TITLE

COUPLING DYES TO CHICKEN IgY ANTIBODIES FOR THE DEVELOPMENT OF IMMUNODIAGNOSTIC TESTS

by

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PREFACE

The experimental work described in this dissertation was carried out in the discipline of Biochemistry, School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, from January 1999 to December 2000, under the supervision of

Professor J.P.D. Goldring.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

Signed:

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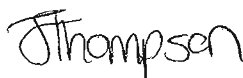

Signed:

Professor J.P.D. Goldring (supervisor).

ABSTRACT:

The aim of this study was to develop a highly simplified, sensitive and specific malarial diagnostic test at the lowest possible cost. Initial work and optimisation of procedures was achieved with chicken antibodies by covalently attaching commercially available dye to them. Chicken antibodies were easily isolated from egg yolk and dye is cheap, easily visible and requires no equipment for identification of results. A dipstick dye-immunoassay was developed with nitrocellulose as the capture phase. The dye-immunoassay is an alternative to the traditional enzyme linked immunosorbent assay (ELISA) technique, which employs the use of an enzyme-substrate reaction.

Numerous dyes were investigated and included Reactive black 5, trypan blue, Cibacron Blue, Congo red, Acid-black 2, dianix blue, dianix red, para-nitroaniline and primulin. Most of these dyes have dark colours which are essential for good contrast on nitrocellulose and in a microtitre plate. Some dyes contain amino (NH_2) groups, which are targeted in a covalent linking step and attached to the lysine residues on antibody molecules or to the carbohydrate groups on antibody molecules.

Attachment of dye molecules to antibodies with glutaraldehyde was the chief coupling method explored and conditions were optimized in this study. Unbound dye was removed by dialysis. Reactive black 5 is sensitive down to 50 nanograms of antigen on nitrocellulose.

A second covalent coupling method was investigated by means of attaching dye to the carbohydrate moieties on the antibody. Reactive black 5 was sensitive down to 50 nanograms of antigen. The carbohydrate method appears to be more sensitive than the glutaraldehyde method at lower antibody concentrations.

Primulin, a yellow dye, was similarly investigated. This dye does not have a dark colour initially, but can be diazotized to change its colour to orange or purple. It also fluoresces under ultra-violet light. This dye was sensitive down to 500 nanograms of antigen with both the glutaraldehyde and carbohydrate coupling techniques.

A dye-linked immunosorbent assay (D-LISA) protocol for direct antigen detection has been developed whereby the dye-antibody solution (dianix blue dye) acts as the primary antibody and substrate respectively. Sensitivity levels compare with traditional ELISAs. Dianix blue is sensitive down to 25 nanograms of antigen in a microtitre plate.

Unique protein staining abilities of the dyes used in this study were indicated by staining IgY in electrophoretic gels. Acid-black 2 indicated better protein staining abilities than that of Coomassie brilliant blue.

Evidence shows that dye was successfully covalently attached to antibodies and that antigen detection is possible by visualising the dye developed spots. Although malarial antibodies were not used, all procedures with chicken antibodies were optimised. Highly simplified, sensitive and specific diagnostic tests were developed.

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LIST OF ABRREVIATIONS

ABTS	2, 2'-azo-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AMCA	aminomethylcoumarin
BSA	bovine serum albumin
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
CαRA	affinity purified chicken anti-rabbit albumin
Da	daltons
DIA	dye immuno-assay
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	phenylisothiocyanate
HRP	horseradish peroxidase
IgY	immunoglobulin Y
IgG	immunoglobulin G
IgM	immunoglobulin M
kDa	kilo daltons
M	molar
NHS	N-hydroxysuccinimidobiotin
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid schiff
PBSM	phosphate buffered saline-milk
PBSMT	phosphate buffered saline milk tween (0.2% tween)
PcAbs	polyclonal antibodies
PDMS	poly-dimethylsiloxane
PEG	polyethylene glycol
PVDF	polyvinylidene difluoride
RB5	Reactive black 5
RER	rough endoplasmic reticulum

SDS	sodium dodecyl sulfate
TBST	Tris buffered saline-tween (0.2% tween)
TBSM	Tris buffered saline-milk (5% milk)
TLC	thin layer chromatography
UV	ultraviolet
VLDL	very low density lipoprotein

CHAPTER 1: INTRODUCTION

1.1 Chicken Immunoglobulin - (IgY)

The antibody in chicken egg yolk, Immunoglobulin Y (IgY), was chosen as the subject antibody. The university has excellent housing facilities for chickens and Kwazulu-Natal is well known for its chicken farms, so the chickens themselves are easy to obtain. There is no need to bleed animals as chicken antibodies are present in the egg yolk in concentrations very similar to that of serum antibody levels (Landon, 1995). The ease of collecting eggs and isolation of IgY compared to the costs of raising monoclonal antibodies makes it viable for this study (Polson *et al.*, 1980).

1.1.1 Why IgY instead of IgG?

It has been reported that the concentration of IgG in the hen's blood is very similar to that of the concentration of IgG in the yolk (Landon *et al.*, 1995). Immunoglobulins G (IgGs) which exist in the hen's systemic circulation are transported across the oviduct and into the egg yolk (Landon *et al.*, 1995). The embryo would thus receive these antibodies which are vital to protect it until its immune system develops. There are two known types of IgG in the hen. The first is structurally similar to mammalian immunoglobulin M (IgM) which exists as a pentamer made up of five dimeric subunits of IgM and has a molecular mass of approximately 900 000 daltons (Da). The other which occurs in the egg is known as IgY and is structurally similar to mammalian IgG and has two heavy (H) and two light (L) chains. There are, however, a number of important differences between IgG and IgY that should be borne in mind when constructing immunoassay procedures. The molecular mass of IgY has been reported to be approximately 180 000 (Landon *et al.*, 1995; Kim and Li-Chan, 1998; Shimizu *et al.*, 1988) as compared to the molecular mass of IgG of 160 000. IgY has a preponderance of non-polar, uncharged amino acids such as glycine, alanine and leucine compared to IgG. This confers an isoelectric point of one pH unit lower than mammalian IgG. The pH optima for use of these two molecules under *in vitro* conditions therefore differs. The Fc portion of IgY also does not bind to complement, protein G, protein A, rheumatoid factor

or macrophages so immunoassays should not be based on the interaction of IgY with any of these molecules (Landon *et al.*, 1995).

The amino termini of the heavy and light chains of immunoglobulins are associated into variable domains which form the antigen-binding site. This site has portions of greatly varying amino acid sequences and the name hypervariable regions or complementarity determining regions (CDRs) has been ascribed to these regions. The manner in which these heterogenous sequences "shape" the antigen binding sites dictates the way in which a specific antibody will interact with its antigen.

1.1.2 IgY isolation

Vitellines are proteins found in the egg yolk and are made up of various lipoproteins (complexes of lipid and proteins) (Polson, 1990). These include low-density lipoproteins that are synthesized in plasma from very low-density lipoprotein (VLDL) and have the highest content of cholesterol of any plasma lipoproteins. High-density lipoprotein is synthesized in the liver and reverses the transport of cholesterol from tissues to the liver (Oxford Dictionary of Biochemistry, 1997). The other protein elements of yolk are called the levitins and consist of alpha levitin (mostly ovalbumin), beta levitin (mostly alpha-2-glycoprotein) and gamma levitin, or importantly the IgY fraction (Hatta *et al.*, 1990).

Methods to separate the antibody from the lipid components of yolk use a number of different approaches. These include lipid precipitation with polyethylene glycol 6000 (PEG) (Polson *et al.* 1980), dextran sulfate and calcium chloride (Jensenius *et al.*, 1981) or magnesium chloride and phosphotungstic acid (Viera *et al.*, 1984). Ultracentrifugation and the use of organic solvents such as chloroform (Aulisio and Shelokov, 1967) and pre-cooled propane-2-ol (Bade and Stegemann, 1984) have been used. A large number of gums that are commonly found to be food additives have a slight negative charge and can bind with and aggregate lipoproteins that are positively charged. The aqueous phase that is left behind is lipoprotein clear and contains the immunoglobulins which can be used for many applications (Landon *et al.*, 1995). The method in the present study employed the addition of PEG to a volume of diluted yolk (Polson *et al.*, 1980; Goldring and Coetzer, 2003) and after centrifugation, three phases were observed in the centrifuge tubes. A surface fluid containing a lipid layer, a clear layer and a semi-solid phase of vitellin. The surface fluid was filtered through cotton wool to trap the

lipids and the addition of more PEG to the clear filtrate caused the precipitation of the IgY after another centrifugation step. The IgY pellet was resuspended in buffer before use.

1.1.3 IgY stability

The stability of the IgY molecule is important as it cannot be subjected to extreme conditions during experimentation and retain its native structural and functional characteristics. Antibody or immunoglobulin molecules are composed of two light (L_2) and two heavy chains (H_2) held together by noncovalent interactions as well as a number of disulfide bonds, (Figure 1.1) (Johnstone and Turner, 1997).

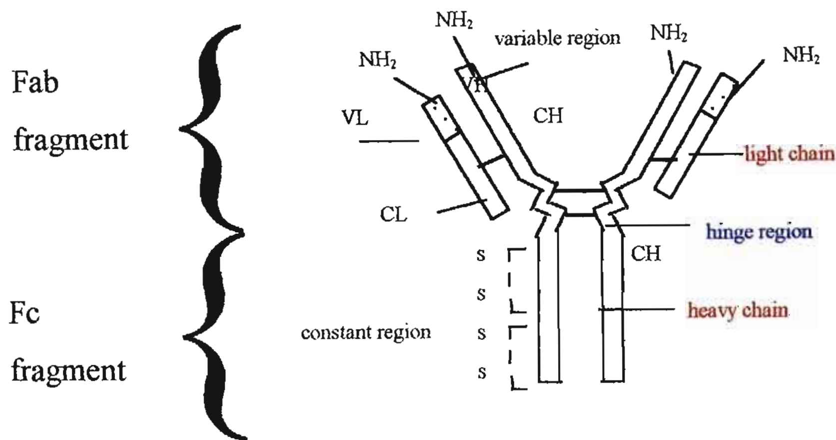


Figure 1.1: Basic H_2L_2 structure of an immunoglobulin-G molecule.
The antibody consists of two heavy protein chains, and two light protein chains. The Fab fragments are the variable regions and bind antigen while the Fc portion is known as the constant region.

The two halves are identical, each consisting of one heavy (H) and one light (L) chain. Inter-chain disulfide bonds connect the halves. The light chains are disulfide bonded to the heavy chains in the CL (constant light) and CH (constant heavy) regions, and the heavy chains are in turn disulfide bonded to each other in the hinge region (Johnstone and Turner, 1997). A number of different classes of immunoglobulins are characterized by structural differences in the H chains (Clark, 1991). Humans have five principle classes namely IgA (immunoglobulin A), IgD, IgE, IgG and IgM. Their heavy chains are designated α, δ, ε, γ, and μ respectively (Voet and Voet, 1995). Not only are there different types of heavy chains but two different types of light chains, κ and λ, but these are found in immunoglobulins of all classes.

Shimizu *et al.* (1992; 1993; 1998) have done a significant amount of work on the molecular stability of IgY. They compared the molecular stability of IgGs from chicken and four mammalian species (cow, pig, goat and rabbit) from an immunochemical and biochemical aspect. They reported that IgG from bovine, pig and goat showed similar stability against heat, acid and denaturation treatments, while rabbit IgG showed higher stability than the rest, suggesting a more stable molecular structure (Shimizu *et al.*, 1988). Conformational stability of chicken IgY was lower than mammalian IgGs, suggesting a more unstable molecular structure. Chicken IgY can, however, withstand temperatures of up to 60°C; pHs as low as 3.5, and concentrations of guanidium chloride, a denaturant, of 2.5 M.

1.1.4 Conjugation strategies with IgY

In the present study, IgY was used for conjugation by covalent linking to dye particles. Gribnau *et al.* (1982) previously described a procedure whereby antibody (human IgG) was linked to dye. Antibodies were raised against a specific hormone, coated with dye particles and used for an agglutination assay of this hormone (dye-immunoassay/agglutination). A means of ionically coupling dye molecules to antibodies for microtitre plate assays was also developed by Gribnau *et al.* (1982). The chromophoric properties of the dye particles were applied by using them as a label in sandwich-type ELISA immunoassays (dye-immunoassay/sandwich).

Glutaraldehyde was used as a chemical coupling agent between antibody and dye in the present study because it has two available aldehyde groups that are able to couple to amino groups on proteins. Briand *et al.* (1985) recommended a ratio of 40:1 for the carrier peptide to protein, or which could be adapted to, dye to antibody.

There are various ways to modify both the antibody and the dye molecules in order to achieve a linking reaction between the two. Other than glutaraldehyde, an alternative conjugation approach is to modify the antibody (IgY) itself, by periodate oxidizing the carbohydrate entities consisting of asparagine-linked oligosaccharides (Matsuura *et al.*, 1993) found on the heavy chain (Fc portion) of the immunoglobulins.

1.2 Dyes

1.2.1 An overview

Dyes are chemical entities which give colour to what would otherwise be a dull existence. Used in the textile and clothing industries, for paint, in food, and found in plants and our natural surroundings, dyes also have countless medical and histological uses. Dyestuffs give colour to the material onto which they are anchored, by selectively retaining some of the wavelengths of light falling upon the surface.

Table 1.1: Table of wavelengths of visualized light (taken from Trotman, 1984).

Wavelength absorbed (nm)	Absorbed colour	Visible colour
400 – 435	Violet	Yellowish-green
435 – 480	Blue	Yellow
480 – 490	Greenish-blue	Orange
490 – 500	Blueish-green	Red
500 – 560	Green	Purple
560 – 580	Yellowish-green	Violet
580 - 595	Yellow	Blue
595 – 605	Orange	Greenish-blue
605 – 750	Red	Blueish-green

Table 1.1 indicates the visual spectrum of light, which wavelengths are absorbed and the actual colour visualized. At least one chromophore is required for unsaturated aromatic compounds to become coloured in ordinary light. A chromophore is an arrangement of chemical functional groups including one or more multiple bonds (Gurr, 1965). The function of a chromophore is to change the absorption range of the molecule into longer wavelengths and, in so doing, deepens and intensifies the colour depending on whether one or more chromophores are present (Gurr, 1965).

There is a difference between auxochromes and chromophores. An auxochrome is described as a sub-atom or sub-group that involves an increase in the intensity of absorption of light due to the presence of a chromophore. In other words, the function of an auxochrome is similar to

that of another chromophore (Gurr, 1965). A special kind of auxochrome is called a colligator and it is because of this colligator that dyes are able to chemically unite with other compounds. Acidic colligators allow acidic dyes to attach themselves to basic substrates and basic colligators allow basic dyes to attach themselves to substances that are mostly acidic.

All synthetic dyes are derivatives of benzene and can be classified as aromatic organic chemical compounds; groups such as nitro, nitroso, azo and carbonyl groups are chromophores. The quinone ring is regarded as a chromophore (Figure 1.2).

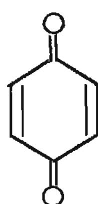


Figure 1.2: The quinone ring which is considered to be a chromophore.

The quinone ring is a derivative of the benzene ring structure with additional functional groups.

1.2.2 Classification of dyes

Dyes are molecules that have two main components: i) the inorganic coloured ion (although not all dyes have coloured inorganic ions) and ii) the aliphatic (a compound in which the carbon-atom backbone forms an open structure, i.e. is non-cyclic) organic ion which normally has an opposite charge. This confers an ionic nature to the dye particle. Gurr (1965) developed a classification system for dyes. The classification system is based on the presence or absence of charges on dyes. Three initial groups of dyes were assembled and each of these subdivided further (Table 1.2).

The sulfonic group (-SO_3) makes the dye water-soluble and has very little, if any, influence on the colour of the dye. Other auxochromes in the molecular structure of the dye change the steric conformation of the entire molecule and the intensity of dye colour will increase if more auxosomes are present.

Table 1.2: A dye classification scheme (Gurr, 1965).

Group 1: Non-ionic dyes	Group 2: Cationic dyes	Group 3: Anionic dyes
Class 1 Acids, e.g. phenols	Class 1 Wholly basic dyes	Subgroup 1 Wholly Acid Dyes
		Class 1 Carboxylated dyes without hydroxyl groups
Class 2 Bases, e.g. imino or amino bases	Class 2 Basic dyes having acidic side chains	Class 2 Carboxylated dyes with hydroxyl groups
Class 3 Neutral		Class 3 Sulfonated dyes without hydroxyl groups
		Class 4 Sulfonated dyes with hydroxyl groups
		Class 5 Sulfonated dyes with carboxyl groups and/or with hydroxyl groups
		Subgroup 2 Weakly amphoteric dyes
		Class 1 Without hydroxyl groups
		Class 2 With hydroxyl groups
		Subgroup 3 Moderately or strongly amphoteric dyes
		Class 1 Without hydroxyl groups
		Class 2 With hydroxyl groups

1.2.3 Classes of dyes

1.2.3.1 Basic dyes

The charge on a basic dye is positive, and basic dyes are also known as cationic dyes. Figure 1.3 shows the general structure expected for basic dyes.

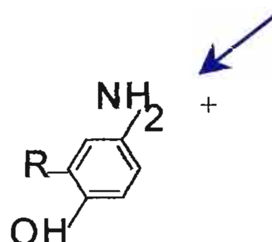


Figure 1.3: The general chemical formula of a basic dye.

The arrow indicates a typical amino group colligator and the 'R' refers to any carbon-containing group.

Colligators present on basic dyes are, for example, the amino group (NH_2), the amide group ($CONH_2$) or just the nitrogen atom (N). Colligators are necessary in order that the largely positive basic dye molecule may adhere to substances that are acidic or negative in nature. The basic part of the molecule doesn't contain a chromophore and so is not coloured. The colour only appears on salt formation with other ions and, when this happens, the resultant brightness and the intensity of their colours is a distinguishing property of the basic dyes (Kulkarni *et al.*, 1986). An example of a basic dye used in this study is para-nitroaniline.

1.2.3.2 Acidic dyes

Acidic colligators may aid in the identification of acidic dyes and examples of acidic colligators are; sulfate (SO_3), hydroxy (OH), carboxylate (COOH), sulfite (SO_3) and bisulfite (HSO_3). For example, in Figure 1.3, the amino group (NH_2) would be replaced by an SO_3 group in an acidic dye. A dye is classified as acidic when its overall charge is negative. Acidic dyes may also be called anionic dyes. The reason acidic dyes are so called is because they are added to a mixture of solutions mostly containing acids, and also that most are purchased as sodium salts of organic acids. The anion, (negatively charged component) contains the coloured section (Kulkarni *et al.*, 1986). A particular characteristic of acidic dyes is that they

usually bear from one to three sulfonic acid groups which renders them particularly water soluble. An example of an acidic dye used in this study is Acid-black 2.

1.2.3.3 Direct (substantive) dyes

Direct dyes are also known as substantive dyes and are strongly attracted to cellulose and also, coincidently, to proteins (Trotman, 1984). Cellulose assumes a negative charge when placed in water and this repels the similarly charged ion on the direct dye. To overcome this, electrolytes from a sodium salt are added to the dye solution and these cancel out the charge on the cellulose, allowing the dye molecules to adhere by hydrogen bonding.

The direct dyes were named as such because they do not require mordants (a mordant is a substance to which another compound is able to attach) in the way they are applied. They are applied and bond directly to the substrate involved by electrostatic forces (Kulkarni *et al.*, 1986). When direct dyestuffs are dissolved in water, they dissociate into cations and anions. One of the methods of attachment of direct dyes to their substrates is by a weak acid-base interaction that involves van der Waals forces or hydrogen bonding. This occurs between the hydroxyl groups of the cellulose and dye molecules that contain reactive groups, e.g. amines (Trotman, 1984).

Direct dyes are similar to acidic dyes but there is one important difference. Direct dyes normally have their aromatic rings arranged in a planar form. This makes it possible for the dye molecule to lie parallel to the substrate surface and allows hydrogen bonding to take place. Acidic dyes have their aromatic rings grouped together making this type of interaction impossible (Kulkarni *et al.*, 1986)

The characteristics of this type of dye were considered useful for the present study. Amino groups exist on both antibodies and dyes. Congo red and primulin were amongst the first direct dyes to be discovered (Trotman, 1984) and were used in this study. Another direct dye used in this study is trypan blue, otherwise called Direct blue 14 in the colour index.

1.2.3.4 Mordant and metallocomplex dyes

Mordant dyes chelate (a chelator is a substance that combines a metal ion to groups that contains two or more electron donors) metallic oxides to form insoluble coloured regions, called lakes. Numerous classes of dyes have or use mordant materials to bind to fibres. Metallic salts are called mordants because they "bite" into certain fibres (e.g. textile fibres)

thus giving adhesion to dyes which couldn't do so on their own. There are a number of metal salts used to treat unmetallized dyes: this increases their molecular mass and in so doing, deepens the colour (Kulkarni *et al.*, 1986). An example of a mordant dye is Mordant orange 1.

1.2.3.5 Azoic dyes

Azoic dyes have the functional group (- N ≡ N -), known as the azo group, and are similar in chemical composition to the azo dyes in other application classes. Dyes of this class were discovered when impregnation of cellulose with certain dyes and then development with a coloured diazo component, produced a greatly improved method with which to dye cellulose with brighter colours. Azoic dyes are produced in small amounts only because of environmental factors and their use has mostly been replaced with direct dyes. They are strikingly similar to direct dyes; both require a linear molecular structure and hydrogen-bond forming groups (Trotman, 1984).

Diazotized amines are unstable in storage. Fast salts (stabilized diazonium salts) were subsequently developed. They are soluble in water and are ready for immediate application because of their simple application and fast, bright colours. Fast salts also minimize contact with harmful waste compounds and toxic fumes (Kulkarni *et al.*, 1986). An example of an azoic dye is Fast red PDC salt.

1.2.3.6 Sulfur dyes

As the name of this class of dyes suggests, their characteristic feature is that they contain sulfur linkages in their structures and can be detected by the release of hydrogen sulfide when treated with reducing agents. They are insoluble in water but dissolve in solutions containing sulfuric acid or sodium sulfide. These act as reducing agents, breaking the sulfur linkages and reorganizing molecules into simpler components. The smaller components are soluble in water and dye cellulose. Their application is based on a balance of interactions between the water soluble reduced state and the insoluble oxidized form. Sulfur dyes are applied to the textile in the reduced form in solution and, after entering the fibre, oxidized into the insoluble form. Post-treatment with metallic salts assists in developing brighter colours more rapidly. A chelating agent is required to prevent the textile from developing a harsh texture (Kulkarni *et al.*, 1986). A stable reduced state of sulfur dyes renders a water-soluble type of sulfur dye and their dyeing properties are similar to those of the direct dyes, but they do not dye cellulose

without the presence of a base (Trotman, 1984). An example of a sulfur dye is Sulfurrhodamine 101 hydrate.

1.2.3.7 Disperse dyes

Disperse dyes arose from workers who tried to find an easy and commercially viable way to dye cellulose acetate and other synthetic fibres. Acetylation of cellulose makes the substrate hydrophobic and direct dyes do not respond to it at all.

Disperse dyes consist of water insoluble pigments held in suspension. They are prepared in powder form by having the pigment milled with a dispersing agent and water and this is dried and given a diluent such as Glauber's salt (sodium sulfate). The disperse dyes can either penetrate the fibres or form a surface-water interface (Kulkarni *et al.*, 1986). They have low water solubilities but are slightly soluble because there are no sulfonic acid groups and their non-ionic nature is necessary to engage stable dispersions. Examples of disperse dyes used in this study are dianix blue FBLN-SE 300 and dianix red.

1.2.3.8 Reactive dyes

When applied under alkali conditions, reactive dyes produce a covalent bond with cellulose. Note that all the other dyes bind to cellulose only by hydrogen bonding, van der Waal's or ionic interactions. Reactive dyes have two electron deficient carbon groups that are able to effect a nucleophilic attack and therefore bond with the hydroxyl ions of cellulose. Reactive dyes have a small molecular size and are able to enter fabric fibres easily, they are available in a wide range of colours and they can dye various types of fabrics.

