DEVELOPMENT OF A NOVEL DIAGNOSTIC SYSTEM BASED ON THE USE OF THE COMPACT DISC AS A PLATFORM FOR BIOLOGICAL ANALYSIS.

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By

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ABBREVIATIONS

AP	alkaline phosphatase
BCIP/NBT	5-Bromo-4-Chloro-3-Indoyl Phosphate/NitroBlue tetrazolium
BSA	bovine serum albumin
CD	compact disc
4C1N	4-Chloro-1-Napthol
CV	coefficient of variation
DAB	3,3'-Diaminobenzidine
DELFIA	Dissociation Enhanced Lanthaninide Fluorescence Immunoassay
DNP	dinitrophenol
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
FgD	human fibrinogen (fragment D)
FIA	fluoroimmunoassay
FITC	fluorescein isothiocyanate
β-Gal	β-Galactosidase
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HRP	horseradish peroxidase
HSA	human serum albumin
hTSH	human thyroid stimulating hormone
IFMA	immunofluorometric assay
IFN	Interferon-α-1
^{125}I-BSA-Ar36	^{125}I- labelled arsenate conjugated bovine serum albumin.

IRMA	immunoradiometric assay
OD	optical density
OPD	orthophenylenediamine dihydrochloride
PBS	phosphate buffered saline
PCR	polymerase chain reaction
POC	point of care
POCT	point of care testing
PSA	prostate-specific antigen
PVLA	polyvinylbenzyl lactonoylamide
RAST	radioallergosorbent
RIA	radioimmunoassay
RNase A	bovine ribonuclease A
TMB	3,3', 5,5'- Tetramethylbenzidine

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SUMMARY

The aim of this project was to develop a novel diagnostic system based on compact discs as a platform for biological analyses - in effect a diagnostic disc. This would allow a blood or serum sample to be tested against a panel of clinically relevant analytes or diseases thus aiding diagnosis and saving time. It would also allow such tests to be decentralised from a large testing laboratory and performed at the point of care such as in field hospitals, clinics and health centres. This has been developed initially for immunoassays with a view to moving away from the standard 96-well plates, where only one analyte can be tested per sample, to multianalyte testing on the same sample. This would then be read using compact disc technology in a specially adapted laser reader, producing optical density (O.D.) values. The initial aim of the project was to show that a panel of immunoassays could be performed in parallel without cross-reaction or cross-contamination. This would then be extended to a more clinically relevant panel for example, parasitic diseases.

The system was originally based on the use of sectors, which resembled miniaturised microplates. These contained 44 wells of approximately 3.5ul in volume. The sectors are held within a "spider" structure based on a compact disc. Each spider holds eight sectors giving a total of 352 wells. The ELISAs performed were initially as on a 96-well plate, the main difference being that due to the spinning of the disc while being read, a substrate had to be found that would produce an insoluble end-product. A basic ELISA was developed that could be used while the sectors and spider were being developed. Once the final structure had been determined the sectors were moulded from medical grade polystyrene. Work was then carried out on plain plastic discs while the sectors were being moulded; primarily to determine the sensitivity of insoluble 3,3', 5,5'-Tetramethylbenzidine (TMB) - a substrate not usually used in ELISAs. A novel washing procedure, called "flood and fill" was developed which allowed the wells to be washed simultaneously while the sectors were held in the spider. A multianalyte test was constructed using seven different ELISAs which involved detecting the complementary antibody to the following commercially available antigens, Human Serum Albumin, α-1-

Antitrypsin, α-1-Macroglobulin, Antithrombin III, Catalase, α-1-Antichymotrypsin and Plasminogen. There was no cross-reaction or cross-contamination between wells except for a low-level cross-reaction between anti-Antithrombin III and α-2-Macroglobulin. There was very low non-specific binding. The incubation times were greatly reduced with a total assay time of one hour, excluding washing. There was inter-sector and inter-disc variation in colour, which was reduced by pre-coating the sector with a silicone spray. Evaluation of colour was mainly by visual examination with the sectors due to problems with the development of the reader

There were two main problems associated with the sectors. The first was how to keep them in the spider while the disc was being read and the second was how to prevent the liquid left in the wells at the end of the assay from escaping the disc. There was a requirement for a "snap-up" mechanism to be added to the sector that, at the end of the assay, would both hold the sector in the spider and also create a seal to prevent the leakage of any liquid. This would have been prohibitively expensive and therefore a second prototype platform was developed. Earlier work had shown that the multianalyte assay could be performed on a compact disc without separate wells. A compact disc had a black label applied such that the pattern of wells was the same as the sector. The antigen and blocking agent had to be dried on to the wells to prevent "streaming", an effect whereby there was cross-contamination between wells and the blue colour produced at the end of the assay was not localised in the appropriate wells. It was found that the multianalyte ELISA could be performed in this way and at the end of the assay, the disc was simply allowed to air-dry and then inserted in the reader. The O.D. values were significantly higher with the black disc as compared to the sectors and it was used as the platform for the remainder of the project.

There was significant variation in O.D. values between wells and discs and this made it difficult to draw conclusions about assay sensitivity and the effect of changing assay conditions such as incubation times and temperatures. This was primarily due to the antigens being coated on to

the disc by hand-pipette (1μl per wells), thus introducing a large degree of error. In addition the insoluble TMB did not produce quantitative results as shown by with poor gradation in colour when the antigen and antibody concentrations were varied. Alternatives under consideration include blue bead conjugated second antibody or changing the signal entirely from colorimetric to fluorescence.

Future work will involve comparing results from the disc with those from microtitre plates using clinically defined sera and from there to constructing logical panels of immunoassays for example parasitic immunoassays that can be used in the field.

CHAPTER 1

INTRODUCTION

1. 1 Overall objectives in medical and veterinary diagnostics.

Medical diagnostics have been going through a period of rapid growth and change in the last decade due to the demands of scientists and clinicians for rapid and more informative tests, increasing scientific knowledge and improved information technology systems. The common trend has been the development of tests for the point of care (POC) whether that is in the home, health centre, hospital or field testing in developing countries. The aim of such tests is to provide reliable, rapid results without the need for highly trained laboratory staff thus aiding patient care and treatment. There are other aspects to point of care tests such as quality control that have to be considered but this is a rapidly growing area that many see as ultimately ending with much publicised the "lab-on-a-chip". This refers to the miniaturisation by microengineering of any chemical or biochemical process for the purpose of analysis, synthesis or research and development. Microengineering allows the precise and reproducible construction of structures ranging from simple channels to pumps complete with moving parts. Originally these were constructed on silicon chips but glass and most recently plastic have proved cheaper. Microengineering offers the benefit of reduced size including reduced diffusional distances for improved efficiency of for example, mixing and heat transfer. The commercial applications of "lab-on-a-chip" include environmental monitoring, diagnostics, laboratory measurement, and chemical production and drug discovery.

Immunoassays are widely used in medical diagnostics and scientific research. It is a highly sensitive and precise technique due to the highly specific interaction between antigen and antibody. As knowledge about this interaction has increased, especially at the molecular level, the sophistication and sensitivity of the immunoassay has also dramatically improved leading to automation and multianalyte testing, where a single sample is tested for more than one antigen or antibody.

The first attempt to automate immunoassays came with radioimmunoassays came in the late 1970's but these had limited throughput, reliability and cost-effectiveness[1]. Subsequent systems used non-isotopic labels and had improved analytical performance with increased sensitivity,

precision, dynamic range and clinical performance. Immunoassay analysers nowadays have features such as random access, bar coding, extensive menus and autodilution. There are currently over 25 immunoassay systems on the market using a range of spectrophotometric and electrochemiluminescent signals. Labels used include enzymes and chemluminescence with separation methods such as coated beads, tubes, wells and magnetic particles among others. Some analysers have test menus for over 75 analytes for tests such as thyroid function, fertility, cardiac, tumour markers, and drugs of abuse, toxicology, infectious disease and cytokines. By incorporating speciality testing into a routine analyser it was possible to connect the analyser to other high throughput instruments. The goal is the integration of immunoassays with other clinical systems potentially integrating pre- and post- analytical functions. For example, this could mean the incorporation of sample (serum or plasma) preparation and interpretation of results.

In this introduction I will give a history of the development of immunoassay and then how this can be modified for use at the point-of-care (POC). I will then describe how the microplate can be incorporated into a compact disc (CD). The work in this thesis will show how it is possible to use a modified CD player as an instrument for rapid analysis of immunoassay results. The use of compact disc technology would be appropriate as a new and innovative platform not least because of its ability to store and disseminate information. Multianalyte testing has always been a goal in immunodiagnostics. This has the advantage of testing for more than one analyte at the same time and has been increasingly incorporated into point of care and other immunoassay systems although not without initial problems. The development of this new system had two parts – the chemistry which will be discussed in this thesis, and a modified compact disc player and specific software for reading and interpretation of the chemistry on the disc which was developed by colleagues.

1.2 Development of immunoassays.

Immunodiagnostics development began in the late 1950's with semi-quantitative assays. These had progressed to fully quantitative assays by the early 1960's and by the late 1960's there was

the development of solid-phase technologies and sandwich assays. Hybridoma and monoclonal antibody technology in the 1970's advanced assay specificity. The 1980's saw an increasing variety of detection technologies decreasing detection limits while the 1990's have shown advances in automation thus reducing assay times[1]. Control of the antigen-antibody reaction by, for example molecular biology techniques to mutate the antibody binding site were used to optimise assay conditions. Detection limits have increased since the mid-1980's with assays detecting analytes at nanomole concentrations with reduced assay times. For example an assay for thyroid stimulating hormone has a detection range in the attomole to femtomole range in a time of 5-10 minutes. There have been theoretical claims of zeptomolar and below but this has not achieved clinical reliability. Immunosensors are devices that incorporate antigens or antibodies coupled to a signal transducer that detects the binding of a complementary species and will prove to be a novel analytical too especially in conjunction with simultaneous multianalyte methods and miniaturisation. Multianalyte testing was first developed in the late 1970's and early 1980's with the simultaneous measurement of thyroid hormones[2] and has continued with the ambient analyte technique by Ekins[3]. Nanotechnology where every atom is known and is in a selected position will produce very high performance system but there is no commercial product as yet

Microtitre plates are the traditional workhorse of the immunoassay, especially the 96-well plate. There are a number of disadvantages with this system including the expense of the plates, reagents and specific readers and the fact that they can only be performed in the laboratory. There has been increasing interest in developing diagnostic tests that can be used at the point-of-care whether that is in the health centre, intensive care unit or in the home. Tests of this type aim to produce qualitative or quantitative results as required in a fast and reliable manner, which would provide results that are meaningful for the user.

1.2.1 Radioimmunoassays.

Immunoassays belong to a class of assays known as ligand binding assays. This class of assay relies on the observation of the products of the binding reaction between an analyte and a specific binding reagent. In the case of immunoassays an antibody is directed against the antigenic sites on the analyte molecule. Earlier immunoassays were based on the direct observation of the antigen-antibody complex or "precipitate". The limitations of this technique provided the stimulus for the further development of immunoassays. The development of radioimmunoassay (RIA) took place in the mid-1950's independently in New York and London. Yalow and Berson[4,5,6] investigated the quantitative aspects of the reaction between insulin and antibody and the species specificity of available antisera[7]. They followed the disappearance of beef-pork insulin administered intravenously to rabbits. Paper electrophoresis showed the presence of ^{131}I-insulin and antibody complexes. Following this, beef, pork and horse were quantitatively measured by competitive inhibition using labelled and unlabelled insulin. The lack of pure human insulin prevented its use as an animal immunogen. However it was found that human insulin strongly cross-reacted with guinea pig anti-beef insulin antibodies. This provided a method of detecting and measuring human insulin at normal concentrations in the plasma of normal subjects. The results of these experiments therefore resulted in a way of measuring plasma insulin in humans[5]. RIA is based on determining the concentration of unknown unlabelled antigen by comparing its inhibitory effect on the binding of radioactively labelled antigen to specific antibody with inhibitory effects of known standards[8]. The RIA principle can be extended to other systems where there is a specific binding substance such as an enzyme or receptor. This was first applied to the use of thyroxine binding globulin as a specific receptor for measurement of serum thyroxine as described below with Ekins[9]. These radioisotopic techniques resulted in the concept of small molecules for example; vasopressin and oxytocin being antigenic in some species and also that equilibrium constants can be up to 10^{14}l mol^{-1}.

About the same time Ekins was working at the Middlesex Hospital Medical School in London on thyroid physiology. A patient in the department with a massive thyroid carcinoma was routinely treated over a year with massive doses of ^{131}I. Ekins observed that the radioactive material was protein-bound which was confirmed chromatographically. Simple electrophoresis showed that there were changes in distribution between labelled T4 in bound (i.e. thyroxine-binding globulin) and free (albumin-bound) moieties following addition of varying amounts of unlabelled T4. This enabled response curves to be constructed relating the observed distribution to unlabelled T4 concentration[3]. Previous work on the lack of analytical methods to measure hormone concentration in blood had shown that simple analysis of the laws of mass action produced equations, which allowed the dose-response curves in this situation to be predicted. Over the next two years blood samples from the thyroid carcinoma patient was tested. In addition, serum vitamin B12 was also assayed and the results published in 1962 as a description of "saturation analysis"[9,10]. This was a general procedure and therefore included all immunoassays and not just specifically RIAs.

By the late 1960's, RIA had become a major tool in hormone studies including their regulation, secretion and interrelationships and was important in the discovery of new forms of hormones in blood and tissues such as proinsulin and proparathyroid hormone[8]. It had become evident that the sensitivity and specificity of RIA meant that a large number of samples could be assayed using less than 1 ml of sample. Multiple samples could also be tested which meant that for the first time alterations in the circulating hormone could be followed after the administration of a stimulus for example, the effect of glucose feeding on plasma insulin levels over a period of time. Radioimmunoassay was first applied to measurement of peptide hormones in plasma because it provided the specificity and sensitivity to measure at the very low concentrations required (10^{-10}-10^{-12} M) in the presence of the much higher concentrations of plasma proteins. Such was the success of the technique that it was soon applied to the measurement of non-peptide hormones[11] which had the advantage of being at a higher concentration in plasma. The sensitivity, specificity and comparative ease of RIA meant that with the availability of reagents and equipment, many biologically significant substances could

be measured. This would otherwise have been very difficult or impossible[8]. Today radioimmunoassays are used in a large range of non-hormonal substances including viruses[12] and pharmacological agents[13].

The major milestones in immunoassay development are shown in Table 1.1. The next stage in immunoassay development was in the late 1960's with the development of the Immunoradiometric Assay (IRMA)[14]. In conventional RIAs the antigen for example, insulin is radioactively labelled and is present in excess and there is competition between labelled and unlabelled insulin for a limited amount of antibody in the sample (serum). In 1968, Miles and Hayes postulated that in order to obtain maximum sensitivity and precision, all the unknown should be reacted and the amount of product should be assayed by a procedure which gives a low background and shows changes in direct proportion to the change in product concentration. Standard RIA s did not fulfil these criteria because often large proportion of the unknown remains unreacted and a change in the amount of free or bound antigen (hormone) is measured against a large background giving a small relative change against possibly a large relative change in the amount of unlabelled antigen bound to antibody. The main difference between RIA and IRMA was the use of excess labelled antibodies converting all antigen to a labelled derivative meaning that the signal is detected against a theoretically zero background thus maximising the signal to noise ratio and improving detection limits[15]. In the early 1970's Addison and Hales coupled an additional antibody to a particle or other solid-phase producing a two-site IRMA or "sandwich" assay where the antigen forms a bridge between solid-phase antibody and a second labelled antibody to further improve sensitivity and precision[16]. The assertion that IRMA was more sensitive than conventional RIA was challenged in the early 1970's by Rodbard and Weiss[17], although in the early 1980's Ekins challenged this again regarding immunometric assays[18].

DATE	IMMUNOASSAY DEVELOPMENT
Mid-Late 1950's	1956, 1957 - Initial development of competitive radioligand assays.
Early 1960's	*Berson & Yalow, 1960 – Insulin RIA* *Ekins, 1960 - Thyroxine "Saturation Assay"*
Late 1960's - Early 1970's	Initial development of radiolabelled antibodies and "sandwich assays". *Wide, 1967 – Allergen Antibodies, "RAST" Test.* *Miles and Hales, 1968 – Insulin IRMA.*
Early 1970's	*Rubenstein, 1972 – Homogeneous drug assays, "EMIT".*
Mid-Late 1970's	Initial development of ultrasensitive immunoassays with high specific activity non-isotopic labels.
Early 1980's	*Ekins/Wallac collaboration – "Delfia" System, 1983.*
1990's	Initial development of microarray multianalyte assays. *Ekins, 1990,1991 – Microspot Immunoassay.* Development of "lab-on-a-chip" and other microarray systems.

Table 1.1 : Major developments in immunoassays from the late 1950's to the late 1990's.

At the same time as Miles and Hales were investigating IRMA, a similar technique was being demonstrated in the diagnosis of allergy. Wide developed the radioallergosorbent or RAST test for the detection of allergen-specific antibodies[19]. The allergen was coupled to an insoluble dextran polymer, which is added to serum. If allergen-specific antibodies are present they react with the allergen. The dextran is washed and ^{125}I-labelled antibodies added which bind to the anti-allergen antibodies on the dextran. The uptake of labelled antibodies is proportional to the amount of allergen antibodies in the sample.

The development of IRMAs was held back by the lack of purified antibodies for labelling and also solid phase antigens which were used to remove excess labelled antibody[15]. It was another 10-15 years before researchers and kit manufacturers were interested in this technique although this was also due to its perceived lack of sensitivity. In the mid-1970's the development of the hybridoma technique that led to monoclonal antibody production meant that large quantities of antibodies could be produced[20]. There was also an increase in label development producing radioisotopes with higher specific activities therefore increasing analytical sensitivity. The sensitivity of these assays is limited by the affinity and non-specific binding of the labelled antibody. The detection limit of RIA is limited mainly by the equilibrium constant (Ka) of the reaction and technical errors of reagent manipulation such as pipetting and separation. Antibody reactions rarely exceed a Ka of 10^{-12} mol l^{-1} and this is generally taken as a detection limit. The potential detection limits of RIA and IRMA are shown in Table 1.2. IRMA has similar constraints to RIA and has a potential sensitivity of 10^{-16} mol l^{-1} which is reduced to 10^{-14} mol l^{-1} in practice. RIA can potentially be used to measure any substance in a biological medium in the range 10^{-6}-10^{-14} mol l^{-1}. The precision in RIA is related to the negligible radioactive background in biological samples and the fact that disintegration is not related to factors in the biological medium for example, pH, ionic strength or colour.

Immunoassay	Potential Sensitivity (Ka)
Radioimmunoassay	$10^{-6} - 10^{-14}$ mol l^{-1}
Immunoradiometric Assay	10^{-16} mol l^{-1}

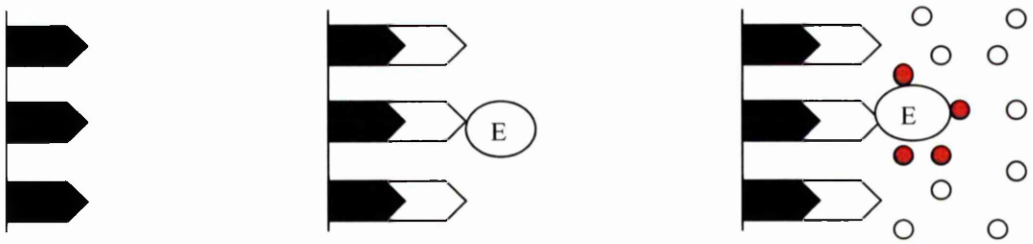
Table 1.2 : Potential detection limit of immunoassays.

Immunoassays can be classified by a number of criteria, which can lead to confusion. For the purpose of this work they will be described as reagent limited or reagent excess for either antigen or antibody. Reagent-limited assays are also called competitive assays where a limited amount of antibody is used which is insufficient to bind all the antigen. A fixed amount of labelled antigen competes with the sample antigen for the limited antibody. Reagent-excess assays are also called non-competitive or immunometric assays where antigen binds to an excess of antibody. A further important classification is the separation requirement of bound from free label. Heterogeneous immunoassays require a physical separation step for example, microtitre plates while in homogeneous immunoassays there is a detectable change in the label signal when antigen is bound compared to when it is free. Heterogeneous assays are usually more sensitive than homogeneous assays but there are some situations where their use is an advantage for example, assay is usually faster[21].

1.2.2 Enzyme immunoassays.

Radioimmunoassay had produced a technique for the sensitive and quantitative analysis of antigens and antibodies. However the main drawback was the use of radioisotopes themselves with the inherent health risk and waste disposal problem. Therefore there has always been a drive for alternative labels of high sensitivity and specificity without the associated radioisotope problems. The first alternative was enzymes. They had previously been used in immunohistochemistry[22] and immunoelectrophoresis[23]. These were first used in immunoassay in 1971 by Van Weeman and Schuurs[24] who used purified horseradish peroxidase conjugated

human chorionic gonadotropin (hCG) for an enzyme immunoassay (EIA) of both antigen and antibody. The early 1970's were a very important time in the development of enzyme immunoassay with several important papers being published in the period 1970-1972. Engvall and Perlmann used an alkaline phosphatase conjugated rabbit IgG to determine rabbit IgG concentration in a competition assay[25]. The sheep anti-rabbit Ig was conjugated to microcrystalline cellulose. The unlabeled rabbit IgG competes with labelled rabbit IgG for the cellulose-conjugated anti-rabbit IgG. This was found to be comparable to RIA with respect to sensitivity and precision. In the same year Engvall and Perman[26] used antibody-coated tubes to determine antigen concentration as shown in Figure 1.1. They applied the competition principle of RIA but used antibodies immobilised on polystyrene tubes making separation of free and bound antibody-antigen complexes easier.



1) Antibody attached to tube.

2) Incubate with enzyme-labelled antigen in presence or absence of standard antigen or unknown sample

3) Incubate with substrate.

Figure 1.1: Competitive immunoassay using immobilised antibody in tubes and enzyme-conjugated antigen.

This procedure produced assays with very high sensitivity, which was comparable to RIA and could also be used for antibody quantitation and was followed in 1972 by more work on this technique[27]. Antigen-coated tubes were incubated with antibody and then alkaline phosphatase -labelled anti-IgG. After washing the remaining enzyme is a measure of the amount of specific antibody in the serum. This was a non-competitive method similar in principle to the RAST technique described previously.

In 1972 Rubenstein produce a method for a homogeneous enzyme immunoassay for hapten detection based on steric hindrance to control enzyme activity[27]. The control of enzyme activity by antibodies is related to size of the enzyme substrate. When an antibody is attached to an enzyme-conjugated antigen, it sterically inhibits substrate access to the active site of the enzyme. This could also happen if a hapten is attached near the active site of the enzyme. The binding of a hapten-directed antibody may have the same effect as binding the antibody directly to the enzyme. Enzyme activity could therefore be regulated by the amount of antibody available for binding to enzyme-bound hapten. Free antibody could in turn be controlled by addition of free hapten, which would compete with enzyme-bound hapten for antibody binding sites. The enzyme activity of enzyme and antibody mix would be directly related to the amount of free hapten and could be used in an immunoassay. No separation is required and adding the unknown increases the competition for antibody binding sites therefore more enzyme-hapten is released and more substrate converted to product. The advantage of this type of homogenous assay is its simplicity, reagent stability and ability to be quantified. This work in the early 1970's resulted in most of the basic EIA techniques that have been developed and refined ever since. As work progressed, the use of enzyme linked immunosorbent assays (ELISA) were used to detect other substances such as serum proteins and infectious diseases. The main impact of ELISA was in the detection of antibodies as shown by Engvall[25]. Throughout the 1970's, the ELISA technique was extended to a wide range of antibodies and antigens for example, Rubella, Herpes Simples Virus, Salmonella O antigens, E. coli, Streptococcal M protein, Malaria and Schistosome antigens[28].

There are a number of advantages of using an enzyme as a label[1]. Of all the non-isotopic methods, enzymes are measurable by the greatest number of methods and also have the possibility of secondary amplification thus increasing assay sensitivity. Enzymes are therefore very versatile and can be used in a wide range of assay formats from the disposable point of care to the automated. However there a number of disadvantages of which the most important is the high molecular mass of the enzyme. Large enzyme-conjugates diffuse slowly and can have

a greater tendency to bind non-specifically. Enzymes are also more susceptible to inactivation by environmental factors. There are several advantages of enzyme immunoassays including: their sensitivity and wide applicability, the equipment required is relatively cheap and widely available, reagents are relatively cheap and have a long shelf-life, manipulations are simple, assays can be very rapid, a separation step may not be required, multiple simultaneous assays can be performed, there is the potential for automation and there are no radiation hazards[29].

The most common enzyme label is horseradish peroxidase (HRP), closely followed by alkaline phosphatase (AP). HRP has a high turnover number and is suitable for a range of diverse conjugation procedures. It also has a low molecular size compared to other enzymes and can be used in a range of sensitive assay systems including colorimetric, fluorometric, luminometric, electrochemical and photodensitometric (1). Gosling[30] showed that in 1990 enzymes as labels have increased in popularity from use in 20% of new assays to about 35%. HRP was used in half of all new assays while AP was used in a quarter of new assays. Enzymes can also be used as secondary labels to provide an amplification factor that results in accurate and sensitive detection[28]. For example fluorescein isothiocyanate (FITC) is used to prepare a primary label then the concentration of FITC-antigen or FITC-antibody is determined by enzyme-linked monoclonal antibody to FITC[31]. Another common system involves conjugating biotin to antigen, antibody or hapten as primary label and then an enzyme-linked avidin or streptavidin as a secondary label. Multienzyme complexes can be used to maximise the final signal by attaching multiple molecules of label to each immune complex to be detected. This is usually accomplished by the use of antibodies to the label. However these systems are hindered by steric effects of large label-containing complexes and high non-specific binding and are not really suitable for assays with low detection limits[1]. As an alternative to this, liposomes can be loaded with many enzymes as primary labels resulting in amplification when lysis of each liposome releases many enzyme molecules[32].

Enzyme activity can be measured by a variety of methods[1]. Perhaps the easiest method is by visual assessment where a change in colour density, shade, area, location indicates a positive reaction. These assays are qualitative or semi-quantitative and are commonly used in point-of care over the counter kits for example, pregnancy tests. Most are carried out on a paper strip or membrane with the final product being an insoluble precipitate on the solid-phase. Colorimetry is perhaps the most common method of measuring the enzyme activity. It is simple, well understood and adequate for most applications. Dedicated plate readers are used to measure the optical density of the final product and the limitations of the end-point detection is due to the limitations of colorimetry itself. These disadvantages of using colorimetry are not found when flourometric measurements are made to determine enzyme activity. Measurements of very small concentrations of pure fluorescent compounds are very precise because measurement is in a complete absence of light and a very sensitive light detection method can be used. Ishikawa[33] showed that using a fluorometric measuring system for HRP, β-Galactosidase and AP increased the detection limit from 25, 1000 and 10000 attomoles respectively with a colorimetric method to 5, 0.2 and 10 attomoles using a fluorescent method. Luminometry is most often used with HRP although several assays with AP and β-Galactosidase have been developed. Enhanced luminescence can be used where certain compounds are added to the assay buffer to enhance and prolong light emission from the reaction catalysed by the enzyme. In some cases this can increase light emission by one thousand times[1] and prolong emission for several minutes. The use of electrometry to determine enzyme activity has a number of advantages for example, cloudy or opaque solutions can be used and the detection electrode can be a tiny remotely located probe. The amplification of systems can also be accomplished by using multi-stage assay systems such as the one developed by Self in 1985. This system used the dephosphorylation of $NADP^+$ to NAD^+ and a specific redox reaction to amplify the reaction and is widely used in research and commercially. This system has been extended by Cook and Self in 1993 to incorporate fluorescent detection which extended the range of measurements to 1/1000 attomole i.e. 1 zeptomole of AP per microtitre well[34].

Enzyme labels can often be used in multianalyte systems of which there are two formats – multiple enzymes to distinguish between intermixed binding sites[1] or one enzyme against spatially different groups. With the former method, it is difficult to distinguish between even two enzyme labels colorimetrically. In addition, different enzymes required different reaction conditions, which have to be compromised in an enzyme mixture. In the latter method only the number of sites on the solid-phase limits the number of analytes. This has been developed for many commercial applications for example; MAST® system currently available commercially has 38 individual allergen-coated cellulose threads in a pipette-like chamber that measures picomole amounts of allergen-specific IgE. The binding of anti-human IgE – peroxidase conjugate to the threads is detected by a peroxidase-dependent enhanced luminescent reaction. New enzyme labels are being sought with improved thermal stability and high turnover numbers[35]. Although enzymes are the most popular of labels, there is a wide range of other non-isotope labels also in use such as fluorescent, chemiluminescent, and electrometric.

1.2.3 Fluoroimmunoassays.

Fluoroimmunoassays (FIA) involve labelling an antigen or antibody with a fluorescent molecule and during the late 1970's and 1980's became increasingly popular as an alternative to radioimmunoassays. Labelling with a fluorescent molecule allowed heterogeneous and homogeneous assays to be developed. The high sensitivity of the fluorescence measurement combined with its sensitivity to changes in environment makes it suitable for both heterogeneous assay development and especially homogeneous assays where no separation step is required. The main problem with this type of label is that its high sensitivity is reduced by the concomitant high background but this has been greatly reduced in the past decade by the development of solid phase separation systems, new fluorescent probes and new instruments that have resulted in lowered background and in sensitivity comparable to RIA and IRMA[36]. FIAs have become more widely applied with the introduction of solid-phase based "sandwich" assays characterised by an excess of antibody over antigen. This shortens incubation times and potentially increases sensitivity. The solid phase facilitates separation and the signal is directly

proportional to the amount of antigen[37]. There are four main problems when using fluorescence - light scattering, background fluorescence, quenching and inner filter effects. The most common fluorescent molecules used are organic in nature – fluorescein and rhodamine and their derivatives. Others used include the umbelliferones and the rare earth chelates. The isothiocyanate conjugate of fluorescein is most commonly used, FITC[38]. A number of alternatives to fluorescein and rhodamine are also being investigated including fluorescent microparticles, natural porphyrins and chlorophylls, the aim being long emission rates, large Stokes' shifts (the difference between absorbance and emission spectra) and long fluorescence decay times. In fluorimetry, the sample is detected against a zero background producing a much more sensitive procedure theoretically detecting to 10^{-14}M while colorimetric measurements can detect down to 10^{-8}M.

In metalloimmunoassay, antigen and antibody are labelled with a metal ion. After the reaction is completed, the metal ion is detected by a number of methods for example, atomic absorption spectrophotometer or fluorimetry. Fluorescent rare earth ions and their chelates have unique fluorescent properties, high detection sensitivity (10^{-14} mol l^{-1}) and have been widely applied in time–resolved fluorimetry. Rare earth ions for example Europium (Eu^{3+}) and Terbium (Tb^{3+}) form highly stable luminescent chelates with suitable organic ligands. Energy transfer follows light absorption by the ligand from the excited singlet state through its triplet state to the energy resonance levels of the lanthanide ion[39]. Luminescent lanthanides differ from other fluorescent molecules in that they have a much longer Stokes shift and decay times are much longer at 10^{-5} – 10^{-2} seconds. This longer lived fluorescence means that any background fluorescence from for example, serum has decayed. There are two different systems using time-resolved fluoroimmunoassays. The DELFIA (Dissociation Enhanced Lanthanide Fluorescence Immunoassay) system was developed by Wallac[40] while Diamandis[41,42] produced an alternative system based on time-resolved fluorescence measurement in the solid phase. This method uses biotinylated antibodies labelled with a streptavidin-europium chelator reagent.

Today, time-resolved fluorometry with lanthanide chelate labels is well established in diagnostics. It is increasingly used as a tool for drug screening in both heterogeneous and homogeneous formats. It combines temporal resolution with high spatial resolution, which should lead to miniaturisation, multi-labelling and increased sample throughput[43]. There has been increasing work on developing one-step time-resolved assays. Merio[44] used this format to measure free and total prostate-specific antigen. The development of new chelates meant that fluorescence could be read directly from the well in approximately 15 minutes making it ideal for automation. Similar one-step assays were tested with hCG, alpha-fetoprotein and progesterone. The detection limit was 0.5 nanomoles for progesterone[45]. Development of FIAs is certain to proceed with the development of rapid and simple homogeneous assays which are all well-defined, simple to use and rapid. Immunofluorometric assays (IFMA) using low background solid-phases reduces endogenous background fluorescence[46]. Assay sensitivity is often less than IRMA but there are the benefits of not using radioisotopes.

1.2.4 Chemiluminescent immunoassays.

Chemiluminescence is the emission of light that occurs as a result of a chemical reaction. There is no initial absorption of light as in fluorescence therefore measurements are made against a lower background giving greater sensitivity of detection[47]. There a several advantages of using chemiluminescence as a label in immunoassay. Sensitivities of attomole and sub-attomole level can be attained with the signal being generated in several seconds and remaining stable for several hours. The procedures are relatively simple and generally non-hazardous reagents are used[48] . Recent advances in molecular biology techniques have allowed the specific bioluminescent compounds to be synthesised and modified for use in immunoassays[45]. Among the non-bioluminescent systems perhaps the best known is luminol or isoluminol[49] which are often used with enzyme amplification systems for example, microperoxidase and haemin[50]. Since 1983 a range of enzymes have been used for example, alkaline phosphatase and horseradish peroxidase[48] but acridinium salts[51] are about the most successful as labels in immunoassays and are found in some commercial systems for example the ACS: 180

Immunoassay systems by Ciba Corning Diagnostics[47]. Immunoassays utilising these compounds as labels produce sensible robust assays for measuring direct chemiluminescence and usually use simple reagents. The use of enzyme amplification systems with chemiluminescence results in a highly sensitive system and some chemiluminescent immunoassays can yield higher specific activities than radioisotopes when used as direct labels in non-competitive assays and therefore could provide the basis for ultrasensitive non-competitive assay technologies[52].

1.2.5 Light-scattering immunoassays.

Light-scattering immunoassays are based on the formation of aggregates between antigen and antibody that are large enough to scatter light more than the reaction constituents. The polyvalent antigens react with the divalent antibodies to form a large complex leading to the formation of a precipitate, which depends on the colloidal stability of the constituents and their relative proportions. The reaction is monitored using nephelometry or turbidimetry. Other light-scattering immunoassay techniques include particle counting, ellipsometry and surface plasmon resonance spectroscopy[53]. Particle-enhanced immunoassays are widely used and have a number of benefits including the enhancement of sensitivity by increasing the relative light scattering signal. There are a number of different types of particles used in these assays including erythrocytes, metal sols (for example, gold, silver iodide etc) and latex particles. Light scattering immunoassays are used with a wide range of analytes but mainly in the detection of therapeutic drugs, drugs of abuse or hormones where the detection limit is in the nanometre range[54,55] and mainly uses turbidimetric monitoring. Protein assays using turbidimetry have been incorporated into clinical chemistry analysers and also developed for the point of care market in disposable cartridges[56,57].

1.2.6 Electrochemical immunoassays.

Electrochemical immunoassay measures the signal by utilising a simple current or voltage change and is often incorporated into biosensors as described below. Amperometric

measurement offers a way of increasing the dynamic range and sensitivity of an immunoassay as compared to spectrophotometric measurement[58]. It also has the advantage that because it is a non-optical system, whole blood samples can be used. Enzymes can be used as amplification labels with the enzyme monitored by the measurement of an electroactive product or substrate. This is the most common method although electroactive compounds themselves can also be use directly as labels[59]. This type of immunoassay has been important in the development of ultrasensitive, simplified and micro-multianalyte immunoassays. The latter involves photolithography and dry etching on silicon wafers[53] and silicon micromachining with the formation of micropumps and microelectrode arrays[60] and will result in the development of ultrasensitive, small-volume and simplified immunoassays.

1.2.7 Immunosensors.

Immunosensors are devices composed of antigens or antibodies coupled to a signal transducer, which detects the binding of a complementary species[61]. Methods include the detection of binding by a change in potential difference, current resistance, mass, heat or optical properties and can be used for real-time monitoring of the antigen-antibody interaction. Detection limits for a wide range of molecules is commonly from 10^{-9}-10^{-13} mol l^{-1} and are found in a few commercial where the antigen or antibody is immobilised on the transducer surface which converts the binding event to an electrical signal which is detected optically. There a variety of electronic, acoustic and optical techniques used to construct immunosensors. There is increasing interest in the application of this type of immunoassay to clinical diagnostics and environmental monitoring. Most commercial sensors are based on optical devices for example, Pharmacia Biosensor AB produce the BIAcore™ family of products that use surface plasmon resonance with microfluidics and have a detection range of 10^{-3}-10^{-12} mol l^{-1}. Immunosensors provide a powerful tool that could be used to monitor a range of reactions for example, the presence or absence of substances, active concentration measurements and relative or comparative binding patterns for example, surface binding phenomena or monoclonal binding affinities. Multianalyte immunosensors will be produced in the future for example based on

antibodies with different cross-reactions to produce a response pattern that provides information on the presence and structurally similar analytes such as the triazine group of pesticides[62].

Single and multilayer films are becoming more widely used in immunoassays. These devices contain most or all of the reagents required to carry out an immunoassay in a thin single element and use dry-chemistry technology[63]. Large areas of the thin films are produced and the assay works by the application of the sample rehydrating dried reagent and initiating the immunoassay. There are four main zones on these thin films - the support zone; analytical zone, reflective zone and spreading zone. Single layer devices have all the zones on one layer for example, filter paper while a multilayer device has as many layers as required for the assay. There are several commercial systems that use these thin film devices for example, Dade Stratus Immunochemistry Assays, which is a heterogeneous immunoassay used for therapeutic drug monitoring and allergy testing among others. Thin film immunoassays are generally more rapid, more stable and less wasteful of reagents compared to other immunoassays although the sensitivity is not as good.

1.2.8 Microfabricated and multianalyte immunoassay devices.

Disposable immunoassay devices and point of care devices were developed in tandem but not exclusively, in order that people without formal training in analytical techniques could use an analytical device. There are several types of disposable devices i.e. encapsulated wet chemistry has all the components in a complex disposable plastic unit requiring only sample to be added[64]. A simple immobilised analyte capture device is a flow-through device where the capture antibody is immobilised on a solid porous matrix to which the sample is added[65]. Lateral flow devices involve chromatographic and liquidic systems where the reaction components are impregnated or immobilised on porous solid phase, which is brought into contact with the sample in sequence after addition of diluent[66]. The final type of disposable were mentioned previously i.e. immunosensors. Early versions of disposable devices were the over-the-counter (OTC) pregnancy tests, which were multistep assays based on latex

agglutination or colour formation for the monitoring of enzyme-labelled conjugates. Further development of for example, monoclonal antibodies and improved plastic production greatly improved and increased these tests. Today solid porous matrices are used for a wide variety of analytes for example, infectious diseases, cardiac markers or drug screening. Most current disposable devices utilising immunoassays have one reagent bound to a solid membrane for example, nitrocellulose[67] and are either the flow through type for example, the capture antibody immobilised onto a membrane producing a heterogeneous immunometric assay with a coloured result. An example of this is the qualitative Helisal Rapid Blood Test for Heliobactor pylori antibodies which, use 20μl of whole blood and takes twenty minutes. The other most popular disposable device use lateral flow for example, the TropT System for Boehringer Mannheim which measures Troponin T in 150μl of whole blood and takes 20 minutes. Devices have also been constructed for multianalyte detection for example, Biosite Triage™ for drugs of abuse. The sensitivity can have increased by the use of highly sensitive labels for example, enzyme amplification although both the qualitative and semi-quantitative assays compare well with reference procedures.

Microfabricated devices have also been developed for use at the point-of-care. The impetus behind this was the exploitation of new automatable technology for example, multilayer films making the assay suitable for the POC. Low complexity devices perform a single analytical function and usually involve capturing an analyte by immobilised antibody. Sandwich assays are most common with the reagent being in excess. Dipsticks are often used and are composed of a nylon, polycarbonate or nitrocellulose strip on which the reagents are dotted and dried and then affixed to a cellulose-acetate stick. There are sequential incubations and washings and a signal often colour is generated. This could be used for multianalyte testing for example, Quidel Mast® system for screening allergens. A multizone dipstick is incubated with a serum sample, anti-human IgE- alkaline phosphatase and substrate. Blue dye is deposited indicating a positive reaction. The most sophisticated microfabricated devices are the multifunction and

multi-feature devices often as a cassette or cartridge. There are usually combinations of analytical steps including analyte capture, sample measurement, flow control and reagent addition. One of the most complicated devices is the Biotrack 516 from Boehringer Mannheim[64], which is a cassette device for measurement of theophylline using 20μl whole blood and takes less than 3 minutes. The cassette is a 3-part injection moulded device that measure and dilutes sample, mixes, adds reagents and produces optical measurements.

