of detection of the present assay, ferritin standards ranging from 4.1 to 525 μg/l, plus a blank, were assayed in replicates of five (Section 4.3.2). Imprecision values obtained were less than 5% for all concentrations tested, thus establishing the working range at 4.1 to 525 μg/l. The minimum detectable dose was calculated as 0.7 μg/l, equivalent to $1.56 \times 10^{-12}$ mol/l or $3.11 \times 10^{-16}$ mol. However, this was considered as only a potential sensitivity, since ferritin concentrations at this level were not assessed for (im)precision. Therefore, the detection limit had to be set at 4.1 μg/l or $9.11 \times 10^{-12}$ mol/l ($1.82 \times 10^{-15}$ mol). In any case, there is little need to assay concentrations below 4.1 μg/l, since this is well below the clinical decision limit for ferritin of 15 μg/l.

In addition, since it was intended to reuse the columns after assays, it was necessary to assess how the regeneration process would affect the detection limit and assay precision. The latter was of particular interest, since there was no guarantee that the process would affect all the columns to the same degree. Such an occurrence would inevitably lead to an increase in imprecision. However, results obtained for a further nine assays using the same columns allayed this initial fear (Section 4.3.2). The coefficients of variation for the standards rarely rose above 5%, indicating that the regeneration process affected all the columns to equivalent degrees. Moreover, the limit of detection remained at the subfemtomolar level throughout.

Although imprecision values obtained for standards over the ten assays were impressive, one could not assume that serum samples would respond with such consistency. Therefore, it was necessary to assess the within-run precision of serum samples. For this purpose, three serum pools, representing different levels of the working range, were assayed in replicates of ten using freshly prepared columns
(Section 4.3.3). As expected, precision matched that of standards at each of the levels tested. In addition, since the within-run precision of standards was maintained over nine regeneration processes (ten assays), it was assumed that the same would be true for the samples. In support of this assumption, favourable inter-assay precision values were obtained when the three serum pools were analysed singly for a further nine assays using the same columns (Section 4.3.4).

Standards for the present assay were prepared in a Tris-HCl buffer, pH 7.4, containing 0.1% BSA (w/v) and 0.035% Tween 20 (v/v). Due to obvious differences between the standard matrix and that of serum samples, there was no guarantee that serum samples would be measured accurately. The accuracy of the system was determined using recovery, linearity, and correlation studies. For analytical recovery, human serum devoid of ferritin was spiked with known amounts of ferritin standard and analysed using the present assay on columns used once previously (Section 4.3.5). The recovery values for the eight samples tested (5-480 μg/l) were 91-110%, with a mean of 101.9%, indicating that serum samples reacted with the system in the same way as standards. This conclusion was strengthened further by results of the linearity studies (Section 4.3.6). Three serum samples with high ferritin concentrations were diluted in the standard matrix and the dilutions of each sample, together with the neat serum, were assayed using freshly prepared columns. When the volume of sample was plotted on the x-axis against the measured concentration on the y-axis for each sample, no significant deviation from linearity was found. In addition, plotting the dilution (x-axis) against specific absorbance (y-axis) for the standard and the samples gave a series of parallel curves. However, according to Micallef and Ahsan (1994), the latter method can hide even pronounced effects. Finally, the assay’s ability to measure serum samples with accuracy was further highlighted by the results of the correlation study (Section
4.3.7). The measured ferritin concentrations obtained for thirty clinical samples showed excellent correlation ($r = 0.995$, $y = 1.084x - 1.388$) to results obtained using the chemiluminescence immunoassay performed on the Ciba Corning ACS:180.

The assay protocol includes an initial addition of a 3 ml volume of carrier buffer after storage solution (approx. 2 ml of dH$_2$O) has drained completely from the columns. However, this process is **ONLY** required when using columns directly from storage. When columns are to be used immediately after regeneration, the equilibration step can be excluded from the procedure, since the regeneration process leaves the columns ready to receive sample or standard. Consequently, the time of sample analysis is reduced slightly in such a case. The assay protocol also includes a lengthy 30-min incubation step for the detection of the bound alkaline phosphatase label. To overcome this limitation, one could use a substrate that is converted into a product detectable with a high degree of sensitivity, e.g., 4-methylumbelliferyl phosphate. Therefore, one could use a shorter substrate incubation time to achieve the same assay sensitivity. Alternatively, a label could be used that does not require an incubation step for its detection, e.g., fluorescent tag.