The reactive group on the dye is usually a double bond or a nucleophilic chlorine atom which then reacts with the available amino or hydroxyl groups on cellulose to form covalent bonds (Kulkarni *et al.*, 1986).

Reactive dyes are highly water-soluble and are similar to azoic dyes: they provide a stable link to both the chemical groups on the fabric and to the chromogen. Examples of reactive dyes used in this study are Reactive black 5 and Cibacron Blue 3GA.

1.2.4 Why are dyes used in laboratories?

There are many dyes that make the presence of proteins visible to the human eye by imparting colour to them. The extent to which the proteins are coloured may be measured, amongst others, by a spectrophotometer in order to quantitate how much protein is present. The Lowry or Folin-Ciocalteu method uses the Folin-Ciocalteu reagent, a mixture of sodium tungstate, molybdate and phosphate (Wilson and Walker, 1996). This compound is able to interact with the aromatic ring of tyrosine residues and produces a blue-purple colour complex that is measured at 660 nm. The principle of the assay is the formation of cuprous ions in solution, which reduce the Folin-Ciocalteu reagent.

The bicinchoninic acid (BCA) reagent has been developed as an alternative to the Lowry assay to detect cuprous ions in solution as it is quicker and more reproducible. BCA gives a blue-purple colour with cuprous ions with an absorbance maximum at 562 nm.

The biuret method (another protein quantification alternative) uses a copper sulfate solution containing sodium potassium tartrate. Cupric ions form complexes with the peptide bonds to give a blue coloured complex.

The Bradford method uses the dye Coomassie brilliant blue which complexes with proteins under acidic conditions to give an absorption maximum at 595 nm. The concentration of unknown proteins can be deduced from a calibration curve constructed from a suitable standard (Wilson and Walker 1996).

Proteins can be stained quantitatively after electrophoresis, also using Coomassie brilliant blue. This dye, in the presence of methanol and phosphoric acid, obtains a largely negative charge. Incubating the gel in an acidic "fixing" solution causes the proteins to precipitate in the gel and to have a positive charge. Coomassie brilliant blue binds to the positively charged proteins.

Bromophenol blue is a dye so small that it can move through an electrophoretic gel unretarded. This may therefore act as the electrophoretic front and the progress of electrophoresis can be monitored by following its progress.

Amido black is a dye with an overall negative charge that, when dissolved in an acidic solution, stains proteins by being attracted to the positive charges.

Procion S (Ponceau S) is a red dye also used to stain inert proteins, for example on nitrocellulose, particularly after blotting. This is a reversible stain and incubation of the

nitrocellulose in concentrated sodium hydroxide washes the stain away. This is especially useful when the nitrocellulose is required for use in a western or other type of blot.

One of the most popular ways to stain proteins in PAGE gels is silver staining that uses silver nitrate as the protein stain. Silver may also be used to detect nanogram amounts of protein in solution (Krystal, 1987) Silver also stains proteins on nitrocellulose (Draber, 1991). A modified silver stain is used to stain polypeptides in SDS-PAGE gels and the proteins stain different shades of blue, yellow, red and grey (Nielsen and Brown, 1984)

Another precious metal used to stain proteins is colloidal gold, the so-called "AuroDye", which forms a quantitative test for proteins and that offers an enhancement in sensitivity. There are various ways of executing this technique, as described by Egger and Bienz (1987) and Ciesiolka and Gabius (1988).

The development of a near infrared dye immunoassy (NIRDIA) is another alternative to this theme as described by Boyer *et al.*, (1992). A near infrared dye was derivatized with an isothiocyanate functional group and conjugated to specific antibodies. Laser diode detection effects quantitative immunoassay with enhanced sensitivity.

1.2.5 Dyes used in the present study

The goal of the present study was to structure a method for the easy detection of antigens by means of a visible, coloured assay. The colour was derived from numerous dyes that were covalently linked to antibodies using various coupling techniques. The above staining techniques (Section 1.2.4) generally require stringent acidic conditions, usually in the presence of alcohol, which was not suitable for the purposes of this study. In order to retain antibody activity, physiological conditions have to prevail. The coupling methods therefore have to be such that native antibody structure and function are maintained while achieving covalent coupling of dye particles to the antibody.

Specific dyes were chosen as subjects of investigation in this study. The dyes have particular properties, some of which are not published, and this is one of the reasons for having chosen them. It is important to note that these dyes have particular functional groups. The amino groups, particularly, are of interest and are easily targeted when coupling to other molecules. A description of each of the dyes used in the study with their chemical and characteristic properties follows.

1.2.5.1 Reactive black 5

Reactive black 5 (Figure 1.4) has an absorption maximum of 597 nm and a molecular mass of 991,82 Da. This dye has not been used for protein applications, but is classified as a reactive dye and is known to produce covalent bonds (Section 1.2.3.8). It has only one amino group and is classified as a wholly acid anionic dye: sulfonated with a hydroxyl group (Section 1.2.3.2) (Gurr, 1965). This dye was used because it is a very dark blue, almost black, colour and should provide excellent contrast on a white background for detection purposes.

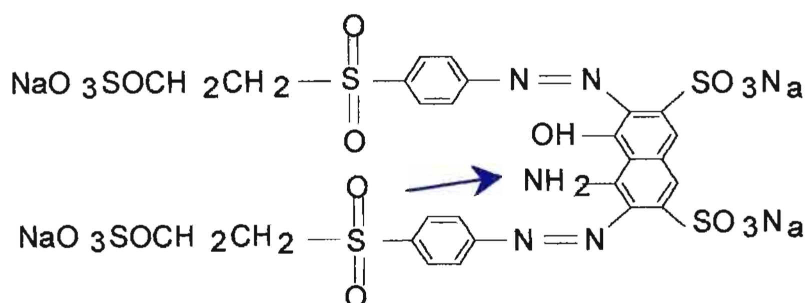


Figure 1.4: The chemical structure of Reactive black 5.
The arrow emphasizes the position of the amino group.

1.2.5.2 Cibacron Blue 3GA

Cibacron Blue 3GA (Figure .5) has an absorption maximum of 620 nm and a molecular mass of 774,2 Da. Refer to Figure 1.5 for the structure of Cibacron Blue 3GA. It contains only one amino group and is classified as a basic dye: cationic with acidic side chains (Section 1.2.2) (Gurr 1965). This dye was chosen for investigation because it has been applied to affinity chromatography by covalently attaching the dye to the primary hydroxyl groups in cellulose. (Scopes, 1987; Baird *et al.*, 1976; Atkinson *et al.*, 1981). The dye molecules mimic natural ligands and provide opportunities for other interactions with protein surfaces. Cibacron Blue is also a potent inhibitor of quinone reductase and is classified as a reactive dye (Prestera *et al.*, 1992). This dye has thus been proven to interact with proteins and therefore is likely to interact with antibodies.

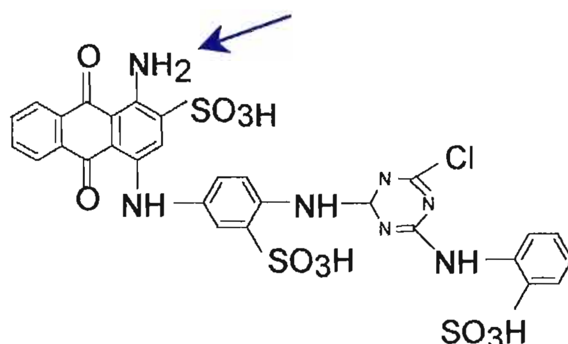


Figure 1.5: The chemical structure of Cibacron Blue 3GA.

The arrow emphasizes the position of the amino group.

1.2.5.3 Primulin

Primulin (Figure 1.6) has an absorption maximum of 229 nm and a molecular mass of 475,5 Da. It only has one amino group and is classified as an amphoteric anionic dye – an acidic dye with weak basic properties (sulfonated without a hydroxyl group) (Gurr 1965). A.G. Green developed primulin in 1887 (Trotman, 1984), a yellow, substantive dye with poor light fastness. As can be seen from the structure of the dye, it has a primary amino group attached to an aromatic nucleus. Trotman classified primulin as a direct dye and states that the dye can be diazotized when it is treated in a cold solution of sodium nitrite in the presence of hydrochloric acid. This forms an intermediate compound that can be used to couple to various phenols or aromatic amines to form new azo dyes. The addition of a chromophore causes the molecule to undergo a colour change. Trotman (1984) lists phenol, β-naphtol and resorcine as a few of the additional chromophoric compounds used in the diazotization reaction.

According to the literature, primulin revealed yet another interesting and unique characteristic. Taki *et al.*, (1994) investigated the quantitative transfer of glycolipids and phospholipids to a PVDF membrane (Taki *et al.*, 1994). Their lipid detection reagent was primulin solution. They also used primulin for general detection of neutral glycosphingolipids separated by thin layer chromatography (TLC) (Taki *et. al.*, 1994) (Lowenstein, 1975). Primulin is fluorescent under ultraviolet (UV) light as indicated in the lipid detection tests.

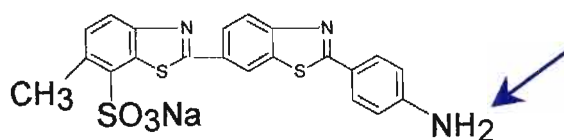


Figure 1.6: The chemical structure of primulin.

The arrow emphasizes the position of the amino group.

1.2.5.4 Para-nitroaniline

Para-nitroaniline (Figure 1.7) is a standard dye used in laboratory assays and has a molecular mass of 138.1 Da. It is classified as a cationic dye (Section 1.2.2). It is normally linked to a substrate, and there are many reports of microassays that measure the release of para-nitroaniline from the substrate at 405 nm (Lee and Anstee, 1994). The dye is used in plasminogen/plasmin assays (Kulisek *et al.*, 1988; Kulseth and Helgeland, 1992), to determine phospholipids in plasma (Wagenvoord *et al.*, 1994; White *et al.*, 1998), and for aspartyl proteinase assay (Filippova, 1996). Para-nitroaniline's solubilities are reported by Huyskens (1998).

Hyman (*el al.*, 1983) described the development of a spot test for urinary methylmalonic acid which involves diazotized para-nitroaniline as part of the assay. The diazotization reaction is entirely chemical and the formation of diazonium salts from primary aromatic amines is one of the popular synthetic protocols in organic chemistry (Trost and Fleming, 1991; Barton and Ollis, 1979). Part of this chemical reaction involves the nitrosation of the amine with nitrous acid in aqueous solution. However, if the amine is basic enough, as are the dyes in our case, they can form a salt with dilute acids in aqueous solution. That is to say that the diazotization method of adding sodium nitrite in aqueous solution to a solution of the amine salt is good enough to obtain the diazo reaction (Trost and Fleming, 1991). Compounds that incorporate the azo group usually have bright colours because of the extended conjugation in their molecules (Johnson, 1999).

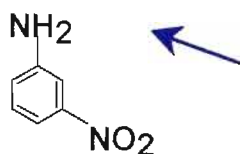


Figure 1.7: The chemical structure of para-nitroaniline.

The arrow emphasizes the position of the amino group.

1.2.5.5 Congo red

Congo red (Figure 1.8) has an absorption maximum of 497 nm and a molecular mass of 696,7 Da. It has two amino groups and can be classified (Section 1.2.2) as an anionic dye: sulfonated without a hydroxyl group and is moderately or strongly amphoteric (Gurr, 1965). Congo red was chosen because it has a bright red colour and would provide excellent contrast on a white background. Trotman (1984) identified Congo red as a direct dye. It is also a popular microscopy stain used to stain tissues and cell types (Gurr, 1965).

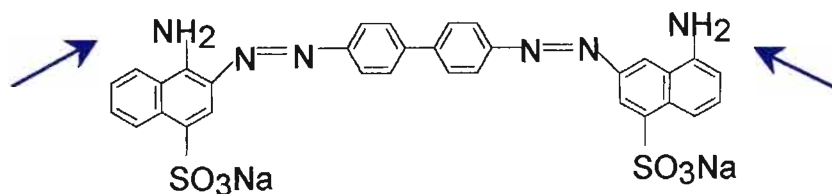


Figure 1.8: The chemical structure of Congo red.

The arrows emphasize the position of the amino groups.

1.2.5.6 Trypan blue

Trypan blue (Figure 1.9) has an absorption maximum of 607 nm and a molecular mass of 960,8 Da. This dye also has two amino groups and can be classified (Section 1.2.2) as an anionic dye: sulfonated with hydroxyl and amino groups and can be moderately or strongly amphoteric (Gurr, 1965). This dye was used because it has a dark blue colour and is known to stain a variety of proteins. It is used in cell culture work where dye exclusion methods are used for cell viability studies (Sigma-Aldrich Catalogue, 2002). Viable cells do not take up the dye and non-viable cells do.

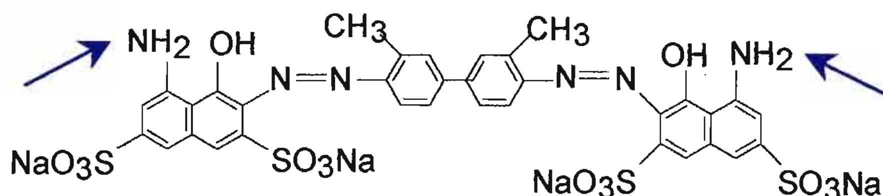


Figure 1.9: The chemical structure of trypan blue.

The arrows emphasize the position of the amino groups.

1.2.5.7 Acid black 2

Acid black 2 is also known as Nigrosin and has an absorption maximum of 540nm. Its structure is not known and, therefore, its molecular mass is also not known. It was chosen for investigation because it very clearly stains gluten proteins that have been resolved on a PAGE gel (Lee, 1963). It also has a very dark colour to once again show good contrast on a light background.

1.2.5.8 Dianix blue FBLN-SE 300

Dianix blue (Figure 1.10) has an absorption maximum of 605 nm and is a disperse or colloidal dye. This dye complexes in solution and exists as multiple different combinations of the basic structure. The preparation of the dye involves numerous centrifugation steps and hence the molecular mass of the particles used in this study are unknown. The molecular mass of the basic unit is 519 Da. An ionic interaction of dye with antibody to form a coloured antibody solution allows this class of dyes to be applied as a label in immunoassays (Gribnau *et al.*, 1982). The properties of the dye mimic the properties of gold particles in a protein staining technique. The method of Gribnau *et al.* describes an ionic interaction between dyes and antibodies, whereas the investigations in this study with the other dyes have involved covalent attachment. Snowden and Hommel (1991) continued to investigate this class of dyes by developing an assay on nitrocellulose using capture antibodies in ELISAs and dot blots, with the colloidal particle linked antibodies for multiple antigen detection

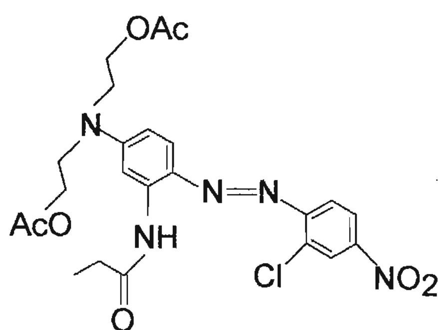


Figure 1.10: The chemical structure of dianix blue FBLN 200.

1.2.5.9 Dianix red

Dianix red (Figure 1.11) has an absorption maximum of 510 nm and, like dianix blue, is a colloidal dye. Rabello *et al.*, (1993) used dianix red (otherwise known as Samaron red or dianix rubine) as the dye conjugated to soluble egg antigen (SEA) and keyhole limpet haemocyanin (KLH). Both of these proteins share carbohydrate epitopes with the surface proteins of schistosomula. They developed an immunoassay to differentiate between acute and chronic forms of schistosomiasis (bilharzia). The methodology they used follows that of Snowden and Hommel (1991), who also used this dye in their experiments The molecular mass of this dye is 417 Da.

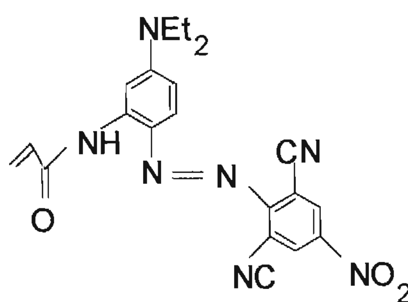


Figure 1.11: The chemical structure of dianix Red.

1.3 Biochemical conjugations and current detection assays

The discovery of the enzyme-linked immunosorbent assay (ELISA) system has simplified many routine laboratory assays. In this type of assay, the antigen is adhered to a microtitre plate, usually consisting of 96 wells. Enzyme-linked antibodies are subsequently incubated in the wells and the corresponding substrate is able to provide a detectable (usually spectrophotometric or fluorimetric) reaction. This assay has been used to prepare antibody-enzyme conjugates to detect any small protein or analyte. It is relatively easy to raise antibodies to just about any protein or analyte and the isolation of the antibodies is also an easy task. Enzymes such as alkaline phosphatase, β-galactosidase, horseradish peroxidase and glucose oxidase are used and have replaced the use of radioisotopes because of the hazards involved in their use (Hermanson, 1996). Fluorescent compounds have also had many applications in this regard. Some of the more commonly used compounds are phenylisothiocyanate (FITC), fluorescein, rhodamine, aminomethylcoumarin (AMCA), and

phycoerythrin (Hermanson, 1996). Each of the fluorescent dyes has its own excitation wavelength whereby light is absorbed and becomes excited, and emits light at another wavelength known as fluorescence.

Another example of an interaction that can be exploited in immunoassays, is that of the avidin-biotin complex. The biotin molecule (vitamin H) acts as a label on the antibody and provides a reactive site for avidin (a glycoprotein found in egg white) or streptavidin to bind (Hermanson, 1996). The biotin binding protein may then be labelled with an enzyme or fluorescent molecule and in so doing, provides a detection system.

The goal behind the covalent coupling techniques in this study was to develop a dye-based immunoassay (DIA) for antigen detection. The antibody is covalently linked to the dye which has an obvious visible colour and serves as the detecting reagent.

1.3.1 Targeting specific functional groups for biochemical conjugation via covalent coupling methods

Antibodies are proteins made up of constituent amino acids; near the surface if they are hydrophilic. These may, in turn, provide a number of sites that may be modified and to which conjugation with other molecules may be effected. Any conjugation protocol is designed around certain functional groups (hence a reactive site on the cross linking or derivatizing reagents). The presence of functional groups on the target molecule that is to be modified is also of great importance. The two sets of functional groups should chemically complement each other so that conjugation may be effected. A recommendation when working with antibodies is to chose reagents and conditions that conserve antigen-binding activity.

There are numerous mechanisms by which reactive groups are able to couple to specific target groups. For instance, amino acids containing ionizable side chains such as aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine and tyrosine, are important in modification and conjugation protocols. Groups within proteins can be in one of two ionisation states: protonated or unprotonated. It is important to consider the pH of the buffer involved in the conjugation methods. At pH values above the pKa of the carboxylate group, the amino acid is ionized and has a negative charge. Similarly, a pH below their pKa values renders the amino acids protonated and they have a positive charge. Different ionizable groups in proteins will have different pKa values influenced by factors such as the presence of salts, elevated temperatures, ionic strength and the nature of the solvent solution.

Proteins with groups of amino acids that contain an overall negative charge are identified as nucleophiles (any atom that contains an unshared pair or an excess of electrons, able to partake in covalent bond formation) that could take part in addition reactions. The source for many coupling reactions in chemical modifications is the nucleophilic attack by electron rich atoms toward other atoms that contain an electron deficiency (or have a positive charge). The hydrophilic, amine-containing side chains in lysine, arginine and histidine are normally on the surface of proteins and can be readily derivatized. Although these amino residues normally appear on the surface of proteins, they may sometimes be drawn into the centre of the protein molecules' tertiary structure depending on the number of hydrophobic amino acids present and their distance and spacing between hydrophilic amino acids (Voet and Voet, 1995).

When the pH of the reaction is bought closer to the pKa of the ionizable amino acid, they are able to couple to a greater degree.

Stable products with good preservation properties and high yields are important aims when considering any conjugation protocol. This is often done by incorporating additives (for example, glycerol in enzyme conjugates) to protect the activity of enzymes or by using site-directed reactions that will result in modifications away from the active centre.

1.3.2 Dye-Antibody conjugation with glutaraldehyde

A number of chemical reactions can be applied to produce antibody-enzyme conjugates. For the purposes of this study, antibody-dye conjugates will be produced. The use of cross-linking reagents in conjugation protocols can be extremely useful. If the molecules to be conjugated are too "bulky" and steric hindrance is an obstacle, this may be overcome by using a cross-linker of sufficient length (Hermanson, 1996). This problem was not anticipated because antibodies are large biological molecules compared to the dye molecules. Zero-length cross-linkers such as glutaraldehyde are applied to molecules that do not have this problem, and was the preferred cross-linker for this study. When a homobifunctional reagent (a compound that has identical chemical groups at both ends) such as glutaraldehyde is used to join together two proteins, a clumping of the product can occur. The reagent could react with either one of the proteins and form an "active intermediate" (Hermanson, 1996). This could then interact with the other protein or with another molecule of the same protein, until a huge cross-linked conglomeration of product is obtained. These problems are unfortunately magnified in single-step reactions that use homobifunctional reagents because single-step

reactions usually require the addition of all the reagents together at the same time. It follows that the resulting yield of desired conjugated product may be small (Hermanson, 1996). There is a way to circumvent this problem. Two-step or multi-step protocols have been designed for use with homobifunctional reagents. One of the protein molecules is "activated" with a cross-linking reagent and any unbound reagents are removed in the first steps of this method. The second protein is added, and again any unbound reagents are removed. This will lead to minimal clumping and maximal conjugation. The resulting yield of desired conjugate is considerably higher compared to that of single-step methods (Hermanson, 1996).

Glutaraldehyde was one of the first and is still the most commonly used cross-linking reagents (Hadju and Frierich, 1975; Briand *et al.*, 1985). It may be applied with almost any protein and its use in fixing samples in preparation for electron microscopy is well documented.
Primary amines (R-NH_2) are found on lysine residues on proteins, including antibodies, and are organic derivatives of ammonia. An imine (nitrogen analog of an aldehyde or ketone) is formed when an aldehyde or ketone interacts with the primary amine. Imines are commonly called Schiff bases. A simplified schematic reaction in shown in Figure 1.12 (Loudon, 1995).

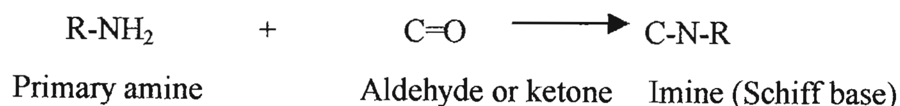


Figure 1.12: A schematic representation of the interaction of a primary amine with an aldehyde or ketone to form an imine.

The cross-linking process using glutaraldehyde proceeds via Schiff base formation and reduction to form a secondary amine. Glutaraldehyde enables a moderate degree of cross-linking and leaves the conformation and biological activity of the protein largely unaltered (Hadju and Friedrich, 1975). Glutaraldehyde, a homobifunctional agent, has the chemical structure shown in Figure 1.13.

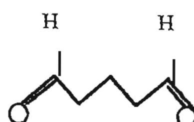


Figure 1.13: The chemical structure of glutaraldehyde.

Glutaraldehyde has two aldehyde groups that can react with primary or secondary amines to form a Schiff base via a dehydration reaction to yield an imine (Figure 1.14).

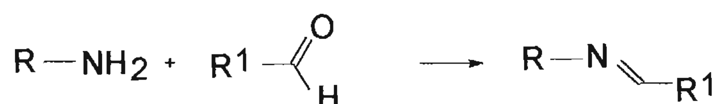
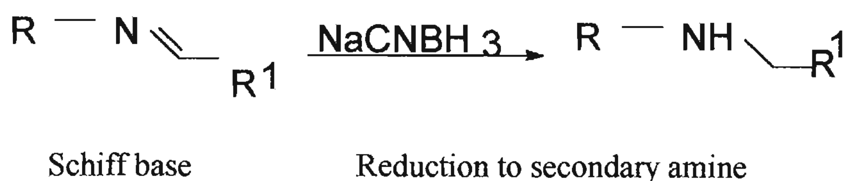


Figure 1.14: Reaction to show the formation of an imine from a primary amine group.