Micromachined microfabricated devices provide the possibility of a "total analytical system". This uses micromachining which is the construction of complex micrometre sized inter-connecting structures for example, valves and pumps. It can be integrated with electronic sensors and controls on a microchip producing a micro-total analytical system[68]. The advantages of producing micromachined devices include flexibility of design and ease of manufacture especially with silicone because the microelectronics industry already set up for high volume and low cost manufacture. The internal volume in the devices are in the microlitre to picolitre range with reduced reagent and sample consumption which is especially beneficial in paediatric and neonatal medicine and leads to reduced waste generation. Response times are also faster which could lead to the production of hand-held POC analysers. The development of multianalyte immunoassays has paralleled micromachining and microfabrication and these devises are ideal for this type of testing. Finally due to the encapsulation of the device, it is not as vulnerable to humidity and temperature changes.

Recent immunoassay development has been towards point-of-care methodologies i.e. home or doctors' surgeries, ultrasensitive assays, immunosensors and multifunctional and random access analysers'[69]. Common to all these is the development of multianalyte immunoassays. Immunoassays were usually performed as discrete tests i.e. one analyte per tube or well. In immunoassay development the aim has been dual or simultaneous assays where two or more analytes are measured in a single assay. This has the advantage of work simplification, increased test throughput and a reduction in the cost per test[70]. Multianalyte testing was most

attractive for logical panels of tests such as thyroid function tests and has been extended to use in allergy testing, screening transfused blood, genetic diagnosis and in determining different isoforms of molecules for example, hormones that may have different physiological functions[69]. Various assay formats were tried such as using a combination of label but there were problems with this method such that it has not gone beyond two labels for example, ^{125}I and ^{131}I or europium and samarium in time resolved fluorescence assays[71]. The use of different labels did not produce good results due to the different requirements for optimum enzyme activity resulting in compromised assay conditions. The overlap of signals led to a rapid loss of accuracy in quantifying individual signals as the number of labels increased[69]. Metal ions for example, bismuth and indium have been used as labels in a simultaneous dual analyte immunoassay using human serum[72]. The most successful multianalyte assays used sophisticated solid phases. As early as 1976, colour coded beads were coated with different antibodies[73] although dual radioisotope assays were performed in 1966[74]. The most popular way of producing multianalyte assays was to produce arrays of individual reaction zones on a single solid phase. This uses only one label but the detection system has to be capable of serial or simultaneous quantification of signals associated with each zone.

Spatially separated areas can be achieved by a number of means such as dotting, entrapping or chemically immobilising different antigens on membranes. Donohue et al developed a system using an enzyme immunoassay on a test card where multiple test areas at individual locations on a membrane solid phase so that simultaneous determinations from a single sample are possible[75]. Kakabakos et al also used spatial separation to develop a time-resolved fluoroimmunoassay for quantifying lutropin, choriogonadotropin, prolactin and follitropin in serum[76]. Four antibody-coated discs were attached to a stick with two discs on each side. A fluorescent europium chelate bound to each disc and was detected by laser-excited time resolved fluorometry in two sequential measurements of side one then side two of the stick. There are a number of possible problems in the development of multianalyte immunoassays such as possible cross-reactions leading to false positive results. There may also be difficulty in optimising assay ranges for

individual analytes and quality control can also be an issue. The immobilisation of a number of antigens or antibodies on a solid phase as "dot" or drop necessarily leads to a reduction in scale of the immunoassay with a number of reactions immobilised on for example, a dipstick or membrane. This miniaturisation has led to the concept of "microspot" antibody technology by Ekins[77]. In this, there may be 100 different immunoassays geometrically arrayed on a cross-sectional area of a human hair. This in conjunction with improved solid substrates and microspotting techniques could yield assays of greater sensitivity with shorter incubation times. This system could also incorporate a variety of quality control and safeguard not feasible in conventional technology for example, spots detecting possible cross-reactions. Ekins had previously shown that the only means of increasing assay sensitivity was to use non-isotopic labels of very high specific activity in non-competitive assays preferably using monoclonal assays. Collaboration between Ekins and Wallac eventually produced the DELFIA system in the early 1980s which was the first commercially available "ultrasensitive" immunoassay using the principles described above.

The microspot theory put forward for immunoassays found that a far smaller amount of immobilised antibody than previously thought was required. Such a small number of antibodies could be located on a membrane or other solid support within a "microspot" leading to the construction of microspot arrays each directed against a different analyte. This would allow the simultaneous measurement of thousands of different analytes from a single drop of blood. The smaller scale antibody arrays and oligonucleotide arrays will make powerful combination and is bound to have a major influence on medical diagnostics in the future[69]. Developing from the microspot theory was the ambient analyte immunoassay in which the analyte concentration is independent of sample volume and the amount of antibody present. There are a wide range of labels for the sensor and developing antibodies used in this technology including radioisotopes, enzymes and chemiluminescence. Fluorescence is useful because arrays of microspots can be scanned leading to multianalyte assays on the same sample. Chemiluminescence is also very useful as a high specific activity label for multianalyte immunoassays[70]. Microspot assays are

more sensitive and rapid than conventional systems and challenges the precepts regarding immunoassay. It would be very useful in environmental and food monitoring in addition to medical diagnostics particularly in the development of the "lab-on-a-chip" which could be linked to computers.

1.3 The solid phase in immunoassays.

Immunoassay techniques depend on the separation of labelled antigen or antibody from unlabelled. This led to the development of different immunoassay "platforms" on which the assay was performed for example, microtitre plates or dipsticks. The aim of this work is to add the compact disc to this list. The first techniques used in immunoassay separation were thin-layer chromatography and electrophoresis and used physicochemical differences between the antigen, antibody and the antigen-antibody complex. However in the late 1970s and 1980s secondary anti-species antibodies became widely available as polyclonal and monoclonal antibodies which could be coupled to a solid-phase and are now used almost exclusively in heterogeneous immunoassays. The disadvantages of this system include using relatively large amounts of antibody, long reaction times and washing the precipitate requires great care. These could be reduced by the development of microtitre plates and tubes and solid phases. Solid phases can be either particulate or solid matrices. Particulate solid phases can be of two main types. Non-magnetic particles can be used for example, latex, glass, Sepharose, Sephadex, beads etc. The choice of particle depends on the relative coupling capacity of the different plastics and the size and density of the plastic. This type of assay is used commercially in the Abbott ImX system, which uses latex-coated particles for the antigen-antibody reaction. These are then captured on a glass-fibre membrane for separation[78]. Magnetic particles for example, paramagnetic ferrous oxide can be incorporated into a cellulose matrix as more recently has chromium dioxide. This avoids a centrifugation step and has the advantage of high surface area with rapid analyte capture and efficient separation and washing. The most recent advances in this area are the ferrofluids, which act almost like liquids but are particles of less than 50 nm in size. Magnetic particles are used in several commercial systems such as the Bayer Immuno-1 format.

Solid matrices have three main formats – fibres (membranes), tubes and microtitre plates[79]. Fibres are usually cellulose, nitrocellulose or glass fibres and have a high surface area with a high antibody binding capacity. It is widely used commercially such as in the Dade Stratus®[80]. Nitrocellulose membranes are commonly used for dot ELISAs. Coated tubes have the lowest capacity of solid matrices and their effective use depends on reproducing the coupling of the antibody to the tube. Microtitre plates are the most popular solid phase but have the lowest antibody-binding capacity. They are produced in a range of plastics that are treated in a number of ways to increase coupling capacity, which depends on the application. Capacity can be increased by irradiating the plate[81] or by drying the antigen on the plate. Microtitre plates can be used with a number of labels for example, enzymes, chemiluminescence and fluorescence. However there a number of problems associated with them such as drift across the plate due to pipetting delays, temperature gradients, batch-to-batch difficulties and quality control issues. Kricka[82] showed that "edge effects" were apparent in microtitre plates i.e. the wells at the edges absorb more protein compared to those in the middle of the plate. In a study by Gosling in 1990 70% of new immunoassays were using the solid phase[30]. The use of microbeads or particulate solid-phase had fallen from 40% of new assays in the early 1980s to 10% in the late 1980s. In the early 1980s antibody-coated tubes accounted for 15% of new assays. But this had fallen due to the increased use of microtitre plates whose use had increased from 15% to 70% in the late 1980s.

Microtitre plates are the traditional workhorses of the immunoassay, especially the 96-well plate. There are a number of disadvantages in this system including such as the expense of the plates, reagents and specific readers and the fact that they can only be performed in the laboratory.

1.4 Structure of the compact disc.

In this project the compact disc is developed as an alternative platform for performing biological assays initially immunoassays. There are a number of reasons that make compact discs suitable platforms for immunoassays and biological analysis in general. They are made of biocompatible plastic and it was found that protein would attach readily to the surface of the compact disc. The plastic is easily moulded and has high strength, toughness, heat resistance and excellent dimensional and colour stability. The CDs have a large surface area and have an optically high quality in reflection and have very good laser scanning. The optics are relatively low cost and there is high resolution for imaging. Using CDs in this manner would require the CD reader to be modified with the addition of another laser to read the chemistry on the top of the disc. Even with this modification, the instrument would be very low cost and compatible with a standard computer loaded with the appropriate software. In addition digital information such as protocols, references, calibration and others could be stored on the underside.

Most of the above properties are due to the structure of the compact disc. The development of the CD was a result of digital technology, which resulted in high quality sound, compared to the analog system of records and tapes and was the result of collaboration between Sony and Phillips[83,84]. Digital technology describes the music signal in the form of binary notation called bits. The bits are recorded on the disc in small grooves called pits and lands, which represent 0's, and 1's respectively. From the digital numbers the CD player reconstructs the original audio signal. Compact discs are 12 cm in diameter plastic discs, 1.2 mm thick and can hold 650 megabytes of data. Information is encoded in a spiral track nearly three miles long and they have a silver coloured surface that reflects laser. The CD has several layers that are built up in a defined order[85,86]. Data is generated and set up to be accessible in the order required. It is then separated into logical files or separate tracks. The pre-mastering step involves the addition of control codes for error correction, synchronisation, address/mode indication and disc rotation speed. The next stage is mastering which involves burning the pits and lands onto a glass master by laser. Electroformation involves the deposition of nickel onto the developed surface of the glass master in a process similar to electroplating. Once the metal master has been

formed, multiple CDs can be achieved, however since the metal master is the original it is usually placed back in the electroplating process to create replicas called "metal mothers". These are exact replicas of the glass master. To be useful these have to go through the electroforming process again to produce "sons" which are exact replicas of the metal master. This process usually produces about 50 "stampers" or "sons" from one metal master. The most common method of forming CDs is by injection moulding of polycarbonate onto stampers. This is followed by metalization where aluminium is deposited onto the polycarbonate mould. This is required to give the disc the reflective properties required for data playback. Finishing is the application of an acrylic or lacquer coating to the disc surface to prevent aluminium oxidation and surface scratches. Compact discs have moved into other areas due to their ease of use and longevity and have also been used for information storage, data transfer and database storage. New writing and reading lasers have been designed which allow more information to be put on the same size of compact disc. This allows vast amounts of data to be stored in a tiny amount of space while being able to access and organise it very quickly.

The collaboration between Sony and Philips also led to the design and development of the CD player[86]. Information on a CD is stored digitally. To allow data to be stored on a CD it has to be encoded. The CD player has to decode this data. There are five main components in a CD player. The disc drive system spins the CD at the correct speed while in the laser pick-up system the compact disc is scanned by the laser beam which is reflected from a land to a photoelectric cell. From the photodetector, the signal goes to the data decoder and servo system, which is responsible for the focusing and tracking of the laser beam. The data decoding system demodulates and decodes the data string of 0's and 1's and converts it into a more recognisable form for example, music. The control and display system processes the subcode embedded on the CD. The subcode shows information such as how many tracks, time left on CD etc. Within this project the development of specific software and the modification of a standard CD player to detect the immunoassay results was carried out by other collaborators.

1.5 Immunoassays at the point of care.

The increased specificity and sensitivity of immunoassays as described above has led to the development of rapid, reliable and straightforward immunoassays for use outwith the central laboratory. Point of care (POC) testing can be defined as any laboratory test performed outside a central hospital laboratory and is usually performed close to the patient. It is also called near-patient testing, decentralised testing, alternate site testing, patient-focussed testing, ancillary testing or bedside testing and reflects the wide variety of POC tests that can be delivered[87]. The classic POC test is found in glucose testing where a capillary blood sample from a patient is analysed on a small handheld meter. This type of testing arose from demands made by clinicians for improvements to existing tests with respect to speed and high levels of analytical sensitivity[88].

There are different "levels" of POC as shown by the most common operating areas for example, casualty department, intensive care, operating theatre, inpatient or outpatient departments, nursing homes, home health care, pharmacies, health centres, doctors' surgeries and field testing. These different levels reflect varying degrees of complexity and the skill level of the operator. For example tests for use in the home have to be very simple and are often in the style of dipsticks. A positive or negative reaction is signified by a change of colour that is observed visually or compared to a calibration chart. Tests used in casualty departments or health centres may be of higher complexity for use by medical staff. This may include POC tests for monitoring cardiac enzymes in suspected myocardial infarction cases, which have reduced the turnaround time for such tests. The usefulness of these tests must be balance by immediacy of results i.e. speed, reliability, cost, staff impact i.e. the complexity of the test[88].

The first POC test involved measuring arterial blood gases, sodium, potassium, calcium, magnesium, haemoglobin, haematocrit, glucose and lactate for use at the hospital bedside. More recently they have also been developed for therapeutic drugs, drugs of abuse and cardiac enzymes as described above. The advantages of point of care testing (POCT) include the use of

whole blood or plasma thereby minimising sample manipulation and sample loss and cost-effectiveness in either improving patient outcome or decreasing the cost per test. The other main advantage is multianalyte testing where a small sample of whole blood or plasma can provide results for a group of analytes for example, to detect viral or bacterial pathogens quickly, thus leading to better patient treatment[89]. POCT is the fastest growing sector of the diagnostics area with improvements such as sensor miniaturisation, use of solid state reagents in unique matrices, plastic disposable devices and microfabricated devices[88]. Miniaturisation introduces scaling issues i.e. physical size and scale of reaction volumes. POC devices are often packaged into hand-held devices such as a holder or cartridge providing a convenient way of introducing the sample. However this reduction in sample volume may mean that there are very few molecules of analyte in sample and care has to be taken with evaporation effects that may affect sample or reagent volumes although pipetting systems have been designed that automatically replace lost fluid. Alternatively the chip may be enclosed in a controlled environment. One of the main benefits of miniaturisation is integration of steps usually by interconnection on the chip surface. The aim is the development of a "lab-on-a-chip".

There are numerous examples of POC tests that are available commercially. One of the areas these were first developed for was qualitative screening kits for drugs of abuse. An example of this is the EZ-Screen Test kit produced by Editek Inc, which tests for cocaine, cannabinoids and opiates in urine and showed comparable efficiency with the Adx Abbott standard machine[90]. One of the most popular POC formats is the reagent strip for example, Clinitek from Bayer. This has a novel dip and read device for the quantitation of the albumin: creatinine ratio in urine providing individual concentrations of both. This was compared with a lateral flow device from Roche – the Micral II. It was found that the Clinitek system provided a reliable means to screen semi-quantitatively for microalbuminuria. One way to reduce turnaround time in POC tests was to use whole blood. Merenbloom and Oberhardt produced a whole blood immunoassay using test cards and a small instrument for use at the POC. This was a steric hindrance immunoassay to detect biotin[91]. A clinical chemistry analyser was developed for multianalyte detection in less

then 10 minutes from less than 10µl of whole blood[92]. The blood is applied directly to a single-use plastic rotor of 7.9 cm in diameter. The rotor contains liquid diluent and a range of 4-12 tests in 1-2 mm diameter reagent beads. The rotor is placed in the instrument and as it spins, capillary and rotational forces processes the blood into diluted plasma, which is distributed to cuvettes with the reagent beads. The instrument monitors the reaction at nine different wavelengths and calibration data is read for each reagent from a bar code on the outer edge of the rotor. The instrument also calculates stores and prints results and there are several different disc menus for both versions of the instrument. It is portable, lightweight and can be rum from a 12-volt battery or car cigarette lighter (with an adapter). This system is available commercially from Abaxis as either the "Vetscan" for use in veterinary surgeries or the "Piccolo" for hospitals, health centres or doctors' surgeries and is in use in the USA and Europe.

The concept of using the compact disc system for biological analysis originated with Dr John Gordon[93,94] and "Molecular Drives", a small company that originated in the University of Glasgow. In the system described here, the modified compact disc is developed as both a platform for performing immunoassays and a method of storing and retrieving information regarding the assays. This could be references, protocols, calibration data or any other important information. The use of a modified CD player to read and specific software to interpret the results provides a self-contained system that could be connected to any computer carrying the correct software – a "plug and play" peripheral for the computer. "Gamera Bioscience", a company based in the USA is developing a "LabCD" based on compact discs and CD player technology for use in diagnostic testing. This system is based on microfluidics on the disc and operates at a much smaller level than the system proposed in this thesis. Although not on the market as yet, this technology will be capable of performing a range of laboratory procedures and will use whole blood. It could also be used for DNA diagnostics.

From the development of immunoassays as described above and the requirements of the immunodiagnostics market especially at the point of care, it is evident that a new immunoassay platform that also incorporates a method for storing and transmitting data would fill an obvious gap in the market for clinical diagnosis and scientific research. This thesis will show that it is possible to perform a multianalyte immunoassay using a compact disc as a novel platform for immunoassay and how with further development this system could become as ubiquitous as the microtitre plate.

1.6 Aim of project

The aim of this project was to develop a new platform for immunoassays using compact disc technology, that could be used for multianalyte testing. The development of this new system had two parts – the chemistry which will be discussed in this thesis, and a modified compact disc player and specific software for reading and interpretation of the chemistry on the disc which was developed by colleagues.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and materials.

2.1.1 Sectors and discs.

There were three types of disc used, all based on the compact disc (CD). These were 1) plain or transparent plastic discs, 2) aluminium-covered discs and 3) black discs, all of which were purchased from Nimbus Manufacturing. The original test discs were plain and had holes drilled in them, which were sealed with "Transpaseal" (Medical Illustration, Institute of Biomedical and Life Sciences, University of Glasgow) to simulate wells. The sectors had various formats, which are described in chapter 3. The final format is shown in Figure 2.1.

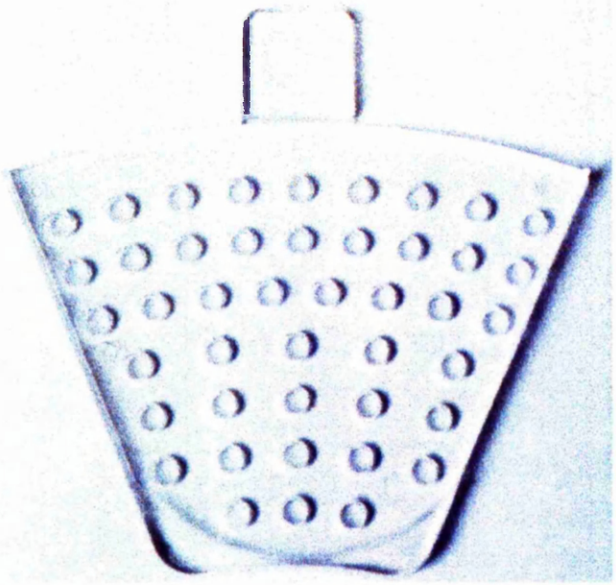


Figure 2.1 : Final format of the sector.

Pascoe Engineering, Glasgow, made these and the final moulded sectors. The medical-grade polystyrene used for the final format of the sector was from Elf Atochem UK Ltd. The holding mechanism for the sectors was called a "spider" and was a combination of two plain or aluminium discs. The discs were held together by a "separator" which divided the spider into eight segments. It was cut from a single piece of plastic then coated with sticky tape on both sides. There was a filling hole at the base of each segment on the upper disc to allow washing and application of reagents to individual sectors. The separators were constructed and the

spiders assembled by the Department of Mechanical Engineering, University of Paisley. The Mechanical Workshop, Institute of Biomedical and Life Sciences, University of Glasgow constructed the spider with the clip mechanism and the modified spider used with the black disc in later work. Silicone grease (Dow Corning High Vacuum Grease) was used to coat spiders and sectors to prevent leakage but was later replaced by a silicone spray (Wholesale Automotive (UK) Ltd). Poly-L-Lysine, 0.1% w/v (Sigma Bioscience, P-8920) was used to coat some sectors.

2.1.2 Dinitrophenol (DNP) and rabbit IgG ELISAs.

DNP-bovine serum albumin (BSA) and anti-DNP-BSA (rabbit) was from the Department of Immunology, University of Glasgow. The blocking agent was 50mg/ml bovine serum albumin (Sigma Bioscience A-9647) in phosphate buffered saline pH7.2-7.4 (BSA/PBS). The second antibody was Goat Anti-Rabbit IgG (whole molecule) Alkaline Phosphatase Conjugate (Sigma Bioscience A-0418), Goat Anti-Rabbit IgG (whole molecule) Peroxidase conjugate (Sigma Bioscience A-9169) or Amdex™ Goat Anti-Rabbit IgG-Horseradish Peroxidase Conjugate (Amersham Pharmacia Biotech, RPN 4301). The antigen coating buffer was PBS, pH 7.2-7.4. The antibody diluting buffer was 0.05% BSA/PBS and the wash buffer was PBS /0.05%Tween 20 (Sigma Bioscience P-1379). Rabbit IgG was from Sigma Bioscience (I-8140) with the second antibodies, buffers and blocking agent as for the DNP ELISA.

2.1.3 Multianalyte ELISA.

Antigen coating buffer, antibody diluting buffer and wash buffer were as described for the DNP ELISA. The main blocking agent was 50mg/ml BSA/PBS although skimmed milk (Safeway) was also used. The antigens and antibodies for the multianalyte ELISA were purchased from Calbiochem.

The antigens were Human Serum Albumin (Cat. No. 126658), Human Plasma α-1-Antitrypsin (Cat. No. 178251), Human Plasma α-2-Macroglobulin (Cat. No. 441251), Human Plasma Antithrombin III (Cat. No. 169756), Human Erythrocyte Catalase (Cat. No. 219008), Human Plasma α-1-Antichymotrypsin (Cat. No. 178196), Human Plasma Plasminogen, Glu-Type (Cat. No. 528180), Human Serum α-1-Acid Glycoprotein (Cat. No. 112251) and Human Plasma α-2-Antiplasmin (Cat. No. 178221).

The corresponding primary antibodies were all raised in rabbits. Anti-Human Serum Albumin (Cat. No. 126582), Anti-Human α-1-Antitrypsin (Cat. No. 178256), Anti-Human α-2-Macroglobulin (Cat. No. 441253), Anti-Human Antithrombin III (Cat. No. 8200493), Anti-Human Erythrocyte Catalase (Cat. No. 219010), Anti-Human α-1-Antichymotrypsin (Cat. No. 178216), Anti-Human Plasminogen (Cat. No. 527557), Anti-α-1-Acid Glycoprotein (Cat. No. 122164) and Anti-Human α-2-Antiplasmin (Cat. No. 178234). Normal Rabbit Serum (SAPU, Product Code S030-220) was used as negative control.

The second antibodies used were Goat Anti-Rabbit IgG (whole molecule) Peroxidase Conjugate (Sigma Bioscience A-9169), Amdex™ Goat Anti-Rabbit IgG-Horseradish Peroxidase Conjugate (Amersham Pharmacia Biotech, RPN 4301), Donkey Anti-Rabbit IgG Peroxidase Conjugate (SAPU, Product Code SO83-201) or Donkey Anti-Rabbit IgG-Fluorescein Isothiocyanate Conjugate (SAPU, Product Code S076-201).

2.1.4 Substrates.

2.1.4.1 Soluble

Two soluble substrates were used in conjunction with peroxidase-conjugated second antibodies. Orthophenylenediamine (OPD) Dihydrochloride (Sigma Bioscience P-8287)) Tablets with Phosphate–Citrate Buffer Capsules (Sigma Bioscience P-4922) and 3,3', 5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma Bioscience T-8665).

2.1.4.2 Insoluble.

Four insoluble substrates were tested, three with peroxidase-conjugated second and one with alkaline phosphatase-conjugated second antibody. Sigma *Fast*™ 5-Bromo-4-Chloro-3-Indoyl Phosphate/Nitro Blue Tetrazolium Tablets (Sigma Bioscience B-5655) was used with alkaline phosphatase-conjugated antibodies. Sigma *Fast*™ 3,3'-Diaminobenzidine (DAB) with Metal Enhancer Tablets (Sigma Bioscience D-0426) was used with Urea Hydrogen Peroxide Tablets (Sigma U-4756). The final two substrates were 4-Chloro-1-Napthol (4C1N) tablets (Sigma Bioscience C-6788) and Insoluble 3,3', 5,5'-Tetramethylbenzidine (Calbiochem, 613548). The latter three substrates were used with peroxidase-conjugated second antibodies.

2.2 Dinitrophenol (DNP) ELISA.

This was a standard indirect ELISA for the detection of antibody. Antigen was coated on to the solid-phase and an enzyme-conjugated second antibody that reacts with a substrate to produce colour detects the sample antibody. Both soluble and insoluble substrates were used.

2.2.1 Microtitre plates.

Microtitre plates (96-well) were coated with DNP-BSA (100µl/well) and left overnight at 4°C. All other incubations were at room temperature. After washing four times with 0.05%Tween/PBS (wash bottle), the excess was tapped out of the wells and the plate dried by "knocking" vigorously on paper towels. Washing took place after each incubation stage. Blocking was with 50mg/ml BSA/PBS (150µl, 30 minutes), followed by the primary antibody, anti-DNP (100µl, 30 minutes), and then enzyme-conjugated anti-rabbit IgG (100µl, 30 minutes). After washing for the final time, either soluble or insoluble substrate was added, (100µl/well) and incubated for the appropriate time after which the colour was analysed for intensity.

2.2.2 Discs and sectors.

The protocol remained essentially the same as for microtitre plates except the volumes in the wells were reduced and insoluble substrates used.

2.2.2.1 Single and double discs.

After sealing the wells with Transpaseal, the wells were coated with DNP-BSA (5-10µl/well) and incubated for 2 hours at room temperature. The other incubations were for 1 hour at room temperature excluding the substrate, which had a shorter incubation period. The disc was washed with 0.05% Tween/PBS and a wash bottle by filling and emptying the wells four times. Then discs were dried by tapping on paper towels taking care not to disturb the well surface. This was repeated after each stage of the assay Incubation with 50mg/ml BSA/PBS (5-10µl/well) was followed by anti-DNP-BSA (5-10µl/well) and enzyme-conjugated anti-rabbit IgG (5-10µl). Finally, the appropriate insoluble substrate was added and after the incubation period, the wells were washed with water. The intensity of colour was determined by visual examination.

2.2.2.2 Plain discs.

The plain discs were used while the sectors were being moulded. "Wells" were marked on the disc using black marker pen. The DNP-BSA antigen (10-20µl) was coated onto the wells and left to incubate for 2 hours. All other incubations were also at room temperature. Due to the nature of the plastic, the antigen sat as a droplet on the surface. Washing was with a wash bottle and PBS/0.05%Tween. After this, as much excess liquid as possible was removed from the disc surface using paper towel, without disturbing the antigen. This took place after each stage of the assay. Blocking agent, 50mg/ml BSA/PBS (10-20µl/well) was applied to the wells for 30 minutes. As the surface of the disc was now wet, the applied reagents tended to merge.

Although this did not matter at the blocking stage, it was difficult to separate different primary and secondary antibody dilutions applied to different wells at later stages in the assay. Blocking was followed by the primary antibody (anti-DNP-BSA, 10-20µl/well, 30 minutes) at the appropriate dilution. After washing again the peroxidase-conjugated second antibody (10-20µl/well) was applied for 30 minutes followed by insoluble TMB. It was flooded across the entire area containing the wells for 20 minutes after which the disc was washed with distilled water and air-dried. A strong blue colour was deposited at the site of a positive reaction, which was examined visually.

2.2.2.3 Sectors.

All incubations were at room temperature. Wells were coated with DNP-BSA. In the original sectors (Transpaseal), the coating volume was 4µl while in the moulded polystyrene sectors it was 2µl. The underside of the sector was coated with silicone grease before inserting in the spider and the incubation time was 2 hours. Washing was initially with a 1ml plastic syringe filled with PBS/0.05%Tween which flooded across the sector, pulled up and down the syringe three times and discarded. This was repeated four times. The syringe was later changed to a 1ml pipette. Washing took place in this way after each stage of the assay. Blocking agent (50mg/ml BSA/PBS, 1ml), was added by flooding across the sector and pulling the liquid up and down the syringe three times. This ensured that all the wells were filled and was later called "flood and fill". The washing and application of reagent protocols were accomplished by the same technique. Incubation with BSA/PBS was for 30 minutes followed by anti-DNP-BSA (30 minutes) and peroxidase-conjugated second antibody (30 minutes) followed this. Finally the substrate, insoluble TMB, was added, and incubated for 20 minutes after which the sector was washed with distilled water. The sectors were removed from the spider; excess water tapped from the wells and allowed to air-dry.

2.3 Rabbit IgG ELISA.

2.3.1 Microtitre plate.

This was a direct ELISA with rabbit IgG coated on to the solid phase, reacting with enzyme conjugated anti-rabbit IgG. Rabbit IgG in PBS, 100µl/well was coated on to a microtitre plate and left overnight at 4°C. All other incubations were at room temperature. After washing four times with 0.05% Tween/PBS, blocking agent, (50mg/ml BSA/PBS, 150µl) was added and incubated for 30 minutes. After washing again, dilutions of enzyme-conjugated anti-rabbit IgG were added to the wells and incubated for 30 minutes followed by a suitable substrate (100µl). The developed colour was examined for intensity.

2.3.2 Discs and sectors

2.3.2.1 Single and double discs

All incubations were at room temperature. Rabbit IgG (5-10µl) was coated on to the wells and incubated for 2 hours. Washing was with PBS/0.05%Tween by filling and emptying the wells four times and took place between each stage of the assay. Tapping on a paper towel dried the discs. The blocking agent (50mg/ml BSA/PBS, 5-10µl/well) was incubated for 1 hour followed by the anti-rabbit-IgG-peroxidase or alkaline phosphatase conjugate (5-10µl/well) which was also incubated for 1 hour. The substrate depended on the enzyme conjugate and was incubation was for 30 minutes. The discs were then washed with distilled water and air-dried before the colour intensity was examined visually.

2.3.2.2 Plain discs

This procedure was similar to the DNP-BSA ELISA (section 2.2.2) with the same problem of keeping the "wells" separate. Rabbit IgG (10-20µl) was coated on to the marked wells and incubated for 2 hours at room temperature. All incubations were at room temperature and washing was as for the DNP-BSA ELISA on plain discs. Blocking agent (50mg/ml BSA/PBS,

10-20µl/well) was incubated for 30 minutes followed by goat anti-rabbit-IgG-peroxidase conjugate for 30 minutes. The substrate used was insoluble TMB, 10-20µl/well, for 20 minutes followed by washing with distilled water. The disc was then air-dried. The deposition of blue colour in the "wells" was evidence of a positive reaction, which was examined visually.

2.4 Multianalyte ELISA

2.4.1 Microtitre plates.

Each ELISA used to construct the multianalyte immunoassay was first tested on a microtitre plate using insoluble TMB as substrate. A range of antigen concentrations (10-200/ml) and primary antibody dilutions ($1/1x10^3$-$1/6.4x10^4$) were tested for the combination that produced the best intensity of colour. All incubations were at room temperature. The plate was coated with antigen (100µl/well) and incubated for 2 hours. Washing was with a wash bottle containing PBS/0.05%Tween 20 and took place between each incubation stage of the assay. The blocking agent was 50mg/ml BSA/PBS, 150µl/ well with 30 minutes incubation. This was followed by primary antibody at the appropriate dilution in 0.5mg/ml BSA/PBS then Goat anti-Rabbit–IgG-peroxidase conjugate. Both were incubated for 30 minutes. Incubation with insoluble TMB was for 20 minutes followed by washing with distilled water. The colour intensity was evaluated visually from which the appropriate antigen and primary antibody concentrations were determined for use on the sectors. Alpha-1-Antichymotrypsin was tested on a sector due to the small quantity of antigen available.

2.4.2 Sectors.

This was performed on 44-well sectors held within a "spider" with all incubations at room temperature. The sectors were coated with seven antigens (2µl/well). The antigens were Human Serum Albumin, α-1-Antitrypsin, α-2-Macroglobulin, Antithrombin III, Catalase, α-1-Antichymotrypsin and Plasminogen. Each antigen was coated in blocks of four wells starting from top left hand side of sector, with every fifth well coated with PBS only. Once all seven antigens were coated, there was still room to load them in the sequence shown above with the

final two wells being PBS only. The sequence of antigen coating depended on the number of antigens being used and on the experiment. The sectors were placed in a spider, incubated for 15 minutes, then washed by the flood-fill technique. This involved pipetting 1ml of wash buffer (PBS/0.05% Tween) across the sector via the filling hole in the upper disc of the spider, pipetting up and down three times and then discarding. This was repeated three times for each sector giving a total of four washes per sector. Washing between each stage of the assay was by flood-fill and was also used for the application of reagents. The blocking agent was bovine serum albumin (50mg/ml in PBS) which was incubated for 15 minutes. The combination of primary antibodies applied to the sectors depended on the antigens used in the experiment. This was called the "primary antibody mix" and was applied to the sectors by flood-fill and incubated for 10 minutes. Second antibody (peroxidase-conjugated) was incubated for 10 minutes followed by five washes to remove all trace of second antibody. Insoluble TMB was then applied to the sectors but instead of withdrawing, the liquid was left flooded across the sector for 10 minutes after which the insoluble TMB was removed and the sectors washed out four times with distilled water. The sectors were removed from the spider and excess water tapped out of wells. After air drying, the sectors were evaluated visually and then stored in the dark.

2.4.3 Black discs.

Sectors on the black disc were coated with seven antigens, 1µl/well. The antigens were Human Serum Albumin, α-1-Antitrypsin, α-2-Macroglobulin, Antithrombin III, Catalase, α-1-Antichymotrypsin and Plasminogen and were all dried at room temperature (RT) on to the well. Blocking agent (50mg/ml BSA/PBS, 1µl/well) was then applied directly on top of the antigen and also dried at RT. All other incubations were at RT. The disc was placed in an adapted spider structure, which incorporated a silicone rubber version of the "separator" allowing the sectors to be separated on the disc. This meant that different combinations of primary antibodies could be applied to different sectors. Washing with PBS/0.05%Tween was carried

out as described for the multianalyte ELISA on sectors except that without wells, the sector surface was simply flooded. The number of washes was increased to six per sector between each stage of the assay. Application of reagents was by flooding and leaving on the sector surface for the duration of the incubation, which was 10 minutes for the three remaining steps in the assay. After blocking, primary antibody mix was flooded across the sector followed by second antibody (peroxidase-conjugate) and finally insoluble TMB, after which the sector was washed with distilled water. Deposition of blue colour in wells indicated a positive reaction. The disc was then air-dried and placed in the modified reader where readings were made at 650nm.

2.5 Reading of the black discs and standard analysis.

Development of the reader and specific software was carried out by Aileen Fleming. Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 and from left to right as shown below in Figure 2.2.

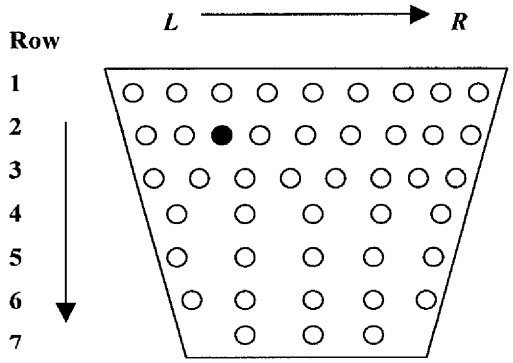


Figure 2.2: Layout of sector on the black disc showing method of reading from rows 2-7 and from left (L) to right (R).

At this time the optics on the reader did not detect row 1. The black well in row 2 had to be coloured with a black marker pen, as it was required for the reader to track correctly across the disc. Each sector had to have a black well in this position. The black disc had a silver outside edge and a silver rectangle above one of the sectors, which also had to be coloured black apart from a small square section that had to be left silver, again for correct tracking. The laser tracked across the disc by rows i.e. row 2 in all eight sectors was read first followed by row 3, then row 4 until row 7 had been read. Measurements were made with a red laser at 650nm, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to optical density (OD) values. Nine greyscale readings were taken in the centre of each well in the pattern of a 3×3 square. The average and standard deviation for the greyscale values were determined and from these the O.D. value and the Coefficient of Variation (CV) for the well were determined as shown below;

$$\text{OD Value} = \text{Log10}\left(\frac{255}{\text{Average Greyscale Value}}\right)$$

$$\text{Coefficient of Variation} = \left(\frac{\text{Standard Deviation}}{\text{Average Greyscale Value}}\right) \times 100\%$$

In some experiments the wells were read twice in order to compare the OD and CV values. For example if 19 wells on the sector were used in an experiment, and each well was measured twice, this resulted in a total of 38 data points on the graphs of OD and CV values.

CHAPTER 3

DEVELOPMENT OF THE COMPACT DISC AS AN IMMUNOASSAY PLATFORM.

The development of the compact disc (CD) into a suitable platform for immunoassays can be divided into three main sections - development of sectors, development of a "holding mechanism" for the sectors and the development of a disc without wells. The development of the sectors and the holding mechanism were mutually dependent and proceeded together.

The initial idea in the development of this new platform was to produce a scaled down version of the microtitre plate on a compact disc. Plain compact discs (without the aluminium layer) had holes drilled in them to simulate wells. The well were in a pattern of four segments each of 48 wells. "Transpaseal", a thick transparent sticky tape, was securely stuck over each segment and moulded onto the wells by using the heat of a hairdryer. This sealed most of the wells but not all and leakage was a regular problem. Preliminary experiments with this disc involved determining if it could be used to perform an ELISA. Rabbit IgG (10μg/ml, 10μl/well) was loaded into the wells and left overnight at 4^{o}C. Next day the wells were washed out by filling with wash buffer (0.05% PBS/Tween) and emptying four times. Discs were dried by tapping on a paper towel. The ELISA was continued as usual - blocking with 50mg/ml BSA/PBS, washing, then serial dilutions of anti-rabbit IgG-horseradish peroxidase (HRP) added to each of the four segments and development of colour was with Diaminobenzidine (DAB). At first the discs had incubation times of one hour and colour was evaluated by visual examination. It was found that the ELISA did work using this set-up with very little non-specific binding which could have been a problem give the "sticky" nature of the Transpaseal. There was however an accumulation of coloured product around the rough edges of the well probably due to greater antigen attachment but this was reduced by decreasing the antigen coating volume to 5ul. There was little gradation between the different anti-rabbit IgG-HRP dilutions, possibly because this substrate was not designed for use with an ELISA. The nature of this compact disc-based system means that once the assay has been completed, the disc is placed in a modified compact disc player with an additional red laser so that the colour produced by the assay can be quantified. Several substrates were tested in order to produce a strong blue colour and both alkaline phosphatase and horseradish peroxidase conjugated second antibodies were

used with a number of substrates. Finally an insoluble 3,3',5,5'-Tetramethylbenzidine (TMB) was found to produce the desired colour (see chapter 6).

Following on from the single disc was a double disc system was devised. The aim was to coat the antigen on a plastic rather than a sticky surface. The lower disc was a plain CD without wells and the upper disc was a single disc as described as above i.e. four segments of forty eight wells. The discs were stuck together using a number of substances eg Araldite glue, 3M Hi Performance Adhesive and Display Mount. None of these techniques were particularly successful due to the capillary action of the liquid between the plastic layers. This resulted in extensive leaking when liquid was applied to the wells. Even with the leakage it was possible to show that the antigen would attach to the plastic in this system. The next variation of the double disc was the incorporation of a "filling hole" in the upper disc in order to use the problem of capillary action to manipulate fluid. The lower disc was sealed with Transpaseal as before while the upper disc had four holes drilled in the area of each well segment. The upper disc was then glued to the lower disc. In different discs, the filling hole was aligned over several different areas of the segment. The hole was to be used for washing and adding reagents to the wells. Initially discs were completely sealed with glue but it was found that the outer edge of the disc had to be left open to allow for air displacement when liquid was applied over the segment Leaving another hole in the upper disc had not worked well because it allowed liquid to escape. The best location for the filling hole was at the base of the segment therefore when liquid was applied using a 1, 5 or 10ml syringe, it flooded across the wells. It was then withdrawn and the wells were left filled with liquid. This provided a system that could fill a number of wells simultaneously and was the subject of a patent application (93). This method of manipulating liquid in the system was called "Flood and Fill".