As a consequence, the final system permits the simultaneous measurement of up to forty samples and nine standards in approximately 1.5 h, with the potential to process up to 400 samples per day, if one allows approximately 60 min for the regeneration of the columns after each batch. Such a sample throughput compares very favourably with some of the flow-through systems described previously in Section 1.13. However, for the measurement of a single sample (or very small numbers of samples) all of these assays tend to be faster.

The HPIAC sandwich ELISA developed by de Alwis and Wilson (1985) requires only approximately 15 min for the analysis of a single sample. Subsequent to determination,
a further 15 min are required for immunoreactor regeneration and equilibration prior to measurement of another sample. Therefore, when measuring a large batch of samples one requires 30 min per analysis. For instance, to assay a batch of forty samples continuously would take in the region of 20 h. According to Janis and Regnier (1989), their DCIA has a similar sample throughput, requiring only 30 min per sample or 24 h for forty to fifty. The HPIAC system designed by Hage and Kao (1991) for the measurement of parathyroid hormone requires 1 h 6 min per sampling, allowing at least 1 h for pre-incubation of sample and labelled antibody and 6 min per plasma injection. Despite this pre-incubation, the authors state that up to 240 samples may be analysed per day. The flow-ELISA developed by Nilsson et al. (1993) permits the analysis of a single sample in 330 s or up to 260 samples in 24 h, while the displacement flow-injection assay for cortisol designed by Kronkvist et al. (1997) requires about 8 min per sampling, giving a daily throughput of approximately 180. Their competitive flow-injection assay has a sample throughput of twenty per hour, if one excludes the 1-h incubation of sample and labelled antibody prior to injection. If the timing of these incubations is staggered 3 min apart, one could potentially measure approximately 450 samples per day. The non-competitive ACMIA designed by Freytag et al. (1984) had a total analysis time of 18 min, however, since the number of columns analysed simultaneously was not stated, it is difficult to assess the sample throughput. In any case, even if only ten samples were analysed together every 18 min, the sample throughput would be thirty per hour or approximately 800 per day. However, by far the fastest assay with the greatest sample throughput was developed by Cassidy et al. in 1992. Using KICQA immunoassay, they were able to analyse samples for albumin in under 1 min, giving a potential sample throughput of up to 1,440 per day.
The detection limit of the present assay was set at $1.82 \times 10^{-15}$ mol, which is equivalent to $4.1 \, \mu g/l$ or $9.11 \times 10^{-12}$ mol/l when a 200 $\mu l$ assay volume is used. Such sensitivity compares very favourably with some of the flow-through immunoassays described earlier (Section 1.13). The HPIAC immunoassay developed by de Alwis and Wilson (1985) had the ability to measure 1 pmol of bovine IgG using a 30 $\mu l$ volume ($3.33 \times 10^{-8}$ mol/l), while Janis and Regnier (1989) measured 0.7 pmol of transferrin in a 20 $\mu l$ volume ($3.5 \times 10^{-8}$ mol/l) using DCIA. The ACMIA designed by Freytag et al. (1984) for digoxin and the KICQA immunoassay developed by Cassidy et al. for albumin could measure $5.11 \times 10^{-11}$ mol ($2.56 \times 10^{-7}$ mol/l) and $1.5 \times 10^{-14}$ mol, respectively. For Kronkvist et al. (1997), the best sensitivities were achieved when using the competitive flow-injection immunoassay for budesonide, which allowed measurement of 0.02 pmol ($8 \times 10^{-8}$ mol/l) using amperometric detection. However, in this case, assessment with real samples (serum/plasma) was neglected. While the sensitivities quoted fail to match the sensitivity achieved by the flow-through system presented here, others have succeeded in determining analytes at the attomolar level using column-based systems. For example, the HPIAC assay for parathyroid hormone designed by Hage and Kao (1991) measured as little as 16 amol in a 66 $\mu l$ assay volume ($2.4 \times 10^{-13}$ mol/l), while Lejeune et al. (1990) measured as little as 0.1 amol of human growth hormone using RACIA. It should be stated, however, that the latter achieved such a sensitivity using standards prepared in buffer and using a 3.25-h revelation time for the amplified assay of alkaline phosphatase activity. From a calibration curve prepared using the same amplification procedure (Section 4.3.8), it appeared that a 1 $\mu g/l$ sample could be measured with confidence using the present assay, even after thirteen regenerations of the columns. In addition, the sample volume

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used was only 50 μl, giving approximately a 16-fold increase in sensitivity ($1.82 \times 10^{15}$ mol vs $\sim 1.11 \times 10^{16}$ mol) in the same assay time.