'R' and 'R[1]' indicate any other carbon containing groups.

Schiff base formation is a reversible interaction and the product is relatively unstable in that it is easily cleaved in aqueous solution. Reductive amination describes the interaction of a reducing agent (either sodium borohydride or sodium cyanoborohydride) with an imine to produce a stable amine. Reductive amination thus reduces the Schiff bases to secondary or tertiary amine bonds. Once reduced, these bonds are stable and will not easily hydrolyze in aqueous solution (Figure 1.15). This is known as a zero-length cross-linking reaction where glutaraldehyde offers no extra atoms to introduce spaces between the molecules (Hermanson, 1996).



Schiff base Reduction to secondary amine

Figure 1.15: Sodium cyanoborohydride reduction of a Schiff's base.

A simplified reaction scheme. 'R' and 'R[1]' indicate any other carbon containing groups.

According to Shimizu *et al.,* (1988), IgY has 3.5% molar lysine residues. The ε-amine group on lysine residues is the functional group targeted by glutaraldehyde and amine groups are the targets on the selected dyes. The dye is "activated" and antibody is added after the removal of unbound reagents. Formation of a covalent cross-link between the two molecules is the aim.

1.3.3 Dye-antibody conjugation by means of carbohydrate residues

Antibody molecules have carbohydrate molecules attached to their Fc region (constant portion of the heavy chain) (Hermanson, 1996). According to Wasserman and Capra (1977), the polypeptide chains of immunoglobulins are synthesized on polyribosomes of the rough endoplasmic reticulum (RER). The carbohydrate groups are assembled in a stepwise process at various subcellular sites during transport of the immunoglobulin through plasma cells. The RER adds the glucosamine and mannose residues, the smooth ER and golgi the galactose and sialic acid residues and the antibody is only secreted once it contains all its carbohydrates. (Wasserman and Capra, 1977). Matsuura *et al.*, (1993), determined the presence and analysed asparagine-linked oligosaccharides on IgY from quails (a close relative of the chicken). Glycoproteins such as IgY can be defined as large molecules with a polypeptide backbone with one or more carbohydrate moieties attached. Glycoproteins can be subdivided into groups according to which carbohydrate-peptide bond pre-dominates (Schultz *et al.*, 1969). The asparagine-N-acetyl glucosamine linkage is the dominant form found on IgY. The sugar concerned in the N-glycosidic linkage is N-acetyl glucosamine and the amide group of an asparagine residue supplies the nitrogen atom. The heterosaccharide is often mannose or galactose (Schultz *et al.*, 1969). These glycoproteins are potential covalent cross-linking sites and can thus be targeted as such. The sugar residues may be periodate oxidized to form reactive aldehyde residues. These aldehyde residues can be used to couple to amino groups (on the dyes) to form Schiff bases which may be reduced to form stable products.

1.4 IgY conjugation to horseradish peroxidase

IgY was conjugated to horseradish peroxidase (HRP) in order to compare the dye-antibody reactions in this study to a traditional enzyme system. HRP is a plant enzyme from horseradish roots and has a pH optimum of 7 and a molecular weight of 40 kDa. (Hermanson, 1996). HRP catalyzes the reaction of H_2O_2 with a variety of electron–donating dye substrates to produce coloured products which may be used as detection reagents (Hermanson, 1996). Commercially available substrates may be obtained either to form soluble products for spectrophotometric detection systems such as ELISAs or insoluble forms for staining techniques, applied particularly to nitrocellulose.

HRP is a glycoprotein that contains enough carbohydrate to produce a significant yield of conjugated product (Hermanson, 1996). Periodate oxidation of the sugar residues produces reactive aldehyde residues that may be used for conjugation to amine groups on the antibody. Reductive amination with sodium cyanoborohydride is necessary to produce a stable compound that may be stored at 4°C for months without significant loss in activity.

There are disadvantages to using HRP. It is susceptible to inactivation by many anti-bacterial reagents, such as azide, and may be reversibly inhibited by cyanide and sulfide (Hermanson, 1996). It has only two amine groups, which could limit its ability to be activated by amine-reactive cross-linking reagents (Hermanson, 1996).

1.5 IgY biotinylation

Another comparative immunoassay procedure was developed using the avidin-biotin system. Avidin is a glycoprotein from egg white. It has four identical sub-units each with a binding site for biotin (or vitamin H). Biotin and avidin bind to produce an extremely stable complex. Their interaction is comparable to an antigen-antibody interaction but avidin and biotin have a much greater affinity for each other than an antibody has for its antigen (Hermanson, 1996). The disadvantage to using avidin is that it may bind non-specifically to proteins other than biotin because it has a high pI value and the fact that it contains carbohydrate residues that may be bound by carbohydrate binding proteins on cells.

Biotinylation reagents (a reagent that is used for the modification of proteins by conjugating it to biotin) all have a bicyclic biotin ring and a valeric acid side chain of $_D$-biotin at one end of the structure and a functional group at the other end that may be used to couple to other molecules. N-hydroxysuccinimidobiotin (NHS-Biotin) was the biotinylation compound used in this study. The valeric acid side chain of $_D$-biotin may be activated to yield an NHS ester. The NHS-ester acts by initiating a nucleophilic attack of an amine-containing moleclule on the carbonyl group present in its structure. The NHS group is released and a resultant stable amide linkage is formed. NHS-biotin is, however, insoluble in aqueous solutions and must first be dissolved in DMF. The addition of the NHS-biotin in DMF should not exceed 10% organic solvent to aqueous solvent otherwise it precipitates.

1.6 Study objectives

In this chapter the theoretical aspects of dye-antibody conjugation for diagnostic development were explored. The objectives for this study were as follows:

- Nitrocellulose strips formed the stationary support where antigen was immobilized.
- The nitrocellulose strips were then incubated in a coloured dye-antibody solution and the results visualised by eye.
- By dot-blotting antigen onto nitrocellulose and detection by means of the coloured antibody solution, an antigen detection assay was to be established.
- The development of a dye based, dipstick immunoassay, with a sensitivity comparable to two routinely used enzyme-substrate systems (horseradish peroxidase and biotinylated antibodies).
- This study aimed to evaluate and optimise different conjugation methods with different dyes.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 General reagents:

Sodium cyanoborohydride, rabbit albumin, dalton mark IV SDS molecular mass markers, dialysis tubing (diameter of 20 mm and exclusion limit of 20 000 daltons) were obtained from Sigma Chemical Company (St. Louis, USA). Polyethylene glycol (PEG) 6000 Da and 20 000 Da was from Merck NT laboratory supplies (Darmstadt, Germany). Glutaraldehyde, sodium periodate and mannose were from BDH Laboratory Supplies (Poole, England). Nitrocellulose was from Micron Separations Inc. Bovine serum albumin (BSA) was from Boehringer Mannheim (Mannheim, Germany). AminoLink® resin was from Pierce (Rockford, Illinois). Elite non-fat milk powder was purchased from the local supermarket and was discarded after 3 months of use. 96 well Nunc Maxisorp ELISA plates were from Nunc/Amersham (Roskilde, Denmark). Ultra pure water was obtained from the Milli Q Plus Ultra-pure water system (Millipore, Marlboro, USA).

All chemicals used were of the purest analytical grade.

This study was ethically approved by the Animal Ethics Committee of the University of Natal, Pietermaritzburg, (Hyaline brown chickens), (Animal ethics project number #GOLD/99)

2.2 Laemmli SDS-PAGE

Electrophoresis describes the migration of charged protein molecules in an electric field. This technique is often applied as an analytical method in biochemistry to assess the purity and size and/or charge protein samples. Various gel supports are utilized, for example: polyacrylamide, agarose and cellulose acetate. Differences in composition of cross-linking reagents can lead to the formation of highly cross linked gels for separating small proteins, or gels with a low degree of cross linking for the separation of large proteins. Laemmli (1970) described an SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) system which was used in the present study to analyze chicken IgY and sheep IgG preparations.

2.2.1 Materials

Monomer solution: [30% (m/v) acrylamide, 2.7% (m/v) *N, N'*-methylenebisacrylamide].

Acrylamide (73 g) and *N, N'*-methylenebisacrylamide (2 g) were dissolved and made up to 250 ml with distilled water and stored in an amber coloured bottle at 4°C. This solution was filtered through Whatman No. 1 filter paper before use.

Running gel buffer (1.5 m Tris-HCl, pH 8.8).

Tris (18.17 g) was dissolved in 80 ml distilled water, adjusted to pH 8.8 with HCl and made up to 100 ml. This solution was filtered through Whatman No. 1 filter paper before use.

Stacking gel buffer (500 mM Tris-HCl, pH 6.8).

Tris (3 g) was dissolved in 40 ml distilled water, adjusted to pH 6.8 with HCl and made up to 50 ml. This solution was filtered through Whatman No. 1 filter paper before use.

10% (m/v) SDS.

10 g of SDS was dissolved in 100 ml distilled water with gentle heating if necessary.

10% (m/v) ammonium persulfate.

Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8].

Stacking gel buffer (2.5 ml), 10% SDS solution (4 ml), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with distilled water.

Tank buffer [250 mm Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3.

Tris (15 g) and glycine (72 g) were dissolved and made up to 5 litres with distilled water. Prior to use, 2.5 ml of 10% SDS stock solution was added to 250 ml for use in the Mighty Small II apparatus.

Molecular mass markers.

Molecular mass standards from Sigma were: bovine albumin (66 kDa), egg albumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). Lyophilised markers were reconstituted in 1 ml of the reducing treatment buffer.

Molecular mass standards from Pharmacia were: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Lyophilised markers were reconstituted in 200 μl reducing treatment buffer and diluted 1:5 for application to gels.

2.2.2 Procedure

For SDS-PAGE, the BioRad Mini-PROTEAN II® electrophoresis apparatus was cleaned thoroughly before use and set up according to the manufacturer's specifications. The two glass plates were assembled in the gel casting clamp assembly and separated by 1.5 mm polyethylene spacers. The reagents for two gels to be cast were pipetted into a small erlenmeyer flask as described in Table 2.1.

Table 2.1: Reagents for casting two Laemmli gels in the Bio-Rad Mini-PROTEAN II® caster

Reagent	Stacking gel (4%)	Running gel (10%)	Running gel (12.5%)
Monomer solution (ml)	0.94	5	6.25
Running gel buffer (ml)	-	3.75	3.75
Stacking gel buffer (ml)	1.75	-	-
10% SDS solution (μl)	70	150	150
Distilled water (ml)	4.3	6	4.75
Ammonium persulfate (μl)	35	75	75
TEMED (μl)	30	7.5	7.5

A mixture of the ammonium persulfate initiator solution and *N, N, N', N'*-tetramethylenediamine (TEMED) causes polymerization and the gel begins to solidify. The separating gel solution was pipetted into the space between the two glass plates until 3 cm from the top of the plate and overlaid with distilled water (excludes oxygen from the gel surface as it prevents efficient gel polymerization). The gel was allowed to set (approximately 1 h), and the stacking gel was poured on top of the running gel and a sample application well-forming comb inserted between the two plates. Once this gel had set, the comb was removed and the wells rinsed with distilled water. The whole gel sandwich was transferred to the electrophoretic unit, creating an upper and lower buffer chamber. Laemmli gels use the same buffer in both chambers.

To a volume of the protein samples, an equal volume of reducing treatment buffer was added and this was boiled for 90 s in eppendorf tubes. Bromophenol blue (4 μl) was used as a marker dye as it migrates with the buffer front and was added to each sample just before loading onto the gel. The rate of electrophoresis can be monitored and the completion of the experiment will be evident when the dye reaches the bottom of the separating gel. A BioRad Model 3000 XI computer control electrophoresis power supply pack was used and the gels were run at 18 mA per gel. The gels were removed and placed in an appropriate staining solution.

2.3 Coomassie blue R-250 stain of proteins in an electrophoretic gel

Coomassie blue R-250 is a dye used to stain proteins in slightly acidic solution in electrophoretic gels. The dye stains proteins with good sensitivity and is a total protein stain (Voet and Voet, 1995).

2.3.1 Materials

Stain stock solution [1% (m/v) Coomassie blue R-250].

Coomassie blue R-250 was dissolved in 100 ml distilled water and filtered through Whatman No.1 filter paper.

Staining solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid.

Stain stock (12.5 ml) was mixed with methanol (50 ml) and acetic acid (10 ml) and made up to 100 ml with distilled water

De-staining solution I [50% (v/v) methanol, 10% (v/v) acetic acid].

Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with distilled water.

De-staining solution II [7% (v/v) acetic acid, 5% (v/v) methanol].

Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with distilled water.

2.3.2 Procedure

The gel was carefully removed from its sandwich between the two glass plates using a spatula and placed in the staining solution for 4 h. It was then rinsed with distilled water and left in de-stain I overnight, and in de-stain II to complete the de-stain process until the background was almost completely clear.

2.4 Primulin stain of proteins in an electrophoretic gel

The Coomassie blue protein staining procedure was used to model a method to stain proteins in electrophoretic gels with primulin. The stained proteins were also subjected to a diazotization reaction, to determine if protein detection sensitivity levels increased.

2.4.1 Materials

Dye stock solution [1% (m/v) primulin].

Primulin dye (1 g) was dissolved in 100 ml distilled water.

Dye staining solution [0.125% (m/v) primulin, 50% (v/v) methanol, 10% (v/v) acetic acid].

Primulin dye stock solution (12.5 ml) was mixed with methanol (50 ml) and acetic acid (10 ml) and made up to 100 ml with distilled water.

β-Naphtol developing reagent.

β-Naphtol (1 g) was dissolved in 6.6 ml boiling water containing NaOH (1 g) and made up to 26.4 ml with boiling water.

1.18 M potassium nitrite.

KNO_2 (1 g) was made up to 10 ml in distilled water.

Concentrated HCl/potassium nitrite solution.

Concentrated HCl (1.35 ml) was added to the potassium nitrite solution.

2.4.2 Procedure

The electrophoretic gel was incubated in the primulin staining solution for 16 hs (overnight) with constant agitation. After destaining the gel was viewed under UV light to determine protein detection levels. The gel was then placed in the diazotization solution to ascertain whether protein detection levels improved. Mixing 0.45 ml of the developer with 0.45 ml of the potassium nitrite solution and adding 1.35 ml concentrated HCl made up the diazotization reagent, this was made up to a final volume of 30 ml in distilled water. The gel was incubated

in the diazotization solution for 40 mins at 4°C and then rinsed extensively in distilled water. The protein detection levels could now be recorded under normal light conditions.

2.5 Silver stain of proteins in an electrophoretic gel

The silver stain uses the chemical reduction of silver nitrate as the protein staining medium in a synonymous manner to developing photographic prints. Blum *et al.*, (1987) improved the current silver staining techniques by making use of sodium thiosulfate. Pre-treatment in a solution containing this chemical compound produces enhanced image quality on the gel and it also provides the formation of soluble silver complexes which aids in the reduction of non-specific background staining. Detection of proteins in the 10-20 nanomolar ranges.

2.5.1 Materials

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde].
At the completion of electrophoresis, the gels were fixed in 50 ml methanol, 12 ml acetic acid and 50 µl formaldehyde made up to 100 ml, overnight.

Washing solution [50% (v/v) ethanol].
100 ml ultra pure water was mixed with 100 ml ethanol.

Pre-treatment solution [4 mg/ml $Na_2S_2O_3.5H_2O$].
Sodium thiosulfate (0.2 mg) was dissolved in 100 ml ultra pure water.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.75% (v/v) 37% formaldehyde].
Silver nitrate (0.2 g) and 37% (m/v) formaldehyde (37.5 µl) were made up to 100 ml with ultra pure water.

Developing solution [60 g/l Na_2CO_3, 0.5% (v/v) 37 % formaldehyde, conc. $Na_2S_2O_3.5H_2O$].
Sodium carbonate (6 g) was mixed with 95 ml distilled water, 2 ml of the pre-treatment solution and 50 µl of the formaldehyde solution were made up to 100 ml.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid].

Methanol (50 ml) and acetic acid (12 ml)were added to 38 ml ultra-pure water

2.5.2 Procedure

All steps were carried out on an orbital shaker in glass containers that had been washed with 70% ethanol and then rinsed with de-ionised water in order to minimize background staining. Following the overnight fixing step, gels were washed (3 × 20 min) in 50 % ethanol. The gel was soaked in the pre-treatment solution for 1 min and rinsed in distilled water (3 × 20 sec). It was soaked in the impregnation solution for 20 min and again rinsed in distilled water (2 × 20 sec). The gel was incubated in the developing solution until the first protein bands became visible and the developing solution was immediately replaced with distilled water until enough colour had developed. Immersing the gel in stopping solution stopped protein stain development. The gel was washed and stored in washing solution in polyethylene zip-seal bags.

2.6 Periodic-acid Schiff (PAS) stain

Antibodies are glycoproteins that have identical carbohydrate moieties on their heavy chains. These carbohydrate entities are potential covalent cross-linking sites. Periodate oxidation cleaves a carbon-carbon bond in the hexose ring structure of the sugar molecules to yield reactive aldehyde residues (Hermanson, 1996). A complementary amine group may be reacted with these aldehyde groups to form Schiff bases. Upon reductive amination of the Schiff base, stable secondary amines are formed, thereby forming a stable covalent link which can be used for further experimentation. In this study, the carbohydrate groups on the antibody were oxidized and the complementary amine group was supplied by the addition of dye molecules that contain amine groups.

2.6.1 Materials

Schiff's reagent (Fuschin-sulfate solution).

Potassium metabisulfate (2 g) and sodium thiosulfate (7.5 g) were dissolved in 250 ml distilled water. Concentrated HCl (2,6 ml) and basic fuschin (1 g) were added and stirred at

room temperature for 4 h. Decolourising charcoal was added, stirred, and filtered 15 min later. The filtrate was stored at 4°C.

Fixing solution [25% (v/v) isopropanol, 10% (v/v) acetic acid).

Isopropanol (25 ml) and acetic acid (10 ml) were made up to 100 ml with distilled water.

7.5% (v/v) Acetic acid solution.

Glacial acetic acid (7.5 ml) was added to 92.5 ml distilled water

0.2% (v/v) Periodic acid solution.

Periodic acid (0.2 ml) was made up to 100 ml with distilled water

0.5% (m/v) Potassium metabisulfite solution.

Potassium metabisulfite (0.5 g) was added to 100 ml distilled water.

2.6.2 Procedure

After electrophoresis, the gel sandwich was dismantled, the slab gel removed and placed in the isopropanol-acetic acid fixing solution overnight. It was then soaked in the 7.5% acetic acid solution for 30 min. The gel was immersed in the 0.2% periodic acid solution at 4°C for 60 min and this was covered with aluminium foil. The gel was then immersed (without washing) in neat Schiff's reagent for 60 min at 4°C in the dark. The gel was then fixed in three changes of 0.5% metabisulfite, 10 min each, in the dark. The gel was rinsed and stored in 7.5% acetic acid.

2.7 Generation and isolation of antibodies

Antibodies were isolated from sheep serum, chicken egg yolk or affinity purified from chicken egg yolk. PEG precipitation was used to purify the IgY (Polson *et al.*, 1980).

Proteins of a molecular mass greater than 5000 Da readily stimulate an immune response in animals. In other words, antibodies can easily be raised in chickens, rabbits or sheep injected with an antigen emulsified with an adjuvant. Smaller molecules, between the 1000-5000 Da

(haptens), may be conjugated to larger proteins or carrier proteins. The hapten-carrier linkage site on the hapten should ideally be furthest away from the functional group of the carrier i.e. the hapten acts as a spacer. There are various hapten-carrier conjugation methods, of which glutaraldehyde is the most popular.

Hapten-carrier conjugates are mixed with an adjuvant before immunization of the animal. Adjuvants effectively stimulate the immune system by forming a localized immunogen store at the site of immunization. The hapten-carrier conjugate is slowly released into the bloodstream. Adjuvants assist in reducing the toxic effects of the immunogen and stimulate circulation through the lymph tissue thus increasing the immune response. Adjuvants may be purchased as different types; inorganic adsorbents, mineral oils or bacterial cell wall components. Freund's complete adjuvant (FCA) consists of mineral oil and detergent plus heat inactivated *Mycobacterium tuberculosis*. FCA is usually used for the first immunization as it gives a better stimulation of a local response. Freund's incomplete adjuvant (FIA) consisting of only mineral oil and detergent was used for booster immunisation (Johnstone and Turner, 1997).

200 µg of peptide was conjugated to rabbit albumin at a molar mass ratio of 40:1 peptide to carrier (the peptide was human C_{3a} C-terminal peptide consisting of amino acids HARASHLGLA) was prepared with a glutaraldehyde conjugation method. Equal amounts of immunogen (rabbit-albumin-C3a peptide) and FCA were used to prepare a stable water-in-oil emulsion by trituration. The chickens were immunised by injection of 325 µl of immunogen in either side of the breast muscle at week 0, 2, 4 and 6 followed by two fortnightly injections and then monthly booster injections. Eggs were collected daily and antibody titres were established by ELISA protocols to monitor peak protein concentrations.

Proteins may be purified by precipitation with PEG 6000 (Polson *et al.,* 1985; Goldring and Coetzer, 2003). PEG acts by competing with the protein for solvent molecules. Its water-soluble characteristics ensure very little protein denaturation occurs (Ingham, 1990) and high yields of purified protein are obtained. PEG was used for the isolation of IgG from sheep serum and IgY from chicken egg yolk.

2.7.1 Isolation of IgY by PEG precipitation

PEG was employed in the IgY isolation procedures. Other IgY isolation methods were also explored in an attempt to see whether PEG interfered with IgY conjugation techniques to dye molecules.

2.7.1.1 Materials

100 mM Sodium phosphate buffer, 0.02% (m/v) sodium azide.

$NaH_2PO_4.H_2O$ (13.8 g) was dissolved in 950 ml distilled water, pH titrated to pH 7.6 using NaOH and made up to 1 litre. NaN_3 (0.2 g) was subsequently added.

PEG 6000

Added as final concentrations of 3.5%, 8.5%, and 12%

2.7.1.2 Procedure

Egg yolks were separated from egg white and washed under a gentle stream of running water to remove any albumin. The yolk sac was punctured with a sharp object and discarded and the volume of yolk measured. Two volumes of sodium phosphate buffer were added to the volume of yolk and mixed. Solid PEG 6000 was added to a final concentration of 3.5% (m/v) and dissolved by stirring. The vitellin fraction, the semi-solid yellow phase, was removed by centrifugation (4420×g, 30 min at room temperature). The supernatant was filtered through absorbent cotton wool to remove any lipids. The PEG concentration was increased to 12% (m/v) by adding a further 8.5% (m/v) PEG, mixed thoroughly and centrifuged (12 000×g for 10 min at room temperature). The supernatant was discarded and the pellet dissolved in phosphate buffer at a volume equal to the volume obtained after filtration. The PEG concentration was increased to 12% (m/v), stirred and centrifuged (12 000×g for 10 min, at room temperature). The final supernatant solution was discarded and the pellet was solubilised in 1/6 of the original egg yolk volume in sodium phosphate buffer. To calculate the IgY concentration, a 1 in 40 dilution of the IgY was made in phosphate buffer and its A_{280} measured. The concentration of the undiluted sample was calculated by using the extinction coefficient for IgY. At 1 mg/ml IgY's extinction coefficient is 1.25. The antibody solution was stored at 4°C.

2.7.2. IgY isolation with sodium sulfate

Removal of the lipid fraction is the most challenging step in IgY isolation. Various methods have been employed to do this and the PEG isolation procedure described above does this quite efficiently. The different approaches discussed below were explored for comparison of the yield of IgY obtained and ease and cost of materials used for routine IgY isolation.