The next logical step from the double disc was to produce individual sectors which could be removed from a holding mechanism in which the ELISA was performed. The wells in the original single disc were too large and it was envisaged that the system would comprise eight sectors each containing a number of small wells. These sectors could be placed in a holding

mechanism consisting of two plain compact discs held together by a "separator" such that when the sectors were inserted there was a filling hole at their base to apply and withdraw fluid. The bottom disc was completely plain initially but later an aluminium coated compact disc was used. The upper disc was plain apart form the filling hole drilled at the base of each sector. Glue was used to attach the separator to the discs but this proved too inflexible and also leaked. Instead a double-sided sticky tape was used that attached to both discs. Although this was not ideal it provided a degree of flexibility and reduced leakage. Due to the use of eight sectors, the holding mechanism was called a "spider".

3.1 Sector development.

There were several versions of the sector. Originally there were 46 wells which still required to be sealed with Transpaseal. If all eight sectors were used in the spider this would give a total of 368 wells. The new sectors were tested in the spiders using a 1ml syringe to flood 1% Trypan Blue/PBS across the sectors via the filling hole. The wells filled easily but if excess dye on the sector surface was not removed, washing with 0.05% PBS/Tween meant that large air bubbles were left in the wells and on the sector surface. On re-filling with dye, wells were left empty. If the excess dye was removed washing was easier and there was fewer air bubbles. Another problem was that once the sector was wet, liquid easily travelled underneath raising possible contamination issues. This could be alleviated by coating the underside of the sector with silicone grease. The use of a thicker sector showed that there had to be a certain amount of clearance between the sector and spider to ensure that the wells were filled. This sector also had rounded outer edges which allowed liquid to stream out without all the wells being filled.

The next version of the sector had outer edges that matched those of the spider and additional side walls to prevent fluid travelling underneath the sector. However it was found that even though leakage was reduced, some did still get underneath when no silicone grease was used to coat the sector underside. Fluid also seemed to collect in the bottom corners of the sectors, adjacent to the filling hole and could not be retrieved. Using the DNP ELISA to test progress

showed that coloured product was localised in the wells. The final adjustment made to the sectors involved the bottom corners being slightly blocked off to prevent the fluid collecting as described above, adding a handle to facilitate removal from the spider, and also adding a back wall to reduce leakage. Blocking the corners did reduce but not eliminate fluid accumulation. This reduced the number of wells to 44 per sector giving a total of 352 wells per disc as shown in Figure 3.1.

Testing this sector, it was found that the surface of the sector had to be wet for the wells to fill properly. To investigate this further, two sectors were coated with bovine serum albumin (50 mg/ml in PBS), 4µl/well. One was dried using a hairdryer and the other was placed directly in the spider with the wells full. The dried sector was re-hydrated by washing briefly in PBS/Tween using a wash bottle, with the excess buffer being removed from the wells by tapping the sector on a paper towel. The dried sector was then placed in the spider. Both sectors were then flooded with Trypan Blue/PBS. The wells of the non-dried sector filled first time while the wells of the dried sector only filled completely after flooding three to four times. The effect of freezing liquid in the wells and then attempting to fill the wells was examined by freezing (-20^{o}C) two sectors filled with 50mg/ml BSA/PBS for one hour. After thawing, one sector was placed directly in a spider and the other washed with PBS/Tween before also being placed in a spider. After flooding with dye, both sectors filled completely first time. This was important because it meant that sectors could be stored frozen and then placed directly in the spider for use; the small amount of liquid present on the sector surface being sufficient to ensure all the wells were filled and washed properly. The use of poly-L-lysine as a wetting agent to facilitate filling the wells was investigated and did seem to improve filling empty wells slightly at a concentration of 0.005% poly-L-lysine in PBS i.e. wells did not fill first time but was better than in the absence of poly-L-lysine. At higher concentrations i.e. 0.01% poly-L-lysine in PBS, all but one of the wells filled easily. A wetting agent however eg poly-L-lysine which is charged may not suit all antigens therefore some other agent that is neutral may produce better results.

At the end of the investigations into sector structure it was decided to produce a moulded plastic version of the sector with handle, side and back walls and the bottom corners blocked off slightly. The moulded sectors would not require to be sealed at the bottom and were made of medical grade polystyrene as shown in Figure 3.1.

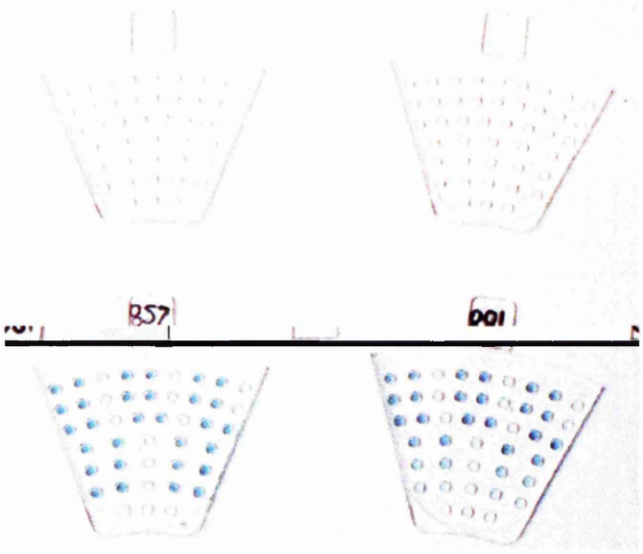


Figure 3.1: Sectors of 44 wells each with handle and side walls. Bottom sectors have been used in an ELISA with insoluble TMB as substrate.

There were a number of alterations that could be made to the sector eg more of a "V"-shape was required at the bottom to prevent fluid collection and improving the fit of the sector in the spider by incorporating grooves and a rubber seal. However, it was decided to continue with the modifications already made especially because the intention was to reduce the sector size at some point in the future which would automatically make the sector more of "V"-shape. At this time other modifications could then be made. The sector and spider technique was the basis for another patent application (94).

3.2 Spider development.

Spider development involved fewer changes than the sectors. As previously mentioned, the separator was initially glued to the upper and lower discs but this was changed to double-sided

tape to increase flexibility and reduce leakage. The spider used in this initial test had the filling hole at the left of the sector base which resulted in several of the wells in the top right of the sector not being filled. This was moved so that it would be in the middle of the sector base which allowed easier flow of liquid over the sector surface. A syringe was used to apply and withdraw fluid through the filling hole but it was found to be difficult to manipulate and did not fit properly. As an alternative, a standard 1ml pipette was tested. This would have the advantage of greater accuracy and ease of use. In addition, using pipette tips instead of syringes for every new reagent or sample to be applied to the sector, would reduce costs. A spider was constructed with the filling holes reduced in size to accommodate a blue tip. Using Trypan Blue/PBS, the sectors were flooded using a 1ml Gilson pipette which was found to be easy to manipulate and quicker than a syringe. It was decided to use the pipette in place of the syringe although it should be noted that a 1ml syringe could also be used with these smaller holes but with a less control over the flow of the liquid. The bottom disc of the spider was completely plain in early work but later an aluminium coated compact disc was used that functioned in the same way and was more like the intended final product (Figure 3.2). In the final structure of the disc, the spider would have an aluminium-coated disc for tracking and information storage.

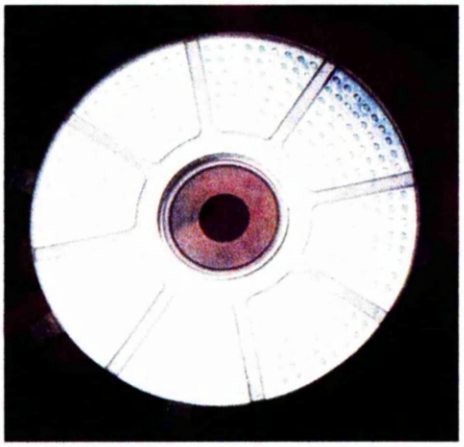


Figure 3.2 : Spider structure shown with sectors after an ELISA

The final change to the spider involved the incorporation of a clip mechanism into the bottom disc. This was to aid holding the sector in place and to increase fit thus reducing leakage. This spider was not constructed from plastic in the University of Glasgow, IBLS, Mechanical Workshop. The bottom disc and the separators were a complete piece of plastic with a clip mechanism to hold each sector in place. Individual pieces of plastic containing the filling hole were then glued over the top of each clip giving a complete spider. This was used purely for performing the assay and not in the modified CD player and therefore did not have an aluminium lower disc or a hole in the centre for placing in the machine.

There had been a number of problems with this sector/spider based system. Once the ELISA had been completed, the sectors were removed from the spider, dried and glued to an aluminium-coated compact disc before being placed in the reader. There were two reasons for this. The first was that as the disc is spinning while being read, the sectors are not secured tightly enough in the spider to prevent them coming out of the spider. The second reason is that due to the nature of the flood-fill technique there is always liquid left in the wells, which would spin out of the wells while the disc was being read. A way of securing the sectors in the spider and preventing the liquid from getting into the CD reader had to be found. The problem was that in order for the sectors to be held tightly, they would then be unable to be removed from the spider. One solution was a clip mechanism that could snap up and hold the sector in place. The sector could then be removed from the spider after reading had taken place. The question of preventing the liquid from escaping into the machine was thought to require a snap-up back wall that would seal the sector. The solution to both problems could possibly have been incorporated into one. However since producing another mould of the sectors with these additional requirements was going to be very expensive and take a significant length of time, it was decided to pursue another area.

3.3 Development of the black disc.

While the original sectors were being moulded, a series of experiments were carried on plain plastic compact discs to refine the assay protocol. There were no separate wells on these discs

and the reagents were applied directly to the surface. Following on from this a spider was taken apart and the bottom disc was used for carrying out an ELISA. Drops (4μl) of antigens (Human Serum Albumin, α-1-Antitrypsin and Antithrombin III) were applied directly to the surface of four "sectors" and left overnight at 4°C. An acetate cover was made with filling holes in the appropriate place. The remainder of the ELISA was carried out as usual with the "sectors" being left flooded during each incubation. Washing was by a modified flood-fill method using a 5ml syringe. It was found that the individual primary antibodies from the panel of could be detected in each of the three "sectors" with very low background and little cross-reaction. The fourth sector showed a positive reaction for all four antibodies as expected. This showed that it was possible to carry out a multianalyte ELISA on a modified compact disc without the presence of wells. At the time this result was not explored further, but the problems with the sectors and spider forced further examination.

A batch of aluminium-coated discs had previously been adapted for another purpose with a black label and the pattern of eight sectors.

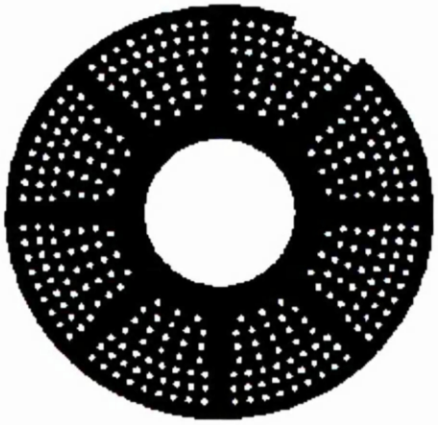


Figure 3.3 : Black disc showing pattern of eight sectors of 44 wells each.

They had not been used for their designated purpose. This provided a disc with "wells" and "sectors" already designated that could be used for further investigation. The advantage of this would be that once the ELISA was completed, the disc could be dried and placed directly in the

reader without the need for a securing mechanism and there would be no excess liquid left. The disadvantage was the loss of the flexibility the sectors provided. Initial results were encouraging with much higher OD values being produced compared to the sectors so it was decided to use the black discs for the remainder of the project.

A spider-like structure had to be constructed for the application, removal and incubation of reagents. This was done by the, Mechanical Workshop of the Institute of Biomedical and Life Sciences (IBLS), University of Glasgow. Once coated with antigen, the black disc is placed in the spider, the silicone rubber separator is placed between the "sectors", the lid is then placed on top and clamped tightly using the screws. This was found to be a very effective method of keeping the sectors separate and there was no apparent cross-contamination. The filling hole was in its usual place and there was a series of holes at the outer edge of the sector to allow for air displacement. A 1ml pipette was used as before for applying and removing reagents. Once the assay is complete, the screws and silicone rubber are removed and the disc allowed to dry before being placed directly in the reader.

3.4 Modified compact disc player.

This was a standard compact disc player with a red laser incorporated to read the blue colour produced by a positive reaction in the ELISA as shown in Figure 3.4.

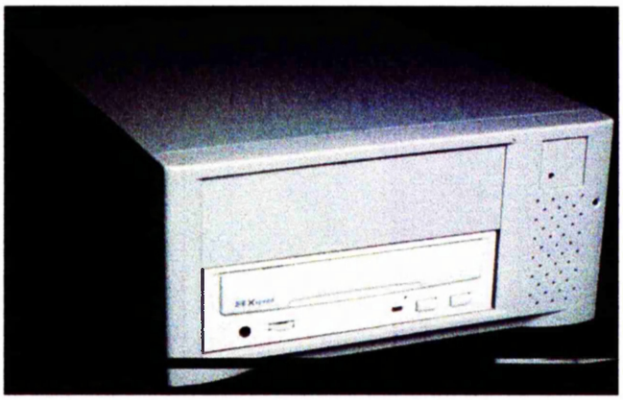


Figure 3.4: Prototype compact disc player

The existing laser in the machine is still present and is used to detect tracking information to allow the disc to run. The black disc has a strip of silver running around its outer edge. This has to be removed by the use of a black marker pen such that a only a small silver strip remains above a designated sector. Currently the machine only reads from well five (reading from the left hand side) in row two (reading from outer to inner wells). This requires that well three of row two be black for the tracking to proceed. This will be altered so that all the wells can be detected in future work.

In conclusion the development of the compact disc to a suitable platform for immunoassays has had three main forms – the original disc with Transpaseal-coated wells, the sectors and spiders and the black disc without removable sectors. The best results were produced with the latter due to problems with keeping the sectors in the spider while in the reader and detection of colour in the wells. The disadvantage of not using the sectors was that there was no longer any element of choice in what sectors were used in the spider. The major advantage of using sectors was that they could be used in two ways – either one patient tested against eight different panels of immunoassays e.g. for screening purposes or eight different patients tested against the same panel. A whole series of sectors could be constructed consisting of logical panels of tests. This advantage was lost with the black disc, although different sectors on the disc could be coated with different immunoassay panels.

CHAPTER 4

ATTACHMENT OF ANTIGEN TO THE SOLID PHASE.

In the next six chapters each individual step in the ELISA will be described in terms of its background and progress towards the development of the compact disc as a platform for immunoassays. These chapters will cover antigen attachment to the solid phase, blocking of non-specific binding, primary antibody binding, secondary antibody binding and colour development via a substrate. There is some overlap between the chapters as the steps are linked in this type of assay and it is difficult to examine them individually. This first chapter deals with antigen attachment.

4.1 Plastic.

The plastic used for the construction of the sectors was medical grade polystyrene although the quality was not the same as that of microtitre plates. This led to problems that will be discussed later and was part of the reason for moving to the black discs without wells. The plain plastic compact discs are made of polycarbonate. This plastic has high strength, toughness, heat resistance, excellent dimensional and colour stability. It is also naturally transparent with light transmission of up to 92% which is nearly that of glass. Biological molecules adsorb well to polycarbonate but its high price has restricted its use in microtitre plates.

4.2 Dinitrophenol and Rabbit IgG ELISAs on prototype discs and sectors.

The DNP and Rabbit IgG ELISAs were tested to show that they would be suitable for use in the development process. The aim was to have a straightforward, routine assay that could be tested on the various developmental stages of the sectors. The two assays were simple - the DNP ELISA required a secondary goat anti-rabbit IgG-horseradish peroxidase or alkaline phosphatase conjugate to detect the anti-DNP primary antibody colorimetrically. The rabbit IgG ELISA was simpler and required only an alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG to detect the coated antigen. The use of two second antibodies conjugated to two different enzyme labels extended the range of possible substrates. Initially the DNP ELISA was tested with Orthophenylenediamine Dihydrochloride (OPD) as substrate which produces an orange-brown colour. It was established that these assays would be suitable and were used for investigations into a suitable substrate. The aim was

to find a substrate that would produce an intense blue colour suitable for use in the modified CD player. Two types of microtitre plates were used in these experiments - specific ELISA 96-well plates and cell culture plates to investigate any differences with the insoluble substrates on the different surfaces but none were observed. The term "insoluble substrate" is used to describe those substrates that do not produce a soluble end product i.e. a precipitate is left on the plastic. All estimations at this stage were made by visual examination of the blue colour produced. The substrates tested were originally developed for immunohistochemistry and immunoblotting (see chapter 8).

An early finding was that the colour produced by the insoluble substrates on the plastic was not very intense, which increasing the antigen concentration did not improve. This was later found to be true for most of the other substrates used possibly due to their lack of sensitivity in determining different antibody concentrations in this technique. The first ELISA attempted on disc was in its original format i.e. a plain disc with four segments each consisting of 48 wells sealed with Transpaseal. Wells were coated with rabbit IgG (10µl, 10µg/ml) and although the assay was successful, there was little gradation between the serial dilutions of anti-rabbit IgG-HRP. A range of antigen concentrations from 10-100µg/ml was tested using HRP and alkaline phosphatase-conjugated anti-rabbit IgG, to increase colour density. The coating volume was also reduced from 10µl to 5µl per well to reduce binding to the rough edges of the well. Increasing the antigen concentration did not appear to substantially increase colour density but reducing the coating volume reduced the "edge effect" produced by the rough edges. This was confirmed by using bovine serum albumin conjugated to fluorescein isothiocyanate (BSA-FITC) and poly-L-lysine conjugated to fluorescein isothiocyanate (poly-L-lysine-FITC). They were coated on a disc with "wells" drilled in it and showed much greater fluorescence in the areas of the rough edges. The same experiment repeated on a 96-well plate showed a similar coating density as the disc which indicated that the antigen was attaching properly to the plain disc surface.

Insoluble 3,3', 5,5'-Tetramethylbenzidine (TMB) was found to produce a suitable blue colour for the assay (see chapter 8) and the effect of antigen concentration on colour density was examined on plates and disc (with Transpaseal). On both, a range of rabbit IgG concentrations from 10-500μg/ml was tested. On the plate there was no significant alteration in colour density while with the disc there did appear to be some increase in colour but its significance could not be determined and may have been due to the sticky nature of the Transpaseal surface. When repeated on plain discs there appeared to be a very limited range of colour produced with both rabbit IgG and DNP. This appeared to indicate that the insoluble TMB was not quantitative when used in this technique. Testing various combinations of antigen and antibody concentrations with both DNP and rabbit IgG ELISAs showed similar results - the colour produced did not vary greatly with antigen or antibody concentration apart from below a threshold level below which colour was very pale. This was also repeated on the prototype sectors prior to moulding and similar results were produced. The drawback of using these sectors was that they were at this point still sealed with Transpaseal which, could have produced erroneous results. The effect of coating poly-L-lysine on to the surface of the sector or plate prior to antigen coating was not found to have any significant effect even when incubated at 37°C.

4.3 Antigens in multianalyte ELISA.

Once the moulded sectors had been produced they were tested with the DNP ELISA and insoluble TMB. This produced a strong blue colour that at the time was thought to be intense enough for the reader. The construction of a multianalyte or panel ELISA followed to show that it was possible to carry out more than one ELISA using the sectors. A panel of seven ELISAs was constructed by sequential addition of each to the sector. Each antigen and corresponding antibody were first tested on a 96-well plate with insoluble TMB to determine the appropriate concentrations and then transferred to the sector. Human Serum Albumin was tested first, then α-1-Antitrypsin, α-2-Macroglobulin, Antithrombin III, Catalase, α-1-Antichymotrypsin and Plasminogen. These were all human antigens with the corresponding antibodies raised in rabbits. Human Serum Albumin is the major blood protein present at high concentration with a

molecular weight of 69000. Alpha-1-Antitrypsin is a serine protease inhibitor of molecular weight 52000, that also acts as a major physiological regulator of elastase. It is a single polypeptide chain of 394 amino acids with three carbohydrate side chains linked to asparagine residues and is present at 290mg/100ml in acute phase plasma[95]. Alpha-2-Macroglobulin[96] is a major serum protein. It is a broad range irreversible protease inhibitor that forms a "trap" around proteases in the clotting and fibrinolytic system and also controls macrophage nitric oxide synthesis. It is a very large molecule of molecular weight 725000. Antithrombin III[97] is a single chain glycoprotein that forms complexes with serine proteases of the blood coagulation system e.g. thrombin, kallikrein. Its potency is enhanced by heparin and its molecular weight is 65000. Catalase[98] controls the levels of oxygen-derived free radicals in mammalian cells together with superoxide dismutase and glutathione peroxidase and in combination may function as a somatic oxidant defence mechanism. The molecular weight of Catalase is 256000. Alpha-1-Antichymotrypsin[99] functions as a specific inhibitor of chymotrypsin-like serine proteases and is present at a concentration of 45mg/100ml in plasma. It is found clinically in inflammatory conditions, some malignancies, Crohn's disease, ulcerative colitis and burn injuries and has a molecular weight of 68,000. Plasminogen[100] is a single chain glycoprotein zymogen, which is synthesised in the liver and circulates in the plasma at a concentration of 2.4uM. It is converted to plasmin by urokinase, streptokinase or tissue plasminogen activator and has a molecular weight of 90000.

On the addition of each antigen to the panel, it was checked for cross-reaction with the others. There was a slight cross-reaction with anti-Antithrombin III producing a faint positive reaction with α-2-Macroglobulin. This was at a low level and it was thought that the reader could be calibrated to allow for this. This is likely to be a problem in other more clinically relevant multianalyte ELISAs especially when serum is in use. The concentrations used in the ELISAs were designed to produce the most intense blue colour with insoluble TMB. At this time the reader was not operational and the required intensity of the coloured end product was not known. In producing the strongest colour it was felt that it could be reduced at a later stage when the laser in the reader had been calibrated.

With all the antigens, the coating concentration was tested over a range that had produced a strong blue colour with insoluble TMB in earlier experiments. Human Serum Albumin coating concentration was tested from 10μg/ml-1mg/ml in a 96-well plate with insoluble TMB. There was good gradation between the different antigen concentrations up to 100μg/ml. Alpha-1-Antitrypsin and α-2-Macroglobulin coating concentrations were tested over the range 10-200μg/ml due to the results with HSA. If there were gradation over 100μg/ml this would have been shown by the 200μg/ml wells being significantly higher in colour. For both α-1-Antitrypsin and α-2-Macroglobulin there appeared no significant difference between coating concentrations of 20-200μg/ml therefore initially 50μg/ml was used. Antithrombin III was also tested over 10-200μg/ml and produced good gradation. The best range of colour was produced from 50-200μg/ml and the best Antithrombin III concentration was judged to be 50μg/ml. Catalase was tested over the concentration range 10-100μg/ml and produced a very strong reaction resulting in 20μg/ml being used as the coating concentration. The final two antigens, α-1-Antichymotrypsin and Plasminogen were also tested using the concentration range 20-200μg/ml and again 20μg/ml was used. A further two antigens – α-2-Antiplasmin and α-1-Acid Glycoprotein - were also tested but produced no colour on microtitre plates or sectors. Alpha-2-Antiplasmin[101] is the primary inhibitor of fibrinolysis. It is a single chain glycoprotein of 453 amino acid residues and forms a covalent complex with plasmin thus causing its inactivation. It has a molecular weight of 70000. Alpha-1-Acid Glycoprotein[102] consists of about 40-45% carbohydrate and is present in plasma at concentrations of 55-140mg/ml. It is an acute phase reactant that with haptoglobin indicates acute inflammation and is useful in diagnosing liver problems. It has a molecular weight of 44100. The reason for the lack of colour with the latter two antigens was unclear but in the case of α-1-Acid Glycoprotein, it may have been due to its high carbohydrate content inhibiting binding to the microtitre plates and sectors. As initial proof of principle, it was decided that seven analytes in a multianalyte ELISA were sufficient and no more were added at this time.

The antigen coating concentrations of Human Serum Albumin, Antithrombin III, Antitrypsin and Macroglobulin were reduced from 50 to 20μg/ml as no significant difference could be observed visually between them. Later experiments showed that with all the antigens tested, colour was produced down to a concentration of 3μg/ml. When this was reduced to 1μg/ml there was very little colour. Colour appeared to decrease dramatically after 10μg/ml, with 5μg/ml antigen concentration being the lowest concentration likely to be detected in the reader. It should be remembered that this was using a low primary antibody dilution (see chapter 6). Colour was produced with some antigens eg Catalase at a concentration of 500ng/ml after which colour decreased dramatically, but this was not reproducible. For most of the work on sectors an antigen coating concentration of 20μg/ml was used as it produced strong colour with all antigens. The idea was to reduce this later once the reader was operational and had been calibrated. One of the most important problems encountered during the work was that of the variability in the colour produced which made it difficult to decide on antigen and antibody concentrations and also incubation times and meant that experiments had to be repeated to confirm results. This seemed to be primarily due to the reaction between the peroxidase conjugated second antibody and the insoluble TMB which produced a variety of colours from green to blue. In addition at high primary antibody concentrations there was an "over-reaction" with insoluble TMB that resulted in a grey colour being deposited in the wells (see chapter 8). This resulted in a lack of reproducibility between sectors and the production of a range of colour for the same ELISA on different sectors. For example, the reproducibility of Antithrombin III, Antitrypsin and Macroglobulin ELISAs at a coating concentration of 50μg/ml was tested. Four sectors were coated with the above three antigens and then exposed to a mixture of all three primary antibodies. There was a great variation in the colour produced both between and within sectors with the outer wells appearing bluer and the inner wells more green. This made it difficult to determine visually if a change in colour under different experimental conditions was relevant. This variation did not seem to appear when microtitre plates were used with insoluble TMB as substrate with a more stable and reproducible blue colour being deposited on the wells with very little variation. On the sectors the colour faded to green then grey. On the plates the colour remained stable for several months with little change in colour.

4.4 Antigen coating volume.

Initially the antigen coating volume in the sectors was 4µl per well although it was known that the minimum coating volume was 2µl. A smaller volume than this did not cover the bottom the well thus preventing adequate washing with air bubbles collecting in the wells. Bubbles in the wells or on the sector surface were found to reduce washing efficiency. To determine the best minimum coating volume, antigen coating volumes of 2, 3 and 4µl were tested with the Human Serum Albumin ELISA and it appeared that the smaller the coating volume the deeper the blue colour produced. When this was repeated and tested with the other antigens it was found that reducing the antigen volume to 2µl did not reduce the colour intensity, as observed visually.

4.5 Incubation times.

One of features of this system is the reduction in incubation times for all steps of the assay. The antigen incubation time was overnight at 4^{o}C or two hours at room temperature. For the initial testing of antigens on sectors the same protocol as the microtitre plates was followed and antigens were coated for the times above. This was gradually reduced to 60, 30, 20, 15, 10 and 5 minutes at room temperature. The colour produced was still significant even with only 5 or 10 minutes incubation time, but due to the variability and reproducibility problems a standard 15 minute incubation was decided upon because it consistently produced an intense blue colour. It was intended that once the reader was working the incubation times could be determined accurately.

4.6 Use of silicone spray on sectors and spiders.

One of the problems associated with using this type of system was getting the liquid to flow properly between the sector surface and upper disc when using the flood-fill technique. It was found that bubbles were produced when using Tween 20 in the wash buffer or BSA in the antibody diluting buffer that could get trapped in the wells. This was due to the nature of the flood-fill process and the hydrophobic nature of the two surfaces. Tween 20 was present at a concentration of 0.05% and the effect of reducing its concentration or removing it from the concentration resulted in higher non-specific binding. Any air bubbles in the wells could not be

removed and meant that the well had to be discounted. Air bubbles on the sector surface, commonly in the bottom corners, meant that wells in that region could not be washed properly and the colour produced was not reliable.

Originally silicone grease was smeared over the surface of the upper disc in contact with the liquid. This did help fluid movement and reduced the number of bubbles but had a number of disadvantages. It had to be re-applied periodically and some of the reagents appeared to "stick" during flood-fill i.e. antibodies, which was found to interfere with the assay. An alternative was to coat the upper disc of the spider with silicone spray before use. This was in effect a silicone polish with the excess being removed by wiping with a paper towel. This was found to allow liquid to flow somewhat easier and also reduced the formation of bubbles in the wells and on the sector surface but was not as effective as the silicone grease. To further enhance the flow of the liquid, the sector surface was also coated with silicone spray. Liquid did indeed flow more easily but a striking by-product was the intense blue colour produced in the wells with insoluble TMB on performing the multianalyte ELISA. This was repeated with individual antigens and again found to significantly enhance colour production with an intense blue colour being produced with all antigens. There was no green or grey colour produced, the colour did not fade as quickly and it appeared more like that produced on microtitre plates. It was decided to use the silicone spray as it did improve liquid flow but more importantly increased colour intensity and stability. It was important not to spray a heavy layer of silicone on the sector surface or this accumulated in the corners of the wells thus reducing the area of the blue colour, although it did not affect colour intensity. It was postulated that the silicone was making the reaction more hydrophobic and stabilising the colour produced by the reaction between insoluble TMB and peroxidase-conjugated second antibody. However when tested with the α-2-Antiplasmin and α-1-Acid-Glycoprotein there was still no colour produced.

It was later found that also contributing to the lack of reproducibility were the "spiders". These were held together with double-sided sticky tape covered separators and were re-used numerous times. This led to the separators becoming less sticky and leakage occurring between different

parts of the spider. This made washing very difficult and cross-contamination inevitable and was the reason for producing the spider with continuous separator and bottom disc. A comparison of colour production and background when the same spider was used three times was made and showed an increase in background, the more the spider was used.

4.7 Use of fluorescent second antibody.

At this time the sectors producing a strong positive reaction were being glued onto a silver disc and placed in the reader, which appeared to be detecting colour at a very low level. There were several possible reasons for this including the plastic of the sectors and related to this the well surface. If coating was not over the entire well surface, this may explain the poor detection by the reader, although at this time the optics of the reader had not been optimised. In order to test coverage of the well surface by antigen, the multianalyte ELISA was performed with a fluorescent second antibody (FITC-anti-rabbit IgG) and a peroxidase-conjugated second antibody (anti-rabbit IgG-HRP) to confirm the protocol was working with insoluble TMB. The sectors were analysed under a fluorescent microscope and showed either no fluorescence or very patchy "dots" of fluorescence. The control sectors produced a positive result indicating the protocol was working. Sectors coated with and without silicone were tested and showed similar results. Examination of sectors under the microscope showed that the surface of the sectors was scratched, uneven and that the plastic contained debris. They were therefore of optically reduced quality as compared to the plastic of microtitre plates. This would affect antigen coating and detection of colour by the reader. The sectors were sonicated in detergent for forty-five minutes to remove any surface debris and left overnight at 4^{o}C with FITC-bovine serum albumin in the wells at concentrations of 5 and 20μg/ml. Microtitre plates and plain discs were also tested with BSA-FITC for comparison. Even though there was not even coverage with BSA-FITC on the plates and plain discs there was significantly less with the sectors - fluorescence was localised in the scratches and edges of the wells and was very patchy. Sonicating the sectors made no difference to the coverage. Antigen concentration would also affect the coverage and coating at a high concentration of 20μg/ml could result in the surface of

the well being too overcrowded. Previous work had shown however that such a coating concentration was required to maintain colour intensity. A range of coating concentrations (10 ng/ml-100μg/ml) was tested using Catalase in microtitre plates, siliconised and non-siliconised sectors. With the plates the wells coated with the highest concentration of antigen over-reacted but on examination under the microscope, well coverage was good, gradually reducing as the antigen concentration decreased. In non-siliconised sectors, green colour was produced while with siliconised sectors the colour was blue. On examination under the microscope the siliconised sector had large regular white circles that interrupted the colour. These were not present in the non-siliconised sectors where the coverage was more even but there were shades of green rather than one shade of blue. The white circles in the siliconised sector were due to the aerosol nature of the silicone spray and were deposits of silicone that could not be removed due to the small size of the wells. These deposits prevented antigen binding. Using liquid silicone in the wells still left some droplets and it was difficult to remove the excess from the wells. It would therefore appear that when using the sectors, silicone is required for development of a strong blue more reproducible colour with insoluble TMB but it affects the antigen coating by silicone aerosols being deposited in the wells that can not be removed.

4.8 Black Disc

The finding of this stimulated the move to the black discs as described in chapter 3. Initially these discs were tested with Catalase (20μg/ml) with 1 or 2μl of antigen coated on each silver "well". The standard incubation times on the sectors was used. With no "spider", a wash bottle was used between steps, which was not ideal. Later, the new spider described in chapter 3 was used in place of the wash bottle. Antigen was washed off after fifteen minutes incubation and the assay completed using a separator from an old spider to separate sectors. An intense blue colour was produced in all wells containing Catalase with the 1μl wells appearing slightly less intense. Once the ELISA was completed the disc was air-dried and placed in the reader for analysis. Figure 4.1(a) shows the OD values and Figure 4.1(b), the coefficient of variation (CV) produced.

A higher OD value was found with a coating volume of 2µl. The CV was slightly higher with the 1µl coating volume although this was not found to be reproducible. At the start of the experiments with the black disc, the CV was very high at over 15%. This CV was within one well i.e. the variation between readings in the same well. At the time, the aim was to investigate the CV between wells and then between sectors on the black disc but this was not accomplished in the duration of this project. The intra-well CV was eventually reduced to below 6% for most of the wells but again there was great variation. The coating volume was investigated further at a later date. The main problem with the black discs appeared to be a "streaming effect" from the wells coated with antigen. This produced streaks of blue colour following the direction of the antigen when it was washed off indicating that the antigen must be attaching very quickly to the disc. This was tested by applying Catalase to all wells in a "sector" on a black disc and washing off immediately using a wash bottle. The antigen was left on for one minute twenty seconds (coating time for one sector). The ELISA was then completed as normal. The colour produced was a strong blue, slightly paler than the colour after fifteen minutes antigen incubation, with extensive streaming. This showed that the antigen is attaching very quickly to the disc surface. A number of strategies were tested to remove the streaming. Using flood-fill with an upper disc and separator from an old spider still produced streaming, as did removing excess antigen with a pipette. Removing the antigen and using combinations of primary and secondary antibodies did not produce streaming indicating that it was the antigen and not non-specific binding of another assay component. It appeared that the best way to do this was to dry the antigen onto the well. Catalase (20µg/ml) was coated onto a black disc in 0.5 or 1µl volumes, dried at 55^{o}C or room temperature and without washing, assayed as usual. This produced some streaming but less than the previous disc. A higher OD value was produced with the sectors dried at room temperature. For the black discs to work without wells, there had to be no streaming and therefore no cross-contamination between wells. The best option appeared to be drying the antigen then drying the blocker (50mg/ml BSA/PBS) directly on top of the antigen at room temperature. This required to be washed off before adding primary antibody was applied and the assay continued as normal. This did not produce any streaming.

Increasing antigen coating time to thirty minutes was tried as a way to improve the colour but it was not significantly increased.

In order to compare the colour production and hence OD values on sectors and black disc, Catalase (20μg/ml) was coated onto a black disc (1μl/well) and eight siliconised sectors (2μl/well). Antigen and blocker were dried onto the black disc while in the sectors they underwent the standard incubation times of fifteen minutes each. Strong blue colours were produced on both sectors and the black disc but the OD values were much higher with the black disc (0.4-0.6 absorbance units) as shown in Figure 4.2(a). The OD values with the sectors were less than 0.1. Figure 4.2(b) shows that the CV values were lower with the black disc. This proved that the black discs produced much better OD values even though on visual examination the colour produced by both sectors and black disc was similar. There was still a lack of reproducibility between wells and between sectors.

Figure 4.1(a): Comparison of the OD values produced with antigen coating volumes of 1 and 2µl.

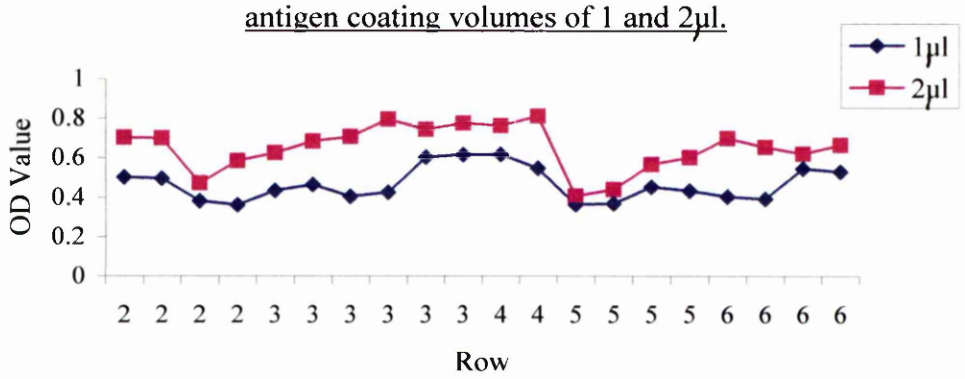
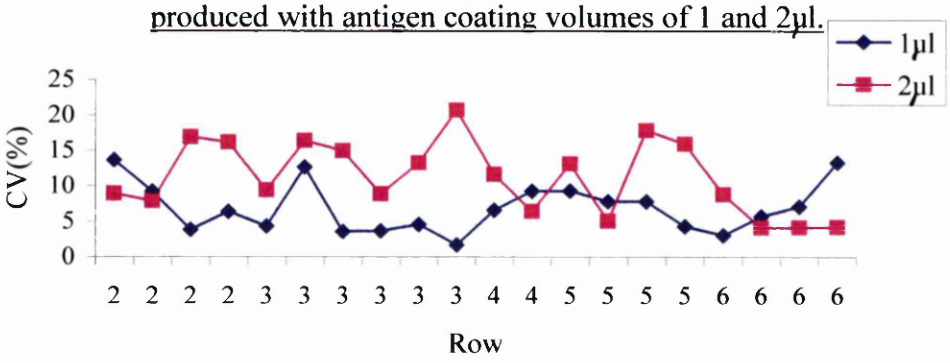
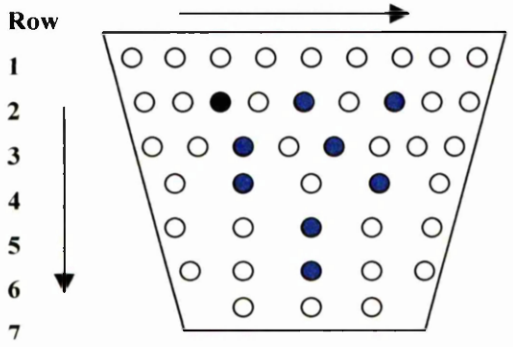


Figure 4.1(b): Comparison of the coeficient of variation produced with antigen coating volumes of 1 and 2µl.





The above diagram shows the well layout for Figure 4.1(a) and 4.1(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 16 data points on the graphs from 8 wells.

Figure 4.2(a) : Comparison of the absorbance produced when using sectors or black discs (no sectors).

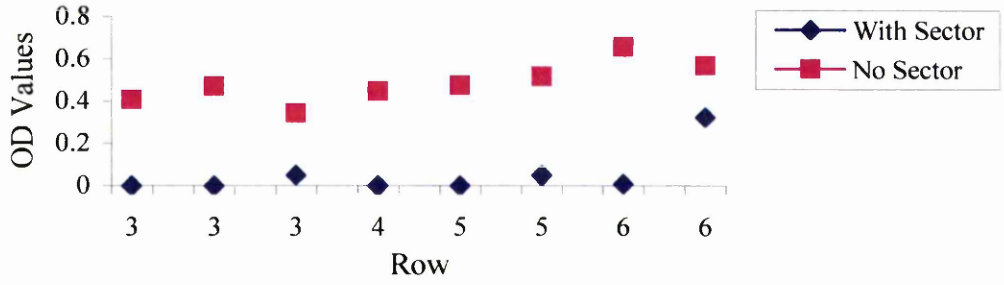
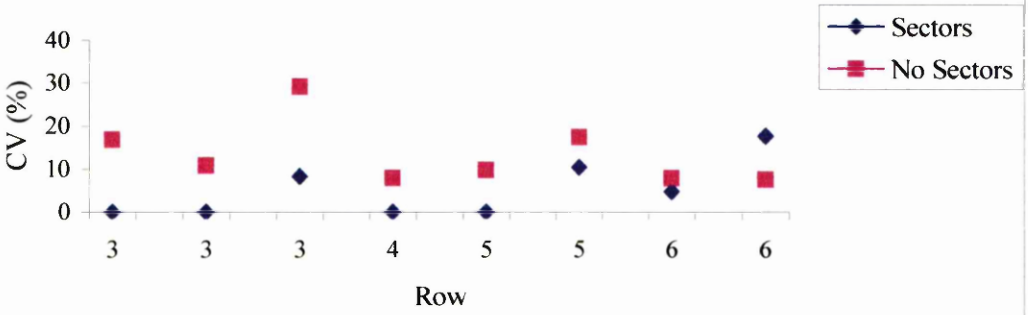
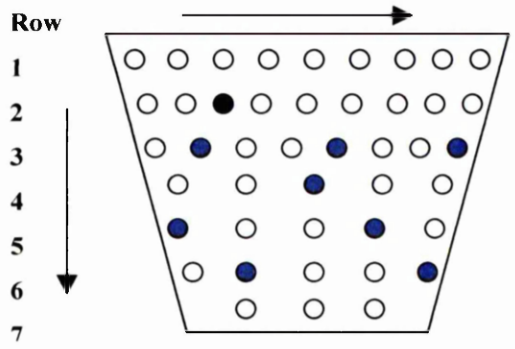


Figure 4.2(b) : Comparison of the coefficient of variation produced when using sectors or black discs (no sectors)





The above diagram shows the well layout for Figure 4.2(a) and 4.2(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured once in this way giving a total of 8 points on the graphs from 8 wells.