An attractive feature of the present assay system is that the columns can be regenerated using a low pH buffer. This presents a deviation from the conventional immunoassay system where the solid phase tends to be used only once. However, such a disregard for economy seems foolish, since more than ten immunoassay cycles can be realised with the same antibody-coated microtitre plates (Ilchmann et al., 1990). Similarly, at least ten assays can be performed with the same columns without apparent adverse affects to the reliability and sensitivity of the system. However, there is no reason to doubt that a far greater number of assays could be performed, since both de Alwis and Wilson (1985) and Janis and Regnier (1989) were able to use their immunoreactors for at least 500 assays using similar elution buffers. Similarly, Hage and Kao (1991) achieved 200-250 injections per column, while Nilsson et al. (1994) were able to perform fifty assays, and in some cases several hundred assays, using the same immunoreactor. However, the system adopted by Kronkvist et al. (1997) potentially allows the immunoreactor to be reused for even more assays, since the columns do not require regeneration after each assay. Moreover, the enzyme-substrate reaction is carried out in a post-column reactor, thus the immunochemical equilibrium of the column is not disturbed by the substrate buffer used.

In conclusion, the affinity-column-mediated ELISA described here is suitable for use in the determination of ferritin in serum samples with concentrations below, within, and above the clinical decision limits. The assay shows a level of reliability and sensitivity comparable to the flow-through systems presented in the literature. The sensitivity, however, is far lower than that achieved elsewhere for ferritin determination using conventional immunoassay (Ishikawa et al., 1982; Hashida and Ishikawa, 1990). The
sample throughput of approximately 400 per day is higher than most of the flow-through systems described, however, for the analysis of a single sample or a small number of samples the latter are superior. In addition, since no lengthy incubations are required for reaction of antigen with its specific antibodies, the overall analysis time tends to be faster than conventional systems. However, the sample throughput of the latter can be well over 1,000 per day, especially if microtitre plate technology is used with automation. In addition, the same columns can be used over ten assays without affecting the integrity of the columns. Presumably, the columns could be used a greater number of times. Finally, the procedure is simple and can be potentially applied to the measurement of any analyte measurable through conventional immunoassay.
6 References
6 REFERENCES


Butler J.E., Ni L., Nessler R., Joshi K.S., Suter M., Rosenberg B., Chang J.,
antibodies adsorbed on polystyrene” *Journal of Immunological Methods* **150**:77-90

Cabral J.M.S., Kennedy J.F. 1991. “Covalent and co-ordination immobilization of
proteins” In: *Protein Immobilization. Fundamentals and Applications* (R.F. Taylor,
ed.), Marcel Dekker, Inc., New York, 73-138

fluorescence excitation transfer immunoassay for measurement of specific proteins”
*Journal of Immunological Methods* **86**:249-256

monitoring specific binding reactions with cofactor labeled ligands” *Analytical
Biochemistry* **72**:271-283

“Development of a urodilatin-specific antibody and radioimmunoassay for urodilatin in
human urine” *Clinical Chemistry* **43**:638-643

addition immunoassays using protein A affinity chromatography” *Analytical Chemistry*
**64**:1973-1977

Chlang C.S., Grove T., Cooper M., Cuan J., Kowalski A., Parcells K.,
Tsunokawa M., Rosenberg M., Arcuri E., Franklin S., Smith T., Debouck C.
antibodies” *Clinical Chemistry* **35**:946-952


Esser P. 1991. “Blocking agent and detergent in ELISA” In: *Nunc Bulletin* (No.9), Nunc Laboratories, Roskilde, Denmark


180


Gibbs J. 1995a. “Immobilization principles - Selecting the surface” In: ELISA Techniques Bulletin (No.1), Corning Costar Corporation, Kennebunk, Maine