Sodium sulfate precipitation entailed the removal of yolk lipid at low ionic strength using distilled water and adjustment of the pH with 0.1 M NaOH to pH 7.0 (euglobin precipitation). Care must be taken to remove all traces of egg white with contaminating proteases, ovalbumin and avidin (Jensenius and Koch, 1997). If turbid supernatants are encountered (indicating residual lipids) the lipids may be extracted with dextran sulfate and calcium chloride before salt precipitation with sodium sulfate. An IgY isolate of high purity is obtained when precipitated with sodium sulfate compared to precipitation with ammonium sulfate (Jensenius and Koch, 1997). A disadvantage to this method is any cold temperature during centrifugation precipitates the salt in solution.

2.7.2.1 Materials

0.1 M NaOH.

Sodium hydroxide (0.4 g) was dissolved in 100 ml distilled water.

0.4 M sodium phosphate buffer, pH 7.6.

NaH_2PO_4 (5.52 g) was dissolved in 80 ml distilled water, adjusted to pH 7.6 with sodium hydroxide and made up to 100 ml.

36% (w/v) Na_2SO_4.

This is a supersaturated solution. Na_2SO_4 (36 g) was dissolved in 100 ml distilled water by boiling. The solution is cooled to 30-40°C before being added to the protein solution at room temperature.

10 mM Tris-buffer, 0.14 M NaCl pH 7.4, 15 mM NaN_3 (TBS).

NaCl (8.1 g), Tris (1.21 g) and sodium azide (0.98 g) were dissolved in 800 ml distilled water, was adjusted to pH 7.4 with HCl and made up to 1 litre.

2.7.2.2 Procedure

The yolks of four eggs were diluted with 9 volumes of distilled water and mixed thoroughly. The pH was adjusted to 7.0 with 0.1 M NaOH. This suspension was frozen at -20°C overnight. The suspension was thawed and centrifuged (20 min, 4°C, 2000 *g*) (Beckman J2-21) with the brake off. The supernatant was harvested and 1 ml 0.4 M phosphate buffer added per 100 ml supernatant. At this stage, the supernatant was turbid but no dextran sulfate was available the lipid removal step was omitted. The yolk solution was stirred and 20 g Na_2SO_4 was slowly added and allowed to stand at room temperature for 30 min. This was re-centrifuged (20 min, 4°C, 2000 *g*) with the brake off. The supernatant was discarded and the sediment was dissolved in 10 ml TBS. This was centrifuged as before and the pellet discarded. The supernatant was stirred and 8 ml 36% (w/v) Na_2SO_4 added and allowed to stand at room temperature for 30 min. The solution was re-centrifuged as before and the supernatant discarded. The pellet was dissolved in 5 ml PBS and dialysed against changes of the same buffer for 72 h. Any precipitate was removed by filtration.

A sample of the final preparation was analysed for purity by reducing SDS-PAGE (Section 2.2).

2.7.3 IgY isolation with propane-2-ol and acetone precipitation

The IgY isolation method proposed by Bade and Stegemann (1984) involves the removal of yolk lipids by the addition of ice-cold organic solvents, propane-2-ol and acetone. This is a rapid IgY isolation procedure as only a few washing steps with organic solvents are required to remove yolk lipids and to precipitate the IgY itself.

2.7.3.1 Materials

Pre-cooled (-20°C) propane-2-ol.

100 ml ice-cold propane-2-ol per yolk was required.

Pre-cooled (-20°C) acetone.

100 ml ice-cold acetone per yolk was required.

10X Phosphate buffered saline (PBS), pH 7.2.

NaCl (80 g), $Na_2HPO_4.2H_2O$ (11.4 g), KCl (2 g) and KH_2PO_4 (2 g) were dissolved in 1 litre distilled water.

2.7.3.2 Procedure

The yolk of 4 eggs were broken, and without the viteline membrane, slowly poured into pre-cooled (-20°C) propane-2-ol (100 ml per yolk) with thorough vortex mixing. The precipitated protein was allowed to settle for 3-5 min before the supernant was decanted. The sediment was washed again with the same volume propane-2-ol, for the complete removal of lipids and the supernatant decanted. This was followed with 3 washes in pre-cooled acetone (100 ml per yolk). The final residue was collected on a Buchner funnel (Whatman No 1 filter paper) washed with a small amount of cold acetone and allowed to air dry. The dry power was extracted with PBS (10 ml per yolk) with stirring for 1 h at room temperature and was centrifuged at 25 000 ×g, 10°C for 15 min. The supernatant was stored at 4°C. Further purification by ion-exchange chromatography was attempted (Section 2.7.2).

2.8 Enzyme-linked immunosorbent assay (ELISA)

This technique is popular as it is easy to execute and is safer than using radio-isotopes. The ELISA uses an enzyme coupled to an antibody that is used to detect antigen or an antigen-antibody complex depending on the type of ELISA. A substrate solution is added to the conjugate and the enzyme converts the substrate into a coloured solution that may be measured spectrophotometrically.

Antigens absorb to the plastic wells of the microtitre plate. The mechanism of adsorption is not well understood. At high protein coating concentrations the protein molecules may to bind to each other instead of to the plate, as the protein binding capacity of the microtitre plates is limited (Johnstone and Turner, 1997).

A non-competitive (indirect) ELISA system was used whereby the microtitre plate was coated with antigen and bound antigen was detected with enzyme-labelled antibody. ELISAs were used in this project to ascertain whether sensitivity levels for dot blots and D-LISAs (dye-linked immunosorbent assay) were comparable to the traditionally used enzyme systems. This

was done by preparing enzyme conjugates with the affinity-purified antibodies used in this study. Two enzyme systems were prepared for comparative purposes, namely horseradish peroxidase (HRP) and the avidin-biotin system.

2.8.1 Horseradish peroxidase conjugation to IgY

2.8.1.1 Materials

0.1 M $NaIO_4$

Sodium periodate (0.021 g) was added to 1 ml reaction solution.

0.001 M Sodium acetate, pH 4.4.

$NaCH_3COO$ (0.082 g) was dissolved in 10 ml distilled water. Acetic acid (57 μl) was added to 10 ml distilled water. 1/3 of the sodium acetate solution was mixed with 2/3 acetic acid solution and diluted 1:100 to give 1 mM.

0.1 M Sodium carbonate buffer, pH 9.5.

Sodium carbonate [Na_2CO_3 (2.12 g)] was dissolved in 100 ml distilled water. Sodium hydrogen carbonate [$NaHCO_3$ (1.68 g)] was dissolved in 100 ml distilled water. Approximately 6.4 ml sodium carbonate solution was added to 18.6 ml sodium hydrogen carbonate solution.

Sodium borohydride (4 mg/ml).

Sodium borohydride (0.004 g) was dissolved in 1 ml distilled water.

0.1 M Sodium borate buffer, pH 7.4.

$Na_2B_4O_7.10H_2O$ (9.54 g) was dissolved in 250 ml distilled water. Boric acid (24.73 g) was dissolved in 4 litres distilled water. Approximately 115 ml borate solution was added to 4 litres boric acid solution until the pH reached 7.4.

2.8.1.2 Procedure

Horseradish peroxidase (4 mg) was dissolved in 1 ml distilled water. To this, 200 μl of the freshly prepared sodium periodate solution was added and gently stirred for 20 min at room temperature. The mixture turned a greenish colour. This was dialysed against sodium acetate buffer at 4°C overnight. Sodium carbonate buffer (20 μl) was added to raise the pH of the reaction mixture to approximately 9 - 9.5. The affinity purified IgY fraction (1 ml) [7.12 mg/ml] was added immediately and allowed to stand at room temperature for 2 h. 100 μl of the freshly prepared sodium borohydride solution was added to the mixture and incubated at 4°C for 2 h. This was finally dialysed extensively against borate buffer and stored in an equal volume of 60% glycerol in borate buffer at 4°C.

2.8.2 Biotinylation of IgY

Avidin is a glycoprotein found in egg white. It has four identical subunits that each have a binding site for biotin (vitamin H). Biotin and avidin bind to each other to produce an extremely stable complex.

N-hydroxysuccinimidobiotin (NHS-Biotin) was the biotinylation compound used in this study to biotinylate IgY according to the method of Wilcheck and Bayer (1990).

2.8.2.1 Materials

N-hydroxysuccinimidobiotin (2 mg/ml) in dimethylformamide.

NHS-biotin (2 mg) was dissolved in 1 ml DMF.

Chicken anti-rabbit albumin in 0.1 M sodium bicarbonate.

Chicken anti-rabbit albumin (2 mg) was suspended in 1 ml of sodium bicarbonate (0.084 g dissolved in 10 ml distilled water).

0.15M NaCl.

Sodium chloride (17.53 g) was dissolved in 2 litres distilled water.

2.8.2.2 Procedure

NHS-biotin solution (25 μl) was added to the antibody solution of 2 mg/ml in 0.1 M sodium bicarbonate. This mixture was allowed to stand at room temperature for an h without stirring. The solution was dialysed against several changes of 0.15 M NaCl at 4°C. and finally against PBS. The biotinylated antibody is stable and can be stored at 4°C.

2.8.3 ELISA protocol for IgY as primary antibody

2.8.3.1 Materials

Phosphate buffered saline (PBS), pH 7.2.
$NaH_2PO_4.2H_2O$ (1.15 g), NaCl (8 g), KH_2PO_4 (0.2 g) and KCl (0.2 g) were made up to 1 litre with distilled water.

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS), pH 7.2.
BSA (0.5 g) was dissolved in 100 ml PBS.

0.1% PBS-Tween, pH 7.2.
Tween 20 (1 ml) was made up to 1 litre in PBS.

0.15 M Citrate-phosphate buffer.
Citric acid (2.1 g) and $Na_2HPO_4.2H_2O$ (3.56 g) were made up to 200 ml with distilled water.

Soluble substrate solution for HRP: [0.05% (m/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer.
ABTS (7.5 mg) and H_2O_2 (7.5 μl) were made up to 15 ml in citrate-phosphate buffer.

2.8.3.2 Procedure

Rabbit albumin was coated onto the microtitre plate (150 μl per well) at various concentrations as indicated in each individual experiment (4°C overnight, or 37°C for 2 h). Non-specific binding of the enzyme-labelled antibody was prevented by the incubation of each well in a 0.5% BSA-PBS blocking solution (200 μl, 1h, 37°C). The plate was washed 4

times by inversion, washing with PBS-Tween and tapping dry on a piece of absorbent paper. Tween assists in blocking any non-specific hydrophobic association of the secondary antibody to the microtitre plate. Enzyme-labelled secondary antibody (Section 2.8.1 and Section 2.8.2) (120 μl) was added at this stage at appropriate dilutions (1:700 for HRP and a 1 μg/ml solution of biotinylated antibody) in PBS-BSA and incubated at 37°C for 1 h. For the biotinylated antibody prepared in Section 2.8.2, this was added to the antigen coated microtitre plate and incubated at 37°C for 1 hr. Another 4×PBS-Tween washing step followed. Enzyme-labelled avidin (horseradish peroxidase) was added to the microtitre plate as the secondary antibody at a dilution of 1:20 000 and incubated at 37°C for 1 hr. Freshly prepared substrate solution was added to the bound, enzyme-labelled secondary antibodies (150 μl) in each well and coloured product was allowed to develop in the dark for approximately 20 min. The A_{405} of each well was measured with a Titertek ELISA plate reader.

2.8.4 Capture enzyme-linked immunosorbent assay (ELISA) on nitrocellulose

Dye sensitivity was compared using a capture dot blot procedure with horseradish peroxidase coupled chicken anti-rabbit albumin.

2.8.4.1 Materials

5% TBSM.

Low fat milk (5 g) was dissolved in 100 ml TBS.

Tris-buffered saline (TBS), [20 mM Tris, 200 mM NaCl, pH 7.6].

Tris (2.42 g) and NaCl (11.69 g) were dissolved in 950 ml distilled water adjusted to pH 7.6 with HCl and made up to 1 litre with distilled water.

0.5% BSA-TBS.

BSA (0.5 g) was dissolved in 100 ml TBS.

Substrate solution [0.06% (m/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2].

4-chloro-1-naphthol (0.03 g) was dissolved in 10 ml methanol. 2 ml of this solution was diluted to 10 ml with TBS and 35% hydrogen peroxide (4 μl) was added.

2.8.4.2 Procedure

Chicken anti-rabbit albumin (CαRA) was spotted in duplicate onto nitrocellulose strips at concentrations similar to the dye capture dot blot experiment (2 μg, 1 μg, 0.5 μg, 0.1 μg, 0.05 μg). The strips were blocked in 5% TBSM solution for 30 min. After rinsing in TBS, the strips were incubated in antigen diluted in TBS at concentrations of 1 μg/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml and 0 ng/ml (TBS) for 1 h. The strips were washed in TBS (3 x 20 min) and incubated in a 1:100 dilution of CαRA-HRP in 0.5% BSA-TBS for 1 h. After the last TBS rinsing step (3 x 20 min), 1 ml substrate solution was added to each nitrocellulose strip and allowed to develop in the dark until sufficient colour development occurred.

2.9 Affinity chromatography of IgY

The principles of affinity chromatography rely on the association of a protein with a specific ligand immobilized on a chromatography matrix (Scopes, 1982). Many examples of protein-ligand interactions exist, namely, enzyme-substrate, hormone-receptor and in this case, antigen-antibody. A protein solution is applied to the affinity matrix and while binding of the antibody will take place to the antigen that is immobilized on the column, non-specific antibodies and other proteins will be washed through the column. Elution of the protein of interest occurs with a change in pH or ionic strength of the buffer.

Sheep IgG and rabbit albumin were the ligands coupled to the affinity matrices respectively. Special affinity columns were purchased from Pierce and are sold under the name of AminoLink® coupling gels. These columns consist of an agarose support and they are specifically activated to yield aldehydes. The aldehydes react with primary amine groups on proteins to form the resultant Schiff's bases. If these bases then undergo reductive amination, a stable covalent linkage is formed with a minimal leak of immobilized protein. According to the manufacturer's instructions, the AminoLink® gel is active over a wide pH range and efficiently couples proteins of diverse molecular weight and isoelectric point. The AminoLink® gel is uncharged which reduces the chance of non-specific binding.

Affinity purified antibodies were used in this project because of their greater specificity to antigen compared to IgY not specific to any antigen. This means that any antibody present in the antibody solution will be specific for its antigen. Eliminating any non-specific IgY present in the antibody solution thus increases sensitivity.

2.9.1 Materials

0.1 M Sodium phosphate buffer, 0.05% sodium azide, pH 7.0.

$NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.5 g) were dissolved in 950 ml distilled water, titrated to pH 7 using NaOH and made up to 1 litre.

1 M Sodium cyanoborohydride in water.

$NaCNBH_3$ (0.625 g) was dissolved in 10 ml distilled water.

1 M Tris-HCl, pH 7.4.

Tris (12.11 g) was dissolved in 100 ml distilled water and titrated to pH 7.4 with HCl.

1 M Sodium chloride (NaCl).

NaCl (5.84 g) was dissolved in 100 ml distilled water.

2.9.2 Preparation of affinity column matrix

The protein of interest (sheep IgG or rabbit albumin) was dissolved in the 0.1 M sodium phosphate (Na_3PO_4) at concentrations of 1-20 mg/ml.

The gel is supplied as a 50% slurry in 0.05% sodium azide (NaN_3) and an aliquot of preferred amount was placed into a sintered chromatography column. The liquid was sucked off the gel until only a wet cake of gel remained. The gel was washed three times with an equal volume of sodium phosphate buffer, and the gel cake transferred to an appropriate sized affinity tube (Bio Rad, Hercules, CA).

An equivalent volume of the protein to be immobilized was added to the gel cake. A fume hood was used to add 0.05 ml of the $NaCNBH_3$ per milliliter of the slurry. The tube was sealed and mixed by gentle end-over-end rotation for 2 h at room temperature and incubated for another 4 h at room temperature.

A sintered chromatography column was used to drain off and wash the gel cake twice with one volume of Tris-buffer. Another 0.05 ml of $NaCNBH_3$ per millilitre of slurry was added in the fume hood, and mixed for 30 min at room temperature.

The gel was drained and washed with 10 bed volumes of NaCl, 10 bed volumes of NaN_3 and refrigerated in sodium phosphate buffer.

2.9.2.1 Materials

100 mM Na_3PO_4 buffer, pH 7.6; 0.02% NaN_3.

$NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) was dissolved in 950 ml distilled water; titrated to pH 7.6 using NaOH and made up to 1 litre.

Elution buffer: 0.1 M glycine-HCl, pH 2.8.

Glycine (0.75 g) was dissolved in 80 ml distilled water; titrated to pH 2.8 using HCl and made up to 100 ml.

1 M Sodium phosphate buffer, pH 8.5.

$NaH_2PO_4.H_2O$ (13.79 g) was dissolved in 80 ml distilled water, titrated to pH 8.5 using NaOH and made up to 100 ml.

2.9.2.2 Procedure

The column was equilibrated to room temperature, connected to a BioRad Econo-pump and washed with 10 bed volumes of phosphate buffer. The IgY solution was filtered through Whatman No. 1 filter paper to remove any insoluble particles, and cycled through the column overnight, at a flow rate of 0.56 ml/min. The whole volume of the IgY sample was passed through the column twice, to ensure all possible binding to the column matrix. A washing step of 10 bed volumes was used to remove any unbound IgY. A set of eppendorfs containing 100 µl of 1 M sodium phosphate buffer, pH 8.5, was arranged and the column eluted with glycine-HCl buffer, pH 2,8. 900 µl fractions were collected and A_{280} readings taken immediately to monitor the protein peak elution.

2.10 Isolation of IgY by ion exchange chromatography

Separation of protein molecules during ion exchange chromatography depends on the reversible adsorption of charged particles to an ion exchange group, of the opposite charge, immobilized onto a chromatographic support. The ion exchange matrix consists of an insoluble porous matrix to which charged groups are covalently bound. The charged groups are associated with mobile counter ions and these counter ions can be reversibly exchanged with other ions of the same charge. The ion exchanger is equilibrated in terms of ionic strength and pH. Upon application of the sample, the desired protein with the appropriate charge diffuses to the exchange sites on the matrix, displaces the counter ions and binds reversibly to the matrix. Unbound proteins and other molecules are washed from the column with the sample buffer. The protein of interest can now be eluted from the column by changing the conditions, with either a pH change or a salt gradient. The increasing ionic strength of the elution buffer releases protein molecules from the column in the order of their binding strengths. The column is washed once again to remove any substances not eluted in the last step and to re-equilibrate for the next purification.

2.10.1 Materials

Pharmacia Sephacel (DEAE cellulose) ion exchange matrix, wet bead diameter 40-160 μm.
The column matrix is supplied as a slurry.

Low salt buffer: 0.015 M Potassium phosphate buffer, pH 8.0.
KH_2PO_4 (2.04 g) was dissolved in 800 ml distilled water, adjusted to pH 8.0 with KOH and made up to 1 litre.

High salt buffer: 0.3 M Potassium phosphate buffer, pH 8.0.
KH_2PO_4, (12.24 g) was dissolved in 250 ml distilled water, adjusted to pH 8.0 with KOH and made up to 300 ml.

1 M NaOH.
Sodium hydroxide (2 g) was dissolved in 50 ml distilled water.

2.10.2 Procedure

A column (1.6cm×9cm) was packed with SEPHACEL and washed with one column volume of 1 M sodium hydroxide to rid the column of any residual proteins. It was rinsed with 10 column volumes of distilled water and equilibrated with 10 column volumes of 0.015 M potassium phosphate buffer, pH 8.0. A linear flow rate of 0.3 ml/min was used throughout. The sample (IgY) was dialysed overnight against 3 changes of low salt buffer and loaded onto the column. The column was washed with low salt buffer until A_{280} measurements showed that all unbound sample had eluted. Bound proteins were eluted with a linear gradient of 0.015-0.3M potassium phosphate buffer in 3 column volumes. Fractions of 2.3 ml were collected on a fraction collector and A_{280} values monitored for the IgY elution profile. The column was once again washed with low salt buffer until A_{280} readings returned to zero.

A sample of the eluted fraction was analysed by reducing SDS-PAGE gel (Section 2.2) for analysis of purity.

2.11 Concentration of proteins by PEG 20 000

Samples were concentrated (when necessary) by dialysis against solid PEG 20 000. This polymer is too large to enter the pores of the dialysis tubing and as a result, draws solution out of the tubing by osmosis, thus reducing the volume and concentrating the protein solution.

2.12 Dialysis of dye samples

During the dye-antibody conjugation procedures, the removal of unbound reagents and unwanted by-products was achieved with dialysis. The activated dye samples were placed into dialysis tubing against distilled water to remove unbound glutaraldehyde. Section 2.12 will describe how unbound glutaraldehyde was detected in the dialysis solution. When unbound dye and sodium cyanoborohydride needed to be removed, the dye-antibody solution was placed in dialysis against PBS (the dye solute).

2.12.1 Spot test for the presence of aldehydes

The 2-step glutaraldehyde conjugation method consists of an activation step of the dye with glutaraldehyde. Unbound glutaraldehyde needs to be removed by dialysis to prevent the formation of high molecular weight polymers as a result of uncontrolled cross-linking. The presence of unbound glutaraldehyde was detected in the solution outside the dialysis tubing. The principle is that triphenylmethane dyes are decoloured by sulphurous acid or other similar derivatives (Feigl, 1946). The colour is restored by the addition of aldehydes, as the dye is converted from a triphenylmethane dye to a quinoid dye. Done as a spot test on filter paper, the development of a green spot indicates the presence of glutaraldehyde.

2.12.2 Procedure

Malachite green (0.4 g) was suspended in a little water (100 ml) and brought into solution by adding sodium sulphite (1.5 g) and warming the solution slightly. Further sodium sulphite (1 g) was added and the solution was filtered through Whatman No.1 filter paper. The filter paper was soaked in the cooled yellowish liquid and dried in the air. Spots of sample solution were applied to the dry filter paper and observed for a green colour showing positive presence of glutaraldehyde.

2.13 Glutaraldehyde conjugation of dye to antibody

The 1-step glutaraldehyde coupling reaction involves the addition of all the reaction solutions together, a reduction step, and dialysis to remove unbound dye. The effects of different ratios of dye to antibody were investigated, starting at a molar mass ratio of 40:1 (forty dye molecules to every one antibody molecule) as suggested by Briand *et al.*, (1985), which is the ratio they used to raise anti-peptide antibodies. The 2-step coupling protocol has the dye "activated" with a final glutaraldehyde concentration of 1.25% and allowed to incubate overnight. Excess or unbound glutaraldehyde was dialyzed out before the other reagents were added.

2.13.1 Materials

10X Phosphate buffered saline (PBS), pH 7.2.

NaCl (80 g), $Na_2HPO_4.2H_2O$ (11.4 g), KCl (2 g) and KH_2PO_4 (2 g) were dissolved in 1 litre distilled water.

Reactive black 5 solution.

Reactive black 5 (2 mg) was dissolved in 1 ml PBS.

25% Glutaraldehyde.

Obtained from Sigma-Aldrich as a 25% (m/v) solution.

1.25% Glutaraldehyde.

Obtained from Sigma-Aldrich as a 25% (m/v) solution and used at a total concentration of 1.25%.

2.13.2 Procedure

1-step coupling protocol

The dye, antibody and glutaraldehyde were incubated together as indicated in table 2.2 with PBS buffer added to make up equal volumes. The dye concentration was 2 mg/ml and the final antibody concentration was 2 mg/ml. These reagents were incubated at 4°C for 2 h.

Sodium cyanoborohydride was added at a concentration of 10 mg/ml (0.01 g per 1000 µl), the solution incubated for 1 h at 4°C and dialyzed against PBS overnight at 4°C to remove unbound dye.

Table 2.2: Volume of reagents for the one-step glutaraldehyde conjugation with dye.