The next step was to test the multianalyte ELISA on the black disc. This was first of all done without drying the seven antigens (1μl/well) and blocker (2μl/well) and produced serious cross-contamination between wells with antigens streaming from inner to outer wells. When this was repeated with drying down all seven antigens and blocker, a strong blue colour was produced with no streaming or cross-contamination. Therefore using this drying protocol, the multianalyte ELISA could successfully be performed on the black disc without the presence of separate wells. Several different antigen coating volumes were tried. Previous work had shown 2μl to be the best antigen coating volume but this was prior to drying the antigen on the wells. The minimum volume tested was 0.5μl but this proved difficult to pipette successfully onto the disc. Volumes of 1, 2 and 5μl were tested using Catalase (20μg/ml) which was dried at room temperature followed by blocker which was also dried at room temperature.

Figure 4.3(a) shows the very high variability produced - OD values varied from 0.05-0.35 absorbance units, although most of the CV values were below 6% (Figure 4.3(b)) which was very much reduced from the early experiments on black discs. It was decided to use 1μl of antigen as the antigen coating volume, as this was easy to pipette and seemed to produce less variable OD and CV values.

Figure 4.3(a): Absorbance values produced on drying different volumes of antigen onto a black disc.

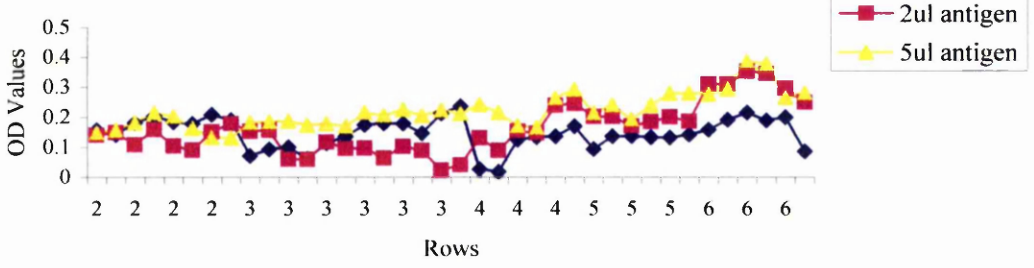
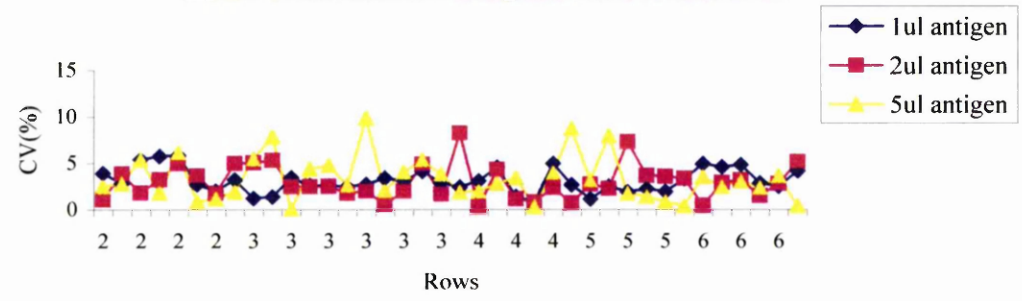
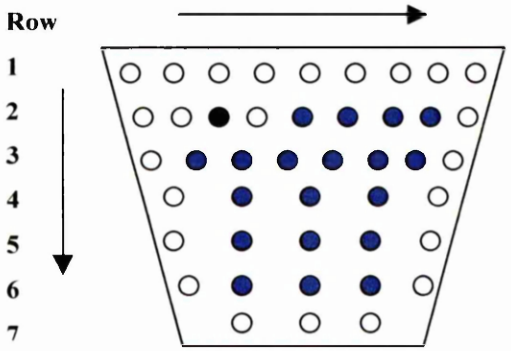


Figure 4.3(b) : Coefficient of variation produced on drying different volumes of antigen on to a black disc.





The above diagram shows the well layout for Figure 4.3(a) and 4.3(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 38 points on the graphs from 19 wells

4.10 Effect of temperature on antigen coating.

The effect of coating antigen at different temperatures was also investigated. Drying at room temperature (22°C) took about forty-five minutes and it was thought that increasing the temperature might serve to enhance antigen binding. A hairdryer (72°C) was used to dry the antigen only and then antigen plus blocker using Catalase (20μg/ml, 2μl). Streaming was produced when only the antigen was dried and seemed to produce a slightly higher but more variable OD value. Comparison with drying at room temperature showed a significantly reduced OD value when the hairdryer was used. Incubating the antigen and blocker for fifteen minutes before using the hairdryer (22+72°C) appeared to produce higher OD values than drying without incubation. Comparing the CV values of drying with hairdryer, drying with hairdryer plus incubation and drying at room temperature showed that drying at room temperature had the highest CV with the other two being difficult to separate. This was due to the great variability in values. An antigen coating temperature of 37°C was tested and comparison with the other coating temperatures (Figure 4.4(a)) showed that for all sectors, the highest OD value was with drying at room temperature.

The next best temperature was 37°C, although this was not found with all sectors. The other temperatures produced similar OD values but with reduced coefficient of variation (Figure 4.4(b)). The temperature order varied between sectors and the results above reflect the majority although those dried at room temperature were always best. The final coating temperature tested was overnight at 4°C. This produced slightly higher OD values compared to room temperature, although this was not considered significant enough to use this coating temperature routinely, although it was used for some experiments.

Figure 4.4(a) : Effect of different antigen coating temperatures on absorbance.

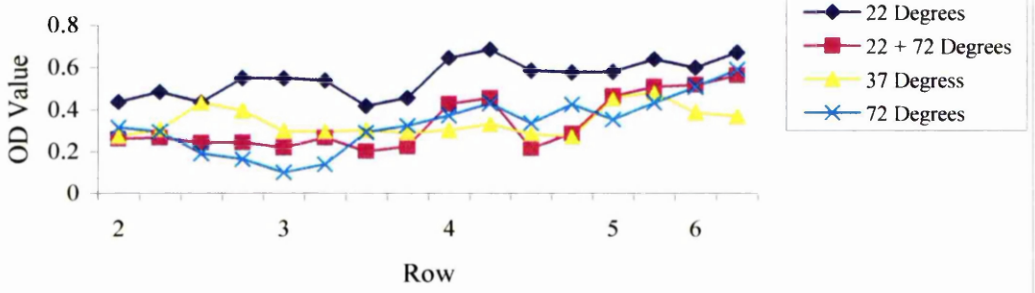
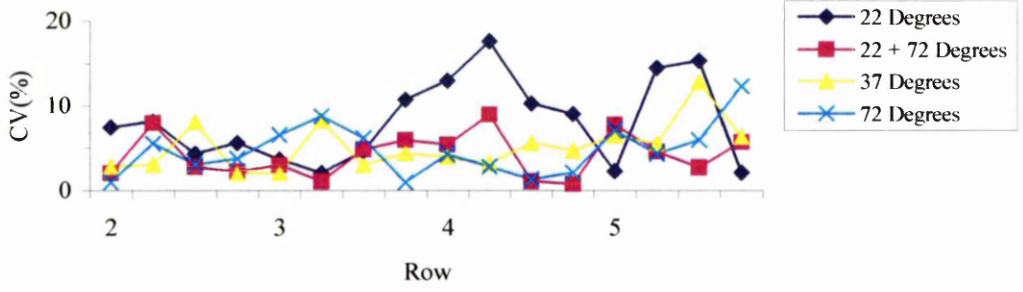
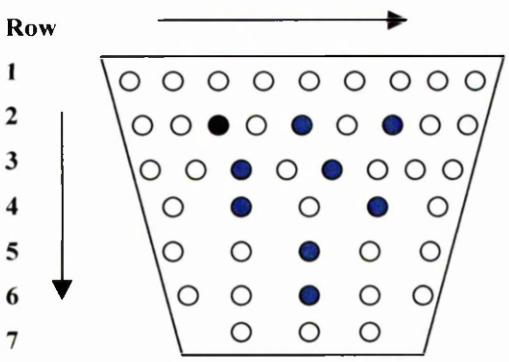


Figure 4.4(b) : Effect of Different Antigen Coating Temperatures on the Coeficient of Variation





The above diagram shows the well layout for Figure 4.4(a) and 4.4(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 16 points on the graphs from 8 wells

Below is an example of the variation over a black disc dried at room temperature. Each sector produced a range of OD values sometimes varying by as much as 0.48 absorbance units. The disc shown in Table 4.1 shows the range of OD values produced in eight sectors for the same ELISA.

Sector	OD range	Change in OD value
1	0.42 – 0.68	0.26
2	0.38 – 0.72	0.34
3	0.42 – 0.82	0.40
4	0.40 – 0.62	0.22
5	0.26 – 0.65	0.39
6	0.56 – 0.74	0.18
7	0.36 – 0.80	0.44
8	0.12 – 0.60	0.48

Table 4.1: Range of OD values produced over eight sectors on a black disc.

The changes in OD value within and between sectors made it very difficult to draw any conclusions from the results. There was thought to be several reasons for the variability and lack of reproducibility on the black discs. Hand pipetting 1µl of antigen on to the wells of all the sectors on the disc was likely to introduce a large error between wells. For accurate coating of antigen automatic deposition of antigen would be required for example in an ink-jet process. Drying the antigen could introduce error because any excess is not removed by washing before blocker is applied. Other reasons include the reader itself, because the optics in the reader was not optimised and was found to have contributed to the variation. The use of insoluble TMB may also introduce variability, as it may not be suitable for quantitative estimations on a plastic surface. An automatic loading device and altering the optics proved too expensive for the duration of this project and the antigen continued to be dried to prevent streaming.

4.11 Improving homogeneity of antigen coating.

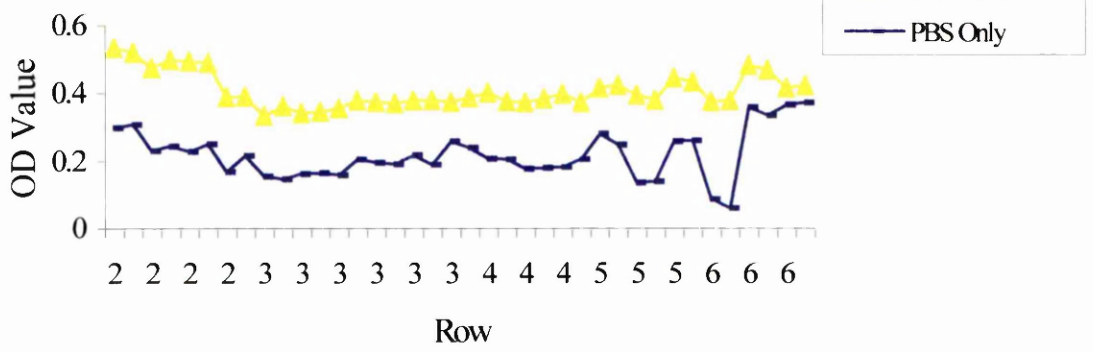
When the antigen is coated it assumed a droplet formation on the disc surface due to the nature of the plastic and did not spread properly. Encouraging the antigen to spread would increase homogeneity of binding and increase reproducibility. This was tried by using a shaker set at a

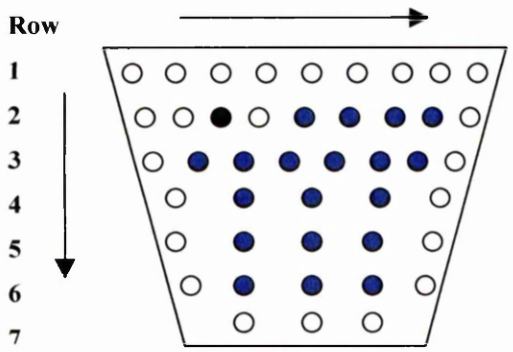
low rate to encourage even antigen distribution, but this did not reduce variation. Further investigations centred on adding different substances to the antigen coating buffer, phosphate buffered saline (PBS), to reduce surface tension of the droplet and encourage spreading. Substances tested included Tween 20, ethanol and bovine serum albumin (BSA). Tween 20 is present in the wash buffer at a concentration of 0.05% and this concentration in addition to 0.1% and 0.25% were added to PBS and used as antigen coating buffer for Catalase (20μg/ml, 1μl/well). As a control, PBS without BSA was used. The antigen and blocker were dried at room temperature. It was found that adding Tween 20 to the coating buffer did not decrease the variation in OD or CV values. The highest OD and lowest CV values were found with PBS only as coating buffer. Ethanol was added at a range of concentrations (0.005%-1%) to the coating buffer and used to with Catalase (20μg/ml, 1μl/well) which in addition to the blocker was dried at room temperature. The results showed that given the variability in OD and CV values, the presence of ethanol did not increase and in some cases appears to decrease OD values. The majority of CV values were below 5%. The third substance tested was bovine serum albumin which was used in the assay as the blocker. It was added to the coating buffer in the range 0.005%-1% in PBS and used to coat Catalase (20μg/ml, 1μl/well) on a black disc. The results showed that including BSA in the coating buffer reduced the variability of both OD and CV values. The best concentration appeared to be 0.1% BSA as shown in Figure 4.5. Visual examination of the disc showed that well coverage was more even with BSA in the buffer. Therefore, 0.1% BSA was incorporated in the antigen coating buffer with all the antigens except Human Serum Albumin . This produced no colour with BSA in the coating buffer.

Varying the antigen concentration on the black discs had shown similar results to the sectors i.e. detectable blue colour produced at 5μg/ml antigen coating concentration. This was repeated to see if the inclusion of 0.1% BSA into the antigen coating buffer changed the minimum antigen coating concentration. Antithrombin III (1μl/well) in 0.1% BSA/PBS, was coated onto a black disc at concentrations of 1, 5, 10 and 20μg/ml. Both antigen and blocker were dried and after washing, the ELISA was completed. An Antithrombin III concentration of 1μg/ml was not

detected. The other concentrations were detected with 5μg/ml being the lowest with an average OD value of 0.2 absorbance units. With 10 and 20μg/ml similar values were produced with an approximate average of 0.3. That these OD values were produced with a high concentration of both primary and secondary antibodies and yet relatively low OD values were still produced even with an antigen coating concentration of 10 or 20μg/ml. This may be due to the limitations of the insoluble TMB. Alpha-2-Antiplasmin and α-1-Acid-Glycoprotein were tested on the black discs, drying both the antigen and blocker, but produced no colour.

Figure 4.5: OD values produced with 0.1%BSA added to the antigen coating buffer.





The above diagram shows the well layout for Figure 4.5. Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 38 points on the graphs from 19 wells.

4.12 Variability in OD and CV values.

The problem with the variability in OD and CV values was thought to be due to the antigen coating and drying technique used. It may also have been caused by the laser in the reader. This was a standard red laser that had not been calibrated for use in the reader and may have been contributing to the variation. To test the laser and optics system, a black disc was covered with blue acetate. The colour was picked to match as near as possible that produced by the insoluble TMB. This resulted in a black disc with all wells an identical colour that would produce identical OD and CV values without the variation produced by the antigen coating and drying and insoluble TMB. Figure 4.6(a) shows the comparison in OD values and Figure 4.6(b) the CV values for sectors seven and eight on the acetate-covered disc. The OD values are still low and there is still large variation between wells on the same sector and also between sectors. This indicated that there were problems in the optics system to be addressed and that the variation being produced was not only due to the antigen coating and drying. Previous work had examined the effect of leaving empty wells between those coated with antigen. It was thought that there might have been interference from adjacent wells thus producing variation. Several experiments were carried out leaving one, two and three wells empty but there did not seem to be a significant improvement in OD or CV values. This was confirmed using the acetate-covered discs, where there was no significant difference in OD or CV values with two or three empty wells between those being used.

Figure 4.6a : Absorbance values produced by a black disc covered with blue acetate - comparison of sectors 7 and 8.

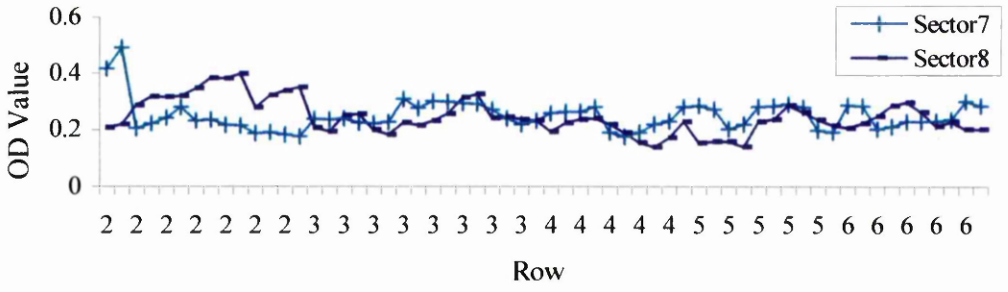
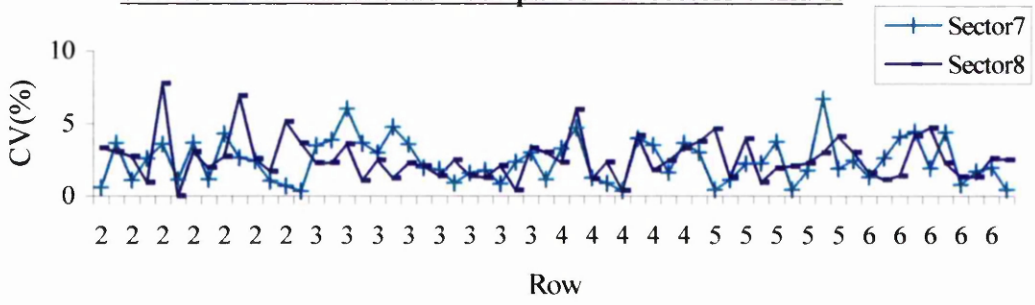
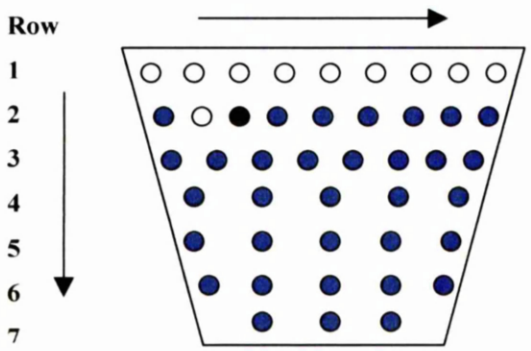


Figure 4.6b : Coefficient of variation produced by a black disc covered with blue acetate - comparison of sectors 7 and 8.





The above diagram shows the well layout for Figure 4.6(a) and 4.6(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left ro right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 60 points on the graphs from 30 wells.

In conclusion the antigens could be used to a concentration of 5μg/ml to produce colour that could be detected by the reader although the OD values was very low even with a high primary antibody concentration. Visual examination showed that colour was detectable to an antigen concentration of 3μg/ml but the best coating concentration was 10 or 20μg/ml which although high produced the best colour for use with a limited range of primary antibody concentrations. The coating volume in the sectors was 2μl with an incubation time was fifteen minutes. On the black discs the antigen coating volume was 1μl and had to be dried to prevent streaming which was thought to make a major contribution to the variation in OD and CV values.

The next step of the ELISA involves blocking of sites on the plastic sectors and discs to which antigen has not adsorbed. This prevents the non-specific binding of antibodies at later stages in the assay.

CHAPTER 5

BLOCKING OF NON-SPECIFIC BINDING.

The aim of blocking is to reduce any non-specific binding to the solid phase by antigen and antibodies. In the ELISA protocol used in this work, bovine serum albumin was used as the blocking agent. In the preparatory DNP ELISA this was at a concentration of 50 mg/ml in phosphate buffered saline, pH 7.2 (50 mg/ml BSA/PBS). Very low background was produced and this concentration was used with the Transpaseal-covered discs where again there was little background. Using the plain discs it was found that the whole disc had to be flooded with blocker to prevent non-specific binding. The wells had to be marked out using black marker pen with antigen coated on these "wells". Due to the hydrophobic nature of the plastic, once the antigen was washed off it was difficult to apply blocker or any of the other reagents to just the "wells". In addition, if blocker was not applied over the entire area where the wells were located, blue colour was produced outside the well due to the non-specific binding of primary or secondary antibodies. This was important later when the black discs were being used. As previously described, early work focused on the production of a blue colour that would be suitable for the reader. A normal rabbit serum was tested as a negative control in the DNP ELISA and also to investigate the use of sera on plain discs without wells. Blocking time with 50 mg/ml BSA/PBS was increased from thirty minutes to one hour on both microtitre plates and plain discs to compare colour production. The presence of serum may have caused higher background requiring more intensive blocking. However this was found not to be the case with similar blue colour being produced with both incubation times while using insoluble TMB as substrate.

5.1 Blocking of non-specific binding on sectors.

When the moulded sectors had been prepared the ability of them to be stored coated with antigen and blocked was investigated using the DNP ELISA. If this new platform were to be used commercially, the sectors would be sold pre-coated and pre-blocked in a way similar to commercially available ELISA kits. Sectors were coated with DNP-BSA (2 hours) and blocked with 50mg/ml BSA/PBS. After incubation for thirty minutes the sectors were placed at -20^{o}C for seventy-two hours. On removal, the sectors were washed, the assay completed and the colour compared with a sector that not been stored in this way. There was no apparent

difference in colour (visual examination). This experiment was repeated with the multianalyte ELISA and it was found that the sectors could be stored for at least six months at -20°C without any apparent loss of colour. A further advantage to a commercial product would be to use the sectors directly from storage at -20°C. In order to test if this was feasible, the sectors were coated with all seven antigens used in the multianalyte ELISA, washed and incubated with 50 mg/ml BSA/PBS then washed again and stored for eighteen days at -20°C. On removal the assay was completed without first washing the sectors. This produced a blue and green mixture of colours but this was in the absence of the silicone pre-coat on the sectors. It was also possible to store the sector after the primary antibody stage, but a weaker colour was produced.

As described in the previous chapter, there were some problems with higher background that were eventually solved using a combination of silicone spray to pre-coat the sectors and spiders that did not leak. One of the options explored during these investigations was the use of another blocker. Skimmed milk is commonly used in ELISA techniques so this was tested at a concentration of 2% in PBS. Incubation times were as for the BSA i.e. thirty minutes. The antigens used were Human Serum Albumin, Antitrypsin and Macroglobulin in a multianalyte ELISA with the appropriate primary antibodies both individually and together as a primary antibody mixture the substrate was insoluble TMB. There did not appear to be any significant differences in background or colour when using skimmed milk and BSA except that a more uniform green was produced with the skimmed milk but as this was before the use of silicone spray which produced a very intense blue colour with BSA as blocker this was not considered significant.

5.2 Incubation time.

As with antigen coating, it was found that incubation times with BSA/PBS could also be dramatically reduced. Blocking time could be reduced to five minutes with slightly increased background. This was carried out using the multianalyte ELISA in conjunction with a reduction in antigen incubation times, and it was found that the best colour (visually) was produced with an antigen coating time of fifteen minutes and a blocking time of ten minutes.

The antigen concentration was 3μg/ml and the volume was 3μl/well. When similar incubation times were tested on a 96-well plate using Human Serum Albumin, at concentrations of 3, 5 and 20μg/ml, very little colour was produced indicating that it was possibly the smaller volumes in the sector that led to the reaction taking place at an enhanced rate. It was very difficult to show any difference between incubation times due to the variation previously described. It was generally found that an incubation time of fifteen minutes for blocking was a good compromise until a quantitative result could be produced. Above this time, there did not seem to be an increase in colour but with five or ten minutes incubation there was greater variation in colour. This was also found with the other incubation times - even though colour was produced with less incubation, it was more variable. To further reduce background, BSA/PBS was left flooded across the sector after the wells had been filled, but this did not have any effect on the colour.

In order to increase the colour, the washing protocol was also tested as any residual blocker left in the wells may interfere with the binding of the antibodies. Experiments with the moulded sectors using Trypan Blue/PBS had shown that four washes left all the wells clear of dye. To investigate this further, the effect of different number of washes after each stage of the assay was tested using the multianalyte ELISA. One, two, three or four washes were used. It was found that at least four washes were required especially with washing out the second antibody prior to the addition of substrate. Using the black discs i.e. without wells this was later increased to six washes to reduce non-specific "cloudiness" over the sectors.

5.3 Non-specific binding on black discs.

The main problem with the black discs was the "streaming" effect. This had previously been described when the multianalyte ELISA was performed without sectors on the bottom disc of the spider. Although this worked very well, with individual antigens being distinguished, a streaming effect was noted with the colour extending towards the outer edge of the disc. This would have occurred during the first wash when the buffer travels across the sector removing excess antigen. With the black discs, drying the antigen and blocker were found to be the only

way to prevent streaming. This was carried out at room temperature, 37°C, using a hairdryer and at 55°C to increase the drying time. Catalase (20μg/ml) was used as antigen. Drying at room temperature could take up to one hour for 2μl of 50mg/ml BSA/PBS while with a hairdryer it was found to take approximately three minutes, eighteen minutes at 37°C and twenty minutes at 55°C. Initially a volume of 2μl was used to block the wells on the black discs. This was to ensure the blocking of all sites and also that the blocker was present in excess compared to the antigen which was coated at a volume of 1μl. Later work however showed that this could be reduced to 1μl without increasing background. Results (Figure 4.4(a), page 72) showed that drying at room temperature produced the higher OD values with the, 37°C next, then the hairdryer and 55°C producing similar but lower OD values. This could be improved by incubating the antigen and blocker for fifteen minutes each before drying with the hairdryer. This appeared to produce a colour comparable to that at room temperature but no time was really saved by incubating for these times.

Due to the nature of the plastic, both the antigen and the blocker sat as a droplet on the surface therefore there did not appear to be a significant increase in blocked areas using a volume of 2μl. The blocker appeared in this system to "anchor" the antigen and prevent streaming. The new spider for the black discs was developed at this time and was used to compare drying antigen and blocker with the hairdryer. Before this the discs had been washed with a wash bottle containing buffer. These results, although very variable, showed that higher OD values were produced when using the wash bottle. This may have been due to the wash bottle removing more of the excess dried blocker than the new spider. The nozzle of the wash bottle could be aimed at each well while in the spider the liquid flowed over the surface of the disc with less force. It was possible, therefore, that not all the blocker had been removed.

The effect of changing the blocking agent was examined by using 50mg/ml skimmed milk in PBS as blocker with Catalase (20μg/ml, 1μl/well) as antigen, both being dried at room temperature. A much paler colour was produced than with 50mg/ml BSA/PBS. It was also noted that it was very much more difficult to wash the dried skimmed milk after drying, with

some remaining on the wells. It was concluded that at this concentration skimmed milk was not suitable for the black discs, but lower concentrations may be easier to remove.

5.4 Volume of blocking agent on black disc.

As previously mentioned the volume of blocker used initially was 2μl, with 1μl of antigen, to ensure complete blockage of non-specific sites. In an attempt to reduce variation, one cause of which was thought to be hand-pipetting such a small volume of antigen (1μl), the coating volume was increased. To further investigate, varying volumes (1, 2 and 5μl) of Catalase (20μg/ml) were coated onto a black disc with 2, 3 and 6μl of 50mg/ml BSA/PBS respectively used as blocker. A further 1μl of Catalase was coated but blocked with only 1μl of 50 mg/ml BSA/PBS. Both antigen and blocker were dried on to the wells. The results showed high variability although most of the CV values were below 6%. The variation was not reduced with the higher coating volumes. Using only 1μl 50mg/ml BSA/PBS also did not increase non-specific and appeared to show better OD values than blocking the corresponding antigen coating volume with 2μl of blocker. From these results it was decided to use 1μl antigen to coat and 1μl blocker until the variation could be reduced and then re-examined.

The concentration of BSA/PBS was also investigated. It was thought that if the concentration of BSA could be reduced without increasing background, this may aid in colour production as less dried material would require to be washed from the well. Using the multianalyte ELISA with antigen concentrations of 20μg/ml in 0.1% BSA/PBS and a coating volume of 1μl/well with 0.5, 1, 5, 10, 20, 30 and 50mg/ml BSA/PBS as blocker showed that there was no significant difference between 20, 30, and 50mg/ml BSA/PBS and that a concentration of 20mg/ml could be used.

One of the main problems with the black disc was the formation of a non-specific "cloudiness" over the entire sector surface. This was blue in colour and was due to the non-specific attachment of antibodies on the sector surface, reacting with the insoluble TMB. The entire surface of the sector was not blocked with BSA/PBS, therefore primary and secondary

antibodies could attach non-specifically to these non-blocked areas. Blocker was applied dierctly on top of the dried antigen and then alowed to air-dry. Flooding the blocker over the whole sector after dring the antigen caused streaming. In order to reduce the non-specific binding, which could affect the reading of the disc, flooding the sector with blocker after drying the antigen was investigated. Antithrombin III (20μg/ml, 1μl/well) was dried at room temperature onto the wells followed by 20 or 50mg/ml BSA/PBS (1μl) which was also dried at room temperature. Following this, one of the sectors was flooded (15 minutes) with 20mg/ml BSA/PBS. There was a reduction in non-specific binding across the sector surface with flooding but a few wells showed slight streaming. Figure 5.1(a) shows that these OD values appeared lower compared to the sectors where the BSA/PBS was dried without flooding. The different concentrations of BSA/PBS showed relatively similar OD values. Figure 5.1(b) shows that the majority of CV values were below 5% with no method or concentration producing significantly higher or lower values.

In conclusion, there were relatively few problems with non-specific binding in the development of this new immunoassay platform, most of which came from using spiders that leaked. With the black discs there was non-specific "cloudiness" produced over the disc. This was at a fairly low level and did not appear to significantly interfere with the OD values but would have to be reduced or eliminated. The main blocking agent used, was bovine serum albumin at a concentration of 50mg/ml in phosphate buffered saline although this was later reduced to 20mg/ml. Skimmed milk was also tested as a possible blocking agent but did not have any significant advantage.

The next stage is the addition of primary antibody. This reacts specifically with the immobilised antigen with the blocking stage described in this chapter reducing or eliminating any non-specific binding between antibody and solid phase.

Figure 5.1(a) : The effect of changing blocking protocol on absorbance.

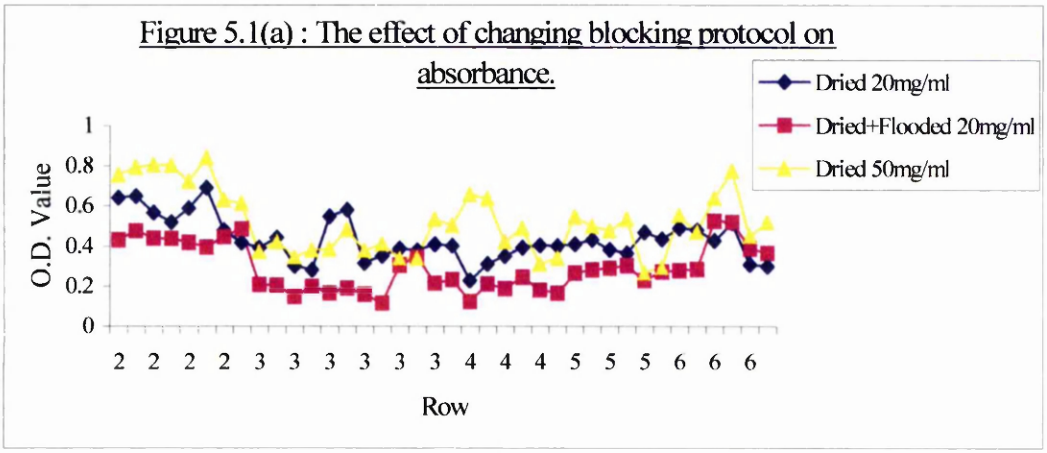
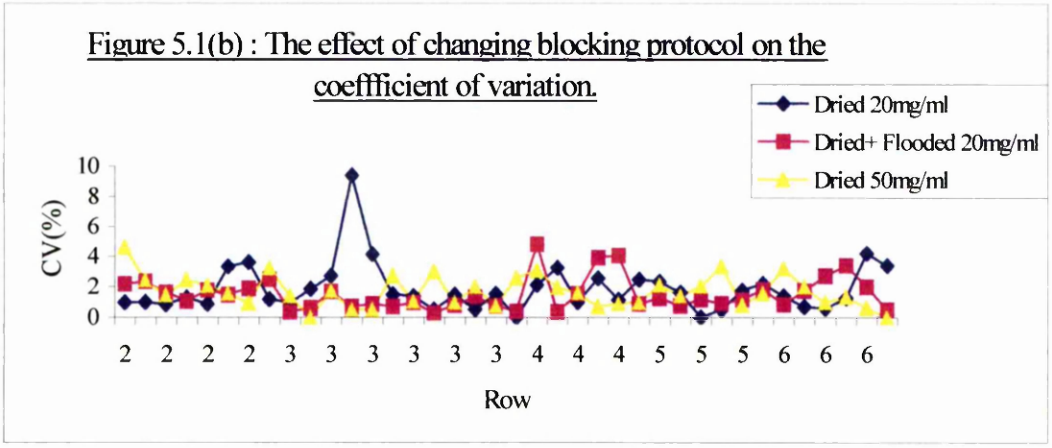
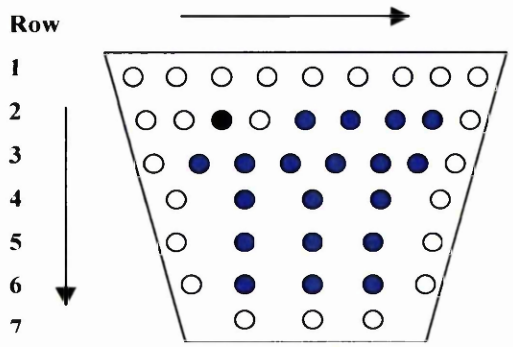


Figure 5.1(b) : The effect of changing blocking protocol on the coeffficient of variation.





The above diagram shows the well layout for Figure 5.1(a) and 5.1(b). Once the assay was completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7, as shown above. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 38 points on the graphs from 19 wells

CHAPTER 6

PRIMARY ANTIBODY

This section of the results corresponds to the sample or unknown to be tested. During this project, development of the assay and platform did not proceed to testing for parasitic or other diseases. For this reason, only standardised antibodies were used corresponding to the specific antigen as described in the materials and methods. The aim throughout was to produce a colour that would be detectable in the reader and at first this concentrated on as strong and as blue a colour as possible. This was later found to restrict the detectable limit of primary antibody.

6.1 Dinitrophenol and rabbit IgG ELISA on sectors and discs.

The DNP-BSA and Rabbit IgG ELISAs were tested for the range of colour produced in 96-well plates using serial dilutions of both antibodies involved. The soluble substrate Orthophenylenediamine Dihydrochloride (OPD) was used and showed that a good range of colour was produced in the range of 0.396-1.99 absorbance units. This was extended to testing insoluble substrates eg Diaminobenzidine (DAB), 5-Bromo-4-Chloro-3-Indoyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) and 4-Chloro-I-Napthol (4C1N) to test for colour intensity. These did not produce the required blue colour. Soluble TMB produced an intense blue colour with peroxidase-conjugated second antibodies and an insoluble version was found that produced a strong blue colour. Visual observation was used to determine colour intensity. This was tested on 96-well plates using the DNP ELISA with serial dilutions of anti-DNP and anti-rabbit IgG-HRP to determine suitable combinations of antibodies that would produce a strong blue colour. The dilutions of primary and secondary antibodies tested found are shown below in Table 6.1. The anti-rabbit IgG-HRP was later used at a constant dilution of 1/1000.

Dilution of anti-DNP	Dilution of anti-rabbit IgG-HRP
1/12800	1/1600
1/12800	1/800
1/25600	1/800

Table 6.1: Dilutions of primary and secondary antibodies in DNP ELISA.

When this was repeated on a Transpaseal-disc some differentiation between different antibody combinations could be observed but due to well leakage this was not as sharply defined as in the 96-well plate. Repeating a similar experiment on a plain disc showed that there was gradation of colour between the different antibody combinations although there was an "over-reaction" at the lower antibody dilutions. An over-reaction occurred when TMB was used as substrate. If the concentration of primary or secondary antibody was too high, the reaction with the HRP-conjugated second antibody proceeded at an accelerated rate. Instead of the desired blue colour, the reaction proceeded to green, orange-yellow. With the insoluble TMB, this left a grey-green wash of colour on the well. The range of primary antibody dilutions used was 1/100 - $1/5.12x10^4$ therefore to remove this over-reaction, the range was changed to remove the lower dilutions. Serial dilutions of $1/5x10^3$-$1/5x10^5$ were used. This produced little apparent gradation between the different dilutions of anti-DNP although there was an obvious difference between the highest and lowest dilutions. Extending this dilution range to $1/5x10^4$-$1/1x10^6$ confirmed this finding with some gradation and fairly strong colour between dilutions of $1/5x10^4$-$1/5x10^5$ but no gradation and a weak "wash" of colour with dilutions of $1/5x10^5$-$1/1x10^6$. This was to be a continuing problem throughout this work. Using insoluble TMB did not produce the range of colour found with conventional ELISA substrates. There appeared to be three groups of colour - weak or pale blue (1 and 2), medium blue (3) and a strong vivid blue (4 and 5) as shown in Figure 6.1, although the division between all three was not always clear.

Figure 6.1: Approximate Range of Colour Produced by Insoluble TMB

Over-reaction in the presence of a high concentration of antibody led to a grey-green wash of colour. It was also found at this time that reducing the blocking and antibody incubation times to 30 minutes on the plain disc did not affect colour production. This was found for both the DNP and Rabbit IgG ELISAs. Further manipulations of the DNP ELISA were tried i.e. diluting the insoluble TMB (see chapter 8), changing concentration of second antibody or using a second antibody designed to enhance assay (see chapter 7) on microtitre plates, plastic discs and Transpaseal-coated sectors. This did not result in better colour gradation between the primary antibody dilutions.

6.2 Multianalyte ELISA on sectors.

The production of the moulded sectors meant that testing could move to a new stage i.e. using a sector with enclosed wells. These were tested using the DNP ELISA with flood-fill for washing and applying reagents. A strong blue-green colour with insoluble TMB was produced showing that these sectors were suitable as a platform for the ELISA. The multianalyte panel was developed as described in Materials and Methods. All the ELISAs were performed first in 96-well plates using insoluble TMB as substrate to determine the best concentration of antigen and antibody for use on the sectors. Antigen concentrations of 10μg/ml -200μg/ml were used and titrated against primary antibody dilutions of $1/1x10^3$ - $1/6.4x10^4$ to determine the best i.e. most intense blue colour for use on the sectors. Human Serum Albumin (HSA) was titrated up to an anti-Human Serum Albumin concentration of $1/2.56x10^5$ using antigen concentrations up to 1mg/ml, but these were reduced to a more viable range with the other antigens. It was found that no colour was produced after a dilution of $1/6.4x10^4$ even at high antigen concentrations. Alpha-1-Antichymotrypsin was titrated on sectors only, due to the small amount of antigen. ELISAs were transferred to sectors and incorporated into the multianalyte panel as described in the materials and methods. The HSA and DNP ELISAs were performed on one sector as a feasibility test for carrying out two ELISAs on the same sector. This worked well without cross-reaction between antigens and antibodies. The only drawback was that a much lower concentration of peroxidase-conjugated second antibody was required for the DNP ELISA compared to the HSA ELISA to prevent the over-reaction of the insoluble TMB. This meant

that colour production in the HSA ELISA was lower. The panel was constructed as described in chapter 4 with the sequential addition of antigens and antibodies to the panel after initial testing on plates to determine appropriate antigen and antibody dilutions. When adding to the panel, each was tested for cross-reaction with the other members of the panel. There was a slight cross-reaction between anti-Antithrombin III and α-2-Macroglobulin but this was at a low level. Testing each primary antibody individually against the panel of antigens confirmed this and showed the low background and lack of cross-reaction between other antigens and antibodies

Primary antibody	Conc. of Stock Solution	Primary Antibody Dilution	Conc. used in ELISA
Anti-Human Serum Albumin	600 μg/ml	1/1000	0.6 μg/ml
Anti-α-1-Antitrypsin	17.67 mg/ml	1/4000	4.4 μg/ml
Anti-α-2-Macroglobulin	4.85 mg/ml	1/4000	1.2 μg/ml
Anti-Antithrombin III	28.2 mg/ml	1/1000	28.2 μg/ml
Anti-Catalase	1.82 mg/ml	1/1000	1.82 μg/ml
Anti-α-1-Antichymotrypsin	16.2 mg/ml	1/1000	16.2 μg/ml
Anti-Plasminogen	1.6 mg/ml	1/1000	1.6 μg/ml

Table 6.2: Range of concentrations in stock primary antibody solutions.