Gibbs J. 1995b. “Optimizing the immobilization of protein and other biomolecules” In: ELISA Techniques Bulletin (No.2), Corning Costar Corporation, Kennebunk, Maine


186


Meyerhoff M.E., Duan C., Meusel M. 1995. “Novel nonseparation sandwich-type electrochemical enzyme immunoassay system for detecting marker proteins in undiluted blood” *Clinical Chemistry* **41**:1378-1384


Novotny M.V. 1996. “Capillary electrophoresis” Current Opinion in Biotechnology 7:29-34


7 Appendix
7 APPENDIX

7.1 Affinity-Column-Mediated Flow-Through ELISA for Ferritin

7.1.1 Summary and explanation of assay

The principles of affinity chromatography and two-site sandwich immunoassay are combined to produce a system capable of rapid (~ 1.5 h) and sensitive quantitation of ferritin in serum. In the assay, samples and standards are passed through separate columns containing immobilised anti-ferritin antibody, and bound antigen is subsequently detected using an anti-ferritin-alkaline phosphatase conjugate. To detect immobilised label, substrate solution is added and product is developed in the column at room temperature. Following elution of product from the column, the absorbance is measured and the columns are regenerated using a low pH elution.

7.1.2 Materials required

a) Carrier buffer: 50 mmol/l Tris-HCl, pH 7.4, containing 0.5 mol/l NaCl, 0.1% (w/v) BSA and 0.035% (v/v) Tween 20.

b) Substrate buffer: 0.1 mol/l Tris-HCl, pH 9.8, containing 1.5 mol/l NaCl.

c) Regeneration buffer: 0.1 mol/l glycine-HCl, pH 2.1.

d) Ferritin from human liver.

e) Mouse monoclonal IgG to human ferritin (Clone B8, Biomerieux, Lyon, France).

f) Monoclonal anti-human ferritin-alkaline phosphatase conjugate (Clone B8, Biomerieux, Lyon, France).
g) Immunoaffinity columns: Polystyrene columns (0.5-2.0 ml) packed with cyanogen bromide-activated Sepharose 4B (0.5 ml), to which anti-ferritin antibody has been coupled (500 µg/g).

h) p-Nitrophenyl phosphate (pNPP).

7.1.3 Test procedures

7.1.3.1 Using the immunoaffinity column

1. To use the column, remove the top cap first. This prevents bubbles being drawn into the gel. Next, remove the bottom cap and place the column in a suitable holder, e.g., 16 x 150 mm test tube. Allow the storage solution (approx. 2 ml of dH₂O) to drain completely from the column.

2. If air bubbles are entrapped in the gel, spin the column first at 1,000 g for 10 min. This procedure is important, since air bubbles significantly reduce flow rates.

7.1.3.2 Quantitation of ferritin

1. Equilibrate⁴ all columns with at least 3 ml of carrier buffer.

2. Pipette 200 µl of standards (ferritin dissolved in carrier buffer) or samples into separate columns and allow them to pass through under gravity. Add 500 µl of carrier buffer to each column to wash antigen further into the gel.

3. Pass 500 µl of the anti-ferritin-alkaline phosphatase conjugate diluted in carrier buffer (1.96 µg/ml) through each column.

4. Wash the excess through using 1 + 1 ml of carrier buffer.

⁴ Equilibration is only required when using columns directly from storage.
5. Add 500 μl of substrate solution (2 mg of pNPP per ml of substrate buffer) to each column and incubate at room temperature for 30 min. The timing of substrate incubation should be started upon addition of substrate to the first column.

6. Transfer the columns to clean test tubes (10 x 100 mm) and elute p-nitrophenol (pNP) product using 1 ml of carrier buffer. To ensure that the timing of incubation of substrate within each column is precise, elution buffer should be added to the batch of columns in the same sequence as the addition of substrate. Therefore, if one assumes that the speed of pipetting substrate approximates the speed of pipetting elution buffer, the time of incubation of substrate should be the same within each column.

7. Pipette 100 μl of the eluate in each tube to a microtitre well and read their absorbances at 405 nm.

8. To regenerate the columns, add 1 ml of regeneration buffer, followed by 2 ml of carrier buffer. Repeat this cycle two more times.