Ratio	Volume of dye (μl)	Volume of glutaraldehyde (μl)	Antibody concentration (mg)	Volume PBS buffer (μl)	Total volume (μl)
40:1	200	10	2	590	1000
80:1	400	10	2	390	1000
160:1	800	10	2	-	1002

2-step coupling protocol

1.25% Glutaraldehyde (50 μl) and dye (200 μl) were incubated overnight in eppendorf tubes at room temperature. This allowed for glutaraldehyde "activation" of the dye for conjugation to antibody. The solution was dialysed to remove any unbound or excess glutaraldehyde. The presence of unbound glutaraldehde was detected outside the dialysis tubing by means of the aldehyde spot test mentioned in Section 2.12.1. The other reagents were then added in a similar manner to that described for the 1-step reaction. The antibody was added, allowed to incubate for 2 hrs at 4°C, followed by a reduction step and dialysis, this time to remove unreacted sodium cyanoborohydride and any unbound dye.

2.14 Conjugation of dye to carbohydrate moieties on antibody

Antibodies are glycoproteins as they have carbohydrate moieties on their heavy chains. These carbohydrate moieties are potential covalent cross-linking sites. Periodate oxidation results in the cleavage of a carbon-carbon bond in the hexose ring structure of the sugar residue to yield reactive aldehydes (Hermanson, 1996). Complementary amine groups may be reacted with these reactive aldehyde groups to form Schiff bases. Reductive amination of the Schiff base yields stable secondary amines. In this study, the carbohydrate groups of the antibody were oxidized and the complementary amine group was supplied by the dye.

2.14.1 Materials

0.1 M sodium periodate.

Sodium periodate (0.002 g) was dissolved in 100 µl distilled water and protected from light in a foil covered test tube.

1 M NaOH.

NaOH (0.4 g) was dissolved in 10 ml distilled water.

5 M cyanoborohydride in 1 M NaOH.

Sodium cyanoborohydride (0.015 g) was dissolved in 50 µl 1 M NaOH.

1 M ethanolamine buffer, pH 9.6.

300 µl ethanolamine was made up to 5 ml with distilled water and adjusted to pH 9.6 with concentrated HCl while being kept cool on ice.

2.14.2 Procedure

The antibody was diluted in PBS to a concentration of 2 mg/ml and added to a tin-foil covered test tube (the next step is light sensitive). Sodium periodate was dissolved in water to a final concentration of 0.1 M, and this was also protected from light. 100 µl sodium periodate solution was immediately added per millilitre of the antibody solution, this was mixed thoroughly and reacted in the dark for 30 min at room temperature. The oxidation reaction was quenched by the addition of 0.1 ml glycerol per millilitre of the reaction solution. The oxidized antibody was used immediately. Volumes of dye were added to the reaction, according to which ratios were being investigated. This was reacted at room temperature for 2 h. Sodium cyanoborohydride was added (10 µl of 5 M sodium cyanoborohydride prepared in 1 M NaOH per millilitre of reaction solution) to reduce the resultant Schiff bases, and reacted for 30 min at room temperature. The unreacted aldehyde sites were blocked by the addition of 50 µl of ethanolamine buffer per millilitre of the reaction solution and reacted for 30 min at room temperature. The conjugate was purified from excess reactants by dialysis against PBS for 16 h at 4°C. Table 2.4 shows the typical manner in which reagents were pipetted for this reaction.

Table 2.3: Volume of reagents for the conjugation of dye to carbohydrate entities on antibodies.

Ratio of dye to ab	Ab (mg)	$NaIO_4$ (µl)	Glycerol (µl)	Dye (µl)	PBS (µl)	$NaCNBH_3$ (µl)	Ethanol-amine (µl)
40:1	2	5.5	6	100	834	10	50.5
80:1	2	5.5	6	200	734	10	50.5
160:1	2	5.5	6	400	534	10	50.5

2.14.3 Phenol-sulfuric acid colour reaction to detect glycoproteins and glycopeptides on nitrocellulose

Wan and van Huystee (1993) describe a means whereby the sugar content of glycoproteins can be determined by the phenol-sulfuric acid colour reaction. The glycoproteins are reacted with a phenol solution and conc. sulfuric acid is added to yield a pinkish solution that is measured spectrophotometrically at 490 nm.

2.14.3.1 Materials

5% Phenol.

Phenol (5 g) was dissolved in 100 ml distilled water. This solution is toxic.

2.14.3.2 Procedure

A standard curve was set up using D-mannose was used as the standard. 250 µl of the sample antibody solution was mixed with 300 µl of the phenol solution. Concentrated sulfuric acid (2 ml) was rapidly added to this mixture in a fume hood. The absorbance of the solution in each test tube was measured at 490 nm after cooling 30 min. The samples investigated were 500 µg each of non-immune IgY, chicken anti-rabbit albumin and sheep IgG.

2.15 Dot blot protocol for antigen detection

Dot blots were used to test the efficacy of dye-antibody binding and its corresponding sensitivity levels.

2.15.1 Materials

Nitrobind nitrocellulose.

Nitrocellulose sheet was divided into squares of 0.5 × 0.5 cm, one for each sample spot.

5% PBSM.

Low fat milk powder (5 g) was dissolved in PBS (100 ml).

Dye-antibody solution.

Prepared by the 2-step reaction as previously described.

2.15.2 Procedure

Gloves were worn when the nitrocellulose was handled to avoid binding of contaminating proteins. The nitrocellulose was cut into rectangular pieces to allow duplicate sample application (square blocks of size 0.5 × 0.5 cm). Antigen solution (1 μl) was spotted onto nitrocellulose in a characteristic dilution series (2 μg; 1 μg; 0.5 μg; 0.1 μg; 0.05 μg; 0.01 μg) and allowed to dry and the nitrocellulose strips were then probed with the dye-antibody solution until visible spots appeared on the nitrocellulose.

A 30 min blocking step in 5% PBSM ensured that all available binding sites were blocked. Washing steps involved rinsing the nitrocellulose strips in distilled water (3 x 10 min). The nitrocellulose strips were each incubated in 1 ml of the dye-antibody solution for 15-30 min until coloured spots were observed. The strips were allowed to air dry, and the results immediately recorded as colour intensity "plus" values.

2.16 Comparative dot blots

The sensitivity levels of antigen detection was determined for the dye conjugated affinity antibodies. The two different dyes, Reactive black 5 and primulin, were both glutaraldehyde

conjugated as well as carbohydrate-conjugated to IgY. Traditional enzyme-substrate antigen detection systems were also used as a control. A further comparison was made by using an anti-chicken secondary antibody to detect antigen and was added to the dye-antibody detection systems to investigate a possible enhancement of the dye signal.

2.16.1 Method

Antigen (rabbit albumin) was spotted onto nitrocellulose as described (Section 2.15). Duplicate spots of the following concentrations of rabbit albumin were applied to the nitrocellulose: 2 μg, 1 μg, 0.5 μg, 0.1 μg, 0.05 μg, 0.01 μg and 0.005 μg.

2.16.1.1 Chicken anti-rabbit albumin-horseradish peroxidase (CαRA-HRP).

The available binding sites on the nitrocellulose strips were blocked with 5% milk-TBS for 1 h and rinsed 3 times with TBS. The CαRA-HRP was diluted 1:100, 1:1000 and 1:10 000 in 0.5% BSA-TBS and the strips were incubated in primary antibody for 1 h and then rinsed 3 times in TBS. The strips were developed in HRP substrate (10 mins in the dark) made up as follows: 0.03 g of 4-chloro-1-naphtol was dissolved in 10 ml methanol. 2 ml of that solution was diluted to 10 ml in TBS and 4 μl of hydrogen peroxide (H_2O_2) was added.

2.16.1.2 Biotinylated chicken anti-rabbit albumin.

The available binding sites on the nitrocellulose strips were blocked with 5% milk-TBS for 1 h and rinsed 3 times with TBS. The biotinylated chicken anti-rabbit albumin was diluted 1:100, 1:1000 and 1:10 000 in 0.5% BSA-TBS and the strips were incubated in primary antibody for 1 h and rinsed 3 times in TBS. The avidin-HRP secondary antibody was diluted to the required dilution by the manufacturer in 0.5% BSA-TBS and the strips were incubated in this for 1 h and rinsed 3 times in TBS. HRP substrate was added as above.

2.16.1.3 Anti-chicken-horseradish peroxidase secondary antibody.

The available binding sites on the nitrocellulose strips were blocked with 5% milk-TBS for 1 h and rinsed 3 times with TBS. The primary antibody, affinity purified chicken anti-rabbit albumin was diluted 1:100, 1:1000 and 1:10 000 in 0.5% BSA-TBS and the strips were incubated in this for an hour and rinsed 3 times with TBS. The anti-chicken secondary

antibody was diluted to the required dilution by the manufacturer in 0.5% BSA-TBS and the strips were incubated in this for 1 h and rinsed 3 times in TBS. HRP substrate was added as above.

2.16.1.4 Reactive black 5.

This dye was conjugated to affinity purified chicken anti-rabbit albumin by means of the 2-step glutaraldehyde protocol as well as the carbohydrate coupling method. The available binding sites on the nitrocellulose strips were blocked with 5% milk-TBS for 1 h and rinsed 3 times with PBS. The strips were incubated in the respective conjugated dye-antibody solutions at antibody concentrations of 2 mg/ml, 1 mg/ml and 0.5 mg/ml. An anti–chicken secondary antibody was also added to a strip of nitrocellulose that had been incubated in glutaraldehyde-conjugated RB5 at a primary antibody concentration of 2 mg/ml.

2.16.1.5 Primulin.

This dye was conjugated to affinity purified chicken anti-rabbit albumin by means of the 2-step glutaraldehyde protocol as well as the carbohydrate coupling method. The available binding sites on the nitrocellulose strips were blocked with 5% milk-TBS for 1 h and rinsed 3 times with PBS. The strips were incubated in the respective conjugated dye-antibody solutions at antibody concentrations of 2 mg/ml, 1 mg/ml and 0.5 mg/ml. An anti–chicken secondary antibody was also added to a strip of nitrocellulose that had been incubated in glutaraldehyde-conjugated primulin at a primary antibody concentration of 2 mg/ml. The results of the primulin dot blots were viewed in a dark box under ultraviolet (UV) light.

2.17 Spectrophotometric quantitation of protein-dye complexes on nitrocellulose

It was necessary to assign a numerical value to quantitate how much dye a "dark" dye spot and a "light" dye spot contained. The antibody-dye complex was spotted onto nitrocellulose and the nitrocellulose was solubilized in DMSO. The spectrophotometric measurement at the maximum absorbance of the dye was recorded. Proteins were also transferred to a nitrocellulose membrane from a SDS-PAGE gel and stained with the dye-antibody solution. This procedure is outlined by Goldring and Ravaioli (1996).

2.17.1 Materials

Standard.

Non-immune IgY-dye (Reactive black 5) was serially diluted on nitrocellulose which was then solubilised in DMSO.

Blank.

BSA serially diluted and spotted onto nitrocellulose, exposed to the dye-antibody solution (chicken anti-sheep IgG) and then solubilised in DMSO.

Sample.

Sheep IgG serially diluted and spotted onto nitrocellulose was exposed to the dye-antibody solution (chicken anti-sheep IgG) and solubilised in DMSO.

2.17.2 Procedure

The standard was spotted onto nitrocellulose in a serial dilution, and the nitrocellulose segments placed into DMSO. The spectrophotometer was blanked against each BSA-blank in DMSO, relative to the antigen concentration spotted onto the nitrocellulose and placed in DMSO, and the absorbance of each sample was read at 597 nm.

2.18 Colloidal dyes

Colloidal dyes are also known as disperse dyes because they are made up of particles of different sizes. They are defined in the Colour Index as being a class of substantially water-insoluble dyes originally utilized to dye cellulose acetate. Acetate is a hydrophobic fibre with a largely electronegative surface. When submerged in water, acetate is not responsive to direct dyes (Kulkarni *et al.*, 1986). Disperse dyes are usually applied from fine aqueous suspensions and are widely used to colour all hydrophobic synthetic fibres (Gribnau *et al.*, 1982).

The novelty of using this particular class of dyes for development of dye-diagnostic assays is that they could replace the expensive colloidal gold particles used as a label in immunoassays.

2.18.1 Preparation of dianix blue FBLN-SE 300 and dianix rubine (red) HBSL-FS for use in D-LISAs and dot blots.

2.18.1.1 Materials

Low-salt buffer: (10 mM sodium phosphate buffer, 2.7 mM NaCl).

NaH_2PO_4 (0.3 g) and NaCl (0.039 g) were dissolved in 200 ml distilled water, adjusted to pH 7.4 with NaOH and made up to 250 ml with distilled water

Centrifugation resuspension solution: (33.3 mM NaH_2PO_4, 0.125 mM NaCl, 5% BSA).

NaH_2PO_4 (0.2 g), NaCl (0.37 g) and BSA (2.5 g) were dissolved and made up to 50 ml in distilled water.

5 mM NaCl, pH 7.4.

NaCl (0.073 g) was dissolved in 250 ml distilled water and the pH was adjusted to 7.4 with NaOH.

30% BSA.

BSA (6 g) was dissolved and made up to 20 ml in 5 mM NaCl, pH 7.4.

2% (m/v) PBSM

Low fat milk powder (2 g) was dissolved in 100 ml phosphate buffered saline

2.18.1.2 Procedure

The dianix blue and dianix red dye solutions were prepared according to the method of Gribnau *et al.* (1982). A 5% (m/v) solution of the dye was dissolved in distilled water in a total volume of x ml. This suspension was centrifuged (100 ×g, 30 min, room temperature). The pellet was discarded and the supernatant centrifuged (10 000 ×g, 30 min, room temperature). The supernatant was discarded and the pellet washed twice and centrifuged (10 000 ×g, 30 min, room temperature) each time. The final pellet was resuspended in ½ x ml distilled water.

A small volume of dianix blue and dianix red dyes were dissolved in ethanol and the peak λ determined as 605 nm for dianix blue and 510 nm for dianix red. by conducting an absorption spectrum from 200-800 nm on the Cary spectrophotometer. The prepared dye was diluted with water to determine the concentration of dye that gave a absorbance value of 1.0 (l = 1 cm). The actual concentration of dye used in dye-antibody reagent was taken as a multiple of 10 of that concentration. An affinity antibody (chicken anti-rabbit albumin) concentration of 10 μg/ml was used in the dye-antibody samples. The low-salt PBS buffer (V volume) was incubated with appropriate volumes of dye and antibody for 1 h to allow for antibody absorption to dye particles. This was spiked with $V/5$ volume of 30% BSA and incubated for 1 h. The dye-antibody mixture was centrifuged (12 000 × g, 20 min, room temperature) and the pellet was resuspended in the centrifugation resuspension solution in a volume to obtain A λmax = 10.

2.18.2 Nitrocellulose dipstick preparation

Numerous pieces of nitrocellulose membrane (1 cm × 2.5 cm) were prepared to accomodate five dots of different antibody concentrations in duplicate. 1 μl samples of affinity purified chicken anti-rabbit albumin antibody was spotted onto the nitrocellulose at concentrations of 2 μg, 1 μg, 0.5 μg, 0.1 μg and 0.05 μg. The nitrocellulose strips were incubated in PBSM (2% milk solution) for 30 min, to block all available binding sites on the nitrocellulose, and then briefly rinsed with distilled water. The strips were incubated in antigen containing PBS solutions (rabbit albumin in PBS) with antigen concentrations of 1 μg/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml (and a control experiment with no antigen containing only PBS) for 1 h to allow for antigen adsorption to the capture antibody. Following another brief rinse in distilled water, the strips were each incubated in 1 ml of the dye-antibody solution for 10-15 min until the coloured spots were sufficiently visible. The strips were rinsed in distilled water and the colour intensity of the developed spots recorded.

2.18.3 Dye-linked immunosorbent assay (D-LISA)

Gribnau *et al.*, (1982) described a method whereby dianix dye-antibody preparations could be used as a dye-sol label in a microtitre plate sandwich assay. The detection limits of capture antibody in their study were between 1 ng/ml and 3 ng/ml for various antibodies. For the

purposes of this study, the dye-sol label was applied in microtitre plates, and called the D-LISA, for the direct detection of antigen.

2.18.3.1 Materials

Dianix blue and dianix red dye-antibody solutions.

Prepared as described previously (Section 2.18.1)

Antigen solution.

Different concentrations of antigen (rabbit albumin) were made up in PBS.

0.5% BSA-PBS.

BSA (0.5 g) was dissolved in 100 ml PBS.

PBS-Tween 20 [0.1% (v/v)].

Tween 20 (1 ml) was added to 1 litre PBS.

2.18.3.2 Procedure

The wells of a microtitre plate were coated (in duplicate) with rabbit albumin diluted in PBS (150 μl) at concentrations 20 μg/ml, 10 μg/ml, 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.6 μg/ml, 0.3 μg/ml and 0.15 μg/ml and allowed to incubate at 4°C overnight to allow for adsorption to the plastic. The plate was inverted and tapped dry on a piece of blotting paper and 200 μl 0.5% BSA-PBS solution added to each well, to block any available binding, sites at 37°C for 1 h. The plate was washed three times with PBS-Tween and 150 μl of the dye-antibody solutions were added to each well with affinity purified CαRA concentrations of 10 μg/ml, 5 μg/ml and 1 μg/ml respectively (separately for both dyes) for 16 h at 37°C. The plate was again inverted, tapped dry on blotting paper and rinsed six times with PBS-Tween. 96% Ethanol (50 μl) was added to each well to solubilise the dye solution on the microtitre plate. The plate with dianix blue was also scanned on a flat bed scanner to indicate the visual sensitivities obtained (Figure 4.1). Dianix red was not as sensitive as the dianix blue, so further studies were conducted with the dianix blue.

in the staining process (Wilson and Walker, 1996). This step also allows the dye to complex to the protein (Voet and Voet, 1995).

Is Coomassie brilliant blue the only dye that can be used to stain proteins post-electrophoretically? Considering the manner in which Coomassie stains proteins, the aim of these experiments was to determine whether the dyes used in this study could not only covalently couple to IgY, but if they could also be utilized to stain proteins.

2.20.1 Materials

Dye stock solutions [1% (m/v)].

The dyes Reactive black 5, trypan blue, acid black 2, (1 g) were respectively dissolved in 100 ml distilled water.

Dye staining solutions [0.125% (m/v) dye, 50% (v/v) methanol, 10% (v/v) acetic acid].

Dye stock solutions (12.5 ml) were mixed with methanol (50 ml) and acetic acid (10 ml) and made up to 100 ml with distilled water, respectively.

Destaining solution I and II.

See Coomassie destaining solutions I and II (Section 2.3)

2.20.2 Procedure

Four 12.5% SDS-PAGE gels (Section 2.2) were loaded with identical decreasing concentrations of IgY. They were electrophoresed at 18 mA each and three gels were stained with solutions of Reactive black 5, trypan blue and acid black 2 for 4 h with constant agitation respectively. One gel was stained with Coomassie brilliant blue as a control.

CHAPTER 3: DYES AND COVALENT INTERACTIONS WITH ANTIBODIES

3.1 Dyes used for covalent interactions

The two dyes used for covalent interactions with antibodies were Reactive black 5 and primulin.

3.1.1 Reactive black 5

Reactive black 5 was the first dye to be investigated in this study. It is a reactive dye with a dark blue/black colour and offers good contrast against a white nitrocellulose background

The dot blots that record the results of this study, although visible, were faint. More colour is seen by eye than some instruments are able to detect. Capturing the sensitivities and reproducing them for the purpose of this document by means of a flat-top scanner or colour-photostatting proved fruitless. Black and white photostats were able to capture similar intensities (to the actual coloured nitrocellulose dot blots) and hence Figure 3.6 is a photostat. Capturing the fluorescence intensities of the primulin dot blots was done by means of colour photographs. The results are mostly presented as a value scale (3+, 2+, 1+, +) whereby the value intensity can be compared to a template, which is included below in Figure 3.1.

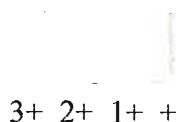


Figure 3.1: Reactive black 5 (RB5) dot dye template showing the visual intensity of spots as they appear in dot blots.

Dots were placed in duplicate and values were arbitrarily assigned according to the intensity of visualised colour, 3+, 2+, 1+, and + in the case of very faint samples.

3.1.2 Primulin

This dye initially attracted attention as it has the ability to undergo a colour change. The light yellow colour of the dye can be altered to a deeper orange or even purple by the addition of

specific chemicals in the diazotization reaction. It fluoresces under ultra-violet light. This characteristic was used to detect glycospingolipids on a TLC plate (Lowenstein, 1975). In the present study, primulin was coupled to affinity purified IgY by means of the glutaraldehyde conjugation method as well as the carbohydrate conjugation method.

3.2 Glutaraldehyde conjugation of dye to antibody

Glutaraldehyde was used as a cross-linking reagent between the dye molecules and the antibodies. Two different ways of utilising the cross-linking properties of glutaraldehyde were investigated in the 1-step coupling (2.11.1) and 2-step coupling (2.11.2) methods. A suitable reducing agent also had to be determined for the reaction.

3.2.1 Influence of different reducing agents on Reactive black 5

Different reducing agents, namely 2-mercaptoethanol (100 µl/ml), dithriothreitol (10 mg/ml m/v) sodium borohydride (10 mg/ml) and sodium cyanoborohydride (10 mg/ml) were added to a solution of Reactive black 5. This dye solution contained a similar concentration of dye as in the 1-step and 2-step coupling methods. The different reducing agents were added in order to establish the effects of the different reducing compounds on the maximum absorption wavelength of the dye, and to draw a comparison between them. The overall change in absorbance was measured over the range of 200-800 nm on the Cary spectrophotometer(Figure 3.2).

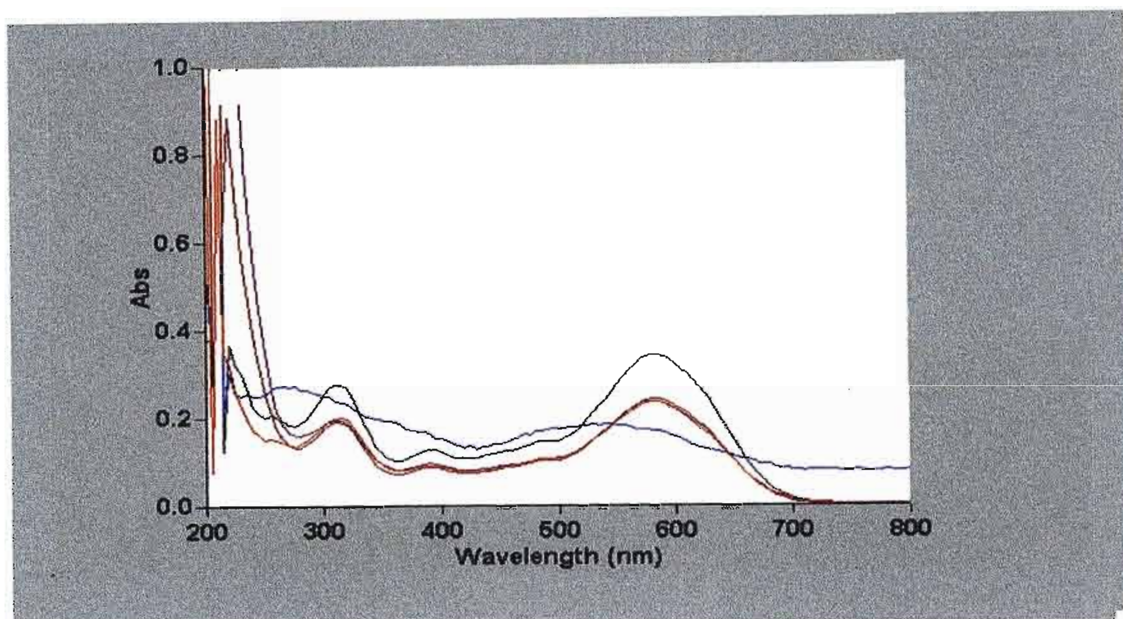


Figure 3.2: The effect of different reducing compounds on the peak absorption wavelength of Reactive black 5:

dye alone, ▬ ; dye + sodium borohydride, ▬ ; dye + dithriothreitol, ▬ ;
dye + β-mercaptoethanol, ▬ ; dye + sodium cyanoborohydride ▬ .