Table 6.2 shows the range of protein concentrations in the stock primary antibody solutions although the dilution factor for anti-α-1-Antitrypsin and anti-α-2-Macroglobulin was increased later to 1/1000 to reduce colour variation. The final concentrations in the ELISA ranged from 0.6 μg/ml for anti-Human Serum Albumin to 28.2 μg/ml for anti-Antithrombin III. With such a wide range in concentrations it may have been expected that there would have been a wider range of primary antibody dilutions that produced colour although this would be depend on the individual antigens in the ELISAs. This shows one of the problems i.e. the apparent insensitivity of insoluble TMB for quantification. All of these were based on visual examination of the colour produced in the sectors.

6.3 Incubation times.

As described above the assays were performed using incubation times of thirty minutes for the primary antibodies. It was found that this could be significantly reduced in a similar way as the antigen coating and blocking. Six sectors were coated with the seven antigens that made up the multianalyte panel - Human Serum Albumin, α-1-Antitrypsin, α-2-Macroglobulin, Antithrombin III, Catalase, α-1-Antichymotrypsin and Plasminogen at a concentration of 20μg/ml and 3μl/well. Incubation was for one hour at room temperature. After washing and blocking (50mg/ml BSA/PBS, 30 minutes), primary antibody mix (see materials and methods) was applied by flood-fill to each of the sectors and incubated for 5, 10, 15, 20, 25 and 30 minutes. After washing, anti-rabbit IgG-HRP was applied to each of the sectors for thirty minutes followed by insoluble TMB for twenty minutes. The resulting colour on the sectors was examined and based on visual examination, showed similar a blue-green colour with little difference in intensity between the five and thirty minutes incubations. This indicated that incubation with the primary antibody could be reduced to five minutes without apparent loss of colour. Further experiments with reduced incubation times showed that there was variation in colour with only a five minute incubation. (Pre-coating sectors with silicone spray was found to increase colour intensity and stability with insoluble TMB). Table 6.3 shows a comparison between a total assay time of 35 and 50 minutes.

Stage in Protocol	Incubations in 35 minute Assay (mins)	Incubations in 50 minute Assay (mins)	Incubations in 60 minute ELISA (mins)
Antigen Coating	10	15	15
Blocking	5	10	15
Primary Antibody	5	5	10
Secondary Antibody	5	5	10
Insoluble TMB	10	5	10

Table 6.3: Incubation times in 35, 50 and 60 minute ELISAs

Both showed a similar pale green colour. A variety of different combinations of incubation times were tested and it was found that the sixty minute protocol produced the most consistent results although colour variation remained a problem.

Even with the sectors pre-coated with silicone spray, there was significantly more variation with lower incubation times especially with the primary and secondary antibodies. The colour produced was also greener instead of the required blue. In the case of the primary antibody incubation, the difference between five and ten minute incubations appeared to be significant. Washing also played an important role in colour development and experiments were performed to determine the minimum number of washes required after each stage of the ELISA. It was found that a minimum of four washes was required with increased background if less were used. This was changed to six washes with the black sectors where up to sixteen washes after each stage was tested. One and two washes did not produce any data and the best OD values were with five, six and seven washes. Above this there was a slight increase in OD value, but compared to the time involved in washing out each sector ten or twelve times by hand after each step of the protocol, this was not thought to be practical. Therefore six washes was considered a good compromise.

When working with the sectors and developing the multianalyte ELISA, the background seemed to increase. This had never been a problem before therefore it was thought that one of the antigens or antibodies in the panel was responsible. Increasing the primary antibody incubation time from five to ten minutes may have been responsible although the background was always low when the standard incubation times were used i.e. thirty to sixty minutes. The background produced by decreasing the primary antibody incubation time to five minutes was examined using six of the seven ELISAs (no α-1-Antichymotrypsin) on sectors. With four washes between each step there was very weak green colour produced. Further experiments involved adding the ELISAs sequentially to the sector with an antigen concentration of 3μg/ml and coating volume of 3μl/well. Each primary antibody was added to the primary antibody mix and the sector was examined for evidence of increased background with the addition of a

specific antibody. The second antibody was Amdex anti-Rabbit IgG-HRP. At this reduced antigen concentration, colour was not going to be at its most intense but it was still strong enough to distinguish from background. There did not appear to be one specific antigen or antibody that increased the background. It was eventually found that the incubation time with the primary antibody did not have a significant effect on the background. The increase in background was due to spiders that leaked. Residual reagents were left in these spiders from each stage of the assay and each wash step, causing interference in colour production and increasing the background.

6.4 Primary antibody detection limit.

It was important to determine the lowest detectable limit of primary antibody. This would be different for each primary antibody due to the differences in stock solution (Table 6.4). In the experiment ten sectors were coated with Human Serum Albumin, α-1-Antitrypsin, α-2-Macroglobulin, Antithrombin III and Catalase (all 20μg/ml and 3μl/well). The primary antibody mix of the specific antibodies of these antigens was prepared using dilutions from $1/1x10^3$-$1/1x10^5$. There was found to be a gradual reduction in colour from the $1/1x10^3$-$1/5x10^3$ dilution which reduced rapidly to the $1/1x10^4$ dilution. After this there was a decrease to the $1/1x10^5$ dilution.

Primary Antibody	**Conc. of Stock Solution**	**Conc. at $1/1x10^3$ Dilution**	**Conc. at $1/5x10^3$ Dilution**	**Conc. at $1/1x10^4$ Dilution**
Anti-Human Serum Albumin	600 μg/ml	0.6 μg/ml	0.12 μg/ml	0.06 μg/ml
Anti-Antithrombin III	28.2 mg/ml	28.2 μg/ml	5.64 μg/ml	2.82 μg/ml
Anti-α-1-Antitrypsin	17.67 mg/ml	17.67 μg/ml	3.53 μg/ml	1.77 μg/ml
Anti-α-2-Macroglobulin	4.85 mg/ml	4.85 μg/ml	0.97 μg/ml	0.485 μg/ml
Anti-Catalase	1.82 mg/ml	1.82 μg/ml	0.36 μg/ml	0.18 μg/ml

Table 6.4: Concentration of primary antibodies at three different dilutions.

Although colour was produced at $1/1x10^4$ dilution for all the ELISAs mentioned above there was a wide range of antibody concentrations and the colour did not appear to vary considerably as determined by visual examination. It was also difficult with the lack of reproducibility between wells and between sectors to rely on the colour produced in one experiment. When the above experiment was repeated with a primary antibody dilutions of $1/1x10^3$, $1/5x10^3$, $1/1x10^4$, $1/2x10^4$ and $1/5x10^4$, colour was produced up to a dilution of $1/2x10^4$ although this was quite pale and not thought strong enough for the reader. On comparison with a previous experiment, the $1/5x10^4$ primary antibody mix dilution had produced a similar colour to the $1/2x10^4$ dilution of this experiment. There were also variations in colour produced at the higher dilutions of $1/1x10^3$, $1/5x10^3$ and $1/1x10^4$ between the two experiments. Using only Human Serum Albumin (20μg/ml, 3μl/well) on the sectors and an anti-Human Serum Albumin dilution range of $1/1x10^3$-$1/2x10^6$ showed that the lowest detectable colour using visual examination was at $1/3.2x10^4$ dilution i.e. an anti-HSA concentration of 1.8ng/ml. However this colour was only just above background. One problem was determining the cut-off point between visual and reader detection i.e. if colour can be observed visually as above background but not by the reader, is it a positive reaction? The effect of the leaking spiders was further shown by testing individual primary antibodies against the panel of antigen in an old and new spider. With the old spider there was high background and cross-reaction with other antigens. With a new spider that did not leak, there was very little cross-reaction except anti-Antithrombin III and α-2-Macroglobulin and background was very low. The colour was also much stronger. This colour was improved even further by pre-coating the sector with silicone spray resulting in an intense blue colour and a reduction in the variation between sectors.

The primary antibodies were tested at first over a wide range of dilutions. These did not produce the required gradation for quantitative results, at least as determined by visual examination of the sectors. This produced a poor range of colour. Attempts to produce a series of multianalyte sectors for showing graded colour for calibration of the reader did not produce the desired results. Using a primary antibody dilution range of $1/1x10^3$-$1/1.28x10^5$ showed that

although colour did decrease from lowest to highest dilution it was not gradual. Effectively there were two stages of colour production as shown in Table 6.5.

Primary antibody dilution	Colour with insoluble TMB
$1/1x10^3 - 1/8x10^3$	Strong Blue
$1/1.6x10^4 - 1/1.28x10^5$	Pale Blue

Table 6.5: Colour produced with different ranges of primary antibody dilution.

A narrower dilution range increasing in smaller increments was tested to produce a more graded response. Eight siliconised sectors were coated with the multianalyte panel of antigens (20μg/ml, 2μl/well) and exposed to the primary antibody mix at a dilution in the range $1/5x10^3$-$1/4x10^4$. Dilutions increased in increments of 1/5000. The best colour was produced with the lower dilutions of $1/5x10^3$ and $1/1x10^4$. From the $1/1.5x10^4$ dilution, the colour produced was not very strong and no further gradation was observed. Although this did not extend the gradation as a whole, some of the antigens i.e. Catalase,

Alpha-2-Macroglobulin and α-1-Antitrypsin did produce stronger colour than the others did although without improved gradation. It appeared that the primary antibody concentration did not significantly affect the colour produced within certain ranges primary antibody dilution. If it did it would be expected that the different concentrations of primary antibody in the primary antibody mix specified by for example $1/8x10^3$ dilution would produce different colours since each antibody was present at a different concentration. It appeared that using insoluble TMB resulted in yes or no result. Usual visual observation was not ideal and at the time it was hoped that using the reader would show the colour gradation to be semi- or fully quantitative.
These experiments were performed using an antigen concentration of 20μg/ml. If these was too high, it may have affected the primary antibody binding and therefore colour production.
Catalase always produced the most intense and stable colour and it was used at coating concentrations of 1, 2, 3, 4, 5, 10 and 20μg/ml and dilutions of anti-Catalase from $1/1x10^3$ -

$1/5.12x10^5$. With all dilutions and Catalase concentrations there was still a sharp cut-off in colour between $1/8x10^3$ and $1/1.6x10^4$. Colour is quite similar at 5, 10 and 20μg/ml although with 5μg/ml the colour was greatly reduced at an anti-Catalase dilution of $1/1.6x10^4$. Although there appeared to be slightly better colour gradation at the lower antigen concentrations of 2-5μg/ml, the colour produced was greatly reduced with a lowered detection limit. At a Catalase concentration of 1μg/ml, anti-Catalase was detectable to $1/3.2x10^4$ while at 20μg/ml it was detectable to a dilution of $1/6.4x10^4$. In both cases the colour was very pale and only just above background. The problem was the sudden decrease in colour between dilution factors of $1/8x10^3$-$1/1.6x10^4$. A higher antigen concentration i.e. 10-20μg/ml was used to maintain the intensity of colour to a higher primary antibody dilution. This reduction in colour was found with all the ELISAs used in the multianalyte sectors.

6.5 Primary antibody detection limits on black discs.

The range of primary antibody dilution was further tested using the black discs. Here was a system where the colour could be determined using the modified reader and not relying on only visual observation.

Using Catalase as antigen (20μg/ml, 1μl/well), a range of anti-Catalase dilutions ($1/1x10^3$ - $1/1.28x10^5$) were applied to different sectors on a black disc. Using the reader, colour was detectable to a dilution of 1/16000 as shown in Figure 6.2. Colour was very intense in the first two dilutions then followed the same pattern as previously described. The Catalase coating concentration was tested between 5ng/ml-10μg/ml using the same anti-Catalase dilutions as above. Colour was detected by the reader with a Catalase concentration of 5 and 10μg/ml and anti-Catalase dilutions of 1/1000, 1/2000 and 1/4000. Colour decreased in intensity with increasing antibody dilution. This shows that the colour produced may be semi-quantitative over a limited range but the variation with the OD values was very large. The maximum OD value was low at about 0.54 absorbance units. This could have been due to the drying of the antigen and blocker because when the antigen was washed off the OD value was higher even with the "streaming effect". The primary antibody dilution range was tested again using

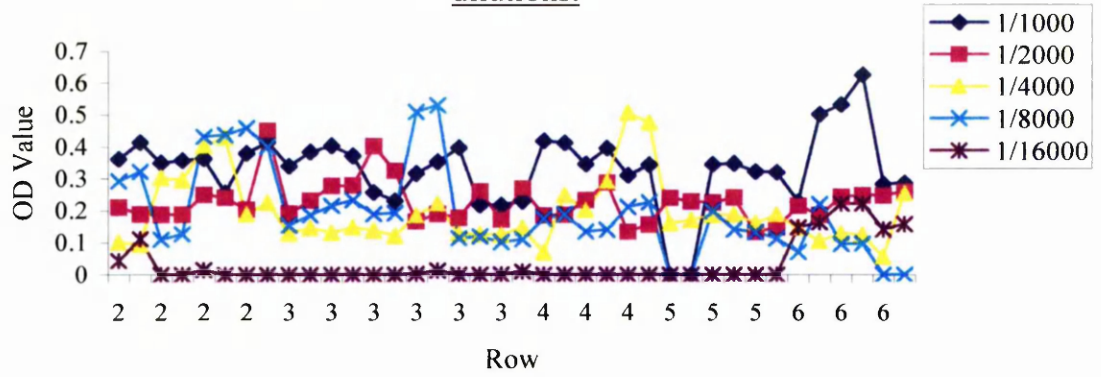
Catalase (20μg/ml, 1μl/well). The anti-Catalase dilution range was $1/6x10^3$-$1/1.8x10^4$ as the lowest detectable limit had been shown to be $1/1.6x10^4$.

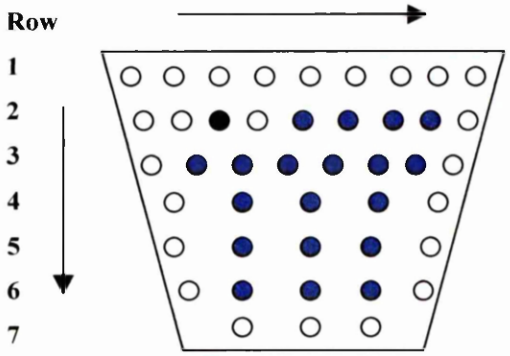
Figure 6.3 shows the OD values when only the four wells row 2 of each sector on the black disc were examined. The $1/6x10^3$ dilution of anti-Catalase produced the highest OD values while the $1/1.8x10^4$ dilution produced the lowest OD values as expected. However the other dilutions within these two limits did not produce OD values according to their dilution as shown in Figure 16. This may have been due to the lack of sensitivity with the insoluble TMB. Even with this the $1/1.6x10^4$ and $1/1.8x10^4$ dilutions had much lower OD values than dilutions in the range $1/6x10^3$-$1/1.4x10^4$. The other rows in the disc also followed this pattern i.e. the variation in OD values across a row in all the sectors.

In conclusion, there did not appear to be quantitative detection of primary antibody in this system although it may have been semi-quantitative up to a certain concentration of antibody. It was difficult to determine this because of the variation in the black discs but a similar pattern was observed in both sectors and black discs. This may have been due to the use of insoluble TMB as substrate, which was not designed for use as an ELISA substrate.

The next stage in the assay is the addition of enzyme-conjugated second antibody. This reacts specifically with the primary antibody and incorporates the label used to produce the colorimetric reaction resulting in the blue colour.

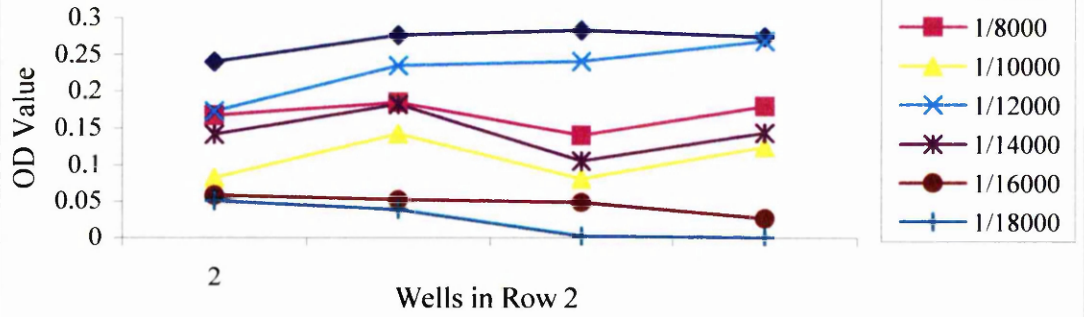
Figure 6.2 : Absorbances produced by a range of anti-Catalase dilutions.

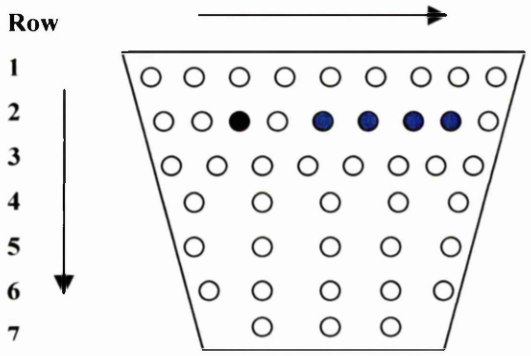




The above diagram shows the well layout for Figure 6.2. Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7, as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 38 points on the graphs from 19 wells.

Figure 6.3 : Absorbance values produced in row 2 of sectors by decreasing primary antibody dilutions.





The above diagram shows the well layout for Figure 6.3. Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7 as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured once in this way.

CHAPTER 7

SECOND ANTIBODY

The initial experiments with the second antibody in the ELISA were with the DNP-BSA and rabbit IgG ELISAs. Two second antibodies were used at first with different enzyme conjugates - horseradish peroxidase (HRP) or alkaline phosphatase (AP) in order to determine which substrate produced the best blue colour. HRP and AP are the most common enzyme conjugates and therefore have the widest range of substrates. Throughout most of this work this work the second antibody was usually a Goat Anti-Rabbit IgG conjugated to either horseradish peroxidase or alkaline phosphatase.

7.1 Enzyme-conjugated second antibodies.

The best working dilution was found to be $1/1x10^3$ for both HRP and AP conjugated antibody. This was found using solid substrates i.e. produced a precipitate on microtitre plates. Diaminobenzidine (DAB) was used with the peroxidase-conjugated antibody and 5-Bromo-4-Chloro-3-Indoyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) with the AP-conjugated antibody. Colour was produced with all thse substrates but it was of low intensity. Results showed that decreasing the second antibody dilution to eg 1/100 did not appear to significantly increase the colour density with either DAB or BCIP. At this time visual examination was used to determine the results and it was often difficult to determine if there was a difference in colour between dilutions. This was tested using microtitre plates and on the Transpaseal-coated discs. The best blue colour was found with a soluble 3,3', 5,5'-Tetramethylbenzidine (TMB) which reacted with the peroxidase-conjugated goat anti-rabbit IgG to produce an intense blue colour. Serial dilutions of anti-rabbit IgG-HRP from $1/10-1/5.12x10^4$ produced a very strong reaction with the high concentrations of antibody, producing a yellow colour within the incubation time indicating an over-reaction. As the blue colour produced by the TMB was a very intense blue, several strategies were followed to attempt to solidify the soluble end-product of the ELISA. None of these were successful. The discovery of an insoluble TMB that deposited colour onto the wells of the microtitre plate, rather than remaining in solution meant that a peroxidase conjugated anti-rabbit IgG was used exclusively for the remainder of the project. There was not much gradation in colour between different concentrations of second antibody using insoluble TMB, although this depended to a certain extent on the concentrations

of antigen and primary antibody. Using a plain disc and the Rabbit IgG ELISA, a second antibody (anti-rabbit IgG-HRP) dilution range of $1/5x10^3$-$1/5x10^4$ produced only limited colour gradation. A similar experiment with the DNP ELISA using a second antibody dilution range of 1/10-$1/1x10^3$ showed that the strongest colour was produced with anti-rabbit IgG-HRP concentrations of 1/750 or $1/1x10^3$. Below this (1/10-1/500), a light "wash" of colour was produced possibly due to the high second antibody concentration. This "wash" effect with insoluble TMB indicated that an "over-reaction" had taken place probably due to the concentration of second antibody being too high. One of the problems with insoluble TMB was later found to be its apparent lack of sensitivity for use in an ELISA. This meant that there was always a limited range of colour produced with not much quantifiable difference between them, even when the reader was used. Colour seemed to be strong, medium or weak with the wash effect really being no colour at all. The disadvantage of using visual examination meant that there might have been differences that were not being detected, but would be with the reader. However this was found not to be so at a later date.

7.2 Enhanced second antibody (Amdex anti-rabbit IgG – HRP)

Although there were sensitivity problems with insoluble TMB, it was used as the substrate of choice because the colour was thought most likely to be detected once the reader was operational. No other insoluble substrate was found that produced anything like the intensity of colour produced by insoluble TMB. One of the options was to find a way to amplify and stabilise the colour. One strategy tried was the use of Amdex goat anti-rabbit IgG-HRP. Amersham Pharmacia Biotech produces this. Standard enzyme conjugated second antibodies have one or two molecules attached to the antibody. The Amdex family of conjugates have a hydrophilic straight chain dextran backbone to which are covalently coupled hundreds of enzyme molecules and also specific antibody molecules. This results in a multifuctional conjugate with significantly enhanced activity. Due to the large number of enzyme molecules within each conjugate molecule, signal can be increased by up to a hundred times thus increasing assay sensitivity and possibly decreasing incubation times. Initial results using microtitre plates and the DNP ELISA were disappointing. With an anti-DNP dilution of

$1/5x10^3$, a range of normal and Amdex Goat Anti-Rabbit IgG-HRP dilutions (1/100-1/8 x 10^8) were tested and showed that a wider range of colour was produced using the normal second antibody. After twenty minutes incubation with insoluble TMB there was only colour in the wells containing $1/3.2x10^3$-$1/2.56x10^4$ Amdex anti-rabbit IgG-HRP. In the presence of normal second antibody, the reaction was much slower and after twenty minutes colour remained in wells containing second antibody in the range $1/1.6x10^3$-$1/1x10^5$. To test if the over-reaction was due to the insoluble TMB or also happened with a soluble substrate, a similar experiment was carried out comparing insoluble TMB and Orthophenylenediamine Dihydrochloride (OPD) in the presence of normal and Amdex second antibody using a dilution range of 1/100-$1/2.56x10^5$. The results from this showed that both insoluble TMB and OPD showed significant differences in the colour produced with Amdex anti-Rabbit IgG-HRP. With the soluble substrate, however there did not appear to be any over-reaction at the low antibody dilutions while at the same time dilutions with insoluble TMB left a grey colour on the plastic. Varying the anti-DNP dilutions (1/100-$1/2.56x10^5$) and using a constant normal and Amdex second antibody dilution of $1/1x10^3$ also produced a wider range colour with the normal second antibody and insoluble TMB. Extending the anti-DNP range from $1/1x10^3$-$1/2.56x10^5$ did not increase the range of colour produced with Amdex anti-IgG-HRP compared with normal second antibody. One possible way to reduce the over-reaction was to dilute the insoluble TMB with distilled water and test it with the Amdex second antibody. Using the DNP ELISA again on a microtitre plate with an anti-DNP dilution range of 1/100- $1/1.28x10^4$, a number of insoluble TMB dilutions were tested with an Amdex second antibody dilution of $1/1x10^3$ but there was still an over-reaction.

The reason that the Amdex second antibody did not appear to be extending the colour range was that the incubation times had not been changed from the standard thirty minutes used for the normal second antibody. This was tested using diluted insoluble TMB and the DNP ELISA. The primary antibody was diluted over a wider range i.e. $1/1x10^3$-$1/1.28x10^5$ and normal second antibody was used with diluted insoluble TMB. The substrate was left for 5-10 minutes this time but there was still a serious over-reaction with high concentration of anti-

DNP. Repeating this with the Amdex second antibody produced more over-reaction and reduced the level of detectable colour down towards the lower anti-DNP concentrations. Colour was detectable with normal second antibody to an anti-DNP concentration of $1/6.4x10^4$, which was extended to $1/1.28x10^5$ with Amdex anti-IgG-HRP. At the other end of the anti-DNP dilution range, normal second antibody detected from an anti-DNP dilution of $1/2x10^3$ and Amdex second antibody from $1/8x10^3$. In all cases, the colour produced was very weak initially and increased in depth to an anti-DNP dilution of $1/6.4x10^4$, after which it reduced. Varying the concentration of DNP and anti-DNP in order to produce a more stable precipitate was tested with normal and Amdex anti-IgG-HRP. The DNP dilution was reduced form $1/1x10^3$ to $1/5x10^3$ with the anti-DNP diluted from 1/100-$1/2.56x10^4$. Both Amdex and normal second antibody were used at a dilution of $1/1x10^3$. No colour was produced with the Amdex second antibody due to over-reaction in all the wells while with normal second antibody colour was produced from an anti-DNP dilution of $1/6.4x10^3$. Over-reaction occurred when the antigen or antibody concentration was too high. The reaction with insoluble TMB proceeded too quickly with the reaction continuing from the desired blue colour to green then orange-yellow. This resulted in a very pale grey-blue precipitate being deposited on the well. Further reductions in the DNP coating dilution to $1/1x10^4$ and $1/2x10^4$ with reduced normal and Amdex second antibody dilutions ($1/1x10^4$-$1/2\ x10^6$) were tried. The reduced coating concentration of DNP seemed to prevent the over-reaction to a certain extent although colour was much fainter. Repeating this experiment using slightly different second antibody concentrations ($1/5x10^3$-$1/1x10^6$) showed no difference between normal and Amdex second antibodies with the strongest colour being produced with a second antibody dilution of $1/1x10^4$ and a DNP dilution of $1/1x10^4$. However it was apparent in both these experiments that over-reaction was still taking place as the second antibody concentration of $1/5x10^3$ produced less colour than $1/1x10^4$ indicating that an over-reaction had taken place.

It was not clear at this time if it was the reduced coating concentration or reduced second antibody concentration that had reduced the over-reaction. Increasing the DNP coating concentration to $1/1x10^3$ did increase the over-reaction. To check if this was an artefact of

using insoluble TMB, the experiment was repeated with soluble TMB, Amdex and normal second antibody. It was found that there was little difference between DNP coating concentrations of $1/1x10^3$ and $1/1x10^4$, and there was also no over-reaction with the DNP concentration of $1/1x10^3$ even with Amdex anti-rabbit IgG-HRP. From these results it appeared that there was no benefit in using Amdex anti-IgG-HRP as a second antibody. At this time it did not significantly increase the range of colour produced and may even reduce it. It also appeared to produce greater over-reaction although this was thought to be due to the use of insoluble TMB because this did not happen with soluble TMB and OPD, which showed gradation in colour. It appeared that with insoluble TMB, the Amdex second antibody was not suitable as in the presence of high primary antibody concentrations it over-reacted and did not increase the colour intensity or range of colour.

With the introduction of the moulded sectors, the second antibody was being used to detect a panel of tests. It was important that all seven ELISAs in the panel were detected by the same second antibody to reduce any possible cross-reactions at this time. Later work showed that it was possible to have more than one second antibody eg anti-rabbit and anti-mouse IgG-HRP but this was not considered ideal. Initially the sectors were incubated for the standard times but as one of the objectives of this project was to produce a rapid multianalyte immunoassay, efforts were made to reduce these. Using normal second antibody, sectors coated with the panel of antigen were exposed to normal anti-rabbit IgG-HRP for 5, 10, 15, 20, 25 and 30 minutes. The colour produced after five minutes did not appear intense enough and the best colour appeared to be after ten minutes incubation. Colour did not appear to increase significantly after ten minutes although this was based on visual examination. In an earlier experiment examining two ELISAs on one sector, Human Serum Albumin (HSA) and DNP had been used. Results from this using normal second antibody had shown that for the DNP ELISA a second antibody dilution of $1/1x10^4$ was optimal compared to $1/1x10^3$ for HSA. The lower dilution was also found to be optimum with the other ELISAs in the panel. This indicated that the DNP ELISA was going to produce very high results and may have been responsible for the problems with Amdex anti-IgG-HRP described above.

The Amdex second antibody was tested with the multianalyte ELISAs on sectors at a dilution of $1/5x10^3$ for ten minutes incubation and was found to produce much more intense colour than normal second antibody. Tests to find the optimum Amdex concentration together with optimum incubation time with insoluble TMB were compared with normal second antibody. Incubating both second antibodies at a dilution of $1/5x10^3$ for ten minutes was followed by a ten minute incubation with insoluble TMB. A pale green colour was produced with the normal second antibody while the Amdex anti-rabbit IgG-HRP produced a much stronger green. Reducing the dilution factor to $1/1x10^3$ for the Amdex second antibody increased the colour intensity to a stronger mixture of blue-green which was also produced with a five minute incubation with second antibody. As previously mentioned, one of the problems was the lack of reproducibility of colour production, which varied a great deal from sector to sector and disc to disc even with identical reaction conditions. In addition the use of visual examination to compare colour was not ideal. This meant that certain experiments had to be repeated several times to confirm results.

The incubation times of all stages in the ELISA were investigated but it was difficult to make a definitive decision at the lower times i.e. five or ten minutes although it was obvious that a significant reduction could be made. The use of Amdex anti-rabbit IgG-HRP as second antibody increased the colour with reduced incubation times and when compared with the same ELISA using insoluble TMB on microtitre plates appeared to extend the colour range by approximately a factor of ten. However with some of the higher concentrations of primary antibody there was an over-reaction that was not present with the normal second antibody. The biggest problem was the variation in colour and the use of silicone spray to pre-coat the sectors as discussed in chapter 4 did appear to reduce this on the sectors at least when using visual observation. Using normal second antibody in the presence of silicone produced a weaker and greener colour with some of the antigens in the multianalyte ELISA. With all the antigens in the panel there was slight differences in the colour production, which were more evident with normal second antibody. This illustrates one of the problems in constructing multianalyte

ELISAs i.e. some of the reaction times eg incubation times may have to be optimised for the panel in general and not just one ELISA. It may be that conditions are similar for all members of the panel but if there is not compatibility between assay some will not be incorporated into the panel. Human Serum Albumin (HSA), however appeared to react much slower and the colour was weaker and more variable. For Catalase this incubation could probably have been reduced to five minutes but HSA required ten minutes. The silicone pre-coat and the use of Amdex anti-IgG-HRP both increased the intensity of colour, making it blue rather than a blue-green and also reduced the variability in colour. For both these reasons, silicone and Amdex second antibody were used with incubation times of 15 minutes for antigen coating and blocking and ten minutes for primary antibody, secondary antibody and insoluble TMB.

7.3 Black discs.

Changing to the black discs meant that a different surface was being used and that quantifiable results were being produced from the reader. Colour production by normal and Amdex second antibody was compared using Catalase (20µl/ml, 1µl/well). Both antigen and blocker were dried onto the wells. Strong blue colour was produced with both normal and Amdex anti-IgG-HRP, with on visual examination, little difference in colour between them. The OD values were similar for both, although there was high variability. This seemed to show that the plastic of the sector might have been causing at least some of the variation and observed visually although there was still great variation when the reader was used.

This was repeated using another normal second antibody, Donkey Anti-Rabbit IgG-HRP. Figure 7.1(a) shows that Amdex second antibody produced slightly higher OD values than both normal second antibodies, which produced similar OD values. The CV values appeared slightly lower with the Amdex second antibody as shown in Figure 7.1(b). From this it was initially decided to use the normal second antibody (Donkey Anti-Rabbit IgG-HRP) because the slight increase in OD value with Amdex second antibody did not really justify its use. This experiment was performed however with Catalase which had always produced very good colour.

The OD values on the black disc were low and seemed to decrease. There was great variation with the OD values produced with all these experiments even with the highest antigen and antibody dilutions. OD values were low compared to a standard ELISA, on average between 0.1-0.5 absorbance units and in later experiments between 0.1-0.3 absorbance units although there were variations e.g. on comparison of OD values on sectors and black discs showed OD values of 0.4-0.6 absorbance units. It was felt that using the Amdex second antibody would boost these low OD values. The incorporation of 0.1% bovine serum albumin (BSA) in to the antigen coating buffer was also tested with normal and Amdex second antibody. Macroglobulin, Plasminogen, Antithrombin III and Human Serum Albumin (HSA) were tested with and without 0.1% BSA in the coating buffer and with normal and Amdex anti-IgG-HRP. All the other antigens produced different but higher OD values with Amdex second antibody and 0.1% BSA/PBS as the coating buffer except the HSA ELISA. It produced no colour in the presence of 0.1% BSA in the coating buffer. The variation (%CV) was reduced with 0.1% BSA in the antigen coating buffer to less than 6%. With such low OD values, Amdex anti-IgG-HRP was used for the remainder of the project. Once the OD values had been increased it was envisaged that normal anti-rabbit IgG-HRP would be used again. One of the problems was thought to be the insoluble TMB which may not be suitable for quantitative measurements on plastic.

Figure 7.1(a) : Absorbance values produced by three different peroxidase-conjugated second antibodies (anti-rabbit IgG).

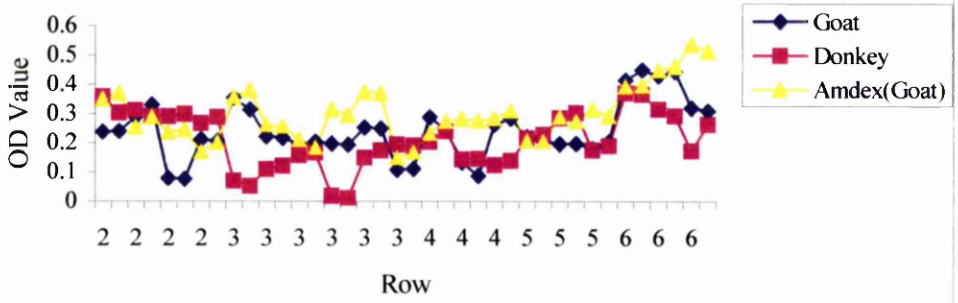
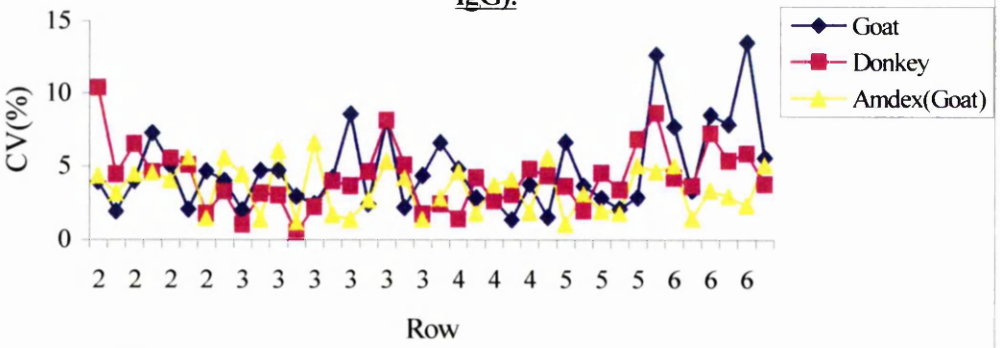
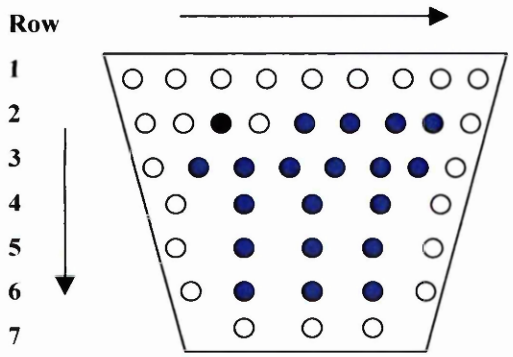


Figure 7.1(b) : The coefficient of variation produced with three different peroxidase-conjugated second antibodies (anti-rabbit IgG).





The above diagram shows the well layout for Figure 7.1(a) and 7.1(b). Once the assay had been completed, the disc was dried and placed in the modified reader for determination of the intensity colour produced in the wells. The wells were read from row 2 to row 7, as shown above, from left to right. The black well in row 2 was required for the correct tracking of the reader. Measurements were made with a red laser, measuring the intensity of colour using a greyscale. This was based on the degree of reflectance from the well, with the greater the intensity of colour having less reflectance and therefore a lower value. An empty well gave the maximum value on the greyscale of 255. These values were converted to OD values. Nine readings were taken in the centre of each well in the pattern of a 3×3 square. From these readings, the OD values and the coefficient of variation between these readings for each well were determined. Each well was measured twice in this way giving a total of 38 points on the graphs from 19 wells.

The use of Amdex anti-rabbit IgG-HRP as second antibody on the plastic sectors increased and stabilised the colour in conjunction with the silicone pre-coat and allowed the incubation time to be reduced. Normal and Amdex second antibodies did produce similar OD and CV values on the black discs but Amdex second antibody was used as it produced slightly better results. The large variation in OD and CV values made it difficult to determine which second antibody was best. The significantly greater cost of the Amdex second antibody meant that it would have been preferable to use the normal second antibody if there was no significant benefit to colour production or variation.

The final stage in the assay is the addition of a substrate that reacts specifically with the enzyme label conjugated to the second antibody. This results in the formation of a coloured product that is proportional to the amount of bound primary antibody.

CHAPTER 8

SUBSTRATES

8.1 Insoluble substrates.

Work on a suitable substrate for use in the sector-based system concentrated on finding one that produced a blue precipitate. From the beginning it was realised that liquid substrates could not be used on a spinning disc in the modified CD reader as the liquid would come out of the wells. The precipitated colour had to be blue because the laser in the modified CD player was red detecting at a maximum of 650nm. A soluble substrate, Orthophenylenediamine (OPD) Dihydrochloride was used to test and confirm ELISAs on microtitre plates particularly the DNP and Rabbit IgG ELISAs. OPD produces a soluble orange-brown end product that is read spectrophotometrically at 450nm. The reaction is stopped by the addition of 3N Hydrochloric acid or 3M Sulphuric Acid[103]. The substrates tested were used for immunohistochemistry and immunoblotting and were chosen on the basis of the colour produced. Diaminobenzidine (DAB) produces a dark blue-black colour with peroxidase activity while 5-Bromo-4-Chloro-3-Indoyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) produces a blue/purple colour with alkaline phosphatase activity. DAB (3,3'-Diaminobenzidine Tetrahydrochloride with Metal Enhancer Tablet Sets) is used in immunohistochemistry and dot blotting to localise peroxidase activity by the deposition of an intense blue to dark blue stain[104]. BCIP/NBT have also been developed for use in immunochemistry for the detection of alkaline phosphatase activity. It is most commonly used in Western Blotting and dot blotting[105].

Both these substrates were tested with both the DNP and Rabbit IgG ELISAs on 96-well plates using Goat Anti-Rabbit IgG second antibody conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase (AP). Colour was deposited in the wells but did not appear to be very intense. DAB produced a brown-black colour and BCIP/NBT a purple colour. This lack of intensity could have been due to the use of a plastic solid phase as opposed to nitrocellulose membranes or glass slides. Increasing the concentration of primary and secondary antibodies and antigen did not appear to significantly increase the colour density of the final product. Increasing incubation times with second antibodies from thirty minutes to one hour also did not increase colour. The ELISAs were also performed using tissue culture 96-well plates and designated ELISA plates but again no increase in colour was observed using these substrates.

One perceived way of increasing colour density was to increase substrate concentration. Both substrates used were in tablet form prepared in a specified volume of buffer or water. Increasing the substrate concentration was a matter of increasing the number of tablets in the specified volume. With DAB and the Rabbit IgG ELISA there was no increase in colour density when the DAB concentration was doubled by dissolving four tablets in 5 ml instead of the normal two tablets. Further increasing the DAB concentration to four times its normal (four tablets in 2.5 mls) also did not have any effect on colour density. Similarly, doubling the concentration of BCIP/NBT with the same ELISA also did not increase colour density.

8.2 3,3', 5,5'-Tetramethylbenzidine.

The problem with DAB and BCIP/NBT was that the colour was not thought to be intense enough or blue enough for use in the reader. The aim at this preliminary state was to find a substrate that produced an intense blue colour on plastic. Once this was established the next step was to produce quantitative readings but at this early stage a detectable colour was the aim. The ELISA substrate 3,3', 5,5'-Tetramethylbenzidine (TMB) is a common and very sensitive ELISA substrate that produces a soluble, blue end product detectable at 370nm or 655nm in the presence of peroxidase[106]. TMB can also be read at 450nm if acidified before reading to produce a yellow colour. TMB is the most sensitive chromogenic substance for the detection of peroxidase. In the presence of HRP and hydrogen peroxide, TMB is first oxidised to a blue cation free radical. On further oxidation with HRP and hydrogen peroxide or on the addition of acid, it is converted to a yellow diimine terminal oxidation product. Using both the DNP and Rabbit IgG ELISAs, colour production with TMB and OPD was compared in microtitre plates with serial dilutions of both primary and secondary antibodies (1/100 - $1/5.12 \times 10^4$). TMB produced a very strong intense blue colour, which was found to over-react with high antibody concentrations producing green then yellow colour. However the blue colour produced was ideal for use in the reader and attempts were made to "solidify" the blue end product. Using the Rabbit IgG ELISA, various gelling agents were added to the blue end product produced by TMB. Agar, agarose and gelatin (1mg/ml) were prepared and added (one drop per 100µl of TMB) to the wells of the microtitre plate. All these agents were dissolved in water by heating

and set on cooling therefore had to be added to the TMB while still warm. This resulted in the TMB turning yellow. By the time it was cool enough to add, all three gelling agents had set. They were difficult to pipette at lower temperatures therefore were added dropwise. It was concluded that this was impractical for use with the disc as it would be impossible to pipette small volumes of gel onto the wells on the disc and the heat of the liquid gel would turn the TMB yellow.