3.2.1.1 Result and discussion

The antibody conjugation methods described by Hermanson (1996) use mostly sodium borohydride as the reduction reagent. The blue trace line (dye plus sodium borohydride) in Figure 3.2 shows an overall reduction in absorbance value and there is no λ max value at 597 nm as compared to the black trace line (dye alone). Adding sodium borohydride to Reactive black 5 yields a vigorous reduction reaction. The solution effervesces (even when kept at 4°C) and Reactive black 5 changes from a dark/blue black colour to a light pink colour. Hermanson (1996) also suggests the use of sodium cyanoborohydride as a reducing agent. Peng *et al.,* (1986) reported that the use of sodium cyanoborohydride was at least five times milder toward immunological activity than sodium borohydride. The pink, brown and red traces on Figure 3.2 (dye plus dithriotheitol, dye plus β-mercaptoethanol and dye plus sodium cyanoborohydride) all show similar absorbances from 400-700 nm. These are most similar to the trace of dye alone. It was decided thus to use sodium cyanoborohydride as the reducing agent for the remainder of the reactions.

3.2.2 1-step coupling of Reactive black 5 to IgY

Due to the fact that Reactive black 5 is darker in colour than primulin and is therefore easier to see when the solution appears darker (i.e. more dye) or lighter (i.e. less dye) the following experiments were conducted with Reactive black 5.

The reaction product, after the 1-step coupling method, was observed by the overall change in absorbance of Reactive black 5 by scanning the completed reaction solution over a range of 200-800 nm on the Varian Cary 50-Bio UV-visible Spectrophotometer (Figure 3.3).

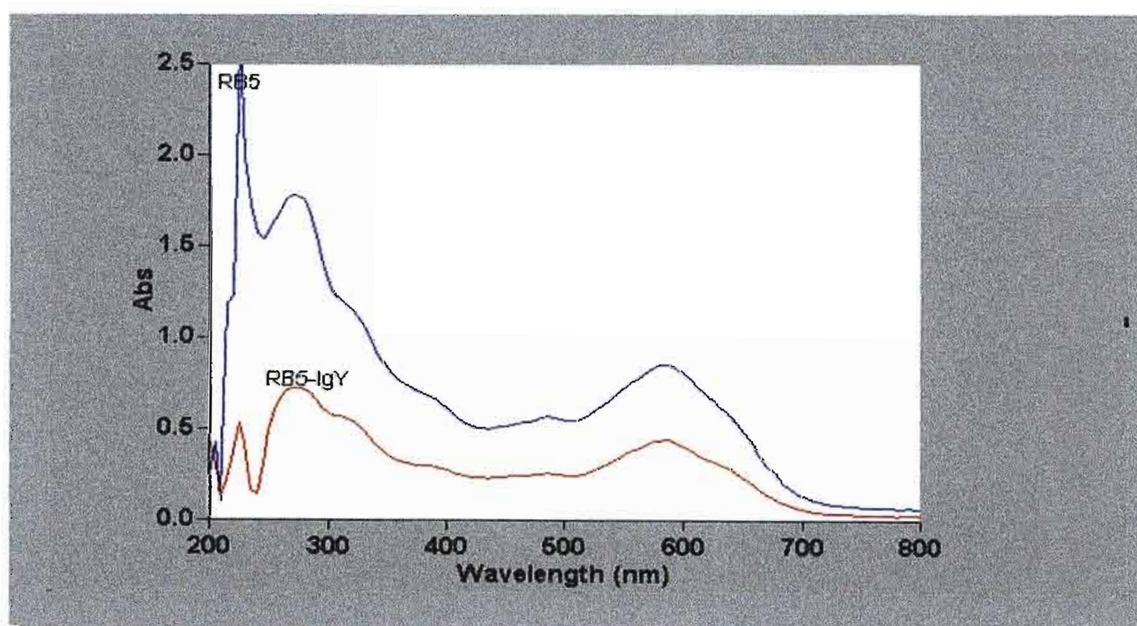


Figure 3.3: Spectrophotometric scan of Reactive black 5 after 1-step glutaraldehyde conjugation.

Reactive black 5 (RB5) ▬ and the dye-antibody (RB5-IgY) complex ▬ from 200-800 nm after 1-step glutaraldehyde conjugation.

3.2.2.1 Result and Discussion

The blue trace on Figure 3.3 indicates the overall change in absorbance for the dye, Reactive black 5, alone. The concentration of dye used was similar to that used in the dye-antibody coupling method and shows an absorption maximum at 597 nm with a maximum absorbance value of 0.85. The red trace or the completed 1-step reaction solution (RB5-IgY) shows an absorption maximum at 597 nm and maximum absorbance value of 0.5. It would appear therefore that the 1-step method allowed a lot of the dye to dialyse out of the reaction

solution. It thus appears that conjugation was not as successful as desired as a significant amount of dye did not conjugate to the antibody. Hermanson (1996) suggested that the 1-step reaction produces high molecular weight complexes as a result of uncontrollable cross-linking when all the reagents are added together. These do precipitate out of solution once left to stand.

3.2.3 2-step coupling of Reactive black 5 to IgY

The reaction product after the 2-step coupling method, was observed by the change in absorbance of Reactive black 5 by scanning the completed reaction solution over a range of 200-800 nm on the Varian Cary 50-Bio UV-visible Spectrophotometer. Refer to Figure 3.4.

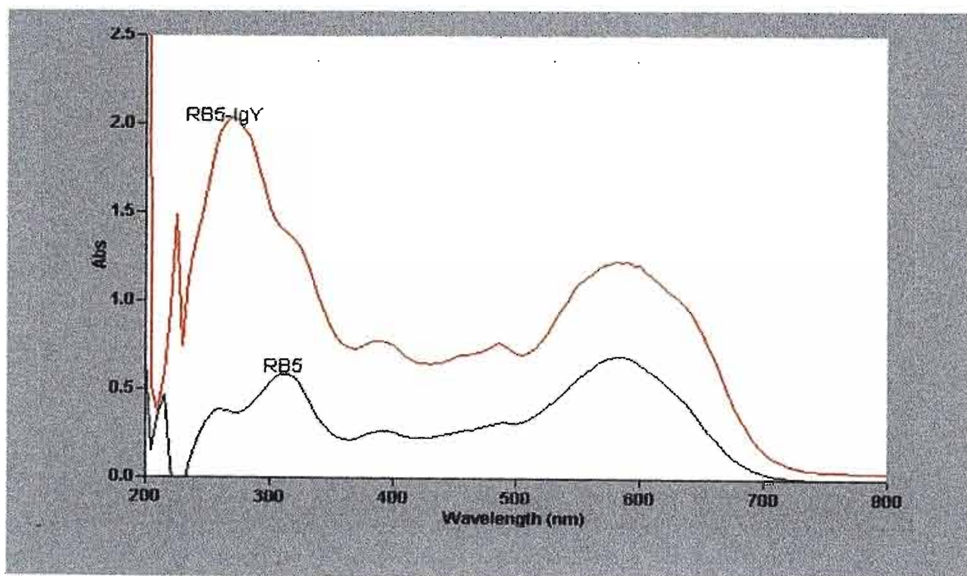


Figure 3.4: Spectrophotometric scan of Reactive black 5 after 2-step glutaraldehyde conjugation.

Reactive black 5 (RB5) ▬ and the dye-antibody (RB5-IgY) ▬ complex after 2-step glutaraldehyde conjugation.

3.2.3.1 Result and discussion

The black trace (Figure 3.4) indicates the overall change in absorbance for the dye, Reactive black 5, alone. The concentration of dye used was similar to that used in the dye-antibody coupling method and shows an absorption maximum at 597 nm with a maximum absorbance value of 0.7. The red trace, for the completed 2-step reaction solution (RB5-IgY), shows an

absorption maximum at 597 nm and maximum absorbance value of 1.2. The completed 2-step glutaraldehyde reaction therefore has a higher maximum absorbance value compared to just dye alone (1.2 vs 0.7). This may suggest that a significant amount of antibody was conjugated to the glutaraldehyde activated dye. As mentioned in Section 2.12, after activation of dye with glutaraldehyde, dialysis was used to remove any unbound glutaraldehyde. The red trace (Figure 3.4) indicates the presence of antibody in the solution at 280 nm by a peak. Hermanson (1996) recommends the 2-step method as it allows for more control over the linking reaction and the high molecular weight complexes that were found in the 1-step method occur. It was thus decided that the 2-step method would be used for all future reactions.

These procedures were not repeated with primulin.

3.3 Conjugation of dye to carbohydrate groups on antibodies

Antibodies are classified as glycoproteins as they have carbohydrate entities on their heavy chains. Conjugation of dye to carbohydrate groups on antibodies (Section 2.14) was done with both dyes, Reactive black 5 and primulin.

3.3.1 Periodic acid-Schiff (PAS) stain

It was necessary to confirm that dye did bind to the carbohydrate portion on antibodies. The periodic acid-Schiff (PAS) stain (Maizels *et al.*, 1991) was used to confirm this (Section 2.6). Kaschnitz *et al.*, (1969) also described a procedure for glycoprotein staining after electrophoresis on acrylamide gels but their procedure was not used in this study.

3.3.1.1 Result and discussion

Two identical SDS-PAGE gels were stained with Coomassie and PAS (Figure 3.5).

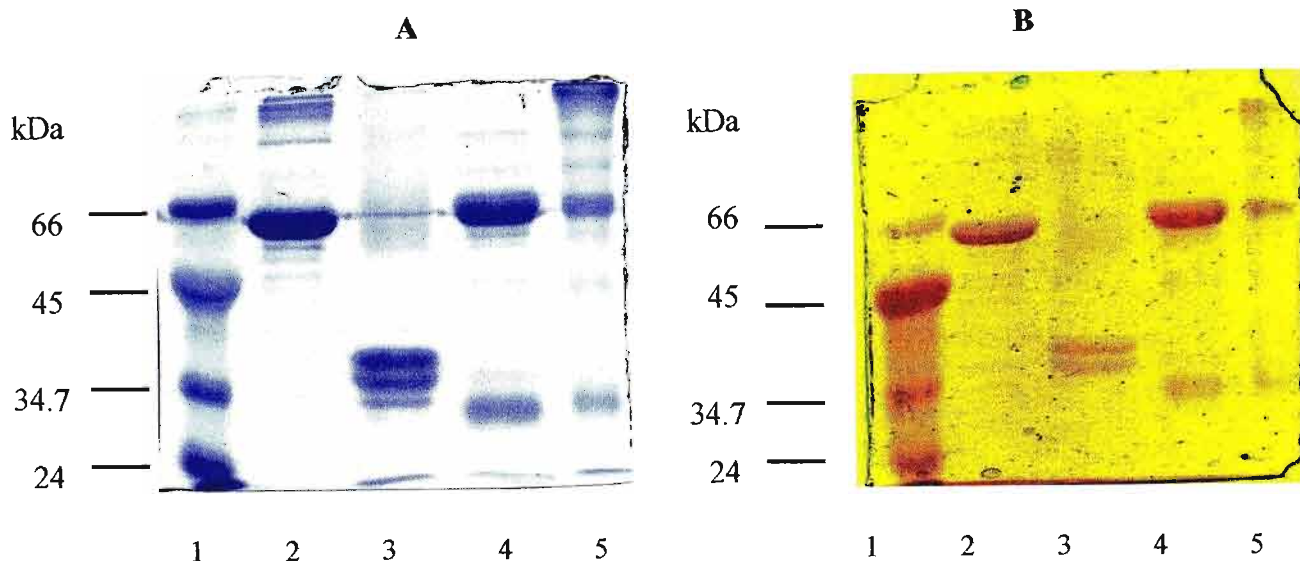


Figure 3.5: Gels stained with Coomassie (panel A) versus PAS (panel B) for carbohydrate.

Gels A and B are 10% Laemmli reducing SDS-PAGE gels and the lanes in both gels contain: Lane 1: Sigma molecular mass markers, Lane 2: BSA (20 μg), Lane 3: casein (20 μg), Lane 4: IgY (20 μg), Lane 5: Reactive black 5 - IgY (20 μg).

The PAS gel (Fig. 3.5 B) was scanned through green cellophane to provide a good contrast of the pink bands against a high (pink) background.

Lane 4 in both gels indicates the intensely stained heavy chain of IgY at approximately 60 kDa. If this is compared to the contents of lane 5, a marked difference is evident because the dye-linked IgY in both gels shows a lesser intense stain of the heavy chain, particularly for the PAS stained dye-linked IgY sample. The PAS stain was in competition for oxidative binding sites at the carbohydrate residues, as dye had already been coupled to those available sites. This suggests that Reactive black 5 has bound to the carbohydrate residues.

Wan and van Huystee (1993) describe a means whereby the sugar content of glycoproteins can be determined by the phenol-sulphuric acid colour reaction (Section 2.14.3). It was found that the affinity purified antibody that is utilised in these experiments (chicken anti-rabbit albumin) has the lowest sugar content compared to other antibodies found in our laboratory. This may thus be a limiting factor with regard to detection sensitivity levels.

3.4 Comparative dot blots

The sensitivity level of antigen detection was determined for the dye conjugated affinity antibodies. The two different dyes, Reactive black 5 and primulin, were both glutaraldehyde-conjugated as well as carbohydrate-conjugated to IgY. Traditional enzyme-substrate antigen detection systems were also used as a method of control. Chicken anti-rabbit albumin-horseradish peroxidase (Section 2.8.1) and the biotinylated chicken anti-rabbit albumin (Section 2.8.2) were used for this purpose. A further comparison was made by using an anti-chicken secondary antibody to detect antigen with the dye-antibody detection systems to investigate a possible enhancement of the dye signal.

3.4.1 Result and discussion

The results for all the above experiments for the comparative dot blots are shown in Figure 3.6. The results are displayed in three panels. Panel A indicates the enzyme-substrate systems: AI - the chicken anti-rabbit albumin-horseradish peroxidase, AII - the biotinylated chicken anti-rabbit albumin and AIII - the detected antigen by anti-chicken secondary antibodies. Panel B indicates the Reactive black 5 systems: BI - the glutaraldehyde conjugated RB5, BII - the carbohydrate conjugated RB5 and BIII - the glutaraldehyde conjugated RB5 with the addition of anti-chicken secondary antibody and the development in substrate. Panel C indicates the primulin systems: CI - the glutaraldehyde conjugated primulin, CII - the carbohydrate conjugated primulin and CIII - the glutaraldehyde conjugated primulin with the addition of anti-chicken secondary antibody and the development in substrate.

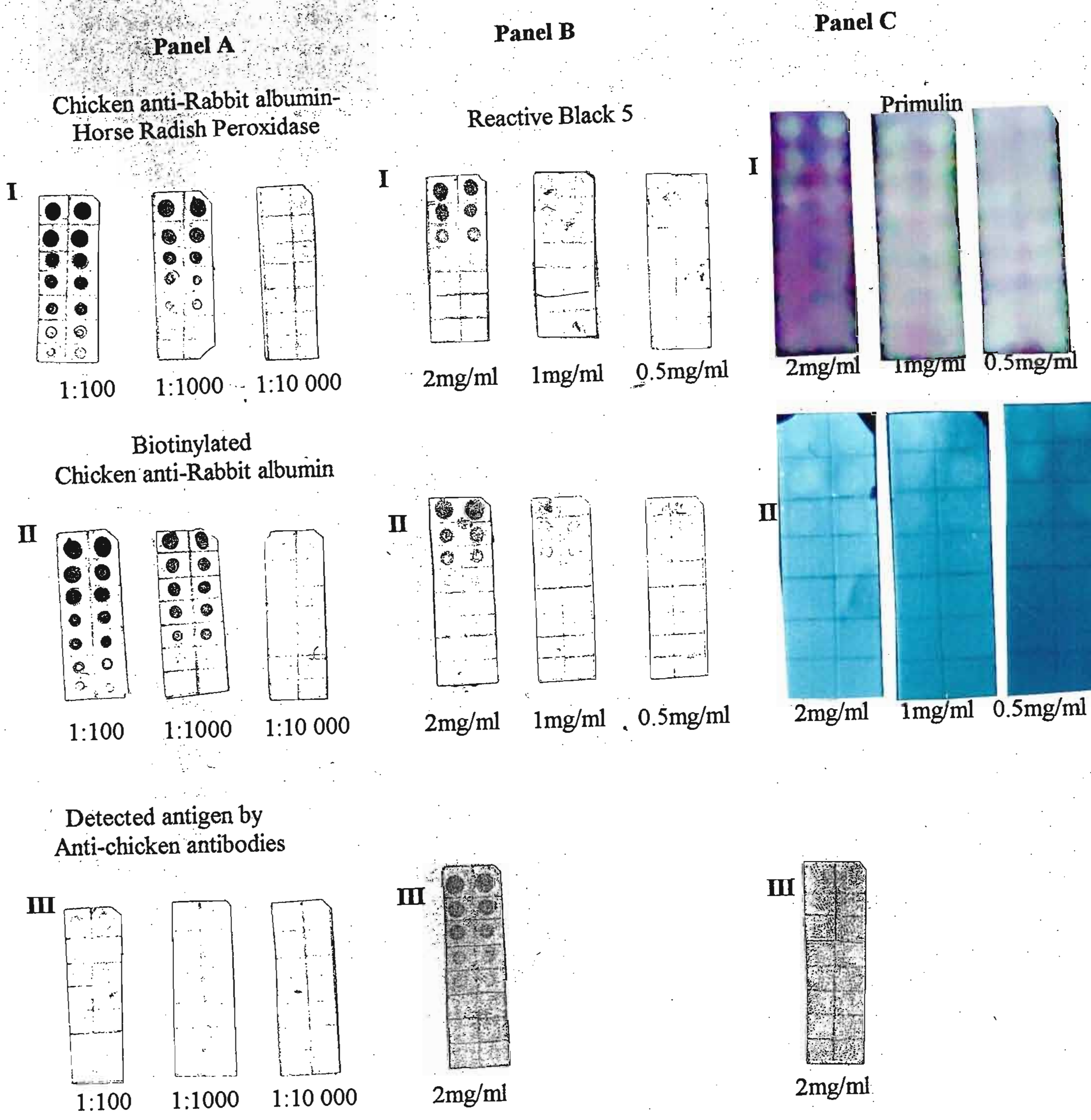


Figure 3.6: A comparative dot blot study on nitrocellulose of detected antigen by two enzymes, horseradish peroxidase, biotinylated antibody with horseradish peroxidase and two dyes, Reactive black 5 and primulin, respectively.

Table 3.1: Sensitivities of the dye and enzyme detection methods for the "dot-blot" immunoassays.

Antigen concentration (μg)	Panel A			Panel B			Panel C		
	I] CαRA-HRP			I] RB5-glutaraldehyde			I] primulin-glutaraldehyde		
	1:100	1:1000	1:10 000	2 mg/ml	1 mg/ml	0.5 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml
2	3+	3+	1+	2+	1+	+	2+	1+	+
1	3+	3+	1+	2+	1+	+	1+	1+	+
0.5	3+	3+	1+	2+	+	+	+	+	-
0.1	3+	2+	+	1+	+	-	-	-	-
0.05	3+	1+	-	+	-	-	-	-	-
0.01	2+	+	-	-	-	-	-	-	-
0.005	1+	-	-	-	-	-	-	-	-
	II] Biotinylated CαRA			II] RB5-carbohydrate			II] primulin-carbohydrate		
	1:100	1:1000	1:10 000	2 mg/ml	1 mg/ml	0.5 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml
2	3+	3+	+	2+	2+	1+	2+	1+	+
1	3+	3+	+	2+	2+	1+	1+	1+	+
0.5	3+	3+	+	2+	1+	1+	+	+	-
0.1	3+	3+	-	1+	+	+	-	-	-
0.05	3+	3+	-	+	-	-	-	-	-
0.01	1+	+	-	-	-	-	-	-	-
0.005	1+	-	-	-	-	-	-	-	-
	Anti-chicken antibodies			Anti-chicken antibodies			Anti-chicken antibodies		
	1:100	1:1000	1:10 000	2 mg/ml			2 mg/ml		
2	1+	+	-	3+			-		
1	+	-	-	2+			-		
0.5	+	-	-	2+			-		
0.1	+	-	-	1+			-		
0.05	+	-	-	+			-		
0.01	-	-	-	-			-		
0.005	-	-	-	-			-		

Table 3.1 is a summary of the developed sensitivities of the experiments as indicated in Figure 3.6 by means of "+" values. The primulin dot blots have "+" values that are indicative of the amount of fluorescence from the antigen-antibody-dye complex.

The results illustrated in Table 3.1 show that each of the dot blots are able to detect the following amounts of antigen:

Panel AI – The 1:1000 dilution is able to detect 10 ng of antigen while the 1:10 000 dilution is able to detect 100 ng of antigen.

Panel AII – The 1:1000 dilution is able to detect 10 ng of antigen while the 1:10 000 dilution is able to detect 500 ng of antigen.

Panel AIII – The 1:100 dilution is able to detect 50 ng of antigen while the 1:1000 dilution is able to detect 2 μg of antigen.

Panel BI – The antibody concentration at 2 mg/ml detects 50 ng of antigen, the antibody concentration at 1 mg/ml detects 100 ng of antigen while the antibody concentration at 0.5 mg/ml detects 500 ng of antigen (although this is not clear from the photostat, the original blot indicates this).

Panel BII - The antibody concentration at 2 mg/ml detects 50 ng of antigen, the antibody concentration at 1 mg/ml detects 100 ng of antigen while the antibody concentration at 0.5 mg/ml detects 100 ng of antigen.

Panel BIII – The RB5-antibody concentration at 2 mg/ml with the addition of a secondary antibody and development in substrate detects 50 ng of antigen.

Panel CI - The antibody concentration at 2 mg/ml detects 500 ng of antigen, the antibody concentration at 1 mg/ml detects 500 ng of antigen while the antibody concentration at 0.5 mg/ml detects 1 μg of antigen.

Panel CII – (The size of the dot blots appear larger due to the size of the photograph on which these blots were developed but they were exactly the same size as the other dot blots) The antibody concentration at 2 mg/ml detects 500 ng of antigen, the antibody concentration at 1 mg/ml detects 500 ng of antigen while the antibody concentration at 0.5 mg/ml detects 1 μg of antigen.

Panel CIII – No antigen detection is visible after incubation in a secondary antibody and development in a substrate.

When the two enyme-substrate systems are compared (chicken anti-rabbit albumin-HRP versus biotinylated chicken anti-rabbit albumin), it appears that the chicken anti-rabbit

albumin-HRP is more sensitive than the biotinylated system as more antigen is detected at the 1:10 000 dilution.

The addition of an anti-chicken antibody to the dot blots did indicate the detection of antigen, although it appears to be less sensitive than the two enzyme systems above. This may be due to the fact that the secondary antibody was a protein from goats raised in chickens instead of a protein from rabbits raised in chickens. When comparing the RB5-glutaraldehyde method with the RB5-carbohydrate method, it appears that the carbohydrate method is slightly more sensitive with antigen detection as described here. The glutaraldehyde method is simpler to execute and further work is necessary to determine whether the increase in sensitivity of the carbohydrate method is worth pursuing.

The addition of a secondary antibody and development in substrate to the dye developed dot blot is interesting in that it shows a deepening of the intensity of the dye developed spots. The sensitivity, however, is not increased.

When comparing the primulin-glutaraldehyde method with the primulin-carbohydrate method, it appears that the sensitivities of the blots under UV light were very similar. More work is needed to determine whether these signals may be improved and to determine if one or the other method is better. The lower the antibody concentration in the dye-antibody solution, the higher the background.

The addition of a secondary antibody and development in substrate to the primulin developed dot blot yields no visual increase in sensitivity as with the Reactive black 5 example. It is possible that due to the fluorescent nature and physical properties of the dye, the secondary antibody was not able to interact with the dye-antibody.

When comparing the two dyes, it appears that Reactive black 5 is more sensitive with regard to the detection of antigen. With the aim of this study in mind, it is also the simpler dye to continue to work with. primulin has unique characteristics but requires UV light to determine the results. The hazards and cost associated with UV light should therefore be considered when compared to Reactive black 5.

An overall comparison of each of the components of Figure 3.6: When considering the 1:1000 dilution of AI and AII, and comparing these to the 2 mg/ml Reactive black 5 and primulin dot blots (BI, BII and CI, CII), they would appear to be comparable with regard to the sensitivity of antigen detection. The enzyme systems are, however, still more sensitive than the dye detection systems.

The detection of antigen by means of these dye-antibody solutions is specific as other antigens (e.g.: BSA, sheep IgG and human IgG) were spotted onto nitrocellulose and no colour development was visualised (data not shown).

The study objectives been achieved. Further work needs to be done on improving the sensitivity of antigen detection however.

3.5 Spectrophotometric quantitation of protein-dye complex on nitrocellulose

In order to quantitate the amount of dye in a "dark" spot or a "light" spot a method outlined by Goldring and Ravaioli (1996) was used. This is discussed in Chapter 6.

3.6 Other dyes investigated for covalent coupling to IgY

A number of other dyes were investigated for covalent coupling to IgY. Each possesses particular structural and chemical properties and it is possibly due to these characterisitics that many of them were unsuitable for the purposes of this study. Table 3.2 summarises the chief disadvantage of each of the listed dyes.

Cibacron Blue 3-GA

This dye lost its colour completely upon reduction with sodium borohydride. Sodium cyanoborohydride made the dye change its colour to a much lighter shade, which was unsuitable for the purposes of this study.

Congo red

This dye unfortunately adhered to the dialysis tubing during the final dialysis step. If the dye stuck to the tubing it is likely that dye-antibody also bound to the tubing. Less dye-antibody would therefore remain available for antigen detection. Congo red also adhered to the particles of a Bio-Gel P_4 column and could therefore not be chromatographically separated from the reaction components and unbound dye (data not shown).

Trypan blue

This dye too, adhered to the dialysis tubing in the final dialysis step. There was negligible colour in the dye-antibody conjugated solution after dialysis.

Acid black-2 (Nigrosin)

The overall structure of acid black-2 is unknown, but it has been shown to associate with proteins (Lee, 1963). This dye unfortunately fades when nitrocellulose dries. This dye also does not dialyse efficiently.

Para-nitroaniline

This is a common laboratory dye used in numerous assay methods. Despite its ability to change colour from light yellow to deep purple, its properties were not rewarding enough for our purposes as it did not change colour successfully after being conjugated to dye.

Dianix red

The disperse dye, dianix red, although it showed results on dot blots (Table 4.1), its sensitivity was lower than that of dianix blue, the other disperse dye investigated in this study.

Table 3.2: A summary of the chief disadvantages of six dyes investigated in this study.

Dye	Associated problem	Sensitivity (ng)
Cibacron Blue 3-GA	Colour disappears upon reduction	-
Congo red	Adheres to dialysis tubing	50
Trypan blue	Adheres to dialysis tubing	100
Acid black-2	Colour fades with drying of nitrocellulose	100
Para-nitroaniline	Does not detect antigen	-
Dianix red	Not sensitive enough	50

CHAPTER 4: COLLOIDAL DYES AND IONIC INTERACTIONS WITH ANTIBODIES

Colloidal dyes belong to a class of dyes also known as disperse dyes as they consist of particles of different sizes. The Colour Index defines them as water-insoluble dyes and their original application was to dye cellulose acetate. Acetate is hydrophobic and has an electronegative surface. When placed under water, acetate repels direct dyes (Kulkarni *et al.*, 1986). These colloidal dyes (dye-sol particles) were used as a physical label in agglutination assays according to the method described by Gribnau *et al.*, (1982). The best results are reportedly obtained using dye-sol particles with a diameter of about 200 nm or less and the shape of the particles appears to be important (Gribnau *et al.*, 1982). Gribnau *et al.*, states that needle shaped particles were less suitable while gravel-like structures yielded good conjugates. The dye for application in this project was centrifuged to separate the smaller particles of interest from larger particles that would not conjugate easily.

4.1 Capture dot blots with dianix blue and dianix red

These experiments combine the theories of sandwhich ELISA, dot blots and the dye-antibody detection reagent in the detection of antigen on nitrocellulose. The affinity purified antibody was coated on to the nitrocellulose to serve as the capture antibody while the same species-specific antibody was used to absorb to the dianix blue and dianix red dyes respectively. As described in Section 2.18.2, the nitrocellulose strips were incubated in different antigen concentrations, which adsorb to the capture antibody and after being incubated in the different dye-antibody solutions were used to determine sensitivity levels.

4.1.1 Result and Discussion

The results of the dianix blue and dianix red capture dot blots are indicated in Table 4.1.

Table 4.1: Dianix blue and dianix red dyes in the antigen capture dot-blot protocol on nitrocellulose.

	Antigen Concentration (µg/ml)									
[Capture antibody] (µg)	1		0.1		0.05		0.01		0	
2	2+	1+	2+	1+	2+	1+	2+	+	1+	-
1	2+	1+	2+	1+	2+	+	2+	+	1+	-
0.5	2+	1+	2+	+	2+	+	2+	+	1+	-
0.1	1+	1+	1+	+	1+	-	1+	-	+	-
0.05	1+	+	1+	-	+	-	+	-	-	-

Note: Dianix blue is in blue and dianix red is in red.

Table 4.1 indicates that dianix blue appears to be more sensitive than the dianix red in a capture antibody protocol. The dianix blue dye detects 10 ng/ml antigen with a capture antibody concentration of 50 ng. The dianix red dye detects 10 ng/ml antigen with a capture antibody concentration of 500 ng. It may be noted that at an antigen concentration of 0 µg/ml for dianix blue, there is still the development of coloured spots on the nitrocellulose, while for dianix red there is not. The dianix blue is involved in a non-specific interaction and attempts were made to eliminate this.

4.1.2 Attempts to eliminate non-specific interactions

The experiments with different buffer systems are summarised in Table 4.2 and the experiments with different blocking systems are summarised in Table 4.3.

Table 4.2: Evaluation of the effects of different buffers and pH on the interaction between dianix blue and antibodies on nitrocellulose.

Different Buffer Systems: **Rabbit albumin** on nitrocellulose, incubated in 2% low fat milk in respective buffers **Probed with dye-antibody**			
Glycine-HCl pH 2.8 milk curdled	$NaCH_3C00$ pH 5.4 milk curdled	PBS pH 7.4 non-specific interaction	$NaCO_3$ pH 9.6 non-specific interaction

Different Buffer Systems: **Rabbit albumin** on nitrocellulose, incubated in 2% low fat milk in respective buffers **Probed with dye alone**			
Glycine-HCl pH 2.8 N/A	$NaCH_3C00$ pH 5.4 N/A	PBS pH 7.4 non-specific interaction	$NaCO_3$ pH 9.6 non-specific interaction

Different Buffer Systems: **IgY** on nitrocellulose, incubated in 2% low fat milk in respective buffers **Probed with dye alone**			
Glycine-HCl pH 2.8 N/A	$NaCH_3C00$ pH 5.4 N/A	PBS pH 7.4 non-specific interaction	$NaCO_3$ pH 9.6 non-specific interaction

Different Buffer Systems: **IgG** on nitrocellulose, incubated in 2% low fat milk in respective buffers **Probed with dye alone**			
Glycine-HCl pH 2.8 N/A	$NaCH_3C00$ pH 5.4 N/A	PBS pH 7.4 non-specific interaction	$NaCO_3$ pH 9.6 non-specific interaction

Table 4.3: Evaluation of different blocking conditions on the interaction between dianix blue and antibodies on nitrocellulose.

Protein on nitrocellulose	**Different Blocking solutions**				
	0.5% BSA-TBS	0.5% BSA-TBS Tween (0.2%)	0.5% Casein-PBS	2% PBS milk Tween (0.2%)	0.5% Gelatin-PB
IgY	non-specific interaction	non-specific interaction	non-specific interaction	eliminates interaction with dye	non-specific interaction
IgG	non-specific interaction	non-specific interaction	non-specific interaction	non-specific interaction	non-specific interaction
Rabbit Albumin	non-specific interaction	eliminates interaction with dye	non-specific interaction	non-specific interaction	non-specific interaction

Other experiments were also conducted. For example, the normal capture dot blot procedure with the affinity antibody spotted onto the nitrocellulose, blocked with 2% PBS milk Tween

(0.2%), and incubated in other protein solutions such as BSA and casein before being probed with the dye-antibody conjugate. This failed to eliminate the non-specific interaction.
The protocol for the dianix dye buffer contains a large amount of BSA in the buffer solution. The BSA was replaced with a similar concentration of IgY. There was no colour development in this instance.

It was thus concluded that the addition of some reagents such as Tween to the blocking step may assist in decreasing the intensity of the non-specific signal. It does not suit our purposes, however, if the non-specific reaction is still present and also reduces the intensity signal of the entire experiment. The group of Kashiwazaki *et al.*, (1994) reported a similar non-specific phenomenon where they obtained pale false-positive dots on negative control dipsticks during a field diagnosis of trypanosome infections in cattle with this colloidal dye immunoassay. They determined the appropriate capture dot concentrations by comparing positive and negative controls. Gribnau *et al.*, (1982) obtained the best results when using dye sol particles with 200 nm or less, and that spherically shaped particles were ideal. The introduction indicates the structure of dianix blue FBLN 200 (van Popering, 1996). The present study used dianix blue FBLN-SE 300 with a diameter of 300 nm, and this could possibly be a factor in the non-specific interaction.

4.2 Capture enzyme-linked immunosorbent assay (ELISA)

The capture dot blot protocol on nitrocellulose was repeated, but instead of the dye-antibody solution, an enzyme-substrate control was used for comparative purposes. The horseradish peroxidase (HRP)-coupled chicken anti-rabbit albumin (Section 2.8.1) was the secondary antibody utilized and 4-chloro-1-naphtol was the substrate used to detect the reaction.

4.2.1 Result and discussion

The results of this capture ELISA protocol on nitrocellulose are indicated in Table 4.4.

Table 4.4: Sensitivities of horseradish peroxidase antibody detection of rabbit albumin.

Chicken anti-rabbit albumin was immobilised on nitrocellulose and probed with different antigen concentrations before being probed with horseradish peroxidase labelled chicken anti-rabbit albumin.

	Antigen concentration (μg/ml)				
[Capture antibody] (μg)	1	0.1	0.05	0.01	0
2	3+	1+	1+	+	-
1	3+	1+	1+	+	-
0.5	3+	1+	+	+	-
0.1	2+	+	+	-	-
0.05	+	-	-	-	-

With the concentrations of immobilised antibody indicated in Table 4.4, the capture dot blot ELISA is sensitive enough to detect 10 ng/ml antigen with 500 ng of capture (immobilised) antibody. This is more sensitive than the dianix red dye, but less sensitive than the dianix blue dye. There is, however, no non-specific interaction as was seen with the dianix blue dye.

Although dianix blue did show non-specific interactions between the dye and the antibodies used in this study, it is possible to use this as a detection method for the presence of antigen. Further experimentation is needed to determine if the non-specific interactions can be eliminated. The horseradish peroxidase enzyme-substrate experiment did not show greater sensitivities than the dianix blue experiment which could possibly be ascribed to a decrease in enzyme activity after a short storage period. This has important implications for the dianix dyes as they do not lose colour or activity after storage periods at 4°C and they may also be frozen to -20°C and thawed or lyophilized for long term storage (Snowden and Hommel, 1991). These properties make the dianix dye-antibody solution ideal for field work purposes.

4.3 Dye-linked immunoassay (D-LISA)

Gribnau *et al.*, (1982) developed a unique method to detect antigen by using the dianix dye-antibody mixture as a dye-sol label in a microtitre plate sandwich assay. The sensitivities they obtained with the dye-immunoassay were at least the same as for radio and enzyme immunoassays. This protocol was repeated here with the dianix blue dye (Section 2.18.3).

4.3.1 Result and discussion

The results of the D-LISA microtitre assay are indicated in Figure 4.1. This representation was chosen to indicate that comparisons can be made with the dot blots mentioned in Section 4.2.

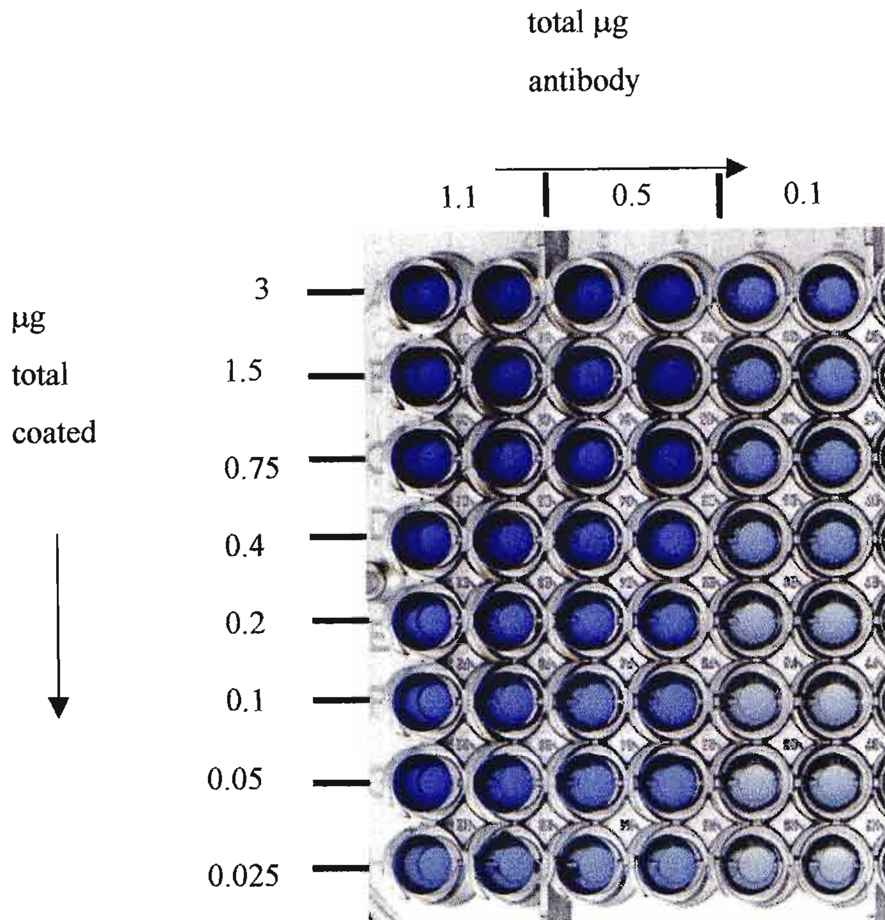


Figure 4.1: A picture of a typical direct antigen detection system with dianix blue in a microtitre plate.

Different concentrations of antigen were coated onto the microtitre plate and probed with three different concentrations of antibody conjugated to dianix blue in duplicate.

Figure 4.1 appears to suggest that intense dye signals are obtained when using antibody concentrations of 10 µg/ml and 5 µg/ml (total antibody concentration of 1.1 µg and 0.5 µg as indicated in Figure 4.1) in the dye-antibody solution. The intensity of the signal decreases from the top to the bottom of the plate as the coated antigen concentration decreases. The signal intensity also decreases across the plate from left to right as the antibody concentration in the dye-antibody solution decreases.

For comparative purposes, the antigen detecting sensitivity of the D-LISA was compared to the antigen detecting sensitivities of conventional ELISAs. The horseradish peroxidase linked

chicken anti-rabbit albumin and biotinylated chicken anti-rabbit albumin (Section 2.8.1) were the secondary and primary antibodies respectively.

4.4 D-LISA versus ELISA's

Three separate experiments were conducted on the same microtitre plate in quadruplicate. The first being the D-LISA described above, the second was the direct detection of antigen (rabbit albumin) with horseradish peroxidase labelled chicken anti-rabbit albumin and the third was the detection of antigen with a biotinylated chicken anti-rabbit albumin as a primary antibody, with avidin-horseradish peroxidase as the secondary antibody. The antibody concentrations in these experiments were similar so that the signal intensity of antigen detections could be compared.

4.4.1. Result and discussion

Figure 4.2 shows a graph of the absorbance values obtained with the different antigen detection systems.

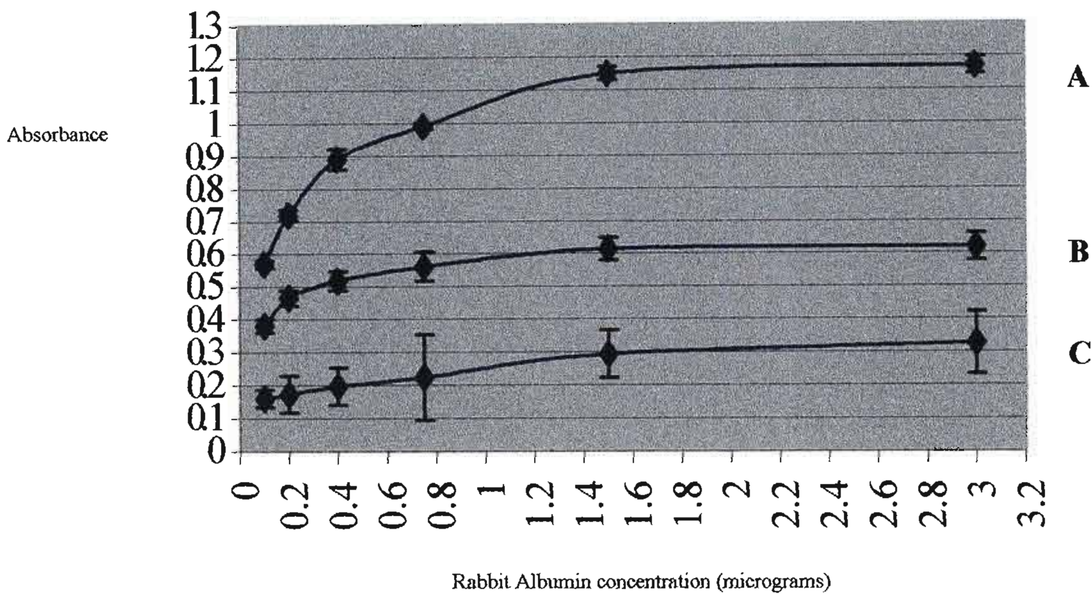


Figure 4.2: A comparison of the direct antigen detection abilities of the D-LISA versus ELISAs in a microtitre plate.

The detection of antigen by chicken anti-rabbit albumin-HRP is represented by graph trace **A**. The detection of antigen by biotinylated chicken anti-rabbit albumin is represented by the graph trace **B** and the detection of antigen by dianix blue dye is represented by the graph trace **C**. The error bars are representative of the standard deviation of the means of the data for each point in the plot.

The sensitivity of the dianix blue detection of antigen is lower than that of its enzyme-substrate counterparts. The HRP-labelled chicken anti-rabbit albumin appears to be the most sensitive method for antigen detection. The avidin-biotin system for antigen detection was less sensitive than the HRP-labeled affinity antibody but more sensitive than the dye-antibody detection system.

The direct detection of antigen by dianix blue becomes difficult to accurately determine below an antigen concentration of 0.4 μg and a total antibody concentration of 0.1 μg.

Gribnau *et al.*, (1982) stated that the sandwich dye immunoassay is at least the same as for enzyme assays. Figure 4.2 indicates that this is indeed the case. The enzyme systems show better sensitivities but a lot of work has been done in the previous decades to obtain these sensitivity levels.

The dianix dye-antibody antigen detection system on a microtitre plate as described by Gribnau *et al.*, (1982) was a successful immunoassay procedure within the scope of this study. Further work is required to investigate whether it is possible to increase the sensitivity of the D-LISA antigen detection level with dianix blue.

CHAPTER 5: NOVEL PROTEIN STAINING IN GELS

Coomassie brilliant blue staining of proteins in electrophoretic gels is a common technique used to visualise all protein present on the gel. When this dye is placed in a solution containing acid and methanol, it adopts an overall negative charge. The Coomassie dye binds to the positively charged amino acids of the protein fragments in the gel.

5.1 Protein staining

The dyes used in this study (Reactive black 5, trypan blue, acid black-2) were similarly investigated not only for their ability to covalently bind to antibodies, but to determine whether they could be utilized to stain proteins. Section 2.20 describes the detail of the procedure.

The ability of the coloured dyes to stain proteins in gels was compared to the protein staining ability of diazotized primulin in gels. Primulin is a yellow dye that doesn't offer the contrast required for the purposes of this study. The uses of primulin dye are not widely documented. Its chief application has been to detect neutral, nonpolar lipids, phospholipids and glycosphingolipids, mostly in thin-layer chromatography. When viewed under ultraviolet light, primulin appears to fluoresce as light blue or yellowish on a dark blue-violet background. Over and above the fluorescent properties of this dye, it also possesses colour-changing abilities by means of the diazotization reaction, discussed briefly below.

Organic chemistry uses the diazotization reaction as a synthetic protocol. It forms diazonium salts from primary aromatic amines. Initially, the amine has to be nitrosated ($R_2N{-}N{=}O$) with nitrous acid in aqueous solution. Nitrous acid is unstable but is prepared in the laboratory by adding sodium nitrite to a strong acid such as HCl or H_2SO_4. If the amine is basic enough, it can form diazonium salts with dilute acids in aqueous solution (Trost *et al.*, 1991), which means that diazotization can occur when sodium nitrite is added to a solution of the amine salt (the two dyes para-nitroaniline and primulin are the amine salts in this instance). When aromatic compounds that contain phenols and amines are used for diazotization, there is an electrophilic aromatic substitution reaction and the terminal nitrogen atom of the diazonium

ion is the electrophile. The derivatives formed have extended conjugated electron systems that reslut in bright colours (Loudon, 1995). Primulin was used to stain a gel and the diazotization reactions as described by Trotman (1984) were used to develop the primulin stain.

5.1.1 Result and Discussion

SDS-PAGE gels (12.5%) were loaded with decreasing concentrations of IgY and electrophoresed as described previously (Section 2.2). The gels were stained as described in Section (2.2). Results for the coloured dye stained gels are indicated in Figure 5.1 and those for or the diazotized primulin stained gels, in Figure 5.2.

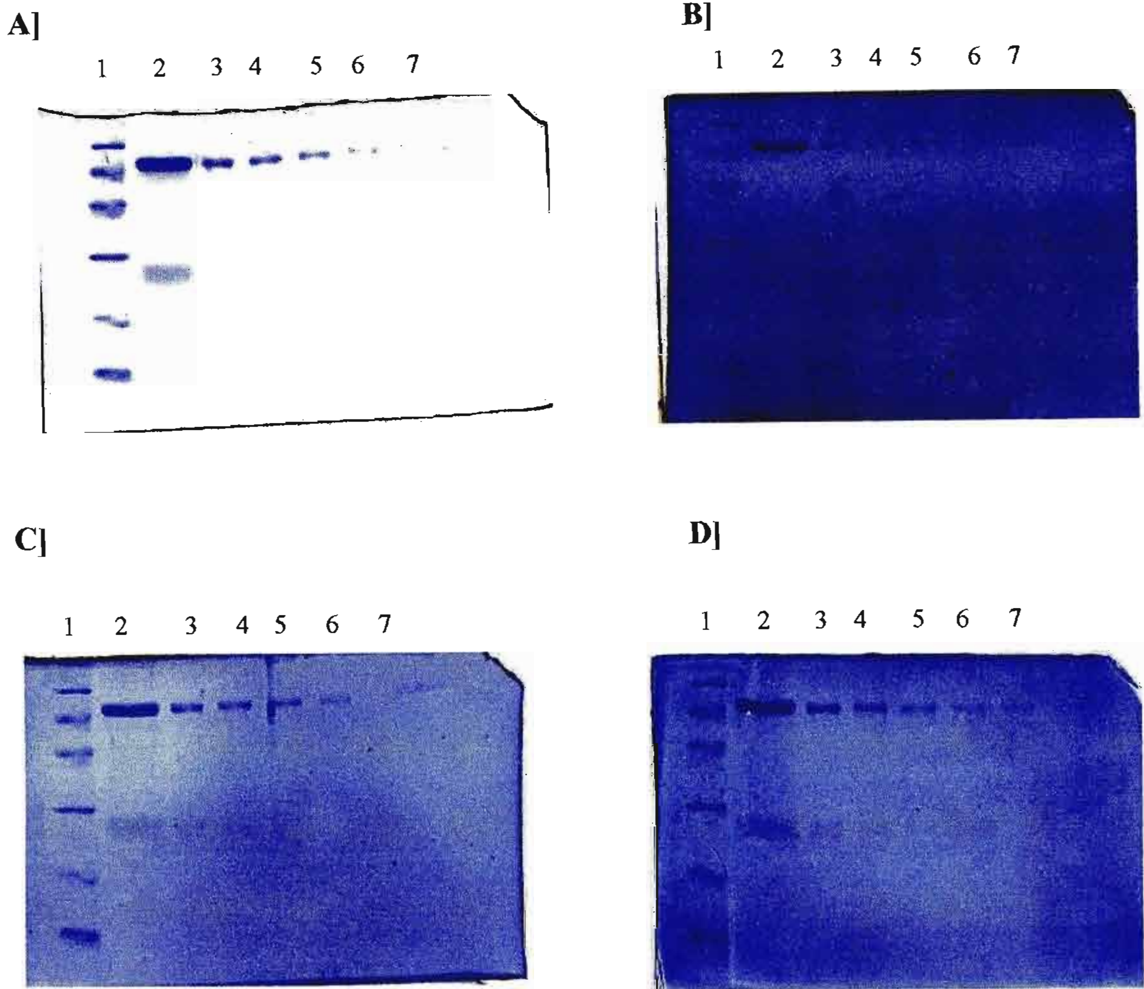


Figure 5.1 : Staining of SDS-PAGE protein gels with 4 dyes used in this study.