Experiments with the first Transpaseal-discs were carried out using the DNP and Rabbit IgG ELISAs and either DAB or BCIP/NBT as substrates. DAB was found to produce very poor colour between different primary and secondary antibody concentrations and there was also accumulation of colour around the rough edges of the wells. A rabbit IgG range of concentrations (10, 20, 50 and 100μg/ml) were used with BCIP/NBT and DAB as substrates. Colour density was greater using BCIP/NBT with gradation between serial dilutions of anti-rabbit IgG-HRP and AP conjugate (1/100- $1/6.4x10^3$). However with both substrates the colour was not intense enough for use in the reader and there was low non-specific binding. One of the problems with this original disc was that the reactions were being performed on Transpaseal and not plastic, which could have affected the colour production. To test this, a disc with wells was attached to a plain plastic disc using Spray Mount. The Rabbit IgG ELISA was carried out using DAB, BCIP/NBT and OPD as substrates. The soluble OPD was used to show colour intensity with a standard ELISA substrate. Although this technique of attaching two discs with adhesive was not generally suitable due to well leakage, this one did work and showed that the strongest colour was produced with OPD. The colour produced with DAB and BCIP/NBT was much weaker, possibly due to the solid phase not being suitable. This experiment also showed that it was not the use of Transpaseal that affected the colour density with insoluble substrates - it was also a problem on plastic. A further insoluble substrate was tested i.e. 4-Chloro-1-Napthol (4C1N) which produces a purple-blue colour in the presence of peroxidase and can be observed visually[107]. It is commonly used in immunoblotting procedures. This was compared with the colour produced using soluble TMB and a Transpaseal-coated disc with the Rabbit IgG ELISA and a range of antibody dilutions (1/100-$1/6.4x10^3$). However 4C1N did not produce

any colour and soluble TMB over-reacted at high antibody concentrations, turning yellow. At the lower antibody an intense blue colour was produced. Using the 4C1N with the DNP ELISA on a similar disc with a range of anti-DNP dilutions (1/100-1/6.4x10) also did not produce any colour.

The final substrate tested was an insoluble TMB. This was a single liquid component system that produces an insoluble aquamarine end product in the presence of peroxidase and is used to significantly increase the detection limits of immunoblotting assays on a number of membranes[108]. Using a Transpaseal-disc, various concentrations of rabbit Ig (10, 20, 50,100μg/ml) were coated on to wells and serial dilutions of anti-rabbit Ig-HRP were used. Insoluble TMB was added to the wells and produced a blue-green insoluble end-product that was much more intense than the insoluble substrates previously tested, although it was not as intense as that produced with soluble TMB. Repeating this on a microtitre plate incorporating the DNP ELISA and serial dilutions of anti-DNP (1/100-1/5.12x10^4) showed what was to be a recurring problem in using insoluble TMB i.e. over-reaction at high antibody concentrations. At the lower antibody dilutions i.e. 1/100-1/400 the colour developed very quickly when the insoluble TMB was added. After the incubation period (20 minutes) no colour remained and only a grey "wash" remained on the well surface. When this was tested using the Rabbit IgG ELISA and extending the range of antibody dilutions (1/100-1/5 x 10^{13}) over the entire microtitre plate. Results showed that there was over-reaction with antibody dilutions of 1/100-1.6x10^3 with no colour after 1/5.12x10^4. Varying the coating concentration of Rabbit IgG did not significantly affect colour production with insoluble TMB or the over-reaction at high antibody concentrations. Even with the over-reaction problem, it was decided to use insoluble TMB as it produced the best colour of all substrates tested. This meant that a peroxidase conjugated second antibody would be required with the ELISAs being used.

8.3 Plain discs.

While the sectors were being moulded, work continued on the ELISA protocols on plain plastic discs. On these it was found that an intense blue colour was produced with insoluble TMB. When the medical grade polystyrene used for the sectors was tested it was found that the colour intensity of the blue colour was less than that on the plain disc. The plain discs were made of polycarbonate. The use of Amdex anti-IgG-HRP was described in the previous chapter. Initially it appeared that it did not increase the detection limit of the assays and seemed to cause more over-reaction with the insoluble TMB. These preliminary experiments with insoluble TMB and Amdex anti-rabbit IgG-HRP appeared to show that it made the over-reaction worse and reduced the range of antibody detected.

To try and reduce the over-reaction observed with normal peroxidase conjugated second antibody; the insoluble TMB was diluted and used in the DNP ELISA on a microtitre plate. Using the diluted insoluble TMB did not alleviate the over-reaction problem with higher antibody concentrations. The reaction seemed to take place as before but the colour was less pronounced and over-reaction took place at the same antibody dilutions as before. Using Amdex second antibody with the diluted insoluble TMB produced similar results as before, just with less colour. Reducing the dilution factor of the insoluble TMB in conjunction with a wider range of anti-DNP dilutions ($1/1x10^3$- $1/1.28x10^5$) still over-reacted at high antibody concentrations even with a high TMB dilution (1:1) and a reduced TMB incubation time from twenty minutes to five or ten minutes. There appeared to be a relatively small range over which the insoluble TMB will produce colour, even when diluted. Varying the coating concentration of DNP from $1/1x10^3$ to $1/1x10^4$ was found to reduce the over-reaction with a wider range of second antibody dilutions ($1/5x10^3$-$1/1 \times 10^6$) and a constant anti-DNP dilution of $1/1x10^3$. Both Amdex and normal anti-rabbit IgG-HRP were used. It was found that with the insoluble TMB, over-reaction occurred with a DNP concentration of $1/1x10^3$, and an anti-DNP concentration of $1/5x10^3$ and $1/1x10^4$ with both Amdex and normal second antibody. No such over-reaction was found with soluble TMB. However repeating this experiment with an increased anti-DNP dilution ($1/5x10^3$) and using only normal second antibody, found that there

was no difference in colour production by insoluble and soluble TMB when the DNP coating concentration was varied ($1/1x10^3$, $1/5x10^3$ and $1/1x10^4$). One important difference between the substrates was the better gradation between antibody dilutions with soluble compared to insoluble TMB. This would be important in later work. Poly-L-lysine (diluted 1:10) pre-coated onto the wells was found to have no effect on colour production.

8.4 Variation in colour.

The main problem was the variation in colour produced on the sectors, which was found to vary from green to green-blue to a strong blue. In order to find out if this was due to inadequate exposure to insoluble TMB, variations on the flood-fill method were tested. Four sectors were coated with Human Serum Albumin, α-1-Antitrypsin, α-2-Macroglobulin and Antithrombin III at a concentration 20μg/ml. At the end of the ELISA, insoluble TMB was added in one of four ways. The usual method of flood-fill meant that liquid was flooded across the sector, pipetted up and down three times and withdrawn leaving only the wells filled. In one of the sectors, liquid was pipetted up and down only once and in another it was twice. A third sector had the wells filled individually by hand and the fourth was filled by flood-fill as usual but the TMB was left across the surface of the sector for the duration of the incubation (twenty minutes). Results showed that the strongest colour was produced with hand filling the wells followed by flooding and leaving the insoluble TMB. Mixing the wells once did not produce any colour and mixing twice produced less than flood and leave. As it was not appropriate to hand-fill every well, it was felt that flooding and leaving the TMB would be the best option. The reason this produced better colour together with the hand loading was due to a larger volume of insoluble TMB being in contact with the peroxidase-conjugated second antibody in the wells. Using flood-fill only meant that 1.5μl of insoluble TMB was left in the wells to react with the peroxidase. Hand-loading the insoluble TMB gave 4μl in each well and leaving it flooded across the surface of the sector meant that all the wells were in contact with 1ml of TMB.

Further experiments using hand loading and flood-fill with varying incubation times (5 to 20 minutes) showed that a range of colour from pale green at five minutes and dark green at

twenty minutes was produced with hand-loading. With flood-fill, the colour was bluer. It was also found that the inner wells of the sector produced darker colour. This was thought to be due to more efficient washing in the inner wells. Filling the wells with Trypan Blue dye and washing out shows that the dye is pushed out of the inner two rows of wells while the outer wells require a further two or three washes before they are completely clear. The effect of mixing and replacing the insoluble TMB during the incubation was also investigated. The TMB was flooded across the sector and mixed every three minutes for thirty minutes and compared with flooding and leaving. Mixing was found to reduce the colour to pale green. Leaving for ten minutes then mixing every three minutes thereafter was also tried but again leaving flooded for thirty minutes produced better colour. Replacing with fresh TMB every three minutes seemed to produce a slightly darker green but would not be practical or cost-effective.

As described in the previous chapter, Amdex second antibody had been tried with the DNP and Rabbit-IgG ELISAs but was not thought to confer any advantage and also made the over-reaction worse. However it was tried again with the moulded sectors in order to reduce the secondary antibody incubation time and was found to produce a much stronger and more intense colour than normal second antibody, although it was still a mixture of blue and green. The multianalyte ELISA was performed on two sectors, one of which was exposed to normal second antibody and the other to Amdex second antibody. The increased colour intensity meant that the second antibody incubation could be reduced to five or ten minutes. The determination of the optimal incubation time for the panel with insoluble TMB was difficult because of the variation in colour. One of the main reasons for this was the use of spiders that leaked and inadequate washing after the second antibody incubation. Leaking spiders meant that liquid could travel throughout the disc and contaminate different sectors, making it very difficult to wash out properly. It had previously been noted that it was imperative to wash out very well after incubation with second antibody and an extra wash had been incorporated into the protocol prior to incubation with insoluble TMB to accomplish this. Any left in the sector would cause over-reaction when it came in contact with the insoluble TMB. Spiders that did

not seal properly often produced large variation in colour. Later work did in fact show that when a new spider was used an intense blue or blue-green colour was produced with all seven ELISAs in the panel. Previously used or in particular wet spiders produced greater variation in colour and often over-reaction caused by leaking spiders. Leaking was caused by the two discs of the spider becoming loose over time and with repeated use. With this in mind a series of experiments were designed where each individual member of the multianalyte ELISA were tested individually with insoluble TMB to test for their optimum incubation time. The coating concentration was 20μg/ml and 3μl/well. The optimum times are as shown Table 8.1.

ELISA	Optimum TMB Incubation (mins)
Human Serum Albumin	20
α-1-Antitrypsin	20
α-2-Macroglobulin	15 - 20
Antithrombin III	10
Catalase	15 - 20
α-1-Antichymotrypsin	15
Plasminogen	15

Table 8.1: Optimum incubation times with insoluble TMB.

From this the incubation time was set at fifteen minutes but this was later reduced to ten minutes as this produced a similar colour as based on visual examination. At the end of the ELISA, insoluble TMB was removed and the sectors washed with water. The observed over-reaction was possibly due to an oxidation reaction therefore the addition of an antioxidant to the final wash might stabilise the reaction and reduce variation. Vitamin C (2mg/ml) was used but did not improve the colour when tested with the Plasminogen ELISA on sectors.

The effect of silicone spray to pre-coat the sectors has been previously discussed. Its use in combination with Amdex anti-rabbit IgG-HRP resulted in an intense blue colour being produced by all the assays in the multianalyte ELISA. There was also significantly less variation between sectors and discs. There still some variation and lack of reproducibility due

to the leaking spiders but this was reduced again by using the spider with the clip structure. It was thought that the use of silicone meant that the insoluble TMB could be flood-filled in a similar way to the other steps in the ELISA instead of flooding and leaving across the sector surface but this was found not to be the case.

8.5 Black discs.

With the black discs, the lack of wells meant that there was no option but to flood TMB across the disc surface. This however produced several problems of its own. The "streaming effect" and its solution were discussed in chapter 4. The lack of wells meant that the primary and secondary antibodies could attach to the sector surface thus causing the insoluble TMB to react with increased non-specific binding. This appeared as a "cloudiness" across the sector surface. To reduce this the entire surface had to be blocked after the antigen and blocker was dried on the well. The colour produced on the black discs was a strong blue even in the absence of silicone. This appeared to indicate that the polystyrene of which the sectors were produced was not of suitable quality as previously discussed. This would agree with the good colour production with insoluble TMB on plain discs.

Insoluble TMB did eventually produce a strong blue colour and the variation on the moulded sectors was likely to be due to the solid-phase. However the main problem was its lack of sensitivity as shown on the black discs. Colour did not appear to be produced in a quantitative manner and was not detected by the reader unless it was present at a fairly high intensity below which little was detected. This is also due to the reader, but it may be that on a plastic solid-phase, insoluble TMB is suitable only as a qualitative or semi-quantitative substrate. The main drawback was the lack of reproducibility making it very difficult to draw any conclusions about antigen or antibody concentrations, incubation times, detection limits etc. The variation produced even with blue acetate on a black disc showed that there is also a problem with the optics of the system but the problems with insoluble TMB and sensitivity have been apparent since early on in the project.

In conclusion, insoluble TMB was found to be the substrate that produced the blue colour considered most suitable for use in the reader. This was based on visual examination of the colour and comparison with other substrates. The intention was to produce as strong a blue colour as possible because at the time there was no way of knowing the range of colour detectable by the reader. Having as strong a colour as possible increased the chance of it being detected. It was found that the insoluble TMB was not quantitative and detected a limited range of blue with relatively low OD values being produced even with the strongest colour. This could have been because this version of TMB was not designed for use on plastic with the ELISA technique, or it may be that a precipitate will not provide the quantitative results that a soluble substrate will under the same assay conditions. To produce a true quantitative result, a different label may be required for example, a fluorescent second antibody.

The addition of substrate and the deposition of colour on the wells was the final stage of the assay. Determination of colour intensity provided a measurement of the primary antibody bound specifically to antigen. This was either by visual examination with the sectors or by using a modified reader to determine the OD values with the black discs.

CHAPTER 9

DISCUSSION

During the developmental process, immunoassays are examined with regards to precision, accuracy, sensitivity and specificity[20]. This is especially true when evaluating a new platform and when performing multianalyte ELISAs as these criteria have to be investigated for each component ELISA.

The precision is the ability to produce the same result when the assay is repeated on the same sample. This depends on the coating stability, low non-specific binding to the solid phase, a good quality solid phase, the quality of reagents and label and careful work.

The sensitivity is the lowest detectable amount of analyte deviating from zero and is influenced by high affinity of antibodies, stability of coating, reagent concentrations, sample size and the reaction time and temperature.

Accuracy is the ability of the analyte to measure the true absolute value of the analyte and depends on the precision, sensitivity and quality of the standard.

The specificity of the immunoassay depends on the specificity of the antigens and antibodies and low non-specific binding to the solid-phase.

All of these have to be examined in the context of the compact disc as a new immunoassay platform. There were several aspects of the ELISA performed on the disc that especially affected the above criteria and these will be discussed later.

The development of the compact disc as a platform for immunoassays started with the idea of producing a miniaturised version of the microtitre plate on a CD. Initially this involved the whole disc with wells drilled in the plastic. The next step was the development of a removable sector, together with the development of a holder for the sectors, which consisted of two modified CDs. This was called a "spider" as it held eight sectors. Later modifications to the

sectors included the addition of back and sidewalls and a handle to aid removal from the spider. The final number of wells in the sector was 44, with a coating volume of 2μl. The sector and spider model was the original prototype on which much of the developmental work on the multianalyte immunoassays was performed. Discussions with clinicians and laboratory workers had indicated that a disc consisting of a greater number of sectors with fewer wells would be required for general use as the average number of tests on one sector would probably be no more than ten or twelve. This would result in a greater number of samples being processed on one disc, and also a smaller sample volume being required. Although the multianalyte ELISA was developed using the sectors, there were several problems with this approach related directly to the spinning of the disc in the modified reader. These included how to prevent the liquid remaining in the wells at the end of the assay leaking into the reader and how to secure the sectors in the spider for reading. The solutions to these problems would have required another mould of the sectors plus higher quality plastic to produce optically improved sectors. This would have been very expensive and for this reason, the sectors were abandoned at this time and the black discs (which had been developed as an alternative for holding the sectors while being read) were used as a replacement. The O.D. values were much improved with the black discs and they were then used as the platform with the required drying of antigen and blocking agent. Although the sectors were not used again in the work described in this thesis, with adequate funding for moulding, suitable plastic and the addressing of the problems described above, they may have a role to play in future multianalyte immunoassays using compact discs.

9.1 Solid phase.

Although other solid-phases are used for immunoassays the most common for the ELISA is the microtitre plate and initially work concentrated on a scaled-down version of this i.e. the sectors. Polystyrene is most commonly used for microtitre plates for a number of reasons. It readily adsorbs biological compounds especially macromolecules and particles, has excellent optical clarity, good mechanical hardness and moulding properties and is resistant to acids, bases, alcohols and many organic solvents. Polystyrene is also commonly used for beads and latexes

in immunoassays[109]. Coating the polystyrene surface is dependent on a number of factors such as the quality of the polystyrene surface. This includes the purity and possible surface modifications, the size and hydrophobicity of the molecule to be adsorbed, the concentrations of biological molecules, the coating time, temperature and pH and the constituents and ionic strength of the buffer solution. The advantage of adsorption is its simplicity and satisfactory performance in most assays. It is also economical with low-cost microplates and microstrips, cheap reagents and simple procedures. The disadvantages include inadequate stability of coating in some assays that require high sensitivity and performance. It can not be used for small or hydrophilic molecules and it may alter the conformation of the molecule making it non-reactive with its respective antigen or antibody, which can also happen if the orientation of the adsorbed molecule is wrong[109].

In microplate immunoassays it is important to determine the binding capacity of the surface. The theoretical maximum binding capacity depends on both the surface area to be coated and the size of the molecule. In practice this is affected by the polystyrene type, coating conditions, molecule to be coated and the method used to estimate binding capacity. The aim was to have the amount of compound bound to the well as uniform as possible from well to well and from plate to plate. Any variation will affect the sensitivity and precision of the assay. The error produced in hand-pipetting 1μl of antigen on to the 44 wells of the sector or 352 wells of an entire disc causes a large part of the poor precision in the assays performed on the black disc. In addition, drying the antigen may also reduce the precision. In a conventional ELISA, unadsorbed antigen is removed by washing after a suitable incubation time, but on the black disc washing only occurs after the blocking agent has been dried on top of the antigen. This may reduce the efficiency of the washing process and any poorly attached antigen may not be removed as compared to a standard incubation where the antigen would be washed off directly. The maximum amount of protein that can be adsorbed as a monolayer on a polystyrene surface is about 300ng/cm^2 depending on the polystyrene surface and the bound molecule. The optimum coating concentration depends on the type of assay for example in sandwich assays as

used in this work; the surface is saturated to give maximum speed and capacity for the assay. Competitive assays require lower coating concentrations for good assay sensitivity[111]. The adsorption process can be followed directly where the adsorbing molecule is labelled radioactively (commonly Iodine-125) or indirectly where the adsorbed molecules are detected using a second labelled molecule. The former method is often used and provides quantitative results without the possible steric effects of the indirect method[111]. The binding capacity of the surface is determined by saturation of the surface with increasing amounts of the absorbing molecule and determination of plateau adsorption. Binding capacity can be affected by the use of detergents as can the stability of adsorption. Detergents increase the polarity of the aqueous solution and can destabilise the adsorption coating. They are generally used to detach and loosely bound molecules to prevent them being released during the assay and also gives a measure of the tight binding capacity of the surface. The total binding capacity is determined by treating the surface with increasing amounts of protein and measuring the amount bound at an infinitely high coating concentration as determined from the plateau of the saturation curve. The limit of independent binding was defined by Cantarero[112], as the highest coating concentration where the proportion of protein adsorbed is independent of the concentration of the coating solution. The theoretical binding capacity of a plain surface can be calculated from the size of the molecule although this will depend on the orientation of the adsorbed molecule. Binding capacity studies can be hampered by the fact that some proteins do not show a distinct maximum binding because of the formation of multilayers at high coating concentrations[113]. This was possibly happening at the high coating concentrations i.e. 10 or 20μg/ml used to coat the sectors and black disc. The coating pH becomes increasingly important as the number of hydrophilic groups on the molecule increases. Many proteins have a very high proportion of charged residues on their surface and adsorption can only be effective when there is minimum surface charge. This means that the pH has to be near their isoelectric point. A range of buffers should have been tested with the multianalye panel – phosphate-buffered saline pH 7.2 (PBS) was used except with the black discs when 0.1% bovine serum albumin was added

(see chapter 4, page 76). Different types of detergent were also not tested – 0.05% Tween 20 in PBS was used throughput. However with both the buffer and detergent, good results were produced in microtitre plates before proceeding to sectors. The aim of the work at this time was to produce a basic panel of ELISAs that could be performed without cross-reaction on a compact disc, although at the time the assay conditions were not ideal. In determining the optimal coating parameters for an antigen or antibody there are four main requirements to fulfil – correct polystyrene surface and pH, optimum coating concentration and a suitable detergent that can be used throughout the assay. For further background on polystyrene in solid phase immunoassays see Appendix 1 (page 160).

9.2 Adsorption to plastic.

Antigen adsorption to a plastic solid phase was used in this project although covalent binding is common for other macromolecules. Both microtitre plates and sectors were produced from medical grade polystyrene, although of different qualities. Microtitre plates are made of optically very high quality while the sectors were of very poor quality with scratches and debris evidnet in the final moulded structure. The plain discs were made of polycarbonate and the black discs had an acrylic or lacquered surface. Adsorption is a non-covalent interaction based on hydrophobic (van der Waals) forces. These are weak interactions; therefore several simultaneous ones are required for stable adsorption. In order for the molecule to be adsorbed properly, it must have a sufficient number of hydrophobic sites on its surface, which is usually found with biological molecules for example, lipids and proteins. The hydrophobic attraction energy between two apolar moieties immersed in water is a result of the hydrogen-bonding energy of cohesion of the water molecules surrounding these moieties. In addition, hydrophobic surfaces do not repel but in fact attract water[114]. The structure of polystyrene is inherently hydrophobic with aromatic benzene rings interconnected with short aliphatic bridges. When exposed to aqueous conditions, hydrophobic groups on molecules avoid contact with the water by conformational change or intermolecular aggregation. When it comes in contact with the polystyrene surface in an aqueous environment, it adsorbs onto the hydrophobic polystyrene

surface with the exclusion of water[113]. The final strength of binding depends on the number of contacts made with the surface, which depends on the size of the molecule and distribution of hydrophobic groups on its surface. For further background on the adsorption of proteins to polystyrene see Appendix 2 (page 163).

The role of protein desorption in polystyrene microtitre plates was examined by Nieto[115] using radioactively labelled arsonate conjugated bovine serum albumin (^{125}I-BSA-Ar36) ELISA and the effect of various experimental parameters such as washing and immune serum on the adsorption/desorption equilibrium. It was found that BSA-Ar36 adsorption to polystyrene microplates was a reversible interaction between a limited number of surface active sites and protein molecules. The Langmuir Isotherm as shown below quantitatively described this interaction:

$$\frac{1}{m} = \frac{1}{b} + \frac{1}{bKc}$$

Where c is the molar concentration of the protein in equilibrium with m grams of adsorbed protein per cm^2, b and K are constants with b corresponding to the maximum amount of protein that can be adsorbed to the solid phase (saturation). K is the association constant corresponding to the reaction between surface active sites and the protein. Nieto postulated that the adsorption constant value in the Langmuir Isotherm might be useful in ELISA design. Washing and the use of antisera was found to promote desorption although the latter was not dependant on antibody concentration with no desorption stimulated differences in ELISA results produced if the value of K was higher than the antibody avidity. The avidity can be regarded as the sum of all the different affinities between the heterogeneous antibodies contained in a serum and the various antigenic sites.

Previous work by Lehtonen and Viljanen in 1980 had also investigated antigen desorption using the amount of antigen on three different solid phases during the stages of an ELISA for chicken anti-BSA antibodies[116]. Polystyrene curettes, nylon rings and cyanogen bromide activated paper were tested. Antigen leakage during the assay was about 30% for the polystyrene, 60% for the nylon and 13% for the activated paper. Incubation with the sample serum appeared to bring about rapid antigen detachment – about half the total leakage with sample antibody took place in the first 15 minutes with the polystyrene cuvettes, although this was found not to be due to the primary antibody binding to the antigen. This could result in competition for the sample antibody between bound and free antigen although the amount of free antigen may be too low for this. This was not found by Nieto[115], as described above. It was also shown that it was important to wash the solid phase after antigen attachment especially at high antigen concentrations – as many as six were recommended to remove all loose antigens. In the sectors, the wells were washed out four times after antigen coating by flood-fill. This had the additional problem of introducing other antigens into the wells as during the pipetting process all antigens in the wells were mixed with the wash buffer. Although at that stage no quantitative reading could be made, the washing was judged to be effective because of the low background produced. With the black discs, antigen and blocker were dried to prevent streaming and therefore were washed off together before the application of primary antibody. Six washes were used but it was found that the O.D. value increased with the number of washes. After about seven washes this increase levelled off and six was chosen as the disc was being washed by hand (see chapter 6, page 96). The covalent coupling of the antigen to the activated paper resulted in greatly reduced desorption and also reduced batch-to-batch variation in the surface concentration of antigen when compared with the nylon and polystyrene solid-phases. With the polystyrene tubes it was found that the surface concentration of antigen varied greatly with a standard deviation about the mean of 50%. This was reduced to about 15% with the different batches of activated paper.

9.3 Microtitre plates.

Investigations into the adsorption of proteins to polystyrene and other plastics had mainly involved tubes. Although they had been used for ELISAs, they did have a number of disadvantages such as the large volume of reagents used and the unsuitability for large-scale use. Voller[117] described the use of a microtitre plate-based ELISA for the study of malaria. These polystyrene plates had 96 wells and were commonly used for haemagglutination studies. They were found to be easier to handle than tubes and were relatively inexpensive. Only small amounts of reagents were required, especially small amounts of antigen to coat the wells. The plates were thought to be suitable for use in the field where they would provide qualitative readings, or in laboratories where quantitative readings could be made. It is interesting to note that all the above reasons for using microtitre plates described in this paper are the same ones, twenty five years later for moving on to the new platform described in this thesis. The main difference between the plate and disc-based system is the benefit of multianalye testing with the latter.

It was envisaged when microtitre plates were introduced that standardisation would be a problem and it was suggested that reference preparations of positive and negative sera be prepared to allow comparable results by different laboratories. This was taken up with internationally recognised standards for many different test sera now available. The use of microtitre plates quickly became the standard for all enzyme immunoassays. Their introduction meant that enzyme immunoassays became increasingly widely used in diagnostics in fields such as endocrinology, immunopathology, haematology, and microbiology and parasitology[118]. One example of this was the development of a rapid quantitative microplate enzyme immunoassay for serum alpha-fetoprotein (AFP) in maternal and non-pregnant sera[119]. This assay system was found to have sensitivity comparable to the radioimmunoassays system without the disadvantages of using radioisotopes. It used simple, readily available laboratory equipment and had low reagent costs. Laboratory personnel time was also reduced.

As previously mentioned the coating of the antigen or antibody on the solid-phase was an important factor in assay sensitivity and reproducibility of results. It had been assumed that adding the same concentration and volume to each well resulted in the same attachment. However Chessum and Denmark[120] reported the uneven attachment of antigen to a several batches of microtitre plates. Identical treatment with the same dilution of antigen, antibody, conjugate and substrate resulted in different levels of attachment to the plates. The results that differed by more than 10% from the arithmetic mean of the total readings were examined. It was found that in some plates there was an edge effect with higher readings in the outer wells while other plates showed high readings in the bottom middle of the plate and low readings at the top. The use of plates showing these effects would result in erroneous results and could have serious consequences diagnostically. Similarly Standefer and Saunders[121] found that in developing an ELISA for gentamicin, some batches of microtitre plates bound as much as half less antibody than usual to their polystyrene surface. In this assay the wells were pre-coated with BSA (2.5g/l) for ten minutes before washing and coating with gentamicin antibody. This was to prevent unequal antibody binding and lower non-specific conjugate binding. It may have worked in a similar manner to adding 0.1% BSA to the antigen coating buffer when using the black discs. The variation between microtitre plates was examined by determining the thermal profile of plates at room temperature and 37°C[122]. A marked edge effect was found for all types of plates tested with the temperature at the edge and in particular the corner of a plate being much higher than in the centre after incubation at room temperature for two hours or overnight. Covering the plate eliminated this variation across the plate but uncovering for more than thirty minutes re-established the temperature gradient. Handling an equilibrated plate also disturbed the temperature and this remained for up to ten minutes. The reason for this difference in temperature was not clear but was possibly due to thermal exchange with the environment. It was thought that this inter-well variation could affect the ELISA in two ways - the coating of the wells and the development of colour. The increased temperature may increase the rate of reaction between the enzyme-conjugated second antibody and substrate thus resulting in

increased higher readings in these wells. Further background into the variability in adsorption to microtitre plates is shown in Appendix 3 (page 168).

A specially formulated polymer that binds proteins, peptides and nucleic acids was used to construct shallow wells in an adaptation of the standard 96-well plate[123]. It was investigated for solid-phase immunoassays and used quantitation of the colour intensity of a dye on the solid phase by means of a simple desk-top scanner coupled to a computer which allows the grey-scale density of the colour to be easily and accurately measured. This is in some ways similar to the system described here. The original readings from the black disc are made in the grey-scale and then converted to OD values. The advantage of the disc system is its use for multianalyte testing.

9.4 Quality control

Any company producing microtitre plates for use in clinical diagnostics has to have rigorous quality control procedures to guarantee their plates. Insufficient antigen or antibody coating can lead to false negative results as was found, in one case, with the HIV-antigen coating of microtitre plates[124]. For example with "Labsystems"[109], the plates are produced in an environmentally controlled area with the raw materials being carefully selected and tested before use. In addition no regrinded material is used i.e. excess material from a batch of plates is not re-used. A 100% optical inspection for all plates is carried out and there are background absorbance tests in addition to antibody or antigen coating tests. The industry standard is ISO 9001. It was particulate polystyrene that was used for the sectors, which was later found to be of very poor quality, possibly as it may have been regrinded. It was later found that the company that produced the moulded sectors (Pascoe Engineering Ltd.) also made microtitre plates for another company and used "virgin" polystyrene for these. The poor quality of the polystyrene used for the sectors obviously affected the antigen coating. Performing binding capacity tests with different detergents and pH conditions may also have resulted in improved antigen adsorption and hence increased assay sensitivity. Coating FITC-conjugated bovine

serum albumin in the wells and using fluorescent second antibody did show that coverage of the wells was not good but this was not quantified. Using the plain discs, which were made of polycarbonate, produced better colour which may have indicated either improved antigen adsorption or improved reaction with the substrate, insoluble TMB, although no quantitative experiment were undertaken to confirm this (see chapter 8, page 121). The surface of the plain disc was smoother and may have contributed to the improved colour production. The black discs have on their surface an acrylic or lacquer coating to which the antigen appeared to attach very quickly - even after one minute incubation period; fairly strong colour was produced. Acrylic microtitre plates are used but have very low binding capacity. They are used when the assay requires no binding to the surface of the wells[109].

The moulded sectors were made of medical grade polystyrene as previously described. There was poor well coverage and a lack of reproducibility between wells and between sectors. The optical quality of the polystyrene was poor – there were obvious scratches on the well surface in addition, no doubt, to the lines produced by crazing (formation of fine lines) during the moulding process. In addition the sectors were not always totally transparent, with debris being evident in the plastic, sometimes even within the well area. Examination of the sectors after the ELISA has been completed showed poor well coverage - the colour was very patchy and when pre-coated with silicone spray, there were white droplets disturbing the colour. This was due to the deposition of silicone aerosols on the surface of the well during pre-coating. Well coverage as dictated by colour was better without the presence of silicone, but this then meant that the colour was not as good i.e. more green than blue. No quality control was carried out on the sectors or polystyrene before moulding and there were no coating or stability experiments. This could have provided information about the adequacy of well coverage although preliminary experiments with FITC-BSA did show that coverage on wells without sectors was not very good with fluorescent accumulations around the edges of the well and in the scratches on the well surface.

It is possible that there was an edge effect as for microtitre plates. Once the sectors had been coated with antigen, they were placed directly in the spider during incubation. This may have reduced any possible edge effects in a similar way to covering the microtitre plates did. The sector was not completely covered, however with the outer edges of the spider open for inserting and removing the spider and this may not have completely removed any edge effects. There was very obvious well-to-well and sector-to-sector variation, which on visual examination appeared to be reduced by the use of silicone. This could have been due the silicone increasing the hydrophobicity of the well surface therefore increasing adsorption between the well and hydrophobic moieties of the protein. No quantitative reading were made to confirm this. A relatively high antigen concentration of 10-20μg/ml had to be used in order to produce a strong blue colour. Colour was produced with a concentration of 3μg/ml but this was very pale and it probably would not have been detectable. On the black discs, a coating concentration of 5μg/ml only produced colour with the highest antibody concentration. Antigen coating concentrations in microtitre plate assays depend on the protein but can be in the low microgram or nanogram range. The reason for the high coating concentration on the sectors and discs was the use of insoluble TMB as substrate which appeared to lack the required sensitivity to produce a range of colour over a defined antigen and antibody concentration range. The coating concentration for all seven antigens in the multianalyte ELISA on sectors and black discs required to be high to produce a limited colour range with a range of primary antibody dilutions.

9.5 Interaction between antibody and solid phase antigen.

The binding of antibody to adsorbed antigen is not the same as the binding of antigen and antibody in solution. Kennel had noted that cell membrane-bound antigens were much more efficient at binding antibody, on a molar basis, than protein antigens bound to polystyrene plates[125]. Using the binding of rat monoclonal antibody to fragment D of human fibrinogen (FgD), the binding of antibody to antigen in solution or on solid supports was analysed.

Binding constants with antigen in solution were found to be similar to solid-phase antigen. The antibody-antigen reaction in solution is monovalent, with only one reactive site per FgD molecule. Possible bivalent interactions on the solid-phase would increase the affinity constant, but this was not observed. There may also be steric effects and difference in diffusion and reaction rate constants. It was also shown that assay sensitivity depended on both the amount of solid phase antigen and its density. FgD bound to polystyrene plates was 3-10 fold less active in binding antibody then when bound to Sepharose beads. This was found even when poly-L-lysine/glutaraldehyde was used to pre-coat plates. The possible reasons for this included steric factors, selective destruction or shielding of the epitope from the antibody due to non-random antigen attachment to the solid support. The use of beads or flat surface may also have affected the nature of the bond between the antigen and solid-phase.

When the black disc was used as the platform, antigen had to be dried at room temperature onto the wells to prevent streaming into other wells. This occurred during the washing step and resulted in cross-contamination between different ELISAs on the disc. The blocking agent (50mg/ml BSA/PBS) had to be dried on top of the antigen to prevent this. Drying the antigen prior to an immunoassay is not a new procedure. It has been perhaps most commonly used on membranes and latex, for example the "Dryspot" kit for detection of the infectious mononucleosis heterophile antibody which uses dried antigen-coated latex[126]. Other formats include dipsticks, which often contain dried reagents. It has become increasingly common however to dry antigen and other assay components onto wells of microtitre plates. For example an assay based on one-step all-in-one dry reagent time-resolved fluorimetry has been developed for monensin, a carboxylic acid ionophore used in poultry to control coccidiosis. All assay components are pre-dried onto microtitre plate wells. Only the diluted serum sample is required to be added. The limit of detection was found to be 14.2ngml^{-1}. In another 96-well microplate plate assay, wells were coated with Brucella abortus smooth lipopolysaccaride antigen and air-dried at room temperature for one hour. The plates were resealed and stored on the bench until use. Consistent results were produced which appeared to indicate that air-drying was suitable

for some antigens[127]. The main difference with the platform described here was that it was being used for multianalyte testing and it was required to physically anchor the antigen to prevent cross-contamination with other wells.

It had been found that there was improved detection of antibody when it was bound to antigen rather than directly adsorbed to a solid phase. Dierks[128] quantified the differential recognition of an antibody adsorbed directly to the solid phase and an antibody bound to antigen on the solid-phase using an ELISA technique and quantitative immunoprecipitation. Two isotypes, IgG and IgA were used and two antigens, ovalbumin and fluorescein. It was found that antibody adsorbed directly to the solid phase was recognised less efficiently in the ELISA than antibody bound to antigen in the solid phase and that this was dependant on the isotype and not on their specificity. As previously found, the introduction of a spacer molecule, linking the antigen to the solid phase, improved antigenic activity and hence antibody binding[125]. In this case glutaraldehyde was used[128]. Displaying the antigen at a greater distance from the solid-phase may reduce problems arising from steric hindrance or inhibitory effects from surface chemistry repulsion. The use of a linker molecule in the disc-based system may improve antigen coating but this may be negated by having to dry the antigens onto the wells. The use of glutaraldehyde caused aggregates of ovalbumin to form, which may have exposed epitopes that are lost when monomer ovalbumin is adsorbed onto the solid phase. The IgG antibodies had to be adsorbed to the solid-phase in the presence of 250ng/ml rabbit serum albumin in the coating buffer. Without this, the guinea-pig anti-rabbit IgG detection antibodies only poorly recognised the IgG when less than 50ng was adsorbed. It was thought that the addition of the rabbit serum albumin increased the net amount of protein on the surface and forced the IgG molecules to orient so that their Fc antigenic determinants were exposed in a manner similar to when IgG is bound to antigen. The addition of 0.1% BSA to the antigen coating buffer on the black discs had the similar effect of producing a higher OD value perhaps due to an effect similar to the one described here. The presence of BSA in the antigen coating buffer increased the number of antigen molecules with the correct epitopic orientation resulting in increased primary antibody

binding and hence higher O.D. values. A similar effect has been found with other systems. Binding of three monoclonal antibodies to recombinant interferon-gamma, immobilised on an ELISA plate surface was enhanced when BSA was added to the coating mixture. This suggested that the amount of interferon-gamma on the plastic surface had increased and that its original structure was preserved[129].

No colour was produced with 0.1%BSA in the buffer on coating Human Serum Albumin (HSA) on to the black discs. This could have been due to several reasons. Firstly, the BSA was present at a concentration of 1μg per well and the HSA at a concentration of 0.02μg per well giving a 50-fold excess of BSA. The high BSA concentration could be preventing the attachment of HSA to the disc, but this did not happen with the other antigens. The addition of BSA to the coating buffer may have altered the pH so that HSA would not bind to the plastic surface. HSA and BSA have a similar heart-shaped structure consisting of three homologous domains with about 67% α-helical content. There is a repeating series of six sub-domains that form a hydrophobic channel and there are ten principal helices in each domain along with a number of disulphide linkages[130,131]. The molecular weights of HSA and BSA are also similar i.e. 69000 and 66000 respectively. The similarity could mean that there is an association between the two molecules in solution that leads to the HSA being "masked" and therefore not being available to bind the primary antibody. It is possible that this is an artefact produced by drying the antigen and blocking agent on the well – it may not happen if the HSA was coated in wells with the excess being washed out after incubation. It has been shown that HSA will in fact also bind to hydrophilic surfaces. In theory, HSA dissolved in water should be repelled from hydrophilic surfaces such as glass and silica resulting in no adsorption at pH 4.85. But it was found that at pH values less than 8, HSA did adsorb moderately well to these surfaces. The reason was thought to be the presence of discrete heterogeneous sites on the hydrophilic surface that are positively charged and electron acceptors (for example Ca^{2+}) that attract negatively charged and electron donor groups on the HSA surface[132].

The use of other surfactants to aid adsorption has also been investigated. In this thesis Tween 20 was present in the wash buffer at a concentration of 0.05%. It reduces non-specific binding and removes any loosely adsorbed antigen and poorly bound antibodies. Adding it to the antigen coating buffer (PBS, pH 7.2) at concentrations of 0.05%, 0.1% and 0.25% did not improve the variation in OD and CV values (chapter 4, page 76). This involved drying the antigen on to the well, which could have affected the result. An investigation into the effect of Tween 20 on albumin adsorption onto silicon surfaces was carried out by Zhang and Ferrari[133]. Protein adsorption was found to depend on the degree of hydrophobicity of the surface and the concentration of the surfactant. A reduction of 90% in albumin adsorption on hydrophobic methylated surfaces by 0.05% Tween 20 was found with only a 15% reduction on hydrophilic surfaces. Similar effects are observed on other surfaces with Tween 20. The effects on desorption of single protein solutions from polyethylene and polyurethane was studied[134]. Single solutions of fibrinogen, IgG, HSA, high density lipoprotein (HDL) and plasma were used. The results showed that the effect of Tween 20 on protein desorption is due to either the displacement of protein or the prevention of protein adsorption onto the surface depending on the protein and the surface. For example, about 40% of adsorbed HSA and 80% of the HDL were desorbed from polyethylene while there was only a small effect on fibrinogen and IgG under the same conditions. See Appendix 4 (page 170) for further background on the adsorption of proteins to other surfaces in ELISAs.