A] Gel stained with Coomassie brilliant blue **B]** Gel stained with Reactive black 5

C] Gel stained with trypan blue **D]** Gel stained with acid black 2. All gels were loaded with IgY in similar manner. Lane 1, Molecular mass markers (Pharmacia); Lane 2, 5 μg IgY; Lane 3, 1 μg IgY; Lane 4, 0.75 μg IgY; Lane 5, 0.5 μg IgY; Lane 6, 0.25 μg IgY;
Lane 7, 0.125 μg IgY

The Coomassie stained gel was used as the control in this instance due to its routine use in laboratories. As shown in Figure 5.1, the Coomassie stained IgY down to a concentration of 0.25 μg (lane 6). The IgY light chains do not stain below concentrations of 5 μg. The Reactive black 5 stained gel shows that IgY is stained at a concentration of 0.5 μg (lane 5). The IgY light chains do not stain below a concentration of 5 μg. The trypan blue stained gel shows that IgY is stained down to a concentration of 0.25 μg (lane 6). The IgY light chains do not stain below concentrations of 0.75 μg. The acid black 2 stained gel shows that IgY is stained down to a concentration of 0.125 μg (lane 7). The IgY light chains do not stain below

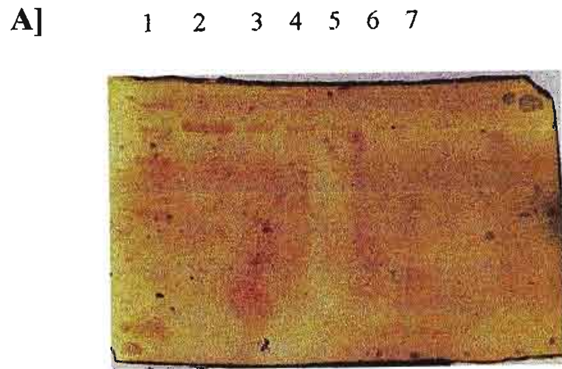
a concentration of 0.25 μg and they are seen as smudgy, diffuse bands. All these gels have high backgrounds when compared to the Coomassie stained gel.

The acid black 2 stained gel (Figure 5.1 **D)** shows greater intensity of protein staining compared to the other stains, including the Coomassie stain (Figure 5.1 **A**). Acid black 2 (otherwise known as Nigrosin) has already been reported to stain wheat gluten proteins in an electrophoretic gel (Lee, 1963). It is also the only stain to show the IgY light chain at low IgY concentrations and these results suggest that this dye possibly has a larger negative charge than the other dyes. The only disadvantage to using this dye is the extended period of time (36 h) required to destain the gel. The method used to destain was as reported by Lee (1963), with methanol, water, acetic acid, and 5% aqueous acetic acid solution to restore the gel to its original size. The methanol incubation step shrinks the gel and makes it brittle and care should be taken when handling the gel.

Trypan blue (Figure 5.1 **C**) has better staining abilities than the Reactive black 5 stained gel. Trypan blue stains IgY light chains to a greater degree. Reactive black 5 (Figure 5.1 **B)** was the least sensitive protein staining dye although it had a lower background compared to trypan blue and acid black 2 (the scanned image shown in Figure 5.1 shows a relatively high background but the gel itself had a low background).

The manner in which Coomassie stains proteins in gels was applicable to the dyes used in this study too. The acidic-methanol conditions rendered the dyes with enough of a negative charge to stain the positively charged IgY in the gel. These staining techniques could possibly be applied for routine protein staining in laboratories.

A]



B]

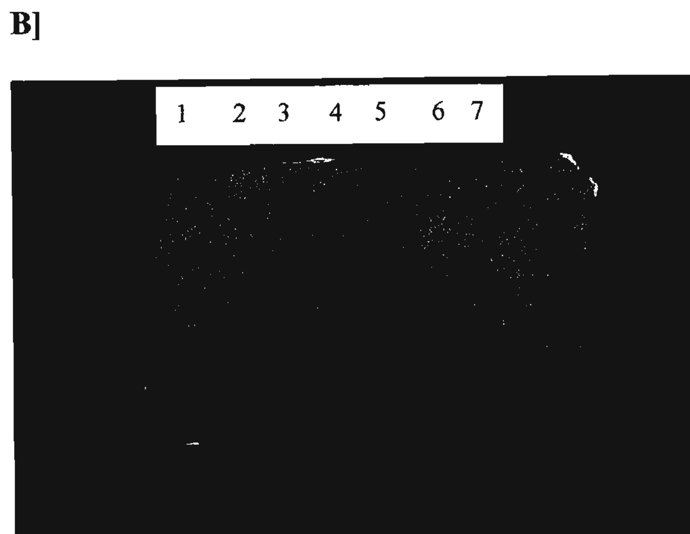


Figure 5.2: SDS-PAGE protein gels stained with primulin and diazotized primulin.

A] Diazotized primulin stained gel viewed under normal light conditions. **B]** Gel stained with primulin viewed under UV light. Both gels were loaded with IgY in a similar manner. Lane 1, Molecular mass markers (Pharmacia); Lane 2, 5 μg IgY; Lane 3, 1 μg IgY; Lane 4, 0.75 μg IgY; Lane 5, 0.5 μg IgY; Lane 6, 0.25 μg IgY; Lane 7, 0.125 μg IgY.

The gel stained with primulin viewed under UV light (Figure 5.2 **B**) allows 0.25 μg IgY in lane 6 to be visualised. The IgY light chains do not stain. Under normal light conditions, before diazotization, the protein bands appear as a light yellow colour against a yellow background. Under UV light, the protein bands appear as grey bands on a blue-purple fluorescent background. The diazotized primulin gel in Figure 5.2 **A** allows a concentration of 0.75 μg of IgY in lane 4 to be visualised. The IgY light chains do not stain. Under normal light conditions the protein bands appear as dark orange bands against a light orange

background. Under UV light, the protein bands appear as dark brown bands against a dark purple background.

The primulin stained gel (Figure 5.2 **B)** fluoresces under UV light to show IgY detection at 0.25 μg. This is comparable to the Coomassie and trypan blue stains. When this dye is diazotized (Figure 5.2 **A**), visual detection of IgY is possible, but is less sensitive than the other dyes with an IgY detection limit of 0.75 μg. The diazotized gel under UV light does not show greater sensitivity than that visualized in white light.

This application of primulin has shown novel characteristics in being able to stain and thus detect protein when viewed under UV conditions in an electrophoretic gel. Furthermore, its colour changing abilities have been clearly evident with the diazotization reaction. Protein detection limits were, however, not improved compared to the Coomassie staining method.

CHAPTER 6: GENERAL DISCUSSION:

The aim of this study was to develop a simplified, sensitive and specific malarial diagnostic test at the lowest possible cost. Common laboratory reagents and simple laboratory techniques were utilised to achieve this goal.

The primary reference and starting point for this study was the article by Gribnau *et al.*, (1982) on "The application of colloidal dye particles as label in immunoassays: disperse dye (D) immunoassays (DIA)". The concept of investigating ionic interactions between dye and antibody molecules as a label in immunoassays was modified to investigate whether covalent interactions were more useful. Reactive groups on both dyes and antibodies were utilised and simple chemistry was manipulated to form stronger, covalent bonds to possibly improve sensitivity levels. Gribnau *et al.*, (1982) also investigated, quantitatively, the amount of antigen that could be detected in this way, as well as the simultaneous determination of two antigens by using dyes with different colours.

The antibody used in this study, chicken anti-rabbit albumin, purified by affinity chromatography, was chosen because it is well characterised in ELISAs, western blots and ochterlony techniques. The rationale was to investigate whether covalent interactions between dye and antibody were possible, to determine if the reaction was specific and to determine whether sensitivity levels could be heightened before moving on to investigations with malarial antibodies. The ultimate aim was to develop a dye-based dipstick immunoassay for the field testing of malaria. This study has developed the necessary ground-work for further investigations with malarial antibodies.

As mentioned in the introduction, immunglobulin from chicken egg yolks (IgY) has many advantages over the use of IgG. Isolation of IgY from egg yolk was simplified in a procedure described by Polson *et al.*, (1980) and eliminated the need to sacrifice the chickens. The stability of IgY is documented by Shimizu *et al.*, (1988, 1992, 1993) and for the purposes of this study was an ideal antibody.

Alternative chromatographic techniques that have been employed to isolate IgY were extensive and subtracted from the original goal of this study. Ingham (1990), states that PEG

is removed during chromatographic steps on ion-exchange or affinity columns to which PEG has no tendency to absorb. Polson *et al.*, (1964) also stated this. However PEG isolation of IgY is sufficient for dye-antibody conjugation purposes as PEG does not interfere with the coupling process.

The dyes used in this study are discussed in detail in the introduction. Most of the dyes were chosen due to the presence of particular amino groups in their structure. Colloidal dyes are the exception. The extent to which each of these dyes was investigated differs mostly due to disadvantages detected in their use in these assays. These disadvantages are summarised in Table 3.2. One dye in particular (Reactive black 5) was investigated rather extensively as it showed versatility with regard to all of the parameters being tested in this study. Para-nitroaniline is a yellow coloured dye, used in microassays that measure the release of this dye from a substrate, which can be detected spectrophotometrically at 405 nm. Para-nitroaniline is used to detect phospholipids (in plasma), just as primulin detects lipids (White *et al*, 1998). A method described by Hyman *et al.*, (1983) indicates that para-nitroaniline could be diazotized. Their methods were replicated and applied here to antigen detection dot blot methods. The diazotization reactions were, unfortunately, unsuccessful in developing para-nitroaniline detected antigen spots and further investigations with this dye were stopped at this point.

The 2-step glutaraldehyde protocol proved to be the most versatile method to covalently couple dye to antibody. The addition of glutaraldehyde to dye to "activate" the dye and its subsequent removal by dialysis proved to be a quick and convenient way to prepare dye-antibody solutions. The glutaraldehyde was shown to dialyse out of the dye-glutaraldehyde mixture and into the surrounding distilled water dialysis solution by using a spot test for aldehydes described in Section 2.12. It cannot be explained at this time, however, why, the removal of glutaraldehyde by dialysing against distilled water, did not result in the outward movement of dye as well (Craig, 1967). To promote the removal of unconjugated dye, the complete reaction mixture was dialysed against PBS, the dye solute.

A second covalent coupling method, of conjugation of dye to carbohydrate entities on the antibodies, was explored. This method was less convenient, but possibly slightly more sensitive than the glutaraldehyde method as suggested by the results indicated in Table 3.1. In order to determine that it was in fact the carbohydrate entities that were involved in the

covalent coupling of dye to antibody, the Periodic Acid Schiff (PAS) stain was used. The PAS stain is a carbohydrate stain, which was developed in the late 1940s. It was originally used as a micro-chemical reaction that resulted in the staining of polysaccharide structures in fixed tissue preparations, especially from plants (Gurr, 1965). It has since been used to stain a wide variety of plant, animal and human carbohydrates, saccharides and glycoproteins, in gels and on nitrocellulose. Fairbanks *et al.*, (1971) used the PAS stain to characterise the major polypeptides of the human erythrocyte membrane, after SDS electrophoresis. Glycoproteins that contain more than 10% carbohydrate show atypical electrophoretic migration when compared to molecular mass markers (Segrest and Jackson, 1972). This is caused by decreased binding of SDS per gram of glycoprotein as compared to the standard proteins. This is a factor to be taken into account when using the PAS stain. The PAS stain can be used to detect glycoproteins separated by nondenaturing PAGE methods (Doerner and White, 1990). The silver stain has been combined with the PAS stain for the enhanced detection of carbohydrates (Dubray and Bezard, 1982; Jay *et al.*, 1990). Dot blots on nitrocellulose can also be stained by the PAS stain to detect glycoproteins (Wan and van Huystee, 1993). Figure 3.5 reports the results of the PAS stain. Comparing lane 4, the IgY and lane 5, the dye bound IgY in the PAS stained gel B to that of the total protein stain in lanes 4 and 5 in gel A, it appears that lane 5 shows less stain in the PAS stained gel B compared to lane 5 in gel A. There appears to have been competition for the carbohydrate moieties by the previously bound Reactive black 5. This suggests that the carbohydrate moieties were indeed involved in the covalent coupling of dye to antibody. Further work is required with this coupling method to determine if it could surpass the versatility of the glutaraldehyde coupling method.

The spectrophotometric quantitation of protein-dye complexes on nitrocellulose was attempted as described by Goldring and Ravaioli (1997). The antibody-dye (Reactive black 5) complex was quantitatively spotted onto nitrocellulose, solubilised in dimethyl sulfoxide (DMSO) and spectrophotometric measurements were taken at the λ max (597 nm). The antibody-dye spots were visible on the nitrocellulose, but when dissolved in DMSO and subjected to spectrophotometry, the readings were negligible. Evidently, there was too little dye for the spectrophotometer to detect.

Proteins were also transferred to nitrocellulose from an SDS-PAGE gel and stained with dye-antibody probe (Reactive black 5-chicken anti-sheep IgG). The result showed extensive non-

specific staining. This was ascribed to the presence of methanol and glycine in the western blotting buffer.

The affinity purified antibody, chicken anti-rabbit albumin was used for several coupling reactions, including with dye. It was also conjugated to horseradish peroxidase and was biotinylated and applied as both soluble and insoluble labels in ELISAs in microtitre plates and on nitrocellulose, respectively. Chapter 3 deals with comparative dot blots on nitrocellulose. As mentioned in Chapter 3, antigen detection and the visualisation thereof with the enzyme systems are more sensitive (with regard to antigen detection) compared to the dyes. The 1:1000 dilution detects 5 ng of antigen in both enzyme systems. Decades of research have perfected the enzyme systems while not much work has been done on the optimisation of a dye based equivalent. In other words, although the enzyme systems are superior, the dye systems offer comparable sensitivities. The most difficult part about using the dyes was to capture and reproduce the sensitivities for the purposes of this document. Reactive black 5 and the carbohydrate coupling method could detect 5 times more antigen than the Reactive black 5 glutaraldehyde coupling method. An advantage of the glutaraldehyde method is the relative ease with which the procedure may be carried out and a disadvantage is the fact that glutaraldehyde is toxic and should be used under a fume-hood. An advantage of the carbohydrate method is the shortened amount of time it takes to prepare dye-antibody conjugates, but a disadvantage is that the early reaction stages are light sensitive and it also requires the addition of more reagents at relatively frequent intervals.

When the antigen detection ability of primulin is compared to the Reactive black 5 and the enzyme systems, it appears to be inferior. The antigen detection ability is less sensitive compared to that of the enzyme systems as well as Reactive black 5, as it cannot detect low concentrations of antigen. The hazards with visualising the results from primulin dot blots under UV light should be considered when working with this dye. primulin is a unique dye with potential for many applications, but the necessity for visualisation of the results under UV light subtracts from the original goal of this study, i.e. maximal simplicity.

Gribnau *et al.*, (1982) initiated the work with colloidal dyes and the capture dot blot method. Since then, a number of workers have used this technique on nitrocellulose. Rabello *et al.*, (1993) used antigen concentrations of 0.5 μg and 0.1 μg of soluble egg antigen and keyhole limpet haemocyanin respectively. The sandwich or capture dot blot technique for microtitre plates and for nitrocellulose (dye and enzyme comparisons) were used and they report no

significant difference between their diagnostic techniques. These antigen concentrations are comparable to those used in the present study.

Table 6.1 highlights the differences between authors who have done similar work using the capture dot blot technique (Section 4.1). It is evident that the type and quality of antibody determines how much is needed for conjugation to the dye(s). This was also suggested by Gribnau *et al.*, (1982). Also, depending on the type of assay required, antigen can be spotted onto nitrocellulose instead of antibody, as done by Rabello *et al.* (1993). The group of Zhu *et al.*, (2002) also used a dipstick dye immunoassay to detect antibodies in the serum of schistosomiasis patients. They reported that there was no significant difference in the sensitivity and specificity levels between their dipstick dye immunoassay and traditional ELISA assays.

The capture dot blots on nitrocellulose done in this study (Section 4.1) could not eliminate the non-specific interaction witnessed in the control sample. Kashiwazaki *et al.* (1994) reported similar findings and they determined appropriate capture dot concentrations by comparing positive and negative controls. Although the capture dot blot technique with colloidal dyes was applied in this study, the non-specific interaction remains a problem. Further experimentation on nitrocellulose was stopped, and continued in a microtitre plate. Gribnau *et al.*, (1982) used colloidal dyes as a dye-sol label in a microtitre plate sandwich assay. This method was modified and optimised to formulate a direct antigen detection assay. The level at which antigen detection becomes difficult with the D-LISA was 0.4 μg of antigen with 0.1 μg of antibody in the dye-antibody solution. The comparison with traditional enzyme systems illustrates that although the D-LISA does not produce as high readings as the enzyme systems, it is able to detect antigen and is not significantly inferior. The D-LISA has the advantage that incubation in secondary antibody is not necessary as in the case of the biotinylated enzyme, and eliminates the incubation step in substrate, thereby reducing assay time.

The ability of Reactive black 5 to detect antigen in a microtitre plate was also determined. The problem is that there is too little dye attached to the antibody, at the concentrations and volumes required for microtitre plates, to make this assay successful. Further work is required to determine if more Reactive black 5 dye can be attached to the antibody for successful antigen detection in a microtitre plate assay.

Table 6.1: Differences between the methodology of Snowden and Hommel (1991), Rabello *et al.*, (1993), Nataraju *et al.*, (1994) and Kashiwazaki *et al.*, (1994).

	Snowden and Hommel	Rabello *et al.*,	Nataraju *et al.*,	Kashiwazaki *et al.*,
Nc membrane before protein is applied.	Used dry	Immersed in PBS before use	Used dry	Used dry
Protein applied to nitrocellulose.	Species specific polyclonal rabbit antisera against human or chicken IgG	Keyhole limpet cyanin and soluble egg antigen	Species specific polyclonal or monoclonal rabbit antisera against BmNPV	Species specific polyclonal or monoclonal antisera against rabbit IgG
Order and type of assay.	Species specific antisera dots ↓ antigen ↓ affinity purified secondary antibody linked to dye	Antigen ↓ test serum ↓ secondary antibody linked to dye	Species specifc antisera dots ↓ antigen ↓ secondary antibody linked to dye	Species specific antisera dots ↓ test whole blood ↓ affinity purified secondary antibody linked to dye
Concentration of protein loaded onto nitrocellulose.	0.5 – 20 μg/ml	50 – 250 μg/ml	1 – 4 μg/dot	30μg/ml, 50μg/ml or 75μg/ml
Amount applied.	3 μl	2 μl	3 μl	2.5 μl
Method of letting nc dry after application of protein.	Air dry	37°C for 30 min	Washed in PBS then air dried	Dried at 4°C
Blocking reagents.	PBS + 2%milk, 30 min room temp.	PBS + 3% milk, 1 h room temp.	PBS + 2% BSA, 30 min at 37°C	PBS + 1% milk, 30 min at 4°C
"Sandwich" protein.	Numerous purified chicken IgG standards or human IgG standards	Human serum	Purified BmNPV	Diluted whole blood (cattle)
Washing steps.	Tap water	Rinse 5 times in PBS containing 0.05% Tween 20	PBS initially then tap water	Initially tap water then 1.6 ml distilled water
Antibody bound to dye.	Rabbit anti-chicken IgG or rabbit anti-human IgG	Goat anti-human IgG	Rabbit anti-BmNPV	Rabbit PcAbs against *Trypanosoma brucei rhodesiense*
Concentration of antibody used in incubation with dye.	10 μg/ml	100 μg/ml	500 μg/ml	20 μg/ml
Length of incubation with dye-antibody.	1 h at room temp	30 min at room temp	37°C for 5 min – 1 h	1 h at room temperature

The novel protein staining abilities of the dyes used in this study were discussed in Chapter 5. The staining of proteins in a gel by primulin itself and also by diazotized primulin added an interesting comparison. When viewed under UV light, the stained protein bands were comparable to those of a Coomassie stained gel. This may add a new application for this dye in laboratories as it appears to be as sensitive as the standard Coomassie stain routinely used in laboratories. The diazotized primulin made the protein bands visible in normal light conditions and the use of UV light is thus optional. When viewing the diazotized gel under UV light, there was no increase in sensitivity with regard to the amount of protein visualised. Primulin as a dye, has many promising applications. As indicated in this document, it can be used for covalently coupling dye to antibody and detect antigen, and can be used as a protein stain in gels, before and after diazotization. For the purposes of this study however, the fact that primulin requires UV light for visualisation of the results does not ensure that it meets all the suitability criteria for a field diagnostic tool.

A look into the future could show a unique application of the dipstick dye immunoassay. Bernard *et al.*, (2001) report a new format for immunoassays. They developed a microfluidic network which places different antigen solutions in narrow stripes (20 micrometers wide), by means of silicon wafers, onto a planar substrate (poly-dimethylsiloxane – PDMS). Different antibody solutions were placed at right angles to the antigens and the result is a mosaic of signals from cross-reacted zones. Their analytes were tagged with fluorescent and enzyme-conjugates. The dye immunoassay would simplify this assay, which the authors report takes 20 min to complete.

The dipstick dye immunoassay was ultimately to be used for the diagnosis of malaria in rural areas where disease diagnosis is difficult. Many diagnostic kits have previuosly been developed, which do minimize costs to an extent, but the kits themselves are very expensive and these patients would be unable to afford the cost of such diagnosis. These kits utilize enzyme-substrate catalyzed reactions which provide coloured reactions which can be quantitated, or simply give a positive or negative result. Malarial test kits e.g.: the ICT (immunochromatographic card test) Malaria Pf™ test kit is an *in vitro* immunodiagnostic kit that tests for histidine rich protein-2 (Pf HRP-2) in circulating blood (Durrheim *et al.*, 1998). This test makes use of two antibodies specific for Pf HRP-2 antigen. The OptiMal test is another malarial diagnostic test based on the detection of an enzyme that the plasmodium

parasites produce (Palmer *et al.*, 1998). Parasite lacate dehydrogenase (pLDH) is an intracellular metabolic enzyme produced by the plasmodium parasites and the OptiMal test can differentiate between the pLDH isoforms (Palmer *et al.*, 1998).

The aim of this study was to develop a simplified, sensitive and specific malarial diagnostic test at the lowest possible cost. Although malarial antibodies were not used in this work, all the described procedures have been optimised. The foundation has thus been laid for further experimentation with malarial antibodies.

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