9.6 Sensitivity of ELISA.

The affinity of the antibody for the immobilised antigen is thought to be one of the most important factors determining the limitation of the sensitivity of the immunoassay. It is apparent from the results presented in this thesis that the sensitivity was limited in detection of the primary antibody. This was also due to the substrate used to produce the blue colour (insoluble TMB). This did not produce a range of colour, as with a soluble ELISA substrate, instead colour was weak, medium or strong blue, with it often being difficult to distinguish

between them (see chapter 6, page 92). Essentially the colour was good enough for a yes/no rather than a quantitative result. This may be good enough when the presence or absence of an analyte is all that is required to be determined but for other analytes such as hormones, an actual amount or concentration is required. The problems with antigen coating would also have affected the sensitivity – a coating concentration of 20μg/ml was likely to be too high but due to the substrate this was required to maintain colour. There was a range of primary antibody concentrations used at various dilutions. These ranged from 0.6μg/ml to 28.2μg/ml at a dilution of $1/1x10^3$ (see chapter 6, page 94). Given this range of concentrations there was little or no difference in OD value produced, emphasising the lack of sensitivity of the substrate. Reducing the primary antibody concentration did not significantly increase the sensitivity with the lowest dilution being $1/1.6x10^4$ before colour was not detectable by the reader. For further background on the theory of immunoassay sensitivity and methods of improving sensitivity see Appendices 5 (page 172) and 6 (page 176) respectively.

9.7 Washing of sectors and discs.

The wash buffer used was phosphate buffered saline (PBS) with 0.05% Tween 20. No other wash reagents were tested because of the very low non-specific binding produced with PBS/Tween. During the development of a multianalyte system, other wash buffers may require to be investigated, including the addition of buffer salts, chaotropes, proteins and other detergents to the wash buffer. Washing is a very important step in an immunoassay of which the physical flushing is only one aspect. Often some or all of the components of the wash buffer are included in the reaction buffer. In the system developed on the black disc, BSA was used in the reaction buffer and Tween 20 in the wash buffer although PBS was used for both washing and reactants. The use of detergents can lead to better precision and accuracy of results, however if too much is present, changes in protein structure can occur in addition to desorption of antigens or antibodies from the solid phase. Some detergents may not be highly purified and can contain peroxides and other impurities that may inhibit the antigen-antibody reaction[135].

Washing removes entrapped label, reduces adsorption of the label and aids removal of the reaction supernatant[79]. This is especially important with a multianalyte ELISA and on the black disc where there were no separate wells.

In immunoassays, the wash technique depends on the solid phase being utilised. Particles, beads and microtitre plates can be washed actively. In general, microtitre plates are washed either by a designated plate washer where the volume and number of washes can be pre-programmed or by the use of a wash bottle. Membranes are usually washed by capillary flow or radial partition, which produces a much slower flow rate increasing the importance of detergents and proteins in the wash buffer to reduce non-specific binding. The use of the flood-fill technique to wash the disc between different stages of the ELISA proved very successful. There was very low non-specific binding and no cross-contamination between wells on both the sectors and black disc. Using the disc as the solid phase allowed for robust washing of the system. It was important however not to pipette wash buffer over the sector surface too vigorously, as this resulted in the formation of bubbles (due to the presence of detergent) which reduced the washing efficiency. It was found that increasing the number of washes increased the O.D. value, but it was decided to use six washes because at the present time, washing was performed by hand. Each sector was washed six times after each reagent incubation. If all eight sectors were being used, this could take up to ten minutes to wash the whole disc, adding considerable time to the assay. It was envisaged that in the future, a modified "washing pipette" could be developed allowing all eight sectors to be washed simultaneously. This would increase the efficiency of the washing process and significantly reduce the overall assay time. One of the advantages of the CD-based system is that it could be used as a stand-alone piece of equipment on the bench for a low volume throughput of samples or as an automated system for high-throughput sample testing. In the latter system, automated washing would be required which could be easily set-up for the disc. No other detergents were tested in the system as Tween 20 produced good results. The type of wash buffer may require to be changed depending of the components of the multianalyte ELISA.

9.8 Substrates.

A number of different substrates were used in order to produce an insoluble blue colour of suitable intensity. In initial studies based on visual examination, this was taken to be a strong blue colour that would be detected by the modified reader. It was thought that the stronger the colour, the more likely it was to be detected by the reader and the more likely it was to be used to produce quantitative results.

The substrates tested were 3,3'-Diaminobenzidine, 4-Chloro-1-Napthol, 5-Bromo-4-Chloro-3-Indoyl Phosphate/NitroBlue Tetrazolium and insoluble 3,3', 5,5'-Tetramethylbenzidine (TMB). Of these only insoluble TMB produced the desired colour and this was used throughout the project. Tetramethylbenzidine is a colourless chromogenic reagent that turns to a blue-green in the presence of peroxidase and hydrogen peroxide. The insoluble TMB used was a propriety all-in-one reagent containing hydrogen peroxide that was applied directly to the sectors as the last stage in the assay. In the presence of the horseradish peroxidase conjugated second antibody, a blue colour was deposited on the wells. The structure of TMB is shown in Figure 9.1.

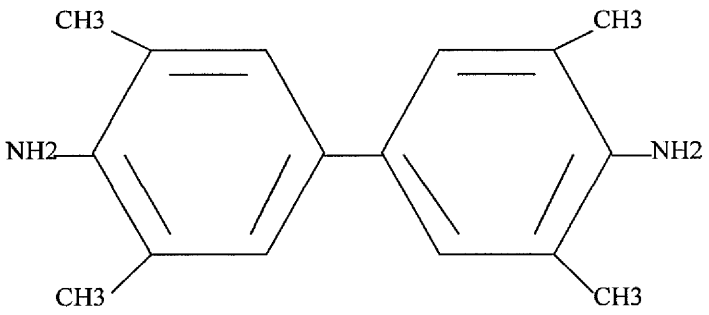


Figure 9.1: The Structure of 3, 3', 5, 5'-Tetramethylbenzidine.

Tetramethylbenzidine is the most sensitive chromogenic substrate used for detection of horseradish peroxidase-labelled probes. It is used routinely in ELISA procedures where its soluble oxidation products are measured spectrophotometrically. The insoluble TMB was designed for use in immunoblotting on a variety of membranes and is often used in preference to the carcinogenic Diaminobenzidine (DAB). A comparison of insoluble TMB and DAB in horseradish peroxidase-dependant immunoblot assays showed that the TMB was more efficient due to its easy preparation increased stability and reduced carcinogenic properties. The TMB was found to be functional after four weeks at 48°C and after eight weeks at room temperature compared to the DAB which was functional after one week at 48°C and four weeks at room temperature[136]. In conventional ELISA techniques, TMB is measured at 655nm but there is some evidence that a double wavelength measurement provides a three-fold enhancement of the measuring range[137]. In order to use TMB for immunoblot assays, enhancers, usually sugar polymers such as dextran sulphate, are used. For example, in a comparison of TMB with Diaminobenzidine (DAB) and 4-Chloro-1-Napthol (4C1N), the membranes were incubated first in 1% dextran sulphate for ten minutes before reacting with the TMB. The colour developed was more stable and the assay more sensitive when TMB was used compared to DAB and 4C1N[138]. The insoluble TMB reagent used with the disc-based system did not use sugar polymers as a proprietary methodology developed by "Calbiochem" eliminated the need for them. This resulted in a single component insoluble TMB system.

9.9 3,3', 5,5'-Tetramethylbenzidine.

In conventional ELISAs, TMB is widely used as a specific peroxidase substrate due to its high sensitivity and relatively low toxicity. It is used in a range of different areas covering environmental, agricultural, veterinary, research and medical diagnostics (see Appendix 6 on page 176).

Insoluble TMB is used mainly on membranes, commonly nitrocellulose, in immunoblotting assays. These type of immunoassays are also used in a wide range of different areas but are

perhaps most recognisable in a dipstick format where the nitrocellulose or other membrane is attached to a plastic holder where the development of colour can be easily observed. In some cases qualitative results are used but where quantitative results are required a colorimeter or similar is used to determine the amount of antigen or antibody present in the sample. Examples of the dipstick format utilising insoluble TMB as substrate include one developed for the rapid detection of the Fusarium T-2 toxin in wheat[139]. In this a direct competitive immunoassay was performed on the membrane which was coated with anti-T-2 toxin antibodies before being assembled into a dipstick. This included exposing the antibody-coated dipstick to a mixture of sample and T-2-toxin-horseradish peroxidase conjugate. After this the strips were placed in a solution of TMB where the deposition of blue colour on the membrane indicated a positive reaction. A portable colorimeter was used to quantify the colour. It was found that the assay could be performed in 45 minutes with a visual detection limit of 0.25ng/ml. Similarly in the detection of ochratoxin A, also in wheat, a flow-through membrane-based system was used where antibody-coated membranes were placed on an absorbent layer in a plastic test device and a sequential competitive enzyme immunoassay was performed in less than fifteen minutes[140]. The dot colour intensity produced had an inverse relationship to the toxin concentration and a portable colorimeter was again used to quantify the colour. For other examples of the use of insoluble TMB on dipsticks and membranes see Appendix 7 (page 181).

9.10 Insoluble TMB on sectors and black discs.

Using insoluble TMB as a substrate provided a simple way of showing that it was possible to perform a multianalyte ELISA on the sectors and black disc. The main disadvantage appeared to be that it did not produce quantitative results. This may have been due to the fact that this substrate was not specifically meant for use on plastic solid phases which could affect its sensitivity. It was found that the colour produced with the insoluble TMB on sectors varied depending on the plastic used. The sectors were made of polystyrene and it was found that the colour production was very variable and tended to be green rather than blue. It also had poor stability as the colour faded relatively quickly to pale green. The use of silicone spray to pre-

coat the sectors greatly improved the intensity, stability and reproducibility of the colour, with a stronger blue being produced which did not fade as quickly (see Chapter 4, page 58). This was thought to be due to the silicone increasing the hydrophobic environment of the well resulting in increased antigen coating and also making a more favourable environment for the reaction of insoluble TMB with the peroxidase-conjugated second antibody. Two different second antibodies were used with insoluble TMB. With the sectors, normal goat anti-rabbit IgG–HRP was used initially. This was later changed to an enhanced second antibody (Amdex anti-rabbit IgG-HRP), where instead of one enzyme molecule being attached to the antibody; many are attached via a dextran backbone (see chapter 7, page 107). The function of this is to increase assay sensitivity and reduce incubation times. The use of the Amdex second antibody increased the colour intensity, although in some cases this meant a stronger green colour. The addition of the silicone pre-coat led to a stronger blue colour with enhanced stability. On the black discs there was less difference in colour produced by normal and Amdex second antibodies. Due to the lack of reproducibility and relatively low O.D. values the Amdex second antibody was used on the black disc, but once the variation was reduced, it was envisaged that normal second antibody could be used (chapter 7, page 112).

The use of insoluble TMB as a substrate has shown that it is possible to perform a multianalyte ELISA on a modified compact disc. It does not appear to be sensitive enough to quantify results although it may be useful in producing a semi-quantitative result. On both the sectors and black disc, it was obvious from visual examination of the colour produced at different antigen and antibody concentration that there was poor gradation of colour as compared with a conventional ELISA substrate such as orthophenylenediamine dihydrochloride (OPD) or even soluble TMB (chapter 8, page 125). With these substrates there is an obvious grading of colour over a range of antibody dilutions, that was not evident with insoluble TMB. This was apparent even in initial experiments when insoluble TMB was used as the substrate in microplate-based ELISAs. The variation and lack of reproducibility in O.D. values produced when the reader was used with the black disc made it difficult to draw significant conclusions. However even

from this it was clear that the insoluble TMB was not quantitative and would perhaps be best utilised for a yes/no result. It would have been interesting to investigate the colour production with insoluble TMB using the individual ELISAs from the multianalyte system with a nitrocellulose membrane as the solid phase to test for increased gradation of colour. As described above there have been several cases where quantifiable results have been produced with insoluble TMB on membranes[139,140], therefore the reagent itself must have a degree of sensitivity that is not being fully exploited on a plastic solid phase possibly due to inadequate dispersion of the antigen.

To produce a quantitative system, it would therefore be necessary to change to a different substrate. One option would be to conjugate a blue bead to the second antibody, eliminating the need for a signal development step. The blue beads would be directly related to the number of second antibodies bound and the current system of optics in the reader could be used. The problem may be producing a colour of suitable intensity as it was found with the insoluble TMB that colour had to be of a fairly high intensity before it was detected. Another alternative is to use a fluorescent second antibody although this would require a change in the optics of the reader, but would almost certainly increase the sensitivity of the system. The reader itself also contributed to the variability and sensitivity problem as shown by the variability in O.D. and C.V. values produced with the blue acetate disc (chapter 4, page 79). The optics in the system including primarily the red laser, had not been optimised or calibrated, therefore this would have affected the sensitivity of the system, although the results from visual observation of the insoluble TMB colour indicated a lack of gradation in colour as previously described.

Therefore to produce a quantitative system, a different substrate or signal is required together with an improved detection system in the reader. These requirements will depend on the nature of the results required from the immunoassays on the disc, which will depend on the analyte being investigated. For example certain parasitic or other diseases may require only a yes/no result whereas a test for a panel of hormones or cardiac enzymes require a fully quantitative

result. It is feasible therefore that the use of insoluble TMB may be good enough for a yes/no test with the result based on visual examination of the disc with a limited requirement for a reader. Fully quantitative tests would require a reader. Expansion of the disc-based system may have two or more different versions; for example, qualitative and quantitative, depending on the tests incorporated on the disc. The incubation times used with the sectors and black disc are shown below in Table 9.1.

Stage in ELISA	Sector Incubation Times (mins)	Black Disc Incubation Times (mins)
Antigen Coating	15	45
Blocking	15	45
Primary Antibody	10	10
Second Antibody	10	10
Insoluble TMB	10	10

Table 9.1: Incubation times for ELISA performed on sectors and black discs.

Antigen coating and blocking with the black disc involved drying at room temperature which took longer than the corresponding fifteen minute incubation times on the sector, where there was also a washing stage as in a conventional ELISA. Although this significantly increased the total assay time, it should be considered from the viewpoint of the end user. If this disc-based system was to be used commercially in, for example, hospitals, clinics or for field testing, the discs would be supplied pre-coated and pre-blocked. The end-user would only be required to wash the disc before applying the sample. Therefore the increased times for antigen coating and blocking with the black disc does not affect the speed of the assay at the point where it is used. The drying stages would only affect the speed of the assay for the end user if they were performing the entire assay as in a research environment. With the incubation times described above, this would give a total assay time of two hours, excluding washing. Once the variability has been reduced it may be possible to reduce the incubation times even more. Early experiments on the sectors found that reducing the incubation times to five minutes was

possible for some stages in the assay, although the variation in colour made it difficult to make a definitive evaluation (chapter 4, page 59).

9.11 Multianalyte immunoassays

Multianalyte immunoassays have become increasingly common especially over the last decade. This has been based on the development of point of care tests for the home or doctors' surgery, resulting in ultrasensitive assays, transducer-based immunosensors and multifunctional and random access analysers[69]. The conventional approach relied on the simultaneous measurement of multiple labels, which did not prove successful beyond two labels. One of the main drives towards multianalyte testing was the molecular heterogeneity of many biologically important substances such as hormones. Meaningful measurement of a mixture of such substances in, for example a serum sample, must measure each isoform of that substance present in the sample in order to produce a meaningful result[141]. This can cause problems with immunoassay validity, standardisation and interpretation. Multianalyte immunoassay was therefore put forward as a solution to some of these problems.

Although early attempts were based on different coloured beads coupled to antibodies, more recently this has evolved into arrays of individual reaction zones on a single solid phase[70]. Spatially separated areas can be achieved by dotting, entrapping or chemically immobilising different antigens onto a suitable membrane. There are a number of possible drawbacks to this type of immunoassay including the possibility of cross-reactions, difficulty in optimising the assay ranges for individual analytes and quality control issues. Following on from this has been the development of miniaturised multianalyte systems allowing for the simultaneous ultrasensitive measurements of a number of analytes. The development of such assays has followed on from the miniaturisation trend in the microelectronics industry. These assays would have a number of benefits for the medical diagnostics industry in which immunoassays have a major role, including, the use of very small samples and moving away from centralised laboratory testing to the point of care[69].

9.12 "Microspot"immunoassays and microarrays.

A direct consequence of the miniaturisation trend was the development of the antibody "microspot" immunoassay. As described by Ekins[142] this is based on the "antibody occupancy principle" of immunoassays and led to the formation of the antibody microspot immunoassay and "ambient analyte immunoassays" where a small amount of antibody is confined to a microspot on a solid support. This then led to the development of the dual-label microspot immunoassay. In this the bound antibody is exposed to an analyte-containing medium which is then removed and a second high concentration "developing" antibody solution is applied. This second antibody can be directed against a second epitope on the analyte molecule if it is large or if it is small, against unoccupied antibody binding sites[143]. The fractional occupancy of the sensor (solid-phase) antibody is determined by measuring the ratio of sensor and developing antibodies with different labels for example a pair of radioactive, enzymic or chemiluminescent markers. Fluorescent labels would allow the use of optical scanning techniques to scan arrays of antibody microspot over a surface, with each microspot directed against a different analyte thus allowing multianalyte assays on the same sample. Ekins also suggested that using the microspot format could result in immunoassays that were more sensitive and rapid than conventional systems leading to a reduction in incubation times. This was because the smaller the microspot of sensor antibody, the lower the diffusion constraints on the velocity of the antibody-analyte reaction so that the kinetics approach those of a homogeneous liquid-phase reaction. In addition the fractional rate at which sensor antibodies on the solid phase become occupied is greater than when a higher antibody concentration is used as in conventional assays[143]. This may also explain the reduced incubation times in the CD- based system described in this thesis. Although the ELISA used had antigen coated on to the solid phase and not antibody as described in the work of Ekins, it was found that incubation times could be drastically reduced – to as low as ten minutes with the antibodies and substrate.

There was a lower limit to the size of the microspot below which there was a loss of precision in measurement. The properties of the label and the measuring instrument also affect this lower limit on size. Conventional fluorophors (Texas Red and FITC) were used as labels with a laser scanning confocal microscope to quantify the fluorescence signals. White microtitre plates were used as the solid phase. Ekins also noted that it was deficiencies in the deposition of antibody on to the solid phase that was the main source of imprecision in assay results and limitation in assay sensitivity. Sensitivity could have been increased with better antibody microspotting techniques[143]. This was also found when using the black disc where the antigen (1μl) was coated by hand. A later commercial method of constructing microarrays was developed by Boehringer Mannheim, which relies on small polystyrene carriers onto which the microspots were deposited using ink-jet technology. Arrays of 100-200 spots can be deposited on the flat bottom (~3mm diameter) of the carrier wells. Each drop of reagent (antigen or antibody) has a volume of <1nl and produces a spot of diameter approximately 80μm. Spot size and array patterns are controlled automatically by a digital imaging feedback device allowing very precise microspot application[3]. This ink-jet method would also be suitable for the deposition of coating antigen on to the black disc where it would reduce the variability due to hand-coating 1μl of antigen. Other microarray immunoassays are described in Appendix 8 (page 184) with further details on multianalye immunoassays on dipsticks in Appendix 9 (page 188).

9.13 Point of care

One of the perceived advantages of multianalyte testing has been its applicability to the area of point of care or near-patient testing. The use of a compact disc-based system, with the familiarity of the compact disc itself and the modified CD player, lent itself to use at the point of care, especially with the advantage of computer compatibility. It was envisaged that such a system could cover several different levels of point of care testing from hospitals to health centres, doctors' surgeries and field-testing.

The general trend towards point of care testing (POCT) has been due to advances in biosensors, microprocessors, and the measurement of undiluted blood and miniaturisation of laboratory analysers. This has in turn led to a decentralisation of laboratory medicine. The advantages of POCT include faster turnaround times, more rapid medical decisions, avoidance of sample identification and sample transport problems and the need for smaller sample volumes. However, these are not always associated with a clear clinical benefit to the patient. The disadvantages of POCT include incorrect handling and maintenance of analysers by untrained staff, inadequate or absent calibrations and quality control, lack of cost-effectiveness due to an increased number of analysers, more expensive reagents, insufficient documentation and different comparability of POCT results with centralised laboratory results[143].

The role of POCT has been undergoing scrutiny and in same cases it is thought that the decision to move to POCT should be made only when necessary and when the quality of the analyses can be guaranteed. In the case of intensive care medicine this was recently suggested to be restricted to vital functions such as haemoglobin, activated whole blood clotting time, blood gases, sodium, potassium, ionised calcium, glucose, creatinine, ammonia and lactate[144]. The anticipated benefits of early POCT have not always happened especially in hospital environments, often due to poor quality control where the results of the tests are acted on before the appropriate quality checks are performed. This was also partly due to the decentralisation of testing also leading to the decentralisation of information[144]. One of the aims of POCT is to provide results as quickly as possible and the use of information technology systems will be vital. It will be important in the future of POCT that any testing system can be integrated into the medical information system. The system described in this thesis could be easily integrated into a medical information system as the reader is run using a standard computer fitted with the appropriate software. In a networked hospital system this would allow results to be accessed by the appropriate clinicians or laboratory personnel. Although quality control was not addressed in this thesis, it was envisaged that in the future, designated wells or sectors on the disc would be

used as control wells. If they did not produce the correct results the user would be alerted by the software and the disc discarded.

It is evident that point of care testing is going to be playing an increasingly important role. This is dependant upon improved quality control and the presence of demonstrable improvements to patient care both clinically and financially. The CD-based system described in this thesis is still in development, but it is has been shown that it is possible to perform a multianalyte immunoassay on a modified compact disc. The results can be determined by a modified reader and delivered by specific software to a computer. As previously described, this makes it highly suitable for integration into existing medical information systems. Although the system is still to be tested on a specific disease panel and there are technical problems such to be addressed this system still represents a novel and effective way of performing immunoassays, delivering results rapidly for a panel of different tests from a small sample volume. The cost of reagents is greatly reduced due to the small volumes being used and the cost of the CD as a platform is also low. In addition the disc can also be used to store information such as protocols, references and troubleshooting guides. This system is a valuable and flexible addition to the growing number of point of care tests. Although the disc-based system has been used initially for immunoassays, it could also be adapted for other multianalyte testing procedures such as clinical chemistry and genomics. In this way there would be a standard testing platform for a whole range of different biological analyses all of which could be performed at the POC with integration into the existing medical information system. See Appendix 10 (page 191) for further background on point of care.

9.14 Conclusion

From the results presented in this thesis, it is shown to be possible to perform a multianalyte immunoassay using a modified compact disc as the solid phase. The seven antigens and antibodies were used to test the system were commercially available and therefore sera, plasma or whole blood samples were not used. However even with this it was possible to perform the

multianalyte ELISA without cross-reaction. Before moving on to testing sera the aim is to eliminate the variation in O.D. and CV values. This is thought to be due to problems with antigen coating and also the substrate as previously discussed. Alternatives to the insoluble TMB include second antibody conjugated to blue beads or a fluorescent second antibody. Changing the label, for example to fluorescence would mean that the optics on the reader would have to be modified. This would be followed by testing the system using defined Rubella sera and specific antigens from the International Reference Laboratory and comparing the sensitivity and specificity with that produced on microtitre plates. Following this, multianalyte tests would be developed for parasitic diseases such as Schistosomiasis and Filariasis. This would result in a fast, reproducible system that could be used in the field providing rapid results for tests that would normally take much longer. In the bigger picture, the utilisation of a computer-compatible system means that a networked system could be set-up allowing access to results and information from field hospitals and clinics in different regions and different countries.

Before the CD-based system could be used in this way the performance characteristics of the assay have to be determined. Suitable controls would need to be worked out for all ELISAs in the multianalyte system and it would also need to be determined, if one assay failed did this affect the rest in the panel. There are a number of studies to be performed in investigating the performance of an immunoassay in a kit system[145]. 1) Reproducibility involves determining intra-assay variation (within run), inter-assay variation (between runs), variation between reagent lots and reproducibility between laboratories. 2) The recovery is determined at several different analyte concentrations representing the range of the assay by spiking a base material with a specified amount of analyte. The recovery is calculated by comparing the observed and expected concentrations after correcting for the endogenous level of analyte. 3) Linearity is determined by diluting several serum samples with elevated levels of the analyte multiple times, which are then assayed. Linear regression statistics are then performed. 4) The sensitivity of the assay is the lowest measurable concentration of analyte, which can be statistically distinguished from the calibrator. There has been debate about the true meaning of sensitivity

as discussed previously. 5) Specificity studies are conducted to identify likely interfering substances and to determine the level of specificity in the test system. 6) Stability studies should be carried out on all individual reagents and components of the assay as well as the finished assay. Reagents should be stored under various conditions and temperatures over different time periods to establish stress in addition to stability. 7) The effect of analyte excess should be investigated to show that the system would not give a false negative result for a sample with elevated analyte being measured. 8) A description of how the instrumentation used should be submitted in a manner that allows easy understanding of the principles of the assay.

It has been shown that it is possible to perform a multianalyte immunoassay on a modified compact disc using commercially available antigens and antibodies. The four criteria with which immunoassays are examined - precision, sensitivity, accuracy and specificity - were described previously.

The precision of an immunoassay is the ability to produce the same result when the assay is repeated on the same sample and depends on a good quality solid phase, coating stability, low non-specific binding to the solid phase, the quality of reagents and label and careful work. The black discs were not tested for quality and suitability for immunoassays and there may have been defects affecting the precision and even though the plastic appeared to be of good quality. The coating of the antigen would also reduce the precision. This was due to uneven drying of the antigen on the black disc and the error incurred in coating 1 µl of antigen by hand, over a sector of 44 wells or a disc of 352 wells. Non-specific binding on both the sectors and the black discs was very low while the quality of the reagents and enzyme labels were high. Both non-specific binding and reagent quality would therefore have comparatively less effect on the precision of the multianalyte assay. The final point to be addressed in determination of the precision is careful work while performing the assay. This would also be affected by hand-coating such a small volume of antigen and by other aspects of the procedure. For example, the rate of washing i.e. the speed of pipetting over the sector surface during flood-fill and ensuring

that all sectors being assayed at the same time had the same number of washes and the same incubation times.

The sensitivity is the lowest detectable amount of analyte deviating from zero. High affinity of antibodies, stability of coating, reagent concentrations, sample sizes and the reaction time and temperature influence the sensitivity. The antigens and antibodies used were commercially available. In a panel of immunoassays, such as for infectious diseases, there may be a range of antibody affinities present in the sample. The antibodies used for the multianalyte ELISA described in this thesis were polyclonal and of the same affinity. The antigen coating would also affect the sensitivity as described above. The reagent concentrations were similar to a conventional ELISA using microtitre plates, the lowest antigen coating concentration that produced detectable colour being 5μg/ml. However the colour was poor even with a low primary antibody dilution, although some standard ELISAs use antigen coating concentrations in the nanogram range. This appeared to be due to the use of insoluble TMB as substrate and changing to another substrate or detection method may result in a reduction in the antigen coating concentration. This may also result in a quantitative system and increased detection limits. The sample size was approximately 1ml, which was flooded across the sector surface. The sample could be plasma, serum, urine or whole blood although none of these were tested on the black disc; the primary antibody was diluted in buffer. A reduction in sample size would be likely in the future, as 44 wells on one sector were found to be too many. Instead of an 8-sector disc, it would be likely to be a 16- or 24-sector disc with approximately 24 or 12 wells respectively, depending on the number of assays required in the multianalyte ELISA, and if replicate tests were performed. This would result in smaller sectors and therefore smaller sample volumes, for example, 0.5ml, 0.2ml or less. A larger number of wells may be required for screening purposes, for example, allergies. The reaction was only carried out at room temperature (22°C) with the effect of temperature not being investigated, with the exception of its effect on antigen coating.

The accuracy of an immunoassay is the ability of the analyte to measure the true absolute value of the analyte and depends upon the precision, sensitivity and quality of the standard. As the precision and sensitivity have been most affected by the coating stability and the solid phase, this will have a knock-on effect on the accuracy. No standards were run in these preliminary tests but in effect the antigen and antibodies used were standards themselves, as they were defined antigens and antibodies with known concentrations. In a standard ELISA, an unknown antigen or antibody concentration would be compared against a standard or series of standards of known concentration.

The specificity of the immunoassay depends on the specificity of the antigens and antibodies and low non-specific binding to the solid-phase. The specificity between the antigens and antibodies used was very high and there was very low non-specific binding except for a very weak cross-reaction between anti-Antithrombin III and α-2-Macroglobulin. This is an obvious disadvantage of multianalyte testing, one which would have to be taken into consideration when producing different panels of multianalyte ELISAs. If there was a strong cross-reaction, it may not be possible to have the assays together in a panel but if it is only a weak cross-reaction it may be possible to allow for this when the sector is being read. There was very low non-specific binding to the solid-phase.

In conclusion, it has been shown that it is possible to perform a multianalyte immunoassay using a modified compact disc as a new platform. This is part of an integrated system comprising the compact disc as platform, a modified compact disc reader to quantify results and specific software to interpret results and to function as an interface between the system and the end-user. The new system is still in development, with a number of issues still to be addressed. Perhaps most importantly is to reduce the variability in O.D. values observed between different wells in the same sector, different sectors and different discs. The most important factor in this is the small antigen coating volume, which ideally requires an automated loading method to reduce variation. There other improvements that could be made to the coating method without

resorting to automation, which would be expensive. For example, the incorporation of a surfactant to encourage more even spreading of the antigen should be investigated further as should the effect of drying the antigen and blocking agent. Once the variation has been reduced, it will be easier to evaluate the effects of different experimental conditions. The lack of quantitative results was thought primarily to be due to the use of insoluble TMB as substrate. Several alternatives such as blue bead conjugated second antibodies and fluorescent second antibodies will be investigated.

It is important to compare this CD-based ELISA system with those performed on microtitre plates and compare the precision, sensitivity, accuracy and sensitivity. Once the technical problems have been solved, a clinically relevant panel of immunoassays will be constructed using Rubella virus antigen and defined patient sera from the International Reference Laboratory in order to compare all four of the above parameters on microtitre plates and disc. From there a logical panel of parasitic immunoassays, for example Schistosomiasis and Filariasis, can be constructed for routine testing using patient sera and tested for use at the point of care, primarily in clinics or field hospitals.

APPENDICES

Appendix 1: Polystyrene as a solid phase in immunoassays.

Although pure polystyrene has been used for a wide range of solid-phase immunoassays, as previously mentioned, it is not always suitable for hydrophilic compounds or for assays requiring very high sensitivity or reproducibility. This has resulted in the development of modified polystyrene microplates that increase the binding of hydrophilic molecules. This is brought about by the incorporation of polar groups onto the polystyrene surface by covalent coupling of oxygen or nitrogen atoms, after gamma or beta irradiation, corona treatment or plasma treatment with polystyrene of very high purity[146]. This can result in a significant increase in protein adsorption. For example the adsorption of Human Albumin on "Labsystems" standard microtitre plate, the Combiplate® Universal was compared with the modified Combiplate® EB plate using Iodine-125 as a direct label. With the Universal plate the tight binding capacity was 10ng/cm^2 while with the modified plate it was 130ng/cm^2. This shows the importance of taking into consideration the nature of the molecule to be adsorbed. Recently, polyvinylbenzyl lactonoylamide (PVLA) has been used to increase binding of hydrophilic proteins to microtitre plates[147]. PVLA is a polystyrene derivative with lactonoylamide chains originally used to promote proliferation and differentiation of cultured hepatocyte cells. Here the hydrophobic polystyrene part of the molecule was used to coat the microplate and the hydrophilic chains, which stick away from the surface, to couple proteins. Seven-fold higher amounts of alkaline phosphatase and mannose-binding protein-A were immobilised using this method. This was found to be a major improvement in the binding of small and hydrophilic proteins but with other proteins there was no increase in immobilisation compared to the adsorption method. One fairly recent development as an alternative to passive adsorption on solid phases is "hydrocoating"[148]. In this technique, molecules for example peptides that are poorly adsorbed on polystyrene are immobilised via an activated hydrophilic molecule. Soluble dextran was activated using tresyl chloride resulting in the activation of hydroxyl groups on the polymer. This activated molecule was then immobilised on a solid

surface containing amino groups leaving a sufficient number of active groups for the binding of a secondary molecule, for example antigen. Peptides that were not detected on polystyrene were easily recognised after hydrocoating and other peptides had improved detection by a factor of 5-10. Also used to pre-coat microtitre plate was methyl vinyl ether-maleic anhydride copolymer (MMAC)[149]. This had been used initially to immobilise small ligands such as peptides and oligosaccharides but was later extended to heparin. This resulted, in a detection limit of 0.1-1 femtomole of peptide per well for an angiotensin peptide and a coating concentration of 20-200 nanomoles of oligosaccharides per well in order to detect lectins.

The use of ultraviolet light has also been explored. Irradiating polystyrene ELISA plates prior to coating with target peptide was found to increase the specific signal in a dose-dependent manner without increasing the background. This method was found to be suitable for murine monoclonal antibodies in addition to rabbit and human polyclonal antibodies. It was found that using this technique, the antigenic structures recognised by the monoclonal antibodies generated against envelope protein gp120 of the human immunodeficiency virus could be localised[150]. However exposing the microtitre plate to ultraviolet light was found to have an adverse effect on an antiphospholipid (aPA) antibody ELISA[151]. Exposing microtitre plates to short wave UV light (254nm) the signal in the ELISA decreased in an UV dose-dependent manner. Using anionic phosphatidylserine as antigen, it was found that its attachment was reduced by 53% in UV exposed plates. It was thought that the UV exposure altered the lipid binding properties of the microtitre plate.

Pre-coating polystyrene microtitre plates to make them more hydrophilic, for example, with polyethylene glycol can aid in the detection of smaller molecules present at low concentrations in biological fluid[152]. The presence of the hydrophilic layer eliminates non-specific binding of other macromolecules. The nature of the hydrophilic layer can affect the orientation of the molecule being bound. For example with epoxide groups, the antigens and antibodies are randomly bound while with acid hydrazide, antibodies are site-specifically bound by their

carbohydrate residues thus retaining greater activity and more of their native antigenic structure. This would therefore be advantageous in assays for minor analytes in a mixture of major constituents.

The use of polystyrene as a non-particulate solid-phase for immunoassays was carried out by Catt and Tregear[153]. Before this polystyrene solid-phases had been in the form of powders, for example Sephadex or small discs. Two types of plastic tubes were tested – polypropylene and polystyrene and both gave satisfactory results. The tubes were coated with antibodies and when applied to the radioimmunoassays of human growth hormone and human placental lactogen, produced sensitivity comparable to other methods of radioimmunoassay. The advantages to using plastic tubes were simplicity and economy with the same tube being used for all stages of the assay. The use of polystyrene tubes was developed further for a sandwich radioimmunoassay for the quantitation of human immunoglobulin[154]. The tubes could be stored at room temperature after binding of antibody. This sandwich technique resulted in enhanced antigen binding capacity compared to a single-layer solid-phase. This was thought to be due to the correct orientation of the antibody in the sandwich method. Adsorbing the antibody directly onto the plastic could have meant that not all binding sites were available for the antigen.

Appendix 2: The adsorption of proteins to polystyrene.

The conformational change in proteins upon adsorption to polystyrene particles has been studied using circular dichroism[155]. Bovine ribonuclease A (RNase A), horseradish peroxidase, sperm-whole myoglobin, human haemoglobin and bovine serum albumin (BSA) were used to determine the secondary structure of protein adsorbed on the ultrafine polystyrene particles. This involved investigating the effects of the properties of the proteins and adsorption conditions on the extent of changes in secondary structure after adsorption. In myoglobin, haemoglobin and BSA, there were significant changes in secondary structure with a marked decrease in alpha helix content. With BSA this was found to decrease with decreasing pH and increase with increasing amount of adsorbed protein. The changes in secondary structure were insignificant in RNase and peroxidase. In an investigation into the protein co-adsorption onto polystyrene latexes with different functional groups, it was found that the presence of protein on the surface latex shifts the isoelectric point of the latex-protein complex to pH values near the isoelectric point of the protein, which is in the majority[156]. Modifications of the coating surface can also reduce the binding of proteins. For example Van Delden found that covalently immobilising various polyethylene oxides onto carboxylated polystyrene lattices resulted in reduced adsorption by plasma proteins especially with the polyethylene oxides of higher molecular weight[157]. Treating antibodies with acid prior to adsorption was also found to increase antigen binding capacity compared to non-treated antibodies. The solid-phases tested had high non-specific protein binding properties for example, polystyrene and silica[158]. It was further found that the length of acid pre-treatment could be used to control the packing efficiency and orientation of the adsorbed antibodies when using a silica surface, treated with dichloromethylsilane to increase hydrophobicity. Acid pre-treatment was thought to lead to increased exposure of hydrophobic sites on the constant region on the antibody resulting in a preferential orientation on the solid surface that results in increased antigen-binding capacity.

Smaller molecules such as haptens and peptides adsorb poorly[110]. Hydrophilic molecules such as carbohydrates also do not adsorb well and require either the surface to be modified or carefully defined adsorption conditions to allow binding. For example the optimal adsorption conditions for type III pneumococcal polysaccharide is at pH 3.5 with a coating concentration of 1.0μg/ml. This was found to give a detection limit of 5-10ng/ml[159]. The molecule to be adsorbed would also have hydrophilic domains with polar or ionizable groups. The distribution and charge of these can affect the adsorption of the molecule as can any alteration in the pH and ionic strength of the solution during the immunoassay[113]. Protein adsorption at a solid–liquid interface has two stages; an initial diffusion-rate limited process followed by a slow association[160]. The slow association rate in the second stage of the adsorption process is thought to be due to re-organisation of the adsorbed layer. This can take place in conjunction with conformational change, which may also take place on its own. The intrinsic rate constant is dependent on the surface concentration of bound protein even at low levels far removed from saturation. Attempts were made to explain this as due to repulsive interactions between protein molecules and experiments with other macromolecules had showed that the repulsion was a weak long-range interaction. This was also found between silica particles at an air-liquid interface. However further work with surface-adsorbed ferritin molecules showed that these interactions were attractive[160].

Some of the most important work on the adsorption of proteins was carried out by Cantarero[112]. This involved studying the adsorption of seven proteins (several bovine immunoglobulins, BSA, ovalbumin and lactalbumin) of different molecular weight and ionic charge to polystyrene tubes using radiolabelled proteins and an ELISA. It was found for all the proteins that there was a range of input where the proportion bound was independent of the amount of protein added. This was called the region of independence and was found to have a maximum of 1000ng (1μg) for the proteins tested. It was also found that the maximum number of moles of protein that could be bound in the range of independence was inversely related to the size of the protein i.e.

the higher the number of moles of protein bound to the polystyrene, the smaller the protein. It appeared that gravimetric rather than molar concentration correlated better with the upper limit of the region of independent binding. Results had also suggested that at the upper limit of the region of independence, a protein layer one molecule thick covered the effective surface of the tube. Cantarero made two deductions from this. Firstly there appeared to be two sets of binding sites. The primary binding sites allow adsorption in the range of independence and the secondary binding sites, which were responsible for adsorption at concentration ranges beyond the range of independent binding. This may include protein-protein associations and the formation of multilayers, which meant that even though more protein was bound there was no increase in the amount exposed to antibodies. The secondary adsorption could also be due to plastic-protein interactions but the molecules are packed too closely together and the antibody can not get access due to steric hindrance. This was deemed unlikely by Stokes radii experiments showing that there was approximately equal surface area available. All proteins appeared to show a limit to the region of independent adsorption at about the same concentration and the total number of moles bound is limited by their size. The different proportions of different proteins that bound in the region of independence was due to the nature of the protein. Adsorption was also found to be affected by the presence of competing proteins, however when the total protein is added at the limit of the region of independence the amount of protein bound is unaffected by competing proteins. Hence all proteins in the mixture will adsorb onto the tube but due to their different affinities not in the same proportion as in the original mixture i.e. protein adsorption to polystyrene depends on the nature of the protein used. Different times and temperatures of adsorption were also found to significantly affect adsorption and that the upper limit of independent binding is also the upper effective antigen-binding capacity in solid-phase immunoassay for polystyrene (under the conditions of the study). The amount of protein bound under constant time and temperature varied depending on the protein. The conclusion from this work was that firstly when a constant amount of protein is added to polystyrene tubes the amount adsorbed would vary with the protein used. This means that the amount of antigen added to ensure antigen excess and maximise the detection range

varies with the protein used. Coating concentrations in the region of independence should be used since above this the proportion bound decreases rapidly and additional bound protein does not increase the working range of the assay. Cantarero recommended adding 500ng per 6.5cm^2. In the black disc, when the antigen was applied to a well it did not cover the entire surface, but was present as a droplet in the middle of the well. The presence of 0.1% BSA/PBS as coating buffer did help to disperse the antigen somewhat but not completely. This meant that not all wells were coated in exactly the same way as sometimes the drop of antigen covered slightly different areas of the well. Cantarero also concluded that adsorption should be standardised to ensure a constant ligand concentration on the surface and when working with mixtures, the amount of protein should be below the upper limit of independent binding to ensure that all proteins in the mixture will be adsorbed to the polystyrene.

Pesce[161] had previously found that the polystyrene surface could be used to bind either antigen or antibody and that the rate of reaction is dependent on the concentration of protein added to the tube. In addition it was noted that dilutions of unpurified antiserum of less that 1/500 was not required because at these concentrations, the protein was saturating the plastic. Walsh later found that radioiodination of IgG influenced the kinetics of IgG adsorption to polystyrene and in a series of experiments investigating the relationship between the amount of protein bound versus the amount added. It was found that in all experiments the amount bound increased until a plateau was reached which represented maximum binding capacity under the conditions used. Use of radioiodinated IgG was found to reduce adsorption by up to 50% compared with the non-labelled IgG.

The covalent attachment of antigen to polystyrene tubes was investigated in 1980 by Rubin[162]. Using a radioimmunoassay it was found that very high concentrations of antigens could be conjugated to polystyrene tubes. This appeared to result in a highly stable association. Low avidity antibodies were better detected using this method than with adsorbed antigen possibly

due to their large dissociation constant or the lower chance of a correct collision with an immobilised antigen. Increasing the antigen concentration increases the chance of a collision.

Appendix 3: Variability in the adsorption properties of microtitre plates.

Similarly, work was carried out on the variability in the adsorption properties of microtitre plates the physical properties of the plates and their surface characteristics by the use of enzyme-labelled proteins and enzyme immunoassays[82]. It was found that absorbance values occurred in groups with the highest values at the edges (edge effect). Increasing the protein concentration during coating did not reduce this. Calibration curves for the same wells from different batches of plates showed large differences with smaller but similar differences within individual plates. In one assay, results for an individual sample varied by ± 18%. The strain in the plastic of different wells was examined using birefringence in red light. Strain in the plastic may cause crazing (formation of tiny cracks) resulting in increased surface area and increased protein attachment. The birefringence showed the presence of different amounts of strain in different wells but this could not be correlated with the observed protein binding in the wells. Physical differences in the dimensions of the wells and surface irregularities due to the moulding procedure were not supported as a cause of the variability as examined by scanning electron microscopy of internal well surfaces. Differential evaporation from the wells could increase the protein concentration adsorbed but the plates tested were covered during incubation, which negated this effect. It was apparent from the different patterns of protein adsorption produced that there were a number of factors involved, not just temperature. This meant that the apparent result for a sample will vary depending on which well the assay was performed and also in which wells the standards were assayed. This study suggested that batches of plates should be screened for use in immunoassays and for variability in protein adsorption and also that the wells at the edge of the plate should not be used.

Shekarchi[163] tested seventeen lots of microtitre plates that differed in lot, batch, plastic and manufacturer in ELISAs for antibodies for measles, toxoplasma and human gamma globulin. Plate-to-plate variation, well-to-well variation, edge effect and the effect of pre-treatment of plates with solvents and cleaning agents were studied. Well-to-well variation was found on all

plates studied. Edge effect was also found but statistical analysis showed that this was an extension of the well-to-well effect and of the two, well-to-well variation was more significant. Edge effect was minimised by incubating covered plates in a moist 37°C chamber. Pre-treatment of plates with cleaning agents was not useful or efficient. Antibody titres found by serial dilution were less susceptible to the difference between plates and well-to-well variation. It was also found that washing the plates with 6N HCl for at least two hours allows them to be re-used. This was also found with an enzyme immunoassay for progesterone, where plates could be recycled and used for the same assay again fifteen times[164]. Rebeski carried out an in-depth study into the level of non-specific background produced on 16 types of microtitre plates from seven commercial sources[165]. Plates were tested for their capacity to adsorb a conjugated antibody in diluent buffer containing 0.05% Tween 20 and 5% skimmed milk protein. Plates with an absorbance value of 0.05 or greater in not more than one well were judged as being within acceptable limits. High binding gamma-irradiated polystyrene plates from all sources where only less than or equal to 30% of plates was acceptable. These showed high non-specific binding with some wells showing absorbance values greater than 2.0. Similar results were obtained when high binding plates were repeatedly irradiated and after gamma irradiation of low binding polystyrene plates. For high binding non-irradiated plates, 70% of plates were acceptable. All low binding polystyrene plates showed 86-100% acceptability. Only one commercial source in three provided acceptable low binding polyvinylchloride plates. From these results, non-specific binding to certain plates could be a serious factor in both the development and application of ELISAs.

Appendix 4: Adsorption of proteins to other surfaces in ELISAs.

The use of other surfaces for the adsorption of proteins in ELISA has been investigated. Pre-adsorbing protein on to aluminium hydroxide before coating the ELISA plate abolished denaturation of the protein often found during coating[166]. The protection against denaturation also depended on the buffer used for example no denaturation was present with carbonate buffer but this was not so with phosphate buffer. This was due to the elution of protein from the aluminium hydroxide or lack of binding to the aluminium hydroxide on the presence of aluminium. It also appeared that the protein had to be bound to the aluminium hydroxide to allow it to bind to the plastic surface. When coating antibodies on to a plastic surface, it is sometimes necessary to modify the coating procedure. For example in an ELISA to detect ferritin in human serum, the colour produced was improved by pre-exposure to low pH, 3M urea and high temperatures (82°C). This was shown to be due to the pre-treatments partially denaturing the antibody structure resulting in the exposure of hydrophobic sites not normally available to bind to the plastic[167]. Heating human haptoglobin to 60°C generated the formation of aggregates which when adsorbed onto polystyrene resulted in a 100-fold increase in signal compared to adsorption on plates pre-coated with albumin and plasma. Unheated haptoglobin produced similar results with all three methods[152]. In a study by Butler, the functional properties of adsorbed antigens and antibodies on polystyrene and silicone were compared with immobilisation by other methods[168]. It was found that less than 20% of polyclonal and 1-2% of monoclonal antibodies remained functional after adsorption as a monolayer. This was more than doubled or in some cases completely reversed when the antibodies were immobilised by a streptavidin bridge (polyclonal) or first-stage polyclonal capture antibody (monoclonal). When using immobilised bovine IgG with polyclonal and monoclonal anti-IgG, increased antigenicity was found when the IgG was immobilised using a streptavidin bridge or when adsorbed on to an albumin monolayer. Denaturation of the bovine IgGs and adsorption with 6M guanidine-HCl did not reduce the antigenic properties. It appeared that using a linker molecule or denaturing the adsorbing molecule increased the antigenic epitopes or antibody binding sites. In addition

the microenvironment of the epitope was also found to be important. This shows the difference in behaviour between antigens and antibodies in solution and on the solid-phase. It may have been useful to use an albumin monolayer to adsorb the antigens as a way of increasing colour production and reducing variability.

Similar results were found with an ELISA for detecting anti-saxitoxin antibodies[169]. Pre-coating the microtitre plates with 5μg/ml bovine serum albumin was followed by the addition of 5μM saxitoxin in 0.01M piperazine-glycylglycine buffer, pH 10. The use of silicone had therefore been shown to be suitable for adsorbing antigens and antibodies. Ellipsometry and radiolabelling were used to investigate the deposition of Human Serum Albumin (HSA) followed by excess polyclonal or monoclonal anti-HSA to methylated silicon. It was found that adsorbed HSA and HSA-monoclonal- anti-HSA layers were rapidly removed by 0.1-0.5% sodium dodecyl sulphate but HSA-polyclonal-anti-HSA layers were not. It was suggested that antigens and polyclonal antibodies cross-link on surfaces thereby stabilising the protein films[170]. Synthetic peptides often do not bind well to the plastic surface of microtitre plates. Pre-treatment of the wells with Alcian Blue in acetic acid prior to coating the target peptide was found to improve the reactivity of synthetic peptides on the solid phase. When using hyperimmune serum, this treatment was found to decrease the amount of antigen required to generate the desired signal for antibody detection. In addition, monoclonal antibodies against human immunodeficiency type 1 (HIV-1) vpu protein which were not detected in conventional ELISAs gave a positive signal after Alcian Blue treatment of the microtitre wells[171].

Appendix 5: Theory of immunoassay sensitivity.

Theories of antigen-antibody interactions in an immunoassay are based on work on the reactions between free haptens and antibodies in the liquid-phase. Under such conditions, the reaction is rapid, reaching a dynamic equilibrium. The interpretation of results from solid-phase immunoassays, for example ELISA have been based on assumed equilibrium conditions. However in these assays, equilibrium is seldom attained due to the relatively short incubation times used and these times are reduced even further in the protocols described in this thesis. Primary antibody incubation times in a standard ELISA can be from 30-60 minutes or even longer whereas using the disc-based system it was reduced to 10 minutes. The total reaction process is the sum of several sub-processes - the transport of reactant from the bulk of solution to the interface, the binding reaction, dissociation of reactants or reaction complexes and the transport of dissociated reagents from the reaction area[160]. The total reaction rate is determined by the slowest of these subprocesses and each one could be diffusion-rate limited or reaction-rate limited. The initial binding of antibody to immobilised antigen at a rate proportional to the surface concentration of antigen, initial concentration of antibody in the sample and the forward rate constant. The forward reaction in a solid-phase immunoassay consumes antibody close to the surface and may deplete the solution of antibody close to the interface. The reaction rate is then limited by the diffusion of antibody from the bulk of the sample to the reaction area. This effect becomes more important with increasing antigen density at the surface, high values of the forward reaction rate, slow diffusion of antibodies in solution and a slow reverse reaction rate. The reaction mechanism is more complex at an interface than in solution. Sensitive assays such as the ELISA measure only the initial binding of antibodies therefore the results are affected by mass transport limitations. It is only the mass transport of antibodies over the depleted layer that is measured by the ELISA.

A theoretical analysis of the sensitivity of solid-phase ELISAs was carried out by Griswold to show that the ELISA will exhibit either antibody affinity dependent or independent behaviour

depending on the assay conditions and the range of affinity of the antibody being used[172]. The analysis showed that the ELISA method would exhibit either affinity independent or affinity dependent behaviour according to the assay conditions and the range of affinity of the antibody being studied. Each IgG molecule has two paratopes, which can combine with antigen. A monovalent antigen may have epitopes that are distributed far apart; therefore one antibody paratope will bind to one epitope. However when the antigen is multivalent and the epitopes are closer together, both paratopes may bind. This increases possible problems due to steric hindrance of epitopes by bound paratopes. The binding reaction has been found to vary depending if one or both paratopes are involved. It was further shown that in a typical sensitivity curve relating minimum detectable antibody concentration to the affinity of the antibody that the maximum sensitivity occurs in the affinity independent region. At low antigen concentrations, the assay is affinity dependent and if the epitopes are closely spaced, both paratopes are involved in binding, making the assay more sensitive and less affinity dependent. Antigen concentration and assay sensitivity can also be improved by using covalent methods to attach antigen to the solid phase. Improvement in the immunoenzymatic detection system will also enhance the sensitivity of the assay for antibodies of all affinities. Some low affinity antibodies may dissociate easily from the antigen and may be removed during the washing procedure. This will decrease the sensitivity of the assay for low affinity antibodies. This was taken further, with highly specific immunoassays for small molecules where the washing steps increased the specificity of the assay. In addition there is the use of a multiple binding format where the analyte is bound to two different primary capture monoclonal antibodies[173]. The theoretical approach however does not always compare favourably with experimental results. O'Connor carried out a series of experiments to determine the dependence of the detection limit of reagent–limiterd immunoassays on antibody affinity[174]. This used a range of anti-progesterone monoclonal antibodies of different affinities and a fixed concentration of tritiated progesterone. The antibody concentration was varied to determine the detection limit for each antibody. In addition, optimum concentrations of both antigen and antibodies were predicted by a mass-action model and used to set up "theoretically optimised " assays. The detection limits

produced by the two different approaches were very close; the concentration of reagents predicted by the theoretical method was up to one thousand times higher than those estimated experimentally. It was thought that experimental determination of actual detection limits may help to identify factors other than antibody affinity or the specific activity of the label that most affect the detection limit of reagent-limited immunoassays. Extending this further it was found that using enzyme-labelled progesterone introduced several other factors into the assay process[175]. The analyte and the enzyme-labelled tracer can be very different in size and it has been shown that a large enzyme tracer with a relatively low affinity can produce a more sensitive immunoassay than a small radioactive label. This and the fact that antigen-antibody reactions at interfaces seldom reach equilibrium as previously discussed contribute to the mass-transport limitation of the reaction. The detection limit in the solid-phase enzyme immunoassay was 200 times less than with a solution-based radioimmunoassay with a tritiated label.

Immunoassays are part of a group of assays known as ligand binding assays. These rely on the observation of the products of a binding reaction between an analyte and a specific binding reagent, in the case of immunoassays, an antibody reacting with the antigenic sites of an analyte molecule. One of the principal aims in immunoassay development has been greater assay sensitivity together with reduced incubation and hence assay times, but there have been disagreements about the concept of "assay sensitivity" which have affected immunoassay design and performance[3]. The first concept defines sensitivity of a measuring system in terms of the slope of the dose-response curve. Yalow and Berson also linked assay sensitivity to the slope of the curve, later relating the fraction of bound analyte to analyte concentration, thus arguing that the response curve slope was indicative of the assay detection limit[6]. In the second concept, Ekins defined the sensitivity of the assay as represented by the (im)precision of measurement of an analyte concentration of zero[3]. This quantity was seen as being the most important factor in determining the lower detection limit of a measuring system and hence its ability to determine small amounts of analyte. The most important difference between the two concepts is that the slope method assumes constant random errors in the response measurement

irrespective of any changes in the immunoassay for example changes in antibody concentration. The second concept, however, requires detailed statistical analysis of assay data to determine the size of random errors in the measurement of response. This proved important in the development of ultrasensitive microspot assays.

Appendix 6: Methods of improving immunoassay sensitivity.

Surface plasmon resonance was used to analyse the affinity and kinetics of immobilised peptide-antibody interaction[175]. Reflectometry was used to monitor peptide adsorption and the resulting antibody binding on a polystyrene surface. When peptides other than the parent protein were applied there was 10 times less antibody binding activity and 100 times faster dissociation and it was this rather than poor adsorption that proved to be critical in determining assay sensitivity. Modifying the peptides chemically or increasing their molecular weight restored and improved antibody binding activity and affinity. The solid phase used can also affect the sensitivity of the assay and in conjunction with other aspects can increase the detection limit. A chemiluminescence immunoassay for estradiol used a homologous combination of a monoclonal antibody to estradiol-6-carboxymethyloxime-bovine serum albumin and estradiol-6-carboxymethyloxime-aminobutylethylisoluminol (E2-ABE1) as chemiluminescent marker[176]. With polyacrylamide as solid phase, the detection limit was 4.9pg of estradiol per cuvette. When the monoclonal antibody was bound to microtitre plates, the detection limit decreased to 0.71pg per well. Delaying the addition of the chemiluminescent marker further improved the detection to 0.37pg per well. In an alternative format, microtitre plates were coated with a second antibody and the monoclonal antibody was incubated with other reagents. In combination with the delayed addition of the chemiluminescent marker, the detection limit was reduced again to 0.25pg per well. In an enzyme immunoassay for human interferon-α-1 (IFN), a polyclonal antibody was used for coating with monoclonal antibodies to IFN as second antibody. A major increase in assay sensitivity was found when the polyclonal antibody was combined with a mixture of three monoclonal antibodies binding to different regions of the IFN molecule compared to the combination of the polyclonal antibody with a single monoclonal antibody[177]. Assay sensitivity has also been improved by using DNA-labelled antibodies and polymerase chain reaction for the amplification of assay response[178]. This was demonstrated with a multianalyte immunoassay for the detection of three analytes (hTSH, hCG,

and β-Gal). Labelled antibodies were prepared by covalently coupling uniquely designed DNA oligonucleotides to each of the analyte-specific monoclonal antibodies. Each DNA oligonucleotide label contained the same primer sequence to facilitate co-amplification by a single primer pair. Assays were performed using a two-antibody sandwich assay format and a mixture of three DNA-labelled antibodies. Analytes were detected at sensitivities exceeding those of conventional enzyme immunoassays by three times. The use of such DNA–labelled antibodies could provide the basis for the simultaneous detection of many analytes at sensitivities greater than those of existing antigen systems. The immune complex transfer immunoassay has also recently been found to improve assay sensitivity[179].

Non-specific adsorption can limit the sensitivity of the assay. For example in an electrochemical enzyme immunoassay for chicken luteinizing hormone, alkaline phosphate was used with p-aminophenyl phosphate as the substrate[180]. The enzyme-generated p-aminophenol was then injected into a flow-injection system and detected amperometrically in a thin-layer flow cell with a glass carbon electrode. However there was a problem with the adsorption of unwanted proteins to the solid phase, reducing the detection sensitivity. The major protein involved appeared to be the biotinylated second antibody. This was cross-reacting with the capture antibody and non-specifically adsorbing to the solid phase. To reduce the non-specific binding, 0.25% mouse serum was added to prevent the cross-reaction and a combination of Tween 20 and bovine serum albumin were used as blocking agents. This decreased the detection limit from 39pg/ml in the original assay to 2.5pg/ml in the electrochemical assay.

It is often important to remove all traces of other assay components to improve assay sensitivity. For example in an ELISA for typing ABH antigens in bloodstains, the antigens had to be solubilised in detergent (n-octyl-β-D-glucopyranoside) which then had to be removed by passing through a Sephadex G-25 mini-column[181]. Excess detergent reduced the sensitivity and

accuracy of the assay probably by removing antibody from the wells. Treatment to remove the detergent increased the sensitivity and accuracy.

In the use of the disc as an immunoassay platform in a hospital laboratory or at the point of care, the analyte to be detected would be in a biological fluid. These fluids can contain other substances that could affect the sensitivity of the assay. In plasma, there may be complement, rheumatoid factor, chelators, polyanions, autoantibodies, drugs and metabolites that could give rise to non-specific effects. This can also occur with the use of serum and in addition whole blood may contain chromogens and enzymes including proteases, hydrolase and those that can generate active oxygen species such as superoxide dismatase, peroxidases and pseudoperoxidases. The latter can especially affect immunoassays with peroxidase as the signal[136]. Other factors include the water used to prepare buffers and other reagents. This can vary in pH greatly from area to area and it is sometimes recommended to use ultrapure water for susceptible ELISAs. Autoclaved tap water can sometimes be substituted as in an indirect ELISA for the detection of antibodies to the pseudorabies virus[182].

In a series of tests to improve the sensitivity and specificity of serum antiganglioside antibodies in ELISA, different types of microtitre plates were tested. The highest background was found with polystyrene tissue culture plates with ELISA plates showing varying background values. This was reduced by gamma irradiation and/or the use of detergent (0.1% Tween 20) in the washing step. The ability of the antibody to distinguish between the epitope of an antigen and epitopes of related antigens also varied with different microtitre plates. The reactivity of the antiganglioside antibodies was also more consistent after detergent treatment[183]. Coating with monoclonal antibodies to increase antigen capture can also improve assay sensitivity. Concentrating the antigen can further enhance this by immunoprecipitation and the removal of any contaminants, for example in the ELISA for Brucella melitensis[184].

Sensitivity is also linked to the coating efficiency of the well and different buffers and additives have been used to improve coating efficiency depending on the substance being immobilised. Greater sensitivity was found in an ELISA with improved coating efficiency of lipopolysaccharide. Addition of 0.2% trichloroacetic acid and incubating for 30 minutes improved sensitivity. Increasing incubation to 16 hours improved sensitivity even more[185].

The effect of convection during incubation of microtitre plates has also been investigated. The incubation period in solid phase immunoassays is used to specifically bind free analyte molecules of low concentration to the immobilised antigen or antibody. Many of these assays use elevated temperature to increase the association of antigen and antibody. However it was found that these were not likely to have any significant effect on the association reaction. In many situations uncontrolled terminally induced free convection played an important role in mass transport of the analyte towards the solid phase. Convection is a function of assay geometry and the properties of the materials used. It has been shown that it is a major source of interassay variation and that applying forced convection during sample incubation of a two-step assay was found to reduce inter-assay variation from 5-10% to 1-2%. Assay sensitivity was found to improve by a factor of 2-6 without modification of assay chemistry[186]. The assay of antibodies is influenced by the affinity of the measured antibodies due to the Law of Mass Action, which can be written as shown below:

$$[AbAg]/[fAb] = K[fAg]$$

Where [AbAg] is the bound antibody, [fAb] is the free antibody, [fAg] is the free antigen and K is the equilibrium constant. In the ELISA, the absorbance reflecting the amount of bound antibodies can be measured in different dilutions of the sample and the dose-response curves constructed thereafter can be interpreted in different ways to determine the amount of antibodies. The antigen density can also affect this interpretation. Lehtonen and Eerola assayed 21 rabbit serum samples for IgG antibodies against 3-iodo-5-nitrophenyl-ε-amino-n-caproic

acid by equilibrium dialysis and ELISA[187]. The results of the ELISA did not correlate with the antibody concentration or average affinity measured by equilibrium dialysis. The expression of ELISA results by a single figure assumes that the dose-response curves are similar in shape although there are studies that show that ELISA dose-response curves from different samples differ from each other. It was shown that the ELISA measures antibodies belonging to different affinity ranges depending on how the results are derived. A low sample dilution produces antibody excess and only antibodies of the highest affinity will bind. In high sample dilutions, antibodies of lower affinity may also bind. High antigen density enhanced the selection of high affinity antibodies when the ELISA was performed at low sample dilution, possibly because high antigen density reduces the immunoreactivity of a solid phase antigen. The analysis of ELISA dose-response curves allows the separate estimation of high affinity antibodies and total antibodies. The smallest analyte concentration an immunoassay can reliably measure is one of its most important properties. Brown unified five of the current mathematical definitions of this concentration to give a standardised value[188]. This unified definition would have important implications for defining positive results for screening and diagnostic tests, setting criteria for immunoassay quality control and optimal design, reliably measuring biological substances at low concentrations and in general measuring small analyte concentrations with calibrated analytic methods. This unified description of smallest analyte concentration was applied to a microparticle capture immunoassay system for prostate-specific antigen (PSA) developed for the Abbott IMx automated immunoassay system. The smallest detectable antigen concentration for this assay using the unified approach was found to be 4.1-7.1 times greater than reported. This would mean that the ability of the assay to reliably measure very small quantities of PSA was overstated. This could be very important for other assays claiming very low detection limits.

Appendix 7: Insoluble 3,3', 5,5'-Tetramethylbenzidine (TMB)

In conventional ELISAs, TMB is widely used as a specific peroxidase substrate due to its high sensitivity and relatively low toxicity. It is used in a range of different areas covering environmental, agricultural, veterinary, research and medical diagnostics. The format is usually as microplate ELISAs, for example in the determination of the herbicide fluazifop in drinking water[191], and the detection of the herbicide imazaquin[190]. It has also been used in a PCR-ELISA for the quantitative detection of Epstein-Barr virus genome[191], immunotoxin investigation[192], and in the quantification of skeletal muscle calpastatin[193]. TMB is also used in ELISAs for measurement of estrone in sows[194], measurement of tumour necrosis factor[195,196], in vitro development of Cryptosporidium parvum[197], measurement of AMP and GMP in biological fluids[198] and in the measurement of human renin[199]. Other assays include the simultaneous ELISA of albumin, transferrin, IgG and α-microglobulin in renal studies[200] and bovine milk progesterone[201] and in measuring cortisol in fish[202]. In addition it is used in assays for the detection of hepatitis B surface antigen[203], quantification of biotin in blood[204], measurement of estradiol in the serum of women[205] and in a highly sensitive sandwich enzyme immunoassay for α-fetoprotein in human saliva[206].

Insoluble TMB is commonly used in a dipstick format. One of the advantages of this is that they produce rapid results and are therefore commonly used in environmental monitoring as above or in analysis of food such as in the development of a rapid dipstick immunoassay for the detection of peanut contamination of food[207]. This was used to specifically detect conarachin, the 7S globulin of the peanut Arachis hypogea and used TMB as substrate in the final step. It was found to be highly sensitive and could detect as little as 0.1% (w/w) of peanut in chocolate and could be used with a range of foodstuffs. In the development of a rapid, low technology ELISA for use in field-testing for Schistosoma mansoni both dipsticks and nitrocellulose membranes were investigated[208]. The dipsticks were Nunc "ImunoSticks" composed of

polystyrene. These were coated with antigen, dried and then exposed to the reagents by immersion in separate tubes of sample, conjugate and finally TMB. A blue colour develops in the substrate tube if a positive reaction has occurred. The colour did not form on the dipstick. Once coated with antigen the assay can be performed in eight minutes. With the nitrocellulose membranes, 3,3'-Diaminobenzidine (DAB) was used as substrate, which deposited a brown colour on the membrane with a positive reaction. This had a total assay time of fifty minutes. Both systems could be used with serum or whole blood. The use of insoluble TMB with the "Immunosticks" in this assay would have resulted in the deposition of blue colour on the polystyrene rather than in the tube. This would have provided a similar assay system to the disc-based one described in this thesis. The production of a soluble end product in the final tube means that the assay is not suitable for multianalyte testing. The insoluble TMB would have provided the system with the advantage of the final reaction and production of colour taking place on a defined area of the solid-phase rather than in a tube. This would then mean that the system could be used for multianalyte testing, perhaps for two or more parasitic or other diseases in addition to S. mansoni. The DAB used with the nitrocellulose membranes could also have been tested on the "Immunosticks", although when tested on early versions of the sectors, it did not produce a very strong colour and therefore may not have been suitable for use on polystyrene. The colour production by DAB and TMB was compared in an enzyme immunoassay for the detection of yellow-head virus from the penaeid shrimp using nitrocellulose as the solid phase[209]. The TMB produced a more distinct and easily recognisable colour with increased sensitivity – 0.4ng viral protein for TMB and 0.8ng viral protein for DAB.

There are other methods of using the membrane in this type of system. For example in an enzyme immunoassay for the detection of Cryptosporidium oocysts, the membrane is coated with specific antibody and is used to filter untreated stool samples[210]. Any oocysts present in the sample bind to the antibody, which is detected using a biotin-streptavidin system and visualised with TMB. Other methods involve microscopic techniques, which were more subjective and also labour-intensive. For the rapid detection of Salmonella enteritidis in eggs, a

polyester cloth is used to capture the lipopolysaccharide antigens of S. enteritidis, which were then detected by specific antibodies and TMB[211]. Alternatives to nitrocellulose membranes are often used. For example in the detection of human chorionic gonadotropin (hCG), an anti-β-hCG subunit was immobilised on a glass microfibre disc in the form of six radially located bars. The dry disc was placed on water-absorbing material in a plastic holder and after binding of hCG a peroxidase-conjugated second antibody was applied followed by TMB. The number of blue bars appearing on the test disc depended on the concentration range of the hCG. This provided a semiquantitative assay that produced results in accordance with those from classical ELISA and two commercial ELISA kits[212]. TMB has also been used with colony lift immunoassays. One such assay is for the detection of Escherichia coli 0157:87 [213]. In this, polyvinylidene difluoride (PVDF) membranes were pre-wet with methanol and used to make replicates of the bacterial colonies on agar plates or filter monitor membranes. The membranes were dried, blocked and exposed to peroxidase-conjugated goat anti-E. coli 0157 antibody. After washing the membranes were exposed to TMB or aminoethyl carbazole (produces red deposits on reaction with peroxidase), rinsed in water and air-dried. Either a blue or red spot identified Colonies of E. coli 0157:H7. The coloured spots were matched to the parent colonies on the agar plates or filter membranes. This provided a simple, rapid and accurate method for confirming the presence of E. coli 0157:H7 with the advantage of being able to test every colony serologically. A similar method was also developed for the detection of Campylobacter jejuni, C. coli and C. lari on agar plates and filter membranes[214], allowing the Campylobacter colonies to be quantified 18-24 hours after sampling. Hydrophobic, high protein-binding membranes were used and treated as described above. A peroxidase-conjugated anti-Campylobacter was used with only TMB to produce the colour. This proved a rapid and simple way of detecting and quantifying the Campylobacter organisms present on agar plates or filter membranes.

Appendix 8: Microarrray-based immunoassays.

A variety of competitive and non-competitive imunoarray systems have been developed comprising multiple sandwich assays, capture antigen assays for detection of serum antibodies, labelled analyte back-titration assays for low molecular weight analytes. Assays have also been constructed for use in endocrinology, allergy and infectious disease and also for the screening of therapeutic drugs. Thyrotropin assays have been of high sensitivity and shown good correlation with other techniques. A number of allergens, for example, house dust mite, bee venom and total IgE have produced assay precision and sensitivity better than commercially available tests. Microspot assays for a number of infectious diseases such as HIV; Hepatitis B surface antigen and Rubella have shown improved performance when compared to the latest commercially available tests[3].

The microspot assays described above are on a much smaller scale than those performed on the compact disc. The disadvantage of the microarray system is the production cost. The system described in this thesis was on a larger scale (coating volume 1μl compared to <1nl) but there are do have similarities between the systems and it would be feasible to use the compact disc in the future as a platform for microarray technology. The CD-based system was seen as a bridge between the old technology of microtitre plates and the new microarray technology.

There has been great interest in the development of miniaturised array-based multianalyte binding assays for immunoassay. This has mainly involved localising the arrays on a microchip, which is potentially capable of determining the amounts of hundreds of different analytes in a drop of blood. Genetic testing using oligonucleotide arrays is also being developed in the US and Europe[215]. A number of different techniques have been tested for the construction of the microarrays including the use of hydrogel[216]. This involves the use of a micromoulded hydrogel "stamper" and an aminosilylated receiving surface. The stamping procedure allowed direct protein deposition and micro patterning and avoided cross-

contamination of the separate patterned regions. Using this method, three different antibodies were stamped in adjacent circular arrays of 50-80μm with retention of activity. The miniaturisation of assays has also brought about the possibility of performing analytical reactions in minute chambers micromachined in small silicon, glass or more recently plastic chips. These disposable micromachined devices are the basis of the "lab-on-a-chip" technology. The advantages of these devices are that they are easily designed, low cost, small, portable, easy and fast to operate, use micro volumes of sample and reagents, can be used for multianalyte testing and provide system integration. Micromachined devices have been constructed for a variety of techniques including semen analysis, in vitro fertilisation, PCR and immunoassay. The PCR chip had a volume of 10μl and was successfully used to amplify a range of targets including bacteriophage lambda and Campylobacter jejuni[217]. The miniaturisation of analytical systems such as immunoassays together with automation and improved information systems came partially from the pressure to contain medical costs. One way to reduce coats would be to move to near-patient or point of care testing, but this would need to meet specific requirements depending on the location and circumstances. One area where it may have a role is in emergency testing sites[218].

An alternative to the ambient analyte immunoassay described by Ekins is a mass sensing microarray system as described by Silzela[219]. In this format, miniaturised assays were shown to "harvest" analyte thus determining the total analyte mass in the sample. The capture reagents (antibodies or biotin) immobilised in 200μm zones were shown to substantially deplete analyte from a liquid sample during a 1-3 hour incubation. The resulting assays sensed the total analyte mass in the sample rather than its concentration as with the Ekins ambient method. Fluorescence imaging on the solid phase using a near infra-red label detected as few as 105 molecules of analyte per zone. This was tested with single and multianalyte mass sensing sandwich assays of the IgG subclasses which showed the sensitivity and specificity of ELISA methods while using less than 1/100 of the capture antibody required by a 96-well plate. The

solid phase used was polystyrene film, which was taped to tractor paper and loaded into a computer printer. The antibody was coated onto the film by using a desktop jet printer, which was ultrasonically cleaned. The volume dispensed onto the film was found to have a mean of 80 picolitres. The printed spots dried within 30 seconds of deposition. The fluorescent infra-red dye used was DBCY5, a dicarbocyanine analog of indocyanine green. For initial studies with an avidin-biotin system, avidin was coated on to the solid phase and biotinylated dye was used to detect the avidin. The detection of fluorescence was with a peltier cooled charge coupled device camera coupled to a x6.5 microscope objective and appropriate excitation and emission filters. An assessment of the sensitivity and reproducibility of the printing and measurement processes was made by printing calibrator solutions of native DBCY5 dye and constructing dose-response curves from the reduced image data. The fact that this system senses the mass of each analyte rather than its concentration was shown by the preparation of multianalyte microarrays containing spots specific for IgG3 and IgG4 with some arrays also containing a third monoclonal antibody that recognised both IgG3 and IgG4. There was found to be interarray competition with the IgG3 and IgG4 signals being reduced, relative to controls in the presence of spots recognising IgG3+IgG4. This would not have happened if the sample solution in contact with the array were not being depleted of analyte and shows that the signals are due to "harvesting" of the analyte on the printed spots. This harvesting maximises the signal: background ratio and allows the detection of analyte molecules without the requirement of enzyme amplification.

One method of depositing the microarray was to use a jet printer or similar, which would have been useful for depositing antigen on to the black disc. The main problem would be aligning the jet printer to match the pattern of wells on the disc. Changing the well layout to suit the printer would require changing the optics in the reader, which were designed to read the specific 44-well layout. Calibrating the printing and measurement using native DBCY5 dye was similar to the blue acetate disc constructed to confirm the variability in colour production. The use of the blue acetate disc showed that even though the variability was reduced, it was not eliminated.

Therefore there was also a problem with the optical system in the reader that had to be addressed, in addition to those associated with the chemistry i.e. antigen coating and the substrate. The coating volume used was 80 picolitres which, dried in 30 seconds compared to 1μl on the black disc which dried in 45 minutes. A faster drying time may help to reduce variation over the well surface, as it was found that increasing the antigen coating temperature decreased the C.V. values slightly. This system also did not require enzyme amplification therefore any error associated with the sensitivity of the colorimetric substrate or the inclusion of another step in the assay was removed. It is clear that this method could be utilised as a dipstick or other method making it more "user-friendly" as a point of care device.

Appendix 9: Multianalyte immunoassays on membranes and dipsticks.

Multianalyte testing can also be performed using a dipstick method. These are used to test a wide range of substances but are perhaps most useful in environmental and field testing, providing a rapid, low-cost method of producing results. A dipstick format was used for determining atrazine and terbuthylazine in water samples, where polystyrol strips were used as the solid-phase for coating antibody[220]. These dipsticks were useful as a qualitative or quantitative field test for identifying positive samples thereby reducing the number of samples to be re-analysed in the laboratory. In the area of infectious disease testing, dipsticks can also be very useful. For example a dipstick ELISA has been used to detect both IgG and IgM antibodies to dengue virus where it was also shown to be useful in detecting seroconversions[221]. Similarly a limited field evaluation for a rapid monoclonal antibody-based dipstick assay for urinary schistosomiasis was found to produce a similar sensitivity and specificity to microscopic methods[222]. Several dipstick methods have been developed where colloidal dyes were used to produce the visible result. A multiple antigen detection dipstick for field diagnosis of trypanosome infections in cattle was developed using polyclonal antibodies bound to Palanil Red dye particles. The presence of coloured dots on the dipstick indicated the presence of trypanosome infection[223]. In the development of a dipstick to detect Dirofilaria immitis infection in dogs, antibody-coated nitrocellulose membrane was mounted on acetate strips. Antibody adsorbed to commercial pink colloidal dye particles served as antigen detecting reagent. On comparison with a microplate ELISA, the dipstick was found to be simpler, rapid, inexpensive and equally sensitive and specific in detecting D. immitis infection in dogs[224].

One of the methods used in dipstick technology is the dot-ELISA. In this technique the antigen or antibody is immobilised on a nitrocellulose membrane, which if it to be made into a dipstick, is then mounted on an inflexible holder and the remaining membrane is blocked to prevent non-specific binding. This technique lends itself very well to multianalyte testing, especially on dipsticks. Dot-ELISAs have been utilised widely, for example in veterinary diagnostics for the

rapid (30 minutes) detection of anti-leishmania antibodies in dogs[225,226], the detection of swine trichinosis[227], bovine brucellosis[228] and gentamicin in the milk of dairy cattle[229]. Also in human diagnostic immunoassays, for example, in the diagnosis of enteric fever caused by Salmonella[230], the detection of antibodies to Toxoplasma gondii antigens[231], in a "cholera diagnostic kit" for the sensitive and rapid detection of Vibrio cholerae-01[232] and in the immunodiagnosis of human neurocysticercosis[233]. It can also used in the serological differentiation of acute and chronic Schistosomiasis mansoni[234] the early detection of bladder cancer[235], and in the detection of anti-amoebic antibodies[236,237]. Although nitrocellulose membranes are the most common solid phase for dot-ELISAs other solid phases have been used, for example, Dacron (polyethylenetherephthalate) plates were tested using covalent linkage of a Yersinia pestis antigen to converted azide groups on the surface. No significant difference was observed when using the Dacron plates compared to a nitrocellulose membrane, however the membrane-antigen preparation was stable over a wider ranger of temperatures than the Dacron-antigen preparation[238]. The sensitivity of dot-ELISAs can be as good as a microtitre plate and can also be used to detect small molecules. Several methods were tried for conjugating peptides to a protein on paper, nitrocellulose or nylon membranes and tested for usefulness in dot-ELISA detection of the peptides. Nitrocellulose was found to produce the most sensitive results, detecting a range of peptides from 4-38 amino acids at a concentration of 2-10 femtomoles[239]. An enzyme immunoassay system using test card disposables, reagents and an instrument was developed for multianalyte testing. A nitrocellulose membrane was embossed with an ultrasonic horn to form multiple isolated islands which gave a pattern of a 5 x 6 array of 2.5mm diameter circles to which the capture proteins were applied and dried. The rest of the membrane was blocked to prevent non-specific binding and after drying was assembled on to a plastic test card. This allowed individual reaction sites for each assay on the membrane so that a panel of specific antibody assays was constructed. The assay protocol had four steps – sample and buffer incubation, conjugate incubation, substrate incubation and dry and read. The specific analyser performs all these steps automatically after reading barcodes on the test card to determine the assay protocol. The substrate produces a colour at the site of a

positive reaction by reacting with an enzyme on the bound conjugate. The colour density is proportional to the amount of antibody bound and a printout is produced of the amount of antibody present[75].

Appendix 10: Point of care testing (POCT).

There have been improvements in the quality of POCT due to increased awareness of the need for strict quality control and reliability. A number of comparative tests have been carried out between POCT and the centralised laboratory testing. For example in one study, the results were compared from POC coagulation equipment with standard laboratory coagulation equipment in patients undergoing cardiovascular surgery[240] where it was found that the POC tests correlated with the results from the laboratory showing a potential advantage of using POCT in this situation. It is important that such comparative tests are carried out, instead of the assumption being made that decentralisation is always the best option. For example, POC tests for cardiac markers are one of the most widely used with a biochemical test being required for 90% of patients to confirm or exclude myocardial infarction. A number of POC tests have been developed which have been shown to be accurate and equivalent to a laboratory-based diagnosis. A randomised controlled trial showed that POC testing had a turnaround time of 20 minutes compared to 72 minutes for laboratory testing. The POC-tested patients also had a significant reduction on hospital stay thus, in this case, aiding cost-effectiveness and rapid diagnosis[241]. There are POC immunoassays for several cardiac markers such as creatine kinase (CK-MB), myoglobin, troponin I and troponin T. These can produce qualitative or quantitative results depending on the test that are comparable to laboratory testing. POC assays combining myoglobin and CK-MB have high sensitivity and specificity for diagnosing acute myocardial infarction and may provide the earliest identification of injury, POC troponin T assays are the most studied POC cardiac marker assay and along with troponin I may provide more sensitive identification of myocardial injury[242]. A future POC device is the blood monitor, which will be used in intensive care units. This requires an arterial catheter and measures blood analytes without removing blood permanently from the patient. They will measure blood gases, electrolytes, glucose, urea nitrogen, lactate, coagulation, cardiac markers, specific inflammatory response markers and others[243].

The same comparative tests would require to be carried out at other levels of POC, depending on where the system was being used. This means that in for example health centres doctors' surgeries or in field testing, the device would have to be thoroughly tested against the system already in place.

Point of care testing for infectious disease, either bacterial or viral could provide important information leading to earlier and more effective treatment for the patient. As before this depends on the immediacy of results, reliability of the test, cost and the impact on medical and laboratory staff i.e. the test complexity[89]. Other areas where POCT has recently been introduced include the determination of glycated haemoglobin in diabetes[244] and the rapid detection of cancer[245].